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# Brain damage and axonal injury in a Scottish cohort of neonatal deaths

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## Summary

**Despite the clinical and medicolegal significance attached to perinatal asphyxia, the neuropathological basis of this condition remains obscure. There are very few studies in the literature which correlate the pathological findings in neonatal brains with detailed epidemiological data, and none which are population based. In a Scotland-wide study of neonatal deaths, 70 brains have been examined. On the basis of glial and macrophage reactions, we previously identified infants with putative antepartum brain damage in this cohort and have related these reactions to signs of birth asphyxia. The present study explores the extent of neuronal/axonal injury in these infants since this is likely to be the basis for neurological deficits in surviving infants. We have also investigated these brains for β-amyloid precursor protein (βAPP) positivity to determine whether this is a useful marker of neuronal injury in neonates. Neuronal eosinophilia and karyorrhexes were detected in 43% and 27% of the cohort, respectively; maximally in the subiculum and ventral pons, but often present elsewhere. White matter damage was detected in 24% of cases but without classic cystic lesions of periventricular leucomalacia. βAPP positivity was present in neuronal soma in 52% of cases and, in axons, in 27% of cases, and was seen from as early as 25-weeks gestation. Axonal bulbs were clearly delineated by βAPP positivity and were usually located in the cerebral white matter and internal capsule, and infrequently in the brain stem. Although white matter damage and βAPP axonal positivity were often detected in the same cases ($P = 0.034$), these features also occurred independently of each other. Both neuronal karyorrhexes and white matter βAPP positivity were significantly correlated with the features of birth asphyxia, particularly a history of seizures. Immunocytochemistry for both βAPP and glial fibrillary acidic protein proved useful in detecting neuropathological features which escaped detection on routine examination, particularly in preterm infants. The presence together of recent and older damage in individual brains suggests that there is an ongoing neuronal response to cerebral insults. We find that βAPP is a useful marker of white matter damage in the neonatal brain. Immunopositivity for βAPP in these circumstances is not attributable to inflicted or accidental trauma. While birth-related trauma cannot be ruled out, hypoxia/ischaemia is a likely cause in these infants. However, the exact pathogenesis of neuronal/axonal injury in the neonatal brain remains unclear.**

**Keywords**: neonatal death; neuropathology; amyloid precursor protein; axonal injury; antepartum brain damage

**Abbreviations**: βAPP = β amyloid precursor protein; GFAP = glial fibrillary acidic protein; H&E = haematoxylin and eosin

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## Introduction

The origins of childhood neurological deficits are still poorly understood. Complications of pregnancy and adverse peripartum events are of uncertain predictive value. Perinatal asphyxia in the majority of cases is not followed either by

neonatal encephalopathy or by cerebral palsy (Hall, 1989; Nelson, 1989; Nelson and Leviton, 1991; Blair and Stanley, 1993; Yudkin *et al*., 1995; Edwards and Nelson, 1998) and there is international consensus (the International Cerebral Palsy Task Force) that encephalopathy and cerebral palsy are infrequently caused by perinatal asphyxia (Bakketeig, 1999; MacLennan, 1999). However preterm delivery is a recognized risk factor for such conditions (Murphy *et al*., 1995, 1997; Wood *et al*., 2000).

Major cohort studies of neonatal encephalopathies and related clinical conditions have shown that the associative factors are surprisingly diverse (Badawi *et al*., 1998*a*, *b*). However, these studies seldom include data on neuropathological findings of the deceased infants. On the other hand, reported perinatal neuropathological studies usually represent the collected experience of specialist referral centres (Gilles and Murphy, 1969; Rorke, 1982; Levene *et al*., 1985; Pape and Wigglesworth, 1989; Squier and Keeling, 1991; Marin-Padilla, 1996, 1997, 1999), or focus on particular age groups such as preterm infants (Skullerud and Westre, 1986; Leviton and Paneth, 1990; Paneth *et al*., 1990; Golden *et al*., 1997; Gilles *et al*., 1998). Only a small number of studies have investigated the clinical correlates of identified neuropathological features (Ellis *et al*., 1988; Mito *et al*., 1993; Gaffney *et al*., 1994). Although in recent years neuroimaging has proved to be a powerful tool for studying the extent and location of lesions in the perinatal brain (Cowan *et al*., 2003), neuropathological examination may be more sensitive for defining the nature of lesions. There is a complete dearth of studies designed to match detailed neuropathology with equally detailed demographic data in unselected groups of neonates.

The Scottish Perinatal Neuropathology Study was set up to investigate these issues and, in particular, to establish the prevalence of brain damage in a national cohort of perinatal deaths, including stillbirths and early neonatal deaths from 24 weeks gestation to 1 week of postnatal age, occurring during a 2 year period in the late 1990s. We recently reported that evidence of antepartum brain damage in the neonates in this cohort was more prevalent in those displaying signs of birth asphyxia than in non-asphyxiated infants (57% versus 8%), particularly in those who had suffered seizures (90%) ($P < 0.005$) (Becher *et al*., 2004). The assessment of brain damage in these neonatal deaths was based mainly on the detection of glial and microglial/macrophage responses. However, the important question of neuronal damage was not explored in our previous study.

In the present study, we further investigated this group of neonates to determine the prevalence of neuronal damage, and particularly of axonal injury, in relation to reactive changes in supporting cells and to the clinical history. One reliable marker of axonal injury, β amyloid precursor protein (βAPP), has rarely been investigated in the human infant brain other than in medicolegal cases (Arai *et al*., 1995; Baiden-Amissah *et al*., 1998; Geddes *et al*., 2001*a*, *b*; Reichard *et al*., 2003). It is recognized that both traumatic and hypoxic axonal injury is associated with βAPP positivity (Geddes *et al*., 2001*a*, *b*). In this study, we examined 70 neonatal brains for the presence of βAPP positivity in relation to other evidence of brain damage and to the clinical history.

## Material and methods

In the Scottish Perinatal Neuropathology Study, we set out to examine the brain in all neonatal deaths (24 weeks gestation to 1 week postnatal) occurring during a two-year period in Scotland. Infants with chromosomal, cardiac and CNS abnormalities and with CNS infections were excluded. Post-mortem examination was achieved in 51% of the eligible cases ($n$ = 137 in total, 70 brains examined). These 70 infants were divided into those who had died within 3 days of the onset of labour ($n$ = 59) and those who had died between 3 and 7 days ($n$ = 11). The three-day cut-off point was selected because changes such as macrophage accumulation and astrocytic hyperplasia are thought to require 3 days to become evident, from which we inferred an antepartum origin for these reactions.

Cases were co-ordinated from all 22 Scottish obstetric units through obstetric, paediatric, paediatric pathology and neuropathology colleagues. Consent for research use was obtained from the parents and the study was approved in each centre by the local research ethics committee.

Clinico-epidemiological data was gathered for each case. Up to 20 paraffin blocks were prepared from each brain according to a standard protocol and included representative samples from the cerebrum and hippocampus, basal ganglia and thalami, midbrain, pons, medulla, vermis and cerebellar hemispheres. Sections were examined after staining with haematoxylin and eosin (H&E), and with antibodies to glial fibrillary acidic protein (GFAP) (Dakocytomation Ltd; Ely, UK; diluted 1:1000, with trypsin pretreatment at 37°C for 20 min), the macrophage marker CD68 (Dako; diluted 1:200, with microwave antigen retrieval) and βAPP (Chemicon Europe Ltd; Chandlers Ford, UK; monoclonal clone 22C11, diluted 1:100, with microwave antigen retrieval).

Sections were assessed independently by two observers (J.E.B. and B.W.) without reference initially to the clinical history or to the results of the other staining procedures. Neuronal damage was assessed in routinely stained preparations by evidence of neuronal eosinophilia and/or karyorrhexis as well as the presence of axonal swellings in the white matter. The presence or otherwise of astrocytic hyperplasia, macrophage accumulation and microglial upregulation as well as βAPP positivity in neuronal soma and axons were scored as positive or negative for each case. Concordance between the two observers was achieved in >90% of cases and discrepant cases were resolved at a multiheaded microscope and by further review (J.K.). The localization and patterns of βAPP immunopositivity was correlated with gestation, clinical history and evidence of glial and macrophage reactions, as well as with evidence of neuronal and white matter damage assessed on routine (H&E) staining throughout the brain.

Statistical analysis was performed using the $\chi^2$ test (Fisher's exact test where sample size was <20).

## Results

### *Clinical details*

Of the 70 neonates enrolled in this study, 26 were mature infants (≥37 weeks of gestation) and 44 were preterm (24–36 weeks) (Fig. 1). Thirty-eight infants were delivered by Caesarean section, performed as an elective procedure in one case and as an emergency in 37 (before the onset of labour in half of these and usually precipitated by cardiotocograph

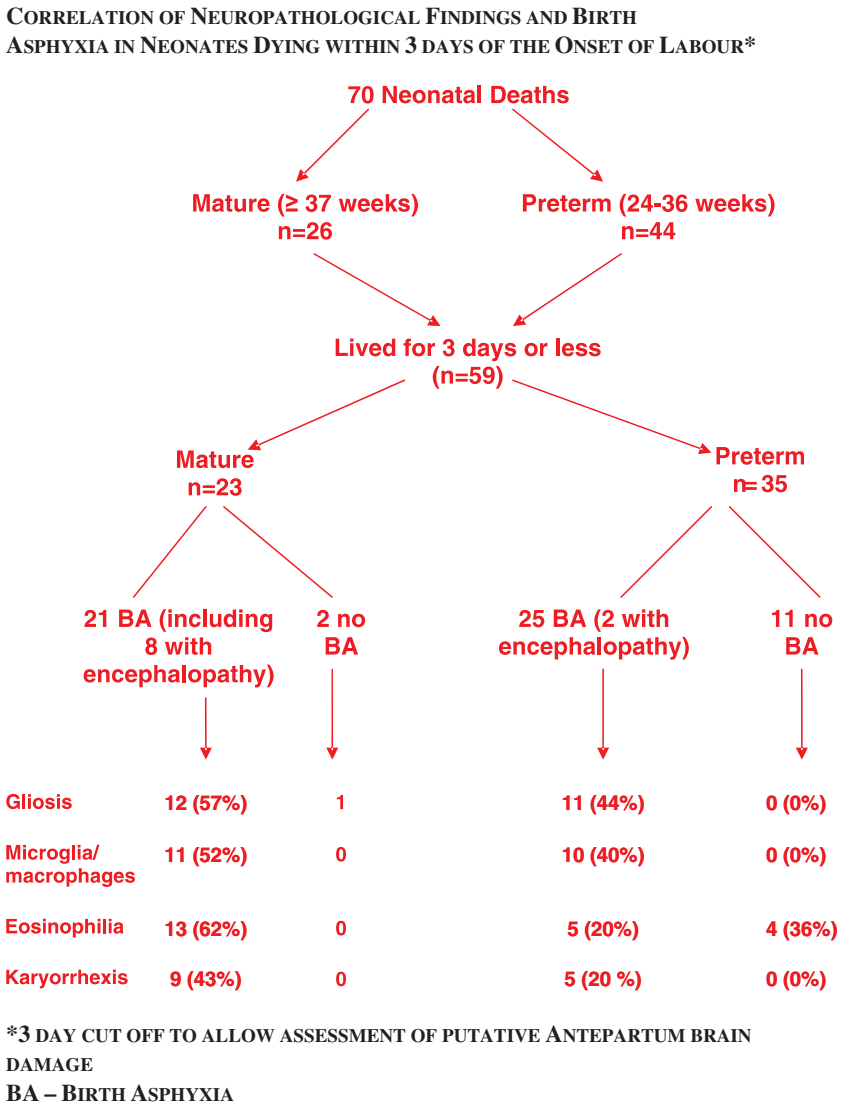


**Fig. 1** Cohort of neonatal deaths.

abnormalities). The mature infants lived for between 15 min and 7 days with only three surviving for >3 days. The preterm infants lived for between 5 min and 5.5 days, with only eight infants surviving for >3 days. Neonatal encephalopathy was observed in 10 mature and five preterm infants. Most infants died of the effects of prematurity, or of congenital anomalies other than those of the CNS (which were excluded from the study), or of 'anoxia'. Fifty-three of the 70 neonates displayed one or more clinical signs of perinatal asphyxia, including 15 with seizures (10 of these infants died aged ⩽3 days), while 17 did not appear to be asphyxiated. Perinatal asphyxia was defined in this study by the presence of one or more of the following: (i) an Apgar score of ⩽5.0 at 5 min; (ii) a cord or initial blood pH of <7.1; and/or (iii) grade 2/3 encephalopathy. Signs of birth asphyxia were associated with a higher prevalence of antepartum brain damage (57% versus 8%, $P < 0.005$), but were not predictive in all cases. Oligohydramnios and meconium staining of the amniotic fluid were also associated with antepartum brain damage, but there was no concordance with placental pathology. A detailed analysis of these and other clinicoepidemiological factors, which may have contributed to neonatal death and to antepartum brain damage in this cohort, has been published previously (Becher *et al*., 2004).

## *Routine neuropathological findings*

Assessment of neuronal damage was first undertaken in sections stained with H&E. Neuronal eosinophilia (Fig. 2A), marking recent onset hypoxic change, was observed in some or all differentiated nerve cell bodies in the cerebral cortex, basal ganglia, brain stem, particularly in the ventral pons and inferior olive, and in the cerebellar dentate nuclei and Purkinje cell layer. Karyorrhectic neuronal nuclei (Fig. 2B) were observed in a similar distribution, but additionally in the dentate gyrus of the hippocampus and in the cerebellar granular layer in occasional cases. If neuronal karyorrhexes were only present in very small numbers, they were considered indistinguishable from the normal neuronal drop-out known to

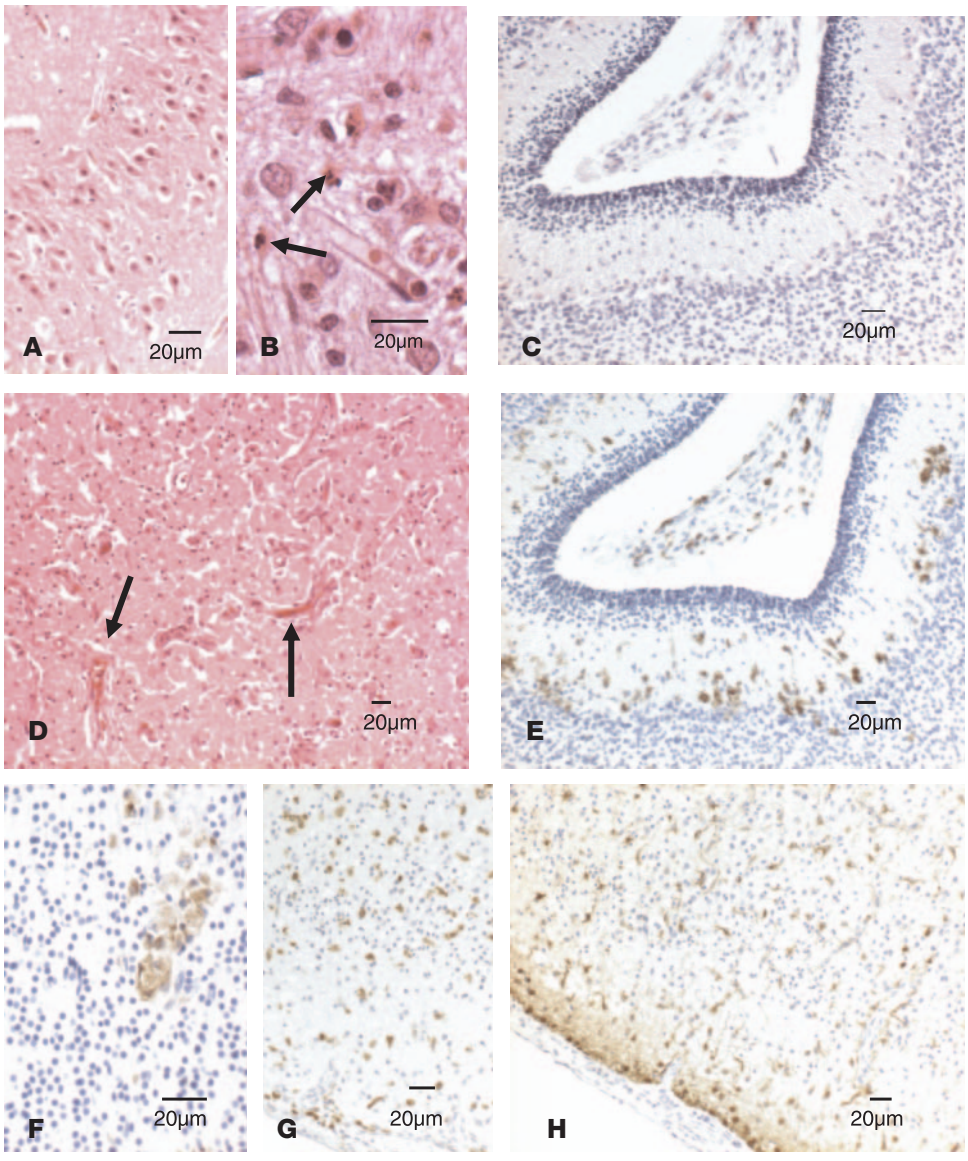


**Fig. 2** Evidence of brain damage in grey matter. (**A**) Eosinophilic hippocampal neurons from a 41-week gestation infant who survived for 1 day. H&E staining. (**B**) Pontine neurons in a 39-week gestation infant who survived <1 day. Karyorrhectic neurons are shown (arrows) between more nearly normal neurons. H&E staining. (**C**) Cerebellar cortex from a 38-week gestation infant who survived just under 2 days. Very few Purkinje cells survive at the interface between molecular and internal granular layers. H&E staining. (**D**) Cerebral cortex from a 42-week gestation infant who survived 7 days and who displayed widespread cortical necrosis. Surviving cortex showed neovascularization (arrows) and the tissue is beginning to disintegrate. H&E staining. (**E**) The cerebellar cortex shown in (**C**) stained for microglia/macrophages using an antibody to CD68. Macrophages are concentrated along the former Purkinje cell layer. (**F**) Cerebral cortex from a 26-week gestation infant who survived for 40 min. A focal infarct, marked by CD68 positive macrophages, is shown in the cortex. (**G**) More generalized cortical CD68-positive macrophage infiltration is present in the cortex of the infant shown in (**D**). (**H**) A GFAP-positive cortical glial response is also present in the case illustrated in (**D**) and (**G**).

occur during development and were disregarded. Even when present in large numbers, the changes affected only a subpopulation of neurons in the neocortex, but were more numerous and concentrated in the subiculum of the temporal lobe (spreading to involve the entorhinal cortex and the neighbouring sectors of the cornu ammonis) and in the ventral pons. The most severely affected cases showed loss of most Purkinje cells (Fig. 2C). Neuronal eosinophilia was identified in 43% of the cohort and karyorrhexes in 27%. Occasional infants showed cortical infarction with prominent neovascularization and perivascular fibrosis (Fig. 2D). Figures 2E–G show focal and diffuse macrophage responses in the context of neuronal loss, not all of which had been suspected on routine staining (Fig. 2F). Cortical damage also results in a conspicuous glial response (Fig. 2H). Case-by-case glial and macrophage reactions are shown in Table 1.

Evidence of white matter damage in routinely stained sections was present in 24% of cases, ranging from eosinophilic homogenization of the neuropil to areas of frank infarction, in relation to which axonal retraction bulbs (Fig. 3A) were

identified in three cases and with more widespread surrounding white matter damage. Moderate numbers of karyorrhectic glial nuclei were identified within damaged white matter, accompanied by macrophage infiltration and astrocytic hyperplasia (Fig. 3B). Even in apparently normal white matter (Fig. 3C), unexpected astrocytic hyperplasia was sometimes revealed by GFAP immunostaining (Fig. 3D). In contrast, Fig. 3E shows normal white matter astrocytic

**Table 1** *Pattern of* βAPP *positivity in 70 neonatal deaths, correlated with glial and macrophage reactions*

**(A) Infants dying within 3 days (*n* = 59)**

| Cases with antepartum damage (*n* = 27) | Gestation (asphyxia at birth) | βAPP neurons | βAPP axons | βAPP mineralizing foci | Metabolic astrocytosis | Reactive astrocytosis | Macrophage reactions |
|---|---|---|---|---|---|---|---|
| 1 (E) | 41 (+) | + | + | − | − | + | + |
| 2 (E) | 41 (+) | + | + | − | + | + | + |
| 3 (E) | 40 (+) | + | − | − | − | + | + |
| 4 (E) | 40 (+) | + | − | + | − | + | + |
| 5 (E) | 39 (+) | + | + + | − | − | + | + |
| 6 (E) | 38 (+) | + | − | − | − | + | − |
| 7 (E) | 38 (+) | + | + | − | + | + | + |
| 8 (E) | 37 (+) | + | − | − | + | + | + |
| 9 (E) | 36 (+) | + | − | − | + | + | + |
| 10 | 40 (+) | + | + | − | − | + | + |
| 11 | 36 (+) | + | − | + + | − | + | + |
| 12 | 35 (+) | + | − | − | − | + | + |
| 13 | 36 (+) | ± | + + | + | − | + | + |
| 14 | 32 (+) | + | ± | + | − | + | − |
| 15 | 28 (+) | − | − | + | + | + | + |
| 16 | 28 (+) | + | − | − | − | + | + |
| 17 | 27 (+) | + | − | − | ± | + | + |
| 18 | 25 (+) | − | + | + | − | ± | + |
| 19 | 26 (+) | − | − | − | − | − | + |
| 20 | 24 (+) | − | − | − | − | + | − |
| 21 | 40 (+) | − | − | − | − | + | ± |
| 22 | 40 (+) | − | − | − | − | + | + |
| 23 | 26 (+) | − | − | − | − | − | + |
| 24 | 25 (+) | + | − | − | − | − | + |
| 25 | 42 (−) | + | − | + | − | + | − |
| 26 | 40 (+) | − | + | − | − | + | − |
| 27 | 40 (+) | + | − | − | + | + | − |
| **Cases with postnatal damage (*n* = 21)** | **Gestation (asphyxia at birth)** | **βAPP neurons** | **βAPP axons** | **βAPP mineralizing foci** | **Metabolic astrocytosis** | **Reactive astrocytosis** | **Macrophage reactions** |
| 28 | 24 (+) | − | − | − | − | − | − |
| 29 | 36 (−) | + | − | − | − | − | − |
| 30 | 28 (−) | ± | − | − | − | − | − |
| 31 | 24 (+) | ± | − | − | − | − | − |
| 32 | 39 (+) | + | − | − | − | − | − |
| 33 | 31 (−) | + | − | + | − | − | − |
| 34 | 24 (+) | − | − | − | − | − | − |
| 35 | 24 (+) | − | − | − | − | − | − |
| 36 | 36 (+) | − | − | − | − | − | − |
| 37 | 40 (+) | + | + | − | − | − | − |
| 38 | 25 (−) | − | − | − | − | − | − |
| 39 | 42 (+) | ± | − | − | − | − | − |
| 40 | 38 (−) | − | − | − | − | − | − |
| 41 | 27 (−) | − | − | − | − | − | − |
| 42 | 29 (+) | + | − | − | − | − | − |
| 43 (E) | 25 (+) | − | − | + | − | − | − |
| 44 | 41 (+) | − | − | − | − | − | − |
| 45 | 30 (−) | NA | NA | NA | − | − | − |
| 46 | 25 (+) | − | + | − | + | − | − |
| 47 | 24 (−) | − | − | − | − | − | − |
| 48 | 38 (+) | − | − | + | − | − | − |

**Table 1** *Continued*

| Cases with no damage ($n$ = 11) | Gestation (asphyxia at birth) | βAPP neurons | βAPP axons | βAPP mineralizing foci | Metabolic astrocytosis | Reactive astrocytosis | Macrophage reactions |
|---|---|---|---|---|---|---|---|
| 49 | 40 (+) | − | + | − | − | − | − |
| 50 | 32 (−) | + | − | − | − | − | − |
| 51 | 35 (−) | − | − | − | − | − | − |
| 52 | 25 (+) | − | − | − | − | − | − |
| 53 | 25 (+) | − | − | − | − | − | − |
| 54 | 27 (+) | − | − | + | − | − | − |
| 55 | 42 (+) | − | − | − | − | − | − |
| 56 | 30 (+) | − | − | − | − | − | − |
| 57 | 26 (−) | − | − | − | − | − | − |
| 58 | 38 (+) | ± | − | − | − | − | − |
| 59 | 25 (−) | + | − | − | − | − | − |

**(B) Infants dying at 4–7 days ($n$ = 11)**

| Damaged and undamaged cases ($n$ = 11) | Gestation (asphyxia at birth) | βAPP neurons | βAPP axons | βAPP mineralizing foci | Metabolic astrocytosis | Reactive astrocytosis | Macrophage reactions |
|---|---|---|---|---|---|---|---|
| 60 | 41 (−) | + | + | + | + | + | − |
| 61 | 27 (+) | + | − | − | − | + | + |
| 62 | 25 (−) | − | + | − | − | − | − |
| 63 (E) | 27 (+) | − | + | + | − | + | + |
| 64 (E) | 42 (+) | + | + | − | − | ++ | ++ |
| 65 | 27 (−) | − | − | − | − | − | − |
| 66 (E) | 36 (+) | + | − | ++ | − | + | + |
| 67 (E) | 29 (+) | + | − | + | − | + | + |
| 68 (E) | 39 (+) | + | + | − | − | + | + |
| 69 | 34 (−) | − | + | − | − | − | − |
| 70 | 26 (+) | + | ++ | − | − | + | + |

E = infants displaying encephalopathy; NA = blocks returned to source hospital before βAPP staining was performed.
−, negative; ±, occasional positive cells or features across the section; +, moderate number of positive cells or features across this section; ++, very numerous positive cells or features across this section.

immunoreactivity in this age group. No foci of cystic periventricular leucomalacia were seen.

Small foci of perivascular mineralization were present in the central white matter in 19% of the total cohort, more commonly in the preterm infants. One case displayed extensive mineralization in the basal ganglia and internal capsule.

In nine cases, glial nuclei in both white and grey matter were enlarged, pale and prominent (Fig. 3F), resembling the Alzheimer type II astrocytes observed in hepatic encephalopathy. Although the cytoplasm of such cells was not generally prominent, they were GFAP positive (Fig. 3G) and, in most of these cases, a reactive astrocytosis was confirmed in the white matter. Some mature neonates showed prominent changes of so-called myelination gliosis (glial cells with small darkly stained nuclei and prominent asymmetric cytoplasm, often arranged in rows) (Fig. 3H), but such cells did not prove to be GFAP reactive in contrast to juxtaposed normal astrocytes (Fig. 3I). Interpretation of such cases sometimes proved difficult on routine staining, especially in the presence of incipient white matter damage, and GFAP staining proved essential for the detection of true astrocytic hyperplasia.

## *Immunoreactivity for βAPP*

βAPP immunopositivity was more prevalent in grey matter (neuronal perikarya, 53%) than in white matter (axons, 27%) (Table 1) Three patterns of βAPP positivity were observed in the cases in this study.

βAPP was detected in neuronal cell bodies in 52% of the study cases; this occurred in both mature and preterm infants (Table 1), varying from global expression in all differentiated neurons to particular subsets of neurons in the cortex (Fig. 4A), basal ganglia, cerebellum or brain stem. The inferior olivary nuclei and neurons in the floor of the fourth ventricle, the dentate nuclei and the Purkinje cells were particularly likely to display βAPP positive neurons. The neurons of the immature cortex in premature babies and the germinal matrix cells and migrating foci were generally negative. Staining of the perikaryon occurred both in morphologically normal cells and in those which proved to be eosinophilic or frankly karyorrhectic on routine staining, although not all karyorrhectic neurons were positive (Fig. 4B). Within the hippocampus, the CA1 sector neurons were occasionally unstained when the rest of the cornu ammonis and the neurons of the subiculum were positive.

Axonal βAPP positivity was detected in 27% of study cases (Table 1) and was seen predominantly in the periventricular

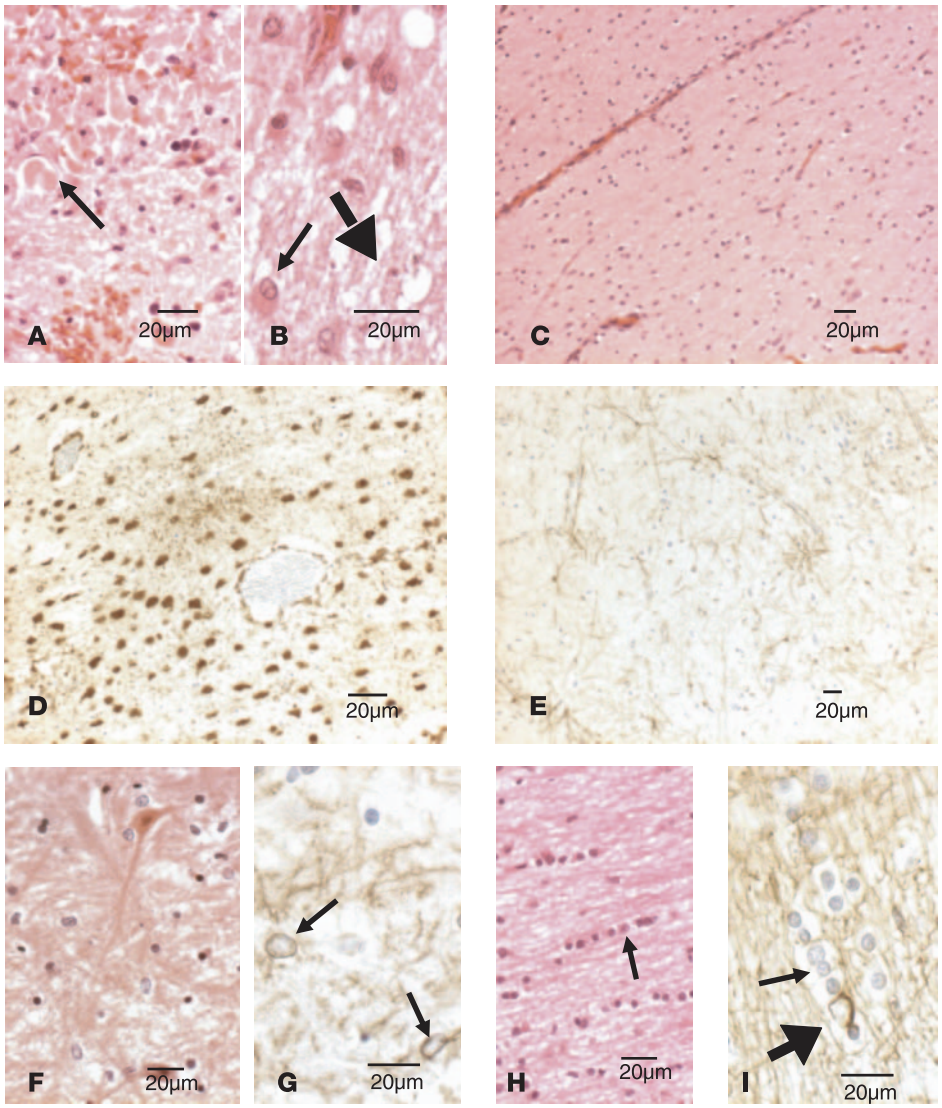


**Fig. 3** Evidence of white matter damage and glial/macrophage reactions. (**A**) A focal area of white matter damage, marked by eosinophilic axonal bulbs (arrowed) is seen in a 28-week gestation infant who survived for nearly 3 days. The area of damage was in the proximity of a germinal matrix haemorrhage. H&E staining. (**B**) White matter damage characterized by astrocytic hyperplasia (fine arrow) and glial karyorrhexes (broad arrow) present in a 42-week gestation infant who survived 7 days. H&E staining. (**C**) Survey of apparently unremarkable white matter in a 42-week gestation infant who survived 4 h. H&E staining. (**D**) Immunostaining with an antibody to GFAP reveals reactive astrocytosis in the infant shown in (**C**). (**E**) Immunostaining for GFAP in an infant age- and stage-matched with the infant shown in (**C**) and (**D**) who did not show white matter astrocytic hyperplasia. (**F**) Glial nuclei in the basal ganglia of a 41-week gestation infant who survived for nearly 2 days. Eosinophilic change is seen in neurons within this field and astrocytic nuclei are pale and somewhat irregular. H&E staining (**G**) Immunostaining of the basal ganglia shown in (**F**) reveals that the pale glial nuclei are associated with GFAP positivity (arrows) without displaying cytoplasmic hyperplasia. (**H**) White matter from an infant of 41-weeks gestation who survived for just over 1 day displaying 'myelination' gliosis. Lines of glial nuclei, some with eosinophilic cytoplasm (arrowed) are shown. H&E staining. (**I**) Same case as (**H**) stained for GFAP. While the majority of cells in the glial columns are GFAP negative (fine arrow), there is an occasional interposed GFAP-positive cell (broad arrow).

central white matter of the cerebral hemisphere and the white matter of the cerebellum. Well-demarcated positive axons were particularly common in the internal capsule (Fig. 4C). Staining patterns varied from smudgy irregular patches of positivity in otherwise apparently normal white matter, to clearly demarcated single axons or bundles of axons, often with evidence of axonal beading. Axonal bulbs and varicosities were most numerous in association with areas of infarction or haemorrhage and were strongly positive for βAPP. The brain stem showed only occasional axonal positivity, particularly in the cerebellar peduncles, in contrast with frequent positivity observed in neuronal soma in the stem.

Perivascular and white matter mineralizing foci were strongly positive for βAPP in all the cases in which this feature had been noted in routinely stained sections (15 out of the 70 study cases; 21%) (Fig. 4D), including the case with significant mineralization in the internal capsule (Fig. 4E). These foci were particularly common in the basal ganglia

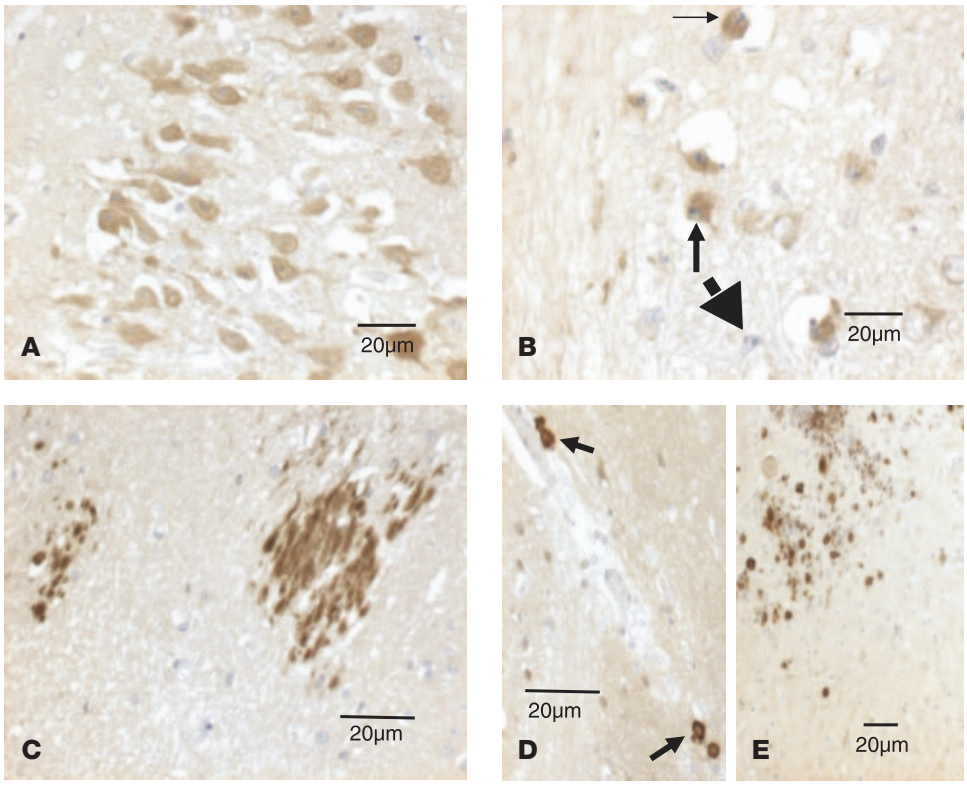


**Fig. 4** βAPP immunoreactivity in the neonatal brain. (**A**) Hippocampal neurons from the infant shown in Fig. 2A displaying positivity for βAPP, co-localizing with eosinophilia. (**B**) Pontine neurons from a 39-week gestation infant who survived 14 h and who displayed prominent neuronal karyorrhexes in the ventral pons. Many karyorrhectic neurons are βAPP positive (fine arrow), but an occasional affected neuron is negative (broad arrow). (**C**) Internal capsule from the infant whose pons is illustrated in (**B**). Bundles of βAPP positive axons are seen and some of these display axonal bulbs. (**D**) Perivascular deposits of mineral in the basal ganglia are positive for βAPP in a 41-week gestation infant who survived 4 days. (**E**) Large focus of mineralization in the internal capsule of a 36-week gestation infant who survived 7 h. The mineralized area is strongly positive for βAPP.

and associated white matter. Mineralized foci did not stain positively with antibodies to GFAP and CD68.

Each of these patterns of βAPP positivity was sometimes found in isolation but much more frequently in association with glial and macrophage responses (Table 1). βAPP positivity was found occasionally in neurons in cases in which no other evidence of damage had been detected. Conversely, in five cases displaying microglial/macrophage and/or glial responses suggestive of antepartum damage, no βAPP positivity was detected. Although faint βAPP positivity was noted in reactive astrocytes, glia and blood vessels were generally negative for this marker.

Table 2 summarizes the prevalence of βAPP positivity in different groups of infants in the present study. One case was not available for βAPP staining, but of those available, 37 (52%) showed neuronal and 19 (28%) showed axonal βAPP positivity. The prevalence of βAPP in the 59 infants dying at $\leqslant$3 days was significantly related to the timing of damage, being higher in both neuronal bodies ($P = 0.08$) and axons ($P = 0.03$) in infants with antepartum damage. Even some infants with no apparent damage on routine staining showed occasional βAPP positivity (27% in neurons and 9% in axons). In the 11 infants dying between 4 and 7 days of age, 36% (and all of the three infants born at term) showed both neuronal and axonal βAPP positivity. In all groups, preterm infants showed a lower prevalence of βAPP positivity in neurons and axons. Table 2 also shows the declining prevalence of βAPP positivity in neonates with postnatal and no observable damage compared with those displaying antepartum damage. In contrast, the infants aged $\geqslant$3 days displayed the highest prevalence of axonal βAPP positivity. White matter damage (24%) and βAPP positivity (27%) were usually present together ($P = 0.034$).

## *Correlation of neuropathological findings with clinical features*

Neuronal eosinophilia was no more common in asphyxiated infants (43%, $n = 53$) than in non-asphyxiated infants (41%, $n = 17$) ($P = 0.87$). However, this feature was detected more frequently in mature (62%, $n = 26$) than preterm (32%, $n = 44$) infants ($P = 0.015$). In contrast with neuronal eosinophilia, neuronal karyorrhexes were significantly increased in asphyxiated infants (36%) compared with non-asphyxiated infants (0%) ($P < 0.01$) and were also more prevalent in mature (27%) than in preterm infants (7%), although not significantly so ($P = 0.08$). Encephalopathic infants showed a particularly high prevalence of neuronal eosinophilia and karyorrhexes

**Table 2** *Neuronal βAPP positivity in the cohort of 70 neonatal deaths*

| **(A) Infants dying within 3 days (*n* = 59)** | | | |
|---|---|---|---|
| | | Localization of βAPP immunopositivity | |
| | | Neurons | Axons |
| Cases with antepartum damage (*n* = 27) (70% neuronal, 33% axonal βAPP positivity in mature and preterm taken together) | Mature (*n* = 14) | 11 (79%) (100% in eight encephalopathic infants) | 6 (43%) (50% in eight encephalopathic infants) |
| | Preterm (*n* = 13) | 8 (62%) (positive in one encephalopathic infant) | 3 (23%) (negative in one encephalopathic infant) |
| Cases with postnatal damage (*n* = 21) (40% neuronal, 10% axonal βAPP positivity in mature and preterm taken together) | Mature (*n* = 6) | 3 (50%) | 1 (17%) |
| | Preterm (*n* = 14) (one case not available) | 5 (36%) | 1 (7%) |
| Cases with no observable damage (*n* = 11) (27% neuronal, 9% axonal βAPP positivity in mature and preterm taken together) | Mature (*n* = 3) | 1 (33%) | 1 (33%) |
| | Preterm (*n* = 8) | 2 (25%) (negative in one encephalopathic infant) | 0 (0%) (negative in one encephalopathic infant) |
| **(B) Infants dying at 4–7 days (*n* = 11)** | | | |
| | | Localization of βAPP immunopositivity | |
| | | Neurons | Axons |
| Cases with recent and older damage (*n* = 11) (64% neuronal, 64% axonal βAPP positivity in mature and preterm taken together) | Mature (*n* = 3) | 3 (100%) | 3 (100%) |
| | Preterm (*n* = 8) | 4 (50%) | 4(50%) |

(90%). White matter damage was significantly more common in encephalopathic (90%) than in non-encephalopathic asphyxiated (29%) and non-asphyxiated (12%) neonates ($P = 0.001$). Infants who survived for ⩾3 days had a higher prevalence of white matter damage than those who survived birth by only a few hours ($P = 0.03$). Encephalopathy was significantly associated with reactive astrocytosis ($P = 0.001$), with macrophage reactions ($P = 0.002$) and with metabolic astrocytosis ($P = 0.024$), but not with deposits of mineral. The minor differences in micromineralization in white matter parenchyma between asphyxiated and non-asphyxiated infants did not amount to significance.

White matter βAPP immunopositivity was confined to the asphyxiated group (12 out of 46 cases) among infants who died at ⩽3 days, but was not most prevalent in the encephalopathic infants. However, somatic neuronal βAPP positivity was significantly more common in encephalopathic infants than in those infants who did not have seizures ($P = 0.012$) (Table 2). A minority of cases showed no evidence of βAPP positivity despite the focal presence of reactive astrocytosis and/or macrophage infiltration of the white matter, suggesting that damaged neurons may already have been removed in these cases. Conversely in infants with postnatal brain damage, mostly in the form of fresh haemorrhage, neuronal βAPP positivity was present in six cases in the absence of astrocytic or macrophage reactions. Overall, there was a significant correlation between white matter damage observed in routinely stained sections and axonal βAPP positivity ($P = 0.034$). The prevalence of axonal βAPP positivity following forceps or Caesarean delivery ($n = 38$) was 37% compared with only 16% in 32 infants delivered normally ($P = 0.01$).

## Discussion

This paper documents the prevalence of neuronal damage and βAPP positivity in a population based cohort of 44 preterm and 26 mature neonatal deaths ascertained in a two-year study. Based on the presence of glial and/or macrophage reactions, we have concluded that brain damage occurred before the onset of labour in 46% of the 59 infants who died at or before 3 days of age (Becher *et al*., 2004). This assessment of the timing of neuropathological damage was grounded in previous observations in human infants (Norman, 1978; Low *et al*., 1989; Squier and Keeling, 1991; Del Bigio and Becker, 1994; Wigglesworth and Bridger, 1994; Norenberg, 1994; Squier, 2001; Kinney *et al*., 2002). However, our previous study failed to identify any pointers which would reliably predict the birth of an infant with antepartum brain damage. We did find an association between brain damage and oligohydramnios, meconium staining of the liquor and an abnormal cardiotocograph, but we found no evidence for an association with prenatal infection or placental inflammation.

This study focuses on neuronal damage. Certain staining and morphological changes (eosinophilia and karyorrhexis) signify irretrievable neuronal death. Most animal studies of timed cerebral insult indicate that neuronal karyorrhexis is visible within 24 h (Ferrer *et al*., 1994; Ferrer, 1996; Tan *et al*., 1998) and estimates of the time lapse between insult and karyorrhexis in the human brain are similar (Friede, 1972; Low *et al*., 1989; Wigglesworth and Bridger, 1994; Squier, 2001). The development of neuronal eosinophilia is thought to require at least 6 h in the rat model (Graeber *et al*., 2002) and possibly longer in the human infant (Norman, 1978; Low *et al*., 1989). From these estimates, it is clearly impossible to infer a definite antepartum origin to the neuronal changes in this cohort, in contrast with the glial and macrophage changes reported previously. With respect to eosinophilia, it is as likely that this was a postnatal event as prenatal in infants surviving for more than a few hours. However, more long-standing neuronal damage in the form of karyorrhexis may have commenced before labour in infants who died during the first day of life.

Apart from two cases of minor cortical dysplasia and one of GM1 gangliosidosis, the damage observed in this study was superimposed on otherwise normally developed brains and is similar to that reported in previous studies (Gilles and Murphy, 1969; Friede, 1972; Norman, 1978; Rorke, 1982; Roessmann and Gambetti, 1986; Skullerud and Westre, 1986; Ellis *et al*., 1988; Low *et al*., 1989; Leviton and Paneth, 1990; Paneth *et al*., 1990; Squier and Keeling, 1991; Mito *et al*., 1993; Del Bigio and Becker, 1994; Gaffney *et al*., 1994; Norenberg, 1994; Wigglesworth and Bridger, 1994; Marin-Padilla, 1996, 1997, 1999; Golden *et al*., 1997; Gilles *et al*., 1998; Squier, 2001; Graeber *et al*., 2002; Kinney *et al*., 2002), although the prevalence of grey matter and neuronal damage is higher in the present cases. Both necrosis and apoptosis have been implicated in perinatal neuronal loss (Edwards *et al*., 1997). Unequivocal signs of cell death are more easily detected in the differentiated neurons of the mature infant brain and were particularly prevalent in asphyxiated babies who suffered seizures. In very few of the present cases were all neurons affected, even within a single target area of the brain. Specific cell surface receptors may confer these differing levels of vulnerability, as well as forming the basis of perinatal patterns of neuronal involvement, as in pontosubicular necrosis (Kinney and Armstrong, 2002). The reactions of the immature brain to hypoxic/ischaemic and other injury differ from those of the adult brain. The white matter appears to be particularly vulnerable in the perinatal period and both white matter infarction and the presence of axonal swellings have been described previously (Norman, 1978; Squier, 2001), although this latter feature has not been widely recognized.

This study reports the first evaluation of βAPP immunoreactivity in a large series of cases with perinatal brain damage. βAPP is known to be upregulated in neuronal stress and is a recognized marker of traumatic axonal damage (Graham *et al*., 2000). Geddes *et al*. (2001*a*, *b*) have described a geographic pattern of axonal βAPP staining in the cerebrum and in the lower brain stem, particularly in the cortico-spinal tracts, in babies with non-accidental head injury. Most of this immunopositivity, with the exception of βAPP positive axonal bulbs, was ascribed to hypoxic rather than traumatic injury. Reichard *et al*. (2003) also described βAPP immunopositive bulbs in a series of 29 medicolegal infant cases, not all of whom had sustained traumatic injury. Results in the present study show that βAPP is a very useful marker for axonal injury in neonates as young as 25 weeks gestation. Similar findings were reported in a previous small study (Baiden-Amissah *et al*., 1998). Inflicted trauma was ruled out in the present cases since virtually all were under close hospital supervision throughout life. The infants displaying βAPP positivity were delivered by a variety of methods including vaginal delivery and, in 19 cases, delivery by emergency Caesarean section, suggesting that axonal injury in these infants originates with antepartum events rather than prolonged birth-related trauma. It is apparent that βAPP staining patterns in the developing brain should be interpreted with caution and that the presence of βAPP positive axonal bulbs should not necessarily be ascribed to a traumatic event.

Since βAPP positivity appears very rapidly after a neuronal insult (Sherriff *et al*., 1994; Baiden-Amissah *et al*., 1998; Graham *et al*., 2000), this feature is unlikely to be of use in ascribing brain damage in the neonate to the antepartum, intrapartum or postnatal period except perhaps in infants who die immediately after birth and when labour has been short. Immunodetection in the neuronal cell body implies upregulation of βAPP expression whereas axonal positivity results from disturbed axonal transport (Sherriff *et al*., 1994). Uncertainties remain as to the duration and/or reversibility of these phenomena. A study of periventricular leucomalacia revealed that βAPP immunoreactive axons which were detected around early lesions were not present in mature lesions (Arai *et al*., 1995). Perivascular amphophilic droplets and foci of micromineralization were consistently βAPP positive in the present study, supporting the thesis that these features may result from breakdown of the blood brain barrier (Squier, 2001), since soluble APP is present in the circulation as well as in CSF (Mattson, 1997). βAPP was not generally observed in immature neuronal cell bodies, unlike preterm axons. Overall, βAPP expression was more frequently observed in mature than preterm brains.

With respect to reactive gliosis, some confusion exists in the literature between the so-called myelination gliosis of normal development and the pathological state of reactive astrocytosis (Squier, 2001; Kinney and Armstrong, 2002). The cells of 'myelination gliosis' are reminiscent of reactive astrocytes in that they have eccentric flares of eosinophilic cytoplasm but they are oriented in regular fashion in white matter of undamaged appearance, which is in the process of myelination. In the present study, we have found such cells to be GFAP negative and infer that they are oligodendroglia. In contrast, astrocytes which prove to be markedly reactive on GFAP immunocytochemistry may be inconspicuous on

routine staining. We conclude that a meaningful assessment of gliosis in the neonatal brain requires GFAP immunocytochemistry. The significance of the apparent Alzheimer type II astrocytosis present in a number of cases is unclear, but has been remarked upon previously in perinatal brains (Kinney and Armstrong, 2002). It may represent the result of metabolic upset and, in our series, was more common in infants with damaged brains.

Correlation between βAPP positive axonal injury and glial/macrophage reactions, although significant ($P = 0.034$), was lower than we had anticipated and quite a number of encephalopathic cases with reactive changes did not display white matter βAPP positivity. This may be a sampling phenomenon, since both βAPP reactive axons and gliosis may be focal phenomena.

With respect to pathogenesis of brain damage in the neonate, many of the pathological features noted in this study have been ascribed classically to hypoxic/ischaemic insults (Rivkin and Volpe, 1993). However, consideration should also be given to other possible mechanisms, including systemic infection and inflammation (Pape and Wigglesworth, 1989; Ellis *et al*., 2000; Wheater and Rennie, 2000)—possibly mediated by pro-inflammatory cytokines (Yoon *et al*., 1997; Duggan *et al*., 2001) or by free radicals (Tan *et al*., 1998; Kinney and Armstrong, 2000). We did not find any evidence of an association between brain damage and intrauterine infection in the Scottish study (Becher *et al*., 2004). The possibility exists that cytokine upregulation in the brain may be a response to neural damage, rather than its cause. Similarly, some of the observed neuronal changes including βAPP expression may be the result rather than the cause of seizures in encephalopathic infants. However, it should be noted that these cellular changes also occurred in infants who had not had seizures. This study highlights the prevalence of recent onset neuronal damage in both mature and preterm infants often co-present with older lesions, suggesting an ongoing neuronal response to cerebral insults. Unfortunately, the pregnancy-associated clinical features that might predict the likelihood of brain damage remain elusive (Becher *et al*., 2004) and the exact pathogenesis of neuronal/axonal injury remains unclear in the neonatal brain.

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