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Impaired Src signaling and post-synaptic actin polymerization in Alzheimer's disease mice hippocampus — Linking NMDA receptors and the reelin pathway

Sandra I. Mota [a,b], Ildete L. Ferreira [a,b], Jorge Valero [a,b], Elisabete Ferreiro [a,b], Ana L. Carvalho [a,b,c], Catarina R. Oliveira [a,b,d], A. Cristina Rego [a,b,d,*]

[a] Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Coimbra, Portugal
[b] Institute for Interdisciplinary Research (IIIUC), University of Coimbra, Coimbra, Portugal
[c] Life Science Department, Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal
[d] Faculty of Medicine, University of Coimbra, Coimbra, Portugal

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ABSTRACT

Early cognitive deficits in Alzheimer's disease (AD) have been related to deregulation of *N*-methyl-D-aspartate receptors (NMDARs) and synaptic dysfunction in response to amyloid-beta peptide. NMDAR anchorage to post-synaptic membrane depends in part on Src kinase, which is also implicated in NMDAR activation and actin cytoskeleton stabilization, two processes relevant for normal synaptic function. In this study we analyzed the changes in GluN2B subunit phosphorylation and the levels of proteins involved in Src related signaling pathways linking the Tyr kinase to actin cytoskeleton polymerization, namely reelin, disabled-1 (Dab1) and cortactin, in hippocampal and cortical homogenates obtained from the triple transgenic mouse model of AD (3xTg-AD) that shows progression of pathology as a function of age versus age-matched wild-type mice. Moreover, we evaluated regional post-synaptic actin polymerization using phalloidin labeling in hippocampal slices. Young (3 month-old) 3xTg-AD male mice hippocampus exhibited decreased GluN2B Tyr1472 phosphorylation and reduced Src activity. In the cortex, decreased Src activity correlated with reduced levels of reelin and Dab1, implicating changes in the reelin pathway. We also observed diminished phosphorylated Dab1 and cortactin protein levels in the hippocampus and cortex of young 3xTg-AD male mice. Concordantly with the recognized role of these proteins in actin stabilization, we detected a significant decrease in post-synaptic F-actin in 3 month-old 3xTg-AD male CA1 and CA3 hippocampal regions. These data suggest deregulated Src-dependent signaling pathways involving GluN2B-composed NMDARs and post-synaptic actin cytoskeleton depolymerization in the hippocampus in early stages of AD.

Introduction

Alzheimer's disease (AD) is the most prevalent form of neurological disorder in the elderly. AD is characterized by the deposition of extracellular amyloid-beta peptide (Aβ, generated from the amyloid precursor protein or APP), forming plaques, and intracellular hyperphosphorylated tau, forming neurofibrillary tangles. Synaptic dysfunction affecting the hippocampus and the cortex has been recognized in AD, leading to early memory and cognitive deficits (Ma and Klann, 2012). Indeed, post-synaptic modifications involve Aβ-mediated effects on *N*-methyl-D-aspartate receptors (NMDARs) (Shankar et al., 2007; Costa et al., 2012; Ferreira et al., 2012). mRNA and protein levels of NMDAR subunits were previously shown to be altered in human *post-mortem* brains of late AD patients, correlating with disease-related cognitive deficits (Sze et al., 2001; Mishizen-Eberz et al., 2004; Jacob et al., 2007). Nevertheless, changes in NMDAR subunits were not the same in all affected brain areas. Levels of GluN1 and GluN2B subunits were decreased in the hippocampus, whereas GluN2A levels decreased in the entorhinal cortex, when compared to control individuals (Sze et al., 2001).

Overactivation of NMDARs linked to excitotoxicity was initially hypothesized to occur at a late-stage of AD; in this respect, memantine, an uncompetitive NMDAR open channel blocker, has

Abbreviations: ApoER-2, apolipoprotein E receptor-2; Dab1, disabled-1; F-actin, filamentous-actin; G-actin, glomerular-actin; GKAP, guanylate kinase-associated protein; NMDAR, *N*-methyl-D-aspartate receptors; PSD-95, postsynaptic density-95; VLDLR, very-low-density-lipoprotein receptor.

* Corresponding author at: Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Largo Marquês de Pombal, 3004-517 Coimbra, Portugal. Fax: +351 239 822776.

E-mail addresses: sandraisab.mota@gmail.com (S.I. Mota), ildetelferreira@gmail.com (I.L. Ferreira), jorge.valero@cnc.uc.pt (J. Valero), beferreiro@gmail.com (E. Ferreiro), alc@cnc.uc.pt (A.L. Carvalho), catarina.n.oliveira@gmail.com (C.R. Oliveira), acrego@cnc.uc.pt, arego@fmed.uc.pt, a.cristina.rego@gmail.com (A.C. Rego).

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been prescribed as a memory-preserving drug for moderate- to late-stage AD patients (Reisberg et al., 2003). However, more recent reports suggest that changes in NMDARs may also occur at early stages of AD (reviewed by Parameshwaran et al., 2008). In young AD triple-transgenic (3xTg-AD) mice, NMDAR-induced Ca^{2+} signals were higher than in non-transgenic (wild-type, WT) mice, suggesting early alterations in NMDAR activity (Goussakov et al., 2010). Moreover, we previously demonstrated the involvement of GluN2B-containing NMDARs in oligomeric $A\beta_{1-42}$-induced intracellular Ca^{2+} rise in cortical neurons (Ferreira et al., 2012), as well as microtubule deregulation (Mota et al., 2012) and ER stress (Costa et al., 2012) in hippocampal neurons. Conversely, evaluation of GluN1 or GluN2 subunit levels in *post-mortem* brains at early stage of AD showed no differences compared with control individuals (Sze et al., 2001), suggesting that pre-AD is not linked to altered NMDAR protein levels.

Phosphorylation of GluN2B subunit at Tyr1472 was previously suggested to constitute a signal for enhanced NMDAR activity by increasing its surface expression at the synapse (Goebel et al., 2005; Goebel-Goody et al., 2009). Src and Fyn Tyr kinases are responsible, in part, for the upregulation of GluN2B-containing NMDARs at the membrane (Xu et al., 2006; Sinai et al., 2010). On the contrary, dephosphorylation of Tyr1472 by $STEP_{61}$, a Tyr phosphatase, leads to decreased NMDAR activity and trafficking (Braithwaite et al., 2006) through internalization of GluN1/GluN2B receptors from the neuronal surface (Kurup et al., 2010).

At the synapse, NMDARs are anchored to the cytoskeleton through several proteins, including cortactin, a protein that promotes actin polymerization (Naisbitt et al., 1999). Cortactin also associates with Src kinase (Okamura and Resh, 1995) and regulates the dendritic clustering of numerous proteins, such as postsynaptic density-95 (PSD-95), a scaffold protein (Catarino et al., 2013). Phosphorylated cortactin binds neuronal Wiskott–Aldrich Syndrome protein (N-WASP) to activate the actin-related protein (Arp)2/3 complex (Tehrani et al., 2007), thus promoting actin polymerization (Uruno et al., 2001). On the other hand, reelin, a secreted glycoprotein involved in synaptic plasticity modulation in the adult, stimulates neurons through the very-low-density-lipoprotein receptor (VLDLR) and apolipoprotein E receptor-2 (ApoER-2) (Weeber et al., 2002), leading to disabled-1 (Dab1) phosphorylation in a Src-dependent manner (Kuo et al., 2005). Once activated, Dab1 induces actin polymerization by directly binding to N-WASP and subsequent activation of the Arp2/3 complex (Suetsugu et al., 2004), similarly as described for activated cortactin. Phosphorylated Dab1 is also able to activate Src kinase family through phosphorylation (Ballif et al., 2003; Bock and Herz, 2003), leading to NMDAR activation (Chen et al., 2005). In agreement, inhibition of reelin pathway significantly decreased the availability of GluN2B subunits at the synapse (Groc et al., 2007). Importantly, reelin was shown to be depleted in AD brain before the onset of Aβ pathology in AD transgenic mice hippocampus and in human frontal cortex at pre-clinical AD stage (Herring et al., 2012).

Considering that NMDARs are key players in the initial stages of AD, we evaluated age-dependent changes in NMDAR subunit (GluN2B and GluN2A) phosphorylation in AD affected brain areas, hippocampus and cortex, using the triple transgenic mouse model of AD (3xTg-AD) that shows progression of pathology as a function of age (Oddo et al., 2003b). This model also reproduces numerous mechanisms involved in AD, such as impairment in intracellular Ca^{2+} homeostasis (Lopez et al., 2008), oxidative stress (Resende et al., 2008), spine degeneration (Bittner et al., 2010) and early cognitive deficits (Billings et al., 2005). Thus, we studied GluN2B Tyr phosphorylation and the activity of Src kinase. Moreover, the signaling pathways that link NMDAR and Src kinase to actin cytoskeleton polymerization, namely cortactin, reelin and Dab1, as well as the levels of polymerized actin (F-actin) in post-synaptic terminals were analyzed in 3xTg-AD mice. Our study shows decreased GluN2B Tyr phosphorylation related to alterations in Src-related signaling in early stages of AD mice brain, which is accompanied by decreased post-synaptic labeling of F-actin. Actin destabilization is suggested to compromise normal synaptic function in AD.

Material and methods

Animals

3xTg-AD and WT strain (C57BL6/129S) mice were a generous gift from Dr. Frank Laferla (University of California, Irvine) and were bred and maintained at CNC-Faculty of Medicine animal house. Animals were housed under a constant temperature, humidity and a 12 h light/dark cycle. In this work, 3-, 6-, 12- and 15-month-old 3xTg-AD or WT males and females mice were used. All procedures using animals were in accordance with the approved animal welfare guidelines and European legislation (European directive 2010/63/EU).

Material

BioRad protein assay reagent was from Bio-Rad (Munich, Germany). Polyvinylidene difluoride (PVDF) membrane was purchased from Chemicon Millipore Merck (Darmstadt, Germany). ECF reagent was obtained from Amersham (Buckinghamshire, UK). Fluorescent dye Hoechst 33342 was purchased from Molecular Probes—Invitrogen (Eugene, OR, USA). VECTASTAIN Elite ABC Kit was purchased from Vector Lab (Burlingame, CA, USA). Alexa® 594 phalloidin was from Cytoskeleton (Denver, CO, USA). Protease cocktail inhibitors and 3,3′-diaminobenzidine (DAB), as well as other analytical grade reagents, were from Sigma Chemical and Co. (St. Louis, MO, USA). Antibodies used in this study are listed in Table 1.

Histological techniques

3xTg-AD or WT mice were deeply anesthetized with 15 μl/g of body weight of a mixture of ketamine (18.3%, Imalgene 1000) and xylazine (10.2%, Rompun 2%) prepared in 0.9% NaCl. Mice were further perfused intracardially with 0.9% NaCl and then with 4% paraformaldehyde in phosphate buffered saline (PBS) (containing in mM: 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 $Na_2HPO_4 \cdot 2H_2O$, pH 7.4). Cerebral hemispheres were further fixed for 3 h in 4% paraformaldehyde, washed with PBS and immersed in 30% (w/v) sucrose until they sank, at 4 °C and further frozen in liquid nitrogen and sliced (40 μm) with a cryostat (Leica, Mannheim, Germany).

Fluorescent immunohistochemistry

Free floating sections obtained from 3 month-old male and female WT and 3xTg-AD were incubated with blocking solution of PBS with 3% BSA and 0.2% Triton X-100 for 1 h at room temperature (RT). Slices were then incubated with primary antibodies (anti-APP, anti-Aβ1–42, anti-PSD-95), diluted in blocking solution, overnight at 4 °C. Sections were further washed with PBS (three times, 10 min) and incubated for 2 h at RT with secondary antibodies Alexa Fluor® 594 goat anti-mouse and Alexa Fluor® 488 goat anti-rabbit prepared in PBS containing 0.2% Triton X-100 in the presence of Hoechst 33432 (1:10,000). For the identification of F-actin, slices were incubated with 50 nM Alexa® 594 phalloidin, a mushroom toxin that has high affinity to polymerized actin (F-actin), overnight, at 4 °C. Finally, slices were mounted with fluoroshield mounting medium. Fluorescent images of hemisphere sections were obtained using an epifluorescence microscope (Axiovert Microscope 200, Zeiss) or with a confocal microscope (Zeiss LSM 510 Meta) for phalloidin and PSD-95 labeling.

DAB immunohistochemistry

In order to label Aβ plaques and neurofibrillary tangles, free floating sections obtained from 15 month-old 3xTg-AD male and female mice versus age- and gender-matched WT mice were washed with PBS