Glycine transporter inhibitors: a new avenue for managing neuropathic pain

Mahmoud Al-Khrasani*, Amir Mohammadzadeh, Mihály Balogh, Kornél Király, Szilvia Barsi, Benjamin Hajnal, László Köles, Zoltán S. Zádori, Laszlo G. Harsing, Jr.

Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, Semmelweis University, Nagyvarad ter 4, P.O. Box 370, H-1445 Budapest, Hungary

Corresponding author.
E-mail address: al-khrasani.mahmoud@med.semmelweis-univ.hu (MAl-K)

Keywords: Neuropathic pain, Spinal cord, NMDA receptors, Glycine transporters, Glycine transporter inhibitors

ABSTRACT

Interneurons operating with glycine neurotransmitter are involved in the regulation of pain transmission in the dorsal horn of the spinal cord. In addition to interneurons, glycine release also occurs from glial cells neighboring glutamatergic synapses in the spinal cord. Neuronal and glial release of glycine is controlled by glycine transporters (GlyTs). Inhibitors of the two isoforms of GlyTs, the astrocytic type-1 (GlyT-1) and the neuronal type-2 (GlyT-2), decrease pain sensation evoked by injuries of peripheral sensory neurons or inflammation. The function of dorsal horn glycinergic interneurons has been suggested to be reduced in neuropathic pain, which can be reversed by GlyT-2 inhibitors (Org-25543, ALX1393). Several lines of evidence also support that peripheral nerve damage or inflammation may shift glutamatergic neurochemical transmission from N-methyl-D aspartate (NMDA) NR1/NR2A receptor- to NR1/NR2B receptor-mediated events (subunit switch). This pathological overactivation of NR1/NR2B receptors can be reduced by GlyT-1 inhibitors (NFPS, Org-25935), which decrease excessive glycine release from astroglial cells or by selective antagonists of NR2B subunits (ifenprodil, Ro 25-6981). Although several experiments suggest that GlyT inhibitors may represent a novel strategy in the control of neuropathic pain, proving this concept in human beings is hampered by lack of clinically applicable GlyT inhibitors. We also suggest that drugs inhibiting both GlyT-1 and GlyT-2 non-selectively and reversibly, may favorably target neuropathic pain. In this paper we overview inhibitors of the two isoforms of GlyTs as well as the effects of these drugs in experimental models of neuropathic pain. In addition, the possible mechanisms of action of the GlyT inhibitors, i.e. how they affect the neurochemical and pain transmission in the spinal cord, are also discussed. The growing evidence for the possible therapeutic intervention of neuropathic pain by GlyT inhibitors further urges development of drugable compounds, which may beneficially restore impaired pain transmission in various neuropathic conditions.

Contents

1. Introduction

Despite the large developments in chronic pain medication, neuropathic pain management is still unsolved. The currently used medications are categorized into three groups: first, second, and third-line drugs (Dworkin et al., 2010). First-line medications include: tricyclic antidepressants, dual reuptake inhibitors of serotonin and norepinephrine, gabapentinoids, and lidocaine transdermal patch. Second-line drugs are classical opioids and drugs having both opioid and non-opioid actions like tramadol. Third-line medications include antiepileptics, topical capsaicin preparations, memantine, and mexiletine.

Neuropathic pain exists in different forms and pathomechanisms, in which the role of glycinergic neurotransmission becomes more and more important based on studies of the past few years (Carland et al., 2014; Zafra et al., 2016). The wide distribution of glycine in the central nervous system turned the attention to the role of glycinergic neurotransmission in pain-mediation encouraging researchers to find novel glycine-based analgesics for the treatment of neuropathic pain. Recently, series of compounds altering glycine transporters have been reported to affect the transduction of neuropathic pain. Inhibitors of glycine transporter type 1 and type 2 (GlyT-1 and GlyT-2), which have primarily roles in regulation of synaptic and non-synaptic glycine concentration, are a new promising way in this respect. In fact, inhibitors of both transporters have been proven to display analgesic action in various experimental conditions (Vandenberg et al., 2014). In this review, we will focus on glycine transporters emerging as novel targets to control neuropathic pain. Recently, Dohi and coworkers (2009) have published an excellent review on the role of GlyTs in pain transmission.

2. Background on pain management

Pain is considered as one of the most ancient health dilemmas that mankind faced with and so far has not been satisfactory solved, especially in the case of chronic neuropathic pain. (Debono et al., 2013). Clinicians commonly face to the problem that the most commonly applied, high efficacy analgesics (e.g. opioids or local anesthetics) show significantly lower efficacy in chronic neuropathic conditions, compared with other pain types (Shaqura et al., 2013; Borbély et al., 2017; Végh et al., 2017; Balogh et al., 2019). The beginning of pain management was based on

sorcery, seeking the help of monks and use of natural products. Egyptians, Chinese, ancient Greek and Roman physicians had achieved a great success in controlling pain by utilizing natural products. However, the breakthrough in pain controlling was the discovery and isolation of opioids by German pharmacist assistant, Friedrich Wilhelm Sertürner in the 19th century (Schmitz, 1985). It was followed by the discovery of other drugs e.g. non-steroidal anti-inflammatory drugs (NSAIDs). At that time, various plants or plant extracts were used to relieve pain, fever, and inflammation. In the mid-19th century salicylates were characterized resulting in discovery of acetylsalicylic acid or Aspirin, considered as a prototype of NSAIDs and as a turning point in development of the current NSAIDs available for clinical use. Despite the development of numerous medications controlling acute, moderate to severe pain, management of neuropathic pain is not solved yet. It is considered as an unmet medical need, affecting 2-3% of the population in developed countries (Field et al., 1999). According to International Association for the Study of Pain (IASP, 2012), neuropathic pain is defined as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system". Based on this definition, it is a result of harms to neurons in peripheral and central nervous system or both. Generally, pain affects 20% of European population (80 million citizens/month) and only in the United States approximately 100 million people are suffering from some kind of chronic pain (Breivik et al., 2006; Debono et al., 2013). This number has exceeded the total number of people with diabetes, heart disease, and cancer. In spite of the availability of pharmacological pain remedies, neuropathic pain usually does not respond well to the current medications. So far, medical management of neuropathic pain is largely based on the symptoms of the disease. It should be kept in mind that less than 50% of patients with neuropathic pain respond to treatment with the currently available drugs, 40% are inadequately treated and 30% do not respond to the treatment (Torrance et al., 2006; Moulin et al., 2014; Van Hecke et al., 2014; Zeng et al., 2017).

3. Pain transmission

Pain transmission from the periphery to the central nervous system is carried out by sensory afferent fibers that are composed of primary, secondary and tertiary ones. Pain sensation moves from the primary to the secondary then to the tertiary fibers via chemical junction points located in the spinal cord and the thalamus,

respectively (Bourne et al., 2014). Aβ, Aδ and C fibers have two terminals, one located in the periphery and the other in the dorsal horn of the spinal cord and named as peripheral (e.g. dorsal root ganglion; DRG) and central terminals of sensonry neurons. Nociceptors are sensory (pain) receptors located on the peripheral ending of primary afferents. Aβ fibers are stimulated by non-noxious mechanical stimuli of low thresholds and are described as light-touch receptors. On the other hand, Aδ and C fibers are stimulated by mechanical, thermal or chemical noxious stimuli. C-fibers are stimulated by multiple modalities including chemical, mechanical (touch, pressure, stretch) and thermal stimuli (Bourne et al., 2014). Pain has both somatosensory and emotional aspects. The transmission of the former is described above, whereas the latter aspect is conveyed by the spinoreticular tract terminating in the reticular formation in the brainstem, where information is further processed to the thalamus and the hypothalamus. Besides the ascending pain pathway, the descending (or inhibitory) pathway is also important in pain processing and pain sensation. Glutamatergic system is of utmost importance for travelling pain from the primary afferent neurons to the secondary afferent neurons at the spinal dorsal horn, because the majority of these primary fibers release glutamate. Besides primary afferent, central terminal glutamate is also a substantial transmitter of prevailing majority of excitatory spinal dorsal horn interneurons (Todd et al., 2003). In neuropathic pain, continuous firing of C-fibers results in a sustained glutamate release, which likely stays beyond the phenomenon called central sensitization indicated by allodynia and hyperalgesia (Neumann et al., 1996; Sandkühler, 2009; Hua and Cabot, 2010). In order to halt chronic pain transmission by drugs influencing the glutamatergic system at this crucial pain traffic point (spinal cord), there are two important mechanisms that could be of great analgesic effects. The first mechanism is to inhibit N-methyl-D-aspartate (NMDA) receptors. This pharmacological intervention has already gained significant clinical attention, since ketamine in subanaesthetic doses has been reported to produce analgesic action in neuropathic pain when it is used acutely or chronically (Leung et al., 2001; Kvarnström et al., 2003; Okie, 2010). However, ketamine and ketamine-like drugs have been proven to cause central side effects such as hallucination, agitation, and panic attacks that largely limit their use (Niesters et al., 2014; Bell and Kalso, 2018). The second mechanism could be to facilitate the active removal of glutamate in the dorsal spinal cord pain synapses upon the robust release.

The evidence mentioned above suggests that the glutamatergic system likely plays a significant role in pain sensation, though the underlying mechanisms are quite complex. In addition, we should also consider that glutamatergic neurons are in tight connection with a number of glycinergic interneurons in spinal dorsal horn.

Herein, we review the impact of novel drugs, that could affect glutamatergic tone at synaptic sites and thereby damping travelling of pain sensation resulted from continuous firing of primary afferent sensory neurons subjected to tissue injury.

4. Neural circuitry in the spinal dorsal horn that mediates neuropathic pain

The dorsal root ganglia contain the cell bodies of the primary afferent neurons (Aβ, Aδ and C), which differ in myelination and sensory information transmitted from the periphery into the spinal cord. The myelinated Aβ fibers process non-noxious, mechanical (superficial light touch, pressure) sensory inputs and project into laminae II and III of the dorsal horn. The central terminals of Aβ fibers operate with glutamate as a neurotransmitter and establish synaptic connections with a number of excitatory and inhibitory interneurons at the spinal dorsal horn (Fig. 1). A subgroup of excitatory interneurons in lamina II expresses protein kinase Cγ (PKCγ) immunoreactivity. These interneurons receive excitatory innervation from Aβ fibers and inhibitory inputs from the parvalbumin positive glycinergic cells and in turn, they process stimulatory inputs to the transient central excitatory interneurons in lamina II (Lu et al., 2013; Vandenberg et al., 2014; Zafra et al., 2016). After nerve injury, parvalbumin-containing glycinergic cell-PKCγ cell synapses are impaired in function (Petitjean et al., 2015). In addition, decrease in parvalbumin-containing cell activity or increased number of PKCγ cells leads to the development of spontaneous pain, hyperalgesia, and allodynia (Petitjean et al., 2015). Aβ fibers also establish synaptic connections with inhibitory glycinergic interneurons in lamina III, which then synapse with excitatory interneurons in laminae I and II. Their inhibitory effect on excitatory interneurons is believed to be critical in blocking flow of sensory information from Aβ fibers to pain-transmismitting neurons in lamina I.

The inhibitory transmission in the spinal dorsal horn, which is mediated by glycinergic and glycinergic/GABAergic neurons, has been increasingly recognized as a key player in the development of neuropathic pain. The principal neurotransmitter in this inhibition is glycine, which has a double neurotransmitter role in the central nervous system, influencing not just inhibitory neurotransmission but, as a coagonist

of NMDA receptors, the excitatory glutamatergic neurotransmission as well. Excitation in the dorsal horn of the spinal cord is mediated via glutamate receptors, which are discussed below.

5. Ionotropic N-methyl-D-aspartate (NMDA) glutamate receptors and their presence in the spinal dorsal horn

There are three major types of ionotropic glutamate receptors (iGluRs): N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors, named after their selective agonists. These receptors share common structural features (e.g. tetrameric architecture with each subunit consisting of an extracellular amino-terminal domain, an extracellular ligand binding domain, four transmembrane domains, and an intracellular carboxyl-terminal domain) placing them into one superfamily (i.e. ionotropic glutamate receptors; others are the cys-loop receptors e.g. nicotinic acetylcholine, 5-HT_3 serotonin, $GABA_A$ and glycine receptors as well as the superfamily of ATP-gated channels). Of the iGluRs, NMDA receptors are composed of three classes of subunits (NR1, NR2A-D, NR3A-B) and their combinations form receptors consising of four subunits (Danysz and Parson, 1998). Three major populations of NMDA NR1/NR2 receptors are present in the spinal dorsal horn neurons: NR2A, NR2B, and NR2D subunits are commonly expressed, whereas NR2C subunit is expressed in a lesser extent in neurons (Karlsson et al., 2002).

A peculiar nature of the NMDA receptor is that its activation requires simultaneous binding of the agonist glutamate to NR2 subunit and the coagonist glycine or D-serine to NR1 subunit (Johnson and Ascher, 1987). NMDA receptors differ in affinity of the two coagonists to the receptors. Therefore, receptor activation is dependent on the coincident agonist and coagonist binding and postsynaptic depolarization (as it relieves the Mg^{2+} blockade - Mg^{2+} largely blocks the NMDA receptor channels at resting membrane potential). These characteristics of NMDA receptor activation are critically involved in its (patho)physiological roles (Köles et al., 2016).

Based upon the subunit composition, NMDA receptors exhibit different locations at synaptic levels and coagonist affinity. The current common view is that NR1/NR2A receptors are located in the pre- and postsynaptic membranes within the synapses and D-serine is the possible coagonist for this subunit composition (Petrenko et al., 2003b; Raiteri and Raiteri, 2010) (Fig. 2). The NR1/NR2B receptors are located primarily extrasynaptically and the coagonist for these receptors is glycine released from neighboring astrogial cells (Zhou et al., 2013; Shibasaki et al., 2017; Pal, 2018). For activation of extrasynaptic NR1/NR2B receptors, glutamate may reach these receptors by spillover mechanism from glutamatergic synapses after its neural release (Wild et al., 2015). It needs to mention that segregation of NMDA receptors as synaptic NR1/NR2A and extrasynaptic NR1/NR2B receptors bears some contradiction as expression of both NR1/NR2A and NR1/NR2B receptors within the synapses was also considered (Paoletti et al., 2013; Pal, 2018).

6. The pivotal role of spinal NMDA receptors and their subunits in neuropathic pain

NMDA receptors in the neural circuitry of the spinal dorsal horn are particularly well characterized since glutamate is one of the neurotransmitters in the non-myelinated C fibers as well as the myelinated Aδ and Aβ fibers. Both excitatory and inhibitory interneurons, which receive glutamatergic innervation from these fibers, express NMDA receptors in the synapse and the extrasynaptic space. In chronic pain states, activation of the extrasynaptic NR1/NR2B receptors occurs as glutamate accumulates extrasynaptically and they have a major influence on mediation of pain transmission (Petrenko et al, 2003a; Vizi et al, 2013). NMDA receptors also participate in neural communications of most glutamatergic excitatory interneurons (PKCγ expressing cells, transient central neurons, vertical cells) localized in spinal laminae I and II (Fig. 1).

Expression of NMDA receptor subunits in the spinal cord has been extensively studied following induction of neuropathic pain by constriction injury of peripheral nerves or in inflammation elicited by formalin or complete Freund's adjuvant (CFA) injection. Yang and coworkers (2009) induced inflammatory pain in mice and found that the total protein level of the NMDA receptor subunit NR1 was not changed in the spinal dorsal horn. NR1 immunoreactivity at synapses was, however, increased by inflammatory pain and it was concluded that increased expression of NR1 at synapses

correlates with development of mechanical allodynia. Roh and coworkers (2008) reported increased number of NR1 and phosphorylated NR1 immunoreactive neurons in laminae I-VI of dorsal horn in rat neuropathic pain model in a time period of 1 to 28 days post surgery. Schlösser and coworkers (2015) reported no alteration in NR1 protein expression (immunofluorescence microscopy) in the dorsal root ganglia of animals with neuropathic. Barthel and coworkers (2014) used the sciatic nerve chronic constriction injury model of pain in rats and found that expression of NR1 subunit (Western blot analysis) was reduced by adding the GlyT-1 inhibitor ALX5407, but not by the GlyT-2 inhibitor ALX1393, whereas both types of GlyT inhibitors acted antineuropathic.

Spinal central terminals of A and C fibers, which synapse to dorsal horn neurons, contain NMDA receptors with NR1/NR2A and NR1/NR2B subunit compositions (Petrenko et al., 2003b). Determination of the occurrence of the various NMDA receptor subunits in the spinal cord indicated that NR2B subunits exhibit the largest expression followed by decreasing proportions of NR2C, NR2A, and NR2D subunits, respectively. In adult mouse spinal cord, NR2A subunits extend to all laminae except for the lamina II, whereas expression of NR2B subunit was preferentially found in that layer (Western blot analysis, Petrenko et al, 2003a).
Karlsson and coworkers (2002) used single cell PCR and reported reduced number of neurons expressing the NR2A subunits following spinal nerve injury in rats. This decrease in NR2A subunits also resulted in an elevated contribution of NMDA receptors containing NR2B and NR2D subunits in neuropathic pain conditions. In the experiments of Petrenko and coworkers (2003b), NR2A subunit gene deletion did not significantly influence nociceptive behavior in nerve-injured and inflammatory neuropathic pain models. This finding suggests that neuropathic pain with various mechanisms can be elicited even in the absence of NR2A subunits in the spinal cord.

Several lines of evidence indicate that NR2B subunits undergo long-term plastic changes in the spinal cord after peripheralnerve injury. In an inflammatory pain model evoked by intraplantar injection of CFA, Yang and coworkers (2009) found an increase in synaptic expression of NR2B subunit, whereas the expression level of NR2A subunit was not altered in mouse spinal dorsal horn. Wang and Yu reported (2016) that bone cancer-induced pain behavior was associated with upregulation of

NR2B protein expression in the spinal cord 5 to 14 days post inoculation of sarcoma cells (Western blot analysis). Geng and coworkers (2010) ligated the L5 spinal nerve in rats and mechanical allodynia was assessed by measuring paw withdrawal threshold. It was found that mechanical allodynia developed in parallel with the increase of brain-derived neurotrophic factor (BDNF) concentrations and activated NR2B subunit was involved in maintenance of persistent pain. It is a tempting assumption, whether increased expression of the extrasynaptic NR1/NR2B receptors is dependent on overproduction of BDNF in spinal neuropathy. In the experiments of Hildebrand and coworkers (2016), BDNF mediated NMDA receptor potentiation through activation of tropomyosin receptor kinase B (TrkB) and phosphorylation of NR2B subunits by Fyn kinase in a peripheral nerve injury model.

Alteration of NR2B subunit expression in neuropathic pain conditions has been reported not only at spinal cord levels but also in supraspinal brain areas (central sensitization). Wu and coworkers (2005) reported that NR2B subunits undergo prolonged upregulation in anterior cingulate cortex neurons after inflammation-related permanent pain. Increase of NR2B subunits (Western blot analysis) in rat arcuate nucleus was also reported in inflammatory pain following CFA injection into the hind paws (Bu et al., 2015).

The molecular mechanism underlying NMDA receptor hyperfunctioning in inflammatory pain is a robust phosphorylation of the receptors (Yang et al., 2009). Guo and coworkers (2002) showed that a rapid and long-lasting tyrosine phosphorylation of the NR2B subunits occurs in the L4-5 segments of rat spinal cord following intraplantar injection of CFA. Similar phosphorylation was not seen in NR2A subunit following hind paw inflammation-evoked hyperalgesia. These findings suggest that neurochemical transmission between primary sensory neurons and spinal interneurons, which is mediated by NR1/NR2A receptors in physiological conditions, is shifted to NR1/NR2B receptor-mediated events in neuropathy. Suo and coworkers (2016) showed that diabetic neuropathy in mice was associated with increased expression of NMDA receptors containing NR2B subunits (Western blot method) and also with Fyn-NR2B interaction in the spinal cord. The time-course on which these events occur in the spinal cord is shown in Fig. 3.

Increase in NMDA receptor subunits expression matches findings showing that NR2B-selective antagonist drugs exhibit antinociceptive effects in animal neuropathic pain models. Thus, hyperalgesia and spontaneous pain behavior observed in the formalin test can be effectively inhibited by MK-801, an NMDA receptor channel blocker or CP 101,606, a selective antagonist of the NR2B subunit (Petrenko et al., 2003b). Zhang and coworkers (2009) performed chronic constriction injury in rats by ligation of the L4-L5 spinal nerves, which led to development of thermal hyperalgesia and mechanical allodynia. In these experiments, chronic nerve injury increased the expression of NR2B subunits in the superficial dorsal horn of the spinal cord. Ifenprodil, an NR1/NR2B receptor selective antagonist, reversed increased expression of NR2B subunits and suppressed thermal hyperalgesia and mechanical allodynia following intrathecal (i.t.) administration. Qu and coworkers (2009) used L5 spinal nerve-ligated neuropathic pain model in rats and reported that i.t. administration of Ro 25-6981, another selective NR2B subunit antagonist, elicited antiallodynic effect. Ifenprodil, elicited similar effect in this study but the drug was without effect on thermal hyperalgesia. These authors concluded that activation of NR2B subunits in the spinal cord may be crucial in development of neuropathic pain. Whether NR1/NR2A-selective antagonists (i.e. NVP-AAM 077) exert beneficial effects on neuropathic pain, need to be determined.

7. The cellular basis of glycine release

As mentioned above, activation of NMDA receptors in the central nervous system requires concurrent binding of glutamate and glycine to NR2A/NR2B and NR1 subunits, respectively (Johnson and Ascher, 1987). Glycine has an obligatory coagonist role with different affinity to NR1/NR2A-D subunit-containing NMDA receptors mediating excitatory glutamatergic neurotransmission. Beside this coagonist function, the inhibitory neurotransmitter role of glycine is also well-characterized in central glycinergic (inter)neurons.

Extrasynaptic glycine concentration is regulated by glycine transporter-1 (GlyT-1) expressed in astroglial cells and its bidirectional operation may increase or decrease the release of glycine into the extrasynaptic space (Harsing and Matyus, 2013). This increase or decrease in glycine concentrations determines the actual saturation of the coagonist binding site at NMDA receptors leading to activation or inhibition of NMDA receptor-mediated glutamatergic neurotransmission. Release of glycine from

astrocytes is a Na^+- but not a Ca^{2+}-dependent process and originates from cytoplasmic stores. In addition, extrasynaptic glycine concentrations can also be influenced by spillover of synaptically released glycine in the spinal cord (Ahmadi et al., 2003). In glutamatergic axon terminals, glycine participates in activation of pre- and postsynaptic NMDA receptors within the synapse (Raiteri and Raiteri, 2010). Thus glycine, which is a necessity for saturation of the strychnine-insensitive glycineB binding site at synaptic and non-synaptic NMDA receptors, may be originated from glial or neuronal sources in the spinal cord.

Glycinergic neurons can be identified almost in all CNS areas, most abundantly in the brain stem and the spinal cord (Vandenberg et al., 2014). Glycinergic neurons in the spinal cord are mainly interneurons located in laminae II and III and they filter incoming sensory information to prevent convergence onto dorsal horn pain neurons. These neurons store glycine as a neurotransmitter presynaptically in vesicles from which its release undergoes by strictly Ca^{2+}-dependent exocytotic process. Following action potential invasion of nerve terminals, released glycine is taken up from the synaptic cleft back to presynaptic axon terminals by glycine transporter-2 (GlyT-2). Once glycine is released into the synaptic cleft it diffuses towards its receptors located in the postsynaptic membranes. The most frequently occurring glycine receptor is the GlyRα3β subtype in the spinal dorsal horn (Morita et al., 2008). Glycine receptors are ligand-gated Cl^- channels and their opening hyperpolarizes the postsynaptic membranes exerting inhibitory influences on synapsing neurons. Glycine may also diffuse out into the extrasynaptic space from synapses of glycinergic interneurons and participate in activation of extrasynaptic NMDA receptors (Dohi et al., 2009). Thus, glycine concentrations in glutamatergic or glycinergic synapses as well as in the extrasynaptic space are regulated by GlyT-1 and GlyT-2 (Fig. 2).

8. The neurobiological characteristics of glycine transporters

The uptake of released glycine into the neighboring glial cells or back to the presynaptic nerve terminals is carried out by two glycine transporters, GlyT-1 and GlyT-2. These plasma membrane carriers belong to the Na^+-Cl^--dependent neurotransmitter transporter family (solute carrier family 6, SLC6). They differ stoichiometrically as GlyT-1 operates with cotransport of $2Na^+$ and $1Cl^-$ and GlyT-2 with $3Na^+$ and $1Cl^-$ cotransport. The difference in ion influx coupled to operation of

GlyTs results in a higher probability for reverse mode operation of GlyT-1 (Roux and Supplisson, 2000; Romei et al., 2016).

Both types of GlyTs comprise several isoforms as there are a, b, c, d, and e variants of GlyT-1 and a, b, and c splicing variants of GlyT-2 (Thomsen, 2006). The biological importance of the various isoforms of GlyTs is not known at present and selective inhibition of them by drugs is also not solved.

There are differences in the distribution of the GlyTs in the central nervous system as was shown by using *in situ* hybridization techniques (Zafra et al., 1995). GlyT-1 is expressed mainly in the cortical areas and hippocampus as well as in the brainstem and spinal cord, whereas GlyT-2 is expressed mainly in the cerebellum and spinal cord. At cellular level, the primary site of GlyT-1 expression has been considered the glial cells, which surround synapses. Besides its glial expression, the location of the GlyT-1 in neurons is also well documented. Cubelos and coworkers (2005) demonstrated that GlyT-1 is presented in glutamatergic synapses in forebrain neurons and can be detected in both pre- and postsynaptic membranes at synaptic level. GlyT-1 in glutamatergic synapses may assure the coagonist glycine for the release-mediating presynaptic NMDA receptors and also for the excitation-mediating postsynaptic NMDA receptors. The role of presynaptic GlyT-1 is to release glycine by transporter reversal, whereas GlyT-1 in the postsynaptic membranes may be associated with potentiation of postsynaptic NMDA receptor function. The expression pattern of GlyT-1 with NMDA receptors also suggests their possible colocalization in the brain (Zafra et al., 2017). Similarly to the central nervous system, GlyT-1 was also found in colocalization with NMDA receptors in the dorsal root ganglia (Schlösser et al., 2015).

A series of experimental release studies showed the neuronal expression of GlyT-1 in the spinal cord (Raiteri et al, 2005). Glycine in these experiments induced glutamate or [^{3}H]aspartate release from spinal cord synaptosomes and this effect was reduced by addition of the GlyT-1 inhibitors NFPS (Table 1) or glycyldodecylamide. These experiments suggest a possible cross-talk between glutamate homotransporters and glycine heterotransporters at glutamatergic nerve endings in the spinal cord. The

heterotransporter function of GlyT-1 was also demonstrated in GABAergic neurons of the spinal cord (Raiteri et al., 2008).

The spinal cord is one of the CNS areas in which both GlyT-1 and GlyT-2 can be found to a greater extent. GlyT-2 distributes in axons and presynaptic axon terminals of glycinergic interneurons in the dorsal horn laminae II and III (Raiteri and Raiteri, 2010; Vandenberg al., 2014). GlyT-2 in the dorsal and ventral horns of the spinal cord is associated exclusively with glycinergic neurons (Zafra et al., 1995). This was also reported by Poyatos and coworkers (1997) who found colocalization of GlyT-2 and glycine immunoreactivities in cultured neurons derived from the spinal cord. Spinal GlyT-2 located at nerve endings of glycinergic interneurons exhibits overlapping expression with strychnine-sensitive glycine receptors (glycineA binding site). Because of this close appearance, it is generally believed that GlyT-2 could be a marker for glycinergic neurons. GlyT-2 serves an essential role in the regulation of inhibitory glycinergic neurotransmission by decreasing synaptic glycine concentration after its impulse-mediated presynaptic release. By its carrier function, GlyT-2 assures the necessary supply of the neurotransmitter glycine for refilling of vesicles in nerve endings (Oyama et al., 2017). Thus, GlyT-2 recycles glycine back into the presynaptic terminals, a process necessary to preserve glycine content inside synaptic vesicles (Zafra et al., 2016).

9. Spinal cord glycine transporters and neuropathic pain

A number of studies examined the expression of GlyTs in neuropathic pain models. Cavaliere and coworkers (2007) made chronic constriction injury of the sciatic nerve to induce persistent pain state in mice. They found development of gliosis in the dorsal horn, reduced expression (after a transient increase) of glial GlyT-1 and glutamate GLT1 transporters, and also increases in glycine and glutamate spinal tissue concentrations (Fig. 3). In contrast to this finding, Schlösser and coworkers (2015) did not find alterations in GlyT-1 and GlyT-2 mRNA expression patterns in the spinal cord following sciatic nerve chronic constriction injury in rats. Similarly, Barthel and coworkers (2014) found that chronic constriction injury of rat sciatic nerve did not result in changes in either GlyT-1 or GlyT-2 protein expression in the spinal cord. In addition, long-term treatments with GlyT-1 and GlyT-2 inhibitors did not alter expression levels of GlyTs in neuropathic spinal cord.

The expression pattern of GlyTs in neuropathic spinal cord is contradictory. However, their distinct locations and the efficacy of their inhibitors to reduce hyperalgesia and allodynia strongly support the regulatory roles of the GlyTs in pain transmission. Inhibitors of GlyT-1 and GlyT-2 that were tested in neuropathy pain models are discussed in detail in the following section.

10. Glycine transporter inhibitors

A great number of glycine transporter inhibitors have been reviewed recently (Gilfillan et al., 2009; Cioffy and Guzzo, 2016). These compounds possess various chemical structures but almost all of them show selective inhibitory effect on GlyT-1 or GlyT-2.

Currently available GlyT-1 inhibitors can be divided into sarcosine- and non-sarcosine-based chemical structures (Harsing et al., 2006). Of the GlyT-1 inhibitors, sarcosine was one of the very first compounds exerting inhibition on this transporter (Table 1). Sarcosine is an alternative substrate for GlyT-1, inhibiting the transporter in a competitive, substrate-type manner. Sarcosine binds to the substrate binding site of the transporter and is taken up into the cytoplasm by normal-mode operation of GlyT-1. The carrier function of GlyT-1 is electrogenic. The resulted increase in intracellular Na^+ concentration leads to reverse mode operation of the transporter and a concomitant glycine release (Thomsen, 2006; Harsing and Matyus, 2013). This release of glycine occurs primarily from astrocytes and perhaps from neural axon terminals, which express GlyT-1 in their presynaptic membranes (Raiteri and Raiteri, 2010; Harsing et al., 2012). Because of the substrate-type inhibitory property of sarcosine, this compound evokes much higher glycine concentration in the extrasynaptic space than other, non-substrate-type GlyT-1 inhibitors (Fig. 4/A). The selectivity of sarcosine to inhibit GlyT-1 is due to the presence of the methyl group on the nitrogen as it fits to the cavity formed by the glycine residue at position 305 in GlyT-1. However, a larger residue is present in equivalent positions of GlyT-2 (Ser481) and the methyl group of sarcosine cannot accommodate within this site resulting in lack of GlyT-2 inhibition (Harsing et al., 2012).

Since sarcosine is a weak inhibitor of GlyT-1 (Liu et al., 1993), several N-substituted sarcosine derivatives have been synthesized in the last two decades. Of note, N-substituted GlyT-1 inhibitors are not substrate-type inhibitors and they do suspend the transporter operation both in normal and reverse directions. Since they do not induce glycine release from neural or glial stores, extracellular glycine concentration is lower than that occurring following substrate-type transporter inhibition (Fig. 4/A). These compounds exhibit high lipophilic physicochemical characteristics and bind to both orthosteric and allosteric binding sites of GlyT-1 (Porter and Dawson, 2015). N-Substituted sarcosine derivatives suspend the glycine-releasing effect of sarcosine (transporter reversal) when they are used in combinations (Fig. 4/B). Of the sarcosine-containing GlyT-1 inhibitors, NFPS (ALX5407), Org-24461, and Org-25935 are the most referred compounds in the literature (Table 1). NFPS is an irreversible non-competitive and Org-25935 is a reversible non-competitive type GlyT-1 inhibitor, which exhibit high selectivity for GlyT-1 and do not inhibit GlyT-2 transporter. In a novel series of GlyT-1 inhibitors, various aromatic rings in NFPS or Org-24461 were replaced by a pyridazinone ring reducing the lipophilic character as well as the toxicity of the compounds (Harsing et al., 2012; 2015).

Neurobiological investigations demonstrated that N-substituted sarcosine derivatives, particularly those inhibiting GlyT-1 irreversibly, cause a number of toxic side effects (respiratory depression, stereotyped running behavior, ataxia, coma) (Harsing et al., 2006; Kopec et al., 2010). Therefore, a great number of selective GlyT-1 inhibitors, which do not contain sarcosine, were synthesized and investigated in various experimental conditions (Depoortere et al., 2005, Lindsley et al., 2006). Compounds with most detailed investigations from this series are bitopertin (non-competitive GlyT-1 inhibitor), SSR-504734 (competitive reversible GlyT-1 inhibitor), and ACPPB (Merck 13-h), which was identified as a competitive, non-substrate type GlyT-1 inhibitor (Table 2) (Mezler et al., 2008).

ACPPB is a benzamide derivative and is particularly interesting because compounds containing this skeleton and substituted with various alkyl or aryl groups exert inhibitory effect also on GlyT-2 (Caufield et al, 2001; Ho et al., 2004). Org-25543 was reported to be an irreversible GlyT-2 inhibitor in HEK293 cells expressing GlyT-2. Mingotance-Le Meur and coworkers (2013) synthesized a series of analogs of Org-

25543 and obtained Compound 1, which was shown to reversibly inhibit GlyT-2 (Table 3). Another analog of Org-25543 (N-[1-(dimethylamino)phenyl][methyl]-3,5-dimethoxy-4-(butoxy)benzamide) inhibited GlyTs with a low selectivity ratio (Harsing, unpublished data).

Some amino acid derivatives also inhibit GlyT-2 selectively. Thus, α-, β-, and γ-amino acid derivatives have been described as potent GlyT-2 inhibitors (Wolin et al., 2004a; b). ALX1393, another amino acid derivative GlyT-2 inhibitor, was particularly well characterized in inhibition of pain transmission (Table 3). The latter compound is a potent inhibitor of GlyT-2, however, it also inhibits GlyT-1 in higher concentrations (Mingotance-Le Meur et al., 2013; Winters et al., 2018).

A series of acylcarnitines and derivatives of the endogenous lipid oleic acid were recently investigated as inhibitors of glycine reuptake (Carland et al., 2013; 2014). Of the oleic acid derivatives, oleoyl-L-carnitine (C18 *cis* ∞9 L-carnitine), N-oleoylglycines (C18 *cis* ∞9 glycine, C18 *cis* ∞8 glycine, and C16 *cis* ∞8 glycine) were identified as selective GlyT-2 inhibitors albeit their efficacy in GlyT-2 inhibition stunted from that of other GlyT-2 inhibitors (Winters et al., 2018). The D-Lys analog (Compound 33) in this series, however, was found to be a potent and selective GlyT-2 inhibitor with good oral absorption and penetration through the blood-brain barrier (Table 4). The compound also showed analgesic effect in a rat neuropathic pain model after intraperitoneal (i.p.) administration (Mostyn et al., 2019). A member of this series, N-arachidonylglycine (NAGly) was shown to be present as an endogenous substrate in the spinal cord. NAGly is a reversible, non-competitive selective GlyT-2 inhibitor, which reduced allodynia in rodents following i.t. injections (Mingotance-Le Meur et al., 2013; Vandenberg et al., 2016). Two lipid-based compounds, N-arachidonylglycine and oleoyl-L-carnitine were shown to directly bind to GlyT-2 (Schumann-Gillett and O'Mara, 2018). With differences in the head group and tail length (C12), glycyldodecylamide (non-transportable inhibitor) and sarcosyldodecylamide were reported to possess weak GlyT-1 inhibitory potential in rat brain synaptosomes (Toth et al., 1986; Harsing, 2013; Cioffi and Guzzo, 2016) although their GlyT-2 inhibitory potentials have not been reported (Table 4).

Inhibitors of GlyTs have been extensively investigated in several animal models of neuropathic pain. These investigations indicate that GlyT-1 inhibitors primarily influence NMDA receptor-mediated glutamatergic neurotransmission and GlyT-2 inhibitors restore impaired glycinergic neurotransmissions in neuropathic spinal dorsal horn (Whitehead et al., 2004; Morita et al., 2008; Tanabe et al., 2008). The antihyperalgesic and antiallodynic effects of GlyTs in experimentally induced neuropathy are summarized below.

11. Glycine transporter inhibitors in neuropathic pain management

Several recent data support the role of GlyT-1 inhibitors in pain modulation (Tables 5/A and 5/B). The selective GlyT-1 inhibitor sarcosine has been reported to produce antinociceptive action in different animal pain models. Intravenous (i.v.) or intrathecal (i.t.) administration of sarcosine (N-methylglycine) or Org-25935 (N-methyl-N-(6-methoxy)-1-phenyl-1,2,3,4-tetrahydronaphthalen-2-ylmethyl)aminomethylcarboxylic acid), another GlyT-1 inhibitor, showed antiallodynic action in neuropathic conditions using the partial ligation of sciatic nerve model in mice (Morita et al., 2008). These results were reassured in animals with knockdown of spinal GlyTs. The same research group showed similar results when the GlyT-2 inhibitors Org-25543 (N-[[1-(dimethylamino)cyclopentyl]methyl]-3,5-dimethoxy-4-(phenylmethoxy)benzamide) and ALX1393 (O-[(2-benzyloxyphenyl-3-fluorophenyl)methyl]-L-serine) were investigated. The onset of antiallodynia effect of the GlyT-1 inhibitors appeared with a delay, this time lag was not seen using GlyT-2 inhibitors. In addition, the antiallodynia effect of Org-25935 or Org-25543 was reversed by the glycine receptor antagonist strychnine or knockdown of glycine receptor GlyRα3 (Morita et al., 2008).

In another study carried out by Hermanns and coworkers (2008), the antinociceptive effects of the GlyT-1 inhibitor ALX5407 (N-[(3R)-3-([1,1'-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine, (+)NFPS) and the GlyT-2 inhibitor ALX1393 were investigated after acute i.t. administration in the chronic constriction injury model (sciatic nerve) of neuropathic pain in rats. In this work, thermal hyperalgesia and mechanical sensitization were both measured by the Hargreaves method and the von Frey filaments, respectively. Interestingly, the highest and the lowest doses of ALX5407 produced analgesia, whereas the medium dose evoked pronociceptive

effects. ALX1393 elicited antinociception at the highest applied dose, yet produced severe adverse effects (respiratory depression and motor deficits).

Tanabe and coworkers measured the effects of the i.t. injected glycine and the selective GlyT-1 inhibitors, sarcosine and NFPS (N-[-3-([1,1-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine, (±)NFPS) in mice using the neuropathic (partial ligation of the sciatic nerve, streptozotocin injection) and inflammatory (formalin-evoked nociceptive behavior) pain models (Tanabe et al., 2008). In nerve-injured animals, both compounds ameliorated thermal and mechanical hypersensitivity and they also reduced mechanical hypersensitivity in streptozotocin-injected diabetic mice. In formalin test, nociceptive reactions were selectively inhibited by GlyT-1 inhibitors in the 2nd phase of acute inflammatory pain. Memory impairment (modeled by determination of long-term potentiation in hippocampal CA1 region) associated with pain conditions was also reversed by NFPS (Tanabe et al., 2008).

Centeno and coworkers tested sarcosine (GlyT-1 selective inhibitor) following two drug-administration routes, namely i.t. and per os (p.o.) in rats with spared nerve injury ligation of the sciatic nerve (Centeno et al, 2009). After acute oral administration, sarcosine exhibited more profound action on the injured compared to the non-injured paws (better dose efficacy). Prefrontal cortex infusions of sarcosine acutely reduced mechanical sensitivity for the injured paw. The repeated oral administration of sarcosine resulted in anti-neuropathic effect days after repeated drug treatments, the measured effects disappeared only few days after treatment cessation. These findings indicate that short- and also long-term inhibition of GlyT-1 results in acute and long-term reduction in neuropathic behavior.

Nishikawa and his coworkers found in another study that only the GlyT-2 selective inhibitors showed analgesic actions in herpetic and postherpetic pain models (Nishikawa et al., 2010). They investigated the effects of the GlyT-2 selective inhibitor ALX1393 on dynamic and static allodynia in mice with herpetic or postherpetic pain after acute i.t. injections. ALX1393, but not the GlyT-1 inhibitor sarcosine, alleviated dynamic and static allodynia at the herpetic and postherpetic stages. They also showed that expression levels of GlyT-1, but not that of GlyT-2 mRNA decreased in the dorsal horn of the lumbar spinal cord at the herpetic and postherpetic stages. These results indicate that GlyT-2 might be a target in patients with herpes zoster and postherpetic neuralgia.

It is a well-known phenomenon that chronic pain might coexist with cognitive deficit and the role of glycinergic neurotransmission is important in these types of cognitive dysfunctions. Indeed, altered glycine concentrations in the brain influence memory functions (Tanabe et al., 2008; Kodama et al., 2011). Kodama and coworkers (2011) measured mechanical hypersensitivity following sciatic nerve ligation in mice and used the novel-object recognition test to detect alterations in memory state. They reported that chronic pain negatively influences recognition ability for novelty in mice. Systemic administration of the selective GlyT-1 inhibitor NFPS abrogated neuropathic pain and cognitive dysfunction was not observed in drug-treated mice. On the contrary, relief of pain after spinal administration of NFPS did not ameliorate impairment of cognitive function suggesting that short-term analgesia leaves disturbed attention and working memory unaltered. These findings further support that increase of extracellular glycine levels by GlyT-1 inhibition may have therapeutic importance in treatment of chronic pain with memory impairment.

The lidocaine metabolite N-ethylglycine was shown to act as a substrate inhibitor of glycine transport (Werdehausen et al., 2015). Indeed, this effect of N-ethylglycine was a result of a selective and competitive inhibition of GlyT-1 expressed in *Xenopus laevis* oocytes, whereas GlyT-2 function was not affected. Using inflammatory (subcutaneous (s.c.) injection of CFA) and neuropathic pain (sciatic nerve ligation) models in C57/BL/6J mice, N-ethylglycine decreased hyperalgesia and allodynia and these effects showed close similarities to the activities of sarcosine. In rats, s.c. injection of N-ethylglycine resulted in parallel increases in the concentrations of the parent compound and glycine in the cerebrospinal fluid. The authors concluded that lidocaine evokes antihyperalgesic effects, at least in part, by metabolizing to N-ethylglycine, which then modulates GlyT-1 activity and increases glycine concentrations in glycinergic synapses in the central nervous system. Of note, local anesthetics also have been reported by other authors to affect the resting catecholamine release in the spinal cord during neuropathic pain, which could also influence pain transmission (Sircuta et al., 2016).

Armbruster and coworkers induced mechanical allodynia and thermal hyperalgesia by chronic constriction injury of the sciatic nerve or inflammation evoked by

carrageenan injections into the plantar surface of the hind paw (Armbruster et al., 2018). The effects of both acute and long-term applications of the selective GlyT-1 inhibitor bitopertin ([4-[3-fluoro-5-(trifluoromethyl)-2-piridinyl]-1-piperazinyl][5-methyl-sulfonyl)-2-[(1S)-2,2,2-trifluoro-1-methylethoxy]phenyl]-methanone) were investigated. Acute drug administration was made p.o., s.c. or i.p., and osmotic minipumps implanted subcutaneously were also used for long-term drug treatment. General activity was analyzed in open field experiments and glycine concentration was measured in the cerebrospinal fluid and blood by HPLC-fluorescent detector. Bitopertin evoked dose- and time-dependent antinociceptive effects on mechanical allodynia and thermal hyperalgesia after acute drug treatment. Bitopertin also showed beneficial effects after long-term application over a 4-week time period. Reaction thresholds to stimuli and general locomotor activity as well as anxiety were not influenced by the test compound in the applied doses. The concentration of glycine was elevated in the cerebrospinal fluid after drug treatment indicating inhibition of glycine transport. These findings point to GlyT-1 as a promising target in the management of different pain conditions.

A number of studies highlight GlyT-2 as a more promising novel target in the management of neuropathic pain. Mingorance-Le Meur and coworkers (2013) investigated the GlyT-2 selective inhibitors ALX1393, Org-25543, and the analogs of the latter one. It was reported that pharmacological inhibition of GlyT-2 by Org-25543 reduced the late phase of formalin-evoked pain in mice, while it exerted only mild effect in the first phase of the test. The inhibitory effect of Org-25543 on GlyT-2 was found to be irreversible. In contrast to Org-25543, ALX1393 exerted only partial inhibitory effect on GlyT-2. The Org-25543 derivative Compound 1 (Table 3) also reduced nociception in the formalin test, however, it proved to be a reversible GlyT-2 inhibitor and showed a more favorable toxicity profile. It was suggested that the biologically reversible GlyT-2 inhibitors might offer a better tolerable balance between efficacy and toxicity.

Barthel and coworkers performed a study to investigate the long-term administration of Gly-T inhibitors and also to detect toxic effects occurring during drug treatment (Barthel et al., 2014). Male Wistar rats were treated with ALX5407 or ALX1393, (selective inhibitors of GlyT-1 or GlyT-2, respectively) via s.c. osmotic infusion pumps for a period of 14 days. Mechanical allodynia and thermal hyperalgesia were assessed before and after chronic constriction injury (sciatic nerve ligation) and every

2nd day during substance application. The expressions of GlyT-1, GlyT-2, and the NMDA receptor subunit NR1 were also analyzed by Western blot in the spinal cord. Both compounds alleviated mechanical allodynia and thermal hyperalgesia in a dose- and time-dependent manner. ALX5407 reduced NR1 subunit expression in the ipsilateral spinal cord whereas the expression of GlyT-1 and GlyT-2 remained unchanged. Toxic or adverse effects were not observed during the experimental period.

Haranishi and coworkers studied the analgesic effects of the GlyT-2 inhibitor ALX1393 following i.t. administration, in acute pain models (tail flick, hot plate, paw pressure, and formalin tests), in rats (Haranishi et al., 2010). ALX1393 inhibited effects of thermal and mechanical stimulations by a strychnine-sensitive manner suggesting the involvement of glycine receptors in antinociception. Pain behavior was also inhibited by ALX1393 in formalin test, whereas it did not alter motor functions in the rotarod test. The authors concluded that GlyT-2 inhibitors may possess novel antinociceptive action.

Motoyama and coworkers have developed a cancer pain model by injection of NCTC 2472 tumor cells into the femur cavity of mice (Motoyama et al., 2014). Both GlyT-1 and GlyT-2 inhibitors (Org-25935 and Org-25543, ALX1393) were administered i.v. or orally and pain-like behaviors (allodynia, withdrawal threshold guarding behavior, and limb-use abnormality) were examined. It was found that both GlyT-1 and GlyT-2 inhibitors exhibited potent and long-lasting pain relief activity. Non-analgesic dose of morphine potentiated the pain-relief effect of Org-25543, this effect was transiently suspended by i.t. administration of strychnine. The pain-relief activity of Org-25543, however, exhibited a phase-dependent pattern suggesting involvement of different mechanisms. Knockdown of spinal GlyTs resulted in a decrease of allodynia scores after tumor cell transplantation, guarding behaviour and limb-use abnormalities were also improved.

In a recent study, Takahashi and coworkers (2015) implanted guide cannula into the right lateral ventricle and ALX1393, a selective inhibitor of GlyT-2, was administered i.c.v. to rats. The formalin test and the chronic nerve constriction injury (sciatic nerve ligation) models were applied to investigate antinociceptive effect of the centrally administered ALX1393. In these experiments, neuropathic pain behaviors

mechanical, cold, and thermal hyperalgesia were assessed by electronic von Frey test, cold plate test, and the plantar test (Hargreaves), respectively. In the formalin test, ALX1393 alleviated the nociceptive reactions without significant effect on the motor functions (rotarod test). In sciatic nerve ligation test, mechanical and cold hyperalgesia were alleviated by ALX1393. Pretreatment with strychnine reversed the analgesic effect of the highest dose of ALX1393 in these tests indicating the involvement of glycine receptors.

In another study, Takahashi and coworkers described novel phenoxymethylbenzamide derivatives as novel selective GlyT-2 inhibitors, which may be taken into account as potential new analgesics (Takahashi et al., 2014). The outcome of this study was the discovery of a promising compound displaying selective inhibition on GlyT-2, yet showing antinociceptive effect in mouse model of partial sciatic nerve ligation. Further investigations of the novel compound, however, are needed (e.g. toxicity).

Omori and coworkers (2015) investigated the analgesic effect of a novel GlyT-2 selective phenoxymethylbenzamide derivate, GT-0198 (Table 3). The new analog showed strychnine reversible analgesic effect after per os, i.v., and i.t. administrations in a model of neuropathic pain elicited by partial sciatic nerve ligation in mice.

It has been previously shown that the endogenous compound, N-arachidonylglycine (NAGly) reduced mechanical allodynia and tonic pain in animal pain models induced by partial nerve ligation and formalin injection, respectively (Carland et al., 2014). It was also demonstrated that NAGly administered i.t. reduced mechanical allodynia and thermal hyperalgesia in a rat inflammatory pain model (Succar et al., 2007). The possible mechanism to elicit analgesia might be inhibition of GlyT-2 activity rather than inhibition of GlyT-1 (Wiles et al., 2006). Mostyn and coworkers (2017) investigated acyl-glycine derivatives that proved to be reversible GlyT-2 inhibitors. They managed to find a novel compound that is 28-fold more potent than NAGly, whereas the selectivity toward GlyT-2 was kept as was determined in *in vitro* biological assays. These or similar compounds might be the first generation of GlyT-2 inhibitors in the clinical practice, yet further investigations (e.g. side effects, toxicity) are needed.

12. Reorganization of neural circuitry in the spinal dorsal horn following peripheral nerve injury and inflammation: The analgesic effects of GlyT inhibitors

Peripheral nerve injury induced by sciatic nerve ligation at the spinal lumbar levels results in a reorganization of the neural circuits in spinal dorsal horn. Mechanisms, which are silent in normal physiological conditions, become operative leading to reduction of the inhibitory tone, whereas the excitatory tone changes to the opposite direction. During this reorganization procedure, physiologically quiescent neural circuits are activated transmitting feed-forward polysynaptic excitation to pain projection neurons in lamina I without receiving glycinergic inhibition. An example for this was given by Lu and coworkers (2013) suggesting that PKCγ-expressing interneurons may activate transient central neurons in neuropathic hyperalgesia, a mechanism not operative under physiological conditions.

Several lines of evidence indicated a marked increase in glutamate release following traumatic brain injury (Dorsett et al., 2017; Perez et al., 2017). Peripheral nerve damage and inflammation may also induce increased glutamate release from central nerve endings of sensory afferent pathways, which then may spillover from the synapses and diffuse into the extrasynaptic space. Increased glutamate release from afferent terminals in the spinal cord also occurs in type-1 diabetic neuropathy (Suo et al., 2016). Glutamate release observed in pathological conditions exhibits sustained tonic characteristics; it is induced by slow irregular neuronal firing and is often directed into the extracellular space (Hinzman et al., 2010). In addition, damage of peripheral nerves following mechanical trauma also leads to rapid glial responses triggering activation of astrocytes and microglial cells in the spinal cord (Calavaliere et al., 2007). Glial activation was indicated by cell hypertrophy, reactive astrogliosis, and increased production of glial fibrillary acidic protein (GFAP) in the spinal cord following traumatic nerve injury (Burda et al., 2016). Molecular cascades in activated glial cells in response to peripheral nerve injury, upregulate a number of transporter proteins in the cells including those for glutamate (GLT1) and glycine (GlyT-1). A frequently observed phenomenon in pathological conditions is that operation of glial GlyT-1 is shifted into the reverse-mode and excess amount of glycine is effluxed into the extrasynaptic space (Harsing and Matyus, 2013; Shibasaki et al., 2017). Thus, tconcentrations of both glutamate and glycine reach high levels in the extrasynaptic

space and they activate non-synaptic glutamate receptors following traumatic nerve injuries, ischemic insults or inflammation (Hanuska et al., 2016).

Extrasynaptic NMDA receptors with NR1/NR2B subunit composition were proposed to primarily mediate neurodegeneration in traumatic injury of neural tissues (Pal, 2018). Activation of extrasynaptic NMDA receptors in response to impairment of peripheral nerves is supported by the fact that NR2B subunits are overexpressed in peripheral neuropathy as was shown in a number of laboratories (Yang et al., 2009; Fakhri et al., 2018; Geng et al., 2010; Suo et al., 2016, Wang et al., 2016). Based on our hypothesis, extrasynaptic NR1/NR2B receptors are silent in physiological conditions but they become upregulated following neural tissue damage of various origins. NR2B subunit was found in particularly high levels in lamina II (Petrenko et al., 2003a) and synaptic connections between the excitatory PKCγ-expressing cells and transient central interneurons are localized also in this lamina (Lu et al., 2013). Therefore, it might be plausible to propose that NR1/NR2B receptors, which are activated in neuropathic pain conditions, are expressed in excitatory interneurons of dorsal horn lamina II.

Moreover, we hypothesize that synaptic NR1/NR2A receptor-mediated glutamatergic transmission becomes hypofunctional following damage of peripheral nerves. Peripheral neural injury causes an increased glutamatergic activity from the central terminals of the Aβ primary afferent neurons and increase of tonic glutamate release may elicit desensitization of postsynaptic NR1/NR2A receptors (Lin et al., 1994; Tong et al., 1995; 2003, Hinzman et al., 2010). The predominant role of extrasynaptic NR1/NR2B receptor mediated-glutamatergic transmission is further asserted by excess glycine efflux from reactivated astroglial cells in neural tissue injury. GlyT-1 inhibitors block both normal and reverse mode operation of the transporter and the latter effect decreases extrasynaptic glycine levels reducing NR1/NR2B receptor activity, which then leads to analgesic effects (Fig. 4B). Accordingly, GlyT-1 inhibitors evoke analgesia indirectly via decreasing the coagonist glycine concentrations at extrasynaptic NR1/NR2B receptors expressed in excitatory interneurons of the dorsal horn. On the other hand, specific NR2B subunit antagonists produce analgesia in neuropathic or inflammatory pain states by blockade of the glutamate binding sites at NR2B subunit-containing NMDA receptors (Qu et al., 2009; Zhang et al., 2009). Our working hypothesis may thus provide explanation for

the seemingly contradictory observations, namely GlyT-1 inhibitors and NR2B subunit antagonists both reduce hyperalgesia and allodynia following peripheral nerve injury.

There is a general agreement that glycinergic inhibition in the spinal cord is reduced in neuropathic pain although the mechanism how it develops is unclear. Bai and coworkers (2019) demonstrated upregulation of GlyT-2 and decreased glycine extracellular concentrations in the spinal cord L3-5 levels on day 14 following induction of knee osteoarthritis in rats. We speculate that tonic increase of synaptic glutamate release from central terminals of Aβ fibers may desensitize postsynaptic NR1/NR2A receptors also in glycinergic interneurons (Tong et al., 1995, Hinzman et al., 2010). As a consequence, synapting glycinergic interneurons become hypofunctional disinhibiting the excitatory interneuronal communication and excitatory influences may invade neurons in lamina I involved in pain transmission (Vandenberg et al., 2014; Zafra et al, 2016). It is quite possible that impaired inhibition mediated by glycinergic interneurons can be restored by inhibition of GlyT-2, a transporter primarily expressed in glycinergic nerve terminals. Enhancing glycinergic neurotransmission in the spinal cord by GlyT-2 inhibition is expected to reduce the advance of non-noxious sensations to the pain projection neurons of the dorsal horn, which then leads to analgesia in neuropathic states. Sequence of events leading to development of neuropathic pain and how GlyT inhibitors or NR2B subunit antagonists influence it is shown in Fig. 5.

Published data adding together speak in favour of increasing excitatory glutamatergic and decreasing inhibitory glycinergic neurotransmissions in the spinal dorsal horn during development of hyperalgesia, allodynia, and spontaneous pain states. This imbalance in neurotransmitter tone may be normalized by using GlyT-1 and GlyT-2 inhibitors. These drugs, however, exhibit a number of differences in development of their analgesic effects. Accordingly, GlyT-1 inhibitors elicit antiallodynic effect with a lag time (Morita et al., 2008). For the reason why this lag time is required, we have speculated that novel otherwise silent neuronal communications are established in the spinal cord following insults of peripheral nerves. Time lag in antiallodynic effect is, however, not apparent in case of GlyT-2 antagonists (Vandenberg et al., 2014) suggesting that neural reorganization does not occur in restoration of glycinergic interneuron-mediated inhibition during analgesia. In addition, the U-shape dose-

response curve of GlyT-1 inhibitors in analgesia suggests that more than one event may be involved in the antinociceptive action of these drugs (Hermanns et al., 2008). It is also worth mentioning that different mechanisms may limit efficacies of GlyT-1 and GlyT-2 inhibitors in treatment of neuropathic pain. Glycine concentration, which is increased by GlyT-1 inhibitors, can elicit synaptic NMDA receptor internalization, whereas GlyT-2 inhibitors were shown to reduce refilling of glycine vesicles in glycinergic axon terminals (Nong et al., 2003; Oyama et al., 2017). Whether these effects influence clinical efficacy of GlyT inhibitors in tranquilizing symptoms of neuropathic pain, remain to be elucidated.

13. Conclusion and further avenues

A great number of experimental data indicate that both GlyT-1 and GlyT-2 inhibitors evoke analgesia in neuropathic pain, albeit the pattern of their pharmacological effects is different. In this regards, most drug-discovery studies aim to develop selective inhibitors for GlyT-1 or GlyT-2. However, one can speculate that the "ideal" analgesic drug for neuropathic pain may inhibit both transporters with identical or at least similar potencies in the spinal dorsal horn. Compounds containing the benzamide skeleton substituted with various aryl or alkyl groups (Tables 2 and 3) possess either GlyT-1 or GlyT-2 inhibitory activity. Therefore, we speculate that this group of compounds may be a possible source to develop non-selective GlyT inhibitors. For this purpose, ALX1393 may also be an investigational drug as it inhibits both isoforms of GlyTs with a smaller magnitude difference in inhibitory potentials (Mingorance-Le Meur et al., 2013). In fact, however, no GlyT inhibitors other than ALX1393 with low selectivity
have been tested in the various neuropathic pain models. Alternatively, combination of GlyT-1 and GlyT-2 inhibitors may indicate whether concomitant inhibition of the two transporters results in any further benefit in pain management. Using such a combination, it is obvious that the pharmacokinetic patterns of the two GlyT inhibitors should be similar or proper technological approach should be applied.

Mechanisms of analgesic actions of GlyT-1 and GlyT-2 inhibitors in terms of how they influence neural circuits in laminae I-III of the spinal cord dorsal horn are different. GlyT-1 primarily regulates the activity of extrasynaptic NR1/NR2B receptors by increasing or decreasing glycine efflux from astrocytes via bidirectional

operation of the carrier. On the other hand, GlyT-2 has a role to set optimal synaptic glycine concentrations required to activate postsynaptic glycine receptors. Thus, GlyT inhibitors exert pharmacological actions both in glutamatergic and glycinergic synapses and these two effects with different sites of actions may exert synergistic blockade of painful sensory transmission in the spinal cord.

Simultaneous use of GlyT inhibitors, however, may be hampered by the narrow analgesic dose-range of GlyT-1 inhibitors and by depletion of vesicular glycine concentrations following GlyT-2 inhibition. Moreover, GlyT inhibitors exhibit characteristic toxicity, which is more pronounced in case of the irreversible inhibition of the transporters. Consequently, the use of reversible GlyT inhibitors with low selectivity for both transporter isoforms would be the preferred choice. At present, we are working to identify GlyT inhibitors with such a pharmacological profile and testing them in neuropathic pain models.

Acknowledgments

This work was supported by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the Neurology thematic programme of the Semmelweis University (FIKP, 2018). ÚNKP-18-3-III (New National Excellence Program, Ministry of Human Capacities, Hungary) and Gedeon Richter Plc.'s Centenáriumi Alapítvány 2019 research grant was awarded to Dr. Mihály Balogh.

Conflict of Interest

The authors report no conflicts of interest in this work.

15. Referred Literature

Ahmadi, S., Muth-Selbach, U., Lauterbach, A., Lipfert, P., Neuhuber, W. L., Zeilhofer, H. U.: Facilitation of spinal NMDA receptor currents by spillover of synaptically released glycine. Science, 2003, 300, 2094-2097. doi: 10.1126/science.1083970

Armbruster, A., Neumann, E., Kötter, V., Hermanns, H., Werdehausen, R., Eulenberg, V.: The GlyT1 inhibitor bitopertin ameliorates allodynia and hyperalgesia in animal models of neuropathic and inflammatory pain. Front. Mol. Neurosci., 2018, 10, 438. doi.10.3389/fnmol.2017.00438.

Balogh, M., Zádor, F., Zádori, Z. S., Mohammed, S., Király, K., Mohammadzadeh, A., Varga, B., Lázár B., Mousa, S. A., Hosztafi, S., Riba, P., Benyhe, S., Gyires, K., Schäfer, M., Fürst, S., Al-Khrasani, M. Efficacy-based perspective to overcome reduced opioid analgesia of advanced painful diabetic neuropathy in rats. Frontiers in Pharmacology, 2019, 10, 347. doi: 10.3389/fphar.2019.00347

Barthel, F., Urban, A., Schlösser, L **,** Eulenberg, V., Werdehausen, R., Brandenburger, T., Aragon, C., Bauer, I., Hermanns, H.: Long-term application of glycine transporter inhibitors acts neuropathic and modulates spinal N-methyl-D-aspartate receptor subunit NR-1 expression in rats. Anesthesiology 2014, 121, 160-169. doi: 10.1097/ALN00000000000000103

Bai, F., Ma, Y., Guo, H., Li, Y., Xu, F., Zhang, M., Dong, H., Deng, J., Xiong, L.: Spinal cord glycine transporter 2 mediates bilateral ST35 acupoints sensitization in rats with knee osteoarthritis. Evidence-Based Complementary and Alternative Medicine, 2019, Article ID 7493286. doi: 10.1155/2019/7493286

Bell, R. F., Kalso, E. A.: Ketamine for pain management. Pain Reports, 2018, 3 p.e674. doi: 10.1097/PR9.000000000000674

Borbély, Z., Csomó, B. K., Kittel, Á., Gerber, G., Varga, G., & Vizi, E. S. Effect of rat spinal cord injury (hemisection) on the ex vivo uptake and release of [^{3}H]

noradrenaline from a slice preparation. Brain Res. Bull., 2017, 131, 150-155. doi : 10.1016/j.brainresbull.2017.04.007.

Bourne, S., Machado, A. G., Nagel, S. J.: Basic anatomy of physiology of pain pathways. Neurosurg. Clin. N. Am., 2014, 25, 629-638. doi:10.1016/j.nec.2014.06.001.

Breivik, H. Collett, B., Ventafridda, V., Cohen, R., Gallacher, D.: Survey of chronic pain in Europe: Prevalence, impact on daily life, and treatment. Eur. J. Pain, 2006, 10, 287-333. doi.10.1016/j.epain.2005.06.009.

Bu, F., Tian, H., Gong, S., Zhu. O., Xu, G.-Y., Tao, J., Hang, X.: Phosphorylation of NR2B NMDA subunits by protein kinase C in the arcuate nucleus contributes to inflammatory pain in rat. www.nature.com/scientific reports/ 2015, 5: 15945/, doi: 10.1038/srep15945

Burda, J. E., Bernstein, A. M., Sofroniew, M. V.: Astrocyte roles in traumatic brain injury. Exp. Neurol., 2016, 275, 305-315. doi: 10.1016/j.expneurol.2015.03.020

Carland, J. E., Mansfield, R. E., Ryan, R, M, Vandenberg, R. J.: Oleyl-L-carnitine inhibits glycine transport by GlyT2. Br. J. Pharmacol., 2013, 168, 891-912. doi: 10.1111/j.1476-5381.2012.02213.x

Carland, J. E., Handford, C. A., Ryan, R. M., Vandenberg, R. J.: Lipid inhibitors of high affinity glycine transporters: Identification of a novel class of analgesics. Neurochem. Internat., 2014, 73, 211-216. doi: 10.1016/j.neuint.2013.08.012.

Caufield, W. L., Collie, I. T., Dickins, R. S., Epemolu, O., McGuire, R., Hill, D. R., McVey, G., Morphy, J. R., Rankovic, Z., Sundaram, H.: The first potent and selective inhibitors of glycine transporter type 2. J. Med. Chem., 2001, 44, 2679-2682.

Cavaliere, C., Cirillo, G., Bianco, M. R., Rossi, F., De Novellis, V., Maione, S., Papa, M.: Gliosis alters expression and uptake of spinal glial amino acid transporters in a mouse neuropathic pain model. Neuron Glia Biol., 2007, 3, 1-14. doi:

10.1017/S1740925X07000695.

Centeno, M. V., Mutso, A., Millecamps, M., Vania Apkarian, A.: Prefrontal cortex and spinal cord mediated anti-neuropathy and analgesia induced by sarcosine, a glycine-T1 transporter inhibitor. Pain, 2009, 145, 176-183. doi:10.1016/j.pain2009.06.014.

Cioffy, C. R., Guzzo, P.: Inhibitors of glycine transporter-1: potential therapeutics for the treatment of CNS disorders. Curr. Top Med. Chem., 2016, 16, 3404-3437. doi: 10.2174/1568026616666160405113340.

Cubelos, B., Gimenez, C., Zafra, F.: Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. Cereb. Cortex, 2005, 15, 448-459. doi:10.1093/cercor/bhh147.

Danysz, W., Parsons, C. G.: Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharm. Rev., 1998, 50, 597-664.

Debono, D. J., Hocksema, I. L. Hobbs, R. D.,: Caring for patients with chronic pain: pearls and pitfalls. J. Am. Osteopath. Assoc., 2013, 113, 620-627. doi:10,7556/jaoa.2013.023. 10.

Depoortere, R., Dargazanli, G., Estenne-Bouhtou, G., Coste, A., Lanneau, C., Desvignes, C., Poncelet, M., Heaulme, M., Santucci, V., Decobert, C., Cudennec, A., Voltz, C., Boulay, D., Terranova, J. P., Stemmelin, J., Roger, P., Marabout, B., Sevrin, M., Vige, X., Biton, B., Steinberg, R., Farncon, D., Alonso, R., Avenet, P., Oury-Donat, F., Perrault, G., Griebel, G., George, P., Soubrie, P., Scatton, B.: Neurochemical, electrophysiological and pharmacological profiles of the selective inhibitor of the glycine transporter-1 SSR504734, a potential new type of antipsychotic. Neuropsychopharmacology, 2005, 30, 1063-1985. doi: 10.1038/sj.npp.1300772

Dohi, T., Morita, K., Kutayama, T., Motoyama, N., Morioka, N.: Glycine transporter inhibitora as a novel drug discovery strategy for neuropathic pain. Pharmacol. Ther., 2009, 123, 54-79. doi: 10.1016/jpharmthera.2009.03.018.

Dorsett, C. R., McGuire, J. L., DePasquale, E. A. K., Gardner, A. E., Floyd, C. L., McCullumsmith, R. E.: Glutamate neurotransmission in rodent models of traumatic brain injury. J. Neurotrauma, 2017, 34, 263-272. doi: 10.1089/neu.2015.4373.

Dworkin, R. B., O'Connor, A. B., Audette, J., Baron, R., Gourlay, G. K., Haanpaa, M. l. Kent, J. L., Krane, E. J., Lebel, A. A., Levy, R. M., Mackey, S. C., Mayer, J., Miaskowski, C., Raja, S. N., Rice, A. S., Schmader, K. E., Sracey, B., Stanos, S., Treede, R. D., Turk, D. C., Walco, G. A., Wells, C. D.: Recommendations for pharmacological management of neuropathic pain: an overview and literature update. Mayo Clin. Proc. 2010, 85, S3-14, doi:10. 4065/ mcp.20090649.

Fakhri, S., Dargahi, L., Abbaszadeh, F., Jorjani, M. Astaxanthin attenuates neuroinflammation contributed to the neuropathic pain and motor dysfunction following compression spinal cord injury. Brain Res. Bull., 2018 143, 217-224. doi: 10.1016/j.brainresbull.2018.09.011

Field, M. J., Bramwell, S., Hughes, J., Singh, L.: Detection of static and dynamic components of mechanical allodynia in rat model of neuropathic pain: are they signalled by distinct primary sensory neurons? Pain, 1999, 83, 303-314.

Geng, S.-J., Liao, F.-F., Dang, W.-H., Ding, X., Liu, X.-D., Cai, J., Han, J.-S.: Contribution of the spinal cord BDNF to the development of neuropathic pain by activation of the NR2B-containing NMDA receptors in rats with spinal nerve ligation. Exp. Neurol., 2010, 222, 256-266.

Gilfillan, R., Kerr, J., Walker, G., Whishart, G.: Glycine transporters and their inhibitors. Top Med. Chem., 2009, 4, 223-247. doi: 10.1007/7355_2009:030.

Guo, W., Zou, S., Guan, Y., Ikeda, T., Tal, M., Dubner, R., Ren, K.: Tyrosine phosphorylation of NR2B subunit of the NMDA receptor in the spinal cord during the

development and maintenance of inflammatory hyperalgesia. J. Neurosci., 2002, 22, 6208-6217.

Hanuska, A., Szenasi, G., Albert, M., Koles, L., Varga, A., Szabo, A., Matyus, P., Harsing, L. G., Jr.: Some operational characteristics of glycine release in rat retina: the role of reverse mode operation of glycine transporter type 1 (GlyT-1) in ischemic conditions. Neurochem. Res., 2016, 41, 73-85.

Haranishi, Y., Hara, K., Terada, T., Nakamura, S., Sata, T.: The antinocicepive efffect of intrathecal administration of glycine transporter-2 inhibitor ALX1393 in rat acute pain model. Anesth. Analg., 2010, 110, 615-621. doi:10.1213/ANE.0b013e3181c7ebbb

Harsing, L. G., Jr., Juranyi, T., Gacsalyi, I., Tapolcsanyi, P. Czompa, A., Matyus, P.: Glycine transporter type-1 and its inhibitors. Curr. Med. Chem., 2006, 13, 1017-1044. doi:10.2174/092986706776360932

Harsing, L. G., Jr., Zsilla, G., Matyus, P., Nagy, K., Marko, B., Gyarmati, Z., Timar, J.: Interactions between glycine transporter type-1 (GlyT-1) and some inhibitor molecules-Glycine transporter type 1 and its inhibitors (Review). Acta Physiol. Hung., 2012, 99, 1-17. doi: 10.1556/Aphysiol.99.2012.1.1

Harsing, L. G., Jr.: An overview on GlyT-1 inhibitors under evaluation for the treatment of schizophrenia. Drugs of the Future, 2013, 38, 555-568. doi: 10.1358/dof.2013.038.08.1965101

Harsing, L. G., Matyus, P.: Mechanism of glycine release, which build up synaptic and extrasynaptic glycine levels: The role of synaptic and non-synaptic glycine transporters. Brain Res. Bull., 2013, 93, 110-119. doi:1016/j.brainresbull.2012.12.002

Harsing, L. G., Jr., Timar, J., Szabo, G., Udvari, Sz., Nagy, K. M., Marko, B., Zsilla, G., Czompa, A., Tapolcsanyi, P., Matyus, P.: Sarcosine-based glycine transporter type-1 (GlyT-1) inhibitors containing pyridazine moiety: a further research for drugs

with potential to influence schizophrenia negative symptoms. Curr. Pharm. Des., 2015, 21, 2291-2303.

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, 214-219. doi: 10.1016/j.neulet.2018.09.012.

Hildebrand, M. E., Xu, J., Dedek, A., Li, Y., Sengar, A. S., Beggs, S., Lombroso, P. J., Salter, M. W.: Potentiation of synaptic GluN2B NMDA currents by Fyn kinase is gated through BDNF-mediated disinhibition in spinal pain processing. Cell Report, 2016, 17, 2753-2765.

Hinzman, J. M., Thomas, T. C., Burmeister, J. J., Quintero, J. E., Huetti, P., Pomerlean, F., Gerhardt, G. A., Lifshitz, J.: Diffuse brain injury elevates tonic glutamate levels and potassium-evoked glutamate release in discrete brain regions at two days post-injury: an enzyme-based microelectrode array study. J. Neurotrauma, 2010, 27, 889-899.

Ho, K.-K., Appell, K. C., Baldwin, J. J., Bohnstedt, A. C., Dong, G., Guo, T., Horlick, R., Islam, K. R., Kultgen, S. G., Masterson, C. M., McDonald, E., McKillan, K., Morphy, J., Rankovic, Z., Sundaram, H., Webb, M.: 2-(Aminomethyl)-benzamide-based glycine transporter type-2 inhibitors. Bioorg. Med. Chem. Lett., 2004, 14, 545-548.

Hua, S., Cabot, P. J.: Mechanisms of peripheral immune-cell-mediated analgesia in inflammation: clinical and therapeutic implications. Trends Pharmacol. Sci., 2010, 31, 427-433.

Johnson, J. W., Ascher, P.: Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature, 1987, 325, 529-531. doi:10.1038/325529a0

Karlsson, U., Sjödin, J., Angery Möller, , K., Johansson, S., Wiksröm, L., Nasström, J.: Glutamate-induced currents reveal three functionally distinct NMDA receptor

populations in rat dorsal horn-effects of peripheral nerve lesion and inflammation. Neuroscience, 2002, 112, 861-868.

Kodama, D., Ono, H., Tanabe, M.: Increased hippocampal glycine uptake and cognitive dysfunction after peripheral nerve injury. Pain, 2011, 152, 809-817. doi:10.1016/j.pain.2010.12.029

Kopec, K., Flood, D. G,, Gasior, M., McKanna, B. A. W., Zuvich, E., Schreiber, J., Salvion, J. M., Durkin, J. T., Ator, M. A., Marino, M. J.: Glycine transporter (GlyT1) inhibitors with reduced residence time increase prepulse inhibition without inducing hyperlocomotion in DBA/2 mice. Biochem. Pharmacol., 2010, 80, 1407-1417.

Köles, L., Kató, E., Hanuska, A., Zádori, Z. S., Al-Khrasani, M., Zelles, T., Rubini, P., Illes, P.: Modulation of excitatory neurotransmission by neural/glial signalling molecules: interplay between purinergic and glutamatergic systems. Purinergic Signal., 2016, 12, 1-24. doi:10.1007./s11302-015-9480-5

Kvarnström, A., Karlsten, R., Quiding, H., Emanuelsson, B. M., Gordh, T.: The effectiveness of intravenous ketamine and lidocaine on peripheral neuropathic pain. Acta Anesthesiol. Scand., 2003, 47, 868-877.

Leung, A., Wallace, M. S., Ridgeway, R., Yaksh, T.: Concentration-effect relationship of intravenous alfentanil and ketamine on peripheral neurosensory thresholds, allodynia, and hyperalgesia of neuropathic pain. Pain, 2001, 91, 177-187.

Lin, F., Stevens, C. F.: Both open and closed NMDA receptor channels desensitize. J. Neurosci., 1994, 14, 2153-2160.

Lindsley, C. W., Zhao, Z., Leister, W. H., O'Brien, J. A., Lemaire, W., Williams, D. L. Jr., Chen, T.-B., Chang, R. S. L., Burno, M., Jacobson, M. A., Sur, C., Kinney, G. G., Pettibone, D. J., Tiller, P. R., Smith, S., Rsou, N. N., Duggan, M. E., Conn, P. J., Hartman, G. D.: Design, synthesis, and in vivo efficacy of novel glycine transporter-1 (GlyT1) inhibitors derived from a series of [4-phenyl-1-(propylsulfonyl)piperidin-4-yl)-

methyl)benzamides. Chem. Med. Chem. 2006, 1, 807-811 doi: 10.1002/cmdc.200600097

Liu, Q. R., Lopez-Corcuera, B., Mandiyan, S., Nelson, H., Nelson, S.: Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. J. Biol. Chem., 1993, 268, 22802-22808.

Lu, Y., Dong, H., Gao, Y., Gong, Y., Ren, Y., Gu, N., Zhou, S., Xia, N., Sun, Y.-Y., Ji, R.-R., Xiong, L.: A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. J. Clin. Invest., 2013, 123, 4050-4062. doi: 10.1172/JCI70026

Mezler, M., Hornberger, W., Mueller, R., Schmidt, M., Amberg, W., Braje, W., Ochse, M., Schoemaker, H., Behl, B.: Inhibitors of GlyT1 affect glycine transport via discrete binding sites J. Pharmacol. Exp. Ther., 2008, 74, 1705-1715. doi: 10.1124/mol.108.049312

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, J. P.: Reversible inhibition of the glycine transporter GlyT2 circumvents acute toxicity while preserving efficacy in the treatment of pain. Br. J. Pharmacol., 2013, 170, 1053-1063. doi:10.1111/bph.12343

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, 32, 633-45. doi:10.1124/jpet.108.136267

Mostyn, S. N., Carland, J. E., Shimmon, S., Ryan, R. M., Rawling, T., Vandenberg, R. J.: Synthesis and characterization of novel acyl-glycine inhibitors of GlyT2. ACS Chem. Neurosci., 2017, 8, 1949-1959. doi:10.1021/acschemneuro.7b00105

Mostyn, S. N., Rawling, T., Mohammadi, S., Shimmon, S., Frangos, Z. J., Sarker, S., Yousuf, A., Vetter, I., Ryan, R. M., Christie, M. J., Vandenberg, R. J.: Development of an N-acyl amino acid that selectively inhibits the glycine transporter 2 to produce

analgesia in a rat model of chronic pain. J. Med. Chem., 2019, 62, 2466-2484. doi:10.1021/acs.jmedchem.8b01775.

Motoyama, N., Morita, K., Shiraishi, S., Kitayama, T., Kanematsu, T., Uezono, Y., Dohi, T.: Relief of cancer pain by glycine transporter inhibitors. Anesth. Analg, 2014, 119, 988-995. doi: 10.1213/ANE.0000000000000388.

Moulin, D. E., Boulanger, A., Clark, A. J., Clarke, H., Dao, T., Finley, G. A., Furlan, A., Gilron, I., Gordon, A., Morley-Forster, P. K., Sessle, B. J., Squire, P., Stinson, J., Taenzer, P., Velly, A.: Pharmacological management of chronic neuropathic pain: Revised consensus statement from the Canadian Pain Society. Pain Res. Manag., 2014, 19, 328-335.

Neumann, S., Doubell, T. P., Leslie, T., Woolf, C. J.: Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature, 1996, 384, 360-364. doi.10.1038/384360a0.

Niesters, M., Martini, C., Dahan, A.: Ketamine for chronic pain: risk and benefits. Br. J. Clin. Pharmacol., 2014, 77, 357-367. doi: 10.1111/bcp.12094.

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, 352-360. doi:10.1254/jphs.09351FP

Nong, Y., Huang, Y.-Q., Ju, W., Kalia, L. V., Ahmadlan, G., Wang, J. T., Salter, M. W.: Glycine binding primes NMDA receptor internalization. Nature, 2003, 422, 302-307. doi: 10.1038/nature01497

Okie, S.: A flood of opioids, a rising tide of deaths. N. Engl. J. Med., 2010, 363, 1981-1985. doi: 10.1056/NEJMp1011512

Omori, Y., Nakajima, M., Nishimura, K., Takahashi, E., Arai, T., Akahira, M., Suzuki, T., Kainoh, M.: Analgesic effect of GT-0198, a structurally novel glycine

transporter 2 inhibitor, in a mouse model of neuropathic pain. J. Pharmacol. Sci., 2015, 127, 377-381. doi:10.1016/j.jphs.2015.02.010

Oyama, M., Kuraoka, S., Watanabe, S., Iwai, T., Tanabe, M.: Electrophysiological evidence of increased glycine receptor-mediated phasic and tonic inhibition by blockade of glycine transporters in spinal superficial dorsal horn neurons of adult mice. J. Pharmacol. Sci., 2017, 133, 162-167. doi: 10.1016/j.jphs.2017.02.011

Pal, B.: Involvement of extrasynaptic glutamate in physiological and pathophysiological changes of neuronal excitability. Cell. Mol. Life Sci., 2018, 75, 2917-2949. doi: 10.1007/s00018-018-2837-5

Paoletti, P., Bellone, C., Zhou, Q.: NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease. Nat. Rev. Neurosci., 2013, 14, 383-400. doi.org/10038/nrn3504

Perez, E., Tapanes, S. A., Loris, Z. B., Balu, D. T., Sick, T. J., Coyle, J. T., Liebl, D. J.: Enhanced astrocytic D-serine underlies synaptic damage after traumatic brain injury. J. Clin. Invest., 2017, 127, 3114-3125. doi: 10.1172/JCI92300

Petitjean, H., Pawlowski, S. A., Fraine, S. L., Sharif, B., Hamad, D., Fatima, T., Berg, J., Brown, C. M., Jan, L.-Y., Ribeiro-da-Silva, A., Braz, J. M., Bashaum, A. I., Sharif-Naeini, R.: Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury. Cell Rep., 2015, 13, 1246-1257. doi: 10.1016/j.celrep.2015.09.080

Petrenko, A. B., Yamakura, T., Baba, H., Shimoji, K.: Unaltered pain-related behavior in mice lacking NMDA receptor GluRε1 subunit. Neurosci. Res., 2003a, 46, 199-204.

Petrenko, A. B., Yamakura, T., Baba, H., Shimoji, K.: The role of N-methyl-D-aspartate (NMDA) receptors in pain: a review. Anesth. Analg., 2003b, 97, 1108-1116.

Porter, R. A., Dawson, L. A.: GlyT-1 inhibitors: from hits to chemical candidates. Top Med. Chem., 2015, 13, 51-100 doi: 10.1007/7355_2014_53.

Poyatos, I., Ponce, J., Aragon, C., Gimenez, C., Zafra, F.: The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons. Mol. Brain Res., 1997, 49, 63-70.

Qu, X.-X., Cai, J., Li, M.-J., Chi, Y.-N., Liao, F.-F., Wan, Y., Han, J.-S., Xing, G.-G.: Role of spinal cord NR2B-containing NMDA receptors in the development of neuropathic pain. Exp. Neurol., 2009, 25, 298-307. doi: 10.1016/j.expneurol.2008

Raiteri, L., Stigliani, S., Siri, A., Passalacqua, M., Melloni, E., Raiteri, M., Bananno, G.: Glycine taken up through GlyT1 and GlyT2 heterotransporters into glutamatergic axon terminals of mouse spinal cord elicits release of glutamate by heterotransporter reversal and through anion channels. Biochem. Pharmacol., 2005, 69, 159-168. doi: 10.1016/j.bcp.2004.08.029

Raiteri, L., Stigliani, S., Usai, C., Diasporo, A., Paluzzi, S., Raiteri, M., Bonanno, G.: Functional expression of release-regulating glycine transporters GLYT1 on GABAergic neurons and GLYT2 on astrocytes in mouse spinal cord. Neurochem. Internat., 2008, 52, 103-112. doi: 10.1016/j.neuint.2007.04.027

Raiteri, L., Raiteri, M.: Functional „glial" GLYT1 glycine transporters expressed in neurons. J. Neurochem., 2010, 114, 647-653. doi:10.1111/j.1471-4159.2010.06802.

Roh, D. L., Kim, H.-Y., Yoon, S.-Y., Seo, H.-S., Kwon, Y.-B., Han, H.-J., Beitz, A. J., Lee, J.-H.: Depletion of capsaicin sensitive afferents prevents lamina-dependent increases in spinal N-methyl-D-aspartate receptor subunit1 expression and phosphorylation associated with thermal hyperalgesia in neuropathic rats. Eur. J. Pain, 2008, 12, 552-563. doi: 10.1016/j.ejpain.2007.09.002

Romei, C., Bonifacino, T., Milanese, M., Usai, C., & Raiteri, L. Colocalization of neurotransmitter transporters on the plasma membrane of the same nerve terminal may reflect cotransmission. Brain Res. Bull., 2016, 127, 100-110. doi: 10.1016/j.brainresbull.2016.08.013

Roux, M. J., Supplisson, S.: Neuronal and glial glycine transporters have different stoichiometries. Neuron, 2000, 25, 373-383. doi: 10.1016/S0896-6273(00)80901-0

Sandkühler, J.: Models and mechanisms of hyperalgesia and allodynia. Physiol. Rev., 2009, 89, 707-758. doi: 10.1152/physrev.00025.2008

Schlösser, L., Barthel, F., Brandenburger, T., Neuman, E., Bauer, I. Eulenburg, V., Werdehausen, R., Harmanns, H.: Glycine transporter GlyT1 but not GlyT2, is expressed in rat dorsal root ganglion-Possible implications for neuropathic pain. Neurosci. Lett., 2015-600, 213-219. doi: 10.1016/j.neulet.2015.06.026

Schmitz, R.: Friedrich Wilhelm Sertürner and the discovery of morphine. Pharm. Hist., 1985, 27, 61-74.

Schumann-Gillett, A., O'Mara, M. L.: Lipid-based inhibitors act directly on GlyT2. ACS Chemical Neuroscience, 2018, 10, 1668-1678. doi: 10.1021/acschemneuro.8b00568.

Shaqura, M., Khalefa, B. I., Shakibaei, M., Winkler, J., Al-Khrasani, M., Fürst, S., Schäfer, M. Reduced number, G protein coupling, and antinociceptive efficacy of spinal mu-opioid receptors in diabetic rats are reversed by nerve growth factor. J. Pain, 2013, 14(7), 720-730. doi: 10.1016/j.jpain.2013.01.776.

Shibasaki, K., Hosoi, N., Kaneko, R., Tominaga, M., Yamada, K.: Glycine release from astrocytes via functional reversal of GlyT1. J. Neurochem., 2017, 140, 385-403. doi: 10.1111/jnc.13741

Sircuta, C., Lazar, A., Azamfirei, L., Baranyi, M., Vizi, E. S., & Borbély, Z. Correlation between the increased release of catecholamines evoked by local anesthetics and their analgesic and adverse effects: Role of K+ channel inhibition. Brain Res. Bull., 2016, 124, 21-26. doi: 10.1016/j.brainresbull.2016.03.009

Succar, R., Mitchell, V. A., Vaughan, C. W.: Action of N-arachidonyl-glycine in a rat inflammatory pain model. Mol. Pain, 2007, 3, 24. doi: 10.1186/1744-8069-3-24

Suo, M., Wang, P., Zhang, M.: Role of Fyn-mediated NMDA receptor function in prediabetic neuropathy in mice. J. Neurophysiol., 2016, 116, 448-455. doi: 10.1152/jn.00229.2016

Takahashi, E., Arai, T., Akahira, M., Nakajima, M., Nishimura, K. Omori, Y., Kumagai, H., Suzuki, T., Hayashi, R.: The discovery of potent glycine transporter type-2 inhibitors: Design and synthesis of phenoxymethylbenzamide derivatives,. Bioorg. Med. Chem. Lett., 2014, 24, 4603-4606. doi:10.1016/J.BMCL.2014.06.059.

Takahashi, Y., Hara, K., Haranishi, Y., Terada, T., Obara, G,, Sata, T.: Antinociceptive effect of intracerebroventricular administration of glycine transporter-2 inhibitor ALX1393 in rat models of inflammatory and neuropathic pain. Biochem. Pharmacol., Behav., 2015, 130, 46-52. doi:10.1016/jpbb.2015.01.001.

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, 929-937. doi:10.1097/ALN. 0b013e31816c9044.

Thomsen, C.: Glycine transporter inhibitors as novel antipsychotics. Drug Discov. Today, 2006, 3, 539-545. doi: 10.1016/j.ddstr.2006.10.016

Todd, A. J., Highes, D. I., Polgae, E., Nagy, G. G., Mackie, M., Ittersen, O. P., Maxwell, D. J.: The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. Eur. J. Neurosci., 2003, 17, 13-27. doi:10.1046/j.1460-9568.2003.02406.

Tong, G., Stepherd, D., Jahr, C. E.: Synaptic desensitization of NMDA receptors by calcineurin. Science, 1995, 267, 1510-1512.

Torrance, N., Smith, B. H., Bernett, M., Lee, A. J.: The epidemiology of chronic pain of predominantly neuropathic origin. Results from a general population survey. J. Pain, 2006, 7, 281-289. doi: 10.1016/j.jpain.2005.11.008

Toth, E., Weiss, B., Banay-Schwartz, M., Lajtha, A.: Effect of glycine derivatives on behavioural changes induced by 3-mercaptoproprionic acid or phencyclidine in mice. Res. Comm. Psychol. Psychiatry Behav., 1986, 11, 1-9.

Van Hecke, O., Austin, S. K., Khan, R. A., Smith, B. H., Torrance N.: Neuropathic pain in the general population: A systematic review of epidemiological studies. Pain, 2014, 155, 654-662. doi:10.1016/j.pain.2013.11.013

Vandenberg, R. J., Ryan, R. M., Carland, J. E., Imlach, W. L., Christie, M. J.: Glycine transport inhibitors for the treatment of pain. Trends Pharmacol. Sci., 2014, 35, 423-430. doi:10.1016/j.tips.2014.05-006

Vandenberg, R. J., Mostyn, S. N., Carland, J. E., Ryan, R. M.,: Glycine transporter2 inhibitors: Getting the balance right. Neurochem. Internat., 2016, 98, 89-93. doi: 10.1016/j.neuint.2015.12.007

Végh, D., Somogyi, A., Bányai, D., Lakatos, M., Balogh, M., Al-Khrasani, M., Fürst, S., Vizi, E. S., Hermann, P. Effects of articaine on [3H] noradrenaline release from cortical and spinal cord slices prepared from normal and streptozotocin-induced diabetic rats and compared to lidocaine. Brain Res. Bull., 2017 135, 157-162. doi: 10.1016/j.brainresbull.2017.10.011

Vizi, E. S., Kisfali, M., & Lőrincz, T. Role of nonsynaptic GluN2B-containing NMDA receptors in excitotoxicity: evidence that fluoxetine selectively inhibits these receptors and may have neuroprotective effects. Brain Res. Bull., 93, 32-38. doi: 10.1016/j.brainresbull.2012.10.005

Wang, D., Yu, J.: Negative regulation of REST on NR2B in spinal cord contributes to the development of bone cancer pain in mice. Oncotarget, 2016, 7, 85564-85572. doi: 10.18632/oncotarget.9447

Werdehausen, R., Mittbacht, S., Bee, L. A., Minett, M. S., Armbuster, A., Bauer, I., Wood, J. N., Herrmanns, H., Eukenberg, V.: The lidocaine metabolite N-ethylglycine has antinociceptive effects in experimental inflammatory and neuropathic pain. Pain, 2015, 156, 1647-1659. doi: 10.1097/j.pain.0000000000000206

Whitehead, K. J., Pearce, S. M., Walker, G., Sundaram, H., Hill, D., Bowery, N. G., Positive N-methyl-D-aspartate receptor modulation by selective glycine transporter-1 inhibition in the rat dorsal spinal cord in vivo. Neuroscience, 2004, 126, 381-390. doi:10.1016/j.neuroscience.2004.04.006

Wild, A R., Bollands, M., Morris, P. G., Jones, S.: Mechanisms regulating spill over of synaptic glutamate to extrasynaptic NMDA receptors in mouse substantia nigra dopaminergic neurons, Eur. J. Neurosci., 2015, 42, 2633-2643. doi: 10.1111/ejn.13075

Wiles, A. L., Pearlman, R. J., Roswall, M., Aubrey, K. R., Vandenberg, R. J.: N-Arachidonyl-glycine inhibits the glycine transporter, GLYT2a. J. Neurochem., 2006, 99, 781-786. doi:10.1111/j.1471-4159.2006.04107

Winters, B. L., Rawling, T., Vandenberg, R. J., Christie, M. J., Bhola, R., Implach, W. L.: Activity of novel lipid glycine transporter inhibitors on synaptic signaling in the dorsal horn of the spinal cord. Br. J. Pharmacol., 2018, 175, 2337-2347. doi: 10.1111/bph.14189

Wolin, R. L., Venkatesan, H., Tang, L., Santillan, A., Jr., Barclay, T., Wilson, S., Lee, D. H., Lovenberg, T. W.: Novel glycine transporter type2 reuptake inhibitors. Part 1: α-amino acid derivatives. Bioorg. Med. Chem. Lett., 2004a, 12, 4477-4492. doi: 10.1016/j.bmc.2004.05.042

Wolin, R. L., Santillan, A., Jr., Barclay, T., Tang, L., Venkatesan, H., Wilson, S., Lee, D. H., Lovenberg, T. W.: Novel glycine transporter type2 reuptake inhibitors. Part 2: β- and γ-amino acid derivatives. Bioorg. Med. Chem. Lett., 2004b, 12, 4493-4509. doi: 10.1016/j.bmc.2004.05.043

Wu, L.-J., Toyoda, H., Zhao, M.-G., Lee, Y.-S., Tang, J., Ko, S. W., Jia, Y. H.,, Shum, F. W. F., Zerbinatti, C. V., Bu, G., Wei, F., Xu, T.-L., Muglia, L. J., Chen, Z.-F., Auberson, Y. P., Kaang, B.-K., Zhou, M.: Upregulation of forebrain NMDA NR2B receptors contributes to behavioral sensitization after inflammation. J. Neurosci., 2005, 25, 11107-11116. doi: 10.1523/JNEUROSCI.1678-05.2005

Yang, X., Yang, H.-B., Xie, Q.-J., Liu, X.-H., Hu, X.-D.: Peripheral inflammation increased the synaptic expression of NMDA receptors in spinal dorsal horn. Pain, 2009, 144, 162-169. doi: 10.1016/j.pain.2009.04.005

Zafra, C., Aragon, L., Oliveres, N. C., Danbolt, C., Gimenez, J., Storm-Mathisen, J.: Glycine transporters are differently expressed among CNS cells. J. Neurosci., 1995, 15, 3952-3969.

Zafra, F., Ibanez, I., Gimenez, C.: Glycinergic transmission: glycine transporter GlyT2 in neuronal pathologies. Neuronal Signaling 2016, 1, NS20160009. doi : 10.1042/NS20160009

Zafra, F., Ibanez, I., Bartolome-Martin, D., Piniella D., Arribas-Blazquez, M., Glimenez, C. : Glycine transporters and its coupling with NMDA receptors. Adv. Neurobiol., 2017, 16, 55-83. doi:10.1007/978-3-319-55769-4.

Zhang, W., Shi, C.-X., Gu, X.-p., Ma, Z.-L., Zhu, W.: Ifenprodil induced antinociception and decreased the expression of NR2B subunits in the dorsal horn after chronic dorsal root ganglia compression in rats. Anesth. Analg., 2009, 108, 1015-1020. doi: 10.1213/ane.0b013e318193ffd2

Zeng, L., Alongkronrusmee, D., van Rijn, R. M.: An integrated perspective on diabetic, alcoholic, and drug induced neuropathy, etiology, and treatment in the US. J. Pain Res., 2017, 10, 219-228. doi:10.214/JPR.S125987

Zhou, X., Ding, Q., Chen, Z., Yun, H., Wang, H. Involvement of the GluN2A and GluN2B subunits in synaptic and extrasynaptic N-methyl-d-aspartate receptor

function and neuronal excitotoxicity. J. Biol. Chem., 2013, 288, 24151-24159. doi: 10.1074/jbc.M113.482000.