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ORIGINAL ARTICLE

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Neuron-specific enolase as an effective immunohistochemical marker for injured axons after fatal brain injury

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Abstract Recently, it has been reported that a diagnosis of diffuse axonal injury in cases with a short survival period can be made with the use of immunolabelling for β-amyloid precursor protein (APP). We examined whether immunostaining for neuron-specific enolase (NSE) can also be a useful marker for the detection of axonal injury in its early stages. Sections of the corpus callosum from 19 cases of head injury and from 9 cases of no head injury were immunostained for NSE and stained by the standard Holmes' silver method. For comparison, serial sections from several cases were immunostained for APP. Immunostaining for NSE as well as for APP, labelled injured axons in head injury cases with as early as 1.5 h survival where Holmes' staining failed to detect any changes of axons. Since NSE and APP labelled only injured axons but not normal axons, the results were readily interpretable. These findings indicate that NSE should be an effective marker for the detection of axonal injury in its early stages.

Key words Head injury · Diffuse axonal injury · Immunohistochemistry · Neuron-specific enolase · Amyloid precursor protein

Introduction

The importance of diffuse axonal injury (DAI) [1] in non-missile head injury is becoming increasingly recognized in forensic medicine [2, 3]. DAI was originally defined by Strich [4] as diffuse degeneration of the cerebral white matter who concluded in a subsequent paper that mechanical forces shearing the fibers at the moment of impact produced the axonal damage [5]. Clinicopathology studies by Adams et al. [6] have endorsed Strich's view and have defined the time course of the structural abnormalities in white matter. Gennarelli et al. [7] have shown that similar abnormalities can be produced experimentally in subhuman primates using angular acceleration of the head. Because of these findings in addition to findings of computer tomography [8], the concept of DAI is widely recognized at present. DAI has been reported to be most common after road traffic accidents [9–11] but can also occur after a fall [12, 13] and assaults [14, 15].

There are three distinctive features in the pathology of DAI in its most severe form: (1) a focal lesion in the corpus callosum, (2) focal lesions in the dorsolateral quadrant or quadrants of the rostal brain stem adjacent to the superior cerebellar peduncles and (3) diffuse damage to axons, i.e. axonal retraction balls (RB), small clusters of microglia, or Wallerian-type degeneration [1, 6]. In patients who sustained milder forms of DAI, there may be no macroscopic abnormalities in the brain [16]. Therefore, the microscopic examination is indispensable for a diagnosis of DAI. However, when using conventional histology techniques, a definite diagnosis of DAI cannot be achieved until 12–15 h after such an injury, at which time classic RBs appear and can be identified [1, 9, 17, 18]. A definite diagnosis of DAI could not be made in patients who survived for only a short time after injury. Therefore, a method for detecting DAI in the early stages would be of great benefit. In this respect, we previously reported that even conventional Holmes' silver staining can demonstrate varicose axons (VA) and waving axons (WA) in cases when more than 5 h have elapsed after a head injury [19]. However, conventional silver staining failed to detect injured axons in cases of shorter survival periods.

On the other hand, it has recently been reported that a diagnosis of axonal injury can be achieved with the use of immunohistochemistry in cases with a shorter period of survival [11, 13, 20–31]. In particular, immunostaining for β-amyloid precursor protein (APP) has been reported to be possibly the most sensitive procedure [20–24]. In a preliminary study, we also tested several antibodies against the neuronal proteins. As a result, it was suggested that

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not only APP but also neuron-specific enolase (NSE) could be a useful marker for injured axons associated with head injury.

The present study was undertaken to evaluate the efficacy of immunohistochemistry for NSE as a marker for the detection of traumatic axonal injury in the early stages.

Materials and methods

From cases of fatal non-missile head injury, 19 were selected to give a wide range of survival times and 9 control cases with no head injury were selected to give a wide range of postmortem periods. The details of the head injury and control cases are summarized in Tables 1 and 2, respectively. Short survival cases represent victims who were revealed by case histories to have died more or less instantaneously. Of the 19 cases of head injury, 17 were from the Department of Legal Medicine, Kagoshima University and 2 from the Second Department of Pathology, Kagoshima University and from the Department of Legal Medicine, University of the Ryukyus. From each case formalin-fixed, paraffin-embedded sections of the corpus callosum were stained with hematoxylin and eosin, Luxol fast blue (LFB)/cresyl violet, immunostained for NSE and by the Holmes' silver method for axons [32]. Sections from 17 head injury cases (cases in Table 1 except cases 16 and 19) and 7 control cases (cases in Table 2 except cases C2 and C4) were also immunostained for APP.

Immunohistochemistry for NSE was carried out on 6 μm sections of the corpus callosum. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in methanol for 30 min. Sections were then rinsed in phosphate-buffered saline (PBS), incubated for 10 min in 20% normal swine serum and for 18 h at 4 °C in pre-diluted polyclonal rabbit anti-NSE antibody (Nichirei, Tokyo, Japan) [33] in PBS. The sections were then rinsed 3 times for 5 min in PBS and incubated for 0.5 h with biotinylated swine anti-rabbit IgG (1/400; Dako, Copenhagen, Denmark). After being rinsed in PBS, they were incubated for 0.5 h with peroxidase-conjugated streptavidin (1/300; Dako). Peroxidase activity was revealed with 0.02% 3,3′-diaminobenzidine and 0.06% H_2O_2 in PBS. Sections were then dehydrated and mounted without counterstaining. Some sections were counterstained with LFB for the purpose of simultaneous examination of axons and myelin.

For comparison, serial sections from 17 head injury cases and 7 control cases were immunostained for APP according to the method described by Sherriff et al. [21] with minor modifications. Briefly, endogenous peroxidase activity was blocked. Sections were rinsed in PBS and incubated for 10 min in 20% normal rabbit serum. They were then incubated for 18 h at 4 °C with monoclonal mouse anti-APP antibody (clone 22C11, Boehringer, Mannheim, Germany) in PBS at a dilution of 1:10. The sections were then rinsed and incubated for 0.5 h in biotinylated 1/200 rabbit anti-mouse IgG (Dako). After being rinsed, they were incubated for 0.5 h with peroxidase-conjugated streptavidin (1/300). Peroxidase activity was detected using the same procedure as described.

Semiquantitation of axonal bulbs (AB), immunopositive round-to-oval substances identical to RB (Fig. 1) [20–24, 27], was performed under × 100 magnification with a Nikon Optiphot microscope (field area calculated as 2.5 mm^2). AB were counted as an average of 18–20 fields in each case.

Table 1 Details of the cases with head injury

Case no.	Age and sex	Survival time	Postmortem period	Cause of head injury	Brain lesions
1	38M	80 d	6 h	Traffic accident	SDH
2	82F	80 d	29 h	Assault	SDH, SAH, ICH
3	60M	7 d	21 h	Traffic accident	EDH, SDH, SAH, CC
4	63M	3 d	37 h	Assault	SDH, SAH, CC
5	49M	2 d	16 h	Assault	SDH, SAH, CC, ICH
6	44M	17 h	20 h	Assault	SDH, SAH, CC
7	40M	9 h	25 h	Assault	SDH
8	56M	9 h	4.5 h	Assault	SDH, SAH, CC
9	32M	9 h	11 h	Assault	SAH
10	29M	8.5 h	11.5 h	Assault	EDH, SDH, ICH, CC
11	56M	5 h	26 h	Assault	SAH, CC, ICH
12	33M	3.5 h	30 h	Helicopter crash	SAH, CC
13	68M	1.5 h	10 h	Traffic accident	EDH, SAH, CC, ICH
14	34M	1.5 h	12 h	Assault	SAH
15	43M	short	16 h	Traffic accident	SAH, CC
16	32M	short	31 h	Traffic accident	SAH, CC
17	31M	short	26 h	Helicopter crash	SAH, CC, ICH
18	54M	short	13 h	Traffic accident	SAH, CC, ICH
19	37M	short	32 h	Traffic accident	SAH, CC, ICH

SDH Subdural hematoma, *SAH* subarachnoidal hemorrhage, *ICH* intracerebral hemorrhage, *CC* cortical contusion

Table 2 Details of the control cases with no head injury

Case no.	Age and sex	Postmortem period	Cause of death
C1	51M	16 h	Exsanguination due to a stab wound of the liver and the inferior vena cava
C2	69M	20 h	Exsanguination due to a stab wound of the branches of the mesenteric arteries
C3	17M	12 h	Strangulation
C4	86M	36 h	Myocardial infarction
C5	53M	54 h	Exsanguination due to rupture of esophageal varices
C6	43M	28 h	Exsanguination due to a stab wound of the intercostal artery and the left lung
C7	22M	3 d	CO intoxication
C8	47M	22 h	Myocardial infarction
C9	28M	11 h	Exsanguination due to a stab wound of the pulmonary artery, the heart and the left lung

Results

Holmes' staining for axons and LFB staining for myelin

Numerous axons were seen in sections of the corpus callosum from both head injury and control cases with Holmes' silver staining for axons. Additionally, classic RB in cases of head injury with more than 2 days survival and VA and WA in cases with more than 5 h survival were detected (Table 3). However, no apparent changes of axons were found in head injury cases with less than 5 h survival, as previously reported [19].

LFB staining could detect myelin globoids, reported to be a form of myelin damage secondary to axonal disruption [28], in cases with a survival of more than 2 days (Table 3). No apparent changes of myelin were found in cases with shorter survival times.

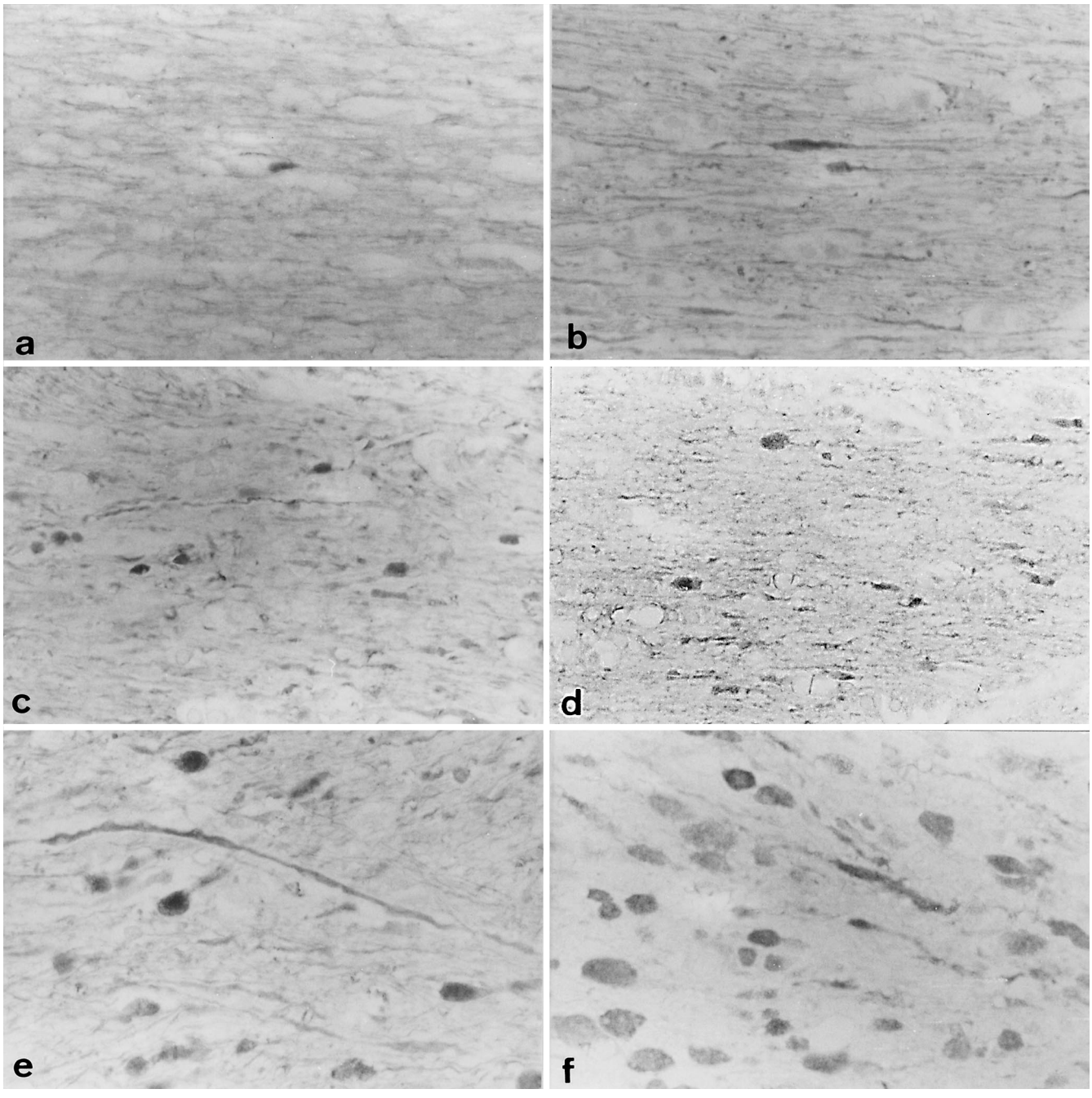

Fig. 1a–f Immunostaining for NSE (× 100) in **a**, control case with no head injury (Case C4 in Table 2), **b**, short survival case with head injury (Case 17 in Table 1), **c**, 1.5 h survival (Case 14), **d**, 1.5 h survival (Case 13), **e**, 9 h survival (Case 8), **f**, 2 day survival (Case 5)

Immunostaining for NSE

In all 9 control cases with no head injury, only less than 0.5 AB per × 100 microscopic field were found (Fig. 1a) regardless of postmortem periods (range 11 h–3 days). Similarly, in 2 out of 5 head injury cases with a short period of survival, only less than 0.5 AB were found (Table 3). In the other 3 cases with a short period of survival, between 0.5 and 1.0 AB per × 100 microscopic field were detected (Fig. 1b). In cases with 1.5 h survival after a head injury, not less than 1 AB per × 100 microscopic field was detected (Fig. 1c–1d). In 10 out of 12 cases with 3.5 h or longer survival times, not less than 1 AB was demonstrated (Table 3) and the longer the survival time, the more apparent AB became (Fig. 1e-f). VA and WA were also detected by NSE staining in head injury cases and usually associated with AB. As a result, immunostaining for NSE detected injured axons in cases with 1.5–3.5 h survival, which were undetectable by Holmes' staining (Table 3). Furthermore, when NSE-immunostained sections were counterstained with LFB, injured axons and myelin globoids were detected in the same sections in cases with not less than 2 days survival (Fig. 2).

Table 3 Histological findings of axons and myelin in the corpus callosum

Case no.	Survival time	Holmes' silver staining		LFB staining	NSE staining	APP staining
		Retraction ball	Varicose axon	Myelin globoid	Axonal bulb*	Axonal bulb*
1	80 d	+	+	+	++	++
2	80 d	–	–	+	–	–
3	7 d	+	+	+	++	++
4	3 d	+	+	–	++	++
5	2 d	+	+	+	++	++
6	17 h	–	+	–	++	+
7	9 h	–	+	–	++	++
8	9 h	–	+	–	++	++
9	9 h	–	+	–	+	+
10	8.5 h	–	+	–	++	++
11	5 h	–	+	–	++	++
12	3.5 h	–	–	–	++	++
13	1.5 h	–	–	–	++	++
14	1.5 h	–	–	–	++	++
15	short	–	–	–	+	–
16	short	–	–	–	–	NT
17	short	–	–	–	+	–
18	short	–	–	–	+	+
19	short	–	–	–	–	NT

NSE, neuron-specific enolase; LFB, Luxol fast blue; NT, not tested
* –, less than 0.5 axonal bulbs (AB), +, between 0.5 and 1.0 AB ++, not less than 1.0 AB per microscopic field (power: × 100)

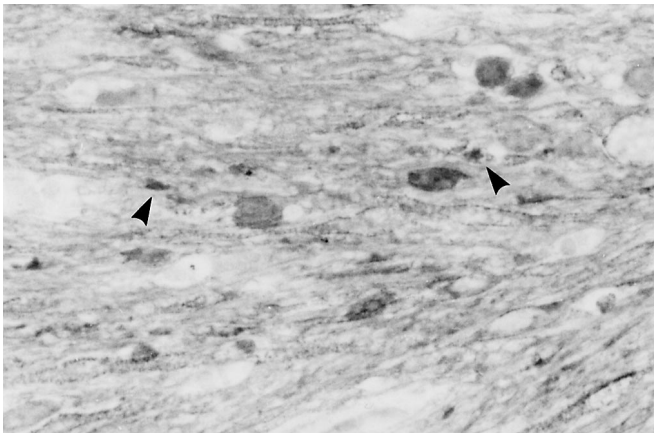

Fig. 2 NSE staining and LFB counterstaining (2 day survival, Case 5 in Table 1, × 100). Axonal bulbs and myelin globoids (arrowheads) are demonstrated in the same section

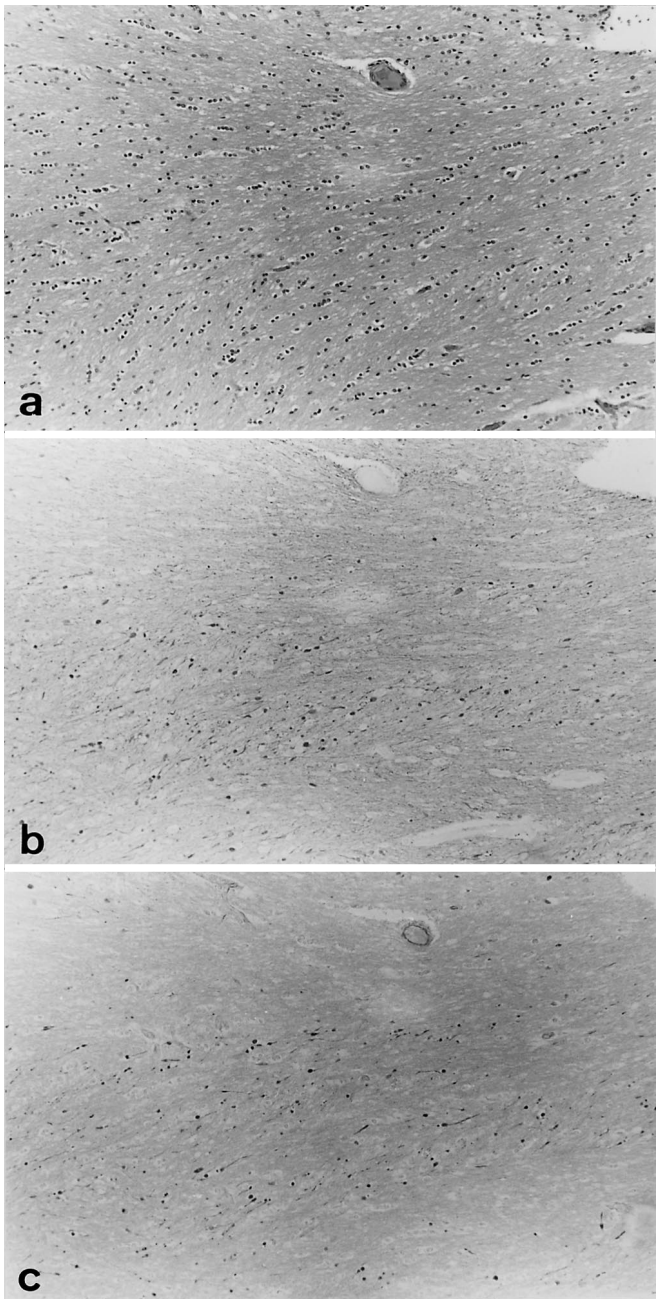

Fig. 3a–c Case of 9 h survival after head injury (Case 8 in Table 1, × 25), **a**, hematoxylin & eosin staining, **b**, immunostaining for neuron-specific enolase, **c**, immunostaining for amyloid precursor protein

Immunostaining for APP

Immunolabelling for APP and NSE was performed in serial sections from 17 head injury cases and from 7 control cases. The APP-stained sections were compared with NSE-stained sections and the numbers of AB stained were almost identical (Table 3). In control cases, only less than 0.5 AB were labelled by APP. In 11 out of 14 cases of head injury with more than 1.5 h survival, not less than 1 AB were labelled by APP. VA and WA were also detected by APP in head injury cases and usually associated with AB. Further, the APP staining pattern was almost identical to the NSE staining pattern in serial sections of every case examined (Figs. 3, 4).

Discussion

It has been reported that immunohistochemical labelling for APP [13, 20–27], neurofilament subunits [28–30], or

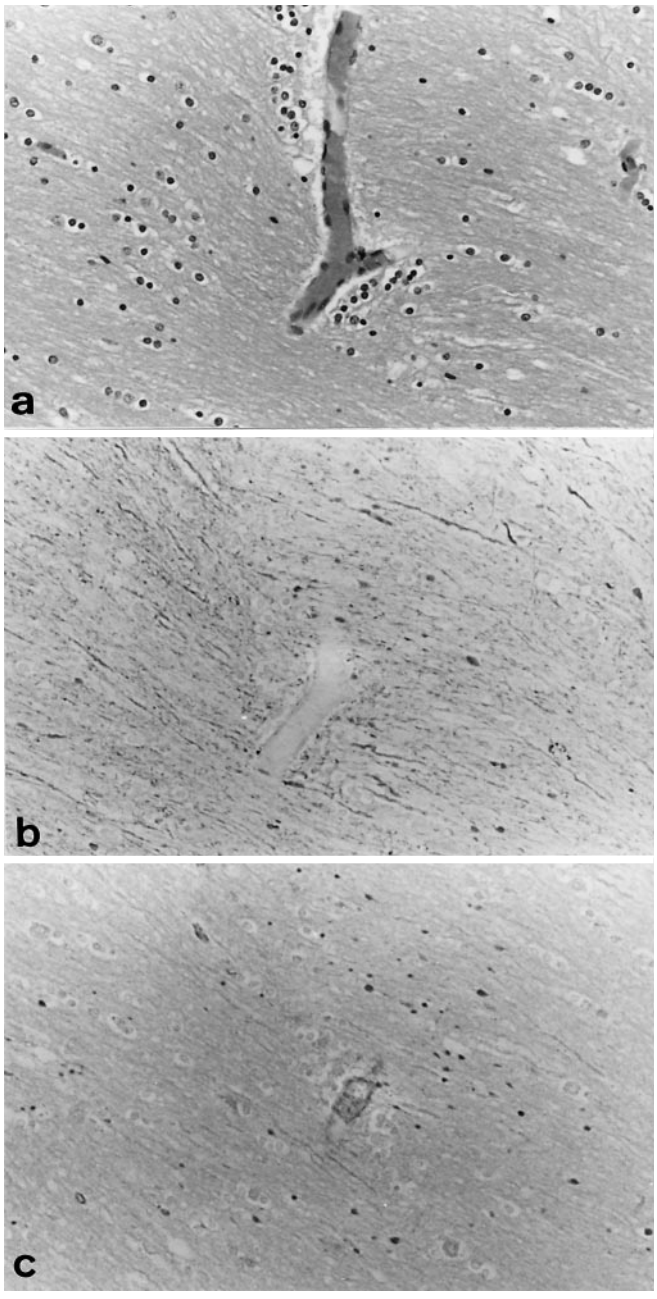

Fig. 4a–c Case of 1.5 h survival after head injury (Case 14 in Table 1, × 50), **a**, hematoxylin and eosin staining, **b**, immunostaining for neuron-specific enolase, **c**, immunostaining for amyloid precursor protein

ubiquitin [31] is far more useful for the detection of axonal injury in its early stages than classic silver staining techniques. In particular, labelling for APP has been reported to be possibly the most sensitive procedure [20–24].

Our studies have indicated that not only APP, but also NSE, was one of the effective markers of injured axons. Since immunostaining for NSE as well as for APP only labelled AB and VA and did not label normal axons, the results were readily interpretable. In all control cases examined, NSE- and APP-labelled AB were few in number regardless of the postmortem period (range 11 h–3 days). Therefore it is suggested that postmortem changes of axons up to at least 3 days might not affect the NSE or APP staining patterns. Furthermore, NSE and APP labelled AB in cases after as little as 1.5 h survival following closed head injury. In addition, staining with the anti-NSE antibody used in this study showed at least as intense immunoreactivity as anti-APP.

NSE is a neuron-specific form of the glycolytic enzyme enolase (E.C.4.2.1.11) [34] and a major protein of human brain tissue, representing about 1.6% of total soluble protein [35]. Immunohistochemistry for NSE has become a marker of neurons and neuroendocrine cells [34, 35]. NSE has been reported to be localized in nerve cell bodies and axons, but not in glial cells [36, 37]. However, the expression of NSE immunoreactivity in reactive astrocytes has been also reported [38–40].

With regard to the mechanisms of axonal injury, the following hypothesis, mainly obtained from experimental studies in animal models of axonal injury, is accepted at present. Only at the greatest levels of injury do axons become sheared or disconnected, that is, undergo primary axotomy and non-disrupted, injured axons undergo a sequence of changes which culminate in secondary axotomy. A focal axonal change, such as a cytoskeletal breakdown, usually occurs possibly due to a calcium influx. Subsequently, this change causes a local interruption of axonal transport. With resultant concentration of transported materials, these substances continue to accumulate in involved parts of axonal segments. At this stage, VA appear and ultimately, axons disconnect to form AB [41–46].

Our study showed that NSE and APP selectively labelled injured axons. This suggests that levels of NSE and APP in non-injured axons which might be undetectable in formalin-fixed tissue by standard immunohistochemical staining are demonstrable by immunohistochemical staining at sites of axonal injury.

Our results have revealed that NSE as well as APP detected both VA and AB in cases of head injury with a short post-traumatic survival period. Since APP is a rapidly transported protein [47], it may accumulate rapidly and reach detectable levels at the site of injury. Although NSE is transported more slowly (classified into the SCb group) [48, 49], its presence at a high concentration is known in rat axons [50]. Thus it is possible that, in humans, slight accumulation of NSE reaches detectable levels.

In this study, NSE and APP labelled injured axons in head injury cases with a survival time of not less than 1.5 h. For APP, these results should be in agreement with the previous findings that immunolabelling of APP was first detected after 1.75–2 h survival in humans [23, 24]. However, no previous reports have suggested that immunolabelling for NSE can also detect injured axons and that labelling for NSE may be as useful a marker for injured axons as APP.

In the control cases with no head injury examined in this study, no false staining features leading to false diag-

nosis of axonal injury were shown by APP and NSE. However, it has been reported that APP immunoreactivity is sometimes seen in cases with no head injury [21, 22, 51]. Geddes et al. [27] emphasized that it might be possible to overdiagnose DAI, particularly in cases with short survival periods, if APP immunopositivity was interpreted uncritically. Furthermore, they proposed that sufficient samples to cover the sites vulnerable to traumatic axonal injury [1, 9, 28] must be taken in order to diagnose DAI. With this in mind, the joint usage of NSE and APP should be useful and more discriminatory for the detection of axonal injury in its early stages.

In 2 out of 5 head injury cases with short survival times, the numbers of AB by NSE staining were as low as those in control cases (Table 3) so that the usefulness for diagnosis has to be called into question. In animal experiments, however, Lewis et al. [52] reported that APP could detect injured axons in sheep as early as 1 h after injury, but this is not comparable to the human experience [24]. While our preliminary studies have indicated that NSE staining detected injured axons in 2 cases with 0.5 and 1 h of survival [53], critical examination and exclusion of inadequate cases showed that a 1.5 h survival period is the limitation of NSE and APP immunostaining. Further studies are required to establish a reliable method for diagnosing DAI in cases with a short survival period.

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