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Research Report

Dopamine induces apoptosis in APPswe-expressing Neuro2A cells following Pepstatin-sensitive proteolysis of APP in acid compartments

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ABSTRACT

A pathological hallmark of Alzheimer's disease (AD) is the presence within neurons and the interneuronal space of aggregates of β-amyloid (Aβ) peptides that originate from an abnormal proteolytic processing of the amyloid precursor protein (APP). The aspartyl proteases that initiate this processing act in the Golgi and endosomal compartments. Here, we show that the neurotransmitter dopamine stimulates the rapid endocytosis and processing of APP and induces apoptosis in neuroblastoma Neuro2A cells over-expressing transgenic human APP (Swedish mutant). Apoptosis could be prevented by impairing Pepstatin-sensitive and acid-dependent proteolysis of APP within endosomal–lysosomal compartments. The γ-secretase inhibitor L685,458 and the α-secretase stimulator phorbol ester elicited protection from dopamine-induced proteolysis of APP and cell toxicity. Our data shed lights on the mechanistic link between dopamine excitotoxicity, processing of APP and neuronal cell death. Since AD often associates with parkinsonian symptoms, which is suggestive of dopaminergic neurodegeneration, the present data provide the rationale for the therapeutic use of lysosomal activity inhibitors such as chloroquine or Pepstatin A to alleviate the progression of AD leading to onset of parkinsonism.

1. Introduction

Alzheimer's disease (AD) is a late-onset neurological disorder characterized by progressive loss of memory and cognitive abilities as a result of excessive neurodegeneration in the hippocampus and cortex (Sabuncu et al., 2011). A pathological hallmark of AD is the presence in the interneuronal space of amyloid plaques formed by aggregates of β-amyloid (Aβ) peptides that originate from an abnormal proteolytic processing of the amyloid precursor protein (APP). APP is a large transmembrane type 1 (cytosolic C-terminal) glycoprotein coded by a gene located on chromosome 21 and giving rise to eight alternative transcripts, of which three are mainly transcribed into the isoforms containing 695, 751 and 770

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DA, dopamine; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; IETD-CHO, acetyl-Ile-Glu-Thr-Asp-aldehyde inhibitor (Ac-IETD-CHO); PI, propidium iodide; PMA, phorbol 12-myristate-13-acetate; L685,458, [(2R,4R,5S)-2-benzyl-5-(Boc-amino)-4-hydroxy-6-phenyl-hexanoyl]-Leu-Phe-NH_2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; ZVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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aminoacids (reviewed in Bekris et al., 2010). APP695 mature protein differs from the whole length APP770 because it lacks the 290–364 sequence comprising the Kunitz-Protease Inhibitor peptide.

In the endoplasmic reticulum and during its transport through the Golgi Complex, nascent APP undergoes co- and post-translational modifications, including N- and O-glycosylation, phosphorylation and tyrosine sulfation, that lead to the so-called mature APP (Perdivara et al., 2009). Mature APP is not permanently resident at the plasma membrane, rather it is subjected to a continuous retrograde trafficking from the plasma membrane to intracellular compartments of the secretory pathway (Vieira et al., 2010), so that at steady state it is more abundant in the Golgi Complex and in endosomes (Koo et al., 1996; Xu et al., 1997; Yamazaki et al., 1996). Moreover, APP is not a stable molecule, as it undergoes proteolysis through multiple and alternative routes. The proteolytic pathways involved in the APP processing and the cellular compartments in which this occurs have been studied in details (for review see Chow et al., 2010; O'Brien and Wong, 2011; Thinakaran and Koo, 2008; Zhang et al., 2011). The order of proteolysis at α, β and γ sites determines whether or not the Aβ peptide will be produced: the sequential action of α- and γ-secretases leads to the production of a soluble APPα fragment (sAPPα), a P3 peptide and an intracellular domain (AICD peptide) at the C-terminus, whereas the sequential action of β- and γ-secretases leads to a soluble APPβ fragment (sAPPβ), the Aβ peptide (of 40 or 42 aminoacids) and the AICD peptide. Thus, proteolysis at β-site is alternative to that at α-site and is fundamental for amyloidogenesis. The main protease responsible for such proteolysis is β-APP cleaving enzyme (BACE), a type-1 transmembrane aspartyl protease mainly localized to endosomes, lysosomes and the Golgi Complex (Cai et al., 2001; Vassar et al., 1999). Another protease with potential β-secretase activity is lysosomal Cathepsin D, which has been shown able to cleave *in vitro* APP and produce Aβ (Chevallier et al., 1997; Higaki et al., 1996), and to be highly expressed in AD brain (Schechter and Ziv, 2008). However, while BACE-deficient mice do not produce Aβ and show normal phenotype (Luo et al., 2001; Ohno et al., 2004), Cathepsin D-deficient mice still produce and accumulate Aβ in hippocampal neurons (Saftig et al., 1996). Amyloidogenic processing of APP has been proved to occur within the Golgi Complex (Xu et al., 1997) and the endosomal compartment (Pasternak et al., 2004). Impairing the internalization of plasma membrane APP reduces the formation of Aβ up to 80% (Koo and Squazzo, 1994), as it does the treatment with drugs that rise the luminal pH of endosomal–lysosomal compartments (Schrader-Fischer and Paganetti, 1996).

To what extent the trafficking and processing of APP *in vivo* occurs constitutively or is affected by the extracellular stimuli, and whether and how neurotransmitters influence the fate of APP and of cells expressing APP is largely unknown. Here, we report on the effect of dopamine (DA), a neurotransmitter diffused in substantia nigra, striatum and other brainstem nuclei, in neuroblastoma Neuro2A cells over-expressing human APP695 (Thinakaran et al., 1996), which is the isoform mainly expressed in human brain (Kang and Müller-Hill, 1990). Neuro2A cells express muscarinis receptors (Edwards et al., 1989) and are prone to cholinergic neuronal differentiation and neurite development (Kojima et al., 1994). Under appropriate stimulation, Neuro2A express tyrosine hydroxylase and produce DA and L-DOPA (Akahoshi et al., 2009) and respond to DA excitotoxicity (Castino et al., 2005). Therefore, Neuro2A cells can be assumed *bona fide* as a valuable *in vitro* model to study the effects of dopamine on APP processing. The data here reported extend the previous knowledge on the relationship between neuronal cell toxicity and endocytosis and processing of APP, and also provide new evidence on the mechanism of DA excitotoxicity in neuronal cells over-expressing APP. The latter may have clinical relevance, given that Parkinson's-like dopaminergic neurodegeneration has been observed in the postmortem brain of AD patients with extrapyramidal signs (Burns et al., 2005; Jellinger, 2003; Schneider et al., 2002).

2. Results

2.1. *Dopamine triggers the intrinsic apoptotic death pathway in Neuro2A cells over-expressing transgenic Human APP*

To address whether the abnormal expression of APP renders dopaminergic neuronal cells susceptible to DA toxicity, we employed an established *in vitro* model system represented by neuroblastoma mouse Neuro2A cells sham-transfected or stably expressing transgenic human APP695 in the Swedish-mutant form (Thinakaran et al., 1996). The cells were exposed to DA and observed under the microscope for gross morphological alterations and cell loss at increasing time of incubation. Evidence of toxic effects was noted starting at 16 h of exposure to DA only in the transfected Neuro2A expressing APP. By this time, nuclei staining with DAPI of cells adherent on sterile coverslips revealed chromatin condensation and fragmentation, typical signs of apoptosis, in samples of Neuro2A-APP exposed to DA (Fig. 1A). TUNEL staining confirmed the occurrence of DNA fragmentation in these samples (Fig. 1B). A quantitative estimation of DA toxicity was obtained by cytofluorometry of the hypodiploid (so-called subG1 peak) cell population, which mirrors late apoptotic cells, in the cultures exposed or not for 16 h to DA. While sham-transfected Neuro2A cells showed negligible sensitivity, Neuro2A-APPswe cells showed high sensitivity to DA toxicity (Fig. 1C). As an additional quantification and proof of the apoptosis induced by DA, we estimated by cytofluorometry the presence of phosphatidyl-serine on the outer leaflet of the plasma membrane (an early marker of apoptosis) in sham- and APP-transfected Neuro2A cells treated in the absence or in the presence of the pan-caspase inhibitor zVAD-fmk. Data showed that as much as 40% of the APP-over-expressing cells treated with DA for 16 h were positive for annexinV (indicative of phosphatidyl-serine exteriorization) and that pre-incubation with zVAD-fmk completely abrogated this effect (Fig. 1D). Taken together, these data demonstrate that chronic DA stimulation, while not toxic to the sham-transfected counterpart, causes apoptotic cell death in Neuro2A cells over-expressing transgenic human APP. Because of the pro-oxidative nature of DA excitotoxicity,