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Coordination of physiologic and toxic pathways in hippocampus by nitric oxide and mitochondria

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1. ABSTRACT

The hippocampus, one of the most vulnerable regions in the brain, has been implicated in learning and memory formation. However, impairment of hippocampal function is observed during normal aging and neurodegenerative processes. Current evidence suggests that mitochondria and NO participate prominently in cellular signaling in the hippocampus integrating physiologic and toxic pathways. Although all isoforms of nitric oxide synthase are expressed in hippocampal cells, the production of NO in the dependency of glutamate receptors is primarily involved in hippocampal physiopathology. Taking into consideration that the biological impact of NO remains largely qualitative, this review discusses generally the regulation of glutamate-dependent NO production in hippocampus with implications in synaptic plasticity and explores mechanisms by which NO and mitochondria coordinate physiologic and toxic pathways, in particular the excitotoxic NO-mitochondrial connection, the excitotoxic-dependent DNA damage and the mitochondrial biogenesis and trafficking.

2. INTRODUCTION

Nitric oxide (NO), synthesized by nitric oxide synthases (NOS), has been identified as an intercellular neuronal modulator implicated in survival and death pathways (1).

The understanding of NO as a diffusible messenger requires the knowledge of, at least, three related issues: 1) the molecular mechanisms that underlie the activation of the distinct isoforms of NOS; 2) the regulation of NOS activity, 3) the diffusion of NO and its reaction with potential targets.

In contrast with the highly regulated mechanism 1) and 2), once synthesized, the reactions of NO in a cellular setting are assumed to be largely unregulated, depending on the local availability of targets, including hemoproteins (hemoglobin, soluble guanylate cyclase, cytochrome c oxidase), radicals (superoxide anion, lipid peroxyl radicals) and thiol groups (GSH, cysteine residues in proteins). Whereas some of these interactions (*e.g.*

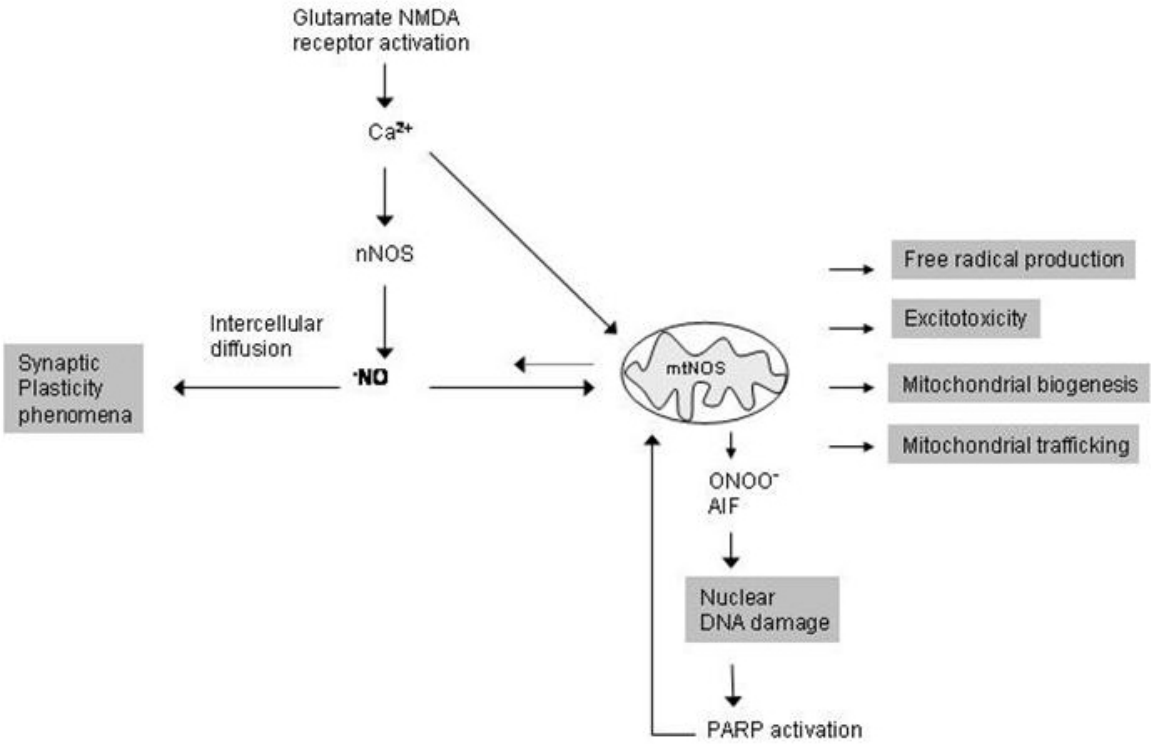

Figure 1. Overview of physiologic and toxic pathways in hippocampus coordinated by NO and mitochondria.

guanylate cyclase, leading to an increase in intracellular cGMP) underlie physiological pathways, others (*e.g.,* superoxide anion ($O_2^{\cdot-}$) leading to the formation of peroxynitrite) may trigger cytotoxicity.

This rationale has formatted the notion that NO concentration determines its actions; the noxious actions of NO being exerted at high (micromolar) concentrations but recent work has challenged the occurrence of such high NO concentrations (2,3). Furthermore, one should emphasize that the evanescent nature of NO makes the evaluation of its concentration dynamics in time and space a difficult task and it is clear that, being a intercellular messenger integrating the activity of neurons in a volume of tissue, the rate and pattern of concentration change is a critical piece of knowledge to gain insights on its modulatory role in the brain. Thus, it should be realized that the biological impact of NO remains largely qualitative, with consequent limitations in the prediction of NO actions as an intercellular messenger. Establishing a quantitative relationship between the profiles of NO with its actions will open a window to see more dynamic events. This can be critically illustrated by a recent study showing that for similar conditions of glutamate NMDA receptor stimulation, distinct profiles of NO concentration dynamics were observed in the different subregions along the trysynaptic loop in hippocampal slices (4).

Notwithstanding the above mentioned notions it is clear that NO plays prominent roles in hippocampal functions, ranging from neuronal plasticity to neurotoxicity (5). At the subcellular level, the interactions of NO with mitochondrial components are likely to support mechanistically many of the NO actions in hippocampus, including the glutamate-dependent neuronal injury. It is now considered that in addition to the essential role in bioenergetics, mitochondria participate in cellular signaling integrating pathways in which NO has a major role (6). The functional coupling of glutamate receptor activation (particularly the NMDA subtype) with NO production, Ca^{2+} load, mitochondrial membrane depolarization and free radical production has been established. In particular, studies in cell cultures and animal models have shown that following Ca^{2+} influx through NMDA receptors, the production of NO, mitochondrial dysfunction, DNA damage and poly(ADP-ribose) polymerase-1 (PARP-1) activation are critical components in the toxic cascade that ultimately leads to the cell death that occurs in neurodegenerative diseases (7-9).

However, the link between NO and mitochondria in neuronal injury goes beyond the mechanisms that trigger mitochondrial dysfunction. Neurons, and cells in general, require biosynthesis and location of mitochondria in appropriate cellular places in order to locally accomplish the metabolic and ion homeostasis demands. This is particular relevant in hippocampal neurons where the distribution of mitochondria is highly heterogenic (10). Thus, the observation that NO inhibits mitochondrial biogenesis (11) and movement in neurons may represent a key event in neurodegeneration (12).

Overall, it may be surmised that NO metabolism and mitochondrial events are interacting phenomena with profound consequences for hippocampal functions, from both, a physiologic and a pathologic standpoint (Figure 1).

3. NITRIC OXIDE PRODUCTION IN HIPPOCAMPUS

The hippocampus is part of the brain medial temporal lobe and has been implicated in learning and memory formation. The hippocampus is one of the most vulnerable regions in the brain. Impairment in this brain region is observed during normal aging and more severe degeneration has been observed in neurodegenerative processes where memory impairment is seen, notably Alzheimer's disease (13).

The principal cells in the hippocampus are organized in two layers, namely the pyramidal cells of the hippocampal proper (consisting mainly in the CA1 and CA3 sub regions) and the granular cells of the dentate gyrus that bend over each other forming two C-shaped structures (14). The hippocampus receives neuronal input from the entorhinal cortex to the granular cells of dentate gyrus which project mossy fibers to the pyramidal neurons of CA3 sub region that, in turn, project the Schaffer collaterals to the CA1 pyramidal neurons. The synapses of this so called "trysinaptic loop" (DG, CA3, CA1 sub regions) are excitatory and use glutamate as a neurotransmitter (13).

In the hippocampus, fast excitatory synaptic transmission is mediated mainly by two classes of ionotropic glutamate receptors, alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-aspartate (NMDA) (15).
As a neural modulator, NO has been associated with the physiological processes in hippocampus, including learning, memory and neurotransmiter release (16). On the other hand, it has been also implicated in the cell death mechanisms that accompany neurodegenerative diseases (5,17).

The current paradigm of NO biology asserts that its actions as a physiological regulator or as a toxin are largely determined by the spatio-temporal variation of its concentration. Being a small highly diffusible signaling molecule, overcoming storage and selective membrane receptor recognition, a transient local production is translated into a cellular response. Thus, the regulation of its synthesis and the biochemical environment of nitric oxide synthase, are crucial determinants of NO bioactivity. Biochemically, NO exerts its effects by reversible binding to hemoproteins (stimulating guanylate cyclase or inhibiting cytochrome oxidase), by post-translational modification of proteins (*e.g.* nitrosation of cysteine residues) or other redox interactions with, for instance, GSH and free radicals. Among the later, the fast reaction between NO and O_2^-, producing the nitrating and oxidizing species peroxynitrite, has received much attention as it is suggested to support many of the toxic effects of NO (18).

However, a general problem concerning the study of NO bioactivity is that research is performed under conditions where the biological samples are, usually, exposed to high non-physiological O_2 tensions; *i.e.* conditions that favor the reaction of NO with O_2 that not only produces highly oxidant derivatives but competes with other biological targets. Also, experiments rely largely on the exposure to NO donors (and not on the endogenous production of NO) and on the use of nitric oxide synthase inhibitors. In this regard, it was recently suggested that up-regulated activity of particular NOS isoforms in the hippocampus of freely moving rats might compensate for the inhibition of the others and, therefore, caution be employed when utilizing nitric oxide synthase inhibitors (19). Finally, indirect measurement of NO (*e.g.* stable derivatives) or measurements that miss the concentration dynamics are typically performed. Under such conditions, not only is unclear what are the levels of NO and its derivatives but also what is the rate and pattern of change in time and space. Consequently, it is difficult to translate data from these experiments into the environment of NO diffusional field in tissues. Technology developments are required for the quantitative understanding of the biological activity of NO as an intercellular diffusional messenger. In this regard, the development of electrochemical sensors to be inserted in brain tissue (20) and fluorescence-based imaging approaches (21,22) are promising approaches in that direction. For instance, it was recently shown by means of an electrochemical microsensor that following stimulation of multiple NMDA receptors, NO can diffuse hundreds of microns in rat hippocampal slices peaking transiently in the nanomolar range (4).

3.1. Constitutive and inducible nitric oxide synthases in hippocampus

Enzymatic production of NO is catalyzed by nitric oxide synthase (NOS) of which there are three well characterized isoforms, products of three distinct genes and initially named after the tissues where they were first isolated; the constitutively expressed neuronal (nNOS or NOS I) and endothelial (eNOS or NOS III) isoforms and the inducible (iNOS or NOS II) expressed upon stimulation. NOS catalyse the conversion of L-arginine to citrulline and NO. Currently, it is accepted that all isoforms can be induced through transcriptional and post-transcriptional mechanisms and can be constitutively expressed (23). For instance, spliced variants of nNOS (in particular nNOSbeta and nNOSgamma) were recognized immunohistochemically and by Western blot analysis in reactive astrocytes (which typically express the iNOS) in the spinal cord of amyotrophic lateral sclerosis patients (24,25).

The NOS reaction is a 5 electron oxidation which requires NADPH and O_2 as co-substrates. All isoforms are homodimeric and require binding of the cofactors FAD, FMN, iron protoporphyrin X heme and tetrahydrobiopterin for activity (23). Also, the three isoforms require Ca^{2+}-calmodulin for activity. However, while the activity of nNOS and eNOS is tightly regulated by intracellular Ca^{2+} level, iNOS binds calmodulin even for very low Ca^{2+} concentrations, so it is usually referred to as being independent (23).

In the central nervous system of mammals, namely in the hippocampus, all three isoforms of NOS are observed. The neuronal isoform was originally isolated

from the cerebellum (26) and it has since been identified in many other brain regions, with different degrees of expression, being observed not only in the different regions, but also in different cell types within the same region. A recent report showed that nNOS is present in the olfactory pathway, in all neocortical regions and cortical layers, cerebellum and striatum (27), thus confirming in three distinct strains of mice what other authors had described for rat (28).

Regarding the hippocampus, initial studies found the expression of nNOS in gabaergic interneurons (29,30), but no nNOS was found in the pyramidal neurons of CA1. This finding greatly conflicted with growing evidence suggesting NO as a major participant in synaptic plasticity process in the CA3/CA1 synapses of the hippocampus. Eventually, nNOS immunoreactivity was demonstrated to exist in dendrites and cell bodies of the CA1 pyramidal neurons in hippocampal sections (31,32) and, furthermore, species and development variations were observed (33). Western blott studies revealed the presence of nNOS in all the sub-regions (CA1, CA3 and dentate gyrus) of hippocampus of young adult rats (34).

The endothelial isoform of NOS, eNOS, initially supposed to be expressed both in the endothelial cells of brain vasculature and in neuronal cells, is likely limited to the blood vessels in rat brain, mainly arteries and arterioles, although low levels of protein were observed in veins, venules and capillaries (35). Interestingly, although in the hippocampus eNOS is confined to the vasculature (33), NO generated from eNOS, in addition to that from nNOS, appears to contribute to synaptic plasticity and, moreover, gene deletion studies in mice suggested that one isoform can compensate for the other (36).

The inducible isoform of NOS is generally expressed by activated macrophages upon exposure to several stimuli such as cytokines. It is a high output enzyme, it produces a high concentration of NO for long periods of time (hours to days, provided substrate and cofactors are available) (23). Some authors have found iNOS expression in astocytes in general, in endothelial cells and even in cytokine-stimulated neuronal cell lines and primary neuronal cultures (24,37-39). However, a recent report showed that iNOS expression in rat hippocampal slice cultures is exclusive of activated microglia, the immunological competent cells of the CNS (3).

A mitochondrial isoform of NOS, the mtNOS, has been described in several tissues, including rat hippocampus (40), where, among other functions, may play a role in the normal physiology of brain development (41). A mtNOS activity of 0.31-0.48 nmol NO/min mg protein has been detected (42), although the production of NO in mouse brain mitochondria has been disputed (43).

3.2. Mechanisms involving glutamate receptors

Glutamate is the main excitatory neurotransmitter in the central nervous system. In the hippocampus, and the CNS in general, NOS activity is intimately related to the activation of glutamate receptors, primarily the NMDA subtype (5). NMDA receptor is highly permeable to Ca^{2+} ions which, following influx through the receptor induces nNOS activation, among a multitude of other cellular processes. Biochemically, receptor and enzyme are linked through protein-protein interactions, both contain PDZ (postsynaptic density-95/Discs large/zona occludens-1) domains which bind to the post-synaptic scaffold protein PSD-95 (44,45). Other proteins, such as CAPON and synapsin 1, are associated with nNOS and may provide regulation of its activity (46).

The co-localization of nNOS, NMDA receptor and PSD-95 in the CA1 pyramidal neurons of rat hippocampus was demonstrated in 2002 by Burette *et al.* (32). The same authors also showed that while this complex is postsynaptic, the main molecular target for NO, soluble guanylyl cyclase, is localized in the presynaptic neuron, thus providing strong support to the hypothesis that NO acts as a retrograde messenger in the mechanisms of synaptic plasticity in the CA1 subregion of the hippocampus.

The arrangement of NMDA receptor with nNOS in a macromolecular cluster rises the possibility of a tight and dynamic regulation of NO production via stimulation of the NMDA receptor, *i.e.* the nNOS is localized within the sphere of increased Ca^{2+} concentrations that flows through the NMDA receptor, thus facilitating an efficient production of NO (47). Given this scenario, it has been argued (48) that activation of nNOS via NMDA receptor is not suitable to tune NO gradients because the only variation of NO concentration via NMDA receptor is towards high pathological, and the prolonged activation of the receptor causes toxicity and neuronal death. In fact, uncoupling the interaction between NMDA receptor and PSD-95 protects neurons against glutamate-mediated toxicity (48).

However, a number of studies point to a more complex picture concerning regulation of NO production via NMDA receptor and, consequently, to the role of NO in NMDA receptor-mediated physiologic and pathologic pathways, as follows. (a) A sustained activity of NMDA receptor-dependent NOS in hippocampal tissue can generate only low nanomolar NO concentrations, which are unlikely to be toxic (49). (b) When following the concentration dynamics of NO in hippocampal slices it has been verified that, even under conditions of sustained stimulation of NMDA receptor, NO increases only transiently peaking at less than hundreds of nanomolar, returning shortly to basal levels (4). (c) Neither endogenous NO, nor exogenous NO in supra-physiological concentrations inhibits synaptic NMDA receptors via a feedback mechanism, as it is claimed to occur via nitrosation of a critical cysteine residue (4,50). (d) Differential stimulation of NMDA receptor exerts distinct effects on nNOS activity, whereas low glutamate reversible inhibits nNOS via phosporylation by the Ca^{2+}-calmodulin protein kinase II (CaMKII), high doses of glutamate stimulate nNOS via dephosphorylation by protein phosphatase 1 (51). If one further considers the differential Ca^{2+} sensitivity of NMDA receptor among the hipoccampal

subregions (*e.g.* CA1 *vs* CA3) (52) it is difficult to predict whether the final outcome on NMDA receptor will be upregulation of nNOS activity to toxic high NO concentrations.

Activation of non-NMDA ionotropic (AMPA/kainate) and metabotropic glutamate receptors have been also implicated in NO production (53) in hippocampus, but involving less characterized pathways. That AMPA receptors are implicated in nNOS activation has been shown years ago in cerebellar slices prepared from adult rats (54). Similarly to NMDA receptor, AMPA receptor (subunits GluR2/3), also contain a PDZ domain on their C-terminal but, conversely to NMDA receptor, this domain interact with proteins (such as GRIP, glutamate receptor interacting protein, and ABP, AMPA-receptor binding protein), other than PSD-95 (55,56). Thus, at variance with NMDA receptor, AMPA receptor is not physically linked to nNOS. It follows that AMPA activation leads to a more subtle NO production (the rise in intracellular Ca2+ required for NOS stimulation can be achieved through permeable AMPA receptors or voltage sensitive Ca2+ channels) being more appropriate for a fine tuning of NO signaling, as compared with the more robust production of NO following activation of the NMDA receptor. In line with this notion, it was recently shown that AMPA and NMDA receptors mediate NO production in a layer specific-manner in rat cerebellar slices and, moreover, that the activation of AMPA receptor leads to a lower production of NO (21).

Finally, it has to be emphasized that regulation of nNOS may be further achieved through synthesis of several nNOS mRNA transcripts. The alternative splicing produces nNOS proteins differing in both enzymatic characteristics and structural features (57). Additionally, NO production may be influenced by other neurotransmitters (GABA, acetylcholine, neuropeptides) through polysynaptic circuits interacting with the glutamatergic system (58).

4. HIPPOCAMPAL PATHWAYS ORCHESTRATED BY NO AND MITOCHONDRIA

4.1. Synaptic plasticity and neuronal development

The notion of synaptic plasticity refers to the change in the strength of synaptic connections, breaking or making of new connections in response to a given stimulus and has been associated to memory and learning in hippocampus. In this context, long-term potentiation (LTP) has been the most studied model (59) as it consists of the enhancement of synaptic strength that can last for a period of hours, days or even weeks, and is seen as the molecular/neural basis of memory and learning in vertebrates. Strong stimulation of the postsynaptic neuron causes LTP and the main trigger for the process is Ca2+ elevation resulting mainly from NMDA receptor activation (60). Enhancement in synaptic strength can result from an increase of transmitter release from the terminal or an increase in postsynaptic sensitivity to released transmitter or both. The latter can be achieved by increasing the receptor density at the membrane.

For some time it remained unappreciated how the increase in postsynaptic Ca^{2+} levels was able to cause an increase in the amount of transmitter released from the synaptic terminal. This phenomenon called for a retrograde messenger to be present; *i.e.* a species that, once produced in the postsynaptic terminal was able to reach the presynaptic neuron and cause such an effect. In 1991 two groups independently showed that NO was produced at the postsynaptic side of the CA1 synapse in result of tetanic stimulation of the Schaffer colateral terminals (61,62). Both groups showed that NO synthesis was required for LTP to be observed and O'Dell *et al* (61) also demonstrated that NO needed to reach the extracellular space. Later it was confirmed that NO was produced in the postsynaptic side of the hippocampal synapse, that it must diffuse into the synaptic cleft and, most importantly, that it reaching the prysynaptic terminal was fundamental for tetanic-induced LTP to be observed (63). More recently, the retroactions of NO, implicating its extracellular diffusion, have been shown in the regulation of synaptic vesicle endocytosis and recycling in hippocampal neurons; specifically, NO generated postsynaptically via NMDA receptor was shown to regulate the presynatic vesicle endocytosis (64,65). Following the initial seminal proposal by Garthwaite *et al.* (64), these experiments consisted in remarkable support for NO as a diffusional intercellular messenger in hippocampus.

Apparently, both nNOS and eNOS play a role in LTP (36,66). More recently, the levels of nNOS expression in the hippocampus of rats were correlated with animal performance during cognitive/memory assessment behavioral tasks (67).

Nitric oxide also plays a part in neuronal development in the CNS in general and particularly in the hippocampus where it modulates neuronal differentiation and maturation as well as local blood flow. In view of this, distinct ontogenic profiles of NOS expression have been described (68). A recent report studied the ontogenic profile of both nNOS and eNOS in the guinea pig hippocampus and found that eNOS expression increased constantly during development, reaching maximal level in the adult. nNOS expression, on the other hand, reached maximal level, equal to that observed in the adult, during the gestational period (69).

Interestingly, this profile differs between species; in the rat hippocampus, for example, eNOS expression is constant during all development (70) and nNOS expression increases gradually only postnatally (70,71). It was also found that, during rat brain maturation, the expression and activity of a mitochondrial isoform of NOS peaked at the late embryonic stages decreasing in the adult stage, a pattern opposite to that found for the cytosolic nNOS (41).

Overall, the results suggest a coordination of NO production via the cytosolic and mitochondrial isoforms with impact in the brain development.

4.2. The excitotoxic NO-mitochondrial pathway

An excessive synaptic release of glutamate with the consequent activation of postsynaptic glutamate receptors triggers excitotoxicity, a phenomenon implicated in the pathogenesis of neurodegeneration occurring in acute

insults and neurodegenerative diseases (72). Whereas, the various subtypes of glutamate receptors have been implicated in excitotoxic cell death, the NMDA subtype is supposed to have a major role owing to its high permeability to Ca^{2+} (47).

It has been known for many years that glutamate kills neurons via pathways that are dependent on Ca^{2+} (73,74). Neurons posses homeostatic mechanisms to maintain a low intracellular Ca^{2+} concentration which, under physiological conditions, mediates several cell signaling pathways, but a sustained increase in the cytosolic Ca^{2+} concentration is believed to trigger downstream events that mediate neuronal death (47). An overload of Ca^{2+} may generally lead to inappropriate activation of proteases, lipases, phosphatases and endonucleases, resulting in a spread damage to cell structures (75). However, although a close relationship between excessive Ca^{2+} influx and neuronal injury has been established (76), the magnitude and temporal change of intracellular Ca^{2+} concentration are not the only determinants for the glutamate-dependent neuronal death. This conclusion can be inferred, for instance, from studies showing that cortical slices from young and adult rats challenged with glutamate exhibited comparable intracellular Ca^{2+} changes but different ADP/ATP and NAD/NADH ratios, as well as different rates of reactive oxygen species formation (77). On the other hand, NMDA currents can be modulated by intracellular Ca^{2+} and this process exhibits hippocampal sub-regional variations; for instance, whereas an increase in intracellular Ca^{2+} levels strongly depressed NMDA currents in CA3 pyramidal cells of hippocampus, only minor effects were observed in CA1 region (52).

Sattler *et al.* (78) suggested that flux pathways, not Ca^{2+} load, determines Ca^{2+}-mediated neurotoxicity, for whereas Ca^{2+} loading via NMDA receptor was highly toxic to cultured cortical neurons, identical Ca^{2+} load via voltage-gated Ca^{2+} channels were harmless. However, it is apparent that other critical factors are crucial in neuronal death associated to excitotocicity. Following Ca^{2+} influx through the NMDA receptor, the localization of sensitive targets in the intracellular Ca^{2+} microdomains and the activation of specific biochemical pathways coupling the rise in cytosolic Ca^{2+} concentration to cell death have to be considered. Nitric oxide metabolism, mitochondrial dysfunction, and oxidative stress (79,80) may represent critical determinants in the progression to cell death. These phenomena may constitute sequential as well as interacting factors that may converge in a final common pathway for neuronal vulnerability, as discussed below.

It is known that mitochondria, acting as local Ca^{2+} buffers, shape Ca^{2+} signals in a spatiotemporal fashion. A well established downstream event of NMDA receptor activation in hippocampal neurons is Ca^{2+} accumulation into mitochondria which causes mitochondrial depolarization, free radical production and initiates cell injury (8,81,82). The process encompasses the release of multiple death-promoting proteins, notably cytochrome c, residing in the mitochondrial intermembrane space (83). Such a role for mitochondria in the pathways of Ca^{2+}-dependent damage is tightened by observations showing that the Inhibition of mitochondrial Ca^{2+} uptake, in spite of the consequent increase of cytosolic Ca^{2+} concentration, protects the cells from death (84). However, linear relationships between mitochondrial Ca^{2+} accumulation and mitochondrial function cannot be unambiguously assumed. Studies in individual neurons revealed that glutamate caused an uniform change in mitochondrial Ca^{2+} throughout the population but the change in both time course and amplitude of mitochondrial membrane potential, $\Delta\Psi_{m,}$ (and thus of oxidative phosphorylation) was highly heterogeneous (80). Interestingly, neurons exposed to NO or NMDA exhibited similar rapid depolarization of mitochondria in a way that was prevented by antagonizing NMDA receptor (85). Also, in hippocampal neurons, inhibition of NOS activity by L-NAME significantly attenuated glutamate-dependent loss of mitochondrial membrane potential (80), implicating NO in the mechanisms of injury.

The involvement of NO in glutamate toxicity has been known for many years and was firstly observed in primary cultured cortical neurons (86). Specifically, the production of NO via activation of the NMDA glutamate receptor subtype in the mechanisms of toxicity was notorious. In fact, these studies showed that inhibition of NOS prevented NMDA-induced toxicity and this effect was reversed by addition of NOS substrate (L-arginine). Inhibition of NOS in coronal slices of rat brain resulted in reduction of NMDA toxicity (87) and, moreover, the neuronal cultures from nNOS knockout mice were resistant to the NMDA toxicity (88). Consistent with a critical role for NO in excitotoxicity *in vivo*, the lesions induced by intrastriatal stereotactic NMDA microinjections were smaller in nNOS knockout mice as compared with the wild-type control animals (89).

Nitric oxide has also been shown to modulate glutamate release in hippocampal synaptosomes, either decreasing the exocytotic release via stimulation of guanylate cyclase or causing a massive release of glutamate through the glutamate carrier, probably due to inhibition of mitochondrial respiration and consequent reduction of ATP/ADP ratio (90). Additionally, under inflammatory conditions, occurring, for instance, in neurodegenerative pathologies, the NO from iNOS in activated microglia might secondarily enhance NO production from nNOS via glutamate release (91). Consequently, a synergistic activity of NO and glutamate in apoptosis of neurons has been proposed (92).

Mitochondria are a target for NO (93,94). At the low nanomolar range NO exerts an acute and reversible inhibition of cytochrome oxidase in competition with O_2 (95), but for persistent higher concentrations, NO acts irreversibly at multiple sites compromising cellular energy metabolism (94,96,97). Additionally, the inhibition of cytochrome oxidase by NO is supposed to evoke the redistribution of O_2 to the neighboring cells (98).

Other actions of NO and its derivatives in mitochondria that are relevant to cell death include the

induction of mitochondrial permeability transition, removal of iron from iron sulfur centers, as well as S-nitrosation of proteins, via nitrosonium ion NO+ and S-nitrosothiols, and the production of oxygen and nitrogen reactive species, such as $O_2^{\cdot-}$, H_2O_2 and peroxynitrite (93). The evidence that such reactive species are critical intermediates of the neuronal toxicity subsequent to glutamate exposure is extensive (99,100,101). In particular, following NMDA receptor activation and NO production, the generation of $O_2^{\bullet-}$, by the respiratory chain in mitochondria may result in the formation of peroxynitrite (102) which via oxidation, nitration and nitrosation reactions may modify critical components in the matrix, inner and outer membrane and intermembrane space (103). DNA, lipids and many proteins including the respiratory complexes, most notably complex I, are targets for peroxynitrite (97,104). Interestingly, however, recent studies reported a role for peroxynitrite in triggering cellular survival signals, in particular those related to neuronal apoptosis (105).

Likely, the formation of peroxynitrite in the biological milieu is in competition with the dismutation of $O_2^{\cdot-}$ catalyzed by superoxide dismutase (SOD) and requires a fine tuning of the radical fluxes of both, $O_2^{\cdot-}$ and NO, as well as their proper compartmentalization. This notion acquires a remarkable relevance if one consider that NO and oxygen free radicals are synthesized by brain cells during normal activity and interact in signal transduction pathways, in which peroxynitrite is not known to play a role (106). In particular, $O_2^{\cdot-}$ produced upon NMDA receptor activation appears to work in combination with NO during induction of LTP in hippocampus (107). More recently, it was also demonstrated the requirement for $O_2^{\cdot-}$ (produced via NADPH oxidase) for the NMDA receptor-dependent activation of extracellular signal-regulated kinase (ERK) in CA1 subregion of hippocampus (108).

In this regard, it is pertinent to note that in the rat brain the localization of Cu/Zn-SOD overlaps with that of NOS in the pyramidal cell layers of CA1, CA3 and dentate gyrus subregions of hippocampus (109). On the other hand, at the hippocampal regional level, the vulnerability to $O_2^{\cdot-}$-mediated oxidative stress is heterogenic, being CA1 pyramidal neurons selectively affected (110). This evidence, in connection with the observation that the CA1 region was found to be the site of the highest production of NO following activation of glutamate NMDA receptors (4), suggest that the higher vulnerability of CA1 region to neurodegeneration may be related to favorable conditions for peroxynitrite formation at higher concentration.

Collectively, the previous considerations suggest that, mitochondria and NO metabolism are central to excitotoxicity. In this regard it is pertinent to address the source of NO. Although the several studies mentioned above support the notion that NO produced by nNOS via NMDA receptor activation can inhibit mitochondrial respiration and dissipate mitochondrial membrane potential (80), the specific role of mtNOS in glutamate-mediated excitotoxicity has been hardly addressed. The mtNOS in rat brain is a nNOS distinct form the nNOS in cytoplasm that responds to Ca^{2+} (41). It is therefore likely that, following overstimulation of NMDA receptor, Ca^{2+} accumulation in mitochondria activates mtNOS and a high local NO flux may participate in mitochondrial dysfunction. However, opposing to the simplicity of this rationale, it was recently shown in cultured hippocampal neurons that while cytosolic NOS contributes to toxicity in mature neurons, in immature neurons mtNOS dissipates membrane potential, by inhibiting mitochondrial respiration, which prevents mitochondrial Ca^{2+} uptake, thus mediating the decreased vulnerability to NMDA in the immature cells (111).

On the other hand, it is also known that activated astrocytes or microglia, producing high concentrations of NO via iNOS, inhibit the mitochondrial respiration in cocultured neurons due to inhibition of cytochrome oxidase and cause neuronal death (91).

That the participation of mitochondria and NO in the mechanisms of excitoxicity may be subtlety intermingled may be further illustrated by studies showing that NMDA receptor activation may induce neuronal death by a pathway involving nNOS dephosphorylation (thus increasing its enzymatic activity) via a calcineurin-dependent mechanism. Calcineurin also activates the proapoptotic protein Bax, which, upon translocation from cytosol to mitochondria, promotes the release of propaptotic proteins from the organelle (51).

Finally, it should be noted that not only NMDA but also AMPA glutamate receptor has been implicated in a neurotoxic pathway involving NO and mitochondria. That AMPA receptors are also permeable to Ca^{2+} and its blockage confer neuroprotection has been known (112). More recently, it was shown that activation of AMPA receptors in cultured rat hippocampal neurons causes inhibition of mitochondrial complex I, likely via peroxynitrite (113).

The detailed molecular mechanisms underlying excitotoxicity remain elusive but it is perceived that the whole process proceeds through a complex signaling pathway that includes the participation of multiple interrelated components which may be integrated on basis of NO and mitochondrial metabolism.

4.3. Excitotoxic-dependent DNA damage

The over activation of poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme that is activated in response to DNA damage and facilitates DNA repair, has been implicated in neuronal death (114). In particular, there is strong evidence for a role of PARP-1 in the pathogenesis of NMDA-mediated neurotoxicity (114). A critical observation supporting this idea is that PARP-1 null mice exhibit a significant neuroprotection against excitotoxic injury (9). That NO may be a key player in the process is suggested, among other studies, by the observation that in nNOS knockout mice the NMDA-dependent DNA damage is much less as compared with wild-type mice (89) and that in the mice lacking nNOS, PARP is not activated (115).

The utilization NAD^+ by PARP-1 results in an ATP decrement that is readily observed following PARP-1

activation in neurons and, typically, a compromised energetic metabolism has been suggested as an underlying mechanism to cell death (116). Initially, the formation of NO and activation of NMDA receptors have been linked to DNA single strand breakage and PARP activation (113) but subsequent studies have demonstrated that the effective trigger of DNA damage is likely peroxynitrite rather than NO (117). Thus, following NMDA receptor activation and NO production, the over activation of PARP-1 may be supported by peroxynitrite-mediated DNA strand breaks (118). The genotoxic effects of peroxynitrite are well-known and involve oxidation and nitration of the nucleobases (119,120). Mitochondria were indirectly implicated in the process by experiments showing that neurons exposed to NO or NMDA exhibited similar rapid mitochondrial depolarization which is inhibited by a superoxide dismutase (SOD) mimetic (85). In line with this observation, over expression of manganese SOD (the enzyme located in mitochondria that removes one of the products, $O_2^{\bullet-}$, for formation of peroxynitrite) provided dramatic protection against NMDA and NO toxicity in cortical neurons (121). Thus, mitochondria appear to mediate NO-dependent PARP activation

More recently, the detailed mechanisms of DNA damage directly implicated mitochondria via the release of AIF (apoptosis-inducing factor) from the organelle with subsequent translocation to the nucleus where it initiates DNA fragmentation and chromatin condensation (122). In fact, it was shown that following excitotoxic NMDA receptor stimulation, the activation of PARP induces the translocation of AIF from the mitochondria to the nucleus with subsequent chromatin condensation, DNA fragmentation and nuclear shrinkage, pointing to AIF as a critical downstream effector of PARP-mediated cell death (17). In this process, NAD+ depletion and mitochondrial permeability transition may be necessary steps linking PARP activation to AIF translocation and cell death (123). Thus a link is established relating NMDA receptor-induced excitotoxicity with NO production, DNA damage, PARP-1 activation, and AIF translocation from the mitochondria to the nucleus (9).

In summary, it is currently thought that a key signaling cascade in NMDA excitotoxicity and neurodegeneration consists in the production of $O_2^{\bullet-}$ in mitochondria and NO from nNOS activation via NMDA receptor, although the production of NO from mtNOS cannot be discarded. Under these conditions, peroxynitrite is formed primarily in mitochondria and in turn, damages nuclear DNA and PARP activation occurs. The signaling events downstream of PARP activation are unclear but, in addition to energy depletion (124), may involve the c-Jun N-terminal Kinase (JNK), AIF translocation from mitochondria to the nucleus and subsequent cell death (125). However, one should note that peroxynitrite formation in mitochondria is distal from nuclear DNA and, in spite of the fact that peroxynitrite can diffuse across biomembranes (126), this concern needs to be addressed as, inside mitochondria, its half-life is short (t1/2 ~ 3-5 ms) due to the abundance of metalloproteins, protein thiols and CO_2 (103).

4.4. Mitochondrial biogenesis and trafficking

The reversible competition of NO with O_2 to cytochrome oxidase in the mitochondrial respiratory chain regulates mitochondrial respiration, as discussed above, and plays a role in acute O_2 sensing and in the cell response to hypoxia (127). In addition to these acute effects on the bioenergetic properties of mitochondria, recent evidences support long-term cellular effects of NO on neuronal viability by interfering with mitochondria biogenesis and trafficking in neurons.

The synthesis of new mitochondria require the coordination of the mitochondrial and nuclear genomes and it has been shown that free radical-mediated damage to rat hippocampus *in vivo* leads to mitochondrial biogenesis in a process that is attenuated by inhibition of nNOS (11). Also, it was demonstrated in other mammalian cells and tissues that NO from eNOS, via activation of guanylate cyclase, leads to biogenesis of functional mitochondria (128). Thus, the role of NO in mitochondrial biogenesis may be understood from a pathological point of view, in which NO-mediated mitochondrial dysfunction, a process associated with neurodegenerative diseases, leads to the generation of new mitochondria as part of the cellular response to damage. Alternatively, it may be consider that the stimulation of mitochondrial biogenesis, resulting in enhanced formation of ATP, help the cells to cope with the energy demanding processes regulated by NO, such as synaptic plasticity and neurotransmitter release in the hippocampus.

Additionally to mitochondrial biogenesis, mitochondrial traffic and delivery to the appropriate location within a neuron likely affects mitochondrial and neuronal functions. The distribution of mitochondria in hippocampal neurons is highly heterogenic being present in filamentous form in dendrites in all hippocampal sub regions but, in contrast, the mitochondrial population in axons were found in the form of discrete bodies (10). It has been shown that activation of glutamate NMDA receptor in primary cultures of rat forebrain results in a fast diminution of mitochondrial movement and also alteration of morphology in a process requiring the entry of calcium (129). Likewise, exposure of primary neurons to a NO donor was shown to inhibit mitochondrial movement, although morphology was not altered (12).

Clearly, considering the role of mitochondria in the integration of intracellular signaling pathways, changes in number, morphology and movement promoted by NO may have consequences for physiology and for neurodegeneration.

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