HIGH AFFINITY LIGANDS FOR THE TARGETING OF SERT AND NET

By

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure

CHAPTER I

INTRODUCTION

1.1 The Mental Health Burden

Neurotransmitters such as serotonin and norepinephrine are important in regulating almost all aspects of human behavior. Incorrect regulation of these neurotransmitters can lead to a variety of mental disorders, many of which are significantly detrimental to the person's general functioning and quality of life. The most common of these include depression, anxiety, autism, and ADHD. According to the NIMH in 2005, there was a lifetime prevalence of 20.8% of the US population that will suffer from a mood disorder, 28.8% that will suffer from an anxiety disorder, 8.1% that will suffer from ADHD, and 1 in 110 children born suffer from some form of autism. In 2006, the NIMH estimated that $57.5 billion was spent by both the healthcare system and individuals on the treatment of mental illness, with 36% of the population seeking treatment. According to their statistics, only 50% or less of the people suffering from the disorders specified received pharmaceutical treatment[1]. It is clear that these disorders are significant burden on the overall and financial wellbeing of our society.

Research on these neurotransmitters has become increasingly prevalent since advent of antidepressant and antianxiety medications, but it is still unknown how exactly this problem with regulation is connected to disease. The diagnosis of these diseases is mainly done through interview and questionnaires developed through years of psychiatric theory. A patient will typically be suffering from their symptoms for months or even years before being considered for treatment. Pharmaceutical treatment is determined

largely by trial and error, sometimes taking months for a patient to find the correct medication and dosage to bring them relief. This process is extremely time consuming, and the cost to both individuals and the health care system increases every year. Studying the behavior of the transporter proteins of these nuerotransmitters on the single molecule level can help to understand the mechanism of disease, develop better pharmaceuticals, and make diagnosis and treatment more accurate and individualized.

1.2 Serotonin and Norepinephrine

The body uses serotonin to regulate learning, mood, memory, sexuality and sleep among other functions. Serotonin transporter protein (SERT) is a 12 domain, transmembrane protein responsible for the clearance of excess serotonin from the synapse after it is released. Serotonin is taken back into the neuron and stored in vesicles until it is released again[2]. SERT malfunction has been linked to many psychological disorders including depression, autism, anxiety and obsessive compulsive disorder[3]. Depression alone is one of the leading causes of disability in the world (WHO)[4], and autism is the fastest growing cause of learning disability in the US (TACA)[4, 5]. Several mutations for SERT have been associated with certain of these diseases, and several cellular components have been shown to affect SERT activity and trafficking[3].

Norepinephrine and its transporter work very similarly to serotonin and SERT. NET protein is almost identical in structure, and not only transports norepinephrine but also dopamine and epinephrine. These all have similar effects as serotonin, but they are also responsible for activating the attention centers of the brain, cognitive ability and

movement. Norepinephrine and epinephrine control the body's "fight or flight" response, and figure heavily in the body's response to pain and pleasure[6]. Since it transports several monoamines, malfunction of the norepinephrine transporter contributes to disorders caused by disregulation of dopamine and norepinephrine. These also include depression, learning disorders, ADHD, schizophrenia, and hypotension[7]. The function of NET and SERT effect the regulation of many monoamines, not just serotonin and norepinephrine, and understanding both along with dopamine transporter is crucial in developing a picture of the whole system.

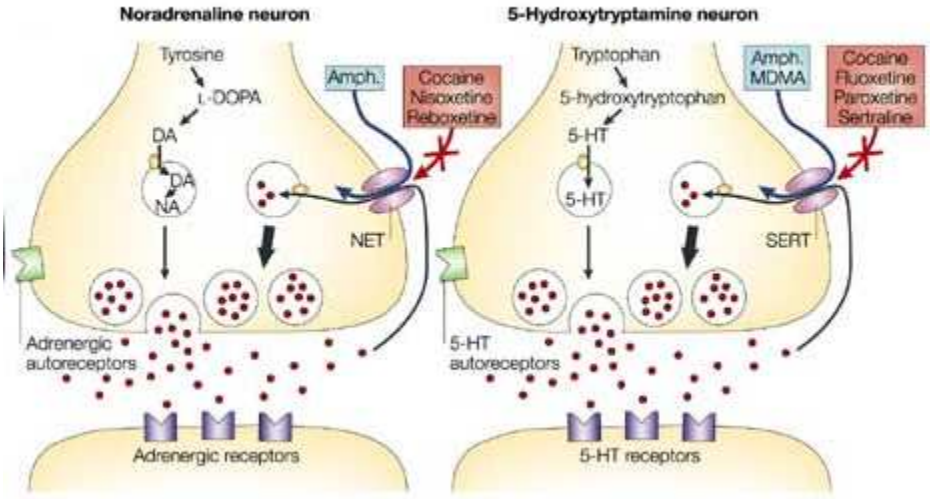


Figure 1.1: Norepinephrine and serotonin in the synapse[8]

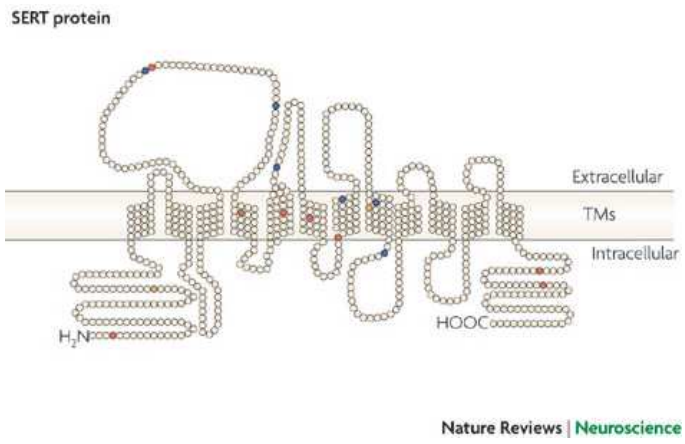
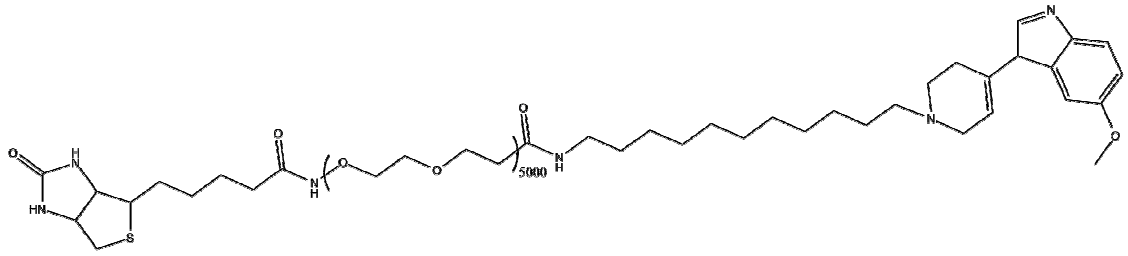


Figure 1.2: SERT protein model[9]

1.3 Targeting SERT and NET

As has already been mentioned, the transporter proteins convey several different monoamines, but they can also bind drugs and other small molecules. This has been a key point in the approach used in the Rosenthal group to study these proteins. Amphetamines, cocaine, some hallucinogenic drugs, and antidepressants all bind to and have various activating or inhibiting effects on these transporter proteins and have all been considered as models for small molecule targets for the ligands used in these experiments. Ian Tomlinson has developed several targeting ligands for monoamine transporters, two of which are currently used to label SERT in cells, IDT318 and IDT361. They both use a high affinity serotonin derivative to target SERT. This small molecule is attached to a PEG5000 chain by an 11 carbon alkyl spacer. The alkyl spacer provides enough distance between the membrane and hydrophilic PEG chain to allow the target to bind to the SERT. Biotin is bound to the other end of the PEG chain to complete the ligand. The IC_{50} of IDT318 is 3.4uM and the IC_{50} of 361 is 0.034uM[10].

A



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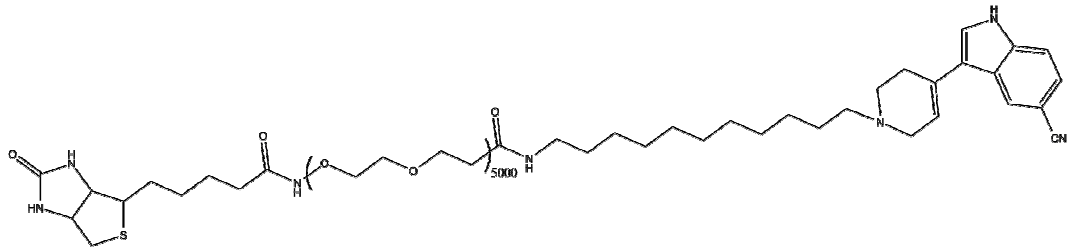

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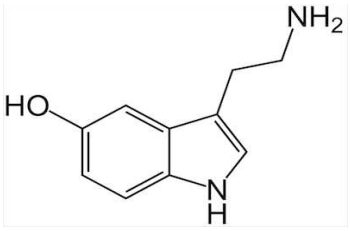


Figure 1.3: (A) IDT318 complete with (left to right) biotin attached to PEG5000 attached to the alkyl spacer with the targeting ligand. (B) IDT361 (C) Serotonin (5-Hydroxytrytophan)

There are several NET targeting ligands Tomlinson has synthesized as well, although thorough testing has not yet been performed. IDT 465 is a talopram derivative, IDT 588 is a tropane derivative, and IDT 545 and 488 are atomoxetine derivatives (See Figure 1.4). All of them can serve as norepinephrine reuptake inhibitors. Screening is

done by first incubating with varying concentrations of the ligand for 30 minutes, then incubating with 10uM IDT307 and imaging. The fluorescence intensity of individual cells can be quantitatively compared to establish the degree of uptake for each concentration of the ligand. After an initial range of effective concentrations is established, they can be tested for labeling.

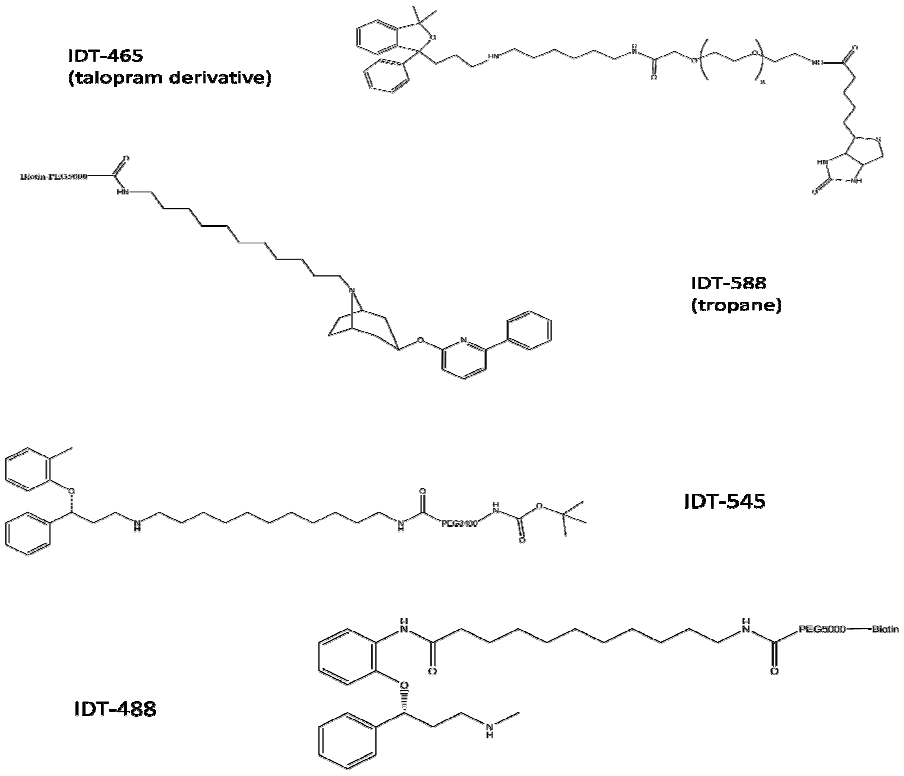


Figure 1.4

1.4 Semiconductor Nanocrystals

Semiconductor nanocrystals or quantum dots (QDs) developed for use in a biological setting have been used since 1998[11]. QDs are ideal for biological application for a number of reasons. Their small size (4-20nm) makes them easy to use in biological

and cellular systems. Due to quantum confinement, they have a small, size dependent fluorescent emission. Being inorganic in composition, they are far less susceptible to photodegradation than organic fluorophores, allowing them to be viewed for much longer periods of time. They have a large molar extinction coefficient and high quantum yields, which gives them an intense fluorescent signal. All of these properties allow for even a single quantum dot to be a powerful tool for observation. QDs also have the unique property of fluorescence intermittency or blinking that allow the observer to ascertain that a single quantum dot is providing the signal, as opposed to an aggregate of dots. This property is especially important in the case of single molecule tracking[12].

Other methods used to study SERT behavior suffer from poor spatial resolution, difficult preparation, high cost, or low photostability. Organic fluorophores are easily obtained, but they photobleach in a matter of seconds. Radiolabled ligands are expensive, require special handling, and also have poor spatial resolution. Antibodies are highly specific in most cases, but they are bulky, expensive, difficult to prepare, and do not target SERT well. Patch clamp measurements give information on the activity of SERT, but do not give positional information[13].

The quantum dots used for these experiments are purchased from Invitrogen. Their peak emission wavelength is 655nm (See Figure 1.5). They are composed of a Cd/Se core with a Zn/S shell. Since the passivating surface ligands present after synthesis are nonpolar and therefore insoluble in water, an ampiphilic polymer interacts with the non polar surface ligands and is crosslinked with itself . The polymer has reactive groups on the outer surface allowing for easy conjugation of targeting moieties (See Figure 1.6). This allows proteins, DNA, peptides, antibodies, and small molecules to be attached to

the surface of the QD. The QDs used are carboxylic acid terminated to allow streptavidin, a protein with high affinity binding to biotin, to be conjugated to the surface. This strong interaction is utilized to bind the quantum dot to a targeting ligand[12].

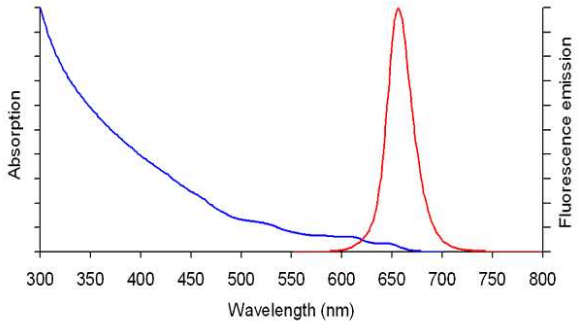


Figure 1.5: Absorption and emission spectrum of QD655 from Invitrogen

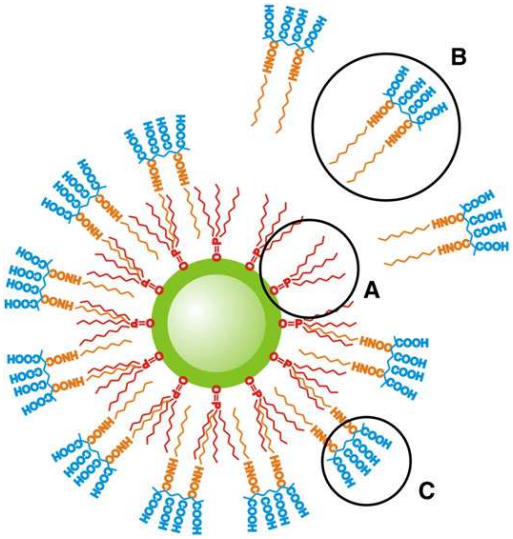


Figure 1.6: Diagram of Quantum Dot. (A) Organic passivating ligands (B) Ampiphilic polymer (C) Carboxylic Acid groups[12]

1.5 The Development of our Labeling Process

The specificity of IDT318 has been well characterized by previous experiments performed by Michael Warnement and Jerry Chang. The ligand was originally tested in an oocyte model. Oocytes are large, easily imaged, and have extremely high expression levels of the protein it is transfected with. Oocytes were injected with a cRNA vector and incubated for several days before testing. The IC_{50} value was determined by incubating the oocyte with IDT318 and tritiated serotonin. The concentration of IDT318 was gradually increased, and the concentration was plotted against the accumulated radioactivity. Patch clamp methods were used confirm inhibition of SERT activity by measuring the difference in the influx voltage when the oocyte was treated with 318 and serotonin and just with serotonin. The current was 80% lower when treated with 318, even after the excess 318 was removed from solution, indicating that the ligand remained bound. Imaging using confocal microscopy was used to determine if the SERTs could be labeled successfully. The oocytes were first incubated with the ligand for an hour, and then incubated with the streptavidin conjugated quantum dots separately for 5 minutes. Nonexpressing oocytes were treated with the quantum dots to determine the degree of nonspecific binding to the oocyte membrane. The SERT transfected oocytes were also imaged with just the quantum dots to determine the degree of nonspecific binding with SERT. SERT transfected oocytes blocked with paroxetine, a high affinity antidepressant that targets SERT, were treated with 318 and the quantum dots to establish that SERT was successfully expressed and labeled. Though all of them show some labeling, the labeling in the SERT transfected oocytes treated with 318 and QD655 is significantly greater than in the controls[14].

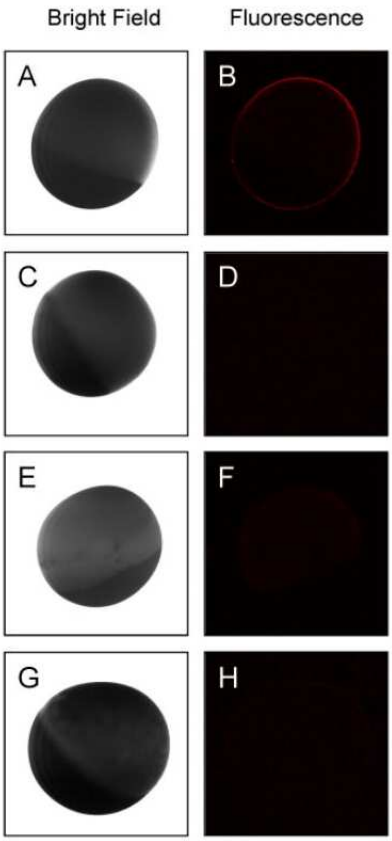


Figure 1.7: Bright field and fluorescent images of the oocytes (A-B) SERT transfected oocyte treated with IDT318 and Streptavidin QD's, (C-D) SERT transfected with just QD's, (E-F) Non-expressing oocyte treated with IDT318 and QD's, and (G-H) SERT transfected and treated with paroxetine prior to IDT318 and QD's[14]

IDT318 was further studied in Hek-293T cells, as they can be easily transiently transfected and lifted from the culture vessel for imaging in suspension. The cells were transfected using a pc3DNA vector and Lipofectamine-2000, a transfection reagent purchased from Invitrogen. A "sham" pc3DNA vector was used as a control to account for effects from the transfection process. IDT307, a small molecule developed in the Rosenthal lab that fluoresces when taken up by monoamine transporters, was used to quickly and easily determine the success of the transfection. The paroxetine block was

also used to establish the expression of SERT since IDT307 can be taken up by several different transporters. The same two step labeling process was used to label SERT in Hek293T cells. The cells were incubated with the ligand, and trypsin was used to detach the cells from the plate before centrifugation and incubation with the quantum dots. SERT specific labeling was observed. Flow cytometry was used to confirm that a SERT labeled population existed that was significantly higher than in sham transfected cells, paroxetine blocked SERT transfected cells, and SERT transfected cells only incubated with the quantum dots. Flow cytometry was also used to determine an optimal incubation time and 318 concentration, ideal alkyl spacer length on the ligand, and specificity of the ligand for SERT over dopamine transporter[15]. The IC_{50} value of IDT361 was determined by patch clamp methods, but information on the the specificty and labeling ability has not been published.

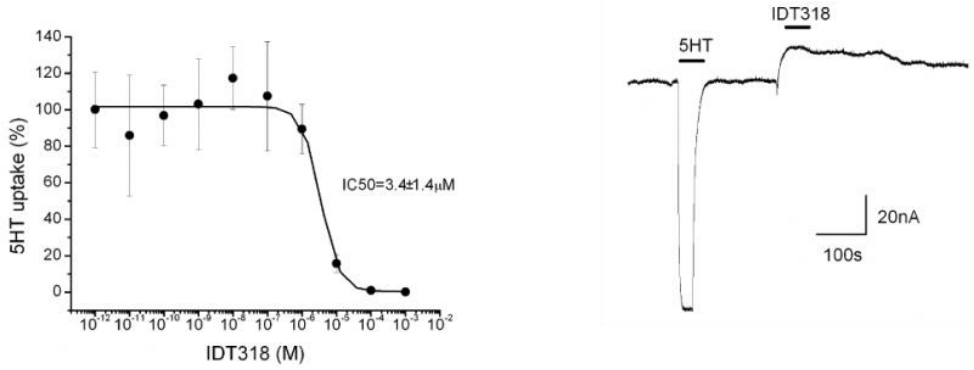


Figure 1.8: IC50 value of IDT318 determined by serotonin uptake and leak[14] current

The purpose of the experiments performed as part of this project was to establish labeling ability of new ligands to target SERT and NET. IDT361 and IDT545 were both tested in Hek293T cells. Since flow cytometery experiments have already been

performed to establish the specificity of IDT361 to SERT, the next step was to show labeling in live cells. Since NET has not been studied in the Rosenthal lab much thus far, the successful amplification of the plasmid and optimum transfection conditions needed to be established before experiments on cells could be done. Then, the initial experiments to show specificity and activity were performed.

CHAPTER II

EXPERIMENTAL

2.1 Cell Maintenance

Cells were maintained according the recommendations of the ATCC. All cells are maintained in a sterile 5% carbon dioxide environment at 32°C. Standard aseptic techniques were followed for all cell maintenance protocols. All cells were obtained as a gift from the Dr. Randy Blakely lab (Vanderbilt University). Hek-293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, 1.0 mM sodium pyruvate and 2 mM L-glutamine with 10% fetal bovine serum and 100ug/mL penicillin and streptomycin. HeLa cells were also maintained in the same way. Cells were passaged after reaching 90% or more confluence. Once the cells reached the desired confluence, the media was removed and a trypsin/EDTA solution (Gibco) was added to induce cell detachment at 37 °C. After the cells detach from the flask, a three fold excess of complete media was then added to neutralize trypsin activity, and the cells were aliquoted to a new flask with an appropriate volume of complete media.

2.2 Plasmid Preparation and Cell Transfection

Transfection was used to induce SERT and NET production in Hek-293T cells and HeLa cells. The plasmid DNA encoding for SERT in a pcDNA3.1 vector was originally obtained as a gift from the Blakely lab. Competent DH5α *E. coli* cells incubated with 1 μL of plasmid DNA, and then heated at 42°C for 30 seconds to induce

the bacteria to take up exogenous plasmid. These heat shocked cultures were subsequently streaked onto LB-ampicillin agar plates and stored overnight at 37°C. The pcDNA3.1 vector has an ampicillin resistance coded into it, so only those *E. coli* cells which have taken up plasmid will be able to survive in the presence of ampicillin. A single colony was picked from the plate, and deposited into 125 mL of LB medium containing ampicillin. These cultures were then incubated overnight at 37°C with vigorous shaking. Afterwards, the plasmid DNA was purified using a plasmid midi kit (Qiagen). An alkaline lysis buffer is used to extract plasmid from bacteria and a series of buffers, purification columns and centrifugation are used to complete purification. Spectroscopic determination of plasmid concentration was then performed using a NanoDrop spectrophotometer. The plasmid was stored in Tris-EDTA buffer at 4°C before use.

Transient transfection was used for SERT production in cells because it typically causes higher expression levels in cells than in stably transfected cells. Lipofectamine 2000 (Invitrogen) is used as the transfection agent, and the suggested protocol for this reagent from the manufacturer was followed. Cells are incubated with 0.8ug plasmid and 2uL Lipofectamine for 24 to 48 hours before use. Transfection efficiency was tested by use of IDT307, a compound that, when transported by monoamine transporters, fluoresces green. Transfections were performed in a 24 well plate format. 0.8 μg plasmid DNA and 2 μL Lipofectamine 2000 were added to each well, according to manufacturer recommendations. Control cells are transfected with an empty pcDNA3.1 vector termed "sham" to eliminate any potential experimental interference resulting from exposure to transfection reagents. Transfections were performed at a confluence of ~90% as the

Lipofectamine 2000 is cytotoxic, and is detrimental to the cell population below this level of confluence.

IDT307 was originally developed in the Rosenthal lab. It is a small molecule selectively transported by monoamine transporters, and only fluoresces inside the cell. IDT307 can be used to assess transporter activity. IDT307 can be added directly to media of the cell culture at 10uM concentration incubated at 37°C for five to ten minutes for successful imaging. A standard GFP filter set can be used to image uptake of IDT307 confirming successful transporter expression. The media is removed and replaced with room temperature PBS just before imaging to ensure no background fluorescence from FBS.

2.3 Transporter Labeling

Transporter labeling is accomplished using a two step process. The cells are cultured in a 24-well plate, and transfected as described. The original media is removed to eliminate background from serum proteins that will bind to the quantum dot. The cells are incubated in phenol free media or PBS with the ligand for an hour. The cells detach from the plate when the PBS is added. After an hour, the cells are transferred to microfuge tubes and a 0.5nM QD655Sav (Invitrogen) solution in PBS is added, and they incubated on ice for 5 minutes. The cells are centrifuged for 5 minutes and resuspended cold cell stripper (Cellgro). They are centrifuged and respended until the desired number of washes have been completed. After the final wash, 50uL of the cell suspension is placed on a glass coverslip for imaging. The cells are washed and imaged in suspension

because it greatly reduces background from unbound quantum dots, and quantum that will adhere to the surface of the culture plate.

Images were obtained using the Zeiss Axiovert 200 Epifluroescent Microscope. The system is located in our lab and is equipped with a mercury vapor lamp as a light source. The objectives used for included images are 20x, 40x, and 100x. The microscope is used in conjunction with Metamorph image acquisition software. The GFP filter set (512nm emission) is used for IDT307 imaging. The QD655 filter set (655nm emission) is used to image labeling of SERT. Imaging is done either directly in the culture plate, or the cells are suspended in buffer and imaged on a glass slide. Imaging is done at room temperature.

2.4 Ligand Screening

The cells were grown up in the desired size plate, usually a 24 wells plate. They were transfected with the appropriate plasmid for the type of targeting ligand that was screened. Screening is done by first incubating with varying concentrations of the ligand for 30 minutes, then incubating with 10uM IDT307 and imaging. The fluorescence intensity of individual cells can be quantitatively compared to establish the degree of uptake for each concentration of the ligand. After an initial range of effective concentrations is established, they can be tested for labeling.

CHAPTER III

RESULTS AND DISSCUSSIONS

3.1 Transfection Efficiency

Initial work was done with HeLa cells, a cell line derived from a cervical cancer sample and now widely used in research. HeLa cells are robust, fast growing epithelial cells that adhere well to plastic plates and flasks. They are easily washed without detaching from the culture surface and are not easily damaged. For these reasons, they are good for labeling experiments but display a greater degree of nonspecific binding with IDT307, which makes them unattractive for uptake and screening assays. Below are images taken with HeLa cells of IDT307 uptake of SERT transfected cells to confirm the expression of the SERT protein. It can be seen that even after adjusting for the background signal, there is a much greater degree of internalization of IDT307 in the SERT transfected cell.

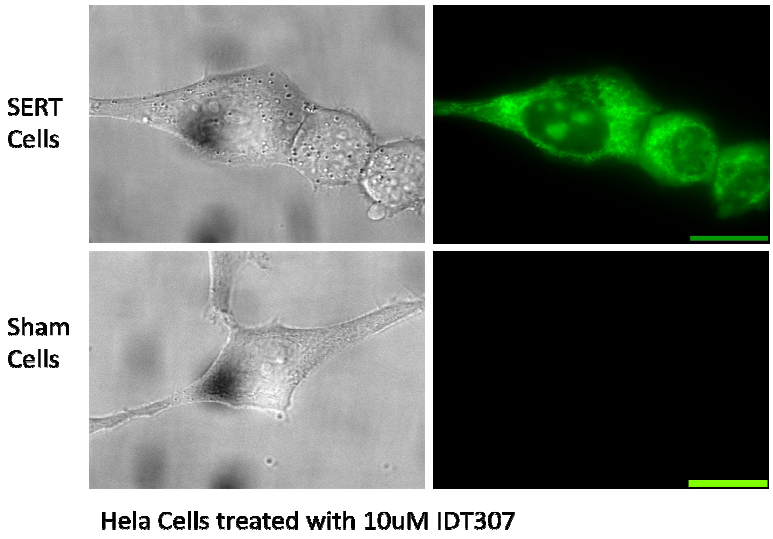
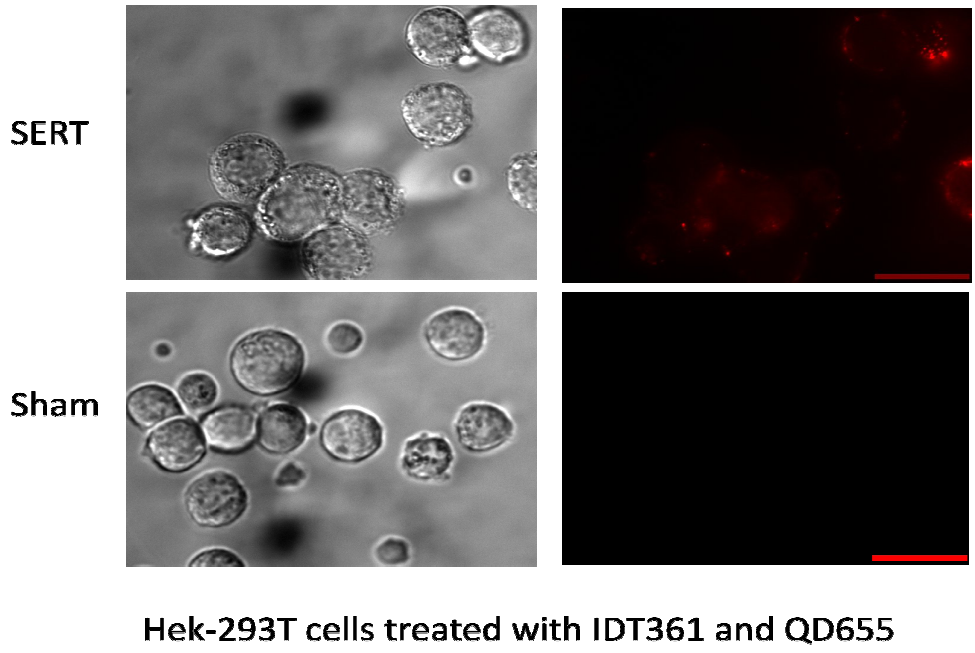


Figure 3.1: Scale bar=20µm

3.2 Ligand Screening

For screening both SERT and NET ligands, Hek-293T cells, human embryonic kidney cells, were used because they are easily transfected and display a lesser degree of nonspecific binding. Hek-293Ts do not show as much background with IDT307, but detach from the plate readily, so washing must be done with care. Even slight changes in temperature can cause them to detach from the plate, so imaging must be done carefully and quickly. Attempts to synthesize more IDT318 have been unsuccessful recently, so testing for a new SERT targeting ligand began with IDT361. It was seen in previous experiments that 361 had an even higher affinity for SERT than IDT318 does. This makes it particularly attractive for single molecule tracking studies. Shown in Figure 11 are HeK293T cells transfected with SERT and a sham plasmid and incubated with IDT361 and QD655. As can be seen in the images, the SERT transfected cells are clearly labeled with quantum dots, whereas the sham transfected cells do not show any labeling above the background.

(A)



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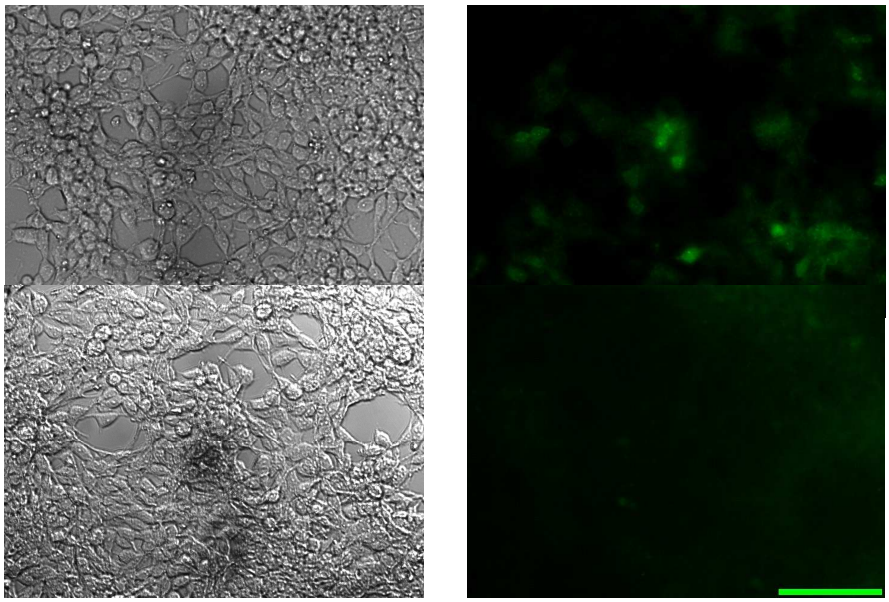

Figure 3.2: Scale bar=20µm, (A) SERT and Sham transfected cells treated with 361 and QD 655, (B) SERT and Sham transfected cells treated with IDT307

3.3 Ideal Transfection Conditions for NET Expression

Ideal transfection conditions for NET expression were tested according to Invitrogen's protocol. Varying volumes of Lipofectamine 2000 and 0.8ug of plasmid DNA were incubated with the cells. IDT307 uptake was used to determine the success of the transfection. Cells were incubated with 10uM IDT307 in PBS for 10 minutes and then imaged. Sham transfected cells were used as a control. As can be seen in Figure 12, the uptake of IDT307 increases with increasing amounts of Lipofectamine, but significant cell damage occurred when more than 4uL was used during transfection.

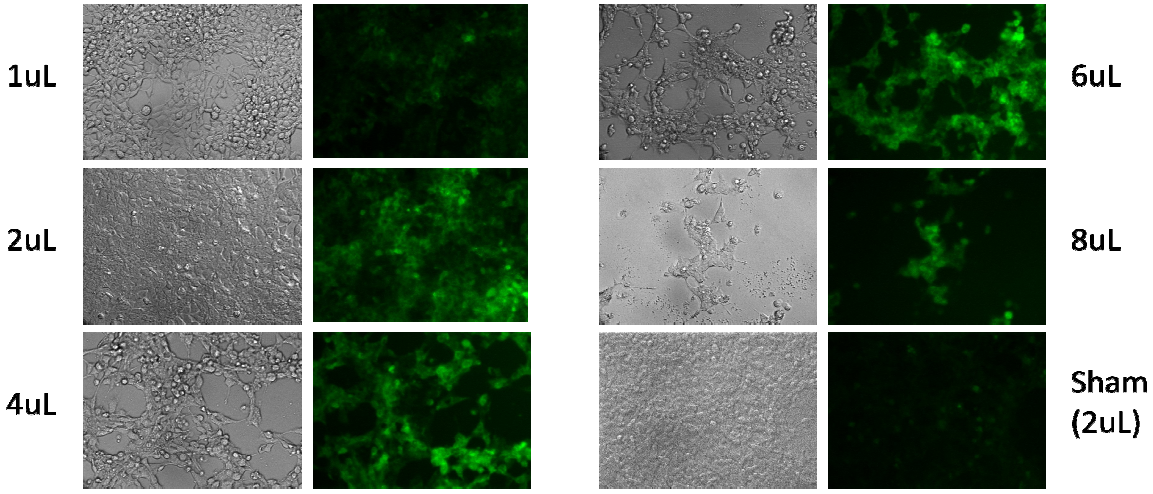

Figure 3.3 NET transfection conditions

3.4 Testing NET Ligand IDT545

To establish the potential of IDT545 to be used for targeting and eventually labeling NET with QD's, Hek293T cells were grown up in a 24 well plate and transfected with NET and sham plasmid. After 24 hours, the cells were incubated with various concentrations of IDT545 for 20 minutes. The concentrations used were chosen because an effective concentration of less (preferably much less) than 10uM is ideal for labeling. The cells were then treated with 10uM IDT307 for 10 minutes. The cell media was removed and replaced with PBS just before imaging. In the images, the ligand does not seem to block the uptake of IDT307 appreciably at any of the concentrations used. Therefore, it is unlikely that this ligand would be used for further study.

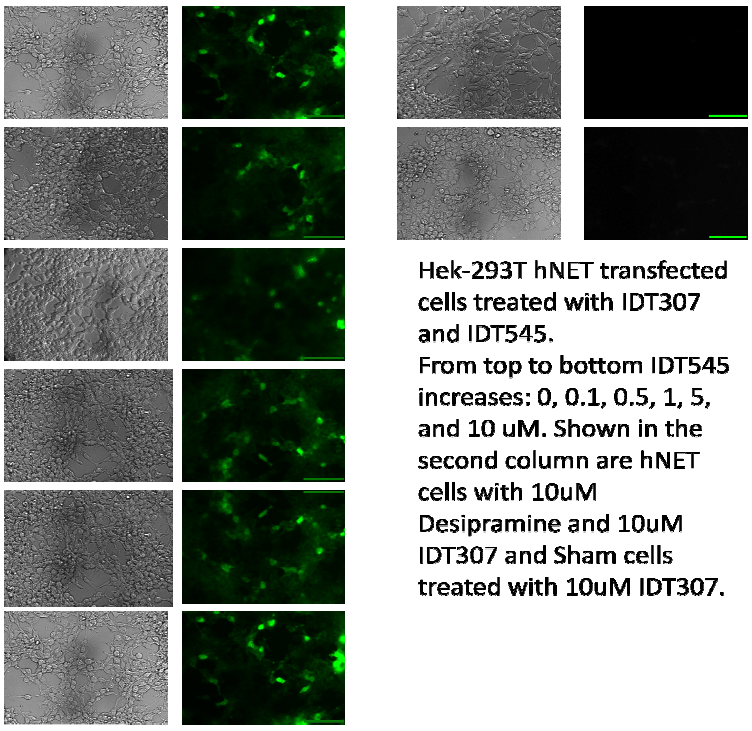

Figure 3.4: IDT545 activity Scale bar=105µm

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Single Molecule Tracking

Single molecule tracking with quantum dots began with the Dahan group in 2003. Quantum dots were used to label glycine receptors in neurons. Using the mean squared displacement and comparing diffusion coefficients of the particles, they were able to distinguish two populations of receptors—ones that were freely moving and ones that were confined to specific areas[16]. In the Rosenthal lab, the statistical analysis has been combined with our labeling process to characterize the behavior of SERT and DAT. The original tracking data is obtained using a process called FIONA, or fluorescence imaging with one nanometer accuracy. It utilizes a point squared function, a two dimensional Gaussian curve comparing the fluorescence intensity on the x axis and the intensity on the y axis. Because of the diffraction limit, objects observed in light microscopy cannot be seen any sharper than 250nm. By using this function, the exact center of the particle (the centroid of the PSF) can be determined on the subpixel scale. In confocal microscopy using a 63x objective, a pixel is $200nm^2$, much larger than SERT or a QD. The use of the PSF not only allows one to determine the position of the particle at the 1nm scale, but causes of noise such as pixilation, camera readout, and fluorescent background are minimized by collecting more photons from a signal. This makes QDs perfect for this type of analysis due to their brightness. PSF measurements are only accurate if it is symmetrical. Spherical aberration and defocusing can cause the center to change up to 50nm[17]. Using single quantum dot tracking, SERT behavior can be quickly

and easily visualized and followed in real time, providing a fluorescent technique that can be used to obtain positional data with high spatial resolution, high, robust signal, and specific targeting.

Jerry Chang has already done an extensive study of the movement and behavior of endogenous SERTs in rat neurons, RN46A[13]. His work has confirmed that SERT is localized in cholesterol rich, lipid rafts by using Alexa Fluor 488-conjugated cholera toxin subunit B, which binds to ganglioside GM1 which has been shown to be in these lipid rafts.[14] The cells showed co-localizion of the CTxB and the QD labeled SERTs. This association was further explored by treating the cells with methyl-ß-cyclodextrin, a cholesterol chelator. After treatment, the CTxB and the SERTs were dispersed. Comparing the tracking data, the instantaneous velocity of the SERTs in the MßCD treated cells was considerably higher than in the control cells. A plot of the log of the mean squared displacement vs. log time indicated that SERTs in the control cells have a confined lateral diffusion whereas in the MßCD treated cells, SERTs display free diffusion. A histogram of the individual diffusion coefficients indicated a single population of SERTs for control cells (as they are all confined), and two populations for MßCD treated cells, one large, fast population, and one small, slower population consistent with the control cells. Protein kinase G phosphorylates SERT after activation by cyclic guanosine monophosphate (cGMP). This has been shown to increase movement and serotonin uptake[18]. 8-bromo-cGMP was used to activate PKG in RN46A cells, and the mean instantaneous velocity of the SERTs increased significantly. The distribution of the individual diffusion coefficients indicated that there were more SERTs with high diffusion compared to the control. The log-log plots indicated that the SERTs still

showed confined lateral movement. PKG activation causes mobilization of intracellular SERTs to the surface of the cell, which may account for the increase in mobility of labeled SERTs[13]. It has also been shown that p38 mitogen-activated protein kinase activation triggered by PKG also causes intracellular SERTs to rise to the surface[18]. To determine whether the increase in SERT trafficking was due to p38 MAPK activation or PKG activation alone, the cells were treated with 8-Br-cGMP and a p38 MAPK inhibitor, SB203580. This significantly decreased the increase in SERT instantaneous velocity from the 8-Br-cGMP treatment. The displacements of the individual SERTs can be better visualized by looking at the lateral trajectories over a 5 second period. These plots from the control, MßCD, and 8-Br-cGMP treatments were compared. These give a clearer picture of the degree of confinement of the SERTs. Control cells showed very little lateral displacement, the MßCD treated cells had very dispersed SERTs, and the 8-Br-cGMP treated cell's SERTs showed similar displacement to the control cells. RN46A cells were treated with an inflammatory cytokine, IL-1ß, to determine the effect of p38 MAPK activation independent of PGK activation on SERTs. The results were similar to those obtained by treating the cells with 8-Br-cGMP. An actin filament disruptor, cytochalasin D, was used to disrupt the cytoskeleton of the cells, which could restrict movement within nearby lipid rafts. While two populations of SERTs were observed in the diffusion rates like with 8-Br-cGMP and IL-1ß treatments, an overall increase in the diffusion rate occurred in both populations. The instantaneous velocity of the lipid rafts themselves also increased, but the SERTs remained co-localized in the rafts. IL-1ß treatment with CytoD did not increase the velocity further, but MßCD treatment with CytoD did. This indicates that the IL-1ß activation of the SERTs is a result of the release

of the transporter from the cytoskeleton, but not the lipid raft[13]. It has been shown that SERT associates with integrins and integrin associated proteins through its C-terminal domain inside the cell. These proteins facilitate interaction between the SERT and the cytoskeleton[18]. To determine whether these interactions could account for restricted motion of the SERTs, the cells were treated with TAT-C-terminal SERT peptides. The results were similar to IL-1ß treatment, and importantly, the treatment caused an increase in SERT activity. This was determined by comparing the uptake of IDT307 in control cells and in C-SERT peptide treated cells. Time lapse images show that uptake of IDT318 in C-SERT peptide treated cells is significantly higher, and increases over a 30 minute period. This indicates that SERT activity is increased when untethered to the cytoskeleton despite still being confined to the lipid raft[13].

However, all of these experiments were done with IDT318, which has not working for the past year. In the future, studies with the RN46A rat neuron cells used for previous SERT studies will be done using IDT361 to establish its transferability. The advantage of using neuron cells is that it is a system that endogenously produces SERT so it can be studied in one of its most natural environments short of actual in vivo studies. However, this system expressed rat SERT, not human SERT, which is not vastly different, but still not the same. The screening process described for IDT318 will also need to be repeated for IDT361 to confirm it's appropriateness for these studies. Hek293T cells will also be used to study IDT361's relationship with SERT.

4.2 SERT Mutations

Our collaborators in the Blakely lab have stably transfected Chinese Hamster Ovary cells with a SERT mutant associated with autism, G56A. This mutation, associated with increased SERT activity, transmits 3:1 to people with autism (to males preferentially) and is also associated with sensory aversion and rigid compulsive traits in these cases. This mutation is located near the N terminus of the SERT (See Figure 14). This variant of SERT has shown no response to PKG and MAPK activation, which usually increases SERT activity. It also increases sensitivity to PKC compared to wild type SERT, which increases activity more than it should. It also shows insensitivity to Ser/Thr protein phosphatase 2A. When this phosphatase is inhibited, SERT is phosphorylated, and its activity is reduced[3]. Using single molecule tracking of these mutated SERTs, the effect of this mutation and these associations could be explained by observing the movement of the protein. The activity could be easily observed under these and other conditions known to effect SERT activity, which could reveal biomarkers that could be used for diagnosis or treatment. Profiling this mutation and others could also lead to the development of gene therapy.

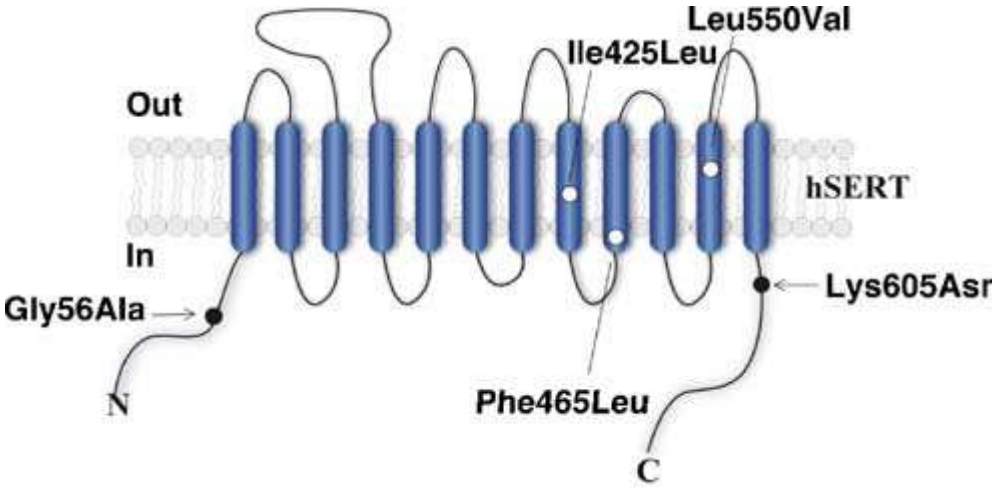

Figure 4.1 SERT mutations [3]

4.3 Targeting NET and Multiplex Experiments

The Rosenthal lab has been studying SERT and DAT for several years now, having already found targeting ligands for them. Much of the work done for SERT has already been described, and a great deal of information has obtained for both SERT and DAT in our group. Without studying NET though, the picture of these monoamine transporters is incomplete. There are still several more NET targeting ligands to be screened and more are being synthesized. A ligand with an IC50 value of 5uM or lower for NET would be ideal for labeling, imaging, and single molecule tracking. With multiple targeting ligands, simultaneous labeling experiments could be done. This would allow for the visualization of not only the behavior of the individual transporters but also their effect on each other.

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