

Original Research Article

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 cholinesterases that is 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 an oviparous lizard, *Calotes versicolor* which exhibits a novel Female-Male-Female-Male (FMFM) pattern of temperature-dependent sex determination (TSD). The eggs were collected during breeding season, padded with moist cotton, and incubated in an incubator at 30° 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 results albeit indirectly suggest 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 [4-8], 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, *Calotes versicolor*. Barring two reports on the topographical distribution of acetylcholinesterase and butyrylcholinesterase in the diencephalon, mesencephalon and cerebral hemisphere of the adult lizard, *Calotes 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.
- the variation 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°, 17'N, 75°, 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 and as approved by the Institutional Animal Ethical Committee [(IAEC), No. 639/GO/02/a/CPCSEA] of the Karnatak University, Dharwad, Karnataka, India.

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°C and stored until for further use.

The whole brain was homogenized in 0.1M phosphate buffer (pH of 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 nitrobenzoate reagent (DTNB - Sigma Aldrich, USA) which acts as chromogen. The change in absorbance at 412 nm was immediately measured using a spectrophotometer (Thermo Scientific™ 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 \pm 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 (Fig. 1). 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).

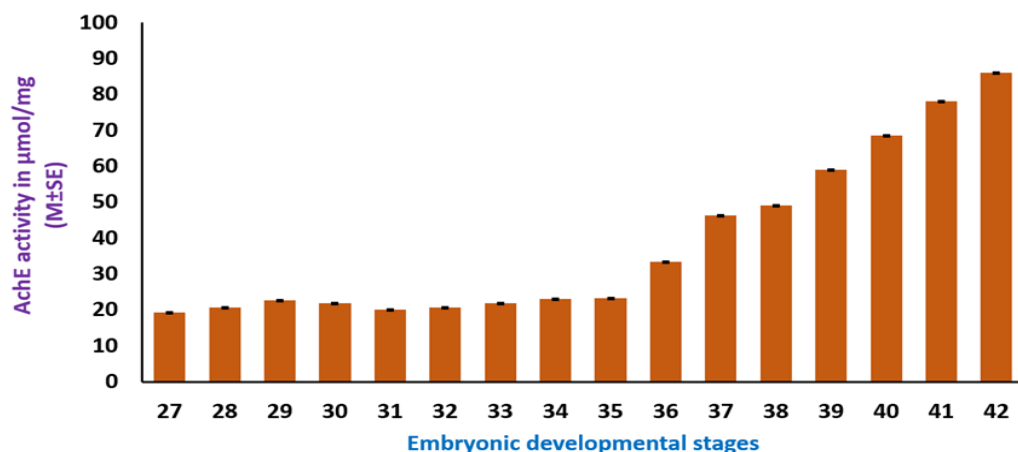


Fig. 1. The AChE activity in the embryonic brain of *C. versicolor* from stage 27 (oviposition) to stage 42 (hatching). The selected variables (data) were presented as mean \pm SE.

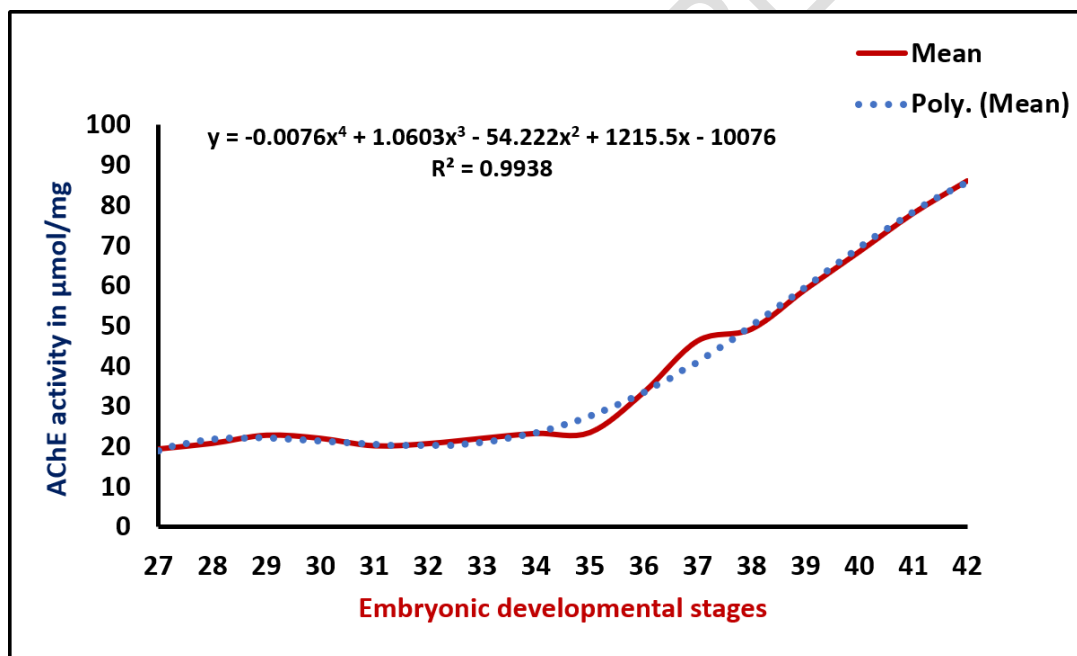


Fig. 2. The Polynomial growth pattern of AChE activity in the embryonic brain of *C. versicolor* from stage 27 (oviposition) to stage 42 (hatching).

4. Discussion

Analysis of Acetylcholinesterase (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 [14] using a spectrophotometer which is the most widely used procedure for measuring enzyme activity with accuracy. 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 throughout the development. 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 the other vertebrates [4-8, 17-18]. 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 *Calotes 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 above results of present investigation reveals a distinct pattern of AChE enzyme activity in the embryonic brain throughout the development of *C. versicolor*.
2. The feeble AChE enzyme activity in the embryonic brain as early as at oviposition suggests the onset 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 AChE enzyme activity in the developing brain of *Calotes versicolor* unveils its involvement in cholinergic neurotransmission which in turn represents a conserved pattern for the cholinergic system in vertebrates.

References

1. Dale, H. The action of certain esters and ethers of choline, and their relation to muscarine. J. Pharmacol Exp. Therap. 1914; 6, 147-190.
2. Soreq H, Seidman S. Acetylcholinesterase--new roles for an old actor [published correction appears in Nat Rev Neurosci 2001 Sep;2(9):670]. *Nat Rev Neurosci*. 2001;2(4):294-302. doi:10.1038/35067589.
3. Lionetto MG, Caricato R, Calisi A, Giordano ME, Schettino T. Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. *Biomed Res Int*. 2013;321213. doi:10.1155/2013/321213.
4. Drews U. Cholinesterase in embryonic development. *Prog Histochem Cytochem*. 1975;7(3):1-52.

5. Lamers WH, te Kortschot A, Los JA, Moorman AF. Acetylcholinesterase in prenatal rat heart: a marker for the early development of the cardiac conductive tissue? *Anat Rec.* 1987;217(4):361-370. doi:10.1002/ar.1092170407.
6. Layer PG. Cholinesterases during development of the avian nervous system. *Cell Mol Neurobiol.* 1991;11(1):7-33. doi:10.1007/BF00712798.
7. Ross LS, Parrett T, Easter SS Jr. Axonogenesis and morphogenesis in the embryonic zebrafish brain. *J Neurosci.* 1992;12(2):467-482. doi:10.1523/JNEUROSCI.12-02-00467.1992.
8. Nakamura T, Ikeda T, Shimokawa I, Inoue Y, Suematsu T, Sakai H, Iwasaki K, Matsuo T. Distribution of acetylcholinesterase activity in the rat embryonic heart with reference to HNK-1 immunoreactivity in the conduction tissue. *Anat Embryol (Berl).* 1994;190(4):367-73. doi: 10.1007/BF00187294.
9. Iqbal Z, Talwar GP. Acetylcholinesterase In Developing Chick Embryo. *J Neurochem.* 1971;18, 1261–1267.
10. Tewari HB, Sethi JS. Topographical distribution of acetylcholinesterase and butyrylcholinesterase in the diencephalon and mesencephalon of the garden lizard (*Calotes versicolor*). *J Neurosci Res.* 1977;3(1):21-35. doi:10.1002/jnr.490030104.
11. Sethi JS. Topographical distribution of acetylcholinesterase and butyrylcholinesterase in the cerebral hemisphere of the garden lizard, *Calotes versicolor*. *J Neurosci Res.* 1979;4(1):35-43. doi:10.1002/jnr.490040106.
12. Muthukkaruppan V, Kanakambika P, Manickavel V, Veeraraghavan K. Analysis of the development of the lizard, *Calotes versicolor*. I. A series of normal stages in the embryonic development. *J Morphol.* 1970;130(4):479-489. doi:10.1002/jmor.1051300407.
13. Inamdar Doddamani LS, Vani V, Seshagiri PB. A tropical oviparous lizard, *Calotes versicolor*, exhibiting a potentially novel FMFM pattern of temperature-dependent sex determination. *J Exp Zool A Ecol Genet Physiol.* 2012;317(1):32-46. doi:10.1002/jez.718.
14. Ellman GI, Courtney KD, Andres V Jr, Feather-Stone Rm. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol.* 1961; 7:88-95. doi:10.1016/0006-2952(61)90145-9.
15. Gorun V, Proinov I, Baltescu V, Balaban G, Barzu O. Modified Ellman procedure for assay of cholinesterases in crude enzymatic preparations. *Anal Biochem.* 1978;86(1):324-326. doi:10.1016/0003-2697(78)90350-0.

16. Torrão AS, Carmona FM, Lindstrom J, Britto LR. Expression of cholinergic system molecules during development of the chick nervous system. *Brain Res Dev Brain Res.* 2000;124(1-2):81-92. doi:10.1016/s0165-3806(00)00113-9.
17. Koenigsberger C, Hammond P, Brimijoin S. Developmental expression of acetyl- and butyrylcholinesterase in the rat: enzyme and mRNA levels in embryonic dorsal root ganglia. *Brain Res.* 1998;787(2):248-258. doi:10.1016/s0006-8993(97)01507-2.
18. Yew DT, Chan WY. Early appearance of acetylcholinergic, serotonergic, and peptidergic neurons and fibers in the developing human central nervous system. *Microsc Res Tech.* 1999;45(6):389-400. doi:10.1002/(SICI)1097-0029(19990615)45:6<389: AID-JEMT6>3.0.CO;2-Z.
19. Hampel H, Mesulam MM, Cuello AC, Farlow MR, Giacobini E, Grossberg GT, et al. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain.* 2018;141(7):1917-1933. doi: 10.1093/brain/awy132.
20. Owasoyo JO, Iramain CA, Egbunike GN. Effect of thyroidectomy on acetylcholinesterase activity in brain areas and adenohipophysis of mature male rats. *IRCS Med Sci.* 1979;7, 57.
21. Gutiérrez-Ospina G, Jiménez-Trejo FJ, Favila R, Moreno-Mendoza NA, Granados Rojas L, Barrios FA, et al. Acetylcholinesterase-positive innervation is present at undifferentiated stages of the sea turtle *Lepidochelis olivacea* embryo gonads: implications for temperature-dependent sex determination. *J Comp Neurol.* 1999; 19;410(1):90-8. doi:10.1002/(sici)1096-9861(19990719)410:1<90:aid-cne8>3.0.co;2-7.
22. Doddamani LS. Development of the adrenal gland in the tropical lizard *Calotes versicolor*. *Gen Comp Endocrinol.* 2000;117(1):89-102. doi:10.1006/gcen.1999.7385.

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