Restoration of the GTPase activity of Gαo mutants by Zn²⁺ in GNAO1 encephalopathy models

Yonika Larasati
University of Geneva, Faculty of Medicine

Mikhail Savitsky
University of Geneva, Faculty of Medicine

Alexey Koval
University of Geneva, Faculty of Medicine

Gonzalo Solis
University of Geneva https://orcid.org/0000-0002-2359-3780

Vladimir Katanaev (vladimir.katanaev@unige.ch)
University of Geneva, Faculty of Medicine https://orcid.org/0000-0002-7909-5617

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Abstract

*GNAO1* encephalopathy is a rare pediatric disease characterized by motor dysfunction, developmental delay, and epileptic seizures\(^1\)\(^-\)\(^3\). *De novo* point mutations in the gene encoding Ga\(^\alpha\), the major neuronal G protein, lie at the core of this dominant genetic malady\(^4\). Half of the clinical case mutations fall on codons Gly203, Arg209, or Glu246 near the GTP binding/hydrolysis pocket of Ga\(^\alpha\)\(^1\)\(^-\)\(^3\). We here show that these pathologic mutations strongly speed up GTP uptake and inactivate GTP hydrolysis by Ga\(^\alpha\), resulting in constitutive GTP binding by the G protein. Molecular dynamics simulations indicate that the mutations cause displacement of Gln205, the key to GTP hydrolysis. Decreased interactions with cellular partners including RGS19 suggest that despite the enhanced GTP residence, the mutants fail to fully adopt the activated conformation and thus transmit the signal. Through a high-throughput screening of approved drugs aiming at correction of this core biochemical dysfunction, we identify zinc pyrithione and Zn\(^{2+}\) ions as agents restoring the active conformation, GTPase activity, and cellular interactions of the encephalopathy mutants, with a negligible effect on wild type Ga\(^\alpha\). We describe a *Drosophila* model of *GNAO1* encephalopathy and show that dietary zinc supplementation restores the motor function and longevity of the mutant flies. With zinc supplements frequently recommended for diverse human neurological conditions, our work spanning from identification of the core biochemical defect in Ga\(^\alpha\) mutants and cellular interactions analysis to high-throughput screening and animal validation of the deficiency-correcting drug defines the potential therapy for *GNAO1* encephalopathy patients.

Background

Heterotrimeric G proteins are the immediate cytoplasmic signaling transducers of G protein-coupled receptors (GPCRs) – the largest receptor class in animals and the major target of modern drugs\(^5\). Composed of the Ga, \(\beta\), and \(\gamma\) subunits, they interact with receptors when the Ga is loaded with GDP to undergo the activated GPCR-induced GDP-to-GTP exchange and dissociation into the Ga-GTP and G\(\beta\gamma\) components, both competent to transmit the signal further downstream\(^6\). With time, the intrinsic GTPase activity of the Ga-subunits leads to GTP hydrolysis – the activity further sped up by the dedicated RGS (Regulator of G protein Signaling) proteins\(^7\). The resultant Ga-GDP can reload with GTP\(^8\) or complex back with G\(\beta\gamma\), thus closing the G protein activation-deactivation loop\(^6\).

Of the 16 human Ga-subunits, Ga\(^\alpha\) is the major neuronal representative, transmitting the signals from numerous GPCRs in developing and adult brain. In 2013, the first cases were reported on pediatric encephalopathy patients harboring *de novo* mutations in *GNAO1* – the gene encoding Ga\(^\alpha\)\(^4\). This discovery was followed by an avalanche of clinical analyses that cumulatively led to the recognition of *GNAO1* encephalopathy as a spectrum of neurodevelopmental disorders manifesting as motor dysfunction, epileptic seizures, and developmental delay first appearing mostly in infancy\(^1\)\(^-\)\(^3\).

As of today, about 200 patients world-wide have been identified to harbor a mutation in *GNAO1* (https://gnao1.org/\(^\)). With the advances in genetic analysis application in clinical practice, *GNAO1* encephalopathy may emerge as one of the most frequent rare diseases. While most of the mutations being single amino acid substitutions spread over the coding sequence of the gene, the codons Gly203, Arg209, and Glu246 emerge as the disease mutation hotspots, together taking the share of 45-68% in recent surveys\(^1\)\(^-\)\(^3\). These amino acid residues are located near the GTP-binding pocket of Ga\(^\alpha\) and play an important role in the adoption of the activated G protein conformation\(^9\) (Fig. 1A).

Apart from symptomatic treatments such as deep-brain stimulation\(^10\), there exists no therapy to *GNAO1* encephalopathy patients. Development of eventual therapies, in turn, is delayed by the lack of understanding of the molecular etiology of the disease. Here, we probe the biochemical deficiency at the molecular core of *GNAO1* encephalopathy, identify a drug correcting this deficiency, validate it in neuronal cells, and finally show that the dietary supplementation of the drug rescues behavioral defects in a *Drosophila* model of the disease, identifying the potential therapeutic avenue to treat human patients.

*Mutations in Gly203, Arg209, and Glu246 result in constitutive GTP binding of Ga\(^\alpha\)*

To seek understanding of the molecular etiology of *GNAO1* encephalopathy, we first probed the basic biochemical properties of the most frequent Ga\(^\alpha\) mutants: GTP uptake and hydrolysis, in comparison to the wild type protein. The GTP uptake analysis has so far been performed to three *GNAO1* encephalopathy mutants: Q52P and Q52R displayed the complete loss of the GTP uptake\(^11\), while
R209H was reported to display a faster GTP uptake\textsuperscript{12}. We have applied the non-hydrolyzable fluorescent BODIPY-GTP\textsubscript{γS} assay\textsuperscript{8,13-17} to monitor the GTP uptake properties of four G\textsubscript{αo} variants: wild type, G203R, R209C, and E246K. This analysis reveals that the three mutants are dramatically faster in uptaking GTP than the wild type (Fig. 1B). The calculated binding rate constant, $k_{\text{bind}}$, increases by the E246K mutation 5-fold, by the R209C mutation – 11-fold, and by the G203R mutation – 28-fold over that of the wild type G\textsubscript{αo} (Fig. 1C).

The faster GTP uptake will lead to the higher GTP residence of the G protein if its GTP hydrolysis rate is not proportionally increased; an accompanying decreased GTP hydrolysis will further aggravate the GTP residence of the G protein. We thus next applied a hydrolyzable fluorescent GTP analog, BODIPY-GTP, whose interaction with an active G protein is seen as a transient rise in fluorescence (indicative of the nucleotide binding) followed by a decay in fluorescence (indicative of GTP hydrolysis due to the lower quantum yield the resultant fluorphore-GDP on the protein)\textsuperscript{8,15,16,18}. Remarkably, we see that all three pathologic G\textsubscript{αo} mutants reveal a dramatically slowed down fluorescent decay rate as compared to the wild type (Fig. 1D). Calculation of the hydrolysis rate constant, $k_{\text{hydr}}$, confirms this assessment: $k_{\text{hydr}}$ of E246K is reduced 65-fold, of R209C – 114-fold, and of G203R – 212-fold as compared to the wild type G\textsubscript{αo} (Fig. 1E).

Thus, mutations in the three most frequently affected \textit{GNAO1} encephalopathy amino acid residues lead to a dramatic increase in the rate of GTP uptake accompanied by a gigantic drop in the rate of GTP hydrolysis. We thus must conclude that biochemically, mutations in Gly203, Arg209, and Glu246 of G\textsubscript{αo} lead to the constitutive GTP-binding state of the G protein – the molecular feature likely at the core of the disease etiology.

\textbf{Defective cellular interactions of the GNAO1 encephalopathy mutants}

The distorted proportion of the GTP/GDP-state residence of the \textit{GNAO1} encephalopathy mutants inferred from the biochemical experiments must have consequences at the cellular level. We thus moved to express the mutants in mouse neuroblastoma N2a cells, frequently used to study the G\textsubscript{αo} function\textsuperscript{13}. In this as other cell lines, wild type G\textsubscript{αo} shows dual localization at the plasma membrane and the Golgi apparatus, as previously reported by us\textsuperscript{13,19}. The G203R, R209C, and E246K mutants show a similar dual localization (Supplementary Fig. S1), indicative of the normal post-translational modifications of the mutants. We next tested for the interaction of the G203R mutant – the most disturbed in terms of the GTP/GDP balance as judged by the biochemical experiments above – with its two cellular interaction partners: G\textsubscript{βγ} that preferentially interacts with the GDP-loaded form of G\textsubscript{αo}, and RGS19 that preferentially binds to the GTP-loaded form\textsuperscript{8}. Using an internal GFP fusion (that does not impede the proper localization nor protein-protein interactions\textsuperscript{11}) of G\textsubscript{αo} and G\textsubscript{αo}[G203R], we first found in the pull-down assay that the mutation significantly reduces the ability of the protein to interact with G\textsubscript{βγ} (Supplementary Fig. S2A, B). This finding agrees with the reduced proportion of the GDP-loaded form of G\textsubscript{αo}[G203R], and goes in the same direction as that observed for the classical constitutively activated point mutant, Q205L (Supplementary Fig. S2A, B).

However, when looking at the interaction with RGS19, we surprisingly found a similar interaction reduction for G\textsubscript{αo}[G203R], opposite to that seen for the constitutively activate Q205L (Supplementary Fig. S2C, D). Puzzled by this finding, we used C-terminally GFP-tagged G\textsubscript{αo}, this time comparing the RGS19 interactions for the wild type and the three mutant variants of the protein. This analysis again revealed that the \textit{GNAO1} encephalopathy mutants display strongly reduced interactions with RGS19 (Supplementary Fig. S2E, see Supplementary Fig. S7 and Fig. 2 below for representative Western blots). These findings, put together with the biochemical data, speak for the following. First, given the lack of cellular interaction with the RGS protein whose function is to speed up GTP hydrolysis\textsuperscript{7,8}, the cellular GTP-residence of the mutants is expected to be even more significant. Second, the mutants display reduced interactions with physiologic G\textsubscript{αo} partners and, by inference, reduced signal transduction capacity in neuronal cells. And third, despite the increased residence in the GTP-bound state, the mutant G\textsubscript{αo} proteins likely do not adopt the truly activated conformation, hence fail to interact with RGS19 (and presumably other signaling partners).

\textbf{Displacement of the GTPase catalytic residue in GNAO1 encephalopathy mutants}
Gαi mutated in the amino acids equivalent to Gao's Arg209 and Glu246, as well as at the Gly204 neighboring the Gly203, have been proposed to fail to adopt the fully activated conformation9, providing a clue to the reduced RGS19 interactions of the Gao mutants we report above. In order to gain insights into the possible structural deficiency in the G203R, R209C, and E246K encephalopathy mutants that lead to the deficient GTPase reaction and incompetent RGS19 interaction, we performed homology modeling followed by molecular dynamics simulations of the GTP-loaded Gao, wild type and the mutants, based on the Gai1-GTPγS structure20. Structural analysis of the energy-minimized state reveals that R209C and E246K, mutations of the amino acids normally forming a salt bridge to fasten switch II and the α3 helix to lock Gα in the active conformation upon the GTP binding9,21, result in a significant displacement of the Glu205 residue – the key to the catalytic GTPase reaction22 – away from the γ-phosphate (Supplementary Fig. S3A, B, D, also see Fig. 3 below). Through molecular dynamics simulations we further find a significant global destabilization of Gao[E246K], in agreement with the similar analysis of the Gai1 structure9, but not of the G203R or R209C mutants (Supplementary Fig. S4A and Supplementary Movie S1). Further, analysis of energy-minimized model of Gao[G203R] reveals a somewhat different arrangement as compared to E246K and R209C mutants: instead of acting through switch II, the substituting Arg residue engages in a direct interaction with the catalytic Glu205, displacing the latter from the γ-phosphate and, importantly, occupying the space normally used by the hydrolysis water molecule and thus inducing its displacement (Supplementary Fig. SC, D, also see Fig. 3 below). These findings suggest a common mechanism of the loss of the GTPase reaction in the three GNA07 encephalopathy mutants as the displacement of the catalytic Glu205. The accompanying changes in Gao structure likely lead to the failure of the G protein to adopt the fully activated conformation and thus make it poorly recognizable by RGS19. Another amino acid residue, T182 playing an important role in the Ga-GTP interaction with RGS proteins23,24, does not show a significant dislocation in the three pathologic Gao mutants (Supplementary Fig. S4C-E).

**High-throughput assay aiming at recovering the GTPase activity of Gao[E246K] identified zinc pyrithione as a drug specifically acting on the mutant but not wild type proteins**

We next argued that since the inability of the three Gao encephalopathy mutant proteins to hydrolyze GTP likely lies at the core of the molecular etiology of the disease and represents an easily measurable biochemical characteristic, one could design an assay to screen for molecules potentially capable of restoring this deficiency. To build such a high-throughput screening (HTS) platform, we employed Gao[E246K] and BODIPY-GTP to monitor the GTP uptake and hydrolysis by the mutant, as described in the first section, and screened a library of 2736 FDA-approved and pharmacopeial drugs (Supplementary Fig. S5A). While wild-type Gao hydrolyzes all BODIPY-GTP provided to it in the biochemical setting within 10 minutes of the experiment, Gao[E246K] fails to do so, resulting in the stable BODIPY fluorescence (see Fig. 1D). Thus, the initial screening was based on the identification of drugs capable of inducing a drop in fluorescence by the 10 minutes incubation of Gao[E246K] with BODIPY-GTP. The screening was followed by hit validation, that resulted in three hit compounds: sennoside A, sennoside B, and zinc pyrithione (ZPT) (Fig. 2A, B and Supplementary Fig. SSB-E). Of those, sennoside A and B were found to decrease GTP uptake by Gao[E246K], rather than GTP hydrolysis by it (Supplementary Fig. S5D, E). In contrast, ZPT restored the GTP hydrolysis (Fig. 2B). When retesting the drugs on wild-type Gao, sennosides were found to equally affect the GTP uptake by it; in contrast, ZPT revealed the action specific for Gao[E246K] but not wild type Gao (Fig. 2C and Supplementary Fig. S5F, G), immediately raising our interest to this molecule.

**Zn2+ is the active component of zinc pyrithione restoring the GTPase activity of the three encephalopathy Gao mutants**

We next found that ZPT revealed a concentration-dependent restoration of the GTPase activity of all the three encephalopathy Gao mutants: G203R, R209C, and E246K (Supplementary Fig. S6A-C). ZPT is a coordinated complex of pyrithione – a membrane-permeable ionophore25 – and Zn2+ (Fig. 2A) and has the primary indication to treat dandruff and seborrheic dermatitis26; other biological activities of ZPT including antifungal, antiviral, and anticancer27-29 have also been reported. Zn2+ ions are poorly penetrant through cellular membranes30, and the pyrithione moiety of the drug serves to deliver the ions inside cells31. We thus found that in our cell-free assay monitoring GTP binding and hydrolysis by Gao, the Zn2+ ions are active in restoring the GTPase activity (Supplementary Fig. S6D-F) unlike the ‘empty’ ionophore (data not shown). We further tested several other metal ions, revealing that none of them recapitulated the effect of Zn2+: Co2+, Fe2+, Ni2+, Mn2+, and Li+ were inactive in restoring the GTPase reaction, while Cu2+ at 100µM appeared to completely inactivate the G protein (Supplementary Fig. S6G). In contrast to the ZPT- or Zn2+-mediated
concentration-dependent restoration of the GTPase activity, the effect of both compounds on wild type Gao was confirmed to be negligible (Fig. 2D, E). An interesting observation refers to the manner the three mutants respond to ZPT / ZnCl₂: while restoration of the GTPase reaction in Gao[R209C] and Gao[E246K] was saturating with the EC₅₀ values around 10µM, the response of Gao[G203R] was rather linear in the concentration range tested (Fig. 2D, E), suggesting the potentially distinct molecular mechanisms of Zn²⁺ acting on the G203 mutant vs. the R209C / E246K mutants.

Potential mechanism of action of Zn²⁺ in restoring the GTPase activity of GNAO1 encephalopathy mutants

We applied structural modeling and molecular dynamics simulations in order to gain insights into the potential mechanism of action of Zn²⁺ in the restoration of the GTPase activity of the three Gao mutants. We argued that Zn²⁺ can replace Mg²⁺ in the Gao's active center upon the interaction with GTP: it is well-known that proteins' Mg²⁺-binding sites are generally unspecific for this ion, which can be substituted by a broad range of other divalent metal ions, with Zn²⁺ being one of the most potent substitutes³². Our analysis shows that the substitution of Zn²⁺ for Mg²⁺ in the active center does not affect the global structure in the energy-minimized state of wild type Gao (Supplementary Fig. S4B, Supplementary Movie S2). Interestingly, the global rearrangement observed in Gao[E246K] was to a certain degree further aggravated in the Zn²⁺-bound protein; no global effect of Zn²⁺ however, was seen for the G203R and R209C mutants (Supplementary Fig. S4B, Supplementary Movie S2), suggesting that it is unlikely to represent the general or main mechanism of Zn²⁺ action to restore the GTPase activity.

We thus next paid special attention to the position and flexibility of the catalytic Gln205 that we found to be placed away from the GTP γ-phosphate in the pathologic mutants (see Supplementary Fig. S3). Our molecular dynamics simulations reveal that for all three mutant variants, distance between the γ-N atom of Gln205 and the γ-P atom of GTP (increased as compared to the wild type Gao) is reduced back to the wild type levels by Zn²⁺ (Fig. 3). It should be noted that in the wild type protein Zn²⁺ also decreases the distance between Q205 and γ-phosphate, but to a considerably smaller extent that apparently does not affect the catalytic activity. This effect is particularly evident for the Gao[E246K] and Gao[R209C] mutants (Fig. 3A-D), agreeing well with the fact that the residues E246 and R209 normally form the salt bridge to fasten the active conformation upon the GTP binding⁹,²¹ and suggesting the common mechanism of action of Zn²⁺ on these two encephalopathy mutants.

The effect of Zn²⁺ on the distance between Gln205 of Gao[G203R] and the γ-phosphate was less pronounced, albeit still visible (Fig. 3E, F). Coming back to the point that the new Arg in position 203 forms a hydrogen bond with Gln205 and that this might contribute to the loss of the GTPase activity in the mutant (see Supplementary Fig. S3C, D), we measured the dynamics of the bond, as the distance between the γ-N atom of Gln205 and the ε-atom of Arg203, in the Mg²⁺- vs. Zn²⁺-bound conformations. Surprisingly, we found that Zn²⁺, if anything, overall stabilized the Arg203-Gln205 interaction (Supplementary Fig. S4F).

Overall, our structural analysis suggests the atomistic mechanism for the action of Zn²⁺ on the restoration of the GTPase activity of the E246K and R209C mutants as the bringing back of the catalytic Gln205 to the γ-phosphate of GTP, otherwise swayed away by the mutations. The GTPase restoration mechanism for the G203R mutation may involve the same principle but could additionally employ other features, as hinted at by altered position of the hydrolytic water molecule (Supplementary Fig. S3C, D) and the different sensitivity of the mutant to ZnCl₂ as compared to the other two mutants (see Fig. 2D, E and the section above).

ZPT restores cellular RGS19 interactions of GNAO1 encephalopathy mutants

Our in vitro data show that ZPT and its ion component, Zn²⁺, are able to restore the GTPase activity of the pathologic Gao. We next wondered whether such a restoration could be seen in vivo, and be reflected in a restored interaction with a Gao partner. To this end, we tested if the mutants’ interaction with RGS19 can be restored in the N2a cells. It is known, however, that Zn²⁺ can either cross the cellular membranes by active transporters³⁰ or with the help of ionophores such as pyrithione³¹. On the other hand, a significant neuronal and other cell toxicity of ZPT has been reported, mainly due to its effectiveness in bringing zinc inside the cells³³-³⁵. Thus, we first investigated the acute cytotoxicity of ZPT, pyrithione, and ZnCl₂ in N2a cells. This analysis confirms the neurotoxicity of ZPT with the IC₅₀ of ca. 5µM; ZnCl₂ in contrast was not toxic up to the concentrations of 100µM (Supplementary Fig. S7A) as presumably
it failed to penetrate the cells in this acute setting. For the subsequent experiments on the restoration of the Gαo-RGS19 interactions in N2a cells, we took the highest tested agents’ concentrations that did not display cytotoxicity: 1μM for ZPT and 100μM for ZnCl₂.

Using these concentrations, and performing the experiments with C-terminally GFP-tagged Gαo as in Supplementary Fig. S2E earlier, we found that ZPT could dramatically recover the ability of Gαo[E246K] to interact with RGS19 to the levels normally seen for wild type Gαo (Supplementary Fig. S7B, C). ZnCl₂, in contrast, was ineffective. The interaction of Gαo wild-type with RGS19 was also stimulated ca. 2-fold by ZPT (Supplementary Fig. S7B, C), which is consistent with the certain effect of Zn²⁺ on it in molecular dynamics simulation described above or might originate from other, indirect, effects. However, this modest stimulation fades away as compared to the effect of ZPT on the mutant Gαo versions: ca. 6-fold for Gαo[R209C], ca. 12-fold for Gαo[E246K], and the dramatic 20-fold for Gαo[G203R] (Fig. 2E, F). We hypothesize that the ionophore-mediated delivery of Zn²⁺ into neuronal cells is capable of restoring the GTPase-competent conformation of the three encephalopathy Gαo variants, the resulting protein-protein interaction(s) and potentially – the signaling functions of the G protein.

**Zn²⁺ dietary supplementation rescues the Drosophila model of GNAO1 encephalopathy**

Animal models have the instrumental role in deciphering the human disease mechanisms and in identifying/validating the treatment routes. The fruit fly *Drosophila melanogaster* represents an excellent model organism for studies in various fields of biology, and our recent work highlights the power of *Drosophila* as the host to model GNAO1 encephalopathy. To establish the GNAO1-encephalopathy model in the fruit fly, we applied the CRISPR/Cas9-mutagenesis together with phiC31-mediated cassette exchange to introduce the pathogenic G203R mutation into the *Drosophila* Gαo (see Methods and Supplementary Fig. S8). We found that the resultant [G203R]/+ flies are viable and fertile yet reveal a number of deficiencies. Specifically, the heterozygous mutant flies manifest a significant motor dysfunction, measured in the negative geotaxis assay as a reduced capacity to climb up the wall (Fig. 4A, B), reminiscent of the motor dysfunction in the human patients. Further, the encephalopathy mutant *Drosophila* display a two-fold reduction in the life span (Fig. 4C).

Dietary zinc supplementation has been applied to treat various human health conditions, including the neurological ones such as depression, epilepsy, psychiatric and neurodegenerative diseases, or sleep disorders, as well as for normal neonatal development. We thus wondered if dietary supplementation of zinc in the *Drosophila* model of GNAO1 encephalopathy may reveal any beneficial effects. Food supplementation of ZnCl₂ to the final concentration of 200μM has been previously shown to rescue the survival of *Drosophila* mutant for dZip1 and dZip2, the gut zinc transporters, providing us a guideline. We also tested 10μM ZPT food supplementation, following the application of up to 15μM ZPT in rats. *Drosophila* lines – G203R/+ and the wild type control (see Methods and Supplementary Fig. S8) – were raised from the egg at the standard food or that supplemented with ZnCl₂ or ZPT. The climbing capacity of the resultant populations were compared, along with their longevitys.

We found that the ZnCl₂-containing food significantly improved the motor function of the G203R/+ flies (Fig. 4D). The effect was particularly strong for female *Drosophila*, bringing the climbing capacity towards the wild type levels (Fig. 4D). Interestingly, the control wild type flies showed instead a clear reduction in the climbing capacity, except for the ability of ZnCl₂ to modestly improve the climbing of female flies (Fig. 4E). Second, ZnCl₂ food supplementation rescued the reduced life span of female G203R/+ flies (Fig. 4F); no effect could however be seen for ZPT or for male flies (Fig. 4F and Supplementary Fig. S9).

These observations reveal strong improvements of the behavioral and life span conditions in the *Drosophila* model of GNAO1 encephalopathy by dietary zinc supplementation. Given the large body of clinical evidence on the dietary zinc supplements for human patients with diverse neurological conditions, our findings may speak for the applicability of such supplementation for the pediatric GNAO1 encephalopathy patients. These issues and the sex-sensitive effects of the dietary zinc supplementation are discussed in the next section.

**Discussion**

GNAO1-dependent pediatric encephalopathy is a recently diagnosed rare yet devastating neurological disease. The number of discovered different – mostly missense point – mutations in GNAO1 causing this malady steadily increases every year since the first
2013’ report\textsuperscript{4}. However, despite some insights\textsuperscript{4,19,46}, the understanding of the molecular etiology underlying the pathological developments has been largely missing. This delay in the understanding blocks development of therapies to treat the patients. Being mostly unresponsive to the conventional anti-epileptic treatments, the sick children have so far demonstrated the best – albeit partial – response to the symptomatic, highly invasive and poorly accessible therapy: the deep-brain stimulation\textsuperscript{10}.

The core molecular dysfunction we have “diagnosed” here for the three most common \textit{GNAO1}\textsuperscript{-}encephalopathy mutations is the constitutive GTP binding state of the mutant \textit{G}α proteins, resulting from a strongly increased rate of GTP uptake concomitant with a dramatically reduced rate of GTP hydrolysis. The failure of the mutants to interact with the GTPase-activating RGS19 is expected to further aggravate this constitutive GTP-bound status of \textit{G}α in \textit{GNAO1}\textsuperscript{-}encephalopathy neurons. Molecular dynamics and structural modeling provide us with the unifying mechanism of this dysfunction: each of the three mutations induces a displacement of the catalytic Gln205; this change is also likely the reason for the defective interaction with RGS19 – and by inference other physiologic partners of \textit{G}α, thus creating a signaling-deficient \textit{G} protein.

It is remarkable that a simple ion, Zn\textsuperscript{2+} emerging from our screening of drug candidates to recover the GTPase deficiency of the mutant \textit{G}α, is efficient in restoring the structural rearrangements induced by the pathologic mutations. Replacing Mg\textsuperscript{2+} from the GTP pocket of \textit{G}α, Zn\textsuperscript{2+} brings back the catalytic Gln205 to the vicinity of the γ-phosphate of GTP. This compensation is stronger for the R209C and E246K mutants and weaker for the G203R mutant, suggesting that additional mechanisms may exist for the restoration of the GTPase activity in \textit{G}α[G203R] that would require more direct – NMR or crystallographic – future structure elucidations. The resulting structural rearrangements are sufficient to recover the GTPase reaction of the three pathologic mutants, their cellular interactions with RGS19, and ultimately provide motor activity and longevity ameliorations in the \textit{Drosophila} model of \textit{GNAO1}\textsuperscript{-}encephalopathy upon dietary zinc supplementation.

Dietary zinc supplements have found numerous applications in human health. Being safe, they have been shown to improve neonatal brain development\textsuperscript{43}, sleep quality in adults\textsuperscript{42}, and to ameliorate health conditions in depression\textsuperscript{39}, epilepsy\textsuperscript{40}, and a set psychiatric and neurodegenerative states\textsuperscript{41}. For example, daily 25mg Zn\textsuperscript{2+} applied for 6 weeks in one study\textsuperscript{47}, and daily 220mg zinc sulphate (providing 50mg zinc) applied for 12 days in another\textsuperscript{48}, was found to positively act on depression patients. The upper limits of dietary zinc with no observed adverse effects, as set by the World Health Organization, are 13 mg/day for the age of 7-12 months, 23 mg/day for the age of 1-6 years, and 45 mg/day for adults\textsuperscript{49}. In this regard, dietary zinc supplementation might be considered as a potential treatment option to improve the conditions of \textit{GNAO1}\textsuperscript{-}encephalopathy patients – at least those carrying the G203R, R209C, and E246K mutations. More studies will show how applicable is the Zn\textsuperscript{2+}-restorable GTPase deficiency mechanism to the other \textit{GNAO1}\textsuperscript{-}encephalopathy mutants.

Despite decades of diverse applications of zinc supplements in human health, the details of bioavailability, pharmacokinetics, pharmacodynamics, and potential toxicities of the dietary zinc are still controversial\textsuperscript{41,50}. Multiple factors confound the efficiency of zinc absorption from the gut, its penetration through the blood-brain barrier, and the ultimate entry and activities within neuronal cells\textsuperscript{41,51,52}. For example, the different efficiency of dietary zinc supplementation in rescuing the motor dysfunction and reduced life span in female vs. male fruit flies (Fig. 4 and Supplementary Fig. S9) might be related to different expression of certain zinc transporters in the two sexes\textsuperscript{53}. Although we consider it unlikely to be reflected in human patients, certain gender differences in the outcome of genetic manipulation of different zinc transporters have been observed in mouse models\textsuperscript{54-57}. Diverse approaches to enhance and control zinc uptake and delivery are being developed, from nutritional chelators and nanoparticle carriers to intravenous, cerebrospinal or intra-brain injections\textsuperscript{41,52,58}, and might be considered in the future prospective applications to \textit{GNAO1}\textsuperscript{-}encephalopathy patients.

To sum up, the study presented here sweeps from the understanding, at the molecular and even atomistic level, of the core biochemical dysfunction seen in the three most frequent \textit{GNAO1}\textsuperscript{-}encephalopathy mutations to establishment and screening for drug candidates to rescue this dysfunction, to be followed by the candidate validation in biochemical and cellular models. Finally, we establish an animal model of \textit{GNAO1}\textsuperscript{-}encephalopathy and show that a dietary supplementation of Zn\textsuperscript{2+} – the active component of the treatment – provides a significant rescue of the movement disorder and life span shortening of the mutant animals. Our work might serve as the ground for recommendation of the dietary zinc supplementation as a treatment option for \textit{GNAO1}\textsuperscript{-}encephalopathy patients.
Materials And Methods

Plasmids and molecular cloning

The plasmids for the Gαo-GFP (C-terminally and internally tagged), mRFP-Gβ1, mRFP-γ3, and His₆-RGS19 were previously described⁸,¹¹. The Gα mutants were obtained by site-directed mutagenesis in the pcDNA3.1 plasmid⁸ using the primers as listed in Supplementary Table S1. The plasmid pET-23a encoding wild-type N-terminally tagged 6xHis-Gαo⁸ was used to create E246K, R209C and G203R mutants through subcloning using restriction sites SphI and EcoRI from the constructs in pcDNA3.1.

Protein production and purification

The Rosetta-gami E. coli strain was transformed with pET23b-Gαo wild type, pET23b-Gαo[G203R], pET23b-Gαo[R209C], or pET23b-Gαo[E246K] and grown at 37°C to OD₆₀₀=0.6 before induction with 1mM IPTG and additional growth overnight at 18°C. Cells then were harvested by centrifugation 3,500 g at 4°C and resuspended in lysis/binding buffer containing 50mM TBS, 1mM PMSF, and 30mM imidazole. Cells were disrupted with High-Pressure Cell Press Homogenizer, the debris was removed by centrifugation at 15,000 g/15min/4°C. The supernatant was applied to the Ni²⁺ resin (Qiagen) overnight in a rotary shaker at 4°C. The Ni²⁺ resin was washed two times with 10 resin volumes of the washing buffer containing 50mM TBS, 1mM PMSF, and 10mM imidazole. On the third wash, the washing buffer was supplemented with 3% glycerol, 10mM MgCl₂, 0.1mM DTT, and 200 μM GDP. The Ni²⁺ resin was washed two more times with 10 resin volumes of the washing buffer. Proteins were then eluted with the buffer containing 50mM TBS, 1mM PMSF, and 300mM imidazole. To subsequently remove imidazole, the protein buffer was exchanged into 50mM TBS using Vivaspin concentrator. Protein concentration was measured using the Bradford assay and the purity was analyzed using SDS-PAGE followed by Coomassie staining.

GTP binding and hydrolysis assay

The GTP binding and hydrolysis assay using BODIPY-GTP (Invitrogen) or BODIPY-GTPγS (Invitrogen) was performed as described⁸. Gαo was diluted to 1μM in the reaction buffer containing 50mM TBS, 10mM MgCl₂, and 0.5% BSA. The mixture was then pipetted into black 384-well plates (Greiner) and BODIPY-GTP or BODIPY-GTPγS (1μM; Invitrogen) was added into the wells. Fluorescence measurements were performed with a Tecan Infinite M200 PRO plate reader with excitation at 485nm and emission at 530nm at 28°C. To trace the fast kinetics of the Gαo[G203R] and Gαo[R209C], the fluorescent nucleotide solution was added using the injector unit followed by immediate measurement. The GTP binding and hydrolysis data were fit to obtain the k_bind and k_hydr rate constants as previously described⁸.

Homology modeling and molecular dynamics analysis

The structure of wild-type GTPγS-bound Gαo was homology modeled using the PDB 1GIA structure²⁰ on the Swiss-Model server with the User Template setting⁵⁹. This structure was used as a base to generate amino acid substitutions in the PyMol software and metal ion substitutions using Check My Metal web interface⁶⁰. The resulting draft PDB models of Gαo mutants bound to Mg²⁺ or Zn²⁺ were directly used in the GROMACS 2021.2 software⁶¹,⁶² to generate both the energy-minimized models and the molecular dynamics runs. To this end, the CHARMM36 all-atom force field was used. The structures were solvated in a cubic box with 1nm distance from protein edges, the phosphate groups charge was neutralized by Na⁺ ions. Subsequently, energy minimization and temperature and pressure equilibration were performed using typical parameters (50ps duration, 2fs step). A 100ns production run was performed on high performance computation (HPC) cluster of the University of Geneva with 2fs step and leap-frog integrator and with 1nm cutoffs for van der Waals and electrostatic cutoffs. Subsequent analysis of the trajectories and structures was performed using both built-in functions of GROMACS package and PyMol using custom scripts.
**High-throughput screening**

High-throughput screening (HTS) for mutant Gαo modulators was performed using the Gαo[E246K] protein and FDA Approved & Pharmacopeial Drug Library (HY-L066, MedChemExpress). DMSO or compounds in DMSO (12.5μM) were mixed with Gαo[E246K] at 1μM in a reaction buffer and BODIPY-GTP at 1μM as described in GTP binding assay above. Reaction was carried out for 10min.

To analyze the data generated by the HTS, two parameters were calculated: (i) binding constant ($k_{\text{bind}}$) and (ii) maximal GTP uptake. For candidates affecting the $k_{\text{bind}}$, the hits were picked if the compound modulated $k_{\text{bind}}$ by $\geq 2$ SD (standard deviation) of DMSO-treated wells. For candidates affecting the maximal BODIPY-GTP uptake, the hits were picked if the compound modulated the maximal GTP uptake by $\geq 3$ SD of DMSO-treated wells. The hits were subsequently validated by performing the GTP binding assay at 50μM of compounds using both Gαo wild type and Gαo[E246K].

**Cell lines and culture conditions**

Male mouse neuroblastoma Neuro-2a (N2a; ATCC CCL-131) cells were maintained in MEM (Thermo Fisher Scientific), supplemented with 10% FCS, 2mM L-glutamine, 1mM pyruvate, and 1% penicillin-streptomycin at 37°C and 5% CO₂.

**Immunoprecipitation (IP)**

Immunoprecipitation of Gαo-GFP constructs was performed as previously described\(^6\). Briefly, N2a cells were transfected with the constructs indicated in the corresponding figures, and after 24h cells were directly harvested or incubated with fresh media supplemented with 1μM ZPT, 100μM ZnCl₂ or DMSO for 3h at normal culture conditions. Cells were harvested with ice-cold GST-lysis buffer (20mM Tris-HCl, pH 8.0, 1% Triton X-100 and 10% glycerol in PBS) supplemented with a protease inhibitor cocktail (Roche). Cell lysates were cleared by centrifugation at 16,000 g for 15min at 4°C, and supernatants were incubated with 2μg of nanobody against GFP\(^6\) on ice for 30min. Then, 20μl of a 50% slurry of Glutathione Sepharose 4B beads (GE Healthcare) was added to the samples and incubated overnight on a rotary shaker at 4°C. Beads were repeatedly washed with lysis buffer and bound proteins were eluted by boiling the beads with SDS-PAGE sample buffer. Samples finally were analyzed by SDS-PAGE followed by Western blot using antibodies against GFP (GeneTex GTX113617), His\(_{6}\)-tag (Qiagen 34650), and mRFP (Santa Cruz sc-101526). Peroxidase conjugated antibodies were from Jackson ImmunoResearch (115-035-062 and 111-035-144). Quantification of blots was done using ImageJ from at least 3 independent experiments and statistical analysis was carried out using Student’s t-test.

**Immunofluorescence and microscopy**

For microscopy, N2a cells were transfected for 7h, trypsinized and seeded on poly-L-lysine-coated coverslips in complete MEM for additional 15h before fixation. Cells were fixed for 20min with 4% paraformaldehyde in PBS, were permeabilized for 1min using ice-cold PBS supplemented with 0.1% Triton X-100, blocked for 30min with PBS supplemented with 1% BSA, incubated with the primary antibody against GM130 (BD Biosciences 610823) in blocking buffer for 2h at RT, washed and subsequently incubated with the secondary antibody and DAPI in blocking buffer for 2h at RT. The Cy3-labelled secondary antibody was from Jackson ImmunoResearch (115-165-146). Coverslips were finally mounted with Vectashield on microscope slides. Cells were recorded with a Plan-Apochromat 63x/1.4 oil objective on a LSM800 Confocal Microscope and further processed using the ZEN blue software (all Zeiss).

**MTT assay**
N2a cells (3000 cells/well) were distributed into a transparent 384-well plate. The medium of each well was replaced by 50μl of fresh medium the next day containing the indicated concentrations of ZPT or ZnCl₂. After incubation for 3h, the medium in each well was replaced by 50μl of 0.5 mg/ml Thiazolyl blue (Carl Roth) solution in 1xPBS. The plates were incubated for 3h at 37°C. Then the solution was removed and 30μl DMSO was added into each well. Absorbance at 570nm was measured in Tecan Infinite M200 PRO plate reader.

**Plasmids for Drosophila dGαo editing**

**Donor Plasmid pGao47-LattP-pBacDsRed-attPR for the CRISPR/Cas9 Step of Transgenesis:**

Donor plasmid pHd-ScarlessDsRed (Drosophila Genomics Resource Center, Bloomington USA, stock #1364) was modified by adding LoxP sequences after dsRed coding region, for which the annealed complementary oligonucleotides GGCCATAACTTGCTATACGAAGTTAT and GGCCATAACTTGCTATACGAAGTTAT were cloned into the NotI site of this plasmid. The resultant plasmid (pScarlessDsRed-lox) was digested with AarI and SapI and assembled with two 110bp attP sequences, using NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, cat. #E5520S). Plasmid pTA-attP (Addgene, #18930) was used as a template for PCR amplifications of attP, which were performed with the primer sets attPfwRl/attPrevHpal and attPfwKpnl/attPrevHpal (see the list of primers below). The resultant pattP-pBacDsRed-lox-attP plasmid contains the pBac transposon with the fluorescent dsRed marker flanked with two inverted attP sequences. The left homologous arm (LHA) was PCR-amplified with the dGao47Lfw and dGao47Lrev primers from Drosophila genomic DNA, producing the 815bp PCR product, which was treated with EcoRI and further cloned into the pattP-pBacDsRed-lox-attP plasmid by the EcoRI site producing the construct pattP-pBacDsRed-lox-attP. The right homologous arm (RHA) was PCR amplified with the dGao47Rfw and dGao47Rrev primers, and the resulting 500 bp PCR product was treated with KpnI and then cloned into the plasmid pattP-pBacDsRed-lox-attP digested with KpnI and SmaI. The resultant donor plasmid pattP-pBacDsRed-lox-attPR contains the dsRed marker flanked with inverted attPs and the 815bp-long LHA and 500bp-RHA. All PCRs were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich USA, cat. #M0530S).

**Plasmids Providing Expression of gRNAs under the Control of the Drosophila U6:2 Promoter:**

CRISPR targets sites were identified using Target Finder⁶⁵ (targetfinder.flycrispr.neuro.brown.edu/). Four targets sites (2 upstream of the ⁴th coding exon and 2 downstream of the ⁷th exon of dGao) were selected. Complimentary oligonucleotides

CTTCGCAATGGGGGCCGTAGCA and AAACGCTAACGCCCCATTCTGC;

TTGCCTCAAGTCTTGGACAGATAC and AAGGTATCTGCCAAGACTTGAGC

were annealed and cloned into pENTR1A-DUAL-CRISPR (kindly provided by A. Glotov, Umea University, Sweden), which was digested with BbsI and SapI (New England Biolabs, cat. #R0539S and # R0569S). The resultant plasmid pDUAL-L1R2 contains two gRNAs for induction of DSBs upstream of exon 4 and downstream of exon 7 of dGao. The same approach was performed to construct pDUAL-R1L2 using the

CTTCGAATGGAGGGAAGGGT and AAACGAGCTTTCACCCGTTACCTC;

TTGCCTCCTCTTATTACCGTTA and AACTAGGTTATTAAAGGAGC oligonucleotides. The plasmids pDUAL-L1R2 and pDUAL-R1L2 combined with the donor plasmid pGao47-LattP-pBacDsRed-attPR were used for CRISPR/Cas9 step of transgenesis (Supplementary Fig. S8).

**Donor plasmids for RMCE (recombinase-mediated cassette exchange):**
Plasmid piB-GFP (Addgene, #13844) containing 2 attB sequences was used as a template for PCR amplification of the 3100bp fragment (plasmid body flanked by attB sequences) with the primer attBcircle. *Drosophila* genomic DNA was used as a template for PCR amplification of 3 fragments with the primer sets attBNco_Gao47L/attBNco_Gao47R (1190bp), attBNco_Gao47L/G203Rrev (367bp) and G203Rfw/attBNco_Gao47R (725bp). The amplified fragments 3100bp and 1190bp were mixed and circulated using the NEBuilder HiFi DNA Assembly Cloning Kit (p2xattB-dGaoWT). The amplified fragments 3100bp, 367bp and 725bp were mixed and treated identically (p2xattB-dGaoG203R). The resultant plasmid p2xattB-dGaoWT has two attB sites which flanking sequences between exons 4-7 of dGao with about 100bp of adjacent non-coding regions. p2xattB-dGaoG203R has the identical structure but bears G203R mutation in exon 5. Both plasmids were used as donor plasmids for RMCE step of transgenesis (Supplementary Fig. S8).

*Drosophila* lines, germline transformation

Flies were maintained at 25 °C on the standard medium. For dietary experiment the food was supplemented with 200µM ZnCl₂ or 10µM ZPT. The line y[1], sc[4], v[1], sev[21]; P[y+[t7.7] v+[t1.8]=nos-Cas9.R]attP2 expressing Cas9 in the germline under the control of the nos promoter (Bloomington *Drosophila* Stock Center (BDSC, Bloomington USA, stock #78782) was used for germline transformation in the CRISPR/Cas9 step of transgenesis (Supplementary Fig. S8). Transgenic flies were selected by red fluorescence in eyes provided by the expression of DsRed under the eye-specific 3xP3 promoter. The resultant fly stock dGao[47dsRed] was combined with P[y+[t7.7]=nos-phiC31\int.NLS]X (BDSC, stock #34770) and balanced over CyO. This stock was used for the RMCE step of transgenesis (Supplementary Fig. S8). Transgenic flies in this case were selected by the absence red fluorescence in eyes. The resultant alleles and dGao[WT-control] were balanced over CyO. The stock dGao[G203R]/CyO is maintained in the heterozygous state as the G203R mutation leads lethality in homozygous. The stock dGao[WT-control] is viable and fertile in the homozygous state. This allele having the identical background to that of the mutant one was used as the wild type control in the negative geotaxis assay and for calculation of the longevity. Germline transformation was performed as described previously37. Fluorescence stereomicroscope (Zeiss SteReEO Discovery.V8, Carl Zeiss, Jena Germany with the Filter Set 43 HE for the DsRed fluorescent dye detection (excitation BP 550/25; emission BP 605/70)) was used for selection of transgenic flies with/without fluorescence in the eyes.

Molecular Analysis of Established fly stocks

Genomic DNA was isolated from individual flies of different genotypes as described previously66. The established transgenic fly strains were verified by PCR with the primers annealing to the neighboring genomic sequences outside of the homologous arms and inside the donor plasmids. PCR analysis was carried out with different primer sets: dGao47Test3(1)/dGao47Rnew1RevLong(2), dGao47Test3(1)/pBacWTlong(3) and pBac_rev(4)/dGao47Rnew1RevLong(2) for dGao[47dsRed]; dGao47Test3(1)/dGao47Rnew1RevLong(2) and dGao47Test3(1)/ G203Rrev(5) for dGao[WT-control] and dGao[G203R]/CyO (Supplementary Fig. S8, Supplementary Table S1). All PCRs were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs) following the manufacturer's instructions. Intactness of inserted sequences via RMCE (stocks dGao[WT-control] and dGao[G203R]/CyO) was verified by sequencing. Proper expression of these alleles was verified by sequencing their cDNA performed as described previously37.

Locomotion Assay and Measurement of Lifespan of *Drosophila*

The negative geotaxis assay was performed as described previously37. Measurement of lifespan was performed as described67; 110 males and 140 females of each genotype and on each supplemented food were monitored.

Declarations

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**Competing interests**

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**References**

1. Schirinzi, T. et al. Phenomenology and clinical course of movement disorder in GNAO1 variants: Results from an analytical review. *Parkinsonism Relat Disord* **61**, 19-25, doi:10.1016/j.parkreldis.2018.11.019 (2019).

2. Kelly, M. et al. Spectrum of neurodevelopmental disease associated with the GNAO1 guanosine triphosphate-binding region. *Epilepsia* **60**, 406-418, doi:10.1111/epi.14653 (2019).

3. A xenen, E. et al. Results of the First GNAO1-Related Neurodevelopmental Disorders Caregiver Survey. *Pediatr Neurol* **121**, 28-32, doi:10.1016/j.pediatrneurol.2021.05.005 (2021).

4. Nakamura, K. et al. De Novo mutations in GNAO1, encoding a Galphao subunit of heterotrimeric G proteins, cause epileptic encephalopathy. *Am J Hum Genet* **93**, 496-505, doi:10.1016/j.ajhg.2013.07.014 (2013).

5. Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B. & Gloriam, D. E. Trends in GPCR drug discovery: new agents, targets and indications. *Nature Reviews Drug Discovery* **16**, 829-842, doi:10.1038/nrd.2017.178 (2017).

6. Oldham, W. M. & Hamm, H. E. Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* **9**, 60-71 (2008).

7. Ross, E. M. & Wilkie, T. M. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem* **69**, 795-827 (2000).

8. Lin, C. et al. Double suppression of the Galpha protein activity by RGS proteins. *Mol Cell* **53**, 663-671, doi:10.1016/j.molcel.2014.01.014 (2014).

9. Knight, K. M. et al. A universal allosteric mechanism for G protein activation. *Mol Cell* **81**, 1384-1396.e1386, doi:10.1016/j.molcel.2021.02.002 (2021).

10. Benato, A. et al. Long-term effect of subthalamic and pallidal deep brain stimulation for status dystonicus in children with methylmalonic acidemia and GNAO1 mutation. *J Neural Transm (Vienna)* **126**, 739-757, doi:10.1007/s00702-019-02010-2 (2019).

11. Solis, G. P. et al. Pediatric encephalopathy: clinical, biochemical and cellular insights into the role of Gln52 of GNAO1 and GNAI1 for the dominat-inat disease. *under review* (2021).

12. Larrivee, C. L. et al. Mice with GNAO1 R209H Movement Disorder Variant Display Hyperlocomotion Alleviated by Risperidone. *J Pharmacol Exp Ther* **373**, 24-33, doi:10.1124/jpet.119.262733 (2020).

13. Solis, G. P. et al. Golgi-Resident Galphao Promotes Protrusive Membrane Dynamics. *Cell* **170**, 939-955, doi:10.1016/j.cell.2017.07.015 (2017).

14. Purvanov, V., Koval, A. & Katanaev, V. L. A direct and functional interaction between Go and Rab5 during G protein-coupled receptor signaling. *Science signaling* **3**, ra65, doi:10.1126/scisignal.2000877 (2010).

15. Egger-Adam, D. & Katanaev, V. L. The trimeric G protein Go inflicts a double impact on axin in the Wnt/frizzled signaling pathway. *Dev Dyn* **239**, 168-183, doi:10.1002/dvdy.22060 (2010).
16  Kopein, D. & Katanaev, V. L. Drosophila GoLoco-protein pins is a target of Galpha(o)-mediated G protein-coupled receptor signaling. *Mol Biol Cell* **20**, 3865-3877, doi:10.1091/mbc.E09-01-0021 (2009).

17  Seguin, L. *et al.* Macropinocytosis requires Gal-3 in a subset of patient-derived glioblastoma stem cells. *Commun Biol* **4**, 718, doi:10.1038/s42003-021-02258-z (2021).

18  Chinn, I. K. *et al.* Short stature and combined immunodeficiency associated with mutations in RGS10. *Science Signaling* **14**, eabc1940, doi:10.1126/scisignal.abc1940 (2021).

19  Solis, G. P. & Katanaev, V. L. Gαo (GNAO1) encephalopathies: plasma membrane vs. Golgi functions. *Oncotarget* **9**, 23846-23847, doi:10.18632/oncotarget.22067 (2018).

20  Coleman, D. E. *et al.* Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. *Science* **265**, 1405-1412 (1994).

21  Iiri, T., Farfel, Z. & Bourne, H. R. Conditional activation defect of a human Gsalpha mutant. *Proc Natl Acad Sci U S A* **94**, 5656-5661, doi:10.1073/pnas.94.11.5656 (1997).

22  Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E. & Sigler, P. B. GTPase mechanism of Gproteins from the 1.7-A crystal structure of transducin alpha-GDP-AIF-4. *Nature* **372**, 276-279, doi:10.1038/372276a0 (1994).

23  Kimple, A. J., Bosch, D. E., Giguere, P. M. & Siderovski, D. P. Regulators of G-protein signaling and their Galpha substrates: promises and challenges in their use as drug discovery targets. *Pharmacol Rev* **63**, 728-749, doi:10.1124/pr.110.003038 (2011).

24  Slep, K. C. *et al.* Molecular architecture of Galphao and the structural basis for RGS16-mediated deactivation. *Proc Natl Acad Sci U S A* **105**, 6243-6248 (2008).

25  Barnett, B. L., Kretschmar, H. C. & Hartman, F. A. Structural characterization of bis(N-oxopyridine-2-thionato)zinc(II). *Inorganic Chemistry* **16**, 1834-1838, doi:10.1021/ic50174a002 (1977).

26  Faergemann, J. Management of seborrheic dermatitis and pityriasis versicolor. *Am J Clin Dermatol* **1**, 75-80, doi:10.2165/00128071-200001020-00001 (2000).

27  Zhao, C. *et al.* Repurposing an antidandruff agent to treating cancer: zinc pyrithione inhibits tumor growth via targeting proteasome-associated deubiquitinases. *Oncotarget* **8** (2017).

28  Park, M., Cho, Y.-J., Lee, Y. W. & Jung, W. H. Understanding the Mechanism of Action of the Anti-Dandruff Agent Zinc Pyrithione against Malassezia restricta. *Scientific reports* **8**, 12086-12086, doi:10.1038/s41598-018-30588-2 (2018).

29  Qiu, M. *et al.* Zinc ionophores pyrithione inhibits herpes simplex virus replication through interfering with proteasome function and NF-κB activation. *Antiviral Res* **100**, 44-53, doi:10.1016/j.antiviral.2013.07.001 (2013).

30  Sekler, I., Sensi, S. L., Hershfinkel, M. & Silverman, W. F. Mechanism and Regulation of Cellular Zinc Transport. *Molecular Medicine* **13**, 337-343, doi:10.2119/2007-00037.Sekler (2007).

31  Ding, W.-Q. & Lind, S. E. Metal ionophores – An emerging class of anticancer drugs. *IUBMB Life* **61**, 1013-1018, doi:https://doi.org/10.1002/iub.253 (2009).

32  Dudev, T. & Lim, C. Principles Governing Mg, Ca, and Zn Binding and Selectivity in Proteins. *Chemical Reviews* **103**, 773-788, doi:10.1021/cr020467n (2003).

33  Chen, M. *et al.* Anti-tumour activity of zinc ionophore pyrithione in human ovarian cancer cells through inhibition of proliferation and migration and promotion of lysosome-mitochondrial apoptosis. *Artificial Cells, Nanomedicine, and Biotechnology* **48**, 824-833, doi:10.1080/21691401.2020.1770266 (2020).

34  Carraway, R. E. & Dobner, P. R. Zinc pyrithione induces ERK- and PKC-dependent necrosis distinct from TPEN-induced apoptosis in prostate cancer cells. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1823**, 544-557,
doi:https://doi.org/10.1016/j.bbamcr.2011.09.013 (2012).

35 Koh, J. Y. & Choi, D. W. Zinc toxicity on cultured cortical neurons: Involvement of N-methyl-d-aspartate receptors. *Neuroscience* **60**, 1049-1057, doi:https://doi.org/10.1016/0306-4522(94)90282-8 (1994).

36 Mohr, S. E. *First in fly: Drosophila research and biological discovery*. (Harvard University Press, 2018).

37 Savitsky, M., Solis, G. P., Kryuchkov, M. & Katanaev, V. L. Humanization of Drosophila Gαo to Model GNA01 Paediatric Encephalopathies. *Biomedicines* **8**, 395, doi:10.3390/biomedicines8100395 (2020).

38 Bateman, J. R., Lee, A. M. & Wu, C.-t. Site-Specific Transformation of Drosophila via ϕC31 Integrase-Mediated Cassette Exchange. *Genetics* **173**, 769-777, doi:10.1534/genetics.106.056945 (2006).

39 da Silva, L. E. M. *et al.* Zinc supplementation combined with antidepressant drugs for treatment of patients with depression: a systematic review and meta-analysis. *Nutr Rev* **79**, 1-12, doi:10.1093/nutrit/nuaa039 (2021).

40 Doboszewska, U. *et al.* Zinc signaling and epilepsy. *Pharmacology & therapeutics* **193**, 156-177, doi:https://doi.org/10.1016/j.pharmthera.2018.08.013 (2019).

41 Grabrucker, A. M., Rowan, M. & Garner, C. C. Brain-Delivery of Zinc-Ions as Potential Treatment for Neurological Diseases: Mini Review. *Drug Deliv Lett* **1**, 13-23, doi:10.2174/2210303111010100013 (2011).

42 Cherasse, Y. & Urade, Y. Dietary Zinc Acts as a Sleep Modulator. *International Journal of Molecular Sciences* **18**, 2334 (2017).

43 Brion, L. P., Heyne, R. & Lair, C. S. Role of zinc in neonatal growth and brain growth: review and scoping review. *Pediatric Research* **89**, 1627-1640, doi:10.1038/s41390-020-01181-z (2021).

44 Qin, Q., Wang, X. & Zhou, B. Functional studies of Drosophilazinc transporters reveal the mechanism for dietary zinc absorption and regulation. *BMC Biology* **11**, 101, doi:10.1186/1741-7007-11-101 (2013).

45 Fliss, H. Zinc I onophores as Anti-Stress Agent. International Patent WO 02/080943 (2002).

46 Muntean, B. S. *et al.* Gαo is a major determinant of cAMP signaling in the pathophysiology of movement disorders. *Cell Rep* **34**, 108718, doi:10.1016/j.celrep.2021.108718 (2021).

47 Nowak, G., Siwek, M., Dudek, D., Zieba, A. & Pilc, A. Effect of zinc supplementation on antidepressant therapy in unipolar depression: a preliminary placebo-controlled study. *Pol J Pharmacol* **55**, 1143-1147 (2003).

48 Salari, S., Khomand, P., Arasteh, M., Yousefzamani, B. & Hassanzadeh, K. Zinc sulphate: A reasonable choice for depression management in patients with multiple sclerosis: A randomized, double-blind, placebo-controlled clinical trial. *Pharmacological Reports* **67**, 606-609, doi:10.1016/j.pharep.2015.01.002 (2015).

49 Gibson, R. S., King, J. C. & Lowe, N. A Review of Dietary Zinc Recommendations. *Food Nutr Bull* **37**, 443-460, doi:10.1177/0379572116652252 (2016).

50 Duan, M. *et al.* Zinc nutrition and dietary zinc supplements. *Crit Rev Food Sci Nutr*, 1-16, doi:10.1080/10408398.2021.1963664 (2021).

51 Frederickson, C. J., Koh, J. Y. & Bush, A. I. The neurobiology of zinc in health and disease. *Nat Rev Neurosci* **6**, 449-462, doi:10.1038/nm1671 (2005).

52 Udechukwu, M. C., Collins, S. A. & Udenigwe, C. C. Prospects of enhancing dietary zinc bioavailability with food-derived zinc-chelating peptides. *Food & function* **7**, 4137-4144, doi:10.1039/c6fo00706f (2016).

53 Lye, J. C. *et al.* Systematic functional characterization of putative zinc transport genes and identification of zinc toxicosis phenotypes in Drosophila melanogaster. *J Exp Biol* **215**, 3254-3265, doi:10.1242/jeb.069260 (2012).
54 Kable, M. E. et al. The Znt7-null mutation has sex dependent effects on the gut microbiota and goblet cell population in the mouse colon. *PLoS One* **15**, e0239681, doi:10.1371/journal.pone.0239681 (2020).

55 McAllister, B. B., Bihelek, N., Mychasiuk, R. & Dyck, R. H. Brain-derived Neurotrophic Factor and TrkB Levels in Mice that Lack Vesicular Zinc: Effects of Age and Sex. *Neuroscience* **425**, 90-100, doi:10.1016/j.neuroscience.2019.11.009 (2020).

56 Thackray, S. E., McAllister, B. B. & Dyck, R. H. Behavioral characterization of female zinc transporter 3 (ZnT3) knockout mice. *Behav Brain Res* **321**, 36-49, doi:10.1016/j.bbr.2016.12.028 (2017).

57 Pound, L. D. et al. The physiological effects of deleting the mouse SLC30A8 gene encoding zinc transporter-8 are influenced by gender and genetic background. *PLoS One* **7**, e40972, doi:10.1371/journal.pone.0040972 (2012).

58 Abdelnour, S. A. et al. Nanominerals: Fabrication Methods, Benefits and Hazards, and Their Applications in Ruminants with Special Reference to Selenium and Zinc Nanoparticles. *Animals (Basel)* **11**, doi:10.3390/ani11071916 (2021).

59 Waterhouse, A. et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res* **46**, W296-W303, doi:10.1093/nar/gky427 (2018).

60 Handing, K. B. et al. Characterizing metal-binding sites in proteins with X-ray crystallography. *Nat Protoc* **13**, 1062-1090, doi:10.1038/nprot.2018.018 (2018).

61 Abraham, M. J. et al. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1-2**, 19-25, doi:https://doi.org/10.1016/j.softx.2015.06.001 (2015).

62 Päll, S., Abraham, M. J., Kutzner, C., Hess, B. & Lindahl, E. 3-27 (Springer International Publishing).

63 Solis, G. P. et al. Local and substrate-specific S-palmitoylation determines subcellular localization of Gao. *bioRxiv*, 2020.08.25.266692, doi:10.1101/2020.08.25.266692 (2021).

64 Katoh, Y., Nozaki, S., Hartanto, D., Miyano, R. & Nakayama, K. Architectures of multisubunit complexes revealed by a visible immunoprecipitation assay using fluorescent fusion proteins. *Journal of cell science* **128**, 2351-2362, doi:10.1242/jcs.168740 (2015).

65 Gratz, S. J. et al. Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in <em>Drosophila</em>. *Genetics* **196**, 961-971, doi:10.1534/genetics.113.160713 (2014).

66 Gloor, G. B. et al. Type I repressors of P element mobility. *Genetics* **135**, 81-95 (1993).

67 Linford, N. J., Bilgir, C., Ro, J. & Pletcher, S. D. Measurement of lifespan in Drosophila melanogaster. *J Vis Exp*, doi:10.3791/50068 (2013).

**Figures**
Figure 1

Mutations in positions 203, 209 and 246 result in constitutive GTP-loading of Gao. (A) Mutated amino acid residues (in red) in the overall structure of Gao. The residues are located in proximity of the switch II region of Gao that performs key functions in uptake and hydrolysis of GTP (shown as a stick structure with standardly colored atoms, complexed with magnesium in magenta). (B, C) Representative curves (B) and quantification of the binding rate constant (kbind, C) of BODIPY-labeled GTPγS binding to Gao, wild-type or mutated. All the mutants demonstrate strongly elevated rates, with G203R being the fastest. (D, E) Representative curves (D) and quantification of the hydrolysis rate constant (khydr, E) characterizing the course of BODIPY-labeled GTP binding and hydrolysis by Gao or its mutants by monitoring the formation and decay of the GTP-bound fraction of Gao. Note the difference between panel (B) where the data are adjusted to the plateau to highlight the differences in the binding rates and panel (D) where the data is shown in raw fluorescence units, as needed for the proper khydr calculation. Note the log scale in the Y-axes in (C) and (E). Data in (B-E) are shown as mean of at least three experiments ± SD. * p<0.05; ** p<0.01, **** p<0.0001 by the T-test.
Figure 2

Zinc pyrithione and Zn2+ restore the GTPase activity and cellular interactions of pathologic Gαo mutants. (A-C) Zinc pyrithione (ZPT, A) restores the GTPase activity of Gαo[E246K] (B) but does not affect the GTP binding and hydrolysis of wild type Gαo (C). Representative curves of BODIPY-labeled GTP binding and hydrolysis in the absence or presence of 50μM ZPT are provided. (D, E) Quantification of khydr of Gαo wild type (WT), Gαo[G203R], Gαo[R209C], and Gαo[E246K] treated with ZPT (D) and ZnCl2 (E). Note the log scale in the Y-axes. (F, G) N2a cells were co-transfected with His6-RGS19 and Gαo-GFP (C-terminally tagged), [G203R], [R209C], and [E246K]. The next day, cells were treated with DMSO or 1µM ZPT for 3h before subjected for immunoprecipitation (IP). IP of Gαo was done using a nanobody against GFP, and the co-precipitation of co-precipitation of RGS19 was analyzed by SDS-PAGE and Western Blot. Abs against GFP and His6-tag were used for the detection of Gαo and RGS19, respectively (F). Quantification of the co-IP of His6-RGS19 by Gαo-GFP G203R, R209C, and E246K (G). Data in (B-E, G) are mean of at least three experiments ± SD. *p<0.05, **p<0.01, ****p<0.0001 by the T-test, ‘n.s.’ – non-significant.
Zn2+ restores the defects induced to the catalytic Q205 by the pathologic Gαo mutations. (A, C, E) Analysis of the distance between the γ-N atom of Q205 residue in Gαo and the γ-P atom of GTP in wild type and mutant proteins over 100ns of molecular dynamics simulation. The panels show the plots of the measured distance between these atoms in wild type (WT) protein containing Mg2+ or Zn2+ and the same in the [E246K] mutant (A), [R209C] mutant (C) and the [G203R] mutant (E). The graphs demonstrate that over the major or significant part of the simulation trajectories in all 3 mutants, the Q205 residue is further away from the active site than in WT protein, which is rescued by presence of Zn2+ in the active site. (B, D, F) 3D structures of the representative states with the maximal removal of Q205 from the active site is shown for the wild type in the Mg2+-bound conformations vs. the mutants in the Mg2+- and Zn2+-bound conformations: [E246K] (B), [R209C] (D) and [G203R] (F). It is evident that upon Zn2+ binding, the mutants’ Q205 is brought back to the γ-P atom of GTP (the hydrogen bonds are indicated). Color coding as for the respective (A, C, E) panels.
Figure 4

Dietary Zn2+ rescues the motor dysfunction and reduced lifespan in a Drosophila model of GNAO1 encephalopathy. (A) Scheme of the negative geotaxis assay. After tapping down, flies start climbing to the top of the vial (blue arrow shows the direction of the negative geotaxis). Climbing pass rate is percent of flies crossed the red line (10 cm from the bottom) in 10 sec. (B) The locomotion behavior of adult flies was measured in the negative geotaxis assay. 100-110 male and female flies (in groups of ca. 15 flies) were measured for each genotype. Data are shown as mean ± sem, n = 10. T-test shows significant differences between the wild-type and G203R/+ flies, p-values < p<0.0001. (C) The lifespan of 250 adult flies (110 males and 140 females for both genotype) was determined during 85 days. Mantel-Cox and Gehan-Breslow-Wilcoxon tests both show highly significant (p-values < p<0.0001) drop in survival of G203R/+ flies, both males and females, as compared to the respective wild type flies. (D) The locomotion dysfunction of G203R/+ flies is rescued by dietary supplementation of ZnCl2, more for females than for male flies; dietary ZPT does not show a consistent effect. (E) Dietary ZnCl2 slightly improves the locomotion behavior of female wild type flies; both ZnCl2 and ZPT worsen the locomotion of male flies. Data presentation and analysis in (D, E) is the same as in (B). (F) The drop in the lifespan of female G203R/+ flies is rescued by dietary supplementation of ZnCl2. Data presentation and analysis as in (C).

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