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Research Paper

Neurovascular uncoupling in the triple transgenic model of Alzheimer's disease: Impaired cerebral blood flow response to neuronal-derived nitric oxide signaling

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ABSTRACT

Nitric oxide (•NO)-dependent pathways and cerebrovascular dysfunction have been shown to contribute to the cognitive decline and neurodegeneration observed in Alzheimer's disease (AD) but whether they represent initial factors or later changes of the disease is still a matter of debate. In this work, we aimed at investigating whether and to what extent neuronal-derived •NO signaling and related neurovascular coupling are impaired along aging in the hippocampus of the triple transgenic mouse model of Alzheimer's Disease (3xTg-AD). We performed a longitudinal study combining behavior studies, *in vivo* simultaneous measurements of •NO concentration gradients and cerebral blood flow (CBF), along with detection of NO synthase (NOS) and markers of nitroxidative stress. Our results revealed an impairment in the neurovascular coupling along aging in the 3xTg-AD mice which preceded obvious cognitive decline. This impairment was characterized by diminished CBF changes in response to normal or even increased •NO signals and associated with markers of nitroxidative stress. The results suggest that impairment in neurovascular coupling is primarily due to cerebrovascular dysfunction, rather than due to dysfunctional •NO signaling from neurons to blood vessels. Overall, this work supports cerebrovascular dysfunction as a fundamental underlying process in AD pathology.

1. Introduction

Neurovascular coupling is a critical process for maintaining brain functional and structural integrity as it orchestrates local adjustments of blood flow and delivery of bioenergetic substrates in accordance to the energetic demands imposed by neural activation. Failure in the regulation of this complex process has been strongly associated with neuronal dysfunction and disease (Iadecola, 2004; Nicolakakis and Hamel, 2011). This may be the case of Alzheimer's disease (AD), a common age-related neurodegenerative disorder characterized by progressive cognitive decline associated to neuronal loss in brain areas linked to memory processing. Classically, research has identified neuropathological features of the disease in the form of accumulated damaged proteins, namely amyloid β peptide (Aβ) and neurofibrilary tangles, but early dysfunctional pathways have remained elusive (Krstic and Knuesel, 2013). Evidence from clinical imaging, epidemiological and pharmacotherapeutical studies have revealed the critical contribution of cerebrovascular dysfunction and impaired neurovascular coupling to the cognitive decline and neurodegeneration observed in AD (Iadecola, 2004; Love and Miners, 2016; Montagne et al., 2016; Zlokovic, 2011). This has led some authors to propose a change in the paradigm of the disease's etiology, identifying AD primarily as a cerebrovascular rather than an inherently neuronal disease (Correia et al., 2012; de la Torre, 2004; Iadecola, 2004). However, while the pathologic importance of cerebrovascular alterations has been gaining strength, the temporal relationship between the onset of hemodynamic dysfunction and the disease progression is not fully elucidated. The controversy stems from the observation that, if on the one hand cerebral hypoperfusion is reported to potentiate Aβ overproduction and accumulation (Iadecola, 2004; Koike et al., 2010), on the other hand Aβ is shown to disrupt the physiological mechanisms regulating cerebral blood flow (CBF) (Iadecola, 2003; Niwa et al., 2000). Thus, although it is accepted that cerebrovascular dysfunction occurs in AD, it is still a matter of debate whether it is a cause or a consequence of the pathology (Stanimirovic and Friedman, 2012).

Nitric oxide is a multifaceted signaling molecule that modulates a wide range of important physiological pathways, but is also implicated in pathological events (Dawson and Dawson, 1998; Knott and Bossy-Wetzel, 2009; Law et al., 2001). It is produced endogenously by a family of nitric oxide synthases (NOS) that exhibit different cellular localizations and are subject to distinct regulation pathways. While the neuronal and endothelial isoforms (nNOS and eNOS, respectively) are

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constitutively expressed, the inducible isoform (iNOS) depends on *de novo synthesis*, which occurs under immunological or inflammatory conditions (Zhou and Zhu, 2009). Although all NOS isoforms are suggested to operate as central mediators in AD, data regarding the constitutive isoforms are highly inconsistent (Limon et al., 2009; Luth et al., 2000; Norris et al., 1996; Yew et al., 1999). Nitric oxide, produced in the neurovascular unit either by neuronal, glial or vascular cells, has been critically implicated in the cerebrovascular regulation. The underlying pathways are complex and its specific role in the regulation of cerebral blood flow likely diverge among different brain regions (reviewed in (Lourenco et al., 2016; Tabatabaei and Girouard, 2014)). In the hippocampus, one of the most severely affected regions in AD, we have recently shown that •NO produced upon glutamatergic stimulation assumes, predominantly *via* the NMDAr-nNOS-sGC pathway, a crucial role as mediator of the neurovascular coupling (Lourenço et al., 2014).

In this work, by using a multidisciplinary approach, combining behavior testing, *in vivo* simultaneous measurements of •NO and cerebral blood flow (CBF) changes, and detection of NOS and markers of nitroxidative stress, in the triple transgenic mice model of AD (3xTg-AD) we aimed to evaluate whether and to what extent the nNOS-related •NO signaling pathway is altered in AD and its correlation with neurovascular coupling.

2. Materials and methods

2.1. Animals

Experiments were conducted in the triple transgenic mouse model of AD (3xTg-AD) developed by Oddo and colleagues. The 3xTgA-D mice expressing $PS1_{M146V}$, APP_{Swe}, and tau_{P301L} transgenes are reported to develop age-dependent and region-specific Aβ and tau aggregations, as well age-related impairment in synaptic plasticity and cognitive function that closely mimic the disease progression in humans (Billings et al., 2005; Oddo et al., 2003). Both 3xTg-AD mice and background strain (C57BL6/129sv mice, Non-Tg) were supplied from a colony implemented in the animal house facilities of the Center for Neurosciences and Cell Biology (Coimbra) obtained from Dr. Frank LaFerla's laboratory at the Department of Neurobiology and Behavior and Institute for Brain Aging and Dementia, University of California at Irvine. Male mice (3–5 littermates per cage) were housed in filter-topped type II Makrolon cages on individually ventilated caging systems (VentiRack Bioscreen ™). The environmental conditions were maintained with a temperature of 22–24 °C, relative humidity of 45–65%, 15 air exchanges per hour and a 12:12 light/dark cycle. Cage bedding of standard corn cob was changed twice a week and environmental enrichment was provided with a piece of tissue paper and a cardboard tube. Mice were fed with a standard chow mice diet (4RF25-GLP Mucedola, SRL, Settimo Milanese, Italy) and chlorinated water was provided *ad libitum*.

All the procedures used in this study were performed in accordance with the European Union Council Directive for the Care and Use of Laboratory animals (2010/63/EU) and approved by local ethics committee (ORBEA). Mice were evaluated in a cross-sectional design in 3 aging groups determined by the expected pathology progression (Billings et al., 2005; Oddo et al., 2003). The young group included animals of 3 months old (reported to have intraneuronal Aβ in cortex) and 6 months old (reported to have intraneuronal Aβ in hippocampus). We included both ages in the same group for simplification due to the lack of differences observed between both ages. The middle-aged group included animals with 12-months of age (reported to have Aβ extracellular in hippocampus) and the old aged group included animals with 18-months of age (reported to have extracellular plaques widespread throughout the hippocampus and cortex). Cages were assigned to each group in a random fashion. A total of 70 mice were used in this study (38 3xTg-AD and 32 Non-Tg).

2.2. Behavior tests

2.2.1. Modified Y-maze test

Spatial memory performance was evaluated using a Plexiglas Y-shape apparatus consisting of 3 equal arms (5 × 35 × 10 cm) with a 120° angle between them. In the trial, animals were placed in the Y-maze with one arm blocked and allowed to explore the two accessible arms for 5 min. After 2 h, the animals were placed back in the maze and allowed to explore the 3 arms for 5 min. The percentage of time spent exploring the novel arm in the test was used as index of memory performance.

2.2.2. Modified Morris water maze test

The spatial reference memory version of the Morris water maze was performed in a large circular tank (1.5 m diameter, 50 cm depth) filled with water at 22 °C. A transparent Plexiglas platform (10 cm diameter) was submerged 1 cm beneath the water surface in a constant position (centered in an imaginary quadrant). Distinctive visual cues were set up on the wall surrounding the tank, positioned in the midpoint of the perimeter of each quadrant. The training session consisted of 10 trials during which, in a pseudo-random order, mice were placed in the water facing the tank at the points defined by the quadrants limits. Animals were allowed 60 s to locate the hidden platform, and in case of failure, were guided by the investigator to the platform. In either case, animals were allowed to remain in the platform 10 s before being removed from the tank. Each trial was delayed 5 min to allow the animal warm up between trials. In each trial, the time required for mice to reach the platform was registered. Retention of the spatial training was assessed after 24 h in a single probe trial consisting in a 60 s free swim in the tank without the platform. The number of times mice crossed the location where the platform was placed in the training trial (platform location crossings) and the percentage of time spent in the quadrant opposite to the target quadrant was determined as index of memory performance.

2.3. Nitric oxide and cerebral blood flow measurements

The •NO and cerebral blood flow dynamics were measured essentially as previously described (Lourenço et al., 2014). Briefly, homemade carbon fiber microelectrodes (tip length of 200 ± 50 μm, 30 μm Ø fiber Textron Lowell, MA) were modified with Nafion® and *o*-phenylenediamine to improve their analytical properties for •NO *in vivo* measurements. Each microelectrode was evaluated for •NO sensitivity and selectivity against major interferents (ascorbate, nitrite, noradrenaline and dopamine) by constant voltage amperometry at +0.9 V *vs* Ag/AgCl using a FAST-16 high-speed electrochemical system (Quanteon, LLC, Nicholasville, KY) in a two-electrode configuration, as extensively described (Barbosa et al., 2008; Lourenco et al., 2014; Santos et al., 2008). Cerebral blood flow was measured using a laser Doppler flowmeter device (Periflux system 5000, Perimed, Sweden) coupled with a needle probe (PF411; outer diameter, 450 μm; fiber separation, 150 μm; wavelength, 780 nm). The •NO microelectrode and the Laser Doppler probe were assembled to an ejection micropipette using sticky wax in a predefined geometric configuration which allowed us to monitor neurovascular coupling in hippocampus with minimal tissue damage (Supplementary Fig. 1). The micropipette was filled with 20 mM L-glutamate or 1 mM adenosine diphosphate (ADP) prepared in NaCl 0.9% using a syringe fitted with a flexible microfilament (MicroFil, World Precision Instruments, UK) prior to insertion into the brain.

2.4. Surgical procedure and in vivo recordings

The animals were anaesthetized by an intraperitoneal injection of urethane (1.5 g/Kg) and placed in a mouse-adapted stereotaxic apparatus (Stoelting Co., USA). Body temperature was maintained at 37 °C and