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Regional Differences in the Distribution of Lipofuscin in Purkinje Cell Perikarya

A Quantitative Pigmentarchitectonic Study of the Cerebellar Cortex of Senile Albino Rats

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Summary. The distribution of lipofuscin in the perikarya of Purkinje cells of vermal and hemispheric lobules has been determined quantitatively in 7 rats, 30–38 months old, by the point-counting method. On the basis of morphologically and statistically significant differences a pigmentarchitectonics of the cerebellar cortex is established. The Purkinje cells of lobule VIa (Larsell 1952) are extremely lipofuscin-rich. The Purkinje cells of the hemispheres, lobules V, VIb+c and VII contain considerable amounts of a finely granular lipofuscin, the Purkinje cells of lobules I–III and VIII–IXa a globular type of lipofuscin. The Purkinje cells of sublobule XI d c and X are lipofuscin-poor cells. Three types of lipofuscin have been identified in the light microscope.

Key words: Lipofuscin – Purkinje cells – Pigmentarchitectonics – Senile rat

Introduction

The role of lipofuscin in the process of aging is still enigmatic. Lipofuscin is regarded as an "Abnutzungspigment" (v. Volkmann 1932) or "wear and tear pigment" (Strehler 1963), but observations on the presence of lipofuscin in childhood (Obersteiner 1903) or on its experimental production in young rats (Sulkin and Srivanij 1960) speak against this point of view. In the cerebellar Purkinje cell layer, lipofuscin can hardly be found in rats younger than 12 months (Reichel et al. 1968). Lipofuscin is easily detected in the Purkinje cells of albino rats older than 24 months by means of fluorescence microscopy (Heinsen 1979) and its presence in the Purkinje cells of other species has also been noted: horse (Kikuchi 1928), guinea pig (Wilcox 1959), rhesus monkey (Tcheng 1964), pig (Whiteford and Getty 1966; Nanda and Getty 1971), man (Leibnitz and Wünscher 1967), mouse (Samorajski Ordy and Rady-Reimer 1968) and cat (Sarnat 1968). Some of these investigators report a progressive increase of lipofuscin with age, and such observations fulfil Strehler's (1963) criteria

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for an aging phenomenon: universality and time dependency. The question of whether the accumulation of lipofuscin is genetically preprogrammed and deleterious could not be resolved, because regional differences in lipofuscin accumulation in vermal lobules X and V have been recently reported (Heinsen 1980). The present study was undertaken in order to obtain further information on the quantitative distribution of lipofuscin in the other vermal lobules and the hemispheres and to compare lipofuscin accumulation in this with that in other brain regions.

Material and Methods

Seven rats, 30–38 months old, were studied (1 male Sprague-Dawley, 3 male Wistar and 3 female Wistar rats). The animals were kept under similar conditions and were allowed water and food (Altrumin®) ad libitum. Only neurologically and physically healthy specimens were used. The animals were deeply anaesthetized by an intraperitoneal injection of Nembutal® and fixed by transcardial perfusion of 200 ml Bouin's fluid (Romeis 1948). The cerebellum was removed six hours after perfusion, postfixed in Bouin's fluid for 24 h, dehydrated in alcohol, cleared in methyl benzoic acid and embedded in paraffin. Care was taken that the cerebella of the individual animals were identically processed and left for equal times in alcohol, methyl benzoic acid and paraffin. The cerebella were cut in serial 8 µm thick sections and the sections stained with gallocyanin-chromalum (Romeis 1948). Four sections from each animal were analysed: 2 parasagittal sections through the vermis about 100 µm to the right or left of the presumed midsagittal plane; the sections in which the three sublobules of crus I, the lobulus simplex, crus II, the lobulus paramedianus dorsalis anterior and the copula were recognizable were chosen for counts of each hemisphere (nomenclature according to Larsell 1952).

Lipofuscin shows an intense autofluorescence when excited by the light of a 200 W mercury lamp and using a BG 12 and BG 38 excitation filter and a 530 nm barrier filter (Fig. 3). The fluorescent lipofuscin stands out sharply against the cytoplasm.

The volume percentage of lipofuscin per Purkinje cell perikaryon was determined by the point-count method. A coherent double lattice test system with 100/2,500 test points (Firma Leitz, Wetzlar) was inserted into a 12.5 × eyepiece and the hits on lipofuscin and the rest of the Purkinje cell perikaryon were counted at a final magnification of 1,250 ×. For further details on counting and adequate testplates see Weibel (1979). Each profile that could be safely identified as a Purkinje cell perikaryon was included in the point counting.

The percentual volume of lipofuscin per Purkinje cell perikaryon was determined by the formula V_{Li} (%) $= (P'_{Lipofuscin}/q^2 \times P_{Purkinjec.} + P'_{Lipofuscin}) \times 100$. P' are the hits on lipofuscin, P the hits on the Purkinje cell perikaryon and $q^2 = 25$. The quantitative data were tested at the Abteilung für Medizinische Dokumentation und Statistik der RWTH Aachen (Head: Prof. Dr. med. Repges) with a BMDP 1 V one way analysis of variance and covariance computer program (vermis – hemispheres) and a BMDP 3 D program (hemispheres – vermis) from the Health Sciences Computing Facility at the University of California (Los Angeles, 1979). Thanks are due to Mrs, Dipl.-Math. G. Henn for performing the statistical tests.

Results

The volume density of lipofuscin per Purkinje cell in the Purkinje cells of the vermis is listed in Table 1 and plotted in Fig. 4a.

With respect to the amount of lipofuscin in the Purkinje cells of the vermis, two extreme regions can be separated. The Purkinje cells immediately rostral and caudal to the primary fissure are rich in lipofuscin and there is 2 to $2^1/_2$ times as much lipofuscin in these cells as in the Purkinje cells of lobules X and IX d c. The Purkinje cells in lobules II–IV accumulate increasing amounts

Table 1. Distribution of lipofuscin in the Purkinje cell perikarya of the vermis. Estimates of means (%), standard errors of the means (s_m) and total number of cells counted in 7 animals (2 sections/animal)

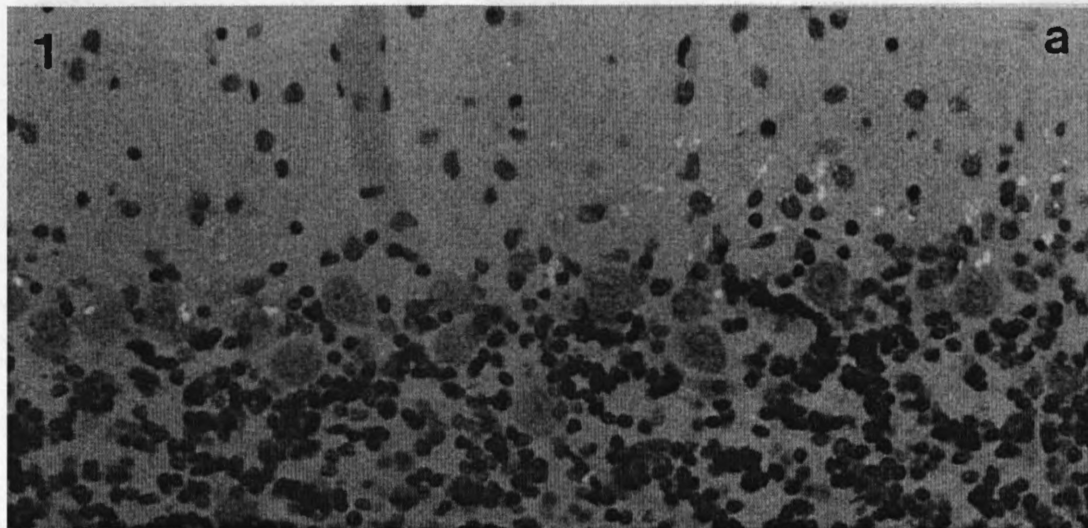
Lobule	I	II	III	IV	V	VIa	
%	12.1821	11.4979	12.5757	14.0821	15.9793	18.1371	
s_m	1.0430	0.6086	0.6144	0.9670	0.9939	1.3486	
Number	551	993	1113	998	1457	1245	
Lobule	VIb+c	VII	VIII	IXa–c	IXdr	IXdc	X
%	14.5407	14.3957	13.7707	10.9293	10.1379	6.9036	6.1043
s_m	1.1076	0.8821	0.9011	0.6317	0.7658	0.4744	0.3689
Number	714	761	1149	1525	348	444	1125

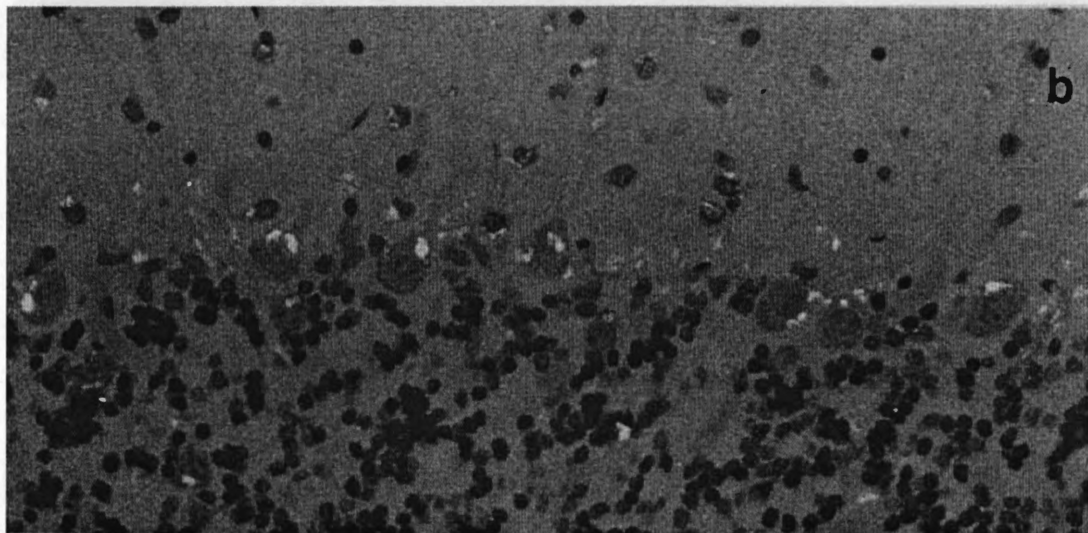
of lipofuscin as one approaches the primary fissure. The Purkinje cells of lobules VIb and c – X show an inverse tendency. The Purkinjc cells of lobule I and IX do not easily fit into this pattern, because the cells in lobule I contain more lipofuscin than the cells in lobule II, and in sublobules IXa–d r clusters of extremely lipofuscin-rich Purkinje cells are found. On the basis of these observations, sublobule IX d had been subdivided into sublobule IX d r and IX d c (Fig. 2). A dramatic decline in volume density of lipofuscin occurs at. the point where the medullary ray of lobule IXc and d emerges from the common medullary trunk of lobule IX (Fig. 2; Table 1). By means of a statistical analysis, 3 distinct regions in the cerebellar vermis can be elaborated in an antero-posterior direction (Table 3). If probabilities of <0.005 are considered as significant, a lipofuscin-rich region, lobule VIa, lobules with an intermediate content of lipofuscin (lobules I–V, VI b+c–IX d r) and lobules with extremely low levels of lipofuscin can be discriminated (Fig. 4a).

The quantitative distribution of lipofuscin in the Purkinje cells of the hemispheres does not exhibit such drastic regional differences as the cells of the vermis (Table 2, Fig. 4b). The estimate for the total mean of the hemispheric lobules is 15.5%. No statistically significant differences were found (Table 4) between the individual hemispheric lobules. Significant differences with $p<0.005$ exist between all hemispheric lobules and L IX d c and L X, differences with $p<0.01$ between the majority of the hemispheric lobules and lobules IX a–c and II. No significant differences were found between the hemispheric lobules and the other vermal lobules.

Besides these significant quantitative regional differences between vermal and hemispheric lobules, qualitative distinguishing features between the lipofuscin of the Purkinje cells in different hemispheric and vermal lobules could be detected. The lipofuscin of the Purkinje cells in lobule X and the caudal part of sublobule IX d is frequently deposited as a flat discoid polygonal body. This type of lipofuscin exhibits a dark yellow fluorescence. The lipofuscin is preferentially situated in the apical region of the perikaryon (Figs. 3a; 1a).

The lipofuscin in the Purkinje cells of lobules I–III and the rostral segment of lobule IV and lobules VIII and IX a shows a similar dark yellow autofluores-





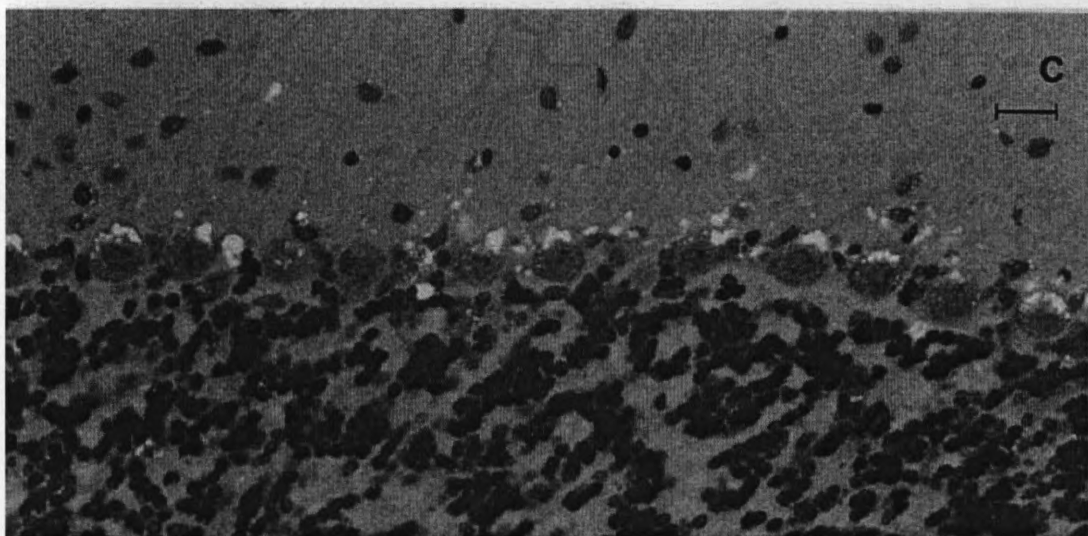
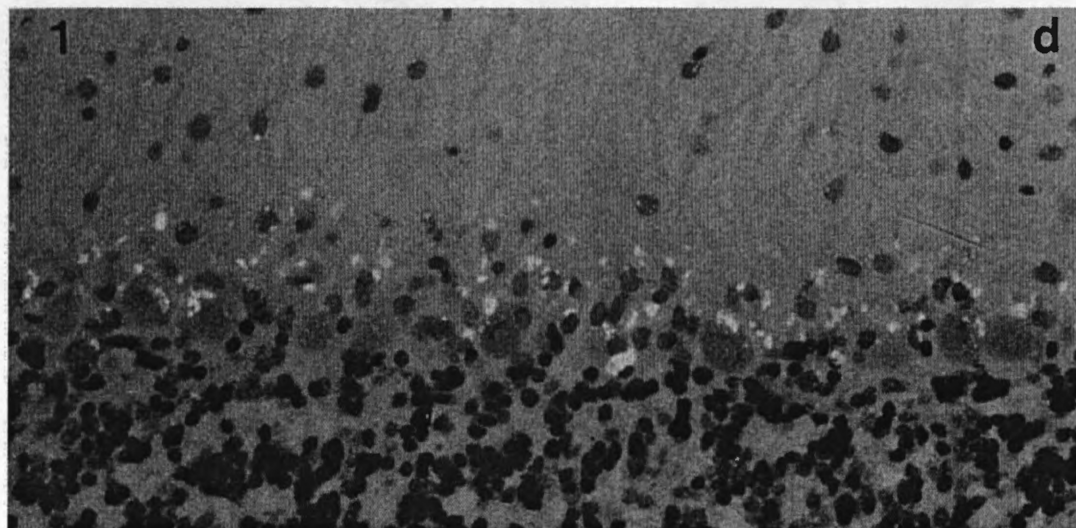


Fig. 1a–f. All photos were taken with a brightfield condensor, 200 W mercury lamp, 2 BG 38, 1 BG 12 and a 530 nm barrier filter at the same magnification. Bar in Fig. 1c represents 20 µm. **a** Lobule X. **b** Lobule I. **c** Lobule VIa, rostral part. **d** Lobule IXa, intermediate part of the lobule. **e** Crus I, rostral part. **f** Lobule IXc, summit of the sublobule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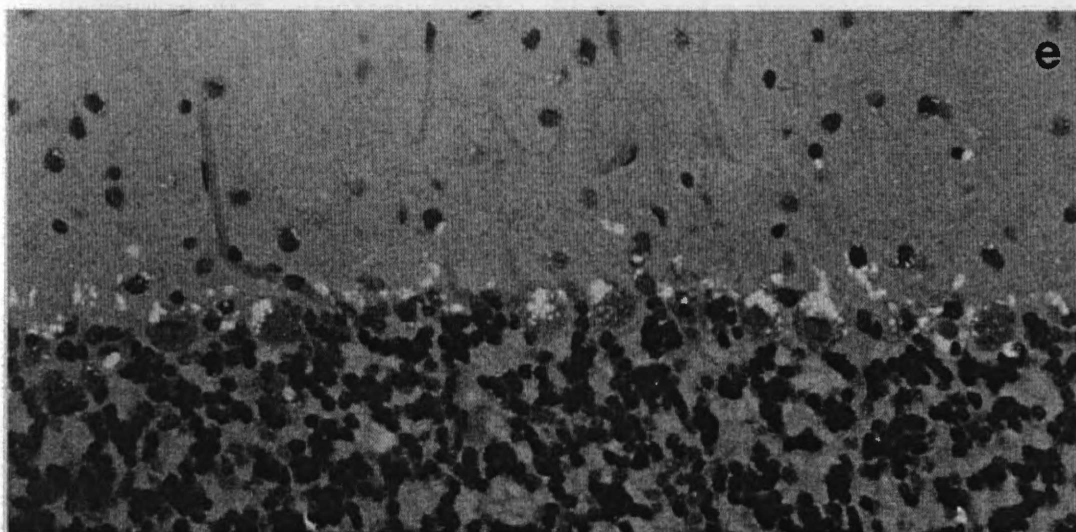
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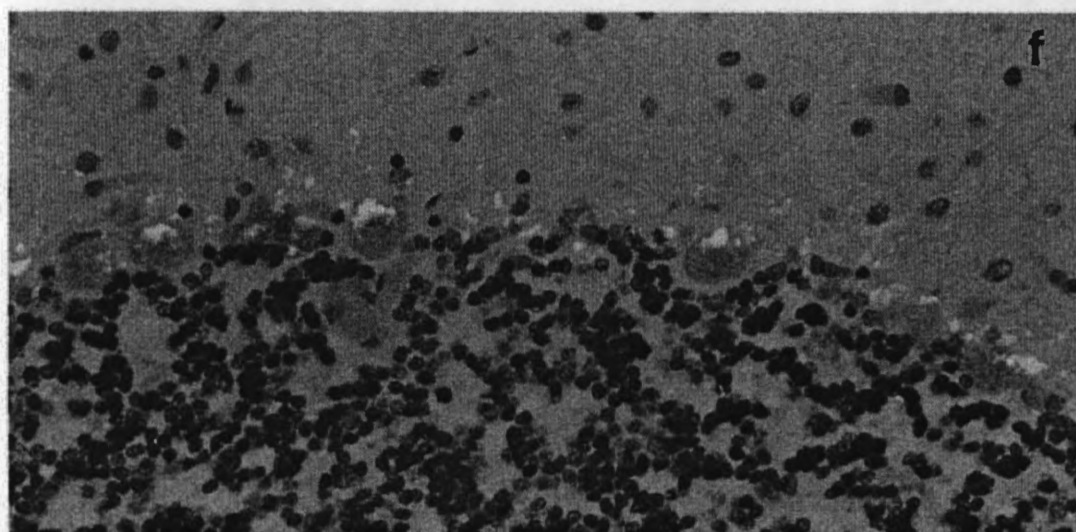
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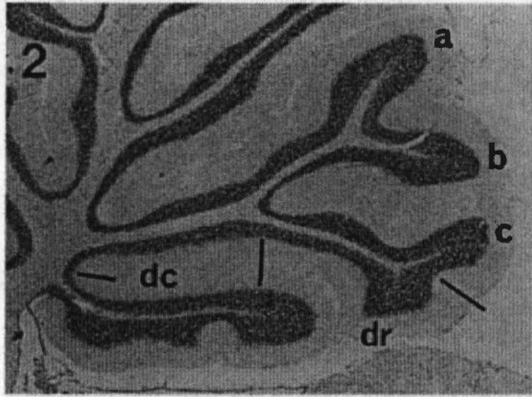


Fig. 2. Lobule IX and its subdivisions. The sublobules *a, b, c* and *d* are demarcated by natural sulci. The sublobule IXd has been subdivided on the basis of the lipofuscin content of its Purkinje cells. The extent of sublobule IX *dr* (rostral) is from the right bar to the bar in the middle of the picture. The extent of sublobule IX *dc* (caudal) is from the bar in the middle of the photo to the left bar. The Purkinje cells of sublobule IX*dc* are lipofuscin-poor cells

Table 2. Distribution of lipofuscin in the Purkinje cell perikarya of the hemispheres. Estimates of means (%), standard errors of the means (s_m) and total number of cells counted in 7 animals (2 sections/animal)

Lobule	L. simplex rostral sublobule	L. simplex caudal sublobule	Crus I	Crus II	L. paramed. dors. ant.	Copula
%	15.6686	16.7164	16.4214	15.6879	14.8686	13.4157
s_m	1.1795	1.2466	1.1149	0.8916	1.0287	0.9441
Number	787	1123	1492	1221	923	520

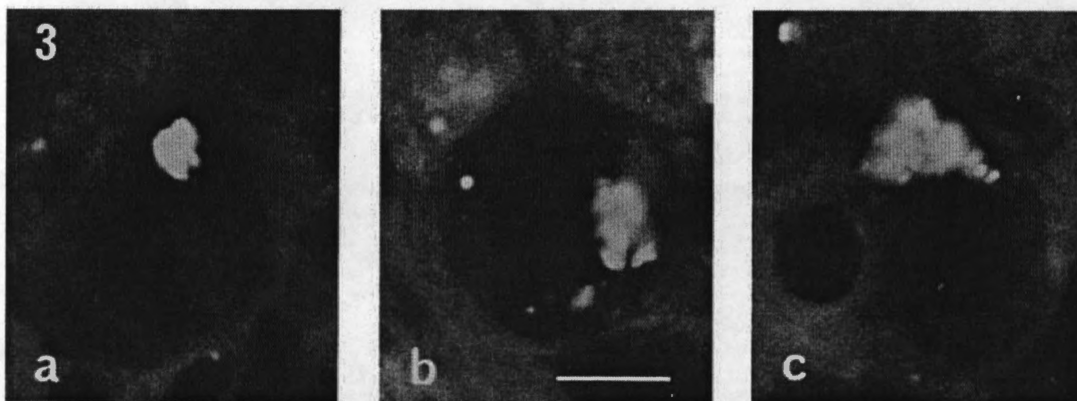
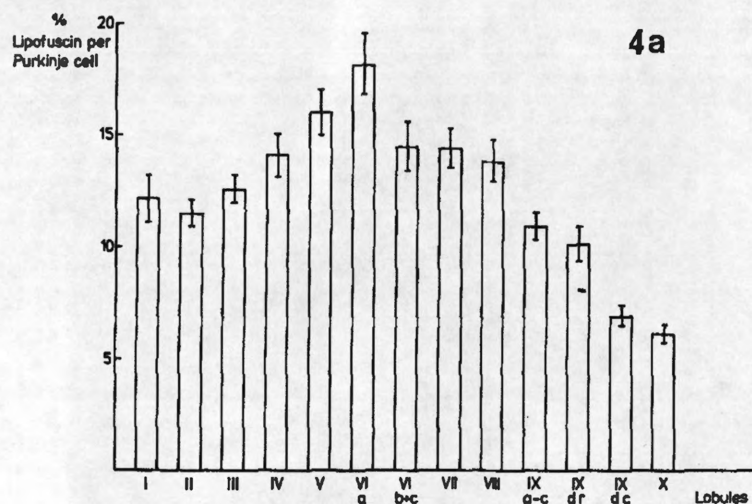


Fig. 3a–c. Autofluorescent lipofuscin photographed with a darkfield immersion condensor and an oil immersion objective 100/1.30. All photos were taken at identical magnification. Bar in 3b represents 10 μm. **a** Purkinje cell from lobule X. **b** Purkinje cell from lobule I. **c** Purkinje cell from the hemisphere. The lipofuscin consists entirely of small granules. The outlines of the granules are blurred because of the minimal depth of focus of the oil immersion objective and the grain of the film

cence. But instead of being discoid the lipofuscin consists of large round globules (Fig. 3b). The globules are encountered in the apical region but also in lower cytoplasmic zones, where they are found to one side or the other of the nucleus (Fig. 1b, d).

The lipofuscin in lobule VIa, the caudal part of lobule V, the hemispheres, lobules VIb+c, VII and the lipofuscin-rich islands of sublobules IXa–d exhibits



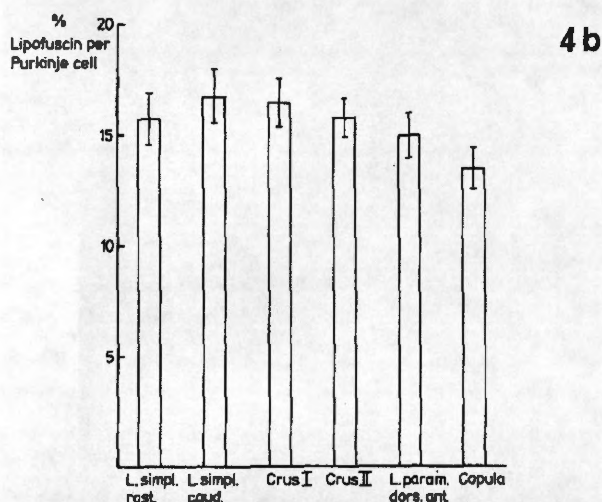


Fig. 4a, b. Bar diagrams of the % content of lipofuscin in the Purkinje cells of the vermal lobules (**a**) and the hemispheric lobules (**b**). Means and standard errors of the means are given in Tables 1 and 2

different morphological and physical properties. Fine granules occupy the apical cytoplasm and frequently form a light yellow fluorescent cone in this part of the Purkinje cell (Fig. 3c). The fine granules are not confined to the apical cytoplasm but are also found in the lower part of the Purkinje cells (Fig. 1c, e, f). The diameter of the granules decreases progressively in the apico-basal direction (Fig. 3c).

As a rule, the dark-yellow fluorescent discoid lipofuscin is encountered in lobule X and sublobule IX d c, a globular lipofuscin in lobules I–III and VIII–IX a and a finely granular light-yellow fluorescent lipofuscin in lobules VIa, V, the hemispheres, lobules VIb+c and VII. Lipofuscin of the globular type is rare in lobule VIa, the caudal part of lobule V and the hemispheres. Lobules IV and VIII seem to be transitional zones where equal proportions of the globular and finely granular lipofuscin are observed.

An additional finding deserves mention. Extraneuronal lipofuscin in Bergmann glial cells is regularly found in lobule IX a (Fig. 1d) and in the caudal

part of lobule X. Here, it extends for a considerable distance into the molecular layer. In all other lobules, the lipofuscin of Bergmann glia is confined to the Purkinje cell layer and is never found in comparable amounts.

Discussion

Hamperl (1934) used fluorescence microscopy to distinguish lipofuscin from melanin. Fluorescence microscopy seems to be a simple and reliable method for the demonstration of lipofuscin. Björkerud (1964) found that the autofluorescent substances in cardiac lipofuscin granules constitute a minimal part of the granules isolated. Using thin layer chromatography, Shimasaki et al. (1977) described an age-related increase in fluorescent substances in nine tissues in 6- and 100-week-old rats. Some properties of the fluorescent components of cardiac lipofuscin were examined by Hendly et al. (1963) and Quadbeck and Weinhardt (1972). Chio et al. (1969) and Chio and Tappel (1969) relate the fluorescent properties of lipofuscin to the peroxidation of polyunsaturated lipids with malonaldehyde as a major reaction product. Malonaldehyde reacts with amino groups of amino acids and the Schiff-base generated is responsible for the autofluorescence of lipofuscin. The fluorescent component seems to be closely linked to the lipofuscin granules because no diffuse cytoplasmic fluorescence could be observed. The shape of the fluorescent lipofuscin granules in the various cerebellar regions coincides well with the morphology of acid-phosphatase positive lipofuscin granules (own unpublished observations). Nevertheless, the ultrastructural localization of the autofluorescent component of lipofuscin is not known.

The significance of lipofuscin is obscure. The accumulation of lipofuscin reflects a basic morphological aging process (Strehler et al. 1959) and, being the only neurocytological age change, is the only consistent alteration that can be correlated with age (Nanda and Getty, 1971). Lipofuscin accumulation is thought to affect the functional integrity of the nerve cells (Brizzee et al. 1976) and ultimately results in nerve cell loss (Brizzee et al. 1974). In the light of these findings the massive accumulation of lipofuscin in the Purkinje cells of the hemispheres and lobules VIa and V would reflect a premature aging process, whereas the Purkinje cells of lobules X and IX d c, which are lipofuscin-poor, are far less prone to the process of aging. A loss of Purkinje cells with progressing age is discussed (for review see Hanley 1974). Hall et al. (1975) and Corsellis (1976) recently reported a 2.5% loss of Purkinje cells per decade in man beyond the 60th year of life, but the authors do not mention regional differences in cell loss.

A linear increase of lipofuscin correlated with aging has been described in various cells and tissues (Strehler et al. 1959; Munnell and Getty 1968; Leibnitz and Wünscher 1967; Sarnat 1968; Dayan et al. 1979). In contrast to myocardial cells (Strehler et al. 1959) and tongue muscle cells (Dayan et al. 1979), Purkinje cells do not uniformly accumulate lipofuscin. The reason for such a differential accumulation is not clear. According to Dapson et al. (1980), who mention statistically significant topographical differences in lipofuscin accumulation in the cerebella of field mice, environmental factors play an important

Table 3. Probabilities for the *t*-values. Purkinje cells of the vermal lobules I–X

Lobule	I	II	III	IV	V	VIa
I	1.0000					
II	0.6595	1.0000				
III	0.7999	0.4881	1.0000			
IV	0.2231	0.0989	0.3333	1.0000		
V	0.0164	0.0049	0.0308	0.2238	1.0000	
VIa	0.0002	0.0001	0.0006	0.0105	0.1671	1.0000
VIb–c	0.1315	0.0528	0.2079	0.7677	0.3554	0.0227
VII	0.1565	0.0648	0.2431	0.8399	0.3092	0.0179
VIII	0.3077	0.1459	0.4422	0.8410	0.1574	0.0061
IXa–c	0.4206	0.7143	0.2906	0.0450	0.0016	0.0000
IXd r	0.1903	0.3821	0.1192	0.0128	0.0003	0.0000
IXd c	0.0010	0.0040	0.0004	0.0000	0.0000	0.0000
X	0.0002	0.0008	0.0001	0.0000	0.0000	0.0000

Lobule	VIb+c	VII	VIII	IXa–c	IXdr	IXdc	X
VIb–c	1.0000						
VII	0.9256	1.0000					
VIII	0.6201	0.6874	1.0000				
IXa–c	0.0222	0.0279	0.0701	1.0000			
IXd r	0.0057	0.0074	0.0214	0.6104	1.0000		
IXd c	0.0000	0.0000	0.0000	0.0111	0.0398	1.0000	
X	0.0000	0.0000	0.0000	0.0025	0.0109	0.6069	1.0000

Table 4. Probabilities for the *t*-values. Purkinje cells of the hemispheric lobules

Abbreviations: *1.0000 = Lobulus simplex, rostral part; *2.0000 = Lobulus simplex, caudal part; *3.0000 = Crus I; *4.0000 = Crus II; *5.0000 = Lobulus paramedianus dorsalis anterior; *6.0000 = Copula

	*1.0000	*2.0000	*3.0000	*4.0000	*5.0000	*6.0000
*1.0000	1.0000					
*2.0000	0.5831	1.0000				
*3.0000	0.6930	0.8770	1.0000			
*4.0000	0.9919	0.5900	0.7005	1.0000		
*5.0000	0.6749	0.3352	0.4171	0.6675	1.0000	
*6.0000	0.2415	0.0896	0.1208	0.2376	0.4475	1.0000

role in lipofuscin accumulation in cerebellar Purkinje cells. Environmental factors can be excluded in the present study because the animals were housed under identical conditions.

Two lines of evidence support the concept of a pigmentarchitectonics of the cerebellar cortex. (1) Statistically significant differences in the amount of lipofuscin per Purkinje cell perikaryon are found (Tables 3 and 4). The present data are 2–3% higher than data published previously (Heinsen 1980). Two reasons may account for these differences. In the previous paper, only the rostral parts of lobules V and X were included in the measurements. The caudal parts of lobules V and X contain more lipofuscin than the rostral parts. Second-

ly, the Purkinje cells of two additional animals have been analysed. (2) Three types of lipofuscin have been observed with the fluorescence microscope (Fig. 3). Braak (1974) supposes that neurotransmitters may play a role in the genesis of lipofuscin. It is not clear whether these three morphological types of lipofuscin in the Purkinje cells reflect different afferent connections with specific neurotransmitters. Eisenman and Noback (1980) demonstrated a ponto-cerebellar projection to the sublobules of lobule IX, which contain lipofuscin-rich islands of Purkinje cells (Fig. 1f). This lipofuscin is morphologically identical to the lipofuscin of the hemispheric Purkinje cells.

The present results allow a differentiation of lipofuscin in the light-microscopical range. Balthasar's (1954/55) observations on the distribution of lipofuscin in two types of Betz' pyramidal cells can be extended to Purkinje cells in the albino rat.

Lipofuscin has proved to be a marker for nerve cells at the macroscopic and microscopic level. At the submicroscopic level it is a valuable tool for the recognition of different nerve and glial cells (Braak 1971; Schlote and Boellard 1975; Heinsen 1979).

Quantitatively and qualitatively different types of lipofuscin point to the existence of at least three subpopulations of Purkinje cells in the cerebellar cortex of albino rats. The physiological role of these subpopulations is not clear, but should be kept in mind when the effects of drugs or toxic substances on the Purkinje cells are evaluated.

Apart from a cytoarchitectonics of the cerebellar cortex (Lange 1970, 1972, 1974, 1975), there exists a pigmentarchitectonics of the Lugaro cells (Braak 1974) and of the Purkinje cells.

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