SUPPLEMENTAL MATERIALS

for:

CIRCRESAHA/2011/262592/R2

Tuning electrical conduction along endothelial tubes of resistance arteries through Ca^{2+}-activated K^+ channels

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Detailed Methods with References

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Detailed Methods

Animal care and use.
All procedures were approved by the Animal Care and Use Committee of the University of Missouri and performed in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were housed on a 12:12-h light-dark cycle at ~23 °C with fresh water and food available *ad libitum*. Experiments were performed on C57BL/6 males bred at the University of Missouri (age, 3–6 months). Each mouse was anesthetized using pentobarbital sodium (60 mg/kg, intraperitoneal injection) and abdominal fur was removed by shaving. Following surgical procedures, the anesthetized mouse was euthanized with an overdose of pentobarbital via cardiac puncture.

Solutions.
Physiological salt solution (control PSS) was used to superfuse EC tubes [(in mmol/L): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10 Glucose]. During SMC dissociation to produce EC tubes, PSS contained 0.1% Bovine Serum Albumin (USB Corp.; Cleveland, OH; USA). During dissection to prepare EC tubes, $CaCl_2$ was absent and 0.01 mmol/L sodium nitroprusside (SNP) was added to PSS (dissection PSS) to relax SMCs. During dissociation of SMCs, SNP was replaced with 0.1 mmol/L $CaCl_2$ (dissociation PSS). All reagents were obtained from Sigma-Aldrich (St. Louis, MO; USA) unless otherwise indicated.

Surgery and microdissection.
A ventral midline incision was made from the sternum to the pubis. While viewing through a stereo microscope (SMZ800, Nikon; Tokyo, Japan), fat and connective tissue superficial to the sternum were removed to expose the proximal ends of abdominal muscle feed arteries (superior epigastric artery) bilaterally. To maintain blood in the lumen and thereby facilitate visualization during dissection, each AFA was ligated along with its adjacent vein using 6-0 silk suture (Ethicon; Somerville, NJ; USA). Abdominal muscles were removed bilaterally and placed in chilled (4 °C) dissection PSS. A muscle was pinned onto transparent silicone rubber (Sylgard 184, Dow Corning; Midland, MI; USA) and the vessel segment (length: ~2 cm) was dissected free from surrounding tissue. Residual blood was flushed from the vessel lumen by cannulating one end with a pipette made from heat-polished borosilicate glass capillaries (G150T-4, Warner Instruments; Hamden, CT; USA) with an outer diameter of 50-80 μm and connected to a static column (height, ~10 cm) of dissection PSS.

Endothelial cell tube isolation and superfusion.
As described,[1, 2] feed arteries were cut into segments (length, ~3 - 5 mm) and placed into dissection PSS containing 0.62 mg/ml papain, 1.0 mg/ml dithioerythritol, 1.5 mg/ml collagenase then incubated for 30 min at 34 °C. Following partial digestion, the PSS containing enzymes was replaced with dissociation PSS and vessel segments were transferred to a 100 x 15 mm Petri dish and gently triturated to remove SMCs using borosilicate glass capillary tubes [1.0 mm outer diameter (OD)/ 0.58 mm ID; World Precision Instruments (WPI), Sarasota, FL; USA] that were pulled (P-97; Sutter Instruments; Novato, CA; USA) and heat-polished (tip ID: 80-120 μm). Following removal of SMCs (confirmed by visual inspection at 200X magnification), an EC tube was transferred to a tissue chamber (RC-27N, Warner) secured on an aluminum platform (width: 14.5 cm, length: 24 cm, thickness: 0.4 cm) containing a micromanipulator (DT3-100, Siskiyou Corp.; Grants Pass, OR; USA) at each end that held a blunt fire-polished micropipette (OD, 60-100 μm) to secure the tube against the bottom (coverslip) of the tissue chamber. The entire preparation was secured on an inverted microscope (Eclipse TS100, Nikon) mounted on a vibration-isolated table (Technical Manufacturing Corp., Peabody, MA; USA) and superfused

at 4 ml/min with PSS. The temperature of the chamber was regulated using an inline heater (SH-27B, Warner) and heating platform (PH6, Warner) coupled to a temperature controller (TC-344B, Warner). Temperature was increased over 30 min to 32 ºC[1, 2] where intact preparations were studied for up to 4 hr. Pharmacological agents were added to the superfusion solution, thereby exposing the entire EC tube to the treatment. Preliminary experiments ejecting blue dye from micropipettes positioned within the chamber confirmed that superfusion flow was laminar in the axial direction of EC tubes.

Intracellular recording.
Membrane potential (V_m) in EC tubes was recorded with an Axoclamp amplifier (2B; Molecular Devices; Sunnyvale, CA; USA) using microelectrodes pulled (P-97; Sutter) from glass capillary tubes (GC100F-10, Warner) and backfilled with 2 mol/L KCl (tip resistance, ~150 MΩ). For experiments testing dye transfer between cells through gap junctions, microelectrodes were backfilled with 0.1% propidium iodide dissolved in 2 mol/L KCl. An Ag/AgCl pellet was placed in effluent PSS to serve as a reference electrode. The output of the amplifier was connected to an analog-to-digital converter (Digidata 1322A, Molecular Devices; Sunnyvale, CA; USA) with data recorded at 1000 Hz on a Dell personal computer using Axoscope 10.1 software (Molecular Devices). For dual simultaneous intracellular recordings[1, 3], a second amplifier (IE-210, Warner) was integrated into the data acquisition system. Current (± 0.1-3 nA, 2 s) was delivered using the Axoclamp electrometer driven by a function generator (CFG253, Tektronix; Beaverton, OR; USA). For current injection, an EC was penetrated at a site located ~150 µm from the downstream (with respect to the direction of PSS superfusion) end of where EC tube was pinned (referred to as Site 1) while recording V_m from an EC at Site 2, which was located at a defined separation distance (50-2000 µm) from Site 1 with reference to a calibrated eyepiece reticle while viewing at 200X magnification. For all experiments (except for length constant determinations), separation distance between microelectrodes was standardized at 500 µm, which corresponds to the distance of ~15 ECs placed end-to-end.

Successful impalements were indicated by sharp negative deflection of V_m, stable V_m for >1 min, hyperpolarization (≥ 20 mV) to 1 µmol/L NS309 (IK_{Ca}/SK_{Ca} activator; Tocris; Bristol, UK), recovery to resting V_m after NS309 washout and return to ~0 mV upon withdrawal from the cell. Correspondence between current injection at Site 1 and V_m responses at Site 2 indicated simultaneous intracellular current injection and V_m recording. Following dye microinjection, using a filter set for rhodamine with illumination provided from a 50W Hg lamp, images of propidium iodide fluorescence were acquired using a 40X objective (Nikon Fluor40; NA: 0.75) and focused onto a cooled CCD camera (MicroFire, Optronics; Goleta, CA; USA).

Pharmacology.
NS309 and SKA-31 (Tocris; Bristol, UK) were used to evoke hyperpolarization in endothelial tubes by direct activation of SK_{Ca}/IK_{Ca} channels [4-6]. Alternatively, the physiological agonist acetylcholine (ACh) chloride was used to indirectly activate SK_{Ca}/IK_{Ca} channels through G_q protein coupled muscarinic receptors.[7] To evaluate the potential effects of nitric oxide synthesis during NS309 or ACh treatment, [8, 9] N^{ω}-Nitro-L-arginine (L-NNA) was applied during select experiments alone (10 min pre-treatment) and subsequently in combination with either NS309 or ACh. To test for the presence of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) and K_{ATP} channels within the endothelium, NS1619 and levcromakalim were applied, respectively [10-12]. The compounds NS309, SKA-31, NS1619 and levcromakalim were dissolved in DMSO and diluted to final working concentrations in PSS on the day of an experiment. Final concentration of DMSO was < 1%. Vehicle controls with DMSO (≤ 2%) in PSS had no effect on V_m or electrical coupling (n=3). Apamin (Alomone; Jerusalem, Israel) and charybdotoxin (Alomone

Jerusalem, Israel or Anaspec; Fremont, CA) were dissolved and diluted into superfusion PSS in combination to block SK_{Ca}/IK_{Ca} channels.[13]

Data analysis.
One EC tube was studied per mouse. Analyses included: 1) Resting V_m (mV) under Control conditions; 2) Change in V_m (Δ mV) = peak response V_m – preceding baseline V_m; 3) Conduction Amplitude (CA, mV/nA) = V_m at Site 2 / current injected at Site 1. With linearity of I-V relationships (*see Results*), -1 nA was used as a standard current for evaluating CA; 4) Fraction of Control CA = CA during treatment / preceding control CA; 5) Conduction Efficiency = CA at each separation distance / CA at 50 µm separation; 6) Length constant (λ) = distance over which the electrical signal decayed to 37% (1/e) of the 'local' value. The shortest separation distance was 50 µm to ensure that both microelectrodes were not in the same EC while recording as close to the signal origin as possible; the length of individual ECs in these freshly-isolated tubes is typically 35-40 μm^2. Fraction of control CA was defined as the CA during NS309 or SKA-31 at respective concentrations divided by CA under control conditions. Linear regression (ΔV_m at Site 2 versus current injection at Site 1), curve fitting (estimates of λ) were performed using GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA; USA). Statistical analyses included repeated measures Analysis of Variance with Tukey or Bonferroni post-hoc comparisons, linear regression and paired Student's -tests (GraphPad Prism). Differences between treatments were accepted as statistically significant with $P < 0.05$. Summary data are presented as means ± S.E.

References

1. Behringer EJ, Socha MJ, Polo-Parada L, Segal SS. Electrical conduction along endothelial cell tubes from mouse feed arteries: Confounding actions of glycyrrhetinic acid derivatives. *Br J Pharmacol*. 2011 *doi: 10.1111/j.1476-5381.2011.01814.x.*
2. Socha MJ, Hakim CH, Jackson WF, Segal SS. Temperature effects on morphological integrity and Ca^{2+} signaling in freshly isolated murine feed artery endothelial cell tubes. *Am J Physiol Heart Circ Physiol*. 2011;301:H773-783.
3. Emerson GG, Segal SS. Electrical coupling between endothelial cells and smooth muscle cells in hamster feed arteries: Role in vasomotor control. *Circ Res*. 2000;87:474-479.
4. Dalsgaard T, Kroigaard C, Simonsen U. Calcium-activated potassium channels - a therapeutic target for modulating nitric oxide in cardiovascular disease? *Expert Opin Ther Targets*. 2010;14:825-837.
5. Kohler R, Kaistha BP, Wulff H. Vascular K_{Ca} channels as therapeutic targets in hypertension and restenosis disease. *Expert Opin Ther Targets*. 2010;14:143-155.
6. Strobaek D, Teuber L, Jorgensen TD, Ahring PK, Kjaer K, Hansen RS, Olesen SP, Christophersen P, Skaaning-Jensen B. Activation of human IK and SK Ca^{2+} -activated K^+ channels by NS309 (6,7-dichloro-1h-indole-2,3-dione 3-oxime). *Biochim Biophys Acta*. 2004;1665:1-5.
7. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: Bringing the concepts together. *Trends Pharmacol Sci.* 2002;23:374-380.
8. Stankevicius E, Lopez-Valverde V, Rivera L, Hughes AD, Mulvany MJ, Simonsen U. Combination of Ca^{2+} -activated K^+ channel blockers inhibits acetylcholine-evoked nitric oxide release in rat superior mesenteric artery. *Br J Pharmacol*. 2006;149:560-572.
9. Sheng JZ, Ella S, Davis MJ, Hill MA, Braun AP. Openers of SK_{Ca} and IK_{Ca} channels enhance agonist-evoked endothelial nitric oxide synthesis and arteriolar vasodilation. *FASEB J*. 2009;23:1138-1145.
10. White R, Hiley CR. Hyperpolarisation of rat mesenteric endothelial cells by ATP-sensitive K^+ channel openers. *Eur J Pharmacol*. 2000;397:279-290.
11. Beleznai TZ, Yarova P, Yuill KH, Dora KA. Smooth muscle Ca^{2+} -activated and voltage-gated K^+ channels modulate conducted dilation in rat isolated small mesenteric arteries. *Microcirculation*. 2011;18:487-500.
12. Takano H, Dora KA, Spitaler MM, Garland CJ. Spreading dilatation in rat mesenteric arteries associated with calcium-independent endothelial cell hyperpolarization. *J Physiol*. 2004;556:887-903.
13. Ledoux J, Bonev AD, Nelson MT. Ca^{2+}-activated K^+ channels in murine endothelial cells: Block by intracellular calcium and magnesium. *J Gen Physiol*. 2008;131:125-135.

Supplemental Table I.

	-1 nA	**-1 nA**	**-2 nA**	**-3 nA**
Distance (μm)	Control ΔV_m2 (mV)	NS309 ΔV_m2 (mV)	NS309 ΔV_m2 (mV)	NS309 ΔV_m2
50	-11.3 ± 0.3	-5.7 ± 0.3*	-11.4 ± 0.6	-17.1 ±0.9
500	-7.7 ± 0.5	-3.1 ± 0.3*	-6.3 ± 0.6*	-9.4 ±0.8
1000	-5.8 ± 0.4	-1.8 ± 0.2*	-3.6 ± 0.3*	-5.4 ±0.5
1500	-3.9 ± 0.3	-1.0 ± 0.2*	-2.1 ± 0.3*	-3.1 ± 0.5
2000	-2.9 ± 0.3	-0.6 ± 0.1*	-1.2 ± 0.3*	-1.8 ± 0.4*

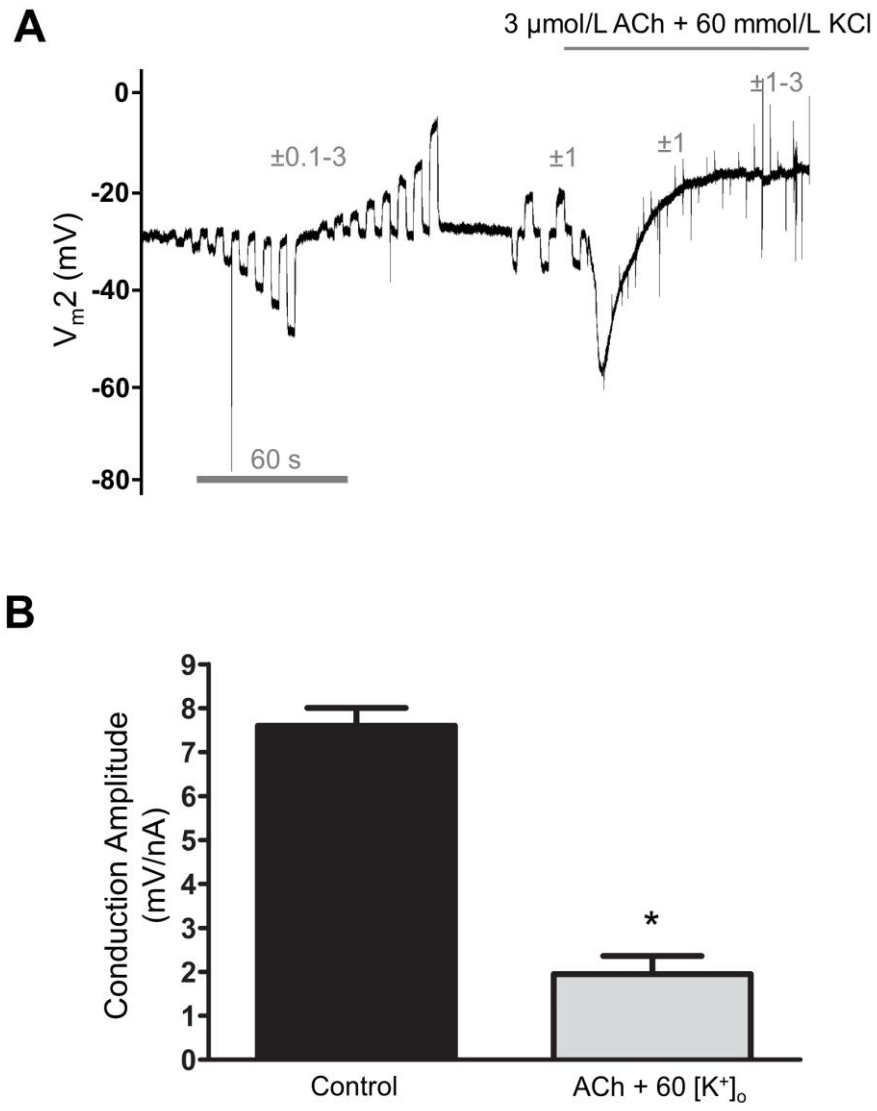
Spatial decay of electrical conduction increases during SK_{Ca}/IK_{Ca} activation with 1 μmol/l NS309. The standard current pulse microinjected at Site 1 to evaluate a change in membrane potential at Site 2(ΔV_m2) at distances of 50-2000 μm was -1 nA (Control, Column 2). Treatment with NS309 reduced ΔV_m2 to -1 nA at each distance (Column 3). To achieve the same ΔV_m2 at 50 μm in the presence of NS309 required twice the current (-2 nA; Column 4); note greater signal loss at 500-2000 μm vs. Control (these data are complementary to Figure 3C). Raising microinjection current to -3 nA during NS309 treatment exceeded Control ΔV_m2 responses at 50 and 500 μm while further illustrating enhanced signal dissipation by 2000 μm during SK_{Ca}/IK_{Ca} activation. *****Significantly less than Control V_m2 responses to -1 nA for the respective distance indicated, $P < 0.05$ (n = 11 at 50 - 1,500 μm; n=7 at 2,000 μm).

Supplemental Table II.

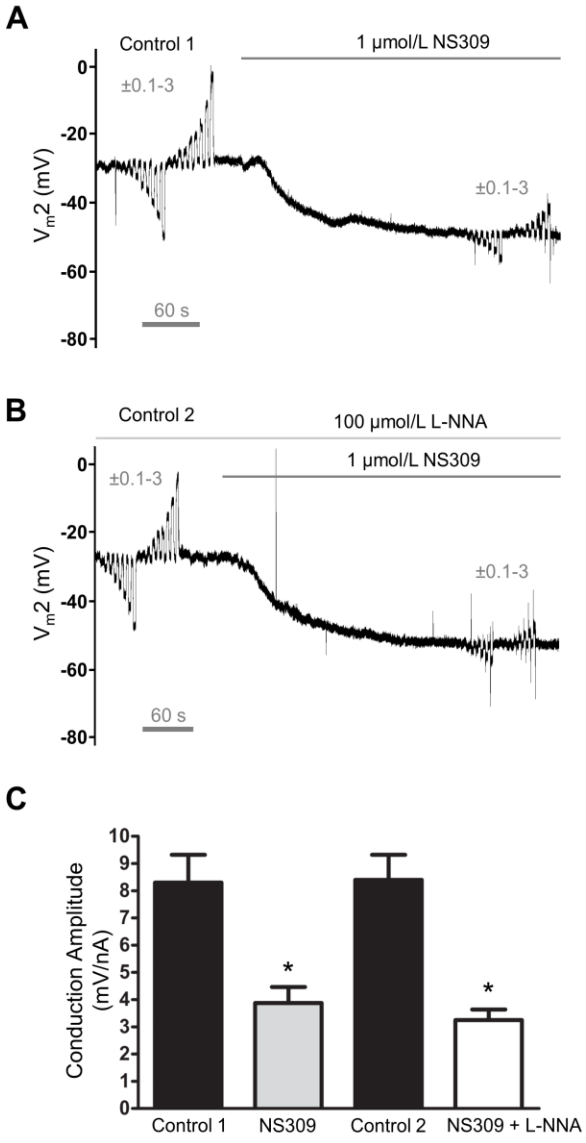
-1 nA		-1 nA	-2 nA	-3 nA
Control ΔV_m2 (mV)	Intervention	Treatment ΔV_m2 (mV)	Treatment ΔV_m2 (mV)	Treatment ΔV_m2 (mV)
-7.4 ± 0.5	**NS309 (1 µmol/L)**	-3.7 ± 0.3*	-7.4 ± 0.7	-11.1 ± 1.1
"	**NS309 (3 µmol/L)**	-1.6 ± 0.1*	-3.1 ± 0.2*	-4.7 ± 0.3*
"	**NS309 (10 µmol/L)**	-0.3 ± 0.1*	-0.7 ± 0.2*	-1.0 ± 0.2*
-7.6 ± 0.6	**SKA-31 (10 µmol/L)**	-4.2 ± 0.6*	-8.3 ± 1.2	-12.5 ± 1.7
"	**SKA-31 (30 µmol/L)**	-2.1 ± 0.4*	-4.3 ± 0.7*	-6.4 ± 1.1
"	**SKA-31 (100 µmol/L)**	-1.3 ± 0.2*	-2.6 ± 0.3*	-3.9 ± 0.5*
-7.0 ± 0.4	**NS309 (1 µmol/L) +KCl (60 mmol/L)**	-3.5 ± 0.3*	-7.1 ± 0.7	-10.6 ± 1.0
-7.1 ± 0.5	**ACh (3 µmol/L)**	-2.0 ± 0.4*	-3.9 ± 0.9*	-5.9 ± 1.3

Effect of SK_{Ca}/IK_{Ca} activation on electrical conduction at a constant reference distance. These experiments evaluated the change in V_m from rest (ΔV_m2 = resting V_m - peak response V_m) at a constant distance (500 µm) from the site of current microinjection and correspond to Figures 4, 6 and 7. Note that ΔV_m2 Treatment responses to -1 nA are significantly less ($P < 0.05$) than ΔV_m2 Control responses during each intervention. During 1 µmol/L NS309 (either alone or with 60 mmol/L KCl) and for 10 µmol/L SKA-31, doubling current to -2 nA increased ΔV_m2 responses to approximate Control values. During 30 µmol/L SKA-31 and 3 µmol/L ACh, tripling current to -3 nA increased ΔV_m2 to approximate Control values but still could not restore ΔV_m2 responses to 10 µmol/L NS309 or 100 µmol/L SKA-31. *****Significantly less than Control ΔV_m2 in response to -1 nA, $P < 0.05$.

Note: During SK_{Ca}/IK_{Ca} blockade with apamin + charybdotoxin, ΔV_m2 responses to -1 nA increased ($P < 0.05$) from -6.5 ± 0.9 to -8.1 ± 0.9 mV, n=6; see Fig. 8B) and less current (-0.8 nA) was required to evoke ΔV_m2 responses (-6.5 ± 0.7) similar to those evoked by -1 nA under Control conditions.



Supplemental Figure I. Acetylcholine inhibits electrical conduction without hyperpolarization. Data are from continuous V_m recordings at Site 2 located 500 µm from current microinjection. **A,** Representative recording illustrating responses to ±0.1-3 nA before and during 3 µmol/L ACh + 60 mmol/L $[K^+]_o$. Note loss of V_m2 responses during 3 µmol/L ACh + 60 mmol/L $[K^+]_o$. **B,** Summary data (means ± S.E.; n=4) for Conduction Amplitude to -1 nA. Plateau V_m during ACh + 60 mmol/L $[K^+]_o$ was -20 ± 1 mV. *Significantly different from Control, $P < 0.05$. Conduction Amplitude recovered to 7.5 ± 0.5 mV/nA (n=4) upon washout of ACh + 60 mmol/L $[K^+]_o$.



Supplemental Figure II. Impaired electrical conduction during SK_{Ca}/IK_{Ca} activation is maintained during inhibition of nitric oxide synthase. Data represent continuous (paired) recordings of V_m2 at 500 µm from site of current microinjection. **A,** Responses to ±0.1-3 nA before and during 1 µmol/L NS309. Note diminished responses during NS309. **B,** As in A with NS309 + 100 µmol/L N□-Nitro-L-arginine (L-NNA). **C,** Summary data for Conduction Amplitude under the initial Control conditions (Control 1), during NS309 alone, following washout of NS309 (Control 2; note reversibility) and during NS309 + L-NNA. The reduction in V_m2 responses to current injection during NS309 + L-NNA was not different from NS309 alone. *Significantly different from Control, $P < 0.05$ (n = 4).

In separate experiments (n=3), complementary results were obtained when comparing ACh (3 µM) alone or in combination with 100 µmol/L L-NNA (also paired continuous recordings) as follows: Conduction Amplitude (mV/nA) at 500 µm separation: Control 1, 7.9 ± 1.4; ACh, 1.9 ± 0.4; Control 2, 7.7 ± 0.7; ACh + L-NNA: 2.0 ± 0.3. Across experiments, treatment with 100 µM L-NNA to inhibit nitric oxide synthase began at least 10 min before addition of NS309 or ACh.