Cell Calcium 73 (2018) 1–10

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

α2 isoform of Na^+,K^+-ATPase via Na^+,Ca^{2+} exchanger modulates myelin basic protein synthesis in oligodendrocyte lineage cells *in vitro*

Jens Hammann, Davide Bassetti, Robin White, Heiko J. Luhmann, Sergei Kirischuk*

Institute of Physiology, University Medical Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, D-55128 Mainz, Germany

ARTICLE INFO

Keywords:
Ca^{2+} signaling
Spontaneous Ca^{2+} transients
Na^+ signaling
Reversal potential

ABSTRACT

Oligodendrocytes in the CNS myelinate neuronal axons, facilitating rapid propagation of action potentials. Myelin basic protein (MBP) is an essential component of myelin and its absence results in severe hypomyelination. In oligodendrocyte lineage cell (OLC) monocultures MBP synthesis starts at DIV4. Ouabain (10 nM), a Na^+,K^+-ATPase (NKA) blocker, stimulates MBP synthesis. As OLCs express the α2 isoform of NKA (α2-NKA) that has a high affinity for ouabain, we hypothesized that α2-NKA mediates this effect. Knockdown of α2-NKA with small interfering (si)RNA (α2-siRNA) significantly potentiated MBP synthesis at DIV4 and 5. This effect was completely blocked by KB-R7943 (1 μM), a Na^+,Ca^{2+} exchanger (NCX) antagonist. α2-NKA ablation increased the frequency of NCX-mediated spontaneous Ca^{2+} transients ($[Ca^{2+}]$t) at DIV4, whereas in control OLC cultures comparable frequency of $[Ca^{2+}]$t was observed at DIV5. At DIV6 almost no $[Ca^{2+}]$t were observed either in control or in α2-siRNA-treated cultures. Immunocytochemical analyses showed that α2-NKA co-localizes with MBP in proximal processes of immature OLCs but is only weakly present in MBP-enriched membrane sheets. Knockdown of α2-NKA in cortical slice cultures did not change MBP levels but reduced co-localization of neurofilament- and MBP-positive compartments. We conclude that α2-NKA activity in OLCs affects NCX-mediated $[Ca^{2+}]$t and the onset of MBP synthesis. We suggest therefore that neuronal activity, presumably in form of local extracellular $[K^+]$ changes, might locally influence NCX-mediated $[Ca^{2+}]$t in OLC processes thus triggering local MBP synthesis in the vicinity of an active axon.

1. Introduction

Myelination of axons by oligodendrocytes in the central nervous system (CNS) is a complex process requiring the synthesis of massive amounts of proteins and lipids. Neuronal activity appears to provide a signal between neurons and oligodendrocyte precursor cells (OPCs), instructing the latter to myelinate active connections [1–3]. Axons are capable of vesicular glutamate [4,5] and ATP release [6], while OPCs express several receptors, including those for glutamate and ATP ([4,7–10], for review [11]). However, neuronal fibers contain only a small number of vesicles and the axon-OPC cleft is relatively wide [4,5]. This leads to the assumption that in addition to neurotransmitters other pathways may exist to mediate neuron-OPC interactions.

Neuronal activity leads to an elevation in the extracellular K^+ concentration ($[K^+]$e). The latter can induce $[Ca^{2+}]$i responses in oligodendrocyte lineage cells (OLCs) through activation of voltage-gated Ca^{2+} channels [12,13] and/or reversal of Na^+-Ca^{2+} exchanger (NCX) [14,15]. Genetic knockout of NCX3 reduces Myelin Basic Protein (MBP) synthesis in OLC cultures and leads to a marked hypomyelination in the spinal cord of NCX3-knockout mice ([16], for review [17]). As NCX is an electrogenic antiporter, the direction of its function depends on Ca^{2+} and Na^+ transmembrane gradients and membrane potential. Recently we have shown that small $[K^+]$e elevation (+5 mM) induces $[Ca^{2+}]$i increase and stimulates MBP synthesis in cultured OLCs in a NCX-dependent manner [18]. We hypothesize that under resting conditions the resting membrane potential is kept close to the NCX reversal potential and neuronal activity can lead to a NCX-mediated Ca^{2+} influx. If this is the case, $[Na^+]$i elevation should also induce $[Ca^{2+}]$i transients and stimulate MBP synthesis. Indeed, a small $[Na^+]$i elevation induced by low dose (500 nM) of ouabain, a Na^+,K^+-ATPase (NKA) blocker, is sufficient to potentiate MBP synthesis [18].

NKA is an ubiquitous plasma membrane enzyme primarily responsible for maintenance of transmembrane gradients for Na^+ and K^+ ions. Minimal functional NKA is a heterodimer of a catalytic (α) subunit

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; OLC, oligodendrocyte lineage cell; OPC, oligodendrocyte precursor cell; OGB1, Oregon Green BAPTA 1; SBFI, sodium-binding benzofuran isophthalate

* Corresponding author.
E-mail address: kirischu@uni-mainz.de (S. Kirischuk).

https://doi.org/10.1016/j.ceca.2018.03.003
Received 31 January 2018; Received in revised form 5 March 2018; Accepted 25 March 2018
Available online 31 March 2018
0143-4160/

and glycoprotein (β) subunit (for review [19,20]). Studies of functional catalytic and transport properties of this enzyme demonstrated the existence of several NKA isoforms. Further investigations revealed four different α subunits [21,22] and three β isoforms; some auxiliary proteins, like γ subunits (FXYD polypeptides), were also reported [23]. Various α subunits of NKA are non-homogeneously expressed in the CNS. α1-NKA isoform is ubiquitous and expressed by all cells. As α1-containing NKAs have high apparent affinities for $[Na^+]$i and $[K^+]$e, it may be assumed to play a housekeeping role in all cells. α3-NKA is expressed by neurons and is supposed to be activated by relatively high $[Na^+]$i occurring after bursts of action potentials. Glial cells (astrocytes and oligodendrocytes) mainly express the α2-NKA isoform. α2 isoform of NKA (α2-NKA) has a lower apparent affinity for $[Na^+]$i and $[K^+]$e and is voltage- and $[Ca^{2+}]$i-sensitive (for review [19,20,24]). Such properties of α2-NKA indicate that it may function as a neuronal activity sensor. Indeed, expression of α2-NKA was reported in neuron-OLC co-cultures and in the optic nerve during the myelination time period but not in OLC monocultures [25], suggesting its involvement in neuron-OLC interactions. Interestingly, in astrocytes α2-NKA distribution in the plasma membrane is identical to that of NCX, indicating that α2-NKA may modulate $[Ca^{2+}]$i signaling in a restricted cytosolic space [26].

MBP mRNA is transported from the nucleus to the plasma membrane in a translationally silenced state in large ribonucleoprotein complexes termed RNA granules [27]. Axon-glial signaling appears to trigger localized oligodendroglial MBP translation and myelination [28–31], but physiological mechanisms controlling local synthesis of MBP remain elusive. We have found that knock-down of α2-NKA with siRNA temporally shifts NCX-triggered spontaneous Ca^{2+} activity to earlier development and increases MBP levels in primary OLC cultures. Interestingly α2-NKA deletion in cortical slice cultures seems not to affect MBP levels but it impairs the axon wrapping.

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. Primary OLC cultures

Primary OLC cultures were prepared from postnatal day (P) 8–10 C57BL/6J mouse brains using MACS Technology (MiltenyiBiotec). The brains were dissociated with the Neuronal Tissue Dissocation Kit (Papain) and the gentle MACSDissociator (MiltenyiBiotec) according to the manufacturer's protocol. Instead of Ca^{2+} and Mg^{2+}-containing Hanks' Balanced Salt Solution (HBSS), the cells were resuspended in DMEM + 1% horse serum (HS). Isolation of OPCs was achieved by using the anti-AN2 (NG2 equivalent in mice) MicroBeads (MiltenyiBiotec). Isolated OPCs were subsequently plated on 0.01% poly-L-lysine-coated wells of 24-well plates (Greiner) in MACS Neuro Medium containing 2% (v/v) NeuroBrew-21 (MiltenyiBiotec), 2 mM L-glutamine and penicillin-streptomycin (100 U/ml). For imaging and immunocytochemistry experiments glass coverslips were placed in the wells before coating. Every second day half of the medium was replaced with new culture medium.

2.2. Antibodies/siRNA

Monoclonal antibodies were used against: CNPase (C5922, mouse; 1:50 for immunocytochemistry (ICC)) and α-tubulin (T6199, mouse, 1:5000 for Western Blot (WB)) purchased from Sigma-Aldrich; MBP (MCA409S, rat, 1:500 WB/IHC; 1:50 ICC) from AbDSerotec and SMI-312 (neurofilament, ab24574, mouse, 1:1000 immunohistochemistry (IHC)) from Abcam (UK). Polyclonal antibodies were used against: ATP1A2 (α2-NKA, 55179-1-AP, rabbit, 1:800 WB, 1:20 ICC) from Proteintech (USA).

Following siRNAs were used in this study: ATP1A2-siRNA (siGENOMESMARTpool siRNA, GCACUCCUCUGCUUCUUAG, GAUCAUCUCUUCCCACGGU, GGACUGGGAUGAUCGGACU, AUAUCUAGGUAUCGUGCUA) or negative control-siRNA (ON-TARGETplus Non-targeting pool, UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA). All siRNAs were purchased from Dharmacon (USA).

2.3. Cell lysis, SDS-PAGE and Western blotting

Cells were washed with cold PBS and afterwards scraped off the culture dish in ice cold lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease and phosphatase inhibitors (cOmplete mini EDTA-free and PhosSTOP, Roche, Switzerland). The lysates were incubated on a rotating wheel for 45 min at 4 °C and then cleared from nuclei and debris by centrifuging at 2000 × *g* and 4 °C for 5 min. The supernatants were transferred to new reaction tubes.

Proteins and molecular mass markers (Bio-Rad or NEB, USA) were separated by SDS-Page using a Mini PROTEAN system (Bio-Rad) with 14% sodium dodecyl sulfate polyacrylamide gels at 175 V for 60 min or a Novex NuPAGE SDS-PAGE Gel system (Life technologies or Thermo Fischer scientific, USA) at 200 V for 50 min (MOPS buffer) and transferred onto Roti-polyvinylidenefluoride membranes (0.45 µm, Roth).Membranes were blocked in Tris buffered saline containing 0.01% Tween 20 and 4% milk powder for 30 min, probed with antibodies 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). Densitometric analysis was performed by using the Image lab software (Bio-Rad).

2.4. Immunostainings of OLC cultures and siRNA transfections

For protein immunostainings OLC cultured were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton-X-100 in PBS. DMEM + 10%HS was used as blocking buffer for 15 min and primary antibodies were diluted in blocking buffer. OLC cultures were incubated in this medium for 1 h. For detection, Cy2, Cy3 and Cy5 coupled secondary antibodies (Dianova) were diluted in blocking medium and incubated for 30 min at room temperature. Nuclei were stained with DAPI (Sigma, 1:1000) and cells were mounted in Mowiol. Images were acquired by using a 40 × /0.75 NA objective lens connected to a monochrome fluorescence CCD-camera (XM10) and using cell^F software (Olympus). Images were adjusted with Adobe Photoshop and ImageJ software.

Cells for western blot analysis and for immunocytochemistry were transfected with 40 pmol siRNA. The tissue for cortical slice cultures was transfected with 80 pmol siRNA. In all cases the transfection was performed at DIV2 using Lipofectamine RNAiMax (Life Technologies).

2.4.1. Evaluation of MBP and α2-NKA co-localization

The co-localization of α2-NKA and MBP in the resulting images was quantified using the JACoP plug-in for ImageJ software ([32], NIH, Bethesda, USA), which allowed the calculation of Pearsońs coefficient. This measure reflects linear correlation between the signal intensities and can range from −1 (mutual exclusion) to 1 (perfect co-localization). Costeś automatic thresholding algorithm was used to exclude from the calculation background pixels in an observer-independent way [33].

Three to four regions of interest with fixed dimensions (100 µm^2) were manually selected in order to isolate specific cellular structures,