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Title: Compound-heterozygous *GRIN2A* null variants associated with severe developmental and epileptic encephalopathy

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Figure 1. Pedigree of a family with two pathogenic *GRIN2A* null variants. The heterozygous parents had mild *GRIN2A*-related phenotypes. The daughter with compound heterozygous *GRIN2A* variants had a more severe phenotype. The brother remains untested but had a clinical phenotype compatible with (heterozygous) *GRIN2A*-related disorder.

Figure 2. Functional studies on h2A-Q661X and L830PX2 variants. A. Expression in oocytes measured by application of 100 mM glutamate and 100 mM glycine for 1 min (Glu/Gly, black bar, inset). The mean and 95% CI (bars) are displayed on top of scatterplots of oocyte maximum current for wild type (WT) and each variant receptor, plus uninjected oocytes. Data are results from two RNA preparations for each variant, and from two separate experiments. *Inset* shows representative inward current traces for each receptor variant. Note that uninjected and h2A-L830PX2 traces overlay each other. *$p<0.01$ from uninjected oocyts by one-way ANOVA and Dunnett's. B. Total and surface receptor protein expression for both GluN2A variants in HEK mammalian cells. *$p<0.05$ from WT control (dashed line) by unpaired t-Test. ND= no detection above background.

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Abstract

We report on an 8-year-old girl with severe developmental and epileptic encephalopathy due to the compound heterozygous null variants p.(Gln661*) and p.(Leu830Pro*fs**2) in *GRIN2A* resulting in a knockout of the human GluN2A subunit of the N-methyl-D-aspartate receptor. Both parents had less severe *GRIN2A*-related phenotypes and were heterozygous carriers of the respective null variant. Functional investigations of both variants suggested a loss-of-function effect. This is the first description of an autosomal recessive, bi-allelic type of *GRIN2A*-related disorder. Still,

there are marked parallels to two previously published families with severe epileptic encephalopathy due to homozygous null variants in *GRIN1* as well as various knockout animal models. Compared to heterozygous null variants, bi-allelic knockout of either GluN1 or GluN2A is associated with markedly more severe phenotypes in both humans and mice. Furthermore, recent findings enable a potential precision medicine approach targeting GRIN-related disorders due to null variants.

Introduction

N-methyl-D-aspartate receptors (NMDAR) are expressed throughout the brain, mediating excitatory neurotransmission important for development, learning, memory, and other higher cognitive functions. NMDAR are di- or tri-heterotetrameric ligand-gated ion channels composed of two glycine-binding GluN1 (encoded by *GRIN1*) and two glutamate-binding GluN2 subunits (encoded by *GRIN2A-D*).[1]

Four genes encoding NMDAR subunits (*GRIN1, GRIN2A, GRIN2B*, and *GRIN2D*) have been linked to human disease. Whereas pathogenic missense variants in all four genes are associated with disease, the pathogenicity of null variants appears to be less stringent.[2] For example, *GRIN1* and *GRIN2D* heterozygous null variants are not disease-causing. By contrast, *GRIN2A* and *GRIN2B* heterozygous null variants (e.g. truncating, frameshift, splice variants) have been unquestionably associated with disease[3,4] and are clearly depleted in the general population (*GRIN2A*: pLI = 1.00; LOEUF = 0.19; *GRIN2B*: pLI = 1.00; LOEUF = 0.06)[2,5]

Still, there is evidence that carriers of pathogenic null variants display a decreased phenotypic severity compared to carriers of pathogenic missense variants. In fact, the clinical spectrum associated with heterozygous *GRIN2A* null variants can be so mild that affected individuals often are able to reproduce, which is why *GRIN2A* null variants

appear to be the only pathogenic variants within the GRIN gene family that are frequently found to be inherited.[4]

In contrast to *GRIN2A* and *GRIN2B*, the pathogenicity of heterozygous *GRIN1* null variants currently remains unclear, as carriers usually do not display clinically relevant phenotypes at all despite the fact that *GRIN1* null variants are depleted in the general population (pLI = 0.98; LOEUF = 0.31).[2] However, homozygous null variants in *GRIN1* resulting in a functional human knockout of GluN1 have been associated with a markedly more severe phenotype than associated with heterozygous *GRIN1* variants.[6,7] Besides these two reports, little to nothing is known on potential human knockouts of other subunits of the NMDAR, i.e. GluN2.

Methods

We reviewed clinical and genetic data of a family with *GRIN2A*-related disorder. Respective family members have agreed to genetic testing and publication of their data. This study has been approved by the ethics committee of the University of Leipzig (224/16-ek, 402/16-ek, 465/19-ek).

Clinical data

We collected phenotypic information on affected family members following a standard clinical questionnaire, which is used in the GRIN Registry (http://grin-portal.broadinstitute.org/).

Genetic data

A venous blood sample of the index girl was analysed by a panel sequencing (TruSight One Sequencing Panel, Illumina, US) within routine diagnostic settings. Parental samples have been tested by conventional Sanger sequencing. The detected *GRIN2A*

variants were classified according to the recommendations of the American College of Medical Genetics and Genomics.[8,9]

Functional data

Introduction of the genetic variants (Quikchange, Stratagene) into the human GluN2A cDNA (NM_000833) and analysis of expression was essentially as described.[10] Briefly, after confirmation by Sanger sequencing (Eurofins) the variant cRNAs were synthesized (Ambion), and separately combined with GluN1 cRNA and injected into *Xenopus laevis* oocytes prepared as described.[11] After incubation for 3-4 days at 15-19°C in Barth's solution, two electrode voltage clamp recordings were performed at V_{hold} -40 mV at room temperature in solution containing (in mM) 90 NaCl, 1 KCl, 10 HEPES, 0.5 $BaCl_2$, and 0.01 EDTA (pH 7.4). Current and voltage electrodes were filled with 3.0 M and 0.3 M KCl, respectively. After stable baseline recordings were achieved, oocytes were challenged with 100 μM L-glutamate and 100 μM glycine for 1 minute to determine maximal GluN2A NMDA receptor responses. In separate studies, the same variants were introduced into cDNAs with β-lactamase fused in frame to the amino terminus of the GluN2A subunit, combined with GluN1 cDNA and co-transfected into mammalian human embryonic kidney (HEK) cells, and surface expression of these variant receptors determined 24 hr later by measuring β-lactamase hydrolysis of nitrocefin as described.[10] Statistical comparisons were made using a one-way ANOVA and Dunnett's multiple comparison test for maximum current in oocytes or an unpaired t-Test for receptor expression in HEK cells.

Results

Clinical description

We describe an 8-year-old girl born to non-related parents. At the age of 9 months, she was diagnosed with delayed motor development and muscular hypotonia (free sitting at 12 months, crawling at 30 months). At the age of 3 years, there was a stagnation of development followed by epilepsy with focal impaired awareness seizures of unknown frequency (including deviation of eyes as well as apathy) at 4 years. EEG at 4 years old revealed generalized slowing and multifocal discharges including bilateral centro-temporal spikes. At age 8 years electrical status epilepticus during slow-wave sleep (ESES) was diagnosed. Additionally, the girl was diagnosed with mild ataxic movement disorder and muscular hypotonia of the trunk. Lamotrigine improved epileptic symptoms and slightly the symptoms of the ataxic movement disorder. After adding Valproate at 8 years, the EEG normalized. MRI at 4 years was normal. At the age of 8 years, she is non-verbal and walks only a few steps unaided. Body measures at 6.5 years were normal: head circumference 53.5 cm (P90-97), weight 23 kg (P75), length 116 cm (P50-75).

Genetic testing revealed two compound heterozygous pathogenic *GRIN2A* null variants: c.1981C>T, p.(Gln661*) and c.2488dupC, p.(Leu830Pro*fs**2).

Family history proofed to be challenging as barely any clinical records were available. However, the mother (37 years) was reported to have had epilepsy with prolonged generalized seizures and complex focal seizures since infancy (3.5 years). Furthermore, she had psychomotor developmental delay with dystonic movement disorder and learning difficulties in childhood. Speech appeared normal. Mood disorders had been treated citalopram. It remains unclear when seizure freedom occurred. However, the EEG remained pathologic and shows mild intermittent slowing right temporal treated with lamotrigine. Segregation analysis revealed the heterozygous *GRIN2A* variant c.1981C>T, p.(Gln661*).

The father (46 years) is mildly intellectually disabled. He never had seizures. Segregation analysis revealed the heterozygous *GRIN2A* variant c.2488dupC, p.(Leu830Pro*fs**2).

The brother (10 years) has speech developmental disorder and ADHD. He spoke first words at 3 years and has a less modulated, monotonous speech with articulation errors and a conspicuous speech melody. Epilepsy is suspected, but an EEG at 10 years remained unremarkable. Genetic testing has not been performed.

A paternal cousin (son of the father's brother) was reported to have had epileptic seizures of unknown semiology between 9 and 11 years and unknown EEG. Genetic testing has not been performed (Figure 1).

Functional analyses

For functional studies we refer to p.(Leu830Pro*fs**2) as h2A-L830PX2 and p.(Gln661*) as h2A-Q661X. Neither h2A-Q661X nor h2A-L830PX2 variants resulted in significant expression of functional NMDA receptors in Xenopus oocytes after 3-4 days incubation (Figure 2A). For the h2A-Q661X variant the mean current size measured after 1 min exposure to maximal glutamate and glycine application (100 μM each) was 11.7±3.7 nA (mean±SEM, n=18) compared to 428±67 nA (n=24) for h2A-WT GluN2A receptors. Similarly, for the h2A-L830PX2 variant the mean current size measured was 2.9±0.5 nA (n=12) and for uninjected oocytes the current magnitude was 2.3±0.4 nA (n=8). Only the h2A-WT receptor current maximum tested significantly different from uninjected oocytes by one-way ANOVA and Dunnett's multiple comparison test. To obtain measureable currents with these *GRIN2A* null variants additional mRNA was injected and when current maximums were normalized to the amount of mRNA injected per oocyte the differences between h2A-WT (1836±200 nA/ng RNA) and h2A-Q661X

(1.2±0.4) and h2A-L830PX2 (0.5±0.01) were further exemplified. Further we also tested higher concentrations of both glutamate and glycine (3 mM each) on the two variants and current magnitudes did not increase greater than those at 100μM/100μM indicating that low current sizes were not due to large shifts in glutamate or glycine potency (data not shown). Thus, current sizes for both variants were deemed too small to measure reliable pharmacological endpoints such as glutamate or glycine EC_{50}'s.

We also conducted β-lactamase-fusion protein studies in mammalian HEK cells (Figure 2B) on both the h2A-L830PX2 and h2A-Q661X variants and although both variants made receptor subunit protein (total protein for h2A-L830PX2 was 74.2±17.4% of WT, n=4; total protein for h2A-Q661X was 44.9±2.8% of WT, n=4), and neither variant was capable of driving significant cell surface expression where h2A-L830PX2 was only 1.9 +1.4% that of WT (n=4, $p<0.05$ from WT control, t-Test) and h2A-Q661X was not detectable above background (n=4). Thus, lack of a current response was consistent with the truncation of the subunit and reduced expression shown by β-lactamase studies.

Thus, both human variants appear to be loss of function based on these results.

Discussion

We describe a girl with biallelic, compound heterozygous *GRIN2A* null variants p.(Gln661*) and p.(Leu830Pro*fs**2) and severe developmental delay with ESES on EEG leading to the diagnosis of epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS). Additionally, ataxic movement disorder and muscular hypotonia of the trunk have been noted. Due to the lack of more detailed information

and clinical descriptions, some of the clinical and epileptologic aspects still remain superficial and will need specification by follow-up reports of additional cases.

Whereas the phenotypic spectrum of the girl's parents with learning disability and mild ID was in perfect agreement to the known range of severity in individuals with heterozygous *GRIN2A* null variants[4], the phenotype of their daughter with compound heterozygous *GRIN2A* null variants is much more severe. In fact, it appeared to be rather comparable to the severe-to-profound clinical phenotypes of individuals with gain-of-function variants. However, functional analyses of both *GRIN2A* null variants [p.(Gln661*) and p.(Leu830Pro*fs**2)] revealed a loss-of-function effect due to strong reduction of surface expression of the GluN2A protein in both mammalian HEK cells and in Xenopus oocytes. These results are consistent with a *GRIN2A* knockout.

Our report is the first case of autosomal recessive *GRIN2A*-related disorder and only the third case where a bi-allelic knockout of a GluN subunit is associated with human disease, with the other cases affecting *GRIN1* c.1666C>T, p.(Gln556*) and c.349-1G>C.[6,7]

In the previously published *GRIN1* families, the knockout phenotype was associated with severe and fatal neonatal epileptic encephalopathy or severe myoclonic epileptic encephalopathy. Similar to our novel *GRIN2A* family, knockout phenotypes were much more severe than the phenotype of the heterozygously affected parents in the *GRIN1* families, who even appeared to be completely healthy.[6,7]

For *GRIN1*, this pattern is perfectly mirrored in rodents, where *Grin1* homozygous knockout mice also suffer neonatal lethality whereas heterozygous mice are healthy.[12,13] However, there is a notable difference, because *GRIN1* knockout mice die from a failure to breathe and suckle milk, not from seizures.

For *GRIN2B*, biallelic null variants have so far not been observed in humans. We speculate that this is due to mainly two reasons. First, individuals with heterozygous *GRIN2B* null variants are so severely affected that so far none is known to have reproduced. Second, human knockout of *GRIN2B* may be pre- or perinatally lethal. In mice, *Grin2b* knockouts die as neonates because they do not suckle.[14]

For *GRIN2A*, the situation appears to be slightly different. *Grin2a* knockout mice have a much milder phenotype than *Grin1* or *Grin2b* knockouts, exhibiting a moderate deficit in spatial learning[15], spontaneous discharges during sleep, and reduced pup vocalization.[16] In humans, this is again potentially mirrored by the fact that the *GRIN2A* knockout phenotype is comprising severe developmental delay and epilepsy but not fatal epileptic encephalopathy as seen in the human *GRIN1* knockout phenotype.

Thus, all above observations and speculations are supported and paralleled by animal models, where murine knockout models of *GRIN1* and *GRIN2B* are lethal[14], whereas *GRIN2A* knockout mice are viable and have the mildest knockout phenotype with a "jumpy" behavior, moderate deficiency in learning, and epilepsy.[15,16]

A recent retrospective observational description of a series of individuals with null variants in either *GRIN2A* or *GRIN2B* revealed beneficial effects of a co-agonistic treatment of the NMDAR with L-serine.[17] It appears conceivable but currently remains unclear whether this treatment will also be beneficial in the context of bi-allelic *GRIN2A* null variants resulting in a knockout of GluN2A as seen in the index case of this study. Independently, we would like to encourage the report of individuals with bi-allelic null variants in any *GRIN* gene to further illuminate the phenotypic spectrum associated with knockouts of any GluN subunit in humans.

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Competing interests

The authors report no competing interests.

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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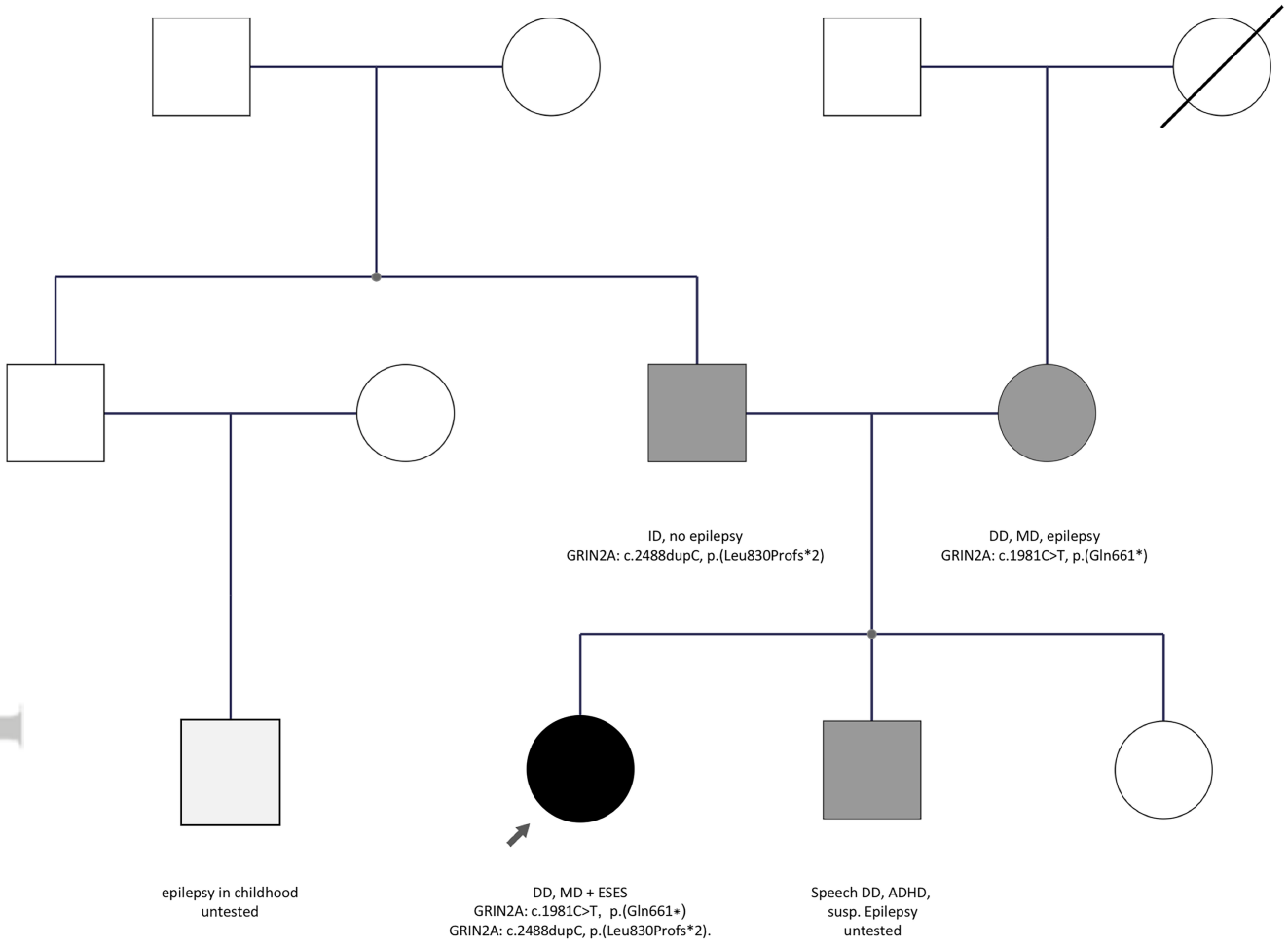

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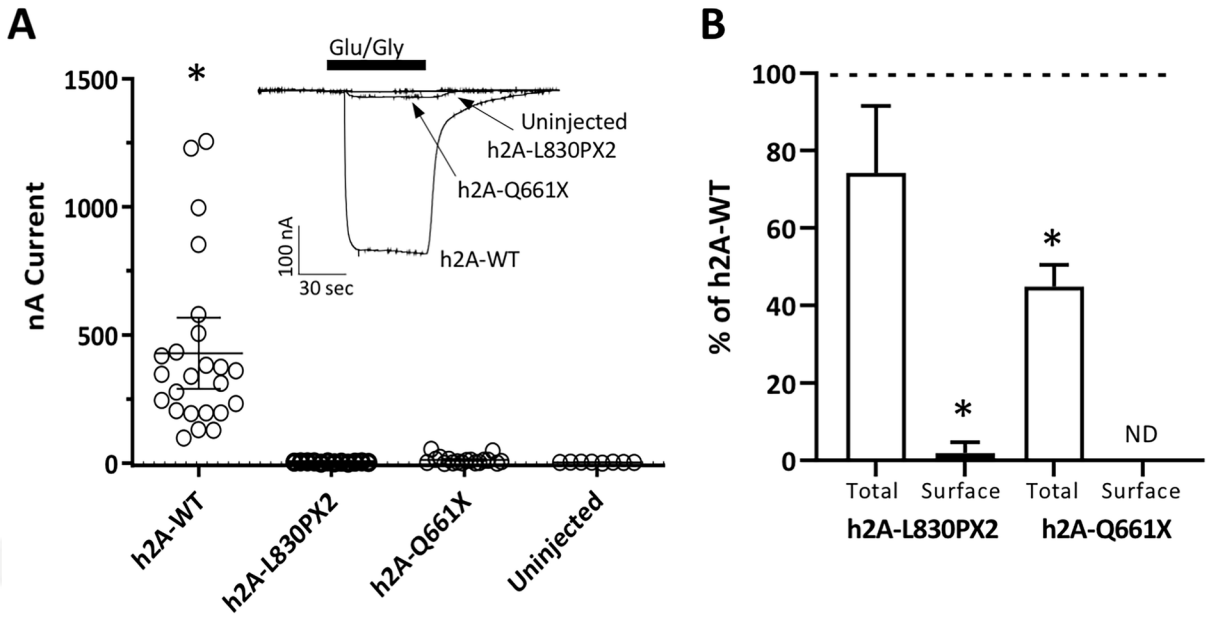

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