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Dimerization of Dopamine (D2) and Adenosine (A2A) Receptors

Elena Vukovic
ev23@uakron.edu

Jackson M. Macatol
The University of Akron, jmm531@uakron.edu

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Jackson M. Macatol

Elena Vukovic

Department of Biology, University of Akron

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Dr. Brian Bagatto

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Abstract

G-Protein Coupled Receptors (GPCRs) are integral for cellular communication, as they trigger an intracellular response to signal molecules. Recently, these receptors were found to link physically with other receptors, forming dimers. The composition of these complex structures significantly alters the binding properties of each of its substructures. This new appreciation of heterodimers led to the increased study of GPCR dimerization, yet there is still debate on heterodimer classification. We used the technique of Pulsed Interleaved Excitation-Fluorescence Cross-Correlation Spectroscopy (PIE-FCCS) to quantify adenosine receptor-dopamine receptor (A2AR-D2R) heterodimerization. We utilized SNAP and CLIP tagging methods in which the fluorescent molecule covalently binds to the protein tag sequence, increasing fluorescence compared to other methods. We tested Wildtype (WT) and Mutant (Mut) A2ARs and their effects on D2R-A2AR heterodimerization and D2R homodimerization. We hypothesized that the three-point mutations (which changed three peptides) in A2AR would significantly alter D2R dimerization. We found that D2R homodimers increased when interacting with A2A Mut, and D2R-A2R heterodimers increased when the cells expressed A2R mutants.

Keywords: GPCR, heterodimer, homodimer, PIE-FCCS

Introduction

Dimerization of Dopamine (D2R) and Adenosine Receptors (A2AR)

GPCRs are the largest class of plasma membrane receptors and ubiquitous in cellular communication (reviewed in Latorraca et al., 2016). GPCR protein function spans from communicating sensory information to the central nervous system to activating metabolic pathways. There are many types of GPCRs, and each has different functions. Even though GPCRs can trigger many different pathways, all GPCRs have similar characteristics and conserved mechanisms during initial signal transduction (Latorraca et al., 2016). The transmembrane protein crosses the membrane seven times and has an extracellular amino terminus (N-terminus) and an intracellular carboxy terminus (C-terminus). The N-terminus serves as an activation site for a ligand, and the C-terminus is associated with a heterotrimeric G-protein (Latorraca et al., 2016). A G-protein has three subunits: alpha, beta, and gamma. An inactivated GPCR will have an intact G-protein with its alpha subunit bound to a guanosine diphosphate (GDP). When a ligand activates a GPCR, the protein conformation changes and prompts the alpha subunit to exchange a GDP for a guanosine triphosphate (GTP) (Yang et al. 2021). Further conformational changes ensue, and the activated G-protein dissociates from the GPCR complex. The protein remains activated until the alpha subunit hydrolyzes a phosphate group from GTP to form GDP. The GTPase activity of the alpha subunit is controlled by specific GTPase activating proteins (GAPs) (Yang et al. 2021). Any reaction step of this signal transduction can break and manifest into complex disorders. Research on GPCR proteins is integral for understanding and, potentially, treating complex health conditions because GPCRs can serve as drug targets.

Many receptors must dimerize to initiate an intracellular response. For instance, a receptor tyrosine kinase dimerizes with itself and autophosphorylates to activate pathways, such as the RAS pathway (Schlessinger, 1993). Dimerization occurs during GPCR activation (Latorraca et al., 2016). With this new information, researchers have characterized these relatively weak interactions at the plasma membrane. Any findings may contribute to the development of therapeutics because small changes in receptor dimerization can manifest into significant changes in signaling (Hauser et al., 2017). This is because GPCR proteins are metabotropic receptors, and they amplify small extracellular signals into large intracellular responses; small changes in a receptor's ability to respond to signals can have large downstream effects (Latorraca et al., 2016). Dimerization is a relatively weak interaction, and it is difficult to measure. Our research used a new technique that utilizes *in vivo* cell imaging to quantify protein coupling. Pulsed Interleaved Excitation-Fluorescence Cross-Correlation Spectroscopy (PIE-FCCS) quantifies the cross correlation in fluorescently tagged receptors to see if they interact once activated. Higher cross correlation indicates interactions between receptors, which is classified as dimerization. Our receptors of interest are dopamine receptors (D2R) and adenosine receptors (A2AR).

The neurotransmitter dopamine is responsible for movement, memory, attention, and emotion, among other functions (reviewed in Ayano, 2016). Dopaminergic neurons express dopamine receptors that respond to dopamine in the synaptic cleft (reviewed in Ayano, 2016). Adenosine is another neurotransmitter that activates receptor proteins in the central nervous system. Depending on the receptor, adenosine often functions to modulate neurotransmitter release and synaptic plasticity (Sheth et al., 2014). A receptor protein can be ionotropic or metabotropic. Ionotropic receptors bind a ligand, change in conformation, and modulate ion flow

through its channel. Metabotropic receptors bind a ligand, which triggers an intracellular cascade, and modulates ion flow through other protein channels (Lemon et al., 2003). A combination of graded potentials from an array of inputs will temporally or spatially summate to increase or decrease the likelihood of an action potential in the postsynaptic neuron. A receptor that increases membrane voltage will cause an excitatory postsynaptic potential (EPSP), and a receptor that decreases membrane voltage will cause an inhibitory postsynaptic potential (IPSP) (Ferster & Jagadeesh, 1992). If a threshold voltage is met for a neuron, action potentials propagate down an axon and elicit the release of neurotransmitter in the synaptic cleft. The neurotransmitter travels across the synaptic cleft and activates receptors in the postsynaptic neuron (reviewed in Kelly, 1993). This activation will elicit an EPSP or an IPSP, which depends on the type of receptor. Receptor proteins vary from environmental and genetic factors (Sanz-Clemente et al., 2013), and differences in receptor composition and expression dictate the efficiency of signal transmission in a neural circuit (reviewed in Kelly, 1993). Therefore, environmental factors and compositional differences in receptor proteins may explain why some people are more susceptible to disorders associated with dopamine and adenosine, such as addiction.

Addictive substances activate the reward center of the brain and can lead to substance abuse. People suffering from addiction accumulate physical changes in their brain because a cell's innate response to repeated activation is to express fewer receptors (Reid & Lingford-Hughes, 2006). As a result, to feel the same euphoric high as before, one must use the substance again. Many suffer from this feedback loop and lose their sense of reality, harming themselves and others (Reid & Lingford-Hughes, 2006). Heterodimer complexes play a role in the mechanism of addiction. Borroto-Escuela et al. (2018) investigated cocaine's effect on A2AR-

D2R heterodimer complexes in the nucleus accumbens of rats. After rats self-administered cocaine, a decrease in A2AR-D2R heterodimers and an increase in A2AR-D2R-Sigma1R heterodimers was reported. They hypothesized that cocaine affects dimerization and causes long-term breaks in receptor proteins, which leads to addiction.

Our research classified the type of dimerization between A2AR and D2Rs. We quantified the cross correlation of membrane bound, fluorescently tagged receptors *in vivo*.

Materials and Methods

Our contributions to the study consisted of culturing, splitting, labeling, and plating cells for imaging and analysis. We used protocols from Dr. Adam Smith's previous project (Asher et al., 2021), which provides a modernized methodology for elucidating heterodimerization in G protein-coupled receptors (GPCRs). Collaborators from Columbia University, St. Jude, and Mayo Clinic provided cell lines: Chinese hamster ovary (CHO) cells with expressions of A2AR, D2R, metabotropic glutamate receptor 2 (mGluR2), and an mGluR2 mutant (Δ2Δ) membrane receptor. We labeled A2ARs with CLIP tags and D2Rs with SNAP tags, which both covalently bind fluorescent molecules to the receptors (Asher et al., 2021).

STABLE SNAP-TAG CHO CELL LINES

To generate SNAP-tag-receptor protein fusion constructs, the gene of interest was integrated into the pcDNA5 SNAP-*fast* FLP Recombination Target (FRT) mammalian expression vector. This expression vector was then transfected into the T-REx-CHO inducible cell line. For multiple constructs, a SNAP-tag ortholog, CLIP-tag, was also produced by inserting the receptor gene into the pcDNA5-CLIP-*fast* frt mammalian expression vector followed by stable transfection into the T-Rex-CHO cell line. To enable the expression of both SNAP-tag and CLIP-tag proteins, cell lines were engineered to incorporate both tags, facilitated

by a P2A peptide cleavage site. These cell lines, provided by the Jonathan Javitch group, constitute a range of stable CHO cell lines that were utilized in the conducted experiments.

CULTURING STABLE CHO CELL LINES

On 10cm culture plates, adherent CHO cells were passaged in Ham's F12 media (Caisson Labs, Smithfield, UT). The stock media of 500mL was supplemented with 10% FBS, 5 mL of 20 mM L-glutamine (Lonza, Basel, Switzerland), and 5 mL of 200X Anti-Anti antibacterial and antifungal solution (Gibco, Waltham, MA). In order to add Ham's F12 media to plates to promote stable CHO cell growth, aliquots of blasticidin S hydrochloride (Alfa Aesear, Haverhill, MA) and hygromycin B gold (Invivogen, San Diego, CA) were thawed and added. Cells were incubated in a 5% CO_2 incubator at 37°C until confluent. When confluent, the cells were split into new passages or frozen for later usage.

INDUCING EXPRESSION AND LABELING OF STABLE CHO CELL LINES

Stable CHO cells were initially cultured on 6 well plates with supplemental Ham's F12 media until reaching approximately 60% confluency in a CO_2 incubator. New media was supplemented with 3.75 µg/mL of tetracycline and left to incubate overnight roughly 18-24 hours before imaging. After this incubation period, the Ham's F12 media was aspirated off and adhered cells were washed with 1X PBS twice. To lift the adhered cells, the plate was incubated with enzyme-free dissociation media (MilliporeSigma, St. Louis, MO). After 4 minutes of incubation, 500 mL of Ham's F12 was added. The media was mixed via pipetting up and down to release cells from the place. The media was transferred to a 1.7 mL epi tube and centrifuged at 500*g for 4 minutes. The cells were left until pellets formed.

Dye solutions were prepared in low light conditions to avoid photobleaching. The dye for heterogeneous SNAP-Tagged GPCR labeling was prepared in 200 µL of Ham's F12. To this, 0.5

µL of 1 µM SNAP-Surface 488 dye and 0.5 µL of 1 µM SNAP-Surface 549 dye (New England Biolabs, Ipswich, MA) were added. For orthogonal GPCR labeling with both SNAP-Tagged and CLIP-Tagged GPCRs being expressed, the same preparation was used except 0.5 µL of 1 µM SNAP-Surface 547 dye (New England Biolabs, Ipswich, MA) replaced the 0.5 µL of 1 µM SNAP-Surface 549 dye. Due to differences in density between green and red dye, the dyes were used in a 2:1 ratio, respectively. Before adding the dye to the cells, they were vortexed.

Once the pellet formed in the 1.7 mL tube, it was isolated by aspirating off the media. The Ham's F12 dye solution was added to the tube and the dye and pellet were pipetted repeatedly to break up the pellet. After this, the epi tube was incubated for 45 minutes. While waiting for the incubation to complete, a MatTek (MatTek Corporation, Ashland, MA) glass bottom imaging dish was prepared for labeled cell plating with fibronectin to improve adhesion.

After incubation, cells were pelleted via centrifuge at 500*g for 4 minutes, washed with 1x PBS three times, and resuspended in 500 µL of Opti-MEM imaging media (Invitrogen, Waltham, MA). Excess fibronectin was removed from the MatTek dish, and 200 µL of the Opti-MEM imaging media and resuspended CHO cells mixture was added to the center of the MatTek dish. An additional 2 mL of Opti-MEM imaging media was also added to the dish. The dish was then incubated for 30 minutes before being ready for imaging and PIE-FCCS experiments.

The different tagging methods allowed us to conduct PIE-FCCS in cell cultures with both A2A and D2 receptor expression. PIE-FCCS analysis (Asher et al., 2021) was used to compare membrane receptor movement *in vivo* to measure cross correlation. Higher cross correlation supported interactions between A2A and D2 receptors when activated. Control groups included mGluR2 receptors, which are known to dimerize (Singh et al., 2021) and delta-2-delta (Δ2Δ)

receptors, which are known to be monomers (Singh et al., 2021). We compared PIE-FCCS data from each treatment group with ANOVA followed by post-hoc Tukey's comparisons.

Results

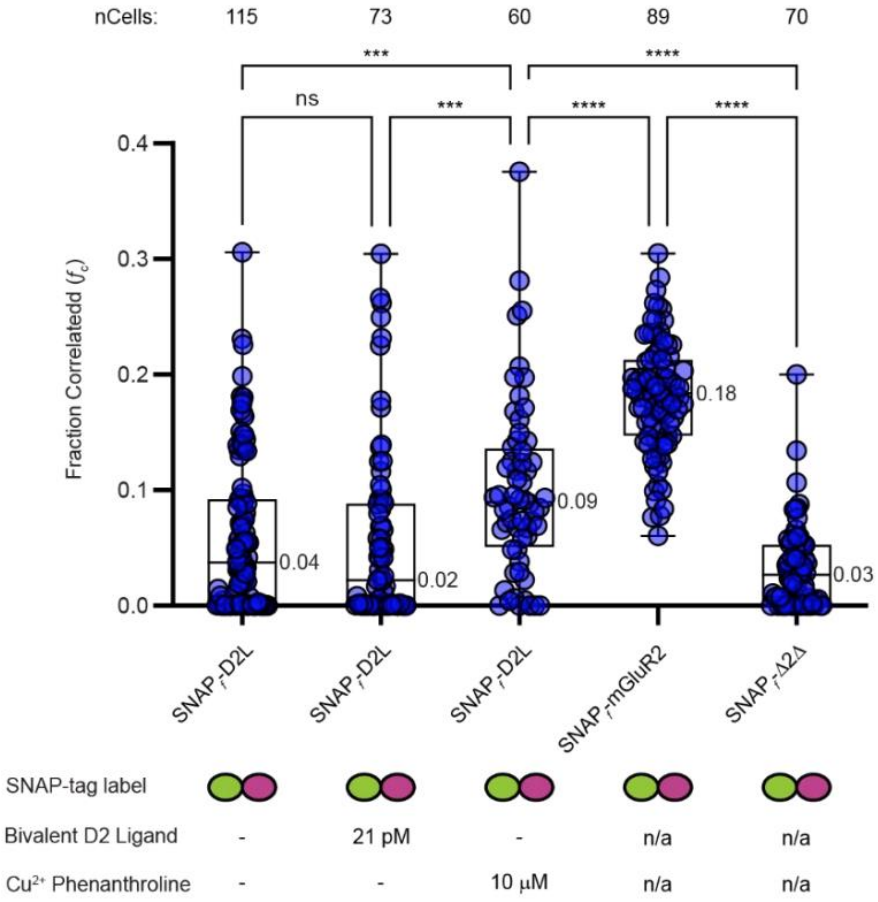

Figure 1. The ability of D2R to form homodimers (schematic of Grant T. Gilmore). SNAP-Surface 488 and SNAP-Surface 547 dyes were used to label D2Rs. We treated cells with Royo's bivalent ligand and Cu2+ phenanthroline and obtained fc values from longitudinal samples before treatment and 30 minutes after treatment. Statistical significance was measured using an ANOVA with post-hoc Tukey's comparison (p=0.05).

D2Rs were measured for their ability to form homodimers among different treatments. The fc of SNAP-D2R was 0.04 (n = 115), and it was not significantly different than the monomer control SNAP-Δ2Δ, which had an fc value of 0.03 (n = 70). Similarly, bivalent ligand treated SNAP-D2R was not significantly different than SNAP-Δ2Δ, having an fc value of 0.02 (n = 73) compared to 0.03. SNAP-D2R when treated with Cu2+ phenanthroline had an fc value of 0.09 (n = 60), which was significantly different than SNAP-Δ2Δ's value of 0.03. The treatment of Cu2+ phenanthroline rendered an increase in SNAP-D2R's fc value, but it is still not significantly different than the dimer control, SNAP-mGluR2, which had an fc value of 0.18 (n = 89).

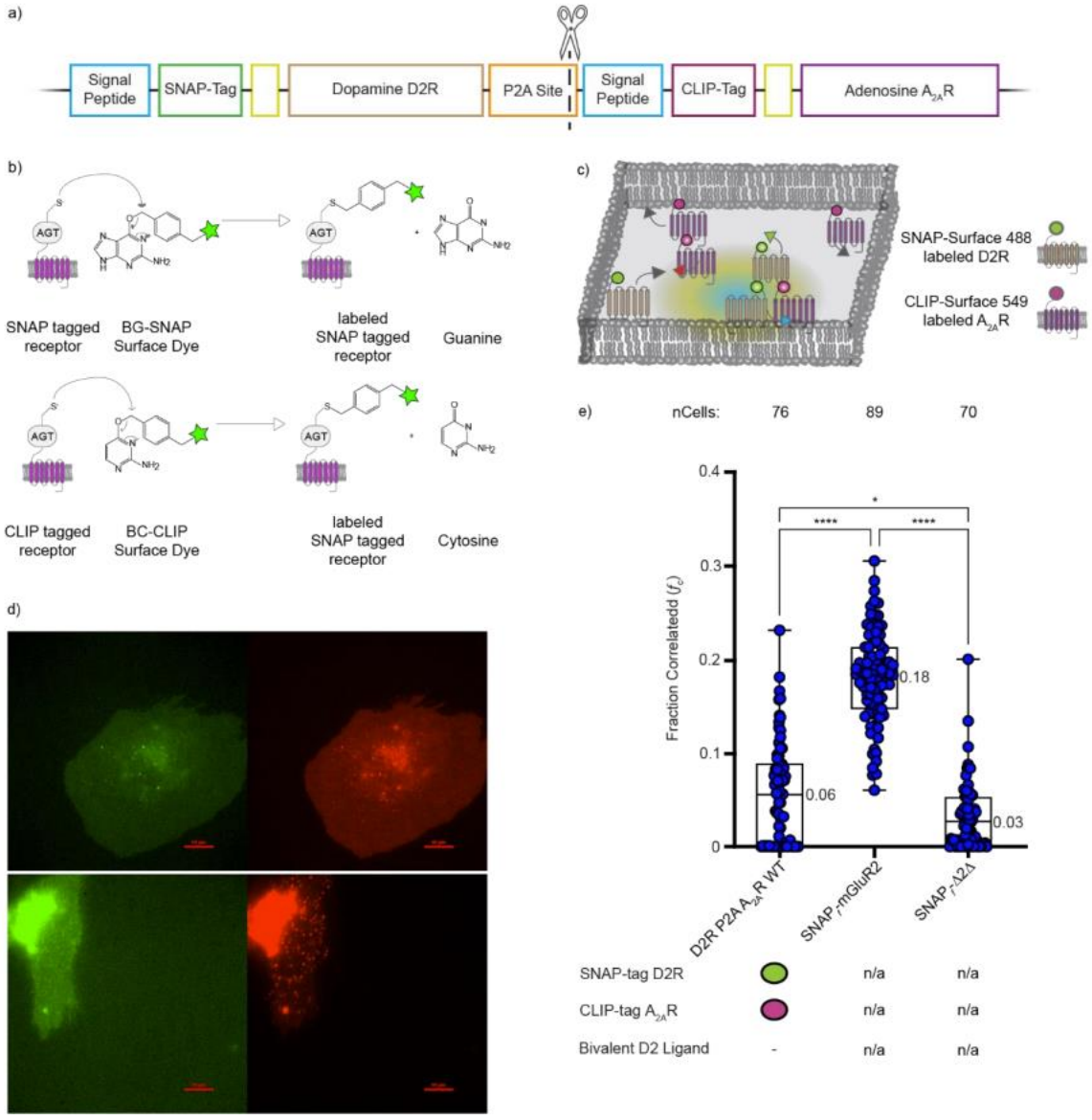

Figure 2. D2R-A2AR heterodimer experimental overview (schematic of Grant T. Gilmore). a) The GPCR primary sequence of D2R and A2AR. During translation, the peptide was cleaved at the P2A site to separate the two receptor proteins. Cleavage assured that the two receptors were independently inserted into the plasma membrane. b) Selective BG-SNAP and BC-CLIP surface dies covalently attach to D2R and A2AR, respectively. c) SNAP-Surface labeled D2R, and CLIP-Surface labeled A2AR were excited by 488nm and 549nm lasers, respectively. After

excitation, the labeled D2Rs emitted photons were detected as green. The labeled A2AR receptors emitted photons were detected as red. d) The fluorescently labelled cell was excited with green and red inducing lasers. The top images were taken before treatment, and the bottom images were taken 30 minutes after treatment of the bivalent ligand. e) The fc values were obtained from the longitudinal samples and compared with dimer and monomer GPCR controls: SNAP-mGluR2 and SNAP-Δ2Δ, respectively. Statistical significance was measured using an ANOVA followed by post-hoc Tukey's comparisons ($p=0.05$).

The fc value for D2R P2A A2AR WT was 0.06 ($n = 76$), which was significantly different than the monomer control, SNAP-Δ2Δ, and the dimer control, SNAP-mGluR2. SNAP-Δ2Δ had an fc value of 0.03 ($n = 70$) and SNAP-mGluR2 had an fc value of 0.18 ($n = 89$). The cross correlation of the treatment group was significantly greater than the dimer control and significantly smaller than the monomer control.

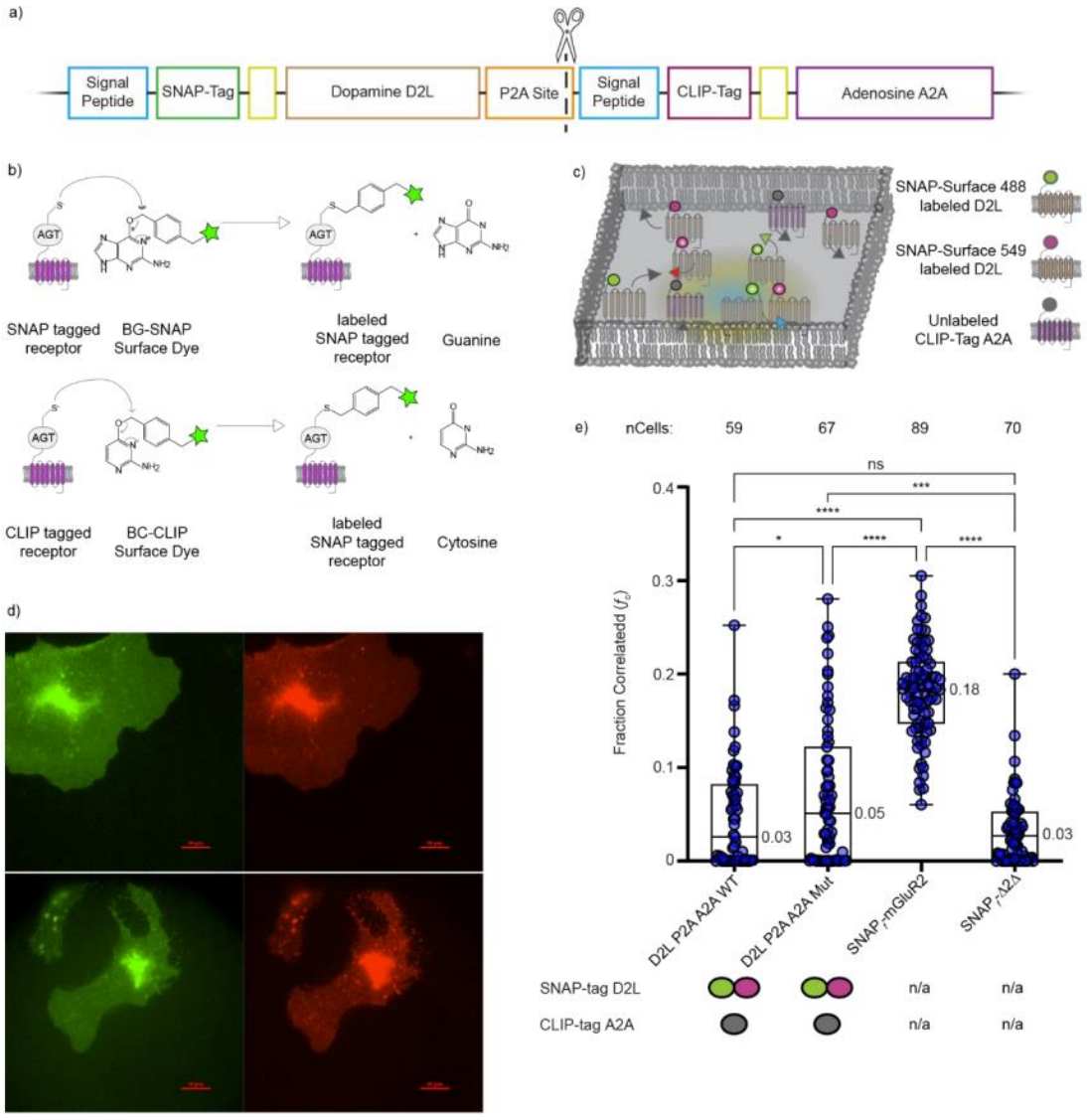

Figure 3. D2R homodimer compared to WT and Mutant A2AR overview (schematic of Grant T. Gilmore). a) The GPCR primary sequence of D2R and A2AR is displayed. During translation, the peptide was cleaved at the P2A site to separate the two receptor proteins. The cleavage assured that the two receptors were independently inserted into the plasma membrane. b) Selective BG-SNAP and BC-SNAP surface dies covalently attached to D2R. c) The SNAP-Surface labeled D2R, and CLIP-Surface labeled D2R were excited by 488nm and 549nm lasers, respectively. Mutant and WT CLIP-tag A2A remained unlabeled. After excitation, the SNAP-

surface 488 labeled D2Rs emitted photons, which were detected as green, and the SNAP-surface 549 labeled D2Rs emitted photons, which were detected as red. d) The fluorescently labelled cell was excited with green and red inducing lasers. The top images were taken before treatment, and the bottom images were taken 30 minutes after treatment of the bivalent ligand. e) The fc values were obtained from the longitudinal samples of D2R P2A A2A WT and D2R P2A A2A Mut, and they were compared with dimer and monomer GPCR controls, which were SNAP-mGluR2 and SNAP-Δ2Δ, respectively. Statistical significance was measured using an ANOVA followed by post-hoc Tukey's comparisons (p=0.05).

Unlabeled Mut A2AR was tested to see if it significantly altered D2R homodimers. The three-point mutations in Mut A2AR rendered an fc value of 0.05, and WT A2AR rendered an fc value of 0.03 (n = 59). The D2R P2A A2AR WT was not significantly different than the monomer control, SNAP-Δ2Δ (fc = 0.03) (n = 70) and was significantly different than the dimer control, SNAP-mGluR2 (fc = 0.18) (n = 89). Unlabeled Mut A2AR significantly increased the fc value of D2R P2A A2AR Mut to 0.05 (n = 67). Additionally, the upper tail is greater than the WT group. The Mut group was significantly different to both the monomer and dimer controls.

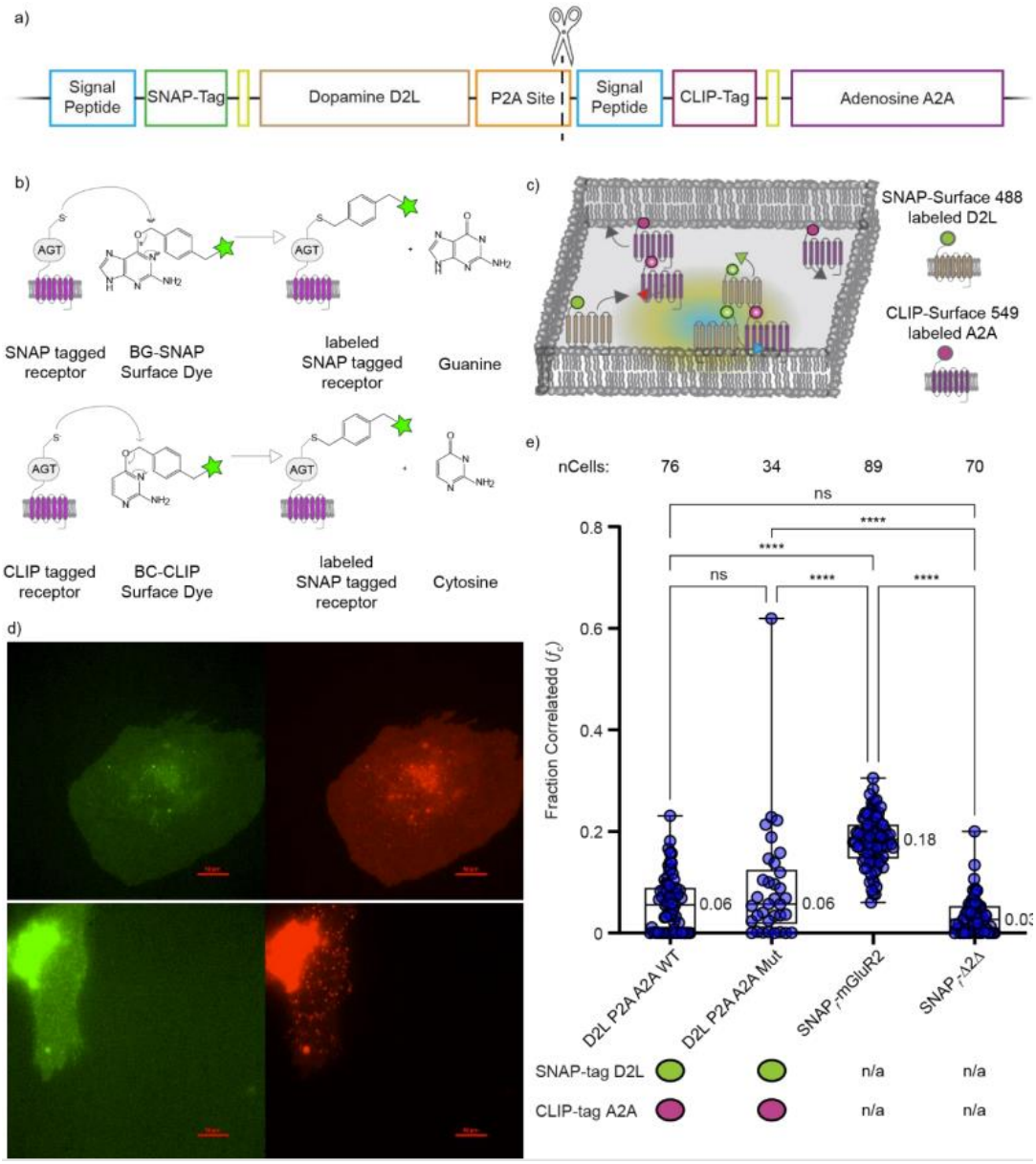

Figure 4. D2R-A2AR heterodimer compared to WT and Mutant A2AR (schematic of Grant T. Gilmore). a) The GPCR primary sequence of D2R and A2AR is displayed. During translation, the peptide was cleaved at the P2A site to separate the two receptor proteins. The cleavage assured that the two receptors were independently inserted into the plasma membrane. b) Selective BG-SNAP and BC-CLIP surface dies covalently attach to D2R and A2AR, respectively. c) The SNAP-Surface labeled D2R, and CLIP-Surface labeled A2AR were excited

by 488nm and 549nm lasers, respectively. After excitation, the labeled D2Rs emitted photons, which were detected as green, and the labeled A2AR receptors emitted photons, which were detected as red. d) The fluorescently labelled cell was excited with green and red inducing lasers. The top images were without treatment, and the bottom images were 30 minutes after treatment of the bivalent ligand. e) The fc values were obtained from the longitudinal samples and compared with dimer and monomer GPCR controls, which were SNAP-mGluR2 and SNAP-Δ2Δ, respectively. Statistical significance was measured using an ANOVA followed by post-hoc Tukey's comparisons ($p=0.05$).

Both D2R and A2AR were labelled, and the Mut and WT A2ARs were tested for their effect on DR2-A2AR heterodimerization. Both groups were not significantly different from each other, yet the Mut treatment group (fc = 0.06) (n = 34) was significantly different to the monomer control, SNAP-Δ2Δ (fc = 0.03) (n = 70). Even though both Mut and WT groups have the same fc value, the upper-tail outlier in the Mut group rendered it significantly different to the monomer control. Both Mut and WT groups were significantly different to the dimer control, SNAP-mGluR2 (fc = 0.18) (n = 89).

Dimerization occurs during intracellular signaling when two receptors physically link to one another. The specific receptors that were investigated were D2 and A2A receptors. To generate SNAP-tag-receptor protein fusion constructs, a mammalian vector was used and transfected into CHO cell lines. The CHO cells were cultured using Ham's F12 media and were incubated in a 5% CO_2 incubator until confluent. The cells were labeled using red and green dye and were prepared on a dish for imaging and PIE-FCCS experiments.

D2Rs were measured for their ability to form homodimers among different treatments and were only found to be significantly different from the monomer control when treated with Cu2+ phenanthroline (**Figure 1**). Different ligand activations prove to affect dimerization differently, which in this case is in D2R homodimers.

D2R P2A A2AR WT was significantly different than the monomer control and the dimer control (**Figure 2**). The cross correlation, fc values, recorded from the treatment group was significantly greater than the monomer control and significantly smaller than the dimer control. We found that WT D2R and A2AR dimerize upon activation, but not as much as the dimer control.

When tested with Mut A2AR, the fc value of D2R P2A A2AR Mut significantly increased (**Figure 3**). The Mut group was significantly different to both the monomer and dimer controls. We interpreted that the three-point mutations, which changed the peptide composition of A2AR Mut, significantly increased interactions in the D2R homodimer compared to the D2R homodimer when interacting with A2A WT.

The D2R-A2AR heterodimer was compared to the WT and Mut A2AR (**Figure 4**). Both D2R and A2AR were labelled, and both groups were not significantly different from each other, and the Mut group was significantly different from the monomer control. This suggests that A2A Mut significantly increased D2R-A2AR heterodimer interaction.

Significant limitations can be found in **Figure 4e** in which the sample size for D2R P2A A2A Mut is 34. The sample size for other treatment groups is about double than the D2R P2A A2A Mut group. If we had an increased number of sampled cells, it may have mitigated the impact of the upper-tail outlier that seems to have skewed the data to be significantly different to the monomer control.

The Mut A2A significantly impacted GPCR dimerization among homodimer D2R and heterodimer A2AR. Our research contributes to the current push to classify different types of GPCRs in terms of dimerization. Differences in GPCR activation and peptide composition prove to affect dimerization differently.

Discussion

A2AR-D2R heterodimer complexes play a role in cocaine addiction in rats (Borroto-Escuela et al., 2017). This study suggests that A2AR-D2R interactions increase D2R activation because breaking these heterodimers decreased the affinity of D2R activation (Borroto-Escuela et al., 2017). D2R activation resulted in strong relapses of cocaine self-administration in rats (Wydra et al., 2014). Additionally, Lisa Briand and colleagues (2008) measured increased elevations of high affinity D2R in rats with chronic cocaine self-administration. Therefore, finding ways to dissociate A2AR-D2R heterodimers may prove to be a potential drug target for addiction. Our study modeled a way in which one may be more susceptible than others to drug addiction. Small genetic differences in receptor genes translate slightly different receptor peptide sequences, which may have different affinities to other receptors. Since receptor-receptor interactions prove to be integral in cocaine self-administration (Borroto-Escuela et al., 2017), these different peptide sequences may make someone more susceptible to drug abuse.

In our study, Mut A2AR increased A2AR-D2R heterodimerization (**Figure 4**) and increased D2R homodimerization (**Figure 3**). Three peptide substitutions in Mut A2AR were enough to elicit significant changes in dimerization. Further study on dimerization may help our field better understand the genetic component of addiction and further uncover how drug use affects molecular mechanisms.

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