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Synapsin I deletion reduces neuronal damage and ameliorates clinical progression of experimental autoimmune encephalomyelitis

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ABSTRACT

The classical view of multiple sclerosis (MS) pathogenesis states that inflammation-mediated demyelination is responsible for neuronal damage and loss. However, recent findings show that impairment of neuronal functions and demyelination can be independent events, suggesting the coexistence of other pathogenic mechanisms. Due to the inflammatory milieu, subtle alterations in synaptic function occur, which are probably at the basis of the early cognitive decline that often precedes the neurodegenerative phases in MS patients. In particular, it has been reported that inflammation enhances excitatory synaptic transmission while it decreases GABAergic transmission *in vitro* and *ex vivo*. This evidence points to the idea that an excitation/inhibition imbalance occurs in the inflamed MS brain, even though the exact molecular mechanisms leading to this synaptic dysfunction are as yet not completely clear. Along this line, we observed that acute treatment of primary hippocampal neurons in culture with pro-inflammatory cytokines leads to an increased phosphorylation of synapsin I (SynI) by ERK1/2 kinase and to an increase in the frequency of spontaneous synaptic vesicle release events, which is prevented by SynI deletion. *In vivo*, the ablation of SynI expression is protective in terms of disease progression and neuronal damage in the experimental autoimmune encephalomyelitis mouse model of MS. Our results point to a possible key role in MS pathogenesis of the neuronal protein SynI, a regulator of excitation/inhibition balance in neuronal networks.

1. Introduction

Multiple sclerosis (MS) is a chronic and progressive inflammatory disease of the central nervous system (CNS) characterized by space- and time-disseminated demyelinating and neurodegenerative lesions known as sclerotic plaques (Compston and Coles, 2008). Auto-reactive T-lymphocytes targeting self antigens from myelin and neurons invade the CNS, causing focal tissue damage (Kebir et al., 2007). This process is further sustained by the abnormal activation of resident innate immune cells, namely microglial cells, and astrocytes, both able to trigger tissue inflammation through the release of pro-inflammatory mediators (Tzartos et al., 2008; Weiner, 2008). The exact causality and the relationship between immune invasion, demyelination, and neurodegeneration are as yet not completely clear.

Inflammation-induced demyelination undoubtedly contributes to axonal degeneration through the loss of trophic support from oligodendrocytes (Funfschilling et al., 2012; Lee et al., 2012). However, increasing evidence has pointed out that white and grey matter alterations in MS patients occur, at least in part, independently (Trapp and Nave, 2008). Magnetic resonance imaging (MRI) studies recently clarified that a reduction of cortical thickness and grey matter atrophy represent early events in disease progression, often preceding cortical demyelination and significant disability, thus suggesting the occurrence of a primary neuronal damage

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(Calabrese et al., 2007; Charil et al., 2007; Charil and Filippi, 2007). This is particularly relevant considering that grey matter atrophy and neuronal loss are probably at the basis of the disabling cognitive alterations that affect ~50% of MS patients and that are manifest at the onset of the pathology (Calabrese et al., 2009; Reuter et al., 2011; Roosendaal et al., 2011).

In addition to overt neuronal degeneration, it is now clear that more subtle alterations of synaptic transmission take place in the inflamed MS brain independently of axonal damage and of demyelination, and probably contribute to the establishment of an early cognitive phenotype (Mandolesi et al., 2015b; Musella et al., 2016). Several studies reported diffuse structural and functional synaptic alterations affecting both glutamatergic and GABAergic systems. In particular, pro-inflammatory cytokines, such as tumour necrosis factor-α (TNFα), interferon-γ (IFNγ), and interleukin-1β (IL-1β), released in the MS brain during acute attacks, are able to increase excitatory and decrease inhibitory synaptic transmission, favouring an excitation/inhibition imbalance and consequent excitotoxic damage (Centonze et al., 2009; de Ceglia et al., 2015; Falco et al., 2014; Mandolesi et al., 2015a, 2012, 2013; Nistico et al., 2013; Rossi et al., 2011; Stellwagen and Malenka, 2006; Zhu et al., 2003). *In vitro* and *ex vivo*, pro-inflammatory cytokines are able to increase the frequency of spontaneous release events at excitatory synapses, thus suggesting a presynaptic involvement (Beattie et al., 2002; Rossi et al., 2012; Stellwagen et al., 2005). Even though the functional outcomes of neuronal exposure to inflammatory insults have been extensively studied, the molecular mechanisms mediating these effects are largely unknown (Mandolesi et al., 2015b; Vezzani and Viviani, 2015).

We used an *in vitro* approach to identify intracellular pathways, activated in the presynaptic compartment of neurons, that could underlie the deleterious effects of pro-inflammatory cytokines. We report that exposure of neuronal cells to a cocktail of pro-inflammatory cytokines leads to the activation of extracellular signal-regulated kinase ERK1/2 kinase and to the phosphorylation of its substrate synapsin I (SynI). SynI is a presynaptic protein involved in the modulation of neurotransmitter release through a complex mechanism, including its phosphorylation-dependent ability to anchor synaptic vesicles to the actin cytoskeleton (Cesca et al., 2010). Phosphorylation by ERK1/2 is able to reduce SynI affinity for actin, thus increasing synaptic vesicle recruitment for exocytosis (Chi et al., 2003; Jovanovic et al., 1996, 2000; Schenk et al., 2005). In addition, by taking advantage of the experimental autoimmune encephalomyelitis (EAE) mouse model (Mix et al., 2010; Ransohoff, 2012), we observed that the ablation of SynI ameliorates disease progression and reduces axonal damage, thus supporting a role for SynI in mediating part of the neuronal response to an inflammatory milieu.

2. Materials and methods

2.1. Animals

Mice were housed under constant temperature (22 ± 1 °C) and humidity (50%) conditions with a 12 h light/dark cycle, and were provided with food and water *ad libitum*. Homozygous SynI knock-out (KO) mice were kindly provided by Prof. Paul Greengard (Rockefeller University, New York, NY) (Chin et al., 1995). SynI KO mice were re-derived on a C57BL/6N background (Charles River Cat# B6NSIFE07S, Calco, Italy). All experiments involving animals followed the guidelines established by the European Community Council (Directive 2010/63/EU of September 22nd, 2010) and were approved by the Institutional Animal Care and Use Committee (IACUC, permission number 690) of the San Raffaele Scientific Institute and by the Italian Ministry of Health. All efforts were made to minimize animal suffering.

2.2. Primary neuronal cultures and cytokine treatment

Primary neuronal cultures were prepared from the hippocampi of embryonic day 17.5 embryos from either wild-type (WT) or SynI KO mice of either sex. Briefly, hippocampi were collected and mechanically dissociated upon incubation with 0.25% trypsin in HBSS (Hanks' balance salt solution) at 37 °C. Neurons were plated on poly-L-lysine (0.1 mg/ml; Sigma-Aldrich, Milan, Italy)-coated 16 mm glass coverslips or plastic wells at a density of 40.000 cells/cm^2. Neurons were maintained in Neurobasal medium supplemented with 2% B-27, 1% glutamax, 1% Pen/Step. All reagents for cell culture were from ThermoFisher (Waltham, MA, USA). Mature hippocampal neurons (14–15 days *in vitro*) received a cocktail of proinflammatory cytokines for 5–60 min in the cell medium. The cytokine cocktail was composed of mouse recombinant TNFα (Peprotech, Rocky Hill, NJ, USA; 100 ng/ml), IFNγ (Peprotech; 20 ng/ml), and IL-1β (Cedarlane, Burlington, Ontario, Canada; 100 ng/ml).

2.3. Western blotting

Neuronal cells were lysed with a buffer containing 1% sodium dodecylsulfate (SDS), 10 mM HEPES, 2 mM EDTA pH 7.4. Spinal cord tissues were lysed in RIPA (RadioImmunoPrecipitation Assay) buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA pH 8, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protein inhibitor, Ser/Thr and Tyr phosphatase inhibitor cocktails (Sigma). Tissues were homogenized by 10 passages through a 25G needle, rotated on a wheel for 30 min at 4 °C and centrifuged at 15,000*g* for 15 min to remove nuclei and cell debris. Protein content was quantified with bicinchoninic acid assay (BCA, ThermoFisher). Laemmli buffer was added to a final 1X concentration (20 mM Tris pH 6.8, 2 mM EDTA, 2% SDS, 10% glycerol, 2% β-mercaptoethanol 0.01% bromophenol blue). Equal protein amounts were loaded on a polyacrylamide gel, subjected to standard SDS-PAGE, using a Standard Vertical Gel Electrophoresis unit (Hoefer, San Francisco, CA, USA), and transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). Blocking of the membranes was performed in 5% milk-TBST (Tris-Base saline-Tween: 150 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.05% Tween 20) for 1 h at room temperature (RT). Primary antibodies were appropriately diluted in 5% milk-TBST and incubated overnight at 4 °C in a humidified chamber. Membranes were washed 3 times in TBST to eliminate primary antibody in excess. Secondary horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies (1:10000, BioRad Cat#170–6516/6515 RRID:AB_11125547 RRID: AB_11125142, Hercules, CA, USA) were diluted in 5% milk-TBST and incubated for 1 h at RT. Membranes were washed 3 times in TBST to remove secondary antibodies in excess. Detection was performed with the enhanced chemiluminescence reaction (ECL; Amersham-GE Healthcare, Buckinghamshire, UK). Signals were visualized on ECL Hyperfilms (Amersham) and scanned with a desktop scanner (Epson Perfection V800 Photo) at 600 dpi. Primary antibodies used: mouse anti-total Syn 19.11 (1:1500, home-made), rabbit anti-pSyn site 4/5 G527 (1:3000, home-made), rabbit anti-pERK1/2 (1:2000, Promega Cat# V8031 RRID:AB_430866, Madison, WI, USA), rabbit anti-total ERK1/2 (1:1000, Promega Cat# V1141 RRID:AB_430839), rabbit anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 1:10000, Cell Signaling Technology Cat# 2118 RRID:AB_561053, Danvers, MA, USA). Home-made antibodies were produced and characterized at the Rockefeller

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