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THE EFFECT OF TOLPERISONE AND L-THEANINE ON GLUTAMATERGIC NEUROTRANSMISSION

PhD thesis

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List of Abbreviations

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
asc-1	alanine-serine-cysteine-1 transporter
ASCT	alanine-serine-cysteine-threonine transporter
ATP	adenosine-triphosphate
CE-LIF	capillary electrophoresis - laser-induced fluorescence detection
CMR	centrally acting muscle relaxant
CSF	cerebrospinal fluid
DAAO	*D*-amino acid oxidase
DL-TBOA	*DL*-threo-β-benzyloxyaspartic acid
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	dimethyl sulfoxide
EAAT	excitatory amino acid transporter
FBS	fetal bovine serum
GlyT	glycine transporter
GS	glutamine synthetase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LTP	long-term potentiation
MDD	major depressive disorder
NBD-F	4-fluoro-7- nitrobenzofurazan
NMDAR	N-methyl-*D*-Aspartate receptor
PPT	paw pressure threshold

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pSNL	partial sciatic nerve ligation
PAG	phosphate-activated glutaminase
SNAT	sodium-coupled neutral amino acid transporter
SR	serine racemase
TBSS	Tris-buffered salt solution
Tris	tris(hydroxymethyl)-aminomethane
S.E.M.	standard error of mean
SLC	solute carrier
VGLUT	vesicular glutamate transporter

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1. Introduction

Glutamate plays crucial roles in several physiological processes in living organisms. As one of the 20 canonical proteinogenic amino acids, it is of fundamental importance in protein structure. It is also a key compound in many biological functions ranging from maintenance of cell redox balance, conjugation of toxic compounds and activation of taste receptors (for review see: (1)). However, its role in central nervous system signaling is considered the most important from pharmacological point of view. A great majority (about 90%) of cortical and hippocampal neurons release glutamate as neurotransmitter (2) and glutamatergic signaling accounts for an estimated 80% of the energy expenditure of the human brain (3). In addition, glutamate may reach remarkably high concentrations (5-15 mmol per kg tissue) in the brain, far exceeding those of other amino acids including GABA and glycine (4). As a result, glutamate is considered not only the primary excitatory mediator but also the most abundant neurotransmitter in the human brain. As expected, glutamate has been associated with a myriad of pathophysiological processes as well. Drugs that either directly or indirectly alter the function of this signaling molecule play an essential part in many pharmacological therapies. Moreover, numerous clinical and preclinical trials are in progress examining new glutamatergic drugs, often with novel mechanisms of action. Repurposing drugs that are already approved for other indications is also a promising path to finding effective and safe medications for treatment of diseases associated with glutamatergic neurotransmission.

1.1. Regulation of Glutamatergic Neurotransmission

Strict regulation of synaptic neurotransmitter concentration is of critical importance in order to prevent neuronal overstimulation and subsequent cell death, known as excitotoxity. This pathological phenomenon is characterized by increased intracellular calcium levels following excessive activation of glutamate receptors and has been associated with numerous neurodegenerative diseases (for review, see: (5)). Synaptic glutamate level is regulated by a series of highly active, compartmentalized processes, collectively known as the glutamate-glutamine cycle. Though some have challenged it (6), it is generally accepted as the primary regulatory system of the glutamatergic

neurotransmission and several of its components have been examined as possible pharmacological targets (7-9).

In short, vesicular release of glutamate from the presynaptic terminals is followed by its rapid uptake from the synaptic cleft. This process is mediated by the excitatory amino acid transporters (EAATs), a family of glutamate transporters (10). Astrocytes are responsible for the majority of neurotransmitter uptake, while neurons only recover a minor fraction of synaptic glutamate (10). The contribution of microglia and oligodendrocytes to glutamate clearance is considered negligible as spill-over from the synaptic cleft is fairly low (5). In astrocytes, about 80% of glutamate is turned into glutamine by glutamine synthetase (GS) (11). With no modulatory effect on glutamate receptors, glutamine can be safely transported to neurons by sodium-coupled neutral amino acid transporters (SNATs) (12). Glutamine in neurons are then converted to glutamate by the phosphate-activated glutaminase (PAG) and stored in the synaptic vesicles, completing the glutamate-glutamine cycle (13).

1.1.1. The Glutamate-glutamine Cycle

High affinity glutamate transporters of the central nervous system are key components of the glutamate-glutamine cycle. While glutamate is substrate of many transporters, EAATs are considered the most important in the mammalian forebrain. All EAATs are members of the large solute carrier (SLC) membrane transporter protein gene family. EAAT2 (SLC1A2; also known as GLT-1 in rodents) is considered the primary glutamate transporter in the central nervous system (14) as it is responsible for the majority (about 95%) of all glutamate transport in the brain of rats and mice (14-16). EAAT1 (SLC1A3, GLAST in rodents) and EAAT3 (SLC1A1, EAAC1 in rodents) are selectively expressed in astrocytes and neurons, respectively, though their role is considered minor (17, 18).

Following its uptake into astrocytes, the majority of glutamate is converted to glutamine by the enzyme GS (4). In adults, GS is exclusively expressed in astrocytes (14, 19). For a long time, glutamate-glutamine transformation was thought to operate on a stoichiometrical basis, with every glutamate molecule removed from the synaptic cleft eventually recycled and restored in the synaptic vesicles of neurons (5). McKenna et. al,

however, showed that a small but not insignificant amount (about 20%) of glutamate is oxidized in astrocytes (20) by enzymes such as glutamate dehydrogenase. The degraded glutamate content is restored through *de novo* synthesis by pyruvate carboxylase in astrocytes (5, 21).

Mechanisms by which glutamine is transported to neurons are not well understood. SNAT3 (SLC38A3) and SNAT5 (SLC38A5) are expressed in astrocytes and thought to contribute to glutamine release from astrocytes (4, 22, 23). Less is known, however, about transporters that facilitate glutamine uptake into neurons as no transporters have been identified in axon terminals yet (4, 14). While SNAT1 (SLC38A1) and SNAT2 (SLC38A2) are expressed in neurons, they are only found in dendrites and cell bodies (4, 14, 24). SNAT7 (SLC38A7) and SNAT8 (SLC38A8) were recently discovered in neurons and may be present in axon terminals, although the results are not yet conclusive (14, 25, 26). In addition, ASCT2 (SLC1A5), a neutral amino acid exchange transporter that belongs to the alanine-serine-cysteine-threonine transporter (ASCT) family (7, 27), also has the ability to transport glutamine (4, 28).

Following neuronal uptake, glutamine is turned into glutamate by PAG, an enzyme that is preferentially localized in neuronal mitochondria (14, 29). As the last step of the glutamate-glutamine cycle, glutamate is diverted from metabolic pathways by vesicular glutamate transporters (VGLUTs). VGLUTs require adenosine triphosphate (ATP) hydrolysis for glutamate uptake into vesicles, where up to 60 mM concentrations can be reached (5, 30).

1.1.2. Glutamate Receptors in the Central Nervous System

Glutamate receptors are divided into ionotropic and metabotropic receptors. All ionotropic glutamate receptors are cation channels as they are permeable to both sodium and potassium ions, and in some cases, calcium ions. They consist of numerous distinct subunits and their composition fine-tunes their activity. Ionotropic receptors are named after their selective agonists, which are all structural analogues of glutamate. Three major receptor-types are recognized: the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), the kainic acid receptor and the N-methyl-*D*-Aspartate receptor (NMDAR) (for review see: (14)). The metabotropic receptors are

also categorized into subgroups based on their intracellular signaling pathways. Eight receptors have been identified to date (for review see: (31)).

1.1.3. The NMDA Receptor

Similarly to other ionotropic glutamate receptors, NMDARs are cation channels that facilitate the influx of sodium ions upon activation. Still, NMDARs possess at least three distinct properties compared to other glutamate receptors: high calcium ion permeability; voltage-dependent channel blockade by magnesium ions and need for co-agonist binding for receptor activation (32).

Functional NMDARs are primarily expressed in neurons, where they are of crucial importance in synaptic plasticity (33). Neuronal NMDARs are divided into synaptic and extrasynaptic receptors based on their structure and function (34). All known NMDARs possess heterotetrameric structure formed by seven distinct subunits (GluN1, GluN2A-D, GluN3A-B) (14, 32). Receptors are generally composed of two GluN1 and two GluN2 subunits that contain the glycine and glutamate binding site, respectively. Receptor activity is primarily regulated by the glutamate-binding subunits (GluN2A-D).

Uniquely among glutamate receptors, NMDAR activation requires the simultaneous binding of glutamate and a co-agonist to their respective binding sites. The co-agonist binding site is located on the GluN1 subunit of receptors. Glycine was long believed to be the only co-agonist of NMDARs, until the discovery by Kleckner et al. that *D*-serine also binds to the co-agonist site with a similar affinity to glycine (35). Nevertheless, the co-agonist binding site is often still referred to as glycine binding site. Kynurenic acid, an endogenous competitive antagonist also binds to the co-agonist site and likely plays a regulatory role in NMDAR activation (36). Despite binding to GluN1 subunits, affinity of glycine and *D*-serine to NMDARs is determined by GluN2 subunits through intrareceptor allosteric modulation (32). Glycine displays higher affinity to GluN2B-containing NMDARs compared to those that contain GluN2A subunit, whereas *D*-serine preferentially binds to GluN2A-containing receptors (37). Naturally, *D*-serine is considered the prevailing co-agonist in brain areas where the expression of GluN2A-subunit dominates, such as the hippocampus or prefrontal cortex (34). Glycine, in turn, is thought to be the co-agonist, where GluN2B expression is favored (37, 38). Besides

differences in brain regions, receptor subunit expression also differs in subcellular localization. GluN2B is abundant in extrasynaptic receptors, while GluN2A is predominantly found in synaptic NMDARs, making *D*-serine the dominant co-agonist of synaptic neurotransmission (32, 34, 39). As co-agonist binding is essential for NMDAR activation, changes in extracellular glycine and *D*-serine levels add another layer of complexity to the regulation of glutamate signaling. Co-agonist levels are delicately balanced by various enzymes and transporters that offer promising pharmacological targets for indirect modulation of NMDAR function.

As mentioned previously, NMDAR plays a crucial role in synaptic plasticity. Activation of AMPARs by glutamate and the subsequent increase in inward sodium current leads to depolarization of neurons allowing the activation of NMDARs. A shift in transmembrane potential removes a magnesium ion from the ion channel pore that otherwise blocks ion transport at resting membrane potential. This is a necessary but not sufficient requirement for NMDAR activation as the binding of glutamate and one of the co-agonists to their respective binding sites is still needed for the opening of the ion channel. Then, a large increase in cytoplasmic free calcium ion level initiates several signaling cascades by activating various phosphatase and kinase enzymes, most importantly calcium calmodulin kinase II, a key enzyme of long-term potentiation (LTP). These cascades elicit numerous changes that facilitate synapse forming. Such an intricate, three-step activation of NMDARs is critical to avoid overstimulation of cells, as increased intracellular calcium levels may also evoke excitotoxic neuronal cell death (40).

1.1.4. *D*-Serine in Glutamatergic Neurotransmission

Due to their critical role in NMDAR activation, both glycine and *D*-serine have been in the focus of numerous studies. There is growing interest in *D*-serine, in particular, as it is thought to play a central role in learning and other cognitive functions associated with NMDARs.

As *D*-amino acids are far less abundant and generally considered less important than their *L*-counterparts, *D*-serine was ignored for a long time until Hashimoto et al. detected its presence in the human brain (41). High concentrations of *D*-serine were

measured in the cerebral cortex, while only low levels were found in the hindbrain (42). Similarly to glutamate, the concentration of *D*-serine, along with that of its precursor, *L*-serine, is determined by several transporters and enzymes in both astrocytes and neurons. This complex regulatory system, known as 'serine shuttle', is responsible for maintaining extracellular *D*-serine level that permits NMDAR activation in the forebrain (for review see: (43)). Its importance can be demonstrated by targeted deletion or pharmacological inhibition of its components, as they lead to impairment of NMDAR-dependent plasticity (43-46).

D-Serine is synthetized from *L*-serine by serine racemase (SR), an enzyme that is predominantly expressed in the cerebral cortex (47). SR was initially thought to be exclusively found in astrocytes and thus *D*-serine was labeled as 'gliotransmitter'. Later studies using more selective immunolabeling not only demonstrated its presence in neurons but proved that the initial astrocytic localization was due to an artefact (for review see: (48)). Today, SR is primarily considered a neuronal enzyme though astrocytic SR may still be important in some conditions.

SR requires constant supply of *L*-serine for continuous synthesis of *D*-serine. *L*-Serine, however, is primarily synthetized from 3-phosphoglycerate by a group of astrocyte-specific enzymes. Undisrupted SR function in neurons therefore demands *L*-serine transport from astrocytes (43, 49). ASCTs, and ASCT1 (SLC1A4) in particular, are known to mediate *L*-serine release from astrocytes (43, 50). Mechanisms responsible for neuronal *L*-serine uptake are not as well understood though several transporters, such as the neutral amino acid antiporter asc-1 (SLC7A10), SNATs (SLC38 family) and system L (SLC7 family) may be involved (28, 43, 51, 52).

Several mechanisms have been identified that regulate SR function in neurons. Kim et al. found that that calcium ion influx after AMPAR activation indirectly enhances SR through the binding of GRIP, an intracellular regulatory protein (48, 53). On the other hand, NMDAR activation leads to nitric oxide production that inhibits SR activity as part of a negative feedback loop (43, 54). In addition, glycine, another substrate of SR, inhibits *D*-serine synthesis. This mechanism is likely important in the spinal cord, brainstem and cerebellum, where glycine is the primarily co-agonist. Inhibition of SR by glycine ensures its dominant role in these areas (43, 55).

Unlike glutamate, *D*-serine is not released from neurons by vesicular exocytosis but by amino acid exchange transporters. Neuronal *D*-serine release is facilitated by asc-1 that also mediates the uptake of neutral amino acids, including *L*-serine (43, 56, 57). Both neurons and astrocytes lack a high affinity reuptake system for the rapid removal of *D*-serine from the synaptic cleft, disproving its role as a fast-acting neurotransmitter (43, 58). As the role of asc-1 in *D*-serine transport is limited to release, the removal of extracellular *D*-serine is mediated by amino acid exchange transporters with only moderate to low affinities (43). Among these transporters, ASCT1 and ASCT2 are known to be expressed on astrocytes (59) and facilitate *D*-serine uptake in rat cortical astrocytes and SH-SY5Y neuroblastoma cells (59, 60). Elimination of synaptic *D*-serine is thus slower than that of glycine or glutamate, which are rapidly removed by the high affinity glycine transporters (GlyTs) and EAATs, respectively. Delayed elimination creates a prolonged presence of *D*-serine in the synaptic cleft. These differences raise the possibility that *D*-serine acts as an autocrine modulator of NMDARs, rather than as a classic co-neurotransmitter (for review see: (48)).

In the forebrain, the half-life of *D*-serine (about 17 hours) (55) is significantly longer than that of glutamate (about 30 min) (61), indicating differences in its rate of degradation as well. *D*-Amino acid oxidase (DAAO) is a peroxisomal enzyme that can degrade *D*-serine. It is predominantly active in astrocytes of the brainstem, cerebellum and spinal cord, where consequentially only low levels of *D*-serine can be detected (62, 63). Although DAAO is expressed in the forebrain, its function is limited in humans. DAAO activity appears to be hindered by low affinity to its co-factor, FAD in this area (64). Interestingly, forebrain *D*-serine may be partially catabolized by its own synthetizing enzyme, SR by α, β-elimination, producing pyruvate and ammonia in the process (65). However, significance of this pathway is controversial, as the synthesis of *D*-serine by racemization is faster than its degradation by α, β-elimination, suggesting the dominance of the former pathway (43). Therefore, astrocytic *D*-serine uptake still remains an important aspect of the serine shuttle.

1.2. Glutamatergic Neurotransmission in Disease

As mentioned previously, glutamate plays a crucial role in central nervous system signaling and has been associated with numerous pathophysiological processes. In the

following two chapters, its involvement in nociception and cognitive functions will be discussed with a focus on its role in neuropathic pain and cognitive dysfunction.

1.2.1. Neuropathic Pain

Glutamatergic signaling is of utmost importance in nociceptive neurotransmission as glutamate acts as the main neurotransmitter in both primary afferent neurons and interneurons of the spinal dorsal horn (66, 67). Furthermore, it plays a critical role in not only acute pain sensation but also in chronic pain and neuropathy due to its involvement in synaptic plasticity.

Neuropathic pain develops following injury of the somatosensory nervous system and its typical symptoms include allodynia (pain following non-noxious stimuli) and hyperalgesia (enhanced sensitivity to pain) (66). Sensory nerve damage leads to central sensitization, a complex central nervous system change that involves numerous signaling mechanisms (for review see: (68)). Enhanced formation of glutamatergic synaptic connections between primary and secondary afferent neurons is one of the most important of these mechanisms. Central sensitization promotes nociceptive neurotransmission by lowering the threshold for the activation of spinal dorsal horn neurons and thus enhances painful stimuli to reach the cortex (68).

Indeed, several studies have shown enhanced glutamatergic neurotransmission in neuropathy. Elevated glutamate levels were detected in the dorsal horn of rats that developed neuropathy after chronic ligature of their sciatic nerve (69). Moreover, increased cerebrospinal fluid (CSF) glutamate concentration was also measured in nerve ligated neuropathic rats, possibly due to spill-over after excessive glutamate release from spinal nerve terminals (70).

Furthermore, several lines of evidence indicate that NMDAR subunit composition is altered during development of neuropathy (for review, see: (71)). The number of GluN2A subunit-containing NMDARs, that appear to play a role in physiological conditions, reportedly decrease following spinal nerve injury in rats, while GluN2B-containing receptor count increases (66, 72). In addition, phosphorylation of GluN2B subunits, but not that of GluN2A subunits was detected in inflammation-induced hyperalgesia (66, 73), which may lead to enhanced activity as receptor phosphorylation

is known to contribute to NMDAR hyperfunction (66, 74). Together, these findings suggest that NMDAR activity shifts from GluN2A-containing receptors to GluN2B-containing ones during the development of neuropathic pain (66). Indeed, GluN2B-selective NMDAR antagonists have antinociceptive effect in nerve injury-induced neuropathic pain, though similar beneficial effects of GluN2A-selective NMDAR antagonists have not been examined yet (66). Though many questions regarding the role of glutamate in the development of neuropathic pain are yet to be answered, its importance is universally accepted forming the basis of new drug research.

1.2.1.1. Direct Inhibition of Glutamatergic Neurotransmission in Neuropathic Pain

Despite progress in the research of new analgesics, the therapy of neuropathic pain remains a major challenge for physicians. Alleviation of nerve damage-induced chronic pain is notoriously difficult and the efficacy of classic analgesics such as opioids and non-steroid anti-inflammatory drugs is limited. Most current pain guidelines suggest the use of various ion channel blockers and neurotransmitter reuptake inhibitors that were originally approved for the treatment of unrelated diseases such as epilepsy or major depression, respectively (75).

Due to its involvement in the pathomechanism of neuropathic pain, NMDAR has become a popular target for drug research. Antagonists of NMDARs can effectively inhibit the depolarization of neurons, thus limiting glutamate release from presynaptic terminals and preventing LTP on the postsynaptic membrane. Though many NMDAR modulators have been identified to date (76), the focus remains on those with safety and efficacy previously demonstrated by clinical trials.

Ketamine is a phencyclidine derivative that was first approved for use in anesthesia. It is a non-competitive inhibitor of NMDARs that decreases channel opening time by binding to an intra-channel site of the receptor (77). Ketamine binds to all known NMDAR subtypes with high affinity and is known to achieve a long-term blockade of receptors (78). Its S-entantiomer has higher affinity to NMDARs and thought to have a better side effect profile compared to the R-enantiomer (77, 78). Many studies have examined the applicability of ketamine in neuropathic pain and the majority of them

showed effective alleviation of pain symptoms (for review, see: (78)). However, some issues may limit its use. Significant analgesic effect was mostly detected in case of intravenous administration, while per os or topical treatment only achieved modest reduction in pain possibly due to the low bioavailability of ketamine (79). Lack of oral or local administration severely restricts its use in neuropathic pain therapy. Furthermore, NMDAR antagonism is known to induce numerous central nervous system side-effects such as hallucinations, alterations in sound and color perception and cognitive deficits that are not tolerated by many patients (80). Moreover, ketamine is a drug of abuse and causes dependence, further limiting its use in clinical practice (80).

Amantadine, and its derivative, memantine are NMDAR blockers used for the treatment of Parkinson's disease and Alzheimer's disease, respectively. The effect of both drugs has been examined in neuropathic pain conditions with limited success (for review, see: (80)). Memantine, a non-competitive inhibitor of NMDARs with a similar effect to that of magnesium ion, failed to alleviate symptoms of neuropathic pain in clinical trials. Amantadine, a unique NMDAR antagonist that acts by accelerating channel closure, also failed to achieve clear analgesic effect, making further trials necessary. On the other hand, dextromethorphan showed more promising results albeit only a few trials have been completed. Numerous drugs such as valproate, methadone, carbamazepine and phenytoin have been shown to inhibit NMDARs and among these, methadone and carbamazepine displayed analgesic effect in certain forms of neuropathy. However, the exact mechanism of action responsible for their analgesic effect is not clear, as both carbamazepine and methadone are known to have other targets, namely voltage-gated sodium channels and opioid receptors, respectively (78).

While direct inhibition of NMDARs may have its advantages, the use of NMDAR antagonists in neuropathic pain is limited by the moderate efficacy and unfavorable side-effect profile of the compounds. In the future, development of new drugs, possibly with selectivity to the GluN2B-subunit or new mechanism of action, may yield better results. For now, drugs that indirectly inhibit glutamatergic neurotransmission remain the first-line medications in neuropathic pain therapy.

1.2.1.2. Indirect Inhibition of Glutamatergic Neurotransmission in Neuropathic Pain

Concurrently with changes in NMDAR expression and activity, neuronal ion channel function is also altered during the development of neuropathy. Upregulation of voltage-gated sodium channel subtype $Na_v1.7$ and the α2δ-subunit of voltage-gated calcium channels have been associated with neuropathy (81-83), while potassium channels, that under normal circumstances counterbalance sodium influx during depolarization, are downregulated (84). These changes collectively lead to the hyperexcitability of neurons, enhancing glutamatergic nociceptive neurotransmission in the spinal cord (81).

Ion channel blockers that can prevent depolarization-induced glutamate release from presynaptic terminals, and thus indirectly inhibit NMDAR activation have been widely used in neuropathic pain therapy. Gabapentinoid anticonvulsant drugs such as pregabalin and gabapentin are considered first line medications in the treatment of neuropathic pain. Both drugs are known inhibitors of the α2δ-subunit-containing calcium channels (75). In addition, sodium channel inhibitors such as carbamazepine and lidocaine can also alleviate pain symptoms following nerve injury (75). Although both gabapentinoids and carbamazepine were originally approved for the treatment of epilepsy, they have been successfully adopted for this new indication, raising the possibility of future re-purposing of similar ion channel blockers.

1.2.1.3. Pharmacodynamics of Tolperisone

Tolperisone is a centrally acting muscle relaxant (CMR) that was developed by Gedeon Richter Plc. in 1959. Its safety and efficacy have been proved by clinical trials for the treatment of painful reflex muscle spasms and post-stroke spasticity (85). As it is known to possess relatively few side-effects (86), tolperisone has been a popular choice for the treatment of muscle-related pains.

Despite extensive use of tolperisone over the decades, its exact mechanism of action still remains elusive. Inhibitory effect on sodium channels was first proposed as possible mechanism of action due the structural similarities between tolperisone and lidocaine, a local anesthetic that is also used in the therapy of neuropathic pain (75). Indeed, numerous studies have reported local anesthetic-like actions of tolperisone. For

instance, Ono et al. found that it exerts a membrane-stabilizing effect on peripheral nerves of rats (87). Inhibition of action potentials by tolperisone on A- and C-fibers of rat sciatic nerves was also reported (88). In addition, Hofer et al. found that tolperisone significantly decreases sodium current in *Xenopus* oocytes expressing voltage-gated sodium channel isoforms $Na_v1.6$, $Na_v1.7$ and $Na_v1.8$ (89). Its potency was found comparable to or even higher than that of lidocaine (88). Inhibition of sodium currents by tolperisone on isolated frog-Ranvier nodes was also reported (89).

Kocsis et al. found that tolperisone and its structural analogues, such as eperisone and silperisone can dose-dependently inhibit spinal reflexes by depression of ventral root potential of isolated hemisected rat spinal cords. A significant suppression of calcium currents was also reported, suggesting that tolperisone, unlike local anesthetics, may affect calcium channels as well (90). Similar concentration-dependent suppression of calcium currents by tolperisone was found on *Achatina fulica* neurons under voltage clamping (91), again indicating an inhibitory effect on calcium channels resembling the effect of gabapentinoids. Interestingly, a possible competitive antagonist action of tolperisone on adrenergic α-receptors has also been reported (92). However, its effect on adrenergic receptors of the central nervous system, especially on α2 receptors that regulate synaptic neurotransmission has not been explored.

Despite the similarity of tolperisone to the above-mentioned local anesthetics and anticonvulsants, its efficacy in neuropathic pain management, to the best of our knowledge, has not been explored yet.

1.2.2. Cognitive Deficits

Similarly to enhanced glutamatergic neurotransmission, decreased glutamate signaling is also implicated in various pathological conditions. As glutamate plays a central role in memory and learning through NMDAR-mediated LTP, the disruption of its signaling can lead to cognitive deficits. Numerous preclinical studies have shown that inhibition of NMDARs, induced by either pharmacological blockade or gene knockout, leads to cognitive dysfunction (for review, see: (93)). Moreover, NMDAR hypofunction has been associated with the cognitive symptoms of schizophrenia and major depressive disorder (MDD). In support of this, altered concentration of *D*-serine has been reported in CSF and blood of patients with either disease (94).

It is well-known that non-competitive NMDAR antagonists such as phencyclidine and ketamine can induce cognitive dysfunction, along with other schizophrenia symptoms, in healthy individuals (93, 95) and exacerbate cognitive deficit in schizophrenia patients (93, 96). The involvement of NMDAR hypofunction in schizophrenia pathomechanism is yet to be elucidated, though many related changes have been detected in patients compared to healthy individuals, ranging from genetic variation of NMDAR subunits (93, 97-99) to increased kynurenic acid levels in brain (93, 100). In addition, a meta-analysis reported decreased blood levels of *D*-serine in patients with schizophrenia compared to healthy individuals (101), suggesting the involvement of the NMDAR co-agonist in the development of the disease. Indeed, *D*-serine improved neurocognitive function of schizophrenia patients when administered either alone or as an adjunct to antipsychotics (102, 103).

Involvement of NMDAR hypofunction in the cognitive symptoms of MDD is more controversial. In fact, growing evidence supports that NMDAR overactivation plays a role in MDD pathomechanism, as subanesthetic doses of ketamine can alleviate MDD symptoms rapidly (104, 105). Many NMDAR antagonists also show promise (106). In contrast with these studies, recent findings show that enhancing NMDAR-activation *via* increasing synaptic co-agonist concentration is beneficial in MDD treatment. A recent meta-analysis (107) found sarcosine, a glycine uptake inhibitor, effective at alleviating MDD symptoms. Moreover, *D*-serine supplementation displayed antidepressant properties in mice and humans (106). Alteration of CSF *D*-serine levels may also occur in MDD patients, although the results are inconclusive as both increase (108) and decrease (109) have been reported.

Reduced NMDAR-activity due to decreased *D*-serine levels may also contribute to age-related cognitive dysfunctions (110). Acute *D*-serine administration was reported to improve some aspects of cognitive performance in a small-scale, double-blind placebo-controlled study that involved elderly individuals (111). Despite this promising result, the use of *D*-serine as a cognitive enhancer is likely limited by its side-effect profile as *D*-serine causes nephrotoxicity after its degradation by DAAO in the kidney, as it was found in rats (112). Therefore, drugs that selectively increase *D*-serine levels in the central nervous system may be a safer approach. The serine shuttle regulatory system provides several pharmacological targets, including ASCTs that mediate *D*-serine

removal from synapses. Inhibition of these transporters could possibly lead to a slight increase in synaptic *D*-serine levels, permitting enhanced NMDAR activity and consequentially improved cognitive functions.

1.2.2.1. Pharmacodynamics of *L*-Theanine

L-Theanine (γ-glutamylethylamide) is a major component of tea leaves (*Camellia sinensis*) (113) and is thought to contribute to the well-known mental and health benefits of tea consumption (114). Numerous studies and meta-analyses have proved its beneficial effects on long- and short-term memory, attention, reaction time and other cognitive functions (114-117). Positive effect on mood have also been reported following *L*-theanine administration (117). Combination of *L*-theanine with other tea components, especially with caffeine, has been found to have particularly favorable effects in cognitive studies (117, 118). In addition, *L*-theanine was shown to augment antipsychotic medication in patients suffering from schizophrenia or schizoaffective disorder, with a reduction measured in positive and general psychopathology (117, 119).

Positive effects of *L*-theanine on cognitive performance have been reported at doses as low as 60 mg, a quantity that is roughly equivalent to the *L*-theanine content of two cups of black tea. Its higher doses (ranging from 200 mg to 400 mg) that have been found to improve mood and alleviate symptoms of stress and anxiety, are therefore well over the amount of *L*-theanine typically consumed with tea. Further studies are needed to assess whether the lower doses of *L*-theanine can induce the reported beneficial effects on mood and anxiety (114). Similarly, only a few of studies have examined the effects of long-term *L*-theanine consumption on cognition. A recent analysis by Baba et al., however, reported significant pro-cognitive effects in middle-aged or older subjects after 12 weeks of regular *L*-theanine intake (120).

Despite its well-described beneficial effects in humans, its exact mechanism of action is yet to be elucidated. *L*-Theanine is known to pass the blood brain barrier through amino acid transporters, achieving significant concentrations in the brain, where it has been suggested to affect glutamate signaling (121). Tamano et al. reported increased hippocampal neurogenesis associated with high brain-derived neurotrophic factor levels in *L*-theanine-treated rats that displayed improved object recognition memory (122). In

a similar study, *L*-theanine significantly prevented stress-induced impairments of long-term potentiation and hippocampal recognition memory in rats (123). Despite these studies, the underlying mechanisms as well as the molecular targets of *L*-theanine are yet to be clarified.

As a structural analogue to glutamate, it was proposed that *L*-theanine may inhibit EAATs and thus elevates extracellular glutamate concentration (121). *L*-Theanine was also shown to bind to all three ionotropic glutamate receptors, albeit with an affinity much lower than that of glutamate (121). Furthermore, activation of $GABA_A$ receptors (124) as well as competitive inhibition of cannabinoid receptors by *L*-theanine have been reported as well (125). Its structure is also similar to that of glutamine, another small neutral *L*-amino acid, that is a known substrate of ASCTs (4, 28). Therefore, *L*-theanine could be a substrate of ASCTs and, as a result, affect extracellular *D*-serine level. The consequentially enhanced NMDAR activity may also contribute to its pro-cognitive effects.

Glutamatergic neurotransmission is of crucial importance in the development of neuropathic pain and cognitive dysfunctions, two major challenges that physicians face today. Central nervous system glutamate signaling is modulated by an intricate regulatory system that provides many pharmacological targets for researchers. Numerous compounds may affect these targets, including drugs that are currently used in unrelated indications or have yet unspecified mechanisms of action. As we gain knowledge about glutamate signaling, the repurposing of these drugs for the treatment of neuropathic pain or cognitive dysfunction, have become a promising path for finding new medications. As these drugs have already proven safety and well-described pharmacokinetics, they may become therapeutic options sooner than newly researched medications.

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2. Objectives

Tolperisone is a CMR with not well-understood mechanism of action. Scarce preclinical data suggests that it may interfere with neurotransmitter release from presynaptic terminals and thus could affect nociceptive glutamate signaling in the spinal cord. The objectives of this study were:

- the examination of the effect of tolperisone on allodynia, using an animal model designed for the study of neuropathic pain (66, 126);
- the investigation of its modulatory role on glutamate signaling, measuring CSF glutamate levels as biomarker;
- and the clarification of the molecular mechanisms, by which tolperisone could affect glutamate signaling, using an *in vitro* synaptosomal release model.

L-Theanine is a dietary supplement and major component of tea leaves, that has been known to enhance cognitive functions. Despite its well-described health benefits, its mechanism of action still remains elusive. Effect on glutamate signaling have long been suggested, however, to date no molecular target have been found that would explain its beneficial effects. Therefore, further objectives of this work were:

- the examination of the effect of *L*-theanine on neuronal glutamate reuptake using the above-mentioned synaptosomal release model;
- and the study of the effect of *L*-theanine on *D*-serine transport using SH-SY5Y neuroblastoma cells, an established *in vitro* model of astrocytic *D*-serine uptake (59). This cell line is known to express functional ASCT transporters that facilitate *D*-serine uptake into cortical astrocytes (59).

3. Methods

3.1. Chemicals

Acetonitrile, acrylamide, 4-aminopyridine, boric acid, calcium chloride, carbamazepine, clonidine hydrochloride, *L*-cysteic acid, dimethyl sulfoxide, *L*-glutamate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), idazoxan hydrochloride, potassium chloride, magnesium chloride, phenobarbital, *D*-serine, sucrose, tris(hydroxymethyl)-aminomethane (Tris), trypan blue, trypsin and verapamil hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, United States). ω-Conotoxin MVIIC, tetrodotoxin and *DL*-threo-β-benzyloxyaspartic acid (*DL*-TBOA) were purchased from Tocris Bioscience (Bristol, United Kingdom). β-Cyclodextrin and 6-monodeoxy-6-mono(3-hydroxy) propylamino -β-cyclodextrin hydrochloride were provided by Cyclolab Ltd (Budapest, Hungary). Sodium chloride and *D*-glucose were obtained from Reanal (Budapest, Hungary). Tolperisone hydrochloride and pregabalin were kind gifts from Meditop Pharmaceuticals Ltd. (Budapest, Hungary). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) and fetal bovine serum (FBS) were provided by Corning (Tewksbury, MA, United States) and Biosera (Nuaille, France), respectively. Stable glutamine was obtained from Pan Biotech (Aidenbach, Germany). 4-Fluoro-7- nitrobenzofurazan (NBD-F) was purchased from Tokyo Chemical Industry (Tokyo, Japan) and was used as a fluorescent reagent. Lidocaine hydrochloride was provided by Egis Pharmaceutical Plc. (Budapest, Hungary). *L*-Theanine was obtained from Abcam (Cambridge, United Kingdom). Ultrapure water from MilliQ Direct 8 water purification system (Merck Millipore, Billerica, MA, USA) was used in all experiments. All compounds were stored and handled as described in the product information sheets.

3.2. Animals

Male Wistar rats were provided by Toxi-Coop Zrt. (Budapest, Hungary). All animals were housed in standard cages in numbers of 4 or 5 animals/cage, depending on their weight. Rats were kept in a room of 20 ± 2 ◦C temperature, with 12 h/12 h light/dark cycle and with water and standard food available ad libitum. A total of 103 animals

were used for Randall–Selitto tests, 80 for capillary electrophoresis analysis and 13 for synaptosome preparation.

3.3. Experimental Protocols of the Animal Study

The experimental protocols used for the assessment of mechanical allodynia were based on a previous work of our research group (70). In short, baseline pain thresholds were determined using Randall-Selitto tests (see section 3.5), then partial sciatic nerve ligation (pSNL) operations were performed on animals (see section 3.4). 14 days after nerve ligation, mechanical allodynia was assessed using Randall-Selitto tests and compounds or vehicles were administered per os (see section 3.6). Mechanical allodynia was again measured 60, 120 and 180 minutes after treatment to assess the acute effects of test compounds, which was quickly followed by sampling of CSF from the animals (see section 3.7).

3.4. pSNL

pSNL was applied for the induction of mononeuropathic pain in rats between 100 and 150 g weight. The operation was based on the Seltzer method (126). Briefly, animals were anesthetized with 60 mg/kg intraperitoneal pentobarbital (2.5 mL/kg volume) and were put on a pillow of 30 ˚C. The sciatic nerve of the right hind paw was exposed under aseptic conditions and without any muscle damage. The nerve was then tightly ligated with an 8-0 silicon-treated silk suture in a way that the dorsal 1/3-1/2 of the nerve thickness was trapped in the ligature. Two stiches were used to close the wound. Sham-operated animals (with the nerve exposed but not ligated) were used as controls.

3.5. Assessment of Mechanical Allodynia

Mechanical allodynia was assessed by paw pressure algesiometry (modified Randall-Selitto test, Ugo-Basile, Comerio, Italy) (127, 128). Paw pressure thresholds (PPTs) were measured in grams following 5 minutes of habituation of animals in the cages. PPT was assessed three times on each paw and the average of the measurements was used for statistical analyses. In case of each animal, a minimum 20% decrease in average PPT value of the operated (right) paw compared to the unoperated (left) paw

was considered as sign of developed mechanical allodynia. Sham-operated animals were again used as controls.

3.6. Treatment of Animals

On the 14th day after pSNL, the animals were treated with a single oral dose of 25, 50 or 100 mg/kg tolperisone or pregabalin. Drugs were dissolved in purificated water and administered *via* an orogastric gavage in a volume of 5 mL/kg. In each treatment group, 3-7 animals were used.

3.7. Capillary Electrophoresis Analysis of Glutamate, *D*-Serine and *L*-Theanine

Glutamate content of CSF samples as well as *D*-serine and *L*-theanine content of SH-SY5Y cell extracts were measured using a capillary electrophoresis laser induced fluorescence detection (CE-LIF) method that was developed in our laboratory (63). The method was extended to and validated for the accurate measurement of *L*-theanine.

Sham-operated and neuropathic rats were sacrificed 14 days after pSNL operation. CSF samples were obtained by cisterna magna puncture and centrifuged at 2 000 x g for 10 minutes at 4°C. Then, the samples were deproteinized by mixing with two volumes of acetonitrile and centrifuged at 20 000 x g for 10 minutes at 4°C. SH-SY5Y cells were deproteinized the same way following *D*-serine uptake experiments in order to determine their intracellular *D*-serine and *L*-theanine content. Deproteinized CSF samples and cell extracts were subjected to derivatization with NBD-F (at a final concentration of 1 mg/mL) in 20 mM borate buffer (pH 8.5) for 20 minutes at 65°C. For the measurement of CSF samples and cell extracts, 1 and 5 µM *L*-cysteic acid were used as internal standard, respectively. Prepared samples were stored at −20 °C until capillary electrophoresis analysis.

Derivatized samples were analyzed using a P/ACE MDQ Plus capillary electrophoresis system coupled with laser induced fluorescence detector (SCIEX, Framingham, MA, United States). The excitation and emission wavelengths were 488 and 520 nm, respectively. Separation took place in fused silica capillaries (i.d.:75 µm; o.d.: 365 µm; 40/50 cm effective/total length; Agilent Technologies, Santa Clara, CA, United States)

coated with linear polyacrylamide using the method of Hjertén et al. (129). 50 mM HEPES solution (pH 7.0) containing 6 mM 6-monodeoxy-6-mono (3-hydroxy) propylamino-β-cyclodextrin was used as separation buffer. Separation of analytes was carried out at 15 ˚C by applying −27 kV constant voltage.

3.8. Glutamate Release from Synaptosomes

Rat brain synaptosomes were prepared using the method of Modi et. al. (130) with some modifications. In short, animals were sacrificed by decapitation and their brain was rapidly removed and homogenized in a buffer containing 0.32 M sucrose and 4 mM HEPES (pH 7.4). The homogenate was centrifuged twice (1 500 x g, 4 ˚C, 10 minutes) and the supernatants were collected and combined. Then, the supernatant was centrifuged twice (20 000 x g, 4 ˚C, 10 minutes) and resulting pellet was resuspended in a buffer containing 0.32 M sucrose and 4 mM HEPES (pH 7.4) with 10% dimethyl sulfoxide (DMSO) and 10% FBS. Synaptosomes were then stored at -80 ˚C until release experiments.

Immediately before experiments, synaptosomal suspensions were thawed and centrifuged (20 000 x g, 4 ˚C, 10 minutes) to remove DMSO and FBS from the buffer. The resulting pellet was resuspended in another buffer (pH 7.4) containing 10 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 5.5 mM glucose. Synaptosomal suspensions were centrifuged to an 8-well strip plate (2500 x g, 4 ˚C, 15 minutes) with each well containing 10 mg of synaptosomes. After centrifugation the supernatant was discarded and the synaptosomes were equilibrated for 2x10 minutes at 37 ˚C before stimulation. In order to elicit depolarization and subsequent glutamate release, a stimulation buffer containing either 1 mM 4-aminopyridine or 33 mM potassium chloride was used. Forty µM *DL*-TBOA, a blocker of EAATs (131), was included in the equilibration and stimulation buffer to inhibit the reuptake of released glutamate. Test compounds were also added during the equilibration periods as pretreatment. Following stimulation, aliquots were taken at 2-minute intervals and stored at −20 ˚C until capillary electrophoresis analysis. Concentration of released glutamate was measured 6 or 8 min after stimulation.

3.9. Capillary Electrophoresis Analysis of Glutamate Released from Synaptosomes

Glutamate content released from synaptosomes was determined using a CE-LIF method developed in our laboratory (132). Samples were derivatized as described previously (see section 3.7.). Separation was carried in polyacrylamide coated fused silica capillaries (i.d.: 75 µm, effective/total length: 10/50 cm), using 100 mM borate buffer pH 8.5, containing 8 mM β-cyclodextrin at 25 ˚C, by applying 18 kV constant voltage.

3.10. Cell Culture

SH-SY5Y cells were acquired from The European Collection of Cell Cultures (Salisbury, United Kingdom) and maintained according to the recommendation of the provider. DMEM/F12 and FBS were used for preparation of cell culture medium.

3.11. Analysis of *D*-Serine and *L*-Theanine Cellular Uptake

On the day of uptake experiments, SH-SY5Y cells were trypsinized and suspended in Tris buffered salt solution (TBSS) or DMEM/F12, at concentration of 1 million cells / 500 µL. For the examination of the effects of *L*-theanine on *D*-serine uptake, cell suspensions were incubated with 25 µM *D*-serine and various concentrations of *L*-theanine (0-20 mM) or sucrose (20 mM) at 37 ˚C for 15 minutes. For the analysis of *D*-serine uptake kinetics, cell suspensions were incubated with increasing concentrations of *D*-serine (25, 50, 100 and 125 µM) in the presence (10 mM) or absence of *L*-theanine at 37 ˚C for 15 minutes. Samples were cooled on ice to terminate incubation and centrifuged (630 x g, 4 °C, 5 minutes). The resulting pellet was washed twice with 4 °C TBSS and was resuspended with acetonitrile:water (2:1; v/v). Precipitated proteins were then removed by centrifugation (3 000 x g, 4 °C, 20 minutes) and the supernatants were stored at -80 °C until analysis.

3.12. Statistical Analysis

All data were presented as mean ± standard error of mean (S.E.M.). Data were analyzed by two-way analysis of variance (ANOVA), where the effect of adrenergic α2 receptor agonists and antagonists was examined on synaptic glutamate release. One-way

ANOVA was applied in case of all remaining experiments. Newman–Keuls (Randall-Selitto), Tukey (CSF glutamate concentration, synaptosomal glutamate release, *D*-serine uptake by neuroblastoma cells) and Sidak (α2 receptor effect) post-hoc tests were used for comparison of multiple groups. In the case of Randall–Selitto tests and CSF glutamate content vehicle-treated animals, while in the release experiments stimulated, uninhibited synaptosomes were considered as control. Differences were considered significant if $p < 0.05$. Data analysis and curve fitting were carried out by statistical software Prism 8.0 (GraphPad Software Inc., San Diego, CA, United States).

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4. Results

4.1. Tolperisone Restores pSNL-induced Mechanical Allodynia in Neuropathic Rats

The antiallodynic effect of oral tolperisone was measured in nerve ligated neuropathic rats. pSNL induced mechanical allodynia, a typical symptom of neuropathic pain (133), that was assessed by Randall-Selitto test and was indicated by a decrease in PPTs. Developed mechanical allodynia was restored by all studied doses of tolperisone (25, 50 and 100 mg/kg; per os) as well as by higher doses of pregabalin (50 and 100 mg/kg; per os) that was used as positive control as it is a first line medication in neuropathic pain therapy (75, 134). Consistent antiallodynic effect was observed in case of both treatments at each tested time points (60, 120 and 180 min following administration). As expected, mechanical allodynia was unaffected in vehicle-treated rats as PPTs of the operated paws remained significantly decreased compared to those of the sham-operated ones (Figure 1).

4.2. Tolperisone and Pregabalin Reduce Elevated Cerebrospinal Fluid Glutamate Level in Neuropathic Rats

A marked elevation in CSF glutamate level was observed in nerve-ligated, neuropathic rats (70). Single-dose tolperisone treatment restored the pSNL-induced increase in CSF glutamate level at all examined doses (25, 50 and 100 mg/kg, per os) 180 minutes after administration (Figure 2A). In addition, 50 and 100 mg/kg single dose treatment of pregabalin also normalized glutamate level in neuropathic rats similarly to tolperisone (Figure 2B).

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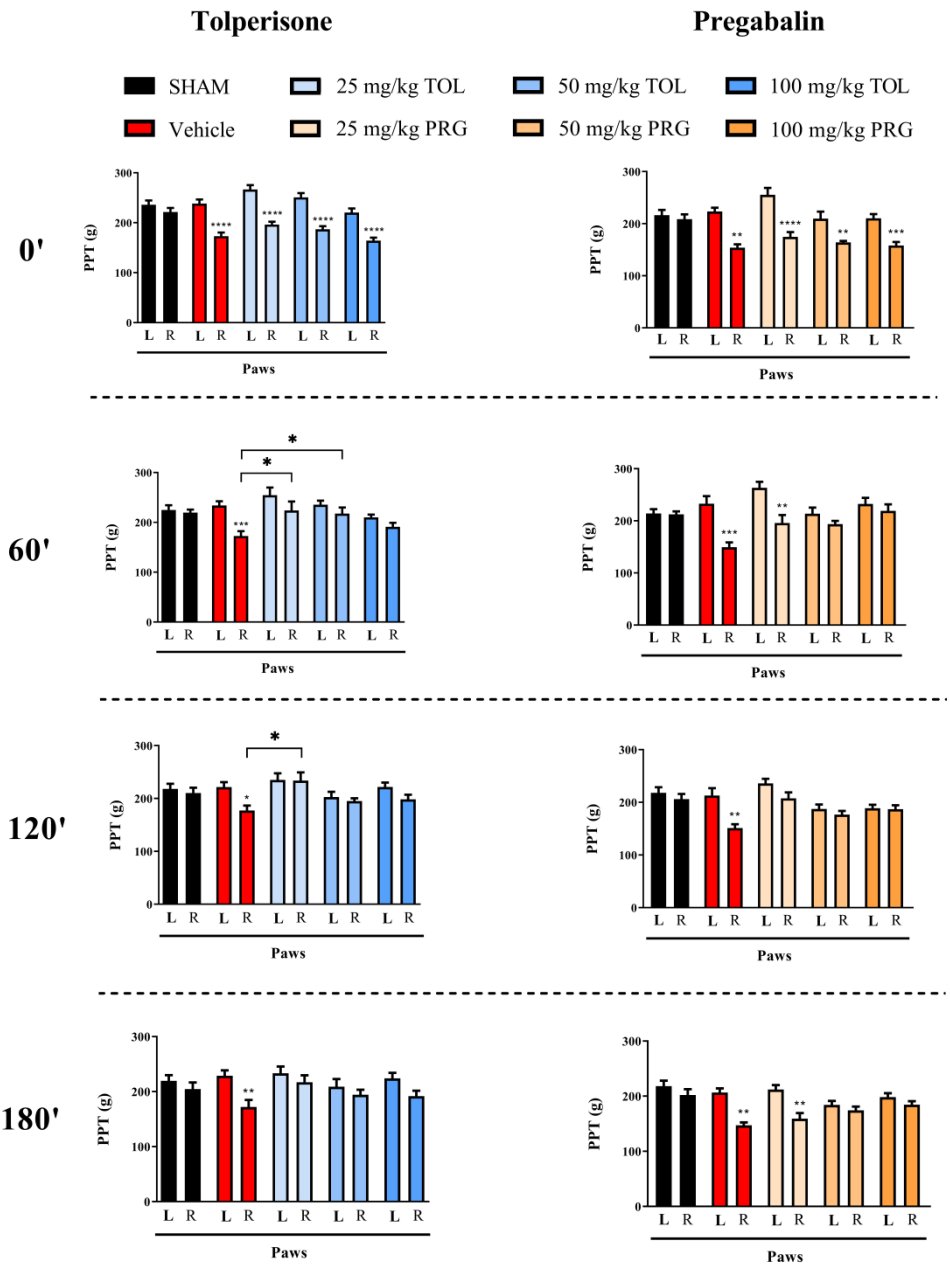

Figure 1. The antiallodynic effect of tolperisone (left column) and pregabalin (right column) following acute treatment (25, 50, 100 mg/kg, per os). Graphs show the means of PPT ± S.E.M. in grams of animals' left (healthy, L) and right (operated, R) paws before (baseline) and after treatment (60 min; 120 min; 180 min) with either tolperisone or pregabalin. Asterisks mark the significant differences compared to left (healthy) paws or vehicle treated group (one-way ANOVA, $F(9, 104) = 16.17$ (tolperisone 0'), $F(9, 104) = 5.182$ (tolperisone 60'), $F(9, 92) = 3.354$ (tolperisone 120'), $F(9, 92) = 3.028$ (tolperisone 180'), $F(9, 82) = 11.15$ (pregabalin 0'), $F(9, 82) = 5.955$ (pregabalin 60'), $F(9, 82) = 5.464$ (pregabalin 120'), $F(9, 82) = 6.234$ (pregabalin 180'), Newman–Keuls post-hoc test; ****: $p < 0.0001$; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$). In each treatment group 6–16 animals were used (135).

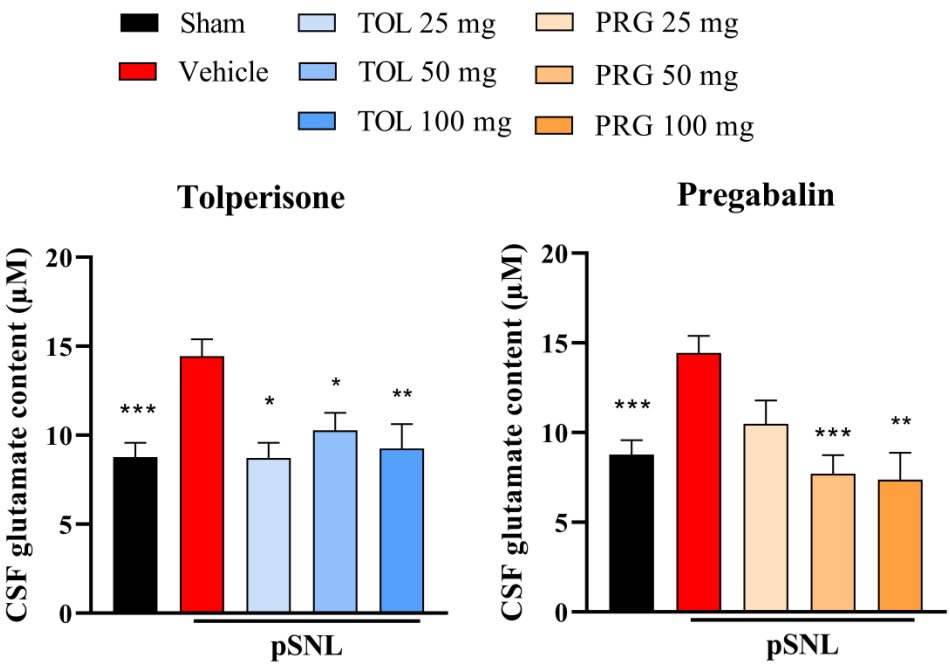

Figure 2. Glutamate content of CSF obtained from pSNL rats 14 days after surgery. Rats were treated per os with 25, 50, and 100 mg/kg dose of tolperisone (TOL) or pregabalin (PRG) or vehicle, and CSF samples were taken 180 min after treatment. Columns represent the mean of amino acid content ± S.E.M. in µM in the indicated groups. Asterisks mark the significant differences compared to vehicle treated group (one-way ANOVA, $F(4, 54) = 6.774$ (A), $F(4, 50) = 8.478$ (B), Tukey post-hoc test; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$). In each treatment group 6–18 animals were used (135).

4.3. Optimalization of a Synaptosomal Release Model

A release model was developed using rat brain synaptosomes in order to examine the possible inhibitory or enhancer effect of drugs on synaptic glutamate release as well as to gain a better understanding of their mechanism of action. Our method was adopted from the works of Kammerer et al. (136) and was adjusted to the examination of glutamate release and its offline measurement by the previously described CE-LIF method.

Glutamate release was induced by the selective A-type potassium channel inhibitor, 4-aminopyridine (137, 138), that can effectively imitate physiological depolarization and neurotransmitter release (137). A concentration-dependent glutamate release was found when synaptosomes were incubated with increasing concentrations (0.01-3 mM)

of 4-aminopyridine. As plateau was reached at 1 mM and further increase in 4-aminopyridine concentration did not elicit greater glutamate release (Figure 3), 1 mM 4-aminopyridine was used in all subsequent release experiments.

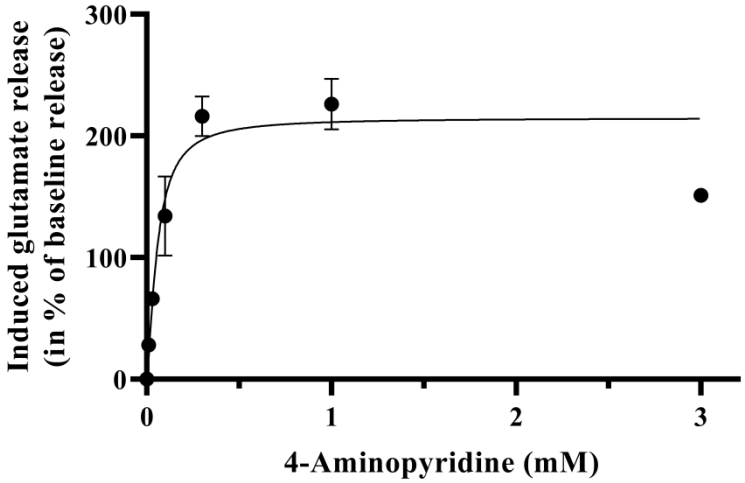

Figure 3. Effect of 4-aminopyridine on glutamate release from rat brain synaptosomes. 4-Aminopyridine was administered after a 20 min incubation period. All data points were normalized using the unstimulated, baseline release and presented as excess glutamate release over the unstimulated one. All data points represent mean of stimulated glutamate release ± S.E.M. in percent. In each treatment group 1–2 experiments were used.

Glutamate release from synaptosomes is counteracted by the reuptake transporters present on the presynaptic membrane that can remove glutamate from the synaptic cleft (131). *DL*-TBOA, a non-selective, non-transportable blocker of EAATs (131), caused a concentration-dependent increase in glutamate release when administered with 4-aminopyridine. As a significant increase was observed at as low as 40 µM *DL*-TBOA (Figure 4), this concentration was used in subsequent experiments.

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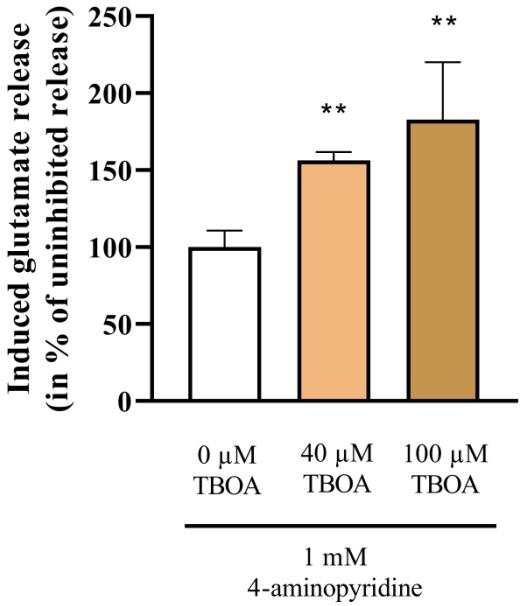

Figure 4. Effect of *DL*-TBOA on measurement of released glutamate from rat brain synaptosomes evoked by 1 mM 4-aminopyridine. *DL*-TBOA was administered as a pretreatment 20 min prior to stimulation. All data points were normalized using the unstimulated, baseline release and presented as percent of the stimulated glutamate release. All columns represent mean of stimulated glutamate release ± S.E.M. in percent in the indicated groups. Asterisks mark the significant differences compared to stimulated glutamate release in the absence of *DL*-TBOA (one-way ANOVA, $F(2, 8) = 8.832$, Tukey post-hoc test; **: $p < 0.01$). In each treatment group 2–6 parallel experiments were used.

4.4. Tolperisone Inhibits 4-Aminopyridine-induced Synaptosomal Glutamate Release

The effect of tolperisone on synaptosomal glutamate release was examined on the above described synaptosomal release model. 4-Aminopyridine-elicited glutamate release was concentration-dependently inhibited by tolperisone, with a significant inhibition achieved by as low as 40 µM tolperisone (Figure 5).

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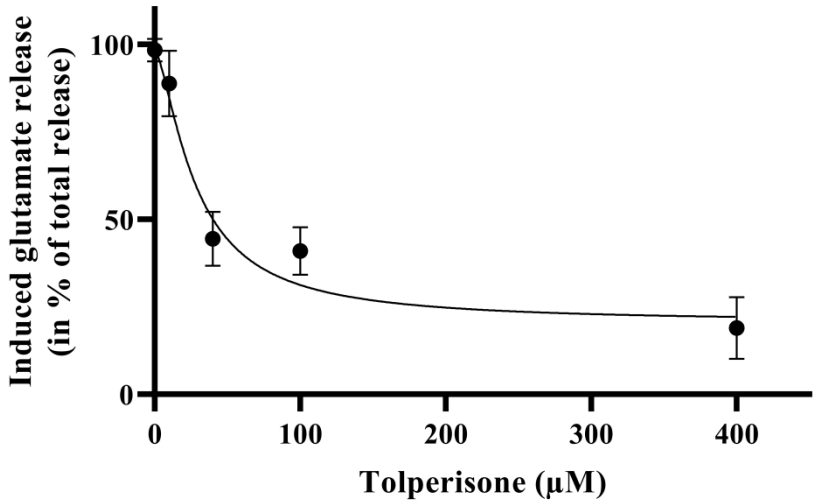

Figure 5. Effect of tolperisone on glutamate release from rat brain synaptosomes evoked by 1 mM 4-aminopyridine. Tolperisone was administered as a pretreatment 20 min prior to stimulation. All data points were normalized using the unstimulated, baseline release and presented as percent of the stimulated glutamate release in the absence of tolperisone. All data points represent mean of stimulated glutamate release ± S.E.M. in percent. Nonlinear regression was fitted using GraphPad Prism 8.0 (r^2 = 0.7580). In each treatment group 4–30 parallel experiments were used.

4.5. Sodium and Calcium Channel Blockers Inhibit 4-Aminopyridine-induced Synaptosomal Glutamate Release

Established voltage-gated sodium channel blockers such as tetrodotoxin, carbamazepine and lidocaine reduced stimulated glutamate release similarly to tolperisone. ω-Conotoxin MVIIC, a selective inhibitor of N-, and P-type voltage-gated calcium channels (139) and high concentrations of verapamil, a calcium channel blocker with low potency and selectivity (140), also inhibited release. Unexpectedly, no significant inhibition was observed in the case of pregabalin (Figure 6), a selective inhibitor of α2δ-subunit-containing calcium channels (141).

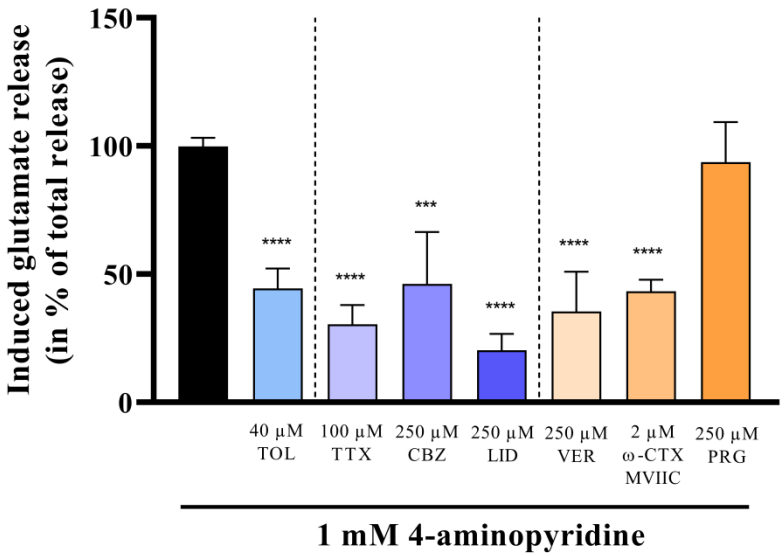

Figure 6. Effect of tolperisone, sodium channel blockers (TTX – tetrodotoxin; CBZ – carbamazepine; LID – lidocaine), and calcium channel blockers (VER – verapamil; ω-CTX – ω-conotoxin; PRG – pregabalin) on glutamate release from rat brain synaptosomes evoked by 1 mM 4-aminopyridine. Tolperisone and channel blockers were administered as a pretreatment 20 min prior to stimulation. All data points were normalized using the unstimulated, baseline release and presented as percent of the stimulated glutamate release in the absence of test compounds (black bar). All columns represent mean of stimulated glutamate release ± S.E.M. in percent in the indicated groups. Asterisks mark the significant differences compared to stimulated glutamate release in the absence of test compounds (one-way ANOVA, $F(7, 50) = 16.83$, Tukey post-hoc test; ****: $p < 0.0001$; ***: $p < 0.001$). In each treatment group 4–30 parallel experiments were used (135).

4.6. Potassium Chloride Elicits Sodium Channel-independent Synaptosomal Glutamate Release

High concentration of potassium chloride (33 mM) was also used to elicit glutamate release from rat brain synaptosomes in order to distinguish between the possible sodium or calcium channel inhibitory effect of tolperisone. High extracellular concentration of potassium chloride induce depolarization and neurotransmitter release by direct activation of calcium currents, thus it is largely independent of the activity of voltage-gated sodium channels (142). Calcium channel blockers, ω-conotoxin MVIIC and verapamil inhibited neurotransmitter release as was expected, while sodium channel

inhibitors, tetrodotoxin, carbamazepine and lidocaine all failed to achieve significant reduction in potassium chloride-elicited glutamate release (Figure 7).

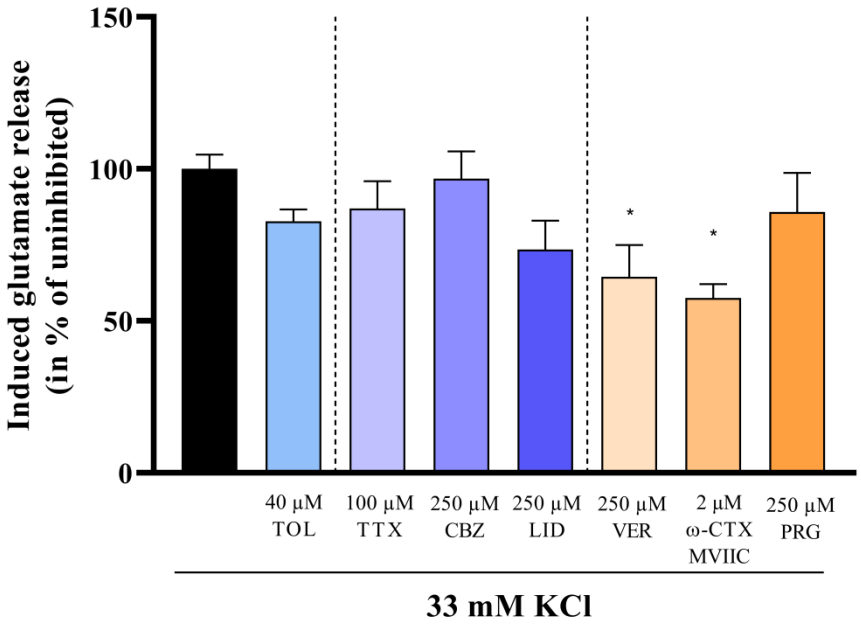

Figure 7. Effect of tolperisone (TOL), sodium channel blockers (TTX – tetrodotoxin; CBZ – carbamazepine; LID – lidocaine), and calcium channel blockers (VER – verapamil; ω-CTX – ω-conotoxin; PRG – pregabalin) on glutamate release from rat brain synaptosomes evoked by 33 mM potassium chloride. Tolperisone and channel blockers were administered as a pretreatment 20 min prior to stimulation. All data points were normalized using the unstimulated, baseline release and presented as percent of the stimulated glutamate release in the absence of test compounds (black bar). All columns represent mean of stimulated glutamate release ± S.E.M. in percent in the indicated groups. Asterisks mark the significant differences compared to stimulated glutamate release in the absence of test compounds (one-way ANOVA, $F(7, 40) = 3.599$, Tukey post-hoc test; *: $p < 0.05$; **: $p < 0.01$). In each treatment group 4–19 parallel experiments were used (135).

4.7. Tolperisone Inhibits Potassium Chloride-induced Synaptosomal Glutamate Release

Concentration-dependent effect of tolperisone on potassium chloride-induced glutamate release was also studied. Inhibitory effect of high concentration of tolperisone was observed. Significant reduction in neurotransmitter release was only achieved at 400 µM, while in case of 4-aminopyridine-evoked release a one order of magnitude lower

concentration (40 µM) was effective (Figure 8). Pregabalin failed to inhibit glutamate release under these circumstances as well (Figure 7).

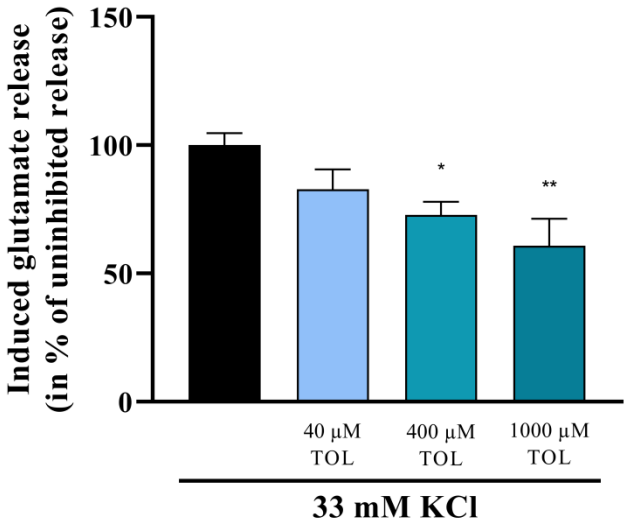

Figure 8. Effect of tolperisone (TOL) on glutamate release from rat brain synaptosomes evoked by 33 mM potassium chloride. Tolperisone was administered as a pretreatment 20 min prior to stimulation. All data points were normalized using the unstimulated, baseline release and presented as percent of the stimulated glutamate release in the absence of tolperisone (black bar). All columns represent mean of stimulated glutamate release ± S.E.M. in percent in the indicated groups. Asterisks mark the significant differences compared to stimulated glutamate release in the absence of tolperisone (one-way ANOVA, $F(3, 29) = 6.983$, Tukey post-hoc test; *: $p < 0.05$; **: $p < 0.01$). In each treatment group 4–19 parallel experiments were used (135).

4.8. The Inhibitory Effect of Tolperisone Is Not Modified by Alpha-2 Antagonist Idazoxan

As some CMRs, such as tizanidine, are agonists of adrenergic alpha-2 receptors (143), the effect of tolperisone on these presynaptic receptors was also examined. Clonidine reduced glutamate release from synaptosomes that could be reversed by idazoxan, an antagonist of alpha-2 receptors (144). Idazoxan, however, failed to alter the inhibitory effect of tolperisone (Figure 9) suggesting that its effect on alpha-2 receptors is negligible.

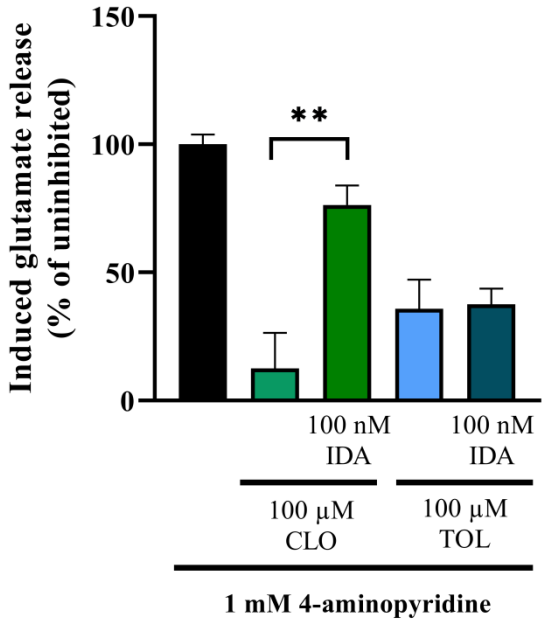

Figure 9. Effect of tolperisone (TOL) and clonidine (CLO), in the presence or absence of idazoxan (IDA), on glutamate release from rat brain synaptosomes evoked by 1 mM 4-aminopyridine. Tolperisone, clonidine and idazoxan were administered as a pretreatment 20 min prior to stimulation. All data points were normalized using the unstimulated, baseline release and presented as percent of the stimulated glutamate release in the absence of test compounds (black bar). All columns represent mean of stimulated glutamate release ± S.E.M. in percent in the indicated groups. Asterisks mark the significant differences compared to stimulated glutamate release in the absence of test compounds (two-way ANOVA, $F(1, 14) = 6.942$, Sidak post-hoc test; **: $p < 0.01$). In each treatment group 4–16 parallel experiments were used.

4.9. *L*-Theanine Enhances 4-Aminopyridine-induced Synaptosomal Glutamate Release

The effect of *L*-theanine on synaptosomal glutamate transport was also studied using the above-described release model. *L*-Theanine failed to inhibit glutamate release at all examined concentrations (Figure 10A). Given the similarity between the chemical structures of *L*-theanine (N-ethyl-*L*-glutamine) and glutamate, a competitive inhibitory effect on neuronal glutamate transporters could be expected. Indeed, when experiments were performed in the absence of *DL*-TBOA (131) a concentration-dependent increase in glutamate release was observed (Figure 10B), suggesting a weak competitive inhibitory effect on neurotransmitter uptake by EAATs.

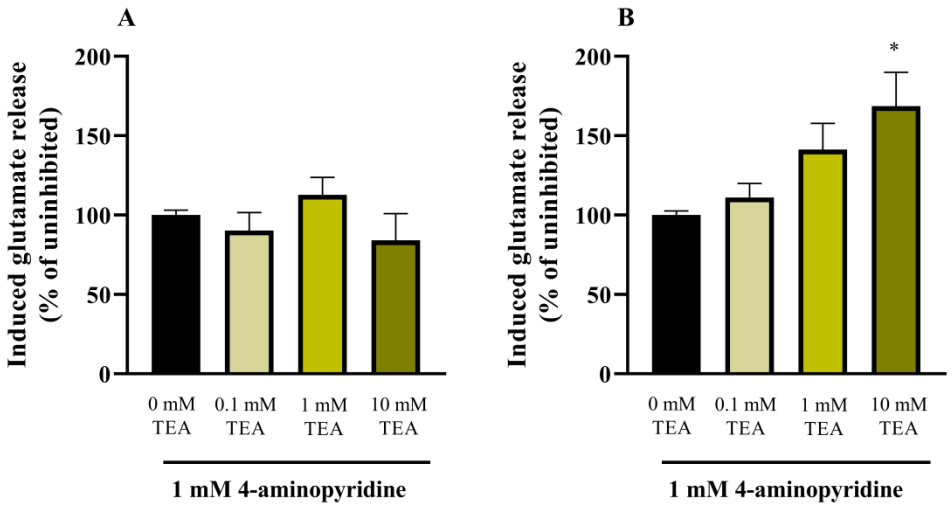

Figure 10. Effect of *L*-theanine (TEA) on glutamate release from rat brain synaptosomes evoked by 1 mM 4-aminopyridine in the presence (A) or absence (B) of *DL*-TBOA. *L*-Theanine was administered as a pretreatment 20 min prior to stimulation. All data points were normalized using the unstimulated, baseline release and presented as percent of the stimulated glutamate release in the absence of *L*-theanine (black bar). All columns represent mean of stimulated glutamate release ± S.E.M. in percent in the indicated groups. Asterisks mark significant differences compared to stimulated glutamate release in the absence of *L*-theanine (one-way ANOVA, $F(4, 20) = 14.57$ (A), $F(4, 29) = 17.38$ (B), Tukey post-hoc test; *: $p < 0.05$). In each treatment group 4–8 parallel experiments were used.

4.10. *L*-Theanine Inhibits *D*-Serine Uptake into SH-SY5Y Cells

In order to examine other possible mechanisms that may contribute to the cognitive enhancer activity of *L*-theanine, its effect on *D*-serine transport was also examined. SH-SY5Y neuroblastoma cells were used to model *D*-serine uptake into astrocytes as this cell line expresses both ASCT-1 and 2 (59), the two neutral amino acid exchange transporters that regulate extracellular *D*-serine concentrations in the central nervous system (145).

L-Theanine caused a concentration-dependent reduction in *D*-serine uptake into SH-SY5Y cells (Figure 11). The highest studied *L*-theanine concentration (20 mM) resulted in significant, about 70% inhibition. The potency of *L*-theanine (estimated IC50 = 9676 µM) was found to be considerably lower than that of neutral *L*-amino acids and ASCT substrates, such as *L*-alanine (174 µM) and *L*-threonine (158 µM) (59, 60).

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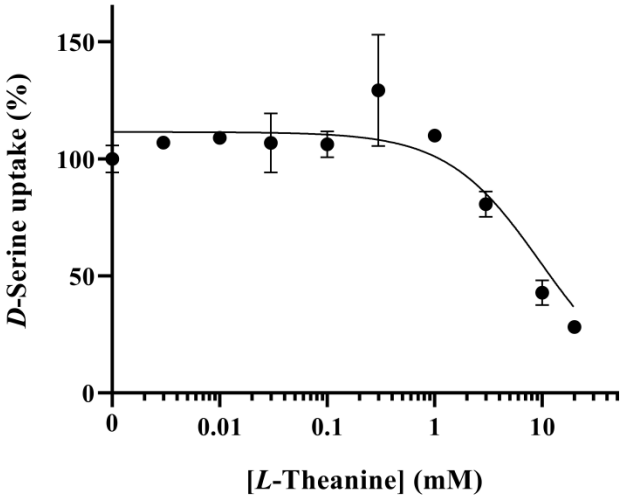

Figure 11. The effect of *L*-theanine on *D*-serine uptake into SH-SY5Y cells. The uptake of 25 μM *D*-serine was measured after 15 min in the presence of various concentrations (0–20 mM) of *L*-theanine. Experiments were carried out using TBSS as background medium. All data points were normalized using *D*-serine uptake rate in the absence of *L*-theanine and presented as percent of the uninhibited uptake. All data points represent mean of *D*-serine uptake ± S.E.M. in percent (n = 3) (146). Copyright © 2020 Lakatos et al., CC BY 4.0.

The possible involvement of the osmotic effect caused by high *L*-theanine concentrations in the inhibition of *D*-serine transport was also examined. As 20 mM sucrose did not alter *D*-serine uptake into cells, this non-specific inhibition was deemed negligible (Figure 12).

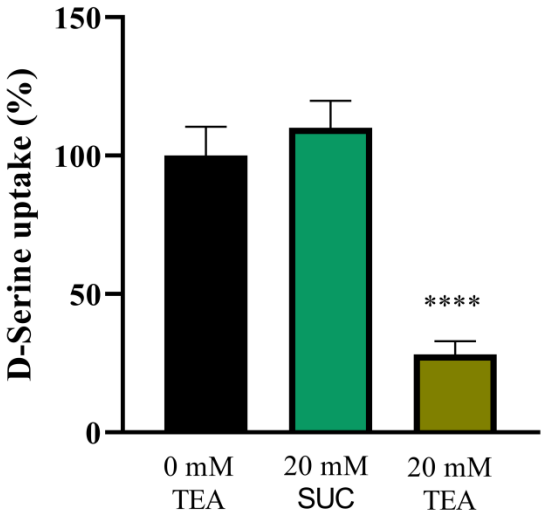

Figure 12. The effect of high concentration *L*-theanine (TEA) and sucrose (SUC) on *D*-serine uptake into SH-SY5Y cells. The uptake of 25 μM *D*-serine was measured after 15 min in the presence of either 20 mM *L*-theanine or 20 mM sucrose. Experiments were carried out using TBSS as background medium. All data points were normalized using *D*-serine uptake rate in the absence of test compounds and presented as percent of the uninhibited uptake (black bar). All columns represent mean of *D*-serine uptake ± S.E.M. in percent in the indicated groups. Asterisks mark the significant differences compared to uninhibited *D*-serine uptake in the absence of test compounds (one-way ANOVA, $F(2,11) = 133.8$, Tukey post-hoc test; ****: $p < 0.0001$). In each treatment group 4-5 parallel experiments were used (146).

4.11. *L*-Theanine Is Taken Up by SH-SY5Y Cells

Concurrently with *D*-serine uptake, a concentration-dependent increase in intracellular *L*-theanine concentrations was found, suggesting that *L*-theanine is taken up by neuroblastoma cells. The simultaneous rise in intracellular *L*-theanine levels and the inhibition of *D*-serine uptake may indicate a weak competitive inhibition of ASCTs by *L*-theanine (Figure 13).

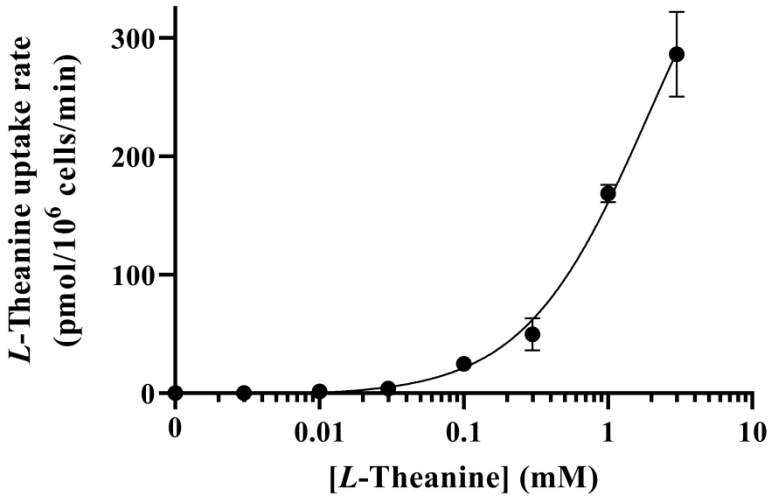

Figure 13. Concentration-dependent *L*-theanine uptake into SH-SY5Y cells. The uptake of 0-3 mM *L*-theanine was measured after 15 min in the presence of 25 μM *D*-serine. Experiments were carried out using TBSS as background medium. All data points are presented as intracellular concentration of *L*-theanine normalized by time of incubation and cell count. All data points represent mean of *L*-theanine uptake rate ± S.E.M. (n = 3) (146).

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4.12. Inhibition of *D*-Serine Uptake by *L*-Theanine Follows Competitive Kinetics

The kinetics of inhibition was examined using Lineweaver-Burk analysis in order to confirm the competitive nature of *D*-serine uptake inhibition by *L*-theanine. Concentration-dependent *D*-serine uptake was measured in the presence or absence of high concentration (10 mM) *L*-theanine, and *D*-serine uptake rate was plotted against its increasing concentrations in the incubation buffer in a double reciprocal manner. Plotting resulted in linear curves suggesting Michaelis-Menten transport kinetics. The presence of *L*-theanine increased the Km value of *D*-serine uptake, while did not affect its V_{max} value confirming the competitive nature of *L*-theanine inhibition of ASCT transporters (Figure 14).

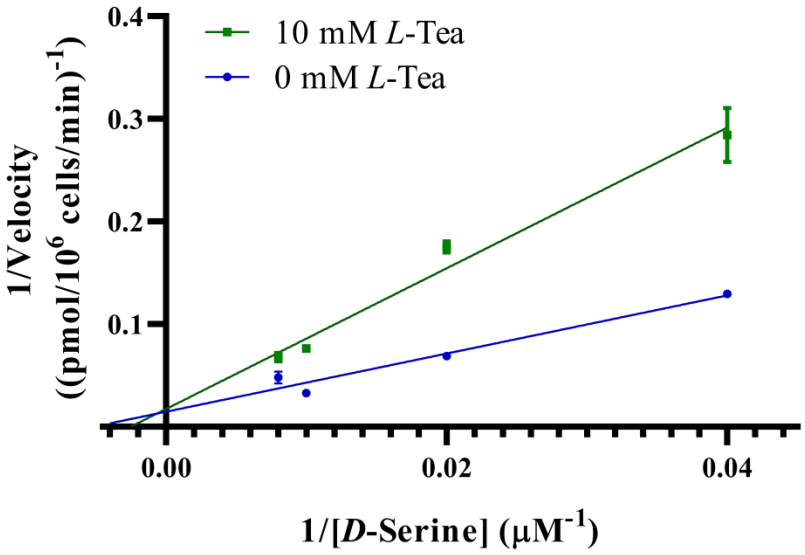

Figure 14. Kinetics of *D*-serine uptake into SH-SY5Y cells in the presence or absence of *L*-theanine (TEA). *D*-Serine uptake velocity was measured after incubating SH-SY5Y cells with increasing concentrations of *D*-serine (25, 50, 100 and 125 μM) in the presence (10 mM) or absence of *L*-theanine for 15 min. Experiments were carried out using TBSS as background medium. Data are plotted in a double reciprocal manner, using Lineweaver–Burk method and presented as mean ± S.E.M. (n = 3) (146).

4.13. *L*-Theanine Inhibits *D*-Serine Uptake in the Presence of Neutral Amino Acids

As neutral amino acids are substrates of ASCTs, a modification of the uptake model was necessary to better model whether *L*-theanine might inhibit *D*-serine transport in the central nervous system. The uptake experiment was performed in DMEM/F12 medium containing neutral amino acids, resembling extracellular milieu of the brain. As expected, *D*-serine uptake was significantly lower (about one quarter less) than in the amino acid-free background buffer (TBSS), likely due to the competition of neutral *L*-amino acids and *D*-serine for the same transporters. Nonetheless, high concentrations of *L*-theanine still achieved significant inhibition (about 22%) of *D*-serine uptake into SH-SY5Y cells (Figure 15).

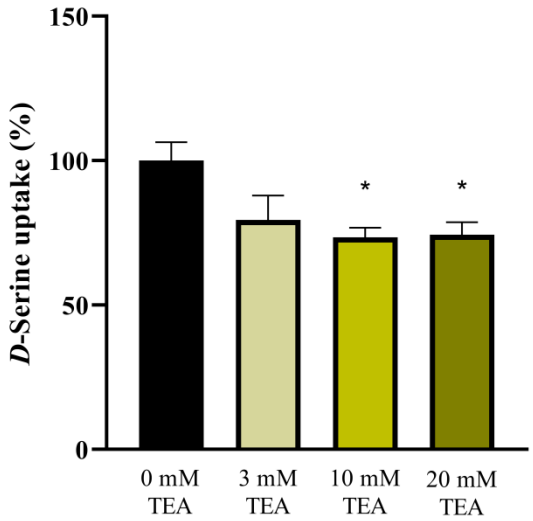

Figure 15. The effect of *L*-theanine (TEA) on *D*-serine uptake into SH-SY5Y cells. The uptake of 25 μM *D*-serine was measured after 15 min in the presence of various concentrations (0; 3; 10; 20 mM) of *L*-theanine. Experiments were carried out using DMEM/F12 as background medium. All data points were normalized using *D*-serine uptake rate in the absence of *L*-theanine and presented as percent of the uninhibited uptake (black bar). All columns represent mean of *D*-serine uptake ± S.E.M. in percent in the indicated groups. Asterisks mark the significant differences compared to uninhibited *D*-serine uptake in the absence of *L*-theanine (one-way ANOVA, $F(3,12) = 4.337$, Tukey post-hoc test; *: $p < 0.05$). In each treatment group 3-4 parallel experiments were used (146).

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5. Discussion

5.1. The Effect of Tolperisone on pSNL-induced Neuropathic Pain in Rats

Chronic pain management in neuropathy is a notoriously challenging task for health care professionals. Numerous drugs have been used for the treatment of neuropathic pain, including calcium channel blocker gabapentinoids and selective noradrenaline serotonine reuptake inhibitors, as well as classic analgesics such as opioids and non-steroid anti-inflammatory drugs (75). Despite the high number of available drugs and non-pharmacological therapeutic options, alleviation of pain symptoms is still not achieved for many patients. Besides the modest efficacy of current medications, slow onset of action, risk of tolerance and inability to achieve analgesia in different types of neuropathy also hinders their success in therapy (147, 148). As a result, new drugs with possible novel mechanisms of action are needed, which has gained attention from researchers. Repurposing of drugs is particularly popular as this method provides new therapeutic options much more rapidly than the development of new compounds.

As a CMR, tolperisone has been known to interfere with neuronal signaling, though this effect was thought to be restricted only to motor functions and muscle contraction. However, its proposed pharmacological profile that involves ion channel inhibition raises the possibility of a modulatory role on nociceptive signaling as well. The major finding of the present work is that single-dose tolperisone treatment significantly restores nerve-damage induced allodynia in neuropathic rats in doses that were proven safe in therapy of spasticity. To the best of our knowledge, no other study has examined the possible beneficiary effect of tolperisone on neuropathic pain.

In our study analgesic effect of tolperisone was observed at doses as low as 25 mg/kg, which is comparable to the antiallodynic effect of carbamazepine previously reported by Smith et. al. Analgesic effect of carbamazepine, an anti-seizure drug and sodium ion channel blocker also used in neuropathy pain therapy, was observed as early as 30 min after administration when assessed by von Frey test in pSNL rats (149). In the same study, antiallodynia was also induced by gabapentin, a calcium channel blocker gabapentinoid that was found effective at 60 mg/kg dose two hours after administration, indicating its lower potency compared to tolperisone (149). On the other hand, potency of tolperisone may be lower than that of duloxetine, a selective serotonin and

noradrenaline reuptake inhibitor, that could significantly restore pSNL-induced allodynia at a dose of 10 mg/kg (150). Analgesic effect of duloxetine, however, was only observed 24 hours after administration (150), while in our study antiallodynic effect of tolperisone showed minimal latency as significantly increased PPTs were measured within an hour of its administration. As it was suggested to block ion channels, tolperisone might effectively reduce neuronal hyperexcitability, a characteristic alteration that occurs in neuropathy (81), explaining its fast onset of action. Hyperexcitability of spinal neurons leads to excessive release of neurotransmitters, especially that of glutamate, the primary neurotransmitter of nociception. Indeed, significant elevation in CSF glutamate concentration was measured by our research group (70) as well as by others (151), making CSF glutamate levels an adequate biomarker of neuropathy. Tolperisone induced a significant decrease in CSF glutamate levels demonstrating that its antiallodynic effect could be the result of inhibition of nociceptive glutamate signaling.

The calcium channel blocker pregabalin was used as positive control as it is a first line medication in neuropathic pain therapy (75). Previous study by Yoneda et al. reported no significant antiallodynic effect of pregabalin at a dose of 10 mg/kg. This appears to be consistent with our results, as in our study pregabalin-induced antiallodynia was only observed at much higher doses (150). Antiallodynic effect of 50 mg/kg and 100 mg/kg pregabalin was found comparable to that of 25 mg/kg tolperisone, showing the considerable analgesic potential of the latter in neuropathy. Similarly to tolperisone, pregabalin also reduced the elevated CSF glutamate level in neuropathic rats, which suggests that both drugs exert their analgesic effect by inhibiting of spinal glutamatergic signaling. CSF glutamate level has been used previously as biomarker of the antinociceptive effect by Suzuki et. al in their study of yokukansan, a traditional Japanese herbal medicine that is approved for the treatment of neurosis and insomnia in Japan (152). Reduction of pain symptoms in chronic constriction injury model of neuropathic pain in rat was observed after yokukansan-treatment, which was accompanied by a decrease in glutamate level of CSF dialysates that had been elevated by painful stimuli (152). Interestingly, neither tolperisone nor pregabalin decreased CSF glutamate level below baseline, indicating selective inhibition of the pathologically elevated glutamate release of hyperexcitable neurons that develop in neuropathy.

Indeed, CSF glutamate level did not decrease in non-neuropathic patients following pregabalin treatment before or after knee replacement surgery (153). Though the effect of carbamazepine on CSF glutamate level in neuropathy has not been examined, it also did not alter CSF glutamate concentration when administered to non-neuropathic, epileptic patients (154).

Tolperisone restored allodynia with a potency that is equal or even higher than that of pregabalin, as its lowest administered dose (25 mg/kg) achieved marked analgesic effect, while pregabalin was found ineffective at the same dose. In addition, in its normal dose range tolperisone does not cause sedation, a characteristic side-effect of pregabalin (155). The present work thus demonstrates that tolperisone could be an effective tool in the treatment of neuropathic pain characterized by mechanical allodynia with possible advantage over pregabalin due to its better tolerable side-effect profile. However, more studies are needed to gain a better understanding of its antinociceptive effects and evaluate its applicability in the therapy of neuropathic pain.

5.2. The Effect of Tolperisone on Synaptic Glutamate Release

In order to elucidate the effects of tolperisone on neuronal ion channels, further experiments were performed using rat brain synaptosomes. As an established *in vitro* model of neuronal functions, synaptosomes have been used extensively in brain research. They are membrane vesicles formed from presynaptic terminals, and thus they enable the study of neurotransmitter release as well as its pharmacological modulation (156). As the *in vivo* results indicated that tolperisone and pregabalin might affect glutamate signaling, their direct effect on glutamate release was examined on synaptosomes. The applied release model was adopted from Kammerer et. al (136) and was used after preliminary experiments for optimalization to study the depolarization-induced glutamate release and its inhibition by the test compounds.

Glutamate release from synaptosomes was elicited by 4-aminopyridine, a selective inhibitor of A-type potassium channels (137). Inhibition of these potassium channels increase the excitability of voltage-gated sodium channels, inducing depolarization and subsequent neurotransmitter release from vesicles. Such an indirect mechanism of action leads to depolarization that closely resembles the physiological process in

neurons (137). Induction of depolarization by other compounds, such as veratridine, a direct sodium channel opener that causes persistent activation of channels (157), is considered less advantageous. In some experiments, high concentration of potassium chloride was used to elicit glutamate release in order to distinguish between voltage-gated sodium and calcium channel function as voltage clamping by high concentrations of potassium chloride is known to induce a considerable shift in transmembrane potential, which directly activates voltage-gated calcium channels (157). Under these circumstances, activation of non-desensitizing calcium channels dominate and neurotransmitter release cannot be inhibited by blockade of voltage-gated sodium channels (142). Therefore, by comparing the effect of tolperisone on glutamate release elicited by 4-aminopyridine or high concentration potassium chloride, the involvement of voltage-gated sodium and calcium channel inhibition can be assessed.

The present work demonstrates for the first time that tolperisone concentration-dependently inhibits 4-aminopyridine-elicited glutamate release from synaptosomes. Significant inhibition was achieved with doses as low as 40 µM, indicating a potent inhibition of glutamate signaling that likely plays a role in the muscle relaxant and analgesic effects of tolperisone. In addition, tolperisone achieved a more effective reduction in glutamate release than high concentrations of tetrodotoxin, an established blocker of voltage-gated sodium channels. Multiple distinct mechanisms may contribute to this marked inhibition of glutamate release. For instance, inhibitory effect on both voltage-gated sodium and calcium channels may explain such potent effect. Another possibility is that tolperisone, similarly to lidocaine (158), can block both tetrodotoxin-sensitive and resistant sodium channel subtypes. Indeed, the inhibitory effect of tolperisone was comparable to that of lidocaine as well as carbamazepine, another sodium channel blocker that is routinely used in the therapy of certain types of neuropathic pain (75).

Tolperisone also inhibited high concentration potassium chloride-induced glutamate release from synaptosomes, albeit only at high concentrations. Under sodium channel-independent circumstances, 40 µM tolperisone failed to reduce glutamate release, even though the same dose caused marked inhibition of 4-aminopyridine-induced release. In contrast, higher doses of tolperisone (400 and 1000 µM) significantly reduced glutamate release, similarly to established calcium channel blockers, ω-conotoxin MVIIC, a

selective inhibitor of N-, and P-type channels (139) and verapamil, a non-selective calcium channel blocker at high concentrations (140). The difference between the effective concentrations indicates that tolperisone inhibits sodium channel-dependent glutamate release more potently than sodium-channel independent release, and thus in clinically relevant concentrations it can be regarded as a relatively selective inhibitor of sodium channels. Though its inhibitory effect on calcium channels may be important in certain cases, the lower potency suggests it is not the primary mechanism involved in tolperisone-induced analgesia.

Drugs that act on noradrenaline signaling such as tricyclic antidepressants and selective noradrenaline serotonin reuptake inhibitors are commonly used in the therapy of neuropathic pain (75). By inhibiting synaptic noradrenaline reuptake, they enhance activation of α2 receptors, and subsequently inhibit release of neurotransmitters including glutamate from presynaptic terminals, alleviating neuropathic pain symptoms. In addition, α2 agonists, clonidine and dexmedetomidine were found effective at relieving pain induced by nerve injury (159) or formaldehyde injection (160). As inhibition of glutamate release by tolperisone could not be reversed by the α2 antagonist, idazoxan (144), its selectivity to sodium channels was further demonstrated.

Though the effects of tolperisone and pregabalin were found remarkably similar *in vivo*, pregabalin failed to inhibit synaptosomal glutamate release elicited by either 4-aminopyridine or high concentration of potassium chloride. Previously Kammerer et al. have also reported that pregabalin does not decrease neurotransmitter release from synaptosomes despite its well-described inhibitory action on calcium channels (136). This apparent discrepancy may have several explanations. It is possible that the inhibitory effect of pregabalin is selective to postsynaptic calcium channels, while presynaptic channels are not affected. As synaptosomes are formed from presynaptic terminals and the postsynaptic membrane is largely lost during preparation, postsynaptic calcium channels do not play a major role in synaptosomal neurotransmitter release. Moreover, another study by Bauer et al. suggested that pregabalin inhibits calcium currents by disrupting the synaptic trafficking of calcium channels (161). Pregabalin, thus may inhibit glutamate release *in vivo* by decreasing the number of calcium channels on the presynaptic membrane, while *in vitro* it remains ineffective as synaptosomes lack channel trafficking. In any case, our data suggest that different

mechanisms are responsible for the antinociceptive effects of tolperisone and pregabalin. These distinct mechanisms of actions raise the possibility of their combination that may provide enhanced antiallodynia though further studies are needed to elucidate their possible complementary effects.

5.3. The Effect of *L*-Theanine on Glutamate and *D*-Serine Uptake

L-Theanine is an active component of tea leaves that likely plays a major role in its positive effects on cognition. Though many possible targets have been proposed, its exact mechanism of action is still unclear. Due to the structural similarity between *L*-theanine and glutamate, EAATs have been suggested as a possible pharmacological targets, though to date no study have confirmed it (121). Enhancement of glutamatergic neurotransmission by competitive inhibition of glutamate uptake might explain the pro-cognitive effects, improved mood, relieved stress and anxiety symptoms associated with *L*-theanine use (114). The present work demonstrates that *L*-theanine can indeed inhibit glutamate uptake into rat brain synaptosomes, albeit only at high concentrations, indicating only weak inhibition of neuronal EAATs. Though a high number of EAAT inhibitors have been synthetized with potencies far exceeding that of *L*-theanine, their therapeutic applicability is severely limited by poor central nervous system penetration or convulsive effects (131, 162). In addition to the well-described central nervous system penetration and favorable side-effect profile of *L*-theanine, its relatively low affinity to EAATs may also present an advantage over these novel transporter inhibitors as marked loss of EAAT2 function by gene knockout has been associated with enhanced excitotoxicity and subsequent cognitive dysfunction (163).

L-Theanine is a known substrate of other amino acid transporters, such as system L that is responsible for its transport through the blood brain barrier. As a structural analogue to *L*-glutamine, *L*-theanine may be a substrate of the neutral amino acid transporters, ASCTs as well. This astrocytic transporter is thought to play an important role in the clearance of extracellular *D*-serine, thus may be a useful target for the modulation of NMDAR activity. *L*-Theanine significantly inhibited of *D*-serine uptake into SH-SY5Y neuroblastoma cells. This cell line was shown to be a good model of astrocytic *D*-serine uptake as it expresses both ASCT subtypes in functional form and its *D*-serine uptake can be inhibited by ASCT substrates (59). System L is unlikely to contribute to the

inhibitory effect of *L*-theanine, as *D*-serine is not a substrate of this transporter (164). Therefore, it can be concluded that *L*-theanine inhibits *D*-serine uptake into SH-SY5Y cells by inhibiting ASCTs. Our results indeed confirmed a competitive inhibition of ASCTs by *L*-theanine.

Though it is tempting to conclude that *L*-theanine alone can enhance NMDAR activity by reducing *D*-serine uptake into astrocytes and glutamate uptake into neurons, thus providing the positive cognitive effects associated with tea consumption, its inhibitory effect on these transporters was found rather weak. Significant reduction in *D*-serine uptake was only achieved when high doses of *L*-theanine were applied and potency of *L*-theanine was found much lower than that of other ASCT substrates. It is unclear whether such high levels of *L*-theanine can be reached in the central nervous system following its intake as a dietary supplement or tea consumption. Concentrations as high as 13 mM and 2.3 mM have been detected in the blood and brain tissue of rats after *L*-theanine administration, respectively (165). On the other hand, when 100 mg *L*-theanine (an equivalent of about 4 cups of green tea (166)) was administered to human volunteers, peak plasma level of *L*-theanine was measured 25 µM (167, 168).

In conclusion, though *L*-theanine can inhibit both glutamate and *D*-serine uptake in the central nervous system, it is unlikely that these mechanisms alone could be responsible for its beneficial effects on cognition. By inhibiting transport mechanisms that regulate the extracellular level of both glutamate and *D*-serine, *L*-theanine could simultaneously increase the synaptic concentrations of the agonist and co-agonist of NMDAR, leading to enhanced glutamate signaling in the brain. Besides this possible complementary effect, its suggested action on other targets such as glutamatergic or cannabinoid receptors, as well as its likely synergism with caffeine (169), and other green tea components could further contribute to the previously described health benefits of tea consumption. Such multifaceted mechanism of action is fairly common among other cognitive enhancers. For instance, multiple active components and numerous targets have been proposed for nootropic compounds such as ginkgo biloba extract with no clear indication of a dominant mechanism of action (170). Moreover, multiple mechanisms involved in the pharmacological action of *L*-theanine may also indicate that the risk of excitotoxicity, and other adverse effects is low, further adding to the benefits

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of *L*-theanine and its pharmacological derivatives in the therapy of cognitive dysfunctions.

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6. Conclusions

Though numerous drugs have been suggested to modulate glutamatergic neurotransmission, their mechanism of action is often unclear, limiting their applicability in the therapy of diseases associated with glutamate signaling. Revealing the processes involved in their pharmacological action can provide us novel therapeutic approaches for new indications. This work demonstrated that tolperisone can alter glutamatergic neurotransmission in neuropathy, where enhanced glutamate signaling is thought to play a major role. Acute tolperisone administration rapidly alleviated allodynia in pSNL animal model, indicating that it could be a promising pharmacological tool in the therapy of neuropathic pain. The induced antiallodynia was comparable to that of pregabalin, an established first line medication in neuropathy. Our results suggest that tolperisone accomplishes its analgesic effect by reducing glutamate release from nerve terminals and thus decreasing nociceptive signaling in the spinal cord. Its potent inhibition of voltage-gated sodium channels may explain the inhibitory effect on synaptic glutamate release. Blockade of calcium channels was also observed albeit only at high concentrations that exceed its normal dose range, while its activity on α2 receptors was found negligible. The inhibitory effect of tolperisone on presynaptic sodium channels rather than postsynaptic calcium channels suggests a mechanism of action that is distinct from that of pregabalin, raising the possibility that their combination has additive antiallodynic effect. More studies are needed, however, to assess the possible benefits of their combined use in the therapy of neuropathic pain.

The effect of *L*-theanine, an analogue of glutamate and one of the suggested pro-cognitive components of green tea leaves, was examined on cellular transport mechanisms. These processes regulate the synaptic concentrations of NMDAR agonist and co-agonist, permitting enhanced receptor activity, and thus improving cognitive functions. *L*-Theanine was identified as a weak inhibitor of EAAT and ASCT, regulatory uptake transporters of glutamate and *D*-serine, respectively. The additive effect of glutamate and *D*-serine transport inhibition, along with its effect on other previously proposed targets may lead to enhanced NMDAR activity, explaining its well-known pro-cognitive effects.

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7. Summary

As the primary excitatory neurotransmitter in the central nervous system, glutamate is of crucial importance in numerous physiological processes. In particular, by activation of its receptor, NMDAR, glutamate plays a central role in synaptic plasticity. However, both increased and decreased glutamate transmission is involved in the pathophysiology of various diseases. Enhanced glutamate signaling has been known to contribute to the development of neuropathic pain, a disease where satisfactory therapy is still not available. On the other hand, decreased NMDAR activity is the primary cause of cognitive dysfunctions, another health deficit that lacks effective treatment. The aim of the present work was to examine the possible antiallodynic effects of tolperisone, a CMR in pSNL-induced neuropathic pain in rats. Mechanical allodynia, a symptom of neuropathic pain was assessed by Randall-Selitto test, while CSF glutamate levels were used as biomarker of drug efficacy to reduce hyperexcitability of glutamatergic neurons. Furthermore, its mechanism of action was studied by *in vitro* glutamate release experiments on rat brain synaptosomes. The effect of *L*-theanine, another potential glutamatergic modulator and an active component of green tea leaves, was examined on cellular amino acid transport mechanisms. These processes regulate extracellular levels of glutamate and *D*-serine, ligands of NMDAR, and thus can modulate receptor activation and synaptic plasticity.

Tolperisone displayed significant antiallodynic effect in neuropathic rats that was comparable to that of pregabalin, a first line medication in neuropathic pain therapy. It likely exerts its antinociceptive effect by a relatively selective blockade of voltage-gated sodium channels in neurons, inhibiting glutamate release. The main findings of this work thus present tolperisone as a promising new agent in reducing neuropathic pain, although further studies are necessary to fully characterize its benefits. *L*-Theanine was shown to significantly inhibit the cellular uptake of glutamate and *D*-serine into brain synaptosomes and SH-SY5Y neuroblastoma cells, respectively. Though its inhibitory effect on either process was found fairly weak, the additive effect of these two mechanisms may contribute to the pro-cognitive effects associated with consumption of *L*-theanine as a dietary supplement.

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9. Bibliography of the Candidate's Publications

Publications Related to the PhD Thesis

1. Lakatos PP, Vincze I, Nyariki N, Bagaméry F, Tábi T, Szökő É. (2020) The Effect of L-Theanine and S-Ketamine on D-Serine Cellular Uptake. Biochim Biophys Acta Proteins Proteom.1868(10):140473.
   IF: 4.125
2. Lakatos PP, Karádi DÁ, Galambos AR, Essmat N, Király K, Laufer R, et al. (2022) The Acute Antiallodynic Effect of Tolperisone In Rat Neuropathic Pain and Evaluation of Its Mechanism of Action. Int J Mol Sci.23(17).
   IF: 6.208

Publications Not Related to the PhD Thesis

1. Vincze I, Lakatos PP, Bagaméry F, Tábi T, Szökő É. (2020) Characterization of a Cell Line Model for D-Serine Uptake. J Pharm Biomed Anal.187:113360.
   IF: 3.571
2. Mohammadzadeh A, Lakatos PP, Balogh M, Zádor F, Karádi DÁ, Zádori ZS, et al. (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).
   IF: 6.208
3. Geda O, Tábi T, Lakatos PP, Szökő É. (2022) Differential Ganglioside and Cholesterol Depletion by Various Cyclodextrin Derivatives and Their Effect on Synaptosomal Glutamate Release. Int J Mol Sci.23(16).
   IF: 6.208
4. Karádi DÁ, Galambos AR, Lakatos PP, Apenberg J, Abbood SK, Balogh M, et al. (2023) Telmisartan Is a Promising Agent for Managing Neuropathic Pain and Delaying Opioid Analgesic Tolerance in Rats. Int J Mol Sci.24(9).
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