Neurochemistry International 75 (2014) 48–53

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Astrocytes of the murine model for Down Syndrome Ts65Dn display reduced intracellular ionic zinc

Raúl Ballestín [a], José Miguel Blasco-Ibáñez [a], Carlos Crespo [a], Juan Nacher [a], Rosa López-Hidalgo [a], Javier Gilabert-Juan [a,b], Dolores Moltó [b], Emilio Varea [a,*]

[a] Neurobiology Unit and Program in Basic and Applied Neurosciences, Cell Biology Department, Universitat de València, Spain
[b] Genetics Department, Universitat de València, INCLIVA, CIBERSAM, Spain

ARTICLE INFO

Article history:
Received 24 January 2014
Received in revised form 23 May 2014
Accepted 29 May 2014
Available online 6 June 2014

Keywords:
Zinc
Ts65Dn
Down Syndrome
Metallothionein 3
Astrocyte
TSQ

ABSTRACT

Zinc is an essential trace element that is critical for a large number of structural proteins, enzymatic processes and transcription factors. In the brain, zinc ions are involved in synaptic transmission. The homeostasis of zinc is crucial for cell survival and function, and cells have developed a wide variety of systems to control zinc concentration. Alterations in free zinc concentration have been related with brain dysfunction. Down Syndrome individuals present alterations in free zinc concentration and in some of the proteins related with zinc homeostasis. We have analyzed the amount of free zinc and the zinc chelating protein metallothionein 3 in the astrocytes using primary cultures of the murine model Ts65Dn. We have observed a higher number of zinc positive spots in the cytoplasm of trisomic astrocytes but a decrease in the total concentration of total intracellular free zinc concentration (including the spots) respect to control astrocytes. Using FM1–43 staining, we found that the endocytic function remains unaltered. Therefore, a possible explanation for this lower concentration of free zinc could be the higher concentration of metallothionein 3 present in the cytoplasm of trisomic astrocytes. The blockade of metallothionein 3 expression using an specific siRNA induced an increase in the concentration of free zinc in basal conditions but failed to increase the uptake of zinc after incubation with zinc ions.

1. Introduction

Zinc is an essential trace element that is critical for a large number of structural proteins, enzymatic processes and transcription factors. The functions of zinc and the effects of its deficiency in the brain and other organs have been continuously studied (Bitanihirwe and Cunningham, 2009). Zinc deficiency affects mainly to proliferative tissues (Hurley, 1981). In adults, the effects of zinc deficiency are more diffuse, affecting the skin, hair, immune function, sexual maturation or neurological function (Tuerk and Fazel, 2009).

In the brain, an excess of zinc ions in the extracellular space induces cell death in cortical neurons (Kim et al., 1999), whereas the experimental chelation of zinc ions may trigger apoptosis (Zalewski et al., 1993). Therefore, a homeostatic balance in the extra/intracellular concentration of zinc ions appear to be necessary. Most cells have developed complex systems to regulate influx and efflux of cytoplasmic zinc: (a) Metallothioneins, among other proteins, are engaged in the intracellular traffic of zinc. They are small, cystein enriched proteins that are able to bind up to seven zinc ions per molecule (Ebadi et al., 1995). (b) Membrane ZIP (Zrt- and Irt-like proteins) transporters carry zinc ions into the cytoplasm (Guerinot, 2000), and (c) Zinc Transporters (ZnT) carry zinc ions either out of the cytoplasm to the extracellular medium (ZnT1) (Palmiter and Findley, 1995) or into different organelles (ZnT2–9) (Lichten and Cousins, 2009); all these systems collectively maintain the intracellular concentration of free zinc within narrow limits.

Despite the broad distribution of zinc in biological systems, only the ionic fraction may be detected by histochemical techniques. In the central nervous system (CNS), some neurons accumulate zinc ions inside synaptic vesicles (Frederickson and Danscher, 1990), as 'synaptic zinc' which is co-released with glutamate during synaptic transmission. In the extracellular space, zinc ions modulate different types of receptors, including the AMPA/kainate receptor, NMDA and GABA receptors as well as voltage-gated ion channels (Izumi et al., 2006; Smart et al., 2004). Nervous tissue should maintain extracellular zinc levels within limits and astrocytes play an important role in the maintenance of the zinc homeostasis in the

* Corresponding author. Address: Neurobiology Unit, Cell Biology Department, Universitat de València, Dr. Moliner, 50, Burjassot 46100, Spain. Tel.: +34 96 354 3783.
E-mail address: emilio.varea@uv.es (E. Varea).

http://dx.doi.org/10.1016/j.neuint.2014.05.013
0197-0186/

CNS. This role is developed by these cells mainly due to their lower sensitivity to a toxic zinc insult compared with neurons (Dineley et al., 2000), their capacity to uptake extracellular zinc (Varea et al., 2006), and their capacity to chelate extracellular zinc by releasing metallothionein (Chung et al., 2008).

Down Syndrome (DS) with an incidence of one in 800 live births (Roizen and Patterson, 2003) is the most common genetic alteration typically associated to intellectual disability. Trisomy of the 21 chromosome induces a variable phenotype that may include immune deficiencies, heart defects, increased risk of leukaemia, and an early development of Alzheimer's disease. The common feature among all DS subjects is the presence of mild to moderate mental retardation. Neural mechanisms underlying this disability may include defects in the establishment of neuronal networks, information processing and brain plasticity.

Several animal models are available to mimic the alterations in DS. The most used is the Ts65Dn mouse. This model is segmentally trisomic for a portion of the mouse chromosome 16 that is orthologous to the long arm of the human chromosome 21. This segment contains approximately 140 genes, many of which are highly conserved between mice and humans (Gardiner et al., 2003). These mice display delay in the acquisition of a number of sensory and motor tasks (Costa et al., 1999; Holtzman et al., 1996), as well as defects in learning and in the execution of memory tasks mediated by the hippocampus (Escorihuela et al., 1998; Holtzman et al., 1996; Reeves et al., 1995), and deficits in long-term potentiation (LTP) (Kleschevnikov et al., 2004).

Zinc metabolism is altered in DS and it has been observed a reduction in the presence of zinc in plasma in children (Lima et al., 2010) and adolescents with DS (Marques et al., 2007). Zinc is a micronutrient required by several cellular processes related to growth and differentiation, transcription, and apoptosis. Therefore, maintenance of intracellular zinc homeostasis is crucial for cell survival and functioning.

Extracellular zinc ions may be captured by cells and confined into vesicles, that have been named 'zincosomes' (Beyersmann and Haase, 2001). Zincosomes have been detected in many cell types (Eide, 2006). Although their nature is still unclear, in mammalian astrocytes, they appear to correlate with endocytic activity (Varea et al., 2006). Therefore, the analysis of those factors that might modulate or alter their functions is of great interest. In fact astrocytes seem to be a target of DS. DS fetuses display a higher density of astrocytes. This excess can be related to the developmental abnormalities present in DS patients (Zdaniuk et al., 2011).

Our aim is to analyze the distribution of zinc in Ts65Dn astrocytes in basal conditions and their ability to uptake extracellular zinc ions in relation with the endocytic activity. We have also checked the cytosolic distribution of metallothionein 3 (MT-3) in order to correlate the changes observed in the cytoplasmic zinc concentration with alterations in the concentration of this metallothionein.

2. Material and methods

2.1. Animals

Experimental mice were generated by repeated backcrossing of Ts65Dn females to C57/6Ei 9 C3H/HeSnJ (B6EiC3) F1 hybrid males. The parental generation was obtained from the research colony of Jackson Laboratory (Bar Harbor, USA). Euploid littermates of Ts65Dn mice served as controls. For this study we have used newborn mice. Cells from each different animal were grown in separate dish plates. The genotypic characterization was established by quantitative polymerase chain reaction (qPCR) using SYBR Green PCR master mix (Applied Biosystems) from genomic DNA extracted of mice tails by mean of the phenol–chloroform method. The genes analyzed where APP (3 copies) and Apo-B (2 copies) as previously used (Liu et al., 2003). The relative amount of each gene was quantified by the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All animal experimentation was conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de València. Every effort was made to minimize the number of animals used and their suffering.

2.2. Primary astrocyte culture

Primary cell cultures of astrocytes were obtained from newborn mice. Animals were decapitated under deep anesthesia (chloral hydrate 4% 1 ml/100 g), and brains were carefully removed and mechanically disaggregated in a Ca^{2+} deficient solution (Hanks balanced salt solution, without Ca^{2+} and Mg^{2+}, supplemented with 1 mM sodium pyruvate and 10 mM HEPES) until cell suspension was reached. The Ca^{2+} concentration was then restored by mixing (2:1) with normal solution, the suspension was centrifuged (1 min at 200*g*) to remove cellular debris and then resuspended in Dulbecco's Minimum Essential Medium, supplemented with antibiotics (penicillin and streptomycin), 2 mM L-glutamine and 5% fetal bovine serum (FBS), and finally cultured in Petri dishes. After reaching confluence, cells were subdivided 1:6 and used for zinc imaging experiments 3–5 days after passage. Under these conditions, cell culture is enriched in astrocytes (at least 95%).

2.3. Transfection procedure

The primary astrocytic cell culture was prepared as described above using a six wells tissue culture plate. Three wells containing coverslips were seeded with the astrocytic culture. Cells grew up to a 70% confluency in a normal growth medium supplemented with 5% FBS. The first well was transfected with a control plasmid (sc-108065, Santa Cruz Biotechnology), the second was transfected with a plasmid containing shMT-3 (sc-934338-SH, Santa Cruz Biotechnology) and the third was maintained in control conditions. The following solutions were prepared freshly:

Solution A, Plasmid DNA Solution: For each transfection, a dilution of 10 μl of resuspended shRNA Plasmid DNA (1 μg shRNA Plasmid DNA control or MT-3) into 90 μl shRNA Plasmid Transfection Medium (sc-108062, Santa Cruz Biotechnology).

Solution B, Plasmid Transfection Reagent: For each transfection, a dilution of 4 μl of shRNA Plasmid Transfection Reagent (sc-108061, Santa Cruz Biotechnology) into 96 μl of shRNA Plasmid Transfection Medium.

The shRNA Plasmid DNA solution (Solution A) was directly added to the solution containing the shRNA Plasmid Transfection Reagent (Solution B) using a micropipette. The solution was mixed gently by pipetting up and down and the mixture was incubated for 45 min at room temperature before transfection.

Then, the cells were washed twice with 2 ml of shRNA Plasmid Transfection Medium. After aspirating the medium, 0.8 ml shRNA Plasmid Transfection Medium were added to each well and then 200 μl shRNA Plasmid DNA/shRNA Plasmid Transfection Reagent Complex (Solution A + Solution B) were added dropwise to each well, covering the entire surface. Afterwards, the medium was mixed it up gently by swirling the plate to ensure that the entire cell layer was immersed in a homogeneous solution. The cells were incubated for 7 h at 37 °C in a CO_2 incubator. Following incubation, 1 ml of normal growth medium containing two times normal serum and antibiotic concentration (2× normal growth medium) were added to the medium and the cells were incubated in this medium for 24 h.