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REVIEW ARTICLE

Role of astrocytes in pathogenesis of multiple sclerosis and their participation in regulation of cerebral circulation

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Abstract

There is about 30% higher risk of the myocardial infarction in patients diagnosed with multiple sclerosis (MS) than in people without MS. Increased risk of cardiovascular disease development positively correlates with levels of serum markers of an endothelial dysfunction, and may give rise to a global cerebral hypoperfusion. It appears that these complications precede progressive loss of axons, which mechanisms are complex and should be linked to a loss of β2 adrenergic receptors on astrocytes of demyelinating lesions. Consequence of this deficiency, the cause of which is not known yet, is a decline in energy metabolism of axons. Moreover, the loss of these receptors is linked to a reduced redistribution of potassium ions by astrocytes, glutamate excitotoxicity and increase of calcium ion concentration in the axon with subsequent activation of necrotic processes. In addition to immunological aspects we should take into account also parameters of the functional state of endothelium when appropriate targeted therapy for patient is considered.

Abbreviations:

MS	- multiple sclerosis
CNS	- central nervous system
IS	- immune system
OPC	- oligodendrocyte-precursor cells
EAE	- experimental autoimmune encephalomyelitis
LIF	- leucemia inhibiting factor
BBB	- blood-brain barrier
VCAM	- vascular cell adhesion molecule
APC	- antigen-presenting cells
STAT	- signal transducers and transcription activators
MHC	- major histocompatibility complex
JAK	- Janus kinase
IRF	- interferon regulatory factors
CIITA	- transactivator class II
TCR	- T-cell receptor
cAMP	- cyclic adenosine monophosphate
PKA	- protein kinase A
CK-BB	- creatine kinase (brain type)
AMPA	- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
DNA	- deoxyribonucleic acid
NGF	- nerve growth factor
BDNF	- brain-derived neurotrophic factor
TNF	- tumor necrosis factor
ROS	- reactive oxygen species
NAAG	- N-acetyl-D-aspartyl-glutamate
NAA	- N-acetyl-D-aspartate
NMDA	- N-methyl-D-aspartate
CBF	- cerebral blood flow
CBV	- cerebral blood volume
VMTT	- vascular mean transit time
CCSVI	- chronic cerebrospinal venous insufficiency

INTRODUCTION

Multiple sclerosis (MS) is a very frequent chronic neurological disease among young adults with a mean age of onset between 20–40 years and a prevalence of 1.3/1000 humans in the developed world (Koch-Henriksen & Sorensen 2010). It is the major cause of the non-traumatic neurological disability in young adults. Neuropathological characteristics of MS are inflammation, demyelination, axonal loss and astrogliosis. Nerve demyelination influences various parts of the CNS (central nervous system) in the white and gray matter, especially nerves in periventricular areas in the brain, brain stem and cerebellum. Demyelinated areas develop into scars in the process of glia proliferation. Historically, MS research has been focused on myelin damage but recently it has become increasingly evident that the axonal degeneration is the key player in the pathogenesis of the progressive disability in MS (Krížová *et al.* 2011).

Autoimmune processes are among the main pathophysiological mechanisms of MS development. Immune system (IS) of the body attacks its own structures, e.g. myelin sheaths, leading to their destruction and demyelination of nerve fibers. It is generally believed that the molecular mimicry phenomenon may play significant part here (Dutta & Trapp 2011). The cause of immune response against myelin still remains unclear. It is assumed that some viruses that have affinity to primary attack nervous system, such as *Herpes simplex virus* and *Morbilli virus* may play significant role in MS development but they do not affect another progress in MS pathology (Dutta & Trapp 2011).

Although MS is not considered to be a hereditary disease, some genetic predispositions are believed to play parts in MS origin. There was observed from 10 to 15 times higher chance for appearing of MS in families with MS in comparison with the rest of population. A great number of genes are supposed to be connected with an increased risk of MS (Frohman *et al.* 2005; Krížová *et al.* 2013a). The most researched and genetically associated with MS is the major histocompatibility complex. The HLA-DRB1 allele is considered to be one of the most important risk alleles. By contrast, expression of some genes was decreased (e.g. HMG-CoA reductase) in MS patients (Lock *et al.* 2002).

The loss of myelin sheath (demyelination) leads to decreased ability of axons to transmit impulses and even to their destruction. In normal, myelinated fibers, Na^+ ion-channels are concentrated at nodes of the Ranvier, allowing saltatory conduction of action potentials. When Na^+ enters into nodal axoplasm, Na^+/K^+-ATPase exchanges it rapidly for extracellular K^+ ions. Na^+/K^+-ATPases are the largest consumers of ATP in the CNS. Thus, myelination not only promotes rapid nerve conduction, but it may be effective in conserving energy (Dutta & Trapp 2011). While demyelination may not really hill axons, it renders them more vulnerable to physiological stress and degeneration by substantially increasing the energy requirements for nerve conduction. Clinically, patients suffer from motorious, sensoric or autonomic deficiency, and often also mental disorders (Čarnická *et al.* 2014). In early stages of MS, approximately 10 to 20 % of axons lose their function and as the disease makes its progress, up to 80 % of axons are damaged (Dutta & Trapp 2011). There are two main stages of myelin loss in demyelinated areas (Kornek *et al.* 2000). During **acute demyelination**, axons are attacked by pro-inflammatory mediators, which increase production of cytokines, free radicals and activate inducible NO synthase. The acute phase lasts from several days to several weeks and it continues as "silent inactive plaques" remission (Kornek *et al.* 2000). In early stages of demyelination, there is a possibility of rebuilding of myelin sheaths during remyelination process. This process is mediated by differentiation of oligodendrocyte-precursor cells (OPC) into mature oligodendrocytes which produce new myelin. **Chronically demyelinated axons** may degenerate due to failure of OPC differentiation because of deficiency of survival factors (e.g. neuralgin produced by well-working astrocytes) or presence of factors that inhibit OPC differentiation (Frohman *et al.* 2005). During chronic stage, disseminated demyelination is present, followed by neurodegenerative alterations associated with widespread axon loss. In chronically demyelinated lesions of the spinal cord, the axon loss was associated with necrotic alterations that included fragmentation of neurofilaments and also significantly decreased amount of mitochondria and microtubules (Dutta *et al.* 2006). Another significant alteration in chronic MS lesions is swelling of axoplasm which is presumably associated with increased Ca^{2+} concentration in axoplasm. The lesions and destruction of axons are considered crucial in MS pathogenesis (Trapp & Stys 2009).

ROLE OF OLIGODENDROCYTES IN MYELINATION

1. Migration of OPC

The first step in use of oligodendrocytes in the process of myelin repair is migration of their precursor cells (OPC) from their "*dormant*" position in the brain parenchyma to demyelinated plaque areas. In these areas, there are hypertrophic astrocytes which express chemoattractants (CXCL1, CXCL2, 3rd grade semaphorines) which are not present in the brain of healthy person (Tsai *et al.* 2002; Spassky *et al.* 2002). It is believed that oversized astrocyte proliferation may lead to the development of physical and biochemical barrier which could stop migration of OPC and interactions between oligodendrocytes and axons. This hypothesis was supported by observation in "experimental autoimmune encephalomyelitis" (EAE) which showed that OPC were gathered among hypertrophic astrocytes

and on borders of chronic demyelinated lesions but not *inside* the lesions (Bannerman *et al.* 2007). In areas with barrier presence, increased accumulation of glucoseaminoglycan hyaluronan was observed, which was produced by T-cells and microglia in acute lesions (low-molecular-weight forms) and by astrocytes in chronic lesions (high-molecular-weight forms). Hyaluronan bound to transmembrane receptor CD44 which was expressed in higher amount on oligodendrocytes and astrocytes in demyelinated lesions present in EAE and MS, which could lead to increased barrier building and to demyelination (Tuohy *et al.* 2004; Back *et al.* 2005).

2. OPC proliferation and survival

After entering demyelinated area, OPC have to proliferate and survive in inflammatory environment to be able to mature. The role of cytokine IL-6 is considered to be significant in process of surviving, migration and differentiation *in vitro* (Louis *et al.* 1993). Higher expression of IL-6 in astrocytes and microglia around demyelinated plaques was detected in biopsy tissue taken from the brains of MS patients. The increased expression correlated with increased oligodendrocytes surviving, what could mean that this cytokine might play a role as the "survival" factor also for oligodendrocytes *in vivo* (Schonrock *et al.* 2000).

3. Differentiation and maturation of OPC

OPC have firstly to mature and differentiate into myelining cells to be able to perform remyelination. In this process, several molecules and factors may be present, including "cell adhesion molecules", extracellular K^+ and also low-molecular-weight soluble axonal signals (Zalc & Fields 2000). ATP produced by axons stimulates purinergic receptors on astrocytes what leads to releasing of cytokine LIF (leucemia inhibiting factor) from IL-6 family, which induces conversion of maturated oligodendrocytes to myelin-producing oligodendrocytes (Ishibashi et al. 2006). The loss of ATP in extracellular compartment may contribute to insufficient myelination by inhibiting this signalization. Extracellular ATP is degraded to adenosine which also stimulates differentiation of OPC to myelin-producing oligodendrocytes by binding to their specific receptors (Stevens *et al.* 2002).

Reactivation of Jagged-Notch signaling pathway in demyelinated plaques in MS may contribute to remyelination disorders (John *et al.* 2002). Jagged 1 expression in astrocytes declines with increasing myelination. Its receptor, Notch 1, is localized on OPC and binding Jagged 1 inhibits OPC differentiation into myelinating oligodendrocytes. Increased Jagged 1 expression was found in astrocytes in demyelinated lesions (but not in remyelinated ones) in the brains of MS patients (John *et al.* 2002). Though inhibition of signalization via Notch receptor led to tissue repairment in EAE (Jurynczyk *et al.* 2005), this hypothesis has to be explored furthermore.

ROLE OF ASTROCYTES IN MS PATHOGENESIS

Altough E. Müller, in 1904, formed a hypothesis that MS is primarily caused by impaired function of astrocytes, their role in MS pathogenesis remained unexplained for long time. But during last decade, information about their possible role in MS development started being collected. Astrocytes are the cells which communicate with neurons and also other glial cells i.e.: oligodendrocytes (that produce myelin sheaths), microglial cells (that have protective function) and ependymal cells (that form inner surface of brain ventricles). Astrocytes play controversial roles in the MS pathogenesis and development. They participate both in axon demyelination as well as in their remyelination. During acute stages of the disease, astrocytes participate in remyelination, but during chronical stages, they block this process.

On one side, astrocytes play their role in providing services for nerve cells: they produce lactate for neurons and oligodendrocytes, express glutamate receptors, glutamate transporters and various growth factors for nerve cells. On the other side, their activation is associated with changes in gene expression of adhesive molecules, cytokines, growth factors, receptors, enzymes (Križová *et al.* 2013b; Williams *et al.* 2007). The loss of β_2 adrenergic receptors on astrocytes in MS lesions is considered to be the crucial cause of the MS pathogenesis. Several various mechanisms were described, explaining the way how the loss of β_2 receptors supported degenerative processes in MS however the cause of the receptor loss is still unknown.

1. Astrocytes transformation into antigen-presenting cells

CD4+ T-cells probably play an important role in the development of inflammatory reaction in MS lesions. These cells are specific for various types of myelin antigens and are present both in MS patients and healthy people (Hellings *et al.* 2001). CD4+ T-lymphocytes activated in peripheral lymphoid organs (e.g. by common bacterial or viral peptides which have similar sequences to myelin antigens) firstly enter the blood-brain barrier (BBB). VCAM-1 (vascular cell adhesion molecule 1) produced by astrocytes is the main receptor for 4-integrin produced in activated T-cells and is needed for their transport through endothelium. After crossing through the BBB, T-cells are activated by interaction with antigen-presenting cells (APCs) which are microglial and dendritic cells. These cells constitutively express some proteins from the major histocompatibility complex class II (MHC II). Non-damaged normal astrocytes are only "candidates" for becoming APCs. Production of MHC II on their membrane is induced by interferon IFN-γ which is produced by activated T-cells (Williams *et al.* 2007). Binding of IFN-γ to its receptor on astrocytes leads to Janus kinase (JAK) activation and phosphorylation of transcription factors – signal transducers and transcription activators (STAT)

in cytoplasm. After dimerization, these molecules proceed into nucleus, bind to DNA's responsive element and consequently, they allow gene transcription, e.g. of interferon regulatory factors (IRF-1 and 2) genes. These together with STAT cooperate in the process of the transactivator class II (CIITA) activation. CIITA is necessary for mutual binding of several transcription factors which are needed for MHC II genes transcription (De Keyser *et al.* 2004). MHC II is necessary for binding myelin antigens to antigen-presenting cells. This complex is then recognized by anti-myelin T-cells receptor (TCR). In this way the inflammatory cascade is activated however, it is well regulated in non-damaged cells. Noradrenaline is an endogenous suppressor of this reaction causing the increase of intracellular cAMP level by binding to β_2-receptors on astrocytes via G_s-protein and adenylate cyclase activation. Consequently, after proteinkinase A (PKA) activation, CIITA is phosphorylated which leads to its inactivation and therefore MHC II expression is blocked (Feinstein *et al.* 2002).

In MS disease, the loss of β_2-adrenergic receptors on astrocytes, after exposure to critical levels of IFN-γ, is responsible for keeping inactive PKA and non-phosphorylated CIITA, which allows MHC II genes expression and therefore initialization of an inflammatory reaction. During the inflammatory reaction, astrocytes become APC which not only produce MHC II but pro-inflammatory cytokines (IL-1), as well. These cytokines activate production of chemokines and adhesive molecules, activate T-cells, B-cells and microglial cells, but they also induce oligodendrocytes and myelin destruction. The loss of β_2-adrenergic receptors may also be a cause of increased inducible nitric oxide synthase expression in MS plaques (Liu *et al.* 2001) and the superoxide radical production.

2. Astrocytes and glutamate excitotoxicity

During inflammation, macrophages and microglial cells produce a higher amount of glutamate which is transported also to astrocytes by Na^+-dependent transporters. Due to the loss of β_2-adrenergic receptors on astrocytes, the gradient of Na^+ is not maintained properly because of the lack of ATP for Na^+/K^+-ATPase function (Danbolt 2001). ATP generation in astrocytes is performed by creatine kinase (CK-BB) the expression of which is stimulated by cAMP.

Extracellular glutamate concentration is maintained by absorption of glutamate by Na^+/K^+-dependent transporters for excitatory amino acids, which are expressed in oligodendrocytes and astrocytes (Vallejo-Illarramendi *et al.* 2006). When Na^+/K^+-ATPase activity in astrocytes is decreased, after the loss of β_2 receptors, opposite glutamate transport is performed, i.e. glutamate flows out from astrocytes (Pitt *et al.* 2000). Axons, such as oligodendrocytes, express AMPA/kainate receptors, and after their activation and Ca^{2+} influx, caspase 3 is activated, DNA is fragmented and finally cells undergo apoptosis and destruction (Ouardouz *et al.* 2009 a,b; Domercq *et al.* 2005).

3. Astrocytes and trophic factors production

Astrocytes also produce various trophic fators (Culmsee *et al.* 1999; Viehover *et al.* 2001). cAMP stimulates production of several of them, for example neuregulin, nerve growth factor (NGF) and brain-derived growth factor (BDNF). In patients with MS, production of these factors is limited in lesions, what causes oligodendrocytes damage or even destruction (they need neuregulin to survive), axon demyelination and other neurodegenerative processes (Lucchinetti *et al.* 2000).

4. Astrocytes as a source of energy for axons

Glycogen as an important energy source for the brain is stored mainly in astrocytes. When higher dose of energy is needed, axons gain ATP from lactate produced by astrocytes in glycogen metabolism (Brown *et al.* 2003). Glycogenolysis in astrocytes is regulated also by noradrenaline through β_2-receptors. Decreased β_2-receptor expression in astrocytes in MS lesions causes that they produce lower amount of lactate. As the result, axons do not have enough energy to maintain Na^+ homeostasis by Na^+/K^+-ATPase. An excessive non-regulated Na^+ influx leads to uncontrolled depolarization, which results into the voltage-gated Ca^{2+} channel opening and reverse function of Na^+/Ca^{2+}-exchanger (Na^+ flows out and Ca^{2+} flows into the cell) in Ranvier nodes (Ransom & Fern 1997). The influx of calcium ions activates degradative enzymes in axons (proteases, phospholipases) which activate a cascade leading to axonal dysfunction. It may also lead to calcium accumulation in mitochondria, another decrease of ATP level and axon loss in MS (Schon & Manfredi 2003).

ROLE OF MITOCHONDRIA IN AXONAL DAMAGE

Both in acute and chronic demyelinated lesions of the white matter, a massive defect in mitochondrion function was observed (Vlček *et al.* 2014; Zambonin *et al.* 2011; van Horssen *et al.* 2012). In early stages of MS lesions development, the increased expression of NADPH-oxidase 1 and 2 was observed, and therefore, the increased production of ROS, as well (Fischer *et al.* 2012). The mitochondrial activity of complex IV has been shown to be increased in chronic MS lesions associated with an increase of mitochondrial mass (Cambron *et al.* 2012). In addition to the activity and density, the mobility of mitochondria within axons may also be influenced by demyelination. The expression of axon-specific mitochondrial docking protein, syntaphilin, in chronic lesions indicates potentially immobile reservoirs which supply the necessary energy in demyelinated axons (Campbell & Mahad 2012). The increase in complex IV activity may be a compensatory mechanism of other mitochondrial complex activities

reduction (complex I and III). However, it still remains unknown if this mechanism is sufficient to sustain the ATP production during a longer period of time. It was hypothesized, that voltage-gated Na^+ channels are redistributed along demyelinated axonal areas in chronic lesions, for restoring conduction in these lesions. In this way Na^+ concentration in axons is regenerated, metabolic demand for Na^+/K^+-ATPase function is increased, which leads to increased amount of mitochondria and complex IV activity. Higher energy demand exhausts mitochondria, their ability of producing ATP is diminished and expression of mitochondrial complex I and III is decreased to 40 to 50 %. Consequently, after Na^+ concentration reaches more than 20 mmol/l Na^+ is transported in higher amounts into extracellular space by reversal Na^+/Ca^{2+}-exchanger, which transports calcium ions inside the axoplasm. This state is called "virtual" hypoxia. An evidence for this mechanism was observed in post-mortem tissue of acute MS lesions where co-localisation of $Na_v1.6$ channels was presented. Increased calcium influx via Na^+/Ca^{2+}-exchanger, in combination with calcium release from mitochondria and also intake of Ca^{2+} by glutamate receptors, activates cascades which leads to axonal degeneration caused by phospholipase A_2, proteinkinase C, NO-synthase and calpain activation. In addition, dysfunctional mitochondria produce ROS in chronic lesions.

In acute MS lesions, N-acetyl-D-aspartate (NAA) production is lowered due to a dysfunction of axonal mitochondria (Aboul-Enein *et al.* 2010; Cader *et al.* 2007). NAA represents storage form of acetyl rests for myelin synthesis. Aspartate is transacetylated by N-acetyltransferase to NAA which is released from neural mitochondria while remaining 2-oxoglutarate may participate in Krebs cycle producing energy. NAA level is considered to be a marker of axonal mitochondria function as well as axonal integrity. Both NAA and NAAG (N-acetyl-aspartylglutamate) are antagonists of NMDA receptors in oligodendrocyte membrane. When NAA level is decreased, the higher sensibility of these receptors for glutamate leads to excitotoxic damage of oligodendrocytes and therefore insufficient axon myelination.

VASCULAR ASPECTS OF MULTIPLE SCLEROSIS

In recent years, several studies have been reported on vascular abnormalities in patients with MS. These abnormalities are diagnosed e.g. by imaging techniques (magnetic resonance, tomography), examination of brain hypoperfusion parameters – CBF (cerebral blood flow), CBV (cerebral blood volume) and VMTT (vascular mean transit time) (Varsik *et al.* 2004a). Many authors have published these findings:

1. a higher risk of ischaemic diseases development – cardiovascular brain stroke in MS patients (Allen *et al.* 2008; Christiansen *et al.* 2010),
2. a decrease of cerebral perfusion in MS patients (Inglese *et al.* 2007; Law *et al.* 2004),
3. a reduced venous blood drainage from the brain and the upper part of the spinal cord (Zamboni *et al.* 2009).

The higher risk of ischaemic diseases in patients with MS should be caused by a dysfunctional vasodilatation and consequently reduced cerebral blood circulation. It is well-known that astrocytes play the main role in regulation of cerebral circulation. In normal conditions, K^+ released at the nodes of Ranvier during axonal discharge, is taken up by astrocytes through Kir ion channels (*inward-rectifying K^+ channels*). K^+ is then intracellulary redistributed in astrocyte cytoplasm and released at perivascular endfeet through Ca^{2+}-activated K^+ channels in a process leading to arteriolar vasodilatation, followed by the increase of cerebral blood circulation (Butt & Kalsi 2006). The energy metabolism in astrocytes is dysfunctional in MS patients. They are deficient in β2-adrenergic receptors and have reduced phosphocreatine metabolism caused by decreased cytosolic creatine kinase B amount and activity. Creatine kinase can generate ATP which is mostly consumed in astrocytic endfeet during axonal electrogenesis on the Na^+/K^+-ATP pump. The reduced energy metabolism in patients with MS might result in lower K^+ recovery in the perivascular space, possibly leading to a state of cerebral hypoperfusion (D'haeseleer *et al.* 2011), as well as to reversal glutamate uptake by glutamate transporter (Cambron *et al.* 2012).

In addition, higher blood concentration of endothelin 1 causing vasoconstriction was measured in patients with MS (Haufschild *et al.* 2001). In oligodendrocytes in MS lesions, higher expression of stress protein p53 was observed, which becomes active in ischaemic brain areas (Wosik *et al.* 2003).

In MS patients, compared to controls, stenosis and other anomalies of *v. jugularis interna* and *v. vertebralis* have been found, which were defined as chronic cerebrospinal venous insufficiency (CCSVI) (Zamboni *et al.* 2009, 2010). These vessels provide draining of most blood from the brain (Varsik *et al.* 2004b). Their anomalies lead to decreased cerebral venous outflow which resulted in the higher intravascular brain pressure, and a partial blood stasis was present. This mechanism, suggested by prof. Zamboni and his colleagues, has not been proven clearly yet as a crucial factor for the MS pathogenesis. Two independent groups of scientists have supported relationship between CCSVI and MS by using Doppler sonography criteria: an abnormal venous outflow was observed in 91% of patients with MS and 90% of MS patients fulfilled diagnostic criteria for CCSVI (Simka *et al.* 2010).

Another typical finding in the brain tissue in MS patients is increased iron level. Although this element is necessary for normal CNS function, ionized Fe contributes to pro-oxidation activity in tissue. However, it

is not clear yet whether the increase of iron level is the *cause* or the *result* of MS. Several hypotheses explaining higher Fe accumulation in the brain of MS patients have been published, and the two of them are the most relevant:

a. the increase of intravascular brain pressure is present due to CCSVI, and consequently endothelial cells move away from each other. Gaps between them get bigger and it leads to higher blood extravasation and erythrocytes degradation followed by iron release in the brain parenchyma (Singh & Zamboni 2009);
b. iron originates in iron-binding proteins of oligodendrocytes, which are excessively destructed in the brain tissue during development of MS inflammatory lesions (Levine & Chakrabarty 2004).

Another possibility for the higher brain iron concentration is the penetration of iron-rich macrophages through the blood-brain barrier and decreased axonal Fe clearance (Lassmann *et al.* 2007).

CONCLUSION

Multiple sclerosis is an "unpredictable" disease, often leading to patient's immobilization at young age and to cognitive complications. A combination of developing gene mutations during patient's life with other endogenous factors, with contribution of environmental impacts, may be crucial for the stage in which the disease will become manifested, how fast it will progress, or whether it will remain chronically latent. The recent therapy is mainly oriented to anti-inflammatory effects. The inhibition of TNFα was a disappointment in MS therapy, although it was effective in the therapy of various autoimmune diseases. Positive effects of cytokines and chemokines which were documented in EAE, were not present in MS. Another problem represents the fact that in each stage of the disease, different cytokine and chemokine mix would be needed as targeted therapy. The best choice would be administering a medicine straight into affected CNS areas, to reach the highest possible effect.

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Conflict of interest statement

Authors declare that they have no financial interest in this manuscript and no affiliations (relationships) to disclose.

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