Neuronal NOS gene expression in gastrointestinal myenteric neurons and smooth muscle cells

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Chakder, Sushanta, Alok Bandyopadhyay, and Satish Rattan. Neuronal NOS gene expression in gastrointestinal myenteric neurons and smooth muscle cells. Am. J. Physiol. 273 (Cell Physiol. 42): C1868–C1875, 1997.—Nitric oxide synthase (NOS) has been characterized in different tissues, and its localization has been suggested in different neuronal tissues, including the myenteric neurons and other nonneuronal cells. The present study examined the distribution of the neuronal NOS (nNOS) mRNA in different tissues of the opossum gastrointestinal tract, internal anal sphincter (IAS) smooth muscle cells, and myenteric neurons using slot-blot and Northern blot hybridization techniques with a specific rat brain nNOS cDNA probe. Significant levels of nNOS gene expression were found in both smooth muscle cells and myenteric neurons of the opossum IAS. This finding was confirmed by reverse transcriptase-polymerase chain reaction analysis of the RNA obtained from cultured opossum IAS smooth muscle cells and myenteric neurons and also from human intestinal smooth muscle and neuroblastoma cell lines. Pyloric sphincter had the highest level of nNOS gene expression compared with other gastrointestinal tissues. There was no significant difference in the nNOS gene expression between other sphincteric and non sphincteric tissues examined. The present study shows the presence of nNOS gene expression in both neurons and smooth muscle cells. The higher levels of nNOS gene expression in the pyloric sphincter compared with other tissues may have pathophysiological significance in some disease conditions.

constitutive nitric oxide synthase; neuronal or brain nitric oxide synthase; vasoactive intestinal polypeptide; internal anal sphincter; nonadrenergic noncholinergic inhibitory neurotransmitter; pylorus

nitric oxide (NO) has been suggested as an inhibitory neurotransmitter in the gastrointestinal tract that includes internal anal sphincter (IAS; see Refs. 21, 27, 28, 31, 33). NO is formed from its precursor by the enzyme nitric oxide synthase (NOS). Two major classes of NOS have been identified. The first class, the constitutive (cNOS), is responsible for rapid biosynthesis of NO and is calcium-calmodulin dependent. One type of cNOS has been demonstrated in the brain and peripheral neurons, is known as neuronal or brain NOS, and is abbreviated as either nNOS, ncNOS, or bNOS (17, 23). The other type of cNOS has been shown to be present in the endothelial cells and is termed eNOS (5, 17). The second class of NOS is responsible for the synthesis of NO in response to an inducible agent, and the synthesis of NO is known to take from hours to days and may occur under protective or pathophysiological conditions. This class of NOS is termed inducible or iNOS (37). The iNOS has been shown to be present in the epithelia, macrophages, mast cells, and different types of hepatic and pancreatic cells and is independent of calcium-calmodulin.

NO and vasoactive intestinal polypeptide (VIP) have been suggested as cotransmitters in the nonadrenergic noncholinergic (NANC) nerve-mediated smooth muscle relaxation (21, 27, 29, 31, 33). Furthermore, a significant interaction between VIP and NO has been reported (13, 15, 27), suggesting that a part of VIP action may be mediated via the release of NO. Immunocytochemical studies have shown the presence of nNOS in the smooth muscle tissues, but it may represent the localization of nNOS primarily in the myenteric neurons, nerve fibers, and terminals (19). The release of NO in response to NANC nerve stimulation may also represent the production of NO primarily from the myenteric inhibitory neurons (9). Interestingly, VIP-induced NO release may come from the myenteric neurons or the smooth muscle cells (SMC; see Refs. 7, 9, 13). The type of NOS responsible for the NO formation in the SMC in response to NANC nerve stimulation and VIP has been suggested to be cNOS (24). The evidence for this is based on calcium-calmodulin dependence of NO production from the isolated SMC. A suggestion has been made that it is an iNOS rather than a cNOS present in the SMC of the blood vessels (12). The presence of mRNA transcripts for cNOS in either the myenteric neurons or the SMC of the gastrointestinal tract has not been investigated before.

Interestingly, in the nNOS gene knockout model of mice, the tissue of major pathophysiological importance was found to be the pyloric sphincter by showing pyloric stenosis and enlargement of the stomach (16). The purpose of the present investigation therefore was twofold: first, to examine the distribution of nNOS mRNA levels in the myenteric neurons and the SMC of the gut and, second, to compare nNOS gene expression in sphincteric and non sphincteric tissues of the gastrointestinal tract.

MATERIALS AND METHODS

Isolation of the sphincteric and non sphincteric tissues of the opossum gastrointestinal tract. After animals were killed under intraperitoneal pentobarbital sodium anesthesia, the abdomen was opened by a midline incision, and different sphincteric (lower esophageal sphincter, pyloric sphincter, sphincter of Oddi, ileocolic sphincter, and IAS) and non sphincteric (body of the esophagus, gastric fundus, antrum, a part of the duodenum adjoining the pyloric sphincter, common bile duct, a part of the duodenum close to the sphincter of Oddi, a part of the ileum adjacent to the ileocolic sphincter, a part of the colon close to the ileocolic sphincter, and a part of the rectum close to the IAS) regions were identified and marked in situ. The tissues were then dissected out carefully, cleaned, and washed in cold sterile saline. To minimize the contaminat-
tivation by different types of cells, the tissues were carefully
cleansed of the extraneous innervation, serosa, blood vessels,
and mucosa. The tissues were cut into small pieces using
sterile scissors and then were processed for total RNA isola-
tion.

Isolation of SMC and myenteric ganglia. Opossum IAS
SMC were isolated following the method described previously
(9). After tissues were cleared from the adjoining blood
vessels and the serosa, they were opened flat and the mucosa
was removed by using fine scissors and forceps. The longitudi-
nal muscle layer was removed by sharp dissection, and only
the circular muscle layer was used for the isolation of SMC.
The circular smooth muscle layer was cut into small pieces
and then was incubated at 31°C in Krebs buffer (pH 7.4)
containing 0.1% collagenase (CLS type I, 150 U/mg; Worthing-
ton Biochemicals, Freehold, NJ), 0.01% soybean trypsin
inhibitor, and mixtures of amino acids and vitamins (1X) for
two successive 45-min periods. The medium was then filtered
through a Nitex (500 µm), the tissues were washed and
incubated for another 30 min in oxygenated Krebs buffer, and
the dissociated cells were isolated from the medium by
filtration.

Myenteric ganglia from the opossum IAS region were
isolated according to the previously described method (9).
After tissues were cut into 2- to 3-cm-long strips, the longitudi-
nal muscle layer along with the myenteric plexus was
isolated by sharp dissection. The tissues were then cut into
small pieces and were incubated in Krebs buffer containing
collagenase (type II, 0.25%), protease (type IX, 0.2%), bovine
serum albumin (BSA, 0.03%), and deoxyribonuclease (type I,
25 µg/ml) at 37°C for 30 min. After the incubation, the tissues
were disrupted mechanically with a pipette, and the myen-
teric ganglia were isolated after centrifugation and filtration.

Culturing the SMC, myenteric plexus neurons, and the
neuroblastoma cells. The opossum IAS SMC and the myen-
teric neurons were cultured for 3–5 days in RPMI 1640
containing 10% fetal calf serum and an antibiotic mixture
(1X; antibiotic antimycotic solution, Sigma Chemical, St.
Louis, MO). The cells were cultured in a humidified incubator
at 37°C under an atmosphere of 5% carbon dioxide. Human
intestinal smooth muscle (HISM) cells and neuroblastoma
cells were grown in Dulbecco’s modified Eagle’s medium
(DMEM) containing 10% fetal calf serum. The progress of the
growth of the cells was examined under the microscope on a
daily basis, and, at the appropriate times of their maximal
growth, the cells were harvested and the RNA was isolated as
described below.

Isolation of total RNA. The total RNA from the tissues and
the cultured cells was isolated using the method of Chomczyn-
ski and Sacchi (10). The tissues were transferred to sterile
polypropylene tubes and were homogenized in 4 M guanidine
thiocyanate, 0.25 M sodium citrate (pH 7.0), 0.5% sarcosyl,
and 0.1 M 2-mercaptoethanol (solution D) with a tissue
homogenizer (Tekmar Tissuemizer; Tekmar, Cincinnati, OH).
The homogenate was centrifuged at 11,000 g for 15 min, and
the supernatant was isolated. The supernatant was treated
with 2 M sodium acetate, pH 4.0, followed by phenol and
chloroform/isooamyl alcohol (49:1) and then kept on ice for 10
min. The mixture was centrifuged, the aqueous phase was
separated, and the RNA was precipitated at – 20°C for 2 h
with an equal volume of isopropanol. The RNA was isolated
by centrifugation, washed with 70% ethanol, and dissolved in
EDTA solution (1 mM) that was stored at – 70°C. For cultured
cells, after cells were harvested, the medium was removed by
centrifugation, the cells were treated with solution D, and the
RNA was isolated as above. The concentration of the RNA
was determined by measuring the absorbance at 260 nm.

Preparation of nNOS cDNA probes. Full-length rat nNOS
cDNA were isolated using the EcoRI site of Bluescript (SK(−1))
and was generously provided by Dr. A.M. Snowman of Johns Hopkins
University, Baltimore, MD. The plasmid was transformed into DH5α bacterial cells and was grown on ampicillin plates.
The positive colonies were isolated and grown in large
quantities of Luria-Bertani broth containing ampicillin. The
plasmid was isolated using Qiagen columns (Qiagen, Chat-
sworth, CA) according to the protocol supplied with the kit.
The full-length nNOS cDNA was isolated by digesting the
plasmid with EcoRI. The cDNA was radiolabeled (1 X 10⁶
counts·min⁻¹·µg⁻¹) using [32P]dATP by the random primer
labeling method.

Northern blot and slot-blot hybridization. For Northern
blot analysis, 20 µg of RNA were denatured with formamide
and were electrophoresed on a 1.2% agarose gel in 3-(N-morfolino)propanesulfonic acid (MOPS) buffer (20 mM
MOPS, 5 mM sodium acetate, and 2 mM EDTA, pH 7.0) along
with proper molecular weight markers. The gel was stained
with ethidium bromide to visualize the RNA bands under
ultraviolet (UV) light for Polaroid photography. The RNA was
then transferred to a nitrocellulose filter using 20× saline-
sodium citrate and was dried at room temperature. The RNA
was cross-linked with the nitrocellulose filter using an auto
cross-linker (Stratalinker; Stratagene, La Jolla, CA). Hybrid-
ization was carried out in a solution containing 5× SSC (1X
SSC = 0.15 M NaCl and 0.15 M sodium citrate at pH 7.0), 5×
Denhardt’s solution (1X Denhardt’s solution = 0.02% BSA,
0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 0.5% sodium
codexyl sulfate (SDS), 0.2 mM EDTA, 20 µg/ml calf thymus
DNA, and 100 ng/ml 32P-labeled nNOS cDNA probe. The
hybridization reaction was carried out at 40°C for 18 h, and
the filters were washed in 2× SSC containing 0.1% SDS for
1 h at room temperature followed by a wash for 1 h at 45°C. The
filters were then dried at room temperature and were exposed
to autoradiographic films (Hyperfilm; Amershams Life
Science) at − 80°C using intensifying screens.

Slot-blot hybridization was used for relative quantification
of nNOS mRNA in opossum IAS SMC, myenteric neurons,
HISM cells, neuroblastoma cells (NG-108), and different
spincteric and nonsphincteric tissues of the opossum gastro-
intestinal tract. For this purpose, 20 µg of RNA were treated
with formaldehyde and heated at 65°C for 15 min. The
denatured RNA was blotted on a nitrocellulose filter using a
filtration manifold (Bio-Dot SF; Bio-Rad Laboratories,
Hercules, CA). The RNA samples were then subjected to hybrid-
ization using 32P-labeled nNOS cDNA probe as described
previously (3).

The inconsistencies in RNA estimation by UV absorption
and in loading and blotting of the RNA during Northern and
slot-blot analyses may introduce inaccuracy in the measure-
ment of total nNOS mRNA in different samples. To minimize
this, the nitrocellulose filters used for nNOS hybridization
were hybridized with radiolabeled glyceraldehyde 3-phos-
phate dehydrogenase (GAPDH) probe after removing the
bound nNOS probes by heating in 0.05× SSC containing 0.01
M EDTA and 0.1% SDS for 30 min as described (30). The
ratio of nNOS to GAPDH mRNAs was used as a relative estimate
of nNOS gene expression in different samples.

Densitometric analysis of the autoradiograms was carried
out using a Lamac thin-layer chromatography scanner II
with SP 4290 integrator (Spectra Physics, San Jose, CA).
Reverse transcription-polymerase chain reaction analysis of
RNAs from opossum IAS SMC, myenteric plexus neurons,
HISM cell line, and neuroblastoma cells. Reverse transcription (RT)-polymerase chain reaction (PCR) was carried out to further confirm the findings from slot and Northern blot that both neuronal cells and SMC express nNOS mRNA. For this purpose, samples of RNA (5 μg) from opossum IAS SMC, myenteric neurons, HISM cells, and neuroblastoma cells were reverse transcribed using random hexanucleotide as a primer with avian myeloblastosis virus RT (Saikagaku America, Rockville, MD) in a total volume of 50 μl. As negative controls, distilled water was used instead of the RNA samples. The RT reaction mixture (10 μl) was subjected to PCR with [32P]dATP-labeled primers in a thermal cycler (Perkin-Elmer Cetus) according to the protocol provided with the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus). The two specific cDNAs primers used were from the coding region of the nNOS cDNA and were of the following sequences: primer 1: 5′-TTC CGA AGC TTC TGG CAA CA-3′ (4111-4130; forward); primer 2: 5′-AGA TCT AAG GCG GTT GGT GTT-3′ (4561-4578; reverse).

The PCR was continued for 40 cycles with each cycle consisting of heating at 94°C for 1 min and 55°C for 2 min followed by 72°C for 3 min. The samples were then rapidly cooled to 4°C for an additional 10 min. The RT-PCR reaction products were analyzed by running 5 μl of the sample on a 2% agarose gel and visualizing the DNA bands under UV light for Polaroid photography. The gels were then dried in a gel dryer (model 583; Bio-Rad) and were exposed to autoradiography films using intensifying screens.

Chemicals, reagents, and cell lines. Restriction enzymes, DNA ligase, polynucleotide kinase, DH5α, and Klenow enzyme were obtained from GIBCO BRL (Gaithersburg, MD). RPMI 1640, DMEM, fetal calf serum, antibiotic antimycotic solution, BSA, SDS, and deoxyribonuclease (type I) were from Sigma Chemical; collagenase (CLS type II) was from Worthington Biochemicals; neuroblastoma cells (NG-108) were a generous gift from Dr. L. Simpson of Thomas Jefferson University. HISM was a commercial source (ATCC) and neuroblastoma cell line (NG-108) were cultured, and the RNAs isolated from these cells were used for slot- and Northern blot hybridization studies. Surprisingly again, similar to the opossum IAS SMC and myenteric plexus neuron, both HISM and NG-108 cells were found to express significant levels of nNOS mRNA as demonstrated by slot- and Northern blot hybridizations (Figs. 2 and 3). The relative expression of nNOS mRNA (calculated on the bases of ratios of nNOS to GAPDH) in HISM, however, was smaller compared with that in the NG-108 (0.20 vs. 0.31). The quantitative data showing the relative expression of nNOS mRNA in opossum IAS SMC, myenteric neurons, HISM, and NG-108 are given in Fig. 2 (n = 3). The data show that both opossum IAS and HISM cells have lower levels of nNOS gene expres-

![Fig. 2. Relative distribution of nNOS mRNA levels in primary cultures of opossum IAS SMC and My. N. Relative distribution of nNOS mRNA in HISM and NG-108. Total RNA (20 μg) from different cell types was blotted on nitrocellulose membrane after denaturation and hybridized with nNOS cDNA probe. Note higher levels of nNOS gene expression in neurons compared with opossum IAS SMC and in NG-108 compared with HISM cells (n = 3).](image-url)
sion compared with that of IAS myenteric neurons and the neuroblastoma cells, respectively.

Figure 3 shows the nNOS band of the Northern blot analysis of RNAs obtained from IAS SMC, myenteric neurons, HISM cells, and the neuroblastoma cells. The presence of the same band in all of the samples supports the findings of the slot-blot experiments that the nNOS gene is expressed in both SMC and the neurons.

RT-PCR analysis of RNAs from opossum IAS SMC, myenteric plexus neurons, HISM cells, and NG-108 cells. To further confirm the presence of nNOS-specific mRNA in the four types of cells, we analyzed the RNAs from these cells by RT-PCR using two oligo primers from the coding region of the nNOS cDNA (from 4111 to 4130 and 4561 to 4578). The RT-PCR analyses of the RNAs from opossum IAS SMC, myenteric plexus neurons, HISM cells, and NG-108 cells using the primers from the coding region of the nNOS cDNA revealed a single DNA band of 468 base pairs on agarose gel electrophoresis (Fig. 4). Whether this band corresponds specifically to nNOS was confirmed by determining the incorporation of [32P]dATP-labeled NOS probe into the NOS cDNA. After this, the exposure of the autoradiographic film to the gel revealed that the same radioactive band was present in all of the samples (Fig. 5). These observations further support the initial findings that nNOS mRNA is expressed in both SMC and myenteric plexus neurons.

Comparison of nNOS gene expression in different sphincteric and nonsphincteric regions of the opossum gastrointestinal tract. NO has been suggested as an inhibitory neurotransmitter in different sphincteric and nonsphincteric regions of the gastrointestinal tract. So, it was of interest to compare the relative expression of the nNOS gene in these diverse regions of the gastrointestinal tract. For this purpose, different sphincteric and the adjoining nonsphincteric tissues as mentioned before were taken, and the total RNAs from
these tissues were analyzed by slot-blot analysis. When the densitometric scanning ratios for nNOS/GAPDH mRNA were compared, no significant difference in the relative expression of nNOS gene between the sphincteric and the adjacent nonsphincteric tissues was observed with the exception of the pyloric sphincter (Fig. 6; n = 3). The relative nNOS gene expression in the pyloric sphincter was found to be significantly higher than in the other tissues examined. Compared with the adjacent gastric and the duodenal tissues, the relative nNOS gene expression in the pyloric sphincter was approximately six and four times higher, respectively.

A typical example of the slot-blot experiment showing the relative distribution of nNOS mRNA in different sphincteric and nonsphincteric regions of the gastrointestinal tract is shown in Fig. 7. All of the regions showed significant levels of nNOS mRNA, but distinctly higher levels of nNOS mRNA were found in the pyloric sphincter.

Figure 8 is an example of the Northern blot analysis of RNAs isolated from opossum IAS and rectal smooth muscle tissues. The data show the presence of an nNOS transcript in the 10-kilobase region in both the IAS and the rectal smooth muscles. The intensities of the bands were similar in both tissues, further suggesting that there is no difference in the nNOS mRNA levels in these tissues.

**DISCUSSION**

The present study demonstrates the gene expression of nNOS in the myenteric plexus neurons at the transcriptional level and supports the role of NO as a NANC inhibitory neurotransmitter in the IAS and other parts of the gastrointestinal tract. The earlier evidence in support of NO as an inhibitory neurotransmitter in the IAS came from the immunohistochemical demonstration of NOS-immunoreactive neurons (20).
and by release of NO by the activation of NANC neurons of the IAS (7). The present data with nNOS mRNA in the myenteric ganglia confirm the site of synthesis and release of NO. The presence of nNOS mRNA in the myenteric plexus neuron was similar to that of the pure cell line of the neuroblastoma cells (NG-108). Compared with the myenteric plexus neuron, the levels of nNOS mRNA in the NG-108 cells were, however, lower.

In addition to the myenteric plexus neuron, significant levels of nNOS mRNA were also present in the SMC of the IAS. An immediate concern for the presence of nNOS mRNA in the SMC be the contamination of the SMC by the myenteric neurons or other nonneuronal structures well known for the presence of NOS and production of NO. Interestingly, the pure cell line of the H1SM cells was also found to express nNOS mRNA transcripts similar to that of the IAS SMC. The exact significance of the presence of the nNOS in the SMC remains to be determined.

The presence of nNOS mRNA in the SMC in the present study is different from the studies on the SMC of the blood vessels in which the NOS type has been shown to be inducible (12). Interestingly, in some parts of the gastrointestinal tract, a specific form of cNOS has been suggested to be present in the SMC (13, 24). It has been theorized that, upon NANC neural stimulation, VIP and NO are released as inhibitory neurotransmitters. A part of the inhibitory action of VIP at the SMC has been suggested to involve the activation of the NOS in the SMC and release of NO. The latter is partially responsible for the relaxation of SMC in response to NANC nerve stimulation and VIP.

Previous studies in the IAS (8, 25, 27) have shown that a major part of VIP-mediated relaxation of the IAS is by its action directly at the SMC and is NO independent. In addition, a part of the VIP-induced relaxation of the IAS may involve the NOS pathway (NO-dependent component of the VIP response; see Ref. 27). The site of NO release responsible for the NO-dependent component of the VIP response has been investigated recently (9). The data suggest that a major part of the NO-dependent component of the VIP response is via the release of NO from the myenteric neurons, and a small part of the NO-dependent component of the VIP response in the IAS may occur in the SMC. The reason for the preferential release of NO by VIP from the myenteric neurons over the SMC remains to be determined. The concept of the myenteric neuronal site of VIP-induced NO release is supported by studies on mouse fundus in which the slow VIP-mediated inhibitory junction potential present in the control mice was absent in the mutant mice that had the nNOS gene knocked out specifically (22).

The presence of nNOS (a cNOS) mRNA transcripts in the nonneuronal cells may not be unique. Interestingly, the presence of nNOS mRNA has been shown in the striated muscle fibers (6) and in a number of other nonneuronal cells. In the skeletal muscle, the presence of nNOS is not simply related to the motor end plates but to the striated muscle cells. Furthermore, nNOS has been shown to be present in the sarcolemma of both visceral and somatic skeletal muscle fibers, and NO has been suggested to play a significant role in the inhibition of contraction (14). The authors concluded that the visceral and somatic striated muscles may constitute the richest and the most important source of NO in the mammalian body. It is possible that NO plays a physiological role in the contractility of the striated muscles, and abnormalities in the nNOS may lead to degeneration of fast-twitch muscle fibers in Duchenne muscular dystrophy (6).

The presence of nNOS-immunoreactive neurons in the gastrointestinal tract is abundant (19, 32, 36). Additionally, it has been suggested that nNOS immunoreactivity may also be present, although sparsely distributed in the circular smooth muscle (4). Whether this NOS immunoreactivity in the circular smooth muscle layer is related to the presence of NOS in the myenteric nerve terminals or the SMC per se is not exactly known. Furthermore, analysis of the presence of NOS activity in the SMC of canine small and large intestine showed primarily the presence of either iNOS or an as yet unidentified type of NOS that is calcium-calmodulin independent (18). Interestingly, other studies showing the presence of cNOS in the SMC have been speculated to be of the eNOS type (24). The present study did not examine the presence of eNOS but clearly demonstrates the presence of nNOS in the SMC. The physiological significance of nNOS in the SMC remains to be elucidated.

Interestingly, among different tissues of the gastrointestinal tract investigated, the higher levels of nNOS were found in the pyloric sphincter reproducibly in all of the animals investigated. Why the pyloric sphincter possesses the highest levels of nNOS is not exactly known. It is possible that it reflects the significant steady-state production of NOS responsible for the synthesis and release of NO and that the basal tone and the inhibitory neurotransmission in the pylorus are critically dependent on the NOS activity. This type of inhibitory innervation may be critical for gastric accommodation and emptying (1, 11, 15, 26). This notion is further supported by the nNOS gene knockout model of the mice where it was found that the animals, otherwise normal, had significantly enlarged stomachs and pyloric stenosis (16). The same observations were confirmed in patients with pyloric stenosis, since these patients had selective loss of NOS immunoreactivity (35).

It is noteworthy that the inhibitory junction potential present in the mouse fundus in response to VIP was absent in the mutant mice with nNOS gene knockout (22). The data also support the importance of nitrergic innervation for the action of VIP in the pylorus (1, 15). The findings are in agreement with our recent studies that show that the NO-dependent component of the VIP response in the IAS may occur via the release of NO preferentially from the myenteric neurons (9). Interestingly, some of the characteristic features of the pylorus are 1) the development of the enteric nervous
system of the human pylorus has been shown to be most advanced (34), and it has been shown to be most abundantly innervated by VIP-immunoreactive neurons (2) and has high levels of VIP gene expression (3).

In summary, nNOS mRNA transcripts are expressed in high levels in the myenteric neurons of the IAS. The data also show the presence of nNOS mRNA in the IAS SMC. This may not be simply due to the contamination of the SMC with the neuronal cells or other nNOS-containing structures, since a pure line of HSM cells also showed similar results. The exact significance of nNOS mRNA in the IAS remains to be determined. In addition, distinctly higher levels of nNOS gene expression were found in the pyloric sphincter compared with other regions of the gastrointestinal tract. The presence of nNOS in the myenteric neurons provides direct support for the role of NO in the inhibitory neurotransmission in the IAS and other areas of the gastrointestinal tract. Characteristically higher levels of nNOS in the pyloric sphincter support the role of the NOS pathway in the regulation and pathophysiology of the sphincter.

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