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Losartan treatment attenuates the development of neuropathic thermal hyperalgesia induced by peripheral nerve injury in rats

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ABSTRACT

Aims: Neuroinflammatory changes in the central nervous system are widely involved in the initiation and maintenance of neuropathic pain after peripheral nerve injury. The present study investigated how losartan treatment may affect the development of neuropathic pain and neuroinflammation.

Main methods: The effect of losartan treatment on the development of peripheral neuropathy was studied in L5 spinal nerve ligation (SNL) model in rats with systemic (100 mg/kg) or intrathecal (10 µl/ 20 µM solution) application of losartan. Electronic von Frey filament and plantar test were used to determine pain thresholds to mechanical and thermal stimulations. At the 7th post-operative day, CD68-positive cells in DRG and dorsal roots were quantified by immunohistochemistry and western blot analyses were used to compare the expression levels of neuroinflammatory markers in lumbar spinal cord (SC).

Key findings: Our data confirmed the presence of SNL-evoked heat hyperalgesia and mechanical allodynia. Losartan application blocked the SNL-induced hypersensitivity to thermal stimuli but failed to prevent mechanical allodynia. No significant difference between systemic and *i.t.* administration of losartan was observed. Immunohistochemistry confirmed the presence of infiltrated macrophages in the ipsilateral DRG that was significantly attenuated with the losartan treatment. Western blot SC tissue analysis revealed that systemic treatment with losartan prevented SNL-induced upregulation of CCR2, TNFα, TNFR1, and OX42 while its effect on CCL2 and AT1R expression was not significant.

Significance: Our results show that losartan treatment attenuates neuroinflammation and neuropathic pain after SNL. These effects of losartan represent an interesting direction for the development of novel treatments of peripheral neuropathy.

1. Introduction

Peripheral nerve injury may induce spontaneous pain sensation, hypersensitivity to normally innocuous stimuli (allodynia) and hypersensitivity to noxious stimuli (hyperalgesia) [1,2]. The diversity of causes, associated with the unclear mechanisms of the development and maintenance of neuropathy are critical for the elaboration of satisfying treatments. Peripheral nerve injury leads to the macrophage activation in the corresponding dorsal root ganglion (DRG) [3] and to local spinal cord inflammation through glial cell activation, immune cell recruitment and release of tumor necrosis factor-*α* (TNFα), interleukin-6 (IL6) or monocyte chemoattractant protein-1 (MCP1) [4–7]. Considering the chronological events in glial activation, microglia plays a prominent role in the initial phase of neuroinflammation *via* early-stage cytokine release [8]. Astrocytes are involved mostly in the maintenance of the neuroinflammation [9,10]. MCP1, also referred as C−C motif chemokine ligand 2 (CCL2), is considered as a key factor in microglial activation and macrophage recruitment. After the nerve injury, CCL2 and its receptor CCR2 expression are upregulated in TRPV1-expressing primary sensory neurons at DRG and in spinal microglia, inducing the recruitment of peripheral immunocompetent cells [11,12]. CD68 is a lysosomal protein in blood-derived immune cells of monocyte line [13], therefore, it is widely used for immunohistochemical labeling of invaded macrophages [3,14,15].

The Renin-Angiotensin system (RAS) is involved in the regulation of blood pressure and fluid and electrolyte homeostasis *via* peripheral somatic [16] as well as CNS-located signaling pathways [17]. Under physiological conditions, Angiotensin II (AngII), the main bioactive component of RAS, is involved in regulation of several functions in the CNS, such as cerebrovascular flow, autonomic and hormonal systems,

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stress, innate immune response and behavior [18]. In pathological states, Ang II signaling participates in inflammatory changes in the CNS [19,20]. At central level, Ang II mediates its effects predominantly through its type 1 receptor (AT1R), which is widely expressed not only in the brain structures, but also in the spinal cord, DRGs and nerve fibers [21]. The use of sartans, specific AT1R antagonists, revealed involvement of AT1R signaling in neuronal inflammation [22], mood disorders [23], Alzheimer's disease [24,25] and neuropathic pain development [26,27]. Direct anti-inflammatory effect of AT1R blockade, independent from the antihypertensive activity was demonstrated in the brain after administration of lipopolysaccharide [20]. Losartan is one of the most used AT1R antagonists among sartans [28]. While losartan was initially developed to treat high blood pressure, it is now also explored for the treatment of other pathological conditions [29]. Additionally to AT1R blocking properties, losartan and few other sartans were shown to activate nuclear peroxisome proliferator-activating receptor γ (PPARγ) [30,31]. Along with its other functions, PPARγ is known to participate in the regulation of neuroimmune response, as well as neuropathic behavior, trough the modulation of neuroinflammation and macrophage polarization in several pathological conditions [32–34].

The present work shows that losartan treatment attenuated neuropathic thermal hyperalgesia, the expression of spinal inflammatory proteins and macrophage accumulation in DRG in a rat model of spinal nerve injury-induced peripheral neuropathy.

2. Materials and methods

2.1. Animals

Adult male Wistar rats, weighing between 250 and 350 g, were used. Animals were kept on a 12-h light/dark cycle, in an environment with adequate temperature and ventilation (22 ± 1 °C) with pellet 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, the European Communities Council Directive of 24 November 1986 (86/609/EEC) and EU Directive 2010/63/EU for animal experiments. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

2.2. Experimental groups

In the present study, 62 rats were randomly assigned to 4 experimental groups. **BPC** (blood pressure control) group ($n = 12$) consisted of unoperated animals, which received losartan *per os* (*p.o.*) treatment and were used for blood pressure and behavioral measurements. 50 rats were subjected to SNL surgery and subdivided to **SNL** group ($n = 24$, without losartan treatment), **LOS** group ($n = 20$, with systemic losartan treatment) and **LOSit** group ($n = 6$, with intrathecal losartan treatment). 32 rats were used for behavioral study: 7 from SNL group, 7 from LOS group, 6 from LOSit group and 12 from BPC group. In each group, baseline data were obtained 1–2 days before the surgery. For behavioral testing, the analyses consisted in the comparison of paw withdrawal latencies (thermal hyperalgesia) and paw withdrawal thresholds (mechanical allodynia) between the operated side (ipsilateral) and the non-operated side (contralateral). After any surgical manipulation (SNL or catheter implantation), animals were placed in individual cages and monitored for healing process and the amount of drinking water or losartan solution consumed. For western blot analyses and immunohistochemistry, we used tissues of rats from the SNL group ($n = 11$ for western blots and $n = 6$ for immunohistochemistry) and from LOS group ($n = 9$ for western blots and $n = 4$ for immunohistochemistry), collected at day 7 after the surgery.

2.3. Surgical procedures and drug administration

2.3.1. 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 a day 0. The ligation of L5 spinal nerve was performed similar to the method described by Kim and Chung [1]. 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.

2.3.2. Intrathecal catheter implantation

Intrathecal catheter implantation was performed as it was described before [35,36]. Catheters were made with two polyethylene tubes 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 longitudinal 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 h before any behavioral testing.

2.3.3. Losartan administration

Losartan was administered *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, the amount consumed was registered and losartan concentration adjusted to fit the average dose of 100 mg/kg/day. For the *i.t.* application - 10 µl of 20 µM solution of losartan (Losartan Potassium, Tocris) was injected into the catheter under isoflurane anesthesia (3%, Forane, AbbVie). This was followed by 40 µl of saline administered into the catheter to clear the catheter dead space. *I.t.* injections were performed daily at the same time starting from the day 0 after the surgery or after the behavioral testing.

2.4. Assessment of mechanical and heat sensitivity

The behavioral tests were performed before the SNL and 1, 3, 5, 7, 9, 12 and 14 days after the SNL operation. Mechanical paw withdrawal threshold (PWT) was assessed on hind paws using electronic dynamic plantar von Frey aesthesiometer (IITC Inc. Life Science). The mechanical withdrawal threshold was the pressure exerted (in grams) that triggered the paw withdrawal. Each stimulus was applied 4 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. Paw withdrawal latencies (PWL) to radiant heat stimuli were determined for both hindpaws. The rats were placed under non-binding, clear plastic cages on a 3-mm thick glass plate and left to adapt for at least 20 min. A focused light source with a halogen bulb was used to deliver heat stimuli (50 W, Dittel). 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 4

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.

2.5. 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 the blood pressure was automatically measured 10 times in consecutive. The averaged value from these 10 measurements was used. BP was monitored in group consisted of control rats with losartan *p.o.* administration in which BP was measured on 3, 7 and 14 day of losartan administration. BP measurement was not performed in the operated animals as the surgery intervened with the BP measurement technique used.

2.6. Western blot assay

The western blot experiments were performed at postoperative day 7 using two groups of rats: SNL ($n = 11$) and LOS ($n = 9$) groups. All rats used for western blot assays exhibited a neuropathic pain-like state, confirmed by prior behavioral testing. Animals were deeply anesthetized with *i.p.* injection of ketamine (100 mg/kg, Narketan, Zentiva) and xylazine (25 mg/kg, Xylapan, Zentiva). The longitudinal incision of the skin was performed at the L3-S1 levels of the spinal cord (SC). After dissection of the muscles and processing of the dorsal part of the spine, the L4-L5 spinal cord section was removed and the left part (ipsilateral) was isolated from the right part (contralateral) by using sterilized blade. The spinal samples were frozen and stored at −80 °C. Each sample was mechanically homogenized with hand-held pellet pestle in CelLytic™ mammalian tissue lysis/extraction reagent (Sigma-Aldrich) prepared with a protease inhibitor cocktail (*w/v* 1:10, Sigma-Aldrich). The samples were then centrifuged (10 min, 5000 rpm, 4 °C), and the supernatants were collected and centrifuged again (20 min, 13,000 rpm, 4 °C). Supernatant protein concentration was determined by Bradford method using BioRad protein assay (BioRad) and the samples were diluted in a reducing sample buffer (1:1, v:v) untill final concentration of 1 μg/μl. Protein samples were boiled for 3 min and separated by SDS-PAGE 4–10% Bis acrylamide (Serva)-Tricine (Sigma-Aldrich) Gel with a sample volume of 20 μl/well. Proteins were then electrotransferred onto a nitrocellulose membrane (0.2 μm, BioRad). The membrane was first saturated by incubation in blocking solution (5% bovine serum albumin and 0.1% Tween 20 in TBS) for 20 min and was then incubated overnight at 4 °C with monoclonal mouse anti β-actin (1:1000, loading control); and rabbit polyclonal anti-TNFα, rabbit polyclonal anti-TNFR1, rabbit polyclonal anti-OX42 (microglial marker), rabbit polyclonal anti-GFAP, rabbit polyclonal anti-AT1R, rabbit polyclonal anti-CCL2, rabbit polyclonal anti-CCR2 or rabbit polyclonal anti-IL6 (1:000, 1:250, 1:500, 1:1000, 1:500, 1:250, 1:250 and 1:1000; respectively) in diluting solution. Blots were rinsed 3 times with 0.1% Tween 20 in TBS and incubated for 90 min with fluorocore-coupled goat anti mouse IRdye 800 and goat anti rabbit licor IRdye 680 (1:5000). Blots were rinsed 3 times with 0.1% Tween 20 in TBS, and then were scanned to reveal the protein bands with the Odyssey System Imager (Li-Cor) coupled to acquisition software. Antibodies were provided by Exbio (anti-CCL2, anti-TNFR1, anti-β-actin), BioVision (anti-CCR2), Neuromics (anti-OX42), Abcam (anti-ATR1, anti-TNFα, anti-IL6), Sigma (anti-GFAP) and Li-cor (anti-mouse, anti-rabbit). The immunoreactivity of proteins of interest was compared with β-actin immunoreactivity values controls and quantified based on scanned images of the blots, with Aida image analyzer software (Aida™). Preliminary analyses were performed to check the variability of β-actin (immunoblotting loading control) levels among the animals and the experiments, using One-way ANOVA statistic test. The results did not show any significant change between the ipsilateral and contralateral levels in all animals.

2.7. Immunohistochemistry

The rats from the SNL ($n = 6$) and LOS ($n = 4$) groups were deeply anesthetized with ketamine (100 mg/kg, Narketan, Zentiva)/xylazine (25 mg/kg, Xylapan, Zentiva) combination on day 7 after the SNL surgery, perfused intracardially with a saline followed by ice cold 4% paraformaldehyde. L5 DRGs and adjacent DRs were removed and post-fixed at 4 °C for 24 h, cryoprotected with sucrose overnight, and cut in cryostat at 16 μm. These sections were then processed for CD68 immunohistochemistry. Briefly, sections were blocked with 3% normal donkey serum for 30 min at room temperature (RT) and incubated overnight at 4 °C with mouse anti-CD68 (1:200; Serotec, Raleigh, NC) primary antibody in 1% NDS with 0.3% Triton X-100. The slides were then washed for 30 min in NDS and exposed to a donkey anti-mouse secondary antibody Alexa Fluor ® 488 (1:400, Jackson Immuno Research Inc., USA) for 2 h. All sections were visualized and captured using fluorescence microscope equipped with a digital camera system (Olympus BX53). For every section, the region of interest (ROI) was outlined and measured (in pixels). For DRG sections, only regions of the sensory ganglia containing sensory neuronal cell bodies (excluding nerve fibers) were outlined. Area of CD68-immunoreactive (IR) cell bodies in this region was measured using ImageJ software (NIH, USA). IR/ROI ratios were calculated and expressed as percentage (IR%).

2.8. Data analysis

During mechanical stimulation with the electronic VF, 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 condition and time point. For statistical analysis two-way repeated measures analysis of variance (RM ANOVA) was used for the ipsilateral *versus* contralateral results comparisons as the between-subject variable and time as the repeated measure, to analyze differences over time between the experimental and control paw in every group of animals. Two-way ANOVA was used to assess statistical differences at different testing time points between the experimental groups. One-way ANOVA was used for the differences over time in blood pressure compared with the basal level. Holm-Sidak *post hoc* test was used to test differences between the ipsilateral and contralateral paws and between the experimental groups at each different time point. For the western blot data analyses, all values are expressed as means ± SEM and were obtained after first standardization of the raw values to β-actin corresponding values and then after normalization using the contralateral spinal cord sample value as reference of 100%. Mann-Whitney Rank Sum Test was used for the statistical comparison of protein levels between ipsi- and contralateral sides, as well as between the SNL group and the LOS group, followed by Bonferroni correction to counteract the problem of multiple comparisons. For immunohistochemistry, IR% ratios were averaged from 4 to 10 sections for each DRG and DR for each animal and averages were further used for statistical analysis. Wilcoxon Signed Rank test was used to compare the differences between ipsilateral and contralateral sides for each group of animals and Mann-Whitney *U* test was used to determine the significance of the difference between SNL and LOS groups, the criterion for statistical significance was $P < 0.05$. All statistical tests were performed using SigmaStat™ software.

3. Results

3.1. Spinal nerve ligation induced thermal hyperalgesia and mechanical allodynia

Unilateral ligation of L5 spinal nerve induced a significant reduction of the paw withdrawal latencies to radiant heat from 22.8 ± 0.3 s

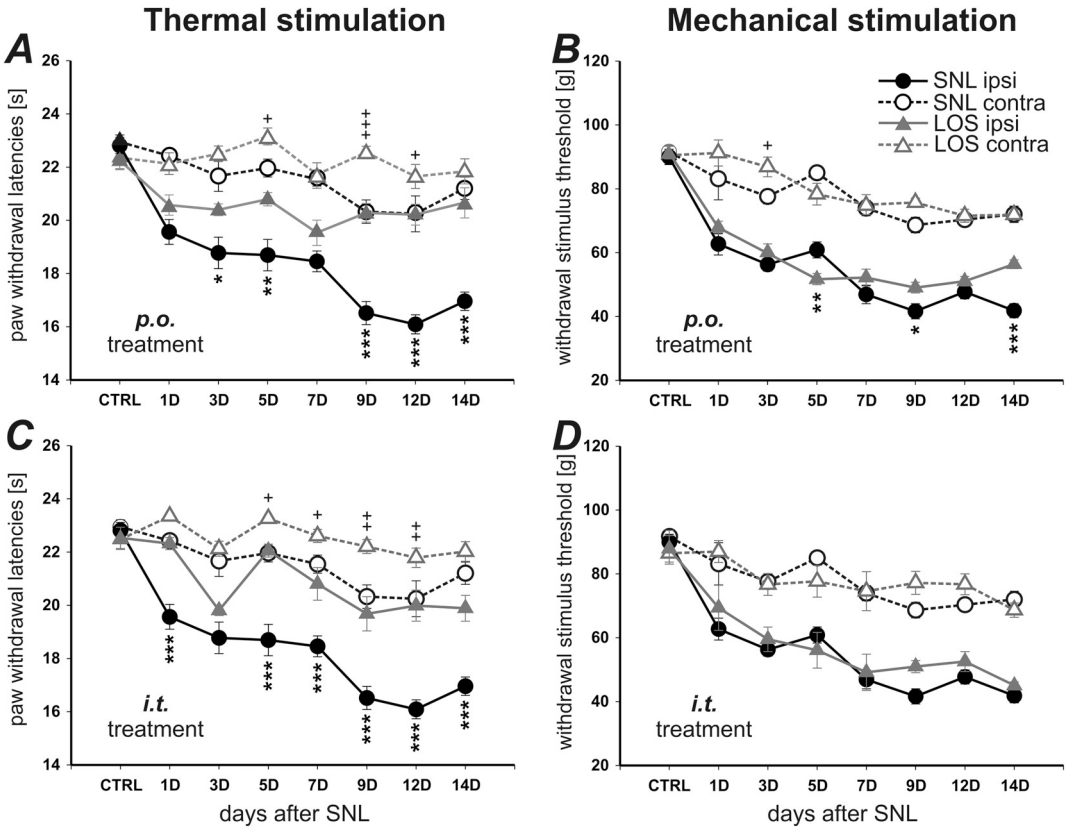

Fig. 1. Losartan treatment attenuated heat hypersensitivity and did not change mechanical allodynia in SNL-induced neuropathy. SNL induced heat hyperalgesia (**A**, **C**) and mechanical allodynia (**B**, **D**) in the tested animals. Losartan, administered *p.o.* (**A**, **B**) or *i.t.* (**C**, **D**) attenuated the development of heat hyperalgesia, but had only minor effect on the SNL-induced mechanical allodynia. Data are presented as means ± SEM. Significant differences between the SNL ($n = 7$) and LOS (n = 7 *p.o.*, $n = 6$ *i.t.*) groups are depicted with asterisks for differences between the ipsilateral and crosses between the contralateral sides (Two Way ANOVA, $^{*}P < 0.05$; $+P < 0.05$; $^{**}P < 0.01$; $++P < 0.01$; $^{***}P < 0.001$; $+++P < 0.001$).

under the control conditions to a minimal value of 16.1 ± 0.4 s, obtained on the 12th day after the surgery. Hypersensitivity of the ipsilateral paw appeared already on day 1 after the SNL and was even more evident 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 contralateral paw ($P < 0.05$) (Fig. 1A, C).

Mechanical allodynia was also present in the SNL group. Already on day 1 after the SNL induction, the thresholds to the electronic von Frey stimulus significantly decreased from 90.0 ± 2.4 g (control value) to 62.7 ± 3.4 g on ipsilateral side. The decrease of the mechanical threshold reached maximum during the second week after the surgery (41.6 ± 2.4 g on the 9th postoperative day). Changes in the mechanical sensitivity on the contralateral side were much less pronounced but also significantly different when compared to the control values before the surgery ($P < 0.05$) (Fig. 1B, D).

3.2. Losartan treatment reduced SNL-induced thermal hyperalgesia

The role of sartan treatment in the development and maintenance of neuropathic pain was tested by losartan administration. Losartan *p.o.* treatment (100 mg/kg/day) significantly attenuated the decrease in the paw withdrawal latencies following the SNL surgery on the ipsilateral side when compared to the control group of animals (Fig. 1A). The PWL to thermal stimulation on the ipsilateral paw in the SNL-operated animals on day 9 was 16.5 ± 0.4 s, while the losartan *p.o.* treated rats (LOS group) exhibited an average of 20.3 ± 0.4 s at the same time point.

Intrathecally administered losartan (10 µl of 20 µM solution every day) also showed antihyperalgesic effect (LOSit group). It attenuated the decrease in PWL on the ipsilateral side which appeared after the induction of peripheral neuropathy on day 9 (16.5 ± 0.4 s in SNL group and 19.7 ± 0.3 s in LOSit group). Antihyperalgesic effects of the losartan treatment were observed during the whole tested period (Fig. 1C).

3.3. Losartan treatment had minor effect on mechanical allodynia induced by SNL

SNL induced a statistically significant reduction of the paw withdrawal thresholds to mechanical stimuli revealing a mechanical allodynia, which was sustained throughout the 14 days of the testing period ($P < 0.05$). Tests with the electronic von Frey filament showed that losartan *p.o.* or *i.t.* treatment did not change the thresholds significantly, when compared to the SNL rats without the treatment at most of the time points (Fig. 1B, D).

3.4. Effect of losartan administration on blood pressure and behavioral responses in un-operated rats

Losartan is a widely used antihypertensive drug. In order to study the possible effects of blood pressure change on the behavioral tests, we performed control experiments with unoperated rats (BPC group, $n = 12$). Losartan was administered *p.o.* (100 mg/kg) for two weeks. The blood pressure levels and behavioral responses to mechanical and thermal stimulations were monitored. As was expected, losartan administration caused a significant decrease of the blood pressure values in the BPC rats. At day 14 of the losartan administration, systolic blood pressure was significantly reduced from 136.6 ± 1.3 mmHg (control value) to 114.9 ± 3.95 mmHg. However, this decrease in BP did not

affect the behavioral responses to thermal and mechanical stimulation ($P > 0.05$). Under the control conditions, the average PWL in this experimental group was 22.4 ± 0.2 s and the PWT was 91.8 ± 1.3 g and after 2 weeks of losartan *p.o.* treatment they were 22.3 ± 0.2 s and 91.3 ± 1.3 g respectively.

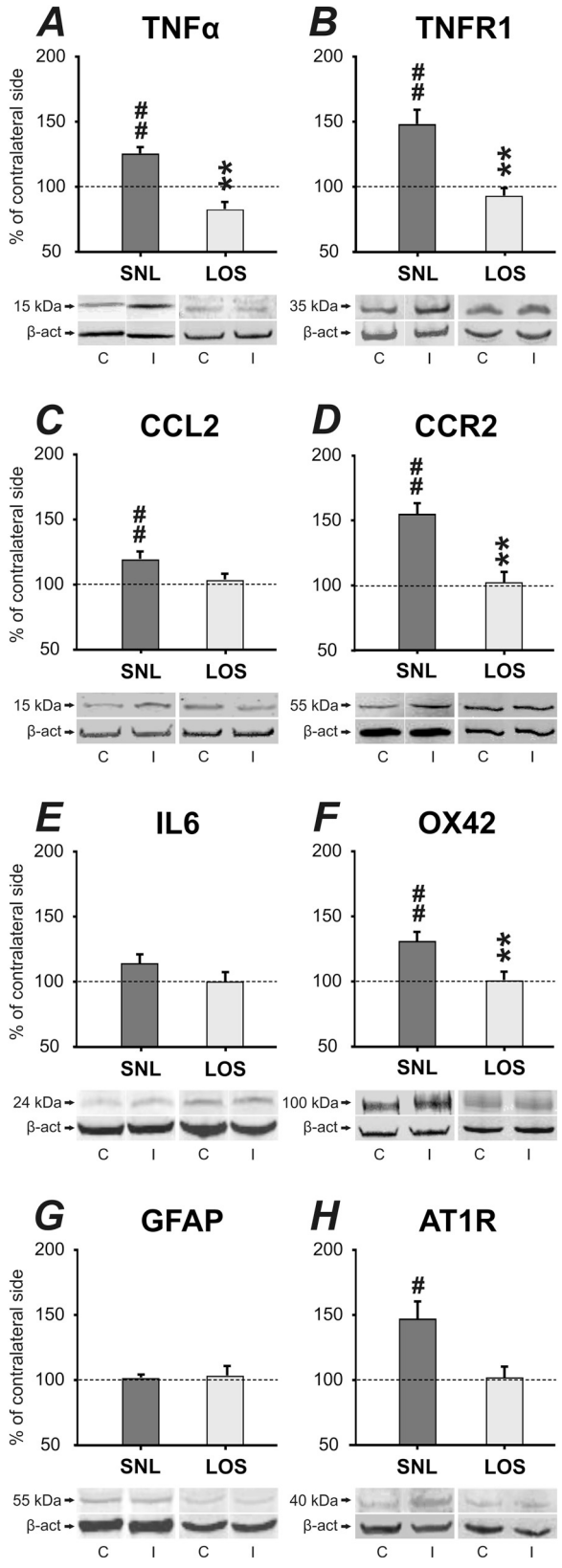

Fig. 2. Losartan *p.o.* treatment diminished ipsilateral increase of neuroinflammatory markers in the spinal cords 7 days after the SNL. Relative protein quantification for comparisons between the SNL-operated rats (**SNL** group, $n = 11$, dark gray bar) and SNL-operated rats with losartan *p.o.* treatment (**LOS** group, $n = 9$, light gray bar). Each value was normalized to the housekeeping protein (β-actin) and SNL ipsilateral spinal cord data are expressed in percentage of the contralateral value in each group. Data are shown as means ± SEM. Mann-Whitney Rank Sum Test was used for the statistical comparison of protein levels between the ipsilateral (I) and contralateral (C) sides (# $P < 0.025$; ## $P < 0.005$), as well as between the SNL and the LOS groups (** $P < 0.005$), followed by Bonferroni correction.

3.5. Effects of SNL on pro-inflammatory markers expression levels in the spinal cord

Our western blot analyses showed that proinflammatory cytokine TNFα (Fig. 2A, 124.9 ± 4.8%) and the chemokine CCL2 (Fig. 2C, 119.1 ± 5.8%) levels were elevated in the ipsilateral side of the lumbar SC after the SNL surgery. Increased levels in SNL-operated rats were also observed for their receptors TNFR1 (Fig. 2B, 147.8 ± 10.6%) and CCR2 (Fig. 2D, 154.3 ± 8.7%) respectively. Cytokine IL6 protein levels were not significantly affected (112.9 ± 6.8%). In addition, neuroinflammatory changes were confirmed by the increase of OX42 protein levels in the ipsilateral side of spinal cord after the SNL (Fig. 2F, 130.4 ± 6.7%), but no significant changes in the GFAP levels were detected (Fig. 2G, 101.2 ± 2.1%), suggesting the occurrence of microglia activation and absence of astrogliosis at the time point of the measurement (day 7 after the SNL surgery).

3.6. Effects of losartan on the expression of pro-inflammatory markers in the spinal cord after the SNL

Losartan *p.o.* (100 mg/kg) abolished the overexpression of TNFα (Fig. 2A, 82.3 ± 5.5%), CCL2 (Fig. 2C, 104.5 ± 3.3%) and their receptors TNFR1 (Fig. 2B, 92.5 ± 5.6%) and CCR2 (Fig. 2D, 102.0 ± 7.8%) in the ipsilateral SC. On the other hand, OX42 levels did not differ between the ipsi- and contralateral sides (Fig. 2F, 100.5 ± 6.5%). The treatment with losartan thus prevented the increased expression of the proinflammatory proteins on the ipsilateral spinal cord after the spinal nerve ligation.

3.7. Effects of SNL and losartan treatment on AT1R expression levels in the spinal cord

SNL induced a significant increase of AT1R levels in the ipsilateral SC (Fig. 2H, 145.8 ± 14.3%) and this increase was prevented by the losartan *p.o.* treatment (101.7 ± 8.6%).

3.8. Effect of losartan p.o. treatment on SNL-induced macrophage accumulation in DRGs and dorsal roots

Analysis of the macrophage accumulation in the DRGs and dorsal roots was based on the expression of CD68-positive cells in the L5 DRGs and adjacent DR. Two sections of DR were compared, the one closer to DRG (approximately 5 mm from DRG - gDR) and the other one closer to the spinal cord (5 mm from spinal cord - scDR). Tight ligation of L5 spinal nerve resulted in enhanced accumulation of CD68-positive macrophages in the L5 DRG ipsilateral to the injury, when compared to the contralateral DRG (Fig. 3A, B, E). In the SNL group the area of CD68 immunopositivity (IR%) measured in the ipsilateral DRGs (2.51 ± 0.63%) was significantly higher when compared to the contralateral side (0.08 ± 0.03%, $P < 0.05$). Macrophage accumulation was also enhanced in the ipsilateral gDR of the SNL animals (0.92 ± 0.37%, $P < 0.05$), while in the contralateral gDR it was very

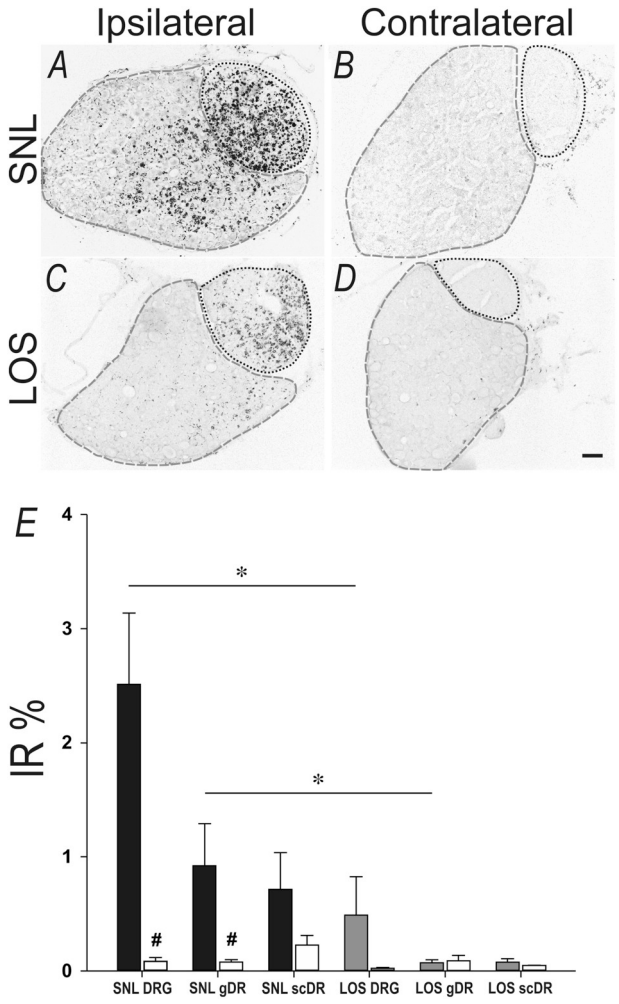

Fig. 3. Losartan *p.o.* treatment decreased SNL-induced DRG macrophage infiltration. Representative immunohistological sections illustrate the accumulation of CD68-positive cells in L5 DRG on the ipsilateral (**A, C**) and contralateral (**B, D**) sides in rats from the SNL (**A, B**) and LOS (**C, D**) groups. DRG bodies are outlined with gray dashed line and the area of dorsal roots is outlined with black dotted line. Scale bar = 100 μm. (**E**) Illustrates the change in CD68-immunoreactive area (IR%) in the ipsilateral DRGs and dorsal roots adjacent to DRG (gDR) and spinal cord (scDR) from the SNL group (n = 6, black bars) and LOS group (n = 4, gray bars) and corresponding contralateral sides (white bars). Data are presented as means ± SEM. Wilcoxon Signed Rank test was used to compare the differences between the ipsilateral and contralateral sides for each group of animals (# $P < 0.05$) and Mann-Whitney U test was used to determine the significance of the difference between SNL and LOS groups (* $P < 0.05$).

low (0.08 ± 0.02%). The difference in CD68 IR% between the ipsilateral and contralateral scDRs was not significant (0.71 ± 0.32% and 0.23 ± 0.09%). Losartan *p.o.* treatment was able to significantly attenuate the macrophage infiltration into DRGs (Fig. 3C, D, E). In the LOS group, CD68 IR% in the ipsilateral DRG and gDRs was significantly lower in comparison to SNL group (0.49 ± 0.34%, and 0.07 ± 0.03%, $P < 0.05$). Losartan treatment also diminished any side to side differences between the DRG, gDR and scDR after the SNL induction.

4. Discussion

Our study confirmed increased sensitivity to thermal and mechanical stimuli and macrophage infiltration into DRG and dorsal roots after SNL and demonstrated increased levels of several proinflammatory proteins in the ipsilateral SC. Administration of losartan (both *p.o.* and *i.t.*) caused a significant reduction of the SNL-induced thermal hypersensitivity, but it failed to prevent the development of mechanical allodynia. Mechanical hypersensitivity was suggested to be associated with CCL2 overexpression [12,36] and in particular with CCL2 produced by DRG primary neurons [37]. In our experiments, losartan treatment attenuated ipsilateral CCL2 overexpression in the SC. It is possible that different origin of CCL2 in the SC and DRG may explain our behavioral data. In the DRG, neurons constitute the main CCL2 source, while at the SC level, glial cells were suggested to mediate CCL2 production [10]. Our data may reflect the fact that losartan was more effective to prevent glial CCL2 upregulation by targeting glial functions, while it was less effective to affect the neuronal CCL2 production. Although macrophage infiltration into the DRG was successfully attenuated by losartan treatment, it was not blocked completely, suggesting reduced presence of chemoattractant substances like CCL2 in the DRG even after losartan treatment. In addition, it is known that the development of thermal hyperalgesia and mechanical allodynia after peripheral nerve injury are modulated by different pathways [38].

The efficacy of the losartan action depends on its ability to penetrate the blood-brain barrier. Pharmacokinetic studies confirmed that losartan administered *per os* leads to satisfactory, but not complete inhibition of centrally mediated actions of angiotensin II, if the administered dose is high enough (30–100 mg/kg) [39]. This is comparable with the relatively high dose of losartan (100 mg/kg, *p.o.*) used in our experiments. Additionally, *i.t.* application of losartan induced similar behavioral effects, suggesting that the effective site of action was within the spinal cord. The main target of losartan, AT1R, was initially characterized as a receptor member of RAS [40], where it is activated by Ang II and is responsible for blood pressure changes. In our control experiments, we have shown that blood pressure changes induced by losartan application did not affect the behavioral responses to thermal and mechanical stimulation. However, losartan dose-response curve was not performed and we cannot exclude the possibility that the results are related to the dose used. Several AT1R inhibitors exhibited neuroprotective activities at very low concentrations, which did not affect blood pressure in normotensive rodents [18,41,42]. These lines of evidence, in addition to our data, suggest that the neuroprotective effects of AT1R blockers were most likely independent of their blood pressure regulatory function.

Protein quantification and immunohistochemistry were performed at day 7 after the SNL, when the behavioral changes and the effect of losartan treatment were most pronounced. Western blot analyses revealed expected overexpression of OX42, indicating microglia activation, while unchanged GFAP levels suggested the absence of astrogliosis at this time point. Both microglia and astrocytes play a major role in the development of behavioral hypersensitivity in animal models of neuropathic pain, notably through the production and secretion of immune mediators [43]. However, in chronic neuropathic process, microglia and astrocytes act at different time points. Microglial cells are activated first and are involved in the initiation phase while astrocytes mediate the maintenance of neuropathic pain after SNL [44]. Day 7 after the SNL in our experiments probably still corresponds to the initiation phase, before the occurrence of astrogliosis. Early upregulation of OX42 along with unchanged GFAP expression was described also in CCI [45] and L5 nerve transection (L5Tx) models [46]. However, other studies revealed astrocytes activation during the development phase of neuropathy in SNL model combined with transection on the distal side of the ligation [47], or an upregulation of OX42 lasting during the maintenance phase after L5Tx [48]. The presence of activated microglia and the lack of astrogliosis at day 7 in our experiments correspond well with increased levels of CCL2, TNFα and their respective receptors CCR2 and TNFR1 after the SNL. These results suggest that microglial activation at this time point was the main source of cytokines and chemokines at spinal level as was described before [49].

Losartan *p.o.* treatment diminished any changes in the expression of CCL2, CCR2, TNFα and TNFR1 when compared both to the

contralateral side and to the untreated animals. These data suggest that losartan application attenuated neuroinflammation on the side of the injury. While it was suggested before that proinflammatory mediators may diffuse also to the contralateral side [50,51], our experiments in control animals confirmed a significant ipsilateral increase of the neuroinflammatory mediators.

IL6 is an important proinflammatory cytokine as IL6 deficient mice did not develop neuropathic behavior after CCI [52]. In our study, the IL6 expression levels in the ipsilateral lumbar SC did not change compared with the contralateral side at day 7 after the SNL. It was suggested before that peak in IL6 expression may be transient and early after the neuropathy induction. Cao et al. [53] observed an elevation of IL6 protein expression with a peak at day 1 after L5Tx, which was maintained at day 3, but no significant difference was detected between the ipsi- and the contralateral SC at day 7. It seems plausible to suggest that the level of IL6 expression has already peaked before the day 7 in our experiments. Our results also showed a reduction of SNL-induced AT1R overexpression in SC after losartan treatment, suggesting a possible involvement of AT1R in the neuroinflammatory process.

Peripheral nerve injury leads to neuroimmune response both in the central and peripheral nervous systems, including activation of resident immune-like cells (microglia in spinal cord or satellite glial cells in DRG) and/or invasion of immunocompetent cells of hematopoietic origin (invaded macrophages). The peak of neuroimmune response may vary in different models of peripheral nerve injury [3], but in the SNL model it is commonly observed approximately one week after the surgery [3,54]. In our study, tight ligation of L5 spinal nerve promoted infiltration of blood-derived macrophages into the ipsilateral L5 DRG and gDR on day 7 after the surgery, as well as microglia activation in SC, reflected in significant increase of the OX42 protein levels.

According to our immunohistochemistry results, systemic losartan treatment significantly attenuated CD68-positive macrophage accumulation in the ipsilateral DRG and gDR. Several research groups used sartans for their experiments of effective anti-inflammatory treatments for different models, for example, dementia [24], atherosclerosis [55] or diabetes [56] and other inflammatory diseases. Notably, losartan acts as a pro-antagonist in the AT1R blockade since it doesn't bind to the Ang II receptor itself. After administration losartan is catabolized into two metabolites: EXP3174 and EXP3179. EXP3174 has been described as the proper antagonist molecule, acting as a competitive antagonist [57], while EXP3179 does not interfere with any Ang II binding sites [58]. We thus cannot exclude the possibility that EXP3179 metabolite may activate different transduction pathways, independently to AT1R antagonism in our experiments. Losartan exhibits a significant affinity to PPARγ receptors [30] and was also shown to activate PPARγ transduction pathways [59]. We hypothesize, that in our study, the effect of losartan treatment on CD68-positive macrophages may be mediated by similar mechanisms, as losartan metabolite EXP3179 binds to PPARγ directly in invaded macrophages [60]. Recently, PPARγ was intensively studied for its role in macrophage polarization. A growing number of studies confirm, that PPARγ activation triggers macrophage polarity shift from M1 (proinflammatory) to M2 (antiinflammatory) phenotype, thus reducing local neuroinflammation and neuropathic pain behavior [32,34].

In a wider view of the possible new neuropathic pain drug development, the emerging data based on other strategies involving the blockade of the angiotensin II signaling seem to be also promising. Two research groups reported analgesic effects of small molecule AT2R antagonists in a peripheral nerve injury and antiretroviral neuropathic pain models in rats [61,62], as well in postherpetic neuralgia patients [63]. In particular, the advanced study of Rice et al. in a randomized, double-blind, placebo-controlled phase 2 clinical trial, showed a superior relief of postherpetic neuralgia, with no serious side effects in patients treated with a small molecule AT2R antagonist (EMA401) at the end of 28 days of treatment [63].

5. Conclusion

Our study has demonstrated a significant anti-inflammatory and analgesic effect of losartan treatment in a model of SNL-induced peripheral neuropathy in rats. Further experiments are needed to identify and describe the specific molecular mechanisms involved in the losartan action. However, losartan based drugs may potentially represent a new treatment approach for neuropathic pain patients.

Author contributions

JP conceived the study, NK performed the surgical procedures, blood pressure measurements, behavioral and immunohistochemistry experiments, MD performed Western blot analyzes. NK and MD wrote the manuscript and all authors discussed the results, read and approved the final version of the manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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