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Histoenzymological Distribution Pattern of Acetylcholinesterase in the Rhombencephalic Brain Centres of *Channa Punctatus*

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Abstract : Rhombencephalon or hind brain of fishes is interesting in many facts as it presents an intricate system of complex neuronal cytoarchitecture, controlling complex physiological and behavioural patterns of the body in very sophisticated way. Study of neuronal architecture of fishes is also of utmost significance because gradual significant modifications in the structure of brain led to complex and highly evolved mammalian brains passing through different vertebrate classes. However, despite a complex cytoarchitecture of fish brain, anatomical differentiation among brain centers and nuclei is poor. Acetylcholinesterase (E.C.3.1.1.7) which is an important brain enzyme belonging to hydrolase group which splits the neurotransmitter acetylcholine in to choline and acetate, thus it is an effective marker of cholinergic and cholinoceptive neurons, giving vivid demarcation of brain areas. Present study has been carried out to histochemically map the different hind brain centers of an Indian non Catfish *Channa punctatus* by employing a modified histochemical technique to visualize the acetylcholinesterase containing neurons. Interestingly, except few most of the hind brain nuclei and centers exhibited strong activity for acetylcholinesterase thereby representing the dominance of cholinergic and cholinoceptive centers. Present study has also been compared with that of other vertebrates studied earlier to trace a phylogenic relationship in the light of recent cytoarchitectonic, and functional studies.

Key Words: Cholinergic, Cholinoceptive, cytoarchitecture, Acetylcholinesterase, Histochemistry.

Introduction

Rhombencephalon or the hind brain of fish consists of the IVth and Vth divisions of the brain called the metencephalon or cerebellum and mylencephalon or medulla oblongata respectively. Metencephalon develops as a large dorsal outgrowth from the hind brain and is only partly visible externally as corpus cerebella. Its main function appears to be the maintenance of the body posture during swimming by coordinating muscular activities. The myelencephalon is well developed in all teleosts and is highly diversified among Catfish and non Catfish (Tripathi and Rahman, 2018) depending upon the relative importance of various senses in them. Fifth to tenth cranial nerves arise from this part of the brain which is therefore associated with both sensory and motor impulses from different body parts.

In the present study by using acetylcholinesterase (AChE) as an effective marker for cholinergic and cholinoceptive neurons, a detailed enzymatic distribution pattern has been studied which exhibited differential grade of activity among different nuclei of the rhombencephalic centres thereby demarcating various brain centers, nuclear groups, fiber tracts and neuropil areas clearly. Previous studies have been done on the distribution pattern of AChE among vertebrate groups including mammals (Krnjevic and Silver, 1964; Bennet *et al.,* 1966; Ishi and Friede, 1967; Bhatt and Tewari, 1978; Giris, 1980), aves (Cavanagh and Lolland,1961; Zuschratter and Scheich,1990; Cookson *et al.,* 1996; Sadananda, 2004) and reptiles(Sethi and Tewari, 1976; Sethi and Tewari,1977; Subhedar, 1990; Tripathi and Srivastava, 2007). Studies available on the distribution pattern of AChE among complex nuclear groups of fish (Contestebile and Zannoni, 1975; Northcutt and Butler, 1993; Tripathi *et al.,* 2013; Tripathi and Rahman, 2014; Tripathi and Rahman, 2018) are inadequate and scattered, particularly in the hind brain nuclei. In addition, The organization, homology and phylogeny of actinopterigian pallial structures has also been reviewed which is reciprocally connected to many hind brain centres (Tripathi and Prakash, 2020). In the present study different rhombencephalic centers of an Indian non Catfish *Channa punctatus* belonging to class Actinopterigii, order Perciformes, family Channaidae, have been mapped for AChE in the light of recent findings and present results have also been compared with other vertebrates studied earlier to establish a homology among brain centers.

Materials and Methods

Twelve male adult *Channa punctatus* of 14 to 16c.m. in length and 35to 45gram in weight were used for present investigation. Prior to their dissection animals were acclimatized according to laboratory conditions at a constant temperature of 30^{0}C. Experimental procedures were performed according to the guidelines of Institutional Animal Ethical Committee (IAEC) of Kulbhaskar Ashram Post Graduate College (KAPG/ZOOL/2012/01). Prior to the dissection of their brains by decapitation method,

fishes were anesthetized with MS-222 (Sigma, St. Lovis, MO). Brains were then fixed in 0.5% paraformaldehyde and 1.5% gluteraldehyde in 0.1M phosphate buffer for 8 hours at 6^0C. The tissue was then given two to four changes in 15% sucrose solution in 0.1M phosphate buffer and stored in the same solution for 3-4 days. 30 micron thick frozen sections were cut by Cryocut (AO Histostat) at -22^0C and stored serially in 0.1 M phosphate buffer. Acetylcholinesterase histochmistry was carried out by employing a modified technique (Hedreen *et al.,* 1985). After washing in 0.1M acetate buffer, pH 6.0, sections were incubated at room temperature for 30 minutes in an incubating mixture comprising 25 mg acetylthiocholine iodide as substrate for enzyme, 32.5 ml 0.1 M acetate buffer (pH 6), 2ml 0.1M sodium citrate, 5 ml 0.03M cupric sulphate, 9.5 ml double distilled water, 1 ml 0.005M potassium ferrycyanide and 0.2m M ethopropazine (sigma) as an inhibitor of non specific esterases. After incubation sections were given five changes of acetate buffer followed by 1% ammonium sulphide. Sections were then given five changes of 0.1M sodium nitrate then exposed briefly to 0.1% silver nitrate followed by five changes of 0.1M sodium nitrate again. Sections were then rinsed in acetate buffer and mounted in glycerin. The dark brown coloured patches in the sections designated AChE activity. Controlled experiments were also performed by omitting the substrate from the incubating mixture. The serial sections after processing were photographed by Computer aided microphotographic camera at different magnifications (4X, 10X).

Results

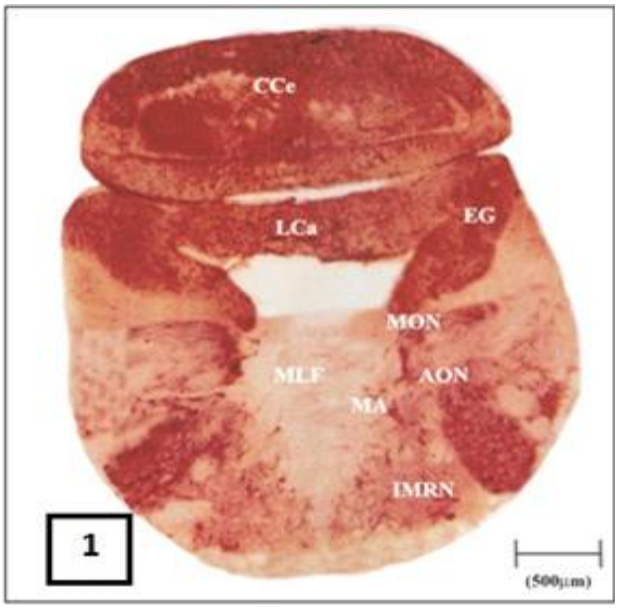
Most of the rhombencephalic nuclei showed intense activity for AChE from rostral to caudal regions. The intensity of AChE activity for different nuclear groups has been shown in table-1.

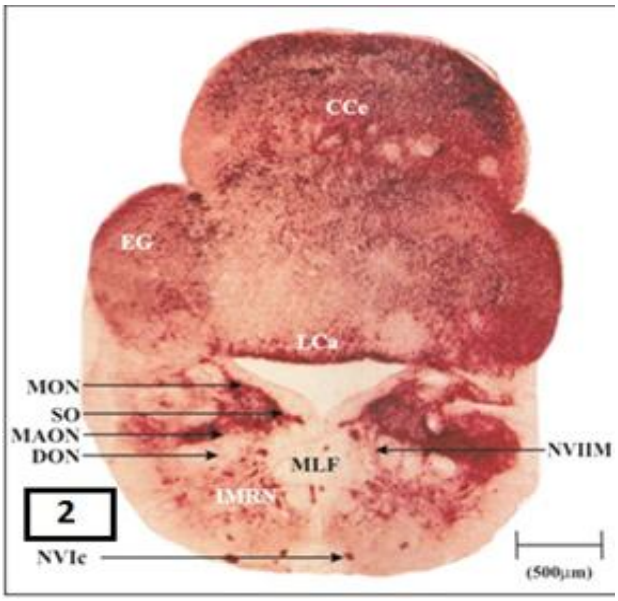
Table: 1-Acetylcholinesterase containing nuclei in the rhombencephalon of *C. Punctatus*

Sl. No.	Name of Nuclei	Abbreviation	AChE Intensity	Figure No.
1	Corpus cerebelli	CCe	+++	1
2	Lobus Caudalis	LCa	+ + +	1,2
3	Eminentia granularis	EG	+ + +	1,2
4	Medial longitudinal fascicle	MLF	--	1,2
5	Facial motor nucleus	NVIIm	+ + + +	6, 7E
6	Intermediate raphe nucleus	IMRN	+ + + +	1-2,
7	Inferior raphe nucleus	IRN	+ + +	3,5,6,7F
8	Caudal abducens nucleus	NVIc	+ + +	2
9	Mauthener cells	MA	+ + +	4,7D
10	Medial octavolateral nucleus	MON	+ + +	1,2,7A
11	Anterior octavolateral nucleus	AON	+ +	1,4
12	Octavolateral area	OA	++	4,5,7D
13	Magnocellular octaval nucleus	MAON	+ + +	2,7A
14	Descending octaval nucleus	DON	+ +	2,6,7A
15	Secondary octaval nucleus	SO	+ + +	2,7A
16	Secondary gustatory nucleus	SGT	+ + + +	6,7B,7C
17	Cerebellar crest	CC	+ + + +	5,6

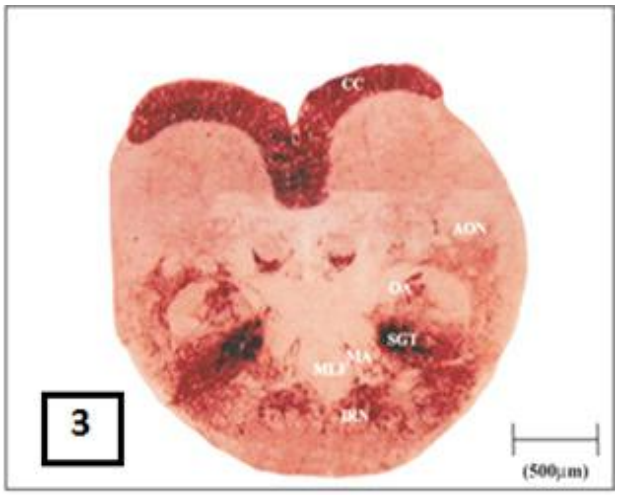
Notation: ++++ = Very Intense; +++ = Intense; ++ = Moderate; +— = Mild; - - = Negative

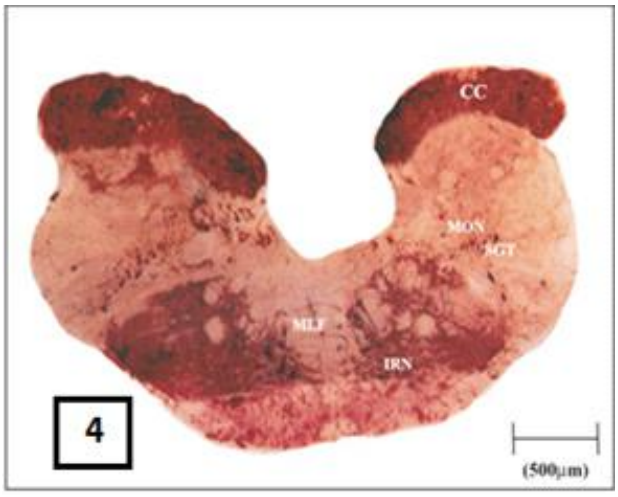
Among the cerebellar subdivisions which are prominent in rostral regions exhibited very intense reaction for AChE. Corpus cerebelli exhibited diffused but very intense reaction (Fig.1-2), though the three layers namely molecular layer, purkinje layer and inner granular layer are not distinct unlike many Catfishes. Lobus caudalis (LCa) which comprises medium sized cells showed intense activity while eminentia granularis (EG) also showed intense reaction for AChE. (Fig. 1-2).



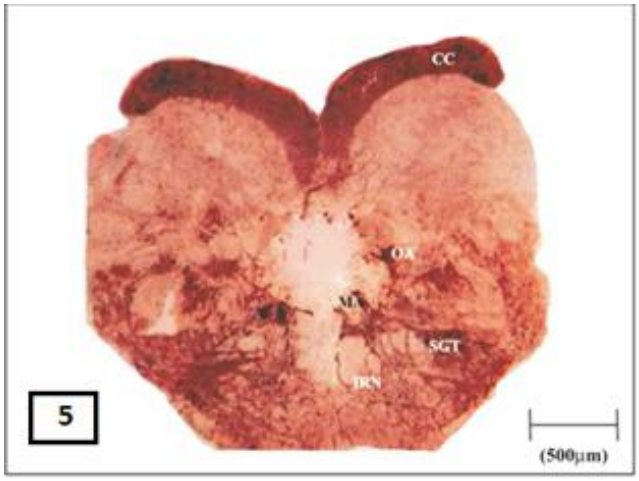


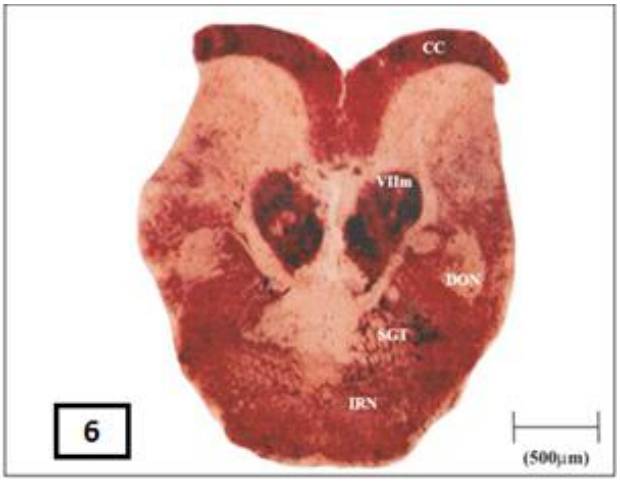
Figs.: 1-2: Photomicrographs of 30 µm thick cryocut transverse sections passing through rostral region of rhombencephalon of C. *punctatus* showing AChE containing nuclei (4X).





Figs. 3 & 4 - Photomicrographs of 30 µm thick cryocut transverse sections passing through middle region of rhombencephalon of C. *punctatus* showing AChE containing nuclei (4 X).





Figs 5 & 6: Photomicrographs of 30 µm thick cryocut transverse sections passing through caudal region of rhombencephalon of C. *punctatus* showing AChE containing nuclei (4 X).

Within the medulla oblongata, the motor nuclei of the cranial nerves, the nuclei of octavolateral area, raphe nuclei and reticular nuclei showed very high intensity for AChE (table-1). Medial octavolateral nucleus (MON) which is located ventral to EG, showed intense activity for AChE particularly in their cell bodies (Fig. 1-2, 7-A). Medial longitudinal fascicle (MLF) which is extended rostro-caudally showed almost negativity for AChE (Fig. 1-2), The fascial motor nucles (nVIIm) which is located ventral to octavolateral area and becomes more prominent in caudal sections showed very high intensity for AChE in their cell bodies as well as neuropil areas (Fig. 6, 7-E). Caudal abducens nucleus ((NVIc) also showed high intensity (Fig. 2).

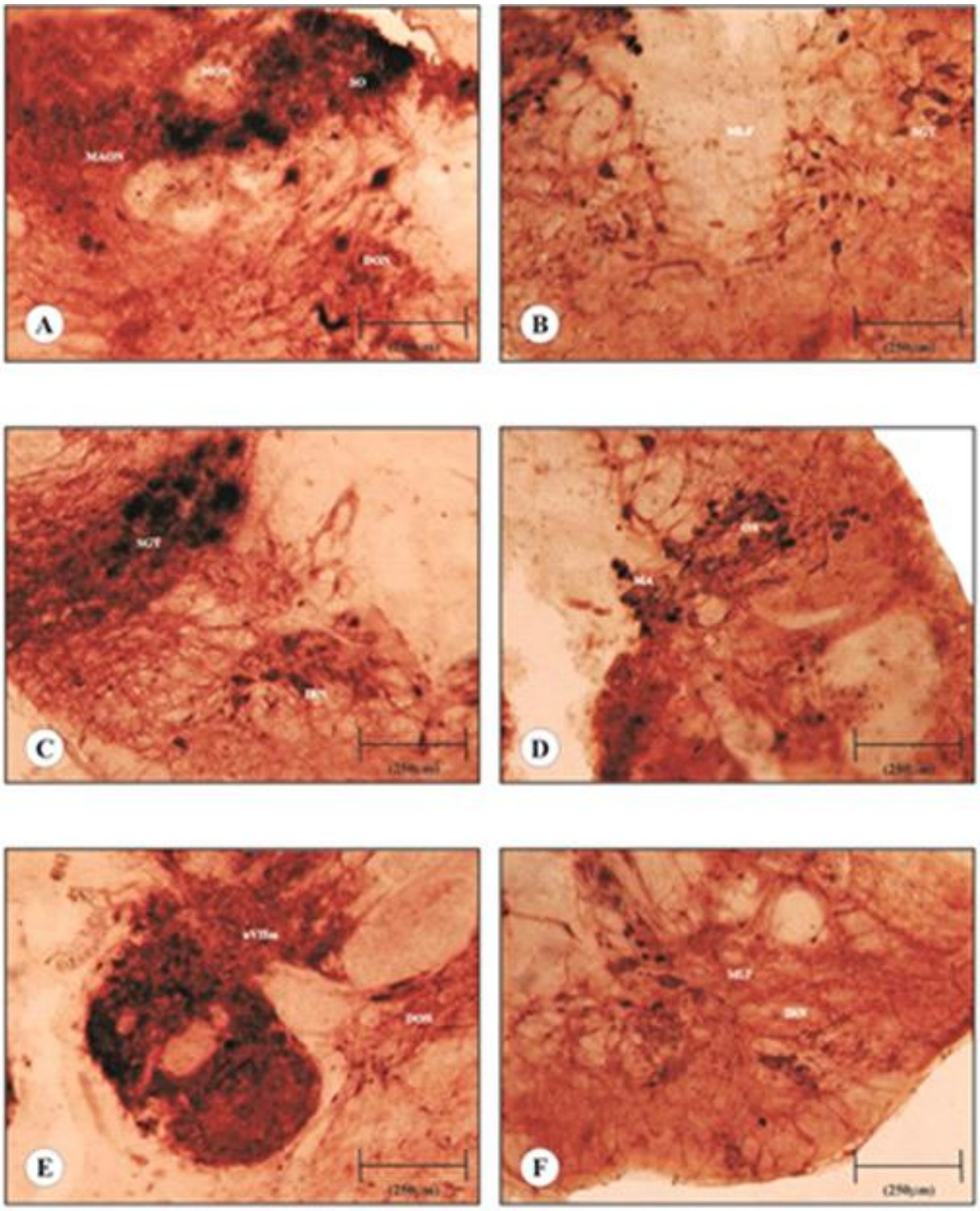


Fig 7- (A-F): Photomicrographs of 30 µm thick cryocut transverse sections in higher magnification, passing through rhombencephalic area showing AChE positive nuclei, dendrites, neuropil and fiber tracts (10X)**.**

The arrangement and intensity for AChE of mauthner cells (MA) was very compact in cell bodies and their dendrites are oriented towards ventrolateral direction of octavolateral area (OA) (Fig. 4 & 7-D). Octavolateral area also showed intense activity for AChE (4-5, 7-D). The inferior raphe nucleus (IRN) and intermediate raphe nucleus (IMRN) are highly prominent subzones in *C. punctatus* and showed highly extended axonal processes and dendritic arborizations to adjacent nuclei with large sized somata. These two regions showed very high intensity (Fig.1-3, 5, 6, 7-F).

Among the octavolateral nuclei, the arrangement of cell bodies and their location is very dense with intense dendritic network. The medial and anterior octavolateral nuclei showed very high intensity for AChE (Fig. 1-2, 4, 7-A). The descending octavolateral nucleus (DON) however showed mild reaction for AChE (Fig. 2, 6, 7-A). The secondary octavolateral nucleus (SO) showed large sized somata with intense activity for AChE (Fig. 2, 7-A). Among the caudal rhombencephalie nuclei, secondary gustatory tract (SGT) was very prominent in rostral sections with large sized somata and ventraly oriented dendrites and it showed moderate reaction in rostral sections while in the caudal parts this nucleus showed high intensity with dense cluster of cell bodies (Fig. 6, 7-B, 7-C). Cerebellar crest (CC) is very prominent which is the dorsal most region of caudal rhombencephalic sections.It showed very compact arrangement of cell bodies and demonstrated very high intensity for AChE (Figs. 5, 6).

Discussion

The results of the present study give an insight on the differential distribution pattern of enzyme acetylcholinesterase in the different subdivisions and nuclear groups of the rhombencephalon of *Channa punctatus.* These results primarily show the intense presence of cholinergic and cholinoceptive neurons. Present observations are comparable to that of other vertebrates in order to trace the phylogeny of the brain. In present results AChE is present almost in all layers of cerebellum in diffused form. It has also been shown to be present in the purkinje cells in zebra fish (Clement *et al.,* 2004), but in mammals it is present despite the absence of cholinergic neurons (Appleyard and Jahnsen, 1992) because it enhances the response of purkinje cells to excitatory amino acids secreted by granule cells of cerebellum. In the present study facial motor nucleus and caudal abducens nucleus showed intense activity, these motor nuclei are reported to be cholinergic in lampreys (Pombol *et al.,* 2001), elasmobranchs (Anadon *et al.,* 2004), teleosts (Ekstrom,1997; Brantley and Bass, 1988), amphibians (Morin *et al.,* 1997) , reptiles (Tripathi and Srivastava, 2010; Tripathi, 2007), birds (Cookson *et al*., 1996; Sadananda, 2004), and mammals (Tago, 1989; Woolf, 1991). It is suggested therefore that motonurons of cranial nerves are cholinergic throughout vertebrate phylogeny. In the present study intermediate raphe nucleus and inferior raphe nucleus showed very strong reaction for AChE like zebra fish studied earlier (Clement *et al.,* 2004) but interestingly in zebra fish cholineacetyl-transferase (ChAT) immunoreactive cells which are the marker of cholinergic neurons, were absent. In cyprinids these two nuclei receive innervations from optic tectum and cerebellum (Grover and Sharma, 1981; Luiten, 1981; Wullimann and Northcutt,1988) Cholinergic neurons are reported to be present in intermediate and inferior raphe nucleus in lampreys (Pombol *et al.,* 2001), elasmobranchs (Anadon *et al*., 2004), teleosts (Ekstrom, 1987; Brantley and Bass, 1988) , amphibians (Morin *et al.,* 1997), reptiles (Tripathi and Srivastava, 2010; Tripathi, 2007), birds (Cookson *et al.,* 1996; Sadananda, 2004), and mammals (Tago, 1989; Woolf, 1991). It is presumed therefore that these two nuclei have cholinergic centres. Mauthner cells which are very prominent in AChE activity and are located adjacent to octavolateral area, mediate fast escape motor responses after receiving vibrational or visual stimuli (Eaton and Bombardieri, 1978) in order to escape from predators. These cells are also AChE positive in zebra fish (Clement *et al,* 2004) showing its complex motor coordination. In the octavolateral area medial, magnocellular and secondary octaval nuclei showed very intense reaction while anterior and descending octaval nuclei showed moderate reaction but in overall picture the entire octavolateral area is rich in AChE. It is reported that the presence of cholinergic nuclei within the octaval region is a conserved phenomenon in most of the fishes (Clement *et al.,* 2004). In other vertebrate groups cholinergic neurons appear in very concrete regions (Cookson *et al*., 1996; Sadananda, 2004; Morin *et al.,* 1997; Tago *et al*., 1989). It is suggested therefore that the presence of cholinergic cells in the octaval region is a primitive feature of vertebrates which might have reduced in tetrapods. In addition, the nuclei of octavolateral area receive ChAT immune-reactive innervations which could reveal the presence of cholinoceptive cells within these nuclei (Anadon *et al.,* 2004). The secondary gustatory nucleus projects to hypothalamus in teleosts (Clement *et al.,* 2004) but also to another diencephalic nuclei that has different names but these are homologically similar (Wullimann, 1988; Yoshimoto *et al.,* 1998; Kanwal *et*

*al.,*1988; Lamb and Caprio, 1993). Further a homology and phylogeny of actinopterigian pallial structures has also been reviewed (Tripathi and Prakash,2020) which reveals the cholinergic connections among pallial and hind brain nuclei. It is presumed therefore that different regions of the secondary gustatory nucleus possess different targets to the diencephalon, which needs further hodological investigation. Furthermore, Many observations have shown that AChE plays many noncholinergic roles in addition to its main role i.e. hydrolysis of acetylcholine. It hydrolyses substance P, met-and leu-enkephalin (Chub *et al.,* 1980; Chub *et al.,* 1982) and could degrade other neuropeptides as well. This could explain the very widespread staining observed in different nuclear areas which are even noncholinergic.

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