Gs- versus Golf-dependent functional selectivity mediated by the dopamine D₁ receptor

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The two highly homologous subtypes of stimulatory G proteins Gₛₛ (Gs) and Gₒₒlf (Golf) display contrasting expression patterns in the brain. Golf is predominant in the striatum, while Gs is predominant in the cortex. Yet, little is known about their functional distinctions. The dopamine D₁ receptor (D₁R) couples to Gₛₛ/olf and is highly expressed in cortical and striatal areas, making it an important therapeutic target for neuropsychiatric disorders. Using novel drug screening methods that allow analysis of specific G-protein subtype coupling, we found that, relative to dopamine, dihydrexidine and N-propyl-apomorphine behave as full D₁R agonists when coupled to Gₛₛ, but as partial D₁R agonists when coupled to Golf. The Gₛₛ/Gₒₒlf-dependent biased agonism by dihydrexidine was consistently observed at the levels of cellular signaling, neuronal function, and behavior. Our findings of Gₛₛ/Gₒₒlf-dependent functional selectivity in D₁R ligands open a new avenue for the treatment of cortex-specific or striatum-specific neuropsychiatric dysfunction.
Functional selectivity is defined as the ability of a ligand to demonstrate a biased profile of potency or efficacy on different signaling pathways. This is distinguished from prototypical uniform activation by general agonism produced by endogenous ligands. In recent years, many ligands with functionally selective properties for G-protein-coupled receptors (GPCRs) have emerged based on the concept that ligands can stabilize specific receptor conformations to which different signaling proteins, such as G proteins and β-arrestins, couple. In addition, accessory proteins to the receptor as well as effector proteins may exert bias in signaling events exhibiting many potential sites for functional selectivity. Thus the concept of functional selectivity has provided a new avenue for the development of drugs with safer therapeutic index, when therapeutic and unwanted side effects are dependent on different signaling pathways.

Several examples of functional selectivity have been reported for dopamine receptor ligands. Dopamine receptors are classified into Gs/Golf-coupled D₁-like receptors (D1R and D5R) and Gi/o-coupled D₂-like receptors (D2R, D3R and D4R). With respect to D₂-like receptors, both G-protein-biased and β-arrestin-biased agonists have been characterized. With respect to D₁-like receptors, biased agonism at G-protein versus β-arrestin signaling has also been reported. We recently found differences in dopamine potency in promoting the coupling of different Giα and Gox (Gi/o) protein subtypes to the D2R, D3R, and D4R. These results suggest the possibility of selectively targeting D₁-like receptor in different brain areas relying on the predominant local expression of certain Gi/o proteins. However, there is no compelling evidence for a differential distribution of Gi/o proteins in the brain. This is in contrast with the clearly distinct distribution of the two subtypes of stimulatory G proteins, Gs and Golf. Golf is by far the most expressed and functions as a signaling G-protein for D1R in the striatum, while Gs is predominantly expressed in cortical and other areas.

In the present study, using a series of novel pharmacological assays, we addressed the possibility of Gs/oilg protein subtype-dependent biased agonism of D1R ligands. Dihydrexidine (DHX) and N-propyl-apomorphine (NPA) behaved as full D1R agonists when coupled to Gs and as partial D1R agonists when coupled to Golf. The significant efficacy bias for Gs-mediated versus Golf-mediated signaling of DHX was further demonstrated with cellular signaling, electrophysiological and psychomotor activation experiments, which enhances our understanding of Golf-signaling in striatal function and psychomotor activity. Moreover, our results highlight the potential use of such functionally selective agonists for treating the “negative” cognitive symptoms of schizophrenia.

Results

Gs- and Golf-biased engagement and activation by D1R ligands. Using the receptor-Gα subunit engagement BRET configuration, the potencies and efficacies of different classes of D1R agonists were compared to dopamine for Gs and Golf coupling (Fig. 1 and Table 1). While the majority of the compounds behaved similarly for the engagement of Gs and Golf, two compounds, dihydrexidine (DHX) and N-propyl apomorphine (NPA), behaved quite differently (Fig. 1c, d; green and yellow curves, respectively). Notably, whereas these compounds behaved as full agonists (relative to DA) for Gs coupling (En_max; DHX, 121.3%; NPA, 111.9%), they displayed only partial agonism for Golf coupling (En_max; DHX, 39.0%; NPA, 67.9%; Fig. 1 and Table 1). To further validate the partial efficacy of DHX for Golf engagement, DHX was tested for its ability to counteract the agonist effect of dopamine (1 µM; Supplementary Fig. 1B). Although much less potent than the commonly used D1R antagonist SCH23390, the Imax value of DHX was in agreement with its Emax value (Supplementary Fig. 1B). The relative potency and efficacy of the different agonists were further tested with the Gα-γ activation BRET configuration (Supplementary Table 1). Similar to results obtained with the engagement assay, DHX and NPA exhibited significantly lower Emax values relative to DA for Golf activation (46.8 and 52.6%; Supplementary Table 1) while retaining Emax values comparable to DA for Gs activation.

Although β-arrestin is essential for GPCR internalization and desensitization, it can also transduce MAPK activation for various receptors, including D1R. Using the same luciferase-fused D1R construct used for the Gα engagement BRET assays, we measured agonist-induced recruitment of Venus-fused β-arrestin-2 to the D1R (Supplementary Table 1). GPCR kinase 2 (GRK2)

Fig. 1 a Scheme for the engagement BRET between D1R-Rluc and Gs-Venus. b Scheme for the engagement BRET between D1R-Rluc and Golf-Venus. c Dose-response curves of drug-induced BRET between D1R-Rluc and Gs-Venus (black dopamine, blue norepinephrine, light orange SKF38393, dark orange SKF81297, brown SKF82958, yellow NPA, green DHX, magenta A77636, black open SCH23390 + 10^{-6} SKF81297). d Dose-response curves of BRET between D1R-Rluc and Golf-Venus (same color scheme). The error bars represent S.E.M.
was co-transfected to enhance β-arrestin-2 recruitment. SKF81297 and SKF38393 showed reduced and minimal β-arrestin-2 recruitment, respectively, confirming their recently reported biased G-protein- versus β-arrestin-dependent signaling12, while A77636 and SKF28958 maintained their high potencies and efficacies in the β-arrestin-2 recruitment assay. DHX and NPA behaved similarly to Gs engagement in terms of efficacy and potency and DHX also showed a significantly higher $E_{\text{max}}$ value than DA (142.4%; Supplementary Table 1). Altogether, these results clearly indicate that DHX and NPA possess biased agonism toward Gs versus Golf but not toward Gs protein versus β-arrestin selectivity.

### Table 1 Pharmacological comparison of Gs and Golf engagement in D1R

|          | $E_{\text{max}}$ (%) | $EC_{50}$ (nM) |
|----------|----------------------|---------------|
|          |                      |               |
| DA       | 100.0 ± 1.3          | 49.0 ± 4.3    |
| NE       | 99.2 ± 2.2           | 102.3 ± 116.3 |
| SKF38393 | 61.6 ± 1.3           | 25.1 ± 4.0    |
| SKF81297 | 95.0 ± 2.7           | 5.4 ± 1.75    |
| SKF28958 | 101.5 ± 5.2          | 0.2 ± 0.19    |
| NPA      | 111.9 ± 2.4          | 145.5 ± 19.5  |
| DHX      | 1213 ± 2.7c          | 24.3 ± 3.5    |
| A77636   | 102.5 ± 3.8          | 0.5 ± 0.15    |

$E_{\text{max}}$ values are means ± S.E.M. of more than five experiments performed in duplicate. $EC_{50}$ values are expressed in μM normalized to dopamine results.

### Gs-D1R interface contribution to bias between Gs and Golf.

We then inspected the sequence differences between Gs and Golf to identify a potential structural element that is responsible for the observed biased agonism. Most of the motifs that interact with D1R are nearly identical between Gs and Golf. However, five residues are divergent at the end of αn helix of G-protein (residues 33–38 in Gs) apposing to the intracellular loop 2 (IL2) of D1R (Fig. 2). The impact of this divergence on the protein-protein interaction was then investigated both in vitro and in silico. To understand the functional impact of this divergence in the D1R-G-protein coupling, a Golf/s chimeric construct was made whereby an ERLAYK to DKQVYR mutation was introduced to the Golf-Venus construct (Fig. 2a). The effect of DHX in the D1R-Ga engagement was then tested with this construct. DHX-induced BRET changes were normalized to the $E_{\text{max}}$ values obtained by DA with the corresponding Ga-Venus constructs (Fig. 2b).

Similar to the results described above, Gs and Golf coupling were 123.3% and 46.8% $E_{\text{max}}$ relative to DA, confirming the biased selectivity of this ligand. In contrast, DHX coupling to the Golf/s chimera was significantly increased ($E_{\text{max}} = 94.04%$ relative to DA). A partial but significant increase in $E_{\text{max}}$ was also observed with NPA (Supplementary Fig. 1D). Thus, our results suggest that the Gs/αn-D1R/IL2 interface is responsible for the biased agonism of DHX on D1R-Gs vs D1R-Golf. As expected, a full agonist effect was observed in Golf/s chimera coupling nearly the same as Gs or Golf with SKF 81297 (Supplementary Fig. 1C).

Further, to exclude the possibility of effects from other non-cognate coupling, Gi1 and Gq engagement to the D1R was tested (Supplementary Fig. 1E, F). DA, SKF81297, or DHX did not cause any coupling of Gi1 or Gq while D2R-Gi1 coupling by DA and M1R-Gq coupling by carbachol were observed as positive controls (Supplementary Fig. 1E, F).

Next, the underlying molecular mechanism for the bias was explored with comparative homology modeling and molecular dynamics simulation. In the simulation of the D1R-Gs and D1R- Golf homology models constructed from β2AR-Gs complex (see Methods), we found that in the D1R-Gs complex, R38 of Gs forms a steady salt bridge interaction with E132 of D1R/IL2 (Fig. 2c), for which the simulation shows the interaction (< 4 Å) holds for 89% of the time (Fig. 2e). In comparison, the D1R-Golf complex has a weaker salt bridge interaction between K40 of Golf and E132 of D1R/IL2 (Fig. 2d) with an intermittent interaction (< 4 Å) of 54% within the simulated time (Fig. 2d). This reduced stability in salt bridge interaction between Golf/αn and D1R/IL2 is consistent with the lower signal observed in D1R-Golf BRET assay.

### GS-biased agonism of DHX at the cellular signaling level.

To confirm the partial agonism of Golf-dependent signaling, DHX was tested in a novel Gs-AC5 coupling assay (Fig. 3 and Supplementary Table 2) in which D1R agonist-induced relative movement (BRET changes) between AC5 and Gs or Golf can be monitored. Assay optimization was first performed by testing various Gs and Golf biosensor constructs with different insertion positions (Supplementary Fig. 2). Ligand-induced BRET changes indicate relative conformational changes between Gs or Golf and AC5, reflecting AC5 activation level. D1R ligands were analyzed for the interactions between Gs and Golf and AC5 (Fig. 3). Again, DHX behaved as a more efficacious agonist than DA with Gs ($E_{\text{max}} = 119.0%$), and as a partial agonist with Golf ($E_{\text{max}} = 37.6%$; Suppl. Table 2 and Fig. 3). To confirm the results and validate this novel assay, we analyzed adenylate cyclase enzymatic activity, cAMP accumulation, in a unique lymphoma cell line (S49 cycl-cells) lacking Gs (Supplementary Fig. 3)20. In this cell line Gs- or Golf-dependent cAMP activation can be separately analyzed by rescuing the expression of either Gs protein subunit. In D1R electroporated cells, a selective full agonist SKF81297 and a selective partial agonist SKF38393 were used as reference compounds to compare with DHX. As expected, when compared to SKF81297 and SKF38393, $E_{\text{max}}$ value for DHX yielded a similar partial efficacy value as SKF38393 with Golf co-transfection (45.1 and 50.3%, respectively), while maintaining partial and near full efficacy values with Gs co-transfection (67.4 and 87.6%, respectively). Forskolin was added to confirm G-protein-independent activation of endogenous AC (Supplementary Fig. 3C, mosaic bar). Increased cAMP production was observed in Golf or Gs electroporated cells compared to the mock electroporated cells due to AC5 co-electroporation in Golf and Gs cells.
GS-biased agonism of DHX in mouse brain tissue. Previous reports have shown contrasting patterns of GS and Golf expression in the brain, with Golf enriched in the striatum but not in the cortex and vice versa for GS.15 Indeed, using single cell RT-PCR analysis in Drd1-tdTomato BAC reporter mice, we were able to confirm a preferential expression of GS and Golf genes in the prelimbic region of medial prefrontal cortex (mPFC) and the shell of the nucleus accumbens (NAC), respectively (Supplementary Fig. 5A, B). Virtually the same results were obtained with tissue punches, confirming the same differential gene expression when also including non-D1-expressing cells (Supplementary Fig. 5C, D). Electrophysiological studies in mouse slices from GS-enriched mPFC and Golf-enriched NAc were performed to confirm the predicted low efficacy of DHX in the striatum. The D1R agonist SKF81297 was used for comparison and expected to behave with full efficacy in both preparations. Both compounds have been reported to bind to D2-like receptors albeit with lower affinity than D131.22. Since D2-like receptors, especially D3R, co-localize with D1R in the brain,23 the ability of both ligands to activate D2R and D3R was also evaluated in BRET-based functional assays. DHX and SKF81297 were about one order of magnitude less potent and less efficacious at D2R and D3R-mediated Go activation than at D1R-mediated GS activation (Supplementary Figs. 4C, D, G, H, 6). Nevertheless, the non-selective D2-like receptor antagonist eticlopride was co-applied with DHX or SKF81297 to completely isolate D1R agonist-mediated effects in the brain slice preparation.

Increased neuronal excitability and cell firing mediated by D1R activation has been reported in the striatum24–26 as well as in the cortex25–28. D1R and NMDA receptors (NMDAR) have been reported to form molecular and functional interactions30–32 and D1R activation has been shown to facilitate NMDAR function via GS/olf-AC-PKA activation33. Differences in GS- and Golf-dependent effects of SKF81297 and DHX were therefore assessed by measuring NMDA-induced firing rates in D1R-expressing neurons using patch-clamp electrophysiology in slices from Drd1-tdTomato mice. First, firing rate was analyzed in D1R-expressing layer V pyramidal neurons in the GS-enriched mPFC (Fig. 4a). The minimal basal spontaneous firing rate (0.010 ± 0.006 Hz) was dramatically increased by NMDA (10 µM; 0.298 ± 0.061 Hz; 2830% of basal), and this was further enhanced by co-application of SKF81297 (0.902 ± 0.229 Hz; 8570% of basal; p < 0.01) or DHX (10 µM; 0.689 ± 0.244 Hz; 6540% of basal; p < 0.05). The enhancement of NMDA-induced firing by SKF81297 and DHX was blocked by the D1R antagonist SCH23390. Spontaneous firing, as well as NMDA-induced firing, was absent in NAc medium spiny neurons owing to their hyperpolarized resting membrane potentials. Therefore, in the Golf-enriched NAc, 200 pA was injected to elicit cell firing (Fig. 4b). NMDA (10 µM) robustly increased the elicited spike frequency.
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![Diagram](image)

**Fig. 3** a) Scheme for the drug-induced interaction BRET between AC5-Nluc and Gs-Venus. b) Scheme for the drug-induced interaction BRET between AC5-Nluc and Golf-Venus. c) Dose-response curves of drug-induced BRET between AC5-Nluc and Gs-Venus (black DA, blue norepinephrine, light orange SKF81297, dark orange SKF81297, green DHX, black open SCH23390 + 10⁻⁶ SKF81297). d) Dose-response curves of drug-induced BRET between AC5-Nluc and Golf-Venus (same color scheme). The error bars represent S.E.M.

The development of D1R pharmacology for over 30 years has verified its potential therapeutic value in various neuropsychiatric disorders, including Parkinson’s disease, schizophrenia and substance use disorders. Intense research in GPCR pharmacology has yielded the concept of functional selectivity, giving rise to the possibility of selective targeting aside from receptor affinity, particularly the ability of ligands to preferentially activate either G-protein-mediated or β-arrestin-mediated signaling. Initial insights into the structural basis for this functional selectivity are beginning to emerge. The present study illustrates a unique paradigm for GPCR functional selectivity; namely, the differential ability of ligands to engage similar but distinct G-protein subtypes. Following the same molecular principle as for the G-protein/β-arrestin functional selectivity, distinct conformations of the GPCR stabilized by a variety of ligands may also achieve G-protein subtype functional selectivity. Motivated by the unique reciprocal expression patterns of Gs and Golf in the cortex and striatum and the functional distinction in these brain areas, the current study was designed to look for D1R ligands that are functionally selective at Gs versus Golf.

** Weak psychomotor activating properties of DHX.** Finally the ability of DHX to produce psychomotor activity by activating striatal D1R was explored in the catecholamine depleted long-term (twenty hours) reserpinized mouse model (Fig. 5). This model allows the in vivo determination of separate striatal postsynaptic activity of selective D1R and D2R agonists without the confounding influence of endogenous dopamine. Thus, either a selective D1R or a D2R agonist produces significant locomotor activation of the akinetic animal. Both DHX and SKF81297 dose-dependently induced significant locomotor activation (Fig. 5a, b; see Supplementary Fig. 8B, C for time course), which was blocked by the D1-like receptor antagonist SCH23390 (0.5 mg/kg), but not by the D2-like antagonist eticlopride (0.5 mg/kg). The same dose of eticlopride, but not SCH23390, blocked quinpirole-induced locomotor activation (Supplementary Fig. 8A). These results demonstrated a selective involvement of D1R versus D2R in the locomotor activation and D1R-specific locomotor effects by DHX and SKF81297. Importantly, and consistent with its partial efficacy on the Golf-coupled striatal D1R, DHX showed a significantly lower locomotor-activating effect compared to the full agonist SKF81297 (Fig. 5c, d; 48.6% at 5 mg/kg or 45.8% at 15 mg/kg).

**Discussion**

The development of D1R pharmacology for over 30 years has verified its potential therapeutic value in various neuropsychiatric disorders, including Parkinson’s disease, schizophrenia and substance use disorders. Intense research in GPCR pharmacology has yielded the concept of functional selectivity, giving rise to the possibility of selective targeting aside from receptor affinity, particularly the ability of ligands to preferentially activate either G-protein-mediated or β-arrestin-mediated signaling. Initial insights into the structural basis for this functional selectivity are beginning to emerge. The present study illustrates a unique paradigm for GPCR functional selectivity; namely, the differential ability of ligands to engage similar but distinct G-protein subtypes. Following the same molecular principle as for the G-protein/β-arrestin functional selectivity, distinct conformations of the GPCR stabilized by a variety of ligands may also achieve G-protein subtype functional selectivity. Motivated by the unique reciprocal expression patterns of Gs and Golf in the cortex and striatum and the functional distinction in these brain areas, the current study was designed to look for D1R ligands that are functionally selective at Gs versus Golf.

DHX was developed in the late 1980s and, based on results obtained with the cAMP assay in monkey and rat striatal tissue and behavioral effects in rats, it was introduced as the first fully efficacious D1R agonist with potential antiparkinsonian effects. However, a clinical trial showed marginal therapeutic efficacy and secondary effects including hypotension and tachycardia. The present study gives a new insight into the potential discrepancies previously observed with this drug and...
antiparkinsonian activity of DHX. We find that DHX indeed behaves as a full agonist at D1R, but only when coupled to Gs, not to the predominant striatal G-protein subtype Golf. Based on our molecular modeling study, we propose that the Gs/αN-D1R/IL2 interface plays a significant role in determining the reported biased agonism, as implicated by previous studies on differential ligand-induced conformations of the IL2 of monoaminergic receptors. Our studies with the chimeric receptor suggest that D1R/IL2 may exist in distinct conformations such that DHX diminishes the already weak Gs/αN-D1R/IL2 interaction of D1R-Golf coupling, compared to DA. Although previous studies have reported a full efficacy profile reported for DHX, several caveats must be considered. First, the DHX efficacy has been reported in heterologous expression systems, which rely mostly on Gs-
mediated readout due to the fact that these cells do not express Golf. Second, the apparent full efficacy of DHX observed in experiments from Golf-rich striatal material \cite{36,37} could be related to the confounding effect of a concomitant Gs-dependent response from D1-like receptors localized in striatal neuronal populations other than medium spiny neurons, such as the large aspiny cholinergic interneurons. Indeed these interneurons are known to contain functional D1-like receptors of D5R subtype \cite{48,49}. Finally, previous in vivo recordings from non-identified ventral pallidal neurons \cite{50,51} compared the effects of DHX with other D1-like receptor agonists. Single cell recording of D1-like receptor-expressing neurons in the striatum and cortex permitted us to better isolate the Golf- and Gs-dependent effects of D1-like receptor ligands. We could in fact confirm the significant respective predominance of Golf and Gs mRNA expression in these brain areas. We were then able to recapitulate by intracellular recordings the same differential pharmacological properties of DHX observed in vitro, namely striatal Golf-dependent efficacy differences between DHX and the full D1R agonist SKF81297.

Locomotor activation in reserpinized mice has been widely used to characterize striatal post-synaptic DA receptor pharmacology. With short-term reserpinization (about 4 h), D1R or D2R agonists produce no or little significant locomotor activation when administered alone, although a strong synergistic effect is observed upon co-administration of D1R and D2R agonists \cite{36,37}. This situation parallels that in non-reserpinized mice, where endogenous dopamine synergizes with the effect of either D1R or D2R agonists. This explains the ability of either D1R or D2R antagonists to counteract the behavioral effects of both D1R or D2R agonists in non-reserpinized and short-term reserpinized mice \cite{36,37}, as reported for DHX \cite{42}. In contrast, with long-term reserpinization, synergistic effects of D1- and D2-like receptor agonists wane and administration of either agonist produces significant locomotion, which allows a more accurate pharmacological characterization of dopamine receptor ligands \cite{36,37}. Long-term reserpinized mice were therefore used to establish the selective D1-like receptor partial agonistic profile of DHX. DHX produced a mild locomotor activation that was counteracted by the D1-like receptor antagonist SCH23390, but not by the D2-like receptor antagonist eticlopride, at the same dose that completely counteracted locomotor activation induced by the D2R agonist quinpirole. This is in spite of previous studies suggesting less potent but efficacious D2-like receptor agonistic properties of DHX \cite{25,52}. A low potency toward D2-like receptors was also observed in the present study using BRET assays of D1R-, D2R-, and D3R-mediated G-protein activation and AC inhibition. In addition, in the G-protein activation BRET assays, DHX behaved as a partial D2R and D3R agonist as compared with the full agonist quinpirole (61.0% and 55.5% of quinpirole respectively), which may not be sufficient to trigger D2-like receptor-dependent locomotor activation in the reserpinized animal. The partial agonism of DHX in the reserpinized mice model thus confirms the key role of striatal post-synaptic Golf-dependent D1R in the mediation of locomotor activation induced by D1R agonists. In agreement with the electrophysiological experiments, we were able to recapitulate in vivo the lower efficacy of DHX, as compared to SKF81297, in an assay that depends on striatal D1R activation. Although differences in bioavailability between these two compounds cannot be ruled out, the lack of full efficacy with DHX is likely not due to poor brain availability, based on prior

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**Fig. 5 a, b** Effect of D1R agonists on locomotion induced in reserpine-treated mice for DHX (a) and SKF81297 (b). Six bar group represents saline injection, 1.5 mg/kg, 5 mg/kg, 15 mg/kg, 5 mg/kg + 0.5 mg/kg eticlopride, 5 mg/kg + 0.5 mg/kg SCH23390 (left to right). c, d Comparison of the ambulatory distance between DHX and SKF81298 at 5 mg/kg (c) and 15 mg/kg (d). Values were statistically analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test. p-values are as indicated: *p < 0.05, **p < 0.01 or ***p < 0.001. The error bars represent S.E.M.
pharmacokinetic studies in rats\textsuperscript{52} as well as nonhuman pri-
mates\textsuperscript{53}. In addition, various functional studies also exhibit a
rapid onset of DHX activity in rodent brain\textsuperscript{30,51,54}.

In summary, the current study provides the first example of a D1R agonist, DHX, that acts as a full agonist with Gs- and a partial agonist with Golf-dependent signaling. Consequently, D1R agonist, DHX, that acts as a full agonist with Gs- and a pharmacological characterization presented here, in combination biosensor construct that contains Rluc and YFP allowing detection of intracellular interaction. (i) Receptor-G activation, (iii) cAMP production, (iv) glutamine (Gibco), and 1 % penicillin streptomycin (Gibco) and kept in an Untagged \( \beta \gamma \) and G receptor constructs. The cAMP sensor with YFP-Epac-Rluc (CAMYEL) was used for locomotor activity experiments. Animals were housed with food and water available ad libitum in temperature-controlled and humidity-controlled rooms and were maintained on a 12 h light/dark cycle. They were experimentally naive at the start of the study and were maintained under the approved protocol of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse.

\textbf{Methods} 

\textbf{DNA constructs.} Human receptor constructs (D1R, D2SR, DSR, and muscarinic M1 receptor [M1R]) were modified N-terminally with in frame fusion of a signal peptide. Cells were cultured in culture with Dulbecco’s modified Eagle’s medium supplemented with 10 % fetal bovine serum (FBS, Atlanta), 2 mM L-glutamine (Gibco), and 1 % penicillin streptomycin (Gibco) and kept in an incubator at 37 \( ^\circ \)C and 5 % CO\textsubscript{2}. The transfection was analysed in a representative IL2 conformation from the lowest energy cluster of the ensemble generated by molecular dynamics. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation.

\textbf{BRET assays.} Variations of bioluminescence resonance energy transfer (BRET) assay were performed to detect receptor ligand-induced events. A constant amount of plasmid cDNA (15 \( \mu g \)) was transfected into human embryonic kidney cells 293 T (HEK-293T) using polyethylenimine (PEI, Sigma) in a 1:2 weight ratio in 10 cm plates. Cells were washed in culture with Dulbecco’s modified Eagle’s medium supplemented with 10 % fetal bovine serum (FBS, Atlanta), 2 mM L-glutamine (Gibco), and 1 % penicillin streptomycin (Gibco) and kept in an incubator at 37 \( ^\circ \)C and 5 % CO\textsubscript{2}. The transfection was analysed in a representative IL2 conformation from the lowest energy cluster of the ensemble generated by molecular dynamics. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation. The optimized models were further investigated by molecular dynamics simulation.
Single cell or tissue extraction RT–PCR. Quantitative real-time RT–PCR was performed and analyzed using LightCycler instrument 480 II (Roche). Coronal slices were prepared as described in slice electrophysiology. For D1-expressing single cell analysis, D1r1-TdTomato mice were used. After identifying D1TdTdTomato positive cells, cytoplasmic content was collected by micro pipet aspiration for single cell analyses. For tissue extracted analysis, wild type slices were obtained in corresponding brain and processed for cell plasm. Collected cell plasm was immuneprof in buffer provided in a PicoPure RNA isolation kit (Invitro LifeTechnologies/Arcturus), and total RNA was isolated according to kit directions. Total RNA was converted to cDNA, then cDNA was amplified to antisense RNA (aRNA) by in vitro transcribe using the Message BOOSTER cDNA Synthesis Kit (Affymetrix, Madison, WI). The aRNA was purified using Qiagen RNeasy MinElute Cleanup kit and transcript to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). PCR reactions were done in a total volume of 20 µL in PCR mix containing 10 µL 2X LightCycler probe master, 500 nM reference gene primer (GAPDH), 500 nM each of sense and antisense primer, 100 nM each of target gene probe and reference gene probe, and 5 µL of 100 ng cDNA filled up to 20 µL with DEPC-treated H2O. Normalization of sample cDNA content was performed using the comparative threshold (ΔΔCT) cycle method, in which the number of target gene copies was normalized to an endogenous reference gene, GAPDH. CT is defined as the fractional cycle number at which the fluorescence generated by probe cleavage passes a fixed threshold baseline when amplification of the PCR product is first detected. The primers and probes were designed using Universal Probe Library Assay Design Center (Roche). Primer sequences and probes are as follows: 5′-caagagaagtacggacct-3′ (Ftos forward), 5′-gtggctgacgctccg-3′ (Ftos reverse), 5′-atcagctaatgctgctg-3′ (Gtos forward), 5′-caggagaggtctgagacccg-3′ (Gtos reverse), 5′-atccatcgctgctggtg-3′ (GAPDH forward), and 5′-aatccatcgctgctggtg-3′ (GAPDH reverse).

Slice electrophysiology. Experiments were performed based on previous report with modifications33. Coronal slices (220 µm) were prepared from male adult D1r1-TdTomato mice (a vibrating tissue slicer (VT-1000S, Leica)). Animals were anesthetized and perfused with modified artificial cerebral spinal fluid (m-aCSF) containing (in mM): 92 NMDG, 20 HEPES, 25 glucose, 30 NaHCO3, 1.2 1048 (2005). 1039–1048 (2006). 1051–1057 (2007). 1057–1062 (2015). 1061–1065 (2009). 1066–1069 (2012). 1070–1073 (2013). 1074–1077 (2014). 1078–1081 (2015). 1082–1085 (2016). 1086–1089 (2017). 1090–1093 (2018). 1094–1097 (2019). 1098–1099 (2020). 1100–1103 (2021).

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