Restoration of the GTPase activity of Gao mutants by Zn^{2+} in *GNAO1* encephalopathy models

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Biological Sciences - Article

Keywords: encephalopathy, GTPase activity, zinc supplements

Posted Date: September 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-900405/v1>

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Version of Record: A version of this preprint was published at Science Advances on October 7th, 2022. See the published version at <https://doi.org/10.1126/sciadv.abn9350>.

Abstract

GNAO1 encephalopathy is a rare pediatric disease characterized by motor dysfunction, developmental delay, and epileptic seizures¹⁻³. *De novo* point mutations in the gene encoding Gao, the major neuronal G protein, lie at the core of this dominant genetic malady⁴. Half of the clinical case mutations fall on codons Gly203, Arg209, or Glu246 near the GTP binding/hydrolysis pocket of Gao¹⁻³. We here show that these pathologic mutations strongly speed up GTP uptake and inactivate GTP hydrolysis by Gao, 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 Zn²⁺ ions as agents restoring the active conformation, GTPase activity, and cellular interactions of the encephalopathy mutants, with a negligible effect on wild type Gao. 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 Gao 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 drugs⁵. Composed of the Gα, β, and γ subunits, they interact with receptors when the Gα is loaded with GDP to undergo the activated GPCR-induced GDP-to-GTP exchange and dissociation into the Gα-GTP and Gβγ components, both competent to transmit the signal further downstream⁶. With time, the intrinsic GTPase activity of the Gα-subunits leads to GTP hydrolysis – the activity further sped up by the dedicated RGS (Regulator of G protein Signaling) proteins⁷. The resultant Gα-GDP can reload with GTP⁸ or complex back with Gβγ, thus closing the G protein activation-deactivation loop⁶.

Of the 16 human Gα-subunits, Gao 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 Gao⁴. 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 infancy¹⁻³.

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 surveys¹⁻³. These amino acid residues are located near the GTP-binding pocket of Gao and play an important role in the adoption of the activated G protein conformation⁹ (Fig. 1A).

Apart from symptomatic treatments such as deep-brain stimulation¹⁰, 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 Gao

To seek understanding of the molecular etiology of *GNAO1* encephalopathy, we first probed the basic biochemical properties of the most frequent Gao 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 uptake¹¹, while

R209H was reported to display a faster GTP uptake¹². We have applied the non-hydrolyzable fluorescent BODIPY-GTP γ S assay^{8,13-17} to monitor the GTP uptake properties of four Gao 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_{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 Gao (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 fluorophore-GDP on the protein)^{8,15,16,18}. Remarkably, we see that all three pathologic Gao mutants reveal a dramatically slowed down fluorescent decay rate as compared to the wild type (Fig. 1D). Calculation of the hydrolysis rate constant, k_{hydr} , confirms this assessment: k_{hydr} of E246K is reduced 65-fold, of R209C – 114-fold, and of G203R – 212-fold as compared to the wild type Gao (Fig. 1E).

Thus, mutations in the three most frequently affected *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 Gao lead to the constitutive GTP-binding state of the G protein – the molecular feature likely at the core of the disease etiology.

Defective cellular interactions of the GNAO1 encephalopathy mutants

The distorted proportion of the GTP/GDP-state residence of the *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 Gao function¹³. In this as other cell lines, wild type Gao shows dual localization at the plasma membrane and the Golgi apparatus, as previously reported by us^{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 $\beta\gamma$ that preferentially interacts with the GDP-loaded form of Gao, and RGS19 that preferentially binds to the GTP-loaded form⁸. Using an internal GFP fusion (that does not impede the proper localization nor protein-protein interactions¹¹) of Gao and Gao[G203R], we first found in the pull-down assay that the mutation significantly reduces the ability of the protein to interact with G $\beta\gamma$ (Supplementary Fig. S2A, B). This finding agrees with the reduced proportion of the GDP-loaded form of Gao[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 Gao[G203R], opposite to that seen for the constitutively activate Q205L (Supplementary Fig. S2C, D). Puzzled by this finding, we used C-terminally GFP-tagged Gao, this time comparing the RGS19 interactions for the wild type and the three mutant variants of the protein. This analysis again revealed that the *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^{7,8}, the cellular GTP-residence of the mutants is expected to be even more significant. Second, the mutants display reduced interactions with physiologic Gao partners and, by inference, reduced signal transduction capacity in neuronal cells. And third, despite the increased residence in the GTP-bound state, the mutant Gao proteins likely do not adopt the truly activated conformation, hence fail to interact with RGS19 (and presumably other signaling partners).

Displacement of the GTPase catalytic residue in GNAO1 encephalopathy mutants

Gai 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 Ga in the active conformation upon the GTP binding^{9,21}, result in a significant displacement of the Gln205 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 Gln205, 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 *GNAO1* encephalopathy mutants as the displacement of the catalytic Gln205. 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 Gα-GTP interaction with RGS proteins^{23,24}, 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. S5B-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 / ZnCl_2 : while restoration of the GTPase reaction in Gao[R209C] and Gao[E246K] was saturating with the EC_{50} values around $10\mu\text{M}$, the response of Gao[G203R] was rather linear in the concentration range tested (Fig. 2D, E), suggesting the potentially distinct molecular mechanisms of Zn^{2+} acting on the G203 mutant vs. the R209C / E246K mutants.

Potential mechanism of action of Zn^{2+} 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 Zn^{2+} in the restoration of the GTPase activity of the three Gao mutants. We argued that Zn^{2+} can replace Mg^{2+} in the Gao's active center upon the interaction with GTP: it is well-known that proteins' Mg^{2+} -binding sites are generally unspecific for this ion, which can be substituted by a broad range of other divalent metal ions, with Zn^{2+} being one of the most potent substitutes³². Our analysis shows that the substitution of Zn^{2+} for Mg^{2+} 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 Zn^{2+} -bound protein; no global effect of Zn^{2+} 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 Zn^{2+} 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 Zn^{2+} (Fig. 3). It should be noted that in the wild type protein Zn^{2+} 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 binding^{9,21} and suggesting the common mechanism of action of Zn^{2+} on these two encephalopathy mutants.

The effect of Zn^{2+} 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 ζ -atom of Arg203, in the Mg^{2+} - vs. Zn^{2+} -bound conformations. Surprisingly, we found that Zn^{2+} , if anything, overall stabilized the Arg203-Gln205 interaction (Supplementary Fig. S4F).

Overall, our structural analysis suggests the atomistic mechanism for the action of Zn^{2+} 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 ZnCl_2 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, Zn^{2+} , 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 Zn^{2+} 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 ZnCl_2 in N2a cells. This analysis confirms the neurotoxicity of ZPT with the IC_{50} of ca. $5\mu\text{M}$; ZnCl_2 in contrast was not toxic up to the concentrations of $100\mu\text{M}$ (Supplementary Fig. S7A) as presumably

it failed to penetrate the cells in this acute setting. For the subsequent experiments on the restoration of the Gao-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 Gao as in Supplementary Fig. S2E earlier, we found that ZPT could dramatically recover the ability of Gao[E246K] to interact with RGS19 to the levels normally seen for wild type Gao (Supplementary Fig. S7B, C). ZnCl₂, in contrast, was ineffective. The interaction of Gao 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 Gao versions: ca. 6-fold for Gao[R209C], ca. 12-fold for Gao[E246K], and the dramatic 20-fold for Gao[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 Gao 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 biology³⁶, and our recent work highlights the power of *Drosophila* as the host to model *GNAO1* encephalopathy³⁷. To establish the *GNAO1*-encephalopathy model in the fruit fly, we applied the CRISPR/Cas9-mutagenesis together with phiC31-mediated recombinase-mediated cassette exchange³⁸ to introduce the pathogenic G203R mutation into the *Drosophila* Gao (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 depression³⁹, epilepsy⁴⁰, psychiatric and neurodegenerative diseases⁴¹, or sleep disorders⁴², as well as for normal neonatal development⁴³. 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 transporters⁴⁴, providing us a guideline. We also tested 10μM ZPT food supplementation, following the application of up to 15μM ZPT in rats⁴⁵. *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 longevities.

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' report⁴. However, despite some insights^{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 stimulation¹⁰.

The core molecular dysfunction we have “diagnosed” here for the three most common *GNAO1*-encephalopathy mutations is the constitutive GTP binding state of the mutant Gao 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 Gao in *GNAO1*-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 Gao, thus creating a signaling-deficient G protein.

It is remarkable that a simple ion, Zn^{2+} emerging from our screening of drug candidates to recover the GTPase deficiency of the mutant Gao, is efficient in restoring the structural rearrangements induced by the pathologic mutations. Replacing Mg^{2+} from the GTP pocket of Gao, Zn^{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 Gao[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 *Drosophila* model of *GNAO1*-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 development⁴³, sleep quality in adults⁴², and to ameliorate health conditions in depression³⁹, epilepsy⁴⁰, and a set psychiatric and neurodegenerative states⁴¹. For example, daily 25mg Zn^{2+} applied for 6 weeks in one study⁴⁷, and daily 220mg zinc sulphate (providing 50mg zinc) applied for 12 days in another⁴⁸, 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 adults⁴⁹. In this regard, dietary zinc supplementation might be considered as a potential treatment option to improve the conditions of *GNAO1*-encephalopathy patients – at least those carrying the G203R, R209C, and E246K mutations. More studies will show how applicable is the Zn^{2+} -restorable GTPase deficiency mechanism to the other *GNAO1*-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^{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^{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 sexes⁵³. 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 models⁵⁴⁻⁵⁷. 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^{41,52,58}, and might be considered in the future prospective applications to *GNAO1*-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 *GNAO1*-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 *GNAO1*-encephalopathy and show that a dietary supplementation of Zn^{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 *GNAO1*-encephalopathy patients.

Materials And Methods

Plasmids and molecular cloning

The plasmids for the Gao-GFP (C-terminally and internally tagged), mRFP-G β 1, mRFP-G γ 3, and His₆-RGS19 were previously described^{8,11}. The Gao 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-Gao⁸ 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-Gao wild type, pET23b-Gao[G203R], pET23b-Gao[R209C], or pET23b-Gao[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 3,500g 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 15,000g/ 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 200μM 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. Gao 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 Gao[G203R] and Gao[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 Gao 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 Gao mutants bound to Mg²⁺ or Zn²⁺ were directly used in the GROMACS 2021.2 software^{61,62} 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 Gao modulators was performed using the Gao[E246K] protein and FDA Approved & Pharmacopeial Drug Library (HY-L066, MedChemExpress). DMSO or compounds in DMSO (12.5 μ M) were mixed with Gao[E246K] at 1 μ M in a reaction buffer and BODIPY-GTP at 1 μ M 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_{bind}) and (ii) maximal GTP uptake. For candidates affecting the k_{bind} , the hits were picked if the compound modulated k_{bind} by ≥ 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 ≥ 3 SD of DMSO-treated wells. The hits were subsequently validated by performing the GTP binding assay at 50 μ M of compounds using both Gao wild type and Gao[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 Gao-GFP constructs was performed as previously described⁶³. 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 1 μ M ZPT, 100 μ M 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 16,000g for 15min at 4°C, and supernatants were incubated with 2 μ g of nanobody against GFP⁶⁴ on ice for 30min. Then, 20 μ l 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), His₆-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 dGao* editing

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

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 GGCCATAACTTCGTATAATGTATGCTATACGAAGTTAT and GGCCATAACTTCGTATAGCATACATTATACGAAGTTAT 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 attPfwRI/attPprevHpaI and attPfwKpnI/attPprevHpaI (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 pLattP-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 pLattP-pBacDsRed-lox-attP digested with KpnI and SmaI. The resultant donor plasmid pLattP-pBacDsRed-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

CTTCGCAGAATGGGGGCGTTAGCA and AAACCTGCTAACGCCCCCATTCTGC;

TTCGCTCAAGTCTTGGCAGATAC and AACGTATCTGCCAAGACTTGAGC

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

CTTCGAATGGACGGGTGGAAGGGT and AAACACCCTTCCACCCGTCCATTC;

TTCGCTGCTCTTTAATACCGTTA and AACTAACGGTATTAAAGAGCAGC 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 flank 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[*], 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

Acknowledgements

The work was supported by the Swiss National Science Foundation grant #31003A_175658 to V.L.K. We thank Sabina Troccaz for the technical assistance and the HumanaFly facility of the Faculty of Medicine, University of Geneva, for hosting the *Drosophila* experiments.

Competing interests

The high-throughput screening and initial hit validation phases of this work were in part financed by a private donation from the family of a *GNAO1*-encephalopath child harboring the [E246K] mutation.

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Figures

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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 (k_{bind} , C) of BODIPY-labeled GTP γ S 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 (k_{hydr} , 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 k_{hydr} 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 $\pm$ SD. * $p < 0.05$; ** $p < 0.01$, **** $p < 0.0001$ by the T-test.

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Figure 2

Zinc pyrithione and Zn²⁺ restore the GTPase activity and cellular interactions of pathologic Gao mutants. (A-C) Zinc pyrithione (ZPT, A) restores the GTPase activity of Gao[E246K] (B) but does not affect the GTP binding and hydrolysis of wild type Gao (C). Representative curves of BODIPY-labeled GTP binding and hydrolysis in the absence or presence of 50 μ M ZPT are provided. (D, E) Quantification of k_{hyd} of Gao wild type (WT), Gao[G203R], Gao[R209C], and Gao[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 Gao-GFP (C-terminally tagged), [G203R], [R209C], and [E246K]. The next day, cells were treated with DMSO or 1 μ M ZPT for 3h before subjected for immunoprecipitation (IP). IP of Gao 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 Gao and RGS19, respectively (F). Quantification of the co-IP of His6-RGS19 by Gao-GFP G203R, R209C, and E246K (G). Data in (B-E, G) are mean of at least three experiments $\pm$ SD. * p <0.05, ** p <0.01, **** p <0.0001 by the T-test, 'n.s.' – non-significant.

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Figure 3

Zn $^{2+}$ restores the defects induced to the catalytic Q205 by the pathologic Gao mutations. (A, C, E) Analysis of the distance between the γ -N atom of Q205 residue in Gao 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 Mg^{2+} or Zn^{2+} 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 Zn^{2+} 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 Mg^{2+} -bound conformations vs. the mutants in the Mg^{2+} - and Zn^{2+} -bound conformations: [E246K] (B), [R209C] (D) and [G203R] (F). It is evident that upon Zn^{2+} 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.

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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 (10cm from the bottom) in 10sec. (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 $\pm$ 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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