

Distinct cortical and striatal actions of a β -arrestin-biased dopamine D2 receptor ligand reveal unique antipsychotic-like properties

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The current dopamine (DA) hypothesis of schizophrenia postulates striatal hyperdopaminergia and cortical hypodopaminergia. Although partial agonists at DA D2 receptors (D2Rs), like aripiprazole, were developed to simultaneously target both phenomena, they do not effectively improve cortical dysfunction. In this study, we investigate the potential for newly developed β -arrestin2 (β arr2)-biased D2R partial agonists to simultaneously target hyper- and hypodopaminergia. Using neuron-specific β arr2-KO mice, we show that the antipsychotic-like effects of a β arr2-biased D2R ligand are driven through both striatal antagonism and cortical agonism of D2R- β arr2 signaling. Furthermore, β arr2-biased D2R agonism enhances firing of cortical fast-spiking interneurons. This enhanced cortical agonism of the biased ligand can be attributed to a lack of G-protein signaling and elevated expression of β arr2 and G protein-coupled receptor (GPCR) kinase 2 in the cortex versus the striatum. Therefore, we propose that β arr2-biased D2R ligands that exert region-selective actions could provide a path to develop more effective antipsychotic therapies.

arrestin | antipsychotics | biased signaling | dopamine D2R | fast-spiking interneurons

G protein-coupled receptors (GPCRs) represent the largest family of receptors in the human genome and are one of the most common targets of pharmaceutical drugs (1, 2). Upon ligand binding, GPCRs activate downstream G protein-dependent signaling pathways followed by phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs) (3). Phosphorylation enhances association of the GPCR with β -arrestins (β arrs), and this combined process mediates desensitization of G-protein signaling (4) and internalization of GPCRs (5–7). Two isoforms of β arrs, β arr1 and β arr2, are widely coexpressed in most tissues in mammals and are 80% identical, but they can have either overlapping or distinct functions (8, 9). It is now firmly established that GPCRs activate downstream signaling pathways through not only canonical G-protein pathways but also, the ability of β arrs to scaffold distinct intracellular signaling complexes (10–12). Elucidation of these distinct G-protein and β arr signaling pathways has provided support for the concept of functional selectivity or biased signaling, wherein each signaling pathway has the ability to mediate distinct physiological responses (13). There are now several physiologically relevant examples of selective engagement of signaling pathways or selective GPCR ligands that target these different signaling pathways (13–15). Therefore, leveraging the concept of GPCR functional selectivity holds promise for the development of more selective therapeutic approaches.

Dopamine (DA) is a catecholamine neurotransmitter that has been implicated in movement, reward, and cognition (16–19) as well as CNS disorders, such as schizophrenia, attention deficit hyperactivity disorder, Parkinson's disease, and obsessive-compulsive disorder (20–23). DA mediates its effects via GPCRs belonging to two major subclasses of receptors: the D1 class [D1 receptor (D1R) and D5 receptor] and the D2 class [D2 receptor (D2R), D3 receptor, and D4 receptor] (24), a classification based on their ability to activate the stimulatory G protein $G_{\alpha_{s/olf}}$ or inhibitory G protein $G_{\alpha_{i/o}}$ signaling pathway, respectively. In the brain, D2Rs activate canonical $G_{\alpha_{i/o}}$ -mediated signaling to inhibit adenylyl cyclase, cyclic adenosine monophosphate (cAMP) production, and the protein kinase A/dopamine and cAMP regulated phosphoprotein M_r 32 KDa (PKA/DARPP32) pathway to mediate many of the behavioral effects of DA (23, 25, 26). However, based on the

Significance

Schizophrenia is a debilitating psychiatric disorder characterized by positive, negative, and cognitive symptoms. Current antipsychotic drugs, including D2 receptor (D2R) partial agonist aripiprazole, antagonize excess striatal dopamine (DA) neurotransmission and reverse positive symptoms but are not efficacious at reversing cortical-related cognitive symptoms. Here, we show using pharmacological, behavioral, and electrophysiological approaches that a β -arrestin2 (β arr2)-biased D2R ligand has opposite antagonist and agonist actions in the striatum and cortex, respectively. This phenomenon is regulated by differential expression levels of signal transducer proteins G protein-coupled receptor kinase 2 and β arr2. Thus, D2R- β arr2-biased ligands have the potential to simultaneously target excess striatal and deficient cortical DA neurotransmission and provide more broadly effective therapies for schizophrenia.

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initial observation that the DA-dependent locomotor response to amphetamine (AMPH) was markedly attenuated in mice globally lacking β arr2 but not β arr1 (9), we provided biochemical and genetic evidence for a β arr2-dependent signaling pathway downstream of D2Rs that is separate from $G\alpha_{i/o}$ signaling (27). We have shown that this D2R- β arr2 signaling pathway inhibits protein kinase B (PKB or AKT) activity, activates glycogen synthase kinase 3 beta (GSK3 β), and can mediate specific DA-dependent behaviors (28–30).

D2Rs are the major target for most antipsychotic drugs (APDs), which are the first-line treatment for schizophrenia (31, 32). APDs do not treat all symptoms of schizophrenia effectively and have several side effects that are thought to be associated with $G\alpha_{i/o}$ signaling (26, 30). APDs that selectively target the D2R- β arr2 pathway could be therapeutically beneficial without producing extrapyramidal side effects. To further investigate the potential role of functional selectivity of the D2R- β arr2 pathway in APD action, we have recently generated β arr2-biased D2R ligands based on the scaffold of the partial agonist Aripiprazole (ARI) (33, 34). These biased ligands show antipsychotic-like profile in pharmacological [phencyclidine (PCP) and AMPH] and genetic (NMDA receptor NR1 subunit knockdown) mouse models of schizophrenia-like phenotypes that depend on their D2R- β arr2 selectivity, because their activity is lost in global β arr2-KO mice (33, 35). Although classical APDs, like haloperidol, and newer APDs, like ARI, reverse the postulated striatal hyperdopaminergic tone associated with schizophrenia, none of these drugs effectively correct cortical dysfunction (36–38). It is currently not known whether targeting D2R- β arr2 signaling might represent an alternative strategy to identify more broadly effective APDs.

In this study, we used β arr2-biased D2R ligands and behavioral and electrophysiological approaches in mice lacking β arr2 in various D2R-expressing neuronal populations to investigate whether region-specific D2R- β arr2 signaling contributes to unique antipsychotic-like effects in vivo.

Results

Determinants of in Vitro D2R- β arr2 Functional Selectivity. Clinically effective APDs are either antagonists or partial agonists at both D2R-G protein and D2R- β arr2 signaling pathways (39, 40). We have previously shown that the β arr2-biased D2R ligands UNC9994A (94A) and UNC9975A (75A), which are based on the scaffold of ARI, are weak selective partial agonists at D2R- β arr2 interactions and have little or no agonist activity at D2R-G protein signaling (33). However, we show in Fig. 1 that the agonist versus antagonist profiles of these ligands at D2R- β arr2 interactions in HEK293 cells can be modulated by the complement of GRK2 and β arr2. In a bioluminescence resonance energy transfer (BRET)-based assay (Fig. 1A, C, and E), compared with DA, ARI, 94A and 75A are very weak partial agonists at mediating D2R- β arr2 interactions, but increasing GRK2 levels in these cells markedly enhanced the partial agonist activity of only ARI and 94A. Consistent with pharmacological principles, when tested as antagonists (Fig. 1B, D, and F), all three ligands fully antagonized DA-mediated D2R- β arr2 interactions, and high GRK2 levels reduced the antagonist efficacy of only ARI and 94A. The profile of 75A was not significantly changed by GRK2, suggesting that it may have slightly different properties than ARI or 94A. However, in a D2R-G protein (GloSensor) assay, ARI and 75A but not 94A behaved as antagonists (Fig. S1 and Table S1), suggesting that only 94A is a completely selective D2R- β arr2 ligand. These results are consistent with the established concept that β arr-dependent GPCR functions are not only dependent on agonist activation but also, enhanced by phosphorylation of the receptor by GRKs (11, 41) and that the expression levels of β arr2 and GRK2 can regulate GPCR signaling and the pharmacological profile of ligands (42–44).

Previous studies have suggested that the levels of GRKs and β arrs can vary significantly between tissues and brain areas (45, 46).

β -arrestin2 BRET

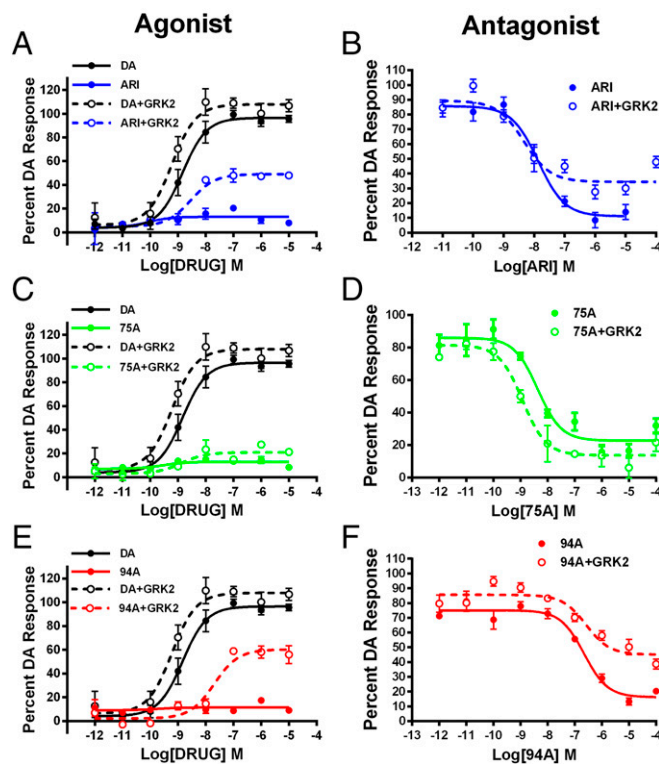


Fig. 1. Effects of GRK2 overexpression on the agonist or antagonist profile of D2R ligands in HEK293 cells. (A) ARI, (C) UNC9975A (75A), and (E) UNC9994A (94A) were tested in agonist mode in a D2R- β arr2 interaction BRET assay with endogenous GRK2 (solid lines) or GRK2 overexpression (dashed lines) compared with DA (black lines) in HEK293 cells. D2R- β arr2 BRET antagonist assays for (B) ARI, (D) UNC9975A (75A), and (F) UNC9994A (94A) with endogenous GRK2 (solid lines) or overexpressed GRK2 (dashed lines) levels in HEK293 cells. Data are presented as inhibition of the total DA response (mean \pm SEM).

Protein levels of β arr2 and GRK2 but not β arr1 are higher in the prefrontal cortex (PFC) compared with the striatum (STR) in mice (Fig. 2A–D) and humans (Fig. S2A and B) (P s < 0.05). Because ARI and 94A gain D2R- β arr2 partial agonism on GRK2 overexpression, we hypothesized that these compounds might behave as agonists in the PFC (high β arr2/GRK2). Previous studies have shown that local injection of the D2R agonist quinpirole can exert antipsychotic-like effects, because it inhibits locomotion induced by the NMDA receptor antagonist PCP (47). PCP-induced locomotion is a pharmacologically induced behavior commonly inhibited by APDs, and the behavioral effects of PCP are thought to be mediated by cortical disinhibition (48). To assess whether PFC D2R- β arr2 agonism can elicit antipsychotic-like effects, we measured the effects of injecting quinpirole, ARI, and 94A locally into the PFC of WT mice on PCP-induced locomotion. Consistent with previous findings, local bilateral PFC injection of the unbiased full-agonist quinpirole significantly inhibited PCP-induced locomotion (Fig. 2E, QUIN) (P < 0.01); 94A mimicked this D2R agonist action (Fig. 2E, 94A) (P < 0.05) and inhibited PCP-induced locomotion, whereas the effect of ARI was weaker and did not reach significance. Coadministration of a pharmacological GRK2 inhibitor [compound 101 (cpd101)] prevented the 94A-mediated inhibition of PCP-induced locomotion (Fig. 2F, cpd101/94A). These results are consistent with our in vitro data, showing that, in a D2R- β arr2 interaction BRET assay, the ability of GRK2 to enhance the agonist effects of both DA and UNC9994A is lost in presence of the GRK2 inhibitor (cpd101) (Fig. S2C). These observations

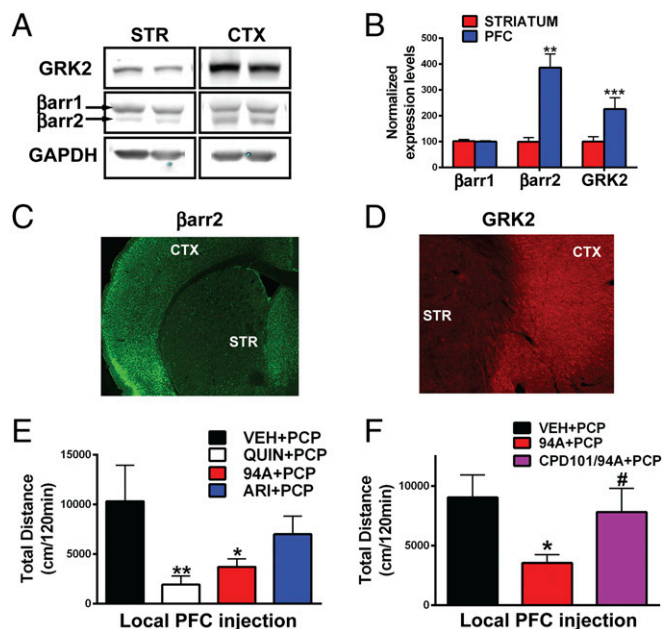


Fig. 2. Cortical and striatal expression patterns of β arr2 and GRK2 as well as D2R PFC agonism. (A) Western blot analysis from WT mice probed with antibodies to GRK2, β arr2, and β arr1 and GAPDH in cortex (CTX) compared with STR and (B) quantification of Western blot band intensities normalized to GAPDH (loading control; $P_s < 0.05$). IHC images of mouse brain sections (cortical-striatal) stained with antibodies to (C) β arr2 and (D) GRK2. Locomotor responses to (E) bilateral local PFC injection 1 μ g per side quinpirole (QUIN), UNC9994A (94A), and aripiprazole (ARI) in WT mice followed by systemic PCP injection (6 mg/kg i.p.; $n = 8-11$) or (F) bilateral local PFC injection of 1 μ g per side UNC9994A (94A) with or without cpd101 (0.5 μ g per side) in WT mice followed by systemic PCP injection (6 mg/kg i.p.; $n = 8-10$). * $P < 0.05$, compared with VEH + PCP; ** $P < 0.01$, compared with VEH + PCP; # $P < 0.05$, compared with 94A + PCP.

show that D2R- β arr2 agonism in the PFC is sufficient to inhibit PCP-induced locomotion, and this effect is dependent on β arr and GRK2.

Distinct Striatal Vs. Cortical Role of D2R on PCP-Mediated Behavioral Response. Although D2R PFC agonism inhibits PCP-induced locomotion, striatal D2R antagonism is thought to be the primary mechanism of action of most APDs (49, 50). To broadly explore this paradoxical role of D2R function behaviorally, we selectively deleted D2Rs in either the PFC by injecting an mCherry-Cre adeno-associated virus (AAV) in the previously described D2R floxed ($D2^{fl/fl}$) mice (51) or the STR by crossing $D2^{fl/fl}$ mice with adenosine 2A receptor Cre (A2aCre) mice (Fig. S3), and we tested the effect on PCP-induced locomotion. We observed that, compared with controls, deletion of D2Rs in the PFC resulted in an enhanced response to acute PCP injection (Fig. 3A) ($P_s < 0.01$), consistent with D2R agonism playing an inhibitory role on the PCP response (47). However, deletion of D2Rs in the STR attenuated the PCP response (Fig. 3B) compared with controls ($P_s < 0.01$), consistent with striatal D2R antagonism inhibiting PCP-mediated responses (49, 50). These pharmacological and behavioral data suggest distinct roles for D2R signaling in the PFC versus the STR in mediating antipsychotic-like effects.

Generation and Characterization of β arr2 Floxed Mice. To further evaluate the potential opposite role of D2R- β arr2 signaling in the PFC and STR in antipsychotic-like effects, we generated a β arr2 floxed (β arr2^{fl/fl}) mouse line for region- and cell-specific Cre-dependent deletion of β arr2 (details are in *Materials and Methods* and *SI Materials and Methods*; Fig. S4A and B); β arr2^{fl/fl} mice were

indistinguishable from C57BL/6J mice in standard behavioral tests (Fig. S4C-E). Deleting β arr2 in all neurons (β arr2^{fl/fl} CMV-Cre) recapitulated the decrease in the AMPH locomotor response originally observed in whole-body β arr2KO mice (27) (Fig. S4F-H). We have previously shown that the AMPH locomotor response is mediated at least in part by D2Rs in a β arr2-dependent manner (27, 29), but D2Rs are expressed in multiple regions of the brain, such as midbrain, STR, and PFC (24, 52-57). To confirm which D2R+ population of neurons is responsible for the AMPH response, we deleted β arr2 in several distinct neuronal populations; β arr2^{fl/fl} mice were crossed with either D2Cre (all D2R+ neurons) or A2aCre (postsynaptic D2R+ striatal neurons) mice as well as the D1Cre (D1R+ neurons) or ChaTCre (cholinergic interneurons) mice as controls. Using immunohistochemistry (IHC) and real-time quantitative PCR techniques, we confirmed selective deletion of β arr2 in appropriate neuronal populations of all Cre lines crossed with the β arr2^{fl/fl} mice (Fig. S5). As shown in Fig. 4, β arr2 in striatal D2R+ neurons seems to play the most prominent role in the AMPH response, because the locomotor response is reduced only in D2R+ or A2aR+ neurons lacking β arr2 (Fig. 4E-H) but not affected by deletion of β arr2 in either D1R+ neurons alone or cholinergic interneurons (Fig. 4C, D, and I). These data suggest that deleting β arr2 in D2R striatal neurons is sufficient to mimic antipsychotic-like activity. Importantly, the mice in which β arr2 is inactivated in select D2R+ neuronal populations provide unprecedented models to critically examine the in vivo antipsychotic-like function of our D2R- β arr2-biased compounds.

Region-Specific Responses of Antipsychotics and β arr2-Biased D2R Ligands. AMPH- and PCP-induced hyperlocomotion are the two commonly used pharmacological models to test APD efficacy. Most APDs are D2R partial agonists or antagonists with varying potencies and efficacies (31, 32, 40, 58) and inhibit either the AMPH- or PCP-induced locomotor response in mice (59). The AMPH-induced locomotor response is dependent on striatal DA release, whereas the behavioral effects of PCP are thought to be mediated by cortical disinhibition and activation of the cortico-striatal pathway (48, 50). We tested the ability of representative first, second, and third generation APDs, such as haloperidol, clozapine, and ARI, respectively, along with the β arr2-biased D2R ligands 94A and 75A in both of these pharmacological models. Optimal doses for APDs and the D2R- β arr2-biased ligands were based on previous studies (30, 33, 35).

For the AMPH-induced locomotor response, AMPH was injected at a dose (3 mg/kg) at which there were no significant

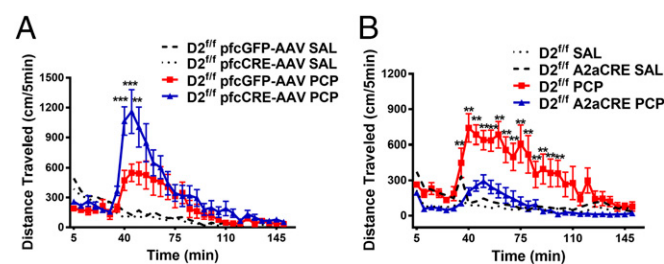


Fig. 3. Distinct contribution of striatal and cortical D2Rs to the behavioral effects of PCP. (A) $D2^{fl/fl}$ mice were injected with a control GFP virus (GFP-AAV) or Cre-AAV in the PFC and 3 wk later, injected systemically with either saline (SAL) or PCP (4 mg/kg i.p.); their locomotor response was recorded ($n = 8-9$). ** $P < 0.01$; *** $P < 0.001$ compare $D2^{fl/fl}$ pfcGFP-AAV with $D2^{fl/fl}$ pfcCre-AAV (PCP) using a three-way RMANOVA [genotype \times treatment \times time interaction, $F(29, 720) = 1.947$, $P < 0.01$] with post hoc Bonferroni tests. (B) $D2^{fl/fl}$ mice were crossed with A2aCre mice ($D2^{fl/fl}$ A2aCre) to delete D2Rs in the STR and injected systemically with either SAL or PCP (6 mg/kg i.p.); their locomotor response was recorded ($n = 8-13$). ** $P < 0.01$; *** $P < 0.001$ compare $D2^{fl/fl}$ with $D2^{fl/fl}$ A2aCre (PCP) using a three-way RMANOVA [genotype \times treatment \times time interaction, $F(29, 840) = 3.722$, $P < 0.001$] with post hoc Bonferroni tests.

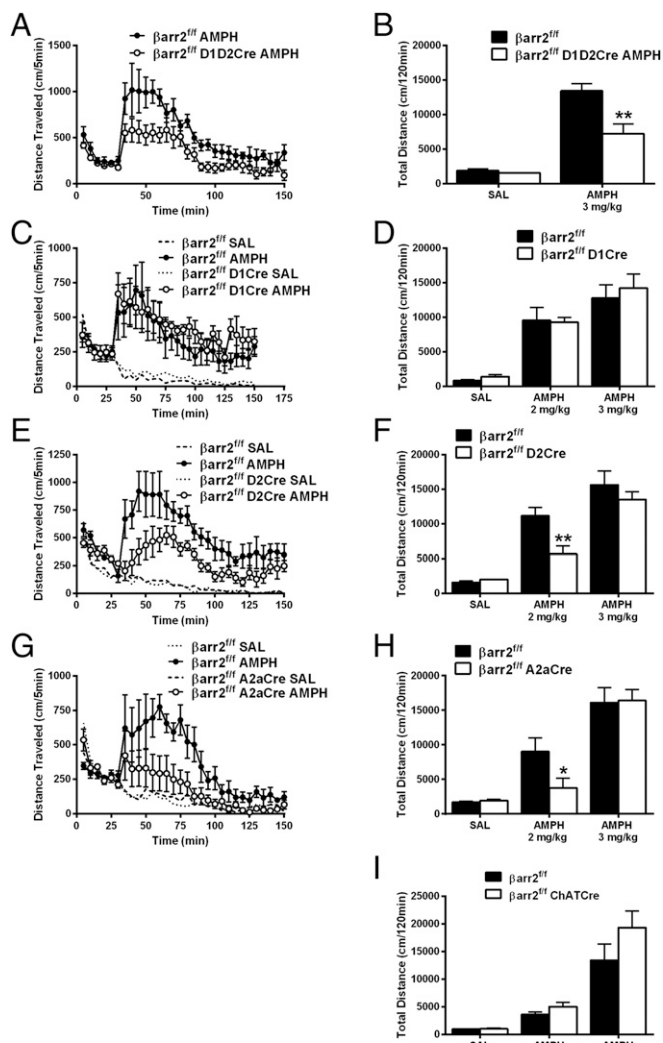


Fig. 4. Deletion of $\beta arr2$ in D2R-expressing neurons inhibits the AMPH response. (A) $\beta arr2^{fl/fl}$ mice were crossed with both D1Cre and D2Cre mice to delete $\beta arr2$ in all striatal neurons simultaneously to generate $\beta arr2^{fl/fl}$ D1D2Cre and injected with 3 mg/kg AMPH; distance traveled was calculated for 120 min after 30 min of habituation compared with control $\beta arr2^{fl/fl}$ mice. Data were analyzed using a two-way RMANOVA test [genotype \times time interaction, $F(29, 145) = 1.956$, $P < 0.01$] followed by Bonferroni comparisons. (B) Total cumulative distance after injection of saline (SAL) and 3 mg/kg AMPH was calculated for $\beta arr2^{fl/fl}$ D1D2Cre and $\beta arr2^{fl/fl}$ controls. $**P < 0.01$, using a two-way ANOVA (Bonferroni) test ($n = 7$ mice for each genotype). (C) $\beta arr2^{fl/fl}$ D1Cre, (E) $\beta arr2^{fl/fl}$ D2Cre, or (G) $\beta arr2^{fl/fl}$ A2aCre and their respective Cre-negative $\beta arr2^{fl/fl}$ controls were injected with SAL or 2 mg/kg AMPH after 30 min of habituation, and locomotor activity was measured for 120 min. Data were analyzed using a three-way RMANOVA for D1 $\beta arr2$ [genotype \times time interaction, $F(29, 116) = 0.4045$, $P = 0.9967$; genotype \times treatment interaction, $F(1, 480) = 3.463$, $P = 0.0634$], A2a $\beta arr2$ [genotype \times time interaction, $F(87, 435) = 4.760$; genotype \times treatment interaction, $F(1, 600) = 47.15$, $P < 0.001$], and D2 $\beta arr2$ [genotype \times time interaction, $F(87, 261) = 3.324$, $P < 0.001$; genotype \times treatment interaction, $F(1, 360) = 66.37$, $P < 0.001$] with post hoc Bonferroni tests. (D, F, and H) Total cumulative (120 min) postinjection distance after SAL or 2 or 3 mg/kg AMPH; $n = 8$ mice for each group. $*P < 0.05$, compared with $\beta arr2^{fl/fl}$ using a two-way ANOVA (Bonferroni) test; $**P < 0.01$, compared with $\beta arr2^{fl/fl}$ using a two-way ANOVA (Bonferroni) test. (I) ChAT-Cre mice were crossed with $\beta arr2^{fl/fl}$ mice to generate $\beta arr2^{fl/fl}$ ChATCre or $\beta arr2^{fl/fl}$ controls, and total cumulative distance after postinjection of SAL or 2 or 3 mg/kg AMPH was calculated; $n = 8$ mice for each group.

genotype differences between mice (Fig. 4 C–H). This higher dose allowed us to achieve better separation of effects when treating with APDs and biased compounds. Consistent with their antagonist

activity, all tested APDs and biased D2R ligands significantly inhibited AMPH-induced locomotion in all control $\beta arr2^{fl/fl}$ mice (Fig. 5, black bars) ($P_s < 0.01$). No genotype differences were observed in mice lacking $\beta arr2$ in D1R+ neurons for all compounds (Fig. 5 A and B) ($P_s < 0.01$). All three APDs and 75A also inhibited the AMPH response ($P_s < 0.01$) in mice lacking $\beta arr2$ in either all D2R+ (Fig. 5C) or striatal A2aR+ neurons (Fig. 5E). However, in these mice, 94A showed markedly diminished antipsychotic-like activity compared with genotype controls ($P_s < 0.05$), consistent with this compound being a selective $\beta arr2$ -biased D2R antagonist. Compared with other APDs, the observations with 94A indicate that D2R- $\beta arr2$ antagonism is sufficient but not necessary for efficacious antipsychotic-like activity in the AMPH pharmacological model, which is consistent with previous observations (60).

For PCP-induced responses, in mice lacking $\beta arr2$ in D1R+ neurons, all drugs significantly inhibited PCP-induced locomotion compared with vehicle (VEH)-treated controls (Fig. 6A) ($P_s < 0.05$), and there were no genotype differences. ARI and 75A also inhibited PCP-induced locomotion in mice lacking $\beta arr2$ in D2R+ striatal neurons (Fig. 6B) ($P_s < 0.05$) or all D2R+ neurons (Fig. 6C) ($P_s < 0.05$) compared with genotype and VEH controls. In contrast to the AMPH response (Fig. 4), however, we observed a loss of 94A activity only in mice lacking $\beta arr2$ in all D2R+ neurons

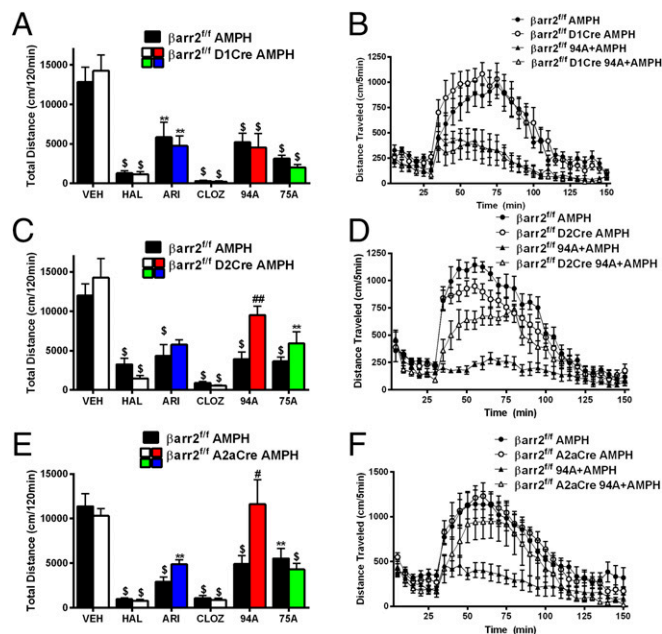


Fig. 5. UNC9994A loses its antipsychotic-like activity in response to AMPH in D2R+ and A2aR+ neuron-specific $\beta arr2$ KO mice. Control mice ($\beta arr2^{fl/fl}$) and (A) $\beta arr2^{fl/fl}$ D1Cre, (C) $\beta arr2^{fl/fl}$ D2Cre, or (E) $\beta arr2^{fl/fl}$ A2aCre mice were injected with vehicle (VEH); the antipsychotics haloperidol (HAL; 0.5 mg/kg), ARI (0.5 mg/kg), or clozapine (CLOZ; 2 mg/kg); or $\beta arr2$ -biased drugs UNC9994A (94A; 2 mg/kg) or UNC9975A (75A; 0.5 mg/kg) followed by 3 mg/kg AMPH injection. Total cumulative distance postinjection of AMPH for 120 min was calculated and shows that all APDs and drugs, except UNC9994A, are able to inhibit the AMPH response in the $\beta arr2^{fl/fl}$ D2Cre and $\beta arr2^{fl/fl}$ A2aCre mice. $**P < 0.01$, compared with respective VEH control; $^*P < 0.001$, compared with respective VEH control; $^{\#}P < 0.05$ compare 94A between genotypes using a two-way ANOVA (Bonferroni) test; $^{\#\#}P < 0.01$ compare 94A between genotypes using a two-way ANOVA (Bonferroni) test. Representative graphs of AMPH inhibition by 94A for (B) $\beta arr2^{fl/fl}$ D1Cre, (D) $\beta arr2^{fl/fl}$ D2Cre, or (F) $\beta arr2^{fl/fl}$ A2aCre mice compared with controls ($\beta arr2^{fl/fl}$); $n = 8$ mice for each group. Data were analyzed by two-way RMANOVA [genotype \times treatment interaction, $F(1, 420) = 2.053$, $P = 0.1526$, for $\beta arr2^{fl/fl}$ D1Cre; genotype \times treatment interaction, $F(29, 420) = 3.858$, $P < 0.05$, for $\beta arr2^{fl/fl}$ A2aCre; and genotype \times treatment interaction, $F(29, 420) = 2.285$, $P < 0.01$, for $\beta arr2^{fl/fl}$ D2Cre] with post hoc Bonferroni tests.

(Fig. 6B) ($P = 0.2526$) but not in striatal D2R+ neurons (Fig. 6C) ($P < 0.05$), suggesting a role for β arr2 outside the STR, because A2aCre is essentially STR-selective. Although the D2Cre is expressed in STR, PFC, and midbrain, a cortical role for β arr2 in 94A-mediated inhibition of PCP-induced locomotion is most likely, because evidence suggests against a role for midbrain DA neuron β arr2 (61) and the primary site of action for PCP is in the PFC. However, to further rule out the contribution of midbrain pre-synaptic β arr2 and confirm a role for cortical β arr2, we deleted β arr2 in PFC and STR or PFC alone by injecting mCherry-Cre AAV in the PFC of β arr2^{fl/fl} A2aCre (PFC + STR β arr2KO) or β arr2^{fl/fl} mice (PFC β arr2KO) (Fig. S6); 94A inhibited PCP-induced locomotion in PFC β arr2KO mice (Fig. 6D) ($P < 0.05$) but lost its antipsychotic-like activity when β arr2 was deleted in both PFC and STR (Fig. 6D), thus confirming a dual dependence on cortical and striatal β arr2. These data suggest that cortical and striatal β arr2 are necessary for the antipsychotic-like effect of 94A. Thus, our behavioral data further support our initial supposition that distinct mechanisms might regulate the antipsychotic-like effect of D2R- β arr2 signaling in the PFC (agonism) versus the STR (antagonism). We next wanted to confirm the neuronal mechanism of these distinct phenomena electrophysiologically in the PFC and STR.

Effect of 94A on Excitability of Cortical D2R+ Fast-Spiking Interneurons.

The PFC comprises multiple neuronal cell types, and many of these neurons, in particular GABA interneurons and pyramidal glutamatergic neurons, express D2Rs (56, 62–64). GABAergic interneurons are thought to play a critical role in schizophrenia pathophysiology in humans and animal models of schizophrenia. Postmortem brain analyses of patients (65–71) and behavioral studies in rodents have implicated GABAergic parvalbumin+ (PV+) fast-spiking interneurons (FSIs) in altered excitation–inhibition imbalance and cognitive impairment in schizophrenia

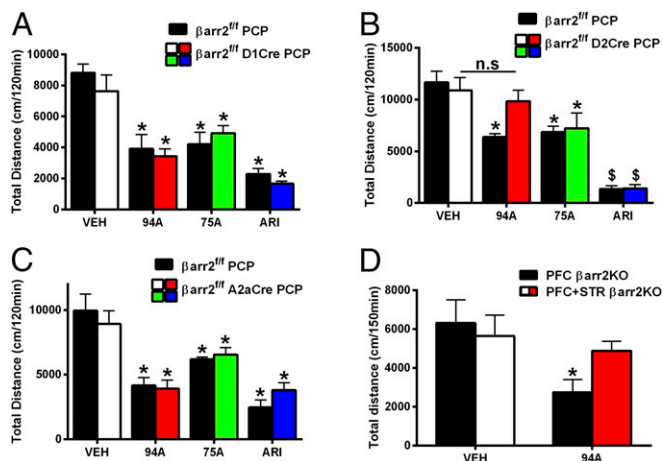


Fig. 6. UNC9994A loses antipsychotic-like activity to PCP in D2R+ but not A2aR+ neuron-specific β arr2KO mice. Control mice (β arr2^{fl/fl}) and (A) β arr2^{fl/fl} D1Cre, (B) β arr2^{fl/fl} D2Cre, or (C) β arr2^{fl/fl} A2aCre mice were injected with VEH, ARI (0.5 mg/kg), or β arr-biased drugs UNC9994A (94A; 2 mg/kg) and UNC9975A (75A; 0.5 mg/kg) followed by 6 mg/kg PCP injection 10 min later. Total cumulative distance postinjection of PCP for 120 min was calculated; $n = 8$ –10 mice for each group. One mouse each from the β arr2^{fl/fl} A2aCre 94A- and 75A-treated groups was discarded based on criterion set in *Statistical Analyses*. * $P < 0.05$, compared with respective VEH controls using a two-way ANOVA (Bonferroni) test; $^{\$}P < 0.001$, compared with respective VEH controls using a two-way ANOVA (Bonferroni) test. (D) mCherry-Cre AAV8 was injected into the PFC of β arr2^{fl/fl} A2aCre (PFC + STR β arr2KO) or β arr2^{fl/fl} (PFC β arr2KO) mice followed by injection 3 wk later with 94A (2 mg/kg i.p.) and PCP (6 mg/kg i.p.). Total cumulative distance postinjection of PCP for 120 min was calculated; $n = 8$ mice for each group. * $P < 0.05$, compared with respective VEH controls using a two-way ANOVA (Bonferroni) test.

(72, 73). The D2R agonist quinpirole increases FSI excitability in adult rodents in a similar fashion as D1R agonists (64), suggesting that this excitatory effect is not mediated by the canonical inhibitory $G\alpha_{i/o}$ activation but presumably, is through a G protein-independent pathway. Our data suggest that the higher levels of β arr2 and GRK2 in the cortex might support this G protein-independent agonist signaling of D2R ligands (Figs. 1 and 2). Additionally, the elevated β arr2 and GRK2 expression is present in PFC PV+ FSI (Fig. S7) and presumably, pyramidal neurons (non-PV+ cells) as well. Given the pharmacological, genetic, and behavioral evidence for the importance of FSIs in schizophrenia pathophysiology, we chose to determine the functional impact of higher cortical levels of β arr2 and GRK2 in FSIs. We performed whole-cell, current clamp slice recordings in prefrontal GAD67+ FSI from control (β arr2^{+/+}) and global β arr2KO mice and assessed the effects of UNC9994A. FSIs were visually identified in acute slices from Gad1-eGFP adult mice and further identified by their responses to hyperpolarizing and depolarizing current injections (Fig. 7A). As expected from previous studies with D2R agonists, like quinpirole (64), ARI and 94A increased prefrontal FSI excitability as measured by changes in FSI action potential frequency in response to minimal amounts of depolarizing current injection. The D2R partial agonist ARI (10 μ M) elicited a modest increase in action potential firing ($+6.2 \pm 1.4$ Hz after 20 min of exposure relative to a 10 min predrug baseline; $n = 6$) (Fig. 7B). This effect was prevented by the D2R antagonist eticlopride (10 μ M) and even led to a slight decrease in FSI excitability (-2.1 ± 2.2 Hz after 20 min of exposure of 10 μ M eticlopride and 10 μ M ARI; $n = 4$) (Fig. 7B). These data suggest that ARI has modest D2R agonist-like activity on FSIs in the PFC and are consistent with the partial agonist activity observed with ARI when GRK2 and β arr2 are overexpressed in HEK293 cells (Fig. 1B, D, and F). Interestingly, 94A (10 μ M) elicited a significantly more robust increase in FSI excitability compared with ARI ($+25.8 \pm 3.8$ Hz after 20 min of 94A; $+6.1 \pm 1.4$ Hz after 20 min of 10 μ M ARI; $n = 4$ and $n = 6$, respectively; $P < 0.01$) (Fig. 7C and D). Bath application of eticlopride (10 μ M) also prevented the increase in FSI excitability by 94A and again, led to a slight decrease in FSI excitability [-5.0 ± 12.6 Hz after 20 min of exposure to eticlopride and 94A (10 μ M); $n = 3$]. Additionally, the effects of 94A were dependent on β arr2-signaling, because UNC9994A failed to enhance FSI excitability in β arr2KO mice [$+1.3 \pm 1.6$ Hz after 20 min of 94A (10 μ M)] (Fig. 7E). Finally, the presence of the membrane-permeable GRK2 inhibitor cpd101 (30 μ M) prevented the increase in FSI excitability elicited by 94A (Fig. 7F) (-2.3 ± 3.2 Hz after 20 min of exposure of cpd101 and 94A; $n = 5$). These data further highlight that 94A requires GRK2 in addition to β arr2 to produce its D2R-dependent, agonist-like effects in cortical FSI.

Effect of 94A on Excitability of Striatal D2R+ Medium Spiny Neurons.

Based on the above behavioral and biochemical observations, because β arr2 and GRK2 levels are significantly lower in the STR than in the PFC, one might expect that the agonist activity of 94A would be reduced in striatal neurons. The D2R agonist quinpirole decreased excitability of striatal medium spiny neurons [MSNs; -6.8 ± 2.2 Hz after 20 min of quinpirole (10 μ M); $n = 8$] (Fig. 8B, black line). These findings are consistent with previous reports showing that D2R agonists reduce the excitability of striatal MSNs (74). Unlike quinpirole, 94A produced a negligible reduction in striatal MSN excitability [-2.6 ± 1.0 Hz after 20 min of 94A (10 μ M); $n = 8$; $P = 0.0137$ compared with quinpirole] (Fig. 8B and C). Thus, 94A lacks agonist-like activity in striatal MSNs, showing that D2R- β arr2-biased ligands can have distinct electrophysiological actions in the PFC and STR.

Discussion

We used neuron-specific β arr2KO mice to characterize the role of β arr2 in striatal and cortical circuits and their involvement in

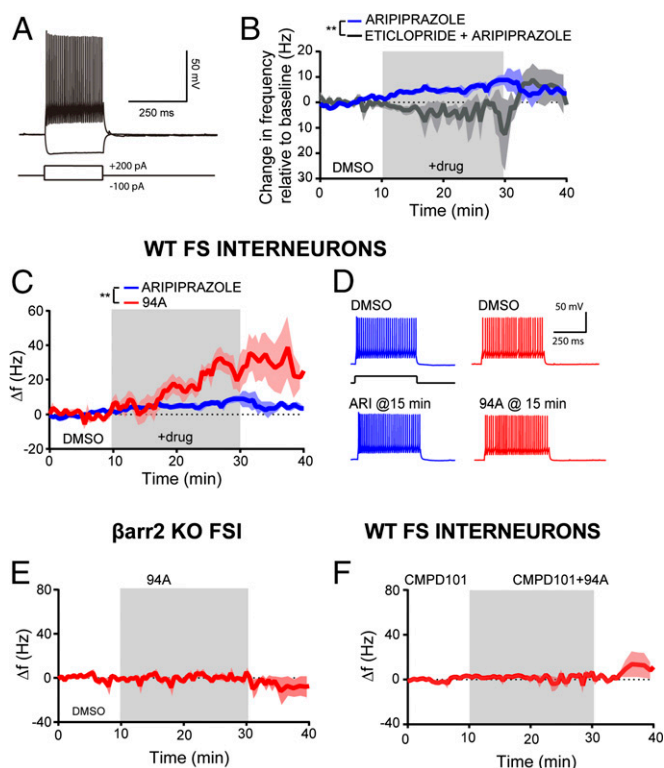


Fig. 7. UNC9994A has β arr2- and GRK2-dependent, agonist-like effects in prefrontal GABAergic FSIs. (A) Sample recording from a prefrontal FSI showing responses to hyperpolarizing or depolarizing current injection. (B) ARI (10 μ M) increases action potential firing in prefrontal FSIs [$+6.2 \pm 1.4$ Hz (relative to a 10-min predrug baseline) after 20 min of exposure; $n = 6$]. Bath application of eticlopride (10 μ M) prevented the increase in excitability elicited by ARI (-2.08 ± 2.2 Hz after 15 min of exposure of eticlopride + ARI; $n = 4$). $**P < 0.01$. (C) UNC9994A (94A; 10 μ M) elicited a greater increase in FSI excitability ($+25.86 \pm 3.8$ Hz after 20 min of exposure; $n = 4$) compared with ARI. Data were analyzed using a standard two-way ANOVA test. $**P < 0.01$. (D) Sample responses of FSIs to depolarizing current pulses in various pharmacologic conditions showing an increase in FSI excitability after bath application of 94A or ARI. (E) The 94A-mediated increase in FSI excitability is absent in β arr2KO mice ($+1.32 \pm 1.68$ Hz after 20 min of exposure; $n = 4$). (F) Bath application of the GRK2 inhibitor cpd101 (30 μ M) prevented the increase in excitability elicited by 94A (-2.3 ± 3.2 Hz after 20 min of exposure of cpd101 + 94A; $n = 5$). FS, fast spiking.

pharmacological models of antipsychotic-like action. The β arr2-biased D2R ligand 94A showed a paradoxical pharmacological profile in biochemical, behavioral, and electrophysiological assays; 94A reversed the hyperlocomotor responses to both AMPH and PCP through both striatal antagonism and cortical agonism. Furthermore, the agonist activity of 94A increased the firing of PFC D2R-expressing FSI in a β arr2- and GRK2-dependent manner but did not mimic quinpirole's ability to inhibit firing of striatal D2R+ MSNs. These contrasting electrophysiological, behavioral, and biochemical effects are consistent with higher PFC expression of β arr2 and GRK2 compared with STR. These data suggest that biased agonism of D2R- β arr2 in the PFC could add a dimension of cortical benefits in addition to the striatal antagonist profile characteristic of clinically efficacious APDs.

The opposite pharmacological action of 94A in the STR and PFC in both behavioral and electrophysiological assays is consistent with the higher expression of β arr2 and GRK2 in PFC compared to STR. Although we show that both ARI and 94A lack basal agonist activity when direct D2R- β arr2 interactions are assessed by BRET in HEK293 cells, the partial agonist activity of these compounds can be revealed when using assays such as the TANGO and DiscoverX (33), which markedly amplify the

signal. Similarly, when the D2R- β arr2 BRET assay is performed in the presence of overexpressed GRK2, which recapitulates PFC expression patterns, both 94A and ARI now show similar D2R- β arr2 partial agonist activity. However, somewhat unexpectedly, 94A is more efficacious than ARI not only at inhibiting PCP-induced locomotion following local PFC injection but also, in enhancing firing of PV+ FSIs of the PFC in a D2R-, GRK2-, and β arr2-dependent manner. This enhanced PFC antipsychotic-like effect and excitability of PV+ PFC FSIs by 94A can be attributed to the relative balance between $G\alpha_{i/o}$ (neuronal inhibition) and β arr2 (neuronal excitation) agonist activities; 94A has no $G\alpha_{i/o}$ agonist activity (33), whereas ARI is a $G\alpha_{i/o}$ partial agonist. In contrast to the PFC, the STR has low expression of GRK2 and β arr2, and we showed in vitro that, with low levels of GRK2 expression, 94A is an antagonist at the D2R- β arr2 pathway. Furthermore, 94A does not mimic the effect of the D2R agonist quinpirole on striatal D2R+ MSN firing and inhibits the striatal AMPH response consistent with its antagonist-like properties in vivo. Therefore, although behaviorally, 94A has the same effect (i.e., inhibition of the locomotor response), it has opposite effects pharmacologically and on neuronal firing in the PFC versus STR. Consistent with the above observations with 94A, D2R deletion also has opposite effects in PFC versus STR on the PCP response. Although the antipsychotic-like effect of 94A is selectively dependent on the D2R- β arr2 pathway, other APDs do not require β arr2 for their antipsychotic-like activity, suggesting that targeting β arr2 is sufficient but not necessary for antipsychotic-like activity (60). However, our data suggest that targeting the D2R- β arr2 pathway may generate unique antipsychotic-like actions that current APDs do not achieve.

D2R antagonist and partial agonist APDs are the primary treatment options for schizophrenia, but there are several disease domains, such as negative symptoms and cognitive deficits, that are unaffected by APDs. The prevalent DA hypothesis of schizophrenia posits that this disorder presents with reduced cortical DA tone but enhanced striatal DA release, with these spatially distinct manifestations being interdependent (20, 75–77). Brain imaging studies

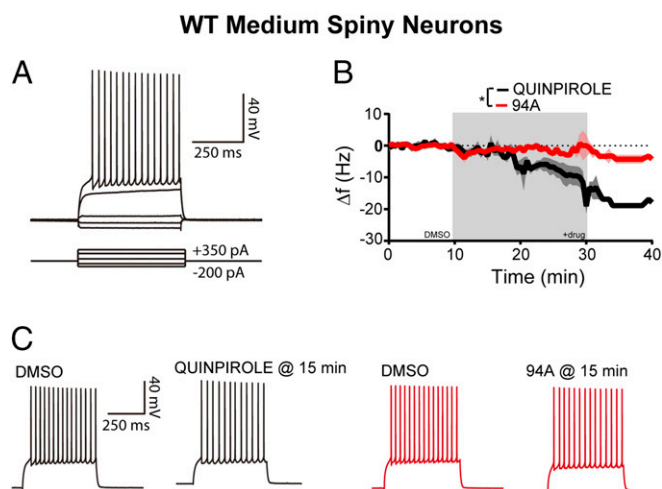


Fig. 8. UNC9994A lacks agonist-like effects in the STR. (A) Sample recording from striatal MSNs showing responses to hyperpolarizing or depolarizing current injection. (B) The D2R agonist quinpirole (10 μ M) decreases action potential firing in striatal MSNs (-6.8 ± 2.2 Hz relative to a 10-min predrug baseline after 20 min of exposure; $n = 8$). UNC9994A (94A; 10 μ M) elicits a significantly smaller decrease in MSN excitability compared with quinpirole (-2.6 ± 1.0 Hz after 20 min of exposure; $n = 8$). Data were analyzed using a standard two-way ANOVA test. $*P = 0.0137$. (C) Representative traces of striatal MSNs to depolarizing current pulses in control conditions and after quinpirole or 94A application.

in schizophrenia patients have shown that AMPH-induced DA release is enhanced in the STR (78) (hyperdopaminergia), whereas a recent study has shown, for the first time, cortical hypodopaminergia in schizophrenia patients (79). Partial agonists, such as ARI, were originally developed to counteract these opposite phenomena but have largely been unsuccessful in correcting cortical dysfunction (80, 81). Our behavioral and electrophysiological data show that the ARI-derived β arr2-biased D2R ligand, 94A, can act as a D2R- β arr2 agonist in the PFC but a D2R- β arr2 antagonist on striatal D2 MSNs, highlighting the feasibility of a pharmacological approach, where a drug could potentially simultaneously counteract both the cortical hypodopaminergia with D2R- β arr2 agonism and striatal hyperdopaminergia with D2R- β arr2 antagonism in schizophrenia.

Our results indicate that 94A acts as an agonist at D2R- β arr2 signaling in GABAergic FSIs. This effect may contribute to the antipsychotic-like action that we observed in PCP-induced locomotion and most likely, will contribute to resetting excitation inhibition balance in cortical circuits. However, we cannot exclude the contribution of D2R-expressing pyramidal neurons that may also have elevated levels of β arr2 and GRK2. However, DA released by PFC-projecting mesocortical DA neurons inhibits pyramidal neurons primarily by activating interneurons (82), suggesting a temporally preceding role for FSIs. To target the exact subtype of cortical neurons for more specific behavioral analyses would require developing novel Cre driver lines involving at least triple-intersectional approaches. Appropriate selectively targeted Cre lines for various D2R neuronal subtypes in the PFC (83, 84) are currently lacking. After appropriate targeting is achieved, signaling effectors downstream of D2R- β arr2 in the PFC can be elucidated. Although the downstream cellular targets of D2R-G protein, such as PKA/DARPP32 signaling, are well-established (23, 25, 85), possible downstream D2R- β arr2 signaling effectors, such as GSK3 β , AMPA, and NMDA receptors, are only now beginning to be revealed (86, 87). In conclusion, our data provide evidence suggesting that combining the concept of functional selectivity and taking into account region- and cell-specific receptor and transducer expression patterns have the potential to generate more effective therapies and at the same time, reduce side effects.

Materials and Methods

Animals and Drugs. All mouse studies were conducted in accordance with the NIH guidelines for animal care and use and through animal protocols approved by the Duke University Animal Care and Use Committee and Pfizer's Institutional Animal Care and Use Committee. All mice were housed in a 12-h light-dark cycle at a maximum of five per cage, provided with food and water ad libitum, and tested at 10–20 wk of age. Details of mouse lines used, generation of β arr2^{fl/fl} animals, and drugs and chemicals used are in *SI Materials and Methods*.

Locomotor Activity. Activity was measured in an Accuscan Activity Monitor (Accuscan Instruments) and performed as described (30, 88). Briefly, mice were allowed to habituate to the open field for 30 min, injected with various drugs, and returned to the open field. Locomotor activity was measured in 5-min intervals, and data were analyzed for the distance traveled in 5-min increments over 120 min. All APDs and tool compounds were administered i.p. and injected 10 min before AMPH or PCP injections.

IHC. Fifty-micrometer-thick vibratome cut sections of formalin-fixed mouse brains were processed for IHC analyses as described previously (30). To assess neuron-specific deletion of β arr2, antibodies to β arr2 (generated in rabbit; at 1:300; gift from Jeff Benovic, Thomas Jefferson University, Philadelphia), Cre recombinase (mouse antibody MAB3120; at 1:500; EMD Millipore), and D2 neuron marker enkephalin (ENK; rabbit antibody; AB5026; at 1:500; EMD Millipore) were used. Because both antibodies to β arr2 and ENK were from rabbit, they were used in combination with antibodies to Cre only. Additionally, because β arr2 levels in the STR are much lower, a TSA Amplification System (Perkin-Elmer) was used to enhance detection of β arr2. Antibodies to PV (1:500; PVG-213; Swant Inc.) and GRK2/3 (1:500; 05-465; EMD Millipore) were used to label PV+ interneurons and assess the levels of GRK2, respectively, in

these cells. All representative images are from IHC analyses from at least two to three mice for each group.

Western Blot. Western blot analyses were performed on postmortem human or drug-naïve mice brain tissue as described previously (30). For human brain tissue, all procedures were carried out in compliance with an approved protocol from the University of Mississippi Medical Center Institutional Review Board. Written informed consent was obtained from legally defined next of kin for tissue collection and informant-based retrospective diagnostic interviews (Table S2). Human or mouse tissue lysates were loaded onto SDS gels followed by transfer onto nitrocellulose membranes. Membrane blots were incubated with primary antibody followed by IR secondary antibody (LICOR), and the blots were developed using an LICOR Odyssey Detection System. Details are in *SI Materials and Methods*.

Stereotaxic Surgeries and Virus. Deletion of β arr2 or D2Rs in the PFC was achieved by a viral approach; β arr2^{fl/fl} or D2^{fl/fl} mice were stereotaxically injected bilaterally with 0.5 μ L AAV serotype 2/8 (UNC Viral Vector Core). β arr2^{fl/fl} (PFC β arr2KO) or A2aCre β arr2^{fl/fl} (PFC + STR β arr2KO) mice were injected with mCherry-Cre AAV, whereas D2^{fl/fl} mice were injected with either GFP or mCherry-Cre AAV at coordinates +2.5 mm anteroposterior (AP), \pm 0.3 mm mediolateral (ML), and –1.8 mm dorsoventral (DV) from bregma to target the prelimbic/infralimbic region of the PFC. Mice were allowed to recover for 3 wk to allow for viral expression of GFP or mCherry-Cre before behavioral testing.

Cannulation and Local PFC Drug Injections. For local PFC injection of drugs, we inserted bilateral guide cannulas (Plastics One) into the PFC of WT mice. The bilateral guide cannulas were inserted at +2.0 mm AP with 1.0-mm spacing (\pm 0.5 mm ML) and –2.0 mm DV and fixed to the skull with dental cement. Mice were allowed to recover for 2 wk, and then, drugs were injected using an automated injection system. Drugs were dissolved in VEH [10% (vol/vol) DMSO and 20% (vol/vol) hydroxypropyl cyclodextrin], and either 1 μ g (Quinpirole and UNC9994A) or 0.5 μ g (cpd101-GRK2/3 inhibitor; Hello Bio, Bristol, UK) per 0.5 μ L were injected per side at a rate of 0.4 μ L/min. After local injection, mice were placed in the activity monitors for 10 min before systemic (i.p.) injection with PCP (6 mg/kg), and then, locomotor activity was recorded.

cAMP Inhibition Assay. To measure D2R G α i-mediated cAMP inhibition, a split luciferase-based cAMP biosensor (GloSensor; Promega) in HEK293T cells was used. The assay was performed in a 384-well plate using a Wallac TriLux Microbeta (Perkin-Elmer) Luminescence Counter. Details are in *SI Materials and Methods*.

β arr BRET Assay. To measure D2R-mediated β arr2 recruitment, a mouse D2_{Long} receptor fused to C-terminal *renilla* luciferase and a Venus-tagged N-terminal β arr2 (a gift from Jonathan Javitch, Columbia University, New York) expressed in HEK293T cells were used in a BRET assay. The assay was performed in a 96-well plate using a Mithras LB940 Multimode Plate Reader (Berthold Technologies). Details are in *SI Materials and Methods*.

Electrophysiology.

Slice preparation. Three hundred-micrometer-thick coronal slices were cut from 7- to 10-wk-old mice of either sex using a Leica VT1200S Microtome. Gad1-EGFP Tg (Jackson ImmunoResearch Laboratories Inc.) and global β arr2^{fl/fl} mice were used in our electrophysiological studies. Acute slices were secured by placing a harp along the midline between the two hemispheres.

Intracellular recording. Whole-cell patch recordings were obtained from visually identified interneurons in layer V of infralimbic or prelimbic cortex or MSNs in the STR using differential contrast video microscopy on an upright microscope (BX51WI; Olympus). Recordings were obtained from FSI or MSNs of Gad1-eGFP adult mice ($n = 1$ per animal), which were further identified by their responses to hyperpolarizing and depolarizing current injections. Recordings were collected using a Multiclamp 700A (Molecular Devices). Patch electrodes (tip resistance = 4–6 M Ω) were filled with the following (in mM): 115 K-Gluconate, 10 HEPES, 20 KCl, 2 MgCl₂, 2 Mg-ATP, 2 Na-ATP, and 0.3 GTP. Slices were submerged in artificial cerebrospinal fluid containing the following: 125 mM NaCl, 25 mM NaHCO₃, 3.5 mM KCl, NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM glucose. All recordings were made at 32.5 \pm 1 $^{\circ}$ C. Series resistance was usually 15–20 M Ω , and experiments were discontinued if the series resistance exceeded 30 M Ω .

Statistical Analyses. Data were analyzed by standard one- and two-way ANOVA or two- and three-way repeated-measures ANOVA (RMANOVA) tests for comparison between genotypes, treatments, or doses. Individual genotypes,

treatments, or doses were compared using a post hoc Bonferroni's test. Data were analyzed for normality with equal variance, and only parametric tests were used. Data points were excluded based on previously established criterion and set to ± 2 SDs from the group mean. Data are presented as mean \pm SEM.

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