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Crosslink of calcium and sodium signalling in health and disease

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New Findings

What is the topic of this review?
This paper overviews the links between Ca\(^{2+}\) and Na\(^{+}\) signalling in various types of cells.

What advances does it highlight?
This paper highlights the general importance of ionic signalling and overviews the molecular mechanisms linking Na\(^{+}\) and Ca\(^{2+}\) dynamics. In particular the narrative focuses on the molecular physiology of plasmalemmal and mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger and plasmalemmal TRP
channels. Functional consequences of Ca$^{2+}$ and Na$^+$ signalling for coordination of neuronal activity with astroglial homeostatic pathways fundamental for synaptic transmission are discussed.

Abstract

Transmembrane ionic gradients, that are indispensable feature of life, are utilised for generation of cytosolic ionic signals that regulate a host of cellular functions. Intracellular signalling mediated by Ca$^{2+}$ and Na$^+$ is tightly linked through several molecular pathways that generate Ca$^{2+}$ and Na$^+$ fluxes and are in turn regulated by both ions. Transient receptor potential (TRP) channels bridge endoplasmic reticulum Ca$^{2+}$ release with generation of Na$^+$ and Ca$^{2+}$ currents. Plasmalemmal Na$^+$/Ca$^{2+}$ exchanger by flickering between forward and reverse mode coordinates influx and efflux of both ions with membrane polarisation and cytosolic ion concentration. The mitochondrial calcium uniporter channel (MCU) and mitochondrial Na$^+$/Ca$^{2+}$ exchanger, NCLX, mediate Ca$^{2+}$ entry into and release from this organelle and couple cytosolic Ca$^{2+}$ and Na$^+$ fluctuations with cellular energetics. Cellular Ca$^{2+}$ and Na$^+$ signalling controls numerous functional responses and, in the CNS, provides for fast regulation of astroglial homeostatic cascades that are critical for maintenance of synaptic transmission.

Key words: Ca$^{2+}$ signalling, Na$^+$ signalling, Na$^+$/Ca$^{2+}$ exchanger, mitochondria, mitochondrial calcium uniporter, TRP channels, astrocytes, neuropathology

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Principles of ionic signalling

Transmembrane ionic gradients are indispensable feature of life; the failure in keeping intracellular ionic composition invariably instigates cell death. Preservation of transmembrane ion gradients consumes the major part of cellular energy recourses: it has been conjectured that the brain, for example, spends 75% of all ATP for restoration of Na\(^+\)/K\(^+\) balance chronically disturbed by synaptic transmission and action potentials (Magistretti, 2009). How transmembrane ion gradients emerged in the early evolution remains a matter of conjecture and speculations; most likely the ionic composition of the primeval cells was defined by concentration of ions in the primordial ocean (which arguably, contained few free Ca\(^{2+}\) ions), by selectivity of membrane aqueous pores (the ancestral ion channels) and by Donnan forces (Donnan, 1911; Case et al., 2007; Plattner & Verkhratsky, 2015; Chatton et al., 2016; Plattner & Verkhratsky, 2016).

In this review we shall concentrate on the signalling links between Ca\(^{2+}\) and Na\(^+\). In both excitable and non-excitable cells fluxes of Na\(^+\) and Ca\(^{2+}\) are coordinated by cationic channels and plasmalemmal and mitochondrial Na\(^-\)-Ca\(^{2+}\) exchangers. Steep transmembrane Na\(^+\) gradients, in addition, furnish the energy for plasmalemmal transport of many molecules including other ions, neurotransmitters or amino acids.

Sodium-calcium exchanger: the key element of Na\(^+\)/Ca\(^{2+}\) signalling

Why is NCX so important for Na\(^+\) and Ca\(^{2+}\) signalling?

Although numerous ion channels and transporters affect the cytosolic concentration of Na\(^+\) and Ca\(^{2+}\), the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) is the only system that directly couples opposite fluxes of Na\(^+\) and Ca\(^{2+}\) across the plasmalemma. This makes the NCX the key molecule integrating Na\(^+\) and Ca\(^{2+}\) signalling pathways. The NCX proteins mediate an electrogenic exchange of 3Na\(^+\) for 1Ca\(^{2+}\), while the net ion-flux can occur either in the forward (Ca\(^{2+}\)-extrusion/Na\(^+\)-entry coupling) or in the reverse (Ca\(^{2+}\)-entry/Na\(^+\)-extrusion) modes (Blaustein & Lederer, 1999; Bers, 2002; Khananshvili, 2014). Since the NCX is electrogenic, membrane depolarisation and increase in [Na\(^+\)], promote the reverse mode of NCX, whereas hyperpolarisation or elevated [Ca\(^{2+}\)], favour the forward mode (Kirischuk et al., 1997; Blaustein & Lederer, 1999; Bers, 2002). Thus, from one side, the Na\(^+\) and Ca\(^{2+}\) gradients tightly control the kinetics and directionality of NCX operation, whereas any changes in NCX activity dynamically affect Na\(^+\) and Ca\(^{2+}\) fluxes. This complex feedback loop involves both thermodynamic...
and kinetic interactions of Na\(^+\) and Ca\(^{2+}\) with ion-transport and regulatory domains of NCX (Bers, 2002; Kirischuk et al., 2012; Kardos et al., 2017; Oheim et al., 2017).

**Forward and reverse modes of NCX – tightrope walking on the acrobat wire**

Dynamic coupling of NCX regulation with Na\(^+\) and Ca\(^{2+}\) signalling is particularly relevant in specific cells types (e.g. in astrocytes) where the reversal potential of NCX (E\(_{\text{NCX}}\)) is close to the resting membrane potential of the cell (E\(_{\text{m}}\) ~ −80 mV). Since the ion-exchange turnover rates of mammalian NCXs are quite high (~5000 s\(^{-1}\)), even small changes in allosteric regulation of NCX (Blaustein & Lederer, 1999; Bers, 2002; Khananshvili, 2014) might instantly affect the coupled Na\(^+\) and Ca\(^{2+}\) signalling (Robinson & Jackson, 2016; Kardos et al., 2017; Oheim et al., 2017). Even small anomalies in allosteric regulation of NCX or coupled ion-transport systems may end-up with devastating outcomes (Wagner et al., 2015; Boscia et al., 2016). For example, Na\(^+\)-driven uptake of neurotransmitters (e.g., GABA or glutamate) elevates \([\text{Na}^+]_i\), thus promoting Ca\(^{2+}\) entry through NCX operating in the reverse mode and increase \([\text{Ca}^{2+}]_i\) with ensuing excitotoxicity (Kirischuk et al., 1997; Boscia et al., 2016; Rose & Verkhratsky, 2016). Moreover, when E\(_{\text{NCX}}\) and E\(_{\text{m}}\) are close, any small changes in the extracellular \([\text{K}^+]_o\) (for example released from neurones during repolarisation) may change membrane potential thus affecting the rates and directionality of NCX.

**Regulatory diversity of NCX variants is structurally predefined**

In mammals, three gene isoforms (NCX1, NCX2, and NCX3) and their splice variants are expressed in a tissue-specific manner, while NCX1 and NCX3 (but not NCX2) undergo alternating splicing (Philipson & Nicoll, 2000; Lytton, 2007). NCX1 is distributed ubiquitously; NCX2 is mainly expressed in the brain and in the spinal cord; whereas NCX3 is found in the brain and in the skeletal muscle. NCX proteins comprise ten transmembrane helices (TM1-TM10 - (Liao et al., 2012)), where a cytoplasmic f-loop (between TM5 and TM6) contains two Ca\(^{2+}\)-binding regulatory domains, CBD1 and CBD2 (Hilge et al., 2006) (Fig. 1). CBD1 of all isoform/splice variants contains a high-affinity allosteric sensor (K\(_d\) ~ 0.2 µM) with four Ca\(^{2+}\) binding sites. The CBD2 domain contains a splicing segment, which controls the affinity (K\(_d\) = 2 - 200 µM) and the number (from zero to three) of Ca\(^{2+}\) binding sites at CBD2 (Boyman et al., 2009; Giladi et al., 2012; Giladi et al., 2015; Lee et al., 2016; Tal et al., 2016; Giladi et al., 2017). Cytosolic Na\(^+\) inactivates NCX1 and NCX3 (but not NCX2) by interacting with yet unknown sites, located outside the CBDs (Giladi et al., 2016a; Giladi et al., 2016b; Khananshvili, 2016). Cytosolic Ca\(^{2+}\) binding to CBD1 activates (up to 25-fold) all mammalian NCXs, whereas Ca\(^{2+}\) binding to CBD2 alleviates Na\(^-\)-induced inactivation in NCX1 and NCX3 (Hilge et al., 2006; Boyman et al., 2009; Boyman et al., 2011; Giladi et al., 2012). Notably, a lack of substantial and long-lasting Na\(^-\)-transients underscores no need for Ca\(^{2+}\)-dependent alleviation of Na\(^-\)-
dependent inactivation of NCX variants in neurones. In contrast, in glia the NCX1 and NCX3 variants undergo Na\(^+\)-induced inactivation and only Ca\(^{2+}\) binding to CBD2 in specific variants can alleviate the activity. Since NCX2 is insensitive to regulatory Na\(^+\), this isoform (forming no splice variants) is capable of retaining its activity in glia at high-amplitude and prolonged Na\(^+\)-transients (Philipson & Nicoll, 2000; Lytton, 2007; Giladi et al., 2012; Giladi et al., 2015; Giladi et al., 2016a; Giladi et al., 2016b; Khananshvili, 2016; Tal et al., 2016).

**So many isoform/splice variants of NCX – are they functionally relevant?**

In NCX1, alternative splicing arises from mixing of six small exons (A,B,C,D,E, and F), where the mutually exclusive exons, A and B, are expressed in excitable and non-excitable tissues, respectively (Philipson & Nicoll, 2000; Lytton, 2007). In NCX3, alternative splicing involves only three exons (A, B, and C), whereas both mutually exclusive exons, A and B, are expressed in neurones and glia (Giladi et al., 2015; Chu et al., 2016; Lee et al., 2016; Tal et al., 2016; Giladi et al., 2017). Exon A and B differentially affect Ca\(^{2+}\) binding to CBD2 in NCX1 and NCX3 variants, which is controlled by single-point mutations at positions 552, 578 and 585 (Fig. 1). More specifically, in NCX1 exon B prevents Ca\(^{2+}\) binding to CBD2, whereas in NCX3, exon A precludes Ca\(^{2+}\) binding to CBD2 (Tal et al., 2016). This also applies to NCX1 splice variants expressed in non-excitable tissues. For example, in the kidney (BD) and in the pancreas (BDF) splice variants of NCX1 do not bind Ca\(^{2+}\) at CBD2, since Ca\(^{2+}\) - dependent alleviation of Na\(^+\)-induced inactivation is not functionally relevant for these NCX variants (Giladi et al., 2012; Giladi et al., 2016b).

**NCX variants differentially shape Na\(^+\) and Ca\(^{2+}\) signalling**

In general, Na\(^+\)-dependent inactivation can transiently inactivate NCX to prevent a toxic Ca\(^{2+}\) entry through NCX operating in the reverse mode (Giladi et al., 2016b; Khananshvili, 2016). For example, a massive and rapid entry of Na\(^+\) through Na\(^+\) channels and Na\(^+\)-dependent transporters can transiently elevate sub-plasmalemmal [Na\(^+\)]\(_i\), which can instantly promote Ca\(^{2+}\) entry through the reverse mode of the NCX. This may occur if a given NCX variant lacks Na\(^+\)-dependent inactivation (e.g. NCX2 or NCX3-AC). This mechanism could be beneficial when a parallel increase in both [Na\(^+\)]\(_i\) and [Ca\(^{2+}\)]\(_i\)], is required. At the same time this may lead to Ca\(^{2+}\) and Na\(^+\) overload if the relevant regulatory systems fail to terminate this process. Different modes of regulation are expected for NCX1-AD, NCX3-B and NCX3-BC variants since they are sensitive to Na\(^+\)-induced inactivation and thus can prevent substantial Ca\(^{2+}\) entry through NCX. Of note, NCX3-B and NCX3-BC may recover from Na\(^+\)-induced inactivation at much lower concentrations of cytosolic Ca\(^{2+}\) than NCX1-AD, since NCX3-B and NCX3-BC bind Ca\(^{2+}\) to CBD2 with much higher affinity than NCX1-AD (Giladi et al., 2012; Tal et al., 2016). Thus, four isoform/splice variants (NCX1-AD, NCX2, NCX3-B and NCX3-BC) expressed in

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neurones and glia have quite diverse and distinct regulatory properties, which can specifically suite for dynamic coupling of Na\(^+\) and Ca\(^{2+}\) movements.

Another important factor that has to be taken into account for dynamic coupling of NCX regulation with Na\(^+\) and Ca\(^{2+}\) fluxes is that mammalian NCX variants show nearly 100-fold differences in slow dissociation of "occluded" Ca\(^{2+}\) from allosteric sensor that has a comparable affinity for Ca\(^{2+}\) binding \((K_d = 0.2-0.5 \, \mu M)\) among NCX variants (Boyman et al., 2009; Giladi et al., 2012; Tal et al., 2016). Moreover, the observed differences in the dissociation kinetics of regulatory Ca\(^{2+}\) match inactivation kinetics of NCX variants upon removal of cytosolic Ca\(^{2+}\) (Giladi et al., 2012; Giladi et al., 2016b; Khananshvili, 2016; Tal et al., 2016). Only one isoform/splice variant is expressed in cardiac (NCX1-ACDEF) or skeletal (NCX3-AC) muscle, whereas a number of variants (NCX1-AD, NCX2, NCX3-B and NCX3-BC) coexist in neurones and neuroglia (Giladi et al., 2012; Giladi et al., 2015; Tal et al., 2016). Moreover, the rate of regulatory Ca\(^{2+}\) dissociation (and thus, the rate of NCX inactivation) is ~10-times slower in the cardiac than in the skeletal variant (Giladi et al., 2015; Tal et al., 2016) and is ~10-times slower in the skeletal variant than in neuronal NCX variants (Tal et al., 2016). These 100-fold differences among NCX variants, expressed in distinct excitable tissues, might have physiological relevance since NCX in the cardiac and skeletal myocytes must clear up (extrude) incomparably much more cytosolic Ca\(^{2+}\) (released from the sarcoplasmic reticum) than in neurones (Giladi et al., 2016b; Khananshvili, 2016).

**Subcellular allocation of NCX variants – a crucial factor for integrating global events?**

Computer-aided modelling reveals NCX can significantly contribute to Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) in cardiomyocytes (Chu et al., 2016), when taking into account experimentally obtained parameters of allosteric regulation of cardiac NCX and high-resolution cell-imaging of subcellular NCX location in cardiomyocyte (Chu et al., 2016). Increasing the fraction of NCX in the dyad and peridyadic (PD) domains decreases frequency and fidelity of Ca\(^{2+}\) sparks, as well as diastolic \([\text{Ca}^{2+}]_i\), although amplitude and duration of Ca\(^{2+}\) sparks are less sensitive to NCX spatial redistribution. Moreover, NCX can promote Ca\(^{2+}\) entry into the dyad to trigger the SR Ca\(^{2+}\) release through RyR at depolarized membrane potentials and elevated local Na\(^+\) concentrations. A relatively high fraction of NCX (~48%) in the dyadic and PD spaces (with a dyad-to-PD ratio of roughly 1:2) promotes the Ca\(^{2+}\)-dependent allosteric activation of NCX as compared with NCX molecules bordering the bulk cytosol. This site-confined allosteric activation of NCX can "functionally localize" exchanger activity to the dyad and PD areas to reduce NCX-mediated Ca\(^{2+}\) extrusion from the bulk of the cytosol. This may protect the cell from acute loss of Ca\(^{2+}\) during diastole (and thus, from arrhythmia), since NCX can generate depolarizing currents through the forward mode in response to "spontaneous" Ca\(^{2+}\) release from the SR.
Transient receptor potential (TRP) channels: Major contributors to cytosolic Na\(^+\) and Ca\(^{2+}\) signals

Because of their unique ability to conduct both Na\(^+\) and Ca\(^{2+}\), transient receptor potential (TRP) channels are particularly important for coordinating signalling between the cell membrane and intracellular organelles. The TRP superfamily includes 28 members, which can be classified into six different families based on sequence homology (Nilius et al., 2005b; Earley & Brayden, 2015). These families are: seven canonical (TRPC), eight melastatin (TRPM), six vanilloid (TRPV), three mucolipin (TRPML), three polycystin (TRPP) and one ankyrin (TRPA) members. The seven TRPCs are called canonical because of their close homology to the founding member, Drosophila TRP (Vazquez et al., 2004; Trebak et al., 2007). All TRP proteins have six transmembrane domains (S1–S6) with intracellular N- and C-termini. They form functional tetrameric channels in humans with the exception of TRPC2 which is a pseudogene. However, TRPC2 is functionally expressed in rodents and plays a crucial role in pheromone sensing (Liman et al., 1999). Substantial controversy surrounds the role of TRP channels (and TRPC in particular) in encoding molecular components of the store-operated Ca\(^{2+}\) entry (SOCE) pathway, which is a ubiquitous Ca\(^{2+}\) entry route into cells activated downstream of phospholipase (PLC) coupled receptors (Trebak & Putney, 2017). SOCE is activated upon endoplasmic reticulum (ER) Ca\(^{2+}\) store depletion following inositol-1,4,5-trisphosphate receptor (InsP\(_3\)R)-mediated Ca\(^{2+}\) release (Trebak & Putney, 2017). The discovery of STIM and ORAI proteins as the bona fide SOCE molecular components has cast a shadow of doubt on the role of TRPC proteins in SOCE (Liou et al., 2005; Roos et al., 2005; Feske et al., 2006; Vig et al., 2006; Zhang et al., 2006). STIM proteins act as Ca\(^{2+}\) sensors in the ER that perceive store depletion and link this depletion through direct protein-protein interactions to ORAI channels at the plasma membrane, thus instigating Ca\(^{2+}\) influx. TRPCs appear to be either concomitantly activated through PLC-mediated production of second messengers such as diacylglycerol (DAG)-mediated activation of TRPC3/6/7 (Trebak et al., 2003) or subsequently activated by Ca\(^{2+}\) microdomains generated by ORAI Ca\(^{2+}\) selective channels (e.g. TRPC1/4/5) (Trebak & Putney, 2017). At the same time TRPC1/3/5 channels seem to be the main molecular component of SOCE in astroglia (Reyes et al., 2013; Verkhratsky & Parpura, 2014).

How Na\(^+\) conductance by TRP shapes Ca\(^{2+}\) signalling in cell microdomains? For example, TRPM4 and TRPM5 channels are essentially impermeant to Ca\(^{2+}\) ions (Liman, 2007). These channels have relatively higher Na\(^+\) permeability compared to K\(^+\) and mediate large inward currents under physiological membrane potentials and buffering conditions. Indeed, TRPM4 have profound depolarizing effects and as such can greatly alter Ca\(^{2+}\) influx. TRPM4 activity is regulated by micromolar concentrations of cytosolic Ca\(^{2+}\) and this regulation is mediated by direct interaction of Ca\(^{2+}\)-calmodulin complex with TRPM4 C-terminal region. Therefore, these interactions are likely to occur at microdomains of high [Ca\(^{2+}\)]\(_i\), near the plasma membrane or at the ER-plasma membrane junctions. TRPM4 channels were shown to be activated as a result of local Ca\(^{2+}\) release from the ER via the InsP\(_3\)R (Gonzales et al., 2010; Gonzales & Earley, 2012). TRPM4-mediated phosphorylation by
PKC was also shown to enhance the sensitivity of TRPM4 to activation by Ca\(^{2+}\) (Nilius et al., 2005a). Na\(^{+}\) signals generated by TRPM4 channels have been shown to impinge on a wide range of physiological functions including membrane excitability of neurons, pancreatic β-cells, smooth muscle cells from bladder and different vascular beds as well as atrial cardiomyocytes (Mathar et al., 2014). Furthermore, TRPM4 is crucial for maintenance of membrane potential in non-excitible cells such as dendritic and hematopoietic cells. Dendritic and mast cells from TRPM4 knockout mice show membrane hyperpolarisation due to lack of depolarizing TRPM4 Na\(^{+}\) currents which enhances the driving force for Ca\(^{2+}\) entry into the cytosol, causes Ca\(^{2+}\) overload and alters cellular function (Mathar et al., 2014).

Another example is endothelial permeability to G protein-coupled receptor (GPCR) agonists which depends on TRPC channels, mainly TRPC1, TRPC4 and TRPC6. Increase in \([\text{Ca}^{2+}]\), was proposed to activate the endothelial actomyosin cytoskeleton to induce stress fiber formation and inter-endothelial cell gaps. However, subsequent studies showed that endothelial SOCE is mediated by STIM1 and ORAI1 independently of TRPC1, TRPC4 or TRPC6 (Abdullaev et al., 2008). Knockdown of ORAI1 or pharmacological abrogation of plasmalemmal Ca\(^{2+}\) entry and Ca\(^{2+}\) release from the ER did not affect the ability of GPCR agonists to modulate the permeability of human microvascular endothelial cells (Stolwijk et al., 2015; Stolwijk et al., 2016). These findings suggest that TRPC role in modulating endothelial permeability could be mediated by Na\(^{+}\) entry through these channels. Therefore, while it is evident that TRP channels contribute to Ca\(^{2+}\) signals that control a wide variety of physiological functions, and while it remains challenging to separate the contributions of Ca\(^{2+}\) versus Na\(^{+}\) signals generated by TRP channels, Na\(^{+}\) entry through TRP channels is likely a major contributor to many if not all of these functions. Unfortunately, dyes and protocols for Na\(^{+}\) measurements within cellular compartments with high temporal and spatial resolution are, as yet, not available. Achieving such measurements will shed light on the contribution of Na\(^{+}\) to cell signalling and function and would enhance our knowledge of mechanisms of physiology and disease.

### Na\(^{+}\) and Ca\(^{2+}\) signalling in regulation of mitochondrial function

**Molecular physiology of mitochondria Ca\(^{2+}\) and Na\(^{+}\) signalling**

Mitochondrial Ca\(^{2+}\) cycles are linked to several critical processes. During physiological cell stimulation, Ca\(^{2+}\)-binding proteins located at the outer mitochondrial membrane sense cytosolic gradients of Ca\(^{2+}\) and move the organelle on the cytoskeletal network rail toward cell regions of intense Ca\(^{2+}\) signalling, the so called Ca\(^{2+}\) hotspots near the endoplasmic reticulum (ER) and plasma...
membrane (Rizzuto & Pozzan, 2006; Pizzo et al., 2012). By regulating local Ca\(^{2+}\) concentrations at these regions, mitochondria can control the activity of multiple ER and plasma membrane channels that are strongly and allosterically modulated by Ca\(^{2+}\) (Giacomello et al., 2010) and thereby contribute to global cellular Ca\(^{2+}\) signals. (Tinel et al., 1999). Mitochondria are also linking cellular Ca\(^{2+}\) signalling to energy production, triggered by the stimulatory actions of Ca\(^{2+}\) on at least three enzymes of the Krebs cycle and F\(_0\)F\(_1\) ATPase (Szabadkai & Duchen, 2008, 2009). Such “coupling” by Ca\(^{2+}\) represents an effective feed-forward mechanism to meet rising energy demand in signalling cells (Hajnoczky et al., 1995; Jouaville et al., 1999). However, mitochondrial Ca\(^{2+}\) signalling also has a dark site. While the elevation of Ca\(^{2+}\) in the mitochondrial matrix plays essential physiological roles if transient, when sustained it leads to Ca\(^{2+}\) overload that in conjunction with oxidative stress represent a primary triggers of cell death (Duchen, 2000). Mitochondrial Ca\(^{2+}\) overload is often linked to impaired mitochondrial Ca\(^{2+}\) handling by both Ca\(^{2+}\) influx and efflux machineries and represents a hallmark event of ischemic and neurodegenerative disorders (Gandhi et al., 2009). A major breakthrough in understanding the link between mitochondrial Na\(^{+}\) and Ca\(^{2+}\) signalling came in 2010 when the gene for the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger was discovered. It was found to be a member of the NCX superfamily and was termed NCLX (Li+-permeable Na\(^{+}\)/Ca\(^{2+}\) exchanger) (Palty et al., 2010). In parallel the components of the mitochondrial Ca\(^{2+}\) uniporter complex (the MCU) were identified (Perocchi, 2010 #171).

**The in vivo pathophysiological role of MCU and NCLX-dependent Ca\(^{2+}\) signalling**

Given that mitochondrial Ca\(^{2+}\) homeostasis regulates many essential aspects of cellular physiology one could easily expect that loss-of-function mutations or genetic ablation of Ca\(^{2+}\) influx and efflux machineries would be incompatible with life. Knockout mouse models of two essential subunits of the uniporter, MCU and EMRE, generated by the International Mouse Phenotype Consortium confirm that a complete block of mitochondrial Ca\(^{2+}\) uptake in C57BL/6 strain leads to embryonic lethality. Surprisingly, MCU\(^{-/-}\) and EMRE\(^{-/-}\) heterozygous mice show quite different phenotypes. The first manifests a decreased cardiac stroke volume, abnormal fasting glucose level and fat amount, whereas the second show neurological defects and impairment of blood glucose homeostasis. Instead, MCU\(^{+/+}\) homozygous mice, which loose dominant-negative regulation on MCU, are viable but show vestibular impairment and electrocardiogram defects. Unexpectedly, the constitutive knockout of MCU in a mixed C57BL/6xCD1 mouse background resulted viable and with the exception of mild muscle weakness the mice lacked any significant phenotype (Herzig, 2013 #189). It remains unclear how the genetic background have such a profound impact but is possible that some kind of compensation (e.g. the activation of alternative routes for mitochondrial Ca\(^{2+}\) uptake) could take place. This concept is supported by results obtained in a heart-specific transgenic mouse model constitutively expressing a dominant-negative MCU isoform (DN-MCU) (Rasmussen et al., 2015; Wu et al., 2015). Although heart mitochondria from DN-MCU mice are unable to take up Ca\(^{2+}\), MCU appears to be dispensable for normal heart function and comes into play only under physiological conditions.
stress, such as during isoproterenol-induced fight or flight response. Conversely, a conditional MCU knockout model in adult heart lacked any fight or flight response and had no major defects in both normal conditions and after cardiac pressure overload induced by transverse aortic constriction (Kwong et al., 2015; Luongo et al., 2015).

In contrast to the mild phenotype of MCU KO a conditional knockout of NCLX led to rapid and fatal heart failure (Luongo et al., 2017). Lethality correlated with severe myocardial dysfunction. The cardiac pathology was largely attributed to mitochondrial Ca\(^{2+}\) overload, leading to enhanced generation of superoxide and necrotic cell death, which was partially prevented by genetic inhibition of mitochondrial permeability transition pore activation. On the other hand overexpression of NCLX in the mouse heart augmented mitochondrial Ca\(^{2+}\) clearance, and similarly to MCU KO it conferred protection against ischaemia-induced cardiomyocyte necrosis and heart failure (Luongo et al., 2017).

The physiological implication of cytosolic and mitochondrial Na\(^{+}\) signalling

One consequence of a 3Na\(^{+}\):1Ca\(^{2+}\) stoichiometry of NCLX is that NCLX is a high capacity mitochondrial Na\(^{+}\) transporter and therefore the major mitochondrial Na\(^{+}\) influx route (Maack et al., 2006). Another important functional feature of NCLX, linking it to global cellular Na\(^{+}\) signalling, is that the apparent \(K_m\) to cytosolic Na\(^{+}\) of ~ 10 mM is very close to resting cytosolic Na\(^{+}\) free concentrations. Hence, even a slight change in cytosolic Na\(^{+}\) will strongly modulate NCLX activity. Thus, NCLX is highly tuned to functionally interact with the Na\(^{+}\) plasma membrane-influx pathways, responding to changes in the cytosolic Na\(^{+}\) concentration (Fig. 3). Several studies underscore the importance of the Na\(^{+}\) signalling interaction of NCLX and the plasma membrane transporters. In pancreatic \(\beta\) cells, glucose-dependent depolarization leads to cytosolic Na\(^{+}\) influx via the voltage-gated Na\(^{+}\) channel (Sekler et al., 2013). This cytosolic Na\(^{+}\) wave is propagated to the mitochondria by NCLX, which is strongly activated by this cytosolic Na\(^{+}\) surge and maintains the mitochondrial Ca\(^{2+}\) level required for the metabolic activity used for ATP production that controls insulin secretion (Nita et al., 2014). The Na\(^{+}\)/K\(^{+}\) ATPase that controls the cytosolic Na\(^{+}\) concentrations participates in this Na\(^{+}\) crosstalk between the plasma membrane and the mitochondria.

Another example for Na\(^{+}\) signalling crosstalk is the interaction of NCLX with the nociceptive noxious heat activated receptor, TRPV1. The latter evokes membrane Na\(^{+}\) and Ca\(^{2+}\) fluxes, thereby triggering the depolarization required for activation of nociceptive neurones. Recent studies show that TRPV1 dependent Na\(^{+}\) fluxes are communicated to the mitochondria by NCLX (Kim & Usachev, 2009; Nita et al., 2016). They are required for activating mitochondrial Ca\(^{2+}\) signalling which in turn control cytosolic Ca\(^{2+}\) which allosterically regulates TRPV1 conductance (Nita et al., 2016).
Finally, the store-operated Ca\(^{2+}\) channel entry (SOCE) is the major Ca\(^{2+}\) influx pathway in non-excitable cells. It is activated by the ER Ca\(^{2+}\) store that harbour the Ca\(^{2+}\) sensor Stim1, which in turn dimerizes, physically interacts and activates the cell membrane Ca\(^{2+}\) channel. A recent study suggested that Na\(^{+}\) influx triggered by Ca\(^{2+}\) store depletion controls communication with the mitochondria by activating NCLX. In support of this model, omission of extracellular Na\(^{+}\), which prevents the cytosolic Na\(^{+}\) rise, inhibits NCLX activity. Activation of NCLX by Na\(^{+}\) flux invoked a mitochondrial redox response, required for preventing Orai1 inactivation by oxidation of its redox sensitive cysteine residue (Ben-Kasus Nissim et al., 2017).

**Astroglial Na\(^{+}\) and Ca\(^{2+}\) signalling and neurotransmission**

Astroglial Ca\(^{2+}\) signalling is generally acknowledged as a substrate for astrocyte excitability (Cornell-Bell et al., 1990; Finkbeiner, 1992; Verkhratsky & Kettenmann, 1996; Verkhratsky et al., 1998; Agulhon et al., 2008; Parpura et al., 2011; Verkhratsky et al., 2012; Volterra et al., 2014; Bazargani & Attwell, 2016). Organisation of astroglial Ca\(^{2+}\) signalling is complex and spatially compartmentalised with both plasmalemmal and intracellular channels and transporters contributing to global and local cytosolic Ca\(^{2+}\) dynamics (Rusakov, 2015; Shigetomi et al., 2016). In astroglial processes, and especially in distal processes, which form the synaptic cradle (Verkhratsky & Nedergaard, 2014), Ca\(^{2+}\) signals occur in a form of localised microdomains, which sometimes are associated with morphological structures such as “appendages” in Bergmann glial cells (Grosche et al., 1999) or perisynaptic leaflets (Shigetomi et al., 2016). Molecular pathways underlying these local Ca\(^{2+}\) signalling involve ER Ca\(^{2+}\) release mediated by all three types of Ins\(_P_3\) receptors (Ins\(_P_3\)R1/2/3) (Kirischuk et al., 1999; Di Castro et al., 2011; Sherwood et al., 2017) as well as plasmalemmal Ca\(^{2+}\) influx. This latter is mediated by ionotropic receptors (NMDA and AMPA glutamate receptors, \(\alpha_7\) acetylcholine receptors or P2X\(_{\gamma5}\) or P2X\(_7\) purinoceptors (Sharma & Vijayaraghavan, 2001; Verkhratsky & Kirchhoff, 2007; Lalo et al., 2011; Illes et al., 2012), TRP channels (Shigetomi et al., 2012; Verkhratsky et al., 2014) and NCX operating in reversal mode (Verkhratsky et al., 2012). Plasmalemmal Ca\(^{2+}\) entry is particularly important for generation of Ca\(^{2+}\) signals in perisynaptic astroglial processes, generally devoid of intracellular organelles including the ER (Verkhratsky & Nedergaard, 2014).

Incidentally, all these plasmalemmal pathways are associated with generating of Na\(^{+}\) fluxes. All astroglial ionotropic receptors, although having relatively high Ca\(^{2+}\) permeability (\(P_{Ca}/P_{monovalent}\) varies between 1 for AMPA receptors, 2-3 for NMDA and P2X1/5 receptors, ~6 for \(\alpha_7\) ACh receptors and > 10 for P2X\(_7\) receptors (Pankratov et al., 2009; Palygin et al., 2010)) mostly produce Na\(^{+}\) currents (reflecting much higher concentration of Na\(^{+}\) in the extracellular space). Similarly all TRP channels expressed in astrocytes (TRPV1, TRPA1 and PTCR1/3/5) are Na\(^{+}/Ca^{2+}\) channels (Reyes et al., 2013; Verkhratsky et al., 2014). Astroglial TRPC channels are main part of the store-operated mechanism.
(Verkhratsky & Parpura, 2014) and hence they link Na\(^+\) influx metabotropic stimulation that causes the depletion of the ER Ca\(^{2+}\) store (Verkhratsky et al., 2014). The second major pathway for Na\(^+\) influx into astrocytes is associated with operation of EAAT1/2 glutamate transporters that couple translocation of single glutamate molecule with influx of 3Na\(^+\) and 1H\(^+\) and countertransport of 1K\(^+\); this stoichiometry makes the transporter electrogenic and generates a substantial Na\(^+\) influx that may elevate [Na\(^+\)]\(_i\) by 10\(^-\)20 mM (Kirischuk et al., 2007; Rose & Verkhratsky, 2016). Astroglial NCX (as has been discussed above) fluctuates between forward and reverse mode (because E\(_{\text{NCX}}\) is close to astroglial V\(_m\)) depending on [Na\(^+\)], and changes in membrane potential. Finally the extrusion of Na\(^+\) and maintenance of resting cytosolic Na\(^+\) concentration (which ranges between 15 and 20 mM, being thus almost 2 times higher compare to neurones (Rose & Verkhratsky, 2016)) is mediated predominantly by the NKA. Resting [Na\(^+\)], may also be influenced by the activity of Na\(^+\)/K\(^+\)/Cl\(^-\) transporter NKCC1 and Na\(^+\)/bicarbonate (NBC) transporter (Kelly et al., 2009).

Powerful Na\(^+\) transporting machinery expressed in astroglial membrane underlies the generation of relatively large and long-lasting [Na\(^+\)], transients upon stimulation. These astroglial Na\(^+\) signals have been observed in astrocytes in cell culture and in brain slices in response to physiological (application of neurotransmitters or neuronal activity) stimulation (Rose & Ransom, 1996; Kirischuk et al., 2007; Kirischuk et al., 2012; Rose & Karus, 2013; Rose & Verkhratsky, 2016). Astroglial [Na\(^+\)], transients last for 10s and 100s of seconds, which may indicate either long-lasting Na\(^+\) entry, or dynamic buffering by binding/unbinding to Na\(^+\) dependent transporters abundantly populating perisynaptic astroglial membranes, or reflect other yet uncharacterised mechanisms (Rose & Verkhratsky, 2016). Cytoplasmic astroglial Na\(^+\) signals can also propagate through the astroglial syncytia with the speed of ~ 60 \(\mu\)m/s; this propagation occurs through diffusion via Cx30 or Cx43 gap junctional channels (Langer et al., 2012).

Sodium signalling regulates astroglial homeostatic function mainly through direct control of plasmalemmal SLC transporters (Fig. 4). Astroglial Na\(^+\) dependent transporters mediate an uptake of glutamate (through EAAT1/2), GABA (GAT1/3) glycine (GlyT1), noradrenaline and dopamine (through noradrenaline transporter NET), and adenosine (through concentrative nucleoside transporters CNT2). Intracellular Na\(^-\) also regulates the main component of glutamate-glutamine shuttle, the glutamine synthetase (which converts glutamate to glutamine) and Na\(^+\)-coupled neutral amino acid transporters SN1/2 which export glutamine. Sodium signals also contribute to the regulation of K\(^+\) buffering by affecting NKA transport; cytosolic Na\(^+\) also controls Na\(^+\)/H\(^+\) and Na\(^+\)/HCO\(_3\)\(^-\) transporters thus contributing to the regulation of pH. By directly defining reversal potential of NCX, astroglial [Na\(^+\)], controls NCX operational mode and contributes to Ca\(^{2+}\) signalling. Finally, [Na\(^+\)], is linked to metabolism, through initiating glycolysis and lactate production (see (Kirischuk et al., 2012; Chatton et al., 2016; Kirischuk et al., 2016; Rose & Verkhratsky, 2016) for detailed account and further references). To conclude astroglial Na\(^+\) signalling represents the fast coordination of neuronal
activity with "homeostatic" response of astroglia mediated through Na⁺-dependent transporters, concentrated in perisynaptic processes.

Conclusions

Intracellular signalling mediated by Ca²⁺ and Na⁺ operates in the majority of cells. Several molecular cascades coordinate movements of these two ions to regulate cellular functions. Despite the emerging importance of Na⁺ signalling in various cell types, number of studies describing cellular and subcellular Na⁺ dynamic, when compared to Ca²⁺, is minimal. A major obstacle for further advancement in the field is the lack of good cytosolic and organellar Na⁺ reporters. There are no known GFP-based Na⁺ reporters. Sadly, the production of the only fluorescent mitochondrial Na⁺ reporter, Corona Red, was recently discontinued, while spectral properties of the few available cytosolic Na⁺ probes are far from being ideal, posing a serious technical challenge to investigators in the field. It is our hope that the exiting new insights gained on the Na⁺ signalling, will lead to the development of such tools.

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Author contributions

All authors participated equally in writing the paper; all authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.
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Mammalian NCXs contain ten trans-membrane helices (TM1-10), where two Ca$^{2+}$-binding regulatory domains (CBD1 and CBD2) are located between the TM5 and TM6. The ion transport sites (cycled by dotted line) are located ~80 Å away from the regulatory CBD domains. Ca$^{2+}$ binding to CBD1 activates (upto 25-fold) NCXs, whereas Ca$^{2+}$ binding to CBD2 alleviates Na$^+$-induced inactivation. In NCX1 and NCX3, the splice segment is exclusively located on CBD2, whereas NCX2 does not undergo splicing. In NCX1, splice variants arise from a combination of six small exons A, B, C, D, E, and F, whereas a mutually exclusive exon (either A or B) appears in every splice variant. B-exon containing variants of NCX1 cannot bind Ca$^{2+}$ to CBD2 and are expressed in non-excitable tissues, whereas exon-A containing variants of NCX1 bind two Ca$^{2+}$ ions to CBD2 (allowing Ca$^{2+}$-dependent alleviation of Na$^+$-induced inactivation) and are expressed in excitable tissues. NCX3 contains only A, B, and C exons. In contrast with NCX1, exon-A containing NCX3 (AC) does not bind Ca$^{2+}$ to CBD2 and is expressed in skeletal muscle, whereas exon-B containing variants of NCX3 (B and BC) bind three Ca$^{2+}$ ions to CBD2 and are expressed in neurones and glia. Residues in positions 552, 578, and 585 form the Ca$^{2+}$-binding sites at CBD2, thereby determining the number and affinity of Ca$^{2+}$ binding sites at CBD2. "Naturally occurring" single-point substitutions at indicated three positions diversify the Ca$^{2+}$-binding capacity at CBD2, thereby resulting functional differences of exon A and B exons in NCX1 and NCX3. It would be interesting to test the effects of relevant substitutions in intact cell NCX variants (e.g., in neurons and glia) with a goal of examining the functional specificity of NCX variants contributions to Na$^+$ and Ca$^{2+}$ signalling.
**Figure 2.** TRP channels mediate Ca$^{2+}$ and Na$^{+}$ entry.

TRP proteins form mostly non-selective channels that are activated by a wide range of intracellular mechanisms and contribute substantial Na$^{+}$ entry into the cytosol. Through membrane depolarization, TRP channels can control the activation of voltage-gated L-type Ca$^{2+}$ channels and thus regulate physiological processes such as neuronal excitability and muscle contraction. Cytosolic signals contributed by TRP channels are likely to affect ionic homeostasis and function of various internal organelles such as mitochondria and lysosomes. Cell depolarization was proposed to regulate Rho proteins which control cytoskeletal rearrangements that occur during cell migration or during intercellular permeability.
Influx of Ca\textsuperscript{2+} into cells is mediated by multiple cell membrane pathways. Among them are store operated Ca\textsuperscript{2+} pathway (SOCE), L-type voltage-gated Ca\textsuperscript{2+} channel (LVGCC) and the cationic channel TRPV1. It is followed by rapid Ca\textsuperscript{2+} influx into the mitochondria by the mitochondrial Ca\textsuperscript{2+} uniporter MCU. Dynamic complexation of MCU with EMRE MICU1 and MICU2 control the affinity and intensity of Ca\textsuperscript{2+} influx via MCU. Mitochondrial Ca\textsuperscript{2+} influx by MCU is followed by a ~100 fold slower Ca\textsuperscript{2+} efflux through the rate limiting mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger NCLX. The removal of mitochondrial Ca\textsuperscript{2+} by NCLX is upregulated by (i) mitochondrial Ca\textsuperscript{2+} rise triggered by MCU and (ii) by cytosolic Na\textsuperscript{+} rise mediated by TRP channels or by the voltage sensitive Na\textsuperscript{+} channel Na\textsubscript{v}. Because of the low affinity of NCLX to cytosolic Na\textsuperscript{+}, cytosolic Na\textsuperscript{+} influx is essential for its activation. This Ca\textsuperscript{2+} and Na\textsuperscript{+} signalling between the cell membrane and mitochondria are linked to multiple physiological processes ranging from insulin secretion to pain sensation. Impaired mitochondrial MCU or NCLX activity leads to mitochondrial Ca\textsuperscript{2+} overload, a major cause for ischemic or neurodegenerative induced damage.
**Figure 4.** Astroglial Na⁺ signals regulate homeostatic functions.

Abbreviations: ASCT2 - alanine-serine-cysteine transporter 2; ASIC - acid sensing ion channels; CNT2, concentrative nucleoside transporters; EAAT - excitatory amino acid transporters; ENaC - epithelial sodium channels; GAT - GABA transporters; GS - glutamine synthetase, GlyT1-glycine transporter. iGluRs - ionotropic glutamate receptors; Na⁺ channels activated by extracellular Na⁺; NAAT - Na⁺-dependent ascorbic acid transporter; NBC - Na⁺/HCO₃⁻ (sodium-bicarbonate) co-transporter; NCX - Na⁺/Ca²⁺ exchanger; NCLX - mitochondrial Na⁺/Ca²⁺ exchanger; H⁺ exchanger; NKCC1 - Na⁺/K⁺/Cl⁻ cotransporter, NET - norepinephrine transporter; MCT1 - monocarboxylase transporter 1; P2XRs - ionotropic purinoceptors; SN1,2 - sodium-coupled neutral amino acid transporters which underlie exit of glutamine; TRP - transient receptor potential channels; ROS - reactive oxygen species; VRAC - volume-regulated anion channels.

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