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ORIGINAL ARTICLE

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Neuronal changes in the arcuate and hypoglossal nuclei of brain stem induced by head injury

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Abstract In head injury, assessing the damage not only to the cerebrum and the cerebellum but also to the brain stem is very important. In this paper, we report neuronal changes of the arcuate nucleus (ARC) and the hypoglossal nucleus (HN) in the brain stem. We investigated these changes immunohistochemically with antibodies against microtubule-associated protein 2 (MAP2), muscarinic acetylcholine receptor (mAChR), c-*fos* gene product (c-Fos), and the 72 kD heat-shock protein (HSP70). We measured the percentage of immunopositive neurons among the total neurons of the ARC and the HN. The investigation of neuronal changes in relation to the type of head injury showed different results. In cases of tonsillar herniation, immunoreactivity to MAP2 and mAChR in the ARC was significantly lower than in the HN ($p < 0.01$). Moreover, MAP2, HSP70 and c-Fos reactivities in the ARC were significantly lower than in other types of head injuries ($p < 0.01$). In the HN, diffuse axonal injury produced slightly higher immunoreactivity to mAChR and c-Fos ($p < 0.1$). Our observations indicate that immunohistochemical examination of brain stem nuclei can provide useful information for estimating damage to the brain stem.

Keywords Head injury · Neuronal damage · Hypoglossal nucleus · Arcuate nucleus · Immunohistochemistry

Introduction

Several types of nucleus in the brain stem are known to play important roles in supporting human life [1, 2, 3]. The arcuate nucleus (ARC) is known to be one of the centres for respiration [1] and the hypoglossal nucleus (HN) also plays a critical role in defining patterns of respiration [2]. Neuronal damage in brain stem nuclei including the ARC and the HN, may be relevant to the failure to support life. However, the relationship between neuronal damage to the nuclei of the brain stem and the cause of death is not yet fully understood. In previous reports, brain stem nuclei in forensic autopsy cases were investigated immunohistochemically [4, 5, 6] and in this paper we describe neuronal changes of the ARC and the HN in the brain stem after immunohistochemical investigation.

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Materials and methods

A total of 28 forensic autopsy cases were divided into 3 groups based on the type of head injury (Table 1). Group I included cases of intracranial head injury (13 cases), group II consisted of cases of tonsillar herniation in addition to head injuries (8 cases), and group III were cases with diffuse axonal injury (DAI) without tonsillar herniation (7 cases). DAI was diagnosed according to previously reported criteria [7, 8, 9, 10, 11, 12]. We also subdivided these cases depending on post-mortem duration and survival duration after assault (see the legend in Table 1).

Heat- and sunstroke damage brain function and cause death [13, 14, 15, 16], so we designated heat- and sunstroke as the control group with brain damage (group HS, 4 cases). Furthermore, as another control group without head injury, internal causes of respiratory failure were adopted (group RF, 3 cases) (Table 2).

The whole brain was fixed in phosphate-buffered formalin for 1 month in a cold room. The brain stem was horizontally dissected at the level of the obex (Fig. 1), embedded in paraffin and sectioned at 4 μm. Sections were incubated with antibodies against microtubule-associated protein 2 (MAP2, 1:400, Amersham, UK), muscarinic acetylcholine receptor (mAChR, 1:200, Transduction Laboratories, USA), c-*fos* gene product (c-Fos, 1:300, Medac, Germany) and 72 kD heat-shock protein (HSP70, 1:1000, Amersham, UK) for 1 h at 37 °C. Immunostaining was carried out using an LSAB kit/HRP (Dako, Denmark) following the manufacturer's instructions. The immunostaining was visualised by incubating the reaction mixture with 0.02% 3,3′-diaminobenzidine (DAB) and 0.03% hydrogen peroxide in PBS. In addition, conventional staining such as hematoxylin-eosin (HE) and Klüver-Barrera (KB) were carried out to examine the morphological changes in the neurons.

The density of neurons and the localisation of their nuclei in the brain stem, neurons in the ARC and the HN were noted (Fig. 1).

Table 1 Summary of examined cases divided into groups I intracranial head injury, II tonsillar herniation and III diffuse axonal injury

Case no.	Group	Age (years)	Sex	Postmortem duration	Survival time	Head injury
1	I	63	M	A	a	BF, BC, SA
2		8 months	M	A	a	SA, BC
3		2	M	B	a	SA, BC
4		13	F	B	a	SA, BC
5		61	M	A	b	SA, BC
6		8 months	M	A	a	SA, BC
7		1	F	A	d	SD
8		56	M	B	a	SD
9		54	M	A	a	SA, BC
10		49	M	B	a	SA, BC
11		59	M	B	a	SA, BC
12		29	M	B	a	SA, BC
13		29	M	B	a	SA, BF
14	II	65	M	B	b	SD
15		67	M	B	c	SD, BC
16		53	M	B	c	SD
17		45	M	B	b	SD
18		51	F	B	a	CH
19		49	F	B	a	SA, RA
20		42	M	A	c	SD
21		37	F	B	b	SA, BC
22	III	61	M	B	d	SD
23		61	M	A	b	SA, BC
24		58	M	A	c	SD
25		52	M	B	a	SD
26		55	F	B	d	SD
27		59	F	B	a	SA, BC
28		72	F	B	c	SA, SD, CH

Postmortem duration (PMD) *A* PMD less than 12 h, *B* 13–24 h.
Survival time after assaults *a* less than 30 min, *b* less than 24 h, *c* 1–7 days, *d* more than 7 days.
Types of head injury *BC* brain contusion, *BF* cranial bone fracture, *CH* cerebellum haematoma, *SA* subarachnoidal haemorrhage, *SD* subdural haematoma, *RA* rupture of basal artery.

Table 2 Summary of control cases (*HS* heat- and sunstroke, *RF* internal causes of respiratory failure)

Group	Age (years)	Sex	Cause of death
RF	64	M	Pulmonary thromboembolism
	58	M	Pleuritis
	29	M	Asthma
HS	46	M	Sunstroke
	3	M	Heatstroke
	29	M	Heatstroke
	1	M	Heatstroke

Using the serial sections of brain tissue, both the total number of neurons and the number of immunopositive neurons in each nucleus were counted using a light microscope (Fig. 2). The percentage of immunopositive neurons compared to total neurons was estimated and differences were analysed statistically by the non-parametric two-way ANOVA test.

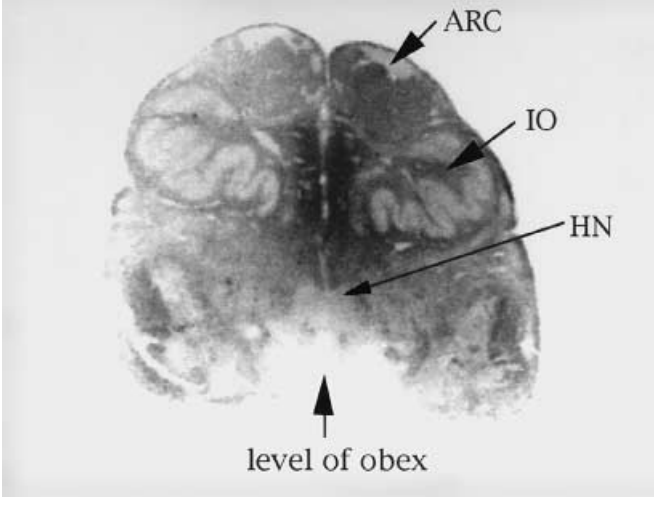

Fig. 1 Plane of section of obex (*ARC* arcuate nucleus, *IO* inferior olive, *HN* hypoglossal nucleus, Klüver-Barrera stain ×1, case no. 22: diffuse axonal injury)

Results

Control groups

In the group RF (Table 3) approx. 80% of neurons showed MAP2 immunoreactivity and for mAChR, immunoreactivity was observed in more than 90% of neurons both in the ARC and the HN. Expression of HSP70 or c-Fos was significantly lower than MAP2 or mAChR ($p < 0.001$). Group HS showed about 60% immunoreactivity against MAP2 and mAChR immunopositivity was about 50%. HSP70 was expressed between 10% and 20% both in the ARC and the HN and c-Fos was observed in about 40% of the ARC and

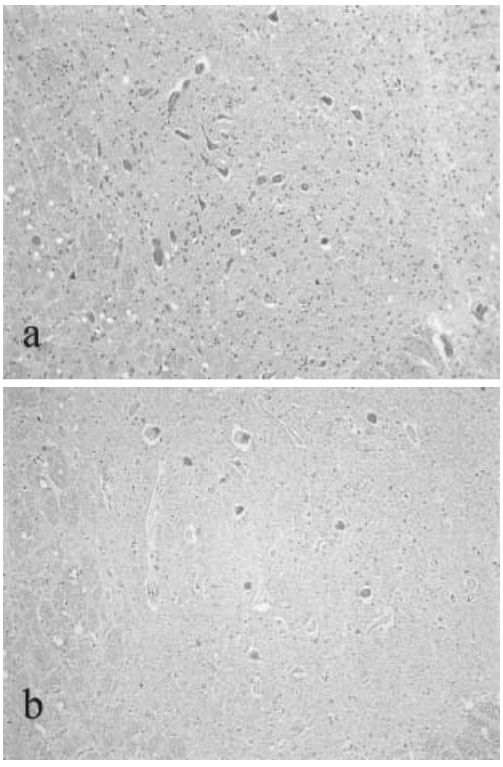

Fig. 2 Immunostaining for mAChR in the HN, **a** HE staining, **b** mAChR immunostaining (× 100), for case no. 22 with diffuse axonal injury and the proportion of immunopositive neurons is 92.1%

Table 3 Percentage of immunopositive neurons in control cases (*RF* cases of respiratory failure, *HS* cases of heat- and sunstroke, values are expressed as the mean ± standard error)

Control cases	Protein			
	MAP2	mAChR	HSP70	c-Fos
Arcuate nucleus				
RF	80.7 ± 5.6	95.1 ± 4.8	15.5 ± 7.5[a]	2.7 ± 1.4[a]
HS	60.0 ± 4.3	49.3 ± 11.3	19.4 ± 5.7	39.1 ± 9.9
Hypoglossal nucleus				
RF	78.5 ± 2.0	90.7 ± 4.6	25.5 ± 7.0	18.9 ± 9.4
HS	57.7 ± 20.9	53.6 ± 19.3	12.3 ± 0.1	13.8 ± 8.5

[a] significantly lower than MAP2 or mAChR ($p < 0.001$)

less than 20% of the HN neurons. In both the RF and HS groups, immunoreactivity to MAP2, mAChR and HSP70 showed no significant differences between the ARC and the HN ($p > 0.05$).

Head injury groups

Significant morphological changes in neurons, such as shrinkage and loss, were not observed in any of the 28 cases.

Postmortem duration and survival time

We investigated the percentage of each immunopositive neuron in relation to postmortem duration. In both the ARC and the HN, there was no significant difference of any immunopositive rate between groups A and B ($p > 0.05$) (data not shown). The percentages of each immunopositive neuron in the ARC and the HN depending on survival time were also investigated but no significant changes were observed among groups a–d ($p > 0.05$) (data not shown).

Immunoreactivity in the ARC

MAP2 was stained in about 50% of neurons in groups I and III, but in group II immunoreactive cells were significantly low in number ($p < 0.05$) (Fig. 3). In mAChR immunoreactivity, there were no significant differences among the head injury groups. HSP70 showed a high rate of about 70% and more in groups I and III but in group II immunoreactivity was less than 40% ($p < 0.05$). The percentage of c-Fos positivity was more than 60% in groups I and III but group II showed a very low rate less than 30% ($p < 0.05$).

Immunoreactivity in the HN

In MAP2 immunostaining there was no statistical significance among these groups (Fig. 4). The muscarinic AChR-immunopositive rate was slightly higher in group III than in groups I and II. HSP70 showed no remarkable differences between any of the groups. A high percentage of c-Fos-positive neurons was observed in group III, but the other two groups showed relatively low c-Fos expression.

Discussion

In considering the course of death in cases of head injury, neuronal damage in the brain stem is very important. Therefore, to assess this we studied the immunohistochemical reactions of neurons of the ARC and the HN .

MAP2 is a cytoskeletal protein of the neuron and alteration in the immunostaining of this protein was generally observed prior to morphological changes [17, 18]. In focal injury, MAP2 degeneration is observed in the lesion and in traumatic brain injury loss of MAP2 occurs selectively in the hippocampus [19], therefore MAP2 was used as a marker of neuronal damage. Muscarinic AChR is localised on the cell surface and mediates the action of the neurotransmitter ACh. The hypoglossal motoneurons are known to be controlled by muscarinic neurotransmission. In a previous study, mAChR binding has been shown to be localised extensively in human ARC [20]. In this study, mAChR was used as a marker of the neuronal membrane and ACh signalling.

HSP70 is known to be expressed in neuronal cells after various stresses, such as heat, trauma and ischemia, thereby protecting neurons against stress-induced damage [21, 22,

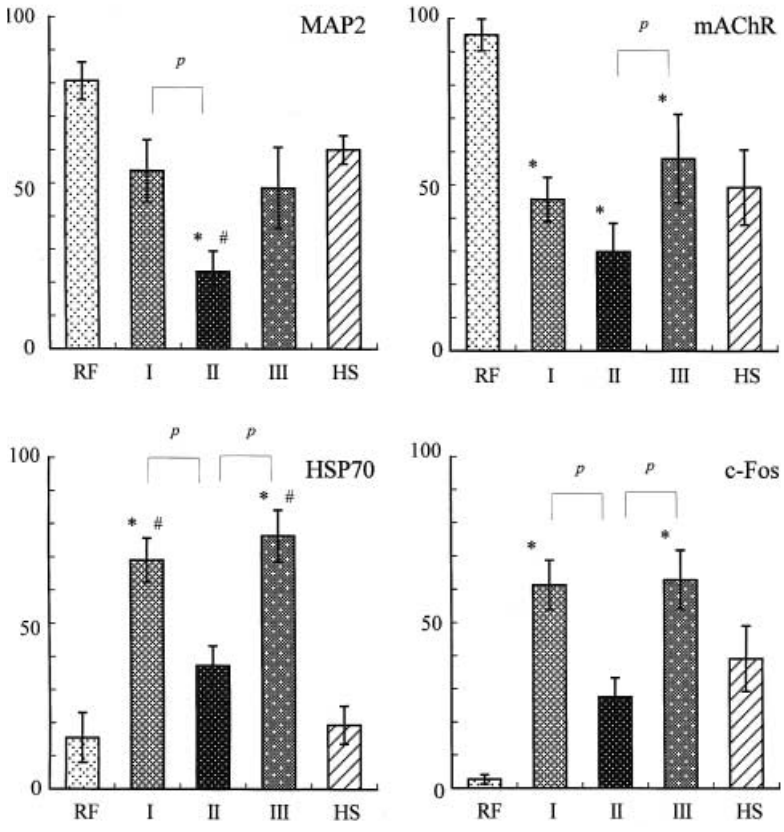

Fig. 3 Percentage of immunopositive neurons in the ARC (*I* intracranial head injury, *II* tonsillar herniation, *III* diffuse axonal injury, *HS* heat- and sunstroke, *RF* internal causes of respiratory failure, values are expressed as the mean ± standard error, significant difference against group RF *, $p < 0.05$, significant difference against group HS #, $p < 0.05$) (*p*), $p < 0.05$

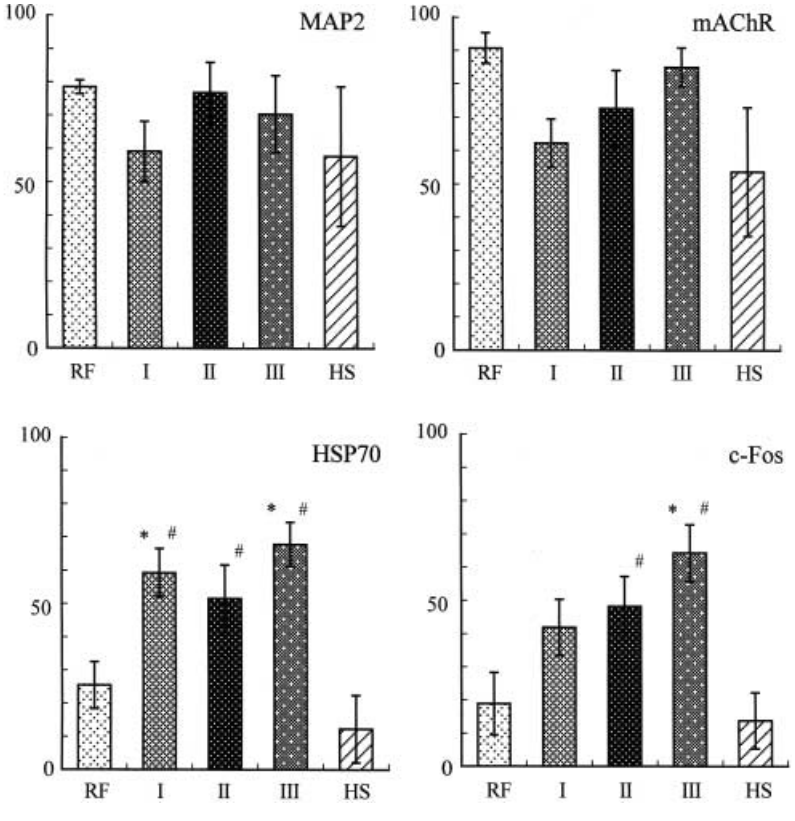

Fig. 4 Percentage of immunopositive neurons in the HN (*I* intracranial head injury, *II* tonsillar herniation, *III* diffuse axonal injury, *HS* heat- and sunstroke, *RF* internal causes of respiratory failure, values are expressed as the mean ± standard error, significant difference against group RF *, $p < 0.05$, significant difference against group HS #, $p < 0.05$)

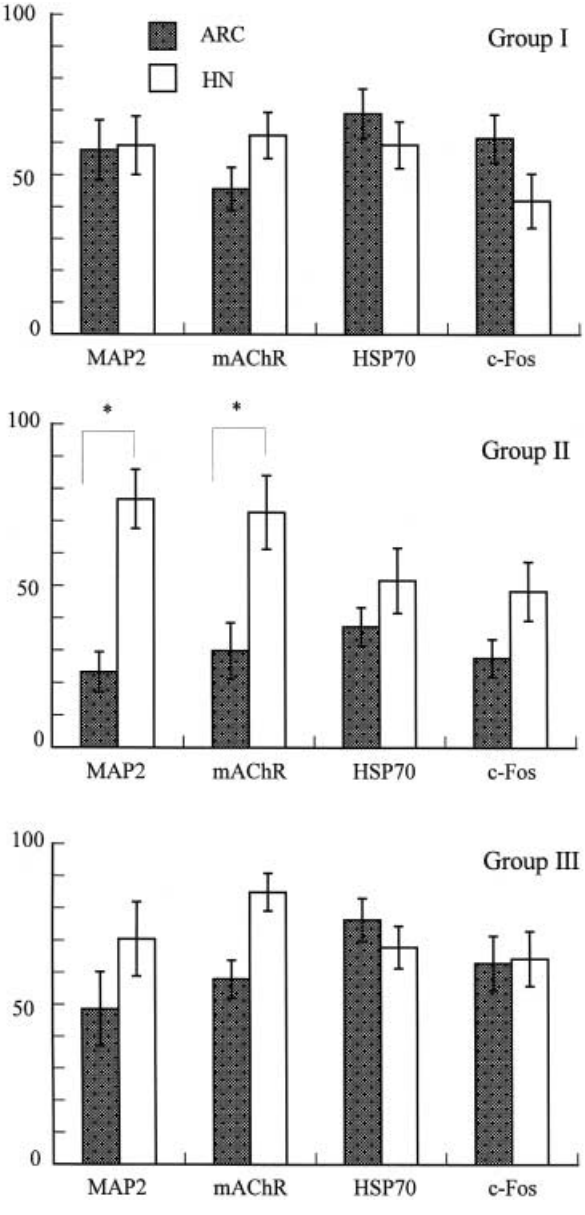

Fig. 5 Comparison of immunopositivity of the ARC with the HN dependent on head injury (*I* intracranial head injury, *II* tonsillar herniation, *III* diffuse axonal injury, values are expressed as the mean ± standard error *, $p < 0.05$)

23, 24, 25]. Like HSP70, c-Fos is considered to be a cellular marker of transcriptional activity in the stress-related circuitry [26, 27, 28, 29]. While the functional significance of HSP70 and c-Fos expression is unclear, it is evident that they are linked to events that will either promote cellular recovery or lead to cell death. Thus, HSP70 and c-Fos were employed as markers of stress- or damage-related events.

In this study, respiratory failure (group RF) and heat- and sunstroke cases (group HS) were used as the control groups (Tables 2 and 3). In group RF, neurons in both the ARC and the HN showed high immunoreactivity of MAP2 and mAChR, indicating that the cytoskeleton and/or cell membrane were not damaged. Furthermore, HSP70 and c-Fos expression was significantly lower than MAP2 or mAChR ($p < 0.001$) and it was concluded that neither nucleus was damaged in these cases. Group HS showed relatively lower immunoreactivity to MAP2 and mAChR than group RF, and HSP70 and c-Fos expression was not significantly different from MAP2 or mAChR, except for an apparent difference between MAP2- and HSP70-immunopositivity in the ARC. Heat- and sunstroke seem to induce neuronal damage in both nuclei as a result of brain stem damage.

In the head injury groups, the effect of postmortem duration was investigated but there was no correlation between the rate of immunoreactivity in neurons and the length of the postmortem period ($p > 0.05$). The percentages of immunopositive neurons in the ARC and the HN dependent on survival time were investigated, but no statistically significant changes were observed ($p > 0.05$). Therefore, in this study, we considered it unnecessary to take account of postmortem changes and survival duration. In addition, the effect of age was investigated. As immunoreactivities showed various patterns (data not shown), it was difficult to estimate the relationship between immunoreactivity and age.

Immunoreactivity in the ARC

In the ARC (Fig. 3), MAP2-immunopositivity of head injury groups was lower than control group RF and group II, especially, showed significantly lower rates ($p < 0.01$). Head injury groups showed significantly decreased mAChR-immunoreactivity compared with that of group RF (group I $p < 0.01$, group II $p < 0.001$, group III $p < 0.05$). HSP70 expression in the head injury groups except group II was increased ($p < 0.001$), and c-Fos in these two groups was also more highly expressed than in group RF ($p < 0.001$) suggesting that the neurons are damaged in head injury cases. Among head injury groups, in group II immunopositivity was decreased for each antibody. MAP2, HSP70 and c-Fos, especially, were significantly lower ($p < 0.01$). These results agreed with the findings of group HS thus suggesting that the ARC damage in tonsillar herniation was sufficiently strong to prevent HSP70 or c-Fos expression.

Immunoreactivity in the HN

In the HN (Fig. 4), MAP2- and mAChR-immunoreactivity in head injury groups were relatively lower than in group RF. HSP70 expression in head injury groups was significantly higher than that in group RF (groups I and III $p < 0.05$). In group III, c-Fos-immunoreactivity was increased compared to group RF ($p < 0.05$). HSP70 and c-Fos expression in head injury cases may be related to neuronal damage. No significant change of immunoreactivity in the HN was observed for all markers among the examined head injury groups ($p > 0.05$). However, in group III, DAI showed

slightly higher immunoreactivity of mAChR ($p = 0.073$) and c-Fos ($p = 0.095$). DAI is a type of head injury to the whole brain that results in disturbance of neurotransmission. In a previous study, we reported that in cases of DAI, HSP70 and c-Fos are expressed in pyramidal cells of the hippocampus [11], so we suspected that c-Fos expression is induced by axonal injury to the hypoglossal nerve system.

Comparison the ARC with the HN

According to the type of head injury (groups I–III), each immunopositive rate in the ARC was compared with those rates in the HN (Fig. 5). In group II, immunoreactivity to MAP2 and mAChR in the ARC was significantly lower than in the HN ($p < 0.01$). Tonsillar herniation is due to an increase in brain mass and brain edema damages neurons through the breakdown of the blood-brain-barrier (BBB). In tonsillar herniation, it was suspected that neurons are damaged both in the ARC and the HN of brain stem. From our results, however, it was considered that the ARC of group II was severely damaged. In tonsillar herniation, the brain stem is compressed and since the ARC is located in the outermost part of the brain stem, the ARC was damaged more severely (and more frequently) than the HN, which is located near the centre of the brain stem. As previously stated, the ARC was considered to be more severely damaged by tonsillar herniation than by other injuries.

In head injury, understanding the damage to the brain stem is very important. Our observations indicate that immunohistochemical examination of brain stem nuclei can provide useful information for estimating the damages to the brain stem.

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