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Signaling-Biased and Constitutively Active Dopamine D2 Receptor Variant

Dayana Rodriguez-Contreras, Alec F. Condon, David C. Buck, Naem Asad, Timothy M. Dore, Dineke S. Verbeek, Marina A. J. Tijssen, Ujwal Shinde, John T. Williams, and Kim A. Neve*

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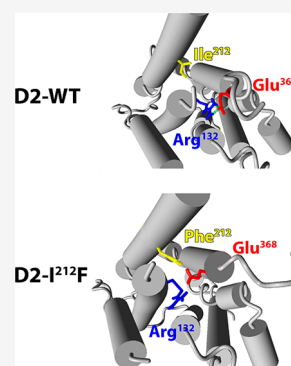
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ABSTRACT: A dopamine D2 receptor mutation was recently identified in a family with a novel hyperkinetic movement disorder. Compared to the wild type D2 receptor, the novel allelic variant D2-I²¹²F activates a $G\alpha_{i1}\beta_1\gamma_2$ heterotrimer with higher potency and modestly enhanced basal activity in human embryonic kidney (HEK) 293 cells and has decreased capacity to recruit arrestin3. We now report that omitting overexpressed G protein-coupled receptor kinase-2 (GRK2) decreased the potency and efficacy of quinpirole for arrestin recruitment. The relative efficacy of quinpirole for arrestin recruitment to D2-I²¹²F compared to D2-WT was considerably lower without overexpressed GRK2 than with added GRK2. D2-I²¹²F exhibited higher basal activation of $G\alpha_{oA}$ than $G\alpha_{i1}$ but little or no increase in the potency of quinpirole relative to D2-WT. Other signs of D2-I²¹²F constitutive activity for G protein-mediated signaling, in addition to basal activation of $G\alpha_{i/o}$, were enhanced basal inhibition of forskolin-stimulated cyclic AMP accumulation that was reversed by the inverse agonists sulpiride and spiperone and a ~4-fold increase in the apparent affinity of D2-I²¹²F for quinpirole, determined from competition binding assays. In mouse midbrain slices, inhibition of tonic current by the inverse agonist sulpiride in dopamine neurons expressing D2-I²¹²F was consistent with our hypothesis of enhanced constitutive activity and sensitivity to dopamine relative to D2-WT. Molecular dynamics simulations with D2 receptor models suggested that an ionic lock between the cytoplasmic ends of the third and sixth α -helices that constrains many G protein-coupled receptors in an inactive conformation spontaneously breaks in D2-I²¹²F. Overall, these results confirm that D2-I²¹²F is a constitutively active and signaling-biased D2 receptor mutant and also suggest that the effect of the likely pathogenic variant in a given brain region will depend on the nature of G protein and GRK expression.

KEYWORDS: Dopamine, D2 receptor, allelic variant, constitutive activity, biased signaling, G protein, arrestin



INTRODUCTION

The dopamine D2 receptor is a G protein-coupled receptor that signals through both $G\alpha_{i/o}$ and arrestin to regulate movement and motivated behavior.^{1–3} The D2 receptor is a target of virtually all antipsychotic drugs currently in use and also a frequent drug target in the treatment of movement disorders such as Parkinson's disease and chorea.^{4,5} The D2 receptor has long (D2_L) and short (D2_S) splice variants; if and in what way the splice variants are functionally distinct is an active area of research.^{6,7}

We recently described a four-generation family with an autosomal dominant genetic disorder characterized by chorea and cervical dystonia, in which affected family members carry the novel D2 receptor missense variant DRD2 (c.634A > T;p.I212F).⁸ Ile²¹² (Ile212^{5,61} according to the Ballesteros-Weinstein numbering scheme)⁹ is in the cytoplasmic extension of the fifth transmembrane α -helix, at the N-terminus of the D2 receptor third cytoplasmic loop. A deep mutational analysis of the β_2 -adrenoceptor identified position 5.61 as being one of the top four mutationally intolerant positions at the β_2 - $G\alpha_s$ interface and also in a part of the receptor where many mutations are activating or inactivating.¹⁰ Mutations intro-

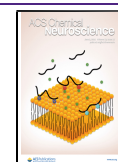
duced in this region of the D2 receptor decrease the binding of at least three D2 receptor-interacting proteins: arrestin,^{11,12} calmodulin,¹³ and S100B.^{14,15} Our initial studies demonstrated that recruitment of arrestin by D2_{L/S}-I²¹²F in human embryonic kidney (HEK) 293 cells is decreased compared to wild type D2_{L/S} (D2_{L/S}-WT), whereas D2_{L/S}-I²¹²F activation of a $G\alpha_{i1}\beta_1\gamma_2$ heterotrimer and inhibition of cAMP accumulation are enhanced.⁸

G-protein-coupled receptor (GPCR) kinases (GRKs) facilitate arrestin recruitment by phosphorylating serine and threonine residues on the intracellular domains of GPCRs, typically leading to receptor desensitization, internalization, and either degradation or resensitization, and also promoting arrestin-mediated signaling.¹⁶ GRK is frequently cotransfected in cellular studies of arrestin recruitment to maximize the

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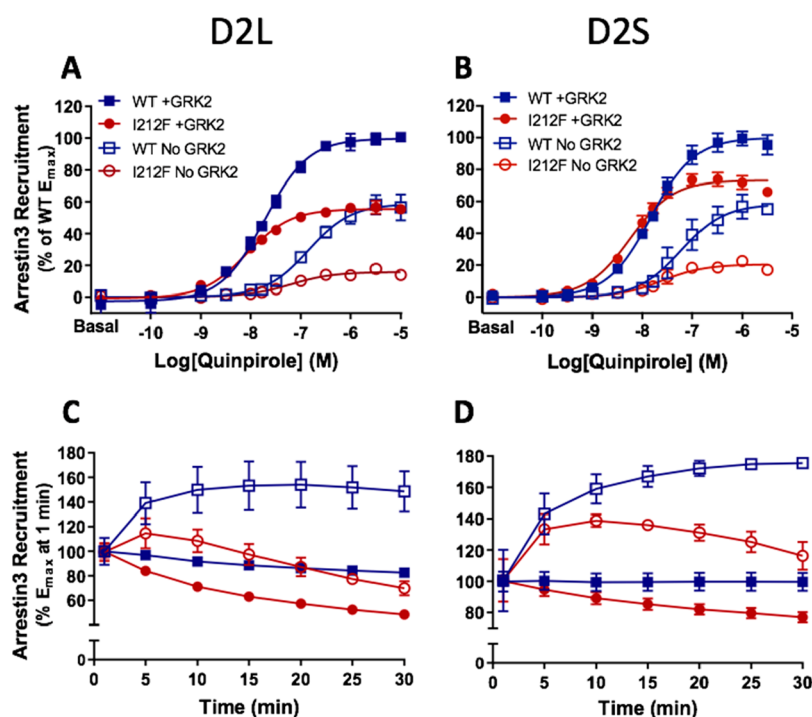


Figure 1. Dose–response curves for quinpirole-induced arrestin3 recruitment mediated by D_{2L/S}-WT and D_{2L/S}-I²¹²F. Arrestin3 recruitment was measured in HEK293 cells cotransfected with GRK2 (+ GRK2) or nonspecific plasmid DNA (No GRK2). Values plotted are the means \pm SD of three to four independent experiments performed in quadruplicate. (A, B) Quinpirole concentration–response curves measured at 10 min. Data from each independent experiment were normalized by subtracting the baseline and expressed as a percentage of maximum arrestin3 recruitment by D2-WT+GRK2. Data for +GRK2 are from the data set described in van der Weijden et al.,⁸ where results were shown after 20 min of agonist stimulation. (C, D) Change in E_{max} values over 30 min, with each condition normalized to E_{max} for that condition at 1 min. Data for +GRK2 were previously described in van der Weijden et al.⁸

Table 1. Arrestin Recruitment: Requirement for Overexpressed GRK2^a

receptor	arrestin recruitment			
	+GRK2 ^b		no GRK2	
	–log EC ₅₀	E_{max} (% of WT+GRK2)	–log EC ₅₀	E_{max} (% of WT+GRK2)
D _{2L} -WT	7.69 \pm 0.05	100 \pm 2	6.85 \pm 0.05 ^{†††}	59 \pm 4 ^{†††}
D _{2L} -I ²¹² F	8.06 \pm 0.03 ^{**}	56 \pm 1 ^{***} (–44%)	7.22 \pm 0.08 ^{**†††}	16 \pm 1 ^{***†††} (–73%)
D _{2S} -WT	7.83 \pm 0.01	100 \pm 3	7.20 \pm 0.13 ^{†††}	59 \pm 2 ^{†††}
D _{2S} -I ²¹² F	8.24 \pm 0.04 ^{**}	73 \pm 2 ^{***} (–27%)	7.59 \pm 0.09 ^{**†††}	21 \pm 0.4 ^{***†††} (–66%)

^aQuinpirole potency is shown as $-\log EC_{50}$. E_{max} was calculated by subtracting basal response from maximal response at 10 min after adding the substrate coelenterazine *h* and is shown as the percentage of D2-WT with added GRK2. For D2-I²¹²F, the percent reduction compared to the corresponding D2-WT E_{max} is included in parentheses. *N* = three to four independent experiments for each condition. Data are presented as mean \pm SEM. ^bFrom the data set described in van der Weijden et al.,⁸ except after 10 min instead of 20 min of agonist stimulation. Statistical differences were calculated by 2-way ANOVA followed by Turkey's posthoc test (^{*}*p* < 0.05, ^{**}*p* < 0.01, ^{***}*p* < 0.001 compared to D2-WT; ^{†††}*p* < 0.001 compared to the corresponding +GRK2 condition).

signal. GRK2/3 are ubiquitously expressed GRKs that are the major subtypes interacting with the D2 receptor.^{17–19} Thus, our previous arrestin recruitment studies were performed with overexpressed GRK2.

Similarly, we assessed D2 receptor activation of a G protein heterotrimer containing $G\alpha_{i1}$ even though the D2 receptor activates both $G\alpha_i$ and $G\alpha_o$.²⁰ $G\alpha_o$ is the most abundant $G\alpha$ subunit in mammalian brain, comprising about 1% of total membrane protein.²¹ Furthermore, $G\alpha_o$ knockout mice have greatly decreased dopamine-stimulated GTP γ S binding and a complete loss of GTP-sensitive dopamine binding in brain, suggesting that $G\alpha_o$ contributes importantly to dopamine signaling.²² Both $G\alpha_i$ and $G\alpha_o$ mediate D2 receptor signaling in brain, with the contribution of specific subtypes varying among brain regions.²³

We now report that D_{2L/S}-I²¹²F receptors have a more stringent requirement than D2-WT for GRKs, so that the novel allelic variant had a more profound loss of arrestin recruitment, compared to that of D2-WT, in the absence of overexpressed GRK2 than when the kinase was overexpressed. We also describe the effect of the mutation on D2 receptor activation of $G\alpha_{oA}$, which differed from $G\alpha_{i1}$ activation in effects on both agonist potency and basal activity. Furthermore, the mutation increased constitutive inhibition of cyclic AMP accumulation in HEK293 cells and increased the apparent affinity of quinpirole for the D2 receptor. In midbrain dopamine neurons expressing D2-I²¹²F, photoactivated sulpiride inhibited a substantial tonic current, consistent with both the constitutive activity and enhanced agonist potency suggested by studies of the novel variant in HEK293 cells. Molecular dynamics (MD)

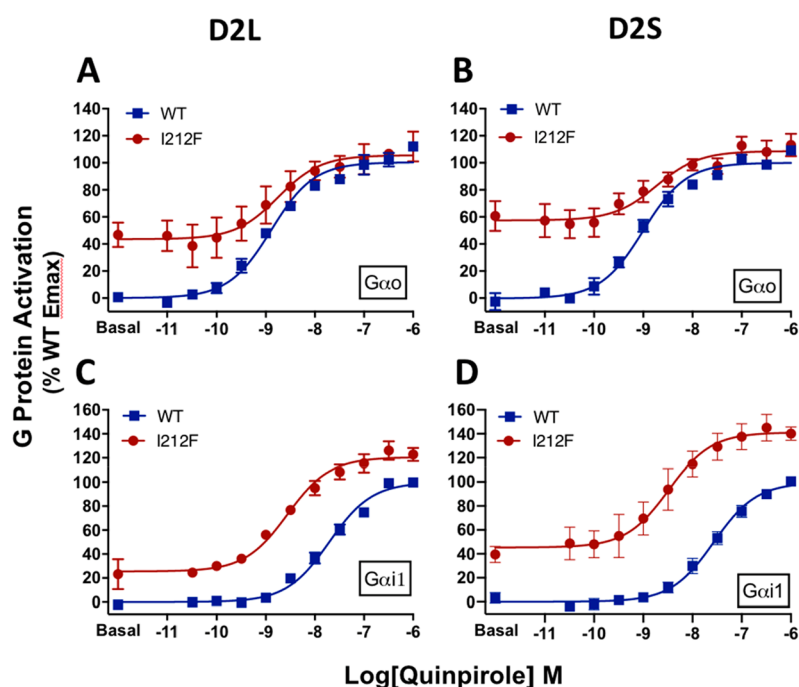


Figure 2. Concentration–response curves for $G\alpha_{i/o}$ protein activation mediated by D2-WT and D2-I²¹²F in response to stimulation with quinpirole. Results are expressed as the percentage of maximum G protein activation by D2-WT, measured 10 min after adding coelenterazine *h*. (A) Activation of $G\alpha_{oA}$ by D2_L-WT/I²¹²F. (B) Activation of $G\alpha_{oA}$ by D2_S-WT/I²¹²F. (C) Activation of $G\alpha_{i1}$ by D2_L-WT/I²¹²F. (D) Data from van der Weijden et al.⁸ for activation of $G\alpha_{i1}$ by D2_S-WT/I²¹²F. Values plotted represent means \pm SD of three (panel C) or four (panels A, B, D) independent experiments performed in quadruplicate.

simulations indicate that these effects of the mutation are associated with the breaking of an “ionic lock” that constrains many unliganded GPCRs in an inactive conformation.

RESULTS AND DISCUSSION

Arrestin3 Recruitment by D2-I²¹²F Depends Heavily on GRK2. We previously investigated the ability of D2_{S/L}-I²¹²F receptors to recruit arrestin3 under the most favorable conditions by overexpressing GRK2, which enhances arrestin recruitment to the D2 receptor.¹⁷ We reported that recruitment of arrestin3 by D2_{S/L}-I²¹²F is reduced by about 30–50% compared to that of D2_{S/L}-WT receptors, whereas the potency of quinpirole is modestly enhanced at D2_{S/L}-I²¹²F.⁸ We now describe arrestin3 recruitment in the absence of GRK2. HEK293 cells were transiently cotransfected with D2_{S/L}-WT or D2_{S/L}-I²¹²F fused with RLuc8 (BRET donor) and mVenus-tagged arrestin3 (BRET acceptor). Previous results with overexpressed GRK2 are shown for comparison. Quinpirole-induced recruitment of arrestin3 by both D2 receptor splice variants was substantially decreased for D2_{L/S}-I²¹²F with or without overexpressed GRK2 (Figure 1A,B; Table 1), whereas the potency of quinpirole was modestly increased for all conditions compared to D2-WT (Table 1). The mutation-induced reduction in maximal recruitment of arrestin was considerably larger in the absence of expressed GRK2 for both splice variants. Thus, with added GRK2, E_{max} for D2_{L/S}-I²¹²F was decreased by 44% (D2_L) or 27% (D2_S), but in the absence of overexpressed GRK2, E_{max} for D2_{L/S}-I²¹²F was decreased by 73% (D2_L) and 64% (D2_S) compared to the corresponding condition for D2-WT (Table 1). In contrast, omitting GRK2 decreased the potency similarly for all variants, ranging from a 4.2-fold decrease for D2_S-WT to a 6.7-fold decrease for D2_L-I²¹²F (Table 1).

In the presence of overexpressed GRK2, maximal arrestin recruitment peaked by the first measurement, (1 min after adding coelenterazine *h*, which was approximately 4 min after addition of quinpirole), whereas maximal recruitment was delayed without overexpressed GRK2, particularly for D2_{S/L}-WT (Figure 1C,D). E_{max} decreased more rapidly for D2_{L/S}-I²¹²F than for D2_{L/S}-WT and more rapidly without overexpressed GRK2 than with GRK2 transfection. Thus, a significant interaction among the three factors of time, GRK2 condition, and genotype was determined by 3-way RM ANOVA (D2_L: F (6, 60) = 17.77, $p < 0.0001$; D2_S: F (6, 60) = 6.689, $p < 0.0001$). This was followed by 2-way RM ANOVAs to assess the interaction between genotype and time (D2_L + GRK: F (6, 24) = 69.15, $p < 0.0001$; D2_L No GRK: F (6, 36) = 81.08, $p < 0.0001$; D2_S + GRK: F (6, 36) = 118.9, $p < 0.0001$; D2_S No GRK: F (6, 24) = 12.49, $p < 0.0001$) and between GRK treatment and time (D2_L-WT: F (6, 30) = 103.7, $p < 0.0001$; D2_S-WT: F (6, 30) = 71.26, $p < 0.0001$; D2_L-I²¹²F: F (6, 30) = 16.62, $p < 0.0001$; D2_S-I²¹²F: F (6, 30) = 19.16, $p < 0.0001$). Consistent with our previous report,⁸ expression of D2_{L/S}-I²¹²F was only 35–40% of D2_{L/S}-WT (Table S1).

Decreased Arrestin3 Recruitment by D2-I²¹²F Was Not Due to Lower Receptor Expression. Based on unpublished data mentioned previously,⁸ we considered it unlikely that the reduced arrestin recruitment by D2_{L/S}-I²¹²F was simply due to lower receptor expression. Furthermore, the mutation-induced instability of the interaction with arrestin (Figure 1C,D) and the greater dependence of arrestin recruitment to D2_{L/S}-I²¹²F on GRK2 are difficult to explain as simply due to lower receptor number. Nevertheless, to confirm that reduced arrestin recruitment by D2-I²¹²F was not a consequence of lower receptor expression, we repeated these

Table 2. $G\alpha$ Protein Activation in HEK293 Cells^a

receptor	-log EC ₅₀		basal activity (% of WT max)	
	Gα _{i1}	Gα _{oA}	Gα _{i1}	Gα _{oA}
D2 _L -WT	7.7 ± 0.05	8.9 ± 0.03	0 ± 0.01	0 ± 0.02
D2 _L -I ²¹² F	8.6 ± 0.04***	8.8 ± 0.09	25 ± 3**	43 ± 6***
D2 _S -WT	7.6 ± 0.05 ^b	9.0 ± 0.02	0 ± 0.01 ^b	0 ± 0.02
D2 _S -I ²¹² F	8.4 ± 0.02 ^{b***}	8.7 ± 0.05**	35 ± 6 ^{b***}	57 ± 5***

^aQuinpirole potency is shown as -log EC₅₀. Basal activity for D2_{L/S}-I²¹²F is expressed as a percentage of the respective D2-WT maximal response. Data are presented as mean ± SEM of three (Gα_{i1}-D2_L) or four (Gα_o-D2_{L/S}) independent experiments performed in quadruplicate. ^bData are from van der Weijden et al.⁸ Statistical differences were calculated by 2-way ANOVA followed by Turkey's posthoc test (***p* < 0.01, ****p* < 0.001 compared to D2-WT).

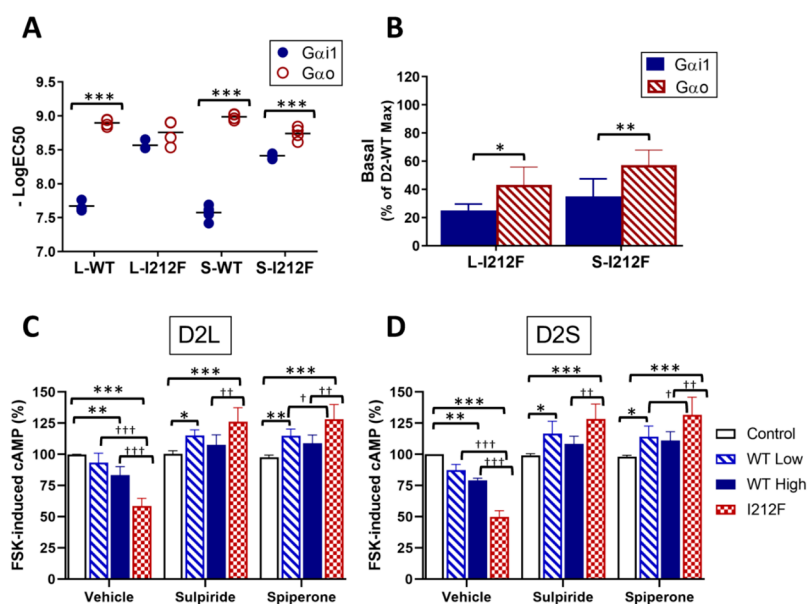


Figure 3. Increased constitutive activity of D2-I²¹²F. Concentration–response curves for Gα_{i/o} protein activation mediated by D2-WT and D2-I²¹²F were analyzed by nonlinear regression to determine quinpirole potency. (A) This was expressed as the -log EC₅₀, and (B) activation in the absence of quinpirole was expressed as the percentage of E_{max} for D2-WT. Data are from Table 2. Statistical differences were determined as described in Table 2 (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). (C, D) Cyclic AMP accumulation was measured in the presence of 10 μM forskolin (FSK) in HEK293 cells transfected with the cyclic AMP biosensor CAMYEL and either control plasmid DNA (control), a high (0.5 μg, WT High) or low (0.2 μg, WT Low) amount of D2_{L/S}-WT DNA, or D2_{L/S}-I²¹²F plasmid DNA (0.5 μg, I212F). Measurements were taken 10 min after addition of either vehicle, sulpiride (10 μM) or spiperone (1 μM), FSK (10 μM), and coelenterazine *h*. Results are expressed as a percentage of cyclic AMP accumulation by control cells treated with the inverse agonist vehicle. Values plotted are mean ± SD of four independent experiments performed in sextuplicate. Statistical differences were determined by 2-way ANOVA followed by Turkey's posthoc test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared to the corresponding control condition; †*p* < 0.05, ††*p* < 0.01, †††*p* < 0.001 compared to D2-WT).

experiments with D2_L under conditions where the wild type and mutant variants were expressed at similar levels and observed a similar mutation-induced decrease in E_{max} (Figure S1). At this lower level of D2 receptor expression, GRK2 overexpression had no effect on E_{max} for D2_L-WT but continued to regulate quinpirole potency at D2_L-WT.

Contribution of Endogenous GRK to Arrestin3 Recruitment. To determine if endogenous GRK2/3 contributes significantly to arrestin recruitment in this assay, we repeated the experiments above with D2_L-WT and D2_L-I²¹²F, adding a condition in which cells were pretreated with the GRK2/3 inhibitor Compound 101 (Cmpd101). In the absence of Cmpd101 (Table S2), results were indistinguishable from those presented above and replicate prior results for D2_L-WT and D2_L-I²¹²F with overexpressed GRK2 (Table 1). Inhibiting endogenous GRK2/3 significantly decreased the maximal response for both allelic variants with (Figure S2A,C) or without (Figure S2B,C) overexpressed GRK2. Nevertheless, maximal arrestin recruitment by D2_L-I²¹²F was always less than

the corresponding condition for D2_L-WT (Table S2). In contrast, quinpirole potency was decreased by either omitting overexpressed GRK2 or adding Cmpd101, but there was no detectable additivity (Figure S2D; Table S2).

GRK2 has both phosphorylation-dependent and phosphorylation-independent effects on D2 receptor function.^{19,24} Inhibition of arrestin recruitment by the active-site inhibitor Cmpd101²⁵ may suggest that at least some of the observed effects of GRK2 require D2 receptor phosphorylation, although it is notable that translocation of GRK2 to the μ-opioid receptor can be inhibited by Cmpd101.^{26,27} Observed effects of overexpressed GRK2 despite the presence of Cmpd101 may reflect phosphorylation-independent processes.

D2-I²¹²F Receptor Expression Increased Basal Gα_{oA} Protein Activation. We assessed G protein activation using a Gα_o energy donor (Gα_{oA}-91-RLuc8), a Gβ₁/Gγ₂ acceptor (mVenus-Gβ₁γ₂), and D2_{S/L}-WT or D2_{S/L}-I²¹²F transiently expressed in HEK293 cells. Quinpirole produced a concentration-dependent increase in Gα_{oA} protein activation for both

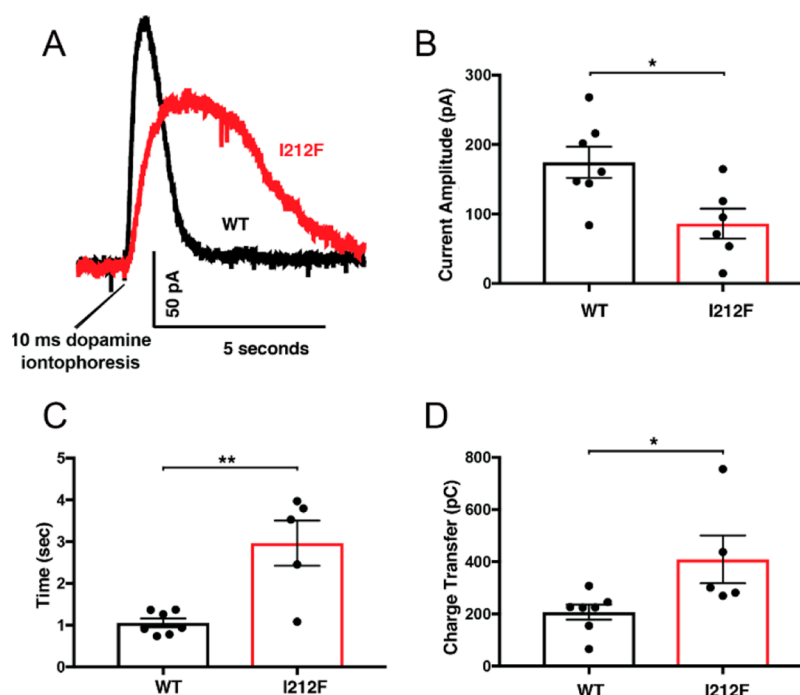


Figure 4. Activation of GIRK currents by dopamine iontophoresis was assessed in mouse midbrain slices. AAV-DIO-D2_S-WT or -D2_S-I²¹²F was used to restore D2 receptor expression in dopamine neurons of auto-D2-KO mice. (A) Representative outward currents in response to iontophoresis of dopamine (1 M) for 10 ms. Mean \pm SEM is shown for (B) current amplitude, (C) peak half-width, and (D) charge transfer. The number of cells differs among panels for D2-I²¹²F, because kinetics in the lowest amplitude response in panel B could not be accurately resolved. Student's *t*-test: **p* < 0.05, ***p* < 0.01.

D2_{S/L}-I²¹²F and D2_{S/L}-WT receptors (Figure 2A,B). For D2_L, no significant difference in the potency of quinpirole at D2_L-WT and D2_L-I²¹²F receptors was observed, whereas quinpirole was slightly but significantly more potent at D2_S-WT (1 nM) than at D2_S-I²¹²F (2 nM; Table 2). On the other hand, basal G α_{oA} activation by D2_L-I²¹²F (43% of maximal stimulation) or D2_S-I²¹²F (57%) was markedly higher than for D2_{S/L}-WT (set as 0%; Figure 2A,B; Table 2). Enhanced basal activity was observed despite lower expression of D2_{S/L}-I²¹²F than D2_{S/L}-WT (Table S1).

Because the effect of the I²¹²F mutation on G α_{oA} protein activation differed in several respects from what we observed previously using G α_{i1} ,⁸ we repeated those experiments with D2_L-WT and D2_L-I²¹²F (Figure 2C; previously published results for D2_S shown in Figure 2D for comparison). Whereas quinpirole potency for activating G α_{oA} was not substantially changed by the I²¹²F mutation, we confirmed our previous observation that the potency of D2_L-I²¹²F for activating G α_{i1} (3 nM) is markedly increased compared to that of D2_L-WT (21 nM; Figures 2C and 3A). Basal activity of G α_{i1} was enhanced by 25% of WT E_{max} in cells expressing D2_L-I²¹²F (Figure 2C). This enhanced basal activity associated with D2-I²¹²F expression was significantly lower than that observed for G α_{oA} for both splice variants (Figure 3B).

Quinpirole was considerably more potent at G α_{oA} than at G α_{i1} for D2-WT, consistent with prior work.²⁸ The G protein subtype-specific effect of the mutation on agonist potency is particularly interesting in light of recent findings that G α_o mediates a relatively high-affinity response to dopamine in the mouse nucleus accumbens that is eliminated by repeated treatment with cocaine.²³ This is in contrast to a lower-affinity, cocaine treatment-insensitive response in the dorsal striatum that is mediated by G α_i . In our results, the mutation-induced

shift in potency of quinpirole at G α_{i1} eliminated the difference between the G protein subtypes (Figure 3A; Table 2). Thus, mice expressing D2-I²¹²F might display higher sensitivity responses to dopamine in both nucleus accumbens and dorsal striatum, responses that would perhaps be unaffected by repeated cocaine treatment.

Constitutive Inhibition of Cyclic AMP Accumulation.

Increased basal activation of G proteins by D2-I²¹²F could be indicative of a higher constitutive activity than that of D2-WT. To test this hypothesis, we measured the ability of D2 receptors to inhibit forskolin-stimulated cyclic AMP accumulation in the absence of agonist. HEK293 cells were transiently cotransfected with the BRET-based cyclic AMP sensor, CAMYEL²⁹ and D2_{L/S}-WT or D2_{L/S}-I²¹²F. Compared to control cells, cells transfected with a higher amount of D2_{L/S}-WT plasmid DNA (0.5 μ g) or D2_{L/S}-I²¹²F showed reductions in cyclic AMP accumulation that were greater for D2-I²¹²F (41–50%) than for D2-WT (17–21%) (Figure 3C,D; D2_L = 83 \pm 7% of control for WT High vs 59 \pm 6% for D2_L-I²¹²F; D2_S = 79 \pm 2% of control for WT High vs 50 \pm 5% for D2_S-I²¹²F). Preincubation with either of the inverse agonists sulpiride and spiperone not only reversed the constitutive inhibition of cyclic AMP accumulation but also yielded significantly enhanced cyclic AMP levels that were highest for D2_{L/S}-I²¹²F (Figure 3C,D; D2_L-I²¹²F = 126 \pm 11% of control for sulpiride and 128 \pm 12% of control for spiperone; D2_S-I²¹²F = 128 \pm 12% for sulpiride and 132 \pm 14% for spiperone). We hypothesize that increased FSK-stimulated cyclic AMP accumulation in the presence of inverse agonists reflects heterologous sensitization of adenylyl cyclase resulting from prolonged constitutive activation of G $\alpha_{i/o}$ by D2-I²¹²F.^{30,31}

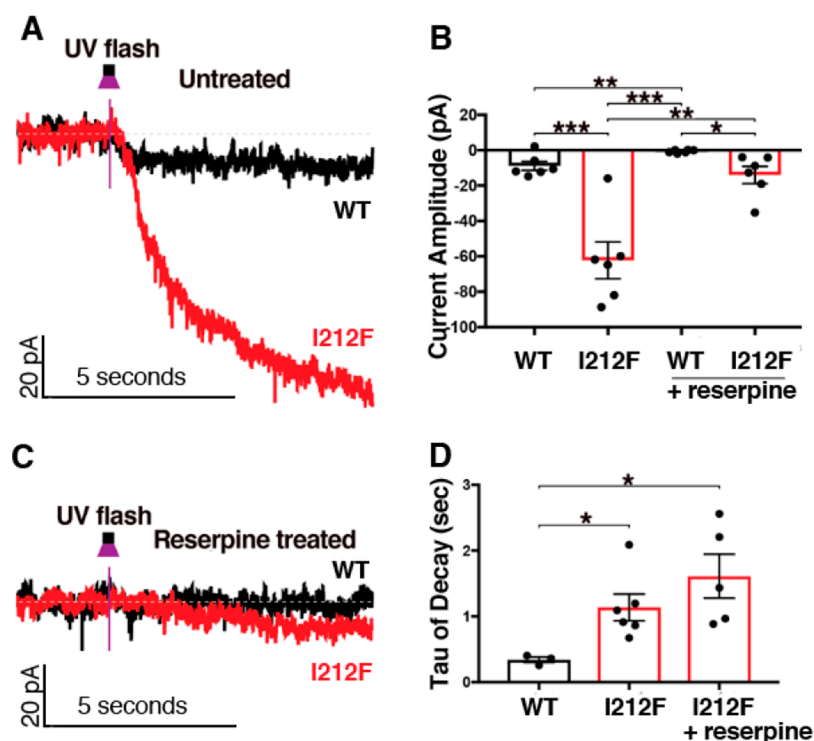


Figure 5. AAV-DIO-D2_S-WT or -D2_S-I²¹²F was used to restore D2 expression in dopamine neurons of auto-D2-KO mice. CyHQ-sulpiride (5 μM) was circulated over a midbrain slice, and photolysis was induced by a 50 ms flash (365 nm) from a 6.5 mW LED light. The left panels depict representative traces for untreated slices (A) and slices incubated with reserpine to deplete endogenous dopamine (C). The right panels depict the mean ± SEM of the decreased current amplitude (B) and the rate of decay of the current after photorelease of sulpiride (D) in control slices and in slices pretreated with reserpine. It was not possible to calculate a decay rate for reserpine-treated slices from mice expressing D2-WT. For some conditions, the number of cells differs between panels B and D, because kinetics could not be accurately resolved in the lowest amplitude responses in panel B. Student's *t* test: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Enhanced Affinity of D2-I²¹²F for Quinpirole. Receptor constitutive activity is commonly reflected in increased affinity for agonists.^{32–34} We carried out competition binding assays to compare the apparent affinity of D2-WT and D2-I²¹²F for quinpirole in membranes prepared from HEK293 cells stably expressing the receptors (Figure S3). In four experiments, the geometric mean for quinpirole K_i decreased from 1.5 to 0.4 μM (D2_L-WT and D2_L-I²¹²F, respectively; *p* = 0.0027) and from 1.9 to 0.5 μM (D2_S-WT and D2_S-I²¹²F, respectively; *p* = 0.0005). Thus, the affinity of D2_{L/S}-I²¹²F for quinpirole was increased roughly 4-fold compared to that of D2_{L/S}-WT.

Altered D2-I²¹²F Receptor-GIRK Currents in Mouse Midbrain Slices. To characterize effects of the mutation in a native environment for brain D2 receptors, we used AAV-mediated expression of DIO-Flag-D2_S-WT or -I²¹²F to restore D2 receptor function in dopamine neurons of auto-D2-KO mice and characterized D2 receptor activation of G-protein-regulated inward-rectifying potassium channels (GIRKs). We used D2_S for these studies because of evidence that this splice variant might contribute more than D2_L to autoreceptor activity.^{6,7} The effect of the mutation on D2 receptor function in HEK293 cells was qualitatively similar for both splice variants. Values for both basal G protein activation and maximal arrestin recruitment were greater compared to D2_{L/S}-WT for D2_S-I²¹²F than for D2_L-I²¹²F, although for most experiments there was neither a significant main effect of splice variant nor an interaction with genotype. The only statistically significant difference among the data presented here was a greater relative efficacy of D2_S-I²¹²F for recruitment of arrestin

in the presence of GRK2 compared to that of D2_L-I²¹²F (*p* = 0.001).

In midbrain slices prepared two weeks after AAV injection, the GIRK response to iontophoretically applied dopamine (Figure 4A) was smaller in amplitude (Figure 4B), slower in rise time, and longer in duration (Figure 4C) for D2_S-I²¹²F-expressing neurons compared to that of D2_S-WT transduced controls. Despite the smaller peak amplitude, the prolonged duration of the response meant that total charge transfer was higher for D2_S-I²¹²F (409 ± 92 pC) than for D2_S-WT (207 ± 29 pC; Figure 4D).

Photolytic release of sulpiride from CyHQ-sulpiride³⁵ produced a small inhibition of a tonic GIRK current in cells expressing D2-WT (−9 pA) and a much larger inhibition in cells expressing D2-I²¹²F (−62 pA; Figure 5A,B). The tonic current could reflect constitutive activation of G proteins, similar to what we observed in HEK293 cells (Figure 2 and Figure 3D), or it could reflect the heightened sensitivity of D2-I²¹²F to agonist that is suggested by data for activation of Gα₁₁ (Figure 2C,D) and inhibition of cAMP accumulation.⁸ To distinguish between these possibilities, we treated slices with reserpine to deplete endogenous dopamine. Reserpine treatment abolished or greatly decreased the response to sulpiride in cells expressing D2-WT or D2-I²¹²F, respectively (Figure 5B,C), indicating that most of the tonic current is due to endogenous dopamine to which D2-I²¹²F is more sensitive but that D2-I²¹²F also displayed some constitutive activity in the presumed absence of dopamine. Because the mutation increased agonist potency for activation of Gα₁₁ but not Gα_o in HEK293 cells, this may indicate that D2 receptor signaling

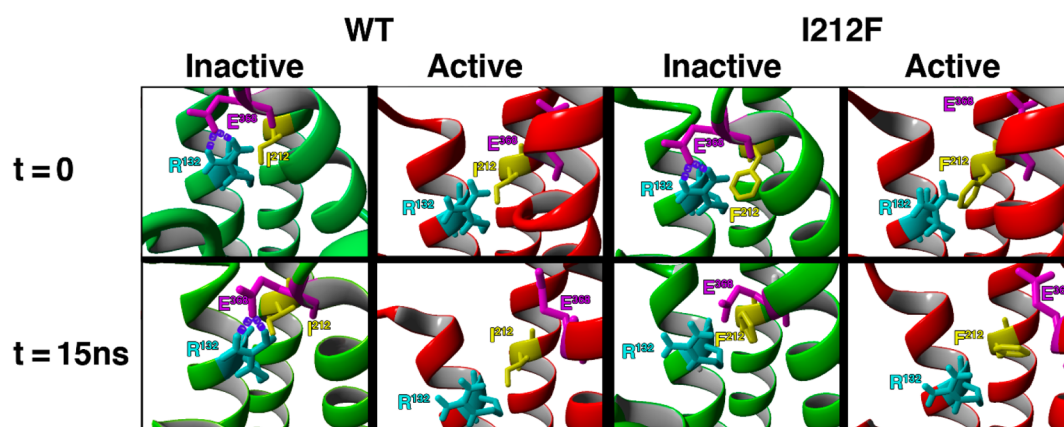


Figure 6. Eight panels show TM3-6 ionic lock residues Arg¹³² (light blue) and Glu³⁶⁸ (magenta), as well as the variant residue Ile/Phen²¹² (yellow). Models are shown before ($t = 0$) and after ($t = 15$ ns) MD simulations for 15 ns. At $t = 0$, the distances between OE1 of Glu³⁶⁸ and HH1 and HH2 of Arg¹³² are short enough to form salt bridges (purple) in both inactive models but are too far apart in both active models. After 15 ns MD simulations, the WT model is essentially unchanged, whereas the presence of Phe²¹² separates the side chains of Arg¹³² and Glu³⁶⁸, preventing maintenance of the ionic lock in inactive D2-I²¹²F. The cytoplasmic face is up.

in SNc dopamine neurons is mediated by $G\alpha_i$. Interestingly, the current decay in response to sulpiride photoactivation was slower for D2-I²¹²F than for D2-WT even following reserpine treatment and the presumed absence of dopamine (Figure 5D), suggesting that relaxation of the receptor or uncoupling of the signaling machinery is inherently slower for the mutant receptor.

Molecular Dynamics Simulations with D2 Receptor Homology Models. One feature of many GPCRs is an “ionic lock” between an Arg residue at the cytoplasmic end of the third transmembrane domain (TM3, Arg¹³² in the D2 receptor) and a Glu residue at the cytoplasmic end of TM6 (Glu³⁶⁸ in the human D2_L receptor).^{36,37} These residues are Arg^{3.50} and Glu^{6.30} in the Ballesteros-Weinstein index.⁹ This ionic lock contributes to maintaining the unliganded receptor in an inactive conformation; the lock is broken in the agonist-activated receptor and, conversely, breaking the lock frequently creates a constitutively active receptor.^{36,38,39} We used crystal structures of the D2 receptor⁴⁰ and other $G\alpha_{i/o}$ -coupled GPCRs to build homology models of the human D2 receptor in both inactive and active conformations, with either Ile²¹² or Phe²¹². As depicted in Figure 6, the side chains of Arg¹³² and Glu³⁶⁸ are in sufficient proximity to form an ionic bond or salt bridge in both inactive models but are too distant for salt bridge formation in both active models. After MD simulations for 15 ns, the Glu³⁶⁸ side chain separated from Arg¹³² in the inactive D2-I²¹²F model, breaking the ionic lock (Figures 6 and 7).

During the MD simulation with the inactive D2-I²¹²F model (Figure 8), a comparison of snapshots obtained at $t = 0.5$ ns (left panel) or 7.5 ns (right panel) shows that, initially, the Phe²¹² side chain extends very close to TM3 residue Ser¹²⁹ (Ser^{3.47}; not shown), whereas Ile²¹² in the inactive D2-WT model is more distant from TM3 (Figure 6). A steric effect of the close Ser¹²⁹-Phe²¹² interaction may provide some of the energy needed to separate the ionic lock residues. As depicted in Figure 8 (right panel), Arg¹³² appeared to strengthen its interaction with Asp¹³¹ (D^{3.49}) likely through ionic interactions.³⁶ Concomitantly, the ionic lock between residues Glu³⁶⁸-Arg¹³² was disrupted as Glu³⁶⁸ moved away from Arg¹³² with the rotation and translocation of TM6, perhaps

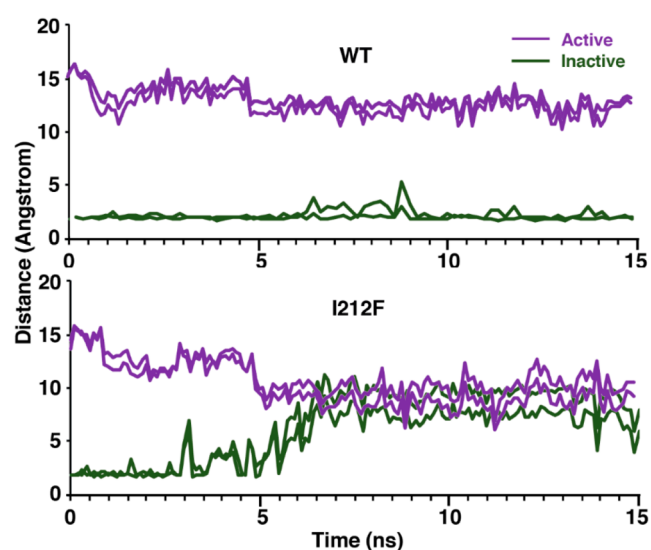


Figure 7. Distances between the atoms that form the two bonds of the ionic lock are shown for all four models during 15 ns MD simulations. Note that the distances are relatively stable for the active and inactive D2-WT models, whereas the distances increased 6–8 Å in the inactive D2-I²¹²F model and decreased ~5 Å in the active model.

interacting with other residues in TM6. The Phe²¹² side chain was reoriented toward a possible interaction with Leu²¹⁶.

These results provide a structural rationale for the effects of the mutation on G protein activation; separation of the lock residues would better enable the conformational rearrangement of TM6 that creates space for binding of $G\alpha$.^{36,37,41,42} Activating mutations of the ionic lock residues also renders the receptor less stable, which is often reflected in decreased expression^{38,39,43} as observed here (Table S1).

Constitutive activation of the receptor potentially explains decreased recruitment of arrestin to D2-I²¹²F, particularly if one speculates that enhanced binding of $G\alpha$ might competitively inhibit binding of arrestin.^{44–46} However, we have also described concurrent reductions in arrestin recruitment and G-protein-mediated signaling for a D2 receptor with a targeted mutation in this part of the receptor,¹² in contrast to

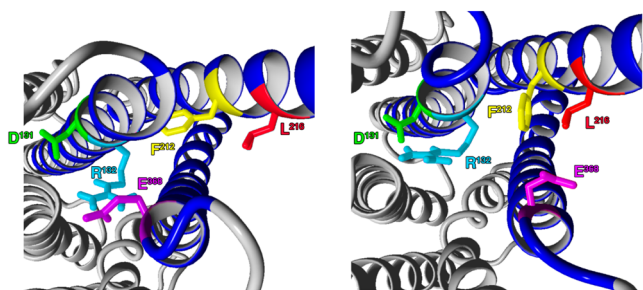


Figure 8. Residues involved in the disruption of the ionic lock are shown at $t = 0.5$ ns (left panel) and 7.5 ns (right panel) during MD simulation with the inactive D2-I²¹²F model. Ballesteros-Weinstein numbering for the colored residues is Asp131^{3,49} (green), Arg132^{3,50} (light blue), Phe212^{5,61} (yellow), Leu216^{5,65} (red), and Glu368^{6,30} (magenta).

the reciprocal effects described here; therefore, it may be that distinct mechanisms underlie the observed effects on arrestin and G protein interaction with D2-I²¹²F. For example, abundant data support a model in which arrestin has separate binding determinants for negatively charged phosphorylated residues on the receptor (“phosphorylation sensor”) and for receptor sites that are exposed by receptor activation (“activation sensor”).^{47–49} We have proposed that non-natural mutations in this intracellular extension of the fifth α -helical domain selectively affect presentation of the activation sensor.¹¹ Decreased engagement of the activation sensor could increase reliance on the phosphorylation sensor, which might explain why arrestin recruitment by D2-I²¹²F has a greater dependence on GRK2 and GRK2-catalyzed phosphorylation.

CONCLUSIONS

The data presented here are consistent with a model in which substitution of Ile²¹² with a Phe residue in the dopamine D2 receptor breaks an interhelical salt bridge that constrains the unliganded receptor. As a result, D2-I²¹²F constitutively activates $G\alpha_{i/o}$ and mediates high-potency agonist activation of at least one $G\alpha_{i/o}$ subtype in HEK293 cells and in dopamine neurons. D2-I²¹²F is biased toward G protein-mediated signaling, because the enhanced $G\alpha_{i/o}$ activation was combined with reduced arrestin recruitment that was particularly profound under conditions where GRK2 activity was limited. A hyperactive D2 receptor would be predicted to cause overinhibition of D2 receptor-expressing medium spiny neurons of the neostriatum and nucleus accumbens. Mice in which the activity of these neurons is genetically inhibited show increased locomotor activity.⁵⁰ Furthermore, overstimulation of G-protein-mediated signaling by the D2 receptor exacerbates, and overexpression of arrestin3 protects against, L-DOPA-induced dyskinesia in mice.⁵¹ This D2 receptor variant c.634A > T;p.I212F is carried by patients with a hyperkinetic movement disorder characterized by both chorea and dystonia;⁸ we speculate that both constitutive activity and G protein bias of D2-I²¹²F contribute to the clinical phenotype and that an effective treatment should target these characteristics of the receptor.

METHODS

Recombinant cDNA Constructs. All human D2 receptor cDNA constructs contained a signal peptide and a FLAG epitope tag at the receptor N-terminus. The wild type (WT) short (SF-hD2_S) and long

(SF-hD2_L) isoforms of human D2 receptors as well as the SF-hD2_L(I²¹²F) and SF-hD2_S(I²¹²F) variants in pcDNA3 and the corresponding RLuc8 fusion proteins for arrestin BRET assays were described previously.⁸ Other plasmids for arrestin3 recruitment BRET assays (human arrestin3 fused to mVenus and human GRK2), for measuring G protein activation ($G\alpha_{i1}$ -91-RLuc8, V1-G β_1 and V2-G γ_2), and for inhibition of cAMP accumulation (pcDNA3L-His-CAMYEL; ATCC MBA-277) were previously described,^{12,29} except for the plasmid $G\alpha_{oA}$ -91-RLuc8^{23,52} that was obtained from Jonathan Javitch (Columbia University, USA). For animal studies, recombinant adeno-associated viral (AAV8.2) vectors containing a Cre recombinase-dependent double-floxed inverted open reading frame (DIO) for hD2_S-WT or -I²¹²F, tagged at their C terminus with a self-cleaving 2A peptide and EGFP, were described previously⁸ and were produced by Virovek, Inc. (Hayward, CA, USA). To generate stable transfected HEK293 cells, pcDNA3.1[SF-hD2_S-P2A-EGFP] was obtained from Jonathan Javitch (Columbia University, USA). Plasmids containing wild type and mutated D2_L isoforms (pcDNA3.1[SF-hD2_L-P2A-EGFP] and pcDNA3.1[SF-hD2_L(I²¹²F)-P2A-EGFP], respectively) and mutated D2_S isoform (pcDNA3.1[SF-hD2_S(I²¹²F)-P2A-EGFP]) were generated by digesting pcDNA3.1[SF-hD2_S-P2A-EGFP] with BstEII and PmlI (New England BioLabs, MA, USA). The purified 6.8 kb fragment was ligated to each 782 bp insert generated by BstEII/PmlI digestion of pcDNA3[SF-hD2_L] and pcDNA3[SF-hD2_L(I²¹²F)] or 695 bp insert of pcDNA3[SF-hD2_S(I²¹²F)], using T4 DNA Ligase (New England BioLabs). All new constructs were verified by DNA sequencing at the OHSU Vollum DNA Sequencing Core Facility (Portland, Oregon, USA).

Cell Culture and Transfection Conditions. HEK293 cells obtained from Caroline Enns (Oregon Health & Science University, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FetalClone I serum (FCS; Thermo Fisher Scientific; Waltham, MA, USA) at 37 °C in a 5% CO₂ atmosphere. New cell cultures were initiated frequently from frozen stocks. Eighteen hours before transfection, HEK293 cells were plated in 100 mm dishes at 60–80% confluence. HEK293 cells were transfected with equal amounts of D2-WT or D2-I²¹²F receptor DNA, except in some arrestin recruitment (Figure S1) and D2-mediated inhibition of cAMP (Figure 3C,D) assays. Transient transfections were performed with polyethylenimine (PEI; MAX 40K reagent, Polysciences, Inc.; Warrington, PA, USA) in Opti-MEM I (Gibco by Life Technologies; Logan, UT, USA). In most cases, two 100 mm Petri dishes per condition were transfected to allow us to carry out BRET and radioligand binding assays using identically treated cells. Transfections were incubated for 5–6 h at 37 °C in the 5% CO₂ humidified atmosphere, after which the medium was replaced by fresh DMEM plus 10% FCS. Cells were harvested 48 h post-transfection for BRET studies and frozen for radioligand binding assays.

To study quinirole affinity of the D2 variants, stable HEK293 cells expressing either SF-hD2_{L/S}-P2A-EGFP or SF-hD2_{L/S}(I²¹²F)-P2A-EGFP were generated by transfecting the respective plasmids with PEI in 12-well plates, as described above. Two days after transfection, cells recovered from each well were plated into two 100 mm dishes in supplemented DMEM containing 500 μ g/mL of G-418 (Gold Biotechnology Inc.; MO, USA). Colonies were initially screened for EGFP expression by immunoblotting. Then, EGFP-positive clones were screened for Flag tagged-D2 expression by immunoblotting, using a rabbit polyclonal antibody anti-DYKDDDDKC epitope tag (Invitrogen; CA, USA), and radioligand binding assays as described below. Stable transfected cells were maintained in DMEM plus 10% FCS, with 500 μ g/mL of G-418.

Bioluminescence Resonance Energy Transfer (BRET) Assays. For arrestin3 recruitment, cells were cotransfected with plasmids containing mVenus-Arr3 (2.5 μ g) and the WT or I²¹²F-mutated D2 receptor fused to RLuc8 (0.25 μ g except for Figure S1, where D2-WT receptor DNA amounts were adjusted to yield similar levels of receptor expression for the allelic variants), with or without hGRK2 (2 μ g). For G protein activation, cells were cotransfected with WT or I²¹²F-mutated D2 receptor (0.5 μ g), the G protein subunits V1-G β_1 (2 μ g) and V2-G γ_2 (2 μ g), and the $G\alpha$ proteins $G\alpha_{i1}$ -91-

RLuc8 (0.2 μg) or G_{α_A-91} -RLuc8 (0.2 μg). For cyclic AMP accumulation, cells were cotransfected with WT (0.2 μg for WT-Low and 0.5 μg for WT-High) or I^{1212F} -mutated D2 receptor (0.5 μg) and the cyclic AMP sensor CAMYEL (2.5 μg). Control cells were transfected with CAMYEL and nonspecific plasmid DNA. After 48 h, cells were harvested, washed, resuspended in PBS containing CaCl_2 , MgCl_2 , and 11 mM D-glucose, plated at 100 000–150 000 cells/well in 96-well OptiPlates (PerkinElmer Life Sciences), and incubated at 37 °C and 5% CO_2 atmosphere for 1 h before adding the agonist quinpirole. Compound 101 (Cmpd101; HelloBio, Princeton, NJ) was initially dissolved in DMSO at 100 mM and subsequently diluted in PBS. For GRK2 inhibition during arrestin recruitment-BRET assays, HEK293 cells were pretreated with 30 μM of Cmpd101 or vehicle (DMSO diluted in PBS) 30 min before agonist addition. For D2-mediated inhibition of cyclic AMP, HEK293 cells were pretreated with the dopamine D1 receptor antagonist SCH 23390 (1 μM) and the β -adrenoceptor antagonist pindolol (0.1 μM ; Sigma-Aldrich; MO, USA) before the addition of inverse agonist (10 μM sulpiride or 1 μM spiperone) and 10 μM forskolin (Sigma-Aldrich; MO, USA). Emission of the donor (460 nm) and acceptor (535 nm) was measured at room temperature several times after adding the luciferase substrate coelenterazine *h*, and BRET ratios were calculated as previously described.^{12,53}

D2 Receptor Radioligand Binding. Membrane expression of the receptors was evaluated exactly as described previously.⁸ Cells were lysed in ice-cold hypotonic buffer (1 mM HEPES, 2 mM EDTA, pH 7.4), scraped from the plate, and centrifuged at 17 000 $\times g$ at 4 °C for 20 min. The resulting pellet was resuspended in Tris-buffered saline (TBS: 50 mM Tris, 120 mM NaCl, pH 7.4) and homogenized for 10 s using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Protein determination was performed using the BCA Protein Assay Kit (Thermo Scientific). Samples were incubated in TBS containing 0.002% BSA and [^3H]spiperone at 37 °C for 1 h in a final volume of 1 mL before addition of ice-cold buffer and vacuum filtration. Nonspecific binding was assessed using (+)-butaclamol (2 μM). Competition binding assays were carried out using membranes prepared from HEK293 cells stably expressing each of the four receptor variants. The ability of various concentrations of quinpirole to inhibit the binding of [^3H]spiperone (~ 85 pM) was analyzed by nonlinear regression. IC_{50} values were converted to K_i according to the method of Cheng and Prusoff.⁵⁴

Mice. All studies were conducted in accordance with the Institutional Animal Care and Use Committees at the VA Portland Health Care System (VAPORHCS) and Oregon Health & Science University (OHSU). Twelve mice (4 male and 8 female, 59–96 days old on day of surgery) were used in this study. Auto-D2-KO mice were bred at the VAPORHCS Veterinary Medical Unit by crossing *Drd2^{loxP/loxP}* mice,⁵⁵ obtained from Jonathan Javitch (Columbia University, USA), with heterozygous *B6.SJL-Slc6a3^{tm1.1(crc)Bkmm}/J* mice⁵⁶ obtained from the Jackson Laboratory (JAX stock no. 006660). All lines are maintained on a C57BL/6 background. Mice were housed in standard plastic containers on a 12 h light/dark cycle with food and water available *ad libitum*. For expression of recombinant D2_S receptors in dopamine neurons, auto-D2-KO mice were immobilized in a stereotaxic alignment system after injection of an anesthesia cocktail consisting of 7.1 mg/kg xylazine, 71.4 mg/kg ketamine, and 1.4 mg/kg acepromazine (10 mL/kg, i.p.). Mice received bilateral 500 nL injections of AAV-DIO-hD2_S-WT or I^{1212F} in the ventral tegmental area, at a rate of 200 nL/min, with the injection needle left in place for an additional 5 min before it was slowly withdrawn. The coordinates for injections were AP -3.26 mm, ML ± 1.2 mm, DV -4.0 mm. After injections, mice recovered in individual (male) or group (female) housing for 2–3 weeks to allow for expression.

Slice Electrophysiology. Mice were deeply anesthetized with isoflurane and euthanized by decapitation. Brains were removed and placed in warm (30 °C) physiologically equivalent saline solution (modified Krebs buffer) containing NaCl (126 mM), KCl (2.5 mM), MgCl_2 (1.2 mM), CaCl_2 (2.4 mM), NaH_2PO_4 (1.4 mM), NaHCO_3 (25 mM), and D-glucose (11 mM) with MK-801 (3 μM) and cut

horizontally (222 μm) using a vibrating microtome (Leica). Slices recovered at 30 °C in vials with 95/5% O_2/CO_2 saline with MK801 (10 μM) for at least 30 min prior to recording. For reserpine treatment, slices instead recovered for 15 min in MK801 followed by 1 h in reserpine (1 μM). Slices were mounted in the recording chamber of an upright microscope (Olympus). The temperature was maintained at 34–36 °C, and modified Krebs buffer was perfused over the slices at 1–2 mL/min. Recordings were obtained with large glass electrodes with a resistance of 1.3–1.9 M Ω when filled with an internal solution containing potassium methanesulfonate (75 mM), NaCl (20 mM), MgCl_2 (1.5 mM), HEPES potassium salt (5 mM), ATP (2 mM), GTP (0.2 mM), phosphocreatine (10 mM), and BAPTA tetrapotassium salt pH 7.35–7.45 (10 mM) at 275–288 mOsm (milliosmole). Cells were voltage-clamped at -60 mV using an Axopatch 200A integrating patch clamp (Axon Instruments). Recordings were made using Axograph 10 and Chart 5.5. D2 receptor-expressing dopamine neurons in the substantia nigra were identified by location, size, firing properties, and EGFP fluorescence.

CyHQ-sulpiride³⁵ was kept as a stock solution in DMSO (10 mM) and diluted to a 5 μM working solution. A Thorlabs M365LP1-C1 LED was used to photolyze CyHQ-sulpiride by means of a 50 ms flash (365 nm) at 6.5 mW. Dopamine iontophoresis (1 M) was done using a thin-walled glass electrode (70–110 M Ω) with its tip placed within 10 μm of the soma. Dopamine was kept in place with a 4 nA backing current and ejected with a 10 ms, 100 nA pulse using an Axoclamp-2a amplifier.

D2 Receptor Homology Models and Molecular Dynamics Simulations. Homology modeling was performed using YASARA Structure⁵⁷ that features a CASP- (Critical Assessment of Structure Prediction) approved protocol.⁵⁸ The inactive state of the D2 receptor was modeled using inactive structures of the β_2 -adrenoceptor (2RH1), the A2A adenosine receptor (3EML and 6GT3), the M2 muscarinic receptor (3UON), bovine rhodopsin (1GZM), and the D2 receptor (6CM4) as templates. The D2 receptor active state model was built using active-state structures of the M2 receptor (4MQS), the β_2 -adrenoceptor (3SN6 and 3P0G), the A2A receptor (2YDV and 5WF6), the CB1 receptor (6N4B), the μ -opioid receptor (6DDE), and rhodopsin (3PQR) as templates. Multiple D2 receptor models for inactive and active states (48 and 45 models, respectively) were obtained, and side chain rotamers were optimized using backbone-dependent probabilities and knowledge-based force fields in YASARA.⁵⁹ The resulting models were further optimized for hydrogen bonding, refined using short molecular dynamics simulations, and ranked. Residue-specific quality graphs were calculated for each model, and a final hybrid model was developed through an iterative process, replacing poorly scoring regions in the best model with the corresponding regions from other models, with the goal of increasing the accuracy beyond each of the contributing models. The stereochemical properties of the homology models were verified using the PROCHECK module⁶⁰ of the PDBSum server, which examines protein quality based on parameters such as percentage of residues lying in favored and allowed regions, the number of glycine and proline residues, the orientation of dihedral angles including phi (φ) and psi (ψ), and backbone conformation. The VERIFY3D⁶¹ server was used to check the compatibility of atomic models (3D) with its own primary amino acid sequences (1D). The RMSD of the α -helical segments of the resulting active-state homology model of the D2 receptor was 1.62 and 1.63, respectively, relative to 6CM4 and the recently published active-state structure of the D2 receptor 6VMS.⁶²

To assess the potential functional impact of the I^{1212F} substitution on the active and inactive D2 receptor homology models, the system was simulated atomistically for 15 ns using the YASARA software package under an NPT ensemble with the AMBER14 force field,⁶³ with a time step of 5.0 fs. Simulation conditions were conducted with periodic boundaries, at 0.9% NaCl concentration by mass, pH 7.4, 298 K, at atmospheric pressure. The water model employed was TIP3 equiv. Snapshots were saved every 100 ps. Structures were visualized using YASARA and the distances between the atom OE1 of E³⁶⁸ and

HH1 and HH2 atoms of R¹³² were monitored during the simulation and plotted using Prism GraphPad software.

Data Analysis and Statistics. Concentration–response curves and radioligand saturation binding curves were analyzed by nonlinear regression using Prism 8 (GraphPad Software Inc.; San Diego, CA, USA). For affinity and potency values, the geometric mean (mean of logK_{d/i} or logEC₅₀) was calculated and used for statistical comparison. Statistical significance between two means was determined using Student's *t*-test and, for comparisons of more than two means, by 2-way ANOVA followed by Tukey's multiple comparisons test except for data shown in Figure 1C,D. The effect of genotype and GRK2 condition on maximal arrestin recruitment over time was assessed using 3-way repeated measures (RM) ANOVA with Geisser-Greenhouse correction, followed by 2-way RM ANOVA, with genotype and/or GRK2 condition as matched values and time (7-time points) as the RM factor.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acchemneuro.0c00712>.

*B*_{max} values for HEK293 studies, regression analyses for Compound 101 experiment–comparison, arrestin recruitment with matched receptor expression levels, concentration–response curves for arrestin recruitment and comparison of ± Compound 101, and quinpirole competition binding curves (PDF)

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Author Contributions

The manuscript is a product of contributions from all of the authors. All authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AAV, adeno-associated virus; BRET, bioluminescence resonance energy transfer; Cmpd101, GRK2/3 inhibitor Compound 101; CyHQ-sulpiride, 1-((8-cyano-7-hydroxyquinolin-2-yl)methyl)-1-ethyl-2-((2-methoxy-5-sulfamoylbenzamido)-methyl)pyrrolidin-1-ium 2,2,2-trifluoroacetate; DIO, double-floxed inverted open reading frame; EGFP, green fluorescent protein; FCS, FetalClone I Serum; FSK, forskolin; GIRKs, G-protein-coupled inwardly rectifying potassium channels; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; HEK, human embryonic kidney; MD, molecular dynamics; RLuc, *Renilla* luciferase; RM ANOVA, repeated measures ANOVA; TM, transmembrane domain; WT, wild type

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