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Histoenzymological Distribution of Acetylcholinesterase in the Rostral Rhombencephalon of *Heteropneustes fossilis*

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ABSTRACT

Acetylcholinesterase (AChE) is an enzyme belonging to hydrolase group which splits the neurotransmitter acetylcholine in to choline and acetate. It is supposed to be a marker of cholinergic and cholinoceptive neurons. In the present investigations, a modified histochemical technique to visualize acetylcholinesterase containing neurons has been employed to map the rostral rhombencephalic nuclei of Heteropneustis fossilis. Based on intensity of reaction, it is interesting to mention that in the present study, most of layers of cerebellum and nuclei of medulla oblongata showed intense activity for acetylcholinesterase.The present results have been discussed in the light of recent cytoarchitectural and hodological studies. In addition, the functional significance of the variable distribution pattern of enzyme has also been discussed.

Key words: *Acetylcholinesterase, Rhombencephalic, Cerebellum, Octavolateral area*

INTRODUCTION

Rhombencephalon of fishes consists of metencephalon or cerebellum and myelencephalon or medulla oblongata respectively. Acetylcholinesterase (AChE) histochemistry is an effective technique to demarcate various centres and nuclear groups of the brain which are often cytologically less differentiated among lower vertebrates including fish. The distribution pattern of cholinesterases has been studied in many mammalian[1-5], avain[6-9] and reptilian[10-13] species. Studies on the distribution pattern of AChE in fishes[14-16] particularly Indian teleosts are in scanty. Authors of the present study have previously described the distribution pattern of AChE in the caudal rhombencephalic nuclei of *Heteropneutes fossilis*[17]. Present work is the extension of the previous study[17].In the present study different layers of the cerebellum and different nuclear groups of medulla oblongata have been thoroughly described. In the last few years a lot of cholinergic[18] and non cholinergic[19-23] roles of AChE, have come in to light, that are functionally correlated with its variable distribution in the different cerebellar and medulla oblongata centres.

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MATERIALS AND METHOD

In the present study, six adult male Heteropneustis fossilis of ideal length and weight were used. Prior to dissection, fishes were acclimatized for laboratory conditions for one week at a constant temperature of 280 C. Guidelines of the Institutional Animal Ethics Committee (IAEC) were followed for experimental procedures. Animals were anesthetized with MS-222 (sigma, st. Lovis, MO) and brains were quickly removed by employing decapitation method. Brains were post fixed in a 0.1M phosphate buffer solution comprising of 0.5% Paraformaldehyde and 1.5% Glutaraldehyde for 6 hours at 40 C. The tissue was then given 2-3 changes in 15% sucrose solution in 0.1M phosphate buffer and stored in the same solution for 2-3 days. 30 micron thick frozen sections were cut by Cryocut (A O Histostat) at - 22^0C and stored serially in 0.1 M phosphate buffer. AChE histochemistry was carried out by using a modified histochemical technique[24]. After washing in 0.1 M acetate buffer, pH 6.0, sections were incubated at room temperature for 30 minutes in an incubating medium made up of 25 mg acetylthiocholine iodide as substrate for AChE, 32.5 ml 0.1 M acetate buffer (pH 6.0), 2 ml 0.1M sodium citrate, 5 ml 0.03 M cupric sulphate, 9.5 ml double distilled water, 1 ml 0.005 M potassium ferricynide and 0.2 m M ethopropazine (sigma) as an inhibitor of non specific esterases. After incubation, sections were given five changes of acetate buffer (pH 6.0) then treated with 1% ammonium sulphide. Sections were then given five changes of 0.1 M sodium nitrate then exposed to 0.1% silver nitrate followed by five changes of 0.1 M sodium nitrate again. Sections were then rinsed in acetate buffer and mounted in glycerine. The dark brown coloured patches appeared in sections which designated AChE activity. Controlled experiments were also performed by omitting the substrate in the present histochemical study to confirm enzyme substrate reaction.

RESULTS AND DISCUSSION

Rostral rhombencephalon comprises cerebellum and medulla oblongata; later further comprise reticular nuclei, raphe nuclei and octavolateral area. Most of the rostral rhombencephalic nuclei showed highest density of AChE activity. AChE positive cells were abundant throughout the isthmus, octavo-lateral area, reticular nuclei and motor nucleus of the cranial nerves. Among the rostral nuclei, the isthmus nucleus (IN) which is diffused with the ventral trigeminal nucleus demonstrated very high intensity (Fig.1). The enzyme activity in the various nuclear groups of rostral rhombencephalon has been shown in table 1.

Cerebellum:

Cerebellum of *Heteropneustis fossilis* comprises of three parts: The Valvula cerebella, the carpus cerebelli and the lobus vesitibulolateralis which is formed by the lobus caudalis and the eminentia granularis. AChE positive neurons were seen in all three parts.

1. Vulvula cerebelli :

It is divisible in to medial, lateral and central sub divisions. Valvula medialis (Vam) showed small to medium sized, ovoid, somata with dense AChE intensity. Vulvula lateralis (val) represented small sized, densely packed cells with very high AChE intensity. Vulvula centralis (Vac) which consists of diffused cells showed highly intensity, for AChE. All the subdivisions are connected through intensely stained dendritic and axonal extensions (Fig. 1).

2. Corpus Cerebelli :

Three distinct layers are present in the corpus cerebelli of *Heteropneustes fossilis*. The outermost thick molecular layer (ML) showed fainted activity for AChE. The irregularly arranged purkinje cells (PC) which constitute the intermediate thin layer, showed strong reaction in their cell bodies which were ovoid or pyriform in shape. The inner granular layer (GL) also demonstrated moderate reaction for AChE (Fig. 1-2).

3. Lobus Vestibulolateralis :

It is composed of lobus caudalis and the eminentia granuleris. Lobus caudalis (LCa) showed intense activity while eminentia granularis (EG) demonstrated moderate

activity in whole rostro- caudal extensions (Fig. 2-3).

Medulla Oblongata (MO): Within the medulla oblongata, the motor nuclei of cranial nerves, the octaval efferent nucleus, the descending octaval nucleus and other nuclei of octavo-lateral area, raphe nuclei and reticular nuclei showed AChE positive neurons.The rostral most portions of both the dorsal and ventral parts of the trigeminal motor nucleus showed very high intensity for AChE (Fig.1). Facial motor nerve (NVIIm) which is located ventral to secondary octaval nucleus in caudal sections showed medium sized somata with ventrally and ventro-laterally oriented dendritic processes. This nucleus demonstrated very high activity for AChE (Fig. 3).

Reticular formation:

It consists of reticular nuclei, raphe nuclei and the mauthner cells. The two rhombencephalic subdivisions (intermediate and inferior) of the reticular nucleus presented AChE positive neurons. This presented very large nuclear area which is rostrocaudally extended adjacent to medial longitudinal fascicle (MLF) (Fig.4). Intermediate reticular nucleus (ImRN) showed very large sized, round or ovoid somata with high dendritic processes extending almost in all adjacent areas including octavolateral area. This nucleus showed very high intensity (Fig. 3-4).

Superior Raphe Nucleus (SRN):

It presents a large group of cells in the rostral rhombencephalic regions. This cell group showed very high intensity for AChE (Fig.1).The medial and the lateral longitudinal fascicle were totally devoid of AChE in entire rostrocaudal extensions (Fig 1-2).

Octavolateral area:

This area of rhombencephalon showed one of the highest densities of AChE positive neurons in presently studied animal (Fig.3). Octavalateral efferent nucleus (OEN) showed numerous and intensely stained pyriform neurons. These cells showed ventrally or ventrolaterally oriented long dendrites (Fig.3). AChE positive neurons of small size were observed in medial octavolateral nucleus (MON); AChE distribution was homogenous in the whole rostrocaudal and mediolateral extension of this nucleus (Fig.2-3). A group of AChE positive cells was observed in the central portion of the anterior octave nucleus (AON) (Fig. 2).In the rostromiddle rhombencephalic parts, magnocellular octaval nucleus (MaON) showed large sized AChE positive neurons and highly ramified axonal and dendritic processes extended to other nuclei of medulla oblongata (Fig. 3-4). Descending octaval nucleus (DON) which is located ventral to MaON showed moderate intensity for AChE (Fig. 3), but it received dendritic processes from secondary octaval nucleus (SO) and magnocellular octaval nucleus (MaON) which were AChE positive (Fig. 3). Secondary octaval nucleus (SO) showed intense activity at all levels (Fig. 3-4)

DISCUSSION

The isthmus nucleus in *Heteropneustes* showed strong activity for AChE in present results. This nucleus is reported to contain cholinergic neurons in all vertebrate groups analysed. The isthmic nucleus is cholinergic in lampreys[25], elasmobranchs[26], teleosts[27-30]amphibians[31], reptiles[32-33] and birds[8-9, 34]. The homologue of the isthmic nucleus in mammals, the parabigeminal nucleus is also cholinergic[35, 36, 37]. A cholinergic connection of isthmic nucleus/parabigeminal nucleus to the optic tectum/superior colliculus has been observed in mammals[37] birds[34] reptiles[32-33] amphibeans[31] and teleosts[27-30]. Thus the cholinergic nature of the isthmic nucleus and its connection of the visual pathway seem to be a well conserved feature throughout phylogeny.The superior reticular nucleus also showed very strong reaction for AChE. Acetylcholinesterase positive cells were also observed in the superior reticular nucleus of zebrafish[27] which also showed ChAT (choline-acetyl transferase) positive cells in the same nuclei. These findings are in agreement with the observations described in other teleosts[27-30]. This nucleus projects to the superficial pretectal nucleus, contralateral preoptic nucleus and optic tectum in other cyprinids[30] andthese nuclei and regions are reported to be

AChE positive in present investigations.Among the cerebellar layers, the purkinje and the granule cells are the only AChE positive/ChAT immune-negative neuronal types studied in other fishes (*Phoxinus*[29] *Danio*[27]).Transient ChAT expressions in purkinje cells have been described in the early postnatal development of the rat[38]. ChAT positive neurons in adult animals have been previously identified as Golgi cells in dog fish and Cat cerebellum[26, 39.] Nonetheless, cholinergic cells were not observed in the cerebellum of teleosts, amphibians, reptiles, birds or mammals[27]. AChE positive granule cells were observed in several fish species[27] and in other vertebrates[40, 41]. However granule cells are not described as cholinergic in any of the fish analysed[26, 27-29]. Our results also suggest about the cholinergic nature of purkinje layer but non cholinergic nature of other two layers. The cerebellum has been described as one region where AChE exists beyond the requirements or in the absence of cholinergic transmission in mammals[42]. In guinea pig AChE enhances the response of purkinje cells to excitatory amino acids released by granule cells[42]. It has been described that fish granule cells use the excitatory neurotransmitter glutamate in their synapses with purkinje cells[43]. We suggest therefore that in presently studied fish AChE may have the above mentioned function as in zebrafish[27]. In addition, the cytoarchitectonic properties of the teleostean cerebellar cortex and its input-output characteristics are so similar to other vertebrates that it probably sub serves functions in motor learning and coordination as well[44].In the medulla oblongata, motor neurons of abducens, fascial and dorsal and ventral parts of trigeminal motor nuclei are reported to be cholinergic in lampreys[25] elasmobranch[26,] teleosts[29-30] amphibians[31] reptiles[32-33] birds[8-9] and mammals[35]. It is suggested therefore that motoneurons of cranial nerves are cholinergic throughout vertebrate phylogeny. Intermediate and inferior reticular nuclei displayed very strong AChE activity in our study. Similar results were obtained in zebrafish[27] but no ChAT immunoreactive cells were detected in zebrafish. In cyprinids, the afferents from optic tectum are reported in these two nuclei[45-46]. These two reticular nuclei also receive afferents from cerebellum[47]. Cholinergic cells are reported to be present in intermediate and inferior reticular nuclei in lampreys[25] elasmobranch[26] teleosts[29-30] amphibeans[31] reptiles[32-33] birds[8-9] and mammals[48-49]. It is presumed therefore that these two nuclei are cholinergic in nature.In the teleosts, studied hitherto, cholinergic cells in the octavolateral area are absent or poorly developed[27-29]. Nonetheless the octavolateral area contains abundant cholinergic cells in dog fish[26]. In other vertebrate groups cholinergic cells appear in very concrete regions[8-9, 31, 35]. It is suggested therefore that the presence of cholinergic cells in the octaval region may be a primitive feature of vertebrates. A reduction of these populations is observed in tetrapods whereas teleosts may have lost these populations secondarily[27] on the other hand AChE activity was displayed throughout the rostrocaudal octavolateral area. Thus AChE positive cells were detected in the medial and posterior octavolateral nuclei, secondary octavolateral nucleus and anterior, magnocellular octavolateral nuclei. Nuclei within the ocavolateral area receive profuse ChAT immunoreactive innervations which could mediate the cholinoceptive nature of the AChE positive neurons within the aforesaid nuclei[27].Many findings have shown that AChE hydrolyses substance P, met and leu-enkephalin and could degrade other neuropeptides as well[19-20]. In addition to it, AChE facilitates neurite growth during embryogenesis[21]. It also acts as neuronal adhesion protein[22-23]. These functions are independents of its role in hydrolysing acetylcholine[18], and explain the wide spread staining, observed in different rostral rhombencephalic nuclei which may be noncholinergic or cholinoceptive in nature.

AChE activity in different rostral rhombencephalic nuclei

Sl. No.	Name of Nuclei	Abbreviation	AChE - activity	Fig. No.
1.	Isthmus Nucleus	IN	+ + + +	1
2.	Vulvula medialis	Vam	+ + +	1
3.	Vulvula lateralis	Val	+ + + +	1
4.	Vulvula centralis	Vac	+ + + +	1
5.	Lobus Caudalis	LCa	+ + +	2-3
6.	Eminentia granularis	EG	+ +	2-3
7.	Facial motor nucleus	NVIIm	+ + + +	3
8.	Intermediate reticular nucleus	ImRN	+ + + +	2-4
10.	Caudal abducens nucleus	NVIc	+ + +	3
11.	Superior reticular nucleus	SRN	+ + + +	1
13.	Octavolateral efferent nucleus	OEN	+ + +	3
14.	Medial octavolateral nucleus	MON	+ + +	2-3
15.	Anterior octavolateral nucleus	AON	+ +	2
16.	Magnocellluler octaval nucleus	MaON	+ + +	3-4
17.	Descending octaval nucleus	DON	+ +	3
18.	Secondary octaval nucleus	SO	+ + +	3-4

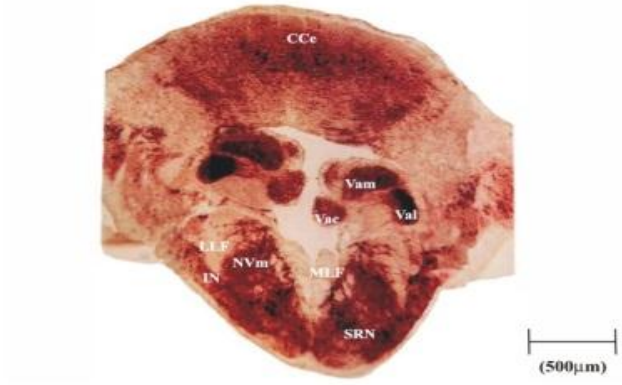

Fig. 1

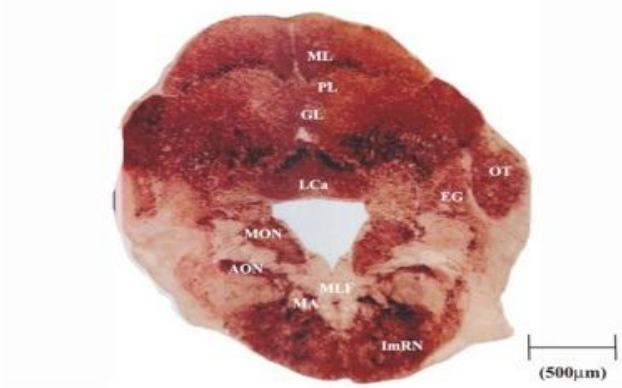

Fig. 2

Fig. 1-2: Photomicrographs of 30µm thick cryocut transverse sections passing through rostral rhombencephalon showing AChE activity in various nuclei. (4X)

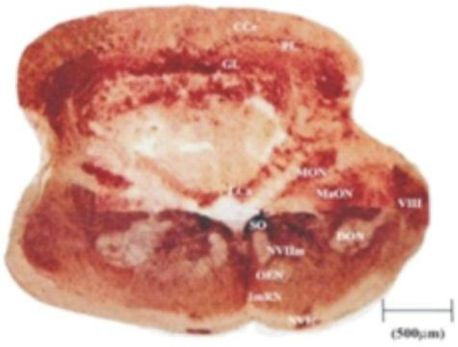

Fig. 3

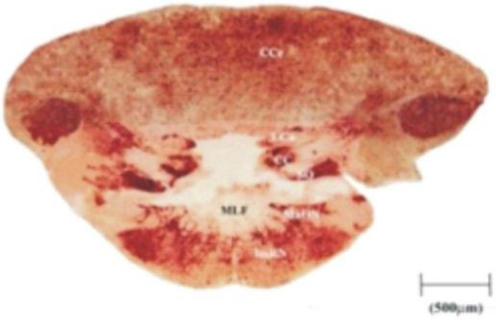

Fig. 4

Fig. 3-4: Photomicrographs of 30μm thick cryocut transverse sections passing through rostro-middle rhombencephalon showing AChE activity in various nuclei. (4X)

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