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REVIEW ARTICLE

NO as a multimodal transmitter in the brain: discovery and current status

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NO operates throughout the brain as an intercellular messenger, initiating its varied physiological effects by activating specialized GC-coupled receptors, resulting in the formation of cGMP. In line with the widespread expression of this pathway, NO participates in numerous different brain functions. This review gives an account of the discovery of NO as a signalling molecule in the brain, experiments that originated in the search for a mysterious cGMP-stimulating factor released from central neurones when their NMDA receptors were stimulated, and summarizes the subsequent key steps that helped establish its status as a central transmitter. Currently, various modes of operation are viewed to underlie its diverse behaviour, ranging from very local signalling between synaptic partners (in the orthograde or retrograde directions) to a volume-type transmission whereby NO synthesized by multiple synchronous sources summate spatially and temporally to influence intermingled neuronal or non-neuronal cells, irrespective of anatomical connectivity.

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Abbreviations

CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; eNOS, endothelial NOS; HCN channels, hyperpolarization-activated, cyclic nucleotide-regulated channels; nNOS, neuronal NOS; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PAPA/NO, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate

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Introduction

Despite its perceived oddity at the time, **NO** rapidly became established as a novel type of signalling molecule in the CNS about 30 years ago (Garthwaite *et al.*, 1988), shortly after being recognized as the endothelium-derived relaxing factor (EDRF) in blood vessels (Furchgott and Zawadzki, 1980; Ignarro *et al.*, 1987; Palmer *et al.*, 1987). NO was not immediately characterized as a neurotransmitter because the prevailing dogma was that a neurotransmitter was stored within vesicles in presynaptic nerve terminals, was released upon depolarization of the terminal, acted on membrane-bound receptors to elicit an electrical response in the adjacent postsynaptic neurone and was subject to a removal/inactivation mechanism to ensure timely termination of the signal. In this context, NO was more like a second messenger, albeit one that diffuses rapidly across membranes to enable intercellular communication rather than being confined intracellularly. With multiple advances in the neurosciences, the previous criteria for the identification of a neurotransmitter had to be loosened, both to recognize alternative modes of release and action of the established neurotransmitters and to incorporate more recently discovered, atypical neural signalling molecules, such as NO and endocannabinoids which are generated 'on demand.' They also often act 'retrogradely' in that they can be synthesized postsynaptically and target presynaptic terminals, for example, to modify the release of conventional neurotransmitters. Hence, it is now justifiable to refer to NO as a neurotransmitter, although given that it is not exclusively generated by neurones nor necessarily target them, the more general term 'transmitter' is preferable.

This volume celebrates the award of the Nobel Prize in Physiology or Medicine given two decades ago for the identification of NO as a signalling molecule in the cardiovascular system. In those pre-molecular biology days, bioassay was a crucially important technique for the identification of biological transmitters. The stage was set almost 100 years ago in the famous experiment of Otto Loewi, who, in an age where many influential researchers had the view that transmission was purely electrical, demonstrated that a saline solution bathing a frog heart undergoing stimulation of its vagus nerve was able to slow a second, denervated heart, implying that a chemical messenger (*vagusstoff*) was released from the stimulated nerve (Valenstein, 2002). The analogy is inescapable between Loewi's experiment and those of Robert Furchgott, over 50 years later, showing that an endothelium-intact preparation of aorta exposed to **ACh** would relax a second endothelium-free preparation held close by, thereby demonstrating the existence of EDRF (Furchgott and Zawadzki, 1980). In the intervening years, analogous techniques were central to the identification of numerous biological transmitters, including ACh (Loewi's *vagusstoff*) and **noradrenaline** (Valenstein, 2002). In the field of chemical transmission, the 20th century can rightly be regarded as the century of the bioassay.

Here, I will summarize how NO came to be discovered to be a transmitter in the brain, experiments in which bioassay yet again proved instrumental, and will then 'fast-forward' to provide a current view of how NO operates as a neural signalling molecule in this most complex of organs.

Discovery of NO as a CNS transmitter

Early findings: glutamate and cGMP

The story started in 1977 when I joined a Medical Research Council laboratory specializing in developmental neurobiology. The laboratory had developed methods for separating different types of cell from the developing cerebellum with the aim of studying the properties of the individual cells in isolation (Cohen *et al.*, 1978). The cerebellum was advantageous because it has only a few distinct cell types and preparations enriched in large Purkinje neurones, the small but very numerous granule cells (the most abundant type of neurone in the brain) and the non-neuronal astrocytes, had been established. One property of interest related to **cGMP**, the levels of which were appreciably higher in the cerebellum than in other brain areas. A prevailing view, based largely on the activity of various drugs *in vivo*, was that cGMP levels in the cerebellum reflected the balance between synaptic excitation and inhibition, becoming raised when activity in the excitatory pathways was increased or when inhibition was reduced and lowered in the converse situations. Although only based on circumstantial evidence, it was widely assumed that the alterations in cGMP were primarily located in Purkinje cells, giving rise to the speculation that **guanylyl cyclase (GC)**, the enzyme generating cGMP may be physically associated with excitatory receptors on these neurones (Biggio and Guidotti, 1976). In this scenario, cGMP was viewed as a second messenger associated with activation of Purkinje cell excitatory neurotransmitter receptors, perhaps even mediating the excitation itself (Guidotti *et al.*, 1975). Partly supporting this scenario were the findings that **glutamate**, which was considered a possible neurotransmitter in the excitatory cerebellar circuitry, was able to raise cerebellar cGMP levels when microinjected *in vivo* (Mao *et al.*, 1974) or applied to slices of the tissue maintained *in vitro* (Ferrendelli *et al.*, 1974). Importantly, however, the cGMP response to glutamate *in vitro* was also shown to be Ca^{2+}-dependent, signifying a more complex mechanism, speculatively one that may include neurotransmitter release (Ferrendelli *et al.*, 1974).

Having access to preparations enriched in Purkinje and other cells offered the opportunity to examine this hypothesis more directly. In intact slices from the developing cerebellum maintained *in vitro*, glutamate gave unprecedentedly large (over 200-fold) increases in cGMP (Garthwaite and Balazs, 1978) but, unfortunately, whether maintained as the starting mixture of cells or as purified cell fractions, the isolated cells responded very variably, often not at all, despite working well in other types of experiment. It turned out that there were two main reasons for these disappointing results. One was that the cells needed to be incubated in high concentrations to generate significant cGMP in response to glutamate, an initially perplexing result that was to provide an important clue as to the underlying mechanism. The other main reason was that the initial experiments were carried out using a standard

bicarbonate-buffered incubation medium gassed with 95% O_2 and 5% CO_2. The high O_2 concentration, which leads to high levels of superoxide ions (which inactivate NO), was the probable culprit.

Importance of NMDA receptors

Although there had been a simmering interest in the idea of glutamate as an excitatory transmitter in the CNS since the 1950s, the late 1970s witnessed a serious intensification, propelled by the identification of agonists whose effects pointed to the existence of different subtypes of receptor for acidic amino acids, of which glutamate and **aspartate** were the prime neurotransmitter candidates. The receptors were broadly classified into those activated by the analogue **NMDA** (**NMDA receptors**) or by other analogues such as kainic acid (collectively called non-NMDA receptors). The subsequent discovery of selective NMDA antagonists and observation of the effects they had in central synapses *in vitro* and *in vivo* boosted the idea that one or more acidic amino acids functioned as excitatory transmitters in at least some central synapses. Initially, aspartate was thought to act mainly at NMDA receptors and glutamate at non-NMDA receptors (Watkins and Jane, 2006).

The pharmacological progress provided an opportunity to investigate the relationship between neuronal excitation and the generation of cGMP. In slices of developing and adult cerebellum, I found that acidic amino acids and their analogues raised cGMP levels through receptors having properties identical to those mediating neuronal excitation (Garthwaite, 1982). Furthermore, having eventually succeeded in getting cell suspensions from the developing cerebellum working reliably (using a high cell concentrations and an air-equilibrated incubation medium), I showed that the cGMP responses to glutamate and aspartate were both mediated exclusively by NMDA receptors, with glutamate being the more potent agonist, contrary to the findings in intact brain tissue (Garthwaite, 1985). Cellular uptake of glutamate was identified as the factor that distorted the pharmacology in the intact tissue. The properties of the receptors mediating cGMP generation revealed in the experiments on isolated cerebellar cells proved uncannily similar to the properties of NMDA receptors as defined in ligand binding experiments on rat brain membranes at about the same time (Olverman *et al*., 1984). Glutamate was soon accepted as the transmitter candidate able to act on both NMDA and non-NMDA receptors.

Glutamate-evoked cGMP elevation depends on cell-to-cell signalling

Two pieces of evidence pointed to the link between activation of excitatory amino acid receptors and the generation of cGMP being very different from the second messenger mechanism that had been promulgated previously.

First, as an alternative to the use of living cell fractions to investigate the location of cGMP responses in the cerebellum, biochemical measurements of the activity of GCs were undertaken. In 1977, two groups had shown that **'soluble' GC** in the CNS could be activated by NO (Arnold *et al*., 1977; Miki *et al*., 1977) and, for the first time, an NO-releaser, **sodium nitroprusside**, was included in our experiments. The cells having the highest levels of both basal and nitroprusside-stimulated GC activity were the non-neuronal astrocytes, rather than the Purkinje neurones, although similarly high levels of activity were also found in an almost pure preparation of specialized cerebellar synaptosomes (Bunn *et al*., 1986). These synaptosome particles predominantly comprise the large nerve terminals of the excitatory mossy fibres, together with attached remnants of dendrites of granule cells with which they form synaptic contact. Furthermore, although stimulation by NO-releasing agents such as nitroprusside was viewed as little more than a pharmacological activation of the enzyme at the time, it was informative that activation by nitroprusside was necessary for the GC activity in homogenates to match the very high initial activity exhibited by those same cells when maintained intact and stimulated by glutamate or NMDA (around 700 $pmol \cdot mg^{-1}$ $protein \cdot min^{-1}$).

Secondly, after moving to the University of Liverpool, our investigations of the mechanisms of neurodegeneration provoked by excessive stimulation of excitatory amino acid receptors ('excitotoxicity') had revealed ways of selectively and rapidly lesioning specific neuronal cell populations in cerebellar slices *in vitro* (Hajos *et al*., 1986). This approach, together with the use of dispersed cell suspensions enriched or depleted in various cell types, offered a new opportunity for finding out where the cGMP responses were located (Garthwaite and Garthwaite, 1987). Importantly, nitroprusside was shown to target the same pool of GC that was used for excitant-induced cGMP elevations. The selective lesioning of a particular class of neurone (granule cells), however, almost obliterated the cGMP response to excitatory amino acids but left the cGMP elevation induced by nitroprusside unaffected. In suspension of cells, moreover, nitroprusside-induced cGMP accumulation was independent of cell concentration, unlike the responses to NMDA or glutamate which progressively dwindled down to nothing as the cells were diluted below about 20 million $cells \cdot mL^{-1}$. No major contribution by Purkinje cells could be detected. These and other results all supported the idea that the cGMP response depended on cell–cell interactions, with glutamate and other agonists acting primarily on a neuronal population (granule cells) but raising cGMP elsewhere, predominantly in astrocytes which were also targeted by nitroprusside. The findings implied the existence of a messenger released by activated neurones to act on astrocytes, but 'attempts to find evidence for a diffusible factor … proved unsuccessful. This may be because the factor is unstable; for example free radicals are potent and effective activators of guanylate cyclase but have only short half-lives' (Garthwaite and Garthwaite, 1987). The latter statement referred, of course, to substances such as NO (Murad *et al*., 1978).

NMDA receptor-linked cGMP elevating factor = EDRF = NO

Many of the results on the isolated cells eventually written up in this latter paper (submitted for publication in 1986) were 5 years old or more but, lacking a clear explanation, had remained undisclosed. An aim now had to be to try to identify the intercellular messenger. From our results, an NO-like substance seemed plausible, a possibility consistent with the findings of Deguchi that an endogenous activator of 'soluble'

GC existed in rat brain and that it stimulated the enzyme similarly to NO-releasing chemicals (Deguchi, 1977). In 1982, the endogenous activator was identified as **L-arginine** (Deguchi and Yoshioka, 1982), but no test to determine if the amino acid elicited cGMP accumulation in intact cells was reported. When we carried out the experiment using cerebellar slices or cell suspensions, L-arginine by itself was found not to stimulate cGMP accumulation significantly. An alternative mechanism had been invoked in studies of the cGMP elevations in neuroblastoma cells brought about by **muscarinic** and **histamine receptor** agonists, in which the Ca^{2+}-dependent generation of **arachidonic acid** and its subsequent conversion to an oxidative metabolite led to the activation of 'soluble' GC (Snider *et al.*, 1984). The search for the chemical identity of EDRF had gone through a similar arachidonic acid metabolite phase (Vanhoutte *et al.*, 1986).

We heard about EDRF in blood vessels possibly being NO prior to any publication, thanks to a lecture by Geoffrey Burnstock (the pioneer of purinergic transmission). After some intense reading-up on the subject, a nice undergraduate student laboratory project took shape. It transpired that some additional evidence linking our mysterious intercellular messenger with EDRF was already in hand: back in 1985, I had found that SOD (with or without **catalase**) enhanced the cGMP response to glutamate in a cerebellar cell suspension (these data were eventually published in figure 1 of Garthwaite *et al.*, 1988). With the very able assistance of a final-year student, Sarah Charles, the results fell rapidly into place. NMDA-evoked cGMP accumulation was subject to the same pharmacological interventions that were effective in the case of EDRF, for example, inhibition by haemoglobin, methylene blue and quinacrine. Most importantly, using a classical bioassay approach, we were able to show with the help of a rapid manual filtration technique that the suspending medium taken from the stimulated cerebellar cell suspension contained an unstable factor (half-life = 18 s in air-equilibrated buffer) that could evoke cGMP accumulation in 'detector' cells, that the factor was stabilized by SOD and that it was released in a Ca^{2+}-dependent manner. Finally, with the help of a cardiovascular expert in a nearby department, Russell Chess-Williams, and a purpose-built, miniaturized organ bath he designed and fabricated, we showed that stimulation of the cerebellar cells with NMDA caused a Hb-sensitive relaxation of arterial smooth muscle. Collectively, the results showed that NMDA receptor activation causing the release of EDRF (Garthwaite *et al.*, 1988), which by then had been identified as NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987).

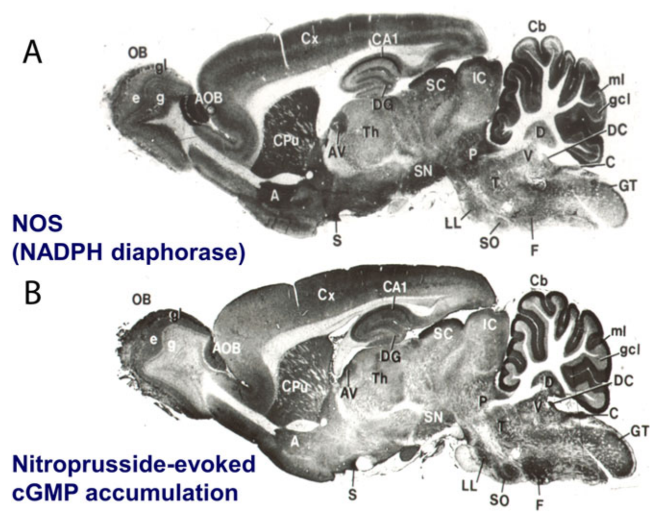

Figure 1

The NO-cGMP signalling pathway in brain. Images are of adjacent para saggital sections from a rat previously perfused *in vivo* with the NO donor, nitroprusside (10 mM, 5 min) and, after fixation, stained for NOS using the NADPH diaphorase histochemical method (A) and for cGMP using immunohistochemistry (B). Images adapted from Southam and Garthwaite (1993).

Tying up some loose ends

Knowing the work by Deguchi identifying L-arginine an endogenous activator for 'soluble' GC in the brain (Deguchi and Yoshioka, 1982) meant that it was no great surprise that this amino acid would turn out to be the substrate for NO-generating enzymes, as was first shown in vascular endothelial cells (Palmer *et al.*, 1988a). As a result of the formidable work by the group of John Hibbs who, in 1987, identified L-methylarginine as an inhibitor of the conversion of L-arginine into **L-citrulline** and nitrite (later shown to derive from NO) by macrophages (Hibbs *et al.*, 1987), the first NOS inhibitor had inadvertently already been found. The compound duly turned out to be an effective inhibitor of NO generation by endothelial cells (Palmer *et al.*, 1988b) and, as part of a collaboration with the group of Salvador Moncada (Wellcome Research Laboratories, UK) who had plentiful supplies of L-methylarginine (curiously, commercial sources were drained of it), we showed the analogue to be a potent inhibitor of NMDA-stimulated cGMP accumulation in cerebellar slices and that its effects could be neutralized by L-arginine which itself augmented the NMDA-induced cGMP accumulation by up to 70% (Garthwaite *et al.*, 1989). L-methylarginine was also found to be effective in the cerebellum *in vivo* against cGMP elevations elicited by exogenous excitatory amino acid agonists as well as by pharmacological agents that activate endogenous excitatory neurotransmission, squaring the new mechanism with the older data (Wood, 1991).

The successful isolation of an **NO-synthesizing enzyme** from rat cerebellum revealed that it was dependent on Ca^{2+}-calmodulin (Bredt and Snyder, 1990), explaining why Ca^{2+} is necessary for NO to be generated in response to glutamate receptor activation. The subsequent molecular cloning of the neuronal enzyme (**neuronal NOS; nNOS**) by this group a year later (Bredt *et al.*, 1991b) ultimately led to an understanding that an intimate coupling between NMDA receptors and nNOS is facilitated at synapses by their mutual binding to postsynaptic density-95 protein (Brenman *et al.*, 1996), an arrangement that positions nNOS only about 18 nm inside the postsynaptic membrane (Valtschanoff and Weinberg, 2001). NMDA receptor-associated ion channels had long been known to have a high permeability to Ca^{2+}, and this localization of nNOS close to the internal mouth of the NMDA receptor channel satisfactorily explained the privileged relationship between NMDA receptors and NO generation.

Finally, while much of the early research on cGMP and subsequently NO had been carried out on the cerebellum, immunocytochemical and *in situ* hybridization studies indicated that nNOS is expressed, often in discrete neuronal types, throughout most of the CNS (Bredt *et al.*, 1991a). As an alternative anatomical method, quite an ancient histochemical technique, NADPH diaphorase, was found to selectively stain NOS in tissue sections (Hope *et al.*, 1991), yielding a closely similar picture to that found using nNOS immunohistochemistry (Bredt *et al.*, 1991a). Identifying the putative targets of NO anatomically was greatly facilitated by the development of an immunohistochemical method for locating formaldehyde-fixed cGMP by Jan de Vente and colleagues (Amsterdam). Pleasingly, when applied to slices of immature and adult cerebellum stimulated by nitroprusside or NMDA, de Vente and colleagues saw cGMP accumulation most prominently in astrocytes (de Vente *et al.*, 1990). Using his special cGMP antibody, we undertook immunocytochemical analysis of the whole brain of adult rats that had been perfused *in vivo* with nitroprusside prior to fixation, with adjacent sections stained for NOS using the NADPH diaphorase histochemical technique (Southam and Garthwaite, 1993). The results showed that nitroprusside-stimulated cGMP accumulation throughout the brain, with a distribution complementary to that of NOS (Figure 1), implying that GC represents a signal transduction pathway for NO throughout the CNS.

Functions of NO in the CNS

Consistent with the widespread distribution of the signalling pathway, NO has been found to participate in numerous central functions, including learning and memory formation, sleeping, feeding, movement, pain, anxiety and reproductive activity (Garthwaite, 2008; Steinert *et al.*, 2010; Chachlaki *et al.*, 2017). Beyond the participation of cGMP and, often, **cGMP-dependent protein kinase**, however, knowledge of the underlying mechanisms at the cellular and subcellular levels remains far from complete. One of the most studied phenomena has been of synaptic plasticity, where transient periods of increased synaptic activity lead to long-term changes in synaptic strength, phenomena that are presumed to be cellular correlates of memory formation. With our evidence in the cerebellum that NO was generated by granule cells and that the excitatory nerve terminals in synaptic contact with them were NO targets immediately prompted the hypothesis that NO functions as a retrograde trans-synaptic messenger, as well as one mediating neurone-astrocyte communication (Garthwaite *et al.*, 1988). Just such a retrograde messenger had been invoked to explain how, in the case of LTP in the hippocampus, enduring presynaptic alterations in glutamate release would result from postsynaptic NMDA receptor stimulation (Bliss and Collingridge, 1993), and despite some initial confusing results, plentiful evidence from many brain areas has accumulated to support such a role for NO (Hardingham *et al.*, 2013), including at the glutamatergic synapses with granule cells in the cerebellum where such a role had originally been envisaged (Maffei *et al.*, 2003). Precisely, how NO elicits enduring increases in neurotransmitter release is unclear, although altering synaptic vesicle dynamics *via* cGMP-dependent protein kinase is a possible contributory mechanism (Eguchi *et al.*, 2012). But NO may also have local postsynaptic effects relevant to synaptic plasticity, including the activation of gene expression (Lu *et al.*, 1999) and an increased trafficking of AMPA receptors into synapses (Serulle *et al.*, 2007). More recently, evidence has emerged that NO also participates in NMDA receptor-independent types of synaptic plasticity. One of these types depends instead on L-type Ca^{2+} channels, which had not traditionally been associated with nNOS activation, and is sustained largely through long-term increases in presynaptic glutamate release (Pigott and Garthwaite, 2016; Haj-Dahmane *et al.*, 2017).

NO transmission: how does it work?

NO receptors

Activation of GC activity remains the only established mechanism of physiological NO signal transduction, and it is better to consider the operative proteins as enzyme-linked receptors for NO, or simply NO receptors, as opposed to 'soluble GC', an archaic name that was adopted specifically to describe the enzyme activity in homogenates, with the explicit recognition that it might not reflect accurately the *in vivo* nature of the enzyme forms (Chrisman *et al.*, 1975). This was a prescient statement in that, of the two known isoforms of NO-activated GC, α1β1 and α2β1, the latter is prominent in brain and is targeted to synaptic membranes by virtue of its binding to the PDZ domains of synaptic scaffold proteins, such as postsynaptic density protein-95 or its presynaptic relatives (Russwurm *et al.*, 2001) and so is not 'soluble'.

Determining the importance of cGMP in NO signal transduction was hindered initially by a lack of useful pharmacological agents. Through mutual interests in the glutamate field, I had often met Tage Honoré who was the Research Director of a Danish pharmaceutical company called A/S Ferrosan (later to be incorporated into Novo Nordisk A/S) that specialized in the discovery of glutamate antagonists. As a screening method, they had measured cGMP in immature cerebellar slices stimulated by glutamate agonists and had found a quinoxaline derivative that potently blocked the cGMP response but, disappointingly for them, was subsequently found to have no effect on glutamate receptors themselves. Tage contacted me to see if I would be interested in taking a look at the compound and, with the help of the group of Bernd Mayer (Graz, Austria), who had access to purified 'soluble' GC, we found it to be a highly selective inhibitor of the NO-stimulated enzyme (Garthwaite *et al.*, 1995). The compound (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was, in fact, related chemically to the quinoxaline-based antagonists for non-NMDA receptors (soon to be called AMPA receptors) that the Danish group had pioneered (Honore *et al.*, 1988), and we called it **ODQ**, omitting the terminal 'X' that was used conventionally for naming the AMPA antagonists (e.g. CNQX for 6-cyano-7-nitroquinoxaline-2,3-dione) to avoid confusion. ODQ inhibits activation of the GC by NO by oxidizing the Fe^{2+} in the haem prosthetic group to which NO binds (Schrammel *et al.*, 1996). In cells, the inhibition is readily reversible (Bellamy and Garthwaite, 2002), with recent evidence indicating that a cytochrome b5

reductase enzyme performs this task ((Rahaman *et al*., 2017). Gratifyingly, ODQ has now been used successfully in a very large number of studies and remains the agent of choice for testing the importance of GC in NO signal transduction, although there remains the need for new inhibitors with a different mode of action to avoid bioavailability problems *in vivo* stemming from binding of ODQ to the very high concentration of haemoglobin in circulating red blood cells.

Valuable complementary approaches for interrogating the roles of NO-activated GC in NO signal transduction have also become established. Most notable are the family of allosteric enhancers, the prototype compound of this class, **YC-1** (Ko *et al*., 1994), being superseded by more potent and selective analogues, including **BAY 41-2272** (Stasch *et al*., 2001) that produce a leftward shift in the concentration–response curve for activation of GC by NO and increase the maximum response. From studies of the purified **α1β1 protein (GC-1)**, the steady-state NO EC_{50} is reduced from 1 nM down to an estimated 4 pM in a saturating BAY 41-2272 concentration (Roy *et al*., 2008a). This remarkable shift is explained by BAY 41-2272 selectively stabilizing the active receptor by binding to it with much higher affinity (K_D = 20 nM) than to the inactive species (K_D = 11 μM). Another useful pharmacological approach takes the form of haem-mimetics, such as **BAY 58-2667** (cinaciguat; Stasch *et al*., 2002), that bind to haem-deficient GC to partially mimic the activated state of the protein, an effect reminiscent of that observed much early using the haem precursor protoporphyrin IX (Ignarro *et al*., 1982). Measurements in freshly isolated rat platelets indicate that about 2% of the protein is haem-deficient, but engaging this small proportion was enough to elicit a physiological response, in this case downstream cGMP-dependent protein phosphorylation (Roy *et al*., 2008b). Compatible with this finding, while the NO EC_{50} for cGMP generation in rat platelets was 10 nM, the curve for cGMP-dependent protein phosphorylation was shifted to the left by 20-fold (EC_{50} = 0.5 nM), results that, along with those from vascular smooth muscle (Mergia *et al*., 2006; Mullershausen *et al*., 2006), emphasize the low degree of NO receptor activity needed to elicit physiological effects. The proportion of haem-free protein may increase in pathological conditions, opening new avenues for therapeutic intervention using haem-mimetic compounds (Stasch *et al*., 2006).

Lastly, a quantitative understanding of how cells decode incoming signals provides a powerful insight into the language of chemical communication and helps identify abnormalities in disease states. To this end, we have developed a formal enzyme-linked receptor mechanism for NO-activated GC based on measurements of the activity of the purified protein under constant, known NO concentrations (Roy *et al*., 2008a). The model accurately replicates all the main functional properties of the protein and provides a thermodynamically sound interpretation of those properties. With minor modifications, the scheme can be used to simulate NO signal transduction in cells where, as mentioned, half-maximal GC activity occurs at 10 nM NO. By using a fluorescent cGMP biosensor, however, cells equipped with NO-activated GC are seen to respond to NO concentrations two to four orders of magnitude lower, even when delivered in the form of brief pulses, with the absolute sensitivity dependent on the level of cGMP **PDE** (Batchelor *et al*., 2010). Although highly unusual for a conventional receptor, responsiveness to such low agonist concentrations is predicted from its enzyme-linked properties which enable it to function most efficiently at low agonist concentrations. For example, based on the receptor model, and in line with experimental findings, 10 pM NO is calculated to produce 10–50 nM cGMP each second in cells (Batchelor *et al*., 2010), a result consistent with multiple independent lines of evidence that physiological NO concentrations lie in the pM to low nM concentration range (Hall and Garthwaite, 2009).

Multiple NO sources and targets in brain

Anatomical studies of nNOS and its targets indicated huge diversity in the possible modes of operation of this signalling pathway. Although nNOS is apparently only present in neurones, there is no consistent pattern of its expression in particular neuronal types, being located in excitatory neurones in some areas and inhibitory interneurones in others. In some brain areas, like the cerebellum, nNOS is found in the majority of neurones (excitatory granule cells and inhibitory interneurones, but not Purkinje cells) whereas in others, like the striatum and cerebral cortex, it is concentrated in discrete interneurone populations comprising only a few percentage of the total but having dense connectivity with other neurones in the region (Vincent and Hope, 1992). A report did appear that **endothelial NOS (eNOS)** is expressed in some brain neurones (Dinerman *et al*., 1994), but this conclusion was later repudiated as an immunohistochemical artefact (Blackshaw *et al*., 2003). Instead, our studies in the rat optic nerve, which lacks synapses, found that eNOS present in blood vessels dynamically signals to the axons to affect their membrane potential and, hence, excitability (Garthwaite *et al*., 2006). The mechanism here depends on the physical proximity between the meshwork of eNOS-expressing microvessels and the axons passing through, with NO eliciting the synthesis of axonal cGMP which acts **on hyperpolarization-activated cyclic nucleotide–gated (HCN) ion channels** to cause membrane depolarization (Figure 2). Of course, microvessels in the brain are always in close proximity to the neural and glial elements and subsequent research on synaptic plasticity in the hippocampus similarly identified eNOS to be the origin of the tonic, low level NO concentrations that, in addition to the acute NO pulse brought about by NMDA receptor-mediated activation of nNOS, is required for LTP of synaptic transmission in this brain region (Hopper and Garthwaite, 2006). Hence, eNOS provides a ubiquitous source of NO throughout the brain, and its absence may explain several neurological and behavioural deficiencies observed in eNOS-knockout mice (Demas *et al*., 1999; Frisch *et al*., 2000). A deficiency in vascular NO signalling to the brain parenchyma may contribute to the pathogenesis of Alzheimer's disease (Austin *et al*., 2013).

Our original finding in the cerebellum that astrocytes constituted a major target for neurone-derived NO is simply explained by cerebellar astrocytes having a very low level of cGMP-hydrolysing PDE enzymes, so that cGMP in these cells can rise to very high levels (approaching millimolar) on exposure to NO (Bellamy and Garthwaite, 2001). Because of this

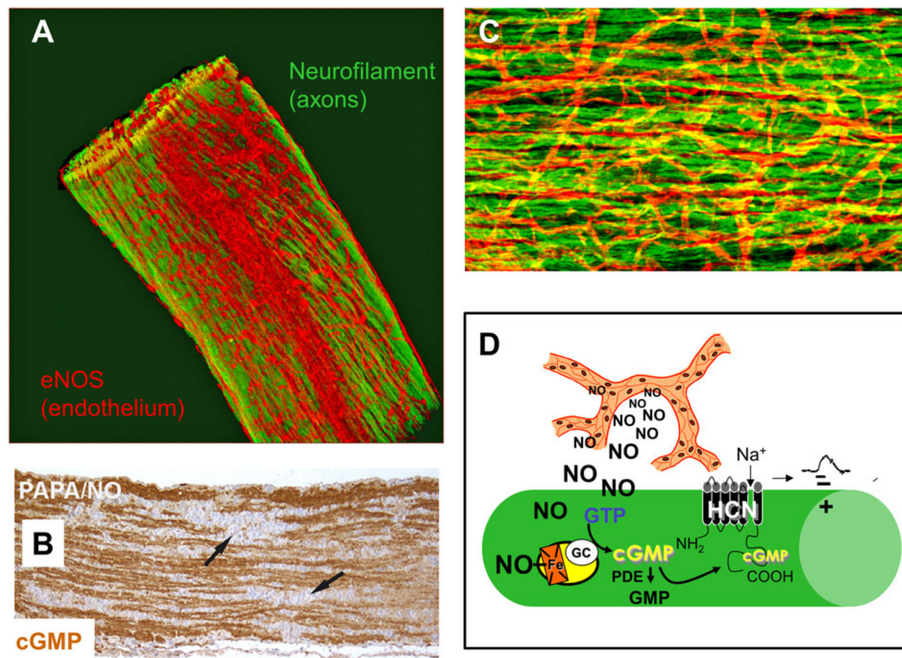

Figure 2

NO signalling from capillaries to axons in optic nerve. (A) Whole-mount preparation of rat optic nerve co-stained for eNOS (red), which labels blood vessels, and neurofilament (green) which labels axons. Image kindly provided by Dr G. Garthwaite. (B) Section of optic nerve immunostained for cGMP after exposure to the NO donor (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate (PAPA/NO, 30 μM, 5 min). Axons show cGMP accumulation (brown) whereas glial cells (arrows) are unstained (Garthwaite *et al.*, 2006). (C) Slice through the specimen shown in (A) showing the intermingled eNOS-expressing microvessels (red) and axons (green). (D) Diagram showing how NO from eNOS in the capillary circulation persistently depolarizes optic nerve axons by raising the level of cGMP, which engages hyperpolarization-activated, cyclic nucleotide-regulated (HCN) channels (Bartus *et al.*, 2007).

property, astrocytes dominate the overall tissue response quantitatively. cGMP immunohistochemistry, likewise, selectively stains cells in which cGMP rises to relatively high levels because the detection limit of the antibody is around 10 μM cGMP, explaining the preferential accumulation of the nucleotide in cerebellar astrocytes using this technique (see above). In alternative approaches, subcellular fractionation techniques showed that NO-activated GC activity is concentrated in synaptic elements (Deguchi *et al.*, 1976; Russwurm *et al.*, 2001), and *in situ* hybridization methodology showed a widespread distribution of the protein RNA in neurones (Matsuoka *et al.*, 1992; Gibb and Garthwaite, 2001), as does immunohistochemistry for the common NO receptor β1-subunit (Ding *et al.*, 2004). When steps are taken to boost its accumulation pharmacologically in slices of the hippocampus, cGMP is seen by immunohistochemistry to have a far wider distribution than was originally evident, with the main classes of pyramidal neurone, interneurones and axons, as well as astrocytes and blood vessels, being prominently labelled (Bartus *et al.*, 2013). As an example, Figure 3 shows the NO-induced accumulation of cGMP in various neurones in a hippocampal pyramidal cell layer. Moreover, in corpus callosum, cerebellum, cerebral cortex and brainstem, oligodendrocytes, which are the cells that wrap axons in myelin, are also targets for NO, as judged by cGMP immunohistochemistry (Garthwaite *et al.*, 2015).

The overall picture to emerge is that, while the sources of NO in any brain area may, in some cases, be relatively limited, the potential targets are a heterogeneous mixture of neuronal and non-neuronal cells, prompting questions as to the circumstances under which these various targets are accessed by endogenously generated NO.

Spread of NO signals from its sources

In the absence of direct evidence, computer modelling provides a conceptual framework for understanding how these various targets might be accessed by neuronal or endothelial sources of NO.

The simplest place to start is with the single synapse under conditions of physiological NMDA receptor stimulation. Electrophysiological investigations suggest that only three to five NMDA receptors are activated under these conditions. If there is a single nNOS molecule associated with each NMDA receptor and it manufactures maximally 10 NO molecules·s^{-1}, then the overall synaptic output of NO corresponds, at best, to 40 molecules·s^{-1}. If the source structure is treated as a disc having a diameter of 400 nm, which corresponds roughly to the shape and size of the postsynaptic density, the resulting spread of NO can be computed and visualized (Garthwaite, 2016). With the time course of NO production set to resemble an NMDA-receptor-mediated synaptic potential, peaking after 40 ms, the maximum NO concentration (in the middle of the source) amounts to only about 60 pM, and it falls steeply on either side such that the signal would be predominantly confined to the dimensions of that individual synapse (Figure 4A). To evaluate the

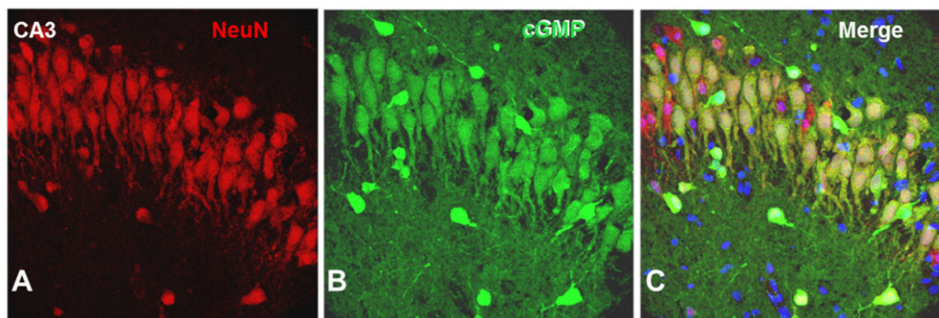

Figure 3

Neuronal targets for NO in hippocampus. Slices of rat hippocampus were exposed for 2 min to the NO donor diethylamine NONOate (10 μM) in the presence of the PDE-2 inhibitor BAY 60-7550 (1 μM) and the allosteric enhancer of NO-activated GC, BAY 41-2272 (10 μM). After fixation, sections were stained for the neuronal marker NeuN (A) and cGMP (B). The images show the CA3 pyramidal layer and are merged in (C) in which nuclei are stained blue using DAPI. Modified from Bartus *et al.*, (2013).

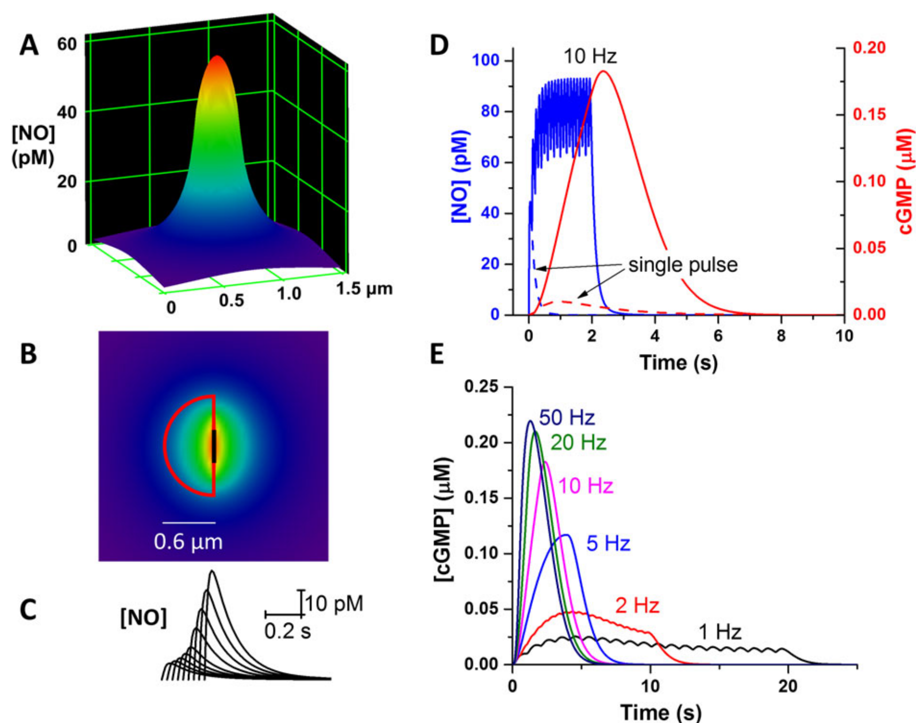

Figure 4

Compartmental model of synaptic NO signal transduction. (A) Plume of NO when generated at a rate of 40 molecules·s^{-1} from a disc of 400 nm diameter, at steady-state. (B) The plume is compressed into two dimensions, superimposed on which is a hemispheric target structure, approximately the size of a presynaptic bouton or dendritic spine head. The black line in the middle signifies the NO-generating zone. For computation purposes, the area is subdivided into multiple concentric hemispheres (not shown). (C) The shape of an applied NO pulse as it diffuses through the target compartments. (D) Inserting NO-activated GC and PDE-5 into the target compartments permits the cGMP concentration to be calculated. The graph shows responses to a single NO pulse and to a train of 20 pulses delivered at 10 Hz. The NO profiles are from the central compartment (adjacent to the source) whereas the cGMP profiles are the same in all target compartments because of the rapid intracellular diffusion of cGMP. (E) Relationship between cGMP generation and frequency of delivery of 20 NO pulses. The fade of the responses seen at 1 and 2 Hz is caused by the time-dependent activation of PDE-5 by cGMP. For full details, see Garthwaite (2016).

consequences of such a signal, the resultant production of cGMP needs to be computed. This problem can be tackled with the help of a compartmental model of the synapse, with the target structure designated as a hemisphere of radius 0.6 μm (Figure 4B). Within the target, the amplitude of the NO pulse diminishes with distance from the source, as expected (Figure 4C). Assuming contents of NO receptor GC and cGMP PDE similar to those found naturally in cells and

activation kinetics derived from our enzyme-linked receptor model (Batchelor *et al*., 2010), it can be seen that a single transient pulse of NO would only generate about 10 nM cGMP in the adjacent target structure (Figure 4D), a concentration probably too low to engage downstream mechanisms. Repeated stimulation, however, leads to temporal summation of the cGMP content, with just 20 pulses delivered over 2 s leading to the generation of almost 200 nM cGMP. As the affinity of cGMP for protein kinases is in the 100 nM range (Francis and Corbin, 1994; Vaandrager *et al*., 2005), a signal of this magnitude is likely to be functionally significant.

The simulations suggest that, at the level of the single synapse, NO is likely to operate in an activity-dependent manner, becoming effective only with repetitive synaptic stimulation. This conclusion is consistent with the role of NO in activity-dependent synaptic plasticity in the brain, which is typically elicited with trains of stimuli (Bliss and Collingridge, 1993), and with NO-mediated orthograde synaptic transmission observed between pairs of snail neurones where, again, repetitive firing of the presynaptic neurone is needed to elicit a response (Park *et al*., 1998). It also resonates with the properties of nitrergic (NANC) transmission in the periphery, where high frequency nerve stimulation is required to elicit smooth muscle relaxation. Indeed, the computed frequency-dependence of cGMP accumulation, peaking at around 20 Hz (Figure 4E) accurately replicates the frequency-response curves typically observed for nitrergic transmission (Rand, 1992).

The picture of how NO distributes at an individual synapse when synthesized within the postsynaptic density gives the impression of a largely synapse-specific signal (Figure 4A, B), but other elements are potentially within range. In particular, the packing density of synapses in the brain means that they are only, on average, about 0.5 μm apart, with some almost touching each other (Rusakov *et al*., 1999; Merchan-Perez *et al*., 2014), suggesting the feasibility of NO-mediated synaptic crosstalk. Moreover, the crosstalk may be between different classes of synapse. For example, in the hippocampus, inhibitory GABAergic nerve terminals can be less than 0.2 μm away from excitatory glutamatergic synapses (Merchan-Perez *et al*., 2009). High levels of nNOS can be found in GABAergic nerve terminals (Aoki *et al*., 1997; Fuentealba *et al*., 2008), raising the possibility of activity in inhibitory circuits being able to influence the functioning of excitatory circuits inter-synaptically through NO.

Volume transmission through NO

By virtue of its physicochemical properties, NO has long been considered a potential 'volume' transmitter, able to accumulate in tissues volumes sufficiently to engage any target cells within it, irrespective of anatomical inter-connectivity (Agnati *et al*., 2010). This potential type of transmission held immediate attraction for theoreticians as a mechanism by which neuronal activity could be coordinated over space and time, with specific implications for the generation of long-term modifications to synaptic function, the control of cerebral blood flow and the establishment and activity-dependent refinement of axonal projections during the later stages of development (Gally *et al*., 1990). Seemingly at odds with the idea of NO being a volume transmitter is the predicted concentration profile of NO around a single active synapse, which suggests a very local signal (Figure 4A, B), but the concentration outside the structure is not negligible (about 5 pM at a 1 μm distance from the source) and, provided there are sufficient sources in close proximity, the intervening NO concentrations summate, ultimately producing a cloud of NO bathing the active regions of the tissue. With an array of small, synapse-sized structures (spheres of diameter 0.4 μm) generating NO from their surfaces, they would need to be less than about 4 μm from each other to enable a volume-type signal whereas if whole cells (diameter 15 μm) were the sources, separation distances less than about 50 μm would be sufficient to provide summated intercellular NO concentrations within the presumed active range (Bellefontaine *et al*., 2014; Garthwaite, 2016). These simulations, therefore, add plausibility to the idea of NO acting as a volume transmitter, but there is one ingredient not hitherto mentioned that needs to be incorporated: a high rate of NO inactivation. If NO is only inactivated slowly, the intercellular NO concentration would not discriminate between a sparse and more dense population of active sources nor would it respond dynamically to alterations in source activity. A suitably high rate of NO inactivation that confers good discrimination and dynamics corresponds to an inactivation rate constant of 100–150 s^{-1} (Bellefontaine *et al*., 2014) which is equivalent to a half-life of 5–7 ms, neatly matching the estimate made for rat cerebellum (Hall and Garthwaite, 2006). An inactivation rate of this magnitude endows constituent cells with a modality switch, permitting local signalling when their activity is sparse or uncoordinated, and a volume signal when there is synchronous population activity.

Having NO serving as a volume transmitter in some situations helps understand why there are multiple NO receptive cells within the brain, often located at a distance from synapses. A good example is provided by oligodendrocytes, the myelinating cells of the CNS, that were found to be targets for NO in several brain areas (Garthwaite *et al*., 2015). Exposing cultured oligodendrocytes to NO leads to a remarkable, cGMP-dependent increase in their growth and arborisation, leading to the hypothesis that a volume NO signal would serve to regulate the myelination of axons by these cells according to the ongoing levels of neuronal activity. Consistent with this hypothesis, inhibition of NOS *in vivo* in the developing rodent reduced myelin density in a major white matter tract (the corpus callosum), an effect that could be overcome by administering inhaled NO, which itself enhanced myelination (Olivier *et al*., 2010).

Another widespread non-neuronal NO target cell, the astrocyte, assists the homeostasis of neurones in many different ways, for example, by clearing the extracellular fluid of neurotransmitters and ions. NO impinges on several astrocytic properties, including the generation of Ca^{2+} transients, cytoskeletal dynamics and the motility of filopodia (Willmott *et al*., 2000; Boran and Garcia, 2007; Sild *et al*., 2016) that, in turn, are likely to have repercussions on synaptic function and maturation. Although in certain situations, astrocyte processes are intimately associated with synapses and so could be the recipients of very local NO signals, it is also probable that, like oligodendrocytes, they react to more global signals with appropriate adjustments to their morphology and function.

The activity of other neurones may also to be subject to volume NO signalling. A good example here is in the preoptic region of the hypothalamus where, according to recent evidence, the hormone leptin evokes widespread activity of the nNOS-expressing neurones, enabling sufficient build-up of intercellular NO concentrations to affect interspersed neuroendocrine neurones. These neurones then release **gonadotrophin-releasing hormone** which ultimately affects gonadal function by releasing **luteinizing** and **follicle-stimulating hormones** from the pituitary gland. In this way, NO helps regulate the onset of puberty and adult fertility (Bellefontaine *et al.*, 2014).

Lastly, although some blood vessels are innervated by individual nNOS-containing axons, the more general increase in blood flow seen with increased neuronal activity (neurovascular coupling) is likely to depend more on generalized neuronal NO formation and to be exerted at the microvascular level through modified smooth muscle cells or pericytes (Attwell *et al.*, 2010). At this level, the vascular system is closely intermingled with the brain neural elements, making it a likely recipient of coordinated neuronal NO synthesis. In accordance, a recent investigation in the cerebellum found that repetitive synaptic activation of granule cells resulted in a rapid and pronounced vasodilation in the region that was dependent on NMDA receptors, NO formation and cGMP (Mapelli *et al.*, 2017). Conversely, the tight network of capillaries is as well placed to supply eNOS-derived NO to the brain parenchyma as it is to supply O_2. In support of this role, computer simulations show how an analogous dense NO-generating network of fibre-like structures is able to produce a volume NO signal when active, a signal that could engage heterogeneous targets to influence brain function (Philippides *et al.*, 2005).

Therapeutic opportunities

Given the propensity of NO in high concentrations to cause cellular damage, an initial impetus was in investigating its participation in neurodegenerative disorders, particularly in the context of 'excitotoxicity', which refers to neuronal loss provoked by excessive activation of glutamate receptors. The influx of Ca^{2+} through NMDA-receptor channels transpired to be the main driver of glutamate neurotoxicity, and the protective effect of NMDA antagonists in conditions of cerebral ischaemia *in vivo* lent weight to the mechanism being of pathological importance (Meldrum and Garthwaite, 1990; Choi, 1992). Initial experiments aimed at investigating the role of NO in NMDA- or glutamate-mediated neurodegeneration gave mixed results, but some clarity emerged from the use of mice lacking individual NOSs subjected to stroke-like ischaemia *in vivo*. The results indicated that nNOS contributed to the early damage whereas eNOS was protective, reflecting its importance in cerebral blood flow and, possibly also, in inhibiting both leucocyte adhesion to the endothelium and platelet aggregation. The inducible isoform **(iNOS),** which becomes expressed in the days following arterial occlusion, appeared to provide additional damage (Iadecola, 1997; Huang, 2000). Translation of these findings to the clinic has not been attempted, partly because of the absence of suitably selective nNOS inhibitors and partly because multiple failures of NMDA antagonists in stroke trials, despite their multiple shortcomings, dampened enthusiasm for pursuing this indication (Ginsberg, 2009). Moreover, several attempts to improve cardiovascular function in stroke patients using the NO donor **glyceryl trinitrate** failed to alter clinical outcome (Bath *et al.*, 2017).

In the context of neurodegeneration, the involvement of NO in many chronic disorders (e.g. Alzheimer's and Parkinson's diseases) is being actively studied at the laboratory level, and some novel therapeutic approaches are emerging (Ben *et al.*, 2016). The expression of iNOS is often considered potentially deleterious because this isoform can generate NO continuously. In the CNS, several lines of circumstantial evidence pointed to a role for iNOS in the pathology of multiple sclerosis, but laboratory results using iNOS inhibitors or animals deficient in iNOS failed to provide a convincing case for clinical progression, possibly because of the concomitant loss of beneficial effects of NO, for example, in limiting the immune response and in quenching noxious free radicals (Smith and Lassmann, 2002). Likewise, in traumatic brain injury, despite circumstantial evidence for iNOS contributing to the damage, functional impairment was worsened in iNOS knockout mice, an effect apparently related to enhanced oxidative stress (Bayir *et al.*, 2005).

Based on preclinical studies, iNOS had also been considered a potential drug target for the treatment of migraine, but clinical trials using the selective iNOS inhibitor **GW274150** failed to show efficacy (Pradhan *et al.*, 2018). This negative result is unlikely to halt interest in targeting NO signalling in migraine, however, because this indication presents probably the most compelling evidence for an involvement of NO signalling in any neurological disorder. The evidence stretches back more than 170 years to when glyceryl trinitrate (or nitroglycerine) was first synthesized and found to produce violent headache, a finding amply confirmed when the compound later became used clinically for the treatment of angina and by the explosives industry in the production of dynamite (Tfelt-Hansen and Tfelt-Hansen, 2009). Glyceryl trinitrate is now commonly used for triggering migraine attacks in humans, and in migraine sufferers, it causes both acute and delayed headache that fulfil the criteria for authentic migraine. That endogenous NO is intimately linked to migraine is supported by clinical evidence that non-selective NOS inhibition with L-methylarginine is an effective treatment and that **sildenafil**, an inhibitor of the cGMP-metabolizing enzyme **PDE-5**, induces migraine in sufferers (Olesen, 2010), consistent with NO signal transduction through cGMP in this condition. The most likely source of the NO is nNOS, which is found throughout the migraine pain pathway and has been implicated by several animal studies (Pradhan *et al.*, 2018), but the lack of selective nNOS inhibitors represents a major setback in pursuing this target further. An alternative and perhaps more feasible option would be to tackle downstream NO signalling, particularly in the light of clinical results with sildenafil (see above) and with animal studies showing that blockade of NO-activated GC with ODQ inhibited all phases of the hyperalgesia induced by glyceryl trinitrate, including pre-established hyperalgesia (Ben *et al.*, 2018), a result consistent with our earlier findings with locally delivered ODQ in a model of neuropathic pain (Salter *et al.*, 1996).

In contrast, enhancing NO signalling may be of benefit in other disorders. In glaucoma, for example, arguments have been made that activation of the NO-cGMP pathway to promote reduction in intraocular pressure, increase ocular blood flow and confer neuroprotection, would provide a novel therapeutic approach for this disease, which is characterized by degeneration of retinal ganglion cells and their axons in the optic nerve (Wareham *et al.*, 2018). Psychiatric disorders may also benefit from augmenting NO signalling. Three broad lines of evidence support this proposal. First, inhibition of NMDA receptors with non-competitive antagonists such as **phencyclidine**, **ketamine** and **dizocilpine (MK-801)** induces schizophrenia-like symptoms in humans, indicating a possible hypofunctioning of NMDA receptors in this condition (Javitt, 2007). Secondly, genetic and pharmacological studies in animals indicated that loss of NO has several behavioural consequences, including impulsivity, aggression and symptoms considered relevant to anxiety, depression and cognitive performance (Freudenberg *et al.*, 2015). Finally, genetic studies in humans have identified nNOS and an associated adaptor protein (NOS1AP) as risk factors in schizophrenia and have suggested that reduction in NO signalling in the prefrontal cortex may be causally involved (Freudenberg *et al.*, 2015). There have been few clinical studies following up these findings, although a pilot investigation found that a single intravenous administration of the NO-releaser, nitroprusside, gave an impressive degree of improvement in psychotic symptoms that was maintained for several weeks (Hallak *et al.*, 2013). A similar small study on patients with more established disease, however, failed to see any clear effect (Stone *et al.*, 2016).

Concluding remarks

The past 20 years have seen major changes in our understanding of the NO-cGMP signalling pathway, both in terms of its overall effects on many different overall brain functions and in the details of how it operates. From the viewpoint of a neurophysiologist/neuropharmacologist, it has been gratifying that NO-mediated transmission can be at last be viewed on a quantitative footing which, for conventional neurotransmission, has long been considered critical to progress. There turn out to be unexpected parallels. While at one time conventional neurotransmission was viewed as a strictly private, point-to-point mode of information transfer at synapses, increasing evidence supports a more multimodal operation in which neurotransmitter released at one synapse can also influence other nearby synapses (synaptic crosstalk) and, should there be several active synapses in a given area, a volume-type signal that may mediate non-synaptic transmission or activation of receptors on astrocytes can be generated (Okubo *et al.*, 2010). Of course, by being able to diffuse through cell membranes, rather than be restricted by them, the three-dimensional spread of synaptic NO is facilitated compared with conventional synaptic neurotransmitters. To register the lower concentrations reaching them, receptors at extrasynaptic locations often have a higher affinity for neurotransmitters than synaptic receptors, but in the case of NO, the only known receptors are similarly sensitive to NO (Russwurm *et al.*, 1998; Gibb *et al.*, 2003). Unlike conventional neurotransmitter receptors, however, the GC-coupled NO receptors can detect a remarkable range of NO concentrations, from low picomolar to mid nanomolar, with graded cGMP output (Batchelor *et al.*, 2010). The level and type of cGMP PDE expressed are likely to be key determinants of NO sensitivity, with lowered PDE activity allowing cGMP to accumulate effectively in targets distant from the NO sources. Perhaps the **α2β1 NO receptor GC (GC-2)** found associated with synaptic scaffold proteins in the brain (Russwurm *et al.*, 2001) preferentially responds to discrete synaptic signals whereas the other (α1β1) isoform, having a broader cytosolic distribution, transduces a more diffuse, volume-type transmission.

In the coming two decades, it can be anticipated that a more precise understanding of these and many other unknowns will surface. One of the biggest unknowns is the mechanism that consumes NO so avidly in brain tissue, giving it an estimated half-life of only about 5 ms (Hall and Garthwaite, 2006). In our hands, the major stumbling block to identify the mechanism was that the NO-consuming activity disappeared after homogenizing the tissue, although some evidence for the participation of cytochrome P450 reductase was obtained (Hall *et al.*, 2009). Deficiency in the operation of this mechanism represents the most likely scenario for NO concentrations to rise to pathological levels (potentially, 100 nM range and above). An ultimate goal must be to visualize NO signalling in the brain in real time and within appropriately small dimensions *in vivo*. Promising new methods for the direct imaging of NO itself in cells using genetically encoded biosensors are being developed (Eroglu *et al.*, 2017), and it has also become possible to image cGMP (Gorshkov and Zhang, 2014) and the engagement of downstream transduction pathways (Mo *et al.*, 2017) in real time. These types of approaches, together with the ability to activate individual NOS-expressing neurones relatively non-invasively using optogenetic methods, are likely to yield radical new insights into the many questions still surrounding NO-mediated transmission in the brain.

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).

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Conflict of interest

The author declares no conflicts of interest.

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