



From Standard of Care to Personalized (Art of) Medicine: Two Novel GABA-A Receptor $\beta 3$ Subunit Mutations Associated With Epilepsy Syndromes

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Synaptic Clustering Differences Due to Different GABRB3 Mutations Cause Variable Epilepsy Syndromes

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GABRB3 is highly expressed early in the developing brain, and its encoded $\beta 3$ subunit is critical for GABAA receptor assembly and trafficking as well as stem cell differentiation in embryonic brain. To date, over 400 mutations or variants have been identified in GABRB3. Mutations in GABRB3 have been increasingly recognized as a major cause for severe pediatric epilepsy syndromes such as Lennox-Gastaut syndrome, Dravet syndrome, and infantile spasms with intellectual disability as well as relatively mild epilepsy syndromes such as childhood absence epilepsy. There is no plausible molecular pathology for disease phenotypic heterogeneity. Here, we used a very high-throughput flow cytometry assay to evaluate the impact of multiple human mutations in GABRB3 on receptor trafficking. In this study, we found that surface expression of mutant $\beta 3$ subunits is variable. However, it was consistent that surface expression of partnering $\gamma 2$ subunits was lower when coexpressed with mutant than with wild-type subunits. Because $\gamma 2$ subunits are critical for synaptic GABAA receptor clustering, this provides an important clue for understanding the pathophysiology of GABRB3 mutations. To validate our findings further, we obtained an in-depth comparison of 2 novel mutations (GABRB3 [N328D] and GABRB3 [E357K]) associated with epilepsy with different severities of epilepsy phenotype. GABRB3 (N328D) is associated with the relatively severe Lennox-Gastaut syndrome, and GABRB3 (E357K) is associated with the relatively mild juvenile absence epilepsy syndrome. With functional characterizations in both heterologous cells and rodent cortical neurons by patch clamp recordings, confocal microscopy, and immunoblotting, we found that both the GABRB3 (N328D) and GABRB3 (E357K) mutations reduced total subunit expression in neurons but not in HEK293T cells. Both mutant subunits, however, were reduced on the cell surface and in synapses, but the Lennox-Gastaut syndrome mutant $\beta 3$ (N328D) subunit was more reduced than the juvenile absence epilepsy mutant $\beta 3$ (E357K) subunit. Interestingly, both mutant $\beta 3$ subunits impaired postsynaptic clustering of wild-type GABAA receptor $\gamma 2$ subunits and prevented $\gamma 2$ subunits from incorporating into GABAA receptors at synapses, although by different cellular mechanisms. Importantly, wild-type $\gamma 2$ subunits were reduced and less clustered at inhibitory synapses in *Gabr3*^{+/-} knockout mice. This suggests that impaired receptor localization to synapses is a common pathophysiological mechanism for GABRB3 mutations, although the extent of impairment may be different among mutant subunits. The study thus identifies the novel mechanism of impaired targeting of receptors containing mutant $\beta 3$ subunits and provides critical insights into understanding how GABRB3 mutations produce severe epilepsy syndromes and epilepsy phenotypic heterogeneity.

Commentary

... And we thought that with precise diagnosis of an epilepsy syndrome we were done ... Epilepsy, despite popular and even more specialized beliefs,¹ is not a single entity but rather a large cluster of seizure disorders. Some of them, though presenting with similar clinical symptomatology, may have completely different etiopathogenesis that would eventually require specific mechanistic treatments. As an example of this, we can use epileptic spasms in infancy (infantile spasms, loosely West syndrome), which clinically look very similar, but

etiopathogenesis in their symptomatic subgroup covers over 200 different causes.² Plus, there is also the cryptogenic subgroup, which slowly rises from its genetic underlayment. Current treatments are far from the mechanistic or rather causative approach: Currently used drugs are antiseizure medications suppressing, if effective, just one component of the syndrome. If we consider efficacy of antiseizure medications, about 33% to 35% of patients with epilepsy (ie, usually with temporal lobe epilepsy) are not responding to treatments.³ This proportion has not changed for the past 30 years,⁴ although new antiseizure



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drugs were introduced in the meantime. Interestingly, the number is quite similar to the number of patients with epilepsy resistant to bromide treatments reported by Gowers in 1881.⁵ Returning to the introductory statement, even new classifications of seizures and epilepsies that went to great lengths to accommodate newly identified genetic causes have not provided a quantum leap in drug efficacy due to more precise diagnosis of syndromes resulting in more refined standards of care. The moral of the story is that we need to figure out (for the beginning) what are the causes of individual epilepsy syndromes and then obtain specific and timely countermeasures.

Identification of GABA (known originally as factor I) as a substance present in the brain including its inhibitory properties dates from 1950^{6,7} with significant input of Takashi Hayashi.⁸ However, the concept of GABA receptors emanates from the works of Curtis et al.,⁹ and the concept of GABA receptor subunits dates even later¹⁰ with molecular unit composition appearing in the early 80s.¹¹ The current study is devoted to 2 single-nucleotide mutations (N328D and E357K) on the gene encoding one of the subunits ($\beta 3$) of the pentameric structure of the GABA-A receptors. A current database search on the *GABRB3* gene from the NIH ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar>) shows a total of 452 mutations, many with severe associated pathologies including various seizure syndromes, autism, and mental deterioration. There are 135 known single nucleotide mutations on the *GABRB3* gene. This illustrates an immense variety of individual and sometimes extremely focal issues on the *GABRB3* gene and resulting GABA-A receptor $\beta 3$ subunit protein.

Characterization of the patients carrying those 2 *GABRB3* gene mutations showed striking differences. The first mutation (de novo N328D) was associated with a generalized tonic-clonic seizure with febrile episode at age 14 months. This seizure type and myoclonic seizures continued despite valproate therapy until 7 years of age when lamotrigine was added. After onset of seizures, moderate developmental and language abnormalities appeared. The E357K mutation was associated with seizure onset (generalized tonic-clonic) at age 14 years and continued with multiple daily absence and myoclonic seizures while the patient was on valproate. Seizures diminished 1 year later with the addition of lamotrigine. The development of the patient was not affected. Interestingly, the same mutation, although clinically silent, was found in the father of this patient.

In their mechanistic investigations, the authors left no stone unturned. Initially, they explored surface membrane expression of receptor subunits β and γ (tagged with hemagglutinin) on various *GABRB3* mutations including those 2 of interest to find whether there is a common outcome. While $\gamma 2$ subunit surface expression was decreased with any $\beta 3$ mutation (except 1 out of 12 as $\gamma 2$ must heteromerize), this was not the case in $\beta 3$ subunits as those subunits can form homopentamers (of unclear physiological function¹²) and get trafficked to the cell surface in that form. Currents passing through GABA receptors with affected $\beta 3$ proteins were attenuated. In the mixed form (receptor containing 1 normal $\beta 3$ and 1 mutant $\beta 3$), a decrease in peak current was smaller than in the full mutation (dose-dependent


effect). N328D mutation presented with a more severe current reduction than the E357K mutation. While the peak current provides useful information, the charge transferred may be more useful¹³ since it considers total number of ions passing through the membrane, which may reveal possible compensatory changes. Unfortunately, the authors did not evaluate area under the curve of the current.

Next, the authors contemplated that the reduced amplitude of the current may be due to reduced surface expression of the receptor, or altered receptor stoichiometry, or altered channel gating. Interestingly, there may also be a presynaptic contribution to the current reduction not investigated here. The expression of all subunits ($\beta 3$, $\gamma 2$, and $\alpha 1$) in heterotrimeric receptors containing $\beta 3$ subunits with mutations was decreased compared to receptors composed of wild-type (wt) subunits. This indicates impaired surface expression due to reduced trafficking from the intracellular compartment to the membrane surface. Puncta of the receptors containing mutated $\beta 3$ subunits had an altered distribution compared to the wt; mutated receptors were mainly found intracellularly in the soma, while the wt receptors had puncta on the processes and cell body surface. Further, there was reduced expression of postsynaptic mutated $\beta 3$ subunits when a coassembly with $\gamma 2$ subunits was investigated. Since $\gamma 2$ subunits are responsible for synaptic clustering,¹⁴ this finding indicates that insertion of postsynaptic GABAA receptors to the synapse is reduced by $\beta 3$ mutations. In addition, mutated $\beta 3$ puncta were reduced at the synapses, which suggests altered stoichiometry of the postsynaptic receptors compared to the wt. Since both mutations were associated with loss of function, a mutant mouse (heterozygous *Gabrb3* knockout) was used to determine expression of $\gamma 2$ subunits (synaptic clustering of the receptors) in the cortical layers. Similar to the in vitro study, there was a decrease in $\gamma 2$ expression in these mice suggesting limited synaptic clustering of GABAA receptors, while gephyrin as the inhibitory synapse marker¹⁵ was not reduced (number of inhibitory synapses was similar to wt, consistent with the in vitro experiments).


There are at least 2 interesting findings that can be pursued further. The first *GABRB3* mutation (N328D) was associated with developmental delay and language deficiency. The authors indicate that this may be the effect of more severe impairment of receptor clustering at the synapse seen with that mutation. An alternative is much longer exposure (compared to the condition of the patient with the other mutation) to the ongoing seizures. Seizure exposure was also running through critical developmental periods of infancy and childhood. This was not the case in the second *GABRB3* mutation (E357K) with seizures observed much later during postnatal development and for significantly shorter period as lamotrigine was titrated much faster. The second surprising finding is a clinically silent mutation (E357K) in the father of the patient with the same mutation (E357K). Here, the investigators have had a tremendous opportunity to determine whether the mutation in a person without seizures also causes deficiency in GABAA receptor clustering in the synapse. If not, this mutation probably requires an additional factor (additional mutation maybe)

for the penetration of clinical symptoms. Alternatively, there may be epigenetic compensation in the clinically silent father. If there was a clustering phenotype yet no clinical manifestations, then we can speculate that GABAA receptor clustering may not be the decisive effect of the mutation required for clinical seizures, or there is another compensatory measure outside the GABAA receptor system. In any case, studying the father who is clinically silent together with the son with epilepsy would bring us closer to understanding how we can compensate for these mutations (partial inheritance of compensatory mechanisms?).

In conclusion, the study indicates that point mutations of the *GABRB3* gene affect synaptic clustering of GABA-A receptors and thereby diminish GABA-A receptor-mediated currents leading to different types of clinical seizures. Yet clinical seizure presentations in both patients though being different respond to lamotrigine. The study underscores the necessity of dissecting causes of individual seizure syndromes and applying individualized treatments.

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