Nitric oxide and autonomic control of heart rate: a question of specificity

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Despite its highly diffusible nature, the gaseous signalling molecule nitric oxide (NO) can exert specific effects within the CNS and PNS. To date, the specificity of the actions of NO remains an unsolved puzzle. There are several plausible mechanisms that might account for this specificity in the context of autonomic regulation of heart rate. NO acts at distinct levels within the autonomic nervous system to control cardiac rate, with opposing effects at different sites. We discuss factors that might contribute to this diversity of action, and conclude that the isoform of enzyme involved in producing NO, the spatial proximity of the NO source to the target, and differences in the intracellular coupling within the target cell are all crucial for encoding the functional action of NO.

Historically, angina has been managed clinically by administering vasodilating drugs (e.g. nitroglycerin) that mediate their effects by releasing nitric oxide (NO). The action of NO in this case has traditionally been explained by its effect upon vascular smooth muscle, into which it can freely diffuse. In addition, NO causes an increase in heart rate, which was believed to originate primarily from the unloading of baroreceptors in response to the decreased arterial pressure. However, it has recently been proposed that some of the effects of nitroglycerin might be, in part, due to actions within the brainstem [1] and directly on the heart itself [2–8].

As NO is well recognized as an endogenous neurotransmitter, neuromodulator and intercellular messenger, it has become clear that endogenous NO might be implicated in control of heart rate, acting at multiple sites (including visceral afferents [9], brainstem neurons that mediate cardiovascular reflexes [10–12] and cardiac autonomic ganglia [13]). Because NO spreads very rapidly and freely from its source in nervous tissue, the question arises of how any sense can be made from this diffusible signal, which penetrates through any membrane and affects cells indiscriminately within a circumscribed area. To achieve some site-targeted action within the bloodstream, NO becomes trapped by erythrocytes and is released only in regions of low oxygen tension, to aid blood flow [14]. However, it is not completely clear whether NO has comparable site-targeted effects within nervous tissue, such as the brainstem or cardiac ganglia, and, if so, what mechanisms might underlie specificity of its action.

To analyse this issue, the role of NO as a signalling molecule in the autonomic control of cardiac rate must be examined. Reflex control of cardiac rate depends on afferent input from various types of visceral afferent, with baroreceptors playing the most important role. It has been demonstrated that NO can affect peripheral afferent excitability [9,15] but there are two other sites for NO modulation: the nucleus tractus solitarii (NTS; the brainstem termination site for baroreceptor afferents) and cardiac autonomic efferents at the level of the heart. At both these sites, there is extensive anatomical evidence to support sophisticated NO-mediated interactions [16–18].

Multiple actions of NO: questioning specificity

Many studies relate the effects of NO to its ability to modulate (usually to increase) release of transmitters, such as glutamate, GABA and ACh [19–21]. If NO plays a physiological role in regulation of a neuronal circuit, a generalized action of increasing release of multiple transmitters (e.g. glutamate and GABA) simultaneously would make little sense. But does this really happen? There might be several ways to achieve a specific action. First, close spatial proximity of the source of NO and its target (e.g. Ref. [22]) could allow discrete actions. Although recent data indicate that, in a brain slice, NO affects targets >150 μm from its source [23], this distance might be much less in the brain *in vivo* and could depend on the ratio between efficacy of NO production and elimination. This raises the second possibility: that the NO synthase (NOS) isoform involved might matter. In normal circumstances, NO in nervous tissue is manufactured by two enzymes – neuronal NOS (nNOS or NOS-I) and endothelial NOS (eNOS or NOS-III) – and presumably these enzymes have very different spatial distributions. In nervous tissue, nNOS is probably confined to nerve cells, but it is unclear whether eNOS is expressed in both neurons and vasculature [24,25] or confined exclusively to blood vessels [26]. Moreover, these enzymes have very different potency in terms of NO production. In peripheral tissues, eNOS produces relatively small amounts of NO, whereas nNOS can produce NO in large quantities (reviewed in Ref. [27]). Hence, activation of nNOS might lead to more diffuse signalling than activation of eNOS. A third factor is the availability and sensitivity of the cellular biochemical targets for NO (reviewed in Ref. [28]) within its diffusion range. For example, the biochemical machinery that regulates release of GABA could be more sensitive to physiological levels of NO than that regulating release of glutamate. It has been demonstrated that the best understood

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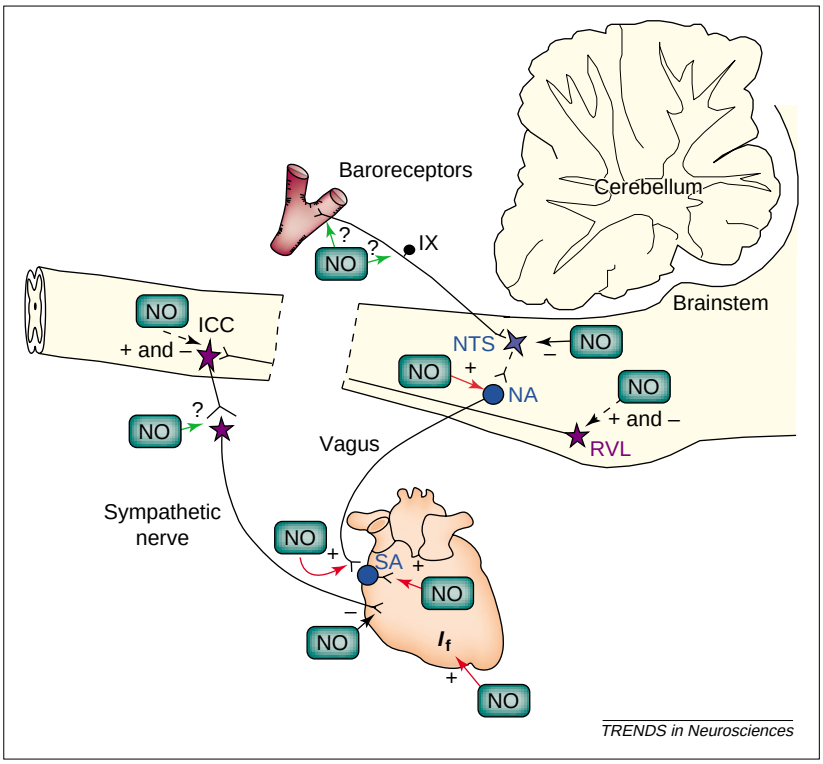

Fig. 1. Site-specific and differential modulation of neuronal activity affecting cardiac function by nitric oxide (NO). The nucleus tractus solitarii (NTS) receives input from barorecptors that is conveyed by glossopharyngeal (IX) afferents. It connects with the nucleus ambiguus (NA), which projects via the vagus to the sinoatrial (SA) node of the heart. The connection from NTS to NA might not be direct, as indicated by the dashed projection. NO has unknown effects (green arrows, labelled with '?') on baroreceptors and IX neurons; it is inhibitory (black arrows, labelled with –) for the NTS and excitatory (red arrows, labelled with +) for the NA [58] and SA node. NO also acts on the rostral ventrolateral medulla (RVL [34,59,60]) and the intermediolateral cell column (ICC) of the spinal cord [61–65], although the reported actions are inconsistent (indicated by a broken black arrow, labelled with '+ and –'). The sympathetic neurons innervated by the ICC are affected by NO in an unknown way, and the cardiac cells these innervate can be inhibited by NO. NO can also activate the pace-making current (I_f) in cardiac myocytes. The effect of NO on the NTS relates only to its modulation of the baroreceptor reflex. The cerebellum is shown for orientation.

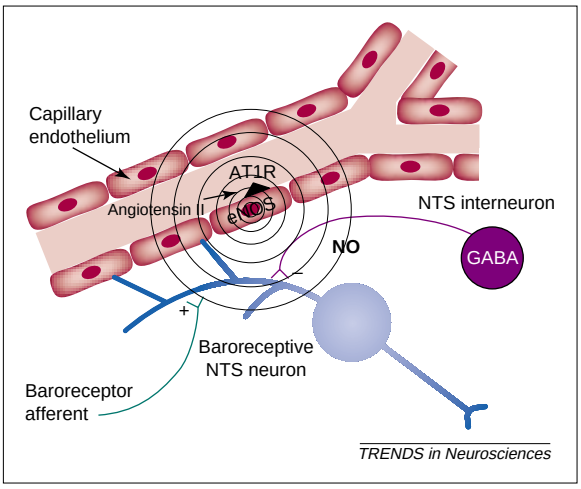

Fig. 2. Vascular–neuronal signaling in the nucleus tractus solitarii (NTS) as a hypothetical explanation for inhibition of the angiotensin-II-induced baroreceptor reflex by nitric oxide (NO). In the NTS, it is proposed that activation of the angiontensin II type-1 receptor (AT1R) by angiotensin II stimulates endothelial nitric-oxide synthase (eNOS) to release NO from the vasculature. This NO diffuses to nearby GABAergic NTS interneurons to enhance inhibition of neurons mediating the baroreceptor reflex [24,39–41]. This indicates that both NOS isoform (eNOS or neuronal nitric-oxide synthase, nNOS) and its spatial proximity to the target could underlie specificity of action. It is also possible that the type of phosphodiesterase within the GABAergic interneuron plays a role in specificity of NO actions.

cellular NO 'receptor' – soluble guanylate cyclase (sGC) – rapidly desensitizes in living cells [29]. This, in concert with variations in the rate of cGMP breakdown, could provide a fundamental way of decoding NO signals under physiological and pathophysiological conditions [29]. Finally, the fact that that some of the actions ascribed to NO might result from the effects of peroxynitrite ($OONO^-$), a product of the reaction between NO and superoxide, should not be overlooked. Peroxynitrite can affect transmitter release independently of NO [19]. Therefore, in conditions of high concentrations of NO (and superoxide), peroxynitrite will form, and this might have completely different actions and affect additional cellular targets. The latter could underlie some of the controversy relating to the presumed effects of NO, which might actually reflect actions of peroxynitrite. This emphasizes the need to establish first that the effect observed is mediated by NO.

NO actions within the NTS

It is known that systemic administration of NOS inhibitors increases the baroreceptor-reflex gain in both normotensive [30] and spontaneously hypertensive animals [31]. It is beyond the scope of this article to consider all possible levels within the autonomic nervous system at which NO might modulate this reflex and affect heart rate, so we consider only the NTS (Fig. 1).

A potent way of affecting heart rate is to modulate the efficacy of baroreceptor-reflex inputs. The NTS is the termination site of multiple visceral afferents, including those from baroreceptors, which regulate cardiac rate on a beat-by-beat basis. Numerous reflex pathways are wired to similar regions of the NTS and can trigger similar autonomic responses. Experiments in which NO donors were microinjected into the NTS (constituting the bulk of currently available information on the role of NO in this tract) yielded inconsistent results (e.g. compare Ref. [32] with Ref. [33]; also reviewed in Ref. [34]). This might relate to the simultaneous activation of multiple reflex circuits, thus making it difficult to interpret these results physiologically. Hence, any change in heart rate and blood pressure elicited from the NTS cannot be equated with a functionally defined reflex circuit. To characterize a circuit in the NTS, one needs to activate a specific reflex pathway. Relatively low concentrations of NO donors microinjected into the NTS powerfully depress the cardiac vagal component of the baroreceptor reflex [24]. In light of the controversy regarding cardiovascular responses elicited from the NTS (and other brainstem sites [34]), microinjection data suffer from two major problems. First, spatial distribution of microinjected NO (donor) might not be even remotely similar to the distribution

Box 1. Cellular and subcellular microdomains of nitric oxide synthase (NOS) in the cardiac myocyte.

At the level of intracardiac ganglia and myocytes, microdomains of nitric oxide synthase (NOS) isoforms are found in the vicinity of their targets (Fig. I). Neuronal NOS (nNOS) is found in the sarcoplasmic reticulum (SR) [a] and endothelial NOS (eNOS) in sacrolemma and t-tubule membranes [b] – areas known for their role in the regulation of Ca^{2+} flux. Emerging evidence shows that nNOS localized to the SR can regulate Ca^{2+} fluxes enhanced by β-adrenoceptor activation and myocardial excitability in the ventricle [c,d]. Although the main functional outcome of NO signaling in parasympathetic control of cardiac rate appears to work through nNOS-dependent presynaptic pathways, evidence suggests that eNOS localized to caveolae is spatially compartmentalized with the β-adrenoceptor and L-type Ca^{2+}channel, allowing eNOS-generated NO to inhibit β-adrenoceptor-mediated contraction. Furthermore, the M_2 muscarinic ACh receptor might also be coupled to eNOS, from which NO modulates ion channels that regulate pace-making. Inhibition of eNOS prevents both the negative chronotropic effects of ACh receptor agonists [e] and ACh-receptor-mediated inhibition of I_{Ca_L} in pacemaking cells [f,g]. This effect is absent in eNOS-knockout mice [h], although others dispute this finding [i] and show that overexpression of eNOS fails to affect the autonomic control of cardiac excitability [j]. The exact role played by eNOS in the cardiac myocyte is clearly not firmly established; however, it is becoming apparent that cellular microdomains of NOS isoforms are involved in a complex interplay in the regulation of cardiac ion-channel function.

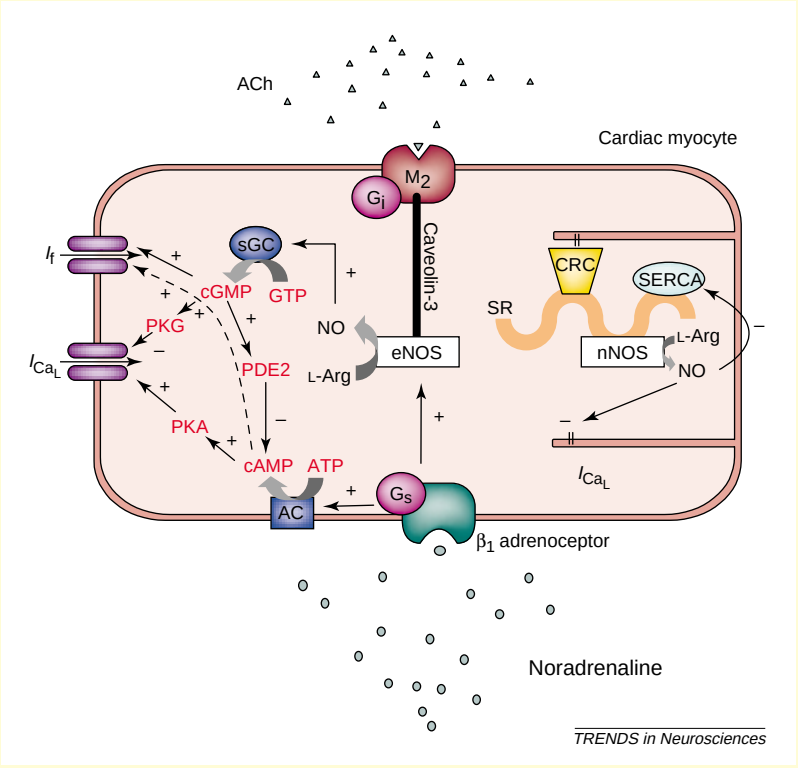

Fig. I. Cellular and subcellular nitric oxide synthase (NOS) microdomains involved in the regulation of second messenger pathways in the cardiac myocyte. Postsynaptically, ACh binds to muscarinic ACh receptors (M_2) on the sinoatrial-node pacemaker cells and, via second messenger pathways, modulates ion channels to reduce heart rate. Note that nitric oxide (NO) might be generated in the pacemaker cell following M_2-receptor activation via caveolin-3 and eNOS to inhibit I_{Ca_L} (current reflecting flow of Ca^{2+} through L-type channels), but it could also potentially activate I_f (the pace-making current) to attenuate the action of excessive M_2-receptor activation. The broken line indicates cAMP coupling to I_f via β_1-adrenoceptor stimulation. Emerging evidence also suggests that when noradrenaline binds to the β_1 adrenoceptor, nNOS localized in the sarcoplasmic reticulum (SR) can regulate Ca^{2+} fluxes [via SR-Ca^{2+} ATPase (SERCA), I_{Ca_L} and ryanodine-sensitive Ca^{2+} release channels (CRC)] to minimize the effect of excessive sympathetic stimulation. Abbreviations: AC, adenylate cyclase; G_i, inhibitory G protein; G_s, stimulatory G protein; PKA, protein kinase A; PKG, protein kinase G; PDE2, phosphodiesterase 2; sGC, soluble guanylate cyclase.

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of NO released from an endogenous source. Second, it is unclear whether injection of NO donors mimics the physiological range of concentrations of NO, and whether in these experiments peroxynitrite, rather than NO, was involved.

In an attempt to mimic endogenous NO release, NOS enzymes can be engaged by administration of the NO precursor, L-arginine. When L-arginine is microinjected into NTS, it elicits bradycardia and hypotension [35], which are indicative of an excitatory neuronal effect. If this response were mediated by baroreceptor-reflex circuitry, one would expect L-arginine to excite or potentiate this reflex too. However, we showed that L-arginine inhibited the baroreceptor reflex [24]. An important difference between these experiments is that Lin *et al.* needed 1000–10 000 pmoles of L-arginine to achieve statistically significant actions [35] (which were suggested to be mediated via the nNOS–guanylate cyclase pathway, as it was sensitive to 7-nitroindazole and LY83583), whereas Paton *et al.* used only 100 pmoles to achieve baroreceptor-reflex inhibition via eNOS [24]. Thus, it is possible that a high precursor load engages mass enzymatic activity of both NOS isoforms to release high concentrations of NO, which have a predominantly excitatory action.

Consistent with this idea, adenoviral vector-mediated non-targeted overexpression of eNOS within the NTS, which probably causes large quantities of NO to be released ectopically, leads to hypotension and bradycardia [36]. By contrast, a more sensitive mechanism that responds to lower levels of NO could operate to activate inhibitory transmission in the NTS. This is probably how NO in the NTS depresses the baroreceptor reflex (Fig. 2).

Perhaps a better way to drive endogenous NO-mediated mechanisms is to use an agonist known to stimulate NO production physiologically. It is well known that angiotensin II triggers NO release in peripheral tissues [37]. Angiotensin II also appears to be a physiological trigger for NO release in the NTS. Indeed, just as with L-arginine, angiotensin II depresses the baroreceptor reflex in NTS and this action requires release of NO by eNOS [24]. Importantly, this action of angiotensin II was mediated by NO itself, rather than peroxynitrite, because adenoviral overexpression of catalase – an enzyme that destroys reactive oxygen species – did not affect the action of angiotensin II in the NTS [38]. Furthermore, indirect evidence suggests that sGC in GABAergic neurons is the likeliest cellular target for the angiotensin-II-triggered NO, which originates from endothelial cells in the NTS [39–41] (Fig. 2). By contrast, in the NTS, nNOS appears to be associated with glutamatergic structures [42] and there is colocalization of nNOS with glutamate [16] and NMDA receptors [43]. To our knowledge, there are no data on the association of eNOS with GABAergic neurons.

NO-mediated modulation of autonomic transmission within cardiac ganglia that affect heart rate

The cardiac autonomic ganglia are another important site for powerful NO modulation [13]. Although the presence of NOS in non-cardiac sympathetic ganglia has been established and NO-related actions have been documented [44,45], little is known about the sympathetic ganglia that innervate the heart. By contrast, there is a wealth of new information available on cardiac vagal ganglia. However, it is difficult to differentiate between effects on the preganglionic terminals and those on the postganglionic neurons or their terminals, or both, because the ganglia are located in close proximity to the neuro–effector junction.

Functionally, the increase in heart rate caused by systemic administration of NO donors is independent of baroreceptor-reflex activation, because the tachycardia persists in isolated working rabbit heart preparations [4], in autonomically denervated and β-adrenoceptor-inhibited rabbit, pig and human hearts [4,8,46], and also in healthy humans in whom blood pressure is held constant [3]. Musialek *et al.* [7] first demonstrated that low doses of NO donors or cGMP increased spontaneous beating by direct activation of the pace-making current, I_f, in myocytes. Neurons of the intrinsic cardiac ganglia can generate NO, and when co-cultured with cardiac myocytes increase their beating rate [2,5]. However, information about NO modulatory actions at the level of the membrane of pacemaker cells is highly complex (Box 1).

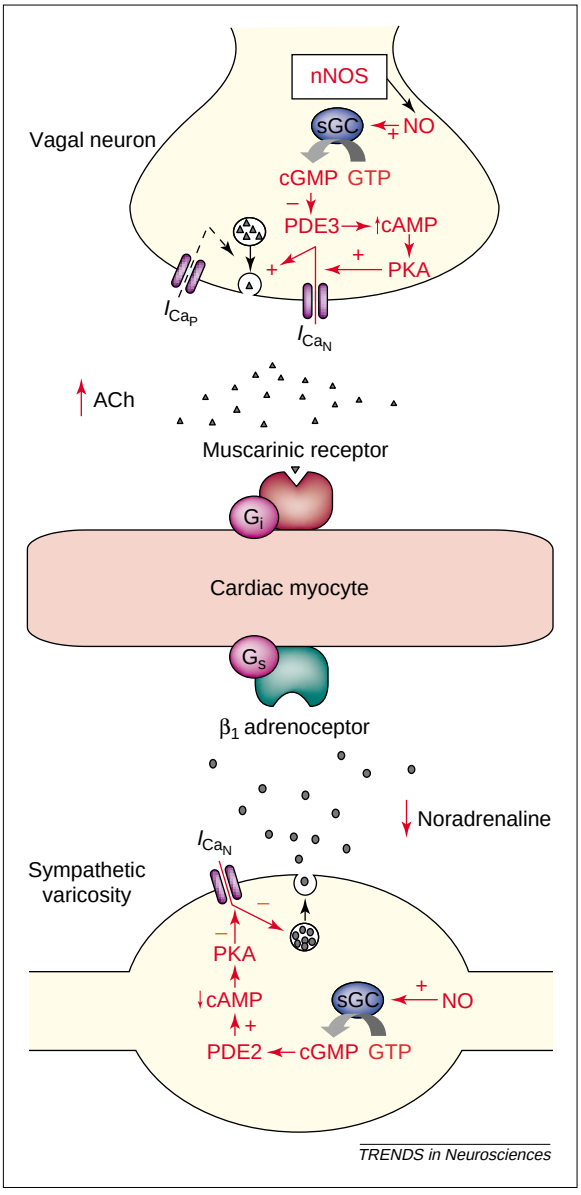

Fig. 3. Action of nitric oxide (NO) in the regulation of cardiac sympatho–vagal activity in the PNS. Proposed model in which NO generated by neuronal nitric oxide synthase (nNOS) increases the heart-rate response to vagal nerve stimulation. NO stimulates presynaptic soluble guanylate cyclase (sGC) to produce cGMP, which inhibits phosphodiesterase (PDE) 3. This elevates cAMP levels and increases protein kinase A (PKA)-dependent phosphorylation of N-type Ca^{2+} channels. Both N- and P-type Ca^{2+} channels (responsible for currents I_{Ca_N} and I_{Ca_P}, respectively) control exocytotic release of ACh (triangles), to activate M_2 muscarinic ACh receptors and inhibitory G proteins (G_i) of cardiac myocytes. When the sympathetic varicosity is activated, NO-dependent stimulation of cGMP might act to decrease neurotransmission, via PDE2-mediated inhibition of cAMP-dependent phosphorylation of the neuronal Ca^{2+}channel to modulate the release of noradrenaline (circles), which activates β_1 adrenoceptors and stimulatory G proteins (G_s) of cardiac myocytes. The source of NO is not known but it might be either an autocrine or a paracrine site (or both).

Nevertheless, there is ample evidence to indicate that NO can act presynaptically to enhance cardiac vagal neurotransmission via an sGC-dependent pathway [21], and facilitates vagal slowing of heart rate (e.g. Refs [47,48]). There is limited information about the source of the NO involved in this modulation, but is likely to come from nNOS within the cholinergic fibres themselves, where nNOS is colocalized with choline acetyltransferase [49]. The potential mechanisms of NO action on ACh release are illustrated in Fig. 3. The downstream events of NO action on sGC include activation of the cGMP–phosphodiesterase-3-dependent pathway and an increase in cAMP–PKA-dependent phosphorylation of presynaptic N-type Ca^{2+} channels [21]. Interestingly, although the involvement of PKA in transmitter release and its modulation by NO has been documented in the CNS [23,50], the possibility that this occurs via activation of phosphodiesterase 3 has not been tested.

In animals with intact vagi, infusion of an inhibitor of nNOS into the artery serving the sinoatrial node increases heart rate and reduces heart-rate variability – further suggesting that neuronally released NO has a vagal facilitatory role in maintaining sinus rhythm [51]. Potentiation of vagal influences on the heart could occur at both pre- and postganglionic levels [21,49,52–54]. However, the system appears plastic and might change in cases of myocardial infarction, when strong upregulation of atrial nNOS is associated with an augmented vagal-induced bradycardia [55].

In contrast to the potentiation of vagal transmission at the level of the heart by NO, the sympathetic system is affected in the opposite way (Fig. 3). For example, inhibition of nNOS or sGC enhances both noradrenaline release and the heart rate responses to peripheral cardiac sympathetic nerve stimulation *in vitro* [56,57] and *in vivo* [53]. This illustrates a point made earlier, that the action of NO might depend entirely on the downstream signalling pathways activated within its physiological targets. It follows that the effects of NO might depend on the presence of specific types of phosphodiesterase located in different cell types within the autonomic nervous system. Although the inhibition of phosphodiesterase 3 by cGMP in cholinergic terminals can stimulate cAMP–protein-kinase modulation of neuronal Ca^{2+} channels to enhance exocytosis [21], it is conceivable that cGMP-mediated activation of phosphodiesterase 2 in the sympathetic varicosities will have the opposite action, because enhanced degradation of cAMP could de-activate PKA and lead to a decrease in Ca^{2+} influx (Fig. 3).

Summary

Using autonomic control of heart rate, the complexity of the actions of NO at distinct levels throughout the autonomic nervous system can be demonstrated. Despite its high diffusibility and the apparent abundance of potential NO targets, the effects of endogenous NO-mediated systems are selective. It is clear that, given the multiple and opposing actions of NO on cardiac control, interpretation of a response after a global intervention of the NO system (e.g. systemic administration of nitroglycerin, a NOS inhibitor or a NOS isoform knockout), will be difficult. Diversity of the intracellular pathways activated by NO and their differential sensitivity to different levels of NO might account for some aspects of specific and opposing effects. Future studies will clarify whether NOS isoforms involved in heart-rate control do indeed have specific physiological 'missions'.

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