ARTICLES

Movement disorder in *GNAO1* encephalopathy associated with gain-of-function mutations

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ABSTRACT

Objective: To define molecular mechanisms underlying the clinical spectrum of epilepsy and movement disorder in individuals with de novo mutations in the *GNAO1* gene.

Methods: We identified all *GNAO1* mutations reported in individuals with epilepsy (early infantile epileptiform encephalopathy 17) or movement disorders through April 2016; 15 de novo mutant alleles from 25 individuals were introduced into the $G\alpha_o$ subunit by site-directed mutagenesis in a mammalian expression plasmid. We assessed protein expression and function in vitro in HEK-293T cells by Western blot and determined functional $G\alpha_o$-dependent cyclic adenosine monophosphate (cAMP) inhibition with a coexpressed α_{2A} adrenergic receptor.

Results: Of the 15 clinical *GNAO1* mutations studied, 9 show reduced expression and loss of function (LOF; <90% maximal inhibition). Six other mutations show variable levels of expression but exhibit normal or even gain-of-function (GOF) behavior, as demonstrated by significantly lower EC_{50} values for α_{2A} adrenergic receptor–mediated inhibition of cAMP. The *GNAO1* LOF mutations are associated with epileptic encephalopathy while GOF mutants (such as G42R, G203R, and E246K) or normally functioning mutants (R209) were found in patients with movement disorders with or without seizures.

Conclusions: Both LOF and GOF mutations in $G\alpha_o$ (encoded by *GNAO1*) are associated with neurologic pathophysiology. There appears to be a strong predictive correlation between the in vitro biochemical phenotype and the clinical pattern of epilepsy vs movement disorder. *Neurology®* **2017;89:762–770**

GLOSSARY

α_{2A} AR = α_{2A} adrenergic receptor; **AC** = adenylate cyclase; **cAMP** = cyclic adenosine monophosphate; **EIEE** = early infantile epileptiform encephalopathy; **GOF** = gain of function; **LOF** = loss of function; **NF** = normal function; **PLOF** = partial loss of function; **PTX** = pertussis toxin; **WT** = wild-type.

Epilepsy is one of the most common neurologic disorders in the United States.[1] Severe early-onset seizures can result in epileptic encephalopathy.[2] There are at least 52 different gene mutations that cause early infantile epileptiform encephalopathy (EIEE).[3] Mutations in the same genes also cause other neurodevelopmental abnormalities.[4,5] A key challenge in genetic epilepsies has been understanding the genotype–phenotype relationships of causal genes; this may require biochemical analysis.[6]

Mutations in the heterotrimeric G protein $G\alpha_o$ (*GNAO1* gene) cause an autosomal dominant epileptiform encephalopathy (EIEE17, OMIM: 615473).[7] In this original article, all 4 mutations were characterized as having loss of function (LOF).[7] More recently, an extended spectrum of *GNAO1* encephalopathies was identified in which individuals had movement disorders but minimal to no seizures.[8,9] Here, we use *GNAO1* encephalopathy to describe the entire clinical spectrum of individuals with pathologic *GNAO1* mutations. A genotype–phenotype correlation was also recently noted[10]; certain mutations (e.g., E246K and several R209 alleles) were found specifically in children with hypotonia, developmental delay, and chorea but no epilepsy. However, the mechanistic basis for this genotype–phenotype correlation remains unknown.

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Supplemental data at Neurology.org

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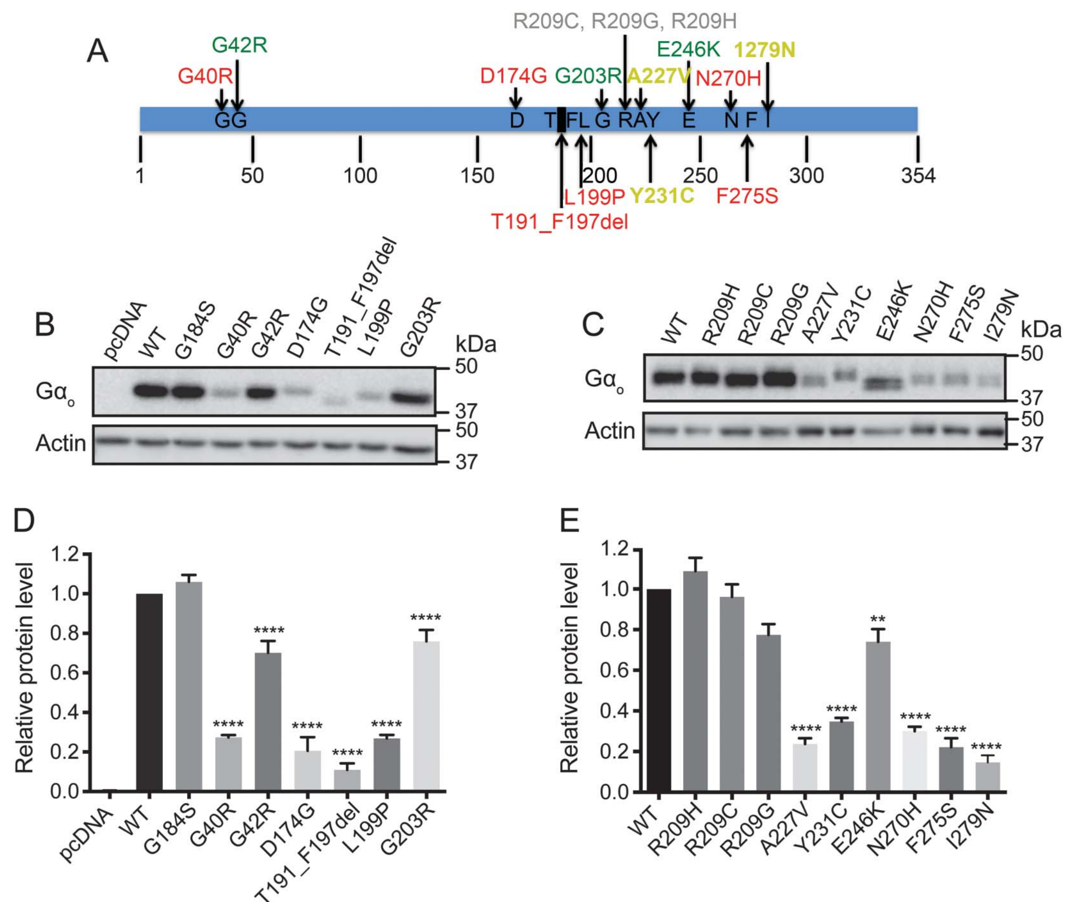
GNAO1 encodes the α subunit of G_o, a heterotrimeric G protein (consisting of α and βγ subunits) that is highly abundant in the CNS, comprising about 1% of brain membrane protein.[11] G_o mediates signals from a wide range of inhibitory receptors including $GABA_B$, α_{2A} adrenergic, adenosine A_1, and dopamine D_2 receptors. A canonical function of $G\alpha_{i/o}$ family proteins is inhibition of cyclic adenosine monophosphate (cAMP).[12] The identification of GOF mutations in *ADCY5* (which encodes adenylate cyclase 5, the enzyme that produces cAMP) in dyskinesia and chorea patients directly links reduced cAMP to involuntary movement disorders.[13–17] This is consistent with GOF behavior of $G\alpha_o$.

Here we assessed expression and function of human mutations in *GNAO1*.[7–9,18–27] Our biochemical analysis identified both LOF and gain-of-function (GOF) behaviors; the latter are associated with movement disorders while the former are primarily found in individuals with epileptiform encephalopathies. This mechanistic insight has important implications for therapies of *GNAO1* encephalopathies.

METHODS The detailed sources of reagents and antibodies, and the methods for mutagenesis, cell culture, transfections, Western blot, and cAMP assays, are described in the e-Methods at Neurology.org. The primer sets used in this study are listed in table e-1.

RESULTS Most pathogenic *GNAO1* mutations cause reduced $G\alpha_o$ protein expression. To evaluate 15 mutations in *GNAO1* (figures 1A and e-1) that were

Figure 1 **Location and protein expression levels of human *GNAO1* mutations related to epileptic encephalopathy**



(A) Location of 15 mutations (G40R, G42R, D174G, T191_F197del, L199P, G203R, R209C, R209G, R209H, A227V, Y231C, E246K, N270H, F275S, and I279N) mapped on the $G\alpha_o$ amino acid sequence. (B, C) Representative Western blots of $G\alpha_o$ protein expression from HEK293T cells transiently transfected with each $G\alpha_o$ mutant. (D, E) Quantification of relative protein levels of each $G\alpha_o$ mutant compared to wild-type $G\alpha_o$. Graphs are the result of 3 independent experiments and data are presented as mean ± SEM. ** $p < 0.01$, **** $p < 0.0001$ using 1-way analysis of variance with Bonferroni post hoc test for pairwise comparison.

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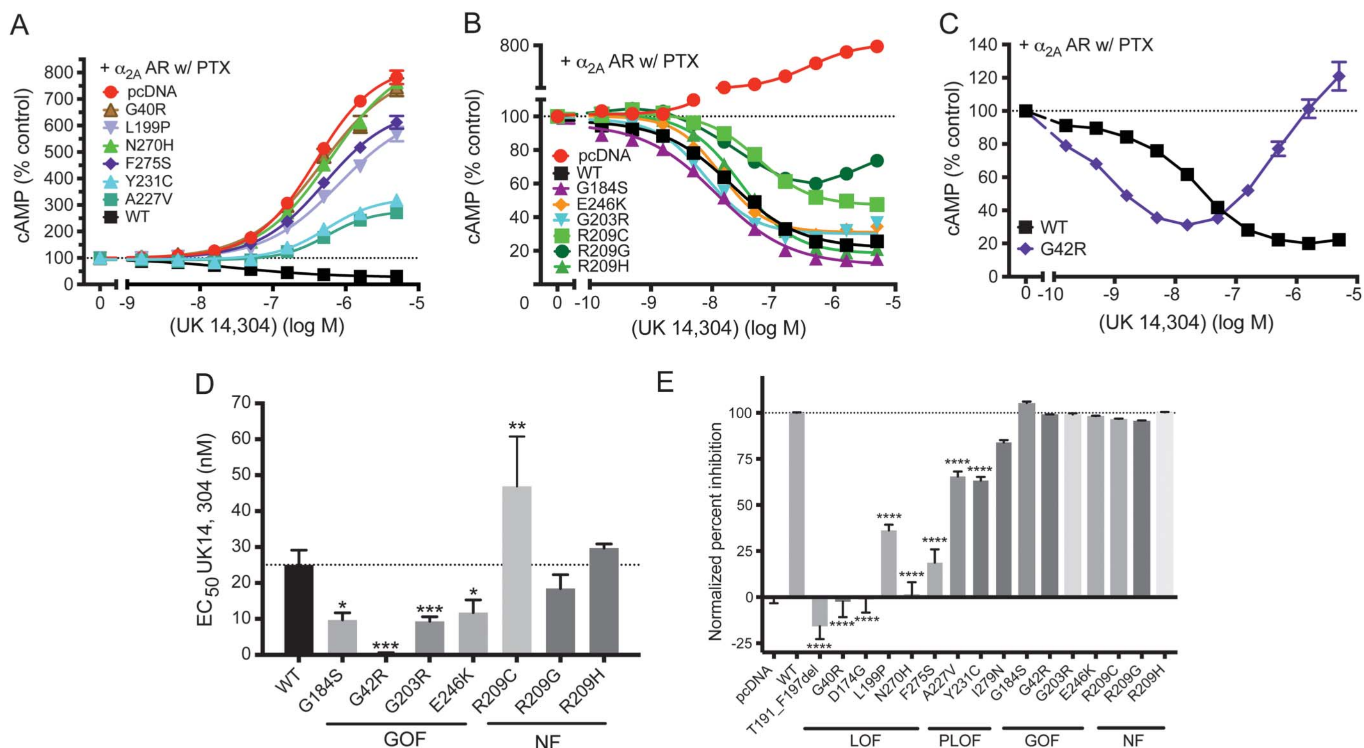
Figure 2 Effect on α_{2A} adrenergic receptor (α_{2A} AR)-mediated cyclic adenosine monophosphate (cAMP) inhibition by *GNAO1* mutants



(A–C) Dose-response curves of representative *GNAO1* mutants. (A) Dose-response curves of loss-of-function (LOF) and partial loss-of-function (PLOF) mutants (G40R, L199P, N270H, F275S, A227V, Y231C) show changes in cAMP production in response to the adenylate cyclase activator forskolin and α_2 AR agonist UK14,304, compared to the positive control (wild-type [WT]) and negative control (pcDNA). (B) Dose-response curves of functioning $G\alpha_o$ mutants show changes in cAMP production in response to the α_2 AR agonist UK14,304. All dose-response curves are shown in comparison with WT and G184S. (C) G42R displays a biphasic dose-response curve with cAMP inhibition at low concentrations (gain of function [GOF]), followed by enhancement of cAMP levels at higher concentrations of UK14,304. (D) Quantification of EC_{50} of functioning $G\alpha_o$ mutants. G42R, G203R, and E246K exhibit significantly increased potency for α_{2A} AR-mediated cAMP inhibition similar to the known GOF mutation G184S. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using paired *t* test between WT and each mutant separately (figure e-4). (E) Percentage of maximum inhibition (n = 5) was normalized to pcDNA (0%; resulting in activation of cAMP) and WT (100%). ****$p < 0.0001$ using 1-way analysis of variance with Bonferroni post hoc test for pairwise comparison. Note that the maximum inhibition of G42R was calculated at UK14,304 of 15.8 nM. NF = normal function; PTX = pertussis toxin.

previously identified in patients with epilepsy or other neurodevelopmental disorders,[7–9,18–27] we performed Western blots in HEK-293T cells transiently transfected with each mutant. The majority of mutants (12) showed significantly lower protein levels than wild-type (WT) $G\alpha_o$, whereas 3 separate Arg^{209} mutant alleles showed essentially normal expression, as did the previously described GOF mutant G184S[28] (figure 1, B–E).

Validation of an in vitro assay to assess function of *GNAO1* mutations. We used inhibition of forskolin-stimulated cAMP levels as a functional readout to allow efficient quantification of $G\alpha_o$ effects with full concentration curves for agonist-mediated signaling. We cotransfected $G\alpha_o$ plasmids with α_{2A} adrenergic receptor (α_{2A} AR) cDNA.[29] Robust inhibition of cAMP by the α_2 adrenergic agonist UK14,304 depends on both the transfected receptor and the $G\alpha_o$ protein (figure e-2, A and B). A pertussis toxin (PTX)–insensitive $G\alpha_o$ (C351G)[30] was used to create all mutant constructs and WT, enabling inactivation of endogenous $G_{i/o}$ proteins using PTX.

When PTX eliminates the inhibitory signaling through $G_{i/o}$, α_{2A} AR couples weakly to G_s and stimulates adenylate cyclase (AC),[31] resulting in increased cAMP levels after PTX treatment in the absence of a transfected $G\alpha_o$ (figure e-2B). Consequently, the fractional inhibition of AC by $G\alpha_o$ mutants was assessed as the decrease from the high control level of cAMP (PTX but no $G\alpha_o$—0%) to the low level with WT $G\alpha_o$ (PTX and PTXi $G\alpha_o$—100%). This is termed normalized % inhibition. PTX treatment did not alter the ability of the PTX-insensitive $G\alpha_o$ to mediate α_{2A} AR-stimulated cAMP inhibition (figure e-2C). Hence, this is a good system to study functional consequences of $G\alpha_o$ mutations.

Nine *GNAO1* mutations result in LOF or partial LOF. Six mutants showed essentially complete LOF with normalized inhibition below 40% (figure 2A and table 1). All of these mutants also showed low

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expression levels (11%–35% of control; figure 1). Three mutants (A227V, Y231C, and I279N; figure 2, A and E, and table 1) had intermediate effects and were classified as partial LOF (PLOF) mutants. The I279N mutation showed a modestly reduced maximal inhibition of cAMP levels (figure 2E and table 1). This mutant also produced a very low EC_{50} value (0.7 nM vs 25 nM for WT $G\alpha_o$), which might explain the discrepancy between the low expression levels (15% of WT) while maintaining good maximal inhibition in the cAMP inhibition assay. Based on the maximum inhibition below 90% of control, however, we classified this mutation as PLOF (table 1).

The dominant nature of the clinical picture in the *GNAO1* encephalopathies raised the question of whether the LOF mutations are actually dominant negative mutations that interfere with the function of the remaining normal $G\alpha_o$ protein expressed in heterozygous individuals. However, in coexpression studies of WT and mutant $G\alpha_o$ at plasmid ratios of 1:1 or 1:2, there was no evidence of a dominant negative action (figure e-3). This suggests that the effect of LOF mutations is through a haploinsufficiency mechanism rather than a dominant negative one.

Six *GNAO1* mutations result in GOF or normal function. Unexpectedly, a significant number of pathologic *GNAO1* mutants showed essentially normal or even GOF behavior (figure 2, B–E, and table 2). As a benchmark for GOF behavior, we used our previously described RGS-insensitive G184S mutant,[28,29,32,33] which shows a mild seizure phenotype in mouse models.[32] It produced a small increase in the maximum inhibition of forskolin-stimulated cAMP levels in response to UK14,304 (table 2). More importantly, it had a significantly more potent response to the α_2AR agonist UK14,304 (figures 2D and e-4A). This represents a 2- to 3-fold increase in signal strength at low agonist concentrations. Three of the human pathologic *GNAO1* mutants also showed GOF behavior by this criterion. The G203R, E246K, and G42R mutants produced robust inhibition of cAMP with significantly lower EC_{50} values for the α_2AR agonist UK14,304 (figures 2D and e-4, and table 2). G203R and E246K showed normal inhibition with modest decreases in EC_{50} (table 2 and figures 2, D and E, and e-4). This is similar to the effect on EC_{50} seen for the bona fide GOF mutant G184S. The G42R mutant showed the lowest EC_{50} of any of the mutants (figure e-4), at least 50-fold lower than the WT protein (figure 2, C and D). However, the inhibition mediated by G42R is followed by activation of cAMP with increasing concentrations of UK14,304 (figure 2C). The calculated normalized % inhibition for the G42R mutant is essentially identical to that of WT $G\alpha_o$, which combined with its very high potency for agonist-mediated inhibition suggests GOF behavior.

Three other patient-derived mutations (R209G, R209H, and R209C) showed almost completely normal function (NF) and nearly normal expression levels and are designated as NF mutants (figures 2, B, D, and E, and e-4, and table 2). The EC_{50} values for R209G and R209H mutant were not significantly different from WT, while the value for R209C was modestly but significantly higher (table 2 and figures 2D and e-4).

Clinical correlation with biochemical behavior of mutant *GNAO1* alleles. To address genotype–phenotype correlations for *GNAO1* encephalopathy, we reviewed the case reports of all 25 individuals who had *GNAO1* mutations that had been reported by April 2016. They have a range of clinical patterns, which extend from early severe epileptic encephalopathy with prominent tonic seizure activity to individuals with a dominant choreoathetotic movement disorder with virtually no evidence of seizures. There are also individuals,[18,21] including one of the original 4 cases[7] (patient 13—G203R; table 3), who had multiple seizures but also showed prominent choreoathetosis.

In 2016, a clinical report[9] described a series of 6 patients with *GNAO1* mutations and a pronounced movement disorder, virtually without seizures. They had global developmental delay and hypotonia from infancy and all developed chorea by ages 4–11 years. In the majority of cases it was intractable, leading to death in 2 cases. Four patients carried the E246K allele, which we have found to be a GOF mutation. The other 2 mutations found in this group (R209G and R209C) exhibited essentially normal function in our cAMP inhibition measurements. There are several other reports[8,9,18–27] of *GNAO1* mutations in individuals with a predominant movement disorder with or without seizures. This distinction of clinical patterns based on certain mutant alleles in *GNAO1* encephalopathy patients was noted very recently[10] but without information about biochemical mechanisms. Table 3 summarizes the $G\alpha_o$ biochemical function from the present report and its relation to seizure disorder or movement disorder in literature reports for these mutations. GOF and NF mutations are nearly always found when movement disorder is the predominant feature of the clinical pattern. Mutations that have pure LOF or PLOF biochemical phenotypes are seen in individuals with epileptic encephalopathy without pronounced choreoathetosis. A number of patients exhibit both seizures and movement disorder. We have indicated in table 3 with major symptoms or minor symptoms which of these features is predominant or less so. Further studies will be needed based on new cases or

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Table 1 Functional data for loss-of-function and partial loss-of-function mutants

Mutations			Expression (% of WT)	cAMP at 5 μM UK14,304 (% of unstimulated)	Normalized % inhibition	$LogEC_{50}$	EC_{50}, nM
pcDNA (no $G\alpha_o$)			0	880 ± 30	0	−6.48 ± 0.05	526
WT			100	23 ± 1	100	−7.71 ± 0.07	25
118G>A	Gly40Arg	G40R	27 ± 1[a]	900 ± 70	−2 ± 8[a]	−6.52 ± 0.13	355
521A>G	Asp174Gly	D174G	21 ± 7[a]	250 ± 20	−1 ± 7[a]	−6.41 ± 0.19	502
517_592del	Thr191_Phe197del	T191_F197del	11 ± 3[a]	290 ± 20	−16 ± 7[a]	−6.36 ± 0.20	525
596T>C	Leu199Pro	L199P	27 ± 2[a]	570 ± 30	36 ± 3[a]	−6.12 ± 0.07	784
680C>T	Ala227Val	A227V	24 ± 3[a]	320 ± 20	65 ± 3[a]	−6.32 ± 0.13	586
808A>C	Asp270His	N270H	30 ± 2[a]	860 ± 60	2 ± 7[a]	−6.42 ± 0.16	499
692A>G	Tyr231Cys	Y231C	35 ± 2[a]	340 ± 20	63 ± 2[a]	−6.34 ± 0.06	472
824T>C	Phe275Ser	F275S	22 ± 4[a]	720 ± 60	19 ± 7[a]	−6.46 ± 0.12	395
836T>A	Ile279Asp	I279N	15 ± 4[a]	100 ± 10	84 ± 1	−9.18 ± 0.15	0.7

Abbreviations: cAMP = cyclic adenosine monophosphate; WT = wild-type.
Values are mean ± SEM.
[a] $p < 0.0001$, one-way analysis of variance.

additional mutations, but there does appear to be a clear pattern emerging about a genotype–phenotype correlation that is driven by a GOF/LOF difference in mutant *GNAO1* alleles.

Location of mutations linked to *GNAO1* encephalopathies in the $G\alpha_o$ protein. To investigate the structural basis for the effects of mutations in $G\alpha_o$, the mutations were modeled onto the published crystal structure of $G\alpha_o$ in complex with RGS16 (PDB: 3C7K; figure e-5). The locations of mutations within the $G\alpha_o$ structure segregated according to their function. The GOF mutations are all near G184S and close to the ribose and phosphate moieties of the bound GDP. The LOF mutants are more broadly scattered throughout the GTPase domain and may destabilize protein folding or stability consistent with their markedly reduced expression levels. The PLOF mutations are clustered in the GTPase domain but away from the bound GDP. This striking structure–function correlation may facilitate prediction of the function of new mutations but ultimately a rigorous

Table 2 Functional data for normal and gain-of-function mutants

Group			Expression (% of WT)	cAMP at 5 μM UK14,304 (% of unstimulated)	Normalized % inhibition	$LogEC_{50}$	EC_{50}, nM
pcDNA (no $G\alpha_o$)			0	880 ± 30	0	−6.48 ± 0.05	525
WT			100	23 ± 1	100	−7.71 ± 0.07	25
550G>A[a]	Gly184Ser	G184S	105 ± 5	15 ± 1	108 ± 0.1	−8.06 ± 0.09[b]	9.7[c]
124G>C	Gly42Arg	G42R	70 ± 6[d]	170 ± 10	99 ± 0.1	−9.34 ± 0.09[e]	0.5[c]
607G>A	Gly203Arg	G203R	72 ± 9[d]	31 ± 3	100 ± 1.3	−8.06 ± 0.06[e]	9.3[c]
736G>A	Glu246Lys	E246K	74 ± 6[f]	39 ± 2	98 ± 0.2	−8.00 ± 0.12[b]	12[c]
625C>G	Arg209Gly	R209G	77 ± 5	74 ± 4	96 ± 0.2	−7.75 ± 0.09	18
626G>A	Arg209His	R209H	109 ± 7	21 ± 1	100 ± 0.1	−7.52 ± 0.02	30
625C>T	Arg209Cys	R209C	96 ± 6	52 ± 2	97 ± 0.2	−7.39 ± 0.08[g]	47[c]

Abbreviations: cAMP = cyclic adenosine monophosphate; WT = wild-type.
Values are mean ± SEM.
[a] Not a human mutation.
[b] $p < 0.05$, paired *t* test.
[c] EC_{50} values significantly different from those of WT.
[d] $p < 0.0001$, one-way analysis of variance.
[e] $p < 0.001$, paired *t* test.
[f] $p < 0.01$, one-way analysis of variance.
[g] $p < 0.01$, paired *t* test.

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biochemical analysis will provide definitive understanding of function.

DISCUSSION The concept of a *GNAO1* encephalopathy has developed based on the identification of at least 15 different mutations in the *GNAO1* gene[7–9,18–27] associated with various combinations of epilepsy, developmental delay, hypotonia, and choreoathetotic movement disorders. Our study demonstrates GOF as well as LOF mutations in *GNAO1* and describes a clear correlation between biochemical and clinical characteristics. The existence of these unexpected GOF mutations has important therapeutic implications. Specifically, one might expect that different approaches to therapy would be needed for different mutations (i.e., agonists for LOF and antagonists for GOF mutants).

We chose inhibition of cAMP production as the functional readout to assess the $G\alpha_o$ mutants because of the robust measurements permitting complete agonist concentration response studies. This was critical to our findings since the GOF mutants were detected primarily through their ability to increase signals at low agonist concentrations (figure 2 and table 2). Our previously studied GOF mutant (G184S), which is insensitive to the inhibitory influence of RGS proteins, shows such a left shift of agonist concentration response curves in vitro[28,34] and in vivo[29,35] and also has a mild seizure phenotype in a mouse model.[32] One might argue that cAMP is not the best choice of functional measures for epilepsy since N-type Ca^{++} channels or the synaptic release mechanism proteins are critical for regulation of neurotransmitter release. However, the apparent correlation of clinical patterns with the biochemical behavior in our cAMP assay suggests that function assessed in this way is relevant to functionality in humans. The clear pathologic effect of

Table 3 **Correlation between cyclic adenosine monophosphate (cAMP) inhibition and clinical diagnosis**

Patient no.	Human *GNAO1* mutations			cAMP inhibition	Epilepsy	Movement disorder	Age	Sex	Ref.
1	124 G>C	Gly42Arg	G42R	GOF		a	Unknown	Female	23
2	736 G>A	Glu246Lys	E246K	GOF		a	13 y	Female	18
3	736 G>A	Glu246Lys	E246K	GOF		a	5.5 y (twins)	Male	9
4	736G>A	Glu246Lys	E246K	GOF		a	5.5 y (twins)	Female	9
5	736G>A	Glu246Lys	E246K	GOF		a	Deceased at 10 y 3 mo	Female	9
6	736G>A	Glu246Lys	E246K	GOF		a	15 y	Male	9
7	625C>G	Arg209Gly	R209G	NF		a	Deceased at 4 y 7 mo	Female	9
8	626G>A	Arg209His	R209H	NF		a	16 y	Male	9
9	626G>A	Arg209His	R209H	NF		a	8 y	Male	24
10	626G>A	Arg209His	R209H	NF		a	6 y	Male	24
11	626G>A	Arg209His	R209H	NF		a	5 y	Male	26
12	625C>T	Arg209Cys	R209C	NF	b	a	18 y	Female	18
13	607G>A	Gly203Arg	G203R	GOF	a	a	8 y	Female	7
14	607G>A	Gly203Arg	G203R	GOF	b	a	14 mo	Female	18
15	607G>A	Gly203Arg	G203R	GOF	a	a	3 y	Female	21
16	692A>G	Tyr231Cys	Y231C	PLOF	a		4 y 9 mo	Female	25
17	680C>T	Ala227Val	A227V	PLOF	a		20 mo	Female	18
18	836T>A	Ile279Asp	I279N	PLOF	a		13 y	Female	7
19	836T>A	Ile279Asp	I279N	PLOF	a		2 y	Male	27
20	118G>A	Gly40Arg	G40R	LOF	a		10 mo	Female	22
21	521A>G	Asp174Gly	D174G	LOF	a		4 y 1 mo	Female	7
22	517_592del	Thr191_Phe197del	T191_F197del	LOF	a	b	Deceased at 11 mo	Female	7
23	596T>C	Leu199Pro	L199P	LOF	a	b	20 mo	Female	8
24	824T>C	Phe275Ser	F275S	LOF	a		9 y	Female	19
25	808A>C	Asp270His	N270H	LOF	a		3 y	Female	20

Abbreviations: GOF = gain of function; LOF = loss of function; NF = normal function; PLOF = partial loss of function.
[a] Major symptoms.
[b] Minor symptoms.

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the R209 mutations (with at least 3 individuals carrying distinct alleles), however, does raise the question of why a protein with normal expression and function would cause pathology. It is possible that the R209 mutations have a selective loss of one of the other functional outputs while retaining a normal ability to inhibit AC. Alternatively, there may be selective alterations in expression or localization in neurons that are not accurately reflected in our HEK-293T cell studies of cAMP regulation. A full understanding of the causal mechanisms in *GNAO1* encephalopathies requires additional studies of these mutant $G\alpha_o$ proteins in neurons and with different functional readouts.

The locations of mutations in the protein structure may partially explain their functional influences. All functioning mutants (NF and GOF) are located around the RGS binding domain, while most of the LOF or PLOF mutants are near the GDP binding region. Two exceptions are D174G and T191_F197del. D174 forms a salt bridge with R162 and mutations in this position may disrupt this interaction. T191_F197del truncates 2 β sheets as well as their linking region, which would be expected to decrease protein stability of $G\alpha_o$.

A dominant genetic effect from GOF mutants is not unusual but the fact that the LOF mutations result in a severe autosomal dominant disorder is a bit surprising. We have ruled out a biochemical dominant negative mechanism of these mutations, at least for cAMP regulation, suggesting a haploinsufficiency mechanism. In mice, homozygous $G\alpha_o^{-/-}$ knockouts exhibit seizures as well as hyperactive turning behavior.[36] We did not, however, observe spontaneous seizures or an increased sensitivity to pentylenetetrazol kindling in heterozygous $G\alpha_o^{+/-}$ knockouts.[32] This suggests that humans are more susceptible to haploinsufficiency of $G\alpha_o$ than are mice. In contrast, we observed enhanced kindling sensitivity and reduced survival in our *Gnao1*$^{+/G184S}$ knock-in mouse model possibly due to seizures.[32] Furthermore, these mice display early neonatal lethality[32] of unclear mechanism, which may be similar to the hypotonia seen in human patients carrying GOF mutations. We do not know whether the abnormalities in these mice are due to brain developmental abnormalities or acute signaling effects. Further studies are needed to better understand this and to determine whether our $G\alpha_o^{+/G184S}$ mutant mouse might represent a useful preclinical model for individuals with *GNAO1* GOF mutations.

To date, all characterized *GNAO1* mutants have been reported as LOF mutations. The G203R mutant in the original article[7] was reported as a LOF mutant for regulation of N-type Ca^{++} channels. Similarly, a G42R mutation in $G\alpha_{i1}$ was reported as a LOF mutant based on biochemical studies.[37] The unique approach that we have taken with detailed cAMP dose–response studies in a mammalian cell model permitted our recognition of the GOF mechanisms (e.g., the $G\alpha_o$ G42R mutation). The patient with the G203R mutation, which we found to have GOF for cAMP inhibition, had a very different clinical pattern than the other 3 patients in the original study.[7] She had a much later onset of disease (7 months) as well as developmental delay and severe chorea with only localized seizures.[7] A similar clinical pattern was observed in 2 more recently described patients with this same mutation.[18,21] All patients carrying the GOF mutations identified here appear distinct from the strict EIEE pattern (table 3). In comparison, patients with LOF or PLOF mutations were diagnosed with either Ohtahara syndrome (Y231C, I279N, D174G, T191_F197del) or EIEE (A227V, L199P, N270H, F275S). Thus GOF and LOF mutations in $G\alpha_o$ appear to result in different disease mechanisms likely requiring different therapeutic approaches.

It has remained challenging to convert knowledge about genetic epilepsy mutations into therapies. The *GNAO1* encephalopathies may be different because of the eminently druggable nature of the receptors that drive $G\alpha_o$ signaling pathways. A critical question then becomes which receptors might be involved. Interestingly, activation of many $G_{i/o}$-coupled receptors is associated with suppression of seizures. Adenosine A1 receptors may play a role in the efficacy of the ketogenic diet[38] and agonists at group II metabotropic glutamate receptors are anticonvulsant in various models.[39] The opposite situation is also seen; $GABA_BR$ agonists exacerbate absence seizures while $GABA_BR$ antagonists suppress them.[40] Identifying which receptors or downstream signaling effectors of $G\alpha_o$ contribute to mechanisms of encephalopathy from LOF or GOF *GNAO1* mutations could therefore reveal potential targets for novel anticonvulsant drug development.

We have identified distinct biochemical mechanisms of pathogenic human *GNAO1* mutations that may improve the understanding of the heterogeneous clinical spectrum of *GNAO1*-associated epilepsy and movement disorders. Furthermore, these results also carry significant implications for personalized therapeutics in *GNAO1* encephalopathies.

AUTHOR CONTRIBUTIONS

Huijie Feng: research design, acquisition, analysis, and interpretation of data, manuscript writing. Benita Sjögren: research design, acquisition, analysis, and interpretation of data, critical revision of manuscript for intellectual content. Behirda Karaj: acquisition of data. Vincent Shaw: interpretation of data. Aysegul Gezer: acquisition of data.

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Richard R. Neubig: research design, interpretation of data, critical revision of manuscript for intellectual content.

STUDY FUNDING

Study funded by the NIH (R01 GM039561 and T32 GM092715).

DISCLOSURE

The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

Received October 26, 2016. Accepted in final form April 17, 2017.

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