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Original Articles

Deep electroacupuncture of neurogenic spots attenuates immobilization stress-induced acute hypertension in rats

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ABSTRACT

Background: Our previous studies proved that neurogenic inflammatory spots (or neurogenic spots) have the same physiological features as acupuncture points and that neurogenic spot stimulation generates therapeutic effects in various animal models. However, it is unclear how deeply the neurogenic spots should be stimulated to generate therapeutic effects.

Methods: The effects of acupuncture at various needle depths below the neurogenic spot were examined in a rat immobilization stress-induced hypertension (IMH) model. Electroacupuncture was applied to a neurogenic spot at depths of 1, 2, or 3 mm using a concentric bipolar electrode.

Results: Electrical stimulation of the neurogenic spot at a 3-mm depth most effectively lowered blood pressure compared with controls and stimulation at 1- and 2-mm depths, which was inhibited by pretreatment with a local anesthetic lidocaine. Electrical stimulation of the neurogenic spot or injection of substance P (SP) at a 3-mm depth significantly excited the rostral ventrolateral medulla (rVLM) compared with superficial stimulation. Electrical stimulation applied at a 3-mm depth on neurogenic spots dominantly caused c-fos expression from rVLM and ventrolateral periaqueductal gray (vlPAG) in IMH rats. Pretreatment with resiniferatoxin (RTX) injection into the neurogenic spot to ablate SP or calcitonin gene-related peptide (CGRP) prevented the effects of 3-mm neurogenic spot stimulation on blood pressure in IMH rats. Conversely, artificial injection of SP or CGRP generated anti-hypertensive effects in IMH rats.

Conclusion: Our data suggest that neurogenic spot stimulation at a 3-mm depth generated anti-hypertensive effects through the local release of SP and CGRP and activation of rVLM and vlPAG.

1. Introduction

Acupuncture, a therapeutic method of traditional medicine, has been used to treat a variety of disorders for centuries. Based on acupuncture theory, there are approximately 360 acupuncture points (or acupoints), most of which lie along the channels connecting the body's surface to internal organs. Each acupoint communicates with internal organs and reflects the status of the internal organs.[1,2] Additionally, internal disorders can be treated by stimulating the acupoints.[1] In support of this, we and others have proven that acupoints become more sensitive under certain pathological conditions of visceral organs and that stimulation of the acupoints can relieve the symptoms of the associated visceral organs.[3,4]

For acupuncture treatment, thin needles are inserted into acupoints, which are specific but poorly defined sites on or under the skin. The depth for insertion of an acupuncture needle is considered the key factor influencing the effect of acupuncture; however, the evidence is limited. The depth of needle penetration for targeting invisible acupoints under the skin is most commonly determined as described in the acupuncture atlas and reference books.[1,2] On the other hand, Hui et al. suggested that for effective acupuncture treatment, the depth of needle penetration can be determined by the needling sensation (i.e., pricking, burning, tingling

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or numbing sensation) or resistance felt by the practitioner.[5] Previous studies have shown that deep needling at the segmental muscle was more effective than shallow insertion in patients with chronic low back pain.[6,7] However, the anatomical depth of acupoints under the skin and the site of needle penetration for effective acupuncture have not been identified scientifically.

Although acupoints have long been thought to be anatomically invisible, our previous studies have suggested that acupoints can be identified as neurogenic inflammatory spots (neurogenic spots) on the skin, which are caused by the convergence of afferent information of somatic afferents and the visceral organs and can be detected by intravenously administered Evans blue dye (EBD).[4,8] Our previous studies have also shown that neurogenic spots share the same physiological features as acupoints: neurogenic spots are found most frequently in the same anatomical locations as traditional acupoints, reveal high electrical conductance and mechanical hypersensitivity, and can produce acupuncture effects on stimulation.[4,9] In acupuncture practice, it is accepted that the important step for effective treatment is to identify the hypersensitive (or sensitized) acupoints which show tenderness or hyperalgesia under certain pathological conditions. Stimulation of the hypersensitive acupoints can effectively relieve the pathological conditions.[10,11] Our previous studies proved that stimulation of most commonly used acupoints, but without neurogenic inflammation, did not produce the therapeutic effects.[4,9] Thus, applying the method used for visibly identifiable neurogenic spots may make it possible to estimate the location of acupoints under the skin and the site of effective needle penetration.

The present study used the rat model of immobilizations stress-induced hypertension (IMH) to determine (1) which area under the skin shows neurogenic inflammation, (2) which site under neurogenic inflammatory skin produces acupuncture-like effects on hypertension when stimulated, (3) which site under neurogenic inflammatory skin activates the rostral ventrolateral medulla (rVLM) or ventrolateral periaqueductal gray (vlPAG), which are known to be important for sensory modulation of blood pressure,[12,13] and (4) whether the acupuncture effects of neurogenic spots are associated with local release of the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) which act as potent vasodilators.[8]

2. Methods

2.1. Animals

All experiments were performed with male Sprague Dawley rats (8–11 weeks old; Hyochang, Seoul, Korea) weighing 240–350 g. The animals were housed under a constant temperature (24±2 °C) and a 12-hour light-dark cycle with free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Daegu Haany University and Yonsei University and conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Chemicals

EBD (1 ml/kg; 50 mg/ml saline; Sigma-Aldrich, USA), resiniferatoxin (RTX; 100 μg/ml vehicle consisting of 0.3 % Tween 80, 10 % DMSO and saline; 10 μl/site; Sigma-Aldrich, USA),[8] human CGRP (a 37-amino acid peptide; 16 pmol/10 μl saline, 10 μl/site; Bachem, USA),[14] SP acetate salt hydrate (0.5 mg/ml saline, 10 μl/site; Sigma-Aldrich, USA),[9] and lidocaine (2 %, 10 μl/site; Huons Pharm, Korea) were used.

2.3. Immobilization stress-induced hypertension and measurement of systolic blood pressure

Hypertension was induced by immobilization with a cone-shaped polyethylene bag, as performed previously.[4] The immobilization was kept over 2 h after initiation of restraint. Systolic blood pressure was measured by a non-invasive computerized tail-cuff system (Model 47, IITC Inc., USA). Each rat was briefly prewarmed in a 32 °C chamber, and an occluding cuff and a pneumatic pulse transducer were placed on the base of the tail. A programmed electrosphygmomanometer (Narco Bio-Systems Inc., USA) was inflated and deflated automatically, and the tail-cuff signals from the transducer were automatically collected every 10 min using an IITC apparatus (Model 47, IITC Inc., USA). The mean of two readings was used for each blood pressure measurement, as described previously.[4, 15]

2.4. Detection of neurogenic inflammatory spots in the skin by EBD injection

Cutaneous neurogenic inflammation was detected using intravenous EBD (50 mg/kg) in rats, as described previously.[4] While the rat was restrained by a cone-shaped bag, the distal portion of the tail was dipped into warm water, and EBD was then injected via the tail vein using a syringe with a 26-gauge needle. Skin color changes were observed up to 2 h after the injection, and the blue-dyed spots on the skin were photographed and compared with a human acupoint chart based on the transpositional method, whereby acupoints are located on the surface of animal skin corresponding to the anatomical sites of human acupoints.[16]

2.5. Electroacupuncture stimulation of neurogenic spots at various depths in IMH rats

Rats were lightly anesthetized with 1.0 %–1.2 % isoflurane, and a concentric bipolar electrode (CBBPE75, FHC, USA) was inserted into a neurogenic spot on the wrist at a depth of 0 mm, 1 mm, 2 mm, or 3 mm. Then, the neurogenic spot was electrically stimulated at various depths according to the group for 10 min at a 3-Hz frequency (0.5 mA, 0.1 ms, triangular pulses) using an electro-stimulator (Han-il Co, Korea). Since previous studies showed that electrical stimulation at low frequency (i.e., 3 Hz) and subthreshold intensity, just below a detectable muscle twitch, effectively reduces hypertension,[4,17] the stimulation condition (3-Hz frequency, 0.5 mA, 0.1 ms triangular pulse, 10 min) was chosen. In the 0-mm group, the electrode was placed on the skin surface and the electrical stimulation was delivered without penetrating the skin. Immediately after terminating the electrical stimulation, the electrode was withdrawn, the isoflurane anesthetic administration was discontinued, and the rat returned to the computerized tail-cuff system for measuring blood pressure. The blood pressure was monitored for up to 120 min after electrical stimulation of the neurogenic spot.

2.6. *In-vivo* extracellular recording of rVLM neurons and electrolytic lesions of rVLM

The rats were anesthetized with urethane (1.5 g/kg), and a carbon-fiber glass microelectrode (0.4–1.2 M Ω, Carbostar-1, Kation Scientific, USA) was placed into the rVLM (stereotaxic coordinate: anterior, −11.96~−12.80 mm; lateral, +1.9~+2.4 mm; deep, 9.8~10.6 mm). Single-unit activity was discriminated from noise, binned at 1-sec intervals, and analyzed via a CED 1401 Micro3 device and Spike2 software (Cambridge Electronic Design, UK). After recording a stable baseline for at least 10 min, a bipolar concentric electrode was inserted into a neurogenic spot near the wrist at a depth of 1 mm or 3 mm, and the responses following electrical stimulation for 2 min were recorded for at least 15 min after stimulation. In another set of animals (n = 5), SP was injected into the neurogenic spot and the responses were recorded.

Electrolytic lesions of rVLM were made before electrical stimulation at a depth of 3 mm, as described previously.[18] Briefly, under isoflurane anesthesia a tungsten electrode, insulated except for 0.5 mm at the tip, was inserted in rVLM. The bilateral electrolytic lesions of rVLM were made by passing ±0.35 mA positive current for 8 s.

2.7. Immunohistochemistry for c-Fos expression in the vlPAG and rVLM of IMH rats

Sixty minutes after immobilization in another set of animals, the rats were sacrificed and perfused with paraformaldehyde. The brains were taken out, post-fixed, cryo-protected, and cryo-sectioned into 30-μm slices. The brain slices were incubated with anti-c-Fos rabbit polyclonal antibodies (1:500) followed by donkey anti-rabbit IgG antibodies (Alexa Fluor 488, USA; 1:500). The slices were then mounted on gelatin-coated slides, photographed, and examined under a confocal laser scanning microscope (LSM700; Carl Zeiss, Germany). The numbers of c-Fos-positive cells in rVLM and vlPAG were blindly counted, and 5–7 slices of brain per animal were analyzed.

2.8. Immunohistochemistry for CGRP or SP expression in the skin after RTX injection into neurogenic spots

The RTX was injected based on previous studies showing that RTX (0.01 %) effectively blocks C/Aδ-fiber or thermal pain over 72 h.[19,20] Seventy-two hours after the injection of RTX into the neurogenic spot near the wrist, skin was taken from the wrists of IMH rats (n = 6) and naïve rats (n = 6). The samples were paraffin-embedded, sectioned (5 μm), and then mounted on gelatin-coated slides. The slides were deparaffinized with a series of different concentrations of xylene or ethanol and then rinsed under cold tap water. The slides were washed in phosphate-buffered saline (PBS) three times (10 min/wash) and incubated in a blocking solution (5 % NGS, 0.3 % Triton X-100, 0.1 M PBS) for 1 hour at 4 °C followed by incubation with anti-CGRP mouse antibody (1:1000; Chemicon, USA) or anti-SP mouse antibody (1:400; Bioss, USA). The sections were then incubated with secondary antibodies (1:1000, Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody; 1:1000, Alexa Fluor 594-conjugated donkey anti-mouse IgG antibody; Thermo Scientific, USA). Skin images were obtained from three sections of each animal with a confocal laser scanning microscope (LSM700, Carl Zeiss, Germany) and quantified using ImageJ software. Pixels with a green fluorescence intensity greater than the cut-off value (100) were counted to quantify positive staining. Data were expressed as the number of positive pixels within a field area of 1280 × 1024 pixels.

2.9. Statistical analysis

Data were presented as the mean ± standard error of the mean and analyzed by one- or two-way measurement analysis of variance (ANOVA), one- or two-way repeated ANOVA, and then *post hoc* testing using the Tukey method or unpaired t-test, as appropriate. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Attenuation of immobilization stress-induced hypertension by superficial or deep electrical stimulation of neurogenic spots

When EBD was administered intravenously, cutaneous neurogenic inflammatory sites (neurogenic spots) began to appear, commonly at

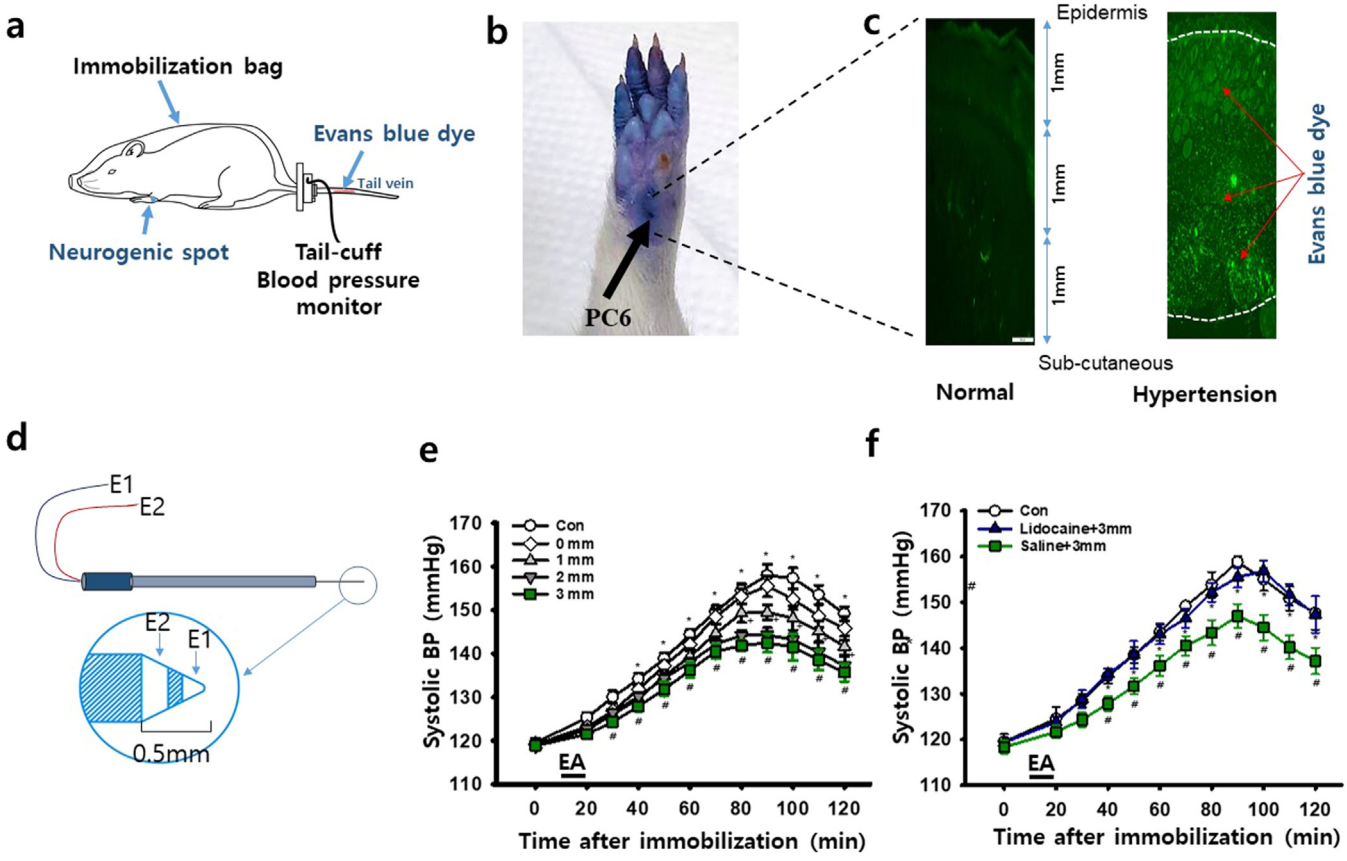

Fig 1. Attenuation of IMH by superficial or deep electrical stimulation of neurogenic spots
(a) Schematics for immobilization and blood pressure measurement in rats. Evans blue dye was injected via the tail vein. (b) A representative neurogenic spot on the wrist. PC6, Pericardium 6 acupoint. (c) Histology of neurogenic spot in the wrist skin. (d) Concentric bipolar microelectrode used for neurogenic spot stimulation. (e) Effect of electrical stimulation of neurogenic spots at different depths on systolic blood pressure in IMH rats (n = 6 rats/group; $^{\#}p < 0.05$, 3 mm vs. Con; $^{*}p < 0.05$, 2 mm vs. Con; $^{+}p < 0.05$, 3 mm vs. 1 mm). (f) Effect of pretreatment with lidocaine on hypertension suppression by electrical neurogenic spot stimulation at a 3-mm depth (n = 6 rats/group; $\#p < 0.05$ vs. Con; $^{*}p < 0.05$, Lidocaine+3 mm vs. Saline+3 mm). Con, control (IMH rats).

the Pericardium 6 acupoint (PC6), within 1 min after IMH (Fig. 1a, b). To identify the location of neurogenic inflammation under the skin, the blue-colored skin stained by EBD was taken out, post-fixed, cryo-protected, cryo-sectioned into 30-μm-thick slices and examined under a green epifluorescence microscope. On microscopic examination, the blue dots displayed a broad diffusion of EBD, from the epidermis (shallow) to subcutaneous (deep) tissue (approx. 3 mm; Fig. 1c), compared with normal tissue.

To identify which area of the neurogenic spot on or under the skin generates therapeutic effects, we used a concentric bipolar electrode for focal electrical stimulation (Fig. 1d), and the needle was inserted into a wrist neurogenic spot (left or right) at a depth of 0, 1, or 3 mm and stimulated electrically in the IMH rats. After initiating immobilization, systolic blood pressure gradually increased over the next 2 h in the IMH rats, reaching at hypertensive levels over systolic blood pressure of 140 mmHg in 1 hour[21] (Fig. 1e). Electrical stimulation of the neurogenic spots at various depths attenuated the development of hypertension in a depth-dependent manner, and the effect of 3-mm stimulation was greater than that of superficial stimulation (Fig. 1e; $n = 6$ rats; two-way repeated ANOVA, $F_{(48,325)}$=1.844; $p < 0.05$, 3 mm vs. control; $p < 0.05$, 2 mm vs. control; $p < 0.05$, 3 mm vs. 1 mm). To identify whether deep afferent nerve fibers mediated the effects, a local anesthetic (lidocaine) was injected into the neurogenic spot at a depth of 3 mm 10 min before electroacupuncture. Although electrical stimulation of the neurogenic spot at a 3-mm depth effectively reduced the development of hypertension following immobilization, the effects were blocked by the injection of lidocaine into the neurogenic spot at a 3-mm depth prior to electroacupuncture (Fig. 1f; $n = 6$ rats; two-way repeated ANOVA, $F_{(22,110)}$=8.224, $p < 0.05$; $p < 0.05$, saline+3 mm vs. control; $p < 0.05$, lidocaine+3 mm vs. saline+3 mm).

3.2. Excitation of rVLM neurons following superficial or deep electrical stimulation of neurogenic spot in IMH rats

To explore which depths of neurogenic spot stimulation affect the excitability of rVLM neurons, a concentric bipolar electrode was inserted into the neurogenic spot at a 1-mm or 3-mm depth and electrically stimulated for 2 min. Electrical stimulation of the neurogenic spot at a 1-mm depth increased the excitability of rVLM neurons by 120 %±5.21 % from baseline within 5 min after stimulation, and the levels returned to baseline within 10 min after stimulation. Neurogenic spot stimulation at a 3-mm depth evoked neuronal excitation of rVLM up to 150 %±8.02 % from baseline within 5 min after stimulation, and the effects lasted up to 10 min after stimulation (Fig. 2b, d; $n = 6$ rats; one-way repeated ANOVA, $F_{(3,10)}$=31.296, $p < 0.05$; $p < 0.05$ vs. baseline). When data for the 5 min after stimulation were analyzed, stimulation of the neurogenic spot at a 3-mm depth significantly enhanced the excitability of rVLM compared with stimulation at a 1-mm depth. To further confirm the activation of rVLM or vlPAG following neurogenic spot stimulation in IMH rats, a concentric bipolar electrode was inserted into either the neurogenic spot or a non-neurogenic spot 3–5 mm away from the neurogenic spot at a 1-mm or 3-mm depth and electrically stimulated the spot for 10 min. Eighty minutes after stimulation, c-Fos expression in rVLM or vlPAG was evaluated. IMH rats showed enhanced expression of c-Fos in rVLM or vlPAG compared with normal rats. Similar to the results of *in-vivo* extracellular recording, electrical stimulation of neurogenic spots

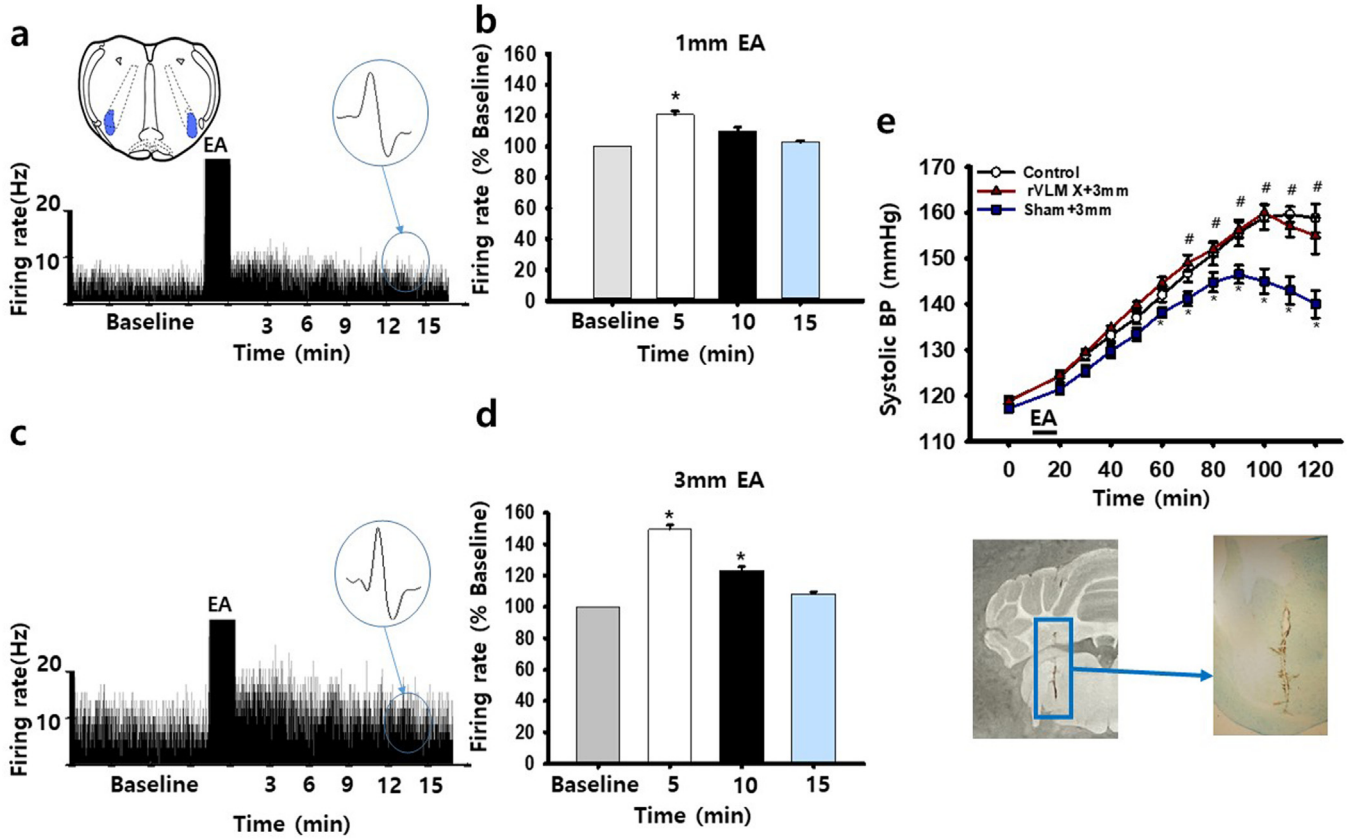

Fig 2. Excitation of rVLM neurons following superficial or deep electrical stimulation of neurogenic spots
(a, b) Effect of electrical neurogenic spot stimulation at a 1-mm depth on the neuronal activity of rVLM. 1-s bin spike rate counts recorded in rat rVLM (a), and 5-min average data before and after stimulation (b; $n = 6$ rats/group; $^{*}p < 0.05$ vs. baseline). (c, d) Effect of electrical neurogenic spot stimulation at a 3-mm depth on the neuronal activity of rVLM. 1-s bin spike rate counts recorded in rat rVLM (c), and 5-min average data before and after stimulation (d; $n = 6$ rats/group; $^{*}p < 0.05$ vs. baseline). (e) Effect of bilateral electrolytic lesion of rVLM on hypertension suppression by electrical neurogenic spot stimulation at a 3-mm depth ($n = 5$ rats/group; $^{*}p < 0.05$ vs. Control; $^{\#}p < 0.05$, rVLM X vs. Sham+3 mml). rVLM X denotes rVLM lesion. Pictures showing electrolytic lesion at rVLM (below).

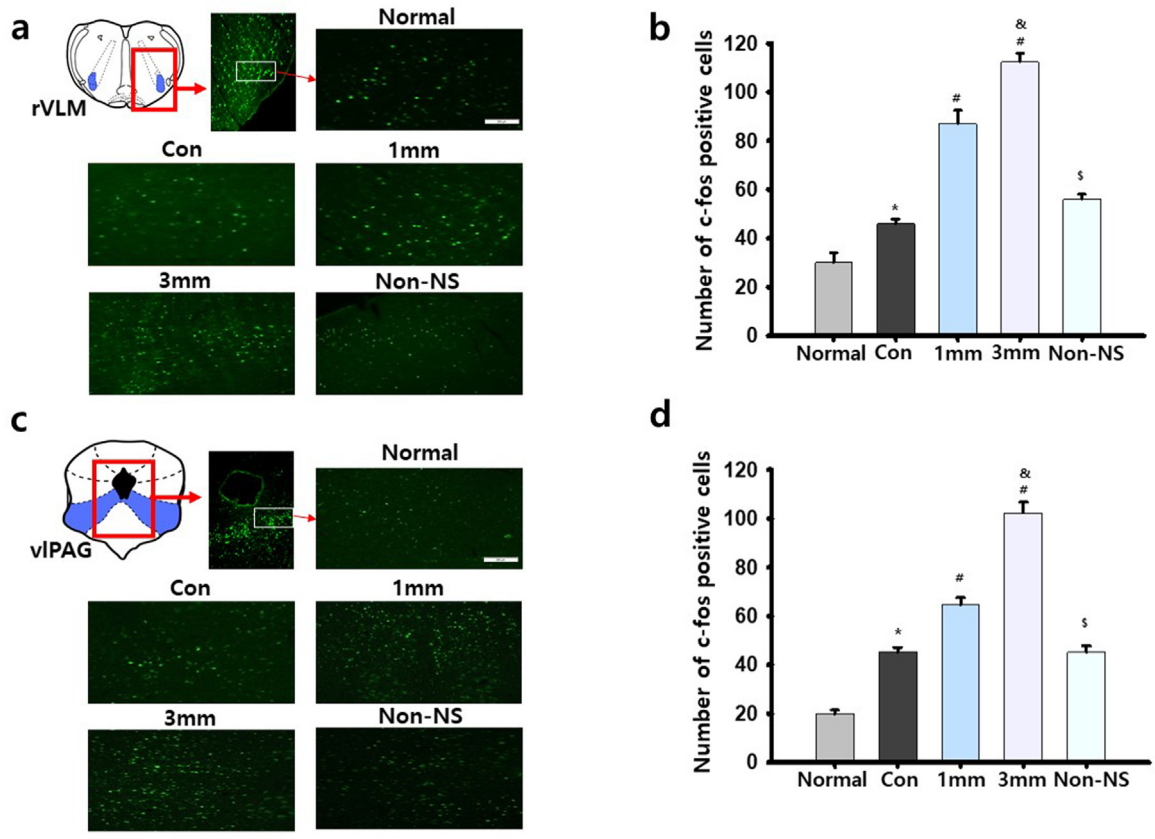

Fig 3. Increased c-Fos expression in rVLM or vlPAG following neurogenic spot stimulation at a 1-mm or 3-mm depth in IMH rats
(a, b) c-Fos expression in rVLM after electrical stimulation of neurogenic spots. Representative pictures (a), and the mean number of c-Fos-positive cells (b). Normal, normal rats; Con, IMH rats; 1 mm, stimulation of neurogenic spots at a 1-mm depth; 3 mm, stimulation of neurogenic spots at a 3-mm depth; Non-NS, non-neurogenic spot 3–5 mm away from the neurogenic spot (n = 6 rats/group). $^{*}p < 0.05$ vs. normal; $^{\#}p < 0.05$ vs. Con; $^{\&}p < 0.05$ vs. 1 mm; $^{\$}p < 0.05$ vs. 3 mm. Bar=200 μm. (c, d) c-Fos expression in vlPAG after electrical stimulation at the neurogenic spot. Representative pictures (c), and the mean number of c-Fos-positive cells (d). Normal, normal rats; Con, IMH rats; 1 mm, stimulation of neurogenic spots at a 1-mm depth; 3 mm, stimulation of neurogenic spots at a 3-mm depth; Non-NS, non-neurogenic spot 3–5 mm away from the neurogenic spot (n = 6 rats/group). $^{*}p < 0.05$ vs. normal; $^{\#}p < 0.05$ vs. Con; $^{\&}p < 0.05$ vs. 1 mm; $^{\$}p < 0.05$ vs. 3 mm. Bar=200 μm.

at a 3-mm depth significantly increased the number of c-Fos-positive cells compared with the normal, control (immobilization only), and 1-mm groups (Fig. 3b, d; n = 6 rats; one-way ANOVA, $F_{(4,25)}$=69.663, $p < 0.05$; $p < 0.05$ vs. normal; $p < 0.05$ vs. control; $p < 0.05$ vs. 1 mm; $p < 0.05$ vs. 3 mm). To see whether rVLM mediates the inhibitory effect of stimulation at a 3-mm depth on hypertension, another set of animals (n = 15) was randomly divided into the following groups; Control (n = 5), immobilization stress only; rVLM X + 3 mm (n = 5), immobilization stress + electrical stimulation at a 3-mm depth in the rats with electrolytic lesion of bilateral rVLM; Sham+3 mm (n = 5), immobilization stress + electrical stimulation at a 3-mm depth in the rats received sham surgery. Electrolytic lesions of LHb were made bilaterally before induction of immobilization stress. Electrical stimulation of neurogenic spot at a 3-mm depth reduced the development of hypertension and these effects were blocked by electrolytic lesions of bilateral rVLM prior to the experiment.

3.3. Small-diameter afferent fiber mediation of the anti-hypertensive effect of deep electrical stimulation of neurogenic spots in IMH rats

To explore whether the anti-hypertensive effects of deep electrical stimulation of neurogenic spots are associated with the local release of neuropeptides SP and CGRP, the potent capsaicin analog RTX was injected into the neurogenic spot 3 days prior to the experiments. Electrical stimulation of the neurogenic spot attenuated the development of hypertension in IMH rats (vehicle), but this result was prevented by pretreatment with RTX prior to the neurogenic spot stimulation (Fig. 4a; n = 6 rats; two-way repeated ANOVA, $F_{(24,233)}$=2.501, $p < 0.001$; $p < 0.05$ vs. control; $p < 0.05$, vehicle vs. RTX). To confirm the role of SP and CGRP in neurogenic spot in the anti-hypertensive effects of neurogenic spot stimulation, SP or CGRP was injected into the neurogenic spot and systolic blood pressure was monitored for up to 120 min after injection. Artificial injection of SP or CGRP in the neurogenic spot prevented the development of hypertension in IMH rats (Fig. 4d; n = 6 rats; two-way repeated ANOVA, $F_{(24,234)}$=3.226, $p < 0.001$; $p < 0.05$, SP vs. control; $p < 0.05$, CGRP vs. control). To confirm whether RTX injection into neurogenic spots depletes the sensory nerves of their mediators, including SP and CGRP, the expression of SP or CGRP was estimated by immunohistochemistry in another set of animals. Green fluorescence intensity levels for SP and CGRP were significantly lower in the neurogenic spots of RTX-treated rats than in those of normal rats (Fig. 4b, c; n = 6 rats; $p < 0.05$ vs. normal).

When SP was injected at a 3-mm depth into a neurogenic spot, in vivo extracellular single-unit recordings showed that the firing rates of rVLM increased by approximately 140 % from baseline compared with the baseline before injection (Fig. 5).

4. Discussion

The present study showed that localized neurogenic inflammation appeared at acupoints near the wrist, such as PC6, in IMH rats. Electrical stimulation of the neurogenic spot at various depths by a con-

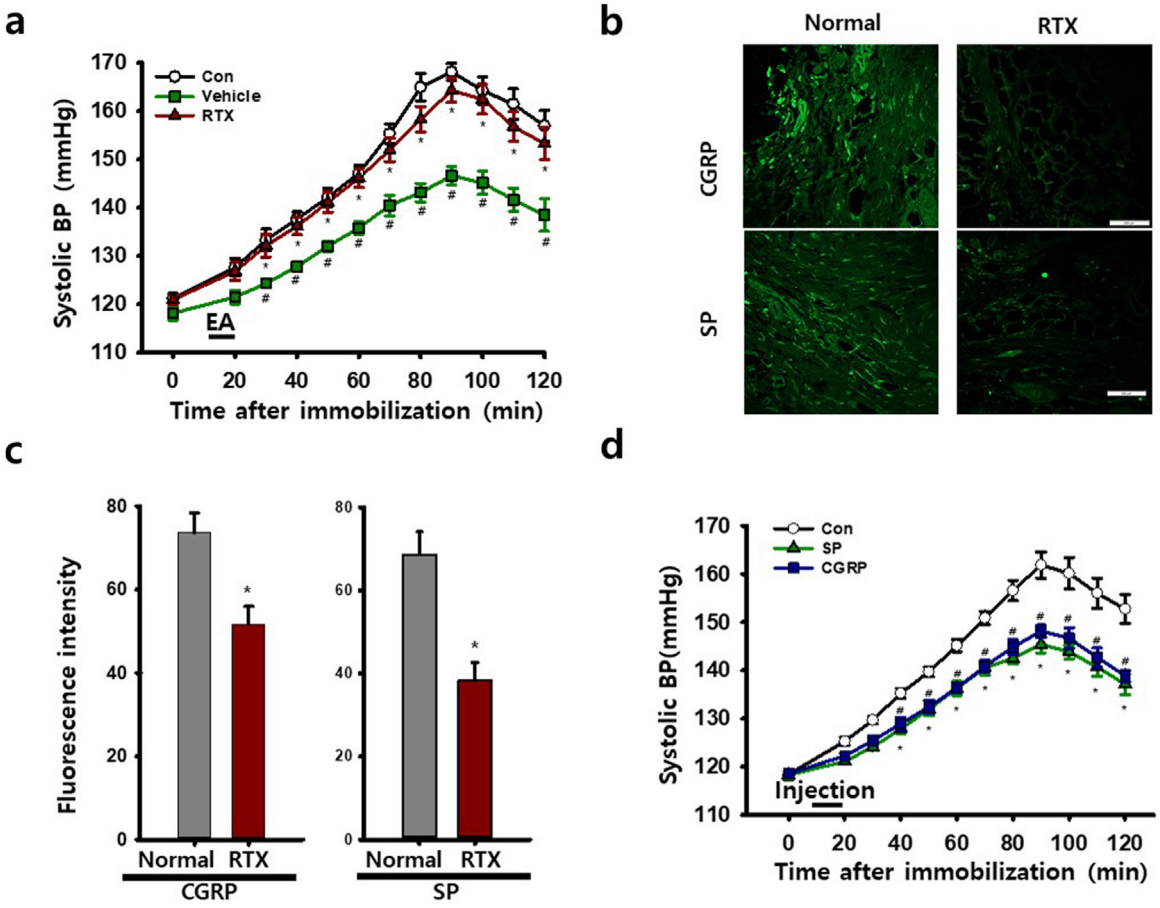

Fig 4. Mediation of neuropeptides SP and CGRP in the anti-hypertensive effect of deep electrical stimulation of neurogenic spots in IMH rats
(a) Effect of RTX injection into neurogenic spots on the suppression of hypertension by deep neurogenic spot stimulation. RTX was injected into the neurogenic spots 72 h before the experiment ($n = 6$ rats/group; $^{*}p < 0.05$, RTX vs. vehicle (saline)). (b, c) Immunohistochemistry for SP or CGRP at the neurogenic spots 72 h after RTX injection into the neurogenic spots ($n = 6$ rats/group). $^{*}p < 0.05$ vs. normal. (d) Effect of CGRP or SP administration into neurogenic spots on systemic blood pressure in IMH rats ($n = 6$ rats/group; $^{*}p < 0.05$ vs. Con; $^{\#}p < 0.05$ vs. Con).

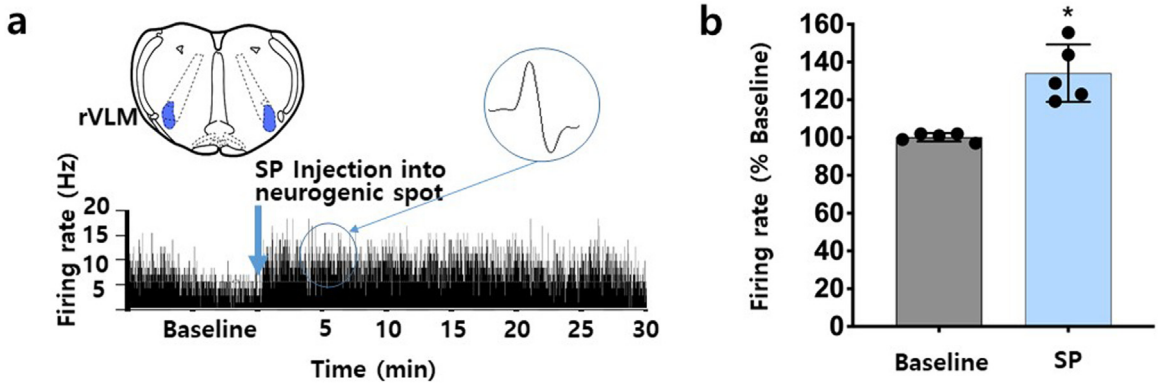

Fig 5. Excitation of rVLM neurons following SP injection into neurogenic spot
(a, b) Effect of substance P (SP) injection into neurogenic spot at a 3-mm depth on the neuronal activity of rVLM. 1-s bin spike rate counts recorded in rat rVLM (a), and 5-min average data before and after SP injection (b; $n = 5$ rats). $^{*}p < 0.05$ vs. Baseline (the values before injection).

centric bipolar electrode attenuated the development of hypertension in IMH rats in a depth-dependent manner. The effects were blocked by pretreatment with a lidocaine injection into the neurogenic spot at a 3-mm depth prior to stimulation. Deep stimulation of the neurogenic spot significantly enhanced the activity of rVLM and vlPAG neurons in IMH rats compared with superficial stimulation. Depletion of sensory nerves, including SP and CGRP, by RTX treatment ablated the effect of neurogenic spot deep stimulation on hypertension. Artificial injection of SP or CGRP produced anti-hypertensive effects in IMH rats. These findings suggest that the anti-hypertensive effects of deep stimulation of neurogenic spots can be attributed to the local release of SP and CGRP in the neurogenic spots and activation of rVLM or vlPAG.

Noxious signals from viscera frequently produce referred pain at somatotopically distinct body surfaces that are generally generated by viscerosomatic convergence at spinal cord segments.[22] Neurogenic inflammatory spots are found in the somatic area of referred pain and

can easily be visualized by intravenously injecting EBD.[4,22] Our previous studies have shown scientific evidence regarding the close relationship between traditional acupoints and neurogenic inflammatory spots.[4,8,9,23–25] In brief, most neurogenic inflammatory spots are found in the same anatomical location as traditional acupoints and reveal the same physiological features as acupoints (i.e., high electrical conductance or mechanical hypersensitivity). We have suggested that traditional acupoints may be identical to neurogenic inflammatory spots occurring on the skin that are associated with visceral disorders. The hypothesis is also supported by other studies.[10,26,27] In our previous study,[4] neurogenic spots in IMH rats were found in the acupoints such as PC6, PC7, HT7, SI3, PC4, LU9, LR3 and BL54 (see Supplementary Table S1 in reference[4]). Approximately 70 % of those spots matched with the acupoints associated with cardiovascular disorders. While most of the neurogenic spots in the hypertensive rats were found in the dermatome of the same spinal segments (C8-T2)[4] that innervate the heart,[28] some spots were also observed over hindlimb such as LR3, BL54 and other non-acupoints, which may be due to the potential influence of immobilization stress on multiple organs. Consistent with our previous study, the present study revealed neurogenic spots most frequently in the acupoints of PC6 over the skin of the wrist. Our previous study also showed that dorsal root ganglions from the cardiac area labeled with a retrograde tracer were double-labeled with another retrograde tracer injected into the neurogenic spots over the wrist in rats and that the neurogenic spots over the wrist were linked to neurogenic spots on the skin to the heart.[4] The present study showed that when a concentric bipolar electrode was inserted into neurogenic spot at various depths, electrical stimulation of the spots attenuated the development of hypertension in a depth-dependent manner, indicating the therapeutic effects of neurogenic spot stimulation on hypertension. Furthermore, the therapeutic effects of deep electrical stimulation of neurogenic spots on hypertension were abolished by pretreatment with lidocaine, a local anesthetic. This finding suggests that deep sensory afferents mediate the effects of neurogenic spot stimulation on hypertension. Previous studies have suggested that internal organ–related acupoints are identical to neurogenic spots that occur on the skin and are associated with visceral disorders.[4,9,11,23] This study showed that deep stimulation of neurogenic spots was more effective than superficial stimulation, which suggests that hypertension-related acupoints, such as PC6, may be located in the deep tissue under the skin rather than in the superficial tissue.

The present study showed that stimulation of neurogenic spots near the wrist activated rVLM and vlPAG. This finding is consistent with previous studies showing that stimulation of neurogenic spots or electroacupuncture at acupoints near the wrist induced c-Fos expression in the rVLM and PAG,[25,29] central sites for the regulation of blood pressure.[30,31] A previous study demonstrated that electrical stimulation of the median nerve over the wrist activates the arcuate nucleus, thereby regulating blood pressure via cardiovascular sympathetic neurons in the vlPAG and rVLM sites.[32] The activity of neurons in the rVLM is regulated by the excitation or inhibition of receptors at different sites, which impacts the perception of pressure and pain stimuli.[33] Activation of vlPAG is mediated by the presympathetic neurons within the rVLM.[31,34] Therefore, we suggest that the inhibition of hypertension by neurogenic spot stimulation is regulated by the activation of neurons in the rVLM and vlPAG regions. Furthermore, we showed that deep stimulation of neurogenic spots significantly enhanced the activity of rVLM and vlPAG neurons—which generated anti-hypertensive effects—compared with superficial stimulation and controls. This result suggests that deep tissue afferents mediate the anti-hypertensive effects of stimulation of neurogenic spots, whereas cutaneous afferents do not. On the other hand, it is known that activation of rVLM causes excitatory responses characterized by an increase in blood pressure.[35] In the present study, it seems paradoxical that neurogenic spot stimulation lowered blood pressure while increasing the neuronal activity of rVLM in IMH rats. Previous studies have shown that acupuncture or neurogenic spot stimulation increases the neuronal activity of rVLM and reduces the elevated blood pressure via endogenous opioid mechanisms.[25,29] In previous studies, acupuncture at PC6 activated rVLM neurons and the most activated rVLM cells were present in close apposition to neuronal fibers containing enkephalin or β-endorphin.[29] Acupuncture increases the release of enkephalin and β-endorphin from the rVLM of rats[36] and can inhibit excitatory cardiovascular reflex responses through activation of opioid receptors in the rVLM.[37] Therefore, we assume that stimulation of the neurogenic spot near PC6 would increase endorphins and enkephalins acting through opioid receptors in the rVLM, inhibit sympathetic outflow and attenuate the development of hypertension in IMH rats, which needs to be explored in future research.

In the present study, ablation of CGRP and SP by RTX treatment prevented the anti-hypertensive effects of deep stimulation of neurogenic spots, and the artificial increase of CGRP or SP generated anti-hypertensive effects, suggesting the mediation of CGRP and SP in neurogenic spot–induced effects on hypertension. Immunohistochemistry revealed increased expression of the neuropeptides SP and CGRP in neurogenic spots. SP increases microvascular permeability and edema formation by activating neurokinin receptors, and CGRP stimulates CGRP1 receptors to dilate arterioles.[38] Our recent study showed that in rat models of hypertension or colitis, neurogenic spots over acupoints released CGRP and SP, causing neurogenic inflammation and plasma extravasation.[4,8] Our previous study has also suggested that elevated SP levels during neurogenic inflammation increase the responses of sensory afferents to acupoint needling and trigger acupuncture, which generates the inhibitory effects of acupuncture on hypertension.[9] In the present study, stimulation of deep afferents at neurogenic spots or artificial injection of CGRP attenuated the development of hypertension in IMH rats and deep injection of SP into neurogenic spot increased the excitability of rVLM. Taken together, these results may suggest that CGRP and SP play important roles in the anti-hypertensive effect of deep neurogenic spot stimulation.

In conclusion, deep stimulation of neurogenic spots attenuates the development of hypertension through activation of rVLM and vlPAG, and the effects are attributed to the local release of SP and CGRP in the deep tissue of neurogenic spots.

Author contributions

Conceptualization: H.Y.K. Data curation, Formal analysis, Investigation: C.Z., H.B.J, D.A., S.C., Y.R., H.K.K, B.H.L, X.G., Y.F., B.H.L. Funding acquisition: H.Y.K. Writing: C.Z., H.Y.K.

Conflict of interest

The authors declare no conflict of interest.

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Ethical statement

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Daegu Haany University and Yonsei University.

Data availability

The data presented in this study are available within the article.

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