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An acetylcholinesterase band-pattern in the molecular layer of the cat cerebellum

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INTRODUCTION

Olivocerebellar fibres terminate as climbing fibres in longitudinal zones in the molecular layer of the cerebellar cortex of the cat (Courville, Faraco-Cantin & Diakiw, 1974; Groenewegen & Voogd, 1975*a*, *b*). A similar longitudinal arrangement of mossy fibre terminals in the granular layer, and of Purkinje cell axons in the cerebellar white matter, has been described (Voogd, 1967; van Rossum, 1967).

It has also been reported that the enzyme 5′-nucleotidase in the molecular layer of the cerebellar cortex is present in a pattern of parallel longitudinal bands (Scott, 1964, 1965; Marani & Boekee, 1973). Ramon-Moliner (1972) described the distribution of acetylthiocholinesterase (AthChE) in the brain stem of four months old cats, and some of his illustrations indicated that AthChE is present in a band-like distribution in the molecular layer of the cerebellum. In this respect, in some parts of the inferior olive, areas of high acetylcholinesterase (AChE) activity (Marani *et al.* in preparation) correspond to the location of cells of origin of olivocerebellar fibres which pass to restricted longitudinal zones in the cerebellar cortex. This suggests a possible relationship between the regional distributions of AChE in the inferior olive and in the cerebellar cortex. The presence of AChE in the molecular layer of the cerebellum of the cat is disputed (for a review see Silver, 1967). With histochemical methods AChE could only be demonstrated during development from the third day till the twentieth day postnatum. Conversely, biochemical determinations in cats showed activity in the molecular layer to be nearly as strong as in the granular layer (Austin & Phillis, 1965; Goldberg & McCaman, 1967).

In the present study the zonal distribution of AChE in the molecular layer of the cerebellar cortex of young and adult cats (see Table 1) has been examined.

MATERIAL AND METHODS

The cats were anaesthetized with ether. The brains were dissected out within 7 minutes and frozen in isopentane at −80 °C in tissue tek (Ames and Co.). One out of ten serial transverse or sagittal cryostat sections (10 μm) was incubated for acetylthiocholinesterase (Gomori, 1952; Karnovsky & Roots, 1964). Preference was given to the 'direct-colouring' thiocholine method (Karnovsky & Roots, 1964) because of its considerably shorter incubation time. In a number of brains, parallel 1:10 series were made and incubated with different concentrations of iso-OMPA, DFP and Eserine (see Table 1). Pre-incubation with inhibitor lasted 30 minutes, except in the case of DFP. During incubation the substrate solutions, with or without inhibitor, were stirred to obtain maximal exchange between substrate and the

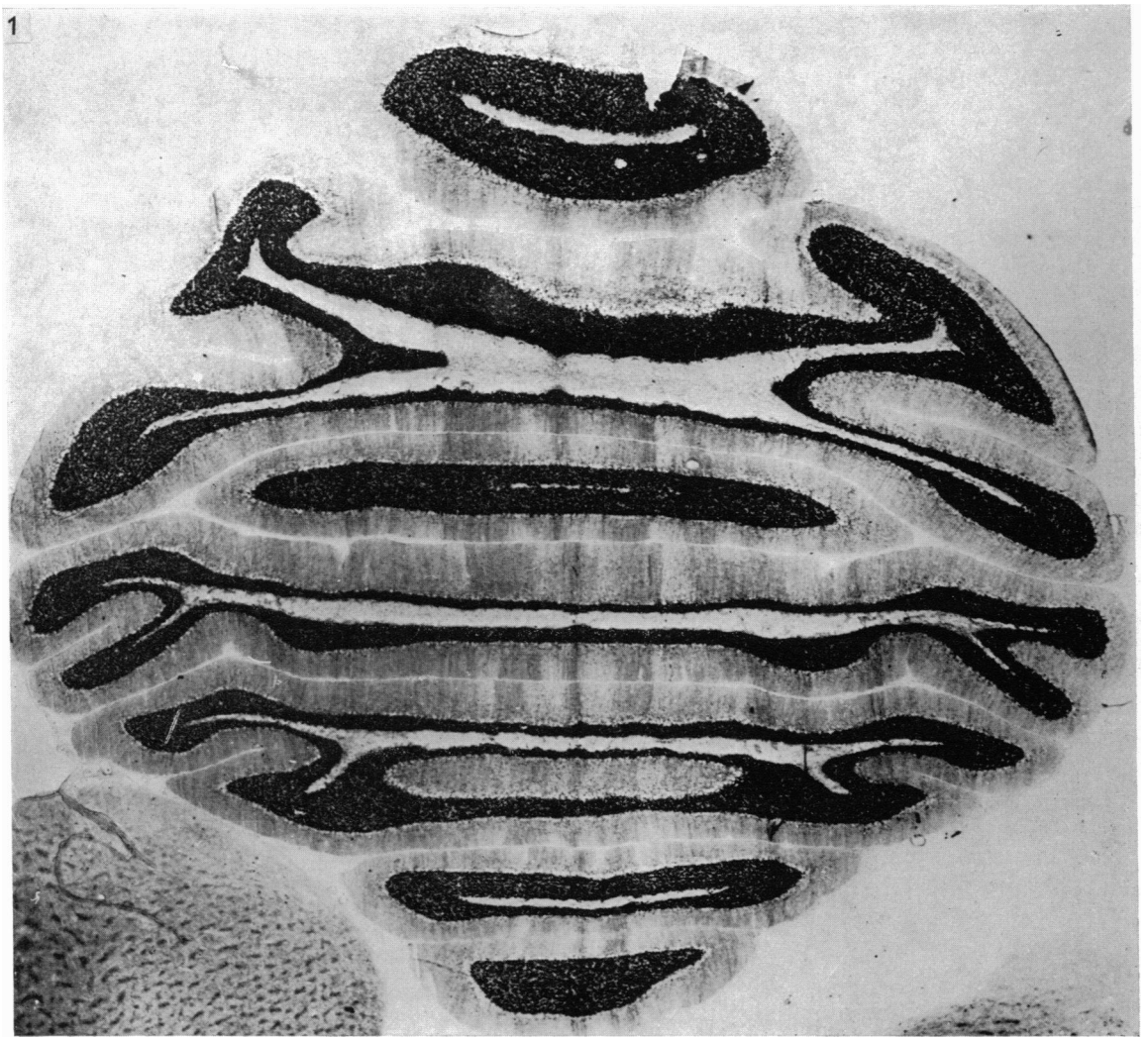

Fig. 1. A representative transverse section from the anterior lobe of animal 8809. Incubation conducted according to the Karnovsky–Roots method and treated with the p-ChE inhibitor iso-OMPA (10^{-5} M). The symmetrical distribution pattern of AChE can be seen restricted to the molecular layer, where it occurs in band-like concentrations.

sections at room temperature. Some series were counterstained with haematoxylin for 30 seconds and rinsed for 5 minutes in tap water. Post-fixation was always performed with Baker's formalin for 10 minutes.

Larsell's (1952) nomenclature is used for the cerebellar lobules.

RESULTS

The distribution of the enzyme was studied in enlarged negative prints of the actual sections. One cerebellum was reconstructed in styropor (Noël, Marquet-Eupen, Belgium).

The band-pattern could be demonstrated with either of the histochemical methods

used. In 15 cats, 2–4 months old, the pattern was present in the vermis of both the anterior and the posterior lobes. In older animals no AthChE could be demonstrated in the molecular layer.

The molecular layer of all lobules of the anterior lobe contained symmetrically disposed acetylthiocholinesterase (AthChE)-positive areas alternating with narrow negative zones (Fig. 1).

Transverse sections indicated that the AthChE activity was present in narrow striations, reaching from the somata of the Purkinje cells to the pial surface (Fig. 1). The somata of Purkinje cells were negative for AthChE, even at the base of AthChE-positive areas in the molecular layer (Fig. 1). The neuropil of the granular layer was strongly AthChE-positive, but the granular cells were negative. No activity was found in the white matter of the cerebellum.

The margins of a midline positive area were ill-defined. It consisted of some closely packed vertically arranged striations. Negative spaces between the striations accumulated laterally to form a narrow negative band. Lateral to these negative bands symmetrically disposed positive bands were present. The transition between these positive areas and the medial negative band was gradual. On the lateral side the border adjoining a second negative band was particularly sharp. Often this lateral border was located opposite a constriction of the white matter that corresponded to the lateral border or the medial compartment A of the white matter (Voogd, 1964, 1967, 1969). The bands deviated progressively lateral from lobule I to lobule V in the anterior lobe (Figs. 1 and 2A). Consequently both negative and positive bands became wider in the dorsal part of the anterior lobe, the two negative bands never attaining the width of the adjoining positive bands. Together the three positive and four negative bands in the medial part of the anterior lobe occupied less than one third of its total width, i.e. they were located in the region commonly designated as the vermis of the anterior lobe. In lobules III and IV the positive area in the lateral two thirds of the folia sometimes appeared to be broken by negative zones (Figs. 2A and B). These negative bands in the lateral part of the anterior lobe sometimes were present as the medial bands and could not be traced throughout the anterior lobe.

In sagittal sections through the anterior lobe the distribution of AthChE activity was more uniform. The striations were either absent, or much wider and less distinct, as compared with transverse sections. Differences in overall enzyme activity in different parts of a folium, or between different folia, could be observed, but the transition between the positive and negative areas was always gradual.

In the posterior lobe the bands had the same appearance as in the anterior lobe. The contrast between positive and negative bands was less obvious, both in negative and positive areas striations being present. The Purkinje cell bodies were negative for AthChE activity. These bands were limited to certain lobules of the posterior lobe of the vermis (Fig. 3). In lobule VI the midline and two lateral bands continued from the anterior lobe. In lobule VII the molecular layer was uniformly positive for AthChE and no negative bands were present. In lobule VIII the midline and lateral positive bands were wide and the negative areas were narrow. Activity was absent in the extreme lateral parts of this lobule. The lateral border of the lateral positive bands therefore presumably corresponded to the border between the zones A and B in the vermis of the posterior lobe (Voogd, 1964, 1967, 1969). In lobule IX the distribution of AthChE activity was slightly different. The midline was occupied by a positive band and on both sides two wide, symmetrically disposed negative

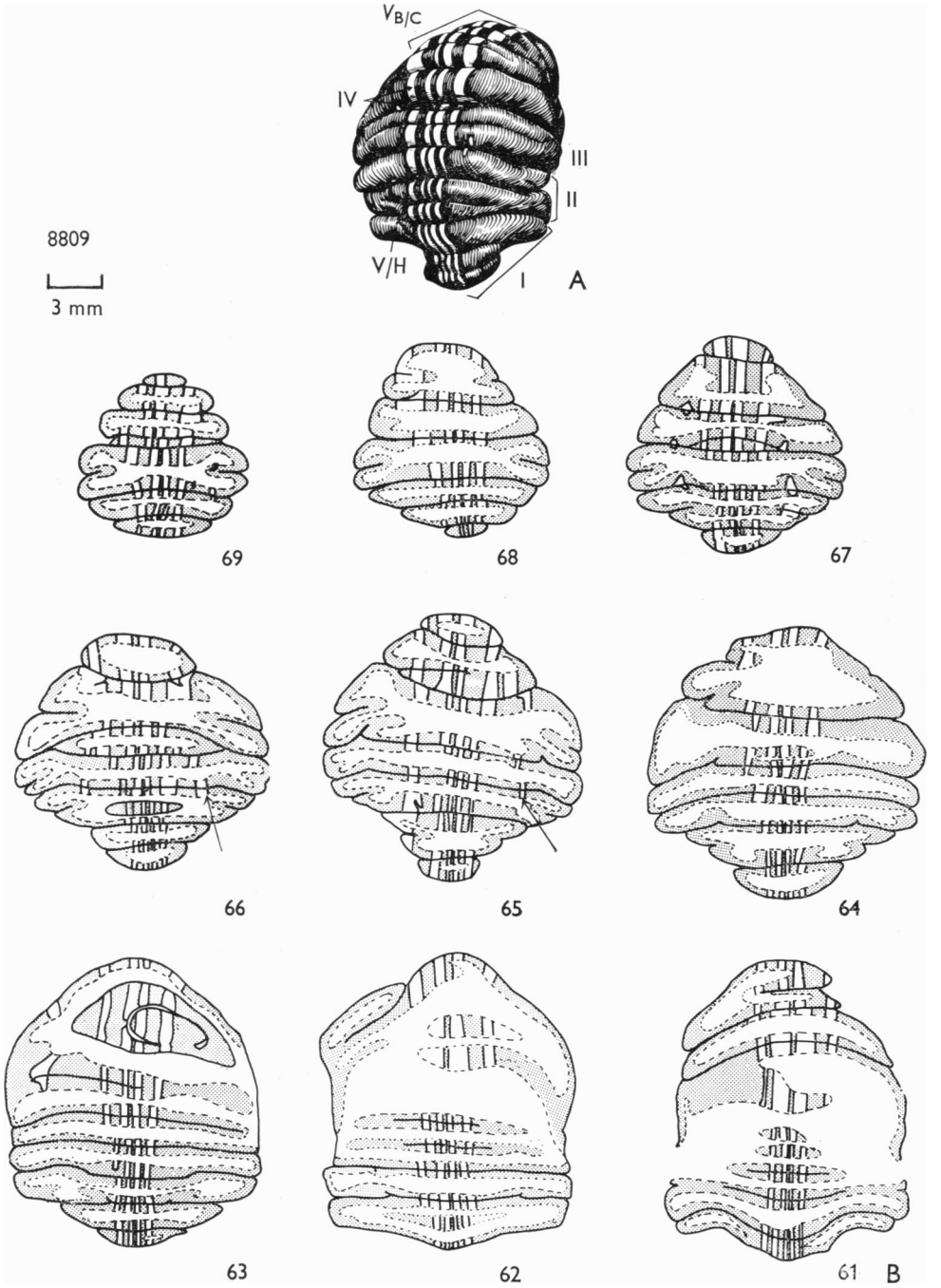

Fig. 2(A) Reconstruction of the anterior lobe of animal 8809 treated with iso-OMPA (10^{-5} M). The borders of the vermis and hemispheres (V/H) are indicated in this figure. The lobules are indicated according to the nomenclature of Larsell (1952). (B) Sequential sections from animal 8809 treated with 10^{-5} M iso-OMPA. The positive areas in the lateral two thirds of the folia of lobules III and IV are shown by arrows.

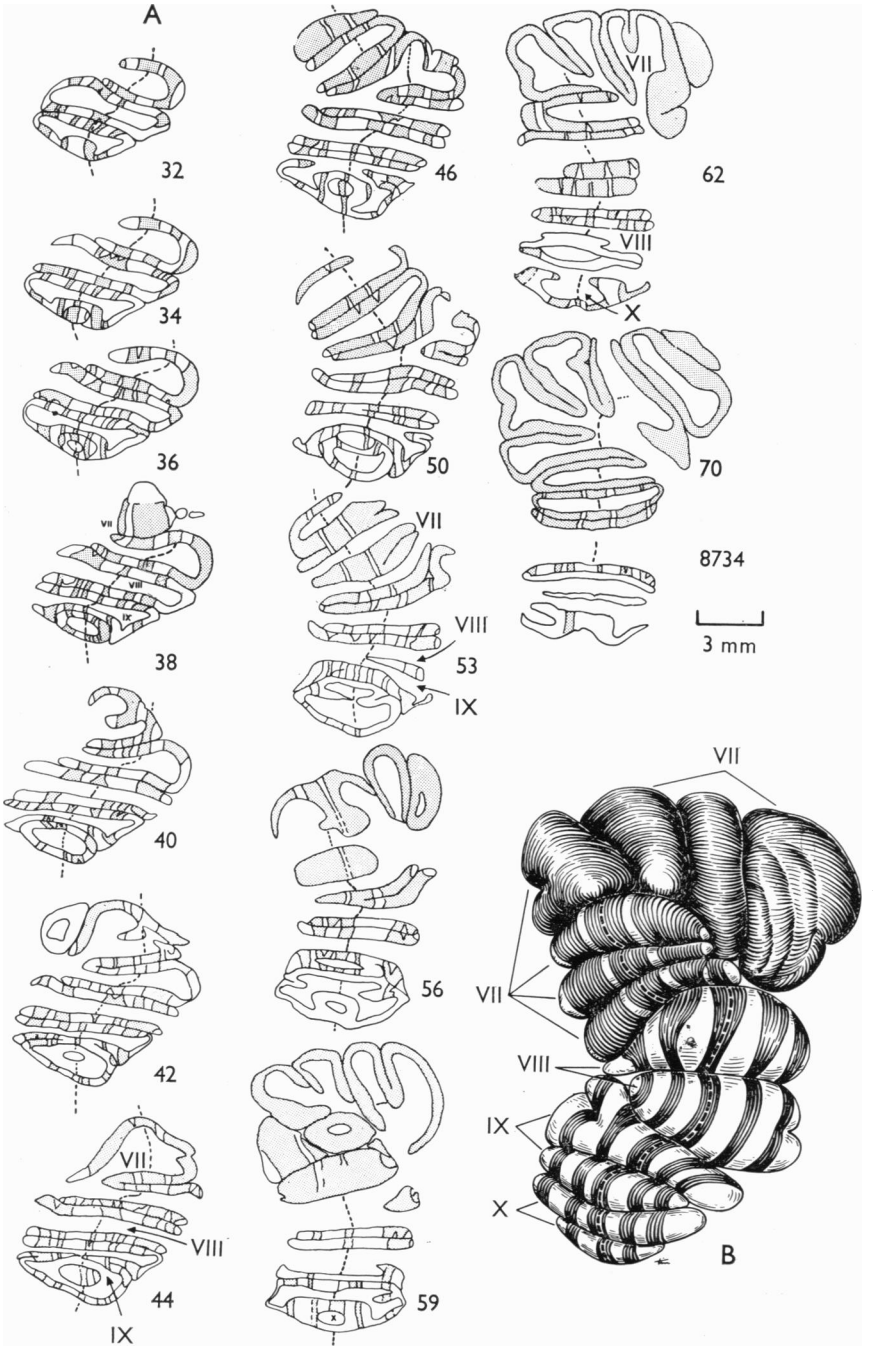

Fig. 3(A) Sections from animal 8734, indicating the band-pattern in the posterior lobe vermis. Incubation conducted according to Karnovsky–Roots method. No inhibitors were added. When the reaction for AthChE was equivocal, the loci in the molecular layer were not stippled. Therefore these sections represent the minimal extent of AthChE-bands. (B) Reconstruction of the posterior lobe vermis of animal 8734. The mid-sagittal plane is indicated by a dotted line. In lobules X to VIII, five positive bands are present alternating with six negative zones. Lobule VII is devoid of alternating areas with and without AthChE activity, except for the most caudal lobules, where three positive and four negative zones are present.

Table 1. *Details of animals and techniques*

Number of animal	Age of animal	Kind of reaction	Parallel series with inhibitor	Concentration	Incubation time (hours)	Direction of cutting	Pattern presence or absence
8896	1–2 days	AChE-K*	iso-OMPA	10^{-3}, 10^{-4}, 10^{-5}	1½ h	Transversal	–
9035	25 days	AChE-K	iso-OMPA	10^{-3}, 10^{-4}, 10^{-5}	1½ h	Transversal	–
8903	7 weeks	AChE-K	iso-OMPA	10^{-6}	1½ h	Transversal	±
8919	7 weeks	AChE-K	iso-OMPA	10^{-6}	1½ h	Transversal	+
8921	7 weeks	AChE-K	iso-OMPA	10^{-6}	1½ h	Transversal	+
8920	9 weeks	AChE-K	iso-OMPA	10^{-6}	1½ h	Transversal	+
8945	12 weeks	AChE-K	iso-OMPA	10^{-3}	1½ h	Transversal	+
8742	4 months	AChE-K	—	—	1½ h	Transversal	+
8734	4 months	AChE-G†	—	—	3 h	Transversal	+
8729	4 months	AChE-G	—	—	3 h	Transversal	+
8865	4 months	AChE-K	—	—	1½ h	Sagittal	+
8809	4 months	AChE-K	iso-OMPA	10^{-5}	1½ h	Transversal	+
8809	4 months	AChE-K	DFP	10^{-6}	1½ h	Transversal	+
8897	4 months	AChE-K	iso-OMPA	10^{-6}	1½ h	Transversal	+
9169	4 months	ButhChE-K	—	—	1½ h	Transversal	–
9169	4 months	AChE-K	Eserine	10^{-3}, 10^{-4}, 10^{-5}	1½ h	Transversal	–
8895	Mature	AChE-K	iso-OMPA	10^{-3}, 10^{-4}, 10^{-5}	1½ h	Transversal	–

* Karnovsky – Roots method.
† Gomori method.

bands were found. In the apex of lobule IX, and in the hemisphere of the posterior lobe, the molecular layer was positive for AthChE and negative bands were difficult to discern.

Specific inhibitors were used to check whether pseudo-cholinesterase (p-ChE) was involved in the band-pattern. Eserine (Long, 1963) is known to inhibit cholinesterase (ChE) activity in the molecular layer. The specific p-ChE inhibitor iso-OMPA (Aldridge, 1953; Long, 1963; Robinson, 1971) was added in concentrations ranging from 10^{-3} M to 10^{-6} M. The band-pattern was retained regardless of the concentration of iso-OMPA. This confirmed previous reports (Silver, 1967) that the molecular layer of the cat cerebellum was nearly devoid of p-ChE and that the acetylthiocholinesterase (AthChE) in the molecular layer could be considered as acetylcholinesterase (AChE).

DISCUSSION

On the basis of our light microscopic histochemical analysis the AChE activity in the molecular layer has no known structural basis in molecular layer. The literature is inconclusive on the presence of AChE in the molecular layer of the cerebellar cortex of cat, or sheep (Palmer & Elleker, 1961; Phillis, 1965*a*; Silver, 1967). In cats, AChE activity was found in the Purkinje cells of neonates, but it had disappeared in three weeks old cats (Sakharova, 1966). In adult cats it has been variously reported as absent (Austin & Phillis, 1965; Kása & Csillik, 1965), or as present only after long incubation (Silver, 1967), or as bound to Purkinje cells (Joo, Savay & Csillik, 1965; Phillis, 1965*a*, *b*). The specific distribution of the enzyme described by us, and previously illustrated by Ramon-Moliner (1972), could only be demonstrated in four months old or younger cats. This, and the disappearance from the molecular layer of AChE activity when cats approach maturity, may explain some of the conflicting opinions expressed in the literature concerning the presence of AChE in the cerebellum.

In the guinea-pig, ChE is bound to the parallel fibres and Golgi cell dendrites; in sagittal sections the Purkinje cell dendrites are negative (Kása, Joo & Csillik, 1965). In the cat the striated appearance of the positive areas in the molecular layer in transverse sections makes improbable the view that the enzyme is bound to parallel fibres and the absence of striations in sagittal sections rules out binding to the Golgi cell dendrites of Bergmann glia. Because glial cells are supposed to contain p-ChE (Kása *et al.* 1965; Silver, 1967), the unaltered appearance of the pattern of positive and negative bands in the molecular layer after iso-OMPA treatment also seems to rule out the Bergmann glia.

The present observations are compatible with a localization of the enzyme in the dendritic tree of the Purkinje cell, or in structures associated with it. This is in accordance with the observations in mature sheep (Phillis, 1965*b*; Silver, 1967) where the Purkinje cells were found to be definitely positive for AChE.

In some rodents and insectivores a similar band-like distribution is present in the molecular layer for the enzyme 5′-nucleotidase (Marani & Voogd, 1973; Scott, 1964, 1965). The 5′-nucleotidase in mouse, rat and shrew is distributed in multiple bands, and in the mouse 5′-nucleotidase compartmentalization can be observed throughout the entire cerebellum. In the cat a 5′-nucleotidase band-pattern could not be demonstrated, the whole molecular layer being uniformly positive for this enzyme. Although the number of the bands, and their presence in the hemisphere in some species distinguishes the 5′-nucleotidase band-pattern from that observed for

AChE in the cat, both in fact seem to be associated with the Purkinje cell dendritic tree (Marani & Voogd, 1973).

Does the similarity in the distribution in the molecular layer of both enzymes also imply a similarity of function in different species?

The ultrastructural localization of both enzymes in the molecular layer is still unknown. However, there is no simple relationship between localization and synaptic transmission in regard to these enzymes. AChE is not only present in synapses of cholinergic neurons, but also has been demonstrated in non-synaptic portions of cholinergic neurons, and in adrenergic cells like those of the locus coeruleus (Lewis & Schon, 1975).

According to the hypothesis of Suran (1974*a*, *b*) and of Marani & Boekee (1973), 5′-nucleotidase plays a role in synaptic transmission by the catalysis of AMP to adenosine.

In agreement with this, direct actions of AMP on the firing rate of rat Purkinje cells (Kostopoulos, Limacher & Phillis, 1975) and of 5′-nucleotides on glutamate-induced muscle potentials (Ozeki & Sato, 1970), have been demonstrated.

According to the hypothesis of Bloom, Hoffer & Siggins (1971, 1974), Hoffer, Siggins & Bloom (1971) and Siggins, Hoffer & Bloom (1971) cAMP is a mediator for norepinephrine effects in rat Purkinje cells, and the breakdown of cAMP by 3′5′-nucleotidephosphodiesterase (Gähwiller, 1976) results in the formation of AMP. Thus the presence of 5′-nucleotidase in or around the Purkinje cell dendrites would determine the amount of 5′-nucleotides available. The existence of medio-lateral gradients of 5′-nucleotidase in the cerebellar molecular layer in the rat and mouse should lead to corresponding gradients in the excitability of the Purkinje cells.

Similarly, the AChE band-pattern in the molecular layer of the cat could play a role in governing Purkinje cell activity. However, in contrast to 5′-nucleotidase, the AChE band-pattern is only present for a limited period in the postnatal development of the molecular layer. The distribution of AChE and other enzymes (e.g. succinic dehydrogenase, Altmann, 1972) shifts from a localization in Purkinje cells in neonates to a diffuse localization in the molecular layer of adults. The significance of these changes in enzyme localization is unknown.

Determining the role of AChE in the cerebellum is complicated by the fact that physiological investigations of acetylcholine in the cat are conflicting. According to Silver (1974) "iontophoretic studies have not provided unequivocal evidence about the existence of cholinergic synapses in the cat cerebellum; indeed they have tended to confuse rather than to clarify the issue". Crawford *et al.* (1966) came to the conclusion that acetylcholine is unlikely to be an excitatory transmitter within the feline cerebellum, even at mossy fibre terminals, despite the presence of relatively high levels of acetylcholinesterase within these terminals. In the same paper these authors reported that Purkinje cells are excited by cholinomimetics and possess choline receptors with muscarine properties. Earlier McCance & Phillis (1964) found both Purkinje cells and granular cells to be sensitive to acetylcholine; however, the action of acetylcholine on Purkinje cells is explained by them as a consequence of prior granular cell excitation, but the presence of acetylcholine receptors on Purkinje cell somata cannot be excluded. These conflicting data could be accounted for by the fact that some Purkinje cells have, and some have not, AChE present in or around their dendritic trees.

In another paper (Marani, Voogd & Boekee, 1976) we described the localization

of AChE in the inferior olive of the cat. It was concluded that some areas of high AChE activity correspond with the origin of olivocerebellar projections to certain longitudinal zones in the cerebellar cortex. Some of these zones can be delimited also on the basis of the distribution of AChE activity in the molecular layer. The lateral borders of the first paramedian band of AChE activity correspond to the border between zones A and B of the vermis of the anterior lobe. Both the caudal half of the medial accessory olive, which projects to the medial zone A of the vermis, and the caudal pole of the dorsal accessory olive, which projects to the more laterally situated zone B (Voogd, 1969; Groenewegen & Voogd, 1975*a*, *b*) show strong AChE activity. Although the distribution of AChE in the inferior olive does not determine the distribution of this enzyme in the molecular layer, the same zonal principle which underlies the projection of the accessory olives to the vermis is evident from the cerebellar distribution of AChE.

SUMMARY

An acetylthiocholinesterase (AthChE) subdivision was found in the molecular layer of the cat cerebellum, using either the 'direct-colouring' thiocholine method or the Gomori method. In 15 cats, 2–4 months old, the pattern was present in the vermis of both the anterior and the posterior lobes. In older animals no AthChE could be demonstrated in the molecular layer.

The molecular layer of all lobules of the anterior lobe and posterior lobe, except for VII, contain symmetrically disposed AthChE-positive areas alternating with narrow negative zones. Transverse sections indicate that the AthChE activity in the positive areas is present in narrow striations, reaching from the somata of the Purkinje cells to the pial surface. The somata of Purkinje cells are negative for AthChE, even at the base of the AthChE-positive areas in the molecular layer.

Specific inhibitors were used to check whether pseudo-cholinesterase (p-ChE) is involved in the band-pattern. Eserine inhibits cholinesterase activity in the molecular layer. The p-ChE inhibitor iso-OMPA was added in concentrations ranging from 10^{-3} M to 10^{-6} M, but the band-pattern was retained regardless of the concentration of iso-OMPA. The AthChE activity in the molecular layer can be considered as true acetylcholinesterase, the more so because, after addition of butyrylthiocholine instead of acetylthiocholine to the incubation medium the band-pattern in the molecular layer is no longer present.

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