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Unravelling the D1R-D2R heteromer

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Unravelling the D1R-D2R Heteromer

by

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Abstract

Dopamine receptors D1R and D2R form a heterooligomeric complex with signaling properties distinct from the individual receptors. Aberrant expression of this protein-protein complex is linked to the etiology of various neuropsychiatric diseases. Formation of the D1R-D2R heteromer is thought to be dependent upon electrostatic interactions occurring between the carboxyl tail of D1R and the third intracellular loop of D2R. Using this interaction site as template, I synthesized several peptides designed to disrupt the minimal area of the D1R-D2R interaction interface and tested these using whole cell lysates of human brain tissue and dopamine receptor constructs. I report that a synthetic peptide with the sequence EAARRAQE is efficient in blocking D1R-D2R interaction, while shorter and more highly charged peptides (EERRAQ, ARRA and AARRAQ) had no effect. This research provides insight into the binding regions involved in D1-D2 heteromer formation, and may aid future drug development efforts that target this receptor complex.

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Introduction

Background and Significance

With over 800 characterized members, the G protein receptor (GPCR) protein superfamily comprises the largest protein superfamily in the mammalian genome.[1] GPCR modulated signal transduction plays a role in physiological processes ranging from head to toe: from olfaction[2] to nail bed growth.[3] GPCR dysregulation is implicated in an equally diverse range of diseases. Diseases whose etiology is linked to perturbations in GPCR signaling include heart disease, diabetes, Parkinson's, and Alzheimer's disease.[4-7] Underscoring the importance of this receptor class, roughly one-third of current drugs target GPCRs.[8-9]

While they exhibit considerable diversity in structure and ligand recognition, all GPCRs share essential structural characteristics. All are integral membrane bound proteins with an extracellular N-terminus and seven intra-membrane α-helices threaded together by a polypeptide loop that passes three times through the extracellular space and three times through the intracellular space until ending at the intracellular C-terminus.[10] A heterotrimeric G protein transiently associates with the intracellular loops and C-terminus of the GPCR. It is this heterotrimeric G protein that will allow signal transduction following receptor occupation. This G protein consists of three subunits: an alpha subunit and a dimer of the gamma and beta subunits. This dimer consists of seven antiparallel β sheets (the beta subunit) and an intrinsically unstable gamma subunit. The alpha subunit is a guanine nucleotide (GTP) binding protein loosely associated with the beta sheets of the βγ dimer.[11] Upon receptor stimulation, the alpha subunit exchanges bound GDP for GTP, rendering the α-subunit-βγ dimer association energetically unfavorable and freeing the α-subunit. The freed α-subunit then exchanges GTP for

GDP in downstream events, triggering various signal transduction pathways before returning to its inactive, GDP binding state, where it associates with another βγ dimer and is ready for the cycle to begin anew. While binding of the alpha subunit to downstream effectors canonically mediates signal transduction, it also is worthwhile to note that the βγ dimer also serves as an independent effector of downstream signaling events, further adding to the diverse effects of GPCR agonization.[12]

The diversity of GPCR signaling is further underscored by the fact that GPCRs undergo ample modification from their initial translation, during which they undergo extensive alternative splicing,[13] and ending with a range of post-translational modifications such as phosphorylation, glycosylation, SUMOylation, and glycosylation.[14] GPCRs can interact with other receptors through the formation of receptor-receptor complexes (hetero or homomers). These heteromers have signaling properties that differ from their component receptors and can further modulate receptor mediated signaling. Notable GPCR heteromers include the adenosine A2A and glutamate mGlu5 receptor heteromer, the serotonin 5-HT2A and glutamate mGlu2 receptor, and the adenosine A2A–cannabinoid CB1–dopamine D2 receptor multimer.[15]

The GPCR landscape is complex and rich, with many unanswered questions and many potential targets for drug discovery efforts. Our research focuses on the family of GPCRs that recognize dopamine, a key neurotransmitter. There are five distinct classes of dopamine (D) receptors, classified as D1-D5. These are further divided into the subclasses of D1-like and D2-like based on the activity of the alpha subunit.[16] In the broad picture of neuronal signaling, D1-like receptors (D1 and D5) are coupled to alpha subunits (G_S and G_{olf}) which activate the regulatory enzyme adenylyl cyclase upon ligand binding to the parent receptor, leading to activation of the second messenger cyclic amp (cAMP) and triggering signal transduction.[17]

Conversely, D2-like receptors (D2R-D4R) are coupled to alpha subunits (G_i and G_o) which inhibit adenylyl cyclase activation and cAMP production.[18] Modulation of cAMP modulates the activity of protein kinase A (PKA), a key regulator of the phosphoprotein DARPP-32. DARPP-32 is something of a master switch protein that is acted upon by multiple signal transduction cascade and whose phosphorylated or unphosphorylated state in turn modulates the activity of the kinases PP-1 and PKA, as well as calcineurin activated pathways, ultimately regulating the transcriptional and electrochemical responses to receptor agonization.[19] As with other GPCRs, the βγ subunit is not merely a passive bystander. For dopaminergic receptors generally, the βγ subunit appears to modulate dopaminergic neurotransmission by regulating activity of the dopamine transporter, an integral membrane protein responsible for maintaining dopamine levels in the synaptic cleft.[20]

Dopamine signaling is further modulated by the ability of the various dopamine receptors to form heterooligomeric complexes with other receptor types. A heteromeric complex exists between D2R and the growth hormone secretagogue receptor (GHSR) and fine-tunes the response to dopaminergic stimulation in neurons (primarily hypothalamic neurons) which co-express the two receptors.[21] The adenosine A1 receptor (A1R) forms a heteromer with D1R, and signaling through this heteromer seems to play a role in regulating D1R sensitization and in trafficking of the two receptors.[22] Similarly, although adenosine A2 receptors (A2R) are coupled to stimulatory alpha subunits and D2R to inhibitory alpha subunits, signaling through a heteromeric A2R-D2R complex modulates D2R signaling by inhibiting D2R internalization.[23]

D1R and D2R are also known to form a heteromeric complex, and understanding the coupling of this D1R-D2R is the focus of this thesis. To our knowledge, the first demonstration of functional linkage between D1R and D2R comes from Lee et al.[24] who demonstrated the

existence of D1R-D2 heteromeric complexes in the striatum of rat brains and linked signaling through these complexes to a G_q mediated signal transduction cascade. Subsequent investigations verified the existence of D1R-D2R heteromers in the human brain[25-26] and have allowed the unique signaling properties of this complex to be understood in some detail.

A simple outline of this pathway is as follows: In response to co-stimulation of D1R and D2R by dopamine, D1R and D2R form a heteromeric complex.[27] Distinct from its constituent receptors, this D1R-D2R heteromer is uniquely coupled to G_q, which dissociates upon signaling through the co-stimulation of D1R-D2R.[25-26] Activated G_q leads to the activation of phospholipase C (PLC) and the subsequent hydrolysis of phosphatidylinositol (PI) into inositol triphosphate (IP3) and diacylglycerol (DAG). The production of IP3 is then associated with the release of calcium from intracellular stores and the autophosphorylation of calcium/calmodulin (CaM)-dependent kinase IIα (CaMKIIα). Phosphorylated CaMKIIα then translocates to the nucleus where it acts as a transcription factor. [26, 28]

D1R-D2R heteromer mediated signaling thus represents a unique addition to the canonical understanding of dopamine signaling. It also provides a point of interplay between dopamine signaling and multiple other signaling pathways. The original studies of D1R-D2R heteromeric signaling revealed that calcium release caused by D1R-D2R heteromer agonization led to upregulation of brain derived neurotrophic growth factor (BDNF).[28] BDNF is a necessary protein for neuron differentiation and growth, and plays a regulatory role in GABAnergic and glutaminergic signaling.[29] Perreault et al.[30] have found evidence that the D1R-D2R heteromer may regulate GABAnergic and glutaminergic signaling in striatal subpopulations of medium spiny neurons. Together, this suggests that D1R-D2R heteromer mediated signaling has an important and yet poorly explored impact on neurotransmission.

Dysfunction of D1R-D2R heteromeric signaling may also help to explain the etiology of some complex diseases canonically linked to dopaminergic signaling. Perreault et al.[31] have provided evidence that the D1R-D2R heteromer is enriched in the paleostriatum of individuals with schizophrenia, and that D_2 receptors in these heteromeric complexes showed higher affinity for the synthetic dopamine receptor agonist SKF 83959 than D_2 receptors in the brains of normal individuals did. Further evidence for a link between D1R-D2R signaling and schizophrenia is seen in mechanistic studies of the antipsychotic clozapine, which exerts its effects through disrupting the D1R-D2R heteromer.[32] Nor is schizophrenia the only disorder of dopamine signaling linked to aberrant D1R-D2R heteromer expression: Studies of Parkinson's disease in animal models revealed that the D1R-D2R heteromer is enriched in striatopallidal neurons of animals with induced Parkinsonian symptoms compared to normal controls.[33] Conversely, signaling through the D1R-D2R heteromer seems to blunt the D1R mediated signaling pathway triggered by cocaine and ameliorates cocaine dependency in rats.[34] Finally, the population of striatal dopamine receptors is found to be shifted towards the D1R-D2R heteromeric state in patients with major depression.[35]

Together, this suggests that signaling through the D1R-D2R heteromer not only mediates neurotransmission in a novel way, but also implicates this complex in the etiology of diseases affecting dopaminergic signaling. One tool for investigating this complex has been the development of synthetic peptides that inhibit or abolish D1R-D2R coupling by mimicking the binding interface between the complex.[34,35]

Contribution of the Current Research

Early research by Łukasiewicz et al.[36] narrowed down the interaction interface between D1R and D2R to a region of the third intracellular loop of D2R containing a pair of adjacent arginines (217RRRRKR222) and a region in the carboxyl tail of D1R containing two adjacent glutamic acids (404EE405). This region was further determined by O'Dowd et al.[37] to be a six amino acid stretch (271–EAARRA). A pair of adjacent arginine residues (274-RR) in this sequence forms a salt bridge with two adjacent glutamic acids in the D1 receptor carboxyl tail, which is essential to heteromer formation.

The present work used this model of the D1R-D2R interaction interface to design a peptide with a minimal sequence but with the capacity to abolish coupling between the two parent receptors. Four peptides with potential to disrupt D1R-D2R coupling were synthesized four peptides aimed at disrupting D1R-D2R heteromerization and tested on both *in vitro* translated D1R and D2R proteins and in whole cell lysates of brain. Of the four sequences tested, one was successful in disrupting the D1R-D2R heteromer. This peptide, bearing the sequence EAARRAQE, is based off the shortest sequence of amino acids in the third intracellular loop of the D2 receptor that O'Dowd et al.[37] implicate in heteromer formation. A mutant peptide with sequence EERRAQ showed some effect but was notably less effective than the longer native sequence EAARRAQE in disrupting heteromer formation. Two shorter peptides (ARRA and AARRAQ) were synthesized with the aim of disrupting the minimal possible interaction site of the four amino acids that form salt bridges between D1R and D2R. These showed little to no effect.

Finally, this work explores the role of a D-isoform of the most successful peptide, EAARRAQE, in disrupting the D1R-D2R heteromer. D-peptides have unique stereochemical

properties that make them potentially more efficacious therapeutic agents than their L-counterparts, and as such are being increasingly explored as potential therapeutic agents.[38] Our results were inconclusive but promising for future study.

By establishing a minimal peptide sequence that abolishes D1R-D2R coupling, this work sheds light upon the structural basis of D1R-D2R interaction. This research aids further investigation of this complex, both in elucidating its structure and in the development of drugs that target the complex. More broadly, this research contributes to the overall development of drugs that inhibit interaction between other GPCR heteromers.

Materials and Methods

Purchase and Isolation of Dopamine Receptor DNA

HaloTagged pFN21A plasmid vectors containing full length human D1R and D2R and ampicillin resistance marker were purchased from Promega. Plasmid transformation into DH5α competent cells was performed using standard protocols. Colonies were picked from a selection plate the following day, expanded, and plasmid purification performed using a Wizard® Plus SV Minipreps Start-Up Kit purchased from Promega.

Expression via Transcription/Translation Kit

D1R and D2R proteins were generated via expression in a TnT® Quick Coupled Transcription/Translation System purchased from Promega, following manufacturer instructions. Briefly, 100 ng of plasmid DNA containing a T7 promoter and encoding the entire dopamine receptor was added to a microfuge tube containing 40 µL TnT® Quick Master Mix, 1 µL of 1 mM methionine, and nuclease free water to a total volume of 50 µL. Tubes were incubated for 80 minutes at 30°C. Correct protein expression was verified via SDS Page. Protein concentration was determined using a Pierce™ BCA Protein Assay Kit purchased from Thermo Scientific.™

Solid Phase Peptide Synthesis

Solid phase peptide synthesis was carried out using a Protein Technologies PS3 synthesizer. Four-fold excess of amino acids was weighed, with reactive amino groups protected by a fluorenylmethyloxycarbonyl (Fmoc) protecting group. For both L- and D- amino acids, the glutamic acid side chains were protected using a tert-butyl ester group, glutamine protected using a trityl group, and arginine protected with a 2,2,5,7,8-pentamethyl-chroman-6-sulphonyl (Pmc) group. All amino acids were purchased from Anaspec, Inc. Peptides were acetylated at the N-terminal and amidated at the C-terminus. These modifications eliminate positive N-terminal

charges and negative C-terminal charges, resulting in a peptide that more closely resembles segment of the native polypeptide chain.

The C-terminal amino acid was attached to a Rink amide linker attached to a MBHA resin support on a 0.1 mmol scale. Rink amide resin was purchased from Nova Biochem. The chemical 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) was used as a coupling agent to induce amide bond formation between amino acids and a solution of 20% piperidine in dimethylformamide (DMF) was used then to deprotect reactive amino groups for the next coupling. The coupling agent HBTU was purchased from Anaspec, Inc. After synthesis, peptides were acetylated with acetic anhydride and then cleaved from the resin by scraping the resin into a beaker containing 10 mL of a solution containing 5% phenol scavenger, 2% triisopropylsilane (TIS), and 5% distilled water in trifluoroacetic acid (TFA). This solution was stirred for two hours at room temperature. Peptides were then precipitated from the solution using diethyl ether chilled to 4°C, filtered with a fritted filter funnel and dissolved in a solution of 35% acetonitrile in water, and lyophilized overnight under vacuum. After lyophilization, peptides were dissolved in neat TFA and purified using reversed-phase high performance liquid chromatography. The stationary phase consisted of a Phenomenex C18 column (25 cm x 2.2 cm), with the aqueous component of the liquid phase composed of 0.1% TFA in water and the organic component of the liquid phase composed of 0.1% TFA in acetonitrile. The gradient ran between 10% to 50% organic phase over a period of two hours, with the majority of the sample eluting within 40 minutes of run start. The solvents acetonitrile and dimethylformamide were purchased from EMD Chemicals, Inc., while piperidine, triisopropylsilane, ether, phenol, acetic anhydride, and trifluoroacetic acid were purchased from Sigma-Aldrich. Tubes containing desired peptide were then lyophilized.

Analytical HPLC to verify purity was performed using a Phenomenex C18 column (25 cm x 4.6 mm). Peptides were then analyzed with paper spray ionization mass spectrometry to confirm molecular weight.

Coimmunoprecipitation and Dot Blotting

A 1000 µM stock solution of peptide was created by weighing the appropriate mass of peptide and dissolving it in a solution of 10% DMSO in phosphate buffered saline (PBS). Powdered phosphate buffered saline was purchased from Sigma-Aldrich. This stock was added to a series of microfuge tubes containing 3 µg of D1R and 3 µG D2R receptor proteins translated using a TnT® Quick Coupled Transcription/Translation System as described above. Phosphate buffered saline at pH 7.0 was used to adjust the volume of each tube to 50 µL, and then 20 µL of pre-washed Protein A/G PLUS-agarose beads purchased from Santa Cruz Biotechnology conjugated to D1R were added to each tube. This solution was incubated overnight at 4 °C on an orbital shaker.

The following day, the samples were precipitated by centrifugation at 3000 xg for 1 minute, and then cleared by washing three times in 400 µL Tris-Buffered Saline and Tween 20 (TBST). Bound protein was released from the beads by boiling for 10 minutes in a 100 ^{o}C water bath. Following release of bound protein, 10 µL of resultant sample was spotted onto a nitrocellulose membrane. Nitrocellulose membranes were purchased from Sigma-Aldrich. This membrane was allowed to dry for 10 minutes at room temperature before being blocked overnight in 5% milk in TBST. Following blocking, membranes were washed three times for five minutes each in TBST, then incubated overnight in 2.5% milk in TBST containing a 1:1000 dilution of primary antibody for D2R, washed in TBST and then incubated overnight again in 2.5% milk in TBST containing a 1:10,000 dilution of secondary antibody for D2R. Blots were developed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate from

ThermoFisher and imaged on a Bio-Rad molecular imager. Quantitation was performed using ImageJ 1.47v software downloaded from the National Institutes of Health and graphed using GraphPad Prism 8.0.

Whole Cell Lysate Testing

Total protein lysate isolated from adult normal frontal and temporal lobe (catalog numbers P1234051 and P1234078, respectively) and depressed temporal frontal and temporal lobe (catalog numbers P1236051Dep and P1236078Dep, respectively) were purchased from BioChain. Institutional Review Board approval was not necessary for the procurement of these protein lysates.

Western Blotting

We incubated 50 µg total of whole tissue lysate overnight in a total volume of 50 µL TBST either alone (control) or containing peptide. After incubating 24 hours, 20 uL of A/G PLUS-Agarose beads conjugated to D1R were added to each tube to capture the complex. These were incubated for 24 hours at 4 °C with shaking. Sample tubes were then centrifuged at 4000 rpm for 1 minute at 4 °C, the supernatant removed, and the beads washed with 800 µL of cold PBS. This process was repeated four times and 10 uL of 5X SSB was added to release the protein complex from the beads. The protein complex was released from the agarose beads by incubation for ten minutes in an 80 °C water bath. Ten microliters of protein were run on a 12% acrylamide gel for 2.5 hours at 100 V and then transferred to western blot and run for one hour. The resulting membrane was blocked overnight in 5% milk in TBST. Following overnight incubation, the membrane was washed three times for five minutes in TBST and then incubated in a solution of 2.5 % milk in TBST containing a 1:10000 dilution of rabbit polyclonal anti-D2R antibody. After an incubation period of 12 hours, the membrane was again washed three times in TBST for five minutes per wash and incubated for 12 hours in a solution of 2.5% milk in TBST

containing a 1:20,000 dilution of goat anti-rabbit IgG HRP tagged secondary antibody. Both antibodies were purchased from Abcam.

Following a 12-hour incubation, the membrane was washed five times in TBST, developed with Pierce™ ECL Western Blotting Substrate and imaged on a BioRad Molecular Imager. Following imaging, the blots were quantitated with ImageJ software and analyzed using Graphpad Prism 8.0.

ELISA

For D1R-D2R binding assays, 60 ng of D2R protein in 100 µL PBS was bound to the wells of Nunc MaxiSorp™ multiwell plates purchased from ThermoFisher. Coated plates were incubated for 24 hours at 4 °C. Subsequently, plates were washed three times with 200 µL TBST and blocked in IB-BSA buffer (1 M Tris-Cl, 1 M $MgCl_2$, 5 M NaCl, 100 µL Tween 20, 1 g bovine serum albumin). After 24 hours, plates were washed as described above and increasing concentrations of D1R protein in PBS were added to each well. The proteins were incubated together for 48 hours, washed three times with TBST (200 µL added to each well, then plates rotated for five minutes on an orbital shaker), and incubated overnight in 50 µL PBS containing a 1:500 dilution of mouse monoclonal primary antibody for D1R. After 12 hours the plate was washed three times with TBST as described above and then incubated for 12 hours in 50 µL PBS containing a 1:10000 dilution of HRP-conjugated anti-mouse (mouse IgGκ binding) secondary antibody. Both antibodies were purchased from Santa Cruz Biotechnology. Plates were then washed three times in TBST, and binding activity assayed via colorimetric reaction with 100 µL room temperature TMB (3,3´,5,5´-tetramentylbenzidine) purchased from Thermo Fisher. After 10 minutes the reaction was quenched by adding 100 µL of 2 M H_2SO_4 and absorbance at 450

nm was measured using a Biotek plate reader. ELISA data was graphed and analyzed using GraphPad Prism 8.0 software.

For peptide interference assays, 1000 µg of L-EAARRAQE was dissolved in 1 mL of a solution containing 10% DMSO in PBS. This stock solution was diluted in PBS to create a final concentration of 60 nM and used to coat the wells of a 96 well Nunc MaxiSorp™ ELISA plate. After a 24-to-48-hour incubation period, the plate was rinsed three times with TBST, and a serial dilution of either D1R or D2R was added to the wells such that concentrations ranged from 1000 nM to 1 nM. After overnight incubation at 4 °C on an orbital shaker, plates were washed three times by adding 200 µL of TBST and then probed using anti-D1R or anti-D2R antibodies. Plates were developed and imaged as per previous protocol.

Results and Discussion

Expression of D1R and D2R Proteins *in Vitro*

Due to the inherent difficulties of expressing and purifying transmembrane proteins, the choice was made to express D1 and D2 receptors in a cell-free system. This procedure is discussed fully in the Materials and Methods. Figure 1 shows that both dopamine receptors were successfully expressed using this system.

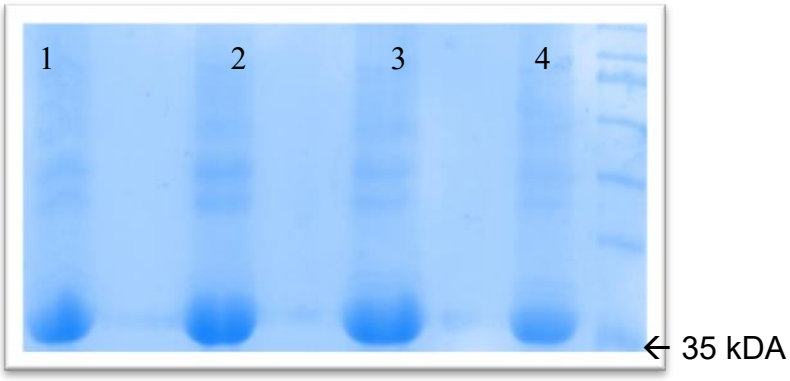

Figure 1. Verification of Properly Expressed Receptors. 3 µg of plasmid DNA encoding D1R (lanes 1 and 2) and D2R (lanes 3 and 4) was expressed using a TnT® Quick Coupled Transcription/Translation Systems kit.

Clean, sharp bands can be observed around 35 kDA for D1R and 38 kDA for D2R. These are slightly lower than the reported sizes of 48 kDA for D1R and 50 kDA for D2R commonly observed in *in vivo* studies of these receptors, a discrepancy likely due to the lack of post-translational modifications for *in vitro* translated receptors. Similar results are seen in Fishburn et al.[39]

The next step was to demonstrate that *in vitro* expressed D1R and D2R form heteromeric complexes. Additionally, extensive literature review revealed little data on the binding affinity

between D1R and D2R (a measure of how easily the interaction between the two happens). To generate this data, we used enzyme-linked immunosorbent assays (ELISA) to demonstrate the ability of our *in vitro* expressed receptors to bind to each other, as well as to determine the affinity of interaction between D1R-D2R. Briefly, a fixed concentration of D2R was bound to the wells of an adsorbent plate, then incubated in the presence of varying concentrations of D1R. Extensive washing removed any unbound protein from the plate, allowing the extent to which the two proteins bind to be quantitated. All ELISA assays were run in triplicate under conditions as described in the Materials and Methods. Results of ELISA assays are seen in Figure 2.

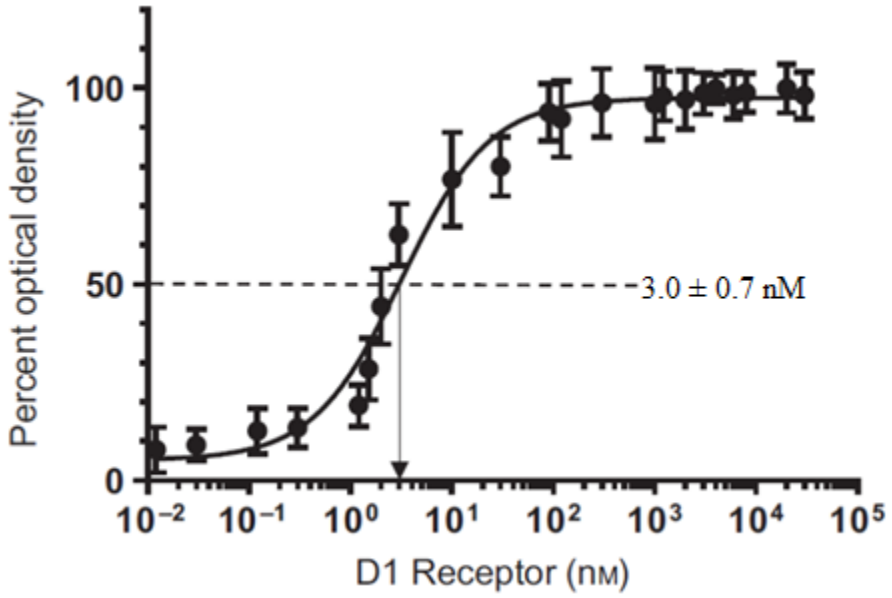

Figure 2. D1R binds to D2R with high affinity. D2R (60 nM) was bound to the wells of a 96 well Nunc Maxisorp Plate. Following overnight blocking, the plate was then incubated with a serial dilution of D1R. The amount of D1R was visualized using anti-D1R specific antibodies and developed using TMB. D1R concentration was expressed as a function of optical density and plotted in GraphPad Prism, with each data point representing the average optical density measurement ± standard deviation over three runs of each assay.

The K_D (a measure of the ease at which the two receptors form a complex) was calculated using GraphPad Prism 8.0, as described in the Materials and Methods. This value was found to be 3.0 ± 0.7 nM. While this data does not demonstrate the binding affinity between native D1R-

D2R, it does suggest that *in vitro* expressed D1R and D2R are capable of forming a complex. This demonstration validates the use of *in vitro* translated receptors as a tool for studying the D1R-D2R complex and provides information about the affinity of interaction between the two. With translated receptors as a tool in hand, we were able to proceed to testing the effects of various peptides on disrupting interaction between D1R-D2R.

Peptide Design and Testing

As discussed, experiments by O'Dowd et al.[37] suggest that D1R-D2R heterooligomerization is mediated by electrostatic interactions occurring along a six amino acid stretch between the third cytoplasmic loop of D2R and the D1R carboxyl tail. This heterooligomerization is particularly dependent upon salt bridge formation between two adjacent arginines in the third cytoplasmic loop of the D2 and two glutamic acids in the D1 receptor carboxyl tail. The proposed interaction site is seen in Figure 3.

D1 receptor (D1R) **D2 receptor (D2R)**

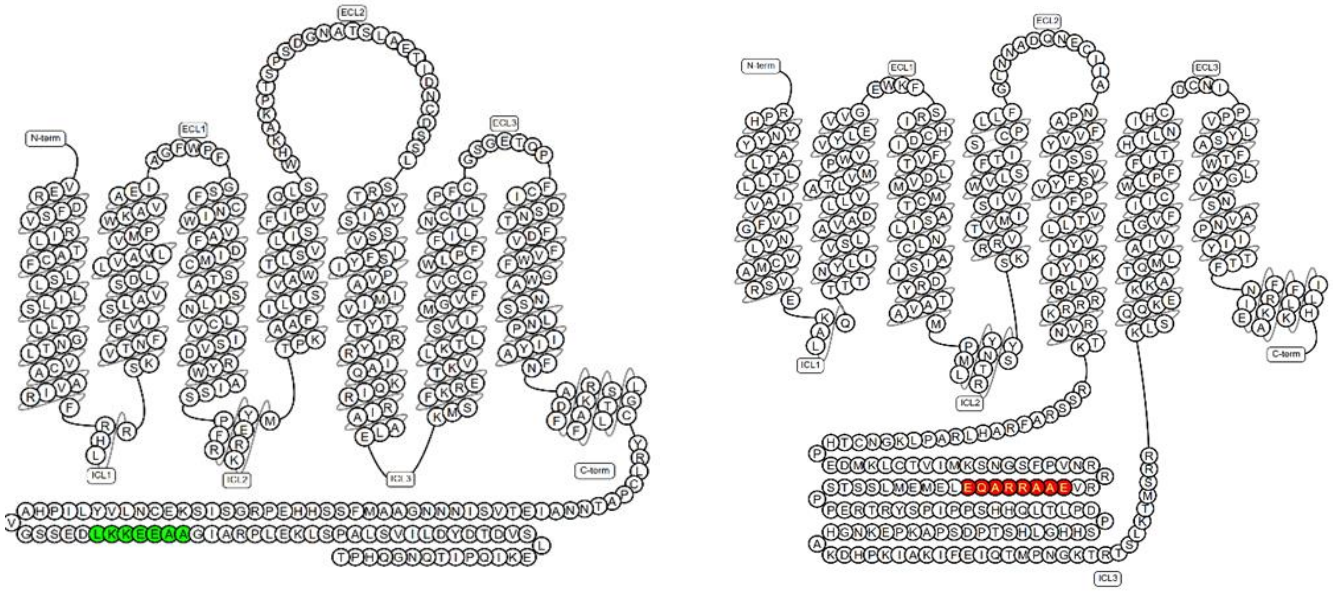

Figure 3. Proposed Interaction Interface between D1R and D2R. Snake plot representation of the D1 and D2 receptors drawn with GPCRdb webserver (http://gpcrdb.org/). This model suggests that binding between D1R and D2R occurs through the interaction of residues within a 6 amino acid long stretch between the carboxyl tail of the D1 receptor and the third cytoplasmic loop of the D2 receptor. Interaction between two adjacent arginines in the D2 receptor and two glutamic acid residues in the D1 receptor is necessary for heteromer formation. Reprinted with permission from ref 40. Copyright 2019 Heyl et al.

Using as template the third intracellular loop of D2R believed to couple to D1R, four peptides were synthesized. The first peptide, EAARRAQE, was designed to fully correspond to the shortest sequence of D2R that most completely contacts D1R. The second peptide, EERRAQ, does not correspond to the native D1R-D2R binding interface but instead is a more highly charged "mutant" with glutamic acid substituted for the wild-type alanine residues. This modification was designed to increase electrostatic interactions between the peptide and a pair of lysine residues in D1R.

Peptide three, ARRA, corresponds to the smallest possible sequence of D2R that preserves the adjacent arginines believed to be critical to D1R-D2R heteromer formation. Peptide four, AARRAQ, was designed to preferentially bind to the six amino acid sequence in

D1R (KKEEAA, Fig. 3) identified by O'Dowd *et al.*[37] as essential to D1R-D2R heteromer formation. Peptide sequences and molecular weights are summarized in Table 1.

Table 1. Synthetic peptide sequences and molecular weights. N-terminal acetyl groups were added to prevent non-native charges. All peptides are carboxamides at the C-terminus.

Sequence	Molecular Weight (Daltons)
Ac-EAARRAQE	971.5
Ac-EERRAQ	829.4
Ac-ARRA	514.3
Ac-AARRAQ	713.4

All peptides were synthesized using a PS3 solid phase peptide synthesizer with Rink amide MBHA resin and an Fmoc protection scheme, as described in the Materials and Methods.

Coimmunoprecipitation Experiments

The possible efficacy of each peptide sequence in disrupting D1-D2 heteromer formation was tested via coimmunoprecipitation as described in the Materials and Methods section. It should be noted that dot blotting for peptides three and four was performed in collaboration with student colleagues. Figure four shows examples of dot blotting results for each peptide tested.

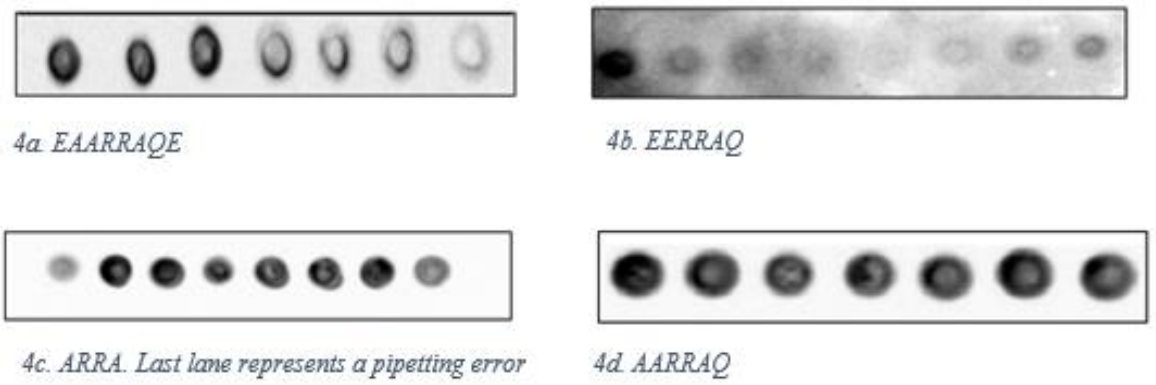

Figure 4. Co-Immunoprecipitation of Expressed D1R and D2R in the Presence of Increasing Peptide. Expressed D1 and D2 receptors were incubated overnight in the presence of increasing concentration of peptide. Receptors were then co-immunoprecipitated with agarose beads conjugated to anti-D1R antibody, then released from the beads by boiling and spotted onto a nitrocellulose membrane. After overnight blocking in 5% milk in TBST, membranes were probed with anti D2R primary and secondary antibodies and imaged. Receptor concentration is directly correlated to density of pixels i.e darkness of the image. From left to right, concentrations are 0, 15, 25, 150, 200, 250, and 300 nM of peptide. All experiments were run in triplicate.

A strong visual trend of decreasing signal intensity with increasing peptide concentration is observed in the blot for EAARRAQE, the peptide designed to most fully cover the native D1R-D2R binding interface. The mutant peptide, EERRAQ, displays a more ambiguous trend: an overall decrease in signal intensity is observed, but in a less dramatic and less linear fashion than for EAARRAQE. ARRA and AARRAQ show little decrease in signal intensity. As a quantitative measure of these results, the concentration at which each peptide displayed half-maximal inhibition was calculated. This value was found to be 72 ±15 nM. Results are seen in Figure 5.

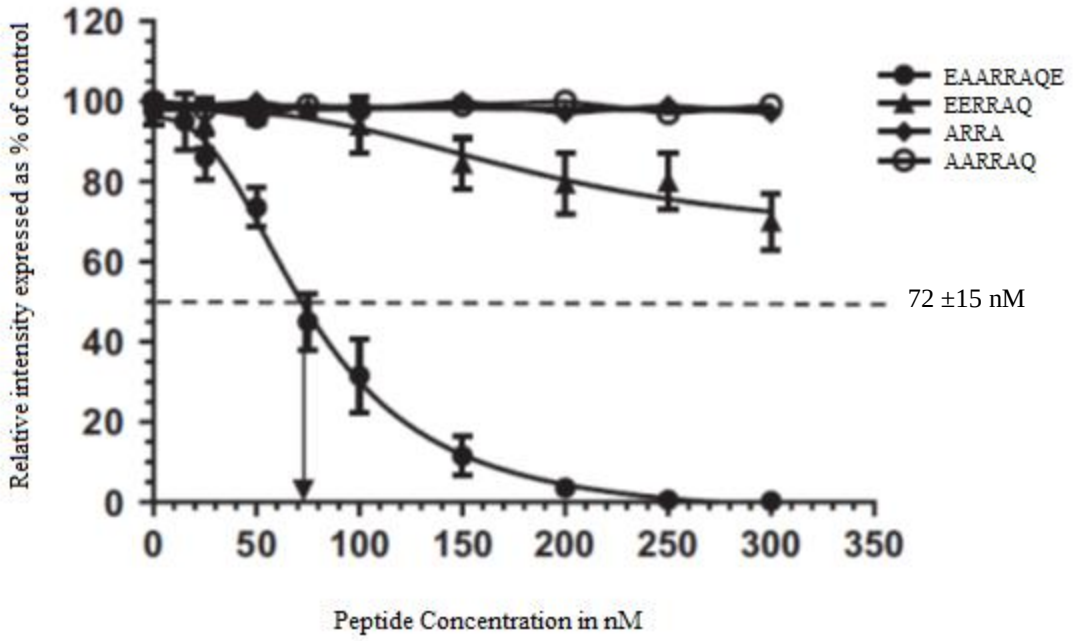

Figure 5. EAARRAQE Disrupts D1R-D2R Interaction. Graphical representation of coimmunoprecipitation experiments. Signal intensity was normalized by plotting ratio of mean signal intensity to maximal binding, then fit to peptide concentration using a nonlinear regression curve. Data was quantitated in ImageJ and processed in GraphPad Prism 8.0. The graph summarizes the results expressed as means ± standard deviation and normalized to control ($P < 0.05$, $n = 5$).

Based on this data, it seems that EAARRAQE is most efficient at interfering with D1R-D2R heteromer formation. While only differing by two amino acid residues, EERRAQ and AARRAQ show very little effect, suggesting that the full coverage of the D1R-D2R interaction interface may be necessary to disrupt the interaction between the two receptors. This trend holds true for both the modified peptide EERRAQ and the native peptide AARRAQ.

However, coverage of the D1R-D2R interaction interface is not sufficient in disrupting D1R-D2R interaction. While equal in length, the highly charged EERRAQ is noticeably better at disrupting D1R-D2R interaction than the native AARRAQ is. This may underscore the importance of electrostatic interactions in forming the D1R-D2R heteromer.

The inability of peptide three (ARRA) to abolish or even diminish D1R-D2R coupling suggests that the EE: RR interaction between D1R-D2R is necessary but not sufficient for heteromer formation. Taken as a whole, the sequence EAARRAQE seemed the most promising for further testing.

D1R and L-EAARRAQE Interact with High Affinity

We next wished to quantitate the extent to which L-EAARRAQE interferes with D1R-D2R coupling. As described in the Materials and Methods, a stock of L-EAARRAQE was used to coat the wells of a Nunc MaxiSorp™ ELISA plate. Following this step, resuspended D1R and D2R was added in increasing concentrations. This assay was repeated for a total of three unique runs, data from which were pooled, normalized, and used to generate a binding curve for D1R and D2R in the presence of L-EAARRAQE. This binding curve is seen in Figure 6.

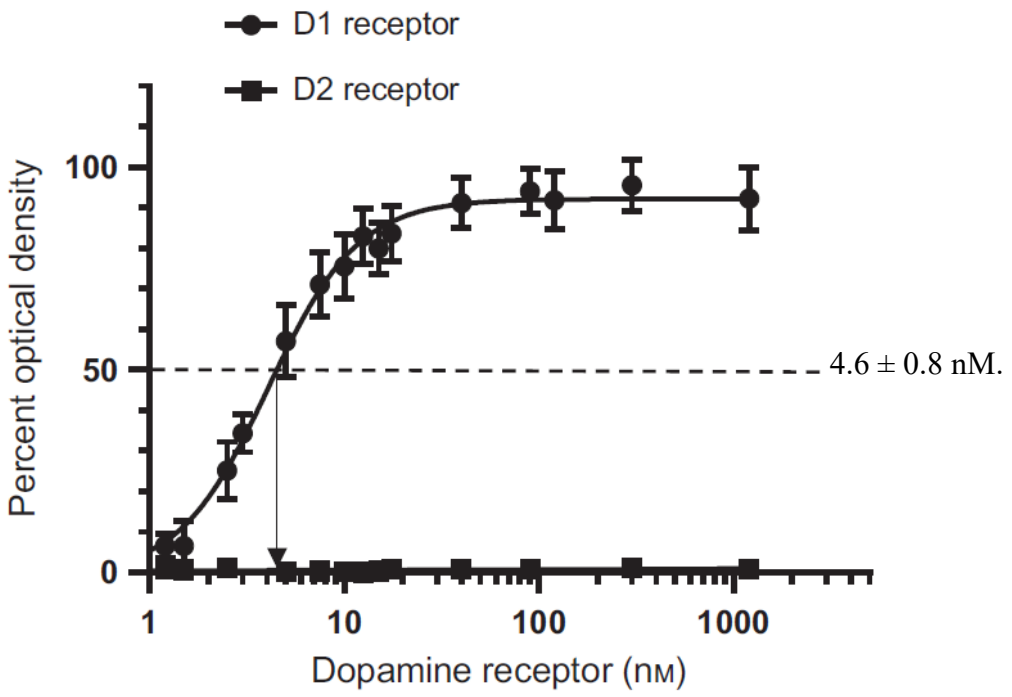

Figure 6. D1R Binds to L-EAARRAQE and D2R with Comparable Affinity. Results of D1R and D2R incubated in 96 well ELISA plate pre-treated with L- EAARRAQE. Levels of D1R or D2R were measured via HRP conjugated antibodies. Each data point represents the pooled mean ± standard deviation of three separately run ELISA assays. K0 was calculated using GraphPad Prism 8.0 and found to be 4.6 ± 0.8 nM.

While D2R and L-EAARRAQE do not bind to each other, D1R and L-EAARRAQE interact with a binding affinity of 4.6 ± 0.8 nM. Although this value is higher than the 3.0 ± 0.7 nM (Fig. 2) between D1R-D2R, it seems to be sufficient to competitively inhibit interaction between the two receptors. This implies that the sequence L-EAARRAQE fully comprises the necessary binding region for native D1R-D2R heteromer formation. These results demonstrate the ability of L-EAARRAQE to effectively inhibit D1R-D2R coupling *in vitro.* However, as previously noted, both D1R and D2R undergo extensive post-translational modification.[13-14, 39] As such modifications would be absent in our *in vitro* model, a full demonstration of the efficacy of L-EAARRAQE in inhibiting D1R-D2R interaction must be made in a model of this system that accounts for such changes. While studying this complex *in situ* was not a feasible option,

whole tissue lysates of human cerebral tissue offered a viable and directly physiologically relevant model.

Total Protein Lysate Testing

Having had success in *in vitro* models, we were interested in whether D1R-D2R heteromers could be detected and disrupted in in tissue lysates. Pei *et al.* originally demonstrated both that the D1R-D2R heteromer is upregulated in striatal tissue isolated from depressed patients. In the same study, the authors demonstrated that administration of peptide designed to disrupt the D1R-D2R heteromer resulted in modulation of behavioral symptoms of depression in rats if administered to the prefrontal cortex, but not if administered to the hippocampus or nucleus accumbens.[35] This tissue specificity was intriguing to us, as the presence of D1R-D2R heteromers is well established in the striatum,[41] but region specific differences in heteromer density are otherwise poorly characterized.

For these reasons, we attempted to analyze two regions where D1R-D2R heteromer density is, to our knowledge, not fully explored. The success of Pei et al.[35] in creating a D1R-D2R disrupting peptide capable of alleviating behavioral symptoms of depression inspired us to seek direct evidence that our own D1R-D2R disrupting peptide might have differential effects *in vivo.* Results of this testing reproduced from Heyl et al.[40] are seen in Figure 7.

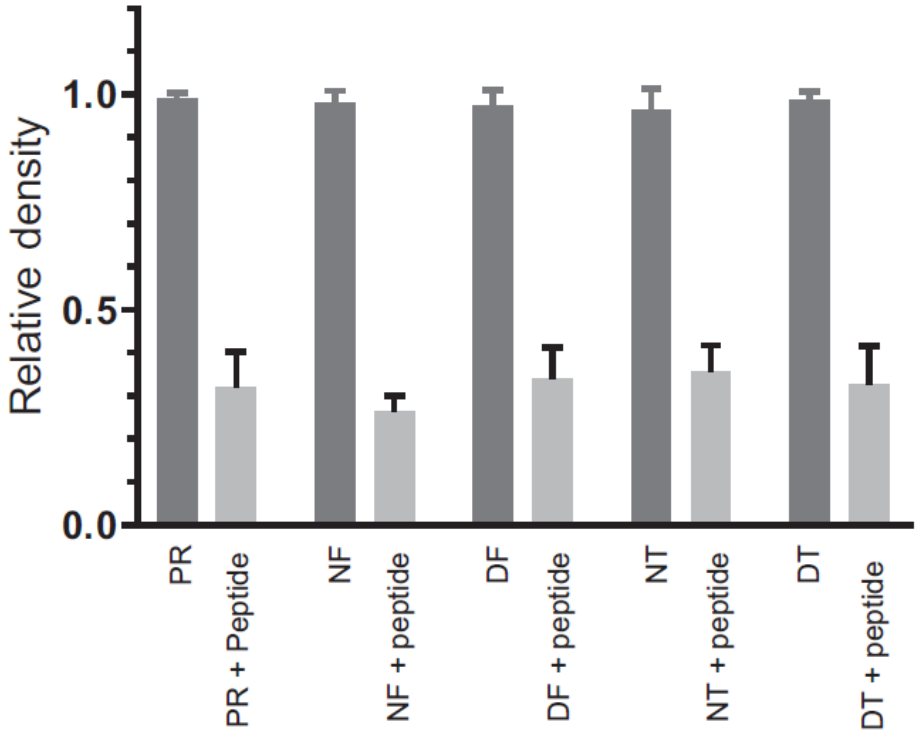

Figure 7. L-EAARRAQE Disrupts D1R-D2R Interaction in Total Protein Lysate. Quantitative summary of D2R-D1R coimmunoprecipitation and western blotting. In vitro expressed receptors (PR), normal frontal lobe (NF), depressed frontal lobe (DF), normal temporal (NT), and depressed temporal (DT) were incubated overnight either in TBST (left) or in a solution of 10 nM of L-EAARRAQE in TBST. Bar graphs represent mean value ± standard deviation of D1R signal intensity in sample treated with L-EAARRAQE relative to D1R signal intensity in untreated controls. Reprinted with permission from ref 40. Copyright 2019 Heyl et al.

These results demonstrate that the D1R-D2R heteromer can be detected in total protein lysate derived from frontal and temporal lobes, suggesting that this protein-protein interaction is not limited to the areas previously discovered. Additionally, they suggest that EAARRAQE effectively prevents D1R-D2R heteromer formation in total protein lysate. Interestingly, it seems to do so equally well in samples obtained from non-depressed subjects as it does in samples obtained from depressed subjects.

D-Isoforms of EAARRAQE

All proteins are built from the stringing together and folding of chains of amino acids, and almost all physiologically relevant amino acids have the same rotational configuration around a single chiral carbon atom. However, 19 out of 20 of the physiological amino acids (excepting glycine, which does not have a chiral carbon) have another form with mirrored symmetry. Due to this mirrored symmetry, D-amino acid peptides have slightly different physiological properties that are sometimes exploited in nature. In humans, D-aspartate is a critical regulator of neonatal brain development and continues to modulate hippocampal neurogenesis through adulthood.[42] Agonization of N-methyl-D-aspartate (NMDA) glutamate receptors requires stimulation both by glutamate and a coactivating second molecule, which can be either glycine or D-serine.[43] More directly relevant to our research, though, is the use of D-amino acids as therapeutic agents. D-amino acids are not targeted by proteases to the same extent as their L-amino acid counterparts are, making D-amino acids a target of research for the development of peptide drugs.[38] This raised the question of whether the D-isoform of EAARRAQE would be effective in blocking the D1R-D2R interaction.

To test this, a D-isoform of EAARRAQE was synthesized using the solid phase peptide synthesis method previously described and tested on total protein lysates concentrations of peptide, then D1R was used to immunoprecipitate D2R as described in the previous experiment with *in vitro* translated receptors, and western blotting performed on the immunoprecipitated complex as described in the Materials and Methods. However, supplies of total protein lysate were limited, and thus only two runs of this experiment were possible. Figure 8 shows representative scans of western blots testing D-EAARRAQE in total protein lysate obtained from patients with (Fig. 8A) or without (Fig. 8B) a history of depression.

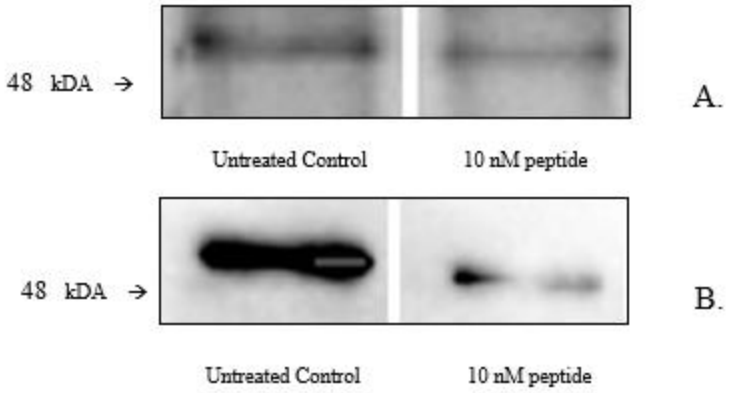

Figure 8. D-EAARRAQE disrupts D1R-D2R interaction in brain tissue total protein lysate. Sample was left untreated (left) or treated with 10 nM D-EAARRAQE (right). A. Western blot of total protein lysate (50 µG) from depressed temporal lobe brain tissue. B. Western blot of total protein lysate (50 µG) from normal temporal lobe brain tissue. Sample was left untreated (left) or treated with 10 nM D-EAARRAQE (right).

The signal intensity of the untreated control (Fig. 8) is notably stronger for untreated controls than for samples treated with 10 nM of D-EAARRAQE. As with the experiments using L-EAARRAQE (Fig. 7) this trend of decreased signal intensity following peptide treatment holds true both for samples obtained from depressed (A) or neurotypical (B) adults. From these preliminary experiments, it would appear that D-EAARRAQE is capable of blocking D1R-D2R interaction in whole tissue lysate samples. Whether it is more efficient at doing so than L-EAARRAQE remains to be seen. A quantitative representation of these western blotting experiments is seen in Figure 9.

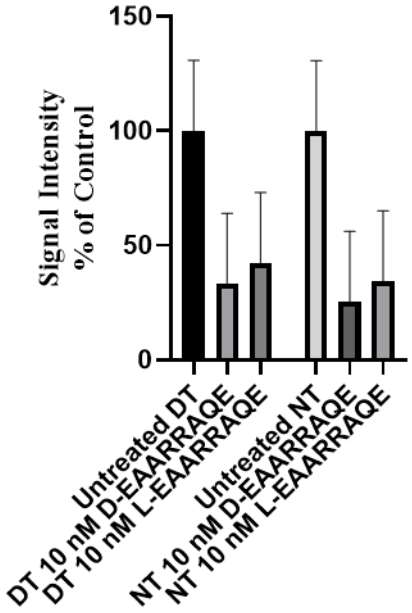

Figure 9. Graphical Summary of L- and D-EAARRAQE western blotting experiments. Bar graphs represent mean value ± standard deviation of D1R signal intensity in sample treated with D-EAARRAQE relative to D1R signal intensity in untreated controls. Values are obtained from two independent western blotting experiments with D-EAARRAQE in each condition and two independent western blotting experiments with L-EAARRAQE in each condition.

This data strongly suggests that D-EAARRAQE is capable of interfering with D1R-D2R interaction. However, it must be noted that each data point represents the average over only two runs of the represented condition. As such, extensive variability exists between each run and so far, no conclusions can be drawn from this data as to whether D-EAARRAQE is more efficacious than its L-counterpart. Unfortunately, as mentioned above, a lack of sample prevented any further testing.

While not yet conclusive, we believe that these initial results warrant further study. If these results were eventually borne out by further tests, this differential effect could be explained by the presence of active peptidases in the total protein lysate sample, to which the D-peptide is less susceptible. It is also possible that the D-isoform has structural features that make it more efficacious at binding to D2R. While the L- and D- isoforms of EAARRAQE should exactly mirror each other, it is possible that the mirrored switch between L- and D- isoforms leads to a

shift of the peptide's backbone dipole. This possibility is being explored by Towse et al.[42] as a general feature of D-peptides. If so, D-EAARRAQE may possess slightly different electrostatic properties than those of L-EAARRAQE. A demonstration that the mirrored symmetry of L- and D-peptides has relevant effects on disrupting the D1R-D2R heteromer would add greatly to our knowledge of peptide therapeutics. For these reasons, we believe that these investigations may be worth pursuing to future researchers, but the present work must conclude with this question unanswered.

Conclusion and Future Directions

The traditional view of dopaminergic signaling has been enriched by the discovery of a novel signaling pathway mediated by a heteromeric complex that forms between dopamine D1 and D2 receptors. Interaction between these two proteins is believed to take place via salt bridge formation between two arginine residues in the third intracellular loop of the D2 receptor and two glutamic acid residues in the carboxyl tail of the D1 receptor. Using this interaction interface as a template, we designed several peptides with the goal of abolishing D1R-D2R coupling. One of these peptides, bearing the sequence EAARRAQE, was successful at disrupting the D1R-D2R interaction both in translated receptor proteins and in total protein lysate derived from human cerebral tissue. This peptide was successful in disrupting D1R-D2R interaction, while a shorter peptide (ARRA) aimed at disrupting the minimal interaction interface between D1R-D2R was unsuccessful. EAARRAQE was also more successful than a shorter native sequence (AARRAQ) and a mutant, highly charged sequence (EERRAWQ). Altogether, this suggests that both full coverage of the binding interface between the two receptors and charge-charge interactions are necessary to disrupt D1R-D2R coupling. While other researchers have created peptide sequences that successfully disrupted D1R-D2R coupling, to our knowledge this is the shortest peptide to successfully disrupt this protein-protein interaction.

Additionally, we have synthesized a D-isomer of this peptide. As mirror images of the physiological amino acids, D-amino acids have slightly different interactions with physiological proteins. Initial studies with D-EAARRAQE are inconclusive due to lack of sufficient sample to fully test the hypothesis that a D isoform of L-EAARRAQE will have differential effects. Further experiments are thus advised in order to determine what, if any, effect exists here. Overall, our findings have relevance to the study of the structural dynamics of

D1R-D2R heterooligomers and may represent a novel tool for investigating potential therapeutic manipulations of this complex.

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