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Axonal injury – a diagnostic tool in forensic neuropathology?[1] A review

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Abstract

We used β-amyloid precursor protein (β-APP) to investigate our own forensic neuropathological case material ($n=252$) in light of the current literature on the phenomenon ''axonal injury'' (AI) to determine the incidence, specificity and biomechanical significance of AI and its significance for determining vitality and survival time. The case material consisted of cases of fatal nonmissile closed-head injury ($n=119$), gunshot injury ($n=30$), fatal cerebral ischemia/hypoxia ($n=51$), brain death caused by mechanical trauma ($n=14$) or nonmechanical injury ($n=18$), and acute hemorrhagic shock ($n=20$). AI was observed in 65% to 100% of cases of closed-head injury, fatal cerebral ischemia/hypoxia, and brain death with a survival time of more than 3 h; AI could not be detected in the cases of acute hemorrhagic shock. A statistically significant difference between traumatically and nontraumatically induced (nondisruptive) AI was not found. There was no statistical evidence of a correlation between AI and the different types of external force, since AI could be demonstrated after both acceleration/deceleration injuries and traumatic impact. Therefore, biomechanical inferences for reconstruction purposes are not possible. On the other hand, β-APP was found to be a definite marker of vitality. In our material, cases with a posttraumatic interval of under 180 min did not express β-APP. Moreover, the literature shows

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that the posttraumatic interval can be determined by other methods for demonstration of AI such as by ubiquitin immunostaining (360 min), silver staining (15–18 h), hematoxylin and eosin staining (about 24 h), or by demonstration of a microglial reaction (about 4 to 10 days) or of a few remaining isolated bulbs, without accompanying fibers, which can be detected after a survival time of up to 17 months.

Keywords: Axonal injury; Traumatic brain injury; Vitality; Survival time; Biomechanics; Diffuse axonal injury

1. Introduction

Axonal injury (AI) has been described as a phenomenon which can be induced as a diffuse change (diffuse axonal injury=DAI) caused by traumatic brain injury [2,24,42] in addition to cerebral edema, ischemia and vascular injury [12,25]. AI can also be observed as single phenomenon in different areas of the brain. Until 1997 the forensic relevance of AI as a traumatically-induced phenomenon was all but ignored in the literature (14th Meeting of the IAFS, Tokyo, 1996, see: [7,13,25]).

We investigated our own case material in light of the recent literature on AI to determine the incidence, specificity and biomechanical significance of AI and its relevance to vitality and survival time. AI was detected by immunohistological demonstration of the β-amyloid precursor protein (β-APP), a neuronal glycoprotein conveyed by rapid anterograde transport [18,39]. This relatively new method has proved to be both extremely sensitive and highly specific [9,38]. The axonal damage involves alterations in the axoplasmic transport and focal accumulations of β-APP at the sites of injury and impaired transport [4,9].

2. Materials and methods

2.1. Cases

The case material is summarized according to age and gender in Table 1. A portion of the material was described and classified in another study (cf. [28,29]). A total of 252 brains were investigated. They were divided into the following case groups:

Total cases of *fatal closed-head injury,* including cases of nonmissile head injury (cortical hemorrhage, subdural hematoma and a combination of both [n=119]) but excluding cases of skull fracture or skull impression. The extent of primary brain injury was not quantified in the individual case.

This group was further divided into the following three subgroups:

Fatal cortical hemorrhage – without subdural hematoma (n=67), i.e. cases with indications of impact as a consequence of external forces.

Fatal subdural hematoma – without cortical hemorrhage (n=26), i.e. cases with indications of an acceleration/deceleration trauma as a consequence of external forces.

Fatal combination of cortical hemorrhage and subdural hematoma (n=26), i.e., cases

Table 1
The entire case material listed according to diagnostic group, age and sex

Diagnosis groups	Age (years)		Sex		Total
	min	max	f	m	*N*
Closed head injury (total cases)	4 m	92 y	41	78	119
Cortical hemorrhages (c.h.)	7 m	92 y	25	42	67
Subdural hematomas (s.h.)	4 m	85 y	9	17	26
Combination of c.h. and s.h.	16 y	71 y	7	19	26
Open head injury (gun shot)	10 y	83 y	6	24	30
Brain death (total)	4 m	72 y	6	26	32
Mechanical trauma	13 y	65 y	3	11	14
Nonmechanical trauma	4 m	72 y	3	15	18
Hypoxia/ischemia (total cases)	11 d	83 y	17	34	51
Hemorrhagic shock (total cases)	18 y	71 y	7	13	20
Total cases	11 d	92 y	77	175	252

with an acceleration/deceleration trauma and indications of impact as a consequence of external forces.

Gunshot injury to the brain ($n=30$): Cases of gunshot injury to the brain are generally characterized by survival periods of unknown duration. Since axon tearing usually occurs parallel to the path of the bullet in such cases [23], the β-APP expression should be particularly pronounced along the bullet's tract. In these cases, therefore, the tissue immediately surrounding the bullet's path was additionally investigated.

Brain death ($n=32$): ''Brain death'' was diagnosed clinically and always accompanied by explantation of the organs. The cases of brain death after closed-head injury due to an external force consisted of dural hematoma ($n=8$) and cortical hemorrhage ($n=6$); the cases of nonmechanically induced brain death involved cerebrovascular insufficiency and/or cutoff of the oxygen supply to the brain ($n=18$). It was therefore necessary to differentiate between two subgroups:

Brain death after *mechanically induced head injury* ($n=14$).

Brain death *without mechanically induced head injury* ($n=18$).

Fatal global cerebral ischemia/hypoxia ($n=51$): In contrast to the cases of brain death, these cases were characterized by subsequent reperfusion; as a consequence, they did not exhibit total brain necrosis.

Acute hemorrhagic shock without traumatic brain injury ($n=20$); this group should be regarded as a control group in which neither mechanical injury of the brain nor a vascular process could have influenced the brain.

2.2. Histological evaluation

Each brain was fixed in toto in 10% formaldehyde for at least two weeks prior to macroscopic examination. For routine investigation blocks of brain tissue were cut from at least eight brain regions, embedded in paraffin, and the paraffin sections subjected to a variety of stains.

Most of the present investigations were carried out on microscopic sections obtained

mainly from the rostral and dorsal parts of the pons, in most cases also from the corpus callosum and the site of injury, and in some cases from the dorsal midbrain. In addition to hematoxylin and eosin, β-APP immunostaining according to the method of Sheriff et al. [38] was performed. Each specimen was subjected to microwave treatment and incubated with the mouse monoclonal antibody against β-APP (Boehringer AG/Mannheim, clone 22 C11, diluted 1:50). The ABC method (horseradish–peroxidase, Dako GmbH, Hamburg, Germany) was used with the secondary antibody (biotinylated rabbit-anti-mouse, Dako GmbH, Hamburg, Germany, diluted 1:200). Peroxidase reactivity was demonstrated using diaminobenzidine (Boehringer AG, Mannheim, Germany) and intensified with 0.5% $CuSO_4$ for 5 min.

2.3. Grading and statistical evaluation

β-APP positivity was assumed in cases in which single axons, axon fragments or axon bulbs were repeatedly detected in one of the brain sections. Evaluation included graphic depiction of the mean percentage of cases with AI in the pons for each posttraumatic interval, together with the confidence interval. The following classification was made:

(0)	No recognizable expression
(+)	Isolated or disseminated β-APP positive axons and/or fragments or bulbs
(++)	β-APP positive axons, fragments or bulbs occur in groups, sometimes in foci, sometimes diffusely

Statistical comparison was made using the test of equality and of two percentages according to Sikal and Rohlf [40].

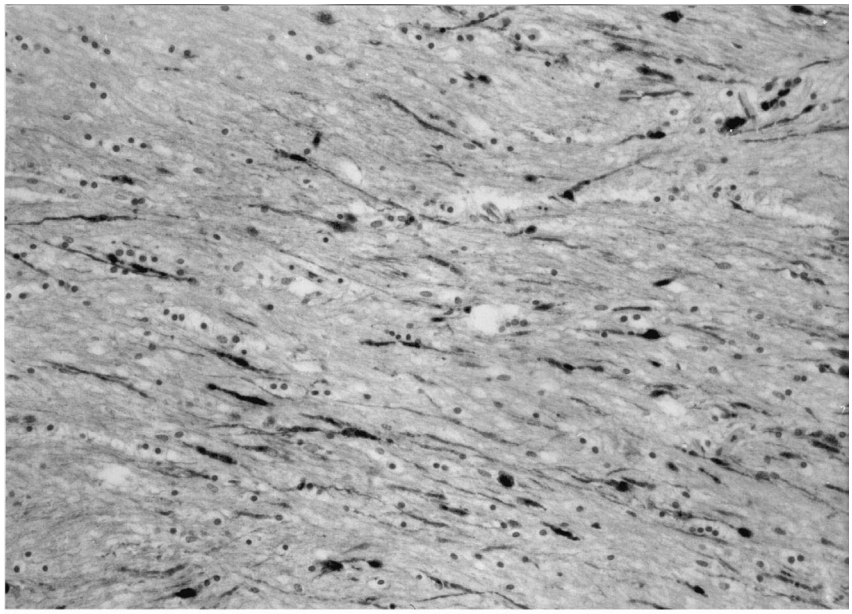
3. Observations and review of the literature

3.1. AI as a morphological phenomenon

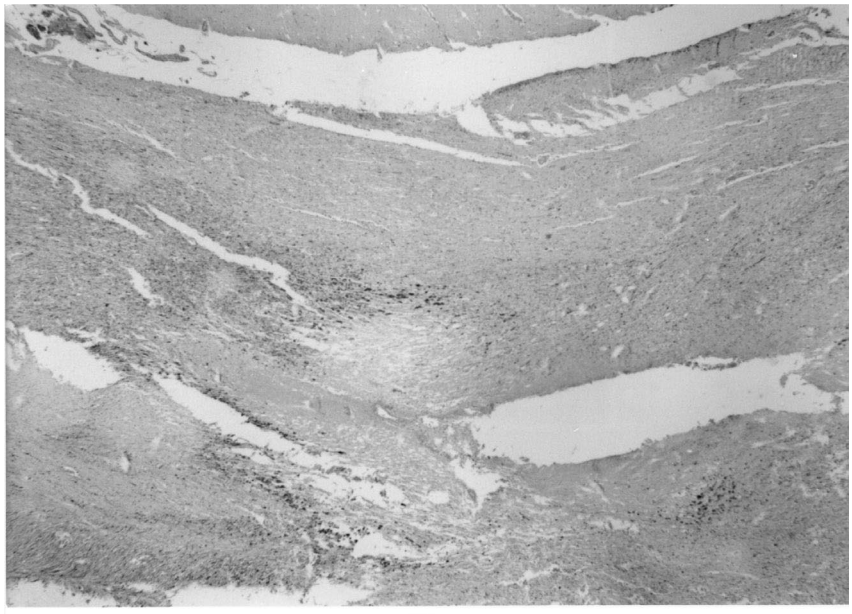
Both fibers and bulbs were found to express β-APP, sometimes disseminated and singly (Fig. 1a), sometimes in large groups (Fig. 1b, 1c), and sometimes in small, diffusely scattered groups. These alterations were mainly found on the midline structures of the whole brain, especially in the corpus callosum and the pons. In the neuropil immediately around the cortical hemorrhages, usually no – or only few – injured axons were detected. They also accumulated in brain areas exhibiting hypoxic damage or diffuse edema. Moreover, it is known that AI is seen especially in cases of local hemorrhage in the corpus callosum (cf. Fig. 2a), in the pons (cf. Fig. 2b), in the thalamic nucleus and in the hippocampal area, as well as in the context of gliding contusions (cf. Fig. 2c).

3.2. The incidence and specificity of AI

Simultaneous investigation of brain sections from different areas of the same brain, especially the sites of maximum damage and the corpus callosum, revealed that AI

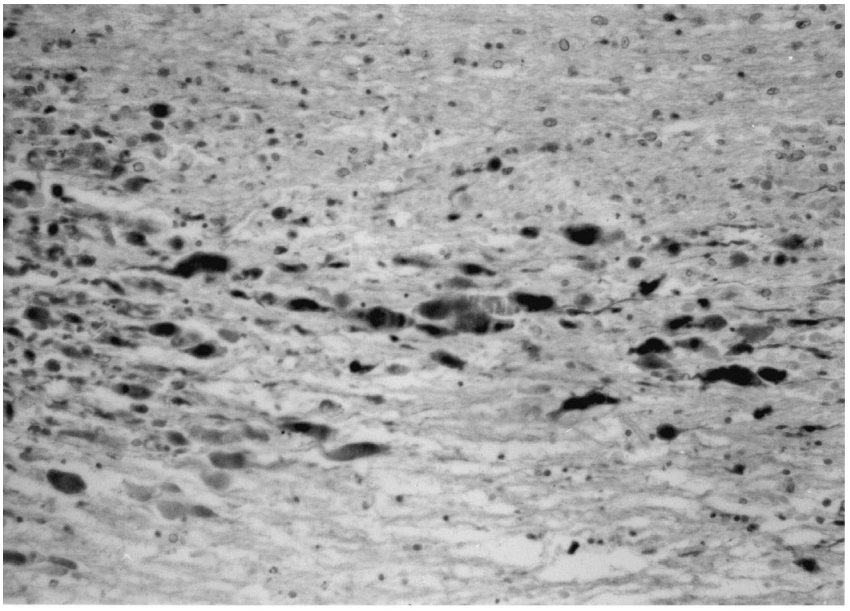


(a)



(b)

Fig. 1. Demonstration of β-APP-reactive injured axons in the corpus callosum. Disseminated and single fibers (a) and groups of bulbs (b and c) can be distinguished (β-APP, hemalum, $b-x$ 50, a and $c-x$ 300).



(c)

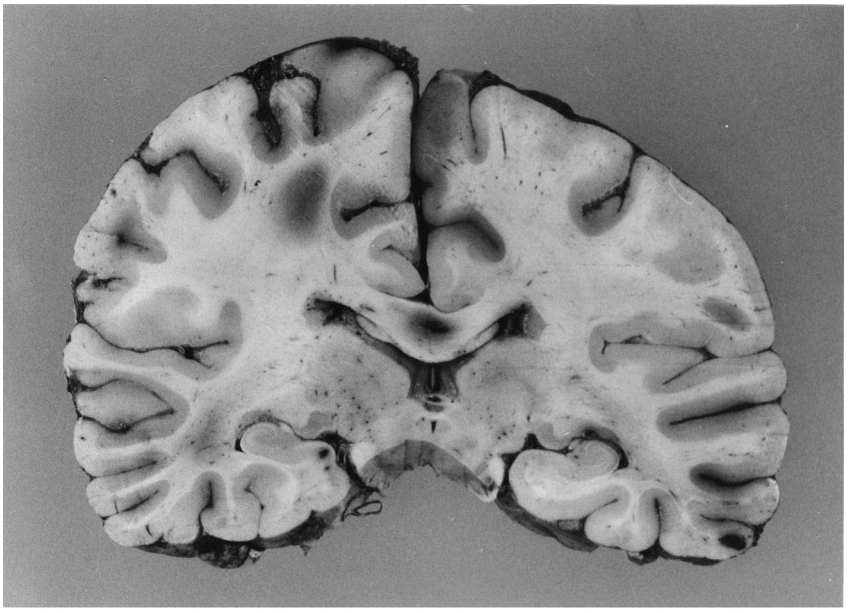
Fig. 1. (*continued*)

expression was both qualitatively and quantitatively most distinct in the pons. Although the following quantitative data pertain predominantly to the pons, they can be taken to apply to a lesser degree to other brain areas as well.

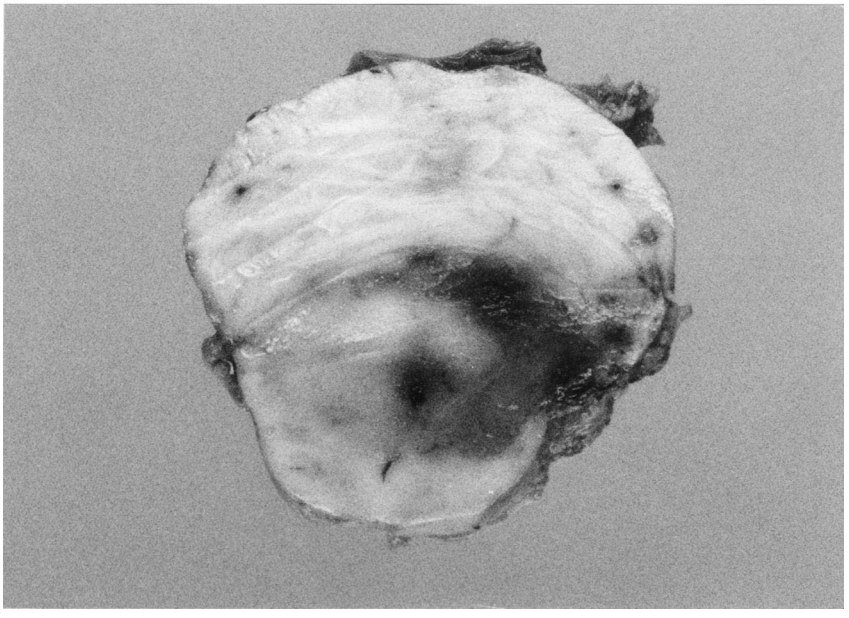
If *all cases of traumatic brain injury* are considered together, 74.5% (range: 70% to 100%) of cases expressed β-APP in the pons regardless of the type of external force (Fig. 3). No fundamental dependence on the age or sex was found; AI occurred alike in children under one year of age and in the elderly. The sole limitation to the detection of AI was the interval between the traumatic event and time of death: AI could not be detected until 3 h after traumatization. Moreover, after 3 h the expression tended rather to rise with increasing survival time. Of the 102 cases surviving for 3 h or more, a total of 84.3% exhibited AI in the pons.

Similar findings were made in cases of *fatal cerebral ischemia/hypoxia* (for a diagnostic differentiation of these cases – see [28]), though a differentiation between the sequelae of hypoxic/ischemic effect and brain swelling was not possible. AI was seen frequently in this group (Fig. 4), although it was present in only 30% of the entire case material. In cases with a survival time exceeding 3 h, the mean percentage of positive cases rose to 64%. No significant difference was evaluated between the traumatic and ischemic/hypoxic groups.

In cases of *intravital brain death* (for diagnostic differentiation of these cases – see [28]) the overall percentage of AI-positive cases (Fig. 5) was nearly 80%, regardless of whether brain death was induced mechanically or nonmechanically. This high incidence

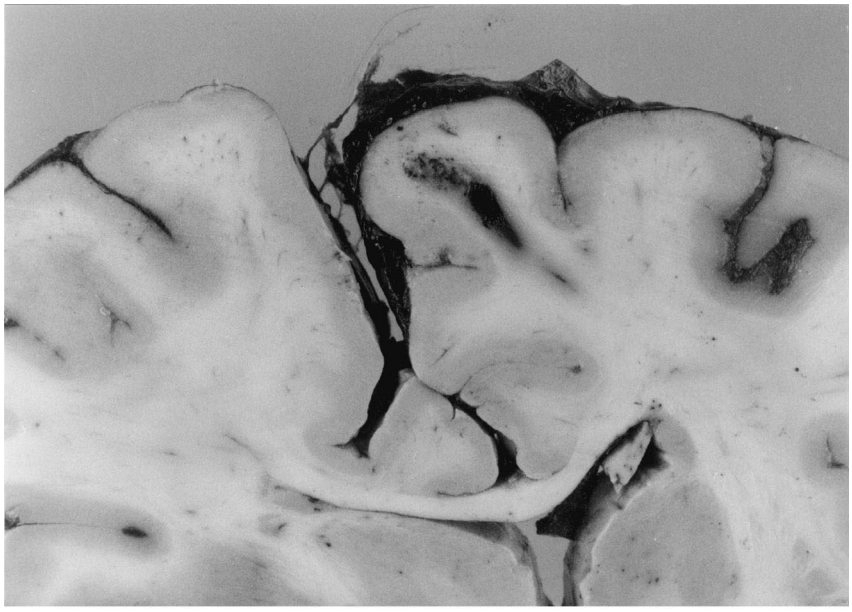


(a)



(b)

Fig. 2. Demonstration of macroscopic alterations accompanying β-APP-reactive axons: Focal hemorrhages in the corpus callosum (a), in the pons (b) as well as in the context of gliding contusions (c).



(c)

Fig. 2. (*continued*)

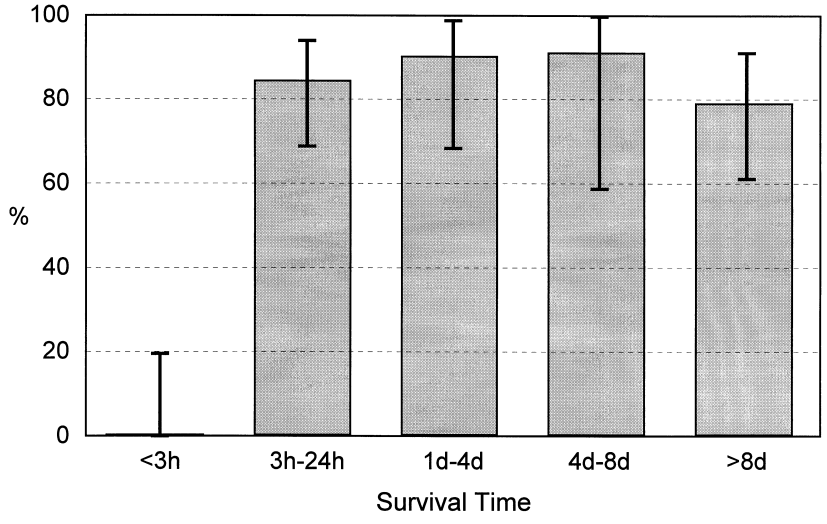


Fig. 3. Incidence of AI as demonstrated by β-APP positivity in the pons of traumatic brain injury cases ($n=119$); percentage of cases expressing AI, including the confidence interval.

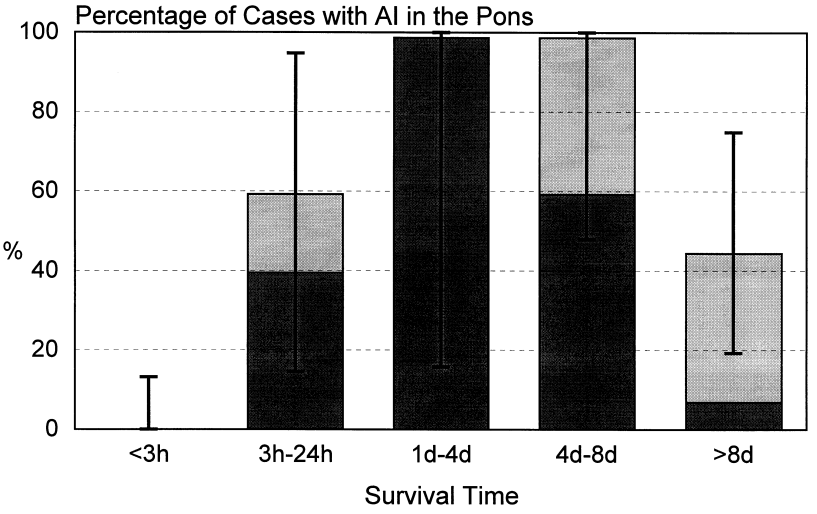


Fig. 4. Cases of fatal cerebral ischemia/hypoxia ($n=51$); percentage of cases expressing AI in the pons, including the confidence interval. The whole column represents the mean percentage of all cases expressing AI (+ and ++); the dark part of the column shows the percentage of cases with distinct AI expression (++).

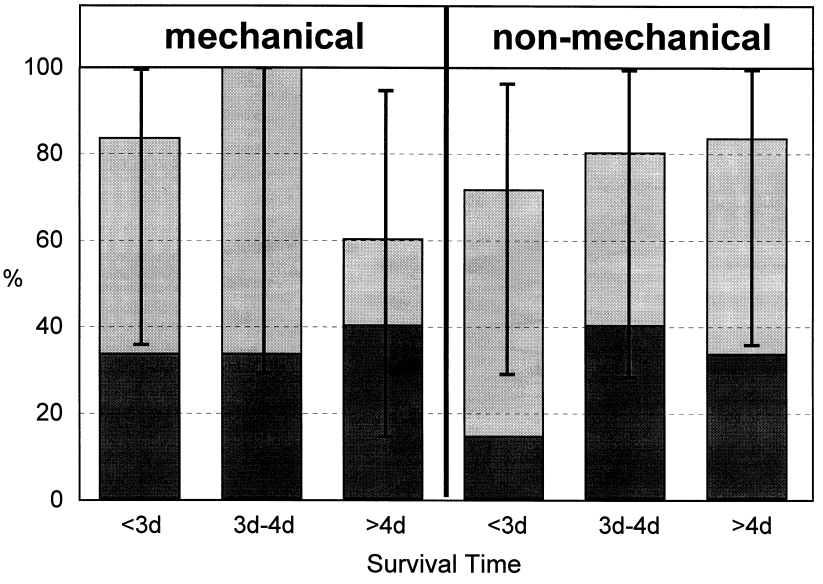


Fig. 5. Percentage of brain death cases imunoreactive to β-APP in axons of the pons: Cases of mechanically-induced brain death (left group of columns), for example by subdural hematoma, are differentiated from cases of nonmechanically-induced brain death (right group of columns), e.g. total brain ischemia. The percentages of cases whose axons show distinct (black parts of columns) or slight (grey parts of columns) β-APP reactivity are shown, as well as the confidence intervals.

may be attributed to the fact that all of these cases had a confirmed survival time exceeding 3 h.

A principal exception was the group of cases of *fatal gunshot injury* to the brain. β-APP could be demonstrated in only 2 of these 30 cases. It must be pointed out, though, that the survival time in this group was almost always considerably less than 3 h, too short for AI to develop. Only the two cases with posttraumatic survival exceeding 3 h exhibited AI in the cerebrum and in the pons.

AI is thought to represent an universal consequence of fatal closed-head injury [10,24,29]. It has been demonstrated 24 h after traumatic event in 90% of such cases [12,20], with peak axonal damage occurring between 10 h and 15 h post injury. Gentleman and coworkers [10] mention a more precise figure of 92% for cases with evidence of bulb formation.

In summary it can be said that β-APP can be demonstrated in the pons after various types of cerebral injury. If the survival time exceeds 3 h, the mean incidences in all diagnostic groups ranged from 60 to more than 90%. It is striking, however, that both systemic ischemia/hypoxia and brain death are assumed to exhibit diffuse processes, whereas mechanical brain injury generally gives rise to local injury. Such local injuries apparently are combined with diffuse changes in the brain which would account for the presence of AI, in the pons in about 80% of the traumatized cases.

The literature contains ample data on the incidence of AI after mechanical brain injury, but little information on its prevalence in cases of ischemia/hypoxia [11,16,31] and none on cases of brain death or gunshot injury. Moreover, other authors have sought to demonstrate AI in all regions of the brain, especially the brain stem and neurogenous tissue surrounding hemorrhages, whereas we focused primarily on the corpus callosum and the pons.

This nonspecificity is understandable in light of the molecular processes leading to the development of AI (cf. [8,33–36]): Mechanical traumatization and ischemia disrupt the neuronal cytoskeleton both by proteolysis of its components and by affecting kinase and phosphatase activities that alter its assembly and are associated with long-lasting membrane leakage and impaired axoplasmic transport. As a consequence, transported materials accumulate at sites of maximal cytoskeletal damage and the axons undergo secondary axotomy.

Meanwhile there exists a consensus regarding the interpretation of reactive or secondary or nondisruptive AI linked to perturbation of axolemma and resulting in disruption of ionic homeostatic mechanisms within the injured nerve fiber [15,19]: There is a loss of axonal microtubules over a period up to 24 h after injury, possibly mediated by a posttraumatic influx of calcium and activation of calmodulin.

3.3. Blood circulation as the precondition of AI

The interval between the traumatic event and circulatory arrest, i.e. the duration of blood circulation in the brain after injury, is known as the survival time. In our study AI could be demonstrated only after 3 h survival time at the earliest – regardless of whether the injury was caused by mechanical trauma, systemic ischemia/hypoxia or intravital brain death. According to these observations AI represents a vital reaction.

The literature on β-APP immunoreactivity as a marker of AI reports even briefer intervals than 3 h: Gentleman and coworkers ([10]; see also [11,20]) describe an interval of 120 min, while Blumbergs et al. [4] observed β-APP after only 105 min. In a systematic study McKenzie et al. [20] first detected β-APP reactive fibers after 2 h survival, axonal bulbs after 3 h survival, and determined that the amount of axonal damage and axonal bulb formation increased in proportion to the length of survival time.

The survival time can be further delimited by utilizing an antibody targeted to ubiquitin, as demonstrated in injured cats: AI could be demonstrated 360 min after injury [37]. Axon bulbs were detected 15 to 18 h after traumatization using the silver staining technique [1,12] and 24 h postinjury using hematoxylin and eosin [38]. Antibodies targeted to the 68 kDa subunit of early neurofilaments showed immunoreactivity within 60 min after mechanical brain injury.

The literature further shows that over time the bulbs become increasingly surrounded by microglial cells and phagocytosed [5]. The demonstration of clusters of microglial cells around individual bulbs may represent an additional aid in determining the vitality of mechanical brain injury. In our own material, such microglial clusters were not present, but a diffuse microglial reaction which included macrophages incorporating β-APP reactive material was evident within 4 to 10 days after the traumatic event.

After 30 days of survival, β-APP immunoreactive fibers had almost disappeared and only axonal bulbs remained, suggesting the existence of a temporal limit. However, axonal bulbs in the pons have been demonstrated in single cases up to 80 days [30] and even up to 17 months after trauma [28]. These observations were made, though, in only a few cases in which the maximum posttraumatic survival time for demonstration of bulbs had not been reached.

A summary of these findings produces the relations depicted in Table 2: The various techniques described here reveal a temporal pattern that enables the determination of survival-time intervals based on the demonstration of AI.

3.4. Biomechanical pathogenesis of AI

As indicated by the various experimental models capable of inducing AI, acceleration and/or deceleration of the head are the chief causes of AI [2,27,29,41]. AI appears to only rarely result from direct blows (for review, [14]). A direct blow to the head is more likely to cause an extra axial collection, whereas acceleration/deceleration injuries more frequently produce diffuse cerebral damage [2,22].

If pure subdural hematomas are mainly caused by acceleration/deceleration mechanisms but cortical hemorrhages by direct impact [32], then it should be possible to demonstrate clear differences in the β-APP reactivity between these two types of injury. A comparison of the two groups (Fig. 6), however, disclosed no fundamental quantitative or qualitative differences, as was statistically confirmed elsewhere [29]. Moreover, comparison of cases of traumatically and nontraumatically induced brain death (Fig. 5) revealed no statistically significant difference (see above).

AI as an isolated local phenomenon with various causes must be distinguished from ‘‘diffuse axonal injury’’ (DAI). AI represents a diagnostic entity chiefly observed after a specific type of traumatizing external force: Demonstration of AI is confined mainly to

Table 2
Time dependency of AI demonstration in paraffin-embedded tissue of the pons

Phenomenon/technique	First expression of β-APP (interval after trauma)	Author(s)
Axonal bulbs and fibers		
68 kD	60 min	[44]
β-APP	105 min	[4]
	120 min	[10]
	180 min	[38]
ubiquitin (in a cat model)	<360 min	[37]
Axonal bulbs		
silver staining	15–18 h	[1]
		[13]
hematoxylin and eosin	24 h	[38]
Microglia clusters	15 h	[32]
around axonal bulbs	24–48 h	[21]
		[43]
Myelin degeneration		
Marchi method	30–60 d	[42]
Axonal bulbs		
maximal survival time	4 weeks	[4]
	3 months	[21]
	17 months	[27]

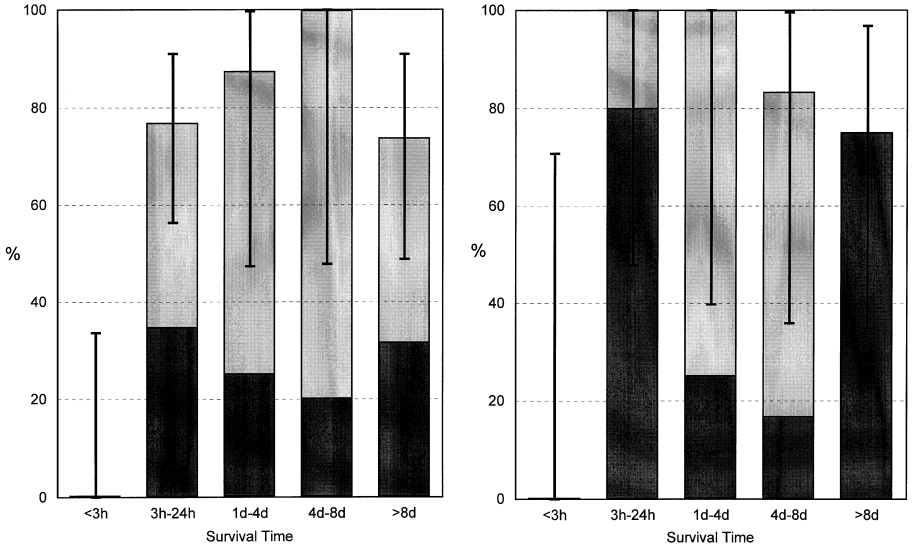


Fig. 6. Incidence of AI as demonstrated by β-APP reactivity in the pons of cases of fatal cortical hemorrhage (n=67 – left group of columns) versus that in cases of fatal subdural hematoma without cortical hemorrhage (n=26 – right group of columns); percentage of cases expressing DAI, including the confidence interval. The whole column represents the mean percentage of all cases expressing AI (+ and ++); the dark part of the column shows the percentage of cases with distinct AI expression (++).

midline structures, the corpus callosum and pons, as well as the paramedial basal ganglia [14].

The literature (for review, [14]) shows that rotatory acceleration is especially capable of inducing DAI; it affirms further that an impact time of 20–25 ms causes DAI, whereas an impact time of 5–10 ms is more likely to produce a subdural hematoma.

These observations show that biomechanical inferences regarding the nature of the traumatizing event cannot be based on the presence of AI in the pons alone, but obviously on DAI in the whole brain. This view is also supported by analysis of the pathogenesis of AI (see above): The type of external force alone is not responsible for AI, but there must be an additional local disturbance of metabolism which requires the occurrence of ischemia/hypoxia and possibly even edema – also in mechanical brain injury. The cumulative effect, moreover, is probably responsible for the secondary events. But since edema and ischemia/hypoxia are always a possible phenomenon in mechanical brain injury survived for at least 3 h, no final biomechanical conclusions can be drawn.

3.5. Clinical correlation

Until now only a few studies have correlated DAI – but not AI – with clinical findings. Katz and Alexander [17] determined that in mild DAI unconsciousness lasts for seconds to minutes (if at all), confusion and posttraumatic amnesia for minutes to hours, and residual neurological dysfunction for days to several months. In more severe DAI there may be days to weeks of unconsciousness, weeks to months of confusion and posttraumatic amnesia, and months to years of residual dysfunction. In the most severe DAI cases, the patient remains in the persistent vegetative state [21]. These observations accord with those of other authors [3,8]. A summary of the observations of Adams and coworkers [3] produces the correlations shown in Table 3. Because DAI can also be demonstrated intravitally using computer and magnetic resonance tomography [6], there is reason to hope that better information on the clinical correlations will soon become available.

Table 3
The results of an analysis of the cases of DAI in the Glasgow data base by [3]

DAI is often combined with
- a road traffic accident
- a fall from a height
- gliding contusions
- small intracerebral hematomas (deep in the hemispheres and the Ammon's horn)
DAI is seldom combined with
- a lucid interval
- a injury to fall (or assault)
- skull fructures
- cerebral contusions
- high intracerebral pressure

Table 4
Summary of the forensic significance of AI

1.	β-App is a highly specific marker of axonal injury (AI).
2.	β-APP expression is an indicator of an intact circulation during the process of axonal injury (vitality).
3.	The estimation of the survival time is possible by the application of different histological techniques (time dependency).
4.	Physical and metabolic processes can induce focal β-APP expression (non specificity).
5.	The phenomenon of diffuse axonal injury (DAI), i.e. a simultaneous demonstration of AI in the corpus callosum and the pons, is an indicator of a physical trauma (specificity).
6.	A rotational accelleration can induce DAI (biomechanical background).

4. General discussion

Taken together, the findings in the literature and our own observations provide a body of information that can be of forensic relevance in cases with verifiable AI (Table 4). The demonstration of β-APP is useful for the detection of injured axons as well as for establishing vitality, especially in cases without hemorrhages. Since AI was present in pons in 70% to 100% of our cases of traumatic brain injury and in about 65% of cases of ischemia/hypoxia-induced brain injury, its demonstration obviously can be used to distinguish intravital from postmortem injury.

It may also be possible to estimate the age of an injury based on the few phenomena whose demonstration depends on the techniques with which AI is expressed. These time-dependent phenomena are classified in a scheme published elsewhere [26,29]. Appropriate studies are lacking which would, for example, enable determination of how early microglial reactions form, when the first signs of Wallerian degeneration appear, or how long the period of time is during which axon bulbs can be demonstrated possibly even beyond the currently known period of 17 months.

It can be further stated that demonstration of AI using β-APP is highly sensitive, but by no means specific for a particular type of injury. In our material, AI occurred after mechanical traumatization and after ischemic-hypoxic injury. This finding is of fundamental importance, since it shows that AI cannot be assumed to result from mechanical injury alone.

At the same time, though, a specific biomechanical force can be excluded as the sole cause of AI. Since AI can only be detected by β-APP after a posttraumatic survival of about 3 h, it cannot be ruled out that intervening additional ischemic-hypoxic injury or edema has produced the secondary or reactive, nondisruptive type of AI. Therefore, AI cannot be interpreted as indicating a rotatory acceleration/deceleration event.

By the same token, it is extremely difficult to establish a clear correlation between the extent and distribution of AI in the brain and the underlying biomechanical process and clinical picture. As mentioned above, correlations do apparently exist; they are, however, loose and do not allow definite conclusions regarding the neurological picture based on morphology. The simultaneous demonstration of AI in the corpus callosum and pons must therefore be regarded as evidence of DAI, a phenomenon that until now was thought to be traumatically induced. The putative cause of DAI is a lateral rotatory acceleration with an impact time of about 20–25 ms.

The forensic significance of AI cannot be doubted. Because AI can be demonstrated even in paraffin sections using monoclonal antibodies against the epitope β-APP and since this method targets only injured-isolated axons, its use is indicated to answer any one of the many forensic-neuropathological questions dealt with in this review.

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