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DISSERTATION

Gene expression and immunohistochemical localization of distinct modulators of inflammation and pain

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1. List of abbriviations

ANOVA……………………………………………………………………analysis of variance

AGE……………………………………….……………for advanced glycation endproducts

CGRP ……………………………………………….…………Calcitonin gene-related peptide

COX-2……………………………………..………………..…….. ….Cyclooxygenase

DRG……………………………………………………………….…Dorsal root ganglion

FPR2……………………………………………………….……... N-formyl peptide receptor 2

GAD65……………....…………………………………... …...glutamate decarboxylase 2

GFAP ………………………………………….……….……..… Glial fibrillary acidic protein

IL-1β……………………………………………………….................…Interleukin-1 beta

IL-10…………………………………………………………..……...…. Interleukin-10

JT ………………………………………………………………………………… Joint trauma

5-LOX…………………………………...…………………………….…5-Lypoxygenase

15-LOX……………………………………………………...……….…15-Lypoxygenase

MR……………………………………………………………....…mineralocorticoid receptors

MOR ………………………………………………………………………. mu-opioid receptor

mPGEs-1………………………………………………. microsomal prostaglandin synthases

mRNA…………………………………………………………Messenger ribonucleic acid

NF200……………………………………………………………………...Neurofilaments

OX42 ……………………………………………………………..Marker for microglia cells

OA……………………………………………………………….. …………Osteoarthritis

PGE2…………………………………………………………………………. Prostaglandin E2

PKC……………………………………………..….Protien kinase c

RA …………………………………………………………………..… Rheumatoid arthritis

Rab7……………………………………………………………..…...........Ras-related protein7

RAGE…………………………..……..……….. Receptor for advanced glycation endproducts

RT-PCR ……………………………………………………….… Real Time quantitative PCR

Ser375…………………………………………………..………………..……..Serine 375

siRNA……………………………...………………………………Small interfering RNA

Thr370………………………………………………………………………… threonine 370

TNFα………………………………………………………..….Tumor necrotic factor- alpha

trkA ……………………………………………………….....…..Tyrosine Receptor Kinase A

TRPV1…………….......... transient receptor potential cation channel subfamily V member 1

2. Abstract

2.1 Abstract (English)

Acute as well as chronic pain leads to suffering and impaired quality of life. This project examined three different ways of pain modulation. In the first study, distinct profiles of pro- versus anti-inflammatory mediators were characterized for patients with various joint diseases. The results showed that upregulated pro-inflammatory mediators mainly regulate the inflammatory process in joint trauma (JT) and rheumatoid arthritis (RA) synovium, whereas upregulated anti-inflammatory mediators regulate the inflammatory process in osteoarthritis (OA) synovium. In the second study mineralocorticoid receptors (MR) as potential pain regulators were identified on peptidergic nociceptive neurons in the spinal cord and dorsal root ganglia as well as in peripheral nerve terminals penetrating the skin of naïve rats. Intraplantar and intrathecal application of MR agonists immediately elicited decreased thresholds of mechanical painful sensation suggesting an enhanced nociceptive sensitization due to rapid non-genomic effects. Consistently, membrane-bound MR in nociceptive neurons could be characterized by radiolabeled ligand binding as putative pain regulators. In the third study pain relieving effects of opioids were impaired in painful diabetic neuropathy. The results demonstrate that enhanced accumulation of advanced glycation products leads to RAGE-mediated PKC activation and subsequent mu-opioid receptor (MOR) phosphorylation and desensitization, thereby, impairing the analgesic efficacy of opioids. Blockade of AGE accumulation or RAGE gene silencing by use of siRNA prevented these diabetes-induced alterations and recovered opioid responsiveness. Taken together, this project identified and characterized three novel mechanisms of pain modulation that is important in human diseases such as arthritis or diabetic neuropathy.

2.2 Abstract (deutsch)

Akute sowie chronische Schmerzen führen zu Leiden und Beeinträchtigung der Lebensqualität. Dieses Projekt untersuchte drei verschiedene Möglichkeiten der Schmerzmodulation. In der ersten Studie wurden für Patienten mit verschiedenen Gelenkerkrankungen unterschiedliche Profile von pro-versus entzündungshemmenden Mediatoren charakterisiert. Die Ergebnisse zeigten, dass hochregulierte pro-inflammatorische Mediatoren hauptsächlich den entzündlichen Prozess bei Gelenktrauma (JT) und rheumatoider Arthritis (RA) regulieren, während hochregulierte entzündungshemmende Mediatoren den entzündlichen Prozess bei Osteoarthritis (OA) regulieren. In der zweiten Studie wurden mineralocorticoide Rezeptoren (MR) als potentielle Schmerzregulatoren auf pepidergischen nozizeptiven Neuronen im Rückenmark und dorsalen Wurzelganglien sowie in peripheren Nervenendigungen, die die Haut naiver Ratten penetrieren, identifiziert. Intraplantare und intrathekale Applikation von MR-Agonisten führten sofort zu verminderten Schwellen mechanischer schmerzhafter Empfindungen, was auf eine verstärkte nozizeptive Sensibilisierung durch membrangebundene MR-vermittelte nicht-genomische Mechanismen schließen lässt. Konsequenterweise konnten membrangebundene MR in nozizeptiven Neuronen durch radiomarkierte Ligandenbindung als potentielle Schmerzregulatoren charakterisiert werden. In der dritten Studie wurde gezeigt, dass schmerzlindernde Wirkungen von Opioiden bei schmerzhafter diabetischer Neuropathie beeinträchtigt waren. Die Ergebnisse zeigen, dass eine erhöhte Akkumulation von fortgeschrittenen Glykations-Endprodukten zu RAGE-vermittelter PKC-Aktivierung und anschließender Mu-Opioidrezeptor (MOR)-Phosphorylierung und Desensibilisierung führt, wodurch die analgetische Wirksamkeit von Opioiden beeinträchtigt wird. Blockade von AGE-Akkumulation oder RAGE-Gen-Silencing unter Verwendung von siRNA verhinderte diese Diabetes-induzierten Veränderungen und stellte die Opiod-Ansprechbarkeit wieder her. Zusammengenommen identifiziert und charakterisiert dieses Projekt drei neuartige Mechanismen der Schmerzmodulation, die bei menschlichen Erkrankungen wie Arthritis oder diabetischer Neuropathie eine wichtige Rolle spielen.

3. Introduction

3.1 Pro- versus anti-inflammatory mediators' contribution to inflammatory pain

Apart from short acting noxious environmental stimuli, a painful sensation is generated as a consequence of tissue injury and a subsequent inflammatory response (Schaible, 2014). In acute tissue injury cell breakdown leads to activation of the processing enzymes cyclooxygenase 2 (COX-2) and microsomal prostaglandin synthases (PGEs-1) which give rise to pain sensitizing products such as prostaglandin E2 (PGE2). In addition, chemokines and cytokines such as IL-1ß, TNF-alpha are released from immigrating immune cells also contributing to the state of enhanced sensitization to painful stimuli (Schaible, 2014). This inflammatory reaction becomes more pronounced and relevant in conditions of persistent inflammation such as arthritis (Schaible, 2014). The more recent literature emphasizes also a counteracting process that leads to the resolution of the inflammatory response and is mediated by anti-inflammatory mediators such as 15-LOX, FPR2 and IL-10 (Buckley, 2014). This process is usually overlapping with the pro-inflammatory process and determines the final healing of the tissue injury.

Noxious environmental stimuli such as heat, mechanical force, and chemical irritants are known to activate specific ion channels at the peripheral nerve ending for example in the skin (Ji RR et al., 2016). These ion channels transduce the noxious environmental stimulus into action potentials, i.e. the language of the nervous system. For example, heat stimuli result in the activation of TRPV1 channels which depolarize the membrane resting potential to a threshold value that triggers the activation of voltage-gated Na channels to finally send action potentials from the peripheral nerve ending to the brain. The response is an immediate reflex-like reaction to withdraw from the source of the painful stimulus, therefore, preventing tissue damage. However, when tissue injury has already occurred, pro-inflammatory mediators (e.g. PGE2, IL-1β, and TNF-α) activate their respective receptors which sensitize peripheral nociceptive neurons resulting in persistent painful sensation (Ji RR et al., 2016). Suppression of the inflammatory response by inhibition of the pro-inflammatory mediators or augmenting the anti-inflammatory mediators leads to a resolution of the inflammatory response and an attenuation of the painful sensation.

This interplay of pro- versus anti-inflammatory mediators plays a crucial role in the different forms of arthritis which is an inflammatory disease or disorder of the joints (NIAMS, 2014).The most common forms of arthritis are osteoarthritis and rheumatoid arthritis (Pelletier et al., 2006; McInnes et al., 2011) which are characterized by an inflammation of the synovium (synovitis)

that often progresses to the destruction of articular cartilage and underlying joints (McInnes et al., 2011). In general clinical signs and symptoms include joint pain, swelling, stiffness, redness, warmth, and dysfunction joints (NIAMS, 2014).The final diagnosis of osteoarthritis or rheumatoid arthritis is made in accordance to the criteria defined by the American Rheumatoid Association and comprises clinical and radiological signs as well as laboratory findings (Altman et al., 1986; Arnett et al., 1988). IL-1β and TNFα are considered the most prominent pro-inflammatory cytokines involved in arthritis (Tracey et al., 1993). In addition, they have a crucial role in the process of cartilage degradation during OA and RA (Youn et al., 2002; Deleuran et al., 1992). 5-Lipooxygenase (5-LOX), a special isoform of lipoxygenase enzyme, has been identified to be involved in the progress of inflammation, and its overexpression has been reported in the synovium of OA and RA patients (Gheorghe et al., 2009). On the other side, anti-inflammatory mediators such as 15-Lipoxygense (15-LOX), formyl peptide receptor2 (FPR2) and IL-10 have shown opposite effects against pro-inflammatory mediators in arthritis (Levy et al., 2014, Corminboeuf et al., 2015). The previous investigation reported that 15-LOX metabolism and FPR2 both have influential anti-inflammatory effects on rheumatoid arthritis (Harada et al., 2003; Dufton et al., 2010; Kao et al., 2014). In a similar way, IL-10 has an important role in limiting the acute and chronic inflammation through inhibition of chemokine and cytokine expression in immune cells like dendritic cells and macrophages (Murray et al., 2005; Moore et al., 2001). Therefore, it was the goal of the first study to characterize the distinct profile of pro- versus anti-inflammatory mediators in various joint diseases with different degrees of an underlying inflammatory process, i.e. in patients with a diagnostic arthroscopy (control), with joint trauma (JT), with osteoarthritis (OA) or with rheumatoid arthritis (RA).

3.2 Neuronal mineralocorticoid receptors' contribution to painful sensation

The persistent inflammatory process and accompanying pain in arthritis can be attenuated by the immunosuppressive effects of steroids such as dexamethasone or methylprednisolone (Garg et al., 2014). The underlying mechanism of this anti-inflammatory effect is through the classical genomic pathway in which the glucocorticoid is bound to its receptor, then the receptor-glucocorticoid complex acts as a nuclear transcription factor within the nucleus and finally activates or inhibits the transcription of specific genes which interrupts the inflammatory process (De Kloet et al., 1998). By this immunosuppressive mechanism is, for example, the enhanced expression and activity of COX-2 inhibited and, thus, the amount of pain sensitizing prostaglandins released into the surrounding tissue significantly reduced (Schaible, 2014). However, glucocorticoids are also known to elicit very rapid effects, e.g on ion channels within

the hippocampus of the brain (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012). Moreover, it was reported that topical corticosteroid application to peripheral neurons immediately blocks the transmission of nerve impulses (Johanson et al., 1990). These actions occur rapidly and cannot be explained by changes in gene expression, underscoring the notion that steroids may also act via non-genomic pathways on specific membrane receptors (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012; Lösel and Wehling, 2003).
Similar to glucocorticoids it was reported that a combination of aldosterone and its respective antagonist spironolactone revealed immunosuppressive effects in allogenic skin grafts (Bachmann et al., 1971), multiple sclerosis (Mertin et al., 1972), and progressive systemic sclerosis (Altmeyer et al., 1985) by a genomic pathway. More recently, treatment with the mineralocorticoid receptor (MR) antagonist spironolactone over three days led to a significant reduction of mechanical pain sensitivity in a rat model of L5 lumbar dorsal root ganglion compression (Gu et al., 2011; Sun et al., 2012). Since the treatment and its effects occurred over a prolonged period of time, they were most likely due to genomic effects of the MR. In addition, Dong et al., (2012) showed a significant reduction in mechanical hypersensitivity following prolonged application of the MR antagonist eplerenone in a model of zymosan-induced L5 dorsal root ganglion inflammation. In this study dorsal root ganglia showed some faint immunohistochemical staining for MR together with the panneuronal cell marker NeuN and eplerenone treatment was shown to reduce the number of activated satellite glia cells. However, up until now, evidence for the exact location of MR in subpopulations of DRG neurons and/or glia cells is still lacking.

Therefore, it was the goal of the second study to identify MR on specific subpopulations of sensory neurons and/or glia cells, to determine whether they are membrane bound and, therefore, subject to fast intracellular signaling and to examine whether peripherally or centrally applied MR agonists show rapid effects on pain sensation which would be consistent with non-genomic effects of these MR.

3.3 Diabetic neuropathic pain's contribution to impaired opioid analgesia

Acute exacerbation of pain is normally treated with analgesic medications among which opioids are the most potent drugs (Stein and Schäfer, 2003). Once administered, opioids enter the central and peripheral nervous system, bind to their respective receptors on neurons and inhibit the propagation of painful stimuli (Stein and Schäfer, 2003). This occurs via G protein coupling

to the mu-opioid receptors (MOR) and subsequent inhibition of intracellular signaling pathways, of voltage-gated calcium channels, and activation of outward rectifying potassium channels to result in the inhibition of pain (Stein and Schäfer, 2003). However, MOR is subject to phosphorylation, desensitization, and internalization after which the receptors are normally recovered by phosphatases and recycled to the cell surface. Only under certain conditions internalized opioid receptors are not recycled but are directed towards their lysosomal degradation which is controlled by GTPases such Rab7 (Mousa et al., 2013). MOR phosphorylation usually occurs within the cytoplasmic tail of the receptor at 363 (Ser363), threonine 370 (Thr370) and serine 375 (Ser375) (Chu et al., 2008; El Kouhen et al., 2011). For example, a previous study demonstrated that PKC activation leads to increased phosphorylation of MOR exclusively on Thr370, but not on Ser375 (Doll et al., 2001). Likely, RAGE receptor in the peripheral sensory neurons can initiate PKC expression and/or activation, e.g. in diabetic neuropathy (Eberhardt et al., 2012; Toth et al., 2007). RAGE is considered as a G-protein coupling receptor which is activated by advanced glycation endproducts (AGE) that accumulate in primary sensory neurons during diabetes (Toth et al., 2007).

It is well known that under certain pain conditions such painful diabetic neuropathy opioids may loose their effectiveness (Bril et al., 2011). With an ever growing population of elderly, obese, and exercise abstinent people the number of patients suffering from diabetes is constantly increasing. One-third of these patients suffer from painful diabetic neuropathy, a disease of the primary sensory neurons which somehow are less responsive to opioid analgesic treatment (Abbott et al., 2001). In the past several studies have assessed the impaired opioid responsiveness following the systemic, spinal, or supraspinal application of opioids (Chen et al., 2003). The various mechanisms have been postulated for this loss in analgesic efficacy, however, the explanations remained controversial and have never been systematically investigated.

Therefore, the goal of the third study was to investigate a putative MOR phosphorylation in primary sensory neurons during diabetes using a specific phosphorylated Thr^{370} MOR antibody. Moreover, this study examined whether primary sensory neurons co-express MOR with specific PKC isoforms as well as with RAGE in rats with experimentally induced diabetes. Finally, this study tested whether the targeted silencing of the RAGE receptor by specific siRNA will prevent PKC activation and subsequent MOR phosphorylation to recover MOR agonist analgesic efficacy.

4. Materials and Methods and results

The following three selected publications are part of my thesis work and are mainly concerned with possible mechanisms of inflammation, pain sensation and pain control.

4.1. Publication: "Comparative expression analyses of pro- versus anti-inflammatory mediators within synovium of patients with joint trauma, osteoarthritis, and Rheumatoid arthritis. "

This project investigated the relative expression of proinflammatory mediators like IL-1ß and TNF-alpha contributing to painful conditions as well as anti-inflammatory mediators such as IL-10 and 15-LOX responsible for the resolution of painful inflammation in different types of human joint diseases.

Study objective: To identify distinct profiles of pro- versus anti-inflammatory mediators in joint diseases with different degrees of an underlying inflammatory process, i.e. in patients with a diagnostic arthroscopy (control), with joint trauma (JT), with osteoarthritis (OA) and with rheumatoid arthritis (RA).

Methods: Following IRB approval synovial tissue samples were freshly taken from different groups of patients scheduled for joint surgery and immediately stored at -80 °C temperature to use for quantitative RT-PCR or fixated in 4% paraformaldehyde to use for immunohistochemistry. For Taqman® RT-PCR primer pairs for IL-1 β, TNFα, 5-LOX, 15-LOX, FPR2, and IL-10 were generated to identify the respective mRNAs. For immunohistochemistry commercial antisera for IL-1β, TNFα, 5-LOX, 15-LOX, FPR2, and IL-10 were purchased to localize respective pro- or anti-inflammatory mediators within synovial tissue. Moreover, markers to characterize different immune cells such as anti-CD15 for granulocytes, anti-CD68 for macrophages, anti-P4HB for fibroblasts, anti-CD3 for Lymphocytes, and anti-Ab-1 for plasma cells were used.

Results: Synovial tissue samples of 5 patients undergoing a diagnostic arthroscopy, of 9 patients following joint trauma, of 11 patients with osteoarthritis, and of 10 patients suffering from rheumatoid arthritis were obtained. These tissue samples showed an increasing number of lining thickness, overall cellularity, and vascularity being lowest in the control group and highest in the rheumatoid arthritis group (control < JT < OA < RA). Double immunohistochemistry identified pro- as well as anti-inflammatory mediators predominantly in granulocytes of JT patients, and in macrophages, lymphocytes, and plasma cells of OA and

RA patients. Quantitative RT-PCR demonstrated a higher abundance of pro-inflammatory IL-1β, TNFα, and 5-LOX specific mRNA in JT and RA patients than OA patients. This was consistent with a higher number of IL-1 β -, TNFα, and 5-LOX-immunoreactive cells in JT and RA than OA patients. However, anti-inflammatory mediator specific mRNA for 15-LOX, FPR2, and IL-10 as well as respective immunoreactive cells within synovial tissue were significantly more abundant in patients with OA than JT or RA.

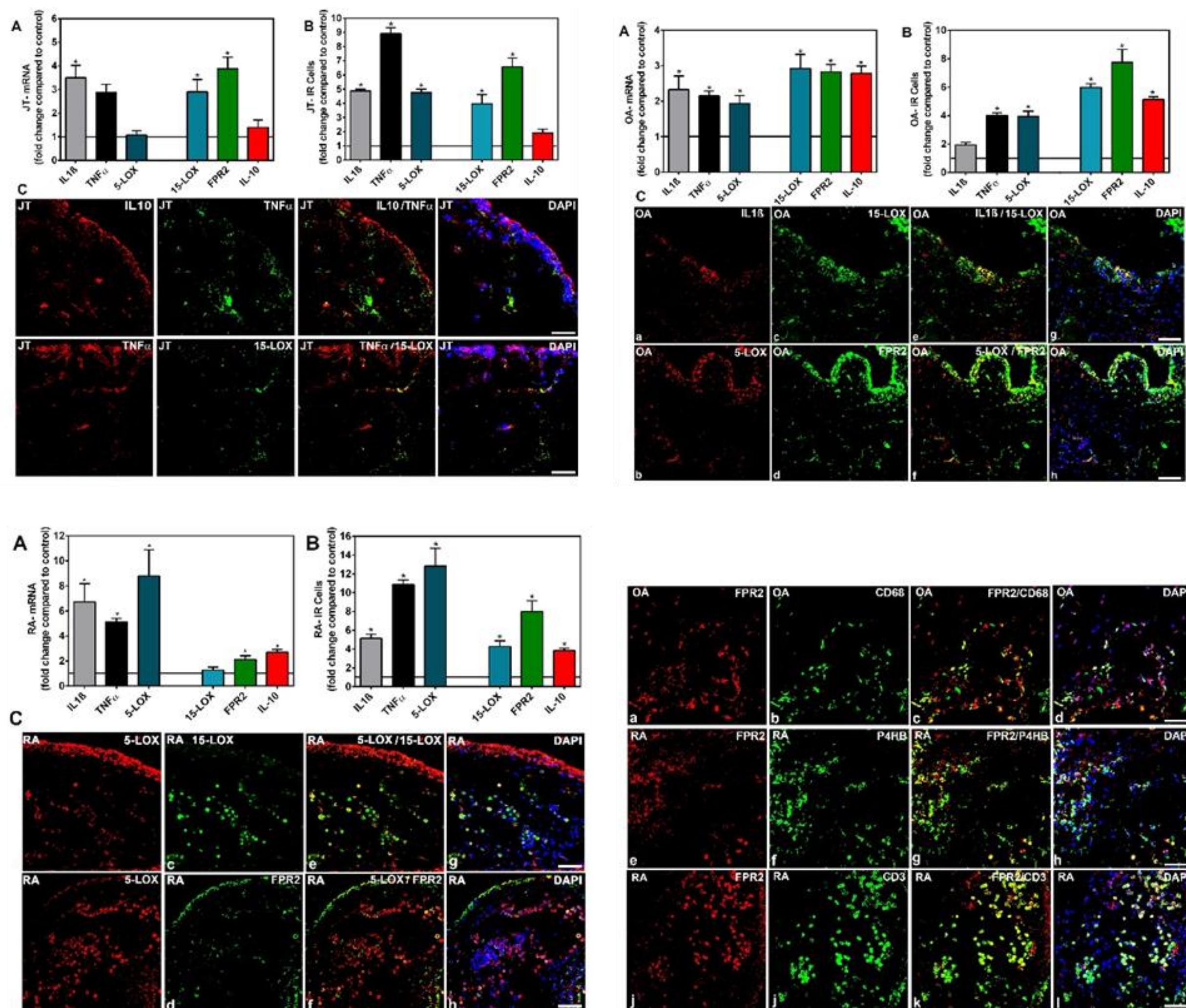

Figure 1. Profiles of pro- and anti-inflammatory mediators determined as mRNA expression and immunoreactive cells within synovial tissue of JT (upper left panel), OA (upper right panel), and RA patients (lower left panel). Synovial tissue was quickly frozen and further processed for quantitative Taqman® RT-PCR with specific primers for pro- and anti-inflammatory mediators and results were calculated by the delta-delta-CT method as –fold change compared to controls (A, B in all panels). In addition, after fixation of synovial tissue immunoreactivity for pro- and anti-inflammatory mediators was determined by use of specific antisera and the number of specifically stained cells per visual field was calculated as –fold change compared to controls (C, D in all panels). In lower right panel: Confocal microscopy of FPR2 (*red fluorescence; a, e, i*) with CD68 (b), P4HB (f) or CD3 (j) (*green fluorescence*) in synovial tissue from patients with osteoarthritis (OA; a-d) and rheumatoid arthritis (RA; e–l) double immunofluorescence. Note that the majority of FPR2 immunoreactive cells coexpress CD68 in OA synovium. e-l shows that the majority of FPR2 immunoreactive cells in RA synovium coexpress P4HB or CD3. Bar=20 µm. Data (n=5 in triplicate) show means ± s.e.m. Asterisk

denote statistically significant differences with a $p<0.05$ (ANOVA and post-hoc Dunnett's test or ANOVA on ranks and post-hoc Tukey test).

Conclusion and relevance: This study shows in various joint diseases with different degrees of an underlying inflammation, i.e. in patients with a diagnostic arthroscopy (control), with joint trauma (JT), with osteoarthritis (OA) and with rheumatoid arthritis (RA), that there is a distinct profile of pro- versus anti-inflammatory mediators contributing to the persistence or resolution of the inflammatory process. These findings may give an incentive to target specific pro- or anti-inflammatory mediators thereby supporting the healing process of different joint diseases.

4.2. Publication "Acute mechanical sensitization of peripheral nociceptors by Aldosterone through non-genomic activation of membrane-bound mineralocorticoid receptors in naive rats. "

This project investigated the distinct localization, expression and functional relevance of the mineralocorticoid receptor (MR) in neurons and/or glia cells of the spinal cord and peripheral sensory neurons, since recent evidence has suggested a role of MR in the modulation of pain.

Study objective: To identify the subtypes of nociceptive neurons and glia cells within the spinal cord and peripheral sensory neurons of naïve rats which express MR and to examine whether its activation by local application of the MR agonist aldosterone results in altered nociceptive behavior via classical genomic or non-genomic mechanisms.

Methods: Following IRB approval kidney, spinal cord, sciatic nerve and innervated skin tissues were freshly taken from naïve rats and immediately stored at -80 °C temperature to use for quantitative RT-PCR and Western blot or fixated in 4% paraformaldehyde to use for immunohistochemistry. For Taqman® RT-PCR primer pairs for MR and the internal control gene 18S were generated to identify the respective mRNAs. For immunohistochemistry commercial antisera for MR, the neuronal marker CGRP, trkA, NF200, trkB, and trkC as well as the glia marker GFAP and OX42 were purchased to localize MR in distinct subpopulations of nociceptive neurons or glia cells within the examined tissue. Following the intraplantar (i.pl. 25-100 μg aldosterone) and intrathecal (i.th., 4-40 μg aldosterone) administration of aldosterone in naïve rats, the sensitivity to mechanical von Frey filament stimulation was tested. To confirm a receptor-specific mechanism aldosterone's effects were antagonized by concomitant application of the MR antagonist canreonate K. Finally, to prove immediate non-genomic

effects by membrane-bound MR saturation binding experiments were performed using the radiolabeled MR ligand [^{3}H] aldosterone.

Results: Similar to MR's well-known localization in kidney tissue, MR mRNA, and protein (107 kDa) were also detected in the spinal cord and dorsal root ganglia of naïve rats. MR immunoreactivity was identified predominantly in Rexed laminae I and II within the dorsal horn of the spinal cord mainly derived from incoming nociceptive CGRP-positive sensory neurons. Intriguingly, MR did not colocalize with GFAP-positive astrocytes and scarcely with OX42-positive microglia of the spinal cord. Consistently, 75% of MR-immunoreactivity was demonstrated in CGRP-positive sensory neurons and 21% of MR-immunoreactivity was shown in NF200 myelinated sensory neurons suggesting its main localization in unmyelinated nociceptive C-fibres. This was confirmed by a similar colocalization of MR with the neuronal marker trkA, but much less with the neuronal markers trkB or trkC. The colocalization of MR with CGRP and trkA extended into the axons of the sciatic nerve and the nerve terminals innervating the epidermal layer of the skin. Local application of the MR agonist aldosterone at the site of the peripheral (i.Pl.) and central (i.th.) nerve terminals resulted in a significant reduction of mechanical withdrawal thresholds suggesting mechanical hypersensitivity. In line with these immediate (within 10 min) effects of the MR agonist aldosterone, MR receptors could be localized on membranes of sensory neurons within the spinal cord and dorsal root ganglia.

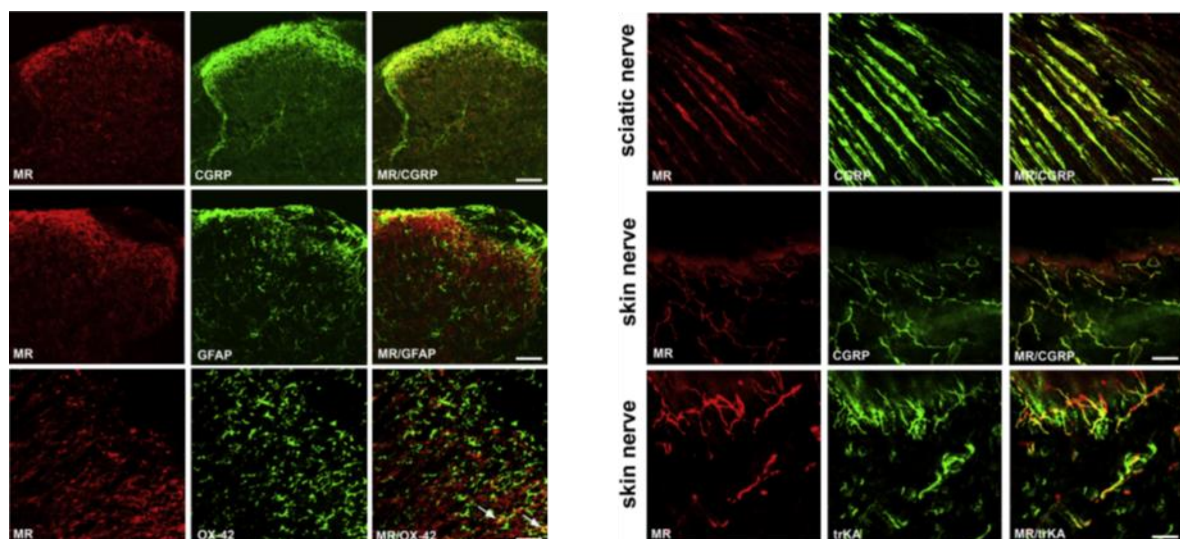

Figure 2. MR specific immunoreactivity in peripheral nociceptive neurons but not glia which activation leads to immediate enhanced nociceptive sensitivity most likely by non-genomic activation of sensory neuron membrane bound MR. Spinal cord, sciatic nerve, and skin tissue were removed from naïve rats and quickly fixated for determination of MR immunoreactivity with the neuronal markers CGRP and trkA or the glia cell markers GFAP and OX42. MR-immunoreactive neurons (*Texas red*) mostly colocalize with nociceptive CGRP- and trkA-positive Neurons (*FITC green*)

but scarcely with glia cells and extend as central terminals into the spinal cord and as peripheral terminals into the skin.

Conclusion and relevance: This study demonstrates the localization of MR primarily on peripheral nociceptive neurons suggesting a functional role in the modulation of incoming painful stimuli. The immediate mechanical sensitization of MR agonists together with the identification of membrane-bound MR suggests its mediation rather by non-genomic than classical genomic pathways. MR antagonists might be an intriguing option for the reduction of enhanced pain sensitivity which needs to be further examined in future studies.

4.3. Publication "Protein kinase C-mediated mu-opioid receptor phosphorylation and desensitization in rats, and its prevention during early diabetes."

This project investigated the underlying mechanisms of impaired opioid responsiveness, such as mu-opioid receptor (MOR) phosphorylation and desensitization, in painful diabetic neuropathy.

Study objective: To examine in a rat model of early streptozotocin-induced diabetes whether the loss of MOR agonist antinociceptive efficacy is due to a reduction in sensory neuron MOR number and/or functional coupling subsequent to enhanced MOR phosphorylation caused by up-regulated PKC expression and activation.

Methods: Following IRB approval Wistar rats were treated with 45 mg/kg i.v. streptozotocin to induce diabetes and nociceptive behavior was tested by paw pressure test following increasing doses of the MOR-agonist fentanyl. Dorsal root ganglia were subjected to Taqman® RT-PCR with specific MOR primers, Western blot, radioligand binding and immunohistochemistry to look for differences in the number, phosphorylation and functional coupling of sensory neuron MOR. In a next step expression and colocalization of different subtypes of the phosphorylating enzyme PKC as well as the receptor for advanced glycation products (RAGE) with MOR and its functional involvement were examined by use of RAGE specific siRNA. To validate colocalization of MOR and phospho-MOR with PKC immunoprecipitation experiments were performed. All data were subject to statistical analysis as outlined in the original publication.

Results: Intravenous streptozotocin treatment revealed a significantly reduced number of insulin-producing pancreatic ß-cells along with elevated blood glucose, diminished weight gain, and impaired antinociceptive efficacy of the MOR agonist fentanyl. At the same time, MOR number in dorsal root ganglia of diabetic rats were not reduced, however, showed decreased functional G protein coupling due to enhanced MOR phosphorylation. Dorsal root ganglion cells showed high colocalization of MOR with specific subtypes of PKC and the receptor for advanced glycation products (RAGE) suggesting that the accumulation of these products activate RAGE which in turn up-regulates the expression and activation of PKC finally resulting in the phosphorylation and desensitization of sensory neuron MOR. Consistently, knock-down of RAGE with specific RAGE siRNA resulted in a complete reversal of all these diabetes-induced alterations.

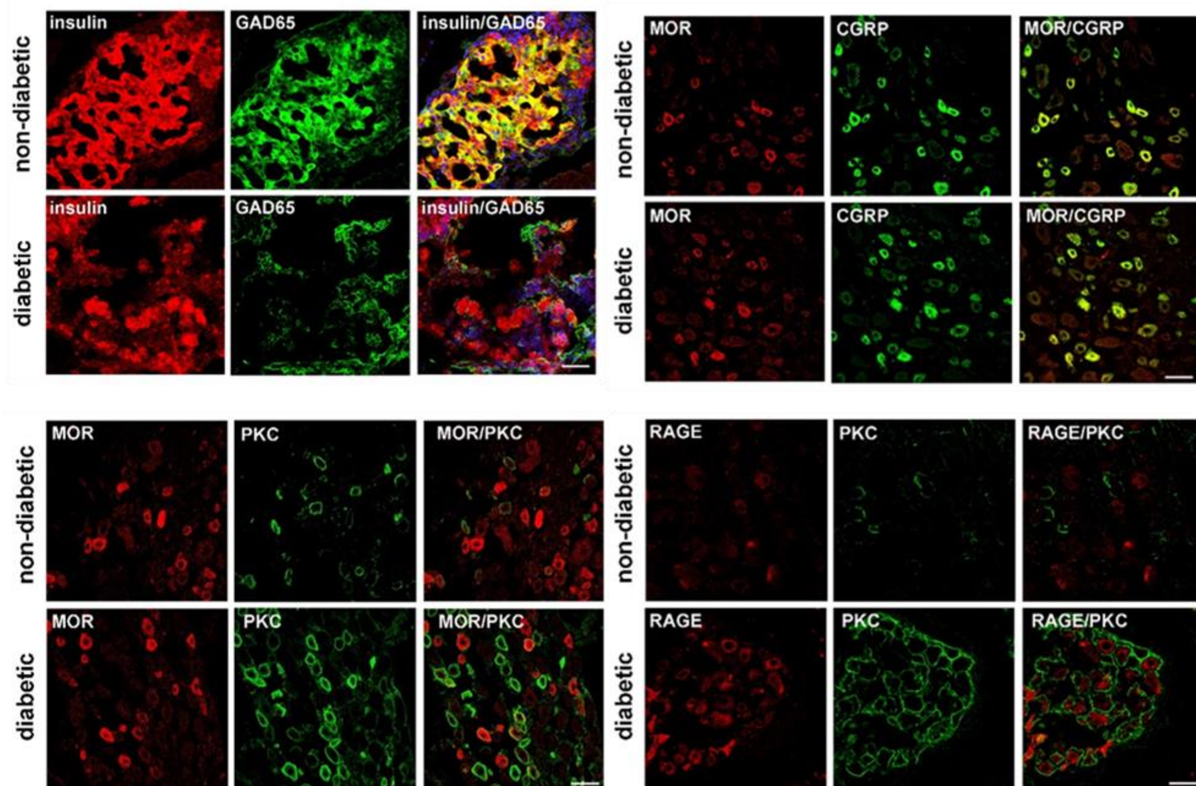

Figure 3. Changes in GAD65-IR or insulin-IR b-cells (upper left panel), not significantly different in number of sensory neuron mu opioid receptor (MOR) (upper right panel), and alteration in PKC (lower left panel) and change also in RAGE-mediated PKC colocalization (lower right panel), during early streptozotocin (STZ)-induced diabetes. Pancreas and DRG tissue were removed from naïve diabetic rats and quickly fixated to peformed the double-immunofluorescence, images showing coexpression of insulin-immunoreactive (Texas red) with GAD65 (FITC green) in numerous pancreatic b-cells with clearly reduction compared to control rats. Colocalized of MOR-IR (Texas red) neurons with CGRP (FITC green) as sensory neuron marker in DRG of diabetic and control rats. Also immunofluorescence colocalized of MOR-IR (Texas red) neurons together with PKC-IR (FITC green). PKC-IR (FITC green) colocalized with sensory neurons (RAGE-IR) (Texas red) and are abundantly increased in DRG of diabetic rats compared to controls.

Conclusion and relevance: This study demonstrates that certain pain conditions such as diabetic neuropathy may impair opioid pain treatment by an underlying mechanism within nociceptive neurons which is based on RAGE-mediated PKC activation and subsequent MOR phosphorylation and desensitization following diabetes-triggered generation of advanced glycation endproducts. Therefore, prevention of AGE accumulation or access to its receptor RAGE may be a new option to recover opioid responsiveness so that patients suffer less likely from opioid side effects due to necessary increased opioid doses during diabetic neuropathy.

5. Discussion

The goal of my project was to elucidate putative mechanisms that contribute to the generation and control of painful conditions. The first study examined the relative contributions of pro- versus anti-inflammatory mediators derived from immigrating immune cells to different painful joint diseases in patients with diagnostic arthroscopy (controls), joint trauma (JT), osteoarthritis (OA) or rheumatoid arthritis (RA). In the synovial tissue of these patients, a distinct profile of immigrated immune cells and expression of pro- versus anti-inflammatory mediators was identified. In summary, IL-1ß, TNF-alpha and 5-LOX mRNA and immunoreactivity representing pro-inflammatory mediators were significantly more abundant in patients with RA and JT than in patients with OA. In contrast, 15-LOX, FPR2, and IL-10 mRNA and immunoreactivity representing anti-inflammatory mediators were significantly more abundant in patients with OA than in those with JT and RA. These findings reveal a distinct profile of pro- versus anti-inflammatory mediators for a different painful joint disease which are presumably based on an apparent imbalance within the so-called inflammatory mediator network.

The results from the first paper demonstrated that IL-1β, TNFα, and 5-LOX specific mRNA, as well as proteins expressed in immigrated immune cells, were significantly more abundant in JT, OA, and RA synovial tissues compared to control but were more prominent in JT and RA patients. These findings are in agreement with high levels of 5-LOX expressed mostly in macrophages, neutrophils and mast cells of RA synovium (Gheorghe et al., 2009). It is well documented that pro-inflammatory mediators such as TNFα, IL-1β, and 5-LOX play key roles in driving the inflammation and synovial cell proliferation during RA-associated joint destruction (Park and Pillinger, 2007). Therefore, the findings of this study suggest that the expression of pro-inflammatory mediators such as TNFα, IL-1β, and 5-LOX is concomitant with the severity of inflammation among patients with JT, OA, and RA. Moreover, this study

provides conclusive morphological evidence of differences in the number and types of cells expressing pro-inflammatory cytokines within each disease and among JT, OA, and RA patients. Double immunofluorescence confocal microscopy showed that most of the CD68 positive macrophages within the synovial lining layer cells contain also anti-inflammatory cytokines such as 15-LOX- and FPR-2-IR. Anti-inflammatory cytokines were expressed predominantly in granulocytes in patients with JT, and in macrophages, lymphocytes and plasma cells in those with OA and RA. These results extend the previous reports of 15-LOX expression in humans (Gheorghe et al., 2009) which did not differentiate the synovial cell types. Interestingly, anti-inflammatory cytokines 15-LOX, FPR-2 and IL-10 specific mRNA, as well as immunoreactive cells, were significantly more abundant in patients with OA than in those with JT and RA. It is known that IL-10 plays a role in preventing exaggerated inflammatory and immune responses and, thus, protects the host from immune-mediated damage (Trinchieri, 2007). IL-10 is a broad spectrum anti-inflammatory cytokine and is produced by different immune cells, like Th1 and Th2 cells, B cells, monocytes, and macrophages. Recently, Vermeij et al., (2016) showed that treatment of an acute joint inflammation with local IL-10 overexpression under the control of disease-regulated promoters inhibited arthritis progression. Consistently, Roybal et al., (2011) showed that early gestational gene transfer of IL-10 by systemic administration of lentiviral vector can prevent arthritis in a murine model. Taken together, the findings of this study are in line with the notion that the upregulated pro-inflammatory mediators regulate the inflammatory process in JT and RA synovium in contrast to upregulated anti-inflammatory mediators which are mainly responsible for lowering the inflammatory process in OA synovium.

The second study, of my project, demonstrated that MR which are commonly known to be present in kidney tissue are also expressed in peripheral unmyelinated nociceptive neurons that colocalized with the peptidergic neuron markers CGRP and trkA. By use of a new highly specific polyclonal rabbit antibody against the rat mineralocorticoid receptor (MR) that does not cross-react with the glucocorticoid receptor (Ito et al,. 2000) it could be demonstrated that MR co-expressed with CGRP a marker for nociceptive C- and Aδ-nerve fibers in neuronal cell bodies of the dorsal root ganglia, their centrally and peripherally directed axons as well as their peripheral (within skin) and central (within spinal cord) nerve terminals. Consistently, this study showed that the majority of MR immunoreactive neurons also colocalized with trkA (Matsumoto et al,. 2012). These findings indicate that MR plays a crucial role in the modulation of nociception. Indeed, our behavioral experiments in naïve animals demonstrate for the first time an immediate and almost 50% decrease in mechanical thresholds, i.e. increased sensitivity

to mechanical stimuli, ipsi- but not contralateral to the intrathecal as well as i.pl. administration of aldosterone. This effect occurred within 10 min, persisted for 40 min and was receptor specific because simultaneous local injection of a mineralocorticoid receptor selective antagonist reversed this effect. These findings are in agreement with previous studies (Ye et al., 2014; Dong et al., 2012) which reported that the MR selective agonist aldosterone dose-dependently increased the number of action potentials evoked by supra-threshold current injection in acutely dissociated dorsal root ganglion neurons of naïve rats. In the other hand, there are a several studies (Ye et al., 2014; Dong et al., 2012; Sun et al. 2012; Gu et al., 2011) have reported antinociceptive effects of a mineralocorticoid receptor antagonist under different pathological conditions such as low back pain (Ye et al., 2014; Dong et al., 2012; Sun et al. 2012; Gu et al., 2011) and diabetic neuropathy (Dong et al., 2013). The acute nociceptive effects in our study occur within a few minutes after local MR agonist application strongly suggest that these effects are due to a non-genomic effect. These findings are consistent with the previous studies by Chatterjee et al., (2014) and Groeneweg et al., (2012) providing evidence that neuronal MR agonists may elicit their effects also by non-genomic ways. The authors suggested that these non-genomic pathways can be elicited either by directly interfering with intracellular signaling pathways or by interfering with membrane-bound structures such as ion channels and G-protein coupled receptors (Chatterjee et al., 2014; Groeneweg et al., 2012). Consistently, this study further substantiated the evidence for a putative non-genomic pathway by binding experiments that demonstrated saturation binding with increasing concentrations of the radiolabeled ligand [^{3}H]aldosterone in the pure membrane fraction of DRG neurons indicating MR specific binding sites. Indeed, immunofluorescence confocal microscopy showed that MR immunoreactivity was highly concentrated in the plasma membranes of DRG neurons. In agreement with these findings, convincing evidence for membrane bound MR is also provided by electron microscopy studies in presynaptic terminals and postsynaptic densities of synaptic areas of the brain (Prager et al., 2010; Johnson et al., 2005).

Painful conditions are typically treated in patients by application of potent analgesic drugs among which opioids are the most powerful. However, in certain painful conditions, such diabetic neuropathy underlying mechanisms within the primary nociceptive neurons counteract their maximum efficacy. Therefore, in the third study of this project, the putative mechanisms that impair the opioid analgesic efficacy in painful diabetic neuropathy were systematically investigated. In the animal model of streptozotocin (STZ)-induced diabetes, rats exhibited reduced peripheral MOR-agonist analgesia which is consistent with previous reports of reduced opioid responsiveness during early diabetes (Chen at al., 2003; Tasertargil and Sadan, 2004;

Nozaki et al., 2005). Interestingly, this study revealed no significant alterations in the number and protein content of MOR on peripheral sensory neurons. Similar findings during early diabetes were obtained by others (Chen et al., 2002; Chen et al., 2003), however, the information about possible mechanisms is still lacking (Mousa et al., 2007). Since opioid antinociception is not dependent only on MOR density but also on functional MOR G protein coupling (Chen et al., 2002; 2003), this study investigated the MOR agonist-induced G protein coupling in DRG by [^{35}S]GTPγS binding. Indeed, the maximal efficacy of MOR G protein coupling (Emax) in the DRG of diabetic animals was significantly reduced compared to controls suggesting that the loss in MOR G protein coupling may be responsible for the impaired opioid responsiveness during early diabetes.

A possible reason for the reduced MOR G protein coupling could be a phosphorylation and consecutive desensitization of sensory neuron MOR (Mann et al., 2014). MOR phosphorylation is a critical process in the regulation of MOR signaling (Feng and Wang, 2011) and interferes with G protein coupling and efficacy. Indeed, this study revealed that the number of phosphorylated Thr^{370} MOR-IR DRG neurons, as well as the total amount of phosphorylated Thr370 MOR protein, was significantly increased in diabetic animals. Since previous reports established that MOR phosphorylation on Thr^{370} occurs through PKC activation (Chu et al., 2010; Doll et al., 2010; Mann et al., 2015), this study examined the functional link between neuronal MOR phosphorylation and PKC activation in diabetic rats. The results identified that inhibition of PKC activation by i.t. calphostin C diminished both PKC and Thr^{370} MOR phosphorylation, improved MOR G protein coupling and rescued MOR-mediated antinociception indicating a functional link. A previous study by Toth et al., (2008) reported that the increased RAGE expression may play a crucial role in the development of diabetic neuropathy and neuronal PKC expression and activation. To proof that RAGE has a major impact on the sensory neuron PKC activation in diabetic animals, the method of i.t. delivery of RAGE specific siRNA was applied to silence the enhanced endogenous expression of RAGE in peripheral sensory neurons (Meloche et al., 2011). Importantly, RAGE siRNA treatment, as well as the oral administration of the AGE inhibitor aminoguanidine, prevented PKC activation and MOR phosphorylation in peripheral sensory neurons of diabetic rats. These findings are supported by a previous study by Toth et al., (2008) showing that the enhanced PKC expression in DRG neurons of SZT-induced diabetes was reversed in RAGE knockout mice. To test the functional consequences of the interaction between MOR with RAGE, phospho-PKC, and phospho-MOR, this study investigated whether two functional parameters, i.e. MOR G protein coupling and peripheral opioid antinociceptive efficacy are affected by RAGE-mediated

enhanced PKC activity. Interestingly, following either oral AGE inhibitor aminoguanidine, i.t. infusion of RAGE siRNA or PKC inhibitor calphostin C treatment, the impairment of MOR G protein coupling was reversed in diabetic rats. Consistent with a functional opioid recovery, the loss in MOR agonist-induced antinociception in diabetic animals was restored following oral aminoguanidine, i.t. RAGE siRNA or calphostin C. Moreover, morphine-induced tolerance and, thus, a loss in antinociceptive efficacy has been shown to be PKC-dependent which could be prevented either by a PKC inhibitor (Granados-Soto et al., 2000; Hull et al., 2010) or by knocking down PKC (Bailey et al., 2009). Together, these findings suggest that sensory neuron MOR phosphorylation is the main cause of the reduced opioid responsiveness in early diabetes. Phosphorylation of receptors limits the physiological response of neurons to external signals and contributes to a loss in agonist efficacy (Yabaluri and Medzihradsky, 1997; Martini and Whistler, 2007; Zöllner et al., 2008).

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Affidavit

I, Mohammed Al-Madol, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic **[Gene expression and immunohistochemical localization of distinct modulators of inflammation and pain]** I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

……………………………

Date: 31.01.2017 Mohammed Ayesh Al-madol

<u>Declaration of any eventual publications</u>

Mohammed Al-Madol, had the following share in the following publications:

Publication 1: [**Mohammed A. Al-Madol,** Mohammed Shaqura, Thilo John, Rudolf Likar, Reham Said Ebied, Michael Schäfer, Shaaban A. Mousa. Comparative expression analyses of pro- versus anti-inflammatory mediators within synovium of patients with joint trauma, osteoarthritis and rheumatoid arthritis. Mediators of Inflammation, 2016.

Contribution in (briefly): I performed all experiments (Taqman® RT-PCR, confocal immunofluorescent experiments, quantification) myself under supervision by colleagues in the lab, I did the data analysis and writing of a first draft of the manuscript which was revised by the supervisors in the lab.

Publication 2: [Mohammed Shaqura, Xiongjuan Li, **Mohammed A. Al-Madol**, Sascha Tafelski , Antje Beyer-Koczorek , Shaaban A. Mousa , Michael Schäfer. Acute mechanical sensitization of peripheral nociceptors by aldosterone through non-genomic activation of membrane bound mineralocorticoid receptors in naive rats. Neuropharmacology 107 (2016) 251-261.

Contribution in (briefly): I performed some of the immunohistochemical experiments myself (all experiments of figure 2 and 5) and participated in the writing and revision of the manuscript.

Publication3: [Shaaban A. Mousa, Mohammed Shaqura, Jens Winkler, Baled. I. Khalefa, **Mohammed A. Al-Madol**, Mehdi Shakibaei, Stefan Schulz, Michael Schäfer. Protein kinase C-mediated mu-opioid receptor phosphorylation and desensitization in rats, and its prevention during early diabetes. PAIN 157 (2016) 910–921.

Contribution in (briefly): I performed some of the immunohistochemically experiments myself (all experiments of figure 1A, G, H, figure 2G, figure 3F, 5A) and participated in the writing and revision of the manuscript.

Signature, date and stamp of the supervising University teacher

----
Prof. Dr. med. Michael Schäfer

Signature of the doctoral candidate

Mohammed Ayesh Al-madol

8. Print copies of the selected publications

1. Publication:

Mohammed A. Al-Madol, Mohammed Shaqura, Thilo John, Rudolf Likar, Reham Said Ebied, Michael Schäfer, Shaaban A. Mousa. Comparative expression analyses of pro- versus anti-inflammatory mediators within synovium of patients with joint trauma, osteoarthritis, and rheumatoid arthritis.

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Hindawi

Research Article

Comparative Expression Analyses of Pro- versus Anti-Inflammatory Mediators within Synovium of Patients with Joint Trauma, Osteoarthritis, and Rheumatoid Arthritis

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Synovial injury and healing are complex processes including catabolic effects by proinflammatory cytokines and anabolic processes by anti-inflammatory mediators. Here we examined the expression of pro- versus anti-inflammatory mediators in synovium of patients with diagnostic arthroscopy (control), joint trauma (JT), osteoarthritis (OA), and rheumatoid arthritis (RA). Synovial samples from these patients were subjected to RT-PCR and double immunofluorescence confocal microscopy of pro- and anti-inflammatory mediators as well as immune cell markers. Interestingly, pro- and anti-inflammatory mediators were expressed predominantly in granulocytes in patients with JT and in macrophages, lymphocytes, and plasma cells in patients with OA and RA. Interestingly, parallel to the severity of inflammation, proinflammatory mediators IL-1β, TNF-α, and 5-LOX specific mRNA as well as immunoreactive (IR) cells were significantly more abundant in patients with RA and JT than in those with OA. However, anti-inflammatory mediators 15-LOX, FPR2, and IL-10 specific mRNA as well as IR cells were significantly more abundant in patients with OA than in those with JT and RA. These findings show that upregulation of proinflammatory mediators contributes to the predominantly catabolic inflammatory process in JT and RA synovium, whereas upregulation of anabolic anti-inflammatory mediators counteracts inflammation resulting in the inferior inflammatory process in OA synovium.

1. Introduction

In inflammatory joint diseases such as joint trauma (JT), osteoarthritis (OA), and rheumatoid arthritis (RA), the inflammatory process within synovial tissue leads to tissue destruction and fibrosis with varying degrees of severity [1–3]. The degree of severity depends on processes that are taking place inside the synovium including catabolic effects by proinflammatory cytokines concomitant with anabolic processes by anti-inflammatory mediators [4]. The most important group controlling the state of disease seems to be inflammatory cytokines including interleukin-1 β (IL-1β) and tumor necrosis factor-alpha (TNF-α). Tumor necrosis factor-alpha (TNF-α), together with IL-1β, is considered a key inflammatory cytokine involved in the pathophysiological processes occurring in the course of chronic disease of human synovium. Also, the lipoxygenase isoform of 5-lipoxygenase (5-LOX) is reported to be overexpressed in synovial tissue of patients suffering from rheumatoid arthritis and osteoarthritis. The 5-LOX is involved in the progress of inflammation [5].

The other group opposing these proinflammatory effects in synovial tissue is formed by anti-inflammatory mediators such as 15-LOX, formyl peptide receptor (FPR2), and IL-10 [6, 7]. Previous studies demonstrated that 15-LOX

metabolites have potent anti-inflammatory actions on rheumatoid inflammation [8]. FPR2, also known as the lipoxin A4 receptor (ALX), belongs to the Gi-protein coupled receptor (GiPCR) family and is activated by lipoxin A4, Annexin A1, and Annexin A1-derived peptides or by the acute phase reactant serum amyloid A resulting in potent anti-inflammatory effects [9, 10]. Similarly, IL-10 inhibits chemokine and cytokine production from macrophages and dendritic cells that leads to a limitation of the acute and chronic inflammation [11–13]. The exact cell types and disease-specific differences in the occurrence of pro- versus anti-inflammatory mediators within human synovium of patients with JT, OA, and RA have not been examined so far.

Therefore, we investigated the expression of proinflammatory (IL-1β, TNF-α, and 5-LOX) and anti-inflammatory (15-LOX, FPR2, and IL-10) mediators in synovium of patients with JT, OA, and RA. We assessed the differences in the number and types of cells expressing proinflammatory and anti-inflammatory mediators by the use of different markers for synovial fibroblasts (P4HB), macrophages/monocytes (CD68), granulocytes (CD15), T lymphocytes (CD3), and plasma cells Ab-1. This study may provide conclusive evidence of a disease-specific profile of proinflammatory versus anti-inflammatory mediators as well as cell types involved that may contribute to a better understanding of these opposing mechanisms and to potential targets for the modulation of these processes.

2. Materials and Methods

2.1. Patients and Synovial Sample Collection. Patients were recruited in three different hospitals: DRK Clinic Westend Berlin, Landeskrankenhaus Klagenfurt, and the University Hospital Regensburg. IRB approval was obtained from all three locations and patients gave their written consent to the participation in this study after they were informed of its purpose (EA). Patients were included when they were scheduled for surgery and had a diagnosis of osteoarthritis, rheumatoid arthritis (according to the criteria of the American College of Rheumatology and to the clinical and radiological criteria of OA) [14, 15], or joint trauma. Patients undergoing a diagnostic arthroscopy were chosen as a control. During surgery, synovial tissues were taken from patients of all four groups and stored at −80°C.

2.2. RT-PCR. Frozen synovial tissue samples were homogenized, and then the total RNA was isolated using the Kit Qiazol Lysis Reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For cDNA synthesis, 500 ng total RNA was isolated by NanoDrop (Peqlab). Then, 500 ng total RNA was converted to cDNA at 42°C for 1 h using Omniscript RT Kit (Qiagen, Hilden, Germany) by incubation with 0.5 mM dNTP, 1 μM Random Primer, 10 units RNase Inhibitor, and 4 units Omnisript reverse Transcriptase. The obtained DNA was stored at −20°C. The following specific primers for IL-1β, TNF-α, 5-LOX, 15-LOX, FPR2, IL-10, and 18s were used (see primers' information in Supplemental Table 1 (see Supplementary Material available online at https://doi.org/10.1155/2017/9243736). Finally, TaqMan® qRT-PCR was performed using a SYBR® Green kit according to the manufacturer's instructions (Applied Biosystems). Amplification was executed with 40 cycles, each consisting of 15 s at 95°C and of 30 s at 60°C. To detect fluorescence specific products for each primer pair, the reaction was carried out at a temperature just below the specific melting temperature (Tm). For statistical analysis, experiments were performed in triplicate in order to determine TNF-α, IL1β, 5-LOX, 15-LOX, and FPR2 mRNA by the delta-delta CT method as described previously [16].

2.3. Tissue Preparation and Histological Evaluation. Intact synovial tissue was fixed in 4% (w/v) paraformaldehyde in 0.16 M phosphate buffer solution (PBS) (pH 7.4) for 4 hours and then cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissue was then embedded in tissue-Tek compound (OCT, Miles Inc. Elkhart, Indiana) and frozen. For histology, 8 μm sections were cut by using a Cryostat (Thermo Fisher, Dreieich, Germany) and were mounted onto gelatin-coated slides. Histological evaluation of intact synovial tissue was performed as previously described [17], identifying the components of lining cell layers, sublining cells. Cell density was determined in 3 fields in each section of at least 4 different tissue samples from each group (hematoxylin-eosin stained) by counting all stained cell nuclei within 3 randomly selected high-power fields of view per section (400x magnification). The lining-layer thickness was determined from at least three sections and analyzed by averaging the number of cells of three fields per section (400x magnification). The number of B cells, macrophages, fibroblast-like cells, granulocytes, and lymphocytes were evaluated in cryosections stained with antibodies against human plasma cells Ab-1, macrophages (CD68), fibroblasts (P4HB), granulocytes (CD15), and lymphocytes (CD3), respectively. The number of stained structures was averaged from 12 randomly selected high-power fields (400x magnification). The number of high-power fields was derived from a histological study by Bresnihan et al., (1998) [18].

2.4. Single and Double Immunofluorescence Staining Procedures. Immunofluorescence staining was processed as described previously [19]. The mounted slide tissue sections were incubated overnight at 4°C with the following primary antibodies (see antibodies' information in supplemental Table 1): single immunofluorescence staining was applied in the following way: (1) anti-IL-1β, (2) anti-TNF-α, (3) anti-5-LOX, (4) anti-15-LOX, (5) anti-FPR2, and (6) anti-IL-10, respectively, in each group (Control, JT, OA, and RA). Double immunofluorescence staining was performed in RA samples in the following way: (1) anti-TNF-α/anti-IL-1β, (2) anti-5-LOX/anti-FPR2, (3) anti-15-LOX/anti-5-LOX, and (4) anti-IL-10/anti-FPR2; in OA samples: (1) anti-5-LOX/anti-FPR2, (2) anti-TNF-α/anti-IL1β, (3) anti-15-LOX/anti-5-LOX, and (4) anti-IL-10/anti-FPR2; in JT samples: (1) anti-TNF-α/anti-IL1β, (2) anti-5-LOX/anti-FPR2, (3) anti-15-LOX/anti-5-LOX, (4) anti-IL-10/anti-TNF-α, and (5) anti-IL-10/anti-FPR2. Antisera specific for certain immune cell types (anti-CD15, anti-CD68, anti-P4HB, anti-CD3, and anti-Ab-1) were applied together with anti-TNF-α, anti-IL1β,

Table 1: Clinical and histological characteristics of patients with joint trauma, osteoarthritis, and rheumatoid arthritis.

Patients	Control ($n = 5$)	JT ($n = 9$)	OA ($n = 11$)	RA ($n = 10$)
Age (years)	65 (±16,8)	48 (±17)	72 (±6)	64.9 (±17)
Sex (F/M)	1/4	9/6	5/2	6/4
Disease duration:				
≤1 year	5/5	4/9	2/11	—
>1 year	—	5/9	9/11	10/10
Drugs; NSAIDs: (Diclofenac, Ibuprofen, Profenid, Paracetamol, Dipyrone)	5/5	8/9	8/11	8/10
Etoricoxib	NA	1/9	3/11	1/10
Prednisolone (Dexamethasone)	NA	NA	NA	7/10
Lining-layer thickness (cell-layers)	1 (1; 2)	2 (1; 3)	3 (3; 4)	4 (3; 5)
Overall cellularity (cells/mm^2)	47 (44; 49)	124 (97; 138)	269 (220; 285)	387 (358; 474)
Vascularity (vessels)	2 (1; 3)	3 (3; 5)	6 (5; 7)	6 (3; 8)

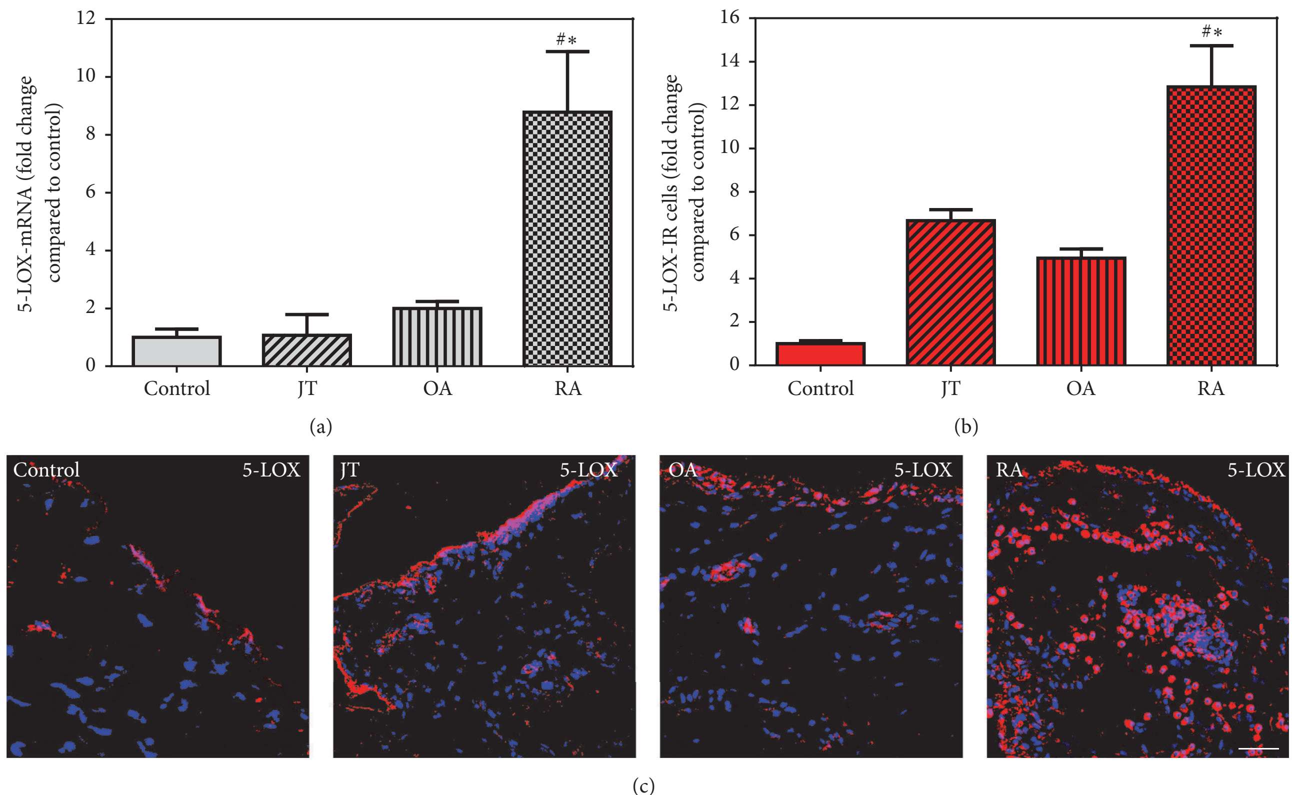

Figure 1: Detection of 5-LOX mRNA (a) and number of 5-LOX-IR cells (b and c) in patients with joint trauma (JT), osteoarthritis (OA), and rheumatoid arthritis (RA). (a) Quantification of 5-LOX mRNA using TaqMan qRT-PCR shows that 5-LOX mRNA was significantly higher in RA compared to JT, OA, and control synovium ($P < 0.05$, one-way ANOVA followed by Tukey's test). (b) Quantitative analysis of immunofluorescence microscopy for 5-LOX-IR cells. *Relative to control, #relative to other groups ($P < 0.05$, one-way ANOVA followed by Tukey's test). (c) 5-LOX-IR cells are more abundant in RA synovium than in JT, OA, and control synovium. Bar = 20 μm.

anti-5-LOX, anti-15-LOX, anti-FPR2, or IL-10 within each group. Then, the slides were washed in PBS and for 2 hours incubated with Alexa Fluor 488 donkey anti-rabbit antibody (Vector Laboratories) or with Alexa Fluor 594 donkey anti-goat or with Alexa Fluor 594 donkey anti-mouse (Invitrogen, Germany) (in single staining). For double staining, slides were incubated with Alexa Fluor 488 donkey anti-rabbit antibody combined with Alexa Fluor 594 donkey anti-goat, Alexa Fluor 594 donkey anti-mouse combined with Alexa Fluor 594 donkey anti-goat, or Alexa Fluor 594 donkey anti-mouse combined with Alexa Fluor 488 donkey anti-rabbit. Finally, the tissues were washed in PBS, and nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) and then mounted in Vectashield (Vector Laboratories) and imaged on a confocal laser scanning microscope, LSM510, as described previously [19]. Then, the photographs were taken

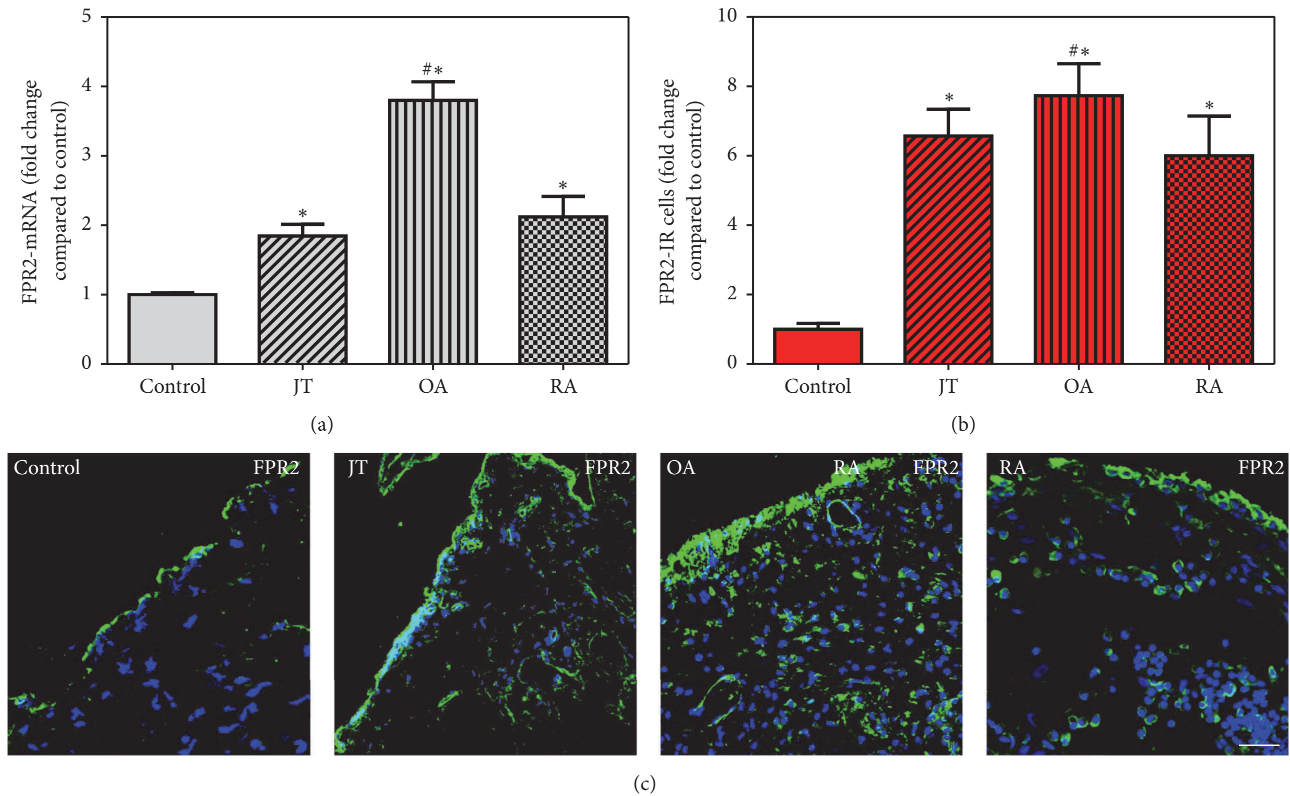

Figure 2: Detection of FPR2 mRNA (a) and number of FPR2-IR cells (b and c) in patients with joint trauma (JT), osteoarthritis (OA), and rheumatoid arthritis (RA). Quantification of FPR2 mRNA (a) and immunofluorescence positive cells (b) shows that FPR2 expression was more prominent in OA compared to RA, JT, OA. *Relative to control, #relative to other groups ($P < 0.05$, one-way ANOVA followed by Tukey's test). Data are shown as means ± SEM. (c) 5-LOX-IR cells are more abundant in RA synovium than in JT, OA, and control synovium. SEM. Bar = 20 μm.

by a confocal microscope (633 nm; Carl Zeiss, Göttingen, Germany) as described in our previous study [20]. To demonstrate specificity of staining, the following controls were included: omission of the primary antisera or the secondary antibodies, as described in our previous studies [19].

2.5. Quantification of Immunostaining. The method for quantification of the number of immunoreactive cells has been described previously [19]. Immunoreactive cells were counted using only intact tissue exhibiting optimal morphology to avoid misleading results. The numbers of proinflammatory (IL-1β, TNF-α, and 5-LOX,) and anti-inflammatory (15-LOX, FPR2, and IL-10) immunoreactive cells were counted by a blinded experimenter in three sections per patient. The value of stained proinflammatory and anti-inflammatory mediators was determined by the formula: stained cells/stained cells of control × 100. Data were obtained from three sections per patient from each group: Control ($n = 5$), JT ($n = 9$), OA ($n = 11$), and RA ($n = 10$).

2.6. Statistical Analysis. Data are represented as means ± SEM. Sample comparisons were made using one-way analysis of variance followed by Tukey test in the case of normally distributed data and Kruskal–Wallis analysis of variance on ranks followed by Dunn's test in the case of data not distributed normally. Differences were considered significant if $P < 0.05$. All tests were performed using Sigma Plot 13.0 statistical software.

3. Results

3.1. Patient Recruitment, Demographics, and Synovial Signs of Inflammation. For this study, a total of 42 patients were screened. Seven patients were excluded during the workup, because tissue samples could not be identified histologically as synovial tissue. The remaining patients were distributed according to their clinical diagnosis among the following groups: Control ($n = 5$), JT ($n = 9$), OA ($n = 11$), and RA ($n = 10$).

Patients' demographics such as patient's age, gender, disease duration, and medications are shown in Table 1. Light microscopic evaluation of patients' synovial tissues for lining-layer thickness, overall cellularity, and vascularity was significantly different for patients with RA but not for patients of the other groups compared to control ($P < 0.05$) (Table 1). Overall synovial cellularity was further characterized in greater detail by immunofluorescent microscopy showing different types of immune cells and fibroblasts. Synovial tissue of patients suffering from OA and RA showed significantly more regional immune cells and fibroblasts than control

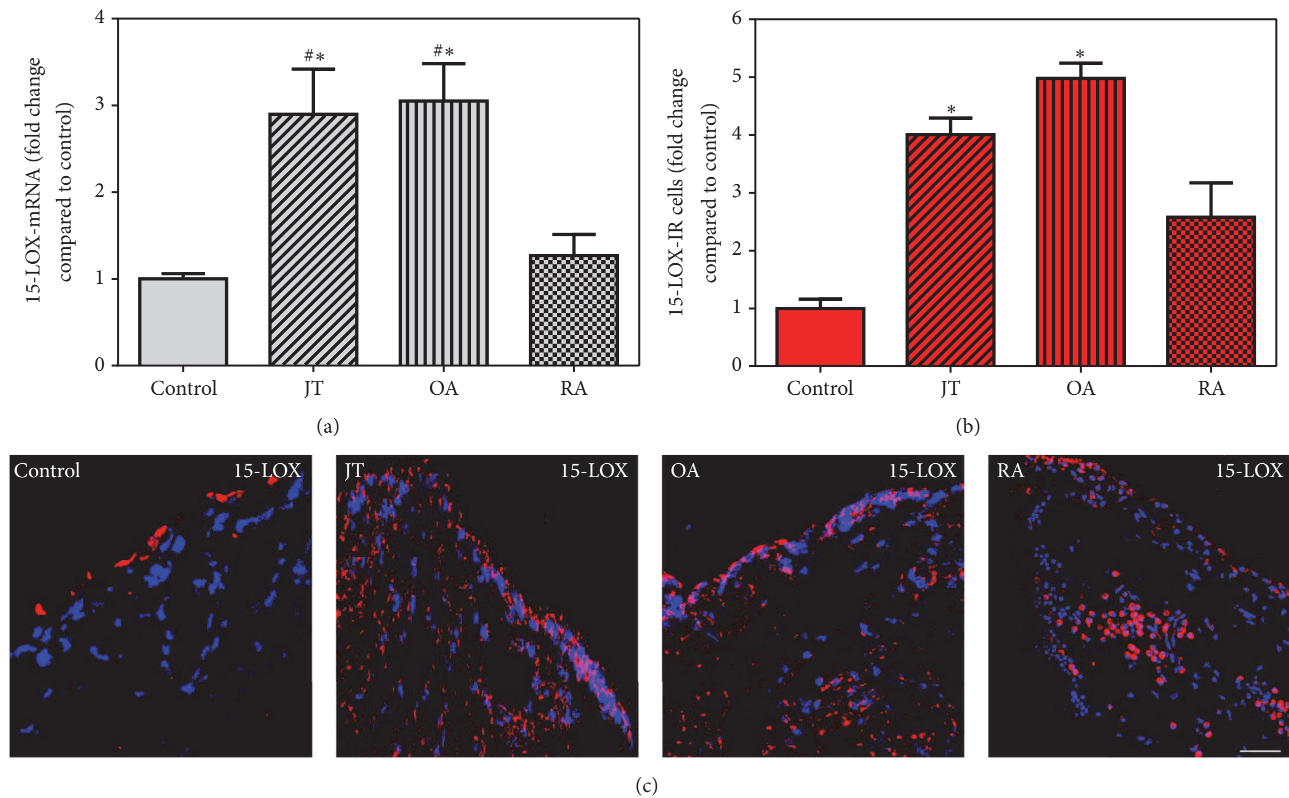

Figure 3: Detection of 15-LOX mRNA (a) and number of 15-LOX-IR cells (b and c) in patients with joint trauma (JT), osteoarthritis (OA), and rheumatoid arthritis (RA). (a) Quantification of 15-LOX mRNA shows that 5-LOX mRNA was significantly higher in OA and JT compared to RA and control synovium ($P < 0.05$, one-way ANOVA followed by Tukey's test). (b) Quantitative analysis of immunofluorescence microscopy for 15-LOX-IR cells. *Relative to control, #relative to other groups ($P < 0.05$, one-way ANOVA followed by Tukey's test). Data are shown as means ± SEM. (c) 5-LOX-IR cells are more abundant in RA synovium than in JT, OA, and control synovium. Bar = 20 μm.

patients and patients with JT ($P < 0.05$). While in patients with JT synovial granulocytes and macrophages were most prominent, in patients with OA fibroblasts and macrophages were the most prominent, and in patients with RA plasma cells, fibroblasts, and macrophages were the most prominent ($P < 0.05$) (Supplemental Figure 1).

3.2. Expression of Synovial Proinflammatory Mediators IL-1β, TNF-α, and 5-LOX. Quantitative RT-PCR analysis demonstrated a significant increase in IL-1β and TNF-α specific mRNA in synovial tissue of JT, OA, and RA patients in contrast to controls. In addition, they were more prominent in JT and RA than in OA patients (Supplemental Figures 2 and 3). Consistently, immunofluorescence confocal microscopy of synovial tissue demonstrated IL-1β and TNF-α expression in layers of synovial lining and sublining cells. Importantly, the number of IL-1β- and TNF-α-IR cells was significantly higher in patients with JT, OA, and RA compared to controls ($P < 0.05$) and was more pronounced in JT and RA than OA patients (Supplemental Figures 2 and 3). Quantitative RT-PCR analysis of 5-LOX specific mRNA revealed a significant increase in synovial tissues of RA patients, while immunofluorescence confocal microscopy showed a significant increase of 5-LOX-IR cells in synovial tissues of JT, OA, and RA patients compared to controls which was more prominent in RA patients ($P < 0.05$, Figure 1).

3.3. Expression of Synovial Anti-Inflammatory Mediators, FPR2, 15-LOX, and IL-10. FPR2 specific mRNA showed a significant increase in synovial tissues of JT, OA, and RA patients compared to controls which was most prominent in OA patients (Figure 2), while 15-LOX mRNA was shown only in JT and OA (Figure 3). However, IL-10 mRNA was prominent only in OA and RA patients (Figure 4). Consistent with these findings, the number of FPR2-IR cells was significantly higher in JT, OA, and RA patients than that of control synovium (Figure 2). However, the number of 15-LOX-IR cells was more prominent in JT and OA than that of RA patients and control synovium (Figure 3), while the number of IL-10-IR cells was higher in RA and OA patients than that of JT and control synovium (Figure 4).

Double immunofluorescence confocal microscopy showed that FPR2 was expressed in CD68-IR macrophages and P4HB-IR fibroblasts in JT, OA, and RA as well as in Ab-1 plasma cells in RA (Supplemental Figure 4). 15-LOX-IR cells were characterized as CD68-IR macrophages and P4HB-IR fibroblasts in OA (Supplemental Figure 5). IL-10-IR cells were mainly CD68-IR macrophages and P4HB-IR fibro-

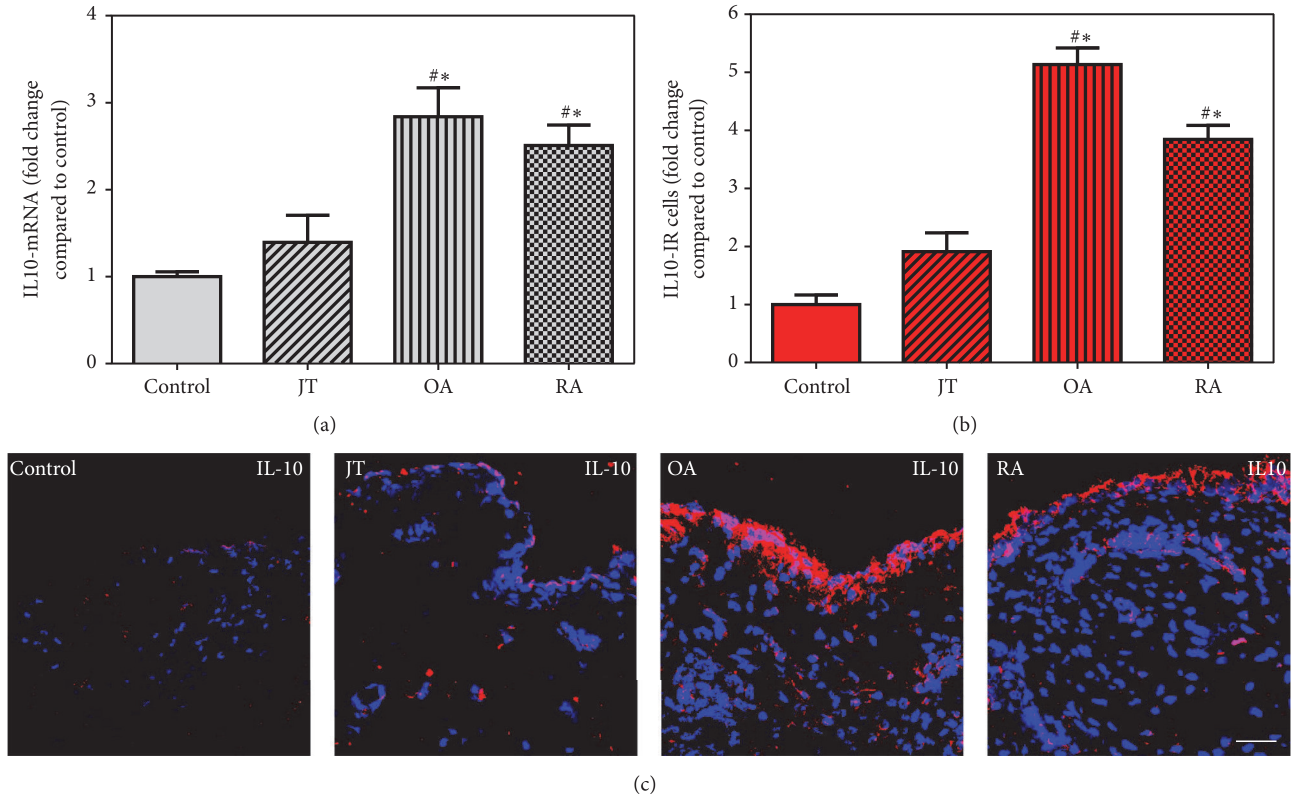

Figure 4: Detection of IL10 mRNA (a) and number of IL10-IR cells (b and c) in patients with joint trauma (JT), osteoarthritis (OA), and rheumatoid arthritis (RA). (a) Quantification of IL10 mRNA shows that IL10 mRNA was significantly higher in RA and OA compared to JT and control synovium ($P < 0.05$, one-way ANOVA followed by Tukey's test). (b) Quantitative analysis of immunofluorescence microscopy for IL10-IR cells. *Relative to control, #relative to other groups ($P < 0.05$, one-way ANOVA followed by Tukey's test). Data are shown as means ± SEM. (c) 5-LOX-IR cells are more abundant in RA synovium than in JT, OA, and control synovium. Bar = 20 μm.

blasts in OA and in RA as well as in Ab-1 plasma cells in RA (Supplemental Figure 6).

3.4. Inflammatory Mediator Profile in JT, OA, and RA. Direct comparison of the mRNA expression profile of pro- versus anti-inflammatory mediators in synovial tissues revealed that cells expressing the proinflammatory mediators IL-1β, TNF-α, and 5-LOX were elevated approximately 4- to 8-fold compared to controls in JT (Figure 5), whereas they were upregulated only 2- to 4-fold in OA (Figure 6). At the same time, the anti-inflammatory mediators 15-LOX, FPR2, and IL-10 were found to be elevated approximately 2- to 6-fold in JT (Figure 5), whereas in OA they were elevated approximately 5- to 7-fold (Figure 6). In contrast, in synovial tissue of RA patients, the proinflammatory mediators IL-1β, TNF-α, and 5-LOX were elevated approximately 5- to 13-fold compared to controls, whereas the anti-inflammatory mediators 15-LOX, FPR2, and IL-10 were upregulated approximately 3- to 7-fold (Figure 7).

4. Discussion

We found the highest amount of synovial lining cells, vascularity, and infiltrating immune cells in patients with RA compared to those with JT and OA. Pro- and anti-inflammatory mediators were expressed predominantly in granulocytes and macrophages in patients with JT and in macrophages, fibroblasts, and plasma cells in those with OA and RA. Overall, proinflammatory IL-1β, TNF-α, and 5-LOX specific mRNA as well as immunoreactive cells were significantly more abundant in patients with RA and JT than in those with OA. In contrast, anti-inflammatory 15-LOX, FPR2, and IL-10 specific mRNA as well as immunoreactive cells were significantly more abundant in patients with OA than in those with JT and RA. These findings provide a morphological evidence of imbalance within the so-called inflammatory factor network between catabolic proinflammatory and anabolic anti-inflammatory mediators among JT, OA, and RA patients.

The lining layer of the synovial membrane contains macrophage-like (type A cells) and fibroblast-like (type B cells) cells [19, 21, 22]. Our double staining demonstrated that most of the lining-layer cells containing proinflammatory cytokines such as IL1β, TNF-α, and 5-LOX were positive for CD68 (macrophages), CD15 (granulocytes), and P4HB (fibroblast-like synoviocytes, type B) [19]. In the sublining layers, we found different patterns of pro- versus anti-inflammatory mediators containing leukocyte subsets in patients with JT, OA, and RA. Pro- and anti-inflammatory mediators were expressed predominantly in granulocytes in patients with JT and in macrophages, lymphocytes, and plasma cells in

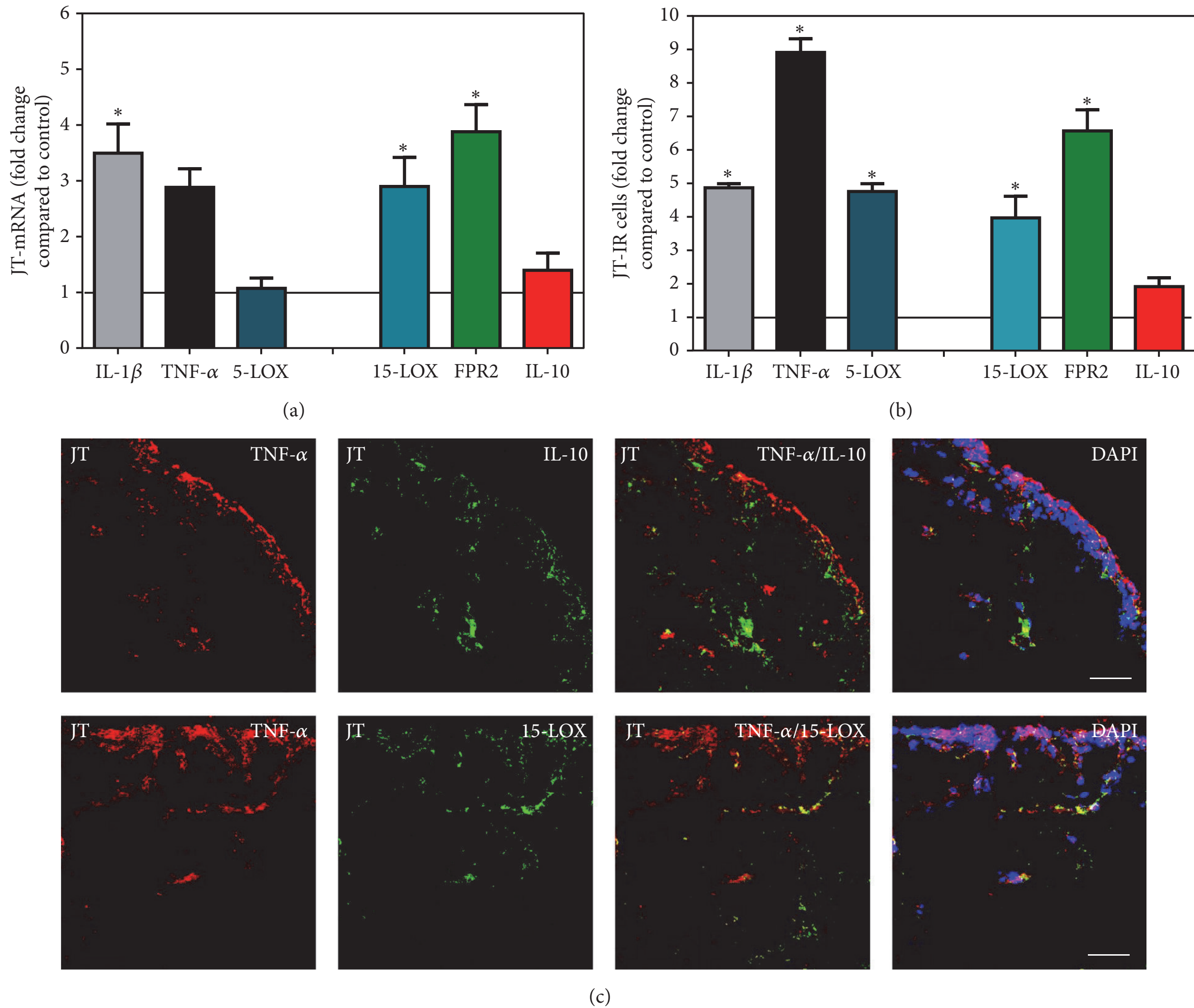

Figure 5: Detection of mRNA (a) and the number of positive cells of inflammatory (IL-1β, TNF-α, and 5-LOX) versus anti-inflammatory (15-LOX, FPR2, and IL-10) mediators (b and c) in patients with joint trauma (JT). (a) Quantification of mRNA of pro- versus anti-inflammatory mediators shows that there is a balance between expressions of pro- versus anti-inflammatory mediators in JT synovium. (b) Quantitative analysis of immunofluorescence microscopy of pro- versus anti-inflammatory mediators shows that the number of cells expressing anti-inflammatory mediators was comparable with those containing proinflammatory mediators in JT synovium. Data are shown as means ± SEM. *Relative to control ($P < 0.05$, one-way ANOVA followed by Tukey's test). (c) Confocal microscopy of pro- (*red fluorescence*; (a), (b)) and anti-inflammatory mediators (*green fluorescence*; (c), (d)) double immunofluorescence (e–h) in JT synovium. Note that anti-inflammatory mediator expression was comparable with those containing inflammatory mediators in JT synovium. Bar = 40 μm.

those with OA and RA. These results extend previous reports of the expression of 5-LOX in macrophages, fibroblasts, and neutrophils [5] and TNF-α in macrophages and monocytes [23] within RA synovium.

Importantly, our results showed that IL-1β and TNF-α and 5-LOX specific mRNA as well as cells expressing these mediators within synovial tissue were more prominent in JT and RA compared to OA patients. These findings are consistent with high levels of 5-LOX reported to be present in RA synovium and mostly expressed in macrophages, neutrophils, and mast cells of the sublining layer [5]. Also, our results are in agreement with the previous study by Park and Pillinger [24], suggesting that the inflammatory mediators such as TNF-α, IL-1β, and 5-LOX play a key role in driving the inflammation and synovial cell proliferation in RA associated joint destruction. Taken together, these results suggest that there is an association between the expression of proinflammatory mediators such as TNF-α, IL-1β, and 5-LOX and the severity of inflammation among patients with JT, OA, and RA [19].

On the other hand, the present results showed that the anti-inflammatory cytokines 15-LOX, FPR2, and IL-10 specific mRNA as well as immunoreactive cells were more abundant in patients with OA than in those with JT and RA. The present results extend the previous reports of 15-LOX

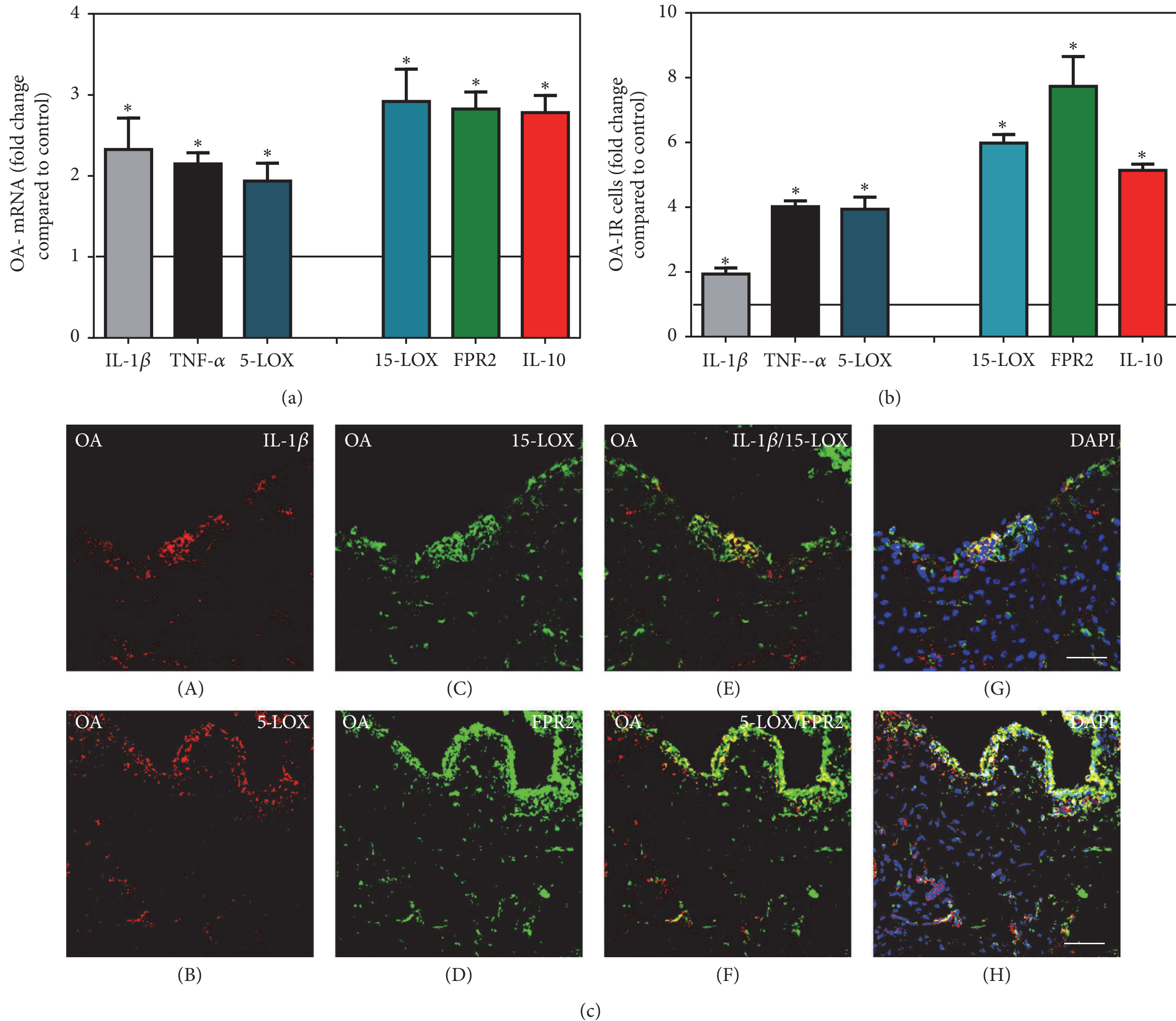

FIGURE 6: Detection of mRNA (a) and the number of positive cells of pro- (IL-1β, TNF-α, and 5-LOX) versus anti-inflammatory (15-LOX, FPR2, and IL-10) mediators (b and c) in patients with osteoarthritis (OA). Quantification of mRNA of pro- versus anti-inflammatory mediators shows that anti-inflammatory mediator expression was more prominent than inflammatory mediators in OA synovium. *Relative to control ($P < 0.05$, one-way ANOVA followed by Tukey's test). (b) Quantitative analysis of immunofluorescent microscopy pro- versus anti-inflammatory mediators shows that the number of cells expressing anti-inflammatory mediators was more prominent than those containing proinflammatory mediators in OA synovium; the asterisks denote significant differences ($P < 0.05$, one-way ANOVA followed by Dunn's test). Data are shown as means ± SEM. (c) Confocal microscopy of pro- (*red fluorescence*; (A) and (B)) and anti-inflammatory mediators (*green fluorescence*; (C) and (D)) double immunofluorescence in OA synovium. Note that anti-inflammatory mediator expression was more prominent than proinflammatory mediators in OA synovium. Bar = 40 μm.

expression in humans [5] that did not differentiate the synovial cell types. IL-10 is a potent immunoregulatory cytokine and plays a role in preventing exaggerated inflammatory and immune responses and thus protects the host from immune-mediated damage [25]. Moreover, IL-10 is a good candidate transgene to suppress arthritis using disease-regulated promoters. Also, IL-10 is a broad spectrum anti-inflammatory cytokine and is produced by different immune cells, like Th1 and Th2 cells, B cells, monocytes, and macrophages [11–13]. Inhibition of several proinflammatory cytokines has been reported; effects were seen for interleukin-1 (IL-1) and TNF-α [26, 27]. Recently, Vermeij et al. [28] showed that treatment of an acute joint inflammation with local IL-10 overexpression under the control of disease-regulated promoters inhibited arthritis progression. Consistently, Roybal et al. [29] showed that the early gestational gene transfer of IL-10 by systemic administration of lentiviral vector can prevent arthritis in a murine model. Taken together, these findings are consistent with the notion that the upregulated proinflammatory mediators support the inflammatory process in JT and RA synovium in contrast to the upregulated anti-inflammatory mediators probably responsible for a counterbalance and, therefore, lower inflammatory process in OA synovium. A potential limitation of this study, however, is that the

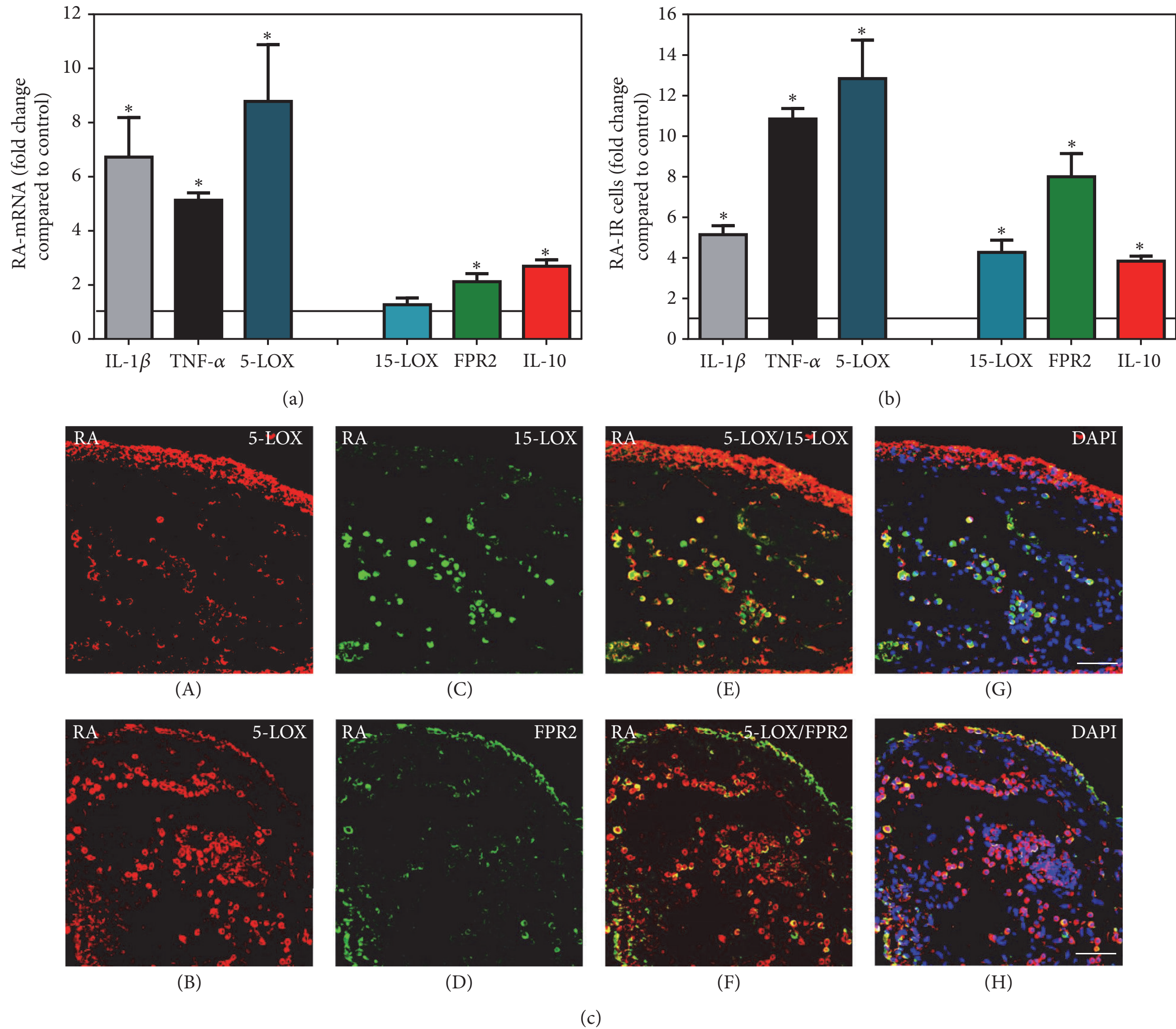

Figure 7: Detection of mRNA (a) and the number of positive cells of pro- (IL-1β, TNF-α, and 5-LOX) versus anti-inflammatory (15-LOX, FPR2, and IL-10) mediators (b and c) in patients with rheumatoid arthritis (RA). Quantification of mRNA of pro- versus anti-inflammatory mediators shows that proinflammatory mediators expressions were more prominent than anti-inflammatory mediators in RA synovium. *Relative to control ($P < 0.05$, one-way ANOVA followed by Tukey's test). (b) Quantitative analysis of immunofluorescence microscopy pro- versus anti-inflammatory mediators shows that the number of cells expressing proinflammatory mediator expression was more prominent than those containing anti-inflammatory mediators in OA synovium; the asterisks denote significant differences ($P < 0.05$, one-way ANOVA followed by Dunn's test). Data are shown as means ± SEM. (c) Confocal microscopy of pro- (*red fluorescence*; (A) and (B)) and anti-inflammatory mediators (*green fluorescence*; (C) and (D)) double immunofluorescence in RA synovium. Note that proinflammatory mediator expression was more prominent than anti-inflammatory mediators in RA synovium. Bar = 40 μm.

observed differences, at least in part, may be also due to apparent differences in age and disease duration of patients influencing the disease-specific pro- and anti-inflammatory balance (see Table 1).

In summary, we found that the upregulation of proinflammatory mediators mediates the predominantly catabolic inflammatory process in JT and RA synovium, whereas the upregulation of anabolic anti-inflammatory mediators may counteract inflammation and be responsible for the inferior inflammatory process in OA synovium. These findings provide a morphological evidence of imbalance within the so-called inflammatory factor network between catabolic proinflammatory and anabolic anti-inflammatory cytokines within each disease and among JT, OA, and RA patients.

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

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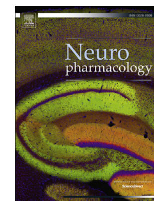

Acute mechanical sensitization of peripheral nociceptors by aldosterone through non-genomic activation of membrane bound mineralocorticoid receptors in naive rats

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ABSTRACT

Recently, there is increasing interest in the role of peripheral mineralocorticoid receptors (MR) to modulate pain, but their localization in neurons and glia of the periphery and their distinct involvement in pain control remains elusive. In naive Wistar rats our double immunofluorescence confocal microscopy of the spinal cord, dorsal root ganglia, sciatic nerve and innervated skin revealed that MR predominantly colocalized with calcitonin-gene-related peptide (CGRP)- and trkA-immunoreactive (IR) nociceptive neurons and only marginally with myelinated trkB-IR mechanoreceptive and trkC-IR proprioreceptive neurons underscoring a pivotal role for MR in the modulation of pain. MR could not be detected in Schwann cells, satellite cells, and astrocytes and only scarcely in spinal microglia cells excluding a relevant functional role of glia-derived MR at least in naïve rats. Intrathecal (i.t.) and intraplantar (i.pl.) application of increasing doses of the MR selective agonist aldosterone acutely increased nociceptive behavior which was reversible by a MR selective antagonist and most likely due to non-genomic effects. This was further substantiated by the first identification of membrane bound MR specific binding sites in sensory neurons of dorsal root ganglia and spinal cord. Therefore, a crucial role of MR on nociceptive neurons but not on glia cells and their impact on nociceptive behavior most likely due to immediate non-genomic effects has to be considered under normal but more so under pathological conditions in future studies.

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1. Introduction

Mineralocorticoid receptors and their endogenous ligand aldosterone are best known for their control of the water and electrolyte balance in the kidney and their involvement in volume and blood pressure regulation (Te Riet et al., 2015). In addition, aldosterone has also been reported to promote inflammation, fibrosis, and remodeling in the heart and vasculature (Ferrario and Schiffrin, 2015; Young and Rickard, 2012). Interestingly in older studies, a combination of aldosterone and its antagonist spironolactone revealed an immunosuppressive effect in allogenic skin grafts (Baethmann et al., 1971), multiple sclerosis (Mertin et al., 1972), and progressive systemic sclerosis (Altmeyer et al., 1985). All these effects occur by the classical pathway at which aldosterone easily diffuses the cellular membrane, binds to its cytoplasmic MR and – upon dissociation of chaperons and formation of MR dimers - is translocated to the nucleus resulting in the enhanced or inhibited expression of several genes (Te Riet et al., 2015). The effects of aldosterone via intracellular MR are usually characterized by a 45-min up to several hours lag period (Funder, 2005). In contrast to these genomic effects more rapid non-genomic effects have also been demonstrated particularly in neurons of the nervous system (Groeneweg et al., 2012). Corticosteroids for example rapidly alter neuronal excitability throughout the brain and as a consequence regulate adaptive behavior and memory (Groeneweg et al., 2012).

Recently, increasing interest has focused on the role of MR in different conditions of pain. In a model of chronic compression of

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the L5 lumbar dorsal root ganglion the twice daily intrathecal injection of the MR antagonist spironolactone over three days resulted in a significant reduction of mechanical allodynia (Gu et al., 2011; Sun et al., 2012). In another model of zymosan-induced local inflammation of the L5 dorsal root ganglion, the combined local application of zymosan with the MR antagonist eplerenone reduced for an extended period of time the mechanical hypersensitivity (Dong et al., 2012). In the same study MR were shown in dorsal root ganglia to colocalize with the pan-neuronal cell marker NeuN and eplerenone treatment apparently decreased the number of activated satellite glia cells. Since these effects occurred with some delay and lasted several days, they were most likely due to genomic effects of the MR. Up to now, evidence for the exact subpopulations of DRG neurons and glia cells that express MR and for putative short lasting non-genomic effects resulting from neuronal membrane bound MR is lacking.

Therefore, we systematically investigated in naive rats i) the presence of MR specific mRNA and receptor protein in spinal cord, dorsal root ganglia and innervating sciatic nerve compared to kidney; ii) the localization of MR in neurons, astrocytes and microglia of the spinal cord, iii) the localization of MR to myelinated, unmyelinated, nociceptive, mechanoreceptive and proprioceptive neurons in dorsal root ganglia; iv) the characterization of MR-ir nerve terminals in the subepidermal and epidermal layer of the skin; v) the changes in nociceptive behavior following the local i.pl. or i.th. administration of a MR selective agonist with and without antagonist; vi) the evidence for membrane bound MR by saturation binding with the radiolabeled MR selective ligand [^{3}H] aldosterone.

2. Methods

2.1. Reagents

The following drugs were used: aldosterone, canreonate-K (Sigma-Aldrich, St. Louis, MO, USA); doses were calculated - where applicable - in terms of the free base. Canreonate-K was dissolved in NaCl 0.9%, aldosterone was dissolved in a vehicle composed of 10% ethanol and 90% normal saline, as described previously (Gravez et al., 2013). Routes and volumes of drug administration were i.t. 20 μL and i.pl. 100 μL. Intrathecal injections were performed under inhalational anesthesia with the rat in the elevated lumbar position. Intraplantar injections were given under inhalational anesthesia into the subcutaneous tissue of the glabrous skin directly proximal to the callosities of the toes. The drug or its solvent were injected into the intrathecal L3-L4 interspace (spontaneous tail movement being a positive indication for correct i.t. positioning) with a 30-gauge needle connected to a 50 μL syringe. In accordance with previous studies (Myers and Van Meerveld, 2009; Khan and Bakshi, 2009), separate groups of animals for each dose and injection technique received i.pl. or i.t. administrations of different doses of: aldosterone (i.pl. 25–100 μg or i.t. 4–40 μg) with and without canreonate-K (150–500 μg). Control animals received vehicle treatment. Experiments were performed in a blinded way to the drugs and doses applied.

2.2. Animals

Experiments were conducted in male Wistar rats (200–250 g) (breeding facility, Charité-Universitätsmedizin Berlin, Germany) after approval by the local animal care committee and in accordance with the European Directive on the protection of animals used for scientific purposes (2010/63/EU).

2.3. Characterization of antibodies

The species, sources, dilutions, and immunogens of the primary antibodies used in this study are summarized in Table 1.

2.4. Tissue preparation

Rats were deeply anesthetized with isoflurane and the subcutaneous paw tissue, sciatic nerve, dorsal root ganglia, spinal cord, and kidney were removed from adult rats for subsequent qRT-PCR and western blot experiments.

2.5. RT-PCR

The total RNA was prepared from kidney, DRG and spinal cord of rats with the commercially available Kit Qiazol Lysis Reagent, (Qiagen, Hilden, Germany) according to the manufacturer's protocol. 500 ng total RNA, measured by Nanodrop (Peqlab) was applied for transcription of cDNA using Omniscript RT Kit (Qiagen, Hilden, Germany) as followed: 0.5 mM dNTP, 1 μM Random Primer, 10 units RNase Inhibitor and 4 units Omnisript reverse Transciptase. Samples were incubated at 42 °C for 1 h and cDNA were stored

Table 1
Characterization of primary antibodies used.

Antigen	Immunogen	Manufacturer, species, type, catalogue number	Dilution used
MR	a 142-amino-acid peptide sequence from the unique DNA-binding domain of the rat MR gene	a gift from M. Kawata (Kyoto Prefectural University of Medicine, Japan), rabbit polyclonal, # Ito et al., 2000	1:2000
MR	amino acids 1–300 of the human MR that is recommended for the detection of mouse, rat and human MR	Santa Cruz Biotechnology (USA), rabbit polyclonal, # sc-11412, Kapoor et al., 2008	1:1000
Calcitonin-Gene Related Peptide	synthetic entire calcitonin gene-related peptide	Peninsula Laboratories (CA, USA), guinea pig polyclonal, # T-5027, Mousa et al., 2013	1:1000
trkA	extracellular domain Ala33-Pro418 of rat trkA	R&D Systems (USA), goat polyclonal, # AF1056, Matsumoto et al., 2012	1:1000
NF200	carboxy terminal tail segment of dephosphorylated NF200	Sigma-Aldrich (USA), mouse monoclonal # N0142/N52, Kestell et al., 2015	1:1000
trkB	extracellular domain Cys32-Thr429 of recombinant mouse trkB	R&D Systems (USA), goat polyclonal, # AF1494, Matsumoto et al., 2012	1:1000
trkC	extracellular domain Cys32-Thr429 of recombinant mouse trkC	R&D Systems (USA), goat polyclonal, # AF1404, Matsumoto et al., 2012	1:1000
GFAP	clone G-A-5	Sigma-Aldrich (USA), mouse monoclonal # G3893, Liu and Chien, 2012	1:1000
CD11b	clone OX-42	AbD Serotec (Germany), mouse monoclonal # MCA275R, Robinson et al., 1986	1:1000

at −20 °C. The following specific primers for MR were used: (Ensemble Accession No: NM_013131.1), Forward primer; : 5′-CCAAGGTACTTCCAGGATTTAAAAAC-3′, Reverse primer; 5′-AAC-GATGATAGACACATCCAAGAATACT-3′ Taqman® qRT-PCR was performed with a SYBR® Green kit following the manufacturer's instructions (Applied Biosystems). Amplifications were carried out for 40 cycles, each consisting of 15 s at 95 °C and of 30 s at 60 °C. A temperature just below the specific melting temperature (Tm) was employed for detection of fluorescence specific products (MR: Tm 76 °C, 18S: Tm 83 °C). MR mRNA was quantified using triplicates of samples and using the delta-delta CT method (Weil et al., 2006). The housekeeping gene 18s (Accession No. NR_046237, Forward primer: CGGCTACCACATCCAAGGAA Reverse Primer: GCTGGAAT-TACCGCGGCT) was used as an internal reference gene.

2.6. Western blot

Kidney, DRG, spinal cord or peripheral (sciatic) nerve from adult rats were solubilized according to Weems et al. (1996). Western blot analysis was performed as previously described (Ji and Rupp, 1997; Mousa et al., 2010). Briefly, the samples were homogenized in RIPA-Buffer and the lysate was centrifuged at 16.000 g for 20 min. The protein concentration of the supernatant was measured using a BCA assay (Pierce, Rockford, IL, USA). Subsequently 10–20 μg protein were denatured in SDS sample buffer (5× loading buffer: 200 mM Tris, pH 6.8, 10% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.05% bromophenol blue; filled up with RIPA buffer) for 10 min at 80 °C. The extracts were separated using 7.5% Mini-PROTEAN TGX Stain-Free Precast Gels (Bio-Rad, Copenhagen, Denmark). After SDS-electrophoresis, the gels were activated using UV light. The activated gel was then electrophoretically transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories GmbH, München, Germany). After blotting the protein transfer was visualized by taken a UV light exposure image. The membranes were blocked in 3% BSA for 2 h and incubated with rabbit anti-MR (Santa Cruz, 1:2.000, in 3% BSA) overnight at 4 °C. This antibody has been proven to be highly specific following MR-transfection and knock-down in different cell lines (O'Hara et al., 2014; Jeong et al., 2009). After incubation with the secondary antibody (peroxidase-conjugated goat anti-rabbit, 1:40.000, Jackson ImmunoResearch) for 2 h at room temperature, reactive protein bands were digitally visualized using ECL solutions (SuperSignal West Pico, Thermo Scientific) in ChemiDoc MP Imager. Finally, the blots were reprobed with monoclonal mouse anti-beta Actin antibody (1:20.000; Sigma Aldrich) as an internal standard. Experiments were repeated three times.

2.7. Immunohistochemistry

2.7.1. Tissue preparation

Adult rats were deeply anesthetized with isoflurane and transcardially perfused with 100 ml warm saline, followed by 300 ml 4% (w/v) paraformaldehyde in 0.16 M phosphate buffer solution (pH 7.4). After perfusion the subcutaneous tissue, sciatic nerve, DRG and spinal cord were removed and fixed in the same fixatives for 90 min, and then cryoprotected overnight at 4 °C in PBS containing 10% sucrose. The tissues were then embedded in tissue-Tek compound (OCT, Miles Inc. Elkhart, IN) and frozen. DRG or sciatic nerve sections (8 μm thick) were mounted onto gelatin coated slides but spinal cord and subcutaneous sections (50 μm thick) collected in PBS (floating sections).

2.7.2. Double immunofluorescence staining

Double immunofluorescence staining was processed as described previously (Mousa et al., 2007). Tissue sections were incubated for 60 min in PBS containing 0.3% Triton X-100, 1% BSA, 10% goat serum (Vector Laboratories, CA, USA) (blocking solution) to prevent nonspecific binding. The sections were then incubated overnight with the following primary antibodies: polyclonal rabbit antibodies against MR (Dr. M. Kawata) (dilution of 1:2000) in combination with a polyclonal guinea pig anti-CGRP (Peninsula Laboratories, 1:1000), anti-NF200 (Sigma, 1:1000), goat polyclonal anti-trkA, anti-trkB or anti-trkC (R&D, 1:500). After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with Alexa Fluor 594 donkey anti-rabbit antibody (Vector Laboratories) in combination with Alexa Fluor 488 goat anti-guinea pig, anti-mouse or anti-chicken antibody (Invitrogen, Germany). Thereafter, sections were washed with PBS, and the nuclei stained bright blue with 4′-6-Diamidino-2-phenylindole (DAPI) (0.1 μg/ml in PBS) (Sigma). Finally, the tissue sections were washed in PBS, mounted on vectashield (Vector Laboratories) and imaged on a confocal laser scanning microscope, LSM510, equipped with an argon laser (458/488/514 nm), a green helium/neon laser (543 nm), and a red helium/neon laser (633 nm; Carl Zeiss, Göttingen, Germany). Single optical slice images were taken using ×10 or ×20 Plan-Neofluar air interface or ×40 Plan-Neofluar oil interface objective lens. The brightness and contrast of the final images were adjusted in Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). To demonstrate specificity of the staining, the following controls were included as described in our previous studies (Mousa et al., 2007, 2010): omission of either the primary antisera or the secondary antibodies. The method of quantification for DRG staining has been described previously (Mousa et al., 2007). For counting of the total number of neurons, only those immunostained neurons containing a distinct nucleus were counted using the microscope (40 × objective). In a similar way, the number of GR/MR-, MR/CGRP-, MR/trkA-, MR/trkB-, R/trkC-, NF200-, GFAP- or OX42-IR neurons was counted and divided by the total number of MR-IR neurons in each DRG section and represented as percentages. Data were obtained from two sections per rat and four to five rats per group.

2.8. Von Frey filament testing

Rats were placed in a plastic cage for half an hour to acclimate until cage exploration and major grooming activities ceased. At the bottom of the cage a wire mesh allowed full access to the paws. Mechanical hind paw withdrawal thresholds were assessed by the application of a calibrated series of von Frey filaments of logarithmic incremental stiffness (Stoelting, Wood Dale, IL, USA) as described previously (Dixon, 1980; Chaplan et al., 1994; Mousa et al., 2016). Von Frey filament testing were firstly performed in all groups before drug application to get baseline values. Then, the nociceptive responses were reassessed at different time-points (0–60 min) following drug administrations to determine drug-related behaviors. Briefly, the resulting pattern of positive and negative responses were tabulated using the convention, X = withdrawal; 0 = no withdrawal, and the 50% response threshold was interpolated using the formula: 50% g threshold $= (10^{(xf+k\delta)})/10{,}000$, where x_f = value (in log units) of the final von Frey filament used; k = tabular value for the pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli. After baseline measurements von Frey thresholds were reevaluated at different time intervals before and 0–60 min after i.pl. or i.t. injection of vehicle, the MR agonist aldosterone alone or in combination with MR antagonist canreonate-K. The mechanical paw withdrawal thresholds were defined as the mean of 5–6 animals performed before and after i.pl. or i.t. drug injections.

2.9. Radioligand binding assay

The following experiments should identify MR specific binding sites in membrane fractions of spinal cord and DRG prepared according to our previous binding studies (Mousa et al., 2010; Shaqura et al., 2004; Zöllner et al., 2003). Membrane fractions from Wistar rat spinal cord or DRG were prepared by homogenizing them in cold assay buffer (50 mM Tris-HCl, 1 mM EGTA, 5 mM $MgCl_2$, pH 7.4) and by centrifuging them at 48,000×*g* at 4 °C for 20 min. The pellet was resuspended in assay buffer followed by 10 min incubation at 37 °C to remove endogenous ligands. The homogenates were centrifuged again at 48,000 g and resuspended in assay buffer. Membranes were aliquoted and stored at −80 °C (for details see Zöllner et al., 2003). Saturation binding experiments were performed using the specific MR agonist [^{3}H]aldosterone (specific activity 77.4 ci/mmol, Hartmann, Germany). 50–100 µg of membrane protein was incubated with various concentrations of 1.25–40 nM [^{3}H]aldosterone and 10 µM of unlabeled aldosterone for 2 h at 30 °C in a total volume of 0.5 ml of binding buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM MgCl2, 100 mM NaCl, 0.2% bovine serum albumin). Nonspecific binding was defined as radioactivity remaining bound in the presence of 10 µM unlabelled aldosterone. At the end of the incubation period, bound and free ligands were separated by rapid filtration over GF/C filters under vacuum using a Brandel cell harvester (Gaithersburg, MD, USA). Filters were washed three times with 4 ml of cold buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction of the filters in 3 ml of scintillation fluid. All experiments were performed in duplicate and carried out at least three times. Nonspecific binding was subtracted from all [^{3}H]aldosterone data. B_{max} and K_d values in saturation binding assays were determined by nonlinear regression analysis of concentration-effect curves using GraphPad Prism (GraphPad Software Inc., CA, USA).

2.10. Statistical analysis

All tests were performed using Sigma Stat 2.03 software (SPSS Inc., Germany). MR mRNA data are expressed as means ± s.e.m. Paw withdrawal thresholds determined by von Frey filament testing were expressed as means ± s.e.m and statistically analyzed by one way or two-way ANOVA and post-hoc by Dunnett's or Tukey test, respectively. For all statistical tests, significance was assumed at $P < 0.05$.

3. Results

3.1. Identification of spinal cord and DRG mineralocorticoid receptor specific mRNA and protein

Using a highly specific primer pair (Fig. 1A) an expected MR specific 85 bp PCR-product was identified in the kidney of naive rats (Fig. 1B, C). Similar to the expression in the kidney the same MR specific PCR-product was also detected in the spinal cord and dorsal root ganglia of these naïve rats (Fig. 1C). This was consistent with the demonstration of a MR specific protein band at 107 kDa not only in the kidney, but also in the spinal cord, dorsal root ganglia, and sciatic nerve (Fig. 1D).

3.2. Localization of spinal MR on central nerve terminals of nociceptive neurons

In the dorsal horn of the spinal cord there was abundant colocalization of MR with CGRP, a marker for nociceptive neurons

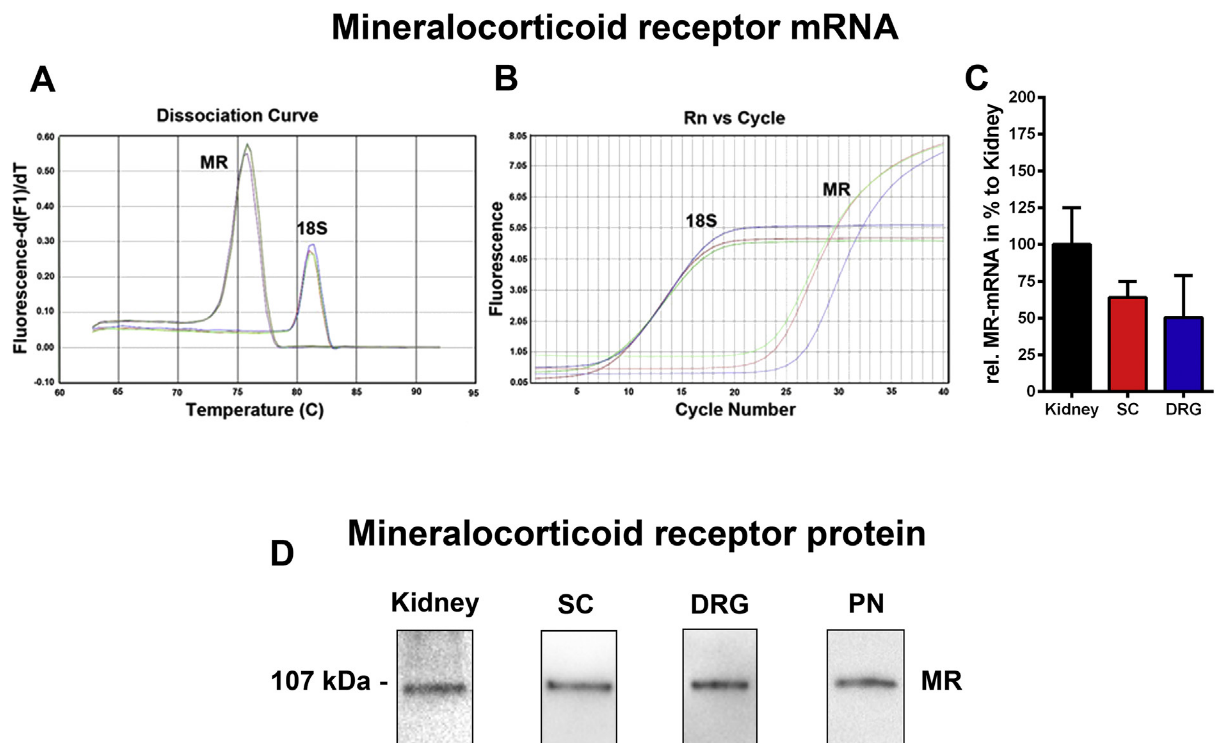

Fig. 1. Detection of MR mRNA and protein in rat kidney, spinal cord (SC), dorsal root ganglia (DRG) and sciatic nerve (SN). Quantification of MR mRNA using Taqman® qRT-PCR in kidney, spinal cord, dorsal root ganglia and sciatic nerve of naïve rats. **A, B**: DNA melting profiles using a 18S- (as internal standard gene) and MR-specific primer pair (A) as well as the amplification profiles of the 18S- and MR-specific cDNA of naïve rats (B). C: column graph represents the per cent of MR mRNA expression relative to the expression in kidneys of naïve rats. **D**: Western blot analysis with a rabbit polyclonal anti-MR antibody shows MR specific protein bands from kidney, SC, DRG and SN of naïve rats with an expected molecular weight of 107 kDa.

(Fig. 2). This colocalization occurred mainly from incoming sensory neurons in Rexed laminae I and II of the grey matter of the spinal cord, whereas few scattered neuronal cells could also be identified in Rexed laminae III and IV (Fig. 2). Interestingly, MR did not colocalize with GFAP, a marker for astrocytes, and only very rarely with OX-42, a marker for microglia, within the spinal cord of naïve rats indicating its main neuronal localization (Fig. 2).

3.3. Colocalization of MR primarily with CGRP and trkA in nociceptive DRG neurons

Double immunofluorescence confocal microscopy showed abundant colocalization of MR with CGRP (74.9 ± 9.7%) (Fig. 3, upper panels) and trkA (55.6 ± 7.3%) (Fig. 4, upper panels); however, there was less colocalization with NF200 (20.8 ± 3.2%) (Fig. 3, lower panels), trkB (27.7 ± 4.0%) (Fig. 4, middle panels) and trkC (15.1± 8.6%) (Fig. 4, lower panels) indicating that the MR is predominantly expressed in nociceptive C- and Aδ-neurons, but much less in mechanoreceptive and proprioceptive neurons (Matsumoto et al., 2012). Approximately 80% of these neurons do not show colocalization with NF200 (Fig. 3, lower panels) indicating that the majority are unmyelinated nerve fibres.

3.4. Localization of skin MR on peripheral nerve terminals of nociceptive neurons

Consistently, MR also colocalized to a high degree with the sensory neuron marker CGRP both in peripheral nerve fibers of the sciatic nerve (Fig. 5, upper panels) and in branching nerve terminals within the subepidermal and epidermal layer (Fig. 5, middle panels), although there were some CGRP-immunoreactive nerve fibers without MR. In addition, MR colocalised to a high degree with the sensory neuron marker trkA in branching nerve terminals of the skin (Fig. 5, lower panels). These nerve terminals extend as

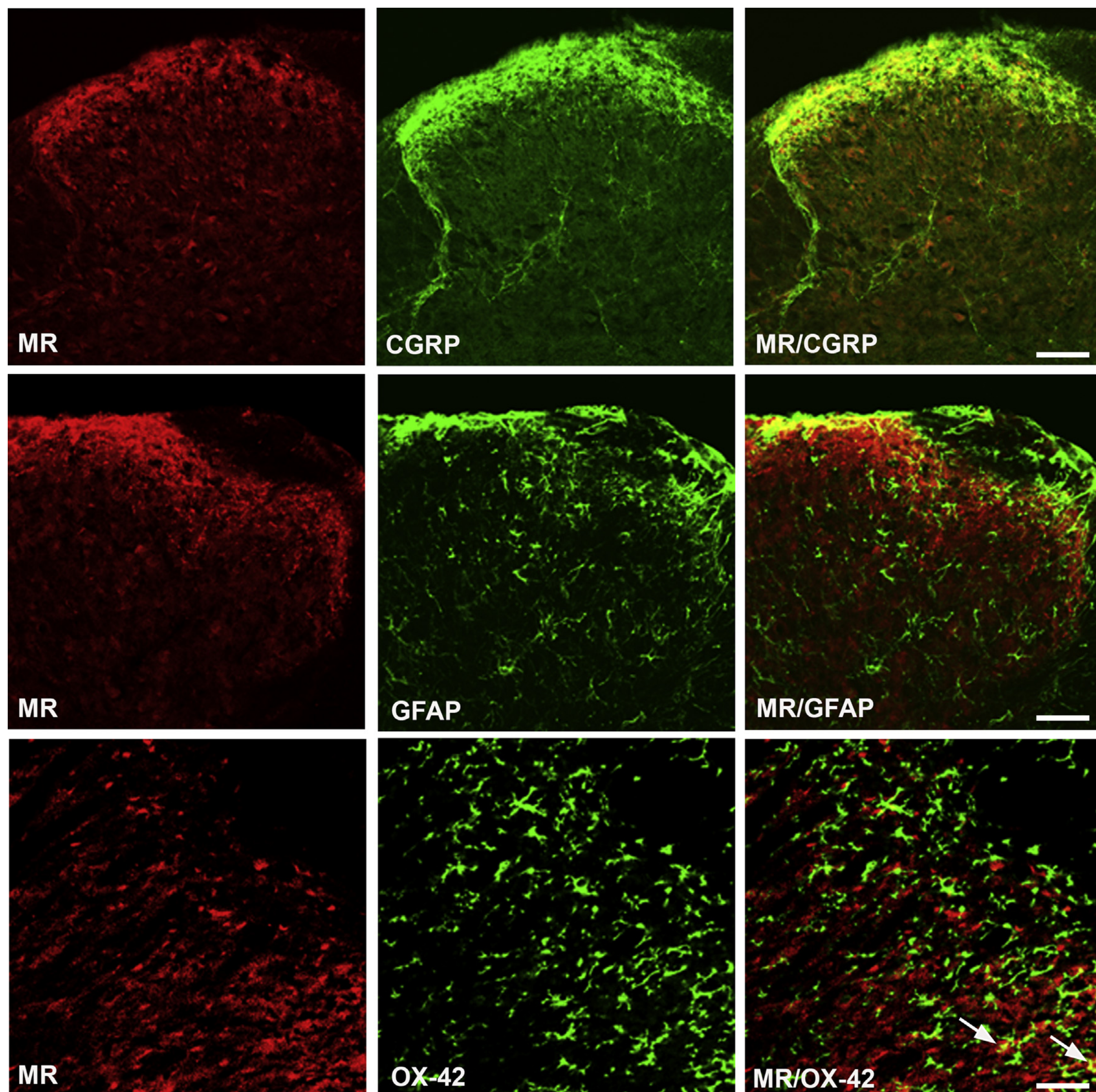

Fig. 2. Confocal microscopy of double immunofluorescence of MR (*red fluorescence*) with CGRP, GFAP or OX-42 (*all green fluorescence*) in the dorsal horn of the spinal cord. **Upper row**: almost complete colocalization of MR- (red) with CGRP- (green) immunoreactivity in Rexed laminae I and II with few, only MR-immunorecative cells in Rexed laminae III and IV (arrow). **Middle row**: lack of colocalization of MR-immunoreactivity (red) with the astrocyte marker GFAP (green) throughout the spinal cord. **Lower row**: almost no colocalization of MR-immunoreactivity (red) with the microglia marker OX-42 (green) except for very few scattered cells with MR- and OX-42 costaining (yellow, arrow). Bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

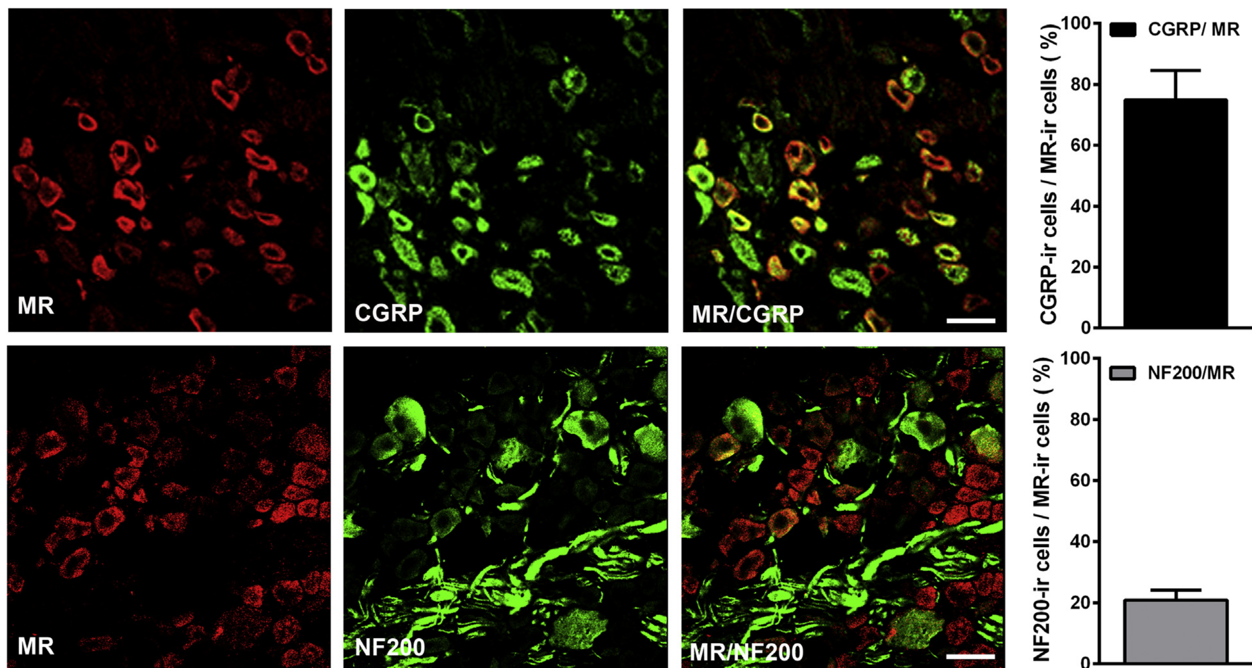

Fig. 3. Confocal microscopy of MR (*red fluorescence*) and CGRP or NF200 (*green fluorescence*) double immunofluorescence in DRG of naïve rats. Upper row: coexistence (double arrow) of MR (red) with CGRP (green) with few cells containing only MR (arrow) or CGRP (arrowhead). Quantitative analysis showed that the number of DRG neurons coexpressing MR with CGRP was 74.9 ± 9.7%. **Lower row**: coexistence (double arrow) of MR (red) with NF200 (green) in a small population of cells with the majority containing only MR or NF200. Quantitative analysis showed that the number of DRG neurons colocalizing MR with NF200 was 20.8 ± 3.2%. Data are shown as means ± SEM. Bar = 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

numerous free nerve endings from the subepidermal into the epidermal layer (Fig. 5, lower panels). Importantly, the MR were not identified over the entire course of the peripheral nerve fibres in accompanying Schwann cells.

3.5. Functional role of spinal cord and DRG mineralocorticoid receptors in nociceptive behavior

To assess the functional relevance of the presence of the MR exclusively on nociceptive fibres we performed von Frey filament testing following the local i.pl. or i.th. administration of increasing doses of the MR selective agonist aldosterone. The results show a significant dose-dependent decrease in mechanical thresholds of up to almost 50% following either the i.pl. or i.th. application of aldosterone (Fig. 6A, B). Co-administration of aldosterone with the MR selective antagonist canreoante K significantly reversed the decrease in mechanical thresholds following both treatments (Fig. 6C, D). Whereas nociceptive effects of i.th. aldosterone administration were observed on both sides, nociceptive effects of i.pl. aldosterone were seen only ipsi- but not contralateral to the injection side indicating a local peripheral effect (data not shown).

3.6. Evidence for membrane bound MR

Following the separation of membrane fractions of DRG neurons and spinal cord by ultracentrifugation MR specific binding sites were identified by use of the MR specific ligand [^{3}H]aldosterone. Increasing concentrations of [^{3}H]aldosterone in the presence of 10 μM unlabeled aldosterone led to a specific saturation curve in membranes of DRG (Fig. 7A) and spinal cord neurons (Fig. 7B) with B_{max} values of 35.9 ± 5.5 fmol/mg protein and 51.8 ± 6.2 fmol/mg protein and K_d values of 24.3 ± 7.3 nM and 11.6 ± 3.4 nM, respectively. In line with these findings and with the antibody specifications (Table 1), MR-immunoreactivity in DRG neurons of naïve rats was not identified in the nuclear but in the cytosol/plasma membrane compartment (Fig. 7C).

4. Discussion

This study systematically examined the exact anatomical localization of MR within the spinal cord and peripheral nervous system of naïve rats. In contrast to previous reports that used different models of persistent pain (Dong et al., 2012; Sun et al., 2012), our results demonstrate that MR are expressed mainly in nociceptive neurons and very rarely in glia cells. In the spinal cord, MR colocalize predominantly with incoming presynaptic CGRP-IR nerve terminals in Rexed laminae I and II. Only few scattered neuronal cell bodies in Rexed laminae III and IV were also positive. In addition, in peripheral nerve fibers of the sciatic nerve and peripheral nerve endings of the skin MR were restricted predominantly to nociceptive neurons such as unmyelinated C-fibers and Aδ-fibers and colocalized only marginally with myelinated mechanoreceptive or proprioceptive neurons (Matsumoto et al., 2012). In Schwann cells, satellite cells, and astrocytes MR could not be identified under normal conditions, though a scant few were found in microglia of the spinal cord. The activation of either spinal or peripheral MR on these nociceptive neurons by local agonist application resulted in a dose-dependent reduction in mechanical thresholds of naïve rats. The use of an MR antagonist demonstrated receptor specificity. These rapid effects in nociceptive behavior together with the presence of neuronal MR specific membrane binding sites in DRG and spinal cord suggest a potential rapid non-genomic mechanism of nociceptive modulation through MR on the plasma membrane of nociceptive neurons.

In recent publications that used different models of persistent pain, MR have been reported to exist in all peripheral neurons (Dong et al., 2012) as well as in glia cells (Sun et al., 2012). However, peripheral neurons were not further distinguished into

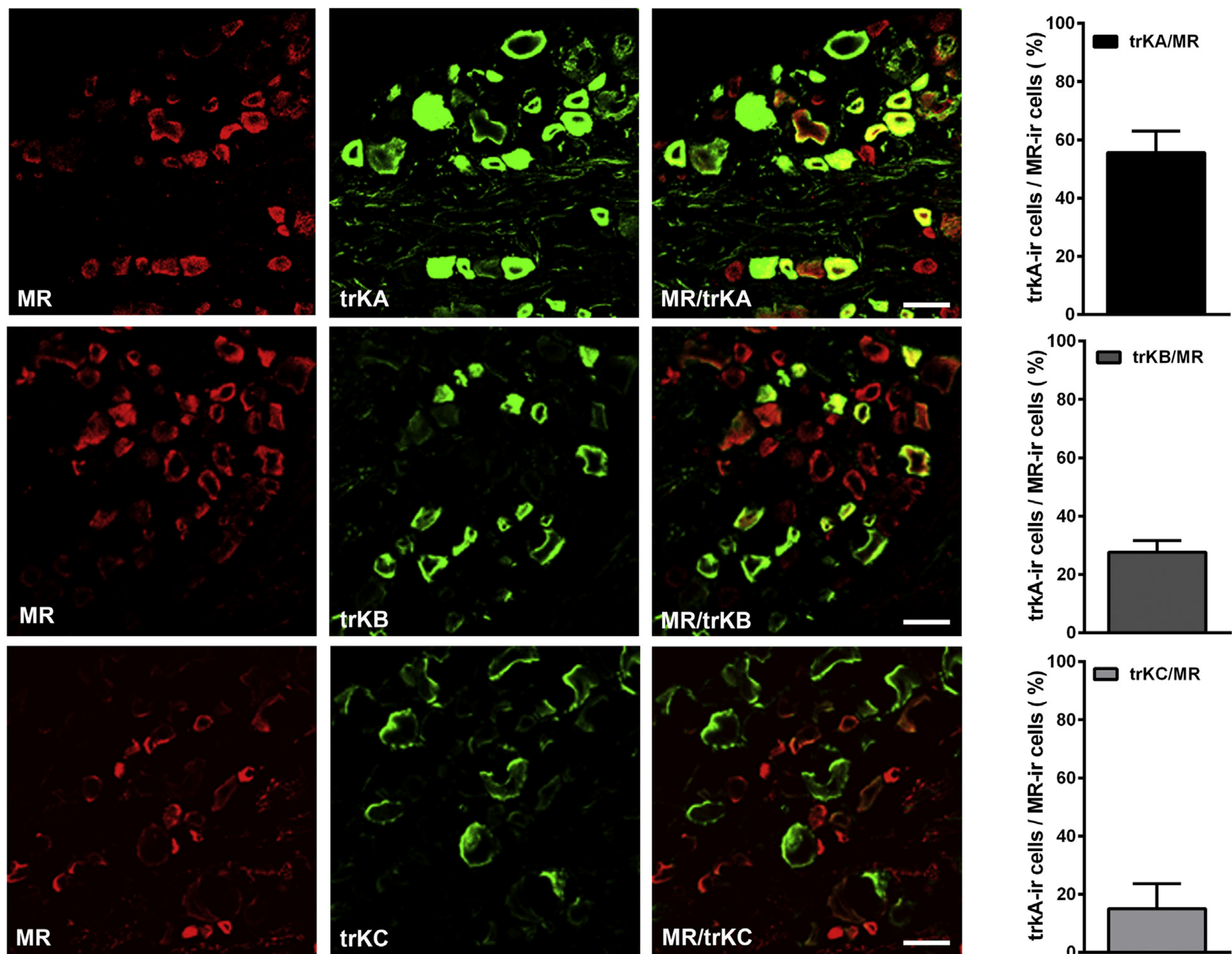

Fig. 4. Confocal microscopy of MR (*red fluorescence*) and trkA, trkB or trkC (*all green fluorescence*) double immunofluorescence in DRG of naïve rats. Note that the population of DRG neurons coexpressing MR with trkA (55.6 ± 7.3%) was much higher than that coexpressing trkB (27.7 ± 4.0%) or trkC (15.1± 8.6%). All data are shown as means ± SEM. Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subpopulations of peripheral neurons and there is no direct proof of MR in glia cells. Evidence for MR on neurons has been known for a long time: earlier binding studies already suggested two different corticoid receptors within the central nervous system (McEwen et al., 1986) and found evidence for type I (i.e. mineralocorticoid receptor) and type II corticosteroid receptor gene expression (Reul et al., 1989). Soon thereafter, type I corticoid receptor (MR)-like immunoreactivity was first reported in the spinal cord (Ahima et al., 1991). However, no data are available on the exact anatomical localization of MR with regards to specific subpopulations of nerve fibers that innervate the periphery. In using a new highly specific polyclonal rabbit antibody against the rat MR that does not cross-react with the glucocorticoid receptor (Ito et al., 2000) we were able to demonstrate MR immunoreactivity in neuronal cell bodies of dorsal root ganglia as well as their peripheral (within skin) and central (within spinal cord) nerve terminals. More importantly, MR immunoreactive neurons strongly colocalized with CGRP, a marker for nociceptive C- and Aδ-nerve fibers. Consistently, the majority of MR immunoreactive neurons also colocalized with trkA (Matsumoto et al., 2012). In longitudinal sections of the innervating sciatic nerve and in sections of arborizing nerve endings at the dermal-epidermal junction of the skin the majority of MR immunoreactive axons colocalized with CGRP which clearly indicates a pivotal role these receptors play in the modulation of nociception.

Indeed in our own behavioral experiments with naïve animals, we could demonstrate for the first time an immediate and almost 50% decrease in mechanical thresholds, i.e. increased sensitivity to mechanical stimuli, ipsi- but not contralateral to the i.pl. administration of aldosterone. This peripheral effect occurred within 10 min, persisted for 40 min and was receptor specific as a simultaneous local injection of a MR selective antagonist reversed this effect. The same effect was observed at central nerve terminals of the peripheral sensory neurons when aldosterone with or without its respective antagonist was given intrathecally. Consistent with such a pronociceptive effect, previous studies have shown that the MR selective agonist aldosterone dose-dependently increased the number of action potentials evoked by suprathreshold current injection in acutely dissociated DRG neurons of naïve rats (Ye et al., 2014; Dong et al., 2012). Interestingly, other studies have described antinociceptive effects of a MR antagonist under different pathological conditions such as low back pain (Ye et al., 2014; Dong et al., 2012; Sun et al., 2012; Gu et al., 2011) and diabetic neuropathy (Dong and He, 2013). Thus in line with these findings of antinociceptive effects by a MR antagonist, we demonstrate that the application of a MR selective agonist induces nociception under non-pathological conditions.

In addition to neuronal MR, it is discussed whether MR on glia cells modulate nociception (Dong et al., 2012). However, whereas MR immunoreactive glia cells in the hippocampus (Hwang et al.,

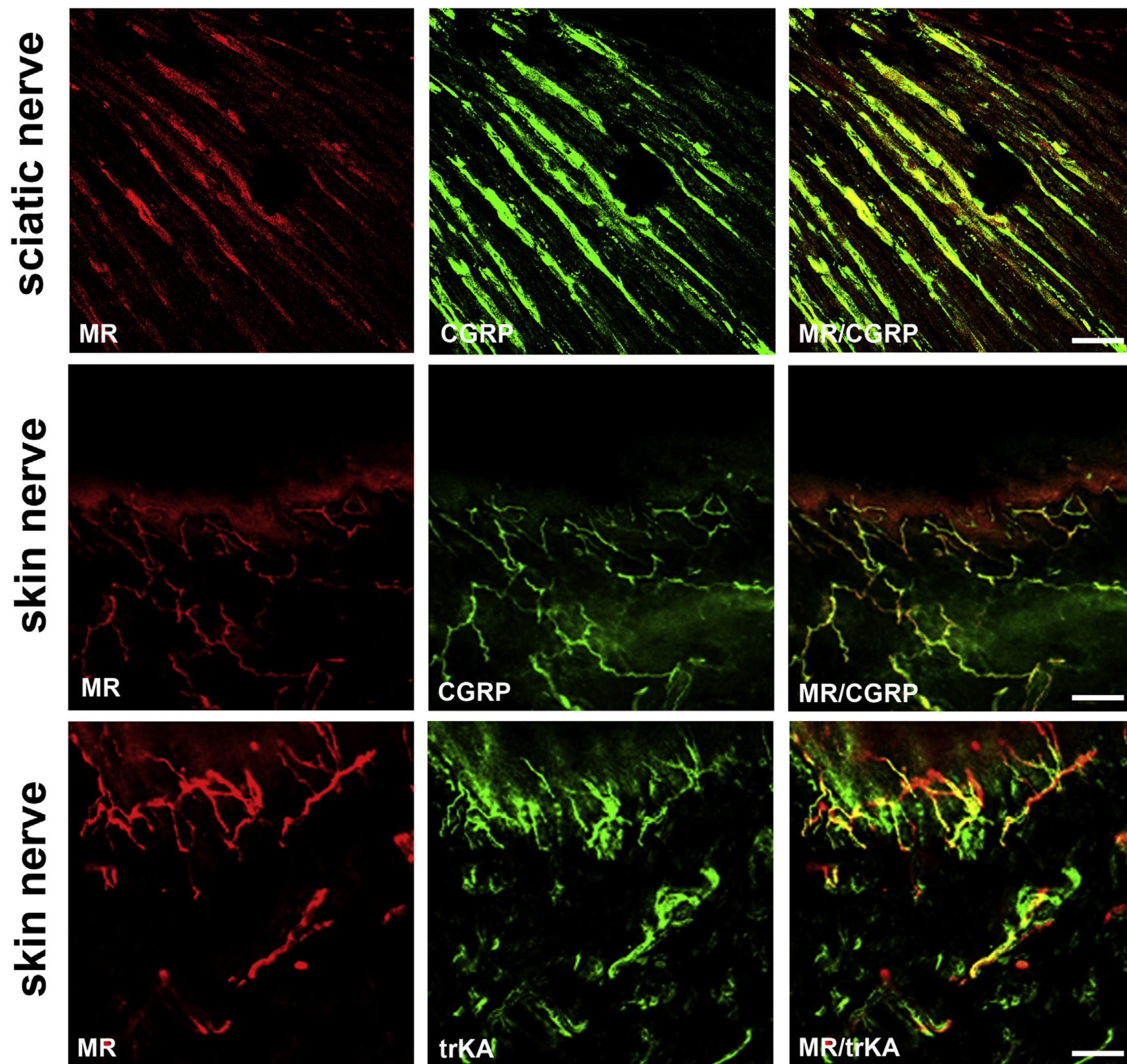

Fig. 5. Confocal microscopy of MR (*red fluorescence*) with CGRP or trkA (*green fluorescence*) double immunofluorescence in sciatic nerve and subcutaneous tissue. Note that the majority of MR immunoreactive nerve fibers coexpress CGRP or trkA in sciatic nerve (upper row) or subcutaneous tissue (middle and lower row). Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2006; Garcia et al., 2004; Bohn et al., 1991) and retina (Golestaneh et al., 2001) have been convincingly demonstrated, there is no direct immunohistochemical evidence of MR in glia cells of the spinal cord or peripheral nerve. Several studies reported the presence of MR in cell cultures of microglia (Girard et al., 2010; Sierra et al., 2008; Tanaka et al., 1997), but the evidence was mainly gained indirectly through MR-mediated effects. Here in naïve rats we could identify a remote colocalization of MR with the microglial marker OX-42 (Fig. 2) and found no presence of MR in astrocytes, satellite cells or Schwann cells (Fig. 2). This might be different under pathological conditions such as nerve injury when expression of MR in glia cells might be up-regulated (Dong et al., 2012), yet direct evidence is still missing.

The observation in our study that acute nociceptive effects occur within a few minutes after local MR agonist application strongly questions a genomic provenance, particularly since MR at the peripheral nerve terminal need to travel retrogradely the entire length of the sciatic nerve in order to reach the cell nucleus and alter gene expression. Instead, recent evidence repeatedly shows that neuronal MR agonists may also elicit their effects through non-genomic ways (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012). These non-genomic pathways can be elicited either by directly interfering with intracellular signaling pathways or by interfering with membrane bound structures such as ion channels and G-protein coupled receptors (Chatterjee and Sikdar, 2014; Groeneweg et al., 2012). Indeed, we further substantiated the evidence for a putative non-genomic pathway in our binding experiments. These demonstrated saturation binding with increasing concentrations of the radiolabeled ligand [^{3}H]aldosterone in the pure membrane fraction of DRG neurons indicating MR specific binding sites. Consistently, our immunofluorescence confocal microscopy showed that MR were not identified in the nuclear but in the cytosol/plasma membrane compartment of DRG neurons. Moreover, we identified MR binding sites in the spinal dorsal horn which might reflect both MR receptors on central endings of primary afferent nociceptors and MR receptors on central neurons. In agreement with our findings, convincing evidence for membrane bound MR is also provided by electron microscopy studies in presynaptic terminals and postsynaptic densities of synaptic areas of the brain (Prager et al., 2010; Johnson et al., 2005). In contrast to genomic MR, membrane bound MR elicit their effects within seconds-to-minutes and have been shown to increase synaptic and

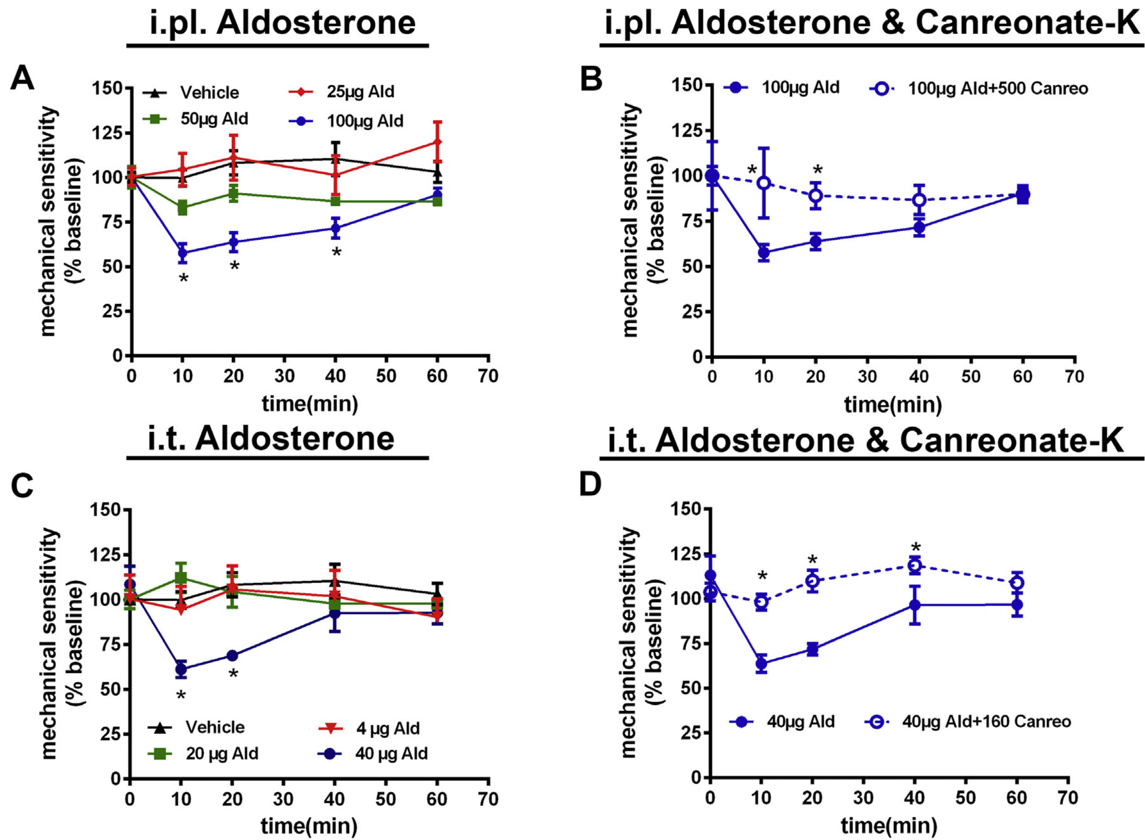

Fig. 6. Von Frey filament testing of mechanical sensitivity following the local i.pl. or i.t. application of aldosterone (A, C) with and without the MR antagonist canreonate-K (B, D). A,B: Local intraplantar (**A**) or i.t. (**C**) injection of increasing doses of the MR agonist aldosterone resulted in the immediate fall of mechanical withdrawal thresholds in hindpaws of naïve rats (*$P < 0.5$, one-way ANOVA, post-hoc Dunnett's test, $n = 6$*). Peak effects occurred at 10 min and by 60 min the mechanical withdrawal thresholds returned to preinjection baseline values. **B, D**: Intraplantar or i.t. injection of aldosterone in combination with the MR antagonist canreonate-K fully reversed MR agonist-induced mechanical hypersensitivity (*$P < 0.5$, two-way ANOVA, post-hoc Tukey test, $n = 6$*). Data are expressed as means ± SEM.

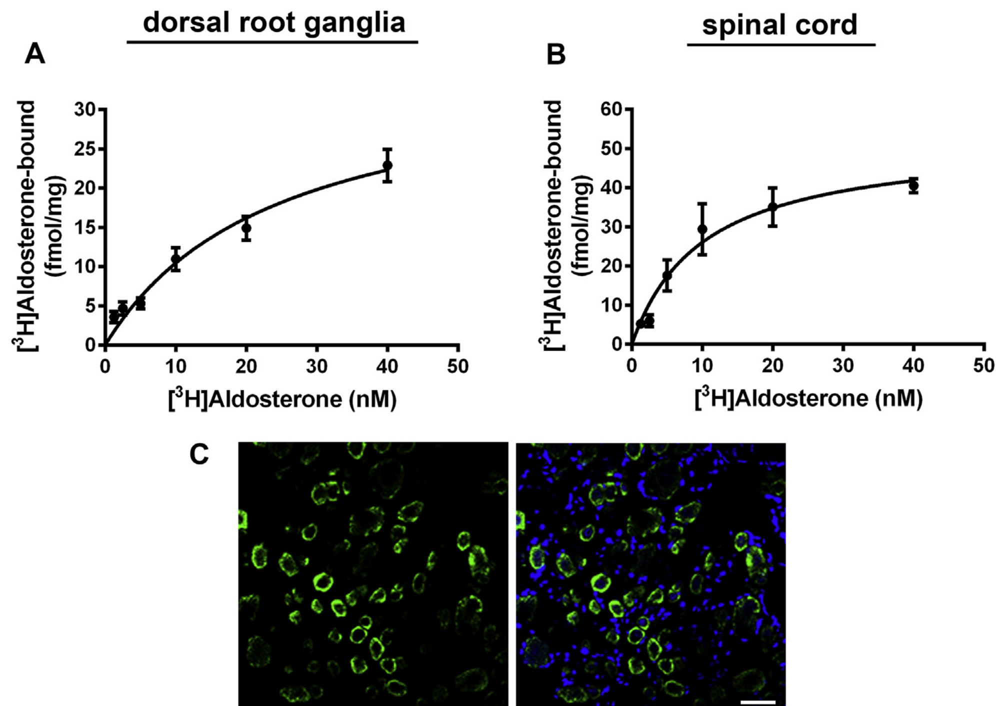

Fig. 7. Saturation binding analysis of MR specific binding sites in cell membranes of DRG neurons (A) and the dorsal horn of the spinal cord (B) with the MR selective radiolabeled ligand [^{3}H]aldosterone. In naive rats, increasing concentrations of [^{3}H] aldosterone (0.25–40 nM) in the presence of excess of cold aldosterone (10 µM, to subtract nonspecific binding) resulted in saturation binding in DRG (**A**) and spinal cord (**B**) neurons with Bmax values of 35.9 ± 5.5 fmol/mg protein and 51.8 ± 6.2 fmol/mg protein and Kd values of 24.3 ± 7.3 nM and 11.6 ± 3.4 nM nM, respectively. Data are expressed as means ± SEM. **C**) Confocal immunofluorescence images show that MR *green fluorescence* labeling was not identified in the nuclear but in the cytosol/plasma membrane compartment. DAPI *blue fluorescence* labeling represents nuclear staining (Bar = 40 µm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neuronal excitability (Prager and Johnson, 2009). In line with this, Dong et al. have shown in dorsal root ganglion neurons of naïve rats that the MR agonist aldosterone dose-dependently increased the number of action potentials evoked by suprathreshold current injection (Dong et al., 2012).

5. Conclusions

Taken together, MR predominantly colocalized with peripheral nociceptive neurons and only marginally with myelinated mechanoreceptive and proprioreceptive neurons underscoring their pivotal role in the modulation of nociception. MR were not detected in most of the glia cells – only scarcely in spinal microglia – indicating no relevant functional role of glia-derived MR at least in naïve rats. However, this might change under pathological conditions that are known to elicit glia activation, e.g. neuropathic pain (Ji et al., 2013). For the first time we can show immediate effects upon activation of MR on peripheral nociceptive neurons that are most likely due to non-genomic mechanisms. This is further substantiated by the identification of membrane bound MR specific binding sites in neurons of dorsal root ganglia and spinal cord. Therefore, the crucial role of MR on nociceptive neurons and their impact on nociceptive behavior most likely due to immediate non-genomic effects has to be considered under normal but more so under pathological conditions in future studies.

Competing interests statement

The authors declare no competing financial interest.

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Publication 3: Shaaban A. Mousa, Mohammed Shaqura, Jens Winkler, Baled. I. Khalefa, Mohammed A. Al-Madol, Mehdi Shakibaei, Stefan Schulz, Michael Schäfer. Protein kinase C-mediated mu-opioid receptor phosphorylation and desensitization in rats, and its prevention during early diabetes. Pain, Volume 157(4), April 2016, p 910–921 2016.

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Curriculum vitae

"My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection"

Complete list of selected publications

Publication 1: Mohammed A. Al-Madol, Mohammed Shaqura, Thilo John, Rudolf Likar, Reham Said Ebied, Michael Schäfer, Shaaban A. Mousa. Comparative expression analyses of pro- versus anti-inflammatory mediators within synovium of patients with joint trauma, osteoarthritis and rheumatoid arthritis. Mediators of Inflammation, 2016.

Publication 2: Shaaban A. Mousa, Mohammed Shaqura, Jens Winkler, Baled. I. Khalefa, Mohammed A. Al-Madol, Mehdi Shakibaei, Stefan Schulz, Michael Schäfer. Protein kinase C-mediated mu-opioid receptor phosphorylation and desensitization in rats, and its prevention during early diabetes. Pain, 2016.

Publication3: Mohammed Shaqura, Xiongjuan Li, Mohammed A. Al-Madol, Sascha Tafelski , Antje Beyer-Koczorek , Shaaban A. Mousa , Michael Schäfer. Acute mechanical sensitization of peripheral nociceptors by aldosterone through non-genomic activation of membrane bound mineralocorticoid receptors in naive rats. Neuropharmacology, 2016.

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