« Human subtlety will never devise an invention more beautiful, more simple, or more direct than does Nature because in her inventions, nothing is lacking and nothing is superfluous... »

Leonardo da Vinci
Acknowledgments

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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Ag/AgCl</td>
<td>Silver/Silver Chloride reference electrode</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4- isoazolepropionic acid</td>
</tr>
<tr>
<td>AMPAR</td>
<td>α-amino-3-hydroxy-5-methyl-4- isoazolepropionic acid receptors</td>
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<tr>
<td>AP</td>
<td>Anterior-Posterior</td>
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<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>Aβ</td>
<td>β-amyloid plaques</td>
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<tr>
<td>Aβ_{1-42}</td>
<td>Aβ-amyloid (fragment 1-42)</td>
</tr>
<tr>
<td>BACE-1</td>
<td>Beta-secretase 1</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CD14</td>
<td>Cluster of Differentiation 14</td>
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<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>EDRF</td>
<td>Endothelium-Derived Relaxing Factor</td>
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<tr>
<td>eNOS</td>
<td>Endothelial isoform of nitric oxide synthase</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
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<td>ERK</td>
<td>Extracellular-Regulated Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
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<td>Flavin Mononucleotide</td>
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<tr>
<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
</tr>
<tr>
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<td>γ-AminoButyric Acid</td>
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<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>H₄B</td>
<td>(6R)-5,6,7,8-tetrahydrobiopterin</td>
</tr>
<tr>
<td>HbO₂</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible isoform of Nitric Oxide Synthase</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KA</td>
<td>Kainate</td>
</tr>
<tr>
<td>K&lt;sub&gt;A&lt;/sub&gt;T&lt;sub&gt;P&lt;/sub&gt;</td>
<td>ATP-sensitive potassium channels</td>
</tr>
<tr>
<td>L-arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>LDF probe</td>
<td>Laser Doppler Flow probe</td>
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<tr>
<td>LO&lt;sup&gt;·&lt;/sup&gt;</td>
<td>Alkoxyl radical</td>
</tr>
<tr>
<td>LOD&lt;sup&gt;·&lt;/sup&gt;</td>
<td>Limit Of Detection</td>
</tr>
<tr>
<td>LOO&lt;sup&gt;·&lt;/sup&gt;</td>
<td>Peroxyl radical</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-Term Depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-Term Potentiation</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>Mitogen-Activated Protein kinase</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle Cerebral Artery Occlusion</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>ML</td>
<td>Medial-Lateral</td>
</tr>
<tr>
<td>mtNOS</td>
<td>Mitochondrial isoform of Nitric Oxide Synthase</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/K&lt;sup&gt;+&lt;/sup&gt;/ATPase</td>
<td>Sodium-Potassium Adenosine Triphosphatase pump</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NFTs</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-Kappa B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartic Acid</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-Methyl-D-Aspartic Acid Receptor</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal isoform of Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>o-PD</td>
<td>o-phenylenediamine</td>
</tr>
<tr>
<td>p38</td>
<td>p38 mitogen-activated protein kinases</td>
</tr>
<tr>
<td>PA</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet Activating Factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein Disulfide Isomerase</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3 kinase</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic Density Protein 95</td>
</tr>
<tr>
<td>RNOS</td>
<td>Reactive oxygen/nitrogen species</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Toll-Like Receptor 4</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor-α</td>
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</table>
Resumo

O cérebro, mais do que qualquer outro órgão, é criticamente dependente de um fornecimento contínuo de sangue. Em resultado da actividade neuronal elevada, necessita de oxigénio e glicose rapidamente e em grande quantidade de um modo espacialmente relacionado com picos de actividade metabólica dos neurónios. Assim, para além da regulação extrínseca do fluxo através do “output” cardíaco, o fluxo sanguíneo necessário para o cérebro é assegurado por um acoplamento espacial e temporal entre a actividade neuronal e a microcirculação cerebral correlacionada, que através de mecanismos específicos, permitem um aumento do fluxo sanguíneo numa determinada região, permitindo deste modo, o suprimento de nutrientes energéticos, bem como a eliminação de resíduos decorrentes da actividade neuronal. Esta fina associação permite deste modo, regular o fluxo sanguíneo cerebral (CBF) local. O paradigma actual para este acoplamento envolve uma ponte astrocitica entre neurónios e vasos. Neste trabalho explora-se uma via alternativa que envolve a difusão isotrópica do óxido nítrico dos neurónios até aos vasos sanguíneos, induzindo aumento localizado de fluxo em função da actividade neuronal.

Por outro lado, é sabido que alterações vasculares, ao interromper estes mecanismos vasoregulatórios que asseguraram um fornecimento adequado de CBF, causam disfunção cerebral, levando a diferentes patologias, nomeadamente em doenças neurodegenerativas como a doença de Alzheimer.

Assim, através de medição directa das dinâmicas de concentração de óxido nítrico in vivo, em tempo real e, mais ainda, em simulatâneo com alterações de CBF no cérebro de animais normais e de animais manifestando condições neuropaotológicas (através da administração de Aβ1-42 assim como de Lipopolissacarídeo), este trabalho fornece novas perspectivas sobre o acoplamento neurovascular, associado ao envelhecimento e neurodegeneração, onde polifenóis provenientes da dieta como a curcumina e a epicatequina, evidenciaram um potencial modulador neste processo de acoplamento.

**Palavras-chave:** Óxido nítrico, Acoplamento Neurovascular, Polifenóis, Doença de Alzheimer, Neuroinflamação.
Abstract

The brain, more than any other organ, is critically dependent on a continuous supply of blood. Due to the high neuronal activity, it needs oxygen and glucose, faster or in greater quantities and in a way that the energy demands imposed by neuronal activity are spatially and temporally correlated with substrate delivery by bloof flow.

Thus, in addition to the regulation of bloof flow by extrinsic factors (cardiac output) the brain has the ability to regulate the blood flow intrinsically. This is ensured by a tight connection between the neuronal activity and cerebral microcirculation, which through specific mechanisms, allow an increase of blood flow in a region (functional hyperemia), thereby enabling the supply of nutrients energy and the disposal of waste arising from neuronal activity.

This neurovascular coupling induces a chain of complex biological mechanisms, resulting in a joint action between neurons, glia and blood vessels, called the neurovascular unit. This fine combination allowing the regulation of cerebral blood flow (CBF) has been mechanistically assigned to than astrocytic bridge between neurons and blood vessels. Here, we explore an alternative hypothesis encompassing the isotropic diffusion of nitric oxide from neurons towards blood vessels, thus inducing vasodilation.

It is known that vascular impairment interrupt these vasoregulatory mechanisms, which ensure the adequate supply of CBF, causing brain dysfunction and leading to different diseases including the neurodegenerative, such as Alzheimer’s. This work aims to clarify the role of nitric oxide as a neuromodulator in the control of neuronal blood flow, as well as explain the influence of dietary polyphenol compounds (curcumin and epicatechin) in the bioavailability of neuronal nitric oxide.

Thus, by means of in vivo and real-time measuring 'NO concentration dynamics in rat brain in simultaneous with measurement of CBF changes in normal animals and animal models of Alzheimer, expressing neuropathological signs (by administration of Aβ1-42 as well as Lipopolysaccharide), this study provides new insights into the neurovascular coupling associated with aging and neurodegeneration, where dietary polyphenols such as curcumin and epicatechin, are evidenced as potential modulator in this coupling process.

Keywords: Nitric Oxide, Neurovascular coupling, Polyphenols, Alzheimer’s Disease, Neuroinflammation.
Chapter 1.

Introduction
I. Nitric oxide

Nitric oxide (NO), a small radical molecule, was identified in biological systems as an important intercellular gas messenger present in the cardiovascular, immune and nervous systems (Koshland, 1992).

During the last three decades, several studies have impart an extraordinary interest to this fascinating molecule, mainly due to its distinct physicochemical properties and diverse bioactivity (Koshland, 1992; Ledo, 2007). In brain, NO plays a dual action, being involved in different physiological and pathological processes. It is implicated in the modulation of neuronal function through signaling pathways involved in synaptic plasticity (Prast and Philippu, 2001) and in the regulation of blood flow (MacMicking et al., 1997). However, NO also acts in different pathways of cell toxicity associated with neurodegenerative diseases and inflammation (Reis, 2006; Pacher et al., 2007).

1. Biochemical Properties and Diffusion of NO

NO is a molecule with only two atoms, being an intermediate between O₂ and N₂. Contrary to what is conventionally found in the literature, NO per se is not very reactive when compared with other O or N-centered radicals (Bonner and Sledman, 1996). In addition to its small size, NO is hydrophobic, highly diffusible and has a short half-life (c.a. 4 s). The high diffusion coefficient (3300 µm²/s) and hydrophobicity allows NO to readily permeate biological membranes and diffuse to considerably high distances to adjacent cells and organelles (Lancaster, 1994; Koppenol, 1998), from its production site, integrating the activity of several cells in a volume of tissue regardless of whether the cells are connected by synapses (Stryer, 1995). Studies have shown that NO can spread to a distance of several microns from its site of synthesis (Miranda et al., 2000). In hippocampal slices, in CA1 subregion, it was demonstrated that NO have the ability to spread approximately 400 µm (Ledo, 2007).

Unlike other neurotransmitters, NO is not stored in vesicles and do not transmit information specifically on basis of its structural characteristics, but instead by changes in it local concentration, mediating a number of physiological pathways quite differently from other neurotransmitters.
Diffusing isotropically in the CNS, the spatio-temporal profile of \(^{\cdot}\)NO concentration is determined by regulation of its synthesis and distribution of its molecular targets (Espey et al., 2002). Also, mechanisms of \(^{\cdot}\)NO degradation are of particular relevance, as physiological processes mediated by \(^{\cdot}\)NO only finish when it removal is complete (Pacher et al., 2007).

The unique chemistry of \(^{\cdot}\)NO allows it to participate in numerous reactions and to be involved in a diverse number of biological mechanisms. The chemical biology of \(^{\cdot}\)NO and its potential reactions can be categorized into two types: direct and indirect (Figure 1).

**Direct effects**

The direct effects rely on the direct reaction of \(^{\cdot}\)NO with molecular targets (metal complexes or radical species) and are usually associated with short exposures and/or low \(^{\cdot}\)NO concentration (Espey et al., 2002).

There are three types of reactions of \(^{\cdot}\)NO with metals, which highlights the reaction between \(^{\cdot}\)NO and heme iron, present in some proteins including soluble guanylate cyclase (sGC), the nitric oxide synthase (NOS) and several P450 enzymes (Espey et al., 2002). Another example is the reaction of \(^{\cdot}\)NO with oxyhemoglobin, forming methemoglobin, which constitutes the main removal pathway from \(^{\cdot}\)NO in biological systems (Lancaster, 1994, Espey et al., 2002, Santos, 2011).

\(^{\cdot}\)NO also directly react with other radical species, particularly with lipid and carbon-centered radicals (alcoxy - \(LO\), peroxyl - \(LOO\), etc.), formed as a result of both oxidative stress and normal metabolism. This reaction lead to the terminating of lipid peroxidation chain reactions, and thus affords protection against peroxide-induced cytotoxicity (Padmaja and Huie, 1993).

**Indirect effects**

Indirect effects are mediated by products of reaction of \(^{\cdot}\)NO with \(O_2\) or superoxide radical (\(O^{2-}\)) to form reactive species of nitrogen/oxygen (RNOS), being related with prolonged and/or high \(^{\cdot}\)NO concentrations and mainly associated to pathological processes.

In aerobic conditions, \(^{\cdot}\)NO reacts with \(O_2\), leading to the formation of several RNOS, such as nitrogen dioxide radical (\('NO_2\)) and nitrogen trioxide (\(N_2O_3\)) (Bruckdorfer, 2005). Both can nitrosate and oxidize different molecules and are primarily responsible for mediating nitrosative stress in vivo.
(Schwartz et al., 1983). However, nitrosation is an important signal transduction pathway, being described as a new mechanism of signal transduction regulated by enzymes, similar to phosphorylation (Mannick et al., 2002). S-nitrosation of regulatory proteins influences their functions, with impact in cellular physiology (Jaffrey et al., 2001).

NO, is also able to react rapidly with O$_2^-$, producing peroxynitrite (ONOO$^-\)$. ONOO$^-$, a very reactive molecule, can directly oxidize protein lipids and nucleic acids and nitrate proteins and lipids, with relevant consequences in several signaling pathways (Radi et al., 1991). It is the main responsible for the cytotoxicity attributed to NO, being involved in neurodegeneration, acute and chronic inflammatory processes, sepsis, ischemia-reperfusion injury and vascular disease (Pacher et al., 2007).

![Figure 1- Reactions mediated by NO in biological systems. Adapted from (Davis et al., 2001).](image)

2. The nitric oxide synthase (NOS)

NO is produced *in vivo* by a family of nitric oxide synthase (NOS), a highly regulated enzyme that uses L-arginine (L-arg) and molecular oxygen (O$_2$) as substrates. The three main isoforms: neuronal isoform (nNOS or NOSI), inducible (iNOS or NOSII) and endothelial (eNOS or NOSIII), are products of different genes and have different cellular localization, regulation, catalytic properties and sensitivity to inhibitors (Tatoyan and Giulivi, 1998). Both nNOS and eNOS are constitutively expressed and regulated by the concentration of calcium through interaction with calmodulin (Mungrue et al., 2003). In turn, iNOS expression depends on immune stimulation and inflammation,
being independent of Ca$^{2+}$ increases. The mitochondrial isoform (mtNOS) despite being a distinct isoform appears to be a variant of the neuronal isoform, resulting from post-transcriptional processing of RNA (Elfering et al., 2002)

2.1. Reductase and Oxygenase Domains

NOS are dymeric enzymes, formed by two identical subunits, each one with two distinct domains (Figure 2):

(A) reductase domain, at carboxyl terminal, which connects two flavin cofactors (FMN, FAD) and NADPH co-substrate. FAD and FMN catalyze the transfer reactions from NADPH to the heme in the oxygenase domain of the opposite subunit of the dimer.

(B) oxygenase domain, at amino terminal, that contains the a heme group (protoporphyrin IX) and binding sites for (6R)-5,6,7,8-tetrahydrobiopterin (H$_4$B) and the substrate L-arginine (L-arg).

Between the two domains there exists a binding site for calmodulin (CaM), which stabilizes the homodimer (MacMicking and Nathan, 1997). Dimerization increases NOS activity by creating high binding affinity sites for L-Arg and BH$_4$, removing the heme group from the solvent phase, and facilitating the flow of electrons between two domains, from FMN in the reductase domain, to the heme group in oxygenase domain (Crane et al., 1999).

![Figure 2](image.png)  
**Figure 2**- NOS structure indicating the oxygenase and reductase domain with binding sites for different cofactors, connected by a calmodulin (CaM) binding site. Adapted from (Cátia Lourenço, unpublished data).
2.2. \textit{NO} Biosynthesis

In essence, \textit{NO} derives from the oxidation reaction of L-arginine catalyzed by NOS to L-citrullin. The electrons flow from NADPH subunits to activate O$_2$ in the heme group of NOS, where L-Arg originates \textit{NO}, L-citrulline and H$_2$O, a reaction that occurs in two steps (Alderton et al., 2001):

a. L-arginine is hydroxylated to N$^\circ$-Hydroxy-L-arginine;

b. N$^\circ$-Hydroxy-L-arginine is oxidized to \textit{NO} and L-citrulline.

\[
\text{L-Arginine} + n \text{NADPH} + m \text{O}_2 \rightarrow \text{L-Citrulline} + \text{NO} + n \text{NADP}^+ 
\]

\[
\text{Step 1: L-arg} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{NOH-L-Arg} + \text{H}_2\text{O} + \text{NADP}^+ 
\]

\[
\text{Step 2: OH-L-Arg} + \text{O}_2 + 1/2 (\text{NADPH} + \text{H}^+) \rightarrow \text{L-citrulline} + \text{NO} + \text{H}_2\text{O} + 1/2 \text{NADP}^+ 
\]

2.3. Regulation of nNOS

The NOS family is one of the most highly and complex regulated enzymes in Biology, envolving transcriptional and post-transcriptional regulation, several substrates and co-factors, protei: protein interaction and subcellular localization.

The regulation of nNOS and subsequent \textit{NO} bioactivity depends on several factors:

- Bioavailability of substrates (ex.: arginine) and cofactors (ex.: H$_4$B and Ca$^{2+}$/CaM) which are crucial for dimerization and activation of NOS, also applicable to eNOS (Jaffrey et al., 1998);

- Concentration of intracellular Ca$^{2+}$ (Jaffrey et al., 1998);

- Coupling of NMDA receptors to the enzyme (PSD-95), which regulates the subcellular localization of nNOS (Jaffrey et al., 1998);

- Coupling of nNOS to different proteins, including CAPON, heat shock protein 90 (Bredl et al., 1992), and phosphofructokinase (Firestein and Bredt, 1999), regulate their location.

- Phosphorylation of nNOS by protein kinases (which suggests that the synthesis of neuronal \textit{NO} is modulated by a myriad of intracellular signaling cascades) (Bredl et al., 1992);

- Binding of \textit{NO} to the heme center to form a stable iron-nitrosyl (Fe$^{2+}$-NO), inactivating nNOS (Cooper, 1999).
3. **Signaling pathways in central nervous system mediated by NO**

NO is present in virtually every area throughout the CNS and has been implicated in the regulation of diverse physiological functions such as visual information, learning and memory, motor planning, cognitive process that drives locomotion (Vincent, 2000), as well as in neurodegeneration. The mechanisms that support the wide range of effects of NO in central nervous system are still being clarified although is well established that NO signaling in CNS is intimately associated to the glutamatergic system.

In glutamatergic synapses, NO synthesis involves the stimulation of ionotropic glutamate receptors, particularly NMDA-subtype to which nNOS is physically coupled, and influx of Ca\(^{2+}\) to the cytosol that, upon binding to calmodulin, activates the enzyme (Figure 3). Once produced, and considering NO reactivity with metal centers, it can control the activity of different heme proteins (sGC, NOS, catalase, cytochrome c oxidase) or non-heme (aconitase (Fe-S)), as well as regulate different cellular pathways via RNOS, with impact on physiology and pathology (Saran et al., 1998).

In hippocampus, a brain region with crucial role in certain kinds of learning and memory processes, NO has been implicated in long-term potentiation (LTP), acting as a retrograde messenger and increasing the neurotransmitter release in the presynaptic nerve terminal (Mizutani et al., 1993; Haley et al., 1996; Prast and Philippu, 2001). The mechanism by which NO promotes neurotransmitter release is considered to be based on the activation of sGC activation, the best characterized signaling target for NO. The binding of NO to the heme of sGC promotes the activation of the enzyme that converts GTP in cGMP (Stone and Marletta, 1996). The cGMP produced, being a secondary intracellular messenger translates the NO signal into a cellular response, namely, activating cGMP-dependent protein kinases (Lucas et al., 2000; Schlossman and Hofmann, 2005), which can regulate vesicle endocytosis (Micheva et al., 2003) and thus modulate neurotransmission.
Additionally, in hippocampus, NO has been implicated in neurodegeneration associated with Alzheimer disease (Contestabile et al., 2003).

4. Bioimaging of nitric oxide

While several techniques were developed to measure NO, the majority is based on indirect methods, not quantifying the radical per se, but instead the products of its decomposition or reaction with other molecules (Figure 4). An example of such technique is the Griess method, a colorimetric assay based on the measurement of nitrite, the oxidation product of NO. The test relies on the conversion of colourless sulphanilamide and N-((1-naphyl)ethylendiamine to a purple azo dye (absorption peak 450 nm) through reaction with nitrite. However, this assay has serious drawbacks, inclusively the lack of sensitivity (LOD > 500 nM). In addition to the colorimetric methods, other methods have been applied to measure NO, such as chemiluminescence, Electron Paramagnetic Resonance (EPR) and fluorescence. Using chemiluminescence, NO can be quantified through reaction with ozone in the gas phase, leading to nitrogen dioxide in the excited state (Fontijn et al., 1970), or by reaction with luminol-H_2O_2 system in the liquid phase, generating a
chemiluminescent compound (Kikuchi et al., 1993). Chemiluminescence has also gained recognition for measuring S-nitrosothiols and it is easily adapted to measure nitrite as a surrogate for \textsuperscript{\prime}NO generation; in this situation, the nitrite-containing sample is injected into an acid-containing mixture in order to generate \textsuperscript{\prime}NO for measurement (Pelletier et al., 2006). By EPR \textsuperscript{\prime}NO can be measured, not directly (although it is a paramagnetic species it is too short-lived and has too broad spectrum to be detected satisfactorily by EPR), but through the use of spin traps, compounds that form paramagnetic adducts with \textsuperscript{\prime}NO, thus allowing the detection of this radical (Tsuchiya et al., 2003). Other methodologies were developed allowing imaging \textsuperscript{\prime}NO in biological preparations based on the use of fluorescent compounds. One of the compounds most commonly used is the DAF-2 (4,5- diaminofluoroscein), a compound that reacts with N\textsubscript{2}O\textsubscript{3}, an oxidation product of \textsuperscript{\prime}NO, giving rise to a highly composite fluorescent DAF-2T (triazolofluorescein) (Kojima et al., 2001). This technique allows both the localization and quantification of \textsuperscript{\prime}NO, but it is limited to the requirement for higher oxides of nitrogen for activation (Suzuki et al., 2002). Although valuable these methods are unable to reflect the \textsuperscript{\prime}NO concentration dynamics in tissues, as they are ex situ techniques, where the analysis is done out of the biological context and the measurements reflect \textsuperscript{\prime}NO concentration at a single time points. Therefore, and considering the previously described properties of \textsuperscript{\prime}NO, particularly that it conveys information through its concentration dynamics, direct detection of \textsuperscript{\prime}NO in situ is crucial for understand its biological actions.

![Figure 4- Techniques available for measurement of \textsuperscript{\prime}NO (Cátia Lourenço, unpublished data).](image)

Figure 4- Techniques available for measurement of \textsuperscript{\prime}NO (Cátia Lourenço, unpublished data).
4.1. Electrodes for NO detection

It is now generally accepted that electrochemical methods, particularly amperometry, associated to NO electrodes, is the only available technique that allow the direct and real time measurement of NO with enough sensitivity to detect relevant NO concentrations. Several electrodes for the direct electrochemical detection of NO have been developed, all based in the principle of NO reacting at an electrode to release electrons to the circuit, which can subsequently be measured as current. The earliest of these electrodes, known colloquially as the "Shibuki electrode", consists in a platinum (Pt) electrode covered with a gas-permeable membrane to successfully monitor endogenous NO production in rat cerebellar slices (Shibuki, 1991). However this sensor is reported to have limited biological usefulness, because it does not respond linearly to concentrations greater than 1 μM and is subject to a destructive buildup of the oxidation products of NO within the enclosed electrolyte surrounding the Pt electrode. Nevertheless, it was an important hallmark because this probe enabled “World Precision Instruments” in 1992 to develop the first widely commercially available NO sensor, the ISO-NO. A second hallmark in the development of sensors for NO detection was the introduction of catalytic electrode surfaces by Malinski and Taha, who used a metalloporphyrin membrane electrochemically deposited on a carbon fiber electrode (Malinski and Taha, 1992). Following this advance, the deposition of polymeric films that catalyse NO oxidation at the electrode surface became an attractive approach and several materials have been proposed, including different types of metalloporphyrins, metallophthalocyanines, copper-platinum microparticles, palladium and iridium oxide. However there are some concerns about the exact chemical mechanism by which these sensors detects NO, since carbon fibers without a porphyrin coating or with a coating of porphyrin without a metal ligand can also detect NO with significant sensitivity. Furthermore, because the surface of the electrode remained in direct contact with the measurement medium a variety of biological species were shown to interfere (i.e. give false responses) during NO measurement. Additionally, various other types of carbon fiber NO sensors that utilize a variety of different coating have been described, such as conducting and non-conducting polymers, multiple membranes, ruthenium, iridium and palladium, heated-denatured cytochrome c; Nafion®, o-phenylenediamine, polylysine, hemoglobin-DNA film and ionic polymers and a-cyclodextrin (Bedioui and Villeneuve, 2003). Although the use of electrodes for NO detection can be endowed of some limitations, as potential
instability in biological medium and compromised selectivity against potential interferents, it has extraordinary advantages over almost all other methods of NO detection, not only because it allows the real time measurement in situ with minimal destruction, but also because of it high sensitivity (Megson and Miller, 2009, GMP book).

II. Neurovascular coupling

Brain areas subject to increased neuronal activity consume increasing amounts of oxygen, glucose and other metabolites (Iadecola, 2004). This high energy consumption translates into higher consumption of ATP, essential for the operation of (Na\(^+\)/K\(^+\) and Na\(^+\)/Ca\(^{2+}\)) pumps by maintain the gradient ion suitable for the generation of action potentials (mechanisms with higher energy costs), as well as the release of neurotransmitters (Drake and Iadecola, 2007). The brain being critically dependent on a continuous supply of blood, the blood flow required to accomplish these needs is ensured by a close link between neuronal activity and cerebral vasculature. This neurovascular coupling, characterized by increased blood flow that occurs when tissues are active in a given region, is called functional hyperemia, and its main goal is to regulate cerebral blood flow (CBF) with high spatiotemporal precision, towards maintain homeostasis (Hamel, 2006; Iadecola and Davisson, 2008, Leite et al., 2009). Thus, the brain is an example of tissue that needs fuel more quickly or in larger quantities, possessing biological mechanisms for this complex and specialized neurovascular coupling, not only through direct interaction of neurons and vessels, but also involving astrocytes that may act as links between blood vessels and neurons (Zonta et al., 2003). Therefore, these neurons are dependent on the hemodynamic response in the brain which is vital in supplying energy nutrients (glucose and oxygen) and residues removal from neuronal activity (CO\(_2\), lactate excess, as well as other metabolites and heat) (Hossmann, 1994; Iadecola, 2004).

Such mechanisms result in an integrated action between neurons, glia, astrocytes and blood vessels (through the myocytes, endothelial cells and erythrocytes), indicating a close anatomical and functional link, the "neurovascular unit" (Figure 5) that acts by acting together, at cellular level to regulate local blood flow (Lo et al., 2003; Iadecola and Davisson, 2008).
Biochemical and neural factors induce changes in blood vessels that result in changes in blood flow, volume and oxygenation. Although extensively investigated, this cascade of events is still poorly understood (Mesquita, 2009). The dependence of brain blood flow is enhanced by the fact that even relatively small reductions in cerebral blood flow (CBF) negatively affects neuronal function, particularly through the inhibition of protein synthesis. If large reductions in CBF occur, ATP synthesis is compromised, the brain cells reduce their ability to fire action potentials, and in severe cases the neurons undergo anoxic depolarization (missing gradinete ion), leading to serious brain injuries and cerebral ischemia (Iadecola, 2004). Moreover, changes in blood vessels (ex.: caused by hypertension), by disrupting vessel regulatory mechanisms that ensure an adequate supply of blood in the brain, manifesting into disruption of control mechanisms, causing brain dysfunction and lead to various diseases, such as Alzheimer's, Parkinson's or other neurodegenerative diseases, cerebral ischemia or stroke. Thus, the investigation of neurovascular coupling is crucial to human health (Iadecola, 2004; Iadecola and Davisson, 2008).

1. **Mechanism of neurovascular coupling**

It was proposed more than a century ago that neurons release vasoactive agents into the extracellular space that, subsequently, reach the blood vessels by diffusion leading to relaxation of vascular smooth muscle. Considerable evidences support the vasoactive neurotransmitters release, especially in the synaptic release of glutamate and GABA, but the mechanisms are much more complex and indirect than simple diffusion to vascular targets (Drake and Iadecola, 2007).

The exact mechanism behind the neurovascular coupling is currently under active investigation and discussion, however it is proposed that this coupling occurs not only indirectly through the astrocytic bridge, but also through direct interaction between vessels and neurons in glutamatergic synapses (Rossi, 2006; Drake and Iadecola, 2007, Stefanovic et al., 2007). The current paradigm establishes an astrocytic bridge involving glutamate binding to metabotropic receptors on astrocytes (Rossi, 2006; Drake and Iadecola, 2007), allowing the (I) entry of calcium into glial cells, also from adjacent cells through gap junctions (GJ); (II) Ca²⁺ diffusion to the astrocytic feet, where it activates phospholipase A2 (PLA2); (III) release of arachidonic acid (AA) from phosphatidylinositol (PI); (IV) which is converted by cyclooxygenase (COX) into vasoactive prostanoids (particularly prostaglandin E2 (PGE2). These prostanoids released by astrocytes play an important role in
regulating contraction and relaxation of smooth muscle, thus allowing greater blood flow when there is dilation. (Rossi, 2006; Drake and Iadecola, 2007).

While 'NO, a diffusible potent vasodilator and produced as a result of glutamatergic activation, has been suggested to mediate the neurovascular coupling, its role has been controversial. Recently, by simultaneous measuring 'NO dynamics and CBF and associated with a pharmacological screening, clear evidences were provided supporting a critical role of neuronal-derived 'NO in matching blood supply with neuronal activity in hippocampus. It was shown that 'NO released upon glutamategic activation in neurons is able to diffuse to the nearby blood vessels, where by activating sGC, promotes vasodilation with consequent increase in CBF (Cátia Lourenço, unpublish data)

**Figure 5**- Neurovascular coupling mediated by isotropic diffusion NO. Adapted from (Drake and Iadecola, 2007).
III. Alzheimer's disease

Alzheimer's disease (AD) is a complex and progressive neurodegenerative disorder associated to neuronal loss in brain areas linked to memory processing, in which NO has been implicated (Fernandez et al., 2010).

In vulnerable brain regions, such as the hippocampus and cortex, there is an accumulation of extracellular neuritic plaques (deposits of differently sized small peptides called β-amloid, Aβ, that are derived via sequential proteolytic cleavages of the amyloid precursor protein, APP) and intracellular neurofibrillary tangles (NFT) which consist largely of hyperphosphorylated twisted filaments of the microtubule-associated protein tau (Buee et al., 2000; Gendron and Petrucelli, 2009), Proteolytic cleavage of APP in the amyloidogenic pathways mainly results in two forms of Aβ: Aβ40 [Aβ-(1–40)] and Aβ42 [Aβ-(1–42)] (Zhang et al., 2010).

In spite of inconsistent results (Law et al., 2001), all NOS isoforms are suggested to operate as central mediators of Aβ action, contributing to the maintenance, self-perpetuation and progression of the disease (Fernandez et al., 2010).

During the development of AD it habe been noticeable the neurotoxic effects of NO supported the pathological mechanisms involving its conversion to more reactive species, namely through the production of RNS and induction of nitrosative stress (due to indirect reaction of NO). In addition to the ability of Aβ itself in generating oxidative stress (Varadarajan et al., 2000), it can act synergistically with NO to induce neuronal damage. Concordantly, high levels of nitrotyrosine have been found in AD patients brains. Moreover, several studies have shown that important proteins are S-nitrosated in AD, such as PDI and dynamin. In addition, cerebrovascular dysfunction detected in AD may be important for the development of the disease, contributing to cognitive decline and neurodegeneration. Indeed, many patients with AD have regional cerebral hypoperfusion that correlates with cognitive decline (Chow et al., 2007). Furthermore, fMRI studies have established that there is an increased delay in the CBF response in patients with increased cognitive impairment especially AD patients (Rombouts et al., 2005). However, NO may have a protective role against the development of AD pathology. For instance, it was shown that NO derived from normal endothelium protects against increases in Aβ, by directly modulating levels of Aβ, APP and BACE-1 (Austin et al., 2010).
Nowadays, several factors have been described to determine the induction of cerebrovascular damage in AD, including endothelial dysfunction promoted by nitro-oxidative stress (a term here used to incorporate the action of both, oxygen- and nitrogen-derived reactive species) (Hamel et al., 2008), loss or abnormal cholinergic innervations of intracerebral blood vessels and accumulation of Aβ on the cerebral blood vessels (cerebral amyloid angiopathy) (Bell and Zlokovic, 2009). However, it is still a matter of debate the pathologic importance of these cerebrovascular alterations and it is unclear whether they were a cause or a consequence of neuronal dysfunction and neurodegeneration (Iadecola, 2004).

1. **Bacteria are powerful stimulators of inflammation and are amyloidogenic**

   It is well known that several bacteria, upon interaction with the mammalian immune-system, induce chronic inflammation and amyloid deposition exacerbating AD. This conclusion is now supported by more than 20 epidemiological studies showing that individuals were protected from AD if they have been taking anti-inflammatory drugs (Veld et al., 2000). Thus, brain inflammation could be induced by systemic administration of LPS mimicking AD. LPS produces a robust initial inflammatory reaction, the innate immune response, in peripheral organs and in the brain (Julius, 2009), leading to activation of microglia, neutrophil infiltration, and mRNA/protein expression of inflammatory mediators (Jeong et al., 2010). A well-studied model of acute inflammation in rodents is the systemic administration of the bacterial inflammatory surface molecule lipopolysaccharide (LPS), a bacterial endotoxin followed by robust innate immune response in peripheral tissues and in the brain. LPS is used world wide in experimental and in vivo models of inflammation and amyloidosis (Veld, 2000). After its recognition by soluble or membrane-bound CD14, LPS binds its signaling receptor, toll-like receptor 4 (TLR-4) (Triantafilou and Triantafilou, 2005) expressed in multiple peripheral sites including brain microvasculature. This is followed by fast and transient release of pro-inflammatory cytokines to the circulation affecting all peripheral organs and the brain (Bosshart and Heinzelmann, 2007). In connection with this notion, AD lesions are characterized by the presence of a series of inflammatory mediators including cytokines, chemokines, proteases, adhesion molecules, free radicals, pentraxins, prostaglandins, anaphylatoxins, and activated complement proteins (McGeer and McGeer, 2002).
Hereupon, bacteria and their toxins are powerful inducers of inflammatory cytokines and activators of the complement pathway (Fox, 1990). It has been known for almost a century that chronic bacterial infections are frequently associated with amyloid deposits in the infected tissues. LPS and bacterial cell wall peptidoglycan are highly resistant to degradation by mammalian enzymes and thus may provide a persisting inflammatory stimulus (Ohanian and Schwab, 1967).

2. Inflammation, NO and polyphenolic pathways

Epidemiological studies have suggested that appropriate consumption of polyphenol-rich natural products may reduce the incidence of certain age-related neurological disorders, including Alzheimer’s disease, and thus modulate multi-factorial events such as neuroinflammation, glutamatergic excitotoxicity, oxidative stress, and depletion of endogenous antioxidants. The beneficial effect has been attributable, at least in part, to their direct effect on blood vessels and in particular on endothelial cells. Indeed, polyphenols have been shown to activate endothelial cells to increase the formation of potent vasoprotective factors including NO and endothelium-derived hyperpolarizing factor (Schini-Kerth et al., 2011).

Polyphenols, in virtue of the plethora of protective effects manifested in various experimental models and clinical trials, seem to be appropriate as dietary supplements for preventing the functional decline of organs with age (Magrone and Jirillo, 2011) by decreasing the risk of a range of diseases, including cardiovascular disease (CVD), certain forms of cancer (Kuriyama et al., 2006) and neurodegenerative diseases. Favoroids (a major class of polyphenols) have also been shown to exert beneficial cognitive effects and to reverse specific age-related neurodegeneration. Polyphenols, including curcumin, epicatechin, and procyanidins, have been proposed in the past to predominantly act via antioxidant activity. The tricyclic structure of the flavonoids determines antioxidant effects that scavenge reactive oxygen species and chelate Fe^{2+} and Cu^{+}, but recently it has becoming clear that polyphenols may participate in the redox modulation of cell functions, including enzyme inhibition and upregulation of antioxidant defenses via changes in gene expression upon interaction with redox-sensitive transcription factors (Katz and Doughty, 2011).

Recently, Han et al., (2006) suggested that the neuroprotective action of various polyphenols could be mediated by the activation of common "receptor" binding sites particularly present at the level of the cellular plasma membrane in the rat brain. It is hypothesized that the binding
polyphenols (able to transverse the blood brain barrier-BBB) to this receptor may be associated with increased NO synthase activity in the brain. Prolonged action of polyphenols on its receptor could, however, lead to decreased receptor sensitivity and/or increased tolerance. Antioxidants are able to increase availability of biologically active NO resulting in a partial decrease of blood pressure. (Han et al., 2006; Schmitt and Dirsch, 2009; Galleano et al., 2010).

The current awareness that oxidative stress plays a pivotal role in the pathophysiologic processes of vascular dysfunction resulted in several treatment strategies to alter ROS levels by decreasing production and/or increasing radical scavenging. Polyphenols appear to involve their interaction with cellular signaling pathways and related machinery that mediate cell function under both normal and pathological conditions. The figure below illustrate their interactions with two such pathways, the MAP kinase (ERK, JNK, p38) and PI3 kinase/Akt signaling cascades, that allow them to impact upon normal and abnormal cell function, thus influencing the cellular processes involved neurodegeneration. For example, their ability to activate ERK in neurons leads to a promotion of neuronal survival and cognitive enhancements, both of which influence the progression of Alzheimer’s disease, whilst ERK activation by polyphenols in vascular endothelial cells influence nitric oxide production and blood pressure (Vauzour et al., 2010).

\[
\text{Figure 6- The interaction of polyphenols with cellular signaling pathways involved in chronic disease. Flavonoid-induced activation and/or inhibition of MAP kinase and PI3 kinase signaling leads to the activation of transcription factors which drive gene expression (Vazour et al., 2010).}
\]
Polyphenols may act to protect the brain in a number of ways, including the protection of vulnerable neurons, the enhancement of existing neuronal function or by stimulating neuronal regeneration (Youdim and Joseph, 2001), to improve memory, learning and general cognitive ability (Vazour et al., 2010). The effects of polyphenols on cognition and against neurodegenerative processes appear to be mediated via their interactions with neuronal and glial signaling pathways that affect gene expression and interfere with the cell death mechanisms (Williams et al., 2004) directly via the inhibition of MAPK signaling cascades, such as p38 or ERK1/2. The effects of flavonoids on these kinases may influence downstream transcription factors, including nuclear factor-Kappa B (NF-κB). This suggests that there may be an interplay between signaling pathways, transcription factors and cytokine production in determining the neuroinflammatory response in the CNS (Figure 6). In this respect, the mechanisms by which polyphenols exert its beneficial function involve interactions with a number of cellular signaling pathways, which are important in the normal functioning of cells. Thus, polyphenols, in particular flavonoids, structurally resemble inhibitors of cell signaling cascades (MAPK/PI3).

Active flavonoid compounds were also found to inhibit NO synthase activity of the three isoforms of the enzyme. It is hard to speculate on the broad ability of flavonoids to inhibit the activity of so many different enzyme systems but such inhibitors fit into the ATP binding pocket of the enzyme and it appears that the number and substitution of hydroxyl groups on the B ring and the degree of unsaturation of the C2-C3 bond are important determinants of this particular bioactivity (Spencer et al., 2003). Yet, it is unlikely that the same three-dimensional orientation would be required by widely different enzymes. Another possibility is that flavonoids bind to proteins, thus changing their orientations and making their active site inaccessible (Middleton et al., 2000).

**IV. Hypothesis**

On basis of the beneficial and diverse effects of polyphenols in brain function, as well as their neuroprotective role against neurodegeneration and further considering that NO produced in glutamatergic neurons diffuses isotropically until blood vessels leading to vasodilation, we
hypothesize that polyphenols, such as curcumin and epicatechin, by interfering with NMDA receptor:nNOS pathway, may modulate glutamate-induced \( \text{NO} \) concentration dynamics, thus affecting the neurovascular coupling.

Therefore, by simultaneously and in real time measuring \( \text{NO} \) dynamics and CBF, we propose to assess:

1) to what extent glutamate-induced \( \text{NO} \) dynamics and coupled cerebral blood flow changes are prone to modulation by polyphenols in physiological conditions;

2) whether glutamate-induced \( \text{NO} \) production is altered under pathological conditions, such as AD and inflammation, and whether the neurovascular coupling is compromised;

3) if polyphenols have any effect over \( \text{NO} \) dynamics and CBF under the previous mentioned pathological conditions.
Chapter 2.

Materials and Methods
The feasibility of this approach is supported by the use of an array consisting of a microelectrode selective for \(\cdot\)NO, an ejection pipette and a laser Doppler flow probe to measure localized cerebral blood flow (CBF). Such an array will be sterotaxically inserted in the brain of the living rat and, upon glutamate stimulus in hippocampus and the dynamics of \(\cdot\)NO and of CBF will be measured in real-time and simultaneously (Figure 7).

![Figure 7- Measurement of CBF and \(\cdot\)NO on rat hippocampus. Array consists of a laser doppler probe for measure cerebral blood flow (CBF), a stimulus micropipette for glutamate injection, and a microelectrode to measure \(\cdot\)NO production.](image)

I. Experimental Design

To study the involvement of neurovascular coupling on brain inflammatory diseases mimicked by rat models of Alzheimer’s Disease (comproved by behavioral tests) as well as administration of lipopolysaccharide (LPS) in male Wistar rats, the project was carried out \textit{in vivo}, to establish potential physiological effects, as well as the modulation of dietary polyphenols in such dementia conditions.
II. Chemicals and Solutions

Curcumin and (-)-Epicatechin and lipopolysaccharide (LPS) used in the study were purchased from Sigma. Aβ$_{1-42}$ peptide was purchased from American Peptides. Ascorbate (AA) and o-phenylenediamine (o-PD) were obtained from Fluka. L-Glutamic acid (Glu) was purchased from Sigma and Nafion1 was purchased from Aldrich. Phosphate buffer (0.05 M PBS) used for microelectrode evaluations was prepared in MilliQ water and had the following composition (mM): 10 NaH$_2$PO$_4$, 40 Na$_2$HPO$_4$, and 100 NaCl (pH 7.4). All drugs ejected into the brain were dissolved in deoxygenated saline (NaCl 0.9%, pH 7.4). Ketamine (IMALGENE® 1000) and xylazine (Rompum 2%) were supplied by the Animal House of the Center for Neurosciences and Cell Biology (Coimbra). Urethane was purchased from Aldrich.

III. In vivo testing

1. NO Microelectrodes

We use carbon fiber microelectrodes fabricated as described elsewhere (Santos, 2008). Briefly, single carbon fibers (30 µm) were inserted into borosilicate glass capillaries and then pulled on a vertical puller. The salient carbon fibers were cut by tweezers under a microscope to obtain an exposed carbon surface with a tip length of 200±50 µm length.

Figure 8- Microelectrodes fabrication (A-B) Pulling of the glass capillary with a carbon fiber inside in a vertical puller. (C) Cutting 200 ± 50 µm of fiber under the microscope (Cátia Lourenço, unpublished data).
Afterwards, a copper wire was introduced, followed by the introduction in the stem end of the microelectrode of conductive silver paint using a syringe, to provide the electrical contact between copper wire and the carbon fiber. The microelectrodes were tested for their general recording properties in phosphate-buffered saline (PBS) medium, by fast cyclic voltammetry (FCV) at a 200 V/s scan rate between −0.4 and 1.2 V for 30 s. Finally, to improve their analytical properties for measuring NO in the rat brain in vivo, the carbon fiber microelectrodes were modified in a two-step protocol with Nafion® and o-PD. Nafion® coating was preformed by immersing microelectrodes tip in the Nafion® solution for 1-2 seconds and drying into an oven at 170-180ºC for 4 min. The procedure was repeated twice. After Nafion coating, the o-PD layer was deposited by electropolymerization by placing the microelectrode tip in a 5 mM o-PD solution and applying a constant potential of 0.7 V vs an Ag/AgCl reference electrode for 30 min.

![Schematic representation of exclusion layers of Nafion® and o-phenylenediamine at the carbon fiber surface.](http://www.cnbc.pt/research/areaC1_2.asp?lg=2).

### 2. Calibration and Selectivity

The analytical performance of modified carbon fiber microelectrodes was assessed in terms of sensitivity, detection limit for NO and selectivity ratios against major interferents: ascorbate and nitrite (Ledo et al., 2002).

Each microelectrode was calibrated with a fresh saturated solution of NO (prepared by bubbling NO gas in MilliQ water until saturation). Typically, the concentration of the saturated NO solution was c.a. 1.9 mM (Barbosa et al., 2011) and was checked by ISO-NOP 2-mm Pt sensor connected to the amperometer ISO-NO Mark II, calibrated by chemical generation of NO from the reaction of NO₂ with excess iodide and sulfuric acid (Mesaros et al., 1997).

The calibration of the modified carbon fiber microelectrode was done by amperometry using the FAST-16 system with an applied potential of 0.9 V vs Ag/AgCl reference electrode. After
stabilization of background current for at least 20 min, the microelectrodes were calibrated. First, 250 µM ascorbic acid (final concentration) is added and then, after current stabilization, three 10 µl consecutive additions of ·NO solution are added with a gas-tight syringe in order to get a final concentration in the range of 0.4 to 2 µM. Finally, nitrite was added at a final concentration of 100 µM (Barbosa, et al., 2008). A typical recording obtained with this procedure is shown in figure 10.

![A](image1.png) ![B](image2.png)

**Figure 10**- Determination of microelectrodes sensitivity for ·NO and selectivity against interferents. (A) Representative recording of microelectrode calibration. Compounds were added at the times indicated by the upper arrows. (B) Linear regression applied to the correlation between the concentration of ·NO added and the current change resulting from each addition.

The calibration parameters calculated for ·NO are the slope (sensitivity), limit of detection (LOD), and linearity (R²). The LOD is defined as the analyte concentration that yields an electrode response equivalent to three times the background noise of the recording system.

Selectivity ratio (·NO vs interferents) is calculated as the ratio of microelectrode sensitivity for NO over interferents and is calculated by dividing the ·NO slope by the interferent slope.

The properties of the chemically modified carbon fiber microelectrodes used are summarized in table 1.

**Table 1**- Calibration parameters and selectivity ratios of carbon fiber microelectrodes coated with Nafion and o-PD. (Data are given as mean±SEM).

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (pA/µM)</th>
<th>304 ± 28 (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity (R²)</td>
<td>0.998 (n=16)</td>
<td></td>
</tr>
<tr>
<td>Detection limit (nM)</td>
<td>8.910 ± 28 (n=16)</td>
<td></td>
</tr>
<tr>
<td>Selectivity ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbate</td>
<td>9452 ± 2293 : 1 (n=16)</td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>2813± 753 : 1 (n=16)</td>
<td></td>
</tr>
</tbody>
</table>
2.1. Neurodegenerative rat models

**Acute model of AD**

The brain AD of rat as well as the memory impairment was developed by a single intracerebroventricular injection of Aβ peptide (fragment 1-42) in rat brain, accordingly to a previously described procedure (Canas et al., 2009). Male rats were anaesthetized with a mixture of ketamine 4:1 xylazine (2,5 mg/Kg, IP), and underwent a intracerebroventricularly (ICV) infusion at a rate of 0.2 µl each 20 seconds of 4 µl of Aβ-amyloid (1-42) (2.257 mg/ml) into the right lateral ventricle, using stereotaxic coordinates: anterior-posterior (AP)- 0.8; medial-lateral (ML)-1.5; dorsoventral (DV)-3.5, according to the atlas of Paxinos and Watson (2007), by microlitre syringe (Hamilton) as previously described (Dall'Igna et al., 2007). Control animals were ICV injected with a similar volume of saline. At the end of the infusion the rat’s scalp was sutured. Animals were allowed to recover and tested after 15 days for behavioral performance. *In vivo* recordings were performed 18-19 days after Aβ infusion.

![Figure 11](image)

**Figure 11:** (A) AD induction by intracerebroventricular injection of Aβ_{1-42}. (B-C) Anesthesia recuperation after surgery, and in detail rat’s scalp sutured.
Inflammatory Animal Model

Additionally, an acute inflammatory brain model was induced by bacterial endotoxin lipopolysaccharide (LPS from Escherichia coli, serotype 0127:B8). Wistar rats were injected intracerebroventricularly (ICV) with LPS (20 μg dissolved in 4 μL of 0.9% NaCl), and in another set of experiments rats were pretreated with a single intraperitoneal injection of LPS (2 mg/kg).

3. Behavior Tests

Behavior tests were performed to address whether the AD models used in this work presented cognitive deficits that resemble those known to exist in AD, namely by related to novelty and memory. Each behavioral test was performed in an isolated room with lighting conditions and environmental cues held constant throughout testing. Animals were delivered to the experimental room the day before the experiment. All behavioral tests were carried out between 10 p.m and 4 p.m, under red-light illumination. To remove the smell traces left by the animals, before each trial the surfaces of the arenas, maze and objects were carefully cleaned. All behavioral tests were carried out between 10 p.m and 4 p.m.

3.1. Open field

The open field locomotion test is primarily used to examine motor function by means of measuring spontaneous activity and exploratory behaviors in an open field arena (Denenberg, 1969). Animals were place in an open field arena (75x60 cm, divided in 20 squares of 15 cm) for 10 minutes and the total number of line crossings (horizontal explorations) and the number of rearings (vertical explorations) were counted.

3.2. Novel-Object recognition test

The object recognition test is based on the natural tendency of rodents to investigate a novel object instead of a familiar one (Squire et al., 2007). In this task, animals were placed in an open field arena (75 x 60 cm) with two identical objects for 5 minutes, during which the animal has habituated to the configuration and properties of the different objects. After 2 hours, animals were
placed in the same arena in which one of the objects was replaced by a new one, with different configuration and color, and left there for 5 minutes. In each trial, the time spent inspecting each object was measured. The index of novel object recognition was calculated as the percentage of time spent inspecting the novel object.

3.3. Y-maze

Another recognition memory test was carried out in a Y-maze apparatus. The Y-maze is constituted by 3 equal arms with an angle of 120 degrees between them. In a first trial, animals were placed in the Y-maze with an arm blocked and the animals were allowed to explore the two arms for 5 min. After 2 hours, the animals were placed back into the maze, this time with all arms open, and scored for 5 minutes. The number of entries and the time spent in each arm were recorded. The percentage of time spent in the novel arm was used as index of cognitive function.

4. Array production

For the simultaneous measurement of NO and CBF the micropipette is attached to the carbon fiber microelectrode using sticky wax, that has been softened by flame. The precise placement is done using a microscope fitted with a reticule to achieve a distance between the tip of the micropipette and the NO microelectrode of 250 ± 50 µm. Laser Doppler probe is finally attached to microelectrode-micropipette array around 500 µm back from the tip.

5. Animals and surgical preparation

In vivo studies were carried out in adult male Wistar rats (8–10 week, weighing 290–350 g) there were maintained in our own animal facilities under controlled environment (23±2°C, 12 h-light/dark cycle, free access to food and water). Experiments were performed in accordance with the European Community Council Directive for the Care and Use of Laboratory Animals (86/609/ECC) and were approved by the local institutional animal care committee. Institutional guidelines were followed during entire experimentation.
For *in vivo* studies, rats were anesthetized with urethane (1.25–1.50 g/kg, IP) and placed in a stereotaxic frame (Stoelting, USA) on an isothermal pad to maintain its body temperature at 37ºC (Burmeister *et al.*, 2002). After exposing the surface of the skull, a small hole was drilled above the recording area and dura matter removed gently to expose the brain surface. An Ag/AgCl reference electrode previously prepared is introduced in the brain. Brain surface was bathed with saline (0.9% NaCl) to prevent drying of the brain surface. The array consisting in the microelectrode-micropippete-LDF probe was then inserted into the rat hippocampus using a hydraulic micromanipulator, using the following coordinates, calculated from bregma (0,0,0) based on the rat brain atlas of Paxinos and Watson (2007): AP- 4.1; ML- 1.5; DV- 3.7.

6. **Stimulation and pharmacological modulation of NO with Curcumin and Epicatechin**

After the insertion of the array into the hippocampus, the baseline current stabilize for at least 30 min. Then NO production was stimulated by ejection of L-glutamate from a micropipette using a Picospritzer III (Parker Hannifin, General Valve Operation, USA), using pressure pulses for 1 s at 7–15 psi. L-glutamate solution 20 mM was prepared in NaCl 0.9%. The volume ejected (low nanoliter range) was calculated by the decrease of the volume of the micropipette measured through a stereomicroscope (Meiji EMZ 13, Japan) fitted with an eyepiece reticule. NO production was modulated with curcumin, and epicatechin. Rats were treated with a single dose (acute administration) of 300mg/kg of curcumin emulsified in dimethyl sulfoxide (DMSO) intraperitoneally or intraventricularly at a concentration of 20 mM µL dissolved in 1µL of DMSO. To examine the neuromodulatory effect of curcumin and epicatechin in terms of neurovascular coupling, we will measure the NO levels and CBF in rats before administration of this dietary phenols (control), regardless the route of administration after suplementation.

7. **Data analysis**

The characterization of *in vivo* signals was assessed by electrochemical and pharmacological verification, and were individually analyzed using the OriginPro 7.5 Software, accordingly to the example depicted in Figure 12. Briefly, the individual NO signals were characterized in terms of 1)
[NO] peak, the peak concentration of the signal; 2) area, calculated as the time integral of the signal; 3) \( T_{\text{rise}} \), the time in seconds necessary to reach the maximum amplitude after the application of the stimulating solution; 4) \( T_{50} \), the time in seconds from maximum amplitude to 50% decay of the signal, 5) \( T_{\text{total}} \), the time in seconds from the application of the stimulation to return to basal levels and 6) Half Width \((dx)\) time by which amplitude reaches half of the maximum levels (Barbosa et al., 2008).

Figure 12- Schematic diagram of a \( \cdot \)NO signal to illustrate how signal parameters were measured and calculated, namely the [NO] peak, area (shadowed), \( T_{\text{rise}}, T_{50} \) and \( T_{\text{total}} \).

Records from CBF were exported from the Perisoft version 2.50 software, averaging 1 point per second, and synchronized with \( \cdot \)NO recorded dynamics based on the markers recorded at the time of stimulations. CBF changes were analyzed in terms of 1) Basal levels, perfusion values previous to stimulation; 2) CBF change, relative change in CBF in respect to the basal levels (percentage), 3) \( T_{\text{rise}} \), the time in seconds necessary to reach the maximum amplitude after the application of the stimulating solution; and 4) \( T_{\text{total}} \), the time in seconds from onset to return to basal levels. Additionally, the delay time of the onset and peak between both dynamics was determined. All statistical analyses were performed using GraphPad Prism 5 Software. Data are presented as mean \( \pm \) SEM. Statistical analyses of the data were performed using one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni’s Multiple Comparison Tests or a Student’s t-test. Differences were considered significant at \( p < 0.05 \).

Figure 13- Experimental Schedule.
Chapter 3.

Results
I. *In vivo* Nitric Oxide and cerebral blood flow coupling dynamics

As previously reported, a localized stimulation with glutamate in rat hippocampus promotes an instantaneous and transient elevation of \( NO \) concentration levels (Lourenço *et al.*, 2010) that in turn is followed, seconds later, by an also transient increase in CBF (Cátia Lourenço, unpublished data).

In figure 14, is shown a typical set of \( NO \) and CBF signals recorded in rat hippocampus upon sequential stimulations with 15-min intervals with L-glutamate (20 mM, 25 nL, 1s). For this particular record, the \( NO \) production was characterized by a maximal peak \( NO \) concentration increase ranging from 1.3 to 0.7 \( \mu \)M, with an average duration of 56 s. The CBF started to increase with an average of 3 s after stimulation, reaching 94 % of the basal level after 39s and returning to baseline after 437 s.

![Figure 14: Representative recording of the simultaneous measurements of \( NO \) produced by local application of L-glutamate on rat hippocampus (bottom, black line) and CBF changes (top, light grey line). L-Glutamate (20 mM, 25 nL) was locally applied at times indicated by the arrows.](image-url)
II. Modulation of neuronal-derived \(\cdot\)NO and neurovascular coupling with Polyphenols

Several lines of evidence suggest the beneficial role of dietary polyphenols by ameliorating brain function and protecting against neurodegeneration. Polyphenols neuroprotection has been ascribed to direct radical scavenging/antioxidant activity, but most significantly by modulation of signaling pathways with significance in cell survival and death (Frade et al., 2005). Based on the above mentioned, it was pertinent to address the potential of polyphenols to modulate glutamate induced \(\cdot\)NO production and cerebral blood flow.

Among a wide variety of polyphenols to which a plethora of protective effects in several experimental models were described, we selected curcumin, the yellow pigment of the widely used spice, turmeric, and epicatechin, a flavanol found, for instance, in green tea, grapes and blueberries. Both were reported to permeate the blood brain barrier and modulate important signaling pathways in the brain (Frade et al., 2005; Lin, 2007; Van Praag et al., 2007).

1. Curcumin

The endogenous \(\cdot\)NO production induced by activation of glutamate receptors and dependent neurovascular coupling was acutely modulated by curcumin via two distinct routes of administration: i) intracerebroventricular (ICV) and 2) intraperitoneal (IP) injection. In order to maximize the reproducibility of the effects, curcumin was only injected after obtaining 2-3 consecutive signals with similar amplitude. Figure 15 shows a typical recording of the effect of curcumin administered ICV (20 mM) in \(\cdot\)NO production and CBF induced by L-glutamate in hippocampus. The parameters obtained by the analysis of individual \(\cdot\)NO peaks and CBF changes before and after curcumin administration are summarized in the Figure 16 and detailed table 2. Globally, we observed that curcumin, once administered ICV, promoted a slight decrease of glutamate-induced \(\cdot\)NO production, particularly significant regarding the duration of \(\cdot\)NO concentration dynamics. The reproducibility of the \(\cdot\)NO signals obtained with repeated stimulations with L-glutamate prior to curcumin administration strengthens the observation that the decrease in \(\cdot\)NO signals is likely due to curcumin. In detail, approximately 20 min after the ICV injection of curcumin glutamate-induced \(\cdot\)NO signals showed decreased amplitude (19%, n=7), as well as
duration (29%, \( p < 0.05 \)). Coherently with the idea that \( \cdot \)NO mediates the neurovascular coupling in hippocampus, the CBF changes coupled to \( \cdot \)NO dynamics also appeared slightly reduced after curcumin ICV administration (CBF change associated to glutamate simulation after curcumin was 25.06% reduced as compared to control levels).

Consistently with the idea that \( \cdot \)NO mediates the neurovascular coupling in hippocampus, the CBF changes coupled to \( \cdot \)NO dynamics also appeared reduced after curcumin ICV administration, although not significantly (CBF change associated to glutamate simulation after curcumin was 10% reduced as compared to control levels).

**Figure 15**- Effect of curcumin in glutamate-induced \( \cdot \)NO dynamics (black line) and CBF changes (grey line) in rat hippocampus. L-Glutamate (20 mM, 25 nL) was locally applied at times indicated by the upward arrows. Curcumin was intracerebroventriculary administrated (20 mM) at the time indicated by the downward arrow.

![Curcumin Effect](image)

**Figure 16**- Quantitative analysis of peak \( \cdot \)NO concentrations (A) and CBF amplitude (B) before and after curcumin intracerebroventricular injection. Data represents mean ± SEM. Statistical analysis was performed by Student’s t-test in relation to control experiments (*\( p < 0.05 \)).
Table 2 - Analysis of glutamate-induced NO signals and CBF changes in hippocampus before and after curcumin injected in intracerebroventricularly.

<table>
<thead>
<tr>
<th></th>
<th>Ctr (n=2)</th>
<th>Cur (n=4)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NO] peak (µM)</td>
<td>2.25 ± 0.10</td>
<td>1.82 ± 0.36</td>
<td>0.4733</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>17 ± 3</td>
<td>18 ± 1</td>
<td>0.3726</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>49 ± 8</td>
<td>35 ± 4</td>
<td>0.0103*</td>
</tr>
<tr>
<td>Half width (s)</td>
<td>20 ± 3</td>
<td>16 ± 2</td>
<td>0.0154*</td>
</tr>
<tr>
<td>CBF increase (%)</td>
<td>100 ± 17.5</td>
<td>90 ± 16</td>
<td>0.6582</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>56 ± 8</td>
<td>62 ± 28</td>
<td>0.3726</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>374 ± 10</td>
<td>352 ± 52</td>
<td>0.6595</td>
</tr>
</tbody>
</table>

Data represents mean±SEM.

As previously mentioned, the effect of curcumin over NO concentration dynamics and coupled CBF changes was further addressed by using intraperitoneal injection as administration route. The effects of curcumin administrated systemically (300 mg/kg) are summarized in figure 17 and detailed in Table 3. Contrary to the effect observed after ICV injection, curcumin injected IP had no significant effect either at the level of NO production or CBF. Although a slight decrease was observed regarding the duration of NO signals (19%, 9 peaks analyzed from 2 individual experiments), NO peak concentration increased after curcumin intraperitoneal injection (15%). The glutamate-induced CBF increase was also slightly higher after curcumin injection (12%).

**Figure 17** - Quantitative analysis of peak NO concentrations (A) and CBF amplitude (B) before and after curcumin intraperitoneal injection. Data represents mean ± SEM. Statistical analysis was performed by Student’s t-test in relation to control experiments (*p < 0.05).
Table 3: Analysis of glutamate-induced NO signals and CBF changes in hippocampus before and after curcumin intraperitoneal injection.

<table>
<thead>
<tr>
<th></th>
<th>Ctr (n=5)</th>
<th>Cur (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{NO}] \text{ peak (µM)})</td>
<td>1.09 ± 0.56</td>
<td>1.25 ± 0.19</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>69 ± 28</td>
<td>52 ± 18</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>137 ± 23</td>
<td>112 ± 24</td>
</tr>
<tr>
<td>Half width (s)</td>
<td>98 ± 19</td>
<td>76 ± 22</td>
</tr>
<tr>
<td>CBF increase (%)</td>
<td>89 ± 11</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>96 ± 18</td>
<td>94 ± 18</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>367 ± 53</td>
<td>293 ± 51</td>
</tr>
</tbody>
</table>

Data represents mean±SEM.

2. Epicatechin

The effects of epicatechin over neurovascular coupling mediated by NO were also evaluated by ICV injection. A representative recording of the effect of epicatechin 100 µM over NO and CBF dynamics in hippocampus is shown in figure 18. The parameters obtained by the analysis of individual NO peaks and CBF changes before and after epicatechin administration are detailed in table 4.

![Figure 18](image_url)

Figure 18: Effect of epicatechin in glutamate-induced NO dynamics (black line) and CBF changes (grey line) in rat hippocampus. L-Glutamate (20 mM, 25 nL) was locally applied at times indicated by the upward arrows. Epicatechin (100 µM) was injected intracerebroventricularly at the time indicated by the downward arrow.
Table 4- Analysis of glutamate-induced NO signals and CBF changes in hippocampus before and after Epicatechin (100µM) locally applied in hippocampus.

<table>
<thead>
<tr>
<th></th>
<th>Ctr (n= 3)</th>
<th>Epicatechin 100 µM (n=7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NO] peak (µM)</td>
<td>1.62 ± 0.05</td>
<td>1.72 ± 0.11</td>
<td>0.4072</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>51 ± 4</td>
<td>52 ± 6</td>
<td>0.8953</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>135 ± 3</td>
<td>120 ± 7</td>
<td>0.3372</td>
</tr>
<tr>
<td>Half width (s)</td>
<td>70 ± 1</td>
<td>73 ± 3</td>
<td>0.4072</td>
</tr>
<tr>
<td>CBF increase (%)</td>
<td>106 ± 10</td>
<td>112 ± 19</td>
<td>0.7780</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>82 ± 9</td>
<td>68 ± 3</td>
<td>0.3181</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>402 ± 16</td>
<td>356 ± 46</td>
<td>0.3372</td>
</tr>
</tbody>
</table>

Data represents mean±SEM.

While in control conditions, average NO peak concentration was 1.62 ± 0.05 µM and CBF change 106 ± 10%, after epicatechin NO peak concentration was 1.72 ± 0.11 µM and the CBF change 112 ± 19%. Although without statistical significance, a slight increase (c.a. 6%) was observed in both NO peak concentration and amplitude of CBF changes, as evidenced in figure 19. As previously observed for curcumin, a slight decrease was also observed regarding the duration of both NO concentration dynamics and CBF changes after epicatechin ICV injection (c.a 11%).

Figure 19- Quantitative analysis of peak NO concentrations (A) and CBF amplitude (B) before and after Epicatechin (100 µM) intracerebroventricular injection. Data represents mean ± SEM. Statistical analysis was performed by Student’s t-test in relation to control experiments (*p < 0.05).
In addition to the effect over glutamate-induced responses, it was interesting to note that the administration of epicatechin promoted an increase in basal CBF levels. As can be observed in figure 18, around 8 min after the injection of the polyphenol CBF increase, reaching a plateau 25% higher that was maintained till the following glutamate stimulation, after which the CBF basal levels were restored.

3. Discussion

NO, a free radical messenger produced upon glutamatergic neuronal activity in brain by nNOS, is implicated both in mechanisms of synaptic plasticity, underlying learning and memory, but also in neuronal degeneration. Because NO is highly diffusible and overcomes specific receptor interaction, it conveys information associated with its concentration dynamics, which implies that it is critical to measure directly NO concentration profiles to unravel its involvement in physiological and pathological processes. Recently, by measuring simultaneously NO and CBF changes, NO was identified as the mediator of the neurovascular coupling in rat hippocampus, establishing a diffusional wireless connection between active glutamatergic neurons and blood vessels (unpublish data). In this work, by using an identical approach we observed that localized glutamate injection in rat hippocampus results in transient elevations of NO levels, which are followed by transient increases in CBF. This observation corroborates the idea that NO match local blood supply with neuronal activity, a critical mechanism for the brain to maintain its structural and functional integrity (Drake and Iadecola, 2007).

Given that natural occurring polyphenols have been described to improve memory, learning and general cognitive ability as well as to afford neuroprotection against brain injury and neurodegeneration (Frade et al., 2005), in this work, we addressed the potential of two polyphenols, curcumin and epicatechin, to modulate glutamate-elicited NO production and coupled cerebral blood flow changes. In addition to their antioxidant activity (Natsume, 2003, Münzel et al., 2010), both curcumin and epicatechin has been shown to modulate several signaling pathways with significance in the context of this work. For instance, curcumin showed to protect against quinolinic acid-induced excitotoxicity in cultured neurons, by inhibiting the quinolinic acid-induced \( \text{Ca}^{2+} \) influx and nNOS activity and to be a potent inhibitor of iNOS (Lin, 2007).

By simultaneous measuring NO concentration dynamics and CBF changes before and after an acute administration of curcumin intracerebroventricularly, we observed that it was able to
promote a slight decrease either in glutamate-induced \textsuperscript{1}NO concentration dynamics as in the coupled CBF changes. This observation may be explained either by a direct scavenging of \textsuperscript{1}NO, decreasing it bioavailability and thus it volume signaling, as well as by an effect along NMDAR-nNOS pathway. Indeed, curcumin has been described to inhibit PKC activity, and subsequent phosphorylation of NR1 of the NMDA receptor, which culminate with a reduced Ca\textsuperscript{2+} influx and decreased nNOS catalytic activity. When the effect of curcumin was evaluated by an acute systemic administration, it was observed that it was not able to decrease the amplitude of \textsuperscript{1}NO and CBF signals, as it did once injected ICV, but instead it promoted a slight increase (although the time course of both dynamics were reduced). We can speculate that this difference observed dependent of the route of curcumin administration relates with the bioavailability of curcumin, which expectedly is lower in the brain after systemic injection. Moreover, through this route, curcumin will be at least partially metabolized and thus curcumin-derivatives may also contribute to modulate both endogenous \textsuperscript{1}NO production and CBF, affecting the same or other signaling pathways.

Regarding epicatechin, a slight increase was observed in both \textsuperscript{1}NO peak concentrations and amplitude of CBF changes after ICV injection. This result agrees with a previously observation that a red wine polyphenolic powder, also containing epicatechin, administered for 4 weeks increased nNOS activity in the cerebral cortex, cerebellum and brainstem (Jendekova et al., 2006). Also, epicatechin shown to stimulate phosphorylation of the cAMP-response element binding protein, a regulator of neuronal viability and synaptic plasticity and up-regulate AMPAR GluR2 subunit (Schroeter et al., 2007), which would have impact over \textsuperscript{1}NO production. However, considering the time window of the observed effects, mechanisms dependent on gene expression are unlikely.

In addition to the effects over glutamate-induced \textsuperscript{1}NO and CBF changes, epicatechin promoted an increase in basal CBF levels. In this respect it should be mentioned that epicatechin is suggested to promote activation of the endothelial isoform of NOS in blood vessels, which expectedly leads to vasodilation. Epicatechin induced eNOS activation is at least partially mediated via the Ca\textsuperscript{2+}/CaMKII pathway (Ramirez-Sanchez et al., 2010).

Based on the \textit{in vivo} insights here presented, iy is apparent that dietary polyphenols may, as a function of the structure, affect the neurovascular coupling process but, clearly, further work should be performed in order to provide robustness to the potential of both curcumin and epicatechin modulate neuronal-derived \textsuperscript{1}NO and neurovascular coupling, which may include the evaluation of a chronic administration of the polyphenols.
III. Dementia and inflammatory animal models

1. Acute model of Alzheimer Disease

The glutamate-induced NO and CBF changes were evaluated in a rat model of AD based on the intracerebroventricular injection of Aβ1-42 peptide. The use of direct Aβ infusion allows the study of the contribution of the amyloidogenic pathway and dissemination of the contribution of each Aβ peptide, in addition reduce significantly the experimental timeline (Lawlor and Young, 2011). In typical late-onset AD there is evidence that Aβ1-42 is a minor Aβ species, but it is also the earliest form and the predominant species deposited in Aβ1-42 the brain parenchyma (Golde et al., 2000).

1.1. Behaviour tests

The model of Aβ1-42 infusion used in this study was previously shown to be characterized by synaptotoxicity and memory impairment (Canas et al., 2009) but, because the synthetic Ab1-42 peptide is difficult to make and given the batch-to-batch variability reported (Lawlor and Young, 2011), behavior tests were performed in Aβ1-42 treated rats 15 days after the injection. In brief, memory performance was evaluated by the novel object recognition and Y-maze tests. We observed that Aβ1-42 injection in rats brain promoted a short-term memory impairment. This conclusion is based on the observation that they spent less time exploring the novel object and in the novel arm when submitted to the novel-object recognition and Y-maze test, respectively. Relatively to the motor function there is no difference in the open field locomotion test as compared to controls (Figure 20)
1.2. CBF and NO Dynamics

The global results of glutamate-induced NO and CBF changes in hippocampus of Aβ1-42-treated and control rats, 20 days after the injection, are represented in figure 21 and a detailed analysis is summarized in table 5. Regarding glutamate-induced NO production, although no significant differences were observed in terms of NO peak concentration between groups, NO signals were briefer in Aβ1-42-treated rat as compared with controls (vehicle treated). Overall, in average, NO concentration dynamic induced by glutamate was characterized by a peak concentration of 1.36 ± 0.06 µM and total duration of 79 ± 4 s in control rat and a peak concentration of 1.40 ± 0.07 µM and total duration of 65 ± 5 s in AD rat (p= 0.7111 and p= 0.0198 respectively, n=31 from 4 animals).

Glutamate-induced CBF changes coupled to NO dynamics were not significantly affected by the Aβ1-42 treatment, at least regarding amplitude (119.56% and 126.05% in Aβ1-42-treated and control rats, respectively, p = 0.5887, n=31 from 4 animals). In turn, CBF changes showed to last shorter in Aβ1-42-treated rats than in control ones (208 versus 267s, respectively, p=0.0474).
Figure 21- Quantitative comparison of the effect of $A\beta_{1-42}$ over glutamate-induced NO dynamics (A) and CBF changes (B) in rat hippocampus. Data represents mean ± SEM. Statistical analysis was performed by Student’s t-test in relation to control experiments (*$p < 0.05$).

Table 5- Analysis of glutamate-induced NO signals and CBF changes in hippocampus of rats injected with $A\beta_{1-42}$ peptide and controls.

<table>
<thead>
<tr>
<th></th>
<th>CTR (n=31)</th>
<th>A$\beta$ (n=29)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[NO] peak (µM)</td>
<td>1.36 ± 0.06</td>
<td>1.40 ± 0.07</td>
<td>0.7111</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>20 ± 3.30</td>
<td>19 ± 3.39</td>
<td>0.8901</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>80 ± 3.55</td>
<td>65 ± 4.92</td>
<td>0.0198*</td>
</tr>
<tr>
<td>Half width (s)</td>
<td>44 ± 3.58</td>
<td>355 ± 5.15</td>
<td>0.1776</td>
</tr>
<tr>
<td>CBF increase (%)</td>
<td>120 ± 6.74</td>
<td>126 ± 9.95</td>
<td>0.5887</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>73 ± 4.79</td>
<td>64 ± 4.05</td>
<td>0.1642</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>267 ± 20.09</td>
<td>208 ± 20.96</td>
<td>0.0474*</td>
</tr>
</tbody>
</table>

1.3. Modulation of neurovascular coupling in AD by Curcumin

Although no statistically significant effects of curcumin were detected over NO-mediated neurovascular coupling under physiological conditions, we aimed to evaluate whether it had any potential to reverse differences observed in $A\beta_{1-42}$-treated rat as compared to controls reported in the previous section. For that purpose curcumin was injected intraperitoneally (300 mg/Kg) and a representative recording is presented in Figure 22. Globally, the results were quite similar to those
obtained in naïve rats (section I), as curcumin injected IP in Aβ₁₋₄₂-treated rats had no strong and significant effect either at the level of NO production or CBF changes evoked by glutamate stimulus. The detailed analysis is presented in table 6. In average, NO concentration dynamics induced by glutamate in Aβ models was characterized by a peak concentration of 3.76± 0.29 µM and total duration of 92.89 ± 27.69 s and a peak concentration of 4.12 ± 0.47 µM and total duration of 53.75 ± 7.74 s, respectively, before and after curcumin, while the corresponding CBF changes were, respectively, 159.08 ± 11.84 % and 168.00 ± 38.27 %. However, a fine tuned observation of the recordings evidences a slight increase in the NO concentration and a decrease in the duration of NO signals after curcumin treatment that were accompanied by an increase in CBF changes.

![Graph showing NO concentration and CBF changes](image)

**Figure 22**: Effect of *in vivo* peripheral administration of curcumin (300 mg/kg, IP) in Aβ₁₋₄₂-treated rats in glutamate-induced NO dynamics (black line) and CBF changes (grey line) in rat hippocampus
Table 6: Analysis of glutamate-induced NO signals and CBF changes in hippocampus of Aβ1-42-treated rats before and after intraperitoneal injection of curcumin

<table>
<thead>
<tr>
<th></th>
<th>Aβ (n= 19)</th>
<th>Aβ_Cur (n=4)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NO] peak (µM)</td>
<td>3.76±0.29</td>
<td>4.12 ± 0.47</td>
<td>0.4456</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>22 ± 4.30</td>
<td>11 ± 1.32</td>
<td>0.2074</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>93 ± 27.69</td>
<td>54 ± 7.74</td>
<td>0.3093</td>
</tr>
<tr>
<td>Half width (s)</td>
<td>43 ± 5.72</td>
<td>34 ± 5.97</td>
<td>0.6161</td>
</tr>
<tr>
<td>CBF increase (%)</td>
<td>159 ± 11.84</td>
<td>168 ± 38.27</td>
<td>0.4191</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>57 ± 3.14</td>
<td>58 ± 12.40</td>
<td>0.7329</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>272 ± 22.15</td>
<td>220 ± 52.25</td>
<td>0.6299</td>
</tr>
</tbody>
</table>

2. Discussion

Neurofibrillary tangles (NFTs), insoluble β-amyloid (Aβ) plaques, neuron loss, NO-related pathways and cerebrovascular dysfunction have been shown to be associated and/or contribute to the progression of AD being the major neuropathological features of this type of dementia (Hardy, 2006). However, the ascertainment of the exact role of each one of these factors still requires investigation.

The experimental approach here used, based on the direct and simultaneously measurement of NO and CBF changes in hippocampus of Aβ1-42-treated rats, aimed to unravel critical mechanisms in AD, namely by providing in vivo critical information on how neuronal-derived NO dynamics and cerebrovascular function occur in AD. Using such approach we observed that in Aβ1-42-treated rats glutamate-induced NO signals were short lasting as compared with controls, although identical regarding the peak amplitude. Previous studies developed by the host group using a triple transgenic mouse model of AD- 3xTg-AD mice also showed that NO peak concentration upon glutamatergic activation remained roughly unchanged between AD mice and controls. Only in aged 3xTg-AD mice (12 month-old) subtle differences were observed in terms of duration of the NO signals, which lasted longer as compared to nonTg mice, evidencing that the pathway of glutamate-induced NO production was not profoundly impaired. In agreement, the results here presented neither support any significant effect of Aβ over nNOS nor implicate nNOS in
the mechanisms of Aβ toxicity and associated cognitive deficits, observed to occur in Aβ1-42-treated rats. At this respect it should be mentioned that Aβ peptide has been described to both inhibit nNOS (Oliveira et al., 2011) as well as stimulate nNOS activity (Stepanichev et al., 2008). Indeed, Rodrigo and colleagues found that while nNOS expression was unaltered, its activity was decreased in the cerebral cortex of 16-month-old Tg2576 mice, as compared to age matched controls (Rodrigo et al., 2004). On the other hand, these authors found an increase in iNOS expression and activity in cortex. Other studies have previously reported that Aβ1-42 infusion, although promoting an impairment in working memory, fail to significantly increase in lipid peroxide levels or MDA and NOx levels (Yamada et al., 1999; Cetin and Dincer, 2007).

Regarding the CBF changes coupled to NO dynamics we observed that in the Aβ1-42-treated rats neurovascular coupling was not significantly different from control animals. In turn, in 3xTg-AD mice previous work demonstrated that glutamate-induced CBF changes were significantly lower in aged 3xTg-AD mice, as compared with non-Tg, suggesting that NO-mediated neurovascular coupling is impaired in the later stages of AD (unpublish data). Moreover, it has been reported that many patients with Alzheimer’s disease have regional cerebral hypoperfusion, which correlates to cognitive decline (Johnson et al., 2005; Ruitenberg, et al., 2005) and that 3xTgAD mice with 11-month have a significant reduction of hippocampal vascular volume (Bourasset et al., 2009). Thus, and considering that Aβ is able to disrupt the physiological mechanisms regulating CBF (Niwa et al., 2000b), it would be expected to obtain some degree of impairment in neurovascular coupling in Aβ1-42-treated rats. By promoting oxidative stress (Park et al., 2004), Aβ should inhibit the production of astrocytic and neuronal derived vasodilating messengers (Sun et al., 2008) and thus decrease functional hyperemia. However, Aβ failed to significant affect NO dynamics as well as that the impairment described to occur in aged 3xTgAD mice is suggested to be largely due to cerebrovascular dysfunction, rather than a dysfunctional NO signalling from neurons to blood vessels. The divergent observations regarding CBF changes may be explained, among other factors, by different levels of soluble Aβ as well as degree and distribution of Aβ deposits between both models. Accordingly, the impairment of CBF increase associated to neuronal activation seems to be more pronounced in the transgenic lines with higher Aβ levels (Niwa et al., 2000b). Also, different Aβ fragments seems differently affect cerebrovascular function, as cerebrovascular alterations observed in transgenic mice are reproduced by superfusion of Aβ1-40, which predominates in vessels, but not by Aβ1-42 (Niwa et al., 2000b). Thus, our data support that Aβ1-42 itself, although
inducing memory impairment, is unable to impair the matching of blood supply with the metabolic demands imposed by increased neuronal activity, suggesting that it deposition may be required to promote cerebrovascular dysfunction as observed in 3xTg-AD mice.

As mentioned before, in Aβ1-42-treated rats glutamate-induced NO signals were not significantly affected regarding peak amplitudes, however, were short lasting as compared with controls, which in part may be due to increased scavenging of NO associated to the Aβ-induced oxidative stress. Indeed, Aβ seems to promote cerebrovascular effects by enhancing oxidative stress, given that the vasoactive action of Aβ is counteracted by antioxidants and scavengers of RONS (Iadecola, 2003). We attempted to counteract the effects observed in Aβ1-42-treated rats by acutely administering curcumin, however no significant effects were observed. Curcumin has been showed to be able to modulate a wide variety of pathways related to AD development. Indeed, curcumin has been described to have anti-amyloidogenic properties, preventing Aβ aggregation through the dienone bridge present in curcumin, which is necessary to reduce plaque deposition and protein oxidation in an Alzheimer's model (Begum et al., 2008). The lack of significant effects here reported however does not minimize the potential neuroprotective role of curcumin, which has been described in several studies. Curcumin extract showed to reduce glutamate-induced excitotoxicity and consequently the neurodegeneration processes in the hippocampus (Pyrzanowska et al., 2010) and that both glutamate and hydroxyl radicals in the hypothalamus caused by central administration of a variety of inflammation inductors, through inhibition of the glutamate-hydroxyl radicals-PGE2 pathways. Additionally it is known that curcumin modulates levels of brain biogenic amines and NO in arsenic-exposed rats, where increased levels of NO in corpus striatum, frontal cortex as well hippocampus in arsenic-treated rats were found decreased in rats treated with curcumin (Yadav et al., 2010). Also, curcumin demonstrated neuroprotective action against focal cerebral ischemic injury, as it significantly diminished infarct volume, and improved neurological deficit in a dose-dependent manner after middle cerebral artery occlusion (MCAO) (Lin, 2004, Zhao et al., 2008), namelly by preventing ONOO− mediated BBB damage (Jiang, 2007). Also, (Shin, 2007) suggest that curcumin is a potent inhibitor of reactive astrocyte expression and thus, prevents hippocampal cell death in mice induced by kainic acid. Thus, further experiments are required, namely be testing it effect chronically and in models of more advanced state of AD, as well by addressing it effect specifically over iNOS, the isoform responsible for the majority of NO-related pathological mechanisms.
3. Lipopolysaccharide (LPS) inflammation induction

NO mediated neurovascular coupling was further addressed under inflammatory conditions. For that purpose, we used lipopolysaccharide (LPS), a component of the Gram-negative bacteria cell wall, which is a potent inducer of inflammation and has diverse effects on cells of the immune system. It was described that the intracerebral injection of LPS activated glial cells in vivo (Andersson, 1992; Szczepanik et al., 1996) and also mimicks AD (Hauss-Wegrzyniak et al., 2000; Hauss-Wegrzyniak and Wenk, 2002; Miklossy, 2008). To accomplish the objective, LPS was either injected ICV (20 μg dissolved in 4 μL of 0.9% NaCl) as IP (2 mg/kg), and its effects evaluated 4.5 and 24 hours later, respectively. In figure 23, it is shown a representative recording of the effects of LPS injected ICV and the effects of both ICV and IP administration routes summarized in table 8. In control conditions, NO production was characterized by a peak concentration of 2.09 ± 0.66 μM with a time rise of 17 ± 2s and a total duration of 77± 17 (6 peaks analyzed from 4 individual experiments). The CBF started to increase 7 ± 2 s after stimulation reaching 103± 22% of the basal level after 56 ± 7s and returning to basal levels after 368± 25s. LPS, 4.5h after ICV injection promoted a slight decrease in duration of NO signals, although the peak concentration was unaffected. Also, regarding the coupled CBF changes, while amplitude was similar after LPS, the transient increase in CBF was short lasting. Intriguing, 24 h after LPS injected IP, while both NO peak concentrations and CBF changes remained similar to the control condition, a significant increase in the time rise was observed in NO concentration dynamics (p < 0.05). A reasonable reason for this event could be related by the fact that pos 24h of inflammation, the levels of iNOS and ROS will be higher as we know, and thus could occur NO scavenging by specialized molecules, as we will discuss later. Also, it should be highlighted the dramatic perturbation of LPS in CBF observed after 6h, shown in Figure 23, where it appears that the increased inflammation over the time induced the crashing of neurovascular regulation and a decoupling between neuronal activity and blood supply.
Figure 23- Intracerebroventricular perfusion of brain with 20 µg of LPS followed by oxidation current records (black line), and cerebral blood changes (grey line) after Glu stimulations during approximately 6 hours of inflammation induction.

Figure 24- Quantitative analysis of the effect of inflammation induced by LPS injected ICV (after 4.5h) and intraperitoneally (after 24 h) over glutamate-induced *NO dynamics (A) and CBF changes (B) in rat hippocampus. Data represents mean ± SEM, (*p < 0.05).
Table 7 - Analysis of glutamate-induced \( \textit{\textsuperscript{\textit{\textit{NO}}}} \) signals and CBF changes in hippocampus before and 4.5 and 24h after LPS-induced inflammation

<table>
<thead>
<tr>
<th></th>
<th>Ctr (n=6)</th>
<th>LPS 4.5 h (n=11)</th>
<th>LPS 24 h (n=4)</th>
<th>( P ) (Ctr vs 4.5)</th>
<th>( P ) (Ctr vs 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \textit{\textsuperscript{\textit{NO}}} ) peak (µM)</td>
<td>2.09 ± 0.66</td>
<td>2.03 ± 0.54</td>
<td>1.85 ± 0.46</td>
<td>0.9485</td>
<td>0.7770</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>17 ± 2.45</td>
<td>14 ± 2.90</td>
<td>31 ± 5.55</td>
<td>0.4161</td>
<td>0.0823</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>77 ± 17.29</td>
<td>59 ± 12.63</td>
<td>62 ± 1.31</td>
<td>0.4172</td>
<td>0.4290</td>
</tr>
<tr>
<td>Half width (s)</td>
<td>31 ± 6.32</td>
<td>27 ± 8.10</td>
<td>30 ± 6.76</td>
<td>0.6359</td>
<td>0.8754</td>
</tr>
<tr>
<td>CBF increase (%)</td>
<td>103 ± 21.9</td>
<td>90 ± 24.88</td>
<td>111 ± 18.16</td>
<td>0.7091</td>
<td>0.7916</td>
</tr>
<tr>
<td>Trise (s)</td>
<td>56 ± 6.97</td>
<td>47 ± 11.37</td>
<td>41 ± 3.66</td>
<td>0.4827</td>
<td>0.0969</td>
</tr>
<tr>
<td>Ttotal (s)</td>
<td>368 ± 24.56</td>
<td>203 ± 34.53</td>
<td>286 ± 5.11</td>
<td>0.0029**</td>
<td>0.0222*</td>
</tr>
</tbody>
</table>

4. Discussion

The bacterial inflammatory surface molecule lipopolysaccharide (LPS) is a powerful inflammatory endotoxin and amyloidogenic factor, being widely used in experimental \textit{in vivo} models of inflammation and amyloidosis (Miklossy, 2008).

Our results of inflammatory condition, induced by ICV injection of LPS, showed that \( \textit{\textsuperscript{\textit{NO}}} \) concentration dynamics in hippocampus were slightly affected (reduced duration), and the same effect was observed for blood flow (Glezer \textit{et al.}, 2003). Also, 24 h after inflammation induced systemically, duration of both \( \textit{\textsuperscript{\textit{NO}}} \) and CBF dynamics were reduced. In turn, the amplitude of CBF changes were less diminished after LPS injected systemically than injected within the brain. This may be associated with the antipyretic effect towards higher supply of blood cells involved in organism defense against inflammation, an elation according to Arreto and its colleagues assigning the pivotal role of macrophages for LPS which induce a dose- and time-dependent neutrophil recruitment accompanied by the generation of a tumour necrosis factor-\( \alpha \) (TNF\( \alpha \))-like activity (Arreto \textit{et al.}, 1997). It has been shown that a considerable part of NF-KB activation by LPS is linked to the NMDA/\( \textit{\textsuperscript{\textit{NO}}} \) pathway in CNS (Glezer \textit{et al.}, 2003). However, the role of NF-KB as a molecule with neurodegenerative or neuroprotector actions is still enigmatic. The relevance of the intracellular cascade linking NMDA pathway to NF-KB activation is also supported by \textit{in vitro} (Burr
and Morris, 2002) and in vivo studies (Madrigal et al., 2001) evidencing the LPS-induced increase of nNOS/eNOS activity (Sánchez-Lemus et al., 2009).

Previous studies described LPS as highly resistant to degradation by mammalian enzymes and thus constituting a persisting inflammatory stimulus. LPS activates many cell types and when administered to animals, a variety of factors are released, such as cytokines, platelet activating factor (PAF), complement-derived C5a anaphylatoxin and NO derived from iNOS. Moreover, LPS injection causes induction of expression of inducible enzyme isoforms, such as the group II extracellular phospholipase A2 (PLA2), the inducible NO synthase (iNOS) and the cyclooxygenase-2 (COX2), all contributing to the hypotensive state in septic shock (Lippolis et al., 2003). Additionally (Standen et al., 1989) have shown the presence of ATP-sensitive potassium (K_{ATP}) channels on vascular smooth muscle cells, ascribing to these channels a role in the regulation of vascular tone.

Particular attention has been focused on the involvement of K_{ATP} channels in both hypotension and vascular hyporeactivity induced by endotoxemia, confirmed latter by (Lippolis et al., 2003) which attribute this channels to be involved in delayed vascular hyporeactivity in rats (24h after Escherichia coli LPS injection). Several authors have shown the involvement of K_{ATP} channels in the early phase (within 5 h of LPS infusion) of endotoxic shock, in anaesthetized (Wu et al., 1995) and unconscious rat (Gardiner et al., 1999), which show the disturbance verified after 4.5 h of inflammation compared to the control.
Chapter 4.

General Conclusions
Globally, by means of *in vivo* and measurements of *NO* concentration dynamics and CBF changes in Aβ1-42-treated rats as well as in an inflammatory LPS rat model of dementia, this work supports a role for neuronal derived-*NO* in cerebrovascular function in neuropathological conditions. Additionally we evaluate whether neurovascular coupling, associated with aging and neurodegeneration, may be modulated by dietary polyphenols such as curcumin and epicatechin. The potential modulation of such a process, that if impaired is related with aging and disease, would critically impact on human health.

The obtained results demonstrated that: i) acute administration of curcumin and epicatechin did not strongly and significantly affect neither *NO* nor CBF profiles under physiological conditions, but insights of potential modulation were provided on basis of a fine tuned observation of the recordings; ii) the neuronal-derived *NO* profiles were not significantly disturbed in Aβ1-42-treated rats (NO signals were short lasting as compared with controls and CBF changes was not significantly different from control animals), evidencing that the pathway of glutamate-induced *NO* production in Aβ1-42-treated rats was not profoundly impaired, although the cognitive deficits found; iii) curcumin did not show significant effects over *NO* dynamics and CBF changes in Aβ1-42-treated rats; iv) glutamate-induced *NO* dynamics and coupled CBF changes were slightly reduced in LPS rat model, showing that the neurovascular coupling is compromised in inflammation rat model.
Chapter 6.

References


