Glycine transporter GlyT1, but not GlyT2, is expressed in rat dorsal root ganglion—Possible implications for neuropathic pain

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1. Introduction

Glycine is a major inhibitory neurotransmitter in the CNS and controls motor and sensory signal transduction. GlyT1 and GlyT2 affect glycinergic neurotransmission by regulating the extracellular glycine concentration [1].

The expression of GlyT2 is confined to glycinergic neurons in caudal regions of the central nervous system (CNS), while GlyT1 is expressed in most regions of the CNS, mainly in glial cells and to a lesser extent in a subpopulation of glutamatergic neurons [2,3]. It is unknown whether GlyTs are also expressed in the peripheral nervous system (PNS). Although inhibitory glycine receptors are absent in the PNS [4], GlyTs might contribute here to the regulation of ionotropic glutamate receptors of the *N*-methyl-D-aspartate (NMDA) subtype [5]. Here, glycine, just as D-serine, acts as an essen-

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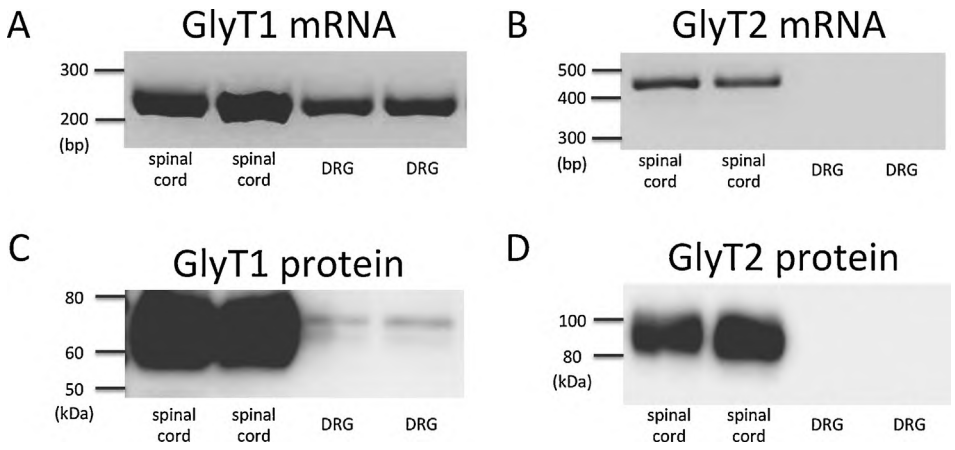

Fig. 1. Reverse-transcription PCR and Western blot analysis of GlyT1 and Glyt2 expression. (A) representative RT-PCR illustrating the presence of GlyT1 (234 bp) in rat spinal cord and DRG. (B) GlyT2 mRNA (424 bp) was found in spinal cord, whereas it was not detected in DRG. Western blot analysis of GlyT1 protein expression (C) revealed high protein levels in spinal cord and lower levels in DRG (GlyT1 70 kDa). GlyT2 protein (D) was expressed in spinal cord (GlyT2 84 kDa).

tial coagonist by binding at the NR1 subunit of the receptor [6]. Interestingly, it has been described that the glycine binding NR1 subunit of the NMDA receptor is upregulated at least in the spinal cord in the context of neuropathic pain [7].

Since changes in glycine-dependent neurotransmission have been shown to play a major role in chronic pain, the role of GlyTs has gained increasing attention recently. The pain-relieving effects of GlyT inhibitors are well documented in a variety of experimental pain models [8]. Yet, whether the development of chronic pain is associated with changes in GlyT activity in the nervous system is still unknown.

In this study, we analyzed whether expression changes for components of glycine-dependent neurotransmission also occur in the DRG. In detail, we show that GlyT1 is also expressed in this PNS structure. Furthermore, we provide evidence that neuropathic pain, does not lead to changes in peripheral NR1 or GlyT1 expression.

2. Methods

2.1. Animal experiments

All experimental experiments were approved by the local animal care and use committee. For Chronic Constriction Injury (CCI) of the sciatic nerve [9], male Wistar rats ($n = 6$ per group) were anesthetized with pentobarbital (60 mg/kg, i.p.). Sham procedure comprised equal treatment without nerve ligation. The development of mechanical allodynia was examined using a Plantar Aesthesiometer (Ugo Basile Inc., Comerio, Italy) with the maximal force applied to the paw set to 50 g. The force at which the paw was withdrawn was registered in four independent measurements before and 12 days after CCI. The observer was blinded to the treatment given to each animal. Subsequently, the spinal cord (L4–L6) and the left DRG L4–L6 were removed under deep pentobarbital anesthesia (150 mg/kg, i.p.) and immediately frozen in liquid nitrogen. Additional tissue samples were dissected from six naïve anesthetized rats to study expression patterns of GlyT1 and NR1 in the spinal cord, DRG and the sciatic nerve.

3. Western blot analysis

Protein samples were prepared by homogenizing and solubilizing the tissue in phosphate buffered saline (PBS) including sodium dodecyl sulfate (SDS 10 mg/ml) and 1 mM phenylmethanesulfonylfluoride (PMSF). After centrifugation (50,000 × g, 15 min, 4 °C) the content was measured using the Lowry method [10]. Equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes in a semidry electroblotting system. Nonspecific protein binding was blocked by incubating the membranes with 5% nonfat milk protein in PBS-Tween. Incubation with primary antibodies (rabbit polyclonal anti-GlyT1 No. 176 (1:1,000) or rabbit polyclonal anti-GlyT2 No. 218 (1:1,000), respectively [2], rabbit polyclonal anti-*N*-methyl-D-aspartate (NMDA) receptor subunit NR1 antibody (1:1000, Cell Signaling, Cambridge, UK) followed. Blots were incubated with horseradish peroxidase-linked anti-rabbit IgG. Equal loading of protein was verified by probing the membrane with anti-tubulin antibody (mouse monoclonal; 1:50,000; Sigma–Aldrich, St. Louis, MO, USA) or anti-GAPDH antibody (mouse monoclonal; 1:40,000), Sigma–Aldrich). Immunoreactive bands were detected using an enhanced chemiluminescence system (Santa Cruz, Dallas, TX, USA). The blots were quantified using GelScan 6.0 software (Decon Science Tec, Frankfurt, Germany). The results are expressed as Differential Integrated Density (DID) and presented as normalized density ratios of the protein to the housekeeper protein. Each experiment was run in duplicates.

4. RNA preparation, cDNA synthesis and RT-PCR

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissues according to the manufacturer's protocol and isolated RNA quantity was determined by UV spectrophotometry (Nanodrop, Thermo Scientific, USA) and RNA integrity was verified by gel electrophoresis using 2.5 μg of total RNA per lane. One microgram of RNA was reversely transcribed to a single-stranded cDNA as described previously [11]. GlyT1 and GlyT2 specific amplicons were amplified in a total volume of 25 μl, containing 50 pmol of reverse and forward primers, 2 mM $MgCl_2$, 2.5 μl 10× PCR buffer, 0.25 U Taq-DNA polymerase (Invitrogen, Carlsbad, CA, USA), 10 mM nucleotide mix (Promega, Madison, WI, USA) and 1 μl cDNA. The primer sequences were: GlyT1 forward 5′-CATACCTCTGCTATCGCAAC-3′, GlyT1 reverse: 5′-AGAAGGCGATGCAGATGAC-3′, GlyT2 forward 5′-CTGCTGGCGGTATTGGGATTTGG-3′, GlyT2 reverse: 5′-CAGACAAATGAGAGACAGGGCAG-3′. The conditions for amplification were: 95 °C for 10 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C (GlyT1) or 62 °C (GlyT2) for 45 s, and elongation at 72 °C for 60 s. PCR products were electrophoresed on 1.5% agarose gels.

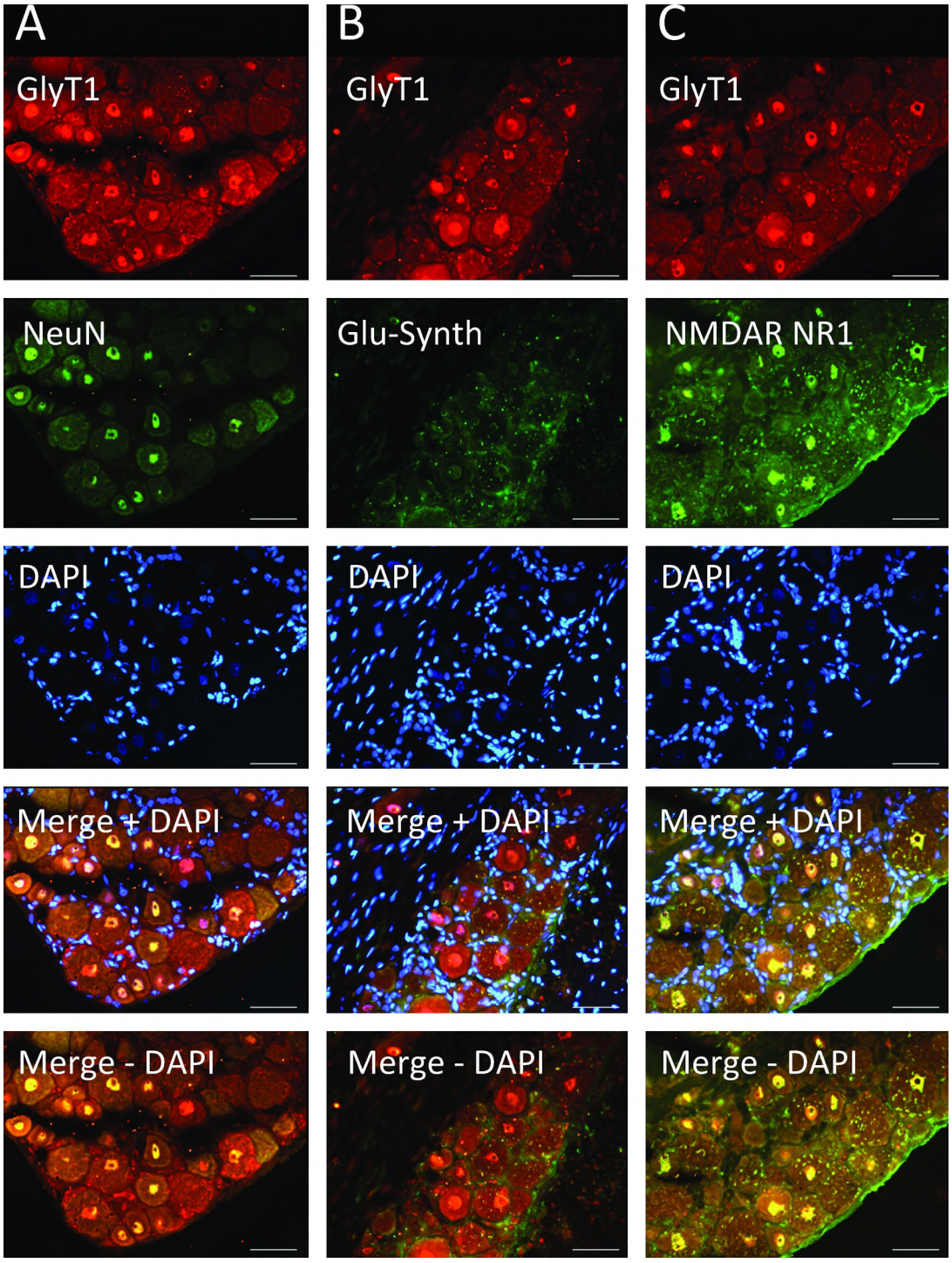

Fig. 2. Immunofluorescence microscopy of glycine transporter 1 (GlyT1) expression in rat dorsal root ganglion cryosections. Nuclei were stained using DAPI. GlyT1 = glycine transporter 1; (A) NeuN = neuron-specific nuclear protein; (B) Glu-Synth = glutamine synthetase; (C) NMDAR NR1 = *N*-methyl-D-aspartate receptor subunit NR1. Scale bar = 10 μm.

5. Quantitative PCR (qPCR)

Quantitative determination of gene expression was performed on an ABI 7300 real time PCR system using TaqMan® universal PCR master mix (Applied Biosystems, Foster City, CA). qPCR assays (Applied Biosystems) for GlyT1 (Assay ID: Rn01416529_m1) and GlyT2 (Assay ID: Rn01475607_m1) were used. ß-Actin (Assay ID: Rn00667869_m1) was used as a reference gene, and relative gene expression was determined according to the ΔDCt method [12] and the relative expression software tool [13]. All samples were run in duplicates and at least in two independent experiment. Data are presented as mean values + SEM.

6. Immunofluorescence staining

For immunostaining of DRG, spinal cord and the sciatic nerve, cryosections (8 μm) were fixated with 4% paraformaldehyde. Subsequently, cryosections were blocked and permeabilized for 1 h

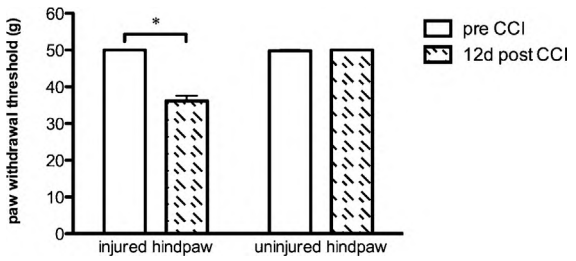

Fig. 3. Mechanical allodynia induced by chronic constriction injury (CCI). Paw withdrawal threshold (g) in the left, injured paw and the right, uninjured paw before (pre) and 12 days after (post) CCI. Means + SEM. $n = 6$. * indicates a $P < 0.05$ (paired Student's *t*-test).

with blocking buffer containing 10% normal goat serum and 0.2% saponine in PBS, respectively.

Primary antibodies (guinea pig anti-GlyT1 antibody, 1:100 raised against the N-terminal domain of GlyT1); mouse anti-NMDA receptor subunit NR1 antibody, 1:50 (Imgenex, San Diego, CA, USA); mouse anti-glutamine synthetase antibody, 1:500 (Abcam, Cambridge, UK); mouse anti-NeuN antibody, 1:300 (Millipore, Billerica, MA, USA) were applied by incubation overnight at 4 °C.

Secondary antibodies (donkey anti-guinea pig IgG indocarbocyanine (Cy3)-conjugated antibody, 1:500 (Dianova, Hamburg, Germany); goat anti-mouse IgG Alexa Fluor 488 antibody, 1:500 (Invitrogen, Carlsbad, CA, USA) were applied for 3–4 h at RT. Subsequently, sections were mounted (ProLong Gold anti-fade reagent with DAPI; Invitrogen), evaluated with a fluorescence microscope and a digital camera (Leica Microsystems, Wetzlar, Germany).

7. Statistical analysis

All data were tested for normality using the Kolmogorov–Smirnov Test for normality. Accordingly, behavioral data, presented as paw withdrawal thresholds before and 12 days after CCI, were compared using the Mann–Whitney *U*-test. Normalized density ratios of the protein expression were analyzed using the unpaired Student's *t*-test (Prism 5, GraphPad Software, La Jolla, CA, USA). Data are presented as means ± SEM. A P value <0.05 was considered to indicate significant differences.

8. Results

8.1. Expression pattern of GlyT in the DRG and spinal cord

To analyze GlyT1 and GlyT2 expression in spinal cord and DRG, cDNA samples were analyzed by RT-PCR. Here, GlyT1 (Fig. 1A) and GlyT2 (Fig. 1B) specific amplicons were identified in samples from spinal cord. In DRG only GlyT1 specific amplicons

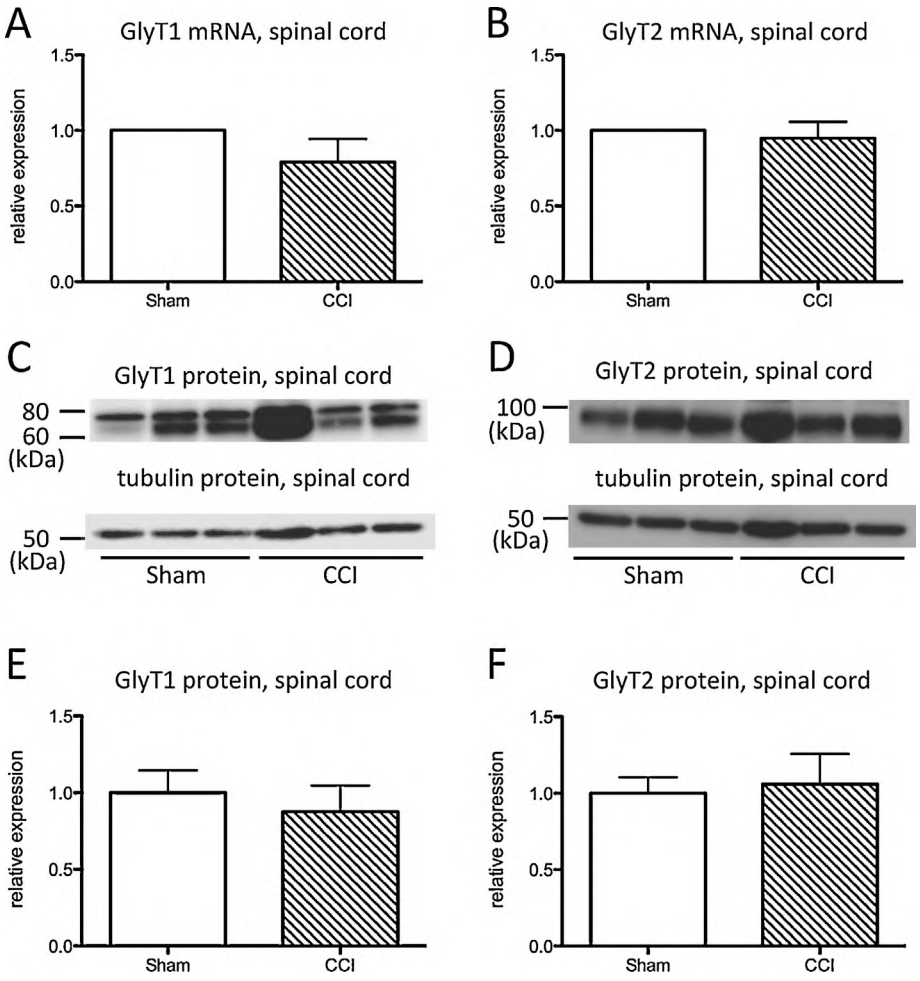

Fig. 4. GlyT1 and GlyT2 expression in spinal cord after chronic constriction injury (CCI). qPCR analysis of GlyT1 (A) and GlyT2 mRNA expression (B) in spinal cord 12 days after sham operation or CCI. β-actin served as reference gene. Representative Western blots analyzing GlyT1 (C) and GlyT2 (D) protein expression (GlyT1 70 kDa, GlyT2 84 kDa) in spinal cord 12 days after sham operation or CCI. α-tubulin (50–55 kDa) was used as loading control (C and D, lower row). Densitometric analysis of GlyT1 (E) and GlyT2 (F) protein expression in rat spinal cord 12 days after sham operation or CCI. Values normalized to sham group. Data are presented as means + SEM ($n = 6$).

were detected (Fig. 1A), whereas, no GlyT2 specific fragment was detected (Fig. 1B).

Protein expression of the both transporters was confirmed in the spinal cord by Western blot analysis (Fig. 1C/D). GlyT1 protein was also found in DRG although the relative amount of GlyT1 protein was lower than in spinal cord. In contrast, GlyT2 protein was not detected in DRG. Immunofluorescence analysis, revealed GlyT1 and GlyT2 specific signals in the spinal cord similar to that described in the literature (data not shown). Consistent with the RT-PCR- data and Western-blot analysis, GlyT1 antibody staining was observed in DRG. Here, GlyT1-immunreactive signals were found predominantly in areas of DRG positive for neuron-specific nuclear protein (NeuN), a specific neuronal marker (Fig. 2A). In contrast, no co-localization was found in structures positive for glutamine synthetase (Glu-Synth), a glial marker (Fig. 2B). Furthermore, GlyT1-related signals were found in areas that stained positive for the NMDA receptor subunit NR1 (Fig. 2C), suggesting a co-localization of GlyT1 and NMDA receptors in DRG. Immunostaining of the sciatic nerve also revealed expression of GlyT1 along myelinated and non-myelinated nerve fibers (Supplement Fig. 1).

9. Behavioral experiments

All animals that underwent CCI developed mechanical allodynia in the left, injured hindpaw 12 days after surgery. Here, the withdrawal threshold decreased significantly from 50.0 ± 0.0 g before CCI to 36.2 ± 1.5 g after 12 days ($P < 0.001$). In the right, uninjured hindpaw (49.8 ± 0.2 g before CCI and 50.0 ± 0.0 g after 12 days) the force until the paw was withdrawn remained constant (Fig. 3). Body weight of the rats increased in the sham group (+9.5 ± 11 g) while it stagnated in the CCI group (−0.5 ± 1.7 g).

10. Expression of GlyT in the spinal cord in response to neuropathic pain

Twelve days after sham injury or CCI, GlyT1 and GlyT2 mRNA expression in rat spinal cord was analyzed using qPCR. Here, no significant changes in expression of the transporters in our CCI model of neuropathic pain compared with sham-operated controls were observed (Fig. 4A and B). Similarly, protein expression, as determined densitometric analysis of Western blots for GlyT1 (representative blot in Fig. 4C) and GlyT2 (representative blot in Fig. 4D), did not reveal any significant difference in the spinal cord of CCI-treated animals compared to sham controls (two membranes with $n = 3$ per group, Fig. 4E and F).

11. Expression of GlyT1 and NR1 in DRG tissue in response to neuropathic pain

In DRG, no changes in mRNA expression for GlyT1 were observed in response to CCI 12 days after surgery when compared to sham controls (Fig. 5A). Similarly, GlyT1 protein expression was not differentially altered 12 days after CCI compared to sham operation (Fig. 5B and C). Additionally, no significant expression changes of NR1 were detected in the ipsilateral DRG (Fig. 6).

12. Discussion

In the present study, the expression of GlyTs and the glycine binding subunit NR1 of the NMDA receptor was investigated in the nervous system in the context of neuropathic pain. We confirmed expression of GlyT1 and GlyT2 in rat spinal cord as described previously [2]. Furthermore, we provide evidence that GlyT1, but not GlyT2, is also expressed in the PNS. GlyT1, but not GlyT2, was identified in DRG. Here, we show that GlyT1 localizes in the vicinity

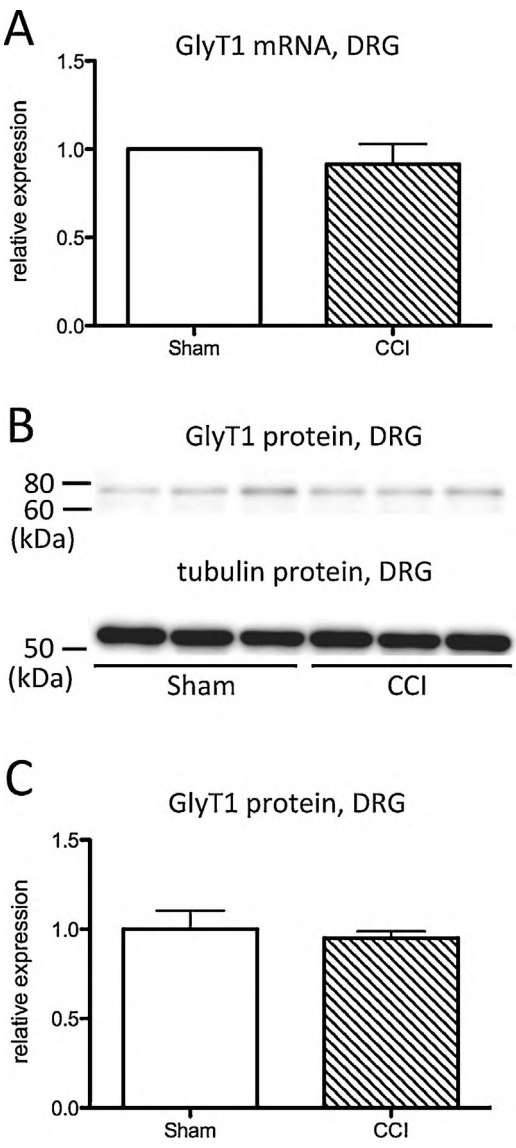

Fig. 5. GlyT1 expression in DRG after chronic constriction injury (CCI). (A) qPCR analysis of GlyT1 mRNA expression in DRG 12 days after sham operation or CCI. β-actin served as reference gene. Data are presented as means + SEM ($n = 6$). Representative Western blots analyzing GlyT1 (B) protein expression (GlyT1 70 kDa) in DRG 12 days after sham operation or CCI. α-tubulin (50–55 kDa) was used as loading control (B, lower row). (C) densitometric analysis of GlyT1 protein expression in DRG 12 days after sham operation or CCI. Values normalized to sham group. Data are presented as means + SEM ($n = 6$).

of peripheral NMDA receptors, as shown by co-localization with the NR1 subunit. A further result of our investigation is that GlyTs are not differentially regulated after induction of neuropathic pain, neither in the spinal cord, nor in the DRG. In contrast to previous findings in the spinal cord [7], no changes in NR1 expression were observed in DRG, indicating that the regulation of NR1 expression differs between spinal cord and DRG.

At present, two glycine transporters have been cloned: GlyT1 and GlyT2, which both exist in multiple isoforms. GlyT1 is expressed in many parts of the CNS, predominantly by astrocytes. Additional expression was shown in a subset of glutamatergic neurons [14]. GlyT2 is exclusively expressed by glycinergic neurons of the CNS [15]. GlyT1 is found in areas without strychnine-sensitive glycine receptors as well [2,3]. Consistently, GlyT1 has been shown to also affect NMDA receptor mediated glutamater-

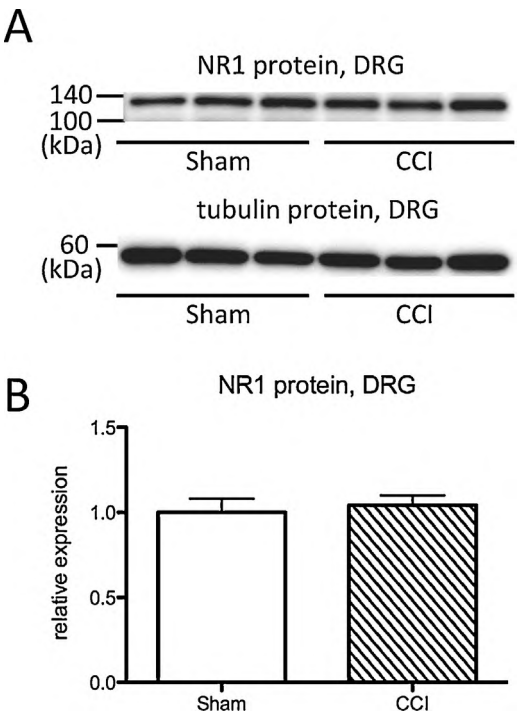

Fig. 6. Western blot analysis of NR1 expression. (A) representative western blots analyzing NR1 protein expression (NR1 120 kDa) in the DRG 12 days after sham injury or CCI. α-tubuline (50–55 kDa) was used as control (B, lower row). (B) densitometric analysis of NR1 protein expression in the DRG 12 days after sham injury or CCI. Values normalized to sham group. Data presented as means + SEM ($n = 6$).

gic neurotransmission in addition to glycine dependent inhibitory neurotransmission [15].

Consistent with the fact that the majority of DRG cells are glutamatergic, and glycinergic cells are absent [16], GlyT2 was not detected, whereas GlyT1 was identified in the vicinity of NR1-positive neurons of DRG.

To our knowledge, up to now there are no studies reporting GlyT expression in the PNS. Our results from immunofluorescence microscopy demonstrate a neuronal localization of GlyT1 protein in DRG. However, the functional role of GlyT1 in the PNS remains unclear. Since GlyT1 seems to be co-localized with NR1, i.e., the subunit binding glycine of the NMDA receptor, it is conceivable that, similar to its function in the CNS, GlyT1 modulates glutamatergic transmission by regulating the glycine concentration at NMDA receptors [14]. Whether NMDA receptors expressed at DRG neuron somata receive synaptic input or whether they participate in chemical neurotransmission between DRG neurons is unclear. Furthermore, GlyT1 and NMDA receptors might also co-express at the spinal terminals of DRG neurons. These questions require further future research.

Numerous studies have reported changes in expression of neurotransmitter transporters in pathological pain. For GABA transporters, however, the results appear inconsistent. Here both an up-regulation [17] as well as a significant down-regulation [18,19] of the GABA transporter GAT-1 has been described in the context of neuropathic pain. Also the spinal expression of the glutamate/aspartate transporter (GLAST) and glutamate transporter GLT-1 was differentially regulated in animal models of neuropathic pain [20,21].

Our analysis did not reveal expression changes of GlyT1 and GlyT2 protein levels in the spinal cord following neuropathic pain. In addition, GlyT1 expression was not altered in the DRG. Hence, regulation of GlyT expression does not seem to contribute significantly to the increased pain sensitivity in neuropathic pain. Whether altered expression of GlyT at earlier or later time points or changes of GlyT activity contribute to the state of hyperexcitability in chronic pain remains unknown.

While NR1 upregulation in the spinal cord is known to contribute to pathological pain [7,22] and furthermore, NR1 expression in the spinal cord has been shown to be reduced by continuous administration of the GlyT1-inhibitor ALX5407 [23], little is known about possible expressional changes of NR1 in DRG. Interestingly, selective knockout of NR1 in DRG was shown to lead to hyperexcitability in DRG neurons and hence to mechanical and thermal hypersensitivity [24], suggesting a complex and possibly antinociceptive effect of NMDA receptor activation in primary sensory neurons. In our study, however, expression of NR1 remained unchanged in DRG of neuropathic animals, while we did not investigate spinal NR-1 expression.

In conclusion, our study provides first evidence for the abundance of GlyTs in peripheral nervous tissue. Although GlyT expression is not differently altered in neuropathic pain, peripheral GlyT might be an interesting target in pain therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2015.06.026

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