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DISSERTATION

Discovery of new regulatory mechanisms of sensory neuron nociception in animal models of inflammatory pain [Erschließung neuer regulatorischer Mechanismen der durch sensorische Neurone-vermittelten Nozizeption in Tiermodellen des Entzündungsschmerzes]

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List of abbreviations

Abbreviation	Meaning
Aldo	Aldosterone
AngI	Angiotensin I
AngII	Angiotensin II
ACE	Angiotensin-converting enzymes
ANOVA	Analysis of variance
Can	Canrenoate-K
CGRP	Calcitonin gene-related peptide (a neuronal marker for peptidergic nociceptive neurons)
CYP11B2	Cytochrome P450 11B2 (18-hydroxylase, aldosterone synthase)
CA3	A subfield of the cornu ammonis in the hippocampus proper
Ctrl	Control group
DRG	Dorsal root ganglion
DAPI	4',6-diamidino-2-phenylindole (a blue-fluorescent DNA stain)
FCA	Freund’s complete adjuvant
FAD286	Fadrozole (aldosterone synthase inhibitor)
FAD	FAD286
FITC	Fluorescein isothiocyanate (a green-fluorescent probe)
i.th	Intrathecal
i.pl	Intraplantar
MR	Mineralocorticoid receptors
NaV1.8 (SNS/PN3)	A tetrodotoxin-resistant voltage-gated sodium channels α subunit
NF200	Neurofilament 200 (a specific marker of myelinated A-type neurons in DRG)
NGF	Nerve growth factor
OA	Osteoarthritis
OCT	Optimal cutting temperature
PPT	Paw pressure thresholds
PBS	Phosphate-buffer saline
SD	Standard deviation
TRPV1	Transient receptor potential vanilloid 1

TrkA	Tyrosine kinase A (a marker for the NGF-dependent peripheral nociceptive neurons)
Texas red	Red fluorescent dye
-ir	-immunoreactive

Abstract

Abstract (English)

In Freund's complete adjuvant (FCA)-induced hindpaw inflammation of Wistar rats, the increased expression of aldosterone synthase, aldosterone and mineralocorticoid receptors (MR) in dorsal root ganglia (DRG) contributes to mechanical sensitivity. The project of my thesis examined in this animal model whether mechanical hypersensitivity was persistent, was due to genomic up-regulation of the expression of certain pain signaling molecules in DRG, and was prevented by the inhibition of aldosterone synthesis in DRG. Nociceptive testing in behavioral experiments showed that intrathecal application of the MR antagonist canrenoate-K in rats with hindpaw inflammation attenuated the inflammation-associated mechanical hyperalgesia, whereas intrathecal injection of the MR agonist aldosterone in normal rats elicited mechanical hyperalgesia. Immunofluorescence microscopy revealed co-localization of MR with the pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA. Both intrathecal treatment with the MR antagonist canrenoate-K and the aldosterone synthase inhibitor FAD286 significantly prevented the hindpaw inflammation-induced up-regulation of these pain signaling molecules, suggesting that this up-regulation is dependent on endogenous aldosterone. Both treatments also resulted in the attenuation of the inflammation-induced mechanical hypersensitivity. A presumed genomic effect was supported by the observation that in DRGs innervating the inflamed painful hindpaw the majority of MR were translocated from the cytosol to the nucleus. In conclusion, local neuronal aldosterone which is regulated by aldosterone synthase within DRG neurons contributes to the up-regulation of the pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA via activation of MR in DRG neurons, and strengthens rats' peripheral inflammatory pain response to mechanical stimuli.

Abstrakt (Deutsch)

Bei Vorliegen einer Freunds complete Adjuvans (FCA)-induzierten Entzündung der Hinterpfote von Wistar-Ratten zeigte sich in früheren Arbeiten, dass die erhöhte Expression von Aldosteronsynthase-, Aldosteron- und Mineralocorticoidrezeptoren (MR) in Spinalganglien zur mechanischen Schmerzempfindlichkeit (Hyperalgesie) beiträgt. Das Projekt meiner Dissertation untersuchte in dem Tiermodell der FCA-induzierten Hinterpfotenentzündung, ob die anhaltende mechanische Hyperalgesie auf einer genomischen Hochregulierung der Expression bestimmter Schmerzsignalmoleküle in Spinalganglien beruht, und ob diese durch Hemmung der Aldosteronsynthese im Spinalganglion verhindert werden kann. Die nozizeptiven Tests in Verhaltensexperimenten zeigten, dass die intrathekale Anwendung des MR-Antagonisten Canrenoate-K bei Ratten mit Entzündung einer Hinterpfote die entzündungsassoziierte mechanische Hyperalgesie abschwächte, während die intrathekale Injektion des MR-Agonisten Aldosteron bei gesunden Ratten eine mechanische Hyperalgesie hervorrief. Immunfluoreszenzmikroskopie ergab eine Kolokalisation von MR mit den Schmerzsignalmolekülen TRPV1, CGRP, Nav1.8 und TrkA. Sowohl die intrathekale Behandlung mit dem MR-Antagonisten Canrenoate-K als auch mit dem Aldosteronsynthase-Inhibitor FAD286 reduzierte signifikant die Entzündungs-induzierte Hochregulation dieser Schmerzsignalmoleküle, was darauf hindeutet, dass ihre Expression von endogenem Aldosteron in DRG-Neuronen abhängt. Dies führte auch zur Abschwächung der Entzündungs-bedingten mechanischen Überempfindlichkeit (Hyperalgesie). Ein vermutlicher genomischer Effekt wurde durch die Beobachtung gestützt, dass in Spinalganglien, welche die entzündete schmerzhafte Hinterpfote innervieren, der Großteil der MR in den Zellkern transloziert wurde. Zusammenfassend lässt sich sagen, dass lokales neuronales Aldosteron, welches durch Aldosteronsynthase in Spinalganglien-Neuronen reguliert wird, über die Aktivierung von MR in Spinalganglien zur Hochregulierung der Schmerzsignalmoleküle TRPV1, CGRP, Nav1.8 und TrkA beiträgt und die periphere entzündliche Schmerzreaktion von Ratten auf mechanische Reize verstärkt.

Synopsis

1. Introduction

1.1. Aldosterone synthase, aldosterone and mineralocorticoid receptors (MR) in dorsal root ganglia

Aldosterone is a mineralocorticoid which is mainly synthesized in the adrenal cortex to control blood pressure and sodium-potassium levels in the body under normal conditions. In contrast, enhanced production of aldosterone outside the adrenal glands causes deleterious effects during heart disease (Reynoso-Palomar et al. 2017; Young and Rickard 2015). It is well known that the last step of aldosterone synthesis is mediated through the enzyme aldosterone synthase expressed within the zona glomerulosa of the adrenal cortex (Bassett et al. 2004). When renal perfusion pressure decreases, renin from the juxtaglomerular cells of the kidney is released into the circulation and transforms angiotensinogen produced by the liver into angiotensin I (AngI), followed by the hydrolyzation of AngI to angiotensin II (AngII) through the angiotensin-converting enzyme (ACE) located in the endothelium of the lungs and kidneys. In the adrenal glomerulosa cells, aldosterone is synthesized through the enzyme aldosterone synthase (CYP11B2) within mitochondria (Bollag 2014; Chow et al. 2018). Aldosterone is the major mineralocorticoid in human and rats, retaining sodium and water, while excreting potassium by reacting with MR in the kidney (MacKenzie et al. 2000).

However, a growing body of recent evidence has shown that other tissues such as the vascular system, heart and brain are able to synthesize aldosterone (Gomez-Sanchez et al. 2010; Reynoso-Palomar et al. 2017; Zhu et al. 2014). Within the central nervous system, the transcription of aldosterone synthase (CYP11B2) was determined in RNA extracts of the whole brain and hypothalamus of adult female Wistar–Kyoto rats (MacKenzie et al. 2000). Moreover, (Gomez-Sanchez et al. 2010) Gomez-Sanchez et al (2010) revealed that aldosterone synthase mRNA expression as well as aldosterone content were higher in the brains and lower in the adrenal glands of Dahl salt-sensitive rats compared to Sprague-Dawley rats. Immunohistochemical staining has also demonstrated aldosterone synthase in the cerebellar cortex of rats, with greatest intensity in the Purkinje cells of the CA3 region of the hippocampus within the brain (MacKenzie et al. 2000). More recently, mRNA and protein of aldosterone synthase (CYP11B2) were identified within dorsal root ganglia (DRG) of the rat (Mohamed et al. 2020). In addition, immunohistochemical studies have shown that CYP11B2-

immunoreactive DRG neurons co-localize with the end product aldosterone as well as with the corresponding receptor MR (Mohamed et al. 2020). The functional relevance of this has been shown in acutely dissociated L4-5 DRG neurons whose excitability was significantly enhanced by the MR agonist aldosterone (Dong et al. 2012). Consistently, preventing the access of endogenous aldosterone to activate its corresponding MR in DRG resulted in an acute, non-genomic reduction of mechanical sensitivity (Li et al. 2018).
Using naive rats, MR mRNA was detected in many tissues such as hippocampus, kidney, heart and cerebellum by Northern Blot Analysis (Reul et al. 1989). In renal collecting duct cells, the binding of cytoplasmic MR and endogenous aldosterone ultimately forms MR dimers, which subsequently translocates to the nucleus, modulates the transcription of genes, such as kinases and ion channels, and has an impact on the balance of water and electrolyte (Te Riet et al. 2015). Immunofluorescent examination found that the immunoreactivity of MR was mainly located in the cytoplasm of neuronal cells of normal rats (Dong et al. 2012). In another group of rats with zymosan-induced DRG inflammation, the MR antagonist eplerenone resulted in long-lasting anti-nociceptive effects (Dong et al. 2012). Indeed, MR co-localized up to 75% with CGRP and up to 60% with TrkA, both markers for peripheral nociceptive neurons, and only up to 19% with NF200, a neuronal marker for myelinated sensory neurons (Shaqura et al. 2016b). These co-localizations suggest that the expression of MR is primarily in nociceptive neurons of DRG. This conclusion has been further supported by behavioral experiments. Apart from these non-genomic effects of MR activation, intrathecal (i.th.) or intraplantar (i.pl.) injection of the MR selective antagonist canrenoate-K significantly reversed the mechanical hypersensitivity as a result of the i.th. or i.pl. administration of the MR selective agonist aldosterone to naïve rats (Shaqura et al. 2016b), implying that the MR contributes to the regulation of nociception. Therefore, my project examined whether neuronal MR and its endogenous ligand aldosterone plays a crucial role in nociception during FCA-induced hindpaw inflammation in rats (Shaqura et al. 2020).

1.2. Specific pain signaling molecules in dorsal root ganglia

Certain pain signaling molecules such as receptors, ion channels, and neuropeptides have been shown to be expressed in peripheral sensory DRG neurons and are known to play a crucial role in nociceptive behavior of animals and humans under normal and various type of pathological conditions. First of all, the transient receptor potential

vanilloid 1 (TRPV1) is a non-selective cation channel expressed by peripheral sensory neurons that is elaborated in pain modulation (Bevan et al. 2014). Indeed, TRPV1 on peripheral DRG neurons is up-regulated during inflammatory condition and consequently contributes to pain sensation (Ji et al. 2002). Second, this also applies to the neuropeptide calcitonin gene-related peptide (CGRP), which is mainly distributed in C- and Aδ- sensory neurons (Russell et al. 2014) and widely accepted as the neuronal marker for peptidergic nociceptive neurons (Mohamed et al. 2020). CGRP is also up-regulated during inflammatory pain (Staton et al. 2007). Consistent with this, a previous study showed that local injection of CGRP to the knee enhanced mechanosensitivity of afferent nerve endings. Increased mechanosensitivity and pain in osteoarthritic (OA) knees can be reduced by peripherally acting CGRP receptor antagonists (Walsh et al. 2015). Moreover, peripherally acting CGRP receptor antagonists reduced the enhanced mechanosensitivity and pain behavior under inflammatory condition reviewed by Walsh et al. (Walsh et al. 2015). Third, the tetrodotoxin-resistant voltage-gated sodium channel NaV1.8 is abundantly expressed in peripheral nociceptive neurons (Benn et al. 2001) and is up-regulated during FCA-hindpaw inflammation (Liang et al. 2013), suggesting that these signaling molecules play a part in chronic inflammatory pain. Moreover, Belkouch et al. reported that NaV1.8 plays a crucial role in Aβ-fibers' excitability and consequently participates in mechanical allodynia under persistent inflammation (Belkouch et al. 2014). Finally, tyrosine kinase A receptor (TrkA), also known as nerve growth factor (NGF) receptor, is a marker for the NGF-dependent peripheral nociceptive neurons that are known to contribute to enhanced nociceptive sensitivity during inflammation (Kiris et al. 2014). Moreover, selective inhibition of TrkA via the specific inhibitor AR786 reduced local inflammation-induced pain behavior in rats (Ashraf et al. 2016). Since these pain signaling molecules play such a crucial role during inflammatory pain, my project investigated whether TRPV1, CGRP, Nav1.8, and TrkA-immunoreactive (-ir) DRG neurons co-localize with MR and whether enhanced aldosterone synthesis and MR activation within DRG neurons might lead to an alteration of their genomic expression and subsequent nociceptive behavior during inflammation.

1.3 Main goals of the study

The main goal of my thesis was to elucidate the role of endogenously synthesized aldosterone in sensory DRG neurons in a rat model of painful FCA-induced hindpaw

inflammation. In behavioral experiments, I examined whether repeated intrathecal injections of the MR antagonist canrenoate-K reduced nociceptive behavior in FCA-treated rats, whereas repeated intrathecal application of the MR agonist aldosterone might *in*duce nociceptive behavior. Using immunohistochemistry, I investigated whether MR co-localized with relevant pain signaling molecules such as TRPV1, CGRP, Nav1.8, and TrkA in the DRG of rats. To assess possible genomic effects of endogenous aldosterone during FCA-inflammation, alterations in the expression of these pain signaling molecules were measured following either antagonism of MR in sensory DRG or inhibition of neuronal aldosterone synthesis. Finally, the impact of these presumed genomic effects on the nociceptive behavior of rats with painful FCA-hindpaw inflammation was assessed.

2. Materials and Methods

2.1 Drugs

A water-in-oil emulsion of killed mycobacteria, i.e. Freund's complete adjuvant (FCA) (Calbiochem, San Diego, CA), was used for this animal model. Anaesthesia was performed with the animal inhaling isoflurane (Abbott, Wiesbaden, Germany) over a nose cone. The water-soluble MR selective antagonist canrenoate-K and the aldosterone synthase inhibitor FAD286 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in 0.9% normal saline. Aldosterone (Novus Biologicals, CO, USA) was dissolved in a vehicle which consisted of 1% dimethyl sulfoxide, 10% ethanol, and 0.9% NaCl (Sigma-Aldrich). These drugs were injected daily into the intrathecal (i.th.) space during brief isoflurane anaesthesia or continuously infused via an i.th. catheter controlled by an osmotic minipump (2000µl, rate 5µl/h) (Alzet Corporation, Cupertino, CA) over 4 days.

2.2 Animals

Experiments were performed on male Wistar rats weighing 180-250 g (breeding facility Charité-Universitätsmedizin Berlin, Germany). Animals were held on a 12 hours' light-dark cycle at 23°C and 75% humidity with standard laboratory rat chow and water ad libitum. All interventions were performed under the inhalation of isoflurane (1.0-2.5 vol%). This research was agreed by the local animal care committee (G0024/14) and followed the European Directive (2010/63/EU) introducing new animal welfare and care guidelines.

2.3 Animal model of FCA-induced hindpaw inflammation

After the Wistar rats were anaesthetized with inhaled isoflurane (1.0-2.5 vol%), each right hindpaw was intraplantarly injected (i.pl.) with 0.15 ml FCA (Mohamed et al. 2020). As a consequence, a sustained local inflammation was generated in the treated paws, accompanied by increased paw size, elevated paw temperature, hyperalgesia and accumulation of various types of immunocytes (Rittner et al. 2001). All experiments were implemented on day 4 after FCA inoculation of the hindpaws.

2.4 Intrathecal injection and intrathecal catheterization

To anaesthetize rats, isoflurane mixed with oxygen was filled in a nose cone which covered the rats' snouts. In the lumbar region, skin above the spinous processes of the L3–L5 vertebrae was cut longitudinally and the respective vertebrae identified (Shaqura et al. 2013). Then, the needle for i.th. injection was slowly advanced into the i.th. space between the L4 and L5 vertebra at a 30° angle. Animals received either repeatedly a single injection or a persistent infusion of drugs through an implanted i.th. catheter (PE 10 tubing attached to PE 60 tubing for attachment to an osmotic pump: Portex Ltd, Hythe, Kent, United Kingdom). Successful penetration into the i.th. space was confirmed by an instantaneous involuntary flicking of the rat's tail or an involuntary movement of the hind limbs.

2.5 Experiment protocols

This study comprised four sets of experiments. In the first set, mechanical hyperalgesia was evaluated by paw pressure thresholds (PPT) before and after intraplantar injection of FCA into the right hindpaws of rats. Rats were repeatedly given i.th. administration of 100μg/20μl of the MR antagonist canrenoate-K or its vehicle (0.9% NaCl) over 4 days. In naive animals, i.th. administration of 40μg/20μl of the aldosterone or its vehicle (1% dimethyl sulfoxide, 10% ethanol, and 0.9% NaCl) over 4 days. Then, the mechanical hypersensitivity was assessed again by the paw pressure test at two hours after i.th. injection. The doses for i.th. injections of the above drugs were applied only once per day which was insufficient to increase plasma concentration (Sadee et al. 1973; Williams et al. 1972) to a level that can produce a systemic effect (Ramsay et al. 1976). The second

set of experiments was designed to examine the co-expression of MR and the pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA in L3-5 DRG neurons obtained from naïve control rats. The third set of experiments mainly studied the expression changes of pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA in L3-5 DRG neurons on the fourth day after FCA administration following chronic i.th. canrenoate-K. The dose-response curves in our previous studies (Li et al. 2018; Shaqura et al. 2016a; Shaqura et al. 2016b) were taken as a reference for the dose selection of each drug. The fourth set of experiments was dedicated to comparing the co-localization of nuclei and MR in L3-5 DRG neurons between the control rats and the rats with FCA-induced inflammation. The final set of experiments focused on the influence of a continuous i.th. delivery of the aldosterone synthase inhibitor FAD286 (1.5μg/5μ/h) over 4 days on the number of neuronal aldosterone, pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA as well as on mechanical PPT in rats with FCA hindpaw inflammation. The dose of FAD286 (1.5 ug/5μ/h) is insufficient to cause systemically effective plasma concentrations (Rigel et al. 2010).

2.6 Mechanical pressure testing

By means of an analgesiometer (Ugo-Basile SRL, Monvalle, Italy), which is used to conduct the Randall-Selitto paw pressure test, paw pressure thresholds (PPT) were measured to evaluate mechanical hyperalgesia of the hindpaws as has previously been shown (Li et al. 2018). Briefly, wrapping in a towel prevented the rat's upper body and head from moving freely and made the animal relaxed and comfortable. The hindpaw of the rat was placed on the meter's plinth after the rat was relaxed and quiet. The rounded tip of the cone-shaped pusher vertically moved downwards to the center of the dorsal aspect of the hindpaw after the meter was started. When the force imposed on the tested hindpaw gradually increased over time, an attempted withdrawal or removal of the hindpaw was recorded as a positive withdrawal reaction indicating mechanical hyperalgesia. A 250 g cut-off value was deemed as the maximal tolerable pressure without any tissue damage. In each test session, each rat was examined three times with a time interval of at least 15 seconds. On the fourth day after hindpaws had been inoculated with either FCA or the vehicle, PPT were determined in all groups. Baseline values of PPT were assessed on both ipsilateral inflamed and contralateral non-inflamed hindpaws before i.th. administration of drugs. Two hours after the last i.th. administration

was completed, the PPT were reevaluated to illuminate the effect of drugs on nociceptive behavior. Means of PPT from 6-10 animals were adopted as the final PPT.

2.7 Immunohistochemistry

In deep anesthesia with isoflurane, rats were transcardially perfused with 100 ml warm saline and then with 300 ml 4% (w/v) paraformaldehyde in 0.16 M phosphate buffer saline (PBS, pH 7.4). Thereafter, L3-L5 DRG post-fixed in the same fixative solution for 90 minutes, and then cryoprotected overnight at 4°C in PBS containing 10% sucrose. The tissues were embedded in tissue-Teck compound (OCT, Miles Inc. Elkhart, IN) and frozen. Subsequently, consecutive sections of a thickness of 8µm were prepared on cryostat (Thermo Fischer, Dreieich, Germany) and mounted onto gelatin-coated slides (Li et al. 2018; Shaqura et al. 2016b). To detect the co-expression of MR and pain signaling molecules in the L3-5 DRG neurons of control rats, the expression changes of pain signaling molecules after treatment, the primary antibodies (see table 1 in (Shaqura et al. 2020) were incubated with tissue sections overnight as follows: mouse antibody against MR (private gift from Prof. Elise Gomez-Sanchez, Jackson, USA) either alone or together with goat anti-TRPV1 (Santa Cruz Biotechnology, CA, USA), guinea pig anti-CGRP (R&D Systems, USA), polyclonal rabbit anti-Nav1.8 (Sigma-Aldrich), or goat anti-TrkA (R&D Systems, USA). In rats with FCA-induced inflammation, tissue sections before and after treatments of canrenoate-K or FAD286 were incubated with primary antibodies of aldosterone (a polyclonal rabbit anti-aldosterone antibody) and the pain signaling molecules TRPV1, CGRP, Nav1.8, or TrkA. After incubation with the corresponding secondary antibodies, the tissue sections were rinsed in PBS, mounted on vectashield (Vector Laboratories, San Francisco, CA, USA) and imaged with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss GmbH, Jena, Germany) as previously described (Li et al. 2018). According to our previous studies (Li et al. 2018; Mousa et al. 2016; Shaqura et al. 2016b), either the primary or the secondary antibodies were omitted in order to clarify specificity of staining.

In the immunofluorescence quantification process (Li et al. 2018; Mousa et al. 2016; Shaqura et al. 2016a), Zeiss Zen 2009 software from Carl Zeiss Micro-Imaging GmbH (Göttingen, Germany) was used to quantify the aldosterone, MR, TRPV1, CGRP, Nav1.8, and TrkA positive neurons in DRG tissue sections. In order to exclude background fluorescence, the images were adjusted to the threshold and then gated to

include only the intensity measurements from positively stained cells. When the total number of neurons was counted, only immunofluorescent cells with a clear nucleus were included. Meantime, the number of aldosterone-ir DRG cells/320μm^2, MR-ir DRG cells/320μm^2, TRPV1-ir DRG cells/320μm^2, CGRP-ir DRG cells/320μm^2, Nav1.8-ir DRG cells/320μm^2, and TrkA-ir DRG cells/320μm^2 cells was counted in each DRG tissue section and represented as percentages. Data were collected from 4-6 rats per group under a 40× objective lens.

2.8 Statistical Analysis

Sigma Stat 2.03 software (SPSS Inc., Chicago, DE, USA) was applied to all tests. When data presented a normal distribution, Student's t-test was used to compare the difference of immunohistochemical results between two groups; otherwise, the Mann–Whitney U test was appropriate for conducting non-parametric comparisons. For multiple group comparisons, a one-way ANOVA was adopted when data were normally distributed, or a Kruskal–Wallis test was carried out when the distribution of data was not normally distributed. Before and after drug injections, paw pressure thresholds (PPT) which were expressed as means±SD were measured within the same group of animals and processed by a repeated measurement-ANOVA, followed by a post hoc Dunnett's test. In all statistical analyses, P<0.05 indicated significance.

3. Results

3.1 MR agonist aldosterone contributes to mechanical hyperalgesia during local hindpaw inflammation in rats

On the fourth day after inoculation of the right hindpaws of rats with Freund's complete adjuvant (FCA), paw pressure thresholds (PPT) of the inflamed hindpaws measured by the Randall-Selitto test were significantly diminished before i.th. vehicle or i.th. canrenoate-K treatment (50.3±2.4 g and 54.3±1.9 g, respectively) indicating mechanical hyperalgesia (P<0.05, repeated measurement-ANOVA, followed by post hoc Dunnett ANOVA, n=6; **Fig. 1A**). However, there were no significant changes in PPT in the contralateral non-inflamed hindpaws (dotted line, **Fig. 1A**). Repeated intrathecal (i.th.) injections of the MR selective antagonist canrenoate-K (over 4 days) resulted in a significant reversal of these FCA-induced diminished PPT (from 50±2.4 g up to 62.0±1.2 g) in inflamed hindpaws (P<0.05, repeated measurement-ANOVA, followed by post hoc Dunnett's test, n=6; **Fig. 1A**), thus, revealing a reduced mechanical hyperalgesia. When

rats with inflamed hindpaws received i.th. vehicle (isotonic saline) instead of the MR antagonist canrenoate-K, the mechanical hyperalgesia was not altered (black line, **Fig. 1A**). In reverse experiments, naïve control rats received i.th. MR selective agonist aldosterone (over 4 days) which resulted in a significant decrease in mechanical paw pressure thresholds (PPT: from 71.4±1.3 g down to 58.9±2.5 g), thus indicating increased sensitivity to mechanical stimuli (hyperalgesia) (P<0.05, repeated measurement-ANOVA, followed by post hoc Dunnett's test, n=6; **Fig. 1B**). No significant alterations in mechanical PPT were observed in naïve control rats when they received an i.th. injection of aldosterone's vehicle (1% dimethyl sulfoxide + 10% ethanol + 0.9% NaCl) (P<0.05, repeated measurement-ANOVA, followed by post hoc Dunnett's test, n=6; **Fig. 1B**).

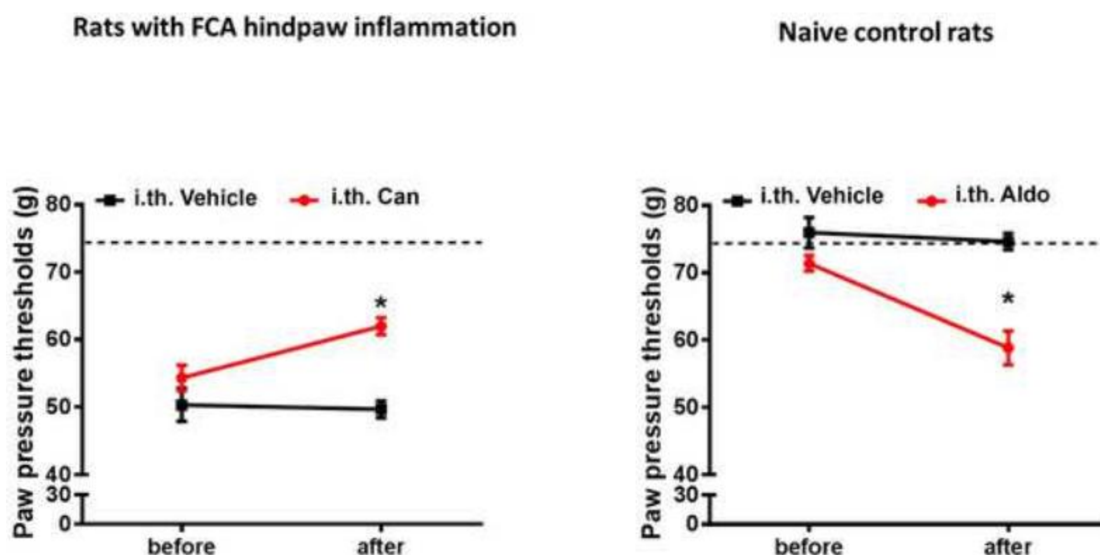

Fig. 1: Reversal of mechanical hyperalgesia in rats with FCA-induced hindpaw inflammation by i.th. MR antagonist canrenoate-K (Can) is consistent with the mechanical hyperalgesia elicited by i.t. MR agonist aldosterone (Aldo) in naïve control rats. A) Mechanical paw pressure thresholds (measured by Randall-Selitto test) of control rats (dotted line) were 75 g, whereas those of rats with FCA-induced hindpaw inflammation (black squares and red squares) were significantly reduced (PPT before i.th. vehicle: 50.3±2.4 g; PPT before i.th. Can: 54.3±1.9 g) (P<0.05, repeated measurement-ANOVA, followed by post hoc Dunnett's test, n=6) and did not change after intrathecal (i.th.) vehicle (0.9% saline) treatment (black squares). In contrast, daily (over 4 days) i.th. treatment of the MR antagonist canrenoate-K (100µg/20µl, red circles) reversed the FCA-induced lowered paw pressure thresholds of the inflamed hindpaws (62.0±1.2 g) (P<0.05, repeated measurement-ANOVA, followed by post hoc Dunnett's test, n=6) indicating a reduction in mechanical hyperalgesia by antagonizing the tonic activation of MR on peripheral sensory neurons. **B)** In naive control rats treated with i.th. vehicle (1% dimethyl sulfoxide + 10% ethanol + 0.9% NaCl, black squares) mechanical paw pressure thresholds were 74.7±1.2 g, whereas those of naïve rats chronically (over 4 days) i.th. treated with the MR agonist aldosterone (40µg/20µl, red circles) were significantly reduced (58.9±2.5 g) (P<0.05, repeated measurement-ANOVA, followed by post hoc Dunnett's test, n=6) indicating an increase in mechanical hyperalgesia by activating MR on peripheral sensory neurons. Data are expressed as means ± SD. (Figure published in Mohammed Shaqura, Li Li et al. 2020).

3.2 Co-localization of MR with various pain signaling molecules such as TRPV1, CGRP, Nav1.8, and TrkA

Double immunohistochemistry with a monoclonal mouse anti-MR antibody together with a polyclonal goat anti-TRPV1 antibody showed MR-immunoreactive (-ir) cells that mostly (68±8%) co-localized with TRPV1 in the DRG of naïve rats (**Fig. 2, panel A**). In addition, immunohistochemistry with a mouse antibody against MR together with a polyclonal guinea anti-CGRP antibody showed abundant co-localization of MR-ir cells with the neuropeptide CGRP (78±11%) (**Fig. 2, panel B**). With a Zeiss LSM 510 confocal laser scanning microscope, it was found that MR-ir neurons, which were identified by a monoclonal mouse anti-MR antibody, highly co-expressed (74±12%) Nav1.8 in the dorsal root ganglion cells of naïve control rats (**Fig. 2, panel C**). Moreover, the fluorescent image of MR largely overlapped TrkA-positive stained DRG neurons (56±4%) that were identified with a polyclonal goat anti-TrkA antibody (**Fig. 2, panel D**).

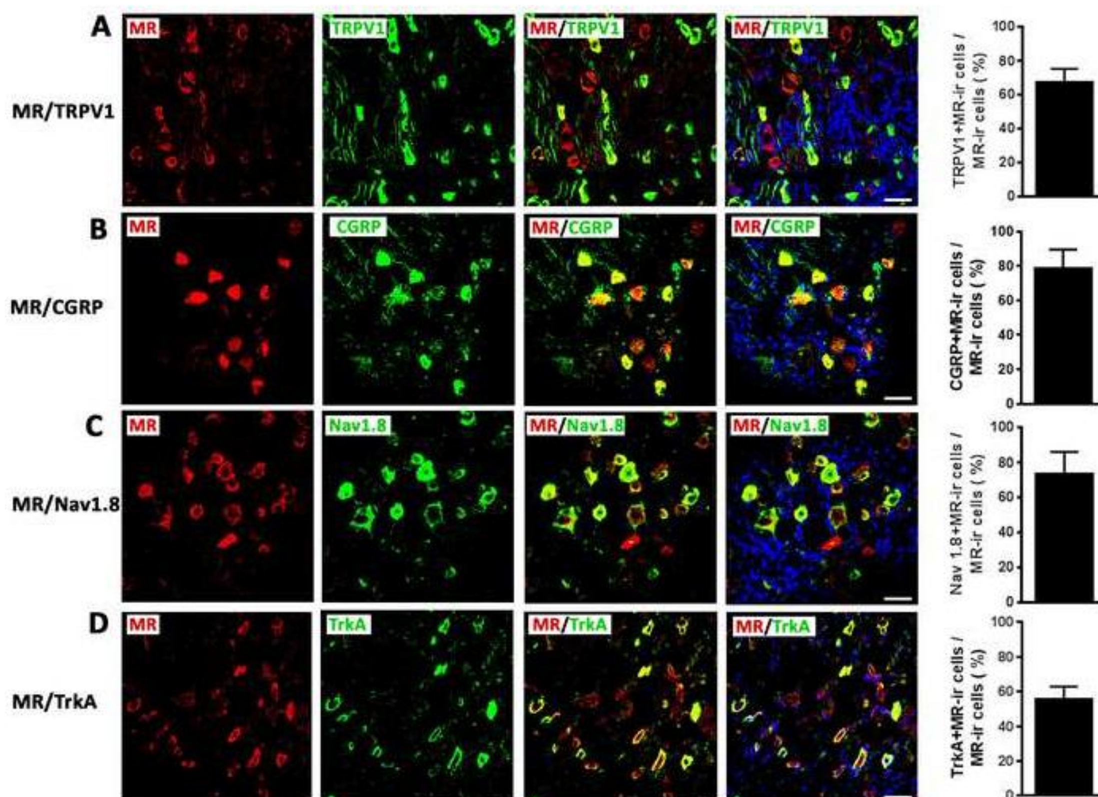

Fig. 2: Co-localization of mineralocorticoid receptors (MR) and pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA in L3-5 dorsal root ganglia (DRG) of naïve control rats under double immunofluorescence confocal microscopy. A-D) Immunohistochemical images were obtained using a Zeiss LSM 510 confocal laser scanning microscope. Texas-red fluorescence, FITC-green fluorescence and yellow (red/green) fluorescence marked MR, various pain signaling molecules and the co-expression

of MR with these pain signaling molecules, respectively. Nuclei were stained with blue DAPI (Bar = 40μm). (**A-D**) Bar graphs show the quantitative assessment of the immunohistochemical pictures with abundant co-existence of MR with the different pain signaling molecules TRPV1 (68±8%), CGRP (78±11%), Nav1.8 (74±12%), and TrkA (56±4%) in DRG neurons. Data are expressed as means ± SD. Bar = 40μm. (Figure published in Mohammed Shaqura, Li Li et al. 2020).

3.3 Intrathecal MR antagonist canrenoate-K reversed the FCA-induced up-regulation of pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA

The immunoreactivity of TRPV1 in ipsilateral L3-5 DRG neurons that reacted with a polyclonal goat anti-TRPV1 antibody significantly enhanced on the fourth day after FCA inoculation compared with TRPV1-ir neurons in control rats. Following consecutive i.th. delivery of canrenoate-K over 4 days in FCA-treated rats, the number of TRPV1-ir cells in DRG neurons significantly decreased ($P<0.05$, one-way ANOVA, followed by post hoc Dunnett's test, n=9-17; **Fig. 3A-D**). In the immunohistochemical examination of CGRP (**Fig. 3E-H**), the number of CGRP-ir neurons in L3-5 DRG recognized by a polyclonal guinea pig anti-CGRP antibody increased 4 days after the injection of FCA when compared to naïve control rats, although this up-regulation was reversed by i.th. administration of canrenoate-K over 4 days. In a similar way, the up-regulated Nav1.8-ir neurons identified by a polyclonal rabbit anti-Nav1.8 antibody on the fourth day after FCA injection were down-regulated as a result of chronic i.th. injections of canrenoate-K over 4 days (**Fig. 3I-L**). Moreover, there was significantly elevated expression of TrkA in L3-5 DRG neurons innervating FCA-induced inflamed hindpaws; however, i.th. administration of canrenoate-K over 4 successive days reversed this elevation of TrkA-ir cells (**Fig. 3M-P**). Notably, the total number of DRG neurons in the same size of field of view (320μm^2) was not significantly different between the control group (Ctrl), FCA inoculation group (FCA), and canrenoate-K-administered group (FCA + Can) (namely Ctrl: 45±8.5; FCA: 45±8.8; FCA + Can: 42±12, $P=0.43$, one-way ANOVA).

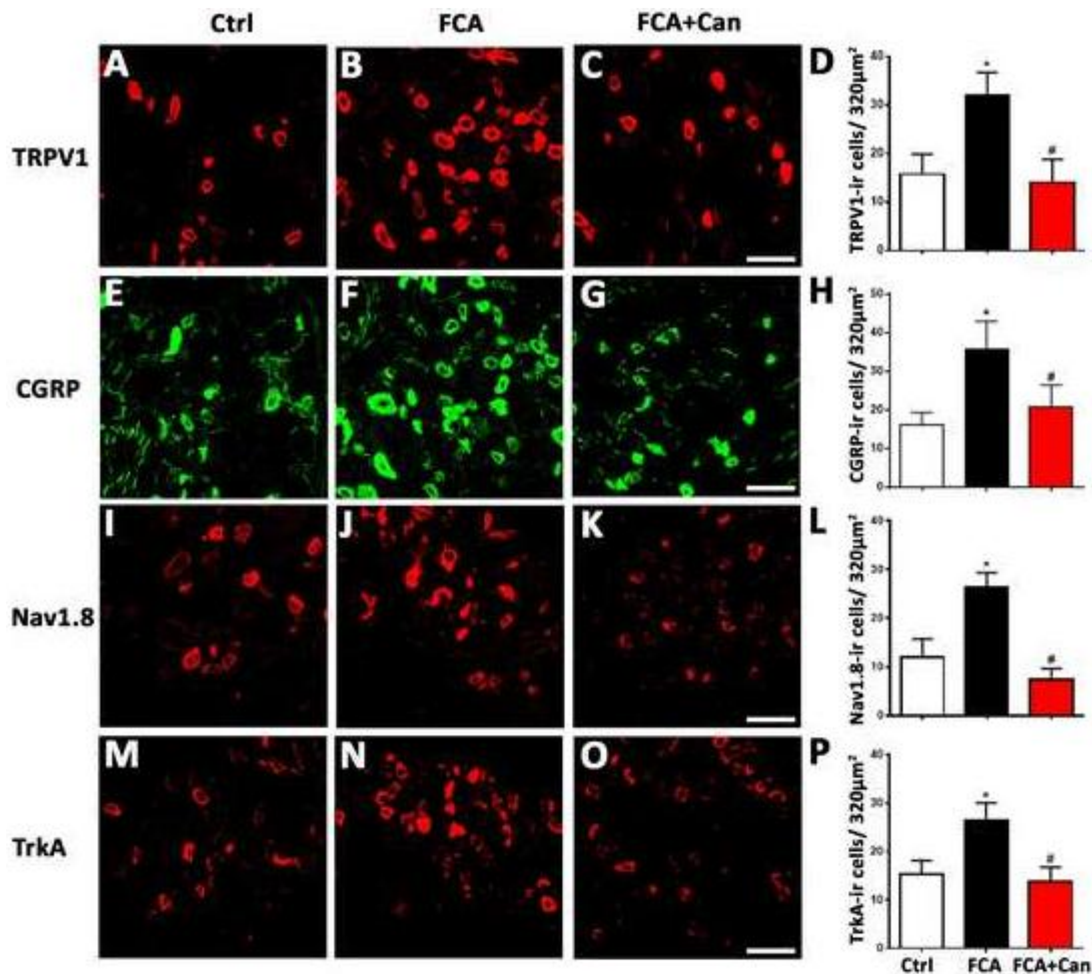

Fig. 3: Reversal of the FCA-inflammation-induced increase in neuronal expression of TRPV1, CGRP, Nav1.8, and TrkA by intrathecal MR antagonist canrenoate-K (Can). A-C) Dorsal root ganglia (DRG) (L3-L5) innervating FCA-inflamed hindpaws (**B**) revealed an increased TRPV1 immunoreactivity (Texas-red fluorescence) compared to DRG (L3-L5) of naïve control rats (**A**). This increase was attenuated in the DRG of rats treated with an intrathecal administration of the MR antagonist canrenoate-K (Can) (100µg/20µl) over 4 days (**C**). In a similar way, FCA-induced increases of CGRP- (**E-G**), Nav1.8- (**I-K**), and TrkA- (**M-O**) immunoreactivity were reversed following the intrathecal administration of the MR antagonist canrenoate-K (100µg/20µl) over 4 days. (Bar = 40µm). **D, H, L, P)** Quantification of the immunohistochemical pictures using Zen 2009 software (Carl Zeiss Micro-Imaging GmbH) revealed that these differences were significant ($P<0.05$, one-way ANOVA, followed by post hoc Dunnett's test), while the total number of dorsal root ganglion cells/320µm^2 was not significantly altered (Ctrl: 45±8.5; FCA: 45±9; FCA + Can: 42±12, $P=0.43$, one way ANOVA). (Figure published in Mohammed Shaqura, Li Li et al. 2020).

3.4 Enhanced transfer of MR to the nuclei of DRG neurons under local inflammation

When compared with control animals, the immunostaining revealed that the overlapping of MR-immunoreactivity and positive-stained nuclei by DAPI in ipsilateral DRG cell bodies increased on the fourth day after FCA was inoculated to the plantar surface of the right hindpaws of rats (**Fig. 4A-B**).

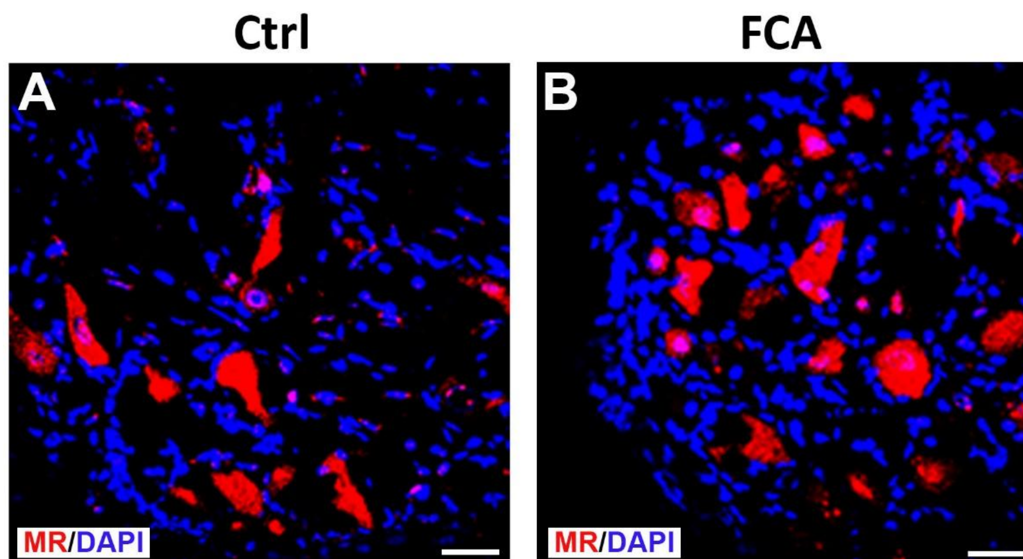

Fig. 4: Increased overlap of immunoreactive mineralocorticoid receptors (MR) with nuclei staining in the dorsal root ganglia (DRG) of rats with FCA inflammation. A, B) The co-localization of MR in nuclei (Texas-red fluorescence) and nuclear DAPI (blue) was identifiable in DRG cells harvested from rats without inflammation (**A**). On the fourth day following intraplantar injection of FCA to the right hindpaws of rats, there was an enhancement of MR that transferred to nuclei and an incremental overlap of MR immunoreactivity and blue-staining nuclei in DRG neurons as a result of FCA inflammation (**B**). Bar = 40µm. (Modified figure published in Mohammed Shaqura, Li Li et al. 2020).

3.5 Intrathecal aldosterone synthase inhibition reverses increased aldosterone expression, up-regulated pain signaling molecules and elevated mechanical sensitivity during inflammation

To identify the relation between aldosterone produced in dorsal root ganglion cells and FCA-elicited inflammatory pain of rats' hindpaws, rats with FCA-inflamed hindpaws were continuously infused with the aldosterone synthase inhibitor FAD286 via intrathecal catheter over 4 days. On the fourth day after FCA inoculation, a polyclonal rabbit anti-aldosterone antibody identified substantial aldosterone-immunoreactive DRG neurons in the DRG ipsilateral to the inflamed hindpaws. Intrathecal administration of the aldosterone synthase inhibitor FAD286 over 4 days significantly reduced aldosterone immunohistochemistry (P<0.05, two-tailed independent Student's t-test, n=7-11; **Fig. 5A-C**), although the total number of dorsal root ganglion cells was not altered (Ctrl: 45±8.5; FCA: 45±8.8; FCA + FAD: 40±6.7, P=0.433, one-way ANOVA). This decrease in aldosterone expression was paralleled by significant reductions in the expression of the pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA. (P<0.05, two-tailed

independent Student's t-test, n=9-17; **Fig. 6A-L**). In the meantime, the ipsilateral PPT on the fourth day after FCA inoculation before i.th. injection of FAD286 or its vehicle (isotonic saline) significantly dropped to 47.0±2.2 g or 50.3±2.4 g, while the baseline value was 75 g. After continuous i.th. therapy with FAD286 over 4 days, PPT of inflamed hindpaws significantly rose to 61.9±2.1 g indicating the anti-nociceptive effect of FAD286 due to the inhibiting of the production of aldosterone by blocking aldosterone synthase; nonetheless, no significant change of PPT was identified when rats were intrathecally treated with the vehicle of FAD286 (P<0.05, repeated measurement-ANOVA, followed by post-hoc Dunnett's test, n=6-10; **Fig. 6M**).

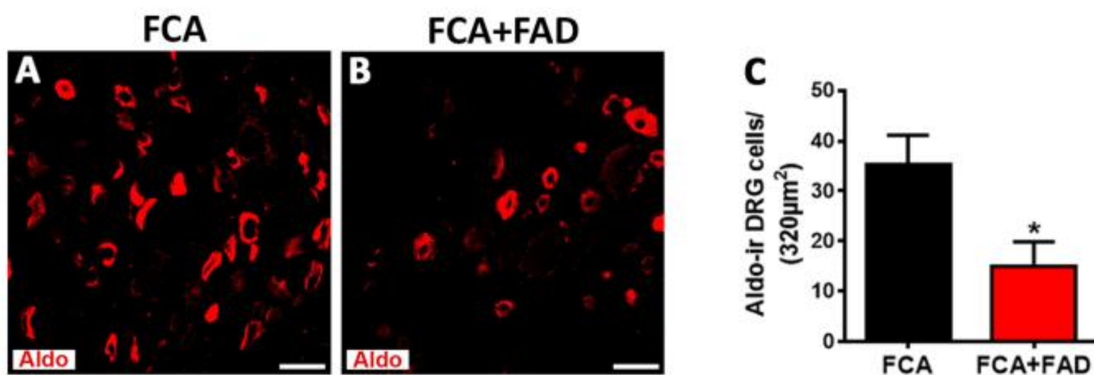

Fig. 5: Reduction of FCA inflammation-induced up-regulation of aldosterone expression in dorsal root ganglia (DRG) by intrathecal aldosterone synthase inhibitor FAD286. A, B) In using a specific anti-aldosterone antibody (Novus Biologicals, CO, USA) for immunohistochemical staining of DRG and a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss GmbH), aldosterone-positive (Aldo) stained neurons (Texas-red fluorescence) were abundantly expressed on the fourth day of FCA-hindpaw inflammation (FCA) **(A)**, while continuous (over 4 days) intrathecal treatment of FAD286 (1.5µg/5µl/h)(FCA + FDA) led to a reduction in aldosterone-positive (Aldo) stained neurons (Texas-red fluorescence) **(B)**. **C)** Quantification of the immuno-histochemical pictures using Zen 2009 software Carl Zeiss Micro-Imaging GmbH (Göttingen, Germany) revealed that the number of Aldo-ir dorsal root ganglion cells in the FCA group (black bar, 35.1±15.0) markedly and significantly declined in the FCA group treated with the aldosterone synthase inhibitor FAD286 (P<0.05, two-tailed independent Student's t-test, n=7-11). Data are expressed as means ± SD. (Modified figure published in Mohammed Shaqura, Li Li et al. 2020).

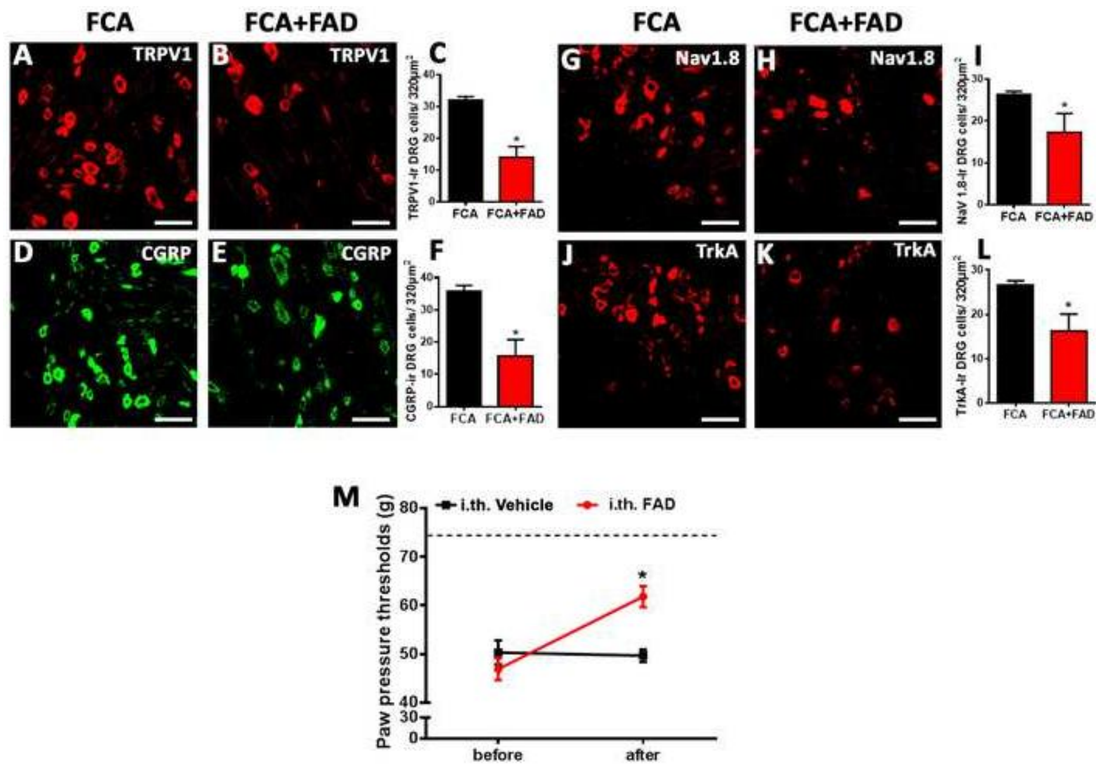

Fig. 6: Reversal of FCA inflammation-induced up-regulation of immunoreactive pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA in L3-L5 dorsal root ganglia (DRG) by intrathecal aldosterone synthase inhibitor FAD286 (FAD) and its consequences for nociceptive behavior. A-C) In the case of persistent intrathecal administration of the aldosterone synthase inhibitor FAD286 (1.5µg/5µl/h) over 4 days, the enhanced TRPV1 immunoreactivity in DRG (Texas-red fluorescence) **(A)**, (black bar) **(C)** was significantly lessened, as shown in graph B (Texas-red fluorescence) and red bar of chart C. Similarly, the increased expression of CGRP (FITC-green fluorescence) **(D)**, (black bar) **(F)**, Nav1.8 (Texas-red fluorescence) **(G)**, (black bar) **(I)**, TrkA (Texas-red fluorescence) **(J)**, (black bar) **(L)** in L3-L5 DRG neurons under FCA inflammation was significantly reversed by chronic i.th. injection of FAD286 (1.5 µg/5µl/h) as depicted in their corresponding images (**E**, **H** and **K**) and red bars **(F, I and L)**. **M)** Before i.th. injection of FAD286 or its vehicle (isotonic saline), the PPT on the fourth day after the injection of FCA into rats' right hindpaws displayed a significant decline (PPT of FAD286: 47.0±2,2 g)(red circle) (PPT of vehicle: 50.3±2.4 g)(black square) in contrast to baseline (PPT: 75 g)(dotted line), indicating FCA-induced mechanical hyperalgesia. However, lasting i.th. infusion of FAD286 (1.5µg/5µl/h) over 4 days inhibited this hypersensitivity and brought about the elevation of PPT (61.9±2.1 g)(red circle), whereas no pronounced hypersensitivity changes (50.0±1.2 g)(black square) were observed when rats with FCA-inflamed paws were treated with i.th. administration of vehicle of FAD286 ($P<0.05$, repeated measurement-ANOVA, followed by post hoc Dunnett's test, n=6-10). These results imply the reduction of aldosterone as a result of inhibited aldosterone synthesis by FAD286 contributes to the weakened mechanical hyperalgesia during FCA-induced hindpaw inflammation. (Figure published in Mohammed Shaqura, Li Li et al. 2020).

4. Discussion

In the rat model of FCA-hindpaw inflammation, the experiments conducted as part of my thesis show that tonic activation of neuronal MR by local aldosterone within dorsal root ganglia (DRG) may lead to an enhanced expression of pain signaling molecules, and that this is most likely due to a genomic mechanism (Shaqura et al. 2020). This subsequently contributes to the inflammation-induced persistent mechanical hypersensitivity. This conclusion is based on the following results of my work (Shaqura et al. 2020): i) i.th. injection of the MR antagonist canrenoate-K caused a significant reduction in mechanical hypersensitivity of FCA-inflamed hindpaws of rats; ii) i.th. administration of the MR agonist aldosterone evoked increased mechanical hypersensitivity in naïve rats; iii) there is abundant co-localization of MR with known pain signaling molecules such as TRPV1, CGRP, Nav1.8, and TrkA in the DRG neurons of naïve rats; iv) these pain signaling molecules were markedly increased in those DRG neurons that were ipsilateral to the FCA-induced rat hindpaw inflammation; v) this inflammation-induced increased expression of pain signaling molecules was attenuated by the i.th. MR antagonist canrenoate-K as well as the aldosterone synthase inhibitor FAD286; and vi) finally, the aldosterone synthase inhibitor FAD286 reduced the inflammation-induced enhanced aldosterone expression as well as mechanical hyperalgesia. These findings suggest that both endogenously and exogenously applied aldosterone contribute to local inflammatory pain via tonic activation of neuronal MR.

In this study, mechanical hypersensitivity due to FCA-induced unilateral hindpaw inflammation was dose-dependently attenuated by i.th. application of the MR antagonist canrenoate-K over 4 days, suggesting a tonic activation of neuronal MR by locally synthesized aldosterone. These findings are in agreement with previous studies showing the inhibition of pain behavior by i.th. application of the mineralocorticoid receptor antagonists eplerenone (Dong et al. 2012) or spironolactone (Gu et al. 2011; Sun et al. 2012). The authors reported that these effects occurred only after 3 days and lasted up to 2 weeks, suggesting a long-lasting genomic effect. However, these findings confirm previous studies from my lab (Mohamed et al. 2020) which showed an immediate and short-lasting inhibition of mechanical hypersensitivity after a single i.th. injection of the MR antagonist canrenoate-K. The effect was immediate and lasted only up to 60 minutes, suggesting a non-genomic effect. Indeed, the inhibition of aldosterone production by an aldosterone synthase inhibitor resulted in the attenuation of mechanical hyperalgesia resulting from FCA-induced hindpaw inflammation (Mohamed et al. 2020). These

findings are supported by our experiments showing an increased mechanical hyperalgesia following an i.th. injection of exogenous aldosterone in naïve animals (Li et al. 2018). Further support for a pronociceptive role of the MR agonist aldosterone comes from an in vitro study which showed that locally applied aldosterone (10 nM) to normal, small-diameter nociceptive DRG neurons triggered and amplified action potentials (Dong et al. 2012). Taken together, these findings suggest that local neuronal aldosterone which activates MR on peripheral nociceptive neurons in a tonic way seems to contribute to inflammation-induced mechanical hypersensitivity.

It has been well established that pain signaling molecules such as TRPV1, CGRP, Nav1.8, and TrkA play a crucial role in pain modulation under various types of pathological condition (Coggeshall et al. 2004; Nahin and Byers 1994; Xu et al. 2009; Liang et al. 2013). Moreover, Schumacher has shown that MR may modulate nociceptive ion-channels in sensory neurons (Schumacher 2012). Interestingly, my double immunofluorescence microscopy analysis revealed an abundant co-expression of MR with those pain signaling molecules such as TRPV1, CGRP, Nav1.8, and TrkA in the neuronal DRG of naïve control rats. TRPV1 is known to be expressed in DRG neurons (Ji et al. 2002) and to be responsible for heat- and proton-evoked nociception (Tominaga et al. 1998). Previous literature has shown that TRPV1 expression was up-regulated in DRG innervating FCA-inflamed hindpaws (Ji et al. 2002). Also, Nav1.8 has been shown to be selectively expressed in DRG neurons and up-regulated in nociceptive neurons following injury (Hameed 2019). CGRP is predominantly expressed in nociceptive DRG neurons and up-regulated in adjuvant arthritic rats. Indeed, the quantification of immunofluorescence of the ipsilateral DRG sections of rats with FCA-induced hindpaw inflammation has revealed that the number of TRPV1- CGRP-, Nav1.8-, and TrkA-ir neurons was significantly increased compared to that of naïve animals. These results align with previous studies showing an up-regulation of pain signaling molecules such as TRPV1, CGRP, Nav1.8, and TrkA (Coggeshall et al. 2004; Liang et al. 2013; Nahin and Byers 1994; Xu et al. 2009) under various types of inflammatory painful condition, suggesting a vital role played by these molecules in pain modulation. The abundant co-localization of MR with these pain signaling molecules within DRG suggests that they may play an important role in pain modulation under inflammatory conditions. Moreover, aldosterone increased Na+, -K+, -ATPase mRNA expression in rat hippocampus (Farman et al. 1994), and chronic intra-cerebroventricular infusion of aldosterone synthase inhibitor FAD286 or the MR blocker eplerenone

inhibited epithelial sodium channel C subunit expression in various brain areas (Mills et al. 2018; Wang et al. 2016). Indeed, the local blockade of the tonic activation of neuronal MR by the i.th.-applied MR antagonist canrenoate-K reversed the up-regulation of pain signaling molecules such as TRPV1, CGRP, Nav1.8, and TrkA during local inflammation. Several previous studies have reported that the genomic effect of aldosterone is dependent on an increased translocation of neuronal MR from the cytosol to the nucleus. In this paper, this notion was confirmed by my immunofluorescence staining of the respective DRG showing an enhanced overlap of MR-immunoreactivity with the nuclear marker DAPI consistent with a previously shown observation of MR nuclear translocation within different brain areas (Kil and Kalinec 2013; Piechota et al. 2017). Similar results were obtained in a model of zymosan-induced DRG inflammation, in which the MR was markedly transferred to the nuclei of DRG cell bodies on the first day after zymosan was administered (Dong et al. 2012). After MR combined with its ligand, the complex translocated to the nucleus and then stimulated gene transcription (Beato and Sánchez-Pacheco 1996). These findings suggest that tonic activation of neuronal MR via enhanced local production of aldosterone enhances the transcription process of certain pain signaling molecules within peripheral nociceptive neurons and that this contributes to inflammation-induced hyperalgesia. Therefore, I examined whether i.th. aldosterone synthase inhibitor FAD286 over 4 days will reduce local aldosterone synthesis and consequently reverse the up-regulation of pain signaling molecules as well as mechanical hyperalgesia under inflammatory conditions. In earlier research, Bassett et al. pointed out that aldosterone synthase converted aldosterone precursors to aldosterone (Bassett et al. 2004). Indeed, i.th. aldosterone synthase inhibitor FAD286 reversed the increased number of aldosterone-ir DRG neurons ipsilateral to the inflamed hindpaw. In fact, the aldosterone synthase (CYP11B2) mRNA, aldosterone synthase protein, and aldosterone were determined in L3-5 DRG of both naïve rats and FCA-inoculated rats (Mohamed et al. 2020). Furthermore, there was remarkably increased expression of both aldosterone synthase mRNA and aldosterone content in DRG in FCA-induced inflammation (Mohamed et al. 2020).

Here, my experiments also illustrated that ongoing i.th aldosterone synthase inhibitor FAD286 over 4 days significantly reduced the up-regulated number of aldosterone-ir DRG neurons concomitant with reversing the enhancement of the pain signaling molecules TRPV1-, CGRP-, Nav1.8-, and TrkA-ir in DRG neurons ipsilateral to the FCA-injection side of rats. Similarly, the aldosterone synthase inhibitor FAD286 not only

inhibited the up-regulation of inflammatory markers in macrophages of mice in vivo, but also reduced the enhanced expression of inflammatory markers in vitro, indicating a direct antagonism of inflammation (Gamliel-Lazarovich et al. 2010). Importantly, i.th. aldosterone synthase inhibitor FAD286 reduced nociceptive behavior in rats with FCA-unilateral inflamed hindpaws. These findings from my thesis strongly suggest the involvement of neuronal aldosterone in the transcriptional regulation of certain signaling molecules involved in pain modulation and subsequent nociceptive behavior, which is in agreement with previous literature (Dong et al. 2012; Jokinen et al. 2017) showing the analgesic effect of a long-lasting blockade of endogenous ligand aldosterone-mediated MR activation through a genomic effect.

In summary, my results revealed that MR predominantly co-localized with the pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA in the peripheral sensory DRG neurons of naïve rats. Moreover, long-lasting blockade of locally synthesized endogenous aldosterone from activating neuronal MR with either a specific receptor antagonist or an aldosterone synthase inhibitor reversed the enhanced expression of the pain signaling molecules TRPV1, CGRP, Nav1.8, and TrkA within peripheral sensory neurons under inflammatory pain and consequently reduced mechanical hypersensitivity. This genomic effect of neuronal endogenous aldosterone during inflammatory pain was supported by the enhanced nuclear translocation of MR in peripheral sensory DRG neurons. Therefore, I conclude that the enhanced local aldosterone synthesis within peripheral sensory DRG neurons results in the up-regulation of pain signaling molecules via tonic activation of neuronal MR through long-lasting genomic effects, and that this consequently plays an important role in ongoing mechanical hypersensitivity.

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Statutory Declaration

"I, Li Li, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Discovery of new regulatory mechanisms of sensory neuron nociception in animal models of inflammatory pain"/ "Erschließung neuer regulatorischer Mechanismen der durch sensorische Neurone-vermittelten Nozizeption in Tiermodellen des Entzündungsschmerzes", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date -------------------   Signature ---------------------

Li Li

Declaration of individual contribution to the following publication

Li Li contributed the following to the below listed publication:
Shaqura, M., Li, L., Mohamed, D. M., Li, X., Treskatsch, S., Buhrmann, C., Shakibaei, M., Beyer, A., Mousa, S., A., & Schafer, M. (2020). Neuronal aldosterone elicits a distinct genomic response in pain signaling molecules contributing to inflammatory pain. J Neuroinflammation, 17(1), 183. doi:10.1186/s12974-020-01864-8

Contribution details:

- Li Li was involved in the design of the experiments
- Li Li performed intrathecal injections, implemented intrathecal catheterizations, and contributed to the harvesting of dorsal root ganglia
- Li Li performed the behavioral tests (Fig. 1 and Fig. 7M)
- Li Li performed under the supervision of Shaaban Mousa the immunofluorescence experiments (Fig. 2, 4, 7 and Fig.5C-D as well as Fig.6A-B)
- Li Li did the data collection and processing
- Li Li contributed to the analysis and discussion of the results
- Li Li wrote parts of the manuscript and revised the entire manuscript

Signature, date and stamp of first supervising university professor / lecturer

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Prof. Dr. med. Michael Schäfer

Signature of doctoral candidate

Li Li

Extract from the Journal Summary list

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"Immunology"** Selected Category Scheme: WoS
Gesamtanzahl: 158 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS IMMUNOLOGY	41,499	44.019	0.080220
2	NATURE IMMUNOLOGY	44,298	23.530	0.094250
3	IMMUNITY	51,051	21.522	0.126090
4	Annual Review of Immunology	17,013	21.429	0.022210
5	Lancet HIV	2,417	14.753	0.014270
6	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY	51,978	14.110	0.076250
7	TRENDS IN IMMUNOLOGY	12,153	13.000	0.025470
8	IMMUNOLOGICAL REVIEWS	15,517	11.292	0.027050
9	JOURNAL OF EXPERIMENTAL MEDICINE	63,983	10.892	0.071790
10	Science Immunology	1,292	10.551	0.007580
11	CLINICAL INFECTIOUS DISEASES	64,031	9.055	0.119010
12	Journal for ImmunoTherapy of Cancer	2,716	8.676	0.011350
13	Cancer Immunology Research	5,420	8.619	0.025380
14	Cellular & Molecular Immunology	4,058	8.213	0.009160
15	AUTOIMMUNITY REVIEWS	9,127	7.716	0.018220
16	CURRENT OPINION IN IMMUNOLOGY	9,164	7.667	0.017440
17	Journal of Allergy and Clinical Immunology-In Practice	4,196	7.550	0.013090
18	JOURNAL OF AUTOIMMUNITY	6,900	7.543	0.015050
19	SEMINARS IN IMMUNOLOGY	5,016	7.358	0.010290
20	Mucosal Immunology	6,990	7.352	0.020730
21	CLINICAL REVIEWS IN ALLERGY & IMMUNOLOGY	3,149	7.328	0.006390

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	Clinical & Translational Immunology	998	7.271	0.003770
23	EMERGING INFECTIOUS DISEASES	30,311	7.185	0.059420
24	Seminars in Immunopathology	3,337	6.804	0.007890
25	ALLERGY	17,873	6.771	0.024250
26	EXERCISE IMMUNOLOGY REVIEW	754	6.455	0.000890
27	Emerging Microbes & Infections	1,941	6.212	0.006720
28	BRAIN BEHAVIOR AND IMMUNITY	14,533	6.170	0.025700
29	Advances in Immunology	2,544	5.771	0.004220
30	Journal of Neuroinflammation	11,767	5.700	0.023240
31	OncoImmunology	7,790	5.333	0.025470
32	Journal of the International AIDS Society	4,530	5.192	0.018770
33	JOURNAL OF INFECTIOUS DISEASES	45,452	5.045	0.076010
34	Allergy Asthma & Immunology Research	1,599	5.026	0.003240
35	npj Vaccines	282	5.020	0.001120
36	BIODRUGS	1,685	4.903	0.003370
37	CANCER IMMUNOLOGY IMMUNOTHERAPY	7,779	4.900	0.012870
38	Virulence	3,557	4.775	0.009120
39	Vaccines	1,077	4.760	0.003910
40	INFECTIOUS DISEASE CLINICS OF NORTH AMERICA	2,765	4.757	0.005160
41	CLINICAL AND EXPERIMENTAL ALLERGY	11,312	4.741	0.012900
42	JOURNAL OF IMMUNOLOGY	127,940	4.718	0.124400
43	Frontiers in Immunology	27,827	4.716	0.085880
44	EUROPEAN JOURNAL OF IMMUNOLOGY	22,037	4.695	0.024730

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RESEARCH Open Access

Neuronal aldosterone elicits a distinct genomic response in pain signaling molecules contributing to inflammatory pain

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Abstract

Background: Recently, mineralocorticoid receptors (MR) were identified in peripheral nociceptive neurons, and their acute antagonism was responsible for immediate and short-lasting (non-genomic) antinociceptive effects. The same neurons were shown to produce the endogenous ligand aldosterone by the enzyme aldosterone synthase.

Methods: Here, we investigate whether endogenous aldosterone contributes to inflammation-induced hyperalgesia via the distinct genomic regulation of specific pain signaling molecules in an animal model of Freund's complete adjuvant (FCA)-induced hindpaw inflammation.

Results: Chronic intrathecal application of MR antagonist canrenoate-K (over 4 days) attenuated nociceptive behavior in rats with FCA hindpaw inflammation suggesting a tonic activation of neuronal MR by endogenous aldosterone. Consistently, double immunofluorescence confocal microscopy showed abundant co-localization of MR with several pain signaling molecules such as TRPV1, CGRP, Nav1.8, and trkA whose enhanced expression of mRNA and proteins during inflammation was downregulated following i.t. canrenoate-K. More importantly, inhibition of endogenous aldosterone production in peripheral sensory neurons by continuous intrathecal delivery of a specific aldosterone synthase inhibitor prevented the inflammation-induced enhanced transcriptional expression of TRPV1, CGRP, Nav1.8, and trkA and subsequently attenuated nociceptive behavior. Evidence for such a genomic effect of endogenous aldosterone was supported by the demonstration of an enhanced nuclear translocation of MR in peripheral sensory dorsal root ganglia (DRG) neurons.

Conclusion: Taken together, chronic inhibition of local production of aldosterone by its processing enzyme aldosterone synthase within peripheral sensory neurons may contribute to long-lasting downregulation of specific pain signaling molecules and may, thus, persistently reduce inflammation-induced hyperalgesia.

Keywords: Aldosterone synthase, Pain signaling molecules, Sensory neurons

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Introduction

Apart from their role of maintaining the homeostasis of the body through regulation of numerous physiologic processes [1, 2], there is increasing evidence for the presence and functional role of glucocorticoid (GR) and mineralocorticoid receptors (MR) within the central nervous system [3–5]. Both genomic and non-genomic effects of these steroid receptors were described contributing to an enhanced plasticity of the central nervous system [3–5].

More recent findings from our laboratory identified MR mainly on peripheral, CGRP-immunoreactive nociceptive neurons [6], and GR on peripheral and spinal nociceptive neurons [7], whereas they only scarcely colocalized with spinal glia cells or astrocytes. The fact that GR and MR are localized predominantly on peripheral and/or spinal nociceptive neurons suggests a pivotal functional role in the processing of painful stimuli. Indeed, application of GR agonists and MR antagonists attenuated nociceptive behavior [7, 8]. This effect occurred immediately and lasted only up to 60 min indicating a non-genomic effect.

Surprisingly, the mRNA and protein of enzyme aldosterone synthase, the enzyme that processes the last step of conversion to aldosterone, were detected in the same nociceptive neurons [9]. Immunohistochemical double-staining showed great overlap between aldosterone synthase, aldosterone, and MR in peripheral nociceptive neurons. Following a localized painful hindpaw inflammation, the expression of aldosterone synthase and the production of aldosterone were upregulated [9]. Intrathecal administration of the MR antagonist canrenoate-K resulted in an immediate and short-lasting (up to 30 min) reversal of mechanical hypersensitivity most likely through a non-genomic effect [9].

In contrast to this short-lasting effect, there is no systematic investigation on the putative genomic effects of MR on nociception, particularly on the transcriptional regulation of certain pain signaling molecules within peripheral nociceptive neurons which contribute to inflammation-induced hyperalgesia. Covenas et al. [10] provided the first evidence supporting the notion that MR may stimulate neuronal peptide synthesis. Moreover, aldosterone selectively increased Na + -K + -ATPase mRNA expression in rat hippocampus [11], and chronic intra-cerebroventricular infusion of aldosterone synthase inhibitor FAD286 or MR blocker eplerenone reduced epithelial sodium channel c subunit expression in supraoptic nucleus [12] and hypothalamus [13].

Thus, this study investigated in rats with Freund's complete adjuvant-induced hind paw inflammation: (i) whether chronic i.t. application of the MR antagonist canrenoate-K attenuates nociceptive behavior, (ii) whether there is great overlap of MR with specific pain signaling molecules and whether their expression is affected by MR receptor blockade, (iii) whether the continuous inhibition of aldosterone's production by an i.t. aldosterone synthase inhibitor will reverse the enhanced expression of pain signaling molecules and subsequently attenuate inflammation-induced hyperalgesia, and (iv) finally, whether the increased production of endogenous aldosterone during FCA hindpaw inflammation leads to enhanced nuclear translocation of MR supporting the occurrence of genomic effects.

Material and methods

Drugs

Freund's complete adjuvant (FCA), a water-in-oil emulsion of killed mycobacteria (Calbiochem, San Diego, CA), isoflurane (Abbott, Wiesbaden, Germany), MR selective antagonist canrenoate-K, and aldosterone synthase inhibitor FAD286 were commercially obtained from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in 0.9% NaCl before application. Drugs were administered intrathecally either as single shot or via an intrathecal (i.t.) catheter as a continuous delivery using Alzet minipumps (Alzet Corporation, Cupertino, CA).

Animals

Following approval by the local animal care committee and according to the European Directive (2010/63/EU) introducing new animal welfare and care guidelines, experiments were performed in male Wistar rats (180–250 g) (breeding facility Charité-Universitätsmedizin Berlin, Germany). Wistar rats received an intraplantar (i.pl.) injection of 0.15 ml FCA into the right hind paw under brief isoflurane (1.0–2.5 Vol%) anesthesia. This treatment consistently produces a local inflammation restricted to the inoculated paw characterized by an increase in paw volume, paw temperature, mechanical hypersensitivity, and infiltration of various types of immune cells as described previously [14]. All experiments were performed on the fourth day of FCA hindpaw inflammation.

Experimental protocols

The first set of experiments examined the potential colocalization of MR with the pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA in sensory L3-5 DRG neurons of naïve control rats. A second set of experiments assessed the impact of i.t. injection of 40 μg/20 μl of aldosterone (over four consecutive days) in naïve rats or 100 μg/20 μl of the MR antagonist canrenoate-K (over four consecutive days) in rats with FCA hindpaw inflammation on mechanical paw pressure thresholds (PPT). The i.t. doses chosen are too low to reach effective plasma concentrations [15, 16] and, thus, will not elicit any systemic effect [17]. The third set of experiments investigated the influence of 4 days FCA hindpaw inflammation on significant changes in the aldosterone, MR,

TRPV1, CGRP, Nav1.8, and trkA expression in sensory L3-5 DRG neurons ipsilateral to the FCA hindpaw inflammation. The doses chosen for each drug were based on generated dose-response curves in our previous studies [6–8]. A final set of experiments investigated the effects of continuous i.t. delivery of the aldosterone synthase inhibitor FAD286 on mechanical PPT and neuronal TRPV1, CGRP, Nav 1.8, and trkA expression after 4 days FCA hindpaw inflammation. For i.t. FAD286 application, Alzet osmotic minipumps (2000 µl, rate 5 µl/h) were filled with 0.9% NaCl with or without 0.3ug/1 µ1 FAD286 and connected to the i.t. catheter to administer FAD286 or vehicle continuously at 5 µl/h; an i.t. dose of 1.5 µg/5 µl/h will not lead to effective plasma concentrations and, thus, will not elicit any systemic effect [18].

For i.t. drug administration, animals were anesthetized with isoflurane in oxygen via nose cone, and a longitudinal skin incision was made in the lumbar region directly above the spinous processes of the L3–L5 vertebrae as described previously [19]. Briefly, the i.t. needle was inserted at a 30° angle between the L4 and L5 vertebra into the i.t. space and either a single injection or the placement of an i.t. catheter (PE 10 tubing attached to PE 60 tubing for attachment to an osmotic pump; Portex Ltd, Hythe, Kent, United Kingdom) was set up. The sign of dura penetration was observed by involuntary movements of the tail or hind limb and verified the i.t. space for drug delivery.

Mechanical hyperalgesia testing

The mechanical hyperalgesia following FCA hindpaw inflammation was assessed by a mechanical pressure apparatus (Ugo-Basile SRL, Monvalle, Italy) with increasing force (measured in grams) applied to the plantar hind paw until a withdrawal reflex was precipitated as described previously [8]. Mechanical paw pressure thresholds (PPT) which triggered a withdrawal response were determined in all groups on the fourth day of FCA hindpaw inflammation. Drug application baseline values were obtained in both inflamed and contralateral non-inflamed hind paws. Then, the PPT were reassessed 2 h following the last i.t. drug administration to determine drug-related behaviors. PPT measurements were performed 3 times consecutively. The final PPT were calculated as the mean obtained from 6–7 animals before and after i.t. drug administration. In all behavioral experiments, drugs were prepared by a different person (M.Sh.), and the examiner (X.L.) was unaware of the treatment that each animal received by chance.

TRPV1, CGRP, Nav1.8, and trkA mRNA detection by quantitative RT-PCR

Total RNA was extracted from L3-5 dorsal root ganglia of Wistar rats (n = 5 per experimental group) using RNeasy Kit (Qiagen, Hilden, Germany) as previously described [8, 20]. The following specific primers were generated and used: for TRPV1, forward primer: AGTGAG ACCCCTAACCGTCA, reverse primer: CGGAAATAGT CCCCAACGGT (Ensembl, Accession Nr: NM_031982.1); for CGRP, forward primer: CCTTTCCTGGTTGTCAGC ATCTT, reverse primer: CAGTAGGCG AGCTTCTTCT TCAC (Ensembl, Accession NM_001033956.1); for Nav.1.8, forward primer: CACGGATGACAACAGGTC AC, reverse primer; GATCCCGTCAGGAAATGAGA (Ensembl, Accession Nr: NM_017247.1); for trkA, forward primer: CCATCCCTGTCT CCTTCTCGC, reverse primer: CCCAAAAGGTGTTTCGTCCTTC (Ensembl, Accession Nr: NM_021589.1). Quantitative real-time PCR (RT-PCR) was performed with a SYBR® Green kit following the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). Amplification was carried out for 40 cycles, each consisting of 15 s at 95 °C. A temperature just below the specific melting temperature (Tm) was employed for detection of fluorescence specific products (TRPV1: Tm 76 °C, 18S: Tm 83 °C). TRPV1, CGRP, Nav1.8, and trkA mRNA were quantified using triplicates of samples using the delta-delta CT method [21, 22].

Aldosterone content measurements in DRG

In deep isoflurane anesthesia rats (n = 7–8 per group) were sacrificed, L3-5 DRG were quickly removed in DMEM–Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific GmbH Berlin, Germany). DRG were prepared as described previously [8, 23]. Briefly, 1 mg/ml collagenase IV was added to the DRG and incubated for 30 min at 37 °C. To stop the collagenase activity, 0.05% trypsin was added and again incubated for 10 min at 37 °C. Finally, the sample was centrifuged at 500×g for 5 min at room temperature and the pellet resuspended in 1 ml RPMI 1640 (GibCO, Thermo Fischer, Dreieich, Germany). After mechanical cell lysis and centrifugation at 500×g for 5 min, the aldosterone content in DRG was determined by a commercial kit from R&D Systems (Minneapolis, MN, USA, Cat. # KGE016). For this, we followed the protocol according to the manufacture's manual and our previous study [8]. Briefly, all reagents and samples were brought to room temperature before use. Determinations of aldosterone in the different samples and standards were done in triplicate as recommended by the manufacture. The optical density of each well was determined within 30 min using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland) set to 450 nm. Then, the average of the triplicate readings for each standard, control and sample were determined and subtracted by the average NSB optical density (O.D.). A standard curve was created using the computer software Magellan Vers. 7.2 (Tecan, Männedorf, Switzerland)

capable of generating a four-parameter logistic (4-PL) curve-fit. The results were calculated by wet weight.

Immunohistochemistry

After transcardial perfusion of rats L3-L5 DRG were removed and further processed as described previously [6, 8], slide mounted tissue sections (8 μm) were obtained by using a kryostat (Microm,Thermo Fischer, Dreieich, Germany). Tissue sections were then incubated overnight with the following primary antibodies (see also supplemental table 1): mouse antibody against MR (private gift from Prof. Elise Gomez-Sanchez, Jackson, USA) was examined alone or in combination with a polyclonal rabbit anti-Nav1.8 (Sigma-Aldrich, USA), guinea pig anti-CGRP, goat anti-trkA (R&D Systems, USA) or goat anti-TRPV1 (Santa Cruz Biotechnology, California): in addition, polyclonal rabbit anti-aldosterone (Novus Biologicals, CO, USA) alone or in combination with the polyclonal guinea pig anti-CGRP or monoclonal mouse anti-MR. The species, sources, dilutions, and immunogens of the primary antibodies used in this study are summarized in the supplemental table 1. Finally, the tissues were washed in PBS, mounted on vectashield (Vector Laboratories), and imaged with a confocal laser scanning microscope, LSM510 as described previously [8]. To demonstrate specificity of staining, the following controls were included as described in our previous studies [7, 8, 20]: omission of either the primary antisera or the secondary antibodies.

The quantification of DRG staining has been described previously [7, 8, 20]. Quantification of immunofluorescence of MR, TRPV1, CGRP, Nav1.8, and trkA in DRG tissue sections was performed by using the Zeiss Zen 2009 software Carl Zeiss Micro-Imaging GmbH (Göttingen, Germany). For counting of the total number of neurons, only those immunostained neurons containing a distinct nucleus were counted. In a similar way, the number of aldosterone-ir DRG cells/320μm^2, MR-ir DRG cells/320μm^2, TRPV1-ir DRG cells/320μm^2, CGRP-ir DRG cells/320μm^2, Nav1.8-ir DRG cells/320μm^2, and trkA-ir DRG cells/320μm^2 cells was counted in each DRG section and represented as percentages. Data were obtained from 4–6 rats per group using × 40 objective lens.

Preparation and immunoblotting of cytosol and nuclear extract

The isolated DRG neurons from control or FCA treated animals were washed three times in ice cold HANKs solution (10,000 rpm/3 min) to remove any remaining medium or buffer from the previous step of cell isolation. For extraction of cytoplasma, the supernatent was carefully removed and the cell pellet resuspended in cytoplasmic extraction buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) containing 0.1 mM DTT, 0.1 mM PMSF, 2 μg/ml leupetin, 2 μg/ml aprotinin, 0.5 mg/ml benzamidin, and incubated on ice for 30 min. Then, 10% NP-40 was added, each sample vortexed for 30 s, centrifuged (10 000 rpm/3 min), and the supernatant (cytosol extract) transferred to a new tube and stored at − 80 °C. For further preparation of the nuclear extract, the remaining pellet (containing the nuclei) was re-suspended in nuclear extraction buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupetin, 2 μg/ml aprotinin, 0.5 mg/ml benzamidin, and incubated for 30 min on ice. Finally, samples were centrifuged (10,000 rpm/3 min), and supernatant containing nuclear extract was transferred to a new tube and stored at − 80 °C. The total protein content in samples was measured using the bicinchinonic acid system (Uptima, France), the samples were reduced with 2-mercaptoethanol, and the protein contents were adjusted. The samples were separated by SDS-PAGE electrophoresis (7% gels), transferred to a nitrocellulose membrane, washed for 2 h with skimmed milk buffer for unspecific blocking, and incubated with primary antibodies for mineralocorticoid receptor (rMR), Poly (ADP-ribose) polymerase (PARP), or β-actin overnight at 4 °C. Finally, the membranes were washed three times with skimmed milk buffer, incubated with secondary antibodies for 2 h, and antibody-antigen complexes visualized by nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (p-toluidine salt; Pierce, Rockford, USA). Experiments were done in 6 animals per group in duplicate.

The Western blot band specific for rMR (107 kDa) or PARP (110 kDa) was quantified by the Java Image processing and analysis software (Image J, open-source image software downloaded from the web[1] [6, 7]. The area and density of pixels within the threshold values representing immunoreactivity were measured, and the integrated density (the product of the area and mean of grey values) was calculated. Integrated immunodensities of controls and treated groups were compared and statistically analysed [19].

Statistical analysis

All tests were performed using the Sigma Stat 2.03 software (SPSS Inc., Germany). Quantitative RT-PCR, Western blot, and immunofluorescence data were analyzed as two group comparisons (FCA treated rats versus controls) by a two-tailed independent Student *t* test in case of normally distributed data. Three group comparisons were done for quantitative RT-PCR and immunohistochemistry experiments by one-way ANOVA followed by a post-hoc Dunnett's test. Paw pressure thresholds (PPT) were determined before and after drug injections within the same group of animals, expressed as means ± SD, and statistically analyzed by a

[1] http://rsb.info.nih.gov/ij/. Last accessed November 28, 2018

repeated measurement-ANOVA, followed by post-hoc Dunnett's test. For all statistical tests, significance was assumed at $P < 0.05$.

Results

Chronic antagonism of endogenous aldosterone or administration of exogenous aldosterone reveals aldosterone's contribution to an enhanced mechanical hypersensitivity

Four days of FCA-induced inflammation of the right hindpaw resulted in diminished mechanical PPT thresholds in inflamed compared to non-inflamed hindpaws (dashed line, Fig. 1) ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test, Fig. 1a). This enhanced mechanical hypersensitivity in inflamed hindpaws was significantly attenuated following daily i.t. administrations of the MR selective antagonist canrenoate-K ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test; Fig. 1a). Canrenoate-K had no effect on the contralateral non-inflamed hindpaw (data not shown), similar to previous reports in naive rats [8]. Conversely in naïve control rats, repeated i.t. administration of the MR selective agonist aldosterone over 4 days resulted in a significant decrease of mechanical PPT thresholds and, thus, enhanced sensitivity to mechanical stimuli ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test; Fig. 1b), while vehicle administration did not significantly alter mechanical PPT ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test; Fig. 1b).

Chronic intrathecal MR antagonism inhibits the enhanced transcription of pain signaling molecules colocalizing with MR during inflammatory pain

Our double immunofluorescence confocal microscopy showed abundant colocalization of MR with several pain signaling molecules such as TRPV1, CGRP, Nav1.8, and trkA (TRPV1 68%; CGRP 79%; Nav1.8 74 %; trkA 56%) (Fig. 2). To investigate potential genomic effects of MR on the expression of these pain signaling molecules, we repeatedly treated rats with FCA hindpaw inflammation over 4 days with i.t. administration of the MR antagonist canrenoate-K (Fig. 3a–d). Indeed, the significantly enhanced mRNA expression of pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA in sensory DRG ipsilateral to the FCA hindpaw inflammation was significantly attenuated by chronic i.t. canrenoate-K delivery ($P < 0.05$, one-way ANOVA, followed by Dunnett's test; Fig. 3a–d). Consistent with these findings, the significantly increased numbers of TRPV1-, CGRP-, Nav1.8-, and trkA-immunoreactive DRG neurons ipsilateral to the FCA hindpaw inflammation were significantly reduced by chronic i.t. canrenoate-K administration ($P < 0.05$, one-way ANOVA, followed by Dunnett's test; Fig. 4a–p), although the total number of DRG cells was not significantly altered (Ctrl 45 ± 8.5; FCA 45 ± 8.8; FCA + Can 42 ± 12, $P = 0.43$ one-way ANOVA).

Enhanced MR translocation to the nucleus of DRG neurons during inflammatory pain

Following several steps of DRG cell extract separation into a nuclear and cytosolic fraction subsequent western blot analysis identified a MR specific protein band at 107 kDa in

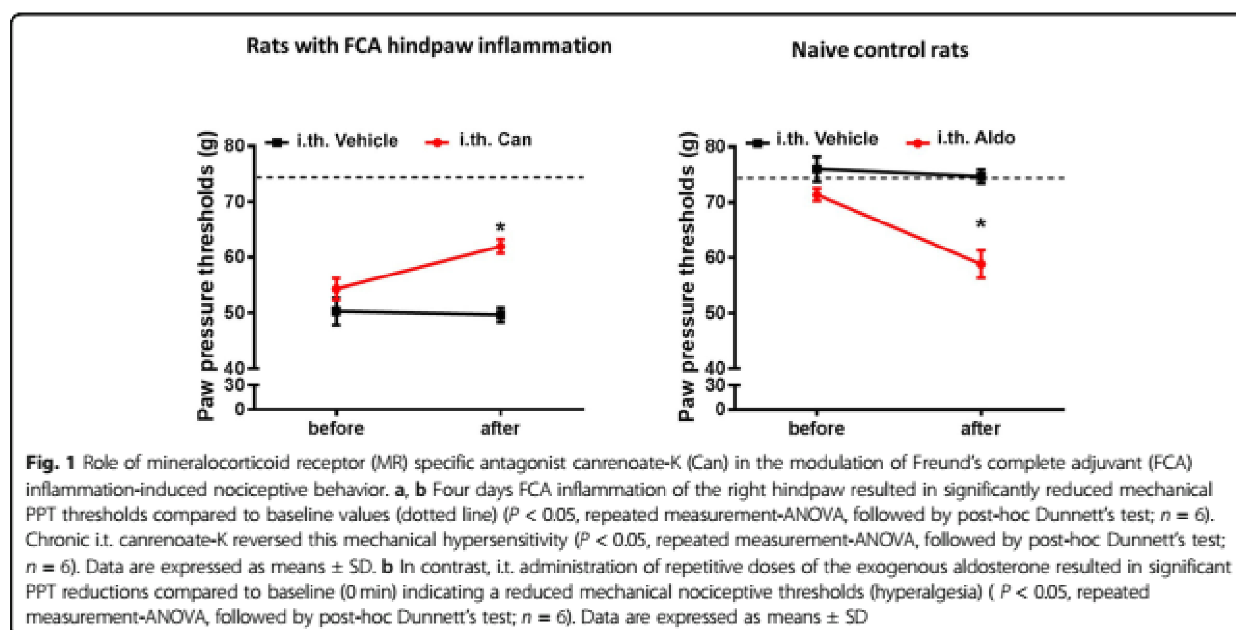

Fig. 1 Role of mineralocorticoid receptor (MR) specific antagonist canrenoate-K (Can) in the modulation of Freund's complete adjuvant (FCA) inflammation-induced nociceptive behavior. **a**, **b** Four days FCA inflammation of the right hindpaw resulted in significantly reduced mechanical PPT thresholds compared to baseline values (dotted line) ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test; $n = 6$). Chronic i.t. canrenoate-K reversed this mechanical hypersensitivity ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test; $n = 6$). Data are expressed as means ± SD. **b** In contrast, i.t. administration of repetitive doses of the exogenous aldosterone resulted in significant PPT reductions compared to baseline (0 min) indicating a reduced mechanical nociceptive thresholds (hyperalgesia) ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test; $n = 6$). Data are expressed as means ± SD

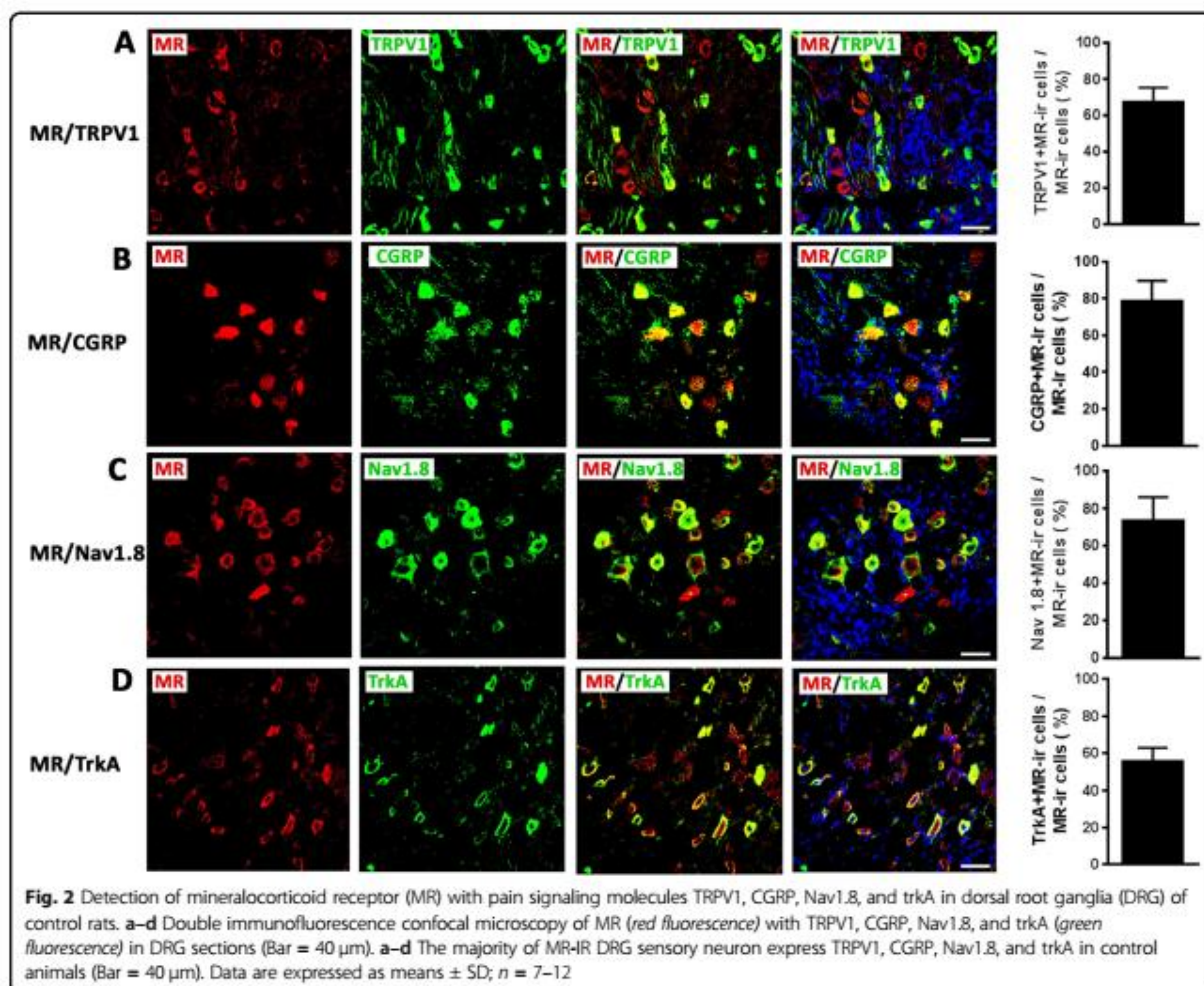

Fig. 2 Detection of mineralocorticoid receptor (MR) with pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA in dorsal root ganglia (DRG) of control rats. **a–d** Double immunofluorescence confocal microscopy of MR (*red fluorescence*) with TRPV1, CGRP, Nav1.8, and trkA (*green fluorescence*) in DRG sections (Bar = 40 μm). **a–d** The majority of MR-IR DRG sensory neuron express TRPV1, CGRP, Nav1.8, and trkA in control animals (Bar = 40 μm). Data are expressed as means ± SD; n = 7–12

both fractions (Fig. 5a, b). The detection of PARP (110 kDa), which is a nuclear specific enzyme for DNA repair, validated the nuclear fraction, whereas the detection of ß-actin verified the cytosolic fraction (Fig. 5a, b). In the nuclear but not cytosolic fraction, DRG innervating the inflamed hindpaws of rats showed a significant increase in the density of the MR protein band compared to those DRG innervating non-inflamed hindpaws ($P < 0.05$, Student's t test; Fig. 5a, b). This was confirmed by immunohistochemistry of the respective DRG showing an increasing overlap of MR-immunoreactivity with the nuclear DAPI staining indicating enhanced nuclear translocation following 4 days of FCA-induced hindpaw inflammmation.

Chronic intrathecal aldosterone synthase inhibition prevents the enhanced expression of pain signaling molecules and the elevated mechanical sensitivity during inflammatory pain

Since aldosterone synthesized in peripheral sensory DRG neurons seemed to contribute to inflammatory pain in rats with FCA hindpaw inflammation, we examined whether continuous i.t. infusion of aldosterone synthase inhibitor FAD286 diminished aldosterone synthesis in DRG of inflamed rats to that of controls. Indeed, i.t. infusion of FAD286 but not vehicle in FCA treated rats significantly reduced the number of aldosterone-IR DRG neurons determined by immunofluorescence confocal analysis ($P < 0.05$, Student's t test; Fig. 6a–c), although the total number of DRG cells was not significantly altered (Ctrl 45 ± 8.5; FCA 45 ± 8.8; FCA + FAD 40 ± 6.7, P = 0.433 one-way ANOVA). Moreover, aldosterone specific ELISA experiments revealed that the increase in aldosterone DRG content of FCA animals was also significantly diminished following i.t. aldosterone synthase inhibitor FAD286 ($P < 0.05$, Student's t test; Fig. 6d). To verify whether the reduced aldosterone content in DRG would affect the enhanced expression of the pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA following FCA hindpaw inflammation, we examined DRG neuron mRNA as well as the number of DRG neurons

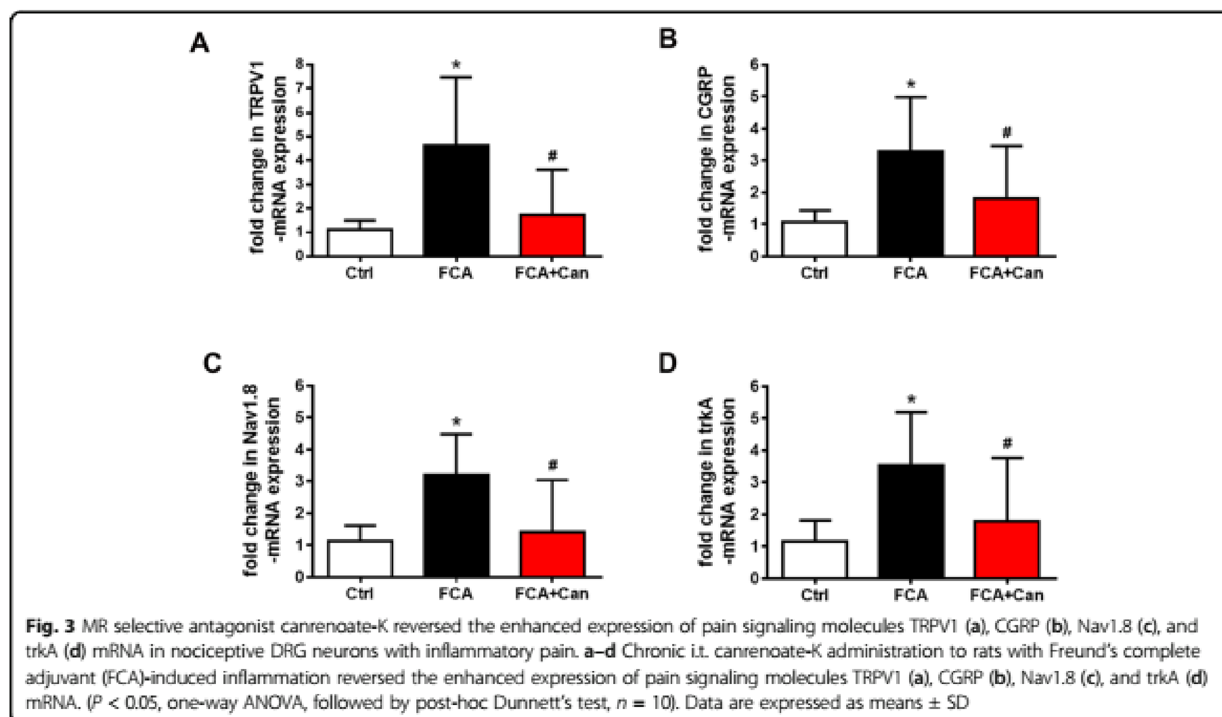

Fig. 3 MR selective antagonist canrenoate-K reversed the enhanced expression of pain signaling molecules TRPV1 (**a**), CGRP (**b**), Nav1.8 (**c**), and trkA (**d**) mRNA in nociceptive DRG neurons with inflammatory pain. **a–d** Chronic i.t. canrenoate-K administration to rats with Freund's complete adjuvant (FCA)-induced inflammation reversed the enhanced expression of pain signaling molecules TRPV1 (**a**), CGRP (**b**), Nav1.8 (**c**), and trkA (**d**) mRNA. ($P < 0.05$, one-way ANOVA, followed by post-hoc Dunnett's test, $n = 10$). Data are expressed as means ± SD

containing these molecules following chronic i.t. administration of aldosterone synthase inhibitor FAD286. This treatment significantly prevented the inflammation-induced increase in mRNA ($P < 0.05$, Student's t test; Fig. 6e–h) and in the number of TRPV1-, CGRP-, Nav1.8-, and trkA-IR nociceptive DRG neurons ($P < 0.05$, Student's t test; Fig. 7a–l). Concomitant with this prevention, the FCA-induced enhanced mechanical hypersensitivity was significantly reduced following i.t. FAD286 ($P < 0.05$, Student's t test; Fig. 7m).

Discussion

For the first time, we have systematically investigated the potential long-lasting genomic effects of endogenous MR agonist aldosterone on the transcriptional regulation of several pain signaling molecules in peripheral sensory DRG neurons during inflammatory pain. Previous studies have demonstrated MR and its endogenous ligand aldosterone in these neurons [8, 9]. Here, chronic i.t. antagonism of endogenous aldosterone during hindpaw inflammation or, conversely, chronic application of exogenous aldosterone in animals without hindpaw inflammation attenuated or evoked mechanical hypersensitivity, respectively. Since the i.t. doses chosen are too low to elicit systemic effects, the findings suggest a persistent endogenous tonic activation of neuronal MR during local inflammation. Interestingly, double immunofluorescence confocal microscopy showed abundant colocalization of MR with several pain signaling molecules such as TRPV1, CGRP, Nav1.8, and trkA. Moreover, continuous (over 4 days) inhibition of endogenous aldosterone production in peripheral sensory neurons by i.t. application of the specific aldosterone synthase inhibitor FAD286 prevented the enhanced transcriptional expression of pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA and subsequently attenuated the nociceptive behavior during hindpaw inflammation. This genomic effect of endogenous aldosterone during inflammatory pain was supported by the demonstration of an enhanced translocation of MR from the cytosol to the nucleus in peripheral sensory DRG neurons.

Previously, we have demonstrated that the final conversion of 18-hydroxycorticosterone into aldosterone by aldosterone synthase also occurs in peripheral sensory DRG neurons [9] confirming preceding reports on the expression of aldosterone synthase outside the adrenal gland, e.g., in brain, heart, aortic endothelial cells, and in vascular smooth muscle [24–26]. Moreover, we have shown that a single systemic or intrathecal administration of the MR antagonist canrenoate-K resulted in an immediate and short-lasting (up to 30 min) reversal of mechanical hypersensitivity most likely through a non-genomic effect [9]. In contrast to this short-lasting effect, we investigated in this study whether chronic antagonism of aldosterone's action or continuous inhibition of aldosterone synthesis (over 4 days) resulted in alterations of the genomic expression of specific pain signaling molecules such as TRPV1, CGRP, Nav1.8, and trkA and

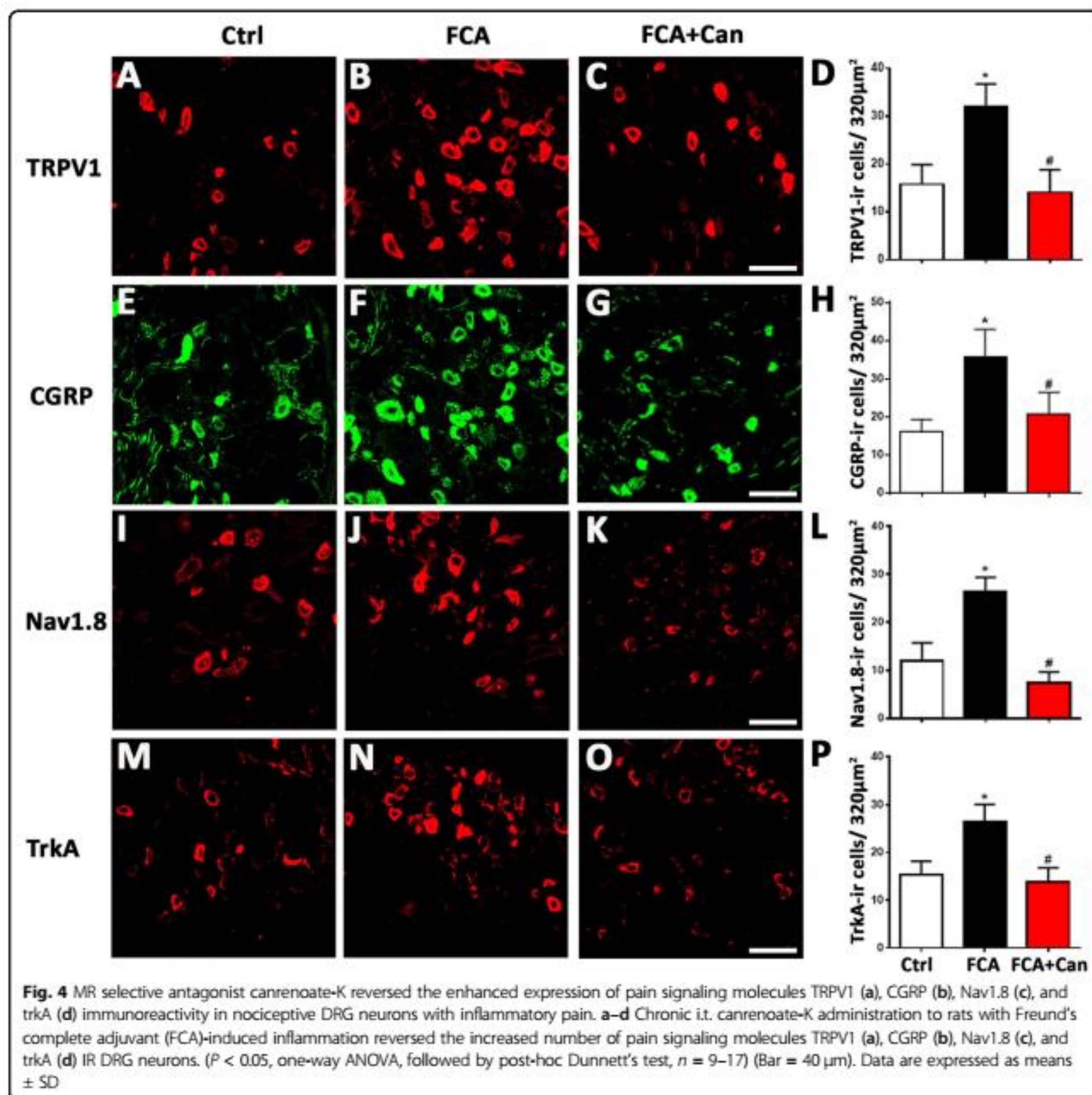

Fig. 4 MR selective antagonist canrenoate-K reversed the enhanced expression of pain signaling molecules TRPV1 (**a**), CGRP (**b**), Nav1.8 (**c**), and trkA (**d**) immunoreactivity in nociceptive DRG neurons with inflammatory pain. **a–d** Chronic i.t. canrenoate-K administration to rats with Freund's complete adjuvant (FCA)-induced inflammation reversed the increased number of pain signaling molecules TRPV1 (**a**), CGRP (**b**), Nav1.8 (**c**), and trkA (**d**) IR DRG neurons. ($P < 0.05$, one-way ANOVA, followed by post-hoc Dunnett's test, $n = 9–17$) (Bar = 40 µm). Data are expressed as means ± SD

whether this resulted in changes of nociceptive behavior following FCA-induced hindpaw inflammation.

First, we demonstrated that chronic i.t. administration of the MR selective antagonist canrenoate-K attenuated mechanical hypersensitivity in inflamed hindpaws suggesting a tonic activation of neuronal MR by endogenous aldosterone. Conversely, chronic i.t. administration of exogenous aldosterone in naïve rats resulted in mechanical hypersensitivity. Previous studies have demonstrated that the MR-immunoreactivity within the dorsal horn of the spinal cord mainly derives from peripherally incoming CGRP-ir nociceptive neurons in Rexed laminae I and II, and only few scattered neuronal cells were identified in Rexed laminae III and IV [6]. Although the corticosterone inactivating enzyme 11ßHSD2 colocalized with sensory neuron MR allows MR activation predominantly by aldosterone [8], the co-activation by corticosterone cannot be fully excluded. Our findings are in agreement with older studies which have shown a long-lasting analgesic effect of MR antagonists [27, 28].

Then, we found that several potential pain signaling molecules such as TRPV1, CGRP, Nav1.8, and trkA showed a 60–75% overlap with MR-immunoreactive DRG neurons, which is consistent with previous studies

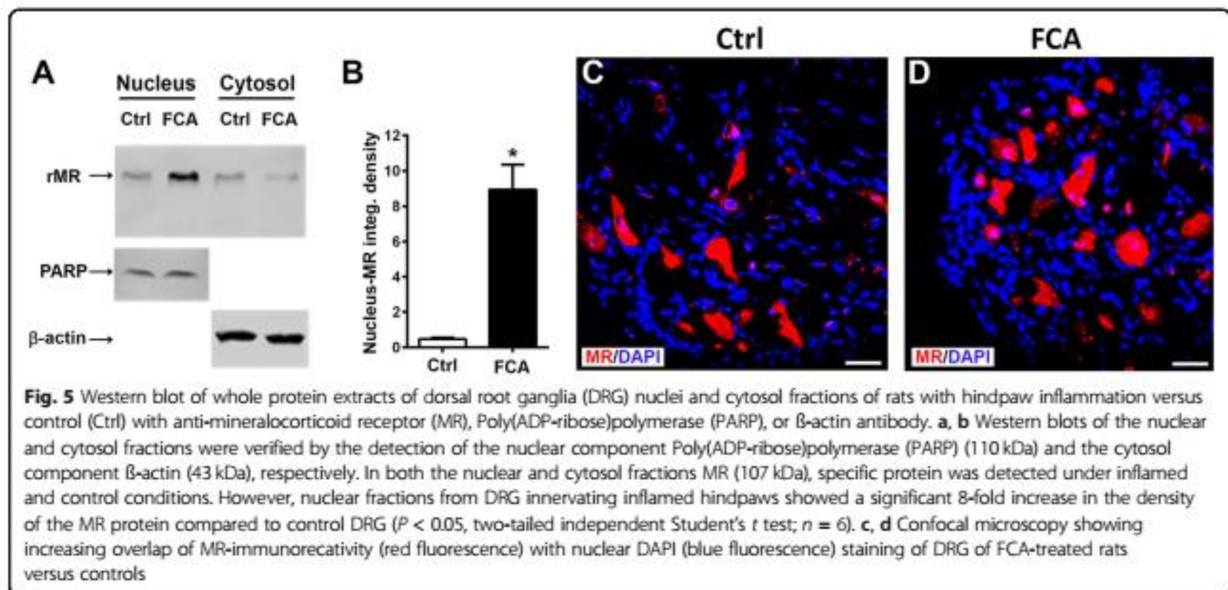

Fig. 5 Western blot of whole protein extracts of dorsal root ganglia (DRG) nuclei and cytosol fractions of rats with hindpaw inflammation versus control (Ctrl) with anti-mineralocorticoid receptor (MR), Poly(ADP-ribose)polymerase (PARP), or ß-actin antibody. **a**, **b** Western blots of the nuclear and cytosol fractions were verified by the detection of the nuclear component Poly(ADP-ribose)polymerase (PARP) (110 kDa) and the cytosol component ß-actin (43 kDa), respectively. In both the nuclear and cytosol fractions MR (107 kDa), specific protein was detected under inflamed and control conditions. However, nuclear fractions from DRG innervating inflamed hindpaws showed a significant 8-fold increase in the density of the MR protein compared to control DRG ($P < 0.05$, two-tailed independent Student's t test; $n = 6$). **c**, **d** Confocal microscopy showing increasing overlap of MR-immunorecativity (red fluorescence) with nuclear DAPI (blue fluorescence) staining of DRG of FCA-treated rats versus controls

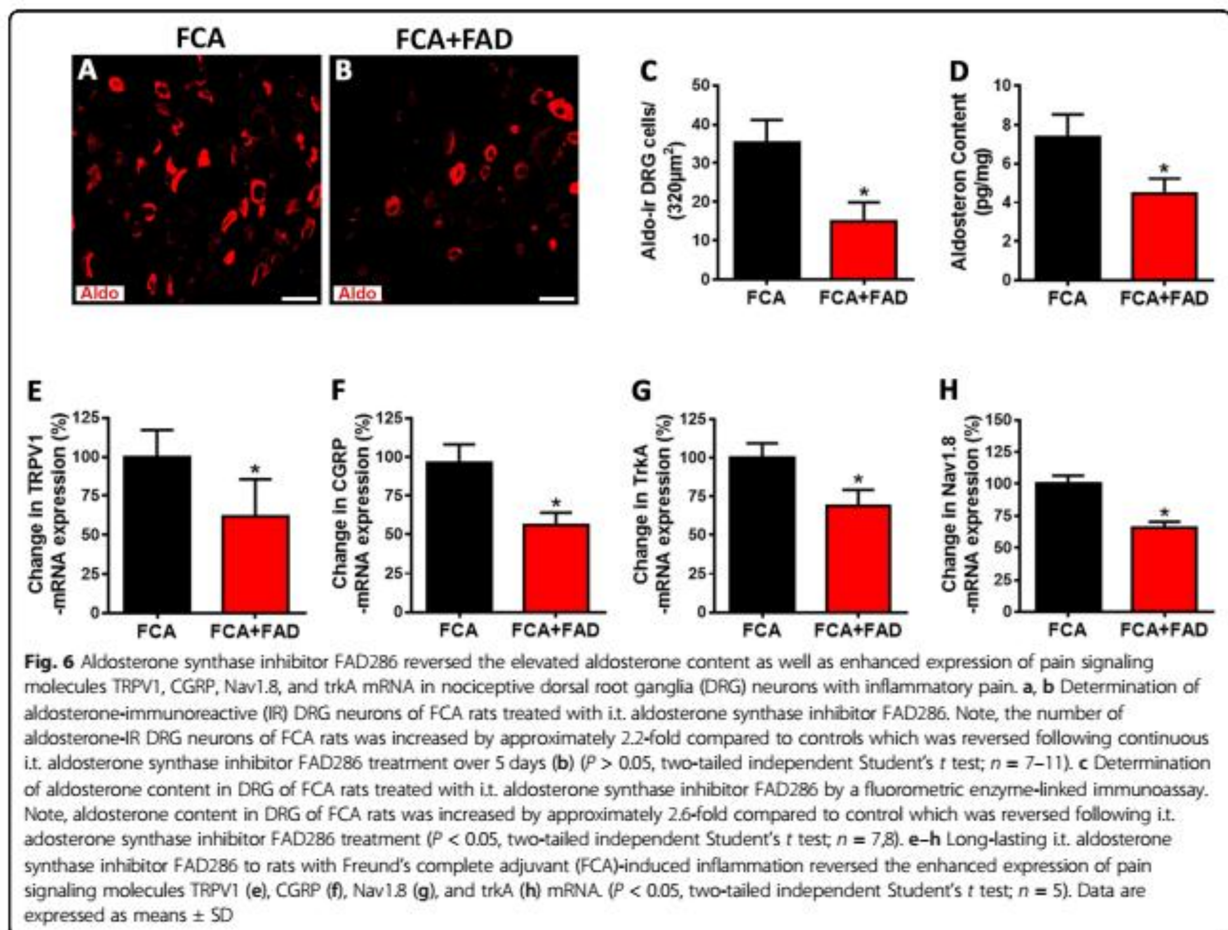

Fig. 6 Aldosterone synthase inhibitor FAD286 reversed the elevated aldosterone content as well as enhanced expression of pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA mRNA in nociceptive dorsal root ganglia (DRG) neurons with inflammatory pain. **a**, **b** Determination of aldosterone-immunoreactive (IR) DRG neurons of FCA rats treated with i.t. aldosterone synthase inhibitor FAD286. Note, the number of aldosterone-IR DRG neurons of FCA rats was increased by approximately 2.2-fold compared to controls which was reversed following continuous i.t. aldosterone synthase inhibitor FAD286 treatment over 5 days (**b**) ($P > 0.05$, two-tailed independent Student's t test; $n = 7$–11). **c** Determination of aldosterone content in DRG of FCA rats treated with i.t. aldosterone synthase inhibitor FAD286 by a fluorometric enzyme-linked immunoassay. Note, aldosterone content in DRG of FCA rats was increased by approximately 2.6-fold compared to control which was reversed following i.t. aldosterone synthase inhibitor FAD286 treatment ($P < 0.05$, two-tailed independent Student's t test; $n = 7,8$). **e**–**h** Long-lasting i.t. aldosterone synthase inhibitor FAD286 to rats with Freund's complete adjuvant (FCA)-induced inflammation reversed the enhanced expression of pain signaling molecules TRPV1 (**e**), CGRP (**f**), Nav1.8 (**g**), and trkA (**h**) mRNA. ($P < 0.05$, two-tailed independent Student's t test; $n = 5$). Data are expressed as means ± SD

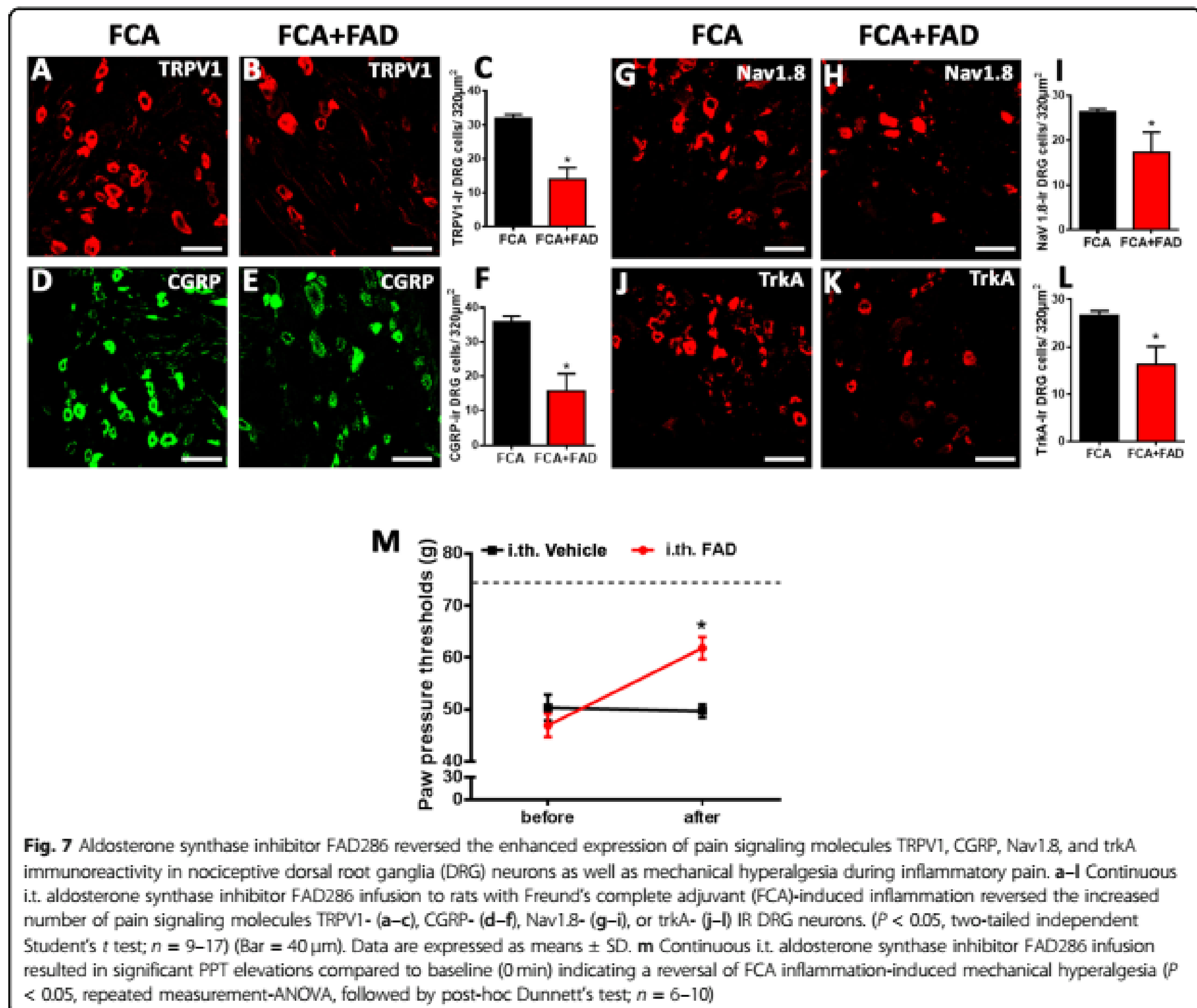

Fig. 7 Aldosterone synthase inhibitor FAD286 reversed the enhanced expression of pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA immunoreactivity in nociceptive dorsal root ganglia (DRG) neurons as well as mechanical hyperalgesia during inflammatory pain. **a–l** Continuous i.t. aldosterone synthase inhibitor FAD286 infusion to rats with Freund's complete adjuvant (FCA)-induced inflammation reversed the increased number of pain signaling molecules TRPV1- (**a–c**), CGRP- (**d–f**), Nav1.8- (**g–i**), or trkA- (**j–l**) IR DRG neurons. ($P < 0.05$, two-tailed independent Student's *t* test; $n = 9–17$) (Bar = 40 μm). Data are expressed as means ± SD. **m** Continuous i.t. aldosterone synthase inhibitor FAD286 infusion resulted in significant PPT elevations compared to baseline (0 min) indicating a reversal of FCA inflammation-induced mechanical hyperalgesia ($P < 0.05$, repeated measurement-ANOVA, followed by post-hoc Dunnett's test; $n = 6–10$)

on the identification of certain subpopulations of MR expressing peripheral sensory neurons [6, 7]. In general, literature on a potential colocalization of neuronal MR with pain signaling molecules is scarce. Derbenev et al. for example reported about a putative colocalization of GR with TRPV1, since whole-cell patch-clamp recordings in the dorsal nucleus of the vagal nerve showed GR-mediated activation of TRPV1 receptors on afferent terminals [29]. In line with older reports [10, 30, 31] about a coexistence of GR with the spinal neuropeptides substance P and CGRP, our previous studies have demonstrated that MR and GR colocalize with CGRP in DRG neurons [6, 7].

Furthermore, our data show that the expression of these pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA is upregulated in DRG neurons ipsilateral to the local hindpaw inflammation consistent with numerous older studies [32–35]. Interestingly, chronic i.t. administration of the MR antagonist canrenoate-K in rats with FCA-induced hindpaw inflammation caused a downregulation of the mRNA and immunoreactivity of these pain signaling molecules in DG neurons suggesting that an intrinsic tonic activation of sensory neuron MR by endogenous aldosterone may contribute to this upregulation. Importantly, this downregulation in various pain signaling molecules was not caused by a decrease in the total number of DRG cells according to our results.

What is the most likely mechanism? It is widely accepted that aldosterone functions by binding to the intracellular MR, a ligand-activated transcription factor and member of the nuclear hormone receptor family, resulting in late genomic effects [36, 37] [10]. provided the first evidence for the notion that the MR agonist aldosterone stimulates the upregulation of substance P and somatostatin after adrenalectomy by a genomic mechanism. Moreover, aldosterone, through its interaction with the MR, increased transient receptor potential canonical 1 and 6 expression in the adrenal medulla

of metabolic syndrome pigs [38–40]. In a similar way, aldosterone selectively increased Na^{+}-K^{+}-ATPase mRNA expression in rat hippocampus [11]. Similarly, chronic intra-cerebroventricular infusion of aldostrone synthase inhibitor FAD286 or MR blocker eplerenone reduced epithelial sodium channel C subunit expression in the supraoptic nucleus [12] and hypothalamus [13]. Taken together, this emerging evidence suggests that neuronal aldosterone may play a potential role in the transcriptional regulation of pain signaling molecules in peripheral sensory neurons.

A genomic effect of neuronal aldosterone is further supported by our demonstration of an enhanced translocation of MR from the cytosol to the nucleus of peripheral sensory DRG neurons. Following fractionation of homogenized DRG into a nuclear and a cytosolic compartment, MR were identified in both DRG of control rats and DRG of rats with an inflamed hindpaw. However, the intensity of the protein band was 8-fold higher in the nuclear than the cytosolic compartment under inflammatory pain conditions indicating an enhancement of nuclear translocation and, thus, supporting a genomic mechanism. This notion was confirmed by immunohistochemistry of the respective DRG showing an increased overlap of MR-immunorecativity with the nuclear marker DAPI and was supported by similar demonstrations of MR nuclear translocation within sensory afferent neurons of the cochlea [41] and striatum [42].

The next question arises: what are the functional consequences of blocking the 18-hydroxycorticosterone conversion into aldosterone via long-lasting inhibition of aldosterone synthase in nociceptive neurons? Since aldosterone content of peripheral sensory DRG was significantly elevated following inflammation, we examined whether i.t. infusion of aldosterone synthase inhibitor FAD286 reduced the aldosterone content in DRG of inflamed rats to control levels. Indeed, chronic i.t. infusion of FAD286 over 4 days significantly reduced aldosterone content as well as the number of aldosterone-IR DRG neurons in FCA rats. Consequently, this FAD286-mediated aldosterone reduction during FCA hindpaw inflammation not only reversed the enhanced expression of the pain signaling molecules TRPV1, CGRP, Nav1.8, and trkA, but also attenuated nociceptive behavior. Our findings of a transcriptional regulation of pain signaling molecules and subsequent nociceptive behavior are consistent with older studies [27, 28] which showed a long-lasting analgesic effect of MR antagonists.

Conclusion

In summary, our present study demonstrates that long-lasting blockade of endogenous aldosterone from activating MR on sensory DRG neurons during local inflammation not only prevents the enhanced transcriptional expression of pain signaling molecules such as TRPV1, CGRP, Nav1.8, and trkA within peripheral sensory neurons but also subsequently attenuates mechanical hypersensitivity. This genomic effect of endogenous aldosterone during inflammatory pain was supported by the demonstration of an enhanced nuclear translocation of MR in peripheral sensory DRG neurons. Together, these findings suggest that local production of aldosterone within peripheral sensory DRG contributes to ongoing mechanical hypersensitivity via continuous activation of neuronal MR, most likely through genomically regulated enhanced expression of pain signaling molecules.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12974-020-01864-8.

Additional file 1: Table S1. Characterization of primary antibodies used

Abbreviations

MR: Mineralocorticoid receptors; FCA: Freund's complete adjuvant; GR: Glucocorticoid receptors; PPT: paw pressure thresholds; DRG: Dorsal root ganglia; Can: Canrenoate-K; PARP: Poly (ADP-ribose) polymerase

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Authors' contributions

MoSh, MS, LL, ST, SM, and AB designed the experiments. MoSh, LL, MeSha, DM, XL, CB, and SM performed the experiments. MoSh, LL, ST, MeSha, DM, XI, MS, and SM performed analysis and interpretation of the experiments. MoSh, LL, SM, and MS wrote and all co-authors critically reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author (Shaaban.mousa@charite.de) on reasonable request. The authors will take responsible for maintaining availability.

Ethics approval and consent to participate

All animal experiments were approved by the local animal care committee (reference No. G0024/14, Landesamt für Gesundheit und Soziales, LaGeSo Berlin) and according to the European Directive (2010/63/EU) introducing new animal welfare and care guideline.

Consent for publication

Not applicable

Competing interests

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

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List of Publications

Shaqura, M., **Li, Li**., Mohamed, D. M., Li, X., Treskatsch, S., Buhrmann, C., Shakibaei, M., Beyer, A., Mousa, S., A., & Schafer, M. (2020). Neuronal aldosterone elicits a distinct genomic response in pain signaling molecules contributing to inflammatory pain. J Neuroinflammation, 17(1), 183.
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Mousa, S., Shaqura, M., Khalefa, B., **Li, Li**., Al-madol, M., Treskatsch, S., & Schäfer, M. (2021). Functional and anatomical characterization of corticotropin-releasing factor receptor subtypes of the rat spinal cord involved in somatic pain relief. Molecular Neurobiology (A preprint in Research Square).
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