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Intracellular ion signaling influences myelin basic protein synthesis in oligodendrocyte precursor cells

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ABSTRACT

Myelination in the central nervous system depends on axon-oligodendrocyte precursor cell (OPC) interaction. We suggest that myelin synthesis may be influenced by $[Na^+]i$ and $[Ca^{2+}]i$ signaling in OPCs. Experiments were performed in mouse cultured OPCs at day *in vitro* (DIV) 2–6 or acute slices of the corpus callosum at postnatal days (P) 10–30. Synthesis of Myelin Basic Protein (MBP), an "executive molecule of myelin", was used as readout of myelination. Immunohistological data revealed that MBP synthesis in cultured OPCs starts around DIV4. Transient elevations of resting $[Ca^{2+}]i$ and $[Na^+]i$ levels were observed in the same temporal window (DIV4-5). At DIV4, but not at DIV2, both extracellular $[K^+]$ ($[K^+]e$) elevation (+5 mM) and partial Na^+,K^+-ATPase (NKA) inhibition elicited $[Na^+]i$ and $[Ca^{2+}]i$ transients. These responses were blocked with KB-R7943 (1 μM), a blocker of Na^+-Ca^{2+} exchanger (NCX), indicating an involvement of NCX which operates in reverse mode. Treatment of OPCs with culture medium containing elevated $[K^+]$ (+5 mM, 24 h) or ouabain (500 nM, 24 h) increased resting $[Ca^{2+}]i$ and facilitated MBP synthesis. Blockade of NCX with KB-R7943 (1 μM, 12 h) reduced resting $[Ca^{2+}]i$ and decreased MBP synthesis. Similar to the results obtained in OPC cultures, OPCs in acute callosal slices demonstrated an increase in resting $[Ca^{2+}]i$ and $[Na^+]i$ levels during development. NCX blockade induced $[Ca^{2+}]i$ and $[Na^+]i$ responses in OPCs at P20-30 but not at P10. We conclude that local $[Na^+]i$ and/or membrane potential changes can modulate Ca^{2+} influx through NCX and in turn MBP synthesis. Thus neuronal activity-induced changes in $[K^+]e$ may *via* NCX and NKA modulate myelination.

1. Introduction

Central nervous system (CNS) myelination of neuronal axons by oligodendrocytes is a complex multi-step procedure requiring the synthesis of huge amounts of myelin lipids and proteins. Recent studies suggest that neuronal activity is a starting point of myelination [1,2]. Axons have been shown to be capable of vesicular glutamate release [3,4] and ATP release [5]. Oligodendrocyte precursor cells (OPCs) express several ionotropic and metabotropic receptors, including NMDA [6], AMPA [3], metabotropic glutamate [7], P2X [8] and P2Y [9] receptors. However, both electrophysiological and electron-microscopy data show that neuronal fibers contain only a small number of vesicles, the axon-OPC cleft is relatively wide and axonal vesicles are found also in the absence of OPC membrane, *i.e.* they are not always co-localized with OPC processes [3,4].

In addition to neurotransmitter release, neuronal activity causes changes in extracellular K^+ concentration ($[K^+]e$). The latter could translate neuronal activity into intracellular ion signaling in OPCs. Indeed, $[K^+]e$ elevation to 15 mM has been shown to reduce intracellular Na^+ concentration ($[Na^+]i$) in OPCs from 15 mM to 4.7 mM [10]. In parallel with the $[Na^+]i$ changes, a $[K^+]e$ rise can induce $[Ca^{2+}]i$ responses through an activation of voltage-gated Ca^{2+} channels [11,12] and/or reversal of Na^+-Ca^{2+} exchanger (NCX) [13,14]. The latter appears to be of crucial importance for myelination, because genetic knock-out of NCX3 was reported to reduce MBP synthesis in OPC cultures, and NCX3-knockout mice show marked hypomyelination of spinal cord [15]. NCX exchanges three Na^+ for one Ca^{2+} ion, *i.e.* NCX is an electrogenic antiporter. Consequently,

Abbreviations: $[Ca^{2+}]i$, intracellular free Ca^{2+} ion concentration; CNS, central nervous system; DIV, day *in vitro*; MBP, myelin basic protein; $[Na^+]i$, intracellular free Na^+ ion concentration; NCX, Na^+-Ca^{2+} exchanger; NKA, Na^+-K^+-ATPase; OPC, oligodendrocyte precursor cell; OGB1, Oregon Green BAPTA 1; SBFI, Sodium-binding benzofuran isophthalate.

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the direction of its operation depends on Ca^{2+} and Na^{+} transmembrane gradients and membrane potential.

Myelin Basic Protein (MBP) is the second most abundant myelin protein and its absence in mutant Shiverer mice and Long—Evans shaker rats results in a severe CNS hypomyelination [16,17]. As the absence of other myelin proteins still allows the formation of compact myelin, MBP has been denoted as the 'executive molecule of myelin' [18]. MBP mRNA is transported from the nucleus to the plasma membrane in a translationally silenced state in large ribonucleoprotein complexes termed RNA granules [19]. Axon-glial signaling seems to trigger localized oligodendroglial MBP translation and myelination [20–23]. Decentralized MBP production appears to be a useful and efficient solution as oligodendrocytes myelinate multiple axonal segments and must locally adjust the myelin synthesis machinery to heterogeneous axonal properties (such as varying diameter or activity). The physiological mechanisms controlling local synthesis of MBP however remain elusive.

Ca^{2+} signaling has been suggested to contribute to OPC maturation and myelination [24,25]. Over-expression of golli myelin basic proteins induces slow Ca^{2+} oscillations in OPCs, stimulating in turn OPC migration [11] but resulting in hypomyelination *in vitro* and *in vivo* [26]. On the other hand, MBP has been reported to inhibit depolarization-induced Ca^{2+} influx in more mature oligodendrocytes [27]. Interestingly, intracellular monovalent ions, including Na^{+}, as well as divalent cations, including Ca^{2+}, have been shown to influence both MBP folding [28] and MBP-lipid interaction [29]. Therefore, it may be hypothesized that changes in local cation concentrations can at least modulate local MBP synthesis, while MBP itself can serve as a negative feedback that fixes ion concentrations at required "mature" levels.

Temporal changes of ion levels in OPCs during development and their impact on MBP synthesis are only poorly investigated. We report that $[Na^{+}]i$ and $[Ca^{2+}]i$ in OPCs both in cultures and in acute brain callosal slices demonstrate developmental changes. As cultured OPCs are capable of synthesizing MBP in the absence of neurons, we asked whether the time-course of MBP synthesis *in vitro* can be influenced by treatments which alter $[Na^{+}]i$ and $[Ca^{2+}]i$. We show that MBP synthesis can be potentiated by elevations of $[Na^{+}]i$ and suppressed by $[Na^{+}]i$ reductions, and these effects are predominantly mediated by NCX. Treatment of OPC cultures with elevated $[K^{+}]e$ stimulates MBP synthesis, indicating that activity-dependent $[K^{+}]e$ changes can potentially modulate local MBP synthesis in OPCs.

2. Materials and methods

All experiments were carried out according to the guidelines for the care and use of laboratory animals of the University Medical Center Mainz and the European Communities Council Directive 86/609/EEC. The study was approved by the local ethical committee (#23177-07/G10-1-010). Experiments were designed to minimize the number of animals used.

2.1. Cell culture

Primary oligodendrocyte precursor cells (OPCs) were obtained from C57BL/6N mice at postnatal day (P) 8–10 by magnetic cell sorting (MACS). Briefly, whole brains were dissociated with the Neural Tissue Dissociation Kit (Papain) in a gentle MACS-Dissociator (MiltenyiBiotec) according to the manufacturer's protocol. Cells were re-suspended in DMEM + 1% horse serum. OPCs were marked with anti-AN2 MicroBeads. Cells were sorted on a magnetic MACS Separator (MACS, MiltenyiBiotec) and washed with DMEM + 1% horse serum. AN2-positive OPCs were removed from the LS columns in culture medium (MACS Neuro Medium containing 2% (v/v) NeuroBrew-21, 100 U/ml Penicillin/Streptavidin and 2 mM L-Glutamine) and plated on 0.1% poly-L-lysine coated culture dishes or glass cover slips. Every second day half of the medium was replaced with fresh MACS Neuro Medium.

2.2. Cell lysates

Cells were harvested by scraping them off the culture dish in cold lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton-X-100, containing protease and phosphatase inhibitor mixtures (Roche)). Lysates were incubated on a rotating wheel for 45 min at 4 °C and cleared from nuclei and cellular debris by centrifugation for 5 min at 2000*g* and 4 °C. The supernatants were transferred to new reaction tubes.

2.3. SDS-PAGE & Western blotting

Samples were separated with 14% sodium dodecyl sulfate polyacrylamide gels using the Mini-PROTEAN Tetra cell system (Biorad) at 175 V for 60 min. Proteins were transferred to polyvinylidene-fluoride blotting membranes at 125 V for 90 min. Membranes were blocked in Tris-buffered saline containing 0.01% Tween 20 and 4% milk powder for 30 min. Antibodies directed against MBP (1:500, Serotec), CNP (1:500, Sigma-Aldrich), GAPDH (1:5000, Bethyl) and Tubulin (1:5000, Sigma-Aldrich) were diluted in blocking buffer, incubated for 1 h at room temperature and detected with horseradish peroxidase (HRP)-coupled secondary antibodies (1:10000, Dianova) in an enhanced chemiluminescence reaction. Images were acquired with a ChemiDoc™ XRS+ System (Bio-Rad) and band intensities were quantified using the Image lab software (Bio-Rad).

2.4. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton-X-100 in PBS. DMEM + 10%HS was used for 15 min of blocking and primary antibodies (ratαMBP, Serotec, 1:50; mouseαCNP, Sigma, 1:50) were diluted in blocking buffer and incubated for 1 h. For detection, Cy2 and Cy3 coupled secondary antibodies (Dianova) were diluted in blocking medium and incubated for 30 min at room temperature. Nuclei were stained with DAPI (Sigma, 1:7500) and cells were mounted in Mowiol. Images were acquired by using a 40x/0.75NA objective connected to a monochrome fluorescence CCD-camera (XM10, Olympus) and using cell F software (Olympus). Images were adjusted with Adobe Photoshop.

2.5. Brain slices preparation

All experiments were conducted with pigmented C57BL/6J mice pups of P10-35. Animals were decapitated under deep isoflurane anesthesia. The brain was removed quickly and transferred into ice-cold saline that contained (in mM): 125 NaCl, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, and 2.5 $MgCl_2$ constantly bubbled with 5% CO_2/95% O_2 mixture (pH = 7.3). Coronal slices (300 μM) were cut on a vibratome (Campden Instruments Ltd., UK). After preparation, slices were stored for at least 30 min at room temperature in artificial cerebrospinal fluid (ACSF) that contained (in mM): 125 NaCl, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 1 $MgCl_2$. pH was buffered to 7.3 by continuous bubbling with 5% CO_2/95% O_2 mixture. ACSF with elevated $[K^{+}]$ was prepared by equimolar replacement of 5 mM NaCl with 5 mM KCl.