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Therapeutic Potential of 17β Estradiol with Tachykinin Neuropeptide NKB and Aβ (25 - 35) on Na^+ - K^+ ATPase Activity in Aging Female Rat Brain

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Abstract

The Na^+ - K^+ ATPase is an enzyme responsible for the active transport of Na^+ and K^+ in most eukaryotic cells. 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 brain synaptosomes of different age groups, by assaying Na^+ - K^+ ATPase enzyme activity. An *in vitro* incubation of isolated synaptosomes with Aβ (25 - 35) showed toxic effects while NKB showed stimulating effect on the Na^+ - K^+ ATPase activity, and the combined NKB + Aβ (25 - 35) incubations showed a partial effect as compared to the Aβ (25 - 35) alone. To understand whether E2 affects the expression of Na^+ - K^+ ATPase molecules, we examined the expression of Na^+ - K^+ ATPase subunit α1 and β2 in E2 treated aging female rat brain synaptosomes. The enzyme was quantified by SDS PAGE in control and E2 treated rat brain. We observed that the expression of α1 and β2 Na^+ - K^+ ATPase molecules increased and reversed to a normal level in E2 treated synaptosomes. These results confirmed that E2 increased turnover of Na^+ - K^+ ATPase molecules in aging rat brain. The present findings also suggest a possible role of NKB with E2 in the age related changes in the brain.

Keywords

Na^+ - K^+ ATPase, Aging, Neurokinin B, Amyloid Beta (25 - 35), Estradiol

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1. Introduction

The brain is vulnerable to oxidative stress, which is associated with age-related brain dysfunction, due to its high content of key compounds for oxidative damage and the relevant scarcity of antioxidant defense systems [1]. Reactive oxygen species (ROS), induced protein damage results in accumulation of misfolded proteins, aggregates something which may cause oxidative stress and increase ROS production [2]-[4]. It is proposed that most of the neurodegenerative disorders like Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and Amyotrophic lateral sclerosis (ALS) result primarily from a loss of trophic peptidergic neurotransmitter, possibly substance P (SP) and related tachykinin neuropeptides [5].

There are multiple isoforms of Na^+ - K^+ ATPase (sodium and potassium adenosine triphosphatase) in the nervous system, three isoforms of α subunit (α1, α2 and α3), and three of the β subunit (β1, β2 and β3). These isoforms exhibit a tissue-specific and developmental pattern of expression that may be important in the maintenance and regulation of Na^+ - K^+ ATPase activity [6]. α subunits contain the active site for ATP hydrolysis and the β subunit required for enzyme assembly [7] [8]. In rat brain, α1 and β2 are found more than any other subunit [9] [10]. The inactivation of Na^+ - K^+ ATPase leads to partial membrane depolarization allowing excessive Ca^{2+} entry inside neurons with resultant toxic events like excitotoxicity [11]. The mechanism of inactivation involves disruption of phospholipid microenvironment of the enzyme or by reactive oxygen radicals [12] [13]. There are reports that the activity of Na^+ - K^+ ATPase declines in the brain of aged animals and that changes in Na^+ - K^+ ATPase activity affect several physiological processes [14] [15].

The ovarian steroid 17β estradiol (E2) hormone is one of the most important hormones, and it can protect neurons against Aβ toxicity, oxidative stress and excitotoxicity [16]-[18]. E2 modulates inflammatory processes in models of human diseases such as arthritis, systemic lupus erythematosus, AD and multiple sclerosis [19] [20]. In females, E2 levels drop abruptly at the time of menopause resulting in a low-grade of systemic inflammation, which can be prevented by chronic treatment with low dose of E2 [21]. E2 has also a neuroprotective action [22] against several toxins that boost production of free radicals including glutamate (which is toxic in high concentrations). Moreover, E2 itself may act as an antioxidant and may help control the onset or progression of AD [23].

Mammalian tachykinins comprise a family of regulatory peptides including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) [24] [25]. They are known to reduce oxidative stress in the brain [26]-[28], and to reverse the neurotoxic effects of Aβ in neurons and a role in neurodegenerative diseases [29] [30]. The SP or related tachykinins exert a neurotrophic action on central neurons and the loss of this input in selective areas results, in the expression of neurodegenerative disorders [31]. The peptides of the tachykinin family have a wide spread distribution in the central and peripheral nervous system and play an important role as excitatory neurotransmitters [32].

The aim of this work was to investigate the effect of different concentrations of NKB with E2 against Aβ (25 - 35) toxicity on the activity of Na^+ - K^+ ATPase in the brain of aging female rats. The quantity of Na^+ - K^+ ATPase molecules was estimated in E2 treated brain of aging female rats.

2. Methods

2.1. Animals

The present study was conducted on female albino rats of Wistar strain in different age groups (3, 12 and 24 months) (n = 8 for each group). 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.

2.2. 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 [33]. 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.

2.3. 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 [34]. 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 0.12 mM dithiothreitol. The pellet obtained after centrifugation at 12,000 (rpm) containing synaptosomes was taken for the present study. The whole procedure was carried out at 4˚C.

2.4. 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.

2.5. Protein Estimation

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

2.6. Measurement of Na^+ - K^+ ATPase Activity

Na^+ - K^+ ATPase activity was measured in the syanptosomes according to the method of Mayanil *et al*. 1982 [34]. The enzyme activity was calculated as the difference of the activity between total ATPase and Mg^{2+} ATPase. The activity of the enzyme is expressed as µmol Pi released/mg protein/min.

2.7. Gel Electrophoresis of Na^+ - K^+ ATPase

The equal amount of estimated protein was mixed with 1X sample buffer (50 mm Tris base, 100 mM dTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol). The protein was subjected to SDS-PAGE on a 12% polyacrylamide gel. The resolving gradient (linear gradient) 12% gel and 5% stacking gel were prepared as described by Towbin and Gordon [36]. Tris-glycine electrophoresis buffer was used for running the gel. The gel electrophoresis revealed bands of 110 and 55 KDa, compatible with the molecular weight of the protein. The intensity of the band for each sample was analyzed by densitometry method.

2.8. 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.

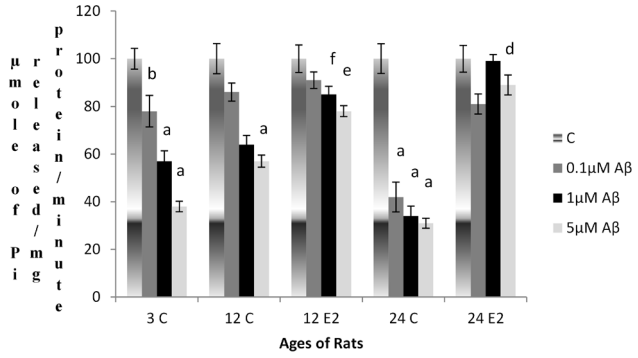
2.9. Chemicals

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.

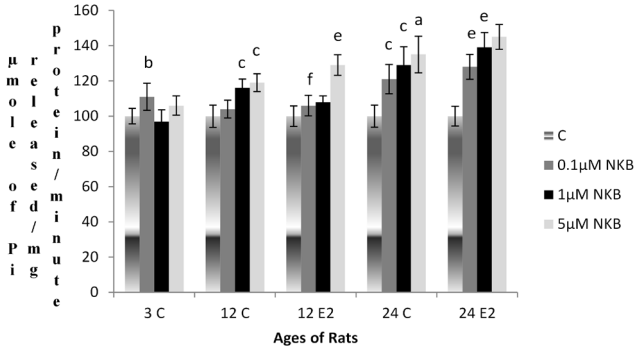
3. Results

3.1. Na^+ - K^+ ATPase

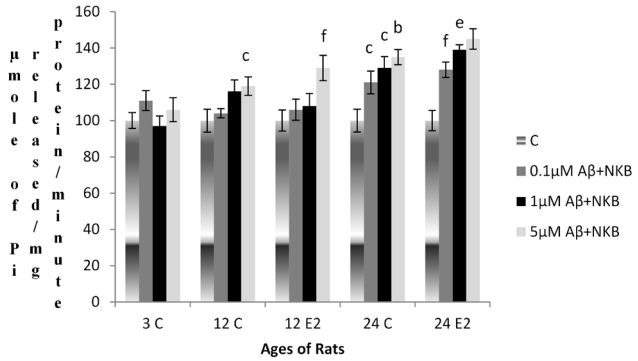
Changes in Na^+ - K^+ ATPase activity were measured in rat brain synaptosomes, in different age groups with and without E2 treatment, at different concentration Aβ (25 - 35), NKB and NKB + Aβ (25 - 35). The Results are shown in **Figures 1(a)-(c)**.



(a)



(b)



(c)

Figure 1. Percentage changes in the Na^+ - K^+ ATPase activity in synaptosomes of 3, 12 and 24 months control (C) and estradiol (E2) treated aging female rats in presence of (a) A*β* (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.

3.1.1. Effect of E2 and Varying Concentrations of A*β* (25 - 35) on Na^+ - K^+ ATPase

The Na^+ - K^+ ATPase activity reduced in the synaptosomes of control rat brain synaptosomes with the incubation of different concentration of A*β* (25 - 35). However, there was less marked decrease in the Na^+ - K^+ ATPase activity, in the synaptosomes of E2 treated rats. In synaptosomes of 12 month E2 treated rats, addition of 1 and 5 µM of A*β* (25 - 35) showed less reduction ($p < 0.05$ and $p < 0.01$) in Na^+- K^+ ATPase activity as compared to age matched control rats. In the synaptosomes of 24 month E2 treated rats, addition of 5µM of A*β* (25 - 35) showed less reduced ($p < 0.001$) Na^+ - K^+ ATPase activity as compared to age matched control rats. Results are shown in **Figure 1(a)**.

3.1.2. Effect of E2 and Varying Concentrations of NKB on Na^+ - K^+ ATPase

The activity of Na^+ - K^+ ATPase increased in all the age groups with NKB incubation. This increase in enzyme activity was more significant in E2 treated rats, with increasing peptide concentration as compared with that of age matched control. The activity increased most significantly with 5 µM of NKB ($p < 0.01$), in the synaptosomes of E2 treated 12 months old rats. Increase in Na^+ - K^+ ATPase activity was highly significant in E2 treated group of 24 months old rat using a dose of 0.1, 1 and 5 µM of NKB ($p < 0.01$, $p < 0.01$ and $p < 0.001$). Results are shown in **Figure 2(b)**.

3.1.3. Effect of E2 and Varying Concentrations of NKB and Aβ (25 - 35) on Na^+ - K^+ ATPase

The changes in Na^+ - K^+ ATPase activity, in control and E2 treated rat brain synaptosomes of different age groups in varying concentration of NKB and Aβ (25 - 35), showed a significant increase in enzyme activity with increasing age from 3 month to 24 month. The Na^+ - K^+ ATPase increased with a combination of NKB and Aβ (25 - 35) in the synaptosomes of control (without E2 treatment) rat brain, but this increase was more significant in synaptosomes of E2 treated 12 and 24 months aging rats. The combined dose of NKB and Aβ (25 - 35) at 5 µM concentrations in 12 month E2 treated rats significantly raised ($p < 0.05$) Na^+ - K^+ ATPase activity as compared to the control of matching age ($p < 0.05$). The combined dose of NKB and Aβ (25 - 35) at 0.1, 1 and 5 µM concentrations in the synaptosomes of 24 month E2 treated rats showed a significant raised in activity as compared to the control of matching age ($p < 0.05$, $p < 0.01$ and $p < 0.001$). Results are shown in **Figure 1(c)**.

3.2. SDS

The age dependent expression of subunit α1 and β2 of Na^+ - K^+ ATPase in the rat brain synaptosomes was analyzed by gel electrophoresis. The bands of α1 and β2 were obtained, and its molecular weight was identified to be of 110,000 and 55,000 KDa. The obtained SDS PAGE results suggest that expression of α1 and β2 in rat brain is age dependent and is differential in expression. The expression of the protein was lower in 3 months old age groups in comparison to older age groups. With an increase in age, the E2 treated 12 months old rat brain synaptosomes showed higher expression of α1 and β2 subunit of Na^+ - K^+ ATPase as compared to any other age group studied. The 24 months old control synaptosomes showed decreased amount of subunits of Na^+ - K^+ ATPase. However in the E2 treated 24 months rats, there were significant increase in α1 and β2 subunit. The gel photograph in **Figure 2** is presented to show the expression of α1 and β2 molecule of Na^+ - K^+ ATPase.

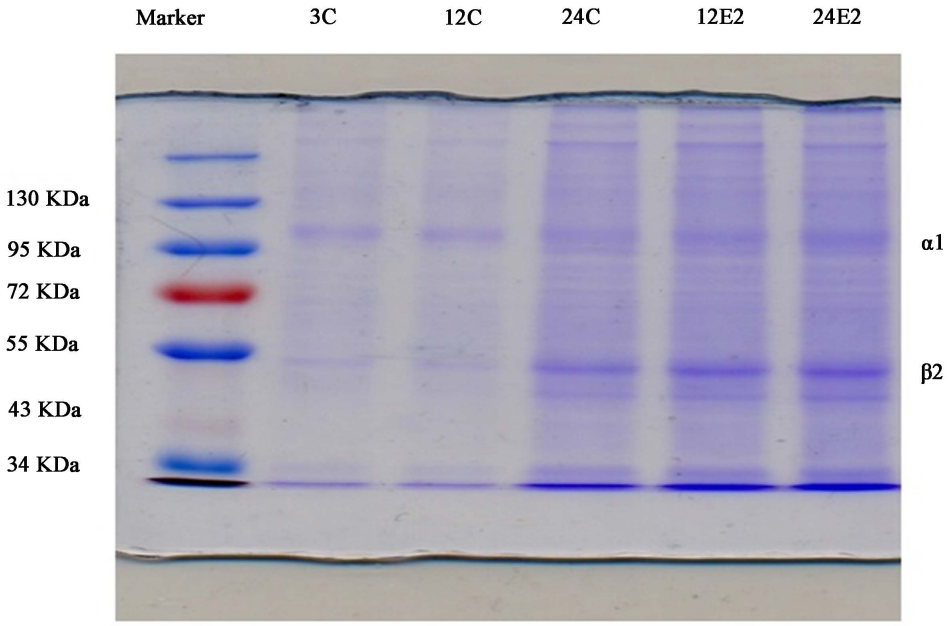


Figure 2. SDS PAGE for the determination of α1 and β2 subunit of Na^+ - K^+ ATPase in control (C) and estradiol (E2) treated aging rat synaptosome.

4. Discussion

The enzyme Na^+ - K^+ ATPase utilizes the energy from ATP hydrolysis to pump out Na^+ from inside the cell and K^+ from outside to cytosol [7]. The pump functions as an antiport and is instrumental in restoring ion-gradients in nerve cells following periods of electrical activity like nerve impulses and synaptic potentials. The energy cost of Na^+ - K^+ ATPase activity is high in the active brain. Energy deficiency and dysfunction of the Na^+ - K^+ ATPase is common consequences of many pathological insults. Na^+ - K^+ ATPase activity in aged rat brains were found to be significantly lower than in the younger age groups of rats. It was suggested that aging-induced inhibitions in the brain Na^+ - K^+ ATPase activity may be implicated in the depression of neuronal excitability and the age-related impairments of cognitive functions.

In the present study, the Na^+ - K^+ ATPase activity was decreased significantly in brain synaptosomes of aged animals when compared to brain synaptosomes from 3 months animals. These observations support Kaur et al. and Chakraborty *et al*. [37] [38]. Decrease in Na^+ - K^+ ATPase activity with an increase in age could result in neurotoxicity resulting in neuronal vulnerability to excitotoxic insults to the neuronal cells. Age related alterations of brain Na^+ - K^+ ATPase activity has been reported earlier [39] [40]. The activity of Na^+ - K^+ ATPase was measured using different concentrations of Aβ (25 - 35), NKB and combined treatment of NKB and Aβ (25-35) in different age groups of control (without E2 treated) and E2 treated rat brain synaptosomes. In different concentration of Aβ (25 - 35) incubation, the enzyme Na^+- K^+ ATPase inhibited in all the age groups when compared with age matched controls. The percentage decrease with Aβ (25 - 35) was lower in the E2 treated rats as compared to control (without E2) 12 and 24 months old rats. These results showed that E2 shows significance response and neuroprotective role against oxidative stress produced by Aβ (25 - 35). The activity of Na^+ - K^+ ATPase increased in all the age groups with an increase in the NKB peptide concentration, as compared with that of age matched control synaptosomes. This result supported the results of Mantha *et al*. [28]. However incubation of synaptosomes of E2 treated rats with various concentration of NKB increased the activity of Na^+ - K^+ ATPase significantly. There was higher increase in the activities of the enzyme in the synaptosomes of NKB incubated E2 treated young and old rats as compared to synaptosomes of without E2 treated rats. These results indicate that NKB showed more significant results with E2 treated synaptosomes as compared to their individual application. The result obtained with NKB suggests its role as antioxidant and free radical scavenger agent towards Aβ (25 - 35) induced deleterious effects in synaptosomes. The combined dose of NKB and Aβ (25 - 35) incubation was effective in increasing the activity of Na^+- K^+ ATPase in the synaptosomes of E2 treated aging rat brain. By suppressing accumulation of oxyradicals, NKB may be slowing down the Aβ (25 - 35) induced neurodegenerative disorders.

Na^+ - K^+ ATPase subunit isoforms are expressed in a tissue-specific manner. The α1 and β2 subunit is expressed predominantly in brain and muscle [41]. In tracts of myelinated axons, some have predominantly α1 [42]. Similarly, β1 and β2 are found in some neurons and some glia. Neurons, consequently, can express any of the Na^+ - K^+ ATPase unique to either neurons or glia, is clearly shown to be expressed in the cerebellar granule cell, which is the single most abundant neuronal cell type in the brain.

In the present study, the age dependent expression of Na^+ - K^+ ATPase sub-unit in the rat brain synaptosomes was analyzed by SDS PAGE. The obtained SDS PAGE results suggest that expression of α1 and β2 subunit of Na^+ - K^+ ATPase in rat brain is age dependent and differential in expression. In 3 months old age groups, the expression of the subunits were lower in comparison to older age groups. With an increase in age, the 12 months old rat brain synaptosomes showed higher expression of α1 and β2 subunit of Na^+ - K^+ ATPase, but 24 month rats show lower expression of subunits. The expression of α1 and β2 were higher in E2 treated rat than without E2 treated control rats. Results of α1 and β2 expression in rat brain synaptosomes indicated that these are present in rat brain synaptosomes but at an age dependent manner. The increase in the expression α1 and β2 subunit of Na^+ - K^+ ATPase in the E2 treated rat brain synaptosomes of different age groups showed that E2 is associated with a clear augmentation of the potency and magnitude of responses to the expression of subunit. These results suggested that estrogen might have a direct regulatory effect on sub-units of Na^+ - K^+ ATPase. In this study, the nature of the response of E2 would depend on the age group of rats.

5. Conclusion

Decrease in Na^+ - K^+ ATPase activity with increase in age may lead to neurotoxicity, resulting in neuronal vulnerability to excitotoxic insults to the neuronal cells. By suppressing accumulation of oxyradicals, estrogen with

NKB may be slowing down the Aβ (25 - 35) induced neurodegenerative cascades. Increase in the expression α1 and β2 subunit of Na^+ - K^+ ATPase in presence of E2 in the synaptosomes of different age groups of rats shows that E2 is associated with a clear augmentation of the potency and magnitude of responses to the expression of subunit. These results suggest that estrogen may have a direct regulatory effect on sub-units of Na^+ - K^+ ATPase.

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