Charles University in Prague

Faculty of Natural Sciences

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DIPLOMA THESIS

The Role of Angiotensin Receptors in Neuropathic Pain

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Prague 2012

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PRONOUNCEMENT

I declare that I have developed this diploma thesis independently, using only the literature registered in the list of references.

Prague, Signature

ACKNOWLEDGEMENTS

I would like to gratefully thank to all the people that contributed to creating my diploma thesis. First of all, I would like to thank my supervisor MUDr. Jiří Paleček, CSc for his thorough guidance and for creating mutually supportive environment. Great thanks belong to co-workers from the Department of Functional Morphology, Institute of Physiology, Academy of Science of Czech Republic, Kateřina Kopecká, RNDr. Gisela Zachařová and RNDr. Tomáš Soukup, CSc. I would also like to thank RNDr. Ivana Vaněčková, CSc. from the Department of Experimental Hypertension for her help with the blood pressure measurment.

ABSTRACT

Neuropathic pain is one of the most debilitating disorders. Currently available treatments for neuropathic pain are still unsatisfactory as they have only limited treatment effect and patients may suffer from unwanted side effects. Mechanism-based approaches to neuropathic pain treatment are considered to be more effective. Therefore multiple studies are dedicated to study the pathophysiological mechanisms of neuropathic pain. One of the possible underlying mechanism that causes neuropathic pain is neuroinflammation. Recent studies suggested that angiotensin II (main effector molecule of the renin-angiotensin system) via its receptors in the central nervous system may be involved in the neuroinflammatory processes. The aim of this study was to investigate the role of angiotensin receptor type 1 in the developement and maintenance of neuropathic pain induced in animal model. Spinal nerve ligation (L5) was used as a model of peripheral neuropathy. Our results showed that treatment with AT_1R blocker losartan markedly reduced thermal hyperalgesia and reduced increased sensitivity to mechanical stimuli in the SNL-operated rats.This indicates a possibly significant role of AT_1 receptors in the development of neuropathic pain, probably due to reduction of neuroinflammation in the nervous system. These findings and further study of the mechanisms by which AT_1R modulate neuroinflammation during peripheral neuropathy may bring new therapeutic approaches for neuropathic pain treatment.

Keywords: neuropathic pain, neuroinflammation, activated astrocytes, SNL, thermal hyperalgesia, mechanical allodynia, angiotensin II, AT_1R, losartan.

ABSTRAKT

Neuropatická bolest je jednou z chorob významně poškozujících pacienty. V současnosti dostupná léčba neuropatické bolesti je stále neúspěšná, neboť má pouze omezený léčebný efekt a pacienti trpí nežádoucími vedlejšími účinky. Zdá se, že vyšší účinnost by mohly vykazovat léčebné postupy založené na mechanismech vzniku neuropatické bolesti. Objasněním patologických mechanismů neuropatické bolesti se proto zabývá řada studií. Jedním z mechanismů, které mohou způsobovat neuropatickou bolest, je neuroinflamace. Nedávné studie naznačují, že angiotensin II (hlavní efektorová molekula renin-angiotensinového systému) může být zapojen v procesu neuroinflamace prostřednictvím svých receptorů v centrální nervové soustavě. Cílem této práce bylo za použití animálního modelu zjistit úlohu angiotensinových receptorů typu 1 na rozvoj a průběh neuropatické bolesti. Jako model periferní neuropatie jsme použili podvázání míšního nervu (SNL, L5). Naše výsledky prokázaly, že aplikace losartanu, působícího jako blokátor AT_1R, výrazně snížila tepelnou hyperalgezii a omezila zvýšenou sensitivitu vůči mechanickým podnětům u SNL-operovaných potkanů. To naznačuje možnou úlohu AT_1 receptorů v rozvoji neuropatické bolesti, pravděpodobně díky snížení neuroinflamace v nervovém systému po poškození nervu. Tyto poznatky a další studie mechanismů, kterými AT_1 receptory modulují neuroinflamaci v průběhu periferní neuropatie mohou mít význam pro vývoj nových terapeutických přístupů při lečbě neuropatické bolesti.

Klíčová slova: neuropatická bolest, neuroinflamace, aktivované astrocyty, SNL, tepelná hyperalgezie, mechanická alodynie, angiotensin II, AT_1 receptor, losartan.

LIST OF SYMBOLS AND ABBREVIATIONS

ACE	angiotensin converting enzyme
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ARB	angiotensin receptor blocker
ATG	angiotensinogen
ATP	adenosine-5'-triphosphate
AT_1R	angiotensin receptor type 1
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
CCI	chronic constriction injury
CGRP	calcitonin gene-related peptide
CNS	central nervous system
DH	dorsal horn
DRG	dorsal root ganglion
ERK	extracellular signal-regulated kinase
GABA	gamma-amino-butyric acid
IL	interleukin
MCP-1 (CCL2)	monocyte chemoattractant protein
NMDA	N-metyl-D-aspartate
NS	nociceptive specific
PAF	peripheral afferent fiber
PAG	periaqueductal gray
PK	protein kinase
PPARγ	peroxisome proliferator-activated receptor gamma
PWL	paw withdrawal latency
RAS	renin-angiotensin system
RVM	rostral ventricular medulla
STAT	signal transducer and activator of transcription
STT	spinothalamic tract
TNFα	tumor-necrosis factor alpha
WDR	wide dynamic dange

CONTENTS

1. INTRODUCTION

Pain is an early-warning physiological protective system. This phenomenon serves to detect and minimize contact with noxious stimuli and thus prevent tissue injury or damage. However under pathological conditions, pain loses its protective function and becomes a maladaptive sensory feeling. One of the difficult to threat pathological chronic pain syndromes often develop during states of peripheral neuropathy. Patients with neuropathic pain exhibit spontaneous sensation of pain, hypersensitivity to noxious stimuli (hyperalgesia) and painful reaction to normally innocuous stimuli (allodynia). The intractable and debilitating nature of these syndromes can substantially affect the quality of life of affected individuals. Currently available treatments for neuropathic pain don't have satisfactory effect. The analgesic therapy used also often brings different unwanted side effects such as nausea, sedation, constipation, tolerance and drug addiction. It is thought that mechanism-based treatment approaches would result in more effective treatment of neuropathic pain. Unfortunately, the mechanisms of development and maintenance of neuropathic pain remain unclear. Multiple studies dedicated to study neuropathic pain suggested several possible mechanisms that may be involved both in the peripheral and central nervous system. Neuroinflammation was suggested as one of the possible mechanisms leading to the neuropathic pain. Recently it was discovered that angiotensin II, the main efector molecule of the renin-angiotensin system, has also pro-inflammatory functions in the CNS. It was suggested that block of the angiotensin II signaling by its receptor antagonist losartan protects against neurodegenerative dysfunctions such as Parkinson's and Alzheimer's disease. I was also documented that angiotensin II via its receptors induces release of pro-inflammatory cytokines by glial cells in the CNS. These findings suggest a possible role of angiotensin II and its receptors in neuroinflammation. Based on these findings, the aim of this study was to investigate possible role of angiotensin receptors in the development of neuropathic pain, using animal model of peripheral neuropathy. Our findings may suggest new approaches for treatment of neuropathic pain patients in the future.

2. THE BASIC MECHANISMS OF PAIN. REVIEW

2.1 The importance of pain

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. The sensation of pain produces a reflexive retraction from the painful stimulus, and tendency to protect the affected body part while it heals and to avoid painful situation in the future. Pain is a very important part of the body's protective system.

Pain can be classified according to its duration, such as acute, subchronic and chronic pain. Acute pain usually lasts only until the noxious stimulus is removed and its intensity may change dramatically over a short period of time. Acute pain is a normal fysiological sensation triggered in the nervous system to alert to possible injury. Cutaneous pain (comes from the skin) evokes motor withdrawal or "flight" reaction. These protective responses help to interrupt exposure to the noxious stimulus and discontinue pain. Acute pain is generated by activation of nociceptors by noxious stimulus. Subchronic pain lasts for hours to days and chronic pain lasts for months to years. These two types of pain are characterized by spontaneous pain, hyperalgesia (increased response to noxious stimuli) and allodynia (pain evoked by normally innocuous stimuli) (Millan, 1999).

Another two categories of pain based on the mechanisms of its development are nociceptive and neuropathic pain. It is important to distinguish between these, as the mechanisms and treatment are different. Nociceptive pain results from tissue damage and can be subdivided into somatic and visceral pain. Neuropathic pain was defined by IASP as a pain resulting from a primary lesion or dysfunction of the nervous system, usually involving an element of sensory dysfunction. Whereas in nociceptive pain the nociceptors are activated by an adequate stimulus, neuropathic pain results from the activation of nervous system, even in absence of nociceptive input (Woolf, 2010).

2.2 Nociception

The Kyoto protocol of the International Association for the Study of Pain (IASP) Basic Pain Terminology clearly defined the term nociception as “the neural processes of

encoding and processing noxious stimuli" (Loeser and Treede, 2008). It is now noted that pain is a subjective experience while nociception is a physiological sensory process.

2.2.1 Transduction and transmission

Transduction is a process of converting stimuli, such as pressure, thermal energy, or chemical irritation, into a nerve signal (e.g., an action potential). It takes place in the periphery at the end of the sensory nerve cell whose terminals are sensitive to this type of activation. These nerve endings, known as nociceptors, are distributed throughout the body. The sensory information is transmissed from the periphery to the central nervous system (CNS) via primary sensory neurons, cell bodies of which are placed in the dorsal root ganglia (DRGs) (Tornsey et al., 2006).

Nerve fibers in the human peripheral nerves can be classified into three types by their diameter, structure and conduction velocity (Figure 2.1)(Millan, 1999):

1. C: thin (0,4-1,2 μm in diameter), unmyelinated and slowly-conducting (0,5-2,0 m sec-1);
2. Aδ: medium (2-6 μm), myelinated and of intermediate velocity (12-30 m sec^{-1});
3. Aβ: large (>10 μm), myelinated and fast (30-100 m sec^{-1}).

C, Aδ and Aβ fibers are typically present in proportions of cca 70, 10 and 20 %, respectively. Peripheral afferent fibers (PAF) also differ in their response properties to the different stimuli. Only C and Aδ, but not Aβ, fibers transmit nociceptive information. The sudden application of a painful stimulus, such as noxious heat, can elicit two distinct forms of pain in human skin: "first" and "second" pain. As Aδ nociceptors are characterised by high conduction velocity, they are responsible for the first pain. The second pain is transmitted by C-fibers (Treede et al., 1995). Aβ- fibers conduct information about innocuous stimuli applied to skin, muscle and joints and do not contribute to pain under normal conditions (Basbaum et al., 2009). Aδ- and C-fibers transmit afferent information about potentially damaging stimuli. Aδ-fibers are thinly myelinated relatively rapid conducting fibers, responding to intense mechanical stimuli and are subdivided into two classes by the responsiveness to intense heat (Miller et al., 2009). Type I high-threshold mechanical nociceptors (HTM) are activated by mechanical and chemical stimuli and are characterized by high heat threshold (>50°C). On the other hand, type II Aδ-nociceptors posess lower heat threshold, but a very high mechanical threshold (Basbaum et al., 2009).

The unmyelinated slowly conducting C-fibers are also polymodal. They are able to respond to noxious thermal mechanical and chemical (e.g. capsaicin) stimuli and inflammatory mediators (Nagy and Rang, 1999). C-nociceptors are subdivided into peptidergic and nonpeptidergic population. Peptidergic C-fibers release the neuropeptides, substance P, calcitonin-gene related peptide (CGRP) and respond to nerve growth factor (NGF). The nonpeptidergic neurons express the c-Ret neurotrophin receptor, which is activated by glial-derived neurotrophic factor (GDNF) (Basbaum et al., 2009). Different sensory modalities are defined by the expression of multiple sensory channels and receptors on the terminal branches of nociceptors that allow them to respond to the specific noxious stimuli (Liu and Ma, 2011).

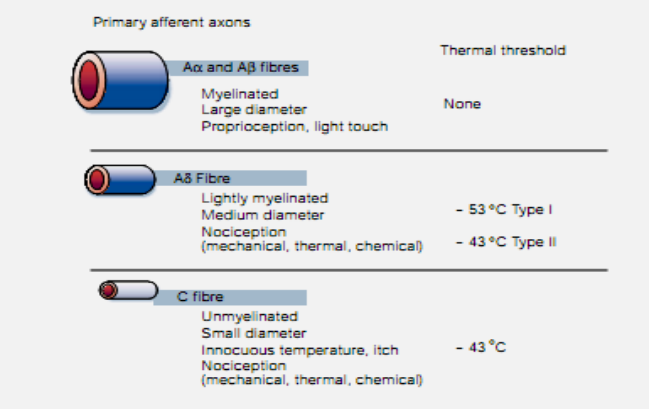

Figure 2.1 Types of peripheral nerves due to their size and myelinisation. Adapted from Julius and Basbaum, 2001.

2.2.2 Dorsal root ganglion cells

Nociceptors, like other somatovisceral sensory receptors, have their cell bodies in dorsal root (or cranial nerve) ganglia. The dorsal roor ganglion (DRG) cells are pseudounipolar cells. They give off a single process, which divides into peripheral and central branches. The peripheral terminals of nociceptors innervate a target organ and the central branch passes into the spinal cord and transmits sensory information from the target organ to the CNS (Fig. 2.2). DRG neurons are classified by their size. Cells with the largest diameters give rise to rapidly conducting, myelinated Aβ- fibers. Small- and medium-diameter neurons give rise to Aδ- and C-fibers respectively (Liu and Ma, 2011).

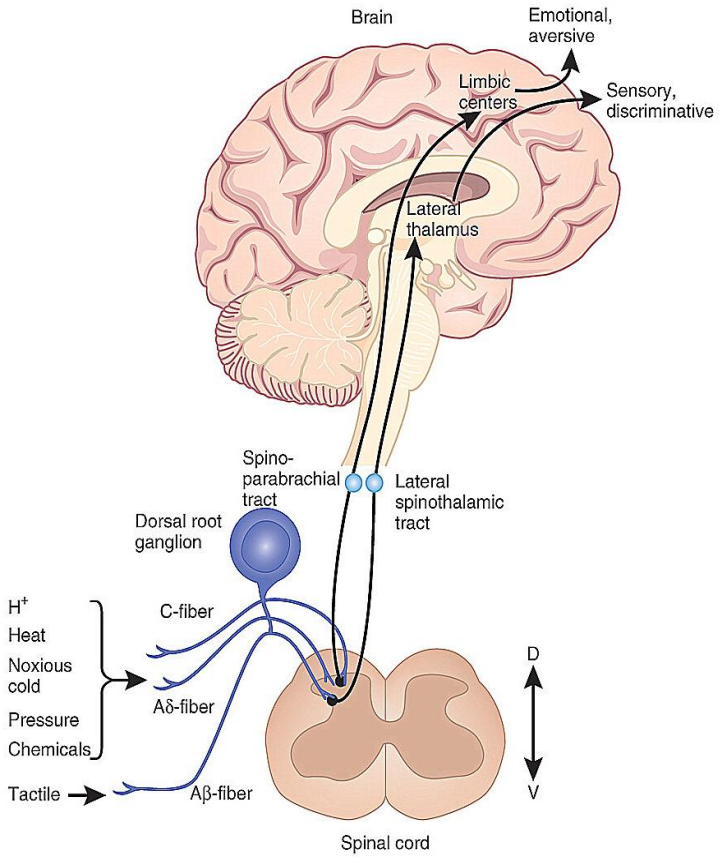

Figure 2.2 Transmission of different sensory modalities from the periphery to the supraspinal centers of the CNS. Adapted from Kuner, 2010.

2.2.3 The spinal nociceptive network

The spinal cord is so-called „gateway“ for the relay of nociceptive information to higher centers of the nervous system. The gray matter of the spinal cord is divided on a cytoarchitectonic basis into 10 laminae. The two most superficial laminae, I and II_o (the outer part of lamina II) , together with deeper laminae, V and VI, and lamina X are predominantly implicated in the reception, processing and rostral transmission of nociceptive information. The peptidergic C-fibers project to lamina I and the most dorsal part of lamina II. The nonpeptidergic afferents terminate in mid-region of lamina II. High threshold Aδ-fibers terminate predominantly in lamina I and II. Both types of nociceptors also provide a comparatively weak input to lamina V and VI (Basbaum et al., 2009). In contrast to small calibre nociceptive fibers, larger, low threshold Aβ fibers selectively innervate deeper dorsal horn laminae (III-IV, less markedly in laminae V/VI) or ascend to

dorsal column nuclei in the brainstem immediately upon entering the spinal cord (Fig. 2.3) (Berger et al., 2011).

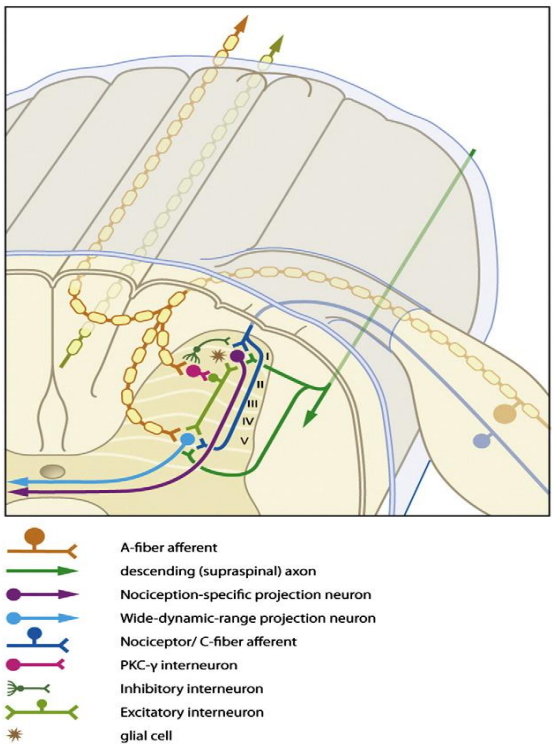

Figure 2.3 The spinal nociceptive network. Adapted from Berger et al., 2011.

There are three types of neurons in the dorsal horn (DH). First, nociceptive-specific (NS) neurones which are activated exclusively by high intensity, noxious stimuli mediated by C and Aδ-fibers. They are most concentrated in the superficial dorsal horn. Secondly, wide-dynamic range (WDR) neurones which are found predominantly in laminae V and respond to both noxious and innocuous peripheral stimuli. WDR neurones are excited by thermal, mechanical and chemical stimuli mediated via both C and Aδ as well as Aβ-fibers. The third class of neuron is non-nociceptive (NON-N). They are found primarily in laminae II, III and IV, but a few may also occur in lamina I (Berger et al., 2011).

It was suggested that between the low-threshold afferents and NS projection neurons there is an „silent“ circuit. The important component of this circuit are the excitatory interneurons in the innermost part of lamina II. These neurons, expressing the γ-isoform of protein kinase C (PKC-γ), receive input from Aβ and C-mechanoreceptors, activated by the innocuous stimuli (Neumann et al., 2008). Under the normal condition PKC-γ-interneurons are inhibited by glycinergic and gamma-amino-butyric acid

(GABA)ergic interneurons and thus information about innocuous stimuli is not gated to NS projection neurons (Berger et al., 2011).

Both types of the projecting neurons (NS and WDR) receive input from the inhibitory neurons. In the spinal nociceptive network there are two inhibitory systems – segmental (glycinergic and GABAergic interneurons) and non-segmental. The non-segmental inhibitory system consists of descending (aminergic) fibers from the higher centers of CNS (Saade and Jabbur, 2008). The serotonergic inhibition originates from the nucleus raphe magnus and noradrenergic inhibitory fibers derive from the locus coeruleus (Berger et al., 2011).

2.2.4 The supraspinal processing of nociceptive information

Projection neurons in the spinal cord transmit impulses from dorsal horn to the higher brain centers. These projection neurons form the ascending pathways. The main ascending pathways are the spinotalamic (STT) and spinoreticulothalamic tracts (Fig. 2.3). STT transmit proprioceptive and mechanoceptive signals to the lateral nuclei of the thalamus. Spinoreticulothalamic tract project to the brainstem structures. The thalamic neurons projecting to the somatosensory cortex provide information about the locatoin and intensity of the noxious stimulus. The cingulate and insular cortices receive information via connections with the brainstem (parabrachial nucleus) and amygdala and maintain the emotional aspect of the pain sensation. Another important structures of the brain nociceptive system are the rostral ventral medulla (RVM) and periaqueductal gray (PAG). These structures, located in the midbrain, control descending feedback systems that regulate output fromthe spinal cord (Fig. 2.4) (Basbaum et al., 2009).

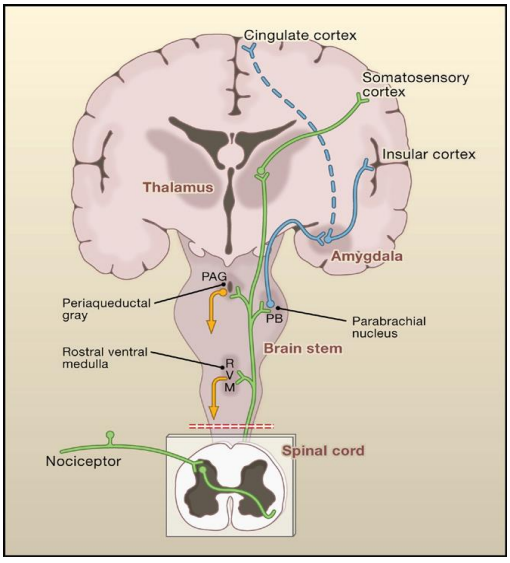

Figure 2.4 The main brain structures involved in supraspinal procession of nociceptive information. Adapted from Bauman et al., 2009.

2.3 Glial cell types in CNS and PNS

The nervous system consists of neuronal and non-neuronal (glial) cells. There are three types of glial cells in CNS - astrocytes, oligodendrocytes and microglia. In PNS Schwann cells perform the functions of oligodendrocytes.

Astrocytes are star shaped cells, isolate neurons and oligodendrocytes to help maintain the invironment of the CNS by regulating extracellular ion concentrations of K+ and Ca2+ as well as neurotransmitter concentrations via uptake. Astrocytes form a matrix that keep neurons in place and isolate synapses (limits the dispersion of transmitter substances released by terminal buttons). Astrocytes also provide a phagocytosis and nourishment to neurons – they receive glucose from capillaries, metabolize it into lactate and release the lactate into the extra cellular fluid surrounding the neurons. They also play a prominent role in establishing and functioning of the blood-brain barrier (BBB). Satellite glial cells surround the sensory neurons in DRGs and are thought to be an equivalent of astrocytes.

Oligodendrocytes produce the myelin sheath which insulates axons. Unlike Schwann cells of the PNS, one oligodendrocyte cell form segments of myelin sheaths of numerous neurons. Satellite oligodendrocytes are functionally different from the rest oligodendrocytes. They do not serve an insulating role, but regulate the extracellular fluid.

Microglia are the smallest glial cells and represent the main form of immune defence in the central nervous system. Resting (ramified) microglia are functionally inactive and uniformly dispersed unlike the other glial cells. BBB prevents most infections from reaching the nervous tissue. If infectious agents are able to cross the blood-brain barrier, a part of ramified microglia undergoes activation to decrease inflammation and to destroy the infectious agents before they damage the sensitive neural tissue (Raivich, 2005). Microglia can be activated by different factors e.g. proinflammatory cytokines, agonists of glutamate receptors, necrosis factors and others. Microglia posess the antigen presenting, cytotoxic and inflammatory mediating signaling of activated non-phagocytic microglia, they are also able to phagocytose foreign materials and present the immunomolecules for T-cell activation. Phagocytic microglia migrate to the site of the injury and secrete pro-inflammatory factors to promote more cells to proliferate.

Schwann cells are the supporting cells in the PNS. They wrap themselves around nerve axons, but unlike the oligodendrocytes single schwann cell makes up a single segment of an axon's myelin sheath.

2.4 Neuropathic pain

Neuropathic pain differs from other pain conditions where the pain sensation is primarily due to nonneural tissues damage injury. Neuropathic pain is induced by a lesion or damage of the nervous system (Woolf, 2010).

Due to the type of damage or the underlying pathological mechanisms neuropathic disorders are classificated to:

- mechanical nerve injury, e.g. carpal tunnel syndrome, vertebral disk herniation;
- metabolic disease, e.g. diabetic polyneuropathy;
- neurotropic viral disease, e.g. herpes zoster, human immunodeficient virus (HIV) disease;
- neurotoxicity, e.g. by chemotherapy of cancer or tuberculosis;
- inflammatory and/or immunologic mechanisms, e.g. multiple sclerosis;
- nervous system focal ischemia, e.g. thalamic syndrome anesthesia dolorosa;
- multiple neurotransmitter system dysfunction, e.g. complex regional pain syndrome CRPS (Zimmermann, 2001).

Neuropathic pain is clinically characterized by spontaneous pain, amplified pain response to noxious stimuli (hyperalgesia) and/or painfull reaction to innocuous stimuli (allodynia) (Baron et al., 2010).

2.4.1 Pathophysiology of neuropathic pain

Neuropathic pain is a common form of the chronic pain and current pharmacological treatments are still unsatisfactory. In clinical practice drugs, such as channel blockers, antidepressants, anticonvulsants or opioids, give limited effect and patients may suffer from unwanted side effects (Attal et al., 2009). Mechanism-based

treatment approaches are considered to be more effective, so multiple studies are dedicated to investigate pathophysiological mechanisms of neuropathic pain.

Neuropathic pain states are characterized by neuroplastic changes that occurs in primary afferent terminals (peripheral sensitization) and also in the spinal cord and brain (central sensitization). These features results in alteration in the processing of sensory information (hyperalgesia and allodynia).

2.4.2 Peripheral mechanisms

Under pathological conditions such as nerve damage, peripheral afferents exhibit ectopic activity and/or hyperexcitability. Ectopia is a spontaneous electrical activity in peripheral afferents, which may originate from blocade of voltage-operated ion channel trafficking during axon damage. It is a result of spontaneous oscillations in the membrane activity and generation of action potentials by the injured fibers (Liu et al., 2000).

Following nerve injury, primary afferent neurons may exhibit hyperexcitability, which occurs either at the peripheral ending of the nerve, or at the level of DRG. These changes include also overexpression and phosphorylation of receptors.

Another mechanism suggested to play a role in neuropathic pain is phenotypic switching of primary afferents. Injury of peripheral neuron triggers de novo expression of neuromodulators such as substance P, brain-derived neurotrophic factor (BDNF) or calcitonin-related peptide (CGRP) in DRG large-sized neurons and subsequently may cause sensitization of dorsal horn neurons (Weissner et al., 2006).

Additionally, the structural plasticity of primary afferents in models of peripheral neuropathy has been reported. Collateral sprouting of afferent fibers in the skin and in the spinal cord, sprouting of sympathetic neurons in the DRG was observed under neuropathic conditions (Berger et al., 2011).

2.4.3 Central mechanisms

Central sensitization plays a key role in the development and maintenance of the neuropathic pain states. This abnormal functional state of neurons and the circuits in the spinal cord dorsal horn is characterized by increased in membrane excitability, synaptic efficacy or reduced inhibition. These changes in the properties of neurons result in

reduction of activation threshold, increased responsiveness to peripheral stimulation and enlargement of their receptive fields.

The abnormal peripheral activity may result also in modulation of synaptic activity in the spinal cord dorsal horn. It is well known, that glutamate is the principle fast transmitter of primary afferent neurons. It binds to several types of ionotropic receptors on the postsynaptic membrane: α-amino-3 hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors (Joshimura and Jessell, 1990). Under neuropathic pain states increased release of glutamate takes place in the spinal cord superficial dorsal horn - the termination site of the polymodal nociceptive C and Aδ fibers.This leads to phosphorylation of glutamate receptors and enhanced transcription of their genes (Woolf and Salter, 2000). Thus, activity-depending synaptic plasticity results in pain hypersensitivity. Under pathological conditions increased release of different active substances into the spinal cord by nociceptors also takes place. Substance P, calcitonin gene-related peptide (CGRP), brain-derived neurotrophic factor (BDNF), bradykinin appears to contribute to central sensitization. These substances binds to their specific receptors and activate intracellular signalling pathways in DH neurons. For example, PKA activated by bradykinin or CGRP, phosphorylates NR1 subunit of NMDA receptor, leading to their increase response to glutamate and, subsequently, to increased membrane excitability and facilitation of synaptic strength (Latremoliere and Woolf, 2009). These features contributes to development of abnormal hypersensitivity by reduction in threshold for activation by peripheral stimuli.

Another mechanism contributing to central sensitization under neuropathic pain states is disinhibition in the spinal cord dorsal horn. Glutamate excitotoxicity induced by changes in functional properties of NMDAR may trigger the apoptosis of inhibitory interneurons (Scholz et al., 2005). BDNF and prostaglandins also may contribute to the reduction of segmental inhibition (Coull et al., 2005). Decrease in the synthesis and release of inhibitory transmitters (GABA and glycine) leads to the state of disinhibition (Suzuki et al., 2004).

One of the possible mechanisms leading to central modulation are suggested structural changes in the spinal cord resulting in alterations of synaptic functions. Peripheral nerve injury can induce the degeneration of C-fiber terminals in lamina II (Arvidsson et al., 1986). Loss of presynaptic input and regenerative response of the injured

neurons trigger the sprouting of myelinated Aβ-fibers from laminae III-IV into the superficial part of DH, where they contact with NS neurons (Shortland et al.,1997).

Functional and structural changes in spinal cord during central sensitization cause functional changes in supraspinal structures involved in nociceptive transmission such as thalamus, amygdala, anterior cingulare cortex, parabrachial nucleus, PAG, superior colliculus and prefrontal cortex (Latremoliere and Woolf, 2009).

It is well known that the injury of peripheral afferent fibers initiates the inflammation response of the neural tissue – neuroinflammation. It's a highly compex process involving neuronal and non-neuronal cells, which undergo pathological alterations (Berger et al., 2011). Neuroinflammation plays a prominent role in induction of neuropathic pain and its mechanisms will be described in details in the next chapter.

2.5 Neuroinflammation

One of the possible underlying mechanisms of neuropathic pain is neuroinflammation. Following peripheral nerve injury immune system becomes activated both in the periphery and CNS. The pathogenesis of neuroinflammation is based on the activation of inflammatory and immune-like glial cells, for example mast cells, neutrophils, macrophages and T-cells at the peripheral level and microglia and astrocytes in the CNS (Moalem and Tracey, 2006).

2.5.1 Changes in the periphery after peripheral nerve injury

Peripheral nerve injury leads to immediate activation of immune cells within damaged nerve. Mast cells residing in the nerve are activated the first and become degranulated. It leads to release of histamine (hist) and tumor-necrosis factor-α (TNF-α) by mast cells. These mediators sensitise nociceptors (Koda and Mizumura, 2002) and on the other hand, induce recruitment of neutrophils and macrophages (Yamaki et al., 1998). The mechanism of the mast cells activation in the peripheral afferent fibers is not clear, but probably can be induced by increased concentration of adenosine and bradykinin released at the site of the injury (Moalem and Tracey, 2006).

Chemokines released by mast cells induce migration and invasion of neutrophils (polymorphonuclear leukocytes) from small blood vessels into the damaged nerve at the site of injury (Perkins and Tracey, 2000). There is some evidence that neutrophils contribute to neuropathic hyperalgesia by releasing cytokines (TNF-α) (Bennett et al., 1998) and defensins contributing to macrophages activation (Scapini et al., 2000).

Under peripheral nerve injury the recruitment of macrophages was also observed. Either resident or blood-derived macrophages are activated by the chemotactic molecules in microinvirontment (moncyte chemoattractant protein-1, MCP-1; macrophage inflammatory protein-1α, MIP-1α; IL-1β) released by immune cells. Macrophages secrete prostaglandins, inducing sensitization of peripheral afferent fibers, and different cytokines, contributing to neuroinflammation (Moalem and Tracey, 2006).

Schwann cells that envelop injured axons also undergo activation. IL-1β released from macrophages induce NGF upregulation in Schwann cells (Heumann et al., 1987). Additionally, those peripheral glial cells synthesize different algesic mediators (TNF, IL-1, IL-6, ATP), but factors regulating the release of these molecules are still unclear (Marchand et al., 2005).

Inflammatory and immune cells also release a range of mediators, such as bradykinin, serotonin, eicosanoids, ATP, reactive oxygen species, neurotrophins (Dray, 1995), which contribute to neuropathy hyperalgesia by sensitization of peripheral afferents.

2.5.2 Changes in the CNS after peripheral nerve injury

The peripheral nerve injury leads to changes in the central processing of sensory information at the spinal cord level. It was suggested that neuroinflammation may play an important part in this process. There are two types of immune cells, involved in the inflammatory process at the spinal level – resident glial cells and blood-derived leukocytes. Extravasation of macrophages or T-cells into lumbar spinal cord occurs after peripheral L5 nerve transaction, but their role in neuroinflammation is still unclear (Sweitzer et al., 2002).

Microglia, oligodendrocytes and astroglia represent glial cells in CNS. Microglial cells plays a prominent role in the initial phase of the neuroinflammation. The mechanisms by which microglia became activated are still unknown, but evidence indicates that those glial cells express P2X4 on their surface. Upon peripheral nerve injury or

neuroinflammation an upregulation of P2X4 receptors occurs especially in the microglial cells. ATP released by injured neurons, binds to P2X4 receptors and results in cytokines expression and release by microglial cells (Le Feuvre et al., 2002). The released cytokines (TNF-α, IL-6) initiate a self-propagating mechanism of cytokine expression by microglial cells (Klein et al., 1997). Fractalkine (CX3CL1) is not expressed in microglia, but these cells constitutively posess receptors for this chemokine, which are upregulated during peripheral neuropathy states (Verge et al., 2004). The monocyte chemoatractant protein-1 (MCP-1 or CCL2) is considered an important molecule signaling microglial activation. Upregulation of CCL2 expression in microglia occurs after peripheral nerve injury (Abbadie et al., 2003). The activation of microglia is associated with phosphorylation of p38 MAP kinase and activation of astrocytes it the same spinal cord segment (Marchand et al., 2005). Morphological changes (proliferation and differentiation) are observed in microglia within a day and in astroglia a few days later after peripheral nerve injury (Romero-Sandoval et al., 2008). Activated astroglia is the main source of CCL2, while central branches of DRG neurons were shown to be a major source of CCL2 after peripheral nerve injury (Van Steenwinckel et al., 2011). Expression of this chemokine is regulated by the activity of MAPK and c-Jun-N-terminal kinase (JNK). Following peripheral nerve injury phosphorylation of JNK in astroglia upregulated CCL2 expression and was observed in DRG as well as in the spinal cord (Gao et al., 2009).

2.6 Angiotensin II in neuroinflammation

The renin-angiotensin system (RAS) is a complex system, regulating blood pressure, vasoconstriction, sodium intake and potassium excretion in mammals. The blood pressure decrease causes the secretion of renin into plasma by renal juxtaglomerular cells. The glycoprotein angiotensinogen (ATG) produced in liver is cleaved by renin into angiotensin I, which is subsequently converted to an active substance angiotensin II (Ang II) by the angiotensin converting enzyme (ACE) (Benigni et al, 2010). ACE is predominantly expressed in endothelial surface of lung vessels and has two principal functions: to convert Ang I to Ang II and to hydrolyze bradykinin into inactive form (Fleming, 2006). Ang II, the essential effector molecule of the RAS, acts through two types of G protein-coupled receptors: angiotensin type 1 and type 2 receptors (AT_1 and AT_2). AT_1 receptors are expressed in kidney, heart, vascular smooth muscule, pituitary gland (Begini et al, 2010) but also in the brain, spinal cord and DRGs (Pavel et al, 2008). Most of the physiological effects of Ang II such as blood pressure increase, aldosterone release,

salt retention and stimulation of the sympathetic nervous system are mediated by AT_1 type receptor. AT_2 receptor is highly expressed in developing foetal tissues and decreases after birth. Activation of these receptors induces vasodilatation and artery remodeling.

RAS components are present not only in the circulatory system but local RAS systems were described in many organs, including nervous system. All the components of the RAS were found in the brain – ATG, renin, Ang I, Ang II, ACE and AT receptors. The brain local RAS appears to play a prominent role in the central blood pressure regulation, maintenance of the blood-brain barrier, food and water intake, regulation of transcription and translation, motor control and behavior and emotions (Paul et al, 2006).

Recent experimental studies suggested hypothesis that RAS in the CNS is involved in the inflammatory processes. The main source of angiotensinogen, the precursor molecule for Ang II in the central nervous system are astrocytes (Stornetta et al., 1988). AT_1 and AT_2 receptors as well as both forms of angiotensin converting enzymes were founded in these glial cells (Gallagher et al., 2006). Ang II via its receptors causes activation of several intracellular signal pathways, including Janus kinase (JAK) 2, signal transducer and activators of transcription (STAT) 3 phosphorylation resulting in IL-6 secretion by astrocytes (Kandalam and Clark, 2009). It is also known that activation of JAK in astrocytes under pathological states leads to enhanced CCL2 expression, hence potentiation of excitatory neurotransmitter signaling in the dorsal horn (Gao et al., 2009). Increased expression of specific chemokines leads to the recruitment of infiltrating inflammatory cells into injured neurons and intencifying of inflammatory response.

Several studies indicated that treatment with blockers of AT receptors has a neuroprotective effect in neurodegenerative deseases, such as the Alzheimer's (Wang et al., 2007) and Parkinson's (Mertens et al., 2010). The neuroprotective actions of AT blocators are probably associated with reduction of oxidative stress – a key player of the disease. It is well established that Ang II stimulates synthesis of oxygen species, that induce mitochondrial disfunction and cellular damage, through activation of NAD(P)H oxidase in vascular smooth muscle cells (Griendling et al., 1994). Similar effect could be induced in the nervous tissue. Additionally, AT blocators activate peroxisome proliferator-activated receptor gamma (PPARγ) (Garrido-Gil et al., 2012). PPARγ has been detected in macrophages, neurons and glial cells and plays a role in microglial activation (Bernardo et al., 2000). It has been established that PPARγ activation inhibits production of

inflammatory cytokines in macrophages and monocytes (Ricote et al., 1998; Jiang et al., 1998).

2.7 Animal models of neuropathic pain

Rodent models have been widely used for various studies of neuropathic pain mechanisms and drug testing (LaBuda and Little, 2005). These models are mainly based on injury of the nervous system either on the periphery or in the CNS. The most frequently used models of peripheral neuropathy are peripheral nerve injuries including (Zimmermann, 2001):

- partial nerve lesion (PNL) - with a tight ligation around part (about 50%) of the nerve fiber (Seltzer et al., 1990);

- chronic constriction injury (CCI) – induced by placing several loose ligatures around the nerve leaving a lumen of less than the diameter of the original nerve (Bennett et al., 2000);

- spared nerve injury (SNI) – ligation/transaction of the tibial and common-peroneal nerve branches of the sciatic nerve with the intact sural nerve (Decosterd et al., 2002; Decosterd and Woolf, 2000);

- Spinal nerve ligation (SNL) - tight ligation or transection of L5 and/or L6 spinal nerve (Kim and Chung, 1992) (Fig. 2.5).

The spinal nerve ligation leads to long-lasting mechanical and cold allodynia, heat hyperalgesia and spontaneous pain (Chung et al., 2004). There are different modifications of the SNL model. In the original model by Kim and Chung (1992) the ventral ramus of the spinal nerve of one or more dorsal ganglia is tightly ligated and/or cut before the spinal nerve joins into the common nerve. Thus, injured and uninjured afferents innervate a particular tissue and interact between themselves. In that case the loss of population of afferents innervating appropriate tissue results in functional changes of neurons that remain (Gold, 2000). Some experimentators used modification of this model, where only L5 spinal nerve is ligated with a cut of the nerve distal to the ligation. This surgery is easier to perform in comparison with combined L5/L6 ligation but also leads to the same symptoms (Li et al., 2000).

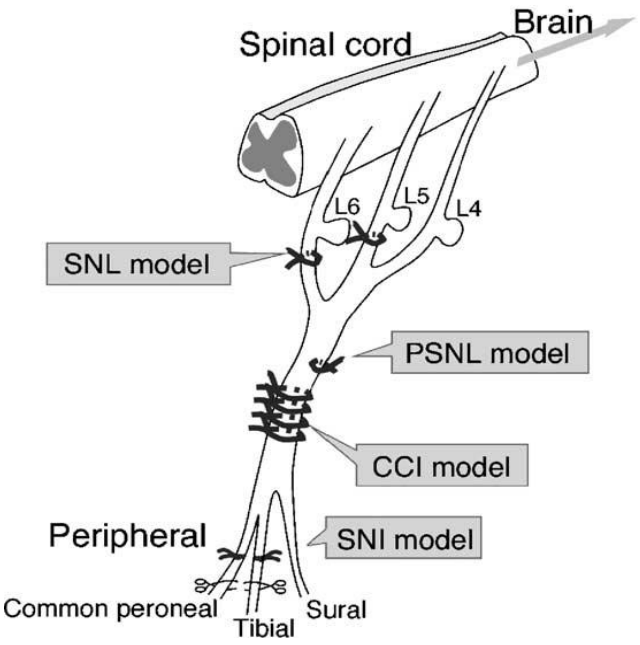

Figure 2.5 Schematic illustration of the most frequently used animal models of neuropathic pain. Adapted from Ueda, 2006.

3. AIMS OF THE STUDY

Neuropathic pain represents a chronic disease that is largely resistant to current options in treatment. The difficulty of the treatment is also due to lack of knowledge about the underlying mechanisms of its development and maintenance. One of the suggested possible mechanisms of neuropathic pain is neuroinflammation in CNS especially in the spinal cord. Activation of glial cells and modulatory effect of several cytokines and chemokines were identified to play a role in this process. Recent studies proposed also participation of angiotensin II and its receptors type 1 in neuroinflammation process in the CNS. In our experiments we wanted to investigate possible effect of AT_1R blocker – losartan, on the development of increased sensitivity to thermal and mechanical stimuli in a model of peripheral neuropathic pain.

Individual goals:

- **Establish the SNL model of peripheral neuropathy in the rat.**
- **Test mechanical and thermal thresholds after SNL induction.**
- **Investigate the effect of losartan administration (p.o. and i.t.) on the development of increased sensitivity to thermal and mechanical stimuli following SNL.**
- **Start to study expression of AT_1R in the DRG.**

4. MATERIALS AND METHODS

Male Wistar rats, weighing between 250 and 350 g, were used. All measures were taken to minimize the number of animals used. Animals were kept on a 12-h light/dark cycle, with food and water *ad libitum*. The experiments were carried out during the light phase of the cycle. All experiments were approved by the local Institutional Animal Care and Use Committee and were consistent with the guidelines of the International Association for the Study of Pain, the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the European Communities Council Directive of 24 November 1986(86/609/EEC).

Surgical procedures

Spinal nerve ligation

The spinal nerve ligation (SNL) was performed on the left side of the rat under ketamine (100 mg/kg i.p., Narketan, Zentiva) and xylazine (25 mg/kg i.p., Xylapan, Zentiva) anesthesia. The day of the surgery is referred to as day 0. The ligation of L5 spinal nerve was performed similar to the method described by Kim and Chung (Kim and Chung, 1992). To create the SNL model, the fur was shaved and the skin was disinfected with antiseptic (Jodisol, Spofa), an incision was made on the left side of the spine at the L4-S1 level. The left transverse process of L6 vertebra was first removed, L5 spinal nerve was exposed and then tightly ligated with 5.0 silk thread. Complete hemostasis was confirmed and the wound was surgically closed in layers.

Intrathecal catheter implantation

Catheters were made with two polyethylene tubings of different size PE-5 and PE-10. PE-10 tube was first bended to the necessary form and then connected on one side with the PE-5 tubing with epoxy glue. Prepared catheter was filled with sterile saline. Catheter implantation was performed under deep anesthesia simultaneously with the SNL operation. The fur was shaved and the skin was disinfected with antiseptic (Jodisol, Spofa) then longitudial incision was made at the region between L3-S1 spinal processes. Spinal muscles were retracted and small opening was made in dura mater with microforceps at the intravertebral area L5-L6. The PE-5 end of the catheter was inserted to the subarachnoidal space and fixed to the vertebral column with dental cement. The wound was then surgically closed with sutures. PE-10 end of the catheter was externalized in the occipital region

and sealed. The animal was allowed to recover from the surgery for 24 hrs before any behavioral testing.

Administration of Losartan

Losartan was administered *per os* (p.o.) or intrathecally (i.t.). For the p.o. administration losartan (Lozap, Zentiva) was dissolved in the drinking water for the use during the experiment. The losartan solution was changed daily and the amount consumed was registered. The average amount of losartan given this way was 100 mg/kg of body weight. For the intrathecal application - 10 µl of 20 µmol solution of losartan (Losartan Potassium, Tocris) was injected into the catheter under aether anesthesia. This was followed by 40 µl of saline administrated into the catheter to clear the catheter dead space. Intrathecal injections were made every day at 11 a.m. from the day 0 after operation after the behavioral testing.

Behavioral test procedures

The behavioral tests were performed before the SNL and 1, 3, 5, 7, 9, 12 and 14 days after the SNL operation.

Testing mechanical responsiveness

Von Frey filaments

Paw withdrawal responses to mechanical stimuli were tested with von Frey (VF) filaments calibrated on a top-loading electronic balance, where the force needed to bend the filament was measured. 4 different von Frey filaments ranging from 40 to 150 mN (40; 60; 100; 150) were used. The rats were placed on a metal mesh floor covered by a non-binding, clear plastic cage and were left to adapt to the testing environment for at least 20 min. Each stimulus was applied from below the mesh floor 5 times, pokes were spaced 2 s apart, and sequential monofilaments were applied in ascending order of stiffness. The number of withdrawal responses to the VF filament stimulation was recorded. Results from each hind paw were averaged and SEMs calculated. Baseline responses were determined in all animals before any experimental procedure.

Testing mechanical responsiveness with electronic von Frey filament

Mechanical withdrawal threshold was assessed on hind paws using electronic dynamic plantar von Frey aesthesiometer (IITC Inc Life Science). The mechanical withdrawal threshold was the maximum pressure exerted (in grams) that triggered the paw withdrawal. Each stimulus was applied 5 times with 5 min between trials. Results from each hind paw were averaged and SEMs calculated. Baseline withdrawal thresholds were determined in all animals before any experimental procedure.

Testing responsiveness to thermal stimuli

Paw withdrawal latencies (PWL) to radiant heat stimuli were determined for both hindpaws. The rats were placed under a non-binding, clear plastic cages on a 3 mm thick glass plate and left to adapt at least for 20 min. A focused light source with a halogen bulb was used to deliver heat stimuli (50 W, Dittel, Prague). The radiant heat was applied to the plantar surface of each hindpaw until a deliberate escape movement of the paw was observed. The PWL was measured by a digital watch with a manual release switch electrically connected with the heat source. A 30 s cutoff time was imposed on the stimulus duration to prevent any tissue damage. The PWL were tested 5 times for each hindpaw with 5 min intervals between the trials. Results from each hind paw were averaged and SEMs calculated. Baseline withdrawal latencies were determined in all animals before any experimental procedure.

Exploratory activity

To measure the exploratory activity of the animals, black painted activity box (40x40 cm) was used. The locomotor activity was monitored and recorded by a digital camera connected with computer during a 30 min period. Data were analyzed offline using EthoVision XT program (Noldus). To analyze the movement of the animal the total distance traveled across the box was used as a main parameter. The animals were tested at the same time during the day in a separate room where no other people or animals were present, with a low noise level. The activity was evaluated over ten consecutive 3min intervals in each rat. In our study we compared total activity of rat during 30 min and activity during first 3 time intervals (9 min). Results from individual animals were averaged and SEMs calculated.

Blood pressure measurement

Systolic blood pressure (SBP) was measured in accordance with the recommendations for BP measurements in conscious animals by tail plethysmography through a tail-cuff multi-channel semiautomated apparatus (Hatteras 4000, USA). The rats were accustomed to this method of indirect BP measurement at least 3 days before the measurements.

For each BP value was the blood pressure measured 10 times in consecutive BP measurements that were automatically performed. The averaged value from these 10 measurements was used. BP was monitored in two groups of animals. The first group consisted of control rats with losartan p.o. administration in which BP was measured on 3, 7 and 14 day of losartan administration. The second group were SNL-operated rats with losartan p.o. treatment. In this group BP was measured on 3, 6, 9, 12 and 14 day after the SNL induction.

Immunohistochemistry

Rats were deeply anesthetized with ketamine (120 mg/kg i.p., Narketan, Zentiva) and xylazine (25 mg/kg i.p., Xylapan, Zentiva) and then perfused through the aorta with 0, 9% saline, followed by ice cold 4% paraformaldehyde in sodium phosphate buffer. The spinal cord and the L3-L5 DRGs were dissected out and postfixed in the same fixative overnight. The DRGs were sectioned at 14-μm on a cryostat (Leica). The sections were rinsed in phosphate-buffered saline (PBS, pH 7,6) three times (10 min each), then blocked with 2% donkey serum that contained Triton X-100 for 30 min at room temperature. The sections were incubated overnight at 4°C with rabbit anti- Angiotensin II Type 1Receptor antibody (1:500; Abcam). The next day the sections were washed with 1% normal donkey serum three times (10 min each) and then incubated for 2 h at room temperature with the secondary antibody conjugated to Texas Red (1:400; Jackson ImmunoResearch). The stained sections were examined with fluorescence microscope (Olympus), and images were captured with a digital camera.

Experimental groups

In our study 4 experimental groups of rats were used: SNL-operated animals (n=7), SNL-operated animals with treatment losartan *per os* (n=7), SNL-operated rats with losartan treatment intrathecally (n=6) and unoperated rats with losartan treatment *per os* (n=4). In each group baseline data were obtained 1-2 days before surgery.

Data analysis

The withdrawal responses evoked during the mechanical stimulation with VF filaments were evaluated as present (1) and absent (0) and a mean value from the 5 trials for each filament strength was calculated. During mechanical stimulation with the electronic von-Frey the withdrawal responses from the 4 trials were averaged. The mean values from all the animals in the group were averaged and means ±SEM were calculated. Paw withdrawal latencies evoked by heat stimuli were averaged from the 4 trials for each hindpaw and mean ±SEM were calculated for each experimental situation and time point. For statistical analysis two-way repeated measures analysis of variance (ANOVA) was used for the ipsilateral versus contralateral results comparisons as the between subjects variable and time as the repeated measure, to assess differences over time between the experimental and control paw in every group of animals. Two-way ANOVA was used to asses statistical differences at different testing period between the experimental groups. One-way ANOVA was used for the differences over time in blood pressure compared with the basal level. Post hoc tests were used to test differences between the ipsilateral and contralateral paws and between the experimental groups at each different time point using Holm-Sidak test. The locomotor activity results were tested for statistical significance by a One Way RM ANOVA with a Holm-Sidak post hoc test. All statistical tests were performed using SigmaStat software.

5. RESULTS

5.1 Spinal nerve ligation leads to thermal hyperalgesia and mechanical allodynia

In our first experiments we have established the model of peripheral neuropathy induced by spinal nerve ligation (SNL) similar to the previously published method (Kim and Chung, 1992). Under the control conditions the average PWL (paw withdrawal latency) to radiant heat was 22,81±0,13s on the ipsilateral and 22,95±0,18s on the contralateral paw (Fig. 1). Ligation of L5 spinal nerve on one side caused significant reduction of the threshold to the heat stimulus reflected by reduced latency of the reflex response (Fig.1). The decreased latency was significantly lower on the ipsilateral side already on the first day after the SNL induction. The decrease was even more evident through the next days and during the second week after the surgery. The PWL decrease was significantly different both from the original control value before the SNL surgery and also from the values measured on the intact colateral paw (Fig. 1). The contralateral hind paw exhibited smaller but also significant decrease in the PWL to thermal stimulation from the 9th day after the SNL, when compared to the control value (Fig.5.1).

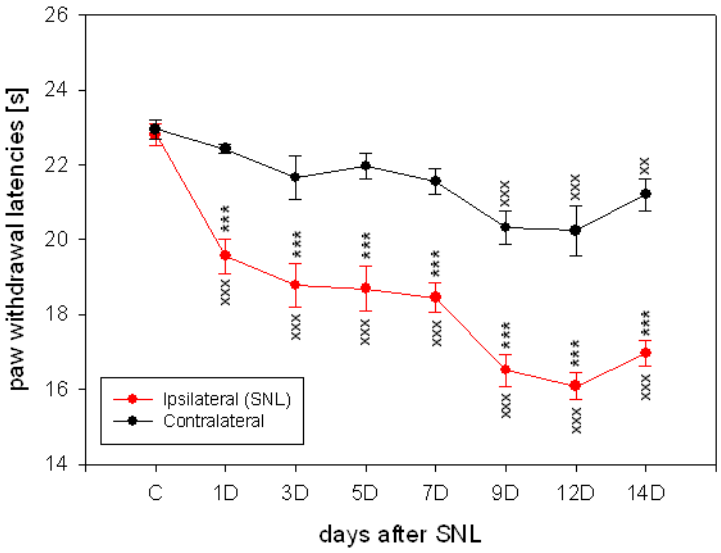

Figure 5.1 The PWL to radiant heat in the SNL group (n=7). Data are represented as means±SEM. Asterisks indicate significant differences between ipsilateral and contralateral hind paws (TW RM ANOVA, ***P<0,001). Crosses represent significant differences between the control values compared with the latencies after the SNL surgery (TW RM ANOVA, ^{xx}P<0,01; ^{xxx}P<0,001).

The responsiveness to mechanical stimulation was tested using two different methods: with electronic von Frey apparatus and mechanical von Frey filaments. Under the control conditions, before the SNL induction, the threshold to the electronic von Frey stimulus was 89,99±0,89 g on the ipsilateral and 91,61±0,7 g on the contralateral paws. On the side ipsilateral to the SNL there was a significant decrease (62,71±0,66 g) in the threshold to mechanical stimulus already on the first day after the surgery (Fig. 5.2). The decrease of the mechanical threshold reached maximum during the second week after the SNL induction. The decrease of the mechanical threshold on the ipsilateral side was significantly different both from the control value and from the values on the contralateral side. Changes in the mechanical sensitivity on the contralateral side were much less pronounced but also signififcantly different when compared to the control values before the surgery.

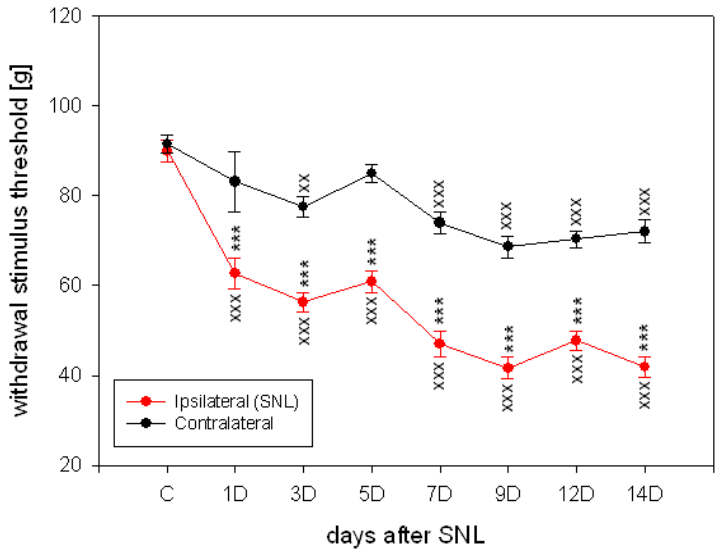

Figure 5.2 The threshold for withdrawal response induced by mechanical stimulus measured with the electronic von Frey in the SNL group (n=7). Data are presented as means±SEM. Asterisks indicate significant differences between ipsilateral and contralateral hind paws (TW RM ANOVA,***P<0,001). Crosses represent significant differences between the control values compared with the latencies after the SNL surgery (TW RM ANOVA, ^{xx}P<0,01; ^{xxx}P<0,001).

Tests with the mechanical von Frey filaments were designed to test possible presence of both mechanical allodynia and hyperalgesia. The low bending force filaments (40 and 60 mN) did not evoke any or only minimal response under the control conditions, while the stronger filaments evoked on the average 1,5 and 2,7 responses out of the 5 tested before the SNL induction (Fig. 5.3). On the ipsilateral side there was a robust

increase in number of evoked responses from the first day after the SNL induction, suggesting presence of mechanical allodynia (filaments 40 and 60 mN) and hyperalgesia (100 and 150 mN) (Fig. 5.3). This increased sensitivity to mechanical stimuli was even more pronounced during the second week after the SNL induction. The changes were significantly different when compared both with the control values before the surgery and with the responsiveness of the contralateral paw. The changes in mechanical sensitivity on the contralateral hindpaw were also significant, but not as robust when compared to the ipsilateral hindpaw (Fig. 5.3).

Our results confirmed that unilateral SNL under our experimental conditions evoked strong thermal and mechanical hyperalgesia and mechanical allodynia. The increased sensitivity to thermal and mechanical stimuli was most pronounced during the second week after the SNL induction.

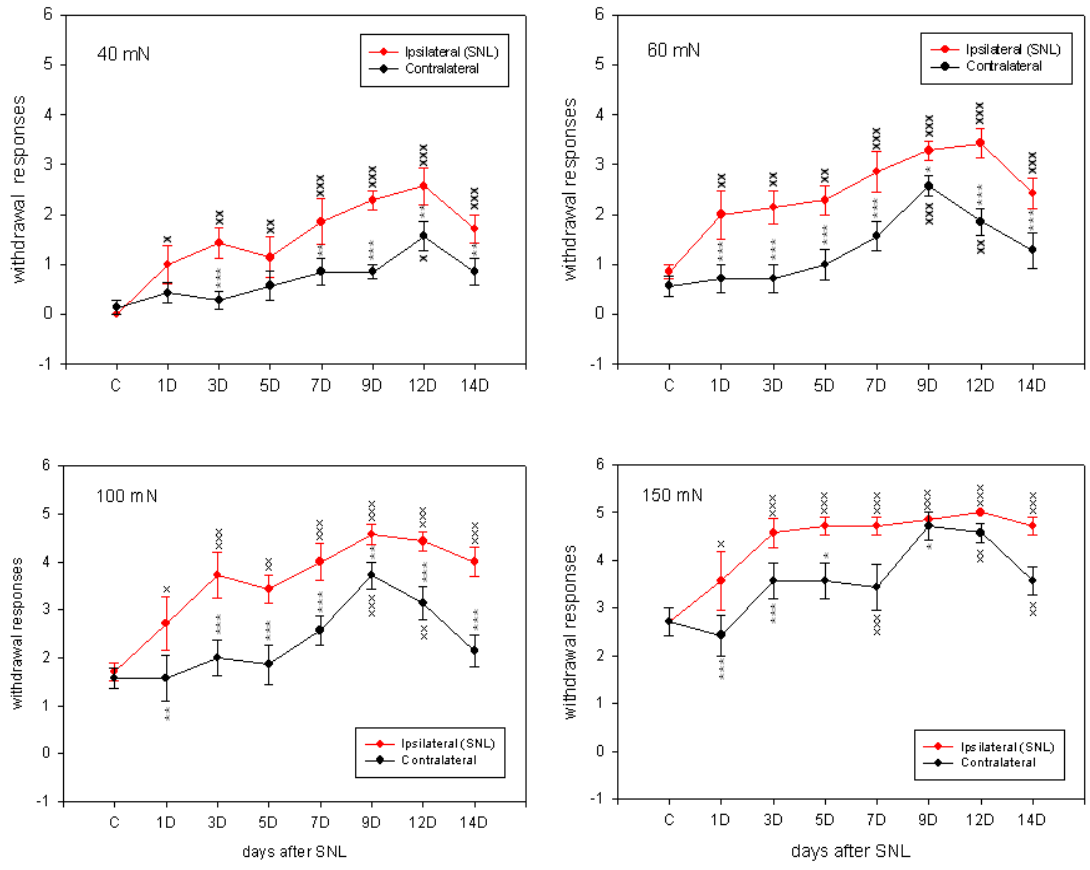

Figure 5.3 Withdrawal responses to mechanical stimuli measured with von Frey filaments in the SNL group (n=7). Data are presented as means±SEM. Asterisks indicate significant differences between ipsilateral and contralateral hind paws (TW RM ANOVA, *P<0,05; **P<0,01; ***P<0,001). Crosses represent significant differences between the control values compared with the latencies after the SNL surgery (TW RM ANOVA, ^{x}P<0,05; ^{xx}P<0,01; ^{xxx}P<0,001).

5.2 Losartan treatment reduces SNL-induced thermal hyperalgesia

In order to verify the effect of losartan treatment on the SNL-induced thermal hyperalgesia losartan was administrated perorally and intrathecally. In SNL-operated animals without treatment maximal decrease of the thermal threshold was detected on the 9th postoperative day and lasts till the end of the experiment (14D). Losartan p.o. treatment (100 mg/kg/day) significantly attenuated decrease in paw withdrawal latencies following the SNL surgery on the ipsilateral side when compared to the control group of animals (Fig. 5.4). Indeed, the PWL to thermal stimulation on ipsilateral paw in the SNL-operated animals on 9th postoperative day was 16,51±0,11s while in the perorally losartan treated rats it was on average 20,28±0,16 s. Losartan treatment attenuated changes in PWL also on the contralateral side. On the 9th day after the SNL induction the PWL contralaterally to the injury decreased to 20,33±0,14 s and in the SNL group with losartan *per os* treatment PWL were 22,55±0,09 s (Fig. 5.4).

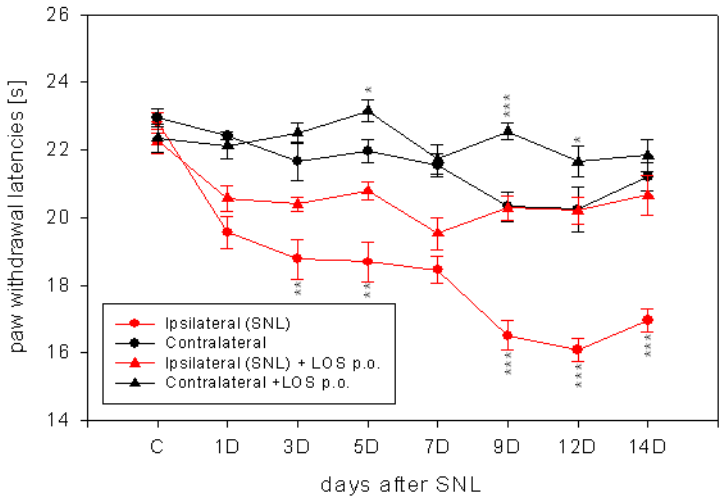

Figure 5.4 PWL to heat stimuli in SNL group and in a SNL group with losartan p.o. treatment (n=7 in both groups). Data are presented as means±SEM. Asterisks indicate significant differences between the two groups (Two Way ANOVA, *P<0,05; **P<0,01; ***P<0,001).

Intrathecally administered losartan (10µl of 20 µM solution every day) also showed anti-hyperalgesic effect. It atennuated decrease in PWL on the ipsilateral side which appears after induction of peripheral neuropathy on day 9 (16,51±0,11 in SNL group

and 19,68±0,13 in SNL group with intrathecal treatment). Anti-hyperalgesic effect of the losartan treatment was observed during all the testing period (Fig. 5.5).

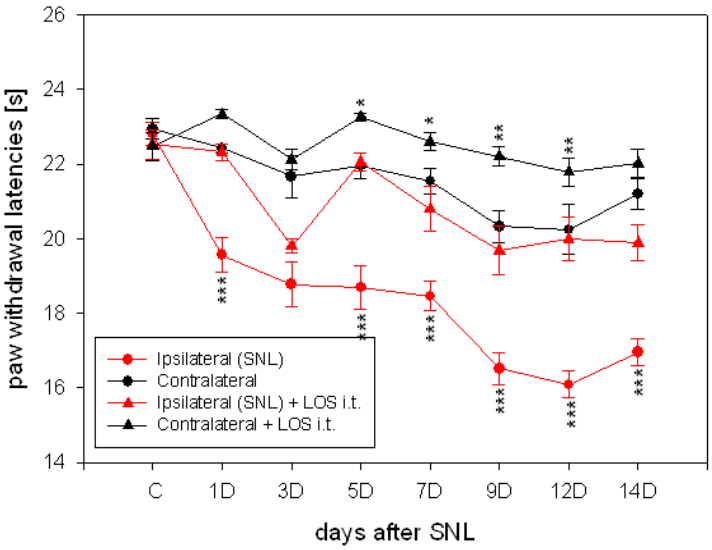

Figure 5.5 PWL to heat stimuli in SNL group and in a SNL group with losartan i.t. treatment (n=6, n=7 respectively). Data are presented as means±SEM. Asterisks indicate significant differences between two groups (Two Way ANOVA, *P<0,05; **P<0,01; ***P<0,001).

The differences in paw withdrawal latencies between neuropathic animals with peroral and intrethecal losartan treatment are shown in Fig. 5.6. A significant difference in PWL in the different groups was observed only at several time points during first week after the SNL induction. However, during the second week after the SNL surgery, when the animals exhibited maximal thermal hyperalgesia (9-14D), there was no statistical difference observed between the two groups (Fig. 5.6).

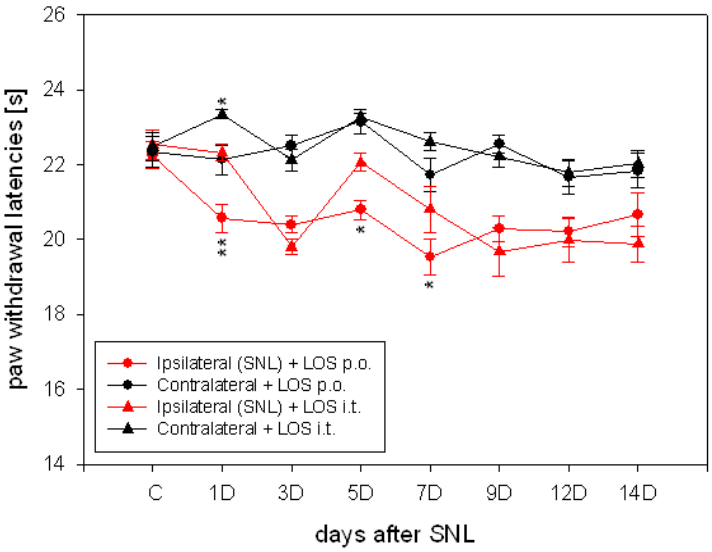

Figure 5.6 PWL to heat stimuli in a SNL group with losartan p.o. treatment and in a SNL group with losartan i.t. treatment (n=7, n=6 respectively). Data are presented as means±SEM. Asterisks indicate significant differences between two groups (Two Way ANOVA, *P<0.05; **P<0.01).

5.3 Losartan treatment partially reduced SNL-induced mechanical allodynia

Spinal nerve ligation induced a statistically significant reduction of the paw withdrawal thresholds to mechanical stimuli indicating pesence of mechanical allodynia, which was sustained throughout the 14 day testing period. Tests with the electronic von Frey showed that losartan p.o. treatment (100 mg/kg/day) did not change the threshold when compared to the SNL rats without the treatment under most time points (Fig. 5.7). The attenuation of the responsiveness induced by losartan treatment was observed during the second week after SNL.

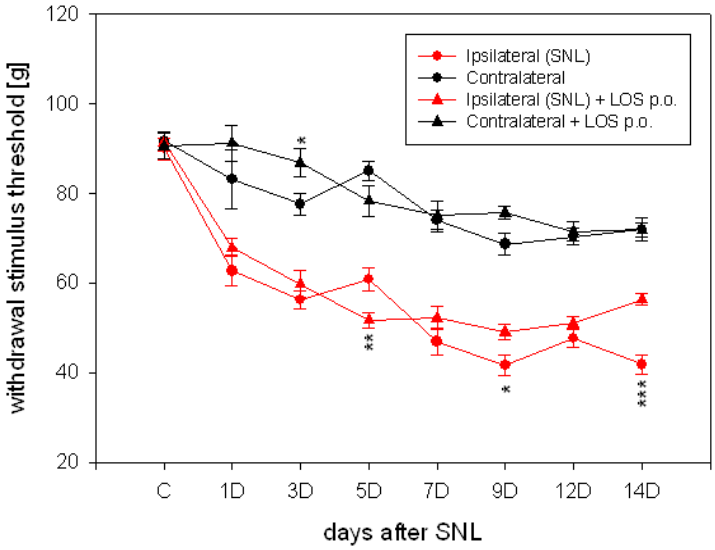

Figure 5.7 The threshold for withdrawal response induced by mechanical stimulus measured with the electronic von Frey in SNL group and in a SNL group with losartan p.o. treatment (n=7 for both groups). Data are presented as means±SEM. Asterisks indicate significant differences between two groups (Two Way ANOVA, *P<0,05; **P<0,01; ***P<0,001).

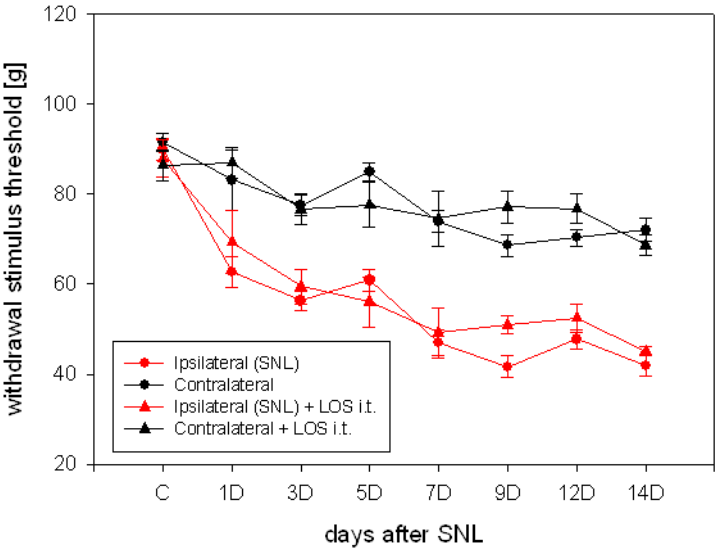

Figure 5.8 The threshold for withdrawal response induced by mechanical stimulus measured with the electronic von Frey in SNL group and in a SNL group with losartan i.t. treatment (n=6, n=7 respectively). Data are presented as means±SEM. Asterisks indicate significant differences between two groups (Two Way ANOVA, *P<0,05).

There was no significant effect on the mechanical thresholds in the group with the intrathecal losartan treatment (Fig. 5.8). Also there was no significant difference detected in the SNL groups, which were treated with losartan either perorally or intrathecally (Fig. 5.9).

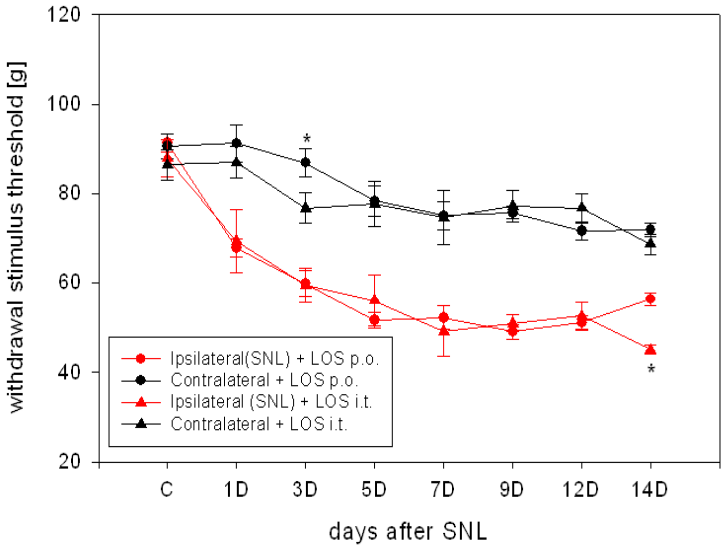

Figure 5.9 The threshold for withdrawal response induced by mechanical stimulus measured with the electronic von Frey in a SNL group with losartan p.o. treatment and in a SNL group with losartan i.t. treatment (n=7, n=6 respectively). Data are presented as means±SEM. Asterisks indicate significant differences between two groups (Two Way ANOVA, *P<0,05).

Tests of sensitivity to mechanical stimuli with the mechanical von Frey filaments detected attenuation of the increased responsiveness after the SNL induction in the animals treated with losartan p.o. (Fig. 5.10).This difference at most instances did not reach statistical significance. Most robust effect was detected in tests with VF filament 100 mN (Fig. 5.10).

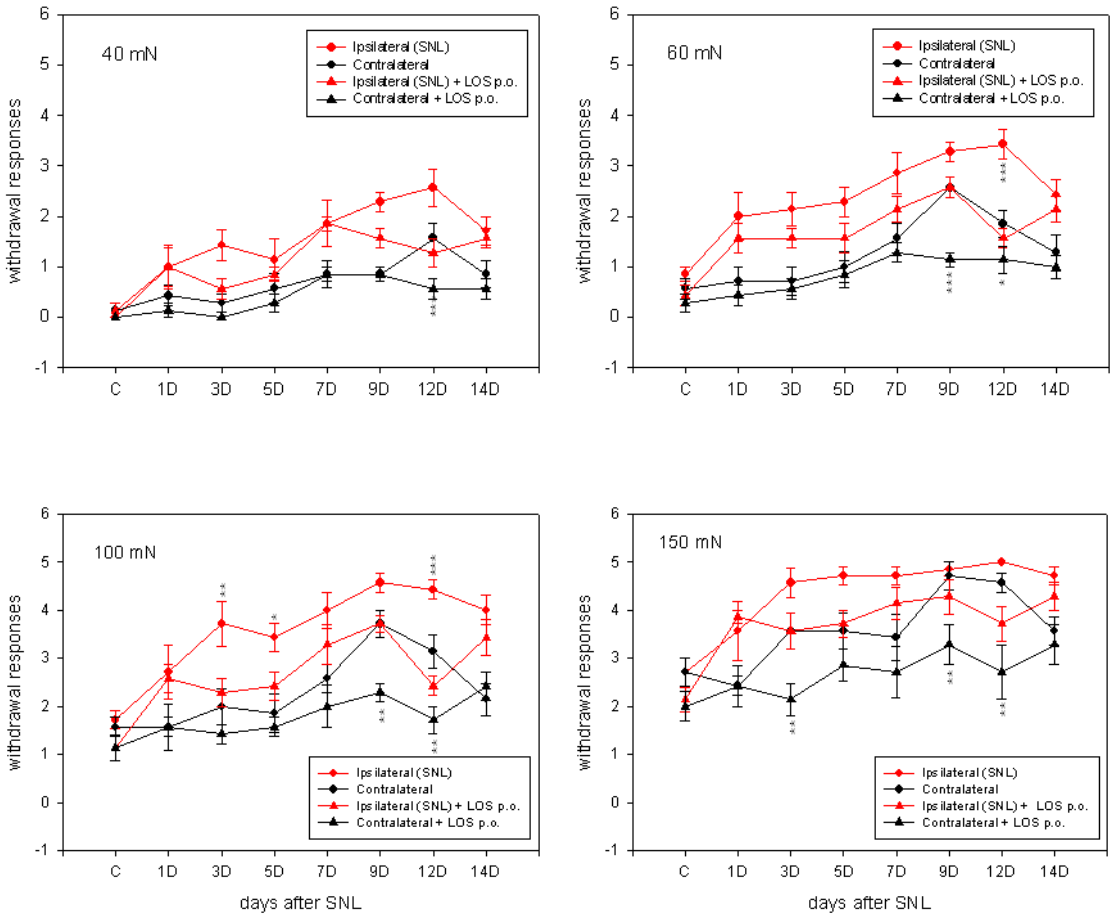

Figure 5.10 Withdrawal responses to mechanical stimuli measured with von Frey filaments in a SNL group and a SNL group with losartan p.o. treatment (n=7 for both experimental groups). Data are presented as means±SEM. Asterisks indicate significant differences between two groups (Two Way ANOVA,*P<0,05;**P<0,01).

The number of withdrawal responses to mechanical stimulation with VF filaments obtained in the SNL-operated rats with losartan p.o. treatment was significantly lower when compared to those in the SNL-operated group with losartan i.t. treatment (Fig. 5.11). These results suggested that intrathecally administrated losartan had minimal effect on the increased mechanical sensitivity after SNL surgery.

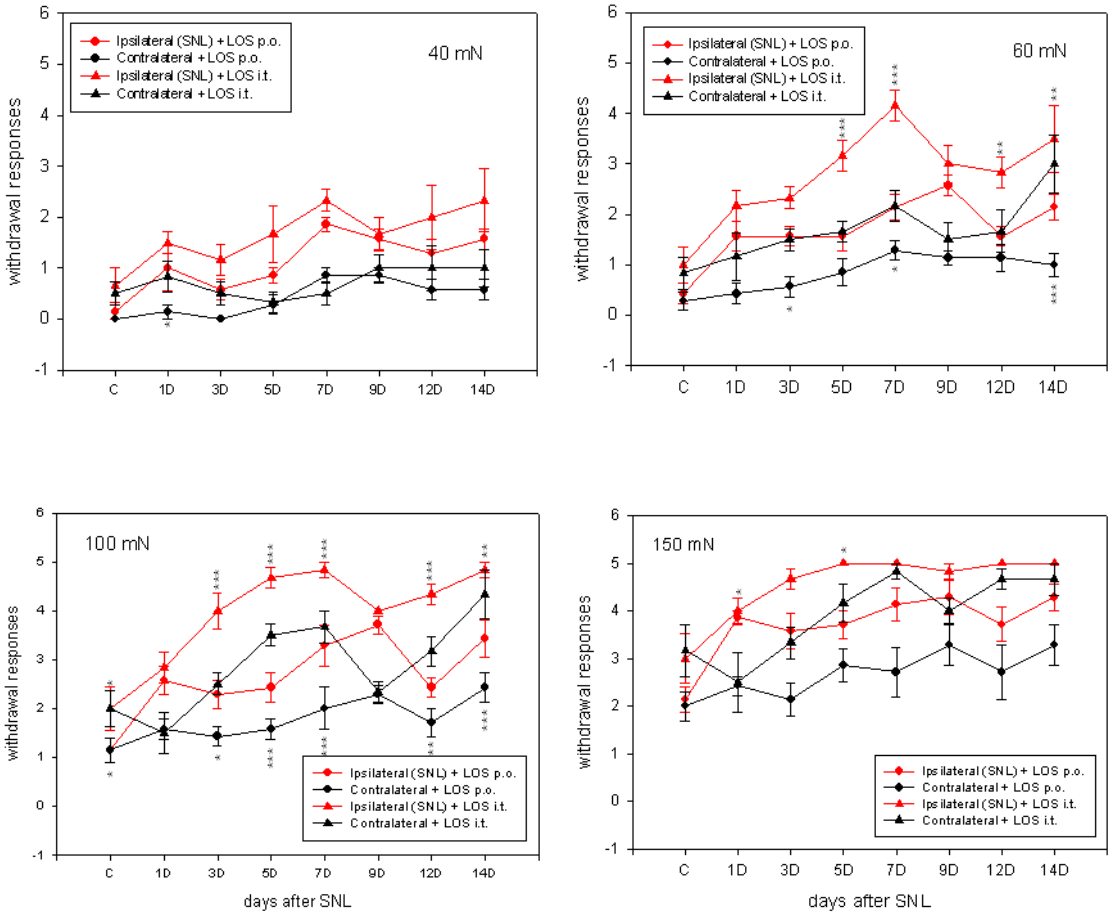

Figure 5.11 Withdrawal responses to mechanical stimuli measured with von Frey filaments in a SNL group with losartan p.o. treatment and a SNL group with losartan i.t. treatment (n=7, n=6 respectively). Data are presented as means±SEM. Asterisks indicate significant differences between two groups (Two Way ANOVA,*P<0,05;**P<0,01).

As it was previously described, maximal reduction in paw withdrawal latencies and withdrawal stimulus thresholds was observed during the second week (9-14D) after the SNL induction. SNL-operated rats exhibited significant thermal hyperalgesia (Fig. 5.12). The average PWL during the second week after the SNL was significantly reduced when compared to control value or with control group with only losartan treatment. Treatment with losartan either perorally or intrathecally significantly attenuated the reduction in PWL induced by the SNL surgery (Fig. 5.12). Significant decrease of the thresholds to mechanical stimuli after the SNL-operation indicated the presence of mechalical allodynia. The differences in the thresholds to withdrawal responses to mechanical stimuli between treated and untreated experimental groups were statistically different (Fig. 5.13), but were not as robust as the differences in PWL to heat stimuli (Fig. 5. 12).

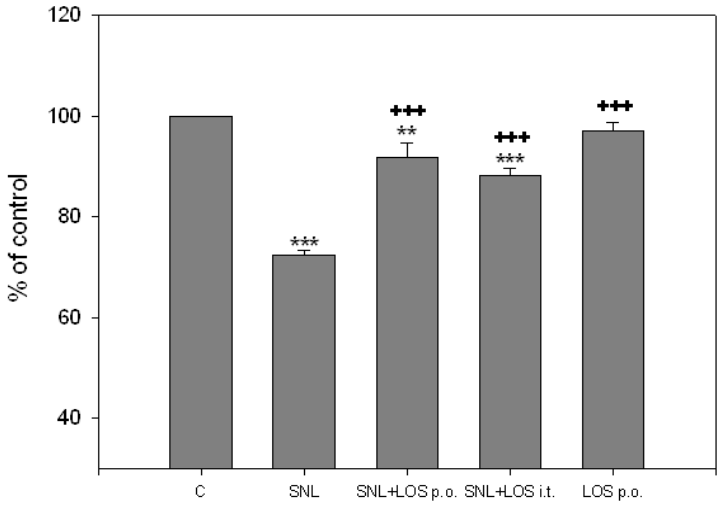

Figure 5.12 PWL to heat stimuli on the ipsilateral paw in different experimental groups averaged for the 9-14 postoperative day period.Data are presented as % from control value (100 %) ±SEM. Asterisks indicate significant differences between the control value when compared with the latencies in experimental groups averaged for the 9-14 postoperative day period (One Way ANOVA, **P<0,01; ***P<0,001).Crosses represent significant diference between latencies in experimental groups when compared to SNL-operated group (One Way ANOVA, **P<0,001).

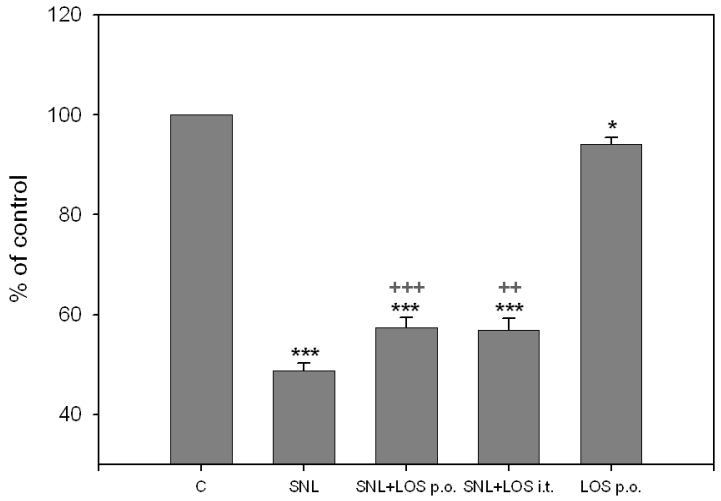

Figure 5.13 The threshold do withdrawal response on the ipsilateral paw induced by mechanical stimulus measured with the electronic von Frey apparatus. Data are presented as % from control value (100 %)±SEM. Asterisks indicate significant differences between the control value when compared with the latencies in experimental groups averaged for the 9-14 postoperative day period (One Way ANOVA, **P<0,01; ***P<0,001). Crosses represent significant diference between thresholds in experimental groups when compared to SNL-operated group (One Way ANOVA, **P<0,001).

5.4 Effect of losartan administration on blood pressure and the behavioral responses in unoperated rats

Losartan is known to induce changes in blood pressure. To test the possible effect of BP change on the behavioral tests, we have performed control experiments. In the first group of animals losartan p.o. treatment (100 mg/kg/day) was used and blood pressure changes were monitored. Under control conditions without losartan treatment the BP in these animals was 132,5±1,58 mmHg. Losartan induced significant blood pressure decrease during the first week of administration (121,25±2,98 mmHg on day 7 of losartan administration). However systolic blood pressure measured on the 14th day of chronic losartan administration (132,5±1,58 mmHg) was not statistically different from the control level (137±1,58 mmHg) (Fig.5.14). The same group of animals were tested for responsiveness to thermal and mechanical stimuli. There was a significant decrease in the PWL in the thermal test on days 7 and 14. There was no change in the responsiveness to mechanical stimuli. These behavioral tests suggested that small decrease in blood pressure induced by losartan administration had only minimal effect on sensitivity to thermal or mechanical stimuli in these tests (Fig. 5.15).

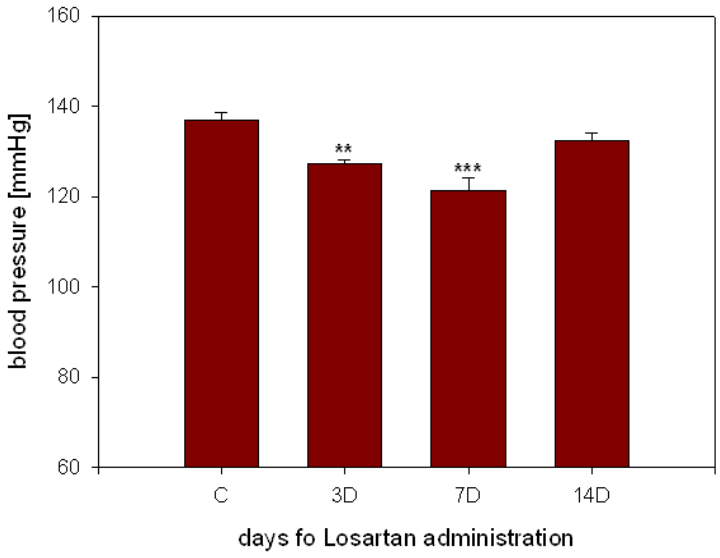

Figure 5.14 Systolic blood pressure in rats with losartan p.o. administration (n=4). Data are presented as means±SEM. Asterisks indicate significant differences in time compared with the control mean (One Way ANOVA, **P<0,01; ***P<0,001).

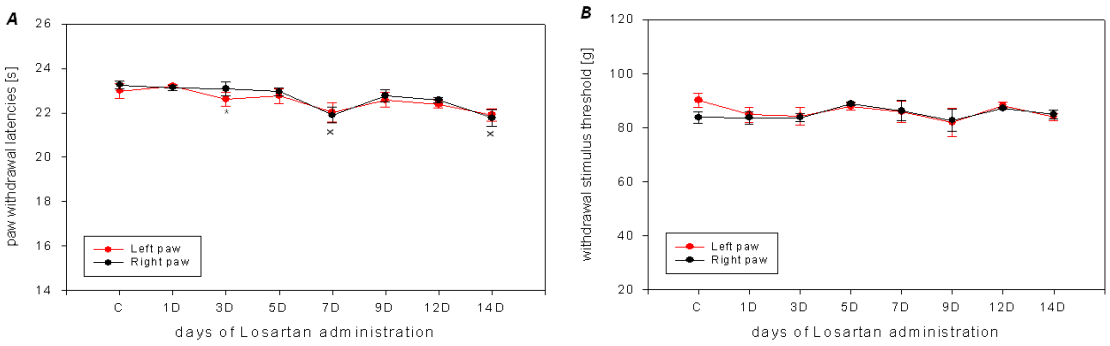

Figure 5.15 Behavioral responses in control rats with losartan p.o. treatment (n=4). **A.** PWL to heat stimulus. **B.** Threshold for withdrawal response induced by mechanical stimulus measured with the electronic von Frey. Data are presented as means±SEM. Asterisks indicate significant differences between ipsilateral and contralateral hind paws (TW RM ANOVA, *P<0,05). Crosses represent significant differences between the control values compared with the latencies after the losartan p.o. treatment (TW RM ANOVA, ^{x}P<0,05).

The second experimental group consisted of SNL operated animals with the losartan p.o. treatment. The blood pressure was measured during the two weeks after the SNL was induced. Changes in the blood pressure induced by the losartan p.o. treatment were not statistically different when compared to the control level (Fig. 5.16). Results of BP measurments from both groups sugested that the minimal changes in BP related to the losartan administration had minimal effect on the behavioral responses measured. The canges seen after losartan in the control group had opposite effect (hyperalgesic) compared to the effect in the SNL rats (analgesic). Confirmation of the effect of losartan administration on blood pressure and possible influence on the behavioral test will need further investigation on larger group of animals.

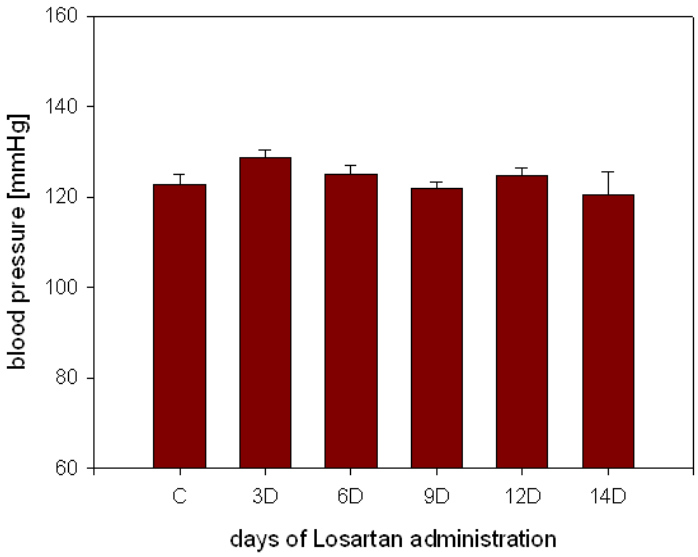

Figure 5.16 Systolic blood pressure in SNL group with losartan p.o. treatment (n=3). Data are presented as means±SEM. Asterisks indicate significant differences in time compared with the control mean (One Way ANOVA).

5.5 Effect of losartan administration on locomotor activity

The locomotor activity of SNL-operated rats in new environment test was measured. Total activity of the rats during 30 min and activity during the first 9 min was evaluated. The differences between distances measured at the different time points after the SNL were not statistical significant when compared to the control value taken before the surgery (Fig. 5.17). Robust decrease of the exploratory activity on the first day after SNL could be due to the recovery period after the surgery. As there was no significant change in locomotor activity in rats after SNL-induction, the testing on the other experimental groups was not performed.

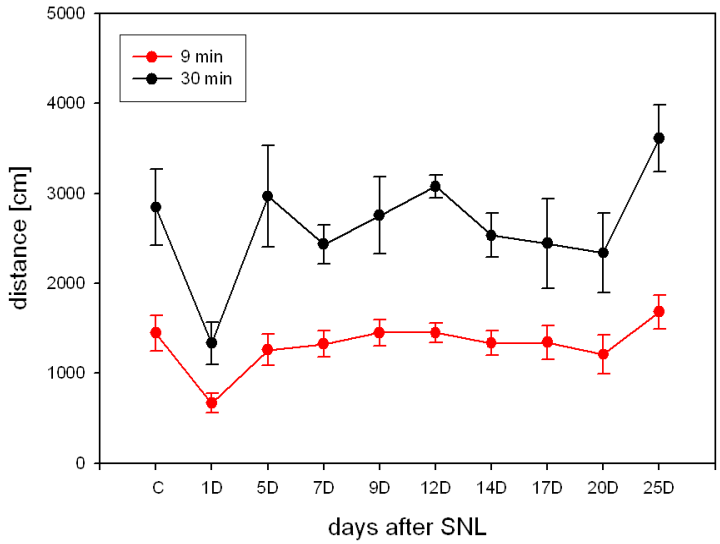

Figure 5.17 The locomotor activity of SNL-operated animals (n=7). Data are presented as means±SEM (One Way RM ANOVA).

5.6 Immunohistochemistry results

Immunohistochemical staining of spinal dorsal root ganglia sections was performed in order to investigate AT receptor type 1 expression. On the DRG slices the number and the size of the neurons was determined and the presence of immunostaining for the AT1R was investigated. Preliminary results of AT1R-immunohistochemistry on ipsilateral L5 dorsal root ganglion are depicted on Fig. 5.18. The figure (5.18A) shows the distribution of the AT1R positive neurons in the DRG histological section. The size and frequency distribution of AT1R positive and negative neurons is given in Fig. 5.18B. This preliminary data suggest that AT1R are expressed predominantly in the small-size DRG neurons.

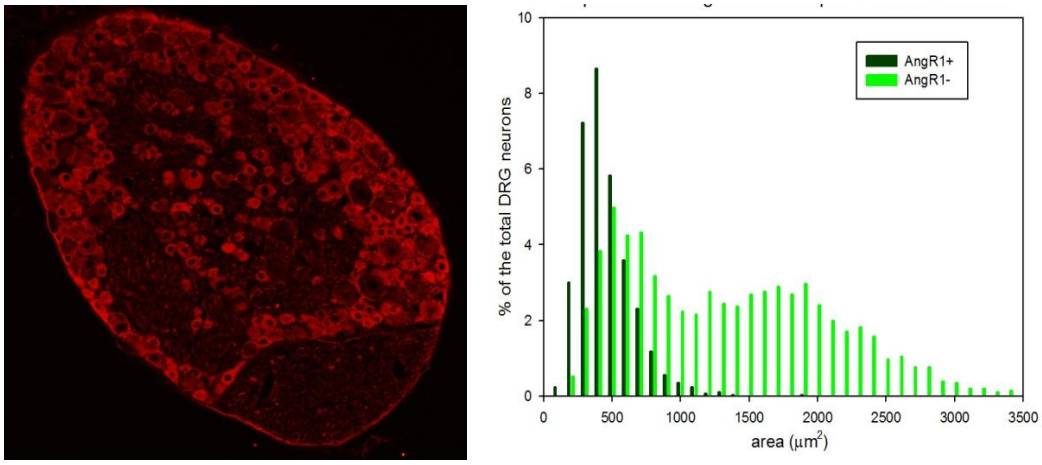

Figure 5.18 A. An example of immunohistochemical section of DRG under fluorescence microscope (foto). **B.** Size distribution of DRG neurons with (+) and without (-) expression of angiotensin receptor 1 detected by immunohistochemistry. Preliminary results.

6. DISCUSSION

Spinal nerve ligation as a model of peripheral neuropathy

SNL-model is one of the most commonly used animal models for studying mechanisms of neuropathic pain, because operated rats exhibit main symptoms of neuropathic pain (spontaneous pain, thermal hyperalgesia and mechanical allodynia). This experimental animal model was first described by Kim and Chung (Kim and Chung, 1992). The original model used tight ligation of L5 and L6 spinal nerves. They also used SNL-model where only L5 spinal nerve was ligated, that we have used also in our study. This modified model is technically easier to perform, nevertheless produces similar behavioral changes with minor differences in time course and magnitude in comparison with the L5-L6 SNL-model (Kim and Chung, 1992). In both models the spinal nerve (or nerves) is tightly ligated at a point before the spinal nerve connects a common nerve, hence injured and uninjured nerve fibers can interact in a peripheral nerve leading to the development of mechanical allodynia (Gold, 2000). The limitation of other peripheral neuropathic models (PNL, CCI, SNI and others) is that the number and type of injured neurons cannot be unequivocally defined. During SNL operation the same spinal nerve is ligated in each animal. The differences in the injury between the experimental subjects are thus minimal. Another uniqueness of the SNL model in comparison with the rest of the models is that the levels of injured and intact spinal segments and ganglions are separated. This feature allows to compare changes in the injured and uninjured structures of the nervous system induced by peripheral nerve damage. Due to these facts SNL model proposed by Kim and Chung is widely used in many different studies. For example, for studies of electrophysiological properties of central and peripheral structures of nervous system (Carpenter et al, 2003; Yang et al, 2011; Ewan et al, 2011); studies of functional alterations in CNS during peripheral neuropathy (Jeon et al, 2008; Liu et al,2010); immunological studies (Levin et al, 2008; Dominguez et al, 2008; Gao et al, 2009); behavioral studies (Funakubo et al, 2011; Guan et al, 2010); pharmacological studies (Mei et al, 2011; Albrecht et al, 2011) etc.

According to data, presented in the original study (Kim and Chung, 1992), animals subjected to the L5 spinal nerve ligation develop thermal hyperalgesia and mechanical allodynia within 24-28 h, peaks at about 7-10 days post-surgery, and sustain throughout

more than 45 days. In our study the SNL-operated rats exhibited hypersensitivity to thermal and mechanical stimuli from the first day after the surgery and maximal decrease of the thresholds was detected at 9-12 day after the operation and persisted for 14 days of the testing period. Similar to the results described by Kim and Chung, the signs of allodynia and hyperalgesia developed not only on the ipsilateral side to the injury, but also on the contralateral intact side. However, the threshold on the contralateral side was higher and the onset time of the changes was slower compared to the ipsilateral side (Fig. 5.1-3).

Effect of the losartan administration on the development of increased sensitivity to mechanical and thermal stimuli

In our experiments treatment with losartan significantly reduced the increased sensitivity to thermal stimuli tested as PWL in the animals after SNL surgery. Losartan was administrated either orally or intrathecally. In both cases losartan had significant anti-hyperalgesic effect (Fig.5.4-6, 5.12).

It was suggested previously that changes in blood flow may affect the PWL responses in this test (Irvine et al., 1995). The pharmacological blockade of the angiotensin type 1 receptor by losartan unquestionably induce blood pressure decrease. In our experiments BP decrease was present in a small control group of rats (Fig. 5.14), while in another group after SNL, losartan administration did not have any significant effect on the BP (Fig. 5.16). In the control group the changes in the blood pressure induced by losartan evoked minor changes in the thermal PWL test and did not affect the mechanical test. Also in this control experiment the losartan application induced decrease of the PWL, suggesting increase of the sensitivity to thermal stimuli (Fig. 5.15). However, as the effect of losartan in the SNL animals was present as a decrease in PWL, it seems plausible to suggest that it was unrelated to the blood pressure change.

There is also some other evidence that losartan administration *per os* may change thermal sensitivity, as it reduced hot plate latencies in normal rats (Irvine et al., 1995). Role for angiotensin II in acute pain was suggested also on the supraspinal level. Spinally projecting neurons of caudal ventrolateral medulla (CVLM, a component of the supraspinal pain modulating system) express AT_1 receptors on their surface. Administration of angiotensin II in the CVLM increased thermal hyperalgesia in acute and inflammatory pain conditions (tail-flick and formalin tests respectively). Hyperalgesic effect of Angiotensin II was prevented by administration of AT_1R antagonist – losartan. The change in

thermal hypersensitivity probably did not depend on cardiovascular effects or body temperature, as Ang II injections did not affect blood pressure in those experiments (Marques-Lopes et al., 2009). Another study suggested analgesic effect of angiotensin II after microinjection to the periaqueductal gray and rostroventromedial medulla (another key structures of supraspinal pain modulatory system), which lead to analgesia, which could be reversed by losartan (Prado et al., 2003).These findings indicate a potential role of angiotensin II in pain modulation, which may be mediated predominantly through supraspinal sites. All these effects seem to be independent of the haemodynamic effects related to the AT_1R activation, but the specific mechanisms need to be further investigated.

Effect of losartan administration on the mechanical allodynia in neuropathic rats in our experiments was not as robust as on the thermal hyperalgesia. The withdrawal threshold to mechanical stimuli was tested with electronic von Frey and responsiveness to graded mechanical stimuli with mechanical von Frey filaments. Mechanical thresholds tested with the electronic von Frey indicated that losartan, administered either perorally or intrathecally, had no major beneficial effect after the SNL injury (Fig. 5.7-9, 5.13). However, tests with mechanical von Frey filaments suggested antiallodynic effect, as neuropathic rats with systemic losartan treatment exhibited significantly lower mechanical allodynia in comparison to SNL-operated animals without the treatment (Fig. 5.10). On the contrary, intrathecal injection of losartan did not affect allodynia-like behavior in the neuropathic rats (Fig. 5.11). The difference in the p.o. and i.t. treatment implies possible other effect of losartan beside at the spinal cord level. These differences will need other experiments to clearly identify the underlying mechanisms.

We assume that AT_1R and their antagonists may affect nociceptive mechanisms under the pathological pain conditions in the central nervous system. So effectivity of the losartan action depends on its concentration in the cerebrospinal fluid (CSF). Pharmacokinetic studies confirmed that losartan, administered *per os*, is rapidly absorbed, reaching its maximal concentration in about 1-2 hours post-administration. Approximately 14 % of the losartan dose is converted to the pharmacologically active E_{3174} metabolite. The important fact is that pharmacokinetics of losartan and its metabolite is linear and does not change with repetitive administration (Sica et al., 2005). The ability of losartan to cross the blood brain barrier (BBB) is dependent on its concentration in plasma, hence, on the administered dose. Losartan administrated orally at dosage 3 mg/kg was shown not to interact with brain AT_1 receptors (Bui et al., 1992). Other studies confirmed that using higher doses of

losartan (30-100 mg/kg) leads to satisfactory, but not complete inhibition of centrally mediated actions of angiotensin II (Culman et al., 1999). It is important to emphasize, that in our study 100 mg/kg/day doses of losartan were used to inhibit AT_1 receptors. This suggests that the losartan dose in our experiments was high enough to penetrate the BBB and block AT_1R in the central nervous system.

Involvement of Angiotensin II and its receptors in neuropathic pain states

A number of studies are dedicated to the cardiovascular effects of angiotensin II via its receptors in the brain. Indeed, the Ang II from the circulatory system interacts with its receptors in circumventricular organs in the brain without crossing the BBB (subfornical organ, organum vasculosum laminae terminalis, area postrema) and participates in regulation of cardiovascular and body fluid homeostasis (Allen et al., 2000). However, angiotensin type 1 receptors are present not only in the circulatory system but also in the CNS behind the BBB. Expression of AT_1 receptors was detected in brain (Moulik et al., 2002), spinal cord, dorsal root ganglia and peripheral nerves. In spinal cord AT_1R were detected predominantly in the superficial dorsal horns (lamina I and II) but also in the central canal region (lamina X) (Pavel et al., 2008). It is well known that these zones are involved in nociceptive transmission. As neither Ang II, nor renin can pass the blood brain barrier, it was considered that local, brain renin-angiotensin system exists. Local brain RAS may regulate both neurological and cardiovascular functions.

Recently it was shown that angiotensin receptors type 1 in the CNS may play an important role in neuroinflammation (Kandalam and Clark, 2009). Neuroinflammation is considered one of the central mechanisms playing an important role in some pathological states, also in the peripheral neuropathy (Berger et al., 2011). Peripheral nerve injury leads to activation of the glial cells in DRGs and spinal cord. Astrocyte activation plays a prominent role in neuroinflammation as activated astroglia produces different signaling molecules. Chemokines and cytokines released by activated astrocytes subsequently facilitate inflammatory response and on the other hand, cause the modulation of the synaptic transmission (Tanga et al., 2004). These glial cells are known to be the main source of angiotensinogen in the CNS and express angiotensin receptors on their surface. Thus, their functions could be influenced by angiotensin II. Indeed, Ang II via AT_1 receptors induces secretion of IL-6 and up-regulation of angiotensinogen by astrocytes (Kandalam and Clark, 2010). IL-6 is a pro-inflammatory cytokine and appears to have a role in the upregulation

of TNF-α and IL-1β release (Schoeniger-Skinner et al., 2007). TNF-α, IL-1β and IL-6 are known as three main pro-inflammatory cytokines released by immune and non-neuronal cells in the CNS under pathological conditions such as peripheral nerve injury (Austin and Moalem-Taylor, 2010). Upregulating of the pro-inflammatory cytokines and their receptors leads to central sensitization. These cytokines modulate different synaptic mechanisms in the neurons of superficial lamina of dorsal horn, thus contributing to the development of hyperalgesia and allodynia (Kawasaki et al., 2008). Studies indicate that Ang II may induce the release of the chemokine CCL2 in monocytes under pathological conditions and this can be prevented by AT_1R antagonists, indicating that angiotensin II complies its anti-inflammatory functions predominantly via AT_1R (Wolf et al., 1998). Another study show that AT_1R blockade by losartan significantly reduced the expression of inflammatory chemokines (CCL2, CCL3, CXCL10) in the spinal cord during autoimmune inflammation (Stegbauer et al., 2009). These findings suggest that blockade of AT_1R may influence the chemokine release by glial cells. This implies our hypothesis that under the conditions of peripheral neuropathy, when neuroinflammation occurs especially at the spinal cord level, AT_1R may modulate nociceptive synaptic transmission due to regulation of glia activation and cytokine release. The results of the behavioral experiments in our study confirmed this hypothesis. However, our results detect more robust effect of losartan administration on thermal hyperalgesia (Fig. 5.4-6, 5.12) when compared to mechanical allodynia (Fig. 5.7-9, 5.13). The possible explanation of this difference is that AT_1 receptors may be expressed on a subtype of DRG neurons that are more involved in mediating thermal hyperalgesia than mechanical allodynia. Indeed the underlying mechanisms of these symptoms may differ (Baron et all., 2010). Co-expression of AT_1R with thermosensitive receptors involved in heat stimuli induced pain such as TRPV1 receptors in DRG neurons may be of great interest. Further studies including double immunohistochemical staining for these receptors should be performed. Our hypothesis will also need confirmation by measurements of the expression and concentration of pro-inflammatory cytokines and their receptors distribution in the CNS during states of peripheral neuropathy and establish possible influence of losartan application.

We assume that anti-hyperlagesic effect of losartan administration in the SNL model of peripheral neuropathy is mediated by blockade of AT_1R in the central nervous system and DRGs. Nevertheless, the difference in the effect on mechanical allodynia between peroral and intrathecal treatment with losartan in neuropathic rats (Fig. 5.7-9, 5.13)

suggests that other sites of losartan action may exist. We also can not rule out completely, that the antihyperalgesic effect of losartan administration in our experiments was due to some unknown effect on other types of receptors, unrelated to the inhibition of AT_1 receptors. This seem unlikely, as losartan is used in clinical practise for many years without any apparent unrelated side effects.

Angiotensin II and its receptors seem to have multiple functions in the CNS, including pronociceptive and antinociceptive effects in different supraspinal structures related to pain modulatory system and play also role in neuroinflammatory processes. Further studies will be needed to determine all the actions of this system in the CNS.

7. CONCLUSIONS

Understanding the mechanisms of neuropathic pain development and maintenence is key to its effective treatment. Neuroinflammation at the spinal cord was suggested to be one of the important elements in neuropathic pain development following peripheral nerve injury. Recent studies suggested that losartan, angiotensin receptor type 1 antagonist, may reduce neuroinflammation in the CNS. In our study we have tested our hypothesis that losartan may reduce pathological pain symptoms such as thermal hyperalgesia and mechanical allodynia due to modulation of neuroinflammatory process at the spinal cord level. We have investigated the effect of losartan administration on the development of increased sensitivity to thermal and mechanical stimuli following induction of peripheral neuropathy model (SNL).

Our results confirmed presence of increased sensitivity to thermal stimuli (heat hyperalgesia) and mechanical stimuli (mechanical allodynia and hyperalgesia) after SNL, similar to other published studies. Treatment with losartan significantly reduced the development of the thermal hyperalgesia both after *per os* and intrathecal treatment, suggesting effect at the spinal cord level. The effect of losartan treatment on mechanical allodynia was much less pronounced and especially after the intrathecal treatment was minimal, suggesting possible smaller involvement of AT_1R in these symptoms and possible role of AT_1R outside the spinal cord. Our preliminary results from immunohistochemical analysis of DRG histological sections indicate expression of AT_1 receptors in small diameter DRG neurons.

These results support our hypothesis that losartan treatment may modulate neuroinflammation at the spinal cord level following peripheral nerve injury and thus diminish some signs of allodynia and hyperalgesia. The exact mechanisms of losartan action under the conditions of peripheral neuropathy will need further investigation. Our study contributes to a better understanding of the neuropathic pain mechanisms development and could lead to better analgesic treatment.

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