Cell death in the embryonic brain of *Gallotia galloti* (Reptilia; Lacertidae): a structural and ultrastructural study

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INTRODUCTION

The cellular death that is observed in the nervous system of vertebrates during embryonic development is a spontaneous phenomenon that occurs at distinct periods specific for each neuronal population. It has been defined as one of the regressive events corresponding to the period of neurogenesis (Cowan, Fawcett, O'Leary & Stanfield, 1984).

Numerous workers have studied the phenomenon of normal cellular death during cerebral ontogenesis of various vertebrates. The majority of the cells that degenerate do so after having migrated to their proper positions (Cunningham, 1982), but cell degeneration has also been observed during the neurulation phase (Kallen, 1955) and at the end of the proliferation period (Panesse, 1976; Hamburger, Brunso-Bechtold & Yip, 1981).

The types of cell death that have so far been described in the nervous system are those called 'nuclear death' and 'cytoplasmic death' by Pilar & Landmesser (1976), or 'Type I' and 'Type II' by Chu-Wang & Oppenheim (1978). These types, morphologically distinct, have been found to correspond to degenerating neurons in early or late maturation phases (Cunningham, 1982), and have been described at the ultrastructural level by numerous authors (Cantino & Sisto, 1972; Pilar & Landmesser, 1976; Chu-Wang & Oppenheim, 1978; Sohal & Weidman, 1978; Giordano, Murray & Cunningham, 1980; Cunningham, 1982; Warton & Jones, 1984). The cellular death found during the early phases of development has been less intensively studied from this standpoint and the importance of this type of cell death for the maturation of the nervous system is now beginning to be recognised (Rager, 1980).

A notable paucity of data referring to reptiles is apparent in this subject. In the present work, we have studied the different appearances of cellular death during embryonic development of three cephalic centres in the Lacertid reptiles, namely the striatum, thalamus and cerebellum. Our objective has been to determine what types of cellular death occur in these centres, what their temporal and spatial patterns are, and to what degree these patterns are similar to those observed in other vertebrates.

MATERIALS AND METHODS

Thirty embryos of *Gallotia galloti* (Reptilia, Lacertidae) were used. The specimens were obtained from pregnant wild females captured during the months of June and July and from eggs collected in the field during July–September. The embryos with a cephalic length greater than 2 mm – Stage 30 (E. 30) onwards – were decapitated
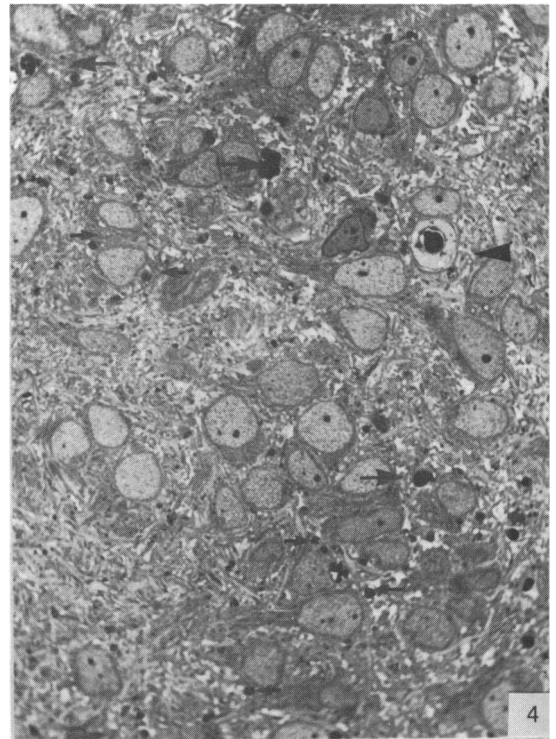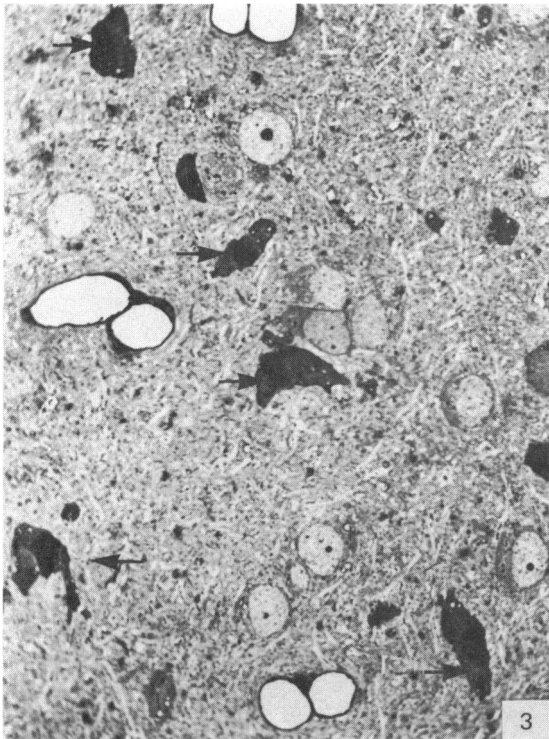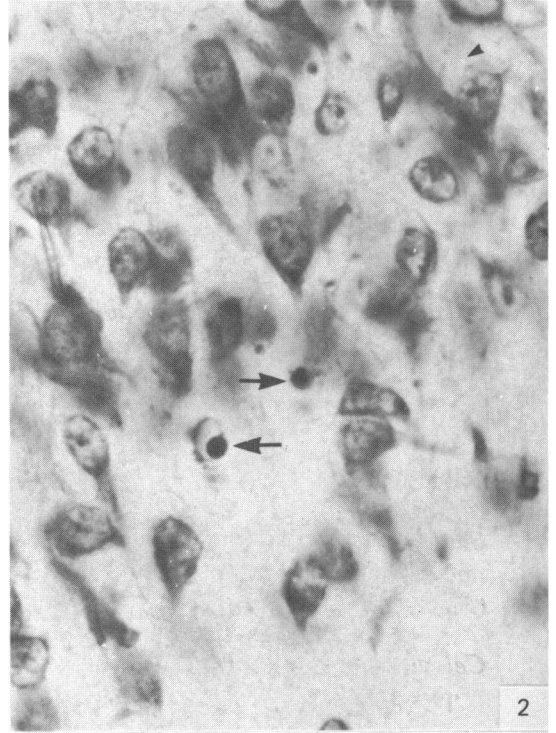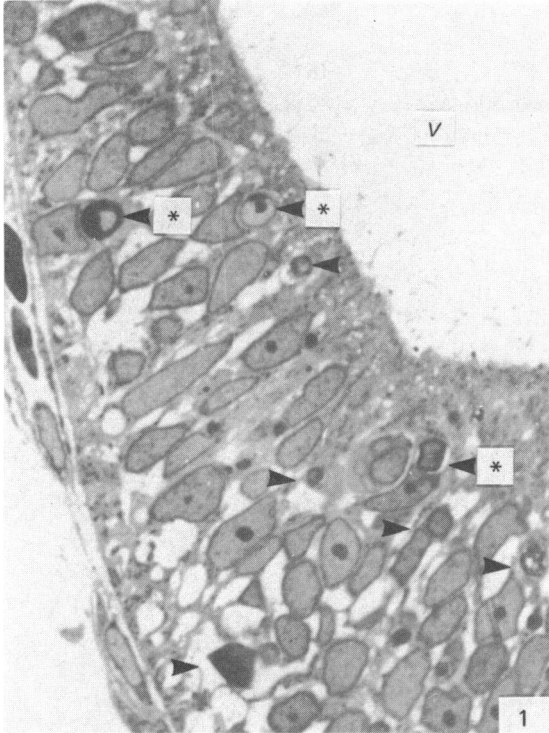
and immediately fixed by immersion. The specimens near to hatching were anaesthetised with Nembutal and perfused.

The embryos were classified on the basis of the developmental table of *Gallotia gallotia*, in which equivalence with the developmental stages of *Lacerta vivipara* (Dufaure & Hubert, 1961) was established.

For light microscopy the embryos were fixed in Bouin's solution and Lillie's formalin, embedded in paraffin wax, sectioned at 7–10 μm and stained with haematoxylin and eosin or toluidine blue and orange-G (Mann–Dominici). For electron microscopy they were fixed with glutaraldehyde in 2·5 % phosphate buffer (0·1 M and pH 7·2), postfixed with 2 % osmium tetroxide in Millonig buffer at 4 °C for 2 hours, dehydrated with acetone and embedded in Araldite. The sections were stained with 1 % toluidine blue.

RESULTS

In the three cerebral centres studied we found four morphological types of normal cellular death. By light microscopy these types can be defined in the following ways: (*a*) Nuclei with a degree of chromatin condensation which frequently appears phagocytosed in the cytoplasm of normal cells (Fig. 1). (*b*) Cells with a pyknotic nucleus and clear cytoplasm (Fig. 2). (*c*) Cells with a dark nucleus and cytoplasm (Fig. 3). (*d*) Small dark 'masses' or 'points' dispersed in the neuropil (Fig. 4).

Cells with condensation of nuclear chromatin

The first type was observed in the ventricular zone or adjacent to it. This type was specially abundant in the thalamus, where from Stage E. 32, such cells were preferentially located in the anterior region of the ventral thalamus. In Stages E. 34 and E. 35 they were mainly observed in the region corresponding to the dorsal thalamic protrusion. As development proceeded their presence became notably less. This type was also found in the ventricular zone and in the external granular layer of the cerebellum in Stages E. 33–E. 36. In the striatum they were detected in the ventricular zone corresponding to the region bordering the nucleus sphericus from E. 32 to E. 37.

Electron microscopy showed the nuclear chromatin condensed into large accumulations that were preferentially positioned towards the periphery (Fig. 5). Granules, similar to glycogen in appearance, were associated with these accumulations and they were frequently seen surrounding the nucleolus. The double nuclear membrane was, in general, conserved but lacked ribosomes on the external surface and it sometimes had invaginations which formed vesicular and lamellar structures. The nucleus was sometimes surrounded by a clear space containing a membranous system that occasionally had dilated vesicles. These apparently contained lipid (Fig. 5).

Fig. 1. Death of undifferentiated neuro-epithelial cells. Degenerated nuclei showing distinct grades of chromatinic condensation (arrowheads). Some have been phagocytosed by neighbouring cells (arrowheads and asterisks). Semithin section (1 μm) corresponding to E. 32, anterior level of ventral thalamus. *V*, ventricle. × 1000.

Fig. 2. The nuclear death type is represented by cells with pyknotic nuclei and clear cytoplasm (arrows). A fine section (10 μm) through the nucleus sphericus, hatching stage, Mann–Dominici stain. × 1000.

Fig. 3. Typical image of the 'cytoplasmic' death type (arrows). Semithin section of the nucleus sphericus, hatching stage. × 1000.

Fig. 4. Cellular debris appears as small dark 'masses' (large arrows) or 'points' (small arrows) scattered in the intermediate zone. A cell with a pyknotic nucleus (arrowhead) also can be observed. Section of nucleus sphericus, E. 38. × 1000.
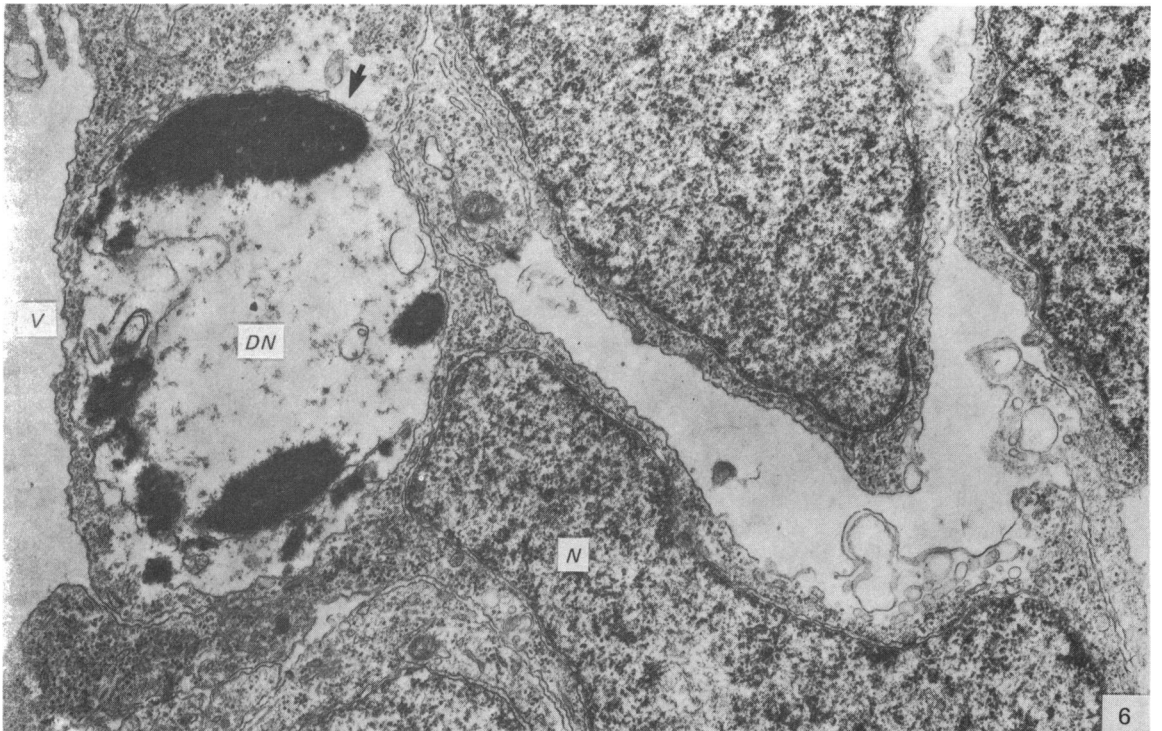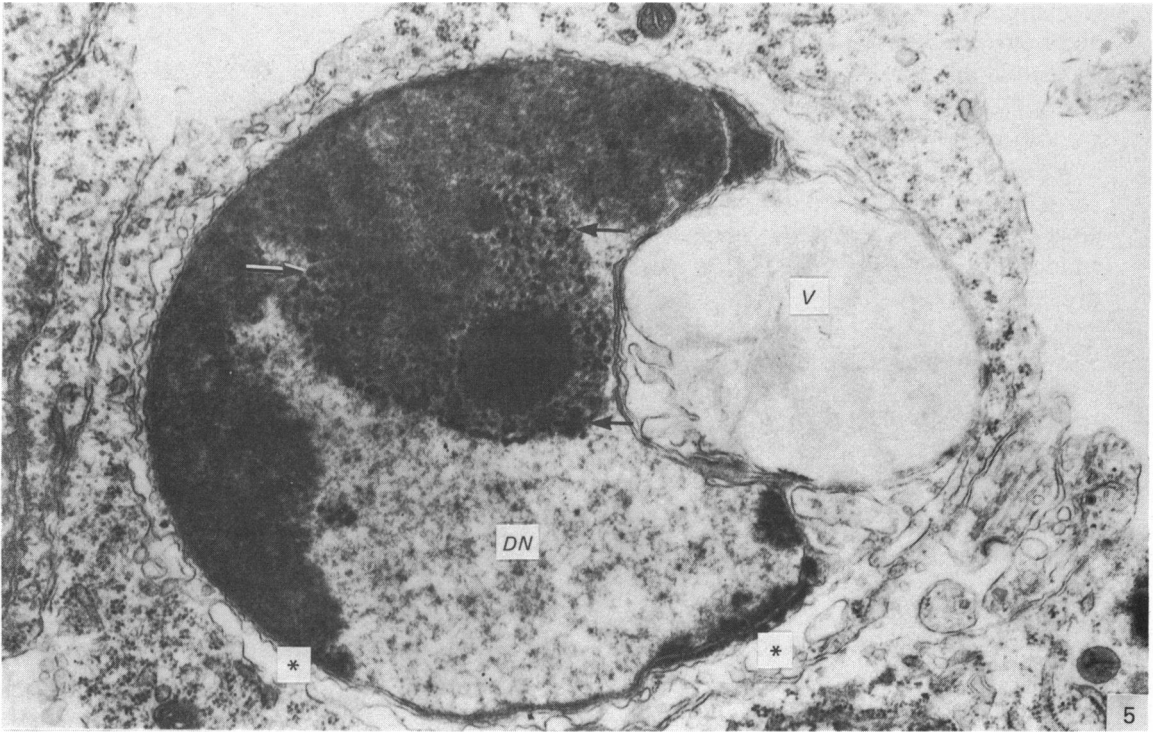
The cell membrane sometimes appeared broken and the sparse cytoplasm was dispersed, leaving the nucleus free. However, there were also a number of cells in which the degeneration affected both nucleus and cytoplasm. There were numerous examples that showed without doubt that the degenerating structures were phagocytosed by neighbouring cells that had the typical appearance of undifferentiated matrix cells (Fig. 6). The final phase appeared to consist of numerous residual masses lying either in the cytoplasm of normal cells, in the intercellular spaces, or even projecting towards the ventricular lumen. The formation of the large lipid drops which characterised the early stages appeared to be associated with the degeneration process.

Cells with pyknotic nuclei

The second type of cell death observed consisted of cells with pyknotic nuclei (Fig. 2) which coincided with the description of Glücksmann (1930, 1951). These cells were dispersed in the normal embryonic brains throughout the three regions studied. In the cerebellum they appeared at about Stage E. 36 in all the zones of the cortex and persisted in the later stages through showing quantitative variations both between the stages and between the cortical layers. Thus, at Stage E. 38 they underwent a notable reduction in number whilst at Stage E. 39 the number of pyknotic nuclei again increased considerably. From Stage E. 35 they were observed in the thalamic nuclei. They increased slightly in number later but were practically non-existent during the hatching stages. In the striatum they were evident from Stages E. 32, E. 35 or E. 37 depending on the nucleus, and remained constant or increased slightly in number in the later phases. In the ventral striatum and anterior dorsal ventricular ridge, the pyknotic cells showed a notable increase in number at the hatching stages.

At the ultrastructural level the process was characterised by a progressive chromatin condensation that affected the whole nucleus (Fig. 7). During this phase, the cytoplasmic content appeared intact, but slightly denser. The nuclear cisternae dilated as the nuclei shrank.

Cells with a dark nucleus and cytoplasm

The first dark neurons were observed at about Stage E. 36 but were few in number. In all regions examined their presence was more constant around Stage E. 38 and from then onwards an increase was observed which, within the embryonic period, reached a maximum in the hatching stages but continued into postnatal and adult life. In general, they were observed alongside normal immature neurons in all areas studied but well spaced out. However, in some nuclei, like the nucleus sphericus, the proportion of these cells was high and degeneration was found to affect groups of neurons jointly.

At an ultrastructural level this type of cell (Fig. 8) contained cisternae of the Golgi complex, dilated rough endoplasmic reticulum, progressive condensation of the

Fig. 5. The neuro-epithelial cell death process. In the foreground a degenerative nucleus (*DN*) with the typical chromatinic 'skull cap'. *V*, Vesicular expansion with apparent lipid content; granules, apparently glucogenic (arrows); membranous cisternae surrounding the nucleus (asterisks). × 23000.

Fig. 6. A more advanced phase of neuro-epithelial degeneration. A degenerate phagocytosed nucleus (*DN*) and a normal nucleus (*N*) can be observed in the same cell. In the degenerate nucleus the double membrane is only partially present (arrow). *V*, Ventricle. × 18000.
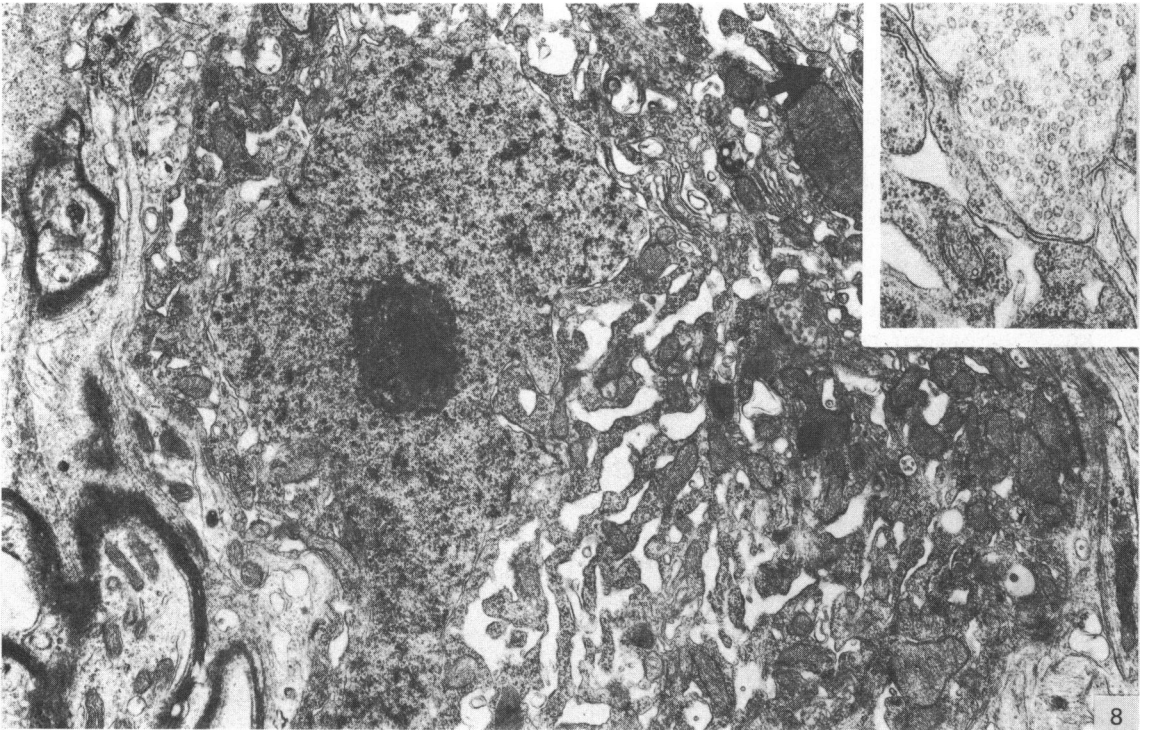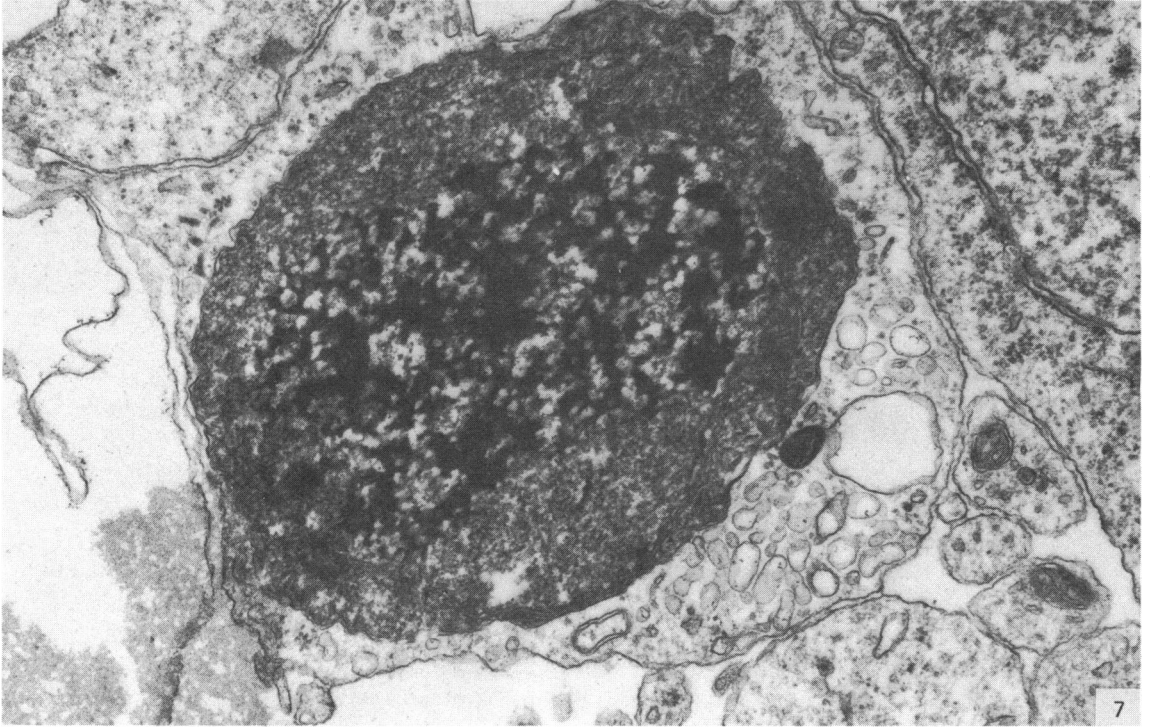
cytoplasm that exhibits lysosomes in different phases of evolution, lipid droplets, degenerated mitochondria and numerous scattered ribosomes. The whole nucleus was condensed and the nuclear membrane finally absent.

Small masses and points

The small masses or dark points that were observed scattered in the neuropil and which at the light microscopy level could be confused with stain precipitate, were present from Stage E. 35, reached their maximum frequency between Stages 37 and 38, and were scarce during the hatching stage. With the electron microscope these masses, which varied in size, were very electron-dense and were clearly identifiable as debris or cellular remains in which it was sometimes possible to identify degenerated cytoplasmic organelles (Fig. 9). They sometimes appeared contained in cytoplasmic islets situated between the fibres of the neuropil or engulfed by normal processes. It is highly probable that these processes belonged to microglial cells as these cells were frequently observed in zones with a high proportion of dark masses (Fig. 10).

DISCUSSION

We have found three types of cellular death during embryonic development both at the light and electron microscopy level. The first affects undifferentiated neuro-epithelial cells, is commonest during the early stages (E. 32–E. 36) and is similar to the nuclear death (or Type I), but with its own characteristics. Morphologically, the early cell death shows similarities to apoptosis as described by Wyllie, Kerr & Currie (1980), but there are some differences related to cytoplasm degeneration and the formation of the apoptotic bodies. Sadler & Cardell (1977) found identical degenerated structures in neuro-epithelial cells of mouse embryos exposed to cytotoxic doses of hydroxyurea, the only difference being the large amount of monosomes dispersed evenly in the cytosome; this was not observed in our case. The phagocytosis of these degenerated cells is carried out by the neighbouring undifferentiated cells. The same process is observed in apoptotic epithelial cells (Wyllie *et al.* 1980), in the anterior neuropore of mouse (Geelen & Langman, 1977) and in embryonic mouse choroid plexus (Sturrock, 1979*a*). This occurs in the early stages in the matrix layer, where there are no microglial cells.

The ultrastructural study of the phagocytic cells shows an absence of lysosomes and thus raises the question as to the localisation of the enzymatic apparatus necessary for the degradation process. The remains of the phagocytosed material, or a portion of them, are degradated until they become transformed into lipid droplets, some of which are voluminous. These droplets are accumulated and they characterise the ventricular zone for a period of time. The association between the different degenerative processes in the brain and the formation of lipids has previously been described (Sturrock, 1980; Franson, 1985) but we do not know its function or the final destination of the lipid droplets of this zone. There is evidence that the droplets are extruded into the ventricle, but we have observed many droplets in the

Fig. 7. Ultrastructural characteristics of a cell affected by a 'nuclear' death process. In this phase, the degeneration affects equally the nucleus and the cytoplasm. This section is from the dorsal thalamus, below the nucleus rotundus. E. 38. × 38000.

Fig. 8. This neuron – a cerebellar Purkinje cell – shows the typical characteristics of cytoplasmic death. The enlargement of the cytoplasmic cisternae and the relative nuclear conservation can be observed. The arrow points out a synapse. The inset shows a magnification of the synapse. × 26000.
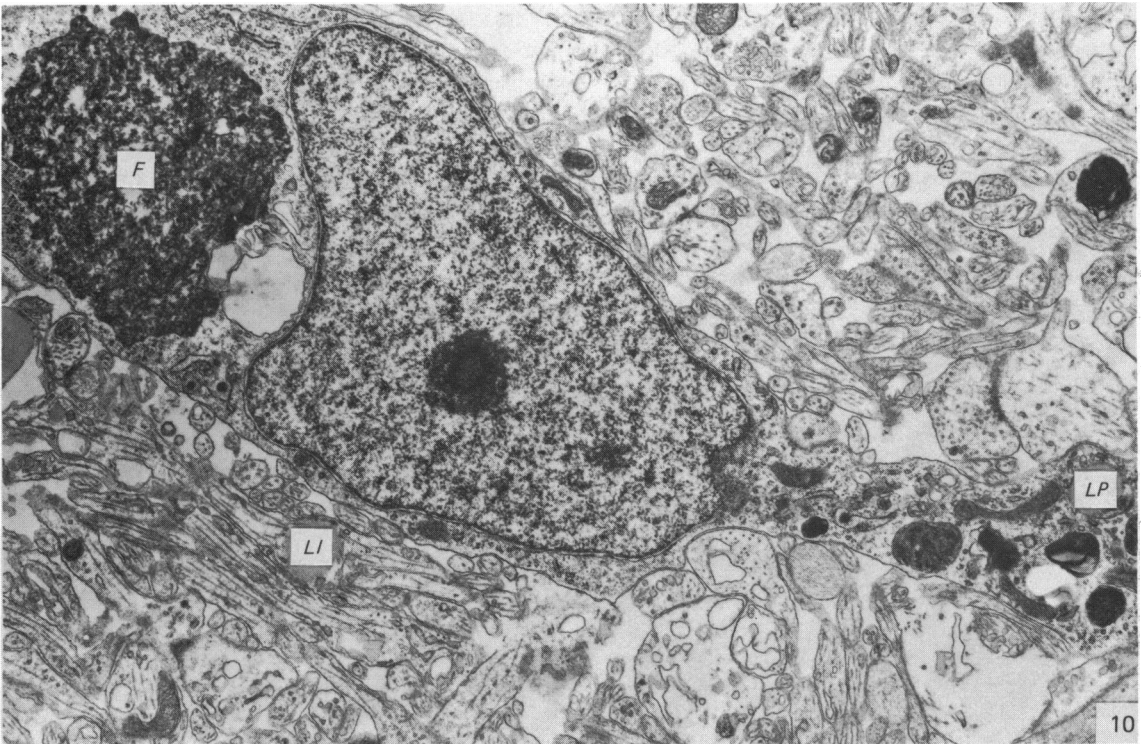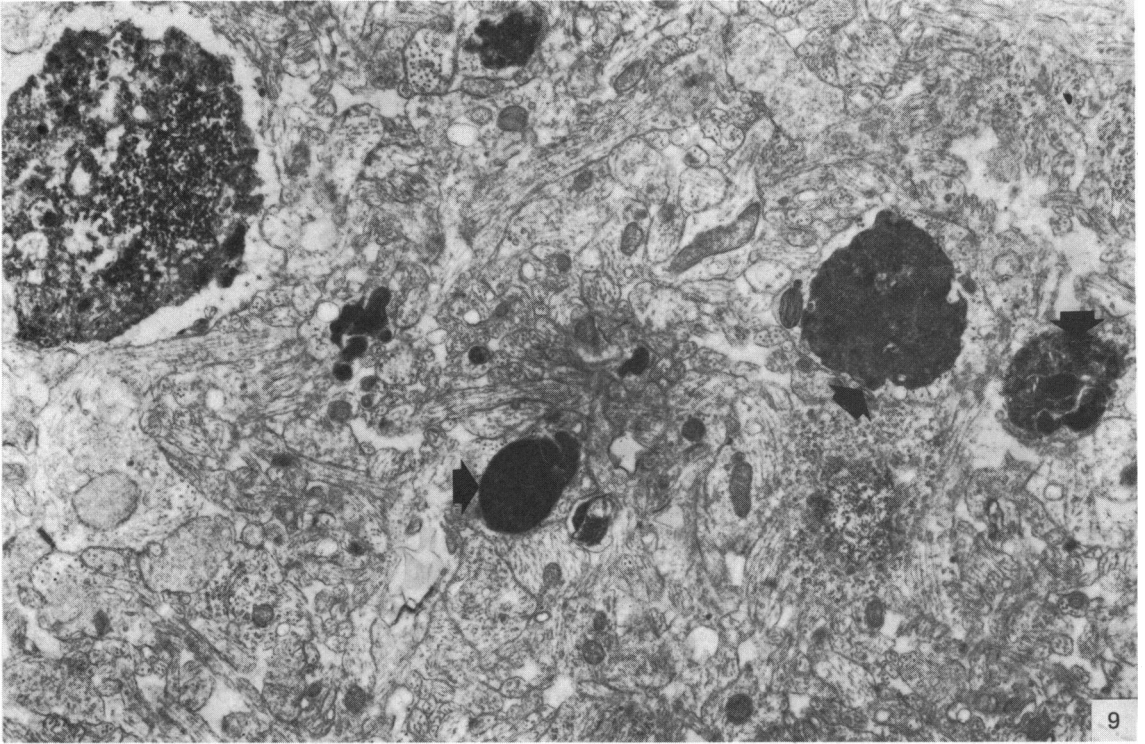
intermediate zone neighbouring the sulcus diencephalicus medius, an area very rich in degenerated structures and also in lipid droplets. This suggests that some of them are transported towards the inner cerebral wall during cellular migration.

The factors involved in the cell death that is characteristic of the proliferative areas must be different to that found later in development and, as suggested by Hamburger *et al.* (1981), is probably due to an inability to terminate the mitotic process correctly. This idea is supported by the findings of Sadler & Cardell (1977) in neuro-epithelial cells after hydroxyurea treatment, a strong inhibitor of DNA synthesis. Cowan *et al.* (1984) mention early neuronal death produced immediately after the degeneration of the cells and used the case of *Caenorhabditis elegans* to explain the role of cellular death in the control of cellular lineages; moreover, they suggest that it is a genetical programmed death in various lineages.

The second type of cell death found corresponds to 'nuclear death' (Pilar & Landmesser, 1976). It is characterised by a very pyknotic nucleus and it reaches a maximum in the middle embryonic periods (Stages E. 37–E. 39).

The neuronal identity of these cells is evident in early stages (E. 32–E. 34) because, in previous studies (Trujillo, 1982–1983) we have observed that the colonisation of the intermediate zone by glial cells is inconspicuous before Stage E. 35. However, in the latter stages, we are not sure that all the cells with pyknotic nuclei observed by light microscopy correspond to neurons, since the nuclei of degenerating glial cells may also become pyknotic (Korr, Schultze & Maurer, 1973, 1975; Korr, 1978, 1980; Sturrock, 1979*b*).

The third type of cell death is similar to 'cytoplasmic death' (Pilar & Landmesser, 1976) and appears in the perinatal stages. At the light microscopic level this type of death can be recognised in cells with a very dark cytoplasm. Without doubt the majority of these cells represent neurons since they possess morphological characteristics identical to the other neurons of the area. Moreover we have found several synapses on these cells.

The ultrastructural pattern of the degeneration process in dark neurons coincides exactly with the description given by several workers (Pilar & Landmesser, 1976; Sohal & Weidmen, 1978; Chu-Wang & Oppenheim, 1978; Giordano *et al.* 1980; Cunningham, 1982). We have observed that these cells are present in greater number about the end of the embryonic period, coinciding with the stages of most advanced synaptogenesis. These cells can coexist with pyknotic cells but pyknotic cells generally appear earlier – when synapses are still not detectable in the intermediate zone – and they reach their maximum frequency earlier than the dark cells. Based on experimental studies, Cunningham (1982) suggests that there are two morphological types of neuronal degeneration and there are two reasons for neuronal death. The first is a decrease in the reception of adequate signals (trophic factors) during the early stages of maturation. The other is that although the neurons may receive adequate initial contacts, the balance of inputs and target contacts is not suitable. All the neurons in the same centre commonly mature simultaneously, but it is not uncommon to find neighbouring neurons with marked differences in their stage of maturation. It is possible that this delay is related to the inadequate reception of trophic factors

Fig. 9. Cellular debris (arrows) within a microglial process. Nucleus sphericus, (E. 38). × 9500.

Fig. 10. A microglial amoeboid cell in the dorsal thalamus (E. 38). *LI*, Lipid droplet; *LP*, lipofuscin granule; *F*, phagosome. × 10000.

as suggested by Cunningham and an indication of the subsequent degeneration process – nuclear death – whilst cytoplasmic death only appears in more mature neurons.

Dark neurons are not only present in the embryonic period. Our observations in postnatal and adult brains of *Gallotia*, albeit in the absence of a quantitative analysis, verify that in the three centres studied, the number of dark neurons increases after hatching. It is possible that all these cells are not degenerating, but in the cases where this is so, one has to consider causes other than those mentioned for the embryonic period and one must conclude that from a particular stage of neuronal maturation the only kind of death produced is cytoplasmic, regardless of the original cause.

There is general agreement regarding the fate of the cellular debris. This appears by light microscopy as small masses or dark points and is indicative of the terminal stages of the cellular death process. There are karyorrhexic nuclei sometimes associated with degenerated cytoplasmic organelles. We have observed that this debris is clearly associated with the degeneration of matrix cells, but we have been intrigued by the large amount of debris in the intermediate zone during the intermediate stages (E. 37–E. 38 mainly). Due to the asynchrony of the events, this debris cannot possibly arise from the dark cells, but more likely corresponds to remains of degenerated matrix cells or to pyknotic cells. As already mentioned, at the ultrastructural level it appears enclosed in microglial cells and, thus, one has to consider that two phagocytic processes are implicated in the death of the matrix cells. The first occurs *in situ* and is by the neighbouring matrix cells; the second is located in the intermediate zone after migration of phagocytic matrix cells and is carried out by microglial cells. We believe that the phagocytic matrix cells have the ability to migrate whilst containing phagocytosed structures. We have observed this in the intermediate zone near the ventricular zone, but we do not know if these cells will transform themselves into 'true' phagocytes or if their contents are expelled into the intercellular space for future phagocytosis by cells of the microglial lineage. It is also possible that the debris arises from pyknotic cells.

SUMMARY

In the striatum, thalamus and cerebellum of a Lacertid reptile, we have found three types of cellular death during embryonic development, both at the light and electron microscopic level. The first affects the undifferentiated neuro-epithelial cells and is commonest during the early stages (E. 32–E. 36). The second corresponds to the type of 'nuclear' death described in the bibliography and reaches a maximum in the middle embryonic period (E. 37–E. 39); nevertheless important variations were observed in different zones. The third is the same as the 'cytoplasmic' death type and appears in the perinatal stages.

Phagocytosis involved in the elimination of dead cells is of two types. One is associated with early death and is carried out by undifferentiated neuro-epithelial cells. The other is carried out by microglial cells which appear around Stage 37. Much cellular debris was observed in the intermediate zone and this was associated with the second type of phagocytosis. In both cases lipid production was associated with the degenerative process.

Comparison of the temporal cellular death pattern with synaptogenesis, gliogenesis and maturation of neuronal processes is consistent with the view that the various types of cellular death found by us had different causes.

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