Nitric oxide as a multimodal brain transmitter

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Abstract

One of the simplest molecules in existence, nitric oxide, burst into all areas of biology some 30 years ago when it was established as a major signalling molecule in the cardiovascular, nervous and immune systems. Most regions of the mammalian brain synthesise nitric oxide and it has many diverse roles both during development and in adulthood. Frequently, nitric oxide synthesis is coupled to the activation of NMDA receptors and its physiological effects are mediated by enzyme-linked receptors that generate cGMP. Generally, nitric oxide appears to operate in two main modes: first, in a near synapse-specific manner acting either retrogradely or anterogradely and, second, when multiple nearby sources are active simultaneously, as a volume transmitter enabling signalling to diverse targets irrespective of anatomical connectivity. The rapid diffusibility of nitric oxide and the efficient capture of fleeting, subnanomolar nitric oxide concentrations by its specialised receptors underlie these modes of operation.

Introduction

The latter half of the 20th century was the golden era for the identification of the major neurotransmitters in the mammalian CNS. Before then, it was generally held that neurotransmission in the brain and spinal cord was primarily electrical but, even when this notion dissipated, it often took decades of incremental advances, often in the face of fierce debate, before candidate neurotransmitters were generally accepted, mostly in the final quarter of that century. While the world had become used to some simpler molecules (e.g. acetylcholine, amino acids and catecholamines) performing a transmitter role, nobody suspected that a molecule as small, chemically unspecialised and freely diffusible as nitric oxide (NO) could function as a major biological messenger. Extraordinarily, it was accorded this status within only a couple of years of the idea being mooted, thanks, in part, to convergent evidence from different biological disciplines, the rapid availability of research tools, and the fact that NO-based treatments for human disorders had already been in use for more than a century, albeit unintentionally. This chapter gives a brief history of the discovery of NO as a signalling molecule in the brain, discusses its current status and mechanisms of action and looks to the future of this humble, fascinating, but still imperfectly understood molecule.

Discovery of NO as brain transmitter

There were a few landmark studies that fed into the research that ultimately established NO as a signalling molecule in the CNS. At the forefront was the pioneering research of J.A. Ferrendelli during the 1970s into the regulation of the second messenger cGMP (Ferrendelli, 1978). Using mainly slices of cerebellum as the experimental material, he discovered the important contribution of excitatory agents, including glutamate, as stimuli for raising cGMP levels. He further found this response to excitation to be Ca^{2+} dependent and speculated (correctly) that the cGMP elevations may be secondary to the release of an unknown transmitter or the Ca^{2+}-dependent production of an intracellular substance. A second key discovery, also during the 1970s, was that in extracts of many tissues, including the cerebellum and cerebral cortex, a variety of nitroso-compounds (compounds such as sodium nitroprusside and nitroglycerine containing an NO-moiety) were powerful stimulators of cGMP synthesis by the so-called soluble guanylyl cyclase enzyme (i.e. the one that was enriched in supernatant tissue fractions after homogenisation and high-speed centrifugation), and that the nitroso-compounds were probably acting through the spontaneous or enzymatic release of NO which was itself shown to be a powerful guanylyl cyclase activator (Katsuki et al., 1977).

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Following the trail laid down by Ferrendelli, and facilitated by recent advances in the pharmacology of glutamate receptors and the attendant growing evidence that glutamate was an excitatory neurotransmitter (Watkins and Jane, 2006), we pursued the possibility that cGMP may represent a second messenger coupled to glutamate receptors. We found that the cGMP response to glutamate was massively higher during the period of major cerebellar development than in the adult (Garthwaite and Balazs, 1978) and that in dispersed cerebellar cells where pharmacological complications imposed by glutamate uptake were greatly diminished, the response was mediated exclusively through NMDA receptors (Garthwaite, 1985). Most importantly, experiments involving the selective lesioning of neuronal cell types in slices, as well as studies in dispersed cell suspensions enriched or depleted in specific cell types, pointed strongly to the cGMP responses to glutamate and NMDA being the result of cell–cell interactions, in contrast to the responses to the NO-releasing agent sodium nitroprusside which appeared to be direct. It was proposed that the primary targets of the excitants were neurones (specifically granule cells) and the cGMP elevations were mainly occurring in astrocytes. Our inability to capture the intervening messenger was attributed to its possibly unstable nature (Garthwaite and Garthwaite, 1987). Once we became aware of research on blood vessels that had developed over the same period, in which an unstable factor released from stimulated endothelial cells caused relaxation of underlying smooth muscle (Furchgott and Zawadzki, 1980), soon shown to be via cGMP, the parallels with our mysterious cell-to-cell signalling mechanism in the cerebellum became inescapable. Following suggestions by Furchgott and Ignarro, and the subsequent evidence that the endothelium-derived factor was indeed NO (Ignarro et al., 1987; Palmer et al., 1987), a few key experiments on cerebellar cell suspensions, and quick pairs of hands, were all that were needed to show that a factor that raised cerebellar cGMP levels was indeed released in response to NMDA receptor stimulation, that it was unstable (half-life in buffer solution = 18 s), that the factor was released Ca^{2+} dependently and that it also had other properties that were identical to the endothelium-derived factor in blood vessels (Garthwaite et al., 1988).

With work by Deguchi several years earlier identifying L-arginine as the precursor of an activator of 'soluble' guanylyl cyclase in the brain (Deguchi and Yoshioka, 1982), it came as no surprise that this amino acid would turn out to be the substrate for NO-generating enzymes, as was first shown in vascular endothelial cells, with L-citrulline being the co-product (Palmer et al., 1988). Fortunately, the first NO synthase inhibitor, L-methylarginine, was waiting in the wings as a result of the ground-breaking work by the group of John Hibbs who, in the year that the first solid evidence for endogenous NO formation was published, identified this derivative as an inhibitor of the conversion of L-arginine into L-citrulline and nitrite (NO_2^-, later shown to originate as NO) by macrophages (Hibbs et al., 1987). This compound duly turned out to be an effective inhibitor of NO generation by endothelial cells and neurones (as well as macrophages) and paved the way for the development of more potent NO synthase inhibitors, such as L-nitroarginine, for use in functional studies. While NO had forged an unexpected convergence in the fields of neuroscience, cardiovascular science and immunology, it soon became clear that NO signalling in each of these areas is largely subserved by

different NO synthase isoforms, so-called neuronal, endothelial and inducible (or immunological) NO synthase (nNOS, eNOS and iNOS, for short) but attempts to discriminate between them pharmacologically proved frustratingly difficult, despite the best efforts of several leading pharmaceutical companies and academic groups.

Purification of all three NO synthases was soon accomplished, with both nNOS and eNOS characterised as Ca^{2+}–calmodulin dependent whereas calmodulin was persistently and tightly bound to iNOS, rendering this isoform Ca^{2+} independent. Histological evidence indicated that neuronal NO-generating capacity was distributed widely in the CNS, often in discrete populations of cells, and the subsequent molecular cloning of nNOS showed that the 'simple' conversion of one of the terminal guanidino nitrogens of L-arginine into NO relied on a large and highly complex enzyme requiring multiple cofactors (Bredt et al., 1991).

NO receptors

Knowledge of the existence of an NO-activated guanylyl cyclase in many types of mammalian cells preceded the discovery of NO as an endogenous signalling molecule by a decade (Katsuki et al., 1977). Few authors dared to suggest that there might be an NO-like molecule functioning to link hormone or transmitter receptor stimulation to cellular cGMP synthesis. Rather, NO was generally considered little more than a pharmacological activator of the enzyme, somewhat analogous to fluoride as a stimulator of adenylyl cyclase, known since the 1960s. Attempts to purify the enzyme, moreover, were confounded by the loss of NO-stimulated activity which, it transpired, was the result of the loss of a prosthetic ferrous (Fe^{2+}) haem group that serves as the NO-binding site. Lacking the chemical specialisation necessary for the usual non-covalent binding of conventional neurotransmitters and hormones to receptors, NO reactivity in biology depends on its radical nature (i.e. its possession of an unpaired electron) which particularly favours covalent binding to transition metals (notably Fe^{2+}). For reasons still not fully understood, the haem NO-binding site effectively excludes O_2 and is only very weakly activated by high concentrations of CO and so it represents a receptor with an astonishing degree of selectivity for NO. A combination of the haem geometry and local protein conformation may explain this selectivity (Montfort et al., 2017) but it is one of the fundamental properties of the receptors that allow NO to operate at extremely low (subnanomolar) concentrations in an environment containing up to a million-fold higher concentration of O_2.

Molecularly, guanylyl cyclase-coupled NO receptors exist as heterodimers made up of a common β1-subunit together with either an α1- or α2-subunit, the main difference being that the α2-subunit possesses extra amino acids that allows binding of the α2β1 isoform to protein PDZ domains, thereby targeting it to the pre- and/or postsynaptic compartment in synapses. In the unstimulated state, a histidine residue of the β1-subunit is coordinated to the haem iron, leaving one coordination site vacant and exposed. Binding of NO to this site is thought to lead to a tilting of the haem that contributes to the breakage of the histidine bond to the protein, triggering a conformational change that propagates to a catalytic site where GTP binds and is converted to cGMP (Koesling et al., 2004; Montfort et al., 2017).

Devising methods for supplying known concentrations of NO either steadily or as brief transients was a prerequisite for unravelling the functional properties of the receptors, the results of which have been incorporated into a formal mechanism-based model, as discussed in detail elsewhere (Garthwaite, 2010). Briefly, with the purified protein, the EC_{50} for NO under standard assay conditions is about 1 nM whereas, in cells, it is 10 nM. The difference is partly explained by cells having a lower GTP concentration than typically used in assays, and also containing ATP, which acts as a mixed inhibitor of the guanylyl cyclase. Offsetting the resulting loss of potency of NO, the receptor kinetics becomes faster. The rate of binding of NO is very rapid, estimated as $3 \times 10^8\,M^{-1}\,s^{-1}$, or about 100 times faster than the rate of binding of glutamate to AMPA or NMDA receptors, a feature that contributes to a swift rate of activation that assists in the capture of brief transient pulses of NO. On removal of NO, deactivation takes about 250 ms. Like most other receptors, cellular NO-activated guanylyl cyclase also desensitises during prolonged agonist exposure, although this phenomenon becomes prominent only with relatively high NO concentrations (>1 nM).

By analogy with other transmitter systems, an EC_{50} for receptor activation in cells of 10 nM might suggest that physiological NO concentrations are of the same order of magnitude. Surprisingly, cells are able to respond to transient NO concentrations up to 1000-fold lower. The explanation is that the receptor in this case is enzyme-linked and it functions most efficiently when the agonist concentration is low compared with the binding affinity (estimated as 20–50 nM). Under these conditions, and with a guanylyl cyclase activity similar to that found naturally in cells, each bound NO molecule stimulates the formation of 5000 cGMP molecules per second, making cells exquisitely sensitive NO detectors (Batchelor et al., 2010).

Through connections in the glutamate field, we were fortunate to have been given the opportunity to test what turned out to be the first selective inhibitor of NO-activated guanylyl cyclase, the compound ODQ (1h-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one). A Danish company, A/S Ferrosan (later to be incorporated into Novo Nordisk A/S), were developing glutamate antagonists and had been using glutamate-stimulated cGMP formation in cerebellar slices as the basis of a screening assay. The compound they synthesised was chemically related to their pioneering AMPA antagonists (e.g. 6-cyano-7-nitroquinoxaline-2,3-dione, or CNQX) and was a potent inhibitor of the cGMP response but, when put through further tests, was found to be inactive against glutamate receptors. The company thought we might be interested in taking a look at the compound, which indeed we were. After exhaustively excluding many alternatives, we were able to pinpoint NO-activated guanylyl cyclase as the sole target (Garthwaite et al., 1995). ODQ acts specifically on the NO-binding site by oxidising the ferrous (Fe^{2+}) haem to a ferric (Fe^{3+}) haem which has a much lower affinity for NO. The inhibition is reversible in cells, presumably because of the action of a haem reductase. Gratifyingly, the compound has stood the test of time and remains the standard pharmacological tool for probing the mechanisms of NO signal transduction in tissues and cells but there remains the need for new compounds, particularly ones that have better bioavailability in vivo where reaction of ODQ with the huge abundance of haemoglobin in the circulation (25 mM) limits access of the compound to tissues when administered systemically.

How does the NO signalling pathway function in the brain?

Seeing as it operates in virtually every brain area, it is unsurprising that NO is involved in many different central functions, such as in learning and memory formation, moving, feeding, sleeping, reproduction, pain and anxiety, as well as in brain development and synaptogenesis (Garthwaite, 2008; Steinert et al., 2010). At the cellular level, NO is generated largely by neurones, often as a result of NMDA receptor activity. The link is facilitated by the nNOS isoform being physically tethered to postsynaptic density-95 protein, positioning it within 20 nm of the mouth of the NMDA receptor channel. Other postsynaptic receptors (e.g. for acetylcholine) may also couple to NO synthesis or, alternatively, NO may be formed presynaptically in some neurones (as it is in autonomic 'nitrergic' nerve fibres innervating many peripheral organs), where voltage-sensitive N-type channels usually serve as the main conduits for the Ca^{2+} that activates the enzyme. In addition, NO from eNOS in blood vessels may access neural elements to alter their function and, under pathophysiological conditions, NO can be generated from iNOS, which is normally absent but can be expressed in microglial cells in response to immune challenge.

The original hypothesis that NO functions as a retrograde trans-synaptic messenger, being generated postsynaptically in response to NMDA receptor activation and acting presynaptically to modify neurotransmitter release (Garthwaite et al., 1988) has received experimental support from many different brain areas and is widely invoked to explain the role of NO in synaptic plasticity, including in long-term potentiation (Hardingham et al., 2013). The potential targets for NO, however, are not exclusively presynaptic. The original experiments on developing cerebellum highlighted a neurone–astrocyte signalling pathway (Garthwaite and Garthwaite, 1987; Garthwaite et al., 1988) simply because the cell suspensions being used contained largely only these two cell types and because cerebellar astrocytes have an extremely low complement of cGMP-degrading phosphodiesterase enzymes, allowing cGMP levels to rise to very high concentrations that influence disproportionately whole tissue cGMP measurements. In several other brain areas, astrocytes are also potential targets, as are presynaptic and postsynaptic elements of different classes of neurone, as well as oligodendrocytes and blood vessels, judging from their ability to raise the level of cGMP in response to endogenous and/or exogenous NO, and to express NO-activated guanylyl cyclase.

The diversity of the prospective lines of communication raises questions of how this signalling system is able to encode a coherent and interpretable transfer of information, particularly since the message itself is not channelled to any target, but is free to diffuse away from its source in three dimensions. Three modes of operation can be envisaged:

1. *The single synapse.* When expressed at glutamatergic synapses, nNOS is frequently found tethered close to postsynaptic NMDA receptors and becomes activated, via calmodulin, when Ca^{2+} near the inner mouth of the channel rises. nNOS can only manufacture NO from L-arginine at quite a slow rate, perhaps 10 molecules/s at best. Furthermore, during normal synaptic transmission, as few as four NMDA receptors become active.

Assuming one nNOS molecule per NMDA receptor, the maximum NO output from a postsynaptic structure during continuous NMDA receptor activity would be about 40 NO molecules/s. To model this situation, the zone of NO generation can be treated as a disc having a diameter similar to that of a postsynaptic density (0.4 μm) and the NO concentrations in and around the synapse with time computed (Garthwaite, 2016). With an input resembling the time-course of an NMDA receptor current with a peak rate of NO synthesis of 40 molecules/s (inset Figure 1(a)), the NO concentration at the source reaches about 60 pM but falls steeply on either side, reaching about 10 pM a distance of 400 nm away, which corresponds roughly to the outer boundaries of the synapse (Figure 1(a) and (b)). Using a compartmental model, cGMP generation in the target structure can be computed assuming a level of NO-activated guanylyl cyclase similar to that found naturally in cells. With a single input pulse, cGMP peaks at about 10 nM which is probably too low to exert physiological effects. Repeated pulses, on the other hand, provide for summated cGMP formation. For example, 10 pulses delivered at 100 ms intervals provide 100 nM cGMP, a concentration in the range capable of activating cGMP-dependent protein kinases to initiate protein phosphorylation. Hence, at individual synapses, NO is an excellent candidate for a retrograde messenger, informing presynaptic nerve terminals when, and how much, postsynaptic NMDA (or possibly other) receptors are activated, or as an orthograde transmitter were it to be formed presynaptically. In either case, NO concentrations are likely to be low and the molecule is likely to operate in an activity-dependent manner, according neatly with experimental findings on peripheral nitrergic transmission and with its participation in events related to the induction of activity-dependent synaptic plasticity in the CNS.

2.	*Synaptic spillover.* Although largely confined within synaptic dimensions, NO produced within an individual synapse is predicted to spread outside these bounds (Figure 1(a) and (b)), possibly in sufficient amounts to act on neighbouring synapses, which can be found only short (submicron) distances away and can even be located side-by-side. Synaptic NO spillover may also impact on astrocytic fibres that ensheath many central synapses.

3.	*Volume transmission.* When multiple NO sources in a tissue volume become active roughly simultaneously and are close enough together, NO in between the sources could rise to active concentrations. Should the sources be of synaptic dimensions, a mean separation of about 3 μm or less should be enough to produce such a scenario (Garthwaite, 2016). There are numerous potential functional roles for this more global type of signalling, for example in providing astrocytes information on overall levels of synaptic activity at any given time, or for signalling to oligodendrocytes to influence myelination. Another potential origin of volume-type signals is the endothelium of blood capillaries which appear to provide a 'basal' NO tone that affects, for example, hippocampal synaptic plasticity or the excitability of optic
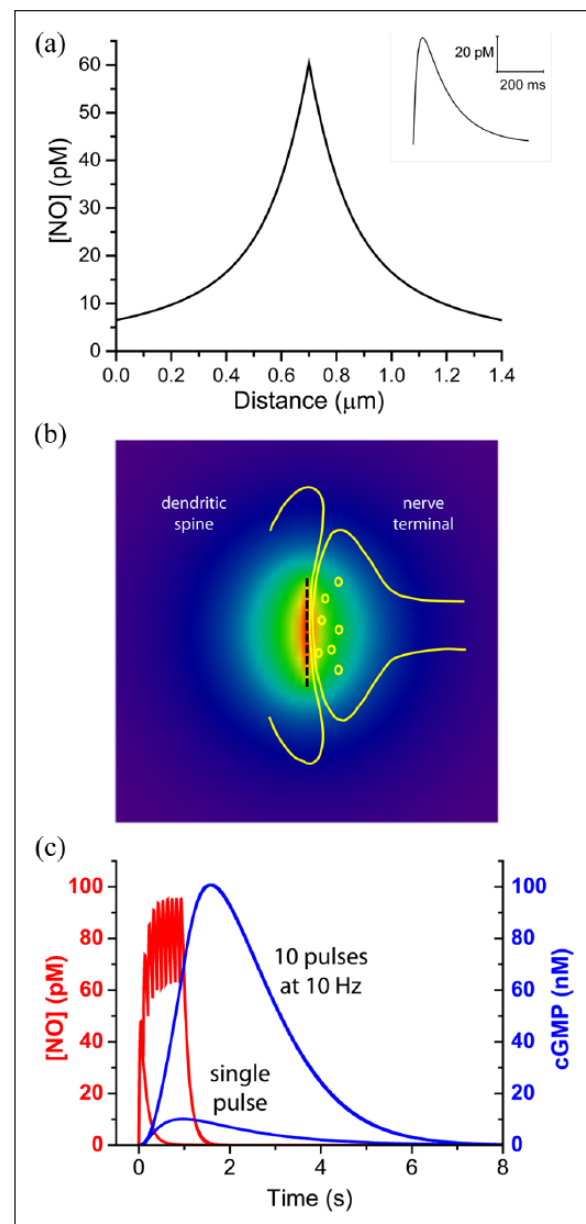

Figure 1. Synaptic NO signalling: (a, b) spread of NO from a disc, simulating a postsynaptic density (depicted as the broken black line in (b)) at the peak of a transient wave of synthesis (inset in (a)) which approximates to the time-course of a synaptic NMDA receptor-mediated rise in postsynaptic Ca^{2+}. The dimensions of B are 1.4×1.4 μm, with a sketch of a typically sized synapse overlaid. (c) Compartmental analysis of NO signal transduction, showing computed cGMP concentrations in a nerve terminal or dendritic spine head, modelled as a hemisphere, following single or multiple NO pulses generated in a disc-shaped zone at its base.

nerve axons. Finally, in some brain areas, the synchronised activity of nNOS neurones may be instrumental in signalling non-synaptically to intervening cells, as appears to be the case in the preoptic area of the hypothalamus where NO generated in response to the hormone leptin diffuses to intervening cells to stimulate

the release gonadotropin-releasing hormone, ultimately affecting fertility (Garthwaite, 2016).

Future prospects

The early incredulity exhibited by many at the notion of NO acting as a transmitter-like molecule in the CNS soon dissipated and, surprisingly, it turns out to operate quite similarly to more conventional transmitters, the major differences being the rapid and unrestrained diffusibility that is unique to NO. While there is a large body of work on NO-mediated phenomena in the CNS, the deeper mechanistic picture remains indistinct. Partly, this deficiency is a consequence of its actions on target tissues being mediated by metabotropic receptors whose effects span different time scales and are frequently not recordable electrophysiologically. It is also difficult to selectively activate endogenous NO sources and to apply exogenous NO locally in physiologically relevant amounts in physiologically relevant places, so new methodology needs to be implemented (e.g. optogenetics and improved photosensitive NO-releasing compounds). A fresh window that allows real-time NO signal transduction to be visualised in individual cells, and even in synapses, using genetically encoded fluorescent cGMP biosensors, has recently opened up and this approach should provide unparalleled insight into many temporal and spatial aspects of NO-mediated transmission that are currently unknown. An improved microanatomical description of the sources and targets of NO will be needed to complement these approaches. Finally, a highly active and apparently enzymatic process for inactivating NO exists in brain tissue such that its half-life is predicted to be as short as 5 ms, but the underlying mechanism remains unknown; the possibility that abnormalities in NO signalling, including defective inactivation, may contribute to brain disorders, including acute and chronic neurodegenerative conditions, has been the subject of a vast literature but there is little understanding of what constitutes an 'abnormality' in this pathway. Establishing what is 'normal' would be a useful antecedent.

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