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RESEARCH PAPER

Oleoyl-L-carnitine inhibits glycine transport by GlyT2

JE Carland*, RE Mansfield*, RM Ryan and RJ Vandenberg

Discipline of Pharmacology, School of Medical Sciences, Bosch Institute, Sydney Medical School, University of Sydney, Sydney, NSW, Australia

Correspondence
Robert J Vandenberg, Discipline of Pharmacology, School of Medical Sciences, Bosch Institute, Sydney Medical School, University of Sydney, Sydney, NSW, 2006, Australia. E-mail: robert.vandenberg@sydney.edu.au

*These authors made equal contributions to the work.

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BACKGROUND AND PURPOSE

Concentrations of extracellular glycine in the CNS are regulated by two Na^+/Cl^--dependent glycine transporters, GlyT1 and GlyT2. Selective inhibitors of GlyT1 have been developed for the treatment of schizophrenia, whilst selective inhibitors of GlyT2 are analgesic in animal models of pain. We have assessed a series of endogenous lipids as inhibitors of GlyT1 and GlyT2.

EXPERIMENTAL APPROACH

Human GlyT1 and GlyT2 were expressed in *Xenopus laevis* oocytes, and the inhibitory actions of a series of acylcarnitines on glycine transport were measured using electrophysiological techniques.

KEY RESULTS

Oleoyl-L-carnitine inhibited glycine transport by GlyT2, with an IC_{50} of 340 nM, which is 15-fold more potent than the previously identified lipid inhibitor N-arachidonyl-glycine. Oleoyl-L-carnitine had a slow onset of inhibition and a slow washout. Using a series of chimeric GlyT1/2 transporters and point mutant transporters, we have identified an isoleucine residue in extracellular loop 4 of GlyT2 that conferred differences in sensitivity to oleoyl-L-carnitine between GlyT2 and GlyT1.

CONCLUSIONS AND IMPLICATIONS

Oleoyl-L-carnitine is a potent non-competitive inhibitor of GlyT2. Previously identified GlyT2 inhibitors show potential as analgesics and the identification of oleoyl-L-carnitine as a novel GlyT2 inhibitor may lead to new ways of treating pain.

Abbreviations

CMC, critical micelle concentration; GlyT1, glycine transporter 1; GlyT2, glycine transporter 2; LiLCarn, linoleoyl-L-carnitine; LLCarn, lauroyl-L-carnitine; NAGly, N-arachidonyl-glycine; NFPS, N[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine; NOGly, N-oleoyl-glycine; OLCarn, Oleoyl-L-carnitine; PLCarn, palmitoyl-L-carnitine; SLCarn, stearoyl-L-carnitine

Introduction

Glycine is an inhibitory neurotransmitter in the spinal cord and brain stem, where it activates strychnine-sensitive glycine receptors (Lynch, 2004). It can also act as an excitatory neurotransmitter throughout the brain and spinal cord, where it is a co-agonist with glutamate at the NMDA subtype of glutamate receptor (Traynelis *et al.*, 2010; receptor nomenclature follows Alexander *et al.*, 2011). Two subtypes of glycine transporters, GlyT1 and GlyT2, are used to regulate extracellular glycine concentrations and have the potential to modulate the dynamics of both inhibitory glycinergic and excitatory glutamatergic neurotransmission (Eulenburg *et al.*, 2005). GlyT1 is expressed in glial cells surrounding both excitatory

and inhibitory synapses, whereas GlyT2 is expressed in presynaptic inhibitory glycinergic neurons (Liu *et al.*, 1993; Kim *et al.*, 1994). Characterization of the different physiological roles of the two GlyT subtypes has opened the possibility of pharmacologically manipulating glycine concentrations to treat schizophrenia (GlyT1 inhibitors) (Atkinson *et al.*, 2001; Aubrey and Vandenberg, 2001; Vandenberg and Aubrey, 2001; Kinney *et al.*, 2003) or pain (GlyT2 inhibitors) (Sur and Kinney, 2004; Vuong *et al.*, 2008; Connor *et al.*, 2010).

Glycine transporter inhibitors can be classified into a number of categories. Sarcosine is a GlyT1-selective substrate inhibitor (Supplisson and Bergman, 1997; Vandenberg *et al.*, 2008) and has provided the basis for the design of potent and selective GlyT1 inhibitors, such as N[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (Atkinson *et al.*, 2001; Aubrey and Vandenberg, 2001). A number of GlyT1- and GlyT2-selective blockers have been designed based on the structures of the tricyclic antidepressants and selective serotonin re-uptake inhibitors (Lechner, 2006). These compounds are likely to bind to the transporters within an extracellular accessible cavity and thereby block access of glycine to its site (Lechner, 2006; Andersen *et al.*, 2009). Our group has investigated the actions of a number of lipid-based GlyT inhibitors. The endogenous fatty acid, arachidonic acid, is a precursor for a large number of lipid compounds, including arachidonyl-amino acids and acyl-amino acids that exert a range of potent biological actions (Connor *et al.*, 2010). We have demonstrated that arachidonic acid inhibits glycine transport by GlyT1 but has no effect on the closely related GlyT2 (Pearlman *et al.*, 2003; Wiles *et al.*, 2006). In contrast, N-arachidonylglycine (NAGly) is a non-competitive inhibitor of GlyT2, with little or no effect on GlyT1 (Wiles *et al.*, 2006; Edington *et al.*, 2009). Furthermore, NAGly is analgesic in animal models of neuropathic and inflammatory pain (Succar *et al.*, 2007; Vuong *et al.*, 2008), and these actions may be mediated by its effects on glycine neurotransmission (Jeong *et al.*, 2009). These observations demonstrate that it is possible to use lipid-based compounds to selectively manipulate GlyT function, and this in turn has prompted our search for more selective and potent compounds.

L-carnitine and acylcarnitines are widely distributed throughout the body and are most commonly associated with the transport of long chain fatty acids into the mitochondria for β-oxidation (Foster, 2004). The acylcarnitine profile is used in neonatal screening to identify in-born errors of metabolism and can be a useful indicator of other disease states, such as ulcerative colitis and diabetes. More recently, carnitine, acetylcarnitine and acylcarnitines have been found to play a range of roles in brain function, by influencing lipid metabolism and modulating membrane protein functions (Jones *et al.*, 2010).

In this study, we investigated the activity of some endogenous acylcarnitines – lauroyl-L-carnitine (LLCarn), palmitoyl-L-carnitine (PLCarn), stearoyl-L-carnitine (SLCarn), linoleoyl-L-carnitine (LiLCarn) and oleoyl-L-carnitine (OLCarn) – and related molecules, on the glycine transporters (Figure 1). We demonstrated that OLCarn was a potent inhibitor of GlyT2 with little or no activity at GlyT1. We characterized the mechanism of OLCarn inhibition of GlyT2 and identified an extracellular loop (EL4) of GlyT2 that is important for determining the potency of OLCarn.

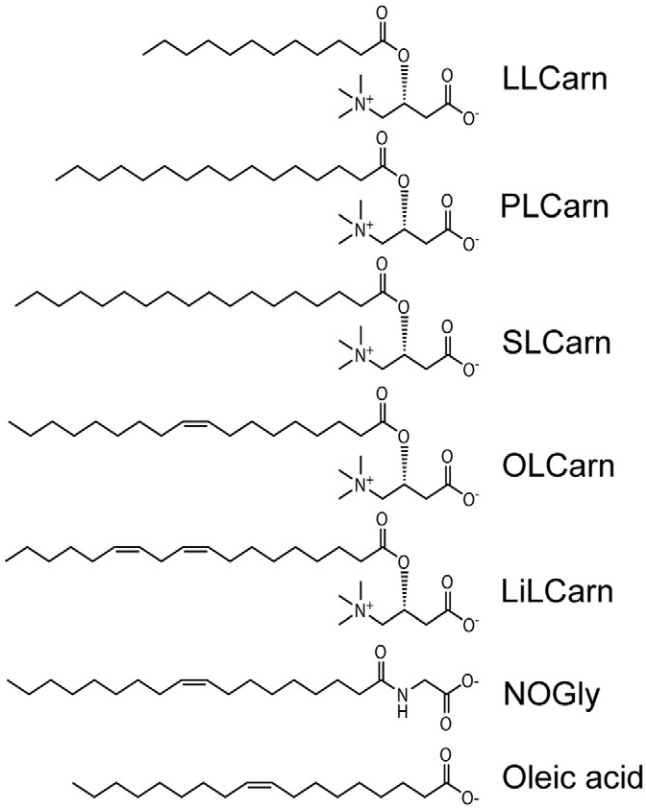

Figure 1
Chemical structures of acylcarnitines investigated in this study. LLCarn, PLCarn, SLCarn, OLCarn, LiLCarn, NOGly, oleic acid.

Methods

Critical micelle concentration (CMC) analysis

Acylcarnitines with long fatty acid tails will spontaneously form micelles in solution at high concentrations, thus decreasing the concentration of free molecules. In the following studies, we have used acylcarnitines at concentrations below the CMC so that we can accurately estimate the concentration of lipids that are interacting with the transporters. The CMCs for LLCarn and PLCarn are 1.2 and 0.075 mM respectively (Doi *et al.*, 2011). We estimated the CMC for OLCarn using the method of Chattopadhyay and London (1984). We first confirmed the utility of the method using dodecyl-β-maltoside (C_{12}M) as a positive control, and CMC values obtained are very similar to published values (see Supporting Information Figure S1). Using this method, the CMC for OLCarn was estimated to be 7.4 ± 1.2 μM (n = 5). The maximum concentration of any acylcarnitine used was 3 μM.

Molecular biology

cDNAs encoding human GlyT1b, GlyT2a and mutant GlyT2a constructs were subcloned into oocyte transcription vector (pOTV). Point mutations were introduced using standard molecular biological techniques, and all constructs were sequenced to confirm fidelity. The plasmids were linearized

with *Spe*I, and mRNA was synthesized using the T7 RNA polymerase mMESSAGEmMACHINE kit (Ambion, Austin, TX). GlyT1b and GlyT2a are referred to as GlyT1 and GlyT2 in the text.

Expression of glycine transporters in Xenopus laevis *oocytes and electrophysiology*

All animal care and experimental procedures were in accordance with the Australian National Health and Medical Research Council guidelines for the use of animals in scientific experiments. Oocytes were harvested from *X. laevis* as described previously (Wiles *et al.*, 2006) and stage V–VI oocytes were injected with mRNA (10 ng) and then stored at 16°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 μg mL^{-1} gentamycin and 100 μg mL^{-1} tetracycline. The storage solution was changed daily.

Recordings of transporter activity were obtained after 3–5 days by two-electrode voltage clamp by means of a GeneClamp 500B amplifier (Axon Instruments, Foster City, CA) interfaced with a Powerlab 2/26 (ADInstruments, Sydney, Australia) and LabChart6 (ADInstruments). Oocytes were voltage-clamped at –60 mV and continually perfused with ND96 solution at room temperature. Current–voltage relationships were measured using a protocol previously described (Vandenberg *et al.*, 1998). Briefly, oocytes were voltage-clamped at –30 mV, and the current–voltage relations were determined by subtraction of steady-state current measurements in the absence of substrate, obtained during 200 ms voltage pulses to potentials between –100 and +60 mV, from corresponding current measurements in the presence of substrate.

Stock solutions (10 mg mL^{-1}) of OLCarn, PLCarn, LLCarn, SLCarn, LiLCarn and NOGly were made in ethanol. Final solutions contained 0.05% ethanol, a concentration shown to have no effect on transporter responses. Stock solutions (10 mg mL^{-1}) of oleic acid and ALX1393 were made in DMSO. Final solutions contained 0.1% DMSO, a concentration shown to have no effect on transporter responses. All compounds were only tested to a maximal concentration of 3 μM because these compounds form micelles at higher concentrations.

Known concentrations of compounds were applied to oocytes in the absence and presence of glycine until a stable current was reached, at which time the oocyte was washed for 3 to 5 min, sufficient time to allow complete recovery of response to a known dose of glycine in the absence of inhibitors. The time course of washout of 1 μM OLCarn was measured by first measuring a control response to 30 μM glycine followed by co-application of 1 μM OLCarn until a stable response was achieved (~5 min). OLCarn and glycine were washed from the bath using the ND96 buffer solution. At 5 min intervals up to 35 min, 30 μM glycine was applied to measure the level of recovery relative to the pre-OLCarn exposure. β-cyclodextrin was also used to investigate factors affecting washout of OLCarn. After measuring the response to 1 μM OLCarn, 1 mM β-cyclodextrin was included in the ND96 washout buffer. After 5 min, responses to glycine were measured.

Analysis of pharmacological data

Current (I) as a function of glycine concentration [Gly] was fitted by least-squares analysis to a derivative of the Michaelis–Menten equation:

$$I = ([Gly] \cdot I_{max})/(EC_{50} + [Gly]),$$

using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA; http://www.graphpad.com). I_{max} is the maximal current, and EC_{50} is the concentration of glycine that generates half maximal current. Glycine current as a function of inhibitor concentration [I] was fitted by least-squares analysis to the equation:

$$I/I_{EC50} = C + (I_{EC50} - C)/(1 + 10^{[I]-\log IC50}).$$

I_{EC50} is the current generated by the EC_{50} concentration of glycine. IC_{50} is the [I] at which half maximal reduction in transport current occurs, and C is the residual current at maximal inhibition of transport.

Data analysis

Full concentration responses for glycine transport by GlyT1, GlyT2 and the various chimeras and mutants were conducted on single cells and values for EC_{50} are presented as mean ± SEM, $n \geq 3$. LLCarn, PLCarn, SLCarn, LiLCarn, OLCarn, NOGly and ALX1393 have very long washout periods, so single concentrations of inhibitor were applied to individual cells and normalized to a pre-drug exposure to a given glycine concentration response. For these experiments, the IC_{50} values are presented as the mean, and 95% confidence intervals (95% CI) are presented. Individual points on the graphs are mean ± SEM, with *n* for each point derived from between three and five cells.

Materials

OLCarn, PLCarn, LLCarn, N-oleoylglycine (NOGly) were obtained from Avanti Polar Lipids (Alabaster, AL). SLCarn, LiLCarn, OLCarn were obtained from Larodan Fine Chemicals (Malmö, Sweden). LiLCarn supplied from Larodan Fine Chemicals was supplied as a solution in chloroform. The chloroform was removed by evaporation under a N_2 stream for 3 h, and then the lipid film was dissolved in ethanol at a concentration of 54 mM. All other lipids were supplied as either a solution in ethanol, or as a powder, which was then dissolved in ethanol. All other compounds and reagents were obtained from Sigma-Aldrich (Sydney, NSW, Australia) unless otherwise stated.

Results

Oleoyl-L-carnitine inhibits GlyTs

Application of glycine to *X. laevis* oocytes expressing GlyT1 and GlyT2, voltage-clamped at –60 mV, generates concentration-dependent inward currents with affinities similar to those reported previously (Vandenberg *et al.*, 2008; Edington *et al.*, 2009) (GlyT2, EC_{50} = 26 ± 4 μM; GlyT1, EC_{50} = 24 ± 3 μM). Application of OLCarn did not generate any currents at either transporter, indicating that it is not a transportable substrate (data not shown). However, co-application of 1 μM OLCarn with 30 μM glycine reduced glycine trans-

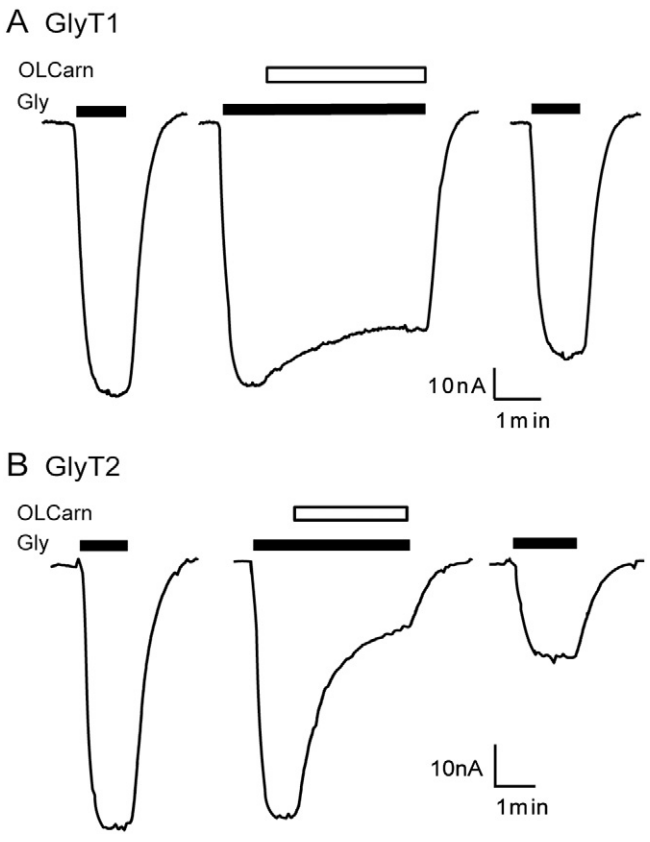

Figure 2

OLCarn is a potent inhibitor of the GlyT2 subtype of glycine transporters. Representative current traces from oocytes expressing (A) GlyT1 and (B) GlyT2 voltage clamped at –60 mV. Glycine (30 μM) generates an inward current that is inhibited by 1 μM OLCarn. After a 5 min washout glycine (30 μM) was reapplied. Data are mean ± SEM ($n \geq 3$).

port currents for both GlyT1 and GlyT2 (Figure 2), with a greater level of inhibition observed for GlyT2. OLCarn had a slow onset of action on GlyT2 (1 μM OLCarn requires 3 min application for a stable level of inhibition) and was not readily reversible (see below). Prolonged exposure of high concentrations of OLCarn (3 μM, ~5 min) also activated an additional current in oocytes expressing GlyT1, GlyT2 and also uninjected oocytes. See supplementary material for further characterization of this response (Supporting Information Figures S2 and S3). In the following studies, we limited the exposure of oocytes to high concentrations of OLCarn to less than 5 min.

The head and tail groups of acylcarnitines influence activity

LLCarn, PLCarn and OLCarn have the same head group (L-carnitine) but differ in lipid tail length and number of double bonds. LLCarn and PLCarn have saturated fatty acid tails that are 12 and 16 carbons long respectively. The fatty acid tail of OLCarn has 18 carbons and contains one *cis* double bond in its backbone structure (Figure 1). The concentration dependence of LLCarn, PLCarn and OLCarn inhibition of

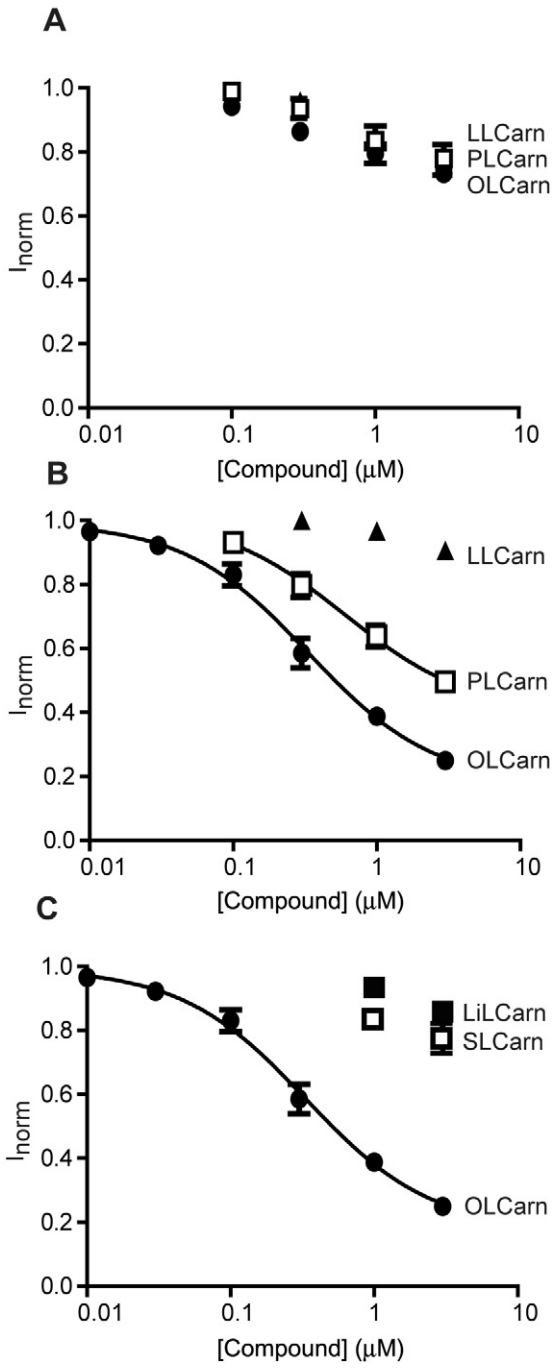

Figure 3

OLCarn is a potent inhibitor of GlyT2. Concentration–response curves for inhibition of 30 μM glycine transport exhibited by the acylcarnitines LLCarn, PLCarn and OLCarn at (A) GlyT1 and (B) GlyT2. (C) Concentration-dependent inhibition of 30 μM glycine transport currents by the acylcarnitines OLCarn, SLCarn and LiLCarn. Current responses were normalized to pre-exposure glycine (30 μM) responses. Data are mean ± SEM ($n \geq 3$).

GlyT1 and GlyT2 transport currents was measured by applying increasing concentrations of the lipid with a fixed concentration of glycine (30 μM) (Figure 3). LLCarn (IC_{50} > 10 μM), PLCarn (IC_{50} > 10 μM) and OLCarn (IC_{50} > 10 μM) displayed a

Table 1

The IC_{50} values for inhibition of GlyT1 and GlyT2 by a range of lipid compounds

	GlyT1	GlyT2	
Compound	IC_{50} (μM)	IC_{50} (μM)	95% confidence intervals (μM)
OLCarn	>10	0.34	0.21–0.53
PLCarn	>10	0.60	0.21–1.7
LLCarn	>10	>10	
SLCarn		>10	
LiLCarn		>10	
NOGly	>10	0.88	0.23–3.3
OA	>10	>10	
NAGly[a]	>30	3.4 ± 0.6	

[a]Data is mean ± SEM (Edington *et al.*, 2009).

similar low level of inhibition at GlyT1 (Figure 3A, Table 1). In contrast, the acylcarnitines exhibited differential activity at GlyT2. LLCarn had minimal inhibitory activity at GlyT2 (IC_{50} > 10 μM), while OLCarn (IC_{50} = 0.34 μM, 95% CI, 0.21–0.53 μM) and PLCarn (IC_{50} = 0.60 μM, 95% CI, 0.21–1.7 μM) were relatively potent inhibitors (Figure 3B, Table 1). The differential sensitivity of GlyT2 to the three acylcarnitines suggested that the fatty acid tail of OLCarn contributed to their activity at GlyT2. We also investigated the effect of different numbers of double bonds in the C18 backbone by comparing the effects of SLCarn (C18:0), LiLCarn (C18:2) and OLCarn (C18:1) on GlyT2. Both SLCarn and LiLCarn were less potent and less effective inhibitors than OLCarn. At concentrations up 3 μM, SLCarn inhibited 30 μM glycine responses by 16.8 ± 6.1%, whilst LiLCarn inhibited the responses by 9.5 ± 3.2% (Figure 3C). Thus, OLCarn (C18:1) provides the optimal inhibitor profile. L-carnitine (up to 300 μM) had no significant activity at either GlyT1 or GlyT2 as a substrate or an inhibitor (n = 3, data not shown). This highlights the importance of both the length and number of double bonds in the lipid tail for mediating the inhibition observed.

We further investigated the nature of the lipid head group required for OLCarn activity at GlyT2 by assessing the activity of the related endogenous compounds, NOGly and oleic acid (Figure 1). In NOGly, the head group is a glycine moiety, and in oleic acid, the head group is a carboxylic acid group. Neither compound generated currents when applied alone to oocytes expressing GlyT1 or GlyT2, indicating that neither were transporter substrates. Oleic acid was a weak inhibitor of both GlyT1 and GlyT2. Oleic acid (1 μM) inhibited the 30 μM glycine transport current of GlyT1 by 17 ± 5% (data not shown) and GlyT2 by 15 ± 5% (Figure 5A). NOGly was inactive at GlyT1 (data not shown) but inhibited glycine transport by GlyT2 (Figure 5A). It is interesting to note that NOGly was more potent than NAGly at GlyT2 (Wiles *et al.*, 2006) but was still approximately twofold less potent than OLCarn (IC_{50} = 0.88 μM, 95% CI, 0.23–3.3). These results suggested that the potency of OLCarn at GlyT2 was due to both the fatty acid tail and the carnitine head group and also demonstrated that the identity of the head group contributed to GlyT subtype selectivity and potency at GlyT2. We also investigated whether OLCarn and NAGly compete for the same site on GlyT2 by first applying a high dose of OLCarn (1 μM) and after the inhibitory response stabilized, adding 10 μM NAGly. If the two compounds interact with GlyT2 at separate sites and the effects were additive, almost complete inhibition would be expected. However, the level of inhibition of the two compounds together was only 12% greater than the compounds added alone (Figure 5B). Similar results were obtained when the order of compound addition was reversed. These results are consistent with the conclusion that NAGly and OLCarn interacting with overlapping sites on GlyT2.

OLCarn shows a mixed mechanism of inhibition of GlyT2

To further characterize the activity of OLCarn at GlyT2, glycine concentration-dependent responses were measured in the presence of varying concentrations of OLCarn (Figure 4). In the absence of OLCarn, the EC_{50} for glycine at GlyT2 is 26 μM (95% CI, 22–30 μM). In the presence of increasing doses of OLCarn (0.1, 0.3, 3 μM), the glycine EC_{50} shows small, but not significant increases [EC_{50} (0.1 μM) = 28 μM (95% CI, 20–5 μM); EC_{50} (0.3 μM) = 41 μM (95% CI, 17–65 μM); EC_{50} (3 μM) = 41 μM (95% CI, 13–69 μM)]. However, the I_{max} of the glycine concentration–response curve is reduced in the presence of 0.3 μM OLCarn to 60% (95% CI, 51–69%) and to 42% (95% CI, 33–51%) in the presence of 3 μM OLCarn. An Eadie–Hofstee transformation of the glycine concentration response curves shows that at low concentrations of OLCarn the mechanism of inhibition appears competitive, but at higher concentrations of OLCarn both competitive and non-competitive features are observed (Figure 4B).

Reversibility of OLCarn inhibition of GlyT2

After washout of OLCarn (1 μM) from the recording chamber, GlyT2 glycine transport currents were still decreased, compared to pre-exposure responses (Figure 2B), demonstrating that OLCarn inhibition of GlyT2 is not readily reversible. The OLCarn concentration- and time-dependence of this effect was investigated by applying a control glycine dose (30 μM) and, once the current had reached its peak, co-applying either a low (0.1 μM) or high (1 μM) dose of OLCarn. Once OLCarn inhibition had stabilized, both glycine and OLCarn were washed from the chamber. Control glycine doses (30 μM) were subsequently applied after 5 and 35 min of washing to estimate the level of recovery. Co-application of glycine with a low dose (0.1 μM) of OLCarn to oocytes expressing GlyT2 reduces the transport current to 72 ± 4%. After washing out of OLCarn, the control responses recovered to 91 ± 3% and 97 ± 3% of control responses after 5 and 35 min respectively (Figure 6). Exposure of oocytes expressing GlyT2 to a high dose of OLCarn (1 μM) reduced the transport currents to 34 ± 3%, and this was unchanged after a 5 min wash (38 ± 8%). Some recovery was evident after 35 min of washing (72 ± 2% of control response; Figure 6). A potential explanation for the slow recovery of glycine transport currents is that OLCarn interacts with the transporter via a lipid phase interaction. We explored this possibility by

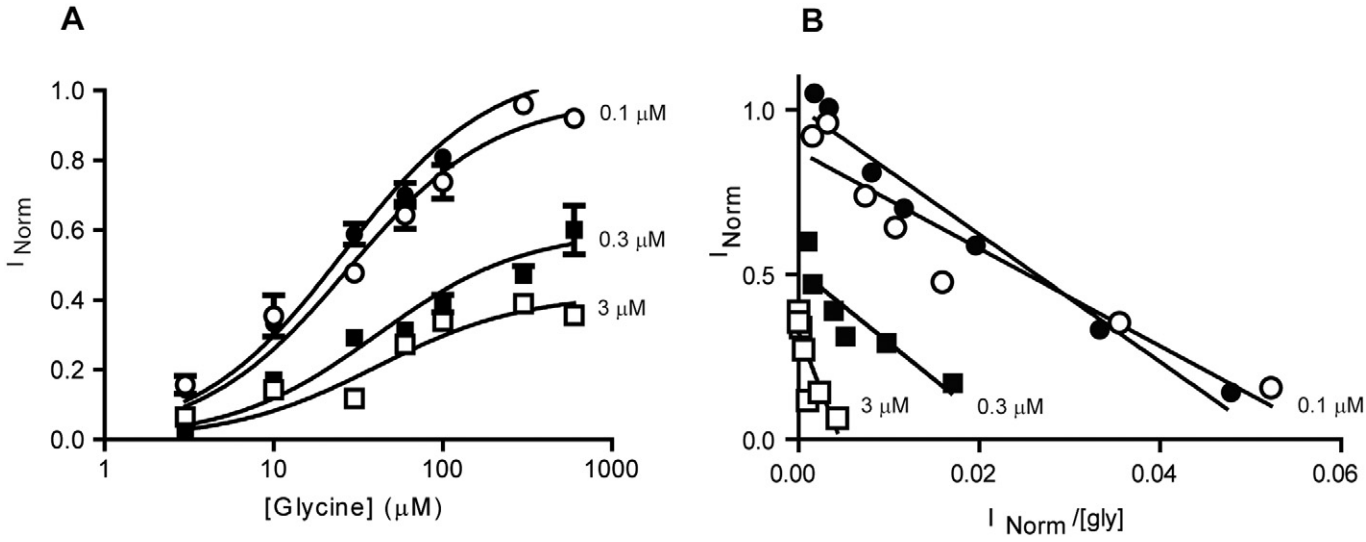

Figure 4

OLCarn acts as a mixed inhibitor of GlyT2. (A) Glycine dose–response curves were constructed alone and in the presence of 0.1 μM,0.3 μM and 3 μM OLCarn. Current responses were normalized to pre-exposure glycine (30 μM) responses. Data are mean ± SEM ($n \geq 3$). (B) Eadie–Hofstee transformation of the data in panel A.

using 1 mM β-cyclodextrin in the wash solution to speed up the extraction of lipids from the membrane (Liu *et al.*, 2009; Gasbarri *et al.*, 2010). Under these conditions, glycine transport currents were fully reversible after 5 min (Figure 6). The faster recovery from inhibition in the presence of β-cyclodextrin suggests that the OLCarn recognition site on GLYT2 may be exposed to the lipid membrane. For oocytes expressing GlyT1, application of a high dose of OLCarn (1 μM) reduced the control glycine response by 23 ± 5%, but in contrast to GlyT2, this response recovered to 93 ± 2% of control after a 5 min washout (data not shown).

Extracellular loop 4 of GlyT2 is required for OLCarn inhibition of GlyT2

Using the crystal structure of a bacterial homologue of the human glycine transporters ($LeuT_{Aa}$) (Yamashita *et al.*, 2005) as a guide, we have constructed chimeric transporters in which either extracellular loop 2 (EL2) or extracellular loop 4 (EL4) were replaced by the equivalent region from GlyT1 (termed GlyT2(EL2) and GlyT2(EL4) respectively; Figure 7A). We have previously shown that both EL2 and EL4 of GlyT2 are important for inhibition mediated by NAGly and N-arachidonyl-γ-aminobutyric acid, but only EL4 is required for N-arachidonyl-L-alanine binding (Edington *et al.*, 2009). As EL2 and EL4 appeared to be important for lipid modulation of GlyT2, we examined whether these loops were also important for OLCarn inhibition of GlyT2 by testing the ability of OLCarn to inhibit glycine transport by these chimeric transporters.

The effect of a range of OLCarn concentrations on glycine transport was measured for each chimeric construct (Figure 7B, Table 2). The GlyT2(EL2) chimera showed a slightly reduced OLCarn response compared with wild-type GlyT2, with an IC_{50} of 0.61 μM (95% CI, 0.26–1.4 μM); however, this difference was not statistically significant. In contrast, GlyT2(EL4) showed a dramatically reduced response, with no observable inhibition up to 1 μM OLCarn. Thus, EL4 appears to contain molecular determinants that influence the differential sensitivity of OLCarn for GlyT1 and GlyT2, which agrees with our previous results for other endogenous lipids (Edington *et al.*, 2009). The GlyT1(EL4) chimera was also tested but showed similar sensitivity to OLCarn as wild-type GlyT1. This suggests that EL4 in GlyT2 is necessary, but not sufficient for OLCarn inhibition.

In contrast to OLCarn and other lipid inhibitors, the structurally unrelated GlyT2 selective inhibitor ALX1393 (Xu *et al.*, 2005; Vandenberg *et al.*, 2008) exerted the same level of

Table 2

The IC_{50} values for OLCarn and ALX1393 inhibition of glycine transport by wild type and GlyT2 mutant transporters

	OLCarn	
Construct	IC_{50} (μM)	95% CI (μM)
GlyT2	0.34	0.21–0.53
GlyT2(EL2)	0.61	
GlyT2(EL4)	>10	0.26–1.4
GlyT1	>10	
GlyT1(EL4)	>10	
R531L	0.43	0.21–0.88
K532G	0.57	0.37–0.87
I545L	>10	
I545A	0.22	0.16–0.31
I545M	0.68	0.35–1.3
I545F	0.44	0.25–0.78

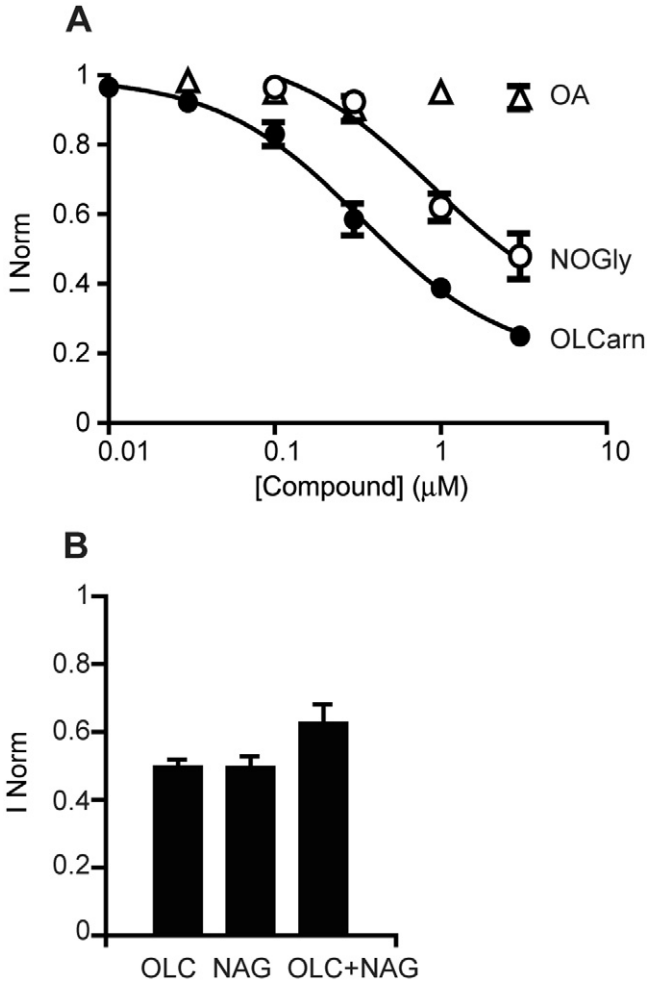

Figure 5

(A) Concentration–response curves for inhibition of 30 μM glycine transport exhibited by OLCarn, NOGly and oleic acid at GlyT2. Current responses were normalized to pre-exposure glycine (30 μM) responses. Data are mean ± SEM ($n \geq 3$). (B) The inhibitory actions of OLCarn and NAGly are not additive. 30 μM Glycine was applied, followed by co-application of 1 μM OLCarn (OLC) and then co-application of 30 μM glycine, 1 μM OLCarn and 10 μM NAGly (OLC + OLC). Similar experiments were conducted with 10 μM NAGly (NAG) applied before OLCarn. The level of inhibition observed for OLC + NAG was the same irrespective of the order of addition.

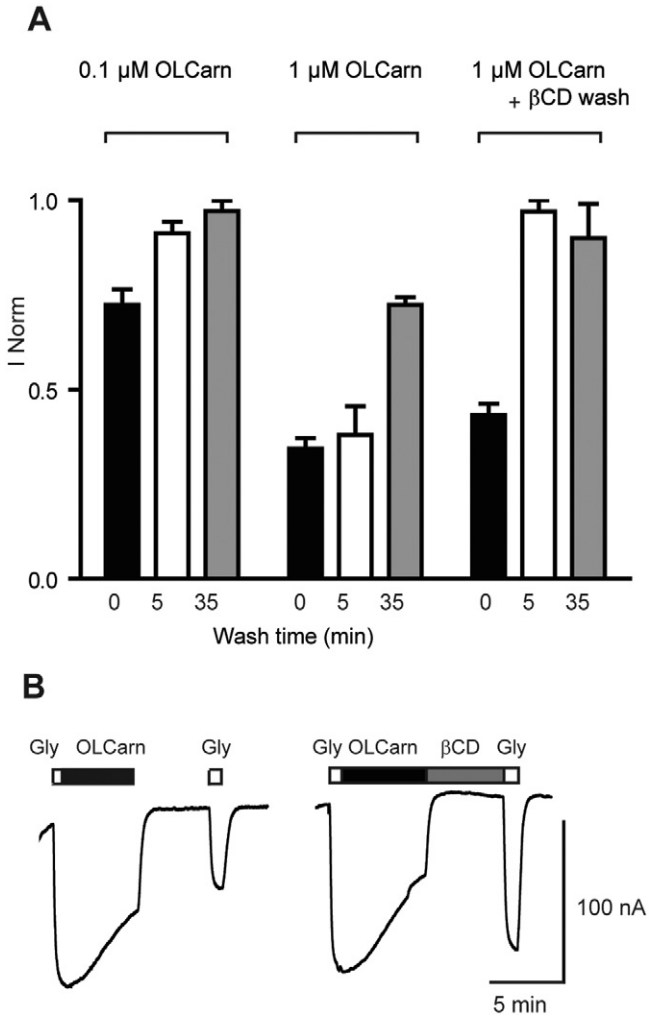

Figure 6

OLCarn inhibition and recovery of the glycine response of GlyT2 is dependent on time and concentration and recovery is enhanced by washing in the presence of 1 mM β-cyclodextrin (βCD). (A) Co-application of glycine (Gly) and OLCarn (0.1 and 1 μM) inhibits the control glycine (30 μM) response. Recovery of response after 5 and 35 min of washing is shown. The rate of recovery was increased by washing in the presence of 1 mM β-cyclodextrin. Current responses were normalised to pre-exposure glycine (30 μM) responses. Data are mean ± SEM ($n \geq 3$). (B) Example current traces for 1 μM OLCarn inhibition of 30 μM glycine transport currents and a comparison of washout of OLCarn in presence and absence of 1 mM β-cyclodextrin.

inhibition on the GlyT2(EL2) and GlyT2(EL4) chimeras as for wild-type GlyT2 (see Supplementary Figure S4 and Table S1), suggesting that these regions are not required for ALX1393 binding.

Residue 545 within EL4 is critical for OLCarn binding

There are 11 residues in EL4 that differ between GlyT1 and GlyT2 (Figure 8A). We have previously shown that individual mutations of three of these residues in GlyT2 to the corresponding GlyT1 residue (R531L, K532G and I545L) significantly reduce NAGly inhibition of GlyT2. To determine whether a similar trend existed for inhibition by OLCarn, these three GlyT2 mutant transporters were tested for inhibition of a 30 μM glycine response over a range of OLCarn concentrations (Figure 8B). Interestingly, while the R531L and K532G mutants were indistinguishable from wild-type GlyT2 [IC_{50} = 0.43 μM (95% CI, 0.21–0.88 μM) and IC_{50} = 0.37 μM (95% CI, 0.37–0.87 μM), respectively; Table 2], the I545L mutant reduced the level of inhibition to a level seen for GlyT1 (IC_{50} > 10 μM). To determine whether the other eight EL4 residues that differ between GlyT2 and GlyT1 also effect OLCarn inhibition, OLCarn (1 μM) inhibition of glycine (30 μM) was measured for each of the remaining

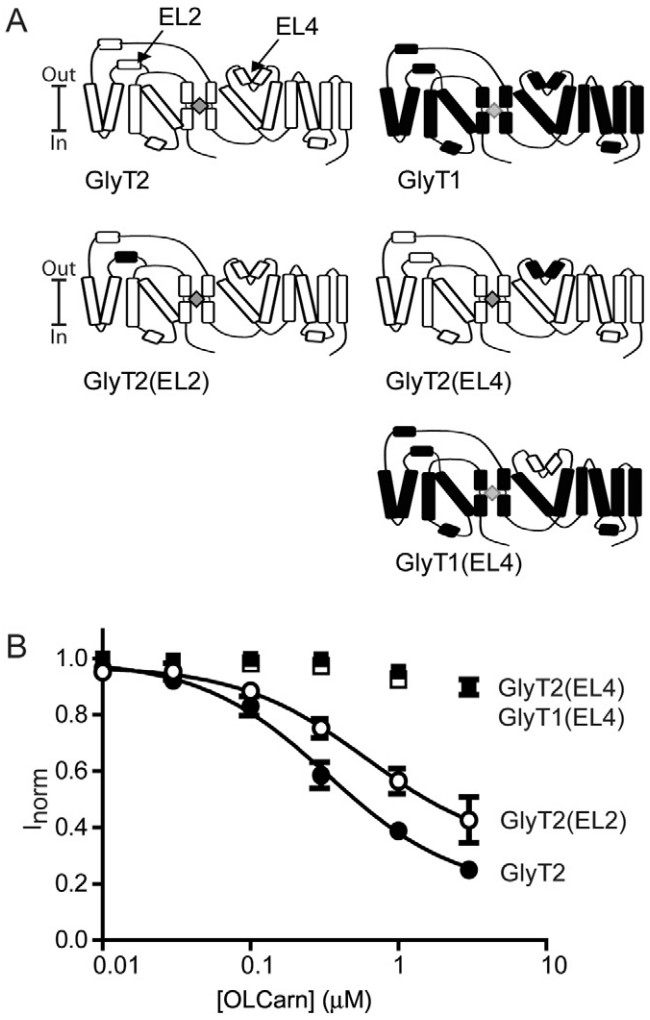

Figure 7

The effect of OLCarn on GlyT2(EL2), GlyT2(EL4) and GlyT1(EL4). (A) Schematic diagram of the chimeric constructs. Portions of GlyT2 sequence are shown in white, and of GlyT1 in black. (B) OLCarn concentration-dependent inhibition of 30 μM glycine transport currents for GlyT2, GlyT2(EL2), GlyT2(EL4) and GlyT1(EL4). Current responses were normalized to pre-exposure glycine (30 μM) responses. Data are mean ± SEM ($n \geq 3$).

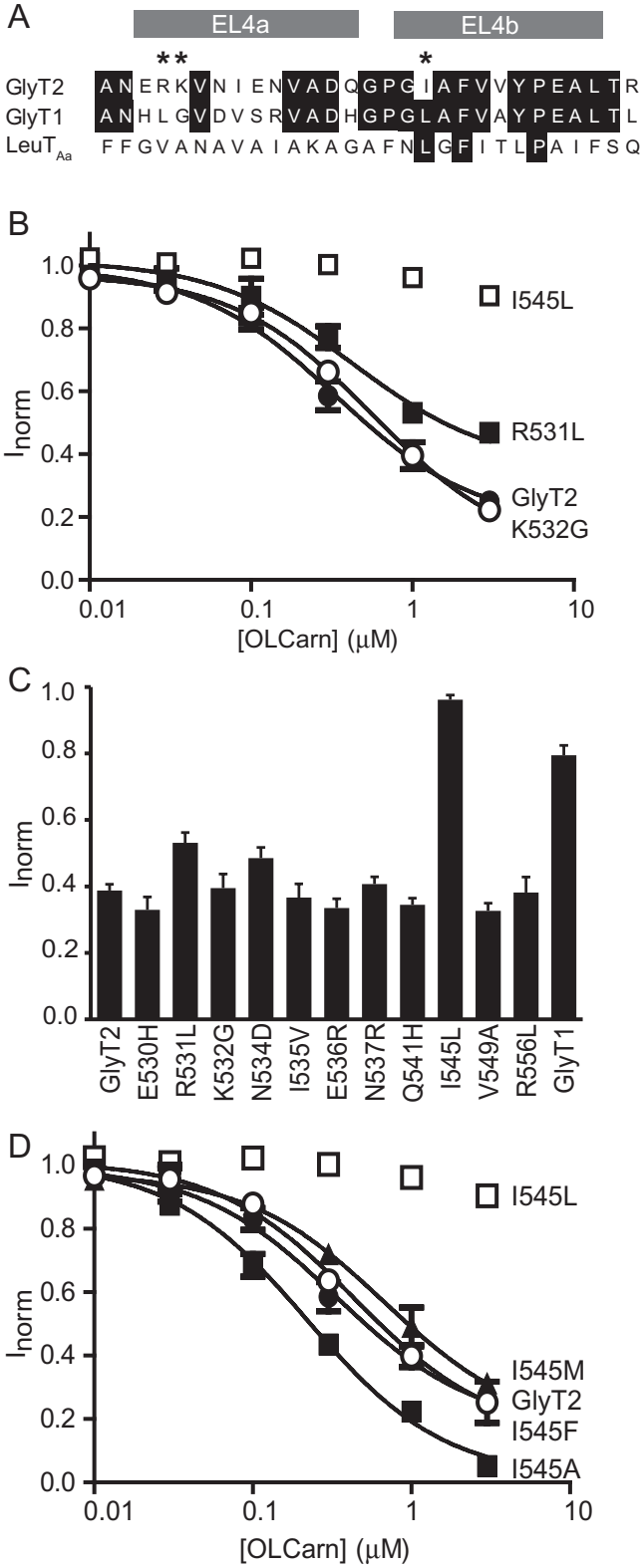

Figure 8

The effect of OLCarn on GlyT2 EL4 point mutants. (A) Sequence alignment of EL4 from GlyT2 (residues 528–556), GlyT1 (residues 408–436) and the bacterial homologue LeuT (residues 305–333). Residues that differ between the two glycine transporters are highlighted in black, and the three residues previously shown to be important for inhibition of GlyT2 by NAGly are indicated by asterisks. Grey bars above the sequence indicate α-helical regions in the crystal structures of LeuT. (B) OLCarn concentration-dependent inhibition of 30 μM glycine transport currents for GlyT2, R531L, K532G and I545L. (C) Inhibition of 30 μM glycine transport currents by 1 μM OLCarn for 11 different GlyT2 EL4 mutants. GlyT1 and GlyT2 responses are included for comparison. (D) OLCarn concentration-dependent inhibition of 30 μM glycine transport currents for GlyT2, I545L, I545A, I545M and I545F. Current responses were normalized to pre-exposure glycine (30 μM) responses. Data in panels B, C and D are mean ± SEM ($n \geq 3$).

mutants (E530H, N534D, I535V, E536R, N537R, Q541H, V549A and R556L). All responses were comparable with wild-type GlyT2 (Figure 8C).

The striking reduction of OLCarn inhibition upon mutation of residue I545 to a leucine is especially remarkable given the small nature of this change, with isoleucine and leucine only differing by the position of a single methyl group. To further investigate the characteristics of residue 545 that allowed or prevented inhibition by OLCarn, a series of further mutations were introduced at this position, namely I545A, I545M and I545F. All mutants were confirmed to be functional, with I545F displaying a glycine response similar to that of wild-type GlyT2 (EC_{50} = 26 ± 4 μM), while I545A and I545M showed a slightly reduced response (EC_{50} = 80 ± 7 and 53 ± 5 μM respectively). OLCarn inhibition of a control glycine (30 μM) response was then determined (Figure 8D). I545A, I545M and I545F were all inhibited by OLCarn to a similar extent to wild-type GlyT2, with IC_{50} values of 0.22 μM (95% CI, 0.16–0.31 μM), 0.68 μM (95% CI, 0.35–1.3 μM) and 0.44 μM (95% CI, 0.25–0.78 μM) respectively (Table 2). These striking results suggested that a single residue in EL4 (I545) was responsible for the ability of OLCarn to selectively inhibit GlyT2, and that mutation of this isoleucine residue to leucine had the most profound effect.

Discussion and conclusions

The GlyT1 and GlyT2 transporters play different roles in the regulation of glycine concentrations in the CNS and this difference has generated considerable interest in the development of subtype-selective GlyT inhibitors for the treatment of neurological disorders. We have previously demonstrated that arachidonic acid inhibits GlyT1 and has no activity at GlyT2, whereas the arachidonic acid derivative, NAGly, inhibits GlyT2 and has no activity at GlyT1 (Pearlman *et al.*, 2003; Wiles *et al.*, 2006). In this study, we have continued our investigations into lipid-based inhibitors of GlyTs and have demonstrated that the acylcarnitines PLCarn and OLCarn were more potent inhibitors of GlyT2 compared to GlyT1. OLCarn had the highest affinity for GlyT2 and was 15-fold more potent than the previously described lipid-based inhibitor NAGly (Wiles *et al.*, 2006; Edington *et al.*, 2009). The differential activity of the compounds tested highlights the importance of lipid structure in determining the activity at GlyTs. Lipid compounds of varying tail lengths have been examined, including C22 (arachidonic), C18 (oleoyl), C16 (palmitoyl) and C12 (lauroyl). Of these, the oleoyl tail had the highest affinity. Furthermore, the number of double bonds in the lipid tail was an important factor in determining efficacy, with the C18:0 and C18:2 derivatives showing markedly less efficacy than the C18:1 derivative, OLCarn. The current study also emphasizes that the identity of the head group was important for the affinity and/or selectivity of lipid compounds for GlyTs, with OLCarn displaying greater potency than NOGly, despite both having a C18:1 tail. The importance of the combination of the tail and head group in lipid activity is highlighted by the fact that neither L-carnitine nor oleic acid, used alone, had any substantial activity at the GlyTs.

OLCarn inhibition of GlyT2 transport exhibits both a slow onset and slow washout. The slow onset of inhibition could be related to the inherent flexibility of OLCarn and it may take some time before the lipid can find the correct conformation required for inhibition. The slow washout of OLCarn inhibition could be attributed to the high affinity of the compound for the transporter. However, it is also important to recognise the potential for OLCarn to interact with the cell membrane. The observation that β-cyclodextrin speeds up the recovery of GlyT2 from inhibition by OLCarn suggests that OLCarn may interact with the transporter via a lipid phase interaction. Previous studies have investigated the ability of acylcarnitines, particularly those with a lipid tail of C12 to C16, to increase membrane permeability to different drugs, such as cefoxitin, gentamicin, cytarabine and α-methyldopa (Fix *et al.*, 1986). These studies suggest that acylcarnitines perturb the lipid order (LeCluyse *et al.*, 1991), possibly by partitioning into the lipid bilayer. Indeed, it has been reported that high doses of PLCarn (25 μM) induced haemolysis of rat erythrocytes (Cho and Proulx, 1971).

Prolonged application of a high concentration of OLCarn (3 μM) to both uninjected oocytes and oocytes expressing GlyT1 and GlyT2 induced a leak current (Supporting Information Figures S2 and S3), suggesting that this current was not directly mediated by the GlyTs. This concentration (3 μM) is close to the CMC for OLCarn (Supporting Information Figure S1), and we propose that the formation of lipid micelles results in the activation of this non-specific leak conductance by altering the integrity of the oocyte membrane. In contrast to this non-specific effect, after prolonged application of a high dose of OLCarn, the slow recovery from exposure to OLCarn appears to be transporter specific, with considerably longer recovery times required for GlyT2-expressing oocytes compared to GlyT1-expressing oocytes or uninjected oocytes (see supplementary data). One potential explanation for this differential washout period is that the cell membrane may form a sink that can accumulate OLCarn. Following removal of OLCarn from the bath solution, the OLCarn that has accumulated within the membrane may diffuse out of the cell membrane and specifically bind to the GlyT2 transporter, maintaining its inhibition of transport. This low residual concentration of OLCarn may not be sufficient to inhibit GlyT1. It is intriguing that SLCarn (C18:0) and LiLCarn (C18:2) are less effective than OLCarn (C18:1) in inhibiting GlyT2, and furthermore that prolonged exposure of high concentrations of SLCarn and LiLCarn did not induce the same non-specific leak current as OLCarn. A possible explanation for the differential effects of OLCarn, SLCarn and LiLCarn on GlyT2 may be that SLCarn and LiLCarn are less efficiently incorporated into the membrane than OLCarn and therefore are less effective inhibitors.

OLCarn is more potent – over 30-fold – at GlyT2 compared with GlyT1. What is the molecular basis for this differential inhibition? We have previously used chimeric constructs of the GlyTs where EL2 and EL4 were switched between GlyT1 and GlyT2 and found that EL4 was required for the selective inhibition of NAGly by GlyT2 (Edington *et al.*, 2009). In this study, we employed the same strategy to probe the molecular basis for the selectivity of OLCarn for GlyT2 and found that EL4 of GlyT2 is essential for OLCarn inhibition. It is clear that EL4 plays an important role in the

transport cycle of the bacterial homologue of the human glycine transporters, $LeuT_{Aa}$. Several crystal structures of $LeuT_{Aa}$ have revealed that during the movement of substrate across the membrane, the transporter undergoes conformational changes from outward-open to occluded, to inward-open (Krishnamurthy and Gouaux, 2012). As part of this process EL4 moves into the extracellular vestibule and this displacement appears to be critical for opening the intracellular gate to allow release of substrate into the cell. In addition, EL4 plays a key role in forming the extracellular gate of $LeuT_{Aa}$, by interacting with residues in adjacent transmembrane domains and undergoing a large movement, to block the substrate binding site from the extracellular side (Krishnamurthy and Gouaux, 2012). Inhibitors of $LeuT_{Aa}$, such as clomipramine and tryptophan, trap $LeuT_{Aa}$ in an outward-facing conformation by preventing movements of EL4 and the collapse of the extracellular gate (Singh *et al.*, 2007; 2008). Interestingly, EL4 of GlyT1 also contains a histidine residue that is required for inhibition by Zn^{2+} (Ju *et al.*, 2004). We propose that lipid compounds, such as NAGly and OLCarn, inhibit GlyT2 in a similar manner, by restricting or preventing the movement of EL4 during the glycine transport process.

The amino acid residue I545 within the loop between EL4a and EL4b (Figure 8A) appears to play a key role in determining the activity of OLCarn on GlyT2. It is intriguing that the conservative mutation of I545L has a marked effect on OLCarn activity, while the less conservative mutations I545A, I545M and I545F do not significantly alter the ability of OLCarn to inhibit glycine transport by GlyT2. The same I545L mutation results in a significant reduction in NAGly inhibition of GlyT2. At this point, we can only speculate as to the structural basis for OLCarn and NAGly selectivity for GlyT2 over GlyT1. Studies investigating the interaction of fatty acids with proteins typically identify hydrophobic cavities as the binding sites for these molecules (Bradbury *et al.*, 2011). Hydrophobic residues within a central cavity of the mitochondrial carnitine/acylcarnitine carrier have been identified as important for binding of the acyl chain (Tonazzi *et al.*, 2012). OLCarn and NAGly may interact directly with EL4 of GlyT2, restricting its movements and thereby inhibiting the conformational changes required for transport. The head groups of OLCarn and NAGly are significantly different, yet their selectivity for GlyT2 over GlyT1 is influenced by the same hydrophobic amino acid residue. An alternative explanation is that OLCarn and NAGly may interact with a membrane-exposed site on GlyT2, which is consistent with our observation that β-cyclodextrin increased the rate of washout of OLCarn from oocytes. In a molecular dynamics study of $LeuT_{Aa}$ interactions with lipids, it was postulated that the head groups of the membrane lipids have specific interactions with the charged residues at the extracellular edges of the transmembrane domains, and that these interactions could influence function (Pantano and Klein, 2009). Thus, it is possible that the head groups of OLCarn and NAGly could also interact with GlyT2 at the extracellular edges of transmembrane domains and in so doing influence the way that EL4 moves in the transport process.

Another issue that this study raises is whether acylcarnitines are likely to be physiologically relevant endogenous modulators of GlyT2 activity. Acylcarnitines are present at high concentrations in the CNS in regions that express GlyT2 and have a number of roles in the CNS. Most actions of acylcarnitines are related to energy metabolism and these actions are thought to be neuroprotective through improved energy utilization (Jones *et al.*, 2010). The related compound L-acetylcarnitine is analgesic in animal models of neuropathic pain (Chiechio *et al.*, 2002), but at this stage, the effects of the longer chain acylcarnitine compounds have not been investigated. NAGly has analgesic effects in both neuropathic pain and inflammatory pain (Succar *et al.*, 2007; Vuong *et al.*, 2008) and it will be of interest to see if OLCarn and PLCarn also provide analgesia in these models of pain. Partial, non-competitive inhibition of GlyT2 would be expected to transiently elevate synaptic glycine levels at inhibitory synapses, which would then enhance glycinergic neurotransmission, thereby enhancing inhibitory tone and reducing nociceptive transmission. The identification of OLCarn as an endogenous and potent inhibitor of GlyT2 may provide leads in the development of new compounds for the treatment of neurological disorders.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 DPH fluorescence of (A) C12-M and (B) OLCarn as a measure of concentration. The DPH fluorescence is greatly enhanced in lipid concentrations above the CMC due to the incorporation of the flurophore into the hydrophobic interior of the micelle. The CMC is obtained from the intersection of the straight line through the RFU measured at low concentrations of compound with the straight line through the RFU measured at the region of rapid fluorescence increase. The CMC for C12-M was calculated to be 163 ± 15 μM ($n = 3$). This is in agreement with technical specifications from the supplier (Affymetrix, Santa Clara, CA). The CMC for OLCarn was calculated to be 7.4 ± 1.2 μM ($n = 5$). Responses were normalised to the relative fluroscence units (RFU) measured at the maximum concentration tested; 500 μM for C12-M and 70 μM for OLCarn. Data are mean ± SEM ($n \geq 3$).

Figure S2 High concentrations of OLCarn induce a leak current in oocytes expressing GlyT2. (A) Representative current trace from an oocyte expressing GlyT2 voltage clamped at –30 mV. Glycine (30 μM) generates an inward current that is inhibited by 3 μM OLCarn. Continued application of OLCarn (3 μM) induces a leak current. Current–voltage measurements were made (a) before application of glycine (control recording), (b) once the glycine (30 μM) response had reached its peak, (c) once OLCarn (3 μM) inhibition had stabilized and (d) after glycine and OLCarn had been applied for 5 min. (B and C) Current–voltage relationships elicited by glycine (30 μM) in the absence (b–a) and presence (c–a) of OLCarn (3 μM). (c) A large leak current is noted after OLCarn application for 5 min (d–a). The current is normalized to the current activated by glycine (30 μM) at –100 mV. (d) Control glycine (30 μM) responses observed following application of OLCarn (3 μM) alone for 1 min and 5 min. Current responses were normalised to pre-exposure glycine responses.

Figure S3 High concentrations of OLCarn induce a leak current in oocytes expressing GlyT1. (A and B) Current–voltage relationships elicited by glycine (30 μM) in the absence (b–a) and presence (c–a) of OLCarn (3 μM). A large leak current is noted after OLCarn application for 5 min (d–a). The current is normalized to the current activated by glycine (30 μM) at –100 mV.

Figure S4 The effect of ALX1393 on GlyT2 chimeras. OLCarn concentration-dependent inhibition of 30 μM glycine transport currents are shown for GlyT2, GlyT2(EL2), GlyT2(EL4) and GlyT1. Current responses were normalized to pre-exposure glycine (30 μM) responses. Data are mean ± SEM ($n \geq 3$).

Table S1 ALX1393 inhibition of glycine transport by GlyT1, GlyT2 and chimeric transporters.