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Eph/ephrin interactions modulate vascular sympathetic innervation

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Abstract

Ephs and ephrins are membrane-bound proteins that interact to modulate axon growth and neuronal function. We tested the hypothesis that eph/ephrin interactions affected the growth and function of vascular sympathetic innervation. Using RT-PCR analyses, we detected both classes of ephs (A and B) and both classes of ephrins (A and B) in sympathetic ganglia from neonatal and adult rats. Both classes of ephs (A and B) and both classes of ephrins (A and B) bound to the cell bodies and neurites of dissociated postganglionic sympathetic neurons. Messenger RNAs encoding for both classes of ephs (A and B) and both classes of ephrins (A and B) were also detected in sympathetically innervated arteries from neonatal and adult rats. These data suggest that ephrins/ephs on nerve fibers of postganglionic sympathetic neurons could interact with ephs/ephrins on cells in innervated arteries. We found that ephA4 reduced reinnervation of denervated femoral arteries. Reinnervation in the presence of ephA4-Fc ($38.9 \pm 6.6\%$) was significantly less than that in the presence of IgG-Fc ($62 \pm 10\%$; $n = 5$; $p < 0.05$; one-tailed unpaired t-test). These data indicate that eph/ephrin interactions modulated the growth of vascular sympathetic innervation. We also found that ephA4 increased basal release of norepinephrine from nerve terminals of isolated tail arteries. These data indicate that eph/ephrin interactions affect the growth and function of vascular sympathetic innervation.

Keywords

sympathetic nervous system; ephrin; eph; axon growth; norepinephrine

Introduction

The sympathetic nervous system is a major contributor to cardiovascular function and homeostasis that contributes to the development and progression of many cardiovascular diseases, including hypertension (11,15,22), atherosclerosis (20) and heart failure (31,33). Cardiovascular effects of the sympathetic nervous system are in large part mediated by postganglionic sympathetic nerves innervating blood vessels. Despite the clear involvement of vascular sympathetic nerves in cardiovascular function and disease, the mechanisms governing the development, maintenance, and function of these nerves are not fully understood.

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Ephrins are a family of membrane-bound proteins that, with their membrane-bound tyrosine kinase receptors, ephs, mediate contact-dependent cell-to-cell interactions (5,25,27). Although the ephrins have been designated ligands and the ephs receptors, there is evidence that these molecules signal bidirectionally (18,21) in that eph activation of ephrins and ephrin activation of ephs modulate cell function. Many studies indicate that ephrin/eph interactions affect the development and function of the central nervous system (10,12,17,21,35). The effects of eph/ephrins on peripheral nerves have not been studied extensively, but evidence suggests that these molecules are likely to affect the development and function of postganglionic sympathetic neurons. Studies of Kasemeier-Kulesa et al. (2006) suggest that ephrinB1 activation of ephB2 on neural crest cells plays a role in the formation of sympathetic ganglia, and studies of Gao et al. (2000) suggest that ephrin A5 activation of ephs on postganglionic sympathetic neurons promotes survival and axon growth. Eph activation of ephrins (reverse signaling) in these neurons has not been reported.

It is well known that interactions between postganglionic sympathetic neurons and their targets modulate postganglionic sympathetic neurons (19,32). Postganglionic sympathetic neurons innervated blood vessels, and vascular-derived signals modulate axon growth (4,23), survival (2), and neurotransmitter/neuropeptide expression (3). Vascular smooth muscle (VSM), which are the primary target for postganglionic sympathetic neurons in blood vessels, express ephrinB2 (7) and ephA4 (26). This led us to hypothesize that vascular ephrins and/or ephs would activate ephs and/or ephrins on perivascular sympathetic nerves and affect their development and function. To begin to test this hypothesis, we assessed the effects of ephrin/eph interactions on reinnervation of denervated arteries and on norepinephrine release from perivascular sympathetic nerves.

Materials and Methods

Animals

The use of animals in this study was approved by the Animal Care and Use Committee at the University of Vermont and was in accordance with the National Institute of Health guidelines for use of animals in research. Tissues were harvested after euthanasia from adult male (250–300 gm), and adult postpartum females (reinnervation studies) and neonatal male and female Sprague Dawley rats. Adult animals were euthanized with an overdose of sodium pentobarbital (150 mg/mg ip). Neonatal rats were euthanized with isoflurane.

Materials

Animals were obtained from Charles River, Canada. Collagenase, hyaluronidase, and trypsin were obtained from Worthington Biochemical. Nuserum, collagen and nerve growth factor were obtained from Collaborative/Becton Dickinson. DMEM/F12, DMEM, penicillin/streptomycin, fetal bovine serum (FBS) were obtained from Invitrogen. Kits from Qiagen were used to isolate RNA for RT-PCR analyses. RNA was reversed transcribed with RETROscript kits and cDNA was amplified with Amplitaq Gold both from Ambion. Recombinant eph, ephrin and control Fc chimeras were purchased from R & D Systems. Binding of Fc chimeras was detected with goat anti-human IgG (Alexa Fluor 568) from Molecular Probes. Gap43 antibody was from Chemicon. GAP43 antibody was detected with donkey-antirabbit secondary antibody (Alexa fluor 555) from Molecular Probes. Tritiated norepinephrine (NE) for NE release assays was from Amersham. All other chemicals were from Sigma.

Tissue Culture

Postganglionic sympathetic neurons were isolated from superior cervical ganglia of male and female Sprague Dawley neonatal rats (3 – 4 days old). Ganglia were collected and

dissociated for 10 minutes at 37°C in a collagenase/hyaluronidase solution (10 mg/ml bovine serum albumin, 4 mg/ml collagenase, 1 mg/ml hyaluronidase) and then for 10 minutes in trypsin (3 mg/ml). Dissociated cells were resuspended in neuronal media (DMEM/F12 supplemented with 5% FBS, 10% NuSerum (Collaborative), 50 ng/ml NGF, and penicillin/streptomycin) and applied to collagen coated tissue culture dishes. The cells were allowed to attach overnight in a humidified 5% CO₂ environment maintained at 37°C. Non-neuronal cells were then growth arrested with mitomycin C (10 µg/ml for 1 hour).

Vascular smooth muscle cells (VSM) were obtained from adult rat femoral arteries as described by Ross (30). The cells were grown in low glucose DMEM supplemented with 10% fetal bovine serum (FBS), 100 units penicillin and 100 units streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ environment. VSM were used for experiments after two passages with trypsin.

RT-PCR

Tissue samples were homogenized and RNA isolated. RNA was reverse transcribed and cDNA was amplified using AmpliTaqGold with 10X PCR buffer II, 25 mM MgCl₂, and dNTP mix. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and visualized with UV light. All PCR reactions included (–) RT and (–) template controls. Data were only included if these controls showed no PCR products. Amplified PCR products were sequenced by the University of Vermont DNA facility to confirm the identity of the DNA. Primer sequences and annealing temperatures are shown (Table 1).

Ephrin/Eph Binding

To determine if ephrins and/or ephs were expressed on the surface of sympathetic neurons, ephrin-Fc and eph-Fc binding was assessed as described by Davy et al. (6). Cultures were washed and incubated at 37°C for 15 minutes with PBS containing 8 µg/ml ephrin-Fc, eph-Fc, or control IgG Fc. Dishes were rinsed with cold PBS and fixed with cold methanol at –20 C for 10 minutes. They were washed twice with PBS and blocked with 5% FBS in PBS at RT for 20 minutes. Binding was detected with fluorescent-labeled goat anti-human IgG Fc (1:500 Alexa Fluor 568 for 1 hour at RT; Molecular Probes, Carlsbad, CA). Dishes were rinsed twice with cold PBS. Binding was visualized on an upright fluorescence microscope (Olympus BX50). Images were captured digitally with an Olympus camera (Model U-ULH) and Magnafire Software and viewed with Adobe Photoshop.

In Vivo Denervation and Reinnervation

Femoral artery denervation and reinnervation were studied as described in Marko and Damon (23). Briefly, adult postpartum female Sprague Dawley rats were anesthetized with a 3% solution of Isoflurane on a Vet Equip Table Top Laboratory Animal Anesthesia System. The animal was placed in a supine position and a small incision was made over the right, distal femoral area. The distal femoral artery was mechanically denervated by severing all nerve fibers adjacent to the artery. An Alzet model 1002 Micro-Osmotic Pump containing 1% Evans Blue and 10µg ephA4-Fc or control IgG-Fc in PBS was then inserted. The opening of the pump was placed adjacent to the denervated artery. The wound was closed using an Ethicon 6-0 Prolene suture. Fourteen days after denervation, denervated and contralateral control femoral arteries were harvested.

Innervation density

Femoral arteries were prepared for GAP43 immunohistochemistry. Perivascular nerves containing GAP43 were visualized on the adventitial surface of the arteries with an Olympus BX50 microscope using a 10X objective. Images were recorded digitally with an Olympus

camera (Model U-ULH) and MagnaFire Software, and analyzed with MetaMorph image analysis software. Total length of GAP43 immunoreactive nerve fibers per unit area was determined. Two density measurements were made per artery and were averaged. Percent reinnervation was determined (denervated innervation density/contralateral control innervations density X 100).

GAP43 Immunohistochemistry

Femoral arteries were fixed for two hours with 4% formaldehyde in phosphate buffered saline (PBS), permeabilized for nine minutes with 0.05% Triton X-100 in PBS and then incubated at room temperature for 20 minutes with 5% FBS in PBS to block non-specific binding of GAP43 antibody. GAP43 antibody (1:1000 dilution) was then added and the arteries incubated overnight at 4°C. Unbound primary antibody was removed with three PBS washes. Samples were blocked for five minutes in 5% FBS in PBS, followed by incubation with donkey anti-rabbit 555 secondary antibody. Four final PBS washes removed any unbound secondary antibody.

Norepinephrine Release

Norepinephrine (NE) release was assessed using tritiated norepinephrine. These assays were performed using HEPES-buffered Krebs solution [122 mM NaCl, 3 mM KCl, 0.4 mM MgSO₄ · H₂O, 1.2 mM KH₂PO₄, 10 mM glucose, 20 mM HEPES, 1.3 mM CaCl₂ · 2H₂O, 1 mM ascorbic acid, 10 μM pargyline (to inhibit monoamine oxidase), pH 7.4]. Arteries were preincubated at 37°C with 100 nM tritiated NE for 60 minutes. The arteries were then washed (6 × 5 minutes) and electrically stimulated (1 min; 4 Hz, 60 mV, 0.5 msec pulse duration (33)). Arteries were then solubilized. Tritiated NE in all samples was collected and analyzed using a Beckman LS6000IC Liquid Scintillation Counter. Basal release was calculated as (cpm in last wash)/(total cpm available for release). Stimulated release was calculated as using (stimulated cpm – basal cpm) / (cpm available for release). NE uptake (total cpm taken up by the artery/wet weight of the artery) was also determined.

Statistical Analysis

Data are presented as means ± standard errors. Unpaired one or two-tailed t-tests were performed to compare data. Differences were considered significant if p-values were less than 0.05.

Results

Eph/ephrin expression

Previous studies suggest that postganglionic sympathetic neurons express ephB2 (16) and ephAs (8). We further characterized the expression of ephs and ephrins in these neurons. Much evidence suggests that class A ephrins predominantly and promiscuously bind to class A ephs, and class B ephrins bind predominantly and promiscuously to class B ephs (28,29). Figure 1A shows representative RT-PCR analyses of eph and ephrin expression in neonatal and adult superior cervical ganglia. For these analyses we examined ephrin A1 as a representative member of the A class of ephrins that would predominantly bind to class A ephs. We examined ephrin B2 as a representative member of the B class of ephrins that would predominantly bind to class B ephs. We examined ephA4, because EphA4 binds ephrin As as well as ephrin B2 and B3. We examined ephB2 as a representative member of the B class of ephs that would predominantly bind to class B ephrins. Figure 1B shows representative binding of ephA5 (which would bind predominantly to ephrin As), ephB2 (which would bind predominantly to ephrin Bs), ephrin A1 (which would bind predominantly to eph As), and ephrin B2 (which would bind predominantly to eph Bs) to

dissociated postganglionic sympathetic neurons from superior cervical ganglia of neonatal rats. These ligands bound to the soma and processes of all neurons in the culture. These data suggest that ephrinAs, ephrinBs, ephAs, and ephBs are expressed on the soma and processes of these postganglionic sympathetic.

In blood vessels, postganglionic sympathetic neurons nerve fibers innervate the adventitial surface of blood vessels. Previous studies indicate that vascular smooth muscle, which are the primary target for the nerve fibers, express ephrinB2 (7) and ephA4 (26). We further characterized eph and ephrin expression in blood vessels. Figure 2 shows representative RT-PCR analyses of eph and ephrin expression in neonatal and adult femoral arteries and in cultured vascular smooth muscle. These data indicate that mRNA encoding for ephrinAs and Bs and ephAs and Bs are expressed by vascular cells. The data in figures 1 and 2 led us to hypothesize that vascular ephs and/or ephrins would bind to and activate ephrins or ephs on perivascular sympathetic nerve fibers.

Eph/ephrin interactions modulate the growth of vascular sympathetic innervation

We tested the hypothesis that eph/ephrin interactions affect the growth of vascular sympathetic innervation (See Fig. 3). To do this, we assessed the effect of EphA4Fc (10 μ g) on reinnervation of denervated femoral arteries. EphA4 binds to all ephrin As and to ephrin B2 and B3 (28,29). Eph A4 binding to ephrinAs, ephrin B2 and ephrin B3 on vascular cells would prevent these ephrins from activating ephs on perivascular sympathetic nerve fibers. EphA4 binding to ephrins on perivascular sympathetic nerves would activate reverse signaling (18,21). Eph/ephrin interactions have been reported to inhibit or promote axon growth. Thus, we assessed the effects of inhibiting eph/ephrin interactions fourteen days after denervation, when the arteries are partially reinnervated (23). Femoral artery reinnervation in the presence of perivascular ephA4-Fc (black bar) was significantly less than reinnervation of control arteries (open bar) that were denervated without implantation of a pump. Reinnervation in the presence of perivascular ephA4 was also significantly less than that in the presence of perivascular IgG-Fc (grey bar).

Eph/ephrin interactions modulate baseline norepinephrine release from vascular sympathetic nerves

Eph/ephrins are also known to modulate the function of neurons (10,12,21,35,). We tested the hypothesis that eph/ephrin interactions modulate neurotransmitter release from perivascular sympathetic nerves. Adult rat tail arteries were isolated and incubated with ephA4-Fc (10 μ g/ml) or control IgG-Fc (10 μ g/ml) for one hour at 37°C. Tritiated NE was then added and the arteries were incubated for an additional hour at 37°C. Basal NE release, electrically stimulated NE release, and NE uptake were then assessed. EphA4 increased basal NE release (figure 4A). Release in the presence of ephA4-Fc (black bar) was significantly greater than that in the presence of control IgG-Fc (open bar). EphA4 did not affect electrically stimulated NE release or NE uptake (figures 4B and 4C). To determine if basal release and if ephA4-Fc modulation of basal NE release was due to neuronal release of NE, experiments were also performed in the presence of 1 μ M desipramine, which inhibits neuronal uptake of NE (Figure 4D). Desipramine increased basal release of NE and eliminated the effect of eph A4-Fc.

Discussion

Ephrin/ephs are membrane associated proteins that signal bidirectionally to modulate or mediate contact-dependent cell-to-cell communication (5,17,18,21,25,27). The present study considers how eph/ephrin interactions affect perivascular sympathetic nerves.

Previous studies report that ephrinA5 and ephrinA2 promote the growth of neurites in cultures of postganglionic sympathetic neurons (8), which suggests that these neurons expressed ephAs. Our studies indicate that postganglionic neurons in the superior cervical ganglia of neonatal and adult rats express both classes of ephs and ephrins (Figure 1). These findings led us to hypothesize that ephs and ephrins would be expressed on perivascular sympathetic nerve fibers. Previous studies suggest that VSM, which are the primary target for vascular sympathetic nerves, express ephrinB2 (7) and ephA4 (26). Our RT-PCR analyses indicate that vascular cells in arteries and cultured VSM also express ephrinA1 and ephB2. The data in figures 1 and 2 led us to hypothesize that multiple ephs and ephrins signal bi-directionally from vascular cells to perivascular sympathetic nerve fibers and/or from perivascular sympathetic nerve fibers to vascular cells and thereby affect the function of the neurons and vascular cells.

Our findings indicate that perivascular administration of ephA4 inhibited reinnervation of denervated femoral arteries and enhanced basal release of norepinephrine from isolated arteries identify novel roles for eph/ephrin interactions. EphA4 mediates its effect by binding to ephrinAs, ephrinB2 and ephrinB3 (28,29) on the nerve fibers and vascular cells. The present studies do not allow us to determine which of these ephrins mediated the observed effects. Our studies also do not allow us to determine if ephA4 inhibited forward signaling or promoted reverse signaling. Gao et al. (8) reported that ephrinA5 stimulated axon growth in cultures of postganglionic sympathetic neurons, which suggests that ephrinA5 activation of an eph, or forward signaling, promoted axon growth. Our finding that ephA4 inhibited reinnervation would be consistent with ephA4 inhibiting ephrin-induced axon growth.

The data in figure 4 indicate that ephA4 increased basal release of NE. Desipramine, an inhibitor of the neuronal norepinephrine transporter (NET; 13), inhibited this increase, which suggests that the effect was dependent upon activity of NET. EphA4 did not decrease uptake of NE into the artery (Figure 4C). Since NET can transport NE bidirectionally (34), our data suggest that ephA4 promotes NE efflux through the NET. The role of basal NE release is unclear, but studies indicate that basal release of other neurotransmitters is physiologically relevant (1,9,24). Thus, it is likely that basal release would affect the concentration of NE at sympathetic neurovascular junctions and thus affect vascular function.

It is well known that targets affect the function of the postganglionic sympathetic neurons that innervate them (2,3,4,19,23,32,37). In most cases, these effects are mediated via soluble target-derived factors (2,3,4,19,23,32). Both ephs and ephrins are cell associated, and thus cell contact is required for eph/ephrin interactions (5). The present studies suggest perivascular eph/ephrin interactions modulate the growth and function of perivascular sympathetic nerves. Our findings thus suggest a novel contact-dependent mechanism for target modulation of sympathetic function.

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References

1. Carter AG, Regehr WG. Quantal events shape cerebellar interneuron firing. *Nat. Neurosci.* 2002; 5:1309–1318. [PubMed: 12411959]
2. Damon DH. NGF-independent survival of postganglionic sympathetic neurons in neuronal-vascular smooth muscle cocultures. *Am. J. Physiol. Heart Circ. Physiol.* 2001; 280:1722–1728.

3. Damon DH. TH and NPY in sympathetic neurovascular cultures: Role of LIF and NT-3. *Am. J. Physiol. Cell Physiol.* 2008; 294:C306–C312. [PubMed: 18032527]
4. Damon DH, teRiele JA, Marko SB. Vascular-derived artemin: a determinant of vascular sympathetic innervation? *Am. J. Physiol. Heart Circ. Physiol.* 2007; 293:H266–H273. [PubMed: 17337595]
5. Davis S, Gale NW, Aldrich TH, Maisonnier PC, Lhotak V, Pawson T, Goldfarb M, Yancopoulos GD. Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science.* 1994; 266:816–819. [PubMed: 7973638]
6. Davy A, Gale NW, Murray EW, Klinghoffer RA, Soriano P, Feurstein C, Robbins SM. Compartmentalized signaling by GPI-anchored ephrin-A5 requires the fyn tyrosine kinase to regulated cellular adhesion. *Genes & Develop.* 1999; 13:3125–3135. [PubMed: 10601038]
7. Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiara TM, McDonald DM, Yancopoulos GD. Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. *Develop. Biol.* 2001; 230:151–160. [PubMed: 11161569]
8. Gao P, Sun C, Zhou X, DiCicco-Bloom E, Zhou R. Ephrins stimulate or inhibit neurite outgrowth and survival as a function of neuronal cell type. *J. Neurosci.* 2000; 60:427–436.
9. Glitsch MD. Spontaneous neurotransmitter release and Ca^{2+} - How spontaneous is spontaneous neurotransmitter release? *Cell Calcium.* 2008; 43:9–15. [PubMed: 17382386]
10. Grunwald IC, Korte M, Adelmann G, Plueck A, Kullander K, Adams RH, Frotscher M, Bonhoeffer T, Klein R. Hippocampal plasticity requires postsynaptic ephrinBs. *Nat. Neurosci.* 2004; 7:33–40. [PubMed: 14699416]
11. Guyenet PG. The sympathetic control of blood pressure. *Nat. Rev. Neurosci.* 2006; 7:335–346. [PubMed: 16760914]
12. Henderson JT, Georgiou J, Jia Z, Robertson J, Elowe S, Roder JC, Pawson T. The receptor tyrosine kinase ephB2 regulates NMDA-dependent synaptic function. *Neuron.* 2001; 32:1041–1056. [PubMed: 11754836]
13. Horvath G, Sutto Z, Torbati A, Conner GE, Salathe M, Wanner A. Norepinephrine transport by the extraneuronal monoamine transporter in human bronchial arterial smooth muscle cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2003; 285:L829–L837. [PubMed: 12807698]
14. Ikushima S, Muramatsu I, Fujiwara M. Effects of 4-aminopyridine on the adrenergic nerve terminals of rabbit arteries. *J. Pharmacol. Exp. Ther.* 1981; 219:792–797. [PubMed: 7299697]
15. Karoon P, Rubino A, Burnstock G. Enhanced sympathetic neurotransmission in the tail artery of 1,3-dipropyl-8-sulphophenylxanthine (DPSPX)-treated rats. *Br J Pharmacol.* 1995; 116:1918–1922. [PubMed: 8528580]
16. Kasemeier-Kulesa JC, Bradley R, Pasquale EB, Lefcort F, Kulesa PM. Eph/ephrins and N-cadherin coordinate to control the pattern of sympathetic ganglia. *Develop.* 2006; 133:4839–4847.
17. Klein R. Eph/ephrin signaling in morphogenesis, neural development and plasticity. *Curr. Opin. Cell Biol.* 2004; 16:580–589. [PubMed: 15363810]
18. Klein R. Bidirectional modulation of synaptic functions by eph/ephrin signaling. *Nat. Neurosci. Rev.* 2009; 12:15–20.
19. Levi-Montalcini R, Booker B. Destruction of the sympathetic ganglia in mammals by an antiserum to the nerve growth-promoting factor. *Proc. Natl. Acad. Sci. USA.* 1960; 46:384–391. [PubMed: 16578497]
20. Lichtor T, Davis HR, Johns L, Vesselinovitch D, Wissler RW, Mullan S. The sympathetic nervous system and atherosclerosis. *J Neurosurg.* 1987; 67:906–914. [PubMed: 3681430]
21. Lim BK, Matsuda N, Poo MM. Ephrin-B reverse signaling promotes structural and functional synaptic maturation in vivo. *Nat. Neurosci.* 2008; 11:160–169. [PubMed: 18193042]
22. Luff SE, Young SB, McLachlan EM. Hyperinnervation of mesenteric arteries in spontaneously hypertensive rats by sympathetic but not primary afferent axons. *J. Vasc. Res.* 2005; 42:348–358. [PubMed: 16015033]
23. Marko SB, Damon DH. VEGF promotes vascular sympathetic innervation. *Am. J. Physiol. Heart Circ. Physiol.* 2008; 294:H2646–H2652. [PubMed: 18408130]

24. McKinney RA, Capogna M, Durr R, Gahwiler BH, Thompson SM. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat. Neurosci.* 1999; 2:44–49. [PubMed: 10195179]
25. Mellitzer G, Xu Q, Wilkinson DG. Eph receptors and ephrins restrict cell intermingling and communication. *Nature.* 1999; 400:77–81. [PubMed: 10403252]
26. Ogita H, Kunimoto S, Kamioka Y, Sawa H, Masuda M, Mochizuki N. EphA4-mediated rho activation via vsm-rhoGEF expressed specifically in vascular smooth muscle cells. *Circ. Res.* 2003; 93:23–31. [PubMed: 12775584]
27. Oike Y, Ito Y, Hamada K, Zhang X, Miyata K, Arai F, Inada T, Araki K, Nakagata N, Takeya M, Kisanuki YY, Yanagisawa M, Gale GW, Suda T. Regulation of vasculogenesis and angiogenesis by EphB/ephrinB2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood.* 2002; 100:1326–1333. [PubMed: 12149214]
28. Pasquale EB. Eph-ephrin promiscuity is now crystal clear. *Nature Neurosci.* 2004; 7:417–418. [PubMed: 15114347]
29. Qin H, Noberini R, Huan X, Shi J, Pasquale EB, Song J. Structural characterization of the ephA4-Ephrin-B2 complex reveals new features enabling eph-ephrin binding promiscuity. *J. Biol. Chem.* 2010; 285:644–654. [PubMed: 19875447]
30. Ross R. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* 1971; 50:172–186. [PubMed: 4327464]
31. Sauzeau V, Sevilla MA, Rivas-Elena JV, de Álava E, Montero MJ, López-Novoa JM, Bustelo XR. *Vav3* protooncogene deficiency leads to sympathetic hyperactivity and cardiovascular dysfunction. *Nature Medicine.* 2006; 12:841–845.
32. Schotzinger RJ, Landis SC. Acquisition of cholinergic and peptidergic properties by sympathetic innervation of rat sweat glands requires interaction with normal target. *Neuron.* 1990; 5:91–100. [PubMed: 2369522]
33. Schrier RW, Abraham WT. Hormones and hemodynamics in heart failure. *New Eng J Med.* 1999; 31:577–585. [PubMed: 10451464]
34. Sitte HH, Freissmuth M. The reverse operation of Na⁺/Cl[−]—coupled neurotransmitter transporters – Why amphetamines take two to tango. *J. Neurochem.* 2010; 112:340–355. [PubMed: 19891736]
35. Takasu MA, Dalva MB, Zigmond RE, Greenberg ME. Modulation of NMDA receptor-dependent calcium influx and gene expression through ephB receptors. *Science.* 2002; 295:491–495. [PubMed: 11799243]
36. Uhrenholt TR, Nedergaard OA. Calcium channels involved in noradrenaline release from sympathetic neurones in rabbit carotid artery. *Pharmacol. Toxicol.* 2003; 92:226–233. [PubMed: 12753410]
37. Wingerd KL, Goodman NL, Tresser JW, Smail MM, Leu ST, Rohan SJ, Pring J, Jackson DY, Clegg DO. $\alpha 4$ integrins and vascular cell adhesion molecule-1 play a role in sympathetic innervation of the heart. *J. Neurosci.* 2002; 22:10772–10780. [PubMed: 12486170]
38. Young HM, Anderson RB, Anderson CR. Guidance cues involved in the development of the peripheral autonomic nervous system. *Auton. Neurosci.: Basic and Clin.* 2004; 112:1–14.

A.

B.

Figure 1. Eph and ephrin expression in postganglionic sympathetic neurons

A) Representative RT-PCR analyses of eph and ephrin mRNA expression in neonatal (n) and adult (a) superior cervical ganglia. B) Representative (n = 2) eph and ephrin binding to dissociated postganglionic sympathetic neurons derived from superior ganglia of neonatal rats.

Figure 2. Eph and ephrin expression in femoral arteries

Representative RT-PCR analyses of eph and ephrin mRNA expression in neonatal (n) and adult (a) femoral arteries and in VSM derived from explants of adult femoral arteries.

Figure 3. Eph/ephrin interactions promote reinnervation of denervated femoral arteries
 Reinnervation of femoral arteries 14 days after denervation in the absence (control arteries; open bars) and presence of IgG-Fc (gray bar) or ephA4-Fc (black bar). * indicates that reinnervation in the presence of ephA4-Fc ($n = 6$) was significantly less than control ($p < 0.05$; two-tailed unpaired t-test). + indicates that reinnervation in the presence of ephA4-Fc was significantly less than that in the presence of IgG-Fc ($n = 5$) ($p < 0.05$; one-tailed unpaired t-test).

Figure 4. Eph/ephrin interactions decrease basal release of norepinephrine

A) Basal release of norepinephrine (NE) from freshly isolated tail arteries incubated for two hours with 10 $\mu\text{g/ml}$ control IgG-Fc (open bars) or ephA4-Fc (closed bars). * indicates that release in the presence of ephA4-Fc was significantly greater than that in the presence of IgG-Fc ($n=4$; $P < 0.05$; one-tailed unpaired t-test assuming unequal variances). B) Stimulated release of NE from freshly isolated tail arteries incubated for 2 hours with 10 $\mu\text{g/ml}$ control IgG-Fc (open bars) or ephA4-Fc (closed bars). C) NE uptake by freshly isolated tail arteries incubated for 2 hours with 10 $\mu\text{g/ml}$ control IgG-C (open bars) or ephA4-Fc (closed bars).

Table 1

PCR parameters

Gene	Primer Sequence	Annealing Temp (C)
<i>ephrin A1</i>	5' AGTTCAAATCCCAAGTTCCGAGAG 3' (S) 5' GCACTGGGTTTCCTGATGGTAGAT 3' (AS)	55
<i>ephrin B2</i>	5' ATCACCCCTAACCTCTCCTGCG 3' (S) 5' GCACAGGACACTTCTCAATGTGG 3' (AS)	54
<i>eph A4</i>	5' CCCTGCACCCGCCACCAT 3' (S) 5' TTCCAGCCAAGCCAGAGCCACACT 3' (AS)	55
<i>eph B2</i>	5' AAAATTGAGCAGGTGATCGG 3' (S) 5' TCACAGGTGTGCTCTTGGT 3' (AS)	54