Review Title: The Janus face of Ouabain in Na^+, K^+-ATPase and calcium signalling in neurons

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Abstract

Na^+,K^+ATPase (NKA), a transmembrane protein essential for maintaining the electrochemical gradient across the plasma membrane, acts as a receptor for cardiotonic steroids (CTS) such as ouabain. CTS binding to NKA, triggers signalling pathways or inhibits NKA activity in a concentration-dependent manner, resulting in a modulation of Ca^{2+} levels, which are essential for homeostasis in neurons. However, most of the pharmacological strategies for avoiding neuronal death do not target NKA activity, due to its complexity and poor comprehension of the mechanisms involved in NKA modulation. The present review aims to discuss two points regarding the interplay between NKA and Ca^{2+} signalling in the brain: NKA impairment causing illness as well as neuronal death due to Ca^{2+} signalling and benefits to the brain by modulating NKA activity. These interactions play an essential role in neuronal cell fate determination and are relevant to finding new targets for the treatment of neurodegenerative diseases.

Keywords: Na^+,K^+-ATPase; Calcium; Brain; Neurons; Ouabain; cardiotonic steroids; cell fate

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Introduction

Na^+,K^+-ATPase in neurons

Neurons are excitable cells that present a resting membrane potential within the range of -70 to -80 mV. The resting membrane potential is defined by the selective permeability of the plasma membrane for ions and electrochemical polarisation. In excitable cells, the plasma membrane is much more permeable to K^+ than to Na^+ ions; thus, the resting membrane potential is closer to the equilibrium potential of K^+ (-90 mV) than that of Na^+ (+65 mV) (Wright, 2004). To maintain this asymmetry, the activity of the transporter Na^+,K^+-ATPase (NKA, also recognized as the Na^+ pump) is essential. When neurons are stimulated, the plasma membrane at the axon hillock may depolarize owing to the opening of voltage-gated Na^+ channels. As Na^+ ions rush back into the cell, they further depolarise the membrane in a positive feedback, known as the action potential (Dobretsov & Stimers, 2005; Wright, 2004).

Once neurons are depolarised, the Na^+ channels close, and repolarisation begins with the opening of voltage-gated K^+ channels. As K^+ moves out of the cell, the membrane potential falls and starts approaching the resting potential. To re-establish the appropriate balance of ions, NKA plays a fundamental role in promoting the movement of three Na^+ ions out and two K^+ ions into the cell at the expense of the energy generated by the hydrolysis of one ATP molecule. The Na^+ gradient promoted by NKA can also support the transfer of other ions, substrates, and neurotransmitters between the intra- and extracellular compartments (Blanco & Mercer, 1998; Dobretsov & Stimers, 2005).

Furthermore, NKA is vital for the modulation of intracellular Ca^{2+} concentration. The levels of intracellular Na^+, strictly controlled by NKA, affect the activity of the Na^+/Ca^{2+}-exchanger (NCX), which removes Ca^{2+} from the cell. As NCX is colocalised with NKA, any alteration in Na^+ would influence Ca^{2+} levels (Juhaszova & Blaustein,

1997). Nevertheless, NKA is not only an ion transporter but also a receptor that activates signalling pathways through protein-protein interactions, which will be discussed in the present review.

The importance of calcium signalling in the synapse

Ca^{2+} is a divalent cation that, in neurons, acts as a second messenger regulating synaptic plasticity, neuronal excitability, the formation of synapses, and neuronal morphology [25]. For instance, Ca^{2+} is important for the long-term potentiation and long-term depression responses, that are forms of synaptic plasticity involved in learning and memory (Brini, Calì et al., 2014).

Most neurons express around 4 to 5 different voltage-dependent calcium channels, such as low-voltage-activated T-type channels and high-voltage-activated channels that include L-type, P/Q-type, N-type, and R-type channels. The activation of calcium channels occurs near the peak of the action potential, and as a result, calcium currents predominate in the hyperpolarization phase (Bean, 2007).

Low-voltage-activated T-type channels are important for neurotransmitter release. When an action potential is triggered along a neuronal fibre, voltage-gated channels localised in the presynaptic terminal open, allowing a transient increase in intracellular Ca^{2+} levels in the active zone. This increase in Ca^{2+} levels results in plasma membrane depolarisation and the fusion of synaptic vesicles with the plasma membrane, which leads to exocytosis of neurotransmitters into the synaptic cleft (Bean, 2007).

The many functions of Ca^{2+} signalling in the synapse are only possible because the concentration gradient for Ca^{2+} ions is tightly maintained in cells, with at least 10,000-fold lower concentrations in the cytoplasm compared to extracellular compartments, and

some intracellular compartments such as the endoplasmic reticulum (ER) (Brini, Calì et al., 2014). In this review, the focus is on ER and NCX.

ER contains two receptors (IP3 receptor and ryanodine receptor) that can release Ca^{2+} in a very controlled manner. ER can either modulate Ca^{2+} levels in specific compartments such as dendritic branches or promote Ca^{2+} waves from the ER that spread through the cell, modulating neuronal gene expression (Hagenston & Bading, 2011). ER is an important buffering system to prevent uncontrolled Ca^{2+}, due to a pump called Ca^{2+}-ATPase (in this case, sarco/endoplasmic reticulum Ca^{2+} -ATPase, or SERCA), which translocates Ca^{2+} from the cytosol to the lumen of the ER restoring homeostatic Ca^{2+} levels (Berridge, 2009; Braet, Cabooter et al., 2004; Brini, Calì et al., 2014).

On the other hand, NCX is a versatile bidirectional membrane ion transporter that transports three Na^{+} ions into the cell in exchange for one Ca^{2+} ion out of the cell. It plays a key role in Ca^{2+} and Na^{+} homeostasis, as the exchanger can reverse and transport Ca^{2+} into the cell depending on the combined effects of Na^{+} and Ca^{2+} gradients in different neuronal environments, such as dendrites, soma, and axon (Yu & Choi, 1997). In the brain, NCX1 interacts with the isoform α2 of NKA (Blaustein & Lederer, 1999), and its activation can be both protective and detrimental according to the situation (Fig. 1). When an increase in intracellular Ca^{2+} levels occurs during excitotoxicity, NCX can be activated in the forward direction exchanging Ca^{2+} out of the cell and Na^{+} inside the cell, even in the presence of a lower extracellular Na^{+} concentration. When ischemia and/or excitotoxic stimulation occurs for a period of time, leading to intracellular levels of Na^{+} beyond a critical point, the exchanger reverses, allowing Ca^{2+} to enter into the cell and thus contributes to cellular damage (Jeffs, Meloni et al., 2007; Sisalli, Secondo et al., 2014; Wolf, Stys et al., 2001).

The detrimental process of excitotoxicity is mainly fired by the glutamatergic system and can lead to neuronal death. Ca^{2+} is important in many aspects of glutamatergic synapses. Glutamate can trigger signalling by ionotropic (iGluRs) or metabotropic receptors (mGluRs), which are found in the membrane of neurons, astrocytes, and oligodendrocytes. Both mGluRs and iGluRs are necessary for synaptic plasticity, linked to memory and learning (Brassai, Suvanjeiev et al., 2015; Debanne, Daoudal et al., 2003; Reiner & Levitz, 2018).

One of the main players in synaptic plasticity is the iGluR NMDA, that once activated, triggers Ca^{2+} currents. These currents modulate not only the membrane potential but also second messenger systems such as cyclic GMP (Nathanson, Scavone et al., 1995). On the other hand, altered NMDA presence and/or function is also relevant in excitotoxicity, and it has been speculated to be associated with many neurodegenerative diseases (Hansen, Yi et al., 2017).

In addition, at postsynaptic sites, ionotropic NMDA activation requires not only glutamate binding but also glycine or serine binding simultaneously at a separate site, and it also displays a degree of voltage dependence due to Zn^{2+} or Mg^{2+} binding in the pore to induce an influx of Ca^{2+} (Berridge, 1998). Additionally, activation of mGluR leads to formation of IP_3, which binds to IP_3 receptors (IP_3R), releasing Ca^{2+} from the ER. A positive feedback loop occurs, and IP_3R, which is also sensitive to Ca^{2+}, releases more Ca^{2+} and propagates Ca^{2+} waves (Mateos-Aparicio & Rodríguez-Moreno, 2020).

The relevance of understanding the crosstalk between NKA and Ca^{2+} signalling goes beyond its classical physiology. Based on several recent observations that NKA, in the presence of specific ligands, can trigger signalling cascades has revolutionized the concept of the NKA in cell biology.

Cardiotonic steroids (CTS)

CTS such as ouabain (OUA), marinobufagenin , and digoxin, are a class of compounds that bind specifically to NKA and can inhibit its activity in a dose-dependent manner (Cherniavsky Lev, Karlish et al., 2015). It is well known that the selective inhibition of NKA activity by CTS enhances intracellular Na^+ and Ca^{2+} concentrations. In cardiomyocytes, the increase in intracellular Ca^{2+} levels leads to contraction; therefore, these drugs have been used for centuries to treat congestive heart failure and arrhythmias (Akera & Brody, 1977; Lingrel, 2010). Different effects can be observed in neurons and glial cells after administration of CTS (Song, Thompson et al., 2013).

Since CTS are structurally different, they have distinct effects, even if they bind to the same enzyme (Klimanova, Petrushanko et al., 2015). However, some outcomes are quite similar; for example, in hippocampal neuronal culture, OUA and digoxin at low concentrations can increase intracellular Ca^{2+} mediated by glutamate, although the effect of OUA seems to be dependent on increased Ca^{2+} storage in the ER, while digoxin does not have a direct effect on ER storage. At low concentrations, there is also an antagonism between OUA and digoxin since the increased glutamate-mediated intracellular Ca^{2+} by OUA can be partially reversed by digoxin and vice versa (Song, Karashima et al., 2014; Song, Thompson et al., 2013).

The function of NKA as a receptor was reported by Zijian Xie and Amir Askari's research group (Xie & Askari, 2002). Through the binding of CTS to NKA, a series of protein–protein interactions occur, triggering diverse signalling pathways. After binding, CTS-induced NKA-mediated stimulation of tyrosine kinase Src evokes transactivation of EGF receptor and, together with the recruitment of adaptor proteins and Ras, enhances the production of ROS and activation of the Raf/MEK/ERK signalling pathway (Xie &

Askari, 2002; Xie & Cai, 2003), both resulting in an increase in systolic and diastolic Ca^{2+} in cardiomyocytes (Tian, Gong et al., 2001). The acute phosphorylation of ERK by OUA has also been demonstrated in a primary culture of rat cerebellar cells, but in long-term OUA incubation, ERK is dephosphorylated (Lopachev, Lopacheva et al., 2016). When activated, ERK can activate NF-κB in cerebellar cells (de Sá Lima, 2013; Kawamoto, Lima et al., 2012). In addition, several other pathways, as well as different cellular effects such as hypertrophy, growth, differentiation, motility, and changes in cell viability have been described (Aperia, 2007; Cui & Xie, 2017).

In the central nervous system (CNS), Anita Aperia´s group showed for the first time that OUA, at non-inhibitory concentrations, could trigger intracellular Ca^{2+} waves (Aizman, Uhlén et al., 2001). These oscillations in intracellular Ca^{2+} concentrations are caused by the physical interaction between NKA and IP_3R, forming a signalling microdomain (Miyakawa-Naito, Uhlén et al., 2003; Zhang, Malmersjö et al., 2006). Indeed, at nanomolar concentrations, OUA can activate the NF-κB signalling pathway in kidney cells (Li, Zelenin et al., 2006), astrocytes (Liu, Miyakawa et al., 2007) and neurons (de Sá Lima, 2013; Kawamoto, Lima et al., 2012; Orellana, Leite et al., 2018). In rat cerebellar primary neuronal culture, NF-κB activated by OUA led to an increase in *Tnf, Il-1, β* and *Bdnf* mRNA levels, while most of the same genes (*Bdnf, iNos, Tnf*, and *Bcl-2*) had augmented mRNA levels in the hippocampus treated with OUA. Thus, OUA treatment promotes the activation of pro-inflammatory pathways (iNOS, TNF, and IL-1β) and neuroprotective pathways (BDNF and Bcl-2) (de Sá Lima, 2013; Kawamoto, Lima et al., 2012) (Fig. 2).

In addition to these findings, it seems that mammals also produce endogenous CTS in the central and peripheral nervous systems, that seem to be a new type of hormones, and several of them have been identified (Schoner & Scheiner-Bobis, 2007).

The adrenal cortex and hypothalamus are considered the main sites of synthesis (Schoner, 2000), but the physiological significance and synthesis pathway are still unclear. Thus, more studies need to be performed to understand the role of endogenous CTS.

In this context, it is important to discuss the most relevant scientific progress in understanding the functional interaction between NKA and Ca^{2+} signalling in neurons under physiological and pathological conditions and the relevance of CTS (mostly OUA) as possible modulators.

The imbalance of α1 and α3 NKA impairs Ca^{2+} signalling leading to illness

NKA is composed of at least two subunits, which are indispensable for NKA activity, α and β. The α subunit is called "catalytic" or "functional", as it is responsible for the transport of ions, hydrolysis of ATP, and has the binding site for the selective ligands CTS.

In the brain, three NKA α isoforms have been identified (α1-3). The α1 isoform is ubiquitously expressed, and it is assumed to play a housekeeping role in the cells. The α2 is mostly expressed in glial cells (astrocytes and oligodendrocytes) and only in embryogenesis, also in neurons. At least in rodents, the α2 isoform has a somewhat higher apparent affinity for ATP and Na^+, which may be important for dissipating extracellular K^+ after the action potential. α2 is also more sensitive to intracellular Ca^{2+} levels, and human isozymes have higher sensitivity to voltage changes (Azarias, Kruusmägi et al., 2013; Blanco, 1998; Hammann, Bassetti et al., 2018; McGrail, Phillips et al., 1991).

α3 is exclusively expressed in neurons (McGrail, Phillips et al., 1991), but a recent study has shown that the mRNA levels of this isoform and α1 are very heterogeneous according to the brain regions and neuronal subtypes. In the hippocampus, parvalbumin-expressing GABAergic neurons express lower levels of α1 mRNA and higher levels of

α3, showing the complexity of the α3 distribution across the brain (Murata, Kinoshita et al., 2020). Furthermore, α3 is activated by high intracellular Na^+ levels that occur after bursts of action potentials and is thought to be critical for subsequent intracellular Na^+ clearance (Azarias, Kruusmägi et al., 2013). Although still obscure, the differential patterns of isoform expression may be related to their physiological roles in each cell type (Blanco, 1998) and during mouse development (Sundaram, Safina et al., 2019).

It has been shown that mutations in NKA isoforms that alter pump operation can lead to neurological disorders due to altered Ca^{2+} signalling. Epileptic seizures are characterized by an increase in intracellular Na^+ and Ca^{2+} concentrations (Raimondo, Burman et al., 2015). A mutation in the housekeeping α1 isoform can lead to large changes in the transmembrane K^+ gradient. Patients carrying a heterozygous mutation in ATP1A1, the gene encoding α1, presented generalised convulsions and severe intellectual disability with limited motor skills. Curiously, molecular analysis of the mutation revealed that the α1 isoform was normally expressed in cellular membranes but had a decreased affinity for Na^+ and K^+. Abnormal cation permeabilities, which cause membrane depolarisation, have also been observed (Schlingmann, Bandulik et al., 2018).

Since NKA is essential for membrane potential maintenance, changes in NKA activity or membrane permeability can induce an electrogenic imbalance in excitatory neurons resulting in seizures (Donaldson, St Pierre et al., 1971; Vaillend, Mason et al., 2002) (Fig. 1B). Some of the ATP1A1 mutations that led to a loss-of-function defect of the NKA, reducing its activity, would probably be associated with reduced efflux of Ca^{2+} via NCX in the axons. These changes could lead to toxic levels of intracellular Ca^{2+}, as described in a group of hereditary peripheral motor and sensory neuropathies called Charcot-Marie-Tooth Type 2. The most common features are the lack of reflexes, sensory loss, and distal weakness and atrophy (Lassuthova, Rebelo et al., 2018).

In addition to mutations that lead to a decrease in α1 NKA activity, mutations that tend to increase expression of NKA are similarly problematic. Angelman syndrome (AS) is a neurodevelopmental disorder that shows an increased expression of the α1 isoform. This increase is observed in the hippocampus and is associated with impaired hippocampal synaptic plasticity, hippocampus-dependent cognitive deficits, epilepsy, and autism symptoms (Kaphzan, Buffington et al., 2013). When α1 levels increase, neurons become less excitable due to a reduction in the resting membrane potential and aberrant Ca^{2+} dynamics (Kaphzan, Buffington et al., 2013; Rayi, Koyavski et al., 2019). Studies in AS model mice suggest that the increased expression of α1 starts at an early age of development (P14), disrupting long-term synaptic transmission and hippocampus-dependent learning in adulthood (Rayi, Koyavski et al., 2019). Genetic manipulations that generated a heterozygous knockout mouse for α1 expression revealed that reduction of α1 re-established homeostasis in the hippocampus, long-term structural plasticity, and hippocampus-dependent memory deficits (Kaphzan, Buffington et al., 2013). In agreement with these findings, pharmacological inhibition of α1 by marinobufagenin in the AS model rescued aberrant α1 activity-dependent dendritic Ca^{2+} gradient and hippocampus-dependent cognitive deficits in AS mice (Rayi, Koyavski et al., 2019). This suggests that impaired NKA activity leading to aberrant Ca^{2+} signalling in neurons is the main molecular mechanism underlying hippocampal deficits and that CTS can be a valuable pharmacological approach.

Furthermore, α1 mutations have functional relevance to neuronal networks. Specific mutations in the protein-coding sequence of ATP1A3 cause alternating hemiplegia of childhood (AHC) and rapid-onset dystonia-parkinsonism. Patients with rapid-onset dystonia-parkinsonism present with not only motor symptoms such as dystonia, but also mood disorders and psychosis. AHC patients present the first symptoms

in infancy, with the most prevalent being dystonia, paroxysmal hemiplegia, and abnormal ocular movements (Heinzen, Arzimanoglou et al., 2014). In both diseases, a reduction in α3 activity points to its role in the suppression of neuronal hyperexcitability (Clapcote, Duffy et al., 2009).

Indeed, neurons differentiated from AHC patient-derived induced-pluripotent stem cells presented less functional NKA. This decrease in pump activity was accompanied by an impaired transmembrane K^+ gradient, attenuated voltage-gated Na^+ channel availability, reduced excitability, and depolarised resting membrane potential (Simmons, Thompson et al., 2018).

Interestingly, both in an animal model that contains the α3 mutation usually found in AHC (Myshkin mouse) and in post-mortem analysis of the prefrontal cortex of bipolar patients, decreased activity of this isoform was detected and related to a behavioural pattern similar to bipolar patients in the manic state (Kirshenbaum, Dachtler et al., 2016; Tochigi, Iwamoto et al., 2008). Primary cortical neurons from the Myshkin mouse model present higher levels of basal intracellular Ca^{2+}, and in the presence of glutamate, transient changes in Ca^{2+} are prolonged (Kirshenbaum, Clapcote et al., 2011).

Regarding neuropsychiatric disorders, some studies have reported that in human post-mortem brains, the expression of the α3 isoform is lower in GABAergic neurons in the frontal cortex of bipolar disorder and schizophrenia patients (Hodes, Rosen et al., 2019). In rodents, high doses of OUA (in the μM concentration range) are used to induce a mania model. Increased locomotor activity, stereotypical behaviour, and decreased anxiety were observed in treated mice after OUA i.c.v. injection, suggesting that D2 dopaminergic receptor activation is a contributing factor (Lopachev, Volnova et al., 2019). In addition, induced maniac-like and depressive-like states by a single OUA

injection was validated as a suitable model for bipolar disorder, presenting the hypothesis that decreased NKA activity is a key factor in triggering mood swing phases (Valvassori, Dal-Pont et al., 2019).

Ouabain (OUA) and NKA functional impairment

Another important interaction of NKA in the CNS is with the TRPC6. All TRPCs are generally receptor-operated nonselective cation channels that are permeable to Ca^{2+} (Sawamura, Shirakawa et al., 2017). Neurotrophic factors such as BNDF or neuropeptides can activate TRPC6 channels either through G-protein-coupled receptors or by receptor tyrosine kinases. In both cases, phospholipase C cleaves PtdIns(4,5)P_2 to produce diacylglycerol (DAG) and IP_3, which can activate TRPC6 by Ca^{2+} directly and indirectly, respectively (Venkatachalam & Montell, 2007).

In general, TRPCs play an important role in synapse transmission, neuronal firing, and gene expression. TRPC6 is localised in excitatory hippocampal synapses at the postsynaptic membrane. It is related to the formation of dendritic spines (Sawamura, Shirakawa et al., 2017), and is associated with hippocampal neuronal dendritic growth during development and in the adult brain via the Ca^{2+} - calmodulin modulated kinases (CaM kinases) - cAMP response element-binding protein (CREB) pathway (Tai, Feng et al., 2008). Remarkably, TRPC6 was shown to physically interact with NKA in the brain (Goel, Sinkins et al., 2005). A high concentration of OUA decreases TRPC6 expression in HEK cells and thus reduces Ca^{2+} entry. This decrease in TRPC6 occurs by its redistribution in the plasma membrane and its degradation in the lysosome; however, the exact mechanism of this regulation is still unknown (Chauvet, Boonen et al., 2015).

The functional relevance of understanding NKA and TRPC6 interplay can be seen in new findings that suggest an important role of TRPC6 in Alzheimer's disease (AD).

Novel evidence suggests that TRPC6 is a γ-secretase modulator, regulating Aβ production due to direct interaction with amyloid-precursor protein (Wang, Lu et al., 2015). It has been shown that amyloid-β (Aβ) peptide 1–40, the most common Aβ isoform, can inhibit NKA activity (Bores, Smith et al., 1998). This inhibition is a consequence of a direct interaction of Aβ with a site between the α1 and β1 subunits (Petrushanko, Mitkevich et al., 2016). In fact, the incubation of neuroblastoma cells (SH-SY5Y) with $A\beta_{1\text{-}42}$ induces acute inhibition of NKA activity and an increase in Src phosphorylation levels. Altogether, it seems that an increase in Aβ peptides can inhibit NKA, which, in turn, decreases TRPC6 expression. As TRPC6 can act as a γ-secretase modulator, its inhibition boosts the generation of Aβ peptides (Wang, Lu et al., 2015).

In addition, spherical Aβ oligomers (amylospheroids) found in the brains of patients with AD could bind to the α3 isoform in neurons, leading to impaired NKA activity, increased N-type voltage-gated Ca^{2+} channel activity, presynaptic Ca^{2+} overload, mitochondrial Ca^{2+} dyshomeostasis, tau abnormalities, and degeneration. This suggests that NKA modulation could be a target to prevent cell death in neurodegenerative diseases (Ohnishi, Yanazawa et al., 2015). Indeed, previous studies have shown impaired NKA activity in the brains of patients with AD (Hattori, Kitagawa et al., 1998; Liguri, Taddei et al., 1990), and an increase in NKA activity in the erythrocytes of patients with AD (Kawamoto, Munhoz et al., 2005).

In Parkinson's disease, α-synuclein also interacts with α3, capturing freely diffusing α3 into α-synuclein clusters. Then, α3 is redistributed through the cell membrane, promoting larger nanoclusters and reducing local densities of α3. The

interaction between α-synuclein and NKA per se implies a decrease in α3 activity, which leads to a higher vulnerability to glutamate toxicity (Shrivastava, Redeker et al., 2015).

Changes in NKA activity have an impact on intracellular Ca^{2+} levels, which are crucial for cell fate. For instance, NKA controls cellular volume and intracellular Ca^{2+} levels (Tiwari, Mohan et al., 2018). Different models of cell death showed that inhibition of NKA activity is followed mainly by an increase in intracellular Ca^{2+} levels, mostly mediated by NCX, leading to excitotoxicity. Indeed, the inhibition of NKA by OUA induces neurite degeneration and, consequently, an increase Na^{+} voltage-gated channels and NCX activation. OUA can cause a reduction in neurite lengths days after treatment with tetrodotoxin (Na^{+} channel blocker), whereas a compound that reverses the transport of NCX (KB-R7943) can prevent the effect observed with inhibitory concentrations of OUA (Persson, Kim et al., 2013).

Many chemical compounds can lead to neuronal toxicity due to an imbalance in NKA activity. A similar pattern was observed in the presence of rotenone, used as an insecticide, that induces ROS production and energy deprivation, which leads to a decrease in mitochondrial ATP production, impairment in NKA increasing Na^{+} influx, and finally causes membrane depolarisation (Persson, Kim et al., 2013). Neuronal death caused by cadmium in cortical neuronal culture was shown to be a consequence of elevated intracellular Ca^{2+} levels and NKA and Ca^{2+}- ATPase inhibition (Yuan, Jiang et al., 2013). Interestingly, the elevation of intracellular Ca^{2+} levels can be reversed by a IP_3R inhibitor (2-APB), suggesting an important role of ER Ca^{2+} storage in this phenomenon. Furthermore, TiO_2 can cause damage to the hippocampus by inducing apoptosis, thus producing memory impairment (Hong, Sheng et al., 2015). These phenomena occur by glutamatergic excitotoxicity, which leads to Na^{+} accumulation and increase in the amount of intracellular Ca^{2+} by activation of voltage-gated Ca^{2+} channels,

reverse mode NCX, and Ca^{2+}-ATPase impairment, thus disturbing NKA activity (Hong, Sheng et al., 2015; Yuan, Jiang et al., 2013).

In addition, glutamate, through NMDA–Ca^{2+} signalling induced nitric oxide (NO) synthase activation, leads to an increase in NO production and stimulation of cyclic GMP and cyclic GMP-dependent protein kinase (PKG) activity, which has been shown to stimulate $\alpha_{2/3}$ NKA activity in the CNS (Munhoz, Kawamoto et al., 2005). Cyclic GMP-PKG pathways can exert specific effects on synaptic transmission (relevant to long-term changes in cell excitability), memory, and actions on energy metabolism (relevant to the pathophysiology of excitotoxicity and aging process) (Kinoshita, Leite et al., 2016; Nathanson, Scavone et al., 1995; Scavone, Munhoz et al., 2005).

Mutations and chemical compounds that cause an imbalance in NKA activity have a pivotal role in illness development and neurodegeneration, raising NKA as an interesting pharmacological target and CTS as exciting tools to better understand NKA (Kinoshita, Leite et al., 2016). Many beneficial effects of modulating NKA activity have been proposed recently.

Ouabain (OUA)/NKA in neuroprotection

Despite their similar chemical structures, all CTSs differ in a range of biological responses (Amaral, Martins Ferreira et al., 2018). As the main representative of this group, OUA displays some singular features when compared to other CTS derivatives. Primarily, OUA performs a rapid onset cardiac action presenting several modulative aspects in heart metabolism, promotes an antagonist axis regarding digoxin response, and occupies a solemn position of being the only CTS able to stimulate NKA in very low

concentrations (Fuerstenwerth, 2014; Fürstenwerth, 2010). Structure-activity and structure-kinetics studies may provide some answers to these differences (Azalim, do Monte et al., 2020).

In the rodent CNS, low doses of OUA blocked LPS-induced neuroinflammation through activation of signalling pathways (Kinoshita, Yshii et al., 2014). Another interesting point is that α2 expression can modulate the responses of astrocytes to LPS, showing the relevance of NKA in the inflammatory response that is present in all neurodegenerative diseases (Kinoshita et al., 2016.

OUA binding to NKA promotes dendritic growth by Ca^{2+} oscillations, MAPK, CaM kinases, CREB, and cAMP response-element -mediated gene expression in embryonic culture of primary cortical neurons (Desfrere, Karlsson et al., 2009). This OUA-NKA effect was also observed in pyramidal neurons from the CA1 and dentate gyrus in the rat hippocampus. In this study, OUA also activated CREB, the Wnt/β-catenin pathway, and NF-κB. The increase in neuronal branching had an impact on animal behaviour by improving long-term spatial reference memory (Orellana, Leite et al., 2018).

In this sense, it has been demonstrated that OUA could be protective in an AD model, leading to autophagy and reducing tau-phosphorylation (Song, Demirev et al., 2019). The OUA-NKA signalling pathways, and/or the increase in NKA activity, could result in cell survival and modulation of intracellular Ca^{2+} levels (Kinoshita, Leite et al., 2016; Orellana, Kinoshita et al., 2016).

In addition, it has been described that the NF-κB activation caused by OUA in the rat hippocampus (Kawamoto, Lima et al., 2012) and in cerebellar cells (De Sá Lima,

Kawamoto et al., 2013) is partially dependent on NMDA activation. Both studies observed an increase in the levels of *Bdnf* mRNA, which is also modulated by CREB, with OUA treatment.

The connection between glutamate receptors and NKA has also been described in different studies. Low-dose ouabain protects against excitotoxic apoptosis and upregulates nuclear Bcl-2. Interestingly, OUA, when administered simultaneously with NMDA, caused an increase in intracellular Ca^{2+} levels. However, these levels declined gradually to the control value by Ca^{2+} extrusion and buffering (Sibarov, Bolshakov et al., 2012). Another study in cortical neurons showed that OUA prevented Ca^{2+} overload caused by kainic acid (Abushik, Sibarov et al., 2013). Thus, this confirms the hypothesis that OUA can protect neurons from Ca^{2+} overload and its relevance to glutamate receptor toxicity (Fig. 3).

The functional interaction between NKA and NMDA can occur via α1 and α3 (Akkuratov, Lopacheva et al., 2015; Akkuratov, Westin et al., 2020). As a result of this interaction, NMDA expression is mediated by α3, while NMDA activation, which increases intracellular Ca^{2+} levels, decreases α1 activity in cerebellar cells. Even though OUA does not change the amount of NKA that interacts with NMDA, showing that NKA-NMDA is not OUA binding-dependent, OUA is capable of modulating NMDA expression in these cells (Akkuratov, Lopacheva et al., 2015). In addition, recent data show that nanomolar OUA induces NKA interaction with NMDA, causing a reduction in the NMDA response on the dendrites of rat hippocampal neurons (Akkuratov, Westin et al., 2020).

Interestingly, targeting the OUA binding site through an antibody (DR-Ab) could modulate α1 activity and protect neurons from glutamatergic excitotoxicity in primary cortical neuronal cultures (Shi, Cao et al., 2018). The hypothesis for this protection relies

on an increase in α1 activity as well as in NCX function that protects cells from Ca^{2+} overload and death. Two main mechanisms have been proposed: increased NCX activity and less conversion of AMPAr from Ca^{2+} impermeable form to Ca^{2+} permeable form (Shi, Cao et al., 2018).

As previously described, the normal function of neurons depends on NKA and Ca^{2+} signalling, especially during the firing of action potentials, repolarisation, and neurotransmitter release, which can modulate neuronal plasticity (de Lores Arnaiz & Ordieres, 2014; Kinoshita, Leite et al., 2016). Taken together, the role of NKA and CTS in neuronal activity and survival are related to the modulation, among other factors, of the intracellular Ca^{2+} levels in neuronal cells. Nevertheless, more studies are necessary to find drugs that can bind specifically to the cerebral isoforms of NKA and that act as biased agonists favouring some signalling pathways that would break the neuronal death process.

Conclusion

The modulation of NKA activity plays a key role in excitability, plasticity, and cell survival. NKA has a well-described function of pump and represents a challenge to be understood as a receptor activating signalling pathways. Both functions have an impact on Ca^{2+} levels in neurons. OUA is an important tool that could be used to understand the role of NKA in excitotoxicity and neuroprotection according to the treatment dose of these compounds. Interaction with Aβ oligomers and α-synuclein reduces NKA activity and causes alterations in Ca^{2+} levels. Thus, NKA could be an important therapeutic target in neurodegenerative diseases.

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Competing financial interests

The authors declare that they have no competing financial interests.

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Legends

Figure 1. Schematic illustration of Ca^{2+} regulation in neurons by endoplasmic reticulum proteins (ER) and Na^+/Ca^{2+}-exchanger (NCX) and its interaction with Na^+,K^+-ATPase (NKA). **(A)** Ca^{2+} regulation in healthy neurons. NKA interacts with NCX, regulating intracellular Ca^{2+}. NKA transports three Na^+ ions out and two K^+ ions into the cell, while NCX uses the Na^+ electrochemical gradient to remove Ca^{2+} from the cytoplasm. Intracellular calcium is also regulated by ER, inositol triphosphate receptor (IP_3R), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), ryanodine receptor (RyR). NKA can interact with IP_3R, modulating intracellular Ca^{2+} concentration. **(B)** Ca^{2+} regulation in neurons with impaired NKA. Loss of NKA transport activity by mutation or induced by high doses/concentrations of OUA leads to an increase in intracellular Na^+. This switches NCX into the “reverse mode”, which transports Ca^{2+} into the cell. OUA may induce the interaction between NKA and IP_3R, releasing Ca^{2+} from the ER. This increase in intracellular Ca^{2+} has detrimental effects on neurons.

Figure 2. Schematic illustration of Na^+,K^+-ATPase (NKA) signalling pathway. Ouabain (OUA) binding to the NKA activates Src which, in turn, evokes transactivation of EGF receptor and, together with the recruitment of adaptor proteins and Ras, leads to the activation of Raf/MEK1/2/ERK1/2 signalling pathway. Ouabain can also induce the physical interaction between NKA and inositol triphosphate receptor (IP_3R), triggering intracellular Ca^{2+} waves. Raf/MEK1/2/ERK1/2 signalling pathway or intracellular Ca^{2+} waves can activate NF-κB. NF-κB activation by OUA induces the expression of neuroprotective and pro-inflammatory genes.

Figure 3. Overview of neuroprotective effects of ouabain (OUA). OUA binding to Na^+,K^+-ATPase (NKA) protects neurons from Ca^{2+} overload and therefore, OUA is relevant against glutamate receptor toxicity, excitotoxicity and apoptosis. Besides, it may promote dendritic growth by Ca^{2+} oscillations, improve long-term spatial reference memory and promote inflammatory balance. Since many beneficial effects of modulating NKA activity have been seen, these interactions may play an essential role in neuronal cell fate determination and are important in finding new targets for the treatment of neurodegenerative diseases. All beneficial effects illustrated above can also interact with each other; therefore, certain phenomenon that can be further related are represented by different line colours in the scheme.

Cover Figure– The Janus face of Ouabain (OUA). OUA binds to Na^+,K^+-ATPase (NKA) to either trigger signalling pathways or inhibit NKA activity in a dose/concentration-dependent manner. Since both NKA activity and OUA-NKA signalling pathways may interfere in intracellular Ca^{2+} levels in the brain, the present review aims to discuss two majors points in the interplay communication between NKA (activity and signalling pathways) and Ca^{2+} signalling in the brain by: beneficial aspects

such as for neuroprotection, cell survival an plasticity by NKA modulation and illness, excitotoxicity and neuronal death due to NKA functional impairment.