Review

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Posted Date: 31 October 2024

doi: 10.20944/preprints202410.2524.v1

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Review

Decoding *GNAO1* Mutations Using *Caenorhabditis elegans* Model System: Past Approaches and Future Prospectives

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Abstract: GNAO1 encephalopathies are a group of neglected genetic disorders primarily occurring due to *de-novo* point mutations in the Gα$_o$ protein-encoding gene in human *GNAO1*. This gene is reported to be highly conserved among *Caenorhabditis elegans (C. elegans)* and humans, with a sequence similarity of nearly 80%. Signaling pathways involved in various neurotransmitters, including GPCR pathways can be easily studied in the *C. elegans* model system. Therefore, using this model system to delineate downstream effectors and clinical targets to Gα$_o$ can be highly advantageous. Mutations that cause GNAO1 encephalopathy can be easily replicated in transgenic *C. elegans* and validated by rescuing phenotypic defects, primarily locomotion and egg-laying defects in worms. Although there are recent technical advancements in understanding the interacting proteins, there are unclear and uncertain hypotheses that explain the effect of Gα$_o$ mutations in humans. Coming to the clinical aspect of this disorder, there are no available approved diagnostic procedures to detect GNAO1 encephalopathy in the early stages of life. The present diagnostic procedures reiterate symptoms and overlap with other neurological symptoms that record neglected data of cases. Therefore, here we provide an overview of past research and a perspective of future work, with the primary objective of focusing on GNAO1 encephalopathy.

Keywords: *Caenorhabditis elegans (C. elegans)*; GNAO1 encephalopathy; Gα$_o$; G-proteins; mutations; disorders; neurotransmitter; phenotype; signaling pathway

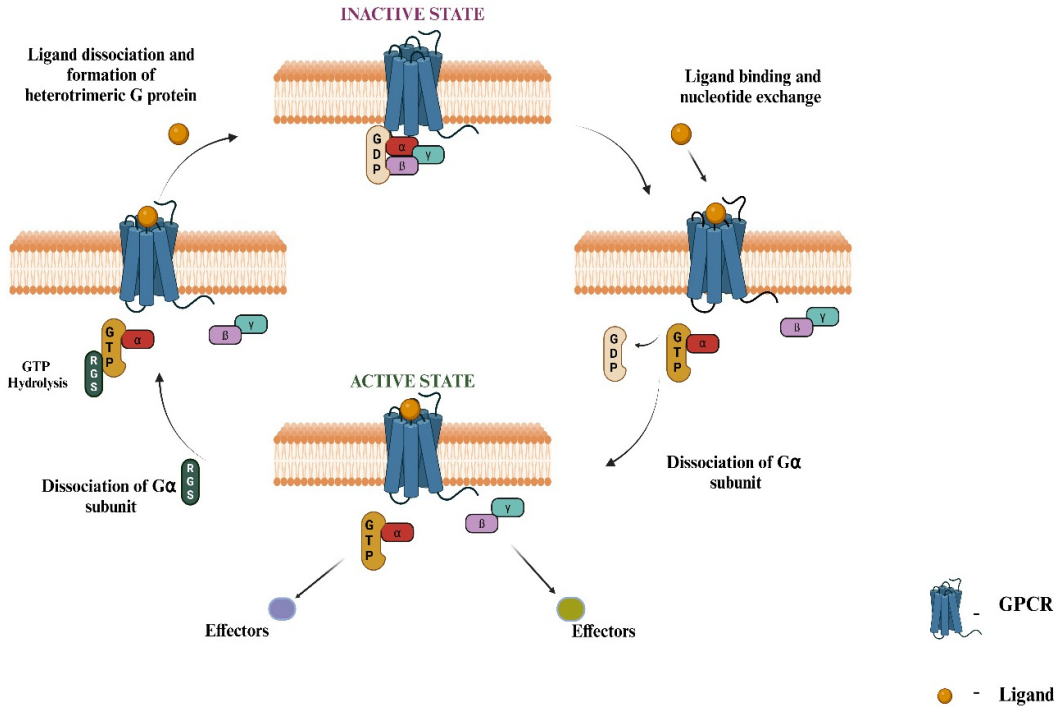
1. Gα$_o$ Signaling Pathway Is Associated with GNAO1 Encephalopathy

G-protein coupled receptors (GPCRs) are the largest class of surface receptors, constituting about 5% of the genome. To date, humans have approximately 1000 GPCRs classified, each with a unique and distinct function [1]. The major functions of GPCRs involve mediating most of the cellular responses to taste, olfaction, and vision [2]. The prominent role of GPCRs is implicated in the fact that they bind to a variety of ligands like neurotransmitters, ions, and hormones and are majorly involved in the transmission of extracellular signals into intracellular responses. The significance of GPCRs lies in their ubiquity and diversity as they are involved in a wide range of biological functions from regulation of blood pressure and heart rate to control of immune responses and release of hormones [3]. GPCRs are coupled with G-proteins (guanosine-binding) proteins, which act as molecular switches, transmitting signals from activated cell surface receptors to intracellular proteins. The activation of G-proteins and their subsequent interaction with effector proteins mediate a wide range of cellular responses, including changes in enzyme activity, ion channel opening or closing, and modulation of intracellular signaling pathways [4]. In its usual conformation, the G-proteins exist as heterotrimeric complexes involving the Gα, Gβ, and Gγ subunits. Neurotransmitter receptors stimulate Gα subunits to exchange bound GDP for GTP. Gα-GTP then separates from the Gβγ, and

 doi:10.20944/preprints202410.2524.v1

these activated G-protein subunits can evoke responses in the cell. Signaling is terminated when the Gα proteins hydrolyze their bound GTP, thus returning to the GDP-bound state and re-associating with Gα **(Figure 1A)** [5]. G-proteins are further classified into four main families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12}$, each mediating different physiological responses depending on the tissue type and signal received [6]**.** Among these, the $G\alpha_{i/o}$ family, $G\alpha_o$ (encoded by the *GNAO1* gene) is the most abundant G-protein found in brain tissue and controls both the development and adult physiology of the brain. It is particularly crucial for regulating neuronal signaling, specifically in neurodevelopment and synaptic transmission [7]. Recently, $G\alpha_o$ has been characterized as an inducer of neuronal differentiation [8]. In *C. elegans*, the homolog of the human $G\alpha_o$ protein is GOA-1. This protein plays a vital role in inhibiting neurotransmitter release by negatively regulating synaptic vesicle exocytosis [9]**.** GOA-1 acts via several signaling pathways, including those involving diacylglycerol (DAG) and protein kinase C (PKC), modulating locomotion and egg-laying behaviors in *C. elegans* [10]**.** Mutations in GOA-1 result in hyperactive neurotransmission that is reflected by hyperactive locomotion and increased egg-laying behavior due to dysregulated synaptic vesicle cycling [11]**.** For the other Gα subunits, the downstream effectors molecules are established using a combination of forward genetics and biochemical analysis. However, $G\alpha_o$ remains the only type of Gα protein in higher eukaryotes for which an effector has not been identified **(Figure 1B)**. This is a remarkable gap in our knowledge since $G\alpha_o$ constitutes 1% of the total membrane protein in the brain, making it orders of magnitude more abundant than other neural G-proteins [12]**.** Over the past two decades, many unsuccessful attempts have been made to classify the effector molecules (signaling proteins) for $G\alpha_o$. The challenge in identifying $G\alpha_o$ effectors through forward genetics could be attributed to several factors. First, the redundancy of multiple functionally similar effectors may mask the effects of mutations in single genes, making it difficult to observe changes in $G\alpha_o$ signaling [13]. Second, if a $G\alpha_o$ effector is vital for survival or reproduction, mutations could result in lethality or impair recovery of such mutants in genetic screens [14]**.**

A.



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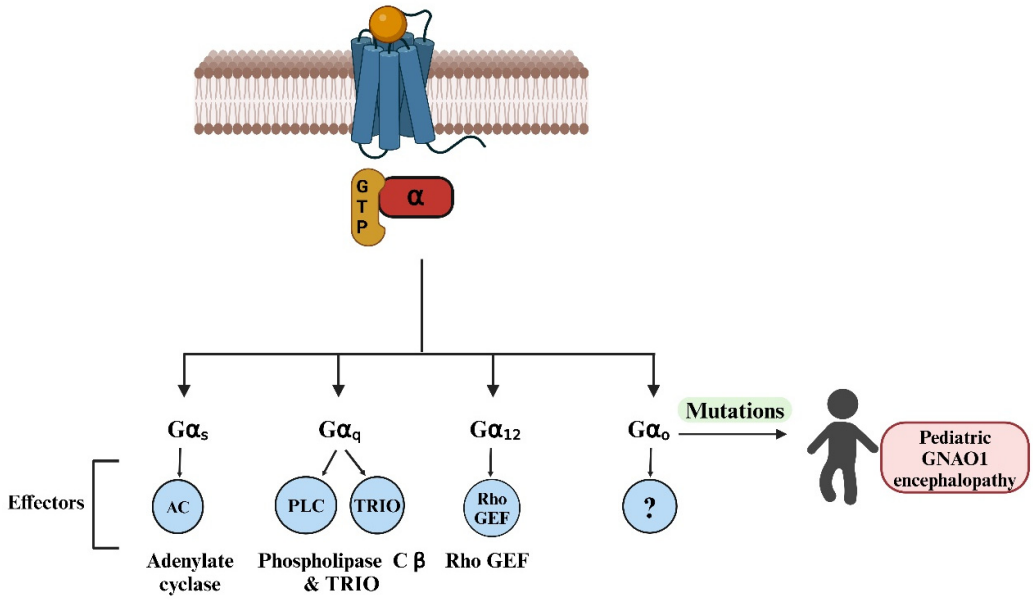

Figure 1. Overview of G-protein-coupled receptor (GPCR) signaling and known G-protein effectors. A) The diagram illustrates the cyclic process of GPCR activation and G-protein signaling. **(Top, left)** In the inactive state, the GPCR is bound to an intracellular heterotrimeric G-protein, composed of Gα, Gβ, and Gγ subunits, with GDP bound to Gα. **(Top, right)** Ligand binding to the extracellular domain of the GPCR induces a conformational change that facilitates GDP release and GTP binding to Gα, activating the G-protein complex. **(Bottom, right)** The Gα subunit dissociates from the Gβγ dimer, and both subunits can interact with downstream effectors, initiating intracellular signaling pathways. **(Bottom, left)** GTP hydrolysis by the **RGS proteins** (**R**egulators of **G**-protein **S**ignaling) leads to the reformation of the inactive heterotrimeric complex and terminates the signaling. This cycle repeats as long as the ligand remains bound to the GPCR. **B)** Four distinct Gα subtypes ($G\alpha_s$, $G\alpha_q$, $G\alpha_{12}$, $G\alpha_o$) are shown, each modulating different downstream effectors: adenylate cyclase (AC) for $G\alpha_s$, phospholipase C β (PLCβ) and TRIO (Trio Rho Guanine Nucleotide Exchange Factor) for $G\alpha_q$, and Rho GEF (Guanine Exchange Factor) for $G\alpha_{12}$. The effector for $G\alpha_o$ is currently unknown (denoted by "?"). Mutations in the *GNAO1* gene, which encodes the $G\alpha_o$ protein, are associated with pediatric GNAO1 encephalopathy, as highlighted on the right side of the diagram. These mutations can lead to severe neurological conditions, underscoring the clinical relevance of $G\alpha_o$ in brain development and function. (Figures created with BioRender.com).

The human *GNAO1* gene encodes $G\alpha_o$, the α subunit of heterotrimeric G-proteins that play important neuro-modulatory functions by coupling with various GPCRs, including dopamine, serotonin, and opioid receptors. [15]**.** Although various signaling events in which $G\alpha_o$ is involved have been described, systematic actions of $G\alpha_o$ in the nervous system have not been fully understood. Numerous *de-novo GNAO1* mutations are associated with neurodevelopmental disorders, collectively termed **GNAO1 encephalopathy-related disorders or GNAO1 encephalopathy,** which mainly include developmental and epileptic encephalopathy 17 (also called early infantile epileptic encephalopathy) (DEE) and neurodevelopmental disorder with involuntary movements (NEDIM) [16]. GNAO1 encephalopathy has a broad, emerging phenotypic spectrum. One core phenotype is weakened movement which can be a characteristic of chorea, dystonia, and dyskinesia, and other altered phenotypes can feature epilepsy and developmental delay. [17]. Evaluation of GNAO1 disorder-associated mutations in mice has reiterated some of the phenotypes of GNAO1 encephalopathy, including impaired movement and seizure susceptibility. Testing pathological GNAO1 mutations in other model organisms will be valuable in assessing the conserved functional effects of these genetic perturbations, and their influence on movement. $G\alpha_o$ is highly conserved in invertebrates including the nematode *C. elegans* where its ortholog, G-protein o-alpha subunit (GOA)-

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1, regulates locomotion and egg-laying circuits [14,18]. The extremely well-defined genetics of GOA-1 in *C. elegans* make this an ideal *in-vivo* system for evaluating the functional impacts of pathological GNAO1 mutations. To date, efforts to characterize pathological mutations of *GNAO1* at the molecular level have yielded promising, but conflicting results in mammalian and rodent systems. An early study evaluated a pertussis toxin-insensitive version of $G\alpha_o$ using a heterologous cell-based assay and placed these pathological mutations in three categories: loss of function, gain of function, and normal function [14]. Recent evaluation of rescued $G\alpha_o$ indicated that *GNAO1* mutations result in loss of function with several mutations reported to antagonize transduction of GPCR signals by acting as dominant negatives [15]. Particularly notable are differing in vitro results with G203R, R209C, and the less well-characterized G42R mutation. These were initially described as gain of function or normal function and were subsequently found to be loss of function and dominant negative. As a result of these differing conclusions, the functional effects, and mechanisms of GNAO1 pathological mutations remain unresolved. Intense interest has emerged in understanding $G\alpha_o$ function in the nervous system and developing intervention strategies for GNAO1 encephalopathy. Thus, there is a persuasive need to use *in-vivo* models to study the behavioral impact of GNAO1 disorder-associated mutations. Here, we highlight the importance of studying *GNAO1* mutations and effectors of $G\alpha_o$, in the context of GNAO1 encephalopathy using *C. elegans* as the model system.

2. Current Status of GNAO1 Encephalopathy

2.1. GNAO1 Encephalopathy: Current Treatments and Clinical Insights

GNAO1 encephalopathy remains considerably rare with only around 400 annual cases reported globally, according to the latest data [19]. However, the number is possibly an underrepresentation of cases, especially from developing countries due to a lack of awareness and diagnostic challenges on genetic diseases. There is no available cure for GNAO1 encephalopathy-related disorders [20]. Investigation on GNAO1-related disorders has identified that deep brain stimulation treatments are efficacious, yet they pose the risk of surgical procedures and failure of medical machinery [21]. A case study by Weihao and his colleagues in 2022 suggested the efficacy of the drug oxcarbazepine in treating GNAO1-related movement disorders. Oxcarbazepine is conventionally used for the treatment of epilepsy as oxcarbazepine is a sodium ion channel blocker. Administration of drugs such as tiapride hydrochloride, phenobarbital, benzodiazepines, and various hormones were found to be ineffective in treating GNAO1-associated movement disorders. Oxcarbazepine is a potent drug to treat movement disorders caused by *GNAO1* mutation which can treat abnormalities like rigidity and twisting of limbs and trunk or chorea particularly in the variant p.Glu237Lys [20]. Clinical investigation performed by Ananth et al., identified the use of neuroleptics and tetrabenazine treatment (commonly used against symptoms of chorea) for 6 patients facing continuous recurring missense mutation of *GNAO1*, analyzed by whole exome sequencing, is only useful to a limited degree as the high dosage treatment only worsened the symptoms of chorea in these patients [22]. Another investigation carried out by Danti et al. 2017 on 7 patients suffering from *de-novo* missense and splice site *GNAO1* mutations, identified by next-generation sequencing highlighted that tetrabenazine was moderately controlled dyskinesia for 2 patients. One patient experienced drug-resistant seizures, and the other five had adequately controlled epilepsy. One patient's life was saved with emergency deep brain stimulation (DBS) [23]. The other emerging area of treatment for genetic mutations is gene therapy where the RNAi approach has been widely explored. RNA interference is the biological process that involves the silencing of gene expression mediated by the formation of double-stranded RNA in the system. This mechanism for the first time was discovered in the *C. elegans* model system [24]. RNAi as a potential approach to gene therapy was described way back in 2003 [25]. For the first time, evidence supporting the successful RNAi-based gene therapy has been shown by targeted nanoparticles in human cancerous cell lines [26]. These advancements have paved the way for RNAi-based therapeutic approaches for genetic diseases causing neuronal abnormalities like epilepsy and movement disorders. One such approach involves the strategy "Silence-and-replace" mechanism where shRNA mediated by Adeno-Associated Vector (AAV)-DJ serotype vectors in primary mouse neuronal cultures that resulted in suppression of endogenous $G\alpha_o$ [27]

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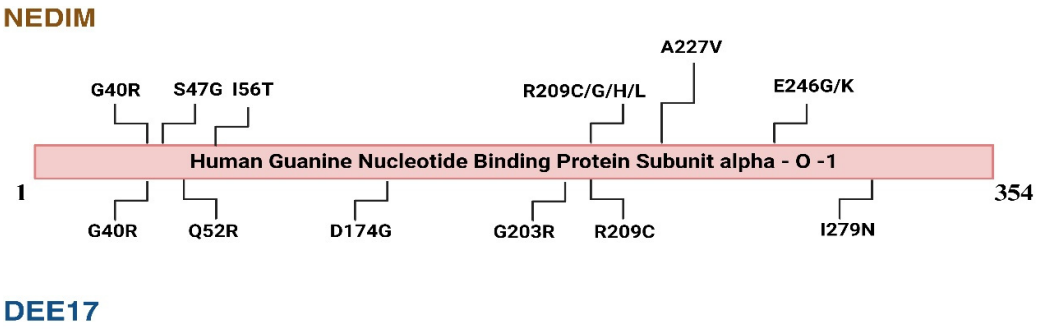
RNAi as a therapeutic approach to GNAO1 encephalopathies is an active area of research in GPCR biology.

2.2. GNAO1 Encephalopathy: Mutation Analysis and Phenotypic Defects

Recent studies suggest that silencing the pathogenic variant at the genetic level can prevent phenotypic defects [28]. All the major mutations occurring in *GNAO1* have been represented in **Figure 2A** which fall under two categories of neurodevelopmental disorders, i.e., NEDIM and DEE17. Among these, studies indicate the occurrence of more frequent G203R, R209C, or E246K mutations in GNAO1 encephalopathies [29]. It has been shown in mice models that contain G203R pathogenic mutant of *GNAO1* are neonatally lethal even in heterozygous conditions [30], but the same heterozygous variant has been successfully silenced using RNA inference in cell-based assays [28]**.** The phenotypic defects of GNAO1 encephalopathies in mouse models are evident in the later stages of their life while in humans, it is pediatric. The mutations are further classified as Loss of function and Gain of Function, affecting the levels of active $G\alpha_o$. As discussed in **Figure 2B**, the defects in humans and mice are indistinguishable making it difficult to assess the outcomes of the mutations. In contrast, *C. elegans* model system, the phenotypic defects are evident with distinguishable locomotory and egg-laying defects. These findings collectively suggest the need for a simpler yet conserved model system to find the potential treatment for GNAO1 encephalopathy. *C. elegans* has proved to be an important *in-vivo* system for assessing the functional genetic effects of *GNAO1* pathological mutations. Prior research has shown evidence that *C. elegans* is useful in investigating the molecular genetic basis of neurodevelopmental disorders [31–34]**.** Indeed, *C. elegans* could be an ideal tool for functionally evaluating the increasing number of *GNAO1* pathological variants identified to date.

A.



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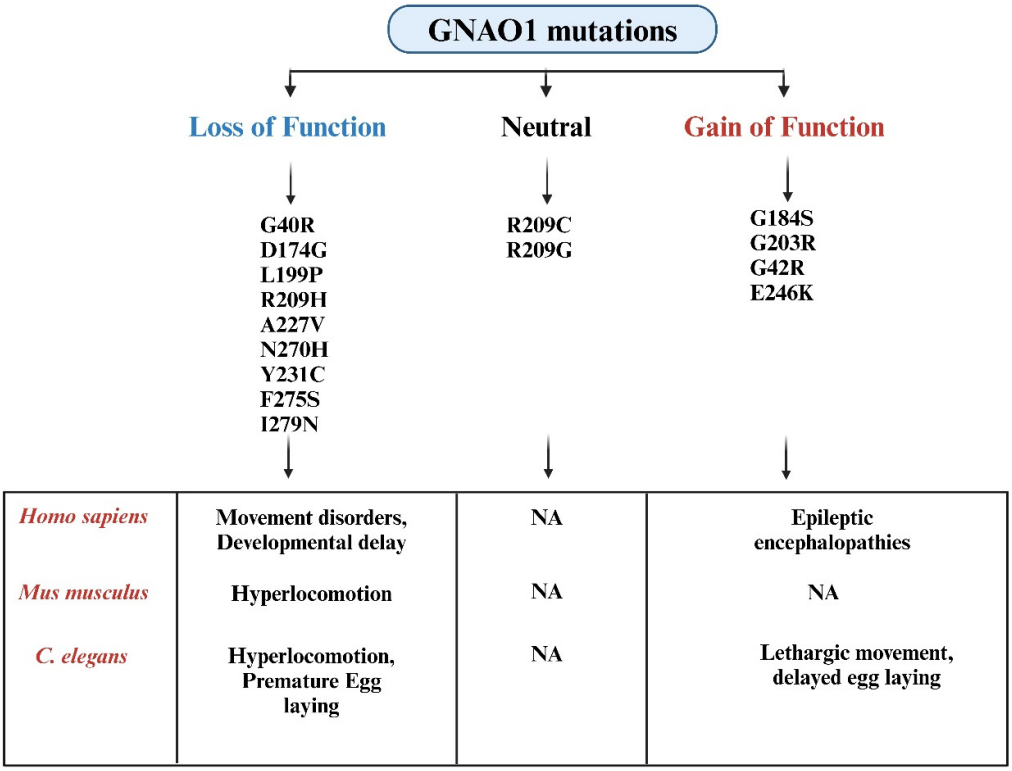

Figure 2. Mutational landscape of *GNAO1* mutations associated with NEDIM and DEE17 syndromes and overview of their phenotypic effects in *Homo sapiens, Mus musculus, and Caenorhabditis elegans* A) The linear schematic represents the hGNA01 protein sequence (1-354 amino acids), highlighting the locations of disease-related mutations. Mutations identified in patients with Neurodevelopmental Disorder with Involuntary Movements (NEDIM) are shown above the protein, while mutations associated with Developmental and Epileptic Encephalopathy 17 (DEE17) are indicated below the protein. Key mutations include G40R, S47G, I56T, and A227V among others, with multiple variations at residue R209. B) Mutations in the *GNAO1* gene are categorized into three groups: loss-of-function mutations (left, blue), neutral mutations (center, black), and gain-of-function mutations (right, red). Each category lists relevant mutations based on their functional impact. Loss-of-function mutations include G40R, D174G, L199P, R209H, A227V, N270H, Y231C, F275S, and I279N, while gain-of-function mutations include G184S, G203R, G42R, and E246K. R209C and R209G represent neutral mutations. The bottom section highlights the phenotypic changes associated with these mutations across different species, including *Homo sapiens*(humans), *Mus musculus* (mice), and *C. elegans* (nematodes). Loss-of-function mutations are associated with movement disorders and developmental delays in humans, whereas gain-of-function mutations cause epileptic encephalopathies. In *Mus musculus*, loss-of-function mutations result in hyperlocomotion, but no phenotypic data is available for gain-of-function mutations. In *C. elegans*, loss-of-function mutations lead to hyperlocomotion and premature egg-laying, while gain-of-function mutations cause lethargic movement and delayed egg-laying. (Figures created with BioRender.com).

3. *C. elegans* as an Advanced Model System to Study GNAO1 Mutations

GNAO1 mutations remain highly understudied because of the non-availability of human subjects due to the extremely low number of reported cases and technical difficulties, permissions, and ethical concerns associated with obtaining human samples. To mitigate the mutations responsible for GNAO1 encephalopathy, researchers have traditionally been using rodent models, particularly mice [30]. There were reported studies of unsuccessful attempts in mice models to study GNAO1-related disorders [35]. Further, in the quest to find a simpler model system, an attempt was made to replace *Drosophila melanogaster*'s $G\alpha_o$ with human *GNAO1* which led to the production of an

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inaccurate nucleotide sequence of the gene. While approximately 75% of human genes associated with diseases have counterparts in *Drosophila*, the proteins they produce differ, which may limit the translation of findings from the *Drosophila* model to human conditions, particularly in drug discovery [36]. These reports establish the need for the use of the alternative model system with the ease of genetic manipulation and evident phenotypic defects. *C. elegans* has been established as a valuable experimental model for understanding GNAO1-related disorders and exploring potential treatments [37]. The researchers created two genetically modified strains with mutations at key positions Glu246 and Arg209, known to be crucial in $G\alpha_o$. These loss-of-function (LOF) mutations caused varied reductions in $G\alpha_o$-mediated signaling, resulting in excessive neurotransmitter release from different neuron types. This led to hyperactive behaviors like increased egg laying and movement [38]. Notably, single-copy mutations demonstrated cell-specific dominant-negative effects, determined by the specific altered residue. Similar to earlier mutants S47G and A221D, caffeine effectively mitigated the excessive movement in animals with the R209H and E246K mutations, implying caffeine's independent action from the mutation type. In contrast, the adenosine receptor antagonist Istradefylline worked in R209H animals but not in E246K worms, suggesting caffeine's multifaceted mode of action [37]. Overall, these findings deepen our understanding of the disease mechanisms and provide more evidence for caffeine's potential efficacy in managing dyskinesia linked to *GNAO1* loss of function mutations. In contrast, models involving the gain-of-function (GOF) mutation of $G\alpha_o$, [G203R] or $G\alpha_o$ [R209C] in striatal neurons led to impaired locomotor behavior in mice which provides insights into G protein functioning and its mutants [39], but its relevance as a disease model is still not clear. Interestingly, hyperactivity seen in C215Y/+ mice is associated with the motor cortex rather than anatomical defects in the striatum [30]. These findings underscore the complexity of modeling GNAO1 encephalopathy and the importance of accurately replicating the disease phenotype in animal models for meaningful research and therapeutic development. *C. elegans* provides an excellent opportunity to define the genetic mechanisms by which *GNAO1* variants affect locomotor behavior and could be key for resolving outstanding mechanistic issues about the molecular pathology of GNAO1 encephalopathy. Moreover, *C. elegans* has the potential to be developed as an *in-vivo* platform capable of evaluating large numbers of *GNAO1* variants and could be used for genetic and small molecule screens targeting *GNAO1*. In *C. elegans* mutations associated with disorders, tested by CRISPR editing of the native GOA-1/$G\alpha_o$ locus, lead to abnormal locomotor behavior also same result was found in previous research in rodents and suggests emerging common principles governing how *GNAO1* pathological variants impact $G\alpha_o$, function in live organisms. The similarities between locomotor behaviors in *C. elegans* and rodent models make this correspondence quite reasonable. Both are sensitive to dopaminergic modulation, the *C. elegans* motor circuit comprising excitatory cholinergic and inhibitory GABAergic motor neurons, and GABAergic striatal neurons (dMSNs and iMSNs) responsible for motor coordination in mice [40–43]. In both cases, loss of $G\alpha_o$ function results in hyperactive locomotion. Furthermore, $G\alpha_o$, signaling serves to inhibit neuronal activity in both *C. elegans* and mammals [44,45]. These conserved features have proven advantageous in the profiling of *GNAO1* variants.

The spectrum of GNAO1 encephalopathy is wide and continually evolving. As mentioned previously, the central aspect of this condition is compromised motor function, along with epilepsy and developmental delay which are prevalent features of this phenotype. $G\alpha_o$ being a major G-protein, is expressed across many brain regions including the cerebellum wherein it is cellularly localized within the Purkinje fibers, basket, and stellate cells which inhibit GABAergic interneurons and Golgi neuronal cells. It forms connections with various crucial G protein-coupled receptors (GPCRs) such as GABA-β, α2 adrenergic, adenosine A1 (A1R), and dopamine D2 (D2R) receptors. These receptors are essential for controlling neurotransmitter release, movement, and neural development functions. There are numerous postulated downstream targets in the signaling pathway of $G\alpha_o$, as well as the other members of the $G\alpha$ family. Many of the targets affected by $G\alpha_o$ signaling are also implicated in disorders related to movement. Mutations in other signaling molecules remain understudies but are of great importance. For example, genetic mutations in ADCY5, the gene responsible for encoding adenylyl cyclase type 5, have been identified in patients

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with dyskinesia and dystonia [46,47]. Acknowledging the significant diversity in clinical manifestations is crucial to comprehend the molecular mechanisms behind GNAO1-related disorders. This diversity encompasses early-onset epileptic encephalopathy, as indicated by [17], as well as patients with intricate movement disorders, some accompanied by epilepsy [22]. The prevailing characteristics among patients harboring *GNAO1* mutations are hypotonia and developmental delay irrespective of their clinical presentation or biochemical traits. After these, choreoathetosis and dystonia are the subsequent most frequent observations [17,48,49]. While a notable proportion of individuals exhibit atypical EEG or MRI results, fewer than fifty percent of those with *GNAO1* mutations displayed distinctly aberrant EEG patterns, primarily among patients with epilepsy and loss-of-function (LOF) mutations. This diversity, encompassing variations in clinical presentation and impact on brain structure/function, suggests an involvement of both neurodevelopmental changes and disruptions in functional signaling. The latter factor seems to be more prominent in patients with gain-of-function (GOF) mutations, who exhibit fewer indications of structural brain abnormalities and display partial positive responses to drug interventions. The potentially uncertain underlying factors contributing to GNAO1-related movement disorders can be elucidated by examining GNAO1 signaling. One potential pathway involves the canonical inhibition of cAMP by $G\alpha_o$, which can be facilitated by $G\alpha_o$ or the liberated $G\beta\gamma$ [50,51]. Notably, mutations in ADCY5, responsible for encoding an AC protein that generates cAMP, also lead to movement abnormalities in human patients. The disruption of cAMP signaling has been linked to impaired brain function [52]. Consequently, disturbances in cAMP levels could perturb the delicately balanced neurodevelopmental system suggesting the operation of $G\alpha_o$ via the cAMP pathway might be important in movement disorders, yet the downstream effectors are not studied [48].

A second theoretical foundation for GNAO1-associated movement disorders pertains to $G\alpha_o$'s involvement in governing neurotransmitter release. The deficiency of crucial neurotransmitters like catecholamines (such as dopamine, epinephrine, and norepinephrine) and serotonin has been extensively studied in the context of movement disorders or seizures [53]. $G\alpha_o$'s presynaptic role in regulating neurotransmitter release presents another potential avenue in the understanding of movement disorder etiology.

A third conceivable perspective, centered on developmental considerations, involves potential alterations in the maturation of neurons, a process vital during appropriate stages of neurological development. Consequently, children with developmental abnormalities might display irregular behaviors. Notably, most individuals with GNAO1-linked movement disorders also suffer from significant developmental delays. Morphologically, MRI scans may reveal widespread atrophy and delayed myelination. In general, genetic factors account for around 40% of cases involving developmental delay, including intellectual disability [54]. Control over cAMP levels and neurotransmitter release can influence ongoing neural functions as well as neurological development. Within this framework, GNAO1-associated movement disorders could arise from disruptions in either or both processes. The former scenario would likely be more amenable to therapeutic interventions compared to the latter.

The conserved nature of G-protein signaling across species enables us to use the *C. elegans* model system, while also highlighting the specific downstream effectors unique to each organism. *GNAO1* mutations significantly impair normal biological functions, manifesting as distinct phenotypes—locomotory defects in *C. elegans* and defective neuronal development in humans. Understanding these pathways can provide critical insights into the molecular mechanisms underlying GNAO1-related diseases and may offer therapeutic targets for treating associated disorders. In *C. elegans*, the $G\alpha_o$ protein is involved in several signaling cascades. Upon activation, $G\alpha$ dissociates from the $G\beta\gamma$ subunits, initiating a downstream signaling cascade that regulates key biological processes. These include serotonin release via *tph*-1, muscle contraction through the hermaphrodite-specific motor neuron (HSN) [55], and GPCR-independent signaling through Ric8 [56], which mediates $G\alpha$ signaling even in the absence of receptor activation. The regulator of G-protein signaling (RGS) protein, egl-10, modulates $G\alpha$ activity by accelerating GTP hydrolysis [57], returning $G\alpha$ to its inactive state bound to GDP. Mutations in *GNAO1* disrupt these pathways, resulting in egg-laying

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and locomotory defects in *C. elegans*, highlighting the critical role of G-protein signaling in neural and muscular functions. In *Homo sapiens*, mutations in *GNAO1* are linked to neurological disorders, including epilepsy and movement disorders. Similar to *C. elegans*, Gα dissociates from Gβγ upon activation by GPCRs. However, in humans, *GNAO1* mutations impact neuronal signaling pathways involved in neurite outgrowth. Specifically, these mutations influence Rho-GTPase signaling, which is modulated by GPRIN (G-protein-regulated inducer of neurite outgrowth) [58]. These proteins possibly interact with GNAO1 and Rho-GTPase via the CDC-42 pathway [59], which regulates cytoskeletal dynamics essential for neurite outgrowth and neuronal morphology. Additionally, the interaction between GNAO1 and Necdin-E2F1 modulates cell cycle regulation, further affecting neuronal differentiation [60]. Defects in these pathways due to *GNAO1* mutations result in impaired neurite development, contributing to the observed neurological phenotypes in patients**. (Figure 3).** A clear picture of all the events is still unknown, opening a plethora of opportunities in the field of GNAO1 biology.

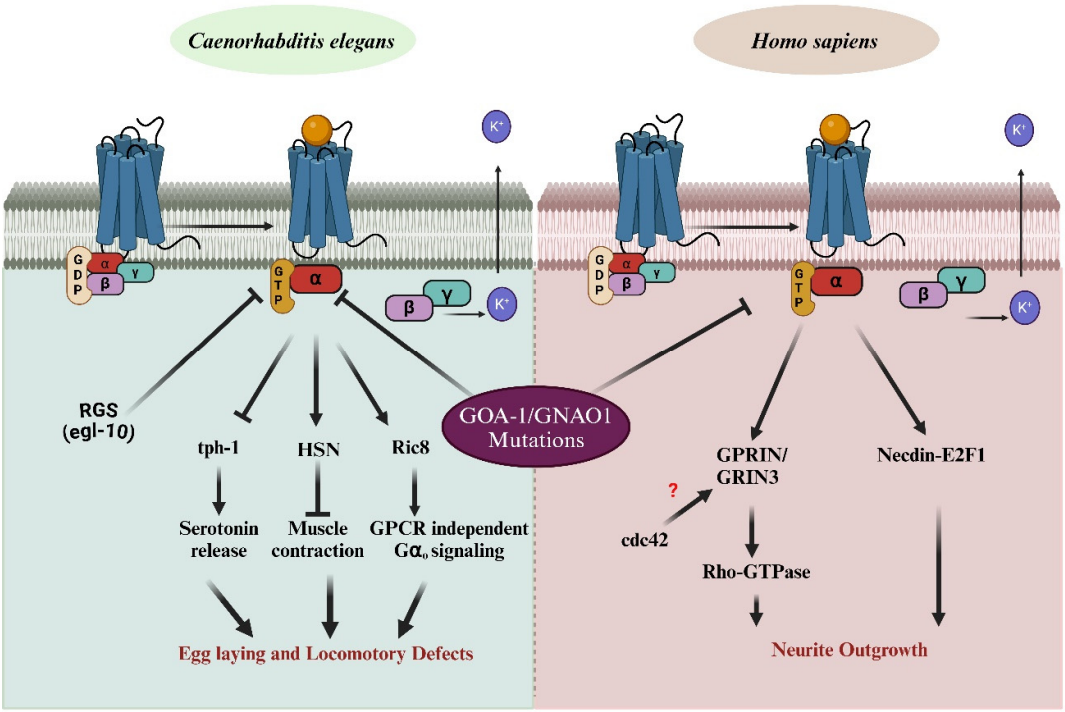

Figure 3. Impact of *GNAO1* mutations on G protein signaling pathways in *C. elegans* and *Homo sapiens*. The left panel illustrates normal G-protein signaling pathways in *C. elegans*, showing the interaction of Gα (bound to GDP) with Gβγ upon GPCR activation. GNAO1 (Gα) regulates processes such as serotonin release, muscle contraction, and egg-laying behavior via downstream effectors such as tbh-1 and Ric-8. Mutations in *GNAO1* lead to defects in egg laying and locomotion. The right panel demonstrates the G protein signaling in *Homo sapiens*. GNAO1 mutations disrupt neurite outgrowth by affecting the interaction with Rho-GTPase signaling, Necdin-EF1, and the GRIN/GRPN complex, with potential involvement of cdc-42, which remains unclear. Arrows indicate pathways affected by GNAO1 mutations, contributing to abnormal neuronal growth and function. RGS—Regulator of G-protein Signaling; tph-1—Tryptophan Hydroxylase; Ric8—Resistance to inhibitors of Cholinesterase; GRIN-1—Glutamate Inotropic Receptor NMDA Subunit -1; cdc-42—Cell Division Control protein–42 (Figures created with BioRender.com).

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4. Conclusion and Perspective

The study of GNAO1 encephalopathy, a genetic disorder caused by mutations in the *GNAO1* gene, which encodes for the $G\alpha_o$ subunit of G-proteins, presents significant challenges due to the complexity of G-protein-coupled receptor (GPCR) signaling pathways in the nervous system [61]. $G\alpha_o$, one of the most abundant membrane proteins in the brain, plays a crucial role in neuro-modulation by interacting with various GPCRs, such as dopamine, serotonin, and opioid receptors [39]. This genetic disorder manifests in patients as a broad spectrum of symptoms, including developmental delays, epilepsy, involuntary movements, and other motor dysfunctions [62]. The exact mechanisms underlying these phenotypes remain poorly understood, and while mammalian models have provided some insight into the effects of *GNAO1* mutations, they often present conflicting results, making it difficult to derive conclusive findings. In this context, *C. elegans* a nematode with a well-conserved G-protein signaling pathway similar to that in humans, has emerged as an ideal *in-vivo* model for studying the functional impacts of *GNAO1* mutations. *C. elegans* $G\alpha_o$ (*goa-1*) shares approximately 80% sequence similarity with the human *GNAO1* gene [63], making it a valuable tool for assessing the consequences of mutations on locomotion, neurotransmitter release, and other nervous system functions.

Several advantages contribute to *C. elegans* being the preferred model system for GNAO1 research. The simplicity of its neural circuit, coupled with its highly conserved genetic pathways, allows researchers to effectively replicate the mutations observed in human GNAO1 encephalopathy. These mutations can be introduced into *C. elegans* using CRISPR editing, enabling precise study of their effects on behavior, such as locomotion and egg-laying, which mirror the impaired movement and motor dysfunctions seen in human patients [38,64]. Additionally, *C. elegans*' rapid life cycle and ease of genetic manipulation facilitate large-scale screenings of *GNAO1* variants, providing a high-throughput platform for studying the functional impact of these mutations. Previous studies in rodent models have confirmed the involvement of $G\alpha_o$ in movement disorders, but *C. elegans* offers the added benefit of being able to dissect the molecular mechanisms of these mutations with greater precision and at a faster pace. For example, recent research using *C. elegans* has demonstrated that mutations in key residues, such as Glu246 and Arg209, lead to hyperactive behaviors like excessive movement and egg-laying, reflecting the overactive neurotransmitter release seen in patients with *GNAO1* mutations [38]. The ability to observe these phenotypic changes in real time makes *C. elegans* an invaluable model for understanding how specific mutations disrupt $G\alpha_o$-mediated signaling pathways.

In terms of therapeutic research, *C. elegans* has proven instrumental in identifying potential treatments for GNAO1 encephalopathy. For instance, studies have shown that caffeine can mitigate the hyperactive phenotypes caused by certain *GNAO1* mutations, offering a potential avenue for pharmacological intervention. This finding highlights the multifaceted nature of caffeine's action, as it was effective across different mutations, suggesting that it may act on multiple pathways involved in $G\alpha_o$ signaling [21]. Similarly, drugs like oxcarbazepine, traditionally used to treat epilepsy, have shown promise in managing movement disorders associated with *GNAO1* mutations, further emphasizing the utility of *C. elegans* in pre-clinical drug screening [20]. These findings underscore the potential of *C. elegans* not only as a model for understanding the genetic basis of GNAO1 encephalopathy but also as a platform for discovering novel therapeutic approaches. Beyond its use in studying movement disorders, *C. elegans* offers a unique opportunity to investigate the role of *GNAO1* mutations in epilepsy, a common feature of GNAO1 encephalopathy. Although much of the research has focused on the motor dysfunctions caused by *GNAO1* mutations, seizures are a significant and often life-threatening symptom of this disorder [65]. *C. elegans'* well-characterized nervous system and responsiveness to various neurotransmitters, including GABA and dopamine, make it an ideal system for studying the mechanisms of seizure induction and progression. This model has already been used to study seizure-like activity in other genetic disorders, and its application to GNAO1 research could provide new insights into how these mutations increase seizure susceptibility. While *C. elegans* provides a powerful model for studying GNAO1 encephalopathy, there remain significant challenges in fully elucidating the downstream pathways

 doi:10.20944/preprints202410.2524.v1

through which $G\alpha_o$ mutations exert their effects. The pathways involving GNAO1 are intricate, with numerous interactions between GPCRs, G proteins, and their downstream effectors, many of which remain undefined. Moreover, the clinical diagnosis of GNAO1 encephalopathy is often delayed due to the overlap of symptoms with other neurological disorders, further complicating efforts to understand the full spectrum of this disease. Future research in *C. elegans* will need to focus on mapping these pathways and identifying specific molecular targets that could be used for early diagnosis and intervention. In conclusion, *C. elegans* serves as an invaluable model for studying the molecular and phenotypic consequences of *GNAO1* mutations. Its conservation of $G\alpha_o$ signaling pathways, combined with its genetic tractability, provides a unique platform for both basic research and therapeutic development. By utilizing *C. elegans* researchers can gain deeper insights into the mechanisms underlying GNAO1 encephalopathy and develop targeted treatments that address the complex interplay of motor dysfunctions, seizures, and developmental delays characteristic of this disorder. As research continues to advance, *C. elegans* will undoubtedly play a crucial role in unraveling the mysteries of GNAO1-related neurological disorders and improving outcomes for affected individuals.

Abbreviations

1. ADCY5—Adenylyl cyclase type 5
2. CRISPR—Clustered Regularly Interspaced Short Palindromic Repeats
3. DAG—Diacylglycerol
4. DBS—Deep brain stimulation
5. DEE-17—Developmental and epileptic encephalopathy-17
6. EEG—Electroencephalography
7. GABA—Gamma-aminobutyric acid
8. GDP- Guanosine diphosphate
9. GNAO1—G-protein subunit alpha-O-1
10. GPCR—G-protein-coupled receptor
11. GTP- Guanosine triphosphate
12. HSN—Hermaphrodite-specific motor neuron
13. MRI—Magnetic resonance imaging
14. NEDIM—Neurodevelopmental disorder with involuntary movements
15. PKC—Protein kinase C
16. RGS—Regulator of G-protein signaling
17. RNAi—RNA interference

Author Contributions: Review Structuring- S.K, S.Y, S.S.V; Literature Searching- S.Y, S.S.V, S.B, S.G, S.N, S.N.B; Manuscript Draft- S.K. S.Y, S.S.V, S.B, S.G, S.N, S.N.B; Manuscript Critical Revision- S.K, Figure Preparation- S.S.V, S.Y.

Acknowledgments: We heartily thank our lab members Ajay Pradhan, Debolina Sarkar, Vandna Maurya, and Lavan Bansal for their critical evaluation and valuable feedback on the manuscript. S.K.'s laboratory is funded by the Department of Biotechnology, India (BT/PR38584/MED/122/247/202), Department of Science and Technology (DST), India (CRG/2021/000732) and DBT/Wellcome Trust India Alliance (IA/I/22/2/506480). S.Y is supported by the ICMR-Junior Research Fellowship.

Conflicts of Interest: The authors declare no competing interests.

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