Assessment of acetylcholinesterase enzyme (AChE) activity in the developing brain of TSD lizard, *Calotes versicolor* (Daud.)

Abstract

Acetylcholinesterase (AChE) is a critical enzyme in the neuronal cholinergic system of vertebrates. It is one of the efficient cholinesterasesthatis involved in the termination of acetylcholine-mediated neurotransmission, rapidly hydrolyzing acetylcholine into acetate and choline. Barring a few reports on the distribution/presence of AChE enzyme activity in the adult reptilian species there are nil reports on the presence of this enzyme activity in the developing brain. Hence, the present investigation aims to evaluate AChE enzyme activity in the developing brain of anoviparous lizard, *Calotes versicolor* which exhibitsa novel Female-Male-Female-Male (FMFM) patternof temperature-dependent sex determination (TSD). The eggs were collected during breeding season, padded with moist cotton, and incubated in an incubator at 30^{o} C. The whole brain from the embryos was collected during different developmental stages from oviposition (Stage 27) to hatching (stage 42). The AChE enzyme activity was quantified in the brain, according to Ellman's protocol using a spectrophotometer. The findings reveal that the onset of AChE enzyme activity is observed as early as at oviposition, which reflects the early action of AChE activity in the developing brain. The observed resultsalbeit indirectlysuggest the involvement of AChE enzyme activity in the morphogenetic process.Further, an exponential increase in AChE activity during the post-gonadal differentiation phase indicates age-related elevation in AChE enzyme activity which in turn unveils its involvement in neuronal transmission of the embryonic brain.Besides, this specific AChE enzyme activity in the developing brain of *Calotes versicolor* represents a conserved pattern for the cholinergic system in vertebrates.

Keywords: Acetylcholinesterase enzyme, oviparous lizard, embryonic brain

1. Introduction

The cholinergic system plays a significant role in neuronal function, transmitting information regarding the peripheral status to the central nervous system (CNS) and vice versa. The central players of this system are acetylcholine (ACh) and its enzyme

acetylcholinesterase (AChE) enzyme. Acetylcholine-mediated neurotransmission [1,2] is fundamental for nervous system functioning. Acetylcholinesterase (AChE) is a critical enzyme in the neurological system of vertebrates, mainly found at neuromuscular junctions and cholinergic synapses [3]. AChE belongs to the family of cholinesterase (ChES), and is involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid thus inactivating acetylcholine, thereby regulating the concentration of the neurotransmitter at the synapses**.**

Further, it has been reported that during development AChE mediates and regulates important morphogenetic and physiological events in rats and chicken [4,5,6,7,8], besides playing a vital role in the pulse transmission process in the developing brains [9].

Many reptiles, including most turtles, some lizards, and all crocodilians, exhibit temperature-dependent sex determination (TSD), in which the incubation temperature and sex steroids determine the sex of the developing embryo. The oviparous lizard, *Calotes versicolor,* is an interesting model to study sex determination and differentiation as it exhibits a novel Female-Male-Female-Male pattern of TSD. Further, in TSD reptiles, the developing brain has been proposed as a sensor of temperature. Since AChE plays a vital role in cholinergic transmission in the central and peripheral nervous system, and it is also used as a marker for cholinergic functions**,** the authors wish to know the onset and functional role of AChE in the neuronal transmission process in the developing brain of the lizard, *C. versicolor*. Barring tworeports on the topographical distribution of acetylcholinesterase and butyrylcholinesterase in the diencephalon, mesencephalon and cerebral hemisphere of the adult lizard, *C. versicolor*[10,11], there are no reports on the possible role AChE in the neuronal transmission process in the developing brain of any reptilian species. Hence, given the above-described scientific background and rationale, the present study aims to know:

- the onset of AChE activity in developing brain of the lizard, *C. versicolor* in order to better understand the evolutionary pattern of the cholinergic system during development.
- thevariation in AChE activity if any, in the brain during different developmental stages

2. Materials and Methods

2.1 The animal model and Developmental stages

Calotes versicolor, a polyautochronic, multiclutched lizard, exhibits an extended breeding phase (May to October). Gravid female lizards possessing oviductal eggs were caught during breeding season from the areas around Dharwad (15^{o}, 17'N, 75^{o}, 30'E),

Karnataka, India. They were maintained in reptile dwellings, food (grasshopper/cockroaches), and water were supplied *ad libitum*. The development of embryos from stage 1 to stage 26 takes place when the eggs are still in the oviduct of the mother. Oviposition occurs at embryonic stage 27 and hatching takes place at stage 42, and it normally takes 60-75 days for hatching [12].

2.2. Collection of Animals and Incubation of egg

In all, 20 clutches of eggs (n=320) from twenty gravid females, were obtained during the breeding season, pooled and randomly assigned,padded with moist cotton, and incubated in the incubator at 30°C. Care was exercised to ensure that fluctuation in the incubation temperature did not exceed 0.5°C with a relative humidity of 62%. Other incubation methods have already been described elsewhere[13]. The eggs were incubated from oviposition (stage 27) to hatching (stage 42). Developmental progress was monitored regularly and the stages of embryonic development were determined as per the criteria previously described for *C. versicolor*[12].All the experiments were conducted by the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines .

2.3.Sample collection and Acetylcholinesterase assay

The whole brain was collected during the breeding season from the developing embryos from oviposition (Stage 27) till hatching (Stage 42). The dissected whole embryonic brain tissue was immediately frozen at -80^{o}c and stored until for further use.

The whole brain was homogenized in 0.1M phosphate buffer (pH 7.6) using 20 mg brain tissue/10 ml of buffer. Following homogenization, the resultant mixture was subjected to centrifugation at 3000 rpm for 10 minutes at 4°C. The supernatant obtained was used as the source for AChE activity. The enzymatic activity was assessed according to the protocol described by Ellman *et al*.,1961 [14] and modified by Gorun *et al*., 1978 [15]. In brief, the incubation mixture comprising of 100µl of the brain homogenate was mixed in a 96 microplate well, followed by the addition of 20µl of the substrate, 8mM Acetylthiocholine iodide (Sigma Aldrich, USA). The incubation period was set at 30 minutes at room temperature. The enzymatic reaction was terminated by adding 180µl of 5', 5'-dithiobis nitro-benzoate reagent (DTNB - Sigma Aldrich, USA) which acts as chromogen. The change in absorbance at 412 nm was immediately measured using a spectrophotometer (Thermo Scientific^{TM} Multiskan Sky Microplate Spectrophotometer, USA). The change in absorbance was eventually converted into enzyme activity by utilizing a previously derived Ellman's formula. All measurements were made in triplicates.

2.4 Statistical analysis

The selected variables (data) were presented as mean ± SE. Statistical analysis was performed using One-way ANOVA, followed by Tukey's post-hoc test, to identify any variations in acetylcholinesterase (AChE) activity across different developmental stages, from oviposition to hatching. The statistical analysis was carried out using SPSS software (Version 25.0). Statistical significance was set at $P < 0.05$. Additionally, growth curve of AChE activity from oviposition to hatching was also analysed through polynomial growth curve model.

3. Results

The Acetylcholinesterase (AChE) activity was noticed throughout the embryonic developmental stages from oviposition (stage 27) to hatching (stage 42) (Fig. 1). During the early stages of development, AChE activity showed minimal variation or remained relatively stable from oviposition to gonadal differentiation (stage 34). Following gonadal differentiation, there was a gradual increase in enzyme activity (stage 36), culminating in a significant rise at hatching (stage 42). The enzyme activity exhibited a progressive increase during post-gonadal differentiation in correlation with embryonic development or age. A substantial difference in AChE activity was noted among different developmental stages ($F_{15,32}$=5450.0, $P=0.05$). Since percentage of variation in AChE enzyme activity was almost 99% (R^2 = 0.9938) the observed growth curve can be best fitted with the polynomial curve (Fig.2).

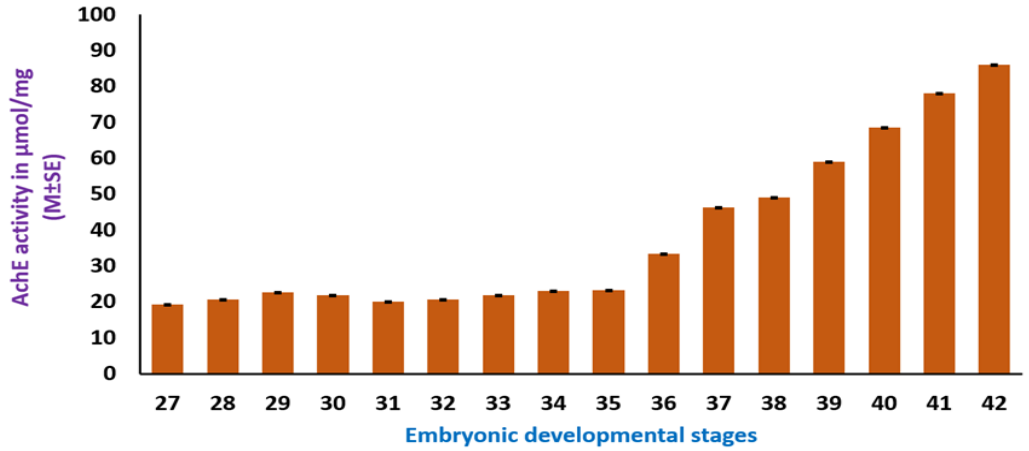

Fig. 1. The AChE activity in the embryonic brain of *C. versicolor* from stage 27 (oviposition) to stage 42 (hatching). The selected variables (data) were presented as mean ± SE.

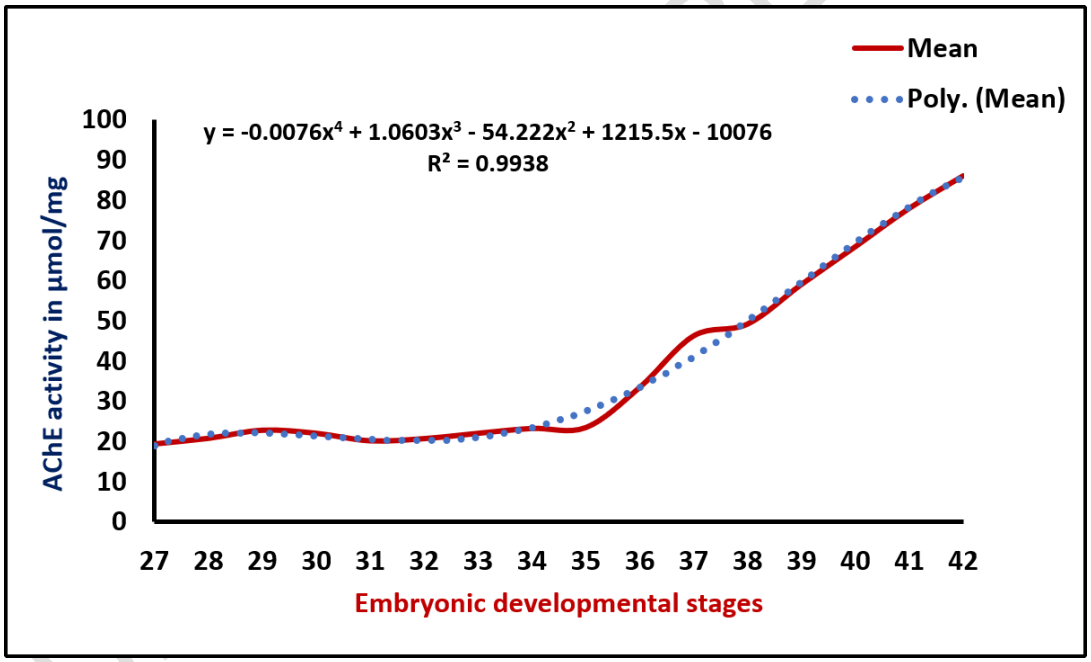

Fig. 2. The Polynomial growth pattern of AChE activity in the embryonic brain of C. versicolor from stage 27 (oviposition) to stage 42 (hatching).

4. Discussion

Analysis ofAcetylcholinesterase (AChE) activity has been the subject of extensive research due to its widespread neuronal and non-neuronal cellular distribution concerning its vital role in cholinergic transmission which is essential for both the vertebrate and invertebrate nervous systems.

The specific activity of AChE was assessed by Ellman's method using a spectrophotometer which is the most widely used procedure for measuring enzyme activity with accuracy [14]. In the present study, the whole brain has been used to quantify AChE enzyme activity during different developmental stages from oviposition to hatching. The findings reveal a distinct pattern of AChE enzyme activity in the embryonic brain during its development stages. The feeble AChE enzyme activity is observed in the embryonic brain as early as at oviposition, suggesting the onset of AChE enzyme activity. The early action of AChE activity demonstrates its non-classical role which corresponds to functional differentiation of the nervous system or brain cell differentiation in turn suggests albeit indirectly its involvement in the morphogenetic process. Similarly, the immuno-expression of AChE activity appeared very early in the ganglia of 3.5-4-day-old chick embryos [16] and in the nervous system of theother vertebrates [4,5,6,7,817,18]. Several lines of research suggest additional roles for cholinergic systems in overall brain homeostasis and plasticity [19].

Further, barring few studies on the topographical distribution of acetylcholinesterase and butyrylcholinesterase activities in the diencephalon, mesencephalon and cerebral hemisphere of the adult lizard, *Calotes versicolor* [10,11]; in the different parts of the adult brain and cerebral spinal cord of *Agama agama* [20] and in neuronal fibers of the undifferentiated gonad of *Lepidochelis olivacea* [21]**,** there are nil reports on the expression/presence of AChE activity in the developing embryos. Further, in the present investigation on the developing brain of *C. versicolor* a slight increase in AChE activity was noticed that remained relatively more or less the same until gonadal differentiation. However, an exponential increase ($F_{15,32}$=5450.0, *P=0.05*) in its activity was noticed from post-gonadal differentiation till hatching. The increased level of AChE activity during the post-gonadal differentiation phases specifies an age-related increase in AChE enzyme activity in the developing brain which in turn unveils its involvement in neuronal transmission. The elevation in AChE activity with the progression of development may reflect the maturation of neural tissue thereby suggesting the augmented cholinergic neuronal transmission. An earlier report on the development of the adrenal gland revealed age-dependent increases in catecholamine-secreting cells specifying an increase in the activity of the sympathetic nervous system [22]. These findings corroborate well with earlier report on chick wherein the onset of AChE enzyme activity in the ganglia commences very early and as development proceeds the developing brain exhibits an increased AChE activity [16]. Likewise, in another study on chick embryos, the specific activity of AChE increased progressively with age in the synaptosomal fraction of the developing brain [9].

Brief findings of the study:

1. Based on the results of present investigation, a distinct pattern of AChE enzyme activity is evident in the embryonic brain throughout the development of *C. versicolor.*
2. The feeble AChE enzyme activity observed in the embryonic brain as early as oviposition indicates the initiation of AChE enzyme activity in the developing brain.
3. The early action of AChE activity demonstrates its non-classical role which corresponds to functional differentiation of the nervous system, in turn suggesting albeit indirectly its involvement in the morphogenetic process.
4. Exponential increase in AChE activity during post-gonadal differentiation phase indicates age-related increase in AChE enzyme activity in the developing brain which in turn unveils its involvement in neuronal transmission.

5. Conclusion:

It is inferred that acetylcholinesterase (AChE) enzyme activity in the developing brain of *Calotes versicolor* unveils its engagement in cholinergic neurotransmission which in turn represents a conserved pattern for the cholinergic system in vertebrates.

Ethical approval:

Ethical approved by the Institutional Animal Ethical Committee [(IAEC), No. 639/GO/02/a/CPCSEA] of the Karnatak University, Dharwad, Karnataka, India.

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