Functional Impact of the G279S Substitution in the Adenosine A1-Receptor (A1R-G279S7.44), 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-G279S7.44, 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-G279S7.44 was enhanced by about 2 and 8 K when compared with wild-type A1R and A1R-Y288A7.53 (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-G279S7.44 (73 ± 23 kJ/mol) than in wild-type A1R (135 ± 4 kJ/mol) or in A1R-Y288A7.53 (184 ± 24 kJ/mol). Consistent with this lower energy barrier, A1R-G279S7.44 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-G279S7.44 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 or A1R-G279S7.44 than in those coexpressing wild-type A1R. These observations predict enhanced inhibition of dopaminergic signaling by A1R-G279S7.44 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 variant of the A1-receptor (A1R-G279S7.44), 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 an 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 from, which associates with synaptic vesicles (Burré et al., 2018). The α-Synuclein can also form protofibrils composed of β-sheets (Burré et al., 2018). It is not clear what triggers β-sheet formation and fibrillar aggregation of α-synuclein.

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 (Polymeropoulous 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-G279S7.44), was identified in a consanguinous Iranian family: two brothers (out of 10 sequenced members) were homozygous for A1R-G279S7.44, and both developed symptoms of Parkinson’s disease during their third decade. None of the other heterozygous members was affected. Hence, A1R-G279S7.44 was identified 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-G279S7.44 in the development of Parkinson’s disease. However, the mechanistic basis remains enigmatic. In this study, we explored the functional consequence of the G279S7.44 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 Biovest (Nuillé, 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 (SC507) were from Sigma Aldrich and from Santa Cruz Biotechnology (Dallas, TX), respectively. AlexaFluor-488–labeled secondary antibody against murine IgG (A2732) 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]Adenosine (specific activity 40 Ci/mmole), [3H]DPCPX (specific activity 164 Ci/mmole), [3H]chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol (specific activity 83 Ci/mmole), and [3S]GTP7S (specific activity 1385 Ci/mmole) were purchased from PerkinElmer (Waltham, MA).

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Materials and Methods

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and plasmids encoding the human wild-type A1R (5.5 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. Non-specific binding was determined in the presence of 10 μM XAC and represented <1% of total binding in the Kd concentration range. In saturation experiments, the IC50 was estimated by fitting the data to the equation for a monophasic displacement curve. The Kd was calculated using the Cheng-Prusoff approximation (Kd = IC50/1 + [L]/Kd). For binding of [3H]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 [3H]GTPγS (≈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% confluent (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 encoding the human wild-type or mutant A1-receptor (A1-R27978S44, 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 A2-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 monoclonal anti-FLAG antibody (1:2000; Sigma) and the secondary mouse anti-mouse IgG 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 FACS Canto 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 a 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-G279S7.44) 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). Precleared heaved 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 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 × 10^6/well) were seeded into six-well dishes. When the cells were about 80% (1 × 10^6/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 × 10^3/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-YF 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 (5 × 10^4 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-G279S7.44). 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 Bmax was 5.1 ± 1.1 and 5.9 ± 1.3 pmol/mg (means ± S.D.) for wild-type A1R and A1R-G279S7.44, respectively. It is also evident from Figure 1A that mutant and wild-type receptor did not differ in their affinity for the radioligand (KD = 1.4 ± 0.3 and 1.5 ± 0.4 nM for wild-type A1R and A1R-G279S7.44, respectively). Similarly, as exemplified in Figure 1B for the A1-selective agonist CPA, the G279S7.44 mutation did not affect agonist affinity (Ki = 0.4 ± 0.1 and 0.3 ± 0.1 µM for wild-type A1R and A1R-G279S7.44, 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 to 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
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 A<sub>1</sub>-Adenosine Receptor.** The substitution of G279<sup>7.44</sup> 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 A<sub>1</sub>-receptor and the changes caused by the G279S<sup>7.44</sup> mutation. We used the solved structure of the adenosine-bound human A<sub>1</sub>-receptor in complex with Gi<sub>2</sub> 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<sup>7.44</sup> interacts strongly with the backbone carbonyl of F275<sup>7.40</sup> (Fig. 2B). A hydrogen bond is much stronger in a hydrophobic environment, where it can provide binding energies up to $\sim 20-25$ 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 A<sub>1</sub>-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 A<sub>1</sub>-receptor was similar for all systems in complex with Gi<sub>2</sub> (Fig. 2C) and without Gi<sub>2</sub> (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 protein subunit. The G279S\textsuperscript{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 A\textsubscript{1}-R-G279S\textsuperscript{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 protein binding was determined by incubating the membranes with [\textsuperscript{3}H]DPCPX on ice. If the heat-induced denaturation was ranging from 50°C to 63°C. Subsequently, the level of residual protein binding was determined by incubating the membranes with [\textsuperscript{3}H]DPCPX on ice. If the heat-induced denaturation was less evident if the incubation time was increased to 20 minutes (Fig. 3B; T\textsubscript{50} = 53.7°C ± 0.5°C and 54.3°C ± 0.8°C for wild-type A\textsubscript{1}R and A\textsubscript{1}R-G279S\textsuperscript{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 A\textsubscript{1}R (Fig. 3C) and A\textsubscript{1}R-G279S\textsuperscript{7–44} (Fig. 3D). As a control, we used A\textsubscript{1}R-Y288A\textsubscript{7–53} (Fig. 2E). This receptor variant has a folding defect, but it can be rescued by pharmacochaperoning (Málaga-Díéguez et al., 2010; Kusek et al., 2015). Accordingly, HEK293 cells were transiently transfected with a plasmid driving the expression of A\textsubscript{1}R-Y288A\textsubscript{7–53} and incubated with 100 \(\mu\) M 3-isobutyl-1-methylxanthine for 16 hours prior to membrane preparation. This suffices to restore folding and cell surface expression of functionally active A\textsubscript{1}R-Y288A\textsubscript{7–53}, i.e., the pharmacochaperoned A\textsubscript{1}R-Y288A\textsubscript{7–53} binds the radioligand [\textsuperscript{3}H]DPCPX and engages G\textsubscript{i} in a manner comparable to wild-type A\textsubscript{1}R (Málaga-Díéguez 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
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-G297S7.44 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 ± 6 kJ/mol for wild-type A1R and 73 ± 2 kJ/mol for A1R-G297S7.44, respectively. As predicted, A1R-Y288A7.53 was inactivated at lower temperatures than wild-type A1R and A1R-G297S7.44 (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-G297S7.44 (cf. squares and circles in Fig. 4A). Thus, the G297S7.44 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 (Pf/Pu) 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-G297S7.44 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-Y288A7.53 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-G297S7.44 has an enhanced thermodynamic stability but a reduced kinetic stability, whereas the reverse is true for A1R-Y288A7.53.

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-G297S7.44, 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 (Bmax = 1.2 ± 0.2 and 1.4 ± 0.2 pmol/mg in the presence of wild-type A1R and A1R-G297S7.44, 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...
via its N-terminal HA tag: in the immunoprecipitated, equivalent levels of wild-type A1R and A1R-G279S7.44 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 communoprecipitation in the absence of receptor activation (Fig. 5B).

**Comparison of G:\ 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-G279S7.44. 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 L2697.34 and A843.29 and at the site of the mutation site, i.e., the distance between T913.36 and H2787.43 (Fig. 7A). These measurements captured the changes in stability, dynamics, and conformations induced by the G279S7.44 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 G2 (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 apo state of the receptor (cf. corresponding second and fourth two-dimensional histograms). The structural change is visible as a shift in the T913.36 – H2787.43 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 G2 (cf. first and third two-dimensional histograms 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). 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 G2 (cf. first and third two-dimensional histograms 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). 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 G2 (cf. first and third two-dimensional histograms 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).
forms on the intracellular side within the transmembrane bundle. A comparison between the active Gα-protein–bound conformation and the receptor without the G protein shows large movements of TM5 to create the space that allows for Gα protein binding (cf. Fig. 8, A and B). We also examined the effect of G279S mutation on the intracellular face of the receptor by measuring distances between I482–43 and V2035.61 (TM2–TM5) and between I482–43 and L2326.33 (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 mutation (brown trace in Fig. 8, C and D) leads to an opening of the binding site for the Gα 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 Gα 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 Gα 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 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-G279S7.44 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]GTPγS (Fig. 9A) and 2) the effect of an inverse agonist on the basal binding of [35S]GTPγS (Fig. 9B) under initial rate conditions (i.e., after 1 minute): when stimulated with the agonist CPA at saturating concentrations, the mutant A1R-G279S7.44 caused a larger increase in [35S]GTPγS 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-G279S⁴⁴. HEK293 cells (1 × 10⁶/well) were transiently cotransfected with a constant amount of plasmid encoding D₁R [1 and 0.2 μg/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 μg/well (A–D) or increasing amounts [0–1.8 μg/well; (E–H)] of plasmid coding for the wild-type (wt A₁R) or the mutant A₁-receptor (A₁R-G279S⁴⁴), 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 μg/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 × 10⁴/well) and allowed to adhere for 15 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, 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 ± S.D. from three independent experiments done in parallel and carried out in duplicate. AUC, area under the curve; Fl, fluorescence; mBU, milli BRET unit.
wild-type A1-receptor (Fig. 9A). In contrast, the concentration required for half-maximum stimulation did not differ (EC50 = 38 ± 6 and 58 ± 19 nM for wild-type A1R and A1R-G279S7.44, respectively). On average, the basal rate of [35S]GTPγS binding was slightly higher in membranes prepared from HEK293 cells transiently expressing A1R-G279S7.44 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

### Table 1

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<th>Incubation</th>
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<th>Maximum BRET signal</th>
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Fig. 7. The mutation G279S7.44 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 Cα atoms (CA) of T913.36 and H2787.43 (x-axes) and of A843.29 and L2697.34 (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 C2. 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 Gαs, CA,
the extent to which a saturating concentration of the antagonist/inverse agonist DPCPX reduced basal $[^{35}S]$GTP$\gamma$S binding in membranes from HEK293 cells transiently expressing wild-type A$_1$R and A$_1$R-G279S$^{7,44}$. It is evident from Figure 8B that DPCPX caused a statistically significant inhibition of basal $[^{35}S]$GTP$\gamma$S binding to membranes harboring A$_1$R-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 $[^{35}S]$GTP$\gamma$S binding to membranes harboring wild-type A$_1$R (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 A$_1$R-G279S$^{7,44}$ is more pronounced than that of the wild-type receptor.

Taken together the data in summarized in Figure 9 suggested that A$_1$R-G279S$^{7,44}$ was more effective in promoting nucleotide exchange on cognate G proteins than the wild-type receptor. The A$_1$-receptor is a prototypical G$_i$/G$_o$-coupled receptor, which engages all isoforms of G$_{xi}$ and G$_{xo}$ (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 D$_1$- and A$_1$-receptors (Ferré et al., 1998). We therefore explored if wild-type A$_1$R and A$_1$R-G279S$^{7,44}$ differed in their ability to inhibit cAMP accumulation induced by the D$_1$-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 $[^{35}S]$GTP$\gamma$S binding (Fig. 8B) translated into modulation of cAMP production. This was the case: in HEK293 cells coexpressing the D$_1$-receptor and the mutant A$_1$R-G279S$^{7,44}$, DPCPX produced a consistent and statistically significantly increased cAMP response to dopamine (by 1.4 ± 0.2-fold; Fig. 10A). In contrast, in HEK293 cells coexpressing the D$_1$-receptor and

**Fig. 8.** Geometry of the G$_\alpha$ protein binding cavity of the wild-type adenosine A$_1$-receptor and of the mutant A$_1$R-G279S$^{7,44}$. Snapshots taken at the end of the simulations shows the wild-type A$_1$-receptor from the cytoplasmic side with (A) and without (B) bound G$_{\alpha}$. The A$_1$-receptor is represented as white cartoon and TM7 is highlighted in yellow, the bound C-terminal helix of the G$_{\alpha}$ protein is shown in transparent red color. Residues 148$^{2,43}$, I232$^{2,33}$ 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$_{\alpha}$ atoms (CA) of I482.43 (TM2) and V203$^{5,61}$ (TM5) (C and E) and of I482.43 (TM2) and I232$^{2,33}$ (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 A$_1$-receptor was simulated using the active form based of the agonist-ligated, 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 A$_1$R-G279S$^{7,44}$ (G279S) were simulated in the absence (apo) and presence of adenosine.

A Mutated A$_1$-Receptor Associated with Parkinson's Disease 261
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-G279S7.44, the concentration-response curve for CPA was shifted to the left of that seen in cells expressing wild-type A1R (Fig. 10B; IC50 = 2.4 ± 0.5 and 0.9 ± 0.4 nM for wild-type A1-receptor and the mutant A1R-G279S7.44, 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-G279S7.44 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 their genes (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 G279S7.44 mutation in the human A1-receptor is the report by Jaberi 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-G279S7.44 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-G279S7.44 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-G279S7.44 than for the wild-type receptor, regardless of whether guanine nucleotide exchange or cAMP accumulation was assessed. 3) The agonist-ligated A1R-G279S7.44 was more efficacious than the wild-type receptor in catalyzing guanine nucleotide exchange. Accordingly, in cells expressing A1R-G279S7.44, the agonist concentration-response curve for

**Fig. 9.** [35S]GTPγS 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 μg/15-cm dish) or the mutant (A1R-G279S7.44, full circles, 5.5 μg/15-cm dish) adenosine A1-receptor and membranes were prepared as outlined in the legend to Figure 1A. (A) Membranes (10 μg) 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 [35S]GTPγS to a final concentration of 1 nM and stopped after 1 minute by rapid filtration as outlined under Materials and Methods. Data are means ± 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 [35S]GTPγS binding (95% confidence interval 91.5–101.3 and 111.5–128.1 fmol/mg for wt A1R and A1R-G279S7.44, respectively). (B) The assay was done as described for (A) in the absence (basal) and presence of the antagonist/inverse agonist DPCPX (10 μM). 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-G279S7.44 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-G279S7.44, the difference between basal [35S]GTPγS binding and binding in the presence of DPCPX was statistically significant (P < 0.02, Friedman test followed by Holm-Sidak post hoc testing).
TM7 and the C terminus must be correctly positioned to allow for docking of ligands during the conformational search. CPA bound with similar affinity to the wild-type A1-receptor, but our observations indicate that the G279S mutation does not affect this interface: wild-type and mutant A1-receptors did not differ in their ability to form complexes with the A2A-receptor (Cheng et al., 2017; Glukhova et al., 2017). The interface in the A1-/D1-receptor heteromer is not known, and to our knowledge, it has not been characterized.

The A1-receptor can form homodimers (Gracia et al., 2013) and heteromers 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 $N \rightarrow U \rightarrow D$, 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 $\Delta G$ (or $\Delta H$) 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 (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 was enhanced; this increase can be

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**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 μg/15-cm dish), and wild-type A1-receptor (wt A1R; 5.5 μg/15-cm dish, triangle down) or the mutant A1R-G279S (5.5 μg/15-cm dish, full circle). After 8 hours, cells were replated into 6-well dishes (0.5 × 10⁶/well) in DMEM medium containing 1 μCi/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 concentrations 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 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 ± S.D. from four independent experiments and the spaghetti plot in the inset shows the IC₅₀ values for wt A1R and A1R-G279S (paired experiments are indicated by the same symbols). IC₅₀ 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; $F = 13.57$, $P = 0.0001$).
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 (Walldhofer 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 (PTHRD1) (Elahi, 2018). PTHRHD1 is a protein of unknown function, which lacks its eponymous activity: although it binds peptidy-l-t-RNA, it does not hydrolyze it (Burks et al., 2016). The role of PTHRHD1 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 PTHRHD1, 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 A2A-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 \(\alpha\)-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 \(\alpha\)-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 at 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 toxic 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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