Activity of novel lipid glycine transporter inhibitors on synaptic signalling in the dorsal horn.

Running title: GlyT2 inhibitors increase spinal glycinergic signalling.

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Abstract:

Background:

Inhibitory neurotransmission plays an important role in controlling excitability within nociceptive circuits of the spinal cord dorsal horn. Loss of inhibitory signalling is thought to contribute to the development of pathological pain. Preclinical studies suggest that increasing inhibitory glycinergic signalling is a good therapeutic strategy for treating pain. One approach to increase synaptic glycine is to inhibit the activity of the glycine transporter 2 (GlyT2) on inhibitory nerve terminals. These transporters are involved in regulating glycine concentrations and recycling glycine into presynaptic terminals. Inhibiting activity of GlyT2 increases synaptic glycine, which decreases excitability in nociceptive circuits and provides analgesia in neuropathic and inflammatory pain models.

Experimental Approach:

We investigated the effects of reversible and irreversible GlyT2 inhibitors on inhibitory and excitatory neurotransmission in the dorsal horn. The effect of these drugs on synaptic signalling was determined using patch-clamp electrophysiology techniques to measure glycine- and NMDA- mediated postsynaptic currents in spinal cord slices *in vitro*.

Results:

We compared activity of four compounds that increase glycinergic tone with a corresponding increase in evoked glycinergic postsynaptic currents. We found these compounds do not deplete synaptic glycine release over time. Interestingly, we found that none of these compounds increased glycine-mediated excitatory signalling through NMDA receptors. Results suggests that these compounds preferentially inhibit GlyT2 over the glycine transporter 1 (GlyT1) with no potentiation of the glycine receptor or spill-over from inhibitory to excitatory synapses.

Conclusions:

GlyT2 inhibitors increase inhibitory neurotransmission in the dorsal horn and have potential as pain therapeutics.

Abbreviations:

ALX1393, O-[(2-Benzyloxyphenyl-3-flurophenyl)methyl]-L-serine; AP5, (2*R*)-amino-5-phosphonovaleric acid; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; Dichlorokynurenic acid, DCKA; GAT1, GABA transporter 1; GlyT1, Glycine transporter 1; GlyT2, glycine transporter 2; IPSC, inhibitory postsynaptic current; NAGly, N-arachydonoyl-glycine; NOGly, N-[3-([1,1-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine, NFPS; N-Oleoylglycine; OL-Carn, Oleoyl-L-carnitine; ORG25543, *N*-[[1-(Dimethylamino)cyclopentyl]methyl]-3,5-dimethoxy-4-(phenylmethoxy)benzamide hydrochloride; SLCC6, sodium-dependent solute carrier family 6 transporters; QX-314.Cl, *N*-(2,6 Dimethylphenylcarbamoylmethyl) triethylammonium chloride.

Introduction:

Regulation of sensory processing in the spinal cord is mediated through the fast inhibitory neurotransmitters glycine and γ-aminobutyric acid (GABA) (Zeilhofer et al., 2012). These inhibitory neurotransmitters play important roles in limiting excitability in the nociceptive circuit of the dorsal horn and when the receptors of these neurotransmitters are antagonized *in vivo,* this leads to the symptoms of pathological pain (Yaksh 1989). Studies from our group and others using animal models of neuropathic pain have shown that loss of inhibitory GABAergic and glycinergic neurotransmission, or disinhibition, occurs in the dorsal horn in peripheral nerve-injury (Yaksh 1989; Sherman, and Loomis 1995, 1996; Sivilotti, and Woolf 1994; Imlach et al., 2016). The crucial role of glycine signalling in the control of nociceptive transmission in the dorsal horn was recently demonstrated in studies where glycinergic neurons were either ablated or activated in mice, which caused pain and analgesia, respectively (Foster et al., 2015). Since glycinergic neurotransmission has such a profound influence on whether or not nociceptive signals are transmitted to the brain, this system is an attractive target for pain therapeutic development (Imlach 2017).

Glycine concentrations at both inhibitory and excitatory synapses in the CNS are regulated by the glycine transporters 1 and 2 (GlyT1 and GlyT2) which are part of the sodium-dependent solute carrier family 6 (SLCC6) transporters. GlyT1 is expressed by astrocytes at inhibitory and excitatory synapses, while GlyT2 expression is restricted to inhibitory glycinergic neurons at presynaptic terminals (Zeilhofer et al., 2005). GlyT2 plays important roles regulating

synaptic glycine concentrations and recycling glycine back to the presynaptic terminals to maintain sufficient stores of glycine-containing vesicles (Zafra et al., 1995). Many of the inhibitory interneurons that regulate nociceptive signalling in the dorsal horn are located in lamina III, an area where GlyT2 is highly expressed (Zeilhofer et al., 2012). GlyT2 more effectively reduces glycine concentration compared to GlyT1 and is able to reduce synaptic glycine to the low nanomolar range, preventing low level glycine receptor activation (Supplisson, and Roux 2002). This fine control of synaptic glycine levels by GlyT2, suggests that inhibiting the function of this transporter would reduce clearance of synaptic glycine and increase basal inhibitory neurotransmission, which in turn would reduce nociceptive signalling in the dorsal horn. However, the balance between complete and partial inhibition of GlyT2 is also an important factor, as too much inhibition reduces synaptic vesicle loading, thus preventing synaptic glycine release (Vandenberg et al., 2014). The effect of complete inhibition has been observed in GlyT2 knockout mice that suffer from severe motor deficiencies by the second postnatal week due to loss of glycine at the neuromuscular junction (Gomeza et al., 2003). Conversely, partial knockdown of the GlyT2 gene using siRNAs results in a normal motor phenotype and the reduction in GlyT2 activity corresponds with reduced allodynia scores in a mouse model of neuropathic pain (Morita et al., 2008), suggesting that partial inhibitors of GlyT2 may avoid side effects caused by loss of synaptic glycine.

Investigations of the therapeutic potential of the early GlyT2 inhibitors, ALX1393 and ORG25543, show they are analgesic when administered intravenously in the mouse neuropathic pain model (Morita et al., 2008). However, ALX1393 also inhibits GlyT1 at higher concentrations, which may exacerbate pain symptoms by increasing glycine at excitatory NMDA receptors and increasing spinal sensitization (Mingorance-Le Meur et al., 2013). The GlyT2 inhibitor ORG25543 has similar antinociceptive effects to ALX1393 in *in vivo* pain models and has been found to increase glycinergic tonic currents and evoked inhibitory post-synaptic current (eIPSC) decay time in spinal cord slices (Bradaia et al., 2004). However, ORG25543 is an irreversible inhibitor of GlyT2 and following an initial increase in glycinergic activity it causes a significant reduction in glycinergic eIPSCs *in vitro* suggesting that presynaptic glycine vesicle recycling is reduced to a level that does not support synaptic signalling (Gomeza et al., 2003). In a study by Wiles et al. (Wiles et al., 2006), the endocannabinoid N-arachydonoyl-glycine (NAGly) was shown to selectively and reversibly inhibit GlyT2 over GlyT1 and GAT1. NaGly has also been shown to act as an allosteric modulator of glycine receptors, with potentiation of $\alpha 1$ GlyR and inhibition of $\alpha 2$ and $\alpha 3$

(Yévenes and Zeilhofer, 2011). NAGly is found in high concentrations in the spinal cord and reduces pain in inflammatory and neuropathic models of pain through a mechanism that is independent of cannabinoid receptor activation (Succar et al., 2007; Vuong et al., 2008).

Recently a number of lipid inhibitors including Oleoyl-L-carnitine (OL-Carn; C18 *cis* ω9 L-Carnitine) and N-Oleoylglycine (NOGly; C18 *cis* ω9 glycine), that are based on the endogenous GlyT2 inhibitor NAGly, have been tested as GlyT2 inhibitors (Carland et al., 2013). OL-Carn and NOGly have significantly different head groups but both have a C18 (oleoyl) tail (Table 1). This difference in head group results in greater potency of OL-Carn at inhibiting GlyT2 expressed in oocytes but less reversibility compared to NAGly (Carland et al., 2013). Lipid compounds with variations in the tail length have also been investigated as GlyT2 inhibitors in oocyte studies and C18 has been found to have the highest affinity. Double bonds in the lipid tail also have an influence on efficacy with C18:0 and C18:2 derivatives showing less efficacy than the C18:1 derivative. In this study we compare the effects of OL-Carn and NOGly with structurally related compounds, to investigate how differences in head and tail groups affect synaptic signalling in the spinal cord. Other compounds used in this study include compounds with different placements of the double bond (C18 *cis* ω8 glycine) and with shortened tail length (C16 *cis* ω3 glycine) (Table 1). These compounds may provide more specific and reversible inhibition of GlyT2 and may lead to more effective therapeutics for targeting glycine transport to treat pathological pain.

Methods:

Animals: Sprague-Dawley rats were housed in groups of 2-4, in individually ventilated cages with access to standard rat chow and 12 hour light-dark cycles. All animal experiments were performed in accordance with the University of Sydney animal ethics committee guidelines and were approved by the committee, University of Sydney Animal ethics approval number: 2015/803. The animals used in this study were the same age, weight and gender. Animals were sourced from the Animal Resources Centre, Western Australia. Animal numbers were reduced as much as possible without compromising the amount of data points.

Preparation of spinal cord slices. Adult male Sprague-Dawley rats (6-8 weeks at the time of slice preparation, ~180-260 g) (n = 29 rats) were anaesthetized with isoflurane, decapitated and the lumbar region of the spinal cord was removed. Parasagittal slices (340 μm thick) of spinal

cord were cut on a vibratome (Leica VT 1200s) in oxygenated ice-cold sucrose-based artificial CSF (sACSF) that contained (mM): 100 sucrose, 63 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 25 glucose, and 25 $NaHCO_3$. Slices were transferred to a submerged chamber containing NMDG-based recovery ACSF (rACSF) for 15 minutes at 34°C, equilibrated with 95% O_2 and 5% CO_2 and composed of (mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 Glucose, 5 Na ascorbate, 2 thiourea, 3 Na pyruvate, 10 $MgSO_4$ and 0.5 $CaCl_2$, and adjusted to pH 7.4 with HCl. Following the recovery incubation, slices were transferred to normal oxygenated ACSF where they were allowed to recover for 1 hour at 34° C and then maintained at room temperature prior to transfer to the recording chamber. Normal ACSF had the following composition (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 25 glucose, and 11 $NaHCO_3$ and was equilibrated with 95% O_2 and 5% CO_2.

Electrophysiology. Slices were transferred to a recording chamber and superfused continuously at 2 ml/min with normal ACSF that had been equilibrated with 95% O_2 and 5% CO_2 and maintained at 34°C with an inline heater and monitored by a thermister in the slice chamber. Dodt-contrast optics was used to identify lamina II neurons in the translucent substantia gelatinosa layer of the superficial dorsal horn. A Cs^+-based internal solution, which should minimise postsynaptic effects, was used to record electrically evoked inhibitory glycinergic post-synaptic currents (eIPSCs) or excitatory NMDA postsynaptic currents (eEPSCs) and contained (mM): 140 CsCl, 10 EGTA, 5 HEPES, 2 $CaCl_2$, 2 MgATP, 0.3 NaGTP, 5 QX-314.Cl, 2 (osmolarity 285-295 mosmol l^{-1}). QX-314 was used in patch electrodes to prevent action potential firing when recording NMDA-receptor mediated currents, as action potential activity can prevent detection of synaptic currents, particularly at the depolarized potentials required for NMDAR currents. We did not observe loss of NMDAR-mediated currents with QX-314. Patch electrodes had resistances between 3 and 5 MΩ. Synaptic currents were measured in whole-cell voltage-clamp (-70 mV for eIPSCs or -40 mV for eEPSCs, not corrected for a liquid junction potential of 4 mV) from lamina II cells. Bipolar tungsten electrodes placed in the inner laminae (lamina III region) were used to elicit eIPSCs using a stimulus strength sufficient to evoke reliable eIPSCs. Neurons ventral to lamina II, in regions that are known to contain glycinergic neurons were stimulated in this study. Glycinergic IPSCs could not be elicited when from more superficial sites or when the stimulating electrode was moved to more ventral regions of the dorsal horn (deeper in lamina IV-V). Electrodes were placed in the dorsal root entry zone to stimulate eEPSCs. All eIPSCs

were recorded in CNQX (10 μM), AP5 (100 μM) and picrotoxin (80 μM). All eEPSCs were recorded in CNQX (10 μM), picrotoxin (80 μM) and strychnine (0.5 μM). IPSC and EPSC decay constants were determined using the fit exponential tool in Axograph X to fit the current from an average of 10 consecutive IPSCs for each condition. For eIPSCs, a mono-exponential function was fitted using a Simplex algorithm. For eEPSCs, the decay course was fitted to a double exponential function: $f(t) = A_1 e(-t/\tau_1) + A_2 e(-t/\tau_2)$ where t is time, A_1/A_2 are peak amplitudes of the fast/slow decay components at $t = 0$ and τ_1/τ_2 are the fast/slow decay time constants respectively. From this we calculated the weighted decay time constant (τ_w) defined by: $\tau_w = (A_1\tau_1)/(A_1+A_2) + (A_2\tau_2)/(A_1+A_2)$. A mono-exponential function was fitted using a Simplex algorithm. Drugs were superfused onto slices at a rate of 2ml/min in normal oxygenated ACSF at 34°C and continued until a stable response was achieved which was followed by 10 consecutive measurements that were averaged for each data point. Vehicle (DMSO) controls were done for each concentration. At the conclusion of each experiment strychnine (0.5 μM) or AP5 (100 μM) were added to the superfusion solution to confirm that recorded currents were glycine- mediated IPSCs or NMDA-mediated EPSCs, respectively. Charge transfer of eIPSCs were determined by measuring the amplitude area using the AxoGraph measure function. The tonic current value (taken just before each eIPSC) was subtracted prior to calculating charge transfer to isolate the eIPSC charge without the tonic current component.

Data analysis. Both tonic current and eIPSC amplitudes were measured from 0 pA. Drug effects were normalized and expressed as % of baseline and presented as mean ± SEM. Data were analysed using Prism 6 (Version 6.03). Statistics were performed on data before it was normalized and normal distributions were confirmed using Prism software. Data is normalized in histograms because the amplitude of evoked currents vary significantly in between recordings. Comparisons of two treatments in the same group were made using a two-tailed paired *t* test. When multiple comparisons were tested, ANOVA with Sidaks or Fishers LSD *post hoc* test to correct for multiple comparisons were used, unless otherwise stated in figure legends. Significance threshold was set at * $P < 0.05$. Group sizes were a minimum of 5 independent values, which is a number determined from previous studies. Data sets were blinded to the experimenter for analyses.

Materials. Picrotoxin, CNQX, NFPS, ORG25543 and strychnine were purchased from Sigma Australia. QX-314 was purchased from Alomone Labs, Israel. AP5 was purchased from Tocris Bioscience, UK. All other chemicals were purchased from Sigma, Australia unless otherwise stated in the text. C18 *cis* ω8 Glycine and C16 *cis* ω3 glycine were synthesised at the Faculty of Science, the University of Technology Sydney. The structural identity and purity of synthesised compounds was determined by ^{1}H and ^{13}C NMR, HRMS, and elemental analysis. Stocks of lipids were made in DMSO.

Nomenclature of targets and ligands. Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16.

Results:

Lipid GlyT2 inhibitors increase synaptic glycinergic tone of lamina II interneurons.

Basal glycinergic tonic currents and electrically stimulated IPSCs were recorded from lamina II interneurons in whole-cell patch voltage clamp configuration in parasagittal spinal cord slices. Stimulating electrodes were placed in lamina III and the glycinergic component of the current was recorded in the presence of glutamatergic and GABAergic inhibitors CNQX (10 μM), AP5 (100 μM) and Picrotoxin (80 μM). Tonic current was calculated as the difference between baseline current before and after superfusion of the glycine receptor antagonist strychnine (0.5 μM), which was applied at the conclusion of the experiment (Fig 1). We investigated the effect of lipid inhibitors (0.1-100 μM) on glycinergic tonic current and found significant increases with all inhibitors except C18 *cis* ω8 Glycine, at concentrations equal or greater than 1 μM (Fig 1). The effects were dose-dependent with NOGly. The increase in eIPSC amplitude followed a similar pattern to the increase in basal tonic current and the two values were not significantly different, which suggests that these inhibitors have no effect on evoked release, but increase activity and therefore amplitude by increasing synaptic glycine levels. The compound that gave the largest significant increase in tonic and evoked currents was NOGly with 35.86 ± 10.2% and 30.7 ± 9.7% increase, respectively. Vehicle (DMSO) controls showed no significant differences in tonic or evoked IPSC amplitudes when compared to baseline (n =

5). Mean baseline eIPSC amplitudes for OL-Carn experiments were 246 ± 30 pA, NOGly were 258 ± 54 pA, C16 *cis* ω3 glycine were 191 ± 25 pA and C18 *cis* ω8 were 238 ± 32 pA.

We have previously found that these compounds have no detectable effect on GlyT1 expressed in oocytes (Mostyn et al, 2017, Table 1). To rule out any alternative modes of action, including activity at the glycine receptor, we performed occlusion experiments in lamina II neurons where slices were incubated with the GlyT2 inhibitor ORG25543 prior and during application of NOGly (Fig 2). There was no significant difference in tonic or evoked current amplitude in the presence ORG25543 and ORG25543 with NOGly. ORG25543 increased tonic current amplitude by 36.4 ± 18.1% and NOGly in the presence of ORG25543 resulted in a tonic current of 49.1 ± 30.1% over baseline (P = 0.59, n = 5). ORG25543 increased evoked current amplitude by 24.5 ± 4.5% and NOGly in the presence of ORG25543 resulted in an evoked current amplitude over baseline of 22.4 ± 7.5% (P = 0.62, n=5).

Effect of lipid GlyT2 inhibitors on decay time and charge transfer of evoked glycinergic IPSCs.

It has been previously shown that the GlyT2 inhibitors NAGly and ALX-1393 increase the decay time of electrically evoked glycinergic IPSCs due to increased synaptic glycine (Jeong et al., 2010). To determine whether the GlyT2 inhibitors used in this study prolong the glycinergic eIPSC decay in lamina II neurons, we fitted exponentials to the decay phase of the current traces of experiments shown in figure 1. In contrast to previously reported data for NAGly and ALX-1393, we found that in most cases there was no change in decay time, with the exception of 10 μM C16 *cis* ω3 glycine which caused a 28.3 ± 6.7% increase compared to pre-drug decay time (Fig 3). To further investigate this lack of effect on decay time constant, we assessed the effects of 10 μM NOGly on spontaneous mIPSC activity (Fig 4). We observed no change in either amplitude or decay in the presence of NOGly (P = 0.8 and 0.3 respectively, n = 7).

Charge transfer was calculated on all eIPSCs with tonic current subtracted. This allowed us to investigate the charge transfer of the eIPSC independent of the tonic current component. No changes were seen with any inhibitors at 0.1 μM, but data showed increases in charge transfer with OL-Carn and C16 *cis* ω3 glycine at higher concentrations. As a percentage of baseline, OL-Carn increased charge transfer with values of 123 ± 7%, 123 ± 3% and 124 ± 8% for 1, 10 and 100 μM respectively (P = 0.04, 0.02 and 0.04, paired t-tests on raw data). NOGly gave no

significant change in charge compared to baseline with mean values of $94 \pm 17\%$, $111 \pm 20\%$ and $102 \pm 10\%$ for 1, 10 and 100 µM respectively. C16 *cis* ω3 glycine gave mean values of charge transfer compared to baseline of $106 \pm 19\%$, $138 \pm 25\%$ and $133 \pm 23\%$ (P = 0.09. 0.04, 0.4, paired t-tests on raw data) for 1, 10 and 100 µM respectively. C18 *cis* ω8 glycine did not change the average charge transfer significantly with mean values compared to baseline of 97 ± 5, 109 ± 13 and 105 ± 9 for 1, 10 and 100 µM respectively.

NOGly does not deplete glycine from presynaptic vesicles following prolonged exposure.

Irreversible inhibitors of GlyT2 such as ORG25543 have been shown to decrease presynaptic glycine vesicle recycling to a level that does not support synaptic signalling (Gomeza et al., 2003). The compounds used in this study are partial inhibitors, so they are more likely to allow sufficient reuptake which would support glycinergic inhibition in the presence of the drug. To investigate vesicle depletion after prolonged exposure to NOGly, we compared mIPSC amplitude and decay before NOGly application and after 20 minute and 2 hours exposure to 10 µM NOGly. Mean amplitude before drug exposure was 28.2 ± 4.0 pA, which was not significantly different to the amplitude at 10 minutes, 27.1 ± 3.6 pA (P = 0.8, n = 6) or 2 hours, 29.3 ± 1.0 pA (P = 0.8, n = 6). There was also no significant difference in delay time constant, which was 4.1 ± 0.4 ms before drug exposure, 3.4 ± 0.3 ms after 10 minutes (P = 0.3, n = 6) and 4.5 ± 0.5 ms after 2 hours (P = 0.5, n =6) (Fig 4).

Excitatory NMDA-mediated currents are not affected by the lipid inhibitors.

At excitatory synapses, glycine acts as a co-activator for NMDA receptor mediated synaptic transmission. During periods of high presynaptic activity in the dorsal horn, glycine released from inhibitory interneurons can escape the synaptic cleft and activate NMDA receptors in the area, which is referred to as spill-over (Ahmadi et al., 2003). Increasing synaptic glycine by inhibition of GlyT2 at inhibitory synapses may increase the chance of enhanced NMDA activity at excitatory synapses if the spill-over is sufficient, which is undesirable as it would have a proalgesic effect. Inhibition of GlyT1 has previously been shown to increase the amplitude of evoked NMDA-mediated EPSCs through the activity of this transporter at excitatory synapses (Raiteri, and Raiteri 2010). In this study, lipid inhibitors were applied in

the presence of the CNQX (10 μM), picrotoxin (80 μM) and strychnine (0.5 μM) to block AMPA-, GABA- and glycine-mediated synaptic currents and EPSCs were elicited by dorsal rootlet stimulation while holding lamina II neurons at -40 mV to prevent voltage-dependent block by endogenous Mg^{2+}. We have previously published data to show that picrotoxin has the same activity as bicuculline on inhibitory currents in spinal cord slices in the concentrations used (Imlach et al., 2016). None of the GlyT2 inhibitors used in this study increase the amplitude or decay time constant of NMDA-mediated eEPSCs (Fig 5).

Previous studies have demonstrated that CNQX (10 uM) does not inhibit potentiation of NMDA-mediated eEPSCs by exogenously applied glycine in spinal cord slices (Bradaia et al., 2004). To confirm the lack of antagonistic activity of CNQX at the glycine binding site of NMDA receptors in our experiments, we performed control experiments under the same conditions with dichlorokynurenic acid (DCKA), an antagonist of the glycine site of the NMDA receptor. We found that 1 μM DCKA reduced NMDA-mediated eEPSCs by 42.7 ± 3.0% in the presence of 10 μM CNQX (n = 5).

To strengthen these findings, we also performed control experiments using the GlyT1 inhibitor, NFPS. Studies by other groups have shown that this inhibitor increases amplitude of NMDA-mediated synaptic currents (Jeong et al., 2010). Our results showed mean time constants were 140 ± 26 ms at baseline and 176 ± 43 ms in the presence of NFPS (P = 0.12, n = 5). NMDA current amplitude increased from 57 ± 6 pA at baseline to 65 ± 7 pA with NFPS (P = 0.03, n = 5).

Discussion and Conclusions.

The aim of this study was to investigate the effect of lipid inhibitors of GlyT2 on synaptic transmission at inhibitory and excitatory synapses within the dorsal horn. Oocyte studies show similar GlyT2 IC_{50} values for the four compounds tested, which range from 0.32 to 0.81 μM (Mostyn et al., 2017; Carland et al., 2013; Wiles et al., 2006). In the dorsal horn all compounds also had similar effects on glycinergic transmission, which is likely to be due to the small differences in GlyT2 IC_{50}. We show that the lipids with glycine head groups retain the effects on inhibitory transmission of the less reversible than OL-Carn, which has a carnitine head group. NOGly which has the same tail as OL-Carn gave maximal increases of 35.86 ± 10.2% and 30.7 ± 9.7% for tonic and eIPSC amplitudes, while OL-Carn increased tonic current by 21.9 ± 5.2 and eIPSC amplitude by 22.6 ± 10.3.

Another measure of increased synaptic glycine is an increased decay time constant of eIPSCs. It has previously been shown that ORG25543 increases both glycinergic tonic currents and the decay time eIPSCs in spinal cord slices (Bradaia et al., 2004). In the current study we found that only one of these compounds, C16 *cis* ω3 glycine, caused a significant increase in decay time, which only occurred at 10 μM. Since the size of the eIPSCs response can mask more subtle effects on decay, we also compared the time constants of spontaneous mIPSC events following an incubation in NOGly. This data showed no difference in mIPSC kinetics or amplitude, which supports the results we obtained when analysing eIPSC events. Interestingly, the increased delay we observed in C16 *cis* ω3 glycine eIPSCs was also reflected in the eIPSC charge transfer in response to 10 μM C16 *cis* ω3 glycine, which was significantly increased. OL-Carn also produced significant increases in eIPSC charge transfer at concentrations between 1-100 μM, which may be due to its longer lasting activity since it is a less reversible compound. The general lack of effect on both eIPSC decay and charge transfer would suggest that these glycine head group partial inhibitors are much less effective on GlyT2 than previously described inhibitors of GlyT2 and GlyT1.

To rule out any alternative modes of action, including activity at the glycine receptor and GlyT1, we performed an occlusion experiment where slices were incubated with ORG25543 prior and during application of NOGly. These results showed no additional increase in glycinergic transmission with NOGly, which suggests there is no potentiation of the glycine receptor. This differs to the structurally similar compound NAGly which has also been shown to act as an allosteric modulator of glycine receptors, with potentiation of α1 GlyR and inhibition of α2 and α3 (Yévenes and Zeilhofer, 2011).

It has been previously shown that irreversible inhibitors of GlyT2 such as ORG25543 can decrease glycinergic signalling due to reduced vesicle recycling. Following an initial increase in glycinergic activity these compounds cause a reduction in glycinergic eIPSCs *in vitro* suggesting that the presynaptic glycine vesicle recycling is reduced to a level that does not support synaptic signalling (Gomeza et al., 2003). To test the effect of lipid GlyT2 inhibitors on rundown, we measure mIPSC amplitude and decay over a two hour incubation in NOGly. We found no change in either amplitude or decay, which suggests that NOGly does not reduce vesicle recycling.

To investigate whether these lipid inhibitors increased glycine-mediated excitatory activity in the dorsal horn, we measured their effect on NMDA-mediated currents. For these experiments we used one concentration of drug (10 µM) that we found to be effective at increasing glycinergic tone in the first set of experiments. At excitatory synapses, glycine acts as a co-activator for NMDA receptor mediated synaptic transmission. Increased glycine at these synapses may result from decreased GlyT2 activity and spill-over to excitatory synapses, or decreased activity of GlyT1 which is involved in glycine transport at excitatory synapses. In this study we found that none of the lipid inhibitors increased eEPSC amplitude or decay of NMDA-mediated eEPSCs. This suggests that there is no spill-over of glycine from inhibitory synapses and these compounds have no effect on GlyT1. These are desirable properties, since NMDA activation is likely to exacerbate pain symptoms by increasing excitation and spinal sensitization (Mingorance-Le Meur et al., 2013).

Modifications in tail length and double bond position with the glycine head group drugs had no significant difference in activity across concentrations. Although these structural changes have the potential to cause subtle differences in activity, none of these modifications abolish or greatly increase activity. This would suggest these compounds may have similar potencies *in vivo*, but this is yet to be tested in animal pain models.

In conclusion, we show that lipid inhibitors of GlyT2 are effective at increasing glycinergic neurotransmission in the dorsal horn and neurotransmission is increased by all compounds with specific activity at inhibitory synapses and not at excitatory synapses. (Fig 6). There is a great need for new therapeutics to treat chronic pain conditions and targeting glycinergic signalling in the spinal cord is a promising strategy. In neuropathic and inflammatory pain conditions, glycinergic signalling is greatly reduced and increasing signalling through glycine receptors in the dorsal horn has antinociceptive effects. Due to relatively localised expression of GlyT2 in the spinal cord, these drugs are likely to have good side effect profiles. Therefore, the GlyT2 inhibitors used in this study have potential as pain therapeutics.

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Author contribution:

WLI and MJC designed and conceived experiments, BLW and WLI performed experiments, TR synthesized lipid compounds, BLW analysed data, WLI coordinated the study and wrote the manuscript. All authors were involved in editing the manuscript.

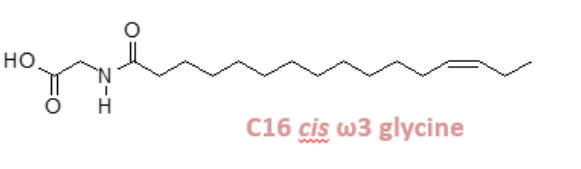**Conflict of interest:** The authors declare no conflicts of interest.
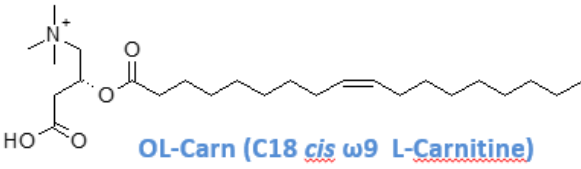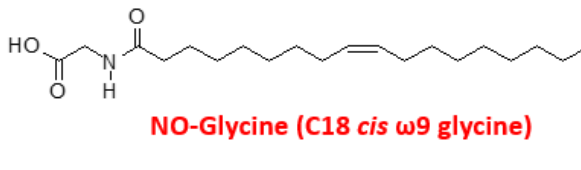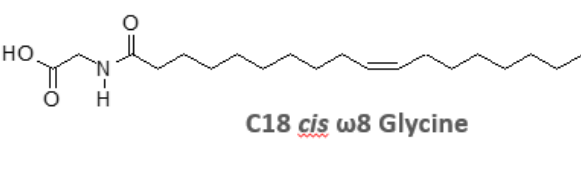
Compound	IC$_{50}$ (µM) (95% CI)		% Max inhibition of GlyT2[a,b]
	GlyT2[a,b]	GlyT1[a,b]	
OL-Carn (C18 *cis* ω9 L-Carnitine)	0.34 (0.21–0.53)	> 10	72 ± 4
NO-Glycine (C18 *cis* ω9 glycine)	0.50 (0.23–1.1)	> 10	66.8 ± 2.79
C18 *cis* ω8 Glycine	0.32 (0.18-0.57)	> 10	61.3 ± 1.69
C16 *cis* ω3 glycine	0.81 (0.46-1.4)	> 10	65.3 ± 1.94

Table 1. Structures and GlyT inhibitor properties of compounds used in this study. IC$_{50}$ data shows inhibition of 30 µM glycine transport by GlyT2 and GlyT1, expressed in Xenopus laevis oocytes from a) Mostyn et al, 2017 and b) Carland et al., 2013. Data presented are mean with 95% confidence intervals for each measurement (CI).
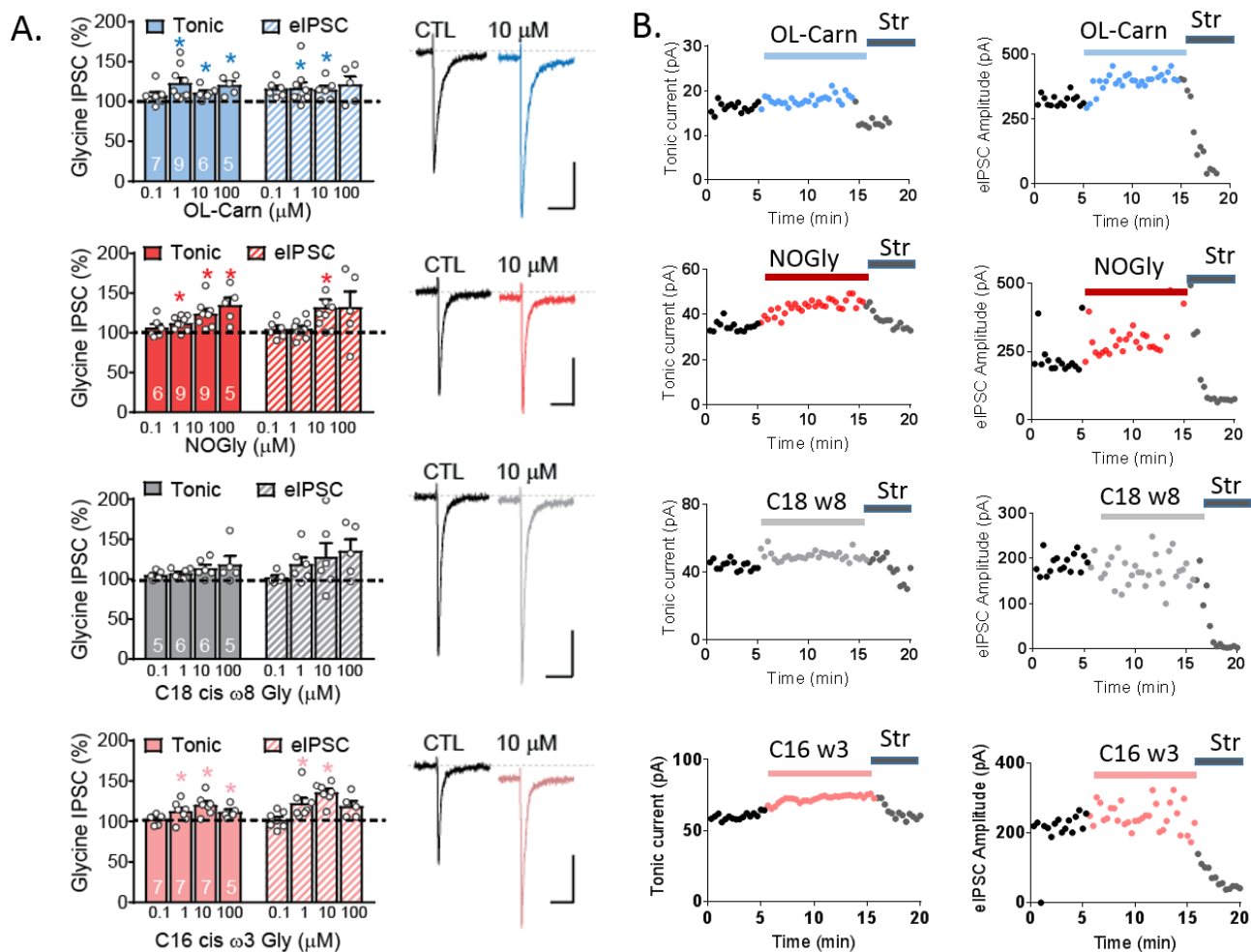
Figure 1. GlyT2 inhibitors increase the amplitude of glycinergic currents in dorsal horn neurons. The effect of GlyT2 inhibitors (0.1-100 μM) on the amplitude of glycinergic tonic and evoked IPSCs were recorded in lamina II neurons. A) Glycinergic currents are normalized to baseline and shown as a percentage. Examples of glycine-mediated eIPSC traces are shown for baseline controls (black) and in the presence of 10 μM GlyT2 inhibitor (coloured) for each drug. N is shown in white text. Dotted lines indicate level of 0 pA. Scale bars are 50 ms and 100 pA. * $p < 0.05$ (paired t test). B) Time plots showing glycinergic tonic current amplitude and eIPSC amplitude in response to 10 mM GlyT2 inhibitor (individual experiments). Coloured bars show period of GlyT2 inhibitor superfusion and the dark grey bar shows period of strychnine application.
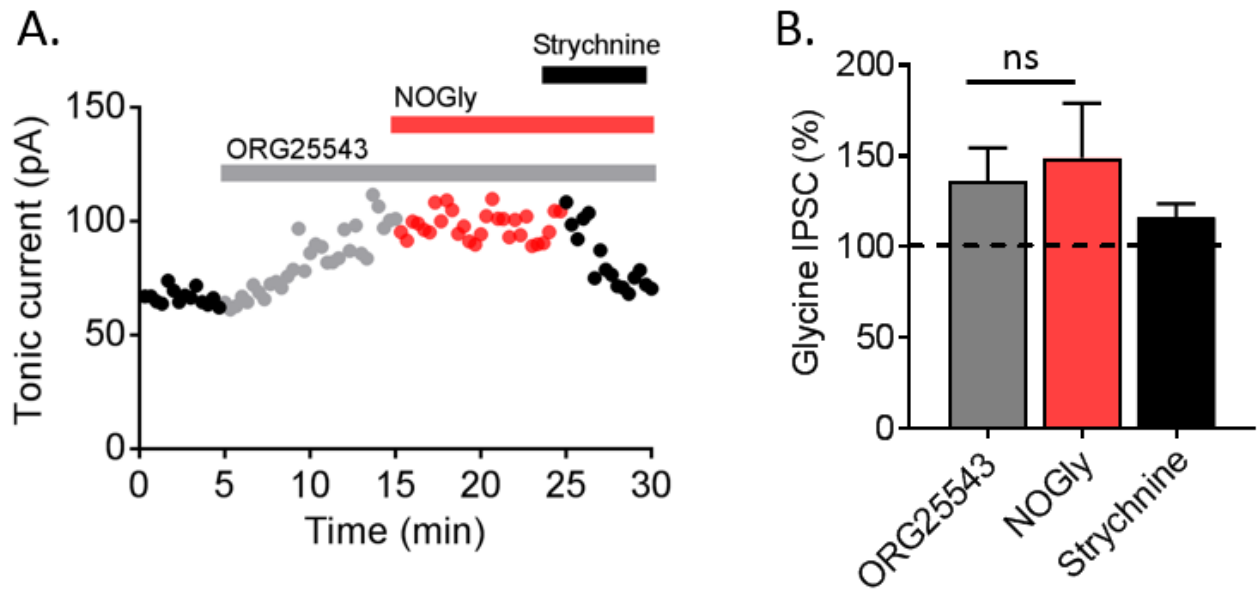
Figure 2. NOGly does not increase glycinergic transmission further in the presence of the GlyT2 inhibitor ORG25543. A) Time plot showing tonic glycinergic current amplitude with baseline (black), followed by 5 µM ORG25543 (grey), 10 µM NOGly (red) and 0.5 µM strychnine (black). B) Histograms show glycinergic tonic current normalized to baseline and shown as a percentage. Data is expressed as mean ± SEM.
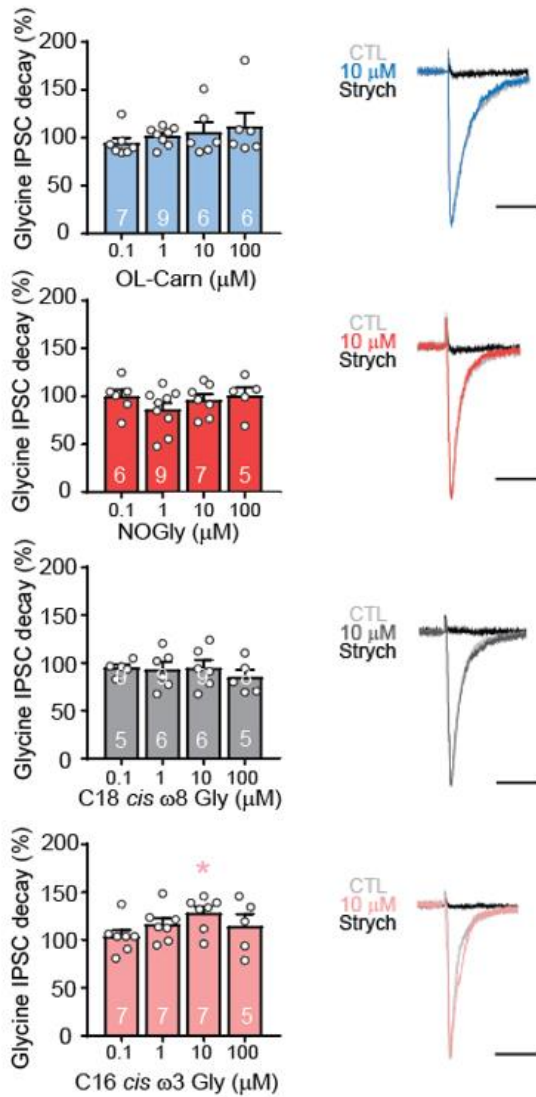
Figure 3. The effect of GlyT2 inhibitors on the decay time of glycinergic eIPSCs. The effect of GlyT2 inhibitors (0.1-100 μM) on the decay time of evoked IPSCs in lamina II neurons were measured by fitting exponentials to the decay phase of the current traces. Histograms show glycinergic current decay times normalized to baseline and shown as a percentage. Examples of glycine-mediated eIPSC traces are shown for baseline controls (grey) and in the presence of 10 μM GlyT2 inhibitor (coloured) for each drug, scaled to baseline amplitude, and with strychnine inhibition of glycine receptors, not scaled. N is shown in white text. Normalized data is presented as mean ± SEM. Scale bars are 25 ms. * $p < 0.05$ (paired t test).
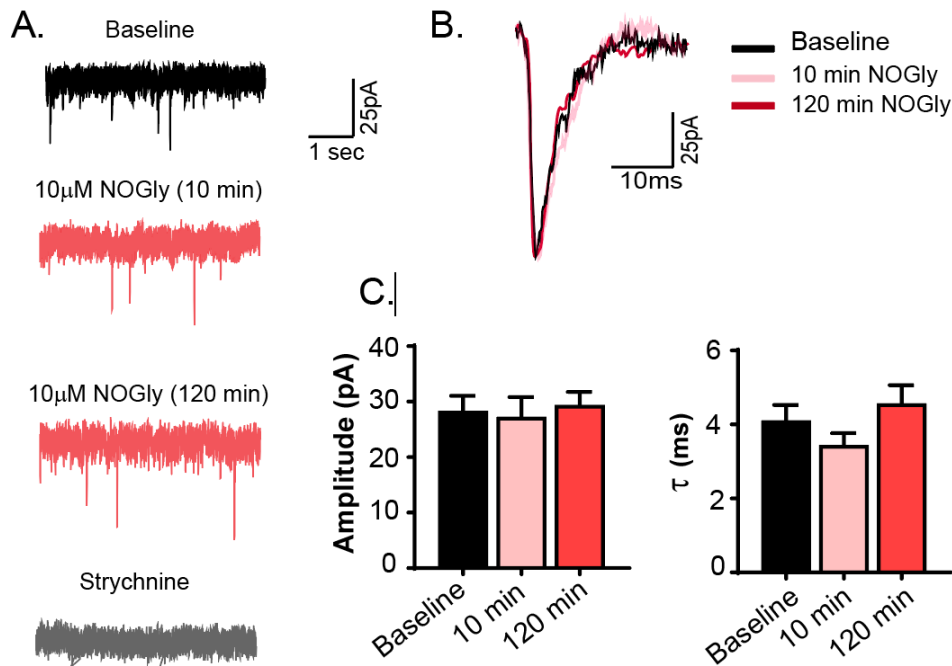
Figure 4. NOGly has no effect on mIPSC amplitude or decay and does not deplete synaptic vesicle refilling after prolonged exposure. A) Traces show mIPSC events prior to exposure to NOGly (10 μM) and post-exposure at 10 and 120 minutes. All mIPSC activity was inhibited by strychnine. B) Traces show averaged mIPSC events from the three time points. C) Histograms show mean mIPSC amplitude and time constant at the three time points, n = 6 for each group. Data is mean ± SEM.
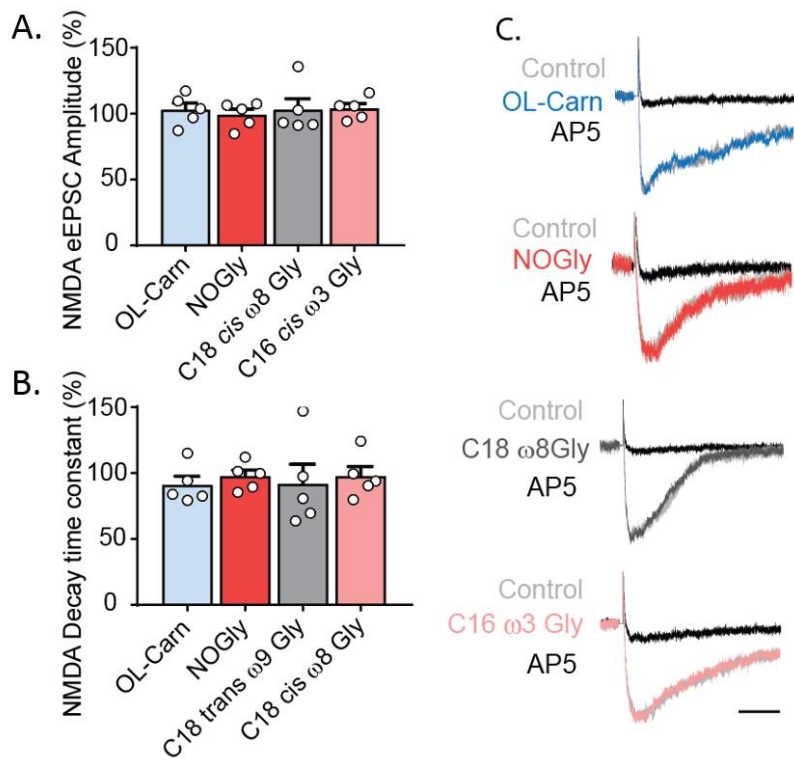
Figure 5. GlyT2 inhibitors do not increase the amplitude or decay time of NMDA-mediated eEPSCs in the dorsal horn. The effect of GlyT2 inhibitors (10 μM) on the amplitude and decay time of evoked NMDA-mediated eEPSCs in lamina II were examined. A) Histograms show eEPSC amplitude shown as a percentage of baseline. B) Decay constants were measured by fitting exponentials to the decay phase of the current traces are shown as a percentage of baseline. C) Examples of scaled NMDA-mediated eEPSC traces are shown for baseline controls (grey) and in the presence of 10 μM GlyT2 inhibitor (coloured) for each drug and with AP5 inhibition of NMDA receptors (not scaled). N = 5 for all data sets. Data is presented as mean ± SEM Scale bars are 50 ms. * $p < 0.05$ (paired t test).
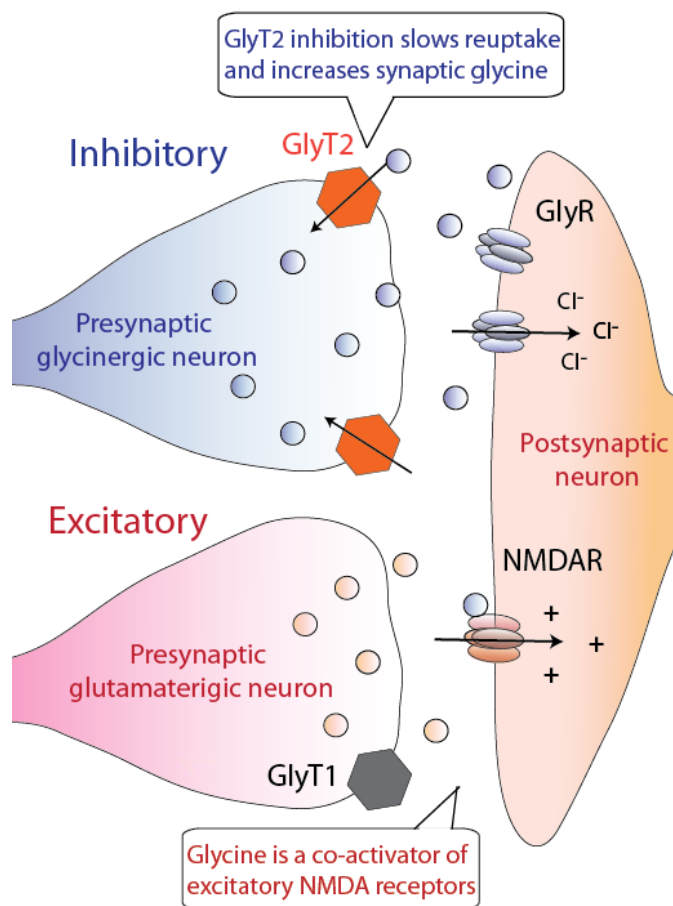
Figure 6. Nociceptive signalling can be reduced by increasing glycinergic activity in the spinal cord dorsal horn through inhibition of the glycine transporter 2 (GlyT2), which increases inhibitory signalling through glycine receptors (GlyRs). Since glycine is a co-activator of excitatory NMDA receptors (NMDAR), it is important that the increased glycine does not spill-over into the excitatory synapses as this may have proalgesic effects.