Severity of *GNAO1*-Related Disorder Correlates with Changes in G-Protein Function

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Objective: *GNAO1*-related disorders (OMIM #615473 and #617493), caused by variants in the *GNAO1* gene, are characterized by developmental delay or intellectual disability, hypotonia, movement disorders, and epilepsy. Neither a genotype–phenotype correlation nor a clear severity score have been established for this disorder. The objective of this prospective and retrospective observational study was to develop a severity score for *GNAO1*-related disorders, and to delineate the correlation between the underlying molecular mechanisms and clinical severity.

Methods: A total of 16 individuals with *GNAO1*-related disorders harboring 12 distinct missense variants, including four novel variants (p.K46R, p.T48I, p.R209P, and p.L235P), were examined with repeated clinical assessments, video-electroencephalogram monitoring, and brain magnetic resonance imaging. The molecular pathology of each variant was delineated using a molecular deconvoluting platform.

Results: The patients displayed a wide variability in the severity of their symptoms. This heterogeneity was well represented in the *GNAO1*-related disorders severity score, with a broad range of results. Patients with the same variant had comparable severity scores, indicating that differences in disease profiles are not due to interpatient variability, but rather, to unique disease mechanisms. Moreover, we found a significant correlation between clinical severity scores and molecular mechanisms.

Interpretation: The clinical score proposed here provides further insight into the correlation between pathophysiology and phenotypic severity in *GNAO1*-related disorders. We found that each variant has a unique profile of clinical phenotypes and pathological molecular mechanisms. These findings will contribute to better understanding *GNAO1*-related disorders. Additionally, the severity score will facilitate standardization of patients categorization and assessment of response to therapies in development.

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Developmental and epileptic encephalopathies (DEEs) are a heterogeneous group of rare neurological disorders defined by early-onset refractory epilepsy, specific electroencephalogram (EEG) abnormalities, developmental delay or regression, and intellectual disability.[1,2] With the advancement of genetic testing, a total of 110 genes associated with DEEs have been identified to date (https://www.omim.org/phenotypicSeries/PS308350). In addition to genetic heterogeneity, there is also a great deal of clinical variability. This is not only because the signs and symptoms are caused by a specific genetic variant, but also because brain physiology is influenced by chronic seizures and prolonged drug exposure.[3] The accurate and timely identification of the genetic etiology of DEEs has the potential to enhance our understanding of the pathophysiology of these disorders, and promote the development of targeted therapies.[4]

DEE 17 (OMIM #615473), caused by variants in *GNAO1*, was initially identified by Nakamura et al. in 2013.[5] Since then, our awareness of the condition has been improved by the extensive description of clinical cases[6–10] and a greater understanding of its pathogenesis.[11–13] This has led to the description of two recognized OMIM phenotypes: (1) developmental and epileptic encephalopathy 17 (OMIM#615473),[5–10] and (2) neurodevelopmental disorder with involuntary movements (OMIM#617493).[8,14] However, growing evidence suggests the severity of the disease lies on a spectrum with intermediate clinical phenotypes.[15]

Although a few studies have examined genotype–phenotype correlation in *GNAO1*-related disorders,[16–18] natural history data or severity scores have not been developed, nor have prognostic factors been identified. Defining subgroups of patients with *GNAO1*-related disorders by severity is a prerequisite for designing more precise natural history studies to identify domains and windows for potential therapeutic interventions, and to plan clinical trials.[4]

The *GNAO1* gene encodes the G protein Gαo subunit. Gαo plays an important role in the control of nervous system function. Among many things, it is involved in cytoskeletal remodeling, and firing of developing neurons, regulation of synaptic function, and neuronal excitability.[11–13,19] Mechanistically, Gαo modulates both inhibitory and stimulatory neuromodulatory signaling to cyclic adenosine monophosphate, a major determinant in the pathophysiology of movement disorders.[18,20] From a molecular perspective, Gαo serves as a transducer of G protein coupled receptor (GPCR) signals as a part of the heterotrimeric complex with Gβγ subunits.[21,22] Understanding how specific variants affect Gαo ability to transduce GPCR signals will be key to the development of interventional strategies. Recent progress in this area suggests that *GNAO1*-associated variants perturb Gαo function by different mechanisms, including loss-of-function, dominant-negative, and debatable gain-of-function effects, which ultimately lead to *GNAO1*-related disorders.[11,15,18,20,23–26] Yet, our understanding of the molecular pathology of *GNAO1* and its relationship with the disease symptomatology is far from complete, and many disease-causing variants remain to be characterized.

In the present study, we investigated 16 individuals carrying de novo missense *GNAO1* variants and deeply analyzed their phenotype, culminating in the development of an all-encompassing disease severity score. We further delineated the molecular pathology of these variants using a molecular deconvoluting platform, and mapped these results onto a disease severity score to facilitate prognosis and the development of precision interventions.

Material and Methods

Standard Protocol Approvals, Registrations, and Patient Consents
This study was approved by the Ethics Committee of the Institut de Recerca Sant Joan de Déu, Barcelona, Spain (PIC-77-21). Written informed consent was obtained from all guardians of participants according to the Declaration of Helsinki.

Patient Ascertainment
A total of 16 individuals with missense variants in *GNAO1* were identified as part of the ongoing study "Prospective and retrospective study of phenotypic and genotypic characterization of patients affected by *GNAO1*-related disorders" at the Hospital Sant Joan de Déu, Barcelona, Spain. This is a prospective 3-year study that annually evaluates children with *GNAO1*-related disorders with a standardized protocol, in addition to retrospectively analyzing their evolution before inclusion in the study. In this article, partial data from the first and second years of assessment are utilized. A case report on 1 patient (P6) was previously published,[27] and data from some of the cohort members were included in the caregiver survey by Axeen et al.[7]

Movement Disorders
O the 16 individuals, 14 were characterized by direct clinical examination, and in all 16, video recordings were independently reviewed by two movement disorder specialists, reaching a consensus agreement. We assessed the baseline movement disorders both at rest and when

performing voluntary movements. During these assessments, the patients were taking their usual medications for epilepsy and movement disorders. Observed movements were classified according to established criteria, and rated using specific and validated severity scales: the Burke–Fahn–Marsden Dystonia Rating Scale for dystonia and the Abnormal Involuntary Movement Scale for chorea or stereotypies. We defined a dyskinetic crises as a sudden and marked exacerbation of abnormal involuntary movements (dyskinesias), which are distinct in onset and duration from the baseline dyskinetic movements of the patient, such as dystonia, chorea, or athetosis. During a dyskinetic crisis, alterations in facial expression may manifest, which are different from epileptic seizures, as there is no loss of awareness or disconnection from the surrounding environment. Additionally, post-episode somnolence is not typically observed, unlike in seizures. These events are typically identified by parents or regular caregivers. Video S1 shows examples of patients with *GNAO1*-related disorders experiencing dyskinetic crises. As the physical examination of children with *GNAO1*-related disorders can be highly variable, we scored each item according to the highest severity observed.

Epilepsy

Seizures were classified according to the International League Against Epilepsy criteria. A total of 10 of 16 patients underwent 24-h video-EEG monitoring in an epilepsy unit during this study. Retrospective data on epilepsy history, seizures, previous video-EEG characteristics, antiseizure medication or other treatments used, and their responses were also collected.

Neurodevelopmental Assessment

Motor and language development were methodically assessed through the Vineland Adaptive Behavior Scales, Second Edition (Vineland-II), the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III), and the Gross Motor Function Measure (GMFM-88).

For each patient, a comprehensive retrospective review of medical records and clinical investigations was also conducted.

Magnetic Resonance Imaging

Brain magnetic resonance imaging (MRI) data were available for all patients. A total of 10 patients underwent brain MRIs during this study. In addition, all previous MRIs available for 14 out of 16 patients were reviewed.

Lumbar Puncture

Available neurotransmitter data from lumbar punctures were collected for 5 out of 16 patients.

Severity of GNAO1-Related Disorders

To develop the *GNAO1*-related disorders severity score, a team of expert pediatric neurologists specializing in movement disorders and epilepsy discussed the items for inclusion in the scale. Several iterations of the scale were constructed and tested, with input from other experts in the field, until the final version was obtained. The process involved a thorough review and analysis of other scales utilized in the assessment of neurodevelopmental disorders and epilepsy, with particular attention given to the unique characteristics and quality of life concerns of individuals with *GNAO1*-related disorders. The final scale reflects the severity of our patient population, and was designed to be reliable and valid for clinical use. We have included the following items: (1) epilepsy, (2) movement disorders, (3) gross motor development, (4) language development, and (5) feeding. For items (1) epilepsy and (2) movement disorders, the average of 4 subitems was taken into account: (a) frequency, (b) intensity and duration, (c) falls or injuries, and (d) medication or therapy. Unless otherwise specified, the condition of the patient for the past year was used when calculating the score. Regarding item (3), gross motor development, infants aged >3 months could be evaluated; otherwise, we computed 0 points. The same applies to item (4), language development. Infants aged >6 months could be evaluated; otherwise, we computed 0 points. The *GNAO1*-related disorders severity score is presented in Table 1. The scores range from 0 to 13. Finally, we categorize the phenotype as mild (total severity score ranging from 0 to 3.9 points), moderate (4 to 7.9 points), or severe (>8 points).

Genetic Analysis

All patients were diagnosed with *GNAO1* missense variants as part of their routine clinical care using available testing, whether through: (1) targeted *GNAO1* gene sequencing, (2) a multiple gene panel for DEEs, or (3) whole exome sequencing.

Functional Studies

Cell Culture and Transfection

HEK293FT cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, minimum Eagle's medium non-essential amino acids, 1 mmol/L sodium pyruvate at 37°C in a humidified incubator containing 5% CO_2. At the time of

TABLE 1. *GNAO1*-Related Disorders Severity Score

GNAO1-related disorders severity score

1. Epilepsy (clinical situation for the past year unless specified)

A. Frequency of seizures
  0: <1 seizure per year
  1: >1 seizure per year, less than one per month
  2: >1 seizure per month, <1 per week
  3: >1 seizure per week or daily
B. Intensity and duration of seizures
  0: No seizures in the previous year
  1: <30 s or without impairment of awareness (low risk of apnea or bronchial aspiration)
  2: 30 s to 3 min (risk of apnea or bronchial aspiration)
  3: >3 min or rescue medication needed
C. Falls or injuries during seizures
  0: No seizures in the previous year
  1: No falls or injuries
  2: Sleep-disrupting seizures or causing falls or injuries
  3: Seizures resulting in apnea or bronchial aspiration
D. Amount of antiseizure medications (ASM)/therapy for epilepsy:
  0: No ASM
  1: 1 ASM/therapy
  2: 2 ASM/therapy
  3: >3 ASM/therapies or rescue medication required in the last 3 months or vagal nerve stimulation implanted or epilepsy surgery performed

2. Movement disorders (clinical situation for the past year unless specified)

A. Frequency of movement disorders
  0: No movement disorders in the previous year
  1: Stable or persistent choreoathetosis and dystonia, stereotypies or other movement disorders
  2: Rare dyskinetic crisis (≤1 episode per month)
  3: Frequent dyskinetic crisis (weekly or daily episodes)
B. Intensity and duration of dyskinetic crisis
  0: No dyskinetic crisis in the previous year
  1: <1 min
  2: 1 to 3 min
  3: >3 min or rescue medication or hospitalization required
C. Falls or injuries during dyskinetic crisis or as a result of any movement disorders
  0: Absence of both movement disorders or dyskinetic crisis in the previous year
  1: No falls or injuries (daily short dyskinetic crisis or persistent movement disorders)
  2: Sleep-disrupting dyskinetic crisis or causing falls or injuries (joint dislocations, bites, etc.) or pain
  3: Movement disorders resulting in rhabdomyolysis, renal or respiratory failure or other life threating conditions
D. Amount of medications/therapy for movement disorders:
  0: No medication needed
  1: 1 drug
  2: 2 drugs
  3: >3 drugs, rescue medication required in the last 3 months or deep brain stimulation implanted

Total item1: (A + B + C + D)/4 =

Total item2: (A + B + C + D)/4 =

3. Gross motor development (this item can be assessed on infants aged >3 months; otherwise, compute 0 points)

  0: Can walk alone or rarely need help
  1: Can walk with support
  2: Can sit without support
  3: Had acquired head control
  4: Absence of gross motor development (not even head control)

4. Language development (this item can be assessed on infants aged >6 months; otherwise, compute 0 points)

  0: Normal or can speak at least two-word phrases
  1: Can speak single words or babbling
  2: Absence of expressive language

5. Feeding

  0: Orally fed
  1: Fed by a gastrostomy or any other type of enteral feeding tube

TOTAL: (items 1 + 2 + 3 + 4 + 5)=

transfection, cells were supplemented with 0.1% Matrigel (Corning, Corning, NY, USA).

For bioluminescence resonance transfer (BRET) assays, cells were seeded in 96-well flatbottomed white microplates (Greiner Bio-One, Kremsmünster, Austria) at a density of 5×10^4 cells/well. At the same time the cells were plated, they were transfected with expression constructs (total 0.09 μg/well), PLUS reagent (0.1 μL/well),

and Lipofectamine LTX (0.5 μL/well). The expression constructs transfected were as follows (number in parentheses indicates the relative amount of DNA, where 1 = 0.015 μg: Flag-D2R (1), GαoA (2), Venus 156–239-Gβ1 (1), Venus 1–155Gγ2 (1), and masGRK3ct-Nluc-HA (1).

For NanoBiT assays, cells were seeded into 6-cm dishes at a density of 4×10^6 cells/dish. After 4 hours, expression constructs (total 2.1 μg/dish) were transfected into the cells using PLUS reagent (7.5 μL/dish) and Lipofectamine LTX (12 μL/dish) reagents. The expression constructs transfected were as follows (number in parentheses indicates the relative amount of DNA, where 1 = 0.42 μg): D2R-mycSmBiT (1), GαoA (0.1), LgBiT-Gβ1 (1), and Gγ2 (1). A calibration curve of wild-type (WT) GαoA cDNA was run, and the amount of GαoA cDNA used in the assay was chosen, so that the assay was not saturated.

BRET Assay

BRET between Venus-Gβ1γ2 and masGRK3ct-Nluc-HA was used to measure trimer formation, agonist-induced G protein activation, and dominant-negative activity of Gαo mutants in living cells.[28,29] At 16 to 24 hours post-transfection, cells were washed once with BRET buffer (phosphate-buffered saline containing 0.5 mmol/L $MgCl_2$ and 0.1% glucose. Cells were harvested with centrifugation at 500 g for 5 min and resuspended in BRET buffer. The substrate for Nano luciferase (Nluc), furimazine (Promega, Madison, WI, USA), was diluted in BRET buffer according to the manufacturer's instructions and added to the cells. BRET measurements were made every 100 ms over a course of 1 minute using a microplate reader (PHERAstar FSX; BMG Labtech, Ortenberg, Germany) equipped with two emission photomultiplier tubes. 100 μmol/L dopamine (Sigma, St. Louis, MO, USA) was added after obtaining a baseline BRET value. All measurements were performed at 37°C. The BRET signal was calculated as the ratio of the light emitted by the Venus-Gβ1γ2 (535 ± 30 nm) over the light emitted by the masGRK3ct-Nluc-HA (475 nm ± 30 nm). The average baseline BRET value recorded before dopamine stimulation was subtracted from the experimental BRET signal values.

NanoBiT Assay

Measurement of bioluminescence caused by complementation between D2R-SmBiT and LgBiT-Gβ1 was performed to examine the interaction between G proteins and the receptor. At 16 to 24 h post-transfection, HEK293FT cells were washed once with BRET buffer and detached by gentle pipetting. Approximately 50,000

to 100,000 cells/well were transferred to a 96-well flatbottomed white microplate (Greiner Bio-One). The substrate for Nluc, furimazine (Promega), was diluted in BRET buffer according to the manufacturer's instructions and added to the cells. Luminescence measurements were made every 0.74 s for 172.66 s using a microplate reader (PHERAstar FSX; BMG Labtech) equipped with 1 emission photomultiplier tubes. Then, 100 μmol/L dopamine (Sigma) was added after obtaining a baseline luminescence value after 55 s. All measurements were performed at 37°C.

Quantification and Statistical Analysis

For clinical studies, we used Spearman's rank correlation to establish the correlation between the *GNAO1*-related disorders severity score and Vineland-II, Bayley-III (cognitive, receptive language and expressive language sub scores), GMFM-88, the Burke-Fahn-Marsden Dystonia Rating Scale, and the Abnormal Involuntary Movement Scale. For functional studies, samples with only pcDNA3.1+ transfected in place of Gαo were used as baseline measurements. The maximum amplitude of these measurements was subtracted from the maximum amplitude of all other measurements. Statistical analysis was performed using GraphPad Prism 9.4 (San Diego, CA, USA). All data are represented as the mean ± SEM. Comparisons were computed using one-way ANOVA. Asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Results

Genetics

We detected many different *GNAO1* variants, all of them de novo and classified as pathogenic according to the ACMG criteria. Although several variants were previously reported in the literature, we found four novel variants: p.K46R, p.T48I, p.R209P, and p.L235P (Table 2). The variants p.T182I (P4 and P5), p.G203R (P11, P12, and P13), and p.R209C (P14 and P15) were identified in multiple patients in this study.

General Clinical, Radiological, and Biochemical Features

Patient ages ranged from 11 months to 15 years and 11 months (median 8.4 years, mean 7.3 years) within the cohort. A total of 56% of individuals were female. We collected retrospective data on P5, who died at the age of 3 years and 2 months due to status epilepticus. Additionally, P6 died during our study at the age of 7 years and 7 months in the context of respiratory failure resulting from pneumonia and a bronchospasm crisis. First symptoms were identified at an age ranging from the prenatal

TABLE 2. Genetics Variants Included in this Study

Patient	Genomic DNA variant (chr16; GRCh37)	cDNA change HGVS NM138736.3	Protein change	Previously reported patients (PMID)	Inheritance	ACMG classification	CADD score	Mutation taster	PROVEAN	SIFT	gnomAD Minor allele frequency
P1	chr16-56226265-G-C	c.118G>C	G40R	28357411 and 26485252	De novo	P (PM2, PM5, PP3, PS1, PM1, PP2, PP5, PS2)	33	Disease causing (1)	Damaging (−6.37, −6.38)	Damaging (0)	0%
P2	chr16-5622650-A-G	c.137A>G	K46R	No	De novo	P (PM1, PM2, PM5, PP2, PP3, PS2)	1.083	Disease causing (1)	Neutral (−2.41)	Damaging (0)	0%
P3	chr16-56226510-C-T	c.143C>T	T48I	No	De novo	P (PM1, PM2, PM5, PP2, PP3, PS2)	27	Disease causing (1)	Damaging (−4.8, −4.81)	Damaging (0)	0%
P4, P5	chr16-56368721-C-T	c.545C>T	T182I	32581362	De novo	P (PM2, PM1, PP2, PP3, PP5, PS2)	28,8	Disease causing (1)	Damaging (−5.44, −5.54)	Damaging (0)	0%
P6	chr16-56370645-T-C	c.596T>C	L199P	27072799 (this patient)	De novo	P (PM1, PM2, PP2, PP3, PP5, PS2)	28,5	Disease causing (1)	Damaging (−4.67, −4.68)	Damaging (0)	0%
P7	chr16-56370675-G-C	c.626G>C	R209P	No	De novo	P (PM1, PM2, PM5, PP2, PP3, PS2)	32	Disease causing (1)	Damaging (−6.59)	Damaging (0.001)	0%
P8	chr16-56370741-A-G	c.692A>G	Y231C	30682224, 28503590, and 27072799	De novo	P (PM1, PM2, PP3, PP5, PP2, PS2)	29,1	Disease causing (1)	Damaging (−8.27, −8.24)	Damaging (0)	0%
P9	chr16-56370753-T-C	c.704T>C	L235P	No	De novo	P (PM2, PM1, PP2, PP3, PS2)	32	Disease causing (1)	Damaging (−6.56, −6.58)	Damaging (0)	0%
P10	chr16-56374893-T-A	c.871T>A	Y291R	30682224, 34139551 (this patient)	De novo	P (PM2, PM1, PP2, PP3, PS2)	26	Disease causing (1)	Damaging (−8.3)	Damaging (0)	0%
P11, P12, P13	chr16-56370656-G-C	c.607G>C	G203R	27864847, 27476654, 26485252, 25966631 and 23993195	De novo	P (PM1, PM2, PS1, PM5, PP5, PP2, PP3, PS2)	32	Disease causing (1)	Damaging (−7.56)	Damaging (0)	0%
P14, P15	chr16-56370674-C-T	c.625C>T	R209C	30103967, 33358199, 32581362, 28688840, 28357411, 27916449, 27864847, 27625011, 27068059, 27476654, 26485252, 26060304, 25966631, 23993195,	De novo	P (PP5, PM1, PM5, PM2, PP2, PP3, PS2)	32	Disease causing (1)	Damaging (−7.55)	Damaging (0)	0%
P16	chr16-56370758-G-A	c.709G>A	E237K	30103967, 29935962 and 32581362	De novo	P (PP5, PM2, PM1, PP2, PP3)	32	Disease causing (1)	Damaging (−3.77, −3.75)	Damaging (0)	0%

Abbreviations: B = benign; LP = likely pathogenic; P = pathogenic; VOUS = variant of unknown significance.

period (P4 and P5 mother described, subjectively, increased fetal movements) to 1.5 years, with hypotonia or motor developmental delay (6 patients, 37.5%), epilepsy (5 patients, 31%), and movement disorder (3 patients, 18.7%) being the most frequently reported (Table 3).

Movement Disorders

Abnormal involuntary movements were present in all patients, in the majority of cases by 12 months-of-age (14 patients, 87.5%). Generalized choreoathetosis and dystonia (13 patients, 81.3%) and stereotypies (3 patients, 18.8%) were the most frequent movement disorders (Video S1, in Supporting Information). A total of 10 patients (62.5%) presented with dyskinetic crises. Six patients (37.5%) were hospitalized specifically for management of their movement disorder. A number of medications were tried in our cohort, with no obvious benefit in many cases (Table 3). Four patients underwent globus pallidus deep brain stimulation that was partially beneficial: dyskinetic crises in P4 were less frequent and severe, avoiding the need for further hospitalizations; generalized dystonia in P14 showed only a slight improvement; P6 demonstrated a significant, but transient, improvement in her dyskinetic crises; whereas P7 had a brief follow-up period after the deep brain stimulation to draw a conclusion. The outcomes of the assessment of dystonia and chorea utilizing the Burke–Fahn–Marsden Dystonia Rating Scale and Abnormal Involuntary Movement Scale rating scales are presented in Table S1.

Epilepsy

Eight patients (50%) were diagnosed with epilepsy, with several seizure types identified and heterogeneous video-EEG findings (Table 3). Of note, the patients with the highest scores on the GNAO1-related disorders severity score had abnormal background activity, with diffuse slowing during wakefulness (5 patients, 31.3%), and absence of physiological sleep elements and periods of diffuse low voltage activity during sleep (3 patients, 18.7%). Variable seizure frequency was observed, with one patient experiencing four seizures over 5 years and others experiencing multiple daily seizures. Six patients (37.5%) met the criteria for drug-resistant epilepsy. Based on interviews with caregivers and previous medical reports, it is not possible to determine which antiseizure medications are more effective. P6 was given a ketogenic diet, which produced a positive, but temporary, response. Other non-pharmaceutical treatments, such as vagal nerve stimulation, were not utilized in any patient.

Neurodevelopmental Outcome

Neurodevelopmental delay was present in all cases. Notably, five patients had a relatively mild phenotype, manifesting normal head growth, independent ambulation, limited spoken language, and purposeful hand function. In contrast, seven patients tended to be more severely affected, with a phenotype more consistent with the classically described GNAO1-related disorder (Table 3). Neurodevelopmental assessment scores of participants (Bayley-III, Vineland-II, and GMFM-88) can be found in Table S2.

Feeding

Five patients (31.2%) required gastrostomies, and 1 patient (6.3%) required a transpyloric tube, whereas the majority of patients were fed by mouth with food of normal or mashed consistency (Table 3).

Brain MRI

Normal brain MRIs were reported for 11 patients (68.6%). Unspecific white matter lesions (1 patient, 6.3%) and global atrophy or widening of the extra axial spaces and ventricular system (2 patients, 12.5%) were frequent findings. Interestingly, P5 showed cortical cytotoxic edema, probably due to status epilepticus versus hypernatremia. No obvious radiological abnormalities of the basal ganglia were detected.

CSF Analysis

CSF neurotransmitter analysis was previously undertaken in 5 patients. Abnormalities, namely, low homovanillic acid and 5-hydroxyindoleacetic acid, were seen in one patient (P15). This patient began L-dopa and 5-OH-thryptophan supplementation with doubtful benefit. It is currently unclear as to why this finding has been observed, and it is possible that this may be a coincidental occurrence.

GNAO1-Related Disorders Severity Score

The range of total severity scores was between 2 and 13 (Table 4). Five patients had a score of mild (from 0 to 3.9 points), four had a score of moderate (from 4 to 7.9 points), and seven had a score of severe (>8 points). The mean score for each subcategory was as follows: (1) epilepsy 0.9 (range 0–3), (2) movement disorders 1.6 (range 0.3–3), (3) gross motor development 2.3 (range 0–4), (4) language development 1.8, and (5) feeding 0.3.

Patients with the same GNAO1 variant had comparable total severity scores (P4 and P5 with p.T182I variant (11 and 13, severe), P11, P12, and P13 with p.G203R variant (10.5, 8 and 9, severe), and P14 and P15 with p.R209C variant (2.5 and 2.8, mild), and were categorized as having the same severity. Video S2 shows examples of

TABLE 3. Summary of Clinical Features in *GNAO1*-Related Disorders Patients

Patient	Sex	Age at last assessment	First symptoms/age at onset	Epilepsy/SZ freq (higher SZ freq/longer SZ free period)	Epilepsy treatments (number tried before/current)	Type of MD/age at onset/dyskinetic crisis	Movement disorder treatment	Gross motor development	GMFM - 88 (%)	Language development	Gastrostomy
P1	F	8.6 y	Epilepsy/NP	Yes/Daily/5 yr	3/LEV + OXC	Choreodystonia + stereotypies/6 mo/No	No	Sits without support	26.23	Absent	No
P2	F	4.7 yr	PDD/18 mo	No / -	-	Dystonia/2 yr/No	No	Walks without support	94.89	Absent	No
P3	F	0.9 yr	Epilepsy and irritability/NP	Yes/Daily	5/LEV + VGB + TPM + pyr	Choreodystonia + stereotypies/NP/No	No	No head control	NA	Absent	No
P4	F	12.2 yr	Increased fetal movements/Prenatal	Yes/Four seizures over a period of 5 yr	2/LEV + LCM	Choreodystonia/Prenatal/Yes	DBS, baclofen, chloral hydrate, clonidine, tetrabenazine	No head control	0	Absent	Yes
P5	F	3 yr	Increased fetal movements/Prenatal	Yes/Daily	4	Choreodystonia/Prenatal/Yes	Baclofen, CBZ	No head control	NA	Absent	Yes
P6	F	7.6 yr	Epilepsy/NP	Yes /Daily (every 15 minutes)/11 mo	12	Choreodystonia/3 yr/Yes	DBS, baclofen, chloral hydrate, trihexyphenidyl, tizanidine,	No head control	0	Absent	Yes
P7	M	8.1 yr	Dystonia and PDD/19 mo	No/-	-	Choreodystonia/17 mo /Yes	CBZ, tetrabenazine	Walks without support	76.29	Single Words	No
P8	M	12.9 yr	PDD/5 mo	Yes/Weekly/ 8 mo	5/VPA + OXC	Dystonia/Yes	Baclofen	No head control	7.67	Absent	No
P9	M	3.6 yr	Hypotonia and PDD/12 mo	No/-	-	Choreodystonia/12 mo/No	No	Walks without support	NA	Babbling	No
P10	M	15.9 yr	Episodes of facial redness during feeding/5 mo	No/-	-	Choreodystonia + stereotypies/12 mo/Yes	Baclofen	Walks without support	35.67	Absent	Yes
P11	F	3.9 yr	Neonatal seizures/NP	Yes/No seizures since NP	LEV	Choreodystonia/3 mo/Yes	Tetrabenazine, CLZ	No head control	6.91	Absent	Yes
P12	F	0.9 yr	Epilepsy/NP	Yes/Daily/2 mo	5/LEV + OXC + CLN	Choreodystonia/6 mo/No	No	No head control	6.82	Babbling	No
P13	M	1.25 yr	MD/2 mo	No/-	No	Choreodystonia /NP/ Yes (daily)	CZP	No head control	NA	Guttural sounds	TP tube
P14	M	9 yr	Dystonia/9 mo	No/-	-	Dystonia/12 mo/Yes	L-dopa, 5-OH-thryptohan	Walks without support	69	Dysarthric speech	No
P15	M	11.5 yr	PDD/12 mo	No/-	-	Choreodystonia/9 mo/No	DBS	Walks without support	NA	Dysarthric speech	No
P16	F	8.8 yr	Irritability/3 mo	No/-	-	Choreodystonia/5 m/Yes	CZP, Baclofen	Head control acquired	8.67	4-5 single words	No

Abbreviations: BRV = brivaracetam; CBZ = carbamazepine; Choreodystonia = choreoathetosis and dystonia; CLB = clobazam; CLN = clonazepam; DBS = deep brain stimulation; F = female; freq = frequency; KD = ketogenic diet; LCM = lacosamide; LEV = levetiracetam; LTG = lamotrigine; M = male; MD = movement disorder; mo = months; NA = not available; NP = neonatal period; OXC = oxcarbazepine; PB = phenobarbital; PDD = psychomotor developmental delay; PER = perampanel; pyr = pyridoxine; RUF = rufinamide; SZ = seizure; TP = transpyloric; TPM = topiramate; VGB = vigabatrin; VPA = valproate; yr = years; ZNS = zonisamide.

patients with varying degrees of severity in the *GNAO1*-related disorders severity score.

Structure: Functional Studies

To understand the molecular mechanism behind the pathology of each uncharacterized variant, we began by mapping their locations onto the structure of Gαo (Fig 1A). We found several variants (p.G40R, p.K46R, and p.T84I) located in the P-loop region. Another variant, p.T182I, directly coordinates the Mg^{2+} ion. Several other variants include proline substitutions. These could be predicted to disrupt secondary structure in regions, such as β3, Switch II, and Switch III, in the cases of p.L199P, p.R209P, and p.L235P, respectively. The remaining two variants, p.Y231C and p.Y391N, are both substitutions of tyrosine residues, with unclear implications for Gαo organization based on the structural considerations.

We have previously developed a pipeline for mechanistic evaluation of disease variants in Gα using a suite of BRET assays that monitor transitions in the G protein cycle.[11,23] We applied this approach to understand the molecular mechanisms underlying the novel Gαo variants uncovered in this study.

We started our examination by assessing the overall ability of the Gαo variants to transmit signals using D2R as a model GPCR that prominently couples to Gαo and is involved in striatal motor control (Fig 1B). For each variant, we observed a significantly reduced maximum response amplitude in response to dopamine stimulation as compared with WT (Fig 1C). Five mutants (p.G40R, p.T48I, p.T182I, p.L199P, p.R209P) completely failed to respond to dopamine stimulation, whereas others displayed varying degrees of deficiency (Fig 1D).

To probe the mechanisms underlying the signaling deficits, we first tested Gαo variants for an ability to form heterotrimers with the Gβγ subunits. This was accomplished by comparing the baseline BRET ratio between NanoLuc-tagged GRK and the Venus-tagged Gβγ (Fig 1E). As BRET between these two molecules is precluded by association of Gαo with Gβγ, we determined the amount of heterotrimer formation based on changes in baseline BRET values induced on the introduction of Gαo. This analysis showed that all of the mutants that were completely unable to transduce GPCR signals (p.G40R, p.T48I, p.T182I, p.L199P) also had significantly reduced ability to form G protein heterotrimers (Fig 1F).

We questioned if there were any folding and/or stability defects in the variants that could cause their reduced ability to form heterotrimers. Western blotting of transfected HEK293FT cell lysates indicated that p.G40R, p.T48I, pT182I, and p.L199P were indeed expressed at significantly lower levels than WT Gαo (Fig 1G). This decrease in expression closely correlates with the reduction in heterotrimer formation observed in these variants, suggesting that loss of protein stability likely underlies the functional deficits in trimer formation and, consequently, transduction of GPCR signals.

We next tested *GNAO1* variants for their dominant negative activity given that it was shown to be present in some previously reported variants.[11] To test this, we expressed the variants alongside WT Gαo, and measured any decrease in dopamine-induced BRET response (Fig 2A). Three variants (p.T48I, p.T182I and p.R209P) displayed a significant decrease in dopamine response, indicating that they interfere with normal activation of Gαo by D2R (Fig 2B, C).

We further interrogated possible mechanisms behind dominant negative effects by studying the recruitment of G protein heterotrimer to the D2R receptor on its activation by an agonist. This was achieved using an assay that measures complementation between SmBit-tagged D2R and LgBit-tagged Gβγ, which reconstitutes Nluc, producing luminescence on Gαo-mediated interaction of Gβγ with the D2R (Fig 2D). Interestingly, with the single exception of p.L199P, we detected interaction with D2R for all mutants with a varying degree of efficiency (Fig 2E, F). The p.G40R, p.Y321C, and p.Y291N variants showed a significantly decreased interaction with the receptor compared with WT, likely explained by the decreased expression of the variants. In contrast, the p.K46R and p.T182I variants displayed a significant increase in interaction with the receptor compared to WT, suggesting that these variants have more nonproductive interactions with the receptor.

Correlation between Clinical and Experimental Measurements

To assess the strength of our experimental measurements in predicting the clinical severity of the different variants, we gave each variant a score ranging from 0 to 3 in the categories of Gαo Expression, Heterotrimer Formation, Loss-of-function, and Dominant Negative phenotype, with 0 indicating no departure from WT values and 3 being the most severe departure from WT values (Table 4). We also gave a score ranging from −3 to +3 for receptor interaction, with positive values indicating increased interaction with the receptor, and negative values indicating decreased interaction with the receptor. These 5 categories were then combined into an overall experimental measurement score. The variants in

TABLE 4. Clinical Severity Scores and Experimental Measurement Scores for Each Patient in the Study

Patient	GNAO1 variant	Current age (years)	Epilepsy					Movement disorders					Gross motor development	Language development	Feeding	GNAO1 - related disorders severity score	mild (0 - 3.9), moderate (4,0 - 7.9), severe (> 8)	Expression	Trimer formation	Loss of function	Dominant negative activity	Receptor interaction	Experimental measure total
			1A	1B	1C	1D	Total	2A	2B	2C	2D	Total											
P1	G40R	8.6	0	0	0	1	0.3	1	1	1	0	0.8	2	2	0	5.0	Moderate	3	2	3	1	-2	7
P2	K46R	4.7	0	0	0	0	0.0	1	0	1	0	0.5	0	2	0	2.5	Mild	0	0	2	0	3	5
P3	T48I	0.91	3	3	2	3	2.8	1	2	1	0	1.0	4	2	0	9.8	Severe	3	3	3	2	-1	10
P4	T182I	12.2	1	2	1	1	1.3	3	3	2	3	2.8	4	2	1	11.0	Severe	2	3	3	2	0	10
P5	T182I	Exitus	3	3	3	3	3.0	3	3	3	3	3.0	4	2	1	13.0	Severe	2	3	3	2	0	10
P6	L199P	Exitus	2	2	3	3	2.5	3	3	3	3	3.0	4	2	1	12.5	Severe	3	3	3	1	-3	7
P7	R209P	8.1	0	0	0	0	0.0	1	1	0	2	1.0	0	1	0	2.0	Mild	0	0	3	3	1	7
P8	Y231C	12.9	3	1	1	2	1.8	1	0	0	1	0.5	3	2	0	7.3	Moderate	1	2	2	0	-1	4
P9	L235P	3.6	0	0	0	0	0.0	1	0	1	0	0.5	0	2	0	2.5	Mild	0	0	2	0	0	2
P10	Y291R	15.9	0	0	0	0	0.0	3	2	2	1	2.0	0	2	1	5.0	Moderate	1	0	2	0	0	3
P11	G203R	3.9	3	1	1	2	1.8	3	3	2	3	2.8	4	2	0	10.5	Severe	0	1	2	1	3	7
P12	G203R	0.91	1	2	1	3	1.8	1	0	0	0	0.3	4	2	0	8.0	Severe	0	1	2	1	3	7
P13	G203R	1.25	0	0	0	0	0.0	3	3	2	2	2.5	4	2	1	9.5	Severe	0	1	2	1	3	7
P14	R209C	11.5	0	0	0	0	0.0	1	0	2	3	1.5	0	1	0	2.5	Mild	0	0	2	1	0	3
P15	R209C	9	0	0	0	0	0.0	3	1	1	2	1.8	0	1	0	2.8	Mild	0	0	2	1	0	3
P16	E237K	8.8	0	0	0	0	0.0	3	2	1	2	2.0	3	1	0	6.0	Moderate	0	0	1	0	2	3

Abbreviations: 1A = frequency of seizures; 1B = intensity and duration of seizures; 1C = falls or injuries during seizures; 1D = amount of antiseizure medications (ASMs)/therapy for epilepsy; 2A = frequency of movement disorders; 2B = intensity and duration of dyskinetic crises; 2C = falls or injuries experienced during dyskinetic crises or as a result of any movement disorders; 2D = amount of medication/therapy for control of the movement disorders.

Muntean et al.[11] were also given an experimental measurement score.

Overall, there was a strong correlation between the clinical severity score and the experimental measurement score ($R^2 = 0.486$; Fig 3A). To further dissect what molecular mechanisms underpinned the clinical severity, we compared the overall clinical severity score with the scores of each individual functional experiment. We discovered a correlation between the clinical severity score and the variant expression levels ($R^2 = 0.301$; Fig 3B) and trimer formation ($R^2 = 0.685$; Fig 3C). The correlations for the loss-of-function, dominant negative, and receptor interaction experiments were much weaker

($R^2 = 0.139$, 0.079, and 0.001, respectively) (Fig 3D–F). We further analyzed the relationship between the highest correlating functional metric of trimer formation and individual clinical traits that contribute to the clinical severity score. This analysis showed the greatest level of correlation with epilepsy measures (Figure S1A–E), and weak, if any, correlation with other measures (Figure S1F–M).

Correlation Analysis with Other Standardized Scales

We used Spearman's rank correlation, and found a significant negative correlation between the *GNAO1*-related
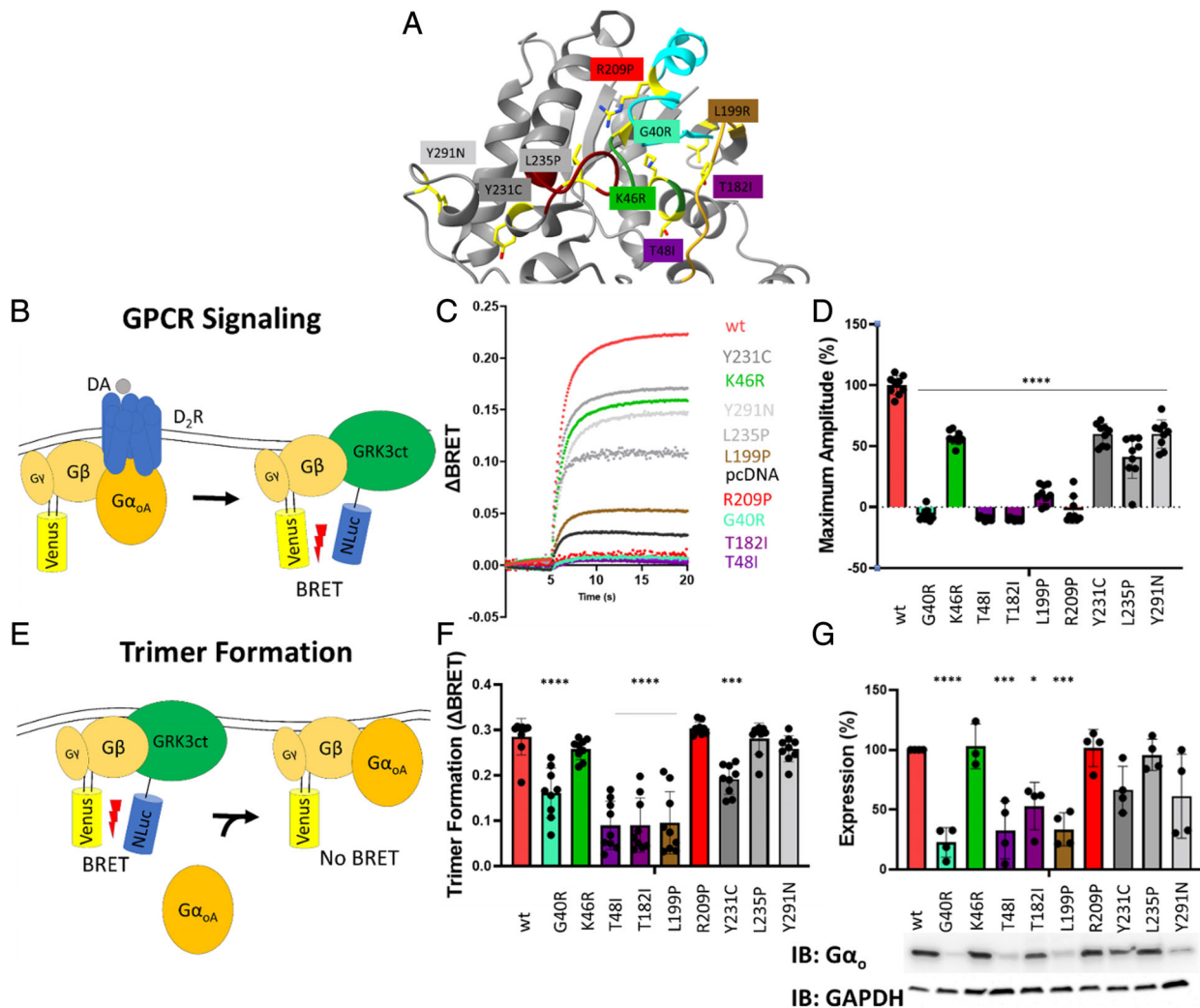
FIGURE 1: All variants show an inhibited agonist response. (A) Location of each variant on Gαo (PDB: 3C7K). Regions are indicated as follows: green, P-loop; goldenrod, Switch I; cyan, Switch II; and magenta, Switch III. (B) Schematic of G protein coupled receptor (GPCR) signaling assay. Dopamine stimulation induces the release of Venus-Gβγ from Gαo, allowing Venus-Gβγ to associate with Nano luciferase (Nluc)-GRK3 and increase the bioluminescence resonance transfer (BRET) signal. (C) Representative traces of variant dopamine BRET responses. (D) The effect of *GNAO1* variants on dopamine induced BRET signal. 0% response was defined as the amount of BRET signal on dopamine activation of the Gαo-free control. A 100% response was defined as the amount of BRET signal upon dopamine activation of the wild-type (WT) Gαo control. (E) Schematic of trimer formation assay. In the absence of Gαo, Venus-Gβγ and NLuc-GRK3 have a high level of basal BRET. The binding of Gαo to Gβγ interferes with BRET between Venus-Gβγ and NLuc-GRK3. (F) The effect of *GNAO1* variants on basal BRET. The amount of basal BRET without Gαo expressed was defined as 0. The amount of trimer formation is determined by subtracting the basal BRET of each variant from the Gαo-free control. (G) Expression levels of each variant. Western blot analysis of each variant blotted with α-Gαo antibody. [Color figure can be viewed at www.annalsofneurology.org]

disorders severity score and Vineland-II ($r = -0.553^*$, $p = 0.032$), Bayley-III Cognitive ($r = -0.811^{**}$, $p = 0.008$), Bayley-III Receptive Language ($r = -0.868^{**}$, $p < 0.001$), Bayley-III Expressive Language ($r = -0.777^{**}$, $p = 0.002$), and GMFM-88 ($r = -0.955^{**}$, $p < 0.001$). In contrast, we found only a weak non-significant positive correlation between *GNAO1*-related disorders severity score and the Burke–Fahn–Marsden Dystonia Rating Scale ($r = 0.191$, $p = 0.532$), and the Abnormal Involuntary Movement Scale ($r = 0.127$, $p = 0.680$). Table S3 shows the correlations

observed between the *GNAO1*-related disorders severity score and other standardized scales.

Discussion

We reported detailed phenotypic data for 16 individuals with *GNAO1*-related disorders harboring 12 distinct missense variants, including 4 novel variants (p.K46R, p.T48I, p.R209P, and p.L235P), thus expanding the genetic variability of the disease. Furthermore, we
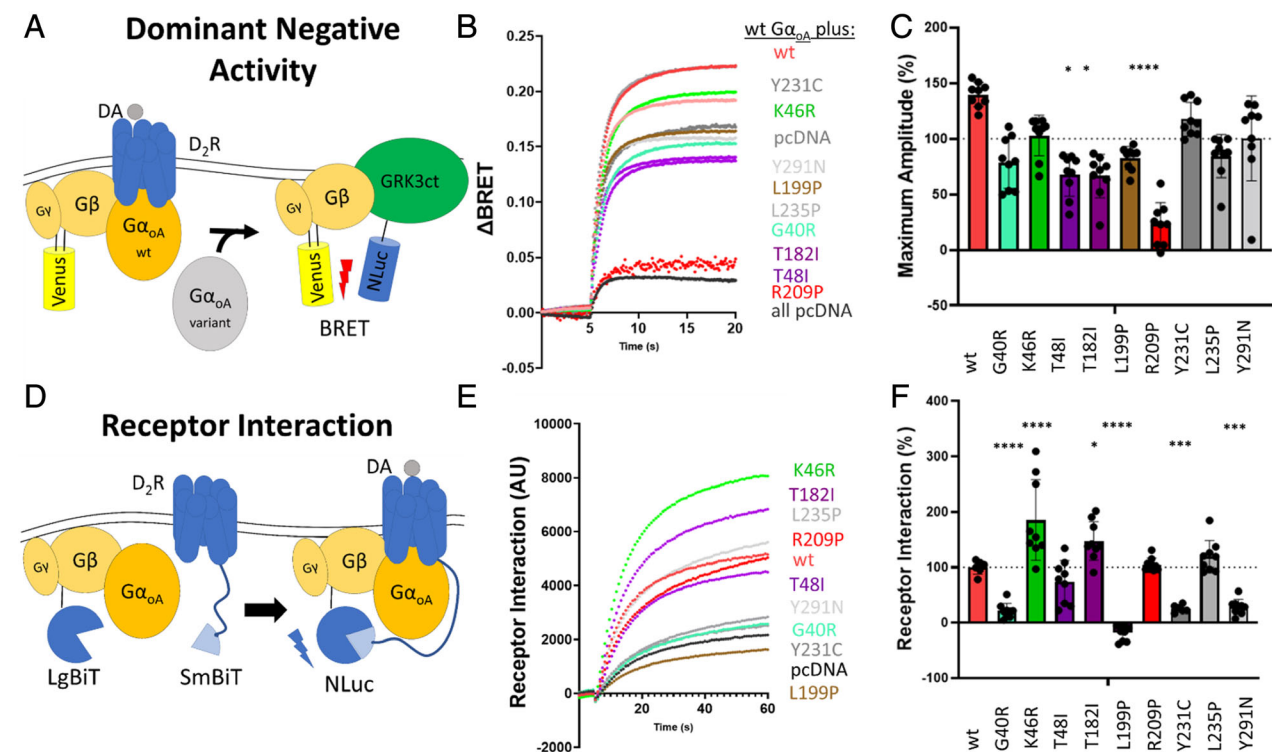
FIGURE 2: Some *GNAO1* variants show dominant negative activity. (A) Schematic of the dominant negative activity assay. variants of Gαo are expressed alongside wild-type (WT) Gαo. Variants with dominant negative activity are able to suppress WT Gαo dopamine-induced activation. **(B)** Representative traces of dopamine BRET responses of GNAO1 variants expressed alongside WT Gαo. **(C)** The effect of *GNAO1* variants on WT Gαo dopamine induced BRET signal. Any condition with a maximum amplitude below the condition expressing pcDNA3.1+ alongside WT Gαo indicates that the variant has dominant negative activity. **(D)** Schematic of the receptor interaction assay. Dopamine stimulation induces the recruitment of G protein heterotrimer to the receptor where the SmBiT tag on the receptor can interact with the LgBiT tag on Gβ. This reconstitutes NLuc and gives a luminescent readout. **(E)** Representative luminescence traces of the interaction of G protein heterotrimer with D2R receptor. **(F)** The effect of *GNAO1* variants on dopamine-induced receptor interaction with G protein heterotrimer. 0% interaction is defined as the amount of luminescence detected when the Gαo-free control was stimulated with dopamine. A 100% interaction is defined as the amount of luminescence detected when WT Gαo was stimulated with dopamine. [Color figure can be viewed at www.annalsofneurology.org]

developed a clinical severity score system to standardize the assessment of individuals with *GNAO1*-related disorders. Additionally, we delineated the molecular mechanisms (receptor-mediated activation of the G protein, trimer formation, dominant negative activity, and receptor interaction) of each variant included in the present study, developed an experimental measurement score for missense *GNAO1* variants, and analyzed its correlation with the clinical score.

Disease-causing missense variants in *GNAO1* are associated with a neurodevelopmental syndrome that ranges from mild to severe, and is characterized by epilepsy, developmental delay or intellectual disability, hypotonia, and movement disorders.[6–10] Using a disease-specific composite score to quantify the severity among different individuals is necessary for establishing truthful phenotype–genotype correlations. The *GNAO1*-related disorders severity score proposed here incorporates the most relevant aspects of this condition, including epilepsy, movement disorders,

neurodevelopmental issues, and the need for a gastrostomy.

Our team, comprised of pediatric neurologists specialized in both movement disorders and epilepsy, developed the *GNAO1*-related disorders severity score after conducting a thorough examination of the phenotype of individuals with *GNAO1*-related disorders. In addition, we carefully reviewed and analyzed other scales utilized in the assessment of neurodevelopmental disorders and epilepsy to inform our design process.[30]

In regard to items (1) epilepsy and (2) movement disorders, we took into account the most significant factors that influence how these conditions impact the quality of life. This encompasses the frequency, duration, and intensity of the condition, as well as the potential for disruptive events, such as falls, sleep disturbances, pain, or injury. Furthermore, we also considered the response of the condition to treatment.

Despite the absence of falls related to movement disorders or seizures in our cohort, we designed the scale to
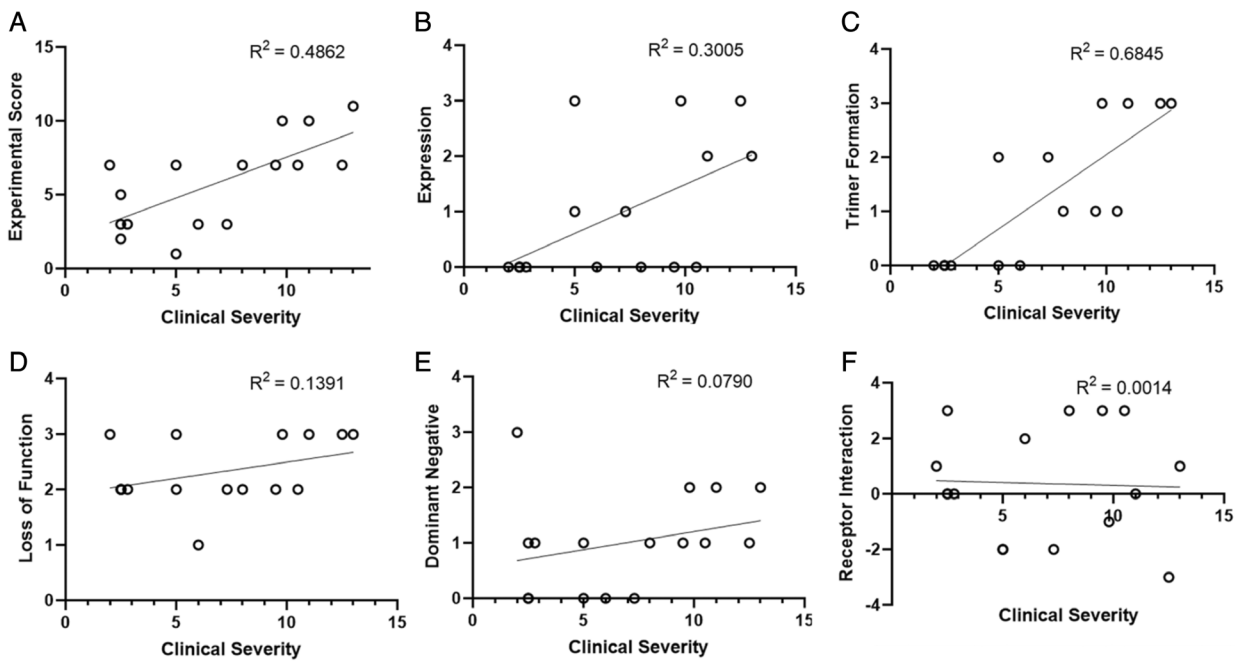
FIGURE 3: Correlations between scores for *GNAO1*-related disorders severity score and experimental measurement. (A–F) Comparisons between clinical and experimental measurements do include repeated measures for patients with the same variant.

be universally applicable to all individuals affected by *GNAO1*-related disorders. Given the possibility that movement disorders and epilepsy may manifest or fluctuate over the clinical course of *GNAO1*-related disorders, it is necessary to undertake additional investigations to confirm the reliability and usefulness of the *GNAO1*-related disorders severity score in larger patient cohorts over an extended time frame. Nevertheless, we observed that in our sample, the severity score appropriately captured the heterogeneous clinical phenotypes and overall severity of each patient, with a wide range of scores and elevated scores consistently correlated with greater disease severity across all domains.

Furthermore, we did not observe any substantial variations in severity levels during the follow-up period of our prospective study. The *GNAO1*-related disorders severity score obtained from our cohort did not show any correlation with age, as we did not observe higher scores in older children. Additionally, in the retrospective analysis of different individuals, we did not identify any significant changes in the severity scores over time. This observation leads us to hypothesize that a significant modification in the severity score of the disorder is more likely to be associated with a therapeutic intervention rather than the natural progression of the condition.

We here present an initial stage in the creation of the *GNAO1*-related disorders severity score. Our aim is to enhance this framework by testing it on a more diverse population, including individuals with varying *GNAO1* variants, to improve the precision of scoring subscales.

Additionally, we are interested in exploring the possibility of applying our severity ranking to individuals with other DEEs, which would be a fascinating avenue of investigation. Limited information on the natural history of specific DEEs,[31] makes disease progression poorly traceable for the majority of DEEs. Yet, as more information becomes available, common severity scoring systems could delineate cross-disorder differences and similarities. Overall, we hope that our *GNAO1*-related disorders severity score will advance the development of therapies for this and related conditions.

The results of our analysis showed significant correlations between the *GNAO1*-related disorders severity score and several standardized scales commonly used to assess developmental and movement disorders (Table S3). Specifically, we found a strong negative correlation between the *GNAO1*-related disorders severity score and the Vineland-II and Bayley-III (cognitive, receptive language, and expressive language sub scores). These findings suggest that as the *GNAO1*-related disorders severity score increases, individuals with *GNAO1*-related disorders tend to show more severe cognitive and language impairments, as well as greater difficulties in adaptive behaviors. In addition, we observed a very strong negative correlation between the *GNAO1*-related disorders severity score and GMFM-88 scale, indicating that as the *GNAO1*-related disorders severity score increases, individuals with *GNAO1*-related disorders tend to have more severe gross motor impairments. Interestingly, we did not find a significant correlation between the *GNAO1*-related disorders

severity score and the Burke–Fahn–Marsden Dystonia Rating Scale or the Abnormal Involuntary Movement Scale, suggesting that the severity of dystonia or involuntary movements may not be strongly related to overall disease severity in this population. In the present study, we expected to find no correlation with the Burke–Fahn–Marsden Dystonia Rating Scale or the Abnormal Involuntary Movement Scale, as we have previously observed in our natural history study that dystonia does not necessarily correlate with the ability to walk (Table S1). There are "mild" phenotypes that are highly dystonic, but still have preserved walking ability. Additionally, we believe that axial hypotonia is a sign that influences motor development more significantly. Another finding that requires further confirmation in a larger cohort is that children with preserved walking ability and generalized dystonia show fewer or no dyskinetic crises compared with hypotonic children with more severe motor impairment.

Remarkably, in our cohort, we identified 3 patients with a very severe phenotype, characterized by marked hypotonia (GMFM-88, 0%), similar to that observed in patients with neuromuscular disease. These patients have very poor voluntary motor control and profound intellectual disabilities, which hinder their ability to follow commands and participate in motor testing. These severe phenotypes are so debilitating that 2 out of the 3 patients have unfortunately died.

The present study is the first to utilize an integrative severity score for each individual with *GNAO1*-related disorders based on a comprehensive clinical scoring system. Similar disease-specific scores have been developed for other rare disorders that have allowed to guide the prognosis of the disease by recognizing clinical or molecular findings as well as establish causal relationships. Brock et al. found a significant inverse correlation between cerebral visual impairment and neurodevelopmental outcome in individuals with DEE 2 due to *CDKL5* variants.[32] Balagura et al. found that the age at the onset of seizures correlates with a poor developmental outcome, as measured by the *STXBP1* (DEE 4) composite developmental score.[33] In the present study, we observed that video-EEG in patients with the most severe *GNAO1*-related disorders showed a remarkable absence of physiological sleep elements and periods of diffuse low-voltage activity during sleep. These characteristics have been identified in other severe DEEs,[34,35] as well as in the previous report of P6.[27] Although more data are needed, this characteristic might be included in the assessment of the severity score for this disorder, or it may even be a potential neurophysiological biomarker for the diagnosis of a *GNAO1*-related disorder. Along the same lines, the mother of P4 and P5, with a severe phenotype, described increased fetal movement.

Although this is somewhat subjective, we believe that rather than epileptic seizures, these movements corresponded to dyskinetic episodes, as both patients with a severe phenotype (11 and 13 points in the severity score, respectively) experience daily dyskinetic episodes. Furthermore, P5 died due to dyskinetic status. Although this is a subjective symptom and needs to be interpreted with caution, this would be the first report of the prenatal onset of movement disorders in *GNAO1*-related disorders. Increased fetal movements suggestive of epileptic seizures have been reported in some neurological disorders; for example, 3-methylglutaconic aciduria.[36] Given these observations, it is likely that increased fetal movement is associated with a more severe phenotype, making the application of prenatal exome sequencing[37] worth considering.

Regarding molecular findings, we found that individuals with the same variant have comparable scores on the *GNAO1*-related disorder severity score (P4 and P5; P11, P12, and P13; and P14 and P15). Furthermore, we found a significant correlation between the clinical severity score and the experimental measurement score of missense *GNAO1* variants. It will be highly interesting if these findings are replicated with a larger sample size, or if genetic modifiers[38,39] or epigenetic factors[40] influence clinical expression as they do for other DEEs. As we have not found significant changes in the severity of symptoms throughout the evolution, it does not appear that changes in the natural history of the disease can significantly modify the correlation with the pathophysiological findings. We have established three levels of severity, namely, mild, moderate, and severe, in our *GNAO1*-related disorders severity score. This stratification takes into account the score variability and aims to group patients in the same range of severity, even if their symptoms may have fluctuated over time.

The results described here support previous findings that any variants in Gαo interfere with receptor-mediated activation of the G protein (Fig 4A). Specifically, it reinforces the assumption that the variants in the P-loop of Gαo reduce the stability of the G protein heterotrimer. Two previously uncharacterized Gαo variants reported here, p.G40R and p.T48I, are located near the P-loop and display decreased interaction with Gβγ. One exception to this observation is the novel variant p.K46R. Although this variant is located in the P-loop, it does not show any significant variation in heterotrimer formation from WT. This is likely due to the conserved nature of the lysine to arginine variant. Additionally, the variants p.T182I, which is located in the Switch I, and p.L199R, which is located on β3, also show a significant decrease in heterotrimer formation. This indicates that in addition to the P-loop, variants in these regions also interfere with

heterotrimer formation. These variants are related structurally to the P-loop, as changes in β3 are passed via α1 to the nucleotide binding pocket.[41] Intriguingly, variants in the Switch II region that directly interact with Gβ, such as p.G203R, p.R209C, and p.R209P, do not interfere with heterotrimer formation. Remarkably, among the categories that comprise the experimental measure score, heterotrimer formation showed the highest correlation with the clinical severity score ($R^2 = 0.685$), thus making it really interesting for predicting the clinical severity of the different variants.

Notably, all the variants studied here interfere significantly with agonist-induced signal propagation. The amount of reduced signal propagation (loss-of-function) gave the strongest correlation with the amount of dominant negative activity presented by a variant (Fig 4B). Surprisingly, the dominant negative activity of a variant had little to do with how strongly it interacted with the receptor. This indicates that the molecular mechanisms of the dominant negative activity for many variants studied remain to be determined. Furthermore, according to our observations, the degree of dominant negative activity is a poor indicator of clinical severity. Indeed, the individual with the only variant showing the most pronounced dominant negative phenotype, p.R209P, only had a mild overall clinical severity score. It is also worth noting that in our study we used the D2 dopamine receptor to model the behavior of Gαo variants. Given the conserved mechanism of G protein activation by GPCRs, we expect similar behavior from Gαo variants with other Gi/o-coupled receptors; however, this assumption remains to be tested.

In our previous studies, we characterized the behavior of p.G203R and p.R209C variants in the endogenous neuronal setting.[11] We found that p.G203R lowered the efficacy of dopamine signaling in indirect-pathway medium spiny neurons (iMSNs), and increased the potency of dopamine signaling in direct-pathway medium spiny neurons (dMSNs). Contrastingly, it reduced adenosine efficacy in dMSNs, and increased potency in iMSNs. In contrast, p.R209C exclusively affected iMSNs by lowering dopamine efficacy, and exclusively affected dMSNs by lowering adenosine efficacy. This difference in response is intriguing, because p.R209C has a mild clinical severity outcome, whereas p.G203R has a severe outcome. It is conceivable that reductions in efficacy in dMSNs and iMSNs are not as pathogenic as increases in agonist potency in these neuronal populations.

Major limitations of the present study include selection bias toward individuals with missense variants, the restricted number of individuals recruited, the limited number of different variants in the GNAO1 gene (12 out of the 60 described to date) included, and the relatively brief period of clinical follow-up. Therefore, further studies are needed to validate the GNAO1-related disorders severity score and verify its usefulness in a generalized clinical routine. Nevertheless, the prospective standardization of data collection and the utilization of the GNAO1-related disorders severity score allow us to address the heterogeneity of these individuals, compare clinical and molecular pathology data, and identify significant correlations.

The majority of current treatments for DEEs focus on individual symptoms, such as seizures or movement disorders, rather than the underlying disease mechanisms.[4] Many individuals with GNAO1-related disorders do not achieve seizure or movement disorder
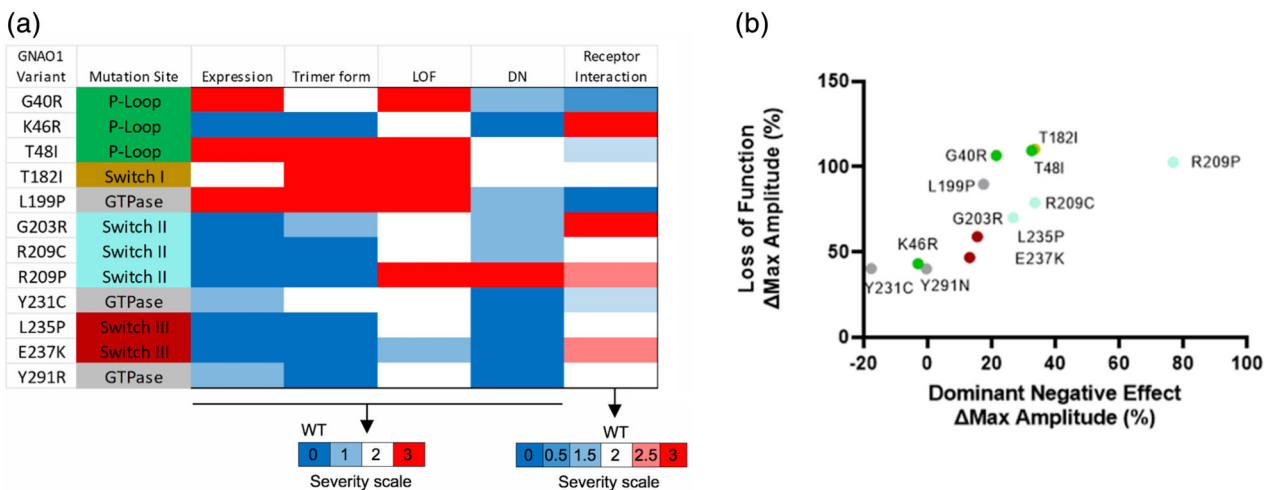

FIGURE 4: Each GNAO1 variant presents a unique functional profile and can be placed on a spectrum of loss-of-function (LOF)/dominant negative activity. (A) Meta-analysis of all GNAO1 variants, combining the results of all functional experiments. (B) Each variant can be placed on a spectrum with loss-of-function and dominant negative properties. Dots are colored according to mutation site as in panel A. [Color figure can be viewed at www.annalsofneurology.org]

control, and even in patients who achieve partial control, neurodevelopmental impairments and other comorbidities frequently continue to be severe.[7] The *GNAO1*-related disorders severity score incorporates the most relevant aspects of this condition, including epilepsy, movement disorders, neurodevelopmental issues, and the need for gastrostomy, and it evaluates the most fundamental aspects of DEEs: epilepsy and movement issues, in a manner that is sensitive to the temporal evolution of these cardinal clinical signs. Our scoring system, together with our molecular findings, can contribute to the design of future trials and studies focusing on natural history. These studies should take into account all of the major aspects of the *GNAO1*-related disorders at different stages among the various subgroups, identify the beneficial endpoints and windows for therapeutic interventions, and determine the optimal timing for therapeutic interventions.[33]

In conclusion, we found that each *GNAO1* variant has a unique profile of clinical and functional phenotypes. Although there is overlap in the clinical outcomes of each variant, each appears to have a unique pathological molecular mechanism. Furthermore, patients harboring the same variants have similar clinical outcomes, indicating that the differences in disease profiles are not due to interpatient variability, but rather, to unique disease mechanisms. As posited previously, each variant lies on a spectrum of loss-of-function and dominant negative activity. Finally, it is likely that the present mechanistic findings will aid in the development of pharmacological interventions for the treatment of *GNAO1*-related disorders. Additionally, the *GNAO1*-related disorders severity score will facilitate standardization of the categorization of patients according to clinical severity and assessment of response to therapies in development.

Author Contributions

J.D.O.E., J.D.C., and K.A.M. contributed to the conception and design of the study; J.D.C., W.G.L., M.J.S., M.F.M., S.B., J.M., D.C.P., I.E., J.D.O.E., and K.A.M. contributed to the acquisition and analysis of data; J.D.C., W.G.L., J.D.O.E., and K.A.M. contributed to drafting the text or preparing the figures. The individuals included in the GNAO1 Study Group have contributed with the data acquisition (including phenotyping and gathering information on patients). They GNAO1 Study Group members and affiliations are included in a Supporting Information Table.

Potential Conflicts of Interest

Nothing to report.

Data Availability

The datasets that support the findings of this study are available from the corresponding authors upon reasonable request.

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