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PATHOPHYSIOLOGICAL AMYLOID CONCENTRATIONS INDUCE SUSTAINED UPREGULATION OF READTHROUGH ACETYLCHOLINESTERASE MEDIATING ANTI-APOPTOTIC EFFECTS

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Abstract—Cholinergically differentiated SH-SY5Y neuroblastoma cells were treated with a pathophysiologically relevant, low (300 nM), and a high (3 μM) dose of amyloid beta 1-42 (Abeta) or 42-1 (revAbeta). At early (1 and 4 h) and late (24 h) time points, the pro- and anti-apoptotic factors – caspase-3 and p53, and B-cell lymphoma 2 protein (Bcl-2), respectively – were assessed together with lactate dehydrogenase (LDH) release as measure of cell viability and ATP levels as marker of mitochondrial activity. The low peptide dose significantly increased Bcl-2 and, time-delayed, caspase-3 and ATP levels, but barely impacted on LDH release, while the high concentration remarkably depressed Bcl-2 levels, depleted ATP and led to increased LDH release. We also monitored acetylcholinesterase (AChE) enzymatic activity and splice variant levels (tailed and readthrough AChE; AChE-T and AChE-R), and assessed choline acetyltransferase (ChAT) and high-affinity choline uptake (HACU). The low Abeta concentration drastically upregulated AChE-R and increased both ChAT and HACU, while the high dose caused cholinergic toxicity. We believe this study offers the first insight into the highly concentration-dependent effects of Abeta on cholinergic dynamics. In particular, it highlights the rescuing role of AChE-R as being, together with mitochondrial activity, involved in cholinergic adaptation to low doses of Abeta.

Key words: amyloid beta, acetylcholinesterase, alternative splicing, neurotoxicity, ATP, cholinergic plasticity.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder among the elderly. Yet, the pathogenic mechanisms underlying this condition continue to be at the centre of animated debate. Specifically, the amyloid hypothesis (Hardy and Selkoe, 2002) suggests that plaques formed by deposits of amyloid beta (Abeta) are at the root of cell death. At the same time, a large body of evidence claims an impaired cholinergic system as at least partly involved in the neurodegenerative processes (Francis et al., 1999; Mufson et al., 2008; Schliebs and Arendt, 2011). This is especially true for two reasons: firstly, the cognitive symptoms related to the condition can be linked to the loss of cholinergic neurons (Coyle et al., 1983; Cummings and Back, 1998), and, thus, the loss of the cholinergic neurotransmitter acetylcholine (ACh); secondly, acetylcholinesterase (AChE) activity (Giacobini, 2003) and AChE isoform composition (Arendt et al., 1992) are altered in AD brain, depending on the patient's cognitive state. Likewise, a remarkable reduction in choline acetyltransferase (ChAT), the enzyme responsible for ACh synthesis, correlates with the severity of dementia (Geula and Mesulam, 1994), while the high-affinity choline uptake (HACU) transporter has been shown to be elevated in AD tissue obtained from rapid autopsy (Slotkin et al., 1994; DeKosky et al., 2002).

Since AChE is known to, firstly, be present in amyloid plaques found in AD brains (Carson et al., 1991), and, secondly, enhance amyloid fibril formation (Inestrosa et al., 1996) and amyloid toxicity (Inestrosa et al., 2008), these two hypotheses have been considered to be closely linked (Auld et al., 2002). In fact, the impact of Abeta has been assessed in a vast array of cellular systems. For example, the peptide affects intracellular ACh concentration in hippocampal neurons (Hoshi et al., 1997), inhibits neurotransmitter release from rat hippocampal slices (Kar et al., 1996), impairs mitochondrial function in PC12 and other cells (Kurz et al., 2010), particularly affecting ATP level depletion (Piaceri et al., 2012), and disturbs cell signalling (Alberdi et al., 2010). Notably, high concentrations of Abeta were shown to lead to a significant reduction in hemicholinium-3 (HC-3)-sensitive choline uptake (Kar et al., 1998; Novakova et al., 2005). The same is true for ChAT activity, which was suppressed by higher Abeta doses (Szutowicz et al., 2000). However, several studies could not detect specific cholinergic toxicity due

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Abbreviations: Abeta, amyloid beta; ACh, acetylcholine; AChE, acetylcholinesterase; AChE-R, readthrough AChE; AChE-T, tailed AChE; AD, Alzheimer's disease; Bcl-2, B-cell lymphoma 2 protein; ChAT, choline acetyltransferase; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FBS, foetal bovine serum; HACU, high-affinity choline uptake; HC-3, hemicholinium-3; KHB, Krebs–Henseleit buffer; LDH, lactate dehydrogenase; mAb, monoclonal antibody; MEM, minimum essential medium Eagle; MW, molecular weight; MWS, molecular weight standard; OD, optical density; pAb, polyclonal antibody; PBS, phosphate-buffered saline; revAbeta, reverse amyloid beta; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEM, standard error of the mean.

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to Abeta (Winkler et al., 1994; Hartmann et al., 2004), and other work demonstrated both neurotrophic and neurotoxic effects of Abeta (Yankner et al., 1990). Yet, if pathophysiological concentrations of Abeta truly exert neurotoxic effects, one should expect, firstly, alterations of caspase-3 and B-cell lymphoma 2 protein (Bcl-2) as parameters classically involved in (anti-)apoptotic processes (Chowdhury et al., 2006), and, secondly, that an alteration of AChE isoforms is detectable in Abeta neurotoxicity models.

This is particularly relevant, since, recently, strong attention has been placed on the characterisation of various AChE isoforms as being involved in the mediation of stress-related responses (Zimmerman and Soreq, 2006). More specifically, the tailed form of AChE (AChE-T) is predominantly detected in cholinergic tissue (Massoulie et al., 2005), while monomeric readthrough AChE (AChE-R) is broadly but only transiently expressed, and implicated in stress-related processes, where it may exert short- to medium-term beneficial effects (Kaufer et al., 1998; Greenberg et al., 2010); the same is true for an N-terminally extended form of AChE-R, which however may be less protective (Toiber et al., 2008). In this context, it is of note that, firstly, changes in AChE-R have been shown to modulate amyloid pathology *in vivo* (Berson et al., 2008) and, secondly, AChE-T enhances the amyloid burden as well as cognitive deficits in mice expressing the human amyloid precursor protein (Rees et al., 2003, 2005). As such, one wonders whether and how AChE isoforms are altered by exposure to Abeta in a cholinergic cell model – especially since AChE has long been hypothesised to exert non-classical functions in AD (Layer, 1995) and is suggested to be involved in the development of a vicious cycle of Abeta dysregulation (Garcia-Ayllon et al., 2011).

Set against this background, the aim of the present study is to characterise the effect of Abeta on the cholinergic system in retinoic acid differentiated SH-SY5Y cells, i.e., a cellular preparation established as a model for cholinergic neurons (Bartolini et al., 2003; Hashemi et al., 2003). Earlier work characterised purely retinoic acid differentiated SH-SY5Y cells as cholinergic in comparison to cells differentiated additionally with phorbol esters (Pahlman et al., 1984, 1995; Xie et al., 2010). In particular, this work focuses on the impact of Abeta on the expression pattern of the two neuronally relevant AChE splice variants, i.e., AChE-T and AChE-R, in the context of addressing the assumption that the peptide induces neurotoxic effects. In order to validate our system, we investigate the effect of Abeta on the classical pro- and anti-apoptotic players – caspase-3 and p53, and Bcl-2, respectively – as well as cell viability. Directly related, we also explore mitochondrial function by means of ATP assessment. A third aim of these experiments is to describe the effect of Abeta on other cholinergic key players, namely, ChAT and HACU. Finally, in view of the above delineated diverging reports on Abeta effects, we chose to compare the impact of a low amount of Abeta (300 nM), which corresponds to concentrations that approach the actual patho-clinical situation (Papassotiropoulos et al., 2003; Li et al., 2004; Tomic et al., 2009), to that of a higher dose (3 μM), which has previously been found to possess strong toxicity also in this cell model (Wakshlag et al., 2006), but likely does not occur *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

Cell culture hardware and reagents/chemicals were obtained from Greiner (Frickenhausen, Germany) and Sigma–Aldrich (Munich, Germany), respectively. Analytical grade buffer substances were from Merck (VWR, Darmstadt, Germany), while sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) chemicals came from Bio-Rad (Munich, Germany).

Amyloid beta preparation

Amyloid beta 1-42 (Abeta) as well as 42-1 (reverse amyloid beta; revAbeta) were obtained from Bachem (Weil am Rhein, Germany). The preparation of Abeta was performed as previously described (Stine et al., 2003). Briefly, 1 mg of peptide was dissolved in 200 μL 1,1,1,3,3,3-hexafluoro-2-propanol, and aliquots evaporated under vacuum for 45 min. The dried film was solubilised in 2 μl dimethyl sulfoxide (DMSO) and, thereafter, diluted in 98 μl HCl (0.01 N) to obtain a working solution of 100 μM. The solution was mixed for 30 s and incubated at 37 °C for 24 h to obtain fibrillary structures.

Cell culture and treatment

Cell culture was performed as described previously (Zimmermann et al., 2004). Briefly, human SH-SY5Y neuroblastoma cells were cultured in minimum essential medium (MEM) containing 10% foetal bovine serum (FBS; PAA, Cölbe, Germany), 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin. 400,000 cells were seeded on 60-mm tissue culture plates, and 24 h afterwards 5 μM of all-trans-retinoic acid was added. This treatment was repeated every 2 days for 6 days to achieve cell differentiation. Differentiation was considered completed following suggestions set out by others and as described earlier (Zimmermann et al., 2004), i.e., considering the length of growth cone-terminated neurites versus cell body diameter (Poongodi et al., 2002; Kraveka et al., 2003): In comparison to not differentiated cells the growth cone-terminated neurites of differentiated cells appeared to be up to three times longer than the diameter of the corresponding cell body. Morphological assessment allows for the assumption that differentiation is near completed after 7 days. Eighteen hours prior to adding Abeta/revAbeta (300 nM or 3 μM, unless otherwise stated), cells were deprived of FBS. Serum was omitted throughout the entire period of peptide application. Cells were treated in the presence of either vehicle (2 μL DMSO plus 98 μL 0.01 N HCl) or Abeta/revAbeta for 1, 4, and 24 h, unless otherwise noted. Vehicle-treated controls were taken at every time point, i.e., Abeta treatment was controlled for both vehicle and peptide amino acid sequence. Fig. 1 gives an overview of all treatment conditions and parameters assessed in this study.

Protein evaluation, SDS–PAGE and Western blot analysis

Cell pellets, as obtained after harvesting in phosphate-buffered saline (PBS) and 5-min centrifugation at 1000*g* and 4 °C, were subjected to the following procedure: to determine AChE,