Abstract

G protein-coupled receptors (GPCRs) are seven-pass transmembrane proteins that facilitate major physiological processes in humans. GPCRs are targets of more than 40% of therapeutic drugs, which lead to activation of intracellular signal transduction proteins (transducers). Drugs targeting GPCRs have recently been shown to have preference for certain transduction pathways, a phenomenon termed functional selectivity. Drugs that are biased to exclusively activate one transduction pathway have been found to be more efficacious and produce fewer side effects. Therefore, understanding the nature of a ligand's preference for a particular transduction pathway may lead to the development of novel drugs with superior therapeutic efficacy. No methods have been developed to specifically characterize the transducer-dependent interaction of a GPCR and its ligand across multiple transducers. The Roth laboratory at UNC is developing a screening platform for this purpose. We utilized this platform and fused the dopamine D2 receptor (D2R), the primary target for antipsychotic drugs, to 17 human transducers. We then performed *in vitro* competitive radioligand binding assays with D2-selective drug, quinpirole, and the D2R endogenous ligand, dopamine. The signaling profiles produced indicated that dopamine and quinpirole exhibit high affinity for D2R fused to canonical $G\alpha_i$ transducers and quinpirole lacks high affinity toward non-canonical G_α transducers. These results align with previous knowledge of the D2 dopamine receptor, and therefore provide validation for the screening platform used. We anticipate these signaling profiles will give insight into functional selectivity that may be utilized for therapeutic advancement of antipsychotic drugs.

Introduction

Until recently, pharmacological theory has postulated that a ligand's ability to occupy a G protein-coupled receptor (GPCR), and therefore activate a response pathway, was solely dependent on the inherent properties of the ligand-receptor pair. This is described as intrinsic efficacy, a phenomenon that has been greatly relied upon for the last half-century (Urban et al., 2007). Intrinsic efficacy depicts a system-independent outcome that is constant for each ligand at a given receptor, regardless of where the receptor is expressed. However, it has been found that some molecules, such as dopamine receptor agonists, do not follow this system-independent pattern for agonist-receptor complexes. These agonists are described as being "biased" toward certain transduction pathways, a phenomenon termed "functional selectivity".

GPCRs are the target of more than 40% of therapeutic drugs (Filmore, 2004), and therefore it is important to consider functional selectivity during drug discovery and development. Since a molecule's efficacy and affinity at a certain receptor may vary depending on environment, it may be possible to find drugs that are more efficacious and/or have less side effects by understanding the nature of the drug's bias for particular signal transduction pathways (transducers). For example, drugs may be optimized to target a specific transduction pathway that is characteristic of a particular tissue to avoid side effects in parts of the body that are not being targeted (Civelli, Bunzow, & Grandy, 1993). A GPCR of pharmacological significance is the D2 dopamine receptor (D2R), encoded by the *DRD2* gene. This receptor is targeted by a variety of traditional and newer drugs to treat mental health conditions such as schizophrenia and Parkinson's disease (Seeman, 2006). In fact, the ability to interact with dopamine D2 receptors is a shared quality of all clinically effective antipsychotics (Masri et al., 2008). Further

comprehension and discovery related to agonists' affinity at GPCRs of interest, such as D2R, will allow for expansion and improvement of drugs and therapies.

Currently, little is known about the relationship between an agonist's ability to signal at a receptor and the transducers bound to that receptor. In order to provide insight to this unknown, the Roth laboratory is developing a screening platform to characterize ligand bias towards individual transducer-coupled receptors. This platform is an expansion of pilot work done by Dr. Ryan Strachan (Strachan et al., 2014). Using methods from Dr. Strachan's study, we fused D2R to 17 human transducers and then performed *in vitro* competitive radioligand binding assays with quinpirole, a D2-selective drug, and dopamine, the endogenous D2R ligand (Figure 1). Drawing upon established knowledge of the dopamine D2 receptor (Montmayeur, Guiramand, & Borrelli, 1993), we predicted that quinpirole and dopamine would exhibit high affinity for D2R bound to its canonical transducer proteins, $G\alpha_i$ (also denoted as Ga(i)), and lack high affinity for D2R bound to non-canonical Gα transducers.

The signaling profiles produced from this assay, as demonstrated in the results section, were consistent with our prediction. This data provides information about the relationship between ligand, receptor, and transducer for the dopamine D2 receptor that is independent of *in vivo* variables, such as the presence of other proteins in a cell's internal environment. Additionally, the results provide validation for the screening platform. We anticipate that these profiles, along with future profiles produced using this platform, will provide insight into functional selectivity that is useful for therapeutic advancement of antipsychotic drugs.

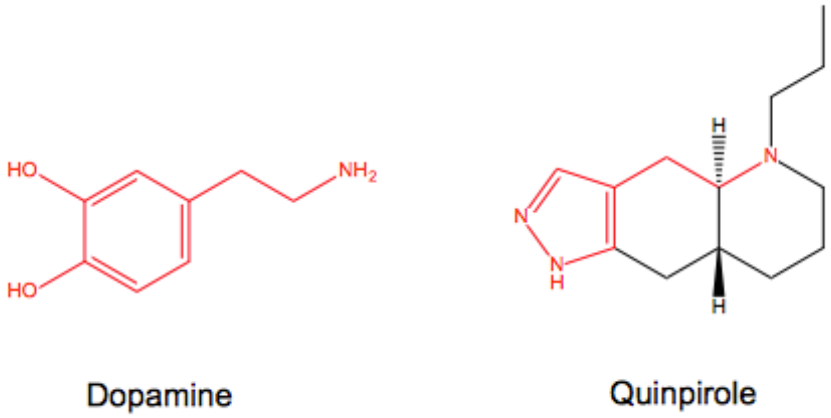


Figure 1. *The chemical structures of dopamine and quinpirole. Dopamine, the endogenous ligand for the dopamine D2 receptor, binds to the D2R active state. Quinpirole, a D2-selective drug, also binds strongly to the D2R active state. Quinpirole lacks free rotating bonds and has structural similarity to dopamine (shown in red), making it a high affinity ligand.*

Methods

Creating D2R-transducer fusions

Prior to fusing the 17 transducer elements to the D2 receptor, each transducer component was PCR amplified using Primestar MAX DNA Polymerase according to manufacturer's instructions. Forward and reverse primers used to amplify each transducer were from Eton Biosciences and are listed in Tabe 1. The amplified transducer elements were verified via gel imaging (Fig. 2) and sequencing (Genewiz) (Fig. 3). Gel imaging was done with 1% agarose gels in 1X TAE buffer and ran for 30 minutes at 150 V. Bands were visualized via UV illumination with 0.5 μg/mL ethidium bromide as the DNA dye.

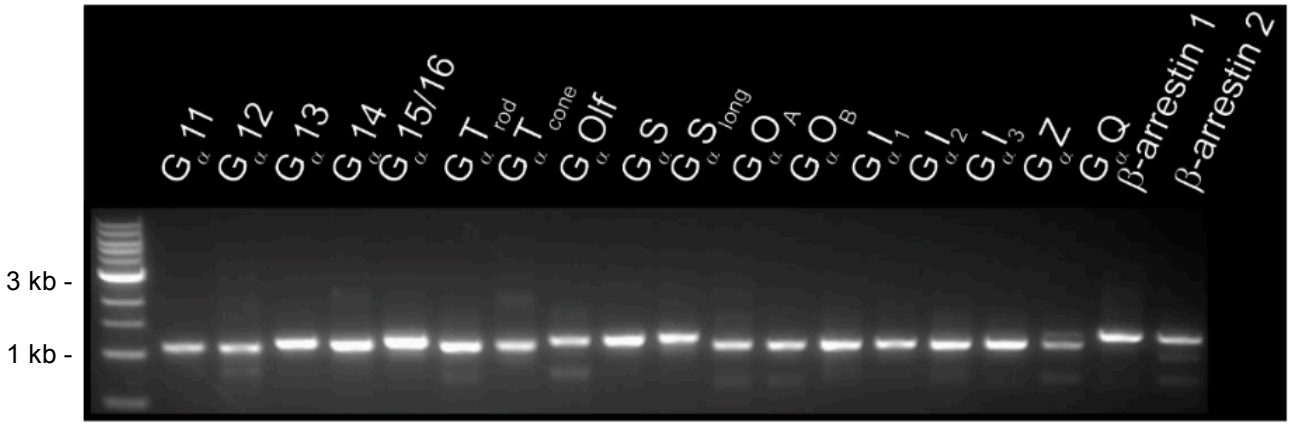


Figure 2. *Gel image of amplified human transducer elements. Elements confirmed to be around 1,000 base pairs with 1 kb ladder as reference (left lane).*

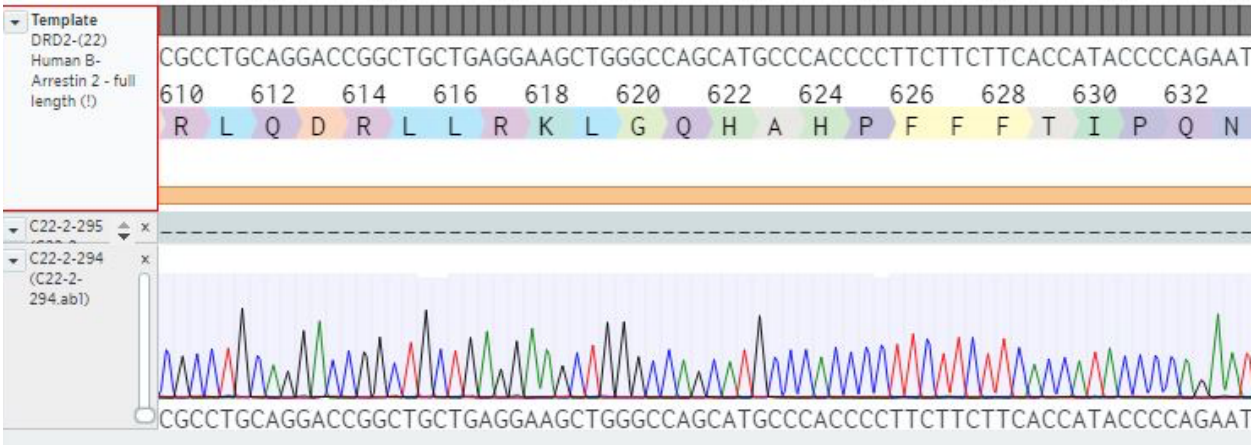


Figure 3. *Example of amplification verification via sequencing, shown for D2-β arrestin 2 fusion with forward primer. Template base code indicated in top row. Clean peaks verify proper sequence in bottom row.*

Transducer	Forward Primer Sequence	Reverse Primer Sequence
Ga11	CTGTACTTCCAGCTAATGACTCTGGAGTCCATGATGGC	CTAGACTCGAGCTATCAGACCAGGTTGTACTCCTTGAG
Ga12	CTGTACTTCCAGCTAATGTCCGGGGTGGTGCGGAC	CTAGACTCGAGCTATCACTGCAGCATGATGTCCTTCAG
Ga13	CTGTACTTCCAGCTAATGGCGGACTTCCTGCCGTC	CTAGACTCGAGCTATCACTGTAGCATAAGCTGCTTGAGG
Ga14	CTGTACTTCCAGCTAATGGCCGGCTGCTGCTGCCT	CTAGACTCGAGCTATTAAACGGGCCCTCTAGACTCGAG
Ga15/16	CTGTACTTCCAGCTAATGGCCCGCTCGCTGACCTG	CTAGACTCGAGCTATCACAGCAGGTTGATCTCGTCC
GaTrod	CTGTACTTCCAGCTAATGGGAAGTGGAGCCAGTGC	CTAGACTCGAGCTATTAGAAGAGGCCGCAGTCCTTG
GaTcone	CTGTACTTCCAGCTAATGGGGGCTGGGGCCAGTGC	CTAGACTCGAGCTATCAGAAGAGGCCACAGTCTTTGAGG
GaOlf	CTGTACTTCCAGCTAATGGGGTGTTTGGGCGGCAA	CTAGACTCGAGCTATCACAAGAGCTCATACTGCTTGAGG
GaS short	CTGTACTTCCAGCTAATGGGCTGCCTCGGGAACAG	CTAGACTCGAGCTATTATAGCAGCTCGTACTGACGAAG
GaS long	CTGTACTTCCAGCTAATGGGCTGCCTCGGGAACAG	CTAGACTCGAGCTATTAGAGCAGCTCGTACTGACGAAGG
GaO A	CTGTACTTCCAGCTAATGGGATGTACTCTGAGCGC	CTAGACTCGAGCTATCAGTACAAGCCGCAGCCCC
GaO B	CTGTACTTCCAGCTAATGGGATGTACTCTGAGCGC	CTAGACTCGAGCTATCAGTAGAGTCCACAGCCCCG
Ga(i) 1	CTGTACTTCCAGCTAATGGGCTGCACGCTGAGCGC	CTAGACTCGAGCTATTAAAAGAGACCACAATCTTTTAGATTATTTTTTATGATG
Ga(i) 2	CTGTACTTCCAGCTAATGGGCTGCACCGTGAGCGC	CTAGACTCGAGCTATCAGAAGAGGCCGCAGTCCTTC
Ga(i) 3	CTGTACTTCCAGCTAATGGGCTGCACGTTGAGCGC	CTAGACTCGAGCTATCAATAAAGTCCACATTCCTTTAAGTTGTTTTTAATG
GaZ	CTGTACTTCCAGCTAATGGGATGTCGGCAAAGCTC	CTAGACTCGAGCTATCAGCAAAGGCCAATGTACTTGAG
GaQ	CTGTACTTCCAGCTAATGACTCTGGAGTCCATCAT	CTAGACTCGAGCTATTAGACCAGATTGTACTCCTTCAGGTTC
β-arrestin 1	CTGTACTTCCAGCTAATGGGCGACAAAGGGACCCG	CTAGACTCGAGCTACTATCTGTTGTTGAGCTGTGGAG
β-arrestin 2	CTGTACTTCCAGCTAATGGGGGAGAAACCCGGGAC	CTAGACTCGAGCTACTAGCAGAGTTGATCATCATAGTCGTC

Table 1*. Primers used for amplification of transducer elements.*

The D2R GPCR (DRD2-Tango from addgene.org) (Kroeze et al., 2015) was amplified using Primestar MAX DNA according to manufacturer's instructions. Primers used for amplification were transfus-tangobbf (TAGCTCGAGTCTAGAGGG) and transfus-tangobbr (TAGCTGGAAGTACAGGTTC).

All DNA amplification was done using 10-beta competent E. coli cells. DNA was purified using Zymo mini-prep kit according to manufacturer's instructions. Zymo midi-prep kit was used according to manufacturer's instructions for full DNA preparation for transfection.

The GPCR-transducer fusions were then generated using homologous recombination cloning with NEBuilder Hi-Fi DNA Assembly Master Mix, according to manufacturer's instructions. Successful incorporation of the transducer was confirmed via gel imaging (Fig. 4) and sequencing (Genewiz) (Fig. 3). Sequencing confirmation was done with primers BGHR (TAGAAGGCACAGTCGAGG), CMV-Forward (CGCAAATGGGCGGTAGGCGTG), TangoTransFusSeq-F(V2tail-F) (GGGTCCCCAAGATGAGTCCTGC) and TangoTransFusSeq-R(V2tail-R) (GCAGGACTCATCTTGGGGACCC). General plasmid design of final fusion constructs is outlined in Figure 5.

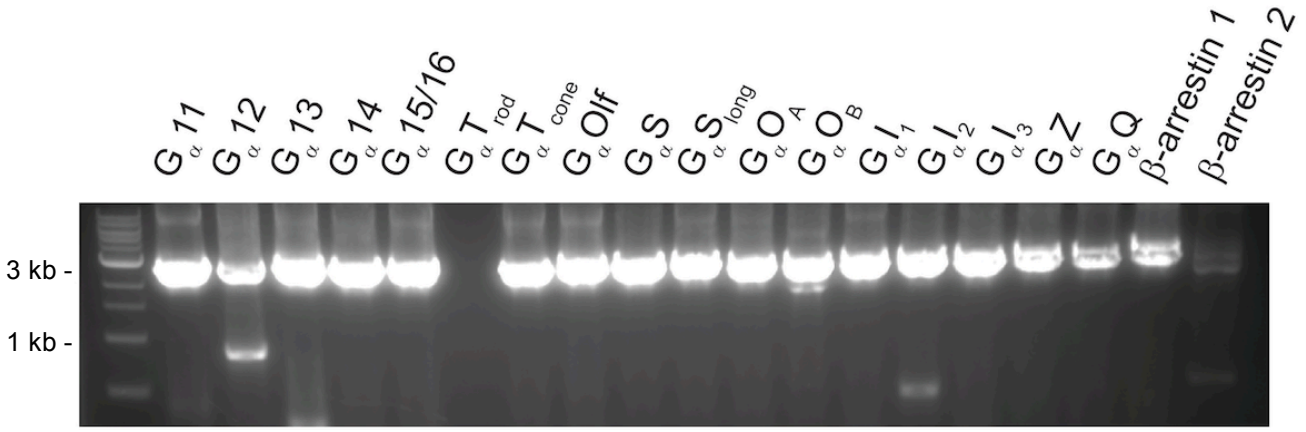


Figure 4. *Gel image of fusion constructs. Successful insertion of transducer into DRD2-Tango backbone is visualized by appropriate size, about 3,000 base pairs. 1 kb ladder used as reference (left lane). Fusions missing in image (D2-GaT$_{rod}$ and β-arrestin 2) were made and confirmed at a later time.*

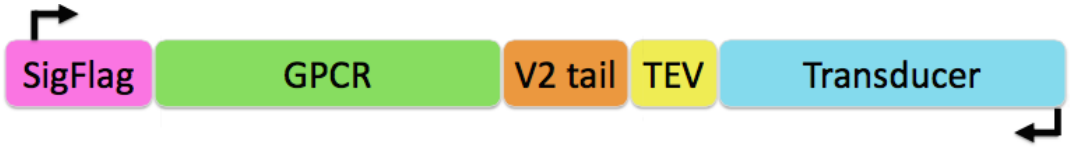


Figure 5. *General plasmid design for GPCR-transducer fusion proteins. Arrows indicate primer sites for PCR verification of fusions used to generate Figure 4. Includes signal for overexpression in membrane, a FLAG affinity tag for visualizing receptor expression, Vasopressin 2 receptor (V2R) C-terminus as a linker and arrestin recruitment domain, and a TEV protease cleavage site to remove fused transducer for control experiments.*

Expression and membrane preparation of DRD2-transducer fusion proteins

HEK293T cell membranes were transfected using a modified calcium phosphate method (Jordan, Schallhorn, & Wurm, 1996). Briefly, cells were transfected with 20 µg of D2 fusion cDNA and allowed to express for at least 48 hours. On the day of membrane preparation, media

was decanted and cells were in 10 mL ice-cold lysis buffer (1 mM HEPES, 2 mM EDTA, pH 7.4) and left at 4°C for at least 10 minutes. Cell lysates were re-suspended, transferred to 30 mL centrifuge tubes, and centrifuged at 30,000xg for 30 minutes at 4°C. Tubes were decanted and membrane pellets were re-suspended in 4 mL/15 cm plate of Standard Binding Buffer (SBB; 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4). Cells were re-suspended using a mechanical homogenizer (Polytron, setting 4) for 5 seconds, and 1 mL aliquots of the re-suspension were transferred to pre-chilled 1.7 mL microcentrifuge tubes. Re-suspended membranes were then centrifuged at 13,000xg for 10 minutes at 4°C. The SBB was decanted and membrane pellets were stored at -80°C until use.

Competitive radioligand binding

Competition binding assays were used to estimate shifts in affinity comparing D2R unfused to D2-fused receptors. Receptors were labeled with radioactive D2R antagonist [3H] N-methyl spiperone (0.1 nM, effective concentration with 50% effect (EC50)) (Figure 6) and tested in competition with 11 concentrations in duplicate to generate a KHi and KLo (Strachan et al., 2014). Binding assays were performed in 96-well plates with 150 µL per well of SBB containing 0.1% bovine serum albumin (BSA) and 0.01% ascorbic acid. Receptors were incubated for at least 3 hours at 25°C in the dark to achieve equilibrium. Reactions are stopped by vacuum filtrations onto 0.3% polyethyleneimine-soaked 96-well filter mats using a 96-well Filtermate harvester, followed by at least 3 washes of cold wash buffer. Scintillation (MeltiLex) cocktail is melted onto dried filters and radioactivity is counted using a Wallac Trilux Microbeta (Perkin

Elmer). Data was quantified and graphed using Prism 6.0 software and methods from Strachan et al., 2014.

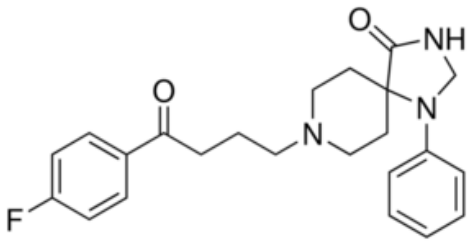


Figure 6*. Chemical structure of radioactive D2R antagonist [3H] N-methyl spiperone.*

Results

[3H]-N-methyl spiperone, the radioligand, showed no significant high affinity shifts for the fusions with canonical transducers of interest, Ga(i)1,2,3 (Fig. 7) . This demonstrates that [3H]-N-methyl spiperone is neutral and nonselective for these transducers. [3H]-N-methyl spiperone is therefore an appropriate radioligand for this assay.

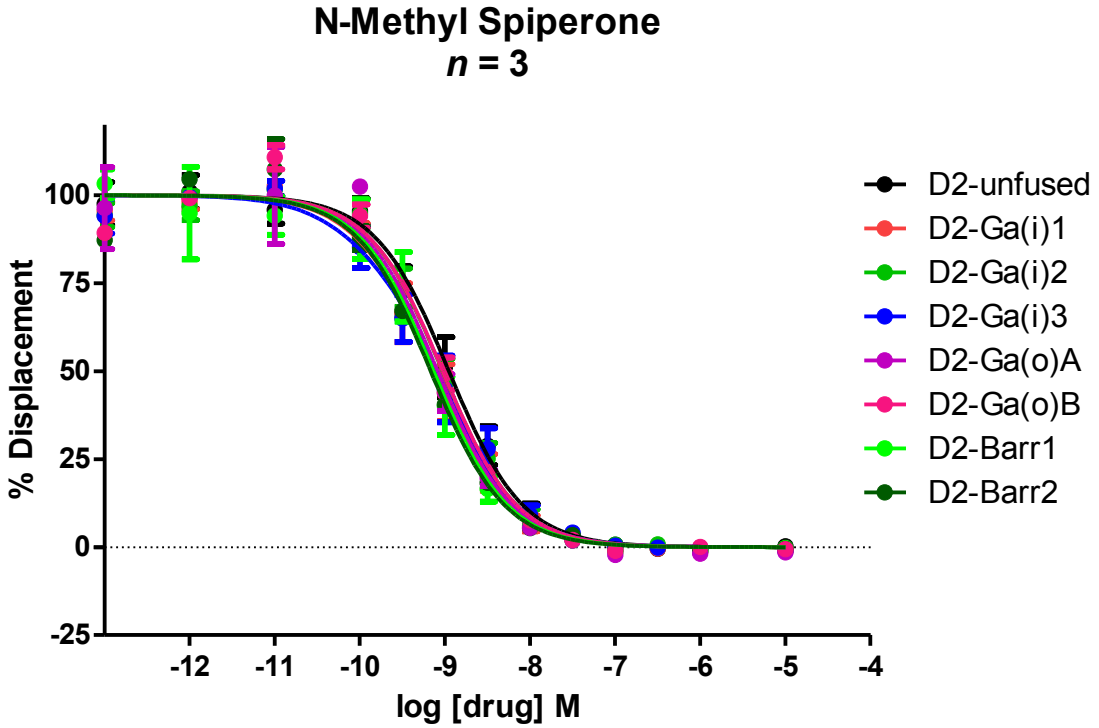


***Figure** 7. Binding curves for [3H] N-Methyl spiperone. No significant high affinity shifts for this radioligand at any fusions with canonical transducers of interest (Ga(i)1,2,3). Data is from three (n=3) separate set-ups and runs with [3H] N-Methyl spiperone.*

Quinpirole, a D2-selective drug, was tested with twelve of the transducer fusions (Figure 8). Quinpirole showed high-affinity site binding shifts for canonical transducers Ga(i)1, Ga(i)2, and Ga(i)3. The high- and low-affinity half maximal inhibitory concentration ($IC50_{Hi}$ and $IC50_{Lo}$, respectively) for the fusion curves was used to determine fold shift from the D2-unfused receptor curve. Quinpirole showed a 30-fold shift with Ga(i)1, a 118-fold shift with Ga(i)2, and a 68-fold shift with Ga(i)3 (Figure 9). These shifts demonstrated significant bias for the canonical Ga(i) transducers.

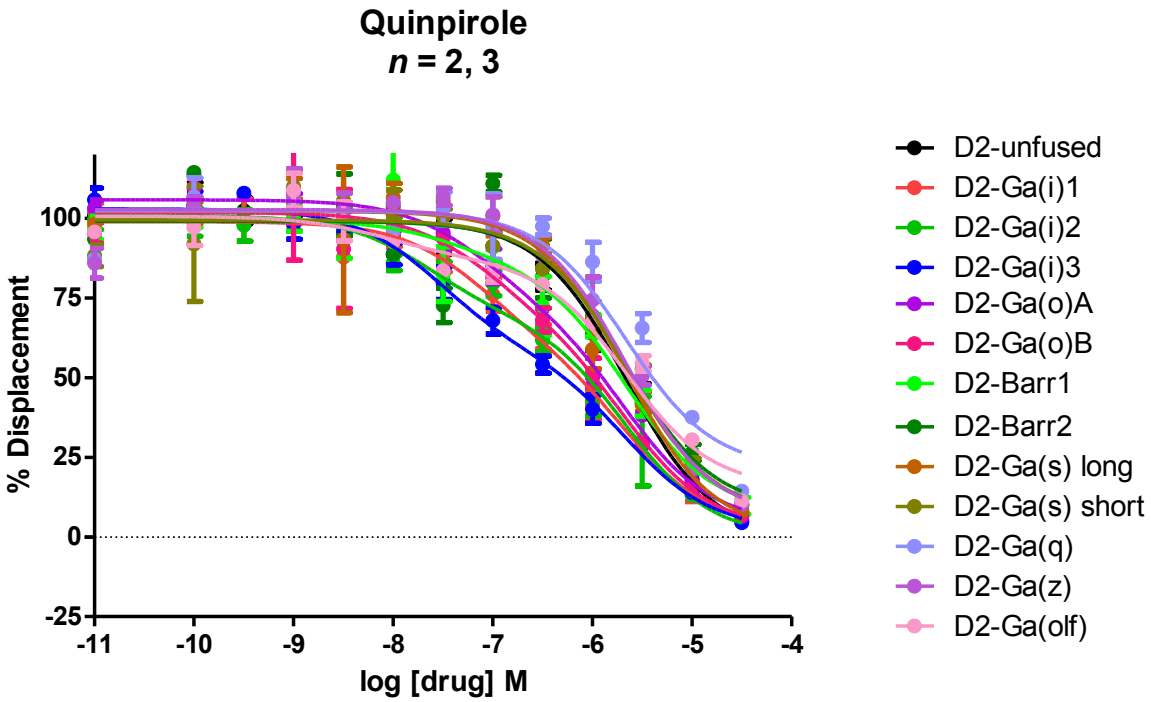
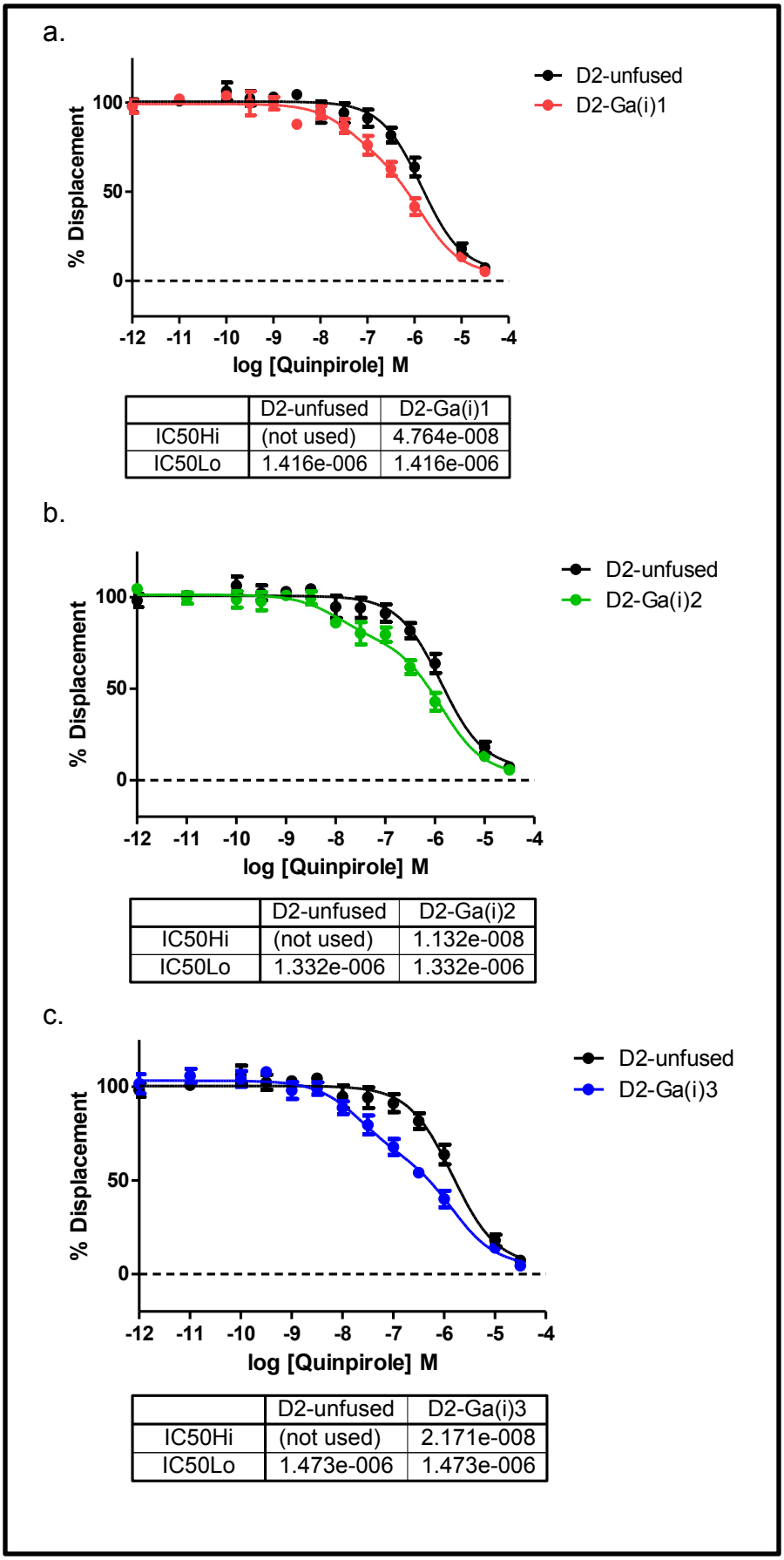


Figure 8. *Quinpirole binding curves for 12 transducer-fusions. Quinpirole showed high-affinity site binding shifts for canonical transducers Ga(i)1, Ga(i)2, and Ga(i)3, taken from three (n=3) sets of data. Significant shifts were not detected for other transducers, performed with two experimental runs (n=2).*



	D2-unfused	D2-Ga(i)1
IC50Hi	(not used)	4.764e-008
IC50Lo	1.416e-006	1.416e-006

	D2-unfused	D2-Ga(i)2
IC50Hi	(not used)	1.132e-008
IC50Lo	1.332e-006	1.332e-006

	D2-unfused	D2-Ga(i)3
IC50Hi	(not used)	2.171e-008
IC50Lo	1.473e-006	1.473e-006

Figure 9. *Quinpirole high-affinity site binding shifts at the Ga(i)1, 2, and 3 transducers. D2-Ga(i)1 fusion showed a 30-fold shift in its high affinity IC50 ($IC50_{Hi}$ = 47.6 nM) relative to the low affinity IC50 ($IC50_{Lo}$ =1416 nM) (a). D2-Ga(i)2 fusion showed a 118-fold shift in its high affinity IC50 ($IC50_{Hi}$ =11.3 nM) relative to the low affinity IC50 ($IC50_{Lo}$ =1332 nM) (b). D2-Ga(i)3 fusion showed a 68-fold shift in its high affinity IC50 ($IC50_{Hi}$ =21.7 nM) relative to low affinity IC50 ($IC50_{Lo}$ = 1473 nM) (c). All three fold-shift values for the Ga(i) transducers were significant in determining high-affinity binding sites for quinpirole. (n=3) for (a), (b), and (c).*

Quinpirole showed no significant affinity shifts for non-canonical transducers, including Ga(s) long, Ga(s) short, Ga(q), Ga(z), and Ga(olf) (Figure 10).

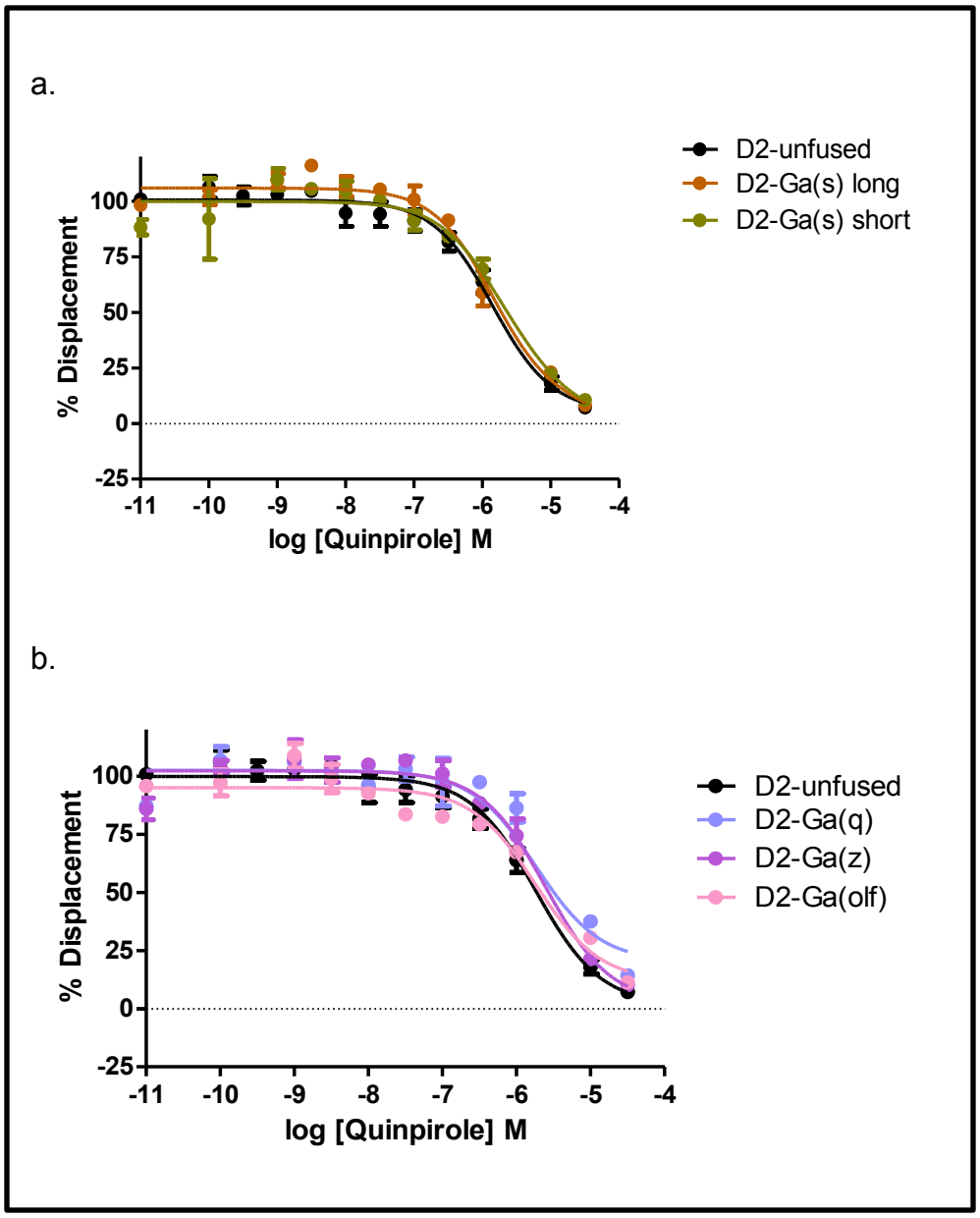


Figure 10. *Binding curves for non-canonical transducers. No significant shifts were detected for D2-Ga(s) long or D2-Ga(s) short fusions (a). No significant shifts were detected for D2-Ga(q), D2-Ga(z), or D2-Ga(olf) fusions (b). (n=2) for (a) and (b).*

Dopamine, the endogenous D2R ligand, was also found to show high-affinity site binding shifts for canonical Ga(i) transducers (Figure 11). The high- and low-affinity half maximal inhibitory concentration for the fusion curves was used to determine fold shift from the D2-unfused receptor. Dopamine showed a 181-fold shift with Ga(i)1, a 81-fold shift with Ga(i)2, and a 19-fold shift with Ga(i)3 (Figure 12). The fold shifts indicate that dopamine significantly showed preference for Ga(i)1, Ga(i)2, and Ga(i)3.

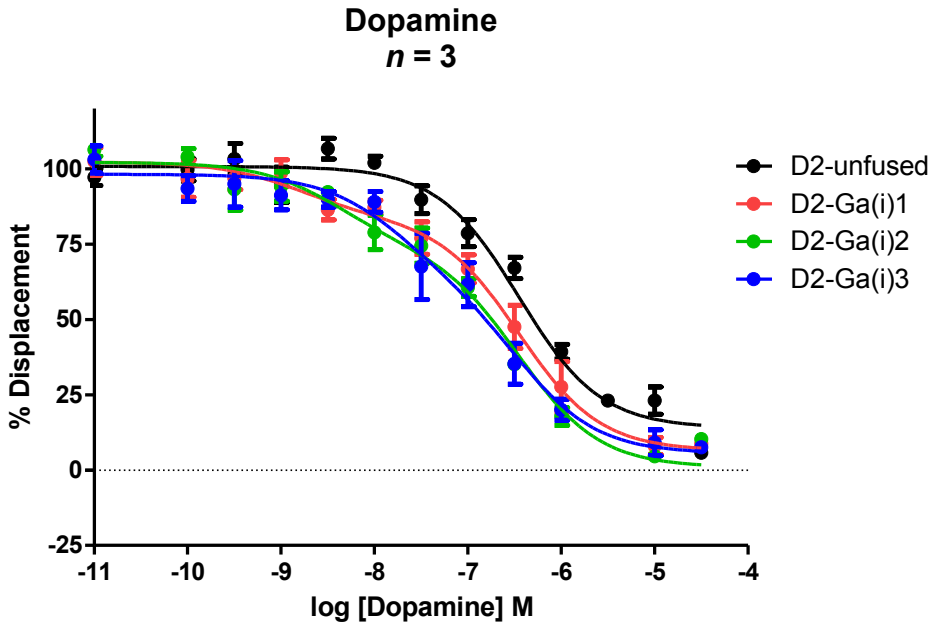
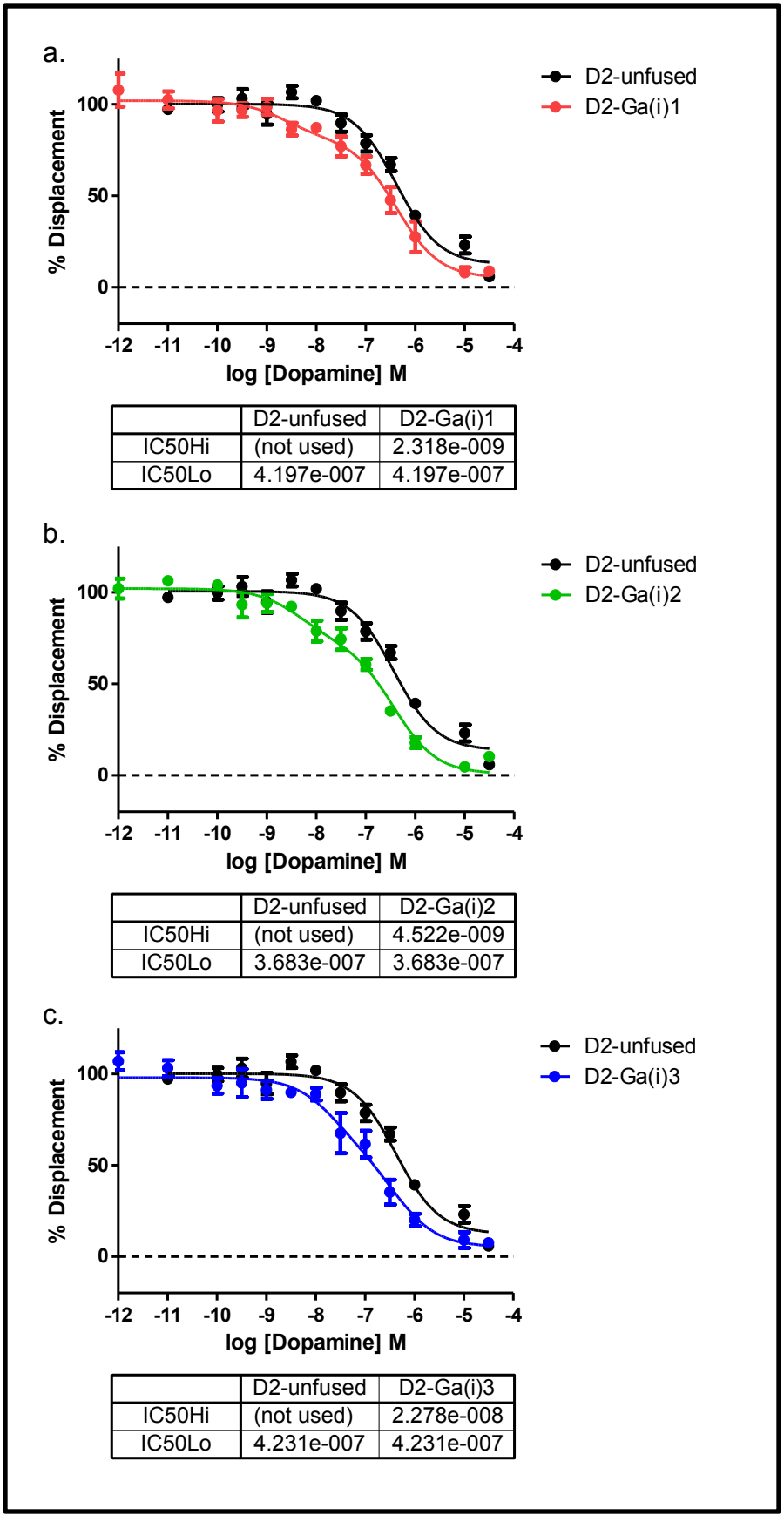


Figure 11. *Dopamine binding curves for canonical Ga(i) transducers. Dopamine showed high-affinity site binding shifts for canonical transducers Ga(i)1, Ga(i)2, and Ga(i)3. Data taken from three (n=3) sets of data.*



	D2-unfused	D2-Ga(i)1
IC50Hi	(not used)	2.318e-009
IC50Lo	4.197e-007	4.197e-007

	D2-unfused	D2-Ga(i)2
IC50Hi	(not used)	4.522e-009
IC50Lo	3.683e-007	3.683e-007

	D2-unfused	D2-Ga(i)3
IC50Hi	(not used)	2.278e-008
IC50Lo	4.231e-007	4.231e-007

Figure 12. *Dopamine high-affinity site binding shifts at the Ga(i)1, 2, and 3 transducers. D2-Ga(i)1 fusion showed a 181-fold shift in its high affinity IC50 ($IC50_{Hi}$ = 2.3 nM) relative to the low affinity IC50 ($IC50_{Lo}$ =419.7 nM) (a). D2-Ga(i)2 fusion showed a 81-fold shift in its high affinity IC50 ($IC50_{Hi}$ =4.5 nM) relative to the low affinity IC50 ($IC50_{Lo}$ =368.3 nM) (b). D2-Ga(i)3 fusion showed a 19-fold shift in its high affinity IC50 ($IC50_{Hi}$ =22.7 nM) relative to low affinity IC50 ($IC50_{Lo}$ = 423.1 nM) (c). All three fold-shift values for the Ga(i) transducers were significant in determining high-affinity binding sites for dopamine. (n=3) for (a), (b), and (c).*

Discussion

Our assay provides insight into the functional selectivity of quinpirole and dopamine at the dopamine D2 receptor. Quinpirole, a D2-selective drug, and dopamine, the D2R endogenous ligand, both showed high affinity shifts for D2R fused to canonical $G\alpha_i$ transducers (Figures 9, 11, 12). Quinpirole did not show high affinity shifts for non-canonical G_α transducers (Figure 10). This data is consistent with our predictions and the accepted knowledge of the nature of the dopamine D2 receptor (Lane, Powney, Wise, Rees, & Milligan, 2007) and therefore provides validation for the developing screening platform used in the Roth laboratory.

The fusion constructs created were designed to express D2R fused to one transducer. The subsequent isolation of the complexes allowed for *in vitro* binding assays that would provide data without *in vivo* variables, such as the presence of other proteins and transducers in a cell's internal environment. Therefore, it is understood that the signaling profiles produced provide data that is result of the definitive receptor-transducer pair. The variations in affinity can be attributed to the presence of a transducer at the receptor, as seen with the quinpirole and dopamine data presented here.

This screening platform can be utilized to investigate ligands' pharmaceutical potential. Expression of transducers is cell- and tissue-type dependent (Sharma et al., 2015). By understanding a ligand's varying preference for transducers, this differential transducer expression could be used to design optimized drugs. For example, transducer Ga(i)3 is found to be highly expressed particularly in mouse optic nerve cells (Sharma et al., 2015). If a drug is found to be functionally selective for Ga(i)3, it may be speculated that it would target optic nerve cells with minimal activity at cells that do not possess Ga(i)3. This hypothetical drug would

likely be more efficacious and trigger less side-effects than a drug that was not characterized as having high affinity for Ga(i)3.

In addition to facilitating new drug design, our platform can uncover novel biology occurring between current pharmaceutical and their targeted receptors. This information will allow for clearer understanding of a drug's preference for particular transducers, therefore prompting speculation of the drug's effects on a body-wide scale. For example, the serotonin 5-HT_{2A} receptor is mostly known for its expression in the central nervous system and is clinically targeted accordingly. However, 5-HT_{2A} is also highly expressed in the intestine, platelets, and endothelial cells (Raote, Bhattacharya, & Panicker, 2007). Using our platform to create affinity profiles for 5-HT_{2A}-targeted drugs may reveal new information about the drug's behavior at the body's different cell types, considering that transducer expression is differential between cells and tissues. This insight may then uncover new uses for the drug or allow for modification of the drug to reduce its effects at untargeted sites of the body.

To further broaden and develop this platform, more ligands should be tested against our D2R transducer fusions. This may include characterizing known clinical antipsychotics that are D2R-targeted, such as aripiprazole which is prescribed to treat schizophrenia (Burris et al., 2002) and bipolar disorder (Kanba et al., 2012). Additionally, other GPCRs of pharmacological importance can be used for fusions in order to create high-affinity profiles for a variety of drugs. For our next step, the Roth lab plans to expand this screening platform by creating fusions with the aforementioned serotonin 5-HT_{2A} receptor, the target of clinical drugs such as mirtazapine which is prescribed to treat depression (Celada, Puig, Amargós-Bosch, Adell, & Artigas, 2004).

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