Cholinesterases Preceding Major Tracts in Vertebrate Neurogenesis

Paul G. Layer

Summary

The role of acetylcholinesterase (AChE) in neurotransmission is well known. But long before synapses are formed in vertebrates, AChE is expressed in young postmitotic neuroblasts that are about to extend the first long tracts. AChE histochemistry can thus be used to map primary steps of brain differentiation. Preceding and possibly inducing AChE in avian brains, the closely related butyrylcholinesterase (BChE) spatially foreshadows AChE-positive cell areas and the course of their axons. In particular, before spinal motor axons grow, their corresponding rostral sclerotomes and myotomes express BChE, and both their neuronal source and myotomal target cells express AChE. Since axon growth has been found inhibited by acetylcholine, it is postulated that both cholinesterases can attract neurite growth cones by neutralizing the inhibitor. Thus, the early expression of both cholinesterases that is at least partially independent from classical cholinergic synaptogenesis, sheds new light on the developmental and medical significance of these enzymes.

Introduction: Two Classes of Cholinesterases – What For?

Two types of cholinesterases are expressed in all vertebrates. They can be easily distinguished on the basis of their substrate and inhibitor specificities. The classical function of acetylcholinesterase (AChE; E.C. 3.1.1.7) is to degrade acetylcholine at cholinergic synapses (for reviews, see refs 1,2). However, apart from neuronal tissues, AChE is expressed in many nonneuronal and embryonic tissues, e.g. the red blood cell membrane, migrating neural crest cells or the retinal pigmented epithelium[3–5], where its role is unclear. Butyrylcholinesterase (BChE; nonspecific, or serum-, or pseudocholinesterase, E.C. 3.1.1.8.) is able to hydrolyse a number of higher choline esters as well as acetylcholine and this enzyme activity is enriched in serum, in the liver, in the heart and the brain (see refs 1,2). In brain it has been considered a marker for glia[1,2,6]. Its physiological role is ambiguous, since the prolonged inhibition of this enzyme produces no clear pharmacological effects.

Recent work is reviewed here that has shown that both classes of cholinesterases are expressed at crucial times and places during early neurogenesis, that these enzymes are excellent markers for studying neurogenetic processes, and moreover that they could possibly be involved in regulating these processes.

AChE Follows BChE Expression Immediately after the Last Division of Young Neuroblasts

Long before any synaptogenesis occurs in the chicken neural tube, BChE can be seen to be diffusely distributed along the ventricular layer, while AChE is localized in cells along the mantle layer (Figs 1, 2)[7]. The appearance of both enzymes correlates consistently with the end of cell proliferation as shown by [^{3}H]thymidine-autoradiographic, histochemical and quantitative methods in different *in vivo* and *in vitro* tissues of embryonic chicken brains and retina[8–10]. In all these studies, the age-dependent decrease in cell proliferation is accompanied by a transient expression

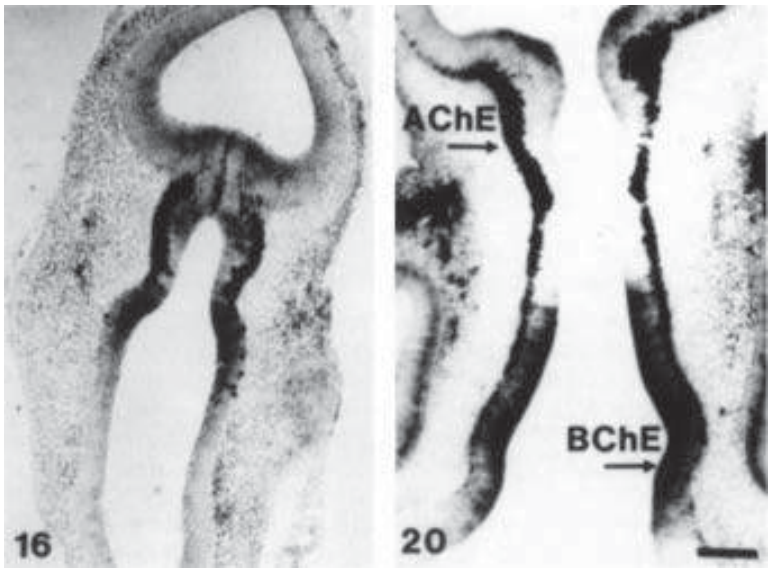

Fig. 1. Both cholinesterases are expressed in close vicinity to each other at early stages of neurogenesis. AChE-labelled cell bodies on the surface have just finished their final mitosis. AChE histochemistry thus reveals the spatial progression of neurodifferentiation (see text; compare stages HH 16[47], left and HH20, right). Preceding AChE, BChE diffusely labels areas of final mitotic activity on the ventricular side of the neural tube (see text and scheme in Fig. 2). Frozen sections showing the diencephalic and midbrain regions of 2 and 3 day-old chicken embryos were stained for both cholinesterases by using acetylthiocholine as substrate. Bar=200 μm.

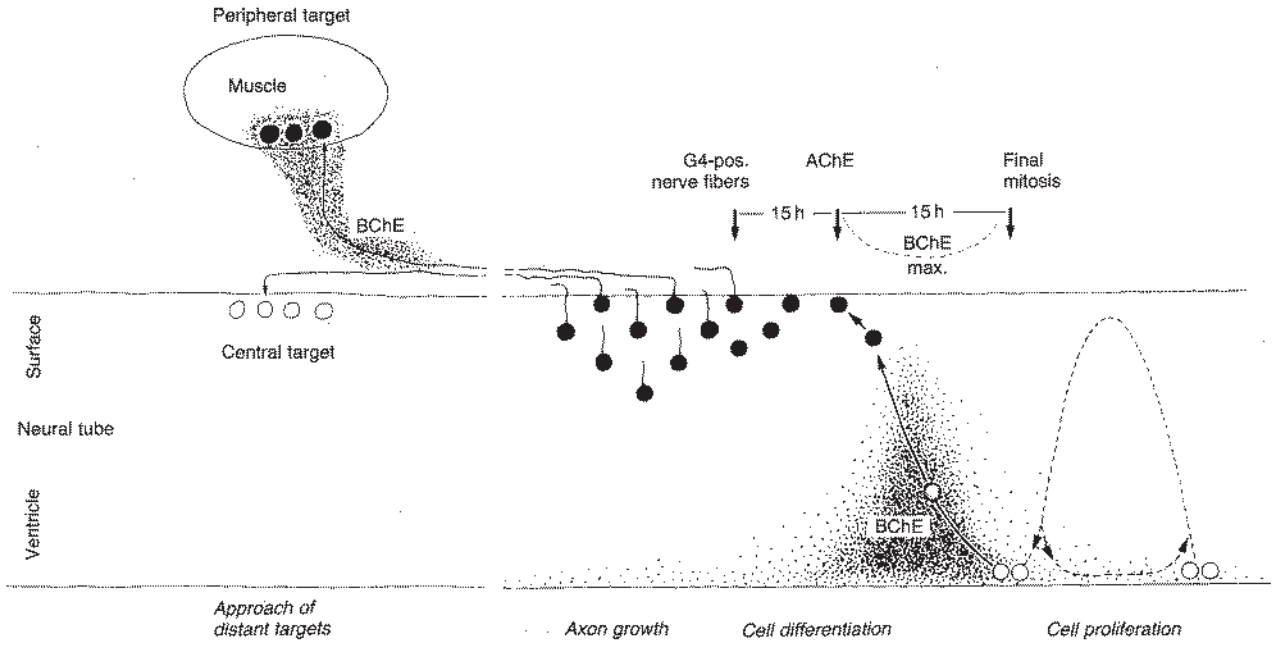

Fig. 2. BChE prepatterns precede cellular AChE expression and axon navigation. At the end of neural proliferation, BChE is transiently increased at the ventricular chicken neural tube. Approaching the outer surface and leaving the BChE field (*dotted*), neuroblasts start to differentiate by expressing AChE. These first postmitotic cells of the neural tube soon will extend long axons, that comprise the primary tracts of the vertebrate brain. Some of them traverse BChE-active zones to reach AChE/BChE-positive peripheral targets (see Figs 3, 4; further details see text).

of BChE, followed by a strong expression of AChE, starting about 10–15 h after the last uptake of thymidine. At this time, the young postmitotic cells are just about to reach the surface of the neural tube (scheme Fig. 2). The molecular forms of BChE expressed in young brain tissues differ from those in older tissues, and they are also distinct from their AChE counterparts[10]. Miki and Mizoguti have distinguished between four different classes of neuroepithelial and myotome cells which express increasing amounts of AChE. They also conclude that a full expression of AChE announces the onset of cell differentiation[5,11]. BChE was not considered in their studies.

AChE as a Postmitotic Marker to Map Patterna of Primary Brain Development

As histochemical detection of the enzyme is very sensitive[12], the appearance of AChE-positive cells along the surface of the neural tube can delineate patterns of spatiotemporal brain differentiation at high resolution. We deduced developmental maps of whole chicken brains by computer reconstructions from AChE-stained serial sections between stages HH11 and HH30[13] and found that the brain differentiates in a polycentric manner; several primary AChE-activation centers can be recognized that do not coincide with the establishment of the primary brain vesicles (see ref. 14). The first AChE-positive cells are detected almost simultaneously in the rhombencephalon and the diencephalon at stage 11, then cells of the mesencephalic trigeminal nucleus follow at stage 15. Telencephalic cells start at stage 18. Only then do the first retinal ganglion cells become postmitotic in the center of the eye. Quantitative biochemical data support this general scheme[10]. This staining technique has also been applied with similar results to whole mounts of chicken[15], fish[16,17], amphibia[18], reptiles and some mammals (L. Puelles, personal commun.). In addition, AChE-staining reveals the segmentally arranged motoneurones of zebrafish[17]. In *Xenopus*, all postmitotic neurons seem to go through a period of AChE-production[18]. For the chick, such a notion holds true for all neurones appearing on the surface of the young neural tube, but the situation may become more complex for later forming interneurones[19]. It has also to be realized that not all of these early cholinesterase-producing cell populations necessarily become classical cholinergic neurons.

The First Long Tracts of Vertebrate Brains Originate from AChE-Producing Cells

The regulation of the complex AChE pattern is not understood. Interestingly, however, the AChE-producing cells are those which are destined to produce a simple scaffold comprising the very first long distance axons[20]. By comparing the patterns of final thymidine uptake, AChE production and neuritogenesis using the G4 antibody[21] at the single cell level and on reconstructed whole brains we have shown that 10–15 h time intervals separate these three differentiative steps

(see scheme Fig. 2). Similarly, in *Xenopus*, the first axon tracts have been shown to originate from AChE-producing neuroblasts[18]. These early systems include central tracts (*e.g.*, the fasciculus mediolongitudinalis), central–peripheral connections including cranial nerves[14] and spinal motor tracts[22], as well as the optic nerve[7,13,20].

Cholinesterases at Origins, at Target Cells and Along Pathways of Spinal Motor Axons

Thus in early brains, AChE-positive cells found *efferent* systems. Whether their central target cells also produce AChE has not yet been established (see Fig. 2). Notably, however, in the adult cat's superior colliculus, AChE patches delineate long distance *afferent* systems[23]. The segmented spinal motor systems provide an excellent model to investigate the expression of both cholinesterases throughout the establishment of a central–peripheral long tract system, since all parts are segmentally (and therefore spatially simply) organized, and moreover motor axons are restricted during their outgrowth to the rostral part of the corresponding sclerotome, while searching for their appropriate myotomal target within each somite[24].

In the chick, the AChE/G4-double staining technique labels the entire segmental motor unit including AChE-positive motoneuronal and myotomal cell bodies and G4-positive motor axons (Fig. 3;[22]). On longitudinal sections, a wave of differentiation of the nerve–muscle complex extending from the ear to the caudal end of the embryo can be followed (see the scheme of Fig. 4). As a very early sign of somite differentiation, BChE expression is spatially elevated on a rostral sector of the differentiating somite. About 2–3 somites more rostral (and thus developmentally later), AChE is expressed in a nonsegmented smooth wave almost simultaneously in motoneuronal cell bodies of the ventral horn and in the corresponding dermomyotomes. Like early BChE expression, AChE is detectable in conjunction with BChE first in a rostromedial sector, thus representing the very first sign of muscle differentiation. Motor axons begin to grow exclusively through the BChE-rich sclerotomal space only after their origins and their target cells have been activated by AChE. At high resolution, a close association of growing neurites with BChE-producing cells can be demonstrated. On motor axons, AChE expression is significantly retarded.

Besides BChE, a number of molecules are localized asymmetrically in one or the other of the sclerotome halves. HNK-1-positive neural crest cells[26], cytotactin[27] and tenascin[28] are found in the rostral sclerotome. PNA lectin-binding proteins are expressed in the caudal part of the sclerotome[29]. But, strikingly, BChE is one of the earliest molecules expressed in the rostral sclerotome, since it is elevated there before immigration of neural crest cells (although having arrived, these cells will contribute significantly to the high amounts of BChE produced in the rostral sclerotome).

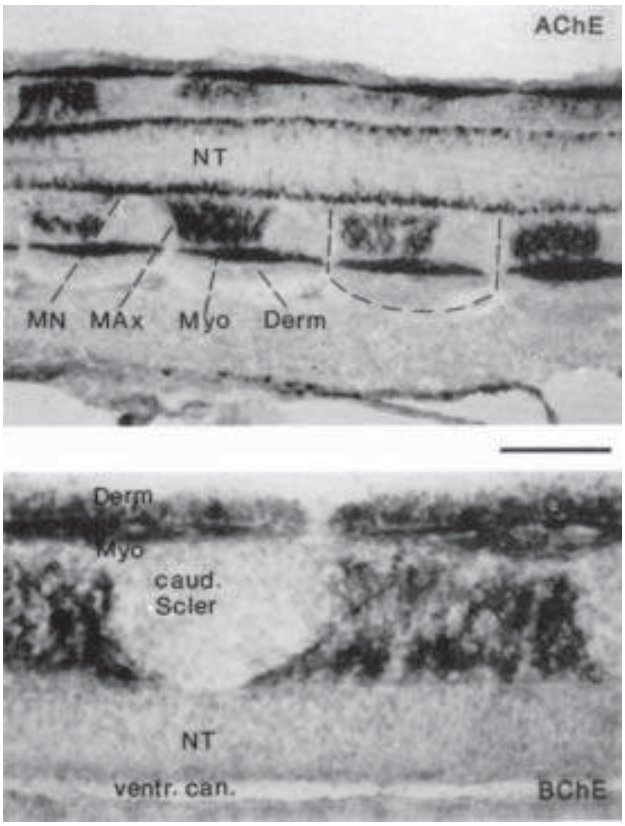

Fig. 3. Cholinesterases outlining the development of segmented spinal motor units. Parallel longitudinal frozen sections of a stage HH 18 embryo are either stained by AChE plus G4-antibody (upper), or BChE plus G4-antibody (lower; G4 is not well visible). AChE (upper) is expressed in source (motoneurones) and myotomal target cells (Myo); BChE (lower) is expressed in target cells and the navigational spaces of motor axons (MAx, detected by the G4 antibody), restricted to the rostral sclerotome. Further description see Fig. 4 and text. Bar=100 μm (upper), =200 μm (lower).

Thus, the entire process of early myotome differentiation, motor axon growth and first establishment of target contacts takes place within the rostral half somite. More importantly, both cholinesterases precede and accompany these processes. In other words, the two spatially separated sides of a neuronally connected system undergo similar steps of differentiation (expression of AChE) at about the same time, *before* they become connected by axons. What regulates this simultaneous expression of AChE in the two sides of the nerve–muscle system ? One possibility is that the motoneurones and the corresponding muscle cells are related by cell lineage and that their differentiation is therefore regulated by a common internal clock. A more likely alternative is that the asymmetrical expression of BChE is due to a segmental gene activation (for review see ref. 25), that induces the concomitant AChE expression at each end of the motor axon (for induction of AChE by BChE, see below). We

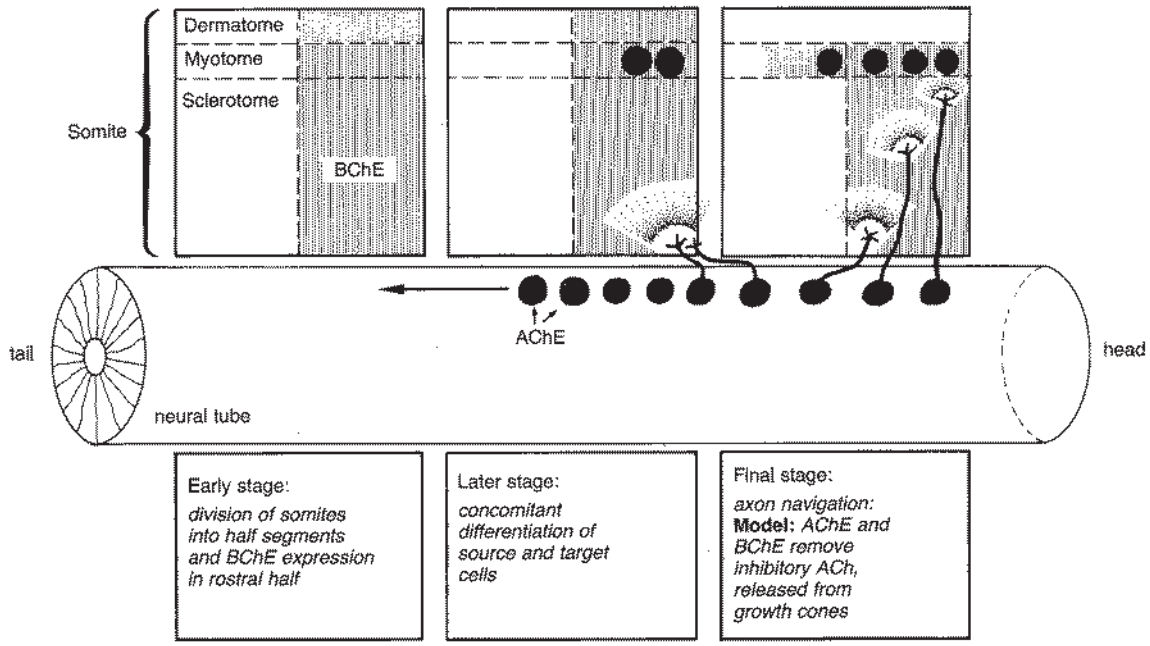

Fig. 4. Sequential differentiation of BChE (*striped fields*) and AChE (*black cells*) within somites (*large quadrangles*) and spinal cord to establish spinal motor units. BChE is initially expressed asymmetrically in the rostral somite (*Early stage*, left), followed by almost simultaneous expression of AChE both in motoneurones and corresponding myotomes (*Later stage*, middle). Then motor axons grow on permissive rostral pathways (*Final stage*, right). The model indicated suggests that motor axon growth cones release AChE (stippled clouds in *middle* and *right*), that inhibits axon elongation (compare ref. 44). Accordingly, AChE and BChE, by effectively removing ACh, would direct axon tips to their targets. Note that the spatial rearrangements leading to the somite subdivisions of dermatome, myotome, sclerotome and rostral and caudal halves are much more complex than indicated in this scheme; see also ref. 22. Further explanations see text.

are presently investigating whether similar center–periphery relationships are also applicable during the spatially much more complex formation of cranial nerves (see also ref. 14).

Do Spatial Prepatterns of a Phylogenetic Older BChE Induce AChE During Ontogenesis?

Is there a causal relationship between AChE and BChE expression? Although both proteins form similar polymorphic molecules[2], they clearly represent independent molecules in the developing brain[10], that are coded for by separate genes. Their phylogeny most likely originated from a gene duplication of an ancestral cholinesterase gene dating back to the emergence of the vertebrates[30,31]. Recent data indicate that the protein coded for by the ancestral gene had intermediate properties of both an AChE and a BChE enzyme*. A possible interpretation of the close spatial and temporal association of the two enzymes is that the BChE that precedes AChE during early development of the chick may resemble the phylogenetically older enzyme. It may lay out diffuse prepatterns during ontogenesis, within which AChE-positive cells subsequently establish refined and interconnected projections (see Fig. 2, e.g. spinal motor units). How this is achieved in molecular terms, remains unexplained. We have shown recently that BChE activity can regulate AChE expression *in vitro*[19,in preparation]. BChE cannot simply be a protein precursor for AChE, as suggested earlier[32]. Rather, it is possible that BChE acts by inducing specific cells to produce AChE. Future research applying molecular technology has to show whether BChE indeed functions as a molecular differentiation switch.

A Role for Cholinesterases in the Modulation of Axon Growth?

Despite many reports on cholinesterases exerting protease, arylacylamidase and neuropeptide producing activitites or interacting with lipoprotein metabolism[33–36], the roles of both AChE and BChE in neurodifferentiation remain unclear. A number of earlier experiments have indicated effects of cholinergic ligands on various morphogenetic processes[37–40]. Recently, axon outgrowth has been shown to be inhibited by excitatory neurotransmitters including acetylcholine[41,42]. Moreover, the blockage of nicotinic receptors by antagonists enhance axon growth[42]. The transmitter ACh itself can either leak from growing axons[42] or be released after their electrical stimulation[43]. Lipton and Kater have reviewed the response of growing neurites and their cell internal signalling machinery to various neurotransmitters[44].

*See L. Pezzementi *et al.*, in: *Proceedings of the Third Int. Meet. On Cholinesterases*, La Grande Motte, France, May 12–16, 1990, ACS Books, in press.

One major mechanism for control of neurotransmitter action during development that has not yet been given appropriate consideration, is their removal by degrading systems. AChE and BChE, because they have very different kinetics[1,2] and are localized at strategic places, could provide highly tunable systems to regulate the levels of ACh. A model for roles of these enzymes in modulation of axon growth is depicted in Fig. 4. The model is based on two assumptions: a) ACh is released from growth cones, and b) it inhibits neurite extension. If these assumptions are correct, cholinesterases would attract and direct neurites by creating ACh-free spaces. In particular for the trunk motor systems, BChE being a partially diffusible and slowly acting enzyme[1] would keep ACh at a slightly decreased level within the entire rostral half somite (long-range action). Very high levels of ACh close to the release sites would also be lowered by BChE, since it is not substrate-inhibited[1,2]. The cell surface-bound, substrate-inhibited AChE would have an extremely rapid but short-range action within a limited range of ACh concentrations. At the immediate outgrowth of axons from motoneurones, ACh levels may be so high that only BChE from the adjacent sclerotomes will work effectively and thus attract motor axons (no BChE is present in motoneurones), whereas on the target side of myotomal cells, AChE acting in conjunction with BChE will provide a pronounced sink of ACh, thereby accelerating the final approach of afferent motoraxon growth cones. Adopting appropriate *in vitro* systems[45], we have started to test whether ACh is released from spinal motoraxons and whether it can inhibit their growth[19].

New Roles of Cholinesterases and Cholinergic Disorders

In a number of neurological disorders, including developmental neural tube defects, mental retardations, dementias and tumours, levels of either one or both of the cholinesterases are significantly different from normal. However, the causal involvement of these enzymes in the pathophysiology of these disorders has not been demonstrated conclusively (for review see ref. 46). These negative results may stem from a general misconception that the defects are due to distortions of cholinergic neurotransmission. Therefore it seems appropriate from a clinical as well as a developmental point of view to investigate the functions of cholinesterases during early neurogenesis. In particular, the regulation of BChE gene expression needs further attention.

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Paul G. Layer is at the Max-Planck-Institut für Entwicklungsbiologie, Spemannstrasse 35/IV, D-7400 Tübingen, FRG.