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Estrogen protects neuronal cells from the cytotoxicity induced by acetylcholinesterase-amyloid complexes

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Abstract The senile plaques present in Alzheimer's disease (AD) are composed of a core of amyloid β-peptide (Aβ) plus several proteins including acetylcholinesterase (AChE). Recently we found that AChE forms complexes with the Aβ peptide in vitro and that these are more cytotoxic than Aβ fibrils alone. Considering that estrogen has been reported to act as a protective agent against Aβ-induced cytotoxicity, the effect of 17β-estradiol was studied in rat pheochromocytoma (PC12) and mouse neuroblastoma (Neuro 2a) cells exposed to either Aβ alone or AChE-Aβ complexes. Estrogen showed a powerful protective effect in response to the challenge of AChE-Aβ complexes as well as with Aβ fibrils. This was also the case for other cytotoxic agents such as glutamate and H_2O_2. Our results suggest a common mechanism for cellular protection by estrogen against the toxicity of both Aβ fibrils and AChE-Aβ complexes, likely avoiding the free radical apoptotic pathway.

Key words: Alzheimer's disease; Amyloid β-protein; Acetylcholinesterase; Estrogen; Neurotoxicity; PC12 cell

1. Introduction

Alzheimer's disease (AD), one of the most common neurodegenerative diseases is clinically characterized by the gradual and progressive loss of cognitive and psychomotor abilities [1]. Recent studies have provided evidence that a lack of the steroid hormone estrogen (estradiol) in postmenopausal women could be a relevant factor in AD pathogenesis [2]. Women who received estrogen replacement therapy (ERT) showed a reduced risk of developing AD [3]. In addition, studies among older women who already had AD showed that ERT was beneficial and decreased the symptoms of the disease [4]. One of the hallmarks of AD is the deposit of amyloid β-peptide (Aβ) as fibril aggregates which form senile plaques [5,6]. Several studies with brains displaying AD lesions have shown that changes occur in the expression and distribution of acetylcholinesterase (AChE), an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine [7]. Furthermore, most of the cortical AChE activity present in AD brains has been found associated with senile plaques, where it co-localizes with Aβ deposits [8]. Moreover AChE promotes the aggregation of Aβ in amyloid fibrils [9] and forms a complex with the Aβ [10] which increases its cytotoxicity [11].

The fact that estrogen was found to significantly increase the neurite outgrowth of AChE-positive fibers from embryonic basal forebrain tissues [12] indicates that this steroid may have a direct trophic effect upon basal forebrain cholinergic neurons. There is also evidence suggesting that the cytotoxicity of Aβ might be mediated by hydrogen peroxide (H_2O_2), thus linking cell death with oxidative stress [13]. Moreover estrogen has been reported to act as a protective agent against Aβ-induced cytotoxicity [14]. In view of our recent finding regarding the enhanced neurotoxicity of amyloid fibrils formed in the presence of AChE [11], we decided to study the protective effect of estrogen in the cytotoxicity caused by the AChE-Aβ complexes in neuronal rat pheochromocytoma (PC12) and mouse neuroblastoma (Neuro 2a) cells.

2. Materials and methods

2.1. Materials

Synthetic peptide $A\beta_{1-40}$ corresponding to residues 1–40 of the human wild-type sequence was purchased from Chiron, Emoryville, CA, USA. 17β-Estradiol was obtained from Sigma (St. Louis, MO, USA).

2.2. Cell lines

PC12 cells [15] were grown on collagen-coated dishes in phenol red-free RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with charcoal-stripped (steroid deficient) sera, namely 10% horse serum and 5% fetal bovine serum, and 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were differentiated with NGF (100 ng/ml) in serum-free RPMI without phenol red for 5 days. The differentiation medium was changed by fresh medium every 48 h [16]. Neuro 2a cells [17] were grown in phenol red-free DMEM medium (Gibco-BRL) supplemented with 5% charcoal-stripped fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin [18]. Neuro 2a cells were differentiated by *N*,2′-*O*-dibutyryladenosine-3′-5′-cyclic-monophosphate (5 mM) in phenol red-free DMEM plus fetal bovine serum (charcoal-stripped serum). The use of lipid-depleted serum in the medium allows only a partial differentiation of Neuro 2a cells.

2.3. Purification of brain AChE

The tetrameric G_4 AChE form (sedimentation coefficient 10.7 S) was purified from bovine caudate nucleus, using an acridine-affinity chromatography as described previously [19].

2.4. Formation of AChE-Aβ complexes in vitro

$A\beta_{1-40}$ peptide was incubated in the absence or presence of AChE under stirred conditions as previously described [10]. Aliquots of peptide stock (70 nmol in 20 μl of dimethyl sulfoxide) were added to phosphate buffer saline (725 μl total volume; pH 7.4). For aggregation experiments with AChE, aliquots of peptide stock were added to buffer containing AChE (100 nM). The solutions were stirred continuously (210 rpm) at room temperature for 48 h [11], then preformed fibrils were washed four times with phosphate buffer saline (PBS) at 14 000 rpm for 30 min to remove remnant soluble Aβ and AChE. The pellets were resuspended in 100 μl PBS and aliquots of 10 μl were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to quantify the concentration of Aβ peptide contained in the fibrils relative to Aβ controls of known concentration by densitometric scanning. Densitometric data were processed using the GS365W program from Hoefer Scientific Instruments (San Francisco, CA, USA).

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Abbreviations: AChE, acetylcholinesterase; Aβ, amyloid β-peptide; AD, Alzheimer's disease

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2.5. Cytotoxicity assays

Cells were seeded in 96-well plates in serum-free medium at a density of 4×10^3 cells/100 μl/ well. AChE-Aβ complexes and Aβ fibrils were added to the wells at a final concentration of 10 μM. 17β-Estradiol, glutamate, H_2O_2, and cholesterol were added at different concentrations using PBS for the respective controls. Cells were incubated for 4 h at 37°C, and viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method [20]. Eleven μl MTT stock solution (5 mg/ml) were added to each well. The reactions were stopped by the addition of 120 μl stop solution: 50% dimethyl-formamide and 20% SDS at pH 7.4. Plates were incubated overnight and MTT reduction was determined in a Labsystem Uniskam I spectrophotometer (Finland) at 540 and 650 nm. Results are expressed as a percentage of control values as described previously [21,22].

3. Results

3.1. Protection of estrogen against the toxicity of glutamate and H_2O_2 in PC12 cells

To evaluate whether estrogen protects PC12 cells against the oxidative damage caused by cytotoxic agents, we first used glutamate and H_2O_2 as positive controls. Fig. 1 shows the decrease in MTT reduction observed in PC12 cells as a function of increasing concentrations of glutamate (Fig. 1A) and H_2O_2 (Fig. 1B). When 10^{-7} M 17β-estradiol was added, cell viability clearly increased in comparison with cells in the absence of the hormone. It is clear from these results that 17β-estradiol protects cells from the damage caused by agents which generate free radical agents.

3.2. Protection of estrogen against the toxicity of Aβ-peptide and AChE-Aβ complexes

Evidence suggests that the toxicity of Aβ could be mediated by free radicals [23]. When cell survival was measured in PC12 cells incubated with either the Aβ peptide or AChE-Aβ complexes, it decreased considerably, in comparison with control cells (Fig. 2A). Moreover, AChE-Aβ complexes were found to be more cytotoxic than Aβ alone, as reported previously [11]. However, either cytotoxicity was partially reversed by the addition of 10^{-7} M 17β-estradiol, although the protective effect of estrogen was more apparent in the case of the AChE-Aβ complex. To ensure that this protection was due to the direct effect of estrogen upon PC12 cells and/or to a free radical scavenger action of the hormone, similar experiments were carried out using the steroidal-hormone precursor cholesterol instead of 17β-estradiol. Under these conditions, no protection against cell damage due to the presence of either Aβ or AChE-Aβ was observed (Fig. 2B). Then the morphology of NGF-treated PC12 cells was studied after incubation with AChE-Aβ complexes alone or in the presence of estrogen. Control differentiated cells show several well developed neurites, with nerve processes connecting groups of cells (Fig. 3A). PC12 cells exposed to the complexes showed an almost complete disappearance of neuronal processes and some cells did not look healthy (Fig. 3B, E), whereas cells incubated with AChE-Aβ complexes plus estrogen showed a normal morphology with neurites well preserved (Fig. 3C, F, see arrows) as observed in control cells (Fig. 3A, D).

The protective effect of estrogen on Neuro 2a cells, a rat neuroblastoma cell line, was also studied. As shown in Fig. 4, cell survival decreased markedly when cells were incubated with either the Aβ peptide or AChE-Aβ complexes in comparison to control cells. Cytotoxicity was particularly clear in the presence of esterase-amyloid complexes. As in the case of PC12 cells, the percentage of neuroblastoma survival rose greatly when 17β-estradiol was added to the cells. The protection conferred by estrogen was also apparent at a morphological level. Control Neuro 2a cells, partially differentiated, show short developed neurites (Fig. 5A, D, see arrows). How-

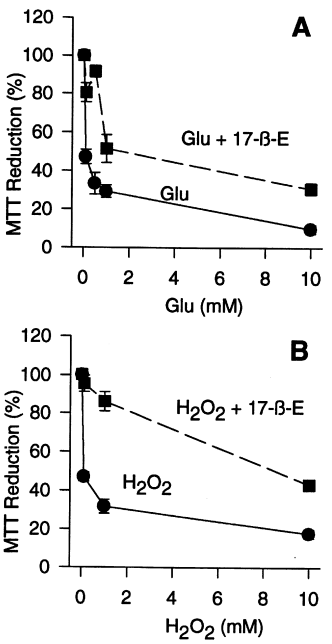

Fig. 1. The effect of 17β-estradiol (10^{-7} M) was studied in PC12 cells treated for 4 h with increasing concentrations of either glutamate (A) or H_2O_2 (B). Inhibition of MTT reduction in PC12 cells was used as a measure of cell viability. MTT reduction assay values are expressed as percentage of control values. Data are mean ± S.E.M. values of 3 independent experiments performed in triplicate.

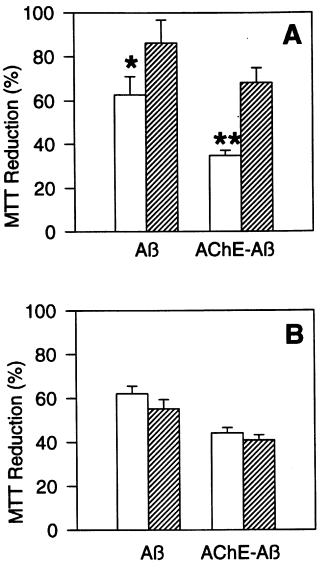

Fig. 2. The protective effect of 17β-estradiol (10^{-7} M) on the cytotoxicity produced by Aβ fibrils added to a final concentration of 10 μM and AChE-Aβ complexes was studied in PC12 cells (A). Controls with cholesterol (10^{-7} M) were performed under the same conditions (B). White bars represent untreated cells whereas grey bars represent cells treated with 17β-estradiol or cholesterol. MTT reduction assay values are expressed as percentage of control values. Data are mean ± S.E.M. values of 3 independent experiments performed in triplicate. $^*P<0.05$; $^{**}P<0.005$.

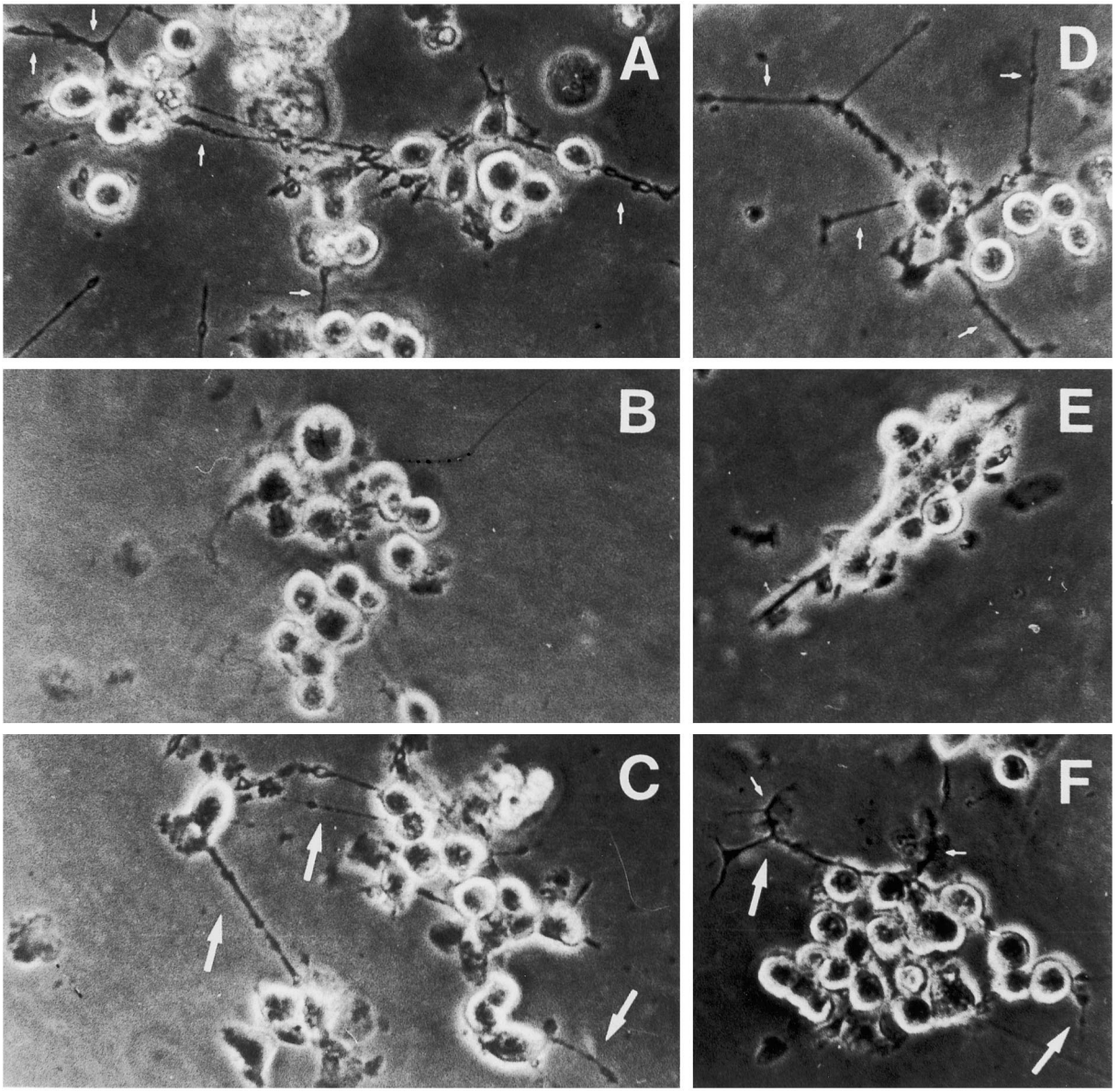

Fig. 3. Estrogen protects NGF-differentiated PC12 cells from the toxic effect of AChE-Aβ. Control cells were seeded at 4000 cells/well and incubated for 4 h (A, D), with 10 μM AChE-Aβ complexes alone (B, E) or with 10^{-7} M 17β-estradiol (C, F). Arrows in A, C, D, F indicate nerve processes. Pictures were taken with a Nikon Optiphot light microscope at 4000× magnification.

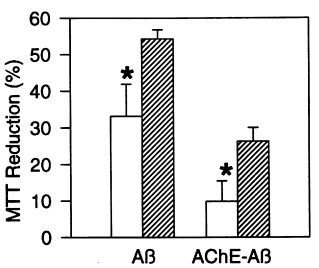

Fig. 4. The protective effect of 17β-estradiol (10^{-7} M) was tested in Neuro 2a cells exposed to Aβ fibrils and AChE-Aβ complexes. Fibrils were added to a final concentration of 10 μM. MTT reduction values are expressed as percentage of control values. Data represent mean ± S.E.M. values of 3 independent experiments performed in triplicate. $^*P < 0.05$.

ever, when cells were exposed to the AChE-Aβ complexes such nerve processes disappeared and cell death was apparent (Fig. 5B, E), whereas in cells incubated with the complexes plus estrogen short neurites are preserved and cells look normal (Fig. 5C, F, arrows, see a long nerve process in F).

4. Discussion

Brains displaying AD lesions present changes in the expression and distribution of AChE [7,24] which is known to colocalize with Aβ deposits, likely forming AChE-Aβ complexes [11]. We have compared the cytotoxicity of the Aβ peptide alone and AChE-Aβ complexes in PC12 and Neuro 2a cells. In both cell types, cell survival was decreased in comparison with control values, particularly in the case of AChE-Aβ complexes, confirming previous results obtained in chick retina

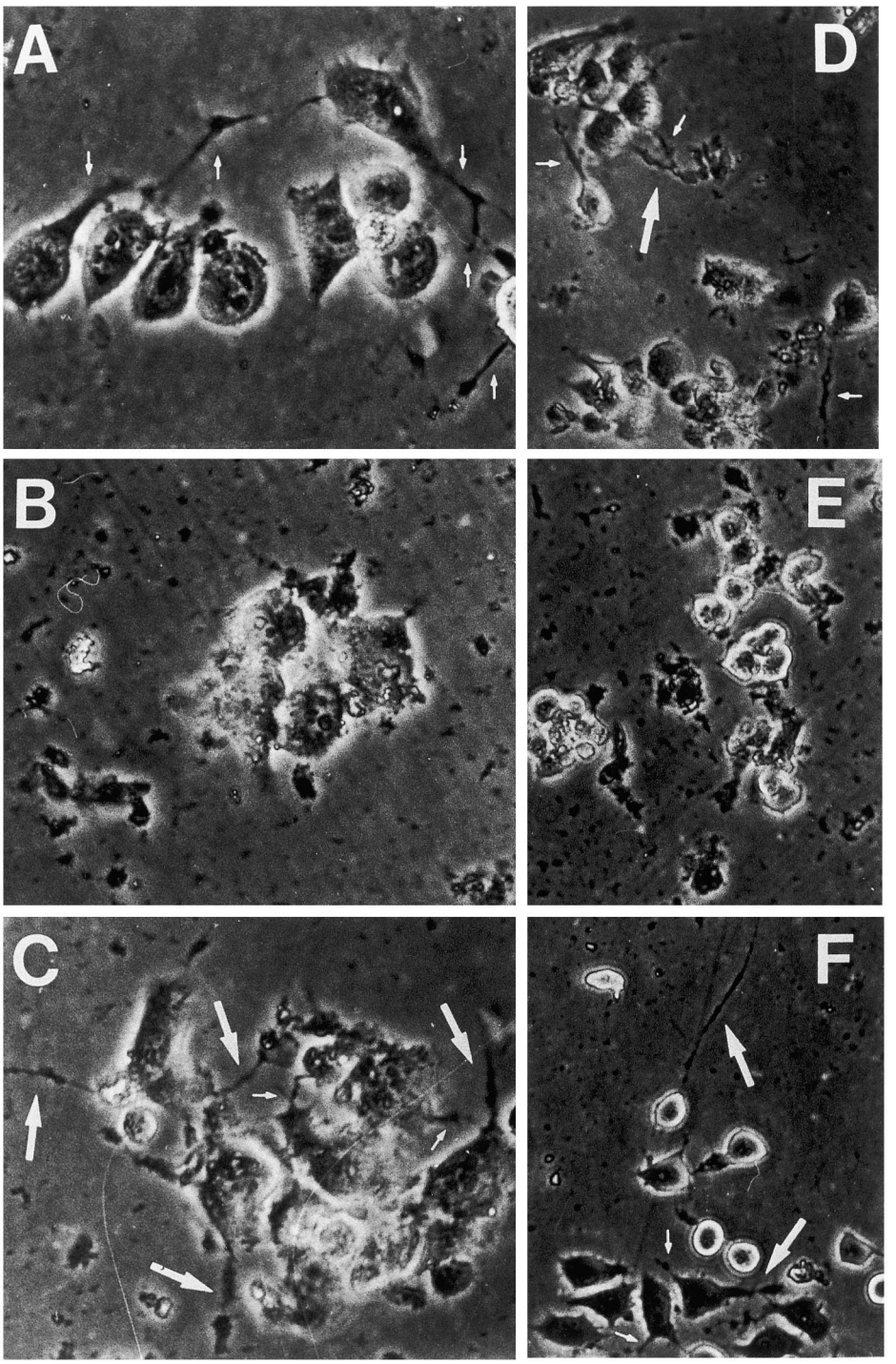

Fig. 5. Estrogen protects cAMP-partially differentiated Neuro 2a cells from the toxic effect of AChE-Aβ. Cells were seeded at 4000 cells/well and incubated for 4 h (A, D) with 10 μM AChE-Aβ complexes alone (B, E) or with 10^{-7} M 17β-estradiol (C, F). Arrows in A, C, D, F indicate the presence of short nerve processes. Note in F a long neurite coming from an estrogen plus AChE-Aβ treated cell. Examination with a Nikon Optiphot light microscope at 5500× (A, B, C) and 3000× (D, E, F) magnification.

cultures [11]. In both cases, 17β-estradiol was able to protect the neuronal cells from the AChE-Aβ toxicity. At the morphological level, the results were very clear for NGF-differentiated PC12 cells. In the case of Neuro 2a cells it was difficult to achieve extensive neuronal differentiation in the presence of delipidated serum; however, despite this problem estrogen protection was also apparent. AChE is likely to become incorporated into nascent Aβ fibers and probably increases Aβ toxicity by acting upon the aggregation of the peptide [9–11,25].

Moreover, the formation of an AChE-Aβ complex may involve high free-radical production, thus explaining its increased toxicity in neuronal cells. There is evidence suggesting that the accumulation of reactive oxygen species in response to Aβ appears to play an important role in the neurodegenerative process which affects neurons in the senile plaques of

AD [26,27]. In this context, it is possible that the association of AChE with the Aβ peptide during fibril formation in senile plaques promotes a higher free-radical generation than that observed in the presence of the Aβ peptide alone and thus enhances oxidative stress. The results presented in this study did not establish the specific mechanism whereby 17β-estradiol protects neuronal cells against AChE-Aβ complex cytotoxicity, although an antioxidant activity is likely, given the comparable results obtained with free-radical producing agents such as hydrogen peroxide and glutamate. On the other hand, cholesterol, the steroidal precursor of estradiol, did not protect cells from the toxicity induced by Aβ or AChE-Aβ complexes, suggesting that the protective effect observed for estrogen is specific for the anti-oxidant function of this hormone. In conclusion, we have demonstrated that 17β-estradiol has a potent neuroprotective capacity in both PC12 and Neuro 2a cells exposed to either Aβ or AChE-Aβ complexes. Furthermore, our findings support the idea that estrogen therapy could be beneficial in delaying the onset and/or slowing the course of the neurodegenerative process in Alzheimer's disease.

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