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Age-related changes of metallothionein 1/2 and metallothionein 3 expression in rat brain

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ABSTRACT

Neurodegeneration is one of the main physiological consequences of aging on brain. Metallothioneins (MTs), low molecular weight, cysteine-rich proteins that bind heavy-metal ions and oxygen-free radicals, are commonly expressed in various tissues of mammals. MTs are involved in the regulation of cell proliferation and protection, and may be engaged in aging. Expression of the ubiquitous MTs (1 and 2) and the brain specific MT3 have been studied in many neurodegenerative disorders. The research results indicate that MTs may play important, although not yet fully known, roles in brain diseases; in addition, data lack the ability to identify the MT isoforms functionally involved. The aim of this study was to analyse the level of gene expression of selected MT isoforms during brain aging. By using real-time PCR analysis, we determined the MT1/2 and MT3 expression profiles in cerebral cortex and hippocampus of adolescent (2 months), adult (4 and 8 months), and middle-aged (16 months) rats. We show that the relative abundance of all types of MT transcripts changes during aging in both hippocampus and cortex; the first effect is a generalized decrease in the content of MTs transcripts from 2- to 8-months-old rats. After passing middle age, at 16 months, we observe a huge increase in MT3 transcripts in both cortical and hippocampal areas, while the MT1/2 mRNA content increases slightly, returning to the levels measured in adolescent rats. These findings demonstrate an age-related expression of the *MT3* gene. A possible link between the increasing amount of MT3 in brain aging and its different metal-binding behaviour is discussed.

1. Introduction

Metallothioneins (MTs) are small, intracellular heavy-metal-binding proteins involved in metal detoxification and protection against oxidative stress [1,2]. Ubiquitously present in living organisms [3,4], the mammalian MT family falls into four subgroups: MT1, MT2, MT3, and MT4 [5]. MT1 and MT2 encode through a series of gene duplication events for multiple isoforms present in almost all tissues, while MT3 and MT4 evolved in a single form to accomplish specific roles in brain and epithelium, respectively [6–8]. In the mammalian brain, MT1 and MT2 are preferentially expressed in astrocytes and activated microglia [9]; MT3 is expressed predominantly in neurons and, to a lesser extent, in astrocytes [10].

It has been demonstrated that MT1 and MT2 protect the central nervous system (CNS) in response to experimentally induced injury [11] and following viral infections [12], whereas MT3 neither affects inflammatory responses nor plays an important antioxidant role [11]. Indeed, the MT3 functional role and regulation in CNS is still a matter of debate [10,13]. MT3 is known as a growth inhibitory factor

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able to inhibit the survival and neurite formation in cultured neurons in vitro [14]. MT3 mRNA and protein are upregulated following brain injury [15] and downregulated in Alzheimer's disease (AD) [14,16].

The known functions of MTs include metalloregulatory roles in cell growth and differentiation [17], so it is not surprising that most mammalian tissues contain age-related basal levels of MTs, with the major amount found in developing cells [18]. Enhanced synthesis of MTs is also observed in rapidly proliferating tissues, stressing their crucial role in normal and neoplastic cell growth [18–20]. However, the MTs' role in aging is controversial: in mammals, MT expression has been found to increase in aged kidney [21], whereas decreased significantly with aging in the skin [22].

Aging is a major risk factor for brain neurodegenerative disorders, including cerebrovascular disease, AD, Parkinson's disease (PD) and cancer [23–26]. Brain aging is accompanied with molecular, functional and genetic changes, leading to increased susceptibility to diseases and cognitive impairments. As it has been proven in the mouse, MTs downregulation results in a progressive neurodegeneration, leading to early aging, morbidity, and mortality [27]; the neurodegenerative alterations are attenuated by MTs overexpression, suggesting the neuroprotective role of MTs in aging [27]. These studies, however, did not include the identification of the MT isoforms functionally involved.

In this study, by using real-time PCR analysis, we examined and compared the expression of *MT1/2* and *MT3* genes during aging in the rat brain. In particular, we evaluated the MT1/2 and MT3 expression profiles in the cerebral cortex and hippocampus of pubertal (2 months), adult (4 and 8 months), and middle-aged (16 months) rats.

Our results demonstrate an age-related expression of the *MT3* gene, thus, suggesting its implication in physiological changes associated with aging.

2. Materials and methods

2.1. Animal and experimental design

Male Wistar rats (Charles River, Calco, Como, Italy) of 2 months of age (adolescent), with the same starting body weight (160 ± 10 g), were individually caged in a temperature-controlled room (23 ± 1 °C) submitted to a 12-h light/12-h dark cycle. Animals were housed in the Animal Care Facility at the Department of Biology, with ad libitum access to water and to a standard diet (Mucedola 4RF21; Settimo Milanese, Milan, Italy) up to 4 (social maturity, group 1), 8 (adulthood, group 2), or 16 (aged, group 3) months. Eight animals of each group were anesthetized with chloral hydrate (40 mg/100 g body wt) and killed by decapitation. Group 4 was comprised 8 adolescent rats sacrificed four days after their arrival in the animal facility. The brains were quickly removed and the cerebral cortex and hippocampus were dissected on ice. Samples of each brain region were snap frozen in liquid nitrogen immediately and stored at −80 °C for subsequent RNA isolation. The livers of the rats belonging to group 4 were also collected, frozen and stored at −80 °C for RNA isolation. The protocols for animal care and use were approved by the Scientific Ethics Committee on Animal Experimentation of the University Federico II of Naples. All experimental animal procedures were carried out in compliance with the national guidelines for the care and use of research animals (D.L.116/92, implementation of EEC directive 609/86). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. RNA purification and cDNA synthesis

Total RNA was extracted according to the TRI-Reagent (Sigma–Aldrich) protocol. The quality of each total RNA was checked by electrophoresis on 2% agarose gel stained with ethidium bromide and by measuring the optical density at 260/280 nm. A ratio of 1.8–2.0 was accepted for further reverse transcription. The QuantiTect Reverse Transcription Kit (Qiagen) was used for the removal of genomic DNA contamination and for the subsequent cDNA synthesis. Approximately 1 μg of total RNA was used, according to the kit's protocol.

2.3. Quantitative real-time PCR analysis

The real-time PCR reactions were carried out in quadruplicate in an Applied Biosystems 7500 Real-Time System by using the Power SYBR Green Master Mix PCR (Applied Biosystems) following procedures recommended by the manufacturer. Each SYBR Green reaction (total volume: 20 μL) contained 12 μL of real-time PCR Master Mix, 1 μL of each of the forward and reverse primers (10 μM), 2 μL of 1:1 diluted cDNA, and 4 μL of nuclease-free water. Nuclease-free water for the replacement of the cDNA template was used as a negative control. For the internal standard control, the expression of *β-actin* gene was quantified. Primer sequences were designed using Primer Express software (Applied Biosystems). A single pair of specific primers for both MT1 and MT2 isoforms was designed on the nucleotide sequences of *Rattus norvegicus* MT1 (NM 138826.4) and MT2 (NM 001137564.1). Specific primers for MT3 were designed on the *R. norvegicus* template NM 053968.3, choosing the most divergent sequence tracts rather than MT1/2 isoforms. β-actin primers were designed on the exon junction 75/76 (forward primer) on *R. norvegicus* template NM031144.2. All the primers used in real-time PCR analysis are listed in Table 1. PCR was performed under the following conditions: holding stage of 95 °C per 10 min; cycling stage (45 cycles): 95 °C × 10 s – 60 °C × 10 s – 72 °C × 10 s; melting stage: 95 °C × 5 s – 65 °C × 1 min – 95 °C × 30 s – 40 °C × 30 s. The melting curve analysis of PCR products was performed in

Table 1
Specific primers used for semiquantitative real-time PCR analyses.

Name	Nucleotide sequence (5′–3′)	Length	Direction
MT1/2_F	ATGGACCCCAACTGCTCCTG	20-mer	Sense
MT1/2_R	CTTTGCAGACACAGCCCTGGG	21-mer	Antisense
MT3_F	CCCCTGTCCTACTGGTGGT	19-mer	Sense
MT3_R	CTGCATTTCTCGGCCTTG	18-mer	Antisense
β-act_F	ACCCGCCACCAGTTCGCCAT	20-mer	Sense
β-act_R	CGGCCCACGATGGAGGGGAA	20-mer	Antisense

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