1521-0103/382/3/246–255$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

dx.doi.org/10.1124/jpet.122.001265
J Pharmacol Exp Ther 382:246–255, September 2022

Peripheral Administration of Selective Glycine Transporter-2 Inhibitor, Oleoyl-$_D$-Lysine, Reverses Chronic Neuropathic Pain but Not Acute or Inflammatory Pain in Male Mice

Bruce S. Wilson, Julian Peiser-Oliver, Alexander Gillis, Sally Evans, Claudia Alamein, Shannon N. Mostyn, Susan Shimmon, Tristan Rawling, MacDonald J. Christie, Robert J. Vandenberg, and Sarasa A. Mohammadi

Faculty of Medicine and Health, The University of Sydney, Sydney, Australia (B.S.W., J.P.-O., A.G., S.E., C.A., S.N.M, M.J.C., R.J.V., S.A.M.) and School of Mathematical and Physical Sciences, University of Technology, Sydney, Australia (S.S., T.R.)

Received April 7, 2022; accepted June 13, 2022

ABSTRACT

Aberrations in spinal glycinergic signaling are a feature of pain chronification. Normalizing these changes by inhibiting glycine transporter (GlyT)-2 is a promising treatment strategy. However, existing GlyT2 inhibitors (e.g., ORG25543) are limited by narrow therapeutic windows and severe dose-limiting side effects, such as convulsions, and are therefore poor candidates for clinical development. Here, intraperitoneally administered oleoyl-$_D$-lysine, a lipid-based GlyT2 inhibitor, was characterized in mouse models of acute (hot plate), inflammatory (complete Freund's adjuvant), and chronic neuropathic (chronic constriction injury) pain. Side effects were also assessed on a numerical rating score, convulsions score, for motor incoordination (rotarod), and for respiratory depression (whole body plethysmography). Oleoyl-$_D$-lysine produced near complete antiallodynia for chronic neuropathic pain, but no antiallodynia/analgesia in inflammatory or acute pain. No side effects were seen at the peak analgesic dose, 30 mg/kg. Mild side effects were observed at the highest dose, 100 mg/kg, on the numerical rating score, but no convulsions. These results contrasted markedly with ORG25543, which reached less than 50% reduction in allodynia score only at the lethal/near-lethal dose of 50 mg/kg. At this dose, ORG25543 caused maximal side effects on the numerical rating score and severe convulsions. Oleoyl-$_D$-lysine (30 mg/kg) did not cause any respiratory depression, a problematic side effect of opiates. These results show the safe and effective reversal of neuropathic pain in mice by oleoyl-$_D$-lysine and provide evidence for a distinct role of glycine in chronic pain over acute or short-term pain conditions.

SIGNIFICANCE STATEMENT

Partially inhibiting glycine transporter (GlyT)-2 can alleviate chronic pain by restoring lost glycinergic function. Novel lipid-based GlyT2 inhibitor ol-$_D$-lys is safe and effective in alleviating neuropathic pain, but not inflammatory or acute pain. Clinical application of GlyT2 inhibitors may be better suited to chronic neuropathic pain over other pain aetiologies.

Introduction

Chronic pain causes terrible physical and psychologic suffering that is often poorly managed by existing treatments. Neuropathic pain is the most difficult type of pain to treat. It results from damage or injury to the somatosensory nervous system and has a prevalence of approximately 18% of all chronic pain patients (von Hehn et al., 2012). Current pharmacotherapies, such as nonsteroidal anti-inflammatory drugs, opioids, anticonvulsants, and antidepressants, only provide clinically adequate analgesia in approximately 40% of sufferers (Breivik et al., 2006), and the utility of each is limited by adverse effects. Of particular concern, opioids are now a leading cause of unintentional death in the United States (Scholl et al., 2018; Kochanek et al., 2019), wherein overdose leads to fatal respiratory depression (Seth et al., 2018).

In the search for improved pain relief options, the glycinergic system is being investigated for novel targets. Glycine is a major inhibitory neurotransmitter that maintains inhibitory tone in spinal pain pathways. In chronic neuropathic pain models, this tonic inhibition is compromised, causing disinhibition, enhanced pain signaling, and inappropriate pain signals in response to non-noxious stimuli (allodynia; Fig. 1; Imlach et al., 2016). In a rat partial nerve ligation (PNL) model of chronic neuropathic pain, a specific population of spinal dorsal horn lamina II interneurons, radial cells, lose inhibitory glycinergic input (Imlach et al., 2016), thus enhancing the efficacy of synaptic transmission of pain signals. The mechanism for this disinhibition was shown to be a reversion of glycine receptor (GlyR) expression to favor the embryonic GlyRα2 subunit, which is important in development, but declines postnatally

This work was supported by the National Health and Medical Research Council Project [Grant APP144429].
A preprint of this article was deposited in Authorea [DOI: 10.22541/au.163256291.16873032/v1].
dx.doi.org/10.1124/jpet.122.001265.

ABBREVIATIONS: CCI, chronic constriction injury; CFA, complete Freund's adjuvant; GlyR, Glycine receptor; GlyT, Glycine transporter; MVb, minute volume; ol-$_D$-lys, oleoyl-$_D$-Lysine; PNL, partial nerve ligation; WBP, whole-body plethysmography.

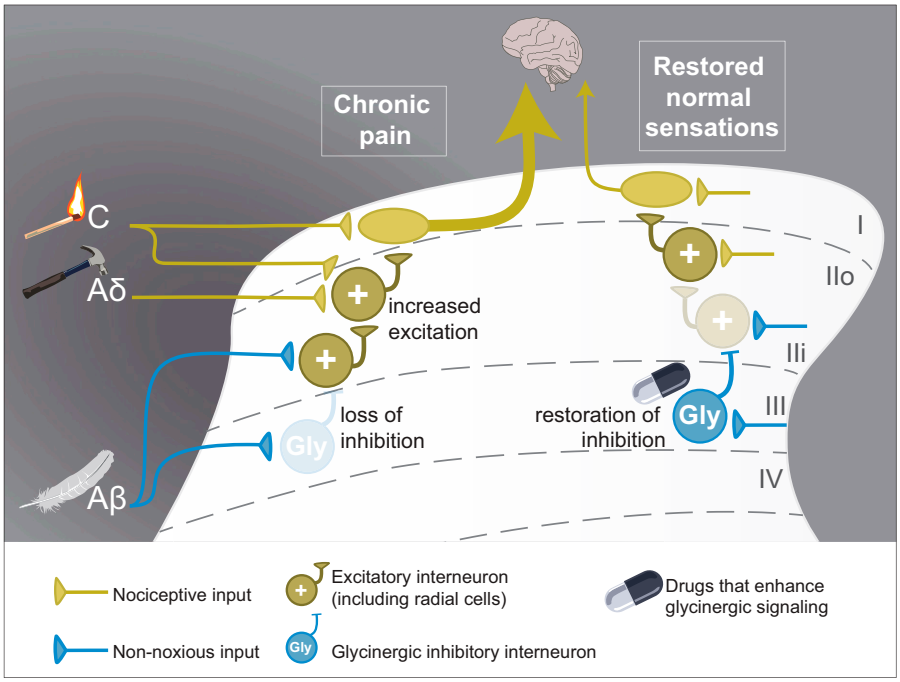

Fig. 1. Glycine inhibition in the dorsal horn is lost in chronic pain states, but may be restored by GlyT2 inhibitors to alleviate pain. Glycinergic inhibition to lamina II excitatory interneurons is lost in chronic pain states, leading to enhancement of nociceptive signals and aberrant activation of the nociceptive pathways in response to non-noxious stimuli such as touch (via Aβ fibers). Drugs that rectify this glycinergic dysfunction, such as GlyT2 inhibitors, are able to alleviate pain by inhibiting the abnormal signaling.

(Malosio et al., 1991). Imlach et al. (2016) demonstrated that, compared with sham controls, the radial cells of PNL rats have altered electrical characteristics reminiscent of GlyRα2-containing receptors; that the PNL radial cells are sensitive to the selective GlyRα2-inhibitor, cyclothiazide; and that GlyRα2 protein could be detected in the dorsal horn via western blot. Furthermore, intrathecal administration of glycine (Tanabe et al., 2008; Cheng et al., 2009) or inhibitors of glycine transporters (GlyTs) (Hermanns et al., 2008; Morita et al., 2008; Mingorance-Le Meur et al., 2013; Motoyama et al., 2014; Takahashi et al., 2015) in rodents can restore the lost inhibitory signaling and attenuate pain responses (reviewed in Dohi et al., 2009; Harvey and Yee, 2013; Vandenberg et al., 2014; Cioffi, 2018). Attenuation of pain responses was similarly observed after genetic knockdown of spinal GlyT expression in mice (Morita et al., 2008; Motoyama et al., 2014), thus establishing the spinal glycinergic system as a feasible target for analgesia. GlyT1 is expressed mostly on glial cells throughout the brain and spinal cord and is involved in excitatory as well as inhibitory signaling. GlyT2 is predominantly expressed on neurons in the spinal cord and brainstem; importantly, the highest densities of GlyT2 are on presynaptic glycinergic inhibitory neurons in lamina III of the dorsal horn (Zafra et al., 1995; Zeilhofer et al., 2005; Betz et al., 2006; Raiteri et al., 2008; Harvey and Yee, 2013; Aroeira et al., 2014; Schlösser et al., 2015; Cioffi, 2018). This distribution in a key location of the pain pathway indicates that GlyT2 is likely to be a promising target for analgesic drugs.

To date, the only blood-brain barrier–permeable GlyT2 inhibitor with selectivity over GlyT1 that is commercially available is ORG25543 (Caulfield et al., 2001). However, on-target toxicity at analgesic doses limit its use in vivo (Mingorance-Le Meur et al., 2013) and safer alternatives are needed. Recently, a lipid-based GlyT2-selective allosteric inhibitor, oleoyl-$_D$-Lysine (ol-$_D$-lys), was developed based on the endogenous lipid, N-arachidonyl glycine (Mostyn et al., 2019). Instead of a glycine amino acid head-group, a lysine head group in the $_D$-configuration was conjugated to an oleoyl tail to produce a metabolically stable, selective, reversible inhibitor of GlyT2. We formerly showed proof of principal analgesic efficacy of intraperitoneally administered ol-$_D$-lys in rats (Mostyn et al., 2019). Here we undertook a detailed comparison of intraperitoneally administered ol-$_D$-lys and ORG25543 in mice to comprehensively characterize the potential therapeutic value of ol-$_D$-lys in multiple pain models.

Materials and Methods

Animals

We used animal models for this project and have adhered to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines in our reporting. We used well established and validated mouse models of pain that are ubiquitous in pharmacological literature. This allows for meaningful comparisons across historical published studies and therefore serves as a reduction technique.

All studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). The study protocols were reviewed and approved by the University of Sydney Animal Ethics Committee (2018/1363).

Experiments were conducted on 357 male C57Bl/6 mice (Animal Resources Centre, Western Australia; Perth, Australia), at 9 weeks of age and weighing 25–30 g on the day of testing. Mice were housed in a specific pathogen-free facility maintained at 22±2°C and 40%–70% humidity with lights on a 12-hour light-dark cycle (06:00–18:00). Mice were housed in individually ventilated cages with corncob bedding, $\alpha\beta\omega\upsilon\theta$ $\phi\omega\upsilon\rho$ mice per cage (between two and six) and ad libitum access to food (irradiated mouse feed, Specialty Feeds; WA) and water. Standard enrichment of a nesting dome, nesting paper, and wooden chew block were provided.

Animals were allowed 7 days acclimatization to the research facility prior to commencement of any procedures and were then acclimatized to the experimenter (male experimenter for analgesia, side effects, and convulsions testing; female experimenter for plethysmography and rotarod), testing room, apparatus, and handling for at least 4 days prior to experiments. Testing was carried out between the hours of 08:00 and 17:00.

A minimum of $n = 6$ animals were used per group, except when a drug dose caused acute toxicity. A single cohort of mice was used to test the numerical rating scores and convulsion scores, while separate cohorts were tested for all other measures.

Materials, Drug Preparation, and Administration

Ol-$_D$-lys (Mostyn et al., 2019) and ORG25543 (Tocris) were dissolved in a vehicle of 1% DMSO (Sigma) and 10% Solutol HS 15 (Sigma) in 0.9% sterile saline. Morphine (Sigma) was dissolved in sterile saline only. A single bolus injection of <10 ml/Kg volume was administered intraperitoneally using 29-G needles (Terumo) and testing was performed half-hourly to capture peak effect and then hourly or two-hourly to capture return to baseline.

Mice were randomly assigned to treatment groups using an online list randomizer (https://www.random.org/lists/) and prefilled needles were labeled with the blinding code by an independent colleague. The experimenters were blind to treatment allocations for the duration of testing and blinding codes were revealed after data were gathered and ready for statistical analysis.

Neuropathic Pain Model

A chronic constriction injury (CCI) model was adapted from Benbouzid et al. (2008), which also appears in Mohammadi and Christie (2014). Under deep isoflurane anesthesia (2%–2.5% saturated oxygen), the left sciatic nerve was isolated from surrounding tissue using blunt dissection. A 2-mm length of polyethylene tubing (Clay Adams BD & Co.; MD; inner diameter: 0.38 mm, outer diameter: 1.09 mm), split lengthwise, was positioned around the sciatic nerve to produce uniform constriction in each animal. The skin incision was closed using tissue adhesive (3M Vetbond) and mice were returned to their home cage to recover. Chronic neuropathic pain developed for 14 days before testing. Paw drooping or autotomy was not observed in any mice over the 14 days postsurgery.

Inflammatory Pain Model

The Complete Freund's Adjuvant (CFA) model of chronic inflammatory pain (Mohammadi and Christie, 2014; Pitzer et al., 2016) was performed via unilateral intra-plantar injection of 20 μL of undiluted CFA using 25-G needles. Injections were given under isoflurane anesthesia (2-2.5% saturated oxygen) and mice were given 3 days to recover from the procedure and to develop the chronic inflammatory pain state.

Allodynia Testing

The von Frey test was used to assess the antiallodynic effects of GlyT2 inhibitors, as previously described (Mohammadi and Christie, 2014). Mice were placed on a mesh-floored chamber and allowed to acclimatize to the apparatus for 30 minutes on the 2 days prior to baseline testing; for 20 minutes or until settled on the day of testing; and for approximately 10 minutes prior to each test time point. A single von Frey filament (Stoelting Co.; Chicago, IL) that elicited a 15% response rate in naïve mice, determined to be 0.4*g*, was used. The filament was pressed to the plantar surface of the mouse's left hindpaw until it just bent. The response of the mouse was recorded as positive (vigorous hindpaw shake or licking of the hindpaw) or negative (no response). The percentage of responses to 10 separate applications of the von Frey hair were recorded as the animals' score. Von Frey thresholds were measured at baseline (presurgery), and 14-days postsurgery on the day of test (see section 1.1 for timing). Exclusion criteria of >30% response rate at baseline or <70% response rate at t = 0 were applied; no animals met these criteria.

Acute Pain Testing (Hot Plate)

Acute pain was tested on a fixed temperature hot plate (IITC Life Sciences) set to 54°C, with a 20-second maximum cutoff time. Mice were placed into Plexiglas chambers (10 cm depth × 15 cm height) with open top; a foot pedal initiated the timer the moment the hindpaws touched the hot plate and stopped the timer the moment an endpoint behavior was observed; mice were then immediately removed from the hot plate (Mohammadi and Christie, 2014). Endpoint behaviors were hindpaw shake or lick and jumping.

Side Effect Testing

Several tests for side effects were employed as listed below. Signs of other abnormal behaviors seen in GlyT2 knockout mice, such as impaired righting responses and hindfeet clasping (Gomeza et al., 2003), were absent and thus not quantified or reported here. These behaviors may be unique to early postnatal mice, with the homozygous knockout fatal around the second postnatal week, or only in cases of complete GlyT2 inhibition.

Numerical Rating Score. Side effects were scored using a severity rating scale from 0 to 3 (0 = no signs; 1 = mild; 2 = moderate; 3 = severe) that assessed eight clinical behaviors: hypoxia, sedation, inactivity, sympathetic nervous hyperactivity (piloerection/shivering), pain/discomfort, dehydration, weak tone, convulsions. A mean score at each time point was generated for each drug dose.

Convulsions Score. Motor convulsions have been reported following the administration of GlyT2 inhibitors in animal models of pain (Mingorance-Le Meur et al., 2013). A dedicated convulsions rating scale was used (Table 1). Average scores were generated for each mouse over the 6-hour testing period.

Motor Incoordination; Rotarod Test. Possible motor incoordination caused by the drugs was tested using the Rotarod (IITC Life Science; CA). Latency to fall from the rotarod was measured using a method adapted from Tung et al. (2016). Briefly, mice were placed on rat-size rods (69.5-mm diameter, 27-cm drop to landing platform) and rotating speed was accelerated from 4 rpm to 40 rpm with a maximum trial length of 180 seconds. The rotarod apparatus automatically detected the fall latency for each animal. Mice were given 5 minutes to acclimatize to the apparatus before each trial. A 3-day training period was given prior to baseline testing to allow acclimatization to the test and eliminate any learning effects, during which three training sessions were given 30 minutes apart. During training, a trial was only counted if the mouse remained on the drum for a period of at least 10 seconds. If the staying period of 10 seconds was not met, the mouse was placed back on the rod and the trial was repeated.

Respiratory Depression. Whole-body plethysmography (WBP) was used in freely moving mice to assess whether ol-$_D$-lys causes respiratory side effects analogous to opioid analgesics. Naïve, uninjured

TABLE 1
Motor convulsion scale (Borges et al., 2003)

Stage	Behavior
0	Normal activity
1	Rigid posture or immobility
2	Stiffened, extended, and often arched (Straub) tail
3	Partial body clonus, including forelimb or hindlimb clonus (seen rarely) or head bobbing
3.5	Whole-body continuous clonic seizures while retaining posture
4	Rearing
4.5	Severe whole body continuous clonic seizures while retaining posture
5	Rearing, falling
6	Tonic–clonic seizures, loss of posture, jumping

mice were used for this test. Mice were placed into the WBP chamber (Buxco, DSI, Harvard Bioscience, Inc.) for 20 minutes to acclimatize, and baseline respiratory frequency was measured prior to drug administration. For each experimental time point, respiratory activity was measured for 10 minutes and averaged into 5-minute bins (Buxco Fineponte); the first bin was excluded from analysis as reacclimation time, while the second bin was used in analysis. The change in respiratory frequency and minute volume (MVb) as a percent of baseline was assessed as follows:

$$\%\ respiratory\ frequency = \frac{post\ injection}{pre\ injection} * 100 \qquad (1)$$

Data and Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in experimental biology Michel et al. (2020). Data were analyzed using GraphPad Prism (version 7.0b for Mac, GraphPad Software; La Jolla, CA). Two-way repeated measures ANOVA was used where two independent factors (time and treatment) were compared. Where statistically significant main effects were observed, multiple comparisons with post hoc Dunnett's correction were used, and statistically significant comparisons to vehicle control groups are reported. For data with non-normal distribution, the nonparametric Wilcoxon matched-pairs signed rank test was used. Non-normalized data are reported throughout, except for the rotarod and respiratory measures, where percent of baseline is used to control for interindividual variance at baseline. Data are represented as mean ± S.D. unless otherwise indicated. Significance level was set at $P = 0.05$ and statistical significance is represented as $^*P < 0.05$.

Results

Analgesic Effects of GlyT2 Inhibitors

Chronic Neuropathic Pain. Systematic administration of ol-$_D$-lys produced dose-dependent antiallodynia in the CCI pain model (main effects of time [$F(6, 240) = 35.26$], treatment [$F(5, 40) = 6.15$] and an interaction effect [$F(30, 240) = 2.618$]) (Fig. 2Ai) with peak effects of the different doses reached between 1 and 2 hours postinjection (Fig. 2Aii). Post hoc analysis showed that the reversal of allodynia compared with vehicle was statistically significant at 60 minutes for 16, 30, and 100 mg/kg doses, and with a peak effect reached at approximately 90 minutes. Statistically significant effects of the 30 mg/kg dose and 100 mg/kg dose lasted for at least 6 hours. A dose response relationship was evident at lower doses, with a peak effect at 30 mg/kg. Doses higher than 30 mg/kg lost the dose-response relationship, with 50 mg/kg and 100 mg/kg producing only moderate analgesic effects compared with vehicle.

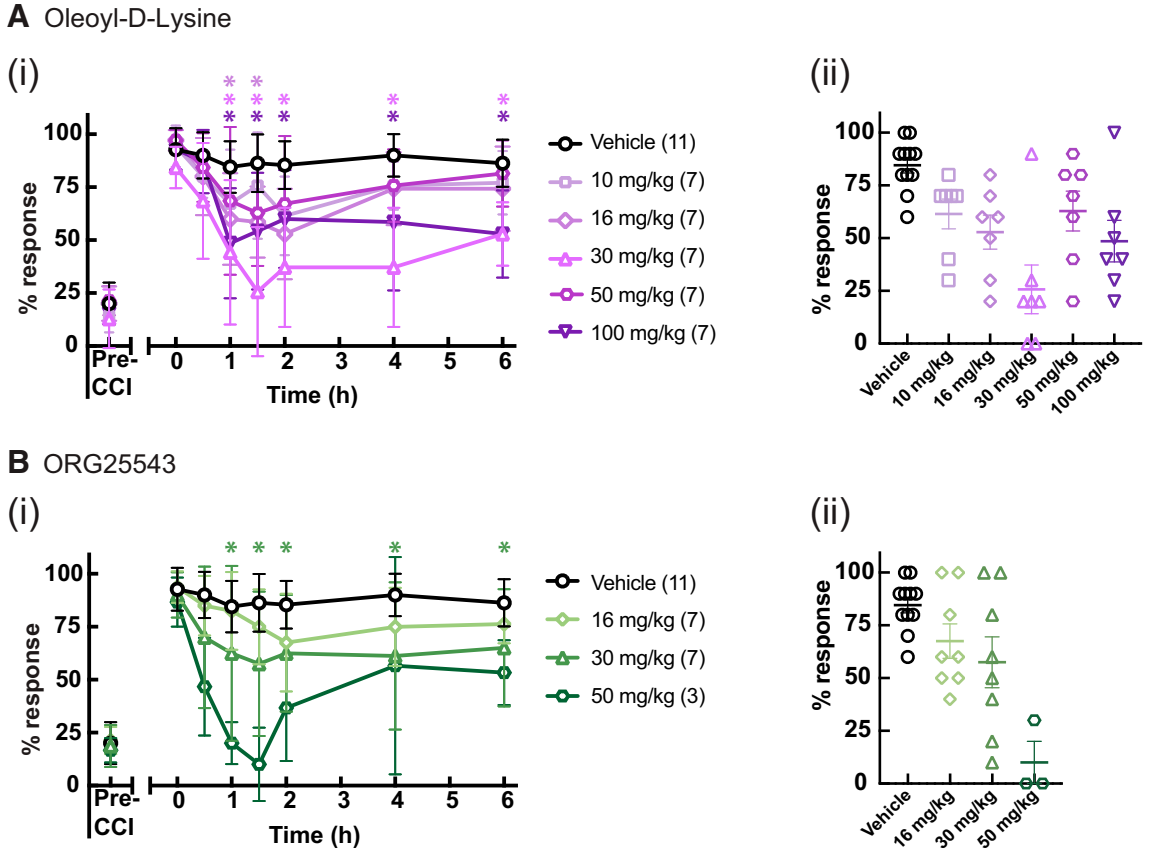

Fig. 2. Analgesia (von Frey) testing in a mouse model of chronic neuropathic (CCI) pain. Time course up to 6 hours (i) and peak effect (mean ± S.E.M.) between 1 and 2 hours (ii) of Ol-$_D$-lys (A, purple) and ORG25543 (B, green) are shown. (A) Ol-$_D$-lys produced analgesia in mice in a dose-dependent manner from 10 mg/kg to 30 mg/kg, at which a peak effect was seen. Higher doses of 50 mg/kg and 100 mg/kg were less effective than the lower dose of 30 mg/kg. (B) ORG25543 produced statistically significant analgesia from 60 minutes to 4 hours at the 30-mg/kg dose. At the highest tested dose of 50 mg/kg, responses to the von Frey stimuli were markedly reduced, however, this coincided with severe side effects and toxicity, which precluded testing the full data set ($n = 3$), and this dose was excluded from statistical analysis. Two-way repeated measures ANOVA was used to compare drug groups with vehicle control and statistically significant post hoc multiple comparisons are indicated by asterisks ($^*P < 0.05$). $n = 7$ for drug groups; $n = 11$ for vehicle. Data for panels (A) and (B) were collected as a single experiment over multiple days and separated here by drug for visual clarity; vehicle group is the same for all panels.

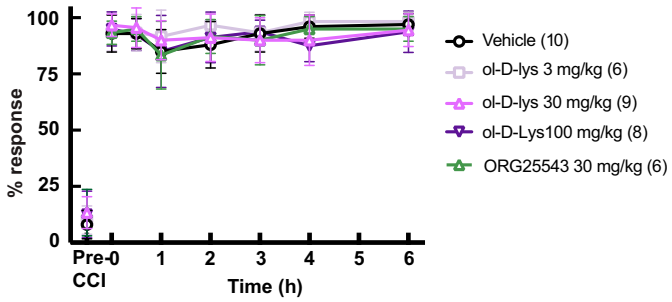

Fig. 3. Analgesia (von Frey) testing in a mouse model of inflammatory (CFA) pain. No analgesia was observed for any dose of ol-$_D$-lys or for 30 mg/kg of ORG25543. n = 6–10.

The analgesic efficacy of ol-$_D$-lys contrasted with that of ORG25543, which also produced main effects of time [F(6, 144) = 7.09] and treatment [F(2, 24) = 3.877]. ORG25543 showed efficacy over a similar period (Fig. 2Bi), with peak effects at 90 minutes to 2 hours postinjection (Fig. 2Bii), but these analgesia scores are likely confounded by severe side effects (see below) and may not reflect a simple antiallodynic effect. The maximum dose of ORG25543 tested was 50 mg/kg. This dose was found to be lethal in one animal during side effect testing, so for ethical reasons only n = 3 was tested here, and this dose was excluded from statistical analysis. For subsequent analgesia testing, only the sublethal dose of 30 mg/kg was used for ORG25543. This dose produced moderate analgesia starting 1 hour postinjection and lasting for the full 6-hour testing duration.

Chronic Inflammatory Pain. In the CFA pain model, 3, 30, and 100 mg/kg of ol-$_D$-lys was tested over 6 hours, compared with vehicle or 30 mg/kg of ORG25543. No significant analgesic effect was observed (significant main effect for time [F(6, 204) = 4.479] but not for treatment or interaction) (Fig. 3). To ensure that this apparent lack of effect was in fact a result of the pain model, rather than other variables, we separately conducted a small head-to-head experiment comparing the effect of 30 mg/kg of ol-$_D$-lys in CCI and CFA pain models with vehicle (n = 3 per group), wherein the CCI model acted as positive control for the pain model. A peak effect was observed for the ol-$_D$-lys (30 mg/kg) CCI group (60 ± 5.7) at 1 hour, while neither the ol-$_D$-lys (30 mg/kg) CFA group (90 ± 5.7) nor the vehicle group (80 ± 5.7) showed significant analgesia at any time point.

Acute Pain. Acute heat pain was not significantly alleviated by either GlyT2 inhibitors ol-$_D$-lys or ORG25543 (Fig. 4A), while the positive control, morphine, did produce near-maximal antinociception, peaking at 30 minutes. Nonparametric Kruskal-Wallis one-way test showed that morphine differed significantly from vehicle at 30 minutes (Fig. 4B). The ORG25532 group exhibited higher variability due to confounding side effects in two animals, however, overall did not differ from the vehicle group.

Side Effects and Convulsions from GlyT2 Inhibitors

Numerical rating scales of categorical endpoints were used to quantify the time course and severity of the general side effects and the convulsions caused by GlyT2 inhibitors ol-$_D$-lys (Fig. 5, Ai–ii and C) and ORG25543 (Fig. 5, Bi–ii and Di–ii) compared with the same behaviors preinjection (t = 0). The 50-mg/kg dose of ORG25543 was lethal in one animal (indicated by the cross symbol in Fig. 5, Bi and Di), so data are missing post 1-hour time point for that animal. Due to this toxicity, we did not attempt to test the full sample size for this dose on any measure.

Figure 5, Ai and Bi, shows the average time course of onset and severity of side effects for ol-$_D$-lys and ORG25543, respectively, both having peak side effects at 1-hour postinjection. The nonparametric Wilcoxon matched-pairs signed rank test was used to compare the behaviors before and after injection for those doses that caused adverse side effects (Fig. 5, Aii and Bii). The 100-mg/kg dose of ol-$_D$-lys and the 50-mg/kg dose of ORG25543 both caused statistically significant side effects at 30 minutes, 60 minutes and 90 minutes postinjection.

Figure 5, C and Di, show the average time course of onset and severity of convulsions for ol-$_D$-lys and ORG25543, respectively. Ol-$_D$-lys did not induce convulsions at any dose, while 50 mg/kg of ORG25543 caused convulsions that peaked at 1 hour postinjection. The Wilcoxon matched-pairs signed rank test was again used to show that significant convulsions were observed at 30, 60, and 90 minutes postinjection (Fig. 5Dii).

Motor Coordination Effects of GlyT2 Inhibitors

The impact of GyT2 inhibitors on rotarod performance was assessed at various doses. For ol-$_D$-lys, significant main effects

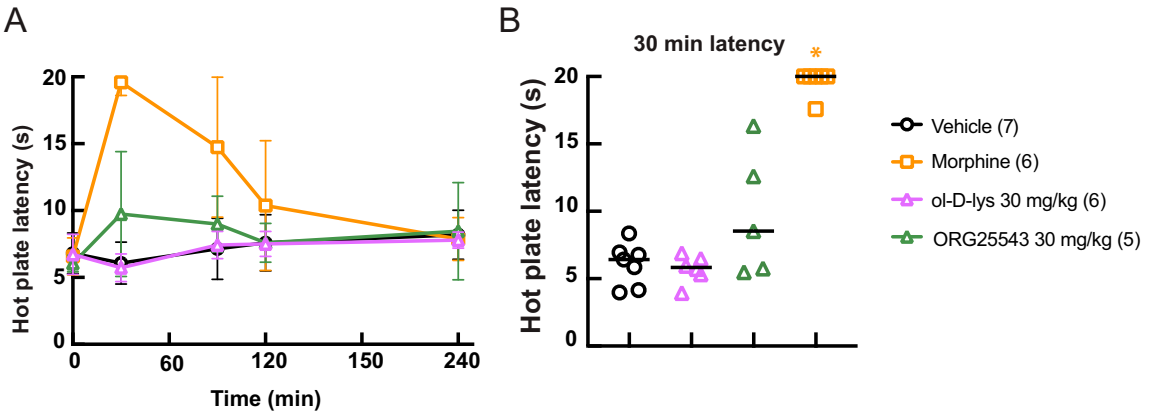

Fig. 4. Acute pain (hot plate) testing in mice. (A) Time course of hot plate testing showed no antinociception for ol-$_D$-lys (30 mg/kg) or ORG25543 (30 mg/kg). (B) Nonparametric Kruskal-Wallis one-way test showed significant antinociception at 30 minutes in the morphine (10 mg/kg) group compared with vehicle control (*$P < 0.05$). n = 5–7.

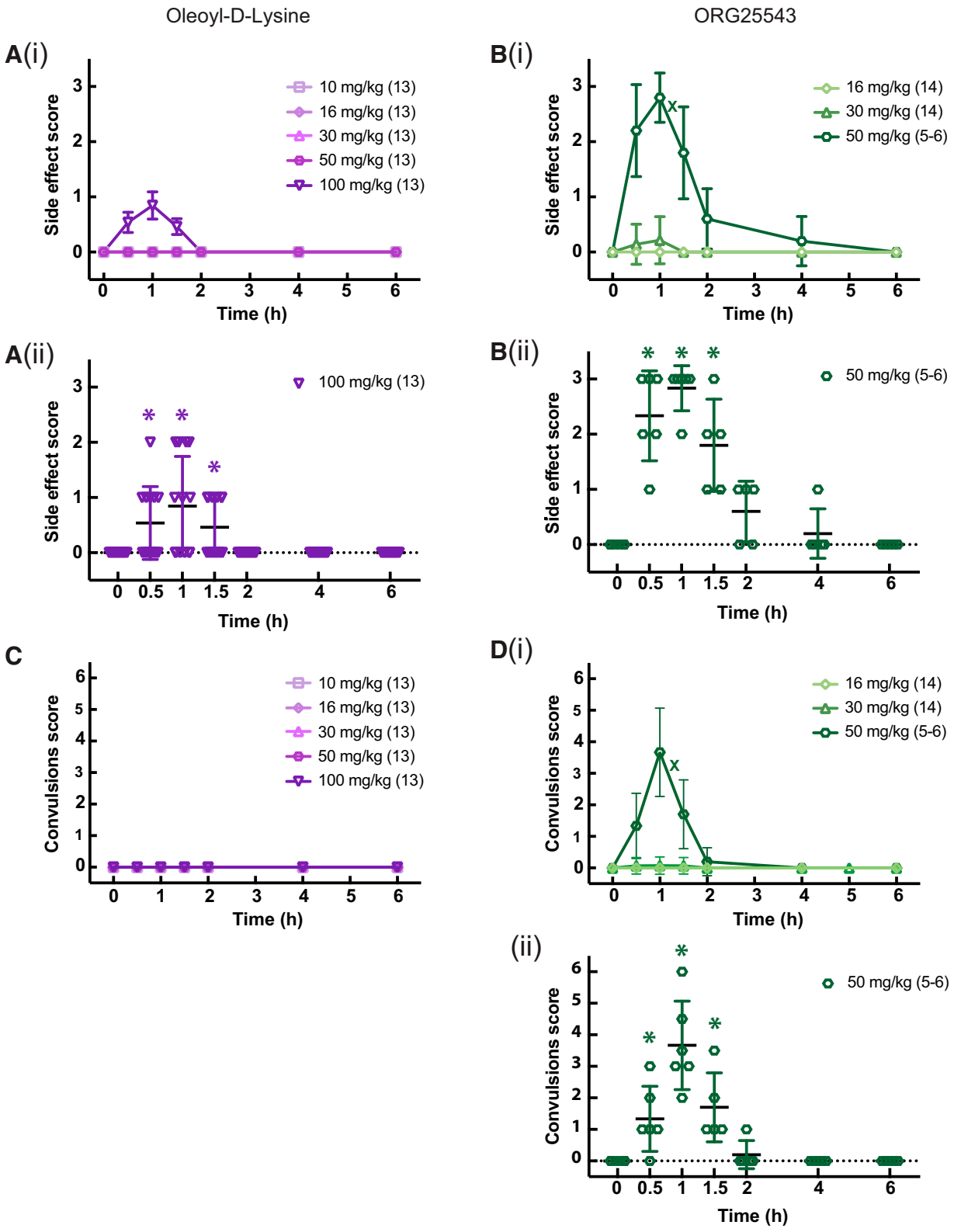

Fig. 5. Side effects and convulsions caused by GlyT2 inhibitors. (Ai–ii) Side effects of ol-D-lys. (Bi–ii) Side effects of ORG25543. (C) No convulsions were caused by ol-D-lys. (Di–ii) Convulsions caused by ORG25543. (Ai, Bi, C, Di) Summary plots show mean ± S.D. (Aii, Bii, Dii) The scatter of the doses that caused significant adverse behaviors compared with t = 0, for which Wilcoxon matched-pairs signed rank test was performed (*$P < 0.05$). The cross marked after 1 hour in (Bi) and (Di) indicates the time at which one animal died. n = 13–14 for all doses except for 50 mg/kg of ORG25543, which was limited to n = 6 due to toxicity.

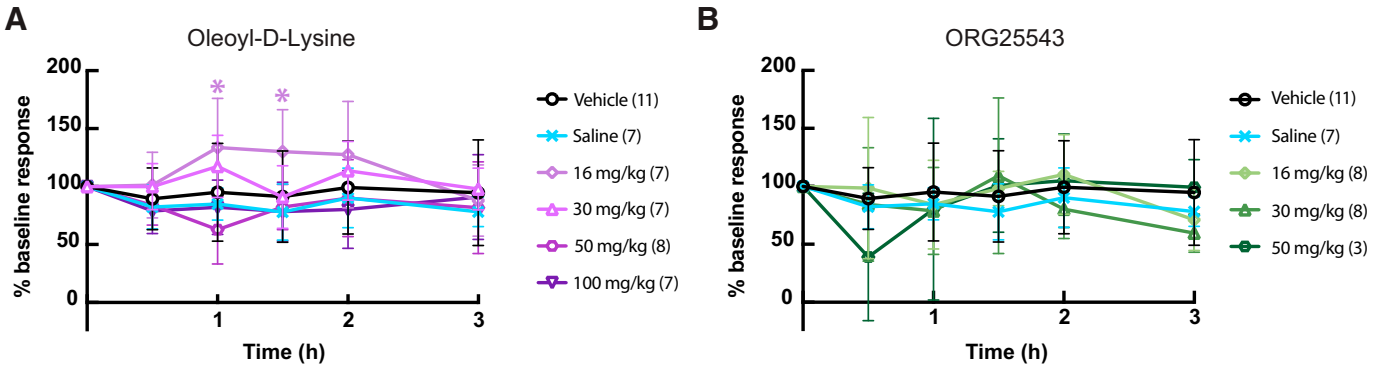

Fig. 6. Motor coordination Rotarod test. (A) Ol-$_D$-lys caused no dose-dependent motor incoordination. However, post hoc multiple comparisons of two-way ANOVA showed an enhanced performance at 60 and 90 minutes, at the 16-mg/kg group (*$P < 0.05$). (B) ORG25543 caused profound and immediate incoordination in two out of three animals at 50 mg/kg, however, due to the low numbers tested, this could not be statistically analyzed. n = 7–8 for all drug groups except for 50 mg/kg of ORG25543, which was limited to n = 3 due to toxicity. n = 11 for vehicle. Data for panels (A) and (B) were collected as a single experiment over multiple days and separated here by drug for visual clarity; vehicle group is the same for both panels.

of treatment [F (5, 38) = 2.783], time [F (5, 190) = 2.375], and an interaction between treatment and time [F (25, 190) = 1.843] were observed. While no detrimental effect of ol-$_D$-lys was observed at any dose, post hoc tests showed an enhanced performance was observed for the 16 mg/kg dose at 1 hour and 1.5 hours (Fig. 6A). For ORG25543, we again limited the number of animals tested at the higher dose of 50 mg/kg for ethical reasons, and thus the analysis is underpowered. The two-way ANOVA showed only a main effect of time [F (5, 125) = 3.203] but not for treatment (Fig. 6B). It would be necessary to collect a larger data set to state with confidence whether 50 mg/kg of ORG25543 had an effect on motor coordination.

Respiratory Effects of ol-D-Lys

Respiratory depression is the primary cause of opioid-related deaths and has been reported to also be produced by ALX-1393, a nonselective GlyT2 inhibitor (Hermanns et al., 2008). In a comparison of ol-$_D$-lys, morphine, and vehicle, morphine produced substantial respiratory depression, as measured by both respiratory frequency (significant main effects of treatment [F(2, 21) = 6.883], time [F(5, 105) = 26.97]), and a main interaction effect [F(10, 105) = 3.921] (Fig. 7A) and MVb (significant main effects of treatment [F(2, 21) = 12.93], time [F(5, 105) = 19.21] and a main interaction effect [F(10, 105) = 3.211]) (Fig. 7B). Multiple comparisons revealed that morphine caused statistically significant depression in both respiratory frequency and MVb at multiple time points compared with vehicle. Ol-$_D$-lys did not cause respiratory depression, and differed from vehicle only at 30 minutes, while respiratory frequency was slightly higher than vehicle.

Discussion

GlyT2 inhibition holds promise as a new analgesic target, however, severe side effects of compounds such as ORG25543 have tempered development. Here, we have shown that a reversible GlyT2 inhibitor is analgesic while eluding serious side effects. Detailed in vivo characterization of ol-$_D$-lys in male mice showed it was an effective analgesic for neuropathic pain, with minimal side effects after systematic administration. We conducted a side-by-side comparison of ol-$_D$-lys and ORG25543—currently the only blood-brain barrier–permeable, GlyT2-selective inhibitor commercially available. While useful as a research tool in vitro, the high toxicity of ORG25543 precludes its use in reliably characterizing the in vivo effects of inhibiting GlyT2. By virtue of its larger therapeutic window, we were able to test ol-$_D$-lys in multiple pain and side effect models and show that inhibition of GlyT2 is a safe and beneficial approach to treating chronic neuropathic pain, but not chronic inflammatory pain or acute antinociception.

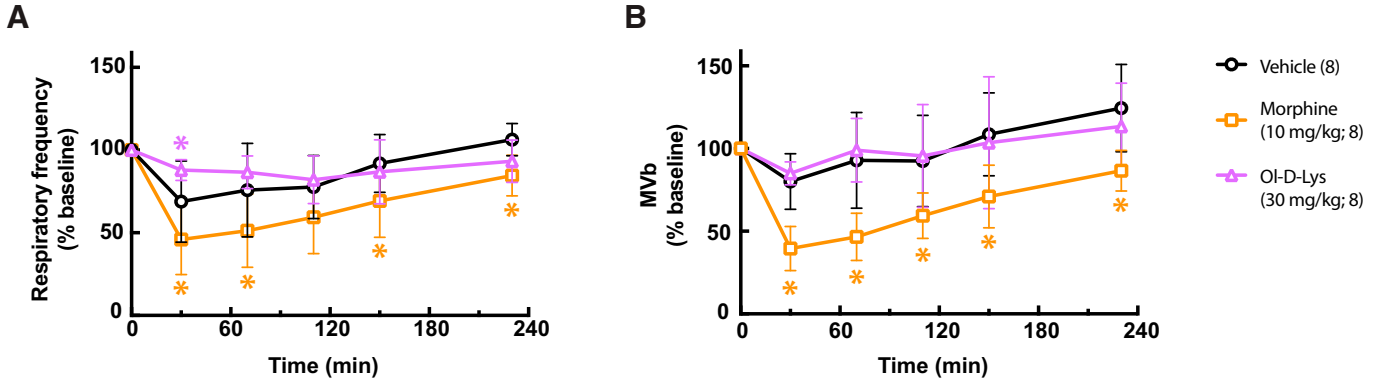

Fig. 7. Respiratory side effects. WBP of freely moving mice showed that a submaximal analgesic dose of morphine caused respiratory depression, measured by respiratory frequency and MVb, but ol-$_D$-lys did not. Significant post hoc multiple comparisons of two-way ANOVA are indicated by asterisks (*$P < 0.05$). n = 8.

Ol-$_D$-lys produced near-complete reversal of allodynia in the mouse CCI neuropathic pain model. A dose-dependent relationship was seen between 10 and 30 mg/kg, while higher doses of 50 and 100 mg/kg were less effective. This may be due to the high lipophilicity and poor aqueous solubility of ol-$_D$-lys, leading to precipitate formation at higher doses, and/or the high degree of binding in the central nervous system, likely due to sequestration to lipid-rich brain tissue after crossing the blood-brain barrier (Mostyn et al., 2019). The analgesic effects of 30 mg/kg of ORG25543 were modest but sustained for the 6-hour duration of testing. At 50 mg/kg, ORG25543 caused mice to reduce response to the stimulus at every time point tested, however, this is confounded by side effects, discussed below. Neither inflammatory pain nor acute thermal pain were alleviated by ol-$_D$-lys or ORG25543. We propose that this is due to the underlying glycinergic changes that occur in chronic pain states, wherein loss of inhibitory glycinergic tone over time leads to increased nociceptive signaling. These changes are the target of GlyT2 inhibitors and may be absent in the shorter-timescale CFA inflammatory model and acute pain model used here.

At the highest dose, ol-$_D$-lys caused mild side effects on only one measure, while ORG25543 caused severe side effects as well as lethal toxicity. Ol-$_D$-lys produced no convulsions at any dose. Mild to moderate clinical behaviors were scored for some mice on the numerical scale, but only at 100 mg/kg—well above the peak analgesic dose. These behaviors included decreased activity, hunched posture, and mild pain behaviors of abdominal constriction, which suggest that a depot of the drug may have formed at the site of injection. Our prediction of precipitate formation at high doses supports this idea of a lipid depot forming and may account for the relatively low and sustained analgesic activity at 100 mg/kg (Zuidema et al., 1994). In contrast, ORG25543 produced severe side effects, convulsions and toxicity at 50 mg/kg, with one mouse dying 1 hour postinjection. Of those animals that were administered the 50 mg/kg dose, most reached the maximum (ceiling) score for the numerical rating scale, and one animal reached the ceiling for the convulsions score.

Ol-$_D$-lys did not impede motor coordination in the rotarod test, with a significant effect only in the positive direction. The nature of such tests that involve subtle behavioral and motivational factors complicates interpretation of this result. Nonmotor factors such as arousal and executive function (e.g., volition and motivation to stay on the rotarod; Yogev-Seligmann et al., 2008) may account for the behaviors, rather than drug-induced motor enhancement per se. With no other observable changes in this group at the time of testing, further experiments are required to determine the true cause of the enhanced rotarod performance. ORG25543 caused profound and immediate aberration of motor coordination in two out of three mice tested at 50 mg/kg. However, due to the low numbers tested, this effect could not be statistically evaluated. The peak effects of ORG25543 on the numerical side effect scale and convulsions score align temporally with the decrease in responses to the von Frey stimulus, thus, confounding the interpretation of the analgesic effects of ORG25543 at this dose. The von Frey test is highly sensitive to state of arousal, alertness, and attention (Callahan et al., 2008). Furthermore, the von Frey test relies on discrete motor behaviors as endpoints for scoring pain thresholds, while ORG25543 caused motor incoordination. Thus, the reduced response to von Frey stimuli likely reflects side effects such as convulsions preventing or distracting the mice from responding, rather than purely analgesic effects of ORG25543.

Respiratory depression is a major side effect of opioids and has been shown to occur following administration of GlyT2 inhibitors (Hermanns et al., 2008). Here, we compared ol-$_D$-lys with morphine, the current putative gold-standard analgesic drug for many types of pain. We observed no respiratory depression caused by the peak analgesic dose of ol-$_D$-lys, 30 mg/kg, compared with vehicle and an analgesic dose of morphine. A comparative increase in respiratory frequency, but not minute volume, at 30 minutes postinjection was observed. ORG25543 was not tested here due to ethical considerations, however, these results support the proposal that reversible and selective GlyT2 inhibitors will not substantially affect respiration.

In this study, as we have previously shown in rats (Mostyn et al., 2019), we have observed severe side effects at minimally analgesic doses following intraperitoneal delivery of ORG25543. Previous reports found complete reversal of allodynia at 100-fold lower doses of ORG25543 than in our study, and vastly differing pharmacodynamics (Morita et al., 2008; Motoyama et al., 2014). These past studies used a variety of pain models, including a similar neuropathic pain model and the same von Frey test as used here. However, those studies found that the analgesic effects peaked from three to 24 hours and lasted several days after a single bolus intravenous injection, with no side effects at analgesic doses. The lack of side effects may be due to the lower doses used compared with our study, although Motoyama et al. (2014) tested a single 1 mg/kg dose, orally administered, without reporting any side effects. There are two key points of difference between these reports and our findings; the route of administration (intravenous or oral, compared with intraperitoneal) and the vehicle (aCSF or saline, compared with 1% DMSO, 10% solutol in saline). While the route of administration may partially account for the differences in effective dose range, it is unlikely to account for this drastic pharmacodynamic difference. ORG25543 has good plasma and hepatic metabolic stability (Caulfield et al., 2001), thus hepatic metabolism upon intraperitoneal administration is unlikely to account for the effects lasting for hours versus days. The vehicle used here was based on the requirements of dissolving the lipophilic ol-$_D$-lys and may have affected the pharmacology of ORG25543. Indeed, an independent study, (Mingorance-Le Meur et al., 2013) also used DMSO (5%) in their vehicle and found that higher doses of ORG25543, 20 mg/kg, were required to reach peak analgesic effect and observed convulsions and lethality at analgesic doses. Our findings indicate ORG25543 to have a narrow therapeutic window with severe on-target side effects at analgesic doses.

The utility of GlyT2 inhibitors as analgesics is being increasingly supported by pharmacological and genetic studies. There is evidence that irreversible blockers of GlyT2 (e.g., ORG25543; Mingorance-Le Meur et al., 2013) will lead to side effects such as convulsions and high toxicity, while reversible compounds (e.g., ol-$_D$-lys) will have a larger therapeutic window. Additionally, the efficacy of compounds may influence side effect liability, with full inhibitors causing more side effects than partial inhibitors. The difference is attributed to the role

of GlyT2 in clearing synaptic glycine and repackaging it into presynaptic vesicles (Jursky and Nelson, 1995); complete depletion of vesicular glycine stores following complete, irreversible GlyT2 inhibition abolishes glycinergic signaling. However, partial or reversible inhibition of the transporter preserves vesicle repackaging while slowing down synaptic clearance and prolonging glycine receptor activation. This concept is mirrored in mouse genetic studies where global deletion of GlyT2 (compare with full/irreversible inhibitor) causes spasticity, tremor, inability to right, and death at postnatal week 2 (Gomeza et al., 2003), whereas partial knockdown of GlyT2 (compare with partial/reversible inhibitor) imparts analgesia with no apparent side effects (Morita et al., 2008; Motoyama et al., 2014).

A limitation of the current study was the exclusive use of male mice, despite the known sex-differences in pain in humans (Bartley and Fillingim, 2013) and rodents [rats (Mapplebeck et al., 2018) and mice (Sorge et al., 2015); see Mogil, (2012) for review]. Systematically administered GlyT2-inhibitors have only been tested in male mice (Morita et al., 2008; Mingorance-Le Meur et al., 2013; Motoyama et al., 2014; Omori et al., 2015) and rats (Antineuropathic et al., 2014; Mostyn et al., 2019). Centrally administered GlyT2 inhibitors have only been tested in male rats (Hermanns et al., 2008; Haranishi et al., 2010; Takahashi et al., 2015) or female mice (Nishikawa et al., 2010). No study has yet to conduct a sex comparison to determine whether sex differences are present in glycine-related pain modulation and GlyT2-mediated analgesia. Such a study would contribute valuable insight to the field.

We observed efficacy of GlyT2 inhibitors in the chronic neuropathic pain model but not the shorter-duration inflammatory and acute pain models. We believe this to be due to the chronicity of pain, rather than the pain model or pain modality, based on the mechanisms of glycinergic inhibition in the spinal cord. Previous studies have shown efficacy of GlyT2 inhibitor ALX1393 (in 100% DMSO) in alleviating mechanical (von Frey) and thermal (Hargreaves test) pain at 12 days post-CCI (Hermanns et al., 2008), albeit with severe respiratory and motor side effects. Another study showed efficacy of ALX1393 in the same mechanical and thermal pain tests using the CCI model after subcutaneous administration, however, since ALX1393 does not cross the blood-brain barrier, these results likely represent an off-target or peripheral mechanism (Barthel et al., 2014). In agreement with our current findings, Morita et al. (2008) found limited efficacy of intrathecal ORG25543 alleviating mechanical allodynia in the CFA model (2–4 days postinjection) while finding complete or near-complete reversal in two chronic neuropathic pain models (PNL 12 days postsurgery; diabetic neuropathy 10 days post-streptozotocin injection). In contrast, Takahashi et al. (2015) administered rats with intracerebroventricular ALX1393 (50% DMSO) and found efficacy in acute (formalin) pain. They also found efficacy in mechanical (von Frey) and cold (cold plate) pain modalities, but not heat (Hargreaves) 7 days after the CCI model. These differences to the present results may be due to the shorter duration of neuropathic pain, the high concentration of DMSO or the supraspinal route of administration.

The potential of targeting GlyT2 clinically is currently being established with the multitarget drug opiranserin (VVZ-149, Vivizon). Opiranserin is a novel analgesic drug, acting as antagonist at both GlyT2 and serotonin receptor 2A (5HT2A; Pang et al., 2012). Opiranserin has successfully completed recruitment for its first phase 3 clinical trial, with recruitment for a second trial underway (https://clinicaltrials.gov/ct2/results?term=vvz-149). All trials for opiranserin have been conducted/planned in postoperative pain. However, our findings suggest that the best indication for a single-target GlyT2 inhibitor is in chronic pain states, where spinal glycinergic changes have become aberrant, and may be corrected with reversible GlyT2 inhibitors. Whether chronic pain of a neuropathic origin is likely to be more receptive over other pain aetiologies requires further confirmation.

The present results build evidence that reversible GlyT2 inhibitors such as ol-$_D$-lys elude side effects observed with irreversible GlyT2 inhibition. Furthermore, GlyT2 inhibitors may offer targeted clinical indications for chronic pain, over acute or shorter-term pain conditions, and are likely to offer relief to often intractable chronic neuropathic pain conditions.

Acknowledgments

The authors thank Dr. Thomas Burton and the University of Sydney's Bosch Animal Behavioural Facility for practical training and assistance on the side effects tests.

Authorship Contributions

Participated in research design: Christie, Vandenberg, Mohammadi.

Conducted experiments: Wilson, Peiser-Oliver, Gillis, Evans, Alamein.

Contributed new reagents or analytic tools: Mostyn, Shimmon, Rawling.

Performed data analysis: Wilson, Peiser-Oliver, Gillis, Evans, Alamein, Mohammadi.

Wrote or contributed to the writing of the manuscript: Mohammadi.

References

Aroeira RI, Sebastião AM, and Valente CA (2014) GlyT1 and GlyT2 in brain astrocytes: expression, distribution and function. *Brain Struct Funct* **219**:817–830.

Barthel F, Urban A, Schlösser L, Eulenburg V, Werdehausen R, Brandenburger T, Aragon C, Bauer I, and Hermanns H (2014) Long-term application of glycine transporter inhibitors acts antineuropathic and modulates spinal N-methyl-D-aspartate receptor subunit NR-1 expression in rats. *Anesthesiology* **121**:160–169.

Bartley EJ and Fillingim RB (2013) Sex differences in pain: a brief review of clinical and experimental findings. *Br J Anaesth* **111**:52–58.

Benbouzid M, Pallage V, Rajalu M, Waltisperger E, Doridot S, Poisbeau P, Freund-Mercier MJ, and Barrot M (2008) Sciatic nerve cuffing in mice: a model of sustained neuropathic pain. *Eur J Pain* **12**:591–599.

Betz H, Gomeza J, Armsen W, Scholze P, and Eulenburg V (2006) Glycine transporters: essential regulators of synaptic transmission. *Biochem Soc Trans* **34**:55–58.

Borges K, Gearing M, McDermott DL, Smith AB, Almonte AG, Wainer BH, and Dingledine R (2003) Neuronal and glial pathological changes during epileptogenesis in the mouse pilocarpine model. *Exp Neurol* **182**:21–34.

Breivik H, Collett B, Ventafridda V, Cohen R, and Gallacher D (2006) Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *Eur J Pain* **10**:287–333.

Callahan BL, Gil ASC, Levesque A, and Mogil JS (2008) Modulation of mechanical and thermal nociceptive sensitivity in the laboratory mouse by behavioral state. *J Pain* **9**:174–184.

Caulfield WL, Collie IT, Dickins RS, Epemolu O, McGuire R, Hill DR, McVey G, Morphy JR, Rankovic Z, and Sundaram H (2001) The first potent and selective inhibitors of the glycine transporter type 2. 1*J Med Chem* **44**:2679–2682.

Cheng W, Yin Q, Cheng MY, Chen HS, Wang S, Feng T, Zeng YM, and Liu GJ (2009) Intracerebroventricular or intrathecal injection of glycine produces analgesia in thermal nociception and chemical nociception via glycine receptors. *Eur J Pharmacol* **614**:44–49.

Cioffi CL (2018) Modulation of glycine-mediated spinal neurotransmission for the treatment of chronic pain. *J Med Chem* **61**:2652–2679.

Dohi T, Morita K, Kitayama T, Motoyama N, and Morioka N (2009) Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. *Pharmacol Ther* **123**:54–79.

Gomeza J, Ohno K, Hülsmann S, Armsen W, Eulenburg V, Richter DW, Laube B, and Betz H (2003) Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. *Neuron* **40**:797–806.

Haranishi Y, Hara K, Terada T, Nakamura S, and Sata T (2010) The antinociceptive effect of intrathecal administration of glycine transporter-2 inhibitor ALX1393 in a rat acute pain model. *Anesth Analg* **110**:615–621.

Harvey RJ and Yee BK (2013) Glycine transporters as novel therapeutic targets in schizophrenia, alcohol dependence and pain. *Nat Rev Drug Discov* **12**:866–885.
von Hehn CA, Baron R, and Woolf CJ (2012) Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron* **73**:638–652.
Hermanns H, Muth-Selbach U, Williams R, Krug S, Lipfert P, Werdehausen R, Braun S, and Bauer I (2008) Differential effects of spinally applied glycine transporter inhibitors on nociception in a rat model of neuropathic pain. *Neurosci Lett* **445**:214–219.
Imlach WL, Bhola RF, Mohammadi SA, and Christie MJ (2016) Glycinergic dysfunction in a subpopulation of dorsal horn interneurons in a rat model of neuropathic pain. *Sci Rep* **6**:37104.
Jursky F and Nelson N (1995) Localization of glycine neurotransmitter transporter (GLYT2) reveals correlation with the distribution of glycine receptor. *J Neurochem* **64**:1026–1033.
Kochanek KD, Murphy SL, Xu J, and Arias E (2019) Deaths: Final Data for 2017. *Natl Vital Stat Rep* **68**:1–77.
Malosio ML, Marquèze-Pouey B, Kuhse J, and Betz H (1991) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J* **10**:2401–2409.
Mapplebeck JCS, Dalgarno R, Tu Y, Moriarty O, Beggs S, Kwok CHT, Halievski K, Assi S, Mogil JS, Trang T et al. (2018) Microglial P2X4R-evoked pain hypersensitivity is sexually dimorphic in rats. *Pain* **159**:1752–1763.
Michel MC, Murphy TJ, and Motulsky HJ (2020) New author guidelines for displaying data and reporting data analysis and statistical methods in experimental biology. *Mol Pharmacol* **97**:49–60.
Mingorance-Le Meur A, Ghisdal P, Mullier B, De Ron P, Downey P, Van Der Perren C, Declercq V, Cornelis S, Famelart M, Van Asperen J et al. (2013) Reversible inhibition of the glycine transporter GlyT2 circumvents acute toxicity while preserving efficacy in the treatment of pain. *Br J Pharmacol* **170**:1053–1063.
Mogil JS (2012) Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nat Rev Neurosci* **13**:859–866.
Mohammadi S and Christie MJ (2014) α9-nicotinic acetylcholine receptors contribute to the maintenance of chronic mechanical hyperalgesia, but not thermal or mechanical allodynia. *Mol Pain* **10**:64.
Morita K, Motoyama N, Kitayama T, Morioka N, Kifune K, and Dohi T (2008) Spinal antiallodynia action of glycine transporter inhibitors in neuropathic pain models in mice. *J Pharmacol Exp Ther* **326**:633–645.
Mostyn SN, Rawling T, Mohammadi S, Shimmon S, Frangos ZJ, Sarker S, Yousuf A, Vetter I, Ryan RM, Christie MJ et al. (2019) Development of an N-Acyl Amino Acid That Selectively Inhibits the Glycine Transporter 2 To Produce Analgesia in a Rat Model of Chronic Pain. *J Med Chem* **62**:2466–2484.
Motoyama N, Morita K, Shiraishi S, Kitayama T, Kanematsu T, Uezono Y, and Dohi T (2014) Relief of cancer pain by glycine transporter inhibitors. *Anesth Analg* **119**:988–995.
Nishikawa Y, Sasaki A, and Kuraishi Y (2010) Blockade of glycine transporter (GlyT) 2, but not GlyT1, ameliorates dynamic and static mechanical allodynia in mice with herpetic or postherpetic pain. *J Pharmacol Sci* **112**:352–360.
Omori Y, Nakajima M, Nishimura K, Takahashi E, Arai T, Akahira M, Suzuki T, and Kainoh M (2015) Analgesic effect of GT-0198, a structurally novel glycine transporter 2 inhibitor, in a mouse model of neuropathic pain. *J Pharmacol Sci* **127**:377–381.
Pang MH, Kim Y, Jung KW, Cho S, and Lee DH (2012) A series of case studies: practical methodology for identifying antinociceptive multi-target drugs. *Drug Discov Today* **17**:425–434.
Pitzer C, Kuner R, and Tappe-Theodor A (2016) EXPRESS: Voluntary and evoked behavioral correlates in neuropathic pain states under different social housing conditions. *Mol Pain* **12**:1–12.
Raiteri L, Stigliani S, Usai C, Diaspro A, Paluzzi S, Milanese M, Raiteri M, and Bonanno G (2008) Functional expression of release-regulating glycine transporters GLYT1 on GABAergic neurons and GLYT2 on astrocytes in mouse spinal cord. *Neurochem Int* **52**:103–112.
Schlösser L, Barthel F, Brandenburger T, Neumann E, Bauer I, Eulenburg V, Werdehausen R, and Hermanns H (2015) Glycine transporter GlyT1, but not GlyT2, is expressed in rat dorsal root ganglion–Possible implications for neuropathic pain. *Neurosci Lett* **600**:213–219.
Scholl L, Seth P, Kariisa M, Wilson N, and Baldwin G (2018) Drug and Opioid-Involved Overdose Deaths - United States, 2013-2017. *MMWR Morb Mortal Wkly Rep* **67**:1419–1427.
Seth P, Rudd RA, Noonan RK, and Haegerich TM (2018) Quantifying the epidemic of prescription opioid overdose deaths. *Am J Public Health* **108**: 500–502.
Sorge RE, Mapplebeck JCS, Rosen S, Beggs S, Taves S, Alexander JK, Martin LJ, Austin JS, Sotocinal SG, Chen D et al. (2015) Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nat Neurosci* **18**: 1081–1083.
Takahashi Y, Hara K, Haranishi Y, Terada T, Obara G, and Sata T (2015) Antinociceptive effect of intracerebroventricular administration of glycine transporter-2 inhibitor ALX1393 in rat models of inflammatory and neuropathic pain. *Pharmacol Biochem Behav* **130**:46–52.
Tanabe M, Takasu K, Yamaguchi S, Kodama D, and Ono H (2008) Glycine transporter inhibitors as a potential therapeutic strategy for chronic pain with memory impairment. *Anesthesiology* **108**:929–937.
Tung VWK, Burton TJ, Quail SL, Mathews MA, and Camp AJ (2016) Motor performance is impaired following vestibular stimulation in ageing mice. *Front Aging Neurosci* **8**:12.
Vandenberg RJ, Ryan RM, Carland JE, Imlach WL, and Christie MJ (2014) Glycine transport inhibitors for the treatment of pain. *Trends Pharmacol Sci* **35**:423–430.
Yogev-Seligmann G, Hausdorff JM, and Giladi N (2008) The role of executive function and attention in gait. *Mov Disord* **23**:329–342.
Zafra F, Aragón C, Olivares L, Danbolt NC, Giménez C, and Storm-Mathisen J (1995) Glycine transporters are differentially expressed among CNS cells. *J Neurosci* **15**:3952–3969.
Zeilhofer HU, Studler B, Arabadzisz D, Schweizer C, Ahmadi S, Layh B, Bösl MR, and Fritschy JM (2005) Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. *J Comp Neurol* **482**:123–141.
Zuidema J, Kadir F, Titulaer HAC, and Oussoren C (1994) Release and absorption rates of intramuscularly and subcutaneously injected pharmaceuticals (II). *Int J Pharm* **105**:189–207.

Address correspondence to: Dr. Sarasa Mohammadi, Charles Perkins Centre, Level 3 West, the University of Sydney, NSW Australia 2006. E-mail: sarasa.mohammadi@sydney.edu.au