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The neuron-astrocyte-microglia triad involvement in neuroinflammaging mechanisms in the CA3 hippocampus of memory-impaired aged rats

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ABSTRACT

We examined the effects of inflammaging on memory encoding, and qualitative and quantitative modifications on proinflammatory proteins, apoptosis, neurodegeneration and morphological changes of neuron-astrocyte-microglia triads in CA3 *Stratum Pyramidale* (SP), *Stratum Lucidum* (SL) and *Stratum Radiatum* (SR) of young (3 months) and aged rats (20 months).

Aged rats showed short-term memory impairments in the inhibitory avoidance task, increased expression of iNOS and activation of p38MAPK in SP, increase of apoptotic neurons in SP and of ectopic neurons in SL, and decrease of CA3 pyramidal neurons. The number of astrocytes and their branches length decreased in the three CA3 subregions of aged rats, with morphological signs of clasmatodendrosis. Total and activated microglia increased in the three CA3 subregions of aged rats. In aged rats CA3, astrocytes surrounded ectopic degenerating neurons forming "micro scars" around them. Astrocyte branches infiltrated the neuronal cell body, and, together with activated microglia formed "triads". In the triads, significantly more numerous in CA3 SL and SR of aged rats, astrocytes and microglia cooperated in fragmentation and phagocytosis of ectopic neurons.

Inflammaging-induced modifications of astrocytes and microglia in CA3 of aged rats may help clearing neuronal debris derived from low-grade inflammation and apoptosis. These events might be common mechanisms underlying many neurodegenerative processes. The frequency to which they appear might depend upon, or might be the cause of, the burden and severity of neurodegeneration. Targeting the triads may represent a therapeutic strategy which may control inflammatory processes and spread of further cellular damage to neighboring cells.

1. Introduction

Brain aging is characterized by decline of cognitive functions along with a variety of neurobiological changes. As the lifespan expectancy of Western population increases, age-related cognitive decline represents a major challenge to the scientific community. Franceschi and coworkers (Franceschi et al., 2007; Deleidi et al., 2015) introduced the term "inflammaging" which describes the progressive changes occurring in the aging brain, characterized by a low-grade chronic up-regulation of certain pro-inflammatory responses and neuroinflammation. Indeed, aging is considered a primary risk factor for Alzheimer's disease (AD), and the onset of low-grade pro-inflammatory conditions observed in senescence is regarded as a prodrome of AD (Giunta et al., 2008; Salminen et al., 2012; Baylis et al., 2013; Salvioli et al., 2013). The association between inflammation, aging and neurodegeneration is based upon complex molecular and cellular changes that we are only just beginning to understand. For instance, it has been demonstrated that increase of pro-inflammatory molecules induces amyloid β

Abbreviations: aCSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; AL, acquisition latency; CA, Cornu Ammonis; CytC, cytochrome *C*; DAB, 3,3′-diaminobenzidine; ERK, extracellular regulated kinase; GABA, gamma-aminobutyric acid; GFAP, glial fibrillary acidic protein; iNOS, inducible nitric oxide synthase; IA, inhibitory avoidance; IBA1, ionized calcium binding adaptor 1; JNK, c-Jun N-terminal kinase/stress activates protein kinase; LPS, lipopolysaccharide; LTM, long term memory; LTP, long term potentiation; MAPK, mitogen activated protein kinase; MCP-1, monocyte chemoattractant peptide-1; MHCI, major histocompatibility class II; NeuN, neuronal nuclei; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; p38, p38 mitogen activated protein kinase; ROS, reactive oxygen species; RL, recall latency; SL, Stratum Lucidum; SP, Stratum Pyramidale; SR, Stratum Radiatum; TNF-α, Tumour necrosis factor-α.

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(Aβ)-deposition on neuron soma (Giunta et al., 2008; Blasko et al., 1999; Sastre et al., 2003; Mercatelli et al., 2015).

Emerging evidence indicates that inflammaging can modify the neuron-astrocyte-microglia interactions (Cerbai et al., 2012; Lana et al., 2014), and this mechanism may be involved not only in brain aging, but also in AD (Mercatelli et al., 2015). Astrocytes are known regulators of brain homeostasis (Wang and Bordey, 2008)and microglial phagocytosis (DeWitt et al., 1998; Yamanaka et al., 2007; Saijo and Glass, 2011; Cerbai et al., 2012; Lana et al., 2014). Both these cell types can recognize "danger signals", which include cellular debris produced from apoptotic or necrotic cells (Milligan and Watkins, 2009), and can clear damaged cells or cellular debris by phagocytosis (Cerbai et al., 2012; Lana et al., 2014). Functional alterations in senescent astrocytes are accompanied by remarkable morphological modifications: we recently demonstrated that senescent astrocytes in the CA1 hippocampal region exhibit morphological traits of clasmatodendrosis (Cerbai et al., 2012; Lana et al., 2014; Mercatelli et al., 2015). The phenomenon of astrocytic clasmatodendrosis, first described by Alzheimer (Penfield, 1928), consists of marked morpho-functional modifications of astrocytes such as beading and disintegration of their distal cell projections along with cytoplasmic vacuolization and swelling (Tomimoto et al., 1997; Hulse et al., 2001; Cerbai et al., 2012; Lana et al., 2014). Clasmatodendrosis has been previously depicted in the white matter of ischemic brains, in AD (Tomimoto et al., 1997) and in mixed Binswanger's/AD disease (Sahlas et al., 2002). Astrocyte clasmatodendrosis is induced in vitro by mild acidosis (Hulse et al., 2001), a micro-environmental condition commonly associated to aging (Ross et al., 2010), ischemia (Sahlas et al., 2002) and Aβ-deposition (Sua and Chang, 2001).

The hippocampus is one of the structures more closely characterized by electrophysiological, structural and morphological changes during aging. The hippocampal regions CA3, CA1 and dentate gyrus have striking anatomical differences (Amaral and Witter, 1989) and distinct functions, contributing uniquely to specific information processing such as novelty detection, encoding, short-term memory, intermediate-term memory and retrieval. It has been demonstrated that CA3 and CA1 neuronal ensembles perform distinct, yet complementary functions in the processing of spatial and contextual information (Vazdarjanova and Guzowski, 2004). In particular, the CA3 subregion of the hippocampus supports processes associated to rapid formation of spatial or contextual memory, especially in the acquisition of novel information and short-term memory (Lee and Kesner, 2002; Lee and Kesner, 2003; Nakazawa et al., 2003; Kesner et al., 2004). Declining hippocampal function is associated with aging and leads to impaired cognitive functions in nearly half of the population over 60 years of age (Small et al., 2002; Hedden and Gabrieli, 2004). With a growing aged population, it is therefore of the utmost importance to understand the age-related neurobiological changes that may contribute to hippocampal dysfunction and to identify target processes/pathways for therapeutic interventions to maintain or restore hippocampal function.

While in the common view neurons were considered to be the basic functional units of the central nervous system and glia only to serve as supportive elements, it is becoming more and more evident that proper functioning of the neuron-astrocyte-microglia "triad" is fundamental for the functional organization of the brain (Barres, 2008; Allen and Barres, 2009; Cerbai et al., 2012; Lana et al., 2014). The interplay among neurons and glia may be responsible for derangements from normal brain aging to neurodegenerative processes (De Keyser et al., 2008; Farfara et al., 2008; Sofroniew, 2009). Recruitment and activation of different astrocytes and microglia in a complex temporal pattern require well organized reciprocal communication between neurons and glia as well as among glial cells. In this study we used normal aged rats in comparison to young rats as controls to understand the interactions among neurons, astrocytes and microglia in the "triad", in physiological conditions and during inflammaging. Our investigation focused on changes in the area CA3 of the hippocampus because of its critical role in memory processing and because it demonstrates significant functional, structural, and morphological alterations with aging and AD (Padurariu et al., 2012). Based on our previous results obtained in the CA1 hippocampal region in different models of neurodegeneration and inflammaging (Cerbai et al., 2012; Lana et al., 2014), here we investigated how the interaction between glial cells and neurons change during normal aging in the three different subregions of the CA3 hippocampus, namely *Stratum Pyramidale*, *Stratum Lucidum* and *Stratum Radiatum* (Amaral and Lavenex, 2007), of aged, memory impaired rats. Our studies show that normal brain aging is characterized by a variety of neurobiological changes that are consistent with a chronic, low-grade inflammatory response. These findings support the recently proposed concept of "inflammaging" in describing progressive changes occurring in the aging brain, and facilitates future research on the role of inflammation in the neuropathology of AD. The actions of astrocytes and microglia may represent either protective mechanisms to control inflammatory processes and the spread of further cellular damage to neighboring tissue, or they may contribute to neuronal damage under pathological conditions. These results can be used to determine whether future therapeutic interventions should attempt to enhance or impair the actions of glia in the aged brain.

2. Materials and methods

Aim of the present research was to investigate howthe interaction between glial cells and neurons change during normal aging in the three different subregions of CA3 hippocampus, namely *Stratum Pyramidale*, *Stratum Lucidum* and *Stratum Radiatum*. We focused on changes in area CA3 of the hippocampus because of its critical role in memory processing and because it demonstrates significant functional, structural, and morphological alterations with aging and AD.

2.1. Animals

Male Wistar rats were used (Harlan Nossan, Milano, Italy). Experimental groups were: rats of 20 months of age (aged) and rats of 3 months of age (young). The animals were housed in Makrolon cages with ad libitum food and water, and were maintained on a 12 h light-12 h dark cycle with light at 7:00 am. The room temperature was 23 ± 1 °C. All rats were kept for at least 1 week in the animal house facility of the University of Florence before experiment. All animal manipulations were carried out according to the European Community guidelines for animal care. Formal approval to conduct the experiments described was obtained from the animal subjects review board of the University of Florence. All efforts were made to use only the number of animals necessary to produce reliable scientific data.

2.2. Step-down inhibitory avoidance task

In the step down inhibitory avoidance task rodents, put on an elevated platform placed by one wall of an arena, learn to associate exploration of the adjacent compartment with a foot shock delivered through the floor grid. On a subsequent exposure to the same environment, the animal will avoid to step down, or will increase the latency before "stepping down" onto the floor grid. We used a standard step-down apparatus placed in a soundproof room. All rats were habituated to the handling procedure the day before testing. On Day 1 (Acquisition trial), each rat is positioned on an elevated platform placed in a dark soundproof compartment facing an open arena with a floor equipped with an electrified grid. Rats were free to step down and explore the open arena. When rats placed all 4 paws on the grid, a foot shock (10 electric shocks, 20 ms/0.5 mA/5 Hz) was immediately delivered through the floor grid. The "Acquisition latency" (AL), i.e. the time spent before stepping down onto the grid was recorded. Rats were immediately removed from the arena and placed in their home cage for 60 min for memory consolidation ("Encoding"). Learning is demonstrated when, on a subsequent exposure to the same environment, the rat avoids