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Post-translational Regulation of Neuronal Nitric Oxide Synthase: Implications for sympatho-excitatory states

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Abstract

Introduction—Nitric oxide (NO) synthesized via neuronal nitric oxide synthase (nNOS) plays a significant role in regulation/modulation of autonomic control of circulation. Various pathological states are associated with diminished nNOS expression and blunted autonomic effects of NO in the central nervous system (CNS) including heart failure, hypertension, diabetes mellitus, chronic renal failure etc. Therefore, elucidation of the molecular mechanism/s involved in dysregulation of nNOS is essential to understand the pathogenesis of increased sympathoexcitation in these diseased states.

Areas Covered—nNOS is a highly regulated enzyme, being regulated at transcriptional and posttranslational levels via protein-protein interactions and modifications viz. phosphorylation, ubiquitination, and sumoylation. The enzyme activity of nNOS also depends on the optimal concentration of substrate, cofactors and association with regulatory proteins. This review focuses on the posttranslational regulation of nNOS in the context of normal and diseased states within the CNS.

Expert Opinion—Gaining insight into the mechanism/s involved in the regulation of nNOS would provide novel strategies for manipulating nNOS directed therapeutic modalities in the future, including catalytically active dimer stabilization and protein-protein interactions with intracellular protein effectors. Ultimately, this is expected to provide tools to improve autonomic dysregulation in various diseases such as heart failure, hypertension, and diabetes.

Keywords

cardiovascular diseases; nNOS; PVN; sympathoexcitation

1. INTRODUCTION

Anomalous regulation of the sympathetic nervous system leading to exaggerated sympathetic nerve activity is associated with many cardiovascular diseases including heart

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Declaration of Interest

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failure (CHF)[1, 2], hypertension, obesity and insulin resistance[3]. The prognosis of patients with elevated sympathoexcitation in cardiovascular diseases is dismal [1, 4, 5]. Despite major advances in the therapy directed to alleviate sympathoexcitation, the morbidity, and mortality related to it are still high for cardiovascular diseases [6, 7]. The increased sympathoexcitation is mediated by a number of important regions within the central nervous system (CNS) including the paraventricular nucleus (PVN) of the hypothalamus and the rostral ventrolateral medulla (RVLM) [8]. The PVN has been suggested to be the highest autonomic control center within the CNS for the regulation of sympathoexcitation [9, 10, 11, 12]. Various neuroanatomical, electrophysiological and functional studies have indicated an important role for the PVN in cardiovascular regulation [10, 13] via sending efferent nerves to RVLM, as well as direct projections to the intermediolateral cell column in the spinal cord[14]. A number of different neurotransmitter systems, excitatory as well as inhibitory have been shown to converge in the PVN to influence ongoing neuronal activity [10]. One important mediator identified to be sympathoinhibitory within the PVN is NO (nitric oxide)[15, 16, 17, 18, 19]. NO is a well-acknowledged, universal signaling molecule, which also acts as a neuromodulator in the central and peripheral nervous system [20, 21]. Cardiovascular actions of NO are not only limited to its direct effects on dilation of blood vessels but also affect the synaptic excitability of pre-autonomic neurons in the CNS. NO modulates the release of several neurotransmitters, such as acetylcholine, catecholamine, excitatory and inhibitory amino acids viz. glutamate and GABA (γ -aminobutyric acid) to influence overall neuronal function [22, 23]. Previous studies from our laboratory have shown that the administration of an NO donor into the PVN decreases renal sympathetic nerve activity (RSNA), whereas administration of nitric oxide synthase (NOS) inhibitor within the PVN increases RSNA [24]. NO-mediated inhibition of neurons within the PVN have been suggested to utilize a GABA-mediated mechanism [25, 26], which is also blunted in rats with CHF [27, 28, 29].

NO is synthesized by the NOS enzyme, which exists in three isoforms, neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). nNOS is the major source of NO in the brain and is expressed in neurons, glia as well as in the adventitia of a subset of rat cerebral blood vessels in CNS [30]. Studies in mammalian, particularly in rodents brains showed that nNOS is abundantly expressed in the various areas including those involved in the regulation of cardiovascular functions, such as, the amygdala, CA1 region, dentate gyrus of hippocampus, the hypothalamus (the supraoptic nucleus and PVN), the thalamus, the RVLM, nucleus tractus solitarius (NTS) and the cerebellum [31, 32]. NO synthesized by nNOS in the brain affects not only PVN but also other central sites, particularly the NTS, and RVLM under physiological and pathological conditions [22]. Lack of NO during various pathological conditions leads to an increase in sympathetic hyperactivity, primarily due to a blunted sympathoinhibitory effect by nNOS, such as CHF[33, 34, 35, 36], hypertension [37, 38], diabetes[39] and in hypertension associated with chronic renal failure[40]. Moreover, oxidative stress has been shown to cause an uncoupling of NOS in the brain during hypertension [41, 42] and CHF [43, 44], thus further depleting the levels of NO and aggravating the disease progression. Until recently, nNOS was considered to be a constitutively expressed enzyme, but current studies [20, 36, 45, 46, 47, 48] suggest that activity and expression of nNOS are regulated, both

transcriptionally and post-transcriptionally by various physiological and pathological stimuli. Although, there are multiple reports on the dysregulation of nNOS in different physiological and pathological conditions [33, 34, 35, 36, 37, 38, 39, 40], there are limited studies examining the molecular mechanism of regulation of nNOS within the CNS. This review highlights the regulation of nNOS in the CNS with particular reference to PVN and with emphasis on post-translational modification and protein-to-protein interactions leading to the regulation of sympathoexcitation using an example of a typical sympatho-excitatory state of chronic congestive heart failure.

2. STRUCTURES AND ACTIVITY OF NO SYNTHASES

Since awarding the Nobel Prize to Furchgott, Ignarro, and Murad for the discovery of NO in 1998, the prevailing literature points towards a significant role for NO in the regulation of neurotransmission within the CNS [15, 16, 17, 18, 19, 24, 26]. NO is an important signaling molecule with a short half-life of 1-5s and is involved in the regulation of the cardiovascular, immune and nervous systems, rather than just a toxic pollutant [30, 45]. NO acts as a novel neural messenger by stimulating soluble guanylyl cyclase, thus increasing the levels of cyclic guanosine 3', 5'-monophosphate in target cells. As mentioned, NO is synthesized by a family of enzymes known as NOS which exist as three genetically different isoforms, coded by their corresponding gene which include NOS1 for nNOS being the isoform first found in neuronal tissues, NOS2 for iNOS being the isoform induced by pro-inflammatory cytokines and inflammation and NOS3 for eNOS expressed in endothelial cell. A unique NOS isoform known as mitochondrial NOS is present in the inner mitochondrial membrane [49]. All three isoforms of NOSs are flavohaem enzymes, which share ~50–60% sequence homology and similar catalytic mechanisms [50]. NOS homodimerize through oxygenase domain and catalyze O₂-dependent, five-electron oxidation of the guanidine nitrogen of L-arginine to NO and citrulline, with N^ω-hydroxy-L-arginine formation as an intermediate [30]. Dimer formation is essential for the catalytic activity of the enzyme. Each monomer exhibits bi-domain structure comprising a carboxy-terminal diflavin-reductase domain and an amino-terminal oxygenase domain, which are separated by a calmodulin-binding motif. Dimerization is assumed to activate the enzyme by sequestering iron, generating high-affinity binding sites for arginine and the essential cofactor (6R)-5,6,7,8-tetrahydrobiopterin (BH4) in the oxygenase domain. The transfer of electrons during the catalytic reaction occurs from the reductase domain of one monomer to the oxygenase domain of the other monomer [46]. Further, Ca²⁺/calmodulin binding support electron transfer. Binding of calmodulin is dependent on calcium in eNOS and nNOS, and therefore, enzyme activity in these isoforms is calcium dependent while calmodulin is tightly bound to iNOS, making this isoform fully active even at basal intracellular calcium concentration [47]. The activity of NOS also depends on the availability of the cofactors BH4, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM), and iron protoporphyrin IX (heme), and nicotinamide adenine dinucleotide phosphate (NADPH) as an electron source (Figure 1A). Calmodulin binding is required to trigger the flow of electrons from the flavins to the active site. As stated previously, regions of high homology characterize the three isoforms of NOS, yet they also have some significant differences in size, as well as each isoform, has distinctive characteristics that make these forms different in their function and

distribution (Figure 1B). The molecular weight of nNOS is 160kDa compared to iNOS (130kDa) and eNOS (135kDa), due to the presence of additional 300 amino-acid sequences at N-terminal containing a PDZ {postsynaptic density protein (PSD95), disc large, ZO-1} domain. The N-terminus of eNOS also has unique myristoylation and palmitoylation sites that regulate the localization of the enzyme to the plasmalemma caveolae.

Since NO has a strong chemical reactivity, short half-life, and high diffusibility, NOS expression, and activity are tightly linked and regulated. Despite the beneficial roles of NO as the neuromodulator, as a messenger and in host defense mechanism, a dysregulated production of NO can give rise to pathological conditions. The modulations in NOS and NO metabolism leads to either decreased the production of NO, which is linked to cardiovascular diseases [35, 36, 37, 39], erectile dysfunction (ED) [51] and gastrointestinal disorders [52] or excess of NO which is related to tumor progression [53] and neurodegenerative diseases[30]. Therefore, the regulation of nNOS activity is strictly controlled to perform the variety of functions, which dictate the specificity of NO signaling and influence on the activity of neurons in regions of the CNS involved in autonomic regulation such as PVN and RVLM.

3. REGULATION OF nNOS

The activity of NOS enzymes is under the complex and integrated control of transcriptional, translational, and post-translational regulation. Hence, understanding of molecular mechanisms involved in dysregulation of nNOS leading to result ultimately in increased sympathoexcitation in various disease states is essential.

3.1 Transcriptional Regulation

nNOS gene located on chromosome 12 has a complex genomic organization, spanning over 160 kbp and consisting of an open reading frame of 29 exons extending from exon 2 to exon 30 in humans [46, 54]. nNOS gene transcribes multiple mRNA transcripts using alternate promoters usage, alternate splicing, cassette insertions/deletions, polyadenylation and different sites for 3'-UTR cleavage making transcriptional regulation of nNOS very complex [46, 55, 56, 57]. Literature suggests that cellular and tissue-specific regulation of nNOS is attributed to various transcription factors, such as the activator protein-2, the transcriptional enhancer factor-1/MCAT binding factor, cAMP response element-binding protein (CREB)/activating transcription factor/c-Fos, nuclear respiratory factor-1, Ets, nuclear factor-1 and NF-kappa B [54, 58]. Binding of CREB to cAMP response element within the nNOS exon 2 promoter makes nNOS a Ca(2+)-regulated gene, which is likely to be involved in the regulation of nNOS in response to neuronal injury and activity-dependent plasticity [58]. Besides that, nNOS is subjected to alternative splicing, and more than ten different spliced variants of nNOS have been reported [59, 60, 61, 62], which potentially encompass unique structural features and catalytic properties. In the brain, the exon 2 – containing nNOS α form is the major splice variant, whereas nNOS μ is predominant splice variant in skeletal muscle and heart [60]. nNOS-2 detected in mouse brain acts as a dominant negative regulator of nNOS activity because of 105-amino acid in-frame deletion of residues 504-608 involved in arginine binding[63]. Both nNOS α and nNOS μ are anchored to subcellular

structures via PDZ domain, whereas another two forms, nNOS β , and nNOS γ are cytoplasmic. These transcriptional and post-transcriptional levels of regulation of nNOS have been reviewed elsewhere [46, 55, 56, 57].

3.2 Post-translational regulation of nNOS

The post-translational modifications including phosphorylation, ubiquitination, and interactions with other structural and regulatory proteins to form functional heterologous complex reveals an additional level of complexity in nNOS regulation.

3.2.1 Regulation of nNOS by phosphorylation—The nNOS enzymatic activity is modulated by phosphorylation at multiple sites and facilitates the cross-talk between NO and other signaling pathways via different protein kinases. Phosphorylation of nNOS can occur at multiple residues, including serine (Ser), threonine (The) and tyrosine (Tyr), which has been associated with either activation or inactivation of the enzyme directly or by modulating other regulatory domains present in nNOS. The best-characterized nNOS phosphorylation site is Ser-847, which is located in the reductase domain of the enzyme. Diverse signaling pathways regulate nNOS phosphorylation/dephosphorylation through specific protein kinases and protein phosphatases, including AMP-activated protein kinase[64] protein kinase C (PKC)[65], cyclic-nucleotide-dependent protein kinases[66] and Ca²⁺/calmodulin-dependent protein kinase (CAMK) [67, 68]. CaMKI and CaMKII phosphorylate nNOS at Ser-741 and Ser-847 residues, respectively, to inhibit its enzyme activity [68, 69]. nNOS has also been shown to have putative consensus sequences for phosphorylation by RSK (p90 ribosomal S6 kinases), which is reported to inhibit nNOS enzyme activity by phosphorylation at Ser 847 in human embryonic kidney cell line over-expressing nNOS and RSK1 and treated with epidermal growth factor [70]. The role of BH4 in precluding PKC- mediated phosphorylation of nNOS dimers has been demonstrated in *in vitro* studies via stabilization of nNOS dimers in neurons that are less susceptible to PKC-dependent phosphorylation [71]. NMDAR mediated transient activation of nNOS involved phosphorylation of nNOS at activating and inhibitory sites viz. Ser-1412 and Ser-847, respectively, in a time-dependent manner [72]. Activation of NMDAR leads to AKT (protein kinase B) dependent phosphorylation at Ser-1412 in the C-terminus of nNOS, resulting in enzyme activation with increased NO and cGMP production[73]. However, phosphorylation at Ser-1412 act in a feedback manner assisting phosphorylation at Ser-847 in the alpha helix auto-inhibitory domain of nNOS by calcium-calmodulin-dependent kinase II, resulting in enzyme deactivation[69]. Further, AngII mediated ERK1/2-RSK-nNOS signaling pathway had been demonstrated in NTS to modulate central blood pressure in spontaneous hypertensive rats[74]. The role of these phosphorylation events in nNOS regulation during other cardiovascular diseases including heart failure remains to be investigated.

3.2.2 Regulation of nNOS via ubiquitination/sumoylation—Ubiquitination and sumoylation have arisen as two major post-translational regulatory mechanisms involved in controlling the levels and function of several neuronal proteins besides phosphorylation. Ubiquitination of proteins is a signal for degradation that leads to delivery and degradation of dysfunctional proteins in the 26S proteasome [75]. The ubiquitin-proteasome system (UPS) regulates the fidelity of many proteins in the post-synaptic density [76]. The UPS is a

key non-lysosomal pathway, which is known to participate in the pathogenesis of various neurodegenerative disorders like Parkinson's, and Alzheimer's disease, and diffuse Lewy body disease [77, 78]. The ubiquitination process is initiated by tagging of cellular proteins with multiple ubiquitin (8kDa peptide) molecules, involving series of enzymes including ubiquitin-activating enzymes, ubiquitin-conjugating enzymes and ubiquitin-isopeptide ligases [79, 80]. Ubiquitin ligases are the critical mediators to provide specificity to the reaction via substrate recognition [81]. As deduced from the literature in a cell-free system, nNOS undergoes enhanced proteolytic degradation due to suicide inactivation with metabolism-based inactivators [82], anti-hypertensive agent guanabenz [83] or by the inhibition of the HSP90-based chaperone system with geldanamycin [84]. Moreover, nNOS is found in ubiquitin conjugates in rat brain homogenates [82] Bender et al., 2000a, strongly suggesting the involvement of UPS in the regulation of nNOS *in vivo*. Importantly, the heme-deficient monomeric form of nNOS is preferentially ubiquitinated compared to heme-bound homodimer form. CHIP (carboxy terminus of Hsp 70 interacting protein) a chaperone-dependent E3 ligase has been proposed to act as E3 ligase for nNOS in human embryonic kidney (HEK) 293T cells [85]. As stated, nNOS requires homodimerization and association with Ca^{2+} -calmodulin for NO production [46]. The monomeric form, however, catalyzes the production of superoxide ($\text{O}_2^{\cdot-}$) that immediately forms peroxynitrite in the presence of NO. Therefore, a decrease in dimer/monomer ratio is thought to reduce NO production and its bioavailability. [82]. Therefore, conditions such as iron heme and BH_4 depletion and addition of a dimerization inhibitor that favors a decrease in dimer/monomer ratio of nNOS will have an effect on the proteolytic degradation of nNOS. This suggests that ubiquitination of nNOS can potentially contribute to the regulated proteolysis of the nonfunctional enzyme [86].

Likewise, the conjugation mechanism of small ubiquitin-related modifier (SUMO ~12Kd) is very similar to those of ubiquitin, but the biological functions of sumoylation are different from ubiquitination. They do not provide a signal for proteasomal degradation, but rather inhibits polyubiquitin-mediated degradation. Levels of sumoylated proteins have been reported to be increased in animal models of neurological disorders such as brain ischemia [87]. Interestingly, studies have shown that components of the sumoylation machinery SUMO-1, Ubc9, and PIASx β are all expressed in cerebellar granular cells of the cerebellum [88] which also express nNOS [89] suggesting that nNOS may be post-translationally modified by SUMO-1 in the brain [90]. This third posttranslational modification besides phosphorylation and ubiquitination might be potentially important in the regulation of nNOS function in the brain, and presents a significant unexplored challenge for future research.

3.2.3 Regulation of nNOS by multiple protein interactions—The catalytic activity of nNOS is influenced by the variety of regulatory and structural proteins [20, 45, 46] Using various methods to investigate protein-protein interactions including co-immunoprecipitation, yeast two-hybrid system, fluorescence resonance energy transfer and mass spectrophotometry, others and we have identified multiple interacting partners of nNOS that modulate its stability as well as catalytic activity [36, 47, 48, 91].

1. Regulation of nNOS by Calmodulin: Ubiquitous calcium binding protein, calmodulin was the first NOS-associated protein identified, which act as an allosteric activator in the isoform-specific manner [92]. Ca^{2+} /calmodulin binding to NOS is non-covalent and reversible and has been shown to be indispensable for electron transfer from the reductase domain to the heme group of oxidase domain of NOS [93]. The threshold levels of 400nM of the cytosolic concentration of calcium facilitate calmodulin and calcium binding and its subsequent interaction with nNOS. The calcium concentration below this level causes dissociation of calmodulin from the nNOS, thus acting as a metabolic switch to turn off the enzyme [20]. Decreased calmodulin levels have been demonstrated in the brains of the genetic model of spontaneously hypertensive rats and deoxycorticosterone acetate (DOCA)-salt rats compared with those in Wistar-Kyoto rats, suggesting that intracellular calcium-dependent regulatory mechanism may be impaired [94]. Similarly, decreased expression of calmodulin has been documented in failing hearts, affecting many Ca^{2+} -dependent processes during the end-stage heart failure but the role of calmodulin in the regulation of nNOS in the brain during heart failure remains to be explored [95].

2. Regulation of nNOS by heat shock proteins: The heat shock proteins (HSP) include a family of molecular chaperones responsible for the proper folding and maturation of proteins [96]. Hsp90/Hsp70 based chaperone machinery regulates nNOS activity and turnover via modulating ligand-binding clefts[97]. The selective inhibition of Hsp90 by geldanamycin leads to inactivation of nNOS [85]. Recently, Hsp90 has been suggested to be responsible for the insertion of heme into monomeric apo-nNOS during the limited availability of heme [84]. Consecutively, the inhibition of Hsp90-based chaperones would favor the monomeric form of nNOS leading to its ubiquitin-mediated proteasomal degradation. In contrast, overexpression of Hsp70 promotes nNOS ubiquitination and degradation leading to reduce levels of NO [98]. Elevated levels of Hsp70 in plasma are correlated with the progression of CHF, which might be used as a potential diagnostic biomarker for early detection of CHF [99]. The role of HSP in the regulation of nNOS in the brain during various cardiovascular disease conditions remains to be explored.

3. Regulation of nNOS by PDZ-domain proteins: PDZ-domain containing proteins such as syntrophin, PSD-95, or PSD-93 play a central role in forming multiprotein signaling complexes and are required for the binding of nNOS to different calcium sources[100, 101]. In skeletal muscles, nNOS is attached to the sarcolemma via syntrophin and dystrophin proteins. While in the CNS, nNOS couples to NMDA-NR₂ cytoplasmic tail through PSD95, which is responsible for calcium influx followed by activation of nNOS [102, 103, 104]. Subcellular fractionation of the brain tissue demonstrates that approximately half of the nNOS in the brain is soluble, and another half is associated with membranous fraction via PSD95 [100]. PSD proteins are predominantly expressed at higher synaptic densities and interact with nNOS through PDZ domains. The nNOS/PSD95 interaction involves the unique β -finger PDZ domain of nNOS and PDZ1 or PDZ2 domain of PSD95. These PDZ domain based interactions create a ternary complex, which triggers the calcium-dependent activation of nNOS[101, 105] and subsequent intracellular signaling cascade[102]. Decreased PSD95 expression and function could contribute to the loss of NO-mediated balance leading to exaggerated glutamatergic/NMDAR driven sympathoexcitation in the

PVN in neurogenic cardiovascular diseases [106]. Studies have shown that nNOS-PSD-95 interaction impairs regenerative repair after stroke in rats. Dissociating nNOS-PSD-95 coupling in neurons promotes neuronal differentiation of neural stem cells and improves stroke outcome by promoting regenerative repair [107] and also can prevent cerebral ischemic damage in rodents [108]. Inhibition of nNOS-PSD-95 complex via ZL006, a drug developed, has been suggested as a potential treatment for stroke [108].

Carboxy-terminal PDZ ligand of nNOS (CAPON) competes with PSD95 for interaction with nNOS in the brain, consequently acting as a negative regulator of NO production and its downstream signaling effects [109, 110]. Hence, over-expression of CAPON results in loss of PSD95/nNOS complexes [110], and uncoupling of NMDA-nNOS complex, resulting in abrogation of NO-mediated signaling pathways. CAPON is also reported to interact selectively with monomeric G-proteins, Dexas1 (Dexamethasone-induced ras protein 1) activated by NO donors [109, 111] through the phosphotyrosine-binding domain, suggesting a potentially important role for CAPON in the regulation of neurotransmitter release and neuronal plasticity under normal and pathological conditions [112]. nNOS-CAPON-Dexas1 association contributes to the modulation of anxiety-related behavior via regulating Dexas1-ERK signaling in the hippocampus of mice subjected to chronic mild stress while targeting of nNOS-CAPON binding via small-molecule blocker Tat-CAPON-12C produced an anxiolytic effect [113]. It has been suggested that reduced CAPON expression in stellate ganglia of SHR rats before the development of hypertension compared to age-matched control rats contributes to a pre-disease neuronal phenotype, that enhances calcium handling and cardiac sympathetic neurotransmission providing a relationship between CAPON and NO-dependent pathway [114]. The role of PSD95, interactions with CAPON and Dexas1 in the regulation of nNOS in the brain during various cardiovascular diseases such as heart failure remains to be explored.

CAPON has been extensively studied in schizophrenia [115, 116] because of its role in glutamate neurotransmission, known to be involved in this disease [117]. Interestingly, nNOS expressing PVN neurons are also significantly reduced in schizophrenic and depressive patients as compared to control patients [118], similar, to decreased expression of nNOS [36] as well as significantly decreased number of NADPH-diaphorase positive neurons in the PVN of rats with heart failure [27]. nNOS expression has also been shown to be altered in other human brain areas of patients with schizophrenia and depression [119, 120]. Our recent studies revealed that enhanced expression of CAPON is associated with a decrease in nNOS in the PVN of rats with CHF (Figure 2 A, B). Further, we have demonstrated that knockdown of CAPON using siRNA in isolated NG108 cells increases the levels of nNOS (Figure 2 C). In a preliminary study, we observed that adenovirus mediated upregulation of nNOS in NG108 cells does not affect the expression of CAPON (Figure 2 D). These data taken together suggest that the decreased levels of nNOS in the PVN of CHF rats is likely due to enhanced levels of CAPON leading to nNOS degradation. These reciprocal changes in CAPON and nNOS in the PVN of rats with CHF are reversed by AT₁R blocker [36]. AT₁R blocker also restores the endogenous levels of functional nNOS demonstrated by typical responses in RSNA to blockade of NOS within the PVN of rats with CHF. This data establish that elevated levels of Ang II in CHF up-regulate the expression of CAPON in PVN, which sequesters and dissociate nNOS from the membrane,

resulting in uncoupling of nNOS from NMDAR. This reduces the NO-mediated inhibitory influence and thus an over-activation of the sympathoexcitatory drive from the PVN in CHF. There is high co-morbidity in patients with depression, anxiety, heart failure, and other heart disease [121, 122]. However, the cause(s) for this significant correlation and coincidence of occurrence and underlying pathophysiological relation are not yet known. Depression is an established risk factor for heart disease [123] and 50% of the patients with congestive heart failure clinically manifest the symptoms of depression [121]. It may well be that the central pathways that are involved in CHF and depression/schizophrenia may utilize nNOS and CAPON as part of the neural circuitry for central processing and alterations in this interaction between CAPON and nNOS in the PVN may be common features involved in the pathogenesis of these diseases. Thus, it seems reasonable to hypothesize that the identification of candidate mediators such as CAPON and nNOS, common to heart failure, hypertension, diabetes and depression/schizophrenia may serve as an important convergence point responsible for frequent co-morbidity. The role of CAPON in the regulation of nNOS in the brain during hypertension and diabetes, both known to exhibit enhanced sympatho-excitatory states remains to be explored.

3.2.4 Regulation of nNOS by endogenous inhibitors—In addition to activity and localization, the other mechanism that regulates NO levels are endogenous NOS inhibitors named asymmetrical dimethyl arginine (ADMA) and its structural isomer, symmetric dimethyl arginine (SDMA). ADMA competitively displaced arginine from the substrate-binding site of NOS and, therefore, impede NOS catalytic activity, while SDMA has been shown not to alter NO production [124]. Limited information concerning the role of ADMA and SDMA in the CNS has been available to date, but in the periphery, ADMA has been associated with cardiovascular risk and clinically correlated with the burden of cardiovascular disease [125]. In renal wrap model of hypertension in rats, decreased expression of nNOS along with reduced SDMA levels, but no significant changes in ADMA levels was reported in the PVN during the onset of hypertension [91]. The role of ADMA and SDMA in the regulation of nNOS in the brain during heart failure and diabetes remain to be explored.

A small molecular weight protein, dynein light chain, also known as PIN was identified by Jaffrey and Snyder in 1996 using the N-terminal domain of nNOS in a yeast two-hybrid system. PIN was reported to bind to a 17-residue peptide fragment of nNOS (Met-228 to His-244) and destabilizes the dimeric structure of nNOS [126] leading to catalytically inactive monomeric form. Because of homology of PIN with the light chain of myosin and dynein, it is conceivable that PIN may be involved in nNOS's association with the neuronal cytoskeleton during axonal transport [127, 128]. PIN has also been shown to be co-expressed and colocalizes with nNOS in the cavernosal and dorsal nerves of the penis as well as in the hypothalamic regions of the rat, particularly in the regions of PVN that controls penile erection [129]. Overexpression of PIN leads to a reduced erectile response to electrical field stimulation while shRNA-mediated knockdown of PIN helps reverses age-related ED [130]. Recently, we have shown AngII-mediated increased expression of PIN in the PVN of CHF rats (Figure 2A, B). PIN colocalizes with nNOS in neurons, and also interacts physically with nNOS as demonstrated by our co-immunoprecipitation studies and

destabilizes the nNOS dimers to catalytically impeded monomers susceptible to degradation[48]. Functional experiments also established the significance of PIN in nNOS regulation as microinjection of plasmid construct of PIN cDNA in the in the rat corpora cavernosa, partially reduced the erectile response to electrical field stimulation of the cavernosal nerve supporting role for PIN in inhibiting the nitregeric transmission for erection by reducing NO synthesis in vivo [129]. Furthermore, RNA silencing of PIN in the kidney in the young SHR attenuated the development of hypertension and restored the normal levels of nNOS [131]. PIN also plays a role in nNOS uncoupling in the RVLM via decreasing the ratio of nNOS dimer/monomer and knockdown of PIN restores nNOS dimer/monomer ratio, restored NO content and alleviated oxidative stress in the RVLM resulting in decreased sympathoexcitation and hypertension associated with metabolic syndrome [132]. Other studies suggest that the function of PIN may be a dynein light chain involved in nNOS axonal [133]. In these experiments with recombinant PIN peptide and nNOS protein showed no alteration in nNOS dimer to monomeric ratio, which leads the authors to conclude that PIN does not have any role in the inhibition of nNOS via interfering with dimerization. This conclusion was based on the fact that the PIN interacting interphase sequence in nNOS lies outside its catalytic core, and thus is not a part of the dimerization region of nNOS [134]. The possibility that PIN may have a role in dimer assembly via interfering with nNOS dimerization even outside the dimerization region or via some yet unknown mechanism/s remains to be explored. Examining the effect of PIN silencing on NO-mediated sympathoinhibition in the PVN of CHF rats, for instance, will help to explore the molecular mechanism further and potentially provide therapeutic targets for intervention in sympatho-excitatory states. The role of PIN, regulated by Ang II, in the regulation of nNOS in the brain during various cardiovascular disease conditions, such as hypertension remains to be explored.

Work from our lab has shown that increased PIN expression (Figure 2A) with concomitant decrease in dimer to monomer ratio in the PVN during CHF (Figure 3A, C), points towards the accumulation of monomeric inactive nNOS enzyme, which leads to ubiquitin-mediated proteolytic degradation, and hence a decrease in levels of nNOS in CHF[48](Figure 3B,C). We also demonstrated an increased accumulation of Ub-nNOS conjugates in the PVN of CHF rats compared to control rats and also provided evidence for Ub-colocalization with nNOS *in vivo* (Figure 3D). Taken together our studies have revealed that nNOS in the PVN is regulated post-translationally by multiple protein-protein interactions as summarized in Figure 4. Monomerization of nNOS via PIN causes the formation of catalytically impaired enzyme leading to reduced levels of NO. Decreased NO level is expected to reduce GABA release and its inhibitory actions [16] resulting in reduced inhibitory influences on neurons in the PVN, and thereby, causing enhanced sympathoexcitation during CHF. NO would also be expected to counteract the enhanced glutamatergic actions observed in rats with CHF [16]. Recently, we demonstrated that up-regulation of nNOS transgenically, significantly decreased the enhanced renal sympathetic activity, arterial blood pressure, and heart rate responses to *N*-methyl-D-aspartic acid in the rats with CHF [23] which suggests that maybe PIN indirectly regulates NMDA receptors via manipulating functional nNOS levels. However, the details of these complex interactions remain to be explored. Nevertheless, taken together, these observations provide a significant insight into the possible

mechanism(s) within the PVN that may contribute to the increased sympathetic drive commonly observed during CHF and offer a novel target for treatment of the enhanced sympathoexcitation in CHF and other cardiovascular diseases.

4. Expert Opinion

Prevailing evidence indicates that the CNS, particularly the PVN is critically responsible for activation of neurohumoral drive in various cardiovascular diseases including CHF [9, 10, 11, 12]. Numerous studies have shown a significant role for nNOS in the regulation of sympathoexcitation [33, 34, 35, 36, 37, 38, 39, 40, 135]. Decrease in nNOS expression and NO bioavailability are evident in several autonomic regions suggesting that decreased nNOS activity may give rise to an overdrive of pre-autonomic neurons within the CNS which have detrimental pathological consequences in these cardiovascular diseases [33, 34, 35, 36, 37, 38, 39]. Despite the intensive research and attempts to develop NO-donors and NOS inhibitors, no significant success in the use of NO-related drugs in cardiovascular medicine has been achieved to date. The studies discussed in this review provide valuable insight into the possible post-translational modifications of nNOS including phosphorylation, ubiquitination, sumoylation, interaction with endogenous proteins viz. CAPON, PIN, HSP etc. in the regulation of nNOS. The importance of these molecules as critical regulator/s of nNOS function suggests that these molecules or their interactions may be the rational therapeutic targets in pathologies associated with dysregulation of nNOS. As literature [30, 35, 36, 37, 52, 53] suggest that NO is a Janus-faced molecule having two contrasting aspects, and incongruous release of this mediator has been linked to a number of pathologies, therefore, a fine regulation of nNOS within specific key brain nuclei such as the PVN is critical for maintaining cardiovascular homeostasis. The knowledge of isoform-specific structural features of the catalytic site, characterization of the interactions with modulating intracellular proteins and application of modern inhibitor design approaches in combination with mutagenesis should result in novel nNOS selective targeting with discrete pharmacological effects. The biochemical pathways known to regulate nNOS in the autonomic nervous system have not been investigated adequately under normal or pathological conditions to date. Our work provides significant insight into the possible molecular mechanism(s), specifically in the PVN, involved in regulation nNOS in CHF via ubiquitin-mediated mechanisms to regulate sympathetic outflow [36, 48]. Although restoration of nNOS centrally via targeted viral vectors or exercise training has shown beneficial effects in animal models [23, 136], further studies are required to determine whether nNOS or factors that regulate nNOS such as CAPON, PIN and CHIP are responsible for the observed benefits. Deciphering the underlying regulatory mechanisms involved in the upregulation of PIN and CAPON in the brain and identification of E3 ligase for nNOS ubiquitination during various cardiovascular diseases will yield further insight into the regulation of nNOS. Targeting nNOS and its upstream, as well as downstream regulatory molecules in the CNS, may be a worthwhile therapeutic strategy in CHF, hypertension, and other diseases associated with increased sympathoexcitation. The design of inhibitors or nanoparticles to target nNOS-CAPON, nNOS-PIN interphase, inhibition of E3 ligase involved in ubiquitination of nNOS or supplementation of food with BH4 to restore the active dimers can help to produce adequate NO in patients. On the whole, understanding

molecular and cellular mechanisms involved in regulation of nNOS and NO signaling may provide novel therapeutic targets to reduce sympathetic outflow for better management of these autonomic abnormalities in patients with cardiovascular diseases.

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Highlights

- Anomalous regulation of the sympathetic nervous system leading to exaggerated sympathetic nerve activity is implicated in a broad spectrum of cardiovascular diseases.
- nNOS is the primary source for NO synthesized in the brain and is abundantly expressed in the various areas including those involved in the regulation of cardiovascular functions, such as the paraventricular nucleus of the hypothalamus.
- Various pathological cardiovascular states are associated with diminished expression of nNOS within key central sites involved in regulation of the central nervous system (CNS) including heart failure, hypertension, diabetes mellitus and chronic renal failure.
- This review focuses on the regulation of nNOS at the posttranslational level within the CNS in the context of healthy and diseased states.
- Gaining further insight into the mechanism/s involved in the regulation of nNOS would provide potential novel strategies for manipulating nNOS directed therapeutic modalities in the future.

Figure 1.

(A) Illustration of the molecular structure of NOS dimer. All NOS monomers include an oxygenase and a reductase domain and calmodulin (CaM) binding site. The oxygenase moiety comprises the L-arginine, heme, and tetrahydrobiopterin (BH4)-binding domains; whereas the reductase moiety includes the flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH)- binding domains. NOS monomers are unable to bind the cofactor BH4 or the substrate arginine to catalyze the NO production. In the presence of heme, NOS can form a functional dimer that can bind to BH4 and L-Arg that allows interdomain electron transfer. (B) Schematic illustration of the protein structure of NOS isoforms.

Figure 2.

(A) Composite data were showing the expression of nNOS, CAPON, and PIN in the PVN of CHF rats. Values are mean $\pm$ SEM (n = 4–5 rats per group). *P < 0.05 vs. Sham. Modified from Sharma *et al* (2011)[36] and Sharma *et al* (2013)[48](B) Immunofluorescent photomicrographs from the sections of the PVN stained for nNOS, CAPON, and PIN. The intensity of nNOS staining (green) is decreased while the intensity of CAPON and PIN as shown in red is increased in the PVN of CHF rats. Yellow staining showed colocalization of nNOS-PIN or nNOS-CAPON in the same neuron. Blue spots showed the nucleus stained by DAPI. Modified from Sharma *et al* (2011)[36] and Sharma *et al* (2013)[48] (C) Effect of silencing of CAPON on nNOS expression in NG108 neuronal cell line. siRNA CAPON or negative control (20 pmol) was transfected in NG108 cells using Lipofectamine 2000 as per the manufacturer's instructions. Values were mean $\pm$ SE from four Independent experiments. From Sharma *et al* (2011)[36]. (D) Effect of upregulation of nNOS on the CAPON expression. Adenovirus vectors encoding nNOS (AdnNOS, 10^5 – 10^8 pfu/ml, 48hrs) or adenovirus vectors encoding EGFP (AdEGFP, 10^5 pfu/ml, 48hrs) were transfected into NG108 cells

Figure 3.

(A) Assessment of monomers and dimers of nNOS represented as a ratio of dimer to monomer and (B) high molecular weight ubiquitinated forms of immuno-detectable nNOS in the PVN of Sham and CHF rats. Dimeric and monomeric nNOS were separated by low temperature-PAGE in the cold room and visualized by immunoblotting using an anti-nNOS antibody. (C) Composite data of Dimer/Monomer ratio and nNOS-Ub in the PVN of Sham and CHF rats. Values are mean $\pm$ SEM ($n = 4-5$ rats per group). * $P < 0.05$ vs. Sham. (D) High magnification images of the coronal sections of PVN (63X) showing the colocalization of Ub(red) and nNOS (green). From Sharma *et al* (2013)[48]

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Figure 4.

Schematic depicting presumptive pathway of nNOS regulation in the PVN of CHF rats. CAPON and PIN are over-expressed due to augmented levels of Ang II and AT₁ receptors in the PVN. Increased CAPON competes with PSD95 for binding to nNOS and sequesters nNOS therefore decreasing NMDAR/PSD95/nNOS complexes at the synaptic membrane. Binding of PIN to nNOS in cytosol destabilizes nNOS dimers, which renders nNOS catalytically inactive, either by interfering with the assembly or dimer stability. Inactive nNOS monomers are susceptible to ubiquitination and subsequent proteasomal degradation. This results in decreased levels of nNOS as well as NO production in the PVN during CHF causing an increase in sympathetic nerve activity (SNA). Modified from Sharma *et al* (2013) [48]