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Themed Section: Nitric Oxide 20 Years from the 1998 Nobel Prize

REVIEW ARTICLE

Nitric oxide and its role as a non-adrenergic, non-cholinergic inhibitory neurotransmitter in the gastrointestinal tract

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NO is a neurotransmitter released from enteric inhibitory neurons and responsible for modulating gastrointestinal (GI) motor behaviour. Enteric neurons express nNOS (NOS1) that associates with membranes of nerve varicosities. NO released from neurons binds to soluble guanylate cyclase in post-junctional cells to generate cGMP. cGMP-dependent protein kinase type 1 (PKG1) is a major mediator but perhaps not the only pathway involved in cGMP-mediated effects in GI muscles based on gene deletion studies. $NOS1^+$ neurons form close contacts with smooth muscle cells (SMCs), interstitial cells of Cajal (ICC) and $PDGFR\alpha^+$ cells, and these cells are electrically coupled (SIP syncytium). Cell-specific gene deletion studies have shown that nitrergic responses are due to mechanisms in SMCs and ICC. Controversy exists about the ion channels and other post-junctional mechanisms that mediate nitrergic responses in GI muscles. Reduced nNOS expression in enteric inhibitory motor neurons and/or reduced connectivity between $nNOS^+$ neurons and the SIP syncytium appear to be responsible for motor defects that develop in diabetes. An overproduction of NO in some inflammatory conditions also impairs normal GI motor activity. This review summarizes recent findings regarding the role of NO as an enteric inhibitory neurotransmitter.

LINKED ARTICLES

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Abbreviations

$[Ca^{2+}]_i$, intracellular Ca^{2+}; $[K^+]_o$, external K^+ concentration; CaCC, Ca^{2+}-activated Cl^- channel; $Ca_V1.2$, L-type Ca^{2+} channel; CPA, cyclopiazonic acid; EDRF, endothelial-derived relaxing factor; EFS, electrical field stimulation; GI, gastrointestinal; ICC, interstitial cells of Cajal; ICC-DMP, interstitial cells of Cajal at the level of the deep muscular plexus; ICC-IM, intramuscular interstitial cells of Cajal; IJP, inhibitory junction potential; IP_3, inositol 1,4,5-trisphosphate; IP_3R1, IP_3 receptor type 1 (encoded by *Itpr1*); IRAG, inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate; $K_{Ca}1.1$, large conductance Ca^{2+}-activated K^+ channel; LES, lower oesophageal sphincter; L-NAME, N^G-nitro-L-arginine methyl ester; L-NNA, N^G-nitro-L-arginine; MLCP, myosin light chain phosphatase; PKG1, cGMP-dependent protein kinase; Rp 8-Br PET cGMPS, bromo-3,4-dihydro-3-[3,5-O-[(R)-mercaptophosphinylidene]-β-D-ribofuranosyl]-6-phenyl-9H-Imidazo[1,2-a] purin-9-one sodium salt; SDK, stretch-dependent K^+; SMC, smooth muscle cell; STIC, spontaneous transient inward current; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide

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Introduction

Thirty years have passed since the introduction of the idea that **NO** is an inhibitory non-adrenergic, non-cholinergic (NANC) neurotransmitter in visceral smooth muscles. Building on findings that NO has similar relaxant properties on smooth muscles as endothelial-derived relaxing factor (EDRF) (Gruetter *et al.,* 1979), a substance like EDRF is produced by cerebellar neurons (Garthwaite *et al.,* 1988), and arginine analogues inhibit synthesis of EDRF (Palmer *et al.,* 1987), the group of John Gillespie reported that N^G-monomethyl L-arginine (l-NMMA) raised tone and blocked inhibitory responses to nerve stimulation in rat anococcygeous muscles, and excess L-arginine reversed this effect (Gillespie *et al.,* 1989). These authors concluded that the NANC neurotransmitter was likely to be NO, and they also noted that the enzymes responsible for synthesizing NO in NANC neurotransmission had somewhat different properties than the enzymes responsible for EDRF.

The concept of NO as an inhibitory neurotransmitter was introduced into the enteric nervous system by a seminal study showing that NANC relaxation of the canine ileocolonic junction is due to a soluble factor with properties and pharmacology like NO (Bult *et al.,* 1990). Later, the non-purinergic portion of electrophysiological responses to NANC nerve stimulation (inhibitory junction potentials; IJPs) was found to be NO, and responses were mimicked by exogenous NO and NO donors in gastrointestinal (GI) muscles of laboratory animals and humans (Dalziel *et al.,* 1991; Stark *et al.,* 1991; Thornbury *et al.,* 1991; Stark *et al.,* 1993). It was then found that NO comes from enteric neurons with Dogiel type 1 morphology, and it is synthesized by the cerebellar isoform of NOS [**neuronal NOS (nNOS)** also known as NOS1] (Bredt *et al.,* 1990; Ward *et al.,* 1992; Young *et al.,* 1992).

The new hypothesis regarding NO as an enteric inhibitory neurotransmitter, however, met with considerable resistance from advocates of vasoactive intestinal polypeptide (**VIP**) in this role (Goyal *et al.,* 1980; Grider *et al.,* 1992). These authors proposed an indirect role for NO, as a secondary, paracrine-like substance generated by smooth muscle cells (SMCs) or released 'in series' from neurons in response to VIP (He and Goyal, 1993; Murthy *et al.,* 1995; Teng *et al.,* 1998). This controversy was debated in a lively manner during the 1990s before NO was generally accepted as an enteric inhibitory neurotransmitter (Sanders and Ward, 1992; Furness *et al.,* 1995; Murthy *et al.,* 1996). This review focuses on progress towards ascertaining the role and mechanisms of NO as an enteric neurotransmitter that have emerged since the Nobel Prize for NO in 1998.

Prejunctional localization and activity of nNOS in enteric neurons

Neuronal NOS protein co-localizes with VIP in enteric neurons in cell bodies and varicose processes that innervate muscle bundles in GI muscles (Ward *et al.,* 1992; Young *et al.,* 1992). Co-localization of these transmitters supports the current idea that there is a single population of inhibitory motor neurons in GI muscles. There is no evidence suggesting that NO is stored in nerve varicosities. NO appears to be produced on-demand when intracellular Ca^{2+} ($[Ca^{2+}]_i$) is elevated in motor neurons, and release of NO is blocked by antagonists of neuronal Ca^{2+} channels (Mashimo *et al.,* 1996). At least six 5′-splice variants of nNOS occur in human GI muscles; three variants of nNOSα, two of nNOSβ and one of nNOSγ (Saur *et al.,* 2000). The α isoforms contain a domain that facilitates membrane associations. The presence of membrane-associated and cytosolic pools of nNOS suggest possible multiple biological functions or warehousing of excess nNOS that can be rapidly recruited into service upon demand.

An interesting question is whether nNOS protein distributed along motor neurons is catalytically active or whether specialized regions of catalytically active nNOS exist in varicosities. Such punctate sources of NO within muscles may spatially limit the effective concentration of NO. Studies of this question with isolated enteric nerve varicosities suggest that active and inactive pools of nNOS are present (Rao *et al.,* 2008). Inactive cytosolic nNOSα associates with proteins, such as dynein light chain 8 (Rodriguez-Crespo *et al.,* 1998), and may, through association with myosin Va, translocate to the plasma membrane (Chaudhury *et al.,* 2011). Mice with partial loss-of-function of myosin Va displayed reduced association of nNOSα with varicosity membranes, attenuated inhibitory junction potentials (IJPs) and reduced hyperpolarization responses to diethylenetriamine-NO in gastric muscles. The nNOS may be localized at varicosity membranes to facilitate interactions with regulatory proteins and a source of Ca^{2+} required for activation of NO synthesis (Chaudhury *et al.,* 2009).

Strains of mice with genetic deletion of *Nos1* (encoding nNOS) have been used to elucidate the role of NO in neurotransmission. The initial knockout targeted exon 2 and produced a phenotype with an enlarged stomach and a hypertrophic pyloric sphincter that was likened to juvenile pyloric stenosis (Huang *et al.,* 1993). Alterations in colonic migrating motor complexes were also noted in this knockout mouse (Dickson *et al.,* 2010). However, residual NOS activity (5%) was present in these mice, and it was later realized that nNOSβ and nNOSγ splice variants do not contain exon 2. Thus, part of the residual NO production after deletion of exon 2 in neuronal tissues may be due to retained nNOS function. Deletion of exon 6, encoding the haem-binding and catalytic domain in nNOS, produced more complete deletion of nNOS and greatly reduced the conversion of [^{3}H]-arginine to [^{3}H]-citrulline (i.e. 0.3% of wild type) (Gyurko *et al.,* 2002). These mice reproduced the phenotype of hypertrophic pyloric stenosis, but, probably due to the feeding and breeding difficulties with these sexually dimorphic mice, further phenotyping of GI abnormalities has not occurred.

Apparatus to transduce NO signals in post-junctional cells

Mice lacking the β1 subunit of soluble guanylate cyclase (sGCβ1) fail to respond to NO donors or to NO released from enteric motor neurons (Groneberg *et al.,* 2011). Blockade of sGC with ODQ inhibits nitrergic IJPs and blocks the inhibition of contractions caused by the release of NO from nerves and NO donors (Franck *et al.,* 1997). ODQ does not affect responses to 8-Br-cGMP. Such observations suggest that sGC

is the sole receptor for NO in post-junctional cells. The receptor for NO is not G-protein coupled, but cytoplasmic, and composed of α (α1, α2) and β (β1) subunits of sGC, α1β1 being the most common (Koesling *et al.,* 2004). Binding of NO to the N-terminal haem group of sGC causes a conformational change and generation of **cGMP** from the catalytic domain at the C-terminus (Ignarro, 1990).

Morphological investigation of the guinea pig GI tract showed the expression of **sGCβ1** in interstitial cells of Cajal (ICC), **PDGFRα**$^+$ cells and enteric neurons within ganglia (Iino *et al.,* 2008). Nearly the same distribution was obtained for **sGCα1**. Immunochemical labelling of sGCβ1 was typically unresolvable in SMCs, but areas lacking intermuscular ICC (ICC-IM) displayed weak sGCβ1-like immunoreactivity in SMCs. The expression of sGC subunits in post-junctional cells in the mouse internal anal sphincter was evaluated, and the relative expression profiles of sGCα1 and sGCβ1 genes were PDGFRα$^+$ cells >ICC >>SMC (Cobine *et al.,* 2014). Levels of cGMP before and after nitrergic stimulation were investigated in canine colonic muscles (Shuttleworth *et al.,* 1993). Stimulation of muscles with exogenous NO increased cGMP in multiple cell types, including SMCs, but electrical field stimulation (EFS) of intrinsic neurons induced cGMP immunoreactivity in ICC, and these responses were blocked by **N^G-nitro-L-arginine (L-NNA)**.

The dominant α subunit expressed in GI muscles is sGCα1, and gastric muscles of *Gucy1a1* knockouts had reduced responses to EFS, NO and **BAY 41-2272**, an sGC agonist. However, inhibitory responses sensitive to **ODQ** were retained in gastric muscles of *Gucy1a1*$^{-/-}$ mice (Vanneste *et al.,* 2007). The effects of ODQ and BAY41-2272 are consistent with responses to NO being mediated by sGC, so it appears that sGCα1 is not the only α isoform of sGC expressed in gastric muscles. Transcriptomic data from expression analysis agree with this conclusion and show a high expression of *Gucy1a1* and much lower expression of *Gucy1a2* (Lee *et al.,* 2017). Despite its low level of expression, *Gucy1a2* may sustain a portion of nitrergic responses when *Gucy1a1* is deactivated.

The increase in cGMP in response to NO is linked to several effectors in cells, including the cGMP-dependent protein kinase, **PKG1**, PDE and nucleotide-gated ion channels (Francis *et al.,* 2010). The expression and abundance of these effectors tend to define the mechanism of action of NO in specific types of cells. Two major splice variants of PKG1 occur, PKG1α and PKG1β (Lincoln *et al.,* 1988). Post-junctional responses to NO are commonly attributed to PKG1. For example, relaxation responses to NO donors and 8-Br-cGMP were greatly attenuated in gastric fundus muscles of *Prkg1*$^{-/-}$ mice (Ny *et al.,* 2000). Another study, however, investigated nitrergic responses in a different *Prkg1*$^{-/-}$ mouse and found that significant NO- and ODQ-dependent responses were retained in the mouse internal anal sphincter (Cobine *et al.,* 2014), suggesting that downstream effectors other than PKG1 contribute to nitrergic relaxation. The effectiveness of the deactivation of *Prkg1* in these studies was demonstrated by parallel positive control experiments performed on mouse aorta. It should be recognized that several PDEs are also expressed in post-junctional cells, including the dual substrate **PDE3A** (Chen *et al.,* 2007) that is expressed in ICC and directly inhibited by cGMP *via* competition with **cAMP** for the active site (Maurice *et al.,* 2003). PDE3A could be a target for cGMP, and therefore, part of the downstream mechanisms causing the inhibitory effects of NO might be cAMP-dependent.

Postjunctional cells that respond to enteric inhibitory neurotransmission

Morphology and connectivity of enteric inhibitory neurons and postjunctional cells

The GI tract is complicated by the fact that there are at least three types of cells that might contribute to the transduction of nitrergic signals. These cells are SMCs, ICC and PDGFRα$^+$ cells. ICC and PDGFRα$^+$ cells are electrically coupled to SMCs, forming an electrical syncytium, known as the SIP syncytium (Sanders *et al.,* 2012). Changes in conductances in any of the SIP cells can influence voltage-dependent processes in the other cells, so neurotransmitter responses can be generated in any of the cells and conduct to other SIP cells. ICC and PDGFRα$^+$ are wound around varicose processes of enteric motor neurons and in close contact with varicosities of excitatory and inhibitory neurons, including those expressing nNOS. Thus, each SIP cell may be exposed to NO released from motor neurons, and there has been a significant effort to determine which cells mediate nitrergic responses.

After discovering that ***Kit*** is expressed in ICC in the GI tract, it was found that mutations in *Kit* (*W* locus in mice) negatively impact the development of ICC (Maeda *et al.,* 1992; Ward *et al.,* 1994; Huizinga *et al.,* 1995; Torihashi *et al.,* 1995). Severe *W* mutations result in complete loss of the tyrosine kinase activity of c-Kit (Nocka *et al.,* 1990), and homozygotic *W* mutants (*W/W*) typically die *in utero* (Russell, 1979). Compound heterozygotes, incorporating less severe mutations in the *W* locus, such as *W/W*^{V}, have one allele that encodes a partially functional tyrosine kinase and offspring with this genotype survive to adulthood (Nocka *et al.,* 1990; Chi and Powley, 2003). These mice have compromised development of ICC and have been used extensively to explore the role of ICC in GI motility (Sanders, 1996).

Morphological studies have shown close contacts between enteric motor neurons and ICC (e.g. Daniel and Posey-Daniel, 1984; Rumessen *et al.,* 1992). This relationship was also observed in the murine gastric fundus where enteric neurons make contacts with ICC-IM (Burns *et al.,* 1996). Immunofluorescence shows extensive tracking of ICC with enteric motor neurons in rodent and primate GI muscles (Wang *et al.,* 1999; Salmhofer *et al.,* 2001; Blair *et al.,* 2012), as illustrated for nNOS$^+$ neurons in Figure 1. Sites of close contact between nerve varicosities and ICC deep muscular plexus (DMP) display pre- and post-junctional specializations. Similar areas of specialization occur between enteric neurons and SMCs; however, these are more rare (Daniel and Posey-Daniel, 1984). Close, synaptic-like contacts between ICC-IM and nNOS$^+$ neurons were also found in the colons of guinea pigs, and contacts of this sort were less common with SMCs (Wang *et al.,* 2000). These observations support the idea that intramuscular types of ICC (ICC-IM and ICC-DMP) are innervated by nNOS$^+$ neurons; however, the presence of similar contacts with SMCs suggests parallel innervation of these cells.

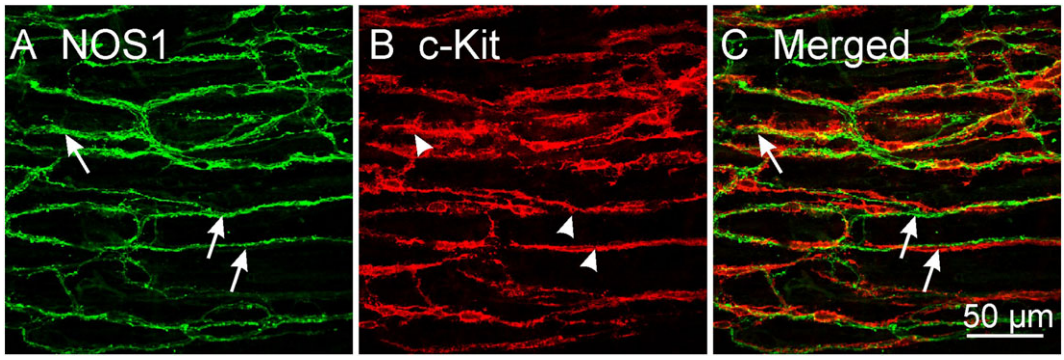

Figure 1

Confocal images of whole-mount preparations showing close relationship between ICC and nNOS$^+$ varicose nerve fibres in the murine proximal colon. Double labelling of the intramuscular class of ICC in colon (ICC-IM) showing varicose processes of (A) nNOS$^+$ neurons (NOS1; arrows) and (B) c-Kit$^+$ ICC-IM (arrow heads). nNOS$^+$ neurons track along ICC-IM for distances greater than 250 μm and form close contacts. (C) Merged image showing the close relationship between nNOS$^+$ neurons and ICC-IM (arrows). Scale bar = 50 μm applies to all panels.

Synapse-like connections between enteric neurons and ICC-IM and cells lining septa separating smooth muscle bundles were investigated in canine antrum (Horiguchi *et al.,* 2003b). Varicosities containing neurotransmitter vesicles were in close contact with ICC (<20 nm), and pre- and post-synaptic densities were observed. Gap junctions were also observed between ICC and SMCs, suggesting that signaling that develop in ICC can be conducted to SMCs. Contacts between enteric nerve varicosities displayed ultrastructural features similar to nerve-nerve varicosities in the CNS. Presynaptic densities contain a complex of proteins, including SNAP-25, syntaxin, synaptobrevin and synaptogamin that are part of the machinery needed for neurotransmitter release *via* exocytosis (Chapman *et al.,* 1994). SNAP-25 and synaptogammin were co-expressed in the varicose processes of nNOS$^+$ motor neurons, and these varicosities adorned ICC-IM along their lengths, suggesting multiple sites of innervation for each cell (Beckett *et al.,* 2005). While vesicular transport is likely not needed for NO, the presence of synaptic proteins and nNOS suggests that release of NO occurs near release sites of other inhibitory neurotransmitters. ICC express postsynaptic density proteins PSD93 and PSD95, and recent transcriptome analysis of ICC from small bowel and colon confirms expression of genes that encode these proteins (i.e. *Dlg2* and *Dlg4*, respectively) (Lee *et al.,* 2017). Transcripts of *Dlg2* and *Dlg4* are found in whole muscles and are depressed significantly in *W/W^V* muscles with reduced ICC (Beckett *et al.,* 2005). Some authors have favoured the idea that neurotransmission occurs by 'volume transmission' in GI muscles (i.e. diffusion of neurotransmitters through the interstitium without synaptic specializations), but the presence of synapse-like morphological structures, pre- and postjunctional specializations and membrane-specific localization of nNOSα (Chaudhury *et al.,* 2009, 2011) suggest that specialized neuro-ICC junctions may be important for enteric motor neurotransmission.

Nitrergic responses in the lower oesophageal sphincter

The lower oesophageal sphincter (LES) maintains tone to restrict movement of gastric contents into the oesophagus. Tone is inhibited during swallowing to allow food to enter the stomach. The main inhibitory neurotransmitter causing relaxation of tone in the swallowing reflex is NO (Kim *et al.,* 1999). The LES contains intramuscular ICC (ICC-IM) that are closely associated with enteric motor neurons. Failure of these cells to develop in *W/W^V* mice impairs nitrergic relaxation, suggesting that a portion of the nitrergic response is transduced by ICC-IM (Ward *et al.,* 1998). Cell-specific genetic deactivation of sGC in SMCs or ICC increased LES tone, suggesting that both types of cells are innervated by nitrergic neurons and contribute to regulation of tone (Groneberg *et al.,* 2015). However, the drop in tone induced by swallowing was compromised only when sGC was knocked down in ICC, suggesting that ICC are important transducers in the swallowing reflex.

Nitrergic responses in the stomach

The proximal stomach serves as a reservoir that relaxes, as food is ingested. This reflex is known as accommodation, and it is driven by nitrergic neurons (Desai *et al.,* 1991). ICC-IM are a prominent population of cells in the gastric fundus, as in the LES. ICC-IM are closely associated with excitatory (cholinergic) and inhibitory (nitrergic) enteric motor neurons (Burns *et al.,* 1996). Loss of ICC-IM has been investigated in several mouse models, the most prominent being *W/W^V* mice. Wild-type mice generate IJPs that are partially inhibited by **N^G-nitro-L-arginine methyl ester (L-NAME)**. IJPs are smaller in *W/W^V* mice and insensitive to L-NAME. EFS causes inhibition of tone in the fundus, and this response switched to a contractile response in *W/W^V* mice. The nNOS$^+$ neurons are distributed normally in fundus muscles of *W/W^V* mice, so the defect in nitrergic responses appears to be related to postjunctional defects. It was also observed that the hyperpolarization response to SNP was greatly reduced or absent in *W/W^V* fundus muscles, but exogenous NO still caused relaxation of the same magnitude as in wild-type muscles. This observation suggests that both ICC and SMCs express sGC and have the intrinsic apparatus to respond to NO; however, bath applied NO does not simulate NO released from enteric inhibitory neurons. Results from studies of *W/W^V* mice suggested that ICC-IM provide a

pathway for transduction of responses to NO released from motor neurons.

Reflex activation of the stomach from the CNS occurs through the vagus nerve that connects with enteric motor neurons. A preparation was developed to record intracellular electrical or mechanical activities from murine fundus or antrum during stimulation of efferent anterior and posterior vagal trunks (Beckett *et al.,* 2017). Stimulation of vagal trunks caused IJPs in the fundus that were enhanced after atropine and nearly blocked by L-NNA. IJPs were absent in most W/W^V fundus muscles, and residual IJPs in others were unaffected by L-NNA. Relaxation responses were caused in fundus and antrum by vagal stimulation, and these were attenuated significantly in W/W^V stomachs (Figure 2).

Some investigators, however, have dismissed the importance of ICC in nitrergic responses, reporting nearly normal functions in muscles with reduced ICC-IM (Goyal, 2016). Comparisons of behaviour in W/W^V muscles can be problematic because we found that lesions in ICC are not uniform, and when a substantial number of ICC remain, nitrergic inhibition is maintained (Sanders *et al.,* 2014). A major argument against a role for ICC in nitrergic responses was that muscle tone in the LES was different in W/W^V mice and $Nos1^{-/-}$. The reasoning was that nitrergic neurotransmission should be deactivated in both mice if ICC have a significant role, but the tone of the LES was hypertensive in $Nos1^{-/-}$ mice and hypotensive in W/W^V mice (Sivarao *et al.,* 2001). This issue can be explained by the fact that only nitrergic responses

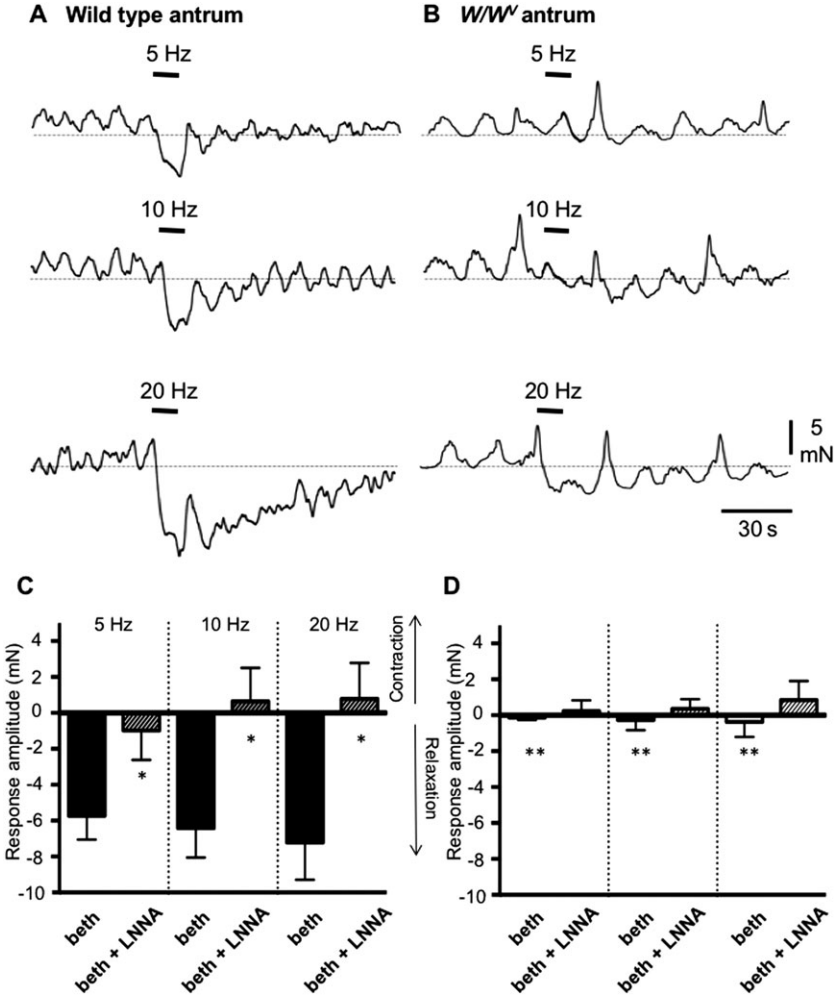

Figure 2

Antral responses to vagal nerve stimulation in wild-type and W/W^v mice. Contractions of wild-type and W/W^V antral muscles were measured in response to electrical vagal stimulation (EVS; 5, 10 and 20 Hz; delivered during periods denoted by black bars). Muscles were pretreated with **bethanechol** (3 μM). (A) EVS elicited relaxation of wild-type muscles, and the amplitude of the relaxation increased with stimulus frequency. (B) EVS caused relaxation of W/W^V muscles, as well, but the responses were of much smaller amplitude than in wild-type muscles. (C, D) Summary of experiments showing average responses to EVS in wild-type (n = 15) and W/W^V (n = 8) antral muscles before and in the presence of L-NNA (100 μM). Data in W/W^V muscles were tested against responses under same conditions in wild-type muscles: *$P < 0.05$; **$P < 0.01$. Redrawn from Beckett *et al.* (2017).

are missing in *Nos1*$^{-/-}$ mice, but cholinergic and nitrergic responses may be reduced in *W/W*^{V} mice (Ward *et al.,* 1998; Ward *et al.,* 2000; Sanders *et al.,* 2002; Sanders *et al.,* 2010). Cholinergic input affects LES tone, so loss of this input may produce hypotensive LES muscles in *W/W*^{V} mice.

The controversy about ICC versus SMCs was addressed in more detail by investigating responses of mice with cell-specific knockouts of sGCβ1 in SMCs and ICC. Knockout of sGC in ICC of the fundus simulated the effects of L-NAME in that relaxation responses to EFS were partially inhibited and nitrergic IJPs were essentially ablated (Groneberg *et al.,* 2013; Lies *et al.,* 2014a). Global knockouts displayed similar effects. These data tend to confirm results from *W/W*^{V} mice, but these authors also found that specific knockout of sGCβ1 in SMCs reduced the durations of IJPs by half and also partially inhibited relaxation responses to nitrergic stimulation. These experiments suggest that cGMP-dependent mechanisms in two cell types may be responsible for nitrergic relaxation in the murine fundus. One mechanism may be voltage-dependent and depend upon ion channels in ICC-IM that are either activated or deactivated in a cGMP-dependent manner. A second mechanism may involve other types of ion channels and a non-voltage-dependent mechanism in which the contractile state of SMC cells, perhaps by modulation of the Ca^{2+} sensitivity of the contractile apparatus, is regulated.

Nitrergic responses in the small intestine

Damaging ICC by cell-specific expression of diphtheria toxin A, caused a sustained depolarized membrane potential and loss of slow waves in small intestinal muscles (Klein *et al.,* 2013). Neither excitatory nor inhibitory junction potentials were elicited in ICC-depleted muscles, and these authors concluded that SMCs are denervated from enteric motor neurons when ICC are lost or damaged. Developmental studies also shed light on the relative role of ICC in nitrergic responses in the small intestine (Ward *et al.,* 2006). ICC-DMP develop after birth in mice, so neural inputs were compared at postpartum day 0 (P0) and at P10. Neural responses were poorly developed at P0, but by P10, both excitatory and inhibitory responses were observed. P0 muscles were put into organ culture and treated with a neutralizing antibody against c-Kit that restricts development of ICC. Neural responses developed in organ culture, but inclusion of the neutralizing antibody led to loss of both cholinergic and nitrergic responses, again suggesting that ICC are a primary site of neurotransmission in the small intestine.

A recent study investigated nitrergic regulation of basal contractions in the small intestine (Voussen *et al.,* 2018). Global and SMC-specific knockout of sGCβ1 had no effect on longitudinal muscles but increased contractions in circular muscle. Significant tone and increased contractile amplitude occurred in response to tetrodotoxin (**TTX**), L-NAME and ODQ in wild-type mice, and these responses were absent in global and SMC-specific sGCβ1 knockout mice. These findings suggest that basal release of NO and exogenous NO affects contractions of SMCs by a mechanism intrinsic to these cells.

Nitrergic responses in the colon

The colon is also under tonic inhibitory drive, and TTX or L-NNA depolarize and generate action potentials, suggesting that tonic inhibition comes from nitrergic neurons (Dickson *et al.,* 2010). L-NNA increased the frequency of contractions in wild-type mice, and this effect of L-NNA was absent in *Nos1*$^{-/-}$ mice. Knockout of sGCβ1 was used as another means of blocking the activity of nitrergic neurons (Lies *et al.,* 2015). Wild-type mice had an irregular pattern of colon contractions, and this activity was also seen in colons with SMC-specific knockout. Global and ICC-specific knockouts of sGCβ1 produced contractions of regular amplitude and duration. Thus, basal release of NO mainly regulates colonic contractile behaviour through its effects on ICC.

The amplitudes of IJPs in colonic muscles were unaffected in mice with SMC-specific knockout of sGCβ1 but reduced in ICC-specific knockouts (Lies *et al.,* 2014a). However, SMC-specific knockout of sGC had a significant effect on the duration of the IJPs, and this effect was not observed in ICC-specific knockouts. These authors hypothesized that there are two components in nitrergic IJPs in colonic muscles, a large amplitude component mediated by ICC and a slower, lower amplitude component mediated by SMCs. This observation suggests that conductances in both ICC and SMC may be targets for nitrergic regulation.

Mechanisms responsible for nitrergic inhibition in GI muscles

As discussed previously, sGC is the receptor for NO and necessary for NO-dependent relaxation in GI muscles, as shown by pharmacological experiments and global gene deactivation of sGCβ1 (Groneberg *et al.,* 2011). The major target for cGMP produced in response to NO released from inhibitory neurons is PKG1. Global knockout of *Prkg1* caused inhibition of NO-dependent relaxation in the stomach (Pfeifer *et al.,* 1998; Ny *et al.,* 2000). *Prkg1* occurs as at least two splice variants in smooth muscles, *Prkg1*α and *Prkg1*β, with *Prkg1*β being dominant (Klein *et al.,* 2013). To generate a quantitative knockout of *Prkg1*, an exon common to both splice variants was targeted (Pfeifer *et al.,* 1998). *Prkg1*$^{-/-}$ mice had grossly distended GI tracts and gastric and pyloric hypertrophy without damage to the enteric nervous system. Nitrergic relaxation responses were absent in muscles of *Prkg1*$^{-/-}$ mice. SMC-specific *Prkg1*$^{-/-}$ mice did not reproduce the phenotype of global knockout animals, so ICC-specific *Prkg1*$^{-/-}$ mice were produced (Klein *et al.,* 2013). Activation of iCre driven by the endogenous promoter for c-Kit in *Prkg1*$^{fl/fl}$ mice resulted in loss of PKGβ1 in only about half of ICC; however, these mice displayed significant GI motor defects, including increased GI transit time, changes in contraction frequency and a reduction in the nitrergic component of IJPs (Figure 3). Results of this study support the idea that ICC transduce neural inputs to GI muscles and explain some of the controversial findings using *W/W*^{V} mice (Goyal, 2016). ICC express relatively high levels of sGC subunits (Iino *et al.,* 2008; Cobine *et al.,* 2014), generate cGMP in response to enteric nerve stimulation (Shuttleworth *et al.,* 1993; Iino *et al.,* 2009) and express *Prkg1* (Klein *et al.,* 2013). If the release of NO from motor neurons is restricted spatially (possibly by amount, binding or metabolism), then the close apposition of nerve varicosities (Burns *et al.,* 1996; Wang *et al.,* 1999, 2000;

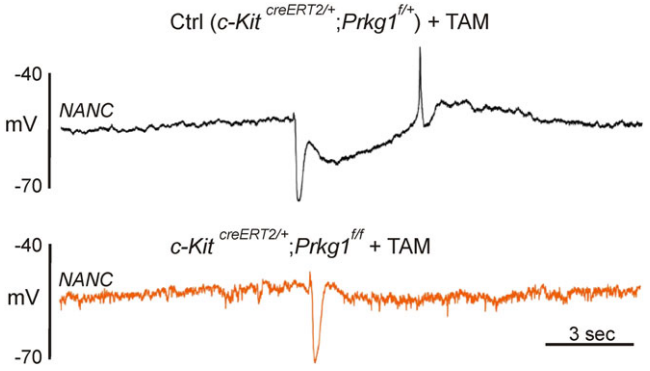

Figure 3

Elimination of slow (nitrergic) IJP in colon of *Prkg1*$^{-/-}$ mice. (A) Intracellular recording of IJP in murine proximal colon from a control [**tamoxifen** (TAM)-treated c-Kit$^{CreERT2/+}$;Prkg1$^{f/+}$] mouse. IJP, evoked by EFS, evoked by EFS, consisting of a fast IJP (fIJP) due to release of a purine, and a slow IJP (sIJP), due to release of NO. (B) IJP evoked in proximal colon of a TAM-treated (3 days) c-Kit$^{CreERT2/+}$; Prkg1$^{f/f}$ mouse. Expression of iCre is limited to ICC in these mice. Note reduction of sIJP in panel (B). PKG1 was not resolved in 40% of cells impaled in the c-Kit$^{CreERT2/+}$; Prkg1$^{f/f}$ animals after 3 days of TAM treatment. Redrawn from Klein *et al.* (2013)

Horiguchi *et al.,* 2003a; Blair *et al.,* 2012) favours responses, particularly electrophysiological responses, to be generated by ICC and conducted to SMCs. Post-junctional mechanisms activated by nitrergic stimuli are complex and incompletely understood. Several possible pathways have been proposed and tested using patch clamp studies, deactivation of specific genes involved in proposed transduction pathways, Ca^{2+} imaging and intracellular electrical recording.

General observations about ion channels that may be affected by NO

Investigators have sought to discover the ionic conductance(s) responsible for electrophysiological responses to NO. Until only recently, it was not possible to isolate and study all three of the cell types of the SIP syncytium, so most experiments have been performed on SMCs. **$Ca_v1.2$** channels, fundamental to voltage-dependent mechanisms regulating contractions, are inhibited by cGMP-dependent mechanisms and increased by an inhibitor of PKG1, Rp 8-Br PET cGMPS (Ruiz-Velasco *et al.,* 1998). This could be a primary mechanism of nitrergic inhibition. Large conductance Ca^{2+}-activated K^+ (**$K_{Ca}1.1$**) channels were reported to be activated by NO in a cGMP-independent manner (Bolotina *et al.,* 1994). However, this finding was not confirmed in studies of $K_{Ca}1.1$ channels from GI muscles, as NO did not affect $K_{Ca}1.1$ channel open probability in inside-out patches from colon SMCs (Koh *et al.,* 1995). $K_{Ca}1.1$ channels were activated significantly when membrane permeable analogues of cGMP or NO were applied to colonic SMCs (Thornbury *et al.,* 1991; Koh *et al.,* 1995). However, it is unlikely that $K_{Ca}1.1$ channels participate in postjunctional nitrergic responses, since $[Ca^{2+}]_i$ is typically low during inhibitory stimulation, voltage-dependent activation of $K_{Ca}1.1$ channels occurs at potentials more positive than membrane potentials during IJPs, and charybdotoxin does not block IJPs. Two additional K^+ channels [called NO1 (82 pS) and NO2 (<4pS) due to their lack of identification in this study] were activated in canine colonic SMCs by NO or dibutyryl cGMP (Koh *et al.,* 1995). Later studies suggested that currents from NO1 are mediated by **$K_{2P}2.1$** channels (Koh *et al.,* 2001).

A role for two-pore K^+ channels in mediating postjunctional responses to NO

Colonic SMCs express stretch-dependent K^+ (SDK) channels encoded by the two-pore K^+ channel, $K_{2P}2.1$ (Koh and Sanders, 2001). Membrane stretch activates 95 pS K^+ channels, as does elongation of single SMCs. These channels have the same properties as the channels referred to previously as NO1 and are activated by sodium nitroprusside (**SNP**) or 8-Br-cGMP (Koh *et al.,* 1995). Openings of SDK channels were potentiated by simultaneous application of NO or 8-Br-cGMP and stretch, suggesting that these stimuli could be synergistic in colonic muscles (Koh and Sanders, 2001). SDK channels, with input from NO released from nitrergic neurons, may be fundamental to maintaining a low degree of SMC excitability, providing a means for the reservoir function of this organ.

SDK channels in colonic muscles are blocked by sulfur-containing amino acids, and **L-methionine** was found to be the most selective antagonist (Park *et al.,* 2005). L-methionine at concentrations effective in blocking SDK channels had little or no effect on other major K^+ currents in colonic SMCs. L-methionine increased the force and frequency of colonic muscle contractions, reversed nitrergic inhibitory responses and depolarized membrane potentials. Responses to L-methionine were blocked by pretreatment of muscles with L-NNA. Nitrergic IJPs were also blocked by sulfur-containing amino acids. How NO activates SDK channels was investigated by expressing $K_{2P}2.1$ channels, the dominant mechanosensitive two-pore K^+ channels in colon SMCs, in COS-7 cells (Koh *et al.,* 2001). $K_{2P}2.1$ channels are activated by stretch as were SDK channels in native cells. $K_{2P}2.1$-mediated currents were also enhanced by SNP or by 8-Br-cGMP. The amino acid sequence of the $K_{2P}2.1$ channel contains two consensus sequences for phosphorylation by PKG1. The response to 8-Br-cGMP was blocked in mutated $K_{2P}2.1$ channels, produced by replacing Ser^{351} with alanine (i.e. S351A). Thus, the proposed pathway by which NO activates $K_{2P}2.1$ channels in postjunctional cells involves cGMP-dependent activation of PKG1 and phosphorylation of $K_{2P}2.1$ at Ser^{351}.

However, other groups have disputed a role for $K_{2P}2.1$ in nitrergic responses (Zhang *et al.,* 2010; Gil *et al.,* 2012). IJPs in oesophageal muscles were compared before and after addition of L-methionine. L-NAME blocked the slow phase of IJPs completely, but L-methionine produced only a small inhibitory effect. However, such a comparison is difficult under the circumstances of this particular experiment, because L-methionine caused substantial membrane depolarization. Thus, if IJPs are due to K^+ channels, the amplitude should increase as the driving force for K^+ current increases. In fact finding that the amplitude was approximately the same before and after depolarization suggests that IJPs were partially inhibited in the latter condition.

Involvement of inward current channels in nitrergic IJPs

IJPs generated by purines were compared with the IJP component attributed to NO in guinea pig colon (Hirst *et al.,* 2004). Increasing the external K^+ concentration ($[K^+]_o$), which decreases the driving force for IJPs due to a K^+ conductance, decreased the amplitude of purinergic IJPs but had little effect on nitrergic IJPs. These authors concluded that purinergic IJPs were due to the opening of apamin-sensitive K^+ channels, but nitrergic IJPs are due to the suppression of the ongoing opening of an inward current. Additional experiments suggested that the inward current was dependent on Ca^{2+}.

A mechanism for IJPs involving Ca^{2+}-activated Cl^- channels (**CaCC**) has also been proposed by several authors, and molecular components necessary for such a response are available in the SIP syncytium (see section on *Effects of nitrergic innervation on Ca^{2+} transients in ICC*). If Cl^- channels are involved in nitrergic responses, this may be a means of determining which cells generate nitrergic IJPs in response to NO released from neurons, because SIP cells each express different types of ion channels. For example, CaCC are expressed in ICC, but not in SMCs and PDGFRα^+ cells.

IJPs evoked in opossum oesophagus were inhibited by TTX, **9-anthroic acid** and **niflumic acid** (Zhang and Paterson, 2002). Ca^{2+} store-active drugs, such as **caffeine**, **ryanodine** and **cyclopiazonic acid** (CPA**)**, blocked nitrergic IJPs in the oesophagus (Zhang and Paterson, 2003), and these authors suggested that Ca^{2+} released from stores may activate CaCC in SMCs. Store-active drugs have multiple effects in intact muscles and may not work exclusively through inhibition of Ca^{2+} release. Caffeine, ryanodine and CPA also blocked nitrergic IJPs in murine colon, but store-active drugs also inhibit $K_{2P}2.1$ channels expressed in COS-7 cells and native SDK currents in colonic myocytes (Hwang *et al.,* 2008). $K_{2P}2.1$ channels are not sensitive to Ca^{2+}, so the effects of these drugs on $K_{2P}2.1$ are unlikely to be due to the effects of the Ca^{2+} released from stores. The effects of caffeine appeared to be due to its well-known actions as a PDE inhibitor, and its inhibitory effects were blocked by dialysis of cells with an inhibitory peptide of PKA. Suppression of CaCC by NO is an attractive hypothesis for the action of NO in GI muscles, but how NO suppresses CaCC and whether CaCC antagonists produce the same magnitude of hyperpolarization as nitrergic stimulation need to be investigated further.

Effects of nitrergic innervation on Ca^{2+} transients in ICC

ICC throughout the GI tract express CaCC encoded by *Ano1* (Chen *et al.,* 2007; Gomez-Pinilla *et al.,* 2009; Hwang *et al.,* 2009), and a similar conductance is not present in other SIP cells. ICC also express genes that encode proteins that facilitate responses to NO, including *Gucy1a1*, *Gucy1b1*, *Prkg1* and *Mrvi1* (Iino *et al.,* 2008; Baker *et al.,* 2018), and these cells generate cGMP in response to nitrergic nerve stimulation (Shuttleworth *et al.,* 1993; Iino *et al.,* 2009). Isolated ICC generate spontaneous transient inward currents (STICs) (Zhu *et al.,* 2011) linked to Ca^{2+} release from stores (Zhu *et al.,* 2015). Dynamic measurements of Ca^{2+} transients and responses to nitrergic agonists, antagonists and NO released from enteric inhibitory neurons have been accomplished recently by imaging ICC-DMP in small intestinal muscles *in situ* from mice expressing GCaMP3 in ICC (Figure 4) (Baker *et al.,* 2018). Spatially limited Ca^{2+} transients occur on an ongoing basis in ICC-DMP, the intramuscular class of ICC in the small intestine. These events are responsible for STICs in ICC-DMP. NOS inhibitors and ODQ increased the frequency of Ca^{2+} transients, and NONOate and **Bay 58-2667**, an sGC agonist, decreased Ca^{2+} transients. EFS caused inhibition of Ca^{2+} transients, and this effect was blocked by L-NNA and ODQ. Taken together, these studies demonstrate that ICC-DMP are innervated by enteric inhibitory neurons and have the molecular machinery to generate ongoing Ca^{2+} transients and STICs *via* activation of CaCC channels. Ca^{2+} transients are inhibited by NO, and this mechanism may provide an explanation for the nitrergic components of IJPs.

The cellular mechanism by which NO inhibits Ca^{2+} release in ICC is still under investigation. The mechanism may involve phosphorylation of inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) by PKG1β. IRAG associates with **IP_3R1** and inhibits agonist-dependent Ca^{2+} release (Geiselhoringer *et al.,* 2004). Gene deactivation of IRAG generated mice with distended stomachs, possibly due to pyloric stenosis, delayed intestinal transit and reduced ability of 8-Br-cGMP to inhibit contractile responses. The phenotype of IRAG$^{-/-}$ mice was dramatic, making it likely that IRAG is involved in nitrergic inhibition. All three SIP cells express *Mrvi1* (gene encoding IRAG) (Lee *et al.,* 2017), but which cell(s) manifests the mechanism dependent upon IRAG is still not known. The expression of *Ano1* in ICC and the reduction in Ca^{2+} release from IP_3R1 in ICC in response to nitrergic signalling could be mechanisms linked to the function of NO and IRAG in GI muscles (Figures 4 and 5). Ca^{2+}-regulated, ongoing inward currents in SMCs that are suppressed by nitrergic mechanisms have not been identified. From the literature available to date, one must conclude that there are substantial uncertainties about the cells and mechanism mediating the Ca^{2+} and voltage-dependent effects of NO in GI muscles.

Nitrergic effects via *modulation of the Ca^{2+} sensitivity of the contractile apparatus*

Another means by which NO regulates GI muscle contractions is by desensitization of the contractile apparatus to Ca^{2+}. The activity of myosin light chain phosphatase (MLCP), the enzyme that dephosphorylates myosin, ends cross-bridge cycling and causes relaxation in SMCs is regulated by G-protein-coupled activation of RhoA/Rho-kinase-dependent phosphorylation of MYPT1, a regulatory co-factor of MLCP (Somlyo and Somlyo, 2003). Desensitization can occur by cyclic nucleotide-dependent kinase-dependent phosphorylation of RhoA, preventing its activation of Rho-kinase (Somlyo and Somlyo, 2003). Another target for Ca^{2+}-induced desensitization by cGMP is **telokin** (smMLCK) which is phosphorylated at Ser^{13} in response to 8-Br-cGMP or forskolin (Walker *et al.,* 2001). Telokin$^{-/-}$ mice displayed increased Ca^{2+} sensitivity and reduced relaxation responses to 8-Br-cGMP (Khromov *et al.,* 2006). Wild-type mice displayed basal phosphorylation at Ser^{13} in telokin, and phosphorylation was increased by SNP and 8-Br-cGMP. Telokin$^{-/-}$ mice showed enhanced contractile responses to

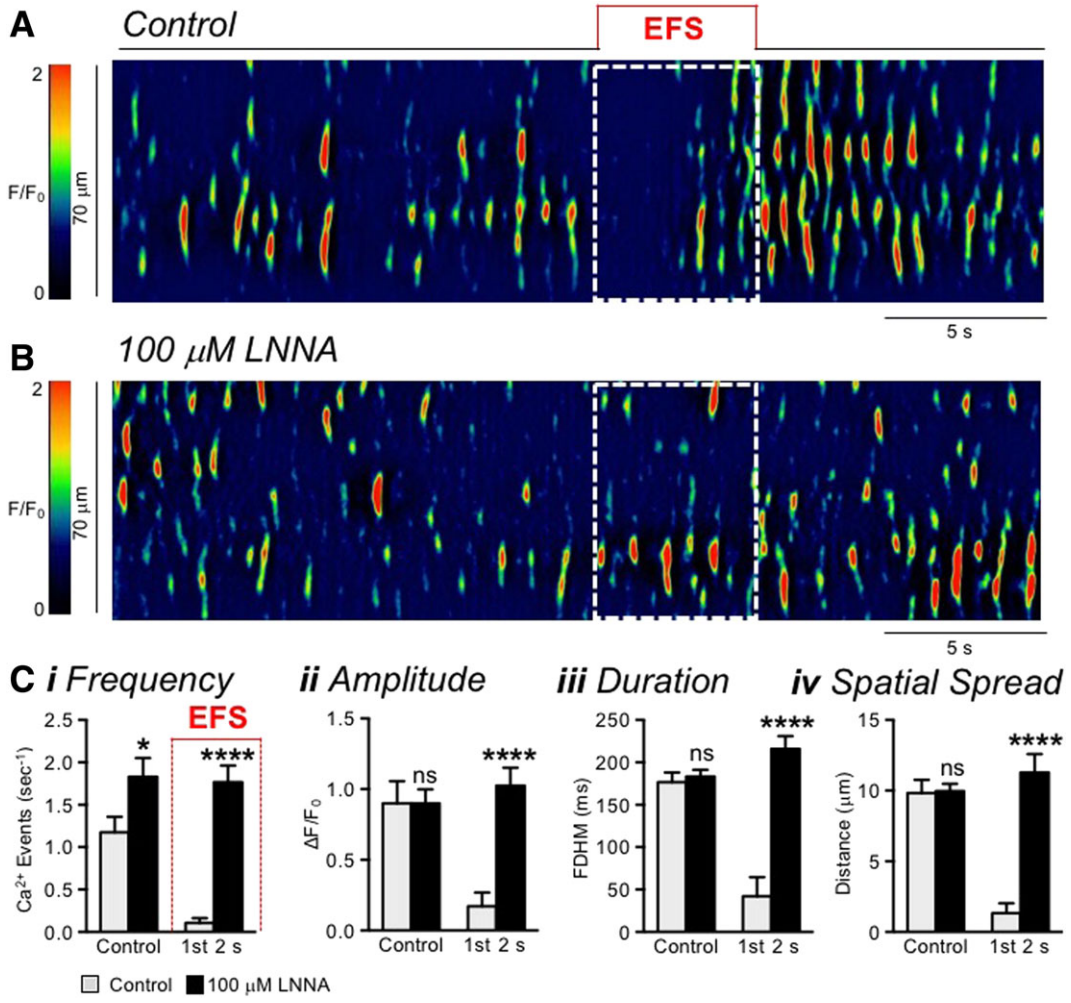

Figure 4

Ca^{2+} transients in ICC-DMP are inhibited by nitrergic neural input. (A, B) Spatio-temporal maps depicting Ca^{2+} transients in an ICC-DMP in the mouse small intestine. Note the transient and spatially restricted nature of these events that are expected to couple to activation of CaCC channels in the plasma membrane. EFS (10 Hz, 0.5 ms pulse duration; 5 s; white dotted box) in panel (A) inhibited Ca^{2+} transients during the initial phase of stimulation (~2 s), and then excitatory input caused restoration of Ca^{2+} release. A rebound period of excitation occurred after cessation of EFS. (B) Addition of L-NNA (100 µM) blocked the inhibition of Ca^{2+} transients during EFS. (C) Tabulation of effects of EFS on Ca^{2+} transient frequency (i), amplitude (ii), duration (iii), and spatial spread (iv) in ICC-DMP during control conditions (pre-EFS) and during the initial 2 s of EFS before and after L-NNA ($n = 5$ animals; 15 cells). ns = $P > 0.05$; *$P < 0.05$; ****$P < 0.0001$. Copied with permission from Baker *et al.* (2018).

elevated $[K^+]_o$, carbachol, and cholinergic neurotransmission. However, no increase in telokin phosphorylation was detected in response to nitrergic neurotransmission or with response-matched concentrations of SNP (An *et al.,* 2015). These data suggest that telokin phosphorylation may be recruited for responses to bath-applied NO but may not be a factor in responses to NO released from neurons.

Mechanosensitive mechanisms linked to NO

Enteric motor neurons are organized to convey local reflexes. For example, initiation of the peristaltic reflex comes from mechanosensitive activation of afferent nerves innervating myenteric ganglia and organizing a stereotypical motor response in the colon. However, cells of the SIP syncytium also demonstrate mechanosensitive responses. For example, stretch of the proximal colon initiates a hyperpolarization response dependent upon the rate at which the stretch is applied (Won *et al.,* 2013). This response was blocked by TTX and L-NNA, suggesting that it might be part of a neural reflex involving activation of nitrergic neurons. However, the inhibitory response to stretch in muscles treated with L-NNA was restored by NO donors, suggesting that a postjunctional mechanism mediating the stretch response is sensitized by NO, which is tonically released from nerves in the proximal colon. ICC appear to mediate the stretch response because muscles treated with a c-Kit neutralizing antibody to disrupt the development of ICC lose the stretch-dependent inhibitory response. This response was also blocked by L-methionine, which blocks $K_{2P}2.1$ channels in GI muscles

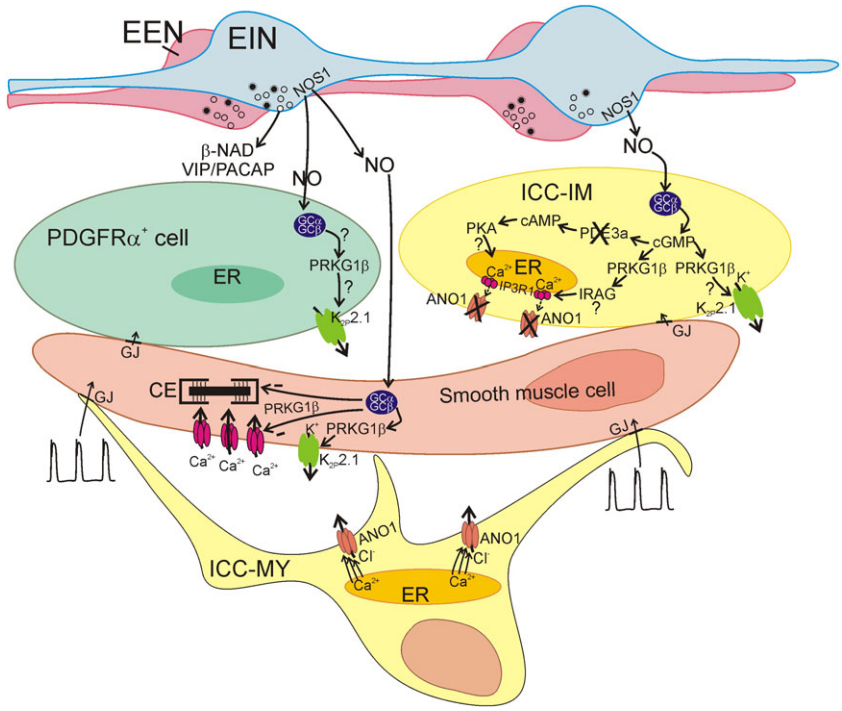

Figure 5

Proposed mechanisms of nitrergic neurotransmission in the SIP syncytium. GI muscles are innervated by excitatory (EEN) and inhibitory (EIN) enteric motor neurons. Major inhibitory neurotransmitters released by EIN are NO, purines (β-NAD shown) and peptides, such as VIP and PACAP (release of β-NAD, and VIP/PACAP is indicated, but mechanisms for these neurotransmitters are not depicted). NO is synthesized by nNOS (NOS1) expressed by EIN and released on-demand by Ca^{2+} influx into varicosities. NO binds to sGC in SIP cells. In ICC-IM, binding of NO to sGC causes formation of cGMP that can interact with PKG1β (PRKG1β) to phosphorylate and activate $K_{2P}2.1$ channels and generate an outward current. PKG1β can also phosphorylate IRAG which interacts with IP_3R1 (IP3R1) and inhibits Ca^{2+} release from stores (see Figure 4). Cessation of Ca^{2+} release causes deactivation of CaCC (ANO1) channels, causing a net gain in outward current in the cells. Activation of $K_{2P}2.1$ channels or deactivation of CaCC (ANO1) can cause hyperpolarization of ICC-IM and, *via* gap junctions (GJ) connectivity with other SIP cells, hyperpolarization of the SIP syncytium. PDE3A is also a possible target for cGMP, and its inhibitory effects on PDE3A may increase the levels of cAMP and cause downstream signalling through PKA. Effectors for this pathway have not been identified. PDGFRα$^+$ cells also express sGC subunits, PKG1 and $K_{2P}2.1$ channels. This pathway in PDGFRα$^+$ cells is speculative at the present time. SK3 channels in PDGFRα$^+$ cells (not shown) produce the outward current that causes the fast IJP phase of inhibitory neurotransmission (Kurahashi *et al.,* 2011) (and see Figure 3). NO also binds to sGC in SMCs where it produces cGMP, activates PKG1β and causes Ca^{2+} desensitization (−) of the contractile apparatus, inhibition (−) of $Ca_V1.2$ channels and activation of $K_{2P}2.1$ channels. Speculative steps are denoted by question marks. ICC-MY are present in most areas of the GI tract and generate pacemaker activity through release of Ca^{2+} from stores and activation of CaCC (ANO1) channels. Pacemaker activity (depicted as slow waves) conducts to SMCs causing periodic depolarization and phasic contractions. Neural inputs are superimposed upon the pacemaker activity.

(Park *et al.,* 2005). The overall mechanism appears to be due to NO, released from nerves, sensitizing $K_{2P}2.1$ channels to muscle stretch.

Defects in nNOS$^+$ neurons or nitrergic neurotransmission lead to GI motor dysfunction

GI dysfunction occurs in a large cohort of patients with long-standing diabetes (Lee and Hasler, 2017; Piper and Saad, 2017). At least part of the motility problems that develop have been attributed to neuropathies in extrinsic nerves and intrinsic (enteric) excitatory, inhibitory and sensory pathways that regulate smooth muscle contraction (Azpiroz and Malagelada, 2016). In keeping with the focus of this review, defects in nitrergic regulation will be discussed. Spontaneous diabetic rats (BB/W), streptozotocin-treated rats and non-obese diabetic (NOD) mice were found to have significantly reduced nNOS$^+$ enteric neurons and *Nos1* transcripts in tissues (Takahashi *et al.,* 1997; Watkins *et al.,* 2000; Choi *et al.,* 2008). Insulin treatment restored the expression of nNOS (Watkins *et al.,* 2000). Responses to nitrergic nerve stimulation are also diminished in GI muscles of diabetic animals and humans (Ordog *et al.,* 2000; Watkins *et al.,* 2000), and these authors attributed the functional effects either to loss of nNOS$^+$ neurons or to reduced nNOS expression. Interestingly, studies of muscles from diabetic human patients found no significant loss of PGP9.5 (a pan-neuronal marker) or of nNOS$^+$ neurons in gastric corpus muscles of 20 diabetic patients; however, about 20% of the diabetic patients displayed a reduction in nNOS$^+$ neurons. These studies all utilized immunofluorescence techniques to quantify nNOS$^+$ neurons. Immunological techniques are limited by threshold-of-detection issues, so one must question whether

reductions in nNOS$^+$ neurons are due to reduced numbers of inhibitory enteric neurons or to reduced expression of nNOS. Co-labelling of tissues with VIP antibodies, a peptide neurotransmitter co-expressed in enteric inhibitory neurons, is a means of further testing the state of this population of neurons. This approach was used with both streptozotocin-treated and NOD mice, and the data suggest that in these models, nNOS expression is reduced, but inhibitory neurons are not (Watkins *et al.,* 2000). While a nitrergic neuropathy appears to be accepted as a cause for the GI motility disorders that accompany diabetes, it is still unresolved whether the reduction in nNOS expression in enteric motor neurons is sufficient to generate a functional reduction in NO release in response to activation of inhibitory neurons. The answer to this question awaits a comparison of NO release in normal and diabetic muscles.

An additional explanation for reduced nitrergic neural regulation of GI muscles in diabetes might be impaired connectivity between motor neurons and postjunctional target cells. As discussed in previous sections of this review, at least a portion of nitrergic neurotransduction is mediated through responses that develop in ICC (Burns *et al.,* 1996; Lies *et al.,* 2014b; Beckett *et al.,* 2017; Baker *et al.,* 2018). Loss of ICC or disrupted connectivity between enteric motor neurons and ICC is the most common histological finding in diabetic tissues of patients with gastroparesis, for example (Faussone-Pellegrini *et al.,* 2012). Lesions of this sort were first reported from studies of a Type I diabetic mouse model (Ordog *et al.,* 2000), but reduced ICC have also been found in GI muscles of human patients with diabetes (He *et al.,* 2001; Faussone-Pellegrini *et al.,* 2012). However, it is still unresolved whether phenotypic changes in ICC (e.g. loss of signalling molecules involved in nitrergic transduction or connectivity with SMCs) occur well before loss of cells. Such phenotypic changes might initiate GI motor defects before a significant reduction in ICC numbers occurs.

Damage to ICC in diabetes has been linked to increased oxidative stress. This appears to be related to the loss of CD206$^+$ macrophages that express **haem oxygenase (HO1)**. At the onset of diabetes, HO1 expression increases in resident macrophages in mice, and this is protective against ICC loss and development of delayed gastric emptying (Choi *et al.,* 2008). Loss of CD206$^+$ macrophages (and concomitant decrease in HO1) results in reduced ICC and the development of gastroparesis. Therapeutic strategies that restore HO1 activity might be protective against some of the GI motor problems associated with diabetes (Farrugia, 2015).

Motor defects related to overproduction of NO

NO is a potent inhibitor of GI muscle contraction, so an overabundance of NO can be detrimental to GI motility. This occurs in some inflammatory conditions. Another gene encoding NOS in cells within the wall of the GI tract is ***Nos2*** [the gene for inducible NOS (iNOS)]. Expression of *Nos2* is normally low but can be induced by exposure to pro-inflammatory cytokines, such as TNF-α, IL-1 and **IFN-γ** in many tissues and cells (Korhonen *et al.,* 2005). Low concentrations of Ca^{2+} facilitate calmodulin binding to iNOS, creating essential conditions of constitutive activation. Abundant NO production for long periods of time can occur after induction of *Nos2*.

Post-surgical ileus is one of the motility disorders that has been associated with an overproduction of NO. Disruptions in ICC networks were observed near sites of small intestinal resections within 5 h after surgery in mice (Yanagida *et al.,* 2004). Slow waves and phasic contractions decreased in regions of muscle with ICC defects. Loss of ICC and compromised slow wave activity decreased as a function of distance from the resection. Muscles from the area near bowel resections were also poorly responsive to **carbachol** or transmural nerve stimulation. ICC networks and slow waves recovered spontaneously near the site of the anastomosis within 24 h. The magnitude of the ICC lesions and recovery periods decreased with presurgical treatment with inhibitors of iNOS and were greatly reduced in *Nos2*$^{-/-}$ mice (Yanagida *et al.,* 2007).

Manipulation of the intestine, as might occur during abdominal surgical procedures unrelated to bowel surgery, activates inflammatory responses. Resident muscularis macrophages are activated by manipulation of the gut (innate response), and these cells initiate a broader inflammatory response leading to recruitment of circulating leukocytes, enhanced release of inflammatory cytokines and increased production of NO and prostanoids (Kalff *et al.,* 2000; Bauer and Boeckxstaens, 2004). Immunohistochemistry showed that iNOS expression increases in phagocytes within the muscularis (Kalff *et al.,* 2000). NO generation from the increased expression of iNOS causes significant inhibition of GI motor activity (Kalff *et al.,* 2000). The overproduction of NO and effects on GI motility were limited in mice with genetic deactivation of *Nos2* (Kalff *et al.,* 2000), by treatment with iNOS inhibitors, when macrophage-deficient mice were utilized for experimental gut manipulation (Wehner *et al.,* 2007) and in *Nos2*-deficient bone marrow chimera mice (Turler *et al.,* 2006). These data show the importance of iNOS in the motility dysfunction elicited by surgical manipulation of the gut.

While NO has direct inhibitory effects on SMCs, it is also probable that part of the deleterious effects of overproduction of NO are mediated by damage to ICC. In a study using jejunal muscle organoids, treatment with IFN-γ and **LPS** for 24 h induced expression of *Nos2* and impaired the pacemaker activity of ICC (Kaji *et al.,* 2016). Pretreatment of the organoids with iNOS inhibitors blocked the damage to ICC induced by IFN-γ and LPS. Antioxidant treatment to reduce oxidative stress caused by NO also reduced the impairment of pacemaker activity, but no benefit was obtained with the blocker of guanylate cyclase, ODQ.

Intestinal manipulation (IM) also impairs ICC networks. Twenty-four hours after IM, the density of ICC in the myenteric region of the small intestine, as determined by c-Kit or *ANO1* immunoreactivity, decreased by about 50%, and the ICC recovered spontaneously by 48 h (Kaji *et al.,* 2018). The speed with which this occurred appeared to be due to loss of these key functional proteins in ICC rather than cell death, as electron microscopy demonstrated retention of a gap junction-coupled network of ICC-like cells in the myenteric region. The appearance of cytoplasmic vacuoles in ICC at 24 h after IM may have indicated the development of autophagy that might have contributed to the decreased

immunoreactivity of common ICC proteins. Treatment of muscles with **aminoguanidine** reduced the disruption in ICC networks, suggesting that the damage was largely due to production of NO *via* iNOS.

Summary and conclusions

Nitrergic regulation is extremely important in GI motility, as NO is a major inhibitory neurotransmitter in nearly every region and a mediator of inflammatory effects. NOS^+ motor neurons are plentiful in GI muscles, and they release NO when activated. Postjunctional responses in cells of the SIP syncytium are activated by NO. Several defined responses have been reported in ICC and SMCs. The apparatus necessary to transduce NO signals is also available in PDGFRα^+ cells (Iino *et al.,* 2008; Iino *et al.,* 2009; Lee *et al.,* 2017); however, no studies have reported effects of NO specific to these cells. The majority of evidence suggests that nitrergic responses are integrated, with contributions from both ICC and SMCs. The actual mechanisms of nitrergic IJPs and relaxation are still controversial with possible contributions from activation of K^+ channels ($K_{2P}2.1$), suppression of activation of Cl^- channels (CaCC/*ANO1*), reduced open probability of Ca^{2+} channels ($Ca_V1.2$) and Ca^{2+} desensitization of the contractile apparatus in SMCs. Relative contributions from these mechanisms may change from region-to-region in the GI tract; however, the ubiquitous expression of CaCC and Ca^{2+} release from stores in ICC throughout the gut suggest the suppression of CaCC activity has a prominent role in nitrergic responses in many regions. A reduced expression of nNOS has been demonstrated in animal models of diabetes and in human GI muscles of diabetic patients. Loss of nitrergic neural signalling may contribute to GI motor disorders occurring in patients with long-standing diabetes. The increased expression of iNOS occurring in inflammatory responses may cause damage to ICC, leading to a pseudo-obstruction-like state or other motor abnormalities. Neither the mechanisms of the muscle inhibition nor the mechanisms causing ICC remodelling are fully understood, so there are many things yet to learn about nitrergic regulation of GI muscles.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.,* 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.,* 2017a,b,c,d,e).

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Author contributions

K.M.S and S.M.W. researched and discussed literature on NO in enteric inhibitory neurotransmission; K.M.S. and S.M.W. developed or redrew the figures; K.M.S. drafted the manuscript; K.M.S. and S.M.W. edited and agreed upon the final draft of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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