*Original Research*
*ISSN (Print) : 0971-913X*
**Journal of Endocrinology and Reproduction,** *Vol 21(2), 67-76, December 2017, DOI: 10.18311/jer/2017/23150* *ISSN (Online) : 2455–1244*

# Rapid *In Situ* Action of Estradiol 17β on Ion Transporter Function in Brain Segments of Female Mozambique Tilapia (*Oreochromis mossambicus* Peters)

**Dafina Wilfred[1], Valsa S. Peter[2] and M. C. Subhash Peter[1,2]**

[1]Department of Zoology, University of Kerala, Kariavattom, Thiruvananthapuram – 695581, Kerala, India; subashpeter@yahoo.com
[2]Inter-University Centre for Evolutionary and Integrative Biology, University of Kerala, Kariavattom, Thiruvananthapuram – 695581, Kerala, India

## Abstract

Being the principal estrogen, estradiol 17β ($E_2$) is essential for normal ovarian function in the vertebrates including fishes. Besides its primary role in reproduction, $E_2$ is also known for its role in many other physiological processes including water and mineral balance. However, it is uncertain, how $E_2$ regulates ion-specific ATPases that drive $Na^+$, $K^+$, $H^+$, $Ca^{2+}$and $Mg^{2+}$ transport in fish brain. We, therefore, examined the short-term *in situ* action of $E_2$ on ion transporter function in the brain segments of freshwater female Mozambique tilapia *Oreochromis mossambicus.* Tilapia were perfused with increasing doses of $E_2$ ($10^{-9}$, $10^{-8}$ and $10^{-7}$ M) for 20 min and sampled for determining $Na^+/K^+$-ATPase, $H^+$-ATPase, $Ca^{2+}$-ATPase, and $Mg^{2+}$-ATPase activities in the prosencephalon (PC), mesencephalon (MC) and metencephalon (MeC) segments of brain. Dose-dependent increase in $Na^+/K^+$- and $Ca^{2+}$-dependent transporter activities after $E_2$ perfusion were found in PC. In MC, $E_2$ treatment, however, produced significant increase in $Mg^{2+}$, $Ca^{2+}$ and $H^+$ transport activities in mitochondria but decreased $Na^+/K^+$- and *v*$H^+$ transporter activities. On the contrary, in MeC, $E_2$ administration while producing increase in $Na^+/K^+$-, mitochondrial- and *v*$H^+$-transport, lowered cytosolic and mitochondrial $Ca^{2+}$ transport. Taken together, the data indicate that $E_2$ has rapid and direct action on ion transporter function that corresponds to the differential activation/inactivation of neuronal clusters in the brain segments of female freshwater tilapia.

**Keywords:** $Na^+/K^+$-ATPase, Estradiol 17β, Fish; Ion Transporter, Ionoregulation, Tilapia brain

## 1. Introduction

Besides having critical role in reproductive and sexual functioning, estradiol 17β ($E_2$), a major ovarian steroid of the hypothalamo-pituitary-gonadal axis, also has other physiological roles in metabolism, osmoregulation and immune function[1-2]. $E_2$ plays a crucial role in the liver by stimulating the synthesis of vitellogenin, the main precursor of the oocyte reserves [3]. Brain also produces $E_2$ that belongs to the group of neurosteroids; it is involved in a variety of neuronal functions[4]. The binding of $E_2$ to membrane receptors is found to cause rapid activation of a broad range of second messengers that lead to alterations in intracellular ion concentrations in a wide variety of cells[5-6]. Besides triggering the classical genomic pathways[7], $E_2$ has also been shown to modulate the activity of ion channels in a diverse range of epithelial tissues[8]. Likewise, an analog of $E_2$, diethylstilbestrol, has been shown to modulate brain $Na^+/K^+$ ATPase activity in *Oreochromis mossambicus*[9]. Furthermore, $E_2$ is found to modify baseline and stress-induced interrenal and corticotropic activities in rainbow trout[1] and zebrafish[10].

Fish brain, one of the most complex and highly heterogenous organs specialized for performing distinct func-

*Author for correspondence
* **Phone:** 352-294-4008, **Fax:** 352-392-8908

tions[11], possesses clusters of neurons that coordinate the body functions[12]. Neurons with their unique ability induce electrical impulses through the semi-permeable and excitable membranes by way of rapid changes in permeability of cations[13]. Modulation of cation distribution on neurons that utilizes energy for maintaining the redistribution of cation gradients is important for neurotransmission via the release of neurotransmitters[14]. Teleost fish brain comprises three segments, namely, prosencephalon (PC) the forebrain, mesencephalon (MC) the midbrain, and metencephalon (MeC) the hindbrain. Prosencephalon includes the telencephalon and diencephalon that consists of dorsal epithalamus, lateral thalamus and ventral hypothalamus. The telencephalon consists of the olfactory lobes and the cerebral hemispheres[15]. The epithalamus contains the choroid plexus and the pineal gland. The ventral part of the hypothalamus consists of the infundibulum and the pituitary gland[16]. Mesencephalon consists of the dorsal optic tectum, mid-ventral torus longitudinalis and the ventral tegumentum[17]. The metencephalon segment includes the cerebellum, medulla oblongata and the brain stem[18- 19].

Brain aromatization occurs in all classes of vertebrates including teleost fish[20- 21]. Ovarian $E_2$ acts in the central nervous system to regulate neuroendocrine events and reproduction[22]. Likewise, $E_2$ regulates gene expression, neuronal survival, neuronal and glial differentiation and synaptic transmission and has anti-inflammatory, protective and reparative properties in the brain[23- 26]. Brain tissue is highly sensitive to oxidative stress due to its high oxygen demand, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses[27-28]. Membrane proteins that control ion gradients across organellar and plasma membranes appear to be particularly susceptible to oxidation-induced changes. Any perturbation in the activities of ATPases affects membrane status by inflicting changes in electrophysiological energetics and normal homeostasis[29].

$Na^+/K^+$-ATPase is responsible for the generation of membrane potential through the active transport of $Na^+$ and $K^+$ ions in the CNS necessary to maintain neuronal excitability[12]. $Na^+/K^+$-ATPase is present at high concentrations in brain consuming about 40–50 % of the ATP generated in this organ [30]. It is implicated in metabolic energy production as well as uptake, storage, and metabolism of catecholamines, serotonin, and glutamate[31]. $Ca^{2+}$-ATPase is responsible for fine-tuning of intracellular $Ca^{2+}$ homeostasis and $H^+$-ATPase is involved in the release and uptake of neurotrasmitter. $Mg^{2+}$-ATPase, on the contrary, is involved in regulating high brain intracellular $Mg^{2+}$ that controls protein synthesis and growth[32]. Intact mitochondrial function, characterized by intact mitochondrial transport mechanisms[19], is essential for cell energy homeostasis. Mitochondrial failure has been implicated in the etiology of several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and Huntington's disease[33-35].

An important mechanism involved in the neuroprotective effects of estrogenic compounds is the regulation of mitochondrial function[36]. There is evidence suggesting that mitochondrial function is regulated by estrogens as estrogen receptor β has been found localized in mitochondria in a variety of cell types including neurons[37, 35]. Despite this information, the role of $E_2$ in ion transport, particularly in the brain segments that holds specific neuronal clusters, has not yet been identified in fish. We, therefore, examined the dose-response *in situ* action of $E_2$ on ion transporter functions in the brain segements of Mozambique tilapia (*Oreochromis mossambicus*).

## 2. Materials and Methods

### 2.1 Animals

Mozambique tilapia, *Oreochromis mossambicus* Peters, belongs to the family Cichlidae and it is native to southern Africa. But it is now found in many tropical and subtropical habitats around the globe. Mozambique tilapia are omnivorous and can live in both brackish and salt water and can survive a wide range of salinity and temperatures[38]. Adult female tilapia in their post-spawning phase, prior to the vitellogenic phase for the next spawning phase, were selected as they retain almost constant low estrogen level throughout the study. The fish were kept as four groups and acclimated in 50 L glass tanks with aerated well water at 28 ± 1 °C (pH 7.2) under natural photoperiod (12L/12D) for three weeks prior to experiment. They were fed with commercial fish feed at a ration of 1.5% of body mass per day. The animal care and the experimentation were strictly according to the regulation of Animal Ethical Committee of the University and there was no mortality during the experimentation.

### 2.2 Experimental Protocol

The dose-responsive *in situ* action of $E_2$ on the ion transporter activities in the brain segments (PC, MC, MeC)

was studied in the present study. Laboratory-acclimated female tilapia, held as four groups of six each, were caught in the net and anaesthetized (8.00 am) in 0.1% 2-phenoxy ethanol solution (Sigma, St. Louis, MO). Blood was drawn from the caudal artery using a heparinized #23 syringe. Fish were then perfused for 20 min following the method as demonstrated earlier[39]. A ventral cut was made to each fish from the anus to the pectoral girdle and infusion was performed by inserting a cannula (PE-50 tubing) into the ventricle through the bulbus arteriosus. Infusion was done with the help of a peristaltic pump (ENPD-100 EnterTech, Mumbai) using a infusion medium (Cortland saline; 119 mMNaCl, 5 mM $NaHC0_3$, 5.4 mM KCl, 0.35 mM $Na_2HP0_4$, 0.44 mM $KH_2P0_4$, 0.81 mM $MgS0_4$, 1.25 mM $CaCl_2$ and 5 mM D-glucose; pH 7.4) at a rate of 0.3 mL $min^{-1}$ for 20 min. $E_2$ was first dissolved in propylene glycol (0.01%) and subsequently diluted with infusion medium[39]. Each fish was infused with varied doses of $E_2$ ($10^{-8}$, $10^{-7}$ and $10^{-6}$ M), respectively, for 20 min. The control fish given infusion lacking $E_2$ was considered as sham control and compared with the other $E_2$-infused fish.

## 2.3 Sampling and Isolation of Brain Segments

After perfusion for 20 min, fish were sacrificed by spinal transsection and the whole brain was excised immediately and sliced into prosencephalon (PC), mesencephalon (MC), and metencephalon (MeC). These three brain segments were kept in ice-cold BME buffer (pH 7.4) and stored at -80 °C for further analysis. We measured $Na^+/K^+$-ATPase in $H_0$ fraction, vacuolar (V) and mitochondrial (mit) $H^+$-ATPase, cytosolic and mitochondrial $Ca^{2+}$-ATPase, and mitochondrial $Mg_2$+-ATPase activities in these brain segments of the fish.

### *2.3.1 Isolation of Brain Mitochondria*

Mitochondria were isolated from the three segments of fish brain following the method of Lee *et al.*[40] and Veauvy *et al.*[41] with modifications. Briefly, each segment of brain was kept in brain mitochondrial extraction (BME) buffer containing 0.25 mM sucrose, 10 mM HEPES, 0.5 mM EDTA, and 0.5 mM EGTA (pH 7.4). The brain tissue was chopped and homogenized (8-10 strokes) using a glass homogenizer. The collected homogenate was first centrifuged (Eppendorf 5430R, Germany) at 2000 xg for 3 min at 4 ºC to separate the membrane constituents from mitochondria and synapses[19]. A portion of this supernatant was used for analyzing the $Na^+/K^+$-ATPase activity. The other portion was then centrifuged at 12,000 xg for 8 min at 4 °C. The supernatant was collected and transferred to an eppendorf tube for analyzing the ion transporters such as $H^+$- ATPase and cyt. $Ca^{2+}$-ATPase. The pellets were then washed in the isolation buffer with BSA and centrifuged at 12,000 xg for 10 min. The pellets were then resuspended in 0.25 M sucrose solution and centrifuged again for 10 min. These final pellets were resuspended and centrifuged again for 10 min. The final pellets were resuspended in sucrose medium and served as the mitochondrial suspension. The purity of mitochondrial suspension was tested by assaying SDH and cytochrome C oxidase activity which showed basal values for intact mitochondrial preparation. The protein contents in the samples were quantified using modified Biuret Assay[42] with bovine serum albumin as standard.

## 2.4 Analyses

### *2.4.1 Quantification of $Na^+/K^+$ -ATPase-Specific Activity*

The ouabain-sensitive $Na^+/K^+$-ATPase-specific activity was quantified in the brain membrane preparation adopting the method of Peter *et al.*[43] modified for microplate assay[44]. Saponin (0.2 mg $protein^{-1}$) was routinely added to optimize substrate accessibility. Samples in duplicates were added to a 96-well microplate containing 100 mM NaCl, 30 mM imidazole (pH 7.4), 0.1 mM EDTA and 5 mM $MgCl_2$. KCl 0.13 mM was used as the promoter and 0.14 mM ouabain was used as the inhibitor. After vortexing, the assay mixture was incubated at 37 ºC for 15 min. The reaction was initiated by the addition of 0.13 mM ATP and was terminated with addition of 8.6% TCA. The liberated inorganic phosphate was measured against phosphate standard at 700 nm in Synergy HT Biotek Microplate Reader (USA). The change in absorbance between promoter and inhibitor assays was calculated and regression analysis was employed to derive the rate of activity of $Na^+/K^+$-ATPase and expressed in micromoles of Pi liberated per min per mg of protein.

### *2.4.2 Quantification of $Mg2^+$-ATPase Activity*

The oligomycin-sensitive $Mg^{2+}$-ATPase activity in brain mitochondria was quantified as described for $Na^+/K^+$-ATPase but using an inhibitor oligomycin.

Mitochondrial samples in duplicate were added to a 96-well microplate with or without oligomycin. The assay mixture was incubated with ATP at 15 min at 37 ºC. The inorganic phosphate content released was measured and expressed in µmol Pi $h^{-1}$ mg $protein^{-1}$.

### *2.4.3 Quantification of Cytosolic and Mitochondrial $Ca^{2+}$ -ATPase- Specific Activities*

The vanadate-dependent $Ca^{2+}$-ATPase activity in cytosolic function and mitochondrial $Ca^{2+}$-ATPase involed in mitochondria was determined as described for $Na^+$/ $K^+$-ATPase but using an inhibitor vanadate. Samples in duplicate were added to a 96-well microplate containing either $CaCl_2$ or vanadate. The assay mixture was incubated with ATP for 15 min at 37 ºC. The released inorganic phosphate content was measured and expressed in µmol Pi $h^{-1}$ mg $protein^{-1}$.

### *2.4.4 Quantification of Vacuolar and Mit $H^+$ATPase Specific Activities*

The bafilomycin-sensitive $H^+$-ATPase activity in the brain cytosolic fraction ($vH^+$ -ATPse) and mitochondria (mito $H^+$-ATPase) were measured as described for $Na^+$/ $K^+$-ATPase using an inhibitor bafilomycin A. The mitochondrial and cytosolic samples in duplicate were added to a 96-well microplate containing bafilomycin A and the reaction was initiated by the addition of ATP and incubated for 15 min at 37 °C. The reaction was terminated by adding 8.6% TCA and the inorganic phosphate content was determined as above and expressed in µmol Pi $h^{-1}$ mg $protein^{-1}$.

## 2.5 Statistical Analysis

Data collected from six female fish from each group were checked for normal distribution and variance homogeneity. Data were subjected to one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test (GraphPad InStat-3; GraphPad Software, Inc., San Diego, CA, USA). Statistical changes between the means were accepted as significant if $P < 0.05$. The significance level in dose-responsive activity of $E_2$ treatments, were represented as "**a**" ($P < 0.05$), "**b**" ($P < 0.01$), and "c" ($P < 0.001$) compared with sham control (0 dose). The difference between the sham control and $E_2$ treated groups was accepted as statistically significant if $P < 0.05$.

# 3. Results

## 3.1 $Na^+$/ $K^+$-ATPase Specific Activity in Brain Segments

Varied doses of $E_2$ ($10^{-8}$, $10^{-7}$, $10^{-6}$M) perfusion for 20 min significantly increased the $Na^+$/ $K^+$- ATPase activity in PC of fish brain (Fig. 1A). In contrast, a significant decrease ($P < 0.05$) in $Na^+$/ $K^+$- ATPase activity was found after $10^{-7}$ M and $10^{-6}$M $E_2$ administration in MC, but $10^{-8}$ M $E_2$ had no effect (Fig. 1A). The $Na^+$/ $K^+$- ATPase activity in MeC significantly decreased ($P < 0.05$) at $10^{-7}$M $E_2$ dose but showed an increase ($P < 0.001$) at $10^{-6}$M $E_2$ dose (Fig. 1A).

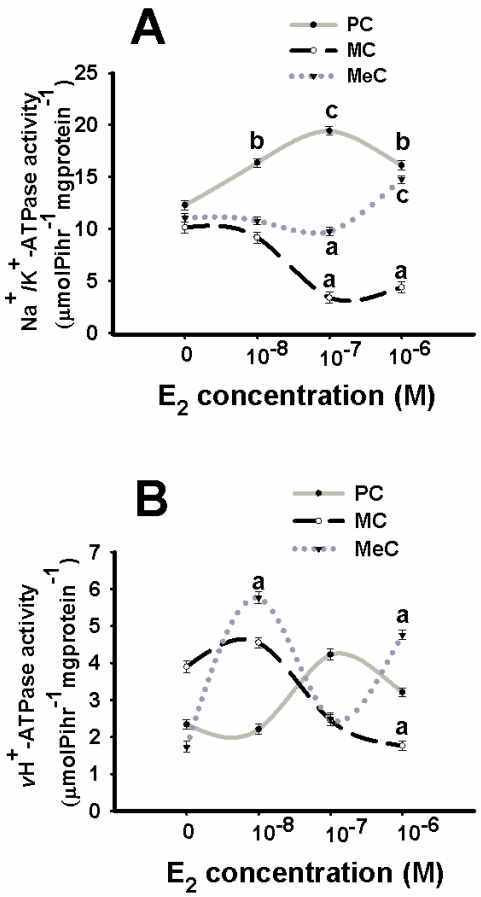


**Figure 1.** Dose-responsive action of *in situ* estradiol 17β ($E_2$; $10^{-8}$, $10^{-7}$, $10^{-6}$ M) treatment for 20 min on $Na^+$/ $K^+$-ATPase activity (A) and $vH^+$-ATPase activity (B) in prosencephalon(PC), mescencephalon (MC) and metencephalon (MeC) brain segments of *Oreochromis mossambicus*. Each point is mean ±SE for six fish. The significance levels are represented as "a" (P<0.05), "b" (P<0.01), "c" (P<0.001) when compared with control fish (0 M $E_2$).

### 3.2 $H^+$-ATPase Specific Activity in Brain Segments

The $vH^+$-ATPase activity in the PC segment of fish brain did not respond significantly to the varied doses of $E_2$ infusion (Fig. 1B). In MC segment, $vH^+$-ATPase activity significantly decreased after $10^{-6}$ M $E_2$ dose, whereas other doses did not produce a significant response (Fig. 1B). In the MeC segment, $vH^+$-ATPase activity significantly increased ($P < 0.05$) after $10^{-8}$ M and $10^{-6}$ M doses of $E_2$, whereas $10^{-7}$ M $E_2$ dose failed to produce response (Fig. 1B). The mitochondrial $H^+$- ATPase activity in PC did not respond to varied doses of $E_2$ infusion (Fig. 3B). In MC, $mH^+$- ATPase activity significantly increased after $10^{-7}$ M ($P < 0.05$) and $10^{-6}$ M ($P < 0.01$) doses of $E_2$ infusion, whereas $10^{-8}$ M $E_2$ infusion failed to respond (Fig: 3B). The MeC segment showed significant increase in $mH^+$-ATPase activity after infusion of all the doses of $E_2$ ($10^{-8}$ M ($P < 0.05$); $10^{-7}$M ($P < 0.001$); $10^{-6}$M ($P < 0.01$; Fig. 3B).

### 3.3 $Ca^{2+}$-ATPase Specific Activity in Brain Segments

Vanadate-sensitive cytosolic $Ca^{2+}$-ATPase activity in PC segment of fish brain showed significant increase after $10^{-8}$ M and $10^{-7}$ M ($P < 0.01$) doses of $E_2$, whereas it did not respond to $10^{-6}$ M $E_2$ dose (Fig. 2A). The $cCa^{2+}$- ATPase activity showed a significant increase ($P < 0.05$) after $10^{-6}$ M dose of $E_2$ infusion in MC, whereas, other doses failed to alter its activity (Fig. 2A). In MeC segment a significant reduction ($P < 0.01$) in $cCa^{2+}$- ATPase activity occured after $10^{-7}$ M and $10^{-6}$ M doses of $E_2$ infusion (Fig. 2A). $10^{-8}$ M $E_2$ dose failed to produce an effect (Fig. 2A). Similar to $cCa^{2+}$- ATPase, the mitochondrial $Ca^{2+}$-ATPase activity in PC and MC segments of tilapia brain increased significantly ($P < 0.01$) after $10^{-7}$ M dose of $E_2$ infusion, though other doses failed to alter its activity (Fig. 2B). In MeC segment, significant ($P < 0.001$) dose-dependent decline in $mCa^{2+}$-ATPase activity was found after $E_2$ infusion (Fig. 2B).

### 3.4 $Mg^{2+}$ ATPase Specific Activity in Brain Segments

In MC segment of fish brain a significant rise ($P < 0.05$) in the mitochondrial $Mg^{2+}$-ATPase activity occurred after $10^{-7}$ M dose of $E_2$ infusion, whereas its activity did not respond to other tested doses (Fig. 3A). The mitochondrial $Mg^{2+}$-ATPase activity remained unaffected after varied doses of $E_2$ infusion in PC and MeC segments of tilapia brain (Fig. 3A).

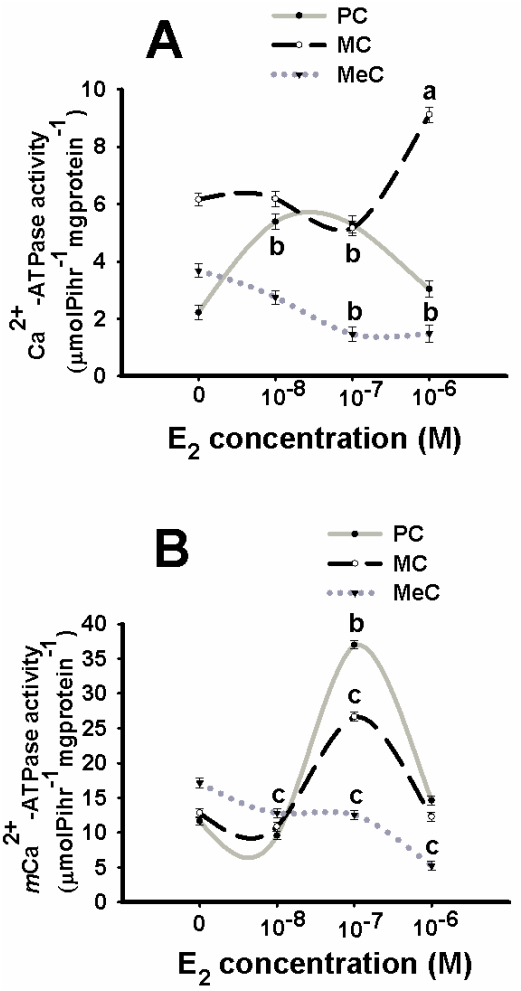


**Figure 2.** Dose- responsive action of *in situ* estradiol 17β ($E_2$; $10^{-8}$, $10^{-7}$, $10^{-6}$ M) treatment for 20 min on $Ca^{2+}$-ATPase activity (A) and $mCa^{2+}$-ATPase activity (B) in prosencephalon(PC), mescencephalon (MC) and metencephalon (MeC) brain segments of *Oreochromis mossambicus*. Each point is mean ±SE for six fish. The significance levels are represented as "a" (P<0.05), "b" (P<0.01), "c" (P<0.001) when compared with control fish (0 M $E_2$).

## 4. Discussion

$E_2$ synthesized and secreted from peripheral endocrine glands, passes through the blood-brain barrier and exerts a critical influence in CNS. In addition, the brain, that also synthesizes neurosteroids including $E_2$, shows neuroprotective actions by attenuating oxidative stress[45]. In

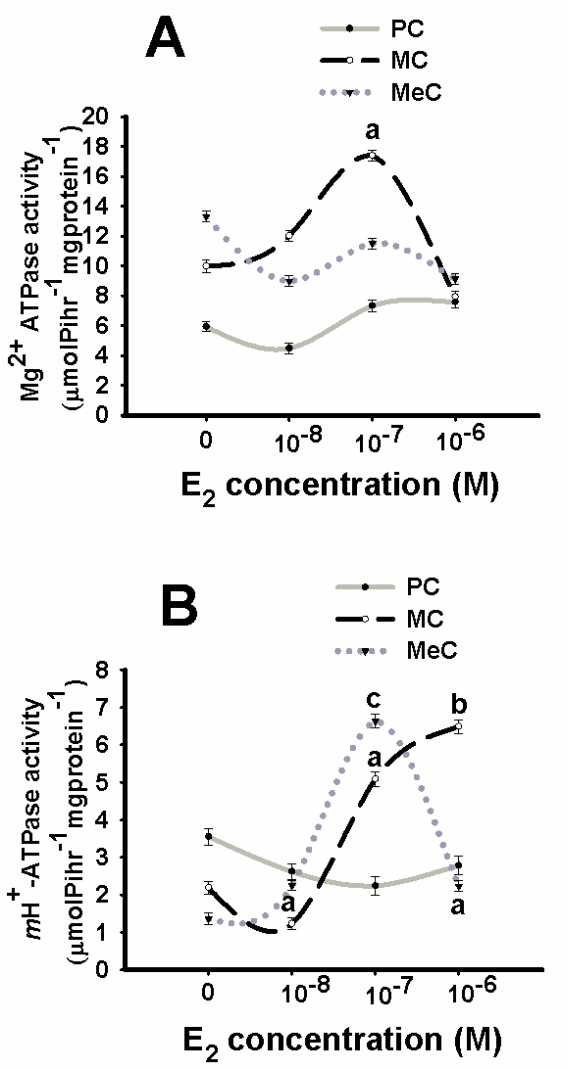


**Figure 3.** Dose-responsive action of *in situ* estradiol 17β ($E_2$; $10^{-8}$, $10^{-7}$, $10^{-6}$ M) treatment for 20 min on $Mg^{2+}$-ATPase activity (A) and *m*$H^+$-ATPase activity (B) in the prosencephalon (PC), mescencephalon (MC) and metencephalon (MeC) brain segments of *Oreochromis mossambicus*. Each point is mean ±SE for six fish. The significance levels are represented as "a" (P<0.05), "b" (P<0.01), "c" (P<0.001) when compared with control fish (0 M $E_2$).

the present study, dose-responsive action *in situ* of $E_2$ was tested on the pattern of ion transporter functions in the brain of female freshwater tilapia. The pattern of tested ion transporters such as $Na^+/K^+$-ATPase, cytosolic and mitochondrial $H^+$-ATPase, cytosolic and mitochondrial $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase activity in the brain segments (PC, MC, MeC) showed significant modification after $E_2$ perfusion for 20 min. The results provide evidence that $E_2$ can exert a rapid action on the iono-regulatory mechanisms in the brain of freshwater tilapia.

$Na^+/K^+$-ATPase, a membrane-bound enzyme that plays a crucial role in neuronal function including the release of neurotransmitters, regulates membrane potential, cell volume and transmembrane fluxes of $Ca^{2+}$. Multiple isoforms of $Na^+/K^+$-ATPase that include three isoforms of $\alpha$ subunit ($\alpha$1, $\alpha$2 and $\alpha$3), and three of $\beta$ subunit ($\beta$1, $\beta$2 and $\beta$3) are found in CNS [46]. 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[46]. It is also involved in the normal cell cycle and differentiation of the nervous system[47]. The $Na^+/K^+$-ATPase activity in the three different brain segments showed dose-dependent and differential modulation in response to the varied doses ($10^{-8}$ M to$10^{-6}$ M) of $E_2$ infusion. The substantial rise in $Na^+/K^+$-ATPase activity in the PC and MeC segments and its decline in MC suggest a direct and rapid action of $E_2$ on this transporter function that modifies the osmotic gradients and transmission potentials, directs neuronal clusters in the brain segments. This differential response of $Na^+/K^+$-ATPase activity to $E_2$ further indicates a dose-specific action of $E_2$ on the release of neurotransmitters in the neuronal clusters of tilapia brain. This further implies the coordinating and integrative mechanisms of $E_2$ to differentially regulate the functions of neuronal clusters in tilapia brain.

The inactivation and activation of $Na^+/K^+$-ATPase found in MC and MeC segments that correspond to modified $Ca^{2+}$-ATPase activity imply a partial membrane polarization/depolarization allowing excessive $Ca^{2+}$ entry inside neurons that result in toxic events like excitotoxicity[48]. It appears that the mechanism of inactivation also depends on the disruption of phospholipid microenvironment of the enzyme or by the higher release of reactive oxygen radicals [49]. However, in brain tissue, $E_2$ has been shown to protect neurons against oxidative stress and excitotoxicity[50]. It has been shown that $Na^+$ gradient, that forms the basis of excitation, also drives many secondary transport systems including glutamate and $Ca^{2+}$-transporters [51]. $E_2$ exerts protective effects against $H_2O_2$-induced toxicity in human neuroblastoma cells by maintaining intracellular $Ca^{2+}$ homeostasis, attenuating ATP depletion, ablating mitochondrial calcium overloading and preserving mitochondrial membrane potential[52]. Calcium efflux from excitable cells occurs through two main systems, an electrochemically driven $Na^+/Ca^{2+}$

exchanger with a low $Ca^{2+}$ affinity, and a plasmalemmal $Ca^{2+}$-ATPase (PMCA), with a high $Ca^{2+}$ affinity[53].

Regulation of cytoplasmic $Ca^{2+}$ is crucial both for proper neuronal function and cell survival as it drives transmitter release, excitability, dendritic integration and synaptic plasticity[54]. Plasma membrane $Ca^{2+}$-ATPase plays a key role in the maintenance of precise levels of intracellular $Ca^{2+}$ essential for the functioning of neurons[55]. Presynaptic $Ca^{2+}$ is the principal regulator of neurotransmitter release, that acts via multiple $Ca^{2+}$-sensing proteins. Mitochondria and plasma membrane $Ca^{2+}$-ATPase have been shown to control presynaptic $Ca^{2+}$ clearance in capsaicin-sensitive rat sensory neurons[56]. The modulating response of cytoplasmic and mitochondrial $Ca^{2+}$-ATPase in the tilapia brain segments indicate that $E_2$ synchronizes these transporters in PC and MeC segments while controlling the intracellular signaling pathways by tightly regulating intracellular calcium levels. It is likely that $E_2$, could thus synchronize the release of $Ca^{2+}$ ions by enthrusting either cytosolic or mitochondrial $Ca^{2+}$-ATPase for maintaining its intracellular levels in the neuronal clusters. Age-related decrease in $Na^+/K^+$ -ATPase and $Ca^{2+}$-ATPase activities in the brains of aging animals usually affect the signal transduction pathway, contractibility and excitability and cellular functions which could lead to the development of neurological disorders [57, 55]. $E_2$ has been shown to modulate the $Na^+/K^+$-ATPase and $Ca^{2+}$-ATPase, activities in the brain of rats, which becomes beneficial in preventing age-related changes in the brain [58]. Further, $E_2$ has been shown to modulate mitochondrial $Ca^{2+}$ flux in rat caudate nucleus and brain stem[59] and exerts neuroprotection through mediating the functioning of $Na^+/Ca^{2+}$-exchanger in cultured cortical neurons of rat[60]. The vacuolar $H^+$-ATPase is ATP-dependent proton pump responsible for both acidification of intracellular compartments and proton transport across the plasma membrane. Intracellular v-ATPase functions in endocytic and intracellular membrane traffic, processing and degradation of macromolecules in secretory and digestive compartments, and coupled transport of small molecules including neurotransmitters and ATP [61-62]. Synaptic vesicles have important roles in the neural transmission at nerve terminals through the storage and the controlled exocytosis of neurotransmitters. In the cytoplasm, neurotransmitters are concentrated inside synaptic vesicles by distinct transport systems driven by the $H^+$ concentration gradient, maintained by the $vH^+$-ATPase[63]. Like $Ca^{2+}$-ATPase, significant modulation was found between $vH^+$-ATPase and $mH^+$-ATPase after varied doses of $E_2$ infusions and that indicates a role for $E_2$ in fine-tuning the physiological pH levels in neuronal cells so as to ensure the release of neurotransmitters. The response patterns of these transporters show high sensitivity to $E_2$ doses and often show opposing actions in the tested brain segments. The wave-like response of these transporters clearly points to the sensitivity of $E_2$ in the modulation of intracellular acidification by regulating proton pump activation/inactivation, which is more evident in MC and MeC segments compared to PC segment. In addition, it is evident that a fine-tuning mechanism operates between cytoplasmic and mitochondrial components to ensure intracellular neuronal pH for the optional release of neurotransmitters particularly in MC and MeC segments. This further suggests a dose-specific action of $E_2$ on neurotransmitter release uptake mechanisms through altered mitochondrial or vesicular membrane pontentials that exists in neuronal clusters of this fish brain.

$Mg^{2+}$-ATPase is a vital in maintaining brain intracellular $Mg^{2+}$ levels because of its role in regulating the rates of protein synthesis and cell growth[32]. Changes of antioxidant enzyme activities was found associated with differential modulation of brain intracellular $Mg^{2+}$, neural excitability, as well as the uptake and release of biogenic amines [31]. In addition, a $Cl^-$ stimulated $Mg^{2+}$-ATPase function has been demonstrated in fish brain which show correlation with the function of gamma-aminobutiric acid (GABA) via GABA receptors, which are linked with $Cl^-$ transport through the postsynaptic membrane[64]. High affinity $Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPases were found in rat brain synaptosomes, synaptic membranes, and microsomes [65]. The altered $Mg^{2+}$ ATPase activity in the fish brain after $E_2$ infusion indicates its specific role in $E_2$ driven Mg balance and in modulating the transport of neurotransmitters. The increased $Mg^{2+}$ ATPase activity in the MC brain segment after $E_2$, infusion suggests a dose-specific and neuronal cluster specific role of $E_2$ in the brain of this fish. $Ca^{2+}/Mg^{2+}$-ATPase are enzymes known to maintain intracellular gradients of ions that are essential for signal transduction in brain. Several neurological diseases are caused primarily by malfunctioning of $Ca^{2+}$ channels or $Ca^{2+}/Mg^{2+}$-ATPase[66]. $Mg^{2+}$-ATPase also exhibited tissue-specific progressive reduction in activity, and it has been known to involve in coupling of ADP + Pi in the biosynthesis of ATP in the mitochondrial system[67]. The continuous firing of action

potentials, transport of nutrients and restoration of resting membrane potential necessitate a very high activity of neuronal membrane ATPases. The present study thus reveals a rapid action of $E_2$ with respect to the excitatory/inhibitory action of neurons in the brain segments that drive many ion-specific ATPases. We found that $E_2$ has a rapid differential action on neuronal clusters of tilapia brain segments as evident in the differential regulation of $Na^+/K^+$ ATPase, $H^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase acivities which are responsible for fine-tuning of intracellular ion homeostasis in maintaining neuronal function in tilapia brain.

## 5. Acknowledgements

Thanks are due to UGC, New Delhi, for SAP-DRS II facility in the Department of Zoology of the University of Kerala, and for UGC-BSR Fellowship to DW. MCSP extends thanks to iCEIB Project of Govt of Kerala. VSP acknowledges Research Associateship Grant of UGC, New Delhi, and Emeritus Scientistship of KSCSTE, Govt of Kerala.

## 6. References

1. Pottinger TG, Carrick TR, Hughes SE, Balm PHM. Testosterone, 11-ketotestosterone, and estradiol-17β modify baseline and stress induced interrenal and corticotropic activity in trout. *Gen Comp Endocrinol*. 1996; 104: 284–295.
2. Cuesta A, Vargas-Chacoff, Garcia-Lopez A, Arjona FJ, Martinez-Rodriguez G, Meseguer J, Mancera JM, Esteban MA. Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream. *Fish Shellfish Immunol.* 2007; 23: 693–700.
3. Flouriot G, Pakdel F, Ducouret B, Ledrean Y, Valotaire, Y. Differential regulation of two genes implicated in fish reproduction: vitellogenin and estrogen receptor genes. *Mol Reprod Dev*. 1997; 48: 317–323.
4. Hu J, Zhang Z, Shen WJ, Azhar S. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab.* 2010; 7: 47.
5. Thomas P. Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. *Gen Comp Endocrinol.* 2012; 175: 367–383.
6. Evans PD, Bayliss A, Reale V. GPCR-mediated rapid, non-genomic actions of steroids: Comparisons between DmDopEcR and GPER1 (GPR30). *Gen Comp Endocrinol.* 2014; 195: 157–163.
7. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev.* 1999; 20: 358– 417.
8. Saint-Criq V, Rapetti-Mauss R, Yusef YR, Harvey BJ. Estrogen regulation of epithelial ion transport: Implications in health and disease. *Steroids.* 2012; 77: 918–923.
9. Sunny F, Oommen OV. Effects of steroid hormones on total brain $Na^+/K^+$- ATPase activity in *Oreochromis mossambicus. Indian J Exp Biol.* 2004; 42: 283–287.
10. Fuzzen LM, Nicholas JB, Glen Van Der K. Differential effects of 17β-estradiol and 11-ketotestosterone on the endocrine stress response in zebrafish (*Danio rerio*). *Gen Comp Endocrinol.* 2011; 170: 365–373.
11. Bone Q, Moore RH. Nervous System. In: Elizabeth Owen. (Ed) *Biology of Fishes*, 3rd edn. Taylor and Francis group, UK. 2008; p. 346–383.
12. Peter MCS, Simi S. Hypoxia stress modifies $Na^+/K^+$-ATPase, $H^+/K^+$-ATPase, $Na^+/NH_4^+$-ATPase, and *nkaα1* isoform expression in the brain of immune-challenged air-breathing fish. *J Exp Neurosci.* 2017; 11: 1–18.
13. McIlwain H, Bachelard H.S. *Biochemistry and the Central Nervous System*. 4th Edn. Chruchill, London. 1971.
14. Bachelard HS. *Outline Studies in Biology: Brain Biochemistry*. Chapman and Hall, London.1974; p. 1-8.
15. Deshmukh PR, Bul SV, Gadhikar YA. Nuclear organization and distribution in the brain regions of the snake headed fish, *Channa marulius. Ann Neurosci.* 2011; 18: 92–99.
16. Lagler KF, Bardach JE, Miller RR. *Ichthyology*, John Wiley and Son, USA.1962; 545pp.
17. Nieuwenhuys R. An overview of the organization of the brain of actinopterygian fishes. *Amer Zool.* 1982; 22: 287–310.
18. Kuhlenbeck H. *The Central Nervous System of Vertebrates, Vol. 4. Spinal Cord and Deuterencephalon.* Basel, Karger, 1975; 4: 388-623.
19. Samuel A, Peter VS, Peter MCS. Effect of L-tryptophan feeding on brain mitochondrial ion transport in netconfined climbing perch (*Anabas testudineus Bloch*). *J Endocrinol Reprod.* 2014; 18: 17.
20. Forlano PM, Schlinger BA, Bass AH. Brain aromatase: new lessons from non-mammalian model systems. *Front Neuroendocrinol.* 2006; 27: 247–274.
21. Do Rego JL, Seong JY, Burel D, Leprince J, Luu-The V, Tsutsui K, Tonon MC, Pelletier G, Vaudry H. Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. *Front Neuroendocrinol*. 2009; 30: 259–301.
22. DonCarlos LL, Azcoitia I, Garcia-Segura LM. Neuroprotective actions of selective estrogen receptor modulators. *Psychoneuroendocrinology*. 2009; 34S: S113-S122.

23. Garcia-Segura LM, Melcangi RC. Steroids and glial cell function. *Glia.* 2006; 54: 485–498.
24. Woolley CS. Acute effects of estrogen on neuronal physiology. *Annu Rev Pharmacol Toxicol.* 2007; 47: 657–680.
25. Spencer JL, Waters EM, Romeo RD, Wood GE, Milner TA, McEwen BS. Uncovering the mechanisms of estrogen effects on hippocampal function. *Front Neuroendocrinol.* 2008; 29: 219–237.
26. Vegeto E, Benedusi V, Maggi A. Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. *Front Neuroendocrinol.* 2008; 29: 507–519.
27. Carbonell T, Rama R. Iron, oxidative stress and early neurological deterioration in ischemic stroke. *Curr Med Chem.* 2007; 14 : 857–874.
28. Venkataraman P, Krishnamoorthy G, Vengatesh G, Srinivasan N, Aruldhas MM, Arunakaran J. Protective role of melatonin on PCB (Aroclor 1254) induced oxidative stress and changes in acetylcholine esterase and membrane bound ATPases in cerebellum, cerebral cortex and hippocampus of adult rat brain. *Int J Devl Neurosci.* 2008; 26: 585–59.
29. Mahendravarma B, Surendrakumar RB. Enhancement of ATPases of fetal brain of mouse exposed to ultrasound. *Biomedicine.* 1996; 16: 27–33.
30. Erecinska M, Silver IA. Ions and energy in mammalian brain. *Prog Neurobiol.* 1994; 16: 37–71.
31. Carageorgiou H, Pantos C, Zarros A, Stolakis V, Mourouzis I, Cokkinos D, Tsakiris S. Changes in acetylcholinesterase, $Na^+/K^+$-ATPase, and $Mg^{2+}$-ATPase activities in the frontal cortex and the hippocampus of hyper- and hypothyroid adult rats. *Metab Clin Exp*. 2007; 56: 1104–1110.
32. Sanui H, Rubin H. The role of magnesium in cell proliferation and transformation. In: Boynton, A.L., McKochan, W.L., Whitfield, J.P., (Eds.), *Ions Cell Proliferation and Cancer*, Academic Press, New York. 1982; pp. 517–537.
33. Simpkins JW, Wang J, Wang X, Perez E, Prokai L, Dykens JA. Mitochondria play a central role in estrogen-induced neuroprotection, *Curr Drug Targets CNS Neurol Disord.* 2005a; 4: 69–83.
34. Simpkins JW, Wen Y, Perez E, Yang S, Wang X. Role of nonfeminizing estrogens in brain protection from cerebral ischemia: an animal model of Alzheimer's disease neuropathology. *Ann N Y Acad Sci.* 2005b; 1052: 233–242.
35. Simpkins JW, Yang SH, Sarkar SN, Pearce V. Estrogen actions on mitochondria-physiological and pathological implications. *Mol Cell Endocrinol.* 2008; 290: 51–59.
36. Brinton RD. The healthy cell bias of estrogen action: mitochondrial bioenergetics and neurological implications. *Trends Neurosci.* 2008; 31: 529–537.
37. Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM, Valencia T, Brun-Zinkernagel AM, Prokai L, Will Y, Dykens J, Koulen P, Simpkins JW. Mitochondrial localization of estrogen receptor beta. *Proc Natl Acad Sci U.S.A.* 2004; 101: 4130–4135.
38. Hwang PP, Lee TH, Weng CF, Fang MJ, Cho GY. Presence of $Na^+$, $K^+$-ATPase in mitochondria-rich cells in yolk-sac epithelium of larvae of the teleost, *Oreochromis mossambicus. Physiol Biochem Zool.* 1999; 72: 138–144.
39. Babitha GS, Peter MCS. Cortisol promotes and integrates the osmotic competence of the organs in North African catfish (*Clarias gariepinus* Burchell): Evidence from *in vivo* and *in situ* approaches. *Gen Comp Endocrinol.* 2010; 168: 14–21.
40. Lee CP, Sciamanna M, Peterson PL. Intact rat brain mitochondria from a single animal: preparation and properties. *Methods Toxicol.* 1993; 2: 41–50.
41. Veauvy CM, Wang Y, Walsh PJ, Perez-Pinson MA. Comparison of the effects of ammonia on brain mitochondrial function in rats and gulf toadfish. *Am J Physiol Regul Integr Comp Physiol.* 2002; 283: R598–R603.
42. Alexander JB, Ingram GA. A comparison of five methods commonly used to measure protein concentration of fish sera. *J Fish Biol.* 1980; 16: 115–122.
43. Peter MCS, Lock RAC, Wendelaar Bonga SE. Evidence for an osmoregulatory role of thyroid hormones in the freshwater Mozambique tilapia, *Oreochromis mossambicus. Gen Comp Endocrinol.* 2000; 120: 157–167.
44. Peter MCS, Leji J, Peter V S. Ambient salinity modifies the action of triiodothyronine in the air-breathing fish *Anabas testudineus* Bloch: effects on mitochondria-rich cell distribution, osmotic and metabolic regulations. *Gen Comp Endocrinol.* 2011; 171: 225–231.
45. Ishihara Y, Takemoto T, Ishida A, Yamazaki T. Protective actions of 17β-estradiol and progesterone on oxidative neuronal injury induced by organometallic compounds. *Oxid Med Cell Longev.* 2015; 1–16
46. Lingrel JB, Orlowski J, Shull MM, Price EM. Molecular genetics of $Na^+$/ $K^+$-ATPase. *Prog Nucleic Acid Res Mol Biol.* 1990; 38: 37–89.
47. Albers RW, Siegel, GJ. Membrane transport, *In*: Brady ST, Siegel GJ, Albers RW, Price D, (Eds.). *Basic Neurochemistry: Principles of Molecular, Cellular and Medical Neurobiology,* 8th edn. Massachusetts, USA: Elsevier Academic Press. 2012; p. 41–62.
48. Beal MF. Aging, energy and oxidative stress in neurodegenerative diseases. *Ann Neurol.* 1995; 38: 357–366.
49. Lehotsky J, Kaplan P, Racay P, Matejovicova M, Drgova A, Mezesova V. Membrane ion transport systems during oxidative stress in rodent brain: Protective effect of stobadine and other antioxidants. *Life Sci.* 1999; 65: 1951–1958.

50. Jha R, Mahdi AA, Pandey S, Baquer NZ, Cowsik SM. Age-related changes in membrane fluidity and fluorescence intensity by tachykinin neuropeptide NKB and A$\beta$ (25 - 35) with 17$\beta$-estradiol in female rat brain. *Am J Exp Clin Res.*2014; 1: 25–30.
51. Man HY. The sodium pump: Novel functions in the brain. *Biochem Anal Biochem.* 2012;1: e116.
52. Wang X, Dykens JA, Perez E, Liu R, Yang S, Covey DF, Simpkins JW. Neuroprotective effects of 17β-estradiol and non-feminizing estrogens against $H_2O_2$ toxicity in human neuroblastoma SK-N-SH cells. *Mol Pharmacol.* 2006; 70: 395–404.
53. Guerini D. The $Ca^{2+}$ pumps and the $Na^+/Ca^{2+}$ exchangers. *BioMetals.* 1998; 11: 319–330.
54. Spitzer NC, Kingston PA, Manning TJ, Conklin MW. Outside and in: development of neuronal excitability. *Curr Opin Neurobiol.* 2002; 12: 315–323.
55. Zaidi A. Plasma membrane $Ca^{2+}$-ATPases: Targets of oxidative stress in brain aging and neurodegeneration. *World J Biol Chem.* 2010; 1: 271–280.
56. Shutov L P, Kim MS, Houlihan P R, Medvedeva Y V, Usachev YM. Mitochondria and plasma membrane Ca2+-ATPase control presynaptic $Ca^{2+}$clearance in capsaicin-sensitive rat sensory neurons. *J Physiol.* 2013; 591: 2443–2462.
57. de Sousa BN, Kendrick ZV, Roberts J, Baskin SI. $Na^+/K^+$-ATPase in brain and spinal cord during aging. *Adv Exp Med Biol.* 1978; 97: 255-258.
58. Kumar P, Kale RK, Baquer NZ. Estradiol modulates membrane-linked ATPases, antioxidant enzymes, membrane fluidity, lipid peroxidation, and lipofuscin in aged rat liver. *J Aging Res.* 2011; 2011: 1–8.
59. Petrovic S, Milosevic M, Drakulic D, Grkovic I, Stanojlovic M, Mitrovic N, Horvat A 17β-Estradiol modulates mitochondrial $Ca^{2+}$ flux in rat caudate nucleus and brain stem. *Neuroscience.* 2012; 220: 32–40.
60. Sanchez J C, Lopez-Zapata D F, Francis L, De Los Reyes L. Effects of estradiol and IGF-1 on the sodium calcium exchanger in rat cultured cortical neurons. *Cell Mol Neurobiol.* 2011; 31: 619–627.
61. Cipriano DJ, Wang Y, Bond S, Hinton A, Jefferies KC, Qi J, Forgac M. Structure and regulation of the vacuolar ATPases. *Biochim Biophys Acta.* 2008; 1777: 599–604.
62. Nelson N. Structure, molecular genetics, and evolution of vacuolar $H^+$-ATPases. *J Bioenerg Biomembr.* 1989; 21: 553–572.
63. Hammond C. Ionic gradients, membrane potential and ionic currents. *Cell Mol Neurophysiol.* 2015; 39–54.
64. Menzikov SA, Menzikova OV. Effects of orthovanadate and genistein on the plasma membrane $Cl^-$-ATPase sensitive to GABA-ergic ligands in the bream (*Abramis brama* L.) brain. *Doklady Biol Sci.* 2002; 385: 334–336.
65. Michaelis EK, Michaelis ML, Chang HH, Kitos TE. High affinity $Ca^{2+}$-stimulated $Mg^{2+}$-dependent ATPase in rat brain synaptosomes, synaptic membranes, and microsomes. *J Biol Chem.* 1983; 256: 6101–6108.
66. Cooper EC, Jan LY. Ion channel genes and human neurological disease, recent progress, prospects, and challenges. *Proc Natl Acad Sci U.S.A.* 1999; 96: 4759–4766.
67. Boyer DD, Chance B, Ernester L, Mitchell P, Racker E, Slater EC. Oxidative phosphorylation and photophosphorylation. *Annu Rev Biochem.* 1977; 46: 955–1026.