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Network and Atomistic Simulations Unveil the Structural Determinants of Mutations Linked to Retinal Diseases

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Abstract

A number of incurable retinal diseases causing vision impairments derive from alterations in visual phototransduction. Unraveling the structural determinants of even monogenic retinal diseases would require network-centered approaches combined with atomistic simulations. The transducin G38D mutant associated with the Nougaret Congenital Night Blindness (NCNB) was thoroughly investigated by both mathematical modeling of visual phototransduction and atomistic simulations on the major targets of the mutational effect. Mathematical modeling, in line with electrophysiological recordings, indicates reduction of phosphodiesterase 6 (PDE) recognition and activation as the main determinants of the pathological phenotype. Sub-microsecond molecular dynamics (MD) simulations coupled with Functional Mode Analysis resolve the improvement of information, showing that such impairment is likely due to disruption of the PDEG binding cavity in transducin. Protein Structure Network analyses additionally suggest that the observed slight reduction of theG99-catalyzed GTPase activity of transducin depends on perturbed communication between RG99 and GTP binding site. These findings provide insights into the structural fundamentals of abnormal functioning of visual phototransduction caused by a missense mutation in one component of the signaling network. This combination of network-centered modeling with atomistic simulations represents a paradigm for future studies aimed at thoroughly deciphering the structural determinants of genetic retinal diseases. Analogous approaches are suitable to unveil the mechanism of information transfer in any signaling network either in physiological or pathological conditions.

Introduction

A number of incurable diseases in the visual system involve one or more components of the phototransduction signaling network (Figure 1). Visual phototransduction is the G protein-mediated process that generates a neural signal following light capture by visual pigments in photoreceptor cells (rods and cones). A unique feature of rod cells, the vertebrate photoreceptors dedicated to dim light vision, is the capability to transduce signals from even single photons due to an extremely efficient amplification not paralleled by other signal transduction pathways [1,2]. The first event in scotopic vision is the absorption of a photon by rhodopsin (R), the cornerstone of family A of the seven-transmembrane G protein-coupled receptors (GPCRs), which leads to the formation of the signaling active state (R*) [3,4]. The latter, in turn, catalyzes the exchange of bound GDP for GTP on the catalytic subunits and two identical α-subunits (GαGTP) dissociates from the βγ-dimer thus stimulating the activation of phosphodiesterase 6 (PDE), a tetramer made of two nearly identical α and β catalytic subunits and two identical γ subunits [5]. The binding of GαGTP to the γ subunit of PDE (PDEγ/γ) releases its inhibitory constraint on the catalytic subunits, thus leading to the hydrolysis of guanosine 3’,5’-cyclic monophosphate (cGMP), followed by a rapid closure of the cGMP-gated ionic channels in the outer membrane and a drop in the circulating current. The lowering in intracellular calcium concentration, associated with cell hyperpolarization ultimately signals the presence of light to the secondary neurons of retina. Signaling shutoff includes at least three calcium feedback mechanisms as well as the simultaneous deactivation of GαGTP and PDE. In this respect, the termination of PDE activation by GαGTP is achieved when the GTP-bound to Gα is hydrolyzed to GDP by the intrinsic GTPase activity of the protein. The latter process is significantly accelerated by a multiprotein complex containing the ninth member of the Regulators of G protein Signaling (RGS) family, hereafter indicated as RGS [6]. As a result of the GTPase Activating Protein (GAP) action of RGS, the GαGTP complex re-associates with the βγ dimer restoring the Gαβγ heterotrimer (i.e. Gt).

Mismisfunctioning of any component of the phototransduction network causes more or less severe vision impairments. Such an example is the Nougaret form of dominant stationary night blindness (Nougaret Congenital Night Blindness, NCNB) caused by a missense mutation, G38D, found in the rod Gα of affected individuals [7–9]. Stationary night blindness is not associated with retinal degeneration and is characterized by the inability to see in the dark, whereas daytime vision is largely unaffected [7–9]. In vitro characterization showed that the aspartate substitution for G38 perturbed the interaction between Gα and Gβγ or activation of transducin by R* [9]. Furthermore, the mutant Gα is character-
Network and Atomic Modeling of Retinal Diseases

Author Summary

Incurable retinal diseases causing vision impairments may be due to spontaneous mutations in one component of the visual phototransduction signaling network. Such alterations include the transducin single point mutation G38D associated with the Noougaret Congenital Night Blindness (NCNB). We combined a systems biology approach with atomistic simulations to gain insights into the structural fundamentals of the NCNB disease. Consistent with in vitro evidence, mathematical modeling suggests reduced effector recognition and activation as the main determinants of the pathological phenotype. Sub-microsecond molecular dynamics simulations improve the resolution of information, suggesting that such impairment is likely due to disruption of the effector binding cavity. Atomistic simulations also suggest that the observed slight reduction of the RGS9-catalyzed GTPase activity of transducin depends on perturbed inter-protein communication involving the nucleotide. The study highlighted manifold effects of a single point pathogenic mutation, thus paving the way for analogous studies towards a thorough understanding of the structural determinants of genetic retinal diseases.

ized by modestly reduced $k_{\text{cat}}$ value for the intrinsic (−2.5-fold) and RGS-catalyzed (−5 fold) GTP hydrolyses. In contrast, biochemical data showed that G38D is totally impaired in its ability to bind and activate PDE [9], whereas suction electrode recordings revealed that homozygous $G_{\alpha\text{GTP}}^{\text{G38D}−/−}$ rods exhibit residual light responses, indicating that the mutation reduced but did not completely abolish effector function [8]. Functional consequences of substituting the homologous amino acid in other G proteins were found to inhibit GTPase activity and to prevent stimulation by GAP in Ras-p21 [10], $G_{\alpha}$ [11], $G_{\alpha}$ [12], and $G_{\alpha}$ [13].

Thus, a single-point mutation in $G_{\alpha}$ seems to elicit a multitude of effects not entirely clarified by in vitro and in vivo experiments and likely involving more than one component in the phototransduction signaling network. In this framework, to gain insights into the molecular bases of the NCNB disease, we integrated the information from in vitro/in vivo experiments with systems-based and atomistic modeling. The systems-based approach relied on a comprehensive quantitative model of phototransduction in rod cells that explicitly includes most of the molecular components of the cascade [14] (Figure 1). In this study, that model was extended to the NCNB pathological phenotype, thus highlighting those reactions and intermolecular interactions perturbed by the $G_{\alpha}$ mutation. The molecular systems involved in those reactions were subjected to atomistic Molecular Dynamics (MD) simulations and included: wild type and mutated $G_{\alpha\text{GTP}}$ taken both in their isolated forms (i.e. $G_{\alpha\text{GTP}}^{\text{WT}}$ and $G_{\alpha\text{GTP}}^{\text{G38D}}$) and in the ternary complex with RGS and PDE7 ($G_{\alpha\text{GTP}}^{\text{WT}}$ and $G_{\alpha\text{GTP}}^{\text{G38D}}$) and in the ternary complex with RGS and PDE7 ($G_{\alpha\text{GTP}}^{\text{WT}}$ and $G_{\alpha\text{GTP}}^{\text{G38D}}$) (Figure 2).

Results

Mathematical modeling of the NCNB phenotype

We presented a dynamical model of the phototransduction signaling network made up of ordinary differential equations, which describe the reactions and their kinetic parameters [14]. The working model used in this study includes also the dynamic scaffolding reactions between dark rhodopsin and Gt [15]. Hence, such model was further extended to describe the heterozygous ($G_{\alpha\text{GTP}}^{\text{G38D}+/−}$) and homozygous ($G_{\alpha\text{GTP}}^{\text{G38D}−/−}$) mutated conditions of $G_{\alpha\text{GTP}}^{\text{G38D}−}$. This was accomplished by introducing the mutated $G_{\alpha}$ protein as an explicit new molecule and adding all the relative reactions in the phototransduction cascade, which concerned the $G_{\alpha\text{GTP}}^{\text{WT}+/+}$ status (Table 1, see Methods). The output of mathematical simulations (i.e. change in photocurrent with respect to dark value, ΔI) was analyzed and compared with the photoreponses of rods from wild type and transgenic mice (Figure 3A, 3B, and 3C). It is worth noting that, due to the significant difference in the species between in vivo (i.e. mammals, Figure 3A, 3B, and 3C) and in silico (i.e. amphibian rods [14], Figure 3D, 3E, and 3F) experiments, the time scales of the photoreponses is different, thus allowing for semi-quantitative comparisons.

The results obtained with our $G_{\alpha\text{GTP}}^{\text{WT}+/+}$ model were in remarkable agreement with in vitro recordings on wild type cells (Figure 3A and 3D).

In order to fit the models onto the pathological NCNB condition, the rates of a number of reactions involving $G_{\alpha\text{GTP}}^{\text{G38D}}$ were systematically reduced by tuning the relative kinetic parameters in decreasing steps (Table 1, Figure 1, see Methods). The reductions were combined into specific heterozygous and homozygous models. At the end, the best fit with electrophysiological recordings of the mutant cells was obtained by making changes in the following reactions:

1) $\text{PDE} + G_{\alpha\text{GTP}}^{\text{G38D}} \xrightarrow{k_{\text{PI}}} \text{PDE} G_{\alpha\text{GTP}}^{\text{G38D}}$
2) $\text{PDE} G_{\alpha\text{GTP}}^{\text{G38D}} \xrightarrow{k_{\text{P2}}} \text{PDE}^{−} G_{\alpha\text{GTP}}^{\text{G38D}}$
3) $\text{RGS} + \text{PDE}^{−} G_{\alpha\text{GTP}}^{\text{G38D}} \xrightarrow{k_{\text{RGS}}} \text{RGS} \cdot \text{PDE}^{−} G_{\alpha\text{GTP}}^{\text{G38D}}$

describing, respectively, a) the binding of one molecule of $G_{\alpha\text{GTP}}$ to one inactive PDE subunit, b) activation of the $G_{\alpha\text{GTP}}$ PDE complex, and c) binding of the RGS complex to a PDE tetramer with one active subunit. The relative changes in the parameters were 35000-fold reduction in $k_{\text{PI}}$ and $k_{\text{P2}}$ and 2-fold reduction in $k_{\text{RGS}}$, with respect to the wild type value. Following such changes, heterozygous cells show a similar behavior to wild type cells under dim flash responses but elongated recovery under brighter flashes associated with slight loss in sensitivity (i.e. a 40% brighter flash is required to generate a half-maximal response, $I_{50}$; Figure 3G and Figure 5B in Moussaïf et al. [8] and Table 2). In contrast, homozygous cells show marked decrease in sensitivity to light (50-fold brighter $I_{50}$, Table 2) and impaired response recoveries for all flash intensities.

Same strengths of flashes delivered to the cells in simulations and electrophysiological recordings [8] result in quantitative differences concerning time scales and sensitivity of the photoreponses, likely due to the different species considered. The higher ΔI elicited by the dimmest flash in computational experiments compared to electrophysiological recordings is exemplar in this respect (Figure 3A and 3D). Differences between the cellular properties of mammals and amphibian rods include temperature and volume, which likely influence initial conditions and concentrations of the molecular species involved in signal transduction [5].

It is worth noting that, in the actual mathematical model, up to two $G_{\alpha\text{GTP}}$ can bind, and in turn activate, either one of the catalytic subunits of PDE, hence leading to a 2:1 stoichiometry. Nevertheless, as previously discussed [14] (see also Table S4 therein), the 2:1 $G_{\alpha\text{GTP}}$PDE complex is detectable only in the presence of light flashes with intensities in the order of $10^{5}$ photons/μm² and, even then, their presence is negligible. As a confirmation of those results, deep reductions of $k_{p3}$ and $k_{p4}$ (regulating, respectively, the binding of the second molecule of $G_{\alpha\text{GTP}}$ to $G_{\alpha\text{GTP}}$-PDE and the activation of both catalytic subunits of the PDE tetramer), in the background of any of the test
models used in this study, did not elicit any change in the photoresponse (Table 1). We cannot, however, exclude that this was due to an inaccurate modeling of this part of the network, whose biochemical detail remains mainly unknown. For example, a finer treatment of the allosteric and regulatory mechanisms may be necessary to recover the role of the second PDE subunit [14]. This might also explain why, with the changes in $k_{P1}$, $k_{P2}$, and $k_{RGS1}$ necessary to reproduce $G_{a_{GTP}}$ photoresponses, loss in sensitivity and delay of the time to peak are more marked in simulated responses compared to in vitro ones (78- vs 47- and 2.4- vs 1.7-fold, respectively, see Table 2).

In line with the statements above, we relied on the fact that, in the present model, changes in $k_{RGS1}$ and $k_{RGS2}$ only affect the formation and activity of the 1:1:1 $G_{a_{GTP}}$-RGS-PDE complex. Noteworthy, as shown in Table 1, $k_{RGS2}$ is a rather coarse parameter, as it describes the RGS-catalyzed GTPase activity in both $G_{a_{GTP}}$-RGS-PDE and $G_{a_{GTP}}$-RGS-PDE-$G_{a_{GTP}}$ complexes as well as disruption of these complexes. For this reason, we couldn’t use the mathematical model to properly evaluate mutational effects on the GAP activity of RGS in the $G_{a_{GTP}}$-RGS-PDE complex.

In summary, consistent with electrophysiological recordings [8] but not with earlier biochemical data [9] mathematical modeling highlights reduction of both PDE binding and activation as the major mutation-induced perturbations in the visual phototransduction signaling network. A marginal reduction in RGS binding helped as well in reproducing the electrophysiological phenotype. On these bases, insights into the structural determinants of such perturbations were searched by atomistic MD simulations targeting both wild type and mutated $G_{a}$ in its isolated form and in ternary complex with both RGS and PDE.$^{\gamma}$

Atomistic simulations on the $G_{a_{GTP}}$ system: The G38D mutant affects the swil/3 cleft, primary determinant in PDE.$^{\gamma}$ binding

Atomistic simulations were firstly carried out on the isolated $G_{a_{GTP}}$ in its wild type and mutated forms ($G_{a_{GTP}}^{WT}$ and $G_{a_{GTP}}^{G38D}$, respectively).
The cartoon representation of GαGTP structure (PDB code: 1TND) is shown. The G protein holds a Ras-like domain and an α-helical domain. The interface between α-helical and Ras-like domain makes the nucleotide binding cleft. The α-helical domain is an orthogonal bundle of six α-helices. The Ras-like domain holds a Rossmann fold, characterized by a 3-layer(a/b) sandwich architecture due to the inversion in the order of the strands β1 and β3 as well as β1 and β4, which are adjacent to each other. The Ras-like domain is colored according to secondary structure (i.e. helices, strands, and loops are, respectively, violet, yellow and white), whereas the α-helical domain is gray. The mutation site is indicated by a cyan sphere centered on the Cα-atom. The GTP nucleotide is represented by sticks colored by atoms type. The nucleotide docks into a binding site contributed by the loops. These are ultraconserved regions also called G boxes 1–5 (G1–G5, colored green). G2 is also called swI (Figure 2). Such mutated position is not involved in backbone-mediated H-bonding interactions with the nucleotide neither in the wild type nor in the mutant, (Supplementary Figure 1A (Figure S1A)). The interaction pattern of the nucleotide remains almost unchanged in the two forms following MD simulations, as also indicated by the patterns of interaction energies between GTP and surrounding residues (Figure S2). Collectively, these data are consistent with the results of in vitro evidence that the mutation elicits a marginal effect on the intrinsic GTPase activity of the protein [8,9].

In contrast to lack of local structural effects, the G38D mutant turned out to affect the intrinsic dynamics of the protein. Indeed, the Cα-atom Root Mean Square Deviation (RMSD) of the mutant is higher than that of the wild type especially over the second half of MD simulation (Figure S3). As expected, Cα-atom fluctuations evaluated in terms of Root Mean Square Fluctuations (RMSFs) show peaks of flexibility in the loops connecting the elements of secondary structure, especially those in the α-helical domain. This effect is greater in the mutant than the wild type (Figure 4). In line with RMSFs, in the mutant form, selected portions of the protein show significant enhancements in their collective motions as inferred from the Principal Component Analysis (PCA) of the trajectories. These portions include linker1, αB/αC loop, αG, αE, αF, inter-switch, and C-term of α3 (Figure 4).

Figure 2. Structure and primary sequence of GαGTP and GαGTP-RGS-PDEγWT. A. The cartoon representation of GαGTP structure (PDB code: 1TND) is shown. The G protein holds a Ras-like domain and an α-helical domain. The interface between α-helical and Ras-like domain makes the nucleotide binding cleft. The α-helical domain is an orthogonal bundle of six α-helices. The Ras-like domain holds a Rossmann fold, characterized by a 3-layer(a/b) sandwich architecture due to the inversion in the order of the strands β3 and β1 as well as β1 and β4, which are adjacent to each other. The Ras-like domain is colored according to secondary structure (i.e. helices, strands, and loops are, respectively, violet, yellow and white), whereas the α-helical domain is gray. The mutation site is indicated by a cyan sphere centered on the Cα-atom. The GTP nucleotide is represented by sticks colored by atoms type. The nucleotide docks into a binding site contributed by the loops. These are ultraconserved regions also called G boxes 1–5 (G1–G5, colored green). G2 is also called swI (Figure 2). Such mutated position is not involved in backbone-mediated H-bonding interactions with the nucleotide neither in the wild type nor in the mutant, (Supplementary Figure 1A (Figure S1A)). The interaction pattern of the nucleotide remains almost unchanged in the two forms following MD simulations, as also indicated by the patterns of interaction energies between GTP and surrounding residues (Figure S2). Collectively, these data are consistent with the results of in vitro evidence that the mutation elicits a marginal effect on the intrinsic GTPase activity of the protein [8,9].
Table 1. Reactions that were changed in the background of the G38D mutation.

| N° | Reaction Equation | Description | O.P. | Min | Max | Effect |
|----|-------------------|-------------|------|-----|-----|--------|
| 1  | $G_{38TP} \overset{\kappa_{Gshutoff}}{\rightarrow} G_{38GD}$ | Intrinsic $G_{38TP}$ GTPase activity | 0.05 | $\times 2$ | 0 | None |
| 2  | $PDE^{-} G_{38TP} \overset{\kappa_{PDEshutoff}}{\rightarrow} PDE + G_{38GD}$ | Intrinsic $G_{38TP}$-PDE GTPase activity | 0.033 | $\times 2$ | 0 | Slowed recovery when $= 0$ |
| 3  | $G_{38TP}^{-} PDE^{-} G_{38TP} \overset{\kappa_{PDEshutoff}}{\rightarrow} PDE^{-} G_{38TP} + G_{38GD}$ | Intrinsic $G_{38TP}$-PDE $G_{38TP}$ GTPase activity | 0.033 | $\times 2$ | 0 | Slowed recovery when $= 0$ |
| 4  | $PDE + G_{38TP} \overset{\kappa_{PDEshutoff}}{\rightarrow} PDE G_{38TP}$ | Binding of $G_{38TP}$ to inactive PDE | $5.5e-2$ | $\times 2$ | $10^3$ | Slowed activation, lower sensitivity and recovery with changes $> 10^3$ |
| 5  | $PDE^{-} G_{38TP} + G_{38GD} \overset{\kappa_{PDEshutoff}}{\rightarrow} G_{38GD} PDE^{-} G_{38TP}$ | Activation of one PDE catalytic subunit | 94.07 | $\times 2$ | $10^4$ | Slowed activation phase with changes $> 10^3$ |
| 6  | $G_{38TP} PDE^{-} G_{38TP} \overset{\kappa_{PDEshutoff}}{\rightarrow} G_{38GD}^{-} PDE^{-} G_{38TP}$ | Activation of the PDE tetramer | 21.09 | $\times 2$ | $10^3$ | None |
| 7  | $RGS + PDE^{-} G_{38TP} \overset{\kappa_{PDEshutoff}}{\rightarrow} RGS PDE^{-} G_{38TP}$ | Binding of RGS9 to $G_{38TP}$-PDE | 1.57e-7 | $\times 2$ | $15$ | Slowed recovery phase |
| 8  | $RGS + G_{38TP}^{-} PDE^{-} G_{38TP} \overset{\kappa_{PDEshutoff}}{\rightarrow} RGS G_{38GD}^{-} PDE^{-} G_{38TP}$ | Binding of RGS9 to $G_{38TP}$-PDE-$G_{38GD}$ | 1.57e-7 | $\times 2$ | $15$ | Slowed recovery phase |
| 9  | $RGS PDE^{-} G_{38TP} \overset{\kappa_{PDEshutoff}}{\rightarrow} PDE + RGS + G_{38GD}$ | GAP activity and disruption of the complexes | 256.07 | $\times 2$ | $10^5$ | Increase in saturating phase for bright flashes with changes $> 10^3$ |

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* N° Reaction number, corresponding to that in Figure 1.
* Description Type of reaction.
* O.P. Original value of the parameter governing the reaction kinetics in the mathematical model [s$^{-1}$] [14].
* Min Minimum and maximum reductions the considered parameters were subjected to in the present study, in order to model the mutated conditions; when “Max R” was 0, the parameter was ultimately set to zero.
* Max Effect of these reductions.
responses to flashes of increasing intensities from wild type GkP1 were generated by changes in the kinetic parameters of photons recordings. The pathological GkP1 in vitro is correlated with the modes describing the essential subspace of the Solvent Accessible Surface Area (SASA) of Y254 observations, Functional Mode Analysis (FMA, see Methods) [8] and provided by Marie E. Burns, refer to mice rods exposed to flashes (C, F) rods are shown. Experimental data, i.e. published in Moussaif et al. (A, D), heterozygous GkP1 WT+/- (A and B) or from 650 to 94000 photons μm⁻² (C). Simulated responses derive from the model of an amphibian rod stimulated with the same light intensities as in vitro recordings. The pathological GkP1 WT+/- and GkP1 WT−/- models were generated by changes in the kinetic parameters kP1, kP2 and kRG1 as described in the text. The dissimilar species justify the time scale difference between in vitro and in silico experiments. The responses were normalized with respect to the maximum photocurrent. G. Normalized simulated light response amplitude is plotted as a function of flash strength. For comparison to in vitro data, see Figure 5B in Moussaif et al. [8]. Flash intensities are the same as in D, E and F.

Figure 3. Flash responses from wild type, GkP1 G38D−/-, and GkP1 G38D−/− rods. Experimental (A, B, C) versus simulated (D, E, F) responses to flashes of increasing intensities from wild type GkP1 WT+/- (A, D), heterozygous GkP1 WT+/- (B, E) and homozygous GkP1 WT−/- (C, F) rods are shown. Experimental data, i.e. published in Moussaif et al. [8] and provided by Marie E. Burns, refer to mice rods exposed to flashes ranging from 5 to 97000 photons μm⁻² (A and B) or from 650 to 94000 photons μm⁻² (C). Simulated responses derive from the model of an amphibian rod stimulated with the same light intensities as in vitro recordings. The pathological GkP1 WT+/- and GkP1 WT−/- models were generated by changes in the kinetic parameters kP1, kP2 and kRG1 as described in the text. The dissimilar species justify the time scale difference between in vitro and in silico experiments. The responses were normalized with respect to the maximum photocurrent.

To investigate whether mutation-induced changes in intrinsic dynamics may affect Gγ portions deputed to RGS and/or PDEγ recognition, we monitored the solvent accessibility of all the Gγ and PDEγ recognition sites on Gγ (indicated, respectively, by orange and green stars in Figure 2), finding more marked effects on the PDEγ sites, in particular Y254 (in the α3/β5 loop). The latter is, indeed, exposed to the solvent in the wild type but buried in the mutant form where it is permanently involved in inter-helical interaction with F211 [2,11], another PDEγ recognition site that becomes no longer available to PDEγ as well (Figure 5). In line with these observations, Functional Mode Analysis (FMA, see Methods) found that the Solvent Accessible Surface Area (SASA) of Y254 is correlated with the modes describing the essential subspace of Gγ.

Incidently, the essential subspace (ES) is given by a variable number of eigenvectors whose associated eigenvalues account for 90% of the total variance of the Gγ-atom displacements in a trajectory. The correlation is already present in the wild type but increases in the mutant form (i.e. the correlation coefficients are 0.73 and 0.86, respectively). Differences in functional modes between wild type and mutant amplify when considering only the first principal component (PC1); in fact, the correlation remains still significant for the mutant (i.e. 0.74) but it drops for the wild type (i.e. 0.39). Collectively, FMA is suggestive of a functional link between protein dynamics and structural environment of Y254. Thus, mutation-induced burying of Y254 results in deformation of the swI/γ3 cleft, which is the primary determinant in PDEγ binding.

We also investigated mutational effects on the structural communication features of Gt by the Protein Structure Network (PSN) analysis, a product of graph theory applied to protein structures (see Methods). The analysis searched for mutation-induced changes in network components (e.g. nodes, hubs, links, shortest communication pathways, etc) on the MD trajectories. The comparative analyses of the Protein Structure Graphs (PSGs) of wild type and mutated Gγ revealed a slight reduction in number of nodes, hubs, and links in the G38D mutant compared to the wild type (Table 3). In contrast, the number of communication pathways and their average length increases in the mutant compared to the wild type. In line with this trend, the maximal, minimal, and average strengths reached by the totality of links in the paths tend to be higher in the mutant compared to the wild type.

To infer a global and coarse view of mutation-induced changes in the communication pathways of Gγ we drew global meta paths, i.e. assemblies of the most recurrent nodes and links in the pool of paths characterized by frequency ≥30% (Figure 6A and 6B, see Methods). In this respect, whereas the wild type is characterized by nucleotide-mediated paths at the interface between Ras-like and α-helical domain, in the mutant form, inter-domain pathways are less frequent as also highlighted by the distribution of linked-node fragments (Figures 6 and S4). This inter-domain uncoupling may be in part related to the fact that, in the mutant, selected portions of the α-helical domain undergo increases in essential dynamics compared to the wild type (Figure 4). Noteworthy, this trend is also evident in the meta paths computed on the sub set of paths made by ≥50% of conserved amino acids (Figure S5A and S5B). Differently from the wild type, in the mutant the most frequent nucleotide-involving pathways transverse essentially β1 and β3.

Table 2. in vitro and in silico flash responses.

|        | in vitro | in silico | in vitro | in silico |
|--------|----------|-----------|----------|-----------|
| GkP1 WT | 48.3 ± 2.1 | 23.64 | 111 ± 6 | 0.96 |
| GkP1 G38D−/− | 77.4 ± 6.0 (1.6) | 31.5 (1.3) | 109 ± 9 (1) | 0.975 (1.01) |
| GkP1 G38D−/− | 2296 ± 125 (47.5) | 1854 (78.4) | 192 ± 43 (1.7) | 2.345 (2.4) |

Flash strength that elicited a half-maximal response. The time scales for in vitro [8] and in silico measurements are ms and s, respectively.

Time to Peak of the flash. In vitro data were taken from the literature [8], while in silico data are the outcome of mathematical simulations done in this study. In brackets, the ratios to the wild type value are shown.

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which describe the essential subspace of the trajectories of Gα, the Noel’s nomenclature [19]. They refer to the 100000 frames constituting the 100 ns trajectory. The secondary structure elements are shown on the abscissa, following the number of PCs used was 111 for Gα.

Figure 4. RMSF profiles and PCA projections. A. The Cα-RMSF profiles from MD trajectories of Gα_GTPWT (violet) and Gα_GTPG38D (green) are shown. They refer to the 100000 frames constituting the 100 ns trajectory. The secondary structure elements are shown on the abscissa, following the linear combination of the ED analysis-derived principal components, which describe the essential subspace of the trajectories of Gα_GTPWT (B and C) and Gα_GTPG38D (D and E) are shown (see text for an explanation of ED). The number of PCs used was 111 for Gα_GTPWT and 74 for Gα_GTPG38D. Cx-atom displacements are highlighted by color ranges from violet to blue for Gα_GTPWT, and from green to blue for Gα_GTPG38D.

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rather than the swII/α3 interface, which is the primary PDEγ binding cleft (Figure 6A and 6B).

In summary, in spite of the lack of significant differences in the interaction pattern of GTP between wild type and mutated forms, Gα_GTPG38D is characterized by increased flexibility of the α-helical domain compared to wild type, which reflects on an apparent inter-domain uncoupling in terms of shortest communication pathways. In contrast, in the wild type, inter-domain pathways localized on the nucleotide binding site cover a significant part of the structural communication modes. Above all, the most significant effect of aspartate substitution for G38 is a structural perturbation in the swII/α3 interface, which is the primary PDEγ binding site.

Atomic simulations on the Gα_GTP-RGS-PDEγ ternary complex: The G38D mutant affects the communication between nucleotide binding site and RGS

Atomic MD simulations on monomeric Gα_GTP highlighted long-distance mutational effects on the Gα regions devoted to PDEγ recognition likely related to the reduced PDE binding and consequently activation inferred from both mathematical simulations and in vitro experiments [8].

In order to clarify ambiguities by in vitro experiments on the GAP activity of RGS towards the G38D mutant [8,9], which couldn’t be properly addressed by mathematical simulations, wild type and mutated Gα were simulated also in the context of the ternary complex with PDEγ and RGS. In this respect, MD simulations on the Gα_GTP-RGS-PDEγG38D ternary complex are justified by the fact that the mutated Gα holds a residual binding to PDEγ [8].

Comparing the dynamics and structural communication features of all the components of the ternary complex in the presence of either wild type or mutated Gα served to infer the effects of the G38D mutation on different structural aspects such as: a) communication and interaction features of the nucleotide, b) intrinsic dynamics of each component of the complex, and c) interprotein communication.

In line with simulations on monomeric Gα_GTP, simulations on the ternary complex show that the interaction pattern of the nucleotide is substantially similar in wild type and mutated Gα (Figures S1 and S2), thus not providing any clue on mutational effects on the GAP activity of RGS.

As for the intrinsic dynamics of the three proteins in the complex, differently from PDEγ, Gα_GTP and RGS are characterized by low mobility in terms of RMSDs (Figure S3). The higher mobility of PDEγ is likely due to the poor intramolecular and intermolecular tertiary contacts made by such protein, which is a 42-amino acid fragment of a small subunit. In deep detail, as for Gα_GTP, the wild type and mutated forms do not differ significantly in terms of RMSDs or RMSFs; major differences concern only the swIII region, which fluctuates less in the mutant than in the wild type (Figures S3 and S6). In line with such behavior, the overlap between the ES of the two Gα forms in the context of the heterotrimer is quite high (0.90), the essential motions of β2/β3 loop and swIII contributing to such differences (Figure S6). RGS shows low mobility as well, its intrinsic flexibility being comparable in the complexes with wild type and mutated Gα (Figure S3). In contrast, the intrinsic flexibility of PDEγ is higher in the complex with mutated Gα_GTP than in the complex with wild type Gα_GTP.
This suggests that the pathogenic Ga mutation increases the intrinsic flexibility of PDEc, which would imply increased instability of the PDEc-GaGTP interface.

According to the crystal structure of the heterotrimeric complex, RGS does not contribute directly to the active site by donating residues or through water-mediated interactions [16]. It is rather thought that RGS would increase the GTP hydrolysis rate by stabilization of the Ga switch regions in their transition state conformation and orientation of the critical Ga carbonyls used to position the nucleophilic water [16,17]. Thus, RGS action is likely due to inter-protein structural communication. On these bases, possible structural relations with the postulated mutation-induced reduction of the GAP activity of RGS were searched by the PSN analysis. Significant differences between the two simulated ternary complexes could be inferred from the analysis of the shortest communication pathways, which were almost halved in the mutated complex compared to the wild type (Table 3). Remarkably, more than 60% of the communication paths that characterize the wild type form hold the GTP-Q200(G3:5)-R:N364 fragment of linked nodes, which is completely absent in the mutant (Figure S4). The global meta path representation clearly shows that the most significant communication in the GaGTP-RGS-PDEcWT involves GTP, Q200(G3:5), R:N364, and E203(G3:8) (i.e. the GTP-Q200(G3:5)-R:N364-E203(G3:8) meta fragment of linked nodes; Figure 6C and 6D). Remarkably, the GTP-Q200(G3:5)-R:N364 connection found in the wild type form is essential for the GAP activity of RGS [16]. Such connection is no longer present in the mutant. In line with path fragment distribution, the most representative nucleotide-mediated paths in the GaGTP-RGS-PDEcG38D complex are intra-Ga located (Figure 6C, 6D, and S4). These differences between wild type and mutant forms are strengthened by the meta paths computed on the subset of paths made by ≥50% of conserved amino acids (Figure S5). Another difference concerning the structural communication of wild type and mutated Gt is that, whereas for the wild type some (4%) of the shortest pathways describe a communication between Ga and PDEc, in the mutant form such communication could not be found, likely due to the increased flexibility of the effector subunit.

In summary, atomistic simulations on the ternary complexes highlight a possible disturbing effect of the pathogenic mutation on the GAP activity of RGS. This would act, at least in part, by destabilizing the Q200(G3:5)-mediated communication between GTP and R:N364. Finally, they strengthen the influence of the mutation on the G protein-effector interface, in line with electrophysiological recordings and mathematical simulations.

Discussion

Mutations in any components of the visual phototransduction signaling network may cause more or less severe impairments in vision. Because of the complexity of such network, any alteration of one of the cascade components would lead to unpredictable and not easily determinable results. Thus, a monogenic disease such as...
NCNB, considered in this study, can result from perturbations not circumscribed to the mutated protein but involving also other members of the network. In this framework, modeling the effects of mutation by systems-based approaches serves to infer how the pathogenic signal propagates through the network and which molecular species are involved. When possible, the latter information is passed to atomistic simulations to gain insights into the structural determinants of the disease.

In this study, we combined visual phototransduction modeling with atomistic simulations to thoroughly investigate the defect associated with the NCNB-causing G38D mutation of Ga. The mathematical model of visual phototransduction was able to reproduce the key features of the behaviors of heterozygous and homozygous rods typical of the NCNB disease. This could be possible upon reducing the constants governing: a) the binding of GaGTP to PDE, b) the activation of the catalytic activity of PDE, and c) the binding of the RGS complex to a PDE tetramer with one active subunit. In fact, a strong reduction in PDE recognition and activation by GaGTP coupled to a two-fold reduction in the RGS binding constant was essential to reproduce the visual responses in Nougaret patients, while decreasing the intrinsic or RGS-catalyzed GTPase activities did not seem to have a significant effect. Thus, mathematical modeling emphasized the formation and activation of the GaGTP-PDE complex as the processes more significantly affected by the aspartate substitution for G38 in Ga, in line with electrophysiological recordings [8].

MD simulations on monomeric GaGTP suggest that such reduction in the PDE recognition ability of mutated Ga is likely due to altered dynamics of the protein associated with changes in the architecture of the swII/a3 cavity, essential recognition point for PDE. A detrimental effect of the mutation on such cavity had been also postulated based upon crystallographic analyses [16]. We ended up independently with this conclusion by individuating also the main actors of this structural effect. In this framework, the Y254 position seems to be particularly sensible to the concerted motions of the protein triggered by the mutation. Indeed, deformation of the swII/a3 cavity results in the burying of Y254 in the a3/b5 loop, preventing both the Y254 itself and F211 (s2:11) from being available to PDE. We also speculated that mutation-induced reductions of the catalytic activity of PDE may derive from the

| Table 3. Network parameters. |
|-----------------------------|
|                            | $G_{\text{aGTP}}^{\text{WT}}$ | $G_{\text{aGTP}}^{\text{G38D}}$ | $G_{\text{aGTP-RGS-PDE}}^{\text{WT}}$ | $G_{\text{aGTP-RGS-PDE}}^{\text{G38D}}$ |
| Imi in (%)                  | 3.24                         | 3.23                         | 3.52                         | 3.58                         |
| Hubstot                      | 34                           | 33                           | 51                           | 51                           |
| Nodes 1st Cls               | 228                          | 192                          | 291                          | 285                          |
| Hubs 1st Cls                | 34                           | 28                           | 47                           | 46                           |
| Links 1st Cls               | 304                          | 265                          | 396                          | 394                          |
| Number of Paths             | 268                          | 330                          | 1930                         | 975                          |
| 1st Cluster Pop             | 57                           | 72                           | 1819                         | 924                          |
| 2nd Cluster Pop             | 27                           | 37                           | 80                           | 36                           |
| 3rd Cluster Pop             | 26                           | 34                           | 22                           | 7                            |
| 4th Cluster Pop             | 22                           | 23                           | 22                           | 4                            |
| 5th Cluster Pop             | 13                           | 19                           | 22                           | /                            |
| Max Length                  | 7                            | 9                            | 16                           | 11                           |
| Avg Length                  | 5.4                          | 5.6                          | 7.7                          | 6.1                          |
| Max Freq                    | 83.6                         | 82.7                         | 85.2                         | 82.3                         |
| LengthMaxFreq               | 5                            | 5                            | 5                            | 5                            |
| Max Score                   | 1.0                          | 1.0                          | 1.0                          | 1.0                          |
| Min Score                   | 0.4                          | 0.6                          | 0.4                          | 0.5                          |
| Avg Score                   | 0.9                          | 0.9                          | 0.4                          | 1.0                          |
| Max SumWgt                  | 79.1                         | 89.2                         | 155.3                        | 127.8                        |
| Min SumWgt                  | 26.2                         | 36.1                         | 32.5                         | 32.7                         |
| Avg SumWgt                  | 51.1                         | 54.9                         | 77.1                         | 62.9                         |
| Max AvgWgt                  | 11.3                         | 12.1                         | 12.3                         | 12.5                         |
| Min AvgWgt                  | 4.3                          | 5.9                          | 5.4                          | 5.5                          |
| Avg AvgWgt                  | 7.9                          | 8.3                          | 8.8                          | 8.9                          |

*Imin values (%) employed for the four simulated systems.
*Total number of hubs.
*Number of nodes, hubs and links in the most populated node cluster.
*Total number of paths characterized by frequency ≥30% and number of paths in the first five most populated path clusters.
*Maximum and average path length excluding the two extremities.
*Maximum path frequency and length of the maximum frequency path (i.e. excluding the extremities).
*Maximum, minimum and average correlation score.
*Maximum, minimum and average path strength obtained by summing the interaction strengths of the links constituting the path.
*Maximum, minimum and average path strength obtained by summing the interaction strengths of the links constituting the path and dividing such sum by the number of links involved in the path.

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NCNB, considered in this study, can result from perturbations not circumscribed to the mutated protein but involving also other members of the network. In this framework, modeling the effects of mutation by systems-based approaches serves to infer how the pathogenic signal propagates through the network and which molecular species are involved. When possible, the latter information is passed to atomistic simulations to gain insights into the structural determinants of the disease.

In this study, we combined visual phototransduction modeling with atomistic simulations to thoroughly investigate the defect associated with the NCNB-causing G38D mutation of Ga. The mathematical model of visual phototransduction was able to reproduce the key features of the behaviors of heterozygous and homozygous rods typical of the NCNB disease. This could be possible upon reducing the constants governing: a) the binding of GaGTP to PDE, b) the activation of the catalytic activity of PDE, and c) the binding of the RGS complex to a PDE tetramer with one active subunit. In fact, a strong reduction in PDE recognition and activation by GaGTP coupled to a two-fold reduction in the RGS binding constant was essential to reproduce the visual responses in Nougaret patients, while decreasing the intrinsic or RGS-catalyzed GTPase activities did not seem to have a significant effect. Thus, mathematical modeling emphasized the formation and activation of the GaGTP-PDE complex as the processes more significantly affected by the aspartate substitution for G38 in Ga, in line with electrophysiological recordings [8]. MD simulations on monomeric GaGTP suggest that such reduction in the PDE binding ability of mutated Ga is likely due to altered dynamics of the protein associated with changes in the architecture of the swII/a3 cavity, essential recognition point for PDE. A detrimental effect of the mutation on such cavity had been also postulated based upon crystallographic analyses [16]. We ended up independently with this conclusion by individuating also the main actors of this structural effect. In this framework, the Y254 position seems to be particularly sensible to the concerted motions of the protein triggered by the mutation. Indeed, deformation of the swII/a3 cavity results in the burying of Y254 in the a3/b5 loop, preventing both the Y254 itself and F211 (s2:11) from being available to PDE. We also speculated that mutation-induced reductions of the catalytic activity of PDE may derive from the
formation of an improperly assembled Gα_{GTP}-PDEγ complex. Along this line, also in the ternary complex with RGS and PDEγ the mutation exerts a long distance effect on the effector binding site resulting in increase in the intrinsic flexibility of PDEγ and lack of communication pathways at the Gα-PDEγ interface. Another clear effect of the Gα mutation is the incapacity to form a stable Q200(G38D)-mediated communication between the nucleotide and N364 of RGS. Such communication is instead present in the wild type and is necessary for the GAP action of RGS [16].

In conclusion, the main structural effects of the G38D mutation turned out to be deformation of the primary effector binding site in monomeric Gα and enhanced flexibility of PDEγ in the ternary complex with RGS and PDEγ.
complex, which would destabilize the Gα-effector interface. Collectively, these effects are connectable with the impaired effector recognition and activation shown by in vitro experiments. Finally, the pathogenic mutation of Gα seems to affect the communication between RGS and nucleotide essential for the GAP activity, thus suggesting that the observed slight reduction of the RGS-catalyzed GTPase activity is a matter of perturbed inter-protein communication.

This study extends the dynamic model of visual phototransduction to the pathological NCNB phenotype and provides insights at the atomic level into the structural bases of the disease. This is an example of a thorough computational investigation employing different scales of description, an approach which should be pursued to unveil the structural determinants of genetic retinal diseases. Analogous approaches are suitable to infer the mechanisms of information transfer in any signaling network either in physiological or pathological conditions.

**Methods**

**Mathematical modeling**

The mathematical model of rod phototransduction (BioModels ID: BIOMD0000000326), employed in the present work for numerical simulations of the phototransduction cascade includes 91 reactions, 71 molecular species, and 63 parameters and was previously developed and validated over a wide range of experimental data on normal and genetically modified rods [14]. The rate of change of the molecular species are monitored by calculating, at given time steps, their rates of production and consumption [14]. The original model was recently modified to account for the postulated R-Gt precoupling in the dark [15], leading to the inclusion of the following reactions, which describe: a) the dynamic formation and b) dissociation of dark R-Gt complexes:

- \[ \text{R} + \text{Gt} \xrightarrow{k_{\text{on}}^{\text{dark}}} \text{R-Gt} \]
- \[ \text{R} + \text{Gt} \xrightarrow{k_{\text{off}}^{\text{dark}}} \text{R} + \text{Gt} \]

The parameters were calculated as relative kinetic constants, following the relationships \( k_{\text{on}}^{\text{dark}} = 1.6 \times k_{\text{on}}^{\text{light}} \) and \( k_{\text{off}}^{\text{dark}} = 315 \times k_{\text{off}}^{\text{light}} \), taken from Surface Plasmon Resonance (SPR) experiments [15]. This led to a \( \approx 20\% \) of Gt to be dynamically precoupled to Rin the dark, consistent with published data [15]. In this study, we built a dynamic model able to describe the GαGTP \( {\text{G38D}}^{+/+} \) and GαGTP \( {\text{G38D}}^{-/-} \) mutated conditions of GαGTP by introducing the mutated G protein as an explicit new molecule and adding all the relative reactions in the phototransduction cascade, which concerned the GαGTP \( {\text{WT}}^{+/+} \) status (Table 1). The concentration of the new species (GαGTP \( {\text{G38D}} \)) was obtained by using its ratio to the normal concentration of Gα in wild type cells taken from expression experiments [15]. Therefore, the total level of Gα \( {\text{G38D}}^{+/+} \) was 25% of which is GαGTP \( {\text{G38D}}^{+/+} \) in heterozygous cells is the same as in wild type rods, while only 35% of the native levels are found in homozygous cells for the mutation. The levels of all the other proteins were kept unchanged and the mutation was assumed to have no effect on rhodopsin-GαGTP \( {\text{G38D}} \) binding [8,9].

In order to fit the models onto the pathological NCNB condition, the rates of a number of reactions involving GαGTP \( {\text{G38D}} \) were systematically reduced by tuning the relative kinetic parameters in decreasing steps (Table 1, Figure 1). The reductions were combined into specific heterozygous and homozygous models. In detail, these reactions refer to: a) intrinsic GTPase activity of GαGTP, b) binding of GαGTP to PDE and resulting PDE activation, and c) shut-off of the photoreponse by RGS binding and RGS-catalyzed GTP hydrolysis. These reactions are highlighted in Figure 1 and listed in Table 1. In line with in vivo electrophysiological recordings on rods from transgenic mice, we monitored the following features of the photocurrent elicited by increasingly stronger flashes of light (Figure 3): a) rate of the activation phase, b) light sensitivity, and c) speed of the recovery phase. In detail, a) the rate of the activation phase was evaluated as the time needed to reach the maximum value of A [A] after the delivery of the flash; b) light sensitivity was taken as the normalized response amplitude as a function of flash strength (Figure 3G and Figure 5B in Moussai et al. [9]); and c) the speed of the recovery phase was evaluated as the time needed for the photocurrent to reestablish its dark value [A] = 0 after a flash. In some cases, the parameters had to be eventually set equal to 0, while in other cases also more limited reductions led to modification of the output (Table 1). All the numerical simulations were carried out by means of Matlab, within the SBIToolbox2 framework [18] (http://www.sbitoolbox2.org/main.php) as already described [14].

**MD simulations: The structural models**

The following PDB structures were selected as inputs of MD simulations: GαGTP \( {\text{WT}}^{/} \) (PDB code: 1TND [19], residue range 27–342), which is the GTP-bound form of Gα, and the GαGTP-RGS-PDE \( {\text{WT}}^{/} \) ternary complex (PDB code: 1FQI [16]) involving GαGTP (amino acids from 28 to 344), RGS (i.e. the RGS domain of RGS9, amino acids 286 to 416) and the 42-amino acid C-terminal fragment of PDE \( {\text{WT}}^{/} \) (residues 46–87). Input structure setup required a number of modifications in the original crystal structures. As for 1TND, the original GTP was analogue was replaced by GTP, as recently reported [20]. As for 1FQI, the original Gα was indeed a chimera identical to Gα except for residues 216–294 which were replaced with the corresponding homologous region of GαJ [21, 22]. Therefore, the GαJ sequence was therefore mutated into the corresponding one in bovine Gα. Moreover, the original GDP was replaced by GDP-βS, which was therefore mutated into the corresponding one in bovine Gα.

All the simulated systems hold the Mg\(^{2+}\) ion together with the coordinating water molecules.

The native G protein in the GαGTP \( {\text{WT}}^{/} \) and GαGTP-RGS-PDE \( {\text{WT}}^{/} \) complexes was finally subjected to the substitution of aspartate for G38, in order to produce the pathogenic mutant (i.e. GαGTP \( {\text{G38D}}^{/} \) and GαGTP-RGS-PDE \( {\text{G38D}}^{/} \)). In vivo mutagenesis was performed by means of the Qsanta software (www.accelrys.com).

**MD simulation: Set-up**

MD simulations on the four systems, GαGTP \( {\text{WT}}^{/} \), GαGTP \( {\text{G38D}}^{/} \) and GαGTP-RGS-PDE \( {\text{WT}}^{/} \)) \( {\text{G38D}}^{/} \), were carried out by using the GROMACS simulation package [21] with the Amber03 all atoms force field [22,23]. The TIP3P water model was employed to describe the solvent, AMBER parameters to describe the GDP and GTP molecules were taken from the literature [24]. Depending on the dimensions of the systems, a variable number of Na\(^+\) and Cl\(^-\) ions were placed at optimum electrostatic positions in order to neutralize the system. In detail, the systems included: 63740 total atoms for GαGTP \( {\text{WT}}^{/} \) (19512 water molecules, 48 Na\(^+\) and 38 Cl\(^-\) ions); 63743 total atoms for GαGTP \( {\text{G38D}}^{/} \) (19511 water molecules, 49 Na\(^+\) and 38 Cl\(^-\) ions); 81906 total atoms for GαGTP-RGS-PDE \( {\text{WT}}^{/} \) (24609 water molecules, 60 Na\(^+\) and 50 Cl\(^-\) ions); 81882 total atoms for GαGTP-RGS-PDE \( {\text{G38D}}^{/} \) (24599 water molecules, 61 Na\(^+\) and 50 Cl\(^-\) ions).
Periodic Boundary Conditions (PBC) were applied by using an octahedric box as a unit cell, imposing a minimum distance of 12 Å between the solute and the box boundaries.

MD simulation setup is the same as the one recently employed to simulate a number of Ras GTPases [20]. All the input crystallographic structures were subjected to energy minimization keeping restricted the positions of the main chain atoms, the nucleotide, the Mg\(^{2+}\) cation and the coordinating water molecules. The systems were then equilibrated at 300 K for 4 ns of backbone restricted MD simulations. The Particle Mesh Ewald (PME) method was employed to compute the electrostatic interactions. Short range repulsive and attractive interactions were computed by using a Lennard-Jones potential with a cutoff of 10 Å. The LINCS algorithm [25] was used to constrain all bond lengths except those in water molecules, allowing for an integration time step of 2 fs through the leap-frog algorithm. The v-rescale thermostat [26] was employed to keep the system at a constant temperature of 300 K, by using a coupling constant (τ\(_v\)) of 0.1 ps. The pressure of the system was kept fixed at 1 atm, using the Berendsen weak coupling algorithm [27] with a coupling constant (τ\(_p\)) of 1 ps. The pre-equilibrated systems were then subjected to 100 ns of unrestrained isothermal-isobaric (T = 300 K, P = 1 atm) MD simulations.

MD analyses of the intrinsic flexibility

MD trajectories were subjected to a variety of analyses aimed at inferring a) the time series of a number of structural descriptors such as the SASA, b) the intrinsic flexibility of the systems (e.g. RMSD, RMSF, and Essential Dynamics (ED) or PCA), and c) potential correlations between structural descriptors and essential motions (i.e. FMA).

As for ED, resting on the assumption that the major collective modes of fluctuation dominate the functional dynamics of a system, information on such global motions can be inferred from the atomic fluctuations by means of the PCA. The latter allows the decomposition of the atomic fluctuations into a set of principal components (eigenvectors of the covariance matrix of positional fluctuations) that describe the concerted motions of these atoms (e.g. the Cα-atoms). The technique is based on the diagonalization of such covariance matrix producing a set of eigenvector and eigenvalue pairs in which the eigenvector and the eigenvalue describe, respectively, direction and amplitude of the concerted atomic motion (a mode). The atomic components of an eigenvector provide a quantitative measure of the participation of each Cα-atom to the collective motion described by the corresponding eigenvector. The subspace spanned by the major modes of collective fluctuations is accordingly often referred to as "essential subspace (ES)". In the same framework, FMA is a technique to identify collective atomic motions related to a specific protein function. Given a large set of structures of one protein, for example from an MD trajectory, the method detects a mode that is maximally correlated to an arbitrary quantity of interest.

Except for FMA, which was carried out by using the GROMACS package, all these MD analyses were performed by means of the Wordom software [28]. As for PCA, the covariance matrices were built on the Cα-atoms of the isolated MD trajectories.

FMA [29] was carried out by using the Linear Mutual Information (LMI) estimator [30]. The structural descriptor correlated with the Principal Components (PCs) was the SASA calculated on selected Cα amino acids involved in Cα-PDEγ and Cα-RGS interactions. A number of PCs were probed.

Non bonded interaction energies for the nucleotide were monitored every 20 ps along the trajectory with GROMACS4.

MD analyses of the structural communication

The structural communication (i.e. PSGs and shortest communication paths) in the four simulated systems was inferred by means of the graph-based approach proposed by Vishveshwara and coworkers [31] and defined as Protein Structure Network (PSN), that was recently implemented in the Wordom software [28]. With this approach, the dynamics of the system is taken into account in terms of occurrence of network components along the trajectory and of correlated motions [32–34].

A graph is defined by a set of points (nodes) and connections (edges) between them. In a PSG, each amino acid is represented as a node and these nodes are connected by edges based on the strength of non-covalent interactions between nodes [31]. The strength of interaction between residues i and j (I\(_ij\)) is evaluated as a percentage given by the following equation:

\[
I_{ij} = \frac{n_{ij}}{\sqrt{N_i N_j}} \times 100
\]

where I\(_ij\) is the percentage interaction between residues i and j; n\(_ij\) is the number of atom-atom pairs between the side chains of residues i and j within a distance cutoff (4.5 Å); N\(_i\) and N\(_j\) are normalization factors for residue type i and j, which take into account the differences in size of the side chains of the residue types and their propensity to make the maximum number of contacts with other amino acids residues in protein structures. The normalization factors for the 20 amino acids were taken from the work by Kannan and Vishveshwara [35]; the normalization values for GTP (derived from 3 heterotrimeric G proteins), Mg\(^{2+}\) (based on 4 heterotrimeric G proteins to properly describe the coordination of such ion in the system under study) and water (based on 5 structures, comprising G proteins and rhodopsin) were 361.3, 23.8 and 27.0, respectively. Thus, I\(_ij\) are calculated for all nodes, excluding i ± n, where n is a given neighbor cutoff of 3. An interaction strength cutoff I\(_{min}\) is then chosen and any residue pair i-j for which I\(_i-j\)=I\(_{min}\) is considered to be interacting and hence is connected in the PSG.

As previously demonstrated [31], the optimal I\(_{min}\) is the one at which the size of the largest cluster of nodes at I\(_{min}\) 0% halves. Incidentally a node cluster is a set of connected nodes in a graph. We approximated the I\(_{min}\) value to the second decimal place. The final I\(_{min}\) cutoffs were: 3.24% for G\(_{α\text{GTP}}\) and 3.23% for G\(_{α\text{GTP}}\) for G\(_{α\text{GTP}}\); 3.52% for G\(_{α\text{GTP}}\) and 3.58% for G\(_{α\text{GTP}}\). To build the PSG, only the edges present in at least 30% of the trajectory frames were used. Those nodes involved in at least four links are named as hubs.

Possible shortest communication paths or optimal paths (OPs) in the different G\(_{α\text{GTP}}\) binary complexes as well as between wild type and mutated G\(_{α\text{GTP}}\) and the other two proteins in the G\(_{α\text{GTP}}\)-RGS-PDEγ ternary complex were searched. All residue pairs except those at sequence distance ≥5 were considered as path extremities (i.e. the first and last amino acids in the path). In detail, the number of intra-G\(_{α}\) amino acid pairs was 49770 for the wild type and mutated forms of isolated G\(_{α\text{GTP}}\) and 50090 for the wild type and mutated forms of G\(_{α\text{GTP}}\) in the ternary complex with RGS and PDEγ. Finally, 56350 amino acid pairs were considered to search all possible communication paths between wild type and mutated G\(_{α\text{GTP}}\) and the two proteins in the ternary complex. Vishveshwara and co-workers implemented also the search for suboptimal paths (SOPs), alternate routes of communication, which can be computed by systematically removing all interactions of an OP node(s) thus forcing the traversal of a less than optimal path [36]. Since our path searches, different from those by Vishveshwara and co-workers [36–38], are not limited to a few
selected node pairs but systematically consider almost all node pairs in a system, the additional search for SOPs would have been too costly in terms of computer time with the high risk to produce more noise than relevant information. For this reason our approach is dedicated exclusively to OPs.

The search for the shortest path(s) between pairs of nodes as implemented in the PSN-path module of Wordom relies on the Dijkstra’s algorithm [39]. They were searched by combining PSN data with cross-correlation of atomic motions calculated by using the LMI method.

Following calculation of the LMI and of correlated motions (by means of the LMI method [30]), for each frame, the procedure to search for the shortest path(s) between each residue pair consists of: a) searching for the shortest path(s) between each selected amino acid pair based upon the stable PSN connectivities, and b) selecting the shortest path(s) that contains at least one residue correlated (i.e., with a LMI cross-correlation $\geq 0.3$) with either one of the two extremities. All the shortest paths that pass the filter of correlated motions are subjected to a further filter based upon path frequency, i.e., number of frames containing the selected path divided by the total number of frames in the trajectory. The relative number of amino acids holding correlated motions with either one of the two extremities is quantified by the correlation score, i.e., the ratio between the number of correlated amino acids and the path length; the latter excludes the two extremities.

Outcome of this stage is the total pool of paths for the system under study. Meta paths made of the most recurrent nodes and links in the path pool (i.e. global meta paths) are worth computing to infer a coarse/global picture of the structural communication in the considered system. In this study, meta paths were computed on the ensemble of paths with frequency $\geq 30\%$. For each link a recurrence score $r$ is calculated using the following equation:

$$r_{ij} = \frac{\sum_{l \in \mathcal{P}} p_{ij}(l)}{\sum_{ij} p_{ij}(l)}$$

where $l$ is a given link present in the considered set of shortest paths, $p_{ij}(l)$ is the total number of shortest paths from node $i$ to node $j$ and $p_{ij}(l)$ is the total number of shortest paths from node $i$ to node $j$ that include link $l$. Finally, only those links with a recurrence score $\geq 30\%$ of the highest score are used in the meta path representation.

Supporting Information

Figure S1 Details of GTP binding modes in G_{cGTP} (top) and G_{cGTP-RGS-PDEγ} (bottom). In both panels, the superimposed structures of wild type (violet) and mutated (green) forms are shown. The nucleotide is always colored by atom type. Only the amino acids that contribute the most to interactions with the nucleotide are shown in sticks. For those amino acids, which form are shown. The nucleotide is always colored by atom type. See the legend to Figure 2 for the labeling scheme. (TIFF)

Figure S2 Nucleotide-protein non bonded interaction energies averaged along the trajectories for G_{cGTP} (A) and G_{cGTP-RGS-PDEγ} (B). In both panels, violet bars refer to the wild type form and green bars to the mutated form. Vertical black bars indicate standard errors. Only the non bonded interactions whose average values along the simulations were smaller than $-20$ kJ mol$^{-1}$ were plotted. (TIFF)

Figure S3 Co-RMSD plots. The time series of the Cz-RMSD with respect to the input structures concerning isolated G_{35GTP} from 1TND, complexed G_{35GTP} from 1FQJ, PDEγ from 1FQJ, and RGS from 1FQJ are shown. Violet refers to the wild type whereas green refers to the mutant. (TIFF)

Figure S4 Fragment analysis on the pool of paths generated by G_{cGTP} (top) and G_{cGTP-RGS-PDEγ} (bottom) structures. In both panels, violet bars refer to the wild type form and green bars to the mutated form. Fragment recurrence is the number of paths containing the given fragment divided by the total number of paths. On the abscissa, the nodes constituting the fragment are numbered according to the secondary structure nomenclature explained in the legend to Figure 2 and used throughout the text. Only fragments of length 3 were taken into account. (TIFF)

Figure S5 Global and coarse view of the communication pathways with high content of conserved amino acids. The meta paths computed over those paths holding $\geq 50\%$ of conserved amino acids are shown. They concern G_{cGTP}NT and G_{cGTP}G38D in their free state (A and B panels, respectively) as well as in ternary complex with both RGS and PDEγ (C and D panels, respectively), colored violet and green respectively. The width of each link is proportional to $r$, while the sphere diameter is proportional to the average $r$ of the connecting link (see Methods for $r$ definition). The $\alpha$-helical and Ras-like domains are dark and light gray, respectively, the PDEγ binding site on GTP is aquamarine, RGS is orange and PDEγ is lemon-green. The mutation site is indicated by the red sphere. (TIFF)

Figure S6 Cz-RMSF profiles and Cz-atom projections. A. The Cz-RMSF profiles from MD trajectories of Gz from G_{cGTP-RGS-PDEγ}NT (violet) and Gz from G_{cGTP-RGS-PDEγ}G38D (green) are shown. They refer to the 100000 frames constituting the 100 ns trajectory. The secondary structure elements are shown on the abscissa, following the Noel’s nomenclature (see [19] in the text). B, C, D, E. The Cz-atom projections along the linear combination of the PCA-derived principal components, which describe the ES of the trajectories of Gz from G_{cGTP-RGS-PDEγ}NT (B and C) and of Gz from G_{cGTP-RGS-PDEγ}G38D (D and E) are shown. The ES is given by a variable number of eigenvectors that describe 90% of the total variance (sum of eigenvalues). The number of PCs was used for B and C, and 103 for D and E. Cz-atom displacements are highlighted by color ranges from violet to blue or and from green to blue, respectively. (TIFF)

Figure S7 Cz-RMSF profiles. The Cz-RMSF profiles from MD trajectories of PDEγ (top) and RGS (bottom) from G_{cGTP-RGS-PDEγ}NT (violet) and Gz from G_{cGTP-RGS-PDEγ}G38D (green) are shown. The secondary structure elements are shown on the abscissa. (TIFF)

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Author Contributions
Conceived and designed the experiments: SM FF. Performed the experiments: SM. Analyzed the data: SM FF. Wrote the paper: FF.

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