Eastern Michigan University

DigitalCommons@EMU

2017

Understanding the interface that enables the interaction between two dopamine receptor subtypes known as D1 and D2 implicated in depression

Adam Baraka

Understanding the interface that enables the interaction between two dopamine receptor subtypes known as D1 and D2 implicated in depression

Abstract

The dopamine receptor subtypes known as DI and D2 have been shown to form a heteromer complex, which is thought to lead to the disruption of a multitude of signaling pathways within the brain. As a result, this has been proposed to lead to diseased states such as Alzheimer's and depression. In previous studies, it has been shown that the use of synthetic peptides corresponding to the surface interface of the interactions (specifically in the third intracellular loop of D2 interface) within the complex have served to compete against the formation of the heteromer. Through *in vitro* biochemical techniques such as SDS-Page and Western blotting, we observed the degree of dissociation of the Dl-D2 complex through a variety of small peptides. These peptides consist of peptide 1, 2, 3 and 4 with the sequences corresponding to Ac-EAARRAQE, AcEERRAQ, Ac-ARRA, and Ac-AARRAQ, respectively. Peptide 1 was found to be the most effective in preventing complex formation.

Degree Type
Open Access Senior Honors Thesis

Department
Chemistry

First Advisor
Dr. Hedeel Guy Evans

Second Advisor
Dr. Deborah Heyl-Clegg

Keywords
Neurochemistry, Depression, Dopamine, Brain, Uncoupling, Heteromer

Subject Categories
Chemistry

Understanding the interface that enables the interaction between two dopamine receptor subtypes known as D1 and D2 implicated in depression

By: Adam Baraka

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

In Partial Fulfillment of the Requirements for Graduation

With Honors in Chemistry

Approved in Ypsilanti, Michigan, on this date Monday, April 10th, 2017

Dr. Deborah Heyl- Clegg

Honors Advisor, Dr. Hedeel Guy Evans

Department Head, Dr. Deborah Heyl-Clegg

Honors Direction, Dr. Rebecca Sipe

TABLE OF CONTENTS

Abstract

The dopamine receptor subtypes known as D1 and D2 have been shown to form a heteromer complex, which is thought to lead to the disruption of a multitude of signaling pathways within the brain. As a result, this has been proposed to lead to diseased states such as Alzheimer's and depression. In previous studies, it has been shown that the use of synthetic peptides corresponding to the surface interface of the interactions (specifically in the third intracellular loop of D2 interface) within the complex have served to compete against the formation of the heteromer. Through *in vitro* biochemical techniques such as SDS-Page and Western blotting, we observed the degree of dissociation of the D1-D2 complex through a variety of small peptides. These peptides consist of peptide 1, 2, 3 and 4 with the sequences corresponding to Ac-EAARRAQE, Ac-EERRAQ, Ac-ARRA, and Ac-AARRAQ, respectively. Peptide 1 was found to be the most effective in preventing complex formation.

Introduction

Depression is a debilitating illness affecting millions with a prevalence of about 4% globally. Individuals suffering depression, especially in major depressive episodes, experience irritability, abnormal sleeping patterns, change in appetite, loss of interest, feelings of guilt or worthlessness, and lower ability for concentration in day to day activities Intervention today consists of using antidepressants known as SSRI's (selective serotonin reuptake inhibitors) and agonists to mimic neurotransmitters involved in mood and energy such as norepinephrine. However, the effectiveness of these treatments are temporary fixes and not all symptoms can be abolished. There are also side effects involved with taking these drugs. These side effects include

3

nausea, increased appetite, insomnia, blurred vision and fatigue (1, 2).One possible explanation could be the deregulation of the dopaminergic system.

The dopaminergic system is involved in locomotion, behavior, learning, and even emotion. The disruption of the dopamine receptor structure that regulates this system leads to disease states such as depression, Alzheimer's, ADHD, drug addiction, and other neuropsychiatric disorders. The dopamine receptors are G-protein coupled receptors (GPCR) (1,2). There are currently five subtypes known with reported subclasses (2,3). Among them are the two dopamine receptors D1 and D2. These receptors, through synergistic interaction, regulate cAMP production. cAMP acts as a crucial secondary messenger (by increasing calcium influx, activating kinase cascades) for many signaling pathways, allowing the rapid action of neurotransmitters, synaptic transmission, as well as neuronal gene expression and differentiation (1,2,3). Generally, the pathway of the expression of D1-D2 leads to the activation CaMKII (calcium calmodulin kinase II α), resulting in an increase of brain derived neurotrophic factor (BDNF) and allowing for the branching of dendrites and an increase in neuroplasticity (6,7). It has been thought that the expression of D1 and D2 in the mesocorticolimbic system may lead to abnormal brain physiology (7). When D1 and D2 are co-activated as a result of constant drug exposure, it causes a surge in dopamine, increasing calcium levels in the cell via cAMP. In the long term, this has been show to upregulate the activation of the transducer G_i, leading to decreased cAMP and decreasing the cell's sensitivity to dopamine (2). Below in Figure 1 is the mechanism of the different cellular cascades that may result after D1-D2 stimulation, through the use of different transducers.
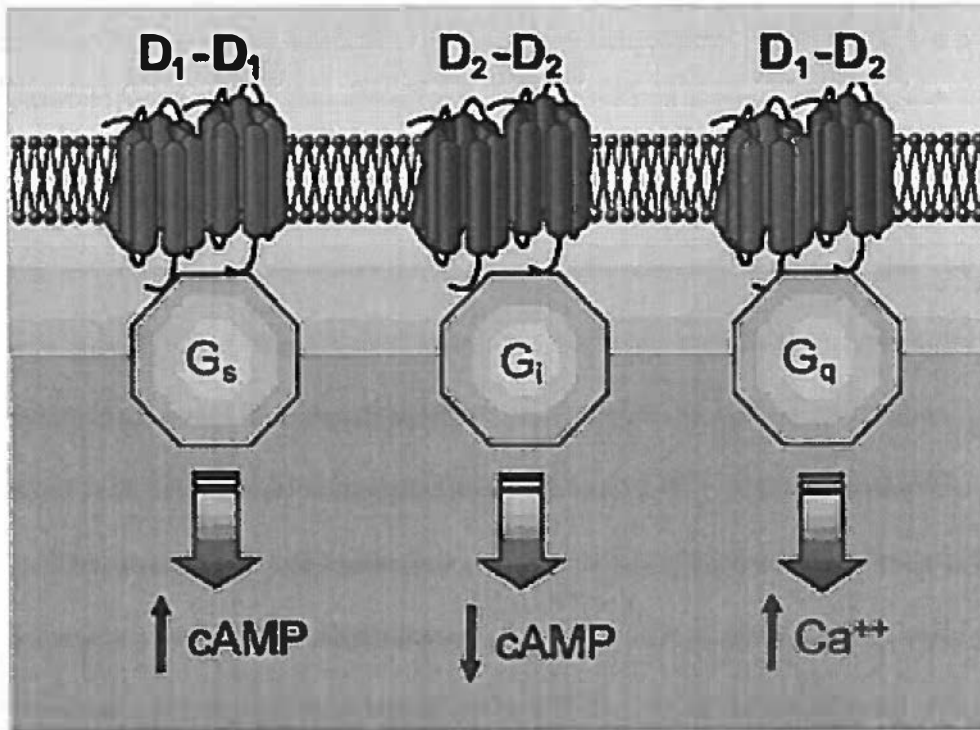
Figure 1: "Dopamine Receptor Complexes Trigger Different Cellular Cascades: Stimulation of the D1-D2 receptor complex (on right) spurs a burst of calcium within the cell—activating the neuron, making the cell more prone to firing again, and ultimately forging new neural connections. Stimulation of the two other complexes influences slower acting biological cascades involving cyclic AMP (cAMP)." (11)

In striatal neurons, D1 and D2 play a significant role in motor behavior and the reward system. The synergistic stimulation of the receptors has been shown to be responsible for GABA release and sensitization to drugs such as cocaine (3). This is most likely caused by the activation of the transducer G_q. The effects of D1 and D2 can be intensified by the use of agonists for both receptors. In one study, a high affinity agonist drug known as SKF89359 increased G_q and cAMP levels leading to synaptic plasticity observed by mice development (5).

5

The uniqueness of these two receptors is that they complex to form a heterodimer, unlike the other classes that exist as monomers. This complexing occurs non-covalently and it is hypothesized that amino acid residues arginine and glutamate, allowing the stable formation of the complex. Specifically, it has been found that the third intercellular loop of D2 and the glutamic acid residues, including the C-terminal tail of D1, tend to have the strongest interactions in the complex (7). Undoubtedly, D1-D2 form a heteromer complex as observed after coimmunopercipitation in rat striatum *in vivo* (4,7). The effects of D1 and D2 have been observed to be inhibited due to heterooligomeric complexing. The complex was shown to be a target of antipsychotic drugs administered to schizophrenic patients. The use of clozapine, an anti-psychotic, was shown to significantly uncouple D1-D2 heteromers.

D1-D2 is shown to change its affinity states in dopamine regulation. The administration of stimulants such as amphetamine, was proven to enhance the presence of D1-D2 as observed in rat striatum via fluorescence resonance energy transfer FRET (7). In postmortem striatum of depression patients, the use of a small peptide from rats was able to disrupt D1-D2 heteromers in pyramidal neurons, but not in the hippocampus. This may indicate that the complex formation of D1-D2 may vary in its interactions throughout the brain (7).

Biochemical analyses confirm the presence of the D1-D2 heteromer complex in postmortem brains of patients suffering from depression. It was shown that the use of a small peptide was able to disrupt the complex by observation of decreased escape failures in learned helplessness tests in rats. Locomotive ability of the rats was not disrupted after administration, making a small peptide a possible alternative to other medical interventions for depression (8).

In a recent study, a peptide 15 amino acids long was derived from the third intracellular loop of D2. This peptide was administered intranasally to rats and showed significant ability to

6

decrease learned helplessness (LH), an indicator of depression. The peptide was successful in uncoupling the complex within the prefrontal cortex as seen by immunoprecipitation (9).

Many of the targets of these small proteins is the surface interface, intracellular loops, amino/carboxyl terminals, and the transmembrane domains between D1 and D2. It has been found that the carboxyl tail residues of the D1 receptor Glu404 and Glu405, were critical in the noncovalent binding with D2 as shown by BRET analysis (4).

In one study, a small interfering peptide of 29 amino acids in the third intracellular loop of the D2 long receptor form was shown to be effective in preventing heteromer formation (10).
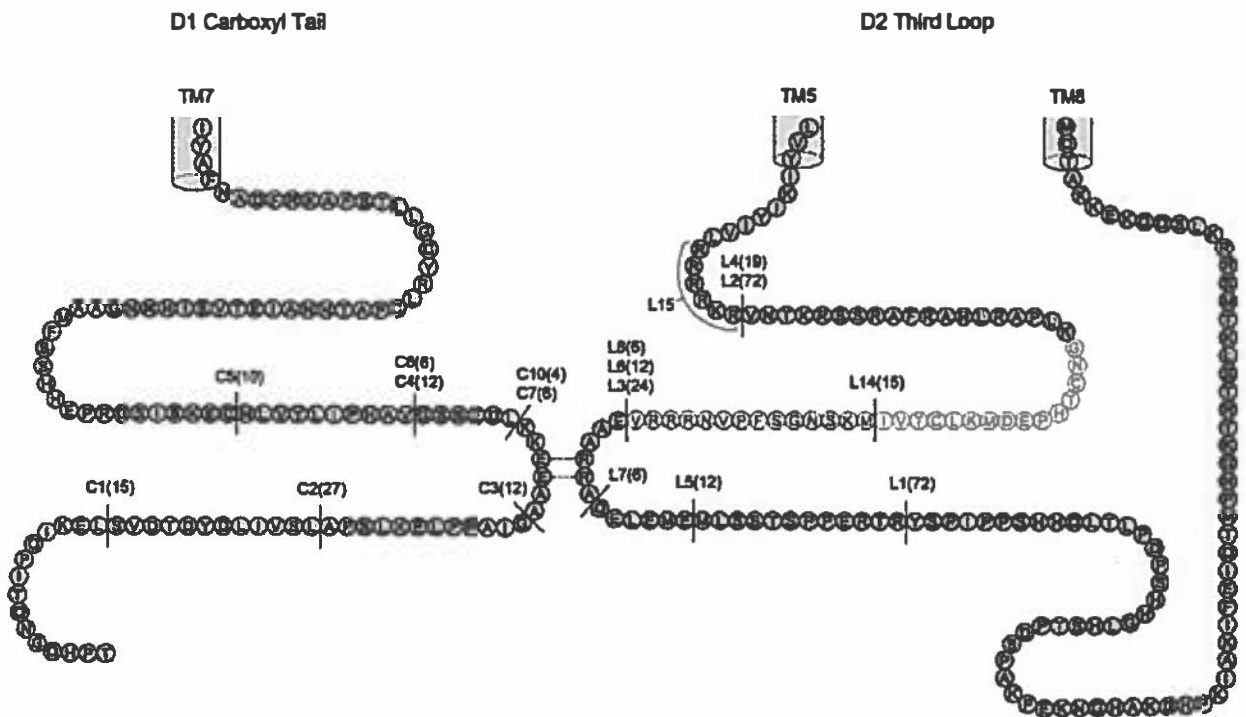

Fig 2: Shows the primary amino acid sequence of the third intracellular loop of D2 and the carboxyl tail of D1. The shaded amino acids represent the inserted 29 amino acid peptide. (10)

In our lab, we plan to synthesize small peptides in order to observe their effectivity in disrupting the D1-D2 heteromer. The four peptides (peptide 1,2,3, and 4) will have the amino acid sequences EAARRAQE, EERRAQ, ARRA, and AARRAQ, respectively and will be acetylated at the N-terminus and carboxamides at the C-terminus to effectively cap them. The peptides' efficiency in the disruption of the complex will be observed through Western Blotting, conventional dot blots, SDS-page, cross-linking, and gel-electrophoresis assays. The discovery of a peptide that may inhibit complex formation in low concentrations can be developed into new pharmaceuticals that may be able to treat depression long-term.

Research Objectives:

1) To determine what regions of the D1-D2 complex are most important for sustaining stability of its formation

2) To synthesize a small synthetic peptide that may be effective in disrupting the D1-D2 interaction

3) To observe the effectivity of said peptide in uncoupling the D1-D2 heteromer

Materials and Methods

Peptide Synthesis

Peptide synthesis was carried out using a PS3 Automated Peptide Synthesizer from Protein Technologies. The protecting groups used for the amino acids was N-α-fluorenyltmethylxycarbonyl (FMOC), on a rink amide resin p-methylbenzhydrylamine (MBHA)

(0.64 mmole/g) on a 0.1 mmole scale. To deprotect the amino-terminus from the FMOC, 20% piperidine in N,N-dimethylformamide (DMF) was used. After the addition of O-(Benzotriazol-1yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU), the amino acid was activated using 0.4 M N,N-diisopropylethylamine (DIEA). The simultaneous deprotection and cleavage of the peptide from the resin was done by treating the resin with 11 mL 90% trifluororacetic acid (TFA)/10% scavenger cocktail (phenol, water, and triisopropylsilane). The reaction was allowed to warm to room temperature, after starting the reaction at $0^{\circ}C$. Uncharged resin was separated and the peptide was allowed to precipitate out with the use of cold diethyl ether. The precipitate was then redissolved in a solution of 70%/30% acetonitrile/water. The peptide was then purified using reverse-phase high performance liquid chromatography (RP-HPLC) on a Jupiter C18 column (2.2 x 25.0 cm, with flow rate of 10 mL/min). A gradient of acetonitirile was employed as follows from 10% acetonitrile (0.1% TFA)/water to 50% acetonitrile (0.1% TFA)/water (0.1% TFA). The sample was then lyophilized.

Peptide Analysis

Purity of the peptides was assessed using RP-HPLC. The peaks were observed at 220 nm, and purity was determined by peak integration. Electrospray mass spectrometry was used to check the molecular weights of the peptides.

D2 and D1 protein synthesis via in vitro translation

Both plasmids, human dopamine receptor 1(DRD1 - HaloTag® human ORF in pFN21A) and human dopamine receptor 2 (DRD2 - HaloTag® human ORF in pFN21A) from Promega, were transformed into DH5α *E.coli*.

Once transformed, DNA was purified from 5ml cultures by the use of PureLink® Quick Plasmid Miniprep Kit from Invitrogen. Using an alkaline solution and sodium dodecyl sulfate (SDS), the cells containing the plasmids were lysed. The lysate was then transferred to a silica membrane selective for the DNA and contaminates were removed using a wash. Tris/EDTA (TE) buffer was used to elute out the DNA. The DNA was then stored at -20 °C.

The human D1 and D2 receptor genes encoded on the plasmids are held on the pFN21A HaloTag® CMV Flexi® Vector. This vector permits constitutive protein expression on mammalian cells by use of human cytomegalovirus (CMV). The vector HaloTag® RNA polymerase promoter sequence is displayed below in Figures 3 and 4 below.
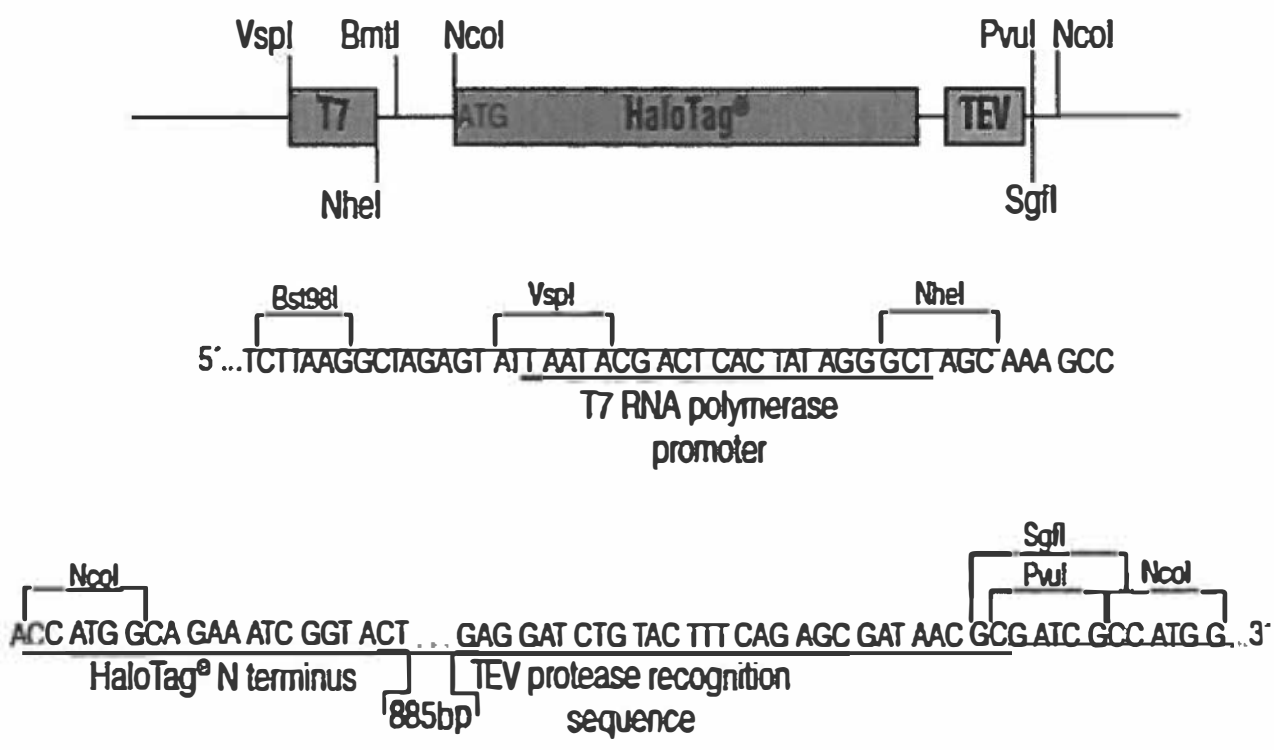
Figure 3: pFN21A HaloTag CMV Flexi Vector sequence upstream and downstream of the HaloTag gene (Promega.com).
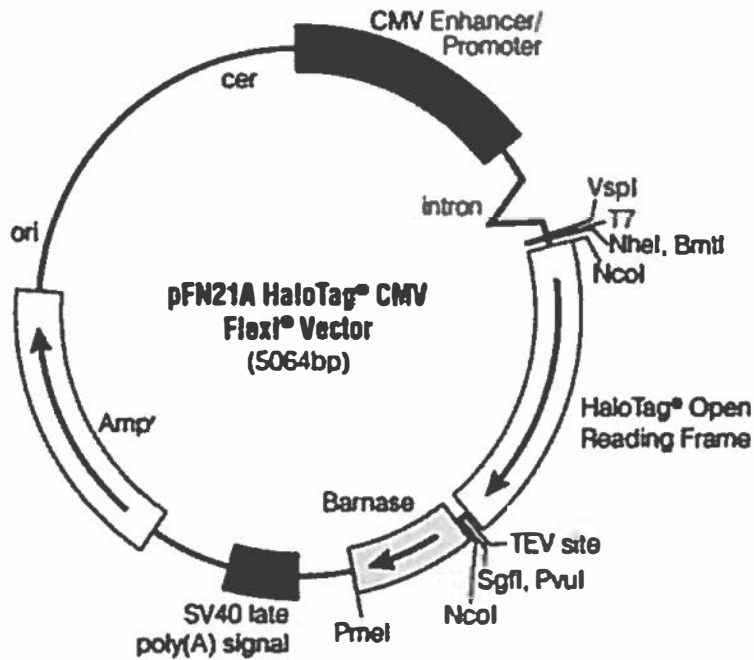
Figure 4: pFN21A HaloTag CMV Flexi Vector circle map and sequence (Promega.com).

For transcription and translation of the system of our gene of interest downstream of T7, a TnT® Quick Coupled Transcription/Translation System from Promega was used. A 40 µL aliquot of TnT® Quick Master Mix was added to 0.2–2.0µg of the template circular plasmid DNA with the addition of 7 µL of nucleus-free water. The reaction was allowed to run 60-90 minutes at a 30°C for protein expression. The TnT® Quick Master Mix couples transcription and translation due to the system having RNA polymerase, various salts and nucleotides, and Recombinant RNasin® Ribonuclease Inhibitor (to prevent nonselective protein expression). After incubation, the mixture was thawed at room temperature then stored in ice. This was done for D1 and D2 receptor separately A summary of the TnT® Quick Coupled Transcription/Translation System from Promega can be found below.
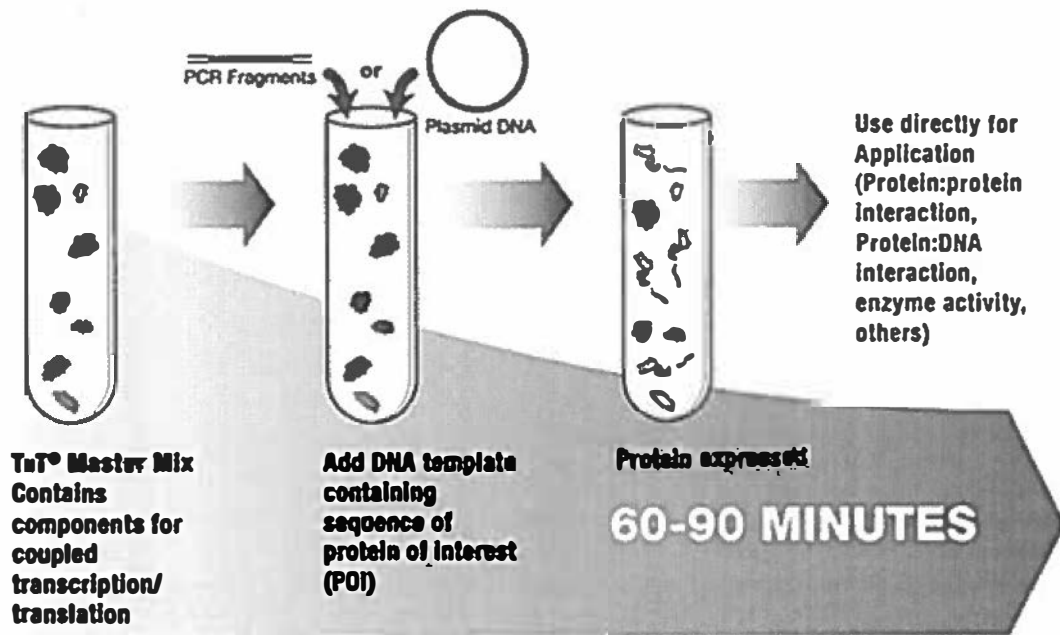
Figure 5: Summary of the TnT® Quick Coupled Transcription/Translation System

Filter cubes were pushed into each compartment below each well to form a small porous opening. Various concentrations of proteins were added to each well including the D1 and D2 receptor.

For *in vitro* analysis of the effectivity of each synthetic peptide in uncoupling the D1-D2 heteromer, a Zoomer dot blot was conducted. Each of the receptor proteins were diluted to 100 μg/mL and centrifuged. D1 and D2 receptors were purchased from Abcam (ab90834 for D1 and ab112281 for D2) and were made using the the TnT® Quick Coupled Transcription/Translation System from Promega. The D1 receptor was diluted to 100 μg/mL in 30 μL PBS after thawing. 5 μl of D1 receptor with the PBS solution was added to each well and each well was then blocked with 50 μL blocking buffer of 10% BSA in PBST (Phosphate Buffered Saline with 20% Tween) to prevent non-specific binding. 1x PBS was made by using 8g NaCl, 0.2 gKCl, The 1% BSA was made by dissolving 5g of BSA and 2.5 ml of 20% Triton-X-100 and 487.5 ml of distilled water. The 10% BSA solution was done diluting 50g of BSA with 2.5 ml of Triton-X-100 and 487.5 ml of distilled water. They were then rinsed 4x with 50 μL rinsing buffer of 1% BSA in PBST. 5 μl of D2 was then added to each well.

Peptides were first diluted by dissolving 0.0010 g in 1ml solution of 100 μl DMSO+900 μl PBS. This was then diluted 10-fold using PBS. The stock concentration (μM) was then determined by multiplying the molecular weight of the peptide to the protein dilution. This concentration was then used to determine the μl of protein of the 10 fold diluted stock per well needed. This was by
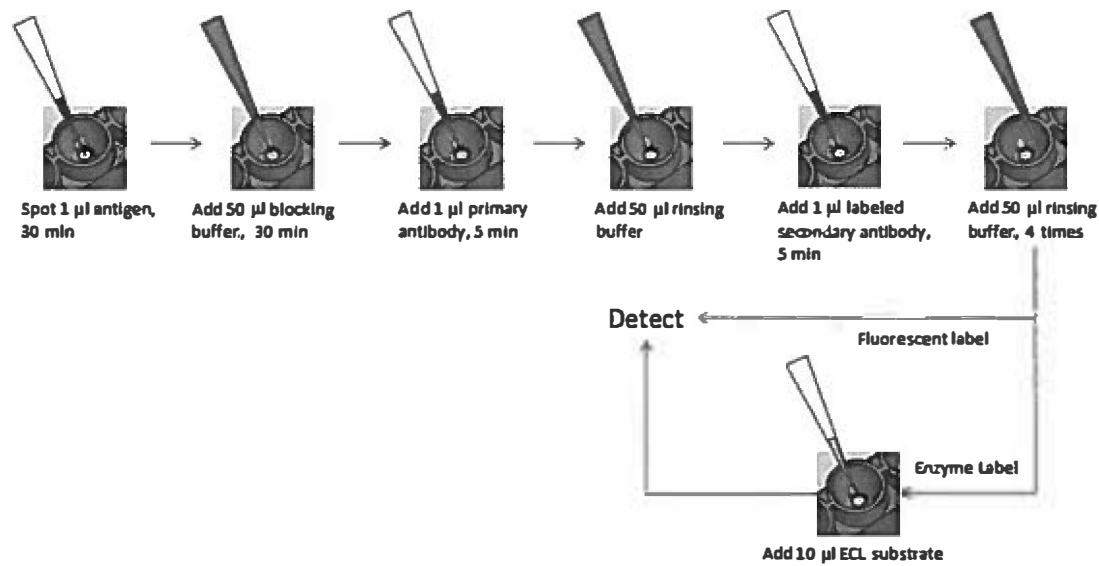
multiplying the needed μM of protein by the total solution in each well (μl) and setting this equal to the stock concentration.

The wells were then allowed to incubate for 2 hours or overnight to allow heteromer complexing. After incubation, 2 μl primary antibody for D2 was added to each well and allowed to incubate for 5 minutes. The wells were then washed once with 50uL rinsing buffer of 1% BSA in PBST. Secondary D2 antibody was then added for visualization, and the wells were allowed to incubate for five minutes. The samples were then washed four times with 50 μl rinsing buffer of 1% BSA in PBST.

For all 8 wells, 10μl of Clarity™ Western ECL mix was added to each well. This was done by mixing 50 μl of peroxide solution and 50 μl of luminol enhancer. The wells were then allowed to sit for 10 minutes and were then imaged. A summary of the reagents and appropriate volumes can be found below in Table 1 for Peptide 1. Figure 6 shows a summary of the zoom dot blot protocol.

Table 1: Solution scheme for each well 1 through 8

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
Peptide concentration	0μM	5 μM	10 μM	15 μM	20 μM	25 μM	30 μM	35 μM
Dilute peptide volume	0 μL	1.0	2.5 μL	4 μL	5 μL	6 μL	7.5 μL	9
Expressed D2	5 μl	5 μl	5 μl	5 μl	5 μl	5 μl	5 μl	5 μl
Expressd D1 in PBS (100 ug/mL)	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL
Cold PBS buffer (pH 7.6)	20 μL	19	17.5 μL	16 μL	15 μL	14 μL	12.5 μL	11 μL
Total Volume	30 μL	30 μL	30 μL	30μL	30μL	30μL	30μL	30μL

Figure 6: Summary of the Zoom Dot Blot Protocol

Western Blotting Co-immunopercipitation

This procedure serves as another quantitative method in observing the effectivity of the synthetic peptides disrupting the formation of the D1-D2 heteromer. Sample preparation was prepared in 8 cuvettes. The sample preparation scheme is shown below in Table 2 for Peptide 2 (note: the µl added of the peptide will vary to match µM). This procedure only differs from the Zoom blot in having no washing or rinsing steps with blocking buffer and rinsing buffer. Protein preparation and dilution remains the same for the stock concentration.

Table 2: Sample Preparation for Western Blotting

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7
Peptide concentration	0 μM	10 μM	20 μM	30 μM	40 μM	50 μM	60 uM
Expressed D1	3 μL	3 μL	3 μL	3 μL	3 μL	3 μL	3 ul
Expressed D2	3 μL	3 μL	3 μL	3 μL	3 μL	3 μL	3 uL
Peptide 2: EERRAQ	0 μL	4.4 μL	8.9 μL	13.2 μL	17.6 μL	22 μL	26.4 μL
1° antibody for D1	3 μL	3μL	3μL	3μL	3μL	3μL	3μL
PBS buffer	44 μL	39.6 μL	35.1 μL	30.8 μL	26.4 μL	22 μL	17.6 μL
Total Volume	53 μL	53 μL	53 μL	53 μL	53 μL	53 μL	53 μL

After samples are prepared, they are incubated overnight in a cold room shaker at 4°C. 20 μl of agarose beads are then added to 8 new cuvettes and washed twice with 300 μl PBS. Washes were done by adding the amount of PBS, centrifuging the tubes for 1 minute at 4,000 rpm, and discarding the supernatant. More PBS was then added to the next wash. The beads must be washed in order to get remove any residuals and contamination off from the beads. The beads are then added to each sample to pull down the D1-D2 complex by binding to the primary antibody. The beads with the sample are then incubated in a cold room shaker at 4°C. The sample tubes were then centrifuged at 4,000 rpm for one minute. 40 μl of supernatant was then drawn from each tube and set aside in new sample cuvettes. The beads were then washed with 80 μl of PBS

at 3,000 rpm, discarding the supernatant each time and adding more PBS after each wash. 10 μl of supernatant in each was then discarded and 10 μl of 5X SSB was added to each sample cuvette containing the agarose beads as well as to the 40 μl cuvettes containing supernatant. All the samples (beads and the supernatant) were then boiled for 10 minutes at 95°C. The samples (beads and supernatant) were then loaded onto SDS-PAGE on 12% acrylamide gels with a 5% stacking gel for 1 hour at 100V. The protein ladder used was Benchmark™ from Invitrogen. The 1x SDS-PAGE buffer for the tank was made with 200 ml of %x Tris/glicne buffer, 750ml of ultrapure water, and 5 ml of 20% SDS. The recipes for the 12% acrylamide and 5% stacking gel is shown below in Table 3.

Table 3: Recipes for 12% separating gel and 5% stacking gel

	12% separating gel	5% stacking gel
H_2O (ml)	3.4	4.19
1.5 M Tris, HCl pH 8.8 (ml)	2.5	0.75
Acryl/Bis 40/1, 30 % (ml)	4	1
SDS 20% (μl)	S0	30
APS 10% (μl)	100	60
TEMED (μl)	10	6
Final Volume (ml	10.06	6.036

Filter paper, sponge, and the nitrocellulose paper were soaked in Western Transfer Buffer. The western transfer buffer was first cooled to about -10°C. The Western Transfer buffer was made with 200 ml of 10x western buffer, 1400 ml of distilled water, and 400 ml ethanol. The gel was then transferred to a sandwich of layers of sponge, filter paper, and nitrocellulose after running SDS-PAGE. The gel is then covered by the nitrocellulose paper. The diagram of the sandwich is shown in figure 7. Proteins in the transfer buffer are negative in charge and they therefore moved from the -ve to +ve pole. So the +ve was above the nitrocellulose and the -ve side is below the gel.
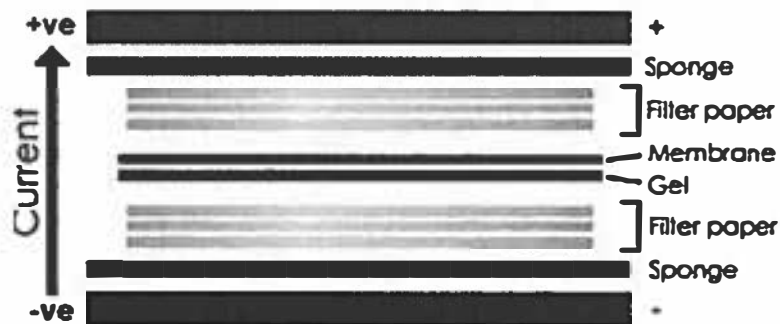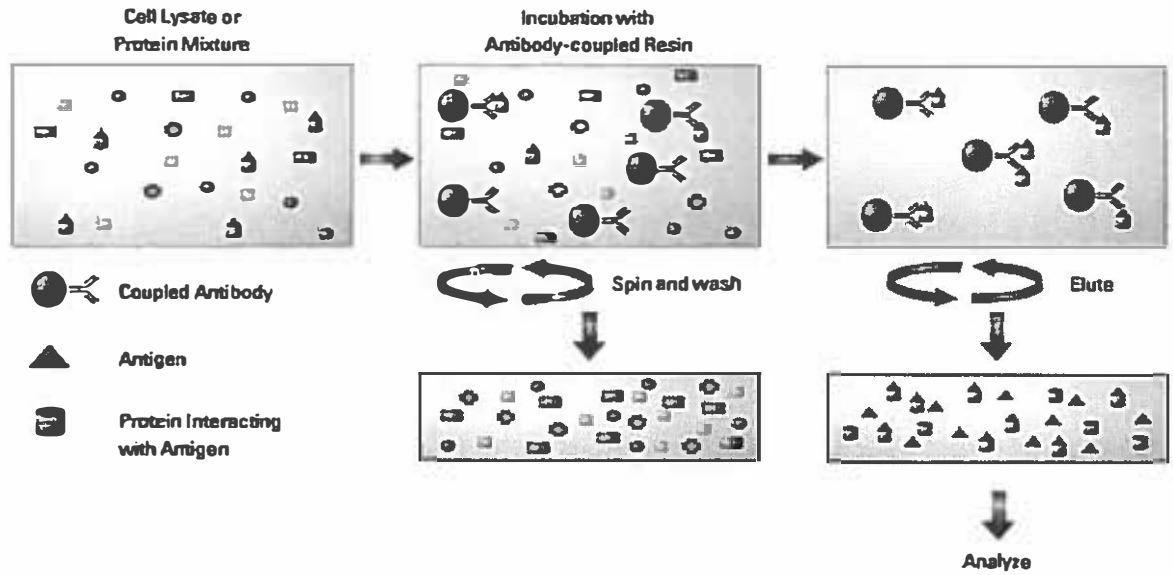

Figure 7: Sandwich for the Western Transfer Buffer.

The tank containing the sandwich was then filled with Western Transfer buffer and the apparatus was allowed to run for 1 hr and 30 min at 100 V. All air bubbles were removed from the sandwich to allow complete transfer of the gel to the nitrocellulose paper. The Western Buffer apparatus was covered with ice to prevent overheating.

After transfer, the nitrocellulose membranes were removed and placed face up in a blotting box, allowing it to soak in the western transfer buffer for 30 minutes. The excess western

transfer buffer was then removed. Each nitrocellulose paper was then washed with 15ml of TBST (Tris Buffered Saline Tween) for three times. TBST was made with 100 ml of TBS 10x, 900 ml distilled water, and 1ml Tween 20%. 5 ml of blocking buffer was then added to each membrane. This was done by diluting 0.5 g of non-fat dry milk in 10 ml of TBST. 2 µl of primary D1 antibody was added to the mix. This was then allowed to block overnight in a cold room at 4℃ on a shaker. The blocking buffers were used to prevent non-specific binding. The membranes were then washed with 15 ml TBST six times each for 5 minutes. Another blocking buffer by adding 2 µl of secondary D2 antibody to 0.5 grams of milk in 10 ml TBST. 5ml was added to each membrane. It was then allowed to incubate overnight in a cold room on a shaker. The membranes were then washed with 15 ml of TBST 6x for 5 minutes. The nitrocellulose membranes were then transferred and imaged with 10µl of Clarity™ Western ECL mix was added to each well. This was done by mixing 1 ml of peroxide solution and 1 ml of luminol enhancer. 1ml was then added to each membrane and the membranes were allowed to develop for 10 minutes. They were then imaged using BioRad™. A summary of the co-immunoprecipitation technique is shown below in Figure 8.
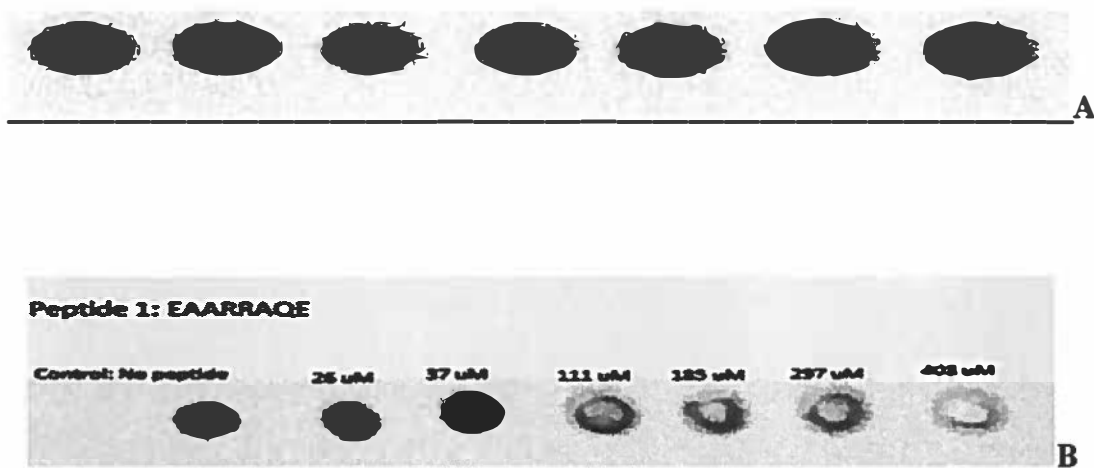
Coupled Antibody

Antigen

Protein Interacting with Antigen

Figure 9: Schematic summary of a standard co-immunoprecipitation

Results

A

Peptide 1: EAARRAQE

Control: No peptide   26 uM   37 uM   111 uM   185 uM   297 uM   408 uM

B

Figure 9A: Co-immunoprecipitaion of 0.5 µg of D1 and D2 receptors in the presence of increasing concentration of peptides. The two proteins were incubated without and with varying concentrations of peptides. D1 was pulled down with D1 specific antibodies, and the amount of D2 bound to D1 was detected by zoom blotting using D2 specific antibodies. Blots were developed, quantitated using Image J (Materials and Methods). Processing of the data was carried out with the GraphPad software using nonlinear regression and a dose response curve fit. The graph summarizes the results expressed as means ± SE and normalized to control ($P < 0.05$, n = 5).

Figure 9B: Upper panel represents an example of dot blotting using peptide 4 and lower panel represents that using peptide 1. Concentrations from left to right are 0, 26, 37, 111, 185, 297, and 408 µM of peptide.

Starting from left to right on figure B of the co-immunoprecipitation dot blot, we have the control, which ideally consists of only the D1-D2 heteromer. It is the darkest dot and ideally, to have an effective complex-uncoupling peptide, the intensity should decrease and get lighter as the peptide concentration increases. As the concentration of peptide 1 increases, the relative intensity of the dots tended to decrease.

In figure A, the same concentration of peptide I was added to each well, showing no change in the relative intensity of the control. This figure served as a comparison for figure B.
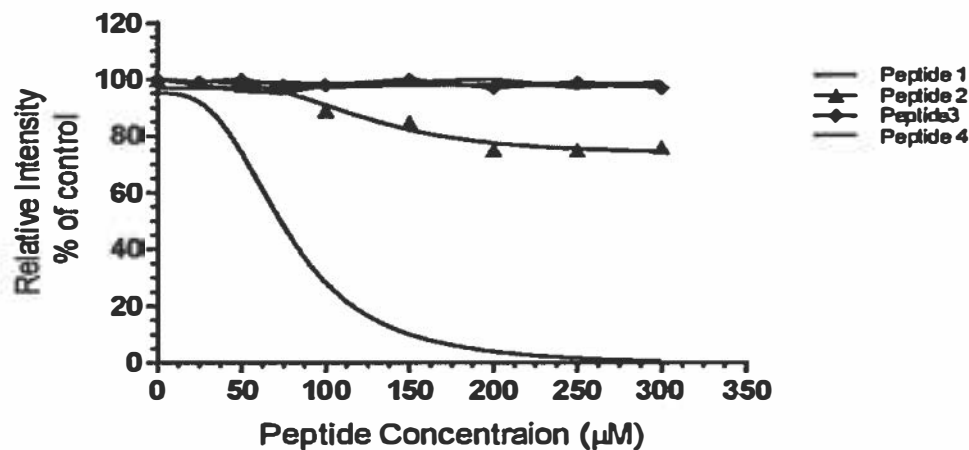

Figure 10: Shows the decrease of the relative intensity of the control to peptide concentration. Concentrations from left to right are 0, 26, 37, 111, 185, 297, and 408 μM of peptide.

It can be shown in Figure 9 that Peptide 1 was the most effective, essentially uncoupling almost all of the D1-D2 heteromer at about 300 μM. Peptide 2 was minimally effective decreasing the intensity of the control by about 20%. Peptide 3 and 4 had little to no effect in uncoupling the heteromer.

These peptides have varying IC_{50} values, which is the concentration of the peptide needed to inhibit 50% of the D1-D2 complex formation, or in this case, the concentration needed to

decrease the relative intensity of 50% of the control. The respective IC_{50} values can be found in Table 4.

Table 4: Summary of each of the peptides IC50 values derived from Figure 10

Name	Sequence	IC_{50} Values (μM)
Peptide #1	Ac-EAARRAQE	75.37
Peptide #2	Ac-EERRAQ	> 350
Peptide #3	Ac-ARRA	> 550
Peptide #4	Ac-AARRAQ	> 550

We can see that Peptide 1 can inhibit 50% of the D1-D2 complex formation at 75.37 μM while peptides 2,3, and 4 needed much higher concentrations. To explore this further, we synthesized a D-version of peptide 1 to test the effectiveness of changing the amino acid stereochemistry.
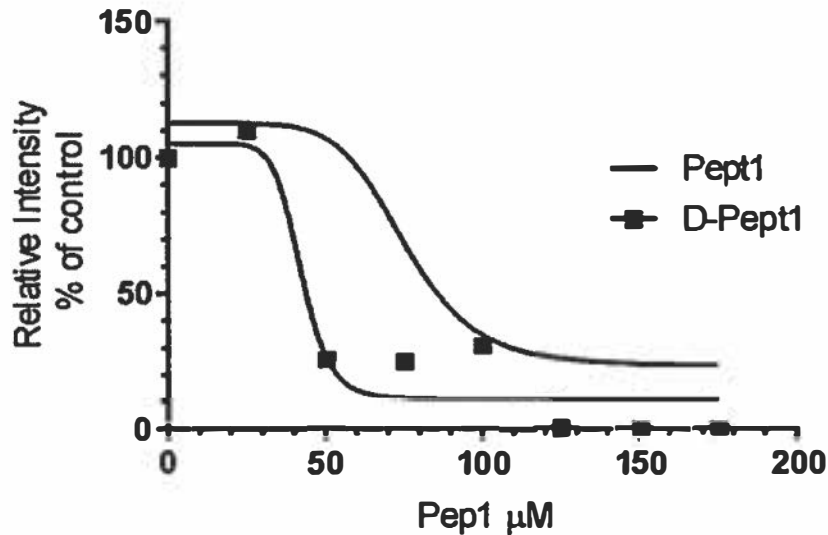
Figure 11: Shows the decrease of the relative intensity of the control to increasing peptide

concentration. Western blot of D1 coimmunoprecipitated in the presence of increasing

concentrations of peptide 1 and D-peptide 1 and visualized using D2 antibodies. Lanes 1-8

correspond to 0, 25, 50, 75, 100, 125, 150, and 175 μM, respectively. Concentrations from left to

right are 0, 26, 37, 111, 185, 297, and 408 μM of peptide 1 and D-peptide 1.

It can be seen that the D-peptide 1 is even more effective than the L-peptide in disrupting the D1-

D2 heteromer. This can also be seen observing the IC_{50} values.

Table 5: Shows the IC50 values for the D and L-peptide 1

Name	Sequence	IC_{50} Values (μM)
Peptide #1	Ac-EAARRAQE	75.37
D-Peptide #1	Ac-eaarraqe	42.11

D-peptide 1 needed almost only half the concentration of the L-peptide 1 to be 50% effective against the D1-D2 heteromer.
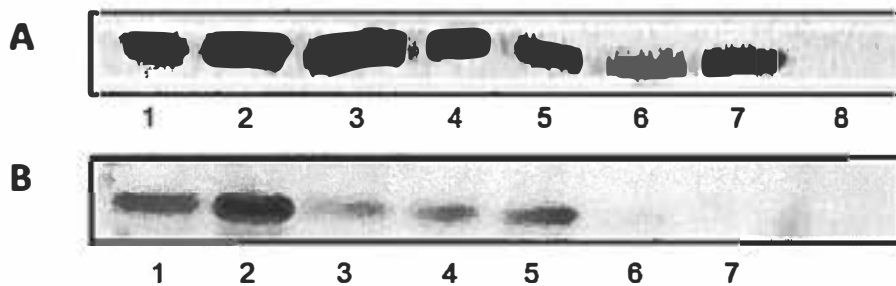

Figure 12: Western blot of D1 coimmunoprecipitated with D2 in the absence (A) and presence (B) of increasing concentrations of D-peptide 1 and visualized using D2 antibodies. Lanes 1-8 correspond to 0, 25, 50, 75, 100, 125, 150, and 175 μM, respectively.

In Figure 12B, lane 2 seems to be an outlier, as the intensity of the dot is higher with a greater concentration of Peptide 1 added. Otherwise, a clear trend is observed where increasing peptide resulted in receptor uncoupling.

Discussion

The four different peptides were synthesized according to the interface identified between the intracellular loop of D2 and the carboxy tail of D1 as indicated by *O'Dowd et. Al. 2012*. This area consists of charged amino acid residues such as glutamate, aspartate, arginine, and lysine. From our results, we can conclude that both the L- and D-Peptide 1 Ac-EAARRAQE prove to be the best inhibitors of the D1-D2 heteromer complex. Interestingly enough, this may indicate that the D-form of other small peptides may be more effective in inhibiting the formation of the heteromer. Peptide 1 only differs from Peptide 4 by two glutamates at the ends, yet much is more effective as indicated by their IC_{50} (75.37 μM Peptide 1 and >350 μM Peptide 4). This may indicate that the crucial interface maintaining theD1 and D2 may be larger than expected between the carboxy tail of D1 and the intracellular loop of D2.

Conclusion

D1 and D2 can be uncoupled by a small peptide as confirmed by other studies (1), but it is not certain if our selected peptides bind between the carboxyl tail of D1 and intracellular loop

3 of D2. There may be other interfaces maintaining the stability of the heteromer. Also our peptides were only shown to bind to D1, to uncouple the heteromer. In the future we may examine other intracellular loops of D2, possibly creating a peptide that may be even more effective in uncoupling D1-D2.

Future Directions

We plan to observe the effects of the D-stereoisomer of the other three peptides on the IC_{50} values. D-peptides may be more effective in heteromer uncoupling. We also plan to test our peptides on HeLa cells *in vivo*, cells that express D1 and D2 endogenously, as well as observe the effects of our peptides on PC12 cells (pheochromocytoma). Developing a peptide that may uncouple the heteromer can possibly serve to alieve the symptoms of depression and act as a possible medical intervention.

Acknowledgements

REFERENCES:

1.   Willipinski-Stapelfeldt, B., Lübberstedt, J., Stelter, S., Vogt, K., Mukhopadhyay, A. K., & Müller, D. (2004). Comparative analysis between cyclic GMP and cyclic AMP signalling in human sperm. MHR: Basic Science of Reproductive Medicine, 10(7), 543–552. https://doi.org/10.1093/molehr/gah065

2.   Abuse, N. I. on D. (2009, October 1). Receptor Complexes Link Dopamine to Long-Term Neuronal Effects. Retrieved April 5, 2017, from https://www.drugabuse.gov/news-events/nida-notes/2009/10/receptor-complexes-link-dopamine-to-long-term-neuronal-effects

3.   Hasbi, A., O'Dowd, B. F., & George, S. R. (2011). Dopamine D1-D2 receptor heteromer signaling pathway in the brain: emerging physiological relevance. Molecular Brain, 4, 26. https://doi.org/10.1186/1756-6606-4-26

4.   Hasbi, A., Perreault, M. L., Shen, M. Y. F., Zhang, L., To, R., Fan, T., … George, S. R. (2014). A peptide targeting an interaction interface disrupts the dopamine D1-D2 receptor heteromer to block signaling and function in vitro and in vivo: effective selective antagonism. The FASEB Journal, 28(11), 4806–4820. https://doi.org/10.1096/fj.14-254037

5.   Rashid, A. J., So, C. H., Kong, M. M. C., Furtak, T., El-Ghundi, M., Cheng, R., … George, S. R. (2007). D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of Gq/11 in the striatum. Proceedings of the National Academy of Sciences of the United States of America, 104(2), 654–659. https://doi.org/10.1073/pnas.0604049104

6.   NIMH » Depression. (n.d.). Retrieved April 5, 2017, from https://www.nimh.nih.gov/health/topics/depression/index.shtml

7.   Perreault, M. L., Hasbi, A., O'Dowd, B. F., & George, S. R. (2014). Heteromeric Dopamine Receptor Signaling Complexes: Emerging Neurobiology and Disease Relevance. Neuropsychopharmacology, 39(1), 156–168. https://doi.org/10.1038/npp.2013.148

8.   Pei, L., Li, S., Wang, M., Diwan, M., Anisman, H., Fletcher, P. J., … Liu, F. (2010). Uncoupling the dopamine D1-D2 receptor complex exerts antidepressant-like effects. Nature Medicine, 16(12), 1393–1395. https://doi.org/10.1038/nm.2263

9.   Brown, V., & Liu, F. (2014). Intranasal Delivery of a Peptide with Antidepressant-Like Effect. Neuropsychopharmacology, 39(9), 2131–2141. https://doi.org/10.1038/npp.2014.61

10.   O'Dowd, B. F., Ji, X., Nguyen, T., & George, S. R. (2012). Two amino acids in each of D1 and D2 dopamine receptor cytoplasmic regions are involved in D1–D2 heteromer formation. Biochemical and Biophysical Research Communications, 417(1), 23–28. https://doi.org/10.1016/j.bbrc.2011.11.027

11.   Whitte, L., National Insitute on Drug Abuse, Oct. 1st 2009, Receptor Complexes Link Dopamine to Long-Term Neuronal Effects https://www.drugabuse.gov/news-events/nida-notes/2009/10/receptor-complexes-link-dopamine-to-long-term-neuronal-effects