Neuronal NOS provides nitrergic inhibitory neurotransmitter in mouse lower esophageal sphincter

CHI DAE KIM,[1] RAJ K. GOYAL,[1] AND HIROSHI MASHIMO[1,2]
[1]*Center for Swallowing and Motility Disorders, Brockton/West Roxbury Veterans Affairs Medical Center, and* [2]*Gastrointestinal Unit, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02132*

Kim, Chi Dae, Raj K. Goyal, and Hiroshi Mashimo. Neuronal NOS provides nitrergic inhibitory neurotransmitter in mouse lower esophageal sphincter. *Am. J. Physiol.* 277 (*Gastrointest. Liver Physiol.* 40): G280–G284, 1999.—To identify the enzymatic source of nitric oxide (NO) in the lower esophageal sphincter (LES), studies were performed in wild-type and genetically engineered endothelial nitric oxide synthase [eNOS(−)] and neuronal NOS [nNOS(−)] mice. Under nonadrenergic noncholinergic (NANC) conditions, LES ring preparations developed spontaneous tone in all animals. In the wild-type mice, electrical field stimulation produced frequency-dependent intrastimulus relaxation and a post-stimulus rebound contraction. NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (100 μM) abolished intrastimulus relaxation and rebound contraction. In nNOS(−) mice, both the intrastimulus relaxation and rebound contraction were absent. However, in eNOS(−) mice there was no significant difference in either the relaxation or rebound contraction from the wild-type animal. Both nNOS(−) and eNOS(−) tissues showed concentration-dependent relaxation to NO donor diethylenetriamine-NO and there was no difference in the sensitivity to the NO donor in nNOS(−), eNOS(−), or wild-type animals. These results indicate that in mouse LES, nNOS rather than eNOS is the enzymatic source of the NO that mediates NANC relaxation and rebound contraction.

nitric oxide; nonadrenergic noncholinergic neurotransmission; endothelial nitric oxide synthase lacking mutant mice

STIMULATION OF THE nonadrenergic noncholinergic (NANC) inhibitory nerves causes relaxation of the lower esophageal sphincter (LES) in all animal species examined, including dog, cat, opossum, guinea pig, mice, frog, as well as in humans (1, 16–18, 24–26, 28). The NANC nerves have been shown to exert their effect via the release of a product of the L-arginine-nitric oxide synthase (NOS) pathway such as nitric oxide (NO) (3, 17). These conclusions are supported by the fact that neurons in the myenteric plexus and the motor nerve terminals express NOS (20). Moreover, NO has been shown to be released with NANC nerve stimulation (16). There is also a similarity of responses of the smooth muscle to NANC nerve stimulation and exogenous NO donors. Both nitrergic transmitter and NO donor elicit hyperpolarization of the smooth muscle cells (6, 25) and cause intracellular accumulation of cGMP (5, 9). The most compelling argument for NO as an inhibitory mediator is the block of nitrergic nerve stimulation by the chemical inhibitors of NOS such as N^{ω}-nitro-L-arginine (L-NNA) or N^{ω}-nitro-L-arginine methyl ester (L-NAME) (16, 25). However, the use of the nonselective inhibitors of NOS fails to identify the type of NOS that is involved in the inhibitory neurotransmission. It also fails to distinguish whether NO is a neurotransmitter released from nerve endings or an intracellular mediator in the effector smooth muscle cells (12).

There are three isoforms of NOS that are products of distinct genes (15). The inducible NOS(iNOS, also called type II) is not normally present in tissues under physiological conditions but is induced during tissue injury and inflammation. iNOS is the source of large quantities of NO that is produced during inflammation. There are two types of constitutive NOS that participate in normal physiological responses, each with specific distribution in the gut. The neuronal NOS(nNOS, also called ncNOS or type I) has been localized to intramural neurons and nerve endings to the smooth muscle cells (4). In contrast, the endothelial NOS(eNOS, also called ecNOS or type III) has been suggested to be localized to smooth muscle cells (13). Targeted disruption of nNOS and eNOS genes has led to production of mutant mice that lack nNOS and eNOS, respectively (10, 11). These mutant mice allow distinction of the roles of nNOS and eNOS in the nitrergic inhibitory neurotransmission in the esophagus and the LES.

We report here our findings that nNOS(−) but not eNOS(−) mice lack electrical field stimulation (EFS)-activated LES relaxation and rebound contraction. These studies show that *1*) nNOS is the source of NO that is responsible for EFS-induced LES relaxation and rebound contraction; *2*) lifelong deficiency of nNOS is not associated with any compensatory changes in the inhibitory neurotransmission; and *3*) because nNOS is localized primarily to nerve ending and nNOS is the source of NO involved in nitrergic neurotransmission and eNOS is present in smooth muscle cells, these studies suggest that NO may serve as an antegrade neurotransmitter in the nitrergic neurotransmission in the LES.

MATERIALS AND METHODS

Mice and tissues. Adult (C57BL/6J X 129/J)F1 mice were used as wild-type mice, and nNOS(−) and eNOS(−) mice were generated as described previously (10, 11). The nNOS(−) mice were prepared by deletion of exon 2 that leads to loss of PDZ binding domain required for membrane association of the enzyme. The PDZ domain also defines the expression of nNOS-α but not the 5′ splice variants nNOS-β and nNOS-γ.

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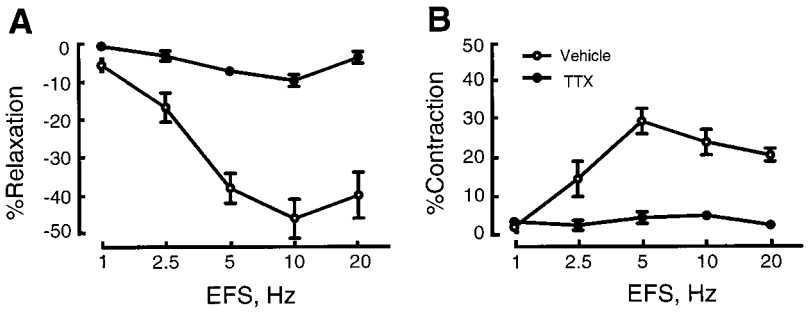

Fig. 1. Effect of TTX (1 μM) on frequency-response relationship for electrical field stimulation (EFS, 1–20 Hz, 60 V)-induced intrastimulus relaxation (*A*) and poststimulus rebound contraction (*B*) in isolated mouse lower esophageal sphincter (LES) ring preparations. Values represent means ± SE from 10 (Vehicle) or 3 (TTX) experiments.

The nNOS(−) mutant mice lack nNOS-α but still express nNOS-β and nNOS-γ (2, 8). These two isoforms account for 5–10% of the residual NOS activity in myenteric neurons in nNOS(−) mutant mice (2). Therefore nNOS(−) mice represent misexpression rather than lack of nNOS. The nNOS-β and nNOS-γ remain soluble cytosolic enzymes with activity that is ~80% and ~3% of the activity of full-length nNOS(2).

Adult mice weighing between 25 and 30 g were killed by cervical dislocation, followed by bleeding from the carotid arteries. The stomach, including a portion of the esophagus, was quickly removed and placed in a Sylgard-bottom petri dish filled with modified Krebs solution (4°C) containing (in mmol/l) 118 NaCl, 4.7 KCl, 0.6 $MgSO_4$, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$, and 11 D-glucose, and bubbled with 95% O_2-5% CO_2. To isolate the LES, the stomach was opened along the greater curvature to reveal the junction between the esophagus and the stomach. The esophagus immediately proximal to the gastroesophageal junction was dissected as a ring 1 mm in width, and mounted in standard organ baths (Radnoti Glass). Among these tissues, ring preparations showing spontaneous contraction were considered as LES and used for the experiments.

LES tension recording. Muscle tension recordings were performed using standard organ bath techniques. Ring preparations of the LES were suspended between two platinum electrodes in 3-ml organ baths containing modified Krebs solution pretreated with atropine (1 μM) and guanethidine (5 μM). One end of the ring preparation was anchored, and the other end was attached with stainless steel hooks to a force transducer (Grass FT03) and recorded by a MacLab acquisition (MacLab/8e AD Instruments) and computer system. The tissues were stretched to a resting tension of 0.2 g and allowed to equilibrate for 90 min. The muscle rings developed spontaneous force that declined after 1–2 h. Inclusion of prostaglandin agonist U-46619 (1 μM) in the bath resulted in increased tone that was steady over several hours. Selective neural stimulation of the tissue was achieved using a pulse generator (Grass Instruments S11) programmed to deliver 30-s duration trains of 60-V square-wave pulses, each of 2-ms pulse duration at 1–20 Hz. All these studies were performed in U-46619 (1 μM)-treated muscle preparations. For each parameter of stimulation, EFS was administered three times at 8-min intervals, and responses were averaged over the series. The relaxation responses by EFS were expressed as %change of active tension before EFS-induced relaxation. Active tension was defined as the force that was abolished by isoproterenol (100 μM). In a separate series of experiments, diethylenetriamine-NO (DNO) was added to assess the responses of LES rings to exogenous NO. At the end of the experiments, isoproterenol (100 μM) was added to attain the maximal relaxation.

Drugs. Drugs used in this study included U-46619 (1 μM), atropine (1 μM), guanethidine (5 μM), isoproterenol (100 μM), and L-NAME (100 μM) obtained from Sigma Chemical and the NO donor DNO from Research Biochemicals International. Each was prepared fresh on the day of the experiment. Tissues were treated with solution containing L-NAME (100 μM) for 45 min before study of its effect.

Statistics. Data are expressed as means ± SE for wild-type, eNOS(−), and nNOS(−) tissues. Differences in the data were evaluated by Student's *t*-test (nonparametric analysis). $P < 0.05$ was considered statistically significant, and *n* represents the number of animals used for each protocol.

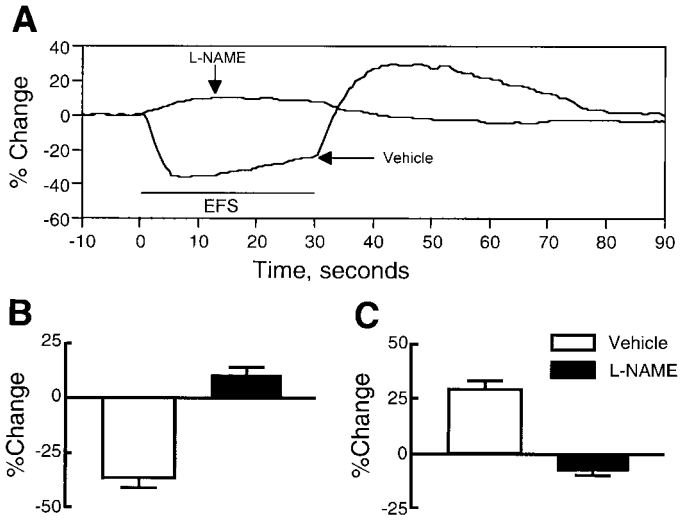

Fig. 2. *A*: representative recordings of EFS (5 Hz, 30 s)-induced intrastimulus relaxation and poststimulus rebound contraction in U-46619 (1 μM)-contracted LES preparations of wild-type mice in absence (Vehicle) and presence of 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME). *B* and *C*: EFS-induced responses of mouse LES ring preparations in absence and presence of L-NAME. %Change is expressed as %change in grams of active tension before EFS-induced relaxation. Values represent means ± SE of results obtained in 10 (Vehicle) or 6 (L-NAME) experiments.

Table 1. *EC_{50} and E_{max} values for DNO-induced relaxations in isolated LES ring preparations of wild-type mice in presence and absence of 100 μM L-NAME*

		DNO	
	n	EC_{50}, M	E_{max}, %
Control	7	$6.9 \pm 1.5 \times 10^{-5}$	94.4 ± 3.2
L-NAME	4	$8.9 \pm 1.0 \times 10^{-5}$	92.8 ± 2.8

Values are means ± SE; *n* = no. of experiments. Maximum relaxation response (E_{max}) by diethylenetriamine-nitric oxide (DNO) represents percentage of maximum relaxation obtained with isoproterenol (100 μM) in each experiment. LES, lower esophageal sphincter; L-NAME, N^{ω}-nitro-L-arginine methyl ester.

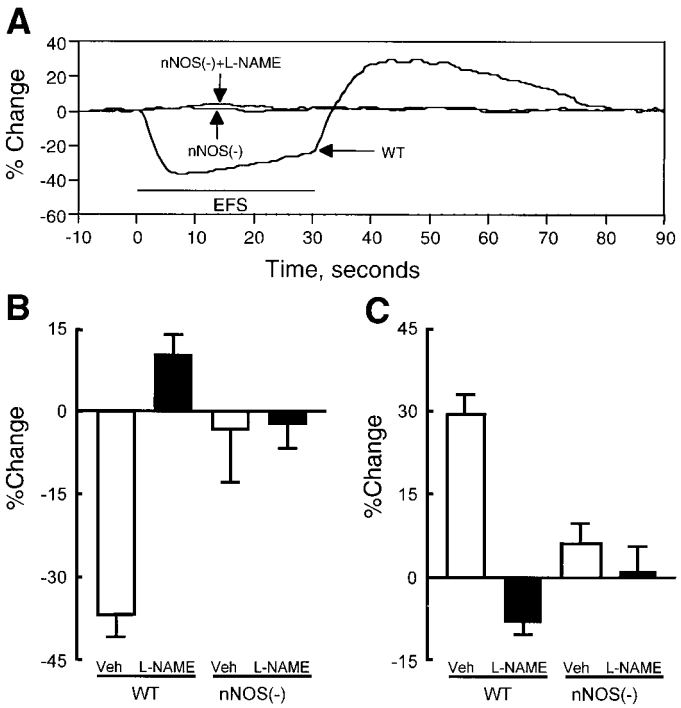

Fig. 3. *A*: representative recordings of EFS (5 Hz, 30 s)-induced intrastimulus relaxation and poststimulus rebound contraction in LES preparations of wild-type (WT) and neuronal nitric oxide synthase mice [nNOS(−)] in absence [nNOS(−)] and presence [nNOS(−) + L-NAME] of 100 μM L-NAME. *B* and *C*: EFS-induced responses of mouse LES ring preparations in absence (Veh) and presence (L-NAME) of L-NAME. %Change is expressed as %change in grams of active tension before EFS-induced relaxation. Values represent means ± SE of results obtained from 5 experiments.

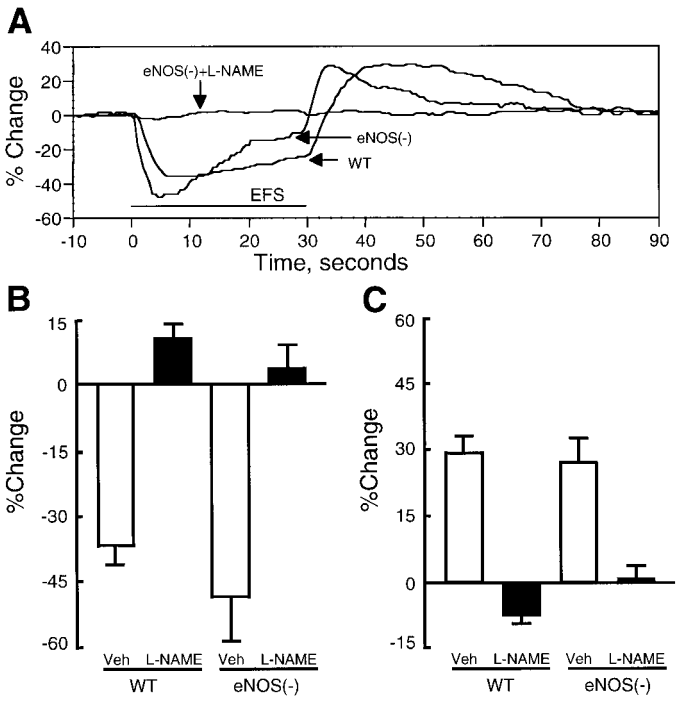

Fig. 4. *A*: representative recordings of EFS (5 Hz, 30 s)-induced intrastimulus relaxation and poststimulus rebound contraction in LES preparations of wild-type (WT) and endothelial NOS mice [eNOS(−)] in absence [eNOS(−)] and presence [eNOS(−) + L-NAME] of 100 μM L-NAME. *B* and *C*: EFS-induced responses in LES ring preparations of eNOS(−) mice in absence (Veh) and presence (L-NAME) of L-NAME. %Change is expressed as %change in grams of active tension before EFS-induced relaxation. Values represent means ± SE of results obtained in 6 experiments.

RESULTS

Responses in control wild-type mice. Ring preparations of LES were stimulated with EFS in the presence of atropine (1 μM) and guanethidine (5 μM) to reveal NANC responses. The muscle rings were contracted by inclusion of a prostaglandin agonist U-46619 (1 μM) to facilitate resolution of EFS-induced relaxation responses.

The amplitudes of both intrastimulus relaxation and poststimulus rebound contraction were frequency dependent over the range of 1–20 Hz in wild-type control tissues (Fig. 1). These responses were abolished by pretreatment with TTX (1 μM). Maximal intrastimulus relaxation and poststimulus rebound contraction were attained at 10 Hz (44.8 ± 5.0%) and 5 Hz (29.6 ± 3.3%), respectively, but higher frequencies of EFS showed diminished responses. The relaxation induced by frequency of 5 Hz applied every 8 min was reproducible. Therefore, experiments in the remainder of this study were carried out with this stimulation. L-NAME (100 μM) abolished the intrastimulus relaxation, converting this response to a contraction, and inhibited the poststimulus rebound contraction (Fig. 2). However, L-NAME did not affect the relaxations induced by direct application of DNO to the tissues (Table 1).

Responses in nNOS(−) mice. To clarify the enzymatic source of NO in the EFS-induced LES responses, LES preparations from nNOS(−) mice were electrically stimulated using the same conditions and parameters (60 V, 5 Hz). EFS-induced responses of LES preparations of nNOS(−) mice were significantly different from those of wild-type mice; these tissues failed to produce EFS-induced relaxation or rebound contraction (Fig. 3) and resembled the responses of wild type treated with L-NAME (100 μM).

Responses in eNOS(−) mice. To study the role of eNOS-derived NO in the EFS-induced LES responses, LES preparations from eNOS(−) mice were electrically

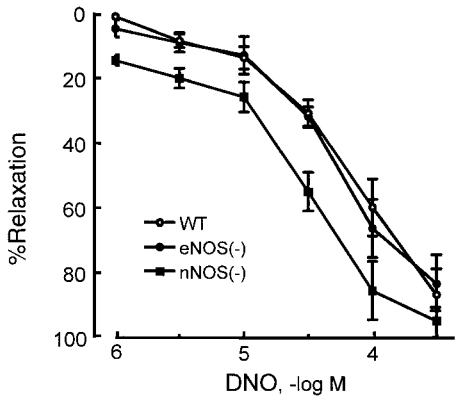

Fig. 5. Relaxation induced by cumulative concentration of diethylenetriamine-nitric oxide (DNO) in LES preparations of wild-type (WT), eNOS(−), and nNOS(−) mice. Values represent means ± SE of results obtained in paired preparations from 4 mice.

stimulated. EFS(60 V, 5 Hz)-induced intrastimulus relaxation and rebound contraction were not significantly altered in the preparations of eNOS(−) mice (48.5 ± 9.6 and 27.1 ± 5.3%, respectively) compared with those of wild-type control tissues (36.9 ± 3.9 and 29.6 ± 3.3%, respectively). In addition, the EFS-induced relaxation and rebound contraction in the eNOS(−) mice were completely abolished by pretreatment with L-NAME (100 μM; Fig. 4).

Comparison of tissue sensitivity to NO donor. In a separate series of experiments, we wished to assess possible differences in LES tissue sensitivity of wild-type, eNOS(−), and nNOS(−) mice to the NO-releasing agent DNO. DNO was used because it is a donor of the NO· redox form of NO that has been shown to be an inhibitory neurotransmitter (9). As shown in Fig. 5, DNO induced relaxation in the LES preparations of these three kinds of mice in a dose-dependent manner. The ED_{50} for DNO in wild-type ($8.05 \pm 1.67 \times 10^{-5}$ M), eNOS(−) ($8.75 \pm 3.44 \times 10^{-5}$ M), and nNOS (−) ($2.20 \pm 1.47 \times 10^{-5}$ M) mice were not significantly different among these three kinds of mice.

DISCUSSION

The mouse LES is similar to that of other animal species. It is composed of smooth muscle fibers and develops spontaneous tone that is not abolished by TTX. Moreover, EFS produces frequency-dependent relaxation that is followed by rebound contraction under NANC conditions. Both the relaxation and rebound contractions are suppressed by NOS inhibitor L-NAME, suggesting that both relaxation and rebound contractions involve NO. The main finding of this study is that both relaxation and aftercontraction in response to EFS under NANC conditions are missing in nNOS-deficient mice. These observations suggest that nNOS is the enzymatic source of NO that is involved in inhibitory nitrergic neurotransmission.

The various structures involved in neuromuscular inhibitory transmission include the motor nerve endings, intramuscular interstitial cells of Cajal (ICC) that may be interposed between the nerve endings and the smooth muscle cells, and the smooth muscle cells themselves (7). In mice, nNOS immunoreactivity has been localized to the myenteric neurons and nerve endings but was not reported in ICC or smooth muscle cells (20). However, Ward and colleagues (25) found NADPH reactivity and NOS staining in ICC but not in smooth muscle cells. In the smooth muscle cells in other species, gene expression for nNOS has been reported in some (4) but not in other studies (19, 23). NOS in the ICC was proposed to provide a major mechanism for amplification of neural signals to the smooth muscle cells. However, it is now thought that the ICC may act to transduce nitrergic chemical signals into electrical hyperpolarization to smooth muscle cells (25). Lack of ICC, as occurs in W^{v} mutant mice, leads to loss of nitrergic relaxation and aftercontraction in the LES even though nitrergic neurons and nerve endings are well preserved in these mutant mice (25).

eNOS has been shown to be localized in some neurons in the central nervous system where NO derived from eNOS may be the physiological mediator (21). eNOS has also been reported to be present in smooth muscle cells (13, 23). Smooth muscle eNOS has been proposed as a major source of NO that is released by the action of putative inhibitory neurotransmitter on the smooth muscle cells (13). The present study shows that nitrergic responses of the LES are not affected in eNOS deficiency.

The localization of nNOS in the nerve endings suggests that the product of L-arginine-nNOS pathway serves as an antegrade neurotransmitter. Recent studies have shown that NO· redox form of NO is the nitrergic neurotransmitter (9). The gastric smooth muscle studies have shown that nNOS-deficient animals have selective loss of nitrergic inhibitory junction potential (IJP), but purinergic IJP is present (14). It was suggested that electrically independent pharmacomechanical inhibition may occur due to alternative mechanisms (14). Moreover, opossum LES relaxation is not fully blocked by L-NNA in vitro and L-NAME in vivo (27). In guinea pig LES L-NNA-resistant IJP was blocked by apamin (28) and in the rat stomach L-NNA-resistant delayed relaxation has been reported to be due to vasoactive intestinal peptide (22).

Mice lacking nNOS revealed complete block of EFS-evoked relaxation and rebound contraction of the LES. The lack of LES relaxatory responses to EFS in the nNOS-deficient mice was qualitatively and quantitatively similar to that produced by acute suppression of NOS by chemical blocker of NOS. The findings suggest that the responses in nNOS(−) mice represent simple lack of NO. These observations are somewhat surprising because we expected lifelong deficiency of nNOS would lead to some alternative or redundant inhibitory pathways. However, no such compensation was observed. We did not perform morphological studies to show any structural changes in the connections of the inhibitory nerves with ICCs. Further morphological and functional studies are needed to identify the presence of possible parallel transmitters and additional role of NOS as an intracellular mediator in the inhibitory neurotransmission in the mouse LES.

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Address for reprint requests and other correspondence: H. Mashimo, Research and Development (151), VA Medical Center, 1400 VFW Parkway, West Roxbury, MA 02132 (E-mail: mashimo@helix.mgh.harvard.edu).

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REFERENCES

1. **Barnette, M. S., M. Grous, C. D. Manning, J. F. Callhan, and F. C. Barone.** Inhibition of neurally induced relaxation of canine lower esophageal sphincter by opioid peptides. *Eur. J. Pharmacol.* 182: 363–368, 1990.
2. **Brenman, J. E., D. S. Chao, S. H. Gee, A. W. McGee, S. E. Craven, D. R. Santillano, Z. Wu, F. Huang, H. Xia, M. F. Peters, S. C. Froehner, and D. S. Bredt.** Interaction of nitric

oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains. *Cell* 84: 757–767, 1996.
3. **Bult, H., G. E. Boeckxstaens, P. A. Pelckmans, F. H. Jordeans, Y. M. van Maercke, and A. G. Herman.** Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* 345: 346–347, 1990.
4. **Chakder, S., A. Bandyopadhyay, and S. Rattan.** Neuronal NOS gene expression in gastrointestinal myenteric neurons and smooth muscle cells. *Am. J. Physiol*. 273 (*Cell Physiol*. 42): C1868–C1875, 1997.
5. **Chakder, S., and S. Rattan.** Involvement of cAMP and cGMP in relaxation of internal anal sphincter by neural stimulation, VIP, and NO. *Am. J. Physiol*. 264 (*Gastrointest. Liver Physiol*. 27): G702–G707, 1993.
6. **Conklin, L., C. Du, J. A. Murray, and J. N. Bates.** Characterization and mediation of inhibitory junction potentials from opossum lower esophageal sphincter. *Gastroenterology* 104: 1439–1444, 1993.
7. **Daniel, E. E., and V. Posey-Daniel.** Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal. *Am. J. Physiol*. 246 (*Gastrointest. Liver Physiol*. 9): G305–G315, 1984.
8. **Eliasson, M. J. L., S. Blackshaw, M. J. Schell, and S. H. Snyder.** Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc. Natl. Acad. Sci. USA* 94: 3396–3401, 1997.
9. **Goyal, R. K., and X. D. He.** Evidence for NO· redox form of nitric oxide as nitrergic inhibitory neurotransmitter in gut. *Am. J. Physiol*. 275 (*Gastrointest. Liver Physiol*. 38): G1185–G1192, 1998.
10. **Huang, P. L., T. M. Dawson, D. S. Bredt, S. H. Snyder, and M. C. Fishman.** Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75: 1273–1286, 1993.
11. **Huang, P. L., Z. Huang, H. Mashimo, K. D. Bloch, M. A. Moskowitz, J. A. Bevan, and M. C. Fishman.** Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377: 239–242, 1995.
12. **Ignarro, L. J., P. A. Bush, G. M. Buga, and J. Rajfer.** Neurotransmitter identity doubt. *Nature* 347: 131–132, 1990.
13. **Makhlouf, G. M., and K. S. Murthy.** Signal transduction in gastrointestinal smooth muscle. *Cell. Signal*. 9: 269–276, 1997.
14. **Mashimo, H., X. E. He, P. L. Huang, M. C. Fishman, and R. K. Goyal.** Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmission. *J. Clin. Invest*. 98: 8–13, 1996.
15. **Moncada, S., R. M. J. Palmer, and E. A. Higgs.** Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev*. 43: 109–142, 1991.
16. **Murray, J., J. N. Bates, and J. L. Conklin.** Nerve-mediated nitric oxide production by opossum lower esophageal sphincter. *Dig. Dis. Sci*. 39: 1872–1876, 1994.
17. **Ny, L., P. Alm, B. Larsson, P. Ekstrom, and K. E. Andersson.** Nitric oxide pathway in cat esophagus: localization of nitric oxide synthase and functional effects. *Am. J. Physiol*. 268 (*Gastrointest. Liver Physiol*. 31): G59–G70, 1995.
18. **Preiksaitis, H. G., L. Tremblay, and N. E. Diamant.** Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. *Dig. Dis. Sci*. 39: 770–775, 1994.
19. **Salapatek, A. M., Y. F. Wang, Y. K. Mao, A. Lam, and E. E. Daniel.** Myogenic nitric oxide synthase activity in canine lower esophageal sphincter: morphological and functional evidence. *Br. J. Pharmacol*. 123: 1055–1064, 1998.
20. **Sang, Q., and H. M. Young.** The origin and development of the vagal and spinal innervation of the external muscle of the mouse esophagus. *Brain Res*. 809: 253–268, 1998.
21. **Son, H., R. D. Hawkins, K. Martin, M. Kiebler, P. L. Huang, M. C. Fishman, and E. R. Kandel.** Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. *Cell* 87: 1015–1023, 1996.
22. **Takahashi, T., and C. Owyang.** Vagal control of nitric oxide and vasoactive intestinal polypeptide release in the regulation of gastric relaxation in rat. *J. Physiol. (Lond.)* 484: 481–492, 1995.
23. **Teng, B., K. S. Murthy, J. F. Kuemmerle, J. R. Grider, K. Sase, T. Michel, and G. M. Makhlouf.** Expression of endothelial nitric oxide synthase in human and rabbit gastrointestinal smooth muscle cells. *Am. J. Physiol*. 275 (*Gastrointest. Liver Physiol*. 38): G342–G351, 1998.
24. **Tottrup, A., L. Ny, P. Alm, B. Larsson, A. Forman, and K. E. Andersson.** The role of the L-arginine/nitric oxide pathway for relaxation of the human lower esophageal sphincter. *Acta Physiol. Scand*. 149: 451–459, 1993.
25. **Ward, S. M., G. Morris, L. Reese, X.-Y. Wang, and K. M. Sanders.** Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. *Gastroenterology* 115: 314–329, 1998.
26. **Williams, S. J., and M. E. Parsons.** Evidence for a role for nitric oxide in relaxation of the frog oesphageal body to electrical field stimulation. *Br. J. Pharmacol*. 122: 179–185, 1997.
27. **Yamato, S., J. K. Saha, and R. K. Goyal.** Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci*. 50: 1263–1272, 1992.
28. **Yuan, S., M. Costa, and S. J. H. Brookes.** Neuronal pathways and transmission to the lower esophageal sphincter of the guinea pig. *Gastroenterology* 115: 661–671, 1998.