Signs of myelin impairment in cerebrospinal fluid after osmotic opening of the blood-brain barrier in rats

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Short title: CSF MBP damage

Summary

A number of clinical neurological pathologies are associated with increased permeability of the blood brain barrier (BBB). Induced changes of the homeostatic mechanisms in the brain microenvironment lead among others to cellular changes in the CNS. The question was whether some of these changes can be induced by osmotic opening of BBB in an *in vivo* experiment and whether they can be detected in cerebrospinal fluid (CSF). CSF was taken via the suboccipital puncture from 10 healthy rats and six rats after the osmotic opening of the BBB. In all 16 animals, concentration of myelin basic protein (MBP ng/ml), Neuron-specific enolase (NSE ng/ml) and TAU-protein (Tau pg/ml) were determined in CSF by ELISA. Values in both groups were statistically evaluated. Significant difference between the control and experimental group was revealed only for the concentration of myelin basic protein (p<0.01). The presented results indicate that osmotic opening of the BBB in vivo experiment without the presence of other pathological conditions of the brain leads to a damage of myelin, without impairment of neurons or their axons.

Key words: myelin basic protein – cerebrospinal fluid (CSF) – CSF sampling - blood-brain barrier (BBB) - osmotic BBB disruption

Introduction

A number of clinical neurological pathologies are associated with increased permeability of the blood brain barrier (BBB). Induced changes of the homeostatic mechanisms in the brain microenvironment lead to cellular changes in the CNS. They are manifested, among others, by signs of structural degradation of myelin (Pokorný *et al.* 2002)

Although myelin basic protein (MBP) also occurs in the peripheral nervous system, it is present mainly in the CNS. His main role is structural. It interacts with lipids of plasma membranes of oligodendrocytes in order to build and maintain the integrity of the

multilayered myelin sheet during the process of myelination in CNS. Its expression is age related and continues from childhood to adulthood.

MBP determines adaptive processes of internal environment to CNS disorders, it is responsible for myelin integrity and it has also other functions. It participates in the transmission of extracellular signals to oligodendrocytes via membrane proteins (e.g. acetin, tubulin), to which it binds. If MBP function in these physiological processes fails, a structural lesion of myelin can develop. Such lesions are accompanied by changes of MBP concentration in CSF (Davies *et al.* 1987, Boggs 2006, Kalwa and Smith 1994, Deber and Reynolds 1991, Simons and Trotter 2007). Also in several other clinical pathologies MBP is a useful marker of impaired internal environment of the brain. In the clinic and in appropriate experimental models in vivo the determination of the concentration of MBP in CSF enables to estimate the degree of impairment of physiological functions. MBP level is estimated primarily in such disorders as multiple sclerosis (MS), cerebral ischemia (stroke), brain injury (traumatic brain injury- TBI) and certain brain tumors. All of these entities are associated with different degrees of increased permeability of the blood brain barrier (BBB). (Engelhardt and Liebner 2014). CSF marker for neuronal damage is neuron-specific enolase (NSE) and the marker for axonal lesion is tau protein (Tau).

The aim of the present study was to determine whether the increased permeability of the BBB itself without the presence of other pathological conditions, may lead to changes in the concentration of CSF markers of structural lesions of the CNS. Vzhledem k výsledkům naší předchozí práce, která prokázala strukturální změny myelinu již za jednu hodinu, zvolili jsme časný odběr likvoru i v této práci (Kozler *et al.* 2010). For this purpose we used the method of osmotic opening of the BBB in experiments on rats. (Rapoport 1970, Kroll and Neuwelt 1998, Kozler and Pokorny 2003).

Material and Methods

Adult male Wistar strain laboratory rats (weight 390-410 g) were used in our experiments. All experiments were approved by the Ethical Committee of the First Faculty of Medicine (Charles University in Prague) and were in agreement with the Guidelines of the Animal Protection Law of the Czech Republic and Guidelines for the treatment of laboratory animals EU Guidelines 86/609/EEC.

Osmotic opening of the BBB

Microsurgical exposure of the internal carotid (ACI):

Animals were put into the state of general anaesthesia using intraperitoneal application of thiopental in the dose of 4 mg/100 g and allowed to ventilate spontaneously throughout the procedure. Starting from a skin incision along the midline between the upper end of the sternum and the mandible, the whole common carotid artery (ACC, arteria carotis communis) was exposed with a standard microsurgical technique and, beyond its bifurcation, also the proximal portions of the ACI and external carotid (ACE, arteria carotis externa), which was ligated close beyond the bifurcation. An intraluminal catheter was introduced into the ACC trunk from the arteriotomy for selective application of mannitol. With the application over and the catheter removed, the ACC was ligated distal to and proximal to the arteriotomy. The operation concluded with a single-layer suture (Kozler and Pokorný 2003).

Osmotic opening of the BBB:

Mannitol 20% (1 098 mosmol/l) in a dose of 5 ml/kg was selectively applied in the ACI at a rate of 0.12 ml/sec (Rapoport 2000). After the surgical intervention, animals were placed in boxes offering standard access to food and drink.

CSF sampling

CSF in amount of 0.15 to 0.2 ml was extracted from cisterna magna by suboccipital puncture in ten healthy rats and six rats after osmotic opening of the BBB. CSF in the experimental

group was taken in time intervals from 95 to 200 minutes after completion of mannitol administration (see Tab. 1).

Table 1. Characteristics of experimental and control group

	number	body weight in grams	time interval between BBB opening and CSF sampling in minutes
control group	10	278-298 ∅ 287,9	
experimental group	6	270-295 ∅ 286,3	95-200 ∅157

Animals were put into the state of general anaesthesia using intraperitoneal application of thiopental in the dose of 4 mg/100 g and allowed to ventilate spontaneously throughout the procedure. During the procedure, animal was fixed in the three-point fixation device of a stereotaxic apparatus (see Fig. 1). Skin was cut at the cervicocranial transition, fascia intersected, nuchal muscles were separated subperiostally, and atlantooccipital membrane explored. Puncture (1.5 mm deep) was performed with a needle attached to a glass syringe fixed at the vertical arm of stereotaxic apparatus at appoint 3 mm laterally from the midline to the left (see Fig. 2). CSF was taken by syringe aspiration. After CSF sampling, the surgical pathway to atlantooccipital membrane was closed in one layer and animal was kept in a tempered boxing until resolution from general anaesthesia. Method of CSF sampling via suboccipital puncture use also other authors (Nirogi *et al.* 2009, Lai *et al.* 1983, van den Berg *et al.* 2002). However, in our modification, CSF was sampled from a small surgical approach with visualization of atlantooccipital membrane. In animals of average weight 400 g (we used in experiments) the total amount of CSF was 0.6 ml with 0.2 ml in the cisterna magna (Rosenling 2008). This means that we took almost the maximum possible amount of CSF obtainable from cisterna magna.

Fig. 1: Animal´s position in stereotaxic apparatus

Fig. 2:   Place of suboccipital puncture
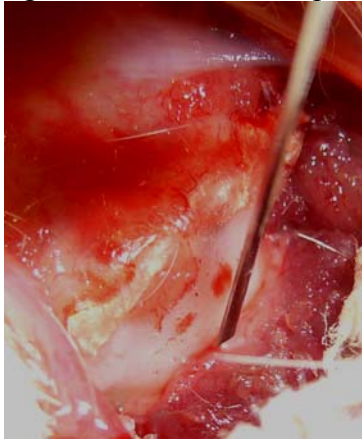
CSF analysis

In all 16 animals, concentration of myelin basic protein in CSF was determined (MBP ng/ml)

by MBP Elisa BECKMAN together with the neuron specific enolase (NSE ng / ml Method:

Elecsys NSE Roche) and the protein Tau (Tau pg/ml, Elisa Total Tau EUROIMMUN).

Statistics

Values of MBP, NSE and Tau protein in both Gross of animals were statistically evaluated

using Kruskal-Wallis and Mann-Whitney non-parametric tests.

Results

Concentrations of individual markers in CFS measured in ten control animals and six animals

after the BBB opening is given in Tab 2.

Tab. 2 CSF composition (average values ± standard deviation)

| | number | Mean±SD | | |
		MBP	NSE	Tau
controls	10	7,119±5,376	0,606±0,418	not present
experimental	6	14,847±0,258	0,942±0,425	not present

The results show that MBP levels in the control group averaged 7.1 ng/l and they had large variability (standard deviation 5.3). Average MBP levels in the experimental group were 14.8 ng/l with very small variability (standard deviation 0.25) *(p < 0,01)* (Fig. 3).

In humans, the normal MBP levels in CSF are reported to be ≤ 4 ng/ml; higher values (4-8 ng/ml) indicate a chronic myelin lesion and values above 9 ng/ml suggest acute damage to myelin (Greene *et al.* 2012). In rats, the concentration of MBP in CSF appears to be several times higher. (Rosenling 2008). The above given values result from the adequate sample dilution recommended by the manufacturer of the laboratory kit (Rosenling 2008).

Contrary to that, NSE concentration in the experimental group remained practically unchanged compared to the control group and Tau protein was not detectable in either group.

Fig. 3: Myelin basic protein concentration in CSF

(control (C) and mannitol administered (MA) rats, concentration of MBP is given in ng/l, ** *= p < 0,01*)
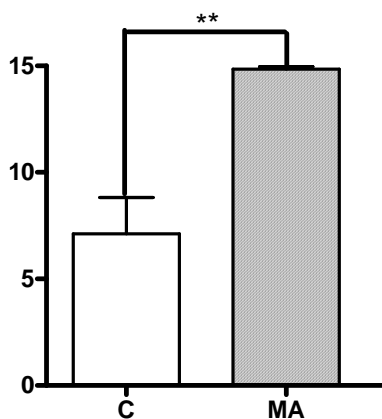
Discussion

Isolation of MBP from the myelin membrane and determination of the sequence of the individual amino acids were accomplished in 1971 (Eylar *et al.* 1971). In the early '80s the method for determining the concentration of MBP in CSF was developed (Delassalle *et al.* 1980). During the next years, increased CSF MBP concentrations in multiple sclerosis (MS), stroke, head injury and brain tumors were reported (Mukherjee *et al.* 1985; Davies *et al.* 1987; Barry *et al.* 1991). Current concept of the pathogenesis of demyelination in multiple sclerosis (MS) is based primarily on results from experimental studies (experimental allergic encephalomyelitis - EAE). It is assumed that the specific autoimmune disease can impair myelin by antigen specific autoreactive T cells (Giovannoni 2006). Experimental models of cerebral ischemia *in vivo* have demonstrated the separation of myelin from the swelling axons. Histopathological evaluation demonstrated an early relative loss of myelin protein immunoreactivity (McIver *et al.* 2010). Liu and co-workers showed that some isoforms of MBP were degraded in cases of experimental injury in vivo (controlled cortical impact in a rat model of TBI). Other in vitro studies have found that MBP is sensitive to calpain, which can cause MBP fragmentation characteristic for TBI (Liu *et al.* 2006).

Elevated concentrations of MBP levels in CSF in cases of oligodendroglial brain tumors (oligodendrogliomas and mixed oligoastrocytomas) are known and they were explained by finding that oligodendrocytes express large amounts of the genes responsible for encoding myelin proteins. MBP thus serves as a molecular biomarker of those tumors (Golfinos *et al.* 1997).

In all the above clinical conditions of demyelination, an increased permeability of the BBB is always demonstrated.

In an experimental model of multiple sclerosis (experimental autoimmune encephalomyelitis - EAE), an increased number of leukocytes in the perivascular space stimulates secretion of

matrixmetalloproteases (MMP-2 and MMP-9), which degrade extracellular matrix with resulting increased permeability of the BBB (Liebner and Engelhardt 2014). In experimental models of cerebral ischemia *in vivo*, permeability disorder has been recently attributed to the dysfunction of newly discovered signalling protein Sonic hedgehog (SHH) (named after the hero of the computer game Sonic the Hedgehog), which occurs during ischemia in higher concentrations and destroys anchoring proteins occludin and laminin (Alvarez *et al.* 2011). In cases of brain tumors the increased permeability of the BBB can be explained by the neovascularization in the growing tumor, with a sharp increase of the expression of vascular endothelial growth factor (VEGF). In brain injury cases the increased permeability of the BBB has a mechanical origin (Engelhardt and Liebner 2014).

In all above pathogenetic categories, an increased BBB permeability is explained by the phenomena which are manifestations of the particular pathological process itself. In our study, the sing of myelin degradation (increased MBP in CSF) appears to be a direct consequence of merely BBB opening without the presence of any other pathological process (significantly higher concentrations of CSF MBP rats in the experimental group compared to healthy animals in the control group (p <0.01). Induced impairment of brain homeostatic leads to significant structural changes in this functionally important protein of CNS.

Concentrations of NSE, an enzyme produced by normal neurons (Tapia *et al.* 1981) were not influenced by the BBB osmotic opening. The tau protein which is responsible for axonal microtubule stabilization was not detectable in either group (Huton and Roder 2007). These results indicate that BBB osmotic opening does not affect the structural integrity of neurons and axons.

Disintegration of myelin after osmotic opening of the BBB was proved already in our previous study (Kozler *et al.* 2010). We do not have yet any explanation for myelin impairment and for the increased MBP concentration in CSF after only BBB osmotic opening

and we found no acceptable explanation in the literature. Increased expression of genes for transcription of the protein, as it is in oligodenroglial tumors, can be certainly excluded. Similarly, the calpain induced degradation of myelin after the mechanical insult is not very likely. Immunoreactivity changes of myelin proteins detectable in the ischemic lesions or effect of yet unidentified antigen-specific substances, which may resemble the damage to myelin in MS cannot be excluded, but their participation in the process of demyelination after the osmotic opening of the BBB is considered only as hypothetical.

Conflict of Interest

There is no conflict of interest

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