Unravelling the mechanisms for recovery from sodium loads in CA1 hippocampal neurons

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"If the brain were so simple that we could understand it, we would be so simple that we couldn't."

Lyall Watson

Zusammenfassung

Die Natriumhomöostase ist von grundlegender Bedeutung für das zentrale Nervensystem. Obwohl es während neuronaler Aktivität zu Fluktuationen in der intrazellulären Natriumkonzentration kommt, wird diese im Mittel durch die Na^+/K^+-ATPase (NKA) konstant gehalten. Natrium ist darüber hinaus ein hochbewegliches, ungepuffertes Ion. Neuste Arbeiten haben gezeigt, dass Diffusion einen Mechanismus zur Rückführung lokaler Natriumerhöhungen auf ein Grundniveau darstellen kann.

Die vorliegende Arbeit ist ein Beitrag zum Verständnis des Natriumtransports aus Somata und Dendriten hippocampaler CA1-Pyramidenzellen der Maus. Durch lokale Druckapplikation von Glutamat wurden intrazelluläre Erhöhungen der Natriumkonzentration während physiologischer neuronaler Aktivität nachgebildet und per Weitfeldimaging aufgezeichnet. Es konnte gezeigt werden, dass transiente Natriumerhöhungen im Soma mit einer Rate von 8 mM min^{-1} auf das Ursprungsniveau zurückgeführt wurden. Durch Perfusion mit Ouabain als spezifischem NKA-Inhibitor ließ sich dieser Vorgang vollständig blocken, während Inhibition der Glykolyse mit Natriumfluorid (NaF) zu einer Verminderung des Transports führte. Dies zeigt, von welch bedeutender Wichtigkeit die NKA zur Aufrechterhaltung einer stabilen Natriumkonzentration ist. Im Vergleich zum Soma erreichte die Natriumextrusionsrate in Dendriten mehr als den zehnfachen Wert und blieb auch bei Gabe von Ouabain oder NaF unverändert. Globale Glutamatapplikation zur Natriumerhöhung in der gesamten Zelle führte zu einer Elimination der Konzentrationsgradienten als treibender Kraft diffusiver Prozesse. Dabei konnten vergleichbare Extrusionsraten in Dendriten und Soma festgestellt werden, die wiederum durch NaF verringert wurden.

Die Aufrechterhaltung einer stabilen Natriumkonzentration in Dendriten und der Ausgleich lokaler Natriumtransienten wird hauptsächlich durch Diffusion gewährleistet. Eine physiologische Funktion der Zelle wird somit auch bei temporärer Energiedeprivation ermöglicht. Ist das Natriumniveau allerdings in weiten Teilen der Zelle erhöht, stellt die NKA vielmehr das maßgebliche System zur Regelung des Natriumhaushaltes dar. Natrium wird zwar in letzter Konsequenz stets durch die NKA aus der Zelle transportiert, der rasche Ausgleich lokaler Natriumerhöhungen durch Diffusion in benachbarte Zellareale sorgt jedoch für eine örtliche Verteilung des Energiebedarfs.

Abstract

Sodium homeostasis is of fundamental importance for the nervous system. Although increases in its intracellular concentration can occur during excitatory activity, baseline levels are kept stable through the action of the Na^+/K^+-ATPase (NKA). Nevertheless, sodium is a very mobile, not buffered ion and a few recent studies have suggested that diffusion can be an additional mechanism for the removal of sodium after local transients.

The present work was designed to elucidate the mechanisms for sodium removal in the soma and dendrite of pyramidal neurons from the CA1 region of the mouse hippocampus. Sodium loads where elicited by pressure application of glutamate in order to simulate physiological neuronal activity and variations in the intracellular sodium concentration were measured by wide-field imaging. In the soma, we showed that sodium transients recovered with a maximum velocity of 8 mM min^{-1}, which was reduced to zero after perfusion with ouabain, a specific inhibitor of NKA. It was also reduced by sodium fluoride (NaF), an inhibitor of glycolysis, revealing the NKA importance for sodium homeostasis. In the dendrites, however, sodium extrusion reached rates more than 10-fold higher when compared to the soma, and was unaffected by ouabain or NaF. Nevertheless, when glutamate was applied globally, inducing a sodium load in the whole cell, dendritic sodium extrusion rates were reduced to values similar to the ones of the soma and decreased by NaF.

This study shows evidences that in the dendrites, the recovery from local sodium increases is dominated by diffusion and the cell is capable of operating efficiently even in times of energy deficits and hence impaired NKA function. However, at a cellular scale, for example when sodium increases globally throughout the cell, concentration gradients as diffusional driving force are missing and the NKA becomes the main mediator of sodium extrusion in dendrites as well. From this point of view, the main relevance of this study is that although sodium will eventually be pumped out by the NKA, the fast diffusion of sodium to neighbour areas might reduce local energy requirements.

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Introduction

The main objective of the present work was to study the various mechanisms for sodium removal in the soma and the dendrites of CA1 hippocampal neurons. As we will see during this introduction, the Na^+/K^+- ATPase is the main responsible for maintaining sodium homeostasis, spending the majority of the energy used by the brain. By its turn, sodium modulates the generation of electrical signalling and provides the energy for a multitude of transport processes, from ion channels to the various pumps. Nevertheless, some recent studies have demonstrated that in some cellular compartments, such as the axon, sodium is mainly removed by lateral diffusion to not stimulated areas.

We will start with a brief introduction about the hippocampus, followed by a description of neuron structure and its various components and functions. With this, we hope to elucidate and clarify how important is sodium homeostasis and energy metabolism in neurons. A disturbance in one of those processes will interrupt many intracellular mechanisms, which can lead to signalling disruption and eventually to cell death.

In the second section of this introduction we will discuss sodium homeostasis and signalling, presenting some of the state of the art about this ion. We will also examine sodium signals, its various properties and characteristics and how they are important for the correct performance and signalling of neurons.

We will follow with a quick look at the Na^+/K^+- ATPase, its structure and function, how it is important for sodium homeostasis and its relation with energy metabolism.

At the last section we will discuss briefly the characteristics of epilepsy and epileptiform activity. We will describe shortly what it is known about the disease nowadays and the cellular mechanisms behind the induction of epileptiform activity.

1. Animal model – Hippocampus and neurons

The hippocampus has been identified as the centre of spatial memory and consolidation (McKenzie & Eichenbaum, 2011) and is one of the best-known cortical structures. Its study has particular relevance since it is the target of various neurodegenerative disorders such as Alzheimer's Disease (Craft *et al.*, 1996) and epilepsy of the temporal lobe. It has also been showed that the hippocampus is quite

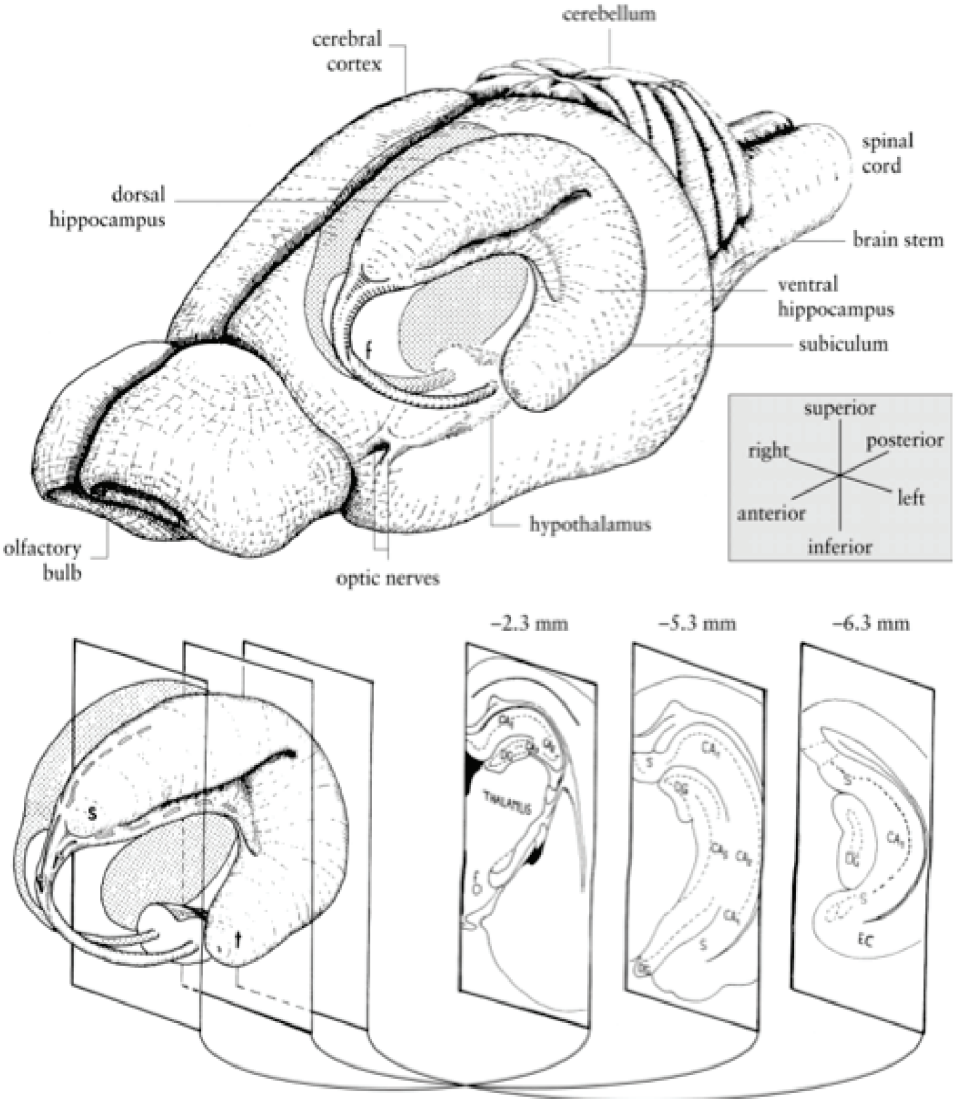

Figure 8- Diagram of a mouse hippocampus. Drawing scheme of a mouse brain emphasizing the tridimentional structure of the hippocampus. Adapted from (Cheung & Cardinal, 2005).

sensitive to energy metabolism and that deficiencies in ATP can induce depression (Fan *et al.*, 2015). There is also evidences that changes in energy homeostasis in the brain and in the periphery can influence dramatically the proliferation of adult neural stem cells and neurogenesis in the hippocampus (Sun *et al.*, 2015). If this is true, then any disturbances to energy metabolism should result in serious neuronal degeneration

and death. Especially if taking into account the correlation between ATP, NKA and sodium (Erecinska & Silver, 1994; Ames, 2000).

The hippocampus is a cylindrical structure situated in each cerebral hemisphere, on the intern side of the temporal lobe, in a dorsal position relative to thalamus and hipothalamus.

The hippocampus can be divided in four regions, usually denominated by CA1-CA4, from Latin *cornu Ammon*, that means horn of Ammon, because of its similitude with ram's horns. The CA1 and CA3 regions comprehend almost the whole hippocampus, in contrast with the CA2 region which is so small in some species that is ignored most times (Cheung & Cardinal, 2005). It is possible to divide it in three layers, the polymorphic layer (*stratum oriens*), pyramidal layer (*stratum piramidale*) and molecular layer (*stratum radiatum* and *stratum lacunosum-moleculare*). The most prolific type of neurons in the hippocampus are the pyramidal neurons which bodies are organized in the pyramidal layer. The cell bodies have a triangular shape (pyramidal) with a diameter of 20-40 μm in the base and 40 to 60 μm in the top. The apical dendrite has a diameter of around 5-10 μm and crosses the entire molecular layer. In addition, they also possess various basal dendrites that form the polymorphic layer. The morphology of the pyramidal neurons changes gradually along the hippocampus, the cell bodies go smaller and smaller while the apical dendrites grow larger as they approach the CA1 region (Alshuaib *et al.*, 2001).

1.1 Neuronal structure

In the nervous system exist two key types of cells, neurons and glial cells. A neuron has four morphologically defined regions: the cell body, dendrites, axon and presynaptic terminals. Each one of these regions has a different and distinct role in the generation and propagation of signals with other nerve cells (Harris *et al.*, 1992; Kandel *et al.*, 2013).

The cell body, or soma, functions as the metabolic centre of the cell. It comprises the nucleus and the endoplasmic reticulum, an extension of the nucleus where proteins are produced (Purves *et al.*, 2012; Kandel *et al.*, 2013). From the cell body, two distinct types of processes develop, several short dendrites and a long, tubular axon. The dendrites spread out in a tree-like fashion and are the cell equipment responsible for receiving incoming signals from other nerve cells (Purves *et al.*, 2012). On the other hand, the axon usually prolongs away from the cell body

transporting electrical signals, also called action potentials, to other nervous cells (Kandel *et al.*, 2013). These action potentials start at a particular region proximal to the origin of the axon, the initial segment, from which they continue down the axon without interruption at speeds of 1 to 100 m/s (Peles & Salzer, 2000).

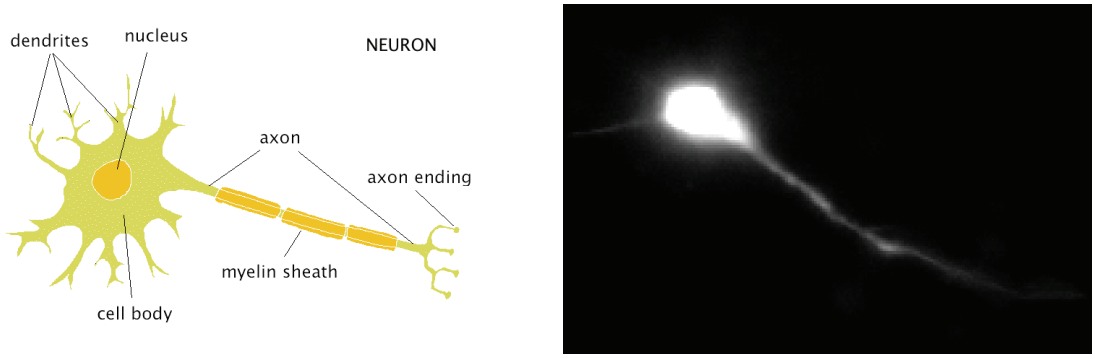

Figure 1- Neuronal structure and morphology. On the left side is visible a schematic drawing of a neuron. Some important regions are identified (adapted from http://webspace.ship.edu/cgboer/theneuron.html). On the right, wide-field image of a pyramidal neuron from the CA1 hippocampal region of a *balb/c* mice. The neuron is filled with sodium-binding benzofuran isophthalate (SBFI) and is an example of one of the cells used during the present work.

Action potentials are the signals by which the brain receives, analyses and transfers information (Kandel *et al.*, 2013). They are highly similar throughout the entirety of the nervous system, although they are initiated by a great array of events and proceedings (Purves *et al.*, 2012). In order to increase the speed of the action potentials, an isolating sheath of myelin, a lipid substance, envelops the axons. At the end of the axon, this process is divided into fine branches that contact other neurons specific zones. These zones are called synapses and is where one cell communicates to the next one (Kandel *et al.*, 2013).

Ramón y Cajal was among the first to understand that the most distinguishable feature between the various types of neuron is form, specially the number of the processes starting at the cell body. Therefore they were separated into three major groups, unipolar, bipolar and multipolar (Ramón y Cajal, 1933). Neurons can also be classified into three principal functional categories: sensory neurons, motor neurons and interneurons (Purves *et al.*, 2012).

As previously said, neurons have the ability to communicate precisely and rapidly with other cells. The major factor for this ability is the capacity of neurons for being excitable, either electrically or chemically. The cell membrane of neurons possess ion channels and receptors that allow the flux of inorganic ions that reorganize the charge and create electrical currents that modify the voltage across the membrane (Mueller *et al.*, 1962; Kandel *et al.*, 2013). The changes in charge induce a wave of depolarization in the form of action potentials.

1.2 Ion channels

Ion channels are proteins that spam the cell membrane and have three important properties, first they distinguish and select specific ions; second, they open and close as a response to various *stimuli* such as electrical, mechanical or chemical signals, and third, they conduct ions across the membrane (Mueller *et al.*, 1962; Hille, 2001; Kandel *et al.*, 2013). The movement of these ions across the membrane produce rapid changes in membrane potential essential for signalling. Each type of channel is selective for one or two types of ions (Hille, 2001). The resting potential of cells, for example, is mainly determined by a class of K^+ channels that are 100-fold more permeable to K^+ than to Na^+ (Purves *et al.*, 2012; Kandel *et al.*, 2013). On the other hand, when action potentials occur, a class of Na^+ channels is activated. In this case, these sodium channels are 10 to 20 times more permeable to Na^+ than to K^+ (Andersen & Koeppe, 1992; Hille, 2001; Kandel *et al.*, 2013).

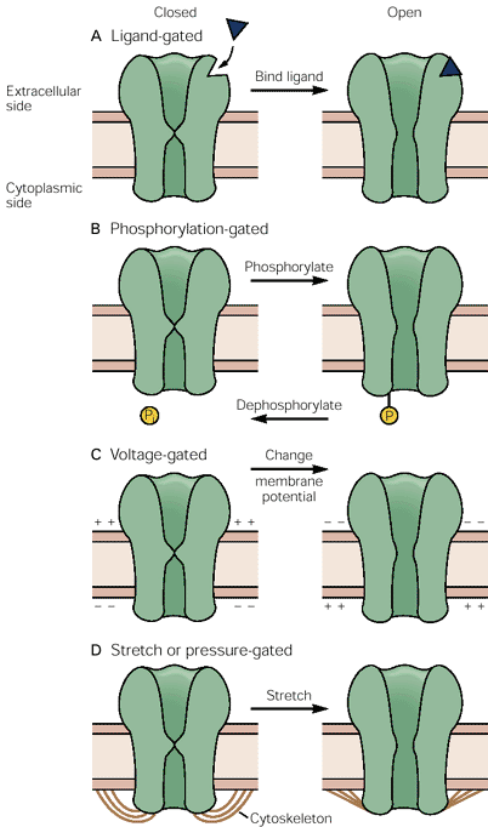

Figure 2- Types of channels gating mechanisms. There are four types of gating mechanisms that control the opening of the various channels. A) Ligand-gated; B) Phosphorylation-gated; C) Voltage-gated and D) Stretch or pressure-gated. These different mechanism control the stated of the channel; resting, active or refractory. Image adapted from the book *Principles of Neural Science 5th Edition* (Kandel *et al.*, 2013).

Ion channels have two or more stable conformational states, representing each one of them, a different functional state. For example, each ion channel has at least one open state and one or two closed states. The transition between these various states is called gating (Hille, 2001; Kandel *et al.*, 2013). As previously stated, the primary function of channels is to create transient electrical signals and three main gating mechanisms have been established to control channel opening (Andersen & Koeppe, 1992; Kandel *et al.*, 2013). Some channels are opened by the binding of chemical ligands (agonists), some of them binding directly to the channel either at an extracellular or intracellular site. Other ligands activate cellular cascades, which can covalently modify a channel through protein phosphorylation (Purves *et*

al., 2012). Some channels are regulated by changes in membrane potential. Finally, other channels are regulated by mechanical stretch of the membrane (Hille, 2001; Kandel *et al.*, 2013). These mechanisms are responsible for the regulation of the channels and control its entry into one of three functional states: closed and activatable (resting), open (active), or closed and nonactivatable (refractory) (Purves *et al.*, 2012; Kandel *et al.*, 2013). The alteration in the functional state of a channel involves the expending of energy (Purves *et al.*, 2012); for example, in voltage-gated channels the movement of a charged region of the channel protein across the membrane's electric field affords the energy for the whole process. This voltage sensor possess basic (positively charged) or acidic (negatively charged) amino acids that induce a net electric charge (Jiang *et al.*, 2002; Kandel *et al.*, 2013). In transmitter-gated channels, on the other hand, the binding of the transmitter to the receptor induces a change in chemical-free energy that in addition stimulates the transformation between the various channel states (Hille, 2001; Gadsby, 2004).

1.3 Synapses

The main objective of the present work is to elucidate the mechanisms for sodium removal after sodium loads in cellular compartments. Sodium loads can arise especially from physiological neuronal activity (Lasser-Ross & Ross, 1992) that occurs at synapses situated in the dendrites and dendritic spines (Rose *et al.*, 1999; Rose & Konnerth, 2001).

Synapse is the dedicated site at which one neuron communicates with another. Synapse transmission is of central importance to neural functions like perception, voluntary movement and learning (Kandel *et al.*, 2013). Usually, neurons have thousands of synapses that they share with other neurons and while some of them are specialized, all synapses use one of two types of transmission: electrical or chemical. In addition, cellular activity can modulate both forms (Carew & Kandel, 1976; Beyer *et al.*, 1987; Hille, 2001; Kandel *et al.*, 2013). This effect, called synaptic plasticity is of extreme importance to memory and other higher brain functions. While electrical synapses are used specially to send rapid and stereotyped depolarizing signals, chemical synapses are capable of more complex activities, for example, they are capable of performing excitatory and inhibitory actions or produce electrical changes that can last several minutes in the postsynaptic cell (Hille, 2001; Kandel *et al.*, 2013). On the other hand, they can also serve to amplify neuronal signals. As a result of this

trait, a small presynaptic nerve terminal can change the response of large postsynaptic cells. In the brain, the large majority of synapses are chemical synapses (Unwin & Zampighi, 1980).

Another major difference concerning both types is that in chemical synapses there is no structural continuity, there is no real connection, between pre- and postsynaptic neurons. In truth, the space that separates both cells at a chemical synapse, the synaptic cleft, is normally wider than the nonsynaptic intercellular space (Ramón y Cajal, 1909) for this reason, the diffusion of the neurotransmitter (the chemical substance that binds to the receptors of the postsynaptic cell) across the synaptic cleft is of critical importance for this type of transmission (Purshpan & Potter, 1957). The neurotransmitters are usually released from specialized swellings of the axon, the presynaptic terminals, which normally contain 100 to 200 synaptic vesicles containing several thousand molecules of the transmitter. The release of neurotransmitters is usually started by the arrival of an action potential, which induces the opening of voltage-gated Ca^{2+} channels (Hille, 2001; Purves *et al.*, 2012). As a consequence, calcium enters the presynaptic terminal, which by its turn initiates a biochemical reaction that causes the vesicles to fuse with the presynaptic membrane releasing the neurotransmitter into the synaptic cleft. Once in the synaptic cleft, the neurotransmitter diffuses away, binding to its specific receptors situated in the postsynaptic cell membrane. The activation of the postsynaptic receptors leads, by its turn, to the opening or closing of ion channels, which results in a flux of ions across the membrane, altering the membrane conductance and potential of the postsynaptic cell (Carew & Kandel, 1976; Kandel *et al.*, 2013).

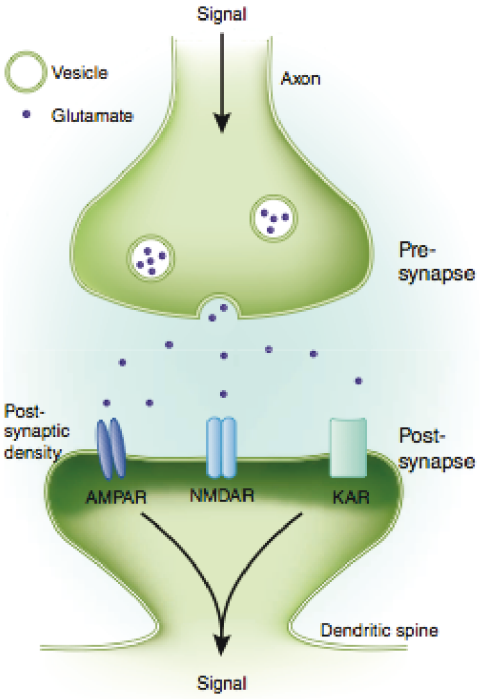

Figure 3- Synapse. Example of a glutamatergic synapse (chemical synapse). Upon activation of a CA1 pyramidal neuron, the cell releases glutamate via exocytosis at presynaptic sites, which, by its turn, activates the glutamatergic receptors of the postsynaptic cell. Adapted from (Gécz, 2010).

Because all these steps have to be performed, there is a delay at chemical synapses that can last several milliseconds. For this reason, signal transmission across

chemical synapses is slower than in electrical ones. Nevertheless, they have the property of amplification. A single synaptic vesicle can release thousand of neurotransmitter molecules. These molecules, in turn, can open thousands of ion channels in the postsynaptic cell. By this method, a small presynaptic nerve terminal, that can only produce a weak electrical current, has the ability to depolarize a large postsynaptic cell (Unwin & Zampighi, 1980; Hille, 2001; Purves *et al.*, 2012; Kandel *et al.*, 2013).

Two main steps are responsible for chemical transmission: first, the presynaptic cell releases the chemical messenger into the synaptic cleft, what is called the transmitting step; and second, after diffusing through the cleft the transmitter molecules bind to the postsynaptic terminal, which by its turn, activates the receptors, this step is called the receptive step (Purves *et al.*, 2012; Kandel *et al.*, 2013). There is an array of chemical substances that can function as neurotransmitters, from small molecules to peptides. Nevertheless, the action of a certain transmitter depends on the characteristics of the postsynaptic receptors that identify and bind the neurotransmitter and not its own chemical properties (Carew & Kandel, 1976; Kandel *et al.*, 2013). For instance, some neurotransmitters can excite some postsynaptic neurons while they inhibit others. The control that a neurotransmitter performs on a certain ion channel can be direct or indirect and it is mediated by different kind of receptors. Receptors that modulate ion channels directly, are usually composed of four or five subunits and contain an extracellular domain that consists of the binding site for the neurotransmitter, and, in addition, a membrane spanning domain that forms the ion conducting pore. These types of receptors are denominated as ionotropic receptors (Purves *et al.*, 2012; Kandel *et al.*, 2013). Once the neurotransmitter binds to the receptor, it experiences a conformational change that opens the channel (Waxman *et al.*, 1972).

On the other hand, receptors that gate ions channels indirectly are often denominated as metabotropic receptors (Purves *et al.*, 2012; Kandel *et al.*, 2013). In this case, receptors are usually composed of seven membrane spanning domains, normally α-helixes, and act by shifting intracellular metabolic reactions. Normally, they are composed of one or two subunits that are different from the ion channels they regulate. The regulation of this ion channels is usually performed by production of second messengers, for example cAMP (Hille, 2001; Kandel *et al.*, 2013). These second messengers activate kinases that, by their turn, can phosphorylate various

substrate proteins, or the ion channels themselves, leading to their opening or closing. While ionotropic receptors induce fast synaptic activities, metabotropic receptors, on the other hand, modulate much slower actions. These slower actions can, however, be very important, since they can modify behaviour by changing neuron excitability and synaptic connections strength. These changes in circuit mediating behaviour, can often act as critical reinforcing pathways, very important to the learning process (Purves *et al.*, 2012).

1.4 Glutamate and its receptors

The amino acid l-glutamate is the main excitatory transmitter in the brain and spinal cord (Purves *et al.*, 2012). The opening of glutamate-gated channels that are permeable to Na^+ and K^+ induces the excitatory postsynaptic potential (EPSP) in spinal cord cell (Eccles, 1964). Glutamate receptors can be divided into two major categories, the ionotropic receptors, channels where the binding of glutamate directly opens the channel; and metabotropic receptors that are directly couple to G proteins that through the production of secondary messengers gate the channel accordingly (Purves *et al.*, 2012; Kandel *et al.*, 2013). There are three main subtypes of ionotropic glutamate receptors, AMPA, kainate and NMDA, named after the synthetic agonists that activate them (Gécz, 2010; Kandel *et al.*, 2013). NMDA receptors are selectively blocked by the drug APV (2-amino-5-phosphonovaleric acid), while AMPA and kainite receptors are not affected by APV, but, on the other hand, they are blocked by CNQX (6cyano-7-nitroquinoxaline-2,3-dione). Ionotropic glutamate receptors usually are excitatory or depolarizing (Gécz, 2010; Purves *et al.*, 2012).

NMDA receptors have many interesting properties. They are permeable to Ca^{2+} as well as to Na^+ and K^+ (Hille, 2001; Kandel *et al.*, 2013). On the other hand, they are the only ligand-gated channels whose opening depends both of transmitter release and membrane voltage. At the resting membrane potential, extracellular Mg^{2+} binds strongly to a site in the pore of the channel, blocking ionic current (Purves *et al.*, 2012). However, after depolarization, usually by activation of AMPA receptors, Mg^{2+} is released from the channel, allowing the entrance of Na^+ and Ca^{2+}. In the majority of central synapses, the postsynaptic terminal contains both AMPA and NMDA receptors and the respective contributions can be dissected by the use of specific pharmacological blockers (Sakmann, 1992; Kandel *et al.*, 2013). As previously stated, at the normal resting potential, Mg^{2+} is blocking the NMDA

receptors. For this reason, the excitatory postsynaptic current (EPSC) is mainly mediated by the charge flowing through AMPA receptors, which generate a current characterized by very fast rising and decay phases (Eccles, 1964). Nevertheless, as the neuron becomes depolarized, Mg^{2+} is expelled from the pore of NMDA receptors, allowing the flow of ions through these channels to increase (Purves *et al.*, 2012; Kandel *et al.*, 2013). For this reason, the current through NMDA receptors is characterize by much slower times either in the rising and decay phases, than the AMPA receptors (Gécz, 2010).

In the present work, physiological synaptic activity was simulated by local pressure application of glutamate in the proximity of the soma or dendrite. With this protocol sodium currents were elicited through activation of both NMDA and AMPA receptors.

2. Ion homeostasis

Like in all tissues, cells maintain an ion gradient across their membrane in the brain. Concentrations of Na^{+}, Ca^{2+} and Cl^{-} are very high when compared to the ones observed in the cytoplasm (Hille, 2001; Kandel *et al.*, 2013). For K^{+} however, the exact opposite is true, the concentration intracellularly is much higher than in the extracellular space. These gradients, as well as the ion permeability of the cell membrane, are the main responsibles for the maintenance of the membrane potential (Purves *et al.*, 2012). In neurons, the resting membrane potential is usually of around -60 to -70 mV (Hille, 2001). As it was previously stated, changes in the membrane potential elicited by the flux of these ions across the cellular membrane are one of the main characteristics of excitable neurons. By the opening of ligand-gated ion channels, neurons generate synaptic potentials (Purves *et al.*, 2012; Kandel *et al.*, 2013). In addition to this fact, activation of voltage-gated ion channels produces regenerative, self-sustaining action potentials. On the other hand, ion gradients and membrane potentials are not only responsible for electrical signalling but as well serve as requirements for many cell functions, for example, transmembrane substrate transport, regulation of cell volume and pH, as well as energy production. Likewise, the Ca^{2+} gradient across the membrane allows this ion to act as a second messenger (Purves *et al.*, 2012; Kandel *et al.*, 2013).

Each ion is subjected to diverse transport processes, including a multitude of channels that serve as paths by which each ion can cross the membrane.

Consequently, there is a leakage of ions that cells need to counteract to maintain the gradients. For this reason, cells spend a lot of the energy generated in the use of ion pumps. Among the most important ones, the sodium potassium ATPase (NKA), the plasma membrane Ca^{2+}- ATPase (PMCA) and the sarcoendoplasmatic Ca^{2+} ATPase (SERCA) (Alberts *et al.*, 2009).

Hence, ion gradients are strictly regulated by homeostatic mechanisms in order to maintain cell function and viability. However, homeostatic processes are interdependent and are modulated by the cell and tissue status. For this reason, the regulatory machinery also serves an adaptive function and, therefore, homeostatic responses may also be interpreted as signals (Purves *et al.*, 2012; Kandel *et al.*, 2013).

2.1 Sodium regulation

The concentration of Na^+ in the extracellular space is around 140-150 mM, while inside the cells is about 10 to 15 mM (Langer & Rose, 2009; Kelly & Rose, 2010*a*; Karus *et al.*, 2015). The maintenance of a low sodium concentration is of critical importance for the generation of electrical signalling in neurons, working as basic charge carriers, while also offering the energy required for a multitude of transport processes at the plasma membrane. For example, the Na^+/Ca^{2+} -exchange (NCX) makes use of the electrochemical gradient generated by sodium for the export of calcium (Rose, 2002).

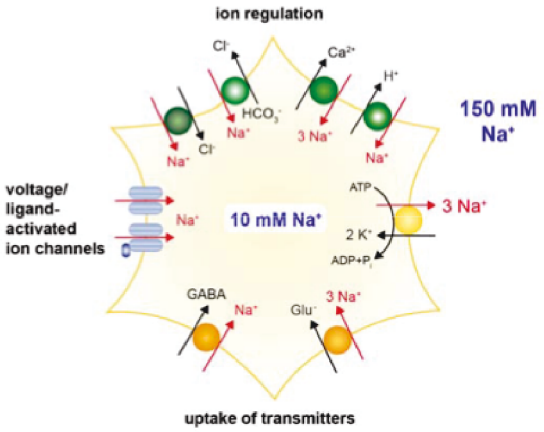

Figure 4- Sodium homeostasis. The sodium concentration in the extracellular space is around 140-150 mM while inside the cells is about 10 to 15 mM. Sodium enter the cells via various channels and transporters. The main mechanism for the removal of sodium from the intracellular space is the Na^+/K^+- ATPase. Image courtesy of Christine Rose.

Many pathological conditions have been described and characterized by simultaneous widespread sodium increases in neurons. These increases of

intracellular sodium induce a decrease in the driving force and the activity of sodium-dependent cellular transport processes, causing, in some cases, their reversal. For instance, an increase in the intracellular sodium concentration in peripheral axons, can cause the reversal of the NCX, which by its turn, induces massive calcium loads, leading, possibly, to cell damage and death (Kelly & Rose, 2010*a*). These increases in sodium intracellular concentration can occur from sodium entrance through glutamate-gated ionotropic receptors, NMDA and AMPA as discussed before; also, it's worth noticing that calcium is of crucial importance for neurotransmitter release. This process, the rise of intracellular sodium concentration, is especially observable in fine processes, such as the axons of vertebrate or fine dendrites (Lasser-Ross & Ross, 1992) and it has been well demonstrated by using sodium-sensitive dyes, for example SBFI (sodium-binding benzofuran isophthalate). Also, it has been demonstrated that in hippocampal neurons, short-burst synaptic stimulation of glutamatergic fibres (Schaffer collaterals) induced local sodium transients in dendrites that amounted to 10 mM, while with a typical LTP induction protocol, dendritic sodium rose by even 45 mM (Rose & Konnerth, 2001). These changes in intracellular sodium concentration are quite surprising since for a long time it was thought that neuronal activity did not induce any measurable fluctuation in sodium (Hodgkin & Huxley, 1952).

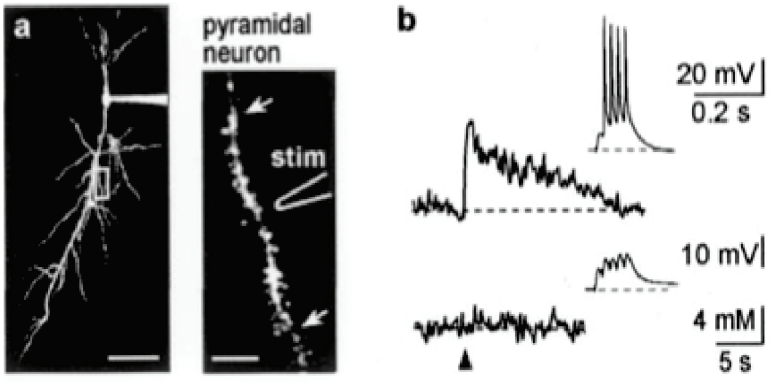

Figure 5- Sodium transients evoked in dendrites. Reconstruction of a stack of 24 optical sections taken at 5 µm intervals from a pyramidal neuron loaded with SBFI. Suprathreshold synaptic stimulation elicits a proeminent sodium load into the dendrite. Image adapted from (Rose & Konnerth, 2001).

In presynaptic terminals, sodium increases can induce the reversal of the NCX. As previously discussed, neurotransmitter release is dependent of calcium, for this reason, the reversal of the NCX imports Ca^{2+}, rising calcium intracellular concentration which by its turn increases transmitter release (Goldman *et al.*, 1994; Bouron & Reuter, 1996; Regehr, 1997). In Bergmann glia cells *in situ*, an increase of

intracellular sodium concentration to around 30 mM by kainite application and the concomitant depolarization were shown to cause reversal of the NCX and the enhance the observed Ca^{2+} signal (Kirischuk *et al.*, 1997).

In the same way, sodium loads can also induce non-vesicular GABA release. An action potential induces the increase in intracellular sodium concentration, which by its turn reverses the GABA transporter GAT-1 (Wu *et al.*, 2007). Some other experiments, this time in initial axon segments, showed that fast sodium transients can be induced by opening of voltage-gated sodium channels during action potential generation and that this transients are not altered by ouabain perfusion (Fleidervish *et al.*, 2010), a specific NKA blocker (Lelievre *et al.*, 1979; Sandtner *et al.*, 2011).

As previously stated, sodium transients have been observed in dendrites, induced by cellular activity, as for example, during back-propagating action potentials (Jaffe *et al.*, 1992), a result also demonstrated in dendritic spines by previous studies in our laboratory, using quantitative multi-photon imaging (Rose *et al.*, 1999). Interestingly, it was also observed that the sodium signals evoked, had different

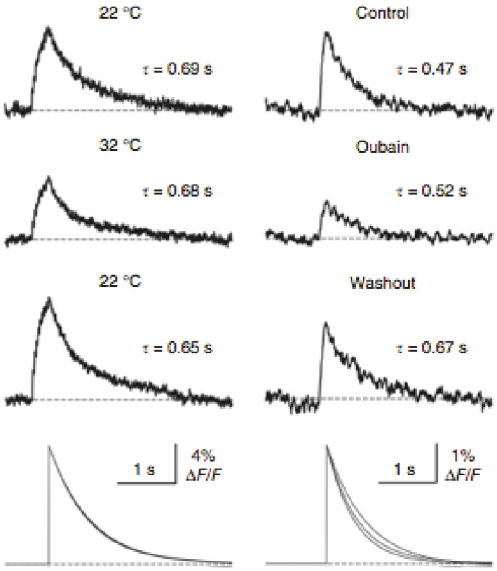

Figure 6- Clearance of sodium in the axon. Active transport cannot account for the swift removal of sodium from the axon. The sodium removal is not affected by temperature and is almost unaltered by the presence of the NKA specific blocker ouabain. Adapted from (Fleidervish *et al.*, 2010).

amplitudes in spines and dendrites, being larger in the first case. These results, which were later corroborated in neocortical neurons (Araya *et al.*, 2007), suggested the presence of voltage-gated sodium channels in spine heads. On the other hand, for example, in cerebellar Purkinje neurons there is no back-propagation of sodium-based action potentials and somatically induced action potentials do not cause sodium transients in the dendritic tree (Lasser-Ross & Ross, 1992).

In CA1 neurons, the main responsible for sodium influx in response to glutamatergic synaptic transmission are NMDA receptors (Rose & Konnerth, 2001).

Once again, Purkinje neurons behave very differently. In these neurons, parallel fibre activation causes local sodium transients in the mM range that are mainly mediated by AMPA receptors (Callaway & Ross, 1997; Bennay *et al.*, 2008). As described in a previous section these receptors act quite differently, exhibiting very distinct activation procedures.

All these studies show clear evidence that sodium transients can reach large amplitudes in very small compartments, as spines or dendrites. For this reason, the removal of sodium from these areas should be of ultimate importance, otherwise sodium homeostasis could be completely disrupted, resulting in serious physiological consequences. Large sodium concentrations in synapses can, for example, decrease the driving force for this ion, affecting all the corresponding currents, which by its turn can induce a state of "dendritic saturation", turning the respective synapse inactive (Bush & Sejnowski, 1994). In addition, large sodium transients, in the range of 10 to 30 mM, have the possibility to modulate the activity of sodium-dependent potassium channels (K_{Na}). This effect can contribute to the after-hyperpolarization following repetitive discharges (Bhattacharjee & Kaczmarek, 2005). In addition, sodium can also modulate synaptic plasticity as it has been demonstrated in Purkinje neurons, where flux of sodium through AMPA receptors is a prerequisite for the induction of LTD (long-term depression) (Linden *et al.*, 1993). Also, the activity of a src-kinase, an enzyme closely associated with NMDA channels and one of the main responsibles for its regulation, is influenced and modulated by intracellular sodium concentration (Yu & Salter, 1998). Another important fact is that sodium changes also affect the time course of activity-induced calcium signals in spines of hippocampal CA1 neurons (Scheuss *et al.*, 2006), which as already discussed is critical for neurotransmitter release . In this case, the accumulation and increase in intracellular sodium induces a reduction in the activity of Ca^{2+} -ATPase and NCX which by its turn reduces calcium extrusion. This result clearly points to the correlation between sodium and calcium homeostasis, indicating that sodium transients can modulate calcium signalling as well as regulate calcium-dependent synaptic plasticity (Rose & Konnerth, 2001).

Even thought the high functional importance and consequences of sodium changes are well known, the properties and determinants of activity-induced sodium signals are still poorly understood, as there are some basic biophysical parameters. For example, the number of studies about its diffusional properties in cellular

microdomains such as dendrites or spines is very limited, as well as there are works about how the amplitude and time course of sodium transients are related to specific activity patterns.

Sodium transients can be elicited experimentally by local application of glutamate. As previously described in this introduction, glutamate activates AMPA and NMDA receptors allowing the entrance of sodium into the cell. In the present work sodium transients were elicited using this exact process, glutamate was applied locally in the proximity of the dendrite or soma via pressure application and recorded using SBFI. Sodium signals induced this way achieved maximal sodium concentration in a couple of milliseconds followed by a decrease. It is possible to fit this decay in sodium concentration with a monoexponential function characterized by a decay time constant of several seconds in dendrites (Rose *et al.*, 1999; Rose & Konnerth, 2001; Meier *et al.*, 2006; Bennay *et al.*, 2008). Usually, this decay time has been assumed to be mainly determined by NKA activity, that is, the velocity at which the NKA extrudes sodium, removing it from the intracellular space. However, sodium possess a diffusion coefficient of about 600 $\mu m^2/s$ (Kushmerick & Podolsky, 1969) and has been described to not be buffered intracellularly (Despa & Bers, 2003; Fleidervish *et al.*, 2010). Comparing it to calcium, for example, sodium transients behave quite differently; first, sodium loads have slower decay times (Kuruma *et al.*, 2003); second, calcium is buffered in the intracellular space (Neher & Augustine, 1992; Neher, 1995) and third, the diffusion coefficient for calcium is lower than for sodium (Allbritton *et al.*, 1992). In addition, our laboratory have showed that sodium signals spread passively between hippocampal astrocytes faster than calcium waves (Langer *et al.*, 2012), most probably because it is not buffered.

In fact, as stated by many studies, the NKA is of central importance in the regulation of intracellular sodium (Rose & Ransom, 1997; Azarias *et al.*, 1998), acting as the main mechanism responsible for the removal of sodium from inside the cells. Inhibition of NKA activity induces an immediate increase in intracellular sodium in central neurons, even at rest, and since sodium transients follow a monoexponential decay, it has been hypothesized that this recovery mainly reflects the pump's activity (Rose & Konnerth, 2001). However, as previously stated, sodium is not buffered intracellularly and shows a relative high diffusion coefficient. In addition, some recent studies have showed that fast sodium transients in the initial segment of axons are not altered upon inhibition of the NKA by ouabain, indicating

that diffusion was responsible for their fast decay (Fleidervish *et al.*, 2010). In the present work it was attempted to answer this question, in CA1 hippocampal neurons is sodium extrusion mainly mediated by the NKA or by diffusion?

3. Na^+/K^+ -ATPase

As previously stated, the maintenance of a steep sodium gradient across the plasma membrane is of critical importance for the function and survival of all eukaryotic cells. The main responsible for the maintenance of the sodium gradient is the enzyme Na^+/K^+ -ATPase (NKA), it is also one of the major subjects of the present work. In order to understand the main mechanisms for sodium removal in CA1 hippocampal neurons, the study of the NKA, its activity, rate of sodium extrusion and its behaviour in the presence of inhibitors, is of vital importance. The NKA moves two potassium ions into the cell, while at the same time, transports three sodium ions to the extracellular space. This process is also accompanied by the breakdown of one ATP molecule, which supplies the energy for the whole process. It is also a major determinant for the neuronal resting potential as well for the excitable properties of muscle and nerve cells (Blanco & Mercer, 1998). The balance of the Na^+ gradient on the other hand, assures that Na^+-coupled transporters, for example, the translocation of ions (H^+, Ca^{2+}, Cl^-, PO_4^{3-}, SO_4^{2-}), substrates such as glucose and aminoacids) and neurotransmitters through the plasma membrane, always have enough energy for their proper function (Glynn, 1993). In the kidney, for example, the NKA plays a primary role in driving the reabsorption of sodium and water. Therefore, this enzyme is vital in the maintenance of body fluid and electrolyte homeostasis (Glynn, 1993; Jorgensen *et al.*, 2003).

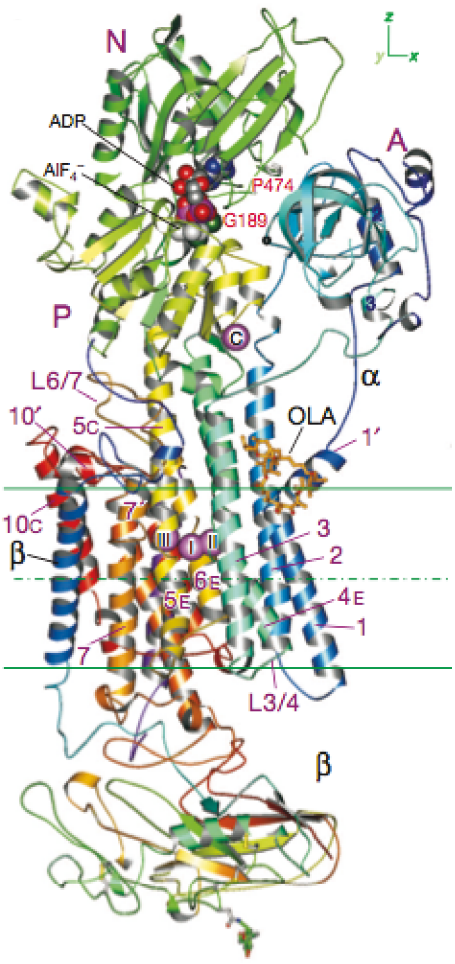

Figure 7- Tridimentional structure of the Na^+/K^+- ATPase. Cristal structure of the NKA in the transition state. The purple spheres represent sodium ions. Image adapted from (Kanai *et al.*, 2013).

The NKA belongs to a family of ATPases, known as P-type, and their main function is to transport various cations through the cell membrane (Blanco & Mercer, 1998). This family of ATPases is located in prokaryotic and eukaryotic cells and transport ions such as H^+, Na^+, Mg^{2+}, K^+, Ca^{2+}, Cu^{2+}, and Cd^{2+} (Lutsenko & Kaplan, 1993). To move the cations against their electrochemical gradient they spend ATP. P-type ATPases have the ability to form a transient state that has a phosphorylated aspartyl residue, and also, in addition, they are characterized by binding, occluding and transporting ions through the cycling between two different conformations, denominated E1 and E2 (Repke & Schön, 1992; Blanco & Mercer, 1998).

Nevertheless, the reaction mechanism (the way they transport the ions across the membrane) is not the only similarity that this family of enzymes share, they also possess equivalent tertiary structures, similar membrane topological organization, as well as several protein domains highly conserved through the enzyme family (Lutsenko & Kaplan, 1993).

The NKA is an oligomeric protein composed of two major polypeptides, the alpha (α) and beta (β) subunits, as well as a third one, gama (γ) (Reeves *et al.*, 1980; Blanco & Mercer, 1998). The α-subunit is a membrane protein with a molecular weight of 112 kDA that traverses the cellular membrane multiple times. In addition, the α-subunit holds the catalytic centre and possess the transport properties of the enzyme, containing the binding sites for the ions, ATP and the specific NKA inhibitor ouabain (Lingrel & Kuntzweiler, 1994). The β-subunit, on the other hand, consists of a polypeptide that only traverses the membrane one time and has a molecular mass around 40 to 60 kDa (Blanco & Mercer, 1998). This mass is, however, dependent on the amount of glycosylation of the polypeptide, which actually varies from tissue to tissue (Blanco *et al.*, 1994; Clapp *et al.*, 1994). The β-subunit also seems to be responsible in the occlusion of K^+ and regulate the enzyme affinity for potassium and sodium. In addition to this fact, the β-subunit can also work as a chaperone by stabilization of the right folding of the α-subunit, to ease its transportation to the plasma membrane in vertebrates (McDonough *et al.*, 1990; Clapp *et al.*, 1994; Blanco & Mercer, 1998).

The third subunit, γ-subunit, is the smallest of the three, with 8 to 14 kDa mass, that possess hydrophobic properties. For some time it was thought to be nothing more than a contaminant of purification (Blanco & Mercer, 1998). Nevertheless it was later discovered that this polypeptide could be covalently labelled by

photoaffinity derivates of ouabain (Forbush *et al.*, 1978; Lowndes *et al.*, 1984). In addition, it has also been showed some colocalization between γ and α-subunits in nephron segments, and also, immunoprecipitation studies demonstrated that the γ-subunit coimmunoprecipitates together with αβ-complexes (Mercer *et al.*, 1993). This subunit has a high level of homology between various species, which usually suggests that the polypeptide in question is important for the respective enzyme function. Some expression experiments have showed that this subunit is not required for the enzyme activity. Nevertheless, some recent studies in *Xenopus* oocytes suggest the voltage dependence of potassium activation can be modify by the γ-subunit (Minor *et al.*, 1998). It has also been implied by some authors, that the γ-subunit has the ability to stabilize the E1 conformation (Therien *et al.*, 1997).

As many essential proteins in cells, the NKA is expressed as several distinct isozymes. Studies by many molecular biology techniques have identified at least three α-subunits in vertebrates (α1, α2 and α3), with each one showing different affinities for sodium and even potassium (Zahler *et al.*, 1997). This three α isoforms are products of three different genes. Of the three NKA subtypes, only α1 and α3 are believed to be neuronal. It appears that α1 is a "housekeeping" isoform in most tissues, being highly expressed in various epithelia with high rates of solute transport (Hieber *et al.*, 1991; McGrail *et al.*, 1991). On the other hand, α2 is found in muscle, adipose tissue, and some studies have also showed some expression in the brain. The expression of α3 has been identified in neural tissue and heart. There is also another different subunit, called α4 that has been acknowledged in testis (Kaplan, 2002; Moseley *et al.*, 2007).

Some recent studies have showed a huge variety in the affinity for sodium of the various α-subunits, having α1 and α2 around 2 to 3 times more affinity than α3 (apparent K_{Na}^{+} α1 = 1.15 ± 0.13 mM; α2 = 1.05 ± 0.11 mM; α3 = 3.08 ± 0.06 mM) (Jewel & Lingrels, 1991). With this data, it has been hypothesized that the α3 isoform may be specifically required for restoration of baseline sodium levels after large transients. The maximum velocity for the extrusion of sodium, that is the removal of sodium from the intracellular space, has been estimated in myocytes to be around 8 mM min^{-1} (Despa *et al.*, 2002*b*, 2004).

In addition to different affinities to sodium ions, the various α-subunits also show various affinities for ouabain, a NKA specific blocker. Inhibition of the NKA activity indicates that α1 has an IC_{50} around 1000-fold higher than α2 or α3,

indicating that the order of ouabain affinity between the different isoforms is α3 > α2 >> α1 (O'Brien *et al.*, 1994; Richards *et al.*, 2007).

With this in mind, is easily understandable the huge importance of the NKA for the intracellular homeostasis and why mutations in its structure can lead to learning and memory deficits (Moseley *et al.*, 2007), parkinsonism (De Carvalho Aguiar *et al.*, 2004), or hemiplegic migraine (Capendeguy & Horisberger, 2004).

3.1 NKA and energy

The balance of extra- and intracellular ion concentrations is of highest functional importance for the brain and requires constant cellular transport activity and energy supply (Erecinska & Silver, 1994; Somjen, 2002). The majority of this energy is used for the transport of sodium to the extracellular space, and as discussed previously, the main responsible for this transportation is the Na^+/K^+- ATPase. As it is well known, the brain is one of the organs that is particularly sensitive to lack of oxygen and in humans is responsible for 20 % of total O_2 consumption, although it accounts for only 2 % of the body weight (Erecińska & Silver, 1989). There is also evidence that sodium transport through the NKA consumes about 50 % of all energy generated in the central nervous system (CNS) (Ames, 2000). For this reason, any disturbances in energy metabolism can induce impairments in the function of the NKA, with massive consequences for neurons.

However, there is some speculation about the origin of the ATP used by the NKA, some studies point to the glycolysis, other ones to mitochondrial respiration. There is proof that glycolytic enzymes are closely associated with NKA. In erythrocytes, for example, it has been showed that glycolytic enzymes within the plasma membrane makes the respective generated ATP immediately available for the energy-demanding transport systems (Proverbio & Hoffman, 1977). In some experiments with exogenous labelled ATP, it was demonstrated that ATP produced intracellularly (unlabelled) is collected in a membrane pool and used by the NKA instead of the ATP applied exogenously (Proverbio & Hoffman, 1977). Another work showed that varying the energy required by the NKA, by increasing extracellular K^+, reducing intracellular Na^+ or adding ouabain, corresponding changes were observed in glycolysis (Paul *et al.*, 1979). Additionally, further evidence for localization of energy metabolism within the cell was provided by the finding that the substrate for glycolysis was separated from the substrate for oxidative metabolism.

On the other hand, glycolysis accounts only for 5 % of the energy generated by the CNS while oxidative metabolism is responsible for around 95 % (Erecińska & Silver, 1989). In addition to this fact, some correlations between NKA and mitochondrial respiration have been found. For example, there is colocalization in the clumping of mitochondria where energy demands are high (Ames, 2000). Using histochemical techniques have demonstrated cytochrome oxidase colocalization at subcellular levels with NKA and NMDA glutamate receptors (Nie & Wong-Riley, 1996). It has also been showed that the level of cytochrome oxidade responded to changes in energy demands, as demonstrated by reversible reductions in the enzyme at sites containing voltage-sensitive Na^+ channels following sustained administration of tetrodotoxin (Wong-Riley, 1989). There is also evidence in the rabbit retina, that only one-tenth of the phosphate used by the NKA was generated glycolytically (Wong-Riley *et al.*, 1998). Cultured astrocytes, for example, maintained their Na^+ gradients when glycolysis was blocked with 2-deoxyglucose, as long as oxidative metabolism remained intact. In addition, it has also been revealed in cultured hippocampal neurons a decrease in glucose uptake upon activation of glutamate receptors and sodium entry (Porras *et al.*, 2004). This is evidence that conversion of glucose in the glycolytic pathway is not essential under these conditions. Another study provided evidence that the NKA is mainly fuelled by ATP produced by oxidative phosphorylation (Fernandez-Moncada & Barros, 2014).

Nevertheless, either if the energy is provided by glycolysis or by mitochondrial respiration, there should be a colocalization between the NKA and ATP synthesis (Proverbio & Hoffman, 1977; Mercer & Dunham, 1981) since diffusion in the dendrites is slower than in the extracellular space (Santamaria *et al.*, 2012) which also applies to ATP (Clegg, 1984). If this is true and the NKA is the main responsible for sodium extrusion, then, all these machinery, glycolytic proteins or mitochondria, should be present at synaptic terminal in dendrites and spines. Unless of course, the NKA is not responsible for sodium removal in these compartments.

4. Epileptiform

Around 0.5 to 1 % of population suffers epilepsy (Thurman *et al.*, 2011), comprehending a large array of disorders. However, some characteristics persist through all forms, the seizures, also called ictus and additional alterations of the

interictal electroencephalogram (EEG) (Fisher *et al.*, 2005; Christensen & Sidenius, 2012), shifting the neural network to a more hyperexcitable state (McCormick & Contreras, 2001). Although epilepsy is a very common disease, its causes are diverse and often unknown.

Recently, various studies have demonstrated that astrocytes, through the release of glutamate, may generate paroxysmal depolarization shifts, making this type of cells responsible for inducing the hyperexcitability observed in this condition (Tian *et al.*, 2005). In various models of epileptiform, increases in the extracellular potassium concentration have been detected (Lux *et al.*, 1986; Wallraff *et al.*, 2006). Also calcium transients have been observed in neurons as well as in astroglia (Carmignoto & Haydon, 2012). Although there are no recordings of intracellular sodium in neurons or astrocytes during epileptiform activity, some studies point to the idea that those could, in fact, exist. For example, reductions of extracellular sodium have been observed in car cortex (Dietzel *et al.*, 1982) and, in addition, some computational studies predicted that intracellular sodium changes could occur (Krishnan & Bazhenov, 2011).

In the present work, hippocampal slices were perfused with Mg^{2+} -free saline containing 10 μM bicuculline methiodide, a known approach for inducing epileptiform activity (Fellin *et al.*, 2006; Rouach *et al.*, 2008). Through this process the magnesium blocking NMDA receptors is released and $GABA_A$ receptors are blocked.

Aim of the present study

The main objective of the present work was to study the major mechanisms for the removal of sodium from various intracellular compartments (soma and dendrites) in CA1 hippocampal neurons. As previously mentioned, previous studies point to some discrepancies. On one hand the recovery from sodium transients follows a monoexponential decay and since the NKA is in theory, the only relevant mechanism for the export of sodium under physiological conditions, it has been hypothesized that this recovery mainly represents the pump's activity (Rose & Konnerth, 2001). The fact that the NKA is the major energy expender in the CNS (Whittam, 1962; Astrup *et al.*, 1981; Ames, 2000), that alterations in its structure induce dramatic issues at the cognitive level (Moseley *et al.*, 2007), and that its inhibition results in immediate intracellular sodium increases (Rose & Ransom, 1997; Kelly & Rose, 2010*a*, 2010*b*), supports this idea.

However, on the other hand, sodium is essentially a non-buffered ion with high mobility. Some studies have calculated an intracellular diffusion coefficient of about 600 $\mu m^2/s$ (Kushmerick & Podolsky, 1969), a value considerably higher than that obtained for free calcium (Allbritton *et al.*, 1992). It has also been demonstrated that during action potentials, fast sodium transients in the initial segment of axons are not affected by inhibition of the NKA by ouabain (Fleidervish *et al.*, 2010). Both these facts indicate that diffusion can be the mechanism responsible for the fast decay of sodium signals.

The present study expects to answer this question, which is the main mechanism for the removal of sodium in each compartment. To address this issue, the recovery to baseline sodium levels was study employing quantitative, ratiometric-imaging using the sodium-sensitive fluorescent dye SBFI. The intracellular sodium loads were induced by application of glutamate in the proximity of the soma or dendrite.

Summary of results and discussion

The present study aimed to explain the mechanisms for intracellular sodium removal from the soma and dendrites of CA1 pyramidal neurons. By using quantitative ratiometric imaging with the fluorescent sodium indicator dye SBFI, it was demonstrated that local glutamate applications induced transient sodium increases. After reaching a maximum amplitude, these sodium transients decayed with an average of $\tau = 63.5 \pm 48.7$ s.

Imaging with SBFI was proved to be a reliable method for the quantitative measurement of intracellular sodium concentrations, as it has already been described by some other authors (Minta & Tsien, 1989; Levi *et al.*, 1994; Rose & Ransom, 1997; Chatton *et al.*, 2000; Diarra *et al.*, 2001). As it is well known, some chemical ion indicator dyes may induce a buffering effect which by its turn influences the shape of the ion signals obtained, for example, maximum amplitude and time course (Neher & Augustine, 1992; Zhou & Neher, 1993). Nevertheless, using the "added buffer approach" it was demonstrated in this work that SBFI does not exert any buffering of sodium with dye concentrations up to 1 mM. While the amplitudes of single-wavelenght fluorescence emission signals at the sensitive wavelength (F_{380}) increases with increasing dye concentrations, the calculated ratios were not altered at all. Although this result was expected since SBFI posses a K_D of around 24 mM (Donoso *et al.*, 1992; Jung *et al.*, 1992; Rose *et al.*, 1999; Sheldon *et al.*, 2004; Meier *et al.*, 2006), a concentration much higher than the ones used, this observation was of critical importance for the interpretation further results on this study.

A new approach was used for the determination of baseline sodium concentration in the soma of CA1 neurons. SBFI ratio values were compared before and after loading a cell with a known concentration of the ion. After obtaining the whole-cell configuration, the soma sodium concentration equilibrates to the one in the patch-pipette and by calculating the difference in the ratio, it was possible to obtain a value of about 13 mM. A value in good accordance with previous studies (Donoso *et al.*, 1992; Langer & Rose, 2009; Kelly & Rose, 2010*b*; Azarias *et al.*, 2012; Karus *et al.*, 2015).

During the course of these experiments, it was found that in the soma of patched cells, after each one of the sodium transients evoked by glutamate, sodium is mainly removed through diffusion into the patch-pipette, achieving maximum

extrusion rates of around 135 mM min^{-1}. A reason for this phenomenon could be that since the sodium concentration in the cell was clamped to the one of the pipette, and that the volume inside it is infinite when compared to the soma, any disturbance to the system will rapidly progress to equilibrium. This fact has already been reported by other authors (Pusch & Neher, 1988). In addition, some recording in frog muscle fibres have revealed a diffusion coefficient for sodium of 600 $\mu m^2/s$ in the cytosol and of 1200 $\mu m^2/s$ in water (Kushmerick & Podolsky, 1969).

For this reason, the extrusion rate was studied in the soma using SBFI-AM, the ester form of the dye. This compound is lipophilic and has the ability to pass the cell membrane. Once inside the cell, cytosolic esterases remove the lipophilic moiety and the polar dye is unable to passively cross back the membrane (Meier *et al.*, 2006; Langer *et al.*, 2012). Although SBFI-AM results in a high background label, therefore only functional for ion signals from the cell body (Schreiner & Rose, 2012), it let us study sodium transients in a less invasive way. Once sodium entered the cells through the NMDA and AMPA receptors activated by our application of glutamate (Rose & Konnerth, 2001; Lamy & Chatton, 2011). However, under this condition, the maximum extrusion rate was 8 mM min^{-1}, a value much more in accordance to those reported in cultured myocytes (Despa *et al.*, 2002*a*, 2004; Despa & Bers, 2003), and a K_M of 19 mM.

The same kind of experiments was performed, this time in the presence of ouabain, a specific blocker of the NKA (Lelievre *et al.*, 1979). After a couple of minutes, sodium baseline concentration started to rise at an average rate of 1.8 ± 0.6 mM min^{-1}. In addition, glutamate induced sodium loads added to the strong on-going increase in "baseline" sodium but no recovery at all was observed. The "baseline" sodium continued to rise until reaching a plateau around 40-50 mM, after this point cells started to loose fluorescence, a clear indication of cell degeneration and death. Thus, in the presence of oubain, sodium extrusion from the soma was completely blocked, suggesting that in the soma, the NKA has a predominant role in the removal of sodium.

If this is true, sodium extrusion should decrease to very low values under low energy conditions, since the NKA activity is strongly dependent on the availability of intracellular ATP (Ames, 2000). To challenge the cellular metabolism, experiments with sodium fluoride (NaF) were performed, a known glycolysis inhibitor (Cox & Bachelard, 1982; Lees, 1991). As expected, perfusion with NaF resulted in a gradual

impairment of recovery from glutamate-induced sodium transients. An increase in the sodium baseline was also observed. Since it is known that glycolysis only produces around 5% of the cellular ATP (Erecińska & Silver, 1989), which is not enough to feed the NKA, the influence of inhibition of glycolysis on sodium export was surprising. Some studies have demonstrated that cultured hippocampal neurons decrease their glucose uptake upon activation of glutamate receptors and sodium entry (Porras *et al.*, 2004). It is also believed that the NKA is mainly fuelled by ATP synthetized via oxidative phosphorylation (Fernandez-Moncada & Barros, 2014). On the other hand, they are evidences of a close relation between glycolytic ATP and the NKA (Paul *et al.*, 1979; Lipton & Robacker, 1983). Various studies suggest that the ATP is deposited in a membrane-associated compartment from which it is used by the NKA (Proverbio & Hoffman, 1977; Mercer & Dunham, 1981).

Taken together, this data clearly indicated that sodium extrusion in the soma of the neurons under study is dependent of energy metabolism and blocked by ouabain, pointing to a major role of the NKA.

Nevertheless, in the dendrites, our results were quite different. First of all, sodium transients induced by glutamate recovered at a rate very similar to the one in the soma of whole-cell patch-clamped cells, and around 10-fold higher that of the somata of non-patched cells. In addition, while perfusion with ouabain resulted in an steady increase in the sodium baseline, indicating the inhibition of NKA, all sodium transients elicited recovered back to baseline at a rate very similar to the control condition. A similar result was previously obtained in axon initial segments were ouabain didn't alter significantly the recovery from sodium transients (Fleidervish *et al.*, 2010). Furthermore, when the cells were submitted to conditions of energy deprivation by the use of NaF, neither the amplitude nor the recovery rate from sodium signals was affected. These data indicate that the reduction of ATP availability does not impair the recovery from local sodium loads in dendrites, at least at this time frame. These results strongly suggest that in the dendrites, recovery from local sodium loads is mediated by lateral diffusion to neighbouring, non-stimulated areas and is independent of the NKA.

To further emphasize this idea, global glutamate applications that cover the entire cell were performed. With this protocol it was expected that global sodium loads were induced and diffusion was reduced to a minimum, by elimination of sodium gradients. Under this condition, there was a large drop in the extrusion rate in

dendrites by around 10 fold, from 10 to 16 mM min^{-1}, probably now reflecting principally dendritic NKA activity. In some previous experiments performed by other authors, the NKA was blocked by removal of extracellular potassium and initial extrusion rates of around 34 mM min^{-1} were obtained in the dendrites (Azarias *et al.*, 2012). Although this value is more than twice the one calculated in the present study, it is important to notice that concentration-based extrusion rates are highly dependent on the surface-to-volume ratio of the compartment under study. When the same non-focal glutamate applications were performed in addition to perfusion with NaF, neuronal dendrites suffered a similar build-up of sodium and failure of sodium extrusion than the soma. This result suggests that reduced ATP availability does not hamper the removal of sodium from local transients. With these results it was possible to assume that local sodium transients will not *per se* induce a local increase in ATP consumption and will not represent a significant local challenge for metabolism. At some point, of course, sodium ions that entered the cytosol will have to be exported through the NKA.

In addition to the main study of the present work, the membrane potential changes during recurrent epileptiform activity were also investigated. After sealing of the cells with a patch-pipette and establishing whole-cell configuration, epileptiform activity was induced by perfusion of the hippocampal slice with Mg^{2+} -free saline containing 10 µM bicuculline methiodide. Before inducing epileptiform activity, cells exhibited a membrane potential of about -65 mV, after around 15 min of perfusion with the solution mentioned above, neurons had a membrane potential of -67 mV from which sustained depolarizations of 20.5 mV superimposed with high-frequency discharges of around 9 action potentials emerged. Wide-field imaging was performed at the same time, using SBFI and revealed that burst discharges were accompanied by transient increases in the somatic sodium concentration ranging from 0.6 to 6.9 mM.

Similar experiments were performed in astrocytes, revealing that under resting conditions, they had a membrane potential of around -87.8 mV. After 15 min of perfusion, astrocytes developed recurring depolarizations of around 10 mV. Interestingly, in the middle of these major depolarizations, smaller ones of around 2 to 3 mV were observed. Similar to neurons, synchronize sodium increases were recorded.

The present work confirms that the NKA is the central mechanism for the removal of sodium ions from neurons. When its function is compromised, either by ouabain or by energy depletion, sodium homeostasis is disrupted and the recovery from sodium loads starts failing or, in some cases, is impossible.

However, the most important result and discovery of this project, was to find that the same principle is not true for the dendrites. In this case, sodium is mainly removed by lateral diffusion to proximal non-stimulated areas, a process that occurs at least 10 times faster than the one mediated by the NKA. As a consequence, local sodium transients will not required a local increase in ATP consumption nor energy metabolism. While sodium ions will have to be extruded to the extracellular space by the NKA at some point, our results suggest that energy requirements will nor surge locally after a local sodium increase, but rather be distributed to and shared by the entire cell.

Nevertheless, when the cell is subjected to global sodium increases, as the one that occur, for example, during epileptiform discharges, the cell will experience some problems to extrude sodium (Karus *et al.*, 2015), if there are any impairments in energy metabolism.

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Publications

"Extrusion versus diffusion: mechanisms for recovery from sodium loads in mouse CA1 pyramidal neurons"

Mondragão MA & Rose CR (2015); *The Journal of Physiology* (submitted JP-RP-2015-271562).

I performed all experiments and analysis. The data I obtained represents 100% of the results presented in the manuscript. I contributed to the experimental design, the interpretation of the data; writing and revision of figures and manuscript.

"Astrocytes restrict discharge duration and neuronal sodium loads during recurrent network activity"

Karus C, Mondragão MA, Ziemens D, Rose CR; *Glia* **63**, 936-57.

I performed the patch-clamp experiments with simultaneous wide-field imaging and contributed to the data analysis. I participated in the critical revision of the manuscript.

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Declaration

I confirm that I made this work independently and have not used any sources or aid than those permitted. I have provided indication of the source for all text passages or illustrations.

The present work was not submitted or presented to another examination office either in part or complete.