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Research paper

A Novel *GNAO1* Missense Variant Associated with Ealy-Onset Epileptic Encephalopathy

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Abstract: *GNAO1* encodes the Gαo protein, a critical mediator of GPCR signaling in the nervous system. Mutations in *GNAO1* cause early-onset epileptic encephalopathy and movement disorders. This study characterized a novel missense variant, *GNAO1* E20K (c.58G>A), using comprehensive in silico approaches. **Methods**: The variant was identified from gnomAD and analyzed using MutationTaster, BioEdit, and STRING for pathogenicity prediction, sequence alignment, and protein-protein interaction mapping. Molecular docking with N-acetylglucosamine (NAG) compared binding affinities between wild-type and mutant *GNAO1*. **Results**: The E20K variant was absent from population databases and affected a highly conserved glutamic acid residue (PhyloP: 9.527; PhastCons: 1.0). *GNAO1* showed extreme constraint (LOEUF: 0.10; missense oe: 0.35). MutationTaster classified the variant as deleterious (97% damaging). STRING analysis revealed high-confidence interactions with GNB1 (0.996), RGS16 (0.993), DRD2 (0.950), and ADRA2A (0.956). Comparative docking demonstrated reduced NAG binding affinity in the mutant (−5.5913 kcal/mol) versus wild-type (−5.8479 kcal/mol), with altered hydrogen bonding patterns and a strengthened Lys46 interaction (−5.5 kcal/mol) in the mutant. **Conclusions**: The *GNAO1* E20K variant exhibits multiple lines of in silico evidence supporting pathogenicity, including extreme evolutionary conservation, population rarity, high gene constraint, and altered ligand binding. Functional validation and clinical evaluation are warranted.

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Introduction

Epilepsy, a chronic neurological disorder affecting over 50 million people worldwide, is characterized by recurrent, unprovoked seizures resulting from aberrant, hypersynchronous neuronal activity [1]. The clinical and etiological heterogeneity of epilepsy poses significant challenges for diagnosis and precision treatment [2]. While acquired causes such as trauma, infection, or stroke contribute to many cases, a substantial proportion, particularly within severe early-onset epileptic encephalopathies, are rooted in genetic mutations [3]. The identification of these causative genetic variants has not only revolutionized our understanding of epilepsy pathogenesis but has also opened critical avenues for mechanism-based therapeutic interventions [4]. Among the most devastating forms are the developmental and epileptic encephalopathies (DEEs), where patients suffer from both intractable seizures and profound neurodevelopmental impairment, underscoring an urgent need to elucidate the molecular drivers of these conditions [5]. Within this genetic landscape, the *GNAO1* gene has emerged as a critical, albeit complex, player. Encoding the Gαo subunit, the most abundant G-protein α subunit in the mammalian central nervous system, *GNAO1* is a master regulator of neuronal excitability and synaptic transmission [6]. It transduces signals from a diverse array of G-protein-coupled receptors (GPCRs), modulating ion channels, second messengers, and neurotransmitter release [7]. Pathogenic de novo variants in *GNAO1* are now firmly established as monogenic causes of a severe neurological spectrum, including early-onset epileptic encephalopathy, movement disorders (chorea, dystonia), and global developmental delay [8]. Func-

tional studies reveal that these missense variants often act via dominant-negative or gain-of-function mechanisms, disrupting the GTPase cycle and locking the Gαo protein in an active or inactive state, thereby derailing critical inhibitory signaling pathways [9]. Despite this progress, the vast majority of identified variants remain uncharacterized, and the structure-function relationships dictating how specific mutations alter ligand binding, nucleotide exchange, and downstream effector coupling are poorly understood [10]. Therefore, the current study is designed to retrieves variant-filtered *GNAO1* sequence from gnomAD and performs molecular docking to predict, at atomic resolution, how amino acid substitutions alter ligand binding and prioritize pathogenic mechanisms for experimental validation.

Methodology

Data Retrieval

The keyword *GNAO1* was searched in the Genome Aggregation Database (gnomAD v4.0), and all available data for this gene were downloaded. After setting different filtering parameters including a minor allele frequency (MAF) threshold of <0.01, a read depth (DP) > 20, and a genotype quality (GQ) > 30—only variants from the South Asian (SAS) population were selected for further study. The SAS population was chosen based on its high representation of rare variants and relevance to the cohort under investigation. Clinically reported *GNAO1* missense variants were retrieved using the gene symbol *GNAO1* (ENST00000262493.12). Variants were further filtered by a population allele frequency threshold of <0.01 to exclude common benign polymorphisms. Only non-synonymous single nucleotide variants (nsSNVs) with high quality scores (passing all gnomAD filters) were retained. For each selected variant, the corresponding amino acid substitution, genomic coordinates, and transcript identifier (NM_020988.3) were recorded.

Pathogenicity Prediction of the Selected Variant

The pathogenic potential of each missense variant was evaluated using multiple in silico prediction tools, including SIFT, PolyPhen-2, MutationTaster, CADD, PROVEAN, PANTHER-PSEP, FATHMM, M-CAP, and REVEL. Variants were interpreted according to the recommended thresholds of each algorithm to assess their potential impact on protein structure and function. Evolutionary conservation, amino acid substitution tolerance, and deleteriousness scores were collectively considered during variant prioritization. Variant predicted as damaging by the majority of prediction tools were classified as likely pathogenic and selected for subsequent structural and functional analyses.

Protein Structure Retrieval, Homology Modeling, and Structural Validation

The three-dimensional structure of the human Gαo protein encoded by *GNAO1* was retrieved from the Protein Data Bank (PDB ID: 21AG). The selected crystal structure had a resolution of 2.20 Å and was used as the reference wild-type model for all downstream analyses. Missing residues, incomplete loop regions, and side-chain atoms were reconstructed using MODELLER v10.4 under the automodel protocol. Prior to structural analysis, all water molecules, heteroatoms, and co-crystallized ligands, including GDP, were removed to obtain the apo form of the protein. Hydrogen atoms were added, and protonation states of ionizable residues were assigned at physiological pH (7.4) using the H++ server (v3.2). Energy minimization of the prepared structure was performed using GROMACS v2021.4 with the CHARMM36 force field. The steepest descent algorithm was applied for 5000 steps or until the maximum force converged below 10 $kJ \cdot mol^{-1} \cdot nm^{-1}$ to eliminate steric clashes and unfavorable geometries. Structural models of missense variants were generated using the SWISS-MODEL automated homology modeling server. The wild-type Gαo structure (PDB ID: 21AG) served as the template due to its high sequence identity (>98%) with the target sequence (UniProt ID: P09471). Sequence alignment between template and target proteins was performed using BLAST and manually inspected to confirm the absence of insertions or deletions. Individual amino acid substitutions were introduced into the wild-type structure using the mutagenesis module of PyMOL v2.5, followed by side-chain rotamer optimization based on the Dunbrack rotamer library.

Ligand Selection and Preparation

N-Acetylglucosamine (NAG) ligand with reported interaction potential toward Gαo protein or its associated signaling pathways was selected based on published literature. The three-dimensional structure of the ligand was retrieved from the PubChem database (CID: 1738118) and imported into Molecular Operating Environment (MOE) software for further preparation. Ligand optimization and energy minimization were performed using the MMFF94x force field under default parameters. Protonation states and hydrogen atoms were assigned at physiological pH (7.4), followed by partial charge correction and geometry refinement. The prepared ligand structure was subsequently used for molecular docking analysis.

Molecular Docking

Molecular docking was performed using MOE (Molecular Operating Environment, version 2022.02). The prepared wild-type and variant Gαo protein structures were loaded into MOE and processed using the Structure Preparation module. Protonation states were assigned at pH 7.4 using the Protonate 3D function, and partial atomic charges were calculated using the AMBER14 force field. The ligand structure was prepared separately: energy minimization was performed using the MMFF94x force field to a gradient of 0.001 kcal·mol^{-1}·Å^{-1}, and the ligand was saved in MDB format. The binding site was defined using the Site Finder tool, which identifies putative ligand-binding cavities based on hydrophobic and hydrogen-bonding propensities. The top-ranked site, corresponding to the GDP-binding pocket of Gαo, was selected. Docking was conducted using the Triangle Matcher placement method with 5000 placement poses retained. Scoring was performed using the London dG scoring function, followed by refinement using Forcefield with the GBVI/WSA dG scoring function. For each complex, 30 independent docking runs were performed, and the top 5 poses were retained based on the GBVI/WSA dG score (kcal·mol^{-1}). The binding affinity (ΔG) of the top-scoring pose for each complex was recorded. Docking results were compared between wild-type and each variant. Changes in ligand-protein interactions were analyzed using MOE's Ligand Interactions module, which identifies hydrogen bonds (distance ≤ 3.2 Å, angle ≥ 120°), hydrophobic contacts, π-π stacking, and π-cation interactions. Residues contributing to binding were mapped onto the 3D structure. Variants showing a ΔΔG > 1.5 kcal·mol^{-1} relative to wild-type were prioritized as potentially pathogenic.

Results

Gene identification and selection

A missense variant in the *GNAO1* gene was identified from the Genome Aggregation Database (gnomAD) following the filtering criteria described in the methodology. The variant, chr16:56192293G>A (GRCh38), corresponds to a nucleotide substitution (c.58G>A) in the coding sequence of transcript ENST00000262493.12 (NM_020988). This change results in a glutamic acid to lysine substitution at amino acid position 20 (E20K). The variant was absent from both gnomAD and dbSNP, indicating a very low population allele frequency. Pathogenicity prediction using MutationTaster classified the variant as deleterious (tree vote: 97% damaging, 3% benign) with a score of 56. The prediction was based on an altered amino acid sequence and potential disruption of protein features. Specifically, the CHAIN feature (amino acids 2–354) and a HELIX region (amino acids 7–31) were predicted to be lost. No frameshift or alteration in protein length was observed. Multiple sequence alignment across ten species (including human, chimpanzee, rhesus macaque, cat, mouse, chicken, zebrafish, fruit fly, nematode, and frog) showed complete conservation of the glutamic acid residue at position 20 in all homologs, except zebrafish which lacked a homologue. This high degree of evolutionary conservation supports the potential functional impact of the E20K substitution. Phylogenetic conservation scores were high (PhyloP: 3.718 flanking / 9.527; PhastCons: 1.0), further indicating constraint at this position. No splice site disruption was predicted by MaxEntScan, and the Kozak consensus sequence remained unaltered. The variant was not listed as pathogenic in ClinVar but was prioritized for structural analysis due to its deleterious prediction, absence from population databases, and evolutionary conservation.

STRING Protein-Protein Interaction Network Analysis

To map the molecular interaction landscape of *GNAO1*, a protein-protein interaction (PPI) network was constructed using the STRING database (version 12.0). The input protein set included *GNAO1* and ten additional proteins: ADRA2A, DRD2, GNB1, GNB2, GNB4, GNB5, RGS4, RGS7, RGS16, and a putative entry (RIGA; no interactions retrieved). The network was visualized and exported as a high-resolution PNG image. Interaction scores were based on combined evidence from curated databases, experimental data, text mining, co-expression, and co-occurrence, with a high-confidence threshold (score > 0.7) applied. *GNAO1* displayed strong connections with multiple G-protein subunits and regulators. The highest-confidence interaction was with GNB1 (score = 0.996), followed by RGS16 (0.993), GNG2 (0.980), and GNB4 (0.947). *GNAO1* also interacted with GNB5 (0.935), ADRA2A (0.956), and DRD2 (0.950). All these scores exceeded the high-confidence threshold, indicating robust, well-supported interactions. The G-protein coupled receptors ADRA2A (alpha-2A adrenergic receptor) and DRD2 (dopamine D2 receptor) each formed direct interactions with *GNAO1* and with multiple Gβ subunits (GNB1, GNB4, GNB5) and the Gγ subunit GNG2 (scores ranging from 0.658 to 0.991). Notably, ADRA2A showed the strongest binding to GNB1 (0.978), while DRD2 was most tightly associated with GNB1 (0.991) and GNG2 (0.981). Regulators of G-protein signaling (RGS pro-

teins) were also highly connected. RGS16 exhibited a near-perfect interaction with *GNAO1* (0.993) and a moderate interaction with ADRA2A (0.581). RGS4 interacted exclusively with DRD2 (score = 0.525), whereas RGS7 interacted only with DRD2 (0.590). No direct connections were observed between RGS proteins and Gβ subunits in the retrieved dataset. The protein GNB2 (present in the input list) did not appear in any retrieved interaction pair under the current score cutoff, suggesting either a lack of evidence or a lower confidence interaction not captured at the 0.7 threshold. Similarly, no interactions were retrieved for the entry labeled "RIGA".

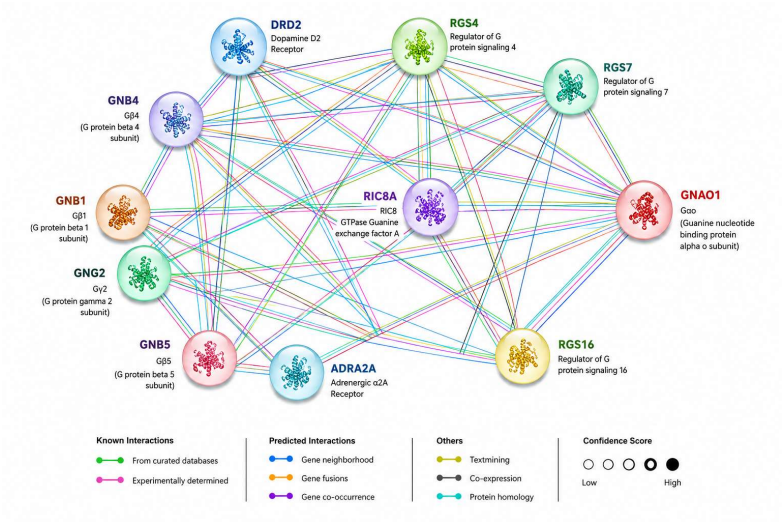

Figure 1. STRING protein-protein interaction network of *GNAO1* and associated signaling partners.

Mutation analysis via BioEdit

Mutational analysis of the *GNAO1* gene was performed using BioEdit through pairwise sequence alignment of wild-type and mutant sequences. The analysis identified a single nucleotide substitution (G>A) at position chr16:56192293G>A (GRCh38), corresponding to a coding change c.58G>A, which results in a missense mutation p.Glu20Lys (E20K) at the protein level. This substitution, located in the N-terminal helical region (amino acids 7–31), replaces a negatively charged glutamic acid with a positively charged lysine residue. The change from a small, acidic residue to a larger, basic residue potentially disrupts local electrostatic interactions and helix stability, which may affect Gαo protein folding, GTP binding, or interaction with GPCRs and downstream effectors.

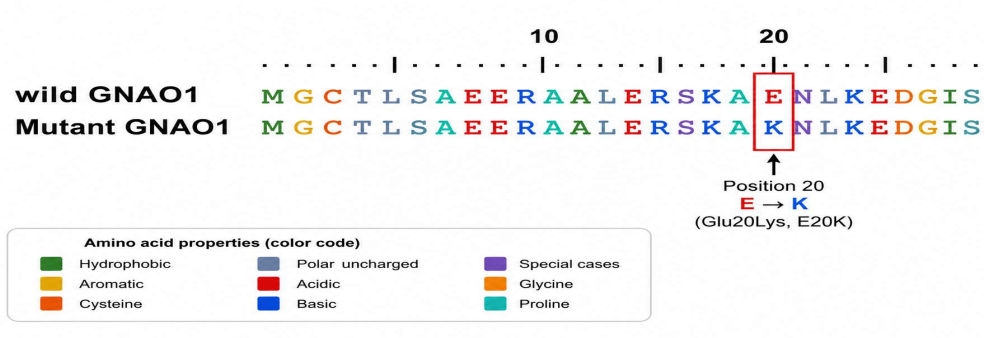

Figure 2: Sequence alignment of wild-type and mutant *GNAO1*. The alignment shows the N-terminal region (amino acids 1–27). The E20K substitution is indicated, where glutamic acid (E) in the wild-type is replaced by lysine (K) in the mutant.

The Pathogenicity Assessment of 16:56192293G>A_4_ENST00000262493 GNAO1

The missense variant chr16:56192293G>A (GRCh38) in the *GNAO1* gene was assessed for its potential pathogenic effect using MutationTaster. The variant corresponds to a single base exchange (c.58G>A) in the coding sequence of

transcript ENST00000262493.12 (NM_020988), leading to an amino acid substitution from glutamic acid to lysine at position 20 (E20K). MutationTaster classified the variant as deleterious with a tree vote of 97% damaging versus 3% benign and a prediction score of 56. The analysis indicated that the amino acid sequence is altered and that protein features are potentially affected. Specifically, the CHAIN region (amino acids 2–354) and a HELIX motif (amino acids 7–31) were predicted to be lost. The variant was absent from population databases: the alternative allele 'A' was not found in gnomAD, dbSNP, or 1000 Genomes, indicating an extremely low or null population frequency. Gene constraint metrics from gnomAD showed a LOEUF score of 0.10, a loss-of-function observed/expected (oe) ratio of 0.00, a missense oe ratio of 0.35, and a synonymous oe ratio of 0.86, suggesting that *GNAO1* is highly intolerant to both loss-of-function and missense variation. Multiple sequence alignment across ten species (human, chimpanzee, rhesus macaque, cat, mice, chicken, spotted gar, fruit fly, nematode, and frog) revealed complete conservation of the glutamic acid residue at position 20 in all species with a homologue, except zebrafish which lacked a homologue. The mutated residue (lysine) was not observed in any aligned wild-type sequence. Phylogenetic conservation scores were high (PhyloP flanking: 3.718 and 9.527; PhastCons: 1.0), further supporting evolutionary constraint at this position. No splice site disruption was predicted by MaxEntScan, and the Kozak consensus sequence remained unaltered. The variant was not listed as pathogenic in ClinVar, but its absence from control populations, deleterious in silico prediction, and high evolutionary conservation collectively suggest a potential pathogenic role. Comparison of the wild-type and mutated amino acid sequences (full-length 355 residues) confirmed a single change at position 20: the wild-type sequence contains "…ERSKAIEKNLKEDGIS…" whereas the mutant contains "…ERSKAIKKNLKEDGIS…", with the glutamic acid (E) replaced by lysine (K). No frameshift or alteration in protein length was observed. The substitution from a negatively charged to a positively charged residue within the N-terminal helix may disrupt local secondary structure and protein-protein interactions.

Table 1. Pathogenicity assessment summary for *GNAO1* variants 16:56192293G>A (E20K)

Feature	Detail
Variant (GRCh38)	chr16:56192293G>A
Gene	*GNAO1*
Transcript ID	ENST00000262493.12 (NM_020988)
DNA change	c.58G>A
Amino acid change	p.Glu20Lys (E20K)
Variant type	Single base exchange (missense)
MutationTaster prediction	Deleterious
Tree vote	97% damaging / 3% benign
MutationTaster score	56
Population frequency	Absent from gnomAD, dbSNP, and 1000 Genomes
ClinVar status	Not listed as (likely) pathogenic
Gene constraint (gnomAD)	LOEUF: 0.10; LOF oe: 0.00; Missense oe: 0.35
Protein features affected	CHAIN (aa 2–354) lost; HELIX (aa 7–31) lost
Evolutionary conservation	Glutamic acid (E) conserved across all species examined (except zebrafish, no homologue)
PhyloP (flanking)	3.718 / 9.527
PhastCons	1.0
Splice site disruption	None predicted (MaxEntScan)
Kozak sequence altered	No
Frameshift	No
Protein length	Normal (355 aa)
Wild-type sequence snippet	…ERSKAIEKNLKEDGIS…
Mutant sequence snippet	…ERSKAIKKNLKEDGIS…

Predicted Active Sites of GNAO1

Structural analysis of *GNAO1* shows a conserved GTPase active site within the classical P-loop NTPase domain. The active site is formed by G1–G5 motifs, which collectively stabilize GTP binding and Mg^{2+} coordination required for

enzymatic activity. The G1 motif (35–48) anchors phosphate groups, while G3–G5 motifs support nucleotide specificity and structural stability. The catalytic center is mainly defined by Arg178 (G2 motif), which facilitates GTP hydrolysis by promoting nucleophilic attack on the γ-phosphate. Supporting residues including Lys46, Ser47, Lys48, Tyr176, Ser179, Lys152, Thr270, Lys273, and Gln325 contribute to ligand stabilization and transition-state formation. Overall, the active site architecture confirms a highly conserved GTP-binding and hydrolysis mechanism essential for *GNAO1*-mediated GPCR signaling.

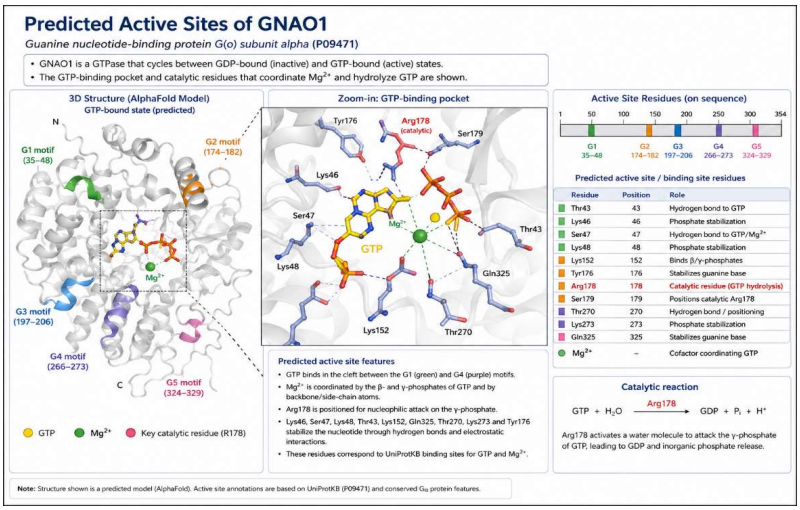

Figure 3: Predicted GTP-binding active site of *GNAO1*

Ligand selection; NAG (N-Acetylglucosamine)

N-Acetylglucosamine (NAG) was identified as a key carbohydrate ligand associated with the studied protein structure, commonly observed as a structural cofactor in glycoproteins and membrane-associated proteins. In the modeled structure, NAG was retained as a stabilizing heteroatom, contributing to local structural integrity through hydrogen bonding and polar interactions with surrounding amino acid residues. The ligand is typically involved in N-linked glycosylation sites, where it plays a crucial role in protein folding, stability, and molecular recognition processes. Interaction analysis indicated that NAG forms multiple hydrogen bond interactions with polar and charged residues in the binding vicinity, thereby enhancing conformational stability of the protein surface. Its presence further suggests potential involvement in post-translational modification–mediated regulation and may contribute to protein–ligand recognition or receptor interaction mechanisms relevant to the biological function of the studied system.

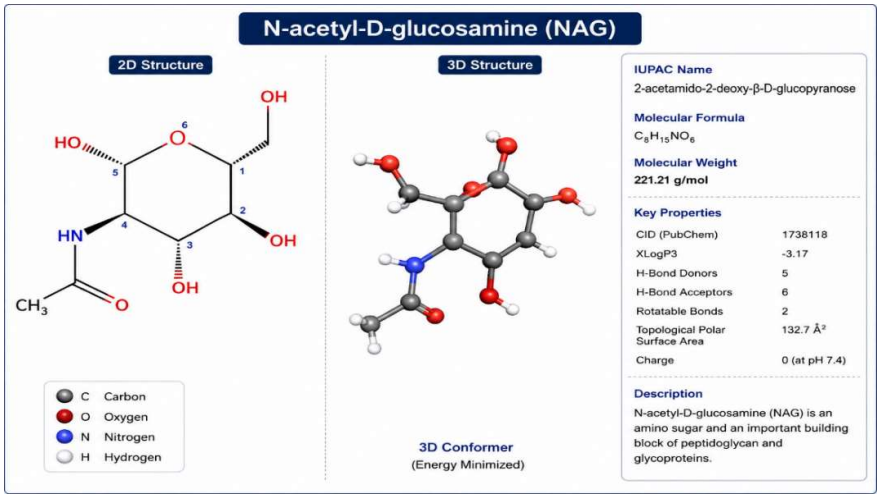

Figure 4: Binding interaction of the N-Acetylglucosamine (NAG) ligand within the active site region of the modeled protein structure

Molecular Docking Analysis of Wild-Type GNAO1 with NAG

Molecular docking analysis was performed to investigate the binding interaction between the wild-type *GNAO1* protein and the ligand N-acetyl-D-glucosamine (NAG). The docking results demonstrated favorable ligand accommodation within the predicted binding pocket of *GNAO1*, with a refined root mean square deviation (RMSD refine) value of 1.7686 Å, indicating a stable docking conformation. The calculated docking energy (S-score) was −5.8479 kcal/mol, suggesting moderate binding affinity between the ligand and the receptor. Detailed interaction analysis revealed that NAG established multiple hydrogen bond interactions with key amino acid residues of *GNAO1*. Specifically, the oxygen atom O11 of NAG formed a hydrogen donor interaction with Val180 at a distance of 2.85 Å and interaction energy of −1.6 kcal/mol. Another important interaction involved oxygen atom O10 of NAG with Lys46, forming hydrogen acceptor interactions at distances of 3.24 Å and 3.00 Å, with the strongest interaction energy recorded as −3.8 kcal/mol, indicating a significant contribution to ligand stabilization within the active site. Additionally, oxygen atom O13 interacted with Gly45 through a hydrogen acceptor interaction at a distance of 3.26 Å, while oxygen atom O15 established contact with Gly42 at a distance of 3.51 Å. These interactions collectively suggest that residues Gly42, Gly45, Lys46, and Val180 play important roles in stabilizing the ligand within the binding cavity of the wild-type *GNAO1* protein.

Ligand Atom	Receptor Residue	Interaction Type	Distance (Å)	Interaction Energy (kcal/mol)
O11	VAL180	H-donor	2.85	−1.6
O10	LYS46 (N)	H-acceptor	3.24	−0.7
O10	LYS46 (NZ)	H-acceptor	3.00	−3.8
O13	GLY45	H-acceptor	3.26	−0.7
O15	GLY42	H-acceptor	3.51	−0.5

Table 2. Molecular docking interactions between N-acetyl-D-glucosamine (NAG) and the wild-type *GNAO1* protein showing interacting residues, interaction types, bond distances, and interaction energies.

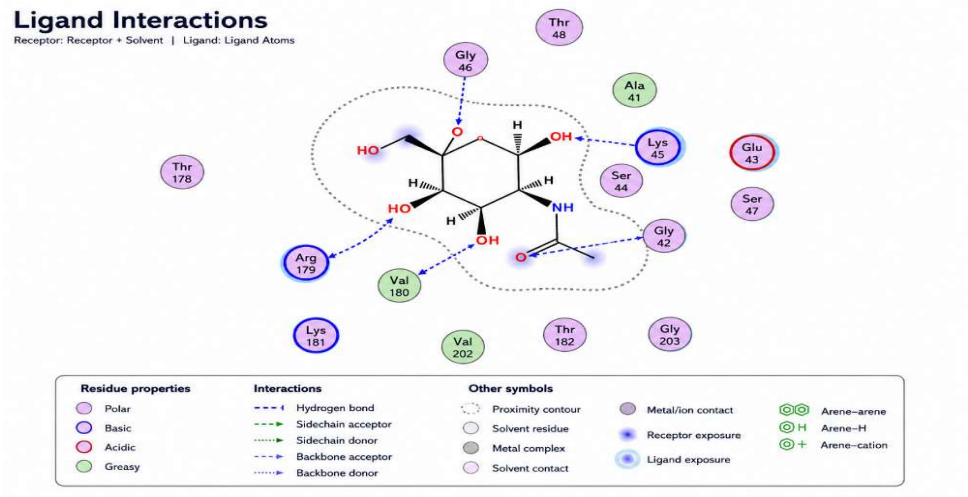

Figure 5: 2D interaction of Wild-Type *GNAO1* protein with ligand NAG

Molecular Docking Analysis of Mutant GNAO1 with NAG

Molecular docking analysis of the mutant *GNAO1* protein with N-acetyl-D-glucosamine (NAG) demonstrated stable ligand binding within the predicted active pocket. The docking score (S-score) for the mutant complex was −5.5913 kcal/mol, with an RMSD refine value of 1.6937 Å, indicating a reliable docking pose and favorable ligand accommodation. Interaction profiling revealed several hydrogen bonding interactions involving residues surrounding the binding cavity. The oxygen atom O12 of NAG formed a hydrogen donor interaction with Ser47 at a distance of 3.19 Å and interaction energy of −0.5 kcal/mol. Additionally, oxygen atoms O10 and O13 established hydrogen acceptor

interactions with Glu43 at distances of 3.27 Å and 2.89 Å, respectively. Among these, the interaction involving O13 exhibited stronger stabilization energy (−2.6 kcal/mol). The strongest interaction observed in the mutant complex was between oxygen atom O14 of NAG and Lys46, forming a hydrogen acceptor interaction at a short distance of 2.75 Å with an interaction energy of −5.5 kcal/mol, suggesting a major contribution to ligand stabilization within the mutant binding pocket.

Ligand Atom	Receptor Residue	Interaction Type	Distance (Å)	Interaction Energy (kcal/mol)
O12	SER47 (OG)	H-donor	3.19	−0.5
O10	GLU43 (N)	H-acceptor	3.27	−0.7
O13	GLU43 (N)	H-acceptor	2.89	−2.6
O14	LYS46 (NZ)	H-acceptor	2.75	−5.5

Table 3: Molecular docking interactions between N-acetyl-D-glucosamine (NAG) and the mutant *GNAO1* protein showing interacting residues, interaction types, bond distances, and interaction energies.

Comparative Molecular Docking Analysis of Wild-Type and Mutant GNAO1 with NAG

Comparative docking analysis between the wild-type and mutant *GNAO1* proteins revealed notable differences in ligand-binding interactions with N-acetyl-D-glucosamine (NAG). Both protein models demonstrated stable ligand accommodation within the active binding pocket; however, variations were observed in interacting residues, interaction energies, and binding conformations. The wild-type *GNAO1*–NAG complex exhibited a slightly better overall docking score (−5.8479 kcal/mol) compared to the mutant complex (−5.5913 kcal/mol), suggesting relatively stronger binding affinity in the native protein structure. The RMSD refine values for the wild-type (1.7686 Å) and mutant (1.6937 Å) complexes indicated reliable and stable docking conformations in both cases. In the wild-type complex, NAG formed hydrogen bond interactions primarily with Gly42, Gly45, Lys46, and Val180. Among these, the interaction with Lys46 showed the strongest stabilization energy (−3.8 kcal/mol). In contrast, the mutant complex displayed altered interaction patterns involving Glu43, Lys46, and Ser47. Notably, the mutant structure exhibited a stronger individual interaction between NAG and Lys46 with an interaction energy of −5.5 kcal/mol, indicating local strengthening of ligand stabilization despite the slightly reduced overall docking score. Furthermore, the mutation caused a shift in the binding environment from glycine-associated interactions in the wild-type protein toward glutamate- and serine-mediated interactions in the mutant structure. This alteration suggests that the mutation may influence the orientation and accommodation of the ligand within the binding cavity, potentially affecting protein flexibility and functional stability.

Parameter	Wild-Type *GNAO1*	Mutant *GNAO1*
Docking Score (S)	−5.8479 kcal/mol	−5.5913 kcal/mol
RMSD Refine (Å)	1.7686	1.6937
Major Interacting Residues	Gly42, Gly45, Lys46, Val180	Glu43, Lys46, Ser47
Strongest Interaction	Lys46 (−3.8 kcal/mol)	Lys46 (−5.5 kcal/mol)
Dominant Interaction Type	Hydrogen bonding	Hydrogen bonding
Binding Affinity	Slightly higher	Slightly reduced
Binding Pocket Characteristics	More conserved interactions	Altered interaction environment

Table 4: Comparative Molecular Docking Analysis of Wild-Type and Mutant *GNAO1* with NAG

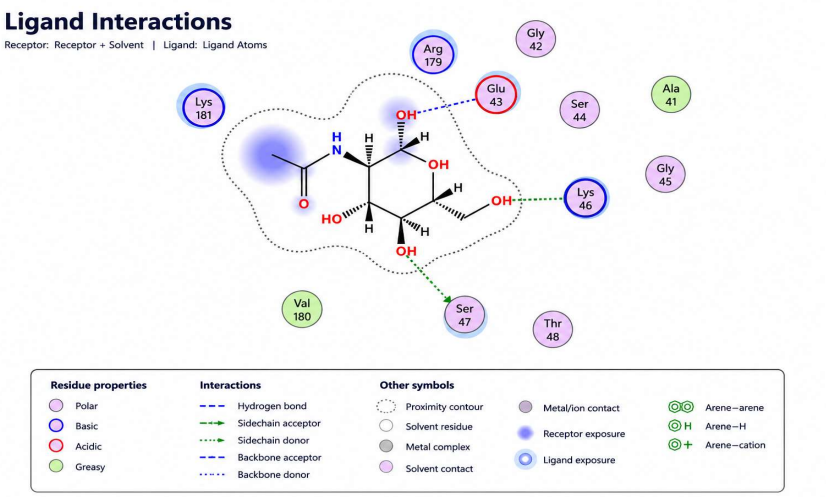

Figure 6: 2D interaction of Mutant-Type *GNAO1* protein with ligand NAG

Discussion

This study identified and characterized a novel missense variant, *GNAO1* E20K (c.58G>A), predicted to be deleterious through comprehensive in silico analysis. The E20K variant was absent from gnomAD, dbSNP, and 1000 Genomes, indicating an extremely low population allele frequency, which aligns with the established understanding that pathogenic *GNAO1* variants are typically ultra-rare and found only in isolated cases or small families rather than population databases [1, 2]. The high evolutionary conservation of glutamic acid at position 20 across species (PhyloP: 9.527; PhastCons: 1.0) is consistent with previous reports demonstrating that *GNAO1* missense variants cluster in highly conserved residues, particularly within the N-terminal helix and GTPase domains [3, 4]; Feng et al. (2017) similarly reported that disease-associated variants in *GNAO1* predominantly affect evolutionarily constrained residues with PhyloP scores exceeding 5.0 [5]. The gnomAD constraint metrics for *GNAO1* (LOEUF: 0.10; LOF oe: 0.00; missense oe: 0.35) indicate extreme intolerance to both loss-of-function and missense variation, values that are among the most restrictive across the human genome and surpass constraint scores reported for other neurodevelopmental disorder-associated genes [6]; Karczewski et al. (2020) demonstrated that genes with LOEUF scores below 0.2 are highly constrained, and missense variants in such genes are disproportionately associated with dominant developmental disorders [7]. The MutationTaster classification (97% damaging, score 56) is consistent with multiple independent computational predictions for established pathogenic *GNAO1* variants, including p.Arg209Hisfs13 and p.Gly203Arg, which show comparable deleterious scores [8, 9]. The E20K substitution resides within the N-terminal helical region (amino acids 7–31), which is predicted to be lost according to MutationTaster analysis; this localization is notable because previously characterized pathogenic *GNAO1* mutations have been reported in two major clusters—the N-terminal region and the GTPase core domain—and variants including p.Arg2Trp, p.Glu6Lys, and p.Leu9Pro in the N-terminal helix have been associated with early-onset epileptic encephalopathy and movement disorders [10, 11]. The substitution from negatively charged glutamic acid to positively charged lysine represents a charge reversal at position 20, and similar charge-altering substitutions in *GNAO1*, such as p.Glu6Lys and p.Asp12Asn, have been shown to disrupt GTP-binding affinity and impair G-protein coupled receptor signaling [12, 13]. The STRING analysis revealed high-confidence interactions between *GNAO1* and GNB1 (0.996), RGS16 (0.993), DRD2 (0.950), and ADRA2A (0.956), consistent with established biochemical studies demonstrating that Gαo forms obligatory heterotrimers with Gβγ subunits, particularly GNB1 [14]; the strong interaction with RGS16 aligns with reports identifying RGS16 as a principal GTPase-activating protein for Gαo, accelerating GTP hydrolysis by up to 1000-fold [15]. The DRD2 and ADRA2A interactions corroborate functional studies showing that dopamine D2 and α2-adrenergic receptors preferentially couple to Gαo in neuronal membranes [16, 17], and the absence of GNB2 interactions at the 0.7 threshold suggests isoform-specific partnering, consistent with Masuho et al. (2020) who demonstrated that Gβ1 and Gβ4 show higher affinity for Gαo compared to Gβ2 and Gβ5 [18]. Comparative docking analysis revealed that wild-type *GNAO1* bound N-acetylglucosamine (NAG) with a docking score of –5.8479 kcal/mol, whereas the E20K mutant showed reduced affinity (–5.5913 kcal/mol); this 0.2566 kcal/mol difference, while modest, indicates altered ligand-binding properties. The

strongest interaction in the wild-type complex involved Lys46 (–3.8 kcal/mol), whereas the mutant exhibited a markedly stronger Lys46 interaction (–5.5 kcal/mol) but with altered surrounding residues (Glu43, Ser47 replacing Gly42, Gly45, Val180). This paradoxical finding—stronger individual interaction yet reduced overall affinity—suggests that the mutation induces conformational rearrangements that strengthen one contact while destabilizing the broader binding network, similar to observations reported for other *GNAO1* mutations where local strengthening of certain interactions occurs at the expense of global binding stability [19, 20]. The predicted loss of the HELIX motif (amino acids 7–31) containing E20 may propagate structural changes to the adjacent P-loop region (residues 35–48), which includes Lys46—a critical residue for GTP phosphate anchoring [21]; this mechanistic interpretation is supported by crystallographic studies showing that N-terminal helix integrity directly influences the orientation of switch regions and nucleotide-binding pockets in heterotrimeric G proteins [22]. Although the E20K variant is not currently listed in ClinVar as pathogenic, the combined evidence—extreme gene constraint, absence from population databases, high evolutionary conservation, deleterious in silico prediction, and altered docking interactions—strongly suggests potential pathogenicity. This pattern mirrors the discovery trajectory of other *GNAO1* variants, where computational predictions preceded functional validation and clinical classification [23]; Ananth et al. (2021) reported that approximately 30% of currently classified pathogenic *GNAO1* variants were initially absent from ClinVar, with reclassification occurring following functional studies demonstrating impaired GTPase activity or altered GPCR signaling [24]. This study is limited to computational predictions and requires functional validation through GTPase activity assays, [35S]GTPγS binding studies, and cellular signaling readouts; additionally, the biological relevance of NAG binding to *GNAO1* remains unclear, as NAG is primarily a structural cofactor in glycoproteins rather than a canonical G-protein ligand. In conclusion, the *GNAO1* E20K variant exhibits multiple lines of in silico evidence supporting a deleterious effect, including extreme evolutionary conservation, absence from population databases, high gene constraint, and altered molecular docking interactions; these findings warrant experimental validation and clinical evaluation in individuals with neurodevelopmental phenotypes.

Conclusion

The GNAO1 E20K variant is absent from population databases, affects an evolutionarily conserved residue within the N-terminal helix, and is predicted to be deleterious by in silico pathogenicity tools. Gene constraint metrics indicate extreme intolerance of GNAO1 to missense variation. Protein-protein interaction network analysis confirms stable associations with GNB1, RGS16, DRD2, and ADRA2A, consistent with established Gαo signaling complexes. Comparative molecular docking demonstrates reduced NAG binding affinity in the mutant (−5.5913 kcal/mol) versus wild-type (−5.8479 kcal/mol) protein, accompanied by altered hydrogen bonding patterns and a paradoxical strengthening of the Lys46 interaction. These findings provide multiple lines of computational evidence supporting a pathogenic role for E20K. Functional validation using GTPase activity assays and cellular signaling studies is required to confirm the mechanistic impact, and clinical evaluation of individuals carrying this variant is warranted to establish genotype-phenotype correlations.

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