ARTICLE

Importance of the commissural nucleus of the solitary tract in renovascular hypertension

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Abstract

The rodent renovascular hypertension model has been used to investigate the mechanisms promoting hypertension. The importance of the carotid body for renovascular hypertension has been demonstrated. As the commissural NTS (cNTS) is the first synaptic site in the central nervous system that receives information from carotid body chemoreceptors, we evaluated the contribution of cNTS to renovascular hypertension in the present study. Normotensive male Holtzman rats were implanted with a silver clip around the left renal artery to induce two-kidney, one-clip (2K1C) hypertension. Six weeks later, isoguvacine (a $GABA_A$ agonist) or losartan (an AT1 antagonist) was injected into the cNTS, and the effects were compared with carotid body removal. Immunohistochemistry for Iba-1 and GFAP to label microglia and astrocytes, respectively, and RT-PCR for components of the renin–angiotensin system and cytokines in the NTS were also performed 6 weeks after renal surgery. The inhibition of cNTS with isoguvacine or the blockade of AT1 receptors with losartan in the cNTS decreased the blood pressure and heart rate of 2K1C rats even more than carotid body removal did. The mRNA expression of NOX2, TNF-α and IL-6, microglia, and astrocytes also increased in the cNTS of 2K1C rats compared to that of normotensive rats. These results indicate that tonically active neurons within the cNTS are essential for the maintenance of hypertension in 2K1C rats. In addition to signals from the carotid body, the present results suggest that angiotensin II directly activates the cNTS and may also induce microgliosis and astrogliosis within the NTS, which, in turn, cause oxidative stress and neuroinflammation.

Keywords renal hypertension · angiotensin II · AT1 subtype receptor · commissural NTS

Introduction

Hypertension is a chronic disease that may cause serious health consequences such as heart attack, stroke and kidney failure [1]. One model of hypertension that has a defined

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cause is renovascular hypertension [2, 3], which is induced by at least 50% renal artery stenosis or occlusion caused primarily by atherosclerosis [2, 3].

A rat model of renovascular hypertension is two-kidneys, one-clip (2K1C) or Goldblatt hypertension, which consists of partial stenosis of the renal artery that supplies one kidney [4, 5]. Renal artery stenosis decreases renal perfusion pressure, causing overproduction of renin and persistent activation of the renin–angiotensin–aldosterone system (RAAS) [5–7]. The overactivity of the RAAS in 2K1C rats produces many peripheral effects that may contribute to hypertension, including vascular and cardiac remodeling, facilitation of adrenergic transmission in peripheral nerves, increase in peripheral resistance, enhanced sodium reabsorption and inflammation [5, 8, 9]. Furthermore, the action of angiotensin II (ANG II) on the central nervous system also causes sympathetic hyperactivity due to an increase in reactive oxygen species (ROS) production, and

inflammation in areas such as the paraventricular nucleus of the hypothalamus (PVN) and the rostral ventrolateral medulla (RVLM), which are essential for the maintenance of hypertension in 2K1C rats [10–12].

The activation of angiotensin type 1 receptor (AT1R) centrally triggers signal transduction via different kinases such as ERK1/2 and MAPK and causes sustained elevations in the transcription of genes such as NF-κB and AP-1 [13, 14]. All of these second messengers are strongly related to the increased production of ROS by NADPH oxidase (Nox) and the increased release of proinflammatory cytokines (PICs) [13], which are important mechanisms for the development of hypertension [11, 15]. Seven members of the Nox family were identified (NOX1-5 and DUOX1-2) [16], of which NOX2 and NOX4 are related to cardiovascular diseases such as hypertension and heart failure [17–19]. The mechanisms by which ROS contributes to cardiovascular diseases are still not clear, but one of the hypotheses is that ROS may activate intracellular signaling mechanisms that lead to an increase in PIC, probably by contributing to microglial and astrocytic proliferation [13]. In the opposite direction, the increase in PIC may cause an increase in ROS production, with IL-1β and TNF-α increasing the activity of NADPH oxidase due to the activation of Rac1, a subunit of NADPH oxidase [20].

Recently, it was demonstrated that carotid sinus nerve denervation (CSD) attenuates hypertension and improves baroreflex function in 2K1C animals [11]. One suggested possibility is that these effects might be the result of central remodeling produced by the removal of the excitatory drive from the carotid body (CB) to the central nervous system (CNS) [11]. In addition, it is also suggested that the increase in angiotensin II (ANG II) levels may activate AT1R located in the glomus cells of the CB [21], producing an intense increase in excitatory drive from the CB to the nucleus of the solitary tract (NTS) contributing to hypertension [22, 23]. However, the mechanism of CB neurotransmission at the level of the NTS has not been studied.

The NTS is an important area of the central nervous system (CNS) involved in cardiovascular control [24]. The commissural region of the NTS (cNTS) is the first synaptic and integrative site for arterial chemoreceptor afferent input [25]. Important pressor mechanisms directly project from the cNTS to sympathetic premotor neurons located in the RVLM [26]. The inhibition or electrolytic lesion of cNTS neurons causes a reduction in blood pressure and sympathetic nerve activity in spontaneously hypertensive rats (SHRs), which suggests that the cNTS is essential for the maintenance of high levels of blood pressure in neurogenic hypertension [27, 28]. The importance of the cNTS for the development and maintenance of other models of hypertension has not been investigated. The cNTS is an area rich in AT1R [29]; however, the possible role of

angiotensinergic signaling within the cNTS in renovascular hypertension is still unknown.

Based on previous studies showing the importance of the cNTS for cardiovascular control and hypertension in SHR, we hypothesized that the cNTS might also have an important role in the maintenance of hypertension in adult 2K1C rats. We further hypothesized that angiotensinergic signaling to the cNTS might be involved in the maintenance of high levels of blood pressure in 2K1C rats. Therefore, in the present study, we investigated the changes in arterial pressure produced by neuronal deactivation or blockade of AT1R in the cNTS in 2K1C rats and compared the effects of NTS blockade with those of carotid body removal. In addition, the changes in microglia; astrocytes; and mRNA expression of RAS components (AT1R, AT2R and ACE), NOX2, NOX4, TNF-α, IL-6, and IL-1β were also analyzed in the cNTS of 2K1C rats.

Methods

Animals

Male Holtzman rats weighing 150–180 g were used in the present study. The animals were group housed in a room with controlled temperature (23 ± 2 °C), humidity (55 ± 10%) and light-dark cycle (12 h/12 h, lights on at 7:00 am), with standard rat chow (Socil, Invivo Nutrição e Saúde Animal LTDA, Descalvado, São Paulo, Brazil) and tap water available *ad libitum*. The experimental protocols were approved by the Ethics Committee for Animal Care and Use of the School of Dentistry of Araraquara, FOAR/UNESP (CEUA 48/2014 and CEUA 05/2014).

Anesthesia and euthanasia

Rats were anesthetized with ketamine [80 mg/kg of body weight (b. wt.)] combined with xylazine (7 mg/kg of b. wt.) for the surgeries. During the surgeries/procedures, the level of anesthesia was monitored by checking the eye blink reflex and the reaction to a paw pinch and was adjusted if necessary. Following the surgeries, the animals received a prophylactic dose of penicillin (50,000 IU, intramuscularly) and a dose of the anti-inflammatory ketoprofen (1 mg/kg of b. wt., subcutaneously). Urethane (1.2 g/kg; iv) was used for experiments in anesthetized rats. Rats were euthanized by placing them under deep anesthesia with sodium thiopental (70 mg/kg of b. wt, i.p.) or an additional infusion of urethane (iv), the kidneys were removed and weighed (wet weight) to confirm atrophy of the clipped kidney and hypertrophy of the contralateral kidney (Table 1). Thereafter, animals were decapitated or transcardially perfused with chilled 0.9%

saline followed by 4% paraformaldehyde, depending on the protocol (see below), and the brainstem was removed.

Renovascular hypertension model

Rats were anesthetized as described above. Laparotomy was performed, and the left kidney was exposed. The renal artery was carefully isolated and partially obstructed using a silver clip of 0.2 mm width in order to reduce renal blood flow and elicit hypertension. Normotensive (NT) animals were subjected to the same surgical procedure without partial renal artery occlusion (sham surgery), as described previously [4].

Arterial pressure and heart rate recordings

For long-term recording, in the third week after the silver clip implantation, telemetry transmitters (transmitter TA11PAC40, DSI, St. Paul, MN, USA) were implanted into the abdominal aorta under xylazine and ketamine anesthesia (as described above) to perform continuous recordings of pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) in a conscious state (DSI, St. Paul, MN, USA). The recordings were performed every second day for 3 weeks and were programmed with the following conditions: 24 h/d, 12 min/h and 1 min segments at a frequency of 1000 Hz.

For acute experiments, rats were anesthetized as indicated above, and a catheter was inserted into the abdominal aorta through the femoral artery to record pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR). A second piece of polyethylene tubing was inserted into the femoral vein for drug administration. Both catheters were tunneled subcutaneously and exposed on the back of the rat. The arterial catheter was connected to a Statham Gould (P23Db) pressure transducer coupled to a pre-amplifier (model ETH-200 Bridge Bio Amplifier) connected to a data acquisition system (model Powerlab 4SP, ADInstruments, Castle Hill, Australia; 1 KHz sampling rate) to record PAP. MAP and HR were derived from PAP signals.

Drug injection into the commissural NTS

Animals were anesthetized with urethane as described above and tracheostomized. A partial craniotomy of the occipital bone was performed, and the dorsal surface of the brainstem was exposed. The drugs were injected into the cNTS using a glass micropipette coupled to a Picospritzer microinjection system (Parker, Cleveland, OH, USA). Each microinjection measured 60 nl in volume and was delivered on the midline, 0.5 mm caudal to the *calamus scriptorius* and 0.4 mm ventral to the dorsal surface of the medulla. At the end of experiments with injections of drugs into the cNTS, the rats received an

Table 1 Ratio of left kidney/right kidney weight (LK/RK) in normotensive (NT), hypertensive (2K1C) and in rats NT or 2K1C rats that had carotid body removed (CBR)

Rat	n	LK/RK
NT	38	0.99 ± 0.01
CBR/NT	6	0.95 ± 0.01
2K1C	37	$0.65 \pm 0.02^*$
CBR/2K1C	8	$0.60 \pm 0.07^*$

Values are means $\pm$ SEM; One-way ANOVA followed by Student–Newman-Keuls test; $p < 0.05$; $n =$ number of animals * different from NT or CBR/NT

injection of 60 nl of 2% Evans Blue into the same area to evaluate the site of drug injection.

Under the same urethane anesthesia conditions, saline followed by 10% buffered formalin was perfused through the heart. The brains were removed, frozen, cut coronally in 50-μm sections using a cryostat (Leica, CM1850, Wetzlar, Hesse, Germany), and stained with Giemsa stain, after which approximately six to eight slices were analyzed by light microscopy (Leica DM5500 B, Wetzlar, Hesse, Germany) to confirm that the injections had been delivered into the cNTS.

Carotid body removal (CBR)

Carotid body removal (CBR) was performed as follows: briefly, one week after the telemetry transmitter implantation, rats were anesthetized with halothane (2% in O_2) and fixed in a supine position. A midline incision (3 cm) was made in the neck to expose the muscles that cover the trachea and carotid bifurcation region. After dissection of the sternocleidomastoid muscle and exposition of the carotid bifurcation, the occipital artery was retracted, and the carotid body was visualized and removed. This procedure was performed bilaterally. Sham-CBR rats were submitted to a midline incision (3 cm) in the neck and dissection of the sternocleidomastoid muscle.

Drugs

Losartan (AT1 receptor antagonist) was purchased from Tocris (USA) and was injected at the dose of 0.6 nmol/60 nL. Isoguvacine (a $GABA_A$ agonist) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and was injected at a dose of 1.2 nmol/60 nL. The doses of the drugs used were based on previous studies [10, 21]. Saline (0.15 M NaCl) with the pH adjusted to 7.4 was used as a vehicle to dissolve all the drugs.

Quantification of gene expression and immunohistochemistry

To evaluate gene expression in the cNTS, we anesthetized the rats as indicated above, then decapitated them, quickly

removed the brains, and froze the collected brains in dry ice for later storage in a −80 °C freezer. The Paxinos & Watson atlas [22] was used as a reference to identify the NTS. Using a surgical microscope, the area postrema (AP) was located as a landmark. Then, two coronal slices 400 μm in thickness were prepared to obtain a 1-mm medial punch of the cNTS (above the central canal). Total RNA was extracted using TRIzol and chloroform and isolated using a PureLink® RNA Mini Kit (Life Technologies, Grand Island, USA). The isolated RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Grand Island, USA), and samples were run in duplicate using a StepOne real-time PCR system, TaqMan Universal Gene Expression Master Mix and validated TaqMan probes (Applied Biosystems, Foster City, CA, USA). The expression patterns of genes of interest were normalized to constitutively expressed GAPDH (Rn01775763_g1), and relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method [23]. The mRNA levels in the iNTS and cNTS of angiotensin type 1 receptor (AT1R; Rn01435427_m1), angiotensin type 2 receptor (AT2R; Rn00560677_s1), angiotensin-converting enzyme (ACE; Rn00561094_m1), NOX2 (Rn00576710_m1), NOX4 (Rn00585380_m1), tumor necrosis factor α (TNF-α; Rn99999017_m1), interleukin-1β (IL-1β; Rn99999009_m1) and interleukin-6 (IL-6; Rn01410330_m1) were evaluated in the NT and 2K1C groups.

Microglia and astrocytes in the cNTS were quantified by immunohistochemistry as described previously [30, 31]. Briefly, animals were deeply anesthetized and perfused as indicated above. One of the 4 brainstem sections (120 μm each) was processed for free-floating immunohistochemistry. Rabbit anti-Iba-1 primary antibody (1:3,000; Wako Chemicals, Richmond, VA, USA) and mouse anti-GFAP (1:500, Chemicon International, Temecula, CA, USA) were used to label microglia and astrocytes, respectively. GFAP immunofluorescence was detected using a Leica DM5500 B Fluorescence microscope (Leica, Wetzlar, Hessen, Germany) using appropriate filters. We used the area postrema (AP), obex, central canal and dorsal nucleus of the vagus nerve (DMV) as anatomical landmarks to define the rostrocaudal and dorsoventral levels of the NTS. The cNTS was located from the obex to 500 μm caudal to the obex. For the assessment of microglial number, 20-μm z-stacks (containing 20 images) were taken through Iba-1-stained sections at 20 × magnification and counted manually. For assessment of GFAP immunoreactivity, images were captured at 20 × magnification and converted into grayscale, and thresholds for black and white balance were adjusted to the same level in all cNTS slices using Leica Application Suite Advanced Fluorescence (Leica, Wetzlar, Hessen, Germany). cNTS was measured in grayscale by area. Photomicrographs to illustrate Iba-1 immunoreactivity were captured at 10 × magnification using a Confocal Fluorescence Microscope (Carl Zeiss LSM 800 with Airyscan).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) and were analyzed by Student's t-test, one-way analysis of variance (ANOVA) or two-way ANOVA followed by a Student–Newman–Keuls post hoc test, depending on the experiment. Differences were considered significant at $p < 0.05$.

Experimental design

Except in the experiment where the carotid bodies were removed, all the experiments were performed 6 weeks after 2K1C or sham surgery. On the day before the experiments, the animals had cannulas implanted into the femoral artery and vein as described in the Methods Section. On the following day, PAP, MAP, and HR were recorded for 30 min in conscious animals, and only the animals with MAP ≥ 160 mmHg were considered hypertensive and used in the experiments.

Effects of carotid body removal (CBR) on the development of 2K1C renovascular hypertension

During the third week after the silver clip implantation, telemetry transmitters were implanted into the abdominal aorta of NT and 2K1C animals. After 4 days of recovery, baseline MAP and HR were recorded for 3 days, and based on studies from Oliveira-Sales et al. [32], only the animals with MAP higher than 145 mmHg were considered for carotid body removal or sham surgery. Four groups of rats were formed: sham-CBR/NT, CBR/NT, sham-CBR/2K1C and CBR/2K1C. MAP and HR were recorded for two weeks after CBR surgery. At the end of the experiment, the femoral artery and vein were cannulated to test the peripheral chemoreflex sensitivity with KCN injections.

MAP and HR in 2K1C hypertensive rats with commissural NTS inhibition

After the baseline levels of MAP were recorded in conscious rats, animals were anesthetized with i.v. urethane, tracheostomized and exposed the NTS, as described in the Methods section. The baseline MAP and HR were recorded again for 30 min, and then saline or isoguvacine (1.2 nmol/ 60 nL) was injected into the cNTS, and MAP and HR recordings continued for an additional 40 min.

MAP and HR in 2K1C hypertensive rats with AT1 receptor antagonism injected in the commissural NTS

The experimental design of this protocol was similar to that described above for isoguvacine injection into the

cNTS, except that losartan (0.6 nmol/60 nL) instead of isoguvacine was injected into the cNTS.

mRNA analysis of RAS components, NOX2, NOX4 and proinflammatory cytokines and quantification of microglia and astrocytes in the NTS of renovascular hypertensive rats

One day after MAP and HR recordings, the brains of the rats were removed as described in the Methods for RT-PCR or immunohistochemistry protocols.

Results

Renovascular 2K1C hypertension in rats subjected to carotid body removal (CBR)

Before CBR surgery, MAP in the 2K1C rats during the light period (pre-CBR/2K1C: 167 ± 11 and pre-sham-CBR/2K1C: 169 ± 8 mmHg) and the dark period (pre-CBR/2K1C: 173 ± 10 and pre-sham-CBR/2K1C: 175 ± 9 mmHg) was significantly elevated compared with the levels in NT rats during the light period (pre-CBR/NT: 100 ± 2 and pre-sham-CBR/NT: 102 ± 3 mmHg) and the dark period (pre-CBR/NT: 102 ± 3 and pre-sham-CBR/NT: 106 ± 3 mmHg) (Fig. 1a, b).

Three days after the CBR, MAP was significantly reduced in CBR/2K1C rats during the light (141 ± 5, vs. sham-CBR/2K1C: 165 ± 6 mmHg) [$F(24, 216) = 0.49$, $p < 0.05$] and dark periods (152 ± 8 mmHg) compared to sham-CBR/2K1C rats (175 ± 7 mmHg) [$F(24, 216) = 0.61$, $p < 0.05$] (Fig. 1a, b, respectively). CBR in NT rats did not modify MAP (Fig. 1a, b). MAP remained reduced until the end of the recordings (13 days after CBR) in CBR/2K1C rats during the light (161 ± 12) and dark periods (176 ± 10) compared to sham-CBR/2K1C rats (189 ± 8 mmHg and 206 ± 6 mmHg, respectively, in the light and dark period) (Fig. 1, b). Although reduced, MAP in CBR/2K1C rats was still elevated compared to the level in normotensive rats (Fig. 1a, b). No consistent change in HR was observed during the light or dark period in CBR rats (Fig. 1c, d). The effectiveness of the CBR was evaluated by KCN injection, which did not produce any change in MAP (CBR/2K1C: -4.8 ± 0.7 or CBR/NT: -3.3 ± 0.4 vs. sham-CBR/2K1C: 58.5 ± 13.7 or sham-CBR/NT: 79.6 ± 4.2 mmHg, $p < 0.05$) or HR (CBR/2K1C: 20 ± 8.9 or CBR/NT: 14.1 ± 1.4 vs. sham-CBR/2K1C: -175.5 ± 61 or sham-CBR/NT: -177.6 ± 26.5 bpm, $p < 0.05$) in CBR rats.

Changes in MAP and HR in 2K1C hypertensive rats with acute commissural NTS inhibition

The baseline MAP and HR levels of 2K1C rats ($n = 11$) were elevated compared to those of NT rats (n = 12) (MAP:

187 ± 7 and 124 ± 2 mmHg, respectively, $p < 0.05$; HR: 373 ± 14 and 345 ± 7 bpm, respectively, $p < 0.05$). Isoguvacine injection (1.2 nmol/60 nl) into the cNTS rapidly reduced MAP in 2K1C rats, reaching a maximum reduction 25 min after the injection (ΔMAP: -37 ± 6, vs. saline: -2 ± 4 mmHg) [$F(27, 190) = 1.76$, $p < 0.05$] (Fig. 2a) and (Figure. 1A and B, supplementary figure). Isoguvacine injection into the cNTS also immediately reduced HR in 2K1C rats (-46 ± 9, vs. saline: -10 ± 7 bpm) [$F(27, 190) = 0.81$, $p < 0.05$], an effect that persisted throughout the experimental period (-39 ± 12, vs. saline: 11 ± 3 bpm at 40 min, $p < 0.01$) (Fig. 2b) and (Figure. 1A and B, supplementary figure). Isoguvacine injection into the cNTS in normotensive rats also reduced MAP (-18 ± 2 vs. saline: -7 ± 2 mmHg at 40 min); however, this reduction was less than that observed in 2K1C rats ($p < 0.05$) (Fig. 2a). No change was observed in HR in normotensive rats (6 ± 18 vs. saline: -6 ± 2.3 bpm) (Fig. 2b).

Figure 2c shows the typical injection site into the cNTS in a rat representative of the animals tested in the present study.

Changes in MAP and HR in 2K1C hypertensive rats that received AT1 receptor blockade in the commissural NTS

The baseline MAP of 2K1C rats ($n = 10$) was elevated compared to that of normotensive rats ($n = 11$) (MAP: 185 ± 8 and 124 ± 3 mmHg, respectively, $p < 0.05$), whereas the baseline HR was similar in 2K1C and normotensive rats (354 ± 14 and 360 ± 9 bpm). 20 min after losartan (0.6 nmol/60 nl) injection into the cNTS, MAP was reduced in 2K1C animals, reaching the maximum reduction 30 min after the injection (Δ MAP: -20 ± 6, vs. saline: -5 ± 3 mmHg) [$F(27, 209) = 19.79$, $p < 0.05$] (Fig. 3a, Figure. 1C, supplementary figure), without changes in HR (Fig. 3b). The injection of losartan into the NTS in normotensive rats produced no significant change in MAP (-9 ± 2, vs. saline: -6 ± 2 mmHg) or HR (-8.1 ± 10.5 vs. saline: -2.2 ± 11.7 bpm).

mRNA expression of RAS components, NOX2, NOX4 and proinflammatory cytokines in the NTS of rats with renovascular 2K1C hypertension

Baseline MAP and HR, respectively, were 200 ± 7 mmHg and 423 ± 21 bpm in 2K1C rats ($n = 7$) and 108 ± 5 mmHg and 357 ± 18 bpm in normotensive rats ($n = 8$). NOX2 mRNA levels increased in the NTS of 2K1C rats compared with normotensive rats (1.9 ± 0.25, vs. normotensive: 1.0 ± 0.15-fold change, $p < 0.05$), without changes in NOX4 mRNA levels (Fig. 4a). TNF-α and IL-6 mRNA levels also increased in the NTS of 2K1C rats (fold changes of 1.7 ± 0.31 and 1.6 ± 0.21, respectively)

Fig. 1 **a**, **b** Mean arterial pressure (MAP) and **c**, **d** heart rate (HR) during the light (**a**, **c**) and dark periods (**b**, **d**) in hypertensive (2K1C) and normotensive (NT) rats that underwent carotid body removal (CBR) or sham carotid body removal (sham-CBR) on day 0 (indicated by the arrows). The results are presented as the means ± SEM. Two-way ANOVA combined with Fisher's LSD test; $p < 0.05$; n = number of rats
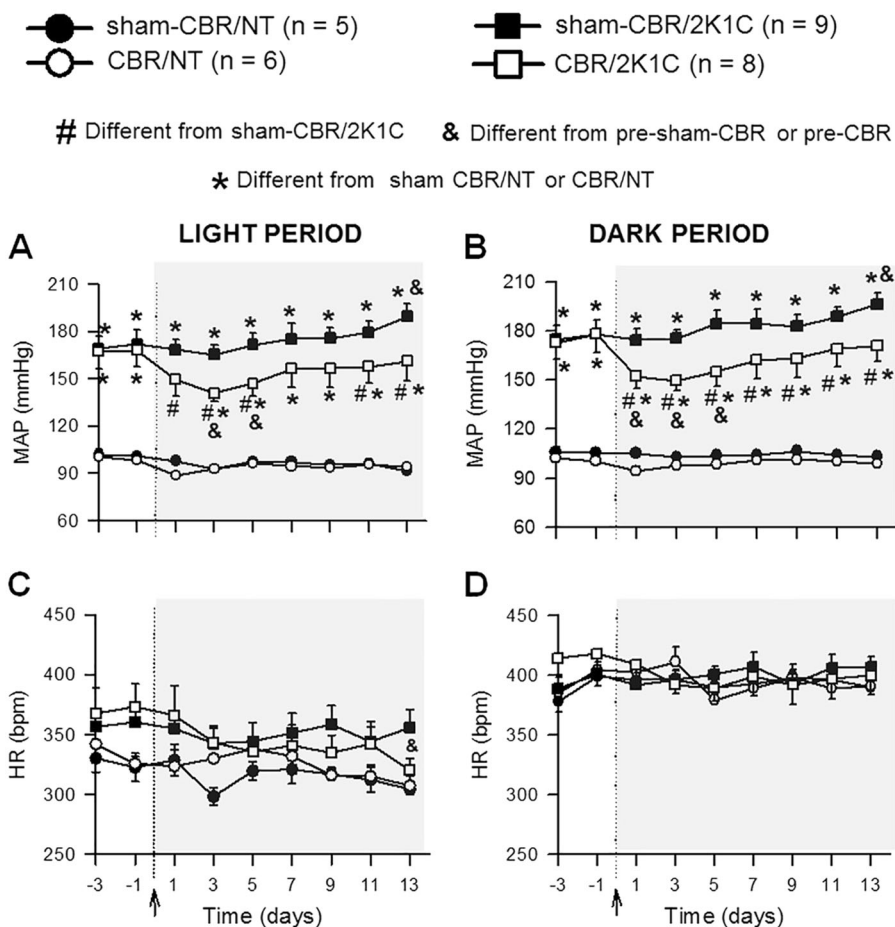
compared to normotensive rats (fold changes of 1.0 ± 0.17 and 1.0 ± 0.12, respectively), whereas IL-1β mRNA levels were not different between groups (Fig. 4b). The levels of AT1R, AT2R and ACE mRNAs in the NTS were similar in 2K1C and normotensive animals (Fig. 4c).

Quantification of microglia and astrocytes in the cNTS of animals with renovascular 2K1C hypertension

The baseline MAP and HR were 191 ± 7 mmHg and 412 ± 26 bpm, respectively, in 2K1C rats ($n = 5$) and 99 ± 3 mmHg and 339 ± 6 bpm, respectively, in normotensive rats ($n = 7$). The number of Iba-1-positive cells in the cNTS increased in 2K1C rats compared to normotensive rats (17.4 ± 4.7 vs. normotensive: 2.1 ± 0.5 cells/section, $p < 0.05$) (Fig. 5a–c, whereas the number of Iba-1-positive cells in the area postrema was similar between 2K1C and normotensive animals (Fig. 5a, d, e). In addition, there was an increase in GFAP-stained area in the cNTS of 2K1C compared to normotensive rats (207.9 ± 20.73, vs. 159.7 ± 7.6 stained area/section, $p < 0.05$) (Fig. 5f–h).

Kidney analysis

The ratio of left kidney to right kidney weight decreased in 2K1C rats compared to normotensive rats, as demonstrated previously [5] (Table 1).

Discussion

The present results demonstrate that neuronal deactivation by injection of isoguvacine into the cNTS strongly reduced arterial pressure in 2K1C hypertensive rats, whereas the same treatment in normotensive rats produced only a slight reduction in arterial pressure. In the same manner, the blockade of the AT1 receptors with losartan injected into the cNTS also reduced arterial pressure in 2K1C hypertensive rats without causing any changes in normotensive rats. These data suggest that the activation of pressor mechanisms, particularly the angiotensinergic mechanisms, present in the cNTS is essential for the maintenance of hypertension in 2K1C rats. In addition, the results showed that 2K1C hypertensive rats have microgliosis, astrogliosis,
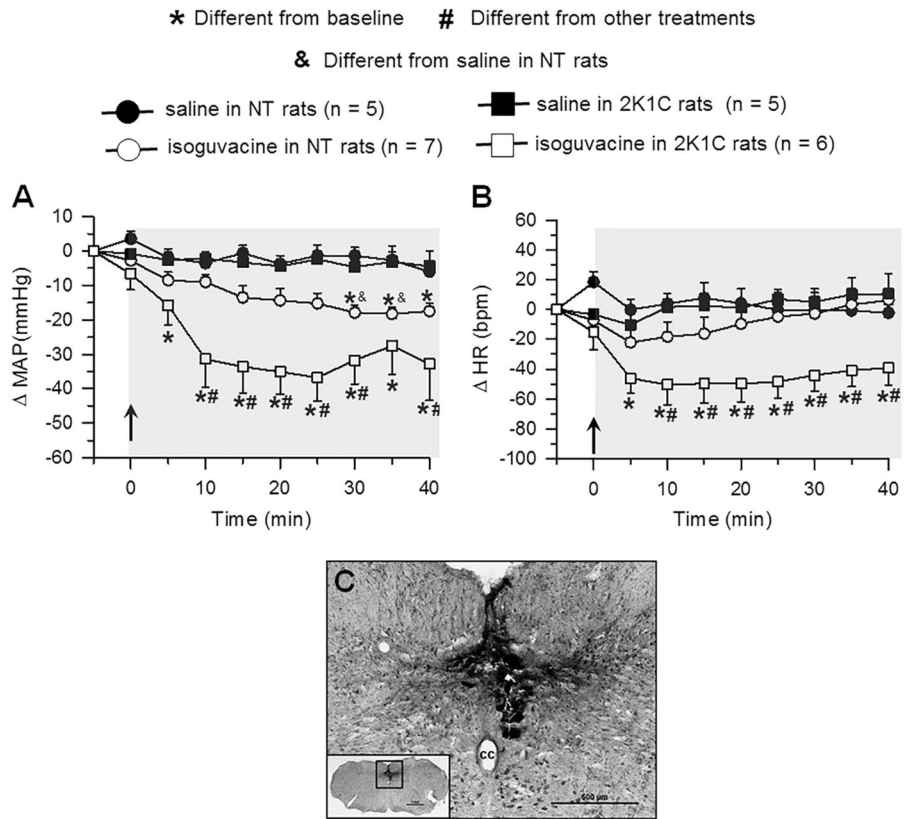
Fig. 2 **a** Change in mean arterial pressure (ΔMAP) and **b** change in heart rate (ΔHR) in normotensive (NT) and 2K1C rats after isoguvacine (1.2 nmol/60 nL) or saline injections into the cNTS. **c** Photomicrograph of a coronal section of the brainstem from a rat representative of those tested in the present study, showing the typical site of injection into the cNTS. cc, central canal. Scale bars = 500 μm and 1 mm, respectively, for higher magnification and inset. The moment of the injections is indicated by an arrow. The results are presented in **a, b** as the means ± SEM. Two-way ANOVA combined with the Student–Newman–Keuls method; $p < 0.05$; n = number of rats

Fig. 3 **a** Change in mean arterial pressure (ΔMAP) and **b** change in heart rate (ΔHR) in normotensive (NT) and 2K1C rats after losartan (0.6 nmol/60 nL) or saline injections into the cNTS. The moment of the injections is indicated by an arrow. The results are presented as the means ± SEM. Two-way ANOVA combined with the Student–Newman-Keuls method; $p < 0.05$; n = number of rats
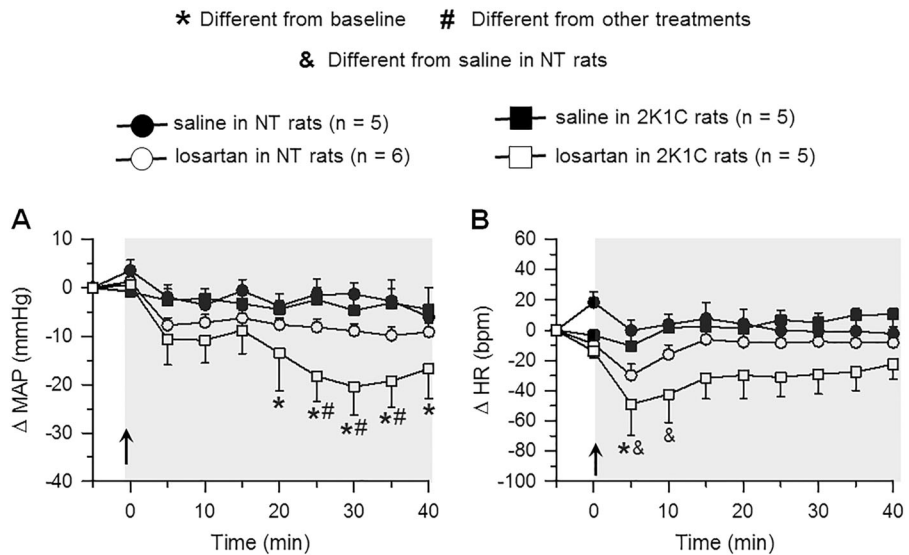

oxidative stress and neuroinflammation in the cNTS, a possible consequence of the increased angiotensinergic action in this area that might be a potential mechanism involved in hypertension.

The results also show that CBR reduced blood pressure chronically in 2K1C rats, an effect similar to that recently demonstrated in rats subjected to carotid body denervation [8]. Glomus cells are rich in AT1R, and the activation of these receptors increases CB activity [33, 34]. Therefore, the overactivity of RAAS in renovascular hypertension may cause hyperactivity of the CB and activation of pressor mechanisms similar to chemoreceptor activation. The present results demonstrated that neuronal inhibition with a GABAergic agonist in the cNTS, the site of the first

Fig. 4 mRNA expression of **a** NOX2 and NOX4, **b** proinflammatory cytokines (TNF-α, IL-6, and IL-1β), and **c** RAS components (AT1R, AT2R, and ACE) in the NTS of normotensive (NT) and 2K1C animals. The results are presented as the mean ± SEM. Student's *t*-test; $p < 0.05$; $n =$ number of rats
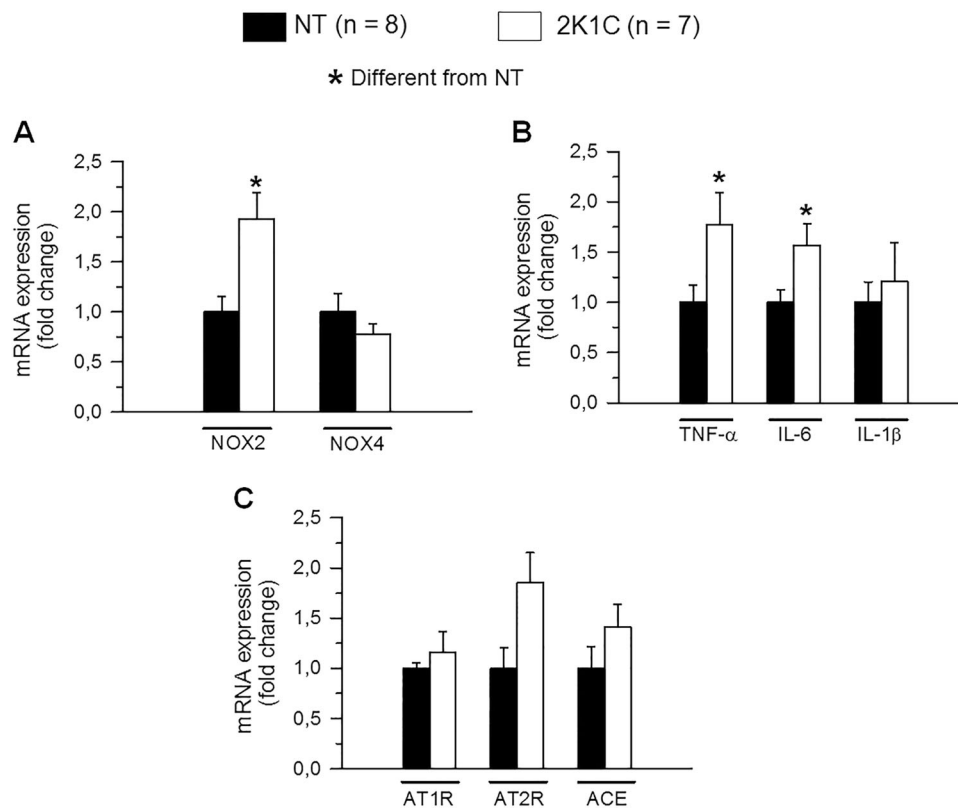
synaptic integration of arterial chemoreceptor afferent input in the CNS, produced an intense decrease in MAP and HR in 2K1C rats, whereas only a slight reduction was produced in normotensive rats, which means that the cNTS is essential to the maintenance of hypertension in 2K1C rats. The importance of the cNTS pressor mechanisms for hypertension was previously demonstrated by studies showing that inhibition or electrolytic lesion of the cNTS produced a decrease in blood pressure and sympathetic nerve activity (SNA) in SHRs [27, 28], suggesting that the excitatory neurons of the cNTS that directly project to sympathetic premotor neurons located in the RVLM [26] are more active in hypertensive rats than in normal rats [27, 28]. The blockade of this pressor mechanism probably causes the decrease in arterial pressure observed in the present and previous studies [27, 28]. The cNTS inhibition also produced bradycardia in 2K1C rats, whereas in normotensive animals, there was a trend towards an increase.

The decrease in MAP caused by CBR was less than that produced by inhibition of cNTS with isoguvacine injection, which suggests that other additional pressor mechanisms, not dependent on the CB, may also modulate the activity of cNTS in 2K1C hypertensive rats. The results showing that the blockade of AT1R with losartan injection into the cNTS in 2K1C animals reduced MAP suggest that in addition to the excitatory input from the CB, angiotensinergic signaling in the cNTS is also

important to maintain high levels of blood pressure in 2K1C rats. In a different direction, Perin et al. (2018) showed that previous exposure to chronic intermittent hypoxia attenuates the development of hypertension in 1-kidney, 1-clip renal hypertensive rats. Therefore, CBR, similar to previous exposure to chronic intermittent hypoxia, reduces renal hypertension (see ref. [8, 35] and the present results) probably affecting a different mechanism. The present results suggest that central pressor mechanisms integrated in the NTS, probably at least some of them dependent on carotid bodies, are important for renal hypertension. On the other hand, the protective effect of exposure to chronic intermittent hypoxia is suggested to depend on enhanced calcium-activated potassium channel (BKCa)-dependent vasorelaxation of the mesenteric artery [36].

In addition, the mRNA expression of NOX2, IL-6, TNF-α, as well as the numbers of microglia and astrocytes, increased in the NTS of 2K1C rats, without changes in mRNA expression of RAS components (AT1R, AT2R, and ACE). Although the expression of AT1R mRNA in the NTS was not modified in 2K1C rats, injection of losartan into the cNTS reduced arterial pressure in 2K1C rats, which suggests that the amount of ANG II acting in the cNTS in 2K1C rats is sufficient to activate pressor mechanisms and possibly other mechanisms related to hypertension such as NADPH oxidase activity, ROS and cytokine production,
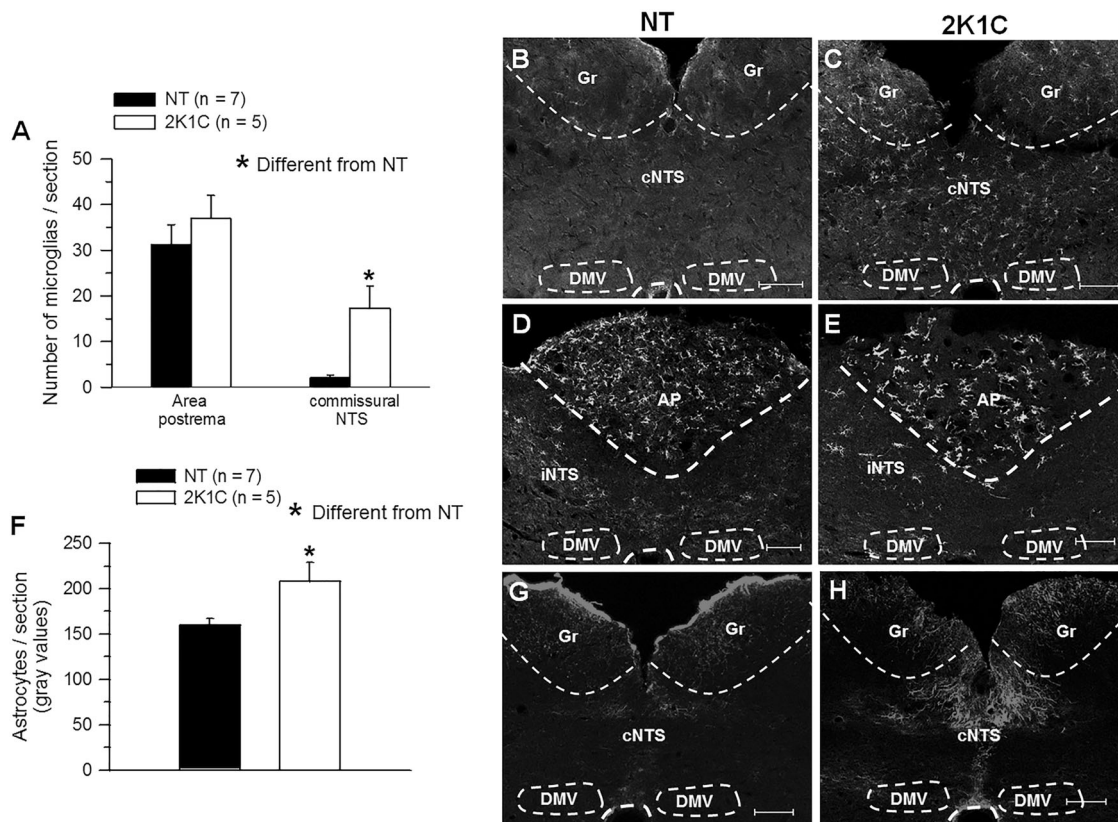
Fig. 5 **a** Numbers of microglial cells in the area postrema (AP) and in the cNTS of NT and 2K1C rats. **b**, **c** Photomicrographs of coronal sections of the brainstem showing Iba-1 immunoreactivity (microglia) in the commissural NTS (cNTS) and **d**, **e** the area postrema in normotensive (NT) (left side) and 2K1C rats (right side). **f** Quantification of the percentage of the area of GFAP-positive staining in the cNTS of NT and 2K1C rats. **g**, **h** Photomicrographs of coronal sections of the brainstem showing GFAP immunoreactivity (astrocytes) in the commissural NTS (cNTS) in normotensive (NT) (left side) and 2K1C rats (right side) 6 weeks after renal clipping. Scale bar = 100 μm. The results in **a**, **f** are presented as the means ± SEM. Student's t-test; $p <$ 0.05; $n =$ number of rats. Gr indicates gracile nucleus; cc, central canal; iNTS, intermediate nucleus of the solitary tract; DMV, dorsal motor nucleus of the vagus nerve

microgliosis and astrogliosis in the NTS, especially in the cNTS. A recent study from our group demonstrated that increased expression of MIF (macrophage migration inhibitory factor) in the intermediate NTS and cNTS of 2K1C rats attenuated the development of hypertension and improved the baroreflex [37], probably as a consequence of the action of MIF as an intracellular inhibitory regulator of the central actions of ANG II [32]. The present study suggests that ANG II acting in the cNTS is critical for the elevation of arterial pressure in 2K1C rats, increasing NADPH oxidase activity, cytokine production, and the numbers of microglia and astrocytes in this area.

The increase in circulating ANG II levels causes hyperactivity in important central areas involved in cardiovascular control, such as the subfornical organ (SFO), organum vasculosum lamina terminalis (OVLT), PVN, and RVLM [10–12]. The present results suggest that the increase in circulating ANG II levels also produces hyperactivity of the cNTS, an effect essential to the maintenance of hypertension in 2K1C animals. Different pathways might lead to hyperactivity of the cNTS neurons. One of them, discussed

above, is that circulating ANG II may activate glomus cells in the CB [33], which, in turn, might stimulate the cNTS neurons through the afferent glossopharyngeal nerve. Another possibility is that circulating ANG II might cross the blood–brain barrier (BBB) [38] and directly activate AT1R located in neurons and in astrocytes and microglia, causing increases in NOX2, IL-6 and TNF-α levels [39].

The increase in the number of microglia and astrocytes may depend on the action of ANG II [39]. On the other hand, astrocytes are one of the main sources of ANG II in the CNS[40]; therefore, the proliferation of astrocytes may increase the levels of ANG II in the NTS. ANG II acting on endothelial AT1R produces phosphorylation of the threonine residue of occludin, reducing the integrity of the tight junctions, which increases the permeability of the BBB [41, 42]. In addition, ANG II inhibits astrocyte GLT1 (glutamate transporter) activity, increasing glutamate in the synaptic site and consequently causing activation of neurons that may increase sympathetic activity [43]. Microglia are one of the main sources of NOX2 and TNF-α in the CNS, and astrocytes and microglia release IL-6 [44]. In the present

study, the mRNA levels of NOX2, TNF-α, and IL-6 and astrocytes and microglia increased in the NTS of 2K1C rats. As suggested by previous studies, these are important mechanisms of hypertension [14, 45, 46]. Therefore, these alterations present in the NTS may contribute to the development and maintenance of hypertension in 2K1C rats.

In conclusion, these results indicate that tonically active neurons in the cNTS that are responsive to ANG II acting on AT1R are essential for the maintenance of hypertension in 2K1C rats. In addition, other hypertensive mechanisms such as NADPH oxidase activity, cytokine production, and the abundance of microglia and astrocytes also increase in the cNTS of 2K1C rats and may contribute to increased sympathetic tonus during the development and maintenance of renovascular hypertension. More studies are necessary to investigate the importance of neuroinflammation, microgliosis, astrogliosis and oxidative stress in the cNTS for the maintenance of hypertension in 2K1C rats, as well as to identify the mechanism that causes these alterations in the cNTS of 2K1C rats.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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