Amphetamine Induced Dopamine Transporter Internalization: A Structure/Function Study

A Major Qualifying Project Report

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Abstract

Dopamine is a neurotransmitter that plays a key role in locomotion and mood, and an imbalance of dopamine levels has found to play a significant role in Schizophrenia, ADHD, and Parkinson's disease. Dopamine is released into the neuronal synapse and taken in by dopamine transporters, which constituently trafficking in and out of the plasma membrane. Using chimeric dopamine/serotonin transporters, this research was aimed at determining the protein sequences necessary for the dopamine transporter trafficking in response to amphetamine. It was shown that amphetamine causes a decrease in dopamine transporter cell surface density and an increase in serotonin transporters. Newly designed dopamine transporter chimeras were made to be used in future experiments.

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Background

Dopamine and Its Trafficking

Dopamine (DA) is a neurotransmitter that plays a key role in locomotion, mood, and neuroendocrine secretions (1). Atypical levels of DA contribute to various neurological diseases and disorders. An imbalance in synaptic DA has been found to be a factor in Schizophrenia, ADHD, addiction, and Parkinson's disease (2), (3), (4). An increase in dopamine in the synapse within the brain is the cause of the pleasant feelings that are associated with drug addiction and reward, therefore the movement of DA in and out of neurons is important to understand for drug addiction, as well as other neurological diseases (5).

Dopamine is made in the neuron by tyrosine hydroxylase which converts the amino acid tyrosine into DA; it is then packaged into vesicles which merge with the presynaptic membrane which releases it into the synapse (6)(7). The post synaptic dopamine receptors are activated which then relays the signal. In order to stop the dopamine from over stimulating the receptors, dopamine transporters (DAT) collect the dopamine from the synapse and pump it back into the presynaptic neuron, as illustrated in Figure 1.

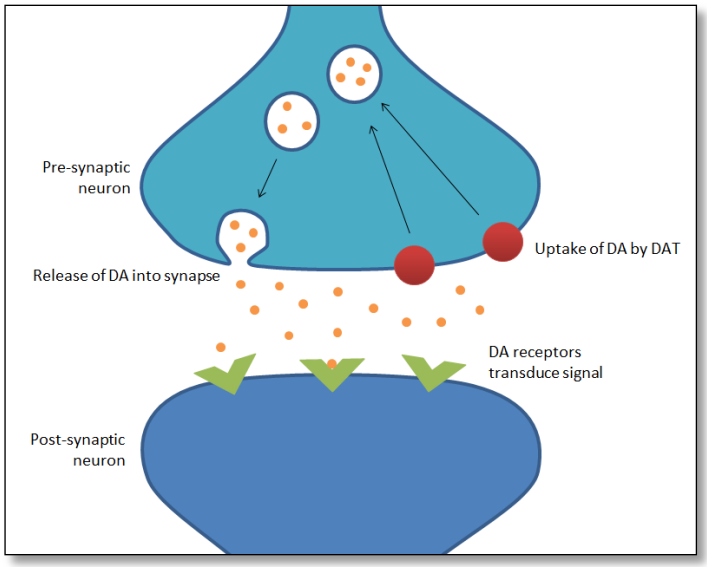

Figure 1: Dopamine Transport within the neuronal synapse

The dopamine transporter (DAT) is a 12 transmembrane protein embedded in the neuron membrane, with intercellular N and C terminus and an extracellular loop; a 3D model of the protein is shown in Figure 2. DAT is a Na^+/Cl^- dependent transporter which is part of the SLC6 gene family that

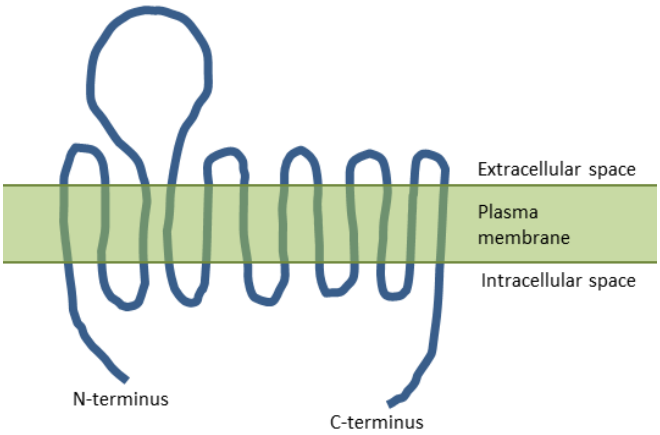

Figure 2: Protein structure of DAT

also includes serotonin and norepinephrine transporters (8). The uptake of DA by the transporter is dependent on the Na^+ gradient; as Na^+ is transported into the cell, DA is as well (8). The transporter is synthesized in the endoplasmic reticulum and processed in the Golgi where it is glycosylated. It is then brought into the plasma membrane where DAT can be regulated by endocytosis and recycled back to the surface by various factors (5). DAT constitutively traffics to and from the plasma membrane, and does not remain static in the membrane (9).

One way DAT trafficking is regulated is by Protein Kinase C (PKC). PKC is molecule necessary for some cell signaling pathways. Activation of PKC by phorbol esters causes internalization and down regulation of DAT (10). This PKC-activated down regulation is dependent on the FREKLAYIA domain, a peptide sequence located in the C-terminus of the protein. This sequence is maintained across the SLC6 gene family (11). PKC-dependent internalization is also dependent on clathrin coated pits in the cell membrane (12).

Amphetamine

Amphetamines (AMPH) are a family of psychostimulants used to treat various mental disorders, such as depression, ADHD and narcolepsy, but are also abused and can lead to drug addiction (13). In DAT cells treated with AMPH, there is an increased internalization rate of DAT, which causes a decrease in the surface level concentration and therefore decreases the neuron's ability to uptake dopamine (14). In addition, AMPH causes an efflux in DA by causing DAT to reverse its transport, trafficking the DA out of the cell into the synapse. It is necessary for the AMPH to enter the cell before it has an effect on DAT;

however the mechanism is not known (14). Experiments have shown that this internalization mechanism is PKC-independent (15).

While increased internalization of DAT occurs in response to AMPH treatment, it has been shown that AMPH has the opposite effect on serotonin transporter (SERT), a member of the SLC6 gene family along with DAT. The cell surface density of SERT is increased in cells treated with AMPH (16). This opposing trafficking response to AMPH is intriguing because DAT and SERT come from the same gene family and both have similar affinities for cocaine (17). I hypothesize that this difference may be due to non-conserved protein regions in the respective transporters. Using this difference, I will determine which peptide sequences may be necessary for DAT and SERT trafficking in response to AMPH.

Purpose

Because the PKC-activation pathway uses a specific protein sequence for trafficking of DAT, I hypothesize that DAT is internalized when treated with AMPH using a similar mechanism. In order to determine if a specific sequence is necessary for this mechanism, I used DAT/SERT chimeras, of which the N and C termini have been switched. Depending on how these chimeras respond to the AMPH treatment, it may allow us to narrow down which segment is necessary for trafficking due to AMPH treatment. How the wild type transporters respond to AMPH is illustrated in Figure 3.

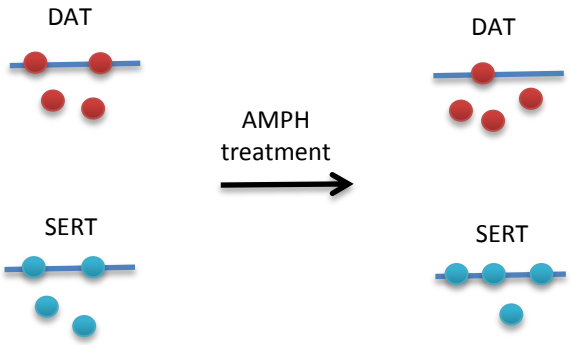

Figure 3: DAT and SERT reactions to AMPH

The nucleotide sequence for the DAT/SERT transporter chimeras are in a pcDNA vector for the experiments, however, for future protein interaction experiments, they must be in a pCFP-C1 vector in

order to observe protein interactions using FRET. I removed the protein nucleotide sequence from the pcDNA vector and ligated it into PCFP-C1 vector which allows the protein to fluoresce and be identified. However, if the insert was cut and placed directly into the PCFP-C1 vector, it would cause a frame shift mutation within the nucleotide sequence for the transporter and would make the protein incorrectly and would not be able to function. Using PCR reactions, a short 5' end of the sequence was added to prevent the mutation and attached before transferred to the PCFP-C1 vector.

Methods

Cell Culture and Transfection: Rat adrenal medulla tumor cells (PC12) were cultured in DMEM; 5% Horse serum; 5% Bovine Calf serum, Supplemented; 2mM Glutamine; 10^2 units/ml penicillin-streptomycin. 1 x 10^6 cells were plated in each well of a 6 well, poly-D-lysine coated plate and were grown in 10% CO_2. Cells were transiently transfected with 4 µg of DNA of each construct using Lipofectamine, according to manufacturer's instructions. Cells were assayed 48 hours post-transfection (18).

Surface Biotinylation Assays: Cells were treated with and without 5µM AMPH in PBS^{2+} (Phosphate buffered saline supplemented with 1.0 mM $MgCl_2$; 0.1 mM $CaCl_2$) for 30 minutes, 37°C after which the cells were rapidly chilled to 4°C to terminate DAT trafficking. Cell surface DAT was labeled by biotinylation, by incubating twice, with 1 mg/mL NHS-SS-biotin each with 15 minute incubations, 4°C. The cells were washed twice in a quench solution (PBS++; 100 mM glycine) and incubated in the quench solution for 15 minutes at 4°C. Cells were washed with PBS++ and lysed for 20 minutes with RIPA lysis buffer/protease inhibitors, 1% phenylmethylsulfonyl fluoride and 1% leupeptin, pepstatin A, aprotinin, 4°C. The samples were transferred to microfuge tubes and spun at 13,000 rpm for 10 minutes to remove insoluble material. A Pierce BCA colorimetric protein assay was run to determine the protein content of each sample compared to BSA standards.

Biotinylated surface proteins were separated from non-biotinylated intracellular proteins by batch streptavidin chromatography, using streptavidin agarose beads. Briefly, each 100µg protein sample was brought up to 200 µL with RIPA and 30 µg of Streptavidin beads were aliquoted into each tube which rotated overnight at 4^{o}C. The totals method was used, where a quarter volume of what was used for the bead sample, 25 µg, was collected and combined with an equal volume of 2x SDS-PAGE loading buffer and frozen at 4^{o}C overnight. The beads were washed with RIPA three times, and after the final wash, 30 µl 2x SDS-PAGE loading buffer was added and the samples were rotated for 20 minutes at 37^{o}C (15).

Immunoblotting: The samples were resolved by a 10% SDS-PAGE electrophoresis gel. Electrophoresed proteins were transferred to nitrocellulose using the Biorad criteria for transfer (19). The nitrocellulose was treated for 45 minutes with a blocking solution (5% nonfat dried milk in PBS, .1% Tween-20). For the transporters containing a DAT N-terminus, a rat α-DAT antibody was used, and for the transporters containing a SERT N-terminus, a mouse α-SERT antibody was used at 1:2000 in blocking solution. These were incubated overnight at 4^{o}C in their primary antibody solutions. The membrane was washed with Blot wash (PBS, 0.1% Tween-20) and a secondary, a goat α-mouse or a horse α-rat antibody, at 1:5000 in blocking solution, was applied, respectively, and incubated at 37^{o}C for 45 minutes. The membrane was washed with blot wash and was developed using Pierce Supersignal Dura, incubating for 5 minutes. Immunoreactive protein bands were imaged using a CCD camera on a Versadoc imaging station (Biorad) and the resulting images were then quantified with Quantity One software (20).

Subcloning: The chimeras for this experiment were made by previous lab members, however in preparation for future experiments, the chimera DNA was removed from the pcDNA vector and ligated into a PCFP-C1 vector. If the chimera DNA had been directly removed from the pcDNA vector and

ligated into the PCFP-C1 vector, a frameshift mutation would have occurred. For this reason, PCR was used to create a short 5' end of the insert; the primers used for the reaction was created by Invitrogen and contained an additional random nucleotide sequence that elongated the insert to prevent the mutation. The products were gel purified on a .7% agarose gel and extracted using the QIAquick Gel Extraction Kit. To add this 5' short end to the chimera, both the PCR created short end and the existing chimera plasmid were digested overnight at 37^{o}C with HindIII and either AgeI or PflMI depending on the construct. The 5' short end digest was purified using the QIAquick PCR Purification Kit and the chimera digest was gel purified. These two components were ligated overnight at 37^{o}C and transformed into DH5α e. coli which were then streaked onto agar plates and grown overnight at 37^{o}C. Colonies were picked and a culture of 3 ml was grown overnight at 37^{o}C; the plasmid DNA was extracted from the bacteria using the QIAprep Spin Miniprep Kit. The DNA was analytically digested for 1 hour at 37^{o}C using HindIII and either AgeI or PflMI depending on the construct. These digests were run on 0.9% agarose gels to determine if the short end had successfully ligated. Once the ligation had been confirmed, the DNA was sent for sequencing by Genewiz to check that it had properly ligated and there were no mutations. When the sequence was determined to be correct, successful bacterial colonies were re-picked and the maxi prep protocol was followed using the QIAfilter Plasmid Maxi Kit where the plasmid DNA was extracted.

In order to remove the chimera DNA from the pcDNA vector, the Hind III and XbaI restriction enzymes digested the constructs overnight at 37^{o}C. The digested products were run on a 0.7% agarose gel, where the insert ran at approximately 2kb and the pcDNA vector ran at approximately 5 kb. The chimera DNA was cut out of the gel and purified using the QIAquick Gel Extraction Kit. Stock CGP-C1 vector was digested overnight with HindIII and XbaI, as well as the chimera DNA. The two products

were ligated together and the final plasmid was transformed into DH5α E. coli to grow up and sequence. The specific sequences used for each chimera can be found in the results section.

Results

In this experiment, I was able to show that AMPH caused a decrease in cell surface density of DAT and an increase of SERT. When I continued on to the chimera testing, I had problems with protein expression and antibody function. Over the course of the experiment I troubleshot various aspects in the experimental process; however I was never able to determine the cause of the problem. Moving on to designing and creating the recombinant DNA, I was successful and have made stocks for future experimentation.

Immunoblotting

One of the first experiments I ran was to make sure the experimental process worked. I treated DAT transfected cells with AMPH and observed how the transporter responded to make sure it would respond as previously shown in other cell types (14), (16). I biotinylated the cells after treating them ±AMPH, and the results are shown in Figure 4. The AMPH decreased the surface DAT to 25% from 55% in the vehicle treated, which has shown to be the usual response.

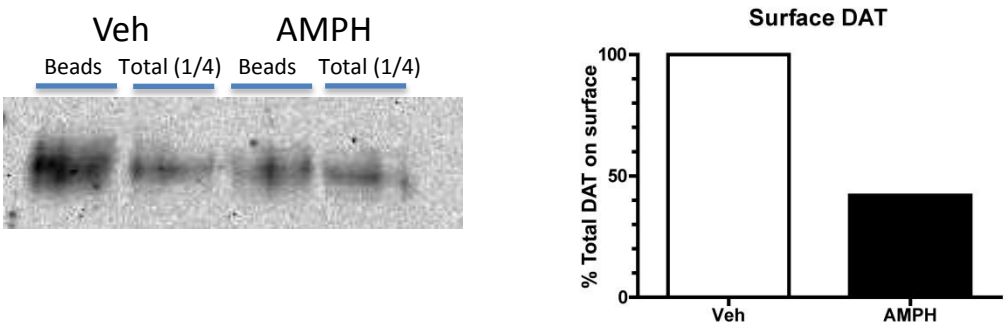

Figure 4: DAT response to AMPH. The first image is the gel resulting from an experiment in which I tested the effects of AMPH on DAT surface levels. The "beads" column is the surface protein that was biotinylated and the "total" column is one fourth the amount of total protein. The graph is a quantification of the bands and is normalized to the vehicle treatment. It shows that when the cells were treated with AMPH, DAT surface levels were about 50% less than they were without the APMH treatment.

I used the same experimental process to analyze the SERT response to AMPH. There was almost a 50% increase, which is to be expected (16).

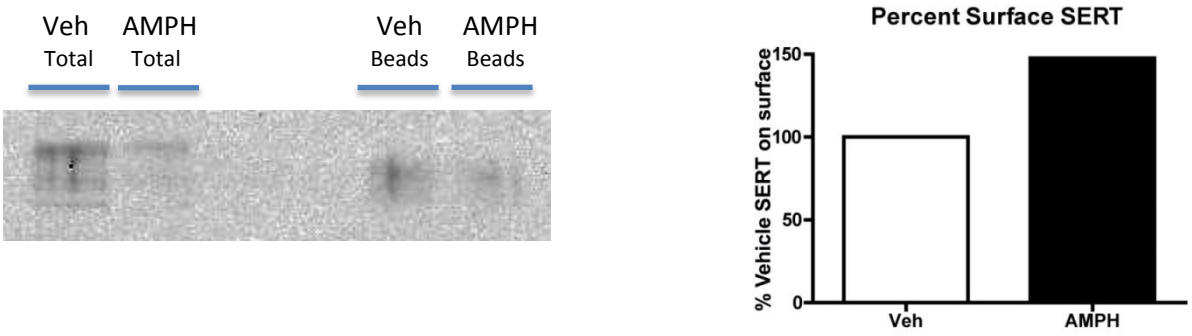

Figure 5: SERT response to AMPH. The first image is the gel resulting from an experiment in which I tested the effects of AMPH on SERT surface levels. The "total" column is the total amount of protein of the cell and the "beads" column is the surface protein that was biotinylated. The graph is a quantification of the bands and is normalized to the vehicle treatment. It shows that when the cells were treated with AMPH, DAT surface levels were about 50% more than they were without the APMH treatment.

Once was it was determined that DAT surface levels would decrease and SERT surface levels would increase in response to AMPH in PC12 cells, I proceeded with the chimera experimentation. This led to a number of blank or spotty blots; either no band of protein showed up or the band was half missing, so no useful data could be drawn from it. To determine the cause of this, I did each step of the experiment individually. I first had my advisor and I both transfect the cells to see if it was something

wrong with my technique, I continued with just simply lysing the cells and running them on the gel,

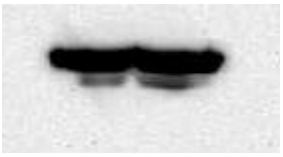

Figure 6: Antibody Test Blot of DAT. Determined the antibody was still effective.

there appeared to be no problem with my transfection technique. I hypothesized that the antibody had become inactive so I lysed the cells and treated them with the antibodies; the blot is shown in Figure 6 , there is a perfectly clear signal coming from the DAT protein. I went through the steps of washing, biotinylating, antibody treatment and development of the blot individually to fix any problems; they all appeared to be worked out. I often ran into issues with only the bead band showing up, leaving the total band blank and nothing to compare. An example of this is shown in Figure 7. After troubleshooting the whole experiment, I did every step, and it resulted in a blank blot, and several more after that. At this point, after discussing it with my advisor, we decided to move onto PCR.

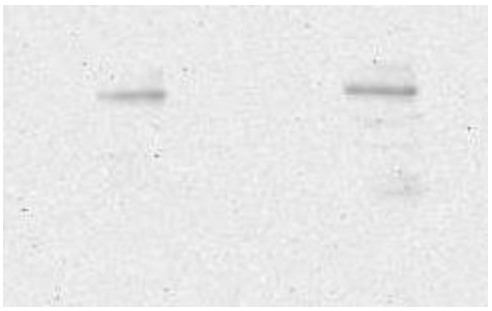

Figure 7: Missing signal in lane to compare

5' short HindIII hSERT claI 3.1(+) in pCFP-C1 vector

This construct started as hSERT claI pcDNA 3.1(+), the ClaI restriction site was added for future chimera creation and was in pcDNA 3.1(+) vector because of its high expression. The 5' HindIII SERT short sequence had already been made, however amplification was needed, so I carried out PCR using the 5'HindIIIshort-F and SERT 1275R primers. The PCR reaction was gel purified and the short sequence was extracted. Both the existing hSERT claI pcDNA 3.1(+) and 5' HindIII short end were digested with HindIII and AgeI, removing the existing 5'end from the hSERT DNA and preparing the short end for ligation. The PCR products were purified and the hSERT was gel purified and extracted. The 5' HindIII short was ligated into the hSERT and the completed sequence was transformed into DH5α e. coli. The bacterial plasmid DNA was extracted and digested with HindIII and XbaI for one hour to see if the insert

had successfully ligated. Once it was determined, the DNA was sequenced to make the official confirmation and check for mutations. Using HindIII and XbaI, both the pCFP-C1 vector and the hSERT in pcDNA were digested to prepare the vector and insert for ligation. They were then gel purified the insert and vector, and ligated them together. DH5α were transformed with the DNA and the plasmid DNA was extracted; the DNA was digested with HindIII and Age-HF to screen for the insert. The XbaI site is methylation sensitive in the pCFP-C1 vector and therefore is inactive in DH5α, so the AgeI site had to be used instead of XbaI (21). When the insert was determined to have successfully ligated, the DNA was sequenced. The methods used are illustrated in Figure 8.

SERT(1-78)/DAT(60-620) in pCFP-C1 vector

The SERT(1-78)/DAT(60-620) sequence had already been created, but as with the hSERT construct, a 5' HindIII SERT short sequence had to be added. I carried out PCR using the 5'HindIIIshort-F and DAT880R because this chimera begins with SERT but the remainder is DAT. The PCR reaction was gel purified and the short sequence was extracted. Both the existing SERT(1-78)/DAT(60-620) pcDNA and 5' HindIII short end were digested with HindIII and PflMI, removing the existing 5'end from the chimera DNA and preparing the short end for ligation. The PCR products were purified using the QIAquick PCR Purification Kit and the SERT(1-78)/DAT(60-620) insert in pcDNA was gel purified and extracted. The 5' HindIII short end was ligated with the chimera and the completed sequence was transformed into DH5α e. coli. The DNA was extracted from the bacteria and analytically digested and run on an agarose gel to see if the insert had successfully ligated. Once it was determined, the DNA was sequenced to make the official confirmation and check for mutations. Using HindIII and XbaI, I digested both the pCFP-C1 vector and SERT(1-78)/DAT(60-620) in pcDNA, to prepare the vector and insert for ligation. I then gel purified the insert and vector, and ligated them together. DH5α were transformed with the DNA and the plasmid DNA was extracted; the DNA was digested with HindIII to screen for the

insert; a 2kb increase in size would indicate the insert had ligated. When the insert was determined to have successfully ligated, the DNA was sequenced. The methods used are illustrated in Figure 8.

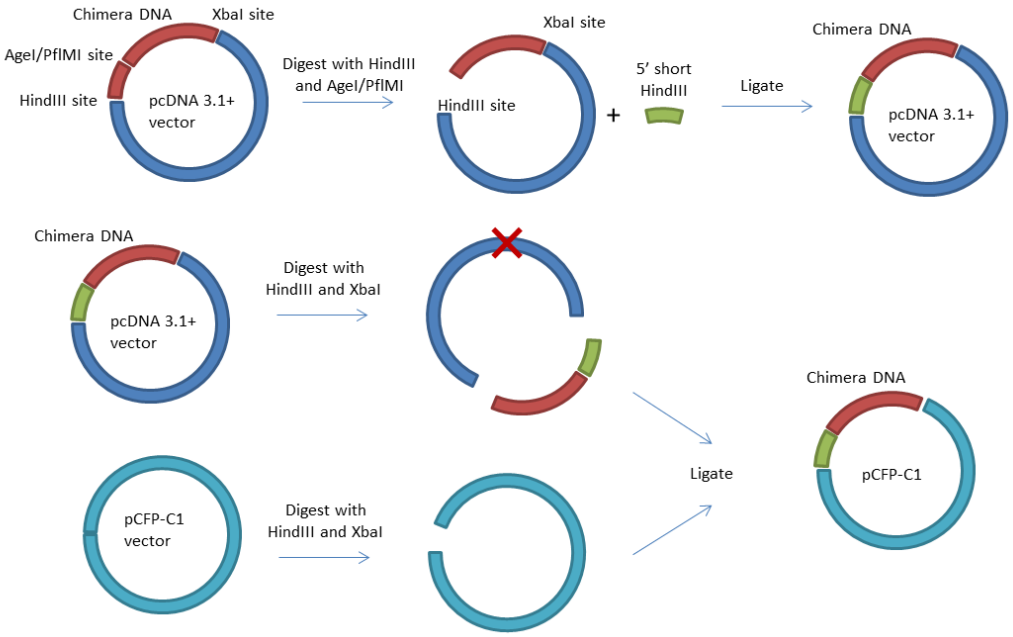

Figure 8: Method for hSERT and SERT(1-78)/DAT(60-620)

DAT(1-59)/SERT(79-630) in pCFP-C1 vector

The DAT(1-59)/SERT chimera had already had a 5' short HindIII site added, so I was able to skip many of the steps I had to do with the other chimeras. Using HindIII and XbaI, I digested both the pCFP-C1 vector and DAT(1-59)/SERT(79-630) in pcDNA, to prepare the vector and insert for ligation. I then gel purified the insert and vector, and ligated them together. DH5α were transformed with the DNA and the plasmid DNA was extracted; the DNA was digested with HindIII and Age-HF to screen for the insert. When the insert was determined to have successfully ligated, the DNA was sequenced. The methods used are illustrated in Figure 9.

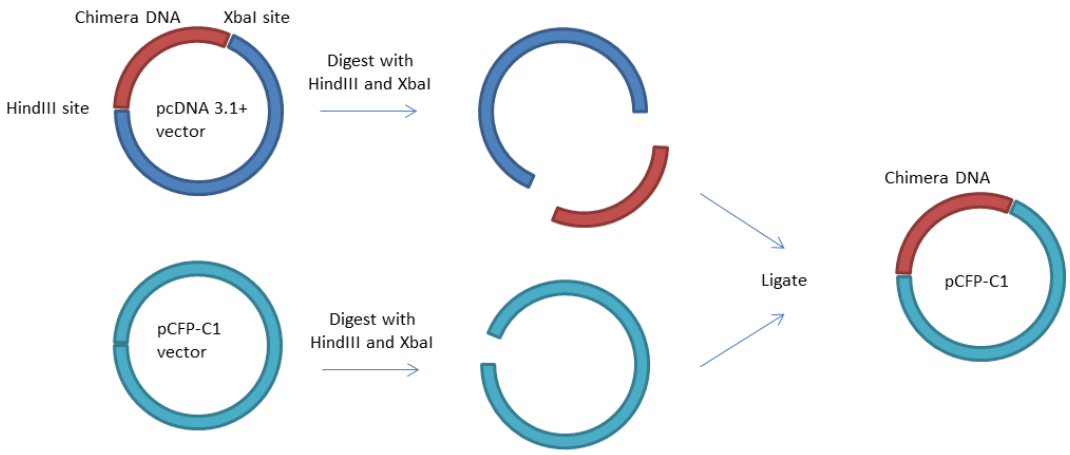

Figure 9: Method for DAT(1-59)/SERT(79-630)

Discussion

My experimentation began with testing the wild type dopamine and serotonin transporter's response to amphetamine. The first experiments did not come out ideal, however I was able to get a few that I was able to determine from that DAT surface levels decrease and SERT levels increase when treated with amphetamine. I then moved on to testing the chimeras and ran into problems. After a month of experimentation and poor results, I began suspecting certain points in the experimental process could be at fault. For a few weeks my nitrocellulose blots were blank, so I thought maybe the DNA I had been using had become denatured by repetitive freezing and thawing; therefore it was not transfecting into the cells. I ran an agarose gel to test the coiling of the DNA, the gel showed streaks of DNA running through the gel at a higher molecular weight than expected, indicating that the DNA could be partially uncoiled. This uncoiling of DNA makes it more difficult to enter the cell and therefore could make a transfection unsuccessful. At this point I performed a maxi prep to make new DNA and ran that DNA on another agarose gel to make sure it was properly coiled, which it was.

Blank blots could also point to a problem with the reactivity of the antibodies, so using the new DNA, I simply ran the transfected cells on a western blot skipping the biotinylation step, and the blot had the expected bands. The antibodies did not appear to be a problem.

The next few experiments I attempted to run were cut short due to low protein concentration; I did not have enough protein to execute the biotinylation. I thought the poly-D-lysine may have become ineffective at adhering the cells to the plate, so I made a new stock and that solved the protein concentration problem.

I began to get some signal from the protein however of the two comparative bead and total columns, the bead column was blank and therefore I had nothing to compare or calculate. After another blank blot, I thought I may not be transfecting the cells correctly, so my PI and I transfected a well of cells each and when I immunoblotted them, they came out the same, so I determined that the transfection was not the problem.

Even though each step seemed to be working, the experiment as a whole was still unproductive. Looking back at my notes, I made a new RIPA buffer around the time things began to go wrong. This buffer lyses the cells to release the internalized transporter, and it was thought that maybe the buffer was not made correctly and therefore was somehow affecting the transporter. But when repeating the experiment using a known working RIPA, it did not change the results.

I determined that each individual portion of my experiment worked, however when I ran it in full, it did not. The one step that was different from all of the troubleshooting experiments was the biotinylation. I may have not had the most skilled technique when it came to biotinylation. I can only hypothesize that this was the problem; I was not able to determine the true error in my experiment. For the sake of my efforts and time, I decided to move my project in another direction.

Instead of trying to find the answer to another question, I made chimera constructs for a future experiment that would help further the research with DAT's response to AMPH. The DAT/SERT chimeras that were made required extensive research into different restriction enzymes and looking at past sequencing reports from my lab. Making the constructs was a lot of trial and error, if the bacteria didn't successfully transform or the DNA didn't ligate correctly, I re-ran the reaction, though I had good luck with the constructs. I rarely had to repeat ligations, the DAT(1-59)/SERT(79-630) construct did give me more difficulty than the rest however, it required two repeated ligations and picking many colonies to find the bacteria that had been properly transformed.

Conclusion

Through this experiment, I have gained many valuable skills as well as knowledge about the experimental processes. While I was not able to answer the hypothesis I set out to discover, I have learned just how unpredictable molecular biology can be. When things don't appear to be working, there is usually a reason which needs to be addressed, there is no use in repeating the same thing if it didn't work the first few times. However, because we do not have a perfect understanding of what is happening at the cellular level, we cannot always understand why things do not work.

Making the chimeric constructs in the pCFP-C1 vectors was helpful for future experiments which will use fluorescence resonance energy transfer to research protein interactions. These experiments will hopefully shed some light on the protein interactions needed for this trafficking in response to amphetamine.

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