Microchannel Electrode Stimulation of Deep Peroneal Nerve Fascicles Induced Mean Arterial Depressor Response in Hypertensive Rats

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Hypertension (HTN) affects over 1 billion people in the world, and while most are treated effectively with pharmacological regimens, 10–30% of them do not show a beneficial response. Electrical stimulation of the renal sympathetic, vagus and carotid sinus nerves has shown depressor effects and has been proposed as an alternative treatment for resistant hypertension (R-HTN). However, these nerves are heterogeneous in afferent/efferent composition, and their stimulation often results in unwanted side effects. We evaluated the possibility of eliciting a depressor response from stimulation of single fascicle of the somatic deep peroneal nerve (fDPN). A microchannel electrode array (μCEA) was used to stimulate the fDPN at low frequency, which induced a significant ($p ≤ 0.03$) transient reduction in mean arterial pressure (MAP) with no significant effects on heart rate. The depressor response was prolonged for several hours by extending the fDPN stimulation to 5 min, which induced significant reduction (17–25%) in MAP for up to 4 h. Immunofluorescence evaluation of the axonal marker, myelin, and active macrophages in the fDPN revealed no indication of nerve damage or overt inflammation in response to the procedure. This study provides evidence supporting the use of μCEA interfacing of small somatic nerve fascicles associated with cardiovascular relevant acupoints to induce significant reductions in MAP and opens the possibility of neuromodulation of small fascicles as an alternative strategy to treat R-HTN with minimal side effects. Further, the μCEA multielectrode array offers an effective tool for neuromodulation of small nerve fascicles, enabling a number of possible future medical bioelectronic applications.
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INTRODUCTION

Hypertension (HTN) affects over 1 billion people around the world (1). The current management of hypertension relies on thiazide-type diuretics, calcium channel blockers, angiotensin-converting enzyme inhibitors and/or angiotensin receptor blockers to maintain normal blood pressure and prevent myocardial infarction, stroke, renal failure and death (2).

However, appropriate dosing is challenging because of the high variability in the patient response, narrow therapeutically acceptable range, adverse reactions and toxicity (3). Moreover, approximately 10–30% of the affected population is resistant to pharmacological treatments, despite the use of combinatorial drug treatments and maximum doses (4,5). Individuals suffering from resistant hypertension (R-HTN) have high rates of cardiovascular complications and few treatment options. Unfortunately, some promising research candidates for the treatment of R-HTN such as renal artery denervation have failed to show efficacy (6,7). Therefore, there is much need for developing alternative methods for R-HTN regulation.

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Vagal nerve stimulation (VNS) was first proposed as a possible method for the treatment of HTN in the late 1990s, but reports of bradycardia and apnea side effects limited its application (8). Recent studies have shown that selective VNS using tripolar cuff electrode stimulation reduces blood pressure from 100 to 80 mmHg in normal rats with minimal side effects (that is, 5–10% in apnea) using 40 Hz, 1 mA and 0.3-ms pulse stimulation for 4 ms (135 pulses) (9). However, apnea increased to 15% when stimulation was applied at 50 Hz. Furthermore, whether this approach can achieve the same therapeutic effect in hypertensive animals was not investigated. In addition, vagal nerve is highly heterogeneous in axonal composition and side effects including cough, hoarseness and voice alteration, and paresthesias have been reported in humans (9,10). Thus, alternative peripheral nerves that can achieve regulation of HTN with significantly less side effects might offer a more appealing treatment for R-HTN.

A recent randomized blind clinical trial demonstrated that active traditional Chinese acupuncture significantly lowers blood pressure in HTN patients compared with sham acupuncture (11), and some report the effect of acupuncture and pharmacological treatment (12). While the working mechanism of this therapeutic effect is unknown, increasing evidence suggests that the acupuncture therapy benefit is based on neuromodulation of the underlying nervous system. Indeed, depressor responses of ~20 mmHg have been reported in hypertensive rats after stimulation of the sciatic nerve (13). Recently, it was shown that electropuncture on a fascicle of the sciatic nerve (the common peroneal nerve, which overlays the acupoint Zuzanli [ST36] and Shangjuxu [ST37]) significantly reduces blood pressure 10–18 mmHg without affecting heart rate (HR) (14).

We hypothesized that electrical stimulation of a fascicle of the somatic deep peroneal nerve (fDPN) provides an effective substrate to induce a significant depressor response in HTN with minimal
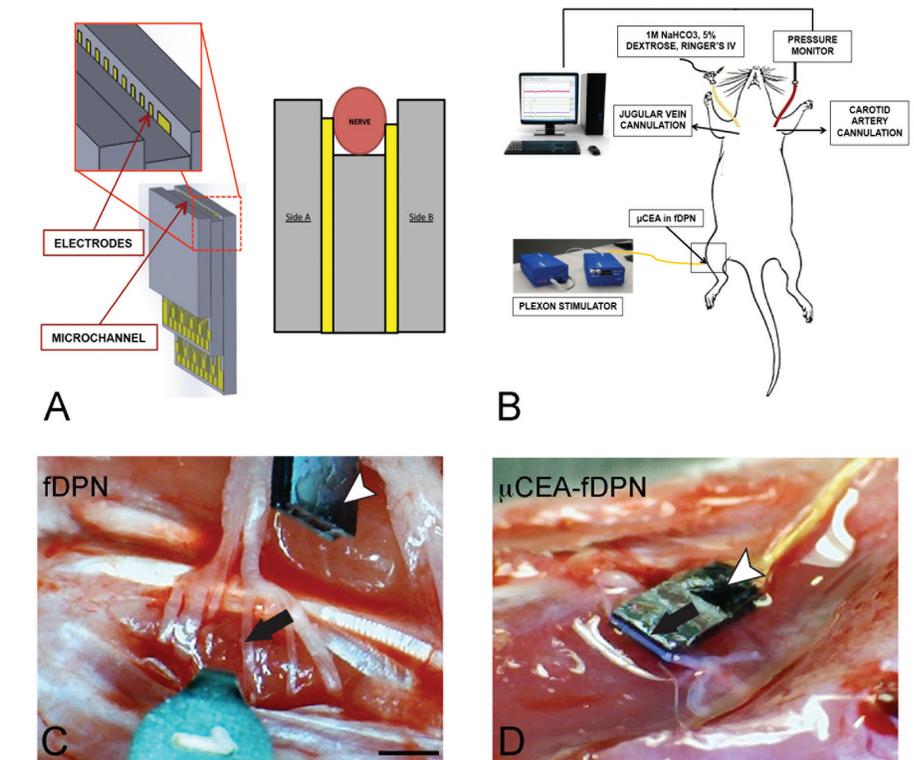
Figure 1. μCEA interfacing of the fDPN. (A) Schematic of the μCEA designed to interface small nerves (50–300 μm OD). (B) Illustration of the experimental animal setup. The fDPN was placed in the μCEA electrode, and the wire connected to a stimulator box and a computer interface. A catheter in the jugular vein was used to replenish liquids, and cannulation of the carotid artery allowed recording of MAP during fascicular stimulation. (C) Photograph showing the isolation of a single fDPN fascicle (black arrow). (D) Placement of the fDPN (black arrow) in the groove of the μCEA (arrowhead). Scale bar = 1 mm.

side effects. A microchannel electrode array (μCEA) specifically designed and fabricated for interfacing with small nerves (that is, 50–300 μm in diameter; autonomic nerve or nerve fascicles) was used to stimulate the fDPN (~150 μm in diameter) (15). We demonstrate that a significant reduction in mean arterial pressure (MAP) in hypertensive rats can be achieved through microelectrical stimulation of a somatic nerve fascicle for up to 4 h without significantly altering HR.

MATERIALS AND METHODS

Microchannel Electrode Array (μCEA)

Large diameter nerves such as the vagus (1–1.5 mm in a rat) are commonly interfaced with cuff electrodes. Because of their size and their tough epineurium,

they withstand electrode implantation procedures that include pulling and stretching the nerve. Conversely, small diameter nerves or fascicles have a thinner epineurium or only perineurium and thus are more susceptible to injury during electrode implantation and chronic testing with current cuff designs. We have previously developed a μCEA to facilitate the interface with small diameter nerves (50–300 μm) with minimal impact on the nerve during implantation. The μCEA electrode was fabricated and characterized as reported previously (15). Briefly, silicon dyes were stacked to form a 2-mm microchannel (that is, 200 μm wide), where 15 gold square electrodes (40 × 50 μm; approximately 2,000 μm^2) were placed bilaterally on the sidewalls (100 μm tall; Figure 1A). These dimen-

sions were optimized for reading the neural output of a small diameter nerve in a rat. The μCEA was coupled to an Omnetics connector by using sonically bonded Parylene-C insulated 25-μm gold wires and further insulated with epoxy (15). Impedance spectroscopy showed values <500 kΩ in at least 50% of the electrodes.

Acute μCEA Implantation onto the Ventral Fascicle of the DPN

Six normotensive Wistar Kyoto (WKY, n = 6) rats and nine spontaneously hypertensive rats (SHR, Harlan, n = 9) were used in this study. Because hypertensive SHR and normotensive WKY rats were both established from the same parental line, WKY rats were used as control rats in studies of SHR (16). The animals were anesthetized with inhaled isoflurane (1–3% in 100% oxygen) from a vaporizer with a scavenger system. An appropriate level of anesthesia was confirmed by the lack of response to noxious stimuli. As previously described (17), intubation via tracheotomy and cannulation of the left carotid artery and right jugular vein was performed. The arterial cannula was interfaced with a flow pressure sensor (Model DTX plus-DT, NN12, Ohmeda) for continuous MAP and HR monitoring (Figure 1B). To stabilize fluid balance and maintain baseline MAP, a continuous infusion of physiological saline (2 mL, 1 mol/L NaHCO$_3$; 10 mL 5% dextrose) was administered into the jugular vein (3–5 mL · h^{-1} · kg^{-1}).

The DPN was exposed in the right hind limb by lateral spreading of the gastrocnemius and peroneus muscles, and a 150–200 μm diameter ventral fascicle (fDPN) was gently separated with a glass rod (Figures 1C, D). The fascicle was then easily inserted into the groove of the μCEA and secured by using a medical grade silicone elastomer (Kwik-Sil, World Precision Instruments).

Animals were then transferred to urethane-based anesthesia and stabilized for 1 h before stimulation, and arterial blood gases and body temperature were maintained at normal values throughout

the experiment. The UTSW Institutional Animal Care and Use Committee (IACUC) approved all procedures, and the studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, used by the U.S. Department of Health and Human Services National Institutes of Health.

Short- and Long-Duration fDPN Electrical Stimulation

We first evaluated the response of the entire DPN to electrical stimulation for the MAP depressor response in WKY and SHR animals by using custom-made hooks with multistranded stainless steel wires (Bioflex wire AS633, Cooner Wire, CA), to determine the optimal range of current intensities (0.1, 0.3 and 0.5 mA), number of pulses (1 and 10 pulses) and stimulation frequency (1 and 100 Hz).

Subsequently, a set of identified conditions (0.1 mA, 10 pulses, 1–2 Hz) were used to stimulate the fDPN by using the μCEA either for 10 s (short duration, n = 6 SHR and WKY) or 5 min (long duration, n = 3 SHR). The biphasic cathodic leading current stimulation pulses were given by using a Plexon Stimulator module (Plexon), and the resultant effects were monitored continuously for either 40 s (short duration) or up to 4 h after stimulation. For consecutive trials, a minimum of 15–20 min of recovery time was maintained to (a) avoid cross-contamination between trials and (b) stabilize the MAP. Continuous MAP and HR data acquisition and analysis were done by using data acquisition software (LabChart, AD Instruments) with the Powerlab analog-to-digital convertor at a 1-kHz sampling rate. Basal measurements were obtained by taking the mean value over 30 s of baseline data immediately before nerve stimulation.

Immunohistochemical Analysis for fDPN upon Repeated Electrical Stimulation

At the end of the recording sessions, the animals were euthanized with an overdose of urethane. The fDPN tissue was collected, post-fixed, cryopreserved

in 30% sucrose in 1× phosphate-buffered saline and cryosectioned at 20-μm sections for immunohistochemical analysis. Contralateral fDPN tissue was collected and used as a control (that is, no electrical stimulation). Potential stimulation-related acute axonal damage was evaluated by triple labeling immunofluorescence studies by using antibodies against the axonal marker β-III tubulin (1:500, mouse IgG; Sigma), myelin (P0) and activated macrophages (ED-1). The tissue sections were blocked with 5% normal goat serum incubated overnight with the primary antibodies and reacted with Cy2 donkey anti-rabbit (1:250) and Cy3 donkey anti-mouse (1:500); Cy5 donkey anti-goat–stained (1:400) (Jackson labs) tissue sections were imaged by using an inverted Nikon Ti Eclipse confocal microscope with a 20× objective.

## Statistical Analyses

MAP and HR from all the trials among different animals were expressed as mean ± standard error of the mean. The data were analyzed by parametric two-tailed Student *t* test followed by Mann-Whitney *post hoc* evaluation using the Prism 4 software (GraphPad Software). Values with $p \leq 0.05$ were considered to be statistically significant.

RESULTS

Cardiovascular Depressor Response by DPN Stimulation

The baseline MAP and HR in normotensive WKY and hypertensive SHR rats was of 128.83 ± 41 and 149.75 ± 35 mmHg and 400.2 ± 38 and 350 ± 83 bpm, respectively. Low-frequency hook-electrode stimulation of the DPN (0.1 mA, 10 pulses, 1–2 Hz) elicited a depressor response in both WKY (–4.36 mmHg) and SHR (–11.28 mmHg). The MAP reduction in these respective animals was more dramatic when 0.3 mA (–21.35 and –27.83 mmHg) and 0.5 mA (–39.64 and –33.52 mmHg) were used. Figure 2A shows the HR (top) and MAP (bottom) in an SHR rat in response to increasing
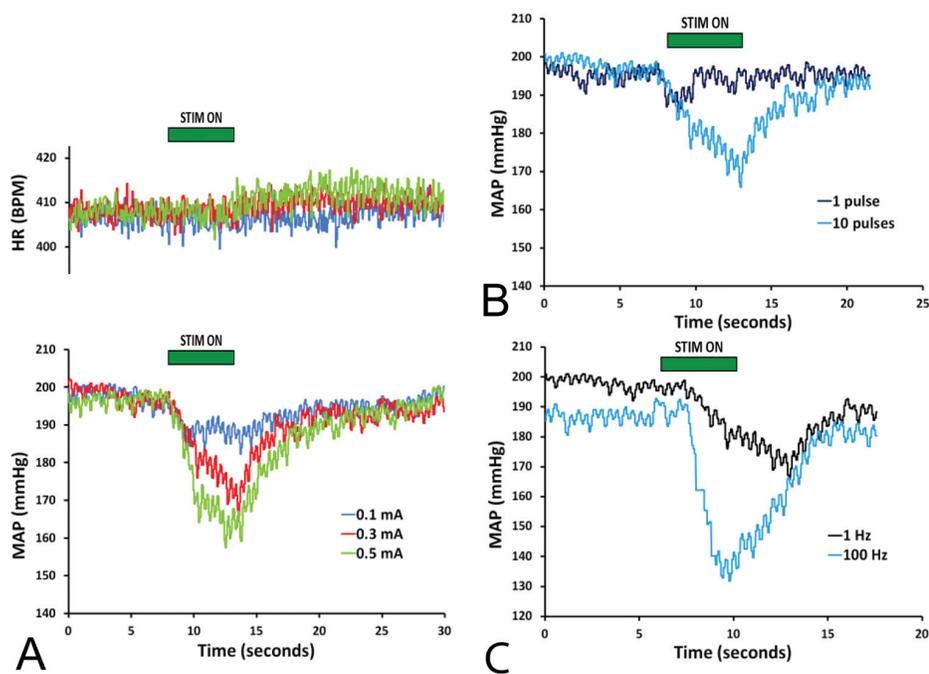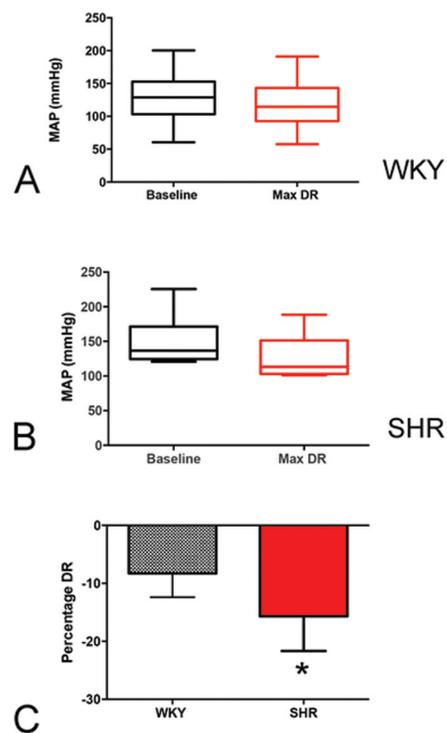
Figure 2. MAP depressor responses induced by DPN stimulation. Optimal stimulation parameters were evaluated in WKY and SHR animals. Representative MAP depressor responses after hook electrical stimulation (STIM) of the DPN nerve of an SHR animal. (A) There was no effect on HR (top) and robust decrease in the MAP (bottom) in response to gradual increment in stimulus intensity under three different parameters. (B) MAP also decreases proportional to the number of stimulation pulses at 0.3 mA and 1-Hz stimulation. (C) MAP depression in response to increased stimulation frequency from 1 to 100 Hz at 0.3 mA, 10 pulses.


Figure 3. Mean MAP depressor responses after brief fDPN stimulation. A 5-s electrical stimulation of the fDPN did not significantly affect the average MAP in normotensive WKY animals (A), but induced a significant ($p < 0.03$) depressor response in SHR hypertensive animals of ~10 mmHg (B, C). The graphs show the mean and standard deviation from six animals in each group.

currents. While the higher stimulus intensity gave larger depressor responses, some muscle contraction was also noted at 0.3- and 0.5-mA intensities. We then used 0.5 mA at 1–2-Hz stimulation parameters to evaluate the effect of pulse number and determined that increasing them from 1 to 10 potentiates the depressor response from –7.05 to –8.98 in WKY and from –33.52 to –39.64 mmHg in SHR (Figure 2B). Finally, we evaluated the effect of stimulation frequency using 10 pulses, at 0.3-mA current intensity. The comparison of 1–100 Hz showed an increment in the depressor response from –33.64 to –40.45 mmHg in WKY and –33.52 to –50.84 mmHg in SHR rats (Figure 2C), respectively. These data showed that 0.1 mA in intensity, 10 pulses and 1–2-Hz stimulation frequency are optimal to be used in fascicular fDPN stimulation using the μCEA, since it achieves

maximal depressor responses with minimal muscle activation.

Significant Depressor Response by μCEA Stimulation of the fDPN without Bradycardia

When we tested short stimulation (that is, 5 s) of the fDPN using the μCEA electrode (0.1 mA, 10 pulses and 1 Hz), the MAP did not change in normotensive rats (118.12 ± 44.8 mmHg; n = 6; Figure 3A). In sharp contrast, SHR showed a maximum reduction in MAP to 126.2 ± 33.3 mmHg (n = 6; Figure 3B). This is a significant 23.6 mmHg drop or a 15.8% reduction ($p \leq 0.03$; Figure 3C). Figure 4 shows the individual response and variability for both HR and MAP responses in WKY and SHR animals (six animals per group) induced by electrical stimulation at fDPN. Baseline HR was similar between control and experimental animals with

values of 400 ± 37 and 401 ± 27 beats per minute (bpm) in WKY and SHR, respectively (Figures 4A, C). Only one animal of the normotensive group showed a reduction in MAP in response to fascicular stimulation (Figure 4B). Conversely, all animals in the SHR group showed a depressor response elicited by stimulation of the fDPN fascicle. After the fDPN stimulation, the SHR returned to hypertensive levels in 5–15 s after stimulation (Figure 4D).

Prolonged Depressor Response by Extended μCEA Stimulation of the fDPN

A separate cohort of animals (n = 3, SHR) was used to evaluate the possibil-
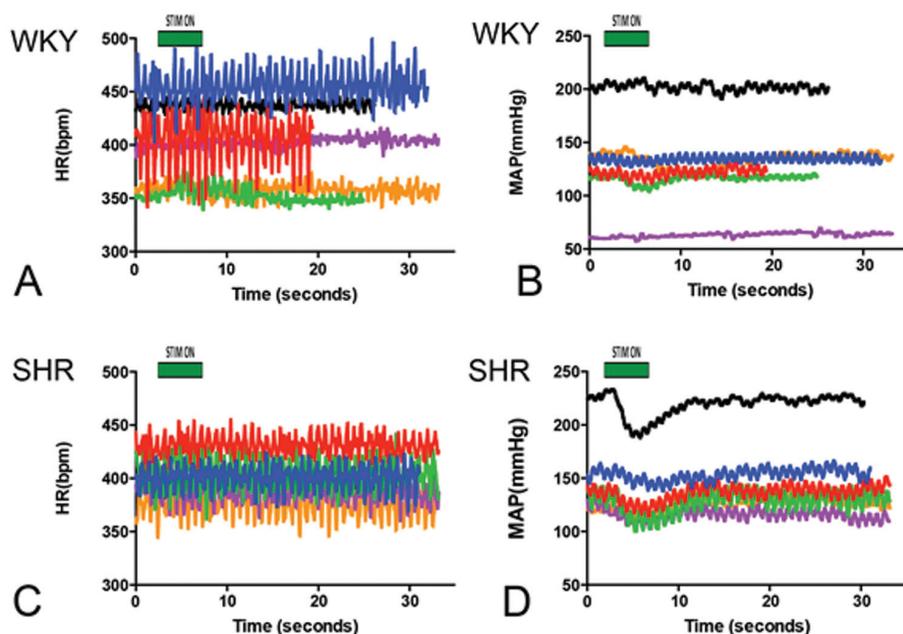
Figure 4. Individual MAP depressor responses in both WKY and SHR rats induced by short fDPN µCEA electrical stimulation. HR and MAP recordings were elicited by 10 pulses (overlaying green trace; 0.1 mA, 1 Hz) of electrical stimulation on the fDPN in six animals per group. No effect was observed on HR in either type of animal (A and C). The depressor response was observed in one WKY rat (green) (B) and in all SHR animals (D). This response returned to baseline values 15 s after stimulation.

ity of extending the depressor response by prolonging the stimulation from a few seconds to 5 min under the same current intensity and frequency. We observed an immediate reduction in MAP during the stimulation (Figure 5A), which persisted for up to 4 h in anesthetized animals (Figures 5B, C). Quantification of the MAP showed a reduction from baseline values (181 ± 6.5 mmHg) to 136 ± 16 and 141 ± 8 mmHg at 1 and 2 h after stimulation, respectively (Figure 6). Such sustained reduction in MAP represents a 24.86 to 17.12% decrease in blood pressure over a 2-h period after the initial 5-min stimulation. The data indicate a prolonged depressor response evoked by a short stimulation of the fDPN.

Repeated Electrical Stimulation Does Not Induce Axonal Damage

No obvious morphological nerve damage and no overt signs of trauma or edema/inflammation were observed during or after fascicular stimulation.

Qualitative immunohistochemical evaluation of the fDPN tissue after 5-min electrical stimulation showed normal histology. The NF-200–labeled axons were normal in morphology and label intensity (Figure 7A). No obvious damage to the myelin sheet was observed (Figure 7B), and no detection of activated macrophages (ED-1–positive cells) was noted (Figure 7C), suggesting that repetitive acute stimulation via µCEA did not cause overt damage to this fDPN tissue.

DISCUSSION

Refractory HTN is characterized by blood pressure that remains above 140/90 mmHg, despite treatments with at least two antihypertensive drugs. Among the surgical therapeutic strategies proposed are renal sympathetic denervation (18), carotid sinus baroreceptor stimulation (19,20) and deep brain stimulation (21). All have shown some promise, but require complicated surgical interventions. Stimulation of autonomic

nerves such as the vagal nerve or the carotid sinus nerve is particularly effective. However, these nerves are heterogeneous in afferent/efferent composition, and their stimulation often results in unwanted side effects such as bradycardia and bradypnea (9,22).

In 1982, Yao *et al.* reported that electrical stimulation of somatic nerves might explain and reproduce the depressor effects of acupuncture in SHR rats. Indeed, acupuncture-like stimulation of the sciatic nerve in SHR animals was reported to induce an initial increase in HR and MAP during constant stimulation at 0.1 mA, 3 Hz for 30 min and a subsequent decrease in MAP from 160 to 135 mmHg, which lasted several hours after stimulation (23). However, this stimulation paradigm also elicited leg twitches, and more specific stimulation strategies were needed. The ST36 acupoint receives sensory innervation from saphenous, superficial peroneal, and lateral sural cutaneous nerves and motor innervation from the deep peroneal and anterior tibialis nerves (24). Stimulation of the DPN for 8 wks has been shown to reduce MAP by 10–18 mmHg without affecting HR (14). In humans, the peroneal nerve has approximately 25–38 fascicles (25) and whether stimulation of a single fascicle can significantly reduce MAP has not been evaluated. Here, we demonstrate that stimulating a single fascicle of the DPN (that is, fDPN) can be used to induce a depressor response in hypertensive SHR without significantly affecting HR. We showed that low-frequency stimulation (1 Hz) of 10 pulses at 0.1 mA induces an immediate MAP drop in a similar range to that observed after sciatic nerve stimulation or ST36-37 electroacupuncture, despite only interfacing a relatively small axonal population. The duration of the depressor response was significantly prolonged by extending the stimulation paradigm for 5 min. This brief stimulation of the fDPN was found to be effective in reducing the MAP in SRH animals for up to 4 h.

The mechanism of action of the observed depressor response in this study
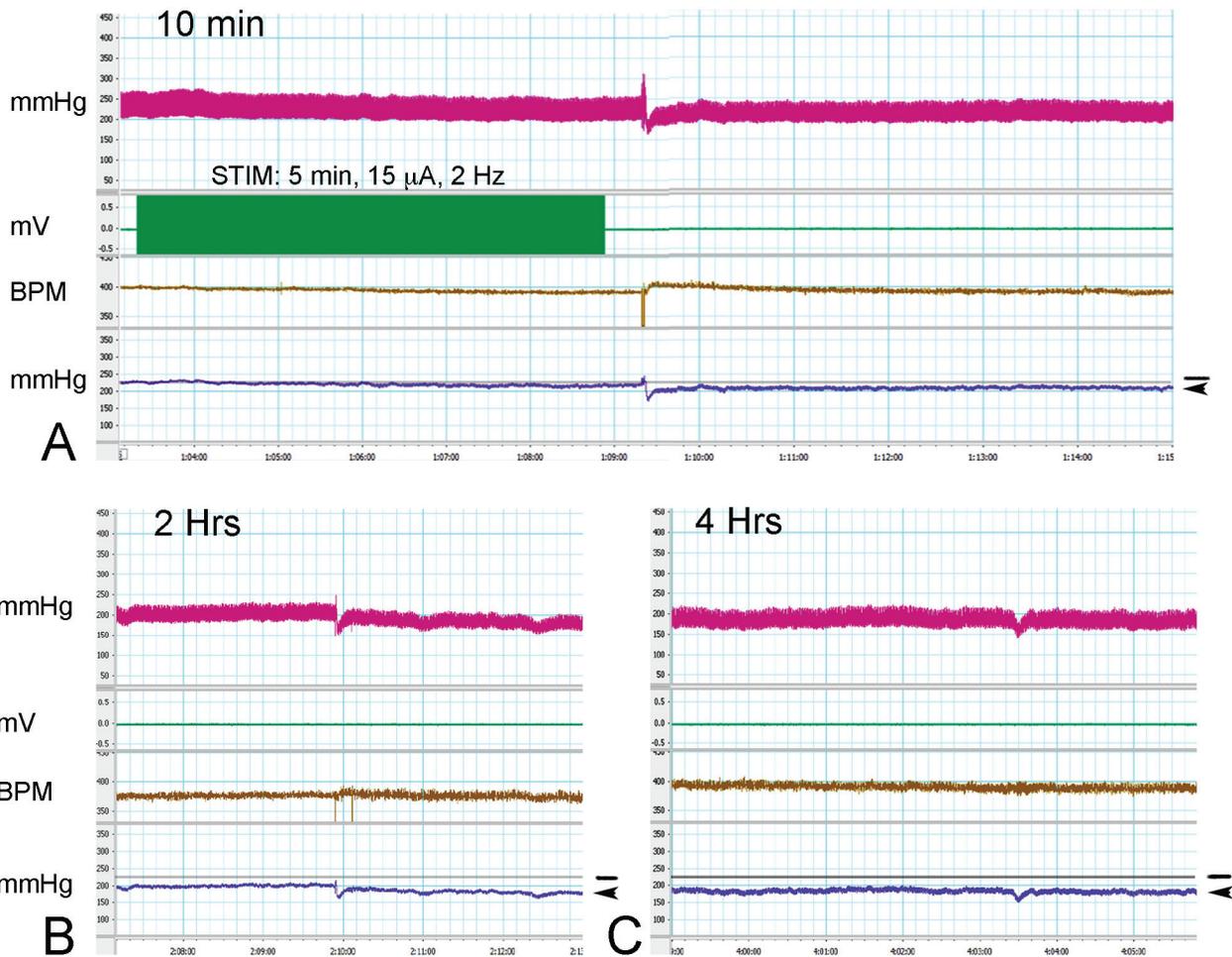
Figure 5. Prolonged MAP depressor response in SHR animals. (A) Representative traces of AP (arterial pressure, red), electrical stimulation (green), HR (brown) and MAP (blue) from a separate cohort of hypertensive rats that underwent 5 min of one-time fDPN stimulation, which produces a prolonged depressor response up to 4 h. Arrowheads indicate the induced MAP levels compared with the baseline (black line) at 10 min (A), 2 h (B) and 4 h (C) after stimulation (arrow heads). The MAP depressor response is evident shortly after stimulation and persists up to 4 h.
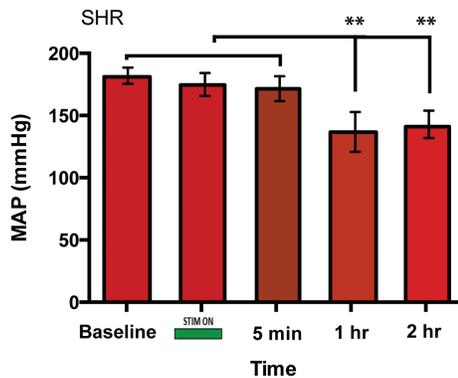
Figure 6. Maximum percentage in MAP depressor responses in SHR animals. Changes in MAP from SRH rats (n = 3), which was significantly reduced at both 1 and 2 h after the initial 5 min stimulation. **$p < 0.001$.

remains to be elucidated. However, the acupuncture stimulation of the ST36 acupoint has been shown to stimulate the DPN and to evoke neural afferent activity from the skin, muscles and joints. These axons terminate in the dorsal horn of the lumbar spinal cord, where they activate GABA-ergic interneurons, which in turn mediate synaptic inhibition of second-order neurons projecting to the brain stem periaqueductal gray, hypothalamic nuclei and rostral ventrolateral medulla (26) regions. Thus, a complex heterosynaptic pathway is likely involved in eliciting the hypotensive effect
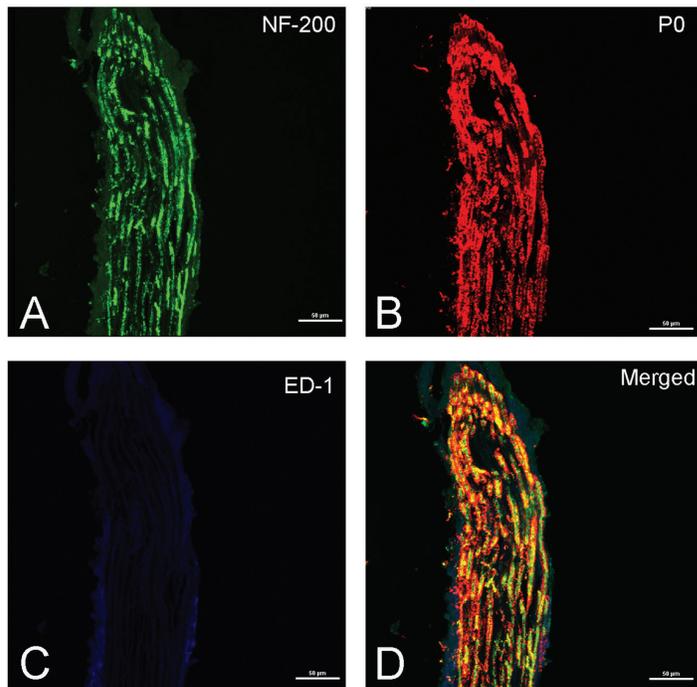
Figure 7. Normal axonal histology in fDPN tissue after 5 min of μCEA electrical stimulation. (A) Representative peroneal nerve fascicle from the animals that were subjected to 5-min stimulation and 4-h monitoring were labeled with the axonal (NF-200, *green*) myelination (P0, *red*) and inflammation (ED-1, *blue*) antibodies. Normal histology was noted, and no overt nerve damage or inflammation was observed. Scale bar = 50 μm.

induced by stimulation of this nerve. This result has been confirmed by recent studies that have demonstrated that damage to the DPN abolishes the depressor response (27). Others have reported that DPN stimulation at 12 Hz and 0.15–0.3 mA excites only Aα, Aβ and Aδ fibers, whereas 0.5–0.8 mA evokes activity in C-fibers (28). Thus, it is likely that the effect that others and we have observed relates to the autonomic regulation of vasodilation in the hind limb.

One of the limitations of the present study is that all experiments were done in anesthetized animals, and thus further studies are warranted to determine whether the fDPN stimulation can be achieved in conscious animals. Such experiments will require not only the use of microelectrode arrays such as the μCEA, but also the development of fully implantable systems. We are currently working on the development of wireless stimulating microelectrodes, which can

be used in the future to evaluate the effect of fDPN stimulation in fully awake animals.

CONCLUSION

In summary, this study provides evidence supporting the use of μCEA interfacing of small somatic nerve fascicles associated with cardiovascular relevant acupoints to induce significant reductions in MAP. The findings open the possibility of clinically relevant neuromodulation of small fascicles, which can be used as a strategy to achieve selective R-HTN therapy with minimal side effects.

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