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Amyloid-β oligomer synaptotoxicity is mimicked by oligomers of the model protein HypF-N

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ABSTRACT

Protein misfolded oligomers are thought to be the primary pathogenic species in many protein deposition diseases. Oligomers by the amyloid-β peptide play a central role in Alzheimer's disease pathogenesis, being implicated in synaptic dysfunction. Here we show that the oligomers formed by a protein that has no link with human disease, namely the N-terminal domain of HypF from *Escherichia coli* (HypF-N), are also synaptotoxic. HypF-N oligomers were found to (i) colocalize with post-synaptic densities in primary rat hippocampal neurons; (ii) induce impairment of long-term potentiation in rat hippocampal slices; and (iii) impair spatial learning of rats in the Morris Water Maze test. By contrast, the native protein and control nontoxic oligomers had none of such effects. These results raise the importance of using HypF-N oligomers as a valid tool to investigate the pathogenesis of Alzheimer's disease, with advantages over other systems for their stability, reproducibility, and costs. The results also suggest that, in the context of a compromised protein homeostasis resulting from aggregation of the amyloid β peptide, a number of oligomeric species sharing common synaptotoxic activity can arise and cooperate in the pathogenesis of the disease.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive decline of cognitive functions. Memory and learning impairments are common features in all AD patients, but at least 1 symptom among aphasia, apraxia and agnosia is also present (Castellani et al., 2010). The 2 distinctive cerebral lesions of AD are extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFTs), together with microglial activation, neuronal loss and alteration, and cerebrovascular amyloid (Altman and Rutledge, 2010; Lee and Landreth, 2010). The SP and NFTs deposits are mainly composed by the amyloid β peptide (Aβ) and tau protein (τ), respectively (Hyman et al., 1989; Masters et al., 1985). These deposits can be found within the association region of the cerebral cortex and within the medial temporal lobes (Arnold et al., 1991; Braak and Braak, 1991), with the earliest pathologic lesions found in the amygdale, subiculum, hippocampal CA1 region, enthorinal cortex, and transenthorinal regions (Castellani et al., 2010).

Extracellular amyloid fibrils formed by Aβ, and particularly the small oligomers forming before the fibrils as on- or off-pathway species, have been found to associate with synapses and cause their dysfunction as an early event in the development of AD (Selkoe, 2008; Stèphan et al., 2001). Indeed, Aβ oligomers have been found to inhibit long-term potentiation (LTP), as shown by electrophysiological experiments performed on cultured neurons, rat hippocampal slices, or rats in vivo (Barghorn et al., 2005; Lambert et al., 1998; Townsend et al., 2006; Walsh et al., 2002; Wang et al., 2003), to co-localize with pre- and post-synaptic markers in cultured primary neurons imaged with confocal microscopy (Deshpande et al., 2006; Lacor et al., 2004; Pitt et al., 2009), and to impair cognitive functions in animal models when microinjected into their brains (Cleary et al., 2005; Lesné et al., 2006; Poling et al., 2008).

Other studies, performed independently of AD, have shown that the protein folding homeostasis network, that is, the cellular machinery dedicated to the maintenance of proteins in their soluble native states, decreases significantly with aging, rendering different

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proteins susceptible to aggregating (Gidalevitz et al., 2006; Kikis et al., 2010; Powers et al., 2009). Moreover, accumulation of misfolded protein aggregates saturates and consequently weakens further the protein homeostasis network (Gidalevitz et al., 2006). In the presence of the aggregates of one or a few proteins, other normally soluble proteins become thereby susceptible to aggregating (Gidalevitz et al., 2006). This scenario is realistic for AD, as aggregates other than those formed by Aβ are found in AD brains; these include NFTs by τ (Goedert et al., 1989; Gotz et al., 2004), Lewy bodies by α-synuclein (Hamilton, 2000; Lippa et al., 1998), intracellular inclusions by the TDP-43 protein (Amador-Ortiz et al., 2007; Uryu et al., 2008), plaques by the prion protein (Ferrer et al., 2001; Schwarze-Eicker et al., 2005), fibrils by lithostathine (Duplan et al., 2001), reticulon 3 aggregates (Heath et al., 2010; Hu et al., 2007), Hirano bodies containing actin and other proteins (Ostrowski et al., 2005), among others.

The recent failures of clinical trials that targeted the release of Aβ by the γ-secretase (Extance, 2010) or, more generally, the production and/or aggregation of Aβ (Mangialasche et al., 2010), has reinforced the idea that production and aggregation of Aβ is one but not the only factor in the origination of AD. According to this idea, oligomers of proteins other than Aβ should be not just generically toxic to cells, but also should cause synaptic dysfunction.

In this work, we used a protein that has no link with AD or other human disease, i.e., the N-terminal domain of HypF from *Escherichia coli* (HypF-N) to show that its oligomers have the potential to target synapses, cause their dysfunction and induce memory impairment, resulting in an overall neurological effect similar to that caused by Aβ oligomers. HypF-N is a good model system for this purpose because it has been shown to form amyloid fibrils morphologically, structurally, and tinctorially similar to those associated with disease (Chiti et al., 2001; Marcon et al., 2005; Relini et al., 2004), because amyloid formation is preceded by the transient accumulation of protein oligomers toxic to cultured cells, as determined using a number of biochemical and cytological tests (Bucciantini et al., 2002; Bucciantini et al., 2004; Campioni et al., 2010; Cecchi et al., 2006; Zampagni et al., 2011), and because oligomer formation can be directed into 2 different forms, found to be toxic (type A) and nontoxic (type B), respectively, providing useful controls for any type of investigations that uses HypF-N oligomers (Campioni et al., 2010; Zampagni et al., 2011).

Herein we will show that HypF-N toxic (type A) oligomers inhibit LTP of rat hippocampal slices, colocalize with post-synaptic markers in cultured rat hippocampal primary neurons, and impair rats' spatial memory when microinjected in their brains. We will also show that native monomeric HypF-N or nontoxic type B HypF-N oligomers formed under different conditions do not have such effects. Overall, these results have implications for providing hypotheses for AD pathogenesis and elect HypF-N type A oligomers as a valid tool to investigate the early synaptic dysfunction typical of the disease.

2. Methods

2.1. Preparation of HypF-N oligomers

HypF-N was expressed and purified as described previously (Campioni et al., 2010). Protein purity was assessed by SDS-PAGE at >95%. The purified protein was stored at −20 °C in 5 mmol/L acetate buffer, 2 mmol/L dithiothreitol (DTT) at pH 5.5 (stock solution). Oligomers were prepared by diluting HypF-N from its stock solution to 48 μmol/L, in 50 mmol/L acetate buffer, 12% (v/v) trifluoroethanol (TFE), 2 mmol/L DTT, pH 5.5 (condition A); or in 20 mmol/L trifluoroacetic acid (TFA), 330 mmol/L NaCl, pH 1.7 (condition B). The samples were incubated for 4 hours at 25 °C, centrifuged for 10 minutes at 16,100 relative centrifugal force (rcf) and resuspended in the same volume of either artificial cerebrospinal fluid (aCSF) for LTP measurements and water maze experiments, or in neurobasal medium (NBM) for the synapse co-localization experiments. The HypF-N stock solutions were diluted further in the same media to reach the desired protein concentrations. Protein preparations were tested for LPS contamination according to the toxin sensor limulus amebocyte lysate (LAL) assay kit protocol (Genscript, Piscataway, NJ), with contamination found to be negligible in all samples (0.02 EU/mL).

2.2. Tapping-mode atomic force microscopy

Aggregates were centrifuged at 16,100 rcf for 10 minutes and resuspended in potassium phosphate buffer, pH 7.0. A 10-μL quantity of each sample was diluted 100 times, deposited on a freshly cleaved mica substrate, and dried under vacuum. Tapping-mode atomic force microscopy (TM-AFM) images were acquired in air using a Dimension 3100 SPM with a "G" scanning head (maximum scan size, 100 μm) and driven by a Nanoscope IIIa controller, and a Multimode SPM equipped with "E" scanning head (maximum scan size, 10 μm) and driven by a Nanoscope IV controller (Digital Instruments–Veeco, Santa Barbara, CA). Single-beam uncoated silicon cantilevers (type OMCL-AC160TS; Olympus, Tokyo, Japan) were used. The drive frequency was between 320 and 340 kHz, and the scan rate was between 0.5 and 2.0 Hz. Other details are as reported elsewhere (Campioni et al., 2010).

2.3. Thioflavin T binding and far UV circular dichroism spectra

Aliquots of 48 μmol/L native HypF-N in 5 mmol/L acetate buffer, 2 mmol/L DTT, pH 5.5 and aliquots of type A and type B oligomers, re-suspended in aCSF at a corresponding monomer concentration of 48 μmol/L after centrifugation for 10 minutes at 16,100 rcf were added to a solution of 25 μmol/L thioflavin T (ThT; Sigma-Aldrich) dissolved in 25 mmol/L phosphate buffer at pH 6.0, to obtain a 3.7-fold molar excess of dye. Final protein concentration was 6 μmol/L. The steady-state intensity of fluorescence emission at 485 nm (excitation at 440 nm) was recorded at 25 °C using a Perkin-Elmer LS 55 spectrofluorimeter (Wellesley, MA). The ratio between the ThT emission in the presence (F) and absence (F_0) of HypF-N is reported. Far UV circular dichroism (CD) spectra were acquired at 37 °C for solutions containing 19 μmol/L native HypF-N or 0.5 mg/mL type A and type B oligomers in aCSF, or in 5 mmol/L acetate buffer, 2 mmol/L DTT, pH 5.5, using a Jasco J-810 Spectropolarimeter (Tokyo, Japan) and a 1-mm path-length cell.

2.4. Cultures of rat hippocampal primary neurons

Cultures of hippocampal neurons were prepared from ED17 fetal Wistar rats (Harlan Italy, Udine, Italy). Both hippocampi were dissociated from rats' brains in sterile Dulbecco's phosphate-buffered saline (D-PBS), and neurons were isolated in the same medium containing trypsine (0.125% in sterile D-PBS) and DNase (5 mg/ml) at 37 °C for 10 minutes (agitating the solution every 5 minutes). After centrifugation at 2000 rcf for 5 minutes, dissociated neurons were resuspended in NBM supplemented with 2% B-27 (Invitrogen, Milan, Italy) and 0.5 mmol/L glutamine, and then plated at a density of approximately 1.3×10^6 per well. Cultures were maintained in NBM at 37 °C in a humidified, 5% CO_2 environment for 21 days. Type A or B oligomers and native HypF-N were diluted in NBM at a final monomer concentration of 1 μmol/L and applied to the cultured neurons for 30 minutes.