Long-term Application of Glycine Transporter Inhibitors Acts Antineuropathic and Modulates Spinal *N*-methyl-D-aspartate Receptor Subunit NR-1 Expression in Rats

Franziska Barthel, V.M.D., Andrea Urban, V.M.D., Lukas Schlösser, M.D., Volker Eulenburg, Ph.D., Robert Werdehausen, M.D., Timo Brandenburger, M.D., Carmen Aragon, Ph.D., Inge Bauer, Ph.D., Henning Hermanns, M.D.

ABSTRACT

Background: Dysfunction of spinal glycinergic neurotransmission is a major pathogenetic factor in neuropathic pain. The synaptic glycine concentration is controlled by the two glycine transporters (GlyT) 1 and 2. GlyT inhibitors act antinociceptive in various animal pain models when applied as bolus. Yet, in some studies, severe neuromotor side effects were reported. The aim of the current study was to elucidate whether continuous inhibition of GlyT ameliorates neuropathic pain without side effects and whether protein expression of GlyT1, GlyT2, or *N*-methyl-D-aspartate receptor subunit NR-1 in the spinal cord is affected.

Methods: In the chronic constriction injury model of neuropathic pain, male Wistar rats received specific GlyT1 and GlyT2 inhibitors (ALX5407 and ALX1393; Sigma-Aldrich®, St. Louis, MO) or vehicle for 14 days *via* subcutaneous osmotic infusion pumps (n = 6). Mechanical allodynia and thermal hyperalgesia were assessed before, after chronic constriction injury, and every 2 days during substance application. At the end of behavioral assessment, the expression of GlyT1, GlyT2, and NR-1 in the spinal cord was determined by Western blot analysis.

Results: Both ALX5407 and ALX1393 ameliorated thermal hyperalgesia and mechanical allodynia in a time- and dose-dependent manner. Respiratory or neuromotor side effects were not observed. NR-1 expression in the ipsilateral spinal cord was significantly reduced by ALX5407, but not by ALX1393. The expression of GlyT1 and GlyT2 remained unchanged.

Conclusions: Continuous systemic inhibition of GlyT significantly ameliorates neuropathic pain in rats. Thus, GlyT represent promising targets in pain research. Modulation of *N*-methyl-D-aspartate receptor expression might represent a novel mechanism for the antinociceptive action of GyT1 inhibitors. **(ANESTHESIOLOGY 2014; 121:160-9)**

IN neuropathic pain, a lesion or disease of the somatosensory system leads to specific pain symptoms.[1] A reduction of inhibitory neurotransmission as well as an exaggeration of excitatory processes in the spinal cord contributes to the development of increased pain sensitivity.[2] Increasing spinal inhibition hence represents a promising therapeutic approach.[3] Glycine transporters (GlyT) tightly regulate the synaptic glycine concentration and thus influence inhibitory glycinergic neurotransmission in the central nervous system.[4] In mutual dependence with the excitatory *N*-methyl-D-aspartate (NMDA) receptor, GlyT regulate pain cognition.[5] The inhibition of GlyT has been shown to reduce nociceptive behavior in various animal pain models, most likely by increasing the glycine concentration at inhibitory glycine receptors (GlyR).[6–9] Yet, in various studies, acute inhibition of GlyT caused serious side effects, such as pareses, respiratory distress, or pronociceptive effects.[6,7,10] Whether a continuous

What We Already Know about This Topic

- Glycinergic neurotransmission is involved in neuropathic pain
- Many agents regulating synaptic glycine availability cause neuromotor side effects

What This Article Tells Us That Is New

- The long-term inhibition of glycine transporters GlyT1 and GlyT2 reduces neuropathic pain–related behavior in a rat model without neuromotor or respiratory side effects
- The modulation of spinal *N*-methyl-D-aspartate receptors seems to contribute to this effect

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systemic application of GlyT inhibitors can ameliorate neuropathic pain without these side effects is unclear.

Furthermore, whether long-term inhibition of GlyT leads to changes of expression in NMDA receptor subunits in the spinal cord has hitherto not been investigated. *In vitro* investigations show that an increased synaptic glycine concentration primes NMDA receptors for internalization.[11,12] Whether this glycine-dependent process also occurs *in vivo* and may lead to a secondary degradation of NMDA receptors is not known.

The aim of the current investigation therefore was to evaluate the effect of long-term application of the GlyT inhibitors ALX5407 (GlyT1) and ALX1393 (GlyT2) (Sigma-Aldrich®, St. Louis, MO) on mechanical allodynia and thermal hyperalgesia in experimental neuropathic pain in rats. Moreover, protein expression patterns of GlyT1, GlyT2, and NMDA receptor in the spinal cord during systemic application of GlyT inhibitors were investigated.

Materials and Methods

Reagents

ALX5407, ALX1393, and (2-hydroxypropyl)-β-cyclodextrin were purchased from Sigma-Aldrich® with a purity of greater than 98% (high-performance liquid chromatography). A polyclonal antibody against the NMDA receptor subunit NR-1 originating from rabbit was purchased from Cell Signaling Technology®, Inc. (Danvers, MA). Polyclonal antibodies against GlyT1 (rabbit no. 176) and GlyT2 (rabbit no. 218) originating from rabbit were described previously.[13] A monoclonal primary mouse-anti-α-tubulin antibody was purchased from Sigma-Aldrich®. Secondary horseradish peroxidase–coupled antibodies were purchased from Dianova (Hamburg, Germany). Unless stated otherwise, further reagents were purchases from Sigma-Aldrich® (Munich, Germany).

Animal Experiments

All animal experiments were performed with approval of the local government (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Recklinghausen, Germany) in accordance with the guidelines of the International Association for the Study of Pain. The role of GlyT in neuropathic pain was investigated in the chronic constriction injury (CCI) model of neuropathic pain[14] in 54 male Wistar rats (weight, 290 to 310 g) as described previously.[6] In brief, the left sciatic nerve was loosely ligated with general anesthesia (pentobarbital, 60 mg/kg; intraperitoneal injection) to induce a peripheral mononeuropathy. Sham operation comprised preparation of the sciatic nerve without ligation. As postoperative treatment, the skin wound was infiltrated with local anesthetic (3 mg/kg ropivacaine) to minimize discomfort.

The degree of thermal hyperalgesia and mechanical allodynia was assessed using a modified Hargreaves method[15] with a mobile radiant heat source (Plantar Test; Ugo Basile, Comerio, Italy) and a modified von Frey–type filament (Plantar Aesthesiometer; Ugo Basile), respectively, as described previously.[16,17]

To analyze the effect of GlyT inhibitors on nociception and spinal protein expression, 54 animals were randomized into nine groups. The required group size was estimated using a power analysis. Preliminary experiments with the highest inhibitor concentration suggested an effect size of at least 0.45. A group size of six thus resulted in a power (1-β) of greater than 0.8 (α = 0.05) using the G*Power 3.1.7 program[18] (Department of Psychology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany) in this case.

All animals underwent CCI and developed typical neuropathic pain symptoms, such as thermal hyperalgesia and mechanical allodynia. Ten days after CCI, animals were randomized to receive 0.2, 2, 20, or 200 μg/kg per day of specific GlyT1 inhibitor (ALX5407), GlyT2 inhibitor (ALX1393) dissolved in 20% (2-hydroxypropyl)-β-cyclodextrin, or vehicle, as control. GlyT inhibitors and vehicle were applied for 14 days with osmotic infusion pumps (Alzet® pump-model 2ML2; DURECT Corp., Sulzfeld, Germany), implanted subcutaneously at the upper neck with a delivery rate of 5 μl/h. The investigator was blinded to treatment. The development of mechanical allodynia and thermal hyperalgesia as signs of neuropathic pain was assessed before, after CCI, and every 2 days during substance application. Four measurements were performed at each time point.

After the last behavioral testing, the lumbar spinal cord (L4–L6) was dissected with deep general anesthesia, separated into ipsilateral and contralateral parts, immediately frozen in liquid nitrogen and stored at −80°C.

To assess potential neuromotor side effects in detail, 18 additional animals were implanted osmotic infusion pumps and randomly received vehicle, ALX1393, or ALX5407 for 14 days (200 μg/kg per day, respectively). During this time, motor coordination was measured using an automated four-lane Rotarod (Rat Rotarod, Model 7750; Ugo Basile) every 2 days during application. The Rotarod consists of a rotating barrel (Ø 4 cm). The rotation started with a speed of 5 rpm and accelerated constantly up to a maximum speed of 20 rpm within 300 s. The fall-off-time in seconds was observed or the Rotarod was stopped when maximum speed was reached (300 s). All animals were trained 1 day in advance of the study for 5 min in the slowest speed.

Furthermore, the Irwin test,[19] modified by Moscardo *et al.*,[20] was performed in each animal, evaluating 39 neurobehavioral and physiological parameters to assess possible unwanted effects. A numerical score was performed to quantify the neurobehavioral data.[20] Physiological status was graded with a score of 4. Variations from a normal score were observed with a score ranging from 0 to 3 or 5 to 8 (see publications by Irwin[19] and Moscardo *et al.*[20]).

Western Blot Analysis

To investigate the effects of glycine transporter inhibitors on protein expression of GlyT1, GlyT2, and NR-1 in the lumbar

spinal cord, samples were pulverized, homogenized in buffer (0.01 M phosphate-buffered saline; 10 mg/ml sodium dodecyl sulfate; and 1 mM phenylmethanesulfonyl fluoride) and centrifuged at 4°C, 50,000*g* for 10 min. The supernatant was collected and the protein content was quantified according to Lowry *et al.*[21] Samples were mixed with loading buffer (sodium dodecyl sulfate-stop-buffer [125 mM SIGMA 7–9; pH, 6.8; 20% glycerol; 9% sodium dodecyl sulfate; 0.05% bromophenol blue] mixed 1:10 with 2-β-mercaptoethanol)[13] and incubated at 37°C for 30 min. Fifty microgram of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis modified according to Laemmli.[22] The gel was run for 85 min at a constant current of 100 V. Proteins were transferred onto polyvinylidene difluoride membrane at 220 mA for 60 min in transfer buffer (26 mM SIGMA 7–9 + 96 mM glycine). The membrane was then blocked with 5% Blotto (5% dried skimmed milk in Tris-buffered saline with 0.1% Tween [TBS-T]) for 2 h at room temperature. The membranes were incubated overnight with a specific primary antibody for GlyT1 (no. 176), GlyT2 (no. 218), or NMDA receptor subunit NR-1. Membranes were washed three times for 10 min in cold TBS-T followed by incubation with a secondary donkey-anti-rabbit immunoglobulin G for 2 h. Subsequently, the blots were washed again in TBS-T and signals were detected with the enhanced chemoluminescent detection method by a digital camera (cool snap HQ2; Photometrics®, Tuscon, AZ). To control for equal protein loading, membranes were incubated with a primary mouse anti-α-tubulin antibody overnight, followed by a secondary goat-anti-mouse immunoglobulin G antibody. Signals were quantified and standardized against tubulin levels by densitometry (GelScan; BioSciTec GmbH, Frankfurt/Main, Germany).

Statistical Analysis

Data are presented as standard error of the mean (SEM). Results from behavioral experiments, that is, the paw-withdrawal thresholds, were compared between the treatment groups with two-way ANOVA followed by Tukey *post hoc* test. *P* value less than 0.05 was considered significant. Densitometric results from Western blot analysis were compared with a two-tailed paired Student *t* test. To correct for multiple testing, in this case *P* value less than 0.025 was considered significant. All calculations were performed using the SPSS program (Version 22.0; IBM Corp., Armonk, NY).

Results

Behavioral Measurements

All experiments lasted 24 days. There were no animal dropouts during the study. The first behavioral measurements were performed on the first day, before CCI, following a second assessment 10 days later as baseline value after development of neuropathic pain. All animals developed thermal hyperalgesia and mechanical allodynia 10 days after CCI in the injured, left hind paw. Nociception of the contralateral hind paw was not affected by surgery. Continuous application of the test substances was started on experimental day 10, and lasted for 14 days, that is, until experimental day 24. A control group of animals (n = 6) received only the vehicle (2-hydroxypropyl)-β-cyclodextrin. The application of vehicle had no impact on nociception, neither on the injured nor on the contralateral hind paw (figs. 1–4). This vehicle group served as control in further comparisons.

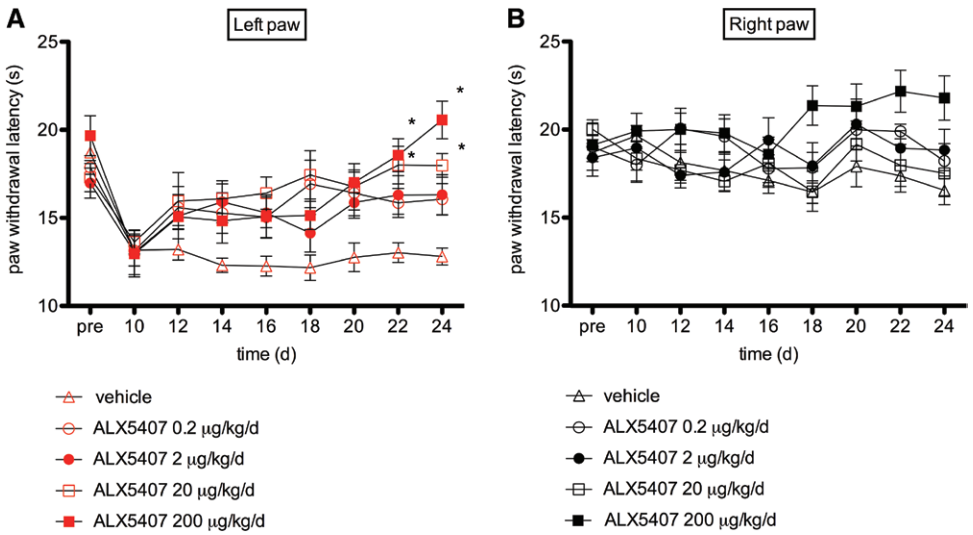

Fig. 1. Effects of ALX5407 on thermal hyperalgesia: Behavioral tests with the Plantar Test (Ugo Basile, Comerio, Italy) in all five groups that received continuous systemic administration of the glycine transporter 1 inhibitor ALX5407 (Sigma-Aldrich®, St. Louis, MO) (0.2, 2, 20, or 200 μg/kg per day, dissolved in (2-hydroxypropyl)-β-cyclodextrin) or vehicle. The *line graphs* show paw-withdrawal latencies (s) as response to a thermal stimulus from the injured left (*A*) and uninjured right paw (*B*) over time during application. Data are presented as mean ± SEM (n = 6). An *asterisk* indicates a significant difference compared with vehicle controls ($P < 0.05$).

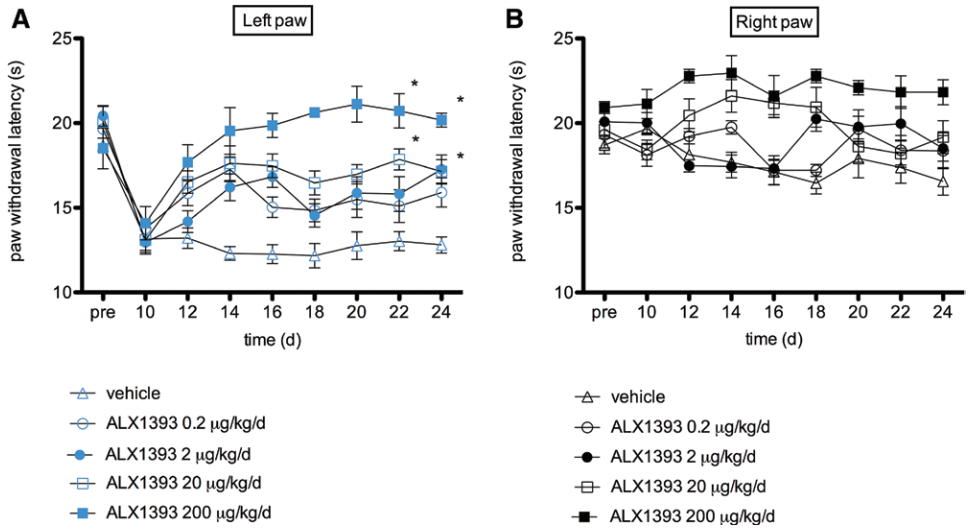

Fig. 2. Effects of ALX1393 on thermal hyperalgesia: Behavioral tests with the Plantar Test (Ugo Basile, Comerio, Italy) in all five groups that received continuous systemic administration of the glycine transporter 2 inhibitor ALX1393 (Sigma-Aldrich®, St. Louis, MO) (0.2, 2, 20, or 200 μg/kg per day, dissolved in (2-hydroxypropyl)-β-cyclodextrin) or vehicle. The *line graphs* show paw-withdrawal latencies (s) as response to a thermal stimulus from the injured left (*A*) and uninjured right paw (*B*) over time during application. Data are presented as mean ± SEM (n = 6). An *asterisk* indicates a significant difference compared with vehicle controls ($P < 0.05$).

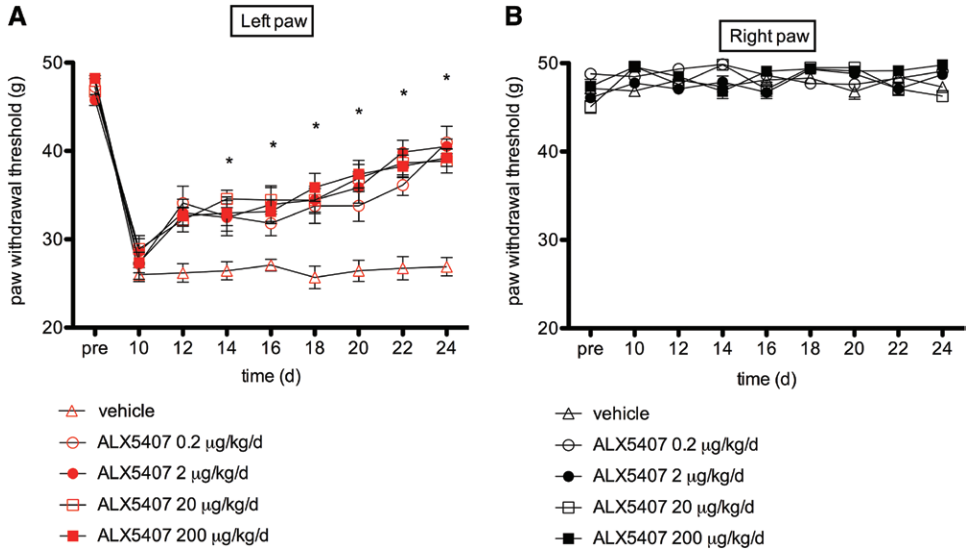

Fig. 3. Effects of ALX5407 on mechanical allodynia: Behavioral tests with the Plantar Aesthesiometer (Ugo Basile, Comerio, Italy) during application of the glycine transporter 1 inhibitor ALX5407 (Sigma-Aldrich®, St. Louis, MO) (0.2, 2, 20, or 200 μg/kg per day, dissolved in (2-hydroxypropyl)-β-cyclodextrin) or vehicle. The *line graphs* show paw-withdrawal thresholds (g) as response to a defined mechanical stimulus from the injured left (*A*) and uninjured right paw (*B*) over time during application. Data are presented as mean ± SEM (n = 6). An *asterisk* indicates a significant difference compared with vehicle controls ($P < 0.05$).

ALX5407 Reduces Thermal Hyperalgesia and Mechanical Allodynia

The GlyT1 inhibitor ALX5407, which was applied continuously, reduced the thermal hyperalgesia in the left, injured paw. Analysis of withdrawal latencies showed that the nociceptive response to thermal stimuli was attenuated within 12 days after onset of systemic continuous application of ALX5407 compared with that in the control group. This effect was statistically significant starting from experimental day 22 at a dosage of 200 μg/kg per day. Furthermore, there was a significant attenuation of thermal hyperalgesia measured at a dosage of 20 μg/kg per day at experimental days 22 and 24 (fig. 1A). ALX5407 had no statistically significant effect on thermal nociception of the contralateral hind paw (fig. 1B).

Continuous systemic administration of ALX5407 diminished mechanical allodynia of the ipsilateral hind paw at all four tested dosages. Withdrawal thresholds were found to be increased with a latency period of 4 days after beginning of

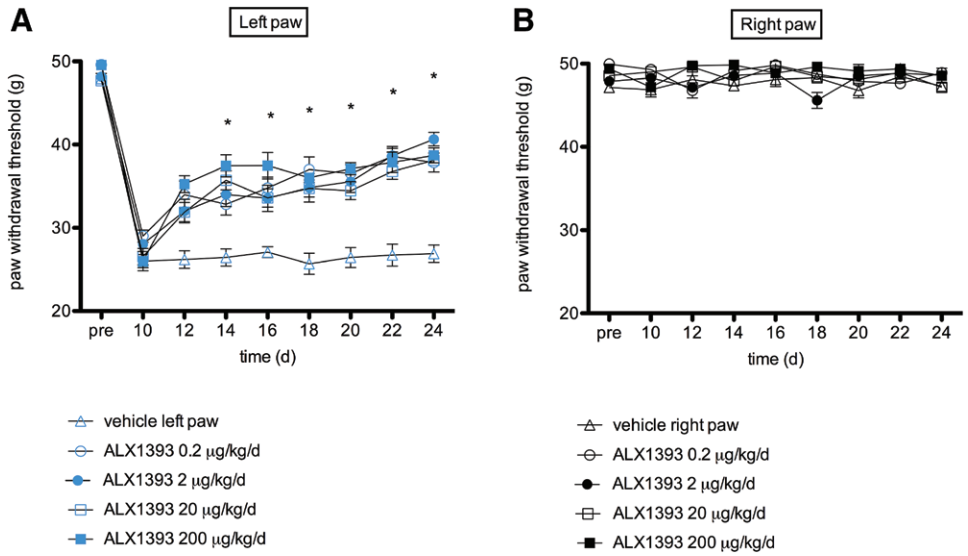

Fig. 4. Effects of ALX1393 on mechanical allodynia: Mechanical allodynia as assessed by the Plantar Aesthesiometer (Ugo Basile, Comerio, Italy) during application of the glycine transporter 2 inhibitor ALX1393 (Sigma-Aldrich®, St. Louis, MO) (0.2, 2, 20, or 200 µg/kg per day, dissolved in (2-hydroxypropyl)-β-cyclodextrin) or vehicle. The *line graphs* show paw-withdrawal thresholds (g) from the injured left (*A*) and uninjured right paw (*B*) over time during application. Data are presented as mean ± SEM (n = 6). An *asterisk* indicates a significant difference compared with vehicle controls ($P < 0.05$).

application when compared with that in controls. This effect was statistically significant from experimental day 14 on in all four doses (fig. 3A). ALX5407 had no effect on the paw-withdrawal threshold as response to mechanical stimuli in the contralateral, uninjured paw in any concentration (fig. 3B).

ALX1393 Reduces Thermal Hyperalgesia and Mechanical Allodynia

Continuous administration of ALX1393, a selective GlyT2 inhibitor, diminished thermal hyperalgesia at the neuropathic extremity. During systemic application of ALX1393 at a dosage of 200 µg/kg per day, thermal hyperalgesia was reduced with a time-of-onset of 12 days. This attenuation was statistically significant from experimental day 22. A lower dose of 20 µg/kg per day also led to a significant amelioration starting from experimental day 22 (fig. 2A). Analogous to the inhibition of GlyT1, the high doses of ALX1393 induced no significant effect on thermal nociception on the right, uninjured hind paw (fig. 2B). Furthermore, continuously applied ALX1393 counteracted mechanical allodynia on the side of the nerve ligation. During application of ALX1393, mechanical allodynia was attenuated with a latency of 4 days in all four tested dosages. From experimental day 14, a statistically significant amelioration was observed (fig. 4A). There were no changes regarding the response to von Frey–type filaments observed on the contralateral hind paw in any dose of ALX1393 (fig. 4B).

ALX1393 and ALX5407 Exert No Significant Neuromotor Side Effects

In contrast to previous studies,[6,10] no serious side effects such as neuromotor disturbances or respiratory distress were observed at any time point during application of the GlyT inhibitors. In naive animals that received the highest dose of the respective inhibitors, the motor behaviour assessed by the Rotarod test did not show any significant difference between the rats treated with the GlyT inhibitors and the vehicle group (fig. 5). Likewise, the systematic multimodal assessment of neurobehavioral effects of GlyT inhibitors with the modified Irwin test showed no difference between the treatment groups within the observation period (see tables 1–3, Supplemental Digital Content 1, http://links.lww.com/ALN/B38).

Continuous Application of ALX1393 or ALX5407 Does Not Alter the Expression of Spinal Glycine Transporters

To evaluate the effect of GlyT inhibitor application on the expression pattern of GlyT1 and GlyT2, the groups that were treated with the highest doses of GlyT inhibitors (200 µg/kg per day) were investigated and compared with the control group that solely received the vehicle. Neither the GlyT1 inhibitor ALX5407 nor the GlyT2 inhibitor ALX1393 had an effect on expression patterns of GlyT1 in the ipsilateral spinal cord (fig. 6A). Likewise, there was no difference in expression of the GlyT1 protein in the contralateral spinal cord (fig. 6B). In addition to this, the expression of GlyT2 was unchanged in the ipsilateral (fig. 6C) and the contralateral (fig. 6D) lumbar spinal cord after application of ALX5407 and ALX1393.

Continuous Inhibition of GlyT1 Reduces the Expression of NR-1

Systemic administration of the GlyT1 inhibitor ALX5407 significantly reduced the expression of NR-1 in the ipsilateral

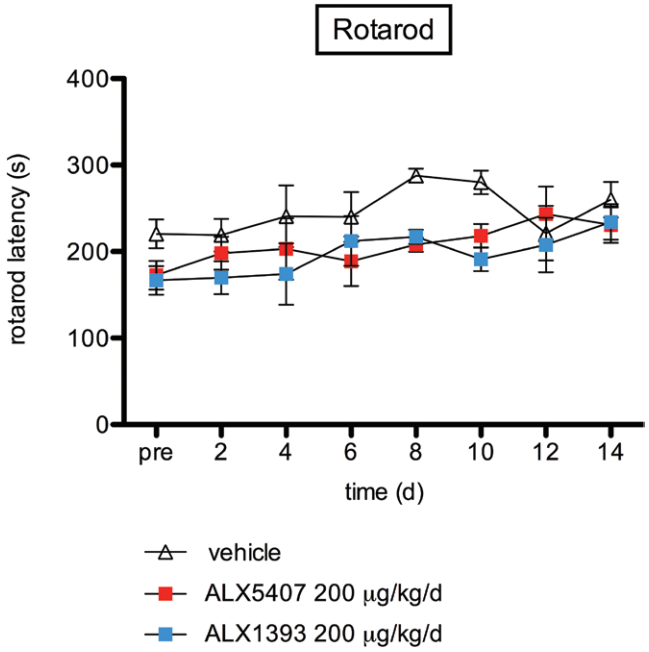

Fig. 5. Effects of ALX5407 and ALX1393 on motor coordination: Motor coordination was assessed by the Rat Rotarod (Ugo Basile, Comerio, Italy) (fall-off-time in seconds over time) during application of either vehicle or the highest dose of the glycine transporter 1 inhibitor ALX5407 (Sigma-Aldrich®, St. Louis, MO) (200 μg/kg per day) or glycine transporter 2 inhibitor ALX1393 (Sigma-Aldrich®) (200 μg/kg per day). Data are presented as mean ± SEM (n = 6).

spinal cord when compared with that in the vehicle group (P = 0.009) (fig. 6E). Yet, there were no statistically significant changes in expression of NR-1 in the contralateral spinal cord during GlyT1 inhibition (fig. 6F). In contrast, the GlyT2 inhibitor ALX1393 did not alter the expression of the NMDA receptor subunit NR-1 in the ipsi- and contralateral part of the spinal cord.

Discussion

In neuropathic pain, an imbalance between spinal inhibitory and excitatory neurotransmission leads to increased responses to noxious stimuli.[23] Among other changes, this imbalance results from a reduction of inhibitory neurotransmission.[2] Besides γ-aminobutyric acid (GABA), glycine is the major inhibitory neurotransmitter. Therefore, a pharmacological restoration of GABAergic or glycinergic inhibition may represent a promising target to find new therapeutic strategies for patients suffering from neuropathic pain.[24] To date, several studies could show that GABA agonists have high antinociceptive potential. Unfortunately, serious adverse effects may occur during GABA agonist application because of the ubiquitary abundance of GABA receptors all over the central nervous system. In contrast to GABAergic neurotransmission, glycinergic inhibition is more restricted to caudal parts of the central nervous system, such as the brain stem or the spinal cord.[25] A pharmacological approach to specifically facilitate glycinergic inhibition might therefore be efficient and bring along fewer adverse effects.[24] Hence, GlyT are considered potential targets of future therapy of chronic pain states.[26]

Indeed, several previous experimental studies could show that inhibition of either GlyT1 or GlyT2 was able to significantly ameliorate neuropathic pain symptoms such as thermal hyperalgesia and mechanical allodynia.[6–9,27–30] Yet, in some of these investigations, especially after bolus application of GlyT inhibitors, serious adverse effects such as pronociceptive effects or neuromotor dysfunction such as respiratory depression occurred.[6,7]

The current study demonstrates that a continuous systemic application of GlyT inhibitors has antinociceptive effects on chronic neuropathic pain in rats. Hyperalgesia and allodynia were significantly attenuated during application of both inhibitors. In contrast to previous studies, no adverse reactions such as pronociceptive effects or neurological impairment were observed. The fact that the paw withdrawal in the contralateral paw was not significantly altered by the inhibitors excludes that relevant neuromotor impairment may account for the increased withdrawal thresholds in the injured paw.

Until today, little is known about the precise mechanisms by which GlyT inhibitors act antinociceptive. Glycine acts as agonist at strychnine-sensitive GlyR[31] and concurrently modulates excitatory neurotransmission by acting as obligatory coagonist at the NMDA receptor.[32] This diverging effect of glycine explains the differential effects of glycine-modulating agents on nociception, including GlyT inhibitors: in principal, increasing synaptic glycine concentration can have both anti- and pronociceptive effects as shown by previous experiments. Furthermore, experimental evidence suggests that the analgesic action of increased glycine concentration

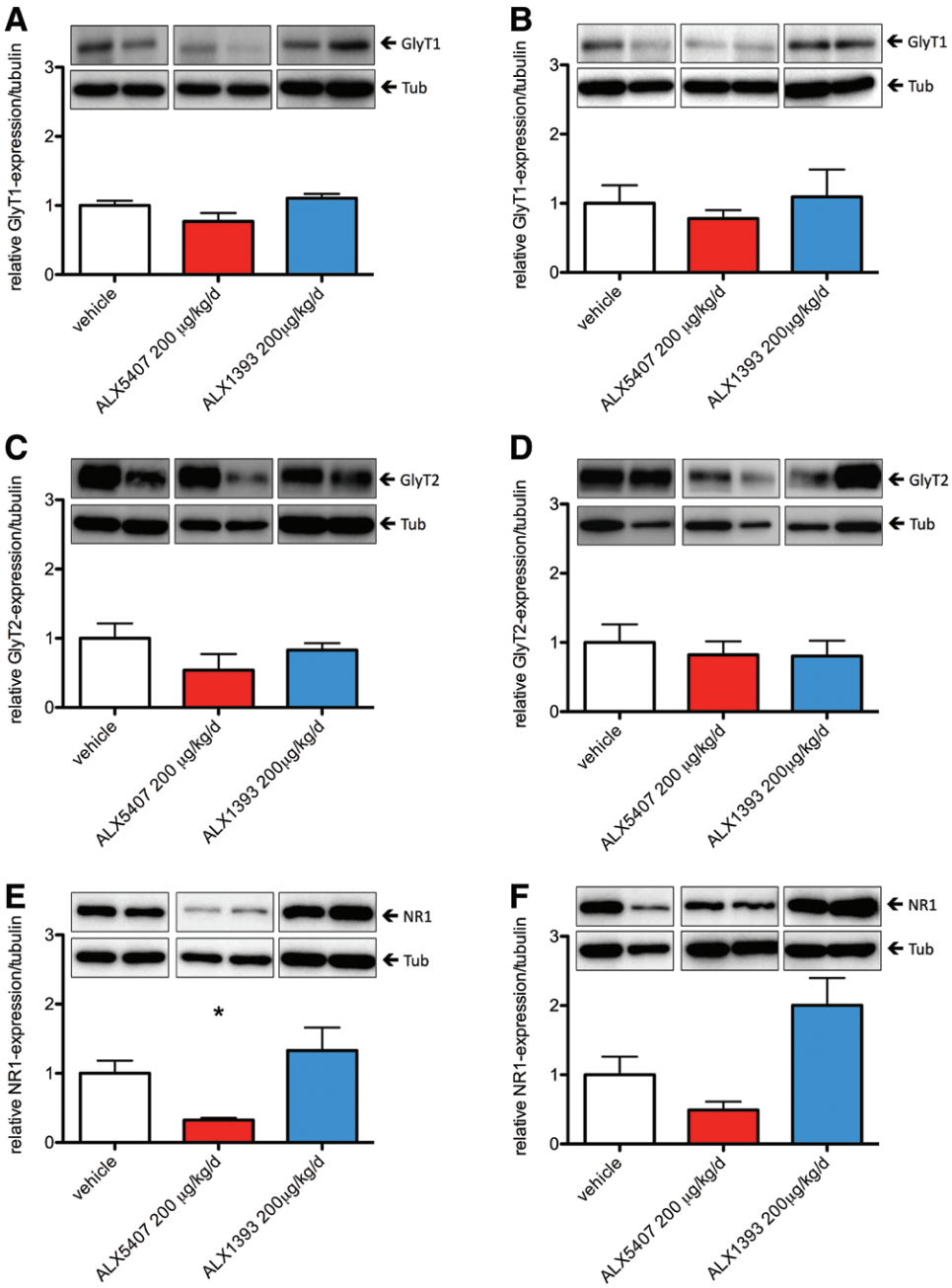

Fig. 6. Effect of GlyT inhibitors on protein expression in the spinal cord: Western blot analyses of glycine transporter 1 (GlyT1) expression (*A* and *B*), glycine transporter 2 (GlyT2) expression (*C* and *D*), and *N*-methyl-D-aspartate receptor subunit 1 (NR-1) expression (*E* and *F*) in the spinal cord of groups that received either vehicle or the highest dose (200 µg/kg per day) of GlyT1 inhibitor ALX5407 (Sigma-Aldrich®, St. Louis, MO) or GlyT2 inhibitor ALX1393 (Sigma-Aldrich®). GlyT1, GlyT2, and NR-1 protein expression was standardized against tubulin (Tub). Data are presented as mean ± SEM (n = 6). An *asterisk* indicates a significant difference compared with controls ($P < 0.025$).

is mediated by inhibitory GlyR and the pain-increasing effects are mediated by increased action at excitatory NMDA receptors.[6,33,34]

To date, two different transporters, GlyT1 and GlyT2, have been discovered.[35–37] GlyT1 is predominantly expressed in glial cells of brain stem and spinal cord.[5,13] Furthermore, GlyT1 was found in glial cells and glutamatergic neurons of other brain regions such as cortex and hippocampus.[13,25,38,39] The primary function of GlyT1 is the glycine clearance from the synaptic cleft into the cytosol although GlyT1 can also release glycine back into the synaptic cleft. This ambivalent function of GlyT1 may explain the differential effect of GlyT inhibitors on nociception. The pronociceptive properties are most likely due to saturation of the glycine-binding sites on NMDA receptors by inhibiting glycine uptake with GlyT1 inhibitors. This mechanism is supported by experiments in

which specific antagonists of the NMDA receptor glycine site reversed this effect.[7] In contrast, the antinociceptive effect results from an increase of the glycine concentration on inhibitory GlyR, which prolongs the duration of the glycinergic synaptic current.[26]

GlyT2 is expressed on glycinergic neurons in the vicinity of inhibitory GlyR. It catalyzes the recycling of glycine from the synaptic cleft to reconstitute glycine vesicles at the presynaptic site.[13,40] Inhibition of GlyT2 enhances glycine-dependent neurotransmission in sensory neurons. Its antinociceptive action can be counteracted by intrathecal injection of strychnine or knockdown of spinal GlyRα3,[7] which suggests that activation of spinal GlyRα3 mediates the antineuropathic effect of GlyT2 inhibitors.

The results of the current study show that a systemic continuous application of the GlyT1 inhibitor ALX5407 significantly decreases the expression of NR-1 in the ipsilateral lumbar spinal cord. NR-1, the glycine-binding subunit of the excitatory NMDA receptor, plays a crucial role in the development of neuropathic pain.[41–43] The observed reduction of spinal NR-1 might result from an internalization of NMDA receptor as a consequence of a continuously increased synaptic glycine concentration. This was shown before in *in vitro* experiments by Nong *et al.*[11] Other groups were able to show that modulation of NMDA receptor subunits, for example in NR-1 knock-out mice, leads to reduced nociception in inflammatory pain models.[44,45] The precise mechanism that led to down-regulation of NR-1 in the ipsilateral spinal cord in response to continuous inhibition of GlyT1 remains unclear. A possible explanation for this phenomenon might be a compensatory increased glycinergic activity in the ipsilateral spinal cord in a state of permanent pathological nociceptive transmission. A further increase of synaptic glycine concentration at GlyR by inhibition of GlyT might hence lead to a synaptic spillover to nearby excitatory synapses[46] and in consequence to an increased glycine concentration and consecutive internalization and secondary degradation of NMDA receptors. This hypothesis may also explain why on the contralateral side, at which there is no increased presynaptic glycinergic activity, a significant reduction of NR-1 expression could not be observed.

In the underlying study, whole cell extracts were used for Western blotting. It is also possible that changes in NR-1 protein expression might be restricted to either the outer or inner membrane. Therefore, further investigations of NR-1 would be necessary to unravel these mechanisms. Furthermore, investigation of expression patterns of the phosphorylated form of NR-1 might serve to elucidate hitherto unknown mechanisms of NMDA receptor–mediated pain chronification.[47] The exact role of GlyT inhibitor–induced NR-1 down-regulation still needs to be further elucidated.

In the current study, thermal hyperalgesia was solely attenuated significantly by the highest doses of both inhibitors after 12 days of treatment. In contrast, mechanical allodynia was reduced by all tested doses from day 4 of treatment, which indicates that allodynia might be more approachable by inhibition of GlyT than thermal hyperalgesia. This phenomenon underlines the fact that different sensory symptoms of neuropathic pain may be a consequence of different pain-generating mechanisms and hence cause differential and individual treatment responses.[48] This preclinical observation is in line with results from clinical investigations. In a multicenter clinical trial, Wallace *et al.*[49] analyzed the efficacy of GV196771, a glycine antagonist at the glycine-binding site of the NMDA receptor, in patients with chronic neuropathic pain. Although the overall effect on pain rating was unchanged and hence further development of the drug was discontinued, a significant reduction of allodynia was reported. These findings show that meticulous definition of the sensory profile of patients with neuropathic pain is crucial and that consideration of the specific pain phenotype may ultimately lead to a more sufficient, individualized pain therapy.[50] Thus, if GlyT inhibitors enter the phase of clinical studies, it may be appropriate to differentiate between different sensory phenomena of neuropathic pain as outcome parameters.

Interestingly, significant antinociceptive effects of both GlyT inhibitors were observed with a delay of several days after commencement of application. In previous studies, an intrathecal bolus application of GlyT inhibitors led to an immediate effect on nociception.[6,34] The reason for the protracted effect in the current study is unknown. Whether an efflux mechanism, an ongoing metabolism, or other mechanisms in the spinal cord leading to a slow increase of glycine account for this phenomenon remains speculative and should be subject of future research. The absence of adverse effects may result from the same mechanism. A slow rise in the presynaptic glycine concentration could consecutively lead to a reduced diffusion of glycine into the spinal ventral horn, where inhibition of GlyT may lead to suppression of spontaneous action potentials.[51] Although we present evidence for a differential mode of action, the reason why both GlyT inhibitors show a similar delay of onset in the antinociceptive action remains elusive. Furthermore, very recent experimental evidence suggests that ALX1393, the GlyT2 inhibitor, used in our study has only limited selectivity on GlyT2 *versus* GlyT1.[52] Further investigations will be needed to clarify this issue, possibly with the help of new-generation GlyT inhibitors.[53]

Further research is necessary to gain more detailed knowledge on the antinociceptive effect of agents that modulate synaptic glycine concentration. Although conventional knock out of either GlyT leads to a lethal phenotype in mice,[54,55] Cre-mediated inactivation of GlyT1 had more differential effects: Inactivation of neuronal GlyT1 does not lead to a change in phenotype. Inactivation of glial GlyT1, however, seems indispensable in early postnatal life, whereas those animals that survived the first postnatal week were did not present any neuromotor abnormalities.[56] This phenomenon suggests that GlyT1 can be inactivated in adults within

a safe margin. Furthermore, GlyT inhibitors, such as sarcosine, have been successfully used in clinical studies in schizophrenia,[57] depression,[58] or obsessive compulsive disorder[59] without side effects.

In summary, our findings show that in neuropathic pain in rats, a systemic continuous inhibition of either GlyT1 or GylT2 can ameliorate neuropathic pain without any side effects. Furthermore, the beneficial effect of GlyT1 inhibition might be mediated by a down-regulation of NMDA receptors. Although further experimental research is required to elucidate the precise molecular mechanisms of GlyT inhibitor–mediated antinociceptive effects, it might be the time to prove this concept of antinociception in a human trial.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Hermanns: Department of Anesthesiology, University Hospital Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany. henning.hermanns@uni-duesseldorf.de. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Treede RD, Jensen TS, Campbell JN, Cruccu G, Dostrovsky JO, Griffin JW, Hansson P, Hughes R, Nurmikko T, Serra J: Neuropathic pain: Redefinition and a grading system for clinical and research purposes. Neurology 2008; 70:1630–5
2. Sivilotti L, Woolf CJ: The contribution of $GABA_A$ and glycine receptors to central sensitization: Disinhibition and touch-evoked allodynia in the spinal cord. J Neurophysiol 1994; 72:169–79
3. Zeilhofer HU, Wildner H, Yévenes GE: Fast synaptic inhibition in spinal sensory processing and pain control. Physiol Rev 2012; 92:193–235
4. Aragón C, López-Corcuera B: Structure, function and regulation of glycine neurotransporters. Eur J Pharmacol 2003; 479:249–62
5. Eulenburg V, Armsen W, Betz H, Gomeza J: Glycine transporters: Essential regulators of neurotransmission. Trends Biochem Sci 2005; 30:325–33
6. Hermanns H, Muth-Selbach U, Williams R, Krug S, Lipfert P, Werdehausen R, Braun S, Bauer I: Differential effects of spinally applied glycine transporter inhibitors on nociception in a rat model of neuropathic pain. Neurosci Lett 2008; 445:214–9
7. Morita K, Motoyama N, Kitayama T, Morioka N, Kifune K, Dohi T: Spinal antiallodynia action of glycine transporter inhibitors in neuropathic pain models in mice. J Pharmacol Exp Ther 2008; 326:633–45
8. Tanabe M, Takasu K, Yamaguchi S, Kodama D, Ono H: Glycine transporter inhibitors as a potential therapeutic strategy for chronic pain with memory impairment. Anesthesiology 2008; 108:929–37
9. Centeno MV, Mutso A, Millecamps M, Apkarian AV: Prefrontal cortex and spinal cord mediated anti-neuropathy and analgesia induced by sarcosine, a glycine-T1 transporter inhibitor. Pain 2009; 145:176–83
10. Perry KW, Falcone JF, Fell MJ, Ryder JW, Yu H, Love PL, Katner J, Gordon KD, Wade MR, Man T, Nomikos GG, Phebus LA, Cauvin AJ, Johnson KW, Jones CK, Hoffmann BJ, Sandusky GE, Walter MW, Porter WJ, Yang L, Merchant KM, Shannon HE, Svensson KA: Neurochemical and behavioral profiling of the selective GlyT1 inhibitors ALX5407 and LY2365109 indicate a preferential action in caudal *vs.* cortical brain areas. Neuropharmacology 2008; 55:743–54
11. Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT, Salter MW: Glycine binding primes NMDA receptor internalization. Nature 2003; 422:302–7
12. Martina M, Gorfinkel Y, Halman S, Lowe JA, Periyalwar P, Schmidt CJ, Bergeron R: Glycine transporter type 1 blockade changes NMDA receptor-mediated responses and LTP in hippocampal CA1 pyramidal cells by altering extracellular glycine levels. J Physiol 2004; 557(Pt 2):489–500
13. Zafra F, Aragón C, Olivares L, Danbolt NC, Giménez C, Storm-Mathisen J: Glycine transporters are differentially expressed among CNS cells. J Neurosci 1995; 15(5 Pt 2):3952–69
14. Bennett GJ, Xie YK: A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988; 33:87–107
15. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988; 32:77–88
16. Muth-Selbach U, Hermanns H, Driehsen C, Lipfert P, Freynhagen R: Racemic intrathecal mirtazapine but not its enantiomers acts anti-neuropathic after chronic constriction injury in rats. Brain Res Bull 2009; 79:63–8
17. Brandenburger T, Castoldi M, Brendel M, Grievink H, Schlösser L, Werdehausen R, Bauer I, Hermanns H: Expression of spinal cord microRNAs in a rat model of chronic neuropathic pain. Neurosci Lett 2012; 506:281–6
18. Faul F, Erdfelder E, Lang AG, Buchner A: G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 2007; 39:175–91
19. Irwin S: Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia 1968; 13:222–57
20. Moscardo E, Maurin A, Dorigatti R, Champeroux P, Richard S: An optimised methodology for the neurobehavioural assessment in rodents. J Pharmacol Toxicol Methods 2007; 56:239–55
21. Lowry EC, Blumberg JM, Rhea RL, Ranson JP: Serum levels of orally administered penicillin. U S Armed Forces Med J 1951; 2:265–70
22. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–5
23. Costigan M, Scholz J, Woolf CJ: Neuropathic pain: A maladaptive response of the nervous system to damage. Annu Rev Neurosci 2009; 32:1–32
24. Zeilhofer HU, Benke D, Yevenes GE: Chronic pain states: Pharmacological strategies to restore diminished inhibitory spinal pain control. Annu Rev Pharmacol Toxicol 2012; 52:111–33

25. Eulenburg V, Gomeza J: Neurotransmitter transporters expressed in glial cells as regulators of synapse function. Brain Res Rev 2010; 63:103–12
26. Dohi T, Morita K, Kitayama T, Motoyama N, Morioka N: Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. Pharmacol Ther 2009; 123:54–79
27. Haranishi Y, Hara K, Terada T, Nakamura S, Sata T: The antinociceptive effect of intrathecal administration of glycine transporter-2 inhibitor ALX1393 in a rat acute pain model. Anesth Analg 2010; 110:615–21
28. Nishikawa Y, Sasaki A, Kuraishi Y: 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 2010; 112:352–60
29. Kodama D, Ono H, Tanabe M: Increased hippocampal glycine uptake and cognitive dysfunction after peripheral nerve injury. Pain 2011; 152:809–17
30. Yoshikawa S, Oguchi T, Funahashi Y, de Groat WC, Yoshimura N: Glycine transporter type 2 (GlyT2) inhibitor ameliorates bladder overactivity and nociceptive behavior in rats. Eur Urol 2012; 62:704–12
31. Lynch JW: Native glycine receptor subtypes and their physiological roles. Neuropharmacology 2009; 56:303–9
32. Johnson JW, Ascher P: Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature 1987; 325:529–31
33. Muth-Selbach U, Dybek E, Kollosche K, Stegmann JU, Holthusen H, Lipfert P, Zeilhofer HU: The spinal antinociceptive effect of nocistatin in neuropathic rats is blocked by D-serine. Anesthesiology 2004; 101:753–8
34. Morita K, Kitayama T, Morioka N, Dohi T: Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice. Pain 2008; 138:525–36
35. Guastella J, Brecha N, Weigmann C, Lester HA, Davidson N: Cloning, expression, and localization of a rat brain high-affinity glycine transporter. Proc Natl Acad Sci U S A 1992; 89:7189–93
36. Liu QR, López-Corcuera B, Mandiyan S, Nelson H, Nelson N: Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. J Biol Chem 1993; 268:22802–8
37. Liu QR, Nelson H, Mandiyan S, López-Corcuera B, Nelson N: Cloning and expression of a glycine transporter from mouse brain. FEBS Lett 1992; 305:110–4
38. Adams RH, Sato K, Shimada S, Tohyama M, Püschel AW, Betz H: Gene structure and glial expression of the glycine transporter GlyT1 in embryonic and adult rodents. J Neurosci 1995; 15(3 Pt 2):2524–32
39. Jursky F, Nelson N: Developmental expression of the glycine transporters GLYT1 and GLYT2 in mouse brain. J Neurochem 1996; 67:336–44
40. Aubrey KR, Rossi FM, Ruivo R, Alboni S, Bellenchi GC, Le Goff A, Gasnier B, Supplisson S: The transporters GlyT2 and VIAAT cooperate to determine the vesicular glycinergic phenotype. J Neurosci 2007; 27:6273–81
41. Garraway SM, Xu Q, Inturrisi CE: siRNA-mediated knockdown of the NR1 subunit gene of the NMDA receptor attenuates formalin-induced pain behaviors in adult rats. J Pain 2009; 10:380–90
42. Da Silva LF, Walder RY, Davidson BL, Wilson SP, Sluka KA: Changes in expression of NMDA-NR1 receptor subunits in the rostral ventromedial medulla modulate pain behaviors. Pain 2010; 151:155–61
43. Tan PH, Chia YY, Chow LH, Chen JJ, Yang LC, Hung KC, Chen HS, Kuo CH: Gene knockdown of the *N*-methyl-D-aspartate receptor NR1 subunit with subcutaneous small interfering RNA reduces inflammation-induced nociception in rats. Anesthesiology 2010; 112:1482–93
44. South SM, Kohno T, Kaspar BK, Hegarty D, Vissel B, Drake CT, Ohata M, Jenab S, Sailer AW, Malkmus S, Masuyama T, Horner P, Bogulavsky J, Gage FH, Yaksh TL, Woolf CJ, Heinemann SF, Inturrisi CE: A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain. J Neurosci 2003; 23:5031–40
45. Cheng HT, Suzuki M, Hegarty DM, Xu Q, Weyerbacher AR, South SM, Ohata M, Inturrisi CE: Inflammatory pain-induced signaling events following a conditional deletion of the *N*-methyl-D-aspartate receptor in spinal cord dorsal horn. Neuroscience 2008; 155:948–58
46. Ahmadi S, Muth-Selbach U, Lauterbach A, Lipfert P, Neuhuber WL, Zeilhofer HU: Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine. Science 2003; 300:2094–7
47. Zhang RX, Liu B, Li A, Wang L, Ren K, Qiao JT, Berman BM, Lao L: Interleukin 1beta facilitates bone cancer pain in rats by enhancing NMDA receptor NR-1 subunit phosphorylation. Neuroscience 2008; 154:1533–8
48. Baron R, Binder A, Wasner G: Neuropathic pain: Diagnosis, pathophysiological mechanisms, and treatment. Lancet Neurol 2010; 9:807–19
49. Wallace MS, Rowbotham MC, Katz NP, Dworkin RH, Dotson RM, Galer BS, Rauck RL, Backonja MM, Quessy SN, Meisner PD: A randomized, double-blind, placebo-controlled trial of a glycine antagonist in neuropathic pain. Neurology 2002; 59:1694–700
50. von Hehn CA, Baron R, Woolf CJ: Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. Neuron 2012; 73:638–52
51. Eckle VS, Antkowiak B: ALX 1393 inhibits spontaneous network activity by inducing glycinergic tonic currents in the spinal ventral horn. Neuroscience 2013; 253:165–71
52. 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, Jnoff E, Courade JP: Reversible inhibition of the glycine transporter GlyT2 circumvents acute toxicity while preserving efficacy in the treatment of pain. Br J Pharmacol 2013; 170:1053–63
53. Carland JE, Handford CA, Ryan RM, Vandenberg RJ: Lipid inhibitors of high affinity glycine transporters: Identification of a novel class of analgesics. Neurochem Int 2013 [Epub ahead of print]
54. Gomeza J, Hülsmann S, Ohno K, Eulenburg V, Szöke K, Richter D, Betz H: Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. Neuron 2003; 40:785–96
55. Gomeza J, Ohno K, Hülsmann S, Armsen W, Eulenburg V, Richter DW, Laube B, Betz H: Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. Neuron 2003; 40:797–806
56. Eulenburg V, Retiounskaia M, Papadopoulos T, Gomeza J, Betz H: Glial glycine transporter 1 function is essential for early postnatal survival but dispensable in adult mice. Glia 2010; 58:1066–73
57. Javitt DC: Glycine transport inhibitors in the treatment of schizophrenia. Handb Exp Pharmacol 2012:367–99
58. Huang CC, Wei IH, Huang CL, Chen KT, Tsai MH, Tsai P, Tun R, Huang KH, Chang YC, Lane HY, Tsai GE: Inhibition of glycine transporter-I as a novel mechanism for the treatment of depression. Biol Psychiatry 2013; 74:734–41
59. Wu PL, Tang HS, Lane HY, Tsai CA, Tsai GE: Sarcosine therapy for obsessive compulsive disorder: A prospective, open-label study. J Clin Psychopharmacol 2011; 31:369–74