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Rapid Communication

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The Beta-Arrestin-Biased Dopamine D_2 Receptor Ligand, UNC9994, Is a Partial Agonist at G-Protein-Mediated Potassium Channel Activation

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Abstract

Background: Previous evidence suggests that UNC9994 is a beta-arrestin2-selective agonist at the dopamine D_2 receptor, lacking ability both to activate and antagonize G protein-dependent signaling. However, this has only been reported by one laboratory using a single assay.

Methods: We used G protein-coupled inward rectifier potassium channel activation in *Xenopus* oocytes to investigate UNC9994-induced modulation of G protein-dependent signaling at dopamine D_2 receptor and dopamine D_3 receptor.

Results: At dopamine D_2 receptor, UNC9994 induced G protein-coupled inward rectifier potassium channel currents that were 15% of the maximal response to dopamine, with an EC_{50} of 185 nM. At dopamine D_3 receptor, the ligand elicited 89% of the maximal dopamine response with an EC_{50} of 62 nM. Pertussis toxin abolished G protein-coupled inward rectifier potassium channel activation. Furthermore, UNC9994 antagonized dopamine-induced G protein-coupled inward rectifier potassium channel activation at dopamine D_2 receptor.

Conclusions: UNC9994 modulates G protein-coupled inward rectifier potassium channel channel activation via pertussis toxin-sensitive G proteins at dopamine D_2 receptor and dopamine D_3 receptor. These findings may have implications for the interpretation of data obtained with this ligand.

KeyWords: antipsychotic, arrestin, beta-arrestin2, biased ligand, electrophysiology

Introduction

A major challenge to effective treatment of psychotic disorders (including schizophrenia, schizoaffective disorder, and bipolar disorder with mania) is the occurrence of adverse reactions, such as extrapyramidal side-effects, to antipsychotic drugs. These side-effects lead to high rates of treatment nonadherence, thus limiting the clinical usefulness of current antipsychotics. Furthermore, whereas existing drugs show efficacy against the so-called positive symptoms of schizophrenia (i.e., delusions and hallucinations), current treatment does not adequately address the negative (e.g., social withdrawal, emotional flattening, avolition) and cognitive symptom domains (Miyamoto et al., 2012).

Current antipsychotics are antagonists or weak partial agonists (which may be functionally selective; see Urban et al., 2007) at dopamine D_2 and D_3 receptors (D_2R and D_3R). These receptors are known to signal via several downstream pathways, which

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Significance Statement

The ability of G protein-coupled receptors to signal not only via G proteins but also via beta-arrestins has gained recent interest. Dopamine D_2 receptor (D_2R) ligands, which selectively modulate the arrestin pathway while leaving G protein signaling undisturbed, have been proposed as a strategy for developing antipsychotics with fewer side-effects and improved efficacy. The compound used in the present study, UNC9994, was reported to be devoid of agonist activity at the G protein pathway and unable to antagonize dopamine-evoked, G protein-dependent signaling. Here, we reassessed these properties of UNC9994 using GIRK channel activation in *Xenopus* oocytes, a time-resolved, G protein-dependent readout of D_2R activity. We find that UNC9994 is a partial agonist at D2R-mediated GIRK channel activation and, as expected of a partial agonist, also antagonizes DA-mediated signaling in the same assay. These findings have implications for the interpretation of data obtained with this ligand, which has been used to interrogate arrestin-dependent dopaminergic signaling.

include the classical G protein pathway and the more recently described arrestin pathway (Beaulieu and Gainetdinov, 2011). G protein-mediated actions include adenylate cyclase inhibition and activation of G protein-coupled inward rectifier (GIRK) channels, whereas arrestin recruitment to the receptor, apart from desensitization and internalization, initiates a distinct signaling cascade, which involves Akt/PKB and glycogen synthase-kinase 3. β-Arrestin2 (also known as arrestin3) has specifically been implicated as the arrestin isoform regulating D_2R downstream signaling (Beaulieu et al., 2005).

The importance of arrestin signaling has come to the forefront of schizophrenia research in recent years (Freyberg et al., 2010). All clinically used antipsychotics antagonize arrestin recruitment to the D_2R (Klewe et al., 2008; Masri et al., 2008), suggesting that this mechanism might be relevant for their therapeutic activity. Indeed, it has been proposed that the clinical benefits of antipsychotics are mediated mainly via inhibition of the arrestin pathway, whereas attenuation of G protein activation would be responsible for extrapyramidal side-effects (Allen et al., 2011). Accordingly, efforts are underway to develop D_2R ligands that specifically modulate arrestin signaling (Urs et al., 2016; Männel et al., 2017; McCorvy et al., 2018).

Recent data suggest that "arrestin-biased" ligands, which act as partial agonists on the arrestin pathway without affecting G protein activity, such as UNC9994 (Allen et al., 2011), are able to restore rodent behavioral deficits induced by phencyclidine (PCP) and amphetamine; 2 pharmacological models frequently used for detecting antipsychotic efficacy (Urs et al., 2016). Acting via D_2R, UNC9994 appears to activate arrestin-dependent signaling preferentially in cortex (Urs et al., 2016), presumably due to the high expression of both β-arrestin2 and G protein receptor kinase-2 (GRK2) in this brain region enhancing D2R coupling to this pathway (Urs et al., 2016; Sahlholm et al., 2017). The resulting action in the striatum, where β-arrestin2 and GRK2 expression is lower, remains less defined but appears to contribute to antipsychotic-like efficacy in the amphetamine and PCP models (Urs et al., 2016; Sahlholm et al., 2018). It has been suggested that such an action could substitute for the frontal cortical hypodopaminergia believed to underlie cognitive and negative symptoms in schizophrenia while simultaneously counterbalancing excessive D_2R signaling in the striatum, thought to cause positive symptoms (Urs et al., 2016). Motor side-effects could potentially be avoided by leaving G protein signaling intact (Allen et al., 2011). However, there are some notable weaknesses and contradictions to these hypotheses; clinical evidence suggests that hypofunction of prefrontal cortical D_1R, rather than D_2R, may be involved in cognitive deficits in schizophrenia (Arnsten et al., 2017), and activation of both cortical and striatal D_2Rs may contribute to the production of positive symptoms (Arnsten et al., 2015). In addition, aripiprazole, which does interfere with G-protein-dependent D_2R signaling (Allen et al., 2011; Urs et al., 2016), has a very favorable motor side-effect profile (Naber and Lambert, 2004).

Remarkably, UNC9994 was reported not only to be completely devoid of agonist activity in assays of D_2R-mediated $G_{i/o}$ protein activation but was also found to lack the ability to antagonize dopamine-induced inhibition of adenylate cyclase activity at D_2R (Urs et al., 2016). It was thus suggested that UNC9994 might be able to interact only with arrestin-coupled- but not G protein-coupled D_2R, making it a truly "arrestin-selective" ligand, which could theoretically allow for a mild side-effect profile as outlined above. However, the lack of antagonistic effects of UNC9994 on D_2R-mediated G protein signaling has been described only on the basis of cAMP accumulation experiments, which lacked temporal resolution (GloSensor, a cAMP assay; Urs et al., 2016). Furthermore, while UNC9994 is a relatively low-potency ligand with an EC_{50} of 0.45 to 1 μM in some arrestin recruitment assays (Allen et al., 2011), only concentrations up to 1 μM of UNC9994 were tested in the GloSensor experiments when competing against dopamine (Urs et al., 2016).

While a completely signaling pathway-selective D_2R ligand would be of great utility to the field of neuropharmacology, purportedly arrestin-selective ligands for other receptors, such the angiotensin-I receptor agonist, SII, have recently been shown to be partial agonists in G protein activation assays (Grundmann et al., 2018), underscoring the need for independent validation. To further investigate the interaction (or lack thereof) of UNC9994 with G protein-dependent signaling, we examined the activity of this ligand toward D_2R- and D_3R-mediated activation of GIRK channels, a sensitive and time-resolved assay that has previously allowed us to study weak partial D_2R agonists (Sahlholm et al., 2011).

Methods

Molecular Biology

Human GIRK1 (Kir3.1) and GIRK4 (Kir3.4) cDNA (provided by Dr. Terence Hebert, University of Montreal, Canada) and Regulators of G protein Signaling (RGS)4 (from the Missouri cDNA Resource Center; www.cdna.org) were in pCDNA3 (Invitrogen). cDNA encoding the human dopamine D_{2L} (long isoform of D_2R) and D_3 receptors and human beta-arrestin2 was in pXOOM (from Dr. Søren-Peter Olesen, University of Copenhagen, Denmark), and the catalytic subunit of PTX (PTX-S1; from Dr. Eitan Reuveny, Weizmann Institute of Science, Israel) was in pGEM-HE. For in vitro transcription, plasmids were linearized (GIRK 1/4, NotI; RGS4, $D_{2L}R$ and D_3R, XhoI; PTX-S1, NheI) and transcribed in vitro using the T7 mMessage mMachine kit (Ambion, Austin, TX). cRNA concentration and purity were determined using a spectrophotometer.

Oocyte Isolation and Injection

Oocytes were surgically isolated from female African clawed toads, *Xenopus laevis*, and injected with cRNA as previously described (Sahlholm et al., 2011). The surgical procedures had been approved by the Swedish National Board for Laboratory Animals and the local ethics committee, Stockholms Norra Djurförsöksetiska nämnd. One nanogram of each GIRK subunit cRNA, 0.2 ng of dopamine D_{2L} or D_3 receptor cRNA, and, when used, 40 nfg of RGS4, 5.6 ng of beta-arrestin2, and 3 ng of PTX-S1 cRNA were injected per oocyte. RGS proteins are GTPase-activating proteins expressed in native tissues, which speed up the G protein cycle such that GIRK channel activity more closely follows receptor occupancy by agonist (Dascal and Kahanovitch, 2015).

Receptor Ligands

Dopamine was purchased from Sigma-Aldrich (St. Louis, MO) and UNC9994 and aripiprazole were from Axon MedChem BV (Groeningen, The Netherlands). Dopamine was dissolved in recording buffer, whereas UNC9994 was dissolved in DMSO. (-)-3PPP was a gift from Astra Zeneca (Södertälje, Sweden). Ligands were diluted in the recording solution to obtain the desired concentrations. The maximum final concentration of DMSO used in any experiment did not exceed 0.1% v/v.

Electrophysiology

The electrophysiological experiments were performed at room temperature (20–22°C) 5 to 7 days after cRNA injection using a 2-electrode voltage-clamp setup (CA-1 amplifier, Dagan, Minneapolis, MN) as previously described (Sahlholm et al., 2011). Data were acquired at 134 Hz using pCLAMP 8 (Molecular Devices) software. A high-potassium solution (in mM: 64 NaCl, 25 KCl, 0.8 $MgCl_2$, 0.4 $CaCl_2$, 15 HEPES, 1 ascorbic acid, adjusted to pH 7.4), giving a K^+ reversal potential of about -40 mV, was used for GIRK current recording. Ascorbic acid was present to prevent the oxidation of dopamine. Single -80 mV pulses were applied to study GIRK current responses to D_2R activity. Ligands were added to the 20-µL recording chamber by superfusion at 1.5 mL/min using a computer-controlled, pressure-driven perfusion system (SmartSquirt, AutoMate Scientific, Berkeley, CA). When evaluating UNC9994 in the agonist mode, 3 to 4 increasing concentrations of the ligand were applied at 35- or 50-second intervals to oocytes expressing D_2R or D_3R, respectively. The UNC9994-evoked current response was determined by subtracting the basal (agonist-independent) current from the experimental record. The UCN9994-induced current responses were subsequently normalized to the mean response to 1 µM dopamine in 4 different oocytes (expressing D_2R or D_3R, respectively, together with RGS4 and GIRK1/4), which had not previously been exposed to UNC. In the antagonist mode, 100 nM dopamine was first applied to provide a baseline response, followed by 3 to 4 applications of increasing concentrations of UNC9994 at 50-second intervals in the continued presence of dopamine. For each oocyte, the current amplitude at the end of each antagonist application interval was normalized to the control response to 100 nM dopamine obtained at the start of the protocol. For dopamine concentration-response data, 4 to 5 increasing concentrations of dopamine were applied at 25-second intervals, ending with a response-saturating concentration (100 µM) of dopamine (supplementary Figure 1).

Data Analysis

Variable slope sigmoidal concentration-response curves were fitted to the concentration-response data using GraphPad (Prism Software). The following equation was fitted to agonist data:

$$Y = top / \left(1 + 10 \wedge \left(\left(\log EC_{50} - X\right) \times n\right)\right),$$

where Y is the response as a fraction of 1, *top* is the maximal response of the agonist expressed as a fraction of 1, *X* is the logarithm of ligand concentration, and *n* is the Hill slope. When analyzing dopamine concentration-response data, *top* was set to 1.

For fitting antagonist data, the fitted equation was:

$$Y = bottom + (1?\,bottom) / (1 + 10((logEC50?\,X) \times n)),$$

where *bottom* is the maximal response inhibition evoked by the antagonist.

Results

First, UNC9994 was applied alone to *Xenopus* oocytes expressing D_2R together with RGS4 and GIRK1/4 channels. The ligand evoked small but clearly detectable inward currents when applied at concentrations between 10 nM and 10 µM (Figure 1A). Such currents were not observed when UNC9994 was applied to oocytes injected with RNA encoding D_2R but not GIRK1/4, nor in oocytes expressing GIRK channels alone (not shown), and were abolished by coexpression of the catalytic subunit of pertussis toxin (PTX-S1; Figure 1A, inset), which inactivates inhibitory $G_{i/o}$ proteins by ADP ribosylation (Vivaudou et al., 1997). The pEC_{50} for UNC9994-induced current activation was 6.73 ± 0.40 (EC_{50} 185 nM; comparable with the K_i of 79 nM of this compound at D_2R, as reported by Allen et al., 2011). The top of the UNC9994 concentration-response curve was $14.5 \pm 2.8\%$ of the mean response to 1 µM dopamine (which evokes a maximal response; see supplementary Figure 1), indicating weak partial agonism of UNC9994 at GIRK activation elicited via D_2R (Figure 1B). The similarly submaximal responses elicited by 10 µM of the known partial D_2R agonists, (-)-3-PPP and aripiprazole, which evoked 27.7 ± 7.5 and $9.2 \pm 2.3\%$ of the mean response to 1 µM dopamine, respectively (supplementary Figure 2), suggest that the receptor reserve under these conditions was low or absent.

When applied to oocytes expressing D_3R together with RGS4 and GIRK1/4 channels, the efficacy of UNC9994 was greater than at D_2R (Figure 1B), evoking $89.1 \pm 24.3\%$ of the maximal response to 1 µM dopamine. In agreement with radioligand competition data reported by Allen et al. (2011), the functional potency of UNC9994 was higher at the D_3R that at the D_2R, with a pEC_{50} of 6.21 ± 0.55 (EC_{50} 62.1 nM). PTX-S1 coexpression prevented UNC9994-induced GIRK activation also in D_3R-expression oocytes (data not shown).

When applied to oocytes expressing D_2R together with RGS4 and GIRK1/4 channels in the presence of 100 nM dopamine, UNC9994 inhibited the dopamine-induced response with an IC_{50} of 630 nM (Figure 1C, D). Assuming a K_d of dopamine of 33 nM based on its EC_{50} value for GIRK channel activation (supplementary Figure 1), the K_i of UNC9994 was estimated as 155 nM, again comparable with the K_i reported by Allen et al. (2011).

Finally, we evaluated the effects of UNC9994 in oocytes expressing D_2R together with GIRK1/4 and beta-arrestin2. The expression levels of beta-arrestin2 were sufficiently high to induce prominent desensitization of the response to 1 µM

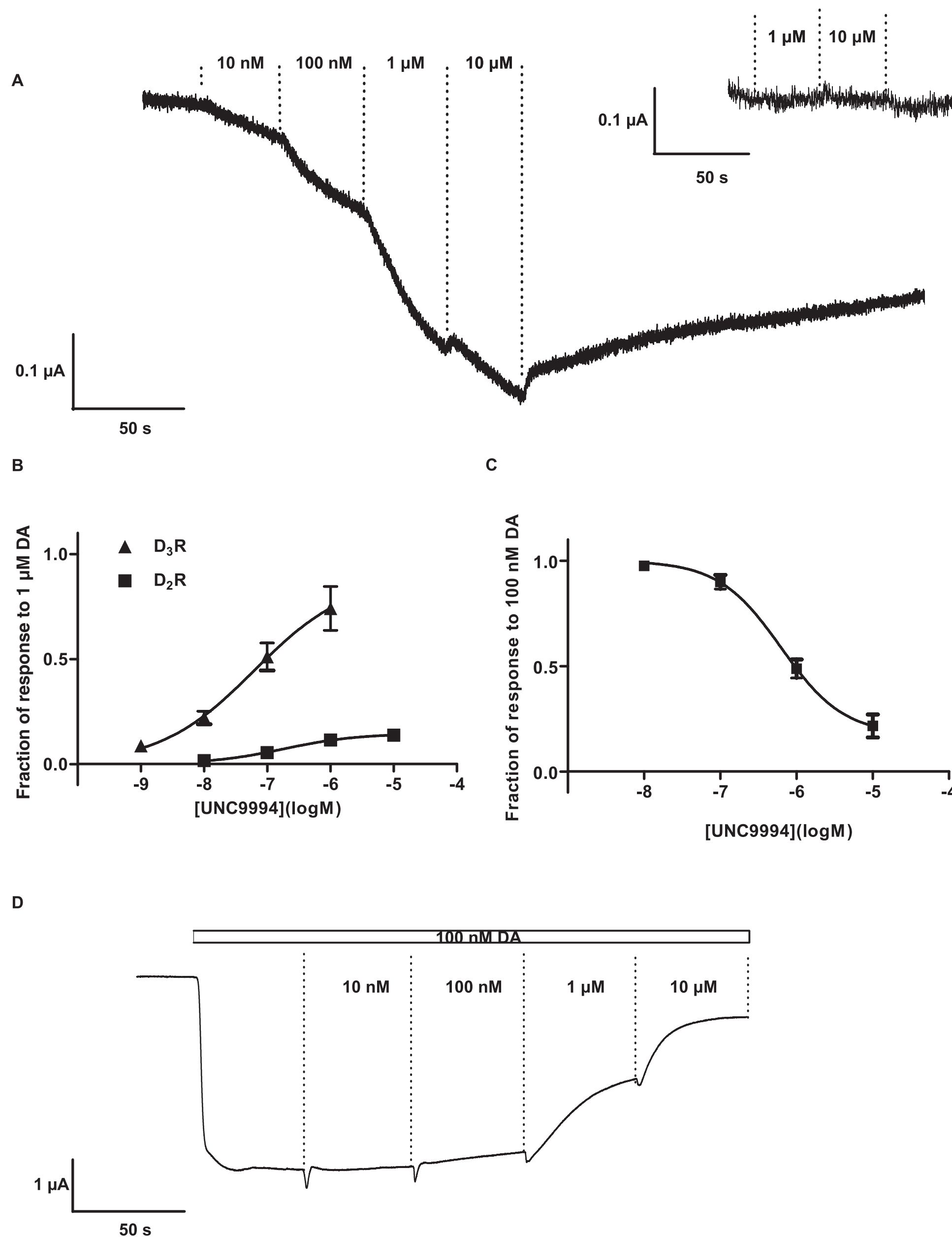

Figure 1. Actions of UNC9994 on G protein-dependent G protein-coupled inward rectifier (GIRK) signaling elicited via dopamine D_2 receptor (D_2R) and D_3R. (A) Representative current trace recorded in an oocyte coexpressing D_2R with Regulators of G protein Signaling (RGS)4 and GIRK1/4 channels, demonstrating GIRK channel activation upon application of increasing concentrations of UNC9994, as indicated. Inset shows the absence of response to 1 and 10 µM UNC9994 in an oocyte coexpressing D_2R with RGS4, GIRK1/4, and the catalytic subunit of PTX. (B) Concentration-response curves for GIRK activation by UNC9994 in oocytes expressing D_2R or D_3R, as indicated, together with RGS4 and GIRK1/4 channels, n = 3 to 5 per data point. The mean current amplitude elicited by each concentration of UNC9994 was normalized to the mean amplitude elicited by 1 µM dopamine in separate cells (n = 4 for both D_2R and D_3R). (C) Concentration-response curve for the inhibition of dopamine-induced GIRK activation by UNC9994. Data were obtained from experiments such as that shown in D, n = 4 to 6 for each data point. (D) Representative current trace recorded in an oocyte coexpressing D_2R with RGS4 and GIRK1/4 channels, showing the inhibition of the GIRK response to 100 nM dopamine by increasing concentrations of UNC9994, as indicated.

dopamine compared with control cells, which had not been injected with RNA encoding beta-arrestin2 (Figure 2A, B). A total of 10 µM UNC9994 was found to induce GIRK activation also under these conditions, which was 21.7 ± 9.1% of the mean response to 1 µM dopamine (Figure 2C, D).

Discussion

UNC9994 has previously been described as an arrestin-biased ligand incapable of activating G protein-dependent signaling downstream of D_2R (Allen et al., 2011) and, even more intriguingly, unable to antagonize dopamine-evoked adenylate cyclase inhibition via this receptor (Urs et al., 2016). It should be emphasized that the present study does not address the relative efficacies of G protein- vs arrestin-dependent signaling elicited by UNC9994. Rather, our results challenge previous reports of a complete lack of interaction of UNC9994 with G protein-dependent signaling, as the present findings strongly suggest that this compound acts as an agonist at GIRK channel activation at D_2R and D_3R. GIRK channels are typically activated by $G_{\beta\gamma}$

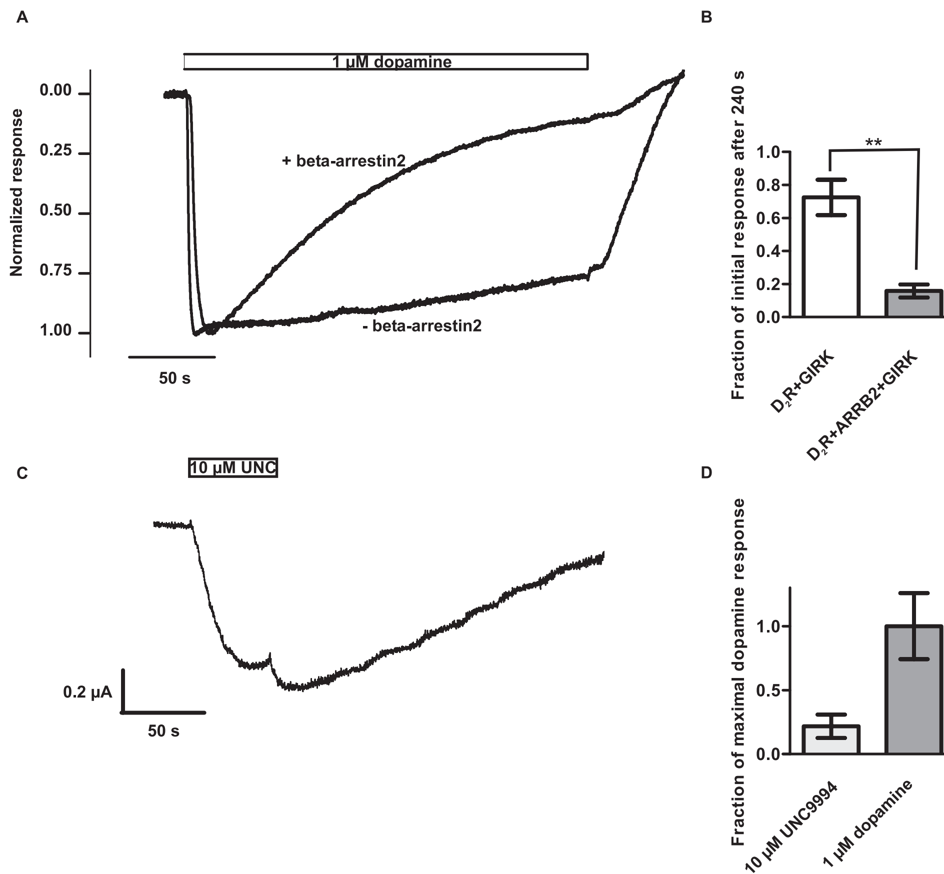

Figure 2. UNC9994 acts as a partial agonist at G protein-coupled inward rectifier (GIRK) activation also in the presence of beta-arrestin2. (A) Representative current traces recorded from oocytes coexpressing dopamine D_2 receptor (D_2R) with GIRK1/4 channels with or without beta-arrestin2, as indicated, demonstrating the impact of beta-arrestin2 coexpression on the time course of the current response to 1 µM dopamine. (B) Ratio between the peak inward current response and the response amplitude at the end of the 240-second application of dopamine. **$P<.01$, Student's t test, n = 5 for each group of oocytes. (C) Representative current trace showing GIRK channel activation elicited upon application of 10 µM UNC9994 (UNC) in an oocyte coexpressing D_2R with GIRK1/4 channels and beta-arrestin2. (D) Average response to 10 µM UNC9994 as a fraction of the average response to 1 µM dopamine in oocytes coexpressing D_2R with GIRK1/4 channels and beta-arrestin2. n = 6 for UNC9994; n = 5 for dopamine.

subunits released from $G_{i/o}$ proteins (Dascal and Kahanovitch, 2015). In accordance with a $G_{i/o}$-dependent process, GIRK activation by UNC9994 was blocked by coexpression of the catalytic subunit of PTX (Vivaudou et al., 1997). Furthermore, we showed that at the D_2R, where the efficacy of UNC9994 is low, this ligand antagonizes the near-maximal GIRK response induced by 100 nM dopamine. *Xenopus* oocytes do not endogenously express functional arrestins (Kovoor et al., 1997); however, we found UNC9994 to elicit GIRK activation also in oocytes expressing human beta-arrestin2.

The reasons for the discrepancy between the present work and earlier published findings by Allen et al. (2011) and Urs et al. (2016) are unclear at present, but may, in part, reflect functional selectivity for GIRK channel activation over adenylate cyclase inhibition. Such selectivity has been reported previously for the antipsychotic aripiprazole and its analogue, SV-III-130s, which are both structurally related to UNC9994. Opposite to UNC9994, aripiprazole and SV-III-130s were found to be partial agonists in adenylate cyclase inhibition assays while acting as neutral antagonists when D_2R-mediated GIRK channel activation was examined (Urban et al., 2007; Luedtke et al., 2012). Here, however, aripiprazole was found to be a partial agonist at GIRK activation, with an efficacy similar to that of UNC9994. Interestingly, aripiprazole and UNC9994 were shown to have similar efficacy also at beta-arrestin signaling (Urs et al., 2017), which could suggest that, when considering GIRK activation as the readout of $G_{i/o}$ activity, there is little difference in arrestin/G protein bias between the 2 compounds. However, bias is context dependent and should be investigated in the same cellular background and at the same time-point following agonist addition (Klein Herenbrink et al., 2016; Onfroy et al., 2017). Measures of biased agonism, as well as antagonism, have been reported to be strongly influenced by the stoichiometry of receptor, G protein, and effector molecules (Onfroy et al., 2017), and these parameters likely differed between our study and those of Urs et al. (2016) and Allen et al. (2011), which may have contributed to the divergent results. For example, a large receptor reserve in the experiments by Urs et al. (2016) may have precluded the detection of antagonism at 1 µM UNC9994, which was the highest concentration tested. In addition, the lack of temporal resolution of the adenylate cyclase assay might have prevented observation of a modulatory effect of UNC9994 (see Klein Herenbrink et al., 2016, for an investigation of the effect of incubation time on measures of ligand efficacy).

Nevertheless, the absence of antagonism of G protein-dependent signaling reported by Urs et al. (2016) stands in sharp contrast to the present data, and our findings may thus warrant reinterpretation of some of the findings reported from studies where UNC9994 was used as an arrestin-specific ligand (e.g., Scarduzio et al., 2017; Pack et al., 2018). The abolition of

antipsychotic-like activity of this ligand in beta-arrestin2 knockout animals certainly suggests an important contribution of the arrestin pathway to such activity (Allen et al., 2011); however, it cannot be excluded that G protein-dependent signaling also plays some role. This is particularly relevant considering that arrestin signaling was suggested to be low or absent in striatal medium spiny neurons (Urs et al., 2016), yet A_{2A}R-expressing neurons, which are mainly of this type, appear to play a role in the ability of UNC9994 to counteract amphetamine- and PCP-induced hyperlocomotion (Sahlholm et al., 2018; Urs et al., 2016). Furthermore, UNC9994 has higher affinity for D_3R than for D_2R (Allen et al., 2011), and the G protein-dependent agonist activity of UNC9994 at D_3R revealed in the present study may confer pro-cognitive properties similar to those described for the similarly D_3R-preferring antipsychotic, cariprazine (Zimnisky et al., 2013; Németh et al., 2017).

In summary, our present findings suggest that UNC9994 is able to induce G protein-dependent GIRK activation via D_2R- and D_3R, acting as a weak partial agonist at the former while being more efficacious at the latter receptor. In addition, contrasting with previous findings examining adenylate cyclase activity, UNC9994 inhibits the G protein-dependent GIRK response to dopamine via D_2R.

Supplementary Materials

Supplementary data are available at *The International Journal of Neuropsychopharmacology* online.

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Statement of Interest

None.

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