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Original Article

Age-related changes in membrane fluidity and fluorescence intensity by tachykinin neuropeptide NKB and Aβ (25-35) with 17β estradiol in female rat brain

Rashmi Jha[1,2] Abbas Ali Mahdi[2] Shivani Pandey[2] Najma Z Baquer[1] and Sudha M Cowsik[1]*
[1]*School of Life Sciences, Jawaharlal Nehru University, New Delhi, India*
[2]*Department of Biochemistry, King George's Medical University, Lucknow, Uttar Pradesh, India*

Abstract. Changes in the fluidity of membrane lipids are known to occur during aging and by lipid peroxidation. It is well documented that the fluidity state of the lipid phase in a membrane is important for the activity of intrinsic membrane proteins. Oxidants and fluidity of membrane lipids play a significant role in aging and age related neurodegenerative diseases. The aim of the present study was to determine the effect of tachykinin neuropeptide, Neurokinin B (NKB) and Amyloid beta fragment Aβ (25-35) on 17β estradiol (E2) treated aging female rat synaptosomes of different age groups. Aging brain functions were measured by membrane fluidity and fluorescent intensity with neuropeptides. An in-vitro incubation of Aβ (25-35) in E2 treated brain synaptosomes showed toxic effects on all the parameters. These effects of aging and Aβ (25–35) on membrane fluidity were restored by NKB and combined NKB and Aβ (25–35) with E2. Furthermore, we measured the Tryptophan (Trp) fluorescence to monitor changes in proteins and to make inferences regarding structure and dynamics. Trp is a sensitive marker of protein oxidation and its fluorescence significantly increased in E2 treated synaptosomes of aging rats. Furthermore, to evaluate the effect of oxidative stress on the membrane and protein conformation, fluorescent probe 1-Anilino-8-Naphthalenesulfonate (ANS) were used. An increase in ANS fluorescence in E2 treated synaptosomes of aging rats indicated that E2 is associated with significant conformational changes and surface hydrophobicity of membranes and proteins.

Keywords: Aging, neurokinin B, amyloid beta (23-35), estradiol

Introduction

Aging is defined as a universal, progressive and deleterious process occurring in cells and tissues, affecting most of the living organisms. During aging, most organs and systems undergo a gradual loss of physiological function usually associated to the imbalance of redox status and alterations in cellular signaling pathways [1]. The free radical or "oxidative stress" theory holds that oxidative reactions are the factors underlying these changes [1]. Highly reactive oxygen species (ROS) cause a wide spectrum of cell damage, including lipid peroxidation, inactivation of enzymes, alteration of intracellular oxidation–reduction state, and DNA damage in the aging brain [2].

The ovarian steroid hormone estradiol (E2) is one of the most important hormones and it can protect neurons against Aβ toxicity, oxidative stress and excitotoxicity [3-5]. Most of the studies showed that E2 is neuroprotective in neurodegenerative disorders such as stroke, Alzheimer disease (AD) and Parkinson disease (PD) [6]. The effect of E2 is primarily mediated by ERα and ERβ which are members of the nuclear receptor superfamily of ligand-activated transcription factors [7]. E2 modulates multiple functions of the brain, via activation of ERα and ERβ including development, cognition and memory [8] highlighting its protective effects against neuronal damage [9].

Mammalian tachykinins comprise a family of regulatory peptides including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) [10, 11]. They are known to reduce oxidative stress in the brain [12-14]; to reverse the neurotoxic effects of Aβ in neurons and play a role in neurodegenerative diseases [15, 16]. NKB has biological importance such as regulatory role in pre-eclampsia [17], neuroprotective agent [16, 18 and 19] and as a potential antioxidant molecule [12, 13]. Several studies have proposed that Aβ binds to the cell surface via direct membrane interactions, thereby initiating both neurotoxicity and plaque formation. Recently, it was demonstrated that Aβ deposition is initiated in a plasma membrane-bound form, resulting in diffuse plaque formation [20].

Many small peptides are able to exist in dynamic equilibria between unfolded and folded structures, depend-

* Corresponding author: Sudha Mahajan Cowsik (scowsik@yahoo.com).

ing on the solvent polarity and their interaction with the membrane phase [21, 22]. This is known for a variety of neurotransmitter, peptides and hormones for which the importance of the ordered structures has been recognized in relation to binding to G protein- coupled membrane receptors [23]. Furthermore, when small peptides permeate the membrane to fulfill physiological requirement, a certain folding may be required for a favorable interaction with the lipid moiety [24]. Decreases in membrane fluidity could hamper the functioning of cell surface receptors and ion channel protein; such decreases have been associated with cellular toxicity [25]. The fluidity parameters of synaptosomal membranes are linked to neuronal signal transduction pathways, channels and enzymes [26]. Changes in the fluidity of membrane lipids are known to occur during aging and by lipid peroxidation. It was evidenced that the fluidity state of the lipid phase in a membrane is important for the activity of intrinsic membrane proteins.

Fluorescence is the result of the three-stage process that occurs in a particular molecule called fluorophores. A fluorescent probe is a fluorophore designed to localize within a region of a biological specimen or to respond to a stimulus. There are three amino acids with intrinsic fluorescence properties, Phenylamine (Phe), Tryptophan (Trp), Tyrosine (Tyr), but only Tyr and Trp are used experimentally because their quantum yield (emitted photons/excited photons) is high enough to give a good fluorescence signal. In a hydrophobic environment, Trp has high quantum yield and fluorescence intensity. In contrast, in a hydrophilic environment its quantum yield decreases leading to low fluorescence intensity. For Trp residue, there is a strong stoke shift dependent on the solvent, meaning that the maximum emission wavelength of Trp depending on the Trp environment. 1-Anilino-8-Naphthalenesulfonate *(*ANS), an amphipathic dye, with hydrophobic naphthalene and phenyl groups and a charged sulfonate group, is frequently used for the investigation of equilibrium, and kinetic protein folding intermediates [27, 28].

E2 treatment had beneficial effects on antioxidant enzymes in aging rat tissues as reported earlier by Jha et al 2013 and Baquer et al 2009 [2, 29]. In the present study, we examine the neuroprotective effect of NKB and E2 against Aβ (25-35) toxicity on the membrane fluidity and fluorescence intensity of the brain synaptosomes of aging female rats.

Materials and methods

Animals

The present study was conducted on female albino rats of the Wistar strain in different age groups (3, 12 and 24 months). Animals were maintained in the animal house facility of Jawaharlal Nehru University (JNU), New Delhi, India at a constant temperature of 25˚ C, humidity 55% and 12h dark and light cycle. The animals were fed standard chow rat feed (Hindustan Leaver Ltd., India) and given tap water until the time of sacrifice. The Institutional Animal Ethics Committee (IAEC) of JNU approved all the animal experiments; all institutional guidelines for care of animals were followed.

Hormone administration

Subcutaneous injections of E2 (0.1 µg/g body weight) were given daily for one month, to the aged rats (12 and 24 months old; n=8 for each group). E2 was dissolved in propylene glycol in appropriate concentrations [30]. Control animals received an equal volume of vehicle. There was no treatment on the day of the sacrifice. Animals of all the groups were sacrificed and brains were isolated for further study.

Preparation of synaptosomes

The animals from control and E2 treated groups were sacrificed by cervical dislocation. The whole brain was excised and washed in ice-cold saline (0.9 % NaCl). Tissue homogenates were prepared as described by Mayanil et al 1982 [31]. Tissues were soaked, dried on blotting paper and weighed, minced and homogenized in nine volumes of homogenizing buffer containing 0.25 M sucrose, 0.02 M triethanolamine (pH 7.4) and 0.12mM dithiothreitol. The pellet obtained after centrifugation at 12,000 (rpm) containing synaptosomes and mitochondria were taken for the present study. The whole procedure was carried out at 4˚C.

Treatment of synaptosomes with NKB and Aβ (25-35)

Each sample containing ~100 µg protein of isolated rat brain synaptosome was incubated with NKB, Aβ (25–35) and NKB+ Aβ (25–35) in microfuge tubes at 37˚C for 60 min in a shaking water bath with 0.1, 1 and 5 µM concentration of each of the peptides. All incubations were performed in four combinations; control (without any peptide), Aβ (25–35), NKB and NKB+Aβ (25–35) in three age groups of control and E2 treated rats at three peptide concentrations.

Measurement of fluorescence Anisotropy

The synaptosomes prepared from the rat brains of different age groups were diluted in 50mM Tris-HCl, to a protein concentration of ~100µg. 1ml of synaptosomal membrane was mixed with 1, 6-diphenyl-1, 3, 5-hexatriene (DPH), a fluorescent probe and the mixture was incubated at 37°C for 30 min and fluorescence intensity was recorded using an excitation wavelength of 365nm and emission wavelength of 428nm [32]. Polarization (p) measurements were carried out on polarization spectrofluorometer as described by Mantha et al. 2006 [13].

Fluorescence Measurement

The fluorescence intensity was measured in the synaptosomes prepared from rat brains of different age groups. Fluorescence measurements were performed in a solution containing 100µg proteins per ml, 10mmol.1 HEPES, 100 mmol/KCl (pH 7.0) at room temperature using Shimadzu RF 540 spectrofluorimeter. The fluorescence emission spectra (from 300 to 450 nm, 5nm slit width) of Trp were measured by excitation at 280nm

(2nm slit width) [33, 34]. ANS fluorescence was measured following 15min incubation of the ANS probe with synaptosomes. The excitation and emission wavelength for ANS measurement were 365 and 480nm, respectively (5nm slit width) [33].

Protein Estimation

Protein was estimated in the synaptosomes by the method of Bradford, 1976 [35] using bovine serum albumin (BSA) as standard.

Statistical Analysis

Data have been presented as mean ± standard error of mean (SEM). The data were analyzed using one way ANOVA to test for differences between different treatments at different age groups. Differences between the means of the individual groups were assessed by Dunnett's multiple comparisons test. A value of $p < 0.05$ was considered to be statistically significant.

Chemicals

All substrates, standards, NKB and Aβ (25–35) peptide fragment were purchased from Sigma Chemicals Company, USA. All other chemicals were of analytical grade and purchased from SRL and Qualigens, India.

Results

Membrane fluidity or Fluorescence Anisotropy

Fluorescence anisotropy is inversely proportional to the membrane fluidity. The changes in the anisotropy (r) monitored by using DPH probe for membrane fluidity were measured in rat brain synaptosomes, in different age groups with and without E2 treatment, at different concentration Aβ (25–35), NKB and NKB+ Aβ (25–35). The results are shown in Figure 1 (A), (B), and (C).

Effect of E2 and varying concentration of Aβ (25-35) on membrane fluidity

The membrane fluidity was found to be decreased in the synaptosomes of control rats (E2 untreated), when incubated with different concentrations of Aβ (25-35). However, this decrease in the activity was less in E2 treated rat brain synaptosomes. In the synaptosomes of E2 treated 12 and 24 month rats, the membrane fluidity decreased with incubation of Aβ (25-35) at 5µM concentration ($p<0.01$ and $p<0.001$). Results are shown in Figure 1 (A).

Effect of E2 and varying concentration of NKB on membrane fluidity

The membrane fluidity was observed to increase in all control age groups with NKB incubation, whereas this increase in membrane fluidity was more significant in E2 treated rats, as compared to age matched control group. The membrane fluidity in the synaptosomes of E2 treated 12 month rats showed an increase when treated with the concentration of 5µM of NKB ($p<0.01$). There was a significant increase in membrane fluidity in the synaptosomes of E2 treated 24 months rats with the incubation of 5µM concentration of NKB ($p<0.001$). Results are shown in Figure 1(B).

Effect of E2 and varying concentration of combined NKB and Aβ (25-35) on membrane fluidity

The membrane fluidity in control synaptosomes (E2 untreated) was observed to increase when treated with a combination of NKB and Aβ (25-35), but this increase was more significant in synaptosomes of E2 treated 12 and 24 months aging rats. There was a significant increase of membrane fluidity in the synaptosomes of 12 months E2 treated rats, with the combined dose of 5µM concentration of NKB and Aβ (25-35) ($p<0.001$). The combined dose of NKB and Aβ (25 - 35) at 5µM concentration in 24-month E2 treated rats showed a significantly raised level in membrane fluidity activity as compared to matched control ($p < 0.001$). Results are shown in Figure 1 (C).

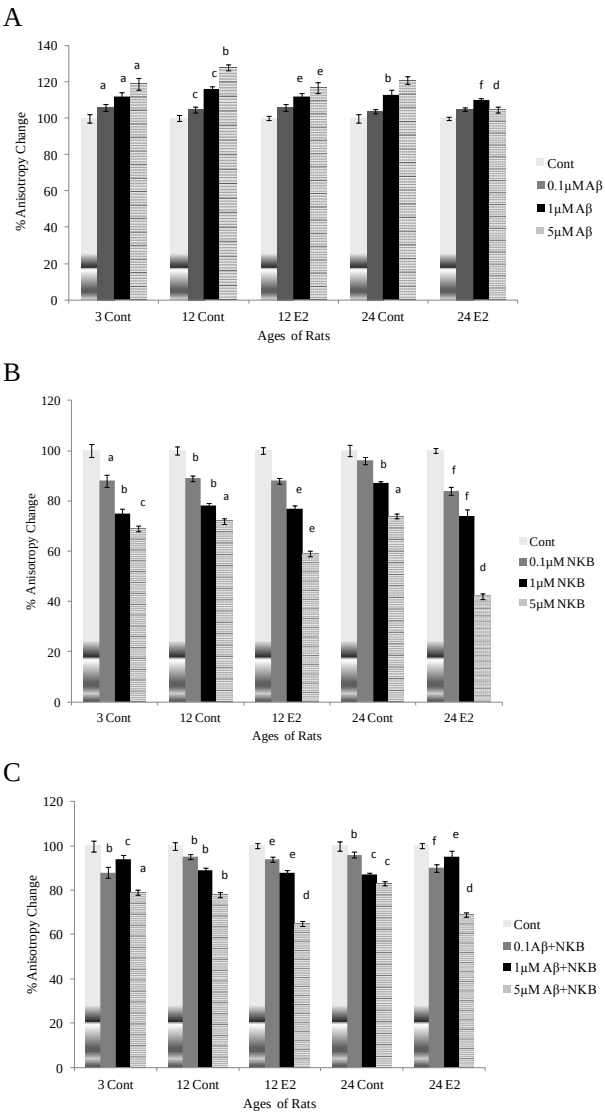

Figure 1: Percentage changes in anisotropy in the synaptosomes of 3, 12 and 24 months control (Cont) and estradiol (E2) treated aging female rats in presence of (A) Aβ (25-35) (B) NKB and (C) NKB+Aβ (25-35). Peptide concentrations are 0.1, 1.0 and 5.0 µM. Statistical significance: [a]$p<0.001$, [b]$p<0.01$, [c]$p<0.05$ comparing age matched control (untreated) versus peptide treated; [d]$p<0.001$, [e]$p<0.01$, [f]$p<0.05$ comparing E2 treated versus peptide treated.

Fluorescence Measurement

The Trp fluorescence was used as a sensitive marker of protein oxidation. Trp fluorescence increased significantly in E2 treated synaptosomes of aging rats. A fluorescent probe ANS was used to evaluate the effect of oxidative stress on the membrane and protein conformation. A change in ANS fluorescence in E2 treated synaptosomes of aging rats indicated that E2 treatment is associated with significant conformational cha nges and surface hydrophobicity of membranes and proteins. Results are shown in Figure 2 (A) and (B).

Fluorescence intensity of Trp in control and E2 treated rat synaptosomes

The Trp fluorescence of control and E2 treated rats is observed to be significantly changed. The level of tryptophan fluorescence intensity increased 76% ($p<0.001$), 87% ($p<0.001$), 81% ($p<0.01$) and 90% ($p<0.01$) in 12 month control, 12 month E2 treated, 24 months control and E2 treated rats respectively when compared with 3 month young rats. Results are shown in Figure 2 (A).

Fluorescence intensity of ANS in control and E2 treated rat synaptosomes

The ANS fluorescence in synaptosomes of aging rats was observed to be significantly changed when compared with synaptosomes from young rats. The fluorescent intensity of ANS probe increased 65% ($p<0.01$), 85% ($p<0.01$), 72% ($p<0.05$) and 88% ($p<0.01$) in 12 month control, 12 months E2 treated, 24 months control and E2 treated rats respectively when compared with 3 month young rats. Results are shown in Figure 2 (B).

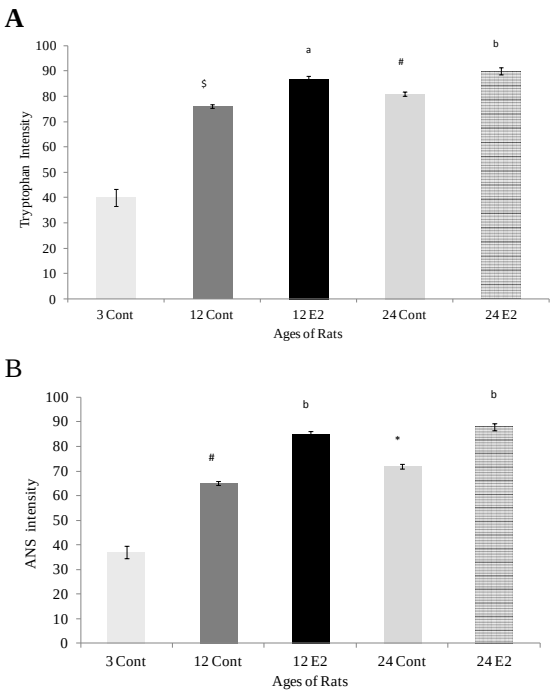

Figure 2: Percentage changes in the fluorescence intensity in synaptosomes of 3, 12 and 24 months control (Cont) and estradiol (E2) treated aging female rats in presence of (A) Tryptophan intensity (B) ANS Intensity. Stastical significane: $^{a}p<0.001$, $^{b}p<0.01$, $^{c}p<0.05$ comparising age matched control verses E2 treatment, $^{\$}p<0.001$, $^{\#}p<0.01$, $^{*}p<0.05$ verses 3 months.

Discussion

Changes in the fluidity of membrane lipids are known to occur during aging and lipid peroxidation. It has been observed that the fluidity state of the lipid phase in a membrane is important for the activity of intrinsic membrane proteins. The fluorescence (polarization) anisotropy of membrane-bound DPH, which is inversely correlated with membrane fluidity, was measured in isolated rat brain synaptosomes at different ages. Results showed a significant decrease in membrane fluidity as a function of age as seen earlier by Muller et al, 2001 [36].

The membrane fluidity was measured at different concentrations of NKB, Aβ (25-35) and combined peptide NKB and Aβ (25-35) in different age groups of control (without E2 treated) and E2 treated rats. In the present study, when we examined the effects of Aβ (25-35) on rat brain synaptosomes of various age group of control (without E2 treated) rats, there was a decrease in membrane fluidity. This result seems to be significant since it has been shown that brain aging enhances amyloid neurotoxicity in a concentration dependent manner for different age groups studied [37]. Aβ (25-35) showed less reduction in fluidity in the synaptosomes of E2 treated rats as compared to age matched control rats. This result suggested that neurotoxic effect of Aβ is probably related to the amplifying effect of Aβ on membrane fluidity or the induction of oxidative stress [13]. The changes in fluidity measured in the presence of NKB in control and E2 treated rat brains of different age groups. Incubation of NKB in the synaptosomes of E2 treated showed a significant increase in fluidity as compared to age match control rats.

The combined treatment of NKB and Aβ (25-35) in the synaptosomes of control and E2 treated rats brain showed an increase in fluidity and this increase are more significant in E2 treated rats as compared to age matched control. These results show that interaction NKB with E2 may represent an important contributing factor in aging and its membrane-mediated functions in the brain tissue.

Electrostatic interactions (including hydration) are vital to the structure and function of proteins [38-40]. Fluorescence from the amino acid Trp has long been known to be sensitive to the polarity of its local environment [41-43]. The intensity, quantum yield, and wavelength of maximum fluorescence emission of Trp are very solvent dependent. The fluorescence spectrum shifts to shorter wavelength and the intensity of the fluorescence increases as the polarity of the solvent surrounding the Trp residue decreases. Trp fluorescence wavelength is widely used to monitor changes in proteins and inferences regarding local structure and dynamics.

In this study, we investigated the potential effects of aging on oxidative modifications of proteins in the synaptosomes of control and E2 treated rats of different age group. We measured Trp fluorescence as a sensitive marker of protein oxidation. The progressive increase in Trp content was observed in the synaptosomes of different age group of rats. An increased fluorescence intensity of Trp was observed in E2 treated brain synaptosomes of aging rats. The results of Trp fluorescence measurements indicated that aging is associated with accumulation of

fluorescent products within the brain and support the view that protein modification mediated Trp. ANS is an amphipathic dye, with hydrophobic naphthalene and phenyl groups, and a charged sulfonate group. It is frequently used for the investigation of equilibrium, and kinetic protein folding intermediates [27, 28]. When ANS is bound to a protein in a nonpolar environment, there is a large increase in the fluorescence quantum yield [44]. ANS has been used extensively as a probe for protein folding intermediates, especially molten globules, because their partially structured nature provides access for ANS to bind exposed hydrophobic regions whereas ANS has a very weak affinity for fully unfolded or folded proteins. ANS has also been widely used as a probe for kinetic intermediates in protein folding [27].

To evaluate the effect of oxidative stress on the membrane and protein conformation we used ANS, an anionic probe, for membrane surfaces and protein cavities. An increase in ANS fluorescence in the synaptosomes of aging rats indicates that aging is associated with significant conformational changes, results in the increased surface hydrophobicity of membranes and proteins. The result showed significant changes in ANS fluorescence in the synaptosomes isolated from E2 treated rats. These results support the view that aging is associated with changes in brain tissue. In summary, this study demonstrates age-dependent increases in protein oxidation in brain synaptosomes of E2 treated rats. This observation suggests protein oxidation may contribute to deterioration of protein, cellular and organ function.

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Conflict of Interest

The authors declare that they have no competing interests.

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