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The Importance of TM1a of GlyT2 in Glycine Transport and Inhibition

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A thesis submitted for the fulfilment of the requirements for the award of Masters of Philosophy (Medicine).

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ABSTRACT

Glycine transporters regulate the concentration of the neurotransmitter glycine within the central nervous system at both excitatory and inhibitory synapses. There are two subtypes of GlyT’s; GlyT1 (primarily expressed on astrocytes adjacent to excitatory and inhibitory glycinergic neurons) and GlyT2 (almost exclusively expressed on inhibitory glycinergic neurons). GlyT2 is responsible for the regulation of glycine concentration and reuptake into presynaptic terminals for repackaging in only inhibitory glycinergic synapses. The mechanism of transport for GlyT2 is unknown though homology models of GlyT2 derived from the crystal structure of a bacterial leucine transporter, LeuT, show a large conformational change in TM1a which allows the subsequent release of substrate in the last step of the transport cycle. NAGly, OLCarn and ALX 1393 are lipid inhibitors selective for GlyT2. The mechanism of inhibition for these lipids are unknown though NAGly has shown promise as an analgesic in animal models of both allodynia and hyperalgesia, which are the major symptoms for neuropathic pain. The aim of this study was to investigate the importance of TM1a during substrate transport via GlyT2 and if lipid inhibitors directly interact with this region to limit conformational changes required for this process. The GlyT2 homology model was adopted to determine the key residues in TM1a that may play an important role in conformational changes or lipid binding interactions. These residues were mutated and expressed in Xenopus laevis oocytes and GlyT2 transport function and inhibitory actions of NAGly, OLCarn and ALX 1393 were investigated using the two-electrode voltage clamp technique. GlyT2 activity remained unchanged with only minor increases in glycine affinity for the transporter. A slight trend showing decreased affinities of GlyT2 for lipid inhibitors NAGly, OLCarn and ALX 1393 (max 3-fold change) were observed though these results were not large enough to reflect a
direct interaction with TM1a. Our results suggest that NAGly, OLCarn and ALX 1393 do not inhibit GlyT2 by direct interactions on TM1a though may interact with surrounding TM’s to hinder the essential movements required for the transport process. Understanding the mechanism of these selective lipid inhibitors is crucial for the identification and development of new novel analgesics for the treatment of neuropathic pain.
STATEMENT

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent had been accepted for the award of any other degree or diploma of a university of higher learning, except where due acknowledgement is made in the text.”

CODE OF ETHICS

All experiments were performed in accordance with the “Australian code of practice for the care and use of animals for scientific purposes.” Australian Government Publishing Service, Canberra, 1990.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BetP</td>
<td>betaine transporter</td>
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<td>cDNA</td>
<td>complimentary DNA</td>
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<td>CMC</td>
<td>critical micelle concentration</td>
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<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>dDAT</td>
<td><em>Drosophila melanogaster</em> DAT</td>
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<td>glycine receptors</td>
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<td>GlyT2</td>
<td>glycine transporter 2</td>
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<tr>
<td>GlyTs</td>
<td>glycine transporters</td>
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<tr>
<td>IASP</td>
<td>The International Association for the Study of Pain</td>
</tr>
<tr>
<td>I_{max}</td>
<td>maximal inhibitory velocity</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>LeuT</td>
<td>leucine transporter</td>
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<tr>
<td>LiLCarn</td>
<td>linoleoyl-L-carnitine</td>
</tr>
<tr>
<td>NAAAla</td>
<td>N-arachidonyl Alanine</td>
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<td>N-arachidonyl GABA</td>
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<td>Abbreviation</td>
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<tr>
<td>NAGly-</td>
<td>N-arachidonyl glycine</td>
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<tr>
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<td>N-methyl-d-aspartate</td>
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<tr>
<td>NMDAR-</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NSAID-</td>
<td>non-steroidal anti-inflammatory drug</td>
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<td>Neurotransmitter Sodium Symporter family</td>
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<td>oleoyl-L-carnitine</td>
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<td>polymerase chain reaction</td>
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<td>peripheral nervous system</td>
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<td>plasmid oocyte transcription vector</td>
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<td>standard error mean</td>
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<td>TCA-</td>
<td>tricyclic anti-depressant</td>
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<td>transmembrane domains</td>
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<tr>
<td>V_{max}</td>
<td>maximal velocity</td>
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<td>WT-</td>
<td>wildtype</td>
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</table>

Single letter codes have been used to represent amino acid residues
1. INTRODUCTION

1.1 Background and Significance

Nearly one in five Australian adults suffer from chronic pain with the cost on the Australian community estimated at $34 billion annually (Holmes)(NSW Ministry of Health, 2012). The prevalence of this condition is comparable to or higher than a number of National Health Priority Areas in Australia such as cardiovascular disease, asthma and hearing loss (NSW Ministry of Health, 2012).

Neuropathic pain is a form of chronic pain that is termed by The International Association for the Study of Pain (IASP) as pain “initiated or caused by a primary lesion or dysfunction in the nervous system” (Harvey, 1995). Neuropathic pain is not the result of ongoing tissue injury and therefore differs from normal nociceptive pain in its mechanism, symptoms and also its treatments (Galer et al., 1997). This chronic pain state is often reported as having a lancinating or continuous burning character and is frequently associated with the appearance of abnormal sensory signs, such as allodynia (pain as a result of a stimulus which does not normally provoke pain) or hyperalgesia (an exaggerated response to a noxious stimulus which is not normally painful)(Campbell et al., 2006).

The causes of neuropathic pain are a complex outcome of multiple pathophysiological changes that develop in the peripheral nervous system (PNS) and central nervous system (CNS) following nerve injury or disease (Devor, 2006). Current therapies for neuropathic pain relief include tricyclic anti-depressants (TCA’s), opioids, non-steroidal anti-inflammatory drugs, and anti-epileptic agents (Gabapentin). A substantial percentage of patients do not respond favorably to treatment with TCAs with no more than 40–60% of patients obtaining partial relief of their pain (Rowbotham et al., 2005). Furthermore, TCAs
cause various side effects such as sedation, dry mouth, constipation, and urinary retention (Rowbotham et al., 2005). Opioids are considered to be the second line treatment for neuropathic pain to TCAs since they produce more side effects than TCAs such as nausea, constipation, and sedation, and have added negative effects of tolerance and dependence (Adams et al., 2006; Raja et al., 2002). Another line of treatment currently prescribed to patients suffering from neuropathic pain is non-steroidal anti-inflammatory drug (NSAID) treatment. The clinical efficacy of these drugs are very low and they exhibit gastrointestinal side effects (Backonja et al., 2003; Vo et al., 2009). Finally, Gabapentin, an anti-epileptic agent has also been noted as a treatment in neuropathic pain though the side effects are significant at doses that provide analgesia (Backonja et al., 2003). There is therefore a great need for new therapies to effectively treat neuropathic pain. One such potential therapy is pharmacological manipulation of the glycineergic pain transmission pathway. Inhibition of the transport of glycine would lead to elevated synaptic glycine concentrations, stimulate glycineergic neurotransmission and may suppress pain and relieve neuropathic pain states.

### 1.2 Glycine Neurotransmission and Glycine Transporters

Glycine is located widely in the CNS and can act as both an excitatory and inhibitory neurotransmitter (Betz et al., 2001). As an excitatory neurotransmitter, glycine acts as a co-agonist with glutamate at glutamatergic synapses, activating the excitatory N-methyl-D-aspartate (NMDAR) subtype of glutamate receptors (Betz et al., 2001). Glycine also acts as an inhibitory neurotransmitter on post synaptic glycine receptors (GlyR). These receptors are located primarily in the brain and spinal cord which, when activated, induces
The synaptic concentrations of glycine are regulated by glycine transporters (GlyTs), which mediate glycine reuptake into nerve terminals and adjacent glial cells (Figure 1) (Euleenburg et al., 2005). GlyTs are Na⁺/Cl⁻ dependent transporters and are members of the Neurotransmitter Sodium Symporter family (NSS) (Euleenburg et al., 2005). They have been characterized into two subtypes, GlyT1 and GlyT2, which share 50% amino acid sequence identity but differ in their pharmacology and distribution (Euleenburg et al., 2005).

Both GlyT1 and GlyT2 function to regulate the concentration of glycine within the synapse and the cellular and tissue expression of glycine transporters co-inside with NMDA receptors and GlyR’s (Aragon et al., 2003). GlyT1 is expressed throughout the CNS at both inhibitory and excitatory synapses, mostly on glial cells surrounding the synapses, but also to a lesser extent on post synaptic neurons (Figure 1) (Cubelos et al., 2005). Unlike GlyT1, GlyT2 is almost exclusively expressed on presynaptic inhibitory glycinergic neurons (Zafra et al., 1995). Glycine is transported against its concentration gradient which is made possible by it being coupled to the co-transport of Na⁺ and Cl⁻ gradients (Roux et al., 2000). Glycine transport by GlyT1 is coupled to the co-transport of 2Na⁺ and 1Cl⁻ for each glycine and functions to remove glycine from the synapse (Euleenburg et al., 2005). Glycine transport by GlyT2 is coupled to the co-transport of 3 Na⁺ and Cl⁻ and is localized in presynaptic glycinergic neurons and transports glycine back into the presynaptic terminal, thereby enabling the refilling of synaptic vesicles (Euleenburg et al., 2005). The
difference in stoichiometry between the two GlyT’s means that GlyT2 has a greater concentrating capacity than GlyT1 (Eulenburg et al., 2005).

**Figure 1:** Schematic diagram of glycine neurotransmission at inhibitory and excitatory synapses. At inhibitory synapses, high cytosolic glycine concentrations are maintained by GlyT2. Upon stimulation of an inhibitory glycinergic neuron, glycine is released into the synapse and will activate postsynaptic GlyR. Glycine is then cleared from the synapses by a combination of diffusion, and uptake by GlyT1 expressed on surrounding astrocytes and GlyT2 in the presynaptic terminal. At excitatory synapses glycine levels are maintained close to an equilibrium concentration set by the activity of GlyT1. GlyT1 is expressed by both astrocytes and in some glutamatergic neurons and will influence NMDAR activity. Glycine may also diffuse from inhibitory glycinergic synapses. Abbreviations: AMPAR, glutamate receptor of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid subtype; (Vandenberg et al., 2014).

Specific inhibitors for both GlyT’s are desirable to fully explore the potential of manipulating glycine concentrations for the treatment of pain (Aragon et al., 2003). GlyT2 is a more relevant target for inhibition to produce analgesic effects since it is present only in glycinergic neurons in association with postsynaptic glycine receptors so it is expected that GlyT2 inhibitors would be useful in the treatment of pain by enhancing glycine receptor systems (Morita et al., 2008). GlyT1 inhibition and subsequent enhancement of
NMDAR’s may compromise the ability of GlyT1 inhibitors to be beneficial for pain control (Morita et al., 2008).

1.3 GlyT2 Knockout and Knockdown Studies

Knockout of GlyT2 causes severe neurological disorders and death two weeks postnatal in animal models (Gomeza et al., 2003; Spike et al., 1997). The knockout mice have severely impaired glycinergic neurotransmission as a consequence of a greatly reduced cytosolic glycine concentration which prevents loading of presynaptic vesicles (Gomeza et al., 2003). These studies suggest that complete pharmacological inhibition of GlyT2 is also likely to deplete cytosolic glycine concentrations, reduce loading of synaptic vesicle, and cause significant side effects and would not be a viable novel pain therapy treatment.

Seltzer et al. developed a nerve ligation method that mimics human pain disorders in rats and has proven to be very useful in animal testing for pain relief therapies (Seltzer et al., 1990). This method involves the ligation of half of the sciatic nerve in the rat thigh and development of spontaneous pain, allodynia and hyperalgesia symptoms (Seltzer et al., 1990). Short-interfering RNA (siRNA) specific for GlyT2 have also been used in animal models to knockdown GlyT2 protein expression (Dorn et al., 2004; Morita et al., 2008). Mice treated with GlyT2 siRNA to cause a 75% reduction in GlyT2 expression show an increased pain threshold following nerve ligation surgery with an absence of any overt unwanted side effects (Morita et al., 2008). The mice display this increased pain threshold until around the 5th day post-surgery and treatment with siRNA which suggests that GlyT2 expression recovers after 5 days, restoring the neuropathic pain symptoms (Morita et al., 2008). Overall, these observations suggest that a 75% reduction in GlyT2 by siRNA
Treatment reduces the clearance rate of glycine from the synapse to enhance GlyR activity, but the remaining 25% of GlyT2 is sufficient to transport glycine and maintain the cytosolic concentration required for synaptic vesicle loading. The results from this study suggest that partial pharmacological inhibition of GlyT2, that may mimic the effects of siRNA-induced partial reduction of GlyT2, has potential for the treatment of neuropathic pain.

1.4 GlyT2 Selective Inhibitors

Several GlyT2 selective inhibitors have been synthesized, which show promise for the treatment of pain. ORG25543 is a selective GlyT2 inhibitor and exhibits anti-nociceptive effects against acute thermal, mechanical and chemical pain (Harvey et al., 2013). The anti-nociceptive effects produced by ORG25543 are fast-acting and are effective at low doses (Dohi et al., 2009). However, not only is ORG25543 an irreversible inhibitor but the doses that produce anti-allodynia effects may also modulate the excitatory glycinergic pathway which reverses the analgesic properties through the activation of the excitatory NMDA receptor pathway. This effect could be explained by excess glycine in the synapse which not only activates the inhibitory GlyR’s but also the NMDAR’s on neighbouring excitatory neurons. Furthermore, ORG25543, at effective analgesic doses, produces excitotoxicity with seizures, tremors and even death (Hermanns et al., 2008; Mingorance-Le Meur et al., 2013). Mingorance et al. developed a GlyT2 selective inhibitor named “compound 1” (Mingorance-Le Meur et al., 2013). Compound 1 is a reversible inhibitor but with reduced affinity compared to ORG25543 and has a sedative and analgesic profile in rodents that is comparable to Gabapentin (Mingorance-Le Meur et al., 2013). Similarly,
ALX1393 is also selective for GlyT2 and exhibits anti-nociceptive effects against acute thermal, mechanical and chemical pain (Harvey et al., 2013). However, ALX1393 has been implicated in causing respiratory depression and motor deficits at doses that provide analgesia. ALX1393 is approximately 20 fold more selective for GlyT2 over GlyT1 (Transport Biology Group unpublished results) and the adverse effects may be a consequence of cross reactivity with GlyT1.

1.5 **Lipid Inhibitors of Glycine Transport**

Endocannabinoids have demonstrated potential as analgesics in animal models of pain and in cancer patients (Nurmikko et al., 2007; Rice et al., 2002). The widespread distribution of this class of compounds in animal tissues suggests a wide range of effects of this family of modulators (Mechoulam et al., 1998). N-arachidonyl glycine (NAGly) is the carboxylic acid congener of the analgesic endocannabinoid, anandamide. NAGly is found at its highest levels in the spinal cord and small intestine, with lower levels generated in brain, kidney and skin in mammals (Huang et al., 2001). This wide distribution is suggestive that it may play a number of roles and interact with multiple targets within the body (Jeong et al., 2010).

Intrathecal injection of NAGly in animals with chronic inflammatory and neuropathic pain symptoms show a reduction in allodynia in several studies with motor function remaining unaffected (Burstein, 1999; Huang et al., 2001; Succar et al., 2007; Vuong et al., 2008). A study by Jeong et al. looked at the modulation of glycine synaptic transmission in lamina II neurons of the superficial dorsal horn of NAGly by using whole patch cell clamp techniques (Jeong et al., 2010). Neurons of lamina II within the spinal cord were studied
using patch clamp techniques recording the effects of NAGly on glycine and β-alanine where evoked currents were measured (Jeong et al., 2010). Both glycine and β-alanine are GlyR agonists, but only glycine is a substrate for the GlyT’s. NAGly had no effect on the GlyR current amplitude but selectively prolonged the time course of the glycine-evoked currents, with no effect on the time course of the β-alanine –evoked currents. The lack of an effect on the amplitude of the GlyR currents suggest that NAGly does not directly modulate GlyR activity, whereas the selective prolongation of the time course of the glycine-evoked currents suggests that NAGly inhibits the transporters. Similar observations were made for the GlyT2 selective inhibitor ALX1393, which suggests that the effects of NAGly are also due to inhibition of GlyT2 to prolong glycine in the synapse and modulate GlyR activity (Jeong et al., 2010). Furthermore, prolonged exposure of the lamina II neurons to NAGly caused a reduction in baseline current, which was reversed by the GlyR antagonist, strychnine. This suggests that NAGly inhibits GlyT’s to elevate the basal glycine concentration and stimulate GlyR’s (Jeong et al., 2010). NAGly also decreased the amplitude of evoked NMDA-mediated excitatory postsynaptic currents which suggests that NAGly may act directly on NMDAR’s as an antagonist at the glycine binding site though further investigation is required (Jeong et al., 2010).

1.6 Structure Activity Studies of Lipid Inhibitors of GlyT’s

Very little is known about how NAGly interacts with GlyT2. One suggestion is that NAGly may interact with the protein on the extracellular edges of transmembrane domains (TM) to restrict conformational changes essential for substrate transport (Carland et al., 2013a; Pantano et al., 2009). The binding site may be exposed to the aqueous phase which
may assist the washout and reversibility properties (Figure 2)(Wiles et al., 2006). Another suggestion is that NAGly binds to the transporter via the lipid phase to alter TM movement in the transport process to produce inhibition (Figure 2)(Carland et al., 2013b). It is conceivable that, since NAGly is a relatively large and flexible molecule, it interacts with GlyT2 in multiple ways, though these interactions need further investigation (Mostyn, unpublished).

![Figure 2: Suggested mechanism of binding of NAGly (blue) to GlyT2 (red) to inhibit transport of glycine. a. NAGly binds transporter via the aqueous phase on the extracellular edges of GlyT2. b. NAGly binds transporter via the lipid phase after the inhibitor diffuses into the lipid membrane.](image)

N-arachidonyl amino acids, like NAGly, contain an arachidonyl tail with the various amino acids as their head groups (Figure 3)(Carland et al., 2013b). Other N-arachidonyl amino acids like N-arachidonyl GABA (NAGABA) and N-arachidonyl Alanine (NAAla) also inhibit GlyT2 with selectivity over GlyT1 though NAGly is the most potent (Figure 3)(Wiles et al., 2006). The differences in potency are likely to be due to the head group of the N-arachidonyl derivatives as the active compounds NAGly, NAGABA and NAAla all contain a nitrogen atom and a carboxyl group in their head group, whilst the inactive compounds, arachidonic acid and anandamide, lack either the carboxyl group.
(anandamide) or the nitrogen (arachidonic acid) (Figure 3) (Wiles et al., 2006). Therefore, it is suggested that the nitrogen atom and carboxyl group in the head group of N-arachidonyl derivatives are required to induce an inhibitory effect (Wiles et al., 2006).

Figure 3: Chemical structures of Arachidonic acid, Anandamide, NAGly, NAAla, NAGABA and Oleoyl-l-carnitine (OLCarn).

OLCarn is widely distributed throughout the body and similarly to NAGly, non-competitively inhibits GlyT2 (Carland et al., 2013b; Foster, 2004). OLCarn is one of a series of endogenous lipid inhibitors of GlyT2 called acylcarnitines which are present in high concentrations within the CNS in similar regions to GlyT2 expression (Carland et al., 2013b). Though OLCarn has many functions within the body, the transport of long chain fatty acids into the mitochondria for β-oxidation and modulation of lipid metabolism and
membrane protein functions in the brain is its main fate (Carland et al., 2013a). Unlike NAGly, OLCarn is essentially irreversible when acting on GlyT2, with electrophysiological studies by Carland et al. showing that sub-maximal doses take more than 35 minutes for partial washout (Carland et al., 2013a). Complete recovery from OLCarn inhibition was achieved by including the lipid extracting agent, β-cyclodextrin which suggests that OLCarn may interact with the transporter via a lipid phase interaction (Carland et al., 2013a). This suggestion is further supported by previous studies that have investigated the ability of acylcarnitines to increase the membrane permeability of drugs and showed that acylcarnitines perturb the lipid order, possibly by partitioning into the lipid bilayer (Fix et al., 1986; LeCluyse et al., 1991). Therefore, the extent of irreversibility could be attributed to the high affinity of the compound for GlyT2 or the potential for the OLCarn tail group to interact with the cell membrane, slowing the washout time (Figure 3)(Carland et al., 2013a). This proposed cell membrane interaction binding mechanism of OLCarn differs from the previously suggested mechanism of NAGly binding GlyT2 on the extracellular edges of certain TM’s to induce inhibition and further investigation is required.

OLCarn exhibits a slow onset of inhibition which could be attributed to the inherent flexibility of OLCarn as it may take some time before the lipid can find the correct conformation required for binding and inhibition (Carland et al., 2013a). Full irreversible inhibition of GlyT2 by OLCarn has the potential to increase the glycine concentrations available to NMDA receptors which increases excitatory activity, reversing the antinociceptive action of GlyT2 (Carland et al., 2013a). The related acylcarnitines stearoyl-L-carnitine (SLCarn) and linoleoyl-L-carnitine (LiLCarn), which differ in tail lengths and a number of double bonds, display reduced inhibitory effects on GlyT2 compared to OLCarn.
(Carland et al., 2013a). This may be due to these molecules being less efficiently incorporated into the membrane than OLCarn to induce the inhibitory effects (Carland et al., 2013a).

1.7 Structure Function Studies of Glycine Transporters

In order to understand the structural basis for lipid inhibition of GlyT’s it is important to not only understand the structure of the transporter but also the nature of lipid interactions with the transporter. GlyT1 and GlyT2 are transporters within the NSS transporter family that includes transporters for biogenic amines (serotonin, dopamine, norepinephrine), amino acids (GABA, glycine, proline, taurine) and osmolytes (betaine, creatine)(Masson et al., 1999). A limitation of current GlyT studies is that the structures of the transporters are not available, and so trying to understand the transport mechanism of these transporters is difficult. We therefore use homology models of transporters based on other transporters that are structurally related to the NSS family. At present, Leucine transporter (LeuT) is the most studied structural model of NSS transporters and has provided very useful information that has been widely used to understand their mechanisms and structure/function relationships (Figure 4)(Krishnamurthy et al., 2012).

LeuT shares approximately 20% amino acid sequence identity with GlyT2 and has been crystalised in three distinct states (Figure 4)(Krishnamurthy et al., 2012). LeuT consists of 12 transmembrane (TM) segments with TM domains 1-10 forming the protein core (Figure 4)(Krishnamurthy et al., 2012). The core folds with a pseudo-two-fold-axis of symmetry in the membrane plane, with TMs 1-5 and TM’s 6-10 being mirror images (Figure 4)(Yamashita et al., 2005). Within this core, the leucine binding site is formed at the
bottom of an extracellularly facing cavity formed by the flexible regions of TM1 & TM6, TM3 and TM8 (Figure 4)(Yamashita et al., 2005). This substrate binding site also coordinates the sodium ions for transport and is a highly conserved region across many transporters in the NSS family, which suggests that they have similar mechanisms of transport (Beuming et al., 2006; Yamashita et al., 2005).

During the first phase of the transport cycle, LeuT exists in an outward-open state, allowing Na\(^+\) ions to bind as well as the substrate to permeate from the extracellular solution to the primary substrate binding site located halfway across the lipid bilayer (Figure 4)(Singh et al., 2008). The substrate is guided into the primary binding site via weak transient interactions of the substrate with residues in the extracellular vestibule (Singh et al., 2008). Once the substrate is bound, R30 and D404 form a salt bridge which is proposed to act as an extracellular gate in the transporter to stabilize and occlude the substrate from the extracellular space (Figure 4)(Singh et al., 2008). Once stabilized, the transporter changes conformation and extracellular loop 4 (EL4) packs into the extracellular facing hydrophobic vestibule (Figure 4)(Krishnamurthy et al., 2012). The transporter is now in the outward-occluded state where the substrate has no exposure to either the extracellular space or the cytoplasm (Figure 4). TM1 contains a central unwound region that provides flexibility to the transporter and following the movement of EL4 into the extracellular vestibule, it has been proposed that the intracellular half of TM1, swings outward, disrupting the substrate binding site, releasing the substrate into the cytoplasm (Figure 4)(Krishnamurthy et al., 2012).
Figure 4: Schematic diagram of LeuT in a. open outward conformation; b. outward occluded conformation (substrate bound [substrate shown in orange labeled “S”]) and; c. inward open conformation. TM’s are numbered and extracellular loop 4 labelled “EL4.” Labelled residue side chains form the important interactions in substrate transport. Na\(^+\) are represented as purple circles which are released with substrate “S” into the cell to complete the transport cycle (Krishnamurthy et al., 2012).

To better understand mechanisms of inhibition of transport in the NSS family of transporters, Ju et al. investigated the molecular basis for Zn\(^{2+}\) inhibition of GlyT1. The Zn\(^{2+}\) binding site is formed by histidine residues in extracellular loops 2 and 4 (EL2 & EL4)(Ju et al., 2004). The dopamine transporter (DAT), which is also a member of the NSS family, also contains a Zn\(^{2+}\) binding site formed by EL2 & 4 and can modulate dopamine transport in this way (Norregaard et al., 1998). This binding suggests that Zn\(^{2+}\) may regulate the rate of glycine transport by interacting with residues in EL2 & 4 of GLYT1, restricting the movement of this entity which may be critical for the transport process (Ju et al., 2004). Since Zn\(^{2+}\) shows similar non-competitive and partial inhibitory properties when acting on GlyT’s as NAGly, it was postulated that EL2 and EL4 may
similarly be involved in NAGly inhibition of GlyT2 (Edington et al., 2009). During transport, EL4 packs against TM1, TM2, TM6 and TM7, causing a large conformational change in the bottom half of TM1 to allow the release of the substrate into the cell (Krishnamurthy et al., 2012).

EL4 is highly conserved between the two GlyT’s but there is minimal sequence identity in the EL2 region (Carland et al., 2013b). Investigation into the molecular basis for the selectivity of NAGly and OLCarn for GlyT2 by Edington et al. and Carland et al. found that EL4 of GlyT2 is essential for both NAGly and OLCarn inhibition (Carland et al., 2013a). In fact, NAGly and OLCarn both have shown disrupted inhibiting properties when a single I545L reside in EL4 of GlyT2 is introduced which may suggest that the lipid inhibitors interact directly with I545 on the transporter (Carland et al., 2013b). Further investigation into these areas, especially in the case of GlyT2, will allow us to potentially uncover the specific interactions required for inhibition of the transport process.

BetP is a related transporter to LeuT/GlyT2 and the NSS transporter family (Ressl et al., 2009). Like LeuT, BetP has also been crystalised in the outward-open, occluded, and inward-open states and conforms to a similar fold as the NSS family of transporters (Ressl et al., 2009). BetP is a highly effective system for the transport of the osmolyte glycine betaine (Ressl et al., 2009). As BetP adopts a similar fold to LeuT but has 2 extra TM helices at the N-terminus, the same numbering of helices has been used between these transporters for simplicity.

One particular region of interest in both LeuT and BetP is the transmembrane region TM1a which, as alluded to earlier, appears to undergo a conformational change to open the cytoplasmic gate and release the substrate into the cell at the end of the transport cycle.
(Perez et al., 2012). Koshy et al. recently used crystallographic techniques to investigate the mechanism of BetP transport and the effects of lipid-protein interactions of various lipids on the transporter during this process (Koshy et al., 2013).

Figure 5: A Lipid Binding Site on BetP. Helices and residues involved in lipid (yellow) coordination are highlighted. TM1 (red) is located between TM5 (green) and TM7 (grey). L7 (yellow) is a lipid created by Koshy et al. that inhibits BetP transport function. Important residues that interact with L7 are labelled and side chains are shown (Koshy et al., 2013).

The structure solved by Koshy et al. revealed how the gating helix TM1a interacts with a lipid (termed L7 in Figure 5) and mutations of residues in TM1 of BetP were shown to reduce rates of betaine transport (Figure 5)(Koshy et al., 2013). Koshy et al. established the interaction of the acyl chain of lipid L7 with M150 as it is nested against TM1 and TM5. When this single Methionine residue at position 150 is mutated to an Isoleucine in the glycine rich unwound region of TM1a, BetP activity is significantly altered in comparison to wild type, which may be due to the lack of flexibility created by the branched chain Isoleucine residue as opposed to the straight chain Methionine residue (Figure 5)(Koshy et al., 2013). It is interesting to note that a neighbouring G153A mutation similarly alters BetP activity and allows choline to also be transported (Figure 5)(Koshy et al., 2013). The conclusion drawn from this study is that the transporter undergoes a
conformational change from the outward to inward state via the closed state and lipid L7 bound to TM1a will influence the rate of conformation changes required for transport (Figure 5)(Koshy et al., 2013). This suggests the importance of TM1a in the betaine transport process and emphasises that maintenance of flexibility is needed for the movement of TM1a in the transport process. Lipid binding to this region may be an important factor in determining the rate of transport (Koshy et al., 2013). The observed lipid-protein interaction sites involving residues important in both transport and regulation of BetP highlight the functional impacts of lipids in the structure-based transport mechanism (Koshy et al., 2013). Therefore, since TM1a has been identified as an important region of BetP for lipid interactions, this region may be a potential binding site for lipid inhibitors on related transporters such as GlyT2 in addition to other interactions on neighbouring regions of the transporter such as TM7 and TM8, the helices that flank EL4.

More recently, the first eukaryotic NSS transporter crystal structure for Drosophila melanogaster DAT (dDAT) was solved which shares greater than 50% amino acid sequence identity with most other mammalian counterparts in this family (Penmatsa et al., 2013; Pörzgen et al., 2001). The crystal structure of dDAT shows many similarities with LeuT and BetP and demonstrates that they are likely to share a common transport mechanism. The structure of dDAT also shows a cholesterol molecule bound to a lipid exposed cavity on the surface of the transporter and earlier studies by Hong et al. have demonstrated that cholesterol inhibits the function of a mammalian DAT by stabilizing the outward conformation of the transporter (Figure 6)(Hong et al., 2010). The cholesterol binding site is formed by TM1, TM5 and TM7, which suggests that cholesterol bound to
this site may inhibit transport by restricting the movement of TM1a and thereby prevent the release of substrate (Figure 6) (Hong et al., 2010; Penmatsa et al., 2013).

Figure 6: Cholesterol positioning in DAT and LeuT. a, Cholesterol (yellow sticks) shown. Residues that interface with the cholesterol group are represented as sticks. b, Potential role of cholesterol in maintaining an outward-open state of transporter. Cholesterol (sticks with transparent surface) sterically clashes with the position of TM1a (red) in the inward-open conformation of LeuT. (Penmatsa et al., 2013).

Leu 276 and Leu 277 in TM5 and Ile 358 in TM7 interact directly with the isoocyt group of cholesterol to anchor it at the junction between these two regions while residues Val 34, Leu 37, Leu 38 and Ile 41 on TM1a interact with cholesterol’s β-face of the sterol ring (Figure 6) (Penmatsa et al., 2013). Also, the α-face of cholesterol interfaces with residues Leu 347 and Ile 351 in TM7 (Figure 6) (Penmatsa et al., 2013). Cholesterol, once bound, appears to sterically clash with the position of TM1a in the inward-open conformation of LeuT (Figure 4), potentially preventing conformational changes in these regions that are essential for substrate transport (Figure 6). TM5, TM7 and TM1 are in very close
proximity to one another in this state and are all interacting with the cholesterol molecule (Figure 6) (Penmatsa et al., 2013). This action of cholesterol interacting with and inhibiting DAT supports the hypothesis that GlyT2 lipid inhibitors may interact with a similar region of GlyT2 to limit the function of glycine transport. By further understanding these specific interactions as well as the interactions of inhibitors on related transporters we may be able to reveal the mechanism of inhibition for these GlyT2 specific inhibitors.

Lipid inhibitor studies conducted by Shannon Mostyn in the Transporter Biology Group in the Discipline of Pharmacology, (unpublished) focused on the regions that are suggested to influence the movement of EL4 during the transport process (Mostyn, unpublished). EL4 is flanked by TM7 and TM8 and it was postulated that movements of these TM domains may influence the movement of EL4 and thereby influence the rate of transport. F515 is located half way along TM7 and is likely to be facing the oily core of the lipid membrane (Mostyn, unpublished). The F515Y mutant displayed marked increases in lipid inhibition of GlyT2 and faster washout properties than wild type (Mostyn, unpublished). L569 in TM8 is not conserved between GlyT1 and GlyT2 and was also investigated as it is a residue that is membrane facing in a cleft-like space between TM5 and TM8 that has the potential to interact with the tail group of lipids (Mostyn, unpublished). The L569F mutant shows marked reductions in both potency and efficacy of inhibition of GlyT2 by OLCarn and N-oleoyl-glycine, but not NAGly (Mostyn, unpublished). This differential effect of OLCarn and N-oleoyl-glycine compared to NAGly suggests that residues in TM8 may influence the specificity of lipid tail: transporter interactions.

Given the similarities between transporters within the NSS transporter family as well as other homologous transporters like BetP, we can hypothesize that TM7, TM8 and TM1a play a role in the way lipids bind and inhibit the transport function of GlyT2. Upon the
binding of lipids, the transporter is stabilized in the outward-open conformational state by
the transmembrane interactions previously discussed in all three of the three transporters;
LeuT, BetP and dDAT (Yamashita et al., 2005). As GlyT2 is structurally similar and has
highly conserved regions shared between these transporters, I will investigate whether
interactions between lipids and GlyT2 in these regions are responsible for the inhibitory
effects on the lipids.

1.8 Scope of this thesis

EL4 is important not only in the transport mechanism of NSS transporters closely related
to GlyT2 but also the specificity of inhibitors for this transporter over other related
transporters (Carland et al., 2013a; Carland et al., 2013b; Krishnamurthy et al., 2012).
Additionally, TM7 and TM8 of GlyT2, which flank the EL4 region, may be important
regions for inhibitor binding (Koshy et al., 2013). Furthermore, the movement of TM1a
during the transport of substrate, a region in close proximity to TM7 and TM8, may be
essential for this process and inhibition of this movement by either direct binding or steric
hindrance could be the underlying mechanism for lipid inhibition of GlyT2.

I hypothesize that the TM1a region of GlyT2 plays an important role in glycine transport
and that inhibition of the conformational changes of TM1a by lipid-based inhibitors may
limit the function of the transporter. To investigate this hypothesis I will perform
electrophysiological studies on GlyT2 with specific single mutations of TM1a incorporated
into the transporter. I will administer glycine in the presence of known GlyT2 specific lipid
inhibitors and record the differences in transporter function by using the two voltage clamp
technique which will reveal any differences in transport activity compared to wild-type GlyT2.
2. MATERIALS AND METHODS

2.1 Overview

The actions of lipid inhibitors NAGly, OLCarn and ALX 1393 on GlyT2 were investigated by expressing wild type and mutant transporters in *Xenopus laevis* oocytes and measuring their effects on transport currents. Site directed mutagenesis was used to probe the roles of individual amino acid residues in mediating the effects of the lipids. The two electrode voltage clamp technique was used to measure currents generated by the co-transport of glycine, Na⁺ and Cl⁻ ions.

2.2 Materials

*Xenopus Laevis* frogs were obtained from NASCO (Wisconsin, USA). NAGly was attained from Sapphire Biosciences (NSW, Australia) and OLCarn was obtained from Larodan Fine Chemicals (Malmo, Sweden). All other chemicals were acquired from Sigma Chemical Co. (Sydney, Australia) unless stated otherwise.

2.3 Assembly of mutant glycine transporters

cDNA (complimentary DNA) from GlyT2a was subcloned into pOTV (plasmid oocyte transcription vector). Site directed mutagenesis using oligonucleotide primers that incorporated the relevant mutations were employed to generate cDNA using the polymerase chain reaction (PCR) technique with a Q5 Site Directed Mutagenesis Kit (New England Biolabs Inc., MA, USA). The products of the PCR were transformed into DH5α chemically competent *Escherichia coli* (*E.coli*) cells that contain an
ampicillin resistant gene. Amplification followed by plating cells onto LB (lysogeny broth) agar bacterial plates containing ampicillin to ensure only E.coli cells with ampicillin resistant gene and mutant cDNA grow overnight in 37°C. A single colony was then picked from the plate and grown in LB containing ampicillin. 700ng of plasmid DNA was sent to the Australian Genome Research Facility (AGRF) for DNA sequencing to confirm that the mutation has been incorporated. Positive sequences that incorporated the relevant mutation were linearised using Spe1 restriction enzyme (New England Biolabs Genesearch, Australia), and used as a template for mutant RNA synthesis using T7 mMessage mMachine RNA Transcription kit (Ambion, Texas, U.S.A).

2.4 Harvesting and injecting of oocytes

All procedures followed the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes. Xenopus Laevis frogs were anethetised with 0.17% 3-aminobenzioc acid and after making an incision in the stomach of the frog, a lobe of oocytes were extracted. Oocytes were manually separated into groups of approximately 5 eggs and incubated within 2 mg/ml collagenase A (Boehringer, Mannheim, Germany) in OR-2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂,6H₂O, 5 mM hemi-Na⁺-HEPES, pH 7.5) for 60-90 minutes to obtain single oocytes. Oocytes were washed with OR-2 and ND-96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM hemi-Na⁺-HEPES, pH 7.5) five times each. Defoliculated stage V-VI oocytes were injected with 4.6ng of RNA encoding the relevant mutant transporter (Drummond Nanoject, Drummond Scientific Co., Broomall, PA, U.S.A). Injected oocytes were
stored for 2-5 days at 18°C in ND-96 solution that contained 50 μg/mL gentamycin and 50 μg/mL tetracycline until transporter expression levels were adequate to measure transport function.

2.5 Electrophysiology

Glycine transport by GlyT2 is coupled to the co-transport of 3Na\(^+\) and 1Cl\(^-\), producing an electrogenic process where the net movement includes three positively charged ions per glycine molecule transported. Two electrode voltage clamp technique was employed to measure glycine transport through change in current. Glycine transport was measured in oocytes sufficiently expressing the relevant mutant transporter at -60mV using GeneClamp 500B amplifier (Axon Instruments, Foster City, CA) in conjunction with Powerlab 2/26 (ADInstruments, Sydney, Australia) for all recordings.

The inhibitory actions of NAGly and OLCarn were investigated using glycine dependent inhibition of glycine transport. For each mutation, 30μM glycine was applied, followed by administration of the relative inhibitor at cumulative doses (NAGly: 0.1, 0.3, 2, 3, 10, 30μM; and OLCarn: 0.01, 0.03, 0.1, 0.3, 1, 3 μM) until the response was observed to plateau. OLCarn is an irreversible inhibitor and does not washout so only a single dose was administered per oocyte. The inhibitory action of ALX 1393 was investigated at a single 0.1μM dose at WT and mutant transporter calculated EC\(_{50}\) concentrations.

OLCarn has a critical micelle concentration (CMC) of 7.4 ± 1.2μM (Carland et al., 2013a) so, to ensure availability of OLCarn molecules, all concentrations used in this study are below the CMC.
2.6 Data analysis

Glycine concentration-dependent transporter current EC\textsubscript{50}’s for mutant and WT GlyT2 were obtained by fitting the curve using the modified Michaelis-Menton equation using GraphPad Prism 6.0 (GraphPad Software, San Diego California, CA):

\[ \frac{I}{I_{\text{max}}} = \frac{[\text{Gly}]}{EC_{50} + [\text{Gly}]} \]

where \( I \) represents current (nA), \( I_{\text{max}} \) is the maximal current generated by glycine (nA), the \( EC_{50} \) is the glycine concentration that generates a half maximal response (μM), and \([\text{Gly}]\) is the glycine concentration applied (μM).

Concentration responses for inhibitors were established using the method of least squares using:

\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1+10^{(X - \text{LogIC}_{50})})} \]

where \( X \) is log[OLCarn or NAGly](μM), \( Y \) is current normalized to glycine absent of inhibitor and “Top” and “Bottom” are the maximal and minimal plateau responses respectively. This equation was constrained to have the bottom value above zero and standard hill slope -1.0.

All values were generated from \( n \geq 3 \) from at least two batches of oocytes. One way ANOVA tests were employed, with a Dunnett’s post-hoc test used to compare each mutants against WT. Statistical significant values were understood to be \( p < 0.05 \).

NAGly IC\textsubscript{50}’s were presented using Standard Error Mean (SEM) as single cells were used to generate full concentration-response curves. OLCarn IC\textsubscript{50}’s were presented using 95% confidence intervals (CI) as multiple cells were used to generate a full
concentration response curve and the results are unlikely to yield identical errors so SEM was not used.
3. **RESULTS**

3.1 **Characterisation of Wildtype GlyT2**

Glycine transport via GlyT2 is an electrogenic process where the transport of one glycine molecule is coupled to the co-transport of 3Na\(^+\) and Cl\(^-\) ions. This ion movement generates glycine concentration-dependent inward currents that can be measured using the two electrode voltage clamp technique. A series of glycine concentrations were tested using this method to produce full concentration response curves (Figure 7A). WT GlyT2 had a glycine EC\(_{50}\) comparable to those reported previously (15.2 ± 0.8μM) (Figure 8A) (Carland et al., 2013a; Mingorance-Le Meur et al., 2013; Vandenberg et al., 2007).

Similarly, compounds that inhibit GlyT2 were investigated using the same 2 electrode voltage clamp technique. NAGly and OLCarn are two lipids that inhibit glycine transport by GlyT2 (Figure 7B & C). Since these lipids are non-competitive inhibitors of GlyT2, elevated concentrations of glycine were used to allow greatest sensitivity in inhibition assays (Carland et al., 2013b; Wiles et al., 2006). Therefore, glycine concentrations used for NAGly and OLCarn inhibition assays were 100μM and 30μM, respectively.

NAGly and OLCarn do not generate any currents when applied alone at concentrations up to 30μM NAGly and 3μM OLCarn, but application of higher concentrations of each lipid causes the cell to become “leaky” and may allow large fluxes of anion across the membrane which can mask the inhibitory effect of the compound on glycine transport. Furthermore, high concentrations of these compounds lead to their formation of micelles in solution which alters the available concentration of the inhibitor (Carland et al., 2013b). Therefore, the maximum exposure time to NAGly and OLCarn was 5 minutes and maximum concentrations investigated were 30μM and 3μM, respectively.
Following the administration of glycine, co-application of the inhibitor reduces the inward transport current, which correlates with a reduction in glycine transport into the cell (Figure 7B & C). This process was repeated using a fixed glycine concentration with a range of inhibitor concentrations to generate inhibitory concentration-response. GlyT2 inhibitors NAGly and OLCarn generated reduced transport currents corresponding to published IC$_{50}$ values (Figure 8B)(NAGly=13.3 ± 3.1μM and OLCarn=0.22 μM (95% CI, 0.07 to 0.7 μM))(Carland et al., 2013b; Edington et al., 2009; Wiles et al., 2006).
Figure 7: Representative traces of concentration responses on WT GlyT2. A. Increasing glycine concentrations were applied to WTGlyT2 expressing oocytes generating an inward current correlating to glycine transport. B. A 100μM glycine concentration (solid bar) followed by a second 100μM glycine dose in the presence of 1 μM NAGly (open bar) until level of inhibition plateaued. C. A 30μM glycine concentration (solid bar) followed by a second 30μM glycine dose in the presence of 3μM OLCarn (open bar) until level of inhibition plateaued.
Figure 8: Glycine and inhibitor concentration responses for WT GlyT2. Data shown is representative of mean ± SEM (n ≥ 3). Where error bars are not visible their location lies within the data point. A. Currents were measured in the presence of increasing glycine concentrations (0.1μM - 300μM). Currents were fitted to the modified Michaelis-Menton equation (see Materials and Methods) and normalized to the calculated V\text{max}. WT GlyT2 EC\text{50}= 15.2±0.8μM. B. The response to a 100μM glycine for NAGly and 30μM glycine for OLCarn concentration in the presence of increasing NAGly (triangle) and OLCarn (circle) concentrations. Currents were normalized to I\text{max}. WT GlyT2 NAGly IC\text{50}= 13.3±3.1μM and WT GlyT2 OLCarn IC\text{50}=0.22 μM (95% CI, 0.07 to 0.7μM)

3.2 Characterisation of Mutant GlyT2

3.2.1 Generation of Homology Model

There are no available structures of GlyT2 so homology models are used to predict the structure of GlyT2 and to investigate the mechanism and function of the transporter. LeuT shares approximately 20% amino acid sequence with GlyT2 and its structure is known so this transporter is widely adopted to understand the mechanism by which GlyT2 functions (Krishnamurthy et al., 2012). In this study, particular focus was put on the TM1a region of GlyT2 as TM1a has been postulated to move into the lipid bilayer as part of the transport
process (see Section 1.7). Therefore, a set of mutations in TM1a of GlyT2 were generated to investigate importance of TM1a in glycine transport (Figure 9). The mutations in TM1a were generated (Table 1) to see if this region also influenced the activity of the lipid inhibitors, NAGly and OLCarn.

Figure 9: TM1a helix of the GlyT2 homology model. Transporter model is rotated ~90° clockwise in plane with the membrane (left) and TM1a highlighted (red). Enlarged TM1a (right) is coloured in reference to the residues where mutations were created; K197 (red), D199 (yellow), F200 (blue), I201 (black), L202 (brown), V205 (pink).
### Table 1: Location, potential interactions and rationale for the generation of each GlyT2 transporter.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location and potential interaction(s)</th>
<th>Rationale for selection of mutant residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>K197L</td>
<td>First residue at the bottom of TM1a that is predicted to move furthest into the lipid bilayer as part of substrate release. This residue also faces toward bottom of TM7, which has been implicated in playing a role in lipid modulation (Vandenberg, Carland, Mostyn unpublished results). The lysine (K) residue contains an amine group which may interact with the phosphate moiety of the lipid membrane head group to stabilize TM1a in the outward open conformation of the transporter.</td>
<td>Leucine (L) is more hydrophobic than lysine (K) so may make movement of TM1a into the lipid bi-layer more favourable during glycine transport. It may also disrupt interaction with the phosphate moiety of the lipid membrane head groups.</td>
</tr>
<tr>
<td>D199A</td>
<td>Third residue from the bottom of TM1a that is adjacent to TM8. TM8 has also been implicated in formation of a lipid binding site in GlyT2 (Vandenberg, Carland, Mostyn unpublished results). The aspartic acid (D) residue contains a carboxyl group that may interact with the choline or ethanolamine moieties of the head groups of membrane lipids to stabilize TM1a.</td>
<td>Alanine (A) is a smaller residue than aspartic acid (D) which may allow for easier movement of TM1a during glycine transport and speed up substrate release, but may also disrupt interactions with the lipid membrane head groups.</td>
</tr>
<tr>
<td>D199S</td>
<td>Serine (S) is a smaller residue than aspartic acid (D) but more polar than alanine, which may allow for easier movement of TM1a during glycine transport and speed up substrate release.</td>
<td></td>
</tr>
<tr>
<td>F200W</td>
<td>Fourth residue from the bottom of TM1a that faces inward towards the hydrophobic core of the protein. The phenylalanine (F) residue contains a hydrophobic benzyl side chain and prefers non-aqueous environments so when TM1a ‘flips’ outward, the F residue may facilitate movement of TM1a into the lipid bilayer and may also interact with lipid inhibitors.</td>
<td>Tryptophan (W) is a residue that has a large side chain which may impair the ability of TM1a to change conformations during glycine transport. Tryptophan residues also allow for the potential binding of lipid compounds via either their head or tail group so may assist in lipid binding for GlyT2 inhibition.</td>
</tr>
<tr>
<td>I201W</td>
<td>Fifth residue from the bottom of TM1a that faces toward TM7. Isoleucine (I) contains a branched hydrophobic hydrocarbon side chain and prefers non-aqueous environments so when TM1a ‘flips’ outward, the I residue may interact with the lipid membrane or the lipid inhibitors.</td>
<td>Tryptophan (W) is a residue that has a large side chain which may impair the ability of TM1a to change conformations during glycine transport. Tryptophan residues also allow for the potential binding of lipid compounds via either their head or tail group so may assist in lipid binding for GlyT2 inhibition.</td>
</tr>
<tr>
<td>L202F</td>
<td>Sixth residue from the bottom of TM1a and is the middle residue on TM1a. This leucine (L) residue faces outward toward the lipid membrane and contains an aliphatic side chain which is hydrophobic in nature and prefers non-aqueous environments. When TM1a ‘flips’ outward L202 may interact with the lipid membrane or lipid inhibitors.</td>
<td>Tryptophan (W) is a residue that has a large side chain which may impair the ability of TM1a to change conformations during glycine transport. Tryptophan residues also allow for the potential binding of lipid compounds via either their head or tail group so may assist in lipid binding for GlyT2 inhibition.</td>
</tr>
<tr>
<td>V205Q</td>
<td>Ninth residue from the bottom of TM1a and fourth residue down from the top of TM1a before the flexible unwound region. Valine (V) consists of a branched hydrocarbon side chain which is hydrophobic in nature so prefers non-aqueous environments. When TM1a ‘flips’ outward it may interact with the lipid membrane and/or lipid inhibitors.</td>
<td>Phenylalanine (F) is a larger than leucine (L) but smaller than tryptophan and may sterically constrain the TM1a movement during transport therefore disrupting the substrate release process and/or interaction with the lipid inhibitor.</td>
</tr>
<tr>
<td>L202W</td>
<td></td>
<td>Glutamine (Q) is a more polar residue than Valine (V) and may disrupt movements of TM1a into the membrane and/or alter lipid interaction.</td>
</tr>
</tbody>
</table>
3.2.2 Glycine Concentration Dependence on Mutants of GlyT2

GlyT2 mutant transporter function was initially tested to ensure that the changes in the protein did not disrupt glycine transport. All mutant transporters were functional glycine transporters though in some cases expression and function was reduced compared to WT (WT GlyT2 EC\textsubscript{50}=15.2±0.8μM). Certain transporters showed changes within both Vmax and Km values from WT and others displayed only one or even no difference in these results. Apart from I201W (EC\textsubscript{50}=18.3±1.0μM), which showed a very comparative glycine affinity to that of WT, each mutant transporter displayed a higher affinity for glycine (Figure 10D). K197L (2.2±0.3μM) exhibited the largest increase in glycine affinity which was approximately 7-fold higher than that of WT (Figure 10A). D199A (EC\textsubscript{50}=4.0±0.7μM), D199S (EC\textsubscript{50}=5.3±0.6μM), F200W (EC\textsubscript{50}=3.3±0.2μM), L202W (EC\textsubscript{50}=6.8±0.5μM), L202F (EC\textsubscript{50}=4.1±0.9μM) and V205Q (EC\textsubscript{50}=8.4±0.8μM) have between 2- and 4-fold increases in glycine affinity compared to WT GlyT2 (Figure 10A, B, C). The small increases in glycine affinity may be the consequence of these mutations disrupting the normal conformational changes in the TM1a region that are required for the transport cycle, resulting in the substrate being bound to the transporter for a longer period of time, which may manifest as a higher affinity.
Figure 10: Glycine dependent transport currents for WT and mutant transporters.
Data shown is representative of mean ± SEM (n ≥ 3). Where error bars are not visible their location lies within the data point. Currents were measured in the presence of increasing glycine concentrations (0.1μM - 300μM). Currents were fitted to the modified Michaelis-Menton equation (see Materials and Methods) and normalized to the calculated V_max. A. Glycine concentration response curves for WT (●), K197L (■), F200W (△) and V205Q (▼). B. Glycine concentration response curves for WT (●), D199A (○) and D199S (♦). C. Glycine concentration response curves for WT (●), L202W (□), L202F (♦) and I201W (▲). D. Calculated EC_{50}±SEM values for WT and mutant GlyT2 transporters (n= number of cells used).
3.2.3 Lipid Inhibition of Glycine Transport by GlyT2 Mutant Transporters

A range of NAGly and OLCarn concentrations were tested in the presence of a fixed glycine concentration in order to investigate the potential binding of the lipid compound to the mutated regions of the transporter. NAGly concentration-response curves were obtained for each GlyT2 mutant transporter (100μM glycine + 0.1, 0.3, 1, 3, 10, 30μM NAGly) except for K197L which displayed currents that were too small to gain reliable results due to low expression of the mutant transporter or reduced substrate transport currents.

The IC$_{50}$ for NAGly inhibition of WT GlyT2 was 13.3±3.1μM and no significant changes in IC$_{50}$ were observed for mutant transporters compared to WT (p>0.05)(Figure 11). D199S (14.3±1.2μM), F200W (13.2±3.4μM), I201W (12.7±2.8μM) and L202F (11.0±0.7μM) displayed the most comparable NAGly IC$_{50}$ concentrations to WT GlyT2. The largest differences in IC$_{50}$ from WT GlyT2 were observed in D199A, L202W and V205Q, which exhibited an approximately 2-fold increase in affinity for NAGly (D199A=5.1±0.5μM, L202W=6.5±1.9μM, V205Q=6.2±1.2μM NAGly) though the differences were not significant (p>0.05)(Figure 11). GlyT2 mutant transporters L202W and D199S depicted a reduced maximal level of inhibition by NAGly with respect to WT though there was no significance in this result (p>0.05)(Figure 11B & C). This decrease in efficacy was unable to be further investigated using higher NAGly concentrations due to the tendency of the lipid to form micelles in aqueous solution.
Figure 11: NAGly concentration response relationship for WT and mutant transporters. A. to C. Transport currents generated by 100μM glycine in the presence of increasing concentrations of NAGly were measured and represented by mean ± SEM (n ≥ 3). Where errors bars are not visible their location lies within the data point. A. NAGly inhibition curves for WT (●), F200W (▲) and V205Q (▼). B. NAGly inhibition curves for WT (●), D199A (○) and D199S (♦). C. NAGly inhibition curves for WT (●), L202W (□), L202F (♦) and I201W (▲). D. Table of NAGly IC$_{50}$ calculations for WT and each mutant transporter represented by mean ± SEM (n= number of cells used).

OLCarn is a GlyT2 selective lipid inhibitor that was also investigated. Inhibitor concentration-response curves were obtained for mutant transporters D199A, D199S,
I201W, L202F and V205Q (30μM glycine + 0.01, 0.03, 0.1, 0.3, 1, 3μM OLCarn)(Figure 12). Due to low mutant transporter expression or lack of function, the effect of OLCarn on K197L, F200W and L202W were unable to be investigated as they displayed small currents (5-10nA) which prevented accurate results to be obtained for analysis.

Figure 12: OLCarn concentration response relationships for WT and mutant transporters. Transport currents generated by 30μM glycine with increasing concentrations of OLCarn were measured and represented by mean ± SEM (n ≥ 3). Where errors bars are not visible their location lies within the data point. A. OLCarn inhibitor concentration-response curves for WT (●), D199A (○) and D199S (♦). B. OLCarn inhibitor concentration-response curves for WT (●), I201W (▲), L202F (+) and V205Q (▼).

The IC$_{50}$ for OLCarn inhibition of glycine transport by WT GlyT2 was 0.22μM (95% CI, 0.07 to 0.7μM), which was comparable to the mutant transporters D199A (0.22μM (95% CI, 0.08 to 0.6μM)), I201W (0.20μM (95% CI, 0.06 to 0.7μM)) and V205Q (0.32μM (95% CI, 0.2 to 0.5μM))(Table 2). Mutant transporter D199S (0.14μM (95% CI, 0.08 to 0.2μM)) showed reduced NAGly maximal inhibition, but displayed a small, but not significant increase in OLCarn affinity compared to WT GlyT2 (p>0.05). The IC$_{50}$ for OLCarn
inhibition of L202F (0.50μM (95% CI, 0.3 to 1.0μM)) was decreased compared to WT GlyT2 but did not reach significance (p>0.05)(Figure 12, Table 2). It is interesting to note that L202W reduced NAGly efficacy similar to that of L202F for OLCarn. These two transporters have a mutation created at the same residue, which is situated on TM1a so that it faces outward toward the lipid bilayer and may potentially be accessible to the lipid inhibitor for direct interactions.

Table 2: IC₅₀ values for OLCarn inhibition of glycine transport by WT and mutant transporters.

<table>
<thead>
<tr>
<th>Mutant Transporter</th>
<th>IC₅₀ (μM)</th>
<th>95% CI (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.22</td>
<td>0.07 to 0.65</td>
</tr>
<tr>
<td>D199A</td>
<td>0.22</td>
<td>0.07 to 0.61</td>
</tr>
<tr>
<td>D199S</td>
<td>0.14</td>
<td>0.08 to 0.24</td>
</tr>
<tr>
<td>I201W</td>
<td>0.20</td>
<td>0.06 to 0.65</td>
</tr>
<tr>
<td>L202F</td>
<td>0.50</td>
<td>0.26 to 0.97</td>
</tr>
<tr>
<td>V205Q</td>
<td>0.32</td>
<td>0.19 to 0.54</td>
</tr>
</tbody>
</table>

3.2.4 ALX 1393 does not bind in TM1a region of GlyT2

ALX1393 is a mixed competitive/non-competitive inhibitor of GlyT2 (Vandenberg, Carland, Mostyn unpublished results) and so the inhibitory effects of this compound on glycine transport by WT and the TM1a mutant GlyT2s were tested at the glycine EC₅₀ concentration for each transporter to allow comparison between transporters with different affinities for glycine. A glycine EC₅₀ concentration was applied, followed by the coadministration of 0.1μM ALX 1393 for WT and each of the mutant transporters (Figure
13). From this, a direct comparison of ALX 1393 affinity between the mutant transporters and WT can be drawn (Figure 13, Table 3).

The mutations created in the transporters may disrupt the conformational changes needed for regular function which may be manifested as a small change in affinity for glycine and/or inhibitors. However, such changes may not necessarily reflect a disruption in a direct interaction between glycine and/or inhibitor and the mutated residue. I have used a cut off of a 10 fold change as most likely to reflect disruptions in glycine and/or inhibitors with the particular residue under study.

An estimated ALX 1393 IC\textsubscript{50} concentration for WT and all mutant transporters was calculated under the assumption that ALX1393 acts by the same mechanism on both the mutant and WT transporters.

![Figure 13: Representative current trace of glycine EC\textsubscript{50}+0.1μM ALX 1393 application for WT GlyT2. An EC\textsubscript{50} glycine concentration (solid bar) was applied followed by a second glycine EC\textsubscript{50} concentration with 0.1μM ALX 1393 (open bar) until a plateau level of inhibition was reached.](image)
Figure 14: Graphical representation of ALX 1393 IC$_{50}$ inhibition of glycine transport by WT and mutant GlyT2. An EC$_{50}$ concentration for WT and mutant transporters was administered followed by 0.1μM ALX 1393 which reduced glycine transport. Current measurements are presented as a proportion of the current in absence of ALX 1393 represented as mean ± SEM (n ≥ 3).

Table 3: IC$_{50}$ values for ALX1393 for GlyT2 WT and TM1a mutant transporters. IC$_{50}$ values were calculated by the equation: $I/I_{max} = 1 - \frac{[I]}{[I] + IC_{50}}$ where $[I]$ is the concentration of ALX (0.1μM) (n= number of cells used).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$I/I_{(EC50)}$(%)</th>
<th>Estimated IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>34 ± 1 (n=3)</td>
<td>52±8</td>
</tr>
<tr>
<td>K197L</td>
<td>54 ± 3 (n=3)</td>
<td>117±19</td>
</tr>
<tr>
<td>D199A</td>
<td>56 ± 2 (n=3)</td>
<td>127±21</td>
</tr>
<tr>
<td>D199S</td>
<td>63 ± 2 (n=3)</td>
<td>170±14</td>
</tr>
<tr>
<td>F200W</td>
<td>51 ± 1 (n=3)</td>
<td>104±8</td>
</tr>
<tr>
<td>I201W</td>
<td>60 ± 3 (n=3)</td>
<td>150±10</td>
</tr>
<tr>
<td>L202W</td>
<td>51 ± 2 (n=3)</td>
<td>104±11</td>
</tr>
<tr>
<td>L202F</td>
<td>38 ± 4 (n=3)</td>
<td>61±32</td>
</tr>
<tr>
<td>V205Q</td>
<td>46 ± 2 (n=3)</td>
<td>85±13</td>
</tr>
</tbody>
</table>
All IC$_{50}$ concentrations of ALX1393 for mutant transporters showed small increases compared to WT (Figure 14, Table 3). The largest increase in IC$_{50}$ concentration was observed in mutant transporter D199S (170nM), which was approximately increased 3-fold compared to WT ALX 1393 IC$_{50}$ (52nM)(Table 3).

Up to 3-fold changes in IC$_{50}$ are unlikely to reflect disruption of a direct interaction between ALX1393 at the various mutated residues. Therefore, it is unlikely that ALX1393 interacts with this region of the transporter. This is consistent with unpublished results from the Transporter Biology Group (Carland, Edington and Vandenberg) which indicate that ALX1393 binds to the glycine binding site on the transporter which is located at the bottom of the external facing vestibule.
4. DISCUSSION

The main aim of this project was to understand the importance of TM1a in GlyT2 for substrate transport and how lipid inhibitors may affect the conformational changes required for this process. I hypothesized that the conformational changes of TM1a required for substrate transport by GlyT2 are a crucial part of the transport process and mutations in this region will disrupt the transport process and alter the actions of lipid-based inhibitors.

4.1 Conformational Changes associated with the transport mechanism

The transport mechanism of GlyT2 is unknown, but there have been considerable advances in knowledge of this mechanism from studies of the related transporter, LeuT, from which it is possible to develop models for GlyT2 (Krishnamurthy et al., 2012). After the substrate binds to the transporter in the outward open state, LeuT progresses to an outward occluded conformation where the substrate is not accessible from either the extracellular or the intracellular space. The final phase of the transport cycle in these models postulate a movement of TM1a flipping outward toward the lipid membrane, disrupting the substrate binding site, and resulting in subsequent substrate release into the cytoplasm (refer to Figure 4c in Introduction)(Krishnamurthy et al., 2012).

BetP is a transporter that is structurally related to GlyT2 and has been crystalized in conformational states similar to that of LeuT; outward open, outward-occluded and inward open (Koshy et al., 2013). Koshy et al. showed that that a specific lipid compound (L7) was able to bind directly to TM1a, TM5 and TM7 of BetP to inhibit substrate transport (refer to Figure 5 in Introduction)(Koshy et al., 2013). L7 has been suggested to induce inhibitory effects by binding to these TM’s of BetP to limit the conformational changes of
TM1a essential for substrate release. The flexibility of TM1a seems crucial for the translocation of substrate as well as substrate release so inhibition of this movement is a viable mechanism of inhibition by this lipid in BetP. As this transporter is structurally related to GlyT2, it was postulated that lipid inhibitors could diffuse into the lipid membrane and interact with GlyT2 in an analogous manner to L7 interactions with BetP.

This postulated mechanism of inhibition in BetP is also shared for inhibition of NSS family transporter dDAT by cholesterol. dDAT shares 50% homology with its mammalian counterparts in the NSS family so provides insights into the potential binding mechanisms of compounds to limit transport function. Crystallographic studies of dDAT by Penmatsa et al. showed that a cholesterol molecule is able to bind in an intracellular cavity coordinated by TM1a, TM5 and TM7 and is lodged into the transporter so that 57% of its solvent-accessible surface area is buried (refer to Figure 6 in Introduction)(Penmatsa et al., 2013). As GlyT2 is part of the NSS family of transporters and is postulated to function in a similar manner to dDAT, lipid inhibitors may hinder transporter function by preventing essential conformational changes required for the transport process.

### 4.2 Mutations in TM1a of GlyT2 did not alter GlyT2 function

In this study, a series of mutations were generated in TM1a of GlyT2 to potentially aid or hinder the movement of TM1a through the substrate transport process, disrupt interactions of TM1a with the lipid membrane, or possibly encourage lipid binding to GlyT2 inhibition (refer to Table 1 in Results). Small increases in substrate affinity were observed for mutant transporters compared to WT GlyT2 but given the magnitude of the changes these results are unlikely to reflect substantial changes in functional properties of the transporter. The
mutations created in TM1a resulted in reduced currents potentially due to small disruptions of conformational changes in TM1a during the transport process. These disruptions may lead to the substrate being bound for a longer period of time which can present as the transporter having a higher substrate affinity, a slower turnover rate and smaller transport currents.

The postulated transport process of GlyT2 involves the essential dipping movement of EL4 into the hydrophobic vestibule to occlude the extracellular pathway. In order for this movement of EL4 to occur, neighbouring TM7 and TM8 regions must also undergo conformational changes to accommodate this concerted movement as any interruption to this process may hinder the transport cycle (Krishnamurthy et al., 2012). Ju et al. showed that Zn$^{2+}$ non-competitively inhibits GlyT1 and identified that H410 and H423 on EL4 are likely to assist in coordinating Zn$^{2+}$ to the inhibitor binding site (Ju et al., 2004). As Zn$^{2+}$ shows similar action to known inhibitors of GlyT2, Edington et al. investigated lipid inhibitors on GlyT2 and observed disrupted effects when I545 on EL4 was mutated (Edington et al., 2009). Although the effects were dramatic, this outcome is unlikely to be the result of direct interaction on this residue but may be the consequence of restrictions in essential conformational changes of surrounding TM’s; TM7 and TM8. This indirect effect may alter the binding site for inhibitors since EL4 undergoes considerable movement through the transport process that TM7 and TM8 must accommodate for. Shannon Mostyn of the Transporter Biology Group investigated EL4 and surrounding TM7 and TM8 regions of GlyT2 in order to understand how these movements may hinder inhibitor activity (Mostyn, unpublished). Membrane exposed residues, F515 on TM7 and L569 on TM8 significantly modulated effects of known GlyT2 inhibitors. Since the inhibitors investigated are relatively large, multiple interactions with a number of regions of the
transporter may be responsible for the inhibitory effects observed. TM1a neighbours TM7 and TM8 and is suggested to undergo the largest conformational change during substrate transport via LeuT (Krishnamurthy et al., 2012). Furthermore, TM1a, TM5 and TM7 also form the binding site for known inhibitor cholesterol in dDAT, a structural homologue of GlyT2 in the NSS family of transporters (Penmatsa et al., 2013). Therefore, TM1a may be an important region for direct binding or steric hindrance by inhibitors. However, the results presented in this thesis did not reflect as hypothesized.

Selective GlyT2 lipid inhibitors NAGly and OLCarn were tested against the WT and mutant transporters in order to understand their potential interactions with the TM1a region of the transporter. These inhibitors did not show any significant differences between their activity on GlyT2 WT and mutant transporters though small trends were observed. Almost all of the mutant transporters displayed a slightly decreased inhibitory effect of NAGly and OLCarn compared to WT. This may be attributed to the non-specific disruption of lipid binding through small alterations in lipid membrane facing residues of the TM1a region of GlyT2; D199, L202 and V205. However, the small decreases in lipid affinity observed in this study are unlikely to reflect disruptions to direct interactions of the lipid with GlyT2. Thus, it may be concluded that NAGly and OLCarn are unlikely to bind to this region of the transporter.

As mentioned earlier, cholesterol can inhibit NSS transporter dDAT by interactions coordinated by TM1a, TM5 and TM7 which may restrict essential conformational changes required for the transport process. The residues on TM1a of dDAT that interact with cholesterol in the inner leaflet of the membrane include L37, L38 and I41. The corresponding residues in the GlyT2 were identified as I201, L202 and V205. In this study, mutations were created to alter the potential interaction of lipids on this region of GlyT2 in
order to understand if known GlyT2 selective inhibitors NAGly and OLCarn exert their inhibitory effect by the same mechanism as cholesterol on dDAT. Mutant transporters did not reveal any significant differences in the inhibitory effects of NAGly or OLCarn from WT GlyT2 so it is unlikely these lipid compounds bind into the cavity coordinated by TM1a, TM5 and TM7 on the intracellular end of the transporter. However, further investigation using mutagenesis of all other residues that face the cavity coordinated by TM1a, TM5 and TM7 is required in order to confidently rule out lipid inhibitor binding to this region to limit transport.

### 4.3 ALX 1393 does not directly interact with TM1a of GlyT2

ALX 1393 is another GlyT2 specific inhibitor whose mechanism is still unknown (Vandenberg et al., 2007). It has been suggested that ALX 1393 may interact with part of the primary GlyT2 substrate binding site as well as potentially interacting at a proposed secondary substrate binding site to inhibit transport therefore making it a mixed inhibitor (Edington A, unpublished results). Shi et al. initially proposed this secondary substrate binding site through their molecular dynamic investigations of LeuT (Shi et al., 2008). The secondary substrate binding site is said to be coordinated by residues in TM1b, TM3, EL4, and TM10 (Shi et al., 2008). In order to ensure ALX 1393 has no direct interactions with TM1a, mutant transporters were generated which consisted of single mutations to residues of TM1a in GlyT2. TM1b is the second helix of TM1 and is located near the postulated secondary substrate binding site so mutations created in TM1a could potentially transmit conformational changes through TM1b, thereby altering this secondary substrate binding site and the subsequent action of ALX 1393.
However, no significant differences were observed in estimated IC$_{50}$ values for ALX 1393 between mutant and WT GlyT2’s which suggests that is does not interact with TM1a and is consistent with previous investigations that this inhibitor may bind at a different location that may overlap with the primary and/or proposed secondary binding site on the transporter (Carland J and Edington A, unpublished results). It may be worth examining the particular residues on TM1b, TM3 and TM10 that are suggested to coordinate the secondary substrate binding site in order to understand if these regions do in fact interact with ALX 1393.

4.4 Limitations of the study

For many of the GlyT2 mutants investigated in this study the glycine transport current amplitudes were relatively small ($I_{\text{max}}$ approximately 20nA, compared to 100nA for WT GlyT2) which made it difficult to reliably measure the inhibitory effects of NAGly and OLCarn. As this study adopted the use of *Xenopus Laevis* ooctyes, variability is observed between each cell. The small amplitudes of mutant transporters limited the reliability of results as it was much harder to determine a true response to the concentration being applied to the cell. Therefore, larger currents allow more reliable results as large fluctuations in current are likely to represent true changes in transporter function. If any hints of altered effect were observed, it would have been desirable to investigate alternate mutations that would yield larger, more reliable transport currents. However, it is worth noting that more conservative mutations are likely to generate larger transport currents but may not alter the functional properties of the transporter so the relevance of these mutations may be limited.
As mentioned previously, OLCarn is an irreversible inhibitor of GlyT2. This property combined with the limitation around the small observed transport currents meant that all OLCarn data was collected through a single dose per cell and the data pooled from many cells (eg. n=42 for V205Q). Cumulative concentration responses were unable to be obtained since the reduced currents observed did not allow for obvious changes in glycine transport between the series of concentrations tested. Single OLCarn concentrations used for each cell meant that greater variability between cells is observed compared to cumulative concentration responses performed on a single cell.

NAGly and OLCarn form micelles at high concentrations (>30μM for NAGly and >10μM for OLCarn) which meant there was a limitation in the range of concentrations investigated. At concentrations above the CMC, the concentration of free lipid is difficult to estimate and will not reach a true concentration higher than the CMC. Thus, it is difficult to estimate the concentration of lipid that reaches the binding site on the transporter. Furthermore, we have very little understanding of how lipid micelles interact with membranes and what the effective concentration of the lipid is if they are able to incorporate into the membrane. However, the observation that cells are unstable after prolonged exposure to high concentrations suggest that lipids can accumulate in the membrane to disrupt membrane structure. Therefore, any interesting results displaying a reduction in maximal inhibition of the compound was unable to be investigated further at higher concentrations because of this property of both NAGly and OLCarn. It was even apparent at the highest concentrations of NAGly and OLCarn that the oocytes being tested were unstable with prolonged (>5 minutes) lipid application.
4.5 Concluding remarks and clinical relevance

Since TM1a is postulated to undergo significant conformational changes during substrate transport, I hypothesized that direct interactions of inhibitors on this region may disrupt the movements required for the transport cycle. However, in this study, mutations created in TM1a of GlyT2 did not significantly alter the transport activity nor greatly modulate the inhibitory effects of known selective GlyT2 inhibitors NAGly, OLCarn and ALX 1393. From this we may therefore conclude that these compounds do not directly interact with TM1a to prevent conformational changes needed for substrate transport. However, previous models have suggested that inhibitory molecules may bind surrounding TM’s of TM1a to sterically hinder this area and disrupt essential conformational changes needed for substrate transport. Therefore, prevention of essential TM1a movements in substrate transport via GlyT2 may be a potential mechanism of inhibition. Further investigation into the surrounding TM’s that may directly interact with lipid inhibitors may be an interesting follow up to this study (Figure 15).
Figure 15: TM helices of GlyT2 homology model for further investigation. TM5 (green), TM7 (red) and TM8 (blue) are highlighted as TM’s that may have residues directly related to transport inhibition. TM5 and 7 form an inner leaflet of the transporter with TM1a that undergoes conformational changes that, for cholesterol in related NSS transporter dDAT, inhibits transport. TM7 and 8 undergoes essential conformational changes during transport to allow EL4 to dip in and occlude the extracellular pathway. If these movements are restricted the transport process may be inhibited.

By understanding the way in which these inhibitors of GlyT2 act we can gain key insights into the properties that may be suitable for the development of novel GlyT2 inhibitors that may be useful for the treatment of conditions such as neuropathic pain. The optimal GlyT2 inhibitor would be a potent, reversible, partial inhibitor that is selective for GlyT2. There is a great need for more relevant and targeted therapies for neuropathic pain since the current treatments are either ineffective or provide an array of undesired side effects that restricts its clinical relevance. Since over 1 in 5 Australians are diagnosed with chronic pain,
improved therapies such as targeted GlyT2 partial inhibition may provide much needed relief for sufferers of this condition (NSW Ministry of Health, 2012).
REFERENCES


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