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Characterization of Potential Genes Encoding for a Glycine Transporter from Drosophila Melanogaster

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CHARACTERIZATION OF POTENTIAL GENES ENCODING FOR A GLYCINE TRANSPORTER FROM *DROSOPHILA MELANOGASTER*

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Dedication

To my crazy, funny, and loving nieces, Lylithe, Lyriss, and Laynne

CHARACTERIZATION OF POTENTIAL GENES ENCODING FOR A GLYCINE TRANSPORTER IN *DROSOPHILA MELANOGASTER*

by

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THESIS

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Abstract

The study of glycine transporters has been neglected compared to other members of the Solute Carrier 6 (SLC6) family of proteins, such as the dopamine and serotonin transporters, even though they are just as crucial to survival. However, glycinergic neurotransmission has been extensively studied in mammals, resulting in vast, valuable information. The same cannot be said for invertebrates, however. In this study, we aim to establish an initial first step in elucidating components of glycinergic neurotransmission in the invertebrate, *Drosophila melanogaster.* We believe this organism possesses a glycine transporter because a glycine receptor has been identified and characterized. Specifically, we hypothesize the candidate gene, CG10804, is a potential gene that encodes for a glycine transporter in *Drosophila*. We used a bioinformatics approach to determine any conserved residues essential for glycine, sodium, and chloride binding between our candidate gene and its mammalian counterpart. Various uncharacterized *D. melanogaster* genes were analyzed by homology analysis, and it was determined CG10804 was the most likely candidate to encode for a glycine transporter based on the number of conserved residues with mammalian glycine transporters. Based on the atomic coordinates of the human GlyT1 crystal structure, the amino acids that participate in glycine, sodium and chloride binding were found to be identical to the corresponding residues in the CG10804 protein. Furthermore, gene expression, immunofluorescence and western blot analyses seemingly demonstrate that CG10804 is localized at the plasma membrane of Porcine Aortic Endothelial (PAE) cells, which fulfills a requirement as a member of the SLC6 family. However, uptake experiments demonstrated an insignificant amount of glycine transport from CG10804, suggesting this may not be a glycine transporter in *D. melanogaster*. However, future studies aim to utilize an insect cell line instead of the mammalian PAE cells, before definitively ruling out CG10804 as the *Drosophila* glycine transporter.

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List of Abbreviations

GLUT1- Glucose Transporter 1

ATP- Adenosine Triphosphate

Na+K+—Sodium, Potassium

NSS- Neurotransmitter Sodium Symporters

SLC6- Solute Carrier 6

TM- Transmembrane Domains

NTT- Neurotransmitter Transporters

GABA- γ-aminobutyric

SERT- Serotonin Transporter

DAT-Dopamine Transporter

GAT- GABA Transporter

IAAT- Insect Amino Acid Transporters

CNS- Central Nervous System

NMDAR- *N*-methyl-*D*-aspartate

GlyT- Glycine Transporter

Dmel- *Drosophila melanogaster*

NE- Norepinephrine

PAE- Porcine Aortic Endothelial

BLAST- Basic Local Alignment Search Tool for Proteins

PCR- Polymerase Chain Reaction

CMV- Cytomegalovirus

PFA- Paraformaldehyde

PBS- Phosphate Buffered Saline

BSA- Bovine Serum Albumin

TBS-T- Tris Buffered Saline with Tween-20

NIH- National Institutes of Health

hGlyT1- Human GlyT1

Introduction

Membrane potential and cell transport

Molecules move in and out of the cell through the plasma membrane in a process known as cell transport. There are two types of cell transport, passive and active, and one or the other is utilized depending on the energetics and molecule being transported (Nelson et al., 2008). Passive transport, which is subdivided into simple (Figure 1A) and facilitated diffusion (Figure 1B), does not require any energy. During simple diffusion, nonpolar and lipid-soluble solutes are able to move through the membrane from regions of high concentrations to low concentrations until both regions reach equal solute concentrations. As polar compounds cannot pass freely through the membrane, proteins are needed to facilitate this compound movement, thus activating facilitated diffusion. These membrane proteins, called transporters or permeases, are able to aid in the movement of solutes by behaving similarly to enzymes, as they are able to lower the activation energy needed for transport. Unlike enzymes, however, the transporters are not chemically altered. There are two types of transporters: carriers and channels. They differ in stereospecificity (with carriers binding substrates at higher stereospecificity) and transport rates (with channels being able to move solutes at a much higher rate than carriers). A common example of a passive transporter is the glucose transporter of erythrocytes (GLUT1), which transports glucose inside the blood cell at a far greater rate than uncatalyzed diffusion.

In contrast to passive transport, substances move against the concentration or electrical gradient, resulting in a solute concentration above equilibrium (active transport). Due to being thermodynamically unfavorable, active transport only takes place with some form of energy expenditure, such as Adenosine Triphosphate (ATP). Furthermore, active transport is subdivided into two categories: primary (Figure 1C) and secondary (Figure 1D). While both categories require

energy to function, primary transport depends directly on ATP, while secondary uses it indirectly (Alpern et al., 2008). During primary active transport, an electrochemical gradient is generated when a cation (not of interest) moves up to build a concentration gradient. This electrochemical gradient is a result of sodium ions entering the cell and the buildup of sodium ion concentration outside the plasma membrane. As the electrochemical gradient is generated, the molecule of interest is now able to move down the gradient using the energy stored in the gradient. This electrochemical gradient is a result of sodium ions entering the cell and the buildup of sodium ion concentration outside the plasma membrane. Though indirectly, secondary transport still relies on ATP to transport substrates (LibreText, 2020). Under secondary transport, two molecules (hence the alternative name, co-transport) are able to move in either the same (symport) or different (antiport) directions of the gradient.

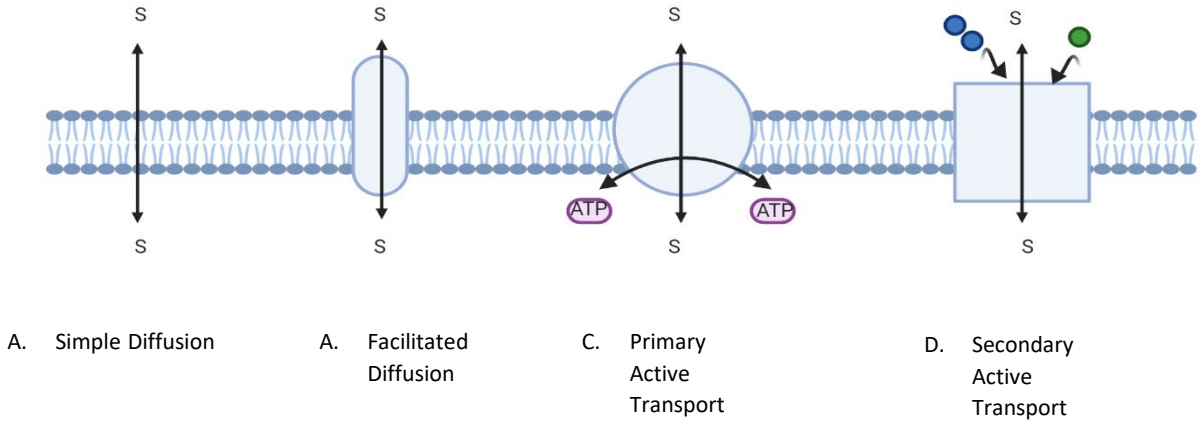

Figure 1: ***Types of transport.*** **A. represents simple diffusion, B. facilitated diffusion, C. primary active transport and D. secondary active transport using a SLC6 member as a model. Blue circles represent sodium ions and green circles represent chloride ions. (Created with BioRender.com)**

Overall, transport is only feasible through facilitation from the membrane potential and the differing ion concentrations both intra- and extra-cellularly (Ramahi & Ruff, 2014). A membrane

potential at rest naturally has a negative ion charge intracellularly and a value at approximately -70mV. A membrane becomes depolarized when the intracellular space becomes less negative in charge. Inversely, hyperpolarization is the result of the membrane potential becoming more negative. In cells, there are two positive ions (sodium and potassium), and one negative ion, (chloride), that contribute to changes in the membrane potential. Of those ions, potassium is found at higher concentrations inside the cell than outside and is the primary ion responsible for keeping a resting membrane potential. Because ions are not able to cross the membrane freely, they must travel via channels. Appropriately, potassium and sodium travel through channels named the potassium channel and sodium channel, respectively.

Along with potassium, sodium additionally contributes to resting membrane potential in neurons. The Na+K+ ATPase known as the sodium-potassium pump facilitates the resting membrane potential of a cell by pumping sodium and potassium across the membrane. Their function is to transport three sodium ions to the outside of the cell for every two potassium ions going to the interior. The sodium gradient is used by voltage-gated Na+ channels to rapidly depolarize the membrane and trigger an action potential. Action potentials are important because they are used in cell-to-cell communication. For example, in neurons, signals are sent through its axon, eventually directing the neuron to communicate with other neurons at the synapse. Though all action potentials are a response to rapid depolarization, they may vary in duration and amplitude (Hammond, 2015). Sodium-dependent action potentials occurring in the somas and axons of neurons have a large amplitude and do not last long. On the other hand, sodium-calcium dependent action potentials of cell bodies and axon terminals last longer with a plateau after the initial peak.

Solute carrier 6 family

Also known as neurotransmitter sodium symporters (NSS), the solute carrier 6 (SLC6) family is composed of secondary active transporters and has been found to be highly conserved across both eukaryotic and prokaryotic organisms. Members of this family are integral membrane carrier proteins used to transport amino acids and other similar substrates (Pramod et al., 2013). As suggested by its alternative name, transport involving SLC6 proteins are primarily sodium-dependent, though chloride is used as well. The most characteristic feature of these carriers is the 12 transmembrane domain (TM) topology possessing intracellular *N*- and *C*- termini (Ribeiro & Patocka, 2013). There is also a large extracellular loop located between TMs 3 and 4. A high number of conserved residues located along TMs 1,6 and 8 in the bacterial leucine transporter (a homolog template used for the study done by Thimgan, et. al. (2006)) were found to be crucial for sodium and substrate binding. In humans, SLC6 proteins are categorized into four subgroups: the neurotransmitter transporters (NTT), the amino acid transporters, the osmolyte transporters, and the creatine transporters. Among these four groups, the NTTs (which include dopamine, γ-aminobutyric (GABA), and glycine transporters, among others) and amino acid transporters (including proline transporters) are the most thoroughly studied, as they have been associated with the manifestation of a multitude of diseases. These transporters are essential for many physiological functions. They are responsible for maintaining osmolyte homeostasis, neurotransmitter signaling in both the central and peripheral nervous systems (Kristensen et al., 2011), etc. To be more specific, SLC6 transporters play a critical role in synaptic transmission termination by the transport of many amino acids and neurotransmitters. Throughout the years, multiple studies have been performed to determine the effects of a malfunctioning or deleted SLC6 member in various organisms. Impaired SLC6 members resulted in deleterious effects (Gomeza

et al., 2003; Quan et al., 2004; Tsai et al., 2004), establishing the importance of the SLC6 family to the physiology and survival of mammals.

Though multiple studies have found SLC6 proteins to be crucial for survival in various organisms, very little was known about the proteins in invertebrates when compared to mammals. Additional insight was gained, however, when the organism *Drosophila melanogaster* was studied in 2006 (Thimgan et al., 2006). Using a bioinformatics approach, by comparing the only previously known and characterized amino acid sequences (such as the serotonin (SERT) and dopamine (DAT) transporters) to *Drosophila* genes, 21 members (same amount as humans) of the SLC6 family were discovered. These 21 members were subdivided into one of five subfamilies (Figure 2), including a newly discovered insect amino acid (IAAT) subfamily and those including the GABA, amino acid, monoamine, and orphan neurotransmitter subfamilies. There were a few members that did not quite fit in any of the five subfamilies and were therefore not classified into any of them. Though a few of the 21 members have been identified and characterized since the publication of this report, other genes such as CG10804 and CG7075 are awaiting to be further elucidated.

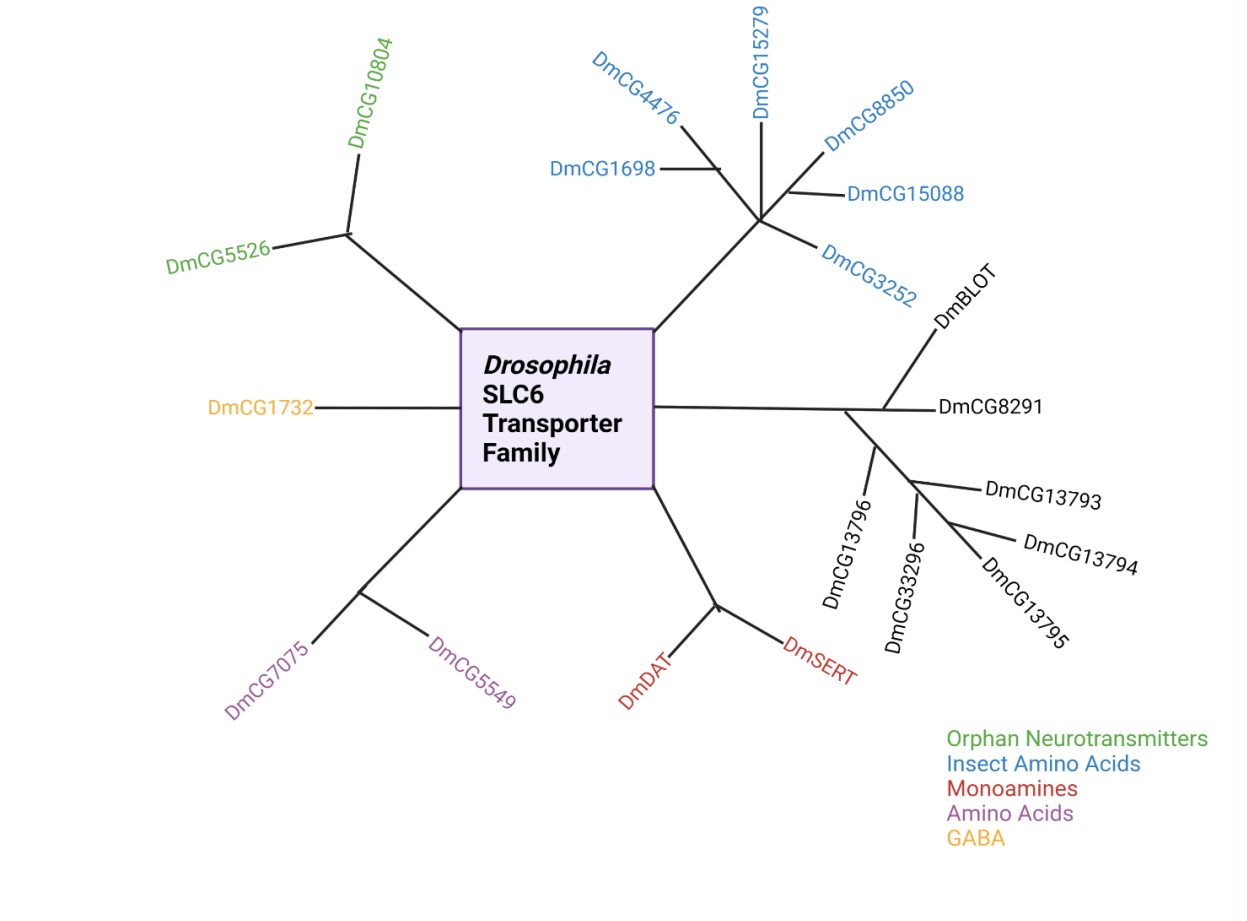

Figure 2: ***A diagram representing the Drosophila SLC6 Transporter Family*****. A similar phylogenetic tree to the one presented by Thimgan et. al. (2006), demonstrating novel Drosophila melanogaster members categorized by subfamilies. Created with BioRender.com**

Glycine transporter characterization and function

The mammalian glycine transporters belong to the SLC6 family and share all the features of the other members of the family. These transporters are primarily expressed in inhibitory glycinergic neurons of the central nervous system (CNS) where they participate in inhibitory neurotransmission, though they may also participate in excitatory neurotransmission as a co-agonist of a glutamate receptor's *N*-methyl-*D*-Aspartate (NMDA) subtype (Johnson & Ascher,

1987). When acting as the traditional inhibitory neurotransmitter, glycine activates glycine receptors usually found in the inhibitory synapses located in the spinal cord, brainstem, and retina. Hyperpolarization of the postsynaptic neuron results from the influx of chloride ions through glycine receptors. NMDA receptor activation by the L-glutamate neurotransmitter is dependent upon glycine binding to the NMDA receptor 1 (NR1) subunit present in all NMDA receptors, thus making glycine an allosteric modulator of calcium-dependent neuronal excitation (Wolosker, 2007).

There are two subtypes of glycine transporters: Glycine Transporters 1 and 2 (GlyT1 and GlyT2). GlyT2 is expressed in the brainstem, spinal cord, and cerebellum and is primarily associated with glycinergic neurotransmission, while GlyT1 is primarily found in the neocortex, thalamus, and hippocampus, and focuses mainly on glutamatergic neurotransmission. Functionally, both subtypes are responsible for synaptic glycine clearance. Another glaring difference is their transport kinetics. For every two sodium ions, GlyT1 transports one chloride ion and one glycine, while GlyT2 uses three sodium ions in exchange for glycine. While the mechanism for glycine transporter expression has not been fully elucidated, it is believed that neurotrophins and calcium signaling have an impact on glycine transporter trafficking.

As members of SLC6, they share the hallmark 12 TM domains (Figure 3), with the large extracellular loop, containing glycosylation sites, and intracellular *N*- and *C*- termini. The one major structural difference between the two subtypes is the length of the *N*-terminus, with the GlyT2 being significantly longer at 201 amino acids, compared to GlyT1's approximate 100 amino acids. Recent studies have indicated this longer tail is essential to PKC-dependent phosphorylation, as activity was found to be completely abolished at certain lengths (Gentil et al., 2020). However, this same study demonstrated that while the difference in tail length did affect

transporter stability at the plasma membrane, there was no change in glycine uptake. Together, these results suggest the GlyT2 *N*-terminus is key for transporter localization at the plasma membrane and also contributes to a scaffold complex. More research needs to be performed to understand what specific role the long N-terminus of GlyT2 plays, however.

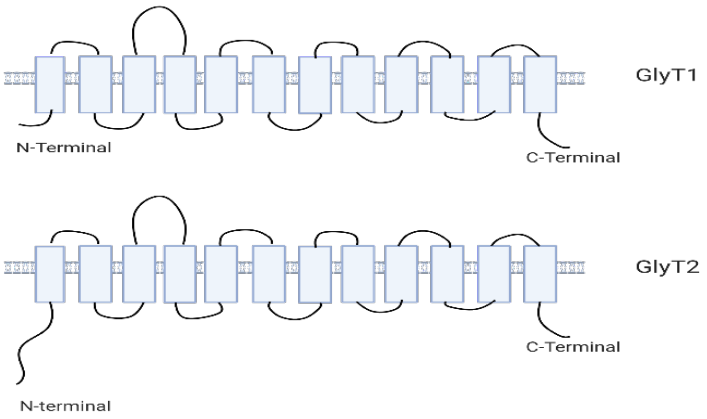

Figure 3: ***The structures of Glycine transporters 1 and 2.*** **Each have the characteristic 12 transmembrane domains with a large extracellular loop between TMs 3 and 4. GlyT2 possesses a much longer N-terminus tail. Created with BioRender.com**

Though they function independently of each other, both GlyT1 and GlyT2 are equally crucial for survival. Mouse models showed that deletion of GlyT1 or GlyT2 leads to excessive glycinergic inhibition, while GlyT2 impairment leads to a deficiency in inhibition. Usually, either abnormal inhibition ultimately proves to be fatal. Examples of phenotypes from disrupted glycine transporters include "sustained inhibition of respiratory centers in the brainstem" and the formation of the rare Startle disease (hyperekplexia), a genetic disorder characterized by an exaggerated startle response to any stimuli (Harvey & Yee, 2013) and other neurological disorders, such as schizophrenia and epilepsy. As a result, studies focusing on the relationship between glycine transporter and these illnesses have become popular in recent years.

Glycine transporters as potential drug therapy targets

As mentioned earlier, glycine transporters have been implicated in many diseases. As such, they have emerged as a popular target for drug therapies. Multiple studies over the last decade have been targeting different avenues of glycine transporters depending on the disease being desired to be treated. Some of these diseases include neurological disorders, such as schizophrenia, pain or epilepsy (Javitt, 2007; Coyle, 2012, Dohi et. al., 2009). However, researchers have been looking at avenues to inhibit the glycine transporter to potentially treat non-nervous system disorders, such as diabetes and obesity (Yue et al., 2016). The possibility of treating non-neurological disorders by targeting glycine transporters further emphasizes just how essential glycine is for overall health and survival.

Multiple groups have targeted glycine transporter inhibition to potentially treat schizophrenia, alcohol dependence, and pain. Interestingly, a malfunctioning glycine transporter is not the direct cause of any of these illnesses, so why look at this transporter? While glycine transporters are generally known to be inhibitory neurotransmitters, they also modulate excitatory neurotransmission at glutamatergic synapses mediated by NMDA (N-methyl-D-aspartate) receptors, thus making them co-agonists. As GlyT1 is the transporter most associated with glutamatergic synapses, this became each study's focus as a potential therapeutic target for the aforementioned disorders. While the dopamine receptor is the most common target to treat the positive symptoms of schizophrenia, it is not effective against negative symptoms. Attempts at targeting the NMDA receptor directly to treat the negative symptoms had been met with devastating side effects. In 2004, Tsai et al. looked to find the consequences GlyT1 hypofunction has on NMDA receptors. They did so by creating mice with an inactivated GlyT1-encoded gene and looking at the results. Overall, they confirmed that GlyT1 mediates NMDA receptor activity

and came to the realization hypofunction of GlyT1 seemed to have created antipsychotic and therapeutic effects as it leads to the hypofunction of the NMDA receptor. Following this discovery, studies dedicated to targeting GlyT1 as a schizophrenia treatment began taking place. Among them was a randomized, double-blind study involving a GlyT1 inhibitor, named Sarcosine or *N*-Methylglycine to determine its therapeutic effects on acute schizophrenia (Lane et al., 2008). Had this compound been successful, it was hoped to be used as an adjuvant therapy option with other existing treatments. Unfortunately, this compound did not produce significant therapeutic effects and the study itself had its limitations, such as a small sample size of 20 people. However, similar studies are still taking place to this day to develop GlyT1 inhibitors for schizophrenia treatment.

Studies suggest alcohol dependence can be treated by targeting GlyT1 in two possible ways. One of those ways is to enhance glycinergic inhibition, resulting in weakening the rewarding effects, caused by dopamine, which is evoked by alcohol consumption (Molander & Soderpalm, 2005). The second was approached similarly to the schizophrenia study, where the glycine-B site was stimulated on the NMDA receptor via GlyT1 inhibition. This resulted in a weakened link between drug cues and reward (Uslaner et al., 2010; Achat-Mendes, et al., 2012; Nic Dhonnchadha et al., 2012). Similarly, this now weak link provides evidence GlyT1 may also aid in the treatment of other disorders, such as Obsessive-Compulsive Disorder and other anxiety disorders (Carlsson, 2000; Wu et al., 2012).

Glycinergic neurons are vital to nociceptive (pain) signaling inhibition and are primarily located in the lamina III of the dorsal horn of the spinal cord, with GlyT2 being more abundant than GlyT1. Impairment of both glycinergic and GABAergic systems is a contributing factor to neuropathic and inflammatory pain (Vandenberg et al., 2014). Vandenberg's team went about

determining which glycine receptor would be the most viable option to potentially treat chronic pain by knocking out both GlyTs in mice. This was met with lethality. After performing an incomplete knockdown, GlyT2 was determined to be the best target. Overall, the study showed an intravenous or intrathecal injection of GlyT2 inhibitors has great potential in alleviating pain, though more research needs to be performed, as there are still significant limitations.

While diabetes and obesity do not typically fall under neurological disorders, Yue et. al. (2016) approached similarly treating these metabolic disorders as the schizophrenia study. The focus of this study was NMDA receptors located in the dorsal vagal complex (DVC) as they can suppress glucose production when activated by hypothalamic nutrient sensing. It was apparent direct administration of glycine (due to its co-agonist properties) to the DVC led to decreased glucose production as NMDA receptors were activated. Administering the amino acid glycine, however, is not a feasible option as they tend to possess poor pharmacokinetics. This then brought up the idea of manipulating glycine transporters to regulate the concentration of glycine, as had been previously done with potential therapies for schizophrenia treatment. The results were optimistic, as they showed GlyT1 inhibition in the DVC led to decreased glucose production, increased glucose tolerance, reduced feeding food intake and lower body weight in both healthy and diabetic rodents, all of which are necessary for maintaining health for those suffering from diabetes and obesity (Yue et al., 2016).

Glycinergic neurotransmission in invertebrates

Neurotransmission in vertebrates has long been studied, while invertebrate research is slowly advancing. Among the most studied invertebrates, regarding neurotransmission, is *Hydra vulgaris*, a freshwater polyp of the *Cnidarian* phylum. The presence of a glycine receptor in this organism was only detected in 2001 through biochemical and behavioral studies (Pierobon et al.,

2001) and significant discoveries about the significance of glycine have been made since then. Among those discoveries is the role that glycine plays in *Hydra* pacemaker activity. It is apparent glycine affects the endodermal rhythmic potential (RP) system (responsible for body column elongation) and ectodermal contraction burst (CB) pacemaker systems (responsible for body column contraction) of *Hydra* through "modulation of the function of the circuits that produce the electrical correlates of behavior" (Ruggieri et al., 2004).

Similar to *Hydra*, glycinergic neurotransmission is implicated in a pacemaker system in a different invertebrate, *Drosophila melanogaster*. *Drosophila* possesses a central circadian pacemaker in the small ventral lateral neurons (sLNvs, also known to be glycinergic neurons) responsible for locomotor activity (Frenkel et al., 2017). Frenkel's group was able to determine downregulation or impaired production of glycine resulted in an altered circadian period length by a nearly one-hour increase and impaired rhythmicity (normal rhythmicity is necessary for functional processing within the neuronal network), ultimately revealing glycine aids in circadian network synchronization, at the very least.

Along with the previously mentioned *Hydra* and *Drosophila* organisms, a multitude of invertebrates were studied to test Dale's law, which essentially states one neuron releases a single neurotransmitter at all synapses. Though this has largely been proven to be incorrect, this theory has been primarily tested on vertebrates (Gillespie et al., 2005; Vaaga et al., 2014; Trudeau & El Mestikawy, 2018; Pedroni & Ampatzis, 2019). Therefore, single-cell transcriptomics was performed on a variety of Metazoans to determine if Dale's law applies to different types of species, both vertebrates and invertebrates. (Brunet Avalos & Sprecher, 2021). Brunet Avalos & Sprecher went about answering their question by analyzing fast-acting neurotransmitters in neurons by analyzing scRNAseq cell databases and comparing the expression of any marker genes

encoding for neuronal identities, such as any vesicular transporters or enzymes involved in the synthesis or transport of neurotransmitters. Overall, the results demonstrated the majority of the neurons from the various organisms studied do adhere to Dale's law, though some did express more than one neurotransmitter, simultaneously, such as was discovered in *C. elegans*. This was true across all species studied in this experiment.

The characterized transporters of *Drosophila melanogaster*

As previously mentioned, very little is known about the genes that are part of the SLC-6 family of Drosophila transporters. However, there have been a few genes that have been confirmed as various transporters, such as the dopamine (Dmel DAT), GABA (Dmel GAT), serotonin (Dmel SERT) transporters, and CG7075, an amino acid transporter. Of these four transporters, Dmel SERT was the first to be characterized by Demchyshyn et al. (1994). Demchyshyn's group first used a bioinformatics approach to determine if there was homology among the previously unknown isolated cDNA and similar mammalian transporters. It was determined the amino acid sequence of the dSERT was most similar to its mammalian counterpart, hSERT at 51%. It also shared a high amino acid sequence homology with both the mammalian dopamine and norepinephrine transporters at 47%. To confirm that the isolated cDNA did encode for dSERT, the cDNA of hSERT was transfected in COS-7 cells and eventually used in 5HT uptake assays performed in HeLa cells in the presence of Na+ and Cl-, as SLC-6 members are known to be sodium and chloride dependent. Accumulation of radioactive 5HT (indicative of dSERT presence) was measured via liquid scintillation spectroscopy. The final overall results confirmed the characterization of dSERT.

The approach used by Demchyshyn et al (1994) to characterize dSERT was similarly performed a few years later to characterize dopamine transporters in two invertebrates *C. elegans*

(Jayanthi et al. 1998) and D. melanogaster (Porzgen et al. 2001). Once again, the cDNA of the gene of interest (dopamine transporter) was isolated and screened for amino acid homology with other transporters. Both the predicted *C. elegans* (CeDAT) and *D. melanogaster* (dDAT) shared the highest homology with SERT, NE, and DAT of various organisms. Interestingly, both CeDAT and dDAT shared a higher percentage of identity with the NE transporter of various organisms. CeDAT cDNA was compared to human, mouse, and bovine DA, SERT, and NE transporters, while dDAT was compared to the same transporters of humans, along with CeDAT. While dDAT shared the most identity with hSERT at 52%, CeDAT is the other most similar gene at 51%, more so than hDAT at 49%. While both invertebrate dopamine transporters were more closely related to serotonin transporters in amino acid sequence, they both functionally behaved as dopamine transporters, thus identifying them as such. This was determined by uptake experiments similar to the dSERT experiment for both CeDAT and dDAT, with the addition of electrophysiology analyses to aid in the characterization of dDAT.

CG7075 has been predicted to be a transporter for glycine, however, it is thought that it is a neutral amino acid transporter, therefore it is able to transport other neurotransmitters or amino acids, and is not specific to just glycine. This protein has been found to be only present in male *Drosophila* flies (Thimgan et. al., 2006) and was later determined to specifically be found in the testes and is responsible for spermiogenesis (Chatterjee et. al., 2011).

The experiments described above aided us in carrying out our own experiments to identify a glycine transporter in Drosophila. As Jayanthi et. Al (1998). and Porzgen et al (2001). demonstrated, it is not enough to just rely on amino acid sequence alignment to predict the function of any gene product, as it may function differently than expected. Therefore, we kept this in mind when we performed our experiments.

Drosophila gene CG5549 eliminated as a candidate for glycine transporter

Originally, it was proposed based on homology analysis that the gene, CG5549 (an amino acid transporter first identified by Thimgan et al (2006) encoded for the glycine transporter in *Drosophila*. In fact, literature today refers to CG5549 as the putative glycine transporter. However, very few experimental studies have been performed to confirm the gene as a glycine transporter. Among these experiments was one performed by Frenkel et al. (2017), in which truncated CG5549 RNA was injected into oocytes with glycine transport subsequently being measured, using radioactive glycine. While glycine transport was observed, it was only a minimal difference (about one-fold) when compared to the control oocytes that were injected with water. Therefore, the results were not convincing enough to definitively conclude CG5549 is the *Drosophila* glycine transporter.

Furthermore, a previous student of Dr. Miranda's performed his own experiments to confirm whether CG5549 is the glycine transporter (Lopez, 2020). He expressed the CG5549 gene in mammalian cells and took the human GlyT1 as positive control and then proceeded with the glycine uptake experiments. Reaction media containing glycine were added to the cell, along with varying concentrations of non-radioactive glycine. Glycine uptake was measured using scintillation spectroscopy, taking the human positive GlyT1 as a control. After repeating the experiment multiple times and increasing the concentration of glycine, the results ultimately revealed no statistically significant difference in glycine transport between the mammalian PAE cells expressing CG5549 and the wild-type PAE cells, once again ruling out CG5549 as a *Drosophila* glycine transporter. Based on these findings, it is likely that another SLC6 family member with high homology to the mammalian glycine transporter is the putative gene.

Hypothesis

Due to the *Drosophila* gene, CG5549, being eliminated as a candidate for a glycine transporter, we, therefore, analyzed other uncharacterized genes based on their similarity to their mammalian counterpart. In this analysis, we looked for overall homology, and similarity in the glycine, sodium and chloride binding sites. The results from this analysis narrowed the candidates to two genes, CG7075 and CG10804. However, it is believed CG7075 is a neutral amino acid transporter, rather than a specific glycine transporter. Therefore, we hypothesize CG10804 encodes for a glycine transporter in *Drosophila melanogaster*. To investigate whether the CG10804 is a glycine transporter, we pursued the following aims:

Aims

Aim A.) Localization of control and candidate genes in mammalian cells through immunofluorescence.

As confirmed members of the SLC-6 family of proteins, mammalian cells expressing our candidate and control genes are expected to be localized at the plasma membrane. To identify the localization of the proteins, the candidate proteins possess the Myc tag, an epitope tag used for protein detection, at the *N*-terminus. The immunofluorescence requires the use of a primary antibody, which specifically recognizes and binds to the Myc tag of the protein. A fluorophore-labeled secondary antibody will then recognize and bind to the primary and will allow the visualization of the expression of the transporters under the microscope by fluorescence. This analysis allowed us to confirm protein expression, as well as the location within the mammalian cell.

Aim B.) Confirm expression of control and candidate genes in mammalian cells with western blot.

While immunofluorescence analyses are a satisfactory initial step to take to confirm we possess the correct protein needed to carry out further experiments, it only elucidates the localization of the protein. Furthermore, the primary antibody will recognize and bind to any protein possessing the Myc tag. As all our genes contain the Myc tag, we performed a western blot to determine the expression levels and the molecular weight of the proteins and compared them to their expected molecular weight based on their amino acid sequence. We were able to ascertain if we possess the correct proteins in their mature, folded state by comparing the experimental weight to the proteins' expected molecular weight based on their amino acid sequence.

Aim C.) Determine if our candidate and control genes encode for a glycine transporter in *Drosophila melanogaster*.

Up to this point, there have been very few studies on glycinergic neurotransmission in invertebrates, compared to mammals. While a few transporters have been characterized in *Drosophila*, such as the dopamine and serotonin transporters, glycine transporters have yet to be characterized. As a glycine receptor has been confirmed in *Drosophila,* we hypothesize there is also a glycine transporter. We performed uptake experiments on our candidate genes, CG10804, and control genes, human GlyT1 (hGlyT1), the original putative Dmel GlyT2, and non-transfected PAE cells. We used radiolabeled glycine in the presence of non-radioactive glycine and sodium and chloride ions and measured uptake via scintillation spectroscopy. With this experiment, we were able to determine if our candidate gene encodes for a glycine transporter in *D. melanogaster*.

Materials and Methods

BLASTp search and Clustal Omega: The Basic Local Alignment Search tool (BLAST) can be found on the National Institutes of Health (NIH) website and essentially functions as a database for previously identified sequences of genomes of various organisms. Specifically, we used the BLASTp (proteins) tool to compare *Drosophila* sequences to known mammalian glycine transporter sequences to narrow and choose our candidate *Drosophila* glycine transporters. Additionally, another bioinformatics tool, Clustal Omega was used to align multiple protein sequences and identify conserved residues.

Plasmid constructs: As *Drosophila's* dopamine (Dmel-DAT) and GABA transporter (Dmel-GAT) are already well characterized, these genes were to be utilized as controls and have been cloned, along with our candidate genes CG7075 and CG10804. Each of the four genes was amplified using the polymerase chain reaction (PCR) technique, using primers that introduced the appropriate restriction sites at the 5' and 3' ends. The restriction sites, KpnI and EcoRV were introduced to Dmel-DAT, Dmel-GAT and CG7075, while KpnI and EcoRI were introduced to CG10804. The products that resulted from PCR were our "inserts." Following amplification, the inserts were ligated in the vector, pcDNA 3.1(+) (also containing the corresponding restriction sites). This vector possesses the human cytomegalovirus (CMV) promoter, as seen in Figure 4, which was useful for the high expression of our fully cloned gene. The now ligated vector and inserts were then transformed in NEB (New England Biology Labs) DH5-alpha competent *E. coli* cells and were plated on Luria-Bertani (LB) agar plates containing ampicillin, as pcDNA 3.1(+) possesses an ampicillin resistance marker, which made it useful to select for the desired plasmid constructs. The resulting colonies were then inoculated in LB media, also containing ampicillin, and incubated overnight at 37 degrees Celsius, shaking at 220 rpm. Plasmid DNA was then

purified according to the Qiagen mini-prep protocol and was verified using restriction enzymes and observed on 1% agarose gel.

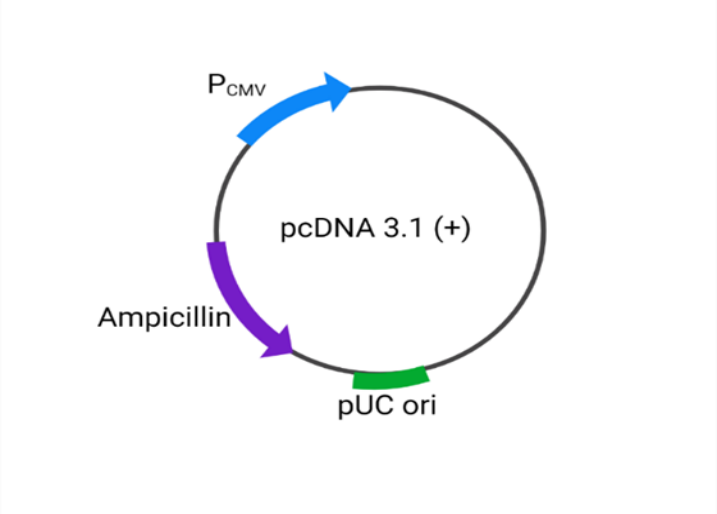

Figure 4: ***Mammalian vector pcDNA 3.1 (+) used for cloning.*** **Our genes of interest were ligated in the mammalian vector pcDNA 3.1 (+) possessing the CMV promoter and ampicillin resistance marker. (Created with BioRender.com)**

Cell Culture and Transfection: Due to its flattened morphology and easy visualization of the plasma membrane, we decided to use Porcine Aortic Endothelial (PAE) cells. The PAE cells were grown at 37° Celsius and 5% CO2 in Ham's F-12 media (containing 10% Fetal Bovine Serum, glutamax, and streptomycin-penicillin antibiotics). The cells were grown to about 80% confluency and transferred to 12-well plates to be used for transfection. In addition to the previously mentioned control genes and candidate genes, an established positive control, the former putative Dmel GlyT2 (CG5549) was transfected in the PAE cells with the lipid, Effectene, used as the transfectant. This was done in accordance with the specifications of the manufacturer of the Effectene Transfection Reagent kit (Qiagen). The transfected cells were then used to isolate and

select individual colonies in 10 cm^2 culture dishes in F-12 media containing G418 antibiotic (100 µg/ml). The media and antibiotic were replaced every three days for a period of three weeks or until several colonies formed that were visible to the naked eye. The colonies were then transferred in half to two separate wells (with one containing a coverslip). One well was utilized to keep growing the cell line and the other (containing the coverslip) was used to confirm the successful transfection of the control and candidate genes via immunofluorescence.

Immunofluorescence: The transfected PAE cells were seeded on coverslips in a 12-well plate and were grown to 20% confluency. In addition to the transfected cells, non-transfected PAE cells were also seeded and utilized for immunofluorescence as a negative control. Once grown to the necessary confluency, the cells were washed three times with I mL of 1X Phosphate Buffered Saline (PBS). Following the final wash, 700 µL of 4% paraformaldehyde (PFA) was added and the cells were incubated at room temperature for 12 minutes. The PFA was applied as a fixing reagent. After the incubation period, the cells were washed twice more with 1X PBS and 800 µL of a solution consisting of 1X PBS, 0.5% Bovine Serum Albumin (BSA) and 0.1% Triton. This solution was used as a permeabilization buffer for the cells and were left to incubate at room temperature for 3 minutes. The solution was then removed, and the cells were washed with 800 µL of 1X PBS containing 0.5% BSA (wash buffer). The cells were then incubated with our primary antibody (anti-Myc monoclonal, mouse; Cell Signaling Technology), which was diluted in a 1:2000 ratio in the wash buffer (0.8 µL of antibody/400 µL of wash buffer). The cells were incubated with the antibody for one hour at room temperature. Following the hour of incubation, the cells were washed three times with the wash buffer and were then incubated with the secondary antibody that was used as a fluorophore (Cy3, mouse) at the same dilution as the primary antibody for one hour at room temperature in the dark. The cells were then washed three more times with

the wash buffer and were incubated with DAPI, a marker that stains the nucleus of the cell, at a 1:1000 ratio (0.4 µL of DAPI in 400 µL of wash buffer) for three minutes at room temperature. The cells were then washed twice more with wash buffer. The coverslips were then dipped in milliQ water to remove any debris and placed on a microscope slide using MWOIL to mount the coverslip. The completed slides were then observed utilizing confocal microscopy.

Gel Electrophoresis and Western Blot: To further confirm the presence of proteins in our control and candidate genes, we performed a western blot. Transfected PAE cells were seeded on a 6-well plate to 100% confluency. We then placed the transfected cells on ice for 10 minutes and then washed them three times with 1X PBS. Following the third wash, the cells were then lysed in TGH buffer (containing 10% glycerol, 25 mM HEPES, 100 mM NaCl, 1% triton and deoxycholic acid, 1 mM EDTA, 1 mM EGTA, and 250 mM PMSF). Using a cell scraper, the cells were then scraped to the wall of the well in the lysis buffer and were then collected and transferred to a pre-chilled Eppendorf tube. The cells in the buffer were then nutated at 4° C for 10 minutes. Following the 10 minutes, the tubes were then centrifuged at top speed for 15 minutes, also at 4° C. The tubes were then placed on the ice again, and the supernatant was transferred to another pre-chilled tube. Once transferred, we added 4X Laemmli Buffer to the samples, finalizing the samples to be used for electrophoresis.

The samples were then loaded on a 10% SDS-PAGE gel to be separated by molecular weight. Following the gel run, the proteins were then transferred to a nitrocellulose blotting membrane for one hour and 30 minutes at 4° C and at 115 V. Once the transfer was completed, the membrane with proteins was washed 3 times for 3 minutes each with 1X Tris Buffered Saline and Tween-20 (TBS-T), which consists of 10 mM Tris base, 150 mM NaCl and 0.1% Tween-20 at pH 7.5. 5% blocking buffer (Carnation dry milk diluted in TBS-T) was then added to the

membrane for one hour and was washed 3 more times for 3 minutes to prevent unspecific binding. The primary antibody from Cell Signaling Technology was then prepared at a 1: 1000 dilution (10 µL anti-Myc monoclonal, mouse in 10 mL TBS-T) and added to the membrane to be incubated overnight at 4° C. The primary antibody was removed, and the membrane was washed at the same specifications as the previous washes. The secondary antibody was prepared at a 1:10000 dilution (1µL anti-mouse DAM HRP in 10 mL TBS-T) and was added and incubated for one hour. The membrane was then washed according to the same procedure and was developed using the ECL western blotting substrate (Thermoscientific) and was imaged using the iBright software.

Glycine Uptake measurement: To measure transporter uptake, we seeded and grew stable PAE cells expressing human GlyT1 (hGlyT1), Dmel GlyT2, CG10804, and non-transfected PAE cells in a 24-well plate to 100% confluency. Duplicates of each cell line were seeded. The cells were then placed in a 37° C water bath and were washed twice with 250 µL of uptake buffer (containing 10 mM HEPES-tris at pH 7.4, 135 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose). Following the washes, half of the wells were treated with 250 µL of uptake buffer containing 4 µCi of [^{3}H] glycine/mL, along with a concentration of 500 µM of non-radioactive glycine. The other wells were treated with the same solution, but also with the addition of 2 µM of ALX 5407, an inhibitor specific to hGlyT1. Each well was incubated with the solution in the water bath for exactly 10 minutes. Following the incubation period, the uptake buffer was removed from all wells and washed twice more with 300 µL of the uptake buffer. To release the radioactivity from the cells, they were lysed with 250 µL 0.2 N of NaOH and placed on a nutator for at least 4 hours. 100 µL of the lysate was then placed in 5 mL of scintillation cocktail and glycine uptake was then measured by spectroscopy.

Results

CG10804 and CG7075 Isoforms

In the mid-1990s, the existence of various isoforms of mammalian GlyT1 were characterized, being a result of alternative RNA splicing (Kim et al., 1994). These isoforms differ in the length of the *N*-terminal tail of the transporter. However, multiple studies have suggested that the different lengths do not result in any type of effect on transport activity. Seemingly, the only difference among these splice variants is the expression pattern in the central nervous system and peripheral tissues (Kim et al., 1994; Borowsky and Hoffman, 1998; Hanley et al., 2000)

Similar to the mammalian glycine transporter, various isoforms of the *Drosophila* candidate genes have been reported. These variants have been confirmed primarily through mRNA analysis. Furthermore, these genes have been regularly revisited and annotated through RNA-seq data, transposable element analysis, and other transcriptomic assays (Mirsa et al., 2002; Matthews et al., 2015) since the *Drosophila melanogaster* genome was sequenced in 2000. It was found from these analyses that CG10804 and CG7075 possess four and two splice variants, respectively (NIH; FlyBase). The difference between CG7075 isoforms A and B is a 48 amino acid longer N-terminus tail possessed by Isoform A (Figure 5). On the other hand, there is slightly more variation among the CG10804 isoforms. CG10804 isoform A has a slightly larger (20 amino acids) extracellular loop between transmembrane domains 7 and 8, while isoform C has a 64 amino acid longer *N*-terminus tail, compared to the others (Figure 6). Curiously, isoforms B and D are identical in sequence conservation, but are still listed as two separate entities, as listed in the NIH databank, though are not listed on FlyBase. To determine the isoform used in this project, we compared all sequences of the isoforms to the sequences of the genes obtained from the

Bloomington Drosophila Stock Center. Based on this comparison, it was determined that the CG10804 splice variant, purchased for our experiments, corresponds to the isoform D (Figure 6, circled in red) and for CG7075 the isoform A (Figure 5, circled in red). Based on the mammalian glycine transporter isoforms where variations in the N- and C-terminal tails do not affect transport but rather trafficking, we can assume that the same is true for the *Drosophila* genes. Just as their mammalian counterparts, we believe the isoforms are expressed in different CNS areas, though at this time we were primarily focused on confirming either or both of our candidate genes as a glycine transporter. If we obtain promising data, future experiments will test if the other isoforms vary by having sequence differences at the N- and C-terminal tails.

CG7075 Isoforms

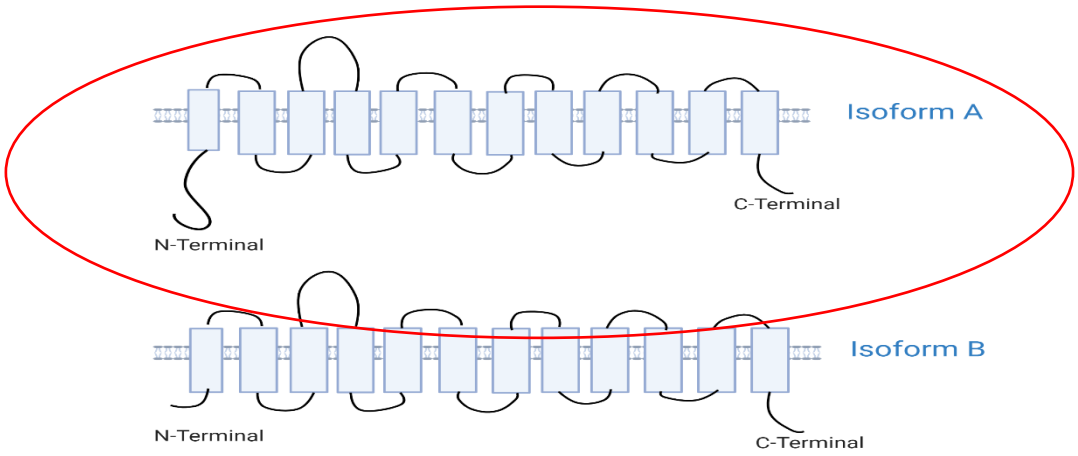

Figure 5: ***The two isoforms of Drosophila melanogaster gene CG7075.*** **Isoform A possesses a longer tail than isoform B. The circled isoform represents the gene we are utilizing in our study. Created with BioRender.com**

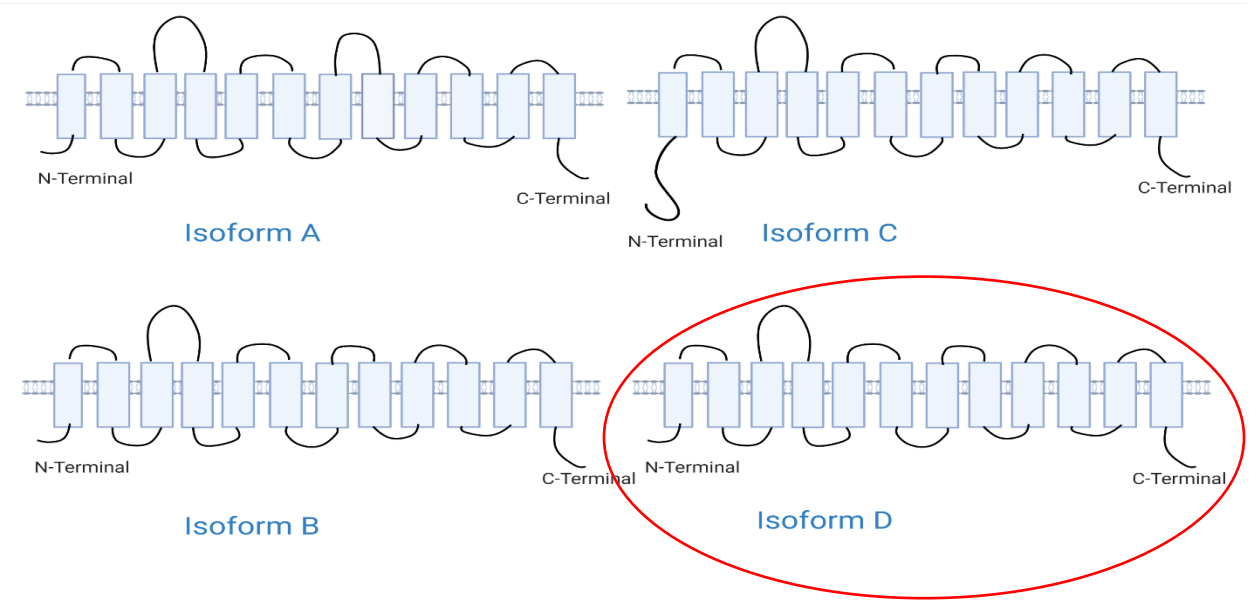

Figure 6: ***The four isoforms of Drosophila melanogaster gene CG10804.*** **Isoform A possesses a larger extracellular loop and Isoform C possesses a longer N-terminus tail. The circled isoform represents the gene we are utilizing in our study. Created with BioRender.com**

Sequence homology between candidate genes and other SLC6 members

As SLC6 sequences are highly conserved among various organisms, we used the BLASTp tool to compare the protein sequences of our control genes (Dmel-DAT and Dmel-GAT) to the human DAT and GAT, followed by our unknown candidate genes (CG10804 and CG7075) to the well-characterized Human-GlyT1and Human-GlyT2 sequences. We found nearly the same overall percent homology between the candidate gene, CG10804, and both Human GlyT1 and GlyT2 at 38%. The other candidate, CG7075, shared a slightly higher homology to Human GlyT1 and GlyT2 at 44% and 41%, respectively. The gene originally thought to be the *Drosophila* glycine transporter shares an overall 48% and 49% homology to GlyT1 and GlyT2, respectively.

The control protein sequences, Dmel-DAT and Dmel-GAT showed homology to their human counterparts at 52% and 39%, respectively. The results from this homology analysis can be found in Table 1 below. In this homology analysis, we also compared the sequences without the N- and C-termini tails, which vary among the same transporters from different species.

Table 1: ***Sequence similarity of control and candidate genes and their human counterparts.*** **Table demonstrating the overall percent homology, homology with the exclusion of N- and C-termini, and homology of just the transmembrane domains. of sequences between the *Drosophila* genes and their human counterparts. Highlighted emphasize candidate genes.**

Gene	Similarity (with tails)	Similarity (without tails)	Similarity (Transmembrane Domains)
Human GlyT1 and Human GlyT2	49%	51%	49%
CG5549 and Human GlyT2	49%	52%	42%
CG10804 and Human GlyT2	38%	40%	41%
CG7075 and Human GlyT2	41%	41%	41%
CG5549 and Human GlyT1	48%	50%	36%
CG10804 and Human GlyT1	38%	38%	41%
CG7075 and Human GlyT1	36%	43%	43%
Dmel-DAT and Human DAT	52%	54%	42%
Dmel-GAT and Human GAT	39%	54%	46%

Residues essential for glycine and ion binding in candidate genes

Through the BLASTp search, we compared the sequence homology of our candidate genes to their mammalian counterparts and other SLC6 members. We wanted to take it a step further and determine if our candidate genes possess the necessary residues needed to interact and bind to glycine and ions, specifically sodium and chloride. Using Clustal Omega, we aligned and

compared the protein sequences of our candidate genes with mammalian GlyT1 and GlyT2, along with other SLC6 transporters. Based on the bacterial leucine transporter (the bacterial homolog of glycine transporters commonly used as a template), it is believed conserved residues located at transmembrane domains (TM) 1, 3, and 6 (Figure 7, highlighted in yellow) of glycine transporters (Zafra & Gimenez, 2008; Lopez 2020) along with residues in extracellular loop 2 (Frenkel et al., 2017) form a binding pocket for glycine uptake. We found CG5549 shares six out of the seven residues needed for glycine binding to mammalian GlyT1 and GlyT2, while CG7075 shares five.

Two binding sites have been confirmed in both mammalian glycine transporters, while a third biding site has been proposed for glycine transporter 2 (Subramanian et al., 2016). The first sodium (Na1) binding site is located between TMs 1,6, and 7 (Figure 8, highlighted in blue), while the second (Na2) site is located between TMs 1 and 8 (Figure 8, in green). Based on the results Subramanian et al. obtained, it is thought the proposed third binding site (Na3) for glycine transporter 2 is located between TMs 3 and 10 (Figure 8, in purple), with glutamate located at residue 648 being the main binding site for sodium. We determined CG1084 is perfectly conserved through all six residues required for the Na1, while CG7075 shares five out of the six residues. For Na2, CG10804 and GlyT1 share three out of four residues and are perfectly conserved with GlyT2. Furthermore, both candidate genes possess glutamate 648 thought to be essential at the proposed Na3, indicating that one or both genes are specifically a glycine 2 transporter.

As chloride ions are also a driving force of SLC6 transporters, we sought to determine if our candidate genes contained the proposed residues needed to interact and bind to chloride. It is thought that serine and threonine residues found in TM 7 (Figure 8, in orange) are required to bind to chloride in many members of the SLC6 family (Zomot et al., 2007). Both of our candidate genes contain these residues, suggesting a chloride dependence on *Drosophila* transporters.

The high number of residues essential for glycine and ion binding shared between our candidate genes and GlyT1 and GlyT2 further validate our hypothesis that one or both of our candidates may encode for a glycine transporter in *Drosophila*. CG10804 shares a slightly higher number of residues than CG7075, making the former a more likely glycine transporter candidate. Furthermore, CG10804 shares more residues with Gly2 than GlyT1 and contains the glutamate residue thought to be needed for a third binding site proposed only for GlyT2, giving indication CG10804 is most likely a glycine transporter 2 in *Drosophila*.

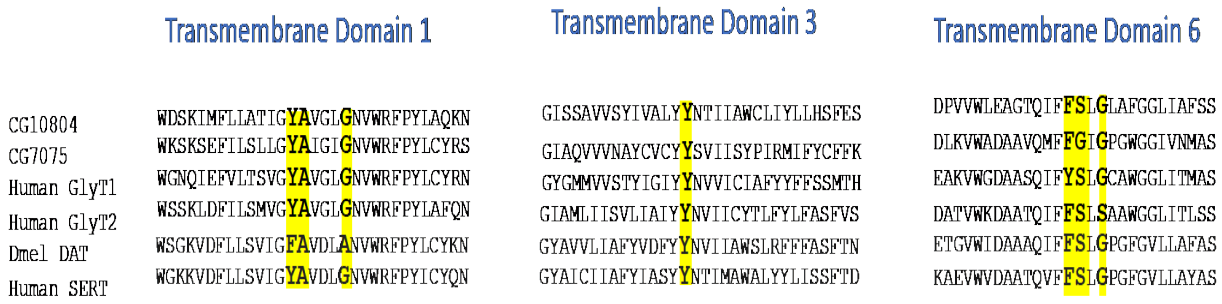

Figure 7: ***Glycine binding sites.*** **Highlighted depicting amino acid residues that interact with and bind to glycine in various SLC6 members.**

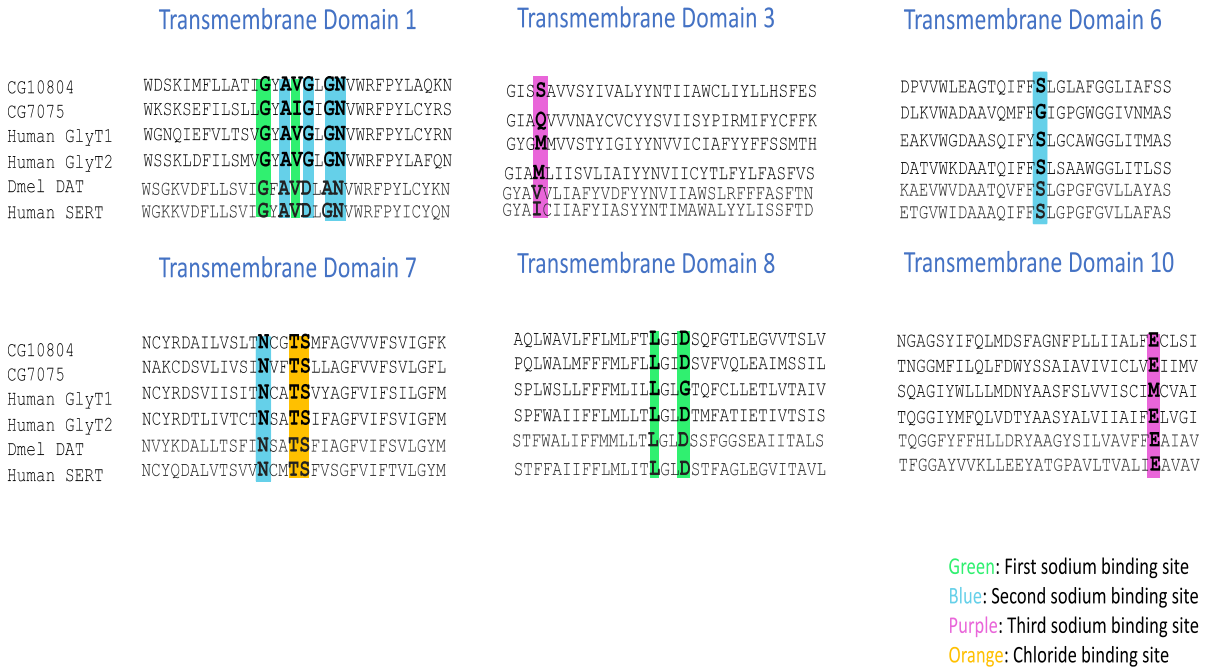

Figure 8: ***Amino acid residues that interact with and bind to sodium and chloride ions.*** **Residues highlighted in green represent the first sodium binding site, blue represent the second sodium binding site. Residues highlighted in purple represent the proposed third binding site in GlyT2. Residues highlighted in orange represents chloride binding site.**

Expression of *Drosophila* genes in PAE cells (immunofluorescence)

Once our control and candidate genes were cloned in pcDNA 3.1(+) containing the N-myc tag, they were stably transfected in PAE cells. We also analyzed PAE cells transfected with vector control, possessing no transporter, and utilized as a negative control. This negative control was confirmed by immunostaining with anti-MYC antibody and confocal microscopy, as seen below in Figure 9A. We also confirmed the expression and localization of the putative proteins by staining with a mouse anti-Myc primary antibody and a mouse Cy3 secondary antibody. The results showed the expression of the corresponding proteins at the periphery, delineating the cell (the putative Dmel-GlyT2), likely at the plasma membrane of PAE cells. Also, perinuclear staining

was observed, representing a newly synthesized transporter in the ER. This pattern of expression has been reported for many mammalian neurotransmitter transporters (Figure 9B). In addition, when PAE cells are transfected with the gene, CG10804, we detected Myc signal at the periphery, presumably at the plasma membrane (Figure 9D), representing expression of the protein at the predicted cell location. To clearly confirm plasma membrane localization, we would need to study co-localization with a plasma membrane marker, a series of experiments that remain to be performed. Interestingly, the other candidate gene, CG7075, showed a perinuclear localization consistent with the endoplasmic reticulum (ER) position, suggesting that this protein is retained in the endoplasmic reticulum (Figure 9C). Two possibilities can explain this expression pattern: the ER localization could be due to protein misfolding, or the protein is rather an ER resident. Given that we expect a plasma membrane localization for a glycine transporter, we did not further characterize this gene product. In addition, immunofluorescence on various resulting colonies of GAT and DAT transfection was shown to have no expression (data not shown). As a result, we were not able to analyze these two proteins as controls.

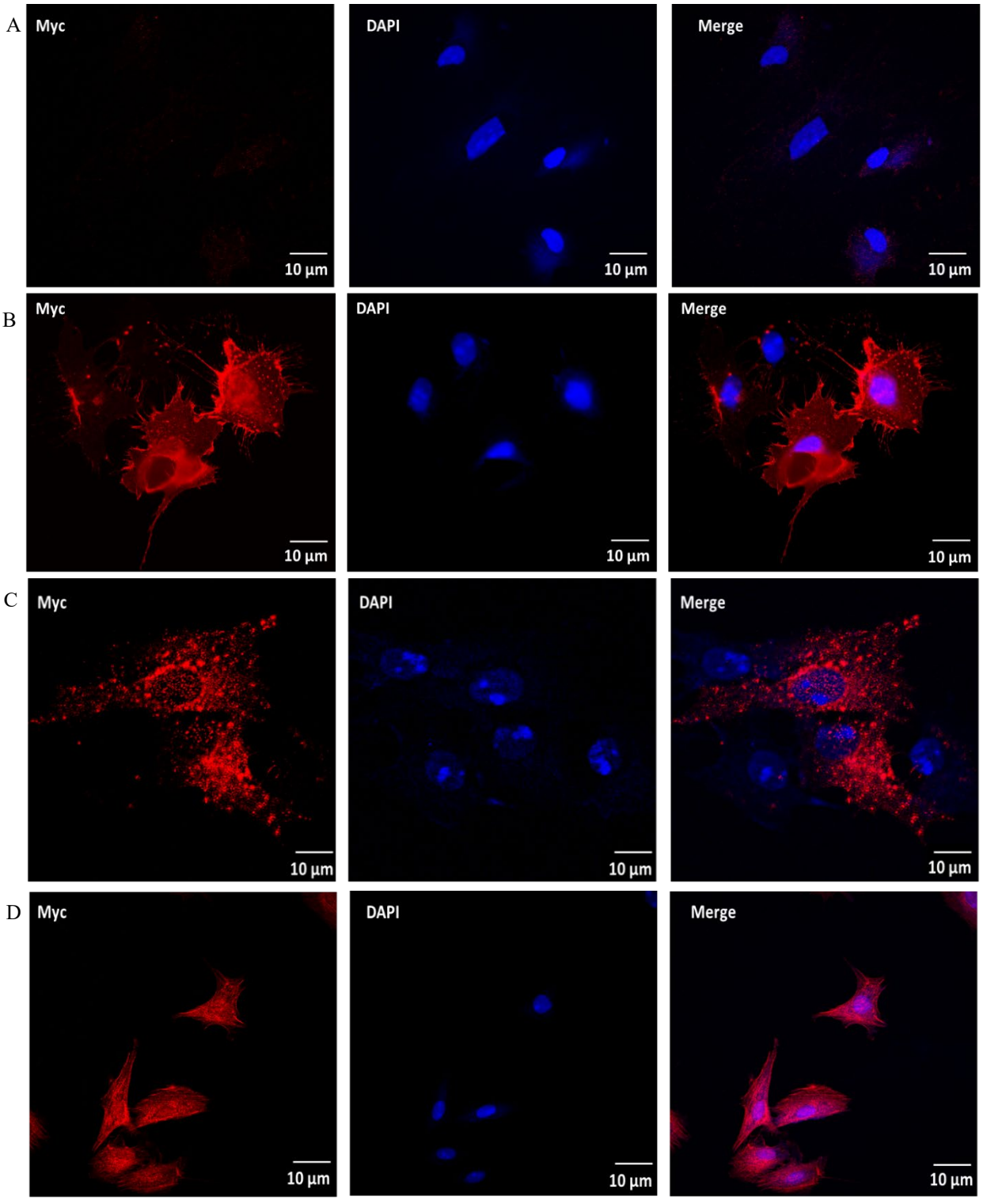

Figure 9: ***Expression and localization of control and candidate genes*****. Row A depicts the anti-Myc, DAPI (blue), and merge images of PAE cells with no protein expression. Row B and D seemingly depict Myc expression (red) of the original, putative Dmel GlyT2 and CG10804, respectively, at the plasma membrane. Row C shows CG7075 with mostly internalized expression. All images were taken at 63X magnification.**

Detection of transporter expression by Western Blot

To analyze the expression of the selected transporters, we took stable expressing PAE cells which were lysed, and cleared by centrifugation, and the lysates were subjected to electrophoresis and western blot analysis. Based on the amino acid sequence of the proteins, Dmel GlyT2 has a predicted molecular weight of around 122 kDa, the Dmel GAT a predicted weight of 72 kDa, Dmel DAT of 70 kDa and CG7075 of 67 kDa. Finally, CG10804 has a predicted weight of 74 kDa. The results of Figure 10 showed immunoreactive bands for CG10804 (Figure 10B) and GlyT2 (Figure 10A, 10B) corresponding to their predicted weight (Table 2) While CG7075 does show levels of expression, it corresponds to about 55 kDa, which is lower than the expected weight. These immunoreactive bands are specific for the corresponding transporter given that PAE cells were utilized as a negative control, and resulted in no immunoreactive bands (Figure 10A). Some weak signal was observed in control PAE that appeared in all the different cell lines but were different from the signal from the transporter protein. Based on this analysis and immunofluorescence staining, we demonstrated that the primary localization of CG7075 is presumably at the endoplasmic reticulum, as opposed to the plasma membrane. Although two studies suggest the expression of this transporter in the testes, we speculate that this transporter functions in the endoplasmic reticulum. As a result, we decided not to use CG7075 in the uptake experiments, as glycine transporters are decidedly plasma membrane proteins.

Table 2: ***Predicted and experimental molecular weight of control and candidate genes.*** **This table demonstrates the predicted weight of the proteins based on amino acid sequence and the weight detected by western blot analysis. The amino acid length of the proteins have been included also.**

Protein	Predicted Molecular Weight (kDa)	Actual Molecular Weight (kDa)	Amino Acid Length
Dmel GlyT2 (CG5549)	122	122	1,188
CG7075	67	50	590
CG10804	74	73	662

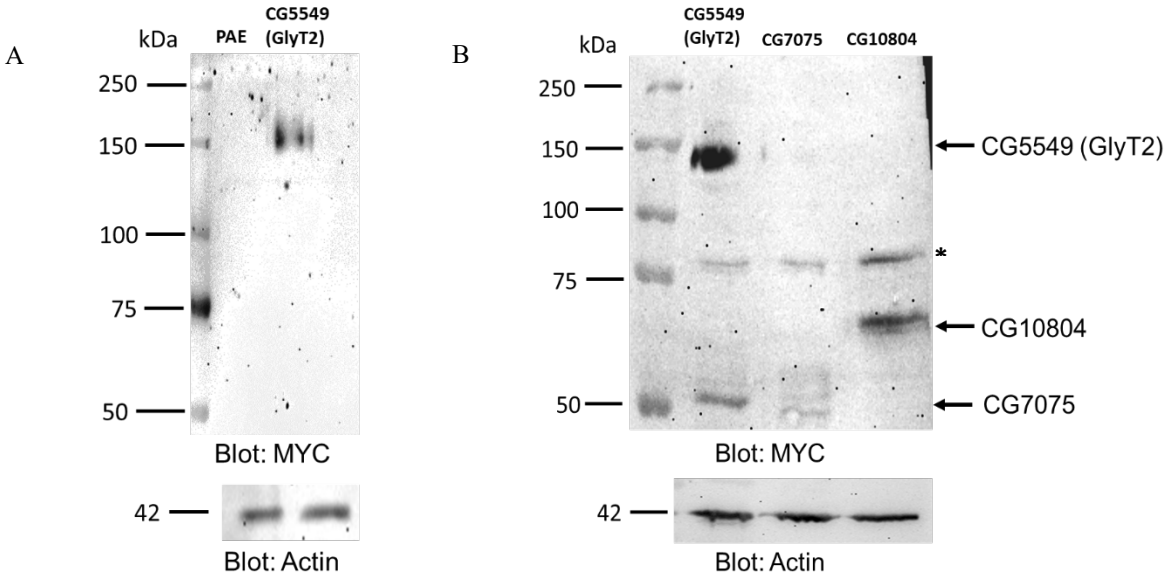

Figure 10: ***Expression and molecular weight of control and candidate genes.*** **Panel A represents the ladder in lane 1, negative control, PAE cells in lane 2 and the original, putative Dmel GlyT2, CG5549 in lane 3. Panel B depicts the ladder in lane 1, CG5549 in lane 2 and candidate genes, CG7075 and CG10804 in lanes 3 and 4, respectively. Actin was used as a loading control for both blots. Asterisk indicates background signal background for all samples at 70 kDa, and not true expression.**

Glycine Uptake

Following confirmation of the correct proteins at their predicted molecular weight, we tested non-transfected PAE cells (with vector) and Dmel GlyT2 (negative controls), hGlyT1 (positive control), and our candidate gene, CG10804 to observe if any glycine uptake took place. This experiment was performed three times and the transport is represented in pmol/min/well. As expected, hGlyT1 resulted in the highest transport capacity in the presence and absence of the mammalian GlyT1 inhibitor, ALX 5407 at 121 and 275 pmol/min/well (Figure 11), respectively. Not surprisingly, non-transfected PAE cells exhibited markedly lower counts for both the inhibited and non-inhibited samples at 61 and 113 pmol/min/well, respectively (Figure 11). As mentioned earlier, Dmel GlyT2 was previously considered and eliminated as a potential candidate for the *Drosophila* glycine transporter in previous uptake experiments performed by Dr. Miranda's lab. The results for Dmel GlyT2 reinforced the previous findings, basal glycine transport values of 65 (inhibitor) and 92 (no inhibitor) pmol/min/well. The candidate gene CG10804 produced similar results to Dmel GlyT2 with uptake values of 70 (inhibitor) and 86 (no inhibitor) pmol/min/well. Altogether, the results show that CG10804 and Dmel GlyT2 have a transport capacity similar to non-transfected PAE cells, suggesting that CG10804 does not transport glycine under the experimental conditions described in the methodology section.

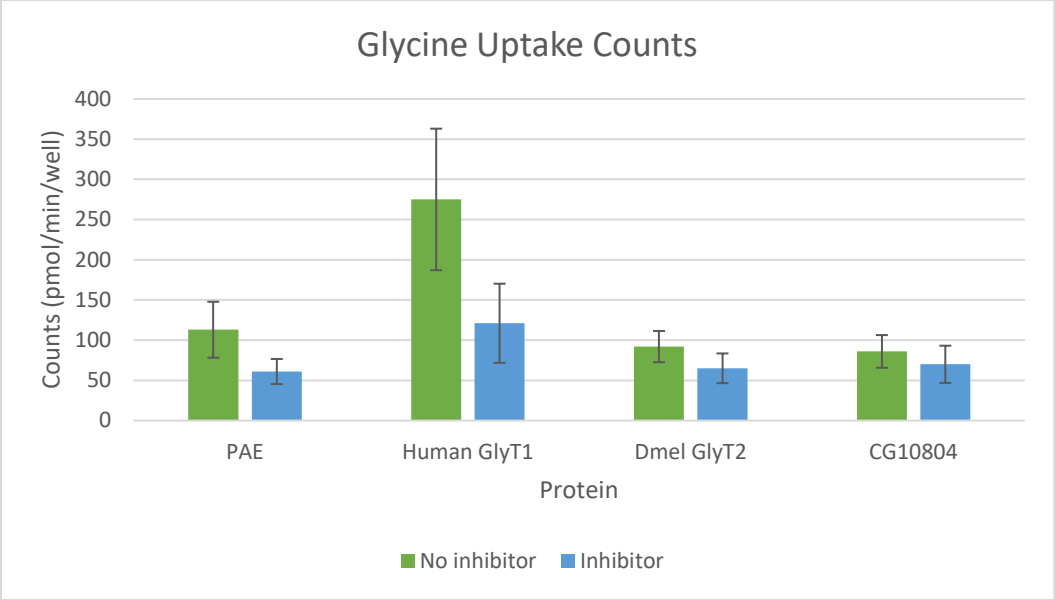

Figure 11: ***Glycine Uptake in PAE cells.*** **This graph includes the counts of non-transfected PAE cells, Human GlyT1, the original putative Dmel GlyT2, and candidate gene. CG10804. Results of cells applied without inhibitor are depicted in green and cells with inhibitor are represented by blue. All counts are expressed in pmol/min/well.**

ALX 5407 was applied to all samples to inhibit GlyT1-mediated glycine transport and was used as an internal control. However, it is important to note this inhibitor is known to be specific to human GlyT1 only. There is not enough evidence to suggest ALX 5407 will inhibit the transporter from other species, nor are there any known inhibitors for the other proteins available. We decided to apply the inhibitor to all cell lines and look for any differences. We observed a reduction of glycine uptake across all proteins as seen above (Figure 11). To find specific transporter counts, we subtracted the inhibitor counts from non-inhibitor counts for human GlyT1 only. Since there is no evidence of inhibitor specificity for the other genes, specific transporter activity was determined by subtracting the transport from the PAE cell from the transport obtained from the candidate genes (Figure 12). These results support the raw data uptake counts and further

suggest CG10804 is not a glycine transporter, as the protein presented specific counts of 27 pmol/min/well, a significant reduction from GlyT1's 154 pmol/min/well.

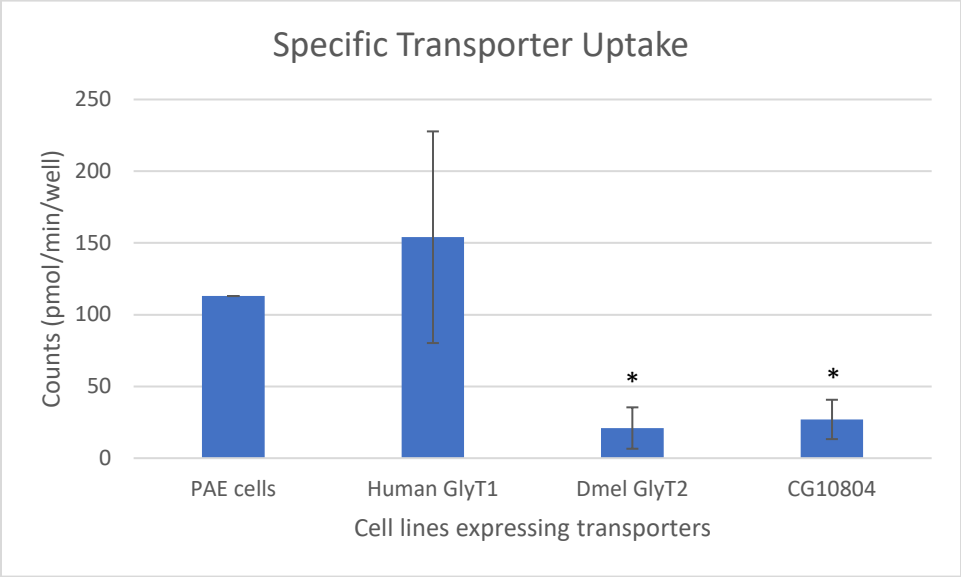

Figure 12: ***Results of Specific Glycine Transporter Activity.*** **This graph includes the counts attributed to specific transporter activity of non-transfected PAE cells, Human GlyT1, the original putative Dmel GlyT2, and candidate gene. CG10804. Asterisk indicates statistical significance as determined by $p<0.05$ (n=3).**

Discussion

Glycine transporters and other SLC6 members have been well-studied and characterized in mammals for many decades. In contrast, however, very little is known about this family member in invertebrates. The *Drosophila* dopamine (DAT) and serotonin (SERT) transporters were discovered in the 1990s and were the only neurotransmitters characterized up until 2006, when 21 additional members in *Drosophila* were identified. Despite the *Drosophila* genome having been sequenced, many family members' functions remain a mystery, even over 15 years after initial sequencing. When the original putative *Drosophila* GlyT2, CG5549 was characterized by our laboratory, we found that expression in mammalian cells did not confer the ability of the transporters to translocate glycine, under our experimental conditions. It is important to mention that the experimental conditions used to characterize the *Drosophila* DAT and SERT are similar to ours and the transporters bound and transported the neurotransmitter. Due to this, we decided to test other potential glycine transporter candidates. By comparing the sequence homology of the unknown *Drosophila* genes to human GlyT1 and human GlyT2 with the BLASTp search, we narrowed the unknowns to our two candidate genes, CG10804 and CG7075. Based on the high percentage of homology and sequence conservation, we hypothesized one or both candidate genes encode for a glycine transporter, with CG10804 being the best candidate for GlyT2. Additionally, detailed alignment analyses showed a high number of conserved amino acid residues essential for glycine, sodium, and chloride binding and a third sodium binding site at position Glu 648 only found in mammalian GlyT2. Given the above information and the presence of a glycine receptor fully characterized in *Drosophila,* the inference of the presence of a glycine transporter is strengthened.

Given that members of the Drosophila SLC6 family are plasma membrane proteins, we performed an immunofluorescence stain to confirm both the mere presence of protein, as well as the location within the PAE cell. With the former putative Dmel GlyT2, CG5549, being used as an established positive control, we were able to confirm the successful transfection of our candidate and control genes. Furthermore, we demonstrated that one of our candidate genes CG10804 is likely localized at the plasma membrane. However, while our other candidate gene, CG7075, was shown to be expressed in the cell, it was unexpectedly localized primarily near the nucleus (possibly the endoplasmic reticulum), as opposed to the plasma membrane. This data goes against the characteristics of the SLC6 family of proteins. We believe this may be due to a couple of factors: the protein had not reached its mature form due to folding difficulties because of the position of the MYC tag, impinging steric hindrance, or CG7075 may not even be a plasma membrane protein but rather a resident protein of the endoplasmic reticulum. Further experiments would need to be performed to test these theories. Because of CG7075 not reaching the plasma membrane, we decided it would be best to omit the protein from the glycine uptake experiments. Similarly, we were not able to produce any viable clones of our intended controls, Dmel DAT and Dmel GAT, as evidenced by both immunofluorescence and western blot, so we decided to use human GlyT1 as a positive control instead.

Western blot analysis was used for quantitative purposes. While the immunofluorescence assay allows us to see protein expression and localization, it does not give us the full picture of the protein. Therefore, we were able to gain more insight into our control and candidate genes by determining the molecular weight of the proteins, as well as their concentration. We were able to determine the results of the western blots supported the results of the immunofluorescence. Both our positive control, the former putative Dmel GlyT2 as well as our candidate gene, CG10804,

correspond to their predicted molecular weights based on their amino acid sequences. However, CG7075 deviated from its predicted molecular weight and was demonstrated to have a lower weight than anticipated.

The human GlyT1 was used as a positive control for the uptake experiments, with non-transfected PAE cells as the negative control. We then determined the capacity of putative Dmel GlyT2 and CG10804 to transport glycine. As expected, the human GlyT1 resulted in the most glycine transport, while the negative control showed very little uptake. Somewhat surprisingly, CG10804 demonstrated insignificant transport similar to Dmel GlyT2, suggesting this protein does not encode for a glycine transporter in *Drosophila melanogaster.* However, we are reluctant to definitively conclude CG10804 is not a transporter due to one major factor. As mentioned before, PAE cells are mammalian cells that function optimally at 37° C. *Drosophila* proteins are synthesized and folded in cells that function optimally at a lower temperature of 30° C. Therefore, future studies aim to repeat uptake experiments at 30° C, instead of the greater temperature. It may also be worth transfecting these proteins in a *Drosophila* cell line or other similar cell lines to carry out the experiments in the endogenous background and at the corresponding growth temperature.

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