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RECEPTOR-PROTEIN INTERACTIONS MEDIATED BY THE FOURTH
TRANSMEMBRANE OF D2R CAN BE ALTERED BY TREATMENT WITH
ANTIPSYCHOTICS

BY
CRAIG MICHAEL IRVING

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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IN
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UNIVERSITY OF RHODE ISLAND

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MASTER OF SCIENCE
IN
INTERDISCIPLINARY NEUROSCIENCE

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UNIVERSITY OF RHODE ISLAND
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ABSTRACT

The treatment of psychological diseases like schizophrenia has depended on the use of antipsychotic drugs many of which were created as far back as the 1970's. These drugs typically act on the D2 dopamine receptor (D2R) to antagonize its signaling and alter the signaling of the mesolimbic and mesocortical dopaminergic pathways in the brain. Several studies have indicated that it may be more important to identify the specific receptor-protein interactions which could prove to be more beneficial drug targets for the treatment of schizophrenia (Magalhaes, Dunn, & Ferguson, 2012). In this study we identified that the fourth transmembrane motif in D2R is responsible for the receptor-protein interaction that was previously shown to decrease the detergent solubility of cellular G β 5. Furthermore, we showed that the biophysical effects of this receptor-protein interaction could be significantly altered by treatment with clozapine but not haloperidol. Clozapine is a first generation antipsychotic that has been shown to have a unique efficacy for the treatment of schizophrenia compared to all other antipsychotics (Attard & Taylor, 2012). This finding identifies both a unique property of clozapine binding to the receptor and a significant alteration of receptor-protein interactions by a drug that previously was only thought to simply antagonize DA signaling at D2R.

ACKNOWLEDGMENTS

I would like to acknowledge a few individuals who have made the work in this thesis possible. First and foremost, Dr. Abraham Kovoov, your guidance and insight over the past three years has been invaluable. You have instilled in me the importance of performing science that will lay the groundwork for future researchers. Every discovery no matter how big or small could be the key to solving a mystery. The research we have conducted I truly hope will rewrite the textbooks and help to develop new antipsychotics that are more effective than the drugs we have today.

I would like to thank my lab mates with whom this work would not have been possible. First I would like to thank Joe Schrader for his training and support as we have worked side by side as graduate students these past three years. I would also like to thank all of the hard work done by the undergraduates in our lab including Joe, Dean and Mihir.

The financial support I received is also a huge part of what made my career at URI so successful. The chemistry department and Dr. Susan Geldart enabled me to teach multiple semesters and provided amazing support and guidance along the way. Not only was this funding important, but the experience I gained from it has led me to following my passion for teaching and helped me be competitive as I applied for jobs in the boarding school setting. Summer funding provided by both INBRE and the Ryan Institute for Neuroscience was also very instrumental in helping me continue my research during the summer and was immensely appreciated.

Dedication

To the friends, family, and loved ones who supported me every step of the way.

PREFACE

This Thesis is being submitted in manuscript format in accordance to the formatting put forth by PlosOne for submission upon completion of degree requirements.

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MANUSCRIPT

This Manuscript has been prepared for submission too PlosOne.

CHAPTER 1

INTRODUCTION

Schizophrenia is a disease of the brain that is classically characterized by its positive, negative and cognitive symptoms. The positive symptoms include delusions and hallucinations while negative symptoms refer to depressive symptoms and a general overall decrease in affect. Cognitively schizophrenia decreases the affected individuals ability to process information and to effectively utilize there working memory. Globally schizophrenia affects about 1% of the population and domestically about 1.2 % of Americans. This translates numerically to approximately 3.2 million Americans who need psychiatric care due schizophrenia (Nemade & Dombeck, 2009). The treatment of schizophrenia is most commonly accomplished via the antagonism of the D2 dopamine receptor (D2R) with the use of antipsychotic drugs (APD) (Stone & Pilowsky, 2007).

D2R, the target of most APD is a G protein coupled receptor (GPCR) that signals within the cell by the activation of a heterotrimeric G protein consisting of a $G\alpha$ and $G\beta\gamma$ subunits. Activation of D2R via the binding of dopamine catalyzes the transfer of a guanosine-5'-triphosphate (GTP) to the $G\alpha$ subunit replacing a guanosine diphosphate, this causes the separation of the $G\alpha$ subunit from the $G\beta\gamma$ subunit allowing them to signal independently of each other. D2 like receptors specifically activate $G\alpha_{i/o}$ proteins (Neve, Seamans, & Trantham-Davidson, 2004)(Bockaert & Pin, 1999). $G\alpha_{i/o}$ proteins comprise a class of $G\alpha$ proteins that is inactivated by

pertussis toxin; these proteins are considered to be inhibitory G proteins due to their inactivation of the adenylate cyclase causing a decrease in cAMP in the cell (Wettschureck & Offermanns, 2005).

The other important signaling molecule released via the activation of D2R is the $G\beta\gamma$ subunit. $G\beta\gamma$ is able to affect its effectors usually located on the plasma membrane (PM) via protein-protein interactions. For example, $G\beta\gamma$ activates the inward rectifying potassium channel. The effectors that are modulated by the $G\beta\gamma$ subunit are diverse and determined by the specific isoforms of $G\beta$ and $G\gamma$ subunits (Srncka, 2008).

The symptom alleviation by APD is thought to be due to the modulation of different pathways in the brain, specifically the dopaminergic pathways. Dysfunction of these pathways and associated symptoms include: the mesolimbic pathway associated with positive symptoms, the mesocortical pathway associated with negative symptoms, the nigrostriatal pathway associated with EPS and tardive dyskinesia and the tuberoinfundibular pathway associated with hyperprolactinemia (Beaulieu & Gainetdinov, 2011; Neve et al., 2004).

There currently are two generations of APDs that both antagonise D2R. First generation (typical) antipsychotics are effective at relieving the positive symptoms of schizophrenia, but prolonged treatment leads to irreversible movement disorders collectively termed as extrapyramidal symptoms (EPS). These symptoms arise from binding to D2R expressed within the striatum, a region of the brain that is important for controlling movements. In the 1970s, a highly efficacious drug, clozapine, was introduced that suppresses both negative and positive schizophrenia symptoms. It also

was found to have very low liability for producing movement disorders (Lieberman & Stroup, 2011). Unfortunately, clozapine produced fatal agranulocytosis in about 1% of patients (Gerson & Meltzer, 2012). It was found that clozapine, has decreased affinity for D2R and increased affinity for the 5'hydroxytryptamine receptor (5HT receptor) compared to first-generation drugs, such as haloperidol. Thus, it was hypothesized that the unique efficacy of clozapine was due to the differential pharmacological binding profile of clozapine compared to other antipsychotics. This encouraged drug companies to develop other molecules with similar pharmacological profile in the hope of producing drugs that recapitulated clozapine's unique efficacy without producing agranulocytosis. However, recent large-scale clinical studies with the second generation APD clearly demonstrated that second generation antipsychotics do not have the same clinical efficacy as clozapine in the treatment of schizophrenia (Attard & Taylor, 2012).

In this thesis, I investigated the unique efficacy of clozapine in regards to the D2R and its receptor-protein interactions, which is supported by two initial findings in our lab. The first finding is that G β 5 when co-expressed with D2R interacts with D2R in a manner that both stabilizes the proteins' expression and moves it into a detergent resistant fraction (Octeau et al., 2014). Secondly, our lab has identified some unique properties of the drug clozapine as it relates to the binding of intracellular D2R. The findings show that APDs increase the detergent solubility of D2R, release of D2R from the endoplasmic reticulum (ER) and subsequently increase insertion of D2R into the plasma membrane. However, clozapine is unique in that it does not increase

receptor detergent solubility and has the smallest increase in plasma membrane insertion of D2R (Schrader, 2017).

My aim is to demonstrate that antipsychotic treatment is able to modulate the expression or solubility of G β 5 as the result of antipsychotic binding of intracellular D2R. Furthermore, to determine the specific receptor-protein interaction between G β 5 and D2R as it relates to D2Rs' ability to alter the biophysical properties of G β 5 by determining the region of D2R that interacts with G β 5. Lastly, I will challenge how we classically think about the mechanism of APDs as they relate to the antagonism of D2R by showing another functional effect at the receptor that is not due to direct antagonism of receptor signaling and postulate a biochemical characteristic of D2R that has never previously been reported.

CHAPTER 2

MATERIALS AND METHODS

Chemicals

Chemicals and reagents utilized in the following methods were obtained from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cell Culture and Transfection

Cell Culture experiments were performed in HEK 293T cells obtained from American Type Culture Collection (ATCC CRL-3216). Cells were maintained according to the ATCC protocols using Dulbecco's modified Eagles media (DMEM) supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml) and 10% Fetal Bovine Serum (Sigma Aldrich) at 37°C and 5% CO₂. Cell transfections were performed with complementary DNA (cDNA) using lipofectamine LTX reagent according to the manufacture's instructions (LTX, Life Technologies). The cDNA constructs transfections were standardized between individual experiments by controlling the amount of cDNA transfected per well and by using the same expression vector for all constructs.

In experiments requiring drug treatment, 24 hours post transfection cells were treated with APDs at a 10 µM concentration for 24 hours. The two drugs utilized in this study were clozapine and haloperidol.

cDNA Constructs

The cDNA constructs used in all cell based experiments were created using standard molecular biology practices or obtained from other labs as cited. PCR was used to amplify, mutate, and add the desired sequences necessary to create the sequences needed for the constructs mentioned below. Amplified cDNA was inserted via ligation, following the protocols put forth by New England BioLabs, of PCR products into PCDNA 3.1+ Zeo, which is a mammalian expression vector allowing us to transiently express the desired proteins into mammalian cell lines (Thermo Fisher Scientific, 2016)(New England Bio Labs, 2016). PCR was carried out according to the specific conditions required by New England Bio lab's (NEBs) for the TAQ polymerase to be used (New England Bio Labs, 2015). Constructs were validated via sequencing at the URI Genomic sequencing center funded by EpSCOR.

The following constructs were utilized for the completion of the mentioned experiments: N-terminally tagged FLAG D2 long (FLAG-D2R) (Glass & Felder, 1997), Gβ5 short isoform construct (Kovoor et al., 2000), endoplasmic reticulum marker mCh-Sec61 beta (Zurek, Sparks, & Voeltz, 2011), N-terminally FLAG-tagged mCherry linked to the D2R fourth transmembrane at the C terminus (D2-4tm mCherry), N-terminally FLAG-tagged 1st -3rd transmembrane of D2R(D2 1-3tm), N-terminally FLAG-tagged 1st - fourth transmembrane of D2R(D2 1-4tm), FLAG-D2R_{D114A} (Mansour et al., 1992), FLAG D2R_{W160A} (Hanson et al., 2008), FLAG D2R_{M155P}(Nilsson et al., 1998).

TX-100 cell lysate fractionation and total protein preparation

After transient expression of the protein into the HEK293-T cells, the cells were collected and lysed according to previously established protocols (Cerver,

Sharma, & Kovoov, 2012). This procedure used a lysis buffer that contains 1X Phosphate buffered saline (PBS) and 2%(v/v) Triton-X 100 supplemented with a SigmaFast protease inhibitor diluted according to manufacturer specifications. Cells were lysed on ice for 1hr, followed by centrifugation at 10,000 g for 10 min to collect the TX-100 insoluble fraction. The soluble protein in the supernatant was then precipitated via a 10% w/v tri-chloroacetic acid precipitation. The protein was then pelleted and washed with ice cold 95% acetone 3X. Both pellets were then resuspended in a SDS sample buffer (2% (w/v) SDS, 50 mM Tris-HCl, 50 mM DTT, 0.1% (w/v) bromphenol blue, pH 6.8) (Sharma, Celver, Oceau, & Kovoov, 2013).

For analysis of whole cell lysate, cells were collected in centrifuge tubes and washed once in 1X PBS. After washing in PBS the cell pellet was resuspended in SDS sample buffer and heated at 65°C for 10 min. After incubation the cells were sonicated 10 x at a power setting of 5 for 0.5 seconds with 2 seconds between each pulse (XL2000-qSonica). Samples were then heated again post sonication at 65°C for 10 min before being resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) followed subsequently by western blotting for protein quantification.

Western Blot Analysis

Protein samples were run on 10% SDS-Page gels and transferred to methanol activated PVDF membranes for antibody based detection. Post transfer the blots were blocked in 5% non-fat milk in PBS. Antibodies used were based on the protein of interest being targeted. Blots were probed with a FLAG-HRP conjugated antibody targeted against the FLAG tag (1:5000 in 10% nonfat milk in 1X PBS) (Sigma

Aldrich, 2016). The G β 5 short isoforms were probed using the rabbit polyclonal antibody CT215 (1:5000 in 5%w/v nonfat milk in PBS)(Watson, Katz, & Simon, 1994). Chemiluminescent signals from the HRP conjugated FLAG antibody and anti-rabbit HRP conjugated secondary antibodies will be imaged using Supersignal West Femto substrate (Pierce-Thermo Fisher Scientific, n.d.) and detected using a Chemidoc XRS Molecular Imager (BIO-RAD, 2016). Images were converted to “.tif” files and analyzed using the ImageJ open source software (Schneider, Rasband, & Eliceiri, 2012).

Immunocytochemistry

Immunocytochemistry was performed on HEK293T cells that were cultured on poly-D-lysine coated cover slips. Cover slips were prepared by coating with poly-D-lysine at a concentration of 50 μ g/ml for 1 hr at 37°C, cover slips were then washed 3 times in sterile PBS according to manufacture guidelines (Sigma). Prepared coverslips were placed into the bottom of 12-well plates and cells were then plated on top of them at a density of 1×10^5 cells/well, and transfected with the appropriate cDNA constructs. APD treatment of the cells occurred 24 hours post transfection. Cells were dosed at a final drug concentration of 10 μ M.

24 hours post drug treatment cells were fixed using ice cold 100% methanol to both fix and permeabilize the cells. Cells were washed 3x in 1X PBS, and blocked in 5% (w/v in 1X PBS) nonfat milk for 1 hr at room temperature under constant agitation. Post blocking cells were probed with appropriate antibodies depending on the protein being identified. FLAG tagged proteins were identified by incubation in M2 Anti-FLAG (1:1000 dilution in 5% nonfat milk in 1X PBS) overnight at 4°C

under constant agitation (Sigma Aldrich, 2016, p. 2). Similarly the G β 5 short isoforms were identified using the rabbit polyclonal antibody CT215 (1:1000 dilution in 5% nonfat milk in 1X PBS) under constant agitation (Watson et al., 1994). After overnight incubation in the primary antibody, cells were washed 2x with 1X PBS for 15 min at room temperature under constant agitation. The cells were then incubated for 1 hour at room temperature in appropriate Alexa Fluor conjugated secondary antibodies (1:5,000 dilution in 5% w/v Nonfat milk in 1X PBS) for imaging under fluorescent microscopy (Thermo Fisher Scientific, 2016). Microscopy was done with the use of a Zeiss LSM 700 confocal microscope, which was coupled to a HSP 120 metal halide lamp allowing for four excitation lines at 405, 488, 555, and 639. Microscopy also utilized the Zeiss Efficient Navigation (ZEN) Software for image processing and colocalization analysis.

Data Analysis

Western blots were analyzed using ImageJ Software and area under the curve was determined for all necessary bands (Schneider et al., 2012). The values obtained from these analyses were then entered into Prism 7 for statistical analysis (*GraphPad Prism 7*, 2017). Values were first used to perform a one-way analysis of variance (ANOVA) if the P value obtained was <0.05 then a Tukey Post-hoc test was also performed to determine which groups were significantly different.

CHAPTER 3

RESULTS

Transiently expressed alone, Gβ5 solubility is unaffected by antipsychotic treatment.

Gβ5 transiently expressed alone into HEK293T cells was fractionated into detergent soluble and insoluble fractions. After western blot analysis it was determined that $72.16\% \pm 4.88$ was fractionated into the soluble fraction of the cell lysate, this repeated previously published data on the solubility of the transiently expressed receptor (Octeau et al., 2014). In order to determine if drug treatment alone was able to alter the solubility of Gβ5, transfected cells were treated for 24 hours with haloperidol and clozapine. Treatment with haloperidol and clozapine did not lead to any differences in the solubility of Gβ5 expressed in APD treated HEK293T cells indicating that drug treatment alone is not sufficient enough to cause an alteration of Gβ5 cellular detergent solubility (Fig. 1 A).

Solubility of transiently expressed Gβ5 co-expressed with D2R is altered by clozapine treatment.

As previously reported, co-expression of Gβ5 with D2R causes Gβ5 to become more detergent insoluble, this effect was determined to be specific to a direct protein interaction with D2R and not through an regulatory of G protein signaling (RGS) intermediary (Octeau et al., 2014). The co-expression of D2R leads to percent detergent solubility of cellular Gβ5 to decrease from $72\% \pm 4.88$ (n=11) to $25\% \pm 5.00$ (n=12). After cells were treated with clozapine and haloperidol for 24 hours, the mean

percent solubility of G β 5 in cells treated with clozapine increased, and was significantly different from both vehicle treated and haloperidol treated cells, which were not statistically different from each other (Figure 1A). While clozapine treatment increased total cellular G β 5 solubility to 51% $\pm$ 5.00, it was also significantly different from cells transfected with only G β 5. This result indicates that alteration of G β 5 detergent solubility is unique to clozapine is dependent on its co-expression with D2R.

Figure 1. Clozapine increases mean cellular Gβ5 detergent solubility in a D2R dependent manner. A) HEK293 cells were transiently transfected with Gβ5 and D2R + Gβ5, cells were treated for 24 hours with APD and then separated into TX100 soluble and insoluble fractions and resolved via SDS-PAGE used for western blotting and subsequent quantification. Bars represent the mean ($n=9 \pm$ S.E.M) solubility of Gβ5, bars not sharing a letter differ significantly at a $P<0.05$ (Tukey Post Hoc Test). B) Representative images from western blots depicting the relative levels of detergent soluble (S) and insoluble (I) Gβ5 (upper panel) and total protein via coomassie blue staining (lower panel).

Protein Stability of transiently expressed Gβ5 co-expressed with D2R.

Co-expression of Gβ5 with D2R has been shown to also increase the protein stability of Gβ5 over time (Otteau et al., 2014). To determine if this effect of D2R on Gβ5 stability was also sensitive to treatment with an APD, HEK293T cells were co-transfected with both Gβ5 and D2R and treated for 24 hrs with either clozapine or haloperidol at a final drug concentration of 10 μM. Figure 2 illustrates the mean increase of Gβ5 stability expressed as a percent increase over cells expressing Gβ5 alone. Results indicate that while co-expression of D2R did increase stability after 24 hours, there was no statistical difference between cells treated with vehicle, clozapine and haloperidol.

Figure 2. Co-expression of Gβ5 with D2R increases Gβ5 stability, but stability is not significantly affected by treatment with APDs. A) HEK293 cells were transiently transfected with D2R + Gβ5, cells were treated for 24 hours with APD and then solubilized in SDS sample buffer and used for western blotting and subsequent quantification. Bars represent the mean ($n=6 \pm \text{S.E.M}$) increase of Gβ5 signal expressed as a percent increase over cells expressing Gβ5 alone; bars not sharing a letter differ significantly at a $P<0.05$ (Tukey Post Hoc Test). **B)** Representative images from western blots depicting the relative levels of total Gβ5 signal (upper panel) and β actin signal (lower panel).

Gβ5 cellular distribution is altered when coexpressed with D2R and treated with haloperidol but not clozapine.

It has been previously shown using immunocytochemistry (ICC) in HEK293T cells expressing D2R that treatment with haloperidol significantly alters the colocalization of D2R with the ER. This can visually be recognized by a clear increase in D2R signal at the PM after haloperidol treatment. Conversely no redistribution of D2R can be visualized after clozapine treatment and close to 90% of D2R instead is colocalized with the ER using a Sec 61 mCherry ER marker, indicating that clozapine is not altering cellular localization (Schrader, 2017; Zurek et al., 2011). In order to determine if this redistribution of D2R by haloperidol could also change the cellular distribution of Gβ5 we used immunocytochemistry to visually examine the distribution within the cell. Visually Gβ5 appears to mainly localize to the cytosol and is evenly distributed within the cell when expressed alone. When coexpressed with D2R and treated with haloperidol though, Gβ5 seems to visually redistribute within the cell. Under these conditions Gβ5 colocalizes more with D2R signal and shows a decrease in overall diffusion within the cytosol. Conversely treatment with clozapine does not visually alter the distribution of Gβ5 coexpressed with D2R (Figure 3). Drug treatment also was able to repeat previously reported results, which can be seen by the dramatic increase in PM D2R signal (Schrader, 2017).

Figure 3. Visual Gβ5 distribution is altered when coexpressed with D2R and treated with haloperidol but not clozapine. Green signal represents the signal from D2R labeled with the Alexa Fluor 488, red signal is from Gβ5 labeled with Alexa Fluor 568. Each column represents either D2R signal alone, Gβ5 signal alone, merged signal, or merged signal with the addition of transmitted signal used to illustrate the border of other cells within the image. Each row describes the transfected cDNA construct and treatment group.

***Gβ5 detergent solubility is unaffected by APD when co-expressed with D2R_{D114A},
Ligand binding deficient mutant.***

In order to determine if the clozapine induced alteration of Gβ5 detergent solubility by D2R is due to specifically to the binding of APD to D2R, a mutant D2R that is unable to bind antagonist was utilized. The D2R mutation was made in the binding pocket and was done by replacing an aspartic acid residue with an alanine residue at the 114 amino acid position of D2R (Mansour et al., 1992). D2R_{D114A} upon co-expression was able to decrease Gβ5 solubility to 46%±4.82(*n*=6), but upon treatment with both haloperidol and clozapine no significant changes were seen in the mean detergent solubility of Gβ5 coexpressed with D2R_{D114A} (figure 4).

Figure 4. Nonbinding mutant D2R_{D114A} is able to decrease Gβ5 detergent solubility, this effect is unaltered by APD treatment. HEK293T cells transiently transfected with D2R nonbinding mutant D2R_{D114A} and Gβ5, were treated for 24 hours with vehicle, clozapine or haloperidol. Cellular proteins were then fractionated into

TX100 soluble and insoluble fractions and run out on SDS-PAGE used for western blotting and subsequent quantification. Bars represent the mean ($n=6 \pm \text{S.E.M}$) solubility of G β 5, bars not sharing a letter differ significantly at a $P<0.05$ (Tukey Post Hoc Test).

Removal of D2R from the ER is important for the interaction of G β 5 with D2R.

D2R when examined under confocal microscopy is often seen inside of the cell and not highly expressed on the PM. Furthermore a number of studies have identified ER retention signals within the 3rd intracellular loop of D2Rs protein sequence (Kubale, Blagotinšek, Nøhr, Eidne, & Vrecl, 2016). Furthermore, other studies have shown that a highly conserved tryptophan residue located in the fourth transmembrane across G protein coupled receptors (GPCR) that is important for cholesterol binding and interactions with other transmembrane domains and ultimately the trafficking of GPCRs within the cell (Dupré, Hébert, & Jockers, 2012; Hanson et al., 2008; Kobayashi, Ogawa, Yao, Lichtarge, & Bouvier, 2009). It has been previously shown that some molecules such are able to act as molecular chaperones, capable of stabilizing β -adrenergic receptor in a conformation that is able to overcome the tryptophan residue mutation and release it from the ER allow for dimerization of the mutated receptor (Kobayashi et al., 2009).

We also have previously reported similar results with D2R_{W160A}, a D2R mutant with a mutated tryptophan that is highly conserved across GPCRs (Schrader, 2017). This trafficking deficient D2R_{W160A} was determined to be expressed exclusively in the ER of HEK293T cells by ICC colocalization with Sec61-mCherry ER marker. Rescue from the ER was accomplished via the treatment of haloperidol and observed by a

2000% increase in surface D2R_{W160A} signal, however this increase in surface signal was not seen with the treatment of cells with clozapine. This suggests that haloperidol is able to act as a molecular chaperone within the cell, but clozapine binding of the receptor imparts a conformation that retains D2R within the ER (Schrader, 2017).

In order to determine if the interaction of Gβ5 with D2R was occurring within the ER or in some intermediary between the ER and the PM we utilized the trafficking mutant D2R_{W160A}. HEK293T cells were co-transfected with Gβ5 and D2R_{W160A}, and dosed with 10 μM clozapine and haloperidol for 24 hrs. Cells were used for both ICC and TX-100 detergent fractionation and western blot quantification of detergent solubility. Vehicle treated and clozapine treated cells expressing D2R_{W160A} and Gβ5 were unable to decrease the detergent solubility of Gβ5. Haloperidol treatment, which has previously been shown to release D2R_{W160A} from the ER, was able to significantly decrease the detergent solubility of Gβ5 to 51%±3.96(*n*=6) compared to 82%±5.8 of vehicle treated cells (figure 5). Furthermore under ICC, Gβ5 appeared to be colocalized with D2R_{W160A} signal (Figure 6).

Figure 5. D2R trafficking mutant, D2R_{W160A}, is only able to effect Gβ5 detergent solubility after treatment with haloperidol. HEK293T cells transiently transfected with trafficking mutant D2R_{W160A} and Gβ5, were treated for 24 hours with either vehicle, clozapine or haloperidol. Cellular proteins were then fractionated into TX100 soluble and insoluble fractions and run out on SDS-PAGE used for western blotting and subsequent quantification. Bars represent the mean ($n=6 \pm$ S.E.M) solubility of Gβ5, bars not sharing a letter differ significantly at a $P<0.05$ (Tukey Post Hoc Test).

Figure 6. Gβ5 distribution visualized by ICC is altered when coexpressed with D2R_{W160A} and treated with haloperidol. Green signal represents the signal from D2R_{W160A} labeled with the Alexa Fluor 488, red signal is from Gβ5 labeled with Alexa Fluor 568. Each column represents either D2R_{W160A} signal alone, Gβ5 signal alone, merged signal, or merged signal with the addition of transmitted signal used to illustrate the border of other cells within the image. Each row describes the transfected cDNA construct and treatment group.

Identification of the D2R protein motif interacting with Gβ5.

The ability of D2R to alter Gβ5 solubility has previously been determined to be due to a specific receptor-protein interaction (Octeau et al., 2014). It is important to try and understand the region of D2R where this interaction occurs, so that it can be determined if it might potentially be able to interact with other proteins as well or possibly be a target for drug compounds. D2R is a classic GPCR in that it is made up of seven transmembrane regions. D2 differs from D1 type receptors due to a large intracellular third loop (Missale, Nash, Robinson, Jaber, & Caron, 1998). This large intracellular domain of D2R has been shown to interact with a number of proteins including calmodulin, β arrestins and calcium dependent protein kinase II (Fukunaga & Shioda, 2012).

For this reason we assumed that it is likely that the 3rd intracellular loop which is located between the fifth and sixth transmembrane domains of D2R is likely the region responsible for the interaction of D2R and Gβ5. We created a truncated version of D2R that specifically removed all amino acid residues after the end of the second extracellular loop. This truncation we called D2 1-4TM, expression of this receptor in HEK293T cells mimicked the detergent solubility of full length D2R and localization within the cell (data not shown). When D2 1-4TM was co-expressed with Gβ5 in HEK293T cells the truncated version of the receptor was still able to decrease the fraction of Gβ5 in the detergent soluble fraction (Figure 7 A) and was able to stabilize the expression of the Gβ5 after 24 hrs (figure 7 B).

Figure 7. The fourth transmembrane region of D2R is necessary for the alteration of both Gβ5 detergent solubility and protein stability. **A)** Protein from HEK293T cells transiently expressing Gβ5 and corresponding D2R variant cDNA construct as noted below each bar were fractionated into TX100 soluble and insoluble fractions and run out on SDS-PAGE used for western blotting and subsequent quantification. Bars represent the mean ($n=6 \pm$ S.E.M) solubility of Gβ5, bars not sharing a letter differ significantly at a $P<0.05$ (Tukey Post Hoc Test). **B)** Total protein from HEK293T cells transiently expressing Gβ5 and corresponding D2R variant cDNA construct as noted below each bar were resolved on SDS-PAGE used for western blotting and subsequent chemiluminescent quantification. Bars represent the mean ($n=3 \pm$ S.E.M) increase of Gβ5 signal expressed as a percent increase over cells expressing Gβ5 alone, bars not sharing a letter differ significantly at a $P<0.05$ (Tukey Post Hoc Test). **C)** Representative images from western blots depicting the relative levels of total Gβ5 signal (upper panel) and β actin signal (lower panel).

This was an interesting result, from previous publications we know that the fourth transmembrane of D2R is also responsible for a number of receptor-protein interactions usually described as dimerization and oligerimization with other receptors or homodimerization (Lee, O'Dowd, Rajaram, Nguyen, & George, 2003; Ng et al., 1996; Scarselli et al., 2001). To determine if the fourth transmembrane might be responsible for interaction with Gβ5 we created another truncated version of D2R that removed the fourth transmembrane domain that we call D2 1-3TM. Co-expression of D2 1-3TM with Gβ5 in HEK293T cells did not cause a decrease in the fraction of

detergent soluble G β 5. It also was unable to stabilize the expression of G β 5 in HEK293T cells expressing D2 1-3TM. This indicates that the fourth transmembrane is important for imparting the decrease in the fraction of soluble G β 5 and also to stabilization of G β 5 expression in HEK293T cells (figure 7 A, B).

To further explore this finding we created two more cDNA constructs. The first was a fusion protein of the fourth transmembrane of D2R that was attached to the C terminus of mCherry a red fluorescent protein. The second construct was a D2R mutant D2R_{M155P}, is a D2R mutant that has a proline residue inserted into the alpha helix of the fourth transmembrane alpha helix causing it to misfold. This mutant has been previously shown to disrupt the topology of the fourth transmembrane and the ability of D2R to form homodimers (Lee et al., 2003).

When expressed with G β 5 the fusion construct 4 TM mCherry was able to both significantly decrease the fraction of soluble G β 5 and stabilize the expression of G β 5 in HEK293T cells. Conversely, the misfolding mutant D2R_{M155P} was not able to change the fraction of soluble G β 5 and was not able to stabilize the expression of G β 5 (Figure 7 A,B).

CHAPTER 4

DISCUSSION

The importance of these findings is directly related to the antipsychotics used, the affects they had, and the identification of a specific region of D2R responsible for the receptor-protein interaction with G β 5. I aimed to illustrate the ability of classic antipsychotic treatment to modulate a D2R receptor-protein interaction. We utilized the biophysical alteration of G β 5 detergent solubility via the D2R-G β 5 receptor-protein interaction as the medium to demonstrate the ability of ADPs to alter receptor-protein interaction. We were able to show that antipsychotics treatment modulated the detergent solubility of G β 5 but were not able to significantly modulate the expression of G β 5.

What was interesting was the manner in which the ADP modulation occurred. D2R when expressed with G β 5 is able to decrease the percent of G β 5 present in the detergent soluble fraction. We know from immunocytochemistry(ICC) studies done (data not shown) and sequence analysis of D2R that the receptor is heavily retained within the ER by a number of protein motifs and that very small amounts of receptor is actually on the surface or in the secretory pathway on the way to the surface compared to total amounts of receptor present in the cell (Kubale et al., 2016; Schrader, 2017). Further, in figure 5 we show that the trafficking mutant D2R_{W160A}, which previously has been shown to be completely retained in the ER, is unable to alter the percent solubility of G β 5 when compared to cells expressing only G β 5. But,

when treated with haloperidol, which we know releases D2R_{W160A} from the ER, enables D2R_{W160A} to alter the percent detergent solubility of Gβ5. This points to the interaction between the two proteins to be occurring outside of the ER where the smallest amount of total D2R receptor is located. We also know from figure 4 that Gβ5 detergent solubility is unchanged when coexpressed with a nonbinding mutant D2R_{D114A} and when treated with haloperidol or clozapine, which means that any changes seen in figure 1 were in fact due to D2R binding by APDs.

The ability of haloperidol to release D2R_{W160A} from the ER indicates that it acts not only as an antagonist of D2R but is also able to act as molecular chaperone able to change the conformation of the receptor in a way that enabled it to also interact with Gβ5. We also showed that clozapine treatment is able to increase the detergent solubility of Gβ5. This is interesting because from what we have determined so far there are only a couple of situations that allow for clozapine treatment to increase solubility. One possibility is that clozapine increases the retention of the receptor in the ER. The other possibility is that the receptor when bound to clozapine is put into a conformation that inhibits the interaction of D2R and Gβ5. We have previously shown that while clozapine increases surface receptor the least, it still increases the insertion of D2R over that of vehicle treated which indicates that clozapine is not increasing the retention of D2R within the ER. This means that the conformation of D2R caused by the binding of D2R by clozapine is most likely a conformation that is not able to readily interact with Gβ5 in a manner that is able to alter the proteins detergent solubility.

We also performed experiments to try and investigate the region of D2R that might be interacting with G β 5. Our ICC data indicates that G β 5 is mostly cytosolic and does not appear to anchor itself within the plasma membrane. For this reason we first started by creating a D2R mutant that would remove the third intracellular loop (3rd ICL) of D2R. The 3rd ICL has previously been shown to interact with a number of proteins including the calmodulin and β arrestins (Ghanemi, 2015; Missale et al., 1998). This approach proved unsuccessful, as the truncated D2 1-4TM was still able to decrease detergent solubility of G β 5. We then continued by creating a construct that removed the fourth transmembrane region, this approach was successful as the new truncated D2 1-3 TM truncation was not able to decrease G β 5 detergent solubility. We continued to test the hypothesis that it was the fourth transmembrane interacting with G β 5 by using 4TM mCherry and D2R_{M155P}. The fusion protein was able to decrease G β 5 solubility while the 4 TM misfolding mutant D2R_{M155P} was unable to significantly decrease the detergent solubility of G β 5 as described in Figure 7.

These results indicate the fourth transmembrane is important for the G β 5-D2R protein-protein interaction. This is a bit of a confusing result as the fourth transmembrane of D2R should not be accessible to interaction with G β 5 which ICC data shows to be mostly cytosolic, as D2R has been inserted into the plasma membrane. It is possible that the surface is exposed during transport to the surface membrane or that there is some other mediator acting between D2R and G β 5. A protein mediator is unlikely to be the cause for a number of reasons. First, is that all RGS proteins expressed in HEK cells has previously been shown to be statistically insignificant due to there extremely low expression especially compared to the amount

transiently expressed G β 5 (Atwood, Lopez, Wager-Miller, Mackie, & Straiker, 2011). Secondly the protein-protein interaction is able to occur both when the 4 TM is inserted in the membrane, which is the case for most of the truncations used and full length D2 receptors utilized in this study, but also when the 4TM is free in the cytosol which we see in the expression of the fusion protein 4TM mCherry. While improbable the data gained from both the misfolding mutant and D2R truncations suggest that at some point after exiting the ER the 4TM of D2R is assessable to G β 5 and able to change the biophysical properties of G β 5.

CHAPTER 5

CONCLUSION

It is clear that D2R makes a protein-protein interaction with G β 5 that results in a biophysical change in G β 5. Most likely this change also alters G β 5s' ability to perform its normal cellular functions of inhibiting G α_i and G α_q mediated signaling with RGS7 (Witherow & Slepak, 2003). Furthermore, we were able to show that clozapine binding of the receptor invokes a conformation that is either unable to interact with G β 5 causing its increased detergent solubility or localizes D2R in a place not assessable to G β 5 interaction. These results illustrate another possible mechanism of GPCR signaling that is independent of dopamine(DA) activation of the receptor. The results show that APD, commonly classified as antagonists of D2R dopamine signaling, alter other D2R cellular consequences that are independent from DA signaling.

An increased awareness of protein-protein interactions and the effects that drugs have on them is needed (Magalhaes et al., 2012). There has been an increase in exploration into the “moonlighting” activities of many proteins that we classically think do one thing but recently have been discovered to also perform other of target actions. Similarly it is important to understand all protein interactions D2R is making and how different drugs might affect them. One theory that I would like to propose is that D2R has the ability to cause other proteins to become insoluble, which is due to its propensity to cause other proteins to aggregate. We know aggregated protein has

been associated with a number of diseases including Huntington's and Alzheimer's'. In these diseases soluble Tau and mutant Huntington protein act as prionogenic proteins, increasing the propensity for other proteins to aggregate (Brundin et al., 2010). In figure 8 I propose a mechanism that explains how soluble D2R may act as a prionogenic protein which soluble form is able to interact with other proteins therefore causing them to aggregate. Furthermore the diagram illustrates how ER retention by clozapine could be neuro-protective preventing D2R from interacting and aggregating proteins in the cell.

Figure 8. We propose D2R acts as a prionogenic protein and that this property is inhibited by clozapine. The above diagram illustrates the ability for free soluble D2R in the right hand panel to freely interact with Gβ5 and cause it to aggregate inducing insolubility. The left hand panel illustrates clozapine binding of D2R increases ER retention of D2R and decrease the ability for D2R to interact with Gβ5, decreasing its' prionogenicity.

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