Mineralocorticoid receptor associates with pro-inflammatory bias in the hippocampus of spontaneously hypertensive rats

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INTRODUCTION

Previous research has indicated that brain damage of the adult spontaneously hypertensive rat (SHR) resembles the neuropathology of mineralocorticoid-induced hypertension, suggesting a similar endocrine dysfunction in both situations. The endocrine abnormalities of SHR include a hyperactive hypothalamic-pituitary-adrenal axis, stimulation of the brain angiotensin- and vasopressin systems and an increased binding capacity and expression of the mineralocorticoid receptor (MR or NR3C2) in the hippocampus and hypothalamus. Augmented MR expression has also been detected in the heart, vascular endothelial cells and kidney of SHR, suggesting that increased MR function is a generalised property of this genetically selected rat strain. Functional evidence for the implication of brain MR in hypertension comes from the enhanced expression of vasopressin and c-Fos in the hypothalamus following mineralocorticoid treatment of SHR rather than the parent strain Wistar-Kyoto (WKY) from which SHR were derived. Furthermore, a decrease of systolic blood pressure occurs after i.c.v. administration of the MR antagonist RU28318, particularly if the SHR rats were sensitised by sodium loading.

In the hippocampus of SHR several alterations characteristic for damage occur, such as myelin loss, reactive astroglia and microglia, expression of the mineralocorticoid receptor in the hippocampus, increased MR expression causes a bias towards a pro-inflammatory phenotype characteristic for hypertensive encephalopathy.

Damage observed in the hippocampus of the adult spontaneously hypertensive rat (SHR) resembles the neuropathology of mineralocorticoid-induced hypertension, supporting a similar endocrine dysfunction in both entities. In the present study, we tested the hypothesis that increased expression of the hippocampal mineralocorticoid receptor (MR) in SHR animals is associated with a prevalent expression of pro-inflammatory over anti-inflammatory factors. Accordingly, in the hippocampus, we measured mRNA expression and immunoreactivity of the MR and glucocorticoid receptor (GR) using a quantitative polymerase chain reaction and histochemistry. We also measured serum-glucocorticoid-activated kinase 1 (Sgk1 mRNA), the number and phenotype of Iba1+ microglia, as well as mRNA expression levels of the pro-inflammatory factors cyclooxygenase 2 (Cox2), Nlrp3 inflammasome and tumour necrosis factor α (Tnfa). Expression of anti-inflammatory transforming growth factor (Tgf)β mRNA and the NADPH-diaphorase activity of nitric oxide synthase (NOS) were also determined. The results showed that, in the hippocampus of SHR rats, expression of MR and the number of immunoreactive MR/GR co-expressing cells were increased compared to Wistar-Kyoto control animals. Expression of Sgk1, Cox2, Nlrp3 and the number of ramified glia cells positive for Iba1+ were also increased, whereas Tgflβ mRNA expression and the NADPH-diaphorase activity of NOS were decreased. We propose that, in the SHR hippocampus, increased MR expression causes a bias towards a pro-inflammatory phenotype characteristic for hypertensive encephalopathy.

KEYWORDS

hippocampus, hypertension, microglia, mineralocorticoid receptor, neuroinflammation
increased oxidative stress, decreased neurogenesis, neuroinflammation, vascular remodelling, neuronal mitochondrial dysfunction, decreased activity of nitric oxide synthase (NOS) and pyramidal neurone atrophy.\textsuperscript{2,12-16} MR activation in brain endothelia induces mRNA of pro-inflammatory cytokines\textsuperscript{17} and stimulates proliferation of the forebrain microglia.\textsuperscript{18} The resulting neuroinflammation is a strong contributor to the pathogenic effects of hypertension.\textsuperscript{19} Thus, although MR is neuroprotective to the effects of chronic stress in hippocampus,\textsuperscript{20,21} it appears that during hypertension MR activation contributes to end-organ damage, which is further aggravated by salt loading.\textsuperscript{2}

The present study aimed to determine whether changes in the expression of the MR and its colocalised glucocorticoid receptor (GR or NR3C1)\textsuperscript{22,23} associate with the immunophenotype of microglia and neuroinflammation markers in the hippocampus of SHR. Accordingly, we used 10 month-old male SHR and WKY rats to study (i) the expression of MR and GR in hippocampus; (ii) the expression of the MR and GR-target gene serum-glucocorticoid-activated kinase 1 (Sgk1 mRNA) as an indicator of receptor functionality\textsuperscript{24}; (iii) the number and phenotype of Iba1+ microglia; (iv) mRNA expression levels of the pro-inflammatory factors cyclooxygenase 2 (Cox2), Nfκb inflammasome and tumor necrosis factor α (Tnfα); (v) expression of Tgfiβ mRNA, a factor endowed with anti-inflammatory properties\textsuperscript{25}, and changes of the NADPH-diaphorase activity of NOS\textsuperscript{7}, an enzyme related to vasodilation, which is suppressed by MR activation.\textsuperscript{26} The results highlight an association between MR, neuroinflammation and hypertensive encephalopathy.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Ten-month-old male SHR and WKY rats were purchased from the animal facility of the Instituto de Biología y Medicina Experimental (Buenos Aires, Argentina). Rats were maintained under a 12:12 hour light/dark cycle (lights on 07:00 h) at 22°C and controlled humidity and given food and water ad libitum. The blood pressure response, as monitored using a tail-cuff method, measured 187.1±5.2 mm Hg in SHR and 111.8±3.0 mm Hg in control WKY rats. For immunohistochemistry (IHC) and immunofluorescence (IF) assays, rats were anaesthetised with isoflurane under a fume hood, and perfused via the heart with 0.9% NaCl in 0.1 mol L\textsuperscript{−1} phosphate-buffered saline (pH 7.4) (PBS) followed by 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) in phosphate buffer at pH 7.4. For real-time polymerase chain reaction (PCR), anaesthetised rats were decapitated and brains collected for further analysis. Animal handling followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Instituto de Biología y Medicina Experimental Animal Facility Assurance Certificate; #N-AS072-01) and was approved by the Institute’s Animal Care and Use Committee. Efforts were made to reduce the number of animals in the different experiments.

2.2 | IHC and IF assays

After perfusion, brains were post-fixed for 24 hours in 4% PFA at 4°C and transferred to 0.1 mol L\textsuperscript{−1} phosphate buffer (pH 7.4) for 2 hours. Using a vibrating microtome, 50-μm coronal sections from plates 27 to 33 of a rat atlas\textsuperscript{27} were cut and processed for free-floating IHC or IF. For IHC, endogenous peroxidase activity was quenched for 20 minutes in 50% methanol/1% hydrogen peroxide in PBS. Sections were washed three times in PBS, blocked with 3.5% bovine serum albumin (Sigma, St. Louis, MO, USA) in PBS – 0.1% Triton X-100 for Iba1 staining, or treated with 10% horse serum in PBS-0.15% Triton X-100 for MR and 10% goat serum in PBS for GR for 30 minutes at room temperature. Subsequently, sections were incubated with primary antibodies against the MR, GR or Iba-1 antigens. Table 1 shows the dilutions, incubation times and antibody sources used for IHC. After two overnight incubations with the respective primary antibody at 4°C, sections were exposed to biotinylated horse anti-mouse immunoglobulin G or goat anti-rabbit immunoglobulin G and further processed using an ABC kit in accordance with the manufacturer’s instructions (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Immunoreactions were visualised in terms of peroxidase activity using diaminobenzidine tetrachloride (0.25 mg mL\textsuperscript{−1}; Sigma) as substrate in the presence of 0.01% hydrogen peroxide for 7 minutes in the dark. The sections were given a final rinse in PBS, placed onto gelatine-coated glass slides, dehydrated in a series of ethanol and xylene, and finally mounted using synthetic Canada balsam (Biopack, Buenos Aires, Argentina).

| Antibody          | Host    | Trademarks                                   | AB No.    | Working dilution |
|-------------------|---------|----------------------------------------------|-----------|------------------|
| Anti-MR           | Mouse   | Gomez Sanchez et al. (2006)                   | #365 4D6  | 1:400            |
| Anti-GR           | Rabbit  | Santa Cruz Biotechnology, Santa Cruz, CA, USA | #M-20, SC1004 | 1:200            |
| Anti-Iba1         | Rabbit  | Wako, Tokyo, Japan                           | #019-19741 | 1:2000           |
| Anti-mouse biotinylated | Horse  | Vector Laboratories, Burlingame, CA, USA    | #BA2000   | 1:200            |
| Anti-rabbit biotinylated | Goat  | Vector Laboratories, Burlingame, CA, USA   | #BA1000   | 1:200            |
| Anti-mouse IGG - Alexa Fluor 488 | Goat  | Invitrogen - Thermo Fisher Scientific Carlsbad, CA, USA | #A11001 | 1:1000           |
| Anti-rabbit IGG - Alexa Fluor 555 | Goat  | Invitrogen - Thermo Fisher Scientific Carlsbad, CA, USA | #A-21428  | 1:1000           |
For dual IF of MR and GR, brain slices were pre-incubated with 5% goat serum in PBS- 0.15% Triton X-100 for 30 minutes at 37°C, and then incubated with primary antibodies (Table 1) prepared in 5% goat serum, 0.3% Triton X-100 in PBS for 48 hours. Following incubation, sections were washed with PBS and incubated for 1 hour at room temperature with a goat anti-rabbit IgG conjugated to Alexa Red 555 and anti-mouse IgG conjugated to Alexa 488. After further washings, sections were placed in gelatine coated glass slides and cover slipped with Fluoromount-G (Southern Biotech, Birmingham, AL, USA). If primary antibodies were omitted, immunofluorescence was negative for MR or GR. Experimental groups were stained simultaneously to eliminate inter-experiment variations and conditions were maintained so that they were rigorously identical throughout the assays. To rule out experimental bias, the appropriate investigators were blinded regarding the source of the materials.

2.3 | RNA purification and real-time PCR of MR, GR and pro-inflammatory mediators

Immediately after death, bilateral hippocampi were removed from the brains of SHR and WKY rats, frozen on dry ice and temporarily stored at −80°C until subsequent RNA purification. Tissues were homogenized with a Polytron homogeniser (Cole Parmer, IL, USA) and total RNA was then extracted using Trizol reagent (Life Technologies-Invitrogen, Carlsbad, CA, USA), precipitated with isopropanol and dissolved in distilled water. The concentration of total RNA was determined by measuring the optical density at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total RNA was subjected to Dnase I (Life Technologies-Invitrogen) treatment (2 U for 10 minutes at room temperature to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesised from 2 μg of total RNA using a SuperScript III RNase H Reverse transcriptase kit (Life Technologies-Invitrogen) for 60 minutes at 50°C in the presence of random hexamer primers. The sequence of primers for amplification in whole hippocampus of rat MR, GR, Sgk1, Tnfα, Nlrp3, Cox2 and Tgfβ is shown in Table 2. Cyclophilin A was chosen as a housekeeping gene based on the similarity of mRNA expression across all sample templates. Primers were designed using primer-blast web software (National Centre for Biotechnology Information, Bethesda, MD, USA) with default parameters. Primer specificity was also confirmed using primer-blast.

PCR analysis was carried out using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems). Gene expression was calculated using the 2^{-ΔΔCT} method and determined as the fold induction compared to its respective control. PCR was performed in triplicate and data concerning amplification, running conditions, cDNA content and primer concentrations of each gene are specified in Table 2. Specificity of PCR amplification and the absence of dimers were confirmed by melting curve analysis. The accuracy of the reaction was confirmed by the linearity and efficiency of PCR amplification. Results are expressed as the mean±SEM (n=7 rats per group).
2.4 | Quantitative analysis of Iba1 immunoreactive cells

Photographic observation and quantitative analysis of Iba1 immunohistochemistry microglial cells was performed in the stratum radiatum beneath the CA1 and CA3 pyramidal layers and in the hilus of the dentate gyrus. Images were captured using a BH2 microscope (Olympus, Tokyo, Japan) coupled to a Panasonic GPKR222 camera at ×600. The total number of Iba1-labelled cells from the three areas was measured and then a morphometric categorisation of microglial phenotypes was performed with respect to cells with: (i) abundant ramified processes and small soma or (ii) hypertrophic phenotype, containing large soma and shortened processes. The hypertrophic phenotype included cells with varying morphologies, including those with low processes and a round ameboid soma shape. The number of Iba1+ cells of each microglia phenotype was quantified and expressed per unit of volume (mm³) (n=5-6 rats per group).

2.5 | Determination of MR and GR immunoreactive area

Images containing the dorsal hippocampus of SHR and WKY rats were acquired at the same magnification (×212.5) using a GPKR222 digital camera (Panasonic Corp., Osaka, Japan) connected to a BH2 microscope (Olympus) and image analysis software BIOSCAN OPTIMAS VI (Bioscan Inc., Edmonds, WA, USA). Immunoreactive MR and GR areas were determined for the CA3 (MR) and CA1 (MR, GR) hippocampal regions, applying a grey-scale threshold, considering that MR extends to all hippocampus subfields, whereas GR is poorly localised in the CA3 region. Digitised images of tissue sections were processed simultaneously under identically operating conditions, such as light beam, wavelength and greyscale threshold, throughout the experiment. The same number of sections (n=6 per rat brain) was studied for each experimental group (n=6 SHR or WKY rats). Results are expressed as total immunoreactive areas per mm², which is a procedure proportional to the total immunoreactive cell number.

2.6 | Analysis of MR and GR co-expression in the hippocampus of SHR and WKY rats

The percentage of MR positive cells (green) that co-express GR (red) was analysed by confocal microscopy. Confocal microphotographs (2048 × 2048 pixels) were acquired using a DS-U1 camera (Nikon, Tokyo, Japan) with ACT-2U software (Nikon) coupled to an Eclipse E800 confocal Microscope (Nikon) at ×600 magnification. Pairs of images were collected simultaneously in the green and red channels. For image processing and image analysis, IMAGEJ, version 1.51h (NIH, Bethesda, MD, USA) was used. After applying a threshold, the total number of MR positive cells (green IF) was counted and the percentage of MR positive cells that also expressed GR (yellow in merged images) was calculated. Cells were counted in n=6 sections from n=6 animals per experimental group.

2.7 | NADPH-diaphorase histochemistry

Activity, based on the diaphorase property of NOS, was determined by the procedure of Vincent and Kimura in 17-μm cryostat sections of the brain at the level of the dorsal hippocampus. Slices were fixed by immersion in 2% PFA in 0.1 mol L⁻¹ phosphate buffer (pH 7.2) for 6 minutes at 4°C. After fixation, the sections were rinsed twice in PBS, and incubated in a solution containing 0.2 mg mL⁻¹ of nitroblue tetrazolium, 2.7 mg mL⁻¹ L-malic acid, 1 mg mL⁻¹ of b-NADPH (all obtained from Sigma) and 0.3% Triton X-100 dissolved in 0.1 mol L⁻¹ Tris-HCl buffer (pH 7.4). After keeping the reaction in the dark for 60 minutes at 37°C, it was stopped by two washes in PBS at room temperature. Sections were then dehydrated briefly in ethanol, air-dried and coverslipped with Permoun (Fisher Scientific, Waltham, MA, USA). Brain areas containing blue formazan staining were analysed by computerised image analysis, as described previously. Results (n=5 animals per group) were expressed as the NADPH-diaphorase active area (in mm²) in the CA1 and CA3 hippocampal regions.

2.8 | Statistical analysis

Student’s t test was used to determine significance between the two groups (SHR and WKY rats). In case variables did not follow normality criteria in accordance with the Shapiro-Wilk test, the Mann-Whitney test was applied (PRISM, version 4; GraphPad Software Inc., San Diego, CA, USA). All results are expressed as the mean±SEM. P<.05 was considered statistically significant.

3 | RESULTS

3.1 | MR, GR mRNA and MR protein expression and co-expression of MR with GR in the hippocampus of SHR and WKY rats

Dual immunofluorescence analysis of the expression of MR/GR in whole hippocampus and the CA1 and CA3 regions revealed strain differences between SHR and WKY rats (Figure 1). Low power photomicrographs (Figure 1A) showed MR staining widely distributed in pyramidal cell areas and the gyrus dentatus from both SHR and WKY rats. Instead, GR staining was low in the CA3 pyramidal cell layer, as reported previously. In the hippocampus, the signal for MR immunofluorescent protein (green colour) and, to a lesser extent, GR (red colour), as well as co-expression with GR (merged, yellow), was stronger in SHR compared to WKY rats. Second, zoom imaging of CA1 (Figure 1B) and CA3 regions (Figure 1C) showed higher number of MR+ cells and a slightly higher number of GR+ cells in both regions of SHR. Figure 1(B,C) (merged, yellow) showed enhanced colocalisation of MR/GR proteins in SHR compared to WKY rats. Furthermore, quantitative analysis of dual immunofluorescence staining of MR/GR positive cells in the CA1 region of SHR demonstrated a higher percentage of MR+ cells with co-expression of GR (Figure 1B, right, SHR vs WKY rats, P<.01). Similarly, a higher percentage of MR+ cells
colocalised with the GR protein in the CA3 region of SHR (P<.05) (Figure 1C, right).

Real-time PCR studies demonstrated that SHR expressed approximately 2.5-fold higher levels of MR mRNA in whole hippocampus compared to WKY rats (P<0.01), in agreement with a previous study\(^7\) (Figure 2A). Quantitative analysis of the MR immunoreactive protein in the CA1 and CA3 regions showed a larger total immunoreactive area mm\(^2\) in SHR compared to WKY rats (P<.05 for CA1 and P<.05 for CA3) (Figure 2B,C). By contrast, GR mRNA levels in whole hippocampus were similar in both strains (Figure 2D), although differences in GR total immunoreactive area were obtained in the CA1 region, favouring SHR over WKY rats (Figure 2E). Thus, we observed stimulated expression of MR mRNA in whole hippocampus and of MR protein in pyramidal cell layers of SHR.

### 3.2 Microglia morphological phenotype in SHR and WKY rats

We used Iba1 immunofluorescence staining and confocal microscopy to compare microglia morphological phenotypes in WKY rats and SHR. Three areas of the hippocampus were selected for the study: CA1 and CA3 pyramidal regions and the hilus of the dentate gyrus. Iba1+ cells were classified into two main types using light microscopy: (i) cells showing a highly ramified processes and small soma and (ii) cells showing a hypertrophied soma with less pronounced processes. The last group also included cells with ameboid-like appearance, although this was present in very minor amounts. Figure 3 (top) shows examples of the ramified (Figure 3A), hypertrophic (Figure 3B) and ameboid (Figure 3C) Iba1+ cells used for morphological classification. Differences in cell types in the noted regions of WKY rats and SHR were analysed using Student’s t test. As shown in Figure 3, SHR showed higher number of ramified microglia in the CA1 region (P<.05 vs WKY) (Figure 3, left) and in the CA3 region (P<.05 vs WKY) (Figure 3, centre), as well as in the hilus (P<.05) (Figure 3, right). Further analysis of microglia phenotypes by ANOVA followed by a post-hoc test demonstrated that hypertrophic microglia outnumbered the ramified form in WKY rats (CA1, not significant; CA3, P<.01; hilus, P<.001). However, a different morphological profile was found for SHR because, in this strain, ramified and hypertrophic
FIGURE 2  Expression of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA and immunoreactive protein in the hippocampus of SHR and WKY rats. MR mRNA was increased in whole hippocampus of SHR compared to WKY rats (A) (***P < .01). Immunostaining for MR protein in the CA1 region (B) (*P < .05) and CA3 region (C) (*P < .05) of the hypertensive rats was higher than in the normotensive strain. (D) GR mRNA was similar in both strains, whereas GR total immunoreactive area (E) was higher in SHR (*P < .05 vs WKY rats). Statistical analysis by Student’s t test

FIGURE 3  Immunophenotype of microglia revealed by Iba1 staining in the hippocampus of hypertensive and normotensive rats. The upper microphotographic images show examples of microglial immunophenotypes found in the CA1 hippocampal region: (A) ramified type with abundant processes; (B) hypertrophic type with round soma and shorter processes; and (C) ameboid. Inside bar = 20 μm. Quantitative analysis by Student's t test showed that the ramified type prevailed in the CA1 region (*P < .05), CA3 region (*P < .05) and the hilus of the dentate gyrus (*P < .05) of spontaneously hypertensive rats (SHR) compared to Wistar-Kyoto (WKY) rats
microglia were equally distributed for all regions of the hippocampus (not significant).

Because morphology by itself may not be the only criterion to discriminate quiescent from activated microglia, we next analysed the expression of pro-inflammatory and anti-inflammatory mediators. This procedure would better establish the ubiquitous immunophenotype of glial cells in SHR and WKY rats.

### 3.3 | Comparative expression of a marker of MR/GR activation and pro-inflammatory and anti-inflammatory factors in the hippocampus of SHR and WKY rats

To investigate whether changes of microglia phenotype were accompanied by markers of neuroinflammation, we measured, by quantitative PCR, several molecules bound to inflammation and signalling in the hippocampus of the two strains. First, we determined SGK1, a determinant of mineralocorticoid and glucocorticoid function\(^\text{24}\) fostering inflammation in the periphery.\(^\text{24}\) In agreement with these effects, the hippocampus of SHR showed a five-fold increase in Sgk1 mRNA compared to WKY rats (\(P<.01\)) (Figure 4A). We also determined Cox2, an enzyme associated with vascular inflammation in SHR.\(^\text{35}\) Cox2 mRNA was increased two-fold in the hippocampus of SHR vs WKY rats (\(P<.05\)) (Figure 4B). Third, the mRNA for the inflammasome component Nlrp3 was higher in SHR vs WKY rats (\(P<.05\)) (Figure 4C). However, the cytokine Tnfα mRNA in SHR was not significantly different compared to WKY rats (Figure 4D). By contrast to the high expression of the pro-inflammatory mediators Sgk1, Cox2 and Nlrp3 mRNAs, levels of the anti-inflammatory molecule Tgfβ were reduced in SHR vs WKY rats (\(P<.05\)) (Figure 4E). Finally, in agreement with findings obtained in the brain of SHR,\(^\text{36}\) the NADPH-diaphorase activity of NOS was significantly lower in the hippocampus CA1 area of SHR vs WKY rats (Figure 4F). Therefore, changes of a MR-responsive gene, pro- and anti-inflammatory cytokines, and the NADPH-diaphorase activity of NOS evolved concomitantly with MR and possibly GR overdrive in the hippocampus of SHR.

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**FIGURE 4** Quantitative analysis of responsive genes and pro and anti-inflammatory mediators by a real-time polymerase chain reaction. The graphs represent SGK1 (A), cyclo-oxygenase 2 (COX2) (B), NLRP3 subunit of the inflammasome (C), tumour necrosis factor (TNF)α (D), transforming growth factor (TGF)β (E) and NADPH-diaphorase activity of nitric oxide synthase (F). Spontaneously hypertensive rats (SHR) showed higher expression of the mRNA of genes linked to neuroinflammation [(A) (**\(P<.01\)), (B) (*)\(P<.05\)] and (C) (*)\(P<.05\)]; decreased transcription of the anti-inflammatory factor TGFβ (E) (*)\(P<.05\); and decreased NADPH-diaphorase activity (F) (*)\(P<.05\) compared to Wistar-Kyoto (WKY) normotensive rats. The slight increment of TNFα in SHR was not significant. Statistical analysis by Student’s t test
4 | DISCUSSION

The present study has demonstrated further differences in the hippocampus of SHR, suggesting a link between increased MR expression and neuroinflammation in this hypertensive strain.

The current data demonstrate that increased hippocampal MR expression is associated with a shift in balance towards increased expression of pro-inflammatory genes at the expense of anti-inflammatory factors. The results concerning MR agree with the higher binding and expression of MR in the hippocampus and heart, kidney and endothelial vasculature of SHR vs WKY rats.8 In the SHR hippocampus, higher MR expression may be related to existing neuropathology, a circumstance that unmasks this receptor as a proverbial "death receptor" under adverse conditions. It is considered that MR activation mediates a switch from vascular health to damage, inflammation and fibrosis in response to cardiovascular risk factors.37-39 Regarding the GR, quantitative PCR did not show strain differences in mRNA levels, although the total immunoreactive GR protein was significantly elevated in SHR vs WKY rats. However, it should be considered that the quantitative PCR data were obtained from whole hippocampus, containing regions of high (CA1, CA3 and dentate gyrus) and low (CA3) GR expression. Instead, the immunostaining shown in Figure 2(E) corresponds to the CA1 region, which shows high GR expression. Immunofluorescence staining revealed further strain differences in receptor localisation. Although MR localised mainly in the cell nuclei, GR labelling was also found in cytoplasm and cell processes. These differences depend on the receptor in question because GR becomes visible as a function of corticosterone concentration, which concentrates the signal in the nucleus. The MR does not encounter such a problem because, as a result of its high affinity it is occupied throughout the day.23 In both cases, higher intensity staining for MR and GR was observed for SHR vs WKY rats. These changes suggest that differences may exist in the mechanism of steroid action in the hippocampus of hypertensive rats, the nature of which awaits further clarification.

Factors implicated in MR overdrive may comprise oxidative stress the novel MR activators small GTP-binding protein Rac1 and adipocyte-derived aldosterone-releasing factor, the renin-angiotensin system and corticotrophin-releasing factor.40-42 Oxidative stress may play an important role because it appears to boost the activation of MR by endogenous glucocorticoids.38,39,43 It is assumed to be a direct consequence of an intracellular redox change and/or absence of the enzyme 11β-hydroxysteroid dehydrogenase type 2 in hippocampus.44 This enzyme normally protects the aldosterone-selective MR in the kidney, vascular endothelial cells and in discrete brain regions.55,66 This condition of elevated MR in the face of risk factors may increase hippocampal vulnerability, as shown by the profound neuropathology found in SHR.1,2,7,12,16,47-48

In the hippocampus, the preferred MR ligand is corticosterone and not aldosterone as a result of the absence of 11-HSD2; corticosterone binds with high affinity to MR and approximately ten-fold lower affinity to the GR.23 MR and GR are co-expressed in hippocampal neurones (Figure 2)31,49 and bind for the same glucocorticoid response elements in the hippocampal genome as homo- and heterodimers.50-52 Although, under certain conditions, corticosterone enhances hippocampal inflammation,53,54 the extent to which GR also played a damaging role in the hippocampus of SHR remains to be established. In the present study, variation in GR immunoreactivity in the nucleus needs to be considered with caution because of uncertainty about the circulating levels corticosterone.55 Nevertheless, corticosterone may be the prevalent steroid binding in SHR brain because serum aldosterone is nine-fold lower in SHR (33.8±12.9 pg mL⁻¹) compared to WKY rats (295±94.5 pg mL⁻¹).7 Enhanced co-expression of MR and GR in neurones in the hippocampus of SHR was detected by dual immunofluorescence analysis and confocal microscopy. In our studies, a strong response of the MR and GR target gene Sgk1 was found in the SHR hippocampus. Although we did not localise the cellular type responsible for the increased Sgk1 mRNA, the kinase has been detected in neurones, oligodendrocytes, astrocytes and microglia.56 Lang et al.57 have shown that MR activation stimulates expression of SGK1 in neurones. Therefore, up-regulation of Sgk1 in the hippocampus of SHR indicates that MR and GR are functionally active and may be involved in neuroinflammation. Proof of receptor activation is the increase of Sgk1 expression, a molecule genomically regulated by mineralocorticoids and glucocorticoids and involved in MR-mediated inflammation.34 However, SGK1 plays other roles than inflammation because it is also implicated in the physiology and pathophysiology of sodium transport.24 This property opens additional possibilities for the role of SGK1 in the hippocampus.

The high expression levels of MR mRNA and protein support MR-sensitive inflammation in the hippocampus of SHR. Previous studies have suggested that imbalance in MR:GR stimulation increases hippocampal vulnerability and worsens inflammation.54,58,59 In SHR, this possibility is supported by analysis of pro-inflammatory and anti-inflammatory factors. The inducible COX isoform (COX2) produces prostanooids related to the pathogenesis of hypertension, microglia activation and neuroinflammation in deoxycorticosterone-salt hypertensive rats,60 a model sharing strong similarities with SHR; COX2 protein levels are elevated in the urine and kidney of SHR,61 supporting a role for COX2 in the pathophysiology of hypertension. In addition, SHR also showed increased hippocampal expression of Nlrp3 subunit of the inflammasome, localised in neurones and microglia62 and astrocytes following inflammation.63 Also relevant to our results, the NLRP3 inflammasome is involved in the vascular effects of aldosterone and is increased in hypertensive patients.64

By contrast to the increased expression of Cox2 and Nlrp3 mRNAs in SHR vs WKY rats, expression of Tgfβ1 and NADPH-diaphorase activity was reduced and Tnfα was unmodified. Acting on microglia, Tgfβ1 reduces the production of reactive oxygen species and pro-inflammatory factors.25 However, even if changes of TGFβ1 protein accompany changes of Tgfβ1 expression, this will not be final proof for an anti-inflammatory role of this factor in SHR. Regarding the decreased NADPH-diaphorase activity, this possibly depends on an enhanced MR activation.26 In support of our findings, low NOS expression has been already reported in the SHR hippocampus,13 suggesting impaired vasodilation. Despite TNFα being an important pro-inflammatory cytokine, its mRNA levels were not significantly different between SHR
and WKY rats. It has been reported that TNFα involvement is involved in hypertensive vascular remodelling in brain regions outside the hippocampus; Tayebati et al. reported increased immunoreactive TNFα in the frontal cortex but not the hippocampus in 8-week-old SHR vs WKY rats. Unchanged levels of TNFα reported by Tayebati et al., as well as in our own study conducted in the hippocampus, suggest a moderate pro-inflammatory environment of this brain region in SHR. In summary, the MR-induced damage to the hippocampus of SHR appears to be mediated by enhanced expression of some, but not all, pro-inflammatory mediators and by decreased expression of vasodilators and putative anti-inflammatory mediators.

On the basis of these results, we propose that, in the hippocampus of SHR, MR activation may drive a vicious circle, starting with the deleterious effects of hypertension on the vasculature. The latter causes hypoxic neuronal damage, followed by microgliosis and astrogliosis, a process promoted via MR, Reactive glial cells respond by producing some pro-inflammatory factors and decreasing anti-inflammatory TGFβ and NOS, processes that exacerbate oxidative stress and neuroinflammation. In addition, increased MR transcription and translation may promote hippocampal glutamate release, leading eventually to excitotoxicity, Hence, MR is changed into a damaging receptor that fosters neuroinflammation and oxidative stress. In addition, the colocalisation of MR/GR in hippocampus of SHR may signal receptor cooperativity at the Sgk1 gene, as postulated for MR:GR heterodimers in the hippocampus of stressed rats by Mifsud and Reul. Given our results, it is possible that increased MR expression and enhanced MR/GR colocalisation in pyramidal neurones is relevant to the risk of hippocampal abnormalities.

Our findings raise a number of questions. First, does higher MR expression cause the increase in pro-inflammatory markers in hippocampus? To address this question, the increase in MR needs to be generalised to glial and vascular endothelial cells in the SHR to show colocalisation with the increased expression of pro-inflammatory genes. Second, does the hippocampal MR pro-inflammatory phenotype translate into an altered behaviour of the SHR rat? It is of relevance to the present study that SHR rats comprise a classical model for studying attention deficit hyperactivity disorder and also show a different coping style compared to WKY controls. Third, how is the increase in ramified microglia related to the increased expression of pro-inflammatory markers? Further analysis of the data from Figure 3 indicate that WKY rats show more hypertrophic than ramified Iba1+ cells, whereas SHR show similar numbers with respect to the ramified and hypertrophic phenotype in the CA1, CA3 and hilus. In this regard, microglia heterogeneity occurs in neurodegenerative diseases. Therefore, we would like to propose that the microglia phenotype of SHR is consistent with a condition of chronic, subtle inflammation as reported in major neurodegenerative diseases including Alzheimer’s disease. In this regard, neurodegenerative changes in the brain of SHR have been demonstrated in several studies. Accordingly, it is possible, although not yet proven, that the morphological changes of microglia in SHR hippocampus may be secondary to adrenal receptor imbalance and oxidative stress.

In conclusion, in the hippocampus of SHR, we have demonstrated a higher expression of MR and more immunoreactive MR and GR positive cells, which results in a bias towards a pro-inflammatory phenotype characteristic for hypertensive encephalopathy. Thus, the current evidence suggests that the MR becomes activated under adverse conditions via an altered intracellular redox mechanism. This increased MR activity promotes priming and activation of microglia in the neurovascular niche of hippocampus, as shown by morphology and Iba1 immunohistochemistry. This condition can be mimicked by excess deoxycorticosterone in the deoxycorticosterone salt-treated animal model. Further work will aim to further define the role of the endothelial and neuronal MR in the context of neuroinflammation and cardiovascular risk factors.

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