Published in final edited form as:

J Neurosci Res. 2010 May 1; 88(6): 1285–1297. doi:10.1002/jnr.22291.

Nitric Oxide Regulates BDNF Release from Nodose Ganglion Neurons in a Pattern-Dependent and cGMP-Independent Manner

Hui-ya Hsieh¹, Carolyn L. Robertson¹, Anke Vermehren-Schmaedick¹, and Agnieszka Balkowiec^{1,2}

¹Department of Integrative Biosciences, Oregon Health and Science University, Portland, OR

²Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, OR

Abstract

Activity of arterial baroreceptors is modulated by neurohumoral factors, including nitric oxide (NO), released from endothelial cells. Baroreceptor reflex responses can also be modulated by NO signaling in the brainstem *nucleus tractus solitarius* (NTS), the primary central target of cardiovascular afferents. Our recent studies indicate that brain-derived neurotrophic factor (BDNF) is abundantly expressed by developing and adult baroreceptor afferents *in vivo*, and released from cultured nodose ganglion (NG) neurons by patterns of baroreceptor activity. Using electrical field stimulation and ELISA *in situ*, we show that exogenous NO nearly abolishes BDNF release from newborn rat NG neurons *in vitro* stimulated with single pulses delivered at 6 Hz, but not 2-pulse bursts delivered at the same 6-Hz frequency, that corresponds to a rat heart rate. Application of L-NAME, a specific inhibitor of endogenous NO synthases, does not have any significant effect on activity-dependent BDNF release, but leads to upregulation of BDNF expression in an activity-dependent manner. The latter effect suggests a novel mechanism of homeostatic regulation of activity-dependent BDNF expression with endogenous NO as a key player. The exogenous NO-mediated effect does not involve the cGMP-protein kinase G (PKG) pathway, but is largely inhibited by N-ethylmaleimide and TEMPOL that are known to prevent S-nitrosylation. Together, our current data identify previously unknown mechanisms regulating BDNF availability, and point to NO as a likely regulator of BDNF at baroafferent synapses in the NTS.

Keywords

Baroreceptor; Electrical field stimulation; Nodose ganglion; S-nitrosylation; Sodium channel

INTRODUCTION

It has previously been demonstrated that nitric oxide (NO) is a potent modulator of visceral sensory function (Page et al., 2009), including baroreflex function (Wecht et al., 2008), acting both at first-order baroafferent neurons (Matsuda et al., 1995; Li et al., 1998; Meyrelles et al., 2003) and baroreflex circuits in the brainstem *nucleus tractus solitarius* (NTS; Sakuma et al., 1992; Ma et al., 1995). NO inhibits sodium currents and decreases excitability in nodose ganglion (NG) neurons (Snitsarev et al., 2002b), including baroreceptor afferents (Matsuda et al., 1995; Li et al., 1998; Bielefeldt et al., 1999; Snitsarev et al., 2002a; Meyrelles et al., 2003). Acting centrally, L-arginine-derived NO modulates the spontaneous discharge

frequency of NTS neurons. While a majority of NTS neurons are inhibited, some show increases in the discharge rate during a blockade of nitric oxide synthesis (Ma et al., 1995; Lemus et al., 2009).

Recent studies from our laboratory demonstrate that brain-derived neurotrophic factor (BDNF) is abundantly expressed in developing and adult NG neurons, including the baroafferent population, *in vivo*. Moreover, the magnitude of native BDNF release from NG neurons *in vitro* is regulated by physiological patterns of baroreceptor activity, with bursting patterns being significantly more effective than tonic stimulation at the same average frequency (Martin et al., 2009). These data, together with the fact that TrkB, the high-affinity receptor for BDNF, is expressed by sensory relay neurons in the NTS (Balkowiec et al., 2000), point to BDNF as a candidate mediator of plastic changes at baroafferent synapses. However, the characteristics of BDNF release in baroafferent pathways remain unclear, including specific factors and conditions that modulate BDNF availability.

Currently available data on NO regulation of BDNF are conflicting. While it has been demonstrated that both activity-dependent BDNF expression (Xiong et al., 1999) and release (Canossa et al., 2002) are downregulated by NO, endothelial NO knockout mice show decreased BDNF expression (Chen et al., 2005). In the hippocampus, NO leads to a down-regulation of BDNF release and this effect is mediated through the cGMP/protein kinase G (PKG) pathway (Canossa et al., 2002). In baroreceptor afferents, however, NO is known to inhibit sodium channels through S-nitrosylation of cysteine groups, independent of the cGMP/PKG pathway (Li et al., 1998). Since regulated BDNF release requires the activity of sodium channels (Balkowiec and Katz, 2000, 2002), these findings raise the possibility that NO affects BDNF release from NG neurons *via* two distinct mechanisms, a cGMP-dependent and a cGMP-independent mechanism.

The present study was undertaken to test the hypothesis that nitric oxide modulates activity-dependent BDNF release from nodose neurons and to examine the underlying cellular mechanisms of NO effects on activity-dependent BDNF release. Portions of this work have previously been published in abstract form (Balkowiec 2003; Robertson and Balkowiec, 2004).

MATERIALS AND METHODS

Animals

Postnatal day (P) 0–1 Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used for this study. All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University, and conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiological Society.

Preparation of NG cultures

P0–1 rat pups were euthanized by intraperitoneal injection of Euthazol (0.1 mg/kg) and decapitated. Nodose ganglia were dissected and dissociated as recently described by our laboratory (Martin et al., 2009). Dispersed NG cells were plated in: i) UV-sterilized, 96-well, flat bottom ELISA plates (MaxiSorp™, Nalge Nunc Int., Naperville, IL) pre-coated with anti-BDNF capture antibody (BDNF E_{max}™ ImmunoAssay System, Promega; for BDNF ELISA *in situ*; Balkowiec and Katz, 2000), ii) 48-well plates (Nunc Surface; Nalge Nunc Int.) pre-coated with poly-D-lysine (0.1 mg/ml; Sigma) and laminin (0.4 µg/ml; Sigma; for BDNF cell content by ELISA), or iii) 48-well (Nalge Nunc Int.) or 24-well (Falcon; Becton Dickinson and Company, Franklin Lakes, NJ) tissue culture-treated polystyrene plates on poly-D-lysine/laminin-coated glass coverslips (for immunocytochemistry and S-nitrosylated protein

detection). For BDNF release, one NG was used per culture well of a 96-well plate, and an average experiment (8–12 treatment conditions) consisted of 24–36 wells (in triplicates), including no-electrode and electrode-fitted but not stimulated controls, each treated with either a vehicle or a drug. To examine changes in BDNF cell content, three dispersed nodose ganglia were plated per one culture well (48-well plates), and 24–36 wells comprised a single experimental set. Similarly, three ganglia were used per well in the immunocytochemical studies of the effects of electrical stimulation on expression of the neuronal isoform of the nitric oxide synthase (nNOS) in NG neurons and the S-nitrosylation assay. The cultures were grown in Neurobasal-A medium (Invitrogen) supplemented with B-27 serum-free supplement (Invitrogen), 0.5 mM L-glutamine (Invitrogen), 2.5% fetal bovine serum (HyClone, Logan, UT), 1% Penicillin-Streptomycin-Neomycin antibiotic mixture (Invitrogen), and in some experiments, 2.5% Nystatin (Sigma), for 3–7 days at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. For cultures grown longer than 3 days, the medium was replaced with fresh medium on day 3 and 6.

Electrical field stimulation

Following the initial incubation, NG cultures were stimulated in 48- or 96-well plates as previously described by our laboratory (Buldyrev et al., 2006). Specifically, the wells were fitted with paired Ag/AgCl (BDNF release; 96-well plates) or platinum (BDNF cell content; 48-well plates) electrodes (0.25 mm wire diameter; one pair per well), connected in parallel (four or six wells per set) to one of four independent outputs of the stimulator (MultiStim System; Digitimer; Welwyn Garden City, Hertfordshire, UK). The stimulation pattern delivered by each of the outputs was controlled by the 8-channel programmable pulse generator Master-8-cp (AMPI, Jerusalem, Israel). Two additional sets of four wells were included on the same plate: one fitted with pairs of electrodes, but not connected to the stimulator (non-stimulated controls), and the other without electrodes (no-electrode controls). There were no significant differences in the levels of BDNF between the non-stimulated and no-electrode controls, independent of drug treatment, indicating that the electrodes per se do not affect activity-dependent regulation of BDNF. The plate was put back to the incubator, and the neurons were stimulated with biphasic rectangular pulses of 0.2 or 0.5 ms duration and amplitude of 80–120 mA per well, delivered at various patterns (see Results) for 1 hour (BDNF release studies) or 24 hours (BDNF expression studies and immunocytochemical evaluation of changes in nNOS expression).

Drug treatment

Cultures were treated with the drugs for 30 min prior to electrical stimulation, and then during the stimulation and post-stimulation incubation (a total of 2.5 hrs and 24.5 hrs for BDNF release and expression, respectively) at 37 °C in humidified atmosphere of 95% air / 5% CO₂). Each drug (or drug combination) and each vehicle (or vehicle combination) were added to both stimulated and unstimulated cultures, for a total of at least four conditions per each pharmacological treatment. NOR3, an *NO* donor (Calbiochem, La Jolla, CA), (±)-S-Nitroso-N-acetylpenicillamine (SNAP), an *NO* donor (Calbiochem), YC-1, an agonist of soluble guanylyl cyclase (Sigma), KT 5823, a blocker of PKG (Sigma), and 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), a cell-permeable radical scavenger (Calbiochem) were dissolved in dimethyl sulfoxide (DMSO) and used at final concentrations of 100 μM, 1 μM, 100 μM, 40 μM, and 100 μM, respectively; the final concentration of DMSO was 0.2, 0.2, 0.25, 0.02, and 0.2 %, respectively. N-ω-Nitro-L-arginine methyl ester (L-NAME), an inhibitor of endogenous *NO* synthases (Tocris, Ellisville, MO), papaNONOate, an *NO* donor (Sigma), 8-Bromoguanosine 3', 5'-cyclic monophosphate sodium salt, a membrane-permeable analogue of cGMP (8-Br-cGMP; Sigma), Thimerosal, a sulfhydryl oxidant (Sigma), and L-cysteine free base (Sigma) were dissolved in PBS and used at final concentrations of 1 mM, 1mM, 0.5 mM,

0.1 mM, and 10 mM, respectively. N-ethylmaleimide, *an inhibitor of S-nitrosylation* (NEM; Sigma) was dissolved in distilled water, and used at the final concentration of 2 mM.

Measurement of BDNF release

BDNF protein level was measured with a modified sandwich ELISA, termed ELISA *in situ*, as previously described (Balkowiec and Katz, 2000; Buldyrev et al., 2006; Martin et al., 2009). Briefly, 96-well ELISA plates were coated with anti-BDNF monoclonal capture antibody (BDNF E_{max}TM ImmunoAssay System, Promega). Dissociated nodose ganglia, prepared as described above, were plated in anti-BDNF-coated wells, and grown for 3–5 days. The BDNF E_{max}TM ImmunoAssay System (Promega) was used according to the protocol of the manufacturer, except that the concentration of the anti-BDNF monoclonal and anti-human BDNF polyclonal antibody was 3 µg/ml and 2 µg/ml, respectively, and the dilution of the anti-IgY-HRP antibody was 1:100. BDNF samples used to generate standard curves were incubated in the same plate as the NG cell culture. Following cell stimulation, pharmacological treatment and a 1-h post-stimulus incubation, plates were extensively washed to remove all cells and cell debris, and the anti-human BDNF polyclonal antibody was applied, followed by subsequent steps according to the manufacturer's protocol.

Measurement of BDNF cell content

NG cultures grown in 48-well plates were treated with 75 µl/well of pre-chilled lysis buffer (20 mM Tris buffer, pH 7.4, 137 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM PMSF, 0.5 mM sodium vanadate, 10 µM aprotinin, 10 µM actinonin, and 100 µM leupeptin) immediately following removal of the culture medium. Next, the cells were scraped off with a pipette tip, and the entire well content was transferred to a siliconized (Sigmacote®; Sigma) and pre-chilled 1.5-ml microcentrifuge tube. Each well was then rinsed with 150 µl of pre-chilled Block & Sample buffer (1x; BDNF E_{max}TM ImmunoAssay System, Promega), and the well content was transferred into the microcentrifuge tube to combine with the cell lysate. The content of two identically treated sister culture wells was combined in one microcentrifuge tube. Next, each sample (the total volume of 450 µl) was sonicated on ice using a microprobe sonicator (2 × 1.5 W, 5 sec each; Sonicator 3000, Misonix, Inc., Farmingdale, NY), followed by transfer to an anti-BDNF-coated 96-well ELISA plate (4 wells/sample). BDNF ELISA was performed according to the manufacturer's protocol (BDNF E_{max}TM ImmunoAssay System, Promega).

Calculations and statistical analysis

Each value of activity-dependent BDNF release and expression was calculated by subtracting levels of BDNF in unstimulated, but treated with vehicle or drug, cultures from levels of BDNF measured in stimulated, and correspondingly treated, cultures. Consequently, the amount of BDNF released (44.03 ± 1.81 pg/ml, $n=22$ cultures) or expressed (14.82 ± 0.84 pg/culture, $n=18$ cultures) over the culture period prior to stimulation was not taken into account. BDNF levels were calculated from the standard curve prepared for each plate, using SOFTmax PRO® vs. 4.3 software (Molecular Devices). The standard curves were linear within the range used (0–500 pg/ml) and the quantities of BDNF in experimental samples were always within the linear range of the standard curve. Data are expressed as mean $\pm$ standard error, and the sample size (n) represents the number of individual cultures. Samples were compared using ANOVA followed by Duncan's or Tukey's multiple comparison procedure, and $P<0.05$ was considered significant.

RNA interference

Rat small interfering RNAs (siRNAs) directed against BDNF and GAPDH mRNA were obtained from Dharmacon, Inc (Chicago, IL). The BDNF siRNA consisted of a pool of four 21-nucleotide duplexes with 3'-UU overhangs (accell SMARTpool). The four BDNF target

sequences were: 5'-GCCUAGGUUUUAUUUAUUU-3', 5'-GAUUUAUGUUGUAUAGAUU-3', 5'-CACGUAGCCUAGAUUGUUU-3', 5'-CGAUA AUGUUGUGGUUUGU-3'. The SMART pool BDNF, accell GAPDH (D-001930-03-05) and accell non-targeting (D-001950-01-05) siRNAs were resuspended in 1X siRNA universal buffer (Dharmacon B-002000-UB-100) to a stock concentration of 100 μ M, divided to aliquots and stored at -80°C .

Neuron-enriched NG cultures were prepared as previously described (Buldyrev et al., 2006; Scanlin et al., 2008; Tarsa and Balkowiec, 2009), except that 5 nodose ganglia were plated per well of a 48-well plate, and no antibiotics were used. One day after plating, cells were rinsed with antibiotic-free culture medium, followed by transfection with 500 nM of BDNF, GAPDH, or non-targeting siRNA. Specifically, each stock siRNA, diluted in OPTI-MEM reduced-serum medium, was first incubated with Lipofectamine RNAiMAX (1 vol%; Invitrogen) for 20 minutes at room temperature to allow complex formation, and then added to NG cultures. On day 4 of culture, neurons were rinsed with antibiotic-free culture medium and fresh siRNA was added as described for day 1. Cultures were processed for BDNF mRNA and protein content on day 7.

Quantitative PCR

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions, followed by re-suspension in 10 mM Tris pH 8.5 and storage at -80°C until used. To make cDNA, 1 μ g of total RNA was reverse transcribed (20 μ l) using Tetro cDNA synthesis kit (Bioline, Taunton, MA). Quantitative PCR was performed with the MX3000P real time PCR system (Stratagene; Cedar Creek, TX) in a final volume of 10 μ l using Sensimix Plus SYBR master mix (Quantace, Taunton, MA), 2 μ l of cDNA (diluted 1:4), and 3 μ M of each forward and reverse primers. Primer sequences for the BDNF protein coding region (forward: 5'-GGTCACAGCGGCAGATAAAAAGAC-3'; reverse: 5'-TTCGGCATTGCGAGTTCCAG-3'), as well as the housekeeping gene β -actin (forward: 5'-TGTCACCAACTGGGACGATA-3', reverse: 5'-GGGGTGTGTAAGGTCTCAA-3') were designed using Primer3 online software and synthesized by Integrated DNA Technologies (Coralville, IA). The real time amplification data were collected continuously and analyzed using the software supplied with the MX3000P real time PCR system.

S-nitrosylated protein detection—Cultures were fixed with 4% paraformaldehyde in Wash Buffer (S-Nitrosylated Protein Detection Kit; Cayman Chemical, Ann Arbor, MI) for 20 min, immediately following the 1-hr post-stimulation incubation. All subsequent steps of the staining protocol were performed according to the protocol for adherent cultured cells described in the manual of the S-Nitrosylated Protein Detection Kit (Cayman Chemical), using fluorescein as the detection reagent.

Double immunostaining of NG cultures for the neuronal isoform of nitric oxide synthase (nNOS) and either BDNF or protein gene product 9.5 (PGP 9.5)—

Cultures were fixed with 2% (for nNOS/BDNF staining) or 4% (for nNOS/PGP 9.5 staining) paraformaldehyde in 0.1 M sodium phosphate buffer, pH=7.4, for 30 min, and stained according to our previously published protocols (Buldyrev et al., 2006; Scanlin et al., 2008; Martin et al., 2009; Tarsa and Balkowiec, 2009). Briefly, following rinsing and blocking steps, cultures were incubated for 2 h in mouse monoclonal anti-nNOS (1:2500; BD Biosciences, San Jose, CA) combined with either chicken polyclonal anti-BDNF (1:50 or 1:100; Promega) or rabbit anti-PGP 9.5 (1:1000; Accurate Chemical). The secondary antibodies: goat anti-mouse IgG-Cy3 (1:200, Jackson ImmunoResearch), goat anti-chicken biotinylated IgG (1:200, Vector Laboratories), and goat anti-rabbit Alexa 488 (Invitrogen) were applied for 1 h in the

dark. Control cultures, in which primary antibodies were omitted, were completely devoid of staining.

Microscopy, digital imaging and cell counts—Stained NG cultures were imaged with Olympus IX-71 inverted microscope (Olympus America Inc., Center Valley, PA), and images captured with Hamamatsu ORCA-ER CCD camera (Hamamatsu, Bridgewater, NJ, CA) controlled by either Wasabi (Hamamatsu) or Olympus Microsuite (vs. 5.0; Olympus America, Inc.) software. At least 4 (20x objective; PGP9.5/nNOS) or 12 (20x and 40x objective; BDNF/nNOS) randomly chosen fields of view were analyzed per experiment. Double PGP9.5/nNOS and double BDNF/nNOS immunoreactivity were determined using ImageJ software (National Institutes of Health, Bethesda, MD). First, all PGP9.5- or BDNF- immunoreactive neurons located within a field of view were selected, counted and marked. Next, nNOS-immunoreactive cells among PGP9.5- or BDNF-immunoreactive cells were marked and counted on sister images. Cell was considered immunoreactive when the staining intensity was clearly above the background level, established independently by each investigator and used for all analyzed images.

RESULTS

It is well established that baroreceptor activity is regulated by neurohumoral factors released from endothelial cells and adventitia (Kunze et al., 1984; Chapleau et al., 1988; Chapleau 1992; Matsuda et al., 1995; Meyrelles et al., 2003). One of the most extensively studied endothelium-derived factors is nitric oxide (NO) for its role in vascular regulation and neuronal plasticity. Therefore, in the present study we examined the effects of NO on activity-dependent BDNF release from nodose ganglion (NG) neurons *in vitro* and began exploring the underlying cellular mechanisms.

To detect changes in native BDNF release, we employed a modified sandwich enzyme-linked immunosorbent assay (ELISA), which is termed 'ELISA *in situ*' based on a close proximity of BDNF-releasing cells to the BDNF capture antibody layer of the ELISA (Balkowiec and Katz, 2000). In order to demonstrate the specificity of the BDNF ELISA kit, used to quantify BDNF protein levels, we applied the RNA interference technique. RNA interference is a new approach that has proven very effective in knocking down a particular gene for short-term consequences (Sandy et al., 2005; Cullen, 2006; Kim and Rossi, 2008). We developed a sensitive and reliable quantitative PCR assay to measure and compare the relative amounts of BDNF mRNA (protein coding region, 188 bp) to the housekeeping gene β -actin (165 bp). Sister cultures of postnatal day (P) 1 NG neurons were treated for 6 days with small interfering (si) RNA constructs targeting transcription of either BDNF or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; housekeeping gene control). The 6-day duration of treatment was chosen in order to maintain low levels of BDNF mRNA and thus effectively reduce the BDNF protein, known to have a relatively long half-life (Nawa et al., 1995). Cultures treated with BDNF-, but not GAPDH-, siRNA showed a significant decrease in BDNF mRNA levels, as determined by quantitative PCR (Fig. 1 A). Consistent with the BDNF mRNA data, BDNF protein levels determined using the ELISA approach showed a severe reduction in cultures treated with BDNF-, but not GAPDH-, siRNA constructs (Fig. 1B). Thus, the BDNF ELISA faithfully reflects changes in BDNF levels in our culture model.

To stimulate NG neurons, we used two 1-h protocols of baroreceptor activity known to regulate the magnitude of BDNF release from these neurons, as demonstrated in our recent study (Martin et al., 2009): (i) single pulses delivered at 6 Hz (once every 166.7 ms; tonic stimulation), and (ii) 2-pulse bursts (inter-pulse interval 27.8 ms; bursting stimulation) delivered at 6 Hz, a frequency corresponding to a rat heart rate, as previously described by Liu et al., (2000).

Following the initial culture period, sister NG cultures were electrically stimulated using the above described protocols in the presence or absence of nitric oxide donors and pharmacological agents known to affect signaling downstream of NO.

Nitric oxide (NO) inhibits BDNF release from nodose ganglion (NG) neurons in a pattern-dependent manner

We first examined the effects of exogenous NO on BDNF release from NG neurons evoked by baroreceptor patterns of stimulation. Addition of 100 μ M NOR3, an NO donor, to NG cultures during their stimulation resulted in a near abolition (84.1% reduction) of BDNF release evoked by a single-pulse stimulation at 6 Hz (18.14 ± 2.29 pg/ml in stimulated control, $n=52$ cultures *versus* 2.89 ± 1.79 pg/ml with NOR3, $n=33$ cultures; $P<0.001$; Fig. 2). To verify that the action of NOR3 is mediated by NO, we examined the effects of two additional, structurally distinct NO donors, papaNONOate (1 mM) and ($\pm$)-S-Nitroso-N-acetylpenicillamine (SNAP; 1 μ M). All donors significantly inhibited BDNF release evoked by the single-pulse 6 Hz stimulation (34.74 ± 2.55 pg/ml in stimulated control, $n=8$ cultures *versus* 12.78 ± 0.92 pg/ml with papaNONOate, $n=4$ cultures, $P<0.05$, and 10.73 ± 1.95 pg/ml with SNAP, $n=12$ cultures, $P<0.001$). None of the three NO donors, i.e. NOR3, papaNONOate or SNAP, had any significant effect on BDNF release from unstimulated cultures (-2.95 ± 1.23 pg/ml compared to vehicle-treated, $n=42$ cultures; $P=0.4990$).

Interestingly, the same treatment applied to cultures stimulated with a bursting baroreceptor pattern (2-pulse bursts applied at 6 Hz) was ineffective (25.26 ± 3.06 pg/ml in stimulated control, $n=23$ cultures *versus* 21.17 ± 4.09 pg/ml with NOR3, $n=26$ cultures; $P=0.4368$; Fig. 2). A similar result was obtained with another NO donor, papaNONOate (1 mM; data not shown).

Together, these data indicate that exogenous NO is a potent inhibitor of activity-dependent BDNF release from NG neurons. However, the NO-mediated inhibition is dependent on the pattern of stimulation, with a 2-pulse bursting pattern showing resistance to the NO effects.

The neuronal isoform of nitric oxide synthase (nNOS) is coexpressed with BDNF in nodose ganglion (NG) neurons and regulates BDNF expression, but does not affect BDNF release *in vitro*

Our immunocytochemistry data indicate that a vast majority of BDNF-immunoreactive NG neurons *in vitro* shows nNOS immunoreactivity ($82.3 \pm 6.65\%$, $n=93$ BDNF-positive cells examined in 12 culture fields) in unstimulated control cultures. Similarly, most of nNOS-immunoreactive cells are also BDNF-immunoreactive ($84.0 \pm 4.08\%$, $n=101$ nNOS-positive cells examined; Fig. 3A). Therefore, we next explored the possibility that endogenous NO inhibits BDNF release from NG neurons in our cultures stimulated with baroreceptor patterns. We hypothesized that if indeed the endogenous NO tonically inhibited BDNF release, blocking activity of nNOS would result in an increase in BDNF release.

Treatment of cultures with 1 mM L-NAME, a specific inhibitor of endogenous NO synthases (Canossa et al., 2002), did not have any significant effect on the magnitude of unstimulated BDNF release ($+1.21 \pm 2.00$ pg/ml compared to vehicle-treated, $n=37$ cultures; $P=0.3697$) or BDNF release evoked by any of the baroreceptor patterns of stimulation examined. This includes 1-hr single-pulse stimulation at 6 Hz (21.35 ± 1.84 pg/ml in stimulated control, $n=36$ cultures, *versus* 22.14 ± 2.54 pg/ml with L-NAME, $n=34$ cultures; $P=0.8002$; Fig. 3B), and 1-hr 2-pulse bursting stimulation at 6 Hz (24.26 ± 1.73 pg/ml in stimulated control, $n=30$ cultures, *versus* 28.28 ± 2.43 pg/ml with L-NAME, $n=25$ cultures; $P=0.1508$).

To rule-out the possibility that the lack of an effect of nNOS blockade is due to the relatively high levels of BDNF release in stimulated control conditions (without L-NAME), we also examined a substantially weaker stimulation protocol, i.e. single-pulse stimulation at 1 Hz for 1 hour. However, application of L-NAME (1 mM) under the lower stimulation frequency remained without any significant effect on BDNF release (10.33 ± 1.78 pg/ml during 1-Hz control stimulation *versus* 13.29 ± 2.73 pg/ml during 1-Hz stimulation in the presence of L-NAME, $n=26$; $P=0.3668$).

According to the results of our immunocytochemical analysis, while more than a half of all neurons express nNOS in unstimulated control cultures ($51.6 \pm 7.39\%$, $n=105$ cells immunoreactive for the pan-neuronal marker protein gene product 9.5, PGP9.5, examined in 4 culture fields), the percentage significantly increases following 24-hr stimulation at 1 Hz ($73.6 \pm 2.51\%$, $n=118$ PGP9.5-positive cells examined in 6 culture fields; $P<0.01$). Therefore, we examined the effects of endogenous NOS on BDNF expression in NG neurons evoked by 24-hr electrical stimulation at 1 Hz. In the presence of L-NAME (1 mM), the levels of BDNF were significantly increased compared to cultures stimulated in the presence of vehicle (5.96 ± 0.49 pg/culture stimulated with vehicle *versus* 8.98 ± 0.43 pg/culture stimulated with L-NAME, $n=18$ cultures; $P<0.001$; Figure 3C). However, the same treatment paradigm did not have any effect on basal BDNF expression in unstimulated cultures (total BDNF cell content: 14.82 ± 0.84 pg/culture with vehicle *versus* 14.35 ± 0.74 pg/culture with L-NAME, $n=18$ cultures; $P=0.6784$). These data indicate that the electrical stimulation may fuel NO synthesis, which otherwise remains below threshold for regulating BDNF expression.

In summary, while endogenous NO potentially inhibits activity-dependent expression of BDNF over a 24-hour period, it does not regulate basal BDNF expression, nor does it play a role in regulation of BDNF release from NG neurons on the significantly shorter time scale of 2 hours.

Nitric oxide inhibition of BDNF release from nodose ganglion neurons is independent of the cGMP signaling pathway

The intriguing discovery that NO inhibits BDNF release from NG neurons in a pattern-dependent manner prompted us to examine the cellular mechanisms of the NO effects. A well-established, classical mechanism of modulation of neuronal function by NO is activation of the cGMP-protein kinase G (PKG) pathway (Ahern et al., 2002, Canossa et al., 2002). Therefore, we first investigated the possibility that NO inhibits BDNF release from NG neurons through a cGMP-dependent signaling mechanism.

We examined the effects of: i) YC1, an agonist of soluble guanylyl cyclase (sGC), which constitutes the endogenous link between NO and increased production of cGMP, ii) 8-Bromo-cGMP (8-Br-cGMP), a membrane-permeable analogue of cGMP, and iii) KT5823, a selective inhibitor of PKG (Canossa et al., 2002), on BDNF release evoked by two of the baroreceptor patterns used in the studies above: 1) a single-pulse stimulation at 6 Hz and 2) bursting stimulation with 2-pulse bursts applied at 6 Hz.

A 30-min pretreatment of NG cultures with 100 μ M YC1 had no significant effect on the magnitude of BDNF release evoked by either single-pulse stimulation at 6 Hz (16.99 ± 3.86 pg/ml in stimulated control, $n=11$ *versus* 17.65 ± 5.22 pg/ml with YC1, $n=12$; $P=0.9212$; Fig. 3A) or the 2-pulse bursting stimulation (23.30 ± 5.27 pg/ml in stimulated control, $n=13$ *versus* 21.63 ± 6.28 pg/ml with YC1, $n=16$; $P=0.8445$; Fig. 4A).

Similarly, treatment with 1 mM 8-Br-cGMP resulted only in a trend toward reduction of BDNF release evoked by 1-pulse stimulation at 6 Hz (19.42 ± 2.14 pg/ml in stimulated control, $n=31$ *versus* 15.65 ± 2.25 pg/ml with 8-Br-cGMP, $n=20$; $P=0.2473$; Fig. 4B) and by the bursting stimulation (23.15 ± 1.38 pg/ml in stimulated control, $n=6$ *versus* 19.50 ± 3.77 pg/ml with 8-

Br-cGMP, $n=5$; $P=0.3532$; Fig. 4B). Clearly, the effect of 8-Br-cGMP on BDNF release evoked by the single-pulse stimulation protocol (19.4 % reduction) was very weak in comparison with the inhibition of 84.1 % caused by exogenous NO (Fig. 2).

To further examine the contribution of the cGMP pathway to the inhibitory effects of exogenous NO on BDNF release, we pretreated NG cultures with 40 μM KT5823, a blocker of PKG, which is a well-documented downstream target of the NO/cGMP pathway in neurons (Canossa et al., 2002). We hypothesized that if the cGMP-PKG pathway mediated the NO inhibition of BDNF release, blocking PKG would prevent the effects of NO.

Consistent with our prior observations that endogenous NO has no significant effect on either basal or activity-dependent BDNF release from NG neurons, KT5823 alone (i.e. without the NO donor) did not have any significant effect on either unstimulated BDNF release (-2.60 ± 7.64 pg/ml compared to vehicle-treated, $n=15$ cultures; $P=0.9949$), or BDNF release evoked by 1-pulse, tonic stimulation at 6 Hz (20.37 ± 1.93 pg/ml in untreated stimulated control, $n=38$, versus 18.50 ± 3.16 pg/ml with KT5823, $n=10$; $P=0.6508$; Fig. 4C).

Application of KT5823 in the presence of the NO donor did not have any significant effect on the NO-mediated inhibition of BDNF release evoked by the single-pulse stimulation pattern (20.37 ± 1.93 pg/ml in untreated stimulated controls, $n=38$, versus 3.70 ± 1.76 pg/ml with NOR3, $n=27$, versus 6.36 ± 3.02 pg/ml with NOR3 and KT5823, $n=20$; $P=0.4236$; Fig. 4C).

Together, these data suggest that NO inhibits activity-dependent BDNF release from NG neurons by another mechanism, independent of the cGMP pathway.

A mechanism involving S-nitrosylation significantly contributes to the nitric oxide-mediated inhibition of BDNF release

Recent studies indicate that, in addition to guanylyl cyclase- and cGMP- dependent signaling, NO can exert its effects on neuronal function acting through modification of sulfhydryl groups, S-nitrosylation (Ahern et al., 2002; Riccio et al., 2006). These data, together with the fact that NO inhibits sodium currents in baroreceptor neurons through S-nitrosylation of cysteine sulfhydryl groups in sodium channel proteins (Li et al., 1998), led us to explore the involvement of S-nitrosylation in NO regulation of BDNF release from NG neurons.

Using a cytochemical approach, we examined whether our treatment with NO donors leads to S-nitrosylation of cellular targets in our cultures. Sister cultures of newborn NG neurons grown on glass coverslips were first pre-treated with NOR3 (100 μM) or vehicle, followed by tonic electrical stimulation at 6 Hz applied for 1 hr to a half of the cultures, and then 1-hr post-stimulation incubation, to mimic the treatment protocol used in our BDNF release studies. Our data indicate that both electrical stimulation and NOR3 applied separately result in marked increases in the signal corresponding to S-nitrosylated proteins in NG neurons. However, the effect of a simultaneous application of electrical stimulation and NOR3 is remarkably stronger (Fig. 5A).

To determine whether S-nitrosylation is involved in the mechanism of NO-mediated inhibition of BDNF release from NG neurons, we first used the alkylating agent N-ethylmaleimide (NEM), which prevents S-nitrosylation by covalently modifying sulfhydryl groups (Li et al., 1998; Renganathan et al., 2002). Treatment of NG cultures with 2 mM NEM resulted in a significant 47.2 % reversal of the NO-mediated inhibition of BDNF release evoked by single-pulse, tonic stimulation at 6 Hz (17.03 ± 3.39 pg/ml in stimulated control, $n=7$ cultures versus 3.25 ± 1.25 pg/ml with NOR3, $n=8$ cultures versus 9.75 ± 2.07 pg/ml with NOR3 and NEM, $n=5$ cultures; $P<0.0153$; Fig. 5B). NEM alone did not have any significant effect on the stimulated BDNF release (14.17 ± 1.83 pg/ml, $n=7$ cultures; $P=0.4727$; Fig. 5B). These data

suggest that, indeed, S-nitrosylation is a likely mechanism of NO-induced inhibition of stimulated BDNF release.

In a recent study exploring differential roles of S-nitrosylation and the cGMP-PKG pathway in cardiac contractility, the authors used another tool, the radical scavenger and nitric oxide spin trap TEMPOL, to successfully block S-nitrosylation mediated by relatively low concentrations (0.1–10 μ M) of SNAP, an NO donor (González et al., 2008). Thus, to further explore whether S-nitrosylation is involved in the NO-mediated inhibition of stimulated BDNF release, we next examined the effects of TEMPOL. Consistent with the role of S-nitrosylation, and similar to the effects of NEM, treatment of NG cultures with 100 μ M TEMPOL resulted in a significant 73.8% reversal of the NO-mediated inhibition of BDNF release evoked by tonic stimulation at 6 Hz (34.74 ± 2.55 pg/ml in stimulated control, $n=8$ cultures *versus* 10.73 ± 1.95 pg/ml with 1 μ M SNAP, $n=12$ cultures *versus* 28.46 ± 1.57 pg/ml with SNAP and TEMPOL, $n=10$ cultures; $P=0.00014$; Fig. 5B). TEMPOL alone did not have a significant effect on the stimulated BDNF release (38.40 ± 1.46 pg/ml, $n=4$ cultures; $P=0.3607$; Fig. 5C).

We have also attempted to examine the effects of exogenous L-cysteine, acting as a substrate which competitively binds to NO, thus reducing the possibility of S-nitrosylation of cellular targets. However, L-cysteine interfered with the ELISA and impaired BDNF detection (data not shown).

Nitrosylation and oxidation of thiol groups of sodium channel proteins similarly affect the channel function (Evans and Bielefeldt, 2000; Song et al., 2000). Therefore, we used thimerosal, a sulfhydryl oxidant and potent inhibitor of sodium channels in sensory neurons (Song et al., 2000), to determine whether an inhibition of sodium channels by means other than S-nitrosylation also affects activity-dependent BDNF release from NG neurons. In these studies, BDNF release evoked by 6-Hz tonic stimulation of NG cultures was reduced by 45.1 % in the presence of 100 μ M thimerosal compared to control cultures (16.41 ± 2.28 pg/ml in stimulated control, $n=13$ *versus* 9.01 ± 1.70 pg/ml with thimerosal, $n=10$, $P=0.0225$). Together, these data strongly suggest that modifications of sodium channel proteins affect activity-dependent BDNF release from neurons. Moreover, S-nitrosylation of sodium channels by NO likely contributes to the NO-mediated inhibition of BDNF release.

DISCUSSION

The present study shows for the first time that nitric oxide (NO) inhibits BDNF release in a pattern-dependent manner. The cellular mechanisms of the NO-mediated inhibition of BDNF release are independent of the cGMP-protein kinase G (PKG) pathway, and likely involve S-nitrosylation of sodium channel proteins.

Our study suggests a novel role for nitric oxide (NO) acting on baroreceptor afferents to regulate BDNF release. Previous studies have indicated that NO plays an important role in the modulation of baroreceptor reflexes, acting at baroreceptor afferents (Matsuda et al., 1995; Li et al., 1998; Meyrelles et al., 2003), and NTS synapses (Dias et al., 2003, 2005; Lin et al., 2007). We found that endogenous nitric oxide synthase (NOS), expressed in BDNF-containing neurons in our cultures (Fig. 3A), did not play a role in the regulation of activity-dependent BDNF release, whereas exogenous NO had a profound inhibitory effect on the release. Although nodose neurons themselves, including the baroreceptor population, express the neuronal isoform of NOS (Aimi et al., 1991; Wang et al., 1993; Hohler et al., 1994; Tanaka and Chiba, 1994; Dun et al., 1995; Li et al., 1998; Atkinson et al., 2003), the neuronal NOS is not expressed in vagal afferent terminals in the brainstem (Atkinson et al., 2003), the presumed site of BDNF release both *in vivo* and in our *in vitro* model. In view of recent studies demonstrating the role of tonic (endothelial NOS-derived) and phasic (neuronal NOS-derived)

NO signals as mediators of synaptic plasticity (Garthwaite et al., 2006; Hopper and Garthwaite, 2006), it is likely that NO regulates BDNF release from vagal afferents in response to NOS activation in either the postsynaptic neuron (Atkinson et al., 2003) or microvascular endothelial cells (Garthwaite et al., 2006). In fact, it has previously been demonstrated specifically for baroreceptor neurons that their activity is affected by neurohumoral factors, including NO, released from one of two layers of the arterial wall, i.e. endothelium and adventitia (Kunze et al., 1984; Chapleau et al., 1988; Chapleau 1992; Matsuda et al., 1995; Meyrelles et al., 2003).

Another potential explanation for the lack of the effect of NOS inhibition on BDNF release evoked by 1-hr electrical stimulation could be that the amounts of generated NO, or the time course of its increase, are not sufficient to affect BDNF release. This possibility is supported by our data showing the effect of endogenous NOS on BDNF expression during a 24-hr stimulation, providing additional mechanism by which NO can regulate BDNF availability. The molecular mechanisms of the NO-mediated regulation of BDNF expression will be the subject of our future studies. The lack of the effect of NOS blockade on BDNF expression in unstimulated cultures suggests that neuronal activity is required for the NO-mediated regulation of BDNF expression. The results of our staining for S-nitrosylated proteins suggest that electrical stimulation alone increases the amount of S-nitrosylation, and the effect of NOR3 is more powerful in the presence of electrical stimulation (Fig. 5A). This information strongly suggests that endogenous NO is promoted by electrical stimulation, consistent with the lack of the effect of NOS blockade in unstimulated cultures.

However, we cannot rule out the possibility that the lack of an effect of endogenous NO on BDNF release from NG neurons in our model, or some other results, are due to the culture conditions. For example, we do not know whether the subcellular sites of BDNF release from cultured neurons match those *in vivo*, and the potential distance between the site of NO synthesis and BDNF release could prove critical. Our data indicate that neuronal activity plays a crucial role in the NO-mediated regulation of BDNF expression and release. The stimulation protocols were designed to mimic activity of NG neurons *in vivo*, but the possibility arises that not all stimulation parameters are ideally suited for the observed phenomena. Also, it is possible that our newborn NG neurons differ in cellular responses from their adult counterparts.

We used patterned electrical stimulation to evoke BDNF release. This approach revealed an unexpected feature of NO-mediated inhibition of BDNF release that is pattern-sensitive. Intriguingly, whereas NO nearly abolished BDNF release from neurons activated by single-pulse stimulation, its effect on cells activated by 2-pulse bursts was insignificant. It is well-established that baroreceptor afferents differ in their discharge characteristics. Some fire single action potentials in response to heart strokes at low blood pressure levels, but discharge in bursts or continuously when the blood pressure increases. Others are activated, usually in form of bursts of action potentials, only when a certain pressure threshold is reached (Chapleau and Abboud, 1987; Seagard et al., 1990; van Brederode et al., 1990; Chapleau 1991; Seagard et al., 1993). Our result suggests that NO inhibits BDNF release selectively from neurons with single discharge characteristic, thus preventing a continuous BDNF release at baroafferent synapses. Given our earlier data showing that BDNF inhibits AMPA currents in second-order sensory neurons in the NTS (Balkowiec et al., 2000), NO is likely to play a very important role in preventing a tonic suppression of excitatory transmission by BDNF. In this scenario, the role of BDNF in modulating synaptic transmission would be limited to the synapses that receive changing afferent inputs, such as those from high-pressure-activated, bursting baroreceptors. Although the exact role of BDNF in blood pressure regulation remains to be elucidated, it has previously been demonstrated that injection of BDNF into the rostral ventro-lateral medulla leads to a significant increase in the arterial blood pressure (Wang and Zhou, 2002).

We also demonstrate a previously unknown mechanism of NO-inhibition of BDNF release *via* S-nitrosylation. In the hippocampus, the NO-induced downregulation of BDNF secretion has been shown to be mediated through the cGMP/PKG pathway (Canossa et al., 2002). In nodose neurons, on the other hand, the NO action can be largely blocked by preventing S-nitrosylation, whereas blocking the cGMP/PKG pathway does not have a significant effect. One potential explanation for this discrepancy is the fact that Canossa and colleagues (2002) used adenovirally-overexpressed BDNF, whereas our study examined release of the endogenous peptide. Another likely explanation is the type of neuronal activation leading to BDNF release. Specifically, Canossa et al. (2002) used a culture model in which over 50% of the detected BDNF was released in a constitutive manner, as compared to patterned electrical stimulation-evoked release in our study. This is particularly important in view of recent data indicating that electrical stimulation has a wide range of intracellular effects on peripheral neurons (Wan and Lin, 2009). The most relevant to the current study is upregulation of BDNF and its receptor, TrkB, expression, that plays a crucial role in nerve regeneration (Al-Majed et al., 2000, 2004; English et al., 2007; Geremia et al., 2007). Our current findings which demonstrate the requirement for neuronal activity in NO-mediated inhibition of BDNF expression suggest a novel mechanism of homeostatic regulation of BDNF expression. Namely, electrical stimulation and, consequently, neuronal activation lead to an increase in BDNF expression that is accompanied by an enhanced inhibitory influence of NO signaling.

Previous studies showed that NO inhibits sodium currents in baroreceptor and NG neurons *via* S-nitrosylation of sodium channel proteins (Li et al., 1998; Bielefeldt et al., 1999; Snitsarev et al., 2002a, b). This discovery, together with the fact that sodium channels are required for BDNF release from NG neurons (Balkowiec and Katz, 2000), supports our current finding that S-nitrosylation contributes to mechanisms of NO-mediated inhibition of BDNF release by single-pulse stimulation. It remains to be elucidated in future studies why BDNF release evoked by the 2-pulse stimulation does not show the same sensitivity to the NO inhibition.

Although sodium channels are a likely target of S-nitrosylation and a reasonable mechanistic link between NO and inhibition of BDNF release, many other targets of S-nitrosylation may be involved in the observed effects. In fact, our cytochemical data in NG cultures subjected to both electrical stimulation and the NO donor, strongly suggest that the number of S-nitrosylated targets is larger than it could be accounted for by sodium channels alone. Our future studies will address whether S-nitrosylation of sodium channels can, in fact, lead to an inhibition of BDNF release, and whether other mechanisms play a role. It is worth noting that inhibitors of S-nitrosylation did not completely reverse the effects of NO, making a potential contribution of other mechanisms all the more likely.

In conclusion, the present study reveals a novel mechanism of nitric oxide inhibition of BDNF release from neurons that is stimulation pattern-sensitive and involves S-nitrosylation.

Acknowledgments

The authors thank Chandler Schaak for help with analysis of BDNF and NOS double-immunostaining.

Grant Information: The project was supported by Award Number R01HL076113 from the National Heart, Lung, and Blood Institute to A.B. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health. Initial experiments were also supported by the American Heart Association grant # 0230095N to A.B.

REFERENCES

Ahern GP, Klyachko VA, Jackson MB. cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. *Trends Neurosci* 2002;25:510–517. [PubMed: 12220879]

- Aimi Y, Fujimura M, Vincent SR, Kimura H. Localization of NADPH-diaphorase-containing neurons in sensory ganglia of the rat. *J Comp Neurol* 1991;306:382–392. [PubMed: 1864999]
- Al-Majed AA, Brushart TM, Gordon T. Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. *Eur J Neurosci* 2000;12:4381–90. [PubMed: 11122348]
- Al-Majed AA, Tam SL, Gordon T. Electrical stimulation accelerates and enhances expression of regeneration-associated genes in regenerating rat femoral motoneurons. *Cell Mol Neurobiol* 2004;24:379–402. [PubMed: 15206821]
- Atkinson L, Batten TF, Corbett EK, Sinfield JK, Deuchars J. Subcellular localization of neuronal nitric oxide synthase in the rat nucleus of the solitary tract in relation to vagal afferent inputs. *Neuroscience* 2003;118:115–122. [PubMed: 12676143]
- Balkowiec A. Nitric oxide modulation of BDNF release from nodose neurons stimulated with physiological patterns of baroreceptor activity. *Soc Neurosci Abstr* 2003;768.8.
- Balkowiec A, Katz DM. Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. *J Neurosci* 2000;20:7417–7423. [PubMed: 11007900]
- Balkowiec A, Katz DM. Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. *J Neurosci* 2002;22:10399–10407. [PubMed: 12451139]
- Balkowiec A, Kunze DL, Katz DM. Brain-derived neurotrophic factor acutely inhibits AMPA-mediated currents in developing sensory relay neurons. *J Neurosci* 2000;20:1904–1911. [PubMed: 10684891]
- Bielefeldt K, Whiteis CA, Chapleau MW, Abboud FM. Nitric oxide enhances slow inactivation of voltage-dependent sodium currents in rat nodose neurons. *Neurosci Lett* 1999;271:159–162. [PubMed: 10507693]
- Buldyrev I, Tanner NM, Hsieh H-Y, Dodd EG, Nguyen LT, Balkowiec A. Calcitonin gene-related peptide enhances release of native brain-derived neurotrophic factor from trigeminal ganglion neurons. *J Neurochem* 2006;99:1338–1350. [PubMed: 17064360]
- Canossa M, Giordano E, Cappello S, Guarnieri C, Ferri S. Nitric oxide down-regulates brain-derived neurotrophic factor secretion in cultured hippocampal neurons. *Proc Natl Acad Sci USA* 2002;99:3282–3287. [PubMed: 11867712]
- Chapleau MW. Types of baroreceptor afferent neurons. *Circ Res* 1991;68:619–620. [PubMed: 1991360]
- Chapleau MW. Are arterial pressure and deformation the sole determinants of baroreceptor activity? Importance of humoral and endothelial modulation in normal and disease states. *Hypertension* 1992;19:278–280. [PubMed: 1548052]
- Chapleau MW, Abboud FM. Contrasting effects of static and pulsatile pressure on carotid baroreceptor activity in dogs. *Circ Res* 1987;61:648–658. [PubMed: 3664974]
- Chapleau MW, Hajduczuk G, Shasby DM, Abboud FM. Activated endothelial cells in culture suppress baroreceptors in the carotid sinus of dog. *Hypertension* 1988;11:586–590. [PubMed: 3134304]
- Chen J, Zacharek A, Zhang C, Jiang H, Li Y, Roberts C, Lu M, Kapke A, Chopp M. Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice. *J Neurosci* 2005;25:2366–75. [PubMed: 15745963]
- Cullen BR. Enhancing and confirming the specificity of RNAi experiment. *Nature Meth* 2006;3:677–681.
- Dias AC, Colombari E, Mifflin SW. Effect of nitric oxide on excitatory amino acid-evoked discharge of neurons in NTS. *Am J Physiol* 2003;284:H234–240.
- Dias AC, Vitela M, Colombari E, Mifflin SW. Nitric oxide modulation of glutamatergic, baroreflex, and cardiopulmonary transmission in the nucleus of the solitary tract. *Am J Physiol* 2005;288:H256–262.
- Dun NJ, Dun SL, Chiba T, Forstermann U. Nitric oxide synthase-immunoreactive vagal afferent fibers in rat superior cervical ganglia. *Neuroscience* 1995;65:231–239. [PubMed: 7538645]
- English AW, Schwartz G, Meador W, Sabatier MJ, Mulligan A. Electrical stimulation promotes peripheral axon regeneration by enhanced neuronal neurotrophin signaling. *Dev Neurobiol* 2007;67:158–72. [PubMed: 17443780]
- Evans JR, Bielefeldt K. Regulation of sodium currents through oxidation and reduction of thiol residues. *Neuroscience* 2000;101:229–236. [PubMed: 11068151]

- Garthwaite G, Bartus K, Malcolm D, Goodwin D, Kollb-Sielecka M, Dooldeniya C, Garthwaite J. Signaling from blood vessels to CNS axons through nitric oxide. *J Neurosci* 2006;26:7730–7740. [PubMed: 16855101]
- Geremia NM, Gordon T, Brushart TM, Al-Majed AA, Verge VM. Electrical stimulation promotes sensory neuron regeneration and growth-associated gene expression. *Exp Neurol* 2007;205:347–59. [PubMed: 17428474]
- González DR, Fernández IC, Ordenes PP, Treuer AV, Eller G, Boric MP. Differential role of S-nitrosylation and the NO-cGMP-PKG pathway in cardiac contractility. *Nitric Oxide* 2008;18:157–67. [PubMed: 18023373]
- Hohler B, Mayer B, Kummer W. Nitric oxide synthase in the rat carotid body and carotid sinus. *Cell Tissue Res* 1994;276:559–564. [PubMed: 7520363]
- Hopper RA, Garthwaite J. Tonic and phasic nitric oxide signals in hippocampal long-term potentiation. *J Neurosci* 2006;26:11513–11521. [PubMed: 17093072]
- Kim DH, Rossi JJ. RNAi mechanism and applications. *BioTechniques* 2008;44:613–615. [PubMed: 18474035]
- Kunze DL, Krauhs JM, Orlea CJ. Direct action of norepinephrine on aortic baroreceptors of rat adventitia. *Am J Physiol* 1984;247:H811–816. [PubMed: 6093596]
- Lemus M, Montero S, Luquín S, García J, De Alvarez-Buylla ER. Nitric oxide in the solitary tract nucleus (STn) modulates glucose homeostasis and FOS-ir expression after carotid chemoreceptor stimulation. *Adv Exp Med Biol* 2009;648:403–10. [PubMed: 19536505]
- Li Z, Chapleau MW, Bates JN, Bielefeldt K, Lee HC, Abboud FM. Nitric oxide as an autocrine regulator of sodium currents in baroreceptor neurons. *Neuron* 1998;20:1039–1049. [PubMed: 9620707]
- Lin LH, Taktakishvili O, Talman WT. Identification and localization of cell types that express endothelial and neuronal nitric oxide synthase in the rat nucleus tractus solitarii. *Brain Res* 2007;1171:42–51. [PubMed: 17761150]
- Liu Z, Chen CY, Bonham AC. Frequency limits on aortic baroreceptor input to nucleus tractus solitarii. *Am J Physiol* 2000;278:H577–585.
- Ma S, Abboud FM, Felder RB. Effects of L-arginine-derived nitric oxide synthesis on neuronal activity in nucleus tractus solitarius. *Am J Physiol* 1995;268:R487–491. [PubMed: 7864245]
- Martin JL, Jenkins VK, Hsieh H-Y, Balkowiec A. Brain-derived neurotrophic factor in arterial baroreceptor pathways: Implications for activity-dependent plasticity at baroafferent synapses. *J Neurochem* 2009;108:450–464. [PubMed: 19054281]
- Matsuda T, Bates JN, Lewis SJ, Abboud FM, Chapleau MW. Modulation of baroreceptor activity by nitric oxide and S-nitrosocysteine. *Circ Res* 1995;76:426–433. [PubMed: 7859388]
- Meyrelles SS, Sharma RV, Mao HZ, Abboud FM, Chapleau MW. Modulation of baroreceptor activity by gene transfer of nitric oxide synthase to carotid sinus adventitia. *Am J Physiol* 2003;284:R1190–1198.
- Nawa H, Carnahan J, Gall C. BDNF protein measured by a novel enzyme immunoassay in normal brain and after seizure: partial disagreement with mRNA levels. *Eur J Neurosci* 1995;7:1527–1535. [PubMed: 7551179]
- Page AJ, O'Donnell TA, Cooper NJ, Young RL, Blackshaw LA. Nitric oxide as an endogenous peripheral modulator of visceral sensory neuronal function. *J Neurosci* 2009;29:7246–55. [PubMed: 19494147]
- Renganathan M, Cummins TR, Waxman SG. Nitric oxide blocks fast, slow, and persistent Na⁺ channels in C-type DRG neurons by S-nitrosylation. *J Neurophysiol* 2002;87:761–775. [PubMed: 11826045]
- Riccio A, Alvania RS, Lonze BE, Ramanan N, Kim T, Huang Y, Dawson TM, Snyder SH, Ginty DD. A nitric oxide signaling pathway controls CREB-mediated gene expression in neurons. *Mol Cell* 2006;21:283–294. [PubMed: 16427017]
- Robertson CL, Balkowiec A. Cellular mechanisms of nitric oxide-mediated inhibition of BDNF release from nodose neurons. *Soc Neurosci Abstr* 2004;726.12.
- Sakuma I, Togashi H, Yoshioka M, Saito H, Yanagida M, Tamura M, Kobayashi T, Yasuda H, Gross SS, Levi R. N^G-methyl-L-arginine, an inhibitor of L-arginine-derived nitric oxide synthesis, stimulates renal sympathetic nerve activity in vivo. *Circ Res* 1992;70:607–611. [PubMed: 1537096]
- Sandy P, Ventura A, Jacks T. Mammalian RNAi: a practical guide. *BioTechniques* 2005;39:215–224. [PubMed: 16116795]

- Scanlin HL, Carroll EA, Jenkins VK, Balkowiec A. Endomorphin-2 is released from newborn rat primary sensory neurons in a frequency- and calcium-dependent manner. *Eur J Neurosci* 2008;27:2629–2642. [PubMed: 18513316]
- Seagard JL, van Brederode JFM, Dean C, Hopp FA, Gallenberg LA, Kampine JP. Firing characteristics of single-fiber carotid sinus baroreceptors. *Circ Res* 1990;66:1499–1509. [PubMed: 2344663]
- Seagard JL, Hopp FA, Drummond HA, Van Wynsberghe DM. Selective contribution of two types of carotid sinus baroreceptors to the control of blood pressure. *Circ Res* 1993;72:1011–1022. [PubMed: 8477517]
- Snitsarev V, Whiteis CA, Abboud FM, Chapleau MW. Mechanosensory transduction of vagal and baroreceptor afferents revealed by study of isolated nodose neurons in culture. *Auton Neurosci* 2002a; 98:59–63. [PubMed: 12144042]
- Snitsarev V, Whiteis CA, Abboud FM, Chapleau MW. Nitric oxide functions as an autocrine negative-feedback inhibitor of membrane excitability in nodose sensory neurons. *Soc Neurosci Abstr* 2002b; 46.24.
- Song J, Jang YY, Shin YK, Lee MY, Lee C. Inhibitory action of thimerosal, a sulfhydryl oxidant, on sodium channels in rat sensory neurons. *Brain Res* 2000;864:105–113. [PubMed: 10793192]
- Tanaka K, Chiba T. Nitric oxide synthase containing neurons in the carotid body and sinus of the guinea pig. *Microsc Res Tech* 1994;29:90–93. [PubMed: 7529074]
- Tarsa L, Balkowiec A. Nerve growth factor regulates synaptophysin expression in developing trigeminal ganglion neurons *in vitro*. *Neuropeptides* 2009;43:47–52. [PubMed: 19019428]
- van Brederode JFM, Seagard JL, Dean C, Hopp FA, Kampine JP. Experimental and modeling study of the excitability of carotid sinus baroreceptors. *Circ Res* 1990;66:1510–1525. [PubMed: 1971534]
- Wan L, Lin X. Electrical stimulation effects on PNS injury and repair are mediated by accelerating intracellular trafficking? *Bioscience Hypoth* 2009;2:65–68.
- Wang ZZ, Bredt DS, Fidone SJ, Stensaas LJ. Neurons synthesizing nitric oxide innervate the mammalian carotid body. *J Comp Neurol* 1993;336:419–432. [PubMed: 7505296]
- Wang H, Zhou XF. Injection of brain-derived neurotrophic factor in the rostral ventrolateral medulla increases arterial blood pressure in anaesthetized rats. *Neuroscience* 2002;112:967–975. [PubMed: 12088754]
- Wecht JM, Weir JP, Goldstein DS, Krothe-Petroff A, Spungen AM, Holmes C, Bauman WA. Direct and reflexive effects of nitric oxide synthase inhibition on blood pressure. *Am J Physiol* 2008;294:H190–7.
- Xiong H, Yamada K, Han D, Nabeshima T, Enikolopov G, Carnahan J, Nawa H. Mutual regulation between the intercellular messengers nitric oxide and brain-derived neurotrophic factor in rodent neocortical neurons. *Eur J Neurosci* 1999;11:1567–76. [PubMed: 10215909]

Figure 1. BDNF ELISA reliably mirrors changes in BDNF mRNA level evoked by RNA interference in cultured nodose ganglion neurons

(A) BDNF protein coding region mRNA levels relative to β -actin in cultures treated for 6 days with siRNA for the housekeeping gene GAPDH (GAPDH siRNA) and BDNF siRNA; $n=3$ cultures. (B) BDNF total protein levels in naïve cultures (control), cultures treated with GAPDH siRNA as a negative control (GAPDH siRNA), and BDNF siRNA; $n=2$ cultures; * $P<0.05$.

Figure 2. Exogenous nitric oxide inhibits BDNF release from nodose ganglion neurons in a pattern-dependent manner

Mean BDNF release during 1-h stimulation with single pulses delivered at 6 Hz (Tonic Stimulation) or 2-pulse bursts, with inter-pulse interval of 27.8 ms, delivered at 6 Hz (Bursting Stimulation), in control (Stim; n=52 and 23 cultures for tonic and bursting stimulation, respectively) and in the presence of NOR3, a nitric oxide donor (Stim + NOR3; n=33 and 26 cultures for tonic and bursting stimulation, respectively). Data are presented as the difference between BDNF levels in electrically stimulated and non-stimulated sister cultures receiving identical treatment (i.e. vehicle or NOR3). *** P<0.001.

Figure 3. The neuronal isoform of nitric oxide synthase does not affect BDNF release *in vitro*, but regulates BDNF expression

(A) A sample culture of newborn rat nodose ganglion neurons, double-immunostained for BDNF (α BDNF) and the neuronal isoform of nitric oxide synthase (α nNOS). Arrows indicate BDNF-positive neurons (α BDNF, black arrows) that are also nNOS-immunoreactive (α nNOS, white arrows). Scale bar, 30 μ m. (B) Mean BDNF release during 1-h tonic stimulation at 6 Hz in control (Stim; n=36 cultures) and in the presence of L-NAME, a specific inhibitor of endogenous NO synthases (Stim + L-NAME; n=34 cultures). (C) Mean BDNF cell content following 24-h tonic stimulation at 1 Hz in control (Stim; n=18 cultures) and in the presence of L-NAME (Stim + L-NAME; n=18 cultures). Data are presented as the difference between BDNF levels in electrically stimulated and non-stimulated sister cultures receiving identical treatment (i.e. vehicle or L-NAME). *** $P < 0.001$.

Figure 4. Nitric oxide-inhibition of BDNF release from nodose ganglion neurons is independent of the cGMP-protein kinase G (PKG) pathway

Mean BDNF release during 1-h electrical field stimulation with single pulses delivered at 6 Hz (Tonic Stimulation) or with 2-pulse bursts delivered at 6 Hz (Bursting Stimulation), in control (Stim) and in the presence of: (A) 100 μ M YC1, an agonist of the soluble guanylyl cyclase (Stim + YC1), (B) 1 mM 8-Bromo-cGMP, a membrane-permeable analogue of cGMP (Stim + 8-Br-cGMP), and (C) 40 μ M KT5823, a selective inhibitor of PKG (Stim + KT5823). YC1 and 8-Br-cGMP were applied only in the absence of the nitric oxide donor NOR3, as they were intended to examine whether an increase in cGMP levels can mimic the nitric oxide-mediated inhibition of BDNF release. KT5823, on the other hand, was applied both in the absence (Stim + KT5823) and presence (Stim + NOR3 + KT5823) of NOR3. The latter was intended to determine whether blocking PKG can prevent the nitric oxide-mediated inhibition of BDNF release. Data are presented as the difference between BDNF levels in electrically stimulated and non-stimulated sister cultures receiving identical treatment (i.e. vehicle, drug or drug combination). *** $P < 0.001$. For sample sizes, please see Results.

Figure 5. S-nitrosylation is involved in nitric oxide-mediated inhibition of BDNF release from nodose ganglion neurons

(A) Staining for S-nitrosylated proteins in sister cultures of newborn rat nodose ganglia in unstimulated, vehicle-treated control conditions (Veh), following treatment with 100 μ M NOR3, a nitric oxide donor (NOR3), following 1-h tonic stimulation at 6 Hz (Stim), and 1-h tonic stimulation at 6 Hz in the presence of NOR3 (Stim+NOR3). (B) Mean BDNF release during 1-h electrical field stimulation with single pulses at 6 Hz in control (Stim; n=7 cultures) and in the presence of: 100 μ M NOR3 (Stim + NOR3; n=8 cultures), a combination of 100 μ M NOR3 and 2 mM N-ethylmaleimide, which covalently modifies sulfhydryl groups to prevent S-nitrosylation (Stim + NOR3 + NEM; n=5 cultures), and 2 mM N-ethylmaleimide alone (Stim + NEM; n=7 cultures). (C) Mean BDNF release during 1-h electrical field stimulation with single pulses at 6 Hz in control (Stim; n=8 cultures) and in the presence of: 1 μ M SNAP, a nitric oxide donor (Stim + SNAP; n=12 cultures), a combination of 1 μ M SNAP and 100 μ M TEMPOL, a radical scavenger and nitric oxide spin trap known to prevent S-nitrosylation (Stim + SNAP + TEMPOL; n=10 cultures), and 100 μ M TEMPOL alone (Stim + TEMPOL; n=4 cultures). Data are presented as the difference between BDNF levels in electrically stimulated and non-stimulated sister cultures receiving identical treatment (i.e. vehicle, drug or drug combination). * $P < 0.05$; ** $P < 0.01$.