ANESTHESIOLOGY

Aldosterone Synthase in Peripheral Sensory Neurons Contributes to Mechanical Hypersensitivity during Local Inflammation in Rats

Doaa M. Mohamed, M.Sc., Mohammed Shaqura, Ph.D., Xiongjuan Li, M.D., Mehdi Shakibaei, Ph.D., Antje Beyer, M.D., Sascha Treskatsch, M.D., Michael Schäfer, M.D., Ph.D., Shaaban A. Mousa, Ph.D.

ANESTHESIOLOGY 2020; 132:867–80

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Aldosterone is believed to be synthesized exclusively in the adrenal gland through the processing enzyme aldosterone synthase
- Mineralocorticoid receptors are predominantly expressed in peripheral nociceptive neurons whose activation leads to increased neuronal excitability and mechanical sensitivity

What This Article Tells Us That Is New

- Extra-adrenal production of aldosterone by aldosterone synthase within peripheral sensory neurons contributes to ongoing mechanical hypersensitivity *via* intrinsic activation of neuronal mineralocorticoid receptors
- Intrathecally-applied aldosterone synthase inhibitor reduced aldosterone content in peripheral sensory neurons and subsequently attenuated enhanced mechanical hypersensitivity resulting from local inflammation

ABSTRACT

Background: Recent emerging evidence suggests that extra-adrenal synthesis of aldosterone occurs (*e.g.*, within the failing heart and in certain brain areas). In this study, the authors investigated evidence for a local endogenous aldosterone production through its key processing enzyme aldosterone synthase within peripheral nociceptive neurons.

Methods: In male Wistar rats (n = 5 to 8 per group) with Freund's complete adjuvant hind paw inflammation, the authors examined aldosterone, aldosterone synthase, and mineralocorticoid receptor expression in peripheral sensory neurons using quantitative reverse transcriptase–polymerase chain reaction, Western blot, immunohistochemistry, and immunoprecipitation. Moreover, the authors explored the nociceptive behavioral changes after selective mineralocorticoid receptor antagonist, canrenoate-K, or specific aldosterone synthase inhibitor application.

Results: In rats with Freund's complete adjuvant–induced hind paw inflammation subcutaneous and intrathecal application of mineralocorticoid receptor antagonist, canrenoate-K, rapidly and dose-dependently attenuated nociceptive behavior (94 and 48% reduction in mean paw pressure thresholds, respectively), suggesting a tonic activation of neuronal mineralocorticoid receptors by an endogenous ligand. Indeed, aldosterone immunoreactivity was abundant in peptidergic nociceptive neurons of dorsal root ganglia and colocalized predominantly with its processing enzyme aldosterone synthase and mineralocorticoid receptors. Moreover, aldosterone and its synthesizing enzyme were significantly upregulated in peripheral sensory neurons under inflammatory conditions. The membrane mineralocorticoid receptor consistently coimmunoprecipitated with endogenous aldosterone, confirming a functional link between mineralocorticoid receptors and its endogenous ligand. Importantly, inhibition of endogenous aldosterone production in peripheral sensory neurons by a specific aldosterone synthase inhibitor attenuated nociceptive behavior after hind paw inflammation (a 32% reduction in paw pressure thresholds; inflammation, 47 ± 2 [mean ± SD] *vs.* inflammation + aldosterone synthase inhibitor, 62 ± 2).

Conclusions: Local production of aldosterone by its processing enzyme aldosterone synthase within peripheral sensory neurons contributes to ongoing mechanical hypersensitivity during local inflammation *via* intrinsic activation of neuronal mineralocorticoid receptors.

(ANESTHESIOLOGY 2020; 132:867–80)

Aldosterone is known to regulate the body's water and electrolyte balance through activation of corresponding mineralocorticoid receptors on epithelial cells of the renal cortical collecting ducts leading to sodium and water retention in exchange for excreted potassium.[1,2] Aldosterone is generated and secreted in the zona glomerulosa of the adrenal gland through local aldosterone synthase (CYP11B2).[1] Aldosterone circulates in the blood unbound (30 to 50% of total aldosterone) or bound to albumin- or corticosteroid-binding globulin (50 to 70% of total aldosterone). It is rapidly inactivated in the liver with a plasma half-life of 15 to 20 min.[3] While initial reports of aldosterone production outside the adrenal glands were challenged,[4] Takeda and others identified

D.M.M. and M. Shaqura contributed equally to this article.

Submitted for publication April 5, 2019. Accepted for publication December 10, 2019. Published online first on January 30, 2020. From the Department of Anaesthesiology and Intensive Care Medicine, Charite Campus Benjamin Franklin, Charité – Universitätsmedizin Berlin, Berlin, Germany (D.M.M., M. Shaqura, X.L., S.T., M. Schäfer, S.A.M.); Department of Zoology, Faculty of Science, Aswan University, Aswan, Egypt (D.M.M.); and the Departments of Anatomy (M. Shakibaei) and Anaesthesiology (A.B.), Ludwig Maximilian University of Munich, Munich, Germany.

 Anesthesiology 2020; 132:867–80. DOI: 10.1097/ALN.0000000000003127

<zdoi;. DOI: 10.1097/ALN.0000000000003127>

aldosterone synthase (CYP11B2) mRNA expression and aldosterone synthesis in rodent cardiovascular tissue.[5,6] This was later confirmed for the failing human heart, as well.[7,8] More recently, further evidence for extra-adrenal aldosterone synthesis has been demonstrated for specific neurons within the brain (*e.g.,* the hypothalamus and rostral ventral medulla) at which aldosterone appears to contribute to the depolarization of neurons.[9–12] Recent functional evidence implies aldosterone may also play a role in the peripheral nervous system. In an animal model of chronic lumbar dorsal root ganglia compression intrathecal aldosterone antagonism *via* delivery of spironolactone-reversed mechanical allodynia 2 days postsurgery.[13] In the same animal model, the antiallodynic effect of intrathecal spironolactone 4 and 7 days postsurgery paralleled the attenuation of spinal microglia activation and cytokine production.[14] In the rat model of zymosan-induced L5 dorsal root ganglion inflammation 500 μg of the mineralocorticoid receptor antagonist eplerenone given to the site of dorsal root ganglia inflammation reversed mechanical allodynia 1 to 13 days after intervention.[2,15] These effects occurred within days and were considered to be genomic, either through a reversal of nuclear mineralocorticoid receptor translocation or through attenuation of satellite cell activation.[2,15] More recently, we identified mineralocorticoid receptors predominantly on the subpopulation of peptidergic unmyelinated nociceptive C-fibers, strongly indicating a role for the modulation of nociception.[16] Indeed, local application of the mineralocorticoid receptor agonist aldosterone at the peripheral and central nerve terminals of nociceptive neurons elicited a rapid increased mechanical sensitivity.[16] Consistent with this pronociceptive effect, previous studies have shown that aldosterone increased the number of action potentials that were evoked in acutely dissociated dorsal root ganglion neurons.[2,15,17] These effects occurred rapidly, were most likely due to nongenomic mechanisms, and were mediated by membrane-bound mineralocorticoid receptors which were identified by membrane saturation binding.[16]

Using the animal model of unilateral Freund's complete adjuvant hind paw inflammation, this study investigated whether: (1) the antagonism of mineralocorticoid receptors on peripheral sensory neurons will result in the attenuation of mechanical hyperalgesia, suggesting an intrinsic tone of endogenous aldosterone; (2) peripheral sensory neurons are a potential source for local aldosterone production; (3) aldosterone is coexpressed with its processing enzyme aldosterone synthase and if their expression is upregulated during inflammatory conditions; (4) there is a functional link between endogenous aldosterone and sensory neuron mineralocorticoid receptors; and (5) the inhibition of aldosterone's production in peripheral sensory neurons by local administration of a specific aldosterone synthase inhibitor will result in the attenuation of mechanical hypersensitivity. The overall hypothesis, therefore, is that antagonism of sensory neuron mineralocorticoid receptors attenuates inflammation-induced mechanical hyperalgesia most likely due to the inhibition of an endogenous tonic activation through aldosterone derived from peripheral sensory neurons. This would have implications for similar human conditions (*e.g.,* arthritis, vertebral disc herniation) that an ongoing endogenous activation of mineralocorticoid receptors might contribute to enhanced mechanical sensitivity.

Materials and Methods

Drugs

Freund's complete adjuvant, a water-in-oil emulsion with killed mycobacteria (Calbiochem, USA); canrenoate-K, and aldosterone synthase inhibitor (FAD286; Sigma-Aldrich, USA) were dissolved in 0.9% NaCl as described previously.[18,19] Drugs were administered subcutaneously in a volume of 1 ml/kg body weight or intrathecally in a volume of 20 μl. Subcutaneous injections were given under the loose skin of the back, just behind and between the shoulder blades.

Animals

Experiments were conducted in male Wistar rats (180 to 250 g; breeding facility: Charité-Universitätsmedizin, Germany) after approval by the local animal care committee and according to the European Directive for animal welfare and care guidelines (2010/63/EU). Wistar rats were anesthetized by brief isoflurane anesthesia and followed by an intraplantar injection of 0.15 ml Freund's complete adjuvant (Calbiochem) into the right hind paw. This treatment consistently produces a local inflammation of the inoculated paw characterized by an increase in paw volume and temperature, mechanical hypersensitivity, and infiltration of various types of immune cells.[20] All experiments were performed between 10:00 AM and 2:00 PM on the fourth day of Freund's complete adjuvant hind paw inflammation.

For continuous intrathecal delivery of drugs, animals were implanted with chronic intrathecal catheters, as described previously by Shaqura *et al.*[21] Briefly, animals were anesthetized with isoflurane in oxygen *via* nose cone. A longitudinal skin incision was made in the lumbar region directly above the spinous processes of the L4–L6 vertebrae. The needle through which the catheter (PE 10 tubing attached to PE 60 tubing for attachment to an osmotic pump; Portex Ltd, United Kingdom) was set up, was inserted at a 30° angle between the L5 and L6 vertebra. Then, the catheter was carefully advanced upward 1 cm into the intrathecal space while rotating it between the thumb and forefinger. The sign of dura penetration was observed by involuntary movements of the tail or hind limb. Finally, the correct location of the catheter was verified by 10 μl lidocaine 2%, which caused reversible bilateral hind limb paresis for 10 to 15 min.

Experimental Protocols

The first set of experiments examined the influence of 4 days of Freund's complete adjuvant hind paw inflammation on changes in the neuronal aldosterone, aldosterone synthase, 11β-hydroxysteroid-dehydrogenase 2, and mineralocorticoid receptor expression in the innervating dorsal root ganglia. Subsequent experiments assessed the impact of systemic (5 to 20 mg/kg) or intrathecal (10 to 100 μg in 20 μl) injections of increasing doses of mineralocorticoid receptor antagonist, canrenoate-K, on mechanical paw pressure thresholds. The doses chosen for canrenoate-K were based on our previous studies.[19,22] A final set of experiments investigated at 4 days of Freund's complete adjuvant hind paw inflammation the effects of the intrathecal delivery of the aldosterone synthase inhibitor, FAD286, on both aldosterone content and on mechanical paw pressure thresholds. Rats received the following intrathecal treatments for more than 5 days, beginning 1 day before Freund's complete adjuvant–induced inflammation. For intrathecal FAD286 delivery, Alzet osmotic minipumps (2 ml; rate, 5.0 μl/h; Alzet Corporation, USA) were filled with 0.9% NaCl with or without FAD286 (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$) and connected to the intrathecal catheter to administer FAD286 or the vehicle continuously at 5.0 μl/h.[23] All tests were performed on day 5 after pump implantation.

Mechanical Hyperalgesia Testing

The mechanical hyperalgesia following Freund's complete adjuvant–induced hind paw inflammation was assessed by a pressure apparatus (Ugo-Basile SRL, Italy) with increasing force (measured in grams) applied to the plantar hind paw until a withdrawal reflex was precipitated as described previously.[19] A 250-g cut-off value was assigned in the absence of a response. Mechanical paw pressure tests were performed on the fourth day of Freund's complete adjuvant–induced hind paw inflammation before (baseline) and after drug application. Subsequently, mechanical paw pressure thresholds were reassessed at different time points (0 to 120 min) after drug administrations to determine drug-related behavior. Each measurement was performed three times consecutively with a time interval of at least 15 s before the next stimulus was applied. In all behavioral experiments, drugs were prepared by a different person (M. Shakibaei), and the examiner (X.L.) was unaware (*i.e.,* blinded) of the treatment that each animal had received. Animals were incidentally allocated to treatments; however, no specific randomization method was used to assign animals to condition.

11β-Hydroxysteroid-Dehydrogenase and Aldosterone Synthase mRNA Detection by TaqMan Quantitative Polymerase Chain Reaction

Total RNA extracted from L3–L5 dorsal root ganglia of Wistar rats using RNeasy Kit (Qiagen, Germany), as previously described,[19,24] was subjected to the TaqMan quantitative polymerase chain reaction using an SYBR Green kit following the manufacturer's instructions (Thermo Fisher Scientific, Germany). The products were amplified using aldosterone synthase CYP11B2 specific primers: forward primer, 5'-TGGCAGCACTAATAACTCAGGG-3'; reverse primer, 5'-ATTGCTGTCGTGTCAACGCT-3' (Ensemble, Accession No: NM_012538.2); for 11β-hydroxysteroid-dehydrogenase: forward primer, 5'-CGCCGC TTCCTACAGAACTT-3'; reverse primer, 5'-TCCTGGGTTGTGTCATGAACA-3' (Ensemble Accession No: NM_017081.2). Amplification was performed with 40 cycles, each consisting of 15 s at 95°C. The gene-specific fluorescent amplified products were detected just below the determined melting temperature for each product (aldosterone synthase CYP11B2: melting temperature, 83°C; 11β-hydroxysteroid-dehydrogenase: melting temperature, 76°C; 18S: melting temperature, 83°C). Aldosterone synthase and 11β-hydroxysteroid-dehydrogenase mRNA products were quantified in replicates of three samples using the delta-delta Ct method.[19] In addition, the housekeeping gene 18s (Accession No. NR_046237) as the internal reference was quantified using TaqMan quantitative polymerase chain reaction with the following primers: forward primer, CGGCTACCACATCCAAGGAA; reverse primer, GCTGGAATTACCGCGGCT.

Aldosterone Quantification in Plasma and Dorsal Root Ganglia

Blood samples were collected by intracardiac aspiration directly into rapid serum tubes (BD Vacutainer; Becton Dickinson, Germany) from the isoflurane anesthesia sacrificed rats (n = 15, 17). The samples were centrifuged at 1,000 relative centrifugal force and snap frozen at –80°C. These were subsequently subjected to chemiluminescence immunoassay by an external commercial laboratory (SynLab Vet, Germany) to measure the aldosterone plasma concentrations. In parallel, dorsal root ganglia were prepared as previously described,[25,26] in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific). Briefly, thoracic and lumbar dorsal root ganglia of male Wistar rats (n = 5) were removed and placed in sterile Dulbecco's Modified Eagle's Medium at 4°C. Dorsal root ganglia were digested with collagenase type 2 in Minimum Essential Medium (Biochrom AG, Germany) at 37°C for 50 min, and with 0.025% trypsin for 10 min at 37°C. After digestion, dorsal root ganglia were carefully dissociated by mechanical agitation, and centrifuged at 500 g for 5 min and then at 300 g for 5 min. The cells were maintained overnight in Minimum Essential Medium growth media supplemented with 10% horse serum and 50 μg/ml penicillin and streptomycin, and plated in 24-well microplates (1.9 cm^2) at 37°C in an atmosphere of 5% CO_2. Then, the aldosterone content in dorsal root ganglia was determined by a commercial enzyme-linked immunosorbent

assay (ELISA) kit (cat. no. KGE016; R&D Systems, USA), according to the manufacturer's specifications and our previous study.[19] Measurements were performed independently in five to six rats and in triplicate using the same sample. The optical density for each well was determined using a microplate reader (LS45; Perkin Elmer, USA) set to 450 nm. The mean of triplicate readings for each standard, control, and sample subtracted by the average nonspecific binding optical density were measured. A standard curve was created by generating a four-parameter logistic curve-fit using the computer software (LS45; Perkin Elmer).

Western Blot

Dorsal root ganglia and spinal cords from adult rats were solubilized and extracted for immunoblotting investigations as previously described.[16,24] After blotting, membranes were blocked in 3% bovine serum albumin for 2 h and incubated with mouse anti-aldosterone synthase (1:1,000 in 3% BSA; anti-CYP11B2; Merck Millipore, Germany) or rabbit anti–11β-hydroxysteroid-dehydrogenase 2 (1:500 in 3% BSA; St. John's Laboratory Ltd., United Kingdom) or sheep anti–11β-hydroxysteroid-dehydrogenase 2 (1:200 in 3% BSA; private gift from Dr. Elise Gomez-Sanchez, Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi). After incubation with the secondary antibody (peroxidase-conjugated goat anti-rabbit, 1:40,000; Jackson ImmunoResearch, USA) for 2 h at ambient temperature, reactive protein bands were digitally visualized using enhanced chemiluminescence solutions (SuperSignal West Pico; Thermo Fisher Scientific) in ChemiDoc MP Imager. Finally, the blots were reprobed with monoclonal mouse anti-beta actin antibody (1:20,000; Sigma-Aldrich) as an internal standard. Experiments were performed in groups of four to five animals.

The Western blot bands specific for aldosterone synthase (CYP11B2, 57 kDa) or 11β-hydroxysteroid-dehydrogenase (11β-HSD2, 70 kDa) were quantified by Java Image processing and analysis software (ImageJ, open-source image software downloaded from the web; http://rsb.info.nih.gov/ij/, accessed March 9, 2018).[16,22] 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 analyzed.[21]

Immunohistochemistry

After transcardial perfusion of rats (4 days after Freund's complete adjuvant inoculation) dorsal root ganglia were removed and processed as described previously.[19,22] Tissue sections were then incubated overnight with the following primary antibodies: polyclonal rabbit anti-aldosterone (1:500; Novus Biologicals, LLC, USA), in combination with a polyclonal guinea pig anti–calcitonin gene-related peptide, monoclonal mouse anti–mineralocorticoid receptor (private gift from Dr. Elise Gomez-Sanchez) or aldosterone synthase (CYP11B2); in addition, polyclonal sheep antibody against 11β-hydroxysteroid-dehydrogenase was examined in combination with the monoclonal mouse anti–mineralocorticoid receptor (private gift from Dr. Elise Gomez-Sanchez). The species, sources, dilutions, and immunogens of the primary antibodies used in this study are summarized in Supplemental Digital Content, table S1 (http://links.lww.com/ALN/C235). Finally, the tissues were washed in phosphate-buffered saline, mounted in Vectashield (Vector Laboratories, USA) and examined by confocal laser scanning microscope (LSM 510, Carl Zeiss, Germany) as described previously.[19] To demonstrate specificity of staining, the following controls were included as previously reported by our group[19,22,24]: omission of either the primary antisera or the secondary antibodies.

The quantification of dorsal root ganglia staining has been described previously.[19,22,24,27] 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 immunoreactive cells divided by the total number of dorsaal root ganglion cells, the number of mineralocorticoid receptor immunoreactive cells divided by the total number of dorsal root ganglion cells, the number of 11β-hydroxysteroid-dehydrogenase immunoreactive cells divided by the total number of total dorsal root ganglion cells, the number of aldosterone synthase (CYP11B2) immunoreactive cells divided by the total number of total dorsal root ganglion cells, the number of aldosterone plus mineralocorticoid receptor immunoreactive cells divided by the total number of total dorsal root ganglion cells, the number of calcitonin gene–related peptide plus aldosterone immunoreactive cells divided by the total number of total dorsal root ganglion cells, the number of calcitonin gene–related peptide plus aldosterone synthase (CYP11B2) immunoreactive cells divided by the total number of total dorsal root ganglion cells, the number of calcitonin gene–related peptide plus 11β-hydroxysteroid-dehydrogenase immunoreactive cells divided by the total number of total dorsal root ganglion cells, the number of total dorsal root ganglia divided by aldosterone plus aldosterone synthase (CYP11B2) immunoreactive cells divided by the total number of total dorsal root ganglion cells were counted in each dorsal root ganglion section and represented as percentages. Data were obtained from two to three sections per rat and four to five rats per group using 40× objective lens.

Coimmunoprecipitation Assay

L4–L5 dorsal root ganglia samples were solubilized according to Shaqura *et al.*,[28] to obtain whole cell lysate. For quantifying membrane-bound *versus* cytosolic mineralocorticoid receptor, subcellular fractionation was performed as previously described.[23] To examine whether endogenous aldosterone and membranous mineralocorticoid receptor or transmembrane molecule integrin-ß1 integrin as a membrane marker interact with each other, we performed coimmunoprecipitation assays as reported earlier.[29]

Briefly, precleared membrane fraction protein extracts were immunoprecipitated with rabbit polyclonal primary antibody against aldosterone (Novus Biologicals, LLC). Then, immune complexes were subjected to Western blot analysis using mouse monoclonal anti–mineralocorticoid receptor (private gift from Dr. Elise Gomez-Sanchez) or anti–transmembrane ß1-integrin (Merck Millipore).

Statistical Analysis

All tests were performed using Sigma Plot 13.0 software (SPSS Inc., Germany). TaqMan quantitative polymerase chain reaction (unit: fold change in delta-delta CT over control), Western blot (unit: fold change in integrated optical density over control) as well as immunofluorescence data (unit: number of immunoreactive cells) were analyzed as two group comparisons (Freund's complete adjuvant–treated rats *vs.* controls) by the two-tailed independent Student *t* test in case of normally distributed data. Paw pressure thresholds were determined before and after drug injections, expressed as mean ± SD, and statistically analyzed by an independent paired *t* test to examine for differences between baseline values and values obtained 4 days after Freund's complete adjuvant injection or to examine for alterations between different drug doses (between factor analysis) and different time intervals (within factor analysis) by two-way repeated measures ANOVA (two-way repeated measures ANOVA) followed by *post hoc* Dunnett test. Multiple comparisons were analyzed by one-way ANOVA and *post hoc* Tukey test. There were no missing data. For the mechanical hyperalgesia testing no statistical power calculation was conducted before the study. The sample size was based on our previous experience with this design. For the biochemical and immunohistochemical experiments that were done in duplicate or triplicate a minimum number of five independent animals were used. Statistical significance was assumed at $P < 0.05$.

Results

Mineralocorticoid Receptor Antagonism Results in Rapid, Nongenomic and Short-lasting Reduction of Inflammation-induced Mechanical Hypersensitivity

Four days of Freund's complete adjuvant–induced inflammation of the right hind paw resulted in significantly reduced mechanical paw pressure thresholds of the inflamed but not the contralateral noninflamed hind paws compared to baseline values ($P < 0.001$; independent paired Student *t* test; fig. 1). This enhanced mechanical hypersensitivity was dose-dependent and rapidly reversed after systemic administration of the mineralocorticoid receptor selective antagonist canrenoate-K ($P < 0.001$; two-way repeated measures ANOVA, *post hoc* Dunnett test; fig. 1A). Intrathecal application of lower doses of canrenoate-K also resulted in rapid and transient attenuation of the Freund's complete adjuvant inflammation–induced mechanical hypersensitivity ($P < 0.001$; two-way ANOVA, *post hoc* Dunnett test; fig. 1B). Intrathecal canrenoate-K had no effect on the contralateral noninflamed hind paws (data not shown), similar to previous reports in naive rats.[19]

Coexpression of Mineralocorticoid Receptors with Its Protecting Enzyme 11β-Hydroxysteroid-Dehydrogenase 2 in Sensory Dorsal Root Ganglia

Since, it is well established that the mineralocorticoid receptor protecting enzyme 11β-hydroxysteroid-dehydrogenase 2[30] immediately converts corticosterone to its inactive metabolite 11-dehydrocorticosterone and ensures mineralocorticoid receptor activation only through endogenous aldosterone, we have examined the coexpression of mineralocorticoid receptors with 11β-hydroxysteroid-dehydrogenase 2 in sensory dorsal root ganglia. TaqMan quantitative polymerase chain reaction (fig. 2, A and B) and western blot (fig. 2, C and D) analysis identified 11β-hydroxysteroid-dehydrogenase 2 specific mRNA and 11β-hydroxysteroid-dehydrogenase 2 specific protein bands (70 kDa) in dorsal root ganglia. Moreover, double immunofluorescence confocal microscopy showed that 11β-hydroxysteroid-dehydrogenase 2 immunoreactivity mainly colocalized with mineralocorticoid receptor–immunoreactive sensory neurons which further increased in dorsal root ganglia ipsilateral to Freund's complete adjuvant–inflamed hind paws compared to controls ($P < 0.001$; two-tailed independent Student *t* test; fig. 2, E to M). In parallel, 11β-hydroxysteroid-dehydrogenase 2-immunoreactive dorsal root ganglia cells predominantly colocalized with the peptidergic sensory neuron marker calcitonin-gene-related peptide but only scarcely with some satellite cell-like structures confirming our previous study; 11β-hydroxysteroid-dehydrogenase 2-immunoreactive dorsal root ganglia cells colocalized with calcitonin-gene-related peptide in 58 ± 12% of control animals and in 62 ± 17% of rats with hind paw inflammation ($P = 0.619$; two-tailed independent Student *t* test; Supplemental Digital Content, fig. S1, http://links.lww.com/ALN/C235).

Inflammation-dependent Increase of Endogenous Aldosterone Primarily in Mineralocorticoid- and Calcitonin Gene–Related Peptide Immunoreactive Sensory Dorsal Root Ganglia, But Not in Plasma

The selective mineralocorticoid receptor antagonist, canrenoate-K, reversed Freund's complete adjuvant–induced mechanical hypersensitivity suggesting intrinsic mineralocorticoid receptor activation by endogenous aldosterone, therefore, we investigated whether aldosterone could be identified in dorsal root ganglia. Double immunofluorescence confocal microscopy showed abundant colocalization of aldosterone with mineralocorticoid receptors (fig. 3, A to F), as well as aldosterone with the sensory neuron marker calcitonin gene–related peptide (fig. 3, J to O) in control animals. Aldosterone—together with either mineralocorticoid receptor (fig. 3, G and I—or calcitonin gene–related peptide immunoreactive dorsal root ganglia

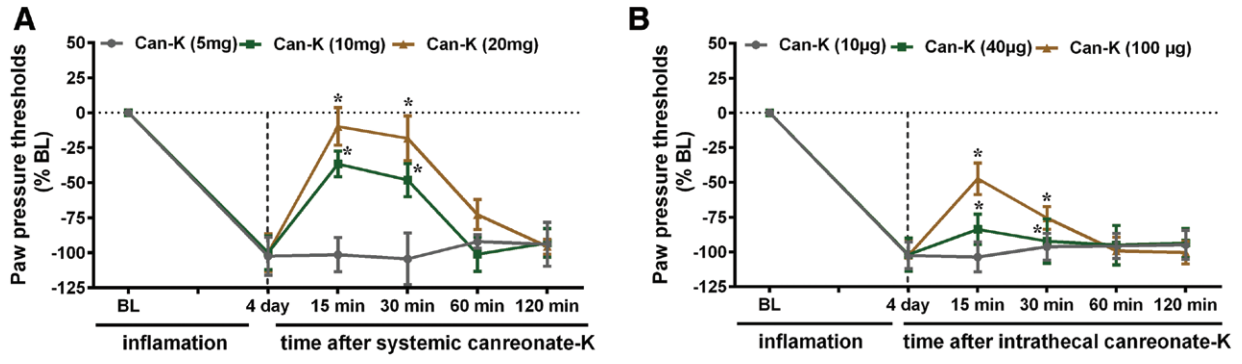

Fig. 1. Role of mineralocorticoid receptor specific antagonist canrenoate-K (Can-K) in the modulation of inflammation-induced nociceptive behavior in the right hind paw. (*A, B*) Four days of Freund's complete adjuvant–induced inflammation of the right hind paw resulted in significantly reduced mechanical paw pressure thresholds (shown as % BL, *i.e.,* % baseline values) compared to baseline values (*dotted horizontal line*; $P < 0.001$; independent paired Student *t* test; n = 7 [*A*], 8 [*B*] rats; experiments in triplicate). Increasing doses of systemic (*A*) or intrathecal (*B*) canrenoate-K reversed this mechanical hypersensitivity within minutes ($P < 0.001$; two-way repeated measures ANOVA, *post hoc* Dunnett test; n = 7 [*A*], 8 [*B*] rats; experiments in triplicate). Can-K shows a transient attenuation of the endogenous tonic activation of mineralocorticoid receptors during hind paw inflammation. Data are expressed as mean ± SD. BL, baseline.

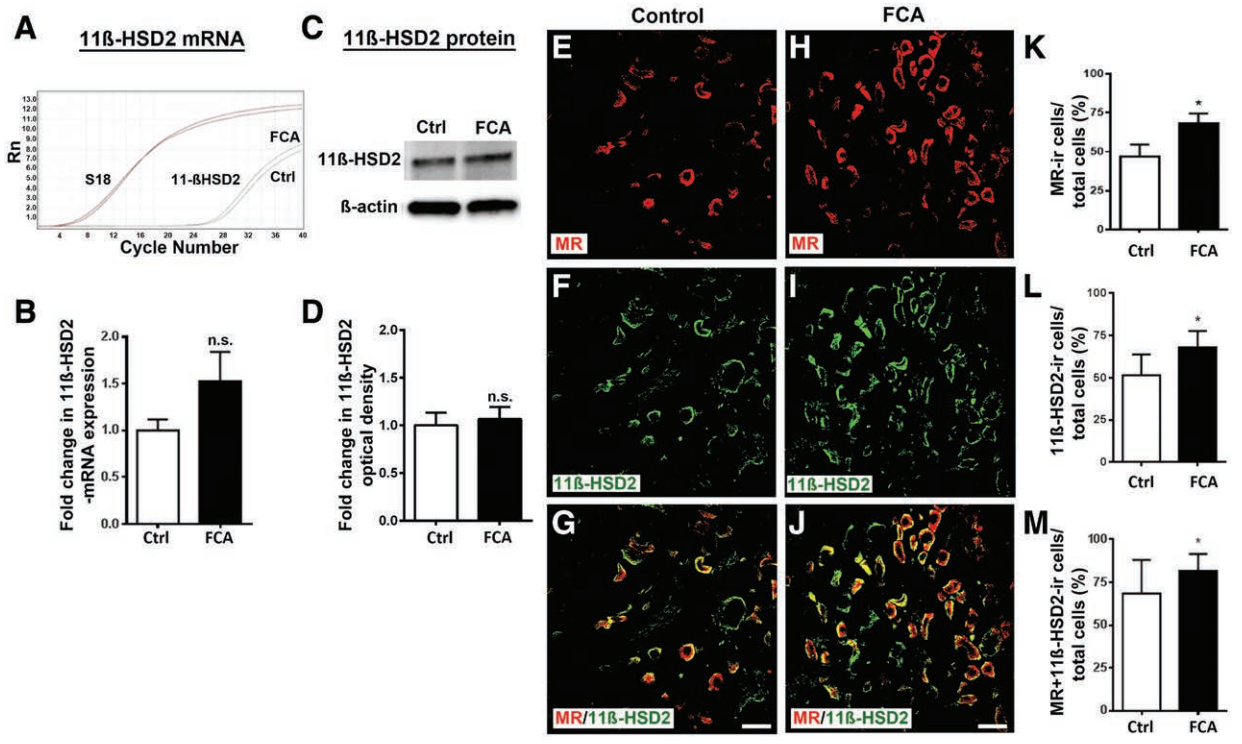

Fig. 2. Detection of 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2) mRNA and protein in dorsal root ganglia of FCA-treated rats compared to controls (Ctrl). (*A* to *D*) Quantification of 11ß-HSD2 mRNA (*A, B*) using TaqMan quantitative polymerase chain reaction and Western blot (*C, D*) shows a significant increase in 11ß-HSD2 mRNA (*A, B*), but not protein (*C, D*) in dorsal root ganglia of FCA treated rats compared to controls ($P < 0.001$; two-tailed independent Student *t* test; n = 5 rats; experiments in triplicate for TaqMan polymerase chain reaction and in duplicate for Western blot). (*E* to *J*) Confocal microscopy of double immunofluorescence of 11ß-HSD2 (*green fluorescence*) with MR (*red fluorescence*) in dorsal root ganglia of FCA treated rats *versus* controls (*K* to *M*). Note that the majority of 11ß-HSD2 immunoreactivity colocalized (*L*) with MR-immunoreactive neurons and significantly increased in FCA treated rats compared to controls (*M*; $P < 0.001$; two-tailed independent Student *t* test; n = 5 rats; 3-4 tissue sections each; *K*). Bar = 40 µm. Data are expressed as mean ± SD. FCA, Freund's complete adjuvant; IR, immunoreactive; MR, mineralocorticoid receptor.

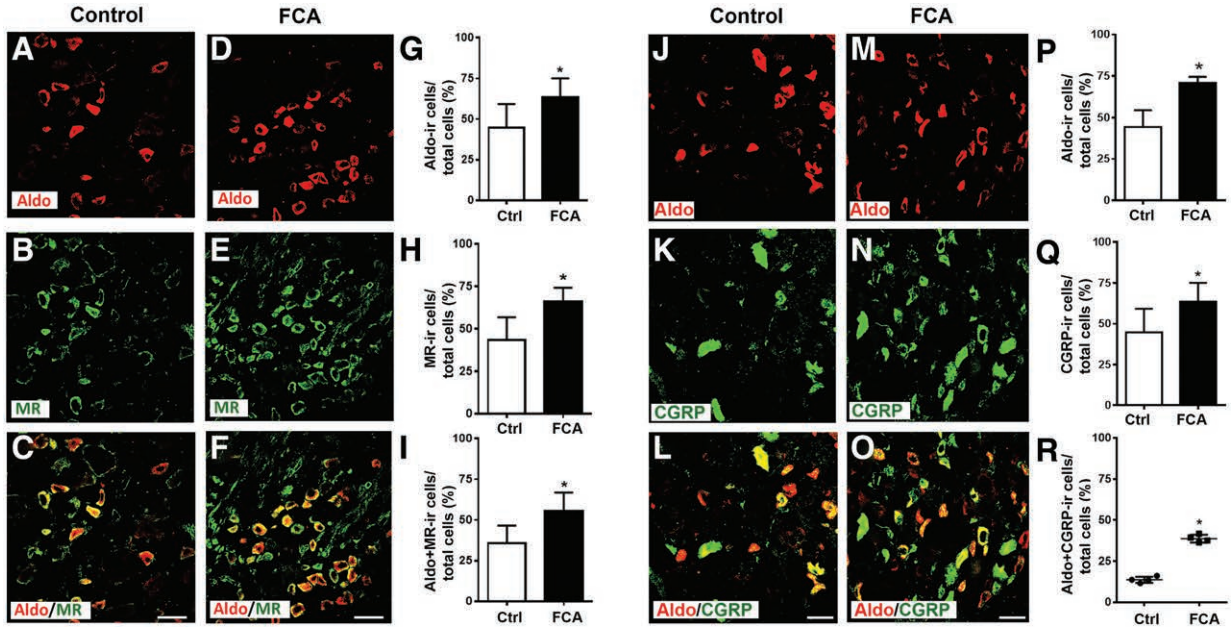

Fig. 3. Detection of aldosterone with MR (*A* to *I*) or CGRP (*J* to *R*) in dorsal root ganglia of FCA-treated rats *versus* controls. (*A* to *F*, *J* to *O*) Double immunofluorescence confocal microscopy of aldosterone (*red fluorescence*) with MR (*A* to *I*) or CGRP (*J* to *R*) (*green fluorescence*) in dorsal root ganglia sections (Bar = 40 μm). The number of Aldo- (*G*, *P*), MR- (*H*, *I*) or CGRP- (*Q*, *R*) immunoreactive neurons in relation to the total number of dorsal root ganglia neurons was significantly increased in FCA treated rats compared to controls ($P < 0.001$; two-tailed independent Student *t* test). In addition, the colocalization of Aldo with MR (*C*, *F*, *I*) or CGRP (*M*, *O*, *R*) in dorsal root ganglia neurons was significantly increased in FCA animals (*I*, *R*; $P < 0.001$; two-tailed independent Student *t* test; n = 4, 5 rats; 3-4 tissue sections each). Bar = 40 μm. Data are expressed as mean ± SD. Aldo, aldosterone; CGRP, calcitonin gene–related peptide; Ctrl, controls; FCA, Freund's complete adjuvant; IR, immunoreactive; MR, mineralocorticoid receptors.

increased significantly (fig. 3, P to R) following Freund's complete adjuvant hind paw inflammation ($P < 0.001$; two-tailed independent Student *t* test; fig. 3).

Using a highly specific aldosterone ELISA, we identified immunoreactive aldosterone in dorsal root ganglia of naïve control animals (2.8 ± 0.7 pg/mg) and demonstrated its significant upregulation after 4 days of Freund's complete adjuvant–inflammation (7.4 ± 1.2 pg/mg; $P < 0.01$; two-tailed independent Student *t* test; fig. 4A). In contrast, aldosterone plasma concentrations in Freund's complete adjuvant–treated rats *versus* controls were not significantly altered ($P = 0.353$; two-tailed independent Student *t* test; fig. 4B).

Aldosterone Synthase mRNA and Protein Expression in Aldosterone- and Mineralocorticoid Receptor–Immunoreactive Dorsal Root Ganglia

Since aldosterone is derived from a final conversion of 18-hydroxycorticosterone into aldosterone by aldosterone synthase, we examined the local expression of this enzyme in sensory dorsal root ganglia. Quantitative TaqMan assay using mRNA specific primers detected aldosterone synthase mRNA in dorsal root ganglia which significantly increased following Freund's complete adjuvant hind paw inflammation ($P < 0.01$; two-tailed independent Student *t* test; fig. 4, C and D). Furthermore, these observations were supported by the identification of aldosterone synthase specific protein bands (57 kDa) in sensory dorsal root ganglia and their increase after hind paw inflammation (fig. 4, E and F). In addition, double immunofluorescence confocal microscopy showed coexistence of aldosterone with its key processing enzyme in neuronal structures of dorsal root ganglia of inflamed and control rats (fig. 5). Moreover, aldosterone synthase colocalized with mineralocorticoid receptor–immunoreactive neurons that further increased following hind paw inflammation ($P = 0.01$; two-tailed independent Student *t* test; fig. 5, G to I, P to R).

Physical Link between Endogenous Aldosterone and Membrane-bound Mineralocorticoid Receptors in Sensory Dorsal Root Ganglia

Immunoprecipitation of whole protein extracts of dorsal root ganglia membrane fractions with anti-aldosterone antibody pulls down multiple proteins that are complexed with aldosterone. Subsequent Western blot analysis of these functional complex formations resulted in the identification of proteins coprecipitated with aldosterone, such as

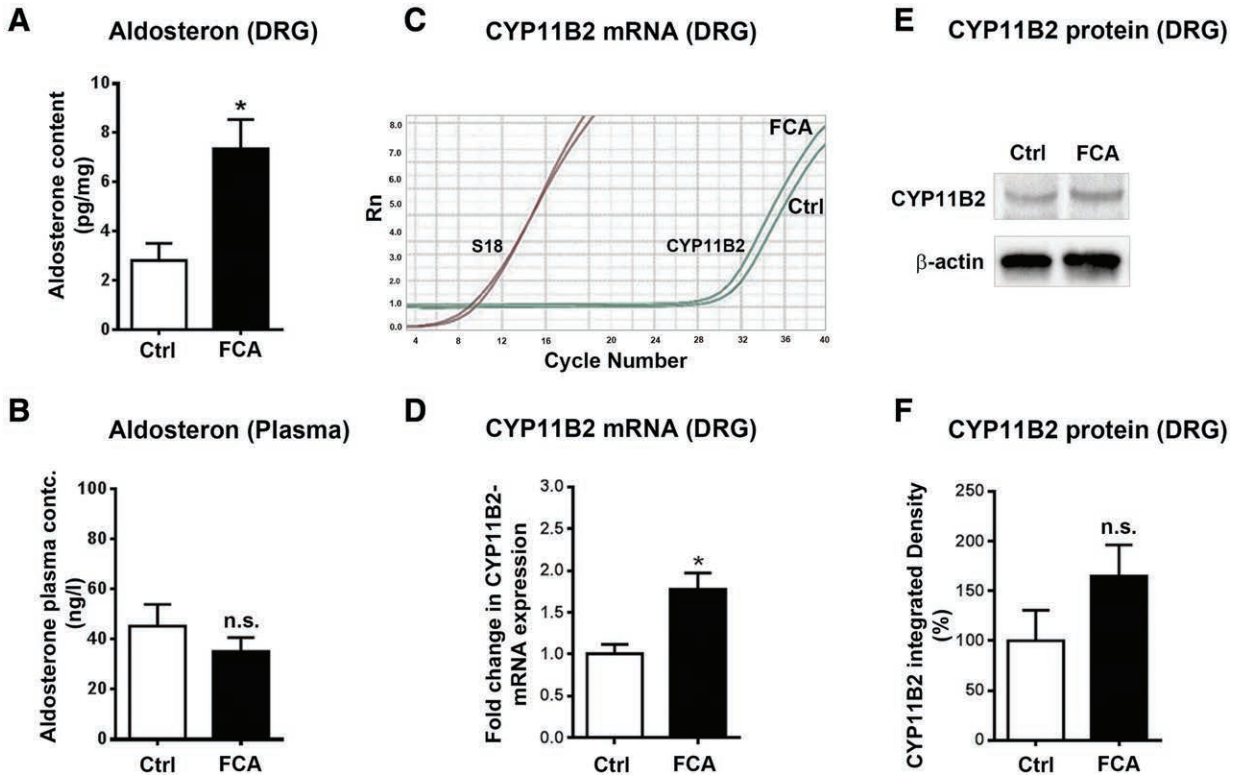

Fig. 4. Detection of aldosterone, as well as its processing enzyme aldosterone synthase, CYP11B2 mRNA, and protein in dorsal root ganglia (DRG) of Freund's complete adjuvant (FCA)–treated rats compared to controls (Ctrl). (*A*) Using an aldosterone specific enzyme-linked immunosorbent assay kit a significant increase in aldosterone content was shown in DRG of FCA-treated rats compared to controls ($P < 0.01$; two-tailed independent Student *t* test; n = 5, 6 rats; experiments in triplicate). (*B*) However, aldosterone plasma concentrations showed no significant differences ($P = 0.353$; two-tailed independent Student *t* test; blood from n = 15, 17 rats). (*C* to *F*) Quantification of aldosterone synthase CYP11B2 mRNA using TaqMan quantitative polymerase chain reaction (*C, D*) and protein using western blot (*E, F*) revealed a significant increase in CYP11B2 mRNA (*D*; $P = 0.043$), but not in protein (*F*; $P = 0.189$) in DRG of FCA-treated rats compared to Ctrl (two-tailed independent Student *t* test; n = 5; experiments in triplicate for TaqMan quantitative polymerase chain reaction and in duplicate for Western blot). Data are expressed as mean ± SD. n.s., nonsignificant differences.

membranous mineralocorticoid receptor and the transmembrane molecule ß1-integrin (fig. 6). Interestingly, the coprecipitated mineralocorticoid receptor protein band showed a higher optical density in dorsal root ganglia innervating inflamed hind paws than in control normal dorsal root ganglia (fig. 6). ß-actin protein bands were not detectable in the membrane fraction but in the cytosolic lysate.

Intrathecal Aldosterone Synthase Inhibitor FAD286 Reduces Aldosterone Content in Dorsal Root Ganglia and Attenuates Inflammatory Mechanical Hypersensitivity

Since endogenous aldosterone content was significantly increased in sensory dorsal root ganglia after inflammation, we examined whether intrathecal infusion of aldosterone synthase inhibitor FAD286 abolished this increase. Indeed, our confocal immunofluorescence analysis showed that intrathecal infusion of FAD286, but not the vehicle, significantly reduced the number of aldosterone-immunoreactive dorsal root ganglia in relation to the total number of dorsal root ganglion cells in Freund's complete adjuvant–treated rats ($P < 0.001$; one-way ANOVA and *post hoc* Dunnett test; fig. 7, A to D). Moreover, our aldosterone-specific ELISA revealed that the increase in aldosterone content in dorsal root ganglia of Freund's complete adjuvant–treated animals was significantly reduced by intrathecal treatment with aldosterone synthase inhibitor FAD286 ($P < 0.001$; one-way ANOVA and *post hoc* Dunnett test; fig. 7E). Consistent with the reduction of aldosterone content in sensory dorsal root ganglia, the enhanced mechanical hypersensitivity was significantly diminished following intrathecal aldosterone inhibitor FAD286 ($P < 0.001$; one-way ANOVA and *post hoc* Dunnett test; fig. 7F).

Discussion

The main findings of this investigation are: (1) blocking an ongoing intrinsic tone of mineralocorticoid receptor activation

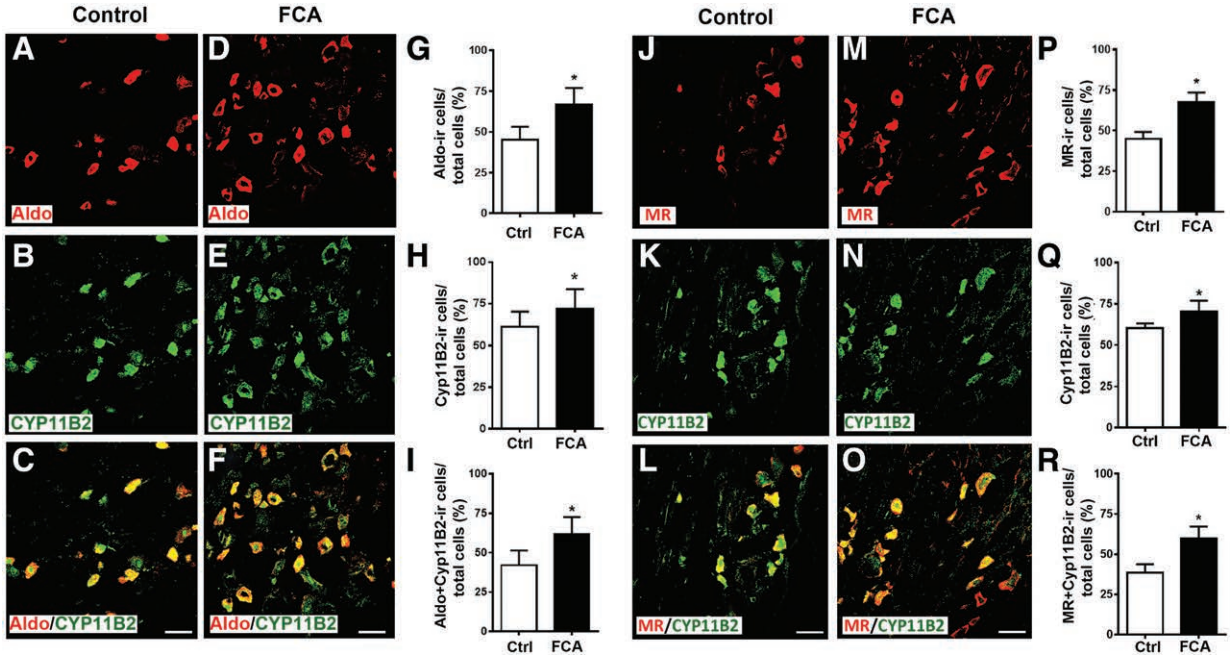

Fig. 5. Detection of aldosterone synthase CYP11B2 with aldosterone (Aldo) and mineralocorticoid receptor (MR) in dorsal root ganglia of Freund's complete adjuvant (FCA)–treated rats *versus* controls (Ctrl). (*A, E*) Confocal microscopy of double immunofluorescence of CYP11B2 (*green fluorescence*) with Aldo (*A* to *I*) or MR (*J* to *R*) (*red fluorescence*) in dorsal root ganglia of FCA-treated rats *versus* controls. Note that the majority of CYP11B2 immunoreactivity colocalized with Aldo- (*C, F, I*) or MR- (*L, O, R*) immunoreactive (IR) neurons. The number of Aldo- (*G*), MR- (*P*), or CYP11B2- (*H, Q*) IR neurons in relation to the total number of dorsal root ganglia neurons was significantly increased in FCA-treated rats compared to controls ($P < 0.001$, two-tailed independent Student *t* test). In addition, the colocalization of aldosterone (*I*) or MR (*R*) with CYP11B2 in dorsal root ganglia neurons was significantly increased in FCA animals ($P < 0.01$; two-tailed independent Student *t* test; n = 5 rats; experiments in triplicate). Bar = 40 μm. Data are expressed as mean ± SD.

in sensory dorsal root ganglia during hind paw inflammation inhibits nociceptive mechanical hypersensitivity; (2) the identification of endogenous aldosterone with its processing enzyme aldosterone synthase in sensory dorsal root ganglia indicates local aldosterone production; (3) the upregulation of aldosterone synthase mRNA and protein is concomitant with an enhanced aldosterone content in sensory dorsal root ganglia after hind paw inflammation; (4) the demonstration of a physical link between the endogenous ligand aldosterone and mineralocorticoid receptor in sensory dorsal root ganglia; and (5) the inhibition of the aldosterone synthase enzyme in sensory dorsal root ganglia attenuates inflammation-induced mechanical hypersensitivity. Overall, these findings suggest that the local production of aldosterone within peripheral sensory dorsal root ganglia contributes to ongoing nociceptive mechanical hypersensitivity *via* intrinsic activation of neuronal mineralocorticoid receptors. Thus, aldosterone synthase (*i.e.*, aldosterone-converting enzyme CYP11B2) represents a novel target for the potential modulation of nociceptive mechanical hypersensitivity during inflammation.

In our animal model of persistent inflammation-induced mechanical hyperalgesia in the right hind paw, the systemic, as well as intrathecal administration of the mineralocorticoid receptor antagonist, canrenoate-K, resulted in rapid (within minutes), short-lasting (30 to 60 min), and transient inhibition of mechanical hyperalgesia. This inhibition of mechanical hyperalgesia most likely indicates a rapid nongenomic effect based on the temporary antagonism of mineralocorticoid receptors on peripheral sensory dorsal root ganglia,[16,22] resulting in the inhibition of intrinsic tonic mineralocorticoid receptor activation by an endogenous ligand that apparently contributes to mechanical hyperalgesia. Our current findings are in line with previous studies reporting the antinociceptive effects of the mineralocorticoid receptor antagonist eplerenone[2,15,17] or spironolactone.[13,14] However, these effects were observed only after 3 days and lasted up to 2 weeks suggesting a slowly developing, but long-lasting genomic effect.

Mineralocorticoid receptors are located mainly on the peptidergic (calcitonin gene–related peptide immunoreactive) subpopulation of peripheral nociceptive neurons.[22] Moreover, previous studies suggested that the endogenous ligand aldosterone facilitates action potentials in dorsal root ganglion neurons[2,15] and elicits mechanical hypersensitivity in naïve animals.[22] Li *et al.*[19] consistently showed that the antagonism of mineralocorticoid receptors attenuates inflammation-induced nociceptive

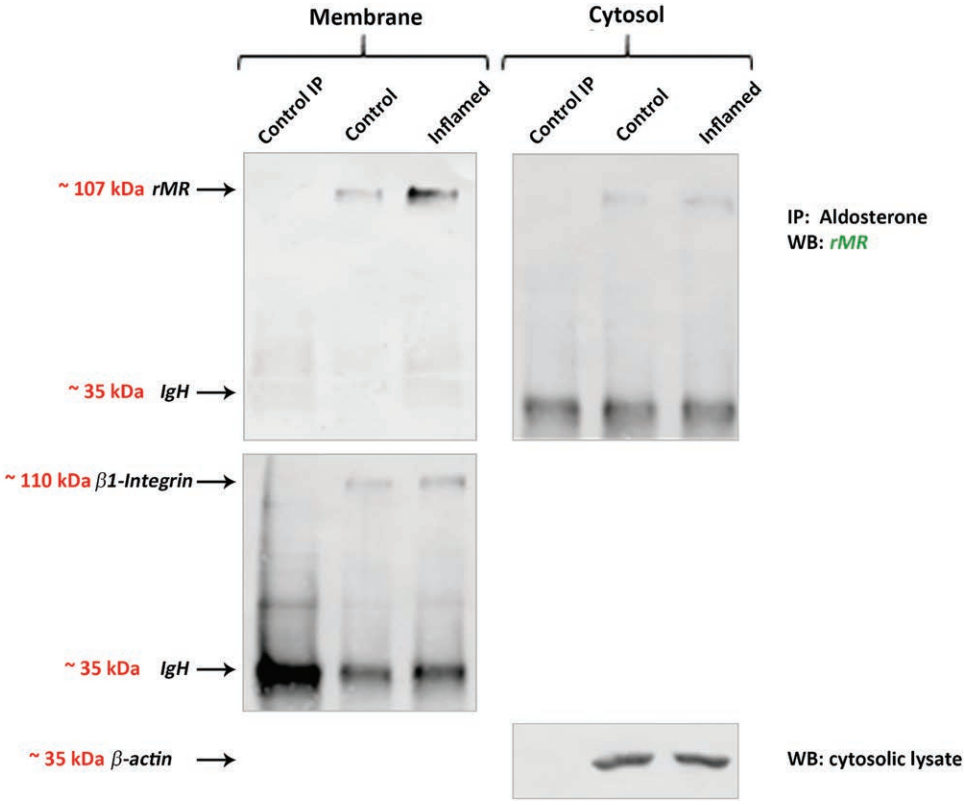

Fig. 6. Immunoprecipitation of whole protein extracts of dorsal root ganglia membrane and cytosol fractions with anti-aldosterone antibody and subsequent Western blot in dorsal root ganglia of rats with hind paw inflammation *versus* controls. L4–L5 dorsal root ganglia membrane and cytosol fraction extracts were lysed and immunoprecipitated with anti-aldosterone and subsequently the immunoprecipitants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblotting using anti–mineralocorticoid receptor and anti-ß1 integrin antibody. The ß-actin blot is shown only for the cytosolic lysate because ß-actin cannot be found in the membrane fraction. IgH, immunoglobulin heavy chain; IP, immunoprecipitation; rMR, mineralocorticoid receptor; WB, Western blot.

behavior by nongenomic mechanisms. Mineralocorticoid receptors in kidney tissue[31] and other peripheral tissues[32] are protected by the enzyme 11β-hydroxysteroid-dehydrogenase 2 from inadvertent activation by corticosterone through its conversion into inactive 11-dehydrocorticosterone.[33] Earlier reports have suggested that 11β-hydroxysteroid-dehydrogenase 2 functions as a gate keeper[33] and ensures mineralocorticoid receptor occupancy only by mineralocorticoids.[32] Interestingly, the current study identified 11β-hydroxysteroid-dehydrogenase 2 specific mRNA and protein in dorsal root ganglia and demonstrated its colocalization with calcitonin gene–related peptide, a marker specific for peptidergic nociceptive dorsal root ganglion neurons. Moreover, we found an approximately 80% coexistence of mineralocorticoid receptors with 11β-hydroxysteroid-dehydrogenase 2-immunoreactive neurons. Taken together these observations imply ongoing intrinsic mineralocorticoid receptor activation is due to endogenous aldosterone.

Recent emerging evidence suggests that extra-adrenal synthesis of aldosterone occurs and has local paracrine effects within the heart[5,7] and brain.[9–11,30] In the current study, the double immunofluorescence confocal microscopy showed abundant aldosterone immunoreactivity in dorsal root ganglion neurons which strongly colocalized with the sensory neuron marker calcitonin gene–related peptide and its corresponding receptor (*i.e.,* the mineralocorticoid receptor). These findings are in agreement with previous reports that suggested a putative paracrine mechanism of aldosterone in contributing to cardiac fibrosis.[34] Several investigators have postulated that the aldosterone may be synthesized in neurons located within the hypothalamus and the rostral ventral medulla of the brain appeared to contribute to their depolarization.[9–11,30] For further support of the local production of aldosterone within the neuron, the current experiments observed that aldosterone-immunoreactive dorsal root ganglion neurons coexpressed to a high degree (90%) the aldosterone synthase which is responsible for the conversion from 18-hydroxycorticosterone to active aldosterone.[9] This is in line with previous studies in hippocampal neurons in which the aldosterone producing enzymes 11ß-hydroxylase

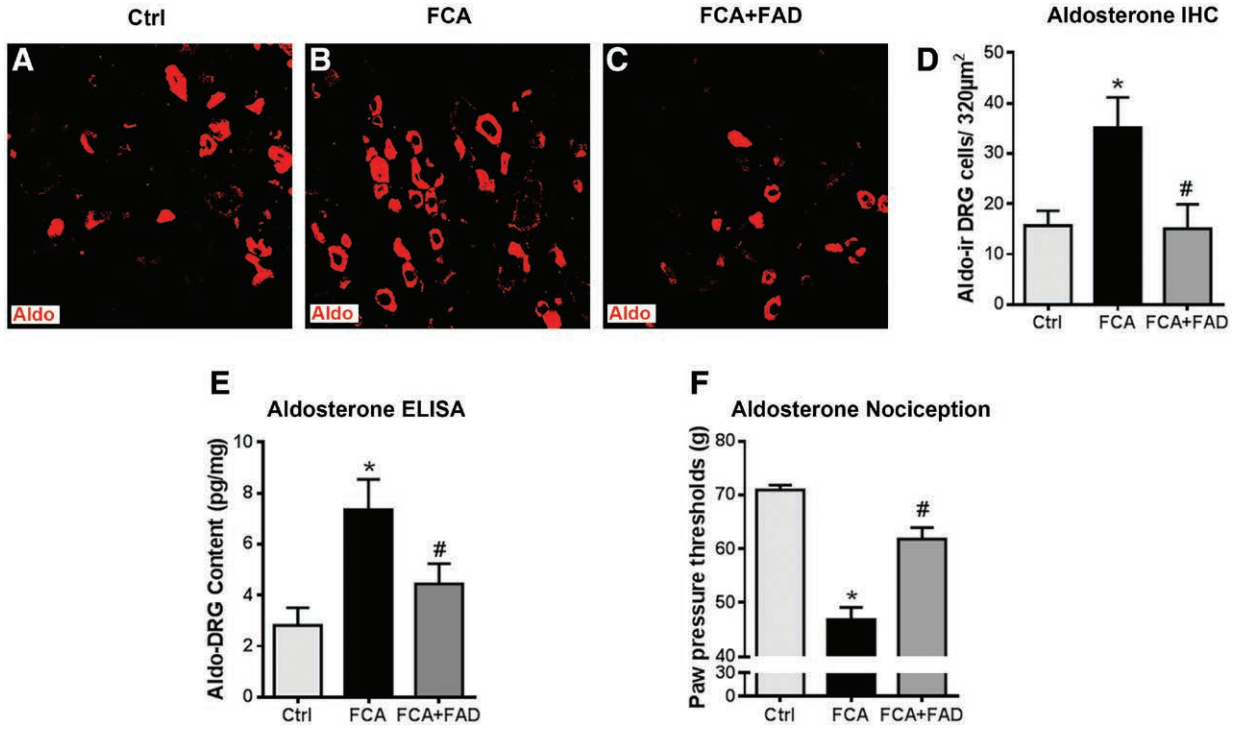

Fig. 7. Impact of aldosterone synthase inhibitor FAD286 on Freund's complete adjuvant (FCA) inflammation–induced aldosterone upregulation in dorsal root ganglia (DRG) (*A* to *E*) and on nociceptive behavior (*F*). (*A* to *D*) Determination of aldosterone-immunoreactive DRG neurons of FCA rats treated with it aldosterone synthase inhibitor FAD286. Note, the number of aldosterone-immunoreactive DRG neurons in FCA rats was increased by approximately 2.2-fold compared to controls which reversed after continuous intrathecal aldosterone synthase inhibitor FAD286 treatment for more than 5 days ($P < 0.001$; two-tailed independent Student *t* test; n = 5 rats, 3 to 4 tissue sections each). (*E*) Determination of aldosterone content in dorsal root ganglia of FCA rats treated with it aldosterone synthase inhibitor FAD286 by a fluorometric enzyme–linked immunoassay. Note, Aldo content in DRG of FCA rats increased by approximately 2.6-fold compared to control which was reversed after intrathecal aldosterone synthase inhibitor FAD286 treatment ($P < 0.001$; two-tailed independent Student *t* test; n = 5, 6, 6 rats; experiments in triplicate). (*F*) Intrathecal FAD286 resulted in significant mechanical paw pressure threshold elevations compared to baseline (0 min), indicating a reversal of FCA inflammation–induced mechanical hyperalgesia ($P < 0.001$; one-way repeated measures ANOVA, followed by *post hoc* Dunnett test; n = 7, 7, 10 rats; experiments in triplicate). Aldo, aldosterone; ELISA, enzyme-linked immunosorbent assay; FAD, FAD286; IHC, immunohistochemistry; IR, immunoreactive.

and aldosterone synthase were identified by reverse transcriptase polymerase chain reaction and immunostaining.[12,35,36] In our animal model of local inflammation, both aldosterone synthase mRNA transcripts and specific protein bands were significantly upregulated in dorsal root ganglia compared to controls. Consistently, both the number of aldosterone-immunoreactive dorsal root ganglion neurons and the aldosterone content of dorsal root ganglia significantly increased under inflammatory conditions, while aldosterone plasma concentrations remained unchanged. Therefore, our findings suggest an enhanced expression of aldosterone-producing enzyme, aldosterone synthase, and subsequent accumulation of converted aldosterone in dorsal root ganglia, which points toward an enhanced intrinsic tone of endogenous aldosterone. Furthermore, immunoprecipitation of dorsal root ganglia membrane fractions with aldosterone antibody followed by anti–mineralocorticoid receptor immunoblotting showed the coprecipitation of functional complex formations of endogenous aldosterone with membrane-bound mineralocorticoid receptors, suggesting a physical link. Our data, showing membrane-bound mineralocorticoid receptors, is supported by electron microscopy studies in presynaptic terminals and postsynaptic densities within certain brain areas.[37,38]

Since aldosterone content in peripheral sensory dorsal root ganglia was significantly increased following inflammation, we examined whether intrathecal infusion of a specific aldosterone synthase inhibitor reestablished baseline aldosterone concentrations in dorsal root ganglia of control animals. Indeed, consistent with aldosterone synthase inhibition within neurons of the brain,[10,11,18] continuous intrathecal infusion of the aldosterone inhibitor FAD286 during 4 days significantly reduced dorsal root ganglion aldosterone content, as well as the number of aldosterone-immunoreactive dorsal root ganglion neurons in Freund's complete adjuvant–treated

rats. Consequently, our behavioral experiments showed that the enhanced mechanical hypersensitivity associated with inflamed hind paws was inhibited after intrathecal aldosterone synthase inhibition. Therefore, our observations showed for the first time that the peripheral sensory dorsal root ganglia are the main site for local synthesis of active aldosterone, which contribute to ongoing mechanical hypersensitivity *via* intrinsic activation of neuronal mineralocorticoid receptors.

Limitations of the study are that demonstration of mineralocorticoid receptors, aldosterone, and aldosterone synthase mRNA by TaqMan quantitative polymerase chain reaction and respective proteins by Western blot in dorsal root ganglia do not necessarily demonstrate their expression in peripheral sensory neurons because dorsal root ganglia not only contain cell bodies of sensory neurons, but also satellite glial cells, fibroblasts, and sparse immune cells. However, our immunohistochemical staining clearly shows that the majority of cells expressing mineralocorticoid receptors, aldosterone, and the processing enzyme aldosterone synthase in dorsal root ganglia are sensory neurons. In addition, evidence for dorsal root ganglia–derived aldosterone has been convincingly demonstrated by different methods (*e.g.,* TaqMan quantitative polymerase chain reaction, Western blot, immunohistochemistry, ELISA, and inhibition of the processing enzyme aldosterone synthase) has shown a reduction in dorsal root ganglia–derived aldosterone with subsequent attenuation of inflammation-induced mechanical hypersensitivity. Differences between the semiquantitative Western blot results and the immunohistochemical counting of immunoreactive cells for the same protein (*e.g.*, 11β-hydroxysteroid-dehydrogenase 2; fig. 2, C and L) might be due to the differences in the assay sensitivity. Another limitation is the relatively small sample size of animals per group and the restriction to male rats, so that the observed significant differences may have occurred by pure chance and may not be detected in female rats. Further limitation might come from the lack of randomization which does not exclude a systematic error due to potential differences among animals even if animals are recruited from an inbred strain.

In summary, our data show that the selective mineralocorticoid receptor antagonist, canrenoate-K, rapidly inhibits mechanical hypersensitivity during inflammatory conditions, suggesting a tonic activation of mineralocorticoid receptors by an endogenous ligand. Indeed, aldosterone synthase mRNA and protein were significantly increased concomitant with enhanced aldosterone content in mineralocorticoid receptor–immunoreactive sensory neurons following local inflammation. Importantly, inhibition of the aldosterone synthase in sensory dorsal root ganglia significantly reduced aldosterone content and consequently attenuated inflammation-induced mechanical hypersensitivity. These findings provide the first evidence that the local production of aldosterone *via* aldosterone synthase within peripheral sensory dorsal root ganglia contributes to ongoing mechanical hypersensitivity *via* intrinsic activation of neuronal mineralocorticoid receptors (Supplemental Digital Content, fig. S2, http://links.lww.com/ALN/C235). This novel concept needs further validation studies in order to be translated eventually into humans.

Acknowledgments

The authors thank Mrs. Petra von Kwiatkowski (Technician, Berlin, Germany) for her technical assistance. The authors are very grateful for the gift of the glucocorticoid receptor and mineralocorticoid receptor antibodies from Drs. M. Kawata (Department of Anatomy and Neurobiology, Kyoto Prefectural University of Medicine, Kyoto, Japan) and E. Gomez-Sanchez (Montgomery Veteran's Affairs Medical Center, Jackson, Mississippi). The incentive of this work has been given the earlier clinical findings of the beneficial role of corticosteroid receptor agonists/antagonists in patients with inflammation and pain by Prof. K. H. René Koczorek (Department of Medicine, Ludwig-Maximilians-Universität München, München, Germany).

Research Support

Supported by the Prof. K. H. René Koczorek Foundation (Neuried, Germany). Doaa M. Mohamed was supported by a scholarship from the Faculty of Science at Aswan University (Aswan, Egypt) and the Egyptian government.

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Mousa: Department of Anesthesiology and Intensive Care Medicine, Charité University Berlin, Campus Benjamin Franklin, Hindenburgamm 30, 12203 Berlin, Germany. shaaban.mousa@charite.de. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Cole TJ, Young MJ: 30 Years of the mineralcorticoid receptor: Mineralocorticoid receptor null mice: Informing cell-type-specific roles. J Endocrinol 2017; 234:T83–92
2. Schumacher MA: Back pain and the mineralocorticoid receptor: Is there a connection? ANESTHESIOLOGY 2012; 117:951–2
3. Gardner DG, Shoback DM: Greenspan's Basic and Clinical Endocrinology. United Kingdom, McGraw-Hill Education, 2017, p 10
4. Lockett MF, Retallack RW: The influence of heart rate on the secretion of a substance closely resembling the 18-monoacetate of D-aldosterone by the hearts of cats under chloralose anaesthesia. J Physiol 1970; 208:21–32

5. Silvestre JS, Robert V, Heymes C, Aupetit-Faisant B, Mouas C, Moalic JM, Swynghedauw B, Delcayre C: Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. J Biol Chem 1998; 273:4883–91
6. Takeda R, Hatakeyama H, Takeda Y, Iki K, Miyamori I, Sheng WP, Yamamoto H, Blair IA: Aldosterone biosynthesis and action in vascular cells. Steroids 1995; 60:120–4
7. Mizuno Y, Yoshimura M, Yasue H, Sakamoto T, Ogawa H, Kugiyama K, Harada E, Nakayama M, Nakamura S, Ito T, Shimasaki Y, Saito Y, Nakao K: Aldosterone production is activated in failing ventricle in humans. Circulation 2001; 103:72–7
8. Yoshimura M, Nakamura S, Ito T, Nakayama M, Harada E, Mizuno Y, Sakamoto T, Yamamuro M, Saito Y, Nakao K, Yasue H, Ogawa H: Expression of aldosterone synthase gene in failing human heart: Quantitative analysis using modified real-time polymerase chain reaction. J Clin Endocrinol Metab 2002; 87:3936–40
9. Colombo L, Dalla Valle L, Fiore C, Armanini D, Belvedere P: Aldosterone and the conquest of land. J Endocrinol Invest 2006; 29:373–9
10. Oshima N, Onimaru H, Takechi H, Yamamoto K, Watanabe A, Uchida T, Nishida Y, Oda T, Kumagai H: Aldosterone is synthesized in and activates bulbospinal neurons through mineralocorticoid receptors and ENaCs in the RVLM. Hypertens Res 2013; 36:504–12
11. Wang HW, Huang BS, White RA, Chen A, Ahmad M, Leenen FH: Mineralocorticoid and angiotensin II type 1 receptors in the subfornical organ mediate angiotensin II - induced hypothalamic reactive oxygen species and hypertension. Neuroscience 2016; 329:112–21
12. MacKenzie SM, Connell JM, Davies E: Non-adrenal synthesis of aldosterone: A reality check. Mol Cell Endocrinol 2012; 350:163–7
13. Gu X, Peng L, Yang D, Ma Q, Zheng Y, Liu C, Zhu B, Song L, Sun X, Ma Z: The respective and interaction effects of spinal GRs and MRs on radicular pain induced by chronic compression of the dorsal root ganglion in the rat. Brain Res 2011; 1396:88–95
14. Sun YE, Peng L, Sun X, Bo J, Yang D, Zheng Y, Liu C, Zhu B, Ma Z, Gu X: Intrathecal injection of spironolactone attenuates radicular pain by inhibition of spinal microglia activation in a rat model. PLoS One 2012; 7:e39897
15. Dong F, Xie W, Strong JA, Zhang JM: Mineralocorticoid receptor blocker eplerenone reduces pain behaviors *in vivo* and decreases excitability in small-diameter sensory neurons from local inflamed dorsal root ganglia *in vitro*. Anesthesiology 2012; 117:1102–12
16. Shaqura M, Li X, Al-Madol MA, Tafelski S, Beyer-Koczorek A, Mousa SA, Schäfer M: Acute mechanical sensitization of peripheral nociceptors by aldosterone through non-genomic activation of membrane bound mineralocorticoid receptors in naive rats. Neuropharmacology 2016; 107:251–61
17. Ye L, Xie W, Strong JA, Zhang JM: Blocking the mineralocorticoid receptor improves effectiveness of steroid treatment for low back pain in rats. Anesthesiology 2014; 121:632–43
18. Deliyanti D, Miller AG, Tan G, Binger KJ, Samson AL, Wilkinson-Berka JL: Neovascularization is attenuated with aldosterone synthase inhibition in rats with retinopathy. Hypertension 2012; 59:607–13
19. Li X, Shaqura M, Mohamed D, Beyer A, Yamada S, Mousa SA, Schäfer M: Pro- *versus* antinociceptive nongenomic effects of neuronal mineralocorticoid *versus* glucocorticoid receptors during rat hind paw inflammation. Anesthesiology 2018; 128:796–809
20. Rittner HL, Brack A, Machelska H, Mousa SA, Bauer M, Schäfer M, Stein C: Opioid peptide-expressing leukocytes: Identification, recruitment, and simultaneously increasing inhibition of inflammatory pain. Anesthesiology 2001; 95:500–8
21. Shaqura M, Khalefa BI, Shakibaei M, Winkler J, Al-Khrasani M, Fürst S, Mousa SA, Schäfer M: Reduced number, G protein coupling, and antinociceptive efficacy of spinal mu-opioid receptors in diabetic rats are reversed by nerve growth factor. J Pain 2013; 14:720–30
22. Shaqura M, Li X, Al-Khrasani M, Shakibaei M, Tafelski S, Fürst S, Beyer A, Kawata M, Schäfer M, Mousa SA: Membrane-bound glucocorticoid receptors on distinct nociceptive neurons as potential targets for pain control through rapid non-genomic effects. Neuropharmacology 2016; 111:1–13
23. Mousa SA, Shaqura M, Khalefa BI, Zöllner C, Schaad L, Schneider J, Shippenberg TS, Richter JF, Hellweg R, Shakibaei M, Schäfer M: Rab7 silencing prevents μ-opioid receptor lysosomal targeting and rescues opioid responsiveness to strengthen diabetic neuropathic pain therapy. Diabetes 2013; 62:1308–19
24. Mousa SA, Shaqura M, Winkler J, Khalefa BI, Al-Madol MA, Shakibaei M, Schulz S, Schäfer M: Protein kinase C-mediated mu-opioid receptor phosphorylation and desensitization in rats, and its prevention during early diabetes. Pain 2016; 157:910–21
25. Bolyard LA, Van Looy JW, Vasko MR: Sensitization of rat sensory neurons by chronic exposure to forskolin or 'inflammatory cocktail' does not downregulate and requires continuous exposure. Pain 2000; 88:277–85
26. Spahn V, Stein C, Zöllner C: Modulation of transient receptor vanilloid 1 activity by transient receptor potential ankyrin 1. Mol Pharmacol 2014; 85:335–44
27. Al-Madol MA, Shaqura M, John T, Likar R, Ebied RS, Schäfer M, Mousa SA: Comparative expression analyses of pro- *versus* anti-inflammatory mediators within synovium of patients with joint trauma, osteoarthritis,

and rheumatoid arthritis. Mediators Inflamm 2017; 2017:9243736
28. Shaqura M, Khalefa BI, Shakibaei M, Zöllner C, Al-Khrasani M, Fürst S, Schäfer M, Mousa SA: New insights into mechanisms of opioid inhibitory effects on capsaicin-induced TRPV1 activity during painful diabetic neuropathy. Neuropharmacology 2014; 85:142–50
29. Mousa SA, Shaqura M, Al-Madol M, Tafelski S, Khalefa BI, Shakibaei M, Schäfer M: Accessibility of axonal G protein coupled mu-opioid receptors requires conceptual changes of axonal membrane targeting for pain modulation. J Control Release 2017; 268:352–63
30. Gomez-Sanchez E, Gomez-Sanchez CE: The multifaceted mineralocorticoid receptor. Compr Physiol 2014; 4:965–94
31. Manders EM, Verbeek FJ, Aten JA: Measurement of colocalization of objects in dual-color confocal images. J Microsc Oxford 1993; 169:375–82
32. Slight SH, Ganjam VK, Gómez-Sánchez CE, Zhou MY, Weber KT: High affinity NAD(+)-dependent 11 beta-hydroxysteroid dehydrogenase in the human heart. J Mol Cell Cardiol 1996; 28:781–7
33. Chapman K, Holmes M, Seckl J: 11β-hydroxysteroid dehydrogenases: Intracellular gate-keepers of tissue glucocorticoid action. Physiol Rev 2013; 93:1139–206
34. Lijnen P, Petrov V: Induction of cardiac fibrosis by aldosterone. J Mol Cell Cardiol 2000; 32:865–79
35. MacKenzie SM, Clark CJ, Fraser R, Gómez-Sánchez CE, Connell JM, Davies E: Expression of 11beta-hydroxylase and aldosterone synthase genes in the rat brain. J Mol Endocrinol 2000; 24:321–8
36. MacKenzie SM, Lai M, Clark CJ, Fraser R, Gómez-Sánchez CE, Seckl JR, Connell JM, Davies E: 11beta-hydroxylase and aldosterone synthase expression in fetal rat hippocampal neurons. J Mol Endocrinol 2002; 29:319–25
37. Prager EM, Brielmaier J, Bergstrom HC, McGuire J, Johnson LR: Localization of mineralocorticoid receptors at mammalian synapses. PLoS One 2010; 5:e14344
38. Johnson LR, Farb C, Morrison JH, McEwen BS, LeDoux JE: Localization of glucocorticoid receptors at postsynaptic membranes in the lateral amygdala. Neuroscience 2005; 136:289–99