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Impact of angiotensin receptor type 1 antagonists in neuropathic pain and opioid analgesic tolerance

Ph.D. thesis

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LIST OF ABBREVIATIONS

ACE1: angiotensin converting enzyme 1

AGTR1a: angiotensin II receptor type 1a (gene)

Ang(1-7): angiotensin(1-7)

AngI: angiotensin I

AngII: angiotensin II

AngIII: angiotensin III

AngIV: angiotensin IV

ANOVA: analysis of variance

ARB: angiotensin receptor type 1 blocker

AT1 receptor: angiotensin receptor type 1

AT2 receptor: angiotensin receptor type 2

autorad: autoradiography

B1R: bradykinin B1 receptor

BDNF: brain derived neurotrophic factor

BW: bodyweight

CALCA: calcitonin related polypeptide alpha (gene)

cAMP: cyclic adenosine monophosphate

CCI: chronic constriction injury

CGRP: calcitonin gene-related peptide

CI: confidence interval

CNS: central nervous system

COX-2: cyclooxygenase 2

CSF: cerebrospinal fluid

D-AP5: D-2-amino-5-phosphonopentanoate

DAMGO: [D-Ala2,N-MePhe4,Gly-ol]-enkephalin

DH: dorsal horn

DM: diabetes mellitus

DMSO: dimethyl sulfoxide

DPA: dynamic plantar aesthesiometer

DRG: dorsal root ganglion

EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ERK1: extracellular signal regulated kinase 1

ERK2: extracellular signal regulated kinase 1

FRAP: fluoride-resistant acid phosphatase

GABA: γ-Aminobutyric acid

GDP: guanosine diphosphate

GPCR: G-protein coupled receptor

GRK: G-protein coupled receptor kinase

GTP: guanosine triphosphate

HEC: hydroxyethyl cellulose

HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

IASP: International Association for the Study of Pain

IB4: isolectin B4

IHC: immunohistochemistry

ip.: intraperitoneal

ISH: in situ hybridization

JAK: Janus kinase

LC: locus coeruleus

MAP: mitogen-activated protein

MOR: μ-opioid receptor

mRNA: messenger ribonucleic acid

NAc: nucleus accumbens

NBD-F: 7-fluoro-4-nitro-2,1,3-benzoxadiazole

NF200: neurofilament protein 200

NGF: nerve growth factor

NK1: neurokinin 1

NMDA: N-methyl D-aspartate

NNT: number needed to treat

NOS: nitric oxide synthase

NP: neuropathic pain

NS: noxious-stimuli specific

NSAID: non-steroidal anti-inflammatory drug

OPRM1: opioid receptor mu-1 (gene)

PAG: periaqueductal gray

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PKA: protein kinase A

PKC: protein kinase C

po.: per os

PPARγ: peroxisome proliferator-activated receptor gamma

pSNL: partial sciatic nerve ligation

PWT: paw withdrawal threshold

RAS: renin-angiotensin system

RB: radio-ligand binding

RIA: radioimmunoassay

RNA: ribonucleic acid

RPM: rotation per minute

S.E.M.: standard error of means

sc.: subcutaneous

SNRI: serotonin-noradrenaline reuptake inhibitors

SP: substance P

SST: somatostatin

TCA: tricyclic antidepressant

TG: trigeminal ganglion

TrkA: tropomyosin receptor kinase A

TRP: transient receptor potential

VGLUT1: vesicular glutamate transporter 1

VH: ventral horn

VTA: ventral tegmental area

WB: western blot

WDR: wide-dynamic range

WHO: World Health Organization

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1. INTRODUCTION

1.1. Pain and its relief

In most cases, pain is a very important indicator of pathology and, therefore, has an essential adaptive function. However, in medical practice today, we are often reminded of the thoughts of Albert Schweitzer, who, in describing his experiences and observations in Equatorial Africa, says: "Pain is a more terrible lord of mankind than even death itself.". Indeed, while certain pain types such as acute nociceptive or inflammatory pain are adequately treatable by modern pharmacological means, chronic pain is a growing epidemiological, economic, and humanitarian problem that has yet to be satisfactorily addressed by pharmaceuticals. A study on the prevalence of chronic pain conducted in Europe has found that 19% of the population had experienced pain lasting for at least 6 months and suffered from pain in the last month and several times during the last week [1]. In addition, chronic pain is one of the most common reasons for seeking medical care [2].

Analgesia, the relief of pain without complete loss of sensation, is not only a humanitarian duty of medical professionals, but also directly contributes to the recovery of patients and to the reduction of their anxiety. The medicinal use of poppy alkaloids for analgesic purposes dates back to antiquity. In the third millennium BC, poppy was called "hul gil", or "the plant of joy" in Sumerian, implying that opium was used as a euphoric agent to enhance religious rituals rather than as a strictly medicinal product. Text of the ancient Egyptian Ebers papyrus dating to ca. 1550 BC implies that Egyptians likely used opium preparations to relieve pain and to induce sedation. Opium is probably mentioned in Homer's Odyssey (around the ninth century BC), when Athena, daughter of Zeus helps Telemachus, and his companions to forget their grief by giving them a certain preparation:

> *"Presently she cast a drug into the wine of which they drank to lull all pain and anger and bring forgetfulness of every sorrow."*
>
> The Odyssey, Homer

From the 8th century onwards, opium began to spread to India and China via Arabic traders, and by the 13th century, it had reached Europe. As its use became widespread around the 16th century, the first descriptions of side effects such as addiction and tolerance appeared in China and in Europe [3,4].

In 1805 Friedrich Sertrüner succeeded in isolating an alkaloid from opium, which he named morphine after Morpheus, the god of dreams [5]. To this day, morphine remains one of the mainstay drugs of pain relief in medicine, used in various clinical scenarios of acute and chronic pain. In the mid-20th century, non-opioid analgesics, such as non-steroidal anti-inflammatory drugs (NSAIDs) among others were developed as alternatives or adjuvants to opioids for pain relief. At present, modern pain management consists of opioid and non-opioid analgesics, as well as other, non-pharmacological tools.

1.1.1. Pain transmission

The normal sensation of pain provides the basis to avoid tissue damage thus it is an indispensable factor of survival. However, under pathological conditions, maladaptive changes may occur, resulting in symptoms such as hyperalgesia (increased sensitivity to painful stimuli) or allodynia (pain evoked by a normally non-painful stimulus). The first step in the processing of pain is “transduction”, and it refers to the phenomenon where an external noxious stimulus is converted into an electrophysiological signal by nociceptors [6]. This process is mediated by specific receptors, such as pressure receptors, temperature coding receptors or acid sensing ion channels among others [7]. The activation of these receptors causes an action potential that is transmitted by sensory fibres, classified based on their diameter, myelination and – consequently – their conduction properties. Pain can be transmitted by two types of sensory fibres: thinly myelinated Aδ or unmyelinated C fibres. The adequate modality of Aδ fibres can be thermal or mechanical stimuli while C fibres are polymodal, as they respond to multiple modalities [8]. The cell bodies of pseudounipolar, primary afferent sensory neurons are located in the dorsal root ganglia (DRG) or trigeminal ganglia [8]. Classically, these DRG neurons can be categorized based on their size (small, medium and large sized neurons) and their neurochemical features. A major distinction is made when subdividing small sized cells between neurons that contain neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP) or somatostatin (SST), hence named

"peptidergic" [8,9] and neurons that do not contain neuropeptides, called "non-peptidergic". The latter ones contain fluoride-resistant acid phosphatase (FRAP) and bind isolectin B4 (IB4) [10]. Peptidergic and non-peptidergic neurons also differ in function. As a neurotransmitter, SP, in addition to glutamate, has a prominent role in facilitating pain transmission [11]. Furthermore, only peptidergic cells express the receptor for nerve growth factor (NGF), tropomyosin receptor kinase A (TrkA) [12,13]. This growth factor is a highly validated factor in pain sensation and the development of hyperalgesia [14–19].

Central axons of DRG primary afferent neurons form synapses with secondary sensory neurons in the dorsal horn of the spinal cord. Peptidergic DRG neurons terminate in lamina I and superficial lamina II, while non-peptidergic ones terminate in deeper lamina II [11]. The most superficial layer, lamina I of the spinal cord contains noxious stimuli specific (NS) neurons, further forming the contralateral lateral spinothalamic tract and wide dynamic range (WDR) cells, that display a large receptive field, with a centre recognizing both noxious and non-noxious stimuli and a surrounding area which exclusively responds to noxious stimuli [11,20]. There is evidence that WDR neurons can create a distinction between noxious and non-noxious stimuli by firing at a higher or lower frequency, which contributes to their proposed integrative function and also under pathological conditions to the development of allodynia by disproportional responsiveness to non-noxious stimuli [8,20,21]. Lamina II, also known as the substantia gelatinosa has the most prominent role in modulating sensory input at a spinal level. This layer contains a number of inhibitory interneurons terminating locally or projecting towards other laminae [8]. These inhibitory neurons can be activated by descending monoaminergic pathways or by collaterals of Aβ fibres ("gate-control of pain"). Activation results in presynaptic inhibition of Aδ and C primary afferents by endogenous opioid, GABA or glycine release [8,22,23]. Disinhibition arising as a consequence of functional loss of lamina II inhibitory neurons seems to be a factor of paramount importance in the development of certain chronic pain states, such as neuropathic pain [23,24].

1.1.2. Neuropathic pain

Neuropathic pain (NP) as defined by the International Association for the Study of Pain (IASP) is “pain that arises as a direct consequence of a lesion or diseases affecting the somatosensory system.”. This condition is most often the result of toxic effects, such as treatment with various chemotherapeutic agents or chronic alcohol intake, but the pathogenic role of high blood sugar in diabetes mellitus, herpes viruses or nerves damaged by surgery or tumours is also important. Hallmark symptoms of NP include allodynia and hyperalgesia and it is often accompanied by paraesthesia, motor dysfunction or autonomic disorders.

Following axonal injury, the disintegration of the myelin sheath is followed by an inflammatory process called Wallerian degeneration. This involves activation of macrophages, granulocytes, and T-cells which is accompanied by the release of proinflammatory mediators and growth factors [25–27]. Following Wallerian degeneration, complex changes in the sensory system can be observed. These include significant alteration in the expression and distribution of ion channels, such as voltage-gated sodium, calcium, and transient receptor potential (TRP) channels [28]. The increase in the expression of the α2δ subunit of voltage-gated calcium channels in the DRG and spinal cord is not only an important factor in the development of allodynia [29,30] but also serves as a pharmacological target for one of the first line treatment options for NP, as described below. Similarly, expression changes of different ion channels and their exploitability as targets for novel analgesics is a highly researched topic.

The spinal nociceptive network undergoes significant changes in neuropathy. As mentioned above, this includes disinhibition caused by the loss of function of GABAergic and glycinergic inhibitory interneurons [23,31], and a decrease in functional opioid receptor number on the pre- and postsynaptic sites of sensory afferent terminals [32–35]. In addition to the impaired function of spinal inhibitory circuits, disinhibition of glutamatergic N-methyl-D-aspartate (NMDA) receptors occurs under neuropathic conditions. This phenomenon is called long-term potentiation, and it is achieved by either repeated depolarisation or stimulation with co-neurotransmitters such as SP or CGRP, released from the central terminal of peptidergic primary afferent neurons. The activation of NMDA receptors manifests in increased intracellular calcium concentration and overall excitability of projection neurons, which makes these cells highly responsive to

stimulation by low level C or Aδ firing or even by stimulation of Aβ fibres, causing hyperalgesia or allodynia, respectively [23,36,37].

1.1.2.1. Current Treatment Options in Neuropathic Pain

Understanding the molecular mechanisms that lead to NP can help to identify novel pharmacological targets for treatment and explain variations in drug response between patients. Each aetiology may present with somewhat unique pathophysiological mechanisms, leading to distinct pain phenotypes. Consequently, these variations in underlying mechanisms contribute to the observed differences in response to first line medications [38,39]. Despite efforts to develop one, individualised pharmacotherapy on a mechanistic basis has yet to be part of the clinical routine [39]. Current first line systemic pharmacotherapy for NP primarily includes tricyclic antidepressants (TCAs), serotonin-noradrenaline reuptake inhibitors (SNRIs), and certain anticonvulsants, such as gabapentin and pregabalin [38,40].

TCAs, particularly amitriptyline are commonly used as treatment in several chronic pain states, and NP is no exception. Their mechanism of action in NP appears to rely on the inhibition of monoamine reuptake, although blockade of voltage-gated sodium channels has also been proposed as a mechanism [41]. In a recent meta-analysis by Finnerup et al., calculated from placebo-controlled trials, combined number needed to treat (NNT) for 50% pain relief value of TCAs was 3.6 (95% CI 3.0-4.4) [38]. Among SNRIs, most guidelines recommend the use of duloxetine or venlafaxine in a number of NP states [38,40,42]. Placebo-controlled trials with duloxetine highlighted its efficacy in diabetic polyneuropathy and chemotherapy-induced NP, while venlafaxine was effective in mixed painful polyneuropathy and diabetic polyneuropathy [42]. Combined NNT value for 50% pain relief was 6.4 (95% CI 5.2-8.4) [38].

Pregabalin and gabapentin bind to the α2δ subunit of presynaptic voltage-gated calcium channels in the spinal cord, causing attenuation of excitatory neurotransmitter release (glutamate and SP) and inducing redistribution of calcium channels away from functional membrane sites [42,43]. Their efficacy is highly validated in several NP types, such as painful polyneuropathy [44,45], spinal cord injury [46,47], or post-herpetic neuralgia [48,49] with a combined NNT of 7.7 (95% CI 6.5-9.4) for pregabalin and 6.3 (95% CI 5.0-8.3) for gabapentin [38].

Largely because of the reduced μ-opioid receptor (MOR) reserve in the spinal cord seen in neuropathy, opioids are less effective in relieving NP than other pain types. Although there is considerable uncertainty about their efficacy in relieving NP and improving quality of life of patients, they are still considered second or third line treatment options in most guidelines [38,40,42]. Until the optimal effect of other agents is achieved, some studies suggest their use in combination with the above-mentioned treatment options [50–52]. However, it is important to bear in mind that these drugs have a high potential for abuse, they can cause serious side effects and the development of analgesic tolerance can hinder their use (see Chapter 1.3). Certain weak opioid agonists, such as tramadol and tapentadol also display monoamine-reuptake inhibitory effects [53,54] which might enhance their efficacy in reducing NP. Tramadol and tapentadol have an NNT value of 4.7 (95% CI 3.6 - 6.7) and 10.2 (95% CI 5.3 - 185.5) respectively, based on a combination of 7 and 1 positive study, respectively [38].

Effective management of NP remains a serious clinical challenge. A significant number of patients treated with current first line agents experience inadequate pain relief or intolerable side effects. Thus, there is urgent need for the development of novel treatment options that provide improved quality of life, adequate pain control and reduced financial burden for society.

1.2. The renin-angiotensin system and its function in the somatosensory system

The renin-angiotensin system (RAS) is a well-known endocrine modulator that regulates systemic blood pressure primarily through the action of its main bioactive octapeptide, angiotensin II (AngII). AngII is synthesised endogenously by a series of enzymatic conversions, starting with the cleavage of angiotensinogen by renin to the inactive intermediate angiotensin I (AngI). AngI is cleaved by the angiotensin converting enzyme 1 (ACE1) to form AngII [55]. Importantly, AngII production is not exclusively mediated by ACE1 as several other enzymes can also catalyse this process, namely cathepsin G, chymase, tonin or neprilysin, among others [56–59]. In addition to AngII, alternative pathways result in other peptides with biological activity, namely angiotensin III (AngIII), angiotensin IV (AngIV) or Ang(1-7) [60]. All the necessary precursors and

enzymes for the formation of biologically active ligands can also be found in the central nervous system (CNS) [59,61]. The central RAS and its components have gained attention for their involvement in emotional processes, learning and memory and sensory functions, among others [59]. The potential therapeutic use of drugs influencing this system has been suggested by numerous publications in, but not limited to, neurodegenerative diseases [62,63] or chronic pain conditions, such as NP [60,64–84]. RAS inhibitors often used in these studies are widely available, inexpensive, and safe drugs that are already in clinical use for the treatment of cardiovascular diseases. These characteristics make them ideal candidates for drug repurposing efforts.

1.2.1 Angiotensin receptors

Angiotensin receptor type 1 (AT1 receptor) and angiotensin receptor type 2 (AT2 receptor) [85] are two mammalian receptors that have been cloned and characterised as targets for biologically active peptides in the RAS. AngII binds with similar affinity to the AT1 and the AT2 receptor [60,76]. These are G protein-coupled receptors (GPCRs) with multiple possible signalling pathways upon activation that can be either heterotrimeric G protein dependent or independent allowing for a wide range of biological effects. The AT1 receptor couples to several types of G_{α} subunits, such as the G_q/G_{11}, G_i/G_o, G_{12} and G_{13} in addition to small G-proteins [76]. Furthermore, activation of AT1 receptors can stimulate growth factor pathways, by activation of mitogen-activated protein (MAP) kinases, Janus kinases (JAK) among others [86]. Their heterodimerisation with AT2 or other receptors has also been noted [76,87]. The signalling pathways of the AT2 receptors are much less understood. The first uncertainty is that although the AT2 receptor had been recognised as a member of the GPCR family, studies have described no change in cytosolic Ca^+ concentration or modulation of cyclic AMP (cAMP) after activation [76,88] and no detectable increase in (^{35}S)GTPγS binding after agonist binding [89]. Controversially, more recent works have demonstrated coupling to G_i proteins, an observation that was further supported by sensitivity to pertussis toxin [90–92]. AT2 receptor activation also results in phosphorylation of endothelial nitric-oxide synthase (NOS) through a protein kinase A (PKA)-dependent pathway, which would suggest G_s coupling [93]. In addition, AT2 couples to phosphatases, resulting in apoptotic and anti-

growth effects via dephosphorylation of extracellular signal regulated kinases 1 and 2 (ERK1 and 2) [94,95].

The angiotensin IV receptor, endogenously activated by AngIV, or the MAS receptor, which is activated by the angiotensin-metabolite heptapeptide Ang(1-7) and primarily works as an antagonist to AngII-AT1 receptorial activity, are outside the scope of the present work and will not be discussed in detail.

1.2.2. Neuroanatomical distribution of angiotensin receptors and endogenous ligands in areas relevant to pain control

Key components of the RAS have been described to be abundant in areas that are involved in pain transmission, including the dorsal horn of the spinal cord [96–101], DRG and identical structures such as the spinal trigeminal tract and trigeminal ganglion (TG) [67,68,83,101–114] or peripheral nerves. In this regard, we have recently reviewed data that provide a detailed description of the neuroanatomical distribution of these structures [76], which are also summarised in Table 1. In this section, only the most important aspects and controversies in the literature are discussed.

Several studies have reported on the presence of AngII in rat and human DRG and TG neurons, satellite cells and T cells [67,99,103,110,115]. Results from some of these studies have shown that in the DRG, AngII was found to colocalise with SP, a peptidergic neuronal marker and regulator of pain transmission [110,115]. In the spinal cord, the highest levels of AngII were found in the superficial dorsal horn, further suggesting an involvement in sensory functions [98,116]. It is noteworthy that AngII levels were found to be elevated in various models of NP (Table 1) [98,106,116].

The neuroanatomical localisation of the AT1 receptor has been well established by numerous studies. Similarly to AngII, it is abundant in areas of importance in pain transmission, such as the sciatic nerve [81,111], DRG [101,102,108,110,111,117,118] and spinal cord [83,97,102,119] alongside of certain supraspinal regions including the spinal trigeminal tract [120], the raphe nuclei [120], the amygdala and the cingulate cortex [121].

Unlike the AT1 receptor, the exact neuroanatomical distribution of the AT2 is a matter of debate. In addition, the controversial results are most likely exacerbated by the fact that commercially available AT2 antibodies have insufficient specificity according

to a recent study [122]. Shepherd and colleagues claim that the AT2 receptor is not expressed on sensory neurons, based on their negative results in finding AT2 mRNA or protein in DRG of mice or humans [99,123]. On the other hand, others have found the AT2 protein [101,106,107,117,124] or mRNA [110,111] in the DRG and sciatic nerve of rats. Recent reviews by Danigo et al. [74] or our research group [76] have speculated that this discrepancy may be due to species differences (Sepherd et al used mice, while other studies have mostly used rats) or a possible gene duplication of AT2 in mice, resulting in a lack of signal in the $Agtr2^{GFP}$ reporter mice used by Sepherd and colleagues.

Table 1. *Neuroanatomical distribution of key ligands and receptors in the renin-angiotensin system with importance in sensory functions*. Abbreviations: p: peptide/protein; r: mRNA; IHC: immunohistochemistry; autorad: autoradiography; PCR: polymerase chain reaction; ISH: in situ hybridization; RIA: radioimmunoassay; RB: radio-ligand binding; WB: western blot; DM: diabetes mellitus; DH: dorsal horn; VH: ventral horn; SP: substance P; CGRP: calcitonin gene-related peptide; NF200: neurofilament protein 200; TRPV1: transient receptor potential cation channel subfamily V member 1; IB4: isolectin B4; NK1: neurokinin 1; NMDA: N-methyl D-aspartate. Hyphen indicates no assessment by the indicated studies.

Ligand/receptor	Species	mRNA /peptide /protein	Method	Details	Changes in neuropathy	References
Peripheral nerves						
Angiotensinogen	rat	p	IHC	present	no data	[83]
AT1 receptor	rat	p	autorad	present	no data	[81]
	rat	r	PCR	present	increased	[125]
AT2 receptor	rat	p	autorad	not present	no data	[81]
	rat	r	PCR	present	increased	[125]
	$Agtr2^{GFP}$ reporter mouse	p	reporter mouse	present in a subset of myelinated $NF200^{+}$ fibres	increased (macrophage infiltration)	[99]
Dorsal root ganglia						
Angiotensinogen	rat	p	IHC	present	no data	[83]
	rat	r and p	PCR and IHC	present	no data	[109]
	rat	r	PCR and ISH	present on all cells	no data	[110]
Angiotensin I	human	p	RIA	present	no data	[110]
Angiotensin II	rat and human	p	IHC and RIA	colocalised with SP and CGRP	no data	[110]
	rat	p	IHC	colocalised with neuronal markers	no data	[104]
	rat	p	IHC and WB	colocalised with SP and NF200	increased	[103]
	human	p	IHC	colocalised with TRPV1 on small and medium neurons	no data	[67]
	rat	p	IHC	on neurons, satellite cells and T cells	increased	[106]
Angiotensin (1-7)	human	p	IHC	not present	no data	[67]
AT1 receptor	rat	r	PCR	present	no change	[125]
	rat	r	PCR	present	no data	[110]
	rat	p	IHC	Schwann cells, satellite cells and neurons	decreased (DM)	[111]

Ligand/receptor	Species	mRNA /peptide /protein	Method	Details	Changes in neuropathy	References
	rat (isolated neurons)	r and p	PCR, WB and RB	present	no data	[102]
	rat	p	IHC	present on small and large neurons	increased	[117]
	rat	p	IHC	present on neurons and satellite cells	no data	[108]
	rat	p	IHC	present on all neurons, higher exp. on small	no data	[101]
AT2 receptor	rat	r	PCR	present	increased	[125]
	rat	r and p	PCR and IHC	present	no data	[109]
	rat	r	PCR	present	no data	[110]
	rat	p	IHC	Schwann cells, satellite cells and neurons	increased (DM)	[111]
	rat (cell culture)	p	WB	present	increased (DM)	[113]
	rat	p	IHC	colocalised with neural markers	no data	[103]
	rat (neonatal)	r and p	PCR, WB and IHC	present on IB4+ neurons	no data	[107]
	rat	p	IHC	present on neurons, satellite cells and T-cells	no change	[106]
	rat	p	IHC	present on all neurons, mostly non-peptidergic C and Aδ, high colocalisation with AT1	no data	[101]
	AgtrGFP reporter mouse and human	r and p	PCR and reporter mouse	not present	no data	[123]
	AgtrGFP reporter mouse	p	reporter mouse	not present	no change	[99]
Spinal cord						
Angiotensin II	mouse	p	IHC	present ubiquitously, highest in laminae I and II	increased	[98]
AT1 receptor	rat	p	IHC, autorad and ISH	present in the superficial DH and on cholinergic neurons in the VH	no data	[96]
	mouse	p	IHC	present in the superficial DH	no data	[97]
AT2 receptor	rat	p	IHC	present in laminae I and II and colocalised with IB4 and SP in	no data	[101]
	AgtrGFP reporter mouse	p	reporter mouse	present in the deep DH and VH and colocalised with neuronal markers	no change	[99]

1.2.3. Mimetics and antagonists of RAS in relation to pain

The impact of ligands that affect the RAS in pain has yet to be fully elucidated, and controversial views pervade the literature on this topic. Several detailed reviews have recently shed light on this system in relation to pain control [60,76,126], so in this chapter we will only focus on the most important elements.

Following the discovery of the central RAS, a number of studies reported on the analgesic effect of ligands *activating* the angiotensin receptors, such as AngII, AngIII, or renin, after central administration in acute pain tests [75,78,127–129]. Notably, these reports offer different possible mechanisms underlying the described effects and all

administered the test compounds directly into the CNS, either intracerebroventricularly (icv.) or intrathecally (ith.). To the best of our knowledge there is only one publication in the literature that has described AngII as having neither pro- nor antinociceptive effects [130]. On the other hand, pronociceptive effect of angiotensin mimetics [98,119,123,131] and antinociception produced by AT1 [64,69,70,82,97,98,108,119,132] or AT2 antagonists [66,67,73,83,99,103,104,106,123] has since been described by a large number of studies conducted on different preclinical pain models and (in case of AT2 antagonist EMA401) in clinical trials as well. Many reports on the beneficial effects of AT1 receptor blockade in NP attribute the analgesic effect to the attenuation of the inflammatory response in the DRG [108,132] or sciatic nerve [69,82] following nerve injury.

1.2.4. Interplay between the central RAS and opioid system in relation to analgesia

The interaction between the RAS and the opioid system was first supported by the study of Haulica and colleagues when they showed that naloxone, an opioid receptor antagonist could block analgesia induced by icv. administered AngII. In this work, the authors speculated that angiotensinergic analgesia is dependent on the endogenous opioid system [133]. They also reported that stress analgesia is sensitive to both naloxone and saralasin, a non-selective angiotensin receptor antagonist/partial agonist [134]. These observations suggest a negative interaction between opioid agonists and angiotensinergic antagonists, which contradicts more recent findings. A bidirectional interaction between the RAS and opioid system is supported by the ability of icv. administered AngII [129], or AT2 receptor activation [131] to reverse morphine antinociception. Recent data of clinical relevance have shown that ACE-inhibition by captopril results in enhanced morphine analgesia and reduced analgesic tolerance development [72]. In our recent review, we have raised that AT2 antagonism could improve opioid efficacy in NP. This hypothesis was based on a publication by Khan et al. describing the ability of AT2 antagonist EMA300 not only to attenuate allodynia in a rodent model of NP but also to restore reduced NGF levels in rat DRGs induced by neuropathy [106]. Previously studies by our group and others have related the reduced levels of NGF in the DRG of neuropathic animals with a reduction in MOR number and the efficacy of opioid analgesia

[35,135,136]. Furthermore, exogenous NGF administration has been shown to enhance opioid analgesia by increasing the functional MOR reserve [35,137].

Possible links between the opioid system and the RAS concerning analgesia are summarized in table 2.

Table 2. *Results of publications suggesting an interaction between the RAS and opioid systems in analgesia.* As mentioned above, there is conflicting literature data and a notable controversy between former (i.e. pre-2000) and later (post-2000) studies. Abbreviations: BV2: ELISA: enzyme-linked immunosorbent assay; CCI: chronic constriction injury; WB: western blot

RAS ligand/receptor	Method	Outcome	Date	Reference
Angiotensin II	rat tail-flick test	AngII mediated analgesia is reversible by naloxone.	1983.	[133]
	rat tail-flick test	AngII is able to attenuate morphine analgesia.	2000.	[129]
Angiotensin converting enzyme	rat tail-flick test	ACE-inhibition cannot influence morphine analgesia.	1984.	[138]
	rat tail-flick and hot plate test	ACE-inhibition enhances morphine analgesia and decreases the development of opioid analgesic tolerance.	2021.	[72]
	ELISA	ACE-inhibiton decreases inflammatory cytokine levels in the DRG of morphine tolerant animals.	2021.	[72]
AT1 receptor	BV2 microglia culture	AT1 blockade (candesartan) reduces inflammatory mediator production induced by morphine treatment.	2022.	[139]
AT2 receptor	mouse tail-pinch test	AT2 activation decreases morphine analgesia	2009.	[131]
	rat tail-flick test	Saralasin decreases stress analgesia.	1986.	[134]
	rat CCI, immunofluorescence, WB	AT2 antagonism restores reduced NGF levels in the DRG of neuropathic animals.	2017.	[106]
		Exogenous NGF administration enhances opioid analgesia by restoring decreased MOR reserve in the DRG in neuropathy.	2013.	[35]

1.3. Opioids and analgesia

At present, opioids are the cornerstone of pain management in clinical practice. Poppy alkaloids have been employed for their pain-relieving properties since the antiquity [140] as discussed earlier. The study of these naturally occurring compounds led to the development of novel semi-synthetic and synthetic opioids, with the intent of improving their potency, efficacy, side effect profile, or pharmacokinetic properties.

The μ-opioid receptor (MOR), δ-opioid receptor (DOR) and κ-opioid receptor (KOR) are the targets of endogenous opioid peptides and exogenous opioid compounds used for analgesic purposes [141]. Most opioid analgesics primarily act by binding to MORs, which are abundantly expressed at regions of utmost importance in pain transmission such as the DRG, dorsal horn of the spinal cord, brainstem, and several other supraspinal centres. Activation of these receptors reduces neuronal excitability and thus modulates the transmission of pain signals. The complex pain modulating effect of opioids occurs at multiple levels, not only limited to dampening the input of pain signals to the CNS, but also altering the emotional response to pain.

In modern medicine opioids have an indispensable role in acute perioperative pain management, management of pain in critical illness, and treatment of chronic pain conditions, including cancer-related pain and non-malignant pain syndromes, among others. It is important to note that opioids may induce a range of adverse effects, including respiratory depression, sedation, constipation, tolerance, physical dependence, and the potential for abuse. Institutional misuse of opioids can lead to a significant public health crisis as seen with the “opioid epidemic” in the US. This highlights the need of opioid dose sparing pain management approaches that may replace or complement the strategies used today.

1.3.1. Neuroanatomical distribution of μ-opioid receptors

The neuroanatomical distribution of MORs has been described in great detail, utilising several experimental techniques. As mentioned above, these structures play a pivotal role in the descending pain modulatory pathway. Accordingly, they were found in the periaqueductal grey matter (PAG), locus coeruleus (LC) and rostral ventromedial medulla [142–146]. They can also be found in the hippocampus, nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala, striatum, thalamus and cerebral cortex, suggesting a role in integrative sensory function and reward. [142–145]. The abundance of these receptors in the dorsal horn of the spinal cord and the DRG underscores their importance in pain processing at the spinal level [146–149].

1.3.2. Opioid analgesic tolerance

The initial analgesic response to opioids diminishes over repeated administration, necessitating higher doses to achieve the same level of pain relief. This phenomenon is called analgesic tolerance. Opioid analgesic tolerance poses a significant clinical challenge as reduced analgesic efficacy and dose-escalation may lead to significant unwanted effects and opioid addiction. Better understanding of the underlying mechanisms of opioid analgesic tolerance may facilitate the development of alternative approaches that minimise the need for dose escalation and its associated risks. Recently, pain research has made significant strides in elucidating the background of opioid analgesic tolerance, however many aspects remain incompletely understood.

Following activation of GPCRs, such as the MOR by an agonist, the magnitude of intracellular signalling is determined by the pharmacodynamic properties of the ligand as well as the duration of time the ligand-receptor pair is bound to each other. The latter is largely governed by the ligand's pharmacokinetic properties and desensitisation and endocytosis of the receptor that occurs after activation. The ability of a ligand to induce desensitisation and endocytosis does not necessarily correlate with its biological activity, thus they can both be viewed as independent factors of the net signalled effect. Phosphorylation of the C-terminal of MORs induced by longer exposure of opioids manifests in the inability to couple with G_i or G_o [150–153]. This phenomenon is called "ligand induced desensitisation" and it can be carried out by a number of kinases on a molecular level. These include G-protein coupled receptor kinases (GRKs), protein kinase C (PKC) or PKA, among others. Importantly, different agonists of the receptor induce site-specific phosphorylation as shown by Doll et al. [154,155] which results in vastly different implications on the activity of the receptor. In general, phosphorylation by GRKs induce beta-arrestin recruitment, causing receptor uncoupling from G proteins [156], scaffolding of other, non-G proteins resulting in the activation various new signal transduction pathways and facilitating receptor endocytosis and either lysosomal degradation or resensitisation through recycling of the receptor to the membrane [157,158]. This process has been mainly described in the context of stimulating the MORs with [D-Ala2,N-MePhe4,Gly-ol]-enkephalin (DAMGO), a selective peptide agonist. On the other hand, when applying continuous treatment with morphine, phosphorylation primarily occurs by PKC [159–161] which leads to more potent desensitiation and

prevention of endocytosis [158]. The fact, that morphine largely lacks the ability to induce internalisation of MORs following PKC-phosphorylation led to the assumption that there is an inverse relationship between receptor endocytosis and the development of tolerance [162]. Indeed, pro-endocytotic efforts, such as overactivation of GRKs or using animals with a mutant MOR that undergoes morphine-induced internalisation have been shown to reduce morphine-induced tolerance [163,164].

As the intracellular signal transduction pathways of G-protein coupled opioid receptors have become better understood, a new direction in opioid research has emerged, namely the development of so-called biased opioid agonist compounds. The idea behind these agents was that by creating a conformational change in which the receptor establishes a G-protein coupled signalling pathway but not beta-arrestin recruitment, an analgesic effect could be achieved, by mitigating some known opioid side effects (such as respiratory depression or emerging tolerance). However, more recent data on these compounds is causing considerable controversy, as several “biased” drugs have been described as non-biased partial agonists with low intrinsic efficacy [165,166].

To avoid the development of opioid analgesic tolerance, the contribution of other CNS pathways has attracted the attention of researchers. In this regard, several studies have shown that NMDA receptor activation plays a prominent role in the development of opioid tolerance [167]. This was first demonstrated by the ability of NMDA antagonists to attenuate analgesic tolerance to opioids [168–171]. According to recent findings, NMDA upregulation in opioid analgesic tolerance is most likely induced by the release of brain derived neurotrophic factor (BDNF) following microglial activation [172]. Indeed, morphine treatment has been shown to induce microglial activation at a spinal level [173,174] accompanied by an increase in pro-inflammatory gene expression and inflammatory mediator release [173,175–177] which contributes to opioid analgesic tolerance and opioid induced hyperalgesia (OIH) [177].

1.3.3. The role of microglial activation in opioid tolerance and neuropathic pain

Activated microglia play a key role in the sensitisation of nociceptors in several pathological conditions affecting spinal sensory circuits (Figure 1) [172,178–186]. This process can be facilitated by endogenous or exogenous factors, such as release of the chemokine fractalkine (CX3CL-1) [179,182,184] or SP [183] from neurons or ongoing

treatment with opioids [172,181,185], respectively. Inflammatory mediators and neurotrophic factors released by the activated microglia contribute to sensitisation via inducing upregulation of NMDA receptors [172], downregulation of potassium chloride co-transporter KCC2, thereby attenuating inhibitory transmission [180,186], or upregulation of glial bradykinin B1 receptors (B1R) [187–189], among others [190]. These mechanistic observations may also provide an exploitable pharmacological target for attenuating spinal nociceptive sensitisation. Inhibition of microglial activation by trifluoro-icaritin [186], peroxisome proliferator-activated receptor gamma (PPARγ) agonists, such as pioglitazone [191,192], AT1 receptor blocker candesartan [139] or microglial inhibitor minocycline [193,194] have been proven to be beneficial in NP or opioid tolerance or both (Figure 1).

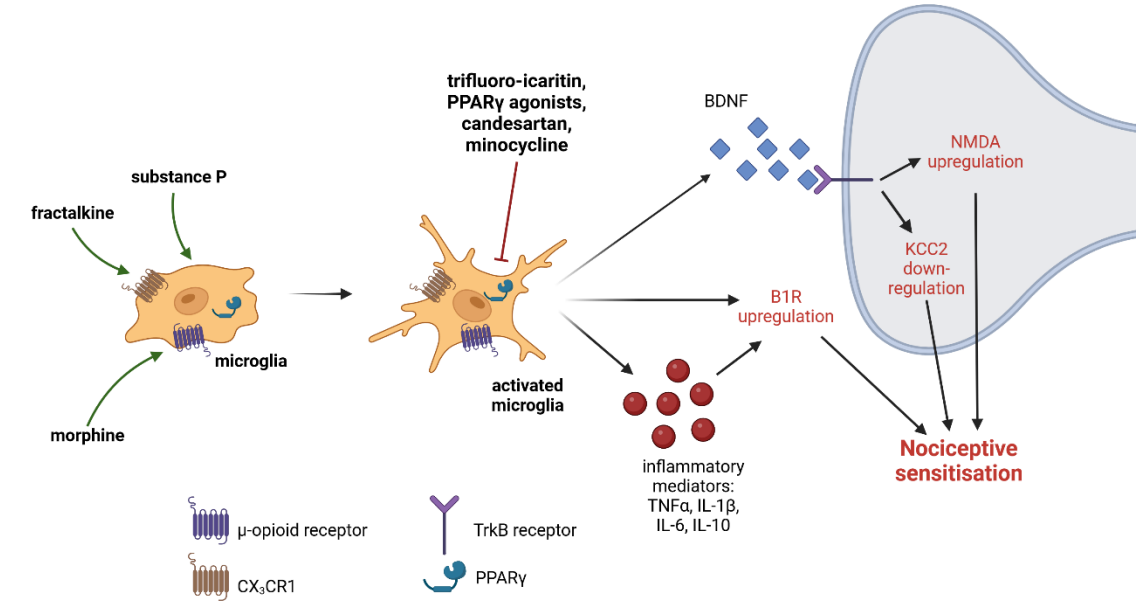

Figure 1. Microglia in the spinal cord can be activated by treatment with opioids such as morphine, or certain chemokines such as fractalkine (CX3CL-1). The former has implications in opioid analgesic tolerance, while the latter may play an important role in the development of certain chronic pain states, including neuropathic pain [182]. This figure was created with BioRender.com.

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2. OBJECTIVES

Current approaches to the treatment of NP are inadequate and associated with side effects in nearly 70% of patients. MOR agonists are essential analgesics for acute and chronic pain, however, their efficacy is compromised under certain conditions, like NP or analgesic tolerance induced by repeated administration.

The present Ph.D. work aimed to investigate:

1. the potential effect of AT1 receptor antagonists, losartan or telmisartan, in reducing allodynia in an acute treatment setting. Parallel with this to evaluate the acute antinociceptive/antiallodynic properties of morphine (for dose determination) as well as opioid analgesic tolerance.
2. to evaluate the combination of AT1 receptor antagonists and morphine in a hope to find multitargeted therapeutic approach for NP.
3. to assess whether co-treatment with AT1 receptor antagonists influences morphine analgesic tolerance.
4. to assess the side effect profile of test compounds regarding motor function in doses achieving promising results *in vivo*.

In order to understand more about the molecular and pharmacological mechanisms of this interaction, we have carried out the following studies:

5. measure the influence of telmisartan on changes observed in morphine-stimulated [^{35}S]GTPγS binding in the spinal cord of rats with neuropathic pain after repeated morphine administration.
6. identifying the potential off-target effect of activating PPARγ for tested AT1 receptor antagonist compounds in the opioid tolerance model.
7. assessment of the degree of microglial infiltration in the spinal cords of chronically treated, opioid tolerant animals and check the effect of AT1 receptor antagonists.
8. mapping spinal neuroanatomical localisation of the target receptors (AT1 and MOR) utilizing RNA Scope® *In-Situ* RNA Hybridisation
9. measure the influence of AT1 receptor antagonists alone or in combination with morphine on CSF L-glutamate and D-serine content in neuropathic and opioid tolerant rats.

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3. METHODS

3.1. Experimental animals

Experimental protocols were performed on male Wistar rats purchased from Toxi-Coop Zrt. (Budapest, Hungary). A total of 123 animals were used for the NP model and 83 animals for the morphine analgesic tolerance model. In addition, 23 animals underwent motor coordination testing. Furthermore, a total of 16 animals were used to obtain histological samples for RNA Scope® *in situ* RNA hybridisation experiments. In total, data from 245 rats were used for this work. The weights of the animals were 120-150 g and 170-200 g at the start of the NP model and the morphine analgesic tolerance experiments, respectively. Animals weighing 170-200g at baseline were used for motor coordination testing and 200-240g for histological sampling for RNA Scope *in-situ* hybridisation. Weight differences were implemented to ensure that the CNS of all animals were fully evolved at the time of interest. Rats were housed in standard cages, with four to six animals per cage in the local animal house of Semmelweis University, Department of Pharmacology and Pharmacotherapy (Budapest, Hungary) and maintained under controlled environmental conditions (12:12 h light/dark cycle, light on at daytime, 20 ± 2 °C temperature). Water and standard rodent chow were available *ad libitum*. Prior to experiments, animals were allowed to acclimatise in the animal house for at least one week. Animal housing and all experiments were performed in accordance with the European Union directive on the protection of animals used for scientific purposes (2010/63/EU), the Hungarian act on the protection and welfare of animals (No. XXVIII of 1998. 32.§) and the local animal care committee (PEI/001/276-4/2013 and PE/EA/619-8/2018). The researchers made every effort to minimise the pain and suffering of the animals and the number of animals used.

3.2. Materials

Telmisartan and losartan-potassium were obtained from TCI EUROPE N.V. (Zwijndrecht, Belgium), morphine-HCl and pentobarbital was obtained from the

Semmelweis University Pharmacy (Budapest, Hungary), and PPARγ antagonist GW9662 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Diethyl-ether, dimethyl sulfoxide (DMSO) and hydroxyethyl-cellulose was purchased from Merck - Sigma-Aldrich (Darmstadt, Germany). Oral administration of AT1 blockers (telmisartan and losartan) was carried out using stainless steel oral feeding needles (purchased from Animalab Hungary Kft., Vác, Hungary) in a total volume of 5 $mL \cdot kg^{-1}$ bodyweight (BW). Telmisartan was suspended in 1% hydroxyethyl-cellulose solution (HEC), while losartan-potassium was dissolved in 0.9% saline. Morphine was administered subcutaneously (sc.), in a total volume of 2.5 $mL \cdot kg^{-1}$ BW. GW9662 was dissolved in 20% DMSO solution and administered intraperitoneally (ip.) in a total volume of 2.5 $mL \cdot kg^{-1}$ BW. Pentobarbital was dissolved in 0.9% saline before being administered ip. in a total volume of 2.5 $mL \cdot kg^{-1}$ BW.

For (^{35}S)GTPγS binding assay DMSO, Tris-HCl, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), NaCl, MgCl2 × 6H2O, GDP and the GTP analog GTPγS were purchased from Merck - Sigma-Aldrich (Darmstadt, Germany). The radiolabeled GTP analog, [35S]GTPγS (specific activity: 1250 Ci/mmol, Cat.No.: NEG030H250UC) and the UltimaGoldTM MV aqueous scintillation cocktail was purchased from PerkinElmer (handled by Per-Form Hungaria Kft., Budapest, Hungary).

For the analysis of CSF neurotransmitter levels by capillary electrophoresis, acetonitrile, boric acid, L-cysteic acid and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) were purchased from Merck - Sigma Aldrich (Darmstadt, Germany). 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) was purchased from TCI EUROPE N.V. (Zwijndrecht, Belgium) and hydroxypropoyl-amino-β-cyclodextrin was purchased from Cyclolab Ltd (Budapest, Hungary).

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

3.3. Experimental protocols

3.3.1. Mononeuropathic pain model

Figure 2 shows a schematic summary of the applied NP model study design. Here we have utilised a method that evokes neuropathic pain by inflicting damage to a single nerve (hence "mononeuropathy"). In the days prior to the start of the experiments, the animals were handled to acclimatise them to the experimental conditions. This consisted of placing the animals in the plastic cages of the experimental apparatus once daily. The development of mechanical allodynia as a symptom of neuropathy was assessed using a dynamic plantar aesthesiometer (DPA 37450, Ugo Basile, Italy) as described before [32,195] with the following settings: incrementation: 10 g/s, maximal force: 50 g. The paw withdrawal thresholds (PWTs) of animals were measured in grams after at least five minutes of habituation in the cage for each animal. Each paw was measured three times, and the mean of the three measurements was used for further analysis. All behavioural studies were performed by the same experimenter.

First, baseline measurements were performed with DPA to determine the pre-operative PWT. Animals were then subjected to partial sciatic nerve ligation (pSNL) using the method described by Seltzer and colleagues and in our previous studies. [195–197]. In brief, the animals were anaesthetised with pentobarbital (60 mg·kg^{-1} BW) and placed on heating units to maintain a body temperature of 37 °C. The right sciatic nerve was exposed at thigh level without muscle damage under aseptic conditions. A size 7-0 silicone-treated silk suture was used to ligate the dorsal half of the nerve tightly. The wound was closed with two sutures. A sham operation, in which the nerve was exposed without subsequent ligation, was performed in a separate group of animals used later as controls.

PWTs were measured on both (operated and unoperated) hind paws of the animals two weeks after surgery. Animals were considered neuropathic if there was a 20% or greater decrease in the average PWT of the operated paw compared to the unoperated one, calculated using the following formula:

$$\frac{(PWT_{unoperated\ paw} - PWT_{operated\ paw})}{PWT_{unoperated\ paw}} \times 100$$

1. Formula to calculate the difference in PWT between operated and unoperated paws.

Randomisation was then used to assign animals to the control and treatment groups. Following randomisation, minor adjustments were made where necessary to ensure that the mean baseline PWT values for each group were close to the same. This was carried out to ensure that any subsequent effects of the test compounds were equally well detected. Following administration of the test compounds or vehicles, the PWTs of the animals were determined again at 60 and 120 min, as shown in Figure 2. A group of animals was used to determine the acute antiallodynic effect of different doses of telmisartan (20, 40 and 80 $\mu mol \cdot kg^{-1}$ BW, po.), losartan (50, 100 and 150 $\mu mol \cdot kg^{-1}$ BW, po.) and morphine (10 and 20 $\mu mol \cdot kg^{-1}$ BW, sc.). In experiments aimed to evaluate the effect of angiotensin receptor type 1 blockers (ARBs) and morphine in combination, the compounds were administered in a time-shifted manner (ARBs at 0 min and morphine at 30 or 90 min). This was done so that the peak effect of the combination elements always coincided in time. The time intervals were chosen based on the data obtained in the first part of the study, namely the acute experiments with different doses of the two ARBs. On the basis of our previous studies, the peak effect of morphine was expected to occur at 30 min after sc. administration [32,198].

A further group of animals received a combination of morphine and an ARB at doses subanalgesic in acute trials (20, 50 and 10 $\mu mol \cdot kg^{-1}$ BW for telmisartan, losartan and morphine, respectively) on the 14^{th} days after Seltzer surgery and for 10 subsequent days. In this group, sc. morphine was administered twice a day, while ARBs were administered po. once a day for 10 days in total. On day 24 after Seltzer surgery, another set of DPA measurements was performed in chronically treated animals. The animals were then sacrificed by overdose of diethyl-ether, after which spinal cord tissue and cerebrospinal fluid samples were collected for further in vitro analyses (see chapters 3.3.3. and 3.3.4.).

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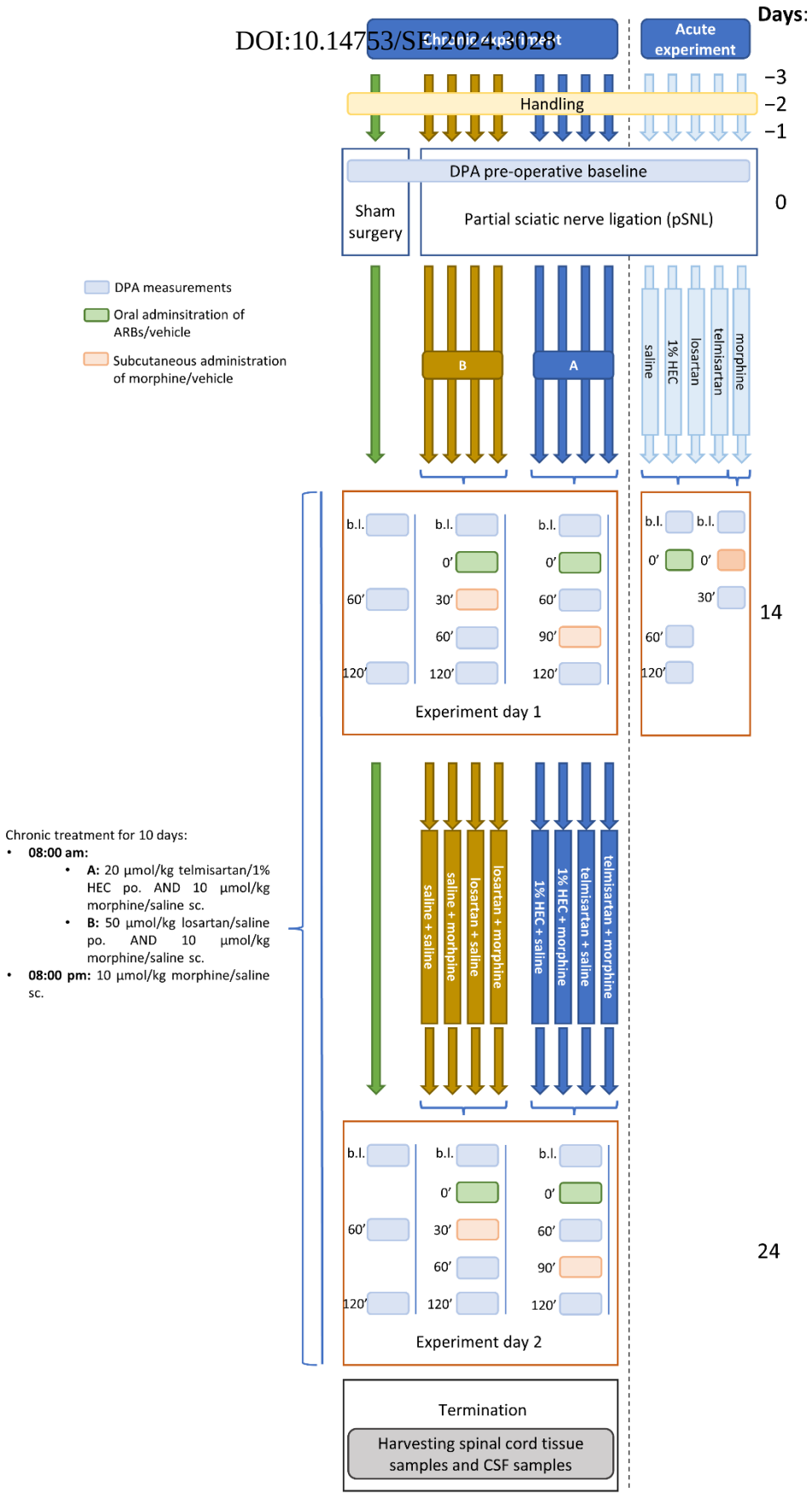

Figure 2. *Schematic representation of the study design applied in the mononeuropathic pain model.* The figure indicates the timeline of the acute and chronic experiments, involving DPA measurements, pSNL, treatment days and termination. Abbreviation "b.l." stands for baseline.

3.3.2. Morphine analgesic tolerance model

Figure 3. shows a schematic summary of the applied morphine analgesic tolerance model study design. To monitor the antinociceptive effect of morphine, acute thermal pain sensation was assessed using radiant heat tail-flick test (IITC Life Science, Woodland Hills, CA, USA) as previously described [199]. Prior to the experiments, a handling procedure was carried out to acclimatise the animals to the experimental conditions. This consisted of placing the animals blindfolded in the tail-flick apparatus once daily. The light intensity was adjusted so that the control tail-flick latency was less than 4 s. The cut-off time was set at 8 s to avoid tissue damage. Tail-flick latency was measured before (baseline) and after test compound or vehicle administration at the depicted time points (see Figure 3). All behavioural studies were performed by the same tester. Following the randomisation of animals into groups, morphine tolerance was established by sc. injections of 10 $mg \cdot kg^{-1}$ (or 31.08 $\mu mol \cdot kg^{-1}$) BW morphine twice daily (8 a.m. and 8 p.m.) for 10 days. Control animals received saline injections (in a total volume of 2.5 $mL \cdot kg^{-1}$ BW) twice daily.

3.3.2.1. Experiments with AT1 antagonists, telmisartan or losartan

In addition to morphine or saline, animals were treated with telmisartan (20 $\mu mol \cdot kg^{-1}$, po.), losartan (50 $\mu mol \cdot kg^{-1}$, po.) or their vehicles, 1% HEC (5 $ml \cdot kg^{-1}$, po.) or saline (5 $ml \cdot kg^{-1}$, po.), respectively, once daily (8 a.m.). Oral treatments were carried out using orogastric gavage. The degree of analgesic tolerance development was determined using the tail-flick test on days 4 and 10. Following this, animals were sacrificed by diethyl-ether overdose and CSF samples were obtained for further *in vitro* analyses (see chapter 3.3.4.).

3.3.2.2. Experiments with PPARγ antagonist, GW9662

In this set of experiments, animals received subcutaneous morphine or saline treatment as described in section 3.3.2., oral treatment with losartan, telmisartan or vehicles in a similar manner as described in section 3.3.2.1, and the selective PPARγ antagonist, GW9662 (2 $mg \cdot kg^{-1}$ or 7.22 $\mu mol \cdot kg^{-1}$ BW) or its vehicle, 20% DMSO (2.5 $mL \cdot kg^{-1}$ BW) ip., once daily. Tolerance development against the analgesic effect of morphine was monitored with tail-flick tests on days 4 and 10. On day 10, animals were

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deeply anaesthetised with pentobarbital (60 mg·kg^{-1}, ip.), transcardially perfused with phosphate-buffered saline (PBS), pH 7.4 and spinal cord tissue samples were collected for further histological analysis (see chapter 3.3.7.).

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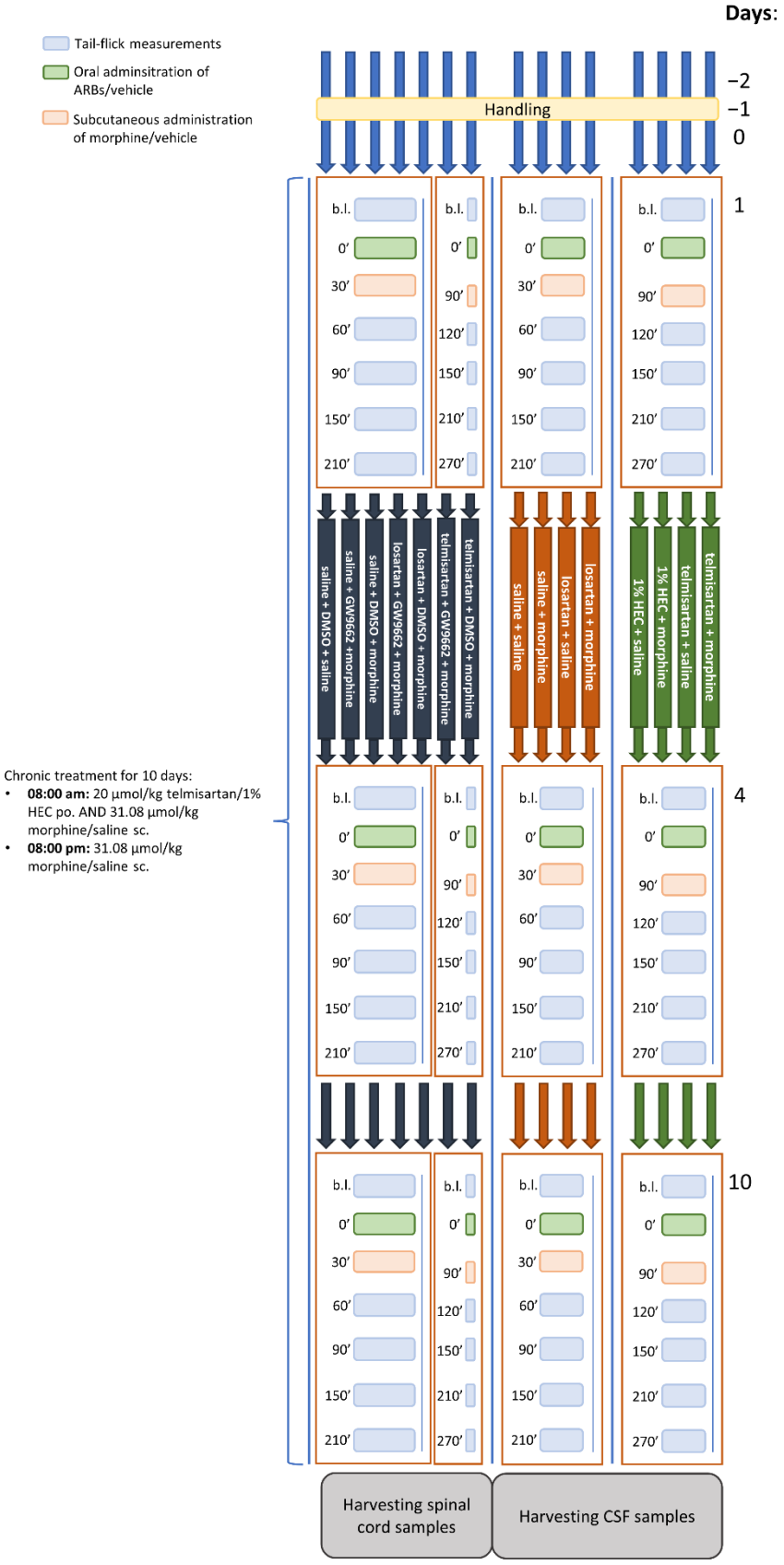

Figure 3. *Schematic representation of the study design applied in the morphine analgesic tolerance model.* The figure indicates the timeline of the experiments, involving tail-flick measurements, treatment days and termination. Abbreviation "b.l." stands for baseline.

3.3.3. Motor Function Testing

The rotarod test (Rat RotaRod, model 7750, Ugo Basile, Italy) was used to assess the effect of telmisartan, morphine and their combination on the motor function of animals. On the day prior to the experiment, the animals were trained to remain on the rotating rod of the apparatus. Rotational speed was set at 16 RPM, and cut-off time was set at 180 s. On the day of the experiment, the animals were treated orally with the highest tested dose of telmisartan (80 μmol·kg^{-1}), the subanalgesic dose of morphine used in the combination experiments (10 μmol·kg^{-1}), their combination or their vehicles (1% HEC or saline, respectively). Animal motor coordination was tested at the time of peak compound effect. Compounds were administered in a time-shifted manner (ARBs at 0 min and morphine at 90 min) to ensure that the peak effect of the combined elements coincided in time. Rotarod latency was recorded in seconds (fall-off time). As a positive control, a high dose of morphine (31.08 μmol·kg^{-1}) was used.

3.3.4. Morphine-stimulated [^{35}S]GTPγS binding assay

Animals with mononeuropathic pain that underwent chronic treatment were sacrificed, as described in section 3.3.1. Their spinal cords were quickly removed, the lumbar portion isolated, frozen in liquid nitrogen and stored at −80 °C until further processing. Tissue samples were prepared for the [^{35}S]GTPγS binding experiments, as described previously [32,195]. In summary, the samples were homogenised in ice-cold TEM buffer containing 50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl2, and 100 mM NaCl, pH 7.4, using a teflon-glass homogeniser. The homogenate underwent centrifugation at 18,000 rotations per minute (RPM) for 20 min at 4 °C. The ensuing supernatant was discarded, and the pellet was incubated at 37 °C for 30 min in a shaking water bath. Centrifugation was then repeated under the same parameters as before. The final pellet was suspended in ice-cold TEM buffer and stored at −80 °C. The protein content of the membrane preparation was assessed using Bradford protein assay and bovine serum albumin (BSA) was employed as a standard.

In the [^{35}S]GTPγS binding experiments, the GDP → GTP exchange of the $G_{\alpha i/o}$ protein was measured in the presence of a given ligand at increasing concentrations. This was done to evaluate the potency and efficacy of the ligand on the tissue sample. A radioactive, non-hydrolysable GTP analogue, [^{35}S]GTPγS was applied to monitor the

nucleotide exchange. Experiments were conducted following the previously established protocols [33,200], with technical modifications implemented when necessary. Briefly, the previously prepared membrane homogenates were incubated at 30 °C for 60 min in TEM buffer containing 20 MBq/0.05 ml [^{35}S]GTPγS (0.05 nM) and 0.1–10,000 nM concentrations of morphine. The experiments were performed in the presence of excess GDP (30 μM). The final volume of the incubation mixture was 1 ml. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. Total binding was measured in the absence of test compounds. The difference between total and non-specific binding represents basal activity. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester, Gaithersburg, MD, USA) separating the bound and unbound [^{35}S]GTPγS, and washed three times with 5 mL ice-cold 50 mM Tris-HCl (pH 7.4) buffer through Whatman GF/B glass fibres. The radioactivity of the filters was detected in UltimaGoldTM MV aqueous scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter (Per-Form Kft, Budapest, Hungary). [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

3.3.5. Immunohistochemistry

Spinal cord tissue samples were obtained as described in section 3.3.2.2. and postfixed in neutral-buffered formalin for 24 hours, followed by cryoprotection in PBS at 4°C overnight. The samples were then dehydrated and embedded in paraffin. 4 μm thick tissue sections were prepared (Leica HistoCore MULTICUT Semi-Automated Rotary Microtome, Leica Biosystems, Deer Park, IL, USA) and subjected to antigen retrieval (Antigen Unmasking Solution, citrate-based, pH = 6, Vector Laboratories, Newark, CA, USA, H-3300) for 30 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS solution, followed by blocking with 2.5% goat serum in PBS and 2.5% milk powder. Primary IBA1 antibodies (019-19741, Anti Iba1, Rabbit (for Immunocytochemistry, FUJIFILM Wako Pure Chemical Corporation, Richmond, VA, USA) were diluted (1:200) in goat serum (2.5%) and were incubated overnight at 4°C. After washing with gentle shaking three times with PBS, the sections were incubated with anti-rabbit IgG secondary antibody (8114S, SignalStain® Boost IHC Detection Reagent (HRP, Rabbit, Cell Signaling Technology, Danvers, Massachusetts, USA) conjugated with horseradish peroxidase. Secondary antibodies were washed with gentle shaking

three times with PBS, and signals were developed with diaminobenzidine (SK-4103, ImmPACT DAB EqV Peroxidase [HRP] Substrate, Vector Laboratories, Burlingame, CA, USA). Staining was analysed and quantified by a blinded analyst using ImageJ "Fiji" Software [201].

3.3.6. RNA Scope® *in-situ* mRNA hybridisation

Naïve Wistar rats weighing 200-240 g were sacrificed, and spinal cord, DRG and brain tissues were harvested after transcardial perfusion with PBS pH 7.4 and 20% formaldehyde solution. Tissue samples were fixed in neutral buffered formalin for 24 h, then dehydrated and embedded in paraffin. 4 μm thick sections were prepared (Leica HistoCore MULTICUT Semi-Automated Rotary Microtome, Leica Biosystems, Deer Park, IL, USA) and used for further analysis.

RNA Scope® *In-Situ* Hybridisation assay was performed using RNA Scope® Multiplex Fluorescent Kit v2 according to the instructions of the manufacturer (Advanced Cell Diagnostics Pharma Assay Services, Newark, CA, United States). Briefly, the formalin-fixed paraffin-embedded tissue sections of 4 μm thickness were pre-treated with heat and protease prior to hybridisation with the following target oligo probes: Positive Control Probe-Rn-Polr2a-C2 (Cat. No.: 312481-C2), 3plex-Rn-Negative Control Probe (Cat. No.: 320871), Rn-Agtr1a-C2 (Cat. No.: 422661-C2), Rn-Agtr2-C2 (Cat. No.: 422671-C2), Rn-Oprm1-C3 (Cat. No.: 410691-C3), Rn-CALCA-C1 (Cat No. 317511), Mm-Slc17a7-C1 (Cat. No.: 416631). The preamplifier, amplifier, and AMP-labelled oligo-probes were then hybridised in sequence, followed by the development of a chromogenic precipitate. A positive control probe specific for the housekeeping genes and a negative control probe were used to quality control each sample for RNA integrity. Pretreatment conditions were optimised for maximum signal-to-noise ratio. Punctate red/green/magenta dots were identified as the specific RNA staining signal. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), which appears as a light purple colour. Imaging was performed with a Leica DMi8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

3.3.7. Capillary electrophoresis analysis of neurotransmitter content

L-glutamate and D-serine content of CSF samples was measured by the capillary electrophoresis laser-induced fluorescence method carried out as described previously [195,197,202] with some modifications.

Animals in our mononeuropathic pain model or morphine analgesic tolerance model were sacrificed after 10 days of treatment, as described above. CSF samples were obtained by cisterna magna puncture and centrifuged at 2000 × g for 10 min at 4 °C. Specimens were frozen immediately and stored at −80°C until processed. On the day of the experiment, samples were deproteinised by mixing with 2 volumes of pure acetonitrile and centrifuged again at 20,000 × g for 10 min at 4 °C. Supernatants were subjected to derivatisation with NBD-F (1 mg/mL final concentration) in 20 mM borate buffer pH 8.5 for 20 min at 65 °C. As internal standard, 1 μM L-cysteic acid was used.

Derivatised samples were analysed using a P/ACE MDQ Plus capillary electrophoresis system coupled with a laser-induced fluorescence detector equipped with a laser source of excitation and emission wavelengths of 488 and 520 nm, respectively, (SCIEX, Framingham, MA, USA). Separations were carried out in polyacrylamide-coated fused silica capillaries (i.d.: 75 μm, effective/total length: 50/60 cm) using 50 mM HEPES buffer pH 7.0 containing 6 mM hydroxypropyl amino-β-cyclodextrin at 15 °C by applying −28 kV constant voltage.

3.4. Statistical Analysis

All values are presented as mean ± standard error of means (S.E.M.). The statistical analysis was performed using GraphPad Prism software (version 8.0.1; GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. Two-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons between related groups. One-way ANOVA followed by Fisher's LSD post hoc test was used to compare independent groups. The post hoc tests were only conducted if F in ANOVA achieved $p < 0.05$. ROUT analysis was performed to identify outliers, with Q value = 0.5%.

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4. RESULTS

4.1. The impact of telmisartan, losartan or their combination with morphine in neuropathic pain evoked by sciatic nerve injury

4.1.1. Oral telmisartan or losartan produces acute antiallodynic effect

In animals showing mechanical allodynia two weeks after pSNL, interpreted as the hallmark symptom of NP, the acute antiallodynic effect of different doses of orally administered losartan and telmisartan was assessed by DPA (Figure 4).

Losartan produced a moderate antiallodynic effect at doses of 100 or 150 μmol·kg^{-1} BW, which peaked at 60 min following systemic administration. However, when compared to the vehicle-treated group, statistically significant analgesia was only achieved in the group receiving the 100 μmol·kg^{-1} BW dose (PWT: 28.19 ± 2.25, n = 9 vs. 16.76 ± 1.80, n = 5; p = 0.013) (Figure 4, panel A).

Compared to its vehicle, telmisartan produced a statistically significant increase in PWT at both higher test doses, namely 40 and 80 μmol·kg^{-1} BW (PWT: 28.47 ± 1.91, n = 9 vs. 16.35 ± 2.30, n = 6; p = 0.013 and 31.48 ± 2.83, n = 6 vs. 16.35 ± 2.30, n = 6; p = 0.014, respectively). Peak antiallodynic effect was achieved 2 hours after systemic administration (Figure 4, panel B).

4.1.2. Morphine cannot increase PWT of neuropathic animals at low doses, but produces acute antinociceptive effect at higher doses

Attenuation of mechanical allodynia by subcutaneously administered morphine alone was also assessed (Figure 4, panel C). The antiallodynic effect was tested at 30 min, which corresponds to the peak effect of morphine [32,198]. In this series of experiments, morphine failed to alleviate NP at a dose of 10 μmol·kg^{-1} BW, which was chosen as the subanalgesic dose for further experiments. At a dose of 20 μmol·kg^{-1} BW, morphine, compared to its vehicle, significantly increased the PWT of neuropathic animals (PWT: 38.08 ± 3.45, n = 5 vs. 19.46 ± 1.99, n = 5; p < 0.0001), consistent with literature data, but also markedly increased the withdrawal threshold of the left (intact) paw to the level of cut-off (Figure 4, panel C).

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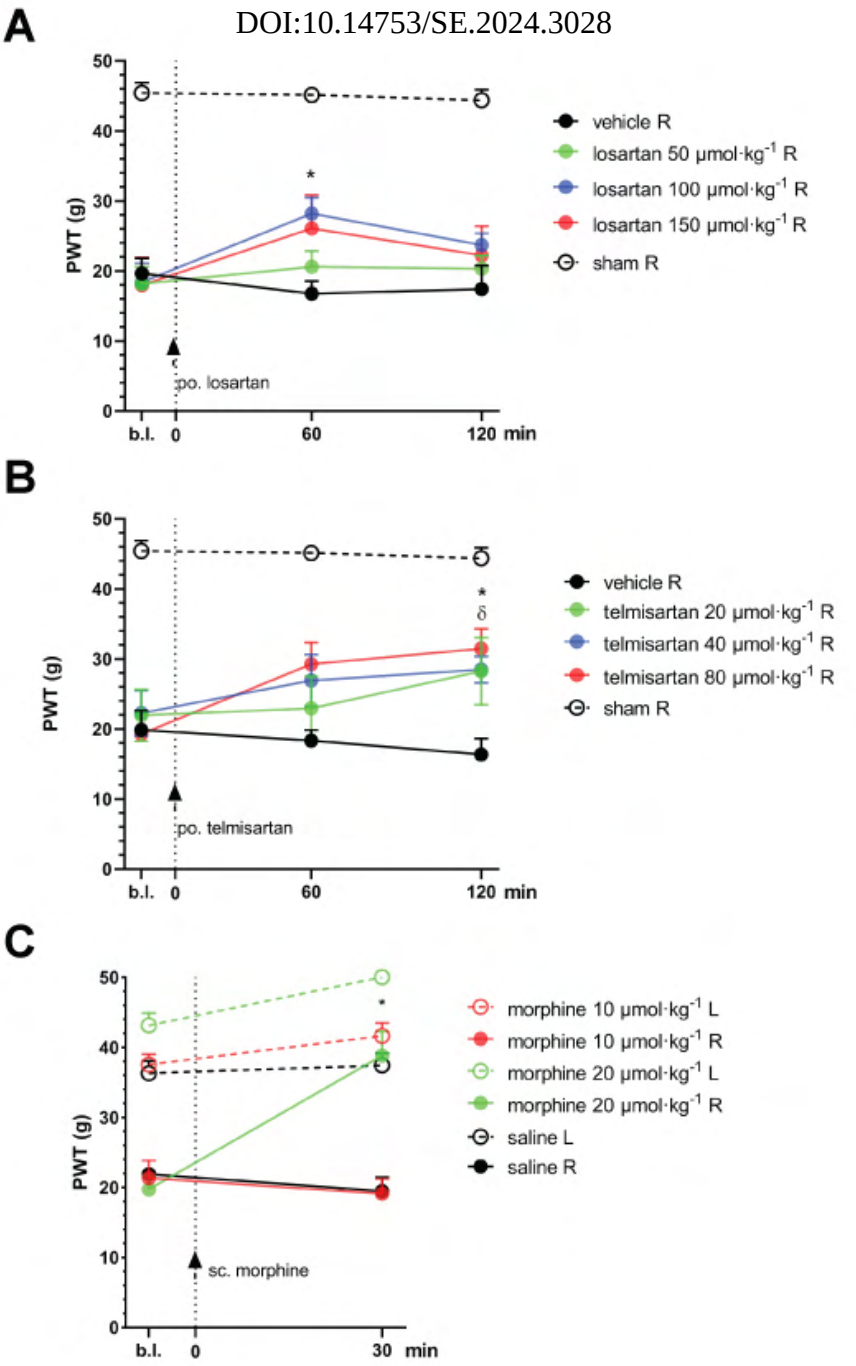

Figure 4. *The acute antiallodynic effect of po. losartan (panel A), po. telmisartan (panel B), and sc. morphine (panel C).* Means of PWT ± S.E.M. are depicted in grams, obtained from the animals' right (operated; R) hind paws on the 14th day after pSNL at the indicated time points. Data obtained from the intact (left) hind paws of the animals are excluded on panels A and B for better visual clarity (however, they are presented in Figure 20). Panel A: * $p < 0.05$ between losartan 100 µmol·kg^{-1} and vehicle, two-way ANOVA followed by Tukey's post hoc test, n = 5–9 per group. F (time; 1.925, 51.98) = 6.607, p = 0.0031. F (treatment group; 4, 27) = 19.44, $p < 0.0001$. Panel B: * $p < 0.05$ between telmisartan 80 µmol·kg^{-1} and vehicle, δ $p < 0.05$ between telmisartan 40 µmol·kg^{-1} and vehicle, two-way ANOVA followed by Tukey's post hoc test, n = 5–11 per group. F (time; 1.847, 51.72) = 7.903 p = 0.0013. F (treatment group; 4, 28) = 12.32 $p < 0.0001$. Panel C: * $p < 0.05$ between morphine 20 µmol·kg^{-1} R and vehicle, two-way ANOVA followed by Tukey's post hoc test, n = 5–12 per group. F (time; 1, 38) = 13.19, p = 0.0008. F (treatment group; 5, 38) = 38.06, $p < 0.0001$. Figures [76,77]

4.1.3. Combination of telmisartan, but not losartan, with morphine at subanalgesic doses produces antiallodynia after chronic treatment

In this series of experiments, doses found ineffective after acute administration were chosen to assess the effect of the combination of test compounds after acute and repeated administration. Thus, 10 μmol·kg^{-1} BW for sc. morphine, 50 μmol·kg^{-1} BW for po. losartan and 20μmol·kg^{-1} BW for po. telmisartan (Figure 5) was used.

First, the combination of subanalgesic doses of losartan and morphine failed to alleviate NP either on the first day of treatment (acute effect) or after 10 days of chronic administration (Figure 5, panel A). On the other hand, the combination of telmisartan and morphine at subanalgesic doses already showed a trend, but not a significant effect, towards the alleviation of NP after acute administration. After 10 days of chronic treatment, this combination alleviated NP in the operated paws. At the peak of the effect of the compounds, a significantly higher PWT of the operated paws was observed in the combination group compared to the vehicle-treated group (PWT: 32.53 ± 3.71, n = 6 vs. 15.46 ± 2.27, n = 4; p = 0.028) or the vehicle plus morphine-treated one (PWT: 32.53 ± 3.71, n = 6 vs. 11.35 ± 2.63, n = 5; p = 0.008) (Figure 5, panel B).

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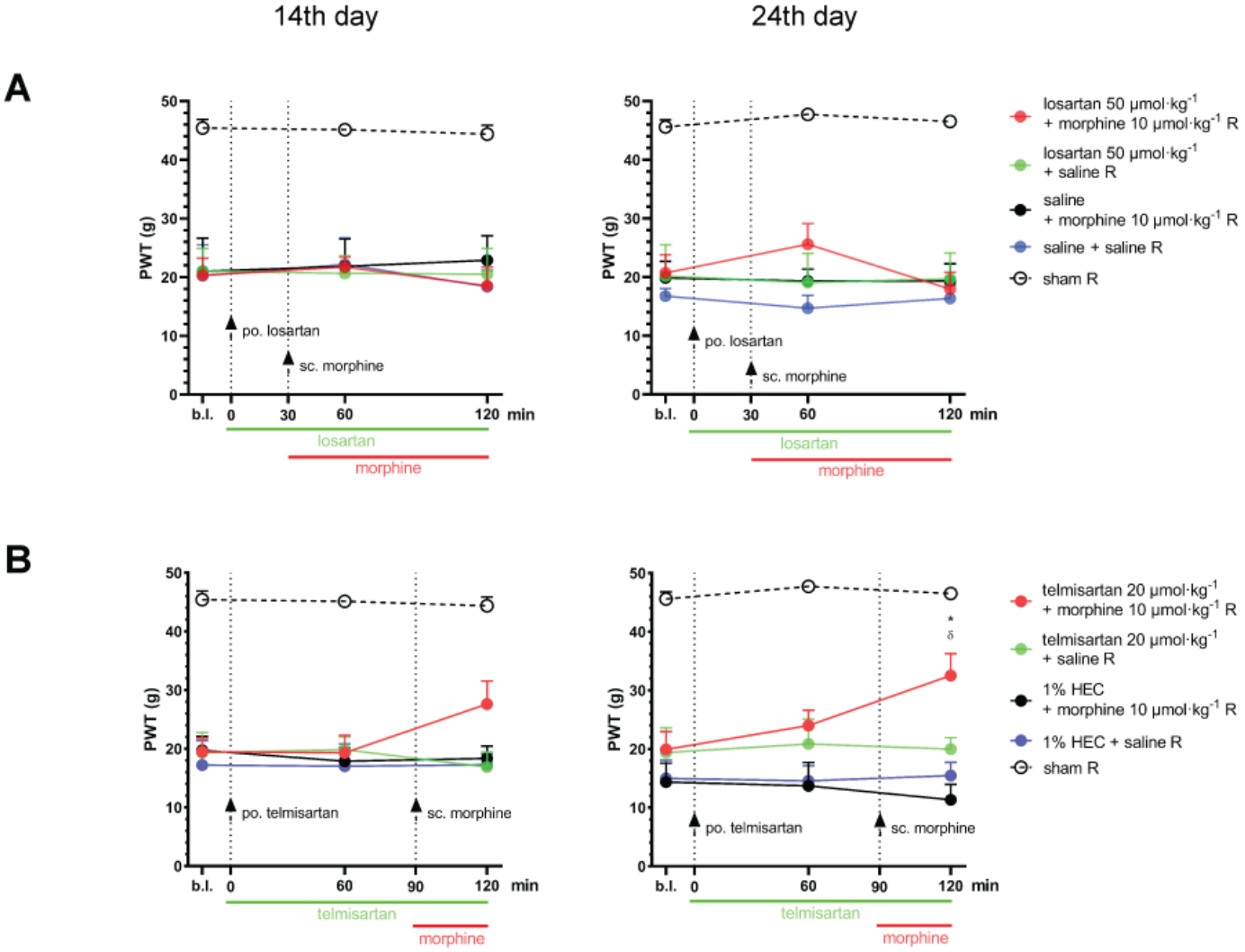

Figure 5. *The antiallodynic effect of po. losartan, sc. morphine and their combination, following acute and chronic treatment on the 14th and 24th day post-pSNL, respectively (A). The antiallodynic effect of po. telmisartan, sc. morphine and their combination, following acute and chronic treatment on the 14th and 24th day post-pSNL, respectively (B).* Abbreviation "b.l." stands for baseline.

Panel A: Two-way ANOVA followed by Tukey's post hoc test, n = 5–6 per group. 14th day: F (time; 1.817, 38.15) = 0.75 p = 0.47; F (treatment group; 4, 21) = 12.93, p < 0.0001. 24th day: F (time; 1.57, 33) = 1.1, p = 0.33; F (treatment group; 4, 21) = 23.87, p < 0.0001.

Panel B: * p < 0.05 versus vehicle, $^{\delta}$ P < 0.05 vs. 1% HEC + morphine 10 µmol·kg^{-1} R, two-way ANOVA followed by Tukey's post hoc test, n = 4–6 per group; 14th day: F (time; 1.711, 35.94) = 0.7 p = 0.48; F (treatment group; 4, 21) = 27.15, p < 0.0001; 24th day: F (time; 1.855, 38.96) = 4.24, p = 0.024; F (treatment group; 4, 21) = 27.06, p < 0.0001. Results are presented as mean ± S.E.M. [77]

4.1.4. Influence of telmisartan on changes observed in morphine-stimulated [^{35}S]GTPγS binding in the spinal cord of rats with neuropathic pain after repeated morphine administration

Specific G-protein coupling of μ-opioid receptors was measured in the spinal cord of neuropathic animals with promising *in vivo* results, using a morphine-stimulated [^{35}S]GTPγS binding assay. The obtained concentration-response curve and binding data are summarized in Figure 6 and Table 3, respectively.

Morphine showed similar efficacy (E_{max}) for G-protein coupling in the spinal cord tissues of animals from either treatment group. On the other hand, the potency of morphine (EC_{50}) with respect to [^{35}S]GTPγS binding was significantly decreased in spinal tissue samples obtained from animals treated chronically with morphine (Figure 6, panel B). This reduction in the [^{35}S]GTPγS specific binding potency of morphine can also be observed as a rightward shift of the morphine concentration-response curve in the indicated group (Figure 6, panel A). However, morphine retained its potency, and its EC_{50} was not significantly different from that of control animals in the spinal cords of animals receiving the combination of subanalgesic doses of telmisartan and morphine.

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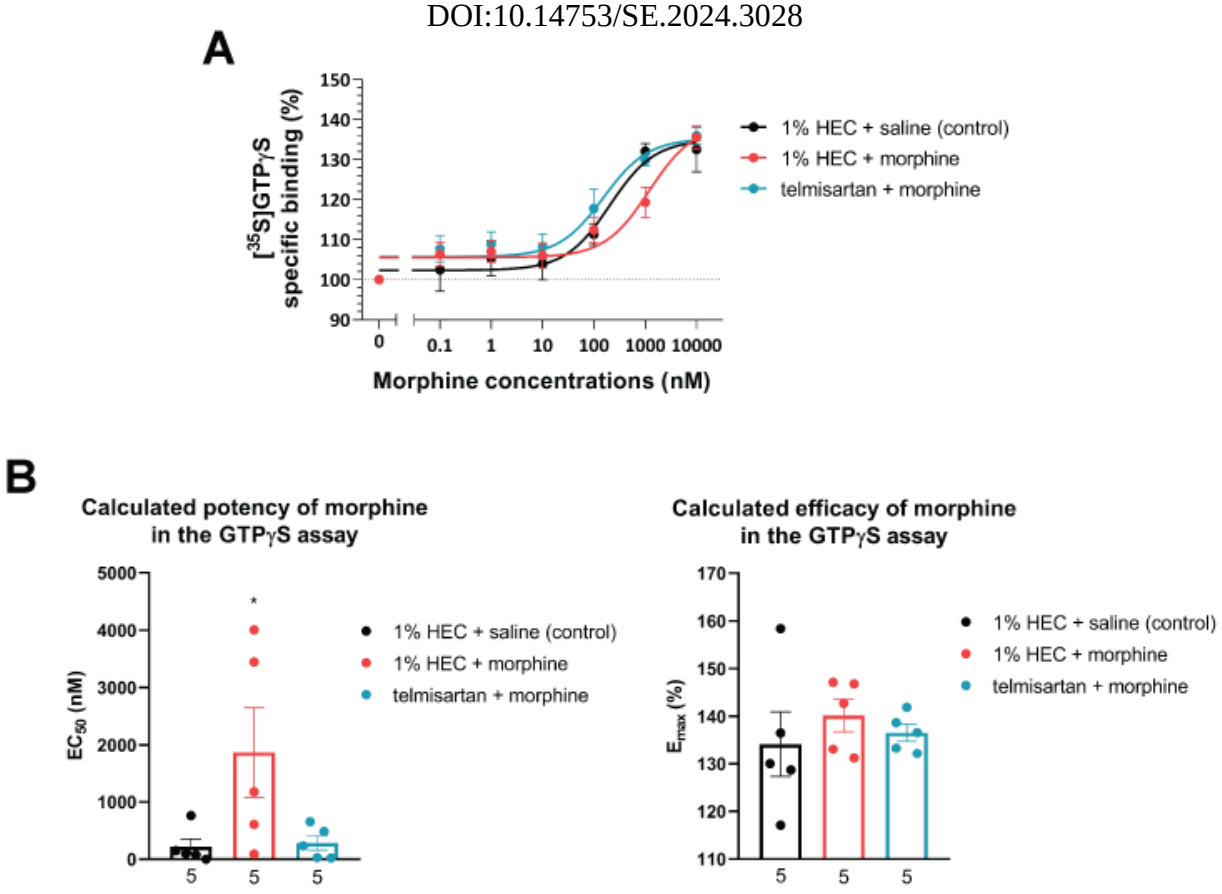

Figure 6. Morphine stimulated G-protein activity in the spinal cords of neuropathic rats treated for 10 days with the indicated combinations (A). Calculated potency and efficacy of morphine on the spinal cords of neuropathic rats treated for 10 days with the indicated combinations (B). * $p < 0.05$ vs. all other groups, one-way ANOVA followed by Fisher's LSD post hoc test, n = 5 per group. EC_{50}: $F(2, 12) = 4.017$, $p = 0.0462$ E_{max}: $F(2, 12) = 0.4538$, $p = 0.6457$. EC_{50} and E_{max} values were calculated individually for each animal and means ± S.E.M. are presented here. [77]

Table 3. Morphine-stimulated [^{35}S]GTPγS binding data on spinal cords obtained from animals undergoing 10 days of chronic treatment. EC_{50} and E_{max} values were calculated individually for each animal sample and means ± S.E.M. are presented here. * $p < 0.05$ vs. telmisartan + morphine; δ $p < 0.05$ vs. control, one-way ANOVA followed by Fisher's LSD post hoc test, n = 5 per group. [77]

Treatment Group (10 Days of Treatment)	**GTPγS Binding**	
	EC50 ± S.E.M. (nM)	**Emax ± S.E.M. (%)**
1% HEC + saline (*n* = 5)	217.2 ± 137.8	134.1 ± 6.8
1% HEC + morphine (*n* = 5)	1864.0 ± 783.5 *$^{\delta}$	140.2 ± 3.4
telmisartan + morphine (*n* = 5)	285.0 ± 125.1	136.5 ± 1.8

4.2. Impact of subanalgesic doses of losartan or telmisartan on the development of morphine analgesic tolerance and accompanying spinal microglial activation

Next, the effect of AT1 blockers was evaluated in a setting of opioid analgesic tolerance produced by chronic treatment with high-dose morphine. Changes in the antinociceptive effect of morphine were monitored using the rat tail-flick test (acute thermal pain).

4.2.1. Telmisartan delays the development of morphine analgesic tolerance in the rat tail-flick assay

Subcutaneous morphine (31.08 μmol·kg^{-1} BW) showed a significant antinociceptive effect indicated by increased latency in the tail-flick test in rats after acute administration (1st day). This effect peaked at 30 min after morphine administration and was maintained when combined either with telmisartan or its vehicle (Figure 7). In contrast, telmisartan (20 μmol·kg^{-1} BW) alone did not show antinociception. Animals in all studied treatment groups showed a similar response on the 4th day of chronic treatment as on the 1st (Figure 7). By the 10th day of treatment, the antinociceptive effect of morphine had disappeared and was not significantly different from the control at any time point, indicating the development of antinociceptive tolerance to morphine. However, when morphine was combined with oral telmisartan, a significant antinociception was maintained on days 4 and 10, peaking at 30 min and lasting up to 3 h (Figure 7).

4.2.2. Losartan delays the development of morphine analgesic tolerance in the rat tail-flick assay

Similar to the experiments with telmisartan described above, the significant antinociceptive effect of sc. morphine (31.08 μmol·kg^{-1} BW) was seen on days 1 and 4 and was maintained when combined with a dose of losartan that did not alter tail flick latency when administered alone (Figure 8). On day 10, morphine alone failed to significantly increase tail flick latency at any time point compared to its vehicle. In contrast, in animals receiving the combination of morphine and losartan a significant

antinociceptive effect of morphine was also observed on day 10, peaking at 30 min and lasting up to 2 h (Figure 8).

4.2.3. PPARγ antagonist attenuates the effect of losartan or telmisartan on the development of morphine analgesic tolerance

In order to determine the degree of the role of PPARγ activation as an off-target mechanism in the morphine analgesic tolerance delaying effect of telmisartan or losartan, the effective treatment groups were combined with a selective PPARγ antagonist (GW9662) or its vehicle (20% DMSO). In this series of experiments, morphine retained its antinociceptive effect in the tail-flick assay on days 1 and 4 but lost it by the 10th day when given alone or in combination with GW9662. When combined with subanalgesic doses of losartan, telmisartan or losartan + GW9662, significant antinociception was also observed on the 10th day. Interestingly, in the group receiving chronic treatment with telmisartan + GW9662 + morphine, on day 10 morphine was unable to significantly increase tail-flick latency compared to the control at any time point (Figure 9). On day 10, the area under the curve (AUC) values of tail-flick latency-time curves were significantly higher solely in groups that received ARBs in combination with morphine and without PPARγ antagonist, as shown in Figure 10, compared to the control group.

4.2.4. Morphine-induced spinal microglial infiltration is decreased by telmisartan or losartan through PPARγ

Animals receiving chronic treatment as described in section 4.4.3. were sacrificed after 10 days and microglial infiltration in their spinal cords was analysed by immunohistochemistry. Chronic treatment with high-dose morphine, with or without PPARγ antagonism, induced microglial activation, seen as a significantly higher number of IBA1-positive cells/mm^2. Co-administration of losartan or telmisartan led to a recovery of the quantity of IBA1-positive cells, indicated by their number not exhibiting significant differences from that of the vehicle (Figure 11). Co-treatment with GW9662 produced a moderate, not significant increase in the number of microglial cells for losartan and a significant increase for telmisartan (Figure 11).

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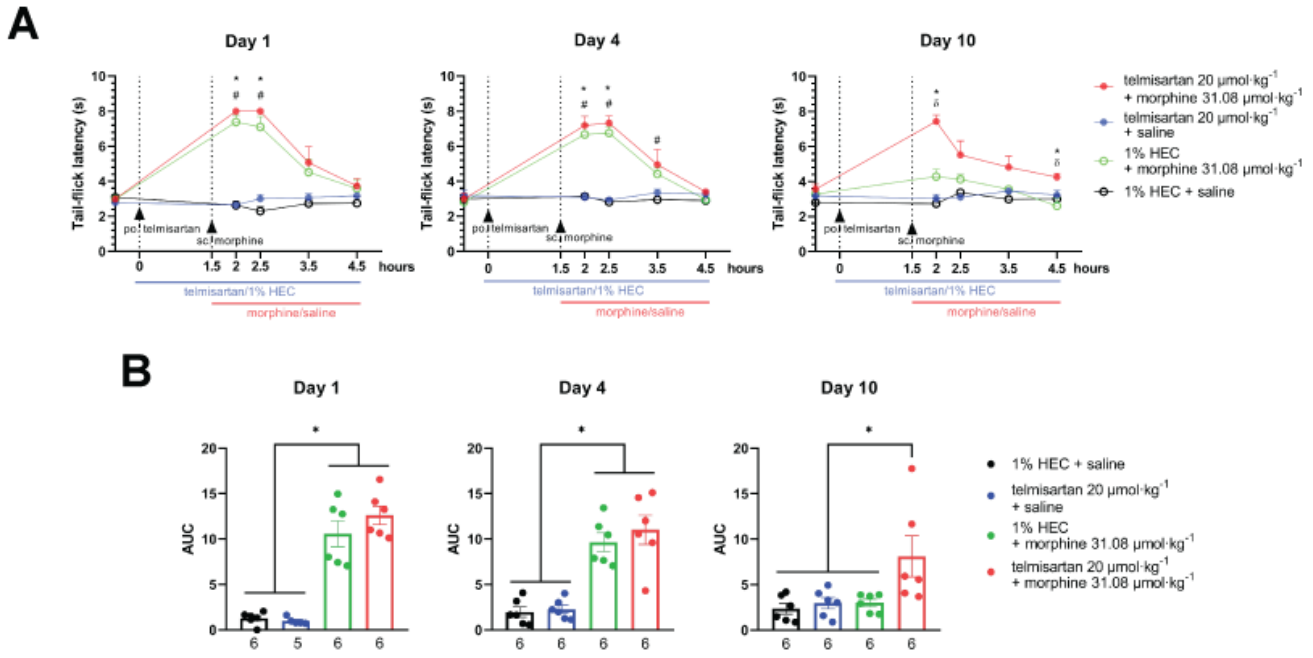

Figure 7. *The effect of morphine, telmisartan or their combination on the tail-flick latency of naïve rats (day 1) and after 4 or 10 days of chronic treatment.* Telmisartan or its vehicle, 1% HEC was administered orally at 0 min, while morphine or saline was administered subcutaneously at 90 min to ensure that the peak effect of the two compounds coincided in time. Data are presented as mean ± S.E.M.
Panel A: * $p < 0.05$ between telmisartan + morphine and 1% HEC + saline, $^{\#}$ $p < 0.05$ between 1% HEC + morphine and 1% HEC + saline, $^{\delta}$ $p < 0.05$ between telmisartan + morphine and 1% HEC + morphine, two-way ANOVA followed by Tukey's post hoc test, n = 6 per group. Day 1: F (time; 2.39, 47.79) = 30.56, $p < 0.0001$; F (treatment group; 3, 20) = 55.15, $p < 0.0001$. Day 4: F (time; 3.07, 61.34) = 34.88 $p < 0.0001$; F (treatment group; 3, 20) = 27.02, $p < 0.0001$. Day 10: F (time; 3.03, 60.63) = 9.79, $p < 0.0001$; F (treatment group; 3, 20) = 22.45, $p < 0.0001$.
Panel B: AUC values calculated individually for each animal of the elapsed time—tail-flick latency curves. For each animal, the tail-flick latency value prior to treatment administration was used as the baseline for calculating AUC values. * $p < 0.05$ between indicated groups, one-way ANOVA followed by Tukey's post hoc test, n = 5–6 per group; exact group sizes are shown in each graph. Day 1: F (3, 19) = 43.3, $p < 0.0001$. Day 4: F (3, 20) = 21.98, $p < 0.0001$. Day 10: F (3, 20) = 4.818, $p = 0.011$. ROUT analysis, with a Q value = 0.5% identified one outlier in the telmisartan + saline group on Day 1. [77]

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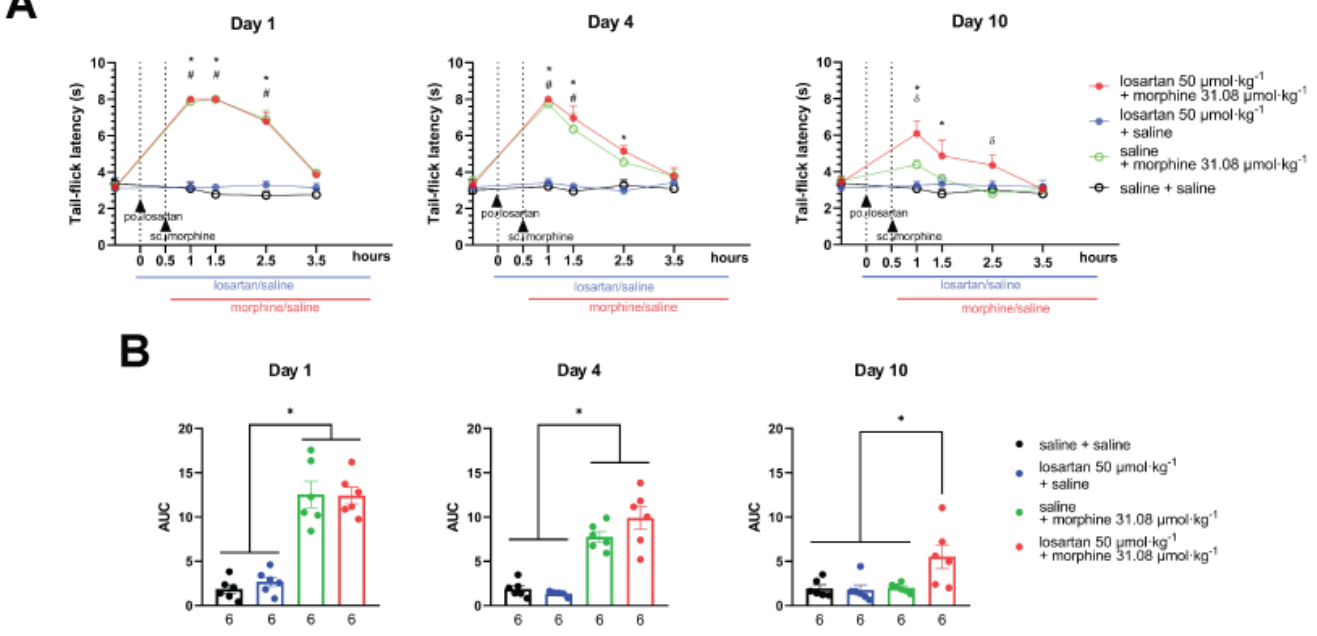

Figure 8. *The effect of morphine, losartan or their combination on the tail-flick latency of naïve rats (day 1) and after 4 or 10 days of chronic treatment.* Losartan or its vehicle, saline was administered orally at 0 min, while morphine or saline was administered subcutaneously at 30 min to ensure that the peak effect of the two compounds coincided in time. Data are presented as mean ± S.E.M.

Panel A: * $p < 0.05$ between losartan + morphine and saline + saline, $^{\#}$ $p < 0.05$ between saline + morphine and saline + saline, $^{\delta}$ $p < 0.05$ between losartan + morphine and saline + morphine, two-way ANOVA followed by Tukey's post hoc test, n = 6 per group. Day 1: F (time; 2.208, 44.16) = 80.64, $p < 0.0001$; F (treatment group; 3, 20) = 163.4, $p < 0.0001$. Day 4: F (time; 2.9, 58) = 38.09 $p < 0.0001$; F (treatment group; 3, 20) = 29.98, $p < 0.0001$. Day 10: F (time; 4, 80) = 9.822, $p < 0.0001$; F (treatment group; 3, 20) = 5.079, $p = 0.0089$.

Panel B: Area under the curve (AUC) values calculated individually for each animal of the elapsed time—tail-flick latency curves. For each animal, the tail-flick latency value prior to treatment administration was used as the baseline for calculating AUC values. * $p < 0.05$ between indicated groups, one-way ANOVA followed by Tukey's post hoc test, n = 5–6 per group; exact group sizes are shown in each graph. Day 1: F (3, 20) = 38.48, $p < 0.0001$. Day 4: F (3, 19) = 30.84, $p < 0.0001$. Day 10: F (3, 20) = 5.584, $p = 0.006$. ROUT analysis, with a Q value = 0.5% identified one outlier in the losartan + saline group on Day 4.

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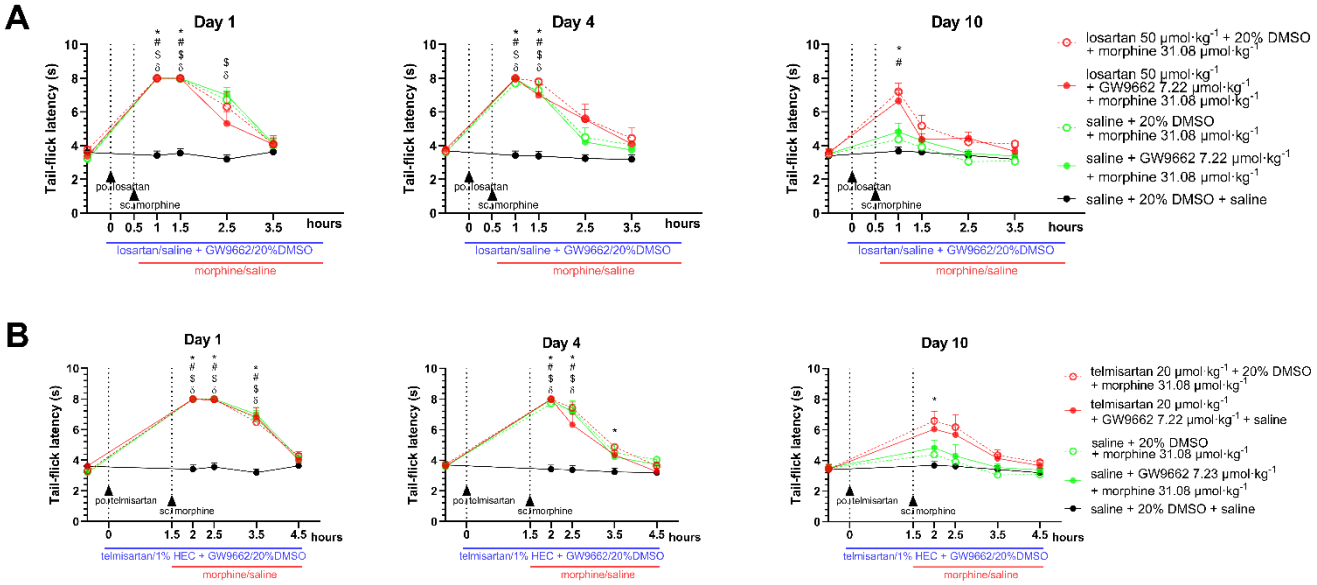

Figure 9. *The effect of morphine with or without GW9662 and losartan on the tail-flick latency of naïve rats (day 1) and after 4 or 10 days of chronic treatment (A). The effect of morphine with or without GW9662 and telmisartan on the tail-flick latency of naïve rats (day 1) and after 4 or 10 days of chronic treatment (B).* Losartan, telmisartan or their vehicle was administered orally at 0 min, GW9662 or its vehicle was administered intraperitoneally at 0 min, while morphine or its vehicle was administered subcutaneously at 30 min (panel A) or 90 min (panel B) to ensure that the peak effect of the two compounds coincided in time. Data are presented as mean ± S.E.M.

Panel A: * $p < 0.05$ between losartan + DMSO + morphine and control, $^{\#}$ $p < 0.05$ between losartan + GW9662 + morphine and control, $^{\$}$ $p < 0.05$ between saline + DMSO + morphine and control, $^{\delta}$ $p < 0.05$ between saline + GW9662 + morphine and control, two-way ANOVA followed by Tukey's post hoc test, n = 5 per group. Day 1: F (time; 1.831, 36.62) = 131.5, $p < 0.0001$; F (treatment group; 4, 20) = 42.99, $p < 0.0001$. Day 4: F (time; 2.738, 54.77) = 95.17 $p < 0.0001$; F (treatment group; 4, 20) = 26.17, $p < 0.0001$. Day 10: F (time; 3.112, 59.12) = 24.54, $p < 0.0001$; F (treatment group; 4, 19) = 8.976, $p = 0.0003$.

Panel B: * $p < 0.05$ between telmisartan + DMSO + morphine and control, $^{\#}$ $p < 0.05$ between telmisartan + GW9662 + morphine and control, $^{\$}$ $p < 0.05$ between saline + DMSO + morphine and control, $^{\delta}$ $p < 0.05$ between saline + GW9662 + morphine and control, two-way ANOVA followed by Tukey's post hoc test, n = 5 per group. Day 1: F (time; 1.986, 39.71) = 173.6, $p < 0.0001$; F (treatment group; 4, 20) = 53.34, $p < 0.0001$. Day 4: F (time; 2.395, 47.89) = 134.5 $p < 0.0001$; F (treatment group; 4, 20) = 31.06, $p < 0.0001$. Day 10: F (time; 2.68, 53.61) = 23.36, $p < 0.0001$; F (treatment group; 4, 20) = 5.652, $p = 0.0033$.

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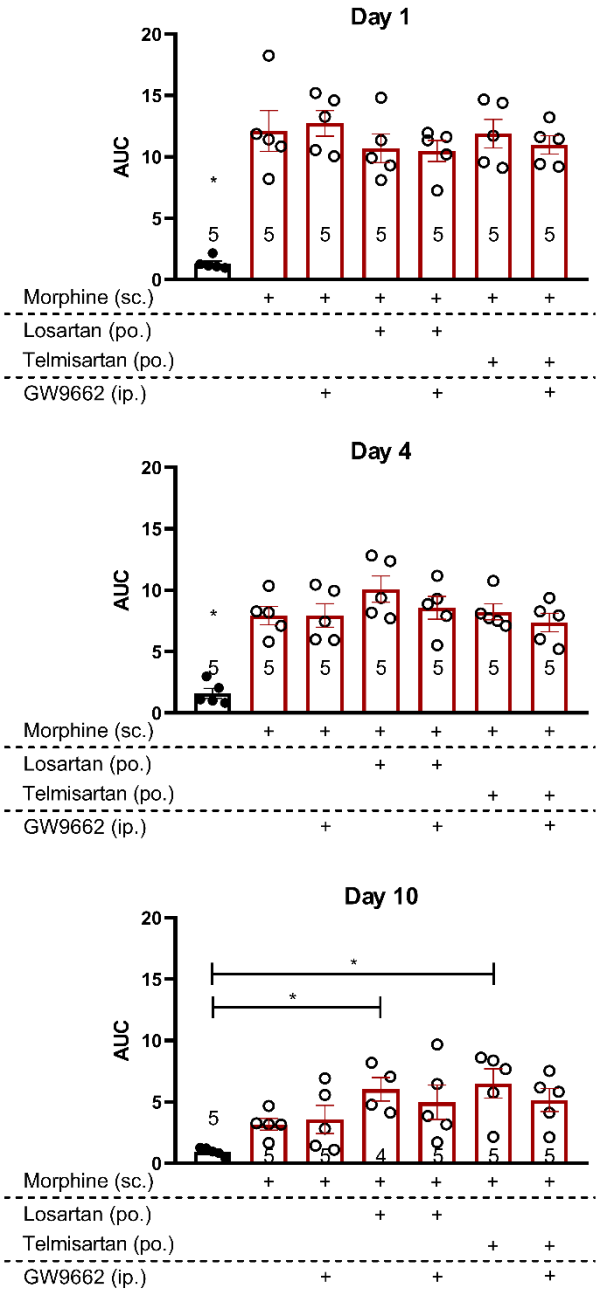

Figure 10. *Area under the curve values calculated from the tail-flick latency – time curves of morphine with or without GW9662, losartan or telmisartan on day 1, 4 and 10 of chronic treatment.* AUC values calculated individually for each animal from the elapsed time tail flick latency curves. For each animal, the tail-flick latency value prior to treatment administration was used as the baseline for calculating AUC values. Note that as some groups received treatment in a time-shifted manner (see above), time points were standardised across all groups prior to AUC calculation to ensure accurate comparison. Days 1 and 4: * $p < 0.05$ between vehicle and all other treatment groups. Day 10: * $p < 0.05$ between vehicle vs. losartan + morphine + DMSO and vehicle vs. telmisartan + morphine + DMSO, one-way ANOVA followed by Tukey's post hoc test, n = 4-5 per group; exact group sizes are shown in each graph. Day 1: $F(6, 28) = 13.71$, $p < 0.0001$. Day 4: $F(6, 28) = 10.98$, $p < 0.0001$. Day 10: $F(6, 27) = 3.785$, $p = 0.0073$.

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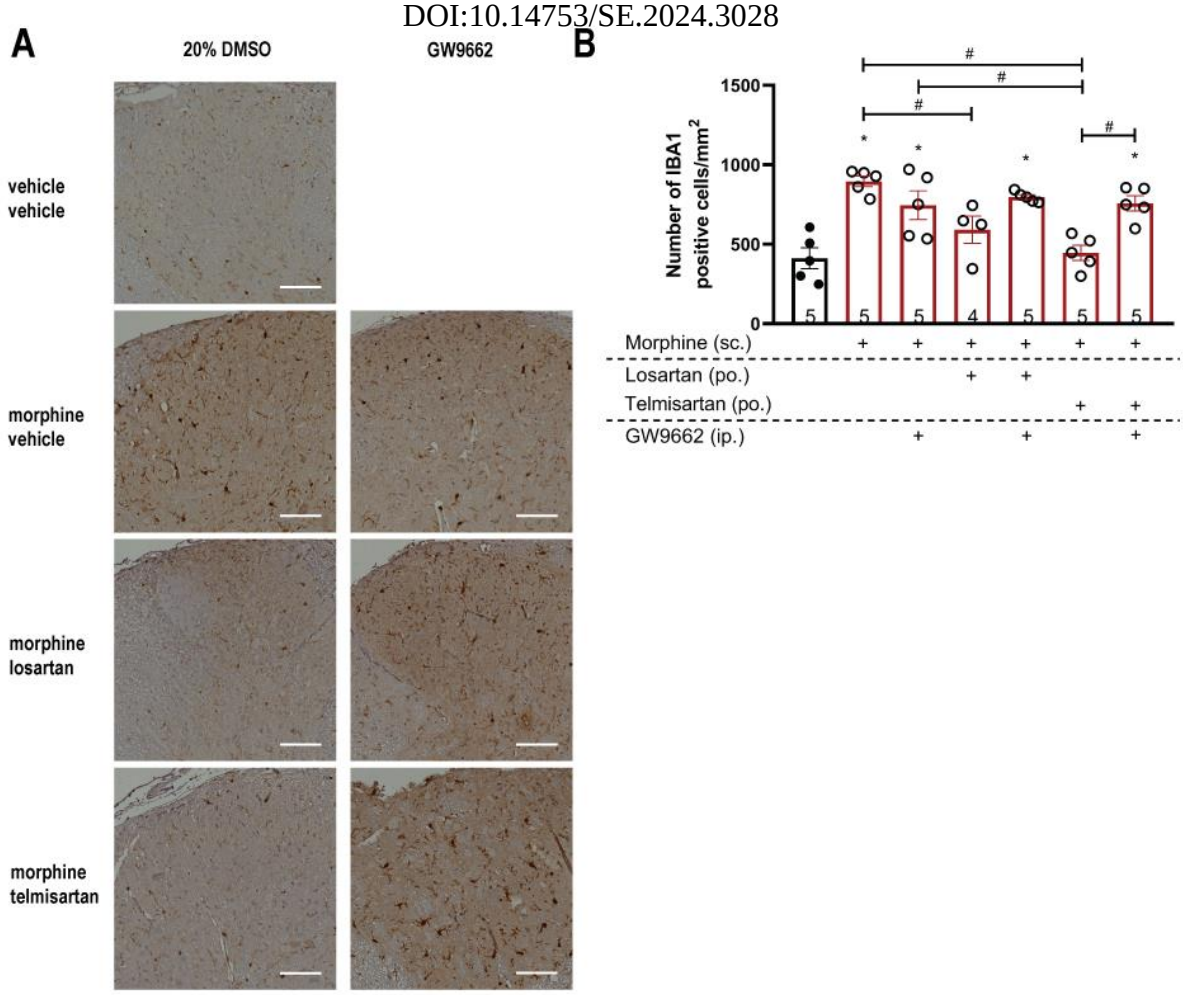

Figure 11. *Representative brightfield microscopy images of IBA1 protein immunohistochemistry in histological spinal cord samples obtained from rats treated for 10 days with the indicated treatment groups (A).* Images are showing the dorsal horns. Signals were developed with diaminobenzidine. Scale bar represents 50 µm. *Number of IBA1 positive cells/mm^2 detected in histological spinal cord samples obtained from rats treated for 10 days with the indicated treatment groups (B).* * $p < 0.05$ vs. vehicle, $^{\#}$ $p < 0.05$ between indicated groups, one-way ANOVA followed by Tukey's post hoc test, n = 4-5 per group; exact group sizes are shown in each graph. $F(6, 27) = 10.06$, $p < 0.0001$.

4.3. Telmisartan and morphine combination is devoid of causing motor dysfunction in rats

Telmisartan (80 µmol·kg^{-1} BW, po.), morphine (10 µmol·kg^{-1} BW, sc.), or their combination failed to exhibit motor dysfunction in rats (Figure 12). Morphine treatment (31.08 µmol·kg^{-1} BW, sc., as positive control) produced a significant disturbance in motor coordination, seen as shorter fall-off times (latency: 19.4 ± 5.67, n = 5 vs. 180 ± 0.00, n = 4).

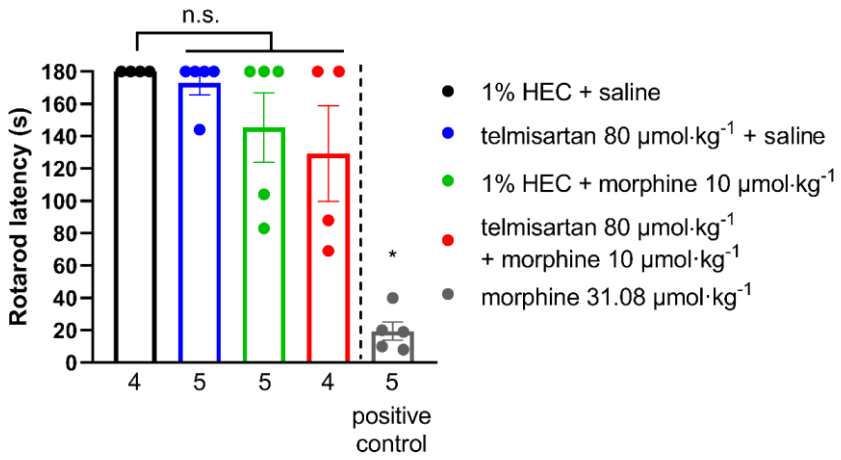

Figure 12. *Effect of systemic (oral or subcutaneous) administration of analgesic doses of telmisartan and its combination with morphine on the motor function of naïve animals.* Columns represent the time latency of animals in the RotaRod assay ± S.E.M. measured at peak effect of test compounds. Abbreviation "n.s." stands for not significant. * $p < 0.05$ vs. all other groups, one-way ANOVA followed by Fisher's LSD post hoc test, n = 4–5 per group; exact group sizes are shown in each graph. $F(4, 18) = 17.13$, $p < 0.0001$. [77]

4.4. OPRM1 mRNA Co-Localises with AGTR1A and AGTR2 mRNA at Key Points of Pain Transmission

RNA Scope® *In Situ* Hybridization assay was performed on DRG samples obtained from naïve rats to simultaneously determine cell-specific localisation of the mRNA of MORs (OPRM1), AT1 receptors (AGTR1A) and AT2 receptors (AGTR2).

4.4.1. Dorsal root ganglia

All three target mRNAs were found in the rat DRG (Figure 13, Figure 14). Certain neurons showed high levels of co-localisation of OPRM1 with AGTR1A (Figure 13, panel A), while the co-localisation between OPRM1 and AGTR2 was less pronounced (Figure 13, panel B). To further characterise DRG neurons with high levels of OPRM1/AGTR1A co-localisation, the peptidergic neuronal marker CGRP (CALCA) and the non-peptidergic/large neuronal marker vesicular glutamate transporter 1 (VGLUT1) [203,204] was used. In this set of experiments, OPRM1/AGTR1A double-positive neurons were also largely positive for CALCA, while OPRM1/AGTR1A/VGLUT1 triple-positive neurons were virtually absent (Figure 14).

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4.4.2. Spinal cord dorsal horn

Consistently with previous reports, AGTR1A and AGTR2 mRNAs were found ubiquitously in the rat spinal cord (see section 1.3.2.), while OPRM1 mRNA was primarily found in the superficial dorsal horn. Here, a moderate level of co-localisation of OPRM1 with both AGTR1A and AGTR2 was observed (Figure 15).

4.4.3. Periaqueductal grey

Similar to the DRG and spinal cord, target mRNAs were found in the periaqueductal grey matter of rats. Comparable to the DRGs, a higher level of co-localisation between OPRM1 and AGTR1a and a moderate level of co-localisation between OPRM1 and AGTR2 were observed (Figure 16).

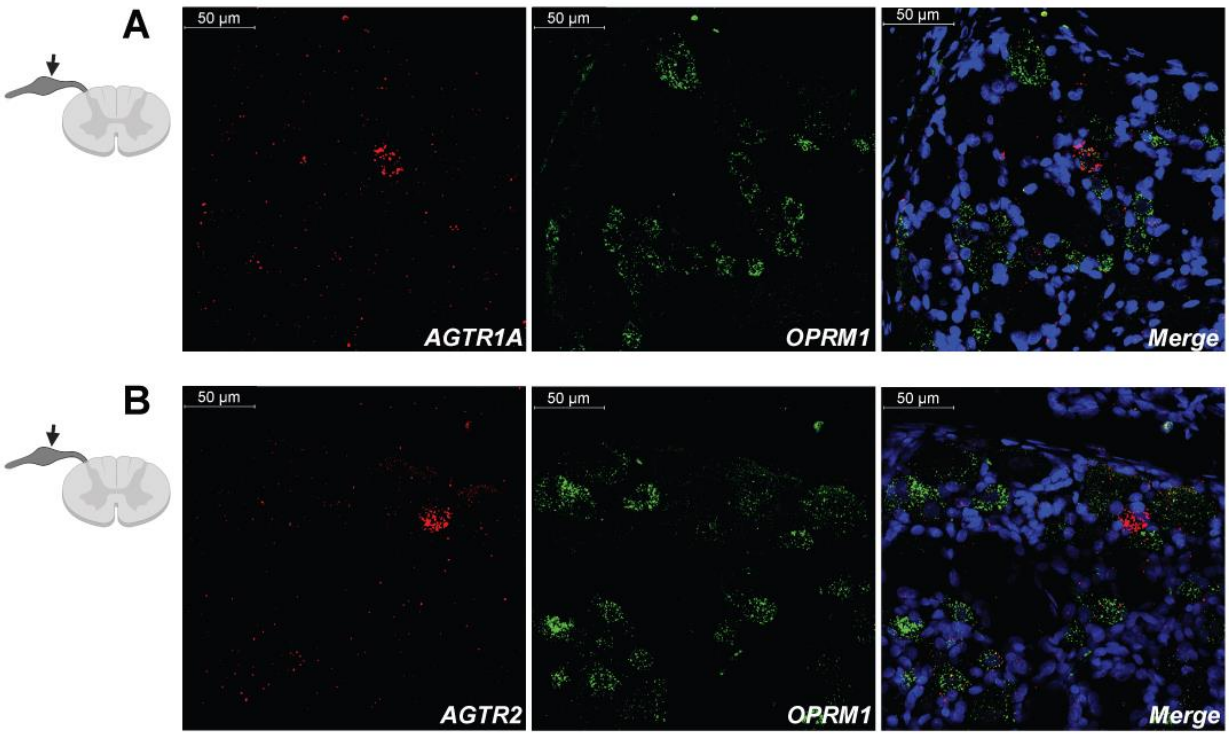

Figure 13. Representative confocal microscopy images of RNA Scope®-AGTR1A-OPRM1 (A) and AGTR2-OPRM1 (B) mRNA expression in histological samples of naïve rat DRGs. Nuclei were stained with DAPI (blue). Cyanine 3- (Cy3-) labelled tyramide (red) was used to visualise mRNA of AGTR1A or AGTR2 and fluorescein-labelled tyramide (green) was used to visualize mRNA of OPRM1 Scale bar represents 50 µm.

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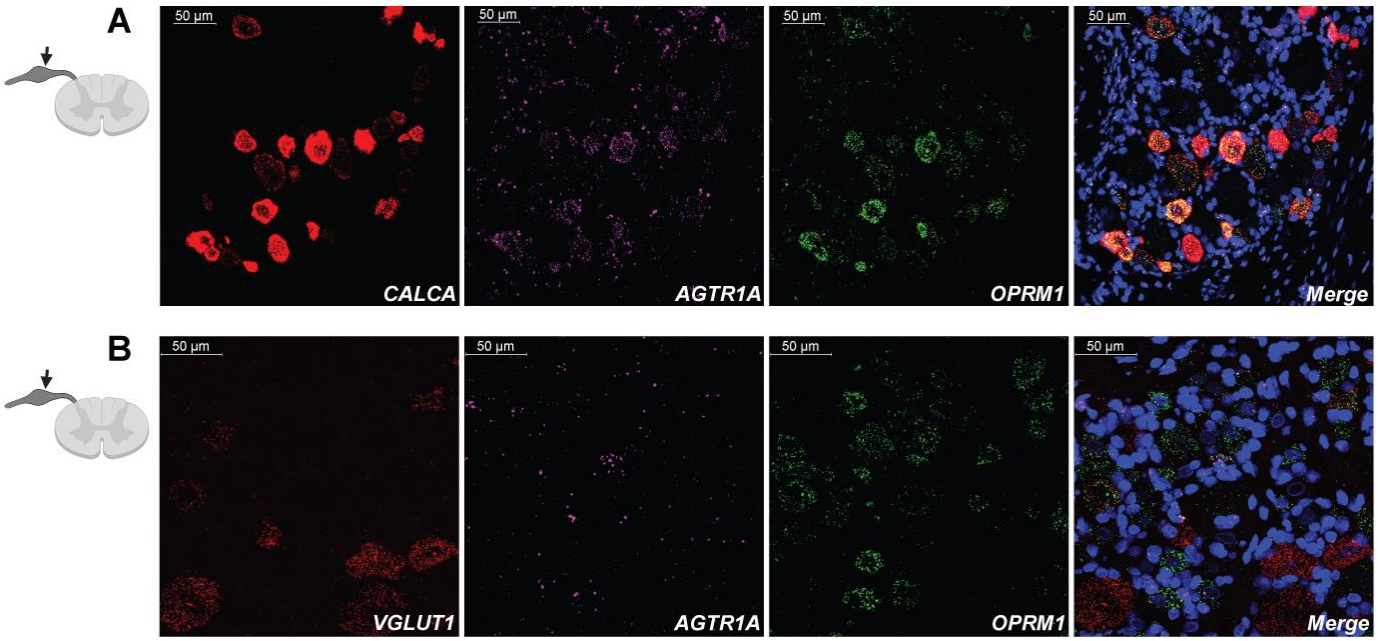

Figure 14. Representative confocal microscopy images of RNA Scope®-CALCA-AGTR1A-OPRM1 (A) and VGLUT1-AGTR1A-OPRM1 (B) mRNA expression in histological samples of naïve rat DRGs. Nuclei were stained with DAPI (blue). Cyanine 3- (Cy3-) labelled tyramide (red) was used to visualize mRNA of CALCA or VGLUT1, Cyanine 5- (Cy5-) labelled tyramide (magenta) was used to visualise AGTR1A and fluorescein-labelled tyramide (green) was used to visualise mRNA of OPRM1 Scale bar represents 50 µm.

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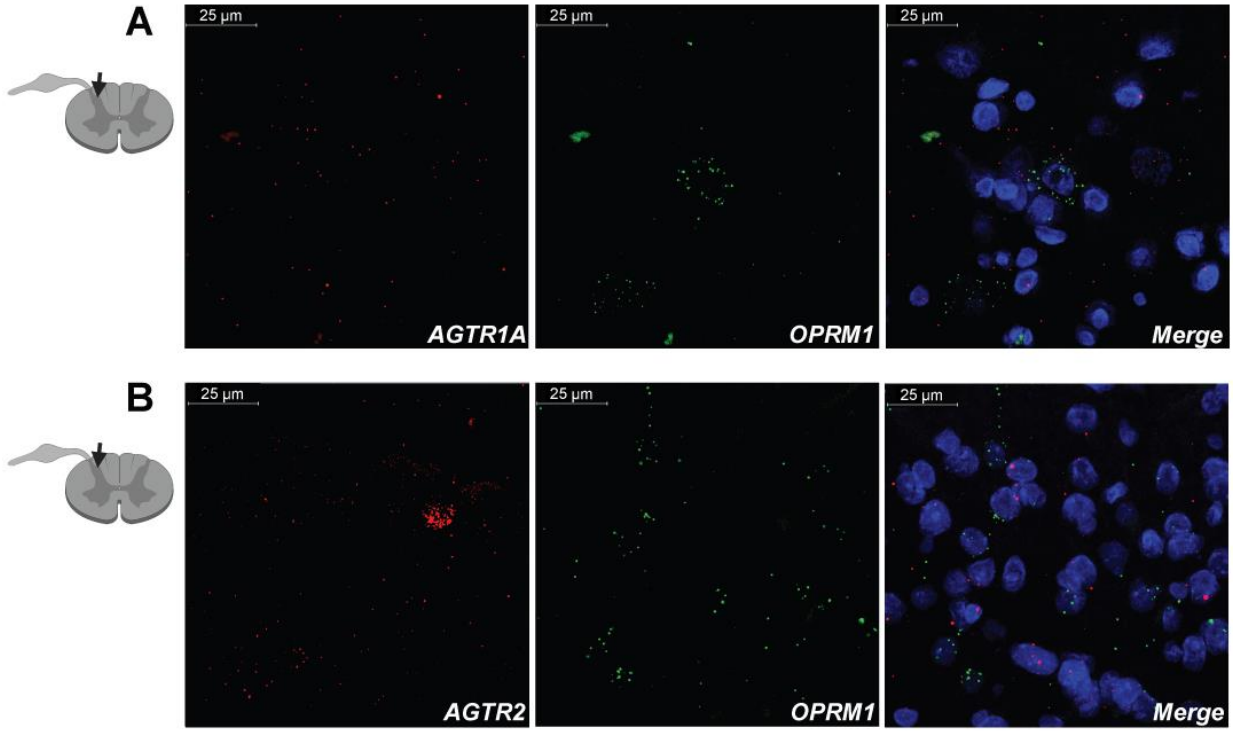

Figure 15. Representative confocal microscopy images of RNA Scope® -AGTR1A-OPRM1 (A) and AGTR2-OPRM1 (B) mRNA expression in histological samples of naïve rat spinal cords. Images are showing the superficial dorsal horn (Rexed laminae I-III). Nuclei were stained with DAPI (blue). Cyanine 3- (Cy3-) labelled tyramide (red) was used to visualise mRNA of AGTR1A or AGTR2, and fluorescein-labelled tyramide (green) was used to visualise mRNA of OPRM1 Scale bar represents 25 µm.

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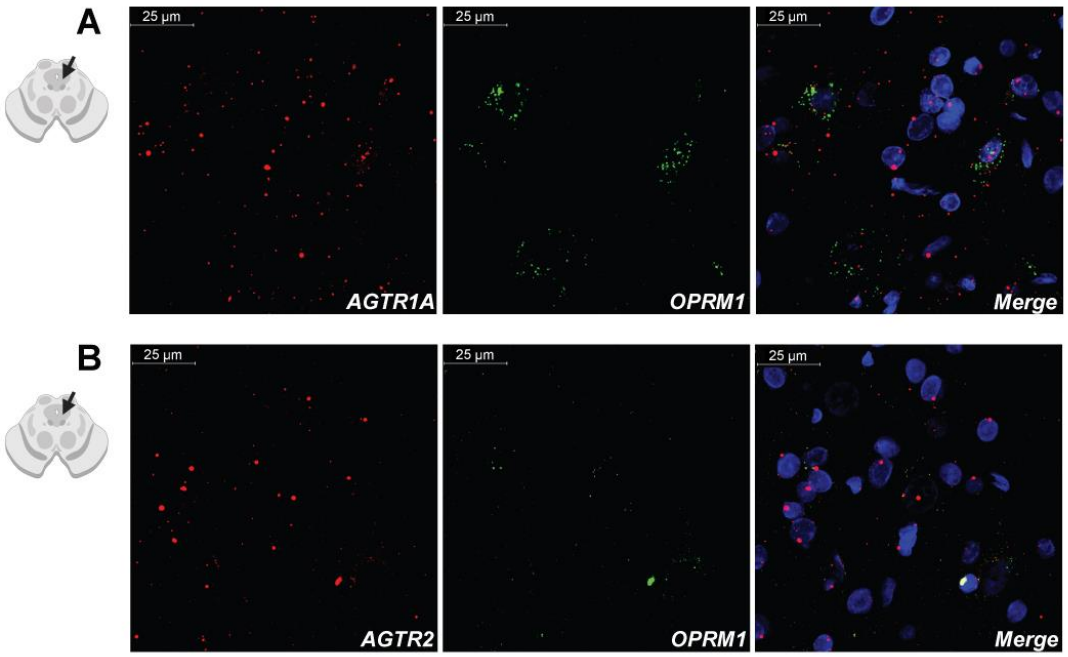

Figure 16. Representative confocal microscopy images of RNA Scope® -AGTR1A-OPRM1 (A) and AGTR2-OPRM1 (B) mRNA expression in histological samples of naïve rat brains. Images are showing the periaqueductal grey matter. Nuclei were stained with DAPI (blue). Cyanine 3- (Cy3-) labelled tyramide (red) was used to visualise mRNA of AGTR1A or AGTR2, and fluorescein-labelled tyramide (green) was used to visualise mRNA of OPRM1 Scale bar represents 25 µm.

4.5. Impact of AT1 receptor antagonists alone or in combination with morphine on CSF L-glutamate and D-serine content in neuropathic and opioid tolerant rats

Following both *in vivo* models described above, CSF samples collected from chronically treated animals were subjected to capillary electrophoresis in order to measure L-glutamate and D-serine content.

In either mononeuropathic or morphine-tolerant animals, only the combination of telmisartan and morphine was able to induce a significant increase in CSF L-glutamate levels compared to vehicle (L-glutamate (µM): 15.84 ± 3.01, n = 6 vs. 9.68 ± 1.09, n = 4; p = 0.04 or 11.11 ± 2.96, n = 5 vs. 5.58 ± 0.63, n = 6; p = 0.033 in the mononeuropathic or morphine tolerance models, respectively). The combination of losartan and morphine

or individual components of either combination failed to do so in either model (Figure 17).

In mononeuropathic animals, the combination of telmisartan and morphine significantly reduced CSF D-serine content when compared to vehicle-treated neuropathic animals (D-serine (μM): 1.23 ± 0.09, n = 5 vs. 1.99 ± 0.49, n = 4; p = 0.026). Similarly, in the morphine tolerance model, animals receiving telmisartan + morphine exhibited significantly lower CSF D-serine levels than those receiving vehicle or morphine alone (D-serine (μM): 1.21 ± 0.13, n = 4 vs. 2.09 ± 0.13, n = 6; p = 0.005 or 1.21 ± 0.13, n = 4 vs. 2.00 ± 0.21, n = 5; p = 0.013, respectively) (Figure 18, panel A). The combination of losartan with morphine had no significant effect on D-serine levels in the neuropathy model, while animals receiving this combination in the morphine tolerance model had similar D-serine levels to those receiving morphine alone (Figure 18, panel B).

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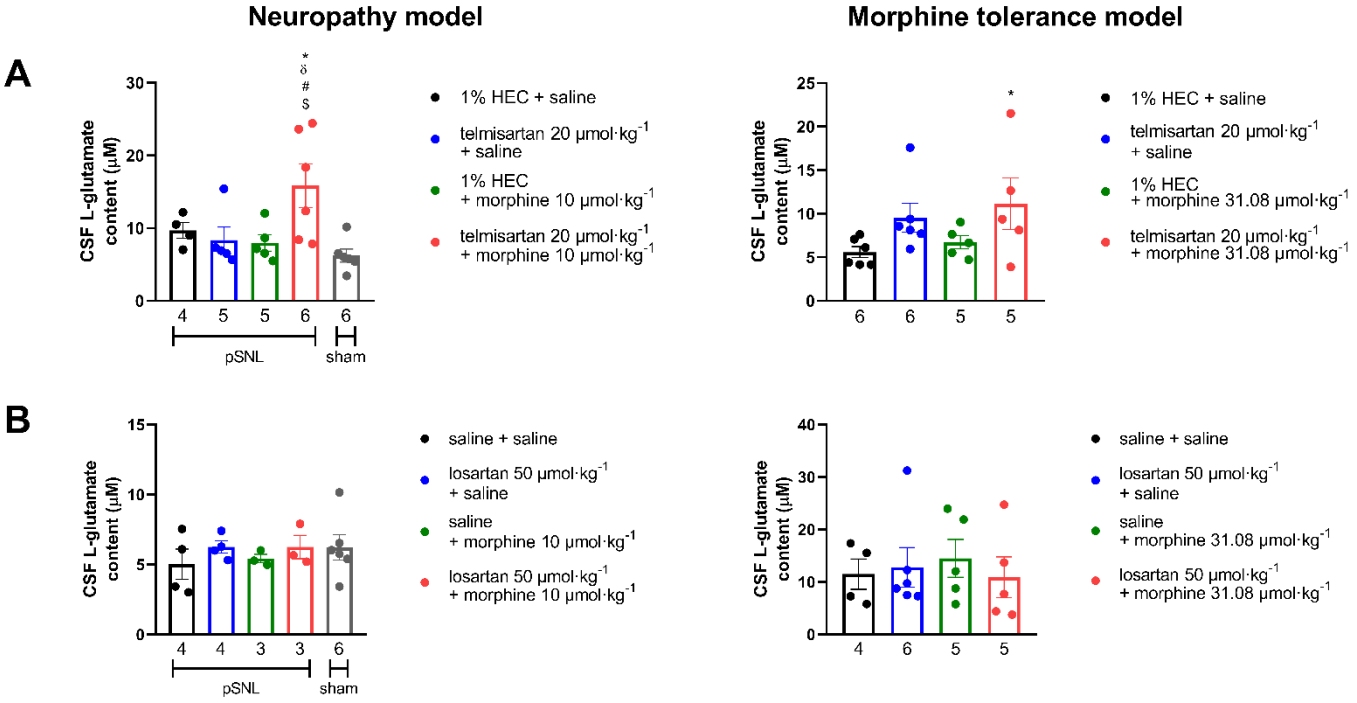

Figure 17. *L-glutamate content in the CSF of animals that underwent chronic treatment with orally administered telmisartan or 1% HEC plus subcutaneously administered morphine or saline (A) or orally administered losartan or saline plus subcutaneously administered morphine or saline (B) in the mononeuropathic pain model or the morphine analgesic tolerance model.* Panel A; "Neuropathy model": * $p < 0.05$ vs. 1% HEC + saline, $^{\#}$ $p < 0.05$ vs. telmisartan + saline, $^{\delta}$ $p < 0.05$ vs. 1% HEC + morphine, $^{\$}$ $p < 0.05$ vs. sham, one-way ANOVA followed by Fisher's LSD post hoc test, n = 4-6 per group; exact group sizes are shown in each graph. $F(4, 21) = 4.238$, $p = 0.0114$. Panel A; "Morphine tolerance model": * $p < 0.05$ vs. 1% HEC + saline, one-way ANOVA followed by Fisher's LSD post hoc test, n = 5-6 per group; exact group sizes are shown in each graph. $F(3, 18) = 2.23$, $p = 0.1198$. Panel B: one-way ANOVA followed by Fisher's LSD post hoc test, n = 3-6 per group; exact group sizes are shown in each graph. [77]

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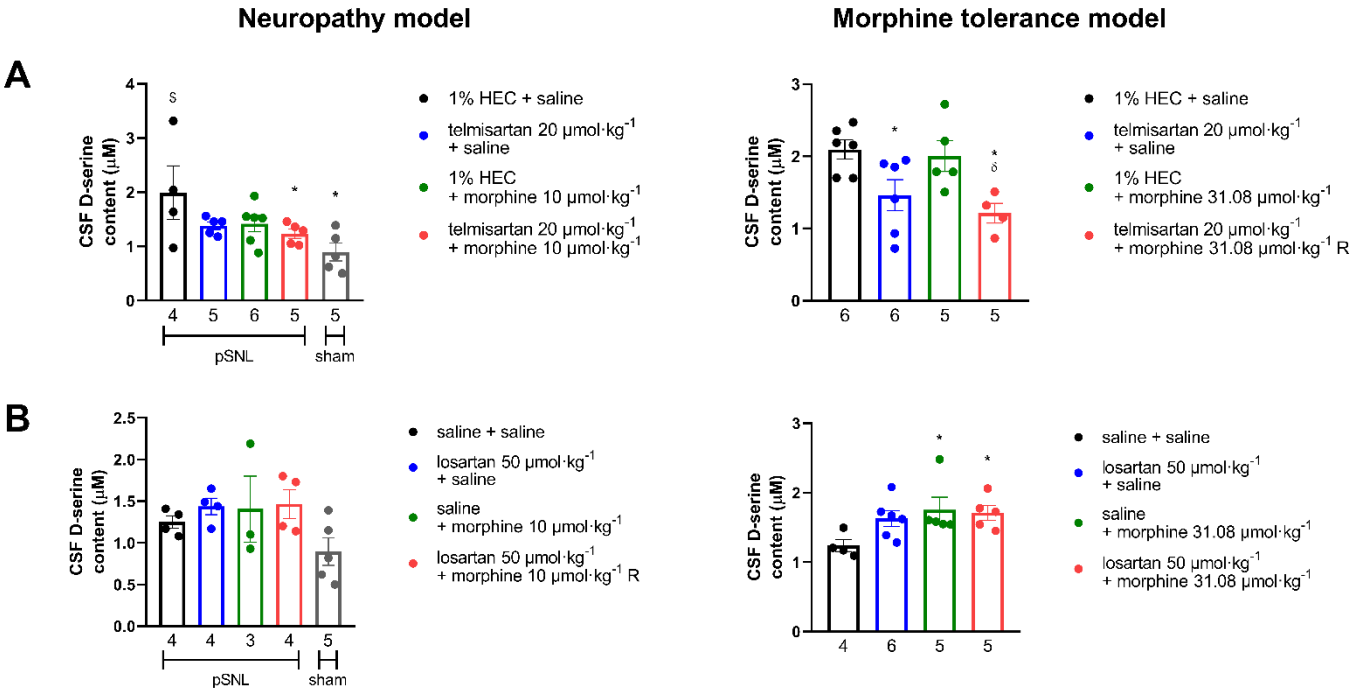

Figure 18. *D-serine content in the CSF of animals that underwent chronic treatment with orally administered telmisartan or 1% HEC plus subcutaneously administered morphine or saline (A) or orally administered losartan or saline plus subcutaneously administered morphine or saline (B) in the mononeuropathic pain model or the morphine analgesic tolerance model.* Panel A; "Neuropathy model": * $p < 0.05$ vs. 1% HEC + saline, $^{\$}$ $p < 0.05$ vs. sham, one-way ANOVA followed by Fisher's LSD post hoc test, n = 4–6 per group; exact group sizes are shown in each graph. $F(4, 20) = 3.142$, $p = 0.0371$. Panel A; "Morphine tolerance model": * $p < 0.05$ vs. 1% HEC + saline, $^{\delta}$ $p < 0.05$ vs. 1% HEC + morphine, one-way ANOVA followed by Fisher's LSD post hoc test, n = 5–6 per group; exact group sizes are shown in each graph. $F(3, 17) = 4.946$, $p = 0.012$. Panel B: "Neuropathy model": one-way ANOVA followed by Fisher's LSD post hoc test, n = 3–5 per group; exact group sizes are shown in each graph. Panel B; "Morphine tolerance model": * $p < 0.05$ vs. saline + saline, one-way ANOVA followed by Fisher's LSD post hoc test, n = 4–6 per group; exact group sizes are shown in each graph. $F(3, 16) = 2.704$, $p = 0.0801$. [77]

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5. DISCUSSION

Current pharmacological treatment options, including opioids and non-opioid analgesics, supplemented by regional anaesthesia and adjunctive agents provide satisfactory relief for most acute pain. However, in chronic pain entities, in particular NP, adequate pain control is not satisfactory even with current treatment options, such as tricyclic antidepressants (TCAs), serotonin and noradrenaline reuptake inhibitors (SNRIs), gabapentinoids, local anaesthetic patches, capsaicin or opioid analgesics. Indeed, the drawback of these medications is not only limited to the efficacy but also a slow onset of action and a progressively worsening side-effect profile with dose escalation. In recent decades, several new pathways and pharmacological targets have been identified in the hope of achieving adequate pain control and decreasing unwanted effects. Repurposing existing medications or exploiting multi-target treatment options or combination treatment regimens that improve the efficacy without worsening the side effects of available analgesic agents has also gained great attention. Emerging as an important novel area of pain research, antagonists of the central RAS, including AT1 receptor antagonists have been suggested to have a promising effect in chronic pain entities, such as NP. In this regard, the present work provides preclinical studies and background for the implication of the AT1 receptor in NP and possible mechanisms involved in the antiallodynic effects of AT1 receptor antagonists. This work also shows that certain AT1 receptor antagonists are able to produce a remarkable antiallodynic effect and delay opioid analgesic tolerance once combined with morphine.

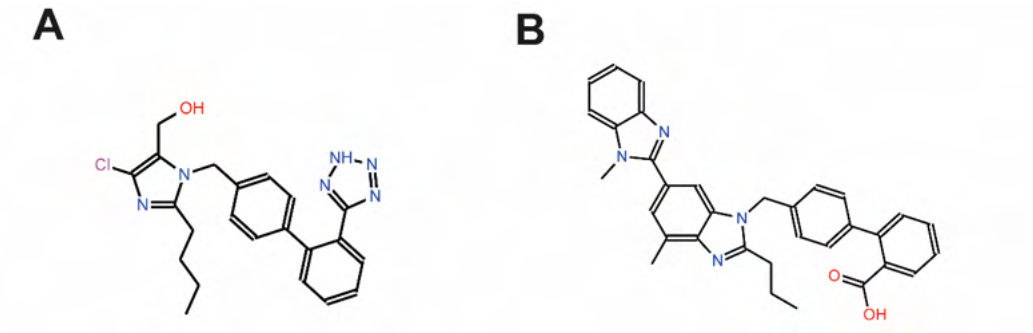

Figure 19. *Molecular structures of losartan (A) and telmisartan (B).*

We first examined the antiallodynic effect of two ARB compounds, losartan and telmisartan alone following acute oral administration. We have chosen both compounds

to be well-known, clinically utilised, inexpensive drugs with tolerable side-effect profiles, making them ideal for potential repurposing endeavours. The molecular structures of the selected ARBs are depicted in Figure 19.

Both inhibitors proved to be effective against allodynia evoked by sciatic nerve ligation (Figure 4, panels A and B). These results are mostly in accordance with previous literature [69,108,116,132], though contradictory results have also been reported on the effect of losartan on NP evoked by sciatic nerve constriction [65]. Indeed, in this latter work by Costa et al., the route of administration for losartan was intraplanar, and a small dose was applied. Furthermore, earlier studies have highlighted the antiallodynic effect of AT1 receptor antagonists in models of polyneuropathic pain (diabetes-induced [69,116] or chemotherapy-induced [108,132]) or peripheral mononeuropathy induced by CCI [82,205]. In the work by Jaggi and Singh, despite the similarity in the route of administration, telmisartan failed to produce an antiallodynic effect following acute administration however after chronic oral treatment in doses of 2 or 5 $mg \cdot kg^{-1}$ it proved to be effective [82]. The applied dose was smaller than that applied in our study, and the magnitude of mechanical allodynia was measured by pinprick test [82]. Applying the same assay, in another study, both losartan and telmisartan were tested for their antiallodynic effect. The authors concluded that both drugs could only produce antiallodynic effect after chronic treatment. Their protocol differs in the applied dose, mononeuropathic pain model (CCI vs. pSNL) and the initiation of treatment course from the present work (starting treatment on the day of surgery vs. starting treatment when symptoms of NP have developed) therefore it is difficult to draw any definitive conclusions in term of similarity or dissimilarity with our results. In fact, our data, to the best of our knowledge, have shown for the first time, that both losartan and telmisartan are able to ameliorate mechanical allodynia, a hallmark symptom of NP evoked by pSNL after a single oral dose. It is noteworthy that the effective dose in our experimental set-up was almost double that used in previous studies. When comparing rodent models of mononeuropathic pain, both CCI and pSNL evoke behavioural responses with a similar time curve; however, the magnitude of mechanical allodynia induced by pSNL is greater [206]. Thus, the difference in effective dosage range of the test compounds can be attributed to the type of surgical methods used to elicit NP. Two notable differences were found between the effects of the two drugs tested. First, losartan had a peak effect at 1

hour, whereas telmisartan had a peak effect at 2 hours. On the other hand, both drugs exhibited a ceiling at the peak effect demonstrated by the higher doses tested: 100 or 150 μmol·kg^{-1} BW for losartan and 40 or 80 μmol·kg^{-1} BW for telmisartan. Even though the effects of the intermediate and high doses of losartan were similar, the intermediate dose produced a statistically significant antiallodynic effect, while the high dose was moderately but not significantly effective. These results suggest that the ceiling effect in the action of losartan at mid-higher doses is more obvious. On the other hand, the mechanical pain threshold of the left (unoperated) paws of the animals remained unchanged following acute treatment with losartan, telmisartan or their vehicles, indicating that the observed effect is of antiallodynic character rather than antinociceptive (Figure 20).

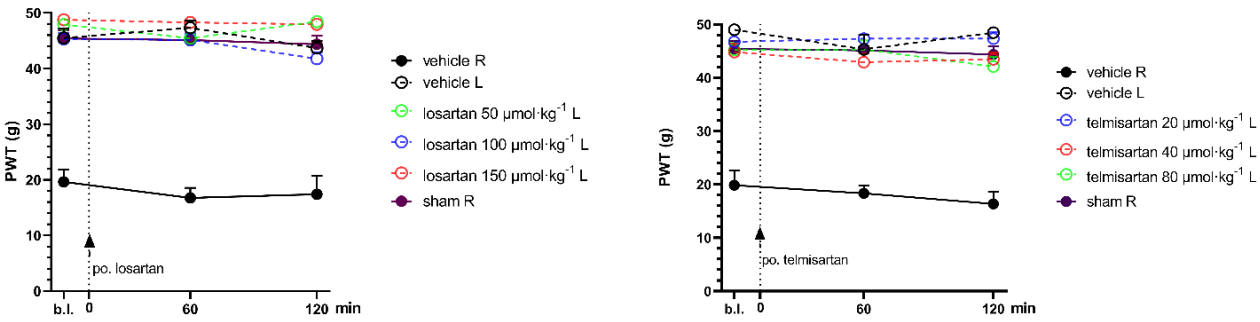

Figure 20. *Mechanical pain threshold of the intact (left, L) hind paws of animals orally treated with losartan or telmisartan or their vehicles in the acute experiments.* Graphs show the means of PWT ± S.E.M. in grams, in the indicated time points, 2 weeks after pSNL.

One of the outstanding results in the present study is that combining subanalgesic doses of telmisartan and morphine showed a remarkable significant antiallodynic effect following chronic treatment, though a trend effect following acute administration was also measured in a model of NP (Figure 5). The efficacy of opioid analgesics in NP is controversial in the literature, and several innovative strategies for improvement have been proposed. Thus, we paid attention to the possibility of improving the analgesic efficacy of morphine in NP by combining it with AT1 receptor antagonists. In addition, we were eager to know whether this combination could open an avenue in hindering opioid analgesic tolerance, ultimately resulting in opioid dose-sparing treatment strategies. To assess this, experiments were initially carried out to ascertain the subanalgesic doses for both tested ARBs. The ineffective dose of telmisartan or losartan

observed in the previous series of experiments was chosen for the combination studies. On the other hand, ineffective morphine dose was also determined under the same experimental conditions. Herein, our results revealed that systemic morphine only at a high dose was able to alleviate mechanical allodynia evoked by pSNL following acute administration. However, at this dose (20 μmol·kg^{-1} BW), the effect of morphine was not restricted to ameliorate allodynia but also caused an impact on the intact paw, indicated by the average PWT of the unoperated paws matching the cut-off value of 50 g (Figure 4, panel C). Indeed, previous important findings are that the number of functional MORs is decreased under neuropathic conditions [32,35] and that acute analgesia is only achievable with large systemic doses of MOR agonists with high intrinsic efficacy [32]. In addition, considering the side effect profile of morphine, this dose (20 μmol·kg^{-1} BW) is known to induce central side effects, such as motor coordination disturbances [207], as described by our group and others.

A further important result of this work is that instead of observing a decrease in the effectiveness of morphine over time, which has been reported in numerous works, in this arrangement, we can report an increase in analgesic effect upon repeated administration. We hypothesise that this effect may be related to the pharmacodynamic profile and possible dynamic interactions between the test compounds without disclosing their pharmacokinetic profiles. Therefore, we have extended our work to examine the activation of opioid receptors by morphine in the spinal cords of neuropathic animals under these conditions. In these experiments, the intrinsic efficacy of morphine (E_{max}) remained unchanged. However, 10 days of treatment with only morphine led to a significant loss of morphine potency (EC_{50}), as demonstrated by a significant rightward shift in the morphine concentration-response curve (Figure 6) in comparison to the control curve. However, when telmisartan was co-administered, morphine potency was retained, as evident from the morphine concentration-response curve in the group treated with the combination, which did not differ from that of control (Figure 6). The seen loss of potency in the morphine-treated group may be explained by the development of analgesic tolerance which result is in line with previous research findings describing no change in opioid E_{max} and increased EC_{50} values in the spinal cord of morphine-tolerant mice [200]. The decreased development of analgesic tolerance to morphine upon co-administration of a sub-analgesic dosage of telmisartan is a notable discovery in this present research.

In the next section of experiments, we further tested whether this effective combination is devoid of side effects related to motor functions. Neither morphine in subanalgesic dose (10 μmol·kg^{-1} BW) nor telmisartan (80 μmol·kg^{-1} BW) plus morphine (10 μmol·kg^{-1} BW) affected the rats' motor function, indicating that the side effect profile of the combination is desirable compared to those seen following higher doses of morphine. As described earlier, a dose of morphine that produced significant antinociception in our experimental arrangement (20 μmol·kg^{-1} BW) has been linked to impairments in motor coordination [207].

To explain the antiallodynic effect of the combination, we extended our study to examine the coexistence of receptors that mediate the observed effect, namely the AT1 receptor and MOR. As discussed in Section 1, the neuroanatomical distribution patterns of AT1 receptors and MORs have been thoroughly investigated and discussed in numerous publications in the past. However, to the best of our knowledge, no study has investigated the co-expression of these structures at the mRNA level in cells that play a crucial role in the transmission and modulation of nociceptive information. In this regard, spinal cord dorsal horns and PAGs of naïve rats were chosen as the most relevant areas to our research question. Applying RNAScope® in-situ RNA hybridisation assay, our results have proven the cellular co-expression of AT1 receptors and MORs in the examined regions (Figures 8-11.). This further supports our findings that there may be cooperation between these receptors at the spinal level and/or supraspinal sites. Next, to judge the source of MORs, we have also examined the DRG, since opioid receptors are mostly synthesised there and transported either to the central terminal of primary afferents or to the periphery, as described previously [208,209]. Indeed, we have identified the highest level of co-expression in the DRGs; further analysis was conducted on neurons exhibiting this trait. This was achieved through the co-detection of mRNA of CGRP (CALCA) and VGLUT1, both of which are important neuronal markers. CALCA/CGRP is a marker of small peptidergic neurons, which are attributed important roles in pain transmission and facilitation of hyperalgesia under pathological states [8,9,11,14,16]. VGLUT1, on the other hand, is mainly localised on large neurons, and it has been shown not to co-localise with CGRP; thus, we used it as a large/non-peptidergic neuronal marker [203]. In this set of experiments, AT1 receptor and MOR-positive neurons in the DRGs were largely positive for CGRP and negative for VGLUT1. As a limitation of our study,

the experimental methodology used here detects these structures at the mRNA level, and thus, future studies are required to validate these results with protein expressional data. In addition, we cannot exclude the interactions between the AT1 receptors and MORs in the periphery, namely the peripheral terminals of primary afferents or immune cells. Future studies are needed to judge whether a peripheral analgesic effect on these sites is producible.

Taken together, our experiments with acute or chronic administration of the tested ARBs show notable differences between them. These differences may be explained by discrepancies in the pharmacokinetic properties of the two agents. Due to its high lipophilicity and ability to penetrate the CNS, telmisartan is considered to be a drug of particular interest among the AT1 receptor antagonists in neurological diseases [210–212]. In a study performed by Michel et al. investigating the properties of different ARBs, telmisartan was found to have a much larger volume of distribution than losartan or its active metabolite, losartan-carboxylic acid (EXP-3174), indicating a greater tissue distribution [213] but, interestingly shows no evidence of CNS penetration by either of the agents. On the other hand, Konno et al. provided evidence of AT1 receptorial effects of telmisartan in the rostral-venterolateral medulla following oral administration in rats, which indicates CNS penetration of the compound [214]. Furthermore, the results of Wang et al. showed that the highly lipophilic AT1 receptor antagonist embusartan, but not losartan, is able to induce a significant CNS effect. [215]. More recently, Kakuta et al. found that telmisartan demonstrated the highest lipophilicity and superior cell penetration capability when compared to other tested ARB compounds [216]. On the other hand, the differences in the effects of telmisartan and losartan may be due to the different pharmacodynamic profiles of the drugs. Telmisartan has an off-target effect in functioning as a high-affinity PPARγ agonist alongside its role as an AT1 receptor antagonist [216]. This dual mechanism of action is a unique feature of this compound, with some studies attributing potent neuroprotective effects to it [217,218]. As mentioned in section 1.3.3. and depicted in Figure 1., PPARγ agonists have been shown to have beneficial effects in NP or opioid analgesic tolerance, explained by their ability to suppress proinflammatory cytokine production, inhibit fractalkine receptors and reduce spinal microglia activation [191,192,219–222]. To date, no study has attributed a similar direct PPARγ-activating effect to losartan or its main active metabolite, EXP-3174

(losartan carboxylic acid). However, a minor metabolite of losartan, EXP-3179, has been reported to have several independent off-target effects, namely blockade of cyclooxygenase 2 (COX-2), phosphorylation of NOS and partial activation of PPARγ [223,224]. This metabolite is formed at low levels and has been shown to be capable of inducing PPARγ activation, the magnitude of which is 51% that of pioglitazone [223]. Whilst the kinetic properties of EXP-3179 have not yet been fully elucidated, Rossi has reported that the plasma levels necessary to produce relevant PPARγ agonism are only transient and are reached briefly following systemic administration [224].

As mentioned above, despite the fact that long-term treatment with MOR agonists results in the development of tolerance, in our experimental setting, this was not the case when low-dose morphine was combined with telmisartan. Therefore, we have assessed the impact of ARBs on the development of opioid analgesic tolerance in another *in vivo* setting. To do this, we have utilised a thermal pain model, the rat tail-flick test, which is a widely accepted assay to monitor opioid antinociceptive activity and efficacy [225,226]. In our experimental setting, as a salient finding, both losartan and telmisartan were able to significantly delay the development of morphine analgesic tolerance (Figures 12 and 13). Previous results support that PPARγ agonists are able to delay the development of morphine tolerance in rodents [222]. Importantly, in this work, both test compounds produced similar results, challenging the hypothesis that the effect of telmisartan solely relies on PPARγ activation. On the other hand, it must also be noted that the losartan metabolite EXP-3179 may also induce PPARγ activation, as discussed earlier. As described in section 1.3.3., PPARγ is abundantly expressed in the spinal cord by neurons and glial cells. A recent study conducted on microglial cell culture implicates that the inhibitory impact of candesartan, an AT1 receptor antagonist, on microglial activation depends on PPARγ-activation [139]. This leads to reduced morphine-induced inflammatory response and thus may be beneficial in opioid tolerance [139]. Accordingly, further experiments were conducted in order to determine the extent to which PPARγ activation contributes to the remarkable morphine tolerance-delaying effect of our test compounds.

In this set of experiments, animals were treated with GW9662, a selective PPARγ antagonist in combination with losartan or telmisartan, as described in section 3.3.2.2. Morphine retained its analgesic effects when combined with losartan, telmisartan or

losartan + GW9662. When it was combined with telmisartan + GW9662, on day 10, morphine caused a moderate but not significant tail-flick latency increase at any time-point compared to control (Figure 13). The AUC values of tail-flick latency-time curves on day 10 were significantly higher than control in the cohorts that were administered ARBs without GW9662 in conjunction with morphine (Figure 10). These results indicate that PPARγ activation contributed to the effect of our test compounds; the magnitude of this contribution is most likely greater in the case of telmisartan than in the case of losartan. This fact is consistent with the notion that PPARγ can be directly activated by only one of losartan's minor metabolites and not by losartan itself [223]. While in the case of telmisartan, the parent structure possesses PPARγ activating capabilities.

Reducing microglial activation is regarded as an important mechanism behind the beneficial effects of PPARγ agonists [192,227]. In this regard, activation and infiltration of spinal microglia have been shown to play an important role in morphine-induced analgesic tolerance [181,193,228,229]. These are consistent with our results regarding spinal microglial infiltration in animals receiving morphine, ARBs, GW9662 or vehicles for 10 days. Namely, chronic morphine treatment increased microglial infiltration, which was decreased by co-treatment with losartan or telmisartan. GW9662 was able to largely reverse the effect of ARBs on microglial cell number (Figure 11).

Taken together, we can hypothesise that PPARγ is considerably involved in the measured effects of losartan and telmisartan regarding delaying opioid analgesic tolerance. This involvement is largely connected to the impact of ARBs on microglial activation. However, AT1 activation or other currently unknown off-target effects of losartan and telmisartan may also be important in the mechanism behind their observed impact on morphine tolerance, since GW9662 was not able to completely abolish their effect. Importantly, it is also possible that the dose of GW9662 used in our experimental protocol was not sufficient to achieve complete inhibition of PPARγ thus future studies may be necessary to fully shed light on the molecular background of morphine-tolerance delaying effect of losartan and telmisartan.

The spinal glutamatergic system and its modulation, especially regarding NMDA receptors, have been described to be fundamentally involved in neuropathic pain [23,195] and opioid analgesic tolerance [167,169,170]. Several works have described the development of opioid analgesic tolerance to correlate with activation of spinal NMDA

receptors containing NR2B subunit [230–234]. It is a given fact that competitive and noncompetitive NR2A-2D containing NMDA receptor blockers such as MK801 or D-2-amino-5-phosphonopentanoate (D-AP5) delayed the development of opioid analgesic tolerance [168,232,233,235]. NR2A-2D containing NMDA receptors encompass a co-agonist binding site (for D-serine and glycine) beside the glutamate binding site. These receptors have been shown to be predominantly located in extra-synaptic positions whereas NR2A-containing NMDA receptors have a synaptic location. Furthermore, these receptor types have a unique feature related to relieving the Mg^{2+} blockade of the channel following simultaneous binding of glutamate and one co-agonist, either D-serine or glycine and coincident depolarisation of the neuron cell membrane.

Therefore, we have extended our experiments with an aim to assess changes in CSF L-glutamate and D-serine levels of neuropathic or morphine-tolerant rats receiving long-term treatment with the tested ARBs alone or in combination with morphine. The combination of telmisartan and morphine produced an increase in the L-glutamate and a decrease in D-serine levels in animals exhibiting improved analgesia or delayed tolerance to opioid analgesia. D-serine is an endogenous co-agonist of NR1 and NR2A-D subunit-containing NMDA receptors as mentioned above. Under neuropathic conditions, it is released from inflammatory astrocytes and mediates extra-synaptic NMDA receptor activation, leading to the development of mechanical allodynia [236–238]. In addition, decreasing elevated D-serine levels in opioid-tolerant animals alleviates the impaired analgesic effect of morphine, as shown by Cao et al. [239]. Taking together, we can hypothesise that decreased D-serine levels in neuropathic or morphine-tolerant animals in our experimental setting led to reduced extra-synaptic, pathological NMDA receptor activation despite high L-glutamate levels. Nevertheless, the mechanism underlying the observed impact of our test compounds *in vivo* and their effect on CSF glutamate and serine content is most likely a complex process involving targets other than NMDA, opioid or angiotensin receptors and PPAR-γ, which targets may directly or indirectly influence neurodegeneration, physiological functions of the central nervous system and, ultimately, analgesia.

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6. CONCLUSIONS

1. The AT1 receptors are likely to participate in the development of neuropathic pain, as AT1 receptor antagonists, **losartan** or **telmisartan alleviate mechanical allodynia**, in **high systemic doses**, following **acute administration**.
2. The antiallodynic effect of **morphine is only achieved** in **high systemic doses**, not devoid of central side effects. This is in line with previous works.
3. Utilizing the co-operation between MOR and AT1 receptor could be a future strategy to treat neuropathic pain, as the combination of **morphine** and **telmisartan** in **subanalgesic doses** produces **significant antiallodynic effect** upon long-term administration.
4. **Chronic combination treatment** with antiallodynic capabilities was **devoid of analgesic tolerance**, the current obstacle to go on with long-term opioid treatment.
5. **Long-term** treatment with **high doses of morphine** results in the development of **opioid analgesic tolerance**, accompanied by the activation of spinal microglia. **Losartan or telmisartan rescues morphine analgesic efficacy** upon long-term treatment by restoring G_i mediated effect of morphine and inhibiting spinal microglial infiltration.
6. Beside blockade of AT1 receptors, activation of **PPARγ likely contributes to the effect of losartan and telmisartan**.
7. The co-operation between **AT1 receptors** and **MORs** is supported by their **colocalisation at key points** related to pain transmission (DRG, spinal cord dorsal horn and PAG). In the DRG, these structures also colocalise with peptidergic neuronal markers.
8. **Inhibition of NMDA receptor overactivation** by decreasing the levels of co-agonist D-serine, is likely to be involved in the beneficial effect of telmisartan.
9. Our results reveal **possible strategies of opioid dose tapering** through concomitant application of AT1 antagonists and opioid analgesics.

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7. SUMMARY

In the present work, we have shown for the first time that losartan and telmisartan, two ARBs with potential for drug-repurposing efforts are able to produce acute antiallodynic effects in animals with mononeuropathic pain evoked by pSNL. Moreover, the combination of subanalgesic doses of telmisartan and morphine produces significant antiallodynia upon long-term administration and prevents the loss of morphine potency at a spinal level. Indeed, as a salient finding of this work, both tested ARBs in subanalgesic doses are able to delay the development of analgesic tolerance in response to long-term, high-dose, systemic morphine treatment in rats.

Our findings may also be supported by the observation that important structures of the RAS and opioid system share features of neuroanatomical localisation at points of crucial role in pain transmission. Our results have directly shown for the first time that receptors of these systems indeed colocalise in the rat spinal cord dorsal horn and PAG. Moreover, in the DRG, which serves as the primary place of synthesis for peripheral and spinal MORs, AT1 and MOR highly co-exist with peptidergic neuronal markers, further supporting involvement in pain transmission.

The indirect activation of PPARγ by AT1 antagonists may contribute to the observed beneficial effects, particularly regarding opioid analgesic tolerance. Complex underlying mechanisms ultimately lead to decreased microglial infiltration of the spinal cord following long-term morphine treatment. A decrease in the concentration of spinal D-serine, a co-agonist of NR2A-D subunit-containing NMDA receptors was also measured in the present work. The prerequisite for the activation as well as overactivation of these receptors is the presence of one co-agonist (glycine or D-serine) and glutamate. This mechanism may also be involved in addition to blockade of AT1 receptors, however future studies are needed to elucidate this issue. Indeed, the involvement of NMDA receptor overactivation in NP or opioid analgesic tolerance is beyond doubt.

Taken together, treatment with telmisartan or losartan may be beneficial in conditions with impaired opioid effect, such as NP or opioid analgesic tolerance. This work may provide the preclinical basis for utilising the combination of AT1 receptor antagonists with MOR analgesics to restore opioid efficacy and reduce the need for opioid dose escalation.

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9.1. The publications of the candidate involved in the current thesis

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9.2. The publications of the candidate not involved in the current thesis

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Review

Shedding Light on the Pharmacological Interactions between μ-Opioid Analgesics and Angiotensin Receptor Modulators: A New Option for Treating Chronic Pain

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Abstract: The current protocols for neuropathic pain management include μ-opioid receptor (MOR) analgesics alongside other drugs; however, there is debate on the effectiveness of opioids. Nevertheless, dose escalation is required to maintain their analgesia, which, in turn, contributes to a further increase in opioid side effects. Finding novel approaches to effectively control chronic pain, particularly neuropathic pain, is a great challenge clinically. Literature data related to pain transmission reveal that angiotensin and its receptors (the AT1R, AT2R, and MAS receptors) could affect the nociception both in the periphery and CNS. The MOR and angiotensin receptors or drugs interacting with these receptors have been independently investigated in relation to analgesia. However, the interaction between the MOR and angiotensin receptors has not been excessively studied in chronic pain, particularly neuropathy. This review aims to shed light on existing literature information in relation to the analgesic action of AT1R and AT2R or MASR ligands in neuropathic pain conditions. Finally, based on literature data, we can hypothesize that combining MOR agonists with AT1R or AT2R antagonists might improve analgesia.

Keywords: μ-opioid analgesics; angiotensin receptors; chronic pain; neuropathic pain

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1. Introduction

Among different types of chronic pain, neuropathic pain is defined by the International Association for the Study of Pain (IASP) as pain caused by a lesion or disease of the somatosensory nervous system (IASP 2012). There are many available treatment approaches for the management of neuropathic pain. Yet, despite these advances, it remains an unmet medical need because most of the treatment approaches intended to halt this pain condition are not effective enough or sometimes effective but limited by side effects. Thus, finding new targets and innovative future strategies that might help to improve neuropathic pain control are of clinical need.

μ-Opioid receptor (MOR) agonists are the mainstay treatment for different forms of chronic pain [1–4]. However, their efficacy in the management of neuropathic pain is a long-standing question of debate. Yet, international guidelines restrict opioids to second- or third-

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line therapy, with no clear consensus on their effect [5–7]. MOR agonists with significantly higher intrinsic efficacy than morphine produced acceptable analgesia in preclinical models of neuropathic pain [8,9]; however, this has not been successfully utilized clinically because clinical trials showed controversial results related to their efficacy and liability for side effects [10–13]. In response to this argument, many studies have been conducted to increase the efficacy and decrease the side effects of opioids when used in the management of neuropathic pain. Some of the encouraging strategies that aim to improve the analgesic effect and decrease the side effects of currently used analgesics, such as opioids, are based on combining two or more different agents. However, so far, clinical research data that is based on combination strategies have not met expectations [14]. Chaparro et al., reviewed clinical trials on the efficacy and safety of various agent combinations for neuropathic pain [14]. Their analysis revealed that the combination of opioids with gabapentin was significantly better than gabapentin alone in reducing the symptoms. However, the number of treated patients that was required for a single patient to benefit was still 9.5, and significantly more participants experienced side effects and thus dropped out of the studies with opioids plus gabapentin than with gabapentin alone [14]. On the other hand, studies assessing the effects of opioids in combination with other sensory-sensitization blocking agents could be of high clinical value. Thus, continuing preclinical research based on the application of multi-target drugs or combination strategies that involve implementing different agents might bring a new treatment option for neuropathic pain. In the former case, for instance, applying opioid receptor ligands that display agonist and non-opioid effects, such as tapentadol, display both the MOR agonist and norepinephrine reuptake inhibitory effects in the same molecule [15]. Recently, our group reported on the promising effect of the combination of glycine transporter 1 and 2 inhibitors in the management of neuropathic pain evoked by sciatic nerve ligation [16]. In such a strategy, we need to consider how the individual drugs affect pain transmission.

Accumulating evidence has proven that drugs affecting the renin–angiotensin system can modulate pain transmission [17–34]. Recent studies have also shown that drugs mimic or antagonize angiotensin type 1 and 2 (AT1R and AT2R) receptor-mediated actions do produce a beneficial analgesic effect in rodent models of chronic pain types [17,20,22,28,29,35–38]. The analgesic effect of ligands affecting angiotensin receptors in neuropathic pain is explained by the contribution of these receptors to neuroregeneration and neuroprotection—partially by reducing neural inflammatory processes [18,24,37,39–41]. Nevertheless, much remains unclear regarding the role and clinical utility of these receptors in analgesia.

This review briefly highlights how the effect of MOR agonist-induced analgesia is altered under neuropathic pain conditions, showing the advantages and drawbacks, as well as principal factors that negatively impact the analgesic effect of MOR analgesics in this pain entity. The next sections review the implication of angiotensin and its receptors in chronic pain, particularly that associated with neuropathy, and also the neuroanatomical overlap between MORs and angiotensin receptors in relation to pain. Finally, according to the reviewed data, perspectives on the future drug combination-based research strategy to treat neuropathic pain are provided. With respect to angiotensin IV and its receptor, the presence of the peptide has been reported in human dorsal root ganglia (DRG) and trigeminal nucleus (TG) [42,43]. However, there are little data related to their analgesic effect. Thus, they will not be discussed in the present review.

2. The Opioid System and the μ-Opioid Receptor in Different Pain Entities

The opioid system is a physiological system for controlling pain, but it also participates in addictive behaviors and immune defense, among others. Mammalian endogenous opioid peptides and exogenous natural, semisynthetic and synthetic opioid agonists can produce their effects through the activation of opioid receptors, namely μ-(MOR), δ-(DOR), and κ-(KOR) opioid receptors. Opioid receptors belong to the class A G-proteins of the pertussis toxin-sensitive Gi/Go family. Their effectors include adenylyl cyclase, N- and L-type Ca^{2+} channels, and inwardly rectifying K^{+} channels. Upon activation, adenylyl

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cyclase and Ca^{2+} channels are inhibited, whereas K^+ channels are activated. Thus, both the limitation of Ca^{2+} entry and the hyperpolarization of the cells may give a tenable explanation for the inhibition of transmitter release at pain traffic points [44,45]. With respect to pain, central MORs are the principal target for mediating the analgesic effects of opioids. As in MOR-knockout mice, selective MOR agonists failed to produce analgesia as well as MOR-induced opioid side effects, such as respiratory depression, gastrointestinal transit inhibition, and addiction liability [46,47]. Since the identification of functional peripheral MORs, it has become obvious that the analgesic effects of opioids do not solely depend on MORs at the central nervous system (CNS) [48]. It is worth noting that achieving peripheral analgesia requires prerequisite factors that are related both to the physicochemical properties of opioid analgesics (limited CNS penetration) and pain entity. In the case of the latter, the pathological state of pain largely reflects the effects of opioid analgesics. In inflammatory or acute non-inflammatory pain, MORs number is increased or maintained at normal level, respectively [9,48–50]. Several opioid researchers have proven that functional MORs in the periphery are targetable, particularly in inflammatory pain types [51–54]. However, under neuropathic pain conditions, several studies have demonstrated the downregulation of MORs in the dorsal spinal cord and DRG [9,55]. The efficacy of currently available MOR agonists in neuropathic pain is a question of debate. Taken together, in cases of acute or inflammatory pain types, opioid analgesics can provide adequate pain control, which is somewhat hampered by above mentioned unwanted effects. However, in the case of neuropathic pain, the desired analgesia itself is often unachievable, consequently demanding dose-escalation, therefore causing more pronounced side effects (Figure 1A) (Karádi and Al-Khrasani, unpublished data) and (Figure 1B) (adopted from our previous work [16]).

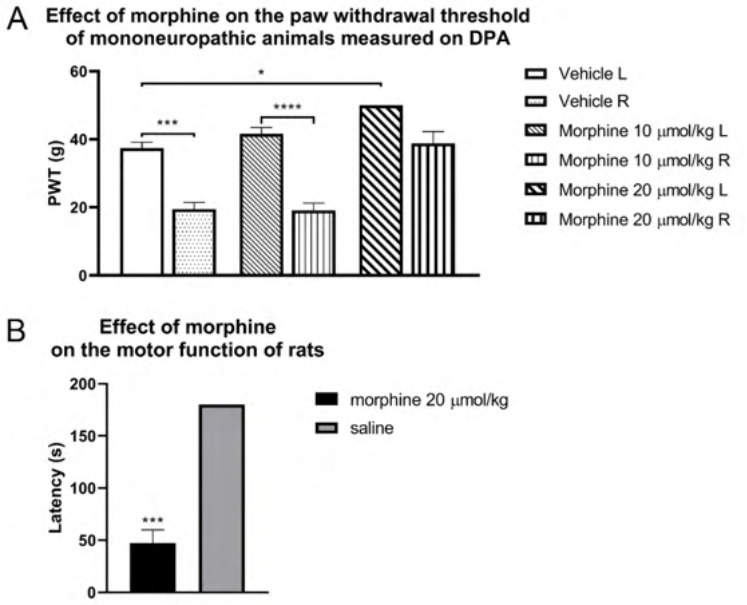

Figure 1. (**A**) The analgesic effect of morphine measured on a dynamic plantar aesthesiometer (DPA) test at 30 min, after s.c. administration to mononeuropathic animals. Columns represent the paw withdrawal threshold of the animals in grams ± S.E.M. Asterisks indicate the significant differences between treatment groups or operated (R) and non-operated (L) hind paws (* $p < 0.05$; *** $p < 0.001$ and **** $p < 0.0001$). Statistical differences were determined with one-way ANOVA and Tukey post-hoc test. Data represent means ± S.E.M (n = 5–12 per group). (Karádi, D.Á.; Al-Khrasani, M.; unpublished data). (**B**) Effect of the systemic administration of morphine to the motor function of rats. Columns represent the time latency of the animals in sec ± S.E.M. at 30 min post-treatment in the rotarod test. Asterisks indicate the significant differences compared to the saline group (one-way ANOVA, Newman–Keuls post-hoc test; *** $p < 0.001$). In each treatment group, 4–7 animals were used. These results were adopted from our previous work [16].

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For restoring the effect of opioids in neuropathic pain, many attempts have been focused on the mechanisms related to changes in the number of functional MORs on sensory neurons in subjects with painful neuropathy. In our and other studies carried out in rats with neuropathic pain induced either by streptozotocin (STZ) or chronic constriction injury (CCI), the number of MORs was found to be decreased in DRG and spinal tissue [9,56,57]. This reduction in MOR number was accompanied by a decrease in the analgesic effects of opioids.

3. Angiotensin Receptor Mimetics and Antagonists in Relation to Pain

3.1. Endogenous Angiotensin Ligands and Angiotensin Receptors

Components of the renin–angiotensin system (RAS) have been previously reviewed or discussed extensively [19,24,58–64]. Nevertheless, the main findings are briefly summarized here for an overview. Among the endogenous peptides of the RAS, neuronal angiotensin II (Ang II) is the most significant in relation to pain. Ang II is an octapeptide derived from the inactive precursor angiotensinogen, which is initially cleaved by renin, resulting in the inactive intermediate angiotensin I (Ang I). Ang II is cleaved from Ang I by the angiotensin-converting enzyme 1 (ACE1). Ang II equally binds to and activates the AT1R and AT2R (see later on). Another relevant endogenous peptide of the RAS to this review is angiotensin 1-7 (Ang (1-7)), which is cleaved by the angiotensin-converting enzyme 2 (ACE2) from Ang II or by ACE1 from Ang I via the intermediate angiotensin 1-9. Ang (1-7) activates the Ang (1-7) receptor or MAS receptor, but it can also bind with lower affinity to AT2R.

There are four angiotensin receptor types known so far within the RAS; namely angiotensin II type 1 and 2 receptors, the angiotensin IV receptor, and the Ang (1-7) receptor or MAS receptor (abbreviated as AT1R, AT2R, AT4R, and AT7R or MASR, respectively). Additionally, in mice and rats, two AT1R isoforms have been identified, namely AT1aR and AT1bR [65,66]. In relation to the RAS, this review will focus on data of AT1R, AT2R, and MASR, with respect to pain, particularly from preclinical studies. They all belong to the rhodopsin-like G-protein coupled receptor family (GPCR); however, they differ significantly in terms of activation of signaling pathways and cellular and tissue distribution patterns. The latter will be discussed in detail in a separate section. The AT1R is a prime example of a GPCR that upon activation can be dependent and independent from heterotrimeric G-proteins, allowing the receptor to have a wide range of signaling responses to Ang II. In terms of G-protein dependent signaling pathways, the AT1R couples to multiple types of Gα, (Gq/11, Gi, G12, and G13), but it also includes the activation of small G-proteins. G-protein independent signaling of AT1R involves β-arrestin 1 and 2, tyrosine kinase-related signaling, reactive oxygen species signaling, receptor-interacting scaffold proteins, or heterodimerization with AT2R or MASR. In the case of AT2R, signaling pathways are still not fully elucidated, in spite of the intensive research. In fact, it is one of the least understood areas of the renin–angiotensin system. Most interestingly, it fails to demonstrate classic GPCR signaling features, such as affecting second messengers (e.g., cAMP, diacylglycerol) or the lack of phosphorylation-induced receptor desensitization, or internalization in most tissue types. However, it has been proven that AT2R is sensitive to GTPγS and pertussis toxin in rat locus coeruleus, indicating Gi/o coupling [67]. AT2R can also stimulate protein phosphatases and nitric oxide production. In addition, AT2R mediates the inactivation of mitogen-activated protein kinase (MAPK) inhibition which is important in the induction of apoptosis [60,67]. The AT2R and Ang II interaction leads to neurite formation and growth via the modulation of polymerized β-tubulin, microtubule-associated proteins (MAP), the activation of the p42/p44 MAPK phosphorylation of trkA. MASR, similar to AT1R and AT2R, can couple to many downstream signaling pathways via Ang (1-7) activation. These include the activation of phospholipase C and A2, arachidonic acid release, or calcium-independent nitric oxide synthase activation. MASR also modulates several kinase-related pathways/effectors, such as the p38 MAPK, ERK1/2, phosphatidylinositol 3-kinase/Akt, RhoA, and cAMP/PKA, in different cell lines. MASR was also demonstrated

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to constitutively couple to Gαi, Gαq, and Gα12/13 [63]. On the other hand, similar to AT2R, in most cases, MASR fails to induce the conventional G-protein mediated signaling response, defined by the levels of classical second messengers, such Ca^{2+}, or inositol trisphosphate (IP3), despite belonging to the GPCR family.

3.2. AT1 and AT2 Receptor Agonists

Following the discovery of the neuronal RAS, numerous studies have reported on the implication of AT1R/AT2R agonists on nociception [27,30,33,68–76]. In spite of the high number of studies conducted, literature data remain highly controversial. Some publications describe the analgesic activity of AngII, AngIII, or renin on acute pain tests following central (intracerebroventricular [27,69,71,72,76] or intrathecal [33]) administration. These reports proposed different possible mechanisms of action behind the observed effects. Many of them indicate the role of the endogenous opioid system as the analgesic activity of test compounds was naloxone-sensitive [27,33,69,71,72]. Next, Shimamura et al., suggested a kinetic interaction between AngIII and met-enkephalin, namely the inhibition of cleavage of the latter [71]. Georgieva et al., found that AngII administered intracerebroventricularly (icv.) produced an antinociceptive effect in the acetic-acid writhing pain model, yet the AngII-induced antinociception was blocked by PD123319, an AT2R selective antagonist but not by losartan, an AT1R antagonist [75]. In this study, the authors concluded that AT2Rs but not AT1Rs are involved in the mechanism behind the analgesic action in acute inflammatory pain. Since then, studies assessing the effects of RAS peptides (angiotensinogen, AngI, AngII, or AngIII) microinjected into different regions of the periaqueductal gray (PAG) were conducted in rats. In these studies, all test peptides were proven to be analgesic on the tail-flick assay, and their effect was AT1R or AT2R antagonist reversible [77]. Another observation is that spontaneously hypertensive rats show longer latency on the hot plate but not on the tail-flick test, when compared to wild-type animals. Moreover, this increase in latency can be reversed by orally administered captopril or losartan, but not by antihypertensive agents which are acting on targets other than the RAS [73]. In contrast to the above-mentioned studies, Cridland et al., reported that AngII failed to show either anti- or pronociceptive effect [72]. However, at present, we cannot judge this issue because, to the best of our knowledge, there is no other study that supports Cridland's observations. It is also worth considering the article of Pavel et al., which examined the effect of AngII and losartan in rats undergoing CCI. In these animals, intraperitoneal AngII was found to be pronociceptive in the von Frey test (mechanical stimuli), constant hot- and cold-plate tests and decremental cold plate test (thermal stimuli). Losartan fully reversed the effect of AngII in case of mechanical stimuli, partially reversed it in case of constant cold-plate test, but further aggravated it in the decremental cold plate test. In the incremental hot plate test, the pain threshold was unchanged both following AngII or AngII + losartan administration [78]. The differences observed in this study between the effect of angiotensin in response to constant or decremental/incremental thermal stimuli is difficult to explain.

Further on, the direct pronociceptive activity of AngII and AngIII was described as spontaneous painful behavior (scratching) was observed following intrathecal administration [40,41]. It is worth noting that the study of Cridland et al., showed neither anti- nor pronociceptive action of AngII, whereas Nemoto and coworkers reported a pronociceptive action. Despite the similar administration route, the phenotype of the animals, as well as the dose applied, was different in these studies [40,41,73]. Therefore, further studies are needed to elucidate the effect of AngII at the spinal level. Indirectly supporting the pronociceptive action of AngII, Kaneko et al., reported icv. administered AngII to attenuate the analgesic activity of morphine in a dose-dependent manner in hot plate and tail pinch tests [69]. Similarly, Yamada et al., found that icv. administrated AngII or the AT2R agonist novokin decreased the antinociceptive effect of morphine in the tail-pinch test [79]. Shepherd et al., also reported an increased mechanical but not thermal allodynia following intraplantar AngII administration in mice after spared nerve injury (SNI) [80].

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There is large literature data on neural regeneration and differentiation mediated by the AT2R, which were recently reviewed by Danigo et al. [24]. From this aspect, activating the AT2R induces positive changes in terms of neural injury. This neuroprotective action linked to the AT2R has been associated with an increase in neuronal BDNF expression by several reports. The AT2R agonist "compound 21" (C21) has been reported to increase neurite growth following spinal nerve injury [81] and to improve survival while attenuating post-stroke neurological deficit in mice [82]. Under these conditions, the common feature was an increase in neuronal BDNF expression. In contrast, increasing BDNF level is not necessarily beneficial in cases of peripheral nerve injury from the aspect of pathological pain, since Madara et al., showed that BDNF could induce glutamate release by enhancing the action of presynaptic NMDA receptors [83]. BDNF release governs the spinal long-term potentiation of C-fibers [84]. Long-term potentiation and a consequently increased glutamatergic tone, involving the increased activity of spinal NMDA receptors, are hallmarks of neuropathic pain or other chronic pain states [85,86]. Furthermore, Chen et al., proved that spinal NMDA receptor-potentiation on primary afferents in neuropathic pain could be blocked either by the BDNF scavenger trkB-Fc or by the trkB receptor antagonist ANA-12 [87]. The contribution of BDNF to pain was validated by Sikandar et al., where they demonstrated that the conditional knockout of BDNF from mouse sensory neurons results in unchanged response to most acute pain types and displayed hypoalgesia in chronic inflammatory or neuropathic pain [88].

3.3. MAS Receptor Agonists

Primarily the Ang (1-7)-MASR branch of RAS acts as an antagonist of the AngII-AT1R activity. The activity linked to AT2Rs is similar in general; however, with respect to pain transmission, this is not the case. The possible analgesic effect of Ang (1-7) was investigated following mostly local (intraplantar [21,23] or intrathecal [34,89–93]) administration. Studies using intraplantar administration reported that Ang (1-7) attenuated PGE2 [21,23,90,91] or carrageenan [23] induced inflammatory mechanical hyperalgesia. The antihyperalgesic effect of Ang (1-7) was lost in MASR KO mice [23] and was reversible by MASR, nNOS, guanylyl cyclase, or ATP-sensitive potassium channel blockers [94] as well as by different adrenergic antagonists [21], but not by naloxone [95].

Intrathecal administration of Ang (1-7) resulted in a decrease in spontaneous nociceptive behavior induced by intrathecal AngII [91], AngIII [92], substance P or NMDA [34]. Furthermore, intrathecal Ang (1-7) showed an antiallodynic and antihyperalgesic effect in neuropathic pain induced by CCI [89], STZ [90], or genetic model of diabetes (ob/ob mice) [93]. Moreover, several authors reported that Ang (1-7) effectively decreased the pathological increased p38 phosphorylation in the spinal cord [90–92,96]. Similar results were reported following intrathecal administration of ACE2 activator DIZE, namely reduced nociceptive behavior in the formalin test and decreased spinal p38 phosphorylation [96]. On the other hand, intraplantar Ang (1-7) was ineffective in the treatment of CCI induced neuropathic pain [23].

The effect of systemic (ip.) administration of Ang (1-7) on bone cancer pain was investigated by Forte et al., In this model, Ang (1-7) reduced spontaneous pain reactions, increased von Frey threshold and tail immersion latency following acute or chronic administration. The authors reported no anti-tumor activity [97].

3.4. AT1 and AT2 Receptor Antagonists

A growing body of literature data supports that antagonists of the AT1R, such as losartan, candesartan, or telmisartan, among others, display analgesic action in different pain models, including acute thermal, inflammatory, or neuropathic pain [17,23,30,35,36,39–41]. With respect to the analgesic effect of telmisartan, our unpublished results also support such findings because it could reduce the partial sciatic nerve CCI-induced allodynia after systemic administration in rats (Figure 2) (Karádi and Al-Khrasani, unpublished data)).

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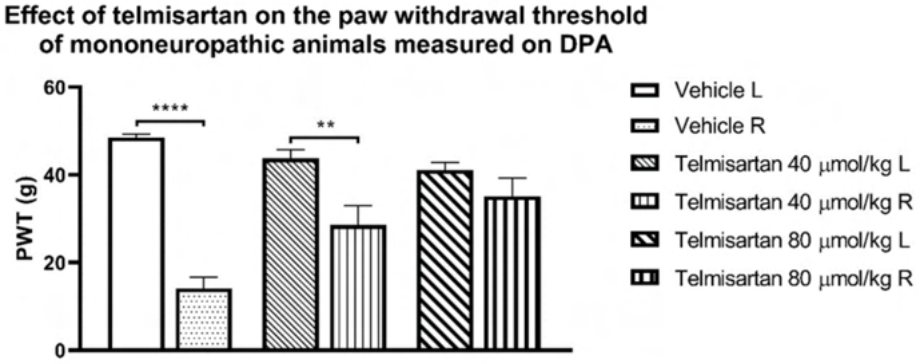

Figure 2. The analgesic effect of telmisartan measured on a dynamic plantar aesthesiometer (DPA) test at 120 min, after p.o. administration to mononeuropathic animals induced by partial sciatic nerve ligation rat model described by Seltzer et al. [98]. Columns represent the paw withdrawal threshold (PWT) of the animals in grams ± S.E.M. Asterisk indicates the significant differences between treatment groups or operated (R) and non-operated (L) hind paws (** $p < 0.01$ and **** $p < 0.0001$). Statistical differences were determined with one-way ANOVA and Tukey post-hoc test. Data represent means ± S.E.M (n = 5 per group). (Karádi, D.Á.; Al-Khrasani, M.; unpublished data).

In addition, intrathecal administration of losartan has been reported to block AngII-induced spontaneous pain [39], both phases of formalin test [41], and STZ-induced allodynia [99]. On the other hand, microinjection of AT1R and AT2R antagonists into the PAG has been reported to aggravate incisional allodynia [26,77]. Local administration of losartan was also investigated by Costa et al., In this study, intraplantar (ipl.) losartan effectively reversed prostaglandin E2 (PGE2) and carrageenan-induced mechanical hyperalgesia but was ineffective in CCI induced neuropathic pain [23]. In contrast, numerous publications have reported that systemic administration of AT1R antagonists to be beneficial [17,20,35,36,79]. Most of these reports suggest that blocking AT1R could also attenuate the inflammatory reaction in DRG [35,36] or the sciatic nerve [17] and elevate the decreased BDNF level in the sciatic nerve [17] following neuronal damage.

Bessaguet et al., investigated the effect of candesartan on resiniferatoxin-induced neurotoxic thermal hypoalgesia in mice and proved that intraperitoneal candesartan was able to reverse the evoked hypoalgesia in this assay, yet the same effect was achieved following the treatment with AT2R antagonist, EMA200 (PD123319). The authors proposed that candesartan may increase the AT2R binding of endogenous AngII, thus lowering the thermal threshold of animals. This proposal is further supported by the lack of efficacy of candesartan in AT2R KO mice [20]. In agreement with these results, Hashikawa-Hobara et al., reported that hypoesthesia caused by fructose induced diabetes was reversible by orally administered candesartan [100]. Obagata et al., showed that intrathecal losartan can attenuate the allodynia evoked by STZ in mice. In addition, they found that Ang II, as well as ACE expression, were increased, indicating the involvement of AngII in neuropathic pain conditions. It has also been reported that candesartan is capable of inducing neuroprotective, anti-inflammatory, and pro-angiogenetic effects accompanied by an increase in BDNF expression [101,102]. In these studies, the beneficial effects of AT1R antagonism were reversible by the AT2 receptor antagonist, EMA200 [101,102]. Similar to the above-mentioned studies, the authors hypothesized that AT1R antagonism causes a shift in endogenous AngII binding from the AT1R to the AT2R, thus indirectly causing AT2R activation.

There are numerous studies indicating that AT2R antagonism can be beneficial in treating different pain entities. In case of inflammatory pain types, the proposal that reduction in hyperinnervation can attenuate pain is in agreement with literature data [103,104]. Chakrabarty et al., reported that EMA200 reduced thermal hyperalgesia, mechanical allodynia, and pathological hyperinnervation of inflamed tissue in a model of inflammatory pain induced by complete Freund's Adjuvant (CFA) [18,22]. The same compound was also effective in the treatment of cancer-induced bone pain, which is mostly an inflam-

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matory pain type, strongly depending on local inflammatory mediators [105]. The most clinically promising results, however, came from the investigation of the analgesic effect of EMA200 and its analogs in neuropathic pain, partially contradicting the above-mentioned data [28,29,37,38,80,106–108]. These include rodent models of mononeuropathic pain and even human clinical trials. AT2R antagonists were shown to be able to attenuate mechanical [37,38,81,107,108] and cold [107] allodynia in different mononeuropathic models, such as CCI or SNI. Moreover, the effect of EMA200 was validated on complex behavioral pain assays as well [109]. The most clinically relevant result, however, is that the analgesic effect of EMA401, the orally available analog of EMA200, was tested in clinical trials for postherpetic neuralgia [28,29] and diabetic neuropathy [28]. The efficacy in attenuating symptoms of the patients enrolled was acceptable in both conditions; however, two of the three studies were prematurely terminated because of preclinical data on the possible hepatotoxic effect of the test compound upon long-term administration [28]. There is no clear consensus whether AT2Rs are expressed on sensory neurons creating a direct pharmacological target for analgesia [18,37,38,106,107,110], or the observed beneficial effect is mediated by immune cells infiltrating injured nerves [80,107]. The neuro-immune cross-talk proposed by the latter studies was recently reviewed by Balogh et al. [19].

4. Neuroanatomical Distribution of the μ-Opioid and Angiotensin Receptors in Areas Related to Pain

4.1. The μ-Opioid Receptor

The neuroanatomical distribution of the MOR is now well-established by immunohistochemistry, autoradiography, in situ hybridization, and fluorescence techniques [109–113]. Accordingly, MORs can be found at supraspinal, spinal, and peripheral levels [114–116]. MORs are enriched in the descending pain modulatory pathway, involving the periaqueductal gray (PAG) matter, rostral ventromedial medulla (RVM), locus coeruleus (LC), and the dorsal horn of the spinal cord [115,117]. In addition, they can be found in brain regions that are strongly related to pain perception and integration, such as the cerebral cortex, thalamus, striatum, amygdala, hippocampus, nucleus accumbens, and the ventral tegmental area (VTA) [115,117]. Within the dorsal horn of the spinal cord, MORs are densely localized in the lamina I-II superficial layers on interneurons and projection neurons [115,118]. The dorsal root ganglia are also a significant locus for MORs attributed to pain [115,119]. MORs can also be found on C- and A-fibers and near primary afferent nociceptors [117].

4.2. Angiotensin Receptors and Endogenous Angiotensin Ligands

The components of neuronal angiotensin system are found in anatomical regions hosting different key points in pain pathways, including the dorsal horn of the spinal cord, dorsal root ganglia (DRG and identical structures, such as the spinal trigeminal tract and trigeminal ganglion), or peripheral nerves. Angiotensinogen mRNA can be found ubiquitously in the mammalian brain [120], spinal cord [99], and almost all cells in the DRG [42,43]. The angiotensinogen level in the CNS is not affected by STZ treatment-induced diabetes; however, it is elevated following peripheral inflammation [22,121].

There are contradictory data in the literature about the localization of neuronal renin, the primary activating enzyme of the renin–angiotensin system [42,43,100]. AngI mRNA is present in the human DRG and trigeminal ganglion (TG) [42,43], whereas its protein form was described in rat DRG [121]. AngII was found in rat and human DRG [18,37,43,106,107], TG [42], neurons, satellite cells, and CD3+ T-cells [106]. The colocalization of AngII alongside components involved in pain sensation, such as substance P (SP) and vanilloid transient receptor potential channels, was reported as well on small and medium neurons [18,37,42,43,106]. In rodent, AngII can be found ubiquitously in the spinal cord; its level was highest in the superficial laminae of the dorsal horn, which could suggest a possible role of AngII in nociception [41,99]. Furthermore, AngII levels have been reported to be increased following mono- or polyneuropathic pain evoked by CCI [106] or

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STZ, respectively [41,99]. Furthermore, this change in AngII levels was also seen in pain conditions induced by intraplantar formalin injection [41,99] or in bone cancer pain [105].

With respect to the receptors, several studies have reported on the distribution of AT1R on key points related to nociceptive transmission both in mice [39,40] and rats [31,36,43,122–129]. These areas include sciatic nerve [31,127,130], DRG [36,43,123, 125,127–131], and spinal cord [22,39,40,129,132]. Moreover, it can be found in different brain regions, such as the spinal trigeminal tract and raphe nuclei [122]. These data also provide strong evidence on a large amount of AT1aR, and smaller amounts of AT1bR mRNA [43,127,129,132], and the receptor protein [31,36,39,40,123,125,127–129] was also shown in the mentioned regions. In the DRG, the receptor protein was found on satellite cells and neurons of all sizes with a greater extent on smaller ones [36,110,128,130]. In the spinal cord, similarly to AngII, AT1R level was the highest in the superficial dorsal horn [39,128].

In contrast to AT1R, AT2R localization and the above-mentioned function in relation to nociception are controversial subjects. At present, little data are available on the ganglional or sensory neural expression of AT2R as many of the currently commercially available AT2R antibodies used for immunohistochemistry seem to show inappropriate specificity [131]. Therefore, it is important to evaluate the results of studies using antibodies with appropriate criticism—especially in case of earlier works.

Early autoradiographic studies found significant inhibition of AngII binding by AT1R but not by AT2R antagonists on the sciatic nerve, spinal cord, and (upper cervical) sensory ganglion [31,128]. AT2 mRNA was found in the DRG and sciatic nerve of rats [43,127]. The receptor protein was found by many research groups on neurons (IB4+ [132]), satellite cells [106,127], and CD3+ T-cells [106] and in the rat DRG as well [101,106,107,110,125,130]. Indeed, in a few studies, the AT2 antibody specificity was verified on AT2R KO mice, further reinforcing the results [37,123]. On the other hand, Shepherd and colleagues were not able to find AT2R mRNA or protein in the DRG of mice or humans [80]. In their study using Agtr2^{GFP} reporter mice, the AT2 positivity in the sciatic nerve was detectable and increased after SNI but because of macrophage infiltration instead of neural expression. Taken together, Shepherd's group claims that AT2R is not expressed on sensory neurons involved in nociception [107]. In contrast, Benitez et al., found AT2 immunoreactivity in rat DRG mostly on non-peptidergic (IB4+) C- and Aδ-fibers showing high colocalization to AT1 yet using an antibody with specificity verified on AT2R KO mice. In their study, the level of AT2 increased in an inflammatory state following treatment with CFA [123]. It is important to mention that mice were used in the study conducted by Shepherd in contrast to rats used by Benitez. A very recent review published in 2021 by Danigo et al., provides detail on how to solve this contradiction and lists species differences as well as the possible gene duplication of AT2R (similar to AT1R) in mice which could cause a lack of signal in the reporter mice [24].

Angiotensin-converting enzyme 2 (ACE2) is a carboxypeptidase enzyme regulating the local levels of AngII and Ang 1-7 (metabolizes AngII to Ang 1-7). Its mRNA and protein were found in human DRG samples, colocalizing with nociceptor neuronal markers [133]. It is also expressed in mouse spinal cord, where it is localized on neurons and microglia but not on astrocytes [93]. Finally, MASR expression was shown in rat DRG [91,92], PAG [134] and in mouse spinal cord [93]. However, to the best of our knowledge, the localization of the Ang (1-7) peptide has not been fully described. The neuroanatomical localization of key elements of the RAS and μ-opioid receptors have been summarized in Table 1.

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Table 1. Neuroanatomical distribution of ligands and receptors in the renin–angiotensin system with importance in pain transmission and the μ-opioid receptor (MOR).

Ligand/Receptor	Species	mRNA /Peptide/ Protein	Method	Details	Changes		References
					Inflammation	Neuropathy	
Peripheral nerves							
Angiotensinogen	rat	p	IHC	detected	increased	-	[22]
AT1 receptor	rat	p	autorad	detected	-	-	[31]
	rat	r	PCR	detected	-	increased	[124]
AT2 receptor	rat	p	autorad	not detected	-	-	[31]
	rat	r	PCR	detected	-	increased	[124]
	AgtrGFP reporter mouse	p	reporter mouse	detected on thick non-peptidergic neurons	-	increased (macrophage infiltration)	[107]
MAS receptor	mouse	p	IHC	detected	-	increased	[135]
MOR	rat	p	IHC	detected	increased	-	[136]
	human	p	IHC	detected on CGRP positive skin sensory nerves	no change	-	[137]
Dorsal root ganglia							
Angiotensinogen	rat	p	IHC	detected	increased	-	[22]
	rat	r and p	PCR and IHC	detected	-	-	[121]
	rat	r	PCR and ISH	detected on all cells	-	-	[43]
Angiotensin I	human	p	RIA	detected	-	-	[43]
Angiotensin II	rat and human	p	IHC and RIA	colocalized with SP and CGRP	-	-	[43]
	rat	p	IHC	colocalized with neuronal markers	increased (bone metastasis)	-	[105]
	rat	p	IHC and WB	colocalized with SP and NF200	-	increased	[37]
	human	p	IHC	colocalized with TRPV1 on small and medium neurons	-	-	[18]
	rat	p	IHC	on neurons, satellite cells, and T cells	-	increased	[106]
Angiotensin (1-7)	human	p	IHC	not detected	-	-	[18]
AT1 receptor	rat	r	PCR	detected	-	no change	[124]
	rat	r	PCR	detected	-	-	[43]
	rat	p	IHC	detected on Schwann cells, satellite cells, and neurons	-	decreased (DM)	[127]
	rat (isolated neurons)	r and p	PCR, WB, and RB	detected	decreased (TNFα)	-	[129]
	rat	p	IHC	detected on small and large neurons	-	increased	[125]
	rat	p	IHC	detected on neurons and satellite cells	-	-	[36]
	rat	p	IHC	detected on all neurons, higher expression on small	increased on large neurons	-	[123]

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Table 1. *Cont.*

Ligand/Receptor	Species	mRNA /Peptide/ Protein	Method	Details	Changes		References
					Inflammation	Neuropathy	
AT2 receptor	rat	r	PCR	detected	-	increased	[124]
	rat	r and p	PCR and IHC	detected	-	-	[121]
	rat	r	PCR	detected	-	-	[43]
	rat	p	IHC	detected on Schwann cells, satellite cells, and neurons	-	increased (DM)	[127]
	rat (cell culture)	p	WB	detected	-	increased (DM)	[100]
	rat	p	IHC	colocalized with neural markers	-	-	[37,105]
	rat (neonatal)	r and p	PCR, WB, and IHC	detected on IB4+ neurons	-	-	[132]
	rat	p	IHC	detected on neurons, satellite cells, and T-cells	-	no change	[106]
	rat	p	IHC	detected on all neurons, mostly non-peptidergic C and Aδ, high colocalization with AT1	increased	-	[123]
	AgtrGFP reporter mouse and human	r and p	PCR and reporter mouse	not detected	-	-	[80]
	AgtrGFP reporter mouse	p	reporter mouse	not detected	-	no change	[107]
MAS receptor	rat	p	IHC	detected	-	-	[95]
	rat	r and p	PCR and WB	detected	-	increased	[89]
	rat	r and p	PCR and WB	detected	-	-	[138]
	mouse	p	WB	detected	increased (bone metastasis)	-	[97]
MOR	rat	p	IHC	detected mainly on small neurons	increased	-	[136]
	rat	p	IHC	detected on small and medium neurons, highly colocalized with CGRP and SP	-	-	[139]
	rat	p	IHC	detected	increased	-	[50]
	rat	r	PCR	detected	increased	decreased	[140]
	human	r	PCR	detected on approx. 50% of neurons, mainly capsaicin-responsive small neurons	-	-	[119]

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Table 1. *Cont.*

Ligand/Receptor	Species	mRNA /Peptide/ Protein	Method	Details	Changes		References
					Inflammation	Neuropathy	
Spinal cord							
Angiotensin II	mouse	p	IHC	detected ubiquitously, highest in laminae I and II	increased	increased	[41,99]
AT1 receptor	rat	p	IHC, autorad, and ISH	detected in the superficial DH and on cholinergic neurons in the VH	-	-	[126,128]
	mouse	p	IHC	detected in the superficial DH	-	-	[39,40]
AT2 receptor	rat	p	IHC	detected in laminae I and II and colocalized with IB4 and SP in	-	-	[123]
	AgtrGFP reporter mouse	p	reporter mouse	detected in the deep DH and VH and colocalized with neuronal markers	-	no change	[107]
MAS receptor	mouse	p	WB	detected	-	-	[93]
	mouse	p	IHC	detected and colocalized with NK1 and NMDA receptors	-	-	[34]
MOR	rat/guinea pig	p	autorad	detected in the superficial dorsal horn	-	-	[113]
	rat	p	IHC	detected on laminae I-II	increased	-	[136]
	rat	p	IHC	present	-	-	[139]
	rat	p	IHC	postsynaptic MOR is restricted to lamina II	-	-	[141]
	rat	p	IHC	detected, half of MOR immunoreactivity in the SC is on primary afferents	-	-	[142]
	rat	r	PCR	detected	no change	no change	[140]
	rat	p	IHC	detected	-	decreased (reversible by NGF)	[57]

Abbreviations: p: peptide/protein; r: mRNA; IHC: immunohistochemistry; autorad: autoradiography; PCR: polymerase chain reaction; ISH: in situ hybridization; RIA: radioimmunoassay; WB: Western blot; DM: diabetes mellitus; DH: dorsal horn; VH: ventral horn; SP: substance P; CGRP: calcitonin gene-related peptide; NF200: neurofilament protein 200; TRPV1: transient receptor potential cation channel subfamily V member 1; IB4: isolectin B4; NK1: neurokinin 1; NMDA: N-methyl D-aspartate. A hyphen indicates no assessment by the indicated studies.

5. Possible Link between MOR Analgesics and Ligands Affecting Angiotensin Receptors in Relation to Pain

Rather than dose escalation of MORs analgesics which is associated with an increase in the incidence of side effects, augmenting MORs-mediated analgesia would be an important strategy in the management of neuropathic pain. In regard to the interaction between opioid and angiotensin systems, to the best of our knowledge, the first study published in 1983 by Haulica et al., described that AngII produced naloxone reversible analgesia following icv. administration in rat tail-flick test; therefore, these results showed the implication of endogenous opioid system in the effect of AngII [68]. In a later study, the same research group also reported that naloxone or saralasin attenuates stress analgesia in rats [70]. Based on another study by Han et al., icv. administered AngII was able to reverse the antinociceptive action of sc. morphine [76]. Similarly, Yamada et al., showed

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that AT2R activation decreases the analgesic effect of morphine [79]. On the other hand, a previous study by Mojaverian et al., reported that orally administered ACE inhibitor enalapril failed to influence morphine analgesia [143]. Recently, Taskiran and Avci reported that systemic captopril alone was able to increase tail-flick and hot plate latency, and it also increased the analgesic effect of systemic morphine. Furthermore, the co-treatment with captopril reduced morphine-induced analgesic tolerance development. Captopril also reduced the inflammatory and endoplasmatic stress response in the DRG caused by acute or chronic morphine treatment [32]. It is important to note however, that ACE inhibition could result in a diverse molecular effect, partly independent from RAS—such as the inhibition of the catabolism of endogenous opioids and peptide mediators, among others. Next, connection between Ang (1-7), MASRs and the opioid system is unclear as to the best of our knowledge there are little data available at present. In this respect, Costa et al., reported that endogenous opioids do not play a role in the analgesic action of Ang (1-7) as it was not sensitive to naloxone [95]. This does not necessarily mean that there are no possible interactions between the two systems. Indeed, there are several reports, indicating opioids are capable of changing physiological parameters, most notably changes in the blood pressure [144–148] or drinking-response to AngII [149–151]. However, regarding the relationship between RAS and the opioid system only a small proportion of these address the role of interactions in analgesia. We have summarized the outcomes of relevant studies in Table 2.

Table 2. Reported connections between the opioid and renin–angiotensin systems in relation to pain.

RAS Ligand/Receptor	Method	Outcome	Reference
Angiotensin II	rat tail-flick test	AngII mediated analgesia is reversible by naloxone.	Haulica et al., 1983 [68]
	rat tail-flick test	AngII is able to attenuate morphine analgesia.	Han et al., 2000 [76]
Angiotensin-converting enzyme	rat tail-flick test	ACE-inhibition cannot influence morphine analgesia.	Mojaverian et al., 1984 [143]
	rat tail-flick and hot plate test	ACE-inhibition enhances morphine analgesia and decreases the development of opioid analgesic tolerance.	Taskiran et al., 2021 [32]
	ELISA	ACE-inhibition decreases inflammatory cytokine levels in the DRG of morphine tolerant animals.	Taskiran et al., 2021 [32]
AT2 receptor	mouse tail/pinch test	AT2 activation decreases morphine analgesia	Yamada et al., 2009 [79]
	rat tail-flick test	Saralasin (AT2 partial agonist) decreases stress analgesia.	Haulica et al., 1986 [70]

Abbreviations: ELISA: enzyme-linked immunosorbent assay.

With respect to neuropathic pain, Khan and coworkers showed that allodynia caused by CCI of the sciatic nerve was attenuated by a systemic single dose of EMA300, a small molecule AT2R antagonist [106]. In this study, the authors also proved that the nerve growth factor (NGF) level was significantly reduced in the ipsilateral lumbar DRGs of neuropathic rats. In addition, treatment with EMA300 could restore the decreased NGF level. Furthermore, several studies have shown that MOR reserve in the spinal cord and DRG is decreased in rodents with neuropathic pain. It is worth noting that administration of exogenous NGF does restore both MOR numbers and their analgesia at main relay points along the pain pathways, such as the spinal cord [58]. These results support a hypothesis on the possible existence of a link between MORs and angiotensin receptor affecting ligands which may provide a new strategy for the treatment of neuropathic pain. Namely, AT2R blockade was reported to restore pathologically decreased NGF levels in neuropathy, which, in turn, could positively influence the MOR number in the DRG and spinal cord, thus restoring the analgesic effect of MOR agonists (Figure 3). An opposing

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viewpoint is the implication of NGF in pain induction which is not the scope of the present review but has been reported by other researchers [152–154]. Finally, whether activation or blockade of AT2R would be of value in managing neuropathic pain, we could propose that AT2R inhibition attenuates pain mediated by largely unidentified pathways. On the other hand, the neural growth and remodeling induced by AT2R activation may be beneficial for neuroregeneration, though undesired effects on the symptoms of neuropathy may occur.

To the best of our knowledge, so far, no publication has investigated the possible connections between the opioid system and the Ang (1-7)—MAS receptor branch of the RAS.

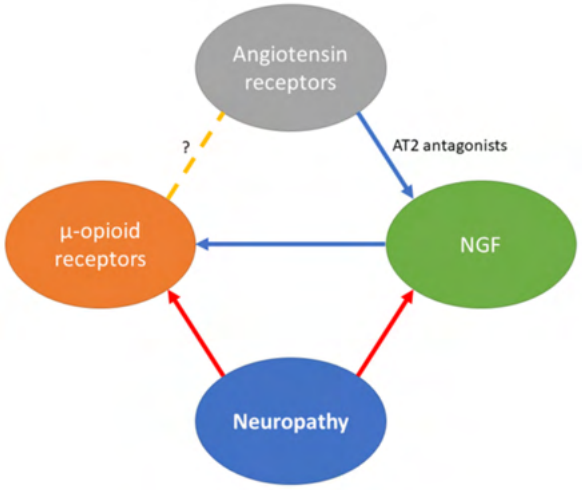

Figure 3. Possible links between neuropathy, the renin–angiotensin system, MORs and NGF. Red arrows indicate a reducing effect, while the blue ones indicate an increasing effect. In neuropathic conditions, the MOR reserve is decreased, resulting in impaired opioid analgesia. The receptor number can be restored by administration of NGF, the level of which is also reduced in the spinal cord in neuropathy. AT2 antagonists are capable of restoring the lowered NGF level, thus possibly restoring the analgesic effect of opioids. To the best of our knowledge, there is no evidence of the direct connection between MORs and the renin–angiotensin system. The figure was constructed based on literature discussed in Section 5.

6. Concluding Remarks and Future Directions

MOR analgesics alleviate neuropathic pain; however, high doses are needed, which, in turn, result in serious side effects both in preclinical and human studies. Current evidence indicates that AT1, AT2, and MASRs are involved in the control of neuropathic pain; however, their mechanism of action related to neuropathic pain has not yet been fully verified. Nevertheless, AT1, AT2, and MASRs are expressed in key areas related to pain where MORs agonists halt pain sensation. In neuropathic conditions, peripheral and central AT1 blockade and spinal MASRs activation appear to be beneficial. Data on the impact of AT2R in neuropathic pain are contradictory, though its activation or inhibition can result in neuroprotection or analgesia, respectively; however, future studies are needed to justify this issue. So far, there are no angiotensin receptor affecting agents that have been utilized clinically; however, there are clinical studies on AT2R inhibitors that have entered phase II trials but did not proceed further due to their toxicity. It is important to note that these clinical studies prove that such AT2R inhibitors showed equipotent efficacy with gabapentin. In neuropathic pain, the MOR receptors and NGF levels are decreased. Treatment with NGF results in restoring MOR and their analgesic activity in preclinical pain studies. On the other hand, there are studies reporting the increase in pain sensation upon NGF use, which is not the scope of the present review. Furthermore, some studies revealed that angiotensin AT2R inhibitors do increase NGF in neuropathic pain and thus normalize MOR levels. Therefore, we can speculate that drugs affecting angiotensin receptors could restore the effect of MOR analgesics, which results in avoiding dose escalation of opioids

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upon the treatment of neuropathic pain. Finally, these strategies might offer a bridge upon titration of drugs with delay in onset used in the treatment of neuropathic pain.

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Article

Telmisartan Is a Promising Agent for Managing Neuropathic Pain and Delaying Opioid Analgesic Tolerance in Rats

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Abstract: Despite the large arsenal of analgesic medications, neuropathic pain (NP) management is not solved yet. Angiotensin II receptor type 1 (AT1) has been identified as a potential target in NP therapy. Here, we investigate the antiallodynic effect of AT1 blockers telmisartan and losartan, and particularly their combination with morphine on rat mononeuropathic pain following acute or chronic oral administration. The impact of telmisartan on morphine analgesic tolerance was also assessed using the rat tail-flick assay. Morphine potency and efficacy in spinal cord samples of treated neuropathic animals were assessed by [^{35}S]GTPγS-binding assay. Finally, the glutamate content of the cerebrospinal fluid (CSF) was measured by capillary electrophoresis. Oral telmisartan or losartan in higher doses showed an acute antiallodynic effect. In the chronic treatment study, the combination of subanalgesic doses of telmisartan and morphine ameliorated allodynia and resulted in a leftward shift in the dose–response curve of morphine in the [^{35}S]GTPγS binding assay and increased CSF glutamate content. Telmisartan delayed morphine analgesic-tolerance development. Our study has identified a promising combination therapy composed of telmisartan and morphine for NP and opioid tolerance. Since telmisartan is an inhibitor of AT1 and activator of PPAR-γ, future studies are needed to analyze the effect of each component.

Keywords: neuropathic pain; neuropathy; opioids; opioid tolerance; morphine; RAS; angiotensin receptor type 1; telmisartan; losartan

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1. Introduction

Management of neuropathic pain (NP) remains a major challenge for clinicians as it affects 7–10% of the general population [1–3]; the effectiveness of currently available drugs is not nearly satisfactory and the treatment is often associated with risk of unwanted side effects [4,5]. Several guidelines are in agreement that the first-line medications in the treatment of NP are tricyclic antidepressants (TCAs), serotonin and norepinephrine reuptake inhibitors (SNRIs) or gabapentinoids [4,6,7]. With respect to effectiveness, the number needed to treat (NNT) values of these agents remain high [5]; moreover, they lack prompt onset of action [8,9]. Therefore, vast efforts are being made in order to identify novel

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pharmacological targets or to reposition already marketed drugs that could be incorporated into the treatment regimens of NP.

Opioid analgesics are the mainstay of acute and chronic pain management [10–12]; however, their use is hampered by their side effects and the development of analgesic tolerance through long-term administration [11]. The effectiveness of opioids in NP remains controversial [13,14]. In the past, it has been shown that a decreased number or G protein coupling of spinal μ-opioid receptors in neuropathy may be responsible for the loss of opioid efficacy [14–17]. In this regard, a decrease in the functional μ-opioid receptor reserve may seem similar to that seen in opioid analgesic tolerance [18].

Since the discovery of the renin–angiotensin system (RAS), it has become a highly exploitable pharmacological target in the management of cardiovascular diseases [19,20]. RAS inhibitors are widely available, cheap, safe and well-tolerated drugs, making them ideal candidates for drug repurposing efforts. The role of angiotensin receptors and ligands in the central nervous system (CNS) and their possible therapeutic use in the alleviation of NP, among other pain types, are emerging as an important area of pain research. Other groups and we ourselves have reported on the connection between the RAS and NP [5,21,22] and possible pharmacological interactions between the opioid and angiotensin systems regarding analgesia [23]. In the present work, we provide, to the best of our knowledge, the first evidence for enhanced analgesia following co-administration of a clinically, widely used, angiotensin receptor blocker (ARB) and morphine in a neuropathic rodent model. In addition, we demonstrate that the ARB telmisartan reduces the development of opioid analgesic tolerance during chronic morphine treatment.

2. Results

2.1. Telmisartan or Losartan Produces Acute Antiallodynic Effect in Neuropathic Pain Evoked by Sciatic Nerve Injury

The acute effect of orally administered losartan (50, 100 and 150 μmol·kg^{-1} body weight (BW)) and telmisartan (20, 40 and 80 μmol·kg^{-1} BW) was assessed by dynamic plantar esthesiometry (DPA) two weeks following peripheral nerve injury, induced by partial sciatic nerve ligation (pSNL), in animals showing significant allodynia, the hallmark symptom of neuropathic pain (Figure 1).

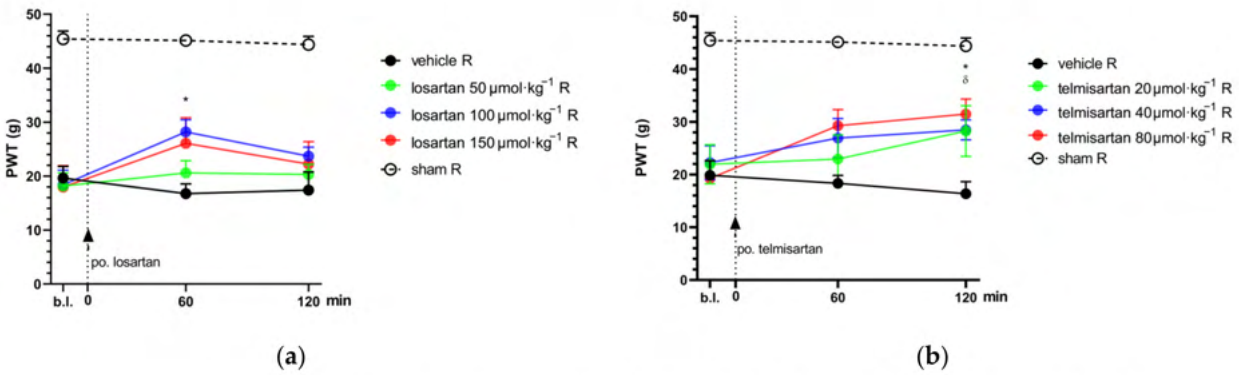

Figure 1. The antiallodynic effect of losartan (**a**) and telmisartan (**b**) following acute oral administration via orogastric feeding tube. Means of PWT ± S.E.M. are depicted in grams, obtained from the animals' right (operated; R) hind paws on the 14th day after pSNL at the indicated time points. Data obtained from the intact (left) hind paws of the animals are excluded for better visual clarity; however, they are presented in Appendix A (Figure A1). Abbreviation "b.l." stands for baseline. Panel (**a**) * $p < 0.05$ between losartan 100 μmol·kg^{-1} BW and vehicle, two-way ANOVA followed by Tukey's post hoc test, n = 5–9 per group. F (time; 1.925, 51.98) = 6.607, p = 0.0031. F (treatment group; 4, 27) = 19.44, $p < 0.0001$. Panel (**b**) * $p < 0.05$ between telmisartan 80 μmol·kg^{-1} BW and vehicle, $^{\delta}$ $p < 0.05$ between telmisartan 40 μmol·kg^{-1} BW and vehicle, two-way ANOVA followed by Tukey's post hoc test, n = 5–11 per group. F (time; 1.847, 51.72) = 7.903 p = 0.0013. F (treatment group; 4, 28) = 12.32 $p < 0.0001$.

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Telmisartan at the two higher test doses (40 and 80 μmol·kg^{-1} BW) showed a significant antiallodynic effect 2 h after systemic (oral) administration when compared to its vehicle, 1% HEC (paw withdrawal threshold (PWT): 28.47 ± 1.91, n = 9 vs. 16.35 ± 2.30, n = 6; p = 0.013 and 31.48 ± 2.83, n = 6 vs. 16.35 ± 2.30, n = 6; p = 0.014, respectively). Losartan in doses of 100 or 150 μmol·kg^{-1} BW produced moderate antiallodynia with a peak effect at 60 min after systemic administration; however, significance was achieved only following the 100 μmol·kg^{-1} BW dose compared to the vehicle treated group (PWT: 28.19 ± 2.25, n = 9 vs. 16.76 ± 1.80, n = 5; p = 0.013) (Figure 1).

2.2. Combination of Telmisartan and Morphine in Subanalgesic Doses Alleviates Neuropathic Pain Evoked by Sciatic Nerve Injury

In this phase of the study, the acute effect of a combination of telmisartan or losartan and morphine was investigated in animals showing allodynia two weeks after pSNL. As stated above, acute treatment with losartan and telmisartan at doses of 50 and 20 μmol·kg^{-1} BW, respectively, did not cause significant analgesic effect (Figure 1). Alleviation of neuropathic pain by morphine alone was also assessed (Figure 2a) following subcutaneous administration, in order to determine the dose of morphine used as part of the combination treatments. In this set of experiments, morphine failed to alleviate neuropathic pain at a dose of 10 μmol·kg^{-1}, which was chosen for further experiments as the subanalgesic dose.

The combination of telmisartan and morphine in subanalgesic doses showed a trend but no significant effect towards the alleviation of neuropathic pain after acute administration. However, following 10 days of chronic treatment, this combination was able to alleviate NP in the operated paws. Namely, at the peak-effect of the compounds, a significantly higher PWT of the operated paws was observed in the combination group when compared with the group treated with their vehicles (PWT: 32.53 ± 3.71, n = 6 vs. 15.46 ± 2.27, n = 4; p = 0.028) or 1% HEC plus morphine (PWT: 32.53 ± 3.71, n = 6 vs. 11.35 ± 2.63, n = 5; p = 0.008) (Figure 2d,e). On the other hand, the combination of subanalgesic doses of losartan and morphine failed to alleviate NP either on the 1st day of treatment (acute effect) or after 10 days of chronic administration (Figure 2b,c).

2.3. Impact of Chronic Telmisartan and Morphine Combination Treatment on Morphine-Stimulated [^{35}S]GTPγS Binding in Spinal Cord Membranes of Neuropathic Rats

Specific G-protein coupling of spinal μ-opioid receptors was measured using morphine-stimulated [^{35}S]GTPγS binding assay and the acquired binding data are summarized in Table 1. Morphine showed similar efficacy (E_{max}) for G-protein coupling in the spinal cord tissues of animals from either treatment group. On the other hand, the potency of morphine (EC_{50}) with respect to [^{35}S]GTPγS binding was significantly increased in spinal tissue samples obtained from animals treated chronically with the combination composed of the subanalgesic doses of telmisartan and morphine compared to the morphine-treated group (1% HEC plus morphine) (Figure 3a,b). This reduction in the [^{35}S]GTPγS specific binding potency of morphine can also be observed as a rightward-shift of the morphine concentration–response curve in the indicated group (Figure 3a).

Table 1. Morphine-stimulated [^{35}S]GTPγS binding data on spinal cords obtained from animals undergoing 10 days of chronic treatment. EC50 and Emax values were calculated individually for each animal sample and means ± S.E.M. are presented here.

Treatment Group (10 Days of Treatment)	GTPγS Binding	
	EC50 ± S.E.M. (nM)	Emax ± S.E.M. (%)
1% HEC + saline (n = 5)	217.2 ± 137.8	134.1 ± 6.8
1% HEC + morphine (n = 5)	1864.0 ± 783.5 *$^{\delta}$	140.2 ± 3.4
telmisartan + morphine (n = 5)	285.0 ± 125.1	136.5 ± 1.8

* p < 0.05 vs. telmisartan + morphine; $^{\delta}$ p < 0.05 vs. control, one-way ANOVA followed by Fisher's LSD post hoc test, n = 5 per group.

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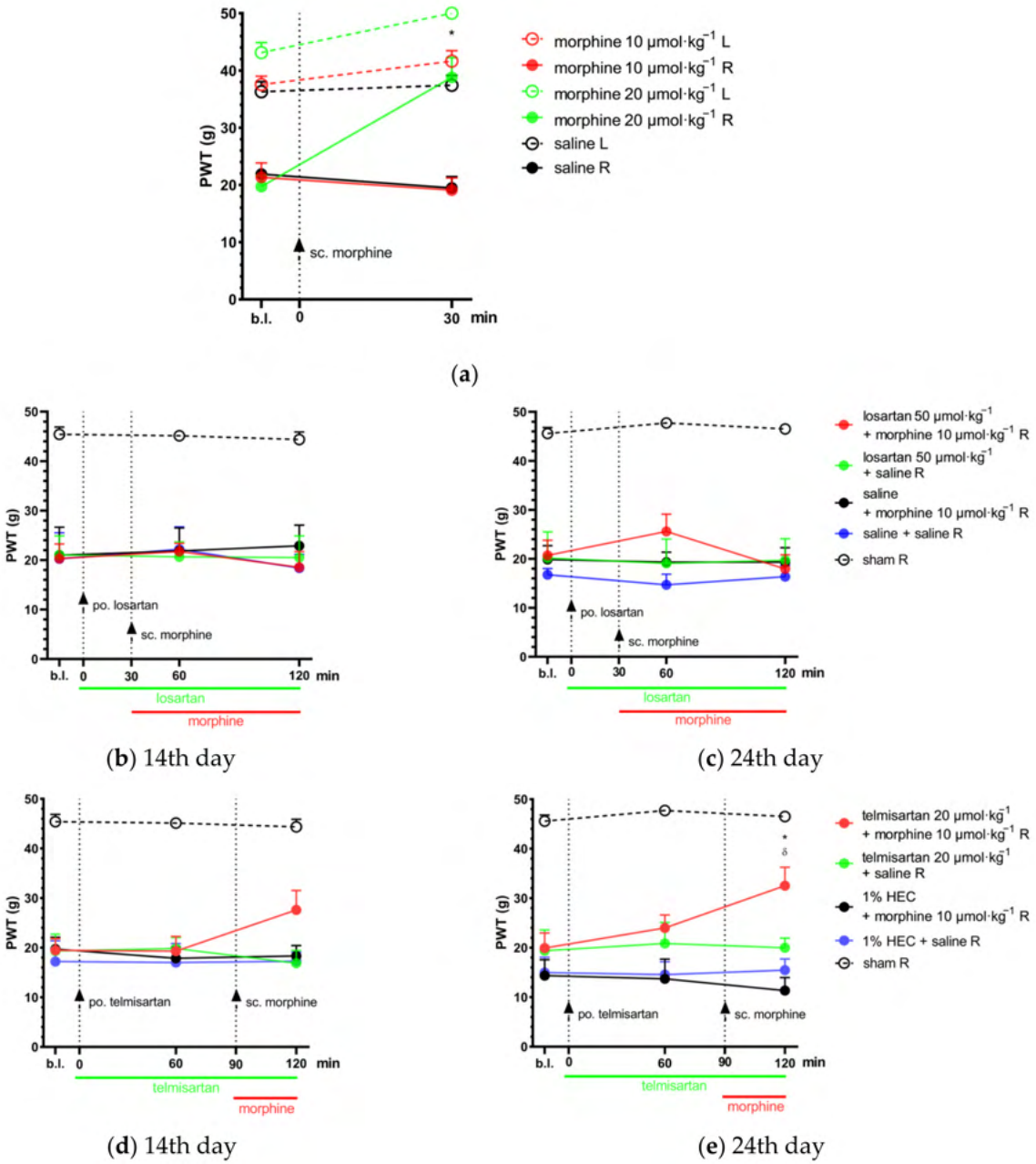

Figure 2. (**a**) Determination of the acute antiallodynic effect of subcutaneous morphine at doses of 10 and 20 µmol·kg^{-1} BW. The antiallodynic effect was tested at 30 min, which corresponds to the peak effect of morphine [14,24]. * $p < 0.05$ versus vehicle, two-way ANOVA followed by Tukey's post hoc test, n = 5–12 per group. F (time; 1, 38) = 13.19, $p = 0.0008$. F (treatment group; 5, 38) = 38.06, $p < 0.0001$. (**b**,**c**) The antiallodynic effect of po. losartan, sc. morphine and their combination, following acute and chronic treatment on the 14th and 24th day post-pSNL, respectively. Abbreviation "b.l." stands for baseline. Two-way ANOVA followed by Tukey's post hoc test, n = 5–6 per group. 14th day: F (time; 1.817, 38.15) = 0.75 $p = 0.47$; F (treatment group; 4, 21) = 12.93, $p < 0.0001$. 24th day: F (time; 1.57, 33) = 1.1, $p = 0.33$; F (treatment group; 4, 21) = 23.87, $p < 0.0001$. (**d**,**e**) The antiallodynic effect of po. telmisartan, sc. morphine and their combination, following acute and chronic treatment on the 14th and 24th day post-pSNL, respectively. * $p < 0.05$ versus vehicle, $^{\delta}$ $P < 0.05$ vs. 1% HEC + morphine 10 µmol·kg^{-1} R, two-way ANOVA followed by Tukey's post hoc test, n = 4–6 per group; 14th day: F (time; 1.711, 35.94) = 0.7 $p = 0.48$; F (treatment group; 4, 21) = 27.15, $p < 0.0001$; 24th day: F (time; 1.855, 38.96) = 4.24, $p = 0.024$; F (treatment group; 4, 21) = 27.06, $p < 0.0001$. Results are presented as mean ± S.E.M.

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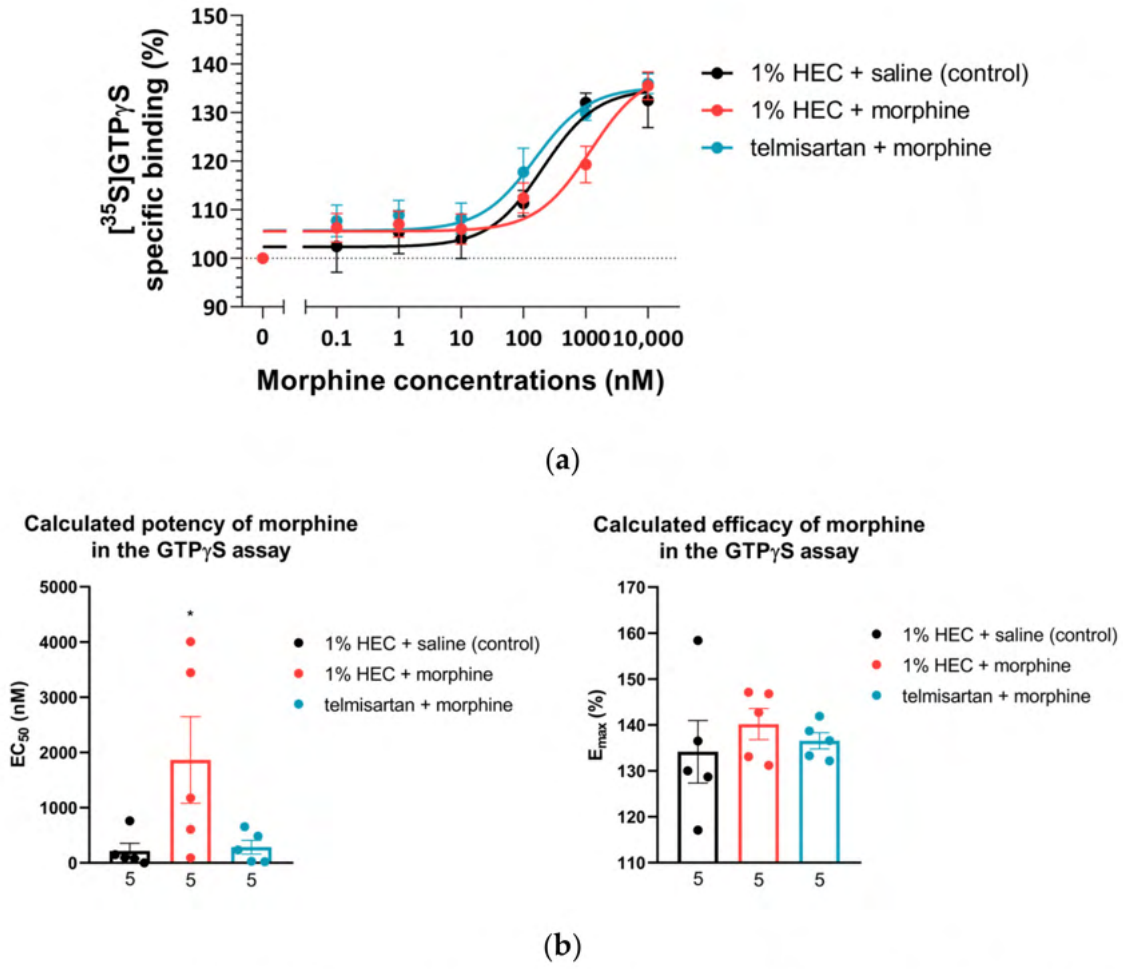

Figure 3. (**a**) Morphine-induced μ-opioid receptor G-protein activity on L4–L6 spinal cord membrane homogenates obtained from animals that had previously undergone 10 days of chronic treatment according to the indicated groups. $n = 5$ per group. (**b**) Calculated EC_{50} and E_{max} of morphine from the morphine-stimulated [^{35}S]GTPγS binding assay on L4–L6 spinal cord samples obtained from animals that had previously undergone 10 days of chronic treatment according to the indicated groups. * $p < 0.05$ vs. all other groups, one-way ANOVA followed by Fisher's LSD post hoc test, $n = 5$ per group. EC_{50}: F (2, 12) = 4.017, $p = 0.0462$ E_{max}: F (2, 12) = 0.4538, $p = 0.6457$. EC_{50} and E_{max} values were calculated individually for each animal and means ± S.E.M. are presented here.

2.4. Telmisartan Delays the Development of Morphine Analgesic Tolerance in Rat Tail-Flick Assay

Subcutaneous morphine (31.08 μmol·kg^{-1} BW) showed a significant antinociceptive effect indicated by increased latency in the tail-flick assay of rats after acute administration (1st day). This effect peaked at 30 min following morphine administration and was retained when combined either with telmisartan or its vehicle (Figure 4). On the other hand, telmisartan (20 μmol·kg^{-1} BW) alone did not show antinociception. Animals in the studied treatment groups showed a similar response on the 4th day of chronic treatment as on the 1st (Figure 4). On the 10th day of treatment, the antinociceptive effect of morphine disappeared and did not significantly differ from the control at any time point, indicating the development of antinociceptive tolerance to morphine. However, when morphine was combined with oral telmisartan, a significant antinociception was maintained on the 4th and 10th day as well, peaking at 30 min and lasting up to 3 h (Figure 4a,b).

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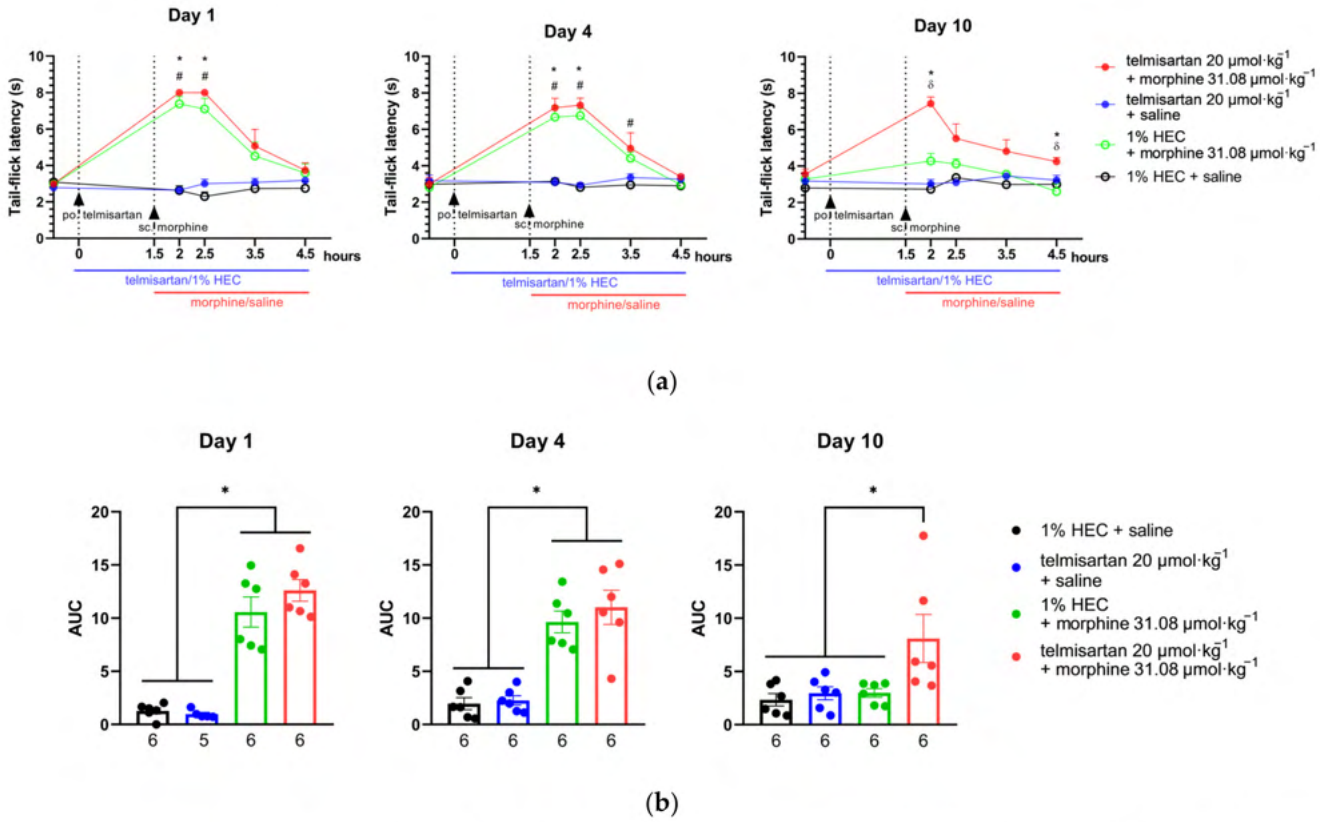

Figure 4. (**a**) The effect of morphine, telmisartan and their combination on the tail-flick latency of *naïve* rats (day 1) and following 4 or 10 days of chronic treatment with the indicated combinations. Telmisartan or 1% HEC was administered orally at 0 min, while morphine or saline was administered subcutaneously at 90 min to ensure that the peak effect of the two compounds coincided in time. * $p < 0.05$ between telmisartan + morphine and 1% HEC + saline, $^{\#}$ $p < 0.05$ between 1% HEC + morphine and 1% HEC + saline, $^{\delta}$ $p < 0.05$ between telmisartan + morphine and 1% HEC + morphine, two-way ANOVA followed by Tukey's post hoc test, $n = 6$ per group. Day 1: F (time; 2.39, 47.79) = 30.56, $p < 0.0001$; F (treatment group; 3, 20) = 55.15, $p < 0.0001$. Day 4: F (time; 3.07, 61.34) = 34.88 $p < 0.0001$; F (treatment group; 3, 20) = 27.02, $p < 0.0001$. Day 10: F (time; 3.03, 60.63) = 9.79, $p < 0.0001$; F (treatment group; 3, 20) = 22.45, $p < 0.0001$. (**b**) Area under the curve (AUC) values calculated individually for each animal of the elapsed time—tail-flick latency curves. For each animal, the tail-flick latency value prior to treatment administration was used as the baseline for calculating AUC values. * $p < 0.05$ between indicated groups, one-way ANOVA followed by Tukey's post hoc test, $n = 5$–6 per group, exact group sizes are shown in each graph. Day 1: F (3, 19) = 43.3, $p < 0.0001$. Day 4: F (3, 20) = 21.98, $p < 0.0001$. Day 10: F (3, 20) = 4.818, $p = 0.011$. ROUT analysis, with a Q value = 0.5% identified one outlier in the telmisartan + saline group on Day 1. Data are presented as mean ± S.E.M.

2.5. Impact of Angiotensin Receptor Blockers and Morphine on the L-Glutamate Content of the CSF

In both in vivo models described above, cerebrospinal fluid (CSF) samples were collected from chronically treated animals and their L-glutamate content was determined by capillary electrophoresis. Based on samples from both mononeuropathic and morphine-tolerant animals, only the combination of telmisartan and morphine significantly increased CSF L-glutamate content. Individual components of the combination failed to do so in either model. (Figure 5).

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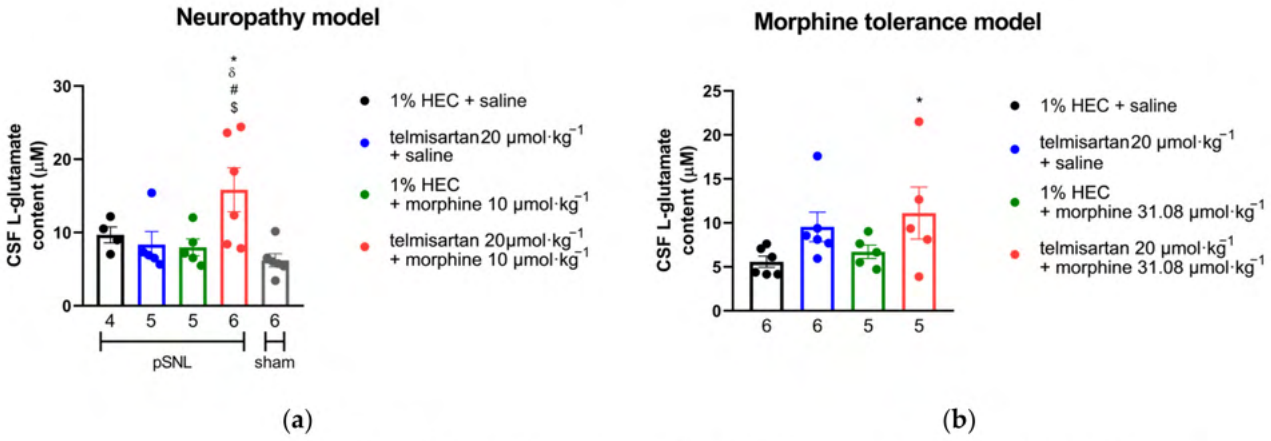

Figure 5. (**a**) L-glutamate content in the CSF of mononeuropathic (pSNL) and sham-operated animals following 10 days of chronic treatment with orally administered telmisartan or 1% HEC plus subcutaneously administered morphine or saline. * $p < 0.05$ vs. 1% HEC + saline, $^{\#}$ $p < 0.05$ vs. telmisartan + saline, $^{\delta}$ $p < 0.05$ vs. 1% HEC + morphine, $^{\$}$ $p < 0.05$ vs. sham, one-way ANOVA followed by Fisher's LSD post hoc test, n = 4–6 per group, exact group sizes are shown in each graph. F (4, 21) = 4.238, $p = 0.0114$. (**b**) L-glutamate content in the CSF of animals following 10 days of treatment according to our morphine analgesic-tolerance protocol. * $p < 0.05$ vs. 1% HEC + saline, one-way ANOVA followed by Fisher's LSD post hoc test, n = 5–6 per group, exact group sizes are shown in each graph. F (3, 18) = 2.23, $p = 0.1198$.

2.6. Impact of Efficient Test Compounds on the Motor Coordination of Rats

Telmisartan (80 μmol·kg^{-1} BW, po.), morphine (10 μmol·kg^{-1} BW, sc.) or their combination failed to exhibit motor dysfunction in rats (Figure 6). Morphine treatment (31.08 20 μmol·kg^{-1} BW, sc., as positive control) produced a significant disturbance in motor coordination, seen as shorter fall-off times (latency: 19.4 ± 5.67, n = 5 vs. 180 ± 0.00, n = 4).

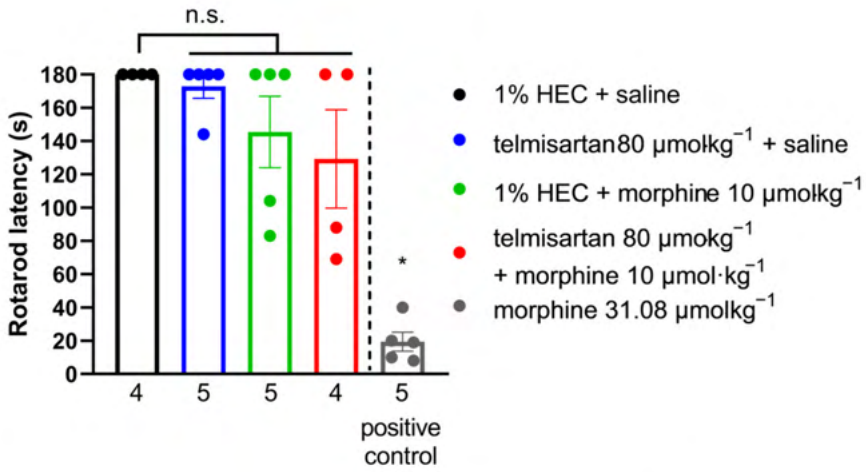

Figure 6. Effect of systemic (oral or subcutaneous) administration of analgesic doses of telmisartan and its combination with morphine on the motor function of naïve animals. Columns represent the time latency of animals in the RotaRod assay ± S.E.M. measured at peak effect of test compounds. Abbreviation "n.s." stands for not significant. * $p < 0.05$ vs. all other groups, one-way ANOVA followed by Fisher's LSD post hoc test, n = 4–5 per group, exact group sizes are shown in each graph. F (4, 18) = 17.13, $p < 0.0001$.

3. Discussion

There are many different pharmacotherapeutic approaches for the treatment of neuropathic pain, but none of these has so far proved fully effective. Furthermore, the analgesic

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effect of current medications is of slow onset, and the side effects are progressively increasing with dose escalation, and thus, limit their further use [5,8]. Therefore, the need to develop novel drugs or new approaches encourages researchers to continue working in this field. In the last decades, many promising drug targets have been identified as potential therapeutic targets for future neuropathic pain treatment, such as angiotensin receptor type 1 [22,23]. The use of combination treatment regimens has also been considered in order to increase the effectiveness without increasing the undesirable effects of the elements of the combination. With respect to the raised issues, we herein present results on the effect of AT1 antagonists, losartan and telmisartan, in a rat neuropathic pain model evoked by pSNL. The antiallodynic effect of these antagonists was then assessed when combined with morphine. Through the present pain assessment model, we have demonstrated that at certain doses both losartan and telmisartan are able to produce significant analgesia in NP after acute oral administration (Figure 1). The pain attenuation effect of losartan or telmisartan is manifested by their ability to ameliorate mechanical allodynia, the hallmark symptom of NP [25]. These results further support previous findings on the analgesic effects of AT1 antagonists in animals with neuropathic pain. There are several studies that demonstrate the analgesic effects of AT1 antagonists in diabetic polyneuropathy [26,27] or chemotherapy-induced neuropathy, among others [28,29]. Indeed, in the peripheral mononeuropathic pain model, previous studies have found that AT1 antagonists such as telmisartan or losartan could attenuate neuropathic pain following chronic treatments in rats that underwent chronic constriction injury (CCI) [30,31]. In this regard, in our present work, we could also demonstrate an analgesic effect for single-dose administration of oral telmisartan or losartan with rat NP evoked by pSNL, but at a dose nearly two-fold higher than in previous studies. In addition, the applied dose range of telmisartan or losartan in previous studies corresponds to the ineffective dose range in our study, tested either after acute or chronic administration. An earlier study by Kim and co-workers showed that CCI of the sciatic nerve, tight ligation of spinal nerves (SNL) and tight partial ligation of the sciatic nerve (pSNL) generally evoked similar time-course behavioral symptoms, but signs of mechanical allodynia were greater in rats subjected to pSNL compared to CCI [32]. At present, the discrepancy in the effect of test compounds regarding the applied dose range can be explained by the type of surgical procedure used to evoke NP in rats (CCI vs. pSNL). Neuropathic pain is a devastating disease evoked by different causes and several underlying pathophysiological mechanisms which have resulted in the existence of multiple therapeutic strategies. Thus, studying the analgesic effects of AT1 blockade is worth doing, although previous work has shown negative results [33]. Indeed, the majority of the findings are promising [26–29] and our results are in line with these findings, because in the present work, we could achieve an acute antiallodynic effect with losartan and telmisartan. However, the doses administered in the experiments here were high when translated to human doses. It means that losartan and telmisartan were able to produce acute analgesic effect in NP but in doses much higher than those being used in animal studies and clinical practice with respect to analgesia or hypertension, respectively. This observed trend corresponds with findings from our previous studies related to the effect of morphine in diabetic rats with polyneuropathic pain [14] and in the present work regarding mononeuropathic pain (pSNL). In this study, the acute antiallodynic effect of morphine was only achieved in high doses. Therefore, the therapeutic strategy of drug combinations was followed to enhance analgesia and decrease deleterious effects. In this regard, we could prove that the subanalgesic dose of telmisartan in combination with the subanalgesic dose of morphine significantly attenuated NP after chronic treatment and produced an analgesic trend after acute administration (Figure 2). On the other hand, the combination of morphine with losartan failed to show pain attenuation following either acute or chronic administration. The differences between the two ARBs in our study may possibly be explained by their different kinetic or dynamic properties as well. In the current literature, telmisartan is considered to be a drug of particular interest regarding its central RAS-influencing effects [34] because of its highest lipophilicity in the class of ARBs and its penetration capabilities

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into the CNS [34–37]. On the other hand, the results of Wang et al. showed that losartan could not induce a significant CNS effect [38]. Furthermore, studies attribute potent neuroprotective effects to telmisartan, explained partly by its AT1 antagonist effect and partly by its unique-in-class peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist effect [39,40]. PPAR-γ agonists have been reported to have relevant suppression on the production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), among others [41]. Peripheral nerve injury-induced microglia activation in the spinal cord results in an increase in these proinflammatory cytokines, which play a profound role in the hyper-excitability and central sensitization that are being seen in neuropathic animals [42–44]. In addition, PPAR-γ inhibited the activation of CX3CR1 (fractalkine receptor) in rats developing neuropathic pain [45]. With respect to the impact of losartan on PPAR-γ, to the best of our knowledge, no study has reported on its direct action yet. However, losartan has several metabolites, among them EXP3179 is a minor one, which has been reported to act as an agonist of PPAR-γ [46]. At present, the lack of antiallodynic effect of losartan in combination with morphine can be explained by low levels of EXP3179, its PPAR-γ agonist metabolite, formed from the low dose of losartan in the combination. With respect to repeated morphine administration, it is known to result in increased spinal glial activation as well as the expression of multiple chemokines and cytokines. Thus, the failure of the combination of subanalgesic morphine and losartan to produce analgesia may be due to a small amount of EXP3179 to activate PPAR-γ and, as a consequence, reduced spinal glial inhibition and increased chemokine and cytokine expression, which contribute to the development of opioid analgesic tolerance and allodynia [47].

Fortunately, the promising analgesic results following the combination of telmisartan and morphine could draw our attention to one particular issue, namely the negligible development of analgesic tolerance to morphine when telmisartan was administered simultaneously. Accordingly, further studies were carried out to shed light on the cellular mechanisms beyond the remarkable analgesic effect seen with the combination of telmisartan and morphine. We have performed additional in vitro experiments utilizing the morphine-stimulated [^{35}S]GTPγS binding assay to demonstrate the G-protein activating capabilities of morphine in the spinal cord of neuropathic animals treated with morphine or the combination of telmisartan and morphine. The obtained results showed no change in the efficacy (E_{max}) of morphine in the spinal samples of either treatment group (Figure 3). However, the salient finding is that in animals receiving telmisartan in combination with morphine for 10 days, morphine retained its potency at the spinal level indicated by the EC_{50} value and attenuated NP evoked by pSNL. On the other hand, 10 days of morphine treatment caused a significant decrease in the potency of the morphine. Previous studies have demonstrated no change in opioid E_{max} values in the spinal cord of mice that develop analgesic tolerance to morphine compared to non-tolerant ones [48]. This is in line with our results; however, we expected a decline in both morphine potency and efficacy in the spinal samples of animals that underwent pSNL, as NP can induce a decrease in the membrane μ-opioid receptor reserve which is reflected by a decrease in the [^{35}S]GTPγS binding E_{max} of morphine, as described previously [14,49]. To explain the results shown here with respect to telmisartan, besides its AT1 antagonist effect, the activation of PPAR-γ [39,40] might also be of importance. Activation of PPAR-γ has been reported to delay the development of opioid tolerance [50] and reduced NP [51]. We have further investigated the impact of chronic oral administration of telmisartan on morphine-induced analgesic tolerance in a thermal pain model, the rat tail-flick assay. Our results are in line with data reported previously, regarding PPAR-γ agonists delaying the development of morphine tolerance in mice [50]. PPAR-γ is expressed by neurons, astrocytes, and microglia in the spinal cord, and it can downregulate CXCRs including CXCR4. CXCR4 antagonists have been reported to enhance the morphine analgesic effect [52,53]. CXCRs activate protein kinase C, which in turn phosphorylates μ-opioid receptors, which results in its uncoupling from the G_i protein. As a consequence, G protein coupled receptor kinase and arrestin are recruited to the μ-opioid receptor, facilitating its internalization and therefore analgesic-tolerance development [52,54].

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These studies proposed a role for a μ-opioid receptor–CXCR4 crosstalk in the spinal cord and the development of opioid tolerance. At present, the mechanism for the measured in vivo and in vitro effects are still not completely understood, and future studies will be needed to elucidate more precisely which factors are beyond analgesic-tolerance control.

Changes in the function of the spinal glutamatergic system have been described in the spinal cord of animals with NP [55,56]. Hence, we assessed the changes in L-glutamate levels in the CSF of rats with NP and treated chronically with subanalgesic doses of telmisartan and morphine separately or in combination. This strategy was also extended to examine the CSF L-glutamate content of rats with developed opioid analgesic tolerance. In these experiments, unexpectedly, the combination of telmisartan and morphine increased L-glutamate content in the CSF of rats showing analgesic response and delayed opioid tolerance. In fact, the mechanism of the observed effect of the combination of telmisartan and morphine related to the increase in glutamate level in CSF is complex and likely involves multiple targets such as AT1, PPAR-γ and opioid receptors which directly or indirectly engage in pain regulation and neuronal protection, among other physiological functions.

The direct action of telmisartan includes an anti-inflammatory effect through activation of PPAR-γ [57] or AT1 inhibition [58], which has been shown in cultured microglia and neurons [39]. Based on these studies, despite the increase in the glutamate content of the CSF in rats with NP and treated with the combination, telmisartan could ameliorate glutamate-induced neuronal-injury-evoked neuropathic pain. It is possible that telmisartan might also protect neuronal cells by decreasing inflammatory response in the spinal cord through either inhibition of AT1 or stimulating PPAR-γ rather than inhibition of glutamate release [39]. Telmisartan has been reported to produce significantly decreased glutamate-induced neuronal injury when applied prior to or concomitantly with glutamate [39]. It is indeed difficult to judge whether neuronal injuries evoked by increased glutamate level upon neuropathy are halted by telmisartan administration. NP and opioid tolerance share overlapping neural changes with respect to glutamate. In this regard, NMDA receptor blockers show an analgesic effect on NP and are able to delay the development of opioid analgesic tolerance [59–62]. However, it is important to note that the elevated CSF L-glutamate content observed in our experiments does not specifically reflect the function of the dorsal horn of the spinal cord, i.e., the spinal sensory system. It is conceivable that the increased glutamate efflux is a consequence of autonomic or other systems and that it does not act exclusively on NMDA receptors. Thus, future studies are required to understand how the telmisartan and morphine combination can ameliorate NP and delay analgesic tolerance. Finally, we compared the antiallodynic advantage of telmisartan to morphine regarding motor function. Even at the highest-applied analgesic dose of telmisartan alone or in combination with a subanalgesic dose of morphine, there was no measurable motor dysfunction—unlike several current medications used in the treatment of neuropathic pain [63,64].

4. Materials and Methods

4.1. Animals

Experimental protocols were carried out on male Wistar rats obtained from Toxi-Coop Zrt. (Budapest, Hungary). Animals weighing 120–150 g or 170–200 g at the beginning of the study were used for the neuropathic pain model or the morphine analgesic-tolerance experiments, respectively. A total of 123 animals were used for the neuropathic pain model and 24 animals for the morphine analgesic-tolerance model. Furthermore, 23 animals underwent RotaRod testing for motor coordination. Thus, a total number of 170 rats were used. Animals were maintained under controlled environmental conditions (20 ± 2 °C temperature, 12:12 h light/dark cycle) in standard cages, holding four to five animals per cage in the local animal house of Semmelweis University, Department of Pharmacology and Pharmacotherapy (Budapest, Hungary). Water and standard rodent chow were available ad libitum. Prior to experiments, animals were allowed to acclimatize for at least 1 week. All

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

4.2. Materials

Telmisartan and losartan-potassium were obtained from TCI EUROPE N.V. (Zwijndrecht, Belgium), while morphine-HCl was obtained from Alkaloida-ICN (Tiszavasvári, Hungary). Telmisartan was suspended in 1% hydroxyethyl-cellulose solution (HEC), while losartan-potassium and morphine-HCl were dissolved in 0.9% saline. Telmisartan and losartan were administered orally (po.), using stainless steel oral feeding needles (purchased from Animalab Hungary Kft., Vác, Hungary) in a total volume of 5 mL·kg^{-1} bodyweight (BW). Morphine was administered subcutaneously (sc.) in a total volume of 2.5 mL·kg^{-1} BW. Pentobarbital was obtained from Semmelweis University Pharmacy (Budapest, Hungary), and dissolved in 0.9% saline before being administered intraperitoneally (ip.) in a total volume of 2.5 mL·kg^{-1} BW. Diethyl-ether was purchased from Sigma-Aldrich (Budapest, Hungary).

For [^{35}S]GTPγS binding assay DMSO, Tris-HCl, EGTA, NaCl, MgCl2 × 6H2O, GDP and the GTP analog GTPγS were purchased from Sigma-Aldrich (Budapest, Hungary). The radiolabeled GTP analog, [^{35}S]GTPγS (specific activity: 1250 Ci/mmol, Cat.No.: NEG030H250UC) and the UltimaGoldTM MV aqueous scintillation cocktail was purchased from PerkinElmer (handled by Per-Form Hungaria Kft, Budapest, Hungary).

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

4.3. Experimental Protocols

4.3.1. Mononeuropathic Pain Model

A schematic summary of the study design of the applied neuropathic pain model is presented in Figure 7. In the days preceding the start of the experiments, handling was performed in order to acclimatize the animals to the experimental conditions. This consisted of placing the animals in the plastic cages of the experimental apparatus once daily. Mechanical allodynia induced by neuropathy was assessed using a dynamic plantar esthesiometer (DPA 37450, Ugo Basile, Italy) as described before [56,65] with the following settings: incrementation: 10 g/s, maximal force: 50 g. The PWTs of animals were measured in grams, following at least five minutes of habituation in the cage in the case of each animal. Each paw was measured three times and the means of the three measurements were used for further analysis. All behavioral studies were performed by the same tester.

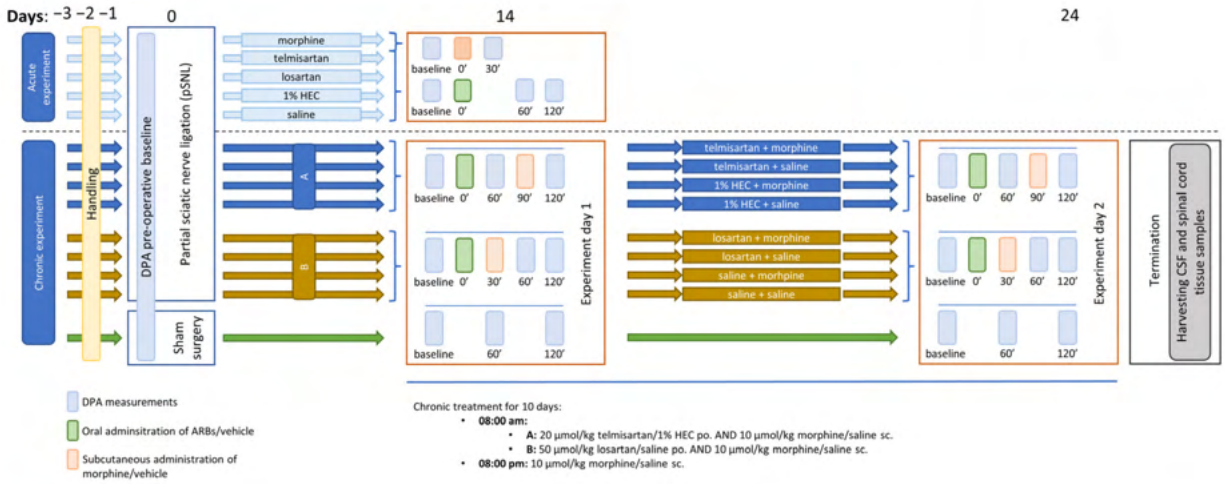

Figure 7. Schematic representation of the study design applied in the mononeuropathic pain model. The figure indicates the timeline of the acute and chronic experiments, involving DPA measurements, pSNL, treatment days and termination.

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First, baseline measurements were performed with DPA to determine the pre-operative PWT. Next, animals underwent partial sciatic nerve ligation (pSNL) based on the method described by Seltzer and colleagues and in our previous studies [56,64,66]. Briefly, under pentobarbital anesthesia (60 mL·kg^{-1} BW), animals were placed on heating pads to maintain 37 °C body temperature. Under aseptic conditions, the right sciatic nerve was exposed without muscle damage at the thigh level. The dorsal 1/2 of the nerve was tightly ligated with size 7-0 silicon treated silk suture. The wound was closed with two stiches. A separate group of animals used later as controls underwent sham-surgery during which the nerve was exposed without subsequent ligation.

Two weeks after the operation, PWTs of both (operated and intact) hind paws of the animals were determined. Animals were considered neuropathic if there was a 20% decrease in the average *PWT* value of the operated (right) paw compared to the unoperated (left) paw, calculated by the following formula:

$$\frac{PWT_{\text{unoperated paw}} - PWT_{\text{operated paw}}}{PWT_{\text{unoperated paw}}} \times 100 \quad (1)$$

Next, randomization was used to allocate animals to control and treatment groups. Following randomization, small adjustments were made where necessary to ensure that the mean baseline PWT values for each group were close to the same. This was carried out to ensure that any subsequent effects of the test compounds were equally well detected. After test compounds or vehicles were administered, the PWTs of animals were determined again at 60 and 120 min as depicted in Figure 1. A group of animals was used to determine the acute antiallodynic effect of different doses of telmisartan (20, 40 and 80 μmol·kg^{-1} BW, po.), losartan (50, 100 and 150 μmol·kg^{-1} BW, po.) and morphine (10 and 20 μmol·kg^{-1} BW, sc.). In experiments aiming to assess the effect of the combination of ARBs and morphine, the compounds were administered in a time-shifted manner (ARBs at 0 min and morphine at 30 or 90 min) so that the peak effect of the combination elements always coincided in time. The time intervals were chosen according to the data obtained in the first part of the study, i.e., the acute experiments with different doses of the two ARBs. Based on our previous studies, the peak effect of morphine was expected at 30 min after subcutaneous administration [14,24].

Another group of animals was subjected to chronic treatment with a combination of morphine and ARBs, using doses that were found to be subanalgesic in acute tests (20, 50 and 10 μmol·kg^{-1} BW for telmisartan, losartan and morphine, respectively). In this group, morphine was administered subcutaneously twice a day, while ARBs were administered orally once a day. On the 24th day following Seltzer surgery, another set of DPA measurements were carried out on chronically treated animals. Following this, animals were sacrificed by diethyl-ether overdose after which spinal cord tissue and CSF samples were obtained for further in vitro analyses.

4.3.2. Morphine Analgesic-Tolerance Model

A schematic summary of the applied opioid analgesic-tolerance protocol is presented in Figure 8. In the days preceding the start of the experiments, handling was performed in order to acclimatize the animals to the experimental conditions. This consisted of placing the animals in the tail-flick apparatus with blindfolds on, once daily. Acute thermal pain sensation was assessed using radiant heat tail-flick test (IITC Life Science, Woodland Hills, CA, USA) as described previously [67,68] with some modifications. Briefly, light-intensity was adjusted to set the control tail-flick latency under 4 s. Cut-off time was set to 8 s to avoid tissue damage. A baseline latency was measured before and after test compound or vehicle administration at the depicted time points (see Figure 2). All behavioral studies were performed by the same tester.

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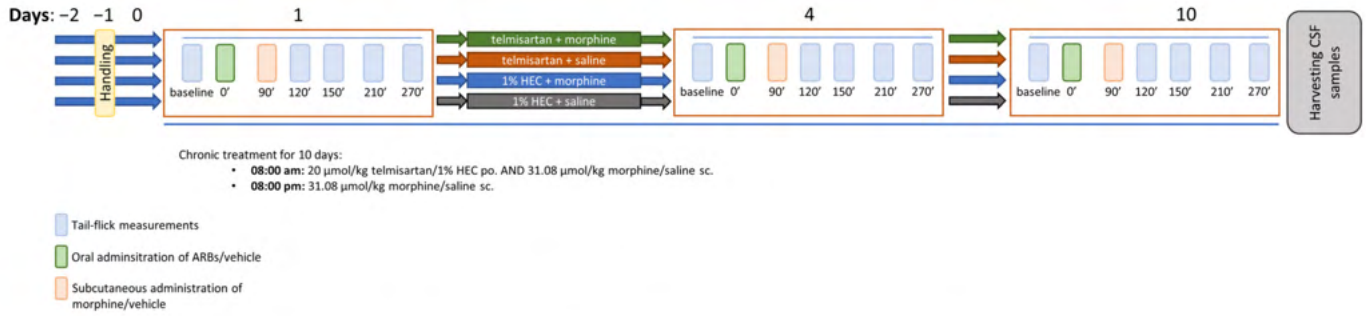

Figure 8. Schematic representation of the study design applied in the morphine analgesic-tolerance model. The figure indicates the timeline of the tail-flick measurements treatment days and termination.

Animals were randomized into groups and rendered tolerant to morphine by subcutaneous injections of 10 mg·kg^{-1} or 31.08 µmol·kg^{-1} BW morphine twice daily (8 a.m. and 8 p.m.) for 10 days. Saline injections (2.5 mL·kg^{-1} twice daily) were used in the control animals. In addition to morphine or saline, the animals also received telmisartan (20 µmol·kg^{-1}, po.) or 1% HEC (5 mL·kg^{-1}, po.) once daily (8 a.m.). The degree of analgesic-tolerance development was determined using the tail flick test on days 4 and 10. Following this, animals were sacrificed by diethyl-ether overdose and cerebrospinal fluid (CSF) samples were obtained for further in vitro analyses.

4.3.3. Morphine-Stimulated [^{35}S]GTPγS Binding Assay

Rats were decapitated and their spinal cords were quickly removed. The spinal cords were prepared for membrane preparation for the [^{35}S]GTPγS binding experiments, as described previously [56]. Briefly, spinal cord samples were homogenized in ice-cold TEM buffer composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl2, and 100 mM NaCl, pH 7.4 with a Teflon-glass homogenizer. The homogenate was centrifuged at 18,000 rpm for 20 min at 4 °C. The resulting supernatant was discarded, and the pellet was further incubated at 37 °C for 30 min in a shaking water-bath. Then, centrifugation was repeated as described above. The final pellet was suspended in ice-cold TEM buffer and stored at −80 °C. The protein content of the membrane preparation was determined by the method of Bradford, BSA being used as a standard [69].

In the [^{35}S]GTPγS binding experiments, we measured the GDP → GTP exchange of the Gαi/o protein in the presence of a given ligand. The nucleotide exchange was monitored by a radioactive, non-hydrolyzable GTP analogue, [^{35}S]GTPγS. The functional [^{35}S]GTPγS binding experiments were performed as previously described [48,49], with modifications. Briefly, the membrane homogenates were incubated at 30 °C for 60 min in TEM buffer containing 20 MBq/0.05 mL [^{35}S]GTPγS (0.05 nM) and 0.1–10 µM concentrations of the GlyT inhibitors (alone or in combination) and DAMGO. The experiments were performed in the presence of excess GDP (30 µM) in a final volume of 1 mL. Total binding was measured in the absence of test compounds, non-specific binding was determined in the presence of 10 µM unlabeled GTPγS and subtracted from total binding. The difference represents basal activity. The reaction was terminated by rapid filtration under vacuum (Brandel M24R Cell Harvester, Gathersburg, MD, USA), and washed three times with 5 mL ice-cold 50 mM Tris-HCl (pH 7.4) buffer through Whatman GF/B glass fibers. The radioactivity of the filters was detected in UltimaGoldTM MV aqueous scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter (Per-Form Kft, Budapest, Hungary). [^{35}S]GTPγS binding experiments were performed in triplicate and repeated at least three times.

4.3.4. Capillary Electrophoresis Analysis of Glutamate Content

L-glutamate content of cerebrospinal fluid (CSF) samples was measured by the capillary electrophoresis laser-induced fluorescence method carried out as described previously [56,70].

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Animals in our mononeuropathic pain model or morphine analgesic-tolerance model were sacrificed after 10 days of treatment as discussed above. CSF samples were obtained by cisterna magna puncture and centrifuged at 2000× *g*, at 4 °C for 10 min. Samples were frozen immediately and stored at −80 °C until further processing. On the day of the experiment, samples were deproteinized by mixing with 2 volumes of pure acetonitrile and centrifuged at 20,000× *g* for 10 min at 4 °C. Supernatants were collected, diluted five times with acetonitrile-distilled water solution (2:1; *v*/*v*) and subjected to derivatization with 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) (1 mg/mL final concentration) in 20 mM borate buffer pH 8.5 for 20 min at 65 °C. Five μM L-cysteic acid was used as internal standard.

Derivatized samples were analyzed using a P/ACE MDQ Plus capillary electrophoresis system coupled with a laser-induced fluorescence detector equipped with a laser source of excitation and emission wavelengths of 488 and 520 nm, respectively, (SCIEX, Framingham, MA, USA). Separations were carried out in polyacrylamide-coated fused silica capillaries (i.d.: 75 μm, effective/total length: 50/60 cm) using 50 mM HEPES buffer pH 7.0 containing 6 mM hydroxypropyl amino-β-cyclodextrin at 15 °C by applying −30 kV constant voltage.

4.3.5. Motor Function Testing

The effect of telmisartan, morphine and their combination on the motor function of animals was assessed using the rotarod assay (Rat RotaRod, model 7750, Ugo Basile, Italy). On the day preceding the experiment, animals were trained to stay on the rotating rod of the instrument. Rotation speed was set at 16 rotations per minute (RPM) and cut-off time was set at 180 s. On the day of the experiment, the animals were treated orally with the highest tested dose of telmisartan (80 μmol·kg^{-1}), the dose of morphine used in the combination experiments (10 μmol·kg^{-1}), their combination or their vehicles (1% HEC or saline, respectively). The motor coordination of animals was tested at the time of peak effect of test compounds. The compounds were administered in a time-shifted manner (ARBs at 0 min and morphine at 90 min) so that the peak effect of the combination elements coincided in time. Latency on the rotarod instrument was noted in s (fall-off time). High dose morphine (31.08 μmol·kg^{-1}) was used as positive control.

4.4. Statistical Analysis

All values are presented as mean ± standard error of means (S.E.M.). The statistical analysis was performed using GraphPad Prism software (version 8.0.1; GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. Two-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons between related groups. One-way ANOVA followed by Fisher's LSD post hoc test was used to compare independent groups. The post hoc tests were conducted only if F in ANOVA achieved $p < 0.05$. ROUT analysis was performed to identify outliers, with Q value = 0.5%.

5. Conclusions

AT1 antagonists attenuated mononeuropathic pain after acute treatment. A subanalgesic dose of telmisartan in combination with a subanalgesic dose of morphine proved to be effective against NP and morphine analgesic tolerance. Concomitantly, telmisartan administration also restored morphine potency in neuropathic rats and delayed morphine tolerance. Thus, in conditions with a loss of opioid efficacy, such as neuropathic pain or the development of opioid analgesic tolerance, telmisartan may make adequate pain control achievable. This can be carried out by finding entirely new pharmacological targets, or by exploiting multitarget treatment options that improve the efficacy of the available agents. These findings may provide the preclinical basis for the use of telmisartan in pain conditions with opioid impairment and raise the possibility of repurposing angiotensin receptor blockers in NP or opioid analgesic tolerance.

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

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

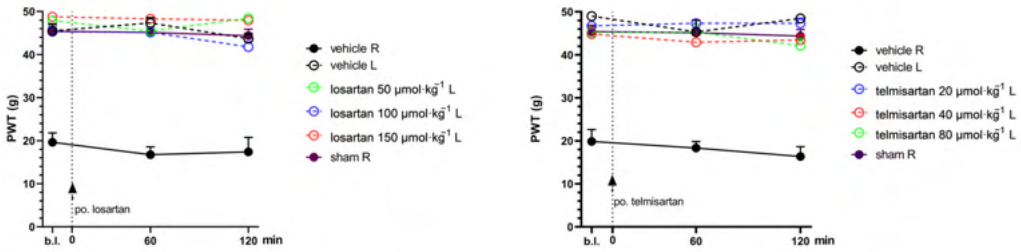

Figure A1. PWT values of the left (intact) paws of animals treated with losartan or telmisartan or their vehicles in the acute experiments. Graphs show the means of PWT ± S.E.M. in grams, after the pSNL in the indicated time points and treatment groups.

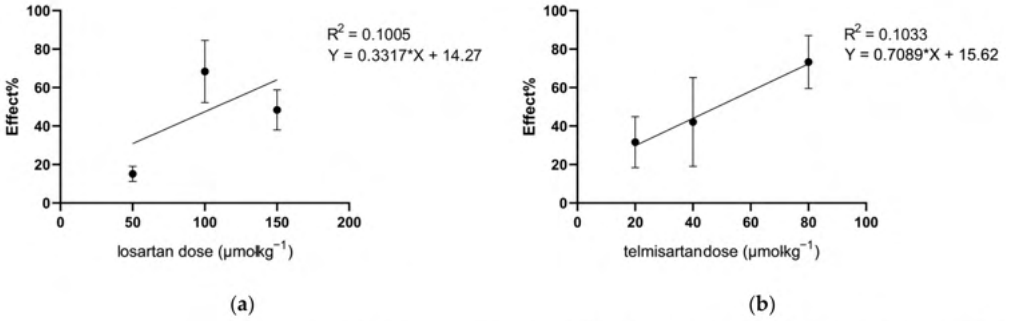

Figure A2. Dose–response curves of losartan (**a**) and telmisartan (**b**) in mononeuropathic animals obtained with DPA. Antinociceptive effect (%) was calculated at peak effect (60 or 120 min for losartan and telmisartan, respectively) as $(PWT_{peak\ effect} / PWT_{baseline}) \times 100 - 100$. ED_{50} values calculated from the linear regression are 107.7 and 48.5 µmol·kg^{-1} BW for losartan and telmisartan, respectively. Graphs show the means of the antinociceptive effect ± S.E.M.

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