Lidocaine Metabolites Inhibit Glycine Transporter 1

A Novel Mechanism for the Analgesic Action of Systemic Lidocaine?

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ABSTRACT

Background: Lidocaine exerts antinociceptive effects when applied systematically. The mechanisms are not fully understood but glycineric mechanisms might be involved. The synaptic glycine concentration is controlled by glycine transporters. Whereas neurons express two types of glycine transporters, astrocytes specifically express glycine transporter 1 (GlyT1). This study focuses on effects of lidocaine and its major metabolites on GlyT1 function.

Methods: The effects of lidocaine and its metabolites monomethylglycinexylidide (MEGX), glycinexylidide, and N-ethylglycine on GlyT1 function were investigated in uptake experiments with [14C]-labeled glycine in primary rat astrocytes. Furthermore, the effect of lidocaine and its metabolites on glycine-induced currents were investigated in GlyT1-expressing Xenopus laevis oocytes.

Results: Lidocaine reduced glycine uptake only at toxic concentrations. The metabolites MEGX, glycinexylidide, and N-ethylglycine, however, significantly reduced glycine uptake (P < 0.05). Inhibition of glycine uptake by a combination of lidocaine with its metabolites at a clinically relevant concentration was diminished with increasing extracellular glycine concentrations. Detailed analysis revealed that MEGX inhibits GlyT1 function (P < 0.05), whereas N-ethylglycine was identified as an alternative GlyT1 substrate (EC50 = 55 μM).

Conclusions: Although lidocaine does not function directly on GlyT1, its metabolites MEGX and glycinexylidide were shown to inhibit GlyT1-mediated glycine uptake by at least two different mechanisms. Whereas glycinexylidide was demonstrated to be an alternative GlyT1 substrate, MEGX was shown to inhibit GlyT1 activity in both primary astrocytes and in GlyT1-expressing Xenopus laevis oocytes at clinically relevant concentrations. These findings provide new insights into the possible mechanisms for the antinociceptive effect of systemic lidocaine.

What We Already Know about This Topic

- Glycine, a major inhibitory neurotransmitter, is rapidly removed after release by glycine transporters, including type 1, expressed in astrocytes.
- Whether lidocaine, which reduces neuropathic pain, acts in part by inhibiting glycine transporter 1 has not been studied.

What This Article Tells Us That Is New

- In cultured astrocytes, lidocaine failed to alter glycine transporter 1 function, but its metabolites inhibited directly or competitively glycine transporter 1 at clinically relevant concentrations, suggesting a possible target for the analgesic action of lidocaine in neuropathic pain.

THE amino acid glycine is, besides γ-aminobutyric acid, the major inhibitory neurotransmitter in the central nervous system. In addition, glycine is involved in excitatory neurotransmission as obligatory coagonist of glutamate at N-methyl-D-aspartate (NMDA) receptors. The synaptic glycine concentration is tightly regulated by the Na+/Cl−...
dependent glycine transporters (GlyT). GlyT type 1 (GlyT1) is predominantly expressed in astrocytes and a subset of presumably glutamatergic neurons, whereas GlyT type 2 (GlyT2) is exclusively expressed in glycineric neurons. At glycineric synapses, GlyT1 removes glycine from the synaptic cleft, while GlyT2 mediates the reuptake of glycine into nerve terminals.

The role of inhibitory, especially glycineric, neurotransmission in pathologic pain is well documented. Spinal synaptic disinhibition is one of the proposed mechanisms of pathologically increased pain sensitivity, and restoring synaptic inhibition may serve to restore physiologic conditions. Likewise, inhibition of glycine transporters has evolved as a potential new treatment of neuropathic pain. In particular, increasing the extracellular level of glycine via blockade of GlyT1 has been proposed very recently as a potential therapeutic approach for chronic pain with cognitive impairment.

The local anesthetic lidocaine has been shown to exert effects in vivo that cannot be explained by its action on voltage-gated sodium channels. These effects comprise, among others, antinflammatory and antinociceptive properties. The significant antineuropathic effect of systemic lidocaine is also well established, although its mechanism remains elusive. Early evidence from in vitro investigations suggested a glycine-like action of lidocaine in the central nervous system. Furthermore, results from a recent study by our own group supported the hypothesis that at least part of the analgesic activity of systemic lidocaine might originate from a glycineric action by lidocaine or one of its metabolites.

Lidocaine is mainly metabolized hepatically by cytochrome P450 isoforms CYP1A2 and 3A4 in a sequential process of oxidative N-dealkylation, although only a small amount (10%) is excreted unchanged renally. Its major metabolites are monoethylglycinexylidide (MEGX), glycine, sarcosine, L-alanine, EG, laminin, poly-D-lysine and tetrazolium salt (XTT) were purchased in their highest available purity from Sigma-Aldrich (St. Louis, MO), Polyclonal antibodies against GlyT1 and GlyT2 originating from rabbit were described previously. Monoclonal mouse anti-glial fibrillary acidic protein and mouse antineuron-specific nuclear protein antibodies were purchased from Millipore (Billerica, MA). A goat antirabbit IgG antibody conjugated to horseradish peroxidase was used as secondary antibody in western blot analysis (Dianova, Hamburg, Germany). Secondary antibodies goat antirabbit and donkey antimouse (Alexa Fluor 488 and 592) for fluorescence microscopy were purchased from Invitrogen (Carlsbad, CA). Phosphate buffered saline without calcium and magnesium was obtained from Invitrogen. Trypsin/EDTA was purchased from Biochrom AG (Berlin, Germany).

Materials and Methods

Reagents

Lidocaine hydrochloride and the metabolites MEGX and GX were provided by AstraZeneca, Research and Development (Södertälje, Sweden). ALX5407, ALX1393, sarcosine, glycine, L-alanine, EG, laminin, poly-D-lysine and tetrazolium salt (XTT) were purchased in their highest available purity from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies against GlyT1 and GlyT2 originating from rabbit were described previously. Monoclonal mouse anti-glial fibrillary acidic protein and mouse antineuron-specific nuclear protein antibodies were purchased from Millipore (Billerica, MA). A goat antirabbit IgG antibody conjugated to horseradish peroxidase was used as secondary antibody in western blot analysis (Dianova, Hamburg, Germany). Secondary antibodies goat antirabbit and donkey antimouse (Alexa Fluor 488 and 592) for fluorescence microscopy were purchased from Invitrogen (Carlsbad, CA). Phosphate buffered saline without calcium and magnesium was obtained from Invitrogen. Trypsin/EDTA was purchased from Biochrom AG (Berlin, Germany).

Astrocyte, Microgial, and Oligodendrocyte Cell Culture

All animal experiments for this study were performed in accordance with the regulations of the local Animal Use and Care Committee (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Recklinghausen, Germany). To screen for GlyT expression in primary cultures of the central nervous system, cells were prepared from cerebral cortices of Wistar rats. In brief, rats were anesthetized using isoflurane (Abbott, Abbott Park, IL), decapitated, and the brains quickly removed. To avoid fibril or endothelial contamination, cortical tissue was carefully isolated from blood vessels and meninges, rinsed with Dulbecco’s modified Eagle medium (DMEM; Invitrogen), dissociated by trypsinization, and suspended in DMEM supplemented with 10% fetal calf serum (Invitrogen), penicillin (80 units/ml), and streptomycin (0.2 mg/ml). Dissociated cells were plated in 75 cm² culture flasks (Corning Incorporated Life Sciences, Lowell, MA). After 5 days, cultures were washed with DMEM to remove cellular debris and maintained until subconfluency. Cellular debris, microglia, oligodendrocytes, and their early precursor cells were then removed by shaking flasks overnight at 250 rpm at 37°C. The remaining cell population harvested from shaking flasks consisted of more than 98% primary rat astrocytes, as determined by immunochemical analysis using antibodies against glial fibrillary acidic protein. For the following glycine uptake experiments, astrocytes were detached by incu-
Neurons were then plated on dishes coated with 0.1 mg/ml for 5 min. Afterward, neurons were resuspended in astrocyte-total volume of 50 ml DMEM and spun down at 1,500 rpm. The reaction was stopped by adding 10 ml of DMEM medium containing 10% fetal calf serum and centrifuged again as described above. Cell pellets were carefully resuspended in 1 ml DMEM and filtered through a sterile nylon mesh (pore diameter: 30 μm). The filtered cell suspension was transferred to a new tube into a 7900 sequence detection system using TaqMan probes (Applied Biosystems). Afterwards, neurons were resuspended in astrocyte-conditioned medium prepared from cortical rat astrocyte cultures after a conditioning period of between 3 and 4 days. Neurons were then plated on dishes coated with 0.1 mg/ml poly-D-lysine and 3 μg/ml laminin.

**Cortical Neuron Preparation**

Pregnant Wistar rats were sacrificed by anaesthetization with isoflurane 15 days after conception, and embryos were removed with sterile technique. Embryonic cortices were removed, gently dissected into small pieces, and collected in DMEM. Following centrifugation (2,000 rpm/30 s), DMEM was discarded, and 10 ml of 0.05% trypsin/EDTA (Invitrogen) were added. The tissue was then incubated for 8 min at 37°C and 10% CO₂. The reaction was stopped by adding 10 ml of DMEM medium containing 10% fetal calf serum and centrifuged again as described above. Cell pellets were then resuspended in 1 ml DMEM and filtered through a sterile nylon mesh (pore diameter: 30 μm). The filtered cell suspension was transferred to a new tube into a total volume of 50 ml DMEM and spun down at 1,500 rpm for 5 min. Afterward, neurons were resuspended in astrocyte-conditioned medium prepared from cortical rat astrocyte cultures after a conditioning period of between 3 and 4 days. Neurons were then plated on dishes coated with 0.1 mg/ml poly-D-lysine and 3 μg/ml laminin.

**Quantitative Real Time Polymerase Chain Reaction**

RNA from cultured cells was purified using the RNeasy kit (Qiagen, Hilden, Germany) following the supplier’s protocols. Isolated RNA was reversely transcribed using the high capacity complimentary DNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Quantitative determination of gene expression levels was performed on an ABI 7900 sequence detection system using TaqMan® universal PCR master mix (Applied Biosystems). TaqMan® probes (Applied Biosystems) for GlyT1 (Assay ID: Rn01416529_m1) and GlyT2 (Assay ID: Rn01475607_m1) genes were selected. Conditions for quantitative real time polymerase chain reaction were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s. GAPDH (Assay ID: Rn01775763_g1) was used as a reference gene and relative gene expression levels were determined according to the comparative CT method. Each sample was measured in quadruplicate.

**Immunofluorescence Microscopy of Glycine Transporter Expression**

To confirm GlyT expression with immunofluorescence microscopy in primary rat astrocytes from the cortical brain, cells were detached by incubation with Accutase (Invitrogen) for 3 min at 37°C, counted, and replated on sterile 8-well Lab-Tek chamber glass slides (Nalge Nunc Int., Rochester, NY). After 24 h, cells were washed with phosphate buffered saline and fixed in 4% paraformaldehyde for 15 min at room temperature. After additional washing with phosphate buffered saline, samples were blocked and permeabilized with blocking buffer containing 1% bovine serum albumin, 3% of normal serum of the second antibody host species, and 0.05% saponine in phosphate buffered saline.

For immunostaining of frozen brain and spinal cord cryosections, 10 μm-thick sections were fixed in 4% paraformaldehyde for 15 min at room temperature and washed with phosphate buffered saline. Subsequently, cryosections were blocked and permeabilized for 1 h with blocking buffer containing 10% normal goat serum and 0.2% saponine in phosphate buffered saline.

Primary antibodies were diluted (1:500) in phosphate buffered saline (containing 1% bovine serum albumin for cell cultures, or 2% normal goat serum and 0.2% saponine for cryosections) and were applied by incubation overnight at 4°C. Secondary antibodies were diluted (1:1,000) in the same buffer and applied for 1 h (between 3 and 4 h for cryosections) at room temperature. Subsequently, glass slides were washed with phosphate buffered saline and mounted to coverslips with mounting medium (for cell cultures: Vectashield; Vector Laboratories Inc., Burlingame, CA; for cryosections: ProLong Gold antifade reagent with DAPI; Invitrogen), evaluated with a Leica DM LB fluorescence light microscope, and documented with a digital camera (Leica Microsystems, Wetzlar, Germany).

**Western Blot Analysis of Glycine Transporter Expression**

To analyze GlyT1 and GlyT2 protein expression, cells were lysed for 20 min in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP-40, 1 μM pepstatin, 0.1 μM phenylmethylsulphonylfluoride, 0.15 μM aprotinin, and 1 μM leupeptin). Lysates were centrifuged at 10,000 g at 4°C for 15 min, and the supernatants were harvested. The protein content was measured using the bicinchoninic acid assay (Pierce, Rockford, IL). Sodium dodecyl sulfate polyacrylamide gel electrophoresis separated equal amounts of protein (20 μg per lane), which were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ). In a transblot SD cell blotting was performed at 1 mA/cm² for 1h (Bio-Rad, Munich, Germany). The membrane was blocked for 2 h with 0.05% Tween 20 in phosphate buffered saline containing 4% bovine serum albumin and incubated with the primary antibodies overnight at 4°C. The secondary antibody was applied for 1 h at room temperature after washing with 0.05% Tween 20 in phosphate buffered saline. The membrane was washed in phosphate buffered saline with 0.05% Tween 20, and bound antibodies were visualized using the enhanced chemiluminescence system (Amersham Buchler, Braunschweig, Germany).
Glycine Uptake Analysis

Primary astrocytes were pretreated for 30 min at 37°C with glycine uptake buffer (120 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.5) alone as negative control. Additional samples contained an excessive concentration of cold glycine (10 mM) for saturation, ALX5407 and sarcosine as positive controls for GlyT1 inhibition. After additional mixing for 3 min the absorbance was measured spectrophotometrically at a wavelength of 450 nm. Resulting values (n = 4 for each condition) for absorbance of untreated controls were subsequently normalized and compared with all other treatments as a measure of metabolic activity.

Detection of Mitochondrial Metabolic Activity

For in vitro determination of mitochondrial metabolic activity as a marker of cell viability, we used the tetrazolium hydroxide (XTT) assay. XTT, a yellow tetrazolium salt, is cleaved to a soluble orange formazan dye, which can be measured by absorbance. To measure cell viability using XTT, prepared with ANOVA followed by post hoc Bonferroni correction (two-tailed testing) using Graph Pad Prism Software. P < 0.05 was considered significant.

Results

GlyT1 Is Differentially Expressed in Primary Central Nervous System Cell Cultures and Tissue

To screen for a primary cultured cell type that allows the analysis of pharmacological effects of lidocaine and its metabolites on GlyT1 function, we analyzed the expression of GlyT based on quantitative real time polymerase chain reaction in different cultured cortical central nervous system cell types. GlyT1 messenger RNA was strongly expressed in astrocytes, while expression levels were considerably lower in neurons and oligodendrocytes.
GlyT1 messenger RNA was barely detectable in microglial cells (fig. 1A). GlyT2-expression was not detectable in either investigated cortical cell type (data not shown). Consistent with these findings, western blot analysis showed robust protein levels of GlyT1 in cultivated astrocytes (fig. 1B), whereas GlyT2 protein was absent in astrocytes (fig. 1C). In addition, GlyT1 expression was found in immunofluorescence microscopy of rat astrocytes (fig. 1D), while GlyT2 was not detectable (fig. 1E).

To exclude that the GlyT1 expression in cultured cortical astrocytes resulted from a tissue culture artifact, we also investigated the expression of GlyT1 in cortical brain coronal sections of the parietal lobe by immunofluorescence microscopy. Here, GlyT1 immunoreactivity was observed in both astrocytes and neurons, as indicated by co-staining with glial fibrillary acidic protein and neuron-specific nuclear protein, respectively (fig. 2A and B). Similar results regarding astrocytes were found in spinal cord cryosections, especially in white matter regions, although neuronal expression of GlyT1 seemed to be less abundant (see Supplemental Digital Content 1, http://links.lww.com/ALN/A790, which is a figure displaying GlyT1 immunoreactivity in spinal cord cryosections).

**Lidocaine Metabolites Reduce Glycine Uptake in Primary Astrocytes**

[^14C]-labeled glycine incorporation assays using cultured cortical astrocytes revealed a high affinity uptake system in these cells. Consistent with an active transporter mediating these effects, [^14C]-labeled glycine incorporation was strikingly reduced by cooling to 4°C (physical inhibition; negative control), or preincubation with a high concentration of glycine. Moreover, glycine uptake activity was considerably reduced by incubation with known inhibitors like ALX5407 or the alternative GlyT1 substrate sarcosine, thus indicating that GlyT1 mediates the majority of the uptake activity. No significant reduction of glycine uptake was found after treatment with the GlyT2 inhibitor ALX1393 (fig. 3A).

Treatment with lidocaine using clinically relevant (1–10 μM) and higher concentrations (100 μM) did not influence glycine uptake. Only a very high and therefore cytotoxic concentration (1 mM) led to a moderate reduction of glycine uptake (fig. 3B). In contrast, all three investigated metabolites of lidocaine led to a significant dose-dependent decrease of glycine uptake (fig. 3B). Evaluation of mitochondrial metabolism with XTT assay and cell morphology by light microscopy indicated no reduction of metabolism and cell viability by all investigated substances and concentrations (data not shown), except with lidocaine at the highest applied concentration (1 mM), which led to a reduction of mitochondrial activity by 24 ± 2% (P < 0.05) compared with untreated controls. Therefore, cytotoxic effects can be ruled out as the mechanism of glycine uptake reduction in our experiments.
Glycine Uptake Inhibition by Lidocaine in Combination with Its Metabolites Depends on Extracellular Glycine Concentration

To investigate the relationship between extracellular glycine concentration and uptake inhibition by lidocaine in combination with its three major metabolites, increasing concentrations of glycine were used (fig. 4). For these experiments, a clinically relevant concentration of lidocaine (4 μM) was combined with a fixed concentration ratio of its metabolites (MEGX 2.5 μM, GX 0.3 μM, EG 30 μM) to mimic the clinical situation, especially after continuous application of lidocaine.32–34 At the lowest glycine concentration (10 μM), glycine uptake was very low and therefore no inhibition could be detected either with ALX5407 as the positive control or with the combination of lidocaine and the three major metabolites. With increasing glycine concentrations (25–100 μM), glycine uptake increased considerably (fig. 4A), whereas the degree of inhibition by ALX5407 decreased from 69 to 40% (P < 0.05; fig. 4B). Similarly, the degree of glycine uptake inhibition by lidocaine combined with metabolites decreased significantly from 43 to 27% (P < 0.05; fig. 4B).

Lidocaine Metabolites Inhibit Glycine Uptake


Fig. 2. Immunofluorescence microscopy of glycine transporter 1 (GlyT1) expression in rat brain cortex coronal cryosections. (A) GlyT1-related signals were found in the most superficial areas of the cortex at the parietal lobe, predominantly in cells that stained positive for glial fibrillary acidic protein, a specific marker for astroglial cells. (B) Less superficial, most GlyT1-related fluorescence was found in cells that sustained positive for neuron-specific nuclear protein. Note that digital overlays (Merge) demonstrate the colocalization of astrocytes and neurons with GlyT1. Nuclei were stained using DAPI. Similar results from spinal cord cryosections can be found in Supplemental Digital Content 1, http://links.lww.com/ALN/A790. GlyT1 = glycine transporter 1; NeuN = neuron-specific nuclear protein.

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The Lidocaine Metabolite N-ethylglycine Is a Substrate of GlyT1

To study the effects of lidocaine metabolites on GlyT1 more directly and thus excluding possible indirect mechanisms, we investigated the effects of the respective substances on GlyT1 in Xenopus laevis oocytes. Here the application of glycine to oocytes expressing GlyT1 resulted in a typical concentration-dependent current response (fig. 5A). Consistent with these currents being exclusively mediated by GlyT1, they were efficiently inhibited by preincubation with 5 μM ALX5407. Moreover, application of sarcosine resulted in inward directed currents similar to those seen after glycine incubation. In noninjected oocytes, no currents were observed with any of the substances applied here.

Lidocaine, MEGX, and GX (each 33 μM and 100 μM) did not lead to a detectable response (data not shown), whereas EG elicited a glycine-like response in a concentration-dependent manner, suggesting that this substance is an alternative GlyT1 substrate (fig. 5A). Similar to glycine-induced currents, the preincubation of the oocytes with ALX5407 resulted in a complete inhibition of EG and sarcosine elicited currents (fig. 5A).

Analysis of concentration-response relationship of EG on GlyT1 resulted in an EC50 of 55 μM (CI 95%: 49, 62 μM) and a shift of the sigmoid concentration-response curve to the right compared with glycine (EC50 27 μM; CI 95%: 23, 31 μM), which was evaluated in the same experimental setup (fig. 5B). EG-induced currents were compared with those of sarcosine, which is known as a naturally occurring inhibitor of GlyT1. At equimolar concentrations, similar GlyT1-mediated currents were observed (fig. 6A), indicating that both
substances act as substrates of GlyT1 and thereby might inhibit glycine uptake. Finally, simultaneous incubation with EG and glycine increased glycine-induced currents in an under-additive and concentration-dependent manner, as would be expected when combining two regular substrates of a transporting system (fig. 6B).

The Lidocaine Metabolite MEGX Has a Direct Inhibitory Effect on GlyT1

In order to identify direct GlyT1-inhibiting effects, glycine-induced currents were recorded before and after incubation with each metabolite of lidocaine for 30 min. Glycine-induced currents (33 μM) were reduced to 63 ± 11% by incubation with MEGX (33 μM) for 30 min (P < 0.05), indicating a direct inhibitory effect of MEGX on GlyT1 (fig. 7A). After thorough washout of MEGX (60 s), glycine-induced currents remained at 58 ± 9% of previous glycine response (P < 0.05). In contrast, incubation with GX (33 μM) did not alter glycine-induced currents (fig. 7B).

Discussion

Aim of the study was to investigate the effect of lidocaine and its major metabolites on glycine transport in vitro to elucidate a possible mechanism by which lidocaine could exert its antinociceptive effects in vivo. Therefore, we first confirmed the predominant expression of functional GlyT1 in rat cortical astrocytes, whereas GlyT2 was barely detectable in this cell type. Furthermore, GlyT1 was found to be expressed not only in astrocytes, but also in neuronal cells of the cerebral cortex. In contrast, expression of GlyT2 was not detected in neuronal cells from the rat cortex, which is in line with previous investigations.

Known inhibitors of GlyT1 diminished glycine uptake in primary astrocytes almost completely, whereas GlyT2 inhibition had no effect on glycine uptake. These results indicate that the glycine uptake in astrocytes is predominantly mediated by GlyT1. The local anesthetic lidocaine did not exert significant effects on glycine uptake in vitro at clinically relevant concentrations. Only at toxic concentrations of lidocaine was glycine uptake slightly diminished. In contrast, all investigated metabolites of lidocaine dose-dependently reduced the GlyT1-mediated uptake by up to 60% at concentrations known to occur after systemic application. In line with the results obtained with glycine uptake assay, data from electrophysiological experiments suggest that lidocaine itself has no effect on GlyT1, although its metabolite MEGX reduces GlyT1 function as demonstrated by inhibition of glycine-induced currents. EG, which is structurally similar to the known GlyT1-inhibitor sarcosine, was shown to be a hitherto unknown substrate for GlyT1.

Numerous effects of lidocaine have been described that cannot be explained by its principal mechanism of action, i.e., the blockade of voltage-gated sodium channels. In vitro
investigations suggest lidocaine-mediated effects among others on Gò protein function, NMDA receptors, thromboxane A2 signaling, and glycineric neurotransmission. Clinically, intravenous lidocaine exerts a variety of effects, some of which are of benefit in perioperative settings. Thus, lidocaine and other local anesthetics have an increasingly broad spectrum of indications beyond analgesia and antiarrhythmic effects, most of which still remain insufficiently investigated.

Furthermore, systemic lidocaine has been shown to act antinociceptive in numerous investigations in animals and humans. The precise mechanism underlying this effect on nociception is still not well understood. In a previous investigation we could demonstrate that at least part of the analgesic effect is mediated by glycineric mechanisms and, consequently, possibly by NMDA receptor signaling.

During continuous systemic or epidural application of lidocaine, unbound plasma levels of lidocaine and MEGX have been reported to remain stable at values of up to 1–6 μg/ml (4.3–25.6 μM) lidocaine and 0.5–0.6 μg/ml (2.4–2.9 μM) MEGX, whereas GX and EG have been reported to accumulate at least for the first 48 h to unbound plasma concentrations of 0.06–0.1 μg/ml (0.3–0.4 μM) and 2.5–3 μg/ml (25–30 μM) respectively. Therefore, especially with continuous application of lidocaine, unbound plasma concentrations of respective metabolites reach levels that lead to significant glycine transport inhibition in vitro.

GlyT regulate the glycine concentration in the synaptic cleft at glycineric inhibitory and glutamatergic excitatory synapses. At glycineric synapses, glycine is removed from the extracellular space by complementary activity of GlyT1-mediated uptake maintaining the glycine concentration below the saturation level of the glycine-binding site of the NMDA receptor.

Inhibition of the glycine transport is thought to have beneficial effects in patients with schizophrenia by indirectly increasing the glycine concentration at glutamatergic synapses and thereby restoring NMDA receptor activation. Therefore, GlyT1 seems to play a crucial role in regulating NMDA receptor function. Accordingly, GlyT1 is present in the vicinity of synapses.
NMDA receptors, which are known to be critically involved in nociception, especially in the context of pathologic pain.49–52 Interestingly, glycine transporter inhibitors have been demonstrated to reduce pain in different in vivo models of neuropathic pain. GlyT1 has been suggested to lower extracellular glycine concentration at glycinergic synapses.23,53 This would be in line with the notion that inhibition of GlyT1 function, leading to accumulation of glycine at the

Fig. 6. (A) Glycine transporter 1 (GlyT1)-mediated currents recorded in *Xenopus laevis* oocytes in response to application of N-ethylglycine (EG) and sarcosine for 30 s were compared. (B) To investigate the additive effect of EG and glycine at GlyT1, EG was applied to *Xenopus laevis* oocytes expressing GlyT1 in mounting concentrations alone or in combination with a fixed concentration of glycine (33.3 μM). Simultaneous application of EG and glycine further increased inducible GlyT1-mediated currents compared with glycine application alone. This under-additive effect further underlines that both substrates compete for transport capacity at GlyT1, as expected for regular substrates. An asterisk indicates a significant difference compared with glycine-induced currents (*P* < 0.05). Values are given as a fraction of glycine-induced currents (33.3 μM). Data are presented as mean ± SD (n = 6).

Fig. 7. Effect of lidocaine metabolites on glycine transporter 1 (GlyT1)-mediated currents. To identify direct GlyT1-inhibiting effects of the lidocaine metabolites monoethylglycinexylidide (MEGX) and glycine (GX) in *Xenopus laevis* oocytes expressing GlyT1, glycine-induced currents were recorded immediately during simultaneous application (first column), after pretreatment of the same oocytes with MEGX and GX respectively for 30 min (second column), and after thorough washout (60 s) of lidocaine metabolites (third column). Whereas MEGX (33.3 μM) led to a significant and prolonged reduction (A), GX (33.3 μM) did not alter glycine-induced currents (B). An asterisk indicates a significant difference compared with initial glycine-induced currents (*P* < 0.05). Values are given as a fraction of initial glycine-induced currents (33.3 μM). Data are presented as mean ± SD (n = 6).
site of inhibitory glycine receptors, can ameliorate pathologic pain. In fact, a study in mice demonstrated that intrathecal strychnine–induced dynamic alldynia was reduced by intrathecal sarcosine,54 supporting the ability of GlyT1 inhibition to enhance glycineergic inhibition. Furthermore, in neuropathic rats after spared nerve injury, GlyT1 inhibition by oral or intrathecal sarcosine has been shown to be effective in reducing pathologic mechanical sensitivity. The authors of this study concluded that inhibition of GlyT1 at multiple central sites induces acute analgesia, as well as acute and long-term reduction in neuropathic pain behavior.11 In addition to that, Tanabe et al. found that an increase of endogenous glycine concentration following GlyT1 inhibition not only leads to inhibition of pain transmission at the spinal level but also to protection against impairment of hippocampal long-term potentiation. Therefore, the authors concluded that GlyT1 inhibition might also be capable of ameliorating the cognitive disturbances often observed in patients with chronic pain.9 However, although numerous further studies point to the importance of GlyT1 inhibition as a potential treatment option for chronic pain therapy,8–10,55 several other studies also underline the relevance of GlyT2 inhibition.8,54,56

Although in our study, the lidocaine metabolite MEGX reduced GlyT1 function and EG was found to inhibit glycine uptake as a novel substrate of GlyT1, the effects on GlyT2 remain unknown. According to our results, a possible mechanism of lidocaine-mediated antinociceptive effects is the increase of extracellular glycine concentration, resulting in enhanced activity at inhibitory glycine synapses by inhibition of GlyT1-mediated glycine reuptake. However, it is also conceivable that an increase of glycine concentration may also enhance the activation of excitatory glutamatergic synapses by local transporter inhibition or neurotransmitter spillover.57 In summary, the direct consequences of a general or selective inhibition of glycine transporters with respect to resulting glycine concentrations and activity of inhibitory and excitatory synapses still remain unclear and need to be further elucidated.

Direct comparison of the chemical structures of GlyT1 inhibitors, like sarcosine, with the molecular structures of lidocaine and its metabolites shows an intriguing similarity. As might have been expected as a result of this structural similarity, we could show that the lidocaine metabolite EG acts as a substrate of GlyT1, whereas MEGX directly interferes with GlyT1 function. Correspondingly, increasing extracellular glycine concentrations led to a reduced glycine transport inhibition by a combination of lidocaine metabolites. The fact that the lidocaine metabolite GX alone induced a significant decrease in glycine uptake in astrocytes, although it did not have an effect on glycine-induced currents, may be attributed to indirect effects on glycine uptake, although this remains speculative.

Hitherto, various explanations for the antinociceptive activity of systemic lidocaine, including both central and peripheral mechanisms, have been discussed.15 Although there is evidence suggesting that systemic lidocaine may have beneficial effects on pathologic pain by suppression of ectopic firing,58,59 the exact mechanism still remains unclear.60 Until now, very few investigations have been conducted to clarify whether lidocaine might exert some of its antinociceptive activity through glycineergic pathways. In contrast to our hypothesis that systemic lidocaine or its metabolites act indirectly through enhancing glycineergic activity by GlyT1 inhibition, another study has proposed that lidocaine and also procaine directly activate glycine and distinct γ-aminobutyric acid receptors mediating the observed antinociceptive effect.61 Whether or not these two proposed glycineergic mechanisms possibly act in concert with each other to produce an antinociceptive effect remains to be clarified.

Of note, various other effects of systemic lidocaine application have been described for the central nervous system.62 Neuropsychiatric adverse effects included dysphoria, depressive mood with or without paranoid ideation, agitation with hallucinations, dizziness, light headache or drowsiness, perioral or limb paresthesia, visual disturbances, confusion, disorientation, and cognitive dysfunction. Since these undesired effects were most likely induced by lidocaine itself or its sodium channel-blocking metabolites MEGX and GX, when aiming for glycineergic enhancement, application of metabolites like EG, which has no relevant effect on sodium channels, could be similarly effective, but associated with a lower probability of inducing undesired effects.

In conclusion, the major lidocaine metabolites, but not lidocaine itself, inhibit the GlyT1-mediated uptake of glycine. While MEGX was found to inhibit glycine-induced response of GlyT1, EG was found to interfere by acting as a previously unknown substrate for GlyT1. This effect can be observed at concentrations of EG that regularly occur systemically during epidural anesthesia or therapeutic systemic application. Furthermore, a combination of lidocaine and its metabolites, mimicking the clinical situation in which the parent drug and the metabolites are present simultaneously, even increases the observed effects. Since inhibition of GlyT1 has been shown to significantly reduce hyperalgesia in a variety of pathologic pain states in vivo, glycine transporter inhibition by lidocaine metabolites might provide a novel molecular mechanism for the well-established antinociceptive effect of systemic lidocaine. However, whether the pharmacological effects observed in our in vitro investigation account for the antinociceptive effect of systemic lidocaine in vivo needs to be further elucidated.

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