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# Piezo2 channel in nodose ganglia neurons is essential in controlling hypertension in a pathway regulated directly by Nedd4-2

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***Running title*:** Nedd4-2 modulates Piezo2 leading to hypertension

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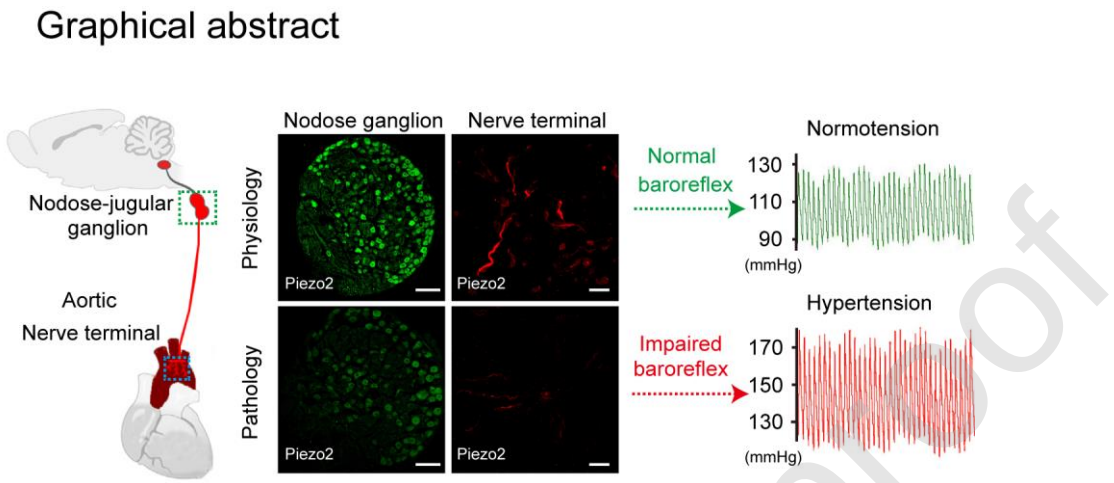
Graphical abstract




## Highlights

- Mechanically-activated Piezo2 channel was highly expressed in baroreceptor nodose ganglia (NG) neurons as well as aortic nerve terminals in normotensive rats and significantly downregulated in hypertensive rats.
- Downregulation of Piezo2 in NG impaired the baroreflex and induced hypertension in rats.
- Nedd4-2 was involved in the downregulation of Piezo2 in NG in hypertensive rats.
- In the future, targeting Piezo2 could be a new strategy in the treatment of hypertension.

## Abstract

Baroreflex plays a crucial role in regulation of arterial blood pressure (BP). Recently, Piezo1 and Piezo2, the mechanically-activated (MA) ion channels, have been identified as baroreceptors. However, the underlying molecular mechanism for regulating these baroreceptors in hypertension remains unknown. In this study, we used spontaneous hypertensive rats (SHR) and NG-Nitro-L-Arginine (L-NNA)- and Angiotensin II (Ang II)-induced hypertensive model rats to determine the role and mechanism of Piezo1 and Piezo2 in hypertension. We found that Piezo2 was dominantly expressed in baroreceptor nodose ganglia (NG) neurons and aortic nerve endings in Wistar-Kyoto (WKY) rats. The expression of Piezo2 not Piezo1 was significantly downregulated in these regions in SHR and hypertensive model rats. Electrophysiological results showed that the rapidly adapting mechanically-activated (RA-MA) currents and the responsive neuron numbers were significantly reduced in baroreceptor NG neurons in SHR. In WKY rats, the arterial BP was elevated by knocking down the expression of Piezo2 or inhibiting MA channel activity by GsMTx4 in NG. Knockdown of Piezo2 in NG also attenuated the baroreflex and increased serum norepinephrine (NE) concentration in WKY rats. Co-immunoprecipitation experiment suggested that Piezo2 interacted with Neural precursor cell-expressed developmentally downregulated gene 4 type 2 (Nedd4-2, also known as Nedd4L); Electrophysiological results showed that Nedd4-2 inhibited

Piezo2 MA currents in co-expressed HEK 293T cells. Additionally, Nedd4-2 was upregulated in NG baroreceptor neurons in SHR. Collectively, our results demonstrate that Piezo2 not Piezo1 may act as baroreceptor to regulate arterial BP in rats. Nedd4-2-induced downregulation of Piezo2 in baroreceptor NG neurons leads to hypertension in rats. Our findings provide a novel insight into the molecular mechanism for the regulation of baroreceptor Piezo2 and its critical role in the pathogenesis of hypertension.

**Keywords:** Hypertension, Baroreceptor, Piezo channel, Nedd4-2, Baroreflex, Nodose ganglia

**Chemical compounds studied in this article**

Ang II (PubChem CID: 172198); L-NNA (PubChem CID: 440005); GsMTx4 (PubChem CID: 146018896); Yoda1 (PubChem CID: 2746822); PE (PubChem CID: 6041); Isoflurane (PubChem CID: 3763); Pentobarbital sodium (PubChem CID: 23676152); benzylpenicillin (PubChem CID: 5904).

## Abbreviations

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; MA, mechanically-activated; RA-MA, rapidly adapting type of mechanically-activated; IA, intermediately adapting; SA, slowly adapting; NG, nodose ganglia; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rats; L-NNA, NG-Nitro-L-Arginine; shRNA, short hairpin RNA; Nedd4-2 (Nedd4L), neural precursor cell-expressed developmentally downregulated gene 4 type 2; nNOS, neuronal isoform of nitric oxide synthase; Ang II, Angiotensin II; GsMTx4, M-theraphotoxin-Gr1a; HR, heart rate; γENaC, epithelial sodium channel γ subunit; ASIC2, acid-sensing ion channel 2; TRPC5, transient receptor potential cation channel classical subtype 5; SP-DiO, 3,3'-Dioctadecyl-5,5'-Di(4-Sulfophenyl) Oxacarbocyanine sodium salt; DiI, 1,1'-dioleyl-3,3,3,'3'-tetramethylindo-carbocyanine methane sulfonate; NE, norepinephrine; PE, Phenylephrine.

## 1 Introduction

In mammals, the arterial baroreceptors play an essential role in triggering the baroreflex to buffer any deviation of arterial blood pressure (BP). Baroreceptors are stretch-sensitive structures that located at the sensory nerve endings in the wall of aorta arch and carotid sinus. These sensory nerve endings are projected by baroreceptor neurons whose cell bodies are located in the nodose and petrosal ganglia [1,2]. Baroreceptors sense the stretch of vascular wall caused by BP changing, and then convert the mechanical signals into electrical signals, which propagate to the cardiovascular center. Ultimately, the heart rate (HR), cardiac output, and vascular tone are adjusted via a sympathetic negative feedback to maintain BP stabilization [2,3]. Baroreflex injury including impairment of baroreceptors leads to overactivity of sympathetic nervous system and results in hypertension [4]. It was reportedly that baroreflex sensitivity impairment is associated with myocardial infarction, heart failure and stroke, and also serve as an indicator for the prognosis of these pathological conditions [5-8]. Electric field stimulation of baroreceptors (also known as baroreceptor activation therapy) elicits a decrease of BP and reduction of sympathetic activity in patients with drug-resistant hypertension [9-11].

The molecular nature of baroreceptor has been studied for decades. Several ion channels such as epithelial sodium channel γ subunit (γENaC) [12], acid sensing ion channel 2 (ASIC2) [8], and TRP channel classical subtype 5 (TRPC5) [13] have been proposed as baroreceptors. However, substantial residual baroreflex is still observed

even though these channels are eliminated [3]. None of them could be directly activated by mechanical stimulation in heterologous expression systems [3,14,15]. Thus, these channels acting as sensors or downstream signals of mechanotransduction remains controversial. Piezo channels, including Piezo1 and Piezo2, are bona fide mechanically-activated (MA) ion channels that have been identified in 2010 and been extensively studied [16,17]. Piezo channels are essential for detecting external mechanical stimuli, as well as mechanical forces within tissues [18-22]. Zeng et al. have identified that Piezo1 and Piezo2 functional together serve as baroreceptors. Double knockout of Piezo1 and Piezo2 results in baroreflex impairment and systolic BP increase in mice [3].

However, this raises new questions. For example, do baroreceptors of other species need Piezo channels to control BP? What is the role of Piezo baroreceptors in hypertensive animals? The underlying molecular mechanisms for regulating Piezo baroreceptors in hypertension also remains to be determined. In this study, we used hypertensive rats to study the role of Piezo1 and Piezo2 in modulation of arterial BP in baroreceptor neurons and their roles in hypertension. We found that Piezo2 was dominantly expressed in baroreceptor neurons and nerve endings in rats. Piezo2 not Piezo1 acts as baroreceptor to regulate rat arterial BP. Downregulation of Piezo2 by Nedd4-2 in baroreceptor NG neurons could induce hypertension in rat.

## 2 Materials and Methods

### 2.1 Animals

Male Wistar-Kyoto rats (WKY; total number, 221; 16 weeks of age; bodyweight, 250-300 g) and male spontaneously hypertensive rats (SHR; total number, 31; 16 weeks of age; weight, 250-300 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). WKY rats and SHR were housed at room temperature (23 ± 1˚C) with a stable humidity (50 ± 5%) and free access to food/water on a 12 h/12 h light/dark cycle. Experimental procedures were approved by the Animal Care and Use Committee of Hebei Medical University (Shijiazhuang, China).

**2.2 Reagents and antibodies**

NG-Nitro-L-Arginine (L-NNA; Cat. no. 2149-70-4) was purchased from Yuan Ye Biotechnology Co., Ltd. (Shanghai, China) and Angiotensin II (Ang II; Cat. no. 4474-91-3) was purchased from Med Chem Express (MEC, New Jersey, USA). GsMTx4 (Cat. no. ab141871), nNOS (Cat. no. ab1376) were obtained from Abcam (Ambridge, UK). Antibodies specific to Piezo1 (Cat. no. 15939-1-AP), β-actin (Cat. no. 66009-1-Ig), and Nedd4-2 (Cat. no. 13690-1-AP) were purchased from Protein Tech Group, Inc. (Wu Han, China; dilution, 1:1,000). Antibodies specific to Piezo1 (Cat. no. NBP1-78446) and Piezo2 (Cat. no. NBP1-78624) were purchased from NOVOUS (Basel, Switzerland; dilution, 1:100). Antibody specific to Piezo2 (Cat. no. ARP49683) was purchased from AVIVA (San Diego, USA; dilution, 1:500); and one self-prepared Antibody specific to Piezo2 (dilution, 1:1000). Antibodies specific to Tuj1 (Cat. no. GTX631836) was purchased from GeneTex (Southern California, USA; dilution, 1:500). Scrambled shRNA (r) Lentiviral Particles (Cat. no. Sc-

108080), Piezo1 shRNA (r) Lentiviral Particles (Cat. no. Sc-27-342-V) and Piezo2 shRNA (r) Lentiviral Particles (Cat. no. Sc-270372-V) were purchased from Santa Cruz (Santa Cruz, USA). Goat anti-rabbit (Fluorescein 5-isothiocyanate, FITC) (Cat. no. 132373) and Goat anti-mouse (Tetramethylrhodamine, TRITC) (Cat. no. 131512) were purchased from Jackson ImmunoResearch Inc. (Baltimore, USA; dilution, 1:400); SP-DiO (Cat. no. D7778) was purchased from Thermo Fisher Scientific (Waltham, USA). DiI (Cat. no. MB4240) was purchased from Dalian Meilun Biotechnology Co. (Dalian, China).

**2.3 Hypertensive animal model**

L-NNA-induced hypertensive animal model: In brief, WKY rats (total number, 30), with body weight of 190-210 g, received intraperitoneal (*i.p.*) injection with L-NNA (7.5 mg/kg/12 hours) for 28 consecutive days [23]. 22 rats had significantly increased BP and were used in the following study; 8 rats were excluded because 5 rats BP fluctuated too much and 3 rats died during the experimental procedure. WKY rats (total number, 30) with the same range of body weight that were intraperitoneally injected with saline (the same volume as L-NNA) for 28 consecutive days were used as control. In control group, 2 rats were excluded because they died during the experimental procedure.

Ang II-induced hypertensive model rats: In brief, WKY rats (total number, 60), with the body weight of 190-210 g, were anesthetized with isoflurane. Osmotic minipumps (Alzet 2002, DURCT, USA) containing either Ang II (400 ng/Kg/min,

ANG II group, n = 30) or the same volume of saline (0.9%, control group, n = 30) were subcutaneously implanted into the animals [24]. benzylpenicillin (19 mg/0.1 mL) was injected into muscular immediately after surgery. Postoperatively, rats were housed individually in plastic cages and supplied with water and food ad libitum. All infusions were performed for 14 days. Arterial BP was measured at the day after 14 days minipump implantation. In Ang II group, 21 rats had significantly increased BP and were used for following study. 9 rats were excluded because 5 rats with damaged osmotic minipumps and 4 rats died during the experimental procedure. In control group, 2 rats were excluded because their osmotic minipumps were damaged.

**2.4 Measurement of the Hemodynamic Parameters in rats**

Carotid catheterization method was used to measure the arterial BP of rats. Briefly, rats were anesthetized with *i.p.* injection of 2% sodium pentobarbital (40 mg/kg). The rats were fixed at supine position and the neck hair was removed. A median incision was made to separate the left carotid artery and vagus nerve. The left carotid artery was separated and two threads were put under the carotid artery for later using. The distal end of the left carotid artery was ligated and the proximal end of the carotid artery was clamped with an arterial clamp. A "V" shaped incision was made between the distal ligation line and the artery clamp by ophthalmic scissors in the centriolar direction at 45°. The "V" shaped incision should not be too large, about one-third of the perimeter of the vessel. The polyethylene catheter (PE 50) filled with 2‰ heparin saline was inserted into the left carotid artery centripetally (about 2 cm) and

connected to the pressure transducer (NL108A; Digitimer Ltd.). The arterial BP of rats was recorded and measured (Data acquisition and analysis system, PowerLab). The heart rates (HR) of rats were measured by electrocardiograph (ECG), which was recorded with three needle electrodes placed subcutaneously on the lower left chest (+), upper right chest (-) and left hind paw (ground), respectively and amplified with a differential amplifier (Data acquisition and analysis system, PowerLab).

**2.5 Immunofluorescence**

Rats were transcardially perfused with 4% PFA under depth of anesthesia (2% sodium pentobarbital, 40 mg/kg). Left NG was removed and stored in 4% PFA followed by embedding in OCT (SAKURA, Japan). Ten micrometers NG sections were cut using a freezing microtome (Leica, Germany). Sections were washed once with 0.1 mol/L PBS (Beijing Solarbio Science & Technology Co., Ltd.) and punched for 30 min in 37˚C with 0.3% Triton X-100/PBS buffer and blocked for 1 hours with blocking buffer (10% goat serum in 0.1 mol/L PBS). Primary antibodies were diluted in 0.1% Triton X-100/PBS buffer before overnight incubation at 4˚C. Antibodies of Piezo1 (Cat. no. NBP1-78446), Piezo2 (Cat. no. NBP1-78624), nNOS (Cat. no. ab1376), Nedd4-2 (Cat. no. 13690-1-AP) and Tuj1 (Cat. no. GTX631836) were used in immunofluorescence (IF) assay. The second day, sections received a further 3 times washes in PBS before incubation with secondary antibodies for 2 hours at 37˚C. Sections were washed with PBS for 3 times and placed on microscope slides in Vectashield with DAPI (Southern Biotech). Staining was visualized and the IF

intensity was measured using a laser scanning confocal microscope (Leica SP5, Leica, Germany).

For aortic arch adventitia, which contains the baroreceptor nerve terminals, was fixed in 4% PFA overnight. Then the adventitia was punched for 3h in 1% Triton X-100. The procedures for immunostaining were the same as described above.

**2.6 Real-Time PCR**

Total RNA was extracted from NG tissues using RNAiso Plus total RNA extraction reagent (Takara Bio, Inc.). cDNA was synthesized using a Prime Script RT reagent Kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan). Genomic DNA is eliminated by treatment with gDNA Eraser for 2 min at 42˚C. The reaction conditions were as follows: 37˚C for 15 min, 85˚C for 5 sec and 4˚C for termination. Subsequently, cDNA was stored at -20˚C. qPCR was performed using a SYBR Premix Ex Taq Real-Time PCR Kit (Takara Bio, Inc.). The reaction conditions were one cycle of initial denaturation at 95˚C for 3 min, followed by 35 cycles of 95˚C for 30 sec, 60˚C for 30 sec. The PCR primer sequences were as follows: Piezo1 forward: 3'-CTCCTGTGGAGAACCGTGAT-5' and reverse: 3'-CTGCGAGTGTTGTAGCCAGT-5'; Piezo2 forward: 3'-TTACATCTGTGCCCTCATCG-5' and reverse: 3'-CATGGGTACTTCCTCCTGTC-5'; GAPDH forward: 3'-GACATGCCGCCTGGAGAAAC-5' and reverse: 3'-AGCCCAGGATGCCCTTTAGT-5'. GAPDH was used as an internal control. Expression data were calculated from the cycle threshold (Ct) value, and the $2^{-\Delta\Delta Ct}$

method was used to calculate RNA level.

**2.7 Co-Immunoprecipitation (Co-IP) and Western blot**

Samples were prepared from NG tissues or HEK 293T cells expressing both Piezo2 and Nedd4-2. A certain volume of RIPA (Beyotime Institute of Biotechnology) (containing protease inhibitor) was added to the NG tissues or the collected cells, lysed on ice for 30 min, centrifuged at 12,000 rpm for 30 min, and the supernatant was collected and the concentrations of proteins were detected using a bicinchoninic acid protein kit (Beyotime Institute of Biotechnology).

For Western blot analysis, proteins were extracted from NG, 30 μg were used, separated by electrophoresis on 8% SDS-polyacrylamide gels, and transferred onto PVDF membranes. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween-20 for 2 hours at room temperature. Membranes were then incubated with primary antibodies overnight at 4˚C. Antibody for β-actin was used as a housekeeping protein. Antibodies of Piezo1 (cat. no. 15939-1-AP; dilution, 1:500), Piezo2 (cat. no. ARP49683; dilution, 1:500) and β-actin (cat. no. 66009-1-Ig) were used for western blot assay. Second day, the blots were probed with secondary antibodies (IRDye 800CW goat anti-rabbit, Cat. no. 926-32210; IRDye 800CW goat anti-mouse, Cat. no. 926-32211; LI-COR Biosciences), and the blots were visualized using the Odyssey Fc System (LI-COR Biosciences). Densitometry of the protein bands was performed using ImageJ 1.50i software (National Institutes of Health). The experiments were repeated at least three times.

200 μg protein samples were used for Co-IP. In this case, the protein was incubated with anti-Piezo2 (self-prepared), anti-Nedd4-2 (Cat. no. 13690-1-AP) and anti-IgG antibodies (each antibody using 3μg) for 16 hours, respectively. Then Protein-G-coated magnetic beads (Sigma, IP50-1KT) were added and the solution was incubated for 12 hours. The protein was denatured with SDS buffer containing 50 mM Tris-HCl, 2% SDS, 100 mM DTT, 10% glycerol and 0.01% bromophenol blue for 10 minutes at 95˚C and then separated by SDS-PAGE. Anti-Piezo2 (self-prepared) antibody and anti-Nedd4-2 (13690-1-AP) antibody were detected. The experiments were repeated at least three times.

**2.8 Retrograde labelling of aortic baroreceptor NG neurons**

The procedures were similar to previously described [13]. Briefly, rats were anesthetized with 2% sodium pentobarbital (40 mg/kg). Nerves in the left cervical area were exposed. 3-4 mm of the aortic depressor nerve was carefully detached from surrounding tissues. The fluorescent dye 1,1'-dioleyl- 3,3,3,'3'-tetramethylindo-carbocyanine methane sulfonate (DiI) crystals were applied around the left aortic depressor nerve with a parafilm underneath, and the area was wrapped by a rapid-curing gel (Kwik-Sil, World Precision Instruments). The incision was sutured afterward. The animals were recovered for 10 days to allow dye to diffuse retrogradely along the aortic depressor nerve to the soma located in NG.

**2.9 Single-cell RT-PCR**

Single-cell RT-PCR was performed as previously described [25]. Retrogradely labeled

NG neurons were aspirated (under a microscope) into a patch pipette using a conventional patch-clamp setup with negatively pressurised pipette holder. The electrode tip was then quickly broken into a 0.2-mL PCR tube containing 0.7 µL of oligo-dT (50 mM), 1 µL of dNTP mixture (10 mM), 0.5 µL of $MgCl_2$ (25 mM), 0.7 µL of RNaseOUT (40 U/mL), and 1.4µL of DEPC-treated water; the mixture was heated to 65˚C for 5 minutes and then placed on ice for 1 minute. Single-strand cDNA was synthesized from the cellular mRNA by adding 0.5 µL of RT buffer, 1.5 µL of $MgCl_2$ (25 mM), 1 µL of DTT (1M), 0.5 µL of RNaseOUT (40U/mL), and 1 µL of Super Script III RT (200 U/mL) and then incubating the mixture at 55˚C for 50 minutes followed by 85˚C for 5 minutes. Synthesis of single-cell cDNA was performed using a C1000 Touch thermal cycler-CFX96Real-time PCR (BIO-RAD, Hercules, CA). First strand synthesis was executed at 95˚C (1 minute) followed by 40 cycles (95˚C for 50 seconds, 60˚C for 50 seconds, and 72˚C for 55 seconds) and a final 10 minutes elongation at 72˚C by adding the specific “outer” primer pairs into each PCR tube (supplementary table 1). Then, 2.5 µL of the product of the first PCR was used in the second amplification round by using specific “inner” primers (final volume 25 µL, supplementary table 1). The second amplification round consisted of heating the samples to 95˚C (1 minute) followed by 40 cycles (95˚C for 50 seconds, 60˚C for 50 seconds, and 72˚C for 55 seconds) and 10-minute elongation at 72˚C. The products of the second PCR were analysed in 2% agarose gels and stained with ethidium bromide. SuperScript III First-Strand Synthesis System Kit and GoTaq

Green Master Mix were obtained from Takara-Clontech (Invitrogen, Carlsbad, CA) and Promega (Madison, WI), respectively.

**2.10 Cells culture and transfection**

Primary cultures of NG neurons were prepared from rats (about 250 g) as previous studies described [26]. In brief, rats were deep anesthetized and then the ganglia were rapidly removed and placed into the D-Hank's solution (Gibco). Ganglia samples were digested at 37˚C with collagenase (2 mg/mL, Worthington) for 30 min. They were subsequently suspended in 10 mL DMEM (Gibco) plus 10% bovine calf serum (Gibco) to stop digestion. Ganglia were then dissociated into a suspension of individual cells and plated onto 13 mm glass coverslips pre-coated with 100 g/mL poly-D-lysine (BD Biosciences). Cells were incubated at 37˚C with a 5% $CO_2$ and 95% $O_2$. Retrogradely labelled NG neurons by DiI dye were used for patch clamp recording within 24 hours.

HEK 293T cells were plated onto 13 mm glass coverslips pre-coated with poly-D-lysine (100 g/mL). Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In addition, EGFP was co-transfected to identify the transfected cells. The Piezo2 plasmid was purchased from Addgene (plasmid #81073). The Nedd4-2 (also known as Nedd4L) plasmid was purchased from Youbao Biological Technology Co., Ltd. (NM-001114386).

**2.11 Electrophysiology**

Whole-cell mode patch clamp recordings were performed at a room temperature of

22-24˚C as previous study described [26]. Coverslips with cultured NG neurons were placed in a 0.5 ml microchamber, mounted on a stage of an Olympus IX70 inverted microscope (Olympus Co, Japan) and continuously perfused at 2 ml/min with bath solution. The bath solution contained (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 glucose with an osmolarity 320 mOsm. The pH was adjusted to 7.35 with NaOH. The internal recording solution contained (in mM) 70 $Cs_2SO_4$, 5 KCl, 2.4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, 5 $Na_2ATP$, 0.33 GTP-Tris salt, pH was adjusted to 7.35 with CsOH and osmolarity was adjusted with sucrose to 320 mOsm. The recording electrodes were pulled from thick wall borosilicate glass capillaries using a Flaming P-97 puller (Sutter Instrument Co. Novato, CA). Resistances of electrodes were 3-5 MΩ after filling recording internal solutions. The protocol used to record MA currents in baroreceptor NG neurons was as follows: the cells that were retrogradely labelled by DiI were selected to record MA currents. The cells were held at -60 mV and cell membranes were displaced by a heat-polished glass probe. The probe, with a ~4 μm diameter tip, was positioned at an angle of 45˚ to the dish surface. Its movement was controlled by a Piezo-electric device (Physik Instrumente, Ltd.). Cells were stimulated with a series of mechanical stimuli in 1 μm increments to elicit MA currents. The moving velocity of the probe was set at 0.5 μm/ms. The probe and the recorded cells were visualized as live images on a monitor throughout the recording. The live images were captured continuously through a CCD camera that was connected to the 40× microscope objective. To record the Piezo2 MA currents,

Piezo2 with or without Nedd4-2 were expressed in HEK 293T cells. The bath solution, internal recording solution and the recording protocol were as same as demonstrated above. Signals were recorded with an Axopatch 700B amplifier, filtered at 2 KHz and sampled at 5 kHz using pCLAMP 10.7 (Axon Instruments; Molecular Devices, LLC).

**2.12 Injection of shRNA Lentivirus or GsMTx4 or Yoda1 in NG**

In brief, WKY rats were anesthetized with *i.p.* injection of 2% sodium pentobarbital (40 mg/kg). A midline incision was made at cervical area of rats. Both nodose ganglions were exposed. The microinjector (Hamilton Co.) contained 2 μL solution of shRNA lentiviruses particles (~$1\times10^6$ copies of Control shRNA, Piezo1 shRNA or Piezo2 shRNA) was focally inserted into both nodose ganglions to a depth of 500 μm. The shRNA lentiviral particles were injected slowly into NG, and the needle was removed 5 minutes after the injection was complete. The incision was closed with sutures. Intramuscular injection of benzylpenicillin (19 mg/0.1 mL) was given immediately after surgery. Postoperatively, rats were housed individually in plastic cages and supplied with water and food ad libitum. After infection for 15 days, the common carotid artery cannulation method was used to measure SBP, DBP, MAP and PP.

Arterial BP was measured from the right common carotid artery cannulation of the rats. The left NG was exposed. After the BP was stabilized, 1 μL GsMTx4 (NS as control) or Yoda1 (5‰ DMSO as control) (100 μmol/L, 0.25 μL/min) was injected

into the NG, and changes in blood pressure were observed after GsMTx4 or Yoda1 injection.

**2.13 Evaluation of baroreflex**

Rats infected with lentivirus particles (Piezo1-, Piezo2- and scrambled shRNA) in NG after anesthetic. 2 weeks later, the rats were fixated at supine position, the left carotid artery was exposed and arterial BP was recorded as above (see 2.4 Measurement of the Hemodynamic Parameters in rats); The HR of rats was measured by ECG (see 2.4 Measurement of the Hemodynamic Parameters in rats). When the BP was stable, PE was slowly administered through the left femoral vein (rate: 200 μL/min; infusion dose: 50 μg/kg). Then, the BP increased sharply and the HR slowed down reflexively. The BP and HR were continuously recorded until the BP recovered. The baroreceptor sensitivity was evaluated by calculating the changes of HR and systolic blood pressure after intravenous injection of PE.

**2.14 Enzyme Linked Immunosorbent Assay (ELISA)**

The peripheral venous blood (about 3 ml) of rats was collected. Samples were incubated at room temperature for 2 hours and then centrifuged at 2000× g for 20 mins. The supernatant was then collected to detect the level of serum norepinephrine (NE) with the ELISA kit (Cloud-Clone corp. Cat. no. HEA907Ge) based on the kit instructions. Briefly, the standards, reagents and samples were prepared before the experiment. 50 μL samples (including standard and sample to be tested) and 50 μL detection reagent A (prepared before using) were added to each well, then incubated

at 37°C for 1 hour. The wells were washed three times using washing solution; then 100 μL detection reagent B was added to each well and incubated at 37°C for 30 minutes. After this, the wells were washed 5 times using washing solution; then 90 μL substrate solution was added to each well and incubated at 37°C for 20 minutes. The reaction was terminated by adding 50 μL stop solution to each well and the absorbance was immediately read at 450 nm.

### 2.15 Statistical analysis

Data were presented as the Mean ± SEM and analyzed using GraphPad Prism 8. Statistical significance was evaluated using either a Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple groups or two-way ANOVA followed by Bonferroni's multiple comparisons test. The $\chi^2$ test was used to assess the differences in the proportion of Piezo2-positive baroreceptor NG neurons between WKY rats and SHR. The $\chi^2$ test was also used to assess the differences in the proportion of RA-MA and non-responsive (NR) neurons in cultured NG neurons transfected with scrambled shRNA and Piezo2 shRNA. $P < 0.05$ was considered to indicate a statistically significant difference.

## 3 Results

### 3.1 Piezo2 was dominantly expressed in NG neurons in WKY rats and downregulated in hypertensive rats

Recent studies have shown that both Piezo1 and Piezo2 MA channels are obviously expressed in the mouse NG neurons [3]. In the present study, SHR and L-NNA- or Ang

Ⅱ-induced hypertensive model rats were used to address the different expression of Piezo1 and Piezo2 in NG between normotensive WKY rats and hypertensive rats. The carotid artery intubation method was used to record the BP of WKY rats and SHR. The SBP, DBP, MAP and PP were all significantly higher in SHR than that of WKY rats (Fig. 1A). qPCR assay showed that the mRNA of both Piezo1 and Piezo2 in the NG of SHR were significantly lower than that of WKY rats by 27.8% ($P < 0.001$) and 15.2% ($P < 0.01$), respectively (Fig. 1B). Immunostaining assays were also performed (Fig. 1C). In the NG neurons of WKY rats, the immunofluorescence (IF) of Piezo2 was obviously displayed, however, the IF of Piezo1 was hardly detected (Fig. 1C). Moreover, the IF intensity of Piezo2 in the NG neurons of SHR was significantly reduced by 25% ($P < 0.001$), while, the IF intensity of Piezo1 had no significant alterations (Fig. 1C & D). Both qPCR and immunostaining assays indicated that Piezo2 expressed at higher levels in the NG of WKY rats and significantly downregulated in the NG of SHR (Fig. 1B-D). Consistent with these results, western blot assay clearly showed that Piezo2 but not Piezo1 dominantly expressed in the NG of WKY rats, and its expression was significantly reduced by 32.2% ($P < 0.001$) in the NG of SHR (Fig. 1E). Indeed, qPCR assay showed that Piezo2 expression was about 12.5-fold higher than that of Piezo1 ($P < 0.001$) at the mRNA level in the NG of WKY rats (Fig. 1F). These results indicate that Piezo2 dominantly expresses in NG neurons of normotensive WKY rats and significantly downregulated in NG neurons of SHR.

We then used drug-induced hypertensive model rats to address whether Piezo2 expression is also downregulated in their NG neurons. As shown in Figure 2A and 2B, L-NNA- and Ang II-induced hypertensive animal models were performed. These two types of hypertensive model rats were characterized with higher SBP, DBP, MAP and PP (Fig. 2A & B). Similar to SHR, qPCR, immunostaining and Western blot assays congruously showed that the expression of Piezo2 in the NG neurons of both L-NNA- and Ang II-induced hypertensive rats was significantly downregulated while Piezo1 kept no changes (Fig. 2C-K). The Piezo2 mRNA in the NG of L-NNA- and Ang II-induced hypertensive model rats were decreased by 24.0% ($P < 0.001$) and 25.6% ($P < 0.05$), respectively (Fig. 2C & D). The IF intensity of Piezo2 in the NG neurons of L-NNA- and Ang II-induced hypertensive rats were reduced by 32.7% ($P < 0.001$) and 15.8% ($P < 0.001$), respectively (Fig. 2G & H). In addition, the Piezo2 protein were decreased by 12.3% ($P < 0.05$) and 30.0% ($P < 0.05$), respectively in the NG of L-NNA- and Ang II-induced hypertensive model rats (Fig. 2I-K). Taken together, these results demonstrate that Piezo2 not Piezo1 dominantly expresses in NG neurons of normal BP rats. Piezo2 is significantly downregulated in hypertensive rats, which strongly indicates that Piezo2 expression in NG neurons may play an essential role in the development of hypertension.

**3.2 The expression and activity of Piezo2 MA channels were downregulated in baroreceptor NG neurons in hypertensive rats**

NG neurons includes different types of sensory neurons innervated the lungs,

gastrointestinal tract, heart and aortic arch [20,27]. The NG neurons innervating aortic arch are named baroreceptor neurons, and its nerve endings express the baroreceptors [2,28]. In addition, NG baroreceptor neurons specifically expressed neuronal isoform of nitric oxide synthase (nNOS) [29,30], therefore nNOS-positive NG neurons could represent baroreceptor neurons. To test the alteration of Piezo1 and Piezo2 in NG baroreceptor neurons in hypertensive rats, immunostaining assays were performed in nNOS positive NG neurons. In WKY rats, Piezo1 and Piezo2 were almost expressed in all nNOS-positive NG neurons (Fig. 3A & B), and 34.8% of Piezo1- and 30.0% of Piezo2-positive NG neurons were overlaped with nNOS (Fig. 3A-C). Neither nNOS/Piezo1- nor nNOS/Piezo2-positive populations of neurons showed significant difference between WKY rats and SHR (Fig. 3C, $P > 0.05$). In SHR, the IF intensity of Piezo2 in nNOS-positive NG neurons was significantly reduced by 30.2% ($P < 0.001$), while no significant changes were observed for Piezo1 ($P > 0.05$) (Fig. 3D).

Piezo1 and Piezo2 were identified as bona fide mammalian mechanically-activated (MA) ion channels [16]. We then performed patch clamp recordings to address the MA currents in aortic baroreceptor NG neurons and to compare the difference in MA currents between WKY rats and SHR. Based on their inactivation kinetics (also known as decay time constant, $\tau$), the MA currents were classified into three types, including rapidly adapting (RA, $\tau < 10$ ms), intermediately adapting (IA, 10 ms $< \tau <$ 30 ms) and slowly adapting (SA, $\tau > 30$ ms) [31,32]. Piezo1 and Piezo2 were sufficient to mediate IA- and RA-MA currents, respectively [16]. In this experiment, retrogradely

labelled aortic baroreceptor NG neurons by DiI [12,13] dye from the aortic depressor nerve were selected for evoking MA currents with mechanical stimulation (Fig. 4A & B). In WKY rats, 46.8% (29 out of 62) of baroreceptor NG neurons showed RA-MA currents. Only small amount of baroreceptor NG neurons (9.7% and 8.1%, respectively) displayed IA- and SA-MA currents (Fig. 4C & D). The proportion of RA-MA type baroreceptor NG neurons was significantly reduced to 20.5% in SHR (Fig. 4C & D, $P < 0.05$). While the proportion of non-responsive (NR) baroreceptor NG neurons was significantly increased from 35.5% in WKY rats to 68.2% in SHR (Fig. 4D; $P < 0.05$). There were no significantly differences in the percentages of baroreceptor NG neurons with IA- and SA-MA currents between WKY rats and SHR (6.9% for IA-MA, $P > 0.05$ and 4.6% for SA-MA, $P > 0.05$) (Fig. 4D). More importantly, the RA-MA current densities of baroreceptor NG neurons were significantly reduced in SHR (Fig. 4C & E). There was ~5-fold reduction on the RA-MA current density at the membrane displacement stimulation of 13 μm (Fig. 4E, $P < 0.05$). Furthermore, single-cell PCR was performed with the retrogradely labelled baroreceptor NG neurons (Supplementary figure 1A). Consistent with the electrophysiological analysis, the proportion of Piezo2-positive neurons was markedly reduced from 89.4% in WKY rats to 66.7% in SHR (Supplementary figure 1B, $P < 0.01$). Taken together, these results indicate that the expression and function of Piezo2/RA-MA channel are downregulated in aortic baroreceptor NG neurons in SHR. Moreover, the Piezo1/IA-MA current densities of baroreceptor NG neurons

have no significant change between WKY and SHR (Supplementary figure 2A & B). While the SA-MA current densities of baroreceptor NG neurons were also significantly reduced in SHR (Supplementary figure 2C & D), which was consistent with the findings of a previous study [33].

To further determine the RA-MA currents were mediated by Piezo2 channels in NG neurons, we recorded the whole-cell MA currents from cultured NG neurons which were transfected with scrambled shRNA and Piezo2 shRNA (n = 33 neurons for scrambled shRNA and n = 48 neurons for Piezo2 shRNA), respectively (Supplementary figure 3). After knocking down the expression of Piezo2 by shRNA, the proportion and current amplitude of RA-MA currents were significantly reduced (proportion in Piezo2 shRNA group, 18.8%, compared with that of scrambled shRNA group, 39.4%, $P < 0.05$, Supplementary figure 3B). While, the proportions of IA- and SA-MA currents kept no significant changes (Supplementary figure 3B).

**3.3 The expression of Piezo2 was downregulated in aortic baroreceptor nerve endings**

We then tested the expression of Piezo1 and Piezo2 and their alterations in the aortic baroreceptor nerve endings in normotensive WKY rats and SHR by immunostaining assay. The aortic baroreceptor nerve endings were specifically labelled by 3,3'-Dioctadecyl-5,5'-Di(4-Sulfophenyl) Oxacarbocyanine, Sodium Salt (SP-DiO). In WKY rats, Piezo2 was obviously identified in aortic baroreceptor nerve endings, whereas Piezo1 was hardly detected (Fig. 5A-C), which was consistent with the

characteristics of Piezo1 and Piezo2 expression in baroreceptor NG neurons. Moreover, Piezo2 expression was significantly downregulated in aortic baroreceptor nerve endings of SHR (70.7 ± 5.1 A.U.) compared with WKY rats (140.9 ± 15.9 A.U., $P < 0.001$), while, Piezo1 expression did not show any alterations (Fig. 5A-C). Taken together, these results strongly suggest that Piezo2 not Piezo1 may act as baroreceptor in the baroreceptive reflex to modulate arterial BP.

**3.4 Knocking down or blocking Piezo2 MA channels in NG resulted in hypertension**

Above results suggested that the Piezo2 could be the modulator of BP. Inhibition of the function of Piezo2 theoretically could upregulate the BP through impaired baroreflex. To test this hypothesis, carotid artery intubation method was used to measure the arterial BP in rats. Firstly, the Piezos inhibitor GsMTx4 (100 μmol/L, 2 μL) [34] was slowly injected into the left NG of WKY rats and the arterial BP was significantly elevated (Fig. 6A & B). The alteration of SBP (ΔSBP), ΔDBP, ΔMAP and ΔPP were significantly increased in GsMTx4 group (21.9 ± 6.5, 14.6 ± 5.3, 19.1 ± 5.0 and 7.2 ± 3.3 mmHg, respectively) compared to that of control group (-3.4 ± 2.0, $P < 0.01$; -1.3 ± 1.6, $P < 0.05$; -2.3 ± 0.9, $P < 0.01$ and -2.0 ± 1.2 mmHg, $P < 0.05$; respectively). (Fig. 6A & B). While Yoda1 (the specific Piezo1 agonist) showed no effects on the arterial BP when it was slowly injected into NG in WKY rats (Supplementary figure 4A). These results indicate that targeting Piezo2 not Piezo1 may modulate arterial BP through affecting baroreflex.

To determine the exact role of Piezo1 and Piezo2 in regulating arterial BP in long term, shRNA lentiviral particles were directly injected into NG to knock down the expression of Piezos. WKY rats was randomly divided into three groups (Scrambled, Piezo1 and Piezo2 shRNA) after evaluation of BP with tail-cuff method (Supplementary figure 4B). Scrambled shRNA lentiviral particles were used as control. After infection for 15 days, the expression of Piezo1 and Piezo2 in NG was significantly reduced by shRNA interference (Supplementary figure 4C & D). Importantly, the BP (SBP, DBP and MAP) and serum norepinephrine (NE) concentrations in Piezo2 shRNA group rats rather than Piezo1 shRNA group rats were significantly elevated (Fig. 6C-E). These results further indicate that Piezo2 not Piezo1 may act as baroreceptor and its downregulation results in hypertension.

To evaluate whether baroreceptor sensitivity was affected by knocking down Piezos in NG, we tested the function of baroreflex in response to PE among the above three groups of rats. Intravenous infusion of PE at a rate of 200 μL/min (50 μg/kg) produced a transient increase in SBP and a consequent decrease in heart rate (HR) through baroreflex (Fig. 6F). In Piezo2 shRNA group rats, the SBP elevation induced by PE (94.5 ± 1.9 mmHg) was significantly higher than control (77.1 ± 1.7 mmHg, $P < 0.05$) (Fig. 6F & G). The PE-induced reduction in HR (287 ± 12 bpm for scrambled shRNA group *vs.* 134 ± 6 bpm for Piezo2 shRNA group, $P < 0.01$) and baroreflex sensitivity (ΔHR/ΔSBP, 3.8 ± 0.2 bpm/mmHg for Scrambled shRNA group *vs.* 1.4 ± 0.1 bpm/mmHg for Piezo2 shRNA group, $P < 0.01$) was obviously attenuated in

Piezo2 shRNA group rats (Fig. 6F & G). Whereas, Piezo1 shRNA group rats did not show any significant alterations in the SBP (76.9 ± 1.8 mmHg, $P > 0.05$), HR (247 ± 8 bpm, $P > 0.05$) and baroreflex sensitivity (3.3 ± 0.1 bpm/mmHg, $P > 0.05$) (Fig. 6F & G). Taken together, these results strongly indicate that Piezo2 rather than Piezo1 act as baroreceptor and its down-regulation significantly impaired the baroreflex and induced hypertension in rats.

**3.5 Nedd4-2 was involved in the downregulation of Piezo2 in NG in hypertensive rats**

Next, we explored the mechanism underlying the downregulation of Piezo2 in baroreceptor NG neurons in hypertensive rats. Piezo2 possesses a conserved PY-motif, PPSY (at the site of 1828-1831, 1796-1799 and 1898-1901 in human, rat and mouse Piezo2, respectively), located in the intracellular domain [16,35] (Fig. 7A). PPSY is well known to interact with the WW domains of E3 ubiquitin ligase Nedd4-2 [36]. Once binding to Nedd4-2, the PY-motif were preferentially orchestrated their internalization and subsequent degradation or recycling by Nedd4-2 [36]. Numerous studies have demonstrated that ion channels possessing PY-motifs (such as KCNQ, ENaC and voltage-gated sodium channel ($Na_v$) (Fig. 7A) were downregulated by Nedd4-2 [37-40]. Therefore, we hypothesis Piezo2 could be downregulated by Nedd4-2.

To test the hypothesis, we first tested the possible interaction between Piezo2 and Nedd4-2 in native NG and in HEK 293T cells co-expressed Piezo2 and Nedd4-2. Co-IP analysis revealed that both Piezo2 and Nedd4-2 bands were detected in IP-Nedd4-2

and IP-Piezo2 experiments either in HEK 293T cells co-expressed Piezo2 and Nedd4-2 (Fig. 7B) or in rat NG (Fig. 7C). Nedd4-2 and Piezo2 obviously pulled down each other both in HEK 293T cells co-expressed Piezo2 and Nedd4-2 (Fig. 7B) and in rat NG (Fig. 7C). In addition, immunostaining assays found that the IF intensity of Nedd4-2 was significantly increased in the nNOS-positive baroreceptor NG neurons of SHR (109.8 ± 5.8 A.U., $P < 0.001$), compared with WKY rats (79.8 ± 4.1 A.U.) (Fig. 7D). These results indicated that Nedd4-2 interacted with Piezo2 in baroreceptor NG neurons and negatively regulated Piezo2 expression in NG neurons of SHR.

To further determine Nedd4-2 negatively regulated Piezo2, whole-cell patch clamp experiments were performed in HEK 293T cells, which were expressed Piezo2 plus Nedd4-2 or alone. Indeed, Piezo2 MA current densities were significantly reduced in Piezo2 co-expression with Nedd4-2 group by 64.8% compared to Piezo2 group under 10-μm mechanical stimulation (Fig. 8A & B). Moreover, the threshold ($P < 0.01$) and latency ($P < 0.05$) for evoking Piezo2 MA currents were significantly increased in Piezo2 co-expression with Nedd4-2 group (Fig. 8C). There was no significant difference in the current decay time between these two groups ($P > 0.05$) (Fig. 8C). Taken together, these results suggested that Nedd4-2 negatively regulated Piezo2 MA channel activities.

## 4 Discussion

The major findings of this study are as following: 1) Piezo2 rather than Piezo1 is dominantly expressed in baroreceptor NG neurons in rats; 2) downregulation of

Piezo2 expression in baroreceptor NG neurons induces hypertension in rats; 3) Nedd4-2, at least partially, is involved in the downregulation of Piezo2 in baroreceptor NG neurons, by which may result in hypertension in rats.

In mammals, baroreceptors initiate the baroreflex to buffer any deviation of arterial BP through the negative feedback control of sympathetic and parasympathetic activities [2,28]. Baroreceptors are mechanotransduction device that located in baroreceptor nerve terminals. It can sense the stretch stimulation from the vascular wall induced by phasic pulsatile changes as well as acute fluctuation of arterial BP and convert these mechanical signals into electrical signals [28,41]. Previous studies have reported that γENaC [12], ASIC2 [8], and TRPC5 [13] are likely to serve as baroreceptors. However, mechanically activated Piezo channels (both Piezo1 and Piezo2) are recently identified as bona fide baroreceptors to regulate arterial BP [3]. Double knockout of Piezo1 and Piezo2 results in labile hypertension and increases BP variability [3]. Thus, the molecular nature of baroreceptor has been re-evaluated [42-44], though some criteria of baroreflex function are not fully satisfied by Piezo1/Piezo2 yet [45]. In the present study, we have further studied the role of Piezo1 and Piezo2 in baroreceptor neurons and nerve terminals in regulation of BP using normotensive and hypertensive rats. Interestingly, our results displayed that Piezo2 is dominantly expressed in NG neurons and baroreceptor nerve terminals. And the majority of baroreceptor NG neurons in rats have rapidly adapting type of mechanically-activated (RA-MA) currents (Fig. 4) which are mediated by Piezo2 [16]. To our best knowledge,

this is the first time to describe RA-MA currents in baroreceptor neurons. Furthermore, Piezo2 expression as well as RA-MA current densities have been significantly reduced in hypertensive rats. In normotensive rats, the arterial BP was significantly elevated either by inhibiting Piezo2 MA channel activity or by knocking down Piezo2 expression in baroreceptor NG neurons. In contrast, the expression of Piezo1 in baroreceptor neurons and nerve terminals is hardly detected in normotensive rats and does not show any difference from that of hypertensive rats. Moreover, knockdown of Piezo1 in baroreceptor NG neurons does not induce any changes in arterial BP. Overall, our study indicates that Piezo2 not Piezo1 may act as baroreceptor to regulate arterial BP in rats. Our study demonstrates the species specificity of Piezo1 and Piezo2 baroreceptors between rats and mice [3]. However, it is hard to know the precise role of Piezo1 and Piezo2 in baroreflex in human due to the limitations. In mouse, Piezo1 and Piezo2 express at similar levels in baroreceptor neurons, but disruption of Piezo1 or Piezo2 alone in baroreceptor neurons has no effect on BP [3]. One plausible explanation is that Piezo1 and Piezo2 may compensate each other under the single gene knockout condition to maintain normal force-sensing function. Increasing evidence demonstrates this view is logical. For example, TRPV4 compensates for the deficiency of TRPV3 in cochlear hair cells to maintain normal hearing in mice [46]. In hypertensive rats, Piezo1 expression may be too low to compensate the downregulation of Piezo2 there by impairs baroreflex.

It has been suggested over time that baroreceptor mechanism only contributes to

short-term (within seconds and minutes) BP regulation [47,48]. This point is originally proposed in the 1970s by Cowley, that complete arterial baroreceptor denervation only leads to an initial increase in arterial BP while the long-term BP is normal [49]. However, this view ignores the resetting mechanism of the central nervous system (CNS) associated with neuroplasticity [50]. Resetting mechanism allows BP to rise to higher levels which perhaps results from an upward resetting of a CNS embodied set-point [50,51]. In fact, resetting mechanism depends upon the reduced (or impaired) baroreflex rather than totally eliminated baroreflex [52]. Indeed, numerous studies have demonstrated that impaired baroreflex results in profound chronic hypertension not only in animals [53,54], but also in human subjects [55-57]. Therefore, increasing the activity of baroreflex or recovering the activity of impaired baroreflex is a valuable strategy to treat hypertension. According to this theory, several studies have suggested that electric field stimulation of baroreceptors (also known as baroreceptor activation therapy) reduces arterial BP in patients with drug treatment-resistant hypertension [9-11]. Thus, determination of baroreceptors' nature is benefit for providing precise target for hypertension therapy. In Zeng et al's research, Piezo1 and Piezo2 are together identified as baroreceptors in mice, double knockout of them in Phox2b-positive neurons results in hypertension [3]. It is worth noting that Phox2b-positive neurons are not exactly the same as baroreceptor neurons [45]. Piezo baroreceptors and baroreflex could be partially remained in Phox2bCre:Piezo KO mice. The remained baroreceptors may induce long-term hypertension through resetting mechanism. In

this study, knocking down the expression of Piezo2 in baroreceptor NG neurons obviously induces hypertension in rats. The remained Piezo2 baroreceptor may induce long-term hypertension through resetting mechanism. Thus, Piezo channel especially Piezo2 may act as baroreceptor to regulate arterial BP in mammals. Piezo2 downregulation results in impaired baroreflex consequently dysregulation of BP including hypertension. In future, discovering small molecules to selectively activate Piezo2 baroreceptors is useful therapeutic treatment for hypertension.

In this study, we have found that Piezo2 expression was downregulated in baroreceptor NG neurons and nerve terminals in hypertensive rats. Moreover, in normotensive rats, downregulation of Piezo2 expression in NG neurons could induce hypertension. Then a question is raised as what mechanism mediates the downregulation of Piezo2 to develop hypertension? Up to now, little is known about the modulation of Piezo2. In the present study, we have found that Piezo2 possessed a PY motif which could be recognized and binded by the E3 ubiquitin ligase Nedd4-2 through its WW domain. Nedd4-2 then induces ubiquitination of target substrates by covalently attaching ubiquitin, a highly conserved polypeptide of 76 AA, to their lysine residues [36,58]. Subsequently, Nedd4-2-induced ubiquitination preferentially orchestrated substrate proteins internalization and degradation or recycling, whereby the degree of ubiquitination determines the expression density of substrate proteins in cell surface [36,58]. In the present study, we have found that Nedd4-2 is interacted with Piezo2 not only in rat NG neurons but also in HEK 293T cells co-expressed with

Nedd4-2 and Piezo2. Piezo2 MA current densities also have been reduced when Piezo2 was co-expressed with Nedd4-2 in HEK 293T cells. Moreover, the Nedd4-2 expression was up-regulated and the RA-MA current densities was downregulated, respectively, in baroreceptor NG neurons of hypertensive rats. In the future, studies on the internalization structure (through the formation of caveolae and/or clathrin-coated pits) used by Piezo2, the degradation product of Piezo2, the degradation pathway (through proteasome and/or lysosome pathway), the cellular mechanosensitivity and its role in other diseases should be addressed. Consistent to our results, previous studies have shown that Nedd4-2 induces ubiquitination and downregulation of numerous ion channels such as KCNQ, ENaC, voltage-gated $Na^+$ channels [37-40]. Knockout of Nedd4-2 leads to up-regulated the membrane expression of voltage-gated $Na^+$ channel (VGSC) $Na_v1.7$ in dorsal root ganglion (DRG) sensory neurons [37]. Thus we speculated that the degradation of Piezo2 in baroreceptor neurons could be induced by Nedd4-2 that ultimately resulted in the elevation of BP in hypertensive rat. To our best knowledge that this study is first time to describe a novel mechanism for downregulation of Piezo2 expression. As a result, our study have found a distinct mechanism in downregulation of Piezo2 expression in baroreceptor neurons in hypertensive rats. Future investigations for suppressing Nedd4-2 in baroreceptor neurons may develop precise treatment of hypertension for increasing the expression of Piezo2 baroreceptor.

## 5 Conclusions

In summary, our study demonstrates that Piezo2 not Piezo1 acts as baroreceptor in rats; downregulation of Piezo2 baroreceptor by Nedd4-2 induces hypertension. Our findings provide novel insight into the molecular basis of baroreceptor and hypertension in mammals that can be applied to the development of strategies to treat hypertension in humans.

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## Author contributions

WZ and ZJ designed the research; LH, YG, DZ, SW, YH, HM, ZY, XQ, RW, DK and HB performed the experiments; LH, YG, DZ, and WZ analyzed the data; ZJ, WZ, HZ and LH wrote the manuscript. All authors approved the final version of the

manuscript.

**Declaration of Competing Interest**

The authors declare no potential conflicts of interest.

**Conflict of interest**

The authors declare no potential conflicts of interest.

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**Figure legends**

Figure 1

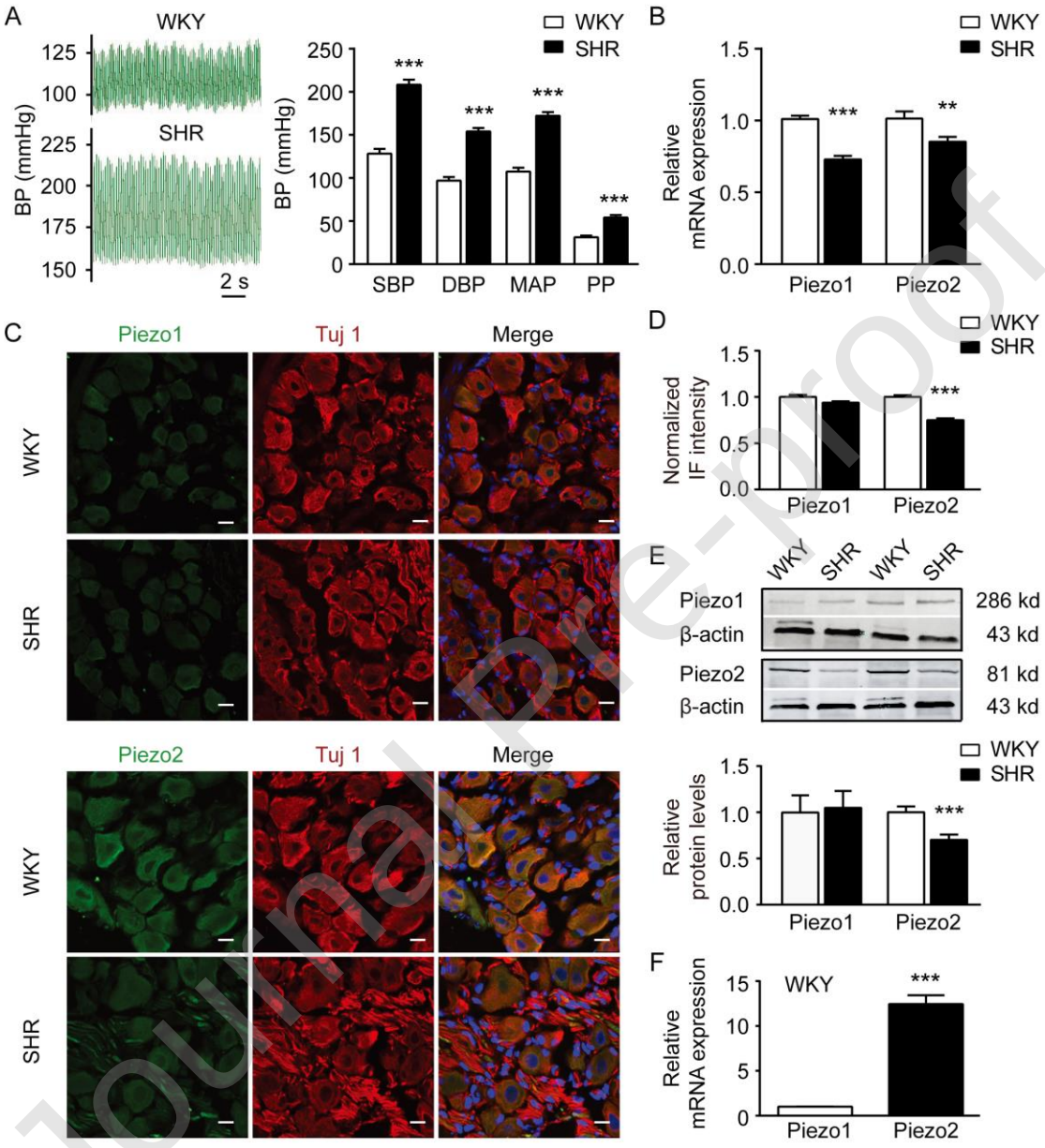


**Fig 1. The expression of Piezo2 was downregulated in NG neurons in SHR.**

(A) Left, Representative blood pressure (BP) traces of normotensive WKY rats (upper) and spontaneous hypertensive rats (SHR) (bottom). Right: Summary data of

systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and pulse pressure (PP) measured by carotid artery intubation method in WKY rats (n = 9) and SHR (n = 10). (B) Relative Piezo1 and Piezo2 mRNA expression in NG in WKY rats (n = 12) and SHR (n = 11). (C) Representative immunofluorescence (IF) images for Piezo1 (top) and Piezo2 (bottom) in NG neurons of WKY rats and SHR. Tuj1 acts as neuronal marker. Scale bar, 25 μm. (D) Summary data of normalized IF intensities for Piezo1 (n = 106 in WKY rats group and n = 155 in SHR group) and Piezo2 (n = 86 in WKY rats group and n = 124 in SHR group). (E) Representative Western blot assay (upper) and summary data (bottom) for the expression of Piezo1 (n = 6 in WKY rats group and n = 6 in SHR group) and Piezo2 (n = 8 in WKY rats group and n = 8 in SHR group) in NG. (F) Relative mRNA expression of Piezo1 and Piezo2 in NG in WKY rats (n = 11). Data are shown as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate SEM.

Figure 2

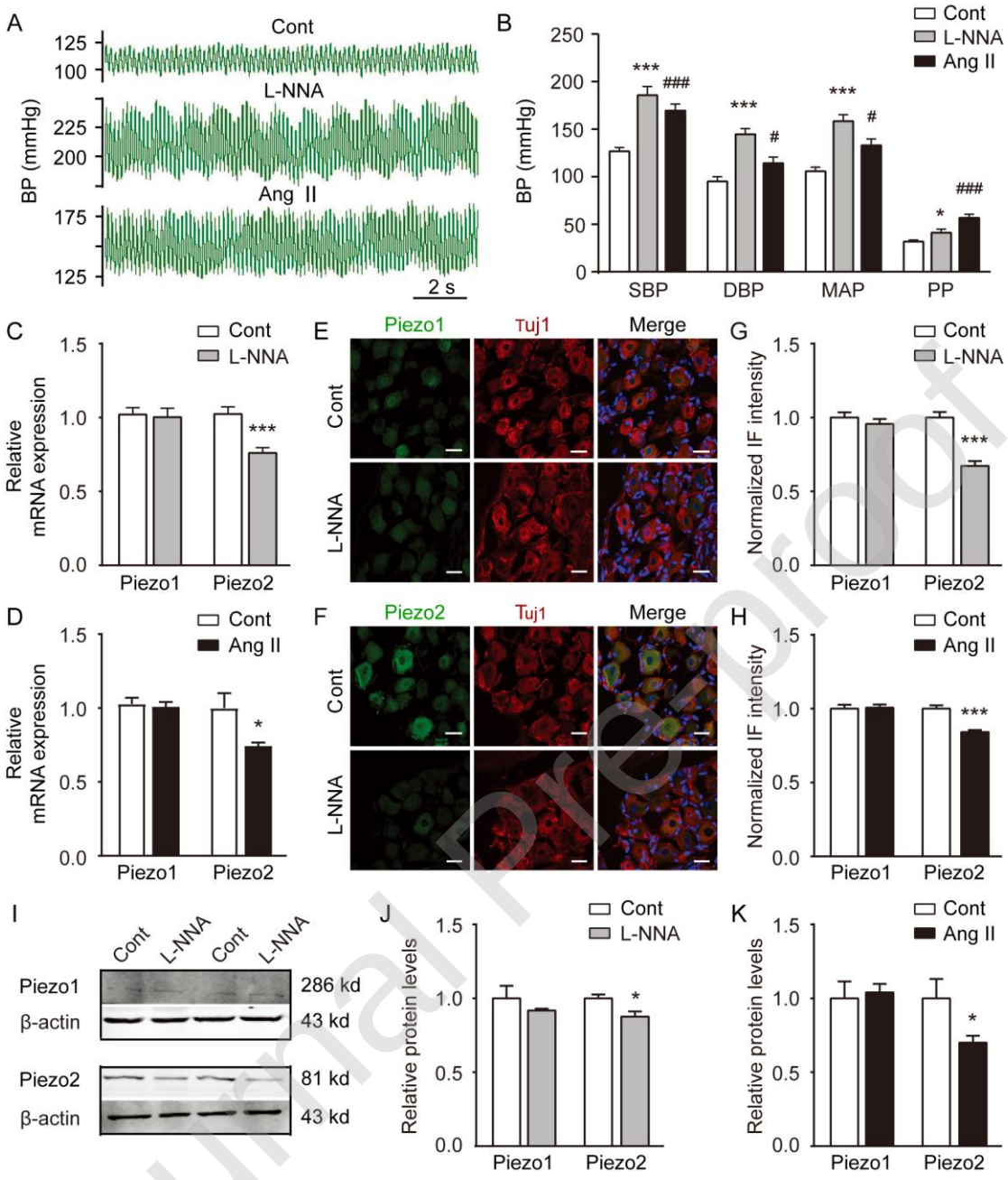


**Fig 2. The expression of Piezo2 was downregulated in NG in hypertensive model rats.** (A) Representative BP traces of control rats (Cont, upper), L-NNA-induced hypertensive model rats (L-NNA, middle) and Ang II-induced hypertensive model rats (Ang II, bottom). (B) Summary data of SBP, DBP, MAP and PP in Cont (n = 12) and L-NNA (n = 13) and Ang II (n = 11) group rats. (C) Relative Piezo1 and Piezo2

mRNA expression in NG in Cont (n = 7) and L-NNA (n = 6) group rats. (D) Relative Piezo1 and Piezo2 mRNA expression in NG in Cont (n = 6) and Ang II (n = 6) group rats. (E, F) Representative IF images for Piezo1 (E) and Piezo2 (F) in NG neurons of Cont and L-NNA group rats, respectively. Tuj1 acts as neuronal marker. Scale bar, 25 μm. (G) Summary data of normalized IF intensity for Piezo1 (n = 64 in Cont group and n = 47 in L-NNA group) and Piezo2 (n = 76 in Cont and n = 87 in L-NNA group) in NG neurons. (H) Summary data of normalized IF intensity for Piezo1 (n = 116 in Cont and n = 301 in Ang II group) and Piezo2 (n = 106 in Cont and n = 294 in Ang II group) in NG neurons. (I, J) Representative Western blot assay (I) and summary data (J) for the expression of Piezo1 (n = 5 in Cont and n = 6 in L-NNA group) and Piezo2 (n =4 in Cont and n = 5 in L-NNA group) in NG. (K) Summary data for the expression of Piezo1 (n = 4 in Cont and n = 3 in Ang II group) and Piezo2 (n = 6 in Cont and n = 6 in Ang II group) in NG in Cont and Ang II group rats. Data are shown as mean ± SEM. $^{*/\#}P < 0.05$, $^{***/\#\#\#}P < 0.001$. Error bars indicate SEM.

Figure 3

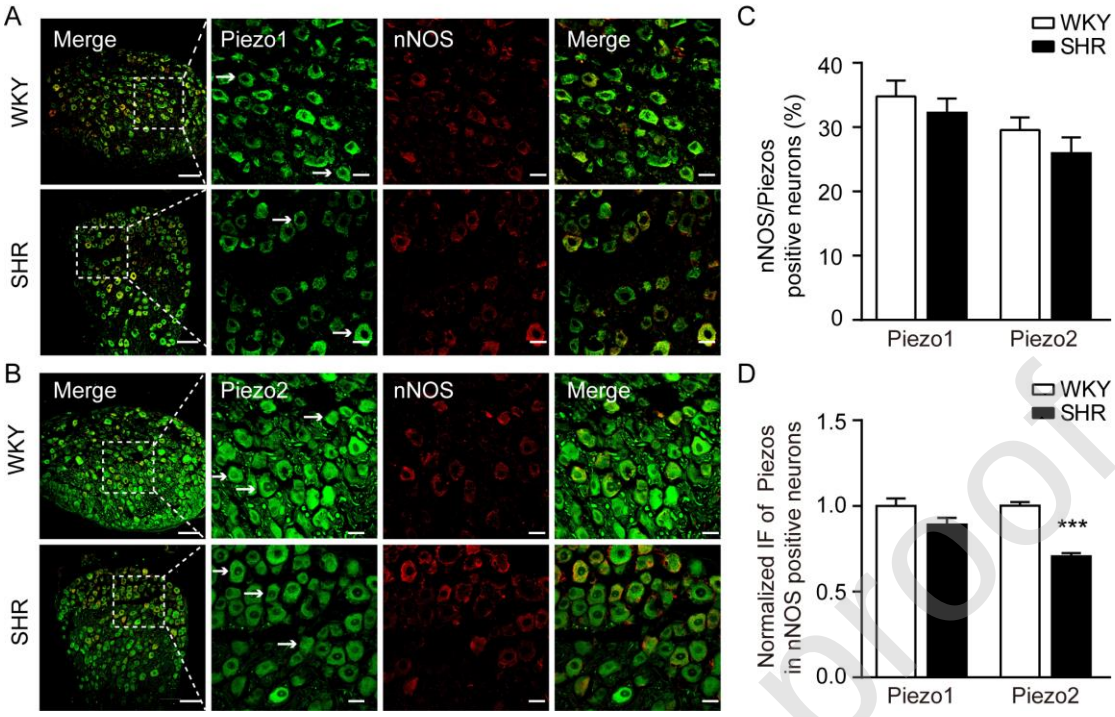


**Fig3. The expression of Piezo2 was downregulated in nNOS-positive baroreceptor NG neurons of SHR.** (A, B) Representative images showing the immunoreactivity to Piezo1 (A, green) and Piezo2 (B, green) in NG neurons in WKY rats (top) and SHR (bottom), respectively. The right three panels were extended from the square in left panel. nNOS (red) acts as the marker of baroreceptor NG neurons. Scale bar, 100 μm and 25 μm, respectively. (C) Histograms of the percentage of nNOS-positive NG neurons in Piezo1-positive and Piezo2-positive NG neurons. (D) Summary data of normalized IF intensity for Piezo1 (n = 72 in WKY rats and n = 54 in SHR) and Piezo2 (n = 154 in WKY rats and n = 127 in SHR) in nNOS-positive NG neurons. Data are shown as mean ± SEM. $^{***}P < 0.001$. Error bars indicate SEM.

Figure 4

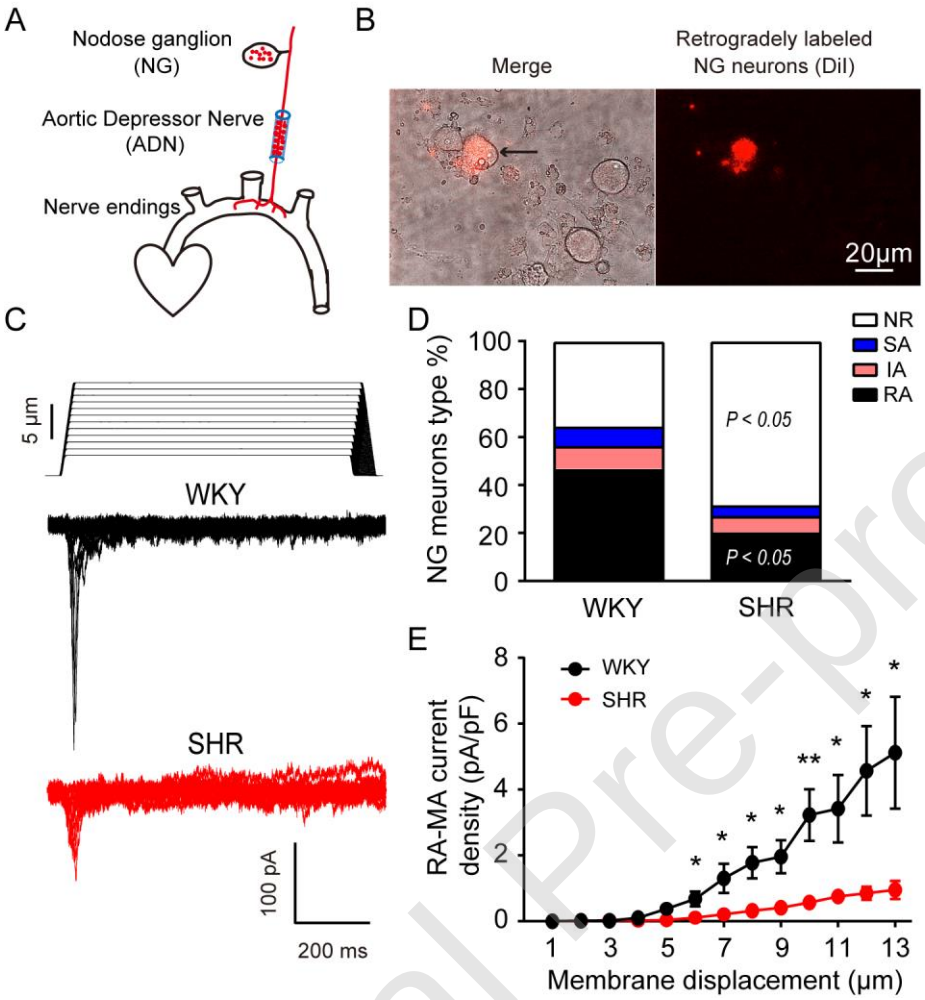


**Fig 4. RA-MA current densities was reduced in baroreceptor NG neurons of SHR.** (A) The schematic of aortic arch baroreceptor nerve terminals afferent to NG, showing DiI retrogradely labelled aortic baroreceptor NG neurons. (B) Representative images showed the retrogradely labelled baroreceptor NG neuron by DiI dye (red) were used to record MA currents. (C) RA-MA current traces in aortic baroreceptor NG neurons in WKY rats (middle) and SHR (bottom) were evoked by the stimulus probe with a series of poking displacement steps (upper). (C) Proportion of MA (RA,

IA and SA) currents in baroreceptor NG neurons in WKY rats (46.8%, 9.7% and 8.1%, respectively) and SHR (20.5%, 6.8% and 4.6%, respectively). NR indicated non-responsive cells. (D) Summary data of RA-MA current densities with different displacement distances in baroreceptor NG neurons in WKY rats (black solid circles) and SHR (red solid circles). Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars indicate SEM.

Figure 5

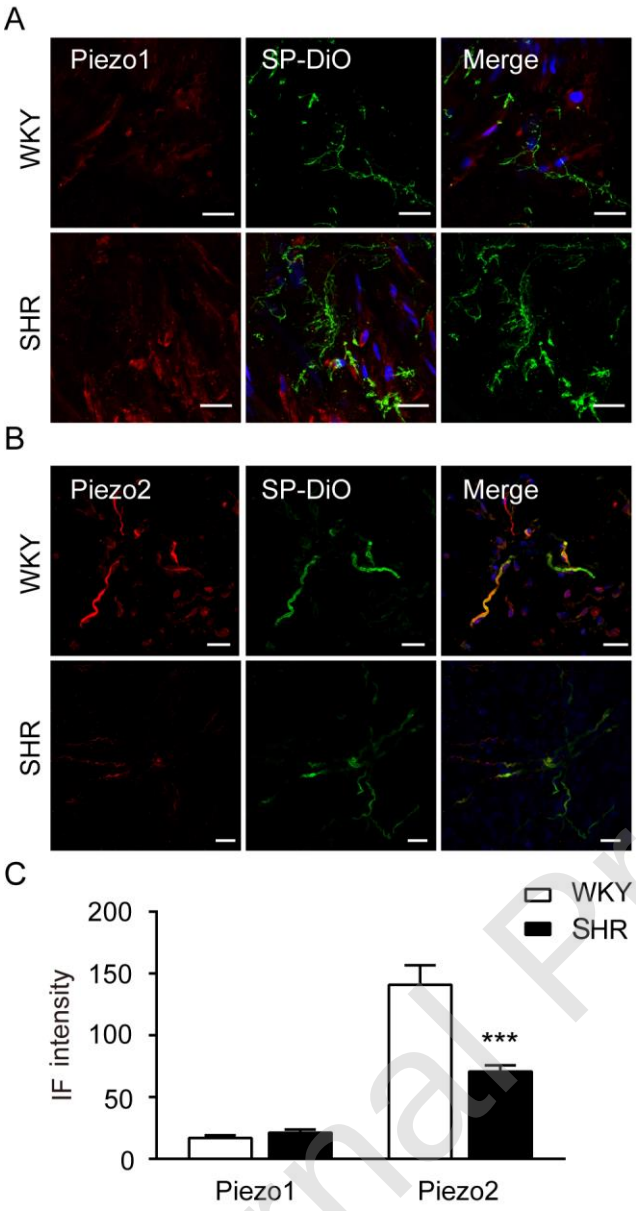


**Fig 5. The expression of Piezo2 was downregulated at aortic arch baroreceptor nerve terminals in SHR.** (A, B) Representative images showed the immunoreactivity of Piezo1 (A, red) and Piezo2 (B, red) at baroreceptor nerve terminals within the aortic arch adventitia in WKY rats (top) and SHR (bottom), respectively. SP-DiO (green) served as a marker of nerve terminals. Merged images of SP-DiO, DAPI (blue) and Piezo1 (A) or Piezo2 (B) were shown. Scale bar: 25 μm. (C) Summary data

of IF intensity for Piezo1 (16.8 ± 2.3 A.U. for WKY rats, n = 8; 21.3 ± 2.4 A.U. for SHR, n = 7) and Piezo2 (140.9 ± 15.9 A.U. for WKY rats, n = 16; 70.7 ± 5.1 A.U. for SHR, n = 14) at aortic arch baroreceptor nerve terminals in WKY rats and SHR. Data are shown as mean ± SEM. $^{***}P < 0.001$. Error bars indicate SEM.

Figure 6

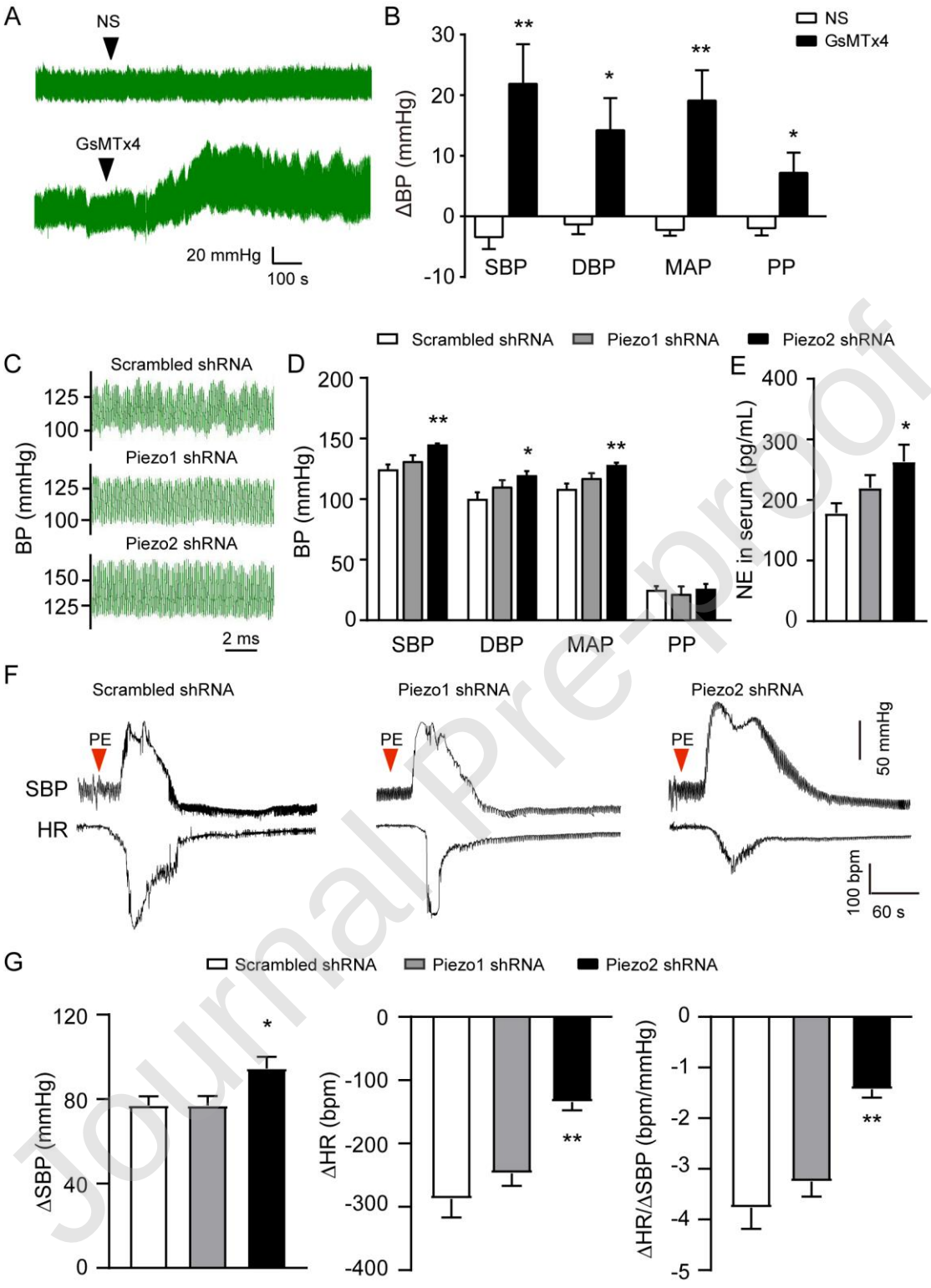


**Fig 6. Inhibition of Piezo2 in NG induced hypertension in WKY rats.** (A)

Representative traces showing that BP changes in WKY rats who were received

injection of Saline (NS, top) and Piezo channel antagonist GsMTx4 (bottom) at NG. (B) Summary data for the changes in SBP (ΔSBP), ΔDBP, ΔMAP and ΔPP induced by NS (open bar, n = 5) and GsMTx4 (solid bar, n = 5). (C) Representative BP traces of WKY rats whose NG were infected with control shRNA lentiviral particles (Scrambled shRNA, upper), Piezo1 shRNA lentiviral particles (Piezo1 shRNA, middle) and Piezo2 shRNA lentiviral particles (Piezo2 shRNA, bottom) for 15 days, respectively. (D) After 15 days infection in NG, the BP were recorded by carotid artery intubation method among the above three groups of WKY rats. The SBP, DBP and MAP of Piezo2 shRNA group rats were significantly increased to 144.3 ± 1.7 mmHg (n = 9, $P < 0.01$), 119.0 ± 4.1 mmHg (n = 9, $P < 0.05$) and 127.4 ± 2.6 mmHg (n = 9, $P < 0.01$). These BP parameters of Scrambled shRNA group rats were 123.8 ± 4.8 mmHg (n = 8), 99.4 ± 6.2 mmHg (n = 8) and 107.5 ± 5.5 mmHg (n = 8), respectively. Piezo1 shRNA group rats (n = 7) did not show any significant alterations on these BP parameters. (E) The concentration of serum NE among the above three groups of WKY rats. (F) Representative BP and HR traces of PE-induced baroreflex in Scrambled shRNA (left), Piezo1 shRNA (middle) and Piezo2 shRNA (right) group of WKY rats. (G) Changes in SBP (ΔSBP), heart rates (ΔHR) and the baroreflex sensitivity (ΔHR/ΔSBP) in Scrambled shRNA (white bar, n = 5), Piezo1 shRNA (grey bar, n = 4) and Piezo2 shRNA (black bar, n = 5) group rats. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars indicate SEM.

Figure 7

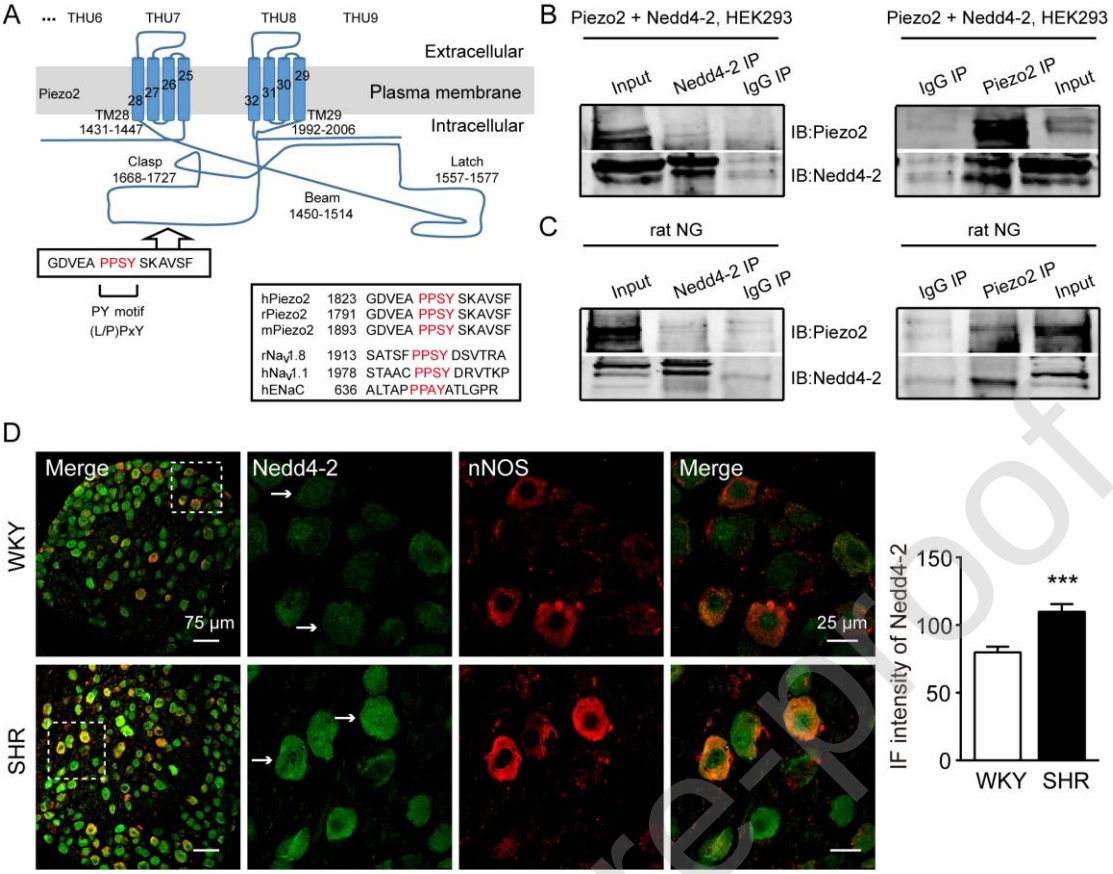


**Fig 7. Nedd4-2 interacted with Piezo2 and was increased in baroreceptor NG neurons in SHR.** (A) Schematic diagram of Piezo2 topology and the sequence of PY-motif in hPiezo2, rPiezo2 and mPiezo2. The PY-motif of rNav1.8, hNav1.1 and hENaC were also shown. (B, C) Representative Co-IP results showing Piezo2 and Nedd4-2 interaction in co-transfected HEK293T cells (B) and rat NG (C). (D) Representative images (left) and IF intensities (right) of the Nedd4-2 (79.9 ± 4.2 A.U. for WKY rats, n = 24; 109.8 ± 5.8 A.U. for SHR, n = 31) in nNOS-positive baroreceptor NG neurons in WKY rats and SHR. Data are shown as mean ± SEM. $^{***}P < 0.001$. Error bars indicate SEM.

Figure 8

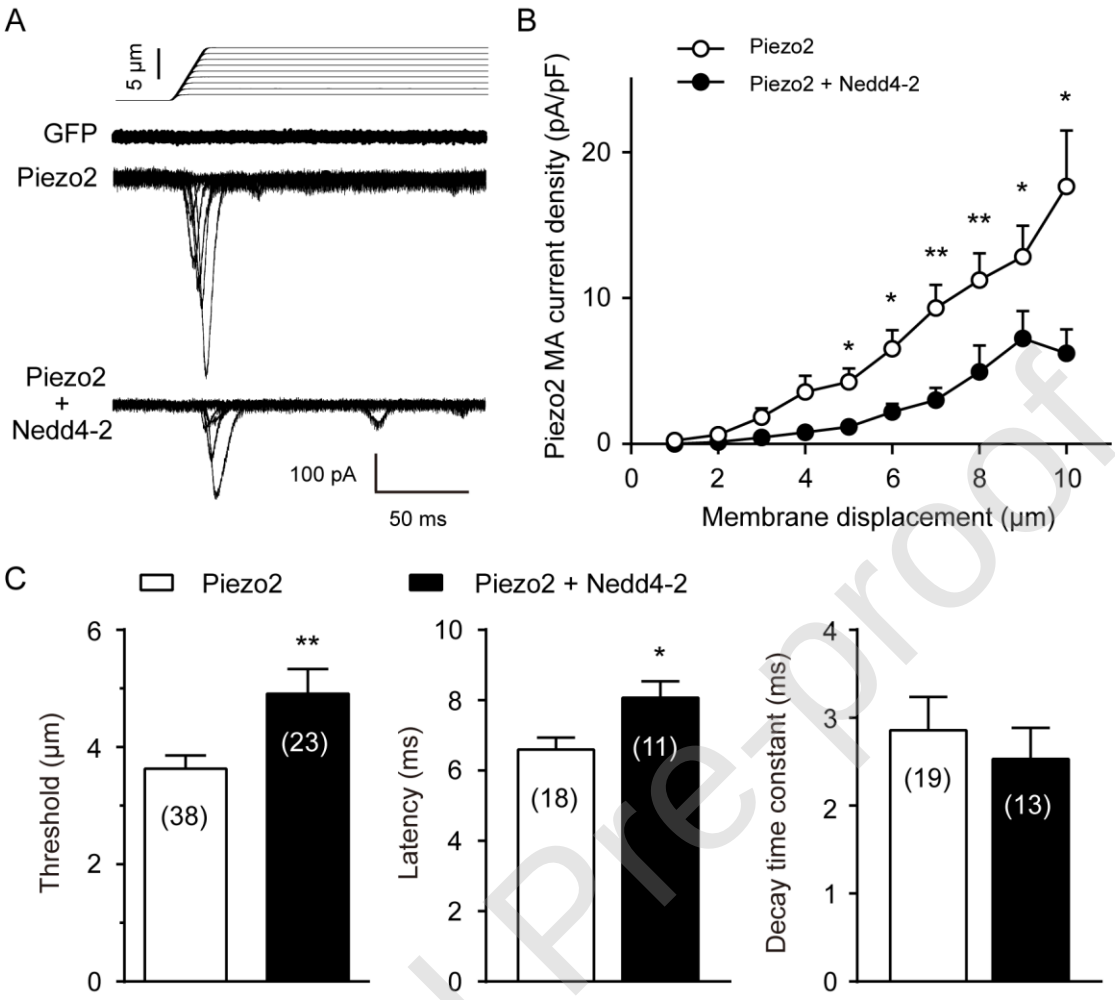


**Fig 8. Nedd4-2 downregulated Piezo2 MA currents in HEK 293T cells.** (A) Representative recording of Piezo2 MA currents in HEK 293T cells who were transfected with GFP (upper), Piezo2 (middle) and Nedd4-2 + Piezo2 (bottom) plasmid. The Piezo2 MA currents were evoked by a stimulus probe with a series of poking displacement steps (top). (B) Summary data of Piezo2 MA current densities with different displacement distances. Empty circles, Piezo2 alone; Solid circles, Nedd4-2 with Piezo2. (C) Summary of the threshold (left), activation latency (middle) for evoking Piezo2 MA currents and the decay time constant (right) of Piezo2 MA

currents. The threshold was significantly increased from 3.6 ± 0.2 μm (Piezo2 alone) to 4.9 ± 0.4 μm (Piezo2 + Nedd4-2). The latency was also elevated to 8.1 ± 0.5 ms for Piezo2 + Nedd4-2 group from 6.6 ± 0.3 ms for Piezo2 group. The decay times were not changed by Nedd4-2 (Piezo2 alone: 2.9 ± 0.4 ms; Piezo2 + Nedd4-2: 2.5 ± 0.4 ms). Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars indicate SEM.