

GLYCINE TRANSPORTER INHIBITORS IN MANAGEMENT OF NEUROPATHIC PAIN

Ph.D. Thesis

Amir Mohammadzadeh

Doctoral school of Pharmaceutical Sciences

Semmelweis University



Supervisor: Mahmoud Al-Khrasani, Pharm.D., Ph.D.

Official reviewers: Kornélia Tekes, Pharm.D., D.Sc.

Eszter Ducza, Pharm.D., Ph.D.

Head of the Complex Examination Committee:

Éva Szökő, Pharm.D., D.Sc.

Members of the Complex Examination Committee:

Péter Petschner, Pharm.D., Ph.D.

Gábor Pozsgai, M.D., Ph.D.

Budapest

2022

Table of contents

Abbreviations	03
1. Introduction	
1.1. Pain transmission	05
1.2. Pain disorders	06
1.2.1. Acute and inflammatory pain	06
1.2.2. Chronic neuropathic pain	07
1.3. Glycine transporters	11
1.4. Glycine transporter inhibitors	12
2. Objectives	14
3. Materials and Methods	
3.1. Animals	15
3.2. Materials	15
3.3. Assessment of acute thermal pain	16
3.4. Assessment of acute inflammatory pain	17
3.5. Assessment of neuropathic pain	17
3.6. Assessment of motor coordination and balance	19
3.7. Assessment of glycine release	20
3.8. Assessment of glycine and glutamate content of cerebrospinal fluid and spinal cord	21
3.9. Assessment of G-protein activity	22
3.10. Statistical analysis	23

4. Results	
4.1. Sarcosine shows analgesic effect after chronic treatment in acute thermal pain model	24
4.2. Sarcosine has no impact in acute inflammatory pain evoked by formalin.....	25
4.3. Sarcosine shows dose related anti-allodynic effect in mono-neuropathic pain model	26
4.4. NFPS shows time lag and dose dependent effect in mono-neuropathic pain model	28
4.5. Org-25543 shows dose dependent effect in mono-neuropathic pain model	29
4.6. Co-administered NFPS and Org-25543 shows augmented anti-allodynic effect in mono-neuropathic pain model	30
4.7. Sarcosine, NFPS and Org-25543 failed to affect motor coordination and balance	30
4.8. Sarcosine increases glycine release	33
4.9. Combination of NFPS and Org-25543 enhances glycine content of cerebrospinal fluid	34
4.10. GlyT inhibitors or their combinations failed to activate $G_{\alpha i/o}$ -protein coupled receptors	36
5. Discussion	37
6. Conclusions	47
7. Summary	48
8. Összefoglalás	49
9. Bibliography	50
10. Bibliography of the author's publications	64
11. Acknowledgements	66

Abbreviations

ACPPB: (S)-2-amino-6-chloro-N-(1-(4-phenyl-1-(propylsulfonyl)piperidin-4-yl)ethyl)benzamide

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BK: Bradykinin

CCI: Chronic constriction injury

CE: Capillary Electrophoresis

CFA: Complete Freund's Adjuvant

CGRP: Calcitonin gene-related peptide

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

DAMGO: Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol

DH: Dorsal Horn

DMSO: Dimethyl Sulfoxide

DPA: Dynamic Plantar Aesthesiometer

DRG: Dorsal Root Ganglion

EPSP: Excitatory post synaptic potential

GABA: Gamma-aminobutyric acid

GC: Glycinergic Cell

GDP: Guanosine 5'-Diphosphate

Glu: Glutamate

Gly: Glycine

GlyR: Glycine Receptor

GlyT: Glycine transporter

GTP: Guanosine 5'-Triphosphate

HPLC: High Purity Liquid Chromatography

IASP: International Association for the Study of Pain

IPSP: Inhibitory post synaptic potential

i.p.: Intraperitoneal

i.pl.: Intraplantar

i.t.: Intrathecal

i.v.: Intravenous

K2P: Two pores potassium channel

MOR: μ -opioid receptor

MPFC: Medial Prefrontal Cortex

NAGly: N-arachidonyl Glycine

NFPS: (N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine)

NGF: nerve growth factor

NMDA: N-methyl-D-aspartate

NMDAR: NMDA Receptor

NSAID: Non-Steroid Anti-Inflammatory Drug

Org-25543:(4-benzyloxy-3,5-dimethoxy-N-[1-(dimethylaminocyclopentyl)-methyl] benzamide)

p.o.: Per os

PKC γ : Protein Kinase C γ

pSNL: Partial Sciatic Nerve Ligation

PV: Parvalbumin

PWT: Paw Withdrawal Threshold

s.c.: Subcutaneous

SNL: Sciatic Nerve Ligation

SP: Substance P

TCC: Transient Central Cell

TRP: Transient receptor potential

TRPM8: Transient receptor potential Melastatin 8

TRPV1: Transient Receptor Potential Vanilloid 1

VC: Vertical Cell

WDR: Wide Dynamic Range

1.Introduction

1.1. Pain transmission

The negative impact of chronic pain in many medical conditions is of no dispute, yet its direct and indirect effects on the society regarding enforcing economic and social burdens makes it a multidimensional problem. Based on the definition of International Association for the Study of Pain (IASP), “pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage”. Pain is categorized to acute and chronic pain. It is estimated that 20% of population worldwide are struggling with chronic pain condition [1, 2]. In spite of high level of occurrence of chronic pain, current medical and pharmacological interventions such as using non-steroid anti-inflammatory drugs (NSAIDs), opioids, gabapentinoids, Na⁺-channel blockers, anti-depressants (mostly serotonin and norepinephrine reuptake inhibitors), topical capsaicin or lidocaine, spinal injection of steroids or local anaesthetics, neuro-stimulation and even surgical intervention are either insufficient for overcoming the pain or their application is limited by their undesirable effects [3, 4]. In this regard investigation of novel compounds such as glycine transporter (GlyT) type 1 and 2 inhibitors in management of neuropathic has been initiated [15, 16, 35].

Nociceptors are sensory receptors of free nerve endings that detect mechanical, thermal and chemical signals occurring at the periphery. The perception of different pain modalities are carried out by primary sensory nerve fibers that have cell bodies located in the dorsal root ganglia (DRG) or in the trigeminal ganglia. In respect to primary afferent (first order) pain neurons that have cell bodies located at DRG, they carry the pain from the periphery to the Rexed lamina I and II in the dorsal horn (DH) of the spinal cord where they synapse with secondary afferent (second order) neurons. The first order pain neurons are either myelinated (A- δ fibers) or unmyelinated (C fibers, polymodal-nociceptive neurons). Next, the second order neurons transmit the pain signal through spinothalamic tract to the reticular formation, thalamus, and mesencephalon where the major process of pain perception occurs. From there, third order (tertiary) neurons branch more connections between lower regions and higher regions of brain such as somatosensory cortex. The modulation of this transmission is through spinal excitatory

and inhibitory interneurons and descending inhibitory pathways. The majority of spinal neuronal excitation is stemmed from the activity of glutamatergic neurons. Glutamatergic neurons by release of the neurotransmitter glutamate activate AMPA and NMDA receptors of post-synaptic membrane, which leads to excitatory post synaptic potential (EPSP). Neuronal inhibition is mostly by the activity of glycinergic, GABAergic and opioid neurons. The released glycine, GABA and endogenous opioid bind to their post-synaptic receptors, namely glycine receptor (GlyR), GABA receptor and opioid receptors respectively, which in turn results in inhibitory post synaptic potential (IPSP) [5, 6].

There are two types of pain regarding the transmission: the first type is the fast pain which is transmitted with velocity of 6 to 30 m/s. The second type is the slow type of pain with 0.5 to 2 m/s of velocity of transmission. Fast pain is more involved in nociceptive type of pain where myelinated A δ fibres are the primary neurons. The slow type of pain that is more involved in sensation of chronic pain and internal pain, which is transmitted primarily by unmyelinated C-fibres. Another type of neurons which are important regarding the neuropathic pain is A β fibres. They are myelinated and fast velocity type of sensory neurons which are responsible for transmission of touch and light pressure signals, but in pathological conditions they contribute to pain sensation [5, 7].

1.2. Pain disorders

1.2.1. Acute and inflammatory pain

Pain is categorized as acute and chronic. In general, acute pain is the result of nociception by any type of pain stimulus within a short duration of time (milliseconds and seconds) while chronic pain is the result of persistence of pain in longer periods of time. As it was discussed in previous section, the high velocity A- δ fibres are related more to transmission of acute type of pain while C-fibres with low velocity are more involved in chronic nociception. However, it is also important to note that sometimes pain have both fast and slow components regarding its transmission. Three types of stimuli for pain induction are mechanical, thermal and chemical stimuli. Mechanical stimuli can affect the stretch of membranes of nociceptors which causes opening of specific sodium channels or activation of some G-protein coupled receptors. Transient receptor potential

(TRP) receptor superfamily is one major type that has been studied for all three types of stimuli. Mechanical stimuli can be external like pressure on skin or visceral such as damage to periosteum. Thermal pain affects nociceptors by increase or decrease of the temperature beyond the physiological tolerance. One of the suggested mechanisms of activation of nociceptors by extreme thermal changes is the change in metabolic rate of cells. The TRP Vanilloid type 1 (TRPV1) receptors are important in regard to transduction of acute thermal pain, while TRP Melastatin 8 (TRPM8) is recognised for its ability of transduction of cold pain. Two-pores potassium channels (K2P) and voltage gated sodium channels also have been linked to sensation of thermal pain. Chemical induction of nociceptors is either through the activation of specific receptors by their agonists or direct disturbance of cellular membrane and increasing the cellular permeability. These chemicals can be external factors such as acids and toxins or endogenous substances from inflammation or damage to tissue. Inflammation leads to release of different types of inflammatory mediators from peripheral tissue or neurons themselves. Some of these inflammatory substances act as agonist for nociceptors. The most important noted endogenous nociceptive inflammatory factors are prostaglandins, leukotrienes, bradykinin, serotonin, histamine, calcitonin gene-related peptide (CGRP), substance P (SP), purines such as ATP, protons, free radicals, lipids, cytokines, chemokines, and neurotrophins such as nerve growth factor (NGF). Bradykinin is known for its direct effect on nociceptors for induction of pain, while prostaglandin plays a role in sensitization of neurons. Different compounds are being used for acute pain. NSAIDs by inhibition of cyclooxygenase enzyme reduce the inflammatory factors and lidocaine by inhibition of sodium channels reduces the pain [5, 94].

1.2.2. Chronic neuropathic pain

Neuropathic pain is “pain caused by a lesion or disease of the somatosensory nervous system.” based on the definition provided by IASP [1]. Neuropathic pain can be generated as a primary condition either from a direct damage to neurons known as trauma, neuroma, and degeneration of neuron, or from a neurological disease (for example Guillain-Barré syndrome and Chronic inflammatory demyelinating polyneuropathy). It can also be generated as a secondary condition after diabetes, herpes zoster, cancer, chemotherapy-induced neuropathy (platinum-, taxanes-, and vinca alkaloids-derived drugs), chronic

infection or after some surgeries. Neuropathic pain is characterised by spontaneous pain, allodynia (pain due to a stimulus that does not normally provoke pain) and hyperalgesia (increased pain from a stimulus that normally provokes pain) [7, 8]. In practical setting it means that sensation of pain after an innocuous stimuli such as fine pressure in case of allodynia and over sensation of pain after a noxious stimuli in case of hyperalgesia.

The principle mechanisms that attribute to the generation of neuropathic pain rely on the modulations in molecular levels and neuroplasticity of neurons in periphery, CNS or both. An example of molecular modifications is when over-stimulation of neurons during chronic pain causes hypersensitivity in primary afferent nociceptive neurons after a phenotypic switch of some subtypes of calcium channels, sodium channels, bradykinin (BK) B1 receptors and capsaicin TRPV1 receptors by upregulation on myelinated primary sensory fibres, while B2 receptors, substance P receptors and some subtypes of sodium channels and opioid receptors undergo downregulation on unmyelinated primary sensory fibres [9]. Many studies have emphasized on the importance of neuronal modifications in dorsal horn (DH) of the spinal cord, the main location for the interactions of first order neurons (C and A δ), second order sensory neurons and interneurons in Lamina I (superficial layer), II (substantia gelatinosa) and III (deep dorsal horn), and the location of A β sensory neurons and wide dynamic range (WDR) projection neurons in lamina III and VI (deep dorsal horn), in development of neuropathic pain [10, 11, 12]. Spinal inhibition in dorsal horn is achieved mostly by glycinergic and GABAergic interneurons. Spinal glycinergic pathway is a major player in the regulation of nociception in dorsal horn of the spinal cord. The concentration of glycinergic innervation is high in deep dorsal horn, and 75% of the inhibition in superficial dorsal horn is achieved through glycinergic pathway as well. Glycinergic neurons generate IPSP on pre and post synaptic membranes of spinal neurons, and hence decreases the degree of pain transmission. In neuropathic conditions however, this regulatory mechanism is compromised, and it fails to establish the level of inhibition required in normal condition and in this way, it contributes to the development of neuropathic pain [9, 12, 13, 14].

One of the main explanations for development of neuropathic pain is based on the gate theory of pain. As it is depicted in Figure 1, spinal inhibition in dorsal horn by parvalbumin positive glycinergic interneurons occurs on protein kinase C γ (PKC γ) expressing excitatory interneurons in lamina II, and hence it reduces the level of EPSP on

secondary afferent neurons. In this way glycinergic interneurons participate in reduction of pain signalling. On the other hand, both PKC γ and glycinergic interneurons could get stimulated by low-threshold mechanosensitive A β fibres. In neuropathic pain condition however, this balance is compromised by reduction in glycinergic activity and elevation in number and activity of PKC γ cells. These changes result in either over-stimulation of pain signalling or stimulation of nociceptive pathway by A β fibres following innocuous stimuli (Allodynia) [15, 16, 17, 29, 35].

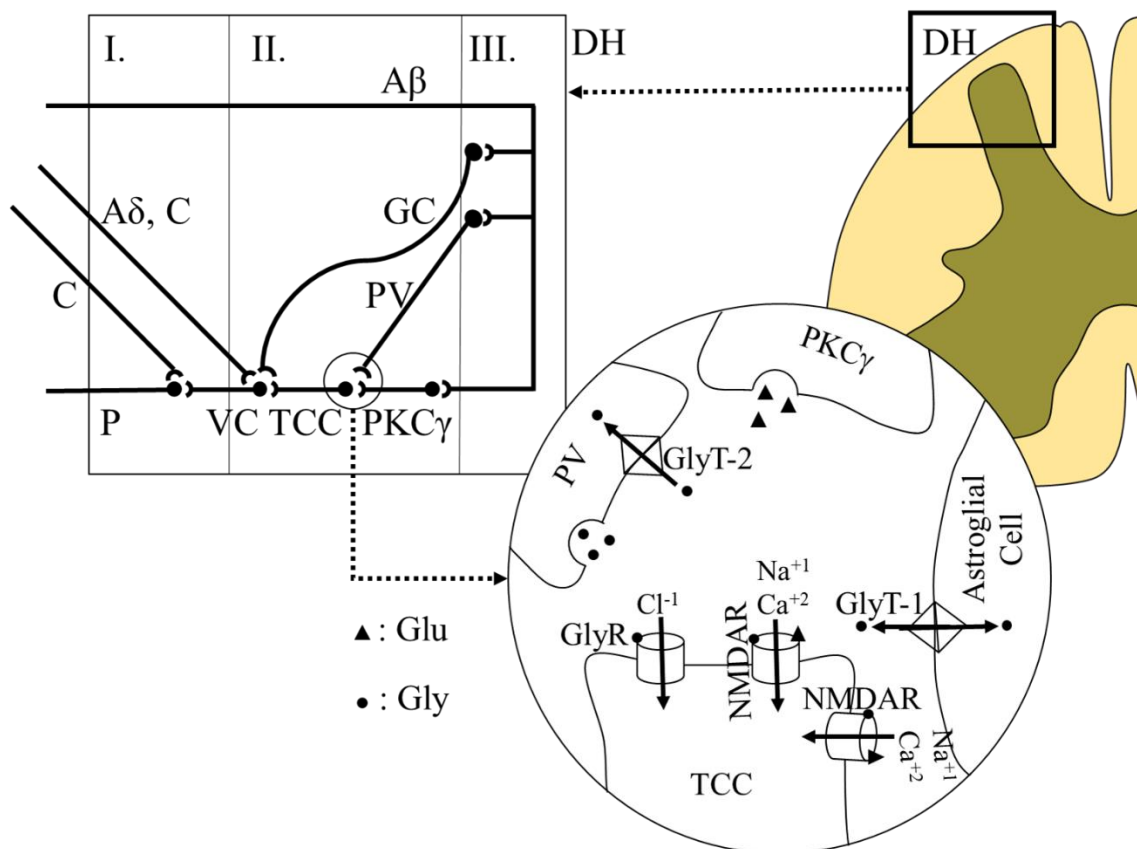


Figure 1. The schematic view of neuronal connections in dorsal horn (DH) laminae I, II, and III. The A β neuron synapses on excitatory and inhibitory interneurons. The activity of excitatory PKC γ expressing interneuron stimulates excitatory transient central cell (TCC) which synapse to excitatory vertical cell (VC) and in consequence VC stimulates secondary afferent neuron (P). On the other hand, A β synapse on glycinergic cell (GC) and parvalbumin (PV) expressing glycinergic cell in which they inhibit excitatory interneurons. A δ and C fibers are primary afferents which directly activate the nociceptive pathway. A schematic of synaptic process is presented as well. Glycinergic neuron by release of glycine activate GlyR, and glutamatergic neurons by release of glutamate, activate synaptic and extra-synaptic NMDA receptor (NMDAR). Activation of these channel-receptors opens the flow of negative and positive charge ions which lead to IPSP and EPSP. Bidirectional GlyT-1 on astroglial cells and unidirectional GlyT-2 on glycinergic neurons regulate the synaptic and extra-synaptic level of glycine [15, 35, 102, 103, 104].

Glycine receptors (GlyRs) are a subfamily of ionotropic receptors superfamily. The most occurring type of GlyR in the spinal cord is GlyR α 3. At receptor level, once glycine release occurs the following events have been reported. It binds to GlyRs, activates them, and allow the entrance of Cl⁻ to the intracellular space which in turn produce IPSP. Glycine also functions as the obligatory co-agonist for ionotropic NMDA receptors. Upon binding of glycine or D-serine simultaneously with glutamate and upon the removal of Mg²⁺ from the channel, the flow of Ca²⁺ and Na⁺ to the intracellular space occurs and consequently EPSP is developed. NMDA receptor has different isoforms. NR1/NR2 is the main form in spinal cord, in which three of its subtypes are more expressed in dorsal horn: NR2A, NR2B, and NR2D. NR1 subunit is the location for the binding of the obligatory co-agonist which is either glycine mostly for extra-synaptic NR1/NR2B subtype or D-serine mostly for synaptic NR1/NR2A subtype [19, 20, 21].

It has been shown that following nerve injury, the level of extra-synaptic NR1/NR2B is up-regulated, while the synaptic NR1/NR2A either remains in the same level or it is down-regulated [22, 23]. This may be the consequence of over activation of central sensory fibres following the increase in glutamate release. The elevation of glutamate level causes desensitization of synaptic NR1/NR2A NMDA receptors and the spill over of glutamate to extra-synaptic space [27, 32, 33]. In this way, in chronic pain, the activity of NR1/NR2B, which is expressed highly in lamina II, is increased by the spilled overed glutamate to extra-synaptic area [24, 26]. Antinociception from NR1/NR2B NMDAR antagonists, CP-101,606 and ifenprodil in nerve injury model points to the major role of this sub-type of NMDA receptor in neuropathic pain [25, 34]. On the other hand, NR1/NR2A gene deletion did not change pain behaviours in animal neuropathic pain model from nerve injury, showing that this sub-type of NMDA receptor doesn't play a major role in pathological over-stimulation of secondary afferent neurons. But still, the desensitization of post-synaptic NR1/NR2A receptors on glycinergic interneurons may play a part in loss of glycinergic inhibition in dorsal horn of spinal cord [25, 32, 33].

Following this overview, the role of glycine in over-stimulation of NMDA receptors during neuropathic pain comes into the focus. In chronic constriction nerve injury, astroglial cells become activated and lead to reverse mode of action of astroglial GlyT-1, which causes more release of glycine in extra-synaptic space. Also, GlyT-1 expression after nerve injury is increased in initial days of nerve injury but it decreases from the third

day, and hence the level of glycine increases. Elevation of glycine and glutamate is detected on day 7 and 14 following nerve injury [28, 30, 31].

Based on above-mentioned facts, we could rationalise that during neuropathic pain, the increase in spinal glutamate and glycine content, the increase in activity and expression of extra-synaptic NR1/NR2B NMDA receptors and the decrease in activity of synaptic NR1/NR2A NMDA receptors lead to the elevation in firing of excitatory glutamatergic interneurons and the reduction in firing of inhibitory glycinergic interneurons in dorsal horn of spinal cord. This imbalance between inter-neuronal excitation and inhibition is in favour of excitatory transmission of pain, and hence it generates neuropathic pain. In this regard, inhibition of glycine transporters seems to be a reasonable method to overcome neuropathic pain, for two reasons: First, to restore the glycine level required for recovery of inter-neuronal inhibition, and second, to block the glycine release to extra-synaptic space in order to reduce inter-neuronal excitation. Antinociceptive effects of GlyT inhibitors have been proven in different studies. [15, 16, 35].

1.3. Glycine transporters

Glycine transporters (GlyTs) belong to solute carrier 6 (SLC6) Na⁺-Cl⁻-dependent membrane transporter family. There are two types of glycine transporters namely, glycine transporter type 1 (GlyT-1) and Glycine transporter type 2 (GlyT-2). The former transporter is expressed highly on astroglial cells, and it is responsible for the clearance of glycine mostly from extra-synaptic space. On the other hand, the later transporter (GlyT-2) is a glycinergic pre-synaptic membrane restricted type, and it is responsible for the reuptake of glycine from synaptic cleft to refill the vesicles. GlyT-1 cotransports 2 Na⁺ and 1 Cl⁻ with glycine bi-directionally, whereas GlyT-2 cotransports glycine, 3 Na⁺ and 1 Cl⁻ unidirectionally. Both types of transporters express an extracellular loop between third and fourth transmembrane domains. Variants of each type shows differences in N and C-terminals of the receptor from alternative splicing [36, 37]. The role of C-terminal is in trafficking and recycling of the transporter. It has been suggested that N-terminal is involved in reverse mode of the transporter. Beside that, N-terminal may play a role in stabilization of the transporter [38].

Both types of glycine transporters exist in the spinal cord, brain stem and cerebellum. However, the GlyT-2 is more expressed in caudal areas of the brain and in the spinal cord. GlyT-1, beside the abovementioned locations, exist in retina, hippocampus, pre-frontal cortex, hypothalamus, and olfactory bulb. GlyT-1 sometimes is not in the vicinity of glycinergic neurons, but instead it is near NMDA receptors to play a role in regulation of their activity by changing the available glycine level around NMDA receptors, since glycine by binding to the allosteric glycine-B binding site of NMDA receptor, acts as an obligatory co-agonist [12, 35, 43]. In regard to differences in location and function of each type of glycine transporters, appearance of different phenotypes from their deletion is expectable. Mice lacking GlyT-1 showed reduction in muscle strength and appearance of respiratory dysfunction [90, 91]. Furthermore, mice lacking GlyT-2 showed tremor and motor dysfunction [93].

1.4. Glycine transporter inhibitors

Based on the selectivity and affinity, glycine transporter inhibitors can be divided into irreversible selective GlyT-1 and GlyT-2 inhibitors or reversible and partial inhibitors of GlyT-1 and 2. In regard to pain research the following irreversible selective GlyT-1 inhibitors have been thoroughly studied: NFPS is also known as ALX5407, Org-25935, bitopertin and N-ethylglycine. Irreversible GlyT-2 inhibitors such as ALX1393 and Org-25543 were also studied in different animal pain models for their analgesic effects. Examples of reversible GlyT-1 inhibitor is sarcosine (N-methyl glycine), and example of reversible GlyT-2 inhibitor is Narachidonylglycine (NAGly) [15, 35, 51, 81].

NFPS (N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine) is a synthetic irreversible inhibitor of GlyT-1 with high degree of selectivity and potency (dissociation $t_{1/2}$: 28 ± 5 min, $IC_{50} = 3$ nM for hGlyT-1). NFPS in dose of 0.3 mM has been reported to inhibit the glycine-induced glycine release by blockage of both direction of glycine movement in GlyT-1. It inhibits GlyT-1 in dose dependent manner [44, 45, 46, 72]. In animal pain models, systemic doses of ALX5407 (NFPS) have shown anti-hyperalgesic and anti-allodynic effects in a neuropathic pain animal model created by nerve constriction injury in rats. In this study, time and dose dependent analgesic effects were achieved on day 4 following treatment initiated 14 days after operation [47]. In another study application of NFPS (i.t.) produced acute antinociception against allodynia in mice

with pSNL or diabetic neuropathic pain, as well as in formalin test [48]. In regard to the side effects, treatment with high doses of NFPS (10-30 mg/kg i.p.) was associated with ataxia, akathisia, respiratory depression and coma in mice [45, 53, 77]. Another example for GlyT-1 inhibitors is bitopertin, which in systemic dose of 1 mg/kg its antiallodynic effect was proved for acute and chronic treatments in mice [75, 81].

Org-25543(4-benzyloxy-3,5-dimethoxy-N-[1-(dimethylaminocyclopentyl)-methyl] benzamide) is an irreversible and selective inhibitor of GlyT-2 with a high potency ($IC_{50} = 16$ nM for hGlyT-2). It is a lipophilic compound with free penetration to CNS and high bonding to brain tissue with only 2% unbound compound available at initial phase after injection [49, 55, 56]. Org-25543 has been reported to produce analgesic effect in the mouse formalin test, pSNL neuropathy, diabetic neuropathy and CFA-induced inflammatory pain models following systemic and/or i.t. administration [43, 49, 50]. ALX1393 (GlyT-2 inhibitor) was also shown to produce analgesic effect in the rat neuropathic pain model following systemic administration [50]. Indeed, severe side effects such as convulsions and lethality have also been associated with dose escalation (e.g. Org-25543 in dose of 20mg/kg i.v. in mice) [49].

On the other hand, few studies have been conducted to assess the analgesic effect of reversible glycine transporter inhibitors. Sarcosine (N-methyl glycine, $IC_{50}=30-60$ μ M) is a reversible endogenous antagonist and substrate for GlyT-1 which can activate the reverse mode of transporter as well [30, 39, 40, 41, 42]. Systemic acute or chronic sarcosine has ameliorated mechanical allodynia in neuropathic rodents. Strychnine (GlyR α 3 antagonist) treatment or knockdown of spinal GlyR α 3 abolished the analgesic effects of sarcosine [43, 54]. A modified analogue of Org-25543 (compound 1) and Narachidonylglycine (NAGly), reversible GlyT-2 inhibitors, showed anti-allodynic effect in neuropathic rodents without side effects [49, 52].

The above-mentioned studies suggest that either GlyT-1 or GlyT-2 inhibitors display analgesic effect, however, appearance of side effects limits their clinical applicability. To the best of our knowledge no study has examined the analgesic effects of a combination of irreversible GlyT-1 and GlyT-2 inhibitors. Therefore, strategy based on the co-administration of sub-analgesic doses of these inhibitors might increase the analgesic effect with possible reduced occurrence of side effects.

2. Objectives

The aims of the study are as follows:

- 1- Evaluation of antinociceptive effect of acute and chronic systemic administration of a reversible GlyT-1 inhibitor (sarcosine) in different pain models (acute thermal pain, acute inflammatory pain, and mono-neuropathic pain model).
- 2- Evaluation of anti-allodynic effects of acute and chronic systemic administration of NFPS and Org-25543, selective and irreversible GlyT-1 and 2 inhibitors respectively in different doses, and the anti-allodynic effect of acute systemic co-administration of sub-analgesic doses of both GlyT-1 and 2 inhibitors (NFPS and Org-25543) in mono-neuropathic pain model.
- 3- Examination of motor coordination and balance following administration of effective analgesic doses of reversible and irreversible GlyT-1 inhibitors (sarcosine and NFPS) and irreversible GlyT-2 inhibitor (Org-25543), and co-administration of sub-analgesic doses of irreversible GlyT-1 and 2 inhibitors (NFPS and Org-25543).
- 4- Evaluation of effect of GlyT-1 inhibitors (sarcosine, NFPS, ACPPB) on the level of in vitro glycine release.
- 5- Comparison of glycine content in cerebrospinal fluid (CSF) between sham operated animals and neuropathic animals treated with vehicle or sub-analgesic doses of NFPS and Org-25543 either separately or in combination (co-administration). Comparison of CSF L-glutamate level between sham and neuropathic animals either treated with combination or with vehicle. Also, the comparison of glycine and L-glutamate content of spinal tissue (Lumbar 4-6) between neuropathic and non-neuropathic (sham) animals.
- 6- Assessment of G-protein activity for all applied test compounds.

3. Materials and Methods

3.1. Animals

For behavioural tests on sarcosine, male Wistar rats (250-300 g for all pain model tests and 170-250 for rotarod test) were obtained from the local animal house (Semmelweis University, Budapest, Hungary). For behavioural tests on effects of NFPS and Org-25543 male Wistar rats (250-300 g for mono-neuropathic pain model, capillary electrophoresis measurements and 170-250 for rotarod test) were obtained from Toxi-Coop Zrt. (Budapest, Hungary). For G-protein binding assay both male and female (50-50%) Wistar rats (250-300 g) were received from local animal house of the Biological Research Centre, Hungarian Academy of Sciences (Szeged, Hungary). Animals were kept in euro-standard cages in numbers of 4-5 animals/cage depending on the weight of animals, in a room of ($20 \pm 2^\circ\text{C}$) temperature, for 12-h/12-h of light/dark cycle in the local animal house of Semmelweis University, Department of Pharmacology and Pharmacotherapy (Budapest, Hungary). In case of G-protein binding assay animals were kept at a temperature-controlled room ($21 \pm 4^\circ\text{C}$) under a 12h/12h light and dark cycle in local animal house of the Biological Research Centre, Hungarian Academy of Sciences (Szeged, Hungary). Water and standard food were available ad libitum.

All housing and experiments were performed in accordance with the European Communities Council Directives (2010/63/EU), the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§) and local animal care committee (PEI/001/276-4/2013 and PE/EA/619-8/2018). Experimenters put their best efforts to reduce the number and suffering of animals.

3.2. Materials

Sarcosine hydrochloride was obtained from Sigma-Aldrich Ltd (Budapest, Hungary), and normal saline was used as its vehicle. NFPS(*N*-[3-([1,1-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine), Org-25543(*N*-[[1-(dimethylamino)cyclopentyl]

methyl]-3,5-dimethoxy-4-(phenylmethoxy)benzamide hydrochloride) were purchased from Bio-Techne R&D System Kft (Budapest, Hungary), and 20% DMSO in normal saline was used as the vehicle. All solutions for behavioral tests were prepared in 2.5 ml/kg volumes. For the capillary electrophoresis analysis glycine, L-cysteic acid, HEPES, acetonitrile and boric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Fluoro-7-nitrobenzofurazan (NBD-F) was obtained from Tokyo Chemical Industry (Tokyo, Japan) and hydroxypropylamino- β -cyclodextrin was provided by Cyclolab Ltd. (Budapest, Hungary). Ultrapure water from MilliQ Direct 8 water purification system (Merck Millipore, Billerica, MA, USA) was used for all experiments.

For G-protein activity assay DMSO, Tris-HCl, EGTA, NaCl, $MgCl_2 \times 6H_2O$, GDP and the GTP analog (GTP γ S) were purchased from Sigma-Aldrich (Budapest, Hungary). Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) which is a highly selective μ -opioid receptor (MOR) agonist enkephalin analog, was obtained from Bachem Holding AG (Budapest, Hungary). The radiolabelled GTP analog, [^{35}S] GTP γ S (specific activity: 1250 Ci/mmol, Lot: 0119) and the UltimaGoldTM MV aqueous scintillation cocktail was purchased from PerkinElmer (handled by Per-Form Hungaria Kft, Budapest, Hungary).

All compounds were stored and handled as described in the product information sheets.

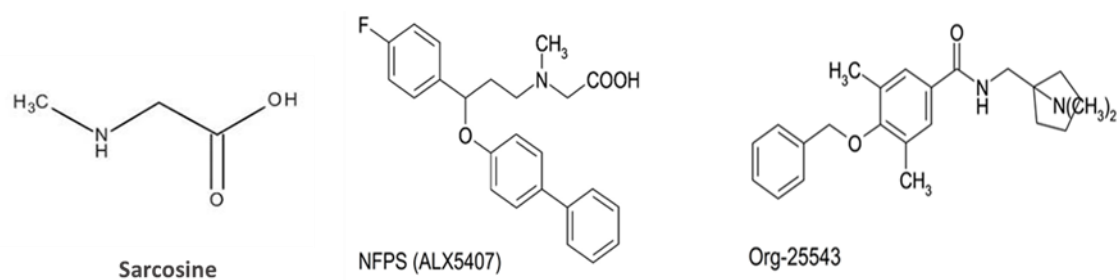


Figure 2. Chemical structural formulas of applied compounds in this study, sarcosine (reversible GlyT-1 inhibitor), NFPS and Org-25543 (irreversible GlyT-1 and 2 inhibitors respectively) [35].

3.3. Assessment of acute thermal pain

To test the effects of sarcosine on acute thermal pain, tail-flick test was performed as it was described before [61]. In this model a beam of light was focused on the dorsal root of the tail of the rat, and when the rat flicked the tail, the time latency in seconds (s) was

written. To avoid damage, 8 seconds cut-off was considered. The anti-nociceptive effect was calculated as a percentage of baseline values (see the formula below). The arbitrary cut-off time was two times of the baseline. The test was performed for the morning doses of sarcosine (500 mg/kg and 1000 mg/kg s.c.) or saline after acute or on day 4 and 8 of chronic treatment (2 times a day for 8 days). In each test, measurements were performed before (baseline, shown as 0' in figures) and after the treatment in time-points of 30', 60', 120', and 180'. Since the acute pain was the objective of this investigation, a broad range of time was considered for chronic treatment.

$$\text{Analgesia (\%)} = \frac{\text{Latency after treatment} - \text{Latency before treatment}}{\text{Latency before treatment}} \times 100$$

3.4. Assessment of acute inflammatory pain

For the assessment of acute inflammatory pain formalin test was used as explained previously [62, 63]. Animals were treated with sarcosine (500 mg/kg s.c.) or saline, and 15' and 180' after treatment, formalin (2.5%, 20 μ L i.pl.) was injected into the right hind paws and animals were placed in a glass box and immediately the number of nociceptive reactions (flinching, licking, elevating of right paws) were measured for each five minutes until 60 minutes. There are two phases of pain induction in formalin test. Phase 1 is the first 10 minutes after the injection of formalin, which is related to the induction of acute pain, and phase 2 is from 10 minutes to about 60 minutes from injection of formalin, which is the consequence of inflammation [64]. To check the chronic effect of sarcosine on acute inflammatory pain a separate group of animals were treated with sarcosine (500 mg/kg s.c.) or saline two times a day and following the morning dose of day 4 the formalin test was performed (formalin injection 180' after injection of sarcosine) in the same way.

3.5. Assessment of neuropathic pain

To induce the mono-neuropathic pain, partial sciatic nerve ligation (pSNL) method was used. In this method based on the Seltzer nerve ligation method, as described before [58, 59], animals undergone anesthesia with 60 mg/kg (i.p.) pentobarbital (in a 2.5 ml/kg volume) and then they were placed on a heating pad (set to 30°C), and under aseptic

conditions the surgery was performed. In this method, sciatic nerve of right leg after exposure (without any muscle damage) at the thigh-high level was tightly ligated with an 8-0 silicon-treated silk suture to trap the dorsal 1/3-1/2 of the nerve thickness. The wound was closed with 2 stiches. In case of sham animals, which represented the absolute control for evaluation of neuropathy development, the nerve was exposed without any ligation. Right legs were the operated side (neuropathic leg, shown as R in figures), and left legs were the unoperated side (healthy leg, shown as L in figures).

To assess mechanical allodynia, paw withdraw threshold (PWT) was measured in grams (g) by DPA (dynamic plantar aesthesiometer 37450; Ugo Basil, Italy). The method of measurement is a modified electronic Von-Frey model based on previous publication, [59, 60] after 5 minutes of habituation of animals in cages of instrument, the centres of paws alternately (right and left) were pressed by a metal filament of 0.5 mm diameter in the instruments, with a force rising from 1 to 50 g (50 g represented the cut-off value) based on the manufacturer's guidelines. Each paw was measured three times in each trial and the averages were used for further analysis. Animals were tested before the surgery and 7 and 14 days after the operation, to evaluate the pain progression. Those animals with 20% reduction in the PWT of operated right paws in comparison to the healthy left paws were chosen for further use in the main experiment. Lack of development of pain in sham operated group proved the correctness of pSNL operation.

In the morning of day 1 of the main experiment (14 days after operation), after baseline measurement (shown as 0' in figures) animals were injected subcutaneously by compounds (sarcosine, NFPS and/or Org-25543) or their vehicles, and then PWT values were measured by DPA at 30', 60' and 180' post-treatment. In case of chronic test, treatments were continued (twice a day for sarcosine and once a day for NFPS and Org-25543) in subsequent days and the measurement was repeated for the morning doses of day 4 and 6 (17th and 19th day from operation, respectively) in the same manner.

Treatments of compounds were as follows: sarcosine (500 and 1000 mg/kg s.c.), NFPS (1, 2 and 4 mg/kg, s.c.), Org-25543 (2 and 4 mg/kg, s.c.), co-administration of NFPS (1 mg/kg s.c.) and Org-25543 (2 mg/kg s.c.), versus their vehicles (normal saline for sarcosine and 20%DMSO for NFPS and Org-25543). Tests for chronic treatments were

performed on both doses of sarcosine and low doses of other compounds (NFPS 1 mg/kg, Org-25543 2 mg/kg s.c.). Figure 3 presents a schematic depiction for this protocol.

Protocol of assessment of allodynia

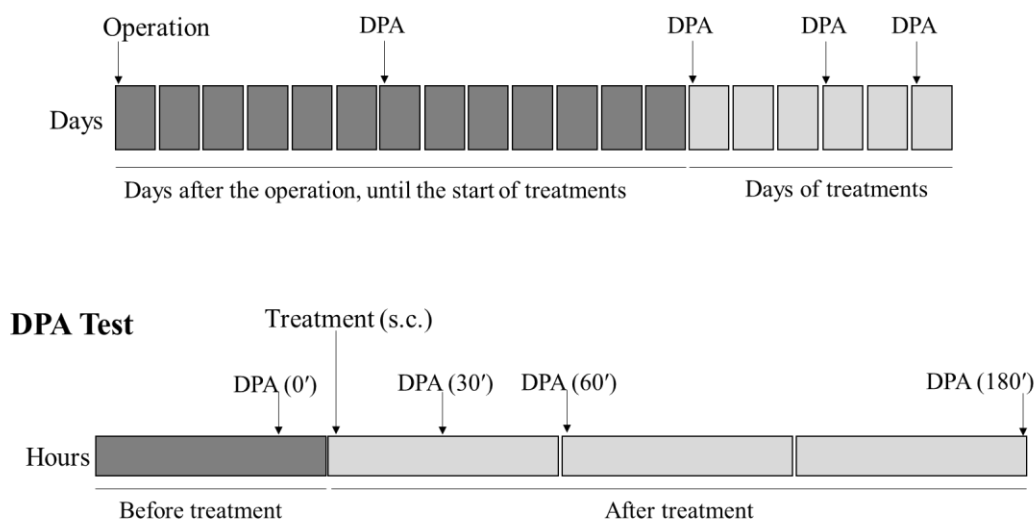


Figure 3. Schematic representation of the experimental protocol for the assessment of allodynia by DPA on pSNL operated animals. After operation, a pilot DPA test was performed to evaluate the pain progression 7 days after operation. 14 days after operation, those animals which developed neuropathic pain were considered for the experiment with the acute treatment with compounds (day 1 of treatment). Tests on chronic treatments were on day 4 and 6 for morning doses. The method of measurement of paw withdraw threshold (PWT) was with DPA (Dynamic plantar aesthesiometer) in grams to assess allodynia before treatment (baseline, shown as 0') and at different time points after the treatment (30', 60' and 180').

3.6. Assessment of motor coordination and balance

In this study, the effect of compounds on motor coordination and balance was evaluated by rotarod test (Rat rotarod, Model 7750; Ugo Basile). The method of measurement was based on previous descriptions [65] and the information sheet of the instrument, with some modifications. In this method animals were located on the rotating cylinder of the instrument and the fall-off time (time latency) was noted in seconds. The speed of rotation was 16 rpm and cut-off time was 180 seconds. Animals were trained one day before the experiment, and on the next day they were tested at different time points after treatment (based on peak effects of each compound in DPA test) for the morning doses and fall-off times were captured. Sarcosine (500 and 1000 mg/kg s.c.) versus vehicle was tested at 60'

for dose 500 mg/kg and at 180' for dose 1000 mg/kg after acute treatment and on day 4 of the chronic treatment (2 times a day for 4 days). NFPS (2 and 4 mg/kg s.c.), Org-25543 (4 mg/kg s.c.), and the co-administration of their sub-analgesic doses (NFPS 1 mg/kg and Org-25543 2 mg/kg s.c.) versus vehicle were assessed at 180' post-treatment. In a separate test, NFPS (4 mg/kg s.c.) and Org-25543 (4 mg/kg s.c.) were measured at 60' post-treatment as well. morphine (6.4 mg/kg s.c.) was tested at the time of its peak effect (30' post-treatment) taken as the positive control for the method.

3.7. Assessment of glycine release

Glycine release was evaluated based on previous descriptions [46]. Rats were decapitated under anaesthetic conditions with isoflurane gas and brain tissues were collected. Hippocampus slices in range of 350 μ m thick were prepared using a McIlwain tissue chopper (The Mickie Laboratory Engineering, Gomshall, UK). Then samples were placed in ice-cold Krebs–bicarbonate buffer, pH 7.4 with the following composition (in mM): NaCl 118, KCl 4.7, CaCl₂ 1.25, NaH₂PO₄ 1.2, MgCl₂ 1.2, NaHCO₃ 25, glucose 11.5, under continuous gas exposure (95% O₂/5% CO₂). After the incubation of samples with [³H]glycine (10 μ Ci/ml) in Krebs–bicarbonate buffer for 30 minutes at 37°C, the tissues were transferred into low volume (0.3 ml) superfusion chambers (Experimetria, Budapest, Hungary) and superfused with aerated and preheated (37°C) Krebs–bicarbonate buffer. The flow rate was kept at 1 ml/min by a Gilson multichannel peristaltic pump (type M312, Villiers-Le-Bel, France). The superfusate of the first hour was discarded and then 22 samples of three-minute fractions were collected by a Gilson multichannel fraction collector (type FC-2038, Middleton, WI, USA). From the tenth fraction, GlyT1 inhibitors were added and maintained throughout the experiment. Following the superfusion, tissues were collected from the superfusion chambers, homogenized, and an aliquot was prepared to determine its radioactivity. To measure the radioactivity released from the tissues, superfusates were mixed with liquid scintillation reagent (Optifluor, Packard, Groningen, The Netherlands) and submitted to liquid scintillation spectrometry. The [³H]glycine efflux was presented as a fractional rate i.e. as a percentage of the amount of radioactivity in the tissue at the time the release was assessed, as it was described in previous work [101]. A computer program (Quattro Pro)

for estimation of fractional rate of [³H]glycine efflux was applied. GlyT-1 inhibitors, sarcosine, ACPFB ((S)-2-amino-6-chloro-N-(1-(4-phenyl-1-(propylsulfonyl)piperidin-4-yl)ethyl)benzamide) and NFPS were investigated.

3.8. Assessment of glycine and glutamate content of cerebrospinal fluid and spinal cord

To evaluate the level of glycine and L-glutamate, capillary electrophoresis-laser induced fluorescence measurement was performed on samples from neuropathic and sham operated animals. Protocol of induction of neuropathic pain in animals and treatments (NFPS 1 mg/kg, Org-25543 2 mg/kg, and their co-administration or vehicle) was based on the protocol used in mono-neuropathic pain model (Figure 3). Animals underwent pSNL operation and after 14 days they were evaluated by DPA test before treatment. Neuropathic animals were treated with compounds and at 180' post-treatment the procedure of CSF sampling was performed. Animals were anaesthetised under isoflurane gas and their CSF was collected from cisterna magna by puncturing with a needle attached to a butterfly collecting tube. Bloodless samples were centrifuged at 2000g, 4°C for 10 minutes and stored in -80°C for further process. Following the CSF sampling, animals were sacrificed by decapitation and spinal cord was isolated with water pressure by hydraulic extrusion method [67]. The lumbar 4-6 ipsi- and contra-lateral sides were collected and frozen immediately and stored in -80°C for later.

The method for glycine and glutamate measurement developed previously by Department of Pharmacodynamics of Semmelweis University with some modifications [66]. For the measurements, spinal cord samples were homogenized for 20 seconds in acetonitrile-distilled water solution (2:1 v/v; 20 µl/mg tissue) by sonication, and then they were centrifuged at 20000g for 10 minutes at 4°C to remove precipitated proteins. The collected CSF samples were deproteinized by adding 2 volumes of pure acetonitrile and they were centrifuged at 20000g for 10 minutes at 4°C temperature. Supernatants of these samples were taken and diluted five times with acetonitrile-distilled water solution (2:1; v/v) and the derivatization with NBD-F (1 mg/mL final concentration) applied for them in 20 mM borate buffer pH 8.5 for 20 minutes at 65°C where 5 µM L-cysteic acid was the internal

standard. Measurement of derivatized samples performed by a P/ACE MDQ Plus capillary electrophoresis system coupled with laser induced fluorescence detector equipped with a laser source of excitation and emission wavelengths of 488 and 520 nm, respectively (SCIEX, Framingham, MA, USA). Polyacrylamide coated fused silica capillaries (i.d.: 75 μm , effective/total length: 50/60 cm) using 50 mM HEPES buffer pH 7.0 containing 6 mM hydroxypropylamino- β -cyclodextrin at 15°C by -30 kV constant voltage were used for the separation.

3.9. Assessment of G-Protein activity

For the preparation of spinal cord membranes, animals were decapitated under anaesthetic conditions and their spinal cords were isolated and membrane preparation was performed as it was described in another study [68] Briefly, samples were homogenized in ice-cold TEM containing 50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl with a Teflon-glass homogenizer. The centrifugation of the homogenate was at 18000 rpm for 20 minutes at 4°C and supernatant was thrown away and the pellet was again incubated at 37°C for 30 minutes in a shaking water-bath. Next, the repetition of the centrifugation in the same manner was performed. The final pellet was poured in ice-cold TEM pH 7.4 (50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl) buffer and kept at -80°C temperature. The protein level of the prepared membrane was assessed by Bradford BSA method and it was used as standard [69].

The functional [³⁵S]GTP γ S binding assay is when the GDP \rightarrow GTP exchange of the G_{ai/o} protein is measured in the presence of a given ligand. The nucleotide exchange was detected by the radioactive non-hydrolysable GTP analogue, [³⁵S]GTP γ S. The functional [³⁵S]GTP γ S binding assay was based on a previously described method [70, 71], with some modifications. Briefly, incubation of membrane homogenates were performed at 30°C for 60 minutes in TEM buffer (pH 7.4, 50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl), containing 20 MBq/0.05 ml [³⁵S]GTP γ S (0.05 nM) and any of GlyT inhibitors (sarcosine 1 mM and 1 M, NFPS and Org-25543 each in increasing concentrations of 0.1 μM , 1 μM , 10 μM and their combination in concentration of 10 μM) or DAMGO (in concentration of 10 μM) or 20%DMSO. The measurement was in

the excess of GDP (30 μ M) in a final volume of 1 mL. Total binding was measured without compounds of study. Non-specific binding was assessed in the presence of 10 μ M unlabeled GTP γ S and subtracted from total binding to yield the basal activity. To terminate the reaction, rapid filtration under vacuum (Brandel M24R Cell Harvester) and washing three times with 5 mL ice-cold 50 mM Tris-HCl (pH 7.4) buffer through Whatman GF/B glass fibers were applied. Then filters were measured for the level of their radioactivity in UltimaGoldTM MV aqueous scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter. Experiments of binding assay were performed in triplicates. Each experiment repeated at least three times.

3.10. Statistical analysis

Statistical analysis and fitting of the graphs were performed by professional software GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Data were presented as \pm SEM value. Normality tests were performed for relevant data. Two-way ANOVA (comparison of two factors) was applied for data analysis of DPA tests, tail-flick tests, and glycine release experiment. One-way ANOVA (comparison of one factor) was considered for data analysis of formalin tests, rotarod tests of NFPS, Org-25543 and their combination, G-protein activity assay and capillary electrophoresis experiments. Rotarod tests on sarcosine and morphine (comparison of 2 columns) were evaluated by t-test (and nonparametric tests). Vehicle treated animals were considered as control for their corresponding compound-treated groups for in vivo studies. The control group for capillary electrophoresis experiment was the sham group. In case of G-protein activity assay data were normalized to basal activity level (100%) in percentage. Glycine releases were compared to levels before incubation of test compounds. For all statistics of in vivo, Newman-Keuls post-hoc test was applied, except for comparison of baselines of chronic sarcosine (Fisher's LSD test) and for sarcosine and morphine rotarod tests (Kolmogorov-Smirnov test). For capillary electrophoresis Holm-Sidak, and for G-protein activity assay and glycine release experiments Dunnett's multiple comparisons post-hoc tests were applied. The difference between the compound-treated groups and control groups were considered significant (indicated by asterisk *) if P value was less than 0.05.

4. Results

4.1. Sarcosine shows analgesic effect after chronic treatment in acute thermal pain model

The effects of sarcosine on acute thermal pain were evaluated by tail-flick test. Results of acute test and test on day 4 did not show any significant antinociceptive effect. However, on day 8 the dose 1000 mg/kg showed analgesic effect at 60' and afterward. (Figure 4C, n=4-5 mean difference of 60': 33.02 ± 14.57 F: 3.526 $P < 0.05$). The trend of baselines of treatment days indicated a decrease in pain sensitivity on day 8 for the higher dose (Figure 4D, n=4-5, Mean difference: 46.02 ± 11.88 F: 6.513 $P < 0.01$).

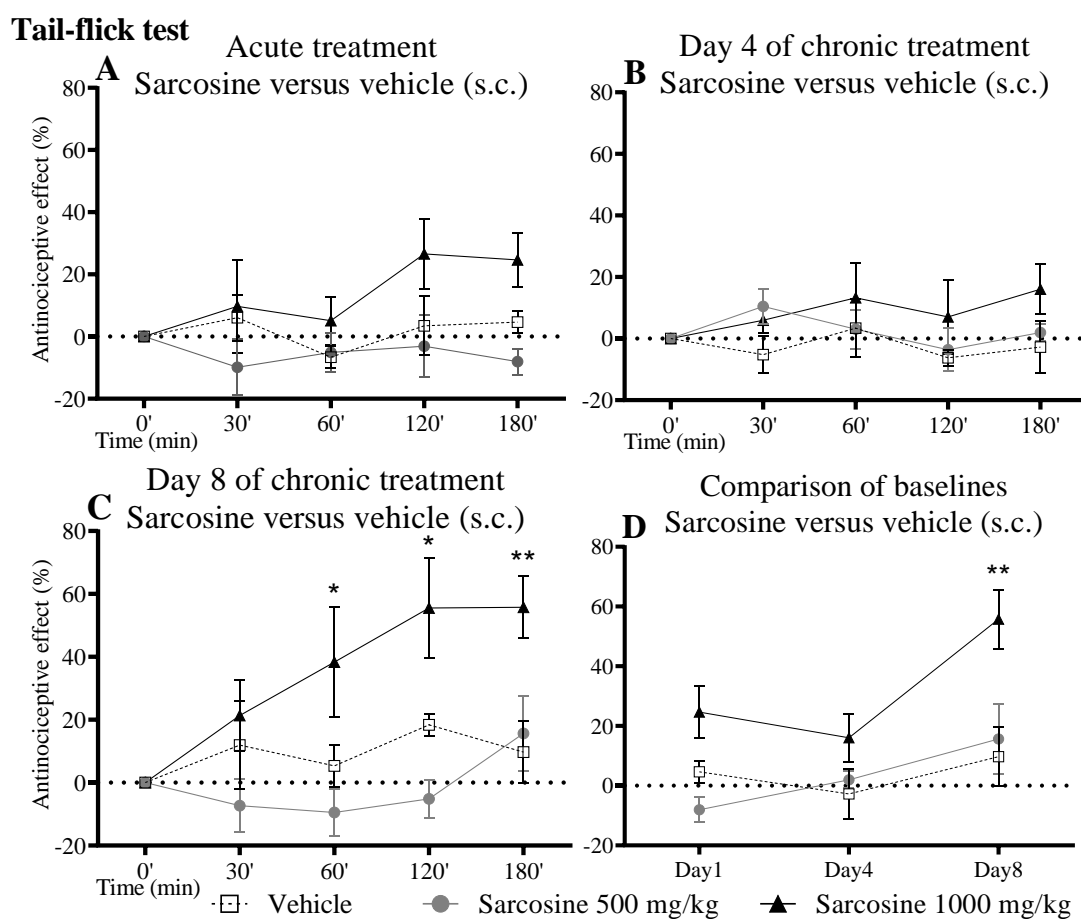


Figure 4. The antinociceptive effect of sarcosine (500 and 1000 mg/kg s.c.) in rat tail-flick test. **A)** Acute treatment **B)** Day 4 of chronic treatment **C)** Day 8 of chronic treatment **D)** Comparison of baseline values of all tests (as percentage of control). Statistical analysis: n=4-5 Two-way ANOVA, Newman-Keuls post-hoc test, values are presented as means \pm S.E.M *: $p < 0.05$, **: $p < 0.01$

4.2. Sarcosine has no impact in acute inflammatory pain evoked by formalin

Effects of sarcosine on acute inflammatory type of pain was assessed by formalin test. In phase 2 when formalin injection was 15' after sarcosine (500mg/kg) treatment, the number of behavioural events increased compared to the control (Figure 5A, n=5 mean difference: 105.4 ± 31.43 F: 109.7 $P < 0.01$) indicating a pro-nociception. However, when formalin injection was applied 180' post-treatment the pro-nociception was absent. Four days of chronic treatment did not show any effect.

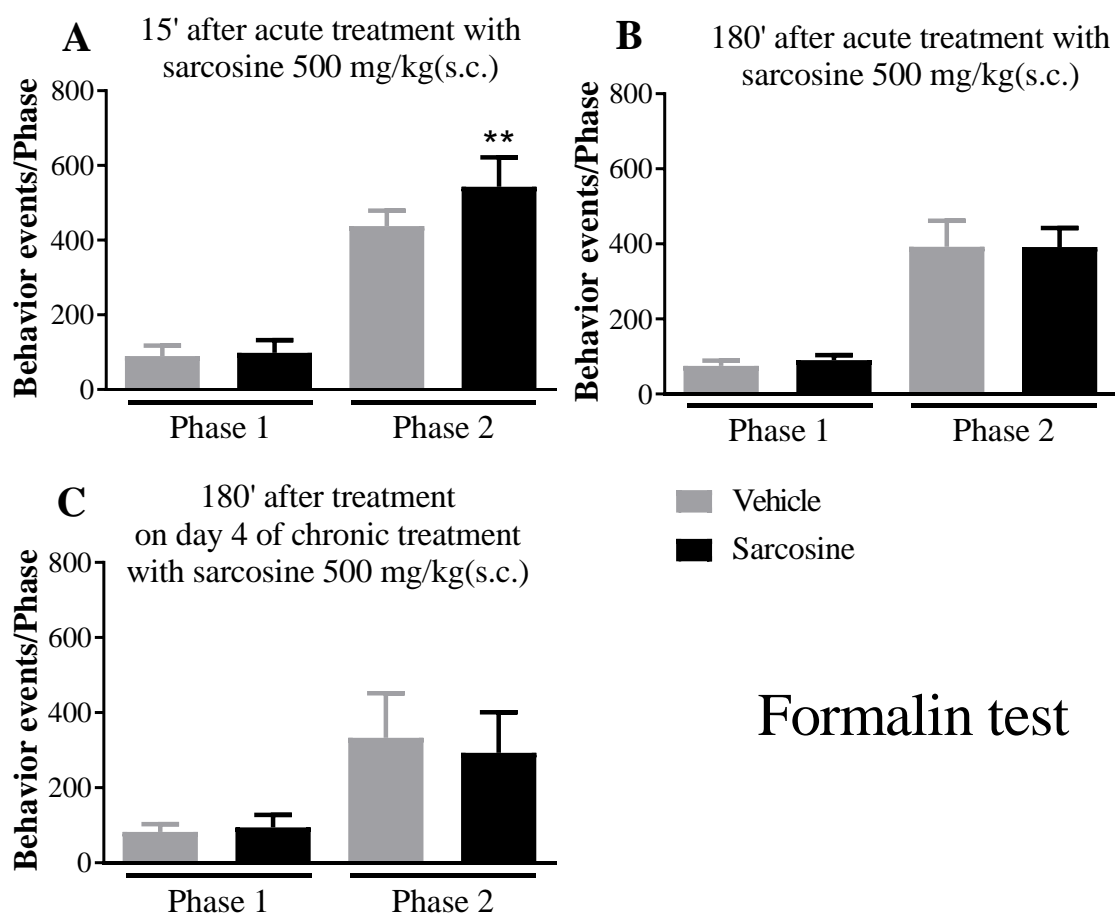


Figure 5. The level of nociceptive behavioural events in formalin test (phase 1 is acute pain and phase 2 is inflammatory pain). **A**) Test started 15' after sarcosine (500 mg/kg). **B**) Test started 180' after sarcosine (500 mg/kg). **C**) Day 4 of chronic treatment, test started 180' after sarcosine. Statistical analysis: n=5 One-way ANOVA, Newman–Keuls post-hoc test, values are presented as means \pm S.E.M **: $p < 0.01$

4.3. Sarcosine shows dose related anti-allodynic effect in mono-neuropathic pain model

The DPA values of paw withdraw threshold (PWT) for 500 and 1000 mg/kg s.c. acute doses of sarcosine on pSNL operated animals showed anti-allodynic effect in both doses. However, the effect of sarcosine 500 mg/kg which showed significant effect after 30' and peaked at 60' (Figure 6A, n=4-5, mean difference: 19.87 ± 3.783 F: 4.156 $P < 0.0001$) was higher than the peak effect of dose 1000 mg/kg at 30' (Figure 6B, n=4-5, mean difference: 12.92 ± 3.566 F: 4.156 $P < 0.01$). Afterwards, treatments were continued 2 times a day for 6 days and allodynia was assessed after the morning doses of day 4 and 6. Result from day 4 showed significantly higher anti-allodynic effect at dose 500 mg/kg with a peak-effect at 60' (Figure 7A, n=4-5, mean difference: 12.57 ± 3.508 F: 0.7595 $P < 0.01$) in comparison to dose 1000 mg/kg which had a mild anti-allodynic effect at 30' and 180' post-treatment (Figure 7B, n=4-5, mean difference: 9.754 ± 3.308 F: 0.7595 $P < 0.05$). On day 6 however, just the lower dose produced anti-allodynia with a peak-effect at 60' (Figure 7C, n=4-5, mean difference: 21.53 ± 4.166 F: 2.484 $P < 0.0001$). In case of baseline values, chronic treatment for the group treated with the lower dose (500 mg/kg) showed a significant elevation on day 4 (Figure 8A, n=4-5, mean difference: 6.526 ± 3.164 F: 16.64 $P < 0.05$) and on day 6. The higher dose (1000 mg/kg) did not show such effect.

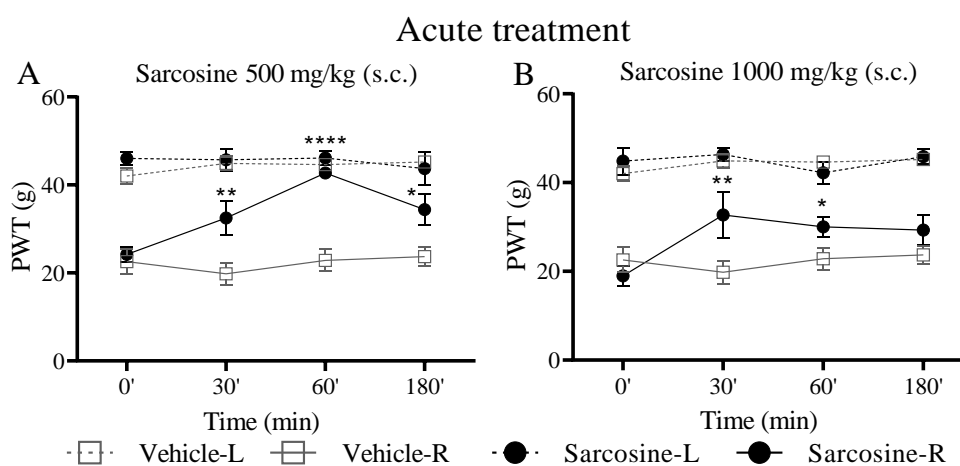


Figure 6. The PWT(g) for healthy (L, left) and neuropathic (R, right) legs of pSNL operated neuropathic rats, before treatment (baseline: 0') and after treatment: **A**) Sarcosine 500 mg/kg (s.c.) **B**) Sarcosine 1000 mg/kg (s.c.), both in comparison to vehicle treated group. Statistical analysis: (n = 4-5) Two-way ANOVA, Newman-Keuls post-hoc test, values are presented as means \pm S.E.M *: $p < 0.05$, **: $p < 0.01$, ****: $p < 0.0001$

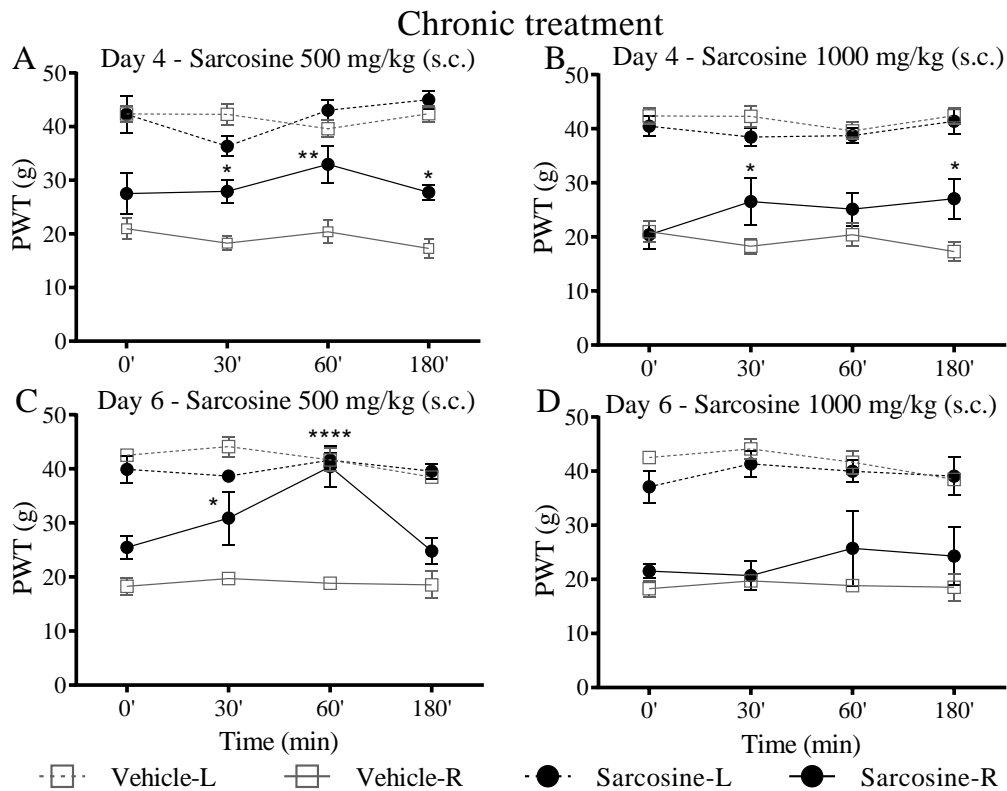


Figure 7. PWT(g) values of healthy (L, left) and neuropathic (R, right) legs of pSNL operated rats after chronic treatment **A**) Day 4 of sarcosine 500 mg/kg(s.c.) **B**) Day 4 of sarcosine 1000 mg/kg (s.c.) **C**) Day 6 of sarcosine 500 mg/kg (s.c.). **D**) Day 6 of sarcosine 1000 mg/kg (s.c.). Statistical analysis: (n:4-5) Two-way ANOVA, Newman-Keuls post-hoc test, values in means \pm S.E.M *:p<0.05, **:p<0.01 ****:p<0.0001

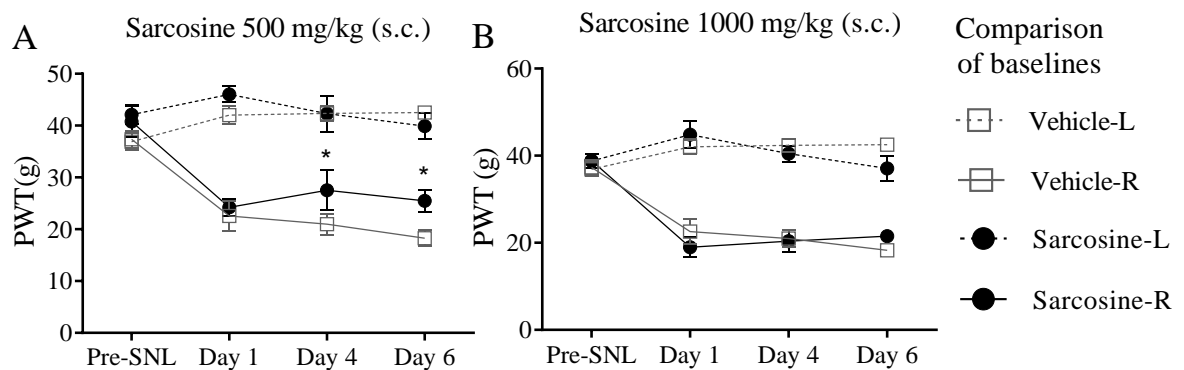


Figure 8. The PWT in grams of healthy (L, left) and neuropathic (R, right) legs of pSNL operated rats for baseline values of DPA tests before operation (shown as Pre-SNL) and in different days of chronic treatment: **A**) Sarcosine 500 mg/kg (s.c.) **B**) Sarcosine 1000 mg/kg (s.c.), both in comparison to vehicle. Statistical analysis: (n = 4-5) Two-way ANOVA, values in means \pm S.E.M, Fisher's LSD test *: p<0.05

4.4. NFPS shows time lag and dose dependent effect in mono-neuropathic pain model

After acute treatment with 3 doses of NFPS (1, 2 and 4 mg/kg s.c.) on mono-neuropathic animals, it was observed that the low doses of 1 and 2 mg/kg were ineffective on mono-neuropathic pain compared to vehicle treated group. However, the higher dose (4 mg/kg) raised the PWT values significantly after 30' and 60' (Figure 9C, 30': n=5 mean difference: 12.79 ± 2.809 F: 2.518, $p < 0.0001$) compared to control. Treatment of dose 1 mg/kg continued once a day for 6 days and on day 4 baseline of PWT increased significantly which raised even further on day 6 (Figure 9D, day 6: n=5 mean difference: 13.29 ± 2.885 F: 0.3925, $p < 0.0001$) indicating its chronic effect.

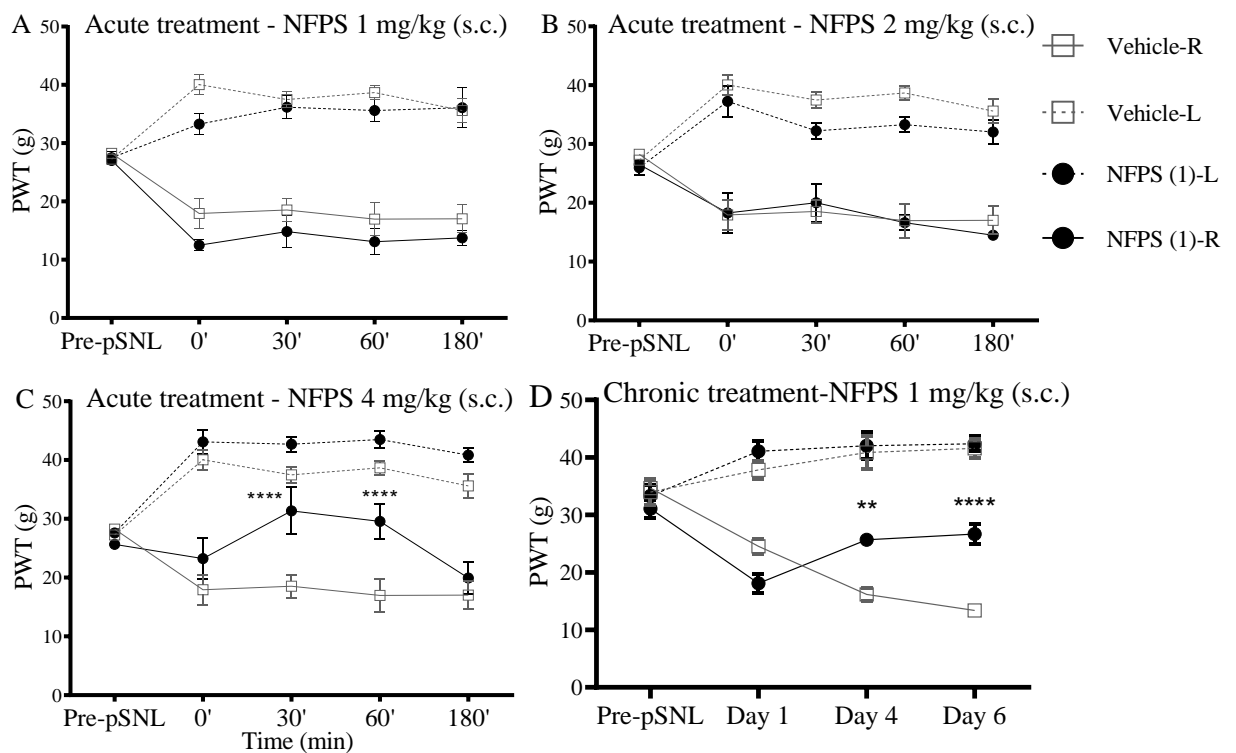


Figure 9. PWT in grams, of healthy (L, left) and neuropathic (R, right) legs of pSNL operated rats, before (baseline, shown as 0') and after administration of NFPS in different doses. **A)** Acute NFPS 1 mg/kg(s.c.) **B)** Acute NFPS 2 mg/kg(s.c.) **C)** Acute NFPS 4 mg/kg(s.c.) **D)** Comparison of baseline values of PWT(g) in different days of chronic treatment with NFPS 1 mg/kg(s.c.) Statistical analysis: two-way ANOVA, Newman–Keuls post-hoc test. Values are presented as means \pm S.E.M, **: $p < 0.01$, ****: $p < 0.0001$

4.5. Org-25543 shows dose dependent effect in mono-neuropathic pain model

In the DPA experiment with Org-25543 two doses of 2 and 4 mg/kg s.c. were examined on mono-neuropathic model in acute and chronic treatment conditions. The lower dose was ineffective in acute test, but the PWT values for the higher dose were increased and reached a significant difference compared to the control at 60' post-treatments (Figure 10B, n=5 mean difference: 7.705 ± 2.933 F: 1.644, $p < 0.01$) which subsided later at 180'. Further on, the dose 2 mg/kg s.c. was administered once a day chronically and baseline values of days 1, 4 and 6 were compared but no significant differences were observed.

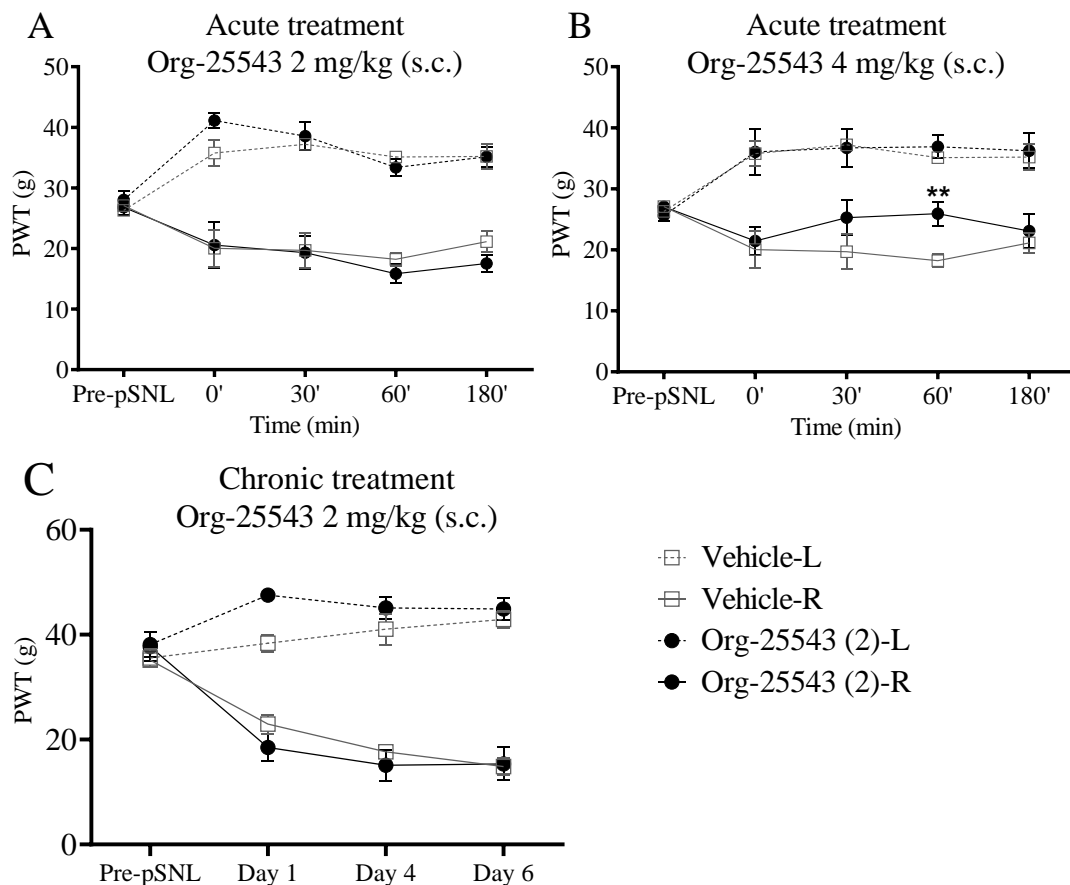


Figure 10. The PWT in grams, of healthy (L, left) and neuropathic (R, right) legs of pSNL operated rats, before (baseline, shown as 0') and after administration of Org-25543 in different doses. **A)** Acute Org-25543 2 mg/kg(s.c.) **B)** Acute Org-25543 4 mg/kg(s.c.) **C)** Comparison of baseline values of PWT(g) in different days of chronic treatment with Org-25543 2 mg/kg(s.c.) Statistical analysis: two-way ANOVA, Newman-Keuls post-hoc test. Values are presented as means \pm S.E.M, **: $p < 0.01$

4.6. Co-administered NFPS and Org-25543 shows augmented anti-allodynic effect in mono-neuropathic pain model

Finally, the acute co-administration of NFPS and Org-25543, GlyT-1 and 2 inhibitors respectively, in low and sub-analgesic doses was examined in DPA test. The systemic co-administration of NFPS 1 mg/kg and Org-25543 2 mg/kg displayed a significant anti-allodynic effect at 60' post-treatment and the effect was further increased at 180' post-treatment (Figure 11, 60': $n = 4-5$ mean difference: 10.04 ± 3.371 F: 0.1225 $p < 0.01$). Additionally, this effect was at similar level to the higher dose of NFPS (4 mg/kg).

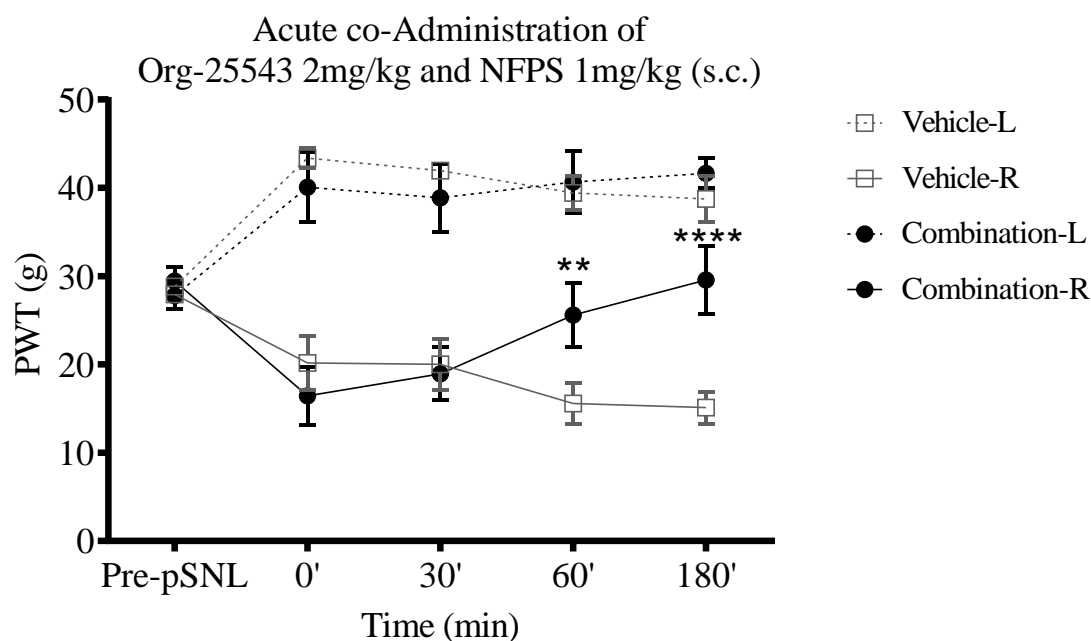


Figure 11. The PWT(g) of healthy (L, left) and neuropathic (R, right) legs of pSNL operated rats, before (baseline: 0') and after co-administration of sub-analgesic doses of NFPS (1 mg/kg s.c.) and Org-25543 (2 mg/kg s.c.) in comparison to vehicle treated group. Statistical analysis: two-way ANOVA, Newman-Keuls post-hoc test. Values are presented as means \pm S.E.M, **: $p < 0.01$ ****: $p < 0.0001$

4.7. Sarcosine, NFPS and Org-25543 failed to affect motor coordination and balance

The rotarod test was performed to assess the possible interference on motor coordination and balance following GlyT inhibitors treatments. None of the applied doses of sarcosine (500 and 1000 mg/kg s.c.) produced any significant motor dysfunction in rats

at their peak effect times (60' and 180' post-treatment for doses of 500 and 1000 mg/kg respectively) following acute or chronic treatments (Figure 12). Application of systemic high dose (4 mg/kg) of NFPS and Org-25543 also did not disturb motor function at their peak effect time of 60' post-treatment (Figure 13B). Similar results were observed following the systemic treatment of NFPS (2 and 4 mg/kg), Org-25543 (4 mg/kg) or their co-administration of sub-analgesic doses (NFPS 1 mg/kg and Org-25543 2 mg/kg) as well as the vehicle treated group (20%DMSO) at 180' (Figure 13A). Morphine group at dose 6.4 mg/kg s.c. displayed a significant reduction in time latency, thus morphine reduced motor functions at its time of peak-effect (30' post-treatment) in comparison to its vehicle group (saline 2.5 ml/kg) (Figure 12E). Morphine treatment represented an adequate positive control for rotarod experiments.

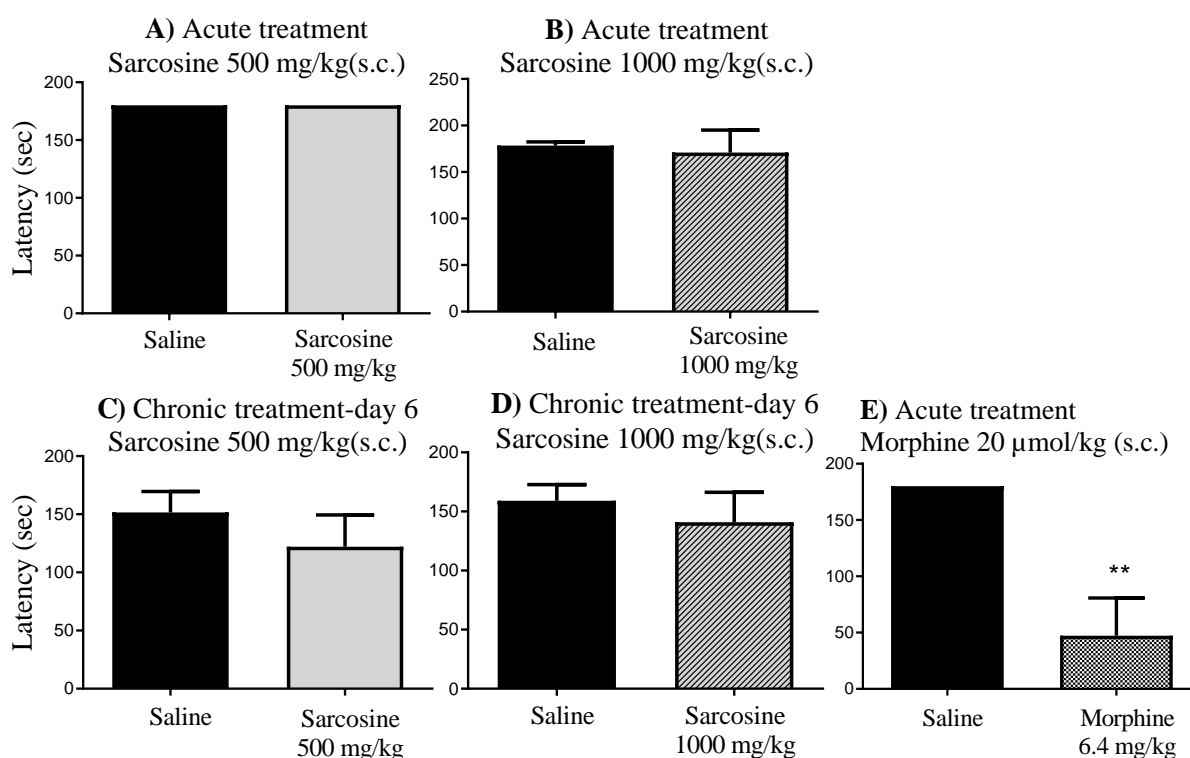


Figure 12. Result of rotarod test for evaluating motor coordination and balance. Comparison of time latencies (sec) from rotarod tests. **A)** Acute treatment with sarcosine (500 mg/kg s.c.), test at 60'. **B)** Acute treatment with sarcosine (1000 mg/kg), test at 180'. **C)** Day 6 of chronic treatment with sarcosine (500 mg/kg s.c.), test for morning dose at 60'. **D)** Day 6 of chronic treatment with sarcosine (1000 mg/kg s.c.), test for morning dose at 180'. **E)** Acute treatment with morphine (6.4 mg/kg, s.c.), test at 30'. All groups were compared to control group (saline 2.5 mg/kg s.c.). Statistical analysis: n= 6-7 t-Test (nonparametric tests) with Kolmogorov-Smirnov test, Values are presented as means \pm S.E.M ** : $p < 0.01$

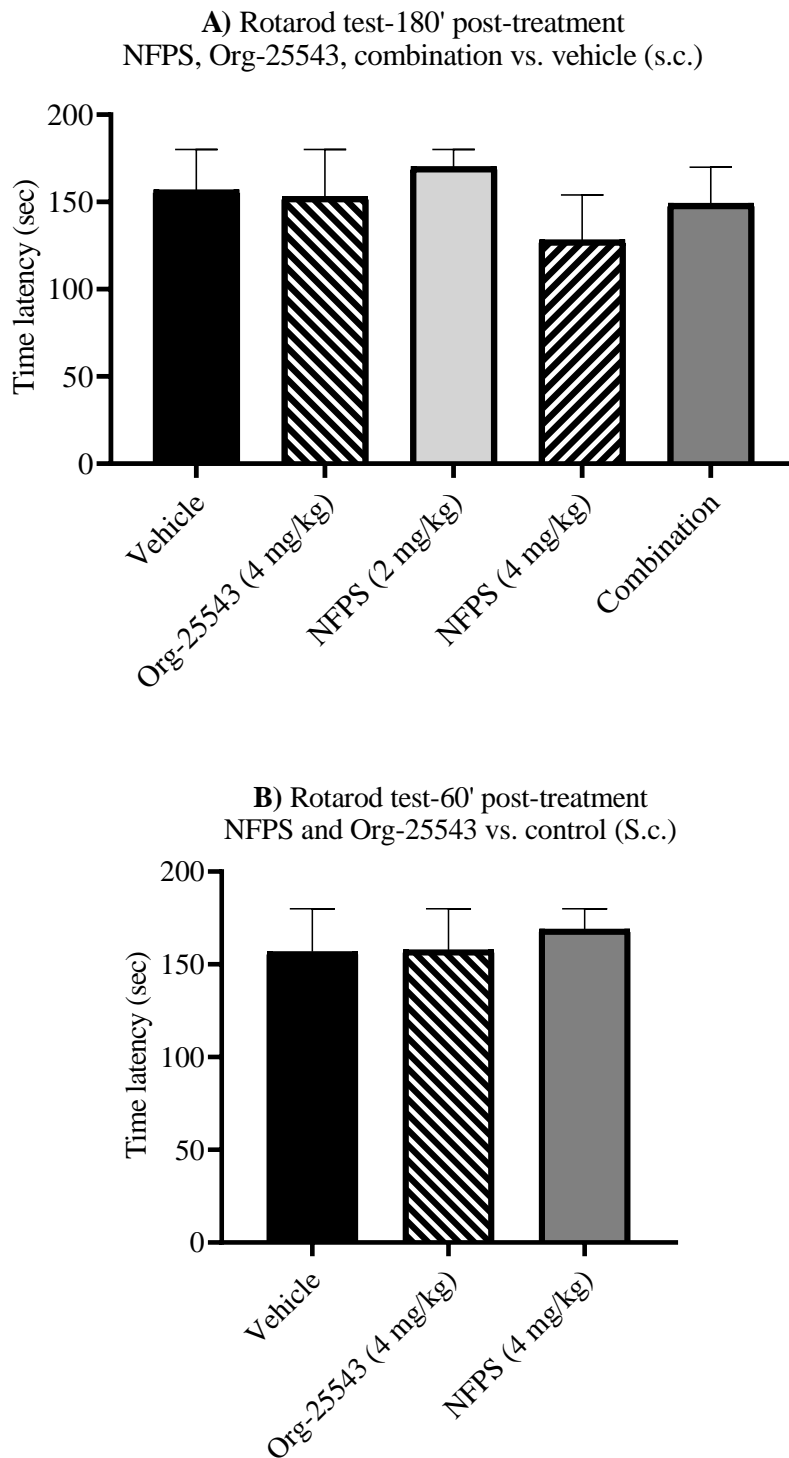


Figure 13. Result of rotarod test for evaluating motor coordination. Comparison of time latencies (sec) from rotarod tests. **A)** Acute systemic treatments with NFPS (2 and 4 mg/kg), Org-25543 (4 mg/kg), Co-administration (combination) of NFPS (1 mg/kg) and Org-25543 (2 mg/kg). n=5-6 **B)** Acute systemic treatments with NFPS (4 mg/kg) and Org-25543 (4 mg/kg) n=4. Statistical analysis: one-way ANOVA, Newman-Keuls post-hoc test, Values are presented as means \pm S.E.M

4.8. Sarcosine increases glycine release

Application of sarcosine (3 and 30 mmol/L), ACPBP (1 μ mol/L) and NFPS (0.1 mmol/L) in isolated hippocampus tissue of rats showed that sarcosine increased the [3 H]glycine efflux (over 50% increase) while the other chemicals (ACPPB and NFPS) did not show this change. Co-application of sarcosine and ACPBP however, subsided the increased glycine release by sarcosine (Figure 14).

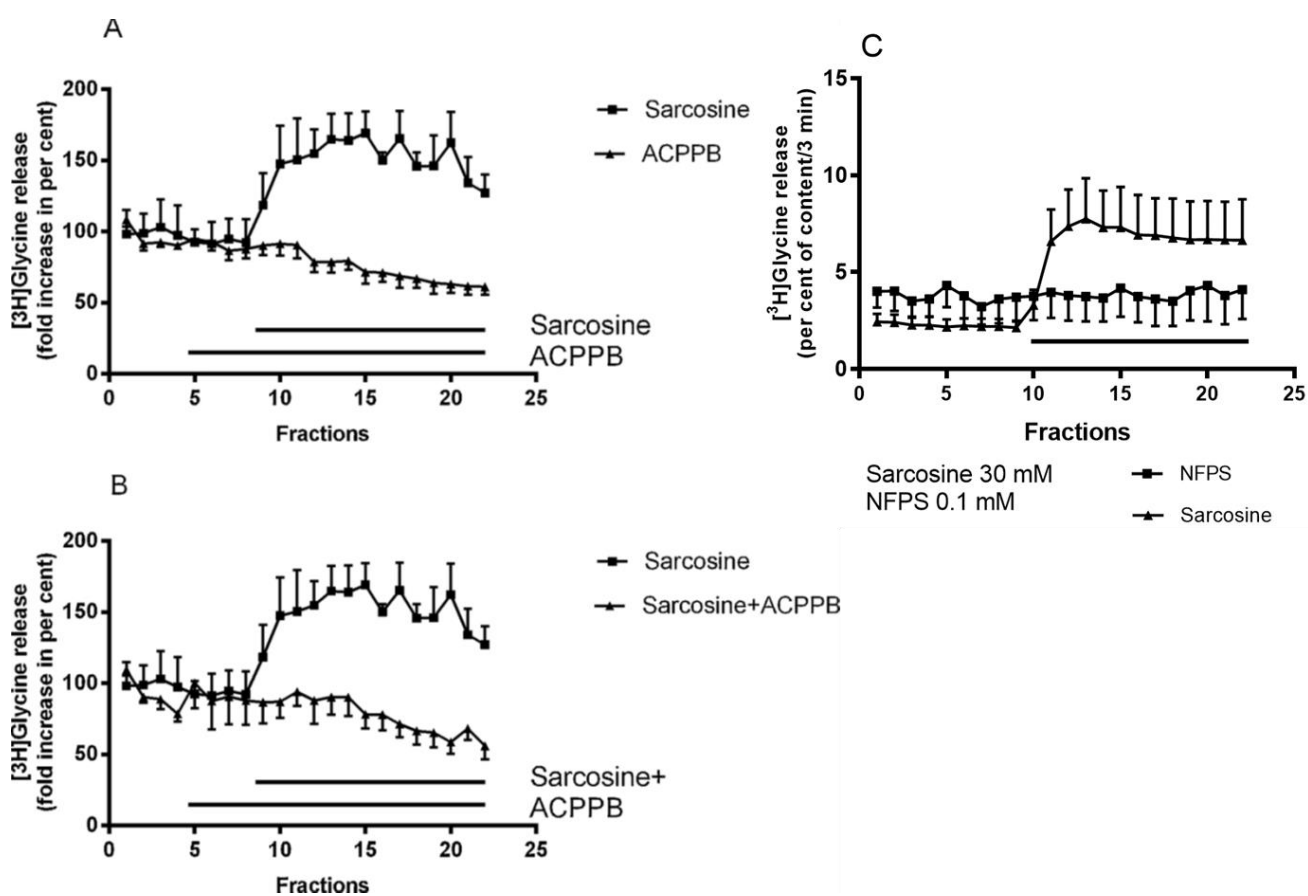


Figure 14. Release experiment with [3 H]glycine in rat hippocampus slices. **A)** Sarcosine (3 mmol/L) and ACPBP (1 μ mol/L) applied separately **B)** Applied sarcosine and ACPBP in combination, in comparison to single dose of sarcosine. **C)** Sarcosine (30 mmol/L) and NFPS (0.1 mmol/L) applied separately. Data are shown as the mean \pm S.E.M., n=4

4.9. Combination of NFPS and Org-25543 enhances glycine content of cerebrospinal fluid

The CSF level of both L-glutamate and glycine was increased in neuropathic animals (vehicle treated), but this increase was significant just for L-glutamate content (Figure 15A, 15B: means $159.3\% \pm 23.28\%$ and $158.4\% \pm 9.21\%$ vs. $100\% \pm 16.08\%$, vehicle: $n = 4$, combination: $n = 5$, sham: $n = 7$, $F(2, 13) = 4.639$, $p < 0.05$, one-way ANOVA, Holm–Sidak post-hoc test).

Co-administration (combination) of systemic low doses of NFPS and Org-25543 (NFPS 1 mg/kg and Org-25543 2 mg/kg) did not change the glutamate levels, but it increased the CSF glycine levels significantly (Figure 15A: means $195.9\% \pm 35.32\%$ vs. $100\% \pm 11.55\%$, combination: $n = 9$, sham: $n = 11$; $F = 2.722$, $p < 0.01$, one-way ANOVA, Holm–Sidak post-hoc test). Additionally, none of the applied single doses showed such level of increase. The measurements on spinal tissue did not show any statistical difference in glycine or glutamate content in sham and neuropathic (vehicle treated) animals for ipsi-lateral (operated side) and contra-lateral (unoperated side) of the spinal cord (Figure 15C, 15D).

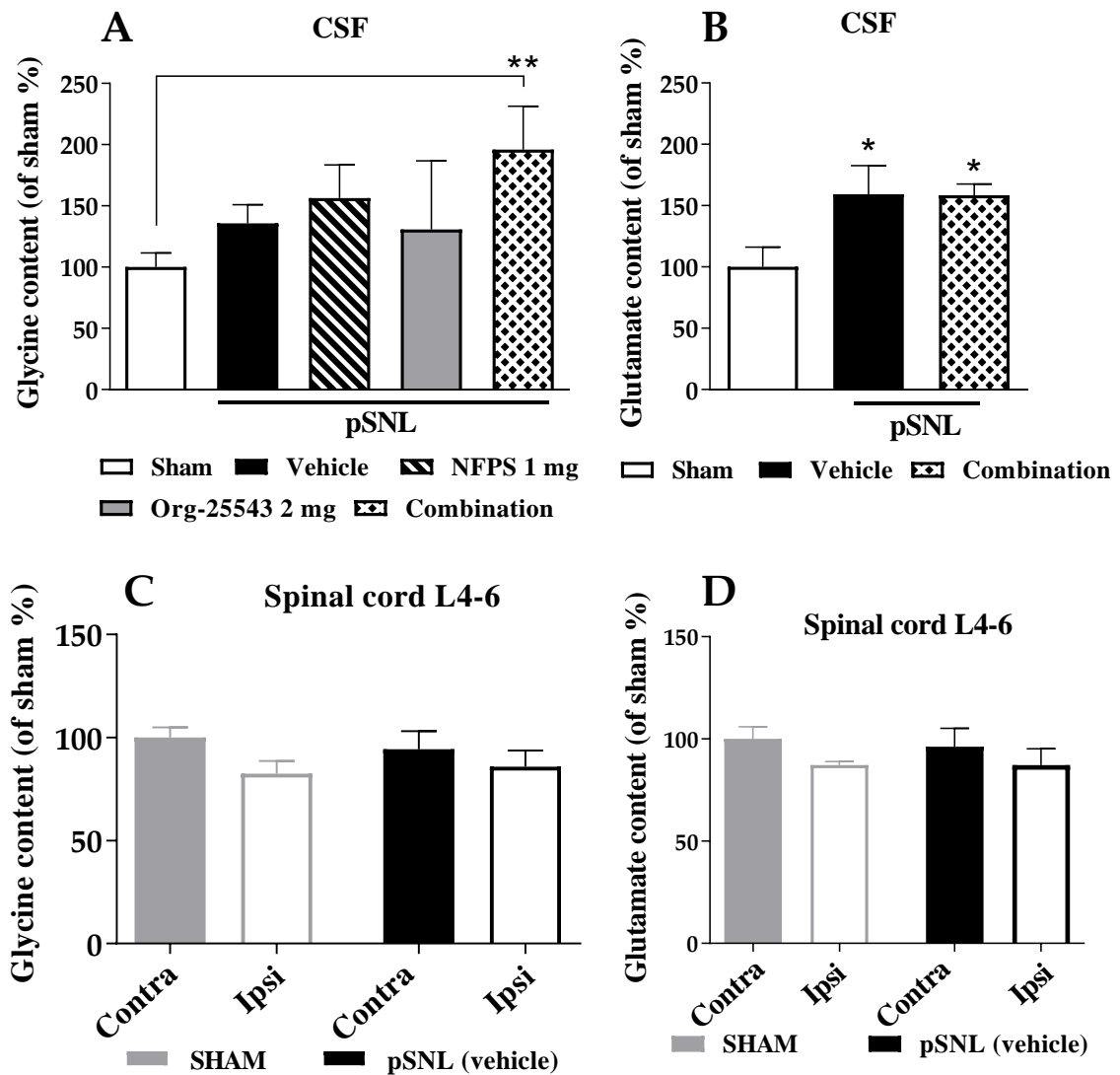


Figure 15. Capillary electrophoresis measurements of glycine and L-glutamate content in CSF n=4-7 (A,B) and in spinal cord L-4-6 ipsi- and contra-lateral side n=9-11 (C,D) obtained 14 days after pSNL operation from sham or neuropathic animals of any of acute treatment groups of NFPS (1 mg/kg s.c.), Org-25543 (2 mg/kg s.c.) or their co-administration (combination) and the vehicle (20%DMSO). Concentration levels were normalized to sham and shown in percentage. Columns represent the given amino acid content in % \pm S.E.M. in the indicated groups. asterisk: marks the significant differences compared to sham group (one-way ANOVA, Holm–Sidak post-hoc test; **: $p < 0.01$, *: $p < 0.05$).

4.10. GlyT inhibitors or their combinations failed to activate $G_{\alpha i/o}$ -protein coupled receptors

Except for the result of the μ -opioid receptor agonist DAMGO (Figure 16A: $140.3\% \pm 1.58\%$ vs. 100% , DAMGO: $n = 4$, basal: $n = 6$, $DF = 42$, $p < 0.001$) which was considered as the positive control, all other test compounds in any of applied concentrations failed to stimulate the $G_{i/o}$ -protein activity significantly over the basal level (100%) (Figure 16).

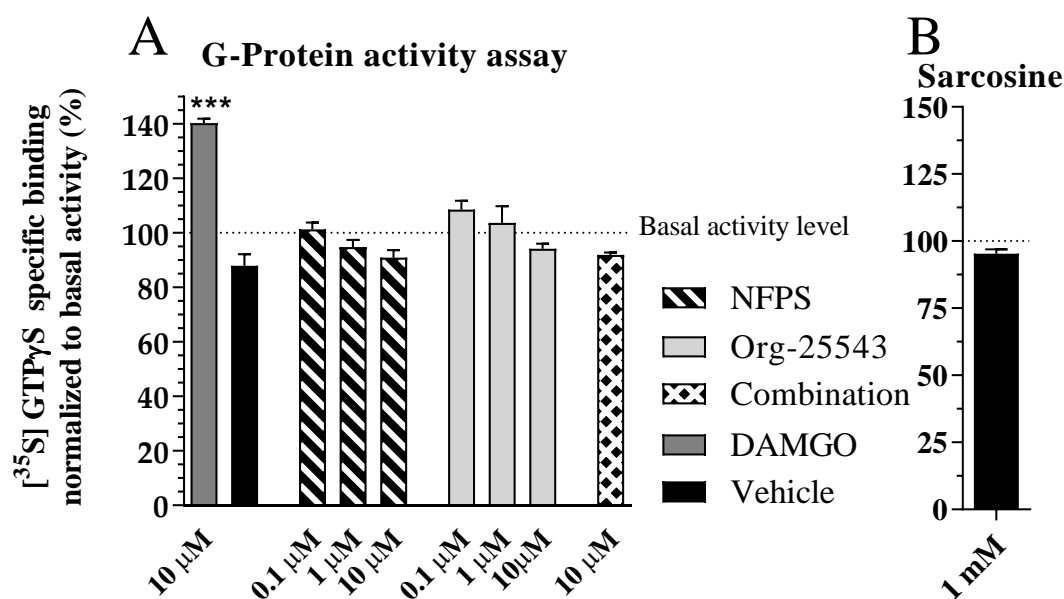


Figure 16. The impact of glycine transporter inhibitors NFPS and Org-25543 (**A**) and sarcosine (**B**) compared to DAMGO, a μ -opioid receptor agonist as positive control (**A**) on G-protein activity in the rat spinal cord membranes. Compounds were added in the indicated concentrations. For comparison, vehicle (DMSO 20%) was also measured. Columns represent the means \pm S.E.M. of the specific binding of [³⁵S]GTPS normalized to basal activity (100%). asterisk: indicates the significant alteration compared to basal activity (one-way ANOVA followed by a Dunnett's multiple comparison test ***: $P < 0.001$). All experiments were repeated at least 3 times, using 3 parallels.

5. Discussion

There are many ongoing research and studies to find better solutions for the management of neuropathic pain, however, most of the available treatment approaches to cure this pain condition are not enough effective or sometimes they are effective but limited by their side effects. Thus, the lack of ideal treatment approach to control this condition has opened a further research path to find novel pharmacological targets such as glycine transporters among others. Pre-clinical studies on inhibitors of glycine transporters have reported on the beneficial effects of these compounds in the management of neuropathic pain, however some disadvantages were raised such as respiratory and motor dysfunctions [77].

The currently available analgesics have different mechanisms of action that interfere with pain transduction, transmission, modulation and perception. Neurotransmitters that commonly participate in the pain ascending pathway within the spinal cord are either excitatory such as glutamate and substance P or inhibitory such as gamma-aminobutyric acid (GABA) and glycine. In addition, endogenous opioids, norepinephrine, serotonin and dopamine are also involved in nociception. Spinal cord dorsal part is a crucial point in pain pathway toward the brain. In this part the central ends of primary afferent neurons ($A\beta$, $A\delta$ and C) which differ in myelination are terminated. The myelinated $A\beta$ fibers process non-noxious mechanical (superficial light touch and pressure) sensory inputs and project into laminae II and III of the dorsal horn. The central terminals of $A\beta$ fibers operate with glutamate as a neurotransmitter and establish synaptic connections with a number of excitatory and inhibitory interneurons at the spinal dorsal horn. In addition, the site of these inter-neuronal interactions is dominantly in lamina II and III of spinal cord [6, 73]. In this part, as mentioned above inhibition is mediated predominantly by GABAergic and glycinergic interneurons, while the excitatory transmission mediated by glutamatergic interneurons. In non-pathological condition the balance between inhibitory and excitatory inter-neuronal activities keeps the pain sensations in normal ranges, but in neuropathic pain condition this balance is changed in favour of final over-excitation of secondary afferent neurons that results in allodynia (perception of pain from innocuous

stimulus) and hyperalgesia (perception of pain with higher intensity than its normal range). One of a possible explanations for this imbalance might be the loss of glycinergic inhibitory interneurons in laminae II and III [13, 30, 74, 75]. In this regard, potentiation of glycinergic pathway by application of glycine transporter inhibitors is a valid approach to reduce the pain, but observation of motor and respiratory side effects from administration of higher doses of more potent and selective compounds, which are mostly irreversible inhibitors with high lipophilicity, have brought an objection for clinical studies [35, 77].

The work in the present thesis was focused on the analgesic effect of GlyT inhibitors following acute and chronic treatments. In this respect, the question was raised whether or not treatment with GlyT-1 inhibitors can alleviate pain of different entities, namely thermal, inflammatory and particularly neuropathic pain in animal pain models of acute and chronic characters. To do that, we have chosen the rat tail-flick test, formalin test and sciatic nerve ligation-induced neuropathic pain model.

Thermal pain models such as tail-flick test are used to assess acute thermal pain sensation in rodents such as rats and mice prior to and after treatment with test compounds [61]. Herein we have assessed the impact of systemically injected sarcosine, a competitive reversible GlyT-1 inhibitor on pain induced by thermal noxious stimuli in rats. Following acute subcutaneous treatment, sarcosine failed to produce significant analgesic effect, but there was a tendency for increasing the pain threshold in animal group treated with the higher dose (sarcosine 1000 mg/kg) compared to vehicle-treated group at 120' or 180' (Figure 4A). Also, following 7 days of chronic treatment (test on day 8) the higher dose of sarcosine showed significant analgesia at 60' and thereafter (Figure 4C). In addition, a significant increase of baseline on day 8 was also detected for chronic treatment with the higher dose (Figure 4D). In these series of experiments, we aimed to focus on the role of glycinergic system on alleviation of acute thermal pain, because to the best of our knowledge no such analysis has been conducted.

Nevertheless, some research groups were interested in the involvement of glycinergic system in the alleviation of acute thermal hyperalgesia and allodynia. In these studies, allodynia was generated by intrathecal strychnine (GlyR α 3 antagonist). According to these authors, following an intrathecal administration of strychnine in rats or mice, in tail

shock vocalization test and tail immersion in a 55°C oil, the pain threshold was significantly decreased and this led to the occurrence of hyperalgesia [97, 98, 99]. The results obtained in this part of our work were new and suggest that sarcosine exhibited a tendency to increase the pain threshold of acute heat-induced pain in rats under the present experimental condition. On the other hand, of note, chronic administration of sarcosine would significantly attenuate thermal pain, which is of clinical value and hence might act as a potential add-on therapy for pain associated with burn.

We continued our work by analysing the effect of sarcosine in a rat model of formalin-induced inflammatory pain. This pain test consists of two phases, namely phase 1 and 2 (early and late). In phase 1 pain behaviours are predominantly stemmed from the activation of C-fibre at the periphery, whereas the pain behaviours of the phase 2 are related to both peripheral inflammatory reaction and functional changes in the spinal dorsal horn [64]. Several analgesic agents have been shown to inhibit either both phases such as opioids or only the inflammatory phase such as non-steroidal anti-inflammatory drugs (NSAIDs) [100]. In our study, systemic sarcosine (500 mg/kg) failed to show antinociception either after acute or chronic treatment. Nevertheless, the chronic treatment lasted for four days in contrast to the above-mentioned tail-flick test where the treatment lasted for eight days. Indeed, it is worth extending this work by continuing the chronic treatment that lasts at least eight days in order to support or deny the analgesic effect of sarcosine upon long-term treatments. In previous work by Tanabe et al, both GlyT-1 inhibitors, sarcosine and NFPS as well as glycine itself reduced the pain behaviours of the second phase of the mouse formalin test, however the route of administration was different (intrathecal) [48]. In our work, when the test was performed just 15' after the acute injection of sarcosine pro-nociception was observed (Figure 5A). However, in another experimental setting, when formalin was injected 180' after acute treatment with sarcosine, either the pro-nociceptive or any other effect were not noticeable (Figure 5B). At the present, it is hard to explain this discrepancy that has been detected and more studies are needed to elucidate it. In studies by Morita and colleagues, they found that sarcosine (20 ng i.t.) could reduce pain up to 44 hours in CFA inflammatory pain model [43], but this pain model does not fully match the concept of the formalin test. Nevertheless, GlyT-2 inhibitor, ALX1393 has been found to inhibit pain behaviours of both phases in formalin test after intrathecal administration but dose

escalation was needed to have the same effect when the drug was administered intracerebroventricularly [18,79]. Systemic and intrathecal administration of Org-25543, a GlyT-2 inhibitor, in inflammatory pain models of formalin test and CFA-induced pain in mouse showed analgesia, as it was mentioned in introduction [43, 49, 50].

The economic burden of neuropathic pain for the society has initiated research to identify novel targets for existing drugs. As antipsychotic agents, glycine transporter-1 inhibitors have progressed to an advanced clinical trials. However, concerns related to low efficacies have suspended their development. Nevertheless, assessment of their effect in other neurological conditions such as neuropathic pain has been continued in pre-clinical studies. Thus, we put a large effort into the current work to assess the analgesic effects of glycine transporter inhibitors, namely GlyT-1 and 2 or their combination in models of neuropathic pain in rat. There are several animal models for preclinical studies that intend to demonstrate the analgesic effect of test compounds in neuropathic pain. In our study we used partial sciatic nerve ligation (pSNL) induced neuropathic pain that described for allodynia by Seltzer et al. [58]. This pain model allows us to measure the developed allodynia in operated paw. In these series of experiments three inhibitors of glycine transporters, sarcosine, NFPS and Org-25543 were tested for their antiallodynic effects.

In experiments designed to assess the antiallodynic effect of sarcosine, two doses (500 and 1000 mg/kg) were administered systemically and the measurements of acute and chronic effects were performed. Under the present experimental conditions, sarcosine in a dose of 500 mg/kg produced significant antiallodynic effect either after acute or chronic administration (on day 4 and 6 of chronic treatment), and also it increased the baseline of pain threshold following 4 days of chronic treatment and there after (Figure 6, 7 and 8). On the other hand, sarcosine 1000 mg/kg produced acute anti-allodynia, but only a minimal effect on pain threshold on day 4 of chronic treatment. Interestingly, ongoing treatment with this dose (1000 mg/kg) failed to produce antiallodynic effect. Results of earlier studies by Centeno and co-workers had shown that sarcosine has a dose dependent analgesia in rats with spared nerve injury (SNI) after acute and chronic (2 times a day) treatments. In addition, in their study acute per os 500 mg/kg sarcosine produced anti-allodynia which fits with the results obtained by us for s.c. 500 mg/kg sarcosine. However, in their study the chronic effect of the highest dose of sarcosine (500 mg/kg, p.o.) was seen on day 7, 10 and 14 of treatment [54]. In addition, the study by Centeno

and co-workers also compared the efficacy of oral sarcosine versus intrathecal or infusion in medial prefrontal cortex (MPFC) in rats with neuropathic pain. In their work the authors found that per os sarcosine caused increase in the pain threshold in both the normal and the neuropathic paws [54]. However, in our study sarcosine (500 mg/kg s.c.) only increased the pain threshold of the neuropathic paws. At the present, it is difficult to justify what made sarcosine to show this discrepancy in the analgesic effect. Thus, further studies are needed to elucidate this issue. Indeed, the route of administration applied by us and Centeno et al. were both systemic route (s.c. and per os), so we can not relate this contradictory result to the difference in the route of administration. Glycine has dual effects, namely activation of inhibitory glycine receptors and co-agonistic effect on NMDA excitatory receptors. On the other hand, the structural similarity between sarcosine and glycine might raise the possibility for sarcosine itself to be involved in the activation of NMDA receptors as a co-agonist. In this regard, however, there is a contradiction in context [95, 96]. Based on these data we can hypothesize that sarcosine in medium doses do inhibit GlyT-1 that results in increase in the concentration of glycine, which acts as a co-agonist on extrasynaptic NMDA receptors NR1/NR2B subtype and promotes its internalization in long term, as described previously [57]. When sarcosine is applied in higher doses in our study (1000mg/kg) it raises the possibility that sarcosine itself acts as a co-agonist at NMDA receptor, though the desensitization of NMDAR by sarcosine is lower than of glycine [95]. Keeping in mind that the presence of glutamate is a prerequisite for the activation of NMDA receptor, and it has been reported to be increased in CNS of rodents with neuropathic pain based on previous works [28, 30, 31] indicating the existence of the crucial prerequisite for the activation of NMDA receptor. Also this condition appears to be met in our present work (Figure 15B). Pro-nociception of GlyT-1 inhibitors has been reported by Hermanns and colleagues (2008), for the medium dose of applied doses (10, 50 and 100 μg i.t) of ALX5407 (Isomer of NFPS) in CCI pain model [53]. The possibility of spill-over of glycine to extra-synaptic space and induction of NMDA receptors was discussed as an explanation for this phenomenon [27, 32, 33]. In our glycine release study the impact of sarcosine, a substrate and reversible GlyT-1 inhibitor, was compared to ACPFB or NFPS, none-substrate irreversible GlyT-1 inhibitors. We could prove that the effect of sarcosine on glycine release differs from that of ACPFB or NFPS. Sarcosine increased the glycine efflux in hippocampus tissue

significantly while ACPPB and NFPS failed to do that, and instead ACPPB inhibited the sarcosine-induced glycine release once combined (Figure 14). Thus, it is possible that sarcosine, as a substrate for GlyT-1, by activating the reverse mode of GlyT-1 and the increase in glycine level in the vicinity of extrasynaptic NMDA receptors promote its internalization as it is reported [57]. In previous study, based on IC₅₀ values from inhibition of [³H]glycine uptake in rat cortical synaptosomes, ACPPB and NFPS were equipotent but much more potent than sarcosine (27 μM for sarcosine, 11 nM for ACPPB and 12 nM for NFPS) [45]. In our in vivo studies we applied irreversible GlyT-1 inhibitor, NFPS for further experiments.

In order to decrease the side effects of drugs, the concomitant administration of small doses of drugs that act at different targets has been followed by many investigators. Herein the targets are glycine transporter 1 and 2 which are unlikely to be equally distributed in CNS regions, accounting for their effects, as it is mentioned in introduction [12, 15, 35, 43]. In this respect, we hypothesized that developing more reversible GlyT inhibitors with lower selectivity or co-administration of existing selective GlyT-1 and 2 inhibitors in lower doses might offer novel therapeutic approach for the treatment of neuropathic pain. Prior to this study, the impact of the co-administration (combination) of sub-analgesic doses of GlyT-1 and 2 selective and potent inhibitors, namely NFPS and Org-25543 on neuropathic pain induced by pSNL in rats was not studied, but the analgesic effect of either NFPS or Org-25543 was analysed separately. In our experiments the acute treatment with low doses of these compounds (NFPS 1 mg/kg or Org-25543 2 mg/kg s.c.) failed to reduce the pain up to 3 hours (Figure 9A and 10A), however the chronic treatment (once a day in the morning) with low dose of NFPS (1 mg/kg) raised PWT baseline values significantly (chronic effect) but this effect did not happen for the low dose of Org-25543 (2 mg/kg) (Figure 9D and 10C). The chronic effect of GlyT-1 inhibitor, NFPS could be the result of reduction of expression of spinal NR-1 NMDA receptors following chronic treatment with this compound, as it was shown by Barthel and colleagues [47], where they did not observe any changes in expression of NMDA receptors from GlyT-2 inhibition by ALX1393. In addition, in their study the level of both types of GlyT expression in spinal cord remained without any changes after chronic inhibition of any of glycine transporters. On the other hand, in another study, prolonged in vitro application of Org-25543 in spinal slices caused reduction in synaptic release of

glycine, indicating its role in reuptake for re-filling of pre-synaptic vesicles [92]. After stepwise doubling of systemic acute doses of GlyT inhibitors, a significant anti-allodynic effect was recorded for both NFPS and Org-25543 at dose of 4 mg/kg with the peak effect at 60' post-treatment (Figure 9C and 10B). Other pre-clinical studies on GlyT inhibitors showed acute and chronic dose-dependent anti-nociceptive effects from their application [43, 47, 48, 50, 79, 75, 80] which has been already mentioned about NFPS and Org-25543 in introduction part.

One of concerns regarding application of irreversible selective inhibitors of glycine transporters is the occurrence of severe adverse reactions on motor function and respiratory system from irreversible blockage of glycine transporters [49]. In experimental mice lacking GlyT-1, extreme muscle weakness and irregular respiration was observed [90, 91]. Mice lacking GlyT-2 showed tremor and motor dysfunction [93]. High doses of NFPS (10-30 mg/kg i.p.) affected motor function and resulted in respiratory depression [35, 53]. The high dose of Org-25543 (20 mg/kg i.v.) produced toxic effects such as spasm, convulsion and death in rodents [49]. The high applied doses in our study were less than half of toxic dose for NFPS and one fifth of toxic dose for Org-25443, and none of these treatments at the time of their peak effect (60' post-treatment) or later at 180' showed any significant motor disturbance and imbalance in rotarod test. Also, co-administration with sub-analgesic doses of NFPS and Org-25543 at time of their peak effect (180') did not produce any motor dysfunction (Figure 13). In addition to this section, applied doses of sarcosine also were tested after acute and chronic treatments and result did not show any motor impairments as well (Figure 12). In all tested treatments during any of experiments we did not observe any suspicious behavioural changes or any observable respiratory distress.

Finally, systemic co-administration of sub-analgesic doses of both compounds (NFPS 1 mg/kg and Org-25543 2 mg/kg) produced anti-allodynia at 60' and the peak effect was at 180' post-treatment (Figure 11). The applied doses are one tenth of the toxic doses claimed by literature, while the effect is higher than two times of the single dose of NFPS (1mg/kg) indicating our hypothesis on potentiation of anti-allodynia from the combination of sub-analgesic doses of both types of inhibitors with a better safety margin. To the best of our knowledge, the approach of combination of sub-analgesic doses of both types of GlyT inhibitors, namely NFPS and Org-25543 in reduction of allodynia and the

measurement of CSF glycine level after treatment with this combination is absent in literature.

To further shed light on the mechanism behind the generation of this effect, we measured glycine and L-glutamate levels in CSF and spinal tissue with capillary electrophoresis. We have succeeded to measure the content of both glycine and L-glutamate either in CSF or spinal tissue. To verify the results of these experiments, thoroughgoing literature has been conducted. Our results were in accordance with the literature where the authors applied similar method to measure the content of glycine in CSF [83].

The measurement of glycine and L-glutamate contents in CSF showed that after induction of neuropathy, there was a tendency of increase in glycine level and a significant increase in L-glutamate compared to sham (Figure 15B). However, comparison of glycine and glutamate contents of Lumbar 4-6 tissue of the spinal cord showed that there was not any difference between operated (ipsi-lateral) and unoperated (contra-lateral) sides, nor between non-neuropathic (sham) and neuropathic (vehicle treated) animals (Figure 15C and 15D). As it was discussed in introduction, neuropathic pain is the result of imbalance between inter-neuronal inhibition and excitation in dorsal horn of spinal cord, and this imbalance is in favour of the excitatory pathway. By time, the high extent of glutamate released from afferent fibres lead to spill-over of glutamate to extra-synaptic space and in consequence it activates extra-synaptic NMDA receptors, and in turn, neuronal excitation increases even further [24, 26, 27, 32, 33]. Our results by showing elevation of CSF glutamate content following neuropathic development without significant increase in glycine content, and by showing the lack of a noticeable difference in tissue levels of these neurotransmitters, confirms the presence of higher amount of glutamate in extra-synaptic area from neuronal over-excitation.

One of solutions to overcome this imbalance is the blockage of glycine reuptake to raise synaptic and/or extra-synaptic glycine level to make it available to synaptic GlyRs and thus to potentiate the glycinergic inhibition in dorsal horn of spinal cord. Different research data confirm the participation of both type of glycine transporters (astroglial GlyT-1 and synaptic GlyT-2) in neurotransmission of glycine in glycinergic inhibition [35, 45, 75,]. Experimental reports from literature data indicate the increase in CSF glycine content following application of GlyT-1 or 2 inhibitors [84, 85, 86]. Results from

HPLC measurements on samples from rats treated with GlyT-1 Inhibitors, ALX5407 (0.1–10 mg/kg p.o) and LY2365109 (0.3–30 mg/kg p.o) shows a dose-dependent increase in CSF glycine content [89]. Inhibition of astroglial GlyT-1 raises the CSF level of glycine and makes it available for over-induction of extra-synaptic NMDA receptors leading to down-regulation of NMDA receptors, as it was already reported by literature [57]. Also, elevated CSF glycine content after GlyT-1 inhibition becomes accessible to GlyRs by spill-over to synaptic areas. In a study, intrathecal injection of strychnine (GlyR α 3 blocker) or knockdown of GlyR α 3 (The dominant sub-type of GlyR in spinal cord and caudal regions of brain) abolished the analgesic effects from GlyT-1 inhibitors without any potentiation of pain. This indicates that anti-nociceptive effects of GlyT-1 inhibitors is the result of induction of glycinergic inhibition by activation of spinal glycine receptors [43, 35, 48, 76, 78]. GlyT-2 is restricted to pre-synaptic membranes and participates in reuptake of glycine back into glycinergic neurons for refilling of vesicles. Inhibition of GlyT-2 increases synaptic level of glycine available for glycine receptors, and thus potentiates glycinergic inhibition. Overall literature data confirm the potentiation of glycinergic inhibition by activation of glycine receptors as a result of elevation of glycine content after blocking glycine reuptake by either GlyT-1 or 2 inhibitors [15, 18, 35, 82]. But other experimental works on assessment of amount of glycine elevation in synaptic space by both types of GlyT inhibitors showed a higher accumulation of glycine in vicinity of synaptic GlyT-2 [87, 88].

In our results, systemic administration of low and sub-analgesic doses of each type of inhibitors alone did not increase the level of glycine in CSF, although a tendency was noted for NFPS, but just the combination of these sub-analgesic doses (NFPS 1 mg/kg and Org-25543 2 mg/kg s.c.) could raise the glycine level significantly compared to control (sham group) and this combination did not affect the L-glutamate level (Figure 15A and 15B). In this regard, it is evident that the potentiation of anti-allodynic effect following treatment with combination of sub-analgesic doses of both types of GlyT inhibitors could be the result of sufficient increase in CSF glycine level which in turn induces glycinergic inhibition and thus alleviates the neuropathic pain. The applied combination of NFPS and Org-25543 lacked any motor side effect. The potentiated anti-neuropathic pain outcome from acute application of low sub-analgesic doses of co-administration of both types of GlyT inhibitors without any observable side effect

presents a new strategy for management of neuropathic pain with higher efficacy and better safety margin. The irreversible inhibition of transporters by applied compounds in this combination is still a concern regarding toxicity following long term treatment with these compounds, and thus reversible inhibition might be a better approach for long term medical treatment. The low potency of sarcosine, a reversible GlyT-1 inhibitor and the observance of undesirable outcomes did not give enough validation for taking this approach in this study, but some recent studies gave promising results from novel synthetic reversible GlyT-2 inhibitors [35, 51]. In conclusion, systemic co-administration of low doses of reversible GlyT-1 and 2 inhibitors could be a future medical intervention for management of neuropathic pain.

A further question was also raised as to the impact of GlyT inhibitors on receptors such as G-protein-coupled receptors (GPCRs). In this regards, NFPS and Org-25543 were tested for their actions on G-protein activity applying radio-ligand G-protein binding assays. To the best of our knowledge only NFPS had been already tested for its G-protein binding by Harsing and colleagues [46] and their result showed a very negligible affinity for G-protein coupled receptors. Subsequently we evaluated the effect of Org-25543, NFPS or their combination on G-protein activity. Our result from G-protein binding assay (Figure 16), excluded any G-protein agonist action for any of studied compounds (20%DMSO, sarcosine, NFPS, Org-25543, combination of NFPS and Org-25543). Thus, the measured analgesic effects for the NFPS, Org-25543 or their combination is likely the consequence of direct inhibition of GlyT transporters, yet in case of sarcosine it is still arguable based on its various interactions with GlyT-1 and NMDAR [40, 95, 96].

6. Conclusions

Anti-neuropathic treatment is a sophisticated subject when it comes to its undesirable outcomes. In neuropathic pain conditions, the interplay of inter-neuronal inhibition and excitation in lamina II and III of dorsal horn of spinal cord is compromised by increase in glutamatergic excitation and decrease in glycinergic inhibition. Application of glycine transporter (GlyT) type 1 and 2 inhibitors potentiates glycinergic neurons by increasing in spinal glycine content and hence reducing the pain, but their high doses show motor and respiratory side-effects. The aim of the study was to initially evaluate sarcosine (reversible GlyT-1 inhibitor) and then NFPS (irreversible GlyT-1 inhibitor) and Org-25543 (irreversible GlyT-2 inhibitor) and their co-administration, to reach a better therapeutic index. Multiple systemic doses (acute or chronic) of these compounds were assessed on allodynia on rats with partial sciatic nerve ligation neuropathic pain model by the measurement of paw withdraw thresholds. Sarcosine was also assessed on acute thermal pain and acute inflammatory pain models. All applied compounds were tested for motor coordination by rotarod test. Glycine and L-glutamate content of CSF and spinal cord (L4-6) were measured with capillary electrophoresis. Glycine release in rat brain tissue was compared between sarcosine, NFPS and ACPFB (irreversible GlyT-1 inhibitor). G-protein activity of compounds were evaluated by G-protein activity assay. Results showed analgesia for sarcosine, a reversible GlyT-1 inhibitor in acute thermal pain and allodynia without any motor side effects. However, the anti-allodynia from the lower dose was more pronounced, probably related to excessive release of glycine, found from in vitro release study. Systemic irreversible GlyT-1 and 2 inhibitors, NFPS and Org-25543 respectively, produced acute anti-allodynia only in high doses, but their co-administration of sub-analgesic doses enhanced acute anti-allodynia. This combination increased the CSF glycine content significantly, which suggests the induction of glycinergic inhibition in spinal cord. NFPS and Org-25543 did not show any motor side effects in rotarod test. Chronic systemic NFPS in low dose increased allodynia threshold. G-protein action was excluded of all test compounds by result of in vitro study. In conclusion, the novel strategy of combination of sub-analgesic doses of GlyT-1 and 2 inhibitors by potentiation of efficacy and avoiding adverse reactions, might be a solution for future clinical application of these compounds in the treatment of neuropathic pain.

7. Summary

The aim of the study was to assess the analgesic effects of different glycine transporter inhibitors, such as sarcosine (reversible GlyT-1 inhibitor), NFPS (irreversible GlyT-1 inhibitor) and Org-25543 (irreversible GlyT-2 inhibitor) in distinct animal models of pain in rats in various treatment conditions. Further goals were to evaluate their locomotor side effects and their impact on glycine and glutamate concentrations and G-protein activity in the central nervous system. The results are summarized below.

1) Chronic systemic high dose (1000 mg/kg) of sarcosine, produced analgesia in acute thermal pain in rat tail-flick test. Systemic acute or chronic sarcosine (500 mg/kg) failed to reduce acute inflammatory pain in rat formalin test. On the other hand, sarcosine showed anti-allodynia in the partial sciatic nerve ligation neuropathic animal pain model after acute and chronic systemic administration (500 and 1000 mg/kg). Additionally, sarcosine increased glycine efflux from rat hippocampus tissue in glycine release experiment. **2)** Rats treated with NFPS or Org-25543 showed anti-allodynia only in the systemic higher acute doses (4 mg/kg) in the partial sciatic nerve ligation neuropathic animal pain model. However, acute systemic co-administration of sub-analgesic doses of NFPS (1 mg/kg) and Org-25543 (2 mg/kg) alleviated pain significantly. The systemic chronic low dose (1 mg/kg) of NFPS also increased the baseline values of allodynia in neuropathic animals. **3)** None of the GlyT inhibitors disturbed motor coordination of rats in rotarod test. **4)** Data from capillary electrophoresis measurements showed the elevation of L-glutamate in cerebrospinal fluid of samples from neuropathic animals, which was not reversed by the combination of systemic sub-analgesic doses of NFPS (1 mg/kg) and Org-2554 (2 mg/kg), but only this combination raised the glycine level significantly. In addition, no changes were observed in glycine or L-glutamate content in spinal cord tissues. **5)** Finally, based on our G-protein activity assay, we can exclude the G-protein coupled activation abilities of either of the test compounds.

In conclusion, augmented anti-allodynia from systemic co-administration of GlyT-1 and 2 inhibitors shows a better therapeutic index, and it could be a novel approach in management of neuropathic pain in future.

8. Összefoglalás

Célunk volt meghatározni több glicin transzporter gátló (GlyT) úgy, mint a szarkozin (reverzibilis GlyT-1 inhibitor), az NFPS (irreverzibilis GlyT-1 inhibitor) és az Org-25543 (irreverzibilis GlyT-2 inhibitor) analgetikus hatását különféle fájdalommodellekben, patkányokon, eltérő kezelési paraméterekkel. További célunk volt megvizsgálni ezen GlyT inhibitorok motoros funkciókra, a központi idegrendszeri glicin és glutamát koncentrációra illetve G-fehérje aktivációra gyakorolt hatását is. Az eredmények összefoglalva az alábbiak:

1) Krónikusan, szisztémásan és magas (1000 mg/kg) dózisban adagolva a szarkozin analgetikus hatást mutatott patkány tail-flick tesztben, viszont a formalin, akut gyulladáshozos tesztekben hatástalan volt 500 mg/kg dózisban, mind akutan, mind krónikusan adagolva. Ugyanakkor antiallodíniás hatást tapasztaltunk akut és krónikus dózisban adagolva (500 és 1000 mg/kg) részleges idegkötést alkalmazó neuropátiás fájdalommodellben. Továbbá a szarkozin megemelte a glicin kibocsátást is patkány hippocampusz mintákban. **2)** NFPS és Org-25543 antiallodíniás hatással rendelkezett, de csak magasabb (4 mg/kg), akut dózisban, viszont alacsonyabb, szubanalgetikus dózisban (NFPS: 1 mg/kg, Org-25543: 2 mg/kg), akutan a két gátlószer kombinálva csökkentette a neuropátiás fájdalmat. Alacsony dózisú (1 mg/kg) NFPS krónikusan adagolva emelte a neuropátiás patkányok alapfájdalom küszöb értékeit. **3)** A vizsgált GlyT inhibitorok közül egyik sem befolyásolta az állatok motoros funkcióit. **4)** A kapilláris elektroforézis kísérletekben neuropátiás állatok agyi-gerincvelői folyadékában magasabb L-glutamát szint volt tapasztalható, mely az NFPS és Org-25543 kombinációjával nem volt visszafordítható. Ezzel szemben, csak a két inhibitor kombinációja emelte meg szignifikánsan az agyi-gerincvelői folyadék glicin szintjét. A gerincvelői mintákban sem a glicin, sem az L-glutamát szintje nem változott. **5)** Végezetül a G-fehérje aktivációs kötési tesztekben egyik GlyT gátlószer sem stimulálta a G-fehérje kapcsolt receptorokat a kísérleti rendszerben.

Konklúzióként elmondható, hogy a GlyT 1 és 2 szisztémás, szubanalgetikus dózisú kombinációja jobb terápiás indexszel rendelkezik az antiallodíniás hatás növelésében, mely egyúttal a jövőben egy új megközelítés lehet a neuropátiás fájdalom kezelésében.

9. Bibliography

1. Merskey H, Bogduk N. (1994) International Organization for the Study of Pain (IASP) IASP Task Force on Taxonomy. Part III: Pain Terms, a Current List with Definitions and Notes on Usage. In Classification of Chronic Pain, 2nd ed. IASP Press: Seattle, 1994: 209–214. <https://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698&navItemNumber=576>
2. Nahin RL. (2015) Estimates of Pain Prevalence and Severity in Adults: United States, 2012. *J. Pain*, 16: 769–780. DOI: <https://doi.org/10.1016/j.jpain.2015.05.002>
3. Varrassi G, Müller-Schwefe G, Pergolizzi J, Orónska A, Morlion B, Mavrocordatos P, Margarit C, Mangas C, Jaksch W, Huygen F, Collett B, Berti M, Aldington D, Ahlbeck K. (2010) Pharmacological treatment of chronic pain - the need for CHANGE. *Current medical research and opinion*, 26(5): 1231–1245. DOI: <https://doi.org/10.1185/03007991003689175>
4. Bates D, Schultheis BC, Hanes MC, Jolly SM, Chakravarthy KV, Deer TR, Levy RM, Hunter CW. (2019) A Comprehensive Algorithm for Management of Neuropathic Pain. *Pain medicine (Malden, Mass.)*, 20(Suppl 1): S2–S12. DOI: <https://doi.org/10.1093/pm/pnz075>
5. Arthur C. Guyton, John E. Hall. (2006) *Textbook of medical physiology*. 11th ed: 598-606
6. Bourne S, Machado AG, Nagel SJ. (2014) Basic anatomy of physiology of pain pathways. *Neurosurg. Clin. N. Am*, 25: 629–638. DOI: <https://doi.org/10.1016/j.nec.2014.06.001>
7. Woolf CJ, Mannion RJ. (1999) Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet (London, England)*, 353(9168): 1959–1964. DOI: [https://doi.org/10.1016/S0140-6736\(99\)01307-0](https://doi.org/10.1016/S0140-6736(99)01307-0)

8. Bridges D, Thompson SW, Rice AS. (2001) Mechanisms of neuropathic pain. *British journal of anaesthesia*, 87(1): 12–26. DOI: <https://doi.org/10.1093/bja/87.1.12>
9. Ueda H. (2006). Molecular mechanisms of neuropathic pain-phenotypic switch and initiation mechanisms. *Pharmacology & therapeutics*, 109(1-2), 57–77. DOI: <https://doi.org/10.1016/j.pharmthera.2005.06.003>
10. Campbell JN, & Meyer RA. (2006). Mechanisms of neuropathic pain. *Neuron*, 52(1): 77–92. DOI: <https://doi.org/10.1016/j.neuron.2006.09.021>
11. REXED B. (1952) The cytoarchitectonic organization of the spinal cord in the cat. *The Journal of comparative neurology*, 96(3): 414–495. DOI: <https://doi.org/10.1002/cne.900960303>
12. Morgan C, Nadelhaft I, de Groat WC. (1981) The distribution of visceral primary afferents from the pelvic nerve to Lissauer's tract and the spinal gray matter and its relationship to the sacral parasympathetic nucleus. *The Journal of comparative neurology*, 201(3): 415–440. DOI: <https://doi.org/10.1002/cne.902010308>
13. Foster E, Wildner H, Tudeau L, Haueter S, Ralvenius WT, Jegen M, Johannssen H, Hösl L, Haenraets K, Ghanem A, Conzelmann KK, Bösl M, Zeilhofer HU. (2015) Targeted ablation, silencing, and activation establish glycinergic dorsal horn neurons as key components of a spinal gate for pain and itch. *Neuron*, 85(6): 1289–1304. DOI: <https://doi.org/10.1016/j.neuron.2015.02.028>
14. Woolf CJ, Salter MW. (2000) Neuronal plasticity: increasing the gain in pain. *Science*. 288(5472):1765-1769. DOI: <https://doi.org/10.1126/science.288.5472.1765>
15. Cioffi CL. (2018) Modulation of Glycine-Mediated Spinal Neurotransmission for the Treatment of Chronic Pain. *Journal of medicinal chemistry*, 61(7): 2652–2679. DOI: <https://doi.org/10.1021/acs.jmedchem.7b00956>

16. Melzack R, Wall PD. (1965) Pain mechanisms: a new theory. *Science* (New York, N.Y.), 150(3699): 971–979. DOI: <https://doi.org/10.1126/science.150.3699.971>
17. Petitjean H, Pawlowski SA, Fraine SL, Sharif B, Hamad D, Fatima T, Berg J, Brown CM, Jan LY, Ribeiro-da-Silva A, Braz JM, Bashaum AI, Sharif-Naeini R. (2015) Dorsal horn parvalbumin neurons are gate-keepers of touch-evoked pain after nerve injury. *Cell Rep.* 13: 1246–1257. DOI: <https://doi.org/10.1016/j.celrep.2015.09.080>
18. Haranishi Y, Hara K, Terada T, Nakamura S, Sata T. (2010) The antinociceptive effect of intrathecal administration of glycine transporter-2 inhibitor ALX1393 in a rat acute pain model. *Anesth Analg*, 110(2):615-621. DOI: <https://doi.org/10.1213/ane.0b013e3181c7ebbb>
19. Karlsson U, Sjödin J, Angery Möller K, Johansson S, Wikström L, Nasström J. (2002) Glutamate-induced currents reveal three functionally distinct NMDA receptor populations in rat dorsal horn-effects of peripheral nerve lesion and inflammation. *Neuroscience*, 112: 861–868. DOI: [https://doi.org/10.1016/S0306-4522\(02\)00140-9](https://doi.org/10.1016/S0306-4522(02)00140-9)
20. Johnson JW, Ascher P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, 325: 529–531. DOI: <https://doi.org/10.1038/325529a0>.
21. Köles L, Kató E, Hanuska A, Zádori ZS, Al-Khrasani M, Zelles T, Rubini P, Illes P. (2016) Modulation of excitatory neurotransmission by neural/glia signalling molecules: interplay between purinergic and glutamatergic systems. *Purinergic Signal*, 12: 1–24. DOI: <https://doi.org/10.1007/s11302-015-9480-5>
22. Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Han HJ, Beitz AJ, Lee JH. (2008) 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*, 12(5): 552–563. DOI: <https://doi.org/10.1016/j.ejpain.2007.09.002>

23. Yang X, Yang HB, Xie QJ, Liu XH, Hu XD. (2009) Peripheral inflammation increased the synaptic expression of NMDA receptors in spinal dorsal horn. *Pain*, 144: 162–169. DOI: <https://doi.org/10.1016/j.pain.2009.04.005>
24. Petrenko AB, Yamakura T, Baba H, Shimoji K. (2003) Unaltered pain-related behavior in mice lacking NMDA receptor GluR ϵ 1 subunit. *Neurosci. Res.*, 46(2): 199–204. DOI: [https://doi.org/10.1016/s0168-0102\(03\)00061-0](https://doi.org/10.1016/s0168-0102(03)00061-0)
25. Petrenko AB, Yamakura T, Baba H, Shimoji K. (2003) The role of N-methyl-D-aspartate (NMDA) receptors in pain: a review. *Anesth. Analg.* 97(4) 1108–1116. DOI: <https://doi.org/10.1213/01.ane.0000081061.12235.55>
26. Vizi ES, Kisfali M, Lőrincz T. (2013) 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: <https://doi.org/10.1016/j.brainresbull.2012.10.005>
27. Lin F, Stevens CF, (1994) Both open and closed NMDA receptor channels desensitize. *J. Neurosci.* 14: 2153–2160. DOI: <https://doi.org/10.1523/jneurosci.14-04-02153.1994>
28. Cavaliere C, Cirillo G, Bianco MR, Rossi F, De Novellis V, Maione S, Papa M. (2007) Gliosis alters expression and uptake of spinal glial amino acid transporters in a mouse neuropathic pain model. *Neuron Glia Biol.* 3: 1–14. DOI: <https://doi.org/10.1017/S1740925X07000695>.
29. Bai F, Ma Y, Guo H, Li Y, Xu F, Zhang M, Dong H, Deng J, Xiong L. (2019) Spinal cord glycine transporter 2 mediates bilateral ST35 acupoints sensitization in rats with knee osteoarthritis. *Evid. Based Complement. Altern. Med.* 2019: 7493286. DOI: <https://doi.org/10.1155/2019/7493286>
30. Harsing LG Jr, Matyus P. (2013) Mechanism of glycine release, which build up synaptic and extrasynaptic glycine levels: the role of synaptic and non-synaptic glycine transporters. *Brain Res. Bull.* 93: 110–119. DOI: <https://doi.org/10.1016/j.brainresbull.2012.12.002>

31. Shibasaki K, Hosoi N, Kaneko R, Tominaga M, Yamada K. (2017) Glycine release from astrocytes via functional reversal of GlyT1. *J. Neurochem.* 140: 385–403. DOI: <https://doi.org/10.1111/jnc.13741>.
32. Tong G, Shepherd D, Jahr CE. (1995) Synaptic desensitization of NMDA receptors by calcineurin. *Science* 267: 1510–1512. DOI: <https://doi.org/10.1126/science.7878472>
33. Hinzman JM, Thomas TC, Burmeister JJ, Quintero JE, Huettl P, Pomerleau F, Gerhardt GA, Lifshitz J. (2010) Diffuse brain injury elevates tonic glutamate levels and potassium-evoked glutamate release in discrete brain regions at two days postinjury: an enzyme-based microelectrode array study. *J. Neurotrauma* 27: 889–899 DOI: <https://doi.org/10.1089/neu.2009.1238>
34. Zhang W, Shi CX, Gu XP, Ma ZL, Zhu W. (2009) Ifenprodil induced antinociception and decreased the expression of NR2B subunits in the dorsal horn after chronic dorsal root ganglia compression in rats. *Anesth. Analg.* 108: 1015–1020. DOI: <https://doi.org/10.1213/ane.0b013e318193ffd2>
35. Dohi T, Morita K, Kitayama T, Motoyama N, Morioka N. (2009) Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. *Pharmacology & therapeutics*, 123(1): 54–79. DOI: <https://doi.org/10.1016/j.pharmthera.2009.03.018>
36. Martínez-Maza R, Poyatos I, López-Corcuera B, N úñez E, Giménez C, Zafra F, Aragón C. (2001) The role of N-glycosylation in transport to the plasma membrane and sorting of the neuronal glycine transporter GLYT2. *The Journal of biological chemistry*, 276(3): 2168–2173. DOI: <https://doi.org/10.1074/jbc.M006774200>
37. Roux MJ, Supplisson S. (2000) Neuronal and glial glycine transporters have different stoichiometries. *Neuron*, 25(2): 373–383. DOI: [https://doi.org/10.1016/s0896-6273\(00\)80901-0](https://doi.org/10.1016/s0896-6273(00)80901-0)
38. Lopez AB. (2020) Insights Into The Structure, Pharmacology, And Evolution Of The Glycine Transporter 2. *Open Access Theses & Dissertations*. 2996. DOI: https://scholarworks.utep.edu/open_etd/2996

39. Thomsen C. (2006) Glycine transporter inhibitors as novel antipsychotics. *Drug Discov. Today* 3(4): 539–545. DOI: <https://doi.org/10.1016/j.ddstr.2006.10.016>
40. Harsing LG, Zsilla G, Matyus P, Nagy KM, Marko B, Gyarmati Z, Timar J. (2012) Interactions between glycine transporter type-1 (GlyT-1) and some inhibitor molecules-Glycine transporter type 1 and its inhibitors (Review). *Acta Physiol. Hung.* 99(1): 1–17. DOI: <https://doi.org/10.1556/Aphysiol.99.2012.1.1>
41. López-Corcuera B, Martínez-Maza R, Núñez E, Roux M, Supplisson S, Aragón C. (1998). Differential properties of two stably expressed brain-specific glycine transporters. *J Neurochem* 71(5): 2211–2219. DOI: <https://doi.org/10.1046/j.1471-4159.1998.71052211.x>
42. Palacín M, Estévez R, Bertran J, Zorzano A. (1998). Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* 78(4): 969–1054. DOI: <https://doi.org/10.1152/physrev.1998.78.4.969>
43. Morita K, Motoyama N, Kitayama T, Morioka N, Kifune K, Dohi T. (2008) Spinal antiallodynia action of glycine transporter inhibitors in neuropathic pain models in mice. *J Pharmacol Exp Ther* 326(2): 633–645. DOI: <https://doi.org/10.1124/jpet.108.136267>
44. Mallorga PJ, Williams JB, Jacobson M, Marques R, Chaudhary A, Conn PJ, Pettibone DJ, Sur C. (2003) Pharmacology and expression analysis of glycine transporter GlyT1 with [3H]-(N-[3-(4'-fluorophenyl)-3-(4'phenylphenoxy)propyl])sarcosine. *Neuropharmacology*. 45(5): 585-593. DOI: [https://doi.org/10.1016/s0028-3908\(03\)00227-2](https://doi.org/10.1016/s0028-3908(03)00227-2)
45. Harsing LG Jr, Juranyi Z, Gacsalyi I, Tapolcsanyi P, Czompa A, Matyus P. (2006) Glycine transporter type-1 and its inhibitors. *Curr Med Chem*. 13(9): 1017-1044. DOI: <https://doi.org/10.2174/092986706776360932>
46. Harsing LG Jr, Gacsalyi I, Szabo G, Schmidt E, Sziray N, Sebban C, Tesolin-Decros B, Matyus P, Egyed A, Spedding M, Levay G. (2003) The glycine transporter-1 inhibitors NFPS and Org 24461: a pharmacological study. *Pharmacol Biochem Behav*. 74(4): 811-825. DOI: [https://doi.org/10.1016/s0091-3057\(02\)01078-x](https://doi.org/10.1016/s0091-3057(02)01078-x)

47. Barthel F, Urban A, Schlösser L, Eulenburg V, Werdehausen R, Brandenburger T, Aragon C, Bauer I, Hermanns H. (2014) Long-term application of glycine transporter inhibitors acts antineuropathic and modulates spinal N-methyl-D-aspartate receptor subunit NR-1 expression in rats. *Anesthesiology*. 121(1):160-169. DOI: <https://doi.org/10.1097/aln.000000000000203>
48. Tanabe M, Takasu K, Yamaguchi S, Kodama D, Ono H. (2008) Glycine Transporter Inhibitors as a Potential Therapeutic Strategy for Chronic Pain with Memory Impairment. *Anesthesiology*, Vol. 108(5): 929–937. DOI: <https://doi.org/10.1097/aln.0b013e31816c9044>
49. Mingorance-Le Meur A, Ghisdal P, Mullier B, De Ron P, Downey P, Van Der Perren C, Declercq V, Cornelis S, Famelart M, Van Asperen J, Jnoff E, Courade JP. (2013) Reversible inhibition of the glycine transporter GlyT2 circumvents acute toxicity while preserving efficacy in the treatment of pain. *Br J Pharmacol*. 170(5):1053-1063. DOI: <https://doi.org/10.1111/bph.12343>
50. Motoyama N, Morita K, Shiraishi S, Kitayama T, Kanematsu T, Uezono Y, Dohi T. (2014) Relief of cancer pain by glycine transporter inhibitors. *Anesth Analg*. 119(4): 988-995. DOI: <https://doi.org/10.1213/ane.0000000000000388>
51. Winters BL, Rawling T, Vandenberg RJ, Christie MJ, Bhola RF, Imlach WL. (2018) Activity of novel lipid glycine transporter inhibitors on synaptic signaling in the dorsal horn of the spinal cord. *Br. J. Pharmacol*. 175(12): 2337–2347. DOI: <https://doi.org/10.1111/bph.14189>
52. Vandenberg RJ, Mostyn SN, Carland JE, Ryan RM. (2016) Glycine transporter2 inhibitors: getting the balance right. *Neurochem. Internat*. 98: 89–93. DOI: <https://doi.org/10.1016/j.neuint.2015.12.007>
53. Hermanns H, Muth-Selbach U, Williams R, Krug S, Lipfert P, Werdehausen R, Braun S, Bauer I. (2008) Differential effects of spinally applied glycine transporter inhibitors on nociception in a rat model of neuropathic pain. *Neurosci. Lett*. 445(3): 214–219. DOI: <https://doi.org/10.1016/j.neulet.2018.09.012>

54. Centeno MV, Mutso A, Millecamps M, Apkarian AV. (2009) Prefrontal cortex and spinal cord mediated anti-neuropathy and analgesia induced by sarcosine, a glycine-T1 transporter inhibitor. *Pain*. 145(1-2): 176-183. DOI: <https://doi.org/10.1016/j.pain.2009.06.014>
55. Benjamin ER, Skelton J, Hanway D, Olanrewaju S, Pruthi F, Ilyin VI, Lavery D, Victory SF, Valenzano KJ. (2005) Validation of a fluorescent imaging plate reader membrane potential assay for high-throughput screening of glycine transporter modulators. *J Biomol Screen*. 10(4): 365-373. DOI: <https://doi.org/10.1177/1087057104274090>
56. Caulfield WL, Collie IT, Dickins RS, Epemolu O, McGuire R, Hill DR, McVey G, Morphy JR, Rankovic Z, Sundaram H. (2001) The first potent and selective inhibitors of the glycine transporter type 2. *J Med Chem*. 44(17): 2679-2682. DOI: <https://doi.org/10.1021/jm0011272>
57. Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT, Salter MW. (2003) Glycine binding primes NMDA receptor internalization. *Nature*. 422(6929):302-307. DOI: <https://doi.org/10.1038/nature01497>
58. Seltzer Z, Dubner R, Shir Y. (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain*. 43(2): 205-218. DOI: [https://doi.org/10.1016/0304-3959\(90\)91074-s](https://doi.org/10.1016/0304-3959(90)91074-s)
59. Balogh M, Varga BK, Karádi DÁ, Riba P, Puskár Z, Kozsurek M, Al-Khrasani M, Király K. (2019) Similarity and dissimilarity in antinociceptive effects of dipeptidyl-peptidase 4 inhibitors, Diprotin A and vildagliptin in rat inflammatory pain models following spinal administration. *Brain Res Bull*. 147: 78-85. DOI: <https://doi.org/10.1016/j.brainresbull.2019.02.001>
60. Balogh M, Zádor F, Zádori ZS, Shaqura M, Király K, Mohammadzadeh A, Varga B, Lázár B, Mousa SA, Hosztafi S, Riba P, Benyhe S, Gyires K, Schäfer M, Fürst S, Al-Khrasani M. (2019) Efficacy-Based Perspective to Overcome Reduced Opioid Analgesia of Advanced Painful Diabetic Neuropathy in Rats. *Front Pharmacol*. 2019 Apr 9; 10: 347. DOI: <https://doi.org/10.3389/fphar.2019.00347>

61. Zádor F, Mohammadzadeh A, Balogh M, Zádori ZS, Király K, Barsi S, Galambos AR, László SB, Hutka B, Váradi A, Hosztafi S, Riba P, Benyhe S, Fürst S, Al-Khrasani M. (2020) Comparisons of In Vivo and In Vitro Opioid Effects of Newly Synthesized 14-Methoxycodine-6-*O*-sulfate and Codeine-6-*O*-sulfate. *Molecules*. 25(6):1370. DOI: <https://doi.org/10.3390/molecules25061370>
62. Coderre TJ, Yashpal K. (1994) Intracellular messengers contributing to persistent nociception and hyperalgesia induced by L-glutamate and substance P in the rat formalin pain model. *Eur J Neurosci*. 6(8):1328-1334. DOI: <https://doi.org/10.1111/j.1460-9568.1994.tb00323.x>
63. Balogh M, Zádori ZS, Lázár B, Karádi D, László S, Mousa SA, Hosztafi S, Zádor F, Riba P, Schäfer M, Fürst S, Al-Khrasani M. (2018) The Peripheral Versus Central Antinociception of a Novel Opioid Agonist: Acute Inflammatory Pain in Rats. *Neurochem Res*. 43(6): 1250-1257. DOI: <https://doi.org/10.1007/s11064-018-2542-7>
64. Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. (1992) The formalin test: an evaluation of the method. *Pain*. 51(1):5-17. DOI: [https://doi.org/10.1016/0304-3959\(92\)90003-t](https://doi.org/10.1016/0304-3959(92)90003-t)
65. Shiotsuki H, Yoshimi K, Shimo Y, Funayama M, Takamatsu Y, Ikeda K, Takahashi R, Kitazawa S, Hattori N. (2010) A rotarod test for evaluation of motor skill learning. *J Neurosci Methods*. 189(2):180-185. DOI: <https://doi.org/10.1016/j.jneumeth.2010.03.026>
66. Jakó T, Szabó E, Tábi T, Zachar G, Csillag A, Szökő E. (2014) Chiral analysis of amino acid neurotransmitters and neuromodulators in mouse brain by CE-LIF. *Electrophoresis*. 35(19):2870-2876. DOI: <https://doi.org/10.1002/elps.201400224>
67. Richner M, Jager SB, Siupka P, Vaegter CB. (2017) Hydraulic Extrusion of the Spinal Cord and Isolation of Dorsal Root Ganglia in Rodents. *J Vis Exp*. (119):55226. DOI: <https://doi.org/10.3791/55226>

68. Zádor F, Kocsis D, Borsodi A, Benyhe S. (2014) Micromolar concentrations of rimonabant directly inhibits delta opioid receptor specific ligand binding and agonist-induced G-protein activity. *Neurochem Int.* 67: 14-22. DOI: <https://doi.org/10.1016/j.neuint.2013.12.005>
69. Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72: 248-254. DOI: <https://doi.org/10.1006/abio.1976.9999>
70. Sim LJ, Selley DE, Childers SR. (1995) In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5'-[gamma-[35S]thio]-triphosphate binding. *Proc Natl Acad Sci U S A.* 92(16): 7242-7246. DOI: <https://doi.org/10.1073/pnas.92.16.7242>
71. Traynor JR, Nahorski SR. (1995) Modulation by mu-opioid agonists of guanosine-5'-O-(3-[35S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol.* 47(4): 848-854. PMID: 7723747 <https://molpharm.aspetjournals.org/content/47/4/848.long>
72. Atkinson BN, Bell SC, De Vivo M, Kowalski LR, Lechner SM, Ognyanov VI, Tham CS, Tsai C, Jia J, Ashton D, Klitenick MA. (2001) ALX 5407: a potent, selective inhibitor of the hGlyT1 glycine transporter. *Mol Pharmacol.* 60(6): 1414-1420. DOI: <https://doi.org/10.1124/mol.60.6.1414>
73. Dinakar P, Stillman AM. (2016) Pathogenesis of Pain. *Semin Pediatr Neurol.* 23(3): 201-208. DOI: <https://doi.org/10.1016/j.spen.2016.10.003>
74. Todd AJ. (2010) Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci.* 11(12): 823-836. DOI: <https://doi.org/10.1038/nrn2947>
75. Zafra F, Giménez C. (2008) Glycine transporters and synaptic function. *IUBMB Life.* 60(12): 810-817. DOI: <https://doi.org/10.1002/iub.128>
76. Zafra F, Gomeza J, Olivares L, Aragón C, Giménez C. (1995) Regional distribution and developmental variation of the glycine transporters GLYT1 and GLYT2 in the rat CNS. *Eur J Neurosci.* 7(6):1342-1352. DOI: <https://doi.org/10.1111/j.1460-9568.1995.tb01125.x>

77. Zeilhofer HU, Acuña MA, Gingras J, Yévenes GE. (2018) Glycine receptors and glycine transporters: targets for novel analgesics? *Cell Mol Life Sci.* 75(3): 447-465. DOI: <https://doi.org/10.1007/s00018-017-2622-x>
78. Harvey RJ, Yee BK. (2013) Glycine transporters as novel therapeutic targets in schizophrenia, alcohol dependence and pain. *Nat Rev Drug Discov.* 12(11):866-885. DOI: <https://doi.org/10.1038/nrd3893>
79. Takahashi Y, Hara K, Haranishi Y, Terada T, Obara G, Sata T. (2015) Antinociceptive effect of intracerebroventricular administration of glycine transporter-2 inhibitor ALX1393 in rat models of inflammatory and neuropathic pain. *Pharmacol Biochem Behav.* 130: 46-52. DOI: <https://doi.org/10.1016/j.pbb.2015.01.001>
80. Omori Y, Nakajima M, Nishimura K, Takahashi E, Arai T, Akahira M, Suzuki T, Kainoh M. (2015) Analgesic effect of GT-0198, a structurally novel glycine transporter 2 inhibitor, in a mouse model of neuropathic pain. *J Pharmacol Sci.* 127(3): 377-381. DOI: <https://doi.org/10.1016/j.jphs.2015.02.010>
81. Armbruster A, Neumann E, Kötter V, Hermanns H, Werdehausen R, Eulenburg V. (2018) The GlyT1 Inhibitor Bitopertin Ameliorates Allodynia and Hyperalgesia in Animal Models of Neuropathic and Inflammatory Pain. *Front Mol Neurosci.*10:438. DOI: <https://doi.org/10.3389/fnmol.2017.00438>
82. Mostyn SN, Carland JE, Shimmon S, Ryan RM, Rawling T, Vandenberg RJ. (2017) Synthesis and Characterization of Novel Acyl-Glycine Inhibitors of GlyT2. *ACS Chem Neurosci.* 8(9):1949-1959. DOI: <https://doi.org/10.1021/acscchemneuro.7b00105>
83. Ogawa S, Ota M, Ogura J, Kato K, Kunugi H. (2018) Effects of L-theanine on anxiety-like behavior, cerebrospinal fluid amino acid profile, and hippocampal activity in Wistar Kyoto rats. *Psychopharmacology (Berl).* 235(1): 37-45. DOI: <https://doi.org/10.1007/s00213-017-4743-1>
84. Pei JC, Hung WL, Lin BX, Shih MH, Lu LY, Luo DZ, Tai HC, Studer V, Min MY, Lai WS. (2019) Therapeutic potential and underlying mechanism of sarcosine (N-methylglycine) in N-methyl-D-aspartate (NMDA) receptor

- hypofunction models of schizophrenia. *J Psychopharmacol.* 33(10): 1288-1302. DOI: <https://doi.org/10.1177/0269881119856558>
85. Alberati D, Moreau JL, Lengyel J, Hauser N, Mory R, Borroni E, Pinard E, Knoflach F, Schlotterbeck G, Hainzl D, Wettstein JG. (2012) Glycine reuptake inhibitor RG1678: a pharmacologic characterization of an investigational agent for the treatment of schizophrenia. *Neuropharmacology.* 62(2): 1152-1161. DOI: <https://doi.org/10.1016/j.neuropharm.2011.11.008>
86. Hofmann C, Pizzagalli F, Boetsch C, Alberati D, Ereshefsky L, Jhee S, Patat A, Boutouyrie-Dumont B, Martin-Facklam M. (2016) Effects of the glycine reuptake inhibitors bitopertin and RG7118 on glycine in cerebrospinal fluid: results of two proofs of mechanism studies in healthy volunteers. *Psychopharmacology (Berl).* 233(13): 2429-2439. DOI: <https://doi.org/10.1007/s00213-016-4317-7>
87. Roux MJ, Martinez-Maza R, Le Goff A, Lopez-Corcuera B, Aragon C, Supplisson S. (2001) The glial and the neuronal glycine transporters differ in their reactivity to sulfhydryl reagents. *J Biol Chem.* 276(21): 17699-17705. DOI: <https://doi.org/10.1074/jbc.m009196200>
88. Poyatos I, Ponce J, Aragón C, Giménez C, Zafra F. (1997) The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons. *Brain Res Mol Brain Res.* 49(1-2): 63-70. DOI: [https://doi.org/10.1016/s0169-328x\(97\)00124-1](https://doi.org/10.1016/s0169-328x(97)00124-1)
89. Perry KW, Falcone JF, Fell MJ, Ryder JW, Yu H, Love PL, Katner J, Gordon KD, Wade MR, Man T, Nomikos GG, Phebus LA, Cauvin AJ, Johnson KW, Jones CK, Hoffmann BJ, Sandusky GE, Walter MW, Porter WJ, Yang L, Merchant KM, Shannon HE, Svensson KA. (2008) Neurochemical and behavioral profiling of the selective GlyT1 inhibitors ALX5407 and LY2365109 indicate a preferential action in caudal vs. cortical brain areas. *Neuropharmacology.* 55(5): 743-754. DOI: <https://doi.org/10.1016/j.neuropharm.2008.06.016>

90. Gomeza J, Hülsmann S, Ohno K, Eulenburg V, Szöke K, Richter D, Betz H. (2003) Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. *Neuron*. 40(4): 785-796. DOI: [https://doi.org/10.1016/s0896-6273\(03\)00672-x](https://doi.org/10.1016/s0896-6273(03)00672-x)
91. Pierrefiche O, Schwarzacher SW, Bischoff AM, Richter DW. (1998) Blockade of synaptic inhibition within the pre-Bötzing complex in the cat suppresses respiratory rhythm generation in vivo. *J Physiol*. 509 (Pt 1): 245-254. DOI: <https://doi.org/10.1111/j.1469-7793.1998.245bo.x>
92. Bradaia A, Schlichter R, Trouslard J. (2004) Role of glial and neuronal glycine transporters in the control of glycinergic and glutamatergic synaptic transmission in lamina X of the rat spinal cord. *J Physiol*. 559(Pt 1): 169-186. DOI: <https://doi.org/10.1113/jphysiol.2004.068858>
93. Gomeza J, Ohno K, Hülsmann S, Armsen W, Eulenburg V, Richter DW, Laube B, Betz H. (2003) Deletion of the mouse glycine transporter 2 results in a hyperekplexia phenotype and postnatal lethality. *Neuron*. 40(4): 797-806. DOI: [https://doi.org/10.1016/s0896-6273\(03\)00673-1](https://doi.org/10.1016/s0896-6273(03)00673-1)
94. Bell A. (2018) The neurobiology of acute pain. *Vet J*. 237: 55-62. DOI: <https://doi.org/10.1016/j.tvjl.2018.05.004>
95. Zhang HX, Hyrc K, Thio LL. (2009) The glycine transport inhibitor sarcosine is an NMDA receptor co-agonist that differs from glycine. *J Physiol*, 587(Pt 13):3207-3220. DOI: <https://doi.org/10.1113/jphysiol.2009.168757>
96. Lee MY, Lin YR, Tu YS, Tseng YJ, Chan MH, Chen HH. (2017) Effects of sarcosine and N, N-dimethylglycine on NMDA receptor-mediated excitatory field potentials. *J Biomed Sci*, 24(1):18. DOI: <https://doi.org/10.1186/s12929-016-0314-8>
97. Lim ES, Lee IO. (2010) Effect of intrathecal glycine and related amino acids on the allodynia and hyperalgesic action of strychnine or bicuculline in mice. *Korean J Anesthesiol*, 58(1):76-86. DOI: <https://doi.org/10.4097/kjae.2010.58.1.76>

98. Sherman SE, Loomis CW. (1996) Strychnine-sensitive modulation is selective for non-noxious somatosensory input in the spinal cord of the rat. *Pain*, 66(2-3):321-330. DOI: [https://doi.org/10.1016/0304-3959\(96\)03063-1](https://doi.org/10.1016/0304-3959(96)03063-1)
99. Beyer C, Banas C, Gomora P, Komisaruk BR. (1988) Prevention of the convulsant and hyperalgesic action of strychnine by intrathecal glycine and related amino acids. *Pharmacol Biochem Behav*, 29(1):73-78. DOI: [https://doi.org/10.1016/0091-3057\(88\)90276-6](https://doi.org/10.1016/0091-3057(88)90276-6)
100. Jourdan D, Ardid D, Bardin L, Bardin M, Neuzeret D, Lanphouthacoul L, Eschalier A. (1997) A new automated method of pain scoring in the formalin test in rats. *Pain*, 71(3):265-270. DOI: [https://doi.org/10.1016/s0304-3959\(97\)03366-6](https://doi.org/10.1016/s0304-3959(97)03366-6)
101. Harsing LG Jr, Sershen H, Lajtha A. (1992) Dopamine efflux from striatum after chronic nicotine: evidence for autoreceptor desensitization. *J Neurochem*, 59(1):48-54. DOI: <https://doi.org/10.1111/j.1471-4159.1992.tb08874.x>
102. Lu Y, Dong H, Gao Y, Gong Y, Ren Y, Gu N, Zhou S, Xia N, Sun YY, Ji RR, Xiong L. (2013) A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. *J Clin Invest*, 123(9):4050-4062. DOI: <https://doi.org/10.1172/jci70026>
103. Vandenberg RJ, Ryan RM, Carland JE, Imlach WL, Christie MJ. (2014) Glycine transport inhibitors for the treatment of pain. *Trends Pharmacol Sci*, 35(8):423-430. DOI: <https://doi.org/10.1016/j.tips.2014.05.006>
104. Zafra F, Ibáñez I, Giménez C. (2016) Glycinergic transmission: glycine transporter GlyT2 in neuronal pathologies. *Neuronal Signal*, 1(1): NS20160009. DOI: <https://doi.org/10.1042/ns20160009>

10. Bibliography of the author's publications

10.1. Author's publications related to this study

1. Mohammadzadeh A, Lakatos PP, Balogh M, Zádor F, Karádi DÁ, Zádori ZS, Király K, Galambos AR, Barsi S, Riba P, Benyhe S, Köles L, Tábi T, Szökő É, Harsing LG Jr, Al-Khrasani M. (2021) Pharmacological Evidence on Augmented Antiallodynia Following Systemic Co-Treatment with GlyT-1 and GlyT-2 Inhibitors in Rat Neuropathic Pain Model. *Int J Mol Sci.* 22(5):2479. DOI: <https://doi.org/10.3390/ijms22052479>
2. Al-Khrasani M, Mohammadzadeh A, Balogh M, Király K, Barsi S, Hajnal B, Köles L, Zádori ZS, Harsing LG Jr. (2019) Glycine transporter inhibitors: A new avenue for managing neuropathic pain. *Brain Res Bull.*152:143-158. DOI: <https://doi.org/10.1016/j.brainresbull.2019.07.008>

10.2. Author's publications unrelated to this study

1. Zádor F, Mohammadzadeh A, Balogh M, Zádori ZS, Király K, Barsi S, Galambos AR, László SB, Hutka B, Váradi A, Hosztafi S, Riba P, Benyhe S, Fürst S, Al-Khrasani M. (2020) Comparisons of In Vivo and In Vitro Opioid Effects of Newly Synthesized 14-Methoxycodine-6-*O*-sulfate and Codeine-6-*O*-sulfate. *Molecules.* 25(6):1370. DOI: <https://doi.org/10.3390/molecules25061370>
2. Balogh M, Zádor F, Zádori ZS, Shaqura M, Király K, Mohammadzadeh A, Varga B, Lázár B, Mousa SA, Hosztafi S, Riba P, Benyhe S, Gyires K, Schäfer M, Fürst S, Al-Khrasani M. (2019) Efficacy-Based Perspective to Overcome Reduced Opioid Analgesia of Advanced Painful Diabetic Neuropathy in Rats. *Front Pharmacol.*10:347. DOI: <https://doi.org/10.3389/fphar.2019.00347>
3. Fürst S, Zádori ZS, Zádor F, Király K, Balogh M, László SB, Hutka B, Mohammadzadeh A, Calabrese C, Galambos AR, Riba P, Romualdi P, Benyhe S, Timár J, Schmidhammer H, Spetea M, Al-Khrasani M. (2020) On the Role of Peripheral Sensory and Gut Mu Opioid Receptors: Peripheral Analgesia and Tolerance. *Molecules.* 25(11):2473. DOI: <https://doi.org/10.3390/molecules25112473>

4. László SB, Lázár B, Brenner GB, Makkos A, Balogh M, Al-Khrasani M, Hutka B, Mohammadzadeh A, Kemény Á, László T, Scheich B, Szabados T, Kenyeres É, Giricz Z, Bencsik P, Varga ZV, Novák J, Helyes Z, Ferdinandy P, Gyires K, Zádori ZS. (2020) Chronic treatment with rofecoxib but not ischemic preconditioning of the myocardium ameliorates early intestinal damage following cardiac ischemia/reperfusion injury in rats. *Biochem Pharmacol.* 178:114099. DOI: <https://doi.org/10.1016/j.bcp.2020.114099>
5. Lázár B, László SB, Hutka B, Tóth AS, Mohammadzadeh A, Berekméri E, Ágg B, Balogh M, Sajtos V, Király K, Al-Khrasani M, Földes A, Varga G, Makra N, Ostorházi E, Szabó D, Ligeti B, Kemény Á, Helyes Z, Ferdinandy P, Gyires K, Zádori ZS. (2021) A comprehensive time course and correlation analysis of indomethacin-induced inflammation, bile acid alterations and dysbiosis in the rat small intestine. *Biochem Pharmacol*, 190:114590. DOI: <https://doi.org/10.1016/j.bcp.2021.114590>

11. Acknowledgements

First, I would like to present my appreciation for the great amount of support I received from my supervisor Dr. Mahmoud Al-Khrasani who helped me to conduct and conclude this project. Also, I am thankful of Prof. Laszlo G. Harsing for his precious contribution in this project and his help for glycine release experiment.

I thank Prof. Péter Ferdinandy, head of the Department of Pharmacology and Pharmacotherapy for the opportunity to work in his institute.

I would like to thank Dr. Zoltán S. Zádori, Dr. Kornél Király and Dr. Pál Riba for their help and support in this study and other research studies.

I am grateful for the cooperation with Dr. Tamás Tábi, Dr. Péter P. Lakatos and Dr. Ferenc Zádor in performing the capillary electrophoresis test and G-protein activity assay.

For assistance in different stages of behavioural studies I am thankful of my colleagues Dr. Mihály Balogh, Dr. Dávid Árpád Karádi, Ms. Anna Rita Galambos, Ms. Szilvia Barsi

I would like to thank all fellow co-workers of the Department of Pharmacology and Pharmacotherapy. I also thank staff of the Doctoral Office for their help.

I am thankful of my family that always gave me motivation and support in this way.

Finally, I present my appreciation for Doctoral School of pharmaceutical sciences of Semmelweis University (Budapest, Hungary) and Richter Gedeon Plc. (Centenárium Alapítvány) for the opportunity and financial support for the present research study.

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) and ÚNKP-18-3-III (New National Excellence Program, Ministry of Human Capacities, Hungary) awarded to Dr. Mihály Balogh.