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Insights Into The Structure, Pharmacology, And Evolution Of The Glycine Transporter 2

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INSIGHTS INTO THE STRUCTURE, PHARMACOLOGY, AND EVOLUTION OF THE GLYCINE TRANSPORTER 2

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Dedication

This dissertation is dedicated to my daughter Paulina.

“Sometimes it is the people no one can imagine anything of who do the things no one can imagine.”

--Alan Turing

INSIGHTS INTO THE STRUCTURE, PHARMACOLOGY, AND EVOLUTION OF THE GLYCINE TRANSPORTER 2

by

ASHLEY BRYAN LOPEZ, B.S.

DISSERTATION

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Acknowledgments

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Abstract of the Dissertation

INSIGHTS INTO THE STRUCTURE, PHARMACOLOGY, AND EVOLUTION OF THE GLYCINE TRANSPORTER 2

By

Ashley B. Lopez

Doctor of Philosophy, Graduate Program in Biological Sciences

The University of Texas, El Paso, April 2020

Dr. Manuel Miranda, Chair

The balance between neuronal excitation and inhibition in the central nervous system is vital for survival. Overexcitation can be toxic and lead to certain manias or even death. On the other hand, extremely low levels of inhibition cause hyperekplexia, pain, and even forms of autism. Neuronal inhibition is, for the most part, achieved by two neurotransmitters, GABA and glycine. Levels of both neurotransmitters in the synaptic cleft are meticulously regulated by the GABA and glycine transporters, respectively, which belong to the solute carrier 6 (SLC6) family of neurotransmitter transporters and share structural similarities with other family members. In **Chapter 1,** we aimed to understand the function of the amino terminus of glycine transporter 2 (GlyT2) by identifying possible interacting partners using biochemical techniques such as immunoprecipitation and Western blots. However, we were unsuccessful at identifying an interacting protein at the N-terminus of GlyT2. We believe that the high complexity of the N-terminal sequence and the

degree of disorder in the structure resulted in transient and weak interactions between N-terminus and interacting proteins. In **Chapter 2** of this dissertation, we aimed to develop specific inhibitors of GlyT2. Using molecular dynamics simulations and docking experiments we predicted four compounds that could inhibit GlyT2 activity, and by using glycine uptake assays we demonstrated the lack of activity of these inhibitors in GlyT1. As a result, we showed the specific inhibition of GlyT2 over GlyT1 *in-vitro*. In **Chapter 3**, we aimed to further elucidate the evolution of GlyT2 through the use of *in-silico* and biochemical functional assays to characterize a gene (CG5549) in *Drosophila melanogaster* that resembles a glycine transporter. However, our results indicated that the lack of efficient glycine transport in CG5549 expressed in a mammalian cell line provides enough evidence to prove that this gene is not a glycine transporter.

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Abbreviations

GluT1-Glucose transporter

SLC-Solute carrier

SLC6-Solute carrier 6

LeuT-Leucine transporter

DAT-Dopamine transporter

NET-Norepinephrine transporter

SERT-Serotonin transporter

GAT-GABA transporter

GlyT2-Glycine transporter 2

GlyT1-Glycine transporter 1

GlyR-Glycine receptor

VIIAT-Vesicular Inhibitory Amino Acid Transporter

ELM-Eukaryote Linear Motif

PKC-Protein Kinase C

PMA-phorbol 12-myristate 13-acetate

PAE-Porcine Aortic Endothelial cells

YFP-Yellow Fluorescent protein

CFP-Cyan Fluorescent Protein

NMDA-N-methyl-D-aspartate

FDA-Food and Drug Administration

CNS-Central Nervous System

Km-Michaelis-Menten equation

Vmax- Maximum transport

dSERT-*Drosophila melanogaster* Serotonin transporter

OAT-Organic Anion Transporter 1

dGAT- *Drosophila melanogaster* GABA transporter

NAT-Nutrient Amino Acid Transporter

General Introduction

Transport across biological membranes and transporters

Cell permeability and the movement of molecules across membrane-bound organelles is mediated by passive and active transport (Lehninger et al., 2013). Passive transport, which includes simple and facilitated diffusion, is driven through concentration gradients (Lehninger et al., 2013). Solutes move across the permeable membrane from regions of high to low solute concentration until equilibrium has been reached (Fig 1A, B). While lipid-soluble and nonpolar molecules can freely cross the phospholipid bilayer during simple diffusion, facilitated diffusion requires channel proteins or passive transporters. For example, glucose transporter 1 (GLUT1) transports glucose from the extracellular space into the cell without energy expenditure (Fig. 1B) (Deng et al., 2014).

Conversely, primary active transport requires energy expenditure in the form of ATP to change protein conformation, this change in structure provides the mechanical force to translocate substrates against their concentration gradient; a classical example of primary active transport is the sodium-potassium pump (Na+/K+ pump, Fig. 1C). In neurons, the Na+/K+ pump re-establishes the high concentration of sodium outside the cell and high potassium levels inside the cell, simultaneously by the usage of ATP (SKOU 1965). Secondary active transporters use concentration gradients of other molecules as the driving force to transport the substrate against its concentration gradient (Fig. 1D). Solute carriers (SLC) are an example of secondary active transporters and constitute the second largest family of transmembrane proteins, following the G protein-coupled receptors (Hediger et al., 2004, Höglund et al., 2011).

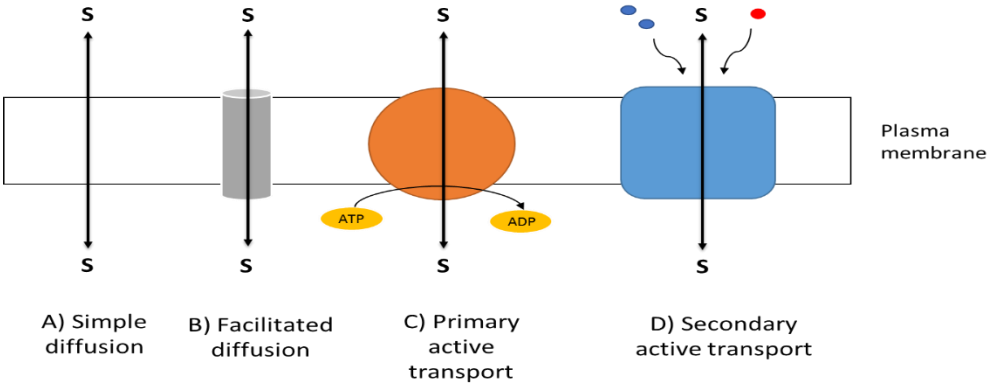


Figure 1: Schematic representation of various type of cell transport. A) Simple diffusion, B) Facilitated diffusion by transporters, C) Primary active transport (Na+/K+ pump), and D) Secondary active transporter (SLC6 family) blue circles: sodium ions, red circles: chloride ion.

The SLC membrane transporters are composed of 52 families with more than 300 members (HUGO gene nomenclature committee, www.genenames.org). The diverse groups of transporters include the following: exchangers, mitochondrial transporters, vesicular, and plasma membrane transporters (Hediger et al., 2004, Höglund et al., 2011). Since the SLC group includes a whole array of transport proteins with different properties, new members must share at least 20-25% of amino acid sequence with their corresponding category to be considered part of the family (Hediger et al., 2004, Höglund et al., 2011).

SLC transporters are important pharmacological targets due to their regulatory role in substrate influx and efflux across cells, particularly the sodium chloride neurotransmitter transporters family or better known as the solute carrier 6 family (SLC6) (Kristensen et al., 2011). SLC6 family members are considered symporters because the ion's movement is coupled to substrate transport (Kristensen et al., 2011). The sodium chloride transporters, as their name

indicate, use the electrochemical gradient of sodium and chloride as a driving force to transport the substrate against its concentration gradient.

Members of the SLC6 family have 12 transmembrane domains and share high sequence conservation and intracellular N- and C- termini. Additionally, they share an elongated extracellular loop between domains III and IV that undergoes N-glycosylation (Olivares et al., 1995, Martinez-Maza et al., 2000, Harvey et al., 2013). Members of this family have a similar mechanism of transport, which was modeled using a bacterial homolog of the SLC6 family: Leucine transporter (LeuT) (Krishnamurthy et al., 2012). The mechanism of transport is composed of three main phases: 1) outward-open, 2) outward-occluded, and 3) inward-open (Krishnamurthy et al 2012) (Fig. 2).

In the outward open structure, sodium and chloride ions are bound to the transporter, waiting for the substrate to bind. The intracellular part of the channel is closed at this stage, and once the ions and substrate are bound to the transporter, its structure changes into the outward-occluded phase. In the outward-occluded phase, extracellular loop 4 serves as a gate that prevents unspecific transport from the outside of the cell. At this point, the substrate and ions are being coordinated by the transporter and the extracellular gate is closed. Subsequently, the transporter transitions to the inward-open state, followed by intracellular channel opening and release of ions into the cytoplasm (Krishnamurthy et al., 2012).

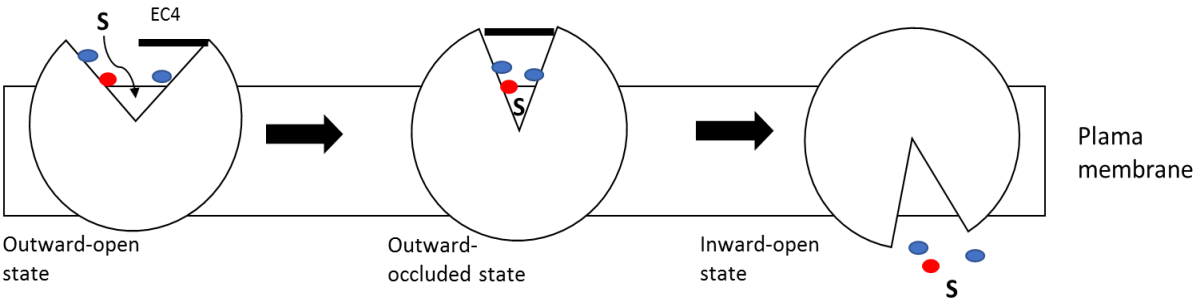


Figure 2: Mechanism of action of the SLC6 family, which consists in 3 stages: outward-open, outward-occluded, and inward-open state. (S) substrate, (EC4) extracellular loop 4, red ovals represent chloride ions and blue ovals, sodium.

Members of the SLC6 family include the serotonin transporter (SERT), dopamine transporter (DAT), norepinephrine (NET), gamma-aminobutyric acid (GABA) (GAT 1, GAT 2, GAT 3, and BGT1), and glycine transporters (GlyT1, and GlyT2) (Kristensen et al., 2011). They represent critical pharmacological targets for the treatment of several neurological disorders such as Alzheimer's disease, Parkinson's disease, schizophrenia, and chronic pain.

Modulation of the neurotransmitter glycine in the synaptic cleft by glycine transporters

The availability of glycine in the synaptic cleft is tightly regulated by glycine transporter 1 and glycine transporter 2 (GlyT1 and GlyT2), which are encoded by two different genes, SLC6A9 and SLC6A5, respectively (Aragón et al., 2005). GlyT1 oversees the clearance of glycine from the synaptic cleft and is expressed in both glial cells and neurons within the thalamus, retina, and several areas of the brainstem and spinal cord (Harvey et al., 2013). On the other hand, the GlyT2 is expressed in neurons and facilitates the reuptake of glycine into the presynaptic neuron. It is extensively expressed in the brainstem, spinal cord, and cerebellum (Zafra et al., 1995). GlyT2 is atypical compared to other members of the SLC6 family since it contains a 200-amino acid N-

terminus, with unknown function. The recycling of glycine by GlyT2 is vital for a continuous supply of glycine for future neuronal release. Once glycine is transported back into the cytosol of the presynaptic neuron, the vesicular inhibitory amino acid transporter (VIAAT) uses a proton gradient to transport glycine back into vesicles for further release of glycine from synaptic vesicles (Aubrey et al., 2007). The VIAAT is also involved in the recycling of GABA by filling synaptic vesicles with the neurotransmitter. Several studies suggest that the VIAAT has no significant variability in its affinity for GABA or glycine. (Aubrey et al., 2007).

GlyT2 regulation of function.

The internalization of the GlyT2 transporter from the plasma membrane is orchestrated by the activation of protein kinase C (PKC). The PKC pathway is activated by phorbol esters, particularly phorbol 12-myristate 13-acetate (PMA) (Fornes et al., 2008). Consequently, treatment with PMA reduces the amount of GlyT2 available at the plasma membrane. Fornes et al., 2004 suggested that the acidic substitution of lysine-422, threonine-419, and serine-420 within intracellular loop 2, abolished PMA-induced internalization. However, it is not clear if PKC directly phosphorylates these specific residues. Post-translational modifications in the transporter tails are believed to cause internalization and degradation of transporters, particularly by ubiquitination (Barrera et al., 2015). Barrera et al., 2015 provided evidence suggesting that the ubiquitination of lysine residues along the amino and carboxyl tails of GlyT1 is non-specific. Therefore, internalization or degradation of GlyT1 does not depend on only one intracellular tail or only one specific lysine residue, and similar properties are assumed to also be present in GlyT2.

The C-terminus of GlyT2 has been associated with a pivotal role in its trafficking to the plasma membrane. Within the C-terminus of other SLC family members, the last three amino acid residues yield a PDZ motif that has been well conserved (Armsen et al., 2007). It is believed that other proteins such as Syntaxin 1A (Geerlings et al., 2000) are interacting with GlyT2 via PDZ domains to provide localization and stability in the plasma membrane. Experiments by Armsen et al., (2007) suggest that truncating and adding alanine's into the C-terminus will perturb the activity and function of PDZ motifs. As a result, the PDZ domain-containing proteins cannot bind to their corresponding motifs, which will interfere with GlyT2 localization at the plasma membrane. It is worth mentioning that the PDZ motif is essential for transporter expression and function in HEK293T cells (Armsen et al., 2007).

The second extracellular loop between domains III and IV of GlyT2 undergoes N-glycosylation on Asparagine (Asn) residues that are located at sites345, 355, 360, and 366. Aragon et al., 2000 performed single/double/triple-mutations at Asn 345, 355, 366. They reported that individual mutations did not affect the transport of glycine or trafficking of GlyT2 to the plasma membrane, but cumulative mutations of Asn residues in the extracellular loop 2 can lead to a misfolded protein, and retention of GlyT2 in the endoplasmic reticulum (Martinez-Maza et al., 2000). Additional findings on post-translational modifications for GlyT2 are vital for the understanding of GlyT2 activity and regulation.

Great progress has been made to understand how GlyT2 function and recognize its critical role in neurotransmission. Therefore, this dissertation investigates the structure, pharmacology, and evolution of GlyT2.

Chapter 1

Characterization of the N-terminus of GlyT2

1.1 Abstract

The N-terminus of GlyT2 is the longest tail sequence in the SLC6 family, composed of 200 amino acids and containing 31 serine/threonine and 23 proline residues. The high abundance of Ser/Thr opens possibilities for post-translational modifications but, together with a disordered state, the function of the N-terminus remains a mystery. To investigate the possible role of this domain, we tried to identify interacting proteins that might shed light on the role of this elongated N-terminus. Previous mass spectrometry data obtained using tissue lysate by our laboratory identified possible GlyT2 N-terminal-interacting proteins. To test whether those interactions were specific, we performed co-immunoprecipitation experiments. Unfortunately, we were not able to co-immunoprecipitate any protein that was a candidate to be interacting with the N-terminus of GlyT2. New experiments are required to address several limitations of our *in-vitro* studies; we strongly believe that using *in-vitro* non-neuronal cell lines has created an artificial environment that deprives GlyT2 of the ability to interact with endogenous proteins. This is important because finding ways to modulate the functionality of GlyT2 can be advantageous to the therapeutic targeting of GlyT2 for alleviating chronic pain.

1.2 Introduction

Intracellular domains of the SLC6 family

The C-terminus of the SLC6 family has been intensively studied and it is well established that this region is critical for transporter trafficking, internalization, degradation, and recycling (Olivares, et al., 1994, Armsen et al., 2007, and de Juan-Sanz et al., 2013). Nonsense or missense mutations cause drastic effects on transporter function and localization in the cell. We now know that all of these transporters are internalized following the ubiquitination of the C-terminus (Barrera et al., 2015). Several well-characterized C-terminus mutants result in the localization of internalized transporter in the rough ER and Golgi apparatus. In contrast to the C-terminus, the N-terminus of the SLC6 family has been less studied and main functions have been elucidated in monoamine transporters such as DAT, SERT, and NET. Several research groups suggest that the reverse transport of neurotransmitter from the cytosol to the synaptic cleft occurs by the work of the N-terminus and certain drugs such as amphetamine (Khoshbouei et al., 2004, Sucic et al., 2010, and Sitte et al., 2015). The function of the N-terminus in GlyT2 and other transporters, however, remains to be elucidated.

Characteristics of the N-terminus of GlyT2

GlyT2 is the only member of the SLC6 family to have an elongated N-terminus of 200 amino acids, compared to the average length of all other members, which is approximately 30-100 amino acids (Figure 1.1).

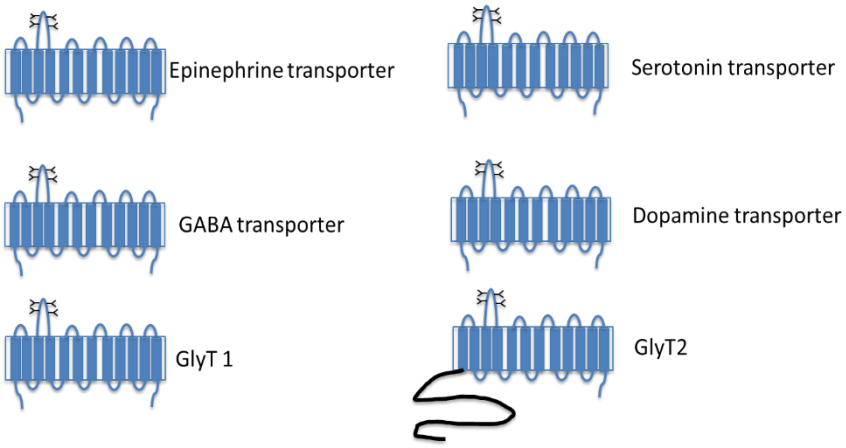


Figure 1.1: Comparison of the N-terminus between GlyT2 and other members of the SLC6 family.

Although some groups have explored the function of the N-terminus, very minimal progress has been made to elucidate the role of this domain. Horiuchi et al. identified Unc-33-like protein 6 as a possible protein that interacts with the amino-terminal of GlyT2 (Horiuchi et al., 2005). Unc-33 is a brain-specific protein that is involved in signaling and axonal guidance; yeast two-hybrid and co-immunoprecipitation assays suggested an interaction between Unc-33 and the N-terminus of GlyT2. However, the role of this interaction and the binding site on GlyT2 remains unknown. Also, there is no evidence indicating that GlyT2 trafficking or function is modified by the lack of Unc-33, leaving important gaps that need to be studied.

Over the past two decades, a large amount of data has been published on the role of GlyT2 at inhibitory synapses, and more recently it emerged as a therapeutic target in the treatment of chronic pain (Vandenberg et al., 2014, Omori et al., 2015). However, several aspects of GlyT2 structure and function remain to be explored. For example, it is unclear what modulates

the activity of GlyT2; is it only the quantal levels of the neurotransmitter in the synaptic cleft or is there an intracellular mechanism that modulates the activity of GlyT2 (such as membrane disruption or lipid-protein interaction). Limited research groups have tried unsuccessfully to unveil the function of GlyT2 N-terminus (Baliova et al., 2003, Horiuchi et al., 2005).

We are interested in the N-terminus of GlyT2 because previous findings from our laboratory suggest that this domain is not involved in glycine transport directly (as demonstrated in GlyT2 N-terminus mutants (Gentil et al., 2020)) or the delivery of GlyT2 to the plasma membrane. However, preliminary data demonstrate that GlyT2 N-deletion is less stable at the plasma membrane and its half-life is significantly reduced in mutants of GlyT2 lacking the N-terminus compared to the wild-type (Gentil et al., 2020). Therefore, **we hypothesize that the N-terminus serves as a binding site for scaffold proteins that stabilize GlyT2 at the plasma membrane**.

1.3 Materials and Methods

Tissue harvest and tissue lysate: Adult mice (*Mus musculus*) were sedated with CO_2 and rapidly decapitated by guillotine. After decapitation, we extracted the brain and placed it in cold phosphate-buffered saline (PBS) to prevent protein degradation. Then, the brain was homogenized (Kontes Glass Co.) in lysis buffer (10% glycerol, 25mM HEPES, 100mM NaCl, 1% Triton, 1 mM PMSF Aprotinin, Leupeptin, 1% Deoxycholic acid, 100 µM EDTA pH 8, 1mM NaF, 1X phosphatase inhibitors, 0.1% of SDS, and 10 mM of dithiothreitol). After homogenization, we collected the cell lysate and placed it in a pre-chilled sterile Eppendorf tube and nutated for 15 minutes at 4 C°. Then centrifuged at 14,500 rpm for 15 minutes at 4 C° to remove insoluble cell debris. The supernatant was transferred to a new pre-chilled Eppendorf tube to quantify the protein using the Bradford protein assay (Bradford 1976) with bovine serum albumin as standard.

Immunoprecipitation of GlyT2 in mouse tissue: 200 µg of mouse brain lysate was incubated with 1 µg of anti-N-GlyT2 antibody in a pre-chilled Eppendorf tube, in a final volume of 700µL. Two antibodies of GlyT2 were used, one recognizing the N-terminus of GlyT2 (Santa Cruz Biotechnology) and the second antibody recognized the C-terminus of GlyT2 (Millipore). For GFP immunoprecipitation we used anti-rabbit Green Fluorescent Protein (Invitrogen). For the control group, we used 1 µg of normalized rabbit IgG (Southern Biotech). The mix was incubated from four hours to overnight on a nutator, at 4 C°. Next, we added 50 µL of sepharose protein A beads (Sigma-Aldrich, St Louis, MO.), followed by an additional incubation period of 1h. We then centrifuged the sample at 14,500 rpm for one minute and discarded the supernatant. Subsequently, we performed 3 washes with lysis buffer and centrifuged it one more time. After

the last centrifugation, we removed the remaining supernatant and eluted the protein in 100 µL of 5X SDS-loading dye, boiled the sample for 10 minutes, and loaded it into an 8% SDS-gel.

Immunoprecipitation of GlyT2 in PAE cells: Cells were plated into a 6-well plate to reach full confluency, then we washed the cells three times with 1x PBS. After washes we added 450µl or 200µl (for total cell lysate) of lysis buffer (10% glycerol, 25mM HEPES, 100mM NaCl, 1% Triton, 1 mM PMSF Aprotinin, Leupeptin, 1% Deoxycholic acid, 100 µM EDTA pH8, 1mM NaF, 1X phosphatase inhibitors). Then we scraped the cells from the bottom of the well, and place the cells with lysis buffer in a pre-chilled Eppendorf tube and leave it nutating at 4 C° for 10 minutes. After 10 minutes, centrifuge 15 minutes at 14,500 rpm at 4 C°, we took the supernatant and placed it into a new pre-chilled Eppendorf tube. Then we added 1 µg of antibody: For GFP immunoprecipitation we used anti-rabbit Green Fluorescent Protein (Invitrogen). For the control group, we used 1 µg of normalized rabbit IgG (Southern Biotech). The mix was incubated overnight on a nutator, at 4 C°. Next, we added 50 µL of Sepharose protein A beads (Sigma-Aldrich, St Louis, MO.), followed by an additional incubation period of 1h. We then centrifuged the sample at 14,500 rpm for one minute and discarded the supernatant. Subsequently, we performed 3 washes with lysis buffer and centrifuged it one more time. After the last centrifugation, we removed the remaining supernatant and eluted the protein in 100 µL of 5X SDS-loading dye, boiled the sample for 10 minutes, and loaded it into an 8% SDS-gel.

FLAG affinity chromatography: In our original DNA construct pcDNA3.1-FHGlyT2, our group introduced a FLAG sequence of 8 amino acids and 10 histidine's. This FLAG protein can allow us to purify our protein of interest, using the available technology for purifying protein using affinity chromatography. We proceeded to immunoprecipitate GlyT2 using FLAG affinity

chromatography. We followed the same protocol as the previous immunoprecipitation of GlyT2 in PAE cells, with only a few modifications. First, in the step where we added the primary antibody recognizing GlyT2 or GFP, we proceed to add an anti-FLAG monoclonal antibody (Millipore-Sigma) that is covalently attached to an agarose bead. We incubated the cell lysate with the FLAG-agarose complex for 3 hours at 4 C°; after the 3 hours incubation, we centrifuged (same as the previous step) and remove the supernatant. We washed the agarose pellet gently four to five times with lysis buffer. After the 4 washes, we eluted with 140µl of 0.1M of glycine pH 2.8 for 2 minutes. After the two minutes we transfer the supernatant into a new pre-chilled tube we added 20µl of 1M Tris pH 8.2 and 40µl of 5x loading dye.

Electrophoresis and Immunoblot: Samples were separated by molecular weight in 8% SDS-PAGE, and the proteins transferred onto nitrocellulose blotting membrane for 1.5 hours (100 volts, 3 amps, and 300 watts). After electro-transfer, the membrane was blocked with 5% milk (Carnation dry milk) in 1X TBS-T (10 mM Tris base, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h. Afterward, the membrane was washed three times for 10 minutes with 1x TBS-T. Then the membrane was incubated with the primary antibody against N- or C- terminus of GlyT2 (dilution 1:1000) followed by three ten-minute washes with 1x TBS-T. Each membrane was then incubated with the anti-rabbit (HRP) secondary antibody (Promega 1: 10,000 dilution) or corresponding secondary antibody. Ultimately, the membrane was washed one last time, following the previously mentioned procedure, and it was then developed using Pierce ECL western blotting substrate (Thermo-Scientific) and radiography films (Phenix research products) or iBright software (ThermoFisher).

1.4 Results

Immunoprecipitation of GlyT2 from porcine aortic endothelial cells.

To better understand the function of the N-terminus of GlyT2, we performed immunoprecipitations in cells grown in culture, specifically in porcine aortic endothelial cells (PAE). In our experiments, we attempted to immunoprecipitate GlyT2 from transfected cell lines (PAE cells) stably expressing wild type GlyT2. To the best of our knowledge, there are only three commercially available GlyT2 antibodies available: 1) against the human GlyT2 N-terminus (Santa Cruz Biotechnology), 2) against the human GlyT2 C-terminus (Millipore), and 3) against the rat GlyT2 extracellular loop (Alomone Labs). The antibody against the extracellular loop (Alomone labs) does not recognize the human GlyT2 protein but only the rodent GlyT2 (data not shown). We used these antibodies for the experiments and the results are shown in the figure: 1.2A. To pull down the antigen-antibody complex, we used Protein A conjugated to sepharose or magnetic beads. Non-immunized rabbit normalized IgG's were used as a negative control. As shown in the western blot of Figure 1.2A, no detection of GlyT2 was obtained in the immunoblot. We detected two bands, 55kD, and 30kD corresponding to the heavy and light chain of the IgG, respectively.

The next two lines on the blot correspond to experimental precipitations with antibodies against the N- or C-terminus of GlyT2 and no immunoreactive bands were detected, suggesting that neither antibody was able to pull down GlyT2. As a positive control for the western blot, we loaded His-tagged GlyT2 purified by nickel-charged affinity chromatography (Ni-NTA), which is based on the binding of histidine for protein purification. Based on these findings, we concluded that none of the antibodies were able to immunoprecipitate GlyT2.

After multiple attempts (N=3) to immunoprecipitate GlyT2 from PAE cells, we began to question the conditions or limitations of the experiment and the ability of the antibody to recognize the antigen. To test our experimental settings, we used antibodies against the dopamine transporter (DAT), another member of the SLC6 family. This antibody has been used for immunoprecipitations of DAT and shown to recognize and bind the bait (Sorkina et al., 2003). In Figure 1.2B, we immunoprecipitated the dopamine transporter using different concentrations of the DAT antibody. The results showed an immunoreactive band corresponding to DAT at the predicted molecular weight. In the negative control, precipitation with normalized Goat IgG's did not detect any signal. As a positive control, we used 15% of the total cell lysate. These results together suggest that GlyT2 N- and C- terminus antibodies do not facilitate immunoprecipitation of the human GlyT2. DAT immunoprecipitation demonstrated that our experimental conditions were not interfering with the antibody binding, but rather our antibodies do not recognize the protein of interest in immunoprecipitation experiments.

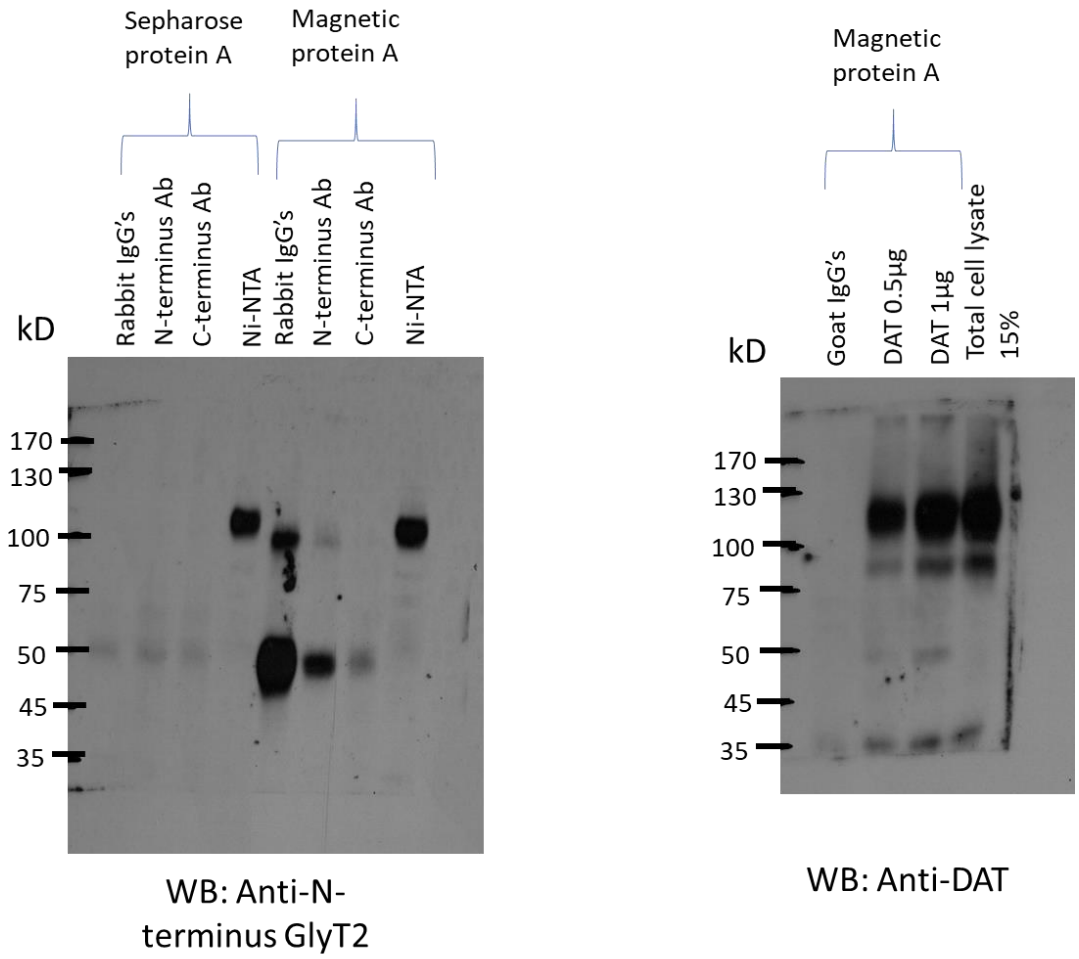


Figure 1.2: Immunoprecipitation of GlyT2 using antibodies against N- and C-terminus. Panel A: Rabbit IgG's negative control, N-terminus GlyT2 antibody, C-terminus GlyT2 antibody and purified GlyT2 (using Ni-NTA as a positive control. B) DAT immunoprecipitation with goat IgG's and total cell lysate as a control.

Immunoprecipitation of GlyT2 using GFP antibodies

Due to the inability of the GlyT2 antibodies to immunoprecipitate GlyT2, we decided to take an alternative approach. Other laboratories have demonstrated the successful immunoprecipitation of dopamine transporters using YFP and CFP proteins (Sorkina et al., 2003) without compromising the functionality of the transporter. By cloning GlyT2 into a YFP vector, we introduced a yellow fluorescent protein that is recognized by GFP antibodies. This YFP gene

was fused to the N-terminus of the GlyT2 gene to produce a fusion protein with YFP followed by the human GlyT2 (Figure: 1.3 A). In Figure 1.3, we demonstrated the ability of GFP antibodies to immunoprecipitate the fusion protein YFP-GlyT2 (lanes: 2 & 4). The addition of a YFP molecule to the N-terminus of GlyT2 results in a molecular weight of 130kD, which is the molecular weight shown in figure 1.3B lanes 2-5, suggesting the successful immunoprecipitation of YFP-GlyT2. To confirm the specificity of the resin along with the GFP antibodies, we performed immunoprecipitation with normalized rabbit IgG's which did not recognize YFP-GlyT2 as shown in Fig 1.3 B, lane 1. Only the normalized IgG's were present in the blot (MW around 50 kD).

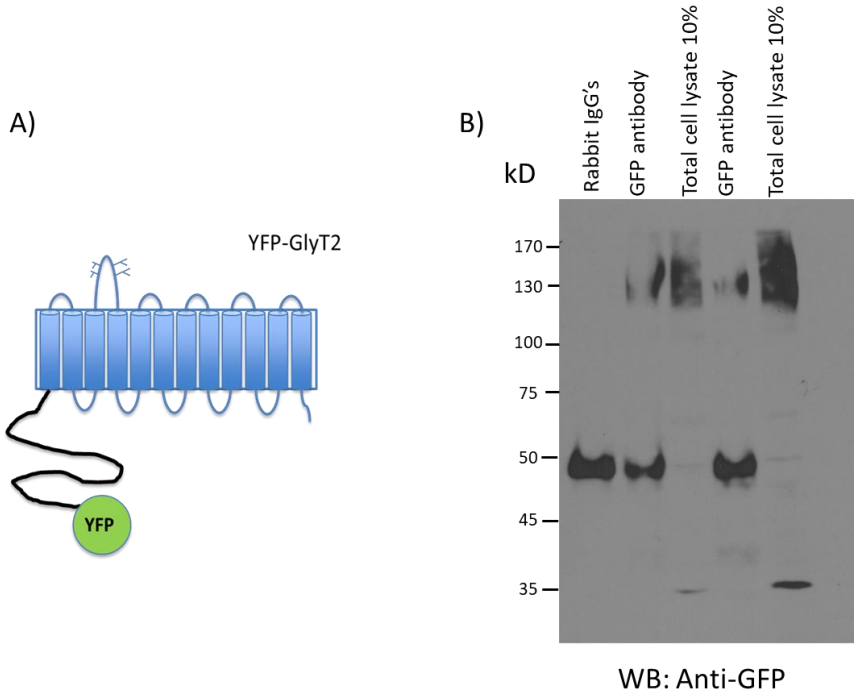


Figure 1.3: Immunoprecipitation of YFP-GlyT2 using GFP antibodies. Panel A: Schematic representation of the DNA construct YFP-GlyT2. B) YFP-GlyT2 immunoprecipitation with normalized rabbit IgG's, GFP antibody, and total cell lysate as a control (GFP antibody immunoprecipitation was duplicated along with their corresponding total cell lysate).

Immunoprecipitation of FH-GlyT2 using FLAG affinity chromatography

While the GlyT2 immunoprecipitation with GFP antibodies was successful, there is still a great concern about the addition of a large protein into an intracellular domain. One concern was that the YFP protein might interfere with the conformational structure of the N-terminus. Also, we noticed that the cells produced low levels of YFP-GlyT2. This low yield in YFP-GlyT2 immunoprecipitation can make it more difficult to detect interacting proteins in co-immunoprecipitations. Therefore, we decided to use a shorter tag and used the original DNA construct of the human GlyT2 which contained a FLAG sequence of 8 amino acids at the beginning of the N-terminus (Figure 1.4A).

We believe that the small size of FLAG protein and the full functionality of the transporter is enough evidence to suggest that the FLAG-Histidine complex is effective for pull-down assays. A FLAG targeting antibody is covalently attached to agarose beads, allowing precipitation and separation of our FLAG-tagged protein from the lysate. Using this method, we were able to immunoprecipitate FLAG-tagged GlyT2 protein; shown in Fig 1.4B, lanes 2 and 3. In Figure 1.4B, we showed the specificity of the FLAG agarose beads using PAE cells not expressing GlyT2 (lane 1), as our negative control. These results showed the successful and specific immunoprecipitation of GlyT2.

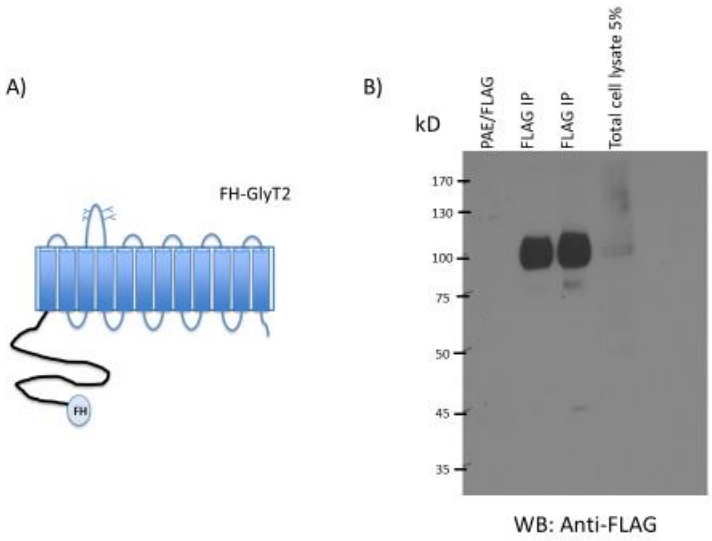


Figure 1.4: Immunoprecipitation of FH-GlyT2 using FLAG antibodies and agarose beads. Panel A: Schematic representation of the DNA construct FH-GlyT2. B) FH-GlyT2 immunoprecipitation with FLAG antibodies in a cell line that does not express FH-GlyT2, then FLAG antibody immunoprecipitation, and 5% of the total cell lysate as a control 5% (FLAG immunoprecipitation was duplicated on the same blot for consistency).

1.5 Discussion

The N-terminal tail of the vertebrate GlyT2 represents an uncharacterized, novel domain within the SLC6 family of transporters. This N-terminus differs from other transporters by the increased number of amino acids comprising it and particularly by the abundance of proline residues it contains, suggesting a poorly structured domain. Based on sequence analysis, we hypothesized that this domain may function as a scaffold to bring proteins in close proximity to the transporter and modulate its functions intracellularly. Traditionally, one of the common approaches for testing protein-protein interactions has been co-immunoprecipitation; unfortunately, technical issues obstructed our ability to immunoprecipitate GlyT2. The experiments shown in the results section suggests that under our experimental conditions, commercial and in-house made antibodies do not recognize the transporter.

This conclusion is supported by immunoprecipitation of DAT from dopamine transporter expressing cells, demonstrating that our experimental conditions were not a problem, but rather GlyT2 antibody did not recognize the antigen in immunoprecipitation (Fig. 1.2). Interestingly, the antibody against the GlyT2 N-terminus functions in applications such as western blots and cell immunofluorescence, but not in immunoprecipitations. When subjecting proteins to denatured electrophoresis and western blot, we disrupt all secondary and tertiary protein structures to produce a linearized chain of amino acids exposing the epitopes. On the other hand, in immunofluorescence, we do not disturb the secondary structure of proteins but fix the cells with 4% paraformaldehyde (PFA). PFA fixation leads to covalent bonds between proteins, flattening and stretching the protein structure by inducing the formation of more covalent bonds and cross-linking between molecules, resulting in the exposure of amino acids for antibody binding (Seong-

Oh et al., 2017). By contrast, in immunoprecipitation, the goal is to maintain the protein in its native conformation and allow normal binding and interaction. The results suggest that our antibody is unable to bind properly to GlyT2 under the experimental conditions used in this study.

By using an alternative strategy, we were able to immunoprecipitate the antigen using different antibodies such as GFP, FLAG, and GlyT2 antibodies. Even though the FLAG antibody showed a stronger immunoprecipitation, we could not co-immunoprecipitate any of the candidates identified in previous studies. In contrast to co-immunoprecipitation, previous studies from our laboratory that identified interacting proteins by Tandem mass spectrometry used a different strategy. Preliminary results were obtained by cloning the N-terminus of GlyT2 into a bacterial vector to yield a GST fusion protein that could be expressed and purified. The purified GST-GlyT2 fusion protein was mixed with a brain lysate following precipitation of the GST fusion protein. As such, we had only the N-terminus of GlyT2, which was incubated with mouse brain lysate. From these experiments, we hoped to pull down only proteins that interact with the N-terminus of GlyT2. This was a good starting point, but certain limitations had to be considered. When a mammalian protein is expressed into bacteria, tpost-translational modifications such as phosphorylation, acetylation, and ubiquitination are absent or different compared to a mammalian expression system.

We know that the N-terminus of the GlyT2 contains 31 serine and threonine residues, in which six of those residues are phosphorylated in native tissue (phosphosite.org). By expressing the domain in bacteria, the phosphorylation of specific residues and the interacting proteins that bind to the phosphorylated serine or threonine residues in the N-terminus will probably be omitted. To succeed in the identification of GlyT2 interacting proteins, we need to develop a

method in which we can immunoprecipitate GlyT2 from tissue lysate and proceed to the identification of neuronal proteins that interact with the N-terminus. Several biochemical assays can be used to identify interacting proteins, one example is the usage of biotin ligase. Biotin ligase labels proteins that are in close proximity to the protein of interest (Kim et al., 2016). Our cell culture model, however, does not accurately represent the native environment of glycinergic neurons and wouldn't allow the use of this approach for identifying interacting proteins.

We are in the process of developing custom rabbit and rat antibodies using GST fused to different regions of the mouse GlyT2, expecting better recognition in mouse tissue. With these new antibodies, we hope to continue our studies using brain lysate. It is worth mentioning that during the initial years of my doctoral training, I tried to identify GlyT2 interacting partners using yeast two-hybrid. However, the excessive amount of false-positive along with other pitfalls of the technique discouraged us from continuing on this path.

Future experiments will be performed using newly generated antibodies for immunoprecipitation. We will generate fusion proteins of the mouse GlyT2: 1) the N-terminus 2) the extracellular domain, 3) the C-terminus and incubate them with mouse brain tissue lysate, then pull down and evaluate samples by SDS-PAGE. Coomassie or silver staining will identify bands specific for the N-terminal domain; these bands will be de-stained and subjected to mass spectrometry. Different GlyT2 N-terminus mutants can be used to validate interactions revealed by mass spectrometry. In conclusion, there is a need for the generation of more specific antibodies for the completion of this research project.

Chapter 2

Pharmacology of Glycine transporters

2.1 Abstract

Glycine transporters are of great pharmacological interest due to their ability to tightly control the amount of glycine available in the synaptic cleft. The inhibition of either of the two glycine transporters results in increased glycine levels in the synaptic cleft. Studies have shown that the inhibition of GlyT2 can lead to an increase in glycine synaptic concentration, subsequently causing prolonged inhibition through the activation of the glycine receptor and a decrease in neuronal activity. GlyT2 has great potential as a pharmacological target due to its anatomical localization, mainly the spinal cord and brainstem, and devoid in other common brain structures associated with pleasure or reward. Other studies have shown that the inhibition of GlyT2 can be a target for the treatment of pain. In this chapter of the dissertation, we collaborated with Dr. Suman Sirimulla to complete a set of experiments using *in-silico* and biochemical assays such as molecular dynamics, docking, and glycine uptake experiments. We provide strong evidence that the inhibition of GlyT2 with selected ZINC molecules is specific and showed no effect on GlyT1.

2.2 Introduction

Pharmacology of SLC6

The SLC6 family members are important pharmacological targets in the treatment of a variety of neurological disorders (Kristensen et al., 2011). Most of the available antagonists bind during the transporter's outward-open conformational state, preventing the transition between the conformational states, thereby inhibiting the translocation of the substrate. In the nervous system, these antagonists lead to a drastic reduction in the reuptake of neurotransmitters from the synaptic cleft. Prozac® and Adderall® are examples of drugs that inhibit the serotonin and dopamine transporters, respectively; and prevent the re-uptake of neurotransmitters. Consequently, increased neurotransmitter levels within the synaptic cleft prolong activation of the corresponding receptors. By mimicking the neurotransmitter structure, these inhibitors lock the transporters into an outward-open state (Figure 2.1).

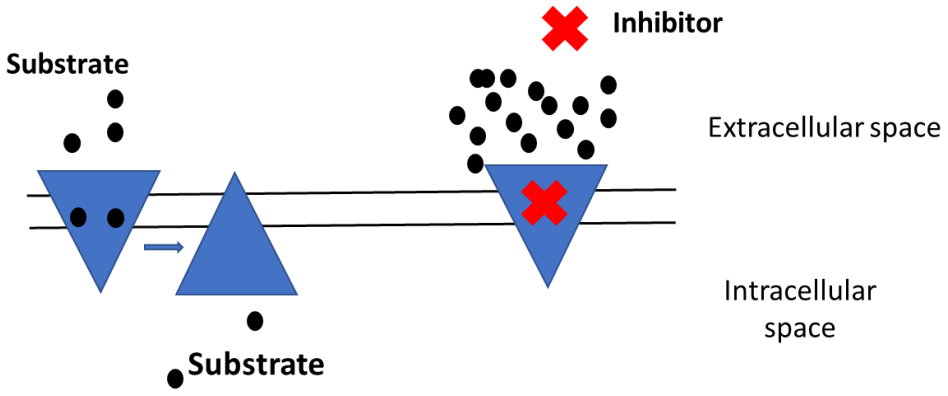


Figure 2.1: Inhibition of the SLC6 family. Inhibitors of the SCL6 lock the transporter in their outward-open state preventing the substrate translocation from the extracellular space to the intracellular space.

The neurotransmitter glycine also acts as a mandatory co-agonist with glutamate at the N-methyl-D-aspartate (NMDA) receptor, a receptor associated with memory and many other functions (Harvey et al., 2013, Aragón et al., 2005). Furthermore, GlyT1 is expressed in both glia and neurons in the retina, brainstem, spinal cord, and the forebrain (Figure 2.2) (Zafra et al., 1995, Harvey et al., 2013, Eulenburg et al., 2018). The main role of GlyT1 is to maintain low levels of glycine at excitatory and inhibitory synapses. The blockage of GlyT1 has been associated with the overstimulation of the NMDA receptor, thus it is a plausible target in the treatment of schizophrenia and has therapeutic potential for NMDA-receptor disorders (Harvey et al., 2013).

As shown in figure 2.2, there is a clear need to develop specific inhibitors to each glycine transporter. Several small compounds have been synthesized to inhibit GlyT1 but despite reaching various stages of clinical trials, none have been approved by the Food and Drug Administration (FDA). Examples are Sarcosine (phase 2), GSK1018921 (terminated, unknown reasons), Bitopertin (phase 3), PF-03463275 (terminated, unknown reasons), PF-02545920 (terminated, unknown reasons), and PF-04958242 (phase 1) (Clinicaltrials.gov, Harvey et al., 2013).

To date, no GlyT2 inhibitors are undergoing clinical trials (clinicaltrials.gov). All available inhibitors have been strictly used in cell lines and animal models. GT-0198, a putative GlyT2 inhibitor, is a phenoxymethylbenzamide derivate that inhibits glycine re-uptake (Omori et al 2015). It has been shown to penetrate the blood-brain barrier and has high specificity for GlyT2 over GlyT1. However, the authors did not provide specific evidence that GT-0198 binds only to GlyT2 and not GlyT1. Two other inhibitors, ORG25543 and ALX 1393 have also been shown to inhibit GlyT2. (Meur et al., 2013). However, ORG25543 binding to GlyT2 is irreversible, suggesting

a slow off-rate and potentially serious consequences such as the disruption of glycine recycling which could deplete the presynaptic neuron of glycine for future release. *In-vitro*, ALX 1393 can inhibit GlyT2 activity in a specific and concentration-dependent manner. However, despite its high efficacy in cell culture conditions, ALX 1393 has limited brain penetration, making it a less-than ideal therapeutic. (Vandenberg et al., 2014).

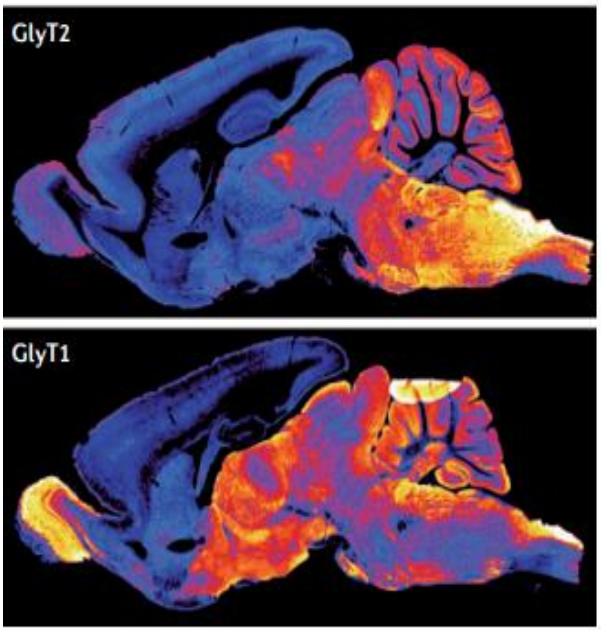


Figure 2.2: The expression of glycine transporters in the rodent (mouse) brain. This image was taken from the work of Harvey et al., 2013

Lipid-based Inhibitors of GlyT2

In the last decade, more attention has been placed on lipids and their role in modulating function and especially structure in membrane proteins (Koshy et al., 2013, LeVine et al., 2016, Landreh et al., 2016, Zeppelin et al., 2018, Schumann-Gillett et al., 2019b). Currently, there are

two theories about how these lipid-protein interactions can occur: 1) specific lipids can alter the membrane fluidity or structure of the membrane adjacent to the protein, thus causing conformational changes in structure that leads to the closing or opening of the transporter2) A direct interaction between specific lipids and protein can result in conformational changes that lead to the closing or opening of the transporter. (Corradi et al., 2019).

In recent years, cholesterol has been identified as a possible allosteric modulator of other members of the SLC6. Even though cholesterol has been detected in many SLC6 atomic structures, there has not been a protein motif identified as the main site for cholesterol-binding (Schumann-Gillett et al., 2019b). In the future, more work is needed to identify the specific protein motif in charge of binding cholesterol, and learn more about the native environment of membrane proteins. Other groups (Schumann-Gillett et al., 2019, Winters et al., 2018, Mostyn et al., 2017) have shown inhibition of GlyT2 by endogenous lipids and lipid-derived arachidonyl-amino acids and acyl-amino acids. However, more evidence is needed to validate the lipid-GlyT2 interaction and the discovery of the interacting motif.

Lipids show great promise as GlyT2 inhibitors due to their ability to be highly metabolized and to partially inhibit GlyT2. Partial GlyT2 inhibition represents an advantage over small chemicals such as ORG25543 (Vandenberg et al., 2016) in that it may allow for the continued replenishing of presynaptic vesicles with glycine. This dissertation chapter aims to find promising and reversible inhibitors for glycine transporters with a high affinity for GlyT2 and no cross-reactivity with GlyT1. To accomplish this objective, we will test the specificity of four compounds (ZINC 6665169, 19862327, 1606495, 30678404) on the mouse GlyT1a.

2.3 Materials and methods

Chemical compounds:

All chemical compounds were screened and identified by our collaborator Dr. Suman Sirimulla and his laboratory.

Glycine uptake experiments

Porcine Aortic Endothelial (PAE) cells stable cell lines expressing the mouse GlyT1a were grown in 24-well plates in high confluency of 90-100%. First, confluent cells were washed twice with 0.25mL of reaction buffer (10mM HEPES pH 7.4, 135mM NaCl, 2mM KCl, 1mM CaCl2, 1mM MgSO4 and 10mM glucose). After washes, we added 0.25mL of reaction buffer with 4 Ci of [3H] glycine per mL, and a final concentration of 500 μM of cold (not-radioactive) glycine along with the inhibitor in increased concentrations for 10 minutes at 37 C°. After 10 minutes, we terminated glycine uptake by washing the cells twice with 0.25mL of cold reaction buffer without [3H] glycine and glucose. Lastly, we extracted [3H] glycine by incubating the cells with 0.25mL of 0.2N NaOH for one hour at room temperature. Then, we proceeded to measure protein concentration using the Bradford method (Bradford 1976). Glycine uptake was measured using scintillation spectroscopy and glycine specific transport was calculated by subtracting the transport of parental wild type PAE cells.

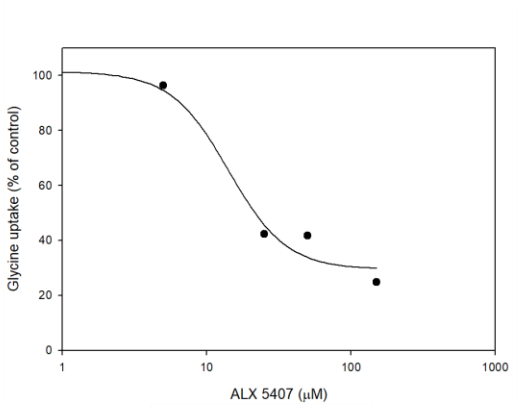
2.4 Results

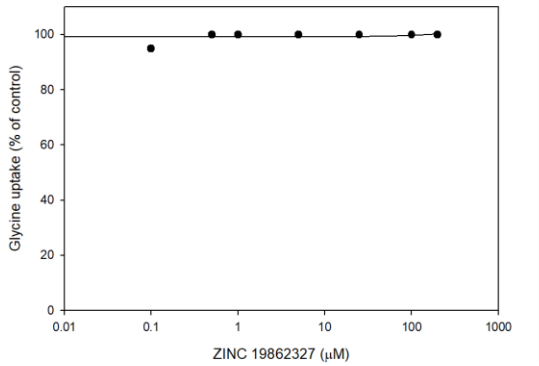
Inhibition assays in cultured cells expressing the mouse GlyT1a.

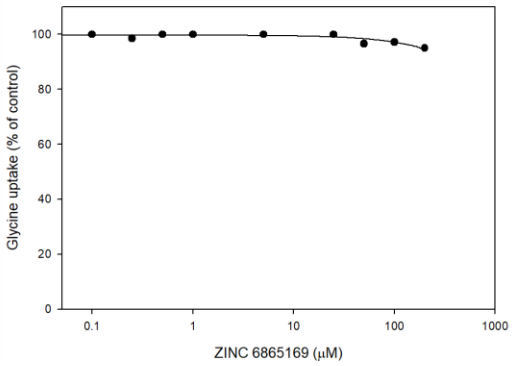
The main goal of these experiments was to test the specificity of predicted GlyT2 inhibitors by assessing their action on the mouse GlyT1a, which has been shown to have a high affinity for glycine. Dr. Suman Sirimulla's laboratory performed virtual screening (pharmacophore) and docking experiments of a library of small molecules and selected those potential molecules predicted to bind to GlyT2 transporter. In these experiments, his research group screened 3.5 million compounds from the ZINC15 library (Sterling, T., & Irwin, J. J. 2015, and Fratev et al., 2019). This ZINC15 library is a public access database for the screening of ZINC molecules that have the potential to be ligands or inhibitors (Sterling & Irwin 2015). Dr. Sirimulla's research group selected the best candidates based on different criteria such as binding capacity and energy requirements (Gibbs free energy). All these procedures resulted in the selection of 20 ZINC molecules that could serve as a potential GlyT2 inhibitor. Out of those 20 ZINC molecules, four were readily available and tested. Those ZINC molecules were: 19862327, 6865169, 30678404, and 1606495. These ZINC molecules will be referred to as Lead 1, 2, 3, and 4 respectively.

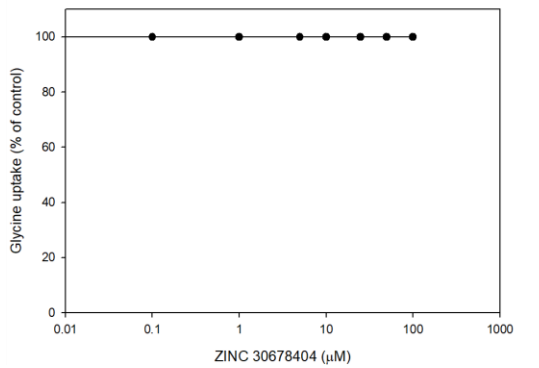
To investigate the properties of these molecules, another laboratory member, Elvia Padilla tested these compounds *in-vitro* using PAE cells expressing the GlyT2. Only Lead 1, 2 were able to inhibit GlyT2 in the mid-nanomolar range (400-400nM). The next step was to test the same compounds for cross-reactivity on the GlyT1 transporter, I was in charge of this part of the project. We tested all four compounds in PAE cells expressing the mouse GlyT1a and measured glycine uptake under different concentrations of the small compounds. We included a positive

control with the specific GlyT1 inhibitor: ALX5407. As shown in Figure 2.3A, ALX5407 inhibited glycine uptake in a concentration-dependent manner, an observation that has been reported by many research groups. As shown in figures 2.3B-E, the ZINC molecules did not inhibit the activity of GlyT1a, even at high concentrations of >100 μM. At this point, these experiments suggest that these molecules do not have any cross-reactivity with GlyT1a but two of them inhibit the GlyT2 (Fratev et al, 2019).









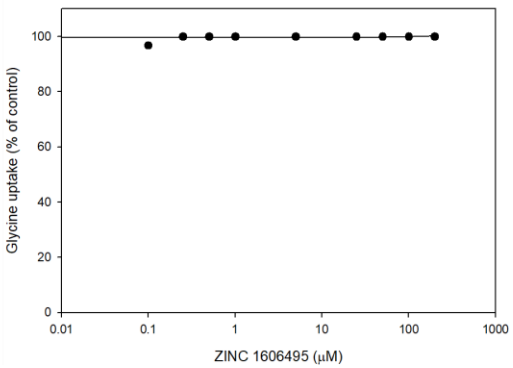


Figure 2.3 Glycine transport inhibition assays in the mouse GlyT1a in the presence of increasing concentration of ZINC molecules. A) mGlyT1a transport in the presence of ALX5407. B- E) ZINC molecules (lead 1-4) as inhibitors in mGlyT1a.

2.5 Discussion

20 years ago, pharmacology was limited by the time-consuming strategy of testing individual molecules based on the protein and molecule structure. With the new era of *in-silico* modeling, we are now able to screen millions of molecules for the search of ligands, allosteric modulators, and inhibitors. In collaboration with Dr. Suman Sirimulla, we were able to select the best candidates for GlyT2 from millions of compounds using *in-silico* techniques. As seen in figure 2.3, we tested selected ZINC molecules (possible inhibitors) and showed no activity on the related transporter GlyT1a compared to our positive control (ALX5407). Cell toxicity assays were done in collaboration with Dr. Armando Varela; the compounds only showed toxicity at extremely high concentration levels of 1mM (data not shown), and are unlikely to be considered good inhibitors at such high concentrations. As a positive control, we used ALX5407, which is a well-established inhibitor for GlyT1 *in-vitro*.

The *in-vitro* assays have some limitations and other factors might contribute to the efficacy of this molecule. One particular limitation is the method of drug delivery, in *in-vitro* studies the inhibitor is added directly to our cells. As a result, we have to test other methods of delivery that are more viable to test in animal models. The difficult task is to test for the compound penetrance into the blood-brain barrier and bioavailability. One particular example is ALX1393 where only 5% of the dosage can reach the brain, as a result, a higher dosage can result in more side effects. In conclusion, we demonstrated the ability of *in-silico* experimentation to identify possible inhibitors from libraries commercially available.

Recently, GlyT2 has emerged as a new potential therapeutic target for the treatment of pain (Vandenberg et al., 2014, Al-Khrasani et al., 2019) . As previously described, GlyT2 is mainly

expressed in the spinal cord and brainstem, more specifically GlyT2 is abundantly expressed in the dorsal horn of the spinal cord (lamina III) (Vandenberg et al., 2014, Al-Khrasani et al., 2019). The lamina III of the dorsal horn of the spinal cord is in charge of receiving sensory signaling from the peripheral nervous system, some of those signaling are the nociceptive or neuropathic pain (pain). The spinal cord function as a relay station for neuropathic pain signaling, in this "relay station" the pain signal will be received from peripheral nervous system and sent directly to the brain. However, glycinergic neurons play a pivotal role in modulating pain signals (Figure 2.4). In the presence of pain, glycinergic neurons will hyperpolarize pain projection neurons by the action of the GlyR.

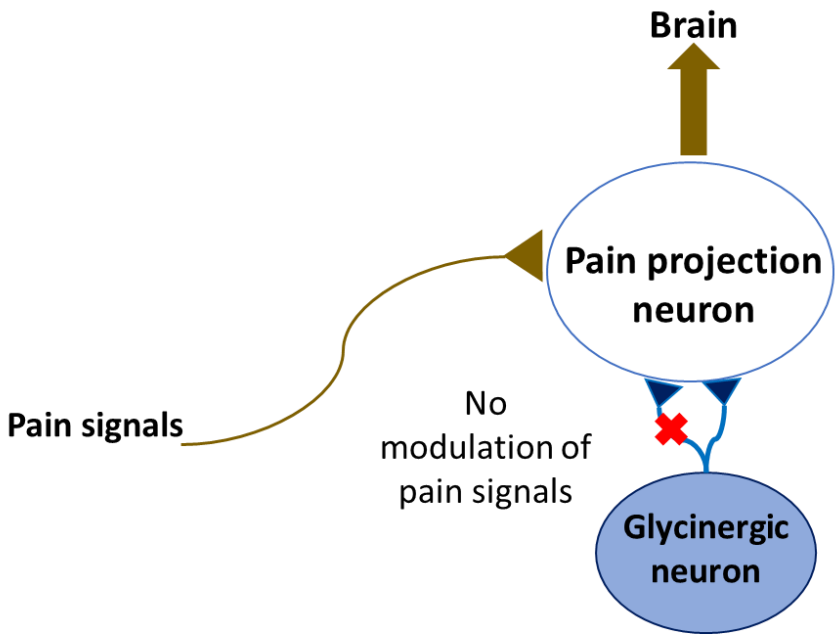


Figure 2.4: Schematic representation role of glycinergic neurons in pain modulation.

Although our body is well prepared to respond to mild and temporary pain, when the pain signal persists for weeks, months, or even years it considered chronic pain (Vandenberg et al., 2014). GlyT2 keeps low levels of glycine in the synaptic cleft to prevent excessive neuronal

inhibition, yet in chronic pain, the release of glycine is not enough to keep pain signals at a minimum. Our goal was simple, to develop a GlyT2 inhibitor. By inhibiting GlyT2, it should increase the glycine in the synaptic cleft, thereby hyper-activating the GlyR and causing a drastic decrease in neuronal activity.

Chapter 3

Characterization of a putative *Drosophila melanogaster* glycine transporter

3.1 Abstract

Although glycine transporters have been established as crucial components for inhibitory neurotransmission in vertebrates, they have been poorly studied in invertebrates such as *Drosophila melanogaster*. Published work by other research groups (Stuart et al., 2006), using sequence alignments of mammalian transporters against the fly genome, predicted the presence of a single glycine transporter. In addition, another research group (Frenkel et al., 2017) reported that this putative glycine transporter can transport glycine, their experimental evidence was not convincing based on the low amount of glycine transported inside the cell. For this project, we used protein modeling and biochemical assays to characterize this putative *Drosophila melanogaster* glycine transporter (Dmel-GlyT2). We measured the affinity of the transporter for glycine and substrate specificity. Based on dynamic simulations, we obtained a 3D model of Dmel-GlyT2 with a similar structural organization to the vertebrate GlyT2 and identification of the possible binding cage for glycine and ions. After the expression of this putative gene in mammalian cells and assay for transport, we found a low affinity for glycine but a higher preference for glutamate. The results suggest that this predicted Dmel-GlyT2 is not a glycine transporter.

3.2 Introduction

Evolution of the SLC6

Extensive research has focused on several aspects of the mammalian CNS, leaving critical gaps in our understanding of the invertebrate CNS. Within invertebrate research, much attention has been given to the biogenic amines such as dopamine, tyramine, octopamine, serotonin, and histamine, which function as neurotransmitters or neuromodulators (Blenau et al., 2001, Wu et al., 2012, Gou et al., 2013). However, during the early 1990s, Norman Davidson performed a full characterization of a member of the SLC6 family in *Drosophila melanogaster* and his group characterized the *Drosophila melanogaster* serotonin transporter (Corey et al., 1994). This opened a new era for brain research as invertebrate models could now be used in understanding homologous neurotransmitter systems.

Ann Stuart's research group from the University of North Carolina at Chapel-Hill was able to predict 21 additional putative members of the SLC6 family using bioinformatics and sequence alignments of the SLC6 bacterial homolog Leucine transporter (LeuT) as a reference (Thimgan et al., 2006). Among those predicted members was a putative glycine transporter 2 (gene CG5549), which comprised 12 transmembrane domains, glycosylation sites at the extracellular domain II, and an intracellular N- and C- terminus (Fig. 3.1). A unique feature of this putative *Drosophila melanogaster* GlyT2 (Dmel-GlyT2) is an extended C-terminus that is unique among the *Drosophila melanogaster* SLC6 family members. The elongated C-terminus of Dmel-GlyT2 C-terminus seems to resemble the N-terminus of mammalian GlyT2 in sequence and size.

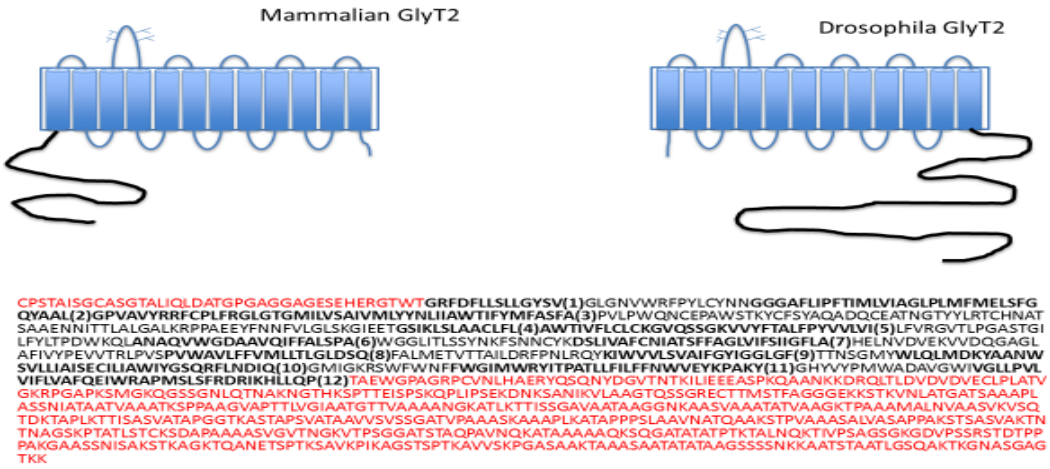


Figure 3.1: Comparison between mammalian GlyT2 and *Drosophila melanogaster* putative GlyT2. The amino acid sequence of Dmel-GlyT2 is given; in bold, transmembrane domains; in red, amino and carboxyl terminals.

Our research group has predicted the 12 transmembrane domains, the N-and C-terminus, and possible glycosylation sites of Dmel-GlyT2 using bioinformatic techniques (Figure 3.1). The group of Frenkel et al., 2017 was able to deorphanize the CG5549 *Drosophila* gene (Dmel-GlyT2) by injecting CG5549 RNA into oocytes and measuring glycine transport using radioactive glycine. However, the difference in transport between the oocytes injected with RNA and the control (injected with water) resulted in only a one-fold difference. This small difference compared to the control did not provide concrete evidence of this gene as a glycine transporter. Additionally, this published work did not show the specificity of this protein for glycine or other amino acids.

Furthermore, Frenkel et al., did not provide concrete evidence about the expression of other glycinergic proteins in *Drosophila* such as the glycine receptor. It is imperative to demonstrate the expression of other glycinergic markers to explain the possible role of this

putative transporter in invertebrates. In their evidence it is still not clear if *Drosophila melanogaster* expresses a glycine receptor. Since both, the GlyR and GABA receptors are hetero-pentameric receptors and chloride ligand-gated channels, more experimental evidence is needed to elucidate the expression of both receptors. We believe it is necessary to provide more concrete data regarding the expression of glycinergic components in *Drosophila melanogaster*. Our experiments test Dmel-GlyT2 for glycine selectivity and catalytic constants such as kinetic constant (Km) and maximal velocity (Vmax) and provide the parameters to assign Dmel-GlyT2 as glycine or another type of transporter.

In collaboration with Dr. Suman Sirimulla and his laboratory, we developed a 3D structure based on molecular dynamics simulation of the gene CG5549 (Dmel-GlyT2). Our preliminary results predicted that CG5549 folds like a vertebrate glycine transporter and docking simulations predicted glycine binding. To fully address the question if this is a glycine transporter, we expressed Dmel-GlyT2 in mammalian cells and provided a full characterization of its kinetic properties.

We also seek to answer the question of whether glycine transporters appear in invertebrates and if they maintain long N- or C-termini. What was the evolutionary pressure to develop a long intracellular tail in vertebrates and whether it is also present in invertebrates? To answer all these questions, we had to follow different steps. First, using bioinformatic algorithms we confirmed that Dmel-GlyT2 has the highest sequence homology with the vertebrate GlyT2. Second, to test if the Dmel-GlyT2 protein sequence has all the amino acids to bind glycine and the necessary ions to transport their substrate. Lastly, to perform glycine uptake assays to

measure the ability of Dmel-GlyT2 to transport glycine, and if this transporter is specific for glycine or recognizes other amino acids.

Other genes resembling a glycine transporter in *Drosophila melanogaster*

The scientific literature contains evidence of the presence of orphan genes from the SLC6 family in *Drosophila melanogaster*. Specifically, it is demonstrated that Dopamine (dDAT), Serotonin (dSERT), Octopamine (OAT), GABA (dGAT) transporters are expressed in *Drosophila melanogaster* (Corey et al., 1994, Leal and Neckmeyer 2002, Pörzgen et al., 2001). Our goal, however, was to determine if additional genes could resemble a glycine transporter aside from gene CG5549. Thimagan et al., 2006 and Romero-Calderon et al., 2007 identified many genes resembling the SLC6 family in *Drosophila melanogaster,* including two that show similarities with the human GlyT2*,* but they are considered orphans. CG7075 is a *Drosophila melanogaster* gene that Thimagan et al., 2006 and Romero-Calderon et al., 2007 identified as a possible member of the SLC6. Northern blots experiments showed that this gene is expressed only in the body of the fly, not the head. This contradicts the expression pattern of a glycine transporter, but more biochemical evidence is needed to demonstrate the capacity of CG7075 to transport glycine.

The second orphan gene CG10804 is now considered a NAT (nutrient amino acid transporter), but also has sequence homology to a glycine transporter. Thimagan et al., 2006 and Romero-Calderon et al., 2007 showed the presence of mRNA mainly in the head but with minimal expression in the thorax. This expression pattern does resemble the expression of the mammalian glycine transporter 2.

The SLC6 family has been proven to be a vital family of transporters, it has been suggested that the SLC6 family has been expressed in animals since the divergence of animals from fungi and plants (Höglund et al., 2011). As a result, understanding the evolution of this family of transporters can help us understand how nature develops these transporters based on their organismal needs. This dissertation chapter aims to identify a glycine transporter in *Drosophila melanogaster*. With this goal in mind, we hope that we can better understand how glycine transporters evolved between invertebrates and vertebrates.

3.3 Materials and Methods

Plasmid constructs: *Drosophila melanogaster* Dmel-GlyT2 (Gene: CG5549) was purchased from GenScript in pcDNA3.1(+)-C-Myc, inserted at XhoI-ApaI restriction sites. Since Dmel-GlyT2 (CG5549) is an uncharacterized protein, there is no commercial antibody available for protein recognition by an antibody and visualization.

Cell culture and transfections: Porcine aortic endothelial (PAE) cells were grown at 5% CO_2 and at 37 °C in Ham's F12 medium (10% fetal bovine serum and antibiotics). For transfection, cells were grown to a confluency of 40-80%. Cells were later transfected with the desired DNA construct Dmel-GlyT2/C-Myc. We used Effectene as a method of transfection, with specifications and directions from manufacturers (QIAGEN). Individual and isolated colonies were selected by growing transfected cells under the presence of the antibiotic G418 (400µG/ml). After the selection of individual colonies, we separated the colonies into two, half to keep growing and the other half were tested for the expression of Dmel-GlyT2-C-Myc using immunofluorescence. In a 12-well plate with coverslips, we seeded the transfected cells at 20-35% confluency. We then removed the media and wash the cells three times with 1mL of PBS. After removal of the last wash, we added 700µl of 4% PFA and incubate for 12 minutes. After the incubation, we proceeded to wash two times with 1mL of 1x PBS. Then the cells were permeabilized using 1x PBS containing 0.5% BSA, 0.1% Triton for 3 minutes at room temperature. After incubation time, the cells were washed twice with 1x PBS containing only 0.5% BSA. The next step was to incubate our cells with our primary antibody. Our primary antibody was diluted and incubated 0.6µl antibody/600µl (1-1000) for one hour at room temperature. After incubation, we washed three times with 1mL of 1X PBS 0.5% BSA buffer and proceed to incubate secondary antibody with the

same dilution for one hour in the dark at room temperature. After secondary antibody incubation, we washed the coverslips three times with 1x PBS-0.5% BSA buffer and after the last wash, we dipped our coverslip in distilled water, dried and placed onto a microscope slide.

BLASTp and Clustal Omega: Basic Local Alignment Search Tool (BLAST) is a bioinformatic algorithm that provides a wide range of tools to align sequences. Specifically, we used BLASTp, which aligns protein sequences. Clustal Omega is another bioinformatic algorithm that aligns protein sequence and delineates residue conservation and provides phylogenetic trees.

3D Model and Molecular Dynamic Simulations: Homology modeling was performed by Drs. Suman Sirimulla and Filip Fratev from the College of Pharmacy at the University of Texas at El Paso. They used both the prime package of Schrodinger software and the I-Tasser server. As a template, they used the high-resolution X-ray structures of Dopamine (pdb id: 4xpt) and Serotonin (pdb id: 5i6x) transporters.

Glycine uptake experiments: Stable PAE cells expressing Dmel-GlyT2 were grown in 24-well plates until cells reach 90-100% confluency. Once the cells reached the desired confluency, cells were placed in a water bath at 37 °C and washed two times with 250µl of reaction media (10mM HEPES pH 7.4, 135mM NaCl, 2mM KCl, 1mM CalCl2, 1mM MgSO4, and 10mM glucose). Then we proceeded to start glycine uptake by the addition of reaction media with 4 µCi of [3H] glycine/mL along with different concentrations of non-radioactive glycine (25 µM-1mM). We incubated the cells with reaction buffer with the isotope for 10 minutes at the physiological temperature of 37 °C. After incubation, we terminated the reaction by removing the reaction buffer and washed the cells two times with cold reaction buffer without glucose (buffer was stored at 4°C). To quantitate

glycine uptake, we lysed the cells using 250 µL of 0.2N NaOH, and we measured [3H] glycine with scintillation spectroscopy. We measured protein concentration by using the Bradford method (Bradford 1976) to normalize our glycine uptake. We calculated glycine uptake of our control positive control GlyT1a and our experimental Dmel-GlyT2 by subtracting the total transport of wild type PAE cells.

Statistical analysis: All statistical analyses for glycine uptake experiments were done using the Michaelis-Menten (hyperbola) equation using Sigma Plot 11.0 software.

3.4 Results

Sequence conservation between members of SLC6 family and Dmel-GlyT2

We aligned the sequences of several SLC6 family members to compare them to our protein of interest, Dmel-GlyT2, and obtained the percentage of homology with BLASTp. Of note, both tails (N- and C- terminus) were excluded from the sequence alignment for simplicity in our analysis. As shown in table 1, Dmel-GlyT2 and human GlyT2 share 52% homology, this is the highest protein sequence homology. The second place in sequence homology was between human GlyT1 and Dmel-GlyT2, which share 50% in sequence homology. The next three transporters were the *Drosophila melanogaster* Dopamine Transporter (Dmel-DAT), human Serotonin transporter (SERT), and bacterial leucine transporter, which demonstrated homology percentages of 44%, 41%, and 27%, respectively. These results suggest that there is higher sequence similarity between human GlyT2 and Dmel-GlyT2.

Table 1. Percent of sequence homologies between some members of the SLC6 family and Dmel-GlyT2 using BLASTp.

Homology %	Dmel-GlyT2	Human-GlyT2	Human-GlyT1	Human-SERT	Dmel-DAT	LeuT
Dmel-GlyT2	100%	52%	50%	41%	44%	27%
Human-GlyT2	52%	100%	51%	43%	48%	27%
Human-GlyT1	50%	51%	100%	43%	44%	22%
Human-SERT	41%	43%	43%	100%	50%	26%
Dmel-DAT	44%	48%	44%	50%	100%	26%
LeuT	27%	27%	22%	26%	26%	100%

Conservation of residues necessary for glycine binding in Dmel-GlyT2

Besides sequence homology, we wanted to determine if Dmel-GlyT2 contained the residues necessary to bind glycine and the chloride/sodium ions to transport glycine. We compared the sequence of Dmel-GlyT2 with the mammalian GlyT1, GlyT2, and other members of the SLC6 family (Yamashita et al., 2005, Penmatsa et al., 2013, Coleman et al., 2016). As shown in Figure 3.2, the sequence alignment highlights the residues that bind glycine (yellow), also suggested to bind glycine by other groups (Subramanian et al., 2016, Ponce et al., 2000). Similar results were obtained by using dynamic simulations from Dr. Suman Sirimulla's laboratory (data not published).

As shown in Figure 3.2, and based on the docking experiments Dr. Sirimulla's lab proposed several residues that are necessary to bind to glycine in the human GlyT2. From this research, we found seven residues that are predicted to form the glycine binding cage. Dmel-GlyT2 had five of those residues out of seven. Furthermore, since our collaborators used the dopamine and serotonin crystal structures to model and predict the structure of GlyT2, we wanted to also include the residues that form part of the binding cage of LeuT. We believe this is important because looking at the chemical structure of glycine and leucine, they are similar in chemical properties. In contrast, dopamine and serotonin are much bigger molecules and only have an amine group and not a carboxyl group. In Figure 3.2 the bold blue and red letters represent the residues that can bind the amine and carboxyl part of the amino acid in LeuT respectively. As shown, there is a conservation of residues that interact with the amine and carboxyl groups between LeuT, GlyT's, and Dmel-GlyT2. Thus, this analysis suggests that Dmel-GlyT2 has the necessary residues to coordinate a substrate that contains amine and carboxyl groups.

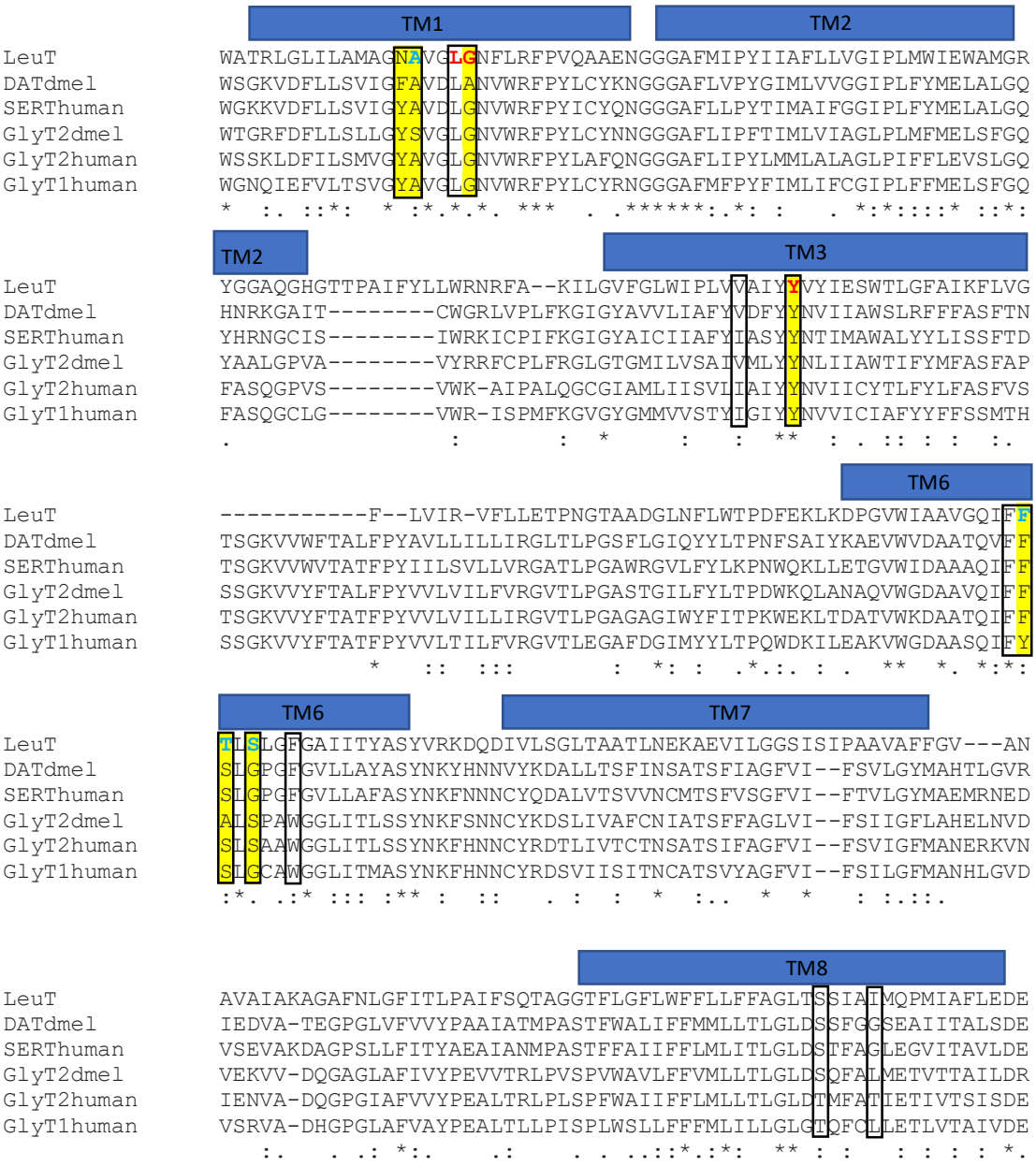


Figure 3.2: Amino acid residues that interact and binds to glycine in GlyT's in yellow. Black boxes indicate residues that coordinate leucine in LeuT, blue bold letters residues that coordinate the amino group of leucine, red the carboxyl group of leucine.

Residues necessary for ion binding between Dmel-GlyT2 and other members of the SLC6 family

If this putative Dmel-GlyT2 is a member of the SLC6 family, then it must also contain the 12 transmembrane domains and binding sites for sodium and chloride that are a hallmark of the

SLC6 family. In Figure 3.3, we show the sequence alignment for the bacterial homolog LeuT, Dmel-DAT, human Serotonin transporter, Dmel-GlyT2, human GlyT2, and human GlyT1 (Yamashita et al., 2005, Penmatsa et al., 2013, Coleman et al., 2016). We highlighted the residues that participate in the binding cage around sodium and chloride ions. These ions are vital since they provide the electrochemical gradient to transport their substrate.

Sodium binding to facilitate glycine transport is another essential component of transporter function. In Figure 3.3, residues highlighted in green contribute to the binding of the first sodium, which is very well conserved. There are only two changes in the first sodium binding site in Dmel-GlyT2, the first one is a serine instead of alanine, and the second an alanine instead of serine. The turquoise highlighted residues in this model interact with and bind to the second sodium. The residues that bind the first and second sodiums are very well conserved among all transporters of the SLC6 family; this is expected because sodium is the driving force for the SLC6 family member function. Consequently, this sequence alignment indicates that Dmel-GlyT2 possesses the necessary residues for the transport of two sodium ions.

Unlike many members of the SLC6 family, the mammalian GlyT2 uses three sodium ions instead of two to transport the substrate (Supplisson et al., 2002, Bröer et al., 2012). This is imperative because it can provide more evidence to validate the idea that Dmel-GlyT2 is glycine transporter 2 and not GlyT1. There are only two members of the SLC6 family that use three sodium instead of two, those are GAT/betaine and GlyT2 (Bröer et al., 2012). Vandenberg et al., 2016 identified the possible residues interacting in the third sodium binding site in GlyT2. Using mutations and transport assays they identified glutamate 648 as responsible for binding the third sodium. Residues highlighted in gray in Figure 3.2 represent the binding sites for the third sodium

of GlyT2. As illustrated, Dmel-GlyT2 has glutamate 648 and absent in GlyT1, which supports our hypothesis that Dmel-GlyT2 is a GlyT2.

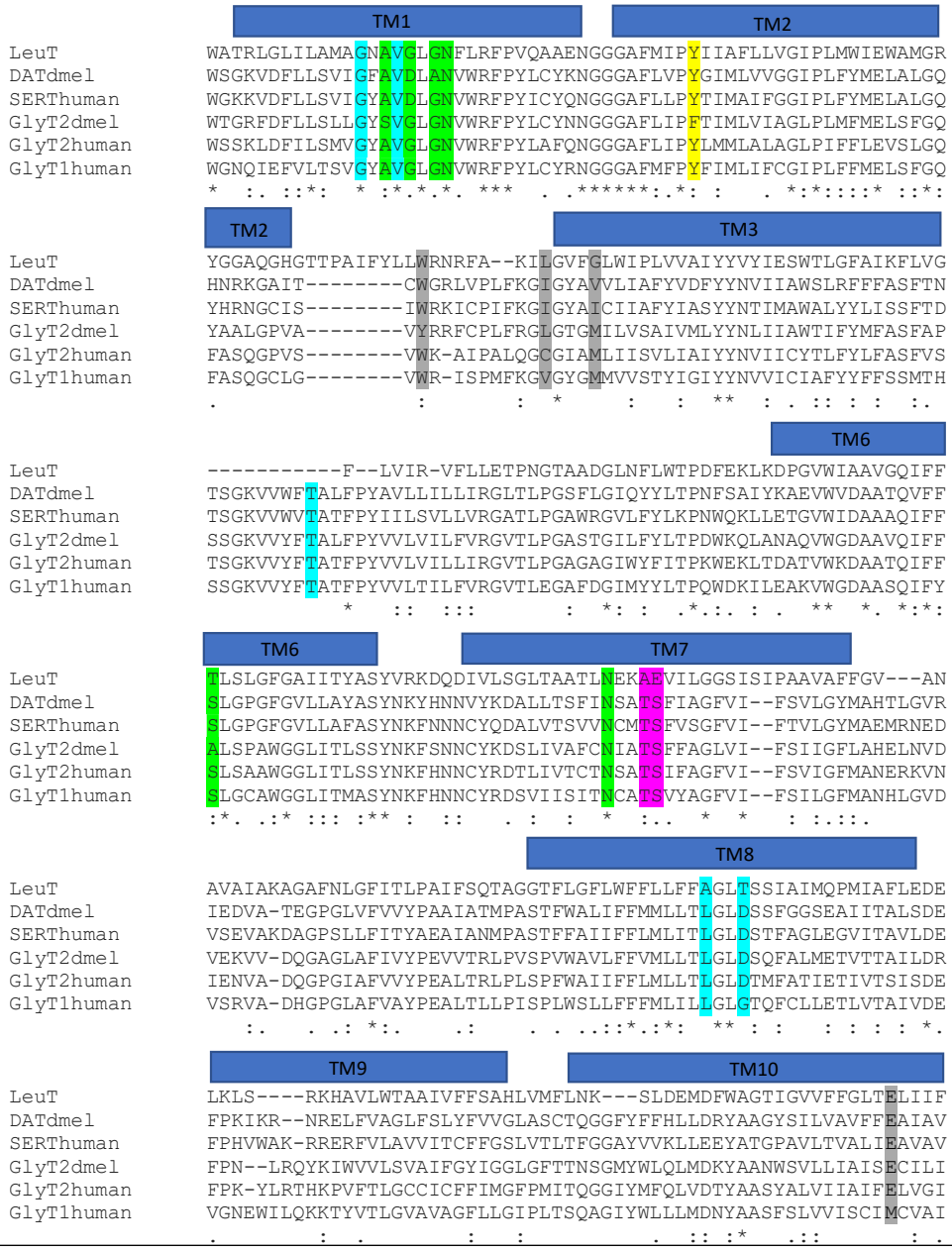


Figure 3.3: Sequence alignment of some members of the SLC6 family using Clustal Omega. (*) conserved residue, (:) R group very similar between residues, (.) R groups with limited similarity. Highlighted in Green (first sodium binding site), turquoise (second binding sodium site), gray (third sodium binding site), purple chloride binding site, and yellow (Dr. Sirimulla's laboratory proposed chloride binding site).

Also, we were interested in the residues that interact and bind to chloride. All eukaryote members of the SLC6 family (vertebrates and invertebrates) contain residues that interact with chloride, except for the bacterial homolog LeuT. Our collaborator, Dr. Suman Sirimulla proposed tyrosine-233 (yellow in Figure 3.3) as an essential residue that binds to chloride. Tyr-233 appears to bind to Cl^- using the hydroxyl group. Importantly, tyrosine (Y)-233 is also conserved in LeuT, but Dmel-GlyT2 has phenylalanine. Further research is required to analyze the importance of this residue Y233 on substrate binding. Kanner et al., 2007 proposed other residues that could bind and interact with Cl^- in different members of the SLC6 family. They suggested a threonine and serine (highlighted in purple in Figure 3.3) that interact with chloride in many members of the SLC6 family but is not conserved in LeuT.

Cumulative data derived from bioinformatic algorithms and sequence alignments support the hypothesis that Dmel-GlyT2 is a type of GlyT2. The overall sequence homology between Dmel-GlyT2 and human GlyT2 was the highest compared to other members of the SLC6 family. Also, the binding cage of glycine is well conserved between Dmel-GlyT2 and both mammalian glycine transporters. From this analysis, we conclude that this putative GlyT2 has conserved residues that can bind the three-essential sodium and chloride ions that are vital for substrate translocation with the SLC6 family.

Molecular dynamics simulations and docking experiments

In collaboration with Dr. Suman Sirimulla's research group, a 3D model of Dmel-GlyT2 was developed and we modeled a similar structure to other members of the SLC6 family (Fig 3.4A). Even though the development of a 3D model is a good start, more evidence is needed due to the

expression of Dmel-GlyT2 at the plasma membrane. As a result, using molecular dynamics simulation, Dr. Sirimulla's group predicted the structure of Dmel-GlyT2 inserted in a plasma membrane. These *in-silico* experiments allowed us to predict the structure of Dmel-GlyT2 at the plasma membrane and its similarity to the vertebrate GlyT2 (3.4 B&C). In Figure 3.4 B and C, we showed the binding cage using docking experiments and find the similarities in the amino acids that bind to the amine and carboxyl group of glycine in the human GlyT2 and Dmel-GlyT2. Dr. Sirimulla group carried out docking experiments using known GlyT2 inhibitors such as ALX1393 in Dmel-GlyT2 and found that ALX1393 can bind to Dmel-GlyT2 in a similar shape as the vertebrate GlyT2 (data not shown).

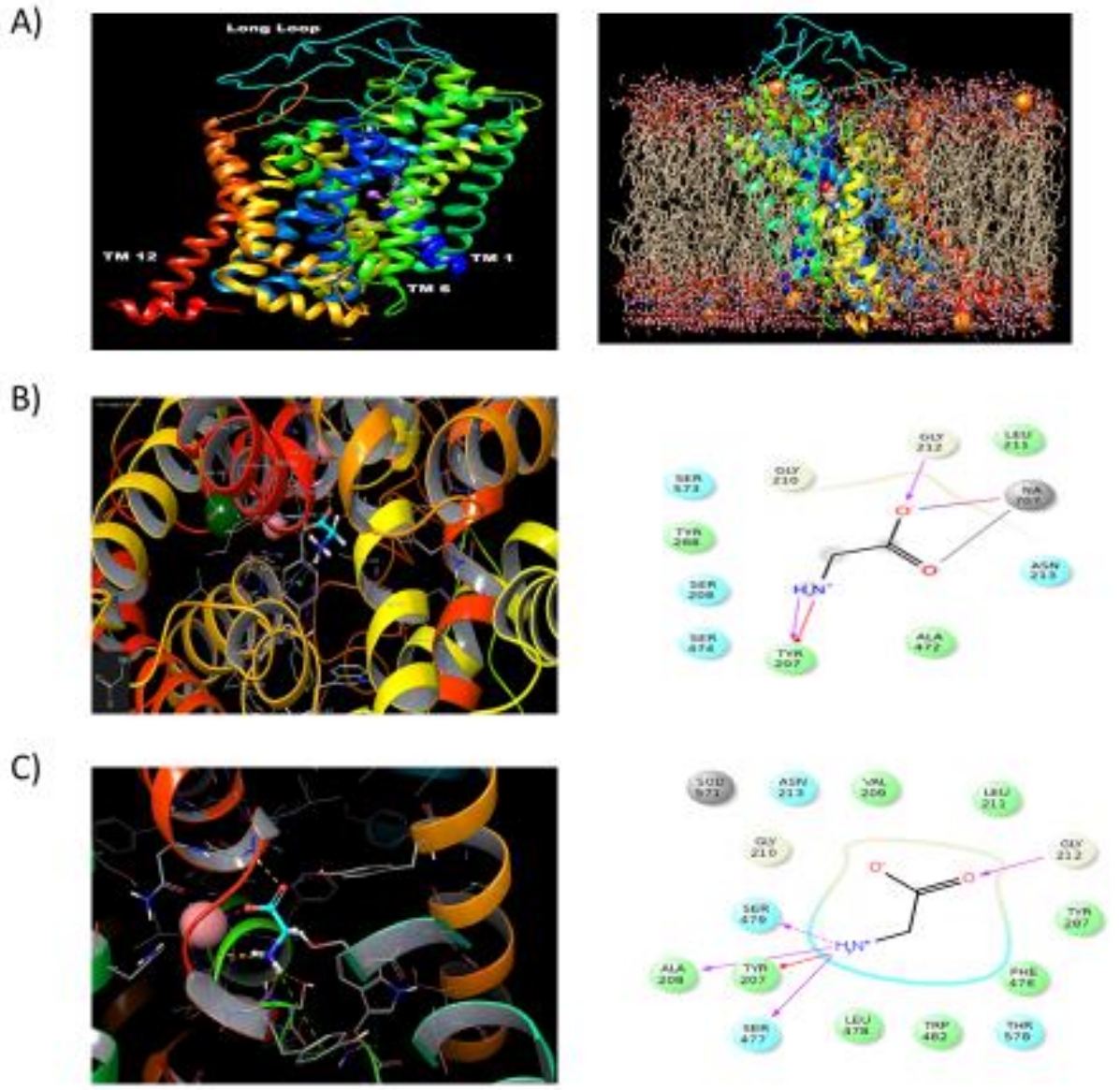


Figure 3.4: A) 3D Model of Dmel-GlyT2, containing 12 transmembrane domains and a long extracellular loop and molecular dynamic simulation of Dmel-GlyT2 at the plasma membrane. B) Docking experiments using glycine as a substrate in Dmel-GlyT2. C) Molecular dynamic simulation of human GlyT2 and docking experiments using glycine as a substrate. (Figure obtained by Dr. Suman Sirimulla's laboratory)

Successful Expression of Dmel-GlyT2-C-Myc in PAE cells

One experimental limitation of introducing a non-endogenous membrane protein into a cell line is the ability of the cell line to successfully express the protein of interest. In this case, Dmel-GlyT2-C-Myc is a *Drosophila melanogaster* gene that was transfected into PAE cells, to confirm the successful expression of Dmel-GlyT2 at the plasma membrane we performed an immunofluorescence assay using Myc antibodies. In Figure 3.5A, we can visualize the expression of Myc in red, in 3.5B is the merged Figure with DAPI which stains the nucleus. With this experiment, we can now validate the expression of Dmel-GlyT2 in PAE cells and the specificity of the Myc antibody (compare the two cells at the center vs surrounding cells).

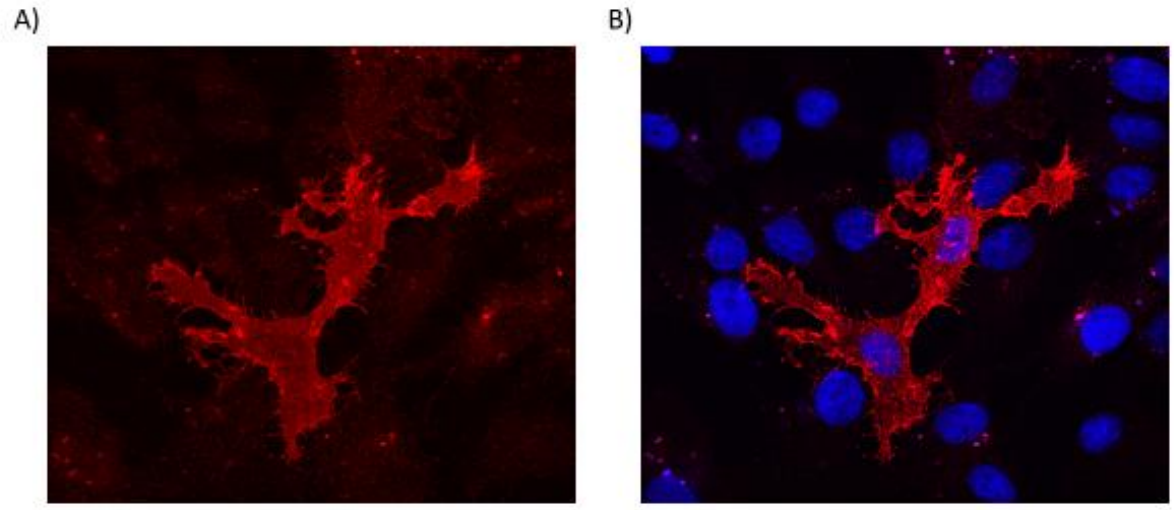


Figure 3.5: Transfection and expression of Dmel-GlyT2-C-Myc in PAE cells. A) Immunofluorescence using anti-Myc antibody followed by staining with anti-rabbit Cy3 labeled secondary antibody (Red). B) Red: Myc, Blue: DAPI.

Glycine uptake experiments

Since our objective was to test if Dmel-GlyT2 is a glycine transporter, we first used mouse GlyT1a as a control to show the kinetic characteristics of a glycine transporter. As shown in Figure 3.6A we demonstrated an efficient and saturable transport of glycine with increasing concentrations. Also, we observed the saturation of GlyT1a around 200 μM of glycine, which is similar to what has been reported in the scientific literature (Guastella et al., 1992, Liu et al., 1993).

To test if Dmel-GlyT2 is a glycine transporter we showed in Figure 3.6 the uptake assays with glycine and with different amino acids. It is worth mentioning that in all uptake experiments the red line represents the wild type PAE cells without the expression of Dmel-GlyT2. In black is the total transport of PAE cells expressing Dmel-GlyT2. In Figure 3.6B we showed the glycine uptake of wild type PAE (red) and PAE expressing Dmel-GlyT2 (black), as we can see there is a very minor difference in glycine transport between background and Dmel-GlyT2. The slight difference in glycine transport became visible at very high glycine concentrations of around 1-3mM, this is a very high concentration for any member of the SLC6 family. Additionally, we observed that glycine uptake experiments in Dmel-GlyT2 were very inconsistent and hard to replicate.

Since Dmel-GlyT2 is not transporting glycine efficiently, we wanted to try other molecules. We proceeded to test the transport of GABA by Dmel-GlyT2 (Figure 3.6C) and glutamate (Figure 3.6D). GABA uptake assays in Dmel-GlyT2 showed no significant difference between PAE and Dmel-GlyT2 even at the high concentration of 5mM. Our next step was to test glutamate

transport (Figure 3.6D), these results suggested a visible glutamate transport between non-transfected PAE cells and Dmel-GlyT2 around 2mM of glutamate. Even though that Dmel-GlyT2 transported glutamate at extremely high concentration, the uptake assay was very inconsistent and the only difference in glutamate transport was achieved in concentrations higher than 2mM. This suggests that Dmel-CG5549 (previously Dmel-GlyT2) is not a glycine transporter.

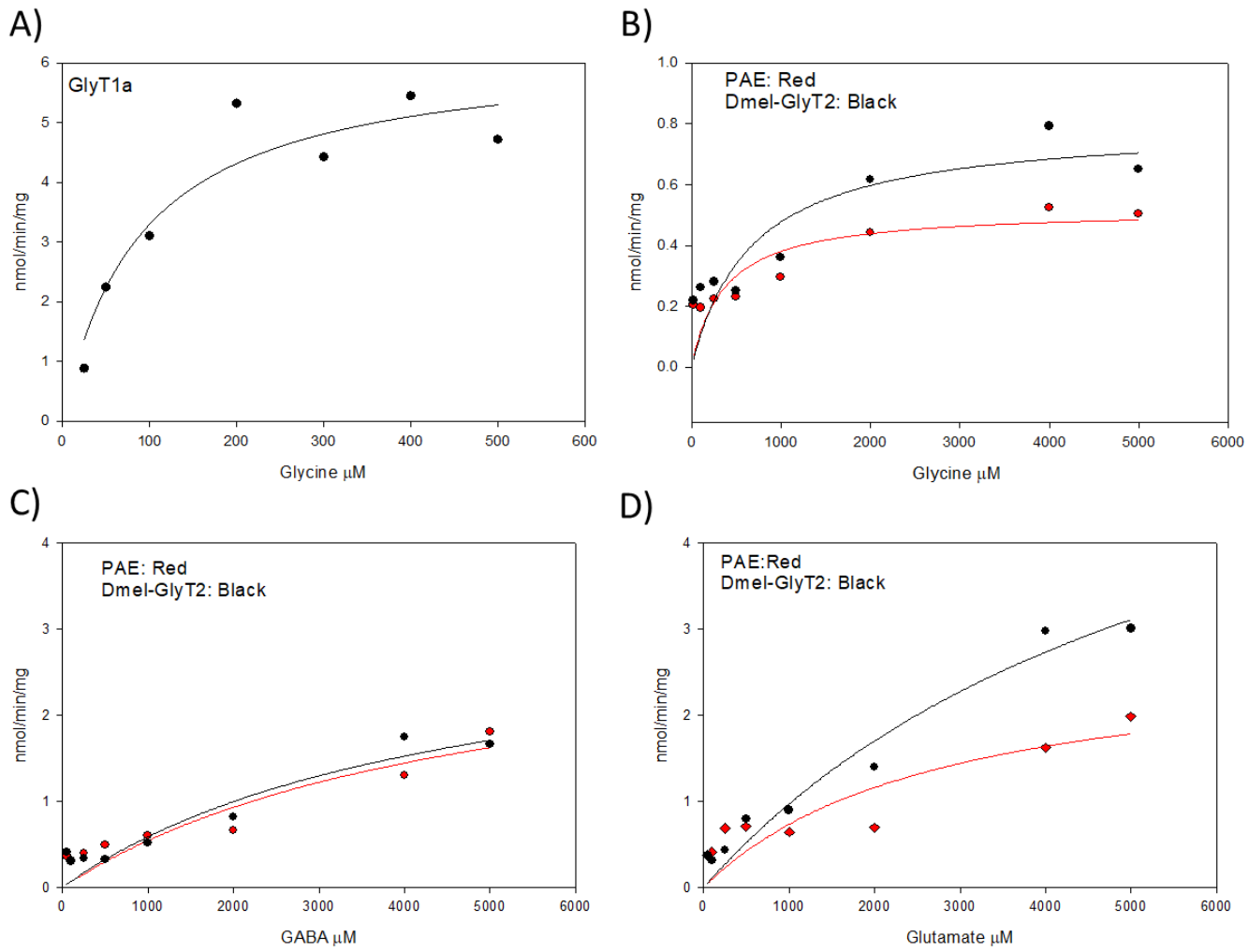


Figure 3.6: A) Glycine uptake experiments in PAE expressing the mouse GlyT1. B) Glycine uptake experiments in PAE expressing Dmel-GlyT2. C) GABA uptake experiments in PAE Dmel-GlyT2. D) Glutamate uptake experiments in PAE expressing Dmel-GlyT2.

3.5 Discussion

In Chapter 3 of this dissertation, we hypothesized that Dmel-GlyT2 is a glycine transporter. Based on sequence alignments and dynamic simulations, we show that Dmel-GlyT2 has all the components necessary to transport glycine. We used bioinformatic tools that supported our initial hypothesis: 1) overall sequence homology between Dmel-GlyT2 and other members of the SLC6 family; 2) conservation of residues that interact and bind to glycine, and 3) conservation in residues that interact and bind the three sodium ions in Dmel-GlyT2, and potentially a chlorine ion. The first first line of evidence was that the sequence of Dmel-GlyT2 revealed that the highest percentage of sequence homology is between human GlyT2 and Dmel-GlyT2 (Table 1). The line of evidence was supported by our sequence alignments in Figures 3.2 and 3.3, these alignments demonstrated that Dmel-GlyT2 has all the residues that have been suggested to bind to glycine and the necessary ions in glycine transporters.

Since the molecular structure of GlyT2 has not been elucidated, for the sequence alignments we used the X-ray structure of bacterial Leucine Transporter (LeuT). Even though that the X-ray crystallization was done more than a decade ago (Yamashita et al., 2005), we still use it as a template due to their relevant similarities to all the members of the SLC6. One of the reasons for using the bacterial LeuT as a control for our sequence alignments is that LeuT uses leucine as a substrate. Leucine and glycine are both amino acids and have a similar structure, unlike serotonin and dopamine that lack a carboxyl group. There is a high degree of amino acid sequence conservation between LeuT and vertebrate GlyT's at residues that interact and bind the amino and carboxyl groups of the amino acid. Based on this conservation, we show that

Dmel-GlyT2 also has conserved residues at positions that could bind the amino and carboxyl groups, suggesting that Dmel-GlyT2 can bind glycine.

Another interesting aspect is the residue that binds the neurotransmitter/substrate between Dmel-GlyT2, vertebrate glycine transporters, and LeuT. One specific example is LeuT, the residues necessary for the binding of leucine are Valine 104, Phenylalanine 259, and Isoleucine 359, but if you take a closer look at the sequence alignment, the amino acids found in Dmel-GlyT2, GlyT2, and GlyT1 are substituted by larger residues. Yamashita et al., 2005 suggested that in GlyT1b, compared to LeuT, there are bigger amino acids to make the binding cage more compact for glycine. However, they only made this suggestion for GlyT1b. As you compare GlyT2 and Dmel-GlyT2 in Figure 3.2 there is a pattern for bigger residues than LeuT.

As a result, this increases the size of the binding pocket that binds to the side chain of the amino acid, suggesting a much smaller binding cage for glycine in GlyT's. Yamashita et al., 2005 also noticed that in dopamine and serotonin transporters structures, the residues of the binding pocket have shorter side chains than in LeuT. For example, Isoleucine 359 in LeuT is substituted by glycine in Dmel-DAT and human SERT, which drastically increases the size of the binding pocket to allow a much larger molecule such as dopamine or serotonin to fit in. There is still more work to be done regarding the binding cage for glycine in Dmel-GlyT2, but the results of Figure 3.2 suggested that Dmel-GlyT2 has conserved residues, suggesting a similar binding pocket.

An exclusive feature of the SLC6 family is its ability to transport substrates using sodium and chlorine ions as an electrochemical gradient to drive transport. Thanks to the crystallization

of two members of the SLC6 family, we now know the residues that bind sodium ions (Yamashita et al., 2005, Penmatsa et al., 2013, Coleman et al., 2016). As expected, there is a high degree of conservation in amino acid residues coordinating these ions.

GlyT2 is one of the two members of the SLC6 family that use three instead of two sodium ions (Supplisson et al., 2002, Subramanian et al., 2016). Since we are hypothesizing that Dmel-GlyT2 is a GlyT2, then Dmel-GlyT2 must have a third sodium binding site. Analysis from Vandenberg et al., 2016 validated the position of the residue that binds the third sodium. Specifically, the third sodium binding site, Glutamate 648, was conserved in GlyT2 and Dmel-GlyT2 but not GlyT1, which further supports our hypothesis that Dmel-GlyT2 is a GlyT2-like transporter.

Different from LeuT, members of the SLC6 family are chloride dependent (Zomot et al., 2007). Based on homology modeling, we found residue conservation among all members of the SLC6 family protein sequence, suggesting the presence of a threonine and a serine that is predicted to bind Cl^- (Zomot et al., 2007). These residues are located at threonine (512) and serine (513) in the human GlyT2. Interestingly, these residues are conserved between members of the SLC6 family but absent in the prokaryote LeuT (chloride independent). In collaboration with Dr. Suman Sirimulla and his research group, homology modeling predicts the residue tyrosine 233 as a key component of the Cl^- binding site. These preliminary results support the idea that Dmel-GlyT2 is a glycine transporter.

Even though the sequence alignments were very useful in determining if Dmel-GlyT2 has sequence homology to the vertebrate GlyT2, many questions were still unanswered. Among one

of the questions was the structure of this putative glycine transporter. It is worth to mention that there is no atomic structure of any glycine transporter. Therefore, we used the Dopamine and Serotonin transporter X-ray crystallization to predict the human GlyT2 and Dmel-GlyT2 structure. Consequently, Dr. Sirimulla's research group developed a 3D model and molecular dynamics simulation of Dmel-GlyT2 (Figure: 3.4A, 3.4B). Inevitable, looking at the predicted structures we were able to compare and identify similarities in their binding cage. Both the human GlyT2 and Dmel-GlyT2 use tyrosine and glycine to interact with the amine and carboxyl group of glycine respectively. Additionally, we were able to observe that in Dmel-GlyT2 glycine is being coordinated/orientated by sodium ions, a true hallmark of the SLC6 family.

Lastly, our final line of evidence to demonstrate that Dmel-GlyT2 is a glycine transporter was to provide evidence of glycine transport. In Figure 3.6B, we demonstrated that there was glycine transport at extremely high concentrations of glycine (5mM), most members of the SLC6 family are saturated with 200 µM of their corresponding substrate. We provided evidence that our protocol is correct by doing glycine uptake experiments in the mouse GlyT1a, we demonstrated specific and saturable glycine uptake very similarly as in the scientific literature. Having demonstrated that our protocol works, we also made sure that the protein was being expressed at the plasma membrane and not being internalized (Figure 3.5). Since Dmel-GlyT2 did not show a strong affinity towards glycine we decided to also do uptake assays with other amino acids. In Figure 3.6C and 3.6D we demonstrated the poor affinity to GABA and glutamate. Another control that we are performing is the expression and uptake assays of a known *Drosophila melanogaster* GABA transporter in mammalian cells. Since the CG5549 gene is coming from an invertebrate, we had concerns over the capacity of the mammalian cell to express an

invertebrate protein. Nevertheless, the poor affinity towards glycine, GABA, Glutamate, leucine (data not shown), and proline (data not shown) suggest that this Dmel-GlyT2 might have another molecule as a substrate. With this body of evidence, we now hypothesized that CG5549 (previously Dmel-GlyT2) is not a glycine transporter, resulting in the ongoing search for a glycine transporter in *Drosophila melanogaster*.

This work has been presented in a Gordon Conference in mechanisms of membrane transport, it received a mixture of feedback, some of them agree that we should continue our search for a glycine transporter in invertebrates and others argue that with my evidence is sufficient to demonstrate the lack of glycine transporters in *Drosophila melanogaster*.

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Vita

Ashley B. Lopez was born in El Paso, Texas. The first son of Dolores Aguirre and Jaime Lopez, he graduated from Riverside High School in El Paso Texas in 2010. After graduation, he attended El Paso Community College (EPCC) for a year and a half. After transferring from EPCC to UTEP and pursuing a Bachelor's of Science in Microbiology, Ashley started researching in the laboratory of Dr. Manuel Miranda-Arango. As an undergraduate, he researched for almost two years, and his research involved the development of viral particles for the delivery of genes of interest. In the Summer of 2014, Ashley graduated from UTEP with a Bachelor's in Science in Microbiology and decided to pursue a Ph.D. in the laboratory of Dr. Miranda. Starting his Ph.D. in Fall 2014 Ashley received the Vulnerability Issues in Drug Abuse (VIDA) fellowship which provided him with a year of student salary and professional development activities that nourished his passion for mental health and the prevention of drug abuse. Afterward, he became a teaching assistant and taught many undergraduate laboratory courses such as General Biology I, Anatomy and Physiology I, General Microbiology I, and Vertebrate Physiology. He was the laboratory instructor of over 200 students ranging from nursing to pre-med, business, and humanities. He was later awarded the Keelung Hong Graduate Fellowship for two years to finish his dissertation. During his Ph.D., Ashley presented his research in leading international conferences such as the Society for Neuroscience (SfN) and a Gordon Conference.

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