

1 Rare coding variants in GABA<sub>A</sub> receptor subunit genes increase the risk for genetic  
2 generalized epilepsies

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### 210 **Competing financial interests**

211 P. N. is a founder, CEO, and shareholder of ATLAS Biolabs GmbH. ATLAS Biolabs GmbH is a  
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214

215 **Generalized epilepsy with genetic etiology (GGE) is the most common type of inherited**  
216 **epilepsy characterized by absence, myoclonic and generalized tonic-clonic seizures typically**  
217 **occurring with generalized spike-and-wave discharges on electroencephalography<sup>1</sup>. Despite**  
218 **its high heritability of 80%, the genetic background is still largely unknown<sup>2-4</sup>. Here we**  
219 **show that whole exome sequencing in a discovery cohort of 196 familial index cases with**  
220 **GGE revealed a significant burden of variants in the ensemble of GABA<sub>A</sub> receptor**  
221 **encoding genes compared to 605 ethnically matched controls. Candidate gene panel**  
222 **sequencing in an independent cohort of 635 familial and sporadic GGE index cases**  
223 **confirmed an overall burden of variants in the same genes compared to independent**  
224 **controls. Functional investigations were performed for seven variants in two selected genes,**  
225 **GABRB2 and GABRA5, using automated two-microelectrode voltage clamping in *Xenopus***  
226 **oocytes. Five of the seven variants showed significant loss-of-function effects with reduced**  
227 **current amplitudes in mutant compared to respective wildtype receptors. In summary, our**  
228 **results suggest that functionally relevant variants in a GABA<sub>A</sub> receptor subunit encoding**  
229 **gene-set constitute a significant risk factor for common forms of GGE.**

230

231 In the recent past, gene discovery in monogenetic diseases, including familial and severe  
232 epilepsy syndromes, has been boosted by next generation sequencing yielding a steadily  
233 increasing number of disease-causing genetic defects. However, unraveling the genetic origin of  
234 complex inherited disorders has been more difficult<sup>5</sup>. In GGE, the prototype of genetic epilepsy  
235 with complex inheritance, a few single nucleotide polymorphisms in genome-wide association  
236 studies and altered copy number variations have been the major common risk factors identified  
237 so far<sup>6-8</sup>. These, however, only explain a small part of the high heritability<sup>2-4</sup>. Furthermore,  
238 single gene defects in larger families with autosomal dominantly inherited GGE have been  
239 identified as disease-causing, e.g. in *GABRA1* or *GABRG2* encoding subunits of GABA<sub>A</sub>  
240 receptors<sup>9-11</sup>, or in *SLC2A1* encoding the glucose transporter type 1<sup>12,13</sup>. Larger candidate gene or  
241 whole exome sequencing (WES) studies have not revealed a significant burden of mutations in  
242 single genes or groups of genes thus far<sup>14,15</sup>. Only recently, a study running in parallel to the one  
243 reported here demonstrated mutational burdens of ultra-rare variants in gene-sets related to  
244 epilepsy<sup>16</sup>.

245 We performed WES in a cohort of 238 independent, mainly familial, cases of classical forms of  
246 GGE, i.e. childhood or juvenile absence epilepsy (CAE/JAE), juvenile myoclonic epilepsy  
247 (JME) and epilepsy with generalized tonic-clonic seizures on awakening (EGTCA), collected by  
248 the Epicure and EuroEPINOMICS-CoGIE consortia (for further details see Supplemental  
249 Methods and Table S1). As controls, we used 728 ethnically matched (Supplemental Fig. S1)  
250 population control individuals from the Rotterdam Study<sup>17</sup>, that underwent WES using the same  
251 enrichment and sequencing procedures, albeit with a somewhat lower coverage than in the GGE  
252 cohort. After quality control (QC) and population stratification, the final dataset consisted of 196  
253 unrelated GGE and 605 unrelated control samples. To adjust for the different coverage between

254 case and control samples, we considered only variants with an average read depth of >10 both in  
255 case and in control samples (see Supplemental Methods and Figs. S2, S3). From the total of 801  
256 samples, 220,522 exonic and splice site variants were called. The mean exonic  
257 transition/transversion ratio equaled 3.47, indicating good data quality. Rare variants  
258 (MAF<0.005) were classified as missense (Nonsyn) and silent (Syn) variants. 91,926 Nonsyn  
259 and 53,967 Syn variants constituted the analysis data set. No single variant or individual gene  
260 achieved genome-wide significance for any variant type (see Supplemental Table S3 and  
261 Supplemental Figures S4, S5). Enrichment for seven disease- or process-specific gene-sets  
262 related to epilepsy (Supplemental Methods and Table S4) was tested individually by variant  
263 types; silent variants were expected to show no difference between cases and controls. We found  
264 a significant burden for missense variants in a set of GABA<sub>A</sub> receptors (19 genes,  $p_{\text{Nonsyn}}=0.008$ ,  
265 OR=2.23, 95% CI=[1.37,3.62]) using the CMC Fisher test<sup>18</sup> test after Bonferroni correction (Fig.  
266 1). Additionally, the genes belonging to the GABAergic pathway (113 genes,  $p_{\text{Nonsyn}}=0.002$ ,  
267 OR=1.88, 95% CI=[1.32,2.67]), genes previously reported to be associated with generalized  
268 epilepsies (28 genes,  $p_{\text{Nonsyn}}=0.002$ , OR=1.89, 95% CI=[1.34,2.68]), genes associated with  
269 epileptic encephalopathies (53 genes,  $p_{\text{Nonsyn}}=0.028$ , OR=1.59, 95% CI=[1.14,2.22]) and the  
270 gene-set representing voltage-gated ion channels (86 genes,  $p_{\text{Nonsyn}}=0.01$ , OR=1.75, 95%  
271 CI=[1.21,2.52]) (Supplemental Table S5) showed a significant variant burden in cases compared  
272 to controls (Fig. 1). For synonymous variants, none of the gene-sets showed a significant  
273 enrichment.

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275 To replicate the finding for the GABA<sub>A</sub> receptors, we designed a targeted enrichment panel  
276 comprising all 19 GABA<sub>A</sub> receptor genes. All genes were sequenced in an independent cohort of  
277 635 individuals with familial or sporadic GGE (Supplemental Methods and Table S2). *GABRR3*  
278 was excluded for QC reasons. Using all missense variants with a MAF<0.005, we confirmed that  
279 GGE patients (94/1,272 alleles) were enriched in the 18 GABA<sub>A</sub> receptor genes against ExAC  
280 (ExAC NFE, 4,064/62,676 alleles) using Fisher's exact test ( $p_{\text{Nonsyn}}=0.034$ , OR=1.23, 95%  
281 CI=[0.98,1.52]). Since individual genotypes are not available for ExAC, we also tested the  
282 GABA panel against the Rotterdam control cohort, and could confirm a significant enrichment  
283 using the CMC Fisher test ( $p_{\text{Nonsyn}}=0.005$ , OR=1.66, 95% CI=[1.14, 2.42], Supplemental Table  
284 S6). No enrichment was observed for synonymous variants. Thus, we can conclude that  
285 enrichment of rare variants in GABA<sub>A</sub> receptor genes is reproducibly present in GGE patients  
286 when compared to controls.

287  
288 The combination of two  $\alpha_1$ -, two  $\beta_2$ - and one  $\gamma_2$ -subunit (genes *GABRA1*, *GABRB2*, *GABRG2*)  
289 represents the most common form of a functional GABA<sub>A</sub> receptor in the brain<sup>19</sup>, and variants in  
290 *GABRA1* and *GABRG2* have been shown to play an important role in familial GGE, febrile  
291 seizures and EE<sup>10,11,20-24</sup>. The  $\alpha_5$  subunit (gene *GABRA5*) is supposed to mediate extrasynaptic  
292 tonic inhibition<sup>25</sup>, and tonic inhibition has been described to be altered in genetic mouse models

293 of epilepsy<sup>26–28</sup>. *GABRB2* and *GABRA5* have not previously been associated with GGE, although  
294 one *GABRB2* mutation was described recently in a patient with intellectual disability and  
295 epilepsy<sup>29</sup>. For functional studies, we selected seven missense variants in *GABRB2* and *GABRA5*  
296 (Supplemental Table S7) for electrophysiological studies in *Xenopus* oocytes (see Methods).  
297 Most of the variants co-segregated with the phenotype of available members in nuclear families,  
298 and one variant (p.R3S) was found in three different French-Canadian pedigrees (Figs. 2a and  
299 3a). All missense variants were predicted deleterious by at least three out of seven missense  
300 prediction tools and were highly conserved. Three of the variants were found in public databases  
301 (dbGAP, 1000G, ExAC) at ultra-rare allele frequencies (less than 1 in 43,000 alleles) in the  
302 European population (Supplemental Table S7). The localization of the variants in the GABA<sub>A</sub>  
303 subunits is shown in Figures 2b and 3b. After application of 1 mM GABA, we observed a  
304 significant reduction in current amplitudes of GABA<sub>A</sub> receptors containing either p.K221R or  
305 p.V316I variants in the  $\beta_2$ -subunit, and p.M1I, p.S238N, or p.E243K in the  $\alpha_5$ -subunit, in  
306 comparison to respective compositions of WT receptors. No significant reductions were  
307 observed for p.R3S in the  $\beta_2$ - and for p.P453L in the  $\alpha_5$ -subunit (Figs. 2c, 2d, 3c, 3d). The  
308 GABA sensitivity was studied by applying different GABA concentrations with no significant  
309 changes observed for receptors containing any of the studied variants (Figs. 2e, 3e). Thus, five  
310 out of seven variants suggest a loss of receptor function predicting postsynaptic or extrasynaptic  
311 neuronal disinhibition. All variants showing significantly reduced current amplitudes co-  
312 segregated with the disease phenotype in family members that were available for testing (Figs.  
313 2a, 3a), corroborating their contribution to the disease phenotypes. In two families, we observed  
314 co-segregating variants in two different GABA<sub>A</sub> receptor subunits: p.V316I in the  $\beta_2$ - and p.M1I  
315 in the  $\alpha_5$ -subunit co-occurred in the same nuclear family, and p.E243K in the  $\alpha_5$ -subunit was  
316 accompanied by a deleterious frameshift mutation in *GABRG2* in another family (Figs. 2a, 3a).  
317 Variants with altered receptor function were all located in the N-terminus containing GABA  
318 binding sites or in the pore region. p.M1I suppresses the start codon such that translation starts  
319 six amino acids later, which shortens the signalling peptide consisting of the first 20 amino acids.  
320 While the peptide is removed and not part of the mature GABA<sub>A</sub> receptor in the plasma  
321 membrane, this alteration could still affect the protein biogenesis and lead to reduced expression  
322 of functional receptors. p.R3S, which also affects the signalling peptide, and p.P453L, located in  
323 the functionally probably less relevant C-terminus, did not lead to a significant change in  
324 receptor function. p.R3S recurred in three French-Canadian families and p.P453L was detected  
325 in only one of several affected members of a larger family indicating that they might represent  
326 benign polymorphisms.

327

328 In summary, our results reveal an excess of rare coding variants in GABA<sub>A</sub> receptor subunit  
329 encoding genes in two independent cohorts of altogether >800 mainly familial GGE index cases.  
330 A few selected variants in two genes, *GABRB2* and *GABRA5*, previously not associated with  
331 GGE (i) clearly changed receptor function and (ii) co-segregated in nuclear families, suggesting



332 an important contribution for the disease phenotype and inheritance in those pedigrees. Whereas  
333 previous studies in smaller cohorts failed to show a significant excess of variants in cases versus  
334 controls either in all ion channel encoding genes<sup>14</sup> or in whole exomes<sup>15</sup>, our findings indicate  
335 that the enrichment of rare genetic variants in a set of inhibitory GABA receptors does play a  
336 significant role in the pathogenesis of GGE, and in the discovery cohort we also found an  
337 enrichment of variants in the group of voltage-gated ion channels. In a parallel study<sup>16</sup>, a similar  
338 effect could be shown for ultra-rare deleterious variants in larger, less selective gene-sets. Both  
339 studies could not identify single genes with a genome-wide significant burden of rare variants  
340 but showed an excess of variants in known epilepsy genes. In addition, our study may  
341 differentiate between a significant burden for GGE in genes associated with generalized  
342 epilepsies and a negative result for genes associated with focal epilepsies. In line with this, there  
343 was no significant burden in excitatory receptors which are known to be mutated in focal  
344 epilepsies<sup>30-33</sup>. These results (i) suggest an epilepsy subtype- and perhaps also pathway-specific  
345 pattern of variants associated with focal and generalized epilepsies, (ii) indicate a genetic overlap  
346 among rare and common forms of epilepsy, and (iii) reiterate a central role of GABAergic  
347 mechanisms in generalized epilepsies<sup>4,9-11,21-29,34,35</sup>.

348

## 349 **Figure legends**

350

351 **Fig.1. Gene-set burden analysis for the CoGIE WES cohort with matched controls from the**  
352 **Rotterdam study.** Gene-set burden analysis using a CMC Fisher Exact test was performed on  
353 seven epilepsy-related gene sets for missense (NONSYN) and synonymous (SYN) variants. The  
354 gene-sets are described in the Supplementary Methods. The red dotted line marks the significant  
355 p-value threshold of 0.05 after Bonferroni correction.

356

357 **Fig. 2. Characterization of *GABRB2* missense variants associated with GGE.** (a) Pedigrees  
358 of the families carrying the respective variants indicating the co-segregation of the variant allele  
359 as far as family members were available for testing. *m/+* denotes one variant and one WT and  
360 *+/+* denotes two WT alleles. Filled symbols represent affected individuals, CAE/JAE:  
361 childhood/juvenile absence epilepsy, EGTC: epilepsy with generalized tonic-clonic seizures. (b)  
362 Schematic representation of the  $\beta_2$ -subunit of the GABA<sub>A</sub> receptor and predicted positions of the  
363 three variants, p.R3S, p.K221R, and p.V316I located in the N-terminal domain or  
364 transmembrane domain 3. (c) Representative examples of GABA-induced currents after 1 mM  
365 GABA application for WT, p.R3S, p.K221R and p.V316I variants. (d) Normalized current  
366 response to 1 mM GABA application for  $\alpha_1\beta_2\gamma_{2s}$  (1:1:2 ratio) WT (n=30), p.R3S (n=24),  
367 p.K221R (n=21) and p.V316I (n=16); \*\*\*p<0.001, One-way ANOVA, Dunn's comparison test.  
368 (e) Dose-response curve for WT (n=30), p.R3S (n=14), p.K221R (n=10), p.V316I (n=7) after

369 application of different GABA concentrations and normalization to the maximal GABA response  
370 for each oocyte.

371

372 **Fig. 3. Characterization of *GABRA5* missense variants associated with GGE.** (a) Pedigrees  
373 of variant carriers as described for Fig. 1a. (b) Schematic representation of the  $\alpha_5$  subunit of the  
374  $GABA_A$  receptor with predicted positions of the M1I, S238N and E243K variants located in the  
375 N-terminal domain, and of P453L located in the C-terminal domain. (c) Representative examples  
376 of GABA-induced currents after 1 mM GABA application for WT, M1I, S238N, E243K and  
377 P453L mutations. (d) Normalized current response to 1 mM GABA application for  $\alpha_5\beta_2\gamma_{2s}$  (1:1:2  
378 ratio) WT (n=43), M1I (n=10), S238N (n=13), E243K (n=14) and P453L (n=11); \* $p < 0.05$ ,  
379 \*\*\* $p < 0.0001$ , One-way ANOVA, Dunn's comparison test. (e) Dose-response curve for WT  
380 (n=37), M1I (n=15), S238N (n=11), E243K (n=8) and P453L (n=8) after application of different  
381 GABA concentrations and normalization to the maximal GABA response for each oocyte.

382

383

## 384 **Online Methods**

385

### 386 **COGIE (Discovery cohort):**

387

#### 388 **Exome Sequencing and Variant calling:**

389 Exome sequencing of 233 patients and 743 controls was performed with the Illumina HiSeq  
390 2000 using the EZ Human Exome Library kit (NimbleGen, Madison, WI). Patients and controls  
391 were sequenced at different locations, cases at the Cologne Center for Genomics, the controls in  
392 Rotterdam. Sequencing adapters were trimmed and samples with  $<30X$  mean depth or  $<70\%$   
393 total exome coverage at  $20X$  mean depth of coverage were excluded from further analysis.  
394 Variant calling was performed by using the BWA-GATK<sup>36,37</sup> (version 3.2) pipeline as described  
395 in the GATK best practices with the GRCh37 human reference genome (see Supplemental  
396 methods). Variants were called within the targeted exonic intervals with 100bp padding followed  
397 by multi allelic variant decomposition and left normalization. Samples were excluded based on  
398 their relatedness, discrepancy with reported sex, excess heterozygosity,  $>3SD$  in any of the  
399 NALT, NMIN, NHET, NVAR, RATE and SINGLETON statistics and ethnicity matching. Low  
400 quality variants that failed variant quality score recalibration and hard filters based on GATK  
401 recommendations were excluded from the further analyses. Only variants with a mean depth of  
402 coverage of  $>10$  in both cases and controls were chosen for further analysis. For individual  
403 genotype filtering a variant was changed to no call if the read depth was  $<10$  or the genotype  
404 quality was  $<20$  and all variants with a missingness  $>3\%$  were excluded. Repeat regions were  
405 downloaded from UCSC and indels within segmental duplications, simple repeat regions, self-  
406 chained regions and homopolymer stretches were excluded.

407

408 **Variant annotation and filtering**

409 Variants were annotated using ANNOVAR<sup>38</sup> version 2015Mar22 with RefSeq gene annotations  
410 and dbNSFP<sup>39</sup> (v3.0) pathogenicity predictions for nonsynonymous variants and conservation  
411 scores (see Supplemental Methods). To investigate ultra-rare variants, we excluded variants  
412 present with minor allele frequency (MAF) > 0.005 in the European populations of the 1000  
413 genomes (<http://www.1000genomes.org>), the Exome Variant Server (EVS;  
414 <http://evs.gs.washington.edu>) and the Non-Finnish European (NFE) data from ExAC<sup>40</sup> (release  
415 0.3).

416

417 **Burden analysis:**

418 All burden tests were performed by using rvtests<sup>41</sup> with default parameters. Only one-sided P-  
419 values were used throughout the analysis. We applied the CMC Fisher test method<sup>18</sup> for gene-  
420 burden analysis, both available in rvtests<sup>41</sup>. P-values were corrected using the Bonferroni method  
421 (as implemented in the R function p.adj) for 17,648 RefSeq protein-coding genes applying a  
422 significance threshold of 0.05. Gene-set burden tests were applied for missense and synonymous  
423 variants for specific groups of candidate genes. Different disease and process-specific gene-sets  
424 were constructed based on their relation to GGE together with control gene-sets not related to  
425 GGE. A description of the gene-set construction is given in Supplemental methods and the gene-  
426 sets are given in Supplemental Table S4. Gene-set burden testing was performed using the CMC  
427 Fisher test. We used the Bonferroni method for P-value correction for seven gene-sets.

428

429 **GABA<sub>A</sub> receptor gene panel (replication cohort):**

430 *Sample recruitment and collection.* Individuals were collected by referral from neurologists or  
431 paediatricians in Quebec, Canada, and in Europe (see Supplemental Methods for cohort  
432 description). Informed consent was obtained from all participants and the ethics committee of the  
433 CHUM-Notre Dame Hospital as well as all local ethical committees approved the study. The  
434 diagnosis of GGE was based on detailed clinical interview and a full neurological examination.  
435 In total, our cohort was constituted of 635 unrelated familial and sporadic cases. Patients were  
436 diagnosed with juvenile myoclonic epilepsy (JME), childhood absence epilepsy (CAE), juvenile  
437 absence epilepsy (JAE) or genetic generalized epilepsy (GGE) not otherwise specified according  
438 to ILAE definitions<sup>2,36</sup>. Only one member per family was selected. All charts and investigations  
439 were reviewed by experienced neurologists (PC, HL, StW, FB, YW, SaW, HH) before making a  
440 final clinical diagnosis. Phenotypic information was centrally databased at CHUM-Notre Dame  
441 Hospital and blood samples were collected and stored at the Research Centre of the CHUM.  
442 DNA was extracted from peripheral blood by using standard protocols.

443 *Design, target enrichment and sequencing.* A total of 19 genes encoding for all known subunits  
444 of GABA<sub>A</sub> receptor were selected for deep sequencing (*GABRA1*, *GABRA2*, *GABRA3*, *GABRA4*,  
445 *GABRA5*, *GABRA6*, *GABRB1*, *GABRB2*, *GABRB3*, *GABRD*, *GABRE*, *GABRG1*, *GABRG2*,  
446 *GABRG3*, *GABRP*, *GABRQ*, *GABRR1*, *GABRR2*, *GABRR3*). Exon targets were generated based

447 on RefSeq, representing 184 exons from 19 genes. Primer design was made using the Primer3  
448 oligonucleotide design tool and In Silico PCR tool for validating the specificity of each  
449 amplicons. Human genome reference sequence (hg19) was used. Target regions were enriched  
450 by PCR using the 48.48 Access Array Integrated Fluidic Circuit (IFC) (Fluidigm, San Francisco,  
451 CA). In the final assay, 185 amplicons targeted the protein-coding sequence of 19 GABRX genes  
452 with an overhang at exon boundaries in order to capture splice site variants. GABRX exon-  
453 specific primers with Fluidigm tags were tested along with materials and reagents as  
454 recommended in the Access Array System User Guide (Fluidigm, South San Francisco, CA).  
455 Finally, *GABRR3* had to be dropped because of QC reasons having not enough good quality  
456 reads covering.

457 *Alignment, enrichment assessment and variant calling.* Using Trimmomatic<sup>42</sup>, all reads were  
458 trimmed and clipped to remove Illumina MiSeq adapters and bad quality bases. BWA mem<sup>37</sup>  
459 was used to align reads to the GRCh37 human reference genome. All alignments files were  
460 stored in the BAM format and the Picard suite was used to merge all alignment in a single file  
461 (<http://picard.sourceforge.net>). GATK<sup>36</sup> suite was used to produce metrics file and to perform  
462 variant calling using UnifiedGenotyper. Finally, ANNOVAR<sup>38</sup> was used to provide gene  
463 annotation information for the classification into missense and synonymous variants and to filter  
464 for rare (allele frequency smaller than 0.5%) variants using the ExAC database. CMC Fisher test  
465 was used for gene-set burden analysis and the Bonferroni method for multiple testing correction.  
466

#### 467 **Functional analysis:**

468 All methods for functional studies have been described previously<sup>21,23</sup> and are summarized in  
469 brief as follows:

470 *Mutagenesis and RNA preparation.* We used the Quick Change kit (Stratagene) to engineer the  
471 missense mutations in the *GABRB2* and *GABRA5* cDNAs (NM\_021911 and NM\_000810,  
472 respectively) inserted in the pcDNA3 vector. Primers are available upon request. Mutations were  
473 confirmed and additional changes were excluded by Sanger sequencing. cRNA was prepared  
474 using the T7 RNA polymerase kit from Ambion.

475 *Oocyte preparation and injection.* Oocytes were obtained from the Institute of Physiology I,  
476 Tübingen, or purchased from EcoCyte Bioscience (Castrop-Rauxel). Experiments were approved  
477 by local authorities (Regierungspräsidium Tübingen, Germany). The preparation of oocytes for  
478 two-microelectrode voltage-clamp recordings included treatment with collagenase (1mg/ml of  
479 type CLS II collagenase, Biochrom KG) in OR-2 solution (mM: 82.5 NaCl, 2.5 KCl, 1 MgCl<sub>2</sub>  
480 and 5 Hepes, pH 7.6), followed by thorough washing and storing at 17°C in Barth solution (mM:  
481 88 NaCl, 2.4 NaHCO<sub>3</sub>, 1 KCl, 0.41 CaCl<sub>2</sub>, 0.82 MgSO<sub>4</sub> and 5 Tris/HCl, pH 7.4 with NaOH)  
482 supplemented with 50µg/ml gentamicin (Biochrom KG) as described previously<sup>21,23</sup>. We injected  
483 a total amount of 70 nl at a concentration of 2 µg/µl of cRNA encoding respective mixtures of  
484 WT or mutant subunits into oocytes using the Robooinject® (Multi Channel Systems). Oocytes  
485 were stored for 1-3 days at 17°C before the experiment. The combination of the different

486 subunits used was  $\alpha_1\beta_2\gamma_{2s}$  or  $\alpha_5\beta_2\gamma_{2s}$  in 1:1:2 ratios. Current amplitudes of WT and mutant  
487 receptors were recorded and compared on the same day using the same batch of oocytes so that  
488 data of different days could be pooled when normalized to the WT.

489 *Automated oocyte two-microelectrode voltage clamp.* GABA-evoked ionic currents in oocytes  
490 were recorded at room temperature (20-22°C) using a Roboocyte2® system (Multi Channel  
491 Systems). Prepulled and prepositioned intracellular glass microelectrodes had a resistance of  
492 0.3–1 MΩ when filled with 1 M KCl and 1.5 M KAc. ND96 was used as extracellular bath  
493 solution (in mM: 93.5 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 5 Hepes, pH 7.5). Currents were  
494 sampled at 1 kHz. To activate the receptors, increasing GABA concentrations (in μM: 1, 3, 10,  
495 40, 100, 300, 1000) diluted in ND96 solution were applied for 15 s each.

496 *Electrophysiological data analysis.* Oocytes were held at a holding potential of -70 mV. The  
497 amplitude of the GABA-induced currents was analyzed using Roboocyte2+ (Multi Channel  
498 Systems), Clampfit (pClamp 8.2, Axon Instruments), Microsoft Excel (Microsoft) and GraphPad  
499 Prism (GraphPad Software) softwares. The current response of each GABA concentration was  
500 normalized to the maximum response evoked by the highest GABA concentration (1 mM). The  
501 following four parameter logistic equation:

$$502 \quad Y(X) = \min + \frac{(max-min)}{1+10^{((LogEC_{50}-X)*nh)}},$$

503 was fit to the normalized GABA responses of each oocyte, with *max* and *min* being the  
504 maximum and minimum evoked responses, X the corresponding GABA concentration, EC<sub>50</sub> the  
505 concentration of the agonist at which half of the maximum response is achieved, and nh the Hill  
506 coefficient reflecting the steepness of the dose-response curve. For each oocyte, EC<sub>50</sub> values  
507 were determined and then averaged for each combination of receptor subunits used. Current  
508 amplitudes in response to 1 mM GABA application for mutant channels were normalized to the  
509 mean value of the WT channel response recorded on the same day.

510 *Statistical analysis.* For statistical evaluation, GraphPad Prism 6 was used. All data were tested  
511 for a Gaussian normal distribution. Groups were compared using one-way ANOVA with  
512 Dunnett's *post-hoc* test for normally distributed data or one-way ANOVA on ranks with Dunn's  
513 *post-hoc* test for not normally distributed data. For unpaired data sets, Student's t test (normally  
514 distributed) or Mann-Whitney rank-sum test (data not normally distributed) were used. All data  
515 are shown as mean ± standard error of the mean (SEM). Statistical differences are indicated in  
516 the figure legends using the following symbols: \*p<0.05, \*\*p<0.001, \*\*\*p<0.0001.

517

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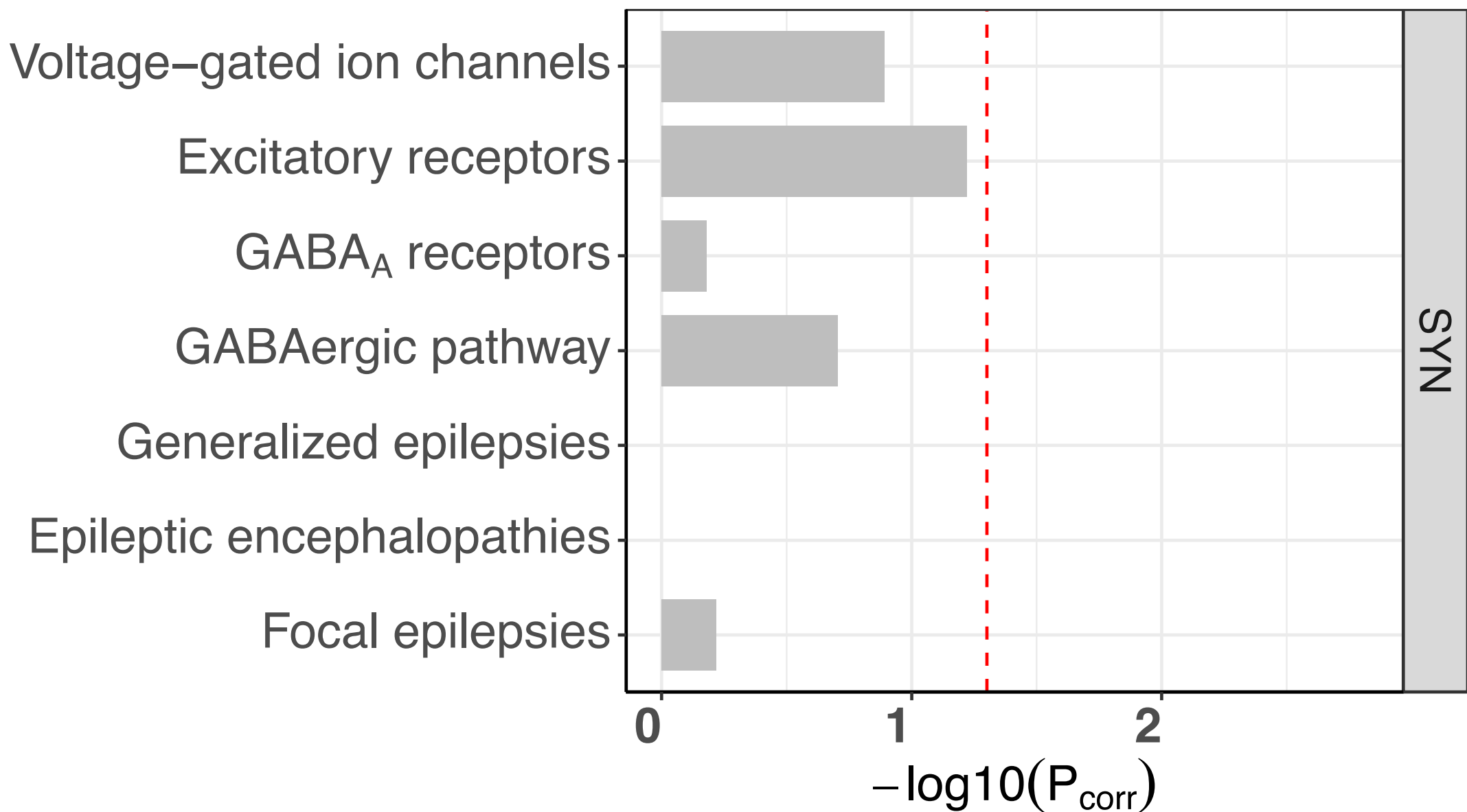
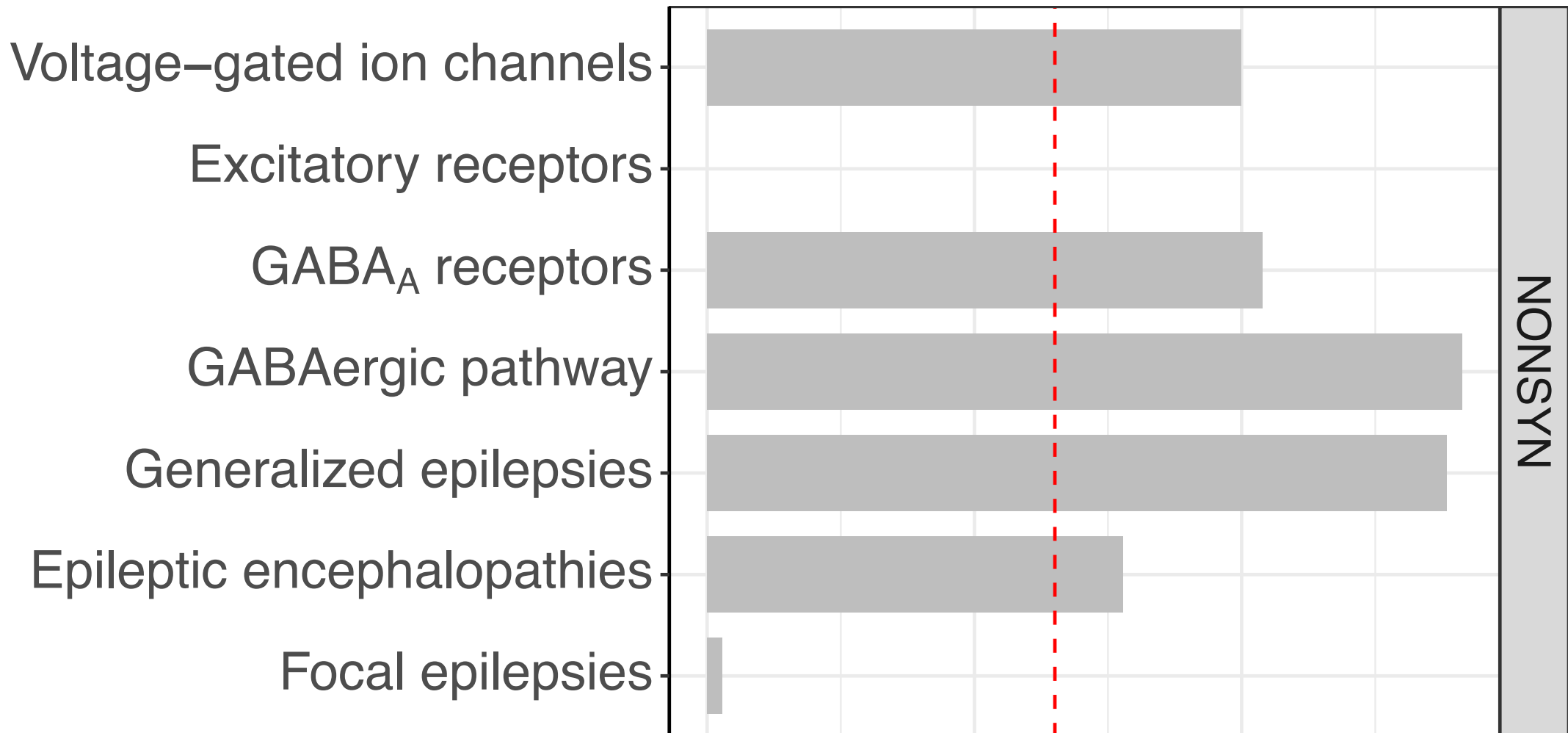
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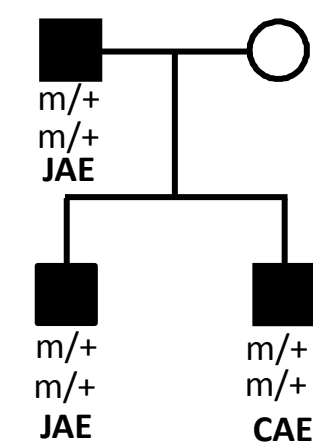
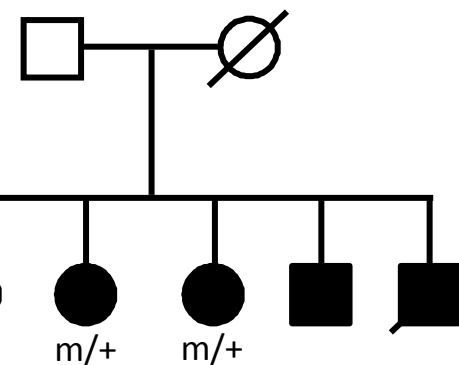
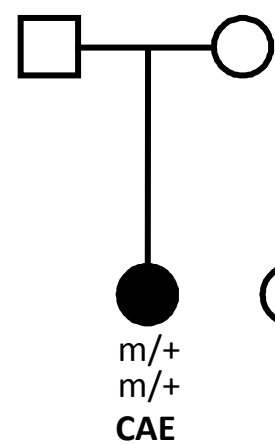
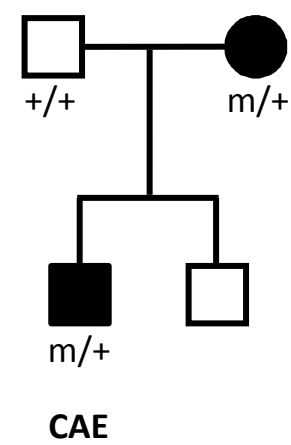
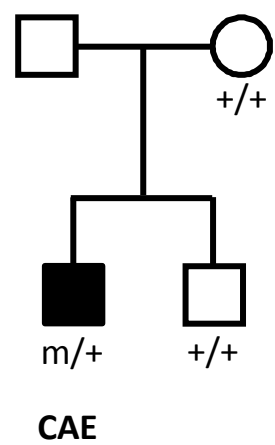
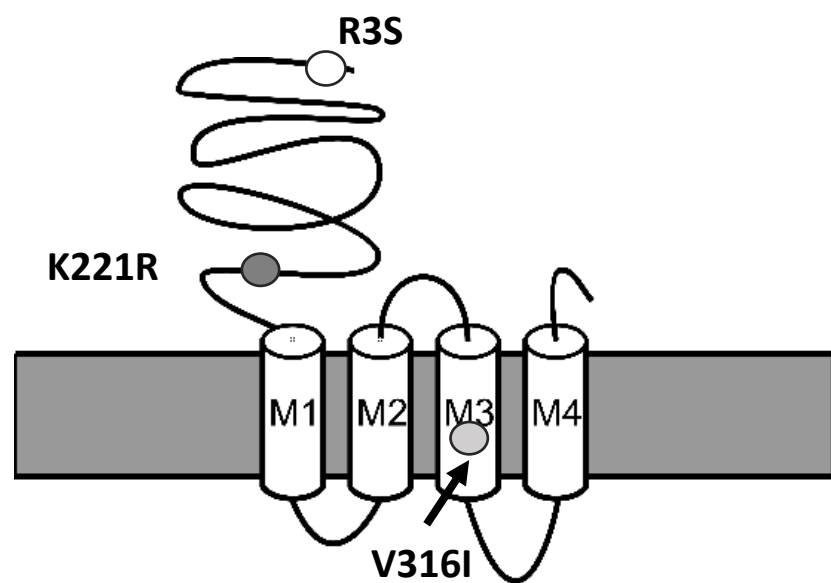
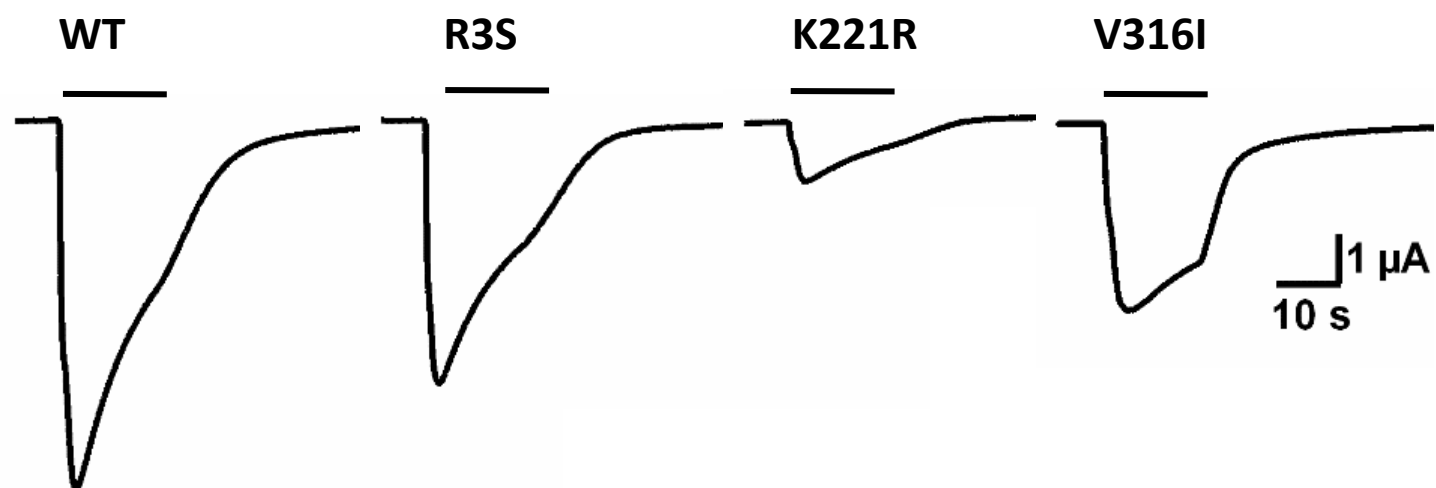
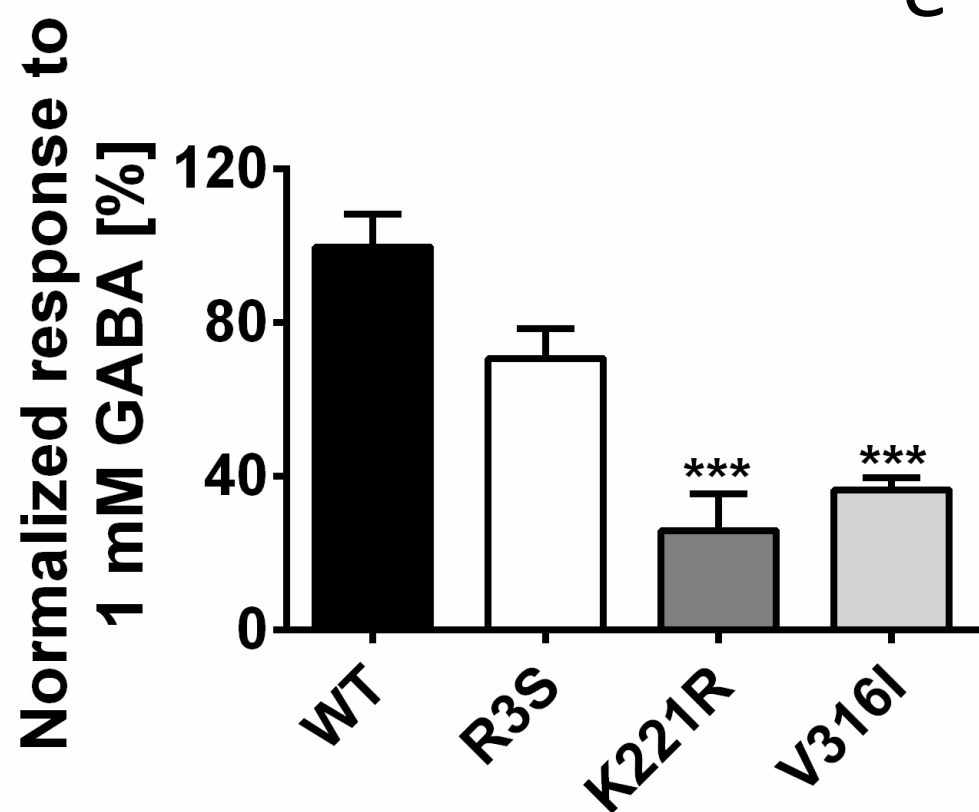
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**a***GABRB2-R3S**GABRB2-R3S**GABRB2-R3S*  
*GABRR2-T151I**GABRB2-K221R**GABRB2-V316I*  
*GABRA5-M1I***b****c****d****e**