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Alterations of acetylcholinesterase activity after traumatic brain injury in rats

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Abstract

Objective: The cholinergic system is highly vulnerable to traumatic brain injury (TBI). However, limited information is available to what extent the degrading enzyme acetylcholinesterase (AChE) is involved. The present study addresses this question.

Method: Thirty-six anaesthetized Sprague-Dawley rats were subjected to sham operation or to TBI using controlled cortical impact (CCI). The AChE activity was histochemically determined in frozen brain slices at 2, 24 and 72 hours after TBI.

Results: High enzyme activity was observed in regions rich in cholinergic innervation such as the olfactory tubercle, basal forebrain, putamen and superior colliculi. Low activity was found in the cortex, cerebellum and particularly in the white matter. A decrease of AchE activity (20–35%) was found in the hippocampus and hypothalamus already at 2 hours after TBI. An increase of ~30% was found in the basal forebrain at 2 and 24 hours. No changes occurred at 72 hours.

Conclusion: The findings are consistent with impairment of the cholinergic neurotransmission after TBI and suggest the involvement of the AChE in short-term regulatory mechanisms.

Keywords: *Traumatic brain injury, acetylcholinesterase, cholinergic system, controlled cortical impact, histochemistry, rat*

Introduction

Traumatic brain injury (TBI) is the leading cause of death and permanent neurological disabilities in children and young adults [1, 2] and is also regarded as an important risk factor for non-familiar Alzheimer's disease (AD) [3, 4]. Like AD, TBI is accompanied by reductions in cholinergic functions [5], partially reflected by decreased availability of acetylcholine in the hippocampus [6–8]. The cholinergic pathway has been implicated in the processes of cognition, arousal and attention. Therefore, acute and chronic changes of this pathway, in particular in the hippocampus and related systems, may contribute to the cognitive deficits seen following TBI [9, 10]. In this regard, TBI is also closely related to AD [11] and for both diseases, a cholinergic hypothesis has been established.

The acetylcholinesterase (AChE) is a fundamental part of the cholinergic signalling, catalysing the hydrolysis of acetylcholine and thereby terminating the synaptic transmission. Beyond this classical function, AChE displays multiple, unrelated biological functions involved in embryogenesis, neuromodulation and stress response. This is attributed to diverse molecular forms, which are generated from a single gene by alternative promoter choices and splicing [12]. An involvement of AChE is also suggested for AD. In an AD patient study with cholinesterase inhibitors, non-treated subjects

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showed a decreased expression of both the monomeric readthrough AChE (R-AChE) and tetrameric synaptic membrane-bound AChE (S-AChE) [13]. Patients treated with AChE inhibitors showed a marked increase of expression.

There is evidence that stress-related events after TBI alter the expression of the R-AChE and experimental attempts in rats and mice have been made to evaluate blockade of AChE as a potential therapeutic approach [14–16]. However, the effects of TBI on the S-AChE have not been studied. The present study was therefore designed to determine the effect of a controlled cortical impact (CCI) in rats on the S-AChE expression in various brain regions and at various time points after the injury.

Methods

Subjects and procedure

All procedures involving animals were carried out with the approval of the institutional animal ethics committee and following national regulations for animal research. Thirty-six male Sprague-Dawley (250–350 g) rats (University of Leipzig in-house breeding) were randomly divided into three groups with different survival times (2, 24 and 72 hours post-injury) and subjected to TBI ($n = 18$) or sham procedure. Rats were housed in a temperature- and humidity-controlled room with a 12 hours dark–12 hours light cycle. They had free access to food and water until the time of the study.

Controlled cortical impact

The model and procedures used are based on a previous study [17], only differing in anaesthesia. Briefly, rats were anaesthetized i.m. with a mixture of fentanyl ($0.005\,mg\,kg^{-1}$, Janssen, Germany), midazolam ($2\,mg\,kg^{-1}$, Ratiopharm, Germany), medetomidin ($0.15\,mg\,kg^{-1}$, Pfizer, Germany) and placed into a stereotactic device. Normal body temperature (37°C) was maintained with a heating pad during surgery until CCI administration. A midline incision was made in the skin and underlying fascia. A unilateral circular craniotomy (~6.0 mm) was performed. The craniotomy was centred over the motor cortex (3.5 mm posterior, +4.0 mm lateral to bregma). Sham-operated animals underwent the identical surgical procedure; however, like in previous studies [18], received no craniotomy or CCI. Because craniotomy is expected to cause minimal trauma [19], surgery is even occasionally avoided in control animals [20, 21].

A contusion injury was performed with a commercially available device (Custom Design & Fabrication, Virginia Commonwealth University, Richmond, VA) with a round stainless steel impactor (5.0 mm diameter) attached to an electromagnetically driven piston. The impactor was accelerated ($4\,m\,s^{-1}$), with a cortical contact time of 100 ms and 2.0 mm impact depth, causing a moderate trauma. Following the contusion, any bleeding was controlled with sterile sponges soaked in cold saline and the craniotomy was closed using the skull fragment and dental cement (TechnoVit® 3040, Kulzer, Germany). The incision was sewed with nylon suture material and the wound was treated with a disinfectant (BetaIsodona solution, Mundipharm Germany). An analgesic was administered (metamizol $0.2\,mg\,kg^{-1}$ i.m., Ratiopharm, Germany). The animals of the 2 hour group did not receive analgesics and were kept in anaesthesia until decapitation. Their body temperature was maintained with a heating unit. All other animals were antagonized with a mixture of naloxon ($0.12\,mg\,kg^{-1}$, Ratiopharm, Germany), flumazenil ($0.2\,mg\,kg^{-1}$, Roche, Germany) and atipamezol ($0.75\,mg\,kg^{-1}$, Pfizer, Germany) injected subcutaneously. Animals returned to their home cage with free access to water (with 0.2 ml metamizol and 5% glucose) and food. Before decapitation, rats were lightly anaesthetized with isofluran (5%, Baxter, Germany). The brain was quickly removed and the hemispheres were separated along the midline. They were then immersed in −35°C 2-methylbutan and frozen for at least 30 seconds. The hemispheres were stored until further use at −55°C.

Histochemistry

All chemicals were purchased from Sigma-Aldrich (Germany) if not otherwise stated.

From each group, six animals were randomly chosen. The frozen traumatized, respectively sham-operated, hemispheres were embedded in Tissue-Tek® (Sakura, VWR Germany). Sagittal sections (12 µm) were cut with a cryostat microtome (MICROM, Walldorf, Germany). The slice coordinates did not exceeded 1.2 mm lateral to the midline. The slices were then mounted onto glass slides, with a total of three sections per slide, dried at room temperature and stored at −25°C for at least 3 days as described in the original method [22] as well as by other authors [23, 24].

For each animal, one slide (three sections) was chosen which preferably matched all others in plane and analysed. Contralateral hemispheres were not investigated.

The method for histochemical detection of the AChE was based on Andrä and Lojda [22], with slight modifications. Briefly, the frozen sagittal slices were dried at room temperature. The slides were then pre-incubated for 30 minutes with 0.1 m Tris-maleate buffer (pH 5.0) at 37°C, followed by

incubation at 37°C for 90 minutes in reaction solution, consisting of 50 mg acetylcholine iodide, 0.1 m Tris-maleate buffer (pH 5.0), 0.4 m sodium citrate, 0.12 m copper sulphate and 0.16 m potassium ferricyanide. The reaction was stopped by removing the solution and washing with 0.1 m Tris-maleate buffer. The slides were dried using an ascending series of ethanol, 5 minutes in 40%, 70% and 96% and 2.5 minutes in absolute EtOH. Finally, the sections were rinsed twice in xylol, air-dried and mounted in Entellan.

Data analysis

Microdensitometric measurements were carried out using a CCD-camera (Nikon, Japan) and a densitometric software (MCID-M4, Imaging Research, Canada). Mean values of optical densities ±SD were calculated, background-corrected and tested for significance ($p < 0.05$) using the two-tailed *t*-test.

Results

The distribution of the AChE in sagittal slices of the rat brain at 2 and 24 hours after sham operation (A, C) and trauma (B, D) is shown in Figure 1. The slices were processed with AChE substrate under identical conditions. Hence, the densitometric measure of the AChE reaction product is directly related to enzyme activity. Cresyl violet stained slices after sham operation (E) and trauma (F) are shown for regional identification. In both groups, high

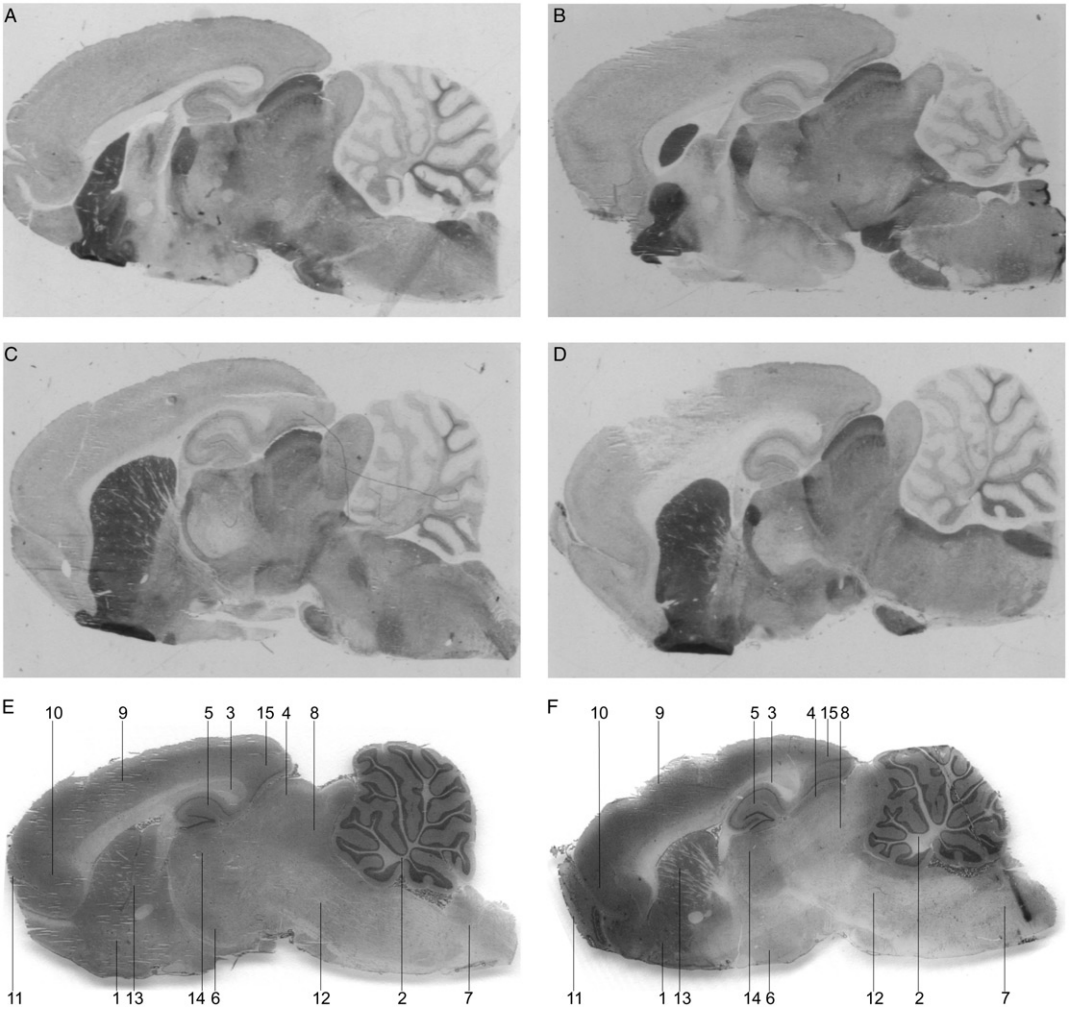

Figure 1. AChE-staining of the rat brain. Photomicrograph showing the regional distribution of the AChE at 2 and 24 hours in a sham-operated (A, C) and a CCI animal (B, D). Additionally cresyl-violet stained sections after sham operation (E) and trauma (F) are provided for regional identification. Regions of interest are labelled with the following numbers. (1) Basal forebrain (accumbens nucleus), (2) Cerebellum, (3) Corpus callosum, (4) Colliculus superior (superficial grey layer), (5) Hippocampus, (6) Hypothalamus (ventromedial hypothalamic nucleus), (7) Medulla (medullary reticular nucleus), (8) midbrain (periaqueductal grey), (9) motor cortex, (10) orbital cortex, (11) olfactory bulb, (12) Pons (pontine reticular nucleus), (13) Caudate Putamen, (14) Thalamus (mediodorsal thalamic nucleus), (15) visual cortex.

enzyme activity (in terms of optical density) was observed in regions rich in cholinergic transmission such as the olfactory tubercle, basal forebrain (medial septal nucleus including vertical and horizontal limbs of the diagonal band of Broca, magnocellular preoptic nucleus, substantia innominata and basal nucleus of Meynert, ventral pallidum), putamen and superior colliculi. Low activity was found in the cortex, cerebellum and particularly in the white matter. This distribution pattern is in concordance with previous findings in rodents [24] and humans [25]. Because of the modification of the original method [22], i.e. omitting the semi-permeable membranes, primarily membrane bound S-AChE was detected, which accounts for more than 80% of the total AChE activity in most human brain regions, except the cortex [25].

Table I shows the relative enzyme activities in 15 brain regions of sham-operated and traumatized animals at 2, 24 and 72 hours post-injury. The values are normalized to the Corpus callosum (=100%).

AChE activity within the sham-operated animals was not significantly altered. However, already 2 hours post-trauma, significant alterations of the AChE activity were found, which were still present after 24 hours, returning to control values at 72 hours. Interestingly, the mode of the alterations was different in the various brain regions. While in most regions no significant changes were observed in both groups, only the basal forebrain of brain-injured animals showed an increase of the AChE activity compared to sham-operated animals. Unlike in sham-operated animals, a post-traumatic decrease was found in the hippocampus and the hypothalamus, i.e. in regions receiving cholinergic input from the basal forebrain [26].

Discussion

The controlled cortical impact model was established in 1988 for ferrets [27] and later adapted to rats [28]. It has been proven to be a suitable tool for the research of focal TBI. The model can induce cognitive and motor impairments [29, 30]. Although behavioural testing was not performed in this study, changes in AChE activity may be involved in the cognitive changes following TBI. Cell losses in the hippocampus, hypothalamus and other regions after TBI have been described in experimental animal models and humans [16, 31–34] and are considered to cause cognitive deficits [16, 31, 35, 36]. The data demonstrate that the enzyme AChE is involved in the sequel of events which follow TBI. They provide further evidence for an impairment of the cholinergic system as it has been previously described with regard to choline uptake and the release and receptor binding of acetylcholine [5–8]. Even mild trauma, which was caused solely by craniotomy, elicited similar effects as TBI (although of lesser magnitude) on acetylcholine and choline dynamics [19]. Therefore, one has abstained from craniotomy in this study. Earlier studies, performed in rats or mice, using closed head injury [14–16] did not investigate alterations of the classical membrane-bound S-AChE activity as done in this study. Instead, they were focusing on total AChE activity [14, 15] or transcripts of the soluble R-AChE [16],

Table I. Relative AChE enzyme activity in various brain regions at different time points after CCI in the rat.

	2 hours post-injury, range (SD)		24 hours post-injury, range (SD)		72 hours post-injury, range (SD)	
Region	Sham	TBI	Sham	TBI	Sham	TBI
Olfactory bulb	193 (6.46)	172 (3.63)	178 (46.1)	174 (16.7)	214 (80.4)	200 (49.9)
Basal forebrain	769 (136)	1025 (102)**	801 (146)	1057 (138)**	978 (167)	895 (136)
Putamen[1]	723 (348)	623 (235)	546 (262)	832 (76.9)	773 (61.5)	645 (241)
Corpus callosum	100	100	100	100	100	100
Orbital cortex	181 (33.1)	197 (29.5)	187 (22.3)	196 (48.2)	198 (32.2)	210 (29.9)
Motor cortex	152 (28.7)	182 (60.5)	190 (20.6)	155 (22.9)*	191 (24.8)	187 (36.9)
Visual cortex	201 (15.1)	190 (94.8)	209 (49.2)	193 (43.1)	245 (58.6)	223 (55.8)
Hippocampus	279 (31.7)	230 (24.5)*	255 (16.5)	229 (16.4)*	269 (39.4)	286 (51.1)
Thalamus	195 (48.0)	188 (35.3)	210 (45.9)	189 (15.1)	210 (38.5)	219 (21.9)
Hypothalamus	215 (43.9)	141 (25.1)**	167 (40.1)	190 (42.8)	166 (33.1)	188 (26.7)
Colliculus superior	519 (46.0)	655 (77.2)	603 (48.1)	635 (35.2)	616 (75.5)	671 (70.7)
Midbrain	376 (42.6)	359 (38.8)	367 (49.5)	370 (24.2)	425 (33.4)	448 (66.2)
Pons	307 (79.7)	354 (80.3)	360 (49.0)	410 (48.0)	368 (114)	376 (79.8)
Cerebellum	226 (43.1)	186 (49.1)	193 (57.1)	186 (42.7)	198 (35.3)	230 (28.1)
Medulla	275 (24.5)	303 (33.8)	274 (47.4)	261 (94.2)	282 (52.3)	294 (65.7)

Values are expressed as mean percentages ±SD normalized to Corpus callosum, $n = 6$, $*p < 0.05$, $**p < 0.025$, significantly different compared with sham-operated animals.
[1] $n = 3$.

which are not necessarily translated into active enzymes [37, 38]. Also in these studies the enzyme activities were measured biochemically using homogenate assays which did not allow a precise identification of regional alterations, especially of small regions, as performed in this study. A previous investigation has shown that histochemical data correspond well with data from biochemical assays of AChE activity and allow detailed studies, e.g. of toxicological influences, in multiple microscopic regions of the rat brain [23]. A limitation of this method is that it cannot precisely differentiate between the two forms of AChE as it is possible to with RT-PCR [37, 39].

The post-traumatic decrease of hippocampal and hypothalamic AChE activity may serve as a very early indicator of neuronal degradation because the most striking changes in AChE activity were already found at 2 hours after TBI. An increase in AChE activity after CCI was observed in the basal forebrain, an area rich in cholinergic neurons, which are the origin of the strong cholinergic innervation of the hippocampus and cortex. These cells are vulnerable to TBI and the number of choline acetyltransferase (ChAT) positive neurons was reduced after TBI in rats [40, 41] and humans [42]. Regarding acetylcholine synthesis, it has been found that choline acetyl transferase (ChAT) activity is diminished in the hippocampus and cortical areas already 1 hour post-trauma [43]. By contrast, the ChAT activity in the medial septal area, as part of the basal forebrain, increased with time following TBI [43], indicating that the neuronal loss is counterbalanced by an increased expression of ChAT.

The mechanism of the special vulnerability of cholinergic neurons is not fully understood. It is hypothesized that constant depolarization of cholinergic neurons causes autocannibalism [44]. TBI is expected to cause neuronal depolarizations by mechanical activation or massive release of other excitatory neurotransmitters. This is supposed to lead to a depletion of ACh. If there is no more free choline to sustain the ACh-production, additional choline from phospholipids in the cell membrane is utilised, which may result in neuronal damage [44].

Also, the continuous ACh-release may trigger the short-term increase of AChE-activity in the basal forebrain via a positive feedback mechanism. It has been suggested that an augmented ACh-release causes AChE-over-expression to restore normal conditions [12, 45]. This is supported by evidence of a stress-related increase of AChE transcription within 1 hour post-trauma, probably mediated via c-Fos as an activator of these regulatory pathways [12, 45, 46]. On the other hand, over-production of AChE as a regulatory response to hippocampal cell loss has been suggested to accelerate neurodeterioration [47] and attempts have been made to counterbalance this by using AChE inhibitors [14] or antisense oligonucleotides [16]. Transgenic mice over-expressing AChE showed progressive cognitive failure, cessation of dendrite branching and spine formation and enhanced high-affinity choline uptake—hallmarks associated with Alzheimer's disease [48]. AChE antisense treatment reduced the mortality of traumatized transgenic mice by more than 50% [16]. Further confirmations of the cholinergic relationship between AD and TBI are shown in a mouse model of AD, were a similar antisense treatment could decrease AChE-activity and improve learning and memory behaviour compared to control animals [49].

The use of AChE inhibitors for treatment of post-traumatic cognitive impairments was introduced into clinical practice [10, 26, 50] although the evidence for the use of AChE inhibitors is not fully developed [10, 26]. Neuroimaging methods, such as positron emission tomography (PET), are regarded as helpful for obtaining *in vivo* measures of cholinergic function [10]. A variety of PET radiotracers is available for AChE imaging and have been primarily used in clinical studies of dementia [51]. It has been shown that cortical AChE activity is associated with attention and working memory [52] and that the degree of cortical enzyme inhibition correlates with changes in executive and attentional functions [53]. Although discussed as an option [26], patients with TBI have not been studied with AChE-PET so far. One may expect that this method is able to differentiate between patients who benefit from cholinergic treatment, such as AChE inhibition, and those who do not.

In conclusion, these data show that experimental TBI induces short-term changes in AChE activity, which are different in various brain regions depending on their involvement in the cholinergic system. These changes may contribute to the cognitive impairments following the acute phase of TBI.

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