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Histochemical and ultrastructural features of neuronal pigment in some encephalic nuclei of ruminants

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Summary. Neuronal pigment in red nucleus, locus coeruleus and substantia nigra from cattle, sheep and goat was examined with the aid of light and electron microscopy. In the red nucleus and locus coeruleus neurons of all the species examined the pigment shows the histochemical and ultrastructural features typical of lipofuscins. The ultrastructural observations indicated that the morphology of pigment granules is related to age and permitted identification of various stages of pigment evolution, which suggested a lysosomal origin of the pigment bodies.

In bovine and sheep the substantia nigra is very reduced and contains no detectable pigment; while in goat, some neurons appear to contain discrete amounts of neuromelanin. Ultrastructurally this kind of pigment appears characterized by the features of a lipofuscin-like granule which stores highly electron dense material identifiable as melanin.

Key words: Central nervous system – Lipofuscin – Neuromelanin – Ruminants – Light and electron microscopy

Introduction

Many authors showed that neuromelanin and lipofuscin pigments contained in the neurons of the central nervous system share the same distribution in all the species so far studied. Melanin was found in substantia nigra,

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locus coeruleus and dorsal vagus nucleus in carnivores (Brown 1943; Levi et al. 1977), primates (Scherer 1939, Barden 1970) and man (Adler 1939; Foley and Baxter 1956, 1958; D'Agostino and Luse 1964; Fenichel and Bazelon 1968; Barden 1969; Levi 1974). The distribution of lipofuscin pigment in the central nervous system has been studied in carnivores (Wolf and Pappenheimer 1945; Sulkin 1955), rodents (Alpert et al. 1960; Duncan et al. 1960; Samorajsky et al. 1965; Brizzee et al. 1969; Nandy 1971; Brunk and Ericsson 1972; Heinsen 1979, 1981) and man (Zeglio 1935; Wolf and Pappenheimer 1945; Bondareff 1959, 1964; Brody 1960; Friede 1962, 1966; Timiras 1972; Braak 1974, 1977; Braak 1976; Braak and Braak 1976). Lipofuscin is present in almost all encephalic nuclei.

Many hypotheses have been put forward on the function and the origin of neuronal pigments. Lipofuscin pigment, which is usually regarded as a wear and tear pigment, represents, in the opinion of many authors, a waste product of cellular metabolism. Almost all the cell organelles are thought to be involved in the genesis of lipofuscin. Among the different theories two are most widely accepted. The first is based upon the concept that swollen degenerated mitochondria transform into pigment particles (Hess 1955; Duncan et al. 1960; Heinsen 1979). The second, supported by many histochemical and UV light microscopical observations, points out the concept that pigment granules are derived from lysosomal bodies and are to be considered as telolysosomes, i. e. terminal nonfunctional lysosomes, which are no longer recycled by the cell (Essner and Novikoff 1960; Malkoff and Strehler 1963; Jamieson and Palade 1964). Even less data are available on the origin of neuromelanin; most studies on this subject have dealt with the differences between neuronal and extraneuronal melanins (Foley and Baxter 1958; Kennedy and Zelickson 1963), but the function of this pigment is still completely unknown.

A review of existing literature shows a lack of available data about neuronal pigment distribution and characteristics in herbivores, particularly in ruminants. In two preliminary studies the existence of lipofuscin pigment was reported for the neurons of the red nucleus and the locus coeruleus in adult cattle (Peirone 1980 a, b). In order to describe the morphological features and the histochemical properties, such as time of appearance and age related changes in neuronal pigment, a thorough study was carried out on some encephalic nuclei of ruminants with the aid of light and electron microscopy.

Materials and methods

For light microscopy, eight bovine brains ranging in age from 8 months to 15 years, eight ovine brains from new born to 10 years and six goat brains form 30 days to 12 years were examined. Gross sections of the cranial portion of the medulla oblongata and of the mesence phalon were fixed in 10% formalin or in Zenker formalin, paraffin embedded and sectioned in toto at 7 µm. Red nucleus, substantia nigra and locus coeruleus were examined with the following techniques: Haematoxylin-Eosin and Toluidine Blue to determine the topography of the nuclei; periodic acid Schiff; Sudan Black B and Oil Red O for the demonstration of lipids; Ziehl-Nielsen for acid fastness; Perls for ferric ion and Lillie for ferrous ion uptake. Some

unstained sections were observed by a blue light fluorescence microscope. The light source consisted of a high pressure mercury vapour burner (Osram HBO-200); the light beam was filtered through a BG 12 exciter filter to obtain a wide band emission ranging from 300 nm to 500 nm.

For electron microscopy, eight bovine brains from animals ranging in age from the new born to 15 years, six ovine brains from the new born to 10 years and four goat brains from 6 months to 12 years were examined. Tissue fragments of 1 mm^3 obtained from the red nucleus, the locus coeruleus and the substantia nigra of all the species were fixed in 2.5% glutaraldehyde buffered at pH 7.4 with 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide, blocked stained with 2% uranyl acetate and embedded in Durcupan. Sections (1 μm thick) were stained with Toluidine Blue to identify the pigment containing cells. The ultrathin sections were stained with Reynolds lead citrate and examined in a Siemens Elmiskop 1A electron microscope.

Results

Light microscopy

Red nucleus. In all the species examined the red nucleus appeared as a conspicuous mass of neurons deeply embedded in the mesencephalic tegmentum at the level of the rostral colliculus. This nucleus contained two distinct cellular populations: a caudal portion (pars magnocellularis) formed by a great number of large neurons and a rostral portion (pars parvocellularis) formed by few small cells. The large neurons of the pars magnocellularis varied in size and shape, some being rounded or ovalshaped, some elongated or with irregular polygonal outlines. The axon hillock was usually well evident. In Toluidine Blue or Haematoxylin stained sections the cell nucleus appeared centrally located within the cell body, the nucleolus stained darkly and Nissl bodies were abundant. In the adult animal these cells contained variable amounts of pigment which generally formed conspicuous clusters within the cell body.

The periodic acid Schiff reaction gave the best results among the histochemical used: the pigment was stained an intense purplish red color and stood out from the pale cytoplasm which surrounded it (Fig. 1). In addition, the pigment stained deeply with lipophilic stains such as Oil Red O and Sudan Black B. The Perls and Lillie reactions were however always negative. The pigment, on the other hand, stained strongly with Ziehl carbolfuscin, thus showing its acid fastness (Table 1).

UV light microscopy demonstrated that the pigment in the red nucleus neurons was always autofluorescent, with a light yellow color. In all the species examined, the amount of neuronal pigment appeared to be correlated to the age of the animals. The first pigment granules appeared, at the end of the first year of life, as brilliant bodies scattered throughout the cytoplasm. With age, there was an increase in the amount of pigment and the granules tend to collect into groups located in one or both neuronal poles. The pigment granules in 6 to 8-year-old animals were larger than in the younger animals and they were clumped in conspicuous clusters; nevertheless we observed minute granules scattered throughout the cytoplasm in these aged animals too. In old animals (10 years and over) the pigment may fill almost entirely the perikaryon, it was sometimes observed within the

axon hillock but never within the dendrites. The most conspicuous pigment clusters were observed in bovine; in the other species the amount of pigment was relatively lower.

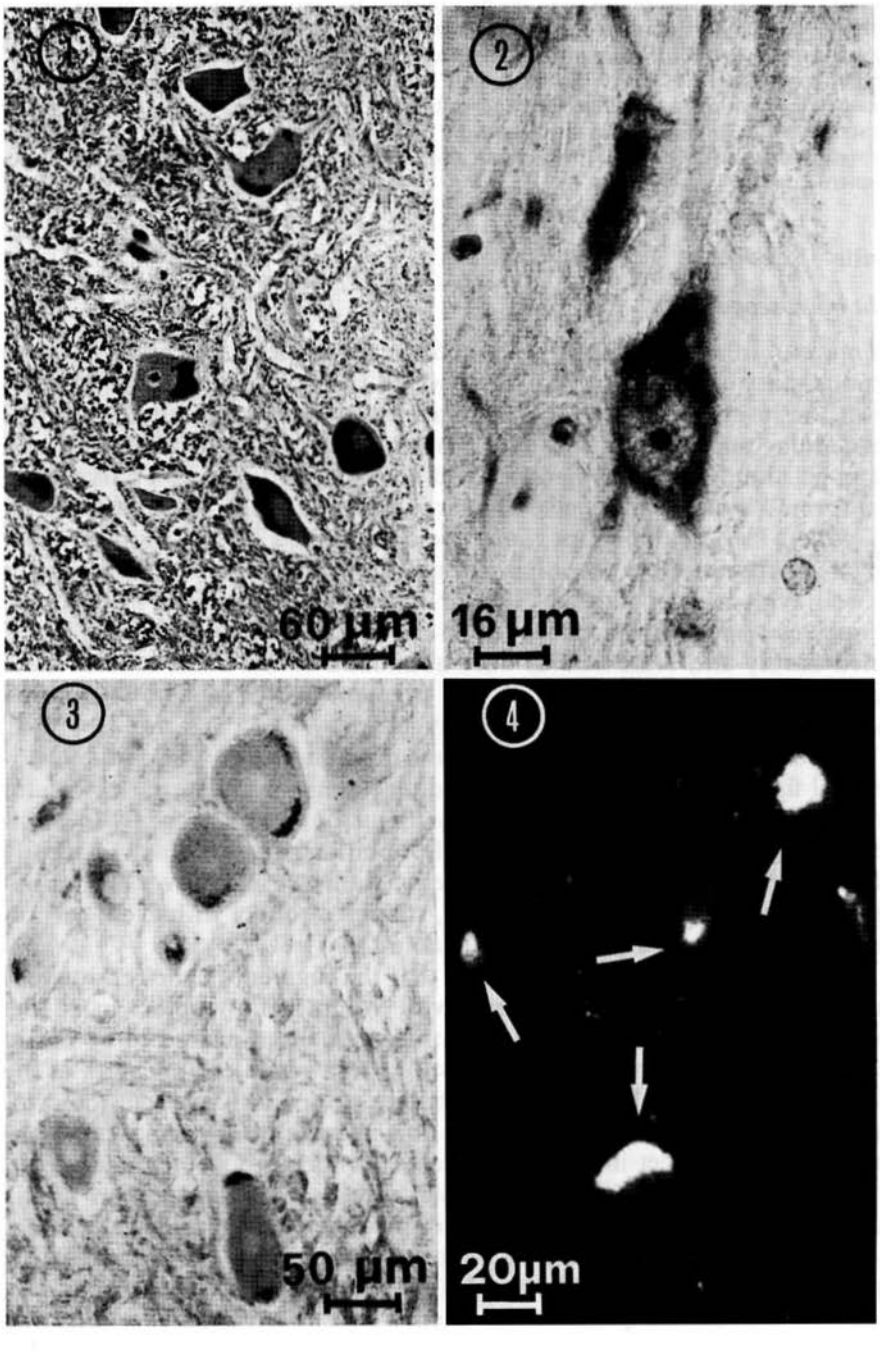

Table 1. Histochemical features of neuronal pigment in the brain stem nuclei examined

	Autofluorescence	P.A.S.	Acid fastness	Sudan Black B	Oil Red O	Perls	Fe^{++} uptake**	
Red nucleus	+	+	+	+	+	−	−	bovine
	+	+	+	+	+	−	−	sheep
	+	+	+	+	+	−	−	goat
Locus coeruleus	+	+	+	+	+	−	−	bovine
	+	+	+	+	+	−	−	sheep
	+	+	+	+	+	−	−	goat
Substantia nigra		no	demonstrable		pigment			bovine
		no	demonstrable		pigment			sheep
	−	−	−	−	−	−	+	goat

** Lillie reaction

Substantia nigra. In ruminants we found that the features of the substantia nigra were somewhat different from those described for man and other species. Bovine substantia nigra was not easily detectable because of the exiguous number of little neurons randomly scattered through the basal portion of mesencephalon. Sheep substantia nigra was even less defined; we found only few little cells scattered here and there on the border between the mesencephalon tegmentum and the crus cerebri. On the contrary, goat substantia nigra was more detectable, it consisted of numerous large polygonal neurons which formed a compact cellular band. We observed no pigment in bovine and sheep neurons, while in unstained or in Toluidine Blue stained sections of goat substantia nigra some cells were found to contain dark brown or black pigment (Fig. 2). Neuronal pigment in goat substantia nigra cells was not autofluorescent; it was also not stained with periodic acid Schiff, lipophilic stains or Ziehl carbolfusin; moreover it was negative for Perls reaction but positive for Lillie ferrous ion uptake (Table 1).

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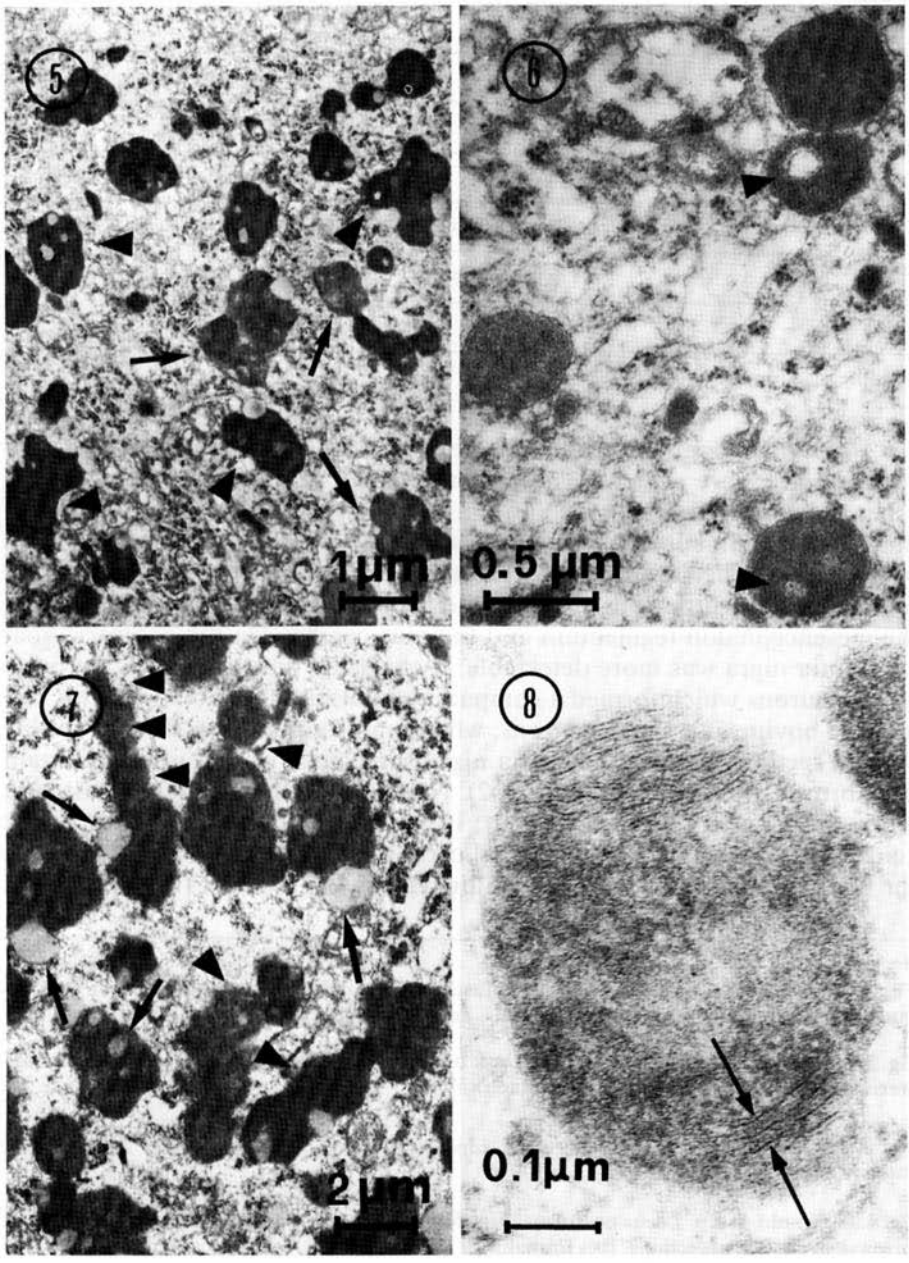
Fig. 1. 12 year-old cow. Red Nucleus. Lipofuscin pigment showing a strong positive PAS cumulates in great amounts in most of the cells. Zenker-formalin. Periodic Acid Schiff. × 625

Fig. 2. 6 year-old goat. Substantia nigra. Dark brown or blackish pigment granules can be appreciated. Zenker-formalin. To luidine blue. × 160

Fig. 3. 2 year-old sheep. Locus coeruleus. Lipofuscin shows a strong affinity for lipophilic stains. Zenker-formalin. Oil Red O. × 190

Fig. 4. 8 year-old sheep. Locus coeruleus. Lipofuscin pigment appears clearly autofluorescent *(arrows)*, in unstained sections. 10% formalin. × 410

Locus coeruleus. The nucleus of the locus coeruleus showed the same topographical and structural features in all the species examined. It was located beneath the floor of the fourth ventricle in the lateral recess at the transverse level immediately rostral to the colliculus facialis. The nucleus of locus coreuleus consisted of ovoid neurons; the cell nucleus was round



and the nucleolus was quite evident. In the perikaryon, at one or both cellular poles, we observed some pigment with histochemical features identical to those described for the red nucleus cells (Table 1; Figs. 3 and 4); whereas UV light microscopy showed that in the locus coeruleus the pigment granules were larger and less numerous. In this site too there is an increase, with age, in the number of pigmented cells and in the amount of pigment in each neuron.

Electron microscopy

Neuronal pigment granules in red nucleus cells were easily detected because of their high electron density (Fig. 5); higher magnifications revealed a complex ultrastructure whose features are closely related to age. Until the third month of postnatal life only small dense round particles, consisting of a homogeneous matrix of a fine granular membrane bounded material, are present in the neuronal perikaryon. These particles strongly resemble lysosomal bodies. From the third month on, in addition to these particles other spheroidal bodies, with a few lamellar structures and one or more light areas in the granular matrix, were also observed (Fig. 6). The pigment particles of adult animals appeared as large irregular membranes-bound dense complexes (Fig. 7). Fully developed pigment granules consisted of: 1) a fine granular matrix formed by 5 nm granules; 2) one or more light areas of varying size, which were identified as lipid containing vacuoles by many authors (Hess 1955; Essner and Novikoff 1960); 3) a periodical lamellar system which was not always clearly evident due to masking by the granular matrix; the fundamental unit of this system was formed by two thin lamellae, separated by a 10 nm space, in the center of which a third thicker and darker lamella was present; the lamellae often formed a sinuous pattern like a finger print (Fig. 8).

In the red nucleus neurons of animals 10 years old or over, we observed many pale granules with scanty granular matrix and very prominent lamellae together with a progressive disappearing of the limiting membrane (Fig. 9).

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Fig. 5. 8 year-old sheep. Red nucleus. The neuronal cytoplasm contains many pigment granules, appearing as dense bodies. Dark *(arrow heads)* and pale *(arrows)* granules are evident. × 10,200

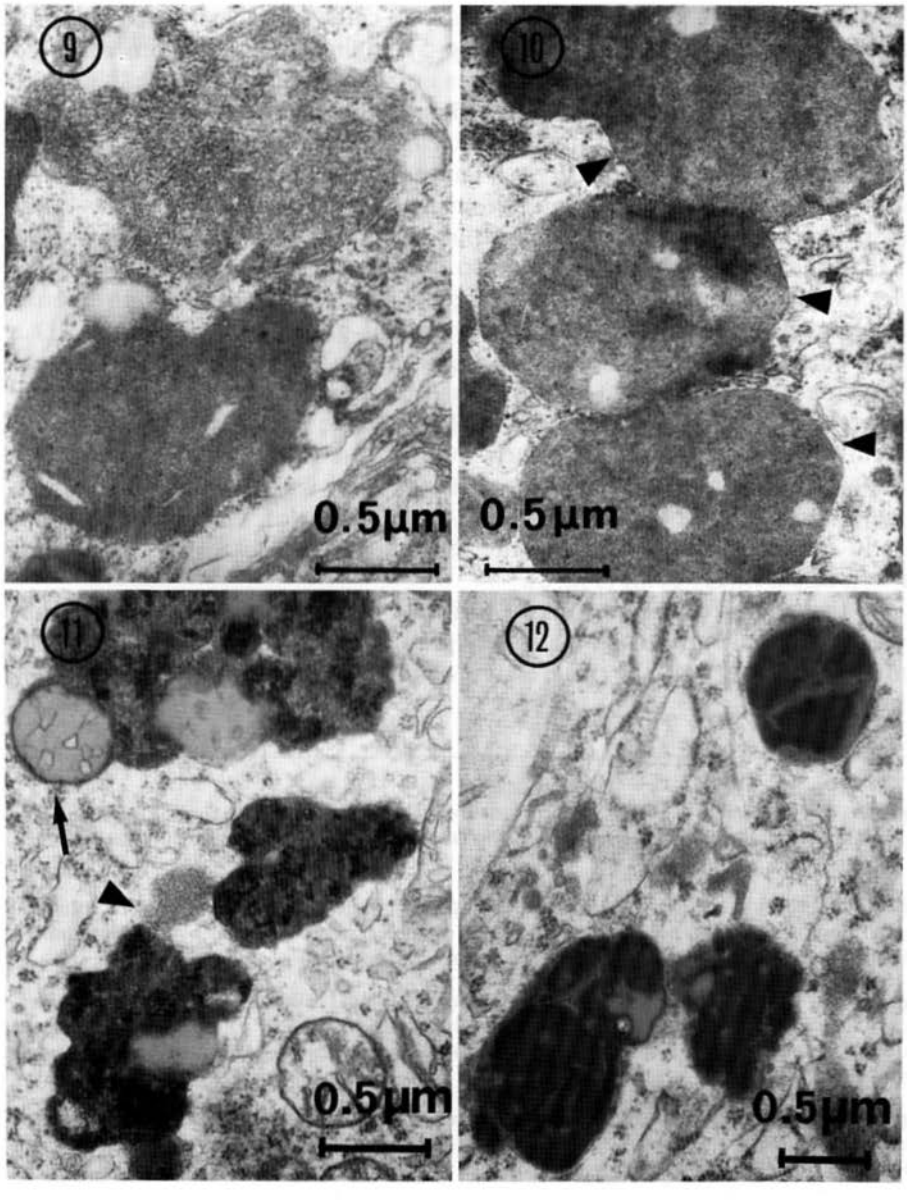
Fig. 6. 3 month-old calf. Red nucleus. Initial stages of pigment granules deposition. Spherical shaped homogeneous granules about 0,5 μm in diameter are surrounded by a single limiting membrane; in two of these a lipid vacuole has formed *arrow heads).* × 32,000

Fig. 7. 6 year-old goat. Red nucleus. Pigment granules typical of adult animals. Note the presence of many lipid vacuoles *(arrows)* and the irregular outlines of the granules due to the fusion of smaller pigment bodies resembling the protopigment described in young animals *(arrow heads).* × 18,000

Fig. 8. 10 year-old cow. Red nucleus. A higher magnification shows the lamellar system; the fundamental unit of this system is formed by a thick lamella with two thin lamellae on both sides. Thick lamellae are well evident *(arrows).* × 130,000

Neuronal pigment granules in the locus coeruleus cells have the same ultrastructural characteristics as in the red nucleus, but they were larger and more regular in shape; the granular matrix was more dense (Fig. 10).

Electron microscopical observations confirmed the lack of pigment granules in the neurons of bovine and sheep substantia nigra. On the other hand we colud easily detect neuronal pigment in goat substantia nigra. Pigment bodies in the substantia nigra of adult goats showed, in addition to



the three ultrastructural components described above in pigment granules of red nucleus and locus coeruleus (i. e. the matrix, the lamellar system and the lipid vacoules), the presence of a highly electron dense material stored in the matrix (Fig. 11). The amount of this material was extremely variable, sometimes being very scanty and sometimes filling almost all the matrix. In the latter case the other structures of the granules were almost completely masked by the electron dense material and the granules appeared as highly electron dense bodies in which no structures was evident, with the exception of a scarce number of lipid vacuoles (Fig. 12). In young animals, pigment granules were small in size and their structure was quite simple. They consisted of a granular matrix with a few lamellae and some lipid vacoules; the matrix always contained a little amount of the electron dense material.

Discussion

In the ruminants examined the features and the distribution of neuronal pigment in the red nucleus and locus coeruleus cells are almost similar; thus they will be discussed together while the substantia nigra will be treated separately.

Red nucleus and locus coeruleus

In the red nucleus and in the locus coeruleus, the neuronal pigment is characterized by bright yellow autofluorescence, positiveness to periodic acid Schiff, acid fastness, strong affinity for lipophilic stains and negativity to Perls and Lillie reactions. The ultrastructural observations on neuronal pigment in these nuclei showed the existence of a triphasic substructure in the pigment bodies. These light and electron microscopical features are distinctive of lipofuscin pigments (Wolf and Pappenheimer 1945, Sulkin 1953, 1955; D'Angelo et al. 1956; Bondareff 1959; Pearse 1960; Samorajsky et al.

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Fig. 9. 15 year-old cow. Red Nucleus. Pigment granules show a different electron density: pale granules show more evident lamellar systems. × 33,000

Fig. 10. 10 year-old ox. Locus coeruleus. Pigment granules are formed of a fine granular matrix from which one or more lipidic vacuoles stand out. The limiting membrane is clearly appreciable at some point of the granule periphery *(arrows heads)*, whilst the lamellar system is basely detectable. × 33,000

Fig. 11. 12 year-old goat. Substantia nigra. Pigment granules are characterized by the presence of an highly electron dense material stored in the matrix. A lipidic vacuole bounded by a rim of the electron dense material is evident *(arrow)*. A protopigment-like particle is present in the center of the micrograph *(arrow head)*. Note the irregular outlines of the granules and the granular appearance of the electron dense material. × 30,000

Fig. 12. 12 year-old goat. Substantia nigra. Another aspect of neuromelanin pigment. Note the regular outlines of the granules and the homogenous appearance of the electron dense material which fills almost completely the granular matrix. × 24,000

1964, 1965; Strehler 1964). The assessment of lipofuscin pigments in ruminant red nucleus is in accordance with the reports in man (Friede 1962, 1966), *Macacus rhesus* (Barden 1970), dog (Sulkin 1955), pig (Nanda and Getty 1971) and rat (Nandy 1971). On the contrary, the existence of lipofuscin pigment in the locus coeruleus points out remarkable differences between these and other species such as carnivores, primates and man where the pigmented cells appear to contain neuromelanin (Adler 1939; Brown 1943; Barden 1970; Levi 1974; Levi et al. 1977).

UV light microscopy pointed out a similarity in the autofluorescent proberties of the pigment granules in both the nuclei, but at the same time the existance of age related differences among the species examined. Our observations suggest that the ultrastructure of the pigment granules undergoes progressive modification with age. Small spherically shaped masses of homogeneous granular content, which are morphologically similar to lysosomal bodies, represent the first stage of formation of the lipofuscin pigment. These small masses could therefore be considered as a protopigment. The lipid vacuoles appear in these granules in the second stage; together with the appearance of the lipid component, the lamellar system differentiates to form the first pigment granules. In a subsequent stage the masses of pigment merge into large complexes which constitute the characteristic elements of the pigment in the adult animals; indeed the observation of some incompletely merged granules, bounded by a single limiting membrane, is quite common. In old animals, pigment granules with a pale matrix and a well defined lamellar system have constantly been detected. This suggests that lipofuscin granules could undergo further morphological changes as a consequence of metabolic processes leading to modification of some of the structures considered metabolically inertial entities by many authors.

We do not share entirely the view of the mitochondrial origin of lipofuscin granules, in fact we seldom observed pigment granules in a spatial relationship with degenerated swollen mitochondria or cell membrane fragments. On the contrary the close morphological similarity between protopigment granules and lysosomes is highly suggestive of the possibility that at least a part of the lipofuscin pigment is derived from lysosomal material. Under this view, the morphological changes observed in the ultrastructure of the pigment granules could be related to the continuous supply of metabolic waste products which are no longer recycled by lysosomal enzymes.

Substantia nigra

Contrary to the observations of Huber et al. (Quoted in Levi et al. 1977), the substantia nigra of the ruminants examined showed different morphological features with respect to other mammalian species. The sections in toto of the brain stem demonstrate that it is impossible to find a typical substantia nigra in cattle and sheep, because of the exiguous number of neurons and the lack of pigment in them.

On the contrary, goats show a definite substantia nigra with a larger number of cells; here some neurons do contain a brownish-black pigment which is characterized by the histochemical properties specific of neuromelanin (Lillie 1955, 1957). The electron micrographs attest the light microscopical observations. the existence of a highly electron dense material, identifiable as melanin, besides the ultrastructural components typical of lipofuscin granules is held to be characteristic of the neuromelanic pigment (Moses et al. 1966; Forno and Alvord 1974). Our observations show the similarity between lipofuscin and neuromelanin at the ultrastructural level. They support the hypothesis of Barden and other (Barden 1981), based on histochemical observations, that neuromelanin is to be regarded as a melanized lipofuscin.

In conclusion, remarkable differences exist in the distribution of neuronal pigment in the brain stem nuclei even among species which are closely related taxonomic entities such as the species examined in this study. The ultrastructural observations clearly demonstrate that the pigment features undergo progressive changes with age and that in a single species these ultrastructural features vary among the different nuclei; moreover these observations suggest a lysosomal origin of lipofuscin. Nevertheless futher observations are to be undertaken to clarify better the relationship between lipofuscin and neuromelanin pigment.

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