Original Article

Acetylcholinesterase (AChE) Expression Demonstrate Differentiation of Nuclei in Rat Brain

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Submission Date: 21-04-2022; Revision Date: 02-05-2022; Accepted Date: 13-06-2022.

ABSTRACT

Acetylcholinesterase (AChE) plays an important role in synaptic transmission by catalysing hydrolysis of the neurotransmitter Acetylcholine (Ach). Earlier studies have demonstrated non-enzymatic role of AChE in the neuron development and differentiation, growth, formation of neuromuscular junctions, synaptogenesis and survival. A direct correlation between endogenous AChE and neurite outgrowth in primary DRG (Dorsal Root Ganglion) neurons has been also reported. In this study, we demonstrated a direct correlation between expression of endogenous AChE and differentiation of neurons in different nuclei of 3, 7, 12, 21 and 30 days old rat pup brains. We have observed differential histochemical expression of AChE in Fronto Parietal Cortex- Motor and sensory area, Amygdala Nuclei, Hypothalamic Nuclei, Thalamic Nuclei, Interior Capsule, Caudate Putamen and Hippocampus area at different developmental stages. We suggest that AChE expression in neuron cell bodies might be important indicator of differentiation of the nuclei. We thus propose that independent of its catalytic activity, AChE might be playing important role in early development and differentiation of the nuclei in the brain.

Keywords: AChE (Acetylcholinesterase), Fronto-parietal cortex, Amygdaloid Nuclei, Hypothalamic Nuclei, Thalamic Nuclei, Caudate Putamen.

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INTRODUCTION

Acetylcholinesterase (AChE) is one of the most efficient enzymes of nervous system that hydrolyse the neurotransmitter acetycholine (Ach) into choline and acetate at cholinergic synapses and neuromuscular junctions.[1] Two groups of cholinesterase have been defined on the basis of substrate specificity, one AChE - relatively specific for Ach and found in abundance in the brain. Other one is Butrylcholinesterase (BChE), has broad substrate scope but lower ACh catalytic efficiency and found primarily in the liver with lower concentration present in the brain. AChE is variously distributed in central and peripheral nervous tissues of different vertebrates.[2-6] It has been localized in non- neuronal tissues and glial cell.[7,8] Recently, a non-classical role in vertebrate gut morphogenesis,[9] in erythropoiesis,[10] in bone development, homeostasis and degeneration for AChE have been also reported.[11]

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DOI: 10.5530/ajbls.2022.11.82

Zachary *et al.*, 202[12] has shown that neurotransmitters are involved in the process of neurotransmission, differentiation, the growth of neurons and development of neural circuit. Neurotransmitter may appear at different points of brain development, like monoamines present before the neurons are differentiated. AChE appears to play an important role in neural development,[13] axonal outgrowth,[14] stimulation of neurite outgrowth,[15] synaptogenesis,[16] cell adhesion[17] and in neuronal migration.[18] Thus, neurotransmitters and the neuromodulators play an important role in the shaping and wiring of the nervous system possibly during critical window period of the development. There is increasing evidences that neurotransmitters in the immature nervous system can act as trophic factors that influence different developmental events such as cell proliferation and differentiation. Work of

Ruediger and Juergen, 2007[19] also demonstrates that neurotransmitters can also influences the targeting of migrating neurons and growing axons during formation of neuronal circuit. Biagioni, S. *et al*., 2000[20] has shown that during early development of the Nervous System Acetylcholine neurotransmitter plays an important role and act as a morphogen and as a modulator of neuronal differentiation. A study identifies Acetylcholine as a potential regulator of the somatotropic axis during the developmental period in the hypothalamic region of brain.[21] Another study also confirmed that cholinergic transmission is essential for adaptive behaviour[22] and mediate fundamental cognitive processes[23] during the embryonal development in rodents and show a rapid age-related increase during the first three week of postnatal development.[24] Another study demonstrate that cholinergic system starts to develop in early pre-natal stages.[25] Earlier experiments using *in-vitro* preparation of several neuronal tissues have shown that neurotransmitters such as- Serotonin, Dopamine, Glutamine and Acetylcholine were able to promote or block neurite outgrowth, depending on the neuronal group and the neurotransmitter involved.[26,27] Different plasticity events namely neurite sprouting, establishment of neuronal connection and exclusion of connections profoundly affect the general organization of the developing mature nervous system. Nicotinic acetylcholinesterase receptors also play role in developmental process such as neurite outgrowth and differentiation.[28]

Idea of role for AChE in development was initially based on *in-vivo* observations that AChE is transiently expressed by neurons throughout the periods of axonal outgrowth prior to synaptogenesis, period during which the classical cholinergic role for AChE in terminating nervous transmission is unnecessary. Robertson 1987,[29] demonstrated that there is transient AChE activity occur in thalamic neurons at a time when their axons are growing in cerebral cortex. Similar observation was reported in developing primates.[30] This expression of AChE also confirmed at the messenger RNA level by *in-situ* hybridization.[31] Different studies confirmed the expression of AChE in pleuripotent stem cells, hippocampal neurons, in Chicken and Zebra fish retina during the process of their development and differentiation.[32-34] In the peripheral nervous system (P.N.S.), AChE is transiently expressed by developing dorsal root ganglion (DRG) neurons[35] and later in their axons and growth cones in the spinal cord.[36,37] All major neurotransmitters such as- Acetylcholine, Glutamate, γ-Aminobutyric acid (GABA), Dopamine and Serotonin can act as regulators for growing neurites and migrating neurons in different species.[19] Cyto-chemical data showed the expression of AChE during very early embryogenesis,[38] during embryonic neurite extension and muscle development and also before synaptogenesis.[39,40] Acetylcholine neurotransmitter is widely distributed in the nervous system and plays a critical role in cerebral cortical development, cortical activity and learning and memory processes.[41] Many studies have shown that hippocampal-dependent learning is associated with an increase in hippocampal Acetylcholine levels, thus the elevation of extracellular acetylcholine is thought to reflect hippocampal dependent memory processes.[42]

In view of these studies, we aim to study the histochemical distribution of the AChE in the brain of 3, 7, 12, 21 and 30-days old rat pups with an objective to elucidate the mechanism of differentiation of various brain nuclei.

MATERIALS AND METHODS

Wistar rats (20) were breed in institutional animal house (CPCSEA order No. 973/ac/06). Pups of age of 3 days, 7 days, 12 days, 21 days and 30 days were used for this study. Rat pups were sacrificed by quick de-capitation method, brain was dissected out as rapid as possible and rinsed in cold distilled water and were placed in Calcium Formol (10%) fixative for 16-24 hr at 4°C. Next day brain tissue was transferred to cold sucrose solution (10%, 20% and 30%) for 24 hr in each dilution. After sucrose treatment tissue were rinsed briefly in cold distilled water. Cryostat sections (free floating) were cut at 15μ thickness and placed in petri-plate containing cold phosphate buffer. A continuous series of selected sections were placed in freshly prepared incubation media for 40-45 min as per Karnovasky and Roots method 1964[43] for staining of AChE. Stained sections were rinsed briefly in distilled water and were placed on albumin coated slides. They were allowed to dry for 30-45 min at room temperature. After drying the slides were passed in alcohol series (30%, 50%, 70%, 90% and Absolute Alcohol) for dehydration (10min. in each) and cleared in xylene (1-2 min). Sections were mounted on glass slide in DPX.

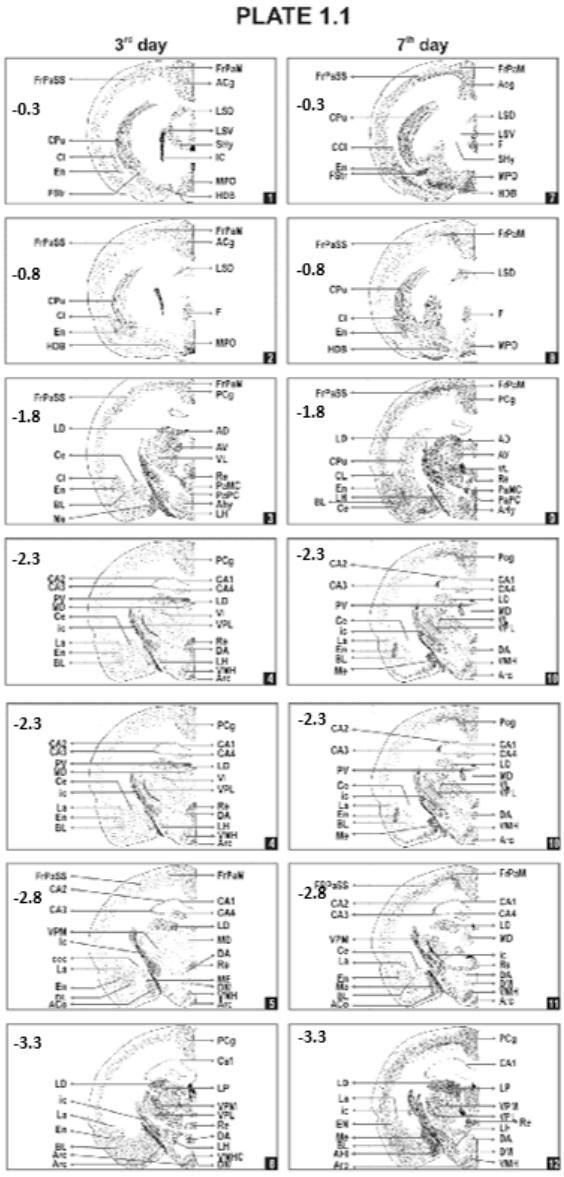
RESULTS

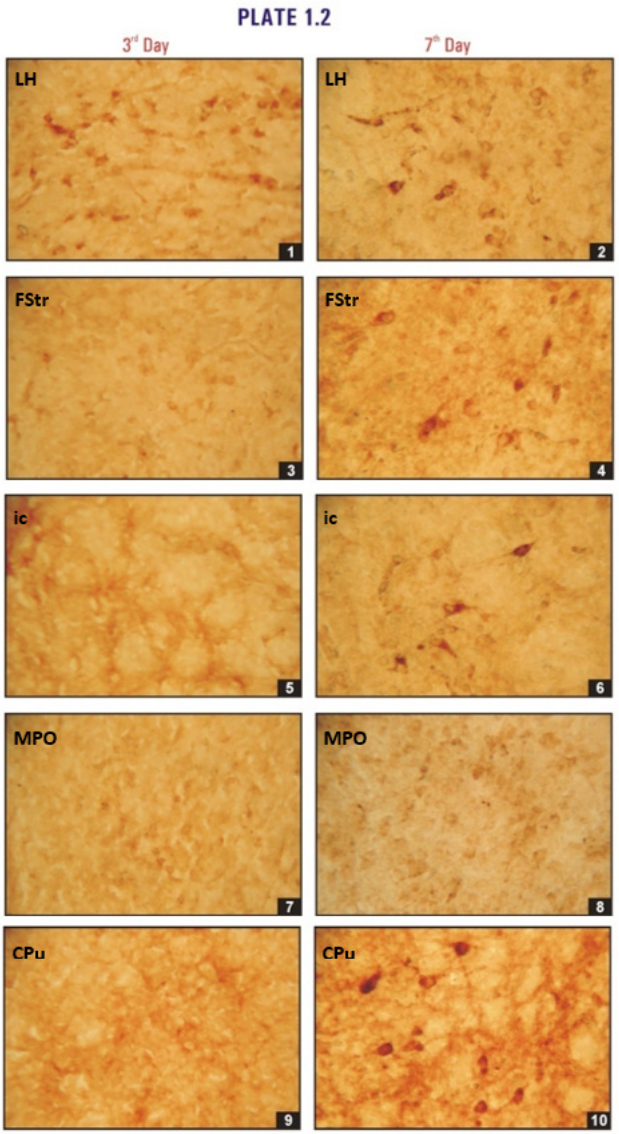
Stained sections passing through different Bregma levels- 0.3mm, 0.8mm, -1.8mm, -2.3mm, -2.8mm and -3.3mm of 3 days, 7 days, 12 days, 21 days and 30 days were selected for analysis. Sections were selected based on presence of following areas- Frontoparietal cortex (Motor and Sensory area), Amygdaloid Nuclei Complex

(Claustrum, Endopiriform, Basolateral, Central, Median and Anterior Cortical Amygdaloid Nuclei), Hypothalamic Nuclei Complex (PaMc- Paraventricular hypothalamic nuclei Magnocellular, PaPC- Paraventricular hypothalamic nuclei Parvocellular, Pe- Periventricular Hypothalamic Nuclei, AHy- Anterior Hypothalamic Nuclei, LH- Lateral Hypothalamic Area), Thalamic Nuclei (AD- Antero Dorsal, Re- Reunion Nuclei, LD- Latero-Dorsal, VL- Ventrolateral, AV- Antero-Ventral, PVA- Paraventricular, Rh- Rhomboid), Interior Capsule Region, Caudate Putamen and Hippocampus Region. Nuclei were identified using Paxinos and Watson rat brain atlas (1986).

Plate 1.1, 1.3, and 1.5

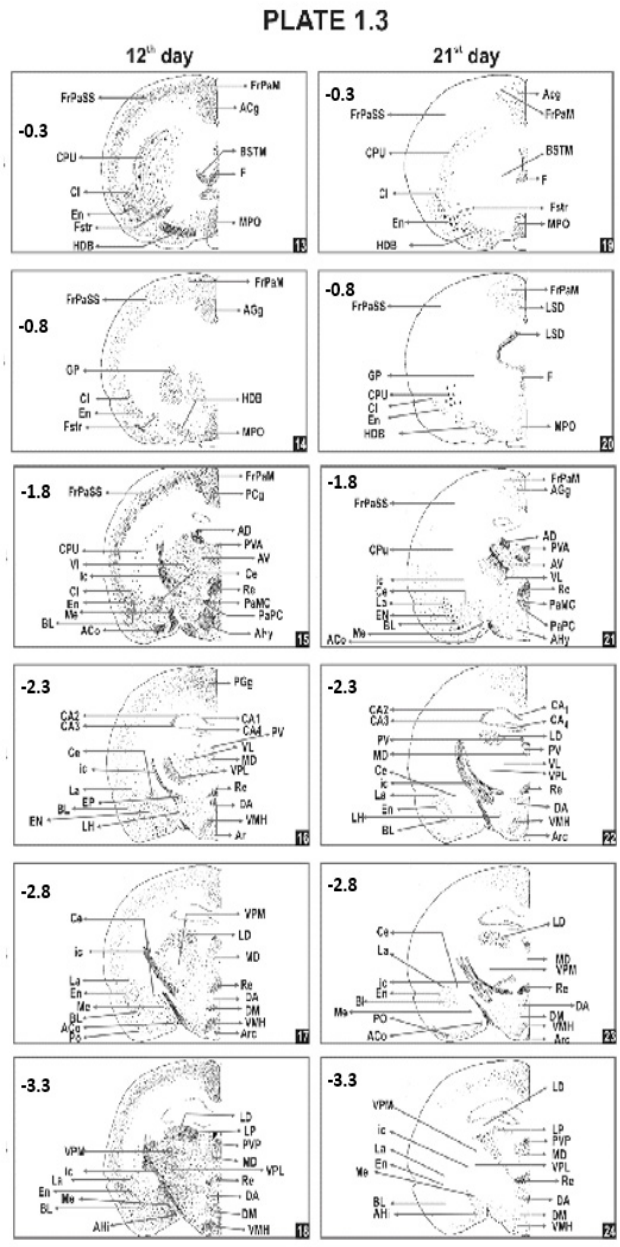
In Frontoparietal cortex (Motor and Sensory area) AChE staining intensity varies from moderate (3rd and

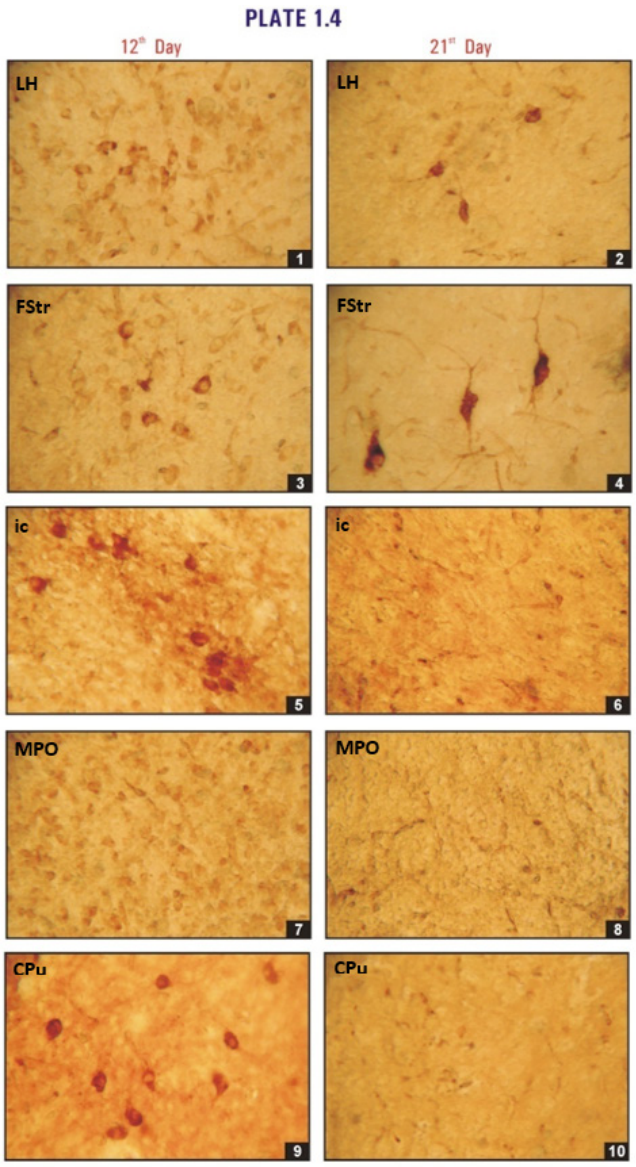




7th day) to Intense (12th day) and weak or absent (21st day and 30th day). Amygdaloid nuclei complex (Cl, En and BL) express moderate to intense AChE activity from 3rd to 30th day but on 21st day very intense AChE activity was observed. In other Amygdaloid Nuclei Ce, Me and ACo showed moderate AChE activity on 3rd and 30th day but on 7th and 12th day very intense and on 21st day weak AChE activity was observed.

Paraventricular Hypothalamic Nucle, Magnocellular (PaMC) showed intense AChE activity in 3rd, 7th, 12th and 30th day but on 21st day it was moderate whereas Parvocellular nuclei (PaPC) showed moderate AChE activity on 3rd, 7th and 30th day but weak activity was observed on 12th and 21st day. In Periventricular hypothalamic area (Pe) AChE intensity varies from moderate to weak from 3rd to 30th day. Anterior Hypothalamic area (AHy) showed weak AChE activity on 3rd, 21st and 30th day but it is moderate on 7th and





intense on 12th day. Lateral Hypothalamic area (LH) showed very intense AChE activity on 3rd, 7th, 12 and 30th day but surprisingly it was weak on 21st day.

Different Thalamic Nuclei like- AD, LD, Re, VL and AV showed intense to very intense AChE activity from 3rd to 30th day of development. In Interior capsule (ic) region very intense AChE activity were observed in neuron cell bodies and intense activity were observed in nerve fibres in 3rd, 7th, 12th and 30th day but both type of activity was totally absent in 21st day of development. Caudate putamen area (CPu) showed moderate AChE activity in 7rd and 12th day but in 3rd, 21st and 30th day activity was absent. Hippocampus area CA1 showed moderate AChE reactivity on 3rd, 7th and 30th day but it was weak on 12 and 21st day, whereas CA3 area showed moderate AChE activity in all age groups and CA2 and CA4 area showed weak AChE reactivity from 3rd to 30th day of development.

Plate 1.2, 1.4, and 1.6

Showed differential expression of AChE intensity in selected rat brain LH, FStr, ic, MPO and CPu areas. Lateral Hypothalamic Area (LH) showed moderate AChE activity in neuron cell bodies as well as in fibers on 3rd and 21st day. On 7th day intense as well as moderate AChE activity was observed in neuron cell bodies only. On 12th day moderate AChE activity was observed in neuron cell bodies. Whereas on 30th day intense AChE activity was observed in neuron cell bodies only.

Fundus Striate (FStr) area showed weak AChE activity in neuron cell bodies as well as in fibres on 3rd day. But on 7th day intense activity were observed in neuron cell and moderate activity were observed in fibres. Intense

PLATE 1.5

30th day

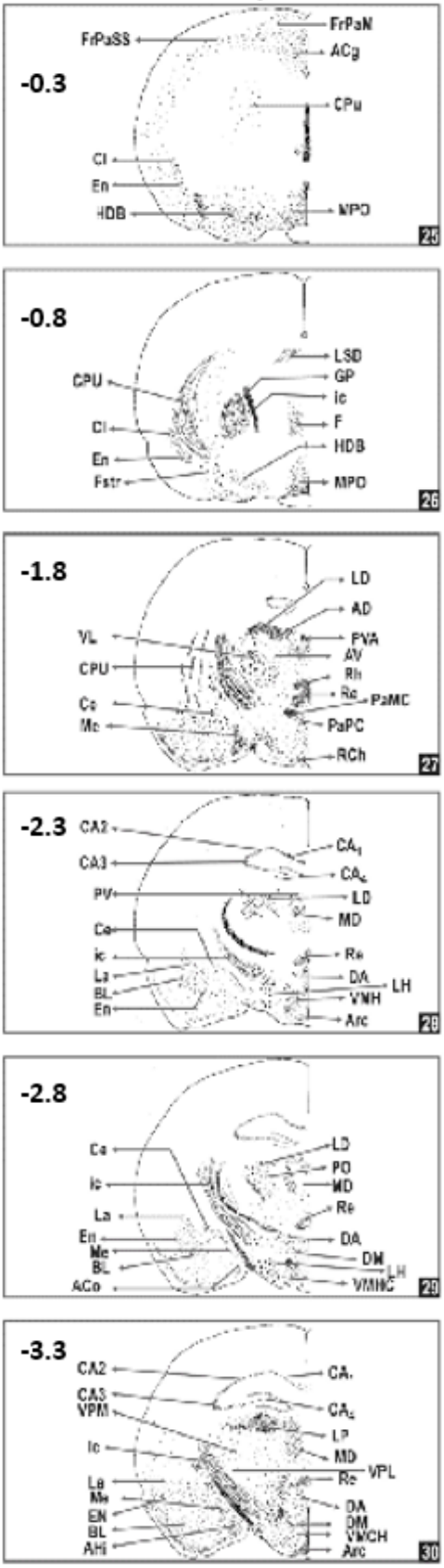
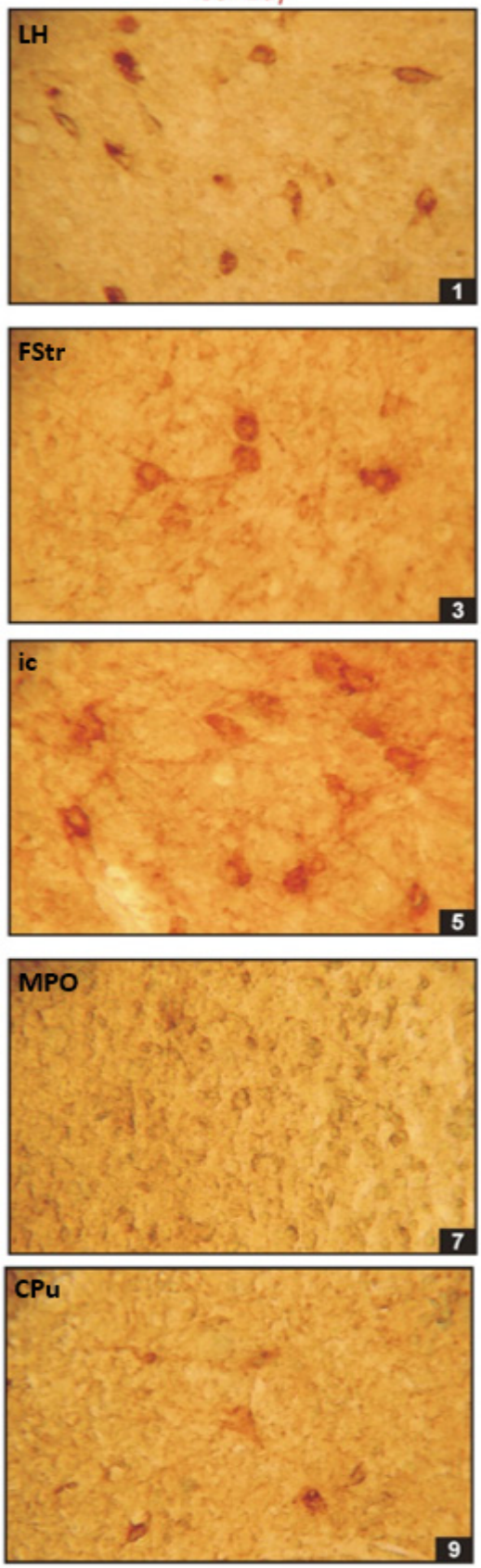


PLATE 1.6

30th Day



as well as weak neuron cell AChE activity were observed on 12th day but moderate activity was observed in neuron cell bodies as well as in fibers on 21st day and intense neuron cell as well as fibers AChE activity were observed on 30th day.

Interior Capsule (ic) region showed moderate AChE activity in neuron cell bodies as well as in fibers on 3rd day but on 7th, 12th and 30th day intense activity was observed in neuron cell bodies and in fibers. On 21st day weak AChE activity was observed. Median Preoptic (MPO) area of thalamic region showed weak AChE activity on 3rd and 21st day, moderate on 7th day and 12th day and 30th day of development. Caudate Putamem (CPu) area showed moderate AChE activity on 3rd and 30th day of development but on 7th day of development intense AChE activity was observed in neuron cell bodies as well as in fibers, whereas only intense neuron cell AChE activity was observed in 12th day. Weak AChE activity was observed on 21st day.

DISCUSSION

Results of our study clearly suggest that AChE expression intensity varies in neuron cell bodies of various nuclei during their development and differentiation. Some nuclei are intense during very early phase of differentiation (Amygdaloid Nuclei- Cl and En, MPO, CPu of 3rd day) but during later development they showed moderate staining or weak staining (Amygdaloid Nuclei- Cl and En, CPu at 7th, 12th, 21st and 30th day), but others may be moderate during early phase of development (Hypothalamic Nuclei- DA, Thalamic Nuclei- LD) at 3rd and 7th day and intense in later development stages (Hypothalamic Nuclei- DA, Thalamic Nuclei- LD) at 21st and 30th day. Neuron cell bodies in some nuclei showed intense staining during middle phase of development (FrPaM, Thalamic Nuclei- SHy, Hypothalamic Nuclei- HDB and AHy and Amygdaloid Nuclei- Me, Ao and BL during 7th and 12 th day) remain weak or moderately intense during early or later phase of development (FrPaM, Thalamic Nuclei- SHy, Hypothalamic Nuclei- HDB and AHy and Amygdaloid Nuclei- Me, Ao and BL during 3rd, 21st and 30th day). These variations in AChE staining/ expression in neuron cell bodies of these nuclei indicate some role of the enzyme in their differentiation or development besides neural transmission. Thus, a pattern of appearance of moderate to intense histochemical reactivity in neuron cell bodies and large processes in few neuron cell bodies in early phases of development and differentiation suggest that in these nuclei neuron cell bodies are fully differentiated and carry out cholinergic activity i.e., secreting Ach and its hydrolysis by AChE. Alternatively, intense nuclei where neuron cell bodies are fully differentiated might be a part of neural network but those which are weak or moderate might not yet develop the synaptic contacts.

Having considered these observations, it is pertinent to analyse the various possible causes for differential staining.

In present investigation most nuclear groups of thalamic regions (AD, CA1, CA3, Re and AV) and hypothalamic region (VMH, Arc, LH, ic, Ce, Me, La and En) were intensely stained for AChE. These might be considered as cholinergic in the light of the finding of Lewis and Shute 1959.[44] Careful analysis of staining pattern in each neuron cell body of different nuclei has shown very interesting observation. In early stages of postnatal development i.e., 7th and 12th day, most of the neuron cell bodies in single nuclei are intensely positive for enzyme reactivity but in later stages of postnatal development mixed population of neuron cell bodies that is neurons in the centre are intensely positive while at periphery are either moderate or weakly positive. Neurons in the central part of nuclei are intensely packed with reactivity but peripheral part showed weak reactivity.

Variation of ACHE reactivity in different nuclei or brain areas could be linked to presence of enzyme species. Fernadez, *et al*., 1999[45] reported presence of multiple AChE species in the brain. There are many AChE species, all of which are encoded by a single gene that is alternatively spliced.[46] Among the multiple mRNAs encoding AChE is one that represents the primary form expressed in brain and muscles. Other forms are present in developing blood cells. AChE mRNAs are present in high abundance in bone marrow, stem cells and in particular peripheral blood cells in certain cases of leukaemia.[47] Bhatnagar and Tewari 1985,[48] demonstrated the presence of AChE isoenzyme expression in brain of 0, 12, 21, 30 and 60 days old rat using PAGE.

Studies of Downs and Granto 2004,[49] Silman and Sussman 2005,[50] have elucidated certain additional non-cholinergic functions of AChE. These wide cholinergic and non-cholinergic roles of AChE provide adequate ground to functionally correlate its distribution in the different nuclei in the brain. Previous evidence also indicate that AChE has extra synaptic function during neural development.[51-53] Robertson and colleagues,[29,53] have demonstrated transient AChE activity in thalamic neurons at a time when their axons are growing in the cerebral cortex. Similar results have been reported by Kristt 1983,[54] in rat and by Kostovic and colleagues

1983,[30] in developing primates. In the chick, transient AChE expression was shown in developing spinal cord neurons which coincided with axonal outgrowths from these cells.[40,51,55] Similar observation was also discussed in the PNS where AChE transiently expressed in developing dorsal root ganglion (DRG) neurons.[35,37,56] Dorsal root ganglion neurons show a transient peak expression of AChE during periods of axonal outgrowth prior to synaptogenesis, suggesting that AChE has a non-enzymatic role during development.[17] It was also observed that over expression of AChE resulted in greater branching at the distal tips of each primary neurite as well as an increase in cell size. These finding further indicated that AChE expressed on the axonal surface of developing DRG neurons may modulate their adhesive properties and thereby support axonal development and suggest that AChE may function in axon extension and cortical differentiation.[57] One more study reported a pathway projecting to the posterior ventral cochlear nucleus (PVCN) of the rat brain that transiently expresses a high level of acetylcholinesterase. AChE is first detectable by postnatal day 3, peeks in expression about P7-P10 and is barely detectable in P15.[58] Result of one more study also confirmed that at the perinatal period both NADPH-d and AChE positive neurons were stained from slight to moderate intensity. During later developmental periods the staining gradually increased and achieved adult level of intensity on the day P2.[59] Another study also confirmed a non-classical role of AChE in the initial formation of axon tracts within the developing vertebrate brain.[60]

Earlier studies on humans also indicated that the immature brain contains many more synapses than the mature brain. For example, within the primary visual cortex, synaptic density increases gradually during late gestation and in early postnatal life. It then displays a steep decrease from 2 to 4 months of age, during which period the density doubles. After 1 year of age, however, there is decline in the synaptic density until adult values (50-60% of the maximum) are attained at about 11 years of age.[61,62] The time course of the decrease in synaptic density varies within different cortical layers. The other cortical areas in the middle frontal gyrus (layer III).[63] This area also shows a postnatal increase in synaptic density followed by decrease, but these changes cut along a long time course in the frontal cortex than in the primary visual cortex. The maximum density of synapses occurs around 1 year of age (compared to 4 months in the visual cortex). Adult values are not obtained until around 16 years of age (compared to 7-11 years for the visual cortex). Overall synaptic density in humans is greater in the frontal and motor cortex than in the visual cortex.[63]

CONCLUSION

In conclusion this study suggested that, in the course of postnatal development from day 1 to day 30th, nuclei and sub-nuclei take their shape as well as get functionally organized. But all this happens in sequence, as few nuclei develop and differentiate early between day 1 to day 7 of postnatal development. Some may develop between day 7 to day 12 of postnatal development and so on. Such development and differentiation of these nuclei is clearly evident by expression of varying AChE reactivity.

ACKNOWLEDGEMENT

This work is highly acknowledged to MLSU Udaipur for providing necessary facilities for the research work.

CONFLICT OF INTEREST

The author declare that there is no conflict of interest.

ABBREVIATIONS

AchE: Acetyl Cholinesterase; **CNS:** Central Nervous System; **LH:** Lateral Hypothalamic Area; **FStr:** Fundus Striate; **ic:** Interior Capsule; **MPO:** Median Preoptic Area; **CPu:** Caudate Putamen; **FrPaM:** Frontoparietal Cortex, Motor Area; **FrPaSS:** Frontoparietal Cortex, Somatosensory Area; **PaMC:** Paraventricular Hypothalamic Nuclei, Magnocelllular; **PaPC:** Paraventricular Hypothalamic Nuclei, Parvocellular; **AHy:** Anterior Hypothalamic Area; **Pe:** Periventricular Hypothalamic Nuclei; **AD:** Anterodorsal Thalamic Nuclei; **Re:** Reunien Thalamic Nuclei; **LD:** Laterodorsal Thalamic Nuclei; **VL:** Ventrolateral Thalamic Nuclei; **AV:** Anteroventral Thalamic Nuclei; **Ce:** Central Amygdaloid Nuclei; **En:** Endopiriform Nuclei; **BL:** Basolateral Amygdaloid Nuclei; **ME:** Median Amygdaloid Nuclei; **ACo:** Anterior Cortical Amygdaloid Nuclei; **HDB:** Nucleus Horizontal Limb Diagonal Band; **DA:** Dorsal Hypothalamic Area; **SHy:** Septo Hypothalamic Nuclei; **Ach:** Acetylcholine; **VMH:** Ventromedial Hypothalamic Nuclei; **Arc:** Arcuate Hypothalamic Nuclei; **DPX:** Ditrene Plasticizer Xylene.

SUMMARY

Result of my study clearly suggest that Acetylcholinesterase histochemical reaction intensity varies in neuron cell bodies of various nuclei distributed

in the whole brain during their development and differentiation. Some nuclei and area Cl, En, ACo, PaMC, PaPC, LH, Rh, ic and MPO are intense during very early phase of differentiation but during later developmeny they show moderate staining or weak staining. Other like FStr, PVA, DA, DM, Po moderate during early phase of development but intense in later development stages. Some nuclei and area like- HDB, Shy, FrPaM, BL, AV, VL, La, Me, Ahi, VMH showed intense staining in neuron cell bodies during middle phase of development but remain weak or moderate during early or late phase of development. In fact only those nuclei show intense AChE reactivity are very well developed during respective development stages. These nuclei also show localized distribution of AChE reactivity in cell body and their respective processes. While which show weak or moderate reactivities in the cell body do not show processes. Anatomically also these nuclei appear fully differentiated when they show intense AChE reactivity in cell body and their processes. These variation in AChE staining in neuron cell bodies of various nuclei clearly indicate some role of the enzyme in their differentiation or development besides neural transmission.

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Cite this article: Anju C, Maheep B. Acetylcholinesterase (AChE) Expression Demonstrate Differentiation of Nuclei in Rat Brain. Asian J Biol Life Sci. 2022;11(2):615-23.