

● INVITED REVIEW

# Targeting neuronal nitric oxide synthase as a valuable strategy for the therapy of neurological disorders

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## Abstract

The management of neurological disorders have huge and increasing human and economic costs. Despite this, there is a scarcity of effective therapeutics, and there is an extreme urgency for new and real treatments. In this short review we analyze some promising advancements in the search of new bioactive molecules targeting neuronal nitric oxide synthase (nNOS), an enzyme deputed to the biosynthesis of nitric oxide (NO). In different conditions of neuronal damages, this molecule is overproduced, contributing to the pathogenesis and progression of neuronal diseases. Two main approaches to modulate nNOS are discussed: a first one consisting in the direct inhibition of the enzyme by means of small organic molecules, which can be also active against other different targets involved in such diseases. A second section is dedicated to molecules able to prevent the formation of the ternary complex N-methyl-D-aspartate (NMDA)-type glutamate receptors, postsynaptic density-95 (PSD95) protein-nNOS, which is necessary to activate the latter for the biosynthesis of NO.

**Key Words:** amidines; carbonic anhydrase; inhibitors; neurological diseases; neuronal nitric oxide synthase; NMDAR; PSD95

## Introduction

A growing number of studies demonstrate that an abnormal nitrergic signaling in the central nervous system (CNS) is a crucial event in the development of neurological disorders, such as ischemic stroke, migraine, acute brain and spinal cord injury, *etc.* (Yuste et al., 2015; Antonaci et al., 2016; Wang et al., 2016). The free radical nitric oxide (NO) is a signaling molecule ubiquitously biosynthesized by the nitric oxide synthases (NOS) family, in a two step reaction involving oxygen and many cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH), flavins and tetrahydrobiopterin. Three NOS isoforms are currently known: the neuronal and endothelial ones are constitutive (nNOS and eNOS, respectively), while the inducible isoform (iNOS) is expressed in response to invading pathogens, or pro-inflammatory stimuli. All of the NOSs have essential physiological roles in the CNS, and their activity is compromised in different neurological states. However, among NOS isoforms, the nNOS is the most implicated in a wide range of functions and pathologies with pleiotropic effects. In the last years, a growing number of molecules able to modulate nNOS activity have appeared, and in this review we focus on nNOS as a possible therapeutic target for neuroregeneration and recovery of neuronal functions.

In the CNS, the nNOS is localized inside the postsynaptic cell membrane and is physically tethered to N-methyl-D-as-

partate (NMDA)-type glutamate receptors, through postsynaptic density-95 (PSD95) protein. Under physiological conditions, mild activation of 'synaptic' NMDARs allows the influx of  $Ca^{2+}$ , which, binding to calmodulin, leads to nNOS catalytic activation. The produced NO exerts different cellular effects, mainly by nitrosylation of the ferrous heme of the metalloenzyme soluble guanylate cyclase, activating the cyclic guanosine monophosphate (cGMP) signaling. Moreover, the reaction of NO and its derived species ( $N_2O_3$ ,  $NO_2^-$ ,  $NO^+$ , *etc.*) with protein cysteine and tyrosine residues, generates S-nitrosothiols and 3-nitrotyrosines, and activates the so called nitrosative signaling (**Figure 1, panel A**) (Heinrich et al., 2013). These events trigger downstream signals, maintaining normal synaptic plasticity and promoting neuronal differentiation or survival. For example, synaptic NO activates the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) pathway, regulating long-term potentiation (LTP, an electrical correlate of learning and memory) and mediating neuronal survival (Lu et al., 1999).

By contrast, accumulating evidences suggest that hyperactivation of 'extrasynaptic' NMDARs can lead to an abnormal  $Ca^{2+}$  influx into the postsynaptic neuron, with a subsequent overstimulation of nNOS, through the ternary NMDAR/PSD95/nNOS complex formation. Thus, nNOS results permanently activated, and the overproduced NO is responsible for excessive protein nitrosation and nitrosylation, determining

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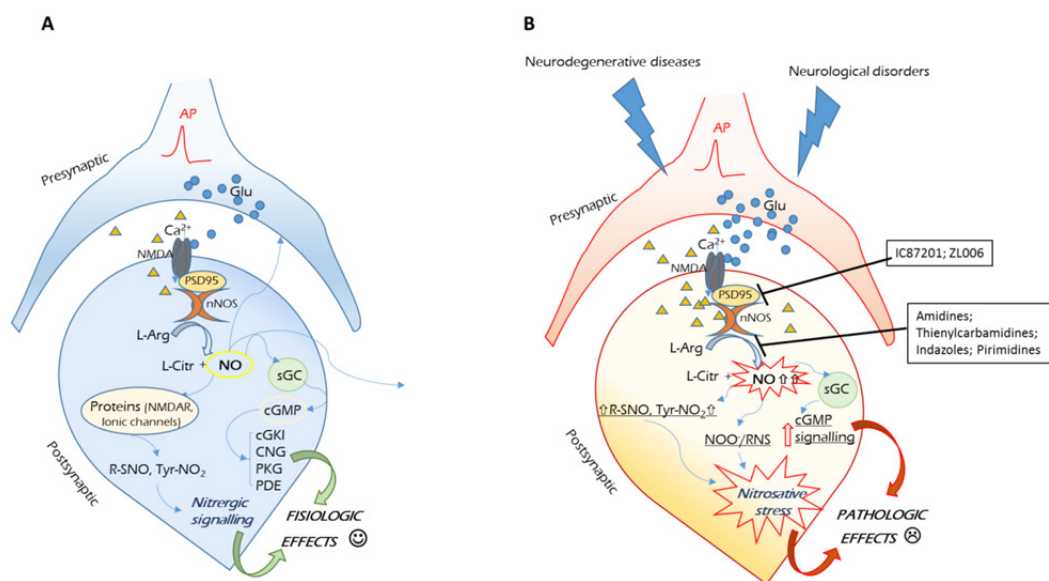
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**Figure 1** Schematic representation of NO synthetic and activity pathways in physiologic conditions (panel A), and pathologic conditions (panel B). (A) Glutamatergic stimulation of NMDA receptors allows  $\text{Ca}^{2+}$  influx. Both  $\text{Ca}^{2+}$ -calmodulin binding and NMDAR-PSD95-nNOS complex formation, lead to mild nNOS activation. Low and controlled amounts of NO are produced, interacting with sGC and different intracellular proteins and ionic channels, to give nitrosothiols (R-SNO) and 3-nitrotyrosines (Tyr- $\text{NO}_2$ ). Both the cGMP and nitrenergic signaling maintain the synaptic plasticity and cellular survival. (B) Both neurodegenerative and neurological disorders lead to excessive glutamatergic stimulation of NMDA receptors. As a consequence,  $\text{Ca}^{2+}$  intracellular concentration increases, leading to a permanently activated nNOS. The overproduced NO is responsible for extensive protein nitrosation and nitrosylation, leading to nitrosative and oxidative stress. This, together with excessive peroxynitrite ion formation, and stimulation of the cGMP signaling, impairs neuronal metabolism and triggers apoptosis. cGMP: Cyclic guanosine monophosphate; NMDA: N-methyl-D-aspartate; nNOS: neuronal nitric oxide synthase; NO: nitric oxide.

nitrosative stress. Additionally, the excessive NO by reaction with superoxide anion ( $\text{O}_2^-$ ), forms the highly reactive oxidant and cytotoxic molecule peroxynitrite ( $\text{ONOO}^-$ ), which triggers lipid peroxidation and damage (Heinrich et al., 2013). As a consequence of these events, neurotransmission is impaired, due to mitochondrial dysfunction and synaptic damage (Figure 1, panel B). In this condition, nitric oxide activates the MAPK p38 and other downstream signaling proteins responsible for neuronal death, inducing apoptosis (Cao et al., 2005).

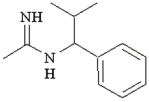
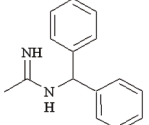
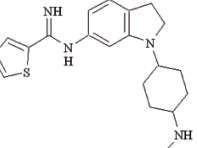
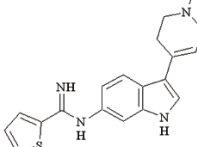
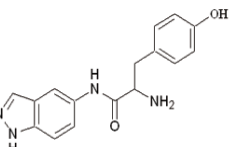
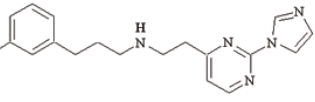
Therefore, blocking overstimulated neuronal nitric oxide synthase can be considered as a target to inhibit neurodegeneration and restore neurological functions. Actually, there are two distinct ways to inhibit NO overproduction from nNOS: 1) blocking the enzyme with inhibitors; 2) inhibiting the NMDAR-PSD95-nNOS complex (Figure 1, panel B).

### Inhibitors of the neuronal NOS

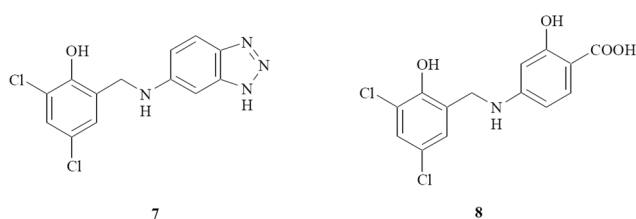
The very first challenge in the discovery of nNOS inhibitors is to obtain selective molecules, which do not interfere with iNOS and eNOS. The difficulty in reaching this goal lies in the fact that the three isoforms have a very similar architecture, and it is necessary to exploit the subtle differences between the catalytic sites of the enzymes, to have selective ligands. Indeed, the early nNOS inhibitors, which were

substrate analogues or arginine based dipeptides, were abandoned, since they showed unacceptable cardiovascular side effects (increase of blood pressure, heart failure, etc.), mainly due to their very low isoform selectivity. In the research of non-aminoacidic compounds able to selectively inhibit nNOS, sterically hindered amidines (**1**, **2**, Table 1) gave interesting results (Maccallini et al., 2013). The phenyl and isopropyl groups of these molecules are the key fragments conferring them the desired selectivity, because they are responsible for the favorable interactions within the nNOS catalytic pocket. Better results have been obtained with thienyl-carbamidines, which proved to be effective against human nNOS and showed neuroprotection in ischemia models and neuropathic pain (**3**, **4**, Table 1) (Vaughan et al., 2010; Annedi et al., 2012a, b). Molecule **4** is currently in Phase III clinical trial for the treatment of acute migraine with aura. Recently, indazole based compounds have been reported as dual agents able to inhibit nNOS and to activate human carbonic anhydrase, another emerging target for the therapy of neurodegeneration. Compound **5** (Table 1) was the most interesting one of this series, showing high potency against nNOS ( $IC_{50} = 0.174 \mu\text{M}$ ), excellent *in vitro* selectivity with respect to eNOS (327 folds), together with a potent and selective activation of the human Carbonic Anhydrase isoform I ( $K_A = 15 \text{ nM}$ ) (Maccallini et al., 2016). Another important chemical scaffold which generated potent and selective nNOS inhibitors was the pyrrolidinomethyl-2-aminopyridine

**Table 1** Inhibitors of neuronal nitric oxide synthase.

Entry	Structure	Activity ( $IC_{50}$ , $\mu$ M)		Target diseases	Clinical trial
		nNOS	eNOS		
1		0.2	100	Neurodegeneration	–
2		0.3	300	Neurodegeneration	–
3		0.37	197	Neurophatic pain	Phase II
4		1.29	64.7	Migraine	Phase III
5		0.174	57	Neurodegeneration	–
6		0.019 <sup>a</sup>	5 <sup>a</sup>	Neurological disorders	–

a: NOS Ki.

**Figure 2** Small molecules able to inhibit neuronal nitric oxide synthase (nNOS) by disrupting the PSD95–nNOS interaction.

one. Several molecules have been published endowed with very interesting biological profiles, but the lack of selectivity at tissue level, together with their poor bioavailability and physicochemical properties, limit their *in vivo* application (Pigott et al., 2013). These unfavorable aspects have been overcome by the 2,4-disubstituted pyrimidine derivatives (**6**, Table 1), which are promising agents for the treatment of NO mediated neurological diseases (Annedi, 2015).

## Inhibitors of the NMDAR-PSD95-nNOS Complex

NMDARs excitotoxicity is mediated by nNOS, which is activated not only by the increased calcium concentration, but also by the binding with the PSD95 protein. Therefore, this protein interaction is a viable druggable target, providing an alternative and selective approach to the direct inhibition of nNOS or NMDAR.

From a medicinal chemistry viewpoint, this is a less investigated area and few compounds have been reported as PSD95/nNOS inhibitors. NR2B9c, a small interference peptide that is supposed to disrupt with high potency both the interactions between the GluN2A subunit of the NMDAR and the PSD95, and between the latter and nNOS, is giving the most encouraging clinical results. It is currently under evaluation in patients with acute cerebral ischemia, in a phase III clinical trial (Aarts et al., 2002). Due to the poor pharmacokinetic profile of NR2B9c, investigations on different compounds able to act

with the same mechanism have been performed. IC87201 is a small molecule identified from a virtual screening of a 150,000-compound library, showing an  $IC_{50}$  of 31  $\mu\text{M}$  (7, **Figure 2**) (Loh et al., 2005). Notably, IC87201 disrupts the PSD95–nNOS interaction without affecting the interaction of PSD95 with other proteins. Based on the presumable structural requirements of the PSD95–nNOS interaction, it was synthesized ZL006 (8, **Figure 2**), another interesting small molecule endowed with high specificity and potency for disrupting the PSD95–nNOS interaction. ZL006 is neuroprotective against NMDAR-mediated excitotoxicity *in vitro* and ischemic neuronal injury *in vivo*, and readily crosses the blood-brain barrier (Zhou et al., 2012).

## Conclusions

In the last decade, the excessive production of nitric oxide by nNOS has been recognized as a major player in the induction and progression of neurological diseases. Medicinal chemists disclosed several molecules able to inhibit this enzyme, although there is still much to do to ameliorate the safety profile of these compounds, as well as their pharmacokinetics. Indeed, only few of the developed nNOS inhibitors have reached clinical trials. Future structure–activity relationship studies are also necessary to identify a pharmacophoric model to develop potent compounds targeting the NMDAR-PSD95–nNOS complex, as a promising strategy to inhibit the downstream death signaling factors responsible of neuronal death.

**Author contributions:** All authors wrote and revised the manuscript.

**Conflicts of interest:** None declared.

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