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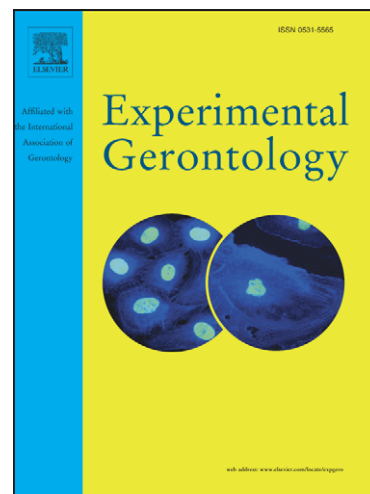
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**ALTERED OXIDANT-MEDIATED INTRANEURONAL ZINC MOBILIZATION IN A
TRIPLE TRANSGENIC MOUSE MODEL OF ALZHEIMER'S DISEASE**

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KEY WORDS: synaptic zinc homeostasis, oxidative stress, 3xTg-AD, PS1_{K1}, presenilin, tau, amyloid, Alzheimer's disease, neuronal death

Abstract:

Alzheimer's disease (AD) is responsible for the most common form of dementia among elderly people. Signature features of the AD brain are intra/extracellular deposits of β -amyloid ($A\beta$) and neurofibrillary tangles composed of hyperphosphorylated tau. Recent evidence indicates that in AD altered Zn^{2+} homeostasis can play an important role in the development of the disease as the cation promotes $A\beta$ oligomerization and plaque formation. In this study, we investigated whether intraneuronal Zn^{2+} homeostasis is affected by known "pro-AD factors" such as mutant forms of the amyloid precursor (APP), presenilin-1 (PS1), and tau proteins. Oxidative stress is a potent trigger for mobilization of intracellular free Zn^{2+} ($[Zn^{2+}]_i$) and we therefore evaluated ROS-driven $[Zn^{2+}]_i$ rises in neurons obtained from triple transgenic AD mice (3xTg-AD) that express mutant APP, PS1 and tau. In this study, $[Zn^{2+}]_i$ rises triggered by prolonged exposure to the membrane-permeant oxidizing agent 2,2'-dithiodipyridine were found to be significantly higher in 3xTg-AD neurons when compared to control cultures, suggesting that neuronal expression of pro-AD factors can facilitate altered Zn^{2+} homeostasis.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder of the aging brain. AD is associated with the appearance of parenchymal deposits of β -amyloid ($A\beta$) and neurofibrillary tangles made of hyperphosphorylated-tau protein (h-tau) surrounded by altered neuritic processes and reactive glia. Growing evidence suggests that alteration in Zn^{2+} levels is an important contributing factor in AD as the cation can induce $A\beta$ oligomerization and plaque formation (Frederickson et al., 2005).

Zn^{2+} is a potent mediator of neuronal death (Sensi and Jeng, 2004) and intraneuronal Zn^{2+} ($[Zn^{2+}]_i$) homeostasis is tightly controlled by a balance between influx, sequestration, buffering, and extrusion. Most Zn^{2+} enters neurons through Ca^{2+} -permeable glutamate receptors (i.e: the NMDA and divalent-permeable AMPA receptors) and voltage-sensitive Ca^{2+} channels (Sensi et al., 1997). Zn^{2+} buffering and sequestration are largely controlled by Zn^{2+} -binding proteins (metallothioneins; MTs) and mitochondria, while extrusion is mediated by the activity of several Zn^{2+} transporters (Sekler et al., 2007).

Zn^{2+} binding to MTs is greatly affected by changes in the redox state of the two Zn^{2+} /Cysteine cluster regions. Recent studies indicate that endogenous reactive oxygen species (ROS) or exogenous oxidants are able to promote harmful Zn^{2+} release from MTs (Aizenman et al., 2000; Bossy-Wetzel et al., 2004; Maret, 2000). MTs are present in the central nervous system in three isoforms (MT1, 2 and 3), but, while MT1 and MT2 are expressed in astrocytes, MT3 is selectively expressed in neurons (Aschner, 1996), thereby representing a potential injurious source of releasable Zn^{2+} inside of neurons (Lee et al., 2003).

Zn^{2+} is sequestered by mitochondria (reviewed in Sensi and Jeng, 2004; Gazaryan et al., 2007; Malaiyandi et al., 2005) and, notably, mitochondrial Zn^{2+} can be re-released into the cytosol in a Ca^{2+} -dependent fashion (Frazzini et al., 2007; Sensi et al., 2003). Mitochondrial Zn^{2+} accumulation is not always a benign process as the cation can potently disrupt mitochondrial function and

promote strong oxidative stress as well as neuronal death (Brown et al., 2000; Gazaryan et al., 2007; Malaiyandi et al., 2005; Sensi et al., 1999).

Recent studies suggest ways by which disruption of Zn^{2+} homeostasis might play a role in AD-related neurodegeneration (Frederickson et al., 2005; Religa et al., 2006). Expression levels of Zn^{2+} transporters such as ZnT1, ZnT4 and ZnT6 are altered in the brain of individuals affected by mild cognitive impairment (MCI) and AD (Lovell et al., 2005; Lovell et al., 2006). Furthermore, studies in AD animal models have shown that genetic ablation of synaptic Zn^{2+} (Lee et al., 2002) or use of Zn^{2+} chelators can have a significant effect on brain accumulation of $A\beta$ (Cherny et al., 2001; Lee et al., 2002; Lee et al., 2004). Further substantiating a role for Zn^{2+} unbalance in AD, a recent phase II clinical trial employing the cell-permeable Zn^{2+}/Cu^{2+} modulator clioquinol has been reported to produce a significant decrease in the rate of cognitive decline in a subset of AD patients (Ritchie et al., 2003).

Oxidative stress is an additional contributing factor in AD (Butterfield et al., 2001) and in this study, we investigated whether expression of pro-AD factors such as mutant forms of the amyloid precursor protein (APP), presenilin-1 (PS1) and tau protein can modulate ROS-mediated $[Zn^{2+}]_i$ rises in cultured cortical neurons obtained from a triple transgenic mouse model of AD (3xTg-AD). 3xTg-AD mice display the major pathological features of the AD brain, including progressive and age-dependent $A\beta$ and tau pathology, and show an early (at 4-6 month of age) intraneuronal accumulation of $A\beta$ in AD-vulnerable hippocampal neurons that well correlates with concomitant appearance of signs of altered synaptic plasticity and cognitive deficits (Billings et al., 2005; Oddo et al., 2003).

MATERIALS AND METHODS

Materials. FluoZin-3 AM and Newport Green diacetate were purchased from Molecular Probes (Invitrogen, Milan, Italy). 2,2'-dithiodipyridine (DTDP) was purchased from Sigma-Aldrich (Milan, Italy). Tissue culture media and serum were from Gibco (Invitrogen, Milan, Italy). All other chemicals and reagents were obtained from common commercial sources.

Animal cell cultures. All animal procedures were approved by our institutional animal care and use committee, and are in accordance with the NIH "Principles of laboratory animal care" and national laws. Murine cortical cultures were prepared from embryonic 3xTg-AD, presenilin-1 knock-in (PS1_{K1}), and non-Tg (CD1) mice. Neurons were plated upon astrocytic monolayers on poly-lysine + laminin coated coverslips as previously described (Sensi et al., 1999).

[Zn²⁺]_i imaging studies. [Zn²⁺] imaging was carried out using an inverted microscope coupled to a xenon lamp, a PC-controlled filter wheel, a 40X, epifluorescence oil immersion objective, and fluorescent cubes. Cultures (at 7 D.I.V.) were loaded in the dark, with Newport Green diacetate (3 μM) or FluoZin-3 AM (5 μM) in a HEPES-buffered medium (HCSS) whose composition was (in mM): 120 NaCl, 5.4 KCl, 0.8 MgCl₂, 20 HEPES, 15 glucose, 1.8 CaCl₂, 10 NaOH, pH 7.4, for 30 min at 25°C, then washed in HCSS and kept in the dark for an additional 30 min; excitation was at 490 nm and emission at 530 nm. Experiments were carried out at room temperature (25°C). Drugs were applied by bath application and removed through a rapid flow exchange system. Images were acquired with a 12 bit digital CCD camera (ORCA-HR, Hamamatsu, Bridgewater, NJ) and analyzed (after background subtraction from a cell-free region of the dish) with Metafluor 6.0 imaging software (Universal Imaging Corporation, Downingtown, PA). Fluorescence values were then converted in [Zn²⁺]_i calibrated values by employing the following equation: $[Zn^{2+}]_i = K_D(F - F_{min}) / (F_{max} - F)$ where K_D is 15 nM (FluoZin-3) or 1 μM (Newport Green). F_{max} was obtained at

the end of each experiment by adding the Zn^{2+} -selective ionophore Na^+ -pyrithione (10 μM) in the presence of 50 μM Zn^{2+} , while baseline fluorescence values were considered as F_{min} .

Experiment replication and statistics. All experiments reported represent at least 3 independent replications. Comparisons were obtained by Student-Newman-Keuls' test ($p < 0.001$).

RESULTS

To evaluate dynamic changes in $[\text{Zn}^{2+}]_i$ levels, cultured (7 D.I.V.) cortical neurons obtained from 3xTg-AD and control mice were loaded with the Zn^{2+} -selective fluorescent probes FluoZin-3 or Newport Green (Gee et al., 2002; Sensi et al., 1999). 3xTg-AD mice have been developed by LaFerla and colleagues by introducing two transgenes (APP_{swe} and $\text{tau}_{\text{P301L}}$) into the germline of the mutant $\text{PS1}_{\text{M146V}}$ knock-in mouse (Oddo et al., 2003), and therefore, in our study, ROS-mediated $[\text{Zn}^{2+}]_i$ rises in 3xTg-AD neurons were compared with both PS1_{KI} and non-Tg cultures considered as controls. Probe loaded cultures were exposed to the oxidizing compound 2,2'-dithiodipyridine (DTDP) and changes in either FluoZin-3 or Newport Green fluorescence units converted in calibrated $[\text{Zn}^{2+}]_i$ values. In pilot experiments, we evaluated DTDP-driven $[\text{Zn}^{2+}]_i$ rises in 3xTg-AD (and control) neurons loaded with Newport Green, a Zn^{2+} -sensitive probe that shows an intermediate affinity ($K_{\text{d}} = 1 \mu\text{M}$) for the cation. In these experiments, exposure to different DTDP concentrations (up to 200 μM) failed to promote any significant Zn^{2+} -dependent fluorescence increase, indicating that DTDP-mediated $[\text{Zn}^{2+}]_i$ rises were in a (sub-micromolar) range not detectable by the probe (data not shown). We then repeated the DTDP challenge in 3xTg-AD, PS1_{KI} and non-Tg neurons loaded with the high affinity ($K_{\text{d}} = 15 \text{ nM}$) Zn^{2+} -selective probe

FluoZin-3 and evaluated $[Zn^{2+}]_i$ rises in the three cultures. In these experiments, DTDP exposure was found capable to promote detectable increases in probe fluorescence. A set of dose-finding experiments (i.e.: DTDP concentrations ranging from 10 to 50 μM) indicated that DTDP at 50 μM is sufficient to bring $[Zn^{2+}]_i$ transients to saturating molar values (> 150 nM) that are not suitable for reliable quantification with FluoZin-3, while optimal $[Zn^{2+}]_i$ excursions could be obtained with prolonged (10 min) exposure to 25 μM DTDP (Fig.1). FluoZin-3 loaded AD and control cultures showed no differences in basal $[Zn^{2+}]_i$ levels, while analysis of peak $[Zn^{2+}]_i$ rises obtained upon exposure to DTDP (25 μM ; 10 min) revealed significantly higher levels in 3xTg-AD neurons (mean \pm SEM: 20.17 ± 3.76 nM) when compared with PS1_{K1} (5.51 ± 0.74 nM) and non-Tg cultures (0.10 ± 0.15 nM; $p < 0.001$; Fig.2A). Peak $[Zn^{2+}]_i$ rises in PS1_{K1} neurons were significantly greater than those observed in non-Tg neurons ($p < 0.001$). To better estimate the extent of DTDP-mediated $[Zn^{2+}]_i$ increases, we also performed an integral analysis of the overall cytosolic Zn^{2+} overload occurring during the 10 minute exposure to the oxidant. Confirming what observed in the case of peak $[Zn^{2+}]_i$ levels, cytosolic Zn^{2+} overload was found to be significantly higher ($p < 0.001$) in 3xTg-AD cultures (Fig. 2B).

DISCUSSION

Results presented in this study indicate that expression of pro-AD factors such mutant APP, PS1, and tau can facilitate perturbation of intraneuronal Zn^{2+} homeostasis.

DTDP can catalyze disulfide exchange and mobilizes Zn^{2+} from MTs in vitro (Jiang et al., 1998) thus, the intraneuronal $[Zn^{2+}]_i$ rises observed upon DTDP exposure are likely due to Zn^{2+} release from MTs. It should be pointed out that altered Zn^{2+} homeostasis, MTs expression, and increased oxidative stress are all playing a critical role in AD and brain aging (Mocchegiani et al., 2005).

In AD, A β generates ROS (Behl et al., 1994) and can thereby promote ROS-driven $[Zn^{2+}]_i$ release. $[Zn^{2+}]_i$ accumulation can, in turn, trigger potent mitochondrial dysfunction and further ROS generation (reviewed in Sensi and Jeng, 2004). Thus, Zn^{2+} -induced oxidative stress can act as a catalyst and enhancer of a self-perpetuating vicious cycle.

AD-associated alterations in $[Zn^{2+}]_i$ homeostasis can have profound implications for the progression of the disease by interfering with A β oligomerization. Recent developments in AD research strongly support the view that the severity of neuronal loss and dementia is mediated by soluble A β oligomers, rather than fibrillar and insoluble deposits of A β (reviewed in LaFerla et al., 2007). There is compelling evidence that, in the brains of AD subjects and AD animal models, levels of soluble A β species correlate better with cognitive decline than plaque density (Gong et al., 2003; Lesne et al., 2006; Lue et al., 1999; Naslund et al., 2000; Oddo et al., 2006a). Studies in brains from AD patients and AD animal models have shown that formation of A β oligomers is initiated intracellularly rather than in the extracellular space (Walsh et al., 2000; Oddo et al., 2006b) and indicated that such process potentially impairs synaptic transmission.

In this scenario, altered levels of intraneuronal Zn^{2+} might exert a critical role in setting the stage for the formation of an early nidus of intrasynaptic oligomerization of A β .

It should be also underlined that brain aging is the major risk factor for AD and is a potentially disrupting Zn^{2+} homeostasis. Animal studies in neurons from aging hippocampi indicate that MT3 expression is more abundant compared to young hippocampal extracts (Giacconi et al., 2003). MTs are potent antioxidants and protective factors against stress conditions and increased MT expression in the aging brain may simply reflect a protective endogenous response to a sub-chronic state of inflammatory and/or oxidative stress. On the other hand, it is conceivable that protective actions of MTs can be overridden by a concomitant increase in ROS-driven $[Zn^{2+}]_i$ levels. As in the aging brain (and even more so in the AD brain) there is an increase in free radical generation, it is possible that ROS induce a chronic MT up-regulation that leads to higher intracellular availability of

releasable Zn^{2+} (Mocchegiani et al., 2001; Mocchegiani et al., 2004). Finally, it should be considered that aging and AD greatly affect mitochondrial capability to cope with both cation loads and oxidative stress and these conditions are associated with increased mutations of mitochondrial DNA as well as expression of defective mitochondrial proteins (Raule et al., 2007; Wallace, 2005), thereby setting the stage for a self-perpetuating vicious cycle.

In summary, in light of the pivotal role played by oxidative stress and $[Zn^{2+}]_i$ unbalance in AD-related neuronal degeneration, our study suggests an intriguing interplay between free radical production and pro-AD factors in the generation of altered $[Zn^{2+}]_i$ homeostasis. Further investigations are needed to elucidate the pathways involved and how brain aging can modulate this pathological process.

Figure legends

Fig. 1. Time course of DTDP-mediated $[Zn^{2+}]_i$ rises in neurons isolated from 3xTg-AD, PS1_{KI}, and non-Tg cortical neurons. FluoZin-3 loaded 3xTg-AD (●), PS1_{KI} (●), and non-Tg (○) neuronal cultures were imaged before and during a 10 min exposure to 25 μM DTDP. Traces show mean (\pm SEM) $[Zn^{2+}]_i$ rises in 15-27 neurons from one experiment representative of 3.

Fig. 2. DTDP promotes different degrees of $[Zn^{2+}]_i$ mobilization in 3xTg-AD, PS1_{KI}, and non-Tg cultured neurons. (A) FluoZin-3 loaded transgenic (3xTg-AD, PS1_{KI}) and non-transgenic cortical neurons were exposed to DTDP (same paradigm as in Fig.1) and peak $[Zn^{2+}]_i$ levels evaluated. Bar graph depicts peak $[Zn^{2+}]_i$ rises (mean \pm SEM) of >48 neurons from the three different cultures (n=3 for each condition). (B) Bar graph depicts the area under the curve of $[Zn^{2+}]_i$ loads (mean \pm SEM) of a 10 min DTDP exposure. * and # indicate differences between 3xTg-AD and PS1_{KI} or PS1_{KI} and non-Tg neurons, respectively ($p < 0.001$).

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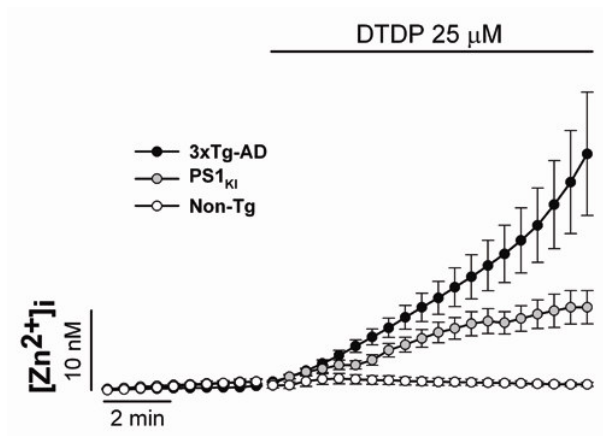
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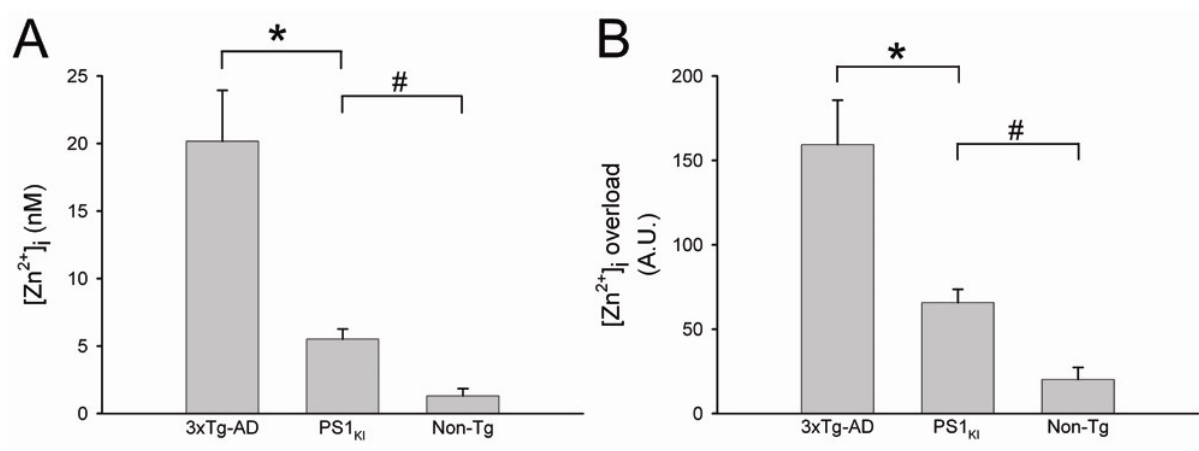
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