Gases as neurotransmitters

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Introduction

A decade ago, who would have believed that two highly toxic, environmentally polluting gases found in the exhaust emissions of cars could be candidates as important signalling molecules in the body? Nevertheless, we reach the late 1990s in just such a position, with nitric oxide (NO) and carbon monoxide (CO) being implicated in many cellular functions, ranging from control of vascular tone and penile erection to mediating pain and learning. Biologists first became aware of this new class of gaseous mediators in 1987, when a vasodilator produced by endothelial cells, termed ‘endothelium-derived relaxing factor’ (EDRF) [1], was identified as being the very simple molecule NO [2]. The following year Garthwaite and colleagues created an entirely new area of research when they suggested that, far from being restricted to the vasculature, EDRF (NO) may also be generated in the central nervous system (CNS) and mediate glutamate receptor stimulation of cGMP production in the cerebellum [3]. Following these two discoveries, the number of scientific publications on NO rose dramatically from less than 100 in 1987 and 1988 to 4304 in 1996! NO has now been implicated in many processes, including smooth muscle relaxation following activation of peripheral non-adrenergic non-cholinergic nerves innervating the anococcygeus and retractor penis muscles, where NO or an NO-like molecule may be a neurotransmitter [4]. NO may also be involved in macrophage cytotoxicity, synaptic plasticity in the CNS and neurotoxicity. It is clear, therefore, that this review cannot possibly cover all aspects of NO and CO function. Instead I will focus on just two areas of CNS research where NO and CO have been investigated: in the generation of long-term potentiation (LTP) in the hippocampus, a cellular model
for some types of learning, and in the processing of nociceptive (pain) signals in the spinal cord.

Why is everyone so interested in these gases?

Although gaseous in the atmosphere, these molecules do not exist as gases within the body, but are instead dissolved within the cell cytosol or extracellular fluid. Being readily soluble but also highly lipophilic, these molecules can pass easily through the cell membrane following their synthesis, which occurs only when they are required. They therefore differ fundamentally from classical neurotransmitters, which are not membrane permeable, are synthesized in advance and are stored in vesicles awaiting release following calcium influx into the presynaptic terminal. This ability to diffuse out of cells allows NO and CO to be ‘released’ from any part of the cell (e.g. from a postsynaptic cell dendrite or cell body), whereas traditional neurotransmitters require the vesicular release machinery present in nerve terminals. In addition, NO and CO can also access the intracellular environment of nearby cells by membrane diffusion, interacting directly with enzymes without needing an extracellular receptor. This provides a flexibility that is not achieved with classical neurotransmitters, and ‘transmission’ can occur in non-classical directions, e.g. from postsynaptic cell to presynaptic cell or from postsynaptic cell to neighbouring postsynaptic cells. NO has the additional property of being short-lived in vivo (the free radical is readily oxidized) with a half-life of about 5 s, thus restricting the region of diffusion and conferring some selectivity of action. CO, however, is much more stable and could, in theory, diffuse greater distances.

NO and CO are formed by enzymes

Structure, regulation and activation of NO synthase (NOS) and haem oxygenase (HO)-2

NOS, which generates NO and citrulline from L-arginine, was cloned in 1991 and is now known to consist of three main isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Figure 1) [5]. NOS is closely related to cytochrome P-450 reductase and appears to be an elongated form of this enzyme. It has the same cofactor-binding sites, but contains some additional binding domains, one of which is for the calcium-binding protein calmodulin (Figure 1). This confers calcium sensitivity to eNOS and nNOS, immediately raising the possibility that they may participate in neuronal signalling. iNOS also contains a calmodulin-binding domain, but is activated in a calcium-insensitive manner. All the NOS isoforms also contain a haem-binding domain near the N-terminus and this could have important implications for inhibitor selectivity, as I shall discuss below. Both nNOS and eNOS contain phosphorylation sites and have the potential to be regulated by calcium/calmodulin-dependent kinase II and protein kinases A, C and G. eNOS has an N-terminal
myristoylation site which is absent from the other isoforms (Figure 1) [5]. Myristoylation sites are found in some proteins that are associated with the plasma membrane, such as G-proteins, and it is thought that myristoylation is required for membrane association. It seems likely, therefore, that eNOS is, or can be, associated with membranes. Indeed, a recent study involving the expression of myristoylation-deficient forms of eNOS seems to confirm this interpretation and suggests that membrane association is required for eNOS to participate in cellular mechanisms [6]. nNOS may also be associated with plasma membrane proteins, but via a different mechanism, as it is able to interact with a postsynaptic density protein (PSD93) which can also interact with the N-methyl-D-aspartate (NMDA) glutamate receptor [7]. It is possible, therefore, that PSD93 could interact simultaneously with nNOS and the NMDA receptor to form large postsynaptic complexes in which the calcium source (NMDA receptor) is coupled tightly to the calcium-sensitive enzyme (nNOS) (see later). iNOS was cloned originally from macrophages and is fundamentally different from eNOS and nNOS as, following cellular stimulation, the enzyme has to be synthesized before it can be activated [5].

Inhibitors of NOS generally act as false substrates, competing for the L-arginine-binding site. While they are specific for NOS, there is little specificity between the various NOS isoforms [8]. A new generation of inhibitors has recently been developed that are derivatives of indazole and are thought to interact with the haem group on NOS. Although these inhibitors are also poorly selective between nNOS and eNOS, one has proved to be a very useful research tool. Although 7-nitroindazole inhibits both eNOS and nNOS in vitro, it is unable to inhibit eNOS in intact endothelial cells (how this occurs is

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**Figure 1. Schematic drawing of the structures of the three classes of NOS: nNOS, eNOS and iNOS**

All three isoenzymes contain binding sites for the calcium-sensitive protein calmodulin (CaM) and for haem (Hm). They require NADPH, and bind FMN and FAD tightly. eNOS is the only isoenzyme that contains a myristoylation site (Myr), and this results in association of the enzyme with the plasma membrane. Both nNOS and eNOS contain phosphorylation sites for protein kinase A (circled P), and nNOS activity can be regulated by calcium/calmodulin-dependent kinase II and protein kinases A and G [5].
poorly understood) and does not, therefore, elevate blood pressure in intact animals, unlike most NOS inhibitors [8]. The selectivity of inhibitors for iNOS is rather better, and several inhibitors are available that do not interact with eNOS/nNOS [8].

CO is a byproduct of the breakdown of haem to biliverdin, catalysed by HO. As with NOS, there is an inducible form of HO (HO-1) and a constitutive form (HO-2), both of which contain a haem-binding pocket [9]. The problem with postulating CO involvement in neuronal function is the apparent lack of an activation signal. NOS contains a calmodulin-binding domain which confers calcium sensitivity to the enzyme, and it is therefore easy to see how NOS may become involved in synaptic modulation. HO-2, however, does not possess such a site; this issue has been largely ignored and really needs to be addressed. Inhibitors of HO also act as false substrates, mimicking the natural substrate haem and competing at the haem-binding site. The inhibitors of HO are not very selective, being able to interact with and inhibit other enzymes containing haem-binding sites, such as NOS and guanylate cyclase (GC) [9].

Localization of NOS and HO-2 within the CNS

Despite the nomenclature used, eNOS is not restricted to endothelial cells, or even to the periphery, and there is now evidence that it can exist in neurons within the CNS, often in the same cell populations as nNOS. Both eNOS and nNOS are present in the olfactory bulb, caudate–putamen, supra-optic nucleus and cerebellum. In the hippocampus eNOS is found in pyramidal neurons, while nNOS is present predominately in interneurons (where its function is not known); it is also present in pyramidal cells, although less abundantly than eNOS [10,11]. Within the spinal cord nNOS is present in the superficial layers of the dorsal horn, where the afferent sensory fibres terminate, and around the central canal in lamina X, which also receives some afferent input [12].

The HO-1 isoform is less abundant in the brain than HO-2, which has a widespread distribution and is present in abundance within the olfactory bulb, cerebellum, brainstem and pyramidal neurons of the hippocampus [9]. As so often happens, the spinal cord has not been included in studies examining the distribution of HO-2 in the brain, and it is not known whether this enzyme is present within the dorsal horn.

What are the targets for NO and CO?

The targets for NO and CO within the CNS are probably very similar, as both molecules can interact with haem moieties. Many enzymes contain haem-binding domains and can become targets for CO and NO. Most extensively studied is GC, the activation of which results in cGMP production. Both NO and CO are activators of GC, although NO is more effective than CO [9]. cGMP has many actions in the CNS: it can open ion channels in the retina, and
can activate a cGMP-dependent protein kinase (protein kinase G) and a cGMP-stimulated cyclic nucleotide phosphodiesterase (which hydrolyses cAMP and cGMP) [3]. NO can also activate other enzymes, such as cyclooxygenase (which also contains a haem moiety), and can stimulate auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase [3,13]. In addition to activating enzymes, NO (and CO) can also inhibit NOS activity by binding at the haem domain (Figure 1), providing (at least in the case of NO) a negative-feedback system. Apart from its effects on enzymes, NO can also inhibit the NMDA receptor by interacting with an extracellular redox site [13]. Given that NOS and HO-2 are found in abundance in the CNS, it seems likely that more NO and CO targets are waiting to be discovered.

LTP in the hippocampus

Why a diffusible messenger is postulated

LTP is a phenomenon that occurs at certain glutamatergic synapses in the CNS, including the CA3 to CA1 synapse in the hippocampus (Figure 2), and is a cellular model for learning and memory. It is induced by high-frequency tetanic stimulation, and also by depolarization of the postsynaptic cell coupled with low-frequency stimulation of the presynaptic fibres (‘paired depolarization’). Following tetanus or paired depolarization there is an increase in the postsynaptic response of the cell. This is observed as an increase in both the amplitude and the speed of depolarization of the excitatory postsynaptic potential (Figure 3), which can last for hours [13]. LTP could result from either an increase in sensitivity of the postsynaptic cell to the neurotransmitter or an increase in neurotransmitter release from the presynaptic cell. There has been controversy over whether a presynaptic or a postsynaptic mechanism is involved in LTP at the CA3 to CA1 pyramidal cell synapse in the hippocampus (the most extensively studied synapse in LTP). Many groups, however, now accept that presynaptic changes can contribute to LTP at this synapse. This leads to a theoretical problem in understanding how LTP is generated, as the activation of postsynaptic NMDA glutamate receptors, and subsequent influx of calcium through this ligand-gated channel, is an absolute requirement for LTP induction at this synapse. How, then, does the presynaptic nerve terminal ‘know’ when NMDA receptors have been activated on the postsynaptic cell? One explanation is that a ‘retrograde messenger’ is released from the postsynaptic cell and diffuses to the presynaptic nerve terminal. Until recently, it was thought the postsynaptic side did not possess release machinery. New evidence challenges this assumption, however, and suggests that exocytosis mechanisms may exist at postsynaptic sites after all. This mechanism may not result in transmitter release, however, but could instead be responsible for inserting new receptors into the postsynaptic membrane [14]. It is necessary, therefore, that any retrograde messenger must be able to diffuse through the plasma membrane and across to the presynaptic cell. There are a very small
Figure 3. LTP induction is prevented in the presence of NOS inhibitors
Tetanic stimulation (at arrow) of the CA3 to CA1 synapse results in an increase in the rising slope of the recorded field excitatory postsynaptic potential (EPSP; ●), which can last for several hours. Following tetanic stimulation in the presence of an NOS inhibitor (nitro-L-arginine; L-NO-Arg), there is an initial increase in the EPSP slope, but this is not sustained (○). Representative waveforms are displayed above; traces a and b were taken before the tetanus, and c and d were taken following tetanus, in the absence and presence respectively of the NOS inhibitor. Adapted, with permission, from Haley, J.E., Schiable, E., Pavlidis, P., Murdock, A. & Madison, D.V. (1998) Basal and apical synapses of CA1 pyramidal cells employ different LTP induction mechanisms. Learn. Mem. 3, 289–295. ©1998 Cold Spring Harbor Laboratory Press.
number of possible retrograde messengers (i.e. that have the necessary physical properties discussed above); these include the gaseous molecules NO and CO.

**NO may be the retrograde messenger**

Using inhibitors of NO and CO production, it was immediately clear that NO and CO are not actually ‘neurotransmitters’ in the hippocampus, as the inhibitors did not alter normal synaptic transmission. NO may, however, have a modulatory role in the CNS, and there is general agreement (although with a few notable exceptions) that NOS inhibitors prevent the induction of LTP in CA1 neurons following either tetanic stimulation or paired depolarization of CA3 axons [13] (Figures 2 and 3). Once LTP has been established, however, NOS inhibitors are without effect, suggesting that, although NO may participate in LTP induction, it is not involved in the maintenance of LTP.

Haemoglobin also blocks LTP induction in these cells, but must do so by binding NO or CO extracellularly, as it is so large that it cannot access the intracellular environment [13]. Once NO or CO has left the postsynaptic cell it could act at nearby presynaptic terminals or postsynaptic neurons. Evidence from dual recordings of hippocampal CA1 neurons suggests that such lateral diffusion does indeed occur, as paired depolarization of one neuron (leading to LTP) results in potentiation in a non-paired neighbouring postsynaptic neuron provided that they are close together (150 μm), an effect that is dependent on NO production [13]. NO may also act at the presynaptic terminal, and induction of LTP in single synaptic pairs in dissociated hippocampal cultures can be blocked by the presence of an NO-sequestering agent in the presynaptic cell [15]. Taken together, these data all indicate that NO is synthesized in the postsynaptic neuron following tetanic stimulation or paired depolarization, and participates in the induction of LTP which can be expressed both in neighbouring neurons and in presynaptic terminals. Moreover, although NO itself cannot induce synaptic potentiation, it can convert weak, non-LTP-inducing tetanic stimulation into long-lasting potentiation [13], indicating that NO may need to combine with some other signal, such as a rise in intracellular calcium, in the postsynaptic or presynaptic cell in order to generate LTP.

More recently, transgenic ‘knock-out’ mice have been generated that lack the nNOS or eNOS gene, or both. These studies have proved to be very revealing, as LTP induction in slices of hippocampus from nNOS knock-out mice appeared to be completely normal [10]. Furthermore, LTP induction in these mice could be blocked by NOS inhibitors. This was rather a surprise, as it was thought that nNOS was the only NOS isoform present in neurons. Subsequent staining with an anti-eNOS antibody, however, revealed high levels of eNOS in hippocampal pyramidal neurons from both the knock-out and wild-type mice [10]. This stunning result suggests that eNOS is present in pyramidal neurons and may be the isoform responsible, alone or in concert with nNOS, for NO production during LTP induction. Transgenic mice were then created that lacked both eNOS and nNOS (double knock-out), and LTP
was significantly reduced in hippocampal slices from these animals [11], providing strong evidence that LTP induction in hippocampal CA1 neurons requires the production of NO. Moreover, it is likely that the eNOS isoform may be dominant in LTP induction in normal hippocampal slices. Overexpression (via an adenovirus expression vector) of a myristoylation-deficient form of eNOS prevents the induction of LTP in CA1 neurons [6]. LTP can be induced, however, if the mutated eNOS is tagged with CD8 (a cell surface protein which inserts into the plasma membrane, dragging the eNOS along as well). Furthermore, LTP under these conditions can be blocked by NOS inhibitors. These data suggest that the eNOS isoform is sufficient to induce LTP and that the enzyme needs to be associated with the plasma membrane in order to function (Figure 4).

It is now becoming clear that NO production is not required for all forms of LTP in CA1 neurons and that NO-dependent and -independent forms of LTP exist. In the apical dendrites of the stratum radiatum (Figure 2), NO-independent LTP can be induced by altering the experimental recording conditions [13]. Furthermore, in the basal dendrites of the stratum oriens only NO-independent LTP is observed, despite the presynaptic neurons arising from the same source in the stratum oriens and the stratum radiatum [16]. This astonishing finding appears to arise from a differential distribution of eNOS in the postsynaptic CA1 pyramidal neuron; it is present in apical but not basal dendrites [10]. The implications arising from the difference in apical and basal LTP mechanisms are interesting, as the majority of the connections on to the stratum radiatum CA1 neuron arise from the ipsilateral hippocampus, while the connections in the stratum oriens arise mainly from the contralateral hippocampus. Is it possible, therefore, that these different LTP induction mechanisms could serve to distinguish between CA3 inputs from different sides of the brain?

Following the discovery that NO may be the retrograde messenger in LTP, interest has also focused on another gaseous molecule, CO. The evidence in support of CO involvement in LTP induction is, however, weak when compared with that for NO. The inhibitors of HO are not selective (see above) and, although a block of LTP induction is seen with some HO inhibitors [17], this does not correlate with their ability to decrease CO production from hippocampal slices [18]. Furthermore, LTP in knock-out mice lacking the HO-2 gene is normal and, although LTP induction in these mice can be prevented by a HO inhibitor [19], it is unlikely that compensation by other HO isoforms is occurring, as there is only one constitutive form of HO. It is more probable, therefore, that the inhibitor is acting on a non-HO target, possibly GC. On the available evidence it therefore seems unlikely that CO is a messenger in hippocampal LTP.

The mechanism by which NO contributes to the stabilization of LTP during induction is not really known. One target of NO is GC and, following tetanic stimulation, cGMP levels in hippocampal slices are increased.
Membrane-permeable cGMP analogues can rescue LTP that has been inhibited by NOS inhibitors [13], and in dissociated hippocampal cultures injection of cGMP into the presynaptic neuron results in LTP when paired with a weak non-LTP-inducing tetanus [20]. This suggests that cGMP production in the presynaptic terminal may be the means by which NO induces LTP (Figure 4). As with NO, some synaptic activity in the neuron is necessary to obtain long-lasting potentiation, as neither NO nor cGMP can elicit potentiation on their own. Another possible mechanism is that of NO-induced auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase (Figure 4). Following tetanic stimulation there is an increase in ADP-ribosylation in hippocampal slices, and inhibitors of mono-ADP-ribosyltransferase can also prevent the induction of LTP [13]. Regardless of whether the effects of NO occur through...
cGMP production or ADP-ribosylation, the final target (e.g. release machinery or receptor) remains unknown.

**Do NO and CO contribute to nociceptive signalling within the spinal cord?**

Hyperalgesia and sensitization within the spinal cord also involve the activation of NMDA receptors by glutamatergic sensory fibres and result in increased excitability of dorsal horn neurons. This can occur following peripheral inflammation produced by chemical agents such as formalin or carrageenan or by peripheral nerve damage, and can be mimicked by the intrathecal administration of NMDA. This form of plasticity within the spinal cord could occur during some chronic pain states, and the underlying mechanisms involved are therefore of considerable interest to clinicians and scientists. Given that both the induction of LTP and spinal sensitization appear to require NMDA receptor activation, it is possible that they have other mechanisms in common, perhaps including NO generation. NO is also unlikely to be a ‘neurotransmitter’ in the spinal cord, as intrathecal administration of NOS inhibitors does not alter acute nociceptive responses to heat or to mechanical or electrical stimulation [21]. NO may, however, be involved in the facilitation of responses caused by peripheral inflammation or nerve damage; following ligation of the sciatic nerve or the intrathecal administration of NMDA, mice appear hyperalgesic and withdraw their tail or paw much faster in response to applied heat or pressure. Intrathecal administration of NOS inhibitors prevents this sensitization from occurring, and the paw/tail withdrawal latencies are the same as for controls [21]. Intrathecal application of NO donor compounds also results in hyperalgesia, mimicking intrathecal NMDA administration. Spontaneous licking of the paw in response to a formalin injection (a response that results from the development of inflammation in the paw and activation of spinal NMDA receptors) is also prevented by NOS inhibitors [21]. These responses in behavioural studies are mirrored in electrophysiological studies, where the increase in dorsal horn neuron firing following injection of formalin into the paw can be prevented by the intrathecal application of NOS inhibitors. These findings each imply that NO within the spinal cord participates in the hyperalgesia generated by peripheral insults or the spinal application of NMDA. Furthermore, NO may diffuse to act on neighbouring cells and terminals, as intrathecal haemoglobin prevents the development of hyperalgesia following spinal NMDA administration [21]. NO may act on GC to elicit hyperalgesia, as GC inhibitors prevent the generation of hyperalgesia following peripheral nerve ligation or intrathecal NMDA administration [21], although it is not known whether cGMP alone can elicit hyperalgesia.

So, all the evidence from studies using NOS and GC inhibitors suggests that NO is produced, following NMDA receptor activation, as a result of peripheral nerve damage, chemical insult or direct application of intrathecal
NMDA agonists, and that NO may activate GC, resulting in hyperalgesia. Unfortunately, a study with nNOS knock-out mice has found that formalin-induced licking behaviour is not altered when nNOS is lacking [22]. Furthermore, NOS inhibitors are without effect in these mice, eliminating up-regulation of eNOS as an explanation. It appears that some other compensation mechanism may be occurring in these mice; perhaps a molecule such as CO may be fulfilling the role vacated by NO. Whether CO has any role in spinal nociception in non-transgenic animals is unclear, as all studies to date have used metalloporphyrin inhibitors of HO and, as mentioned above, these are non-specific, inhibiting both NOS and GC. These HO inhibitors do not mimic NOS inhibitors completely, however, as they prevent the generation of hyperalgesia to mechanical stimuli in preference to thermal stimuli, whereas NOS inhibitors are very effective at preventing thermal hyperalgesia [23]. This suggests that CO may possibly contribute to some forms of hyperalgesia. Further work is therefore required to determine whether NO or CO (or both) contribute to the development of hyperalgesia within the spinal cord.

It is clear that, within the CNS, NO is probably not a neurotransmitter in the classical sense, as it is neither stored in nor released from vesicles in nerve terminals, and it does not participate in synaptic transmission. Nonetheless, it remains an important modulator at some synapses in the CNS and may contribute to various forms of synaptic plasticity that could underlie learning, memory and chronic pain. The role of CO within the CNS is less well defined, and this mainly results from the lack of selective HO-2 inhibitors available; hopefully the development of better tools will allow us to probe the role of CO in the CNS further. Finally, it should not be forgotten that there may well be other members of this family of gaseous molecules, yet to be discovered, that may also contribute to signalling within the CNS.

Summary

- NO and CO are small gaseous molecules that can be synthesized de novo in neuronal tissue and can diffuse readily through the plasma membrane.
- NOS inhibitors prevent the induction of LTP in the hippocampus, and studies with NOS knock-out mice and viral overexpression of mutated NOS indicate that the endothelial form of the enzyme is probably responsible for NO production in these neurons.
- Inhibitors of CO production can block the induction of LTP, but this does not correlate with their ability to prevent CO production in the hippocampus. LTP is normal in mice that lack HO-2 and, furthermore, there is no obvious mechanism by which HO could be activated during synaptic stimulation.
• NO probably diffuses out of the postsynaptic neuron and acts on neighbouring neurons and presynaptic terminals to either instigate, or assist in, the generation or stabilization of LTP, possibly by activating GC.

• There are NO-dependent and NO-independent forms of LTP, and both forms can be found at synapses on to the same neuron. It is therefore possible that subtle discrimination can occur between different inputs on to the same cell.

• NO may also participate in the induction of sensitization within the spinal cord. NOS inhibitors can prevent the development of spinal hyperalgesia due to intrathecal NMDA administration or peripheral nerve injury, and could therefore contribute to some chronic pain states.

References


