Role of Nitric Oxide Synthase in the Function of the Central Nervous System under Normal and Infectious Conditions

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Abstract

Nitric oxide (NO) was discovered as an endothelium-derived relaxing factor more than two decades ago. Since then, it has been shown to participate in many pathways. NO has been described as a key mediator of different pathways in the central nervous system (CNS) in both healthy and diseased processes. The three isoforms of nitric oxide synthase differ in their activity patterns and expression in different cells. Neuronal nitric oxide synthase (nNOS) is localized in synaptic spines, astrocytes, and the loose connective tissue surrounding blood vessels in the brain; eNOS is present in both cerebral vascular endothelial cells and motor neurons; and iNOS is induced in astrocytes and microglia under pathological conditions. During physiological processes, NO produced by eNOS/nNOS, respectively, controls blood flow activation, and act as a messenger during long-term potentiation (LTP). However, under pathological conditions, eNOS appears to be impaired, leading to a reduction in blood flow and, consequently, low oxygen/metabolites delivery, efflux of toxicological agents from the brain tissue and disturbance in the blood-brain barrier. The NO produced by iNOS in glial cells and nNOS, which triggers the NMDA-excitotoxic pathway, combines with superoxide anion and results in peroxynitrite synthesis, a potent free radical that contributes to tissue damage in the brain. Here, we intend to show the controversial role of the nitric oxide delivered by the three isoforms of the nitric oxide synthase in the CNS, assess its impact under healthy/pathological conditions and speculate on its possible sequela, particularly in long-term cognitive decline.

Keywords: nitric oxide, nitric oxide synthase, central nervous system, memory, infection

1. Introduction

Three isoforms of nitric oxide synthase (NOS) have been identified: two constitutive enzymes, neuronal NOS (nNOS) and endothelial NOS (eNOS), and one inducible enzyme (iNOS). These three isoforms of the enzyme nitric oxide synthase (NOS) that are present in the central nervous system (CNS) can produce nitric oxide (NO). eNOS is expressed in the vascular endothelium and choroid plexus; neuronal NOS is mainly expressed in neuronal cell bodies, especially in the cortex, hippocampus, hypothalamus, olfactory bulb, claustrum, amygdala, and thalamus; and inducible NOS is expressed in macrophages, glial cells, infiltrating neutrophils, and, to some extent, neurons [1]. It has also been reported that eNOS can be found in a subset of neurons and astrocytes and that nNOS can be found at low levels in astrocytes [2]. Because these enzymes may have different sites of expression and activation, they have a pivotal role in the divergent functions of NO [3].

Several studies have demonstrated that NO, a freely diffusible gaseous compound, has an important role in a variety of neurobiological processes [4]. Numerous functions of this regulatory molecule have been identified in the CNS, in the process of endothelium-dependent vasodilatation [5–8], in neurotransmission [9, 10], and in host-defense mechanisms [11, 12].

NO is produced from the oxidation of the terminal guanidine nitrogen of the amino acid arginine. This reaction is catalyzed by the NADPH-dependent enzyme, nitric oxide synthase (NOS). After its formation, NO diffuses outside the cell [13]. NO derived from eNOS maintains the CNS microcirculation [14] by inhibiting platelet aggregation and leukocyte adhesion and migration [15]. NO derived from nNOS is an important neurotransmitter related to neuronal plasticity, memory formation, regulation of CNS blood flow, and neurotransmitter release [16, 17].

2. Implications of nitric oxide synthase in the physiological central nervous system

Under physiological conditions, the concentration of NO fluctuates within the range of low values [18] and is produced mainly by nNOS and eNOS. Unlike the other two enzymes, iNOS is not expressed unless it is induced by inflammatory mediators, cytokines, and other agents, such as endotoxins [19]. Due to its calcium-independent activation, iNOS can produce a large amount (100–1000 times greater) of NO in relation to eNOS and nNOS [20]. Until the enzyme is degraded, iNOS constitutively produces NO [21].

NO binds to guanylyl-cyclase, which is a soluble NO receptor and, through cGMP-mediated signaling, acts either as a post- or a presynaptic messenger [22]. As a neurotransmitter, NO may activate the cGMP-dependent protein kinase G (PKG) pathway that phosphorylates synaptophysin, which is critical for fusion of presynaptic vesicles, thereby potentiating and facilitating neurotransmission [22] (**Figure 1**). NO also acts on inhibitory gamma-aminobutyric acid (GABA)-ergic synaptic transmission via cGMP-dependent pathways as well as on ion channels and exchangers [9].
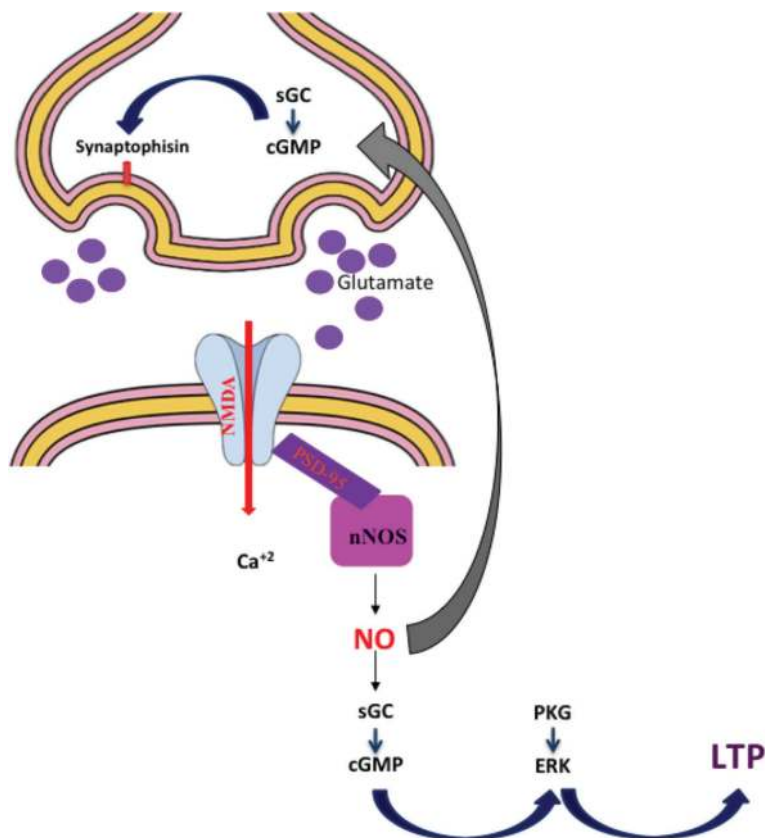
Figure 1. NO act as an unconventional neurotransmitter that is not stored in synaptic vesicles and not released upon membrane depolarization; it releases as soon it is synthesized and does not bind to any receptors, but diffuses from one neuron to another. NO stimulate soluble guanylyl-cyclase to form the second messenger molecule, cyclic guanosine monophosphate (cGMP) either as a post- or a presynaptic messenger. PKG, protein kinase G; ERK, extracellular signal-regulated kinases; LTP, long-term potentiation. Images: https://mindthegraph.com.

The brain relies on a constant and adequate supply of oxygen and glucose that is provided by blood. Cerebral blood flow is altered in response to both neural activity and humoral *stimuli* (e.g., arterial PO_2 and PCO_2). Thus, augmented neural activation results in locally increased cerebral blood flow via functional hyperemia, whereas humoral *stimuli* produce overall increase in cerebral blood flow [23]. The physiological production of endothelium-derived NO by eNOS is protective in hypoxic or ischemic brain injury.

All NOS isoforms have phosphorylation sites for different protein kinases, including protein kinase A, B, and C, and calcium-calmodulin kinase [24]. NOS enzymes are very important for the maintenance of physiological mechanisms within an organism, and the genetic ablation of NOS in mice has been informative for establishing the functional roles of NOS-generated NO in different systems [25]. For instance, nNOS-KO mice present with intense gastroparesis due to impaired vagal innervation of stomach muscle cells [26], decreased apoptosis induced

by striatal N-methyl-D-aspartate (NMDA) microinjections [27], and early dysfunction of hippocampal-dependent spatial memory [28]. Additionally, disrupting the gene that encodes eNOS in mice-induced spontaneous systemic and pulmonary hypertension [29] and inhibited growth factor-mediated angiogenesis [30]. Thus, NO acts through numerous mechanisms of different physiological systems and in living cells.

Shortly after its identification, NO emerged as a possible mediator of neurovascular coupling. Neurovascular coupling is an active mechanism with vessel diameter alterations in response to increased metabolic demands from neuronal activity. Under these conditions, NO acts as a potent vasodilator that is released during enhanced neuronal activity and is well suited to mediate the coupling between neuronal activity and cerebral blood flow [31].

The importance of NO as an intermediary in cell communication in the brain is highlighted by the fact that the excitatory amino acid glutamate is an initiator of the reaction that forms NO. NO can act as a "double-edged sword". Whereas NO supports vascular homeostasis in the endothelium-dependent vasodilatation, its over- or underproduction is linked to pathological conditions [4].

3. Implications of nitric oxide synthase in the pathological central nervous system conditions

NO is also an important mediator under pathological conditions. For instance, in brain ischemia-reperfusion injury, NO formation is initially increased and has a protective function by inducing collateral perfusion as a result of its powerful stimulatory effect on vasodilatation and angiogenesis [32]. NO donors induce neuroprotective effects.

NO can exist in distinct oxidation/reduction states and present dual biological actions as either a neuroprotective or a neurotoxic molecule [4]. Under physiological conditions, nNOS produces hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) in addition to NO [33].

The downstream cascade in the breakdown of the BBB appears to be mediated by eNOS activity; the systemic administration of a selective eNOS inhibitor abrogates VEGF-A-induced BBB disruption and protects against neurologic damage in models of inflammatory disease [34, 35]. Nevertheless, it was suggested that eNOS-derived NO is a neuromodulator that participates in the BBB-mediated control of the cerebellum in experimental models [36].

Curiously, perivascular macrophage-derived iNOS-generated NO, that strategically localizes to leukocytes at brain penetration sites, can serve as a negative feedback regulator that prevents the unlimited influx of inflammatory cells by restoring BBB integrity [37].

In the brain, NOS regulates cerebral blood flow and neurotransmitter release, and the proper operative eNOS/NO system accounts for the microenvironment homeostasis that is essential for the normal functioning of neurons and glial cells [38]. Therefore, the NO produced by NOS results in vasodilation and controls vascular resistance, platelet adhesion and aggregation, leukocyte-to-endothelium interaction, and the maintenance of BBB integrity [36].

Several reports have indicated significant nervous system morbidity due to viral, bacterial, fungal, and parasitic infections (review [39]). During a systemic response to infections, cytokines,

chemokines, and damage-associated soluble mediators of systemic inflammation can gain access to the CNS via blood flow [40]. These mediators can access the brain tissue after the disruption of the BBB. The presence of proinflammatory mediators leads to a disturbance of neuronal and glial homeostasis, with subsequent cognitive and behavioral manifestations that are common during acute infections (anorexia, malaise, depression, and decreased physical activity) and are collectively known as sickness behavior [40]. Although sickness behavior manifestations are transient and self-limited, the cognitive and behavioral changes can become permanent or long-lasting under a persistent systemic inflammatory response. For example, cognitive decline is a common consequence in sepsis and cerebral malaria survivors, as found in both clinical and experimental approaches.

Under healthy conditions, the main cell types that are present in the brain are neurons, oligodendrocytes, astrocytes, and microglia. Neurons connect to each other through long axonal processes with synapses to transmit electrical/chemical signals, thereby generating memory and emotions that are associated with learning, and to control organ and systemic functions. Oligodendrocytes support axons with myelin sheaths. Astrocytes interact with blood vessels to form the BBB and maintain neuronal synapses. Microglia form long processes to phagocyte apoptotic cells and prune inactive synapses without inducing inflammation while maintaining a type of surveillance of neurons. Systemic inflammatory conditions result in the disruption of the BBB and the efflux of proinflammatory cytokines/chemokines as well as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPS), which together activate glial cells. This results in an inflammatory environment due to the release of cytokines/chemokines and reactive oxygen/nitrogen species that exert direct and indirect neuronal cytotoxic effects [40, 41]. Oligodendroglial myelin sheaths can be affected, thereby leading to axonal degeneration. Astrocytosis leads to reduced BBB and synaptic maintenance. Microgliosis results in a proinflammatory microglial phenotype with reduced tissue maintenance functions [41, 42]. Neuronal cells can develop an excitotoxicity process due to excessive glutamate in the synaptic cleft and subsequent extra-synaptic NMDA receptor activation that results in a subsequent increase of Ca^{2+} efflux in neuronal cells and the activation of proteins calpain 1 and neuronal nitric oxide. This results in mitochondrial dysfunction and oxidative damage by reactive oxygen and nitrogen species [43]. Together, these mechanisms lead to neuronal death, thereby contributing to long-term cognitive decline, which has been shown to be a consequence of several infectious diseases.

As shown in **Figure 2**, the gaseous signaling molecule NO has a variety of cellular functions, including neurotransmission, regulation of blood-vessel tone, and immunity. Under pathological conditions, free radicals may deplete NO produced by eNOS through the formation of $ONOO^-$, thus decreasing the vascular bioavailability of NO, which results in BBB dysfunction. This ultimately results in endothelial damage, edema development, and hypoxia. Furthermore, the NO produced by iNOS in glial cells or by nNOS under excitotoxic process can form with free radicals (particularly O_2^-) $ONOO^-$ and produce several deleterious effects on tissue, such as through tyrosine nitration and cysteine oxidation in various proteins. These free radicals can further decompose into highly toxic-free radicals, such as $NO_2^{\bullet}$ and $^{\bullet}OH$ (as reviewed by [44]).

Nitric oxide, as described above, is a key molecule in the regulation of physiological brain homeostasis. Nitric oxide is synthetized by neuronal vessels (eNOS), by neurons (nNOS)
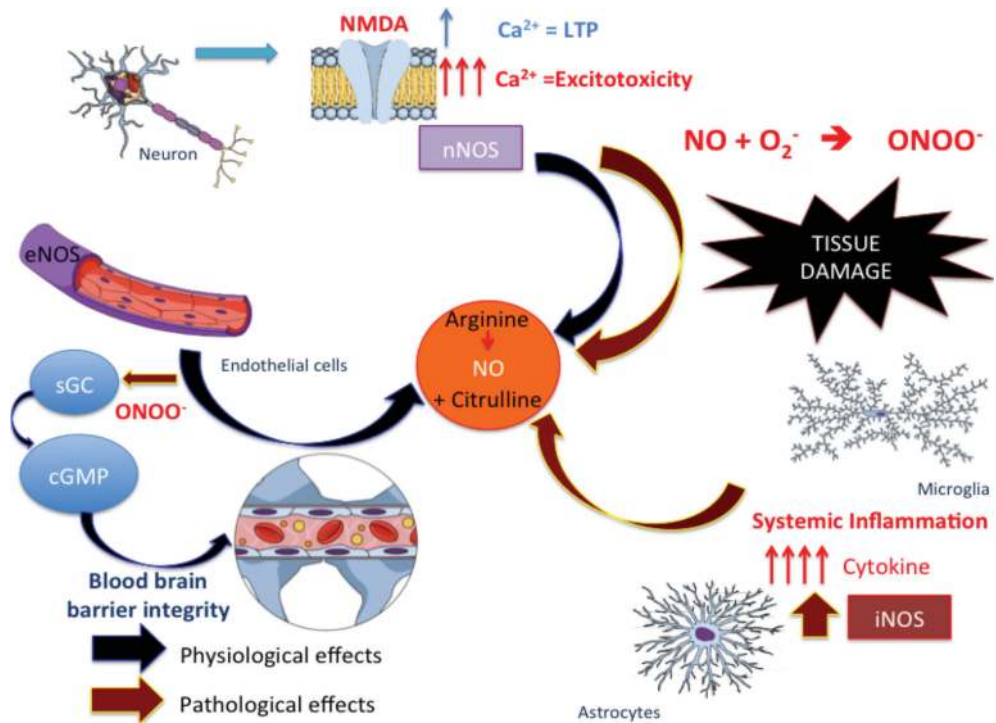
Figure 2. Different steps in the NO signaling cascade under physiological/pathological conditions in the brain. During long-term potentiation, NOS1 or neuronal NOS (nNOS) catalyze the NO synthesis after the activation of the NMDA receptor by Ca^{2+}. Under excitotoxic conditions, excessive Ca^{2+} leads to nNOS hyperactivity, whereas excessive NO production can combine with superoxide to form peroxynitrite, which is responsible for tissue damage due to several biological effects, including blockage of the eNOS pathway and BBB impairment. NO is synthesized following the transcriptional expression of a Ca^{2+}-independent NOS2 or iNOS isoform in glial cells (astrocytes and microglia) after cytokine exposure, thereby contributing to neuroinflammation and tissue damage in the brain. Intracellular Ca^{2+} activates NOS3 or eNOS to release NO from brain microvessels. This NO binds to soluble guanylyl-cyclase (sGC) receptors, which triggers a cGMP-dependent pathway and interacts with its downstream mediators of the physiological regulation of vasodilation and vascular resistance, platelet adhesion and aggregation, leukocyte-endothelial interaction, and BBB integrity maintenance. Images: https://mindthegraph.com.

under physiological/pathological *stimuli*, and by iNOS during inflammatory conditions. It is well known that high levels of nitric oxide (NO) released by the inducible NO synthase (iNOS) are critical for defense against parasites and mediate inflammatory tissue damage. However, the suppression or lack of NO production results in the impaired clearance of some types of bacteria by the host [45].

Our group and others have shown that eNOS is impaired during systemic infection, which leads to brain microcirculation dysfunction [46–48]. We showed that during different infectious diseases, there is a functional capillary rarefaction (**Figure 3**) that can be caused by the impairment of endothelial NO production by a reduction of eNOS activity or by reduced levels of the enzyme cofactor tetrahydrobiopterin—BH4 [49].
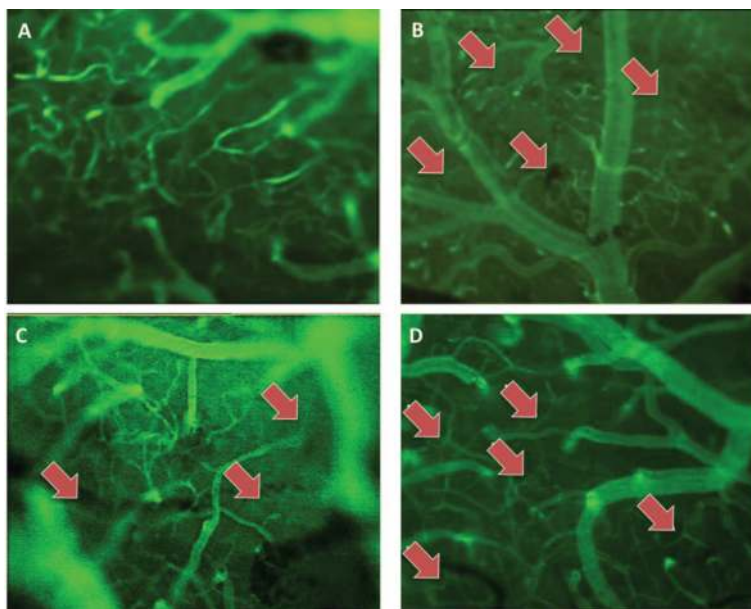
Figure 3. Photomicrography from intravital microscopy showing capillary impairment (red arrows) under healthy (A) and infectious conditions (B) malaria, (C) sepsis, and (D) Chagas' disease [47, 48].

Vascular function can be evaluated by the vasodilator response of cerebral arterioles to acetylcholine (Ach). Our group used intravital microscopy to evaluate the response to Ach in sepsis and Chagas' disease model. The vasodilation-to-Ach test is directly related to the availability of endothelium-derived NO generated from L-arginine by the action of endothelial NO synthase (eNOS). The diffusion of NO to vascular smooth muscle cells and the activation of guanylyl-cyclase resulted in cGMP-mediated vasodilation. However, the vasoconstrictive response to Ach results from a direct muscarinic smooth muscle effect [50]. This vasoconstrictive effect can lead to the slow delivery of oxygen to brain tissue, generating hypoxia and increased glycose consume by glycolytic pathway to generate ATP for neuronal functioning. Consequently, mitochondrial function is compromised by low O_2 concentration [51].

In both sepsis and Chagas' disease models, we observed a vasoconstrictive response to Ach. One mechanism of eNOS "uncoupling" from the reduction of NO production and ROS generation involves the oxidative degradation of the cofactor BH4 (tetrahydrobiopterin), especially by peroxynitrite ($ONOO^-$), which is produced in association with superoxide and nitric oxide. Several studies have shown that there is an increase in impaired eNOS-derived oxidative stress in brain tissue exposed to systemic infection, NO consumption and capillary dysfunction [46, 47, 52, 53].

Interestingly, in experimental models of malaria [48] and sepsis (Reis et al., submitted) blood flow was recovered by treatment with statins. As reviewed by Giannopoulos et al. [54], statins are shown to enhance eNOS expression and can contribute to the restoration of brain capillary function.

Resident glial cells in the CNS (i.e., astroglia and microglia) express inducible nitric oxide synthase (iNOS) and produce high levels of NO in response to a wide variety of proinflammatory and degenerative *stimuli*. Excessive NO production by glial cells evoked by inflammatory signals contributes to the pathogenesis of several neurodegenerative diseases, such as multiple sclerosis, HIV dementia, brain ischemia, trauma, Parkinson's disease, and Alzheimer's disease. NO can also be released in glial cells in response to infectious agent. The inducible form of nitric oxide synthase can be strongly associated with tissue damage, despite its activity as an antimicrobial agent. As reviewed by Saha and Pahan [55], astrocytes and microglia can express iNOS and synthetize NO. According to Radi [56], nitric oxide can combine with superoxide (produced mainly by the NADPH oxidase system and partially by mitochondria) under conditions of oxidative stress. It is common under neuroinflammatory conditions to form peroxynitrite, which binds to tyrosine to produce 3-nitrotyrosine, and a marker of reactive nitrogen species production. The reactions of peroxynitrite with biomolecules can lead to cytotoxic events and may result in apoptotic or necrotic cell death. Peroxynitrite can act via antioxidant enzyme inhibition; antioxidant depletion; protein aggregation (e.g., α-synuclein and microtubule-associated tau protein modifications in the CNS); activation of specific enzymes (e.g., matrix metalloproteinases (MMPs), cytochrome c, glutathione-S-transferase, protein kinase C-ε (PKCε), and fibrinogen); impairment of enzyme cofactors (e.g., BH4); modification of mediator pathways, receptors, and cellular signaling molecules; calcium deregulation; DNA injury; and mitochondrial dysfunction, among other processes (reviewed by [57]).

Data from our group show that the absence of the iNOS enzyme (knockout) or treatment with iNOS-specific inhibitors (e.g., aminoguanidine) has a beneficial effect on the prevention of cognitive impairment (unpublished data) in experimental cerebral malaria. This could be due to the reduced production of the peroxynitrite radical and the subsequent reduction of tissue damage. Other studies are being conducted to clarify the impact of the synthesis of nitric oxide by iNOS in infectious diseases and in the development of cognitive decline. Weberpals et al. also observed that iNOS gene deficiency prevents cognitive decline in addition to promoting a reduction in gliosis (astrocytes and microglia), proinflammatory cytokines TNF-α and IL-1β release, and reduction of synaptic dysfunction in sepsis model [58].

4. NMDA-nNOS pathway and excitotoxicity development

The NMDA receptor is a key regulator during glutamatergic long-term potentiation (LTP) response. During physiological conditions, glutamatergic ionotropic (e.g., kainate, NMDA, and AMPA) and metabotropic receptors (mGlut) are activated, delivering Ca^{2+} into neuronal cells and resulting in depolarization and the triggering of mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways. This then activates phosphoinositide-dependent kinase 1 or 2 (PDK1/2), Akt, and mammalian target of rapamycin (mTOR) downstream signaling [59]. Additionally, calcium-dependent kinase II (CAMKII) is rapidly activated and all events together lead to the

activation of transcription factors (e.g., CREB) and an increase in the synthesis of neurotrophic factors (e.g., BDNF) and proteins associated with the long-term potentiation (LTP) process.

Despite the major activity of NMDA receptors in LTP, several reports have related this receptor with excitotoxicity, which is a neurodegenerative process. This process has been associated with a different class of NMDA receptor: the extra-synaptic receptor. As reviewed by Parsons and Raymond, synaptic NMDA (which expresses the subunit GlutN2A) is associated with LTP, whereas extra-synaptic receptors (which express the subunit GlutN2B) is associated with excitotoxicity and cell death [42]. NMDARs recruit the calcium-dependent enzyme nNOS via PSD95 (postsynaptic density: membrane-associated guanylate kinase (MAGUK) scaffolding protein located in neural postsynaptic densities), which is also associated with the LTP process. This is considered a key contributor to excitotoxicity lesions in both stroke and neurodegenerative diseases [60]. nNOS is activated by calcium/calmodulin signaling and is PSD95 protein-dependent [60]. During excitotoxicity, the activation of NMDA enhances intracellular calcium, leading to nNOS activation and NO production. Additionally, excessive intracellular calcium activates calpain 1, which then disrupts mitochondrial function, thereby triggering the intrinsic apoptotic pathway by releasing cytochrome C and activating apoptosomal protein complex [61]. NO can combine with superoxide, which results in peroxynitrite formation and cellular damage. Peroxynitrite can disturb cellular function by the nitration of proteins, which reduces or eliminates protein function, as described earlier, and by DNA damage via the activation of poly (ADP-ribose) polymerase 1 (PARP-1). The impact of PARP-1 on intracellular concentration of its nicotinamideadenine dinucleotide substrate (NAD), creates a bioenergetic imbalance that culminates with ATP depletion, thereby triggering necrotic neuronal death [62, 63]. In addition, NO can drive the retraction of the synaptic button via the activation of small GTPase RhoA/ROCK signaling through a paracrine/retrograde-signaling pathway [64]. Taken together, these events could contribute to neuronal dysfunction and death associated with cognitive decline.

The roles of NO in neuronal damage following insults, such as hypoxia, traumatic brain injury, and ischemia, have been well established. Recent evidence has implicated an imbalance of ROS and NO signaling in neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, and in cognitive impairments associated with normal physiological aging [65–67]. Whereas mild oxidative/nitrosative (nitric oxide (NO)-related) stress mediates normal neuronal signaling, the accumulation of free radicals is associated with neuronal cell injury or death.

As described above, NO from eNOS modulates blood flow in the brain, and its impairment could be associated with hypoxic events in the brain. Several degenerative and infectious diseases relate hypoxia to neuronal dysfunction and cognitive decline. Using partial eNOS knockout mice model, Tan et al. [68] showed that the development of spontaneous thrombotic cerebral infarction is followed by amyloid protein deposit and cognitive decline. Cognitive decline is a major cause of disability in stroke survivors [69]. We have shown that microvascular impairment in malaria, sepsis, and Chagas' diseases may occur in response to other infectious agents [46–48]. Cerebral metabolism is dependent upon the glucose and oxygen that are

delivered by blood, and it is clear that alterations in endothelial function can disrupt neuronal functions. Additionally, activation of endothelial cells by systemic cytokines, PAMP/DAMP and prooxidant molecules can contribute to eNOS dysfunction [70], blood flow disturbance and neuronal dysfunction, which subsequently results in cognitive decline.

The activation of glial cells that may be related with systemic inflammation associated to host response to pathogens can activate inducible isoforms of iNOS and subsequently generate cellular damage via the generation of peroxynitrite by the combination of NO and superoxide radicals. In this way, the induction of iNOS may result in the development of cognitive impairment [71]. Experimental models of sepsis [58] and malaria (unpublished data) have shown that the inhibition of iNOS has a beneficial effect on the central nervous system, particularly by abolishing cognitive decline.

The main enzyme target in the central nervous system seems to be nNOS. The NO generated by nNOS controls the release of neurotransmitters and is involved in synaptogenesis, synaptic plasticity, memory function, and neuroendocrine secretion. However, the overproduction of NO during NMDA-excitotoxic events can lead to neuronal death and directly impact the cognitive function.

Death pathways are activated in mouse brains during experimental model of sepsis and malaria [72, 73]. Additionally, reduced levels of neurotrophic factors, impairment of neurogenesis, and synaptic dysfunction were observed [74–78]. However, the role of excitotoxicity and nNOS delivery during infectious diseases and long-term cognitive impairment is not yet clear. Neuroinflammation can also induce cell damage/death, and the modulation of the activation of glial cells has been suggested to prevent neuronal damage and cognitive decline. Cognitive impairment is prevented by antioxidants and statin treatment [48, 53, 79], which suggests that the control of oxidative or inflammatory damage is also efficient to avoid cognitive decline.

5. Conclusion

NO is a key molecule involved in the regulation of CNS function in health and disease (**Table 1**). The impairment of enzymatic activity or the overproduction of NO under inflammatory/excitotoxic conditions can contribute to neurological sequela during systemic-infectious diseases (**Figure 3**). The NO synthase complex can be considered a target of pharmacological intervention focusing on the prevention of cognitive sequela; however, this field requires further studies.

NOS isoform	Physiological effect on CNS	Pathological effect on CNS
nNOS	Learning and memory	↑—Neuronal death by excitotoxicity
eNOS	Vasodilation and increased blood flow	↓—Hypoxia
iNOS	Sleep [33]	↑—Tissue damage

Table 1. Nitric oxide synthase enzymes functions on health/pathological conditions.

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