Functional Impact of the G279S Substitution in the Adenosine A₁-Receptor (A₁R-G279S^{7.44}), a Mutation Associated with Parkinson's Disease

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ABSTRACT

In medium-size, spiny striatal neurons of the direct pathway, dopamine D₁- and adenosine A₁-receptors are coexpressed and are mutually antagonistic. Recently, a mutation in the gene encoding the A₁-receptor (A₁R), A₁R-G279S^{7.44}, was identified in an Iranian family: two affected offspring suffered from early-onset L-DOPA-responsive Parkinson's disease. The link between the mutation and the phenotype is unclear. Here, we explored the functional consequence of the G279S substitution on the activity of the A₁-receptor after heterologous expression in HEK293 cells. The mutation did not affect surface expression and ligand binding but changed the susceptibility to heat denaturation: the thermodynamic stability of A₁R-G279S^{7.44} was enhanced by about 2 and 8 K when compared with wild-type A₁-receptor and A₁R-Y288A^{7.53} (a folding-deficient variant used as a reference), respectively. In contrast, the kinetic stability was reduced, indicating a lower energy barrier for conformational transitions in A₁R-G279S^{7.44} (73 ± 23 kJ/mol) than in wild-type A₁R (135 ± 4 kJ/mol) or in A₁R-Y288A^{7.53} (184 ± 24 kJ/mol). Consistent with this lower energy barrier, A₁R-G279S^{7.44} was

more effective in promoting guanine nucleotide exchange than wild-type A₁R. We detected similar levels of complexes formed between D₁-receptors and wild-type A₁R or A₁R-G279S^{7.44} by coimmunoprecipitation and bioluminescence resonance energy transfer. However, lower concentrations of agonist were required for half-maximum inhibition of dopamine-induced cAMP accumulation in cells coexpressing D₁-receptor and A₁R-G279S^{7.44} than in those coexpressing wild-type A₁R. These observations predict enhanced inhibition of dopaminergic signaling by A₁R-G279S^{7.44} in vivo, consistent with a pathogenic role in Parkinson's disease.

SIGNIFICANCE STATEMENT

Parkinson's disease is caused by a loss of dopaminergic input from the substantia nigra to the caudate nucleus and the putamen. Activation of the adenosine A₁-receptor antagonizes responses elicited by dopamine D₁-receptor. We show that this activity is more pronounced in a mutant version of the A₁-receptor (A₁R-G279S^{7.44}), which was identified in individuals suffering from early-onset Parkinson's disease.

Introduction

The motor symptoms of Parkinson's disease—i.e., brady-/akinesia, rigor, tremor, and postural instability—result from a loss of dopamine in the striatum (putamen and caudate nucleus) (Ehringer and Hornykiewicz, 1960). Dopamine is supplied by axonal projections of neurons, which reside in the substantia nigra pars compacta (Hornykiewicz et al., 1968). For reasons that still remain enigmatic, these neurons are

vulnerable and susceptible to degeneration. Hence, the prevalence of Parkinson's disease increases with age (Nussbaum and Ellis, 2003). In most instances, neuronal loss is associated with the accumulation of Lewy bodies and Lewy neurites. These fibrillary aggregates contain α -synuclein (Spillantini et al., 1997) and engulfed organelles (Shahmoradian et al., 2019). α -Synuclein is a small protein, which is largely unstructured in solution, but it adopts a α -helical structure in the presence of highly curved membranes containing acidic phospholipids (Davidson et al., 1998). Thus, under physiologic conditions, α -synuclein is distributed between two pools, a largely unstructured soluble monomeric form and an α -helical oligomeric form, which associates with synaptic vesicles (Burré et al., 2018). α -Synuclein can also form protofibrils composed of β -sheets (Burré et al., 2018). It is not clear what triggers β -sheet formation and fibrillary aggregation of α -synuclein

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ABBREVIATIONS: A₁R, adenosine A₁-receptor; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CPA, N₆-cyclopentyladenosine; D₁R, D₁-receptor; DMEM, Dulbecco's modified Eagle's medium; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; eYFP, enhanced yellow fluorescent protein; GPCR, G protein-coupled receptor; PDB ID, Protein Data Bank identifier; PEI, polyethylenimine; PMSF, phenylmethylsulfonyl fluoride; PTRHD1, peptidyl-t-RNA hydrolase domain containing-1; RMSF, root mean square fluctuation; TM, transmembrane helix; XAC, xanthine amine congener; YFP, yellow fluorescent protein.

in vivo (Giasson et al., 1999), but point mutations can enhance aggregation (Narhi et al., 1999); the mutated variants also nucleate fibrillation of wild-type α -synuclein (Wood et al., 1999). Hence, it is not surprising that they act in a dominant manner. These missense mutations in α -synuclein occur in patients suffering from early-onset, autosomal dominant Parkinson's disease; in fact, they were the first genetic cause identified in Parkinson's disease (Polymeropoulos et al., 1997; Krüger et al., 1998). However, sporadic Parkinson's disease is substantially more frequent than familial forms. Of the many gene loci that have been linked to Parkinson's disease over the past two decades (Chang et al., 2017), only a fraction give rise to Mendelian (monogenic) disease, which can be transmitted in an autosomal dominant or recessive form (Zhang et al., 2018). These hereditary forms of Parkinson's disease have nevertheless shed light on the pathogenesis and on genetic risk factors: mutations in signaling pathways including the endocytotic recycling machinery, in mitochondrial regulators, and in components of the proteostasis network can lead to Parkinson's disease. Thus, Parkinson's disease is heterogeneous in both the clinical manifestation and in the underlying cause: in most instances, environmental factors apparently act in combination with a genetic susceptibility (Singleton et al., 2013; Zhang et al., 2018).

Recently, a mutation in the adenosine A₁-receptor (A₁R), which substituted glycine at the position 279 by serine (A₁R-G279S^{7,44}), was identified in a consanguineous Iranian family: two brothers (out of 10 sequenced members) were homozygous for A₁R-G279S^{7,44}, and both developed symptoms of Parkinson's disease during their third decade. None of the other heterozygous members was affected. Hence, A₁R-G279S^{7,44} was proposed as an autosomally recessive transmitted cause of early-onset Parkinson's disease (Jaberi et al., 2016). The A₁-receptor is a prototypical G protein-coupled receptor (GPCR), which is abundantly expressed in the cerebral cortex and the basal ganglia of the human brain (Fastbom et al., 1987). In the striatum, A₁-receptors reside on the postsynaptic membrane of the medium-sized spiny neurons of the direct pathway, where they antagonize the dopamine D₁-receptor-mediated signaling (Ferré et al., 1994, 1997). In addition, A₁-receptors are also present on dopaminergic neurons of the substantia nigra pars compacta, where they reduce dopamine release by presynaptic inhibition (Yabuuchi et al., 2006; Borycz et al., 2007). Large prospective studies have shown that consumption of caffeine, which blocks adenosine receptors, protects against the development of Parkinson's disease (Costa et al., 2010; Palacios et al., 2012). Thus, there is circumstantial evidence to posit a pathogenic role of the mutant A₁R-G279S^{7,44} in the development of Parkinson's disease. However, the mechanistic basis remains enigmatic. In this study, we explored the functional consequence of the G279S^{7,44} substitution on the activity of the A₁-receptor after heterologous expression in HEK293 cells. We show that the mutation augmented both the basal activity of the receptor and its response to agonist-induced activation due to enhanced conformational flexibility. This translated into more potent inhibition of dopamine-induced cAMP accumulation.

Materials and Methods

Materials. Cell culture media, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), buffers, salts, and standard reagents were purchased from Sigma Aldrich (St. Louis, MO); FBS from Biowest (Nuaille, France);

4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone and xanthine amine congener (XAC) from Tocris (Abingdon, UK); adenosine deaminase and Complete protease inhibitor cocktail from Roche (Mannheim, Germany); N6-cyclopentyladenosine (CPA) from Abcam (Cambridge, UK); and Q5 High-Fidelity 2X Master Mix and NEBuilder HiFi DNA Assembly from New England Biolabs (Ipswich, MA). The mouse monoclonal anti-HA antibody immobilized on agarose (A2095) was from Sigma Aldrich; the rabbit monoclonal anti-HA antibody (C29F4) and mouse monoclonal anti-HA antibody (2367) were from Cell Signaling Technology (Cambridge, UK). Mouse monoclonal (M2 clone; F3165) and rabbit polyclonal anti-FLAG antibodies (SC807) were from Sigma Aldrich and from Santa Cruz Biotechnology (Dallas, TX), respectively. AlexaFluor-488-labeled secondary antibody against murine IgG (A32732) for flow cytometry was from Invitrogen (Carlsbad, CA). A fluorescently labeled secondary antibody (donkey anti-rabbit, 926-32213) from Li-Cor (Lincoln, NE) was used for immunoblotting. [³H]Adenine (specific activity 40 Ci/mmol), [³H]DPCPX (specific activity 164 Ci/mmol), [³H]7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol (specific activity 83 Ci/mmol), and [³⁵S]GTPγS (specific activity 1385 Ci/mmol) were purchased from PerkinElmer (Waltham, MA).

The plasmids encoding the human adenosine A₁-receptor harboring an N-terminal FLAG epitope, the human dopamine D₁-receptor harboring an N-terminal HA-epitope, and NanoLuc luciferase (PNL1.1) were obtained from Sinobiological (Beijing, China), from the cDNA resource center (Bloomberg University), and from Promega (Fitchburg, WI), respectively. The plasmid encoding the human adenosine A₁-receptor with enhanced yellow fluorescent protein (eYFP) fused to its C terminus was a kind gift from Rafael Franco (University of Barcelona, Barcelona, Spain). The G279S^{7,44} mutation was introduced into the cDNA of the FLAG- and eYFP-tagged A₁-receptor by site-directed mutagenesis using the QuikChange II site direction mutagenesis kit (Agilent, Santa Clara, CA). The cDNA coding for the D₁-receptor (D₁R) was fused in frame to the N-terminal sequence of NanoLuc luciferase to generate D₁R-NLuc using NEBuilder HiFi DNA Assembly from New England Biolabs; for polymerase chain reaction amplification of the PNL1.1 vector, standard forward and reverse primers were used; for amplification of the D₁R cDNA, primers were designed to have an additional overhang at their 5' end with the primers used to amplify PNL1.1 vector. The transfection reagent was polyethylenimine (PEI; linear 25 kDa; Santa Cruz Biotechnology), and the working stock solution (1 mg/ml in water) was kept at 4°C (maximum for 2 weeks). For long-term storage up to 12 weeks, the PEI stock solution was kept at -20°C.

Molecular Dynamics Simulations. The adenosine A₁-receptor was simulated using the active form based on the agonist-liganded, G_{i2} protein-bound structure [Protein Data Bank identifier (PDB ID): 6D9H; Draper-Joyce et al., 2018] and starting from the inactive conformation based on the antagonist-bound structure (PDB ID: 5N2S; Cheng et al., 2017). For either conformation, wild-type and mutant receptors were simulated in the presence of absence of adenosine. The missing loop between transmembrane helix (TM) 5 and TM6 (residues 214–222) of the receptor was modeled using Modeler 9.20 (Shen and Sali, 2006; Webb and Sali, 2014) creating 100 structures, which were ranked according to the Discrete Optimized Protein Energy score. The best three were selected for simulations; the G279S mutation was introduced using Pymol (PyMOL Molecular Graphics System, version 1.8.4; Schrödinger, LLC). Eight systems were created for each of these three selected structures, i.e., the receptor with an empty binding site (A₁R and A₁R-G279S^{7,44}) and bound with adenosine (A₁R.ado and A₁R-G279S^{7,44}.ado), each in complex with the G protein (A₁R.G and A₁R-G279S^{7,44}.G; A₁R.ado.G and A₁R-G279S^{7,44}.ado.G) or in the G protein-free state (A₁R and A₁R-G279S^{7,44}; A₁R.ado and A₁R-G279S^{7,44}.ado). Equilibrated membrane embedded systems were created by converting all models into the coarse grain representation of the Martini force field (Monticelli et al., 2008; de Jong et al., 2013; Wassenaar et al., 2015), which allowed for fast membrane equilibration. The proteins were embedded into a phosphatidylcholine (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine)

(POPC):cholesterol membrane (70:30 mol%); the simulation box was filled with water and 150 mM NaCl. The coarse grain systems were simulated for 1 microsecond with the protein structure restrained to avoid conformational changes during membrane equilibration. Next, membrane, water, and ions were converted to an all-atom representation (Wassenaar et al., 2014) while the original receptor structure replaced the coarse grain model. Spurious atom overlaps were relaxed using the *membed* procedure (Wolf et al., 2010). In the all-atom representation, protein, adenosine, and solvent were described using the amber99sb-ildn force field (Lindorff-Larsen et al., 2010), POPC, and cholesterol by Slipid (Jämbeck and Lyubartsev, 2012, 2013). All simulations used GROMACS version 2019.2 (Abraham et al., 2015). The completely assembled systems were energy minimized, and the receptor was released in four steps of 2.5 nanoseconds each by slowly reducing the position restraints (1000, 100, 10, 1 kJ/mol per nanometer) acting on the $C\alpha$ atoms and on adenosine if present. The production runs were carried for 500 nanoseconds for each independently assembled system. The temperature was maintained at 310 K using the v-rescale ($\tau = 0.5$ picosecond) thermostat (Bussi et al., 2007) while separately coupling protein + adenosine, membrane, and solvent. Pressure was maintained at 1 bar using the Parrinello-Rahman barostat (Parrinello and Rahman, 1981) in a semi-isotropic manner and with a coupling constant of 20.1 picoseconds. Long-range electrostatic interactions were described using the smooth particle mesh Ewald method (Darden et al., 1993) applying a cutoff of 0.9 nm. The van der Waals interactions were described using the Lennard-Jones potentials applying a cutoff of 0.9 nm. Long-range correction for energy and pressure were applied. Coordinates of all atoms were recorded every 50 picoseconds. Data for figures were extracted with the GROMACS package and processed in R and Python scripts using the MD Analysis package, version 0.19.2 (Michaud-Agrawal et al., 2011; Gowers et al., 2019). Visual Molecular Dynamics, version 1.9.3 (Humphrey et al., 1996), and Pymol, version 1.8.4, were used for visualization.

Cell Culture. HEK293 cells were plated in growth medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS] in 15-cm dishes or six-well dishes at 37°C in a humidified atmosphere containing 5% CO₂. When the cells were 80% confluent, they were transfected with plasmid(s) of interest using PEI (linear 25 kDa; Santa Cruz Biotechnology) as a transfection reagent. Briefly, DNA and PEI were mixed at a ratio of 1:3 (w:w) in serum-free DMEM and incubated for 15 minutes at 22°C. The mixture was then added in a dropwise manner to the dish. If not otherwise indicated, the total amount of DNA and of PEI used for a typical transfection were 11 µg and 33 µg per 15-cm dish or 2 µg and 6 µg per well, respectively. All assays were done 24 hours after transfection.

Membrane Preparation. HEK293 cells (8×10^6) were seeded in 15-cm dishes. When they were about 80% confluent (about 1.6×10^7 /dish), they were transiently transfected with empty plasmid alone (mock; 11 µg/dish) or the combination of empty plasmid (5.5 µg/dish) and plasmids encoding the human wild-type A₁R (5.5 µg/dish) or A₁R-G279S^{7.44} (5.5 µg/dish), which carried a FLAG-epitope on their N terminus. After 24 hours, the monolayer was rinsed with ice-cold PBS; subsequently, the cells were mechanically detached with a cell scraper, suspended in 5 ml ice-cold PBS containing 0.5 mM phenyl-methylsulfonyl fluoride (PMSF), and harvested by centrifugation at 300g for 5 minutes at 4°C. The cell pellet was resuspended in ice-cold hypotonic buffer containing 20 mM HEPES/NaOH (pH 7.4), 2 mM MgCl₂, 1 mM EDTA, 0.1 mM PMSF, and the Complete protease inhibitor cocktail. Thereafter, the cells were subjected to two freeze-thaw cycles followed by ultrasonication (Sonifier cell disruptor B15, 12 pulses of 0.5-second duration at 50% intensity; Branson Ultrasonics, Danbury, CT). Membranes were pelleted by centrifugation for 15 minutes at 38,000g and at 4°C and subsequently resuspended in buffer containing 20 mM HEPES/NaOH (pH 7.4), 2 mM MgCl₂, 1 mM EDTA buffer (1 ml per 0.2 g of wet pellet). The protein concentration (about 5 mg/ml) was determined by Coomassie Brilliant Blue binding. Membranes were aliquoted, frozen in liquid nitrogen, and stored at -80°C.

Radioligand Binding. For [³H]DPCPX saturation and displacement experiments, membranes (2–5 µg/assay) were incubated in 0.1 ml buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 1 mM EDTA, 0.1 mM GTPγS, and 5 U/ml adenosine deaminase in the absence and presence of ligands (CPA or XAC) and [³H]DPCPX (covering the range of 0.2–8 nM for saturation experiments and ~3 nM for displacement experiments) for 30 minutes at 25°C. The reaction was terminated by rapid filtration through GF/C glass fiber filter (Sartorius Stedim, Göttingen, Germany) followed by three washes with ice-cold wash buffer (10 mM Tris/HCl, pH 7.4, 1 mM MgCl₂) using a Skatron cell harvester. The radioactivity retained on the filters was measured by liquid scintillation. Nonspecific binding was determined in the presence of 10 µM XAC and represented <<10% of total binding in the K_D concentration range. In saturation experiments, the K_D and B_{max} were determined by subjecting the data to nonlinear least-squares curve fitting to the equation for a rectangular hyperbola. In displacement experiments, the IC₅₀ was estimated by fitting the data to the equation for a monophasic displacement curve. The K_i was calculated using the Cheng-Prusoff approximation ($K_i = IC_{50}/(1 + [L]/K_{D,L})$). For binding of [³⁵S]GTPγS, membranes (10 µg/assay) were incubated in a total volume of 80 µl containing 50 mM Tris/HCl (pH 7.4), 5 mM MgCl₂, 1 mM EDTA, 100 mM NaCl, 10 µM GDP, and 5 U/ml adenosine deaminase in the absence and presence of CPA or DPCPX for 30 minutes at 25°C. Thereafter, a solution (20 µl) containing [³⁵S]GTPγS (to ~1 nM final concentration, buffer composition otherwise identical) was added, and the incubation was continued for 1 minute at 25°C. The incubation was terminated by rapid filtration followed by three washes with ice-cold buffer containing 10 mM Tris/HCl (pH 7.4), 1 mM MgCl₂, and 100 mM NaCl. The radioactivity trapped on the filter was determined as outlined above.

Heat Denaturation. Membrane aliquots (5 µg/assay) were incubated in a total volume of 50 µl buffer containing 50 mM Tris-HCl, 2 mM MgCl₂, 1 mM EDTA, and 5 U/ml adenosine deaminase, pH 7.4 at temperatures ranging from 40°C to 65°C for time intervals ranging from 1 to 120 minutes. Thereafter, the reactions were placed on ice for 15 minutes and subsequently incubated in the presence of 3 nM [³H]DPCPX for 1 hour on ice in a final volume of 0.1 ml containing the same buffer as described above.

Flow Cytometry. HEK293 cells (0.5×10^6 /well) were seeded into six-well dishes; when the cells were about 80% confluence (1×10^6 /well), they were transiently transfected with empty plasmid alone (mock; 2 µg/well) or cotransfected with the combination of empty plasmid (1 or 1.5 µg/well) and plasmid (0.5 or 1 µg/well) encoding the human wild-type or mutant A₁-receptor (A₁R-G279S^{7.44}), which carried a FLAG epitope on their N termini. Cells were also cotransfected with plasmids encoding the D₁-receptor (1 µg/well) and wild-type or mutant A₁-receptor (1 µg/well). The transiently transfected HEK293 cells were washed with PBS containing 0.1% bovine serum albumin (BSA) and thereafter incubated with PBS containing 1 mM EDTA for 10 minutes at 37°C to detach the cells and then suspended in ice-cold PBS containing 0.1% BSA to a density of 1×10^6 cells/ml. The single cell suspension was sequentially incubated with the primary mouse M2 monoclonal anti-FLAG antibody (1:2000; Sigma) and the secondary mouse anti-mouse IgG1 antibody conjugated to AlexaFluor-488 fluorophore from Invitrogen (1:2000) for 20 minutes on ice. Thereafter, cells were pelleted by centrifugation (200g for 5 minutes at 4°C), resuspended in PBS containing 0.1% BSA, and injected into the flow cytometer (BD FACSCanto II; BD Biosciences, Franklin Lakes, NJ). Forward versus side scatter was used to identify cell populations and to exclude debris, which was found at the bottom left-hand corner of the forward scatter versus side scatter density plot (data not shown). In addition, backgating was used to ensure that debris and dead cells were not included in the analysis. Single parameter histograms were generated to quantify the staining by AlexaFluor-488 in the gated area; the specific area under the curve was calculated by subtracting the nonspecific area under the curve obtained from cells transfected with empty vector (mock transfection control).

Immunoprecipitation. HEK293 cells transiently coexpressing the HA-tagged D₁-receptor and the FLAG-tagged wild-type or mutant A₁-receptor (A₁R-G279S^{7,44}) were washed thrice with ice-cold PBS, collected in 5 ml PBS containing 0.1 mM PMSF, and harvested by centrifugation for 5 minutes at 300g and at 4°C. The cell pellet was suspended in buffer containing 25 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 1 mM EDTA, 100 mM NaCl, 1% dodecylmaltoside, 0.1 mM PMSF, and EDTA-free Complete protease inhibitor cocktail. Cell lysis was achieved by incubation for 1 hour with end-over-end rotation at 4°C. Thereafter, the solubilized material was retrieved by centrifugation (16,000g for 15 minutes at 4°C). Preequilibrated beaded agarose (0.1 ml of a 50% slurry) containing immobilized anti-HA antibody was added to an aliquot of the lysate (1 mg); the suspension was incubated with end-over-end rotation for 16 hours with at 4°C. Samples were centrifuged (1 minute at 5000g and at 4°C) and washed three times. The bound proteins were eluted with 0.1 ml denaturing sample buffer containing 20 mM dithiothreitol by heating at 60°C for 15 minutes. Thereafter, aliquots (10 µl) were separated by electrophoresis on SDS-polyacrylamide gels; proteins were transferred onto nitrocellulose membrane. Nonspecific binding was blocked by incubating the membranes in 25 mM Tris-buffered saline (pH 7.5), 0.1% Tween 20, and 5% bovine serum albumin for 1 hour at room temperature. After sequential incubation with anti-FLAG or anti-HA antibodies (1:1000 dilution) and fluorescently labeled donkey anti-rabbit antibody (1:1000 dilution), the immunoreactive bands were visualized and quantified on an Odyssey Clx infrared fluorescent imaging system (LI-COR Biosciences). Aliquots of the cell lysates (20 µg) were also electrophoretically resolved and transferred to nitrocellulose to verify comparable levels of receptor expression in the starting material. The pertinent blots were also probed with a rabbit antiserum, which recognizes all G protein β-subunits (Hohenegger et al., 1996), to control for equal loading of individual lanes (1:2000 dilution).

Bioluminescence Resonance Energy Transfer. HEK293 cells (0.5 × 10⁶/well) were seeded into six-well dishes. When the cells were about 80% (1 × 10⁶/well) confluent, the cells were transiently cotransfected with a constant amount of plasmid encoding the human D₁-receptor tagged on its C terminus with NanoLuc luciferase (D₁R-NLuc 0.2 µg/well) and increasing amounts (0–1.8 µg/well) of plasmid coding for the wild-type or mutant A₁-receptor, which were tagged at their C terminus with eYFP. The total amount of plasmid (2 µg/dish) was kept constant by adding the appropriate amount of empty plasmid. After 8 hours, cells were detached, seeded into 96-well dishes (5 × 10⁴/well), and allowed to adhere for 16 hours. After serum withdrawal for 1 hour, vehicle (control) or the indicated ligands (i.e., 10 µM CPA, 10 µM dopamine, or their combination) and luciferase substrate (furimazine = Nano-Glo, Promega; 1:200 dilution) were added, and bioluminescence was recorded for up to 20 minutes. Bioluminescence resonance energy transfer (BRET) readings were taken by simultaneously measuring light emission at 460 nm and at 530 nm in the microplate reader (FlexStation3; Molecular Devices). The BRET unit (BRET) signal was calculated by the ratio of emission at 530 nm [A₁R–yellow fluorescent protein (YFP)] to 460 nm (D₁R-NLuc). Cells expressing BRET donor alone (D₁R-NLuc) were used to determine background. BRET specificity was tested by using human β-arrestin-2 fused at its C termini to NLuc as a donor and A₁R-YFP as an acceptor, which gave equivalent values to that of the cells expressing donor alone. The net-BRET unit was calculated by subtracting background BRET. The data are presented as milli BRET units (net BRET × 1000). Parallel incubations were done with cells solely expressing D₁-receptor tagged with NanoLuc, and the emission recorded from these cells was subtracted.

Accumulation of cAMP. Eight hours after transfection, cells were replated into six-well plates (3 × 10⁵ cells/well) and incubated for 16 hours in DMEM containing 1 µCi/ml [³H]adenine (Waldhoer et al., 1999). Cells were then stimulated with 10 µM dopamine alone or in combination with the indicated concentrations of CPA and DPCPX in a total volume of 1 ml DMEM containing 5 U/ml adenosine deaminase

for 20 minutes at 25°C. Thereafter, the cells were lysed in 1 ml ice-cold 2.5% perchloric acid containing 100 µM cAMP for 15 minutes on ice. The cell extract was then neutralized with 4.2 M KOH. [³H]cAMP was separated by double column chromatography (Johnson et al., 1994).

Statistical Analysis. The first part of the study was exploratory in nature: the pharmacology of the mutant receptor and its expression were characterized without any working hypothesis. Three (CV ≤ 25%) to six experiments (CV ≤ 60%) were considered enough to verify the reproducibility of experimental findings. For experiments examining the hypothesis of constitutive activity (generated by molecular dynamics simulations and the analysis of thermal stability), the number of experiments was adjusted based on the variation observed: if three experiments did not suffice to show statistical significance, the number of required experiments was estimated with a power calculation (>90% probability of finding a statistically significant difference with *P* < 0.025) based on the observed variation. Statistical comparisons were done by paired *t* test (for comparison of two groups), by Friedman test (for paired comparison of multiple groups) followed by Holm-Sidak post hoc testing, or by F-test to compare two curves. Transient transfections with plasmids encoding wild-type and mutant receptors and the subsequent measurements were done in parallel. These parallel samples were considered as paired data because transfection efficiency varied on a day-to-day basis (cf. also Fig. 1D).

Results

Heterologous Expression of Wild-Type (A₁R) and Mutant Adenosine A₁-Receptor (A₁R-G279S^{7,44}). Many mutations affect the ability of GPCRs to undergo folding in the endoplasmic reticulum (Nanoff and Freissmuth, 2012). In fact, a substantial portion of the heterologously expressed wild-type A₁-adenosine receptor is retained and degraded in the endoplasmic reticulum (Pankevych et al., 2003; Kusek et al., 2015). Accordingly, we first examined the impact of the G279S mutation on receptor levels by transient transfection. Transient rather than stable expression was chosen because this approach eliminated possible distortions arising from clonal selection of cells. Comparable levels of receptors were detected with the antagonist radioligand (Fig. 1A): in three independent experiments the number of binding competent receptors B_{max} was 5.1 ± 1.1 and 5.9 ± 1.3 pmol/mg (means ± S.D.) for wild-type A₁R and A₁R-G279S^{7,44}, respectively. It is also evident from Figure 1A that mutant and wild-type receptor did not differ in their affinity for the radioligand (K_D = 1.4 ± 0.3 and 1.5 ± 0.4 nM for wild-type A₁R and A₁R-G279S^{7,44}, respectively). Similarly, as exemplified in Figure 1B for the A₁-selective agonist CPA, the G279S^{7,44} mutation did not affect agonist affinity (K_i = 0.4 ± 0.1 and 0.3 ± 0.1 µM for wild-type A₁R and A₁R-G279S^{7,44}, respectively). We stress that incubations were done in the presence of GTPγS. In the absence of guanine nucleotides, high-affinity agonist binding sites are expected to exist, which reflect ternary complex formation of agonist, receptor, and heterotrimeric G protein. In a buffer devoid of GTPγS, CPA displaced the radioligand with a biphasic curve (data not shown). However, the proportion of high-affinity sites was too low to provide reliable estimates for agonist affinity in the ternary complex. This is to be expected in transient transfections with high expression levels: upon membrane preparation, a large fraction of the receptor accumulates in vesicles, where receptor molecules outnumber G proteins. We also used flow cytometry by detecting the receptors via their N-terminal FLAG-epitope tag to verify that equivalent amounts were delivered to the plasma membrane: there was a variation in surface levels in

Fig. 1. Binding of the antagonist/inverse agonist radioligand [3 H]DPCPX (A) and of the agonist CPA (B) to and surface expression of wild-type (wt A₁R) and mutant A₁-receptors (C and D). (A) Saturation curve. Membranes (5 μ g/assay) prepared from HEK293 cells transiently expressing the FLAG epitope-tagged human wild-type A₁R (triangle down) or A₁R-G279S^{7,44} (full circle) were incubated with the indicated concentrations of [3 H]DPCPX for 30 minutes at 25°C in 0.1 ml buffer. Nonspecific binding was determined in the presence of 10 μ M XAC and subtracted. The data are means from duplicate determinations in a representative experiment. The curves were drawn by fitting the data to the equation for a rectangular hyperbola. (B) Displacement curve. As described for (A), membranes (2–3 μ g/assay) were incubated in the presence of [3 H]DPCPX (2.7 nM) in 0.1 ml buffer containing the indicated concentrations of CPA. Data are means $\pm$ S.D. from three independent experiments carried out in duplicate with membranes prepared from paired transfections. The curves were drawn by fitting the data to the equation for a monophasic displacement. (C and D) Flow cytometry histograms and their quantification. HEK293 cells were transiently transfected with the empty plasmid alone (mock, shaded histogram) or the combination of empty plasmid and plasmid encoding the FLAG epitope-tagged human wild-type A₁R [0.5 or 1 μ g/well, black histogram in (C) and full circle in (D)] or A₁R-G279S^{7,44} [0.5 or 1 μ g/well, red histogram in (C) and triangle down in (D)]. The total amount of plasmid (2 μ g/disk) was kept constant by adding the appropriate amount of empty plasmid. After 24 hours, cells were stained by sequential incubation in the presence of an antibody directed against the FLAG-epitope (1:2000 dilution) and an AlexaFluor-488-conjugated antibody directed against murine IgG1 (1:2000 dilution). The cell-associated fluorescence (FI) was quantified by flow cytometry. The histogram shows a representative result from a paired transfection. The results from four to six paired transfections with 0.5 and 1 μ g plasmid DNA encoding wild-type A₁R or A₁R-G279S^{7,44}, respectively, are summarized in the spaghetti plot shown in (D). AUC, area under the curve.

individual transfections, but in paired experiments there was no appreciable difference between wild-type and mutant receptor (Fig. 1C). In addition, the amount of receptors, which was detected on the cells, was related to the amount of plasmid DNA (Fig. 1D).

Thermal Stability of Wild-Type and Mutant A₁-Adenosine Receptor. The substitution of G279^{7,44} by serine introduces an additional hydrogen bond donor within TM7. We performed molecular dynamics simulations to obtain structural and dynamic insights into the flexibility of wild-type A₁-receptor and the changes caused by the G279S^{7,44} mutation. We used the solved structure of the adenosine-bound human A₁-receptor in complex with G_{i2} as a starting point (Draper-Joyce et al., 2018). Three parallel 500-nanosecond-long simulations were carried out for wild-type and mutant receptor, with and without the G protein and in the

presence and absence of adenosine. Figure 2A shows the membrane exposed orientation of the G279S mutation, located in the middle of TM7. The side chain of G279S^{7,44} interacts strongly with the backbone carbonyl of F275^{7,40} (Fig. 2B). A hydrogen bond is much stronger in a hydrophobic environment, where it can provide binding energies up to ~20–25 kJ/mol (Bowie, 2011). The energetic penalty for opening the hydrogen bond is much higher in a hydrophobic environment because it cannot be replaced by an alternative interaction as would occur in an aqueous environment. We also analyzed the root mean square fluctuations (RMSFs) of the A₁-receptor to quantify the global mobility of the receptor and to detect local changes in protein flexibility (Fig. 2, C and D). The global mobility of the A₁-receptor was similar for all systems in complex with G_{i2} (Fig. 2C) and without G_{i2} (Fig. 2D). The pattern of mobility reflects the secondary

structure of the receptor: the RMSF declines to low values over transmembrane helices, which reflect their rigidity. In contrast, the loops are much more flexible resulting in local maxima of RMSF. Complex formation with G_{i2} has an ordering effect on the intracellular loop 3: the mobility of intracellular loop 3 is strongly reduced when interacting with the G α subunit. The G279S^{7,44} mutation exerts mostly a local effect on TM7 mobility: in the absence of adenosine (i.e., in the apo state), the mobility of TM7 is larger in the mutant than in the wild-type receptor. This is seen in both the receptor complexed to the G protein (cf. brown and dark violet trace in Fig. 2D) and in the absence of the G protein, where the mobility of TM7 is even more pronounced (cf. amber and violet trace in Fig. 2D).

Hydrogen bonds are an important factor contributing to the forces stabilizing membrane proteins (Bowie, 2000; Stockner et al., 2004). Thus, the additional hydrogen bond in TM7 is predicted to increase thermal stability of the A₁-R-G279S^{7,44}. However, TM7 is kinked (cf. Fig. 7A). This bending must be stabilized by helical packing. The additional hydrogen bond introduces a counteracting force, which results in destabilization and hence enhanced flexibility, which is evident from the molecular dynamics simulations, in particular of the G protein-free apo state (amber trace in Fig. 2D). Because of this enhanced flexibility, the mutant receptor also ought to incur a penalty in thermal stability.

These predictions were examined by incubating membranes harboring wild-type and mutant receptors at temperatures ranging from 50°C to 63°C. Subsequently, the level of residual binding was determined by incubating the membranes with [³H]DPCPX on ice. If the heat-induced denaturation was allowed to proceed for 10 minutes, there was a small but consistent difference between wild-type and mutant receptor

(Fig. 3A; T₅₀ = 55.0°C ± 0.6°C and 56.7°C ± 0.5°C for wild-type A₁R and A₁R-G279S^{7,44}, respectively). This difference was less evident if the incubation time was increased to 20 minutes (Fig. 3B; T₅₀ = 53.7°C ± 0.5°C and 54.3°C ± 0.8°C for wild-type A₁R and A₁R-G279S^{7,44}, respectively). There are two components of protein stability, thermodynamic stability and kinetic stability (Sanchez-Ruiz, 2010): thermodynamic stability refers to the equilibrium between the amount of native functional protein and that of unfolded and partially unfolded states. It is high if—at a given temperature—the equilibrium is tilted in favor of the native protein. Kinetic stability is imparted by a high-energy barrier, which prevents the native state from visiting (partially) unfolded states. This energy barrier corresponds to an activation energy and can therefore be extracted from Arrhenius plots. We examined which component was affected by the G279S mutation by measuring the time-dependent loss of binding at different temperatures for both wild-type A₁R (Fig. 3C) and A₁R-G279S^{7,44} (Fig. 3D). As a control, we used A₁R-Y288A^{7,53} (Fig. 2E). This receptor variant has a folding defect, but it can be rescued by pharmacochaperoning (Málaga-Diéguez et al., 2010; Kusek et al., 2015). Accordingly, HEK293 cells were transiently transfected with a plasmid driving the expression of A₁R-Y288A^{7,53} and incubated with 100 μ M 3-isobutyl-1-methylxanthine for 16 hours prior to membrane preparation. This suffices to restore folding and cell surface expression of functionally active A₁R-Y288A^{7,53}; i.e., the pharmacochaperoned A₁R-Y288A^{7,53} binds the radioligand [³H]DPCPX and engages G_i in a manner comparable to wild-type A₁R (Málaga-Diéguez et al., 2010). In all instances, heat led to a biphasic loss of binding competent receptors (Fig. 3, C–E): the curves were adequately described by fitting them to the equation for a biexponential decay. We extracted the rate constants for both the fast and the slow component to

Fig. 2. The G279S mutation enhances TM7 flexibility. (A) Structural overview of the A₁-receptor. TM7 is highlighted in yellow. The inset shows a zoom-in into the structure of the mutant receptor, which highlights the hydrogen bond formed between the hydroxyl group of G279S and the backbone of F275^{7,40} (gray sticks). (B) Frequency distribution of the distance between the carbonyl oxygen of F275^{7,40} and the hydrogen of the hydroxyl group of S279^{7,44}. The histogram shows that—in all simulations (i.e., over 3 × 500 nanoseconds with 1 nanosecond sampling interval)—this hydrogen bond opens very rarely regardless of the state (ligand-bound vs. empty apo state; complexed to G protein or free receptor). (C and D) RMSFs of A₁R with and without G protein, respectively. RMSF plots show average data of three replications, measured with 1 nanosecond temporal resolution. WT and G279S refer to the wild-type and the mutant A₁-receptor, respectively. The apo state corresponds to the empty receptor (binding site devoid of ligand); + G indicates the mutant and wild-type receptor in complex with the G protein G_{i2}. In the apo state [brown and amber traces in (C) and (D), respectively], the upper part of TM7 of A₁R-G279S^{7,44} deviates from all other conformations regardless of whether examined with (C) or without (D) G protein.

generate Arrhenius plots (Fig. 4, A and B). It is evident from Figure 3, C–E, that the rate of the slow component increased with rising temperatures. The corresponding Arrhenius plots in Figure 4A show that this temperature-dependent increase was less pronounced with A₁R-G279S^{7.44} than with the wild-type receptor (cf. circles and triangles in Fig. 4A). From the slope of the Arrhenius plot we calculated an activation energy of 135 ± 4 and 73 ± 23 kJ/mol for wild-type A₁R and A₁R-G279S^{7.44}, respectively. As predicted, A₁R-Y288A^{7.53} was inactivated at lower temperatures than wild-type A₁R or A₁R-G279S^{7.44} (cf. Fig. 3, C–E). However, the slope of the Arrhenius plot was actually steeper, and hence the activation energy (184 ± 24 kJ/mol) was larger than that of A₁R-G279S^{7.44} (cf. squares and circles in Fig. 4A). Thus, the G279S^{7.44} mutation reduced the kinetic stability of the receptor.

Regardless of which variant of the A₁-receptor was examined, the rate of the fast component did not show any appreciable dependence on temperature (Fig. 4B), reflecting the low energy barrier of thermodynamic stability (Sanchez-Ruiz, 2010). Plotting the ratio of the slowly denaturing component over the rapidly unfolding component (P/P_u) as a function of temperature ($1/K$) allows for comparing the thermodynamic stability of the receptor variants: it is evident

from Figure 4C that the x -intercept of A₁R-G279S^{7.44} is shifted to the left (i.e., to a higher temperature) of that of wild-type A₁R; the difference of about 2 K is consistent with the difference in melting temperature seen in Figure 3A. In contrast and as predicted for a folding-deficient mutant, the melting temperature of A₁R-Y288A^{7.53} was lower by some 6.5 K than that of wild-type A₁R (cf. squares and triangles in Fig. 4C). Taken together, these observations show that A₁R-G279S^{7.44} has an enhanced thermodynamic stability but a reduced kinetic stability, whereas the reverse is true for A₁R-Y288A^{7.53}.

Complex Formation between the Dopamine D₁- and Wild-Type and Mutant A₁-Receptors. Adenosine A₁- and dopamine D₁-receptors form heteromeric complexes (Ginés et al., 2000; Rivera-Oliver et al., 2019). When transiently coexpressed with either A₁R or A₁R-G279S^{7.44}, D₁-receptors accumulated to comparable levels as assessed by binding of the antagonist radioligand [³H]7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol ($B_{\max} = 1.2 \pm 0.2$ and 1.4 ± 0.2 pmol/mg in the presence of wild-type A₁R and A₁R-G279S^{7.44}, respectively). Similarly, equivalent amounts of receptors were detected by immunoblotting detergent lysates prepared from cotransfected cells (Fig. 5A). We first assessed complex formation by immunoprecipitating the D₁-receptor

Fig. 3. Heat-induced denaturation of wild-type and mutant adenosine A₁-receptors. (A and B) Membranes were prepared from HEK293 cells transiently expressing the FLAG-tagged human wild-type (wt A₁R, closed triangles) and mutant A₁-receptor (A₁R-G279S^{7.44}, closed circles). Membranes (3–5 μg/assay) were subjected to heat-induced denaturation and incubated for 10 minutes (A) or 20 minutes (B) at the indicated temperatures. Thereafter, the membranes were placed on ice. Binding of the [³H]DPCPX (3 nM) was determined for 1 hour at 0°C. The curves in (A and B) were drawn by fitting the data to a three-parameter logistic equation. (C–E) Membranes harboring FLAG-tagged A₁R-Y288A^{7.53} (closed squares) were prepared from transiently transfected HEK293 cells, which had been incubated in the presence of 100 μM 3-isobutyl-1-methylxanthine to restore folding and surface expression of the receptor prior to cell lysis. Membranes (3–5 μg/assay for wt A₁R and A₁R-G279S^{7.44}; 10–15 μg/assay for A₁R-Y288A^{7.53}) were subjected to denaturation at the indicated temperatures and for the indicated time intervals. Thereafter, binding of [³H]DPCPX (3 nM) was determined as outlined for (A and B). Nonspecific binding was defined in the presence of 10 μM XAC; it was <10% of total binding, did not change with temperature or time, and was subtracted. The 100% reference value is binding to control membranes, which were held on ice throughout the experiment. This binding was 10–15 fmol/assay. Data are means ± S.D. from three independent experiments, which were done in parallel. The curves in (C–E) were drawn by fitting the data to the equation for a biexponential decay.

Fig. 4. Arrhenius plots for the slow (A) and fast component (B) of heat-induced denaturation and the temperature-dependent change of their ratio (C) for wild-type (wt A₁R) and mutant A₁-adenosine receptors. (A and B) The rates of the slow (A) and the fast component (B) were calculated from the individual biexponential decay curves summarized in Figure 2, C–E, and their natural logarithm (means ± S.E.M.) plotted as a function of the reciprocal of the absolute temperature. (C) The relative proportion of the slowly (P_f) and of the rapidly denaturing (P_u) component were calculated from the individual biexponential decay curves summarized in Figure 2, C–E. The natural logarithms of their ratios (means ± S.E.M.) were plotted as a function of the reciprocal of the absolute temperature. The lines were drawn by linear regression.

via its N-terminal HA tag: in the immunoprecipitated, equivalent levels of wild-type A₁R and A₁R-G279S^{7,44} were visualized by immunoblotting for the N-terminal FLAG-epitope (Fig. 5B).

In addition, we examined complex formation in intact cells by bioluminescence resonance energy transfer between a fixed amount of D₁-receptors, which were C-terminally tagged with

a luciferase (NanoLuc), and increasing amounts of YFP-tagged A₁-receptors. This approach allowed for monitoring complex formation in the absence of receptor activation (Fig. 6A) or after stimulation of the receptors' activation by their cognate agonists, i.e., by the A₁-selective agonist CPA (Fig. 6B), dopamine (Fig. 6C), or the combination thereof (Fig. 6D). It is evident that the curves are comparable, i.e., there was no any appreciable difference in the interaction of the dopamine receptor with wild-type or mutant A₁-receptor regardless of whether receptors were activated or not (Table 1). This indicates that the receptor heteromers form in a constitutive manner, an interpretation that is also supported by the coimmunoprecipitation in the absence of receptor activation (Fig. 5B).

Comparison of G_i Activation by Wild-Type and Mutant A₁-Receptor. The analysis of the thermostability suggested that the G279S mutation lowered the energy barrier for conformational changes because its kinetic stability was lower than that of the wild-type receptor (cf. Fig. 4A). The RMSF plots summarized in Figure 2 showed a higher mobility of TM7 in A₁R-G279S^{7,44}. We interrogated the molecular dynamics simulations to search for changes in the energy landscape associated with movements of TM7: we quantified the increased mobility of TM7 by measuring the distances between TM3 and TM7 at the extracellular face of the receptor, i.e., the distance between L269^{7,34} and A84^{3,29}, and at the site of the mutation site, i.e., the distance between T91^{3,36} and H278^{7,43} (Fig. 7A). These measurements captured the changes in stability, dynamics, and conformations induced by the G279S^{7,44} mutation. The two-dimensional histogram (Fig. 7B) and the associated free-energy map (Fig. 7C) visualized the movements of TM7. It is evident that in the apo (i.e., ligand-free) state of the receptor, TM7 visits many more positions distant from TM3 than in the adenosine-bound state: this is true for both the wild-type receptor in the presence (cf. first and third two-dimensional histogram in the top row of Fig. 7B) and absence of G₁₂ (cf. first and third two-dimensional histogram in the bottom row of Fig. 7B) and for the mutant receptor (cf. corresponding second and fourth two-dimensional histograms in Fig. 7B). This observation shows that binding of adenosine restrains the movement of TM7. In fact, the distance between TM3 and TM7 becomes shorter at the bottom of the ligand-binding site as TM7 closes in onto the agonist adenosine. The structural change is visible as a shift in the T91^{3,36}–H278^{7,43} distance (y-axis in Fig. 7, B and C): in the energy basin, the minimum (indicated by an "x" in Fig. 7C) is located at <0.9 nm in the presence of adenosine (two-dimensional histograms in the first and second column of Fig. 7C). In contrast, in the absence of adenosine, the most stable distance is larger than 0.9 nm (two-dimensional histograms in the first and second column of Fig. 7C). The most conspicuous difference between the mutant and the wild-type receptor can be appreciated by comparing the energy minima of the apo state in the presence of G₁₂: in the A₁R-G279S^{7,44}, the basin of low-energy states covers a substantially larger area than in the wild-type receptor (cf. fourth and third two-dimensional histogram in the top row of Fig. 7C). This observation is consistent with a low-energy barrier imparted by the mutation, which allows TM7 and thus the mutant receptor to sample many more conformational states than the wild-type receptor.

In the active state, the receptor has to accommodate the C terminus of its cognate G protein α -subunit in a cavity, which

Fig. 5. Coimmunoprecipitation of the wild-type adenosine A₁-receptor or the mutant A₁R-G279S^{7.44} with the dopamine D₁-receptor. HEK293 cells (1.6×10^7 /15-cm dish) were transiently cotransfected with plasmids encoding the HA-tagged D₁-receptor (5.5 μ g/15-cm dish) and FLAG-tagged wild-type (wt A₁R) or mutant (A₁R-G279S^{7.44}) A₁-receptors (5.5 μ g/15-cm dish). After 24 hours, cells were detached and lysed as described in the *Materials and Methods* section. An aliquot of the lysate (20 μ g/lane) was used to assess the expression of the receptors by immunoblotting with antibodies directed against the epitope tags (A). Lysates were also prepared from HEK293 cells subjected to transfection with empty plasmid (lanes labeled mock). The lysates (1 mg) were incubated with beaded agarose containing immobilized HA-antibody. An aliquot (10%) of the immunoprecipitate was resolved by denaturing electrophoresis and transferred to nitrocellulose membranes. The immunoreactive bands of the D₁-receptor [left-hand blot in (B)] and of wild-type and mutant A₁-receptors

forms on the intracellular side within the transmembrane bundle. A comparison between the active G α protein-bound conformation and the receptor without the G protein shows large movements of TM5 to create the space that allows for G α protein binding (cf. Fig. 8, A and B). We also examined the effect of G279S^{7.44} mutation on the intracellular face of the receptor by measuring distances between I48^{2.43} and V203^{5.61} (TM2–TM5) and between I48^{2.43} and I232^{6.33} (TM2 – TM6) (highlighted as red lines in Fig. 8B). Simulations starting from the inactive conformation reveal that the size of the cavity is sensitive to the mutation: in the apo state, the G279S^{7.44} mutation (brown trace in Fig. 8, C and D) leads to an opening of the binding site for the G α protein. In contrast, the wild-type A₁-receptor remains close to its starting structure in the absence of adenosine (red trace in Fig. 8, C and D). Figure 8, E and F, shows that the bound G α protein restricts receptor movements in both the wild-type and the mutant receptor. In contrast, the inactive conformation of the A₁-receptor is sensitive to the mutation and the presence of the adenosine ligand. Figure 8, C and D, shows that the mutation leads to an opening of the G α protein binding site as compared with the wild-type A₁-receptor, which remains close to its starting structure in the absence of the adenosine ligand. Addition of the adenosine ligand to the wild-type receptor induces a similar conformational change. A comparison between panels D and F of Figure 8 indicates that the time window of the molecular dynamics simulation of 0.5 microsecond is not long enough to observe a complete conversion from the inactive to the active conformation.

In most, if not all, GPCRs TM7 is bent; this is also true for the A₁-receptor. This kink is energetically not optimal and used by the receptor to sense agonist binding. The G279S^{7.44} mutation introduces an additional hydrogen bond donor. This ought to stabilize the protein provided that it optimally fits into the structure. However, the traces in Figure 8D show that, in the absence of the G protein, the additional hydrogen bond prefers or needs a different conformation to fulfill its bonding interactions. As a consequence, in the presence of adenosine, helix TM7 of A₁R-G279S^{7.44} oscillates between two conformations (red trace in Fig. 8D).

We surmised that the increased conformational flexibility (Fig. 7C) and the partial opening of the G protein binding site (Fig. 8, C and D) ought to translate into more effective agonist-induced G protein activation and/or higher basal—i.e., agonist-independent—activity. This prediction was verified by 1) measuring agonist-induced binding of [³⁵S]GTP γ S (Fig. 9A) and 2) the effect of an inverse agonist on the basal binding of [³⁵S]GTP γ S (Fig. 9B) under initial rate conditions (i.e., after 1 minute): when stimulated with the agonist CPA at saturating concentrations, the mutant A₁R-G279S^{7.44} caused a larger increase in [³⁵S]GTP γ S binding than the

[right-hand blot in (B)] were visualized by blotting for the HA- and FLAG-epitope tags, respectively. Arrows point to the receptor-specific immunoreactivity; the lower bands correspond to the ER-resident core glycosylated forms of the D₁-receptor and of the A₁-receptor. We note that there are also receptor aggregates, in particular, of the A₁-receptors (immunoreactive bands at about 70 kDa highlighted by an arrow). Data are from a representative experiment, which was replicated four times in independent, paired transfections. The D₁- and A₁-receptor immunoreactivity was quantified by densitometry, and the ratio seen in these four experiments is shown in (C). AUC, area under the curve; A.U., arbitrary unit; IP, immunoprecipitation.

Fig. 6. BRET between the luciferase-tagged dopamine D₁-receptor and the wild-type adenosine A₁-receptor or the mutant A₁R-G279S^{7,44}. HEK293 cells (1×10^6 /well) were transiently cotransfected with a constant amount of plasmid encoding D₁R [1 and 0.2 μ g/well for (A–D) and (E–H), respectively], which was tagged either on its N terminus with an HA-epitope (A–D) or on its C terminus with a luciferase (NanoLuc) (E–H), and either 1 μ g/well (A–D) or increasing amounts [0–1.8 μ g/well; (E–H)] of plasmid coding for the wild-type (wt A₁R) or the mutant A₁-receptor (A₁R-G279S^{7,44}), which were tagged either on their N terminus with the FLAG-epitope (A–D) or on their C terminus with eYFP (E–H). The total amount of plasmid (2 μ g/dish) was kept constant by adding the appropriate amount of empty plasmid. (A–D) Flow cytometry histograms and their quantification. After 24 hours, cells were detached and divided into two aliquots, which were incubated with murine M2 anti-FLAG antibody (1:2000) or murine anti-HA antibody (1:2000) and then with the secondary anti-mouse IgG antibody conjugated to AlexaFluor-488 (1:2000). The resulting receptor-associated immunofluorescence was quantified by flow cytometry as outlined under *Materials and Methods*. (E–H) BRET recordings in the absence and presence of agonists. Eight hours after transfection, cells were seeded into 96-well dishes (5×10^4 /well) and allowed to adhere for 15 hours. After serum withdrawal for 1 hour, vehicle (control) or the indicated ligands (i.e., 10 μ M CPA, 10 μ M dopamine, or their combination) and luciferase substrate (furimazine, 1:200 dilution) were added, and bioluminescence was recorded for up to 20 minutes. Parallel incubations were done with cells solely expressing D₁-receptor tagged with NanoLuc, and the luminescence recorded from these cells was subtracted. Data are means $\pm$ S.D. from three independent experiments done in parallel and carried out in duplicate. AUC, area under the curve; FI, fluorescence; mBU, milli BRET unit.

TABLE 1

Complex formation between dopamine D₁-receptor and wild-type adenosine A₁-receptor or the mutant A₁R-G279S^{7,44}BRET₅₀ refers to the amount of plasmid encoding wild-type or mutant adenosine A₁-receptor giving half-maximum bioluminescence resonance energy transfer; maximum BRET is the response estimated at saturation. The values (means $\pm$ S.D.) were calculated by fitting the data from three independent experiments (summarized in Fig. 4) to the equation of a rectangular hyperbola.

Incubation	A ₁ -receptor variant	BRET ₅₀ μ g A ₁ R plasmid	Maximum BRET signal milli BRET
Control	Wild-type A ₁ R	0.6 $\pm$ 0.1	177 $\pm$ 9
	A ₁ R-G279S ^{7,44}	0.5 $\pm$ 0.1	160 $\pm$ 30
CPA (10 μ M)	Wild-type A ₁ R	0.9 $\pm$ 0.2	218 $\pm$ 64
	A ₁ R-G279S ^{7,44}	0.9 $\pm$ 0.2	202 $\pm$ 37
Dopamine (10 μ M)	Wild-type A ₁ R	0.7 $\pm$ 0.1	207 $\pm$ 27
	A ₁ R-G279S ^{7,44}	0.8 $\pm$ 0.1	190 $\pm$ 28
CPA and dopamine (10 μ M each)	Wild-type A ₁ R	1.1 $\pm$ 0.3	226 $\pm$ 82
	A ₁ R-G279S ^{7,44}	1.4 $\pm$ 0.6	229 $\pm$ 23

wild-type A₁-receptor (Fig. 9A). In contrast, the concentration required for half-maximum stimulation did not differ ($EC_{50} = 38 \pm 9$ and 58 ± 19 nM for wild-type A₁R and A₁R-G279S^{7,44}, respectively). On average, the basal rate of [³⁵S]GTP γ S binding was slightly higher in membranes prepared from HEK293 cells transiently expressing A₁R-G279S^{7,44} than

those transiently expressing wild-type A₁R. Most G protein-coupled receptors have some basal (i.e., constitutive, agonist-independent) activity, which can be blocked by antagonists, which are in most instances inverse agonists (Schütz and Freissmuth, 1992; Leff, 1995). This is also true for the A₁-receptor (Freissmuth et al., 1991a). Accordingly, we examined

Fig. 7. The mutation G279S^{7,44} lowers the free-energy barrier for TM7 to change conformation. (A) The distances, which were measured during the simulations, are highlighted in the structure of the A₁-receptor. (B) Two-dimensional histogram of the distances between the C α atoms (CA) of T91^{3,36} and H278^{7,43} (x -axes) and of A84^{3,29} and L269^{7,34} (y -axes). (C) Free-energy estimate associated with conformational changes in the distances shown in (B). The minimum position in the free-energy basin is indicated by $\times$. Each plot shows the average of three independent 500-nanosecond simulations with data points sampled at 1-nanosecond intervals. WT and G279S refer to the wild-type and the mutant A₁-receptor, respectively. The apo state corresponds to the empty receptor (binding site devoid of ligand); + G indicates the mutant and wild-type receptor in complex with the G protein G_{i2}. CA, C α .

Fig. 8. Geometry of the G α protein binding cavity of the wild-type adenosine A₁-receptor and of the mutant A₁-R-G279S^{7.44}. Snapshots taken at the end of the simulations shows the wild-type A₁-receptor from the cytoplasmic side with (A) and without (B) bound G α . The A₁-receptor is represented as white cartoon and TM7 is highlighted in yellow, the bound C-terminal helix of the G α_{12} protein is shown in transparent red color. Residues I48^{2.43}, I232^{6.33} and V203^{5.61} are shown as sticks. Red lines indicate the distances measured in (C–F). The inactive receptor is indicated by an asterisk (*). Histograms summarize the frequency at which the indicated distances between the C α atoms (CA) of I48^{2.43} (TM2) and V203^{5.61} (TM5) (C and E) and of I48^{2.43} (TM2) and I232^{6.33} (TM6) (D and F) were observed. Each histogram includes three independent simulations of 0.5 microsecond each. Data points were sampled at 1-nanosecond intervals. The A₁-receptor was simulated using the active form based of the agonist-liganded, G protein-bound structure (+G; PDB ID: 6D9H) and starting from the inactive conformation based on the antagonist-bound structure (PDB ID: 5N2S). For either conformation, wild-type (WT) and mutant receptor A₁-R-G279S^{7.44} (G279S) were simulated in the absence (apo) and presence of adenosine.

the extent to which a saturating concentration of the antagonist/inverse agonist DPCPX reduced basal [³⁵S]GTP γ S binding in membranes from HEK293 cells transiently expressing wild-type A₁R and A₁R-G279S^{7.44}. It is evident from Figure 8B that DPCPX caused a statistically significant inhibition of basal [³⁵S]GTP γ S binding to membranes harboring A₁R-G279S^{7.44} by on average 23.4% (95% confidence interval 12.4%–34.5%). In contrast, there was no appreciable effect of DPCPX on basal [³⁵S]GTP γ S binding to membranes harboring wild-type A₁R (on average 1.1% lower than in the absence of DPCPX; 95% confidence interval from 11.8% lower to 9.7% higher). These observations indicate that the constitutive activity of A₁R-G279S^{7.44} is more pronounced than that of the wild-type receptor.

Taken together the data in summarized in Figure 9 suggested that A₁R-G279S^{7.44} was more effective in promoting nucleotide exchange on cognate G proteins than the wild-type receptor. The A₁-receptor is a prototypical G_i/G_o-coupled

receptor, which engages all isoforms of G α_i and G α_o (Freissmuth et al., 1991b; Jockers et al., 1994). The bidirectional regulation of cAMP formation is the major effector pathway, which is regulated in a mutually antagonistic manner by D₁- and A₁-receptors (Ferré et al., 1998). We therefore explored if wild-type A₁R and A₁R-G279S^{7.44} differed in their ability to inhibit cAMP accumulation induced by the D₁-receptor in transiently cotransfected cells. We first measured dopamine-induced cAMP accumulation in the absence and presence of DPCPX to address the question of whether the different level of constitutive nucleotide exchange activity detected by [³⁵S]GTP γ S binding (Fig. 8B) translated into modulation of cAMP production. This was the case: in HEK293 cells coexpressing the D₁-receptor and the mutant A₁R-G279S^{7.44}, DPCPX produced a consistent and statistically significantly increased cAMP response to dopamine (by 1.4 $\pm$ 0.2-fold; Fig. 10A). In contrast, in HEK293 cells coexpressing the D₁-receptor and

Fig. 9. [35 S]GTP γ S binding to membranes prepared from HEK293 cells transiently expressing wild-type or mutant adenosine A₁-receptors. HEK293 cells were transfected with plasmids driving the expression of the wild-type (wt A₁R, triangle down, 5.5 μ g/15-cm dish) or the mutant (A₁R-G279S^{7.44}, full circles, 5.5 μ g/15-cm dish) adenosine A₁-receptor and membranes were prepared as outlined in the legend to Figure 1A. (A) Membranes (10 μ g) were preincubated at 25°C in the absence and presence of the indicated concentrations of the agonist CPA for 30 minutes; the reaction started by adding [35 S]GTP γ S to a final concentration of 1 nM and stopped after 1 minute by rapid filtration as outlined under *Materials and Methods*. Data are means $\pm$ S.D. from three independent experiments (with different membranes from paired transfections) carried out in duplicate. The curves were drawn by fitting the data to the equation describing the hyperbolic concentration-dependent stimulation of a basal activity. The two curves are better described by separate fits rather than by a fit with shared parameters (F-test based on the extra sum of squares-principle; $F = 16.03$, $P = 0.0004$) because of the difference in the maximal [35 S]GTP γ S binding (95% confidence interval 91.5–101.3 and 111.5–128.1 fmol/mg for wt A₁R and A₁R-G279S^{7.44}, respectively). (B) The assay was done as described for (A) in the absence (basal) and presence of the antagonist/inverse agonist DPCPX (10 μ M). Shown are the results from six independent paired transient transfections; each individual experiment is represented by the same symbol. The lines connect basal binding to the corresponding binding in the presence DPCPX to illustrate the consistent inhibition by DPCPX in membranes harboring the mutant receptor A₁R-G279S^{7.44} and the absence thereof in membranes harboring the wild-type A₁-receptor (wt A₁R). The box plot shows the median and interquartile range; the whiskers correspond to the 95% confidence interval. In membranes carrying A₁R-G279S^{7.44}, the difference between basal [35 S]GTP γ S binding and binding in the presence of DPCPX was statistically significant ($P < 0.02$, Friedman test followed by Holm-Sidak post hoc testing).

the wild-type A₁-receptor, the effect of DPCPX was less pronounced and did not reach statistical significance in spite of the large number of paired observations (increase by 1.2 ± 0.2 -fold; Fig. 10A). Similarly, in cells expressing A₁R-G279S^{7.44}, the concentration-response curve for CPA was shifted to the left of that seen in cells expressing wild-type A₁R (Fig. 10B; $IC_{50} = 2.4 \pm 0.5$ and 0.9 ± 0.4 nM for wild-type A₁-receptor and the mutant A₁R-G279S^{7.44}, respectively). Although the shift was modest, the difference was consistently observed in paired experiments, where cells were subjected to transient cotransfection with plasmids encoding the D₁-receptor and the wild-type or mutant A₁-receptor (cf. inset in Fig. 10B). This difference between wild-type and mutant A₁-receptor is consistent with the higher efficacy of the agonist-stimulated A₁R-G279S^{7.44} in promoting guanine nucleotide exchange (Fig. 9A).

Discussion

GPCRs represent the largest family of mammalian proteins. Hence their genes collectively occupy a large fraction of the protein-coding genome. A survey that covered 107 GPCRs targeted by approved drugs documented that, on average, an apparently healthy individual harbors 68 nonsynonymous coding variations (missense variations) in about one third of these receptors (Hauser et al., 2018). In a data base search (www.ensembl.org), we identified 134 missense variations (relative to the reference genome GRCH38.p13) at 96 positions

in the coding sequence of the A₁-receptor. Fourteen of these variations (at 12 positions) were found in the 2504 apparently healthy individuals covered by the 1000 Genomes Project (Auton et al., 2015). However, the only source describing the G279S^{7.44} mutation in the human A₁-receptor is the report by Jaber et al. (2016), which linked the mutated receptor to early-onset Parkinson's disease. Our experiments were designed to explore the impact of the mutation on the activity of the receptor. Based on our findings, we conclude that the variant A₁R-G279S^{7.44} has an enhanced conformational flexibility, which translates into a higher basal (i.e., agonist-independent, constitutive) activity and an enhanced agonist-induced response. This conclusion is based on three independent lines of evidence. 1) The kinetic stability of A₁R-G279S^{7.44} was about 50% lower than that of the wild-type receptor. Thus the mutation lowered the energy barrier for conformational transitions, and this finding was recapitulated in molecular dynamics simulations. 2) When we probed the receptor in the presence and absence of an antagonist/inverse agonist to define the constitutive activity of the receptor, we consistently observed a larger effect of the antagonist/inverse agonist for A₁R-G279S^{7.44} than for the wild-type receptor, regardless of whether guanine nucleotide exchange or by cAMP accumulation was assessed. 3) The agonist-liganded A₁R-G279S^{7.44} was more efficacious than the wild-type receptor in catalyzing guanine nucleotide exchange. Accordingly, in cells expressing A₁R-G279S^{7.44}, the agonist concentration-response curve for

Fig. 10. Effect of the A₁-antagonist/inverse agonist DPCPX (A) and of the A₁-agonist CPA (B) on dopamine-induced cAMP accumulation in HEK293 cells co-expressing dopamine D₁ and wild-type or mutant adenosine A₁-receptors. HEK293 cells were transiently co-transfected with plasmids encoding D₁R (5.5 $\mu\text{g}/15\text{-cm}$ dish), and wild-type A₁-receptor (wt A₁R; 5.5 $\mu\text{g}/15\text{-cm}$ dish, triangle down) or the mutant A₁R-G279S^{7,44} (5.5 $\mu\text{g}/15\text{-cm}$ dish, full circle). After 8 hours, cells were replated into 6-well dishes ($0.5 \times 10^6/\text{well}$) in DMEM medium containing 1 $\mu\text{Ci}/\text{ml}$ [^3H]adenine and incubated for 16 hours and subsequently stimulated in medium containing 1 μM dopamine alone or combination of 1 μM dopamine and 10 μM DPCPX (A) or increasing concentration of CPA (B) for 20 minutes as outlined under *Materials and Methods*. (A) Shown are the results from 11 independent paired transient transfections; each individual experiment is represented by the same symbol. The lines connect basal cAMP levels to the corresponding level in the presence DPCPX: addition of DPCPX caused a statistically significant increase in basal cAMP levels in cells expressing A₁R-G279S^{7,44} but not in cells expressing wt A₁R ($P = 0.001$ and 0.059 , respectively, Friedmann test followed by Holm-Sidak post hoc testing for multiple comparisons). The box plot shows the median and interquartile range; the whiskers correspond to the 95% confidence interval. (B) Data represent means $\pm$ S.D. from four independent experiments and the spaghetti plot in the inset shows the IC_{50} values for wt A₁R and A₁R-G279S^{7,44} (paired experiments are indicated by the same symbols). IC_{50} -values differ in a statistically significant manner ($P = 0.011$, t test for paired data). The curves were drawn by fitting the data to a monophasic inhibition curve. The two curves are better described by separate fits rather than by a fit to a common curve with shared parameters (F-test based on the extra sum of squares-principle; $F = 13.57$, $P = 0.0001$).

lowering cAMP levels was shifted to the left of that seen in cells expressing the wild-type receptor.

Residues in TM7 contribute to the orthosteric binding site of the A₁-receptor; in fact, T270 is the amino acid critical for binding of ligands, which discriminate between the A₁- and the A_{2A}-receptor (Cheng et al., 2017; Glukhova et al., 2017). G279 is about 2.5 helical turns distal to T270. Our experiments rule out that the G279S mutation has indirect effects on the geometry of the ligand-binding cavity: both the A₁-selective antagonist/inverse agonist DPCPX and the agonist CPA bound with similar affinity to the wild-type A₁-receptor and to A₁R-G279S^{7,44}. Similarly, G279S^{7,44} is about 2.5 helical turns from Y288^{7,53}, which is critical for folding of the A₁-receptor in the endoplasmic reticulum: during the conformational search, TM7 and the C terminus must be correctly positioned to allow for emergence of the native conformation (Pankevych et al., 2003; Málaga-Diéguez et al., 2010). The G279S^{7,44} mutation does not interfere with the folding trajectory of the A₁-receptor. This conclusion is based on our observations that equivalent levels of wild-type and mutant receptors were found on the cell surface. The A₁-receptor can form homodimers (Gracia et al., 2013) and heteromers, in particular with the D₁-receptor (Ginès et al., 2000; Rivera-Oliver et al., 2019), which allows for their reciprocal, mutually antagonistic modulation (Ferré et al., 1998). The interface in the A₁-D₁-receptor heteromer is not known, but our observations indicate that the G279S^{7,44} mutation does not affect this interface: wild-type and mutant A₁-receptors did not differ in their ability to form complexes with the

D₁-receptor, regardless of whether the interaction was assessed by coimmunoprecipitation or monitored by BRET.

We used thermal denaturation to probe the conformational flexibility of mutant and wild-type A₁-receptors. Heating resulted in irreversible loss of binding. Under our experimental conditions, it was not possible to capture the initial reversible unfolding. Hence, a Lumry-Eyring model is applicable, which in its simplest version posits a two-step process $N \rightleftharpoons U \rightarrow D$, where N, U, and D are the native, reversibly unfolded, and irreversibly denatured states, respectively (Lumry and Eyring, 1954). Because the reversibly unfolded state is inaccessible, it is not possible to extract the energy change ΔG (or ΔH) associated with initial unfolding/refolding, i.e., the $N \rightleftharpoons U$ transition. However, the rates of denaturation do shed light on the underlying processes. The rapidly denaturing component reflects a fraction of the A₁-receptor, which visits conformational states that are separated by a low-energy barrier from the unfolded state. This fraction rather than the rate of denaturation increased with temperature. Hence, the resulting Arrhenius plots were flat. We posit that this rapid rate reflects the thermodynamic stability of the protein. Substitution of G279 by serine introduces an additional hydrogen bond donor into TM7. The most likely acceptor is the backbone carbonyl of F275^{7,40} (Cheng et al., 2017; Glukhova et al., 2017): this conjecture was substantiated by the molecular dynamics simulations. When compared with the wild-type A₁-receptor, the thermodynamic stability of the mutant A₁R-G279S^{7,44} was enhanced: this increase can be

accounted for by the extra hydrogen bond donated by the serine residue, which stabilizes TM7. The importance of TM7 for the stability of the receptor is highlighted by the reduced thermodynamic stability of the mutant A₁R-Y288A^{7,53}. In contrast, the second component of thermal denaturation proceeded with a slow rate, which was accelerated with increasing temperature, resulting in steep Arrhenius plots. This second component reflects the kinetic stability of the receptor. The relationship between thermodynamic and kinetic stability is complex: it may range from a perfect correlation to total independence (Sanchez-Ruiz, 2010). Our observations show that, in the A₁-receptor, thermodynamic stability and kinetic stability are not correlated. This is exemplified by both A₁R-G279S^{7,44} and A₁R-Y288A^{7,53}: the energy barrier, which separated the wild-type A₁-receptor in its ground state from the denatured state(s), was larger than that of A₁R-G279S^{7,44} and smaller than that of A₁R-Y288A^{7,53}. We note that the kinetic barrier is substantially smaller in the A₁-receptor (135 kJ/mol) than in rhodopsin (670 kJ/mol; Hubbard, 1958; Corley et al., 2011). This difference is not surprising. Rhodopsin supports vision in dim light and thus requires a high thermal barrier to spontaneous conformational transitions (Guo et al., 2014). However, the kinetic barrier for thermal denaturation of the A₁-receptor is larger than that required for productive ternary complex formation (Waldhoer et al., 1999). In the active, G protein-bound state of the A₁-receptor, the ligand-binding site collapses on the agonist (Draper-Joyce et al., 2018). Our molecular dynamics simulations show that this is due to the movement of several helices, including TM7, which is facilitated by substituting serine for G279^{7,44}. The G279S^{7,44}-induced increased flexibility also allows for rationalizing the reduced kinetic stability, the increase in constitutive activity, and the enhanced agonist-induced response because they can all be linked to lower energy barriers between conformational states.

In Parkinson's disease, the G279S^{7,44} variant of the A₁-receptor is a rare mutation because it does not occur in the large set of whole exome sequencing data of the International Parkinson's Disease Genomics Consortium (Blauwendraat et al., 2017). The interpretation is also confounded by the fact that the affected individuals also harbor a mutation (C52Y) on both alleles of the gene encoding peptidyl-t-RNA hydrolase domain containing-1 (PTRHD1) (Elahi, 2018). PTRHD1 is a protein of unknown function, which lacks its eponymous activity: although it binds peptidyl-t-RNA, it does not hydrolyze it (Burks et al., 2016). The role of PTRHD1 in autosomal recessive Parkinson's disease is supported by two additional reports: an adjacent mutation (H53Y) was found in Iranian patients (Khodadadi et al., 2017), and mutations in PTRHD1, which result in truncation of the protein, were identified in African patients (Kuipers et al., 2018). There are two arguments that support a pathogenic role of A₁R-G279S^{7,44}. First, in the striatum there is mutual antagonism between signaling pathways controlled by dopamine and adenosine; in the direct and indirect pathways, A₁- and A_{2A}-receptors counteract the actions of D₁- and D₂-receptors, respectively (Ferré et al., 1994, 1997; Yabuuchi et al., 2006). Second, although adenosine-induced stimulation of A₁-receptors has been posited to be a priori neuroprotective, this may not be the case upon prolonged stimulation (Cunha, 2016; Stockwell et al., 2017). In fact, prolonged stimulation of A₁-receptors promotes the accumulation of α -synuclein in dopaminergic

neurons of the substantia nigra and impairs motor control of the animals (Lv et al., 2020). Duplication (Chartier-Harlin et al., 2004) and triplication of the α -synuclein gene (Singleton et al., 2003) results in Parkinson's disease, suggesting that increased expression of the protein per se suffices to trigger its fibrillation and Lewy body formation. In the brain, including the striatum, A₁-receptors are expressed to high levels; nevertheless, they do not have an appreciable constitutive activity (Savinainen et al., 2003). We observed that by comparison with the wild-type A₁-receptor, the mutated variant A₁R-G279S^{7,44} had a measurable constitutive activity. This may translate to a tonic long-term activation of signaling pathways favoring neurodegeneration. Thus, at the very least, the A₁R-G279S^{7,44} may represent a disease-modifying gene, which renders individuals more susceptible to insults that impair the activity of the nigrostriatal dopaminergic neurons.

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Authorship Contributions

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