

Histopathological changes of the hippocampus neurons in brain injury

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Summary. The glial fibrillary acidic protein (GFAP) is known as a peculiar marker of mature astrocytes of the central nervous system (CNS). However, we found distinct immunopositivity to a monoclonal anti-GFAP reagent in the hippocampus neurons in head injury fatalities. The present study investigated the neuronal and neuroglial GFAP-immunopositivity in the hippocampus in a series of head injury cases, which included acute and subacute/delayed deaths (n=17 and n=73, respectively), and acute cardiac death (n=13), delayed death due to multiple organ failure from non-head injury (n=6), and pneumonia (n=9) cases were examined as controls. GFAP-immunopositivity in the neurons was frequently observed in CA4, CA3 and CA2 regions in cases of subacute/delayed head injury death that showed marked brain swelling accompanied by secondary brain stem hemorrhages, showing an inverse relationship to that in astrocytes. These findings suggest possible induction of GFAP or a related protein in hippocampus neurons depending on the severity of brain swelling following head injury.

Key words: Forensic neuropathology, GFAP, Immunohistochemistry, Brain injury, Hippocampus

Introduction

Glial fibrillary acidic protein (GFAP) is believed to be a specific marker for astrocytes in the central nervous system (CNS) (Eng et al., 2000). Recently, however, GFAP or GFAP-like antigens were immunohistochemically detected in an increasing number of cell types in the CNS; ependymocytes (Roessmann et al., 1980), immature oligodendroglial cells (Choi and Kim,

1984), pituicytes of neurohypophysis (Salm et al., 1982), and interstitial cells of the pineal gland (Moller et al., 1978). In addition, GFAP-immunoreactivity is observed in oligodendroglial tumors, which possibly contain heterogeneous neoplastic cell populations with transitional cell types between oligodendroglial and astroglial lineages (Matyja et al., 2001a,b). In the peripheral nervous system, enteric glia (Jessen and Mirsky, 1980), non-myelin forming Schwann cells (Jessen et al., 1984), and the olfactory nerve (Barber and Lindsay, 1982) show GFAP-immunoreactivity. Furthermore, GFAP-immunoreactivity was found in non-neural tissues, including perisinusoidal stellate cells of the liver (Gard et al., 1985; Morini et al., 2005) and epithelial cells of the parotid gland (Achstatter et al., 1986). However, there appear to have been no reports on GFAP-immunoreactivity in CNS neurons.

Following CNS injury involving brain tissue damage, ischemia or hypoxia, astrocytes become reactive and rapidly produce GFAP, playing an important role in maintaining the extracellular environment and protecting neurons (Eng et al., 1994; Hatten et al., 1991; Chen and Swanson, 2003). However, we found a distinct immunopositivity to a monoclonal anti-GFAP reagent in hippocampus neurons in head injury fatalities. In the present study, we investigated the neuronal and neuroglial GFAP-immunopositivity in the hippocampus in a series of head injury death cases, and the pathophysiological significance is discussed.

Materials and methods

Materials

Medicolegal autopsy cases within 48 h postmortem at our institute were examined: total, n=118; 93 males and 25 females; 6 months-93 years of age (median, 59 years of age); postmortem interval, 3-48 h (median, 19.5 h), as shown in Table 1. The brain injury cases were

classified as follows: blunt/gunshot head injury (n=90) including acute deaths (survival time <3 h, n=17) and subacute/delayed deaths (survival time, 6 h-3 weeks) under medical care (n=73: cases with/without complications, n=29/44). Clinically diagnosed brain death cases were excluded. The clinical and autopsy data, including macro- and microscopical pathology findings, are shown in Table 2. Control cases without brain injury (n=28) comprised death due to acute cardiac death (ACD) without medical treatment (n=13), multiple organ failure (MOF, n=6) from burns (n=2), septicemia (n=3) and acute myelogenous leukemia (n=1), and pneumonia (n=9) (Table 1). The above-mentioned causes of death were classified on a pathological and toxicological basis, and clearly accountable cases were included.

Tissue specimens

Ten percent formalin-fixed paraffin-embedded hippocampus tissue specimens were used. The cerebral cortex and brain stem were also examined. Serial sections 5 μ m thick were prepared and used for hematoxylin-eosin (HE) and immunostaining.

Immunohistochemistry

Immunoenzyme procedure

The present study counted glial cells smaller than 10 μ m in diameter that were clearly detected in HE staining. Since the glial cells are known to be positive for fibrillary acidic protein (GFAP) (Hausmann et al., 2000), subsequent sections were used in immunohistochemical studies to identify cells. The following primary antibodies were used: monoclonal mouse anti-human glial fibrillary acidic protein (GFAP) (Dako Denmark A/S, clone 6F2, isotype IgG1, diluted 100-fold) (Rutka et al., 1997; Eng et al., 2000), monoclonal mouse anti-human glial fibrillary acidic protein (GFAP) (CRP California, clone SMI-23, isotype IgG2b, diluted 800-fold) (Meikle et al., 2007), polyclonal rabbit anti-cow glial fibrillary acidic protein (GFAP) (Dako Denmark A/S, diluted 400-fold) (Rutka et al., 1997; Viale et al., 1991), and polyclonal rabbit anti-bovine neurofilament H (200KD) (Acris Germany, diluted 500-fold) (Karlsson et al., 1987). They were incubated at 24°C for 12 h in a universal streptavidin/biotin immunoperoxidase detection system (Omni Tags kit)

Table 1. Case profiles (n=118).

Cause of death	n	Male/female	Age (years)		Survival time (h)		PMI (h)	
			range	median	range	median	range	median
Brain injury								
acute death	17	15/2	18-88	55	<0.1-3	0.5	6-32	21.7
subacute/delayed death	73	59/14	0.5-86	60	<6-504	72	3-48	19.3
Acute cardiac death	13	9/4	43-76	62	<0.5-3	0.5	8-35	19.0
Multiple organ failure	6	3/3	28-81	56	<36-504	120	5-33	17.2
Pneumonia	9	7/2	39-84	72	<120-720	168	9-27	21.7

PMI, postmortem interval.

Table 2. Brain injury cases (n=90).

Survival Time	Trauma	Major lesion	*Duret hemorrhage	Complication
<3 h (n=17)	Traffic accident, n=7; Fall, n=3; Blow, n=5; Gunshot wound, n=2	CC, n=8; Brain lacerations, n=9	n.d.	n.d.
6-12 h (n=6)	Assault, n=6	CC, n=1; SDH, n=5	n.d.	n.d.
12-24 h (n=17)	Traffic accident, n=2; Fall, n=4; Blow, n=11	CC, n=5; SDH, n=12	n=10	Pneumonias, n=3
24-72 h (n=12)	Fall, n=4; Blow, n=8	CC, n=6; SDH, n=6	n=7	Pneumonias, n=4
72 h-1 week (n=19)	Traffic accident, n=1; Fall, n=4; Blow, n=14	CC, n=10; SDH, n=8; SAH, n=1	n=10	Pneumonias, n=8
1-3 weeks (n=19)	Traffic accident, n=2; Fall, n=2; Blow, n=15	CC, n=11; SDH, n=8	n=8	Pneumonias, n=14

*Duret hemorrhage, secondary brainstem hemorrhage; CC, cerebral contusion; SDH, subdural hematoma (some cerebral contusion and/or SAH was accompanied to all cases); SHA, subarachnoid hematoma. N.d., not detected.

(Ishikawa et al., 2007). Color development was done with 3,3'-diamino benzidine tetrahydrochloride (DAB, Shandon/Lipshaw/Immunon, Pittsburgh, Penn.) according to the manufacturer's instructions with hematoxylin counterstaining. Endogenous peroxidase was inactivated by incubation with 0.3% hydrogen peroxide for 15 min. The negative control studies were performed by omission of primary antibody, and substitution with mouse IgG or normal rabbit serum at the same dilution in the same staining system. The specificity of the polyclonal reagents was confirmed by an absorption test: The incubation of the sections with antiserum preabsorbed with an excess of the corresponding antigen completely abolished the immunostaining.

Double-color immunofluorescence analysis

Deparaffinized sections were incubated with phosphate buffered saline containing 1% normal goat serum to reduce non-specific reactions. Thereafter, the sections were incubated with pairs of mouse monoclonal anti-GFAP (Dako Denmark A/S, clone 6F2, isotype IgG1, diluted 100-fold and CRP California, clone SMI-23, isotype IgG2b, diluted 800-fold) and polyclonal rabbit anti-neurofilament H (1:500) at 24°C overnight. After incubation with FITC-conjugated anti-mouse IgG pAb (1:50) and Cy3-conjugated anti-rabbit IgG pAb (1:100) at 24°C for 3 h, the sections were observed under a fluorescence microscope.

Quantitative analysis of GFAP-immunopositivity

Neuronal GFAP-immunopositivity was analyzed in CA4, CA3-CA2 and CA1 regions of the hippocampus. The gliocytes were examined in the CA4 region because the localization of gliocytes could not be clearly identified at the border of the CA3, CA2 and CA1 regions.

The total number of neurons and glial cells and the number of neurons and astrocytes in which GFAP-immunoreactivity was detected, respectively, were counted in the CA4 region under 200 x magnification and positive staining was estimated with positive neurons = number of positive neurons/total number of neurons x 100, with positive astrocytes = number of positive astrocytes/total number of astrocytes x 100. Furthermore, in the CA4, CA3-CA2 and CA1 regions, the number of positive neurons (number of positive neurons/total number of neurons x 100) was counted under 200 x.

A Confocal laser scanning microscope (Leica TCS SP) was used to examine the cell reactions. Two channels were used (laser line 1: argon ion 488 nm and laser line 2: krypton 568 nm), allowing for two fluorescent signals (double markers) from the same specimen to be scanned simultaneously or sequentially and then digitally converted into an image. The secondary antibody FITC marked the GFAP positive

cells a green color (ex/em: 488 nm/500–550 nm), while the neurofilaments' autofluorescence appeared in a red color (ex/em: 568 nm/580–630 nm).

Statistical analysis

The Pearson product-moment correlation coefficient was used to compare two parameters, including age, survival time and GFAP-positivity. Comparisons between groups were performed by nonparametric Mann-Whitney U-test, and the Scheffe test was used for analysis involving multiple comparisons. These analyses were performed using the Statview (version 5.0, SAS Institute Inc. SAS Campus Drive Cary). A p-value of less than 0.05 was considered to indicate statistical significance. In Figs. 4, 5 and 7, the results of the data analyses are shown as box-plots, for which 50% of the data is summarized in the box. The line in each box represents the median, and the lines outside of each box represent the 90% confidence intervals.

Results

Distribution of GFAP

In the hippocampus, immunopositivity for GFAP was usually observed in astrocytes (Fig. 1), and some neurons showed positivity in specific cases (Figs. 2, 3), as described below. The specificity was confirmed using two monoclonal, and one polyclonal, anti-GFAP antibodies. Astrocyte GFAP-positivity was mainly observed in the CA4 region, and spread into neighboring regions including CA3, CA2 and CA1. GFAP-immunopositivity in the neurons was granularly detected in the cytoplasm, and three staining patterns were found: (a) accumulation on the opposite side of the axon hillock, (b) distribution around the nucleus, and (c) accumulation in the axon hillock (Fig. 4). Positivity was not detected in the axons. Neuronal GFAP-positivity in the hippocampus was mainly observed in the CA4 and CA3-CA2 regions (Fig. 1), showing a marked correlation between these regions ($r=0.74$, $p<0.0001$). Neuronal GFAP-immunoreactivity was sparse in the CA1 region (Fig. 1), and hardly detected in the cerebral cortex and brain stem.

Quantitative analysis of GFAP-immunopositivity

The total number of glial cells and astrocytes in the CA4 region of the hippocampus, and the total number of neurons in the CA4, CA3-CA2 and CA1 regions did not show age-dependency ($r<0.1$, $p>0.1$). In subacute/delayed death from brain injury, the total number of neurons, glial cells and astrocytes were slightly decreased compared with those in acute death from brain injury ($p<0.05$). In the CA1 region of the hippocampus and neighboring structures, including the alveus, stratum oriens and stratum radiatum, most astrocytes underwent clasmotodendrosis or disappeared.

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However, selective loss of neurons was not observed in the CA1 region. Astrocyte GFAP-immunopositivity in the CA4 region was significantly lower in subacute/delayed death from brain injury, and slightly

lower in acute brain injury death (Fig. 5). Neuronal GFAP-positivity was frequently observed in the CA4 and CA3-CA2 regions of the hippocampus in subacute/delayed death from brain injury, but only

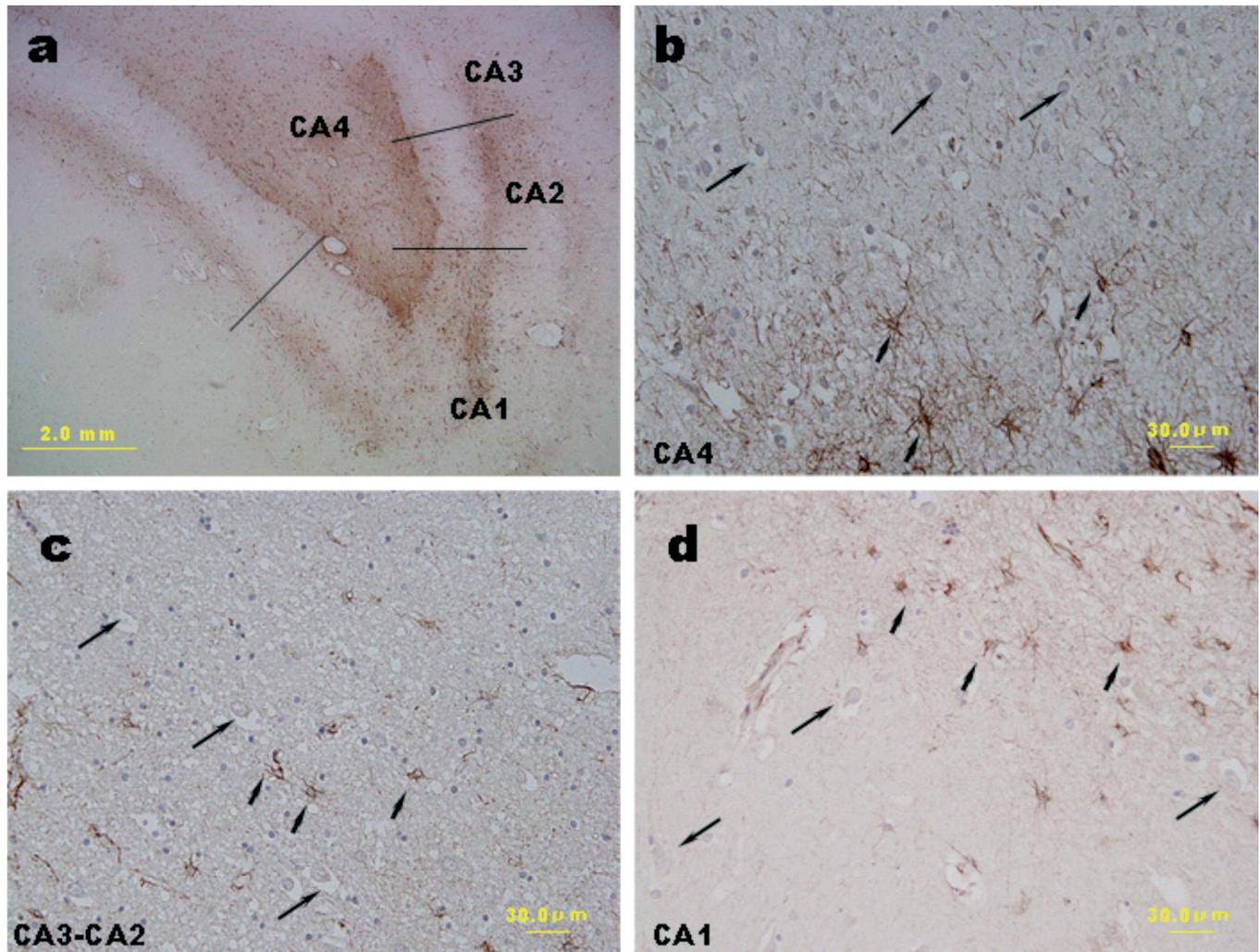


Fig. 1. Immunostaining with a monoclonal mouse anti-GFAP antibody (clone 6F2) in the hippocampus after acute death due to acute cardiac death (71-year-old male; survival time, 0.5 h; 12 h postmortem). **a.** Astrocyte GFAP-immunopositivity is seen in hippocampus. CA4 (**b**), CA3-CA2 (**c**) and CA1 (**d**) regions show GFAP-immunoreactivity in neurons (arrow) and astrocytes (arrowhead).

Table 3. Number of cases showing neuronal GFAP-immunopositivity.

Cause of death	CA4 region (%)		CA3-CA2 region (%)		CA1 region (%)	
Brain injury						
acute death (n=17)	1	5.9	1	5.9	0	0.0
subacute/delayed death (n=73)	53	72.6	52	71.2	20	27.4
Acute cardiac death (n=13)	2	15.4	2	15.4	0	0.0
Multiple organ failure (n=6)	0	0.0	0	0.0	0	0.0
Pneumonia (9)	1	11.0	1	11.0	1	11.0

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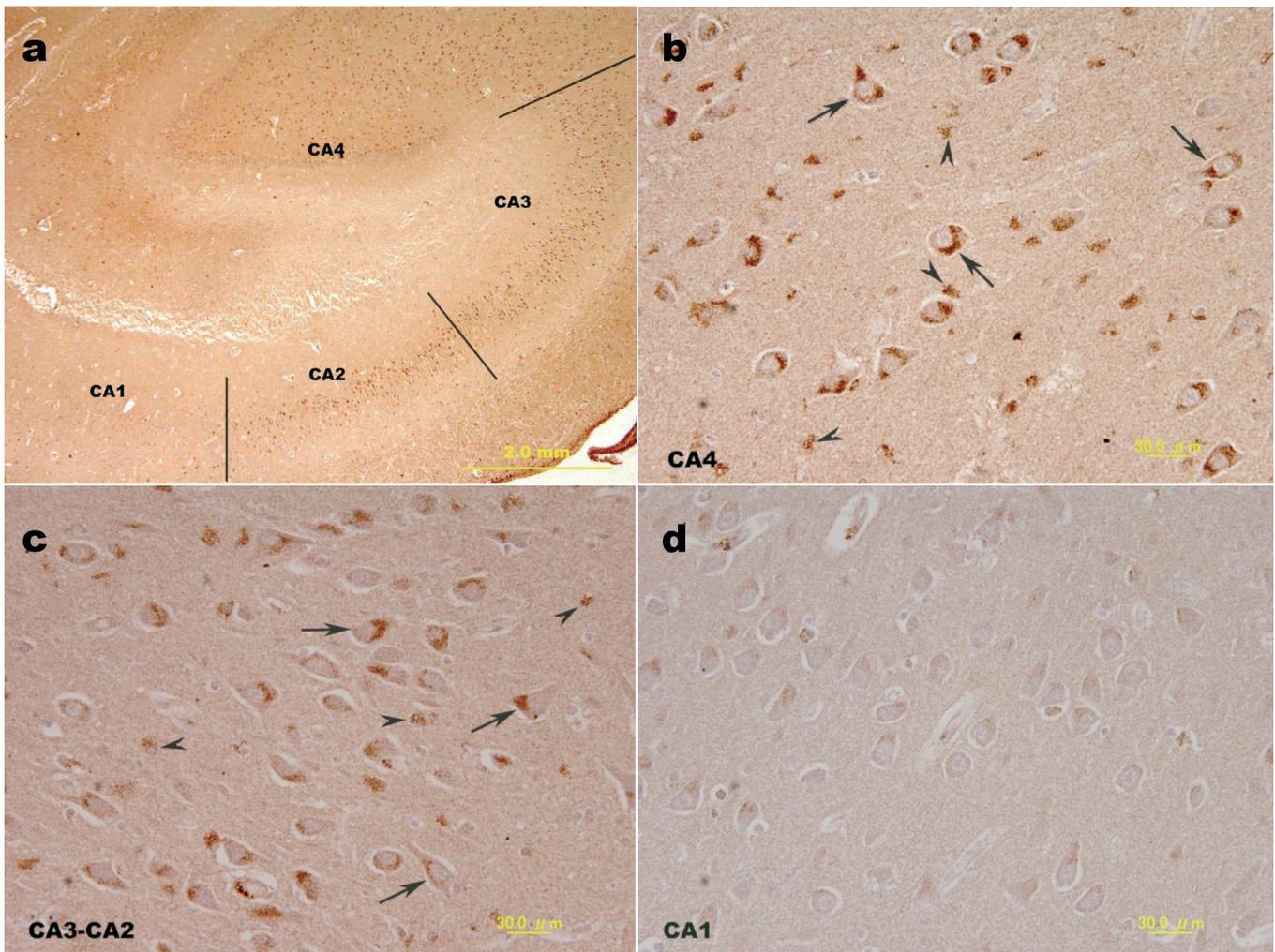


Fig. 2. Immunostaining with a monoclonal mouse anti-GFAP antibody (clone 6F2) in the hippocampus after delayed death due to brain injury (57-year-old male; survival time, 36 h; 19.3 h postmortem). **a.** Neuronal GFAP-immunopositivity is mainly seen in the CA4, CA3 and CA2 regions and is sparse in the CA1 region. Astrocyte GFAP-immunopositivity is mainly seen in the CA4 region, spreads into neighboring regions, and is almost negative in the CA1 region. CA4 (**b**) and CA3-CA2 (**c**) regions show GFAP-immunoreactivity in the neurons (arrows) and astrocytes (arrowheads). In the CA1 region (**d**), neurons and astrocytes are negative for GFAP-immunostaining.

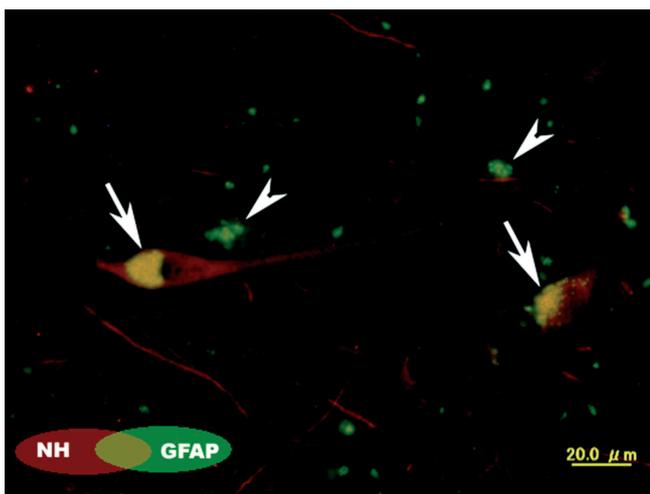


Fig. 3. A double-color immunofluorescence analysis with anti-Neurofilament H and anti-GFAP (Dako). Arrows show neurons and arrowheads show astrocytes.

sporadically in other groups (Table 3, Fig. 6). In brain injury cases, neuronal GFAP-immunopositivity in the hippocampus was detected in cases of survival over around 12 h, in which secondary brain stem hemorrhage (Duret hemorrhage) was often observed (Table 2). There was no difference in neuronal GFAP-immunopositivity between cases with and without complication with pneumonia. For subacute/delayed death from brain injury, positivity in the neurons (y) showed an inverse relationship to that in the astrocytes (x): $y = -0.25x + 17.5$ $n = 73$ $r = -0.45$ $p < 0.0001$ (Fig. 7). In cases with Duret

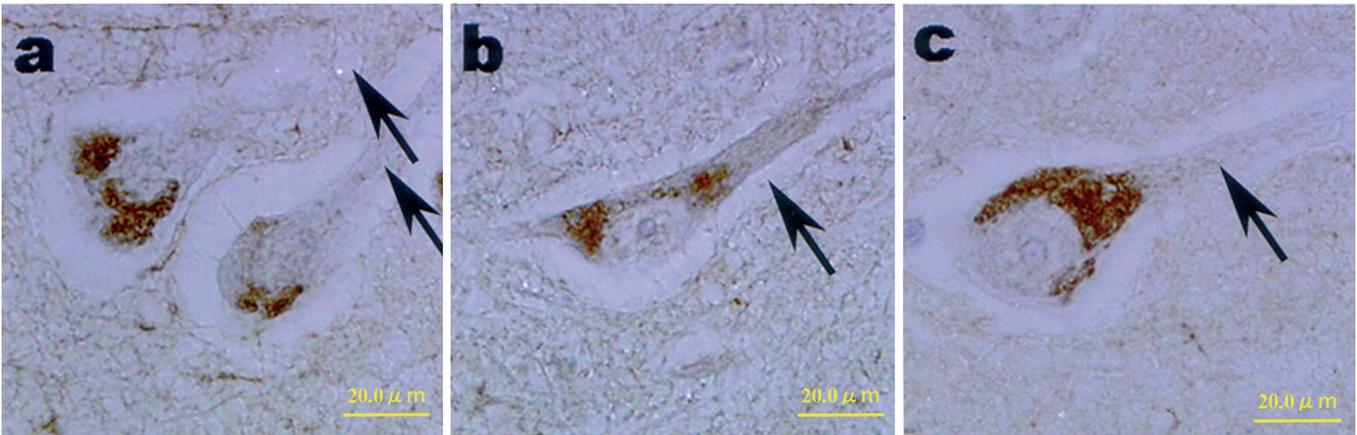


Fig. 4. Monoclonal mouse anti-human GFAP-immunostaining patterns of the neurons. Accumulation on the opposite side of the axon hillock (a), distribution around the nucleus (b), accumulation in the axon hillock (c). Arrows show axons.

hemorrhage, GFAP-immunopositivity was significantly lower for astrocytes ($p < 0.05$) and higher for neurons ($p < 0.05$) (Fig. 8). Cases of hemilateral subdural hematoma accompanied by uncal herniation showed a mild tendency toward higher neuronal GFAP-positivity in the hippocampus on the affected side, although the difference was not significant.

Discussion

In the present study, subacute/delayed death cases with a survival period over 12 h after brain injury showed lower astrocyte GFAP-immunopositivity in the CA4 region, where total numbers of neurons and glial cells were decreased, suggesting post-traumatic neurodegeneration (Raabe and Seifert, 1999; Raghupathi et al., 2000; Li et al., 2006a,b). In these cases, interestingly, GFAP-immunopositivity was also observed in the hippocampus neurons. The staining patterns in the cytoplasm of neurons, showing different localizations around the nucleus from the axon hillock to the other side, suggested that post-transcriptional modulation and redistribution are involved in the function and expression of GFAP or GFAP-like protein. However, GFAP-immunopositivity was hardly seen in the axons. These findings suggest the contribution of GFAP or GFAP-like protein to the response to post-traumatic neuronal injury. Previous studies showed early loss of cytoskeletal protein, including microtubule associated protein 2 (MAP2), in the neurons after brain injury (Kitagawa et al., 1998; Oehmichen et al., 2003). The appearance of GFAP or GFAP-like protein in the neurons may be related to such structural impairment. With respect to these findings and hypotheses, further experimental studies are necessary. GFAP-immunopositivity in the neurons was almost exclusively seen in the CA2, CA3 and CA4 regions in the hippocampus, showing an inverse relationship to the astrocyte GFAP-positivity in the CA4 region. There was

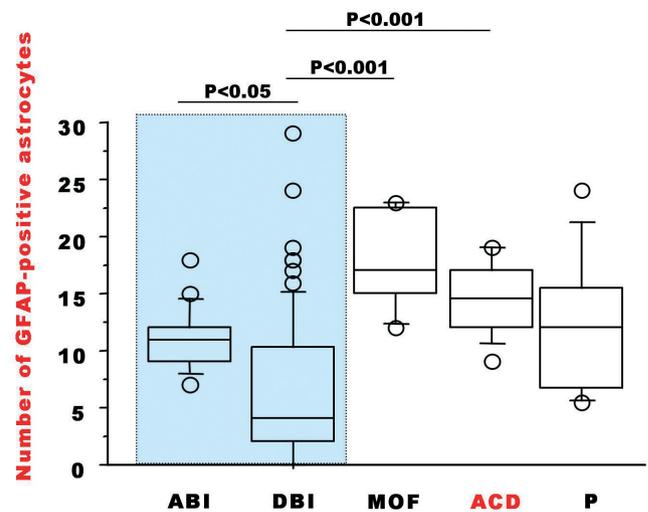


Fig. 5. The number of GFAP-positive astrocytes in the CA4 region of the hippocampus in relation to the cause of death. ABI, acute death from brain injury; DBI, subacute/delayed death from brain injury; MOF, death from multiple organ failure; ACD, acute cardiac death; P, pneumonia. The results of statistical analyses using the Scheffe test are shown. Significantly low: DBI vs. ACD ($p < 0.001$) and MOF ($p < 0.01$). In brain injury cases, DBI shows a significantly low positivity compared with ABI ($p < 0.05$) by Mann-Whitney U test.

no difference in neuronal GFAP-immunopositivity between the CA4 and CA3-CA2 regions. This finding was evident in subacute or delayed death cases showing advanced brain swelling accompanied by Duret hemorrhage, which suggests secondary brain injury due to cerebral compression involving elevated intracranial pressure (Parizel et al., 2002). Furthermore, neuronal GFAP-immunopositivity showed a tendency toward an increase on the side of uncal herniation. However, neuronal GFAP-positivity was hardly detected in the

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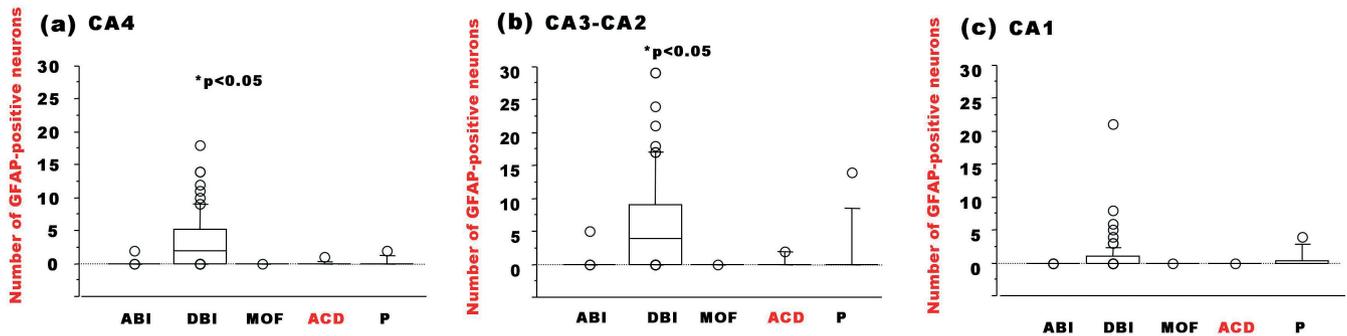


Fig. 6. The number of GFAP-positive neurons in relation to the cause of death. The abbreviations used are shown in Table 3. Significantly high by Scheffe test in the CA4 and CA3-CA2 regions of the hippocampus: *DBI vs. ABI, MOF, ACD and P ($p < 0.05$).

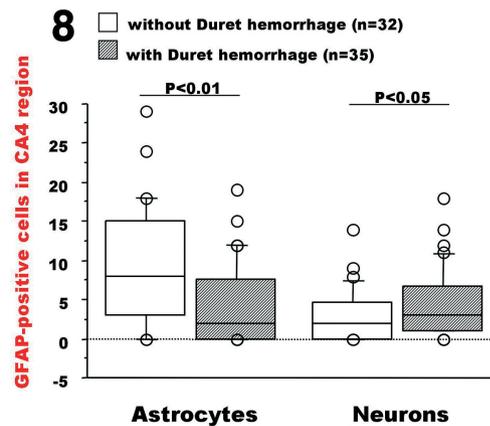
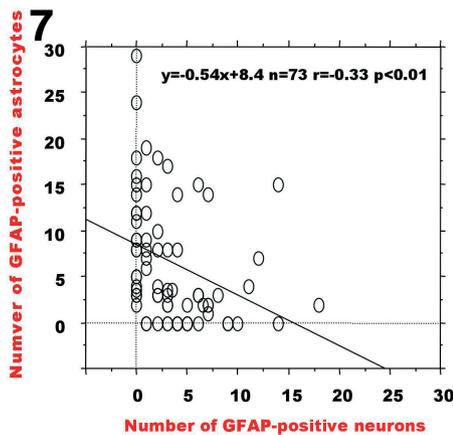


Fig. 7. The relationship between number of GFAP-immunopositive neurons and astrocytes in subacute/delayed death due to brain injury.

Fig. 8. The relationship between number of GFAP-immunopositive cells in the CA4 region of the hippocampus and Duret hemorrhages in cases of subacute/delayed brain injury. In cases of Duret hemorrhage, the number of GFAP-immunopositive astrocytes was significantly lower, while the number of GFAP-immunopositive neurons was significantly higher ($p < 0.01$) according to a Mann-Whitney U-test.

CA1 region, which is the most vulnerable to hypoxia/ischemia (Kirino, 2000). In addition, neuronal GFAP-positivity was hardly seen in cases of multiple organ failure and pneumonia, where the neurons and astrocytes were relatively intact compared with those in brain injury death. These findings suggest that the appearance of GFAP or GFAP-like protein in the hippocampus neurons may be characteristic of delayed neuronal damage due to advanced brain swelling causing cerebral compression, which is closely related to astrocyte injury and independent of cerebral hypoxia. These findings suggest that hippocampus neurons sustain characteristic damage during survival after the onset of brain swelling, accompanied by induction of structural proteins involving GFAP following destruction of glial cells (Pelinka et al., 2004; Sandhir et al., 2008). Although similar findings were previously observed for non- β S100 protein, which diffusely appeared in the neurons of the cerebral cortex in subacute or delayed brain injury death (Li et al., 2006a,b), the relationship with Duret hemorrhages was more evident for GFAP-positivity, as observed in the present study. Moreover, neuronal GFAP-immuno-

positivity was almost completely specific to the hippocampus neurons. Thus, this finding may be closely related to the anatomical or neurological characteristics of the hippocampus, and may be induced by the duration of elevated intracranial pressure at the base of the skull.

In conclusion, we found distinct GFAP-immunoreactivity in the hippocampus neurons in subacute/delayed head injury death, which suggests possible induction of GFAP or a related protein depending on the severity of the brain swelling causing cerebral compression. Although the pathophysiological mechanism of neuronal GFAP-positivity is obscure, it may be closely related to astrocyte injury and be independent of cerebral hypoxia. These findings may be useful for investigating the delayed neuronal changes following head injury causing advanced brain swelling.

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