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Spectrum of neurodevelopmental disease associated with the GNAO1 GTP-binding region

McKenna Kelly, BS* ^{1, 2}

Meredith Park, BS* ¹

Ivana Mihalek, PhD* ³

Anne Rochtus, MD, PhD ¹

Marie Gramm, BS ⁴

Eduardo Pérez-Palma, PhD ⁴

Erika Takle Axeen, MD ^{1, 5}

Christina Y. Hung, MD, FACMG ³

Heather Olson, MD, MS ^{1, 6, 7}

Lindsay Swanson, MS, CGC ⁸

Irina Anselm, MD ^{7, 8}

Lauren C. Briere, MS, CGC ⁹

Frances A. High, MD, PhD ⁹

David A. Sweetser, MD, PhD ⁹

Undiagnosed Diseases Network (UDN)

Saima Kayani, MD ¹⁰

Molly Snyder, MS, CGC ¹¹

Sophie Calvert, MBBS, FRACP ¹²

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Ingrid E. Scheffer, MBBS, PhD¹³

Edward Yang, MD, PhD¹⁴

Jeff L. Waugh, MD, PhD^{7, 8, 15}

Dennis Lal, PhD^{4, 16, 17}

Olaf Bodamer, MD, PhD, FAAP, FACMG**^{3, 17, 18, 19}

Annapurna Poduri, MD, MPH, FAES**#^{1, 6, 7, 17, 20}

*These authors contributed equally to the manuscript, **These authors contributed equally to the manuscript

1. Epilepsy Genetics Program, Department of Neurology, Boston Children's Hospital, Boston, MA
2. currently Dartmouth Medical School, Hanover, NH
3. Division of Genetics and Genomics, Boston Children's Hospital, Boston, MA
4. Cologne Center for Genomics, Cologne, Germany
5. currently Department of Neurology, University of Virginia, Charlottesville, VA
6. Division of Epilepsy and Clinical Neurophysiology, Boston Children's Hospital, Boston, MA
7. Department of Neurology, Harvard Medical School, Boston, MA
8. Department of Neurology, Boston Children's Hospital, Boston, MA
9. Department of Medical Genetics, Massachusetts General Hospital, Boston, MA
10. Department of Pediatrics, Neurology, and Neurotherapeutics, University of Texas Southwestern Medical Center and Department of Neurology, Children's Health, Dallas, TX
11. Department of Neurology, Children's Health, Dallas, TX
12. Neuroscience Department, The Lady Cilento Children's Hospital, Stanley Street, Brisbane, 4101, QLD, Australia.
13. University of Melbourne, Austin Health and Royal Children's Hospital, Florey and Murdoch Children's Research Institute, Melbourne, Australia
14. Department of Radiology, Boston Children's Hospital and Department of Radiology, Harvard Medical School, Boston, MA

15. Department of Pediatrics, University of Texas Southwestern, Dallas, TX
16. Stanley Center for Psychiatric Research, Broad Institute, Cambridge, MA
17. Broad Institute of MIT and Harvard University, Cambridge, MA
18. Department of Pediatrics, Harvard Medical School, Boston, MA
19. Manton Center for Orphan Disease Research, Boston Children's Hospital, Boston, MA
20. F.M. Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA

#Correspondence addressed to:

Annapurna Poduri, MD, MPH, FAES
Epilepsy Genetics Program
Fegan 9, Boston Children's Hospital
Boston, MA 02115
Tel.: 617-355-8656
Fax: 617-730-0463
E-mail: annapurna.poduri@childrens.harvard.edu

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Summary

Objective: To characterize the phenotypic spectrum associated with *GNAO1* variants and establish genotype-protein structure-phenotype relationships.

Methods: We evaluated the phenotypes of 14 patients with *GNAO1* variants, analyzed their variants for potential pathogenicity, and mapped them, along with those in the literature, on a 3-dimensional structural protein model.

Results: The 14 patients in our cohort, including one sibling pair, had 13 distinct, heterozygous *GNAO1* variants classified as pathogenic or likely pathogenic. We attributed the same variant in two siblings to parental mosaicism. Patients initially presented with seizures beginning in the first three months of life (8/14), developmental delay (4/14), hypotonia (1/14), or movement disorder (1/14). All patients had hypotonia and developmental delay ranging from mild to severe. Nine had epilepsy, and nine had movement disorders, including dystonia, ataxia, chorea, and dyskinesia.

The 13 *GNAO1* variants in our patients are predicted to result in amino acid substitutions or deletions in the *GNAO1* GTP-binding region, analogous to those in previous publications. Patients with variants affecting amino acids 207-221 had only movement disorder and hypotonia. Patients with variants affecting the C-terminal region had the mildest phenotypes.

Significance: *GNAO1* encephalopathy most frequently presents with seizures beginning in the first three months of life. Concurrent movement disorders are also a prominent feature in the spectrum of *GNAO1* encephalopathy. All variants affected the GTP-binding domain of *GNAO1*, highlighting the importance of this region for G-protein signaling and neurodevelopment.

Key Words: *GNAO1*, Developmental and Epileptic Encephalopathy, Movement Disorders, Mosaicism

Key Points

- Pathogenic variants in *GNAO1* result in early onset epilepsy and/or movement disorders, with seizures as the most common presenting feature.

- Pathogenic variants in *GNAO1* lead to alterations in the GTP-binding region of the *GNAO1* protein.
- Genotype-phenotype correlations have emerged, with one region of the protein associated with a movement disorder and hypotonia phenotype without seizures.
- Parental mosaicism can lead to siblings carrying the identical *GNAO1* variant that appeared to be *de novo*.

Introduction

GNAO1, or guanosine nucleotide-binding protein G(o) subunit α , is a gene that has been associated with neurodevelopmental disorders, including early onset developmental and epileptic encephalopathy (DEE¹), developmental delay without epilepsy, and a range of movement disorders.²⁻¹⁶ *GNAO1* encodes a G-protein α subunit that, along with dimerized β and γ subunits, forms a heterotrimeric G-protein complex. G-protein α subunits contain GTP-binding sites and dissociate from both the G-protein-coupled receptor and the β - γ dimer of the complex when activated. Alpha subunits are generally classified as stimulatory (s), inhibitory (i), or other (o). *GNAO1* encodes an “o” type α subunit; beyond GTP binding, it has a less well-established role in signaling.¹⁰ *GNAO1* is highly expressed in the central nervous system and is involved in neuronal excitability and neurotransmission.^{2,10}

Here we report 14 previously unpublished patients with heterozygous, *de novo* *GNAO1* variants classified as pathogenic or likely pathogenic, including one variant shared by two siblings attributable to parental mosaicism. Two key components of our *in silico* variant analyses were the comparison of variants in our cohort with those present in the general population and the variant location on the predicted *GNAO1* protein structure. Both analyses demonstrate the importance of the GTP-binding region of the protein. We expand and add evidence to the previously reported phenotypic spectrum of *GNAO1* encephalopathy with the addition of our

14 patients. In addition we identify a region of the GNAO1 protein that correlates with a movement disorder-dominated phenotype without epilepsy.

Methods

Research was conducted with approval from the Institutional Review Board at Boston Children's Hospital (BCH). Patient ascertainment included the Epilepsy Genetics Program, Genetics and Genomics Clinic, and Movement Disorders Clinic at BCH, the National Institutes of Health Undiagnosed Diseases Network, and the Australian Epilepsy Research Centre. Written informed consent was obtained from the parents or legal guardians of all patients included.

Deep phenotyping was performed, including review of medical records, EEG reports, magnetic resonance imaging (MRI) and imaging reports when available, by our multidisciplinary team consisting of pediatric neurologists, including those with training in epilepsy and movement disorders, geneticists, a neuroradiologist, and a genetic counselor.

All 14 individuals had *GNAO1* variants identified through clinical testing, either by whole exome sequencing (WES) (Patients 1-6,8,9,13 and 14) or by targeted next-generation sequencing (NGS) epilepsy panels (Patients 7 and 10-12). Parental testing was completed for all but one patient. In the case of two siblings with the same variant, each reported as *de novo*, we reanalyzed parental WES data for evidence of parental mosaicism using the BCH Connect analysis platform. We conducted a thorough review of the literature (<https://www.ncbi.nlm.nih.gov/pubmed/?term=gnao1>, accessed October 2017) and ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/?term=GNAO1>, accessed March 2018) to identify additional cases for phenotypic comparison, to evaluate whether a given variant was previously reported in the literature or in ClinVar, and to evaluate for potential pathogenicity. Additionally, *in silico* predictions regarding pathogenicity were assessed using a combination of predictors, including the Alamut software suite (<http://www.interactive-biosoftware.com/alamut-visual/>) and Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>). We compared the presence and location of variants in patients, including those in the literature and ClinVar, to those in the

population databases ExAC (www.exac.broadinstitute.org) and gnomAD (<http://gnomad.broadinstitute.org/>).¹⁷ Variant classifications were determined based on the guidelines of the American College of Medical Genetics and Genomics (ACMGG).¹⁸

Using the *GNAO1* protein sequence (canonical transcript ENST00000262494, CCDS10757, NM_020988.2), we analyzed the position of the 13 variants in this report along with 41 additional variants identified through our literature search (Supplemental Table 1), totaling 54 disease-associated variants. For the following analyses, we excluded the intronic variant c.723+1G>A since it cannot be annotated into the protein sequence.¹⁵ To assess variant pathogenicity, we evaluated the missense variant tolerance ratio (MTR) based on regional depletion in the general population.¹⁹ The MTR is a statistically normalized measure of how often a coding region “tolerates” missense variation based on the variant burden observed in gnomAD (n=138,632). We then evaluated the amino acid gene-family paralog conservation (Parazscore, <https://doi.org/10.1101/159780>) to assess for enrichment of patient-related variants compared to gnomAD variants in conserved regions. The Parazscore leverages amino acid conservation across gene-family members assuming that conserved sites are more likely to be important for protein function and thus more likely to be present in patients than controls. Statistical comparison between the variant counts of patients vs. gnomAD was conducted using a two-sided Fisher’s exact test with nominal two-sided p-values <0.05 considered significant.

We assessed genotype-phenotype correlations by comparing the locations of variants and the phenotypic features associated with each variant, with a focus on epilepsy and movement disorders. A model of the *GNAO1* protein structure was generated on a template from the Protein Data Bank (PDB)²⁰ identifier 3C7K,²¹ using SWISS-MODEL²² and mapped on complex structures using deconStruct.²³ Illustrations were generated using PyMol.²⁴

Data Availability

Anonymized data will be made available by request from any qualified investigator.

Results

Genetic variants in *GNAO1*

We identified 13 unique heterozygous *GNAO1* variants in 14 patients (Table 1, Figure 1).

Variants were reported as *de novo* in 13 patients after parental testing; one did not have parental testing performed. Parental mosaicism was suggested in one family by the same *GNAO1* G40E variant present in two siblings, Patients 3 and 4.

Five variants previously reported in the literature, in other patients, are present in Patient 1 (p.G40R)^{4,15}, Patient 6 (p.R209C)¹², Patient 7 (p.R209H)^{6,7,9,11}, Patient 9 (p.Y231C)⁴, and Patient 11 (p.I279N)². Variants in our cohort affecting the previously implicated *GNAO1* G40 amino acid site^{4,15} are present in Patient 2 (p.G40W) and Patients 3 and 4 (p.G40E). We identified six novel variants localized to previously unreported sites in the protein: c.620C>A (p.S207Y), c.662C>A (p.A221D), c.818A>T (p.D273V), c.871T>A (p.Y291N), c.1030_1032delATT(p.I344del), and c.1046_1055del10ins10 (p.R349_G352delinsQGCA). All *GNAO1* variants are annotated based on transcript NM_020988.2.

Assessment of pathogenicity of GNAO1 variants

Ten of the 13 unique variants have been reported in ClinVar, adding support for their pathogenicity (Table 1): five were classified as pathogenic, one with conflicting classification as pathogenic vs. likely pathogenic, two as likely pathogenic, and two as pathogenic vs. uncertain significance (Table 1). The two variants with conflicting classifications as pathogenic or uncertain significance (Patient 1 with p.G40R^{4,15} and Patient 7 with p.R209H^{6,7,9,11}), were *de novo* variants resulting in different amino acid substitutions at residues previously reported as pathogenic in multiple patients; thus, we classified them as pathogenic in accordance with ACMGG variant classification criteria.¹⁸ All variants were additionally evaluated for pathogenicity using Alamut. The three variants not listed in ClinVar were classified as pathogenic based on *in silico* predictions and in accordance with ACMGG criteria (Table 1).¹⁸

Siblings with apparently de novo GNAO1 variants due to parental mosaicism

Patients 3 and 4 were a 20-year-old brother and a 15-year-old sister. Within two hours of life, Patient 3 presented with episodes of apnea and lip smacking. Subsequently, at two months he

developed infantile spasms and hypsarrhythmia as well as focal motor seizures with left eye twitching, left head turning, and left arm extension. His sister also presented with seizures, initially described as bilateral tonic-clonic, on her first day of life. She eventually developed multiple seizure types including focal motor seizures, described as left eye twitching, tonic seizures and infantile spasms. Additional phenotypic features are summarized in Table 2. Family history was notable for febrile seizures in the father, which we consider likely unrelated to the siblings' presentation given their *GNAO1* findings. Extensive genetic and metabolic testing for both siblings was initially non-diagnostic. Initially, a mitochondrial disorder was suspected based on muscle biopsy in Patient 4 suggesting a defect in complex IV of the respiratory chain that was later considered to be a secondary finding. Genetic evaluation included a normal karyotype, chromosomal microarray (CMA), and infantile epilepsy gene panel in 2015. WES performed for both siblings and their parents showed a pathogenic variant in *GNAO1* at c.119G>A (p.G40E) that appeared to be *de novo* in each sibling, leading to the presumption of either germline or gonadal parental mosaicism. Re-evaluation of parental WES data revealed the variant allele (c.119G>A) in one parent in 3 of 150 reads (2%) consistent with germline mosaicism. This level of mosaicism is, however, below the current reporting threshold of CLIA-certified laboratories.

Analysis of *GNAO1* variants in patients compared to controls

Based on the linear amino acid sequence of *GNAO1* (canonical transcript ENST00000262494, CCDS10757, NM_020988.2), we compared the position of the exonic variants in 54 patients with the variants identified in the gnomAD reference database.¹⁷ Collectively, our 14 patients plus 40 from the literature comprise 30 unique variants affecting 23 different amino acids positions (Figure 2), while gnomAD variants affect 95 different amino acids. To further assess variant pathogenicity, we implemented MTR scores and Parazscores for variant interpretation. As expected, both MTR scores (p-value 4.407e-04) and Parazscores (p-value 1.756e-5) from patient variants were significantly lower than those observed in gnomAD controls, indicating population and evolutionary constraint of patient variants, respectively (Figure 2). Further, within the amino acid boundaries of the GTP-binding domain, we observed high intolerance for

missense mutations (i.e., lower MTR scores) and strong conservation across family members (i.e., greater Parazscores). Taken together, these predictions strongly support the pathogenicity of *GNAO1* variants in our cohort, and for patient variants found within the GTP-binding domain.

Phenotypes associated with *GNAO1* variants

Our 14 patients ranged in age from 2-20 years; five were male, and nine were female (Table 2). 9/14 (64%) had epilepsy, 9/14 (64%) had movement abnormalities, and 5/14 (36%) had both epilepsy and movement abnormalities. A diagnosis of epilepsy was reported in nine patients. Eight patients had early onset DEE, with seizure onset under three months, epileptiform activity on EEG, and developmental stagnation or regression (Patients 1-4 and 9-12). The EEGs of all nine patients with epilepsy showed epileptiform activity, including hypsarrhythmia in two patients (Table 2). Patient 13 had a single afebrile seizure at four years of age and a normal EEG. Of the four patients without a history of epilepsy or seizure, three had an EEG: one was normal, one showed background slowing, and one was initially normal but later showed frequent focal spikes during sleep (Table 2).

Hypotonia was reported in all patients. Movement disorders were present in nine patients; five had severe dystonia (Patients 4, 5, 7, 8, 13), two had ataxia (Patients 1, 7), two had dyskinesia (Patients 10, 14), two had chorea (Patients 11, 13), one had akathisia (Patient 11) and one had tremors (Patient 14). Patient 14 had intermittent facial dyskinesia, swallowing dysfunction and dysarthria, which has been reported in four patients with a severe phenotype.

Occipito-frontal circumference (OFC) data were available for 13 of 14 patients. One patient had microcephaly, Patient 3 with OFC 41cm at 10 months (2.8%ile). Brain MR images were reviewed for 10 patients; MRI reports only were available for the remaining patients. The MRIs were either normal or had nonspecific abnormalities, including diffuse atrophy (six patients) and abnormal myelination (two patients). One MRI showed globus pallidus / dentate signal increase, possibly related to vigabatrin use, and one MRI showed signs of mesial temporal sclerosis (Table 2).

All patients had developmental delay and showed a broad spectrum of severity. Five patients showed profound impairment and were nonverbal and non-ambulatory. Patients 5, 13 and 14 had the mildest developmental abnormalities. Patient 5 presented with delayed speech and motor development but was reported to have met motor and language milestones and to have normal cognition at 10 years. Patient 13 presented with language delay, speaking single words at 2.5 years, and full sentences at four years, and mild intellectual disability. Patient 14 presented with oromotor apraxia and moderate intellectual disability. At 11 years, she could recognize colors, some words and numbers, and understand early math concepts but is making very slow academic progress.

We compared the phenotypes of our 14 patients with those of 41 individuals with disease-associated *GNAO1* variants in the literature^{2-16,25} (Supplemental Table 1). Striking phenotypic patterns emerged, despite incomplete phenotype information in some patients.

Epilepsy and movement disorders are common phenotypic features. Forty-two of 55 individuals (76%) had movement disorders, 35/55 (64%) had epilepsy, and 23/55 (42%) had both. The range of movement disorders includes chorea, dystonia, dyskinesia, stereotypies, and ataxia. The 35 individuals with epilepsy most often had DEE (24/35, 69%), including infantile spasms with hypsarrhythmia; seven individuals had focal seizures only, two had generalized seizures only, one had both generalized and focal seizures, one had infantile spasms only, and three reports did not contain enough information as to the type of epilepsy to allow further classification.

All patients had developmental delay in our cohort and the cases in the literature, with a wide range of severity, including our patients with mild delay. Hypotonia was commonly reported (76%) and microcephaly was reported in 20% of those cases for which data were available. MRI was reported to be normal in 20/55 individuals; 16 showed atrophy (both generalized and focal), 12 had corpus callosum abnormalities (most often a thin corpus callosum), seven showed delayed myelination, three had mild hypoplasia of the caudate nuclei, two had bilateral globus pallidus abnormalities (hypointense signal in a case with p.E246K⁶ and hyperintense in

Patient 4 with p.G40E), and one had a left frontal astrocytoma thought to be unrelated to the *GNAO1* variant.¹⁵

Relationship of phenotype with amino acid position and protein domain

Given the high sequence similarity of *GNAO1* to crystallized forms of Gα proteins, its structure is predicted to consist of two domains: one helical and one catalytic with a GTPase-binding pocket. Mapping the disease-associated variants reported to date onto this model structure demonstrates that disease-associated variants are located preferentially in the catalytic domain. This is even more intriguing when taking into account that the two globular domains of *GNAO1* are formed by non-consecutive regions of the protein sequence (Figure 1). In contrast, variants present in the general, unaffected population can be found in both the helical and catalytic domains (Figure 1B, inset).

Evaluating the disease-associated variants on the predicted protein structure, the variants associated with movement disorders exclusively are found outside the catalytic pocket (Supplemental Figure S2), between AA207 and AA221. Reviewing our patients and those in the literature, most patients with variants affecting AA207-AA246 present with movement disorder but not with epilepsy, with the exception of 2 patients with *GNAO1* p.Y231C (Patient 9, this report) and p.A227V.¹² Conversely, any variant in or near the catalytic pocket resulted in epilepsy, either alone or in association with movement disorders.

The most commonly affected amino acids R209 and G40 are both located in the catalytic domain. Examining the phenotypes associated with variants at R209 (p.R209C¹² in Patient 6 and p.R209H^{6,7,9,11} in Patient 7), only 3/10 individuals (30%) were reported to have seizures, one generalized tonic-clonic and two focal. By comparison, variants at G40 produce a far more severe phenotype (p.G40R^{4,15} in Patient 1, p.G40W in Patient 2, and p.G40E in Patients 3 and 4). The G40 site has been implicated in seven patients with epilepsy, including six with DEE. Further, three of these seven individuals are reported to have both movement disorder and epilepsy classified as DEE.^{4,15,25} Six of these seven also had developmental delay and/or intellectual disability, hypotonia, and abnormal findings on MRI.

Discussion

GNAO1 encephalopathy has a broad phenotypic spectrum, most commonly presenting with seizures and less frequently with movement disorders. Developmental delay and hypotonia occur in nearly all cases, although they led less frequently to medical attention. The severe phenotype, occurring in 44% of cases, comprises an early onset profound DEE, with seizure onset soon after birth in 24/55 cases.^{2-5,8,10,12,14-16,26}

MRI studies have not shown major structural abnormalities in patients with *GNAO1* variants. Progressive diffuse atrophy and delayed myelination are noted in some cases with thinning of the corpus callosum.^{2,5,6,8,10,12,14-16} For patients with *GNAO1*-related movement disorders, treatments such as deep brain stimulation have been undertaken^{9,13,15} and require neuroimaging. Therefore, longitudinal assessment of the MRI features of patients with movement disorders will likely be available in the future.

Previous reports mention a slight female predominance in patients with *GNAO1*-related neurodevelopmental disorders¹⁰ and an apparent excess of sibling pairs identified with apparently *de novo* *GNAO1* pathogenic variants.^{9,11} In our cohort, 9/14 (64%) patients are female, and of the 40 cases in the literature identified as male or female, 24 (60%) were female; as more cases continue to accrue, the sex ratio can be more accurately determined. Together with our series, there are now three affected sibling pairs as well as an individual whose brother had previously passed away with the same phenotype.^{9,11} These variants appeared to occur *de novo* based on 'negative' parental testing. However, their recurrence in siblings leads to the hypothesis of parental mosaicism, either in the germline with low allele frequency undetectable by standard clinical sequencing or restricted to the gametes. In our case, reanalysis of the clinical sequencing data identified parental mosaicism in 2% (3/150) WES reads. The phenomenon of parental mosaicism has been observed in association with other disease-associated genes, including those associated with neurodevelopmental disorders.²⁷ At present we do not have evidence to conclude that *GNAO1* is more likely to be associated with parental mosaicism than other genes.²⁸ However, the possibility of parental mosaicism needs to

be taken into account when providing genetic counseling to families who have one child with a *de novo* variant in *GNAO1*. A subsequent child must be considered and, if further children are planned, testing with high depth coverage in the parents offers an opportunity to assess this risk.

GNAO1 encodes a G-protein α subunit highly expressed in the brain. Heterozygous, *de novo* variants are thought to cause a gain of function, as demonstrated in the *Gnao1* +/G184S mutant mouse model.²⁹ However, recent *in vitro* studies suggest that the functional consequences of *GNAO1* variants depend on their location within the gene.³⁰ The G-protein α subunit consists of two sections of a P-loop structure containing a GTP-binding domain. The amino acids between these domains create a helical insertion domain that isolates and stabilizes the guanine nucleotide upon GTP binding and must be displaced for GTP/GDP dissociation to occur. It is in this critical region that the majority of patient variants are located, both in our patients and in the literature.²

We observed interesting correlations between phenotype and genotype across all reported cases according to amino acid position and protein structure. Not surprisingly, the picture was dominated by pathogenic or likely pathogenic variants affecting the GTP-binding regions of the protein in all but one of the published variants, with the only variant identified in the helical insertion domain located at AA174,³¹ a site adjacent to the start of the GTP-binding protein domain. The localization of patient variants in the GTP-binding domain is consistent with our current understanding of the $G\alpha$ function: both the catalytic pocket and interactions with its protein partners are affected through this domain (Supplemental Figure S1). It is possible that the role of the helical domain is simply to function as the 'lid' in the active conformation of the protein, requiring a conserved amino acid sequence. A dysfunctional *GNAO1* catalytic pocket may lead to extended GTP hydrolysis time and consequentially the failure of dissociation between $G\alpha$ and the $G\beta\text{-}\gamma$ dimer. This in turn, through the failure of ion channel regulation exerted by the $G\beta\text{-}\gamma$ dimer, may lead to neuronal hyperexcitability and an epilepsy phenotype; this hypothesis will require further functional testing, as pioneered by Feng et al.³⁰

Many of the shared features identified in the current *GNAO1* patient population are not specific to any single variant or amino acid position. Epilepsy was seen across variants affecting amino acids throughout the protein, and different types of epilepsy seem to be equally distributed across the gene. Movement disorders were associated with amino acid positions across the gene as well, particularly associated with the AA207-246 region, which may act as a 'hotspot' for movement disorder phenotypes typically, though not always, without epilepsy. In addition, it is worth noting that patients with variants affecting *GNAO1* AA40 had particularly severe phenotypic presentations including all features.

As the case number for *GNAO1*-associated neurodevelopmental disorders grows, expansion of the clinical phenotypic spectrum continues to emerge. When *GNAO1* variants are identified in individuals, it is most often by WES rather than by targeted testing. This likely reflects the fact that there is a lag time between gene discovery and inclusion of a given new gene on targeted multi-gene panels as well as a lag in clinician awareness for the gene. Though the number of individuals undergoing WES is increasing, we would support clinical testing laboratories to add *GNAO1* to their panels for early onset epilepsy, DEE, and movement disorders. Earlier genetic identification of a variant associated with DEE might change management, in that epilepsies arising from variants in signal transduction genes are generally not treated by focal brain resection (as had been pursued in one of our patients). Similarly, recognition of a *GNAO1* variant in a patient with a mixed movement disorder should prompt evaluation for therapy with deep brain stimulation, as it has been shown to be effective in a number of cases.^{9,13} Patients with *GNAO1* variants have often been mistakenly diagnosed with secondary movement disorders, which derive much lower benefit from deep brain stimulation,³² in contrast to a *GNAO1*-related disorder.

Conclusions

The phenotypic spectrum of *GNAO1*-related neurodevelopmental disease includes epilepsy and a range of movement disorders, often with epilepsy as the presenting feature. A range of movement disorders is seen in the majority of patients, even if not the presenting symptoms.

Hypotonia and developmental delay were present in all patients in our series with a wide range of severity.

All patients had variants in the GTP-binding region of *GNAO1*, highlighting the importance of this region for normal neurodevelopment. We identified a small cluster of variants apparently associated only with movement disorder but not epilepsy. The presence of two siblings with the same pathogenic variant, with parents who initially appear to lack that variant, highlights the importance of considering parental mosaicism when counseling families.

In addition to expanding and refining the phenotypic spectrum of *GNAO1*-related neurodevelopmental disease in a cohort rigorously analyzed for variant pathogenicity and phenotypic features, we highlight more generally the importance of considering protein structure-phenotype correlations, which may help determine disease prognosis in children with early-onset neurodevelopmental diagnoses. Our findings highlight the importance of pursuing a genetic etiology in patients with a wide range of presenting symptoms, including patients who were previously diagnosed with acquired etiologies for their multi-symptom neurodevelopmental conditions. Particularly when the course of treatment may be altered by the presence of a pathogenic variant in *GNAO1*—currently DBS for movement disorder, but perhaps one day a more targeted treatment addressing the epilepsy and other features of the disorder—early genetic diagnosis should be pursued.

Epilepsia is a member of the Committee on Publication Ethics (COPE), and we adhere to its principles (<http://publicationethics.org/>).

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Figure Legend

Figure 1. Patient variants in *GNAO1* affect the GTP-binding region.

Disease-associated amino acid residues in the *GNAO1* protein localize to the catalytic domain. (A) The protein is shown in linear representation, colored using a 'rainbow' scheme, starting with blue at the N-terminal, and ending with red at the C-terminal. The amino acid substitutions altered by the *GNAO1* variants in our series are depicted here, with colors depicting the neurological features present in each case. (B) The positions of disease-associated variants, from this series and the literature, are shown as spheres on the model of the *GNAO1* structure. The *GNAO1* substrate is also shown as spheres, colored magenta. The same coloring scheme is used in A and B. Patient variants cluster in the region of the catalytic domain (right rectangle), which starts at the very N-terminal aspect of the sequence, weaves into the helical domain, and returns back to complete the catalytic domain structure. The long helix on the N-terminal is not shown, since its position in the active conformation of $G\alpha$ is uncertain, and it carries no reported disease-associated amino acid positions. Inset: the distribution of missense variant amino acids in ExAC¹⁷ covers both structural domains of the protein and does not overlap with the disease-associated amino acid changes in our series.

Figure 2. *GNAO1* patient variant evolutionary conservation and population constrained

assessment. A) *GNAO1* patient variants' paralog conservation score (Parazscore) across the linear protein sequence: Comparing the amino acid sequence of the *GNAO1* protein to that of paralogous proteins in its gene family, the gene family-wise paralog conservation is shown for each amino acid of *GNAO1* protein sequence. Parazscore values range from negative values,

representing less conservation at a given amino acid position, to positive values, representing high conservation, with the highest value depicting identical amino acids are present in all related proteins. B) GNAO1 patient variants' missense tolerant ratio (MTR).¹⁹ The score visualizes the tolerance to missense across the GNAO1 protein sequence based on depletion of variants in population controls from the gnomAD database (ExAC v2).¹⁷ MTR values range from 0 (extremely intolerant to missense variant amino acids) to 1.5 (for positions tolerant to missense variant amino acids). For both graphs, patient variant-related amino acid substitutions not previously reported in the literature (white boxes, n= 9) and patient variant-related amino acid substitutions previously reported in the literature (gray boxes, n=5) are labeled alongside amino acids altered by *GNAO1* variants in the literature (black asterisk, n=40). Altogether, 23 amino acid positions are affected. Amino acids belonging to the GTP-binding domain are marked in blue and represent the vast majority of amino acids affected in patients. Intronic variant c.723+1G>A was not included.¹⁵ All patient variants fall within the boundaries of the GTP-binding domain (blue bars).

Supplemental Figure Legend

Figure S1: Interacting partners of GNAO1 form no interface with its helical domain. Previously published disease-associated variants are shown as white spheres, and the variants reported for the first time in this report are shown in red. Pink indicates the location of the catalytic pocket. GNAO1's interacting partners are shown in surface representation. A: Gβ-γ, modeled after Tesmer et al., 1997,³³ B: GPCR, after Rasmussen et al., 2011,³⁴ C: RGS, regulator of G-protein signaling,²¹ and D: catalytic domains of K⁺ channel.³³ Notably, the helical domain does not participate in any known interactions.

Figure S2: Movement-disorder related variants are located further away from the catalytic pocket than those associated with epilepsy- or combined phenotypes. The backbone of the protein is shown in white, and disease-associated variants and the substrate (pink) are shown as spheres. The color scheme for the disease-associated variants is as follows: blue—epilepsy, red—movement disorder, green—both.

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	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14
Gender	Male	Female	Male	Female	Male	Female	Female	Female	Male	Female	Male	Female	Female	Female
Age (years)	8	2	20	15	10	2	2	15	4	6	2	5	13	10
Variant	c.118G>C (p.G40R)	c.118G>T (p.G40W)	c.119G>A (p.G40E)	c.119G>A (p.G40E)	c.620C>A (p.S207Y)	c.625C>T (p.R209C)	c.626G>A (p.R209H)	c.662C>A (p.A221D)	c.692A>G (p.Y231C)	c.818A>T (D273V)	c.836T>A (p.I279N)	c.871T>A (p.Y291N)	c.1030_1032delATT (p.I344del)	c.1046_1055del10ins10 (p.R349_G352delinsQGCA)
Conservation	High	High	High	High	High	High	High	High	High	High	High	High	N/A	N/A
GVGD	C15	C15	C0	C0	C65	C65	C25	C65	C65	C15	C0	C0	N/A	N/A
SIFT	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	Deleterious	N/A (In-frame deletion)	N/A
Mutation Taster	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	Disease causing	N/A	N/A
Polyphen-2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.989	0.999	0.993	1.0	0.997	N/A	N/A
ClinVar (number of cases)	Pathogenic (1); Uncertain significance (1)	Pathogenic (1)	Pathogenic (1)	Pathogenic (1)	Pathogenic (1)	Pathogenic/Likely pathogenic (3)	Pathogenic (2); Uncertain significance (1)	N/A	Likely pathogenic (1)	N/A	Pathogenic (1)	Likely pathogenic (1)	N/A	N/A
ExAC AF	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GnomAD AF	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 1. Pathogenicity predictions of *de novo* variants in *GNAO1*

AF = allele frequency; *GNAO1* variants were annotated based on transcript NM_020988.2.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14
Age (yr)/Sex	8/M	2/F	20/M	15/F	10/M	2/F	2/F	15/F	4/M	6/F	2/M	5/F	13/F	10/F
Variant	c.118G>C (p.G40R)	c.118G>T (p.G40W)	c.119G>A (p.G40E)	c.119G>A (p.G40E)	c.620C>A (p.S207Y)	c.625C>T (p.R209C)	c.626 G>A (p.R209H)	c.662C>A (p.A221D)	c.692A>G (p.Y231C)	c.818A>T (D273V)	c.836T>A (p.I279N)	c.871T>A (p.Y291N)	c.1030_1032delA TT(p.I344del)	c.1046_1055del1 0ins10 (p.R349_G352del insQGCA)
Novel/Reported	Reported ^{4,15}	Novel	Novel	Novel	Novel	Reported ¹²	Reported ^{6,7,9,11}	Novel	Reported ⁵	Novel	Reported ²	Novel	Novel	Novel
Initial Symptom (age)	Seizures (2.5 mo)	Seizures (5 wk)	Seizures (15 hrs)	Seizures (2 hrs)	Developmental delay (infancy)	Hypotonia (6 mo)	Developmental delay (6 mo)	Developmental delay (9 mo)	Seizure (5 d)	Seizures (2 d)	Seizures (1 hr)	Seizures (2 mo)	Movement disorder (12 mo)	Developmental delay (6 mo)
Epilepsy Type	DEE → LGS	DEE	DEE	DEE	N/A	N/A	N/A	N/A	DEE	DEE	DEE	DEE	N/A	Focal Seizure
Onset	Spasms	Focal Seizure	Spasms	GTC	N/A	N/A	N/A	N/A	Myoclonic Seizure	GTC	Spasms	Focal Seizure	N/A	Focal Seizure
Other sz types	Tonic, Atonic, Myoclonic Seizures, GTC, Spasms	N/A	Myoclonic Seizures, Tonic, GTC, Spasms	Spasms	N/A	N/A	N/A	N/A	Focal Seizure, Myoclonic Seizures	N/A	Focal Seizure	N/A	N/A	
Movement disorder	Ataxia	N/A	N/A	Left arm dystonia	Dystonia	N/A	Dystonia, ataxia	Dystonia	N/A	Dyskinesia	Chorea, akathisia	N/A	Dystonia, chorea	Facial dyskinesia, tremor
Cognition	ID	ID	ID	ID	Normal	ID	MID	MID	ID	ID	ID	ID	MID	ID
Motor	Nonambulatory	Delay	Nonambulatory	Nonambulatory	Delay	Delay	Delay	Delay	Delay	Nonambulatory	Delay	Nonambulatory	Delay	Oromotor apraxia
Speech	Nonverbal	Nonverbal	Nonverbal	Nonverbal	Delay	Delay	Nonverbal	Dysarthria	Delay	Nonverbal	Delay	Nonverbal	Delay	Delay, dysarthria
Tone	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia	Hypotonia
EEG	3yr: slow spike and wave, multifocal spikes	9mo: right temporal seizures, focal spikes, focal slowing	2mo: hypsarrhythmia; 14yr: generalized onset of tonic seizures and epileptic spasms, generalized slowing	9d: multifocal spikes; 14yr: focal spikes and waves, absence of normal awake and sleep features	N/A	15mo: slow posterior dominant rhythm	13mo: normal	11yr: normal 15yr: abnormal during sleep, frequent sharp waves	Neonatal: multifocal epileptiform sharp waves; 20mo: frequent bioccipital spikes	Abnormal epileptiform activity	Neonatal: multifocal sharp waves with high amplitude bursts (not burst suppression); 8mo: modified hypsarrhythmia	1yr: multifocal spikes, focal seizures; 3yr: intermittent posterior slowing	9yr: Normal	7yr: sleep- activated posterior temporal and occipital spikes
MRI	4mo: mildly prominent bifrontal subarachnoid spaces	8mo: bilateral mesial temporal sclerosis, diffuse parenchymal atrophy, delayed	2yr: status post temporal lobectomy, left cerebral atrophy	15mo: nonspecific signal increase in globi pallidi, normal myelination	9yr: Normal	1yr: generalized thinning of corpus callosum, relative paucity of deep white	13mo: frontal lobe volume loss	15yr: Normal	2yr: prominent subarachnoid spaces	Normal	2.5yr: moderate to progressive atrophy with delayed myelination	2.5yr: Normal	8yr: Normal	9yr: Normal

myelination					matter									
Other features	swallowing	gastroesophageal	swallowing	astigmatism,	N/A	N/A	N/A	neonatal feeding	spastic	swallowing	intermittent	hyperopia,	single seizure at	swallowing
	dysfunction,	reflux	dysfunction,	myopia,				difficulties	quadriplegia	dysfunction	exotropia	esotropia	age 4yr	dysfunction
	chronic		cortical visual	alternating exotr										
	respiratory		impairment,	opia, scoliosis										
	dysfunction		scoliosis											

Table 2. Genotype and phenotype of 14 patients with disease-associated variants in *GNAO1*.

All variants are confirmed *de novo* except Patient 10 D273V, for whom parental testing has not been possible. Legend: A = atonic; DEE = developmental and epileptic encephalopathy; F = female; Focal Seizure = focal seizures; GTC = generalized tonic-clonic; ID = intellectual disability; LGS = Lennox-Gastaut syndrome; M = male; MID = mild intellectual disability; Myoclonic Seizure = myoclonic seizures; S = spasms

A)

- Epilepsy
- Intellectual or developmental delay
- Epilepsy + movement disorder
- Movement disorder

B)

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Author/s:

Kelly, M;Park, M;Mihalek, I;Rochtus, A;Gramm, M;Perez-Palma, E;Axeen, ET;Hung, CY;Olson, H;Swanson, L;Anselm, I;Briere, LC;High, FA;Sweetser, DA;Kayani, S;Snyder, M;Calvert, S;Scheffer, IE;Yang, E;Waugh, JL;Lal, D;Bodamer, O;Poduri, A;Adams, DR;Aday, A;Alejandro, ME;Allard, P;Ashley, EA;Azamian, MS;Bacino, CA;Baker, E;Balasubramanyam, A;Barseghyan, H;Batzli, GF;Beggs, AH;Behnam, B;Bellen, HJ;Bernstein, JA;Bican, A;Bick, DP;Birch, CL;Bonner, D;Boone, BE;Bostwick, BL;Brokamp, E;Brown, DM;Brush, M;Burke, EA;Burrage, LC;Butte, MJ;Chen, S;Clark, GD;Coakley, TR;Cogan, JD;Colley, HA;Cooper, CM;Cope, H;Craigien, WJ;D'Souza, P;Davids, M;Davidson, JM;Dayal, JG;Dell'Angelica, EC;Dhar, SU;Dipple, KM;Donnell-Fink, LA;Dorrani, N;Dorset, DC;Douine, ED;Draper, DD;Dries, AM;Eckstein, DJ;Emrick, LT;Eng, CM;Enns, G-GM;Eskin, A;Esteves, C;Estwick, T;Fairbrother, L;Fernandez, L;Ferreira, C;Fieg, EL;Fisher, PG;Fogel, BL;Friedman, ND;Gahl, WA;Glanton, E;Godfrey, RA;Goldman, AM;Goldstein, DB;Gould, SE;Gourdine, J-PF;Grodin, CA;Gropman, AL;Haendel, M;Hamid, R;Hanchard, NA;High, F;Holm, IA;Horn, J;Howerton, EM;Huang, Y;Jamal, F;Jiang, Y-H;Johnston, JM;Jones, AL;Karaviti, L;Koeller, DM;Kohane, IS;Kohler, JN;Konick, S;Koziura, M;Krasnewich, DM;Krier, JB;Kyle, JE;Lalani, SR;Lau, CC;Lazar, J;LeBlanc, K;Lee, BH;Lee, H;Levy, SE;Lewis, RA;Lincoln, SA;Loo, SK;Loscalzo, J;Maas, RL;Macnamara, EF;MacRae, CA;Maduro, VV;Majch-erska, MM;Malicdan, MC;Mamounas, LA;Manolio, TA;Markello, TC;Marom, R;Martin, MG;Martinez-Agosto, JA;Mar-waha, S;May, T;McConkie-Rosell, A;McCormack, CE;McCray, AF;Merker, JD;Metz, TO;Might, M;Moretti, PM;Morimoto, M;Mulvihill, JJ;Murdock, DR;Murphy, JL;Muzny, DM;Nehrebecky, ME;Nelson, SF;Newberry, JS;Newman, JH;Nicholas, SK;Novacic, D;Orange, JS;Orengo, JP;Pallais, JC;Palmer, CGS;Papp, JC;Parker, NH;Pena, LDM;Phillips, JA;Posey, JE;Postlethwait, JH;Potocki, L;Pusey, BN;Reuter, CM;Rives, L;Robertson, AK;Rodan, LH;Rosenfeld, JA;Sampson, JB;Samson, SL;Schoch, K;Scott, DA;Shakachite, L;Sharma, P;Shashi, V;Signer, R;Silverman, EK;Sinsheimer, JS;Smith, KS;Spillmann, RC;Stoler, JM;Stong, N;Sullivan, JA;Tan, QK-G;Tifft, CJ;Toro, C;Tran, AA;Urv, TK;Vilain, E;Vogel, TP;Waggott, DM;Wahl, CE;Walker, M;Walley, NM;Walsh, CA;Wan, J;Wangler, MF;Ward, PA;Waters, KM;Webb-Robertson, B-JM;Westerfield, M;Wheeler, MT;Wise, AL;Wolfe, LA;Worthey, EA;Yamamoto, S;Yang, Y;Yoon, AJ;Yu, G;Zastrow, DB;Zhao, C;Zheng, A

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