The neuromodulatory effect of extracellular ions in the central nervous system

My Forsberg

Department of Physiology
Institute of Neuroscience and Physiology
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2022

Cover illustration: *Action potentials*

The cover illustration depicts evoked action potentials in a CA1 pyramidal neuron, recorded with the patch-clamp technique by the author.

The neuromodulatory effect of extracellular ions in the central nervous system

my.forsberg@gu.se

ISBN 978-91-8009-3 (PRINT)
ISBN 978-91-8009-880-9 (PDF)
http:/hdl.handle.net/2077/71510

Printed in Gothenburg, Sweden 2022
Printed by Stema Specialtryck AB

ABSTRACT

Neurons are the cells that send and receive signals in the central nervous system. Neuronal signaling is the base for all the complex tasks the human brain is capable of, from the detection of a smell to the composition of a symphony. Neurons are continuously receiving input from the periphery and from other neurons and need to integrate these into their own output. One key factor in this process is the neurons' excitability, how prone they are to be excited over a threshold level for the initiation of a signal. Of fundamental importance for the excitability of a neuron is the concentration of ions in the surrounding environment. This PhD project has studied the effects of shifts in the concentration of extracellular ions on fundamental neuronal behavior such as excitation and synaptic transmission. In the first article, we show that the concentration of biologically active calcium in human cerebrospinal fluid (hCSF) is 1 mM, which is about 85% of the total concentration. Since a concentration of 2 mM calcium, or higher, is traditionally used in *in vitro* electrophysiological research, we then demonstrate the marked differences in intrinsic excitability and synaptic transmission that is caused by using 2 mM calcium as opposed to the physiological concentration. We also demonstrate the critical impact of the extracellular concentration of calcium for the induction of long-term potentiation, further underscoring the importance of carefully considering the extracellular calcium concentration. In the second article, we examine the elusive mechanism behind the inhibitory effect extracellular calcium ions have on neuronal excitability. By excluding mechanisms related to changes of the membrane potential, in intracellular calcium and G protein activation, we conclude that the main mechanism in the concentration range between 1.2-2 mM is neutralization of negatively charged residues in the extracellular part of voltage-gated sodium channels. In the last article, we collected hCSF from healthy volunteers after sleep, sleep deprivation or wakefulness and we show that the concentration of potassium in hCSF varies with the circadian rhythm, indicating a role for extracellular ions in the regulation of the behavioral states of sleep and wakefulness.

Keywords: extracellular ions, excitability, synaptic transmission, sleep, wakefulness
ISBN 978-91-8009-879-3 (PRINT)
ISBN 978-91-8009-880-9 (PDF)
http:/hdl.handle.net/2077/71510

SAMMANFATTNING PÅ SVENSKA

Den mänskliga hjärnan består av ett sammansatt nätverk av ca 100 miljarder nervceller som skickar elektriska signaler kortare eller längre sträckor. Precis som att de komplexa uppgifter en dator kan utföra i grunden är baserat på ettor och nollor i datorns kretskort, beror allt den mänskliga hjärnan kan åstadkomma på dessa signalers vara eller icke vara. Redan på 1950-talet föreslogs en modell för uppkomsten och regleringen av nervcellernas elektriska signaler och denna modell är giltig än idag. Modellen slår fast att nervcellens retbarhet, hur benägen den är att skicka en signal, bestäms av två faktorer; skillnaden i koncentration av joner på nervcellens utsida jämfört med koncentrationen på dess insida, samt av genomsläppligheten för dessa joner genom membranet. Genomsläppligheten förändras kontinuerligt genom öppning och stängning av kanaler i nervcellens yttre membran och attraherar mycket uppmärksamhet och forskning. Jonkoncentrationerna är istället relativt konstanta och hur de påverkar hjärnans aktivitet har i jämförelse studerats i väldigt begränsad omfattning. I denna avhandling har vi studerat en viktig jon, kalcium, och fastslagit att det finns ca 1.2 mM av den i nervcellernas omgivning och att ca 85 % av detta är biologiskt tillgängligt (ca 1.0 mM). Vi har vidare använt patch-clamp teknik, en teknik där forskaren kan registrera strömmar över nervcellens membran med hög temporal upplösning, för att jämföra nervcellernas aktivitet när de omges av den naturliga koncentrationen av kalcium med deras aktivitet när de omges av den dubbla koncentrationen, 2 mM vilket ofta används inom forskning. Vi visar bland annat att nervcellerna mer än dubblerar sin spontana aktivitet i naturlig kalciumkoncentration samt att långtidspotentiering, nervcellernas förmåga att lagra information vilken ligger till grund för minne och inlärning, inte verkar fungera som forskare tidigare trott när nervcellen omges av den låga, men naturliga koncentrationen av kalcium. Vidare har vi studerat hur koncentrationer av joner i cerebrospinalvätskan, en vätska som omger hjärnan och fyller alla dess hålrum, varierar under dygnet hos friska människor för att få en ledtråd till regleringen av sömn och vakenhet. Vi fann att koncentrationen av en jon, kalium, var lägre under natten jämfört med under dagtid även om personen inte hade sovit på natten vilket indikerar att det är tiden på dygnet, snarare än sömnen som sådan, som påverkar kaliumkoncentrationen. Framtida forskning kommer att visa om joner spelar någon aktiv roll i sömnreglering och om detta i så fall kan utnyttjas för att främja god sömn och stabilisera vakenhet hos människor.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Forsberg, M., Seth, H., Björefeldt, A., Lyckenvik, T., Andersson, M., Wasling, P., Zetterberg, H., Hanse, E.

Ionized calcium in human cerebrospinal fluid and its influence on intrinsic and synaptic excitability of hippocampal pyramidal neurons in the rat.

Journal of Neurochemistry 2019; 20: 121-133.

II. Forsberg, M., Zhou, D., Jalali, S., Seth, H., Björefeldt, A., Hanse, E.

Evaluation of mechanisms involved in the calcium-dependent regulation of intrinsic excitability in CA1 pyramidal neurons of rat.

Manuscript

III. Forsberg, M., Olsson, M., Seth, H., Wasling, P., Zetterberg, H., Hedner, J., Hanse, E.

Ion concentrations in cerebrospinal fluid in wakefulness, sleep and sleep deprivation in healthy humans.

Journal of Sleep Research 2022 Jun; 31(3):e13522

CONTENT

Abbreviations

aCSF	Artificial cerebrospinal fluid
BAPTA	1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid
CA	Cornu ammonis
CaSR	Calcium sensing receptor
CSF	Cerebrospinal fluid
EEG	Electroencephalogram
GABA	γ-aminobutyric acid
hCSF	Human cerebrospinal fluid
ISF	Interstitial fluid
LTP	Long-term potentiation
NALCN	Sodium leak channel, non-selective
NMDAR	N-methyl-D-aspartate receptor
$NMDG^+$	N-methyl-D-glucamine
NREM	Non-rapid eye movement (sleep)
NSCC	Non-selective cation channel
RAS	Reticular activating system
REM	Rapid eye movement (sleep)
RMP	Resting membrane potential
SWA	Slow wave activity
VLPO	Ventrolateral preoptic nucleus

1 INTRODUCTION

The fundamental role of the cells in the nervous system is to send signals. It can be basic and straightforward signals such as a simple motor command sent from the spinal cord to elicit a single twitch in a skeletal muscle or the very complicated pattern of signals that code for spatial memories in the central nervous system. The signals that neurons send consist of brief changes in voltage across the cell membrane and are called action potentials. Whether or not an action potential is initiated in a neuron is determined by the input to the cell combined with its excitability, a more excitable neuron is more likely and needs less input to send an action potential. Thus, excitability plays a fundamental role in the function of the nervous system.

At the bottom line, two factors determine the excitability of a neuron: the concentration gradients of ions across the membrane and the permeability of those ions through the membrane. The second parameter, the permeability, is determined by the number of, and conductance thorough the channels in the neuronal membrane and is the focus of almost all neurophysiological research concerning excitability. In this thesis, I have studied the ion concentrations, how they affect excitability and how they change in sleep and wakefulness.

1.1 REGULATION OF NEURONAL EXCITABILITY

Neurons are electrical units with an isolating cell membrane across which an electrical voltage ranges. The voltage emerges because of gradients of ion concentrations across the membrane causes diffusion of ions across the membrane. Since ions carry electrical charge, a current across the membrane appears. The driving force across the membrane for each ion is determined by two factors. Firstly, the difference in concentration on the two sides of the membrane and secondly, the voltage of the cell. A positively charged ion will have a higher drive to enter a negatively charged cell and vice versa. This phenomenon is summarized by the Nernst equation:

$$E_{ion} = \frac{RT}{zF} \log \frac{[ion]_e}{[ion]_i}$$

E_{ion} – equilibrium potential, R – ideal gas constant, T – temperature in Kelvin, z –valence of the ion, F – Faradays constant, $[ion]_e$ – extracellular concentration of the ion, $[ion]_i$ – intracellular concentration of the ion

It shows the relationship between the ion concentration gradient and the potential of the cell when the ion is at rest, that is, when no net current of that ion is flowing. The overall voltage of the cell is then determined by the equilibrium potential of each ion, adjusted for the relative permeability through the membrane of that ion. Even if every ion that has a gradient across the membrane contributes to the total voltage, only sodium, potassium, chloride, and to some extent calcium are of practical importance.

It might seem complicated, but at the bottom line, to get a change in the voltage across the membrane (to send an action potential, for example), either the concentration gradient, or the permeability must change. To perform all the dynamic tasks required of neurons in the nervous systems, a wide range of ion channels with varying permeabilities for different ions and varying gating mechanisms have evolved.

1.1.1 THE EFFECT OF CALCIUM IONS

The first review written about the effect of extracellular calcium ions on the excitability of neurons came in 1954 and there it is stated that low concentrations of calcium (0.8 mM and bellow) in the bathing solution destabilizes the membrane (Brink, 1954). The study of the effect of extracellular calcium is thus not particularly new. In this section, I will present the field as it stands 2022.

For most of the time since the question was first asked, the leading explanation as to why extracellular calcium seems to depress neuronal excitability has been the so-called charge screen effect. It was first suggested by A.F. Huxley (Frankenhaeuser and Hodgkin, 1957), and it postulates that calcium ions (and to varying degrees other cations) are attracted to negative charges on the surface of the neuronal membrane and there affect the local electrical field that can be perceived by the voltage sensor of a voltage-gated channel. Although lacking direct evidence, this

theory was supported by the finding that higher concentrations of calcium shift the activation curve of voltage-gated sodium and potassium conductances (the existence of voltage-gated ion channels was not established at this time) in a more depolarized direction (Frankenhaeuser and Hodgkin, 1957, Hille, 1968). In the following decades, the effect of extracellular calcium was studied in a wide range of concentrations, seldom lower than 2 mM and sometimes as high as 100 mM, with effects found on both activation and inactivation of sodium-, potassium-, and even calcium channels (Brink, 1954, Woodhull, 1973, McLaughlin et al., 1971, Hille, 1968, Hahin and Campbell, 1983, Kostyuk et al., 1982, Zhou and Jones, 1995).

Classically, it was thought that it was the negatively charged heads of the phospholipids themselves that influenced the voltage sensors of the channels and could be neutralized by positively charged ions in the extracellular solution. By comparing channel activation in membranes consisting of negatively charged phospholipids with membranes consisting of neutral phospholipids, it has been shown that such an interaction does contribute, albeit with a minor part of the total charge (Bell and Miller, 1984, Moczydlowski et al., 1985, Cukierman et al., 1988). A greater role seems to be played by the channel itself where negative charges can emerge from charged amino acids on the channel and affect the voltage sensing part of the channel (Elinder and Arhem, 1999, Elinder et al., 1998, Elinder et al., 1996). The charges on the channels do not necessarily have to come from amino acids. The surface of the sodium channel is heavily glycosylated with sialic acid and removal of this by neuraminidase treatment shifted the activation curve in a depolarized direction and reduced the effect of changing the concentration of extracellular calcium (Bennett et al., 1997, Recio-Pinto et al., 1990). An alternative interpretation of the importance of calcium ions to the gating mechanism of sodium channels is that the calcium ion acts as a blocker, entering the pore of the channel, and is only removed by depolarization of the membrane (Armstrong and Cota, 1999, Armstrong, 1999). This idea is supported by the fact that blocking calcium entry into the pore greatly diminishes the effect that changes in calcium concentration has on activation kinetics.

For around 100 years, the effect of calcium has mainly been attributed to, and studied in, voltage-gated channels, but over the last decade plenty of articles have been published that attempt to explain the effect partly, or wholly, through mechanisms completely independent of voltage-gated channels.

G protein-mediated cationic leak conductances have emerged as a potential mechanism (Lu et al., 2010). In the neuronal membrane, such leak channels are found coupled to cation sensors such as the calcium-sensing receptor (CaSR) (Chen et al., 2010, Ruat and Traiffort, 2013), whereby a change in extracellular calcium concentration can mediate a change in the conductance of the leak channel. This leak conductance may be carried by the sodium leak channel, NALCN (sodium leak channel, non-selective), which has been shown to react to changes in extracellular calcium both with, and without, the presence of CaSR (Martiszus et al., 2021, Chua et al., 2020).

Intracellular calcium is a well-studied intracellular messenger with diverse effects including activation of potassium channels (Gardos, 1958, Meech, 1972), release of calcium from intracellular stores (Yang et al., 2002) and long-term potentiation (Turner et al., 1982) (for more on intracellular calcium signaling see Berridge et al. (2000)). A possible mechanism for the effect of extracellular calcium on neuronal excitability could therefore be mediated via a change in intracellular calcium concentration (Segal, 2018), for example acting on potassium channels.

These widely varying explanations for the depressive role of extracellular calcium on neuronal excitability seem to conflict, but in this thesis, I will try to reconcile these seemingly contradictory findings and present a common theory of the effect.

1.2 THE REGULATION OF SLEEP AND WAKEFULNESS

The importance of stable states of sleep and wakefulness is demonstrated by the suffering of patients with diseases such as narcolepsy, hypersomnia, or various parasomnias. The first clues to the physiology of this regulation was found by von Economo, an Austrian neurologist who studied an epidemic of sleepiness that swept through Europe and North America in the first decade of the 20th century (Fleming, 1930). He found that many of the patients had lesions in the anterior hypothalamus and called the disorder encephalitis lethargica. Many decades later, in the 70s and 80s (reviewed in Saper et al. (2001), Jones (2003)), nuclei with neurons containing noradrenaline (norepinephrine), serotonin, acetylcholine, dopamine and histamine were identified as part of what was dubbed the

reticular activating system (RAS). RAS neurons project to the thalamus and the cortex and promote activity patterns in these areas that are associated with wakefulness (Aston-Jones and Bloom, 1981, Fornal et al., 1985, Steininger et al., 1999).

If RAS is the wakefulness-promoting formation, the ventrolateral preoptic nucleus (VLPO) in the anterior hypothalamus is the area that counteracts it and promotes sleep. VLPO neurons are active during sleep, contain inhibitory transmitters such as γ-aminobutyric acid (GABA) and galanin (Sherin et al., 1998) and project to the nuclei of the RAS (Sherin et al., 1996, Chou et al., 2002). There is a mutual inhibition between the RAS and VLPO so that when one of them becomes active, the inhibition of the other increases and thus the switch between them is stabilized (Chou et al., 2002).

In 1998, an additional system that acts to stabilize the switch between sleep and wakefulness was discovered in the lateral hypothalamus, consisting of a small group of neurons containing orexin (or hypocretin) (Sakurai et al., 1998, de Lecea et al., 1998). These cells are more active during wakefulness (Estabrooke et al., 2001) and absence, or dysfunction, of these

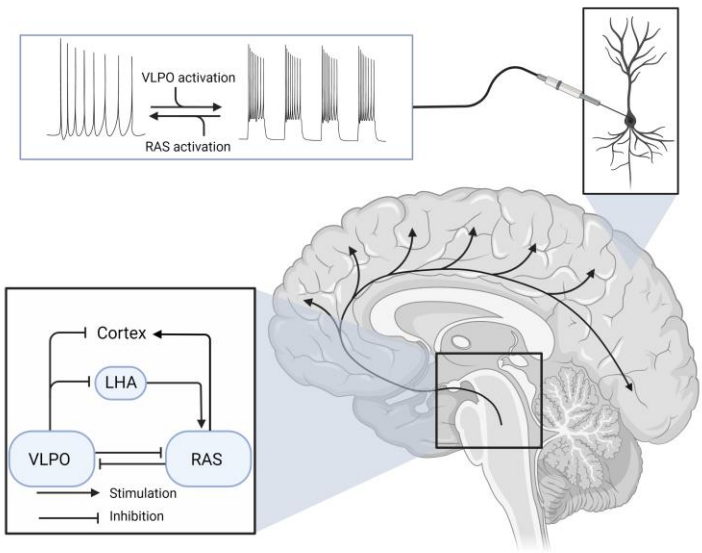

Figure 1. Subcortical nuclei project to the cortex and modulate the firing pattern of individual cortical neurons. LHA – Lateral hypothalamus, VLPO – ventrolateral preoptic nucleus, RAS – reticular activating system

cells cause narcolepsy, a disease characterized by an instability of the switch between wakefulness and sleep, which cause the sufferers to have fragmented sleep and to fall asleep uncontrollably during wakefulness (Lin et al., 1999, Thannickal et al., 2000). The orexin neurons are inhibited by the VLPO (Sakurai et al., 2005, Chou et al., 2002) and project to the nuclei of the RAS and to the cortex where they promote activity associated with wakefulness (Chemelli et al., 1999, Peyron et al., 1998).

During so-called non-rapid eye movement (NREM) sleep, the cells of the cerebral cortex show synchronized firing that collectively gives rise to the oscillatory fluctuations in electrical voltage that can be measured on the scalp with electroencephalography (EEG). EEG has long been used to study sleep and has the great advantage of being non-invasive. However, using more invasive methods, sleep can also be studied on the level of individual cells. During NREM sleep, cortical and thalamic neurons oscillate between periods of depolarization, with intense firing of action potentials, and periods of hyperpolarization with no firing (Steriade et al., 1993, Neske, 2017).

How the subcortical neuromodulatory systems alter the firing patterns of neurons is still debated and mechanisms involving voltage gated potassium channels (Muheim et al., 2019), N-methyl-D-aspartate receptor (NMDAR) (Papouin et al., 2017) calcium-activated potassium channels (Rasmussen et al., 2017) and G protein activated potassium channels (Steinberg et al., 2015) have been reported. There are also several indications that glia cells are important mediators since they have receptors for the modulatory transmitters (Ding et al., 2013, Miyazaki and Asanuma, 2016, Jurado-Coronel et al., 2016, Xu et al., 2018) and can release somnogenic substances such as interleukin-1 (Tobler et al., 1984) or adenosine (Pascual et al., 2005, Halassa et al., 2009). In addition, glia cells can affect the ionic composition of the extracellular space (Wotton et al., 2020, Amzica, 2002).

In 2016, Ding *et al*. provided a new perspective when they showed that the concentration of ions (sodium, potassium, magnesium, calcium and hydrogen) in the interstitial fluid (ISF) and cerebrospinal fluid (CSF) both correlated sharply with, and could induce transitions between sleep and wakefulness in mice (Ding et al., 2016). This perspective is consistent with an earlier finding that modifying the concentrations of calcium and magnesium in the bathing solution could elicit spontaneous rhythmic oscillatory, sleep-like, activity in cortical brain slices (Sanchez-Vives and McCormick, 2000).

How the modification of ion concentrations occurs, and if such a mechanism is present in humans, is still unknown.

1.3 IONS IN THE BRAIN

Ever since Ringer's classical research on frog hearts, it has been clear that the concentration of ions is of critical importance for the function of excitable cells. It is therefore not surprising that the concentrations of the major ions are generally held constant in the extracellular space of the brain. In situations of pathologically high activity, such as seizures, the ion fluxes seem to exceed the capacity of astroglial and neuronal reuptake, causing disturbances of the ion concentrations (Amzica, 2002, Hounsgaard and Nicholson, 1983, Nicholson et al., 1977). Vice versa, pathological conditions such as hypocalcemia or hyperkalemia can cause epileptic activity in the brain (Traynelis and Dingledine, 1988, Han et al., 2015).

An important aspect to keep in mind when considering the concentration of ions in a biological solution is that the ions that are bound to other molecules are not available to affect their surroundings, they are biologically in-active. It is therefore important to consider not only the absolute concentration of an ion but also the concentration of free, ionized ions. For the concentration of calcium ions in the CSF, reports of the ionized fraction span from 50-96% of the total amount (Pedersen, 1971, Schaer, 1974, Goldstein et al., 1979, Robertson and Marshall, 1981), which would place the concentration of ionized, active calcium between 0.6-1.15 mM, a significant difference.

1.4 CEREBROSPINAL FLUID

1.4.1 HUMAN CEREBROSPINAL FLUID

The cerebrospinal fluid (CSF) is a fluid separate from blood, lymph and interstitial fluid. It occupies the ventricles, the cisterns, the sulci and the subarachnoidal space in the brain and spinal cord. It is a clear liquid with low protein content (Segal, 1993), containing nutrients and various neuromodulatory substances in free communication with the interstitial fluid (ISF) (Brightman and Reese, 1969, Jessen et al., 2015).

The existence of a CSF that surrounds the brain is ubiquitous in vertebrates (Brocklehurst, 1979), which indicates important functions, and the CSF seems to have several. Firstly, to allow the brain to float, suspended in a aqueous solution, reduces its relative weigh to about a third and protects it from the mechanical trauma of being pressed against the inside of the scull during sharp accelerations. Secondly, the CSF can act as an extra circulatory system, providing the cells of the brain and spinal cord with nutrients and removing waste products (Segal, 1993). Thirdly, there is growing evidence of active signaling through the CSF. CSF from young mice contains a growth factor that can rejuvenate aged mice brains (Iram et al., 2022) and signaling substances from the CSF is crucial for the normal development of cortical progenitor cells (Lehtinen et al., 2011).

The classical view, which is now somewhat outdated, is that the CSF is produced in the choroid plexus and flows unidirectionally through the brain from the ventricles to the subarachnoidal space where it is reabsorbed by the arachnoid villi. Instead, a much more complex set of motions, driven by the oscillation of blood pressure, respiration and body position together with production and absorption to and from the blood vessels and extracellular space, appear widespread in the central nervous system (Brinker et al., 2014, Oresković and Klarica, 2010). A consequence is that CSF sampled at one location only, might not be representable for the CSF composition in other locations. A well circulated fluid, such as blood, can be sampled from the foot with reasonable confidence that it will not differ from blood sampled from the head. With CSF however, regional differences exist. Protein levels are generally higher in lumbar CSF than in ventricular or cisternal CSF (Hunter and Smith, 1960) and lumbar CSF is richer in peripherally derived proteins (e.g., plasma proteins) than ventricular CSF, which is relatively richer in CNS-derived proteins, e.g., tau, although the concentration differences are not great (Olsson et al., 2019).

1.4.2 ARTIFICIAL CEREBROSPINAL FLUID

When conducting experiments with neuronal tissue *in vitro*, researchers need to use a substitute for the CSF to keep the cells or tissues submerged in (the problems with getting access to real CSF for research are discussed in *Methodological considerations –Lumbar puncture*). The solution used is commonly called artificial cerebrospinal fluid, or aCSF. The goal is (most often) to mimic the real CSF in order to create an environment as similar to *in vivo* as possible. However, the aCSF used is often surprisingly simple. Distilled water with the addition of sugar (glucose) and a handful of ions. It might seem a bit peculiar, considering the discussion in the

previous section, to believe that the exchange of real CSF for this rudimentary solution will not have any major effect on the activity of neurons. Indeed, it has been shown that the *in vitro* excitability of both pyramidal neurons (Bjorefeldt et al., 2015) and interneurons (Bjorefeldt et al., 2016) are markedly increased in human CSF (hCSF) compared to aCSF. The mechanism(s) and mediator(s) behind this increase in excitability is still unknown.

Taking a closer look at the concentration of calcium in aCSF, we see that a wide range of concentrations are still used in research today. In the first progenitor to the aCSF, the original Ringer's solution created by Sydney Ringer in the late 1800s, the concentration of calcium added was about 1.25 mM. When Hodgkin and Huxley did their groundbreaking work on giant axons of the squid, they used sea water, which contained almost 10-fold the concentration of calcium (Hodgkin and Huxley, 1952). Subsequent work done on mammals used a lower, but still higher than physiological, concentration of 2-3 mM (Lancaster and Nicoll, 1987, Turner et al., 1982, Edwards et al., 1989, Bi and Poo, 1998), probably because a higher concentration of calcium facilitates synaptic transmission and suppresses intrinsic bursting which can obscure the recordings. Because of this, very little research has been conducted with physiological concentrations of extracellular calcium and little is known about how this discrepancy has affected the collective knowledge.

2 AIM

In this thesis project, I ask four questions in order to investigate the role that extracellular ions play in regulating the activity in the central nervous system, both *in vitro* and *in vivo*.

I. What is the physiological concentration of ionized extracellular calcium in the central nervous system?
II. How is neuronal excitability affected when a higher than physiological concentration of extracellular calcium is used in neurophysiological research?
III. What is the mechanism behind the inhibitory effect of extracellular calcium on neurons in the central nervous system?
IV. Is there a role for extracellular ions in the regulation of sleep and wakefulness in normal human physiology?

3 METHODOLOGICAL CONSIDERATIONS

The methods are described in detail in the articles. Here I focus on discussing the considerations and decisions regarding the methods.

3.1 ETHICAL CONSIDERATIONS

All experiments in this PhD project were approved by local ethical committees, for details about the permits see the individual articles. The data in this thesis was retrieved from experiments including both humans and animals, with separate ethical considerations. However, some points are universal. Participation was associated with some degree of adverse effects, ranging from mild inconvenience for a part of the human participants, to death for the animals. Keeping this in mind, it is crucial to consider the sample size in each of the experiments and what numbers are required in order to answer the questions asked (sample sizes will be further discussed in the section *Statistical analysis*).

The human participants donated CSF via a lumbar puncture (**article I** and **III**) and spent three nights in a sleep laboratory, two nights with monitored sleep and one night of sleep deprivation (**article III**). They were all given written and oral information about the procedures and provided written and oral consent. The main risk for adverse effect was the typical post-puncture headache that can come after a lumbar puncture. To minimize the risk atraumatic needles were used, which is shown to generate less headache than sharp/cutting tip needles (Arevalo-Rodriguez et al., 2017), and the procedure was carried out by a skilled clinician since multiple attempts also increases the risk for headache (Chordas, 2001).

The animals used were adolescent rats of both sexes (**article I** and **II**). All experiments were carried out in acute brain slices, which are taken from the rat acutely after decapitation. In addition to reduction of the number of rats used, a couple of steps were taken to reduce the suffering of the rats. The animals were bred in house, had free access to food and water and lived in cages with nesting material. Before decapitation the rats were anesthetized deeply enough that they were completely unresponsive to pain stimulation. This is, for now, the best compromise to get access to intact brain tissues (the importance of intact tissue is discussed in the next

section, *The hippocampal brain slice*). Hopefully, in the future, we will have alternatives such as human brain tissues grown from stem cells and thus eliminate the need to involve unconsenting animals.

3.2 THE HIPPOCAMPAL BRAIN SLICE

Large parts of this PhD project are dedicated to studying the functional behavior of neurons in response to varying concentrations of extracellular calcium. The intent is to study physiologically relevant mechanisms and it is therefore of great importance to choose a model system that mirrors the conditions in the intact brain as closely as possible. Neurophysiologists today have multiple model systems to choose from, including human brain tissue removed by neurosurgeons as a part of treatment or diagnosing disease in the brain, human neurons cultured from stem cells, non-neuronal cells such as HEK293 and cells from animals. Animal neurons, most often from mice or rats, are used *in vivo* in the living animal or *in vitro*, after removal from the animal. *In vitro* rodent neurons can be studied either in acutely prepared tissue, functional for several hours after removal from the animal, or as cultured tissue, where the tissue is nurtured for several weeks or months after removal from the animal.

All these models have benefits and disadvantages and in this PhD project, the model used is the acute hippocampal brain slice from rats. This preparation has been extensively used in patch-clamp research for the last 30 years after it first proved possible in 1989 (Edwards et al., 1989). The technique of anesthetizing the animal, rapid decapitation and submersion of the brain in an aCSF with subsequent slicing into thin slices with a mounted razor blade has remained relatively unchanged during this time even if techniques for cooling the tissue and addition various chemicals in the aCSF intended to protect the cells has been added (Ting et al., 2018). The hippocampus has the advantage that it can be cut into slices while preserving a relatively intact synaptic architecture allowing for mono- di- or even trisynaptic transmission due to its lamellar structure (Andersen et al., 1971). Thin slices of tissue let the researcher control the extracellular medium surrounding the cells, a key advantage in this project, as well as facilitating the patch-clamp method that, even though it is possible to do *in vivo*, is much easier with higher success rate in thin slices. Even higher success rate is generally achieved in cultured hippocampal slices because the slice flattens over time and thus presents more cell membrane unobstructed by overlaying tissue. However, culturing the slice *in vitro*

transforms the tissue to some extent. For example, the dendritic anatomy is affected and the number of spines are lower on dendrites of neurons in cultured tissue compared to *in vivo* (Holopainen, 2005). Taken all this in consideration, the acute hippocampal brain slice model was chosen for this project.

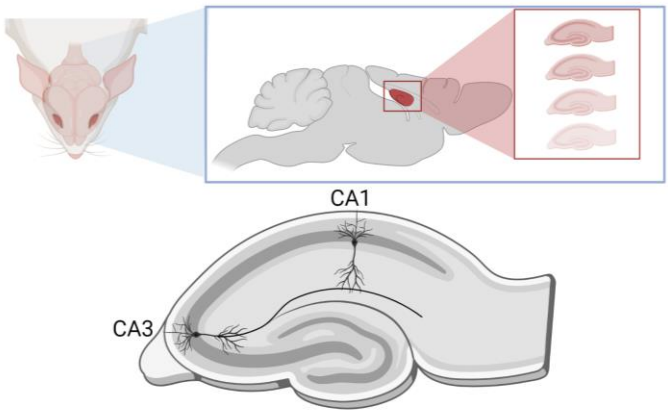

Figure 2. The hippocampus with visualization of the axon of a CA3 pyramidal neuron projecting to the dendritic three of a CA1 pyramidal neuron (bottom) and visualization of the location of the hippocampus in the rat brain (top).

3.3 THE PATCH-CLAMP TECHNIQUE

The patch-clamp method allows the user to record electrical activity in single cells with high temporal resolution. The method was developed by Erwin Neher and Bert Sakmann in the 1970 and 1980s (Neher and Sakmann, 1976, Hamill et al., 1981) and generated discoveries that awarded them a Nobel Prize in 1991.

The method was a significant improvement compared to previous methods where sharp electrodes were used to penetrate the cell membrane to get access to the cytoplasm and record and clamp the voltage of the cell. With the patch-clamp method, the cell membrane is not penetrated. Instead, an

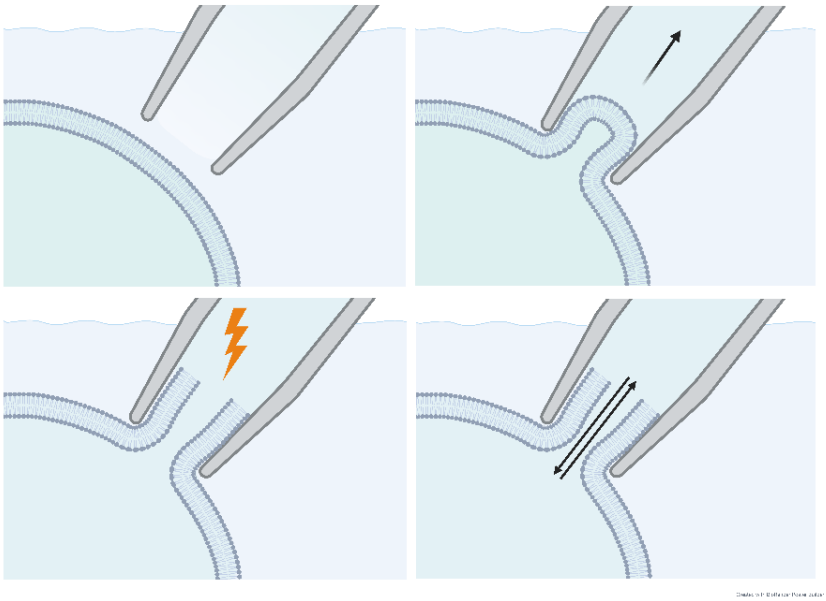

Figure 3. Patching a cell consist of four steps. First, the pipette is located adjacent to the cell membrane (top left). Then a negative pressure is applied to suck a patch of cell membrane into the pipette (top right). A quick zap ruptures the patch of membrane (lower left) before the fluid bridge is established between the cytosol and the electrode in the pipette (lower right).

electrode is inserted into a glass pipette containing a solution made to imitate the cytosol. The pipette is tapered and designed to have a small opening, only a couple of microns in one end. This small opening is placed right adjacent to the cell membrane and when a small suction is applied, a small patch of cell membrane is sucked into the opening and a tight seal is formed. The patch inside the pipette can then be ruptured, letting the solution inside of the pipette come into direct contact with the cytosol, creating a fluid bridge that electrically connects the cell to the electrode (see figure 3). The tight seal between the membrane and the pipette prevents leakage, which improves both cell function and survival as well as providing a better electrical control of the cell with much lower resistance than with previous methods.

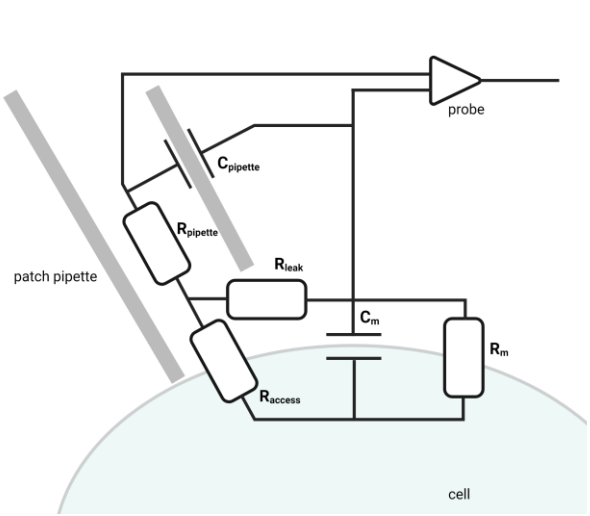

Figure 4. The patched cell is part of an electrical circuit. R_m – membrane resistance, R_{access} –access resistance through the patch, R_{leak} –leak resistance through the patch, $R_{pipette}$ –the resistance in the pipette, C_m –membrane capacitance, $C_{pipette}$ – pipette capacitance

To understand the method and its potential pitfalls, it is helpful to think about the electrical circuit created as illustrated in figure 4. In this model, it is the current passing the membrane though R_m that we want to measure as accurately as possible. All the other components of the circuit confound the output and should ideally be corrected for. For some, this present no great difficulty. The capacitance and resistance of the pipette are constant for a certain pipette and can be measured and corrected for before the pipette makes contact with the cell. The resistance in the patch is, if the patching were successful, small and can be disregarded. The capacitance of the cell is directly proportional to the cell size and can be cancelled or corrected for if necessary.

The last remaining component that needs to be taken into account is the leak resistance (and the corresponding leak current). When the patch is broken and access to the cytosol is established there is no way to directly measure the leakage in the patch and if the leak current changes during the experiment that can confound the data.

One problematic assumption that is made is that the cell is one electrical unit with perfect conductance internally. For a small and spherical cell, this is almost true since the cytosol is a good conductor. For a cell such as a pyramidal neuron with its long and thin protrusions however, this is not the case. This leads to the problem that the distant parts of the cell, such as axon terminals and dendritic bulbs, are neither clamped by, nor perceived by the probe, a problem known as poor space clamp. This is mainly a problem when tight voltage-clamp is needed; in the current-clamp

experiments in this thesis, the results should not be significantly distorted by this limitation.

3.4 LUMBAR PUNCTURE

The method of sampling the cerebrospinal fluid with a needle inserted between the lower lumbar vertebrae into the lumbar sac dates back to the 19th century, first performed by German physician Heinrich Quincke. In short, when a person flexes the lumbar region of the spine, the space between the spinous processes of the vertebrae becomes wide enough to allow access into the lumbar sac where the CSF flows freely. After insertion of a needle into the lumbar sac, the CSF can be allowed to flow passively out into a test tube. This procedure is often used clinically in the emergency room to diagnose intracerebral infections or subarachnoid haemorrhages.

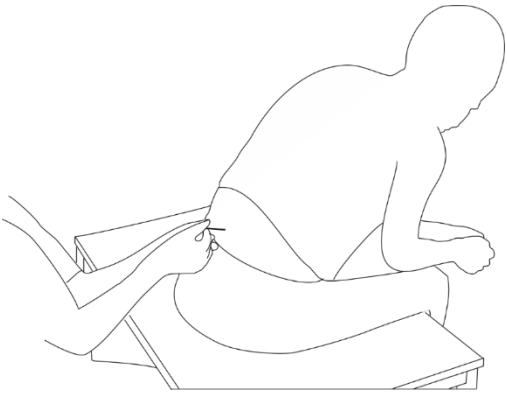

Figure 5. Visualization of the technique for the sitting lumbar puncture

There are a couple of drawbacks with the method. The first is the discomfort of the participant. Even though the procedure is not necessarily more painful than a venous blood sampling, the structure that is targeted is located deeper in the body, hence more skill and knowledge of the anatomy is needed. If performed poorly, the needle will scrape against the skeletal structures, which is painful. The needle can also disturb the nerves in the cauda equina in the spinal canal and activate the nerve, creating a short, painful jolt in the lower extremities (same as hitting the funny bone in your elbow). Beyond the physical discomfort of the participant, there is an acute effect of a lumbar puncture that can be seen in the CSF. There is an increase in both amyloid beta and tau in the days following a lumbar puncture (Olsson et al., 2019). It is not known whether this is due to redistribution of CSF after sampling in the lumbar region or an active increase in these proteins. To avoid repeated punctures when repeated sampling is required,

a small catheter can be inserted into the spinal canal but also this procedure increases the levels of amyloid beta (Bateman et al., 2007).

Lumbar puncture remains, however, the only way to sample CSF from healthy volunteers. Cisternal CSF, from the ventricles of the brain is sometimes drained via shuts in patients suffering from hydrocephalus but the installation of such shunts is very invasive and not an option in healthy volunteers.

3.5 STATISTICAL ANALYSIS

Statistical methods with significance testing are the golden standard in most fields of research and the agreement over a certain set of rules greatly eases the communication between researchers. In our time of easy-to-use software, that instantly execute a multitude of analysis, it is important to remember that these algorithms are not magical. There is no best-for-all statistical test that erases all risk of bias while simultaneously finding all true effects. Instead, we need to know the strength and weaknesses of our data and always use our knowledge and our own good sense when interpreting our data. In addition, the way we present the data in a publication can either help emphasizing the story we want to tell or be neutral and transparent to allow the reader to do an independent interpretation. In this section, I want to discuss both the statistical methods I have chosen to use and the presentation of the data in this thesis.

3.5.1 STATISTICAL METHOD

Every field has its own problems with interpretation of data and the separation of real effects from false ones. The balance between type I problems (rejecting the null hypothesis when it is in fact true, i.e. thinking you have an effect when there is none) and type II problems (failure to reject the null hypothesis when it is false, i.e. not seeing an actual effect that is there) is not an easy task. If we set the bar for significance too high, we risk missing important conclusions and thereby wasting time, resources and human and animal lives. If we set it too low, on the other hand, we will likewise waste time and resources perusing hypothesis that should have been abandoned. You should therefore neither be too lax, nor overly cautious when designing an experiment or analyzing your data.

To thread this line best, knowledge of the strengths and weaknesses of your data is the most important part. Another key feature of scientific work is replication of data in repeat experiments. Ultimately, valid research findings should be replicable between laboratories in completely independent settings.

The most obvious and significant drawback of the methods used in this PhD project from a statistical point of view, is the limited number of data points. The relatively invasive sampling of hCSF from healthy humans (**article I** and **III**) limits the number of subjects that it is reasonable to include from both an ethical and a resources point of view. To record electrical activity in neurons with the patch-clamp method (**article I** and **II**) is also very labor intense, especially when working with intact tissue such as the acute slice. From an ethical point of view the number of cells used should also be held as low as possible since an animal life is sacrificed for each day of recording. Another complication that comes with data collected from cells is the problem with nested data. In my statistical analysis, I treat the individual cells as independent but that is not necessarily the case. Some of the cells come from the same animal (sacrifice of one animal generates 5-10 slices of brain tissue) and some of the animals come from the same litter. Failure to account for nested data can increase the risk of type I error up to 80% in extreme cases (Aarts et al., 2014). However, the paired design (discussed below) and genetic homogeneity of the animals, combined with the limited extent of nesting in my data mitigates the problem.

Fortunately, there are also several advantages with the experimental designs used in this PhD project. Most of the data is collected in a paired manner, meaning that each subject or cell is its own control. This reduces the impact of inter-individual variation. When working *in vitro* as much of the data included in **article I** and **II** is collected, the researcher can control the environment and reduce the number of possible confounders, removing the need of more complicated statistical maneuvers to compensate for such. Most of the parameters analyzed can also be measured quite objectively. The thresholds of action potentials, amplitude of currents and concentrations of ions are all what they are. Exceptions from this are the frequency of minis (post-synaptic currents due to spontaneous release of synaptic vesicles, see **article I**) and estimation of resting membrane potential (see **article II**) which both include a measure of judgement call and can thus be affected by the researchers bias.

Taking these *pros* and *cons* into consideration, most of the statistical analysis in this thesis is carried out with Student's paired t-test (see articles for details).

3.5.2 INTERPRETATION AND PRESENTATION OF DATA

Even a perfectly executed statistical analysis can be misinterpreted and misrepresented. In this thesis, I have used the terminology of significant and non-significant as referring to $p < 0.05$ and $p > 0.05$ respectively. Importantly though, I have not used a "$p < 0.05$"-result as a proof of no effect. When I have concluded the absence of an effect (for example, the failure of various chemicals to block the calcium-dependent neuronal depression in **article II**), this has been based on observation of the data and comparison of effect sizes between groups. It is, of course, not possible to completely rule out effects in this manner but it is possible to conclude that any effect should be very small. I have also, in **article II** and **III**, preferred to give the actual p-values rather than the binary significant/non-significant information. When applicable, I have chosen to present the actual data points in addition to mean and error bars. All these measures are intended to allow the reader a better chance to do an independent interpretation of the data.

4 RESULTS

As many projects, this PhD project started with a simple question asked in an adjacent project. In this case, the question concerned the concentration of ionized, biologically active calcium in the extracellular space. The total concentration of calcium is easy to measure and well known. However, it is the ionized concentration that is relevant to researchers who want to create an aCSF that is as similar to the hCSF as possible. Since the typical aCSF lacks many of the components of the hCSF, there is reason to question whether the fraction of total calcium that is unbound and active is different in an aCSF than in a hCSF. That lead us to the first question I asked in this PhD project:

I. What is the physiological concentration of ionized extracellular calcium in the central nervous system?

In **article I**, we collected hCSF from healthy volunteers via lumbar puncture. The total concentrations of calcium, magnesium, sodium, potassium and chloride were measured. To measure the ionized fraction of calcium we used an ion-specific electrode, which showed that 85% of the total amount of calcium in the samples of hCSF was ionized and free. We did similar measurements for the aCSF used in the rest of the experiments described in this thesis and found a very similar level of calcium binding between aCSF and hCSF. We therefore conclude that adding the same total concentration of calcium to our aCSF as we measured in hCSF would give a physiological concentration of calcium for the neurons studied.

Knowing the concentration of extracellular calcium that neurons are exposed to in their natural habitat, while also knowing that it was only about half the concentration normally used in electrophysiological research, led us to question how this difference affect the neuronal activity we study. Hence the next question I asked was:

II. How does it affect neuronal excitability when a higher than physiological concentration of extracellular calcium is used in neurophysiological research?

In **article I**, we show that lowering the total concentration of extracellular calcium from high (2 mM) to physiological (1.2 mM) has a marked effect on neuronal excitability. Using patch-clamp recordings from CA1 neurons in acute slices from rat, we studied excitability and synaptic transmission. We also recorded extracellular field potentials to study plasticity.

We show that the frequency of spontaneous action potentials increased two and a half times in physiological calcium concentration, which is reversible when the concentration is increased again. By analyzing the response of the neurons to an injected current, we saw that this increase in activity was mainly due to a hyperpolarization of the threshold for action potential firing. Other parameters of the action potential such as amplitude and depolarization and repolarization rates were also affected, whereas the maximal firing frequency was unaffected, pointing to a mechanism involving voltage gated channels.

Influx of calcium ions from the extracellular space is required for synaptic transmission (Dodge and Rahamimoff, 1967), so next we wanted to study how synaptic transmission is affected by a higher than physiological concentration of calcium. Specifically, we wanted to know if there was a difference in the effect on inhibitory and excitatory synapses. If this was the case, it could explain the increased spontaneous activity we previously have shown. By stimulating axons with an extracellular electrode and measuring the post-synaptic currents in the voltage-clamped neuron, we show that both inhibitory and excitatory post-synaptic currents double in amplitude when extracellular calcium is increased from physiological 1.2 mM to 2 mM. By stimulating with paired pulses and analyzing the ratio of the second response to the first response, the pre-synaptic release probability can be estimated (Hanse and Gustafsson, 2002). By this method, we showed that the paired-pulse ratio decreased to the same extent in inhibitory and excitatory synapses, verifying a clear but balanced effect on release probability.

Previous research (Smith et al., 2012) has shown that extracellular calcium, via the CaSR, affect evoked and spontaneous release of synaptic vesicles differently. We therefore wanted to also study spontaneous release and compare the calcium effect on inhibition and excitation. However, neither the amplitude, nor the frequency of spontaneous post-synaptic inhibitory or excitatory currents were affected by a change in the concentration of extracellular calcium.

I therefore conclude that the change in extracellular calcium concentration does not perturb the excitation/inhibition-balance in the model system studied, but it has a significant effect on the amplitude of the postsynaptic response to an evoked stimulus with an increased response in higher concentration of calcium. The induction of long-term potentiation (LTP) is dependent on post-synaptic influx of extracellular calcium (Lynch et al., 1983, Malenka et al., 1992), which in turn is dependent on both the concentration of extracellular calcium and the magnitude of the post-

synaptic depolarization. An artificially high concentration of extracellular calcium should therefore facilitate the induction of LTP. We therefore wanted to induce LTP at high and physiological calcium concentrations. We found no LTP in physiological calcium when we used an induction protocol that induced a clear potentiation in high calcium. Even when blocking inhibition with picrotoxin, no LTP was induced in physiological calcium concentration, bringing into question the relevance of studying LTP in this way.

After mapping out the consequences of using a higher than physiological concentration of extracellular calcium on excitability, synaptic transmission and plasticity, I sought to know why calcium ions have this effect on neurons, which led to the next question in this PhD project:

III. What is the mechanism behind the inhibitory effect of extracellular calcium on neurons in the central nervous system?

I am not the first to ask this question and answers to it has ranged from the charge-screen theory (Hille, 2001, Frankenhaeuser and Hodgkin, 1957), regulation of potassium channels (Vysotskaya et al., 2014, Roshchin et al., 2020), blocking of sodium channels (Chua et al., 2020, Armstrong and Cota, 1991), regulation via the CaSR (Smith et al., 2004, Phillips et al., 2008) and secondary changes in the concentration of intracellular calcium (Segal, 2018).

The variety of explanations may be due to the different concentrations ranges studied, the different cells or species, or the different methods used. In **article II**, I sought to build on the findings of **article I** and investigate what mechanism(s) is responsible for the calcium-related neuronal inhibition in the range between physiological concentration (1.2 mM) and the high concentration (2 mM) often used in electrophysiological research. Building on the physiological approach, we used acute slices rather than cultures. We first repeated the experiments focusing on neuronal excitability from **article I,** but added a G protein blocker to the intracellular solution to block the activity of the CaSR. We found that the effect on excitability of increasing calcium was the same with and without this blocker, ruling out a contribution of the CaSR. To make sure we had a satisfactory effect of the blocker we did control experiments and noted that our blocker effectively blocked the action of carbachol on the medium after-hyperpolarization, a G protein-dependent mechanism (Storm, 1989). We then studied the resting membrane potential (RMP) to examine if a change in sodium leak currents and a subsequent change in RMP could explain the reduced excitability in high calcium. We found no consistent

change in RMP when changing the calcium concentration and the changes that we found did not at all correlate with the magnitude of the change in activity. To test if changes in intracellular calcium is necessary for the effect of extracellular calcium on excitability we added calcium chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid (BAPTA). We found no difference in the effect of changing calcium concentration with or without BAPTA present, leading to the conclusion that the mechanism behind this effect is not mediated via intracellular calcium.

Finally, I wanted to know if there are any physiological processes in the healthy brain that uses changes in the ion concentration as a regulatory mechanism. In 2016, Ding et al (Ding et al., 2016) showed that this is the case in the regulation of sleep and wakefulness in mice, which led me to the final question in this PhD project:

IV. Is there a role for extracellular ions in the regulation of sleep and wakefulness in normal human physiology?

In **article III**, to answer this question, we recruited healthy volunteers who agreed to donate three samples of CSF via lumbar puncture; one sleep-sample (collected in the morning, before getting out of bed), one awake-sample (collected in the afternoon) and one sleep deprivation-sample (collected in the morning after a night of sleep deprivation). We then analyzed the samples for ionic content and found a small, but highly significant reduction in the concentration of potassium in the sleep-samples compared to the awake-samples. The other ions analyzed show a trend towards the same reduction but this trend did not reach significance as it was not as consistent as the reduction in potassium. We also analyzed the osmolality and albumin concentration of the CSF samples to check if there was general dilution of the night CSF but that turned out not to be the case.

5 DISCUSSION

The field of neuromodulation by extracellular ions is huge and only a small fraction of it has been covered in this thesis. In this chapter, I will discuss some of the findings that need to be put into a bigger perspective and highlight some limitations in the interpretation of the data.

5.1 THE EXTRACELLULAR CONCENTRATION OF CALCIUM

Concentrations of calcium in the central nervous system have been reported repeatedly before, but never previously in healthy humans. When ion concentrations are measured with implanted electrodes in the parenchyma, there is always the risk that the trauma may induce differences in the concentrations, both acutely and persistently (Kozai et al., 2015). Even though quick, activity dependent changes in ion concentration will most likely be missed when the sampling is done in the lumbar region, the relevance of the concentrations measured in this way for preparation of an aCSF is clear.

5.2 THE RELEVANCE OF THE CONCENTRATION OF EXTRACELLULAR CALCIUM

Before criticizing research done with unphysiological concentrations of extracellular calcium, it is important to remember that it often is intentional. When the purpose of the study is to investigate the effect of extracellular calcium on neurons, a large concentration difference presumably gives a larger effect, that is more easily detected, as is the case with most of the studies into the charge-screen effect. Studies that have a purpose other than to study the effect of calcium can use unphysiological concentrations in order to suppress spontaneous activity or to facilitate synaptic transmission. It is, however, very important to make an active decision regarding the concentration of extracellular calcium and to be aware of the consequences. The varying concentration ranges used when studying the effect of extracellular calcium is most likely a big contributing

factor to the contradictory claims that have been made in this field, as will be discussed further in the next section.

In research that does not directly concern the effect of calcium, it is equally important to be mindful about the concentration used and the effects on synaptic transmission and excitability. In **article I** we show that an often-used stimulation protocol for induction of LTP, does not produce any potentiation in a physiological calcium concentration. Subsequently, this provoking result has been replicated in another study in which an array of different spike-timing protocols failed to induce LTP in 1.3 mM calcium (Inglebert et al., 2020). Only one protocol during which the pre- and postsynaptic pairing was repeated 100 times at 10 Hz induced a modest LTP in 1.3 mM calcium. Since it is clear that LTP is induced *in vivo*, these results indicate that there are LTP-facilitating mechanisms that are normally not enabled in the slice preparation. Another example from the field of sleep studies is that sleep-like activity can occur spontaneously in brain slices when the concentration of extracellular ions is set to physiological values (Sanchez-Vives and McCormick, 2000). What other phenomena have been missed because normal activity has been suppressed by an unphysiological ionic environment?

5.3 THE MECHANISM BEHIND THE CALCIUM EFFECT

The reason for the numerous explanations regarding the excitability-inhibiting effect of extracellular calcium is probably that the question is incorrectly asked. It is not likely that there is one, unified mechanism that explains every observation that has been made. Instead, a variety of different mechanisms can work synergistically, complementary, antagonistically, or in different concentrations ranges, on different channels or in different species.

Mechanisms involving changes in intracellular calcium and regulation of potassium channels have been proposed by occasional studies, but the two main explanations for the effect of calcium are the relatively recently proposed CaSR – G protein activation – NALCN pathway, and the more traditional charge screen mechanism. The explanation involving the pathway CaSR-G protein activation-NALCN activation (or part of this pathway), fails to explain the clear effect on gating of the voltage sensitive channels. Earlier studies that attempted to explain the effect of calcium

only with the impact it has on voltage gated channels fails to explain why the effect disappears when part(s) of the CaSR-G protein activation-NALCN pathway is missing. This controversy can be explained by the difference in the concentration ranges studied. The CaSR-NALCN mechanism is studied in the range from 0 – 2 mM and seems to be fully saturated at 1.1-1.2 mM. Any concentration above that will add no further activation of the inhibitory mechanism (Lu et al., 2010, Lee et al., 2019). The charge screen, on the other hand, is often studied in the range of 2-100 mM (Armstrong and Cota, 1999, Gilly and Armstrong, 1982a, Hahin and Campbell, 1983, Frankenhaeuser and Hodgkin, 1957). In **article I** and **II**, we have studied the effect of extracellular calcium in the concentration 1.2 – 2 mM, the gap between these two paradigms.

The data presented in **article I** and **II** supports a mechanism involving the voltage-gated channels and speaks strongly against the CaSR-NALCN pathway in this concentration range. Does this mean that the charge-screen theory is the best explanation for our results? Some unanswered questions remain. An electrical screening of the voltage sensor in the ion channel should have the same effect on opening and closing rates. Results regarding this has differed in the literature from identical shifts (Hahin and Campbell, 1983, Behrens et al., 1989) to a larger effect on the opening rate than on the closing rate (Cukierman et al., 1988, Armstrong and Cota, 1999). This may be explained by the species studied where the identical shifts has been found in squids and frogs, whereas differences in the shifts have been found in mammals. An explanation for differing effects on opening- and closing kinetics could be that the ions bind to the channels instead of just screening them. If binding, or interaction, is limited to the open, or closed, channel a differential effect of extracellular ions is possible. Gilly and Armstrong (1982b) suggest such mechanism to explain their finding that extracellular zinc affects opening rate, but not closing rate, of voltage gated sodium channels in the squid. They propose that the zinc ion interacts with parts of the channel that are exposed when the channel is closed, but hidden when the channel is open. This result is to some extent supported from rat voltage-gated sodium channels in planar lipid bilayers where the closing rate was actually affected, but only about half as much as the opening rate (Cukierman et al., 1988). These authors suggest that the closing rate is affected solely by a charge screen of the voltage sensor, but the opening rate of both a charge screen and a direct binding.

What about the idea that calcium ions block the pore of the channel and therefore is directly responsible for the closing mechanism? There are two strong arguments against this idea. The first is that the concentrations of

extracellular ions, among them calcium, affect the activation and inactivation of not only the voltage-gated sodium channels but also the voltage-gated potassium channels (Gilly and Armstrong, 1982a, Frankenhaeuser and Hodgkin, 1957, Hille, 1968), and importantly also the voltage-gated calcium channels (Kostyuk et al., 1982, Zhou and Jones, 1995). It is, however, worth noting that, depending on the number of negative residues at the extracellular mouth of the channel, the effect of calcium varies largely between voltage-gated channels, being clearly largest among voltage-gated sodium channels (Elinder and Århem, 2003). The second argument is that mutation studies in which the channels have been mutated to either increase or decrease the affinity for calcium binding inside the pore have not shown an alteration of the the calcium dependence of their kinetics (Yang et al., 1993, Heinemann et al., 1992, Ellinor et al., 1995).

Finally, it is worth noting that even in the concentration range of 0.1-1 mM, the significance of the CaSR-NALCN pathway in calcium-induced depression of neuronal excitability is controversial. In a recently published study, a research group that previously had shown a CaSR-activated non-selective cation channel (NSCC) in synaptosomes, failed to replicate that finding in whole cells (Martiszus et al., 2021). It is possible that the NALCN is activated by some other G protein coupled receptor than the CaSR in the post-synaptic parts of the neuron. Worth noting is also that there is some doubt whether NALCN is the sodium leak channel it has been proposed to be. Multiple groups have failed to replicate the original finding (Egan et al., 2018, Boone et al., 2014, Sandstrom, 2014) and Senatore and Spafford argue that the properties of the pore points rather to an ion sensor than a conducting channel (Senatore and Spafford, 2013, Senatore et al., 2013). It is also exceedingly difficult to study this kind of channel with the patch-clamp method, as will be discussed in the next section.

5.4 STUDING A NON-SPECIFIC ION CURRENT WITH PATCH-CLAMP

At the bottom line, the patch-clamp probe can only measure the current that passes through the amplifier circuit. It has no way of knowing which path the current has taken. Strategies to remove the components that are of no interest to the researcher are described in a previous chapter (*Methodological considerations – The patch-clamp technique*), but one troublesome component remains, the leak current. Every patch-clamp

recording has a leak current that contains resting conductance through the cell membrane and leak though the seal between patch pipette and cell membrane.

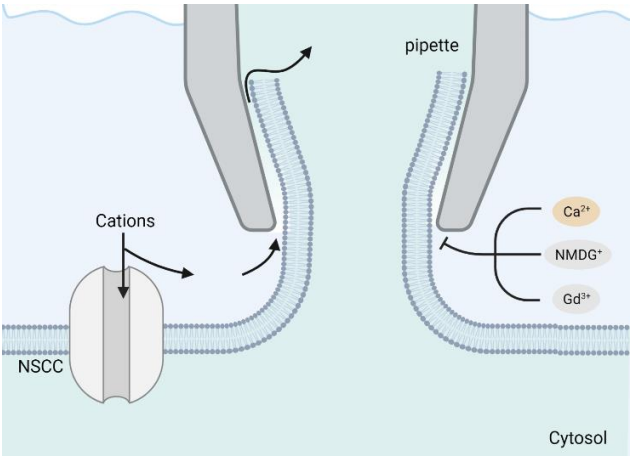

Figure 6. The leak current that passes between the membrane and the pipette is indistinguishable from the current though a non-selective cation channel (NSCC) and is blocked by calcium (Ca^{2+}), gadolinium (Gd^{3+}) and N-methyl-D-glucamine ($NMDG^{+}$)

The leak current can be compensated for by P/N-correction (Molleman, 2003). However, if the leak current changes during the experiment, for example when the extracellular calcium concentration is changed, the correction is no longer valid. If the correction is redone in the new setting, a possible actual current through a leak channel will be obscured. There is simply no way to distinguish between a non-selective leak current with reversal potential around zero, that comes from non-selective cation channels, from a current that is due to a leaky patch. And every patch has some degree of leak. When studying the NALCN, which has been proposed to be such a non-selective leak current, attempts have been made to distinguish between the two by using blockers of the NALCN such as N-Methyl-D-glucamine ($NMDG^{+}$), gadolinium or verapamil (Chua et al., 2020, Martiszus et al., 2021, Lu et al., 2007), but these substances also affect the patch leak current to similar degree (Boone et al., 2014).

What does this mean for the results presented in this thesis? Almost all patch-clamp data in this thesis is from recordings done in current-clamp mode. The exception is the synaptic currents in figure 5 and 6 of **article I**. In current-clamp, we did two types of protocols. In the first type of protocol we injected a current to set the membrane potential to a certain potential (-70 mV) before the stepwise stimulations and then the current that was needed to reach that potential was adjusted before each stepwise stimulation so any change in leak current (either from patch leak or from leak channels) would be compensated for. In the second protocol, we injected no current and recorded the spontaneous action potential activity.

In this case, an increase in leak current would be picked up as a depolarization. The main finding, a hyperpolarization of the threshold for action potentials when the concentration of extracellular calcium was lowered, should therefore possibly, if anything, be underestimated.

Methods for studying changes in membrane potential, without interfering with the membrane to create an extra leak current, but still recording voltage with an adequately high resolution to study the activation and inactivation of voltage-gated channels, are still lacking. In the future, better patch-clamping techniques, or development of other methods such as voltage dependent fluorescence could help to resolve the difficulties I have described here.

5.5 THE REGULATION OF SLEEP AND WAKEFULNESS

In **article III**, we show that there is a small, but highly consistent lowering of the concentration of extracellular potassium in CSF collected in the morning as compared with CSF collected in the afternoon. This lowering was independent of whether the subject had been sleeping, or been awake during the night, indicating a dependence on circadian rhythm rather than on sleep pressure. This is in contrast with the findings by Ding *et al.* (2016) who showed, in mice, that the changes in ion concentrations (including calcium and magnesium, which were unaffected in our study) followed the sleep-wake transition, regardless of whether that transition was naturally occurring or was induced by isoflurane. It seems unlikely that the difference is species-related, considering the universality of sleep in the animal kingdom, and the fact that the wakefulness-promoting pathways from subcortical nuclei to the cortex is similar in rodents and humans (Fort et al., 2009). Since a decrease in extracellular potassium has been shown to facilitate the transition to sleep-like activity in a neuronal model (Rasmussen et al., 2017), one possibility is that the changes in ion concentrations are circadian and pave the way for the transition that is directly controlled by other mechanisms.

We saw a smaller change in the concentration of potassium than was previously reported in mice, and no change in calcium or magnesium. This might be due to the sampling site (lumbar in our study and in the brain parenchyma and the cisterna magna in the previous study on mice). The ion concentrations in lumbar CSF may be affected by the spinal neurons

that show relatively wake-like activity during non-REM and are suppressed during REM (Carli et al., 1967, Soja et al., 2001).

Since sleep is a complex phenomenon and its purpose and regulation are incompletely understood, care should be taken when using proxies such as slow wave activity (SWA) to represent the complete phenomenon. That being said, the finding that SWA occurs spontaneously in cortical slices when the ion concentrations are physiological, opens doors to study sleep regulation *in vitro* (Sanchez-Vives and McCormick, 2000). One prediction would be that the CSF collected for the experiments in **article III** could induce SWA in slices (since it naturally has physiological ion concentrations). Spectral analysis of the oscillatory activity induced by sleep- and awake-CSF would then either strengthen, or weaken, the hypothesis that substances in the CSF (ions or other) mediate the transition from sleep to wakefulness and vice versa.

6 CONCLUSION

The work presented in this thesis has focused on how the concentration of extracellular ions affect neuronal activity and the implications it can have for neurophysiological research. The main conclusions can be summarized in four points:

I. The physiological concentration of ionized extracellular calcium in human cerebrospinal fluid is about 1 mM. The ionized fraction of calcium constitutes about 85% of the total concentration, which is similar in artificial cerebrospinal fluid, indicating that bicarbonate is the major calcium chelator in the cerebrospinal fluid.

II. The higher than physiological concentration of extracellular calcium (2-4 mM) used in neurophysiological research profoundly decreases neuronal excitability. For example, changing the calcium concentration from 1.2 to 2 mM reduces the frequency of spontaneous action potentials by more than 50% and the threshold for the action potential by about 4 mV.

III. The main mechanism behind the decreased excitability of extracellular calcium in the 1-2 mM concentration range appears to be neutralizing negative residues on the extracellular part of voltage-gated sodium channels.

IV. There is a circadian reduction of the extracellular potassium concentration during night in the human central nervous system, which likely contributes to the transition between wake and sleep.

Many questions remain. Are there more phenomena (than LTP) that need to be revisited and studied in a physiological calcium concentration? How are glial cells affected by shifts in ion concentrations? Is there an active process of shifting ion concentrations between behavioral states such as sleep and wakefulness? Future research will provide the answers to these questions and provide many new ones.

ACKNOWLEDGEMENTS

I would like to thank those who have provided funding for this work, Stiftelsen Wilhelm och Martina Lundgrens Vetenskapsfond, Stiftelsen Fru Mary von Sydows. Född Wijk, donationsfond, Stiftelsen för Sigurd och Elsa Goljes Minne, The Swedish Research Counsil and Göteborgs Läkaresällskap.

Figures 1-4 and 6 was created with BioRender.com

There are many persons who have made these years enjoyable and prosperous and whom I would like to thank. First and foremost, **Eric Hanse**, my main supervisor. His encouragement and dedication to the development of others is invaluable for a PhD student, I wish every aspiring researcher got to have such a mentor. Next in line for support, always, was co-supervisor **Henrik Seth**, who have enough energy and capacity for the whole floor. Without you, this project would never have come this far. My other co-supervisors, **Henrik Zetterberg** and **Måns Henningson**, thank you for coming along on this ride and complementing us myopic electrophysiologists.

Thanks to all the **collegues and students at neurophysiology, past and present.** All of you make the 3rd floor the best place to work. I have to mention my PhD student colleagues, **Mats Andersson, Rong Ma, Tim Lyckenvik** and **Thomas Olsson Bontell**. Sharing all the ups and downs of teaching, compulsory courses and experiments gone awry with you have been a blast. **Andreas Björefeldt**, thank you for guiding my first steps in the world of brain slicing and patch-clamping. A special thanks also to the summer students that have worked tirelessly, **Dinna Zhou** and **Shadi Jalali**.

Thank you to the colleagues at **Center for Sleep and Vigilance Disorders**, for introducing me to the fascinating physiology of sleep and wakefulness.

The coffee crew, **Karin Göthner, Staffan Berg** and **Dan Magnusson**, thank you for all the conversations about things other than research, for allowing me to debrief when the research has been mistreating me and for excellent cooperation over botanical matters.

Thank you **Julia Izsak** for inspiring me to work harder and be better. I can't squander the time when working with a colleague as you.

Elin Eriksson Hagberg, even though I couldn't quite keep up with you, thank you for leading the way and being my partner-in-crime during the happy days of our PhD.

My parents, **Eva-Christine** and **Lars**, and brothers, **Christopher** and **Jonathan** for the unconditional love and support and for enduring my stubbornness. Every inch of which has been needed in the fight with the patch-clamp technique.

Finally, **Johan**, thank you for feeding me, supporting me and loving me. I dedicate this work to you and to our little family.

REFERENCES

AARTS, E., VERHAGE, M., VEENVLIET, J. V., DOLAN, C. V. & VAN DER SLUIS, S. 2014. A solution to dependency: using multilevel analysis to accommodate nested data. *Nat Neurosci,* 17**,** 491-6.

AMZICA, F. 2002. In vivo electrophysiological evidences for cortical neuron-glia interactions during slow (<1 Hz) and paroxysmal sleep oscillations. *J Physiol Paris,* 96**,** 209-19.

ANDERSEN, P., BLISS, T. V. & SKREDE, K. K. 1971. Lamellar organization of hippocampal pathways. *Exp Brain Res,* 13**,** 222-38.

AREVALO-RODRIGUEZ, I., MUÑOZ, L., GODOY-CASASBUENAS, N., CIAPPONI, A., AREVALO, J. J., BOOGAARD, S. & ROQUÉ, I. F. M. 2017. Needle gauge and tip designs for preventing post-dural puncture headache (PDPH). *Cochrane Database Syst Rev,* 4**,** Cd010807.

ARMSTRONG, C. M. 1999. Distinguishing surface effects of calcium ion from pore-occupancy effects in Na+ channels. *Proc Natl Acad Sci U S A,* 96**,** 4158-63.

ARMSTRONG, C. M. & COTA, G. 1991. Calcium ion as a cofactor in Na channel gating. *Proc Natl Acad Sci U S A,* 88**,** 6528-31.

ARMSTRONG, C. M. & COTA, G. 1999. Calcium block of Na+ channels and its effect on closing rate. *Proc Natl Acad Sci U S A,* 96**,** 4154-7.

ASTON-JONES, G. & BLOOM, F. E. 1981. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. *J Neurosci,* 1**,** 876-86.

BATEMAN, R. J., WEN, G., MORRIS, J. C. & HOLTZMAN, D. M. 2007. Fluctuations of CSF amyloid-beta levels: implications for a diagnostic and therapeutic biomarker. *Neurology,* 68**,** 666-9.

BEHRENS, M. I., OBERHAUSER, A., BEZANILLA, F. & LATORRE, R. 1989. Batrachotoxin-modified sodium channels from squid optic nerve in planar bilayers. Ion conduction and gating properties. *J Gen Physiol,* 93**,** 23-41.

BELL, J. E. & MILLER, C. 1984. Effects of phospholipid surface charge on ion conduction in the K+ channel of sarcoplasmic reticulum. *Biophys J,* 45**,** 279-87.

BENNETT, E., URCAN, M. S., TINKLE, S. S., KOSZOWSKI, A. G. & LEVINSON, S. R. 1997. Contribution of sialic acid to the voltage dependence of sodium channel gating. A possible electrostatic mechanism. *J Gen Physiol,* 109**,** 327-43.

BERRIDGE, M. J., LIPP, P. & BOOTMAN, M. D. 2000. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol,* 1**,** 11-21.

BI, G. Q. & POO, M. M. 1998. Synaptic modifications in cultured hippocampal neurons: dependence on spike timing, synaptic strength, and postsynaptic cell type. *J Neurosci,* 18**,** 10464-72.

BJOREFELDT, A., ANDREASSON, U., DABORG, J., RIEBE, I., WASLING, P., ZETTERBERG, H. & HANSE, E. 2015. Human cerebrospinal fluid increases the excitability of pyramidal neurons in the in vitro brain slice. *J Physiol,* 593**,** 231-43.

BJOREFELDT, A., WASLING, P., ZETTERBERG, H. & HANSE, E. 2016. Neuromodulation of fast-spiking and non-fast-spiking hippocampal CA1 interneurons by human cerebrospinal fluid. *J Physiol,* 594**,** 937-52.

BOONE, A. N., SENATORE, A., CHEMIN, J., MONTEIL, A. & SPAFFORD, J. D. 2014. Gd3+ and calcium sensitive, sodium leak currents are features of weak membrane-glass seals in patch clamp recordings. *PLoS One,* 9**,** e98808.

BRIGHTMAN, M. W. & REESE, T. S. 1969. Junctions between intimately apposed cell membranes in the vertebrate brain. *J Cell Biol,* 40**,** 648-77.

BRINK, F. 1954. The role of calcium ions in neural processes. *Pharmacol Rev,* 6**,** 243-98.

BRINKER, T., STOPA, E., MORRISON, J. & KLINGE, P. 2014. A new look at cerebrospinal fluid circulation. *Fluids Barriers CNS,* 11**,** 10.

BROCKLEHURST, G. 1979. The significance of the evolution of the cerebrospinal fluid system. *Ann R Coll Surg Engl,* 61**,** 349-56.

CARLI, G., KAWAMURA, H. & POMPEIANO, O. 1967. Transmission of somatic sensory volleys through ascending spinal hindlimb pathways during sleep and wakefulness. *Pflugers Arch Gesamte Physiol Menschen Tiere,* 298**,** 163-9.

CHEMELLI, R. M., WILLIE, J. T., SINTON, C. M., ELMQUIST, J. K., SCAMMELL, T., LEE, C., RICHARDSON, J. A., WILLIAMS, S. C., XIONG, Y., KISANUKI, Y., FITCH, T. E., NAKAZATO, M., HAMMER, R. E., SAPER, C. B. & YANAGISAWA, M. 1999. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell,* 98**,** 437-51.

CHEN, W., BERGSMAN, J. B., WANG, X., GILKEY, G., PIERPOINT, C. R., DANIEL, E. A., AWUMEY, E. M., DAUBAN, P., DODD, R. H., RUAT, M. & SMITH, S. M. 2010. Presynaptic external calcium signaling involves the calcium-sensing receptor in neocortical nerve terminals. *PLoS One,* 5**,** e8563.

CHORDAS, C. 2001. Post–dural puncture headache and other complications after lumbar puncture. *Journal of Pediatric Oncology Nursing,* 18**,** 244-259.

CHOU, T. C., BJORKUM, A. A., GAUS, S. E., LU, J., SCAMMELL, T. E. & SAPER, C. B. 2002. Afferents to the ventrolateral preoptic nucleus. *J Neurosci,* 22**,** 977-90.

CHUA, H. C., WULF, M., WEIDLING, C., RASMUSSEN, L. P. & PLESS, S. A. 2020. The NALCN channel complex is voltage sensitive and directly modulated by extracellular calcium. *Sci Adv,* 6**,** eaaz3154.

CUKIERMAN, S., ZINKAND, W. C., FRENCH, R. J. & KRUEGER, B. K. 1988. Effects of membrane surface charge and calcium on the gating of rat brain sodium channels in planar bilayers. *J Gen Physiol,* 92**,** 431-47.

DE LECEA, L., KILDUFF, T. S., PEYRON, C., GAO, X., FOYE, P. E., DANIELSON, P. E., FUKUHARA, C., BATTENBERG, E. L., GAUTVIK, V. T., BARTLETT, F. S., 2ND, FRANKEL, W. N., VAN DEN POL, A. N., BLOOM, F. E., GAUTVIK, K. M. & SUTCLIFFE, J. G. 1998. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A,* 95**,** 322-7.

DING, F., O'DONNELL, J., THRANE, A. S., ZEPPENFELD, D., KANG, H., XIE, L., WANG, F. & NEDERGAARD, M. 2013. α1-Adrenergic receptors mediate coordinated Ca2+ signaling of cortical astrocytes in awake, behaving mice. *Cell Calcium,* 54**,** 387-94.

DING, F., O'DONNELL, J., XU, Q., KANG, N., GOLDMAN, N. & NEDERGAARD, M. 2016. Changes in the composition of brain interstitial ions control the sleep-wake cycle. *Science,* 352**,** 550-5.

DODGE, F. A., JR. & RAHAMIMOFF, R. 1967. Co-operative action a calcium ions in transmitter release at the neuromuscular junction. *J Physiol,* 193**,** 419-32.

EDWARDS, F. A., KONNERTH, A., SAKMANN, B. & TAKAHASHI, T. 1989. A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system. *Pflugers Arch,* 414**,** 600-12.

EGAN, J. M., PETERSON, C. A. & FRY, W. M. 2018. Lack of current observed in HEK293 cells expressing NALCN channels. *Biochim Open,* 6**,** 24-28.

ELINDER, F. & ARHEM, P. 1999. Role of individual surface charges of voltage-gated K channels. *Biophys J,* 77**,** 1358-62.

ELINDER, F., LIU, Y. & ARHEM, P. 1998. Divalent cation effects on the Shaker K channel suggest a pentapeptide sequence as determinant of functional surface charge density. *J Membr Biol,* 165**,** 183-9.

ELINDER, F., MADEJA, M. & ARHEM, P. 1996. Surface Charges of K channels. Effects of strontium on five cloned channels expressed in Xenopus oocytes. *J Gen Physiol,* 108**,** 325-32.

ELINDER, F. & ÅRHEM, P. 2003. Metal ion effects on ion channel gating. *Quarterly Reviews of Biophysics,* 36**,** 373-427.

ELLINOR, P. T., YANG, J., SATHER, W. A., ZHANG, J. F. & TSIEN, R. W. 1995. Ca2+ channel selectivity at a single locus for high-affinity Ca2+ interactions. *Neuron,* 15**,** 1121-32.

ESTABROOKE, I. V., MCCARTHY, M. T., KO, E., CHOU, T. C., CHEMELLI, R. M., YANAGISAWA, M., SAPER, C. B. & SCAMMELL, T. E. 2001. Fos expression in orexin neurons varies with behavioral state. *J Neurosci,* 21**,** 1656-62.

FLEMING, G. W. T. H. 1930. Sleep as a Problem of Localization. (Journ. of Nerv. and Ment. Dis., March, 1930.) Von Economo, C. *Journal of Mental Science,* 76**,** 563-564.

FORNAL, C., AUERBACH, S. & JACOBS, B. L. 1985. Activity of serotonin-containing neurons in nucleus raphe magnus in freely moving cats. *Exp Neurol,* 88**,** 590-608.

FORT, P., BASSETTI, C. L. & LUPPI, P. H. 2009. Alternating vigilance states: new insights regarding neuronal networks and mechanisms. *Eur J Neurosci,* 29**,** 1741-53.

FRANKENHAEUSER, B. & HODGKIN, A. L. 1957. The action of calcium on the electrical properties of squid axons. *J Physiol,* 137**,** 218-44.

GARDOS, G. 1958. The function of calcium in the potassium permeability of human erythrocytes. *Biochim Biophys Acta,* 30**,** 653-4.

GILLY, W. F. & ARMSTRONG, C. M. 1982a. Divalent cations and the activation kinetics of potassium channels in squid giant axons. *J Gen Physiol,* 79**,** 965-96.

GILLY, W. F. & ARMSTRONG, C. M. 1982b. Slowing of sodium channel opening kinetics in squid axon by extracellular zinc. *J Gen Physiol,* 79**,** 935-64.

GOLDSTEIN, D. A., ROMOFF, M., BOGIN, E. & MASSRY, S. G. 1979. Relationship between the concentrations of calcium and phosphorus in blood and cerebrospinal fluid. *J Clin Endocrinol Metab,* 49**,** 58-62.

HAHIN, R. & CAMPBELL, D. T. 1983. Simple shifts in the voltage dependence of sodium channel gating caused by divalent cations. *J Gen Physiol,* 82**,** 785-805.

HALASSA, M. M., FELLIN, T. & HAYDON, P. G. 2009. Tripartite synapses: roles for astrocytic purines in the control of synaptic physiology and behavior. *Neuropharmacology,* 57**,** 343-6.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch,* 391**,** 85-100.

HAN, P., TRINIDAD, B. J. & SHI, J. 2015. Hypocalcemia-induced seizure: demystifying the calcium paradox. *ASN Neuro,* 7.

HANSE, E. & GUSTAFSSON, B. 2002. Release dependence to a paired stimulus at a synaptic release site with a small variable pool of immediately releasable vesicles. *J Neurosci,* 22**,** 4381-7.

HEINEMANN, S. H., TERLAU, H., STUHMER, W., IMOTO, K. & NUMA, S. 1992. Calcium channel characteristics conferred on the sodium channel by single mutations. *Nature,* 356**,** 441-3.

HILLE, B. 1968. Charges and potentials at the nerve surface. Divalent ions and pH. *J Gen Physiol,* 51**,** 221-36.

HILLE, B. 2001. *Ion channels of excitable membranes,* Sunderland, Mass., Sinauer.

HODGKIN, A. L. & HUXLEY, A. F. 1952. Currents carried by sodium and potassium ions through the membrane of the giant axon of Loligo. *J Physiol,* 116**,** 449-72.

HOLOPAINEN, I. E. 2005. Organotypic hippocampal slice cultures: a model system to study basic cellular and molecular mechanisms of neuronal cell death, neuroprotection, and synaptic plasticity. *Neurochem Res,* 30**,** 1521-8.

HOUNSGAARD, J. & NICHOLSON, C. 1983. Potassium accumulation around individual purkinje cells in cerebellar slices from the guinea-pig. *J Physiol,* 340**,** 359-88.

HUNTER, G. & SMITH, H. V. 1960. Calcium and magnesium in human cerebrospinal fluid. *Nature,* 186**,** 161-2.

INGLEBERT, Y., ALJADEFF, J., BRUNEL, N. & DEBANNE, D. 2020. Synaptic plasticity rules with physiological calcium levels. *Proc Natl Acad Sci U S A,* 117**,** 33639-33648.

IRAM, T., KERN, F., KAUR, A., MYNENI, S., MORNINGSTAR, A. R., SHIN, H., GARCIA, M. A., YERRA, L., PALOVICS, R., YANG, A. C., HAHN, O., LU, N., SHUKEN, S. R., HANEY, M. S., LEHALLIER, B., IYER, M., LUO, J., ZETTERBERG, H., KELLER, A., ZUCHERO, J. B. & WYSS-CORAY, T. 2022. Young CSF restores oligodendrogenesis and memory in aged mice via Fgf17. *Nature,* 605**,** 509-515.

JESSEN, N. A., MUNK, A. S., LUNDGAARD, I. & NEDERGAARD, M. 2015. The Glymphatic System: A Beginner's Guide. *Neurochem Res,* 40**,** 2583-99.

JONES, B. E. 2003. Arousal systems. *Front Biosci,* 8**,** s438-51.

JURADO-CORONEL, J. C., AVILA-RODRIGUEZ, M., CAPANI, F., GONZALEZ, J., MORAN, V. E. & BARRETO, G. E. 2016. Targeting the Nicotinic Acetylcholine Receptors (nAChRs) in Astrocytes as a Potential Therapeutic Target in Parkinson's Disease. *Curr Pharm Des,* 22**,** 1305-11.

KOSTYUK, P. G., MIRONOV, S. L., DOROSHENKO, P. A. & PONOMAREV, V. N. 1982. Surface charges on the outer side of mollusc neuron membrane. *The Journal of Membrane Biology,* 70**,** 171-179.

KOZAI, T. D., JAQUINS-GERSTL, A. S., VAZQUEZ, A. L., MICHAEL, A. C. & CUI, X. T. 2015. Brain tissue responses to neural implants impact signal sensitivity and intervention strategies. *ACS Chem Neurosci,* 6**,** 48-67.

LANCASTER, B. & NICOLL, R. A. 1987. Properties of two calcium-activated hyperpolarizations in rat hippocampal neurones. *J Physiol,* 389**,** 187-203.

LEE, S. Y., VUONG, T. A., WEN, X., JEONG, H. J., SO, H. K., KWON, I., KANG, J. S. & CHO, H. 2019. Methylation determines the extracellular calcium sensitivity of the leak channel NALCN in hippocampal dentate granule cells. *Exp Mol Med,* 51**,** 1-14.

LEHTINEN, M. K., ZAPPATERRA, M. W., CHEN, X., YANG, Y. J., HILL, A. D., LUN, M., MAYNARD, T., GONZALEZ, D., KIM, S., YE, P., D'ERCOLE, A. J., WONG, E. T., LAMANTIA, A. S. & WALSH, C. A. 2011. The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron,* 69**,** 893-905.

LIN, L., FARACO, J., LI, R., KADOTANI, H., ROGERS, W., LIN, X., QIU, X., DE JONG, P. J., NISHINO, S. & MIGNOT, E. 1999. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell,* 98**,** 365-76.

LU, B., SU, Y., DAS, S., LIU, J., XIA, J. & REN, D. 2007. The neuronal channel NALCN contributes resting sodium permeability and is required for normal respiratory rhythm. *Cell,* 129**,** 371-83.

LU, B., ZHANG, Q., WANG, H., WANG, Y., NAKAYAMA, M. & REN, D. 2010. Extracellular calcium controls background current and neuronal excitability via an UNC79-UNC80-NALCN cation channel complex. *Neuron,* 68**,** 488-99.

LYNCH, G., KESSLER, M., HALPAIN, S. & BAUDRY, M. 1983. Biochemical effects of high-frequency synaptic activity studied with in vitro slices. *Fed Proc,* 42**,** 2886-90.

MALENKA, R. C., LANCASTER, B. & ZUCKER, R. S. 1992. Temporal limits on the rise in postsynaptic calcium required for the induction of long-term potentiation. *Neuron,* 9**,** 121-8.

MARTISZUS, B. J., TSINTSADZE, T., CHANG, W. & SMITH, S. M. 2021. Enhanced excitability of cortical neurons in low-divalent solutions is primarily mediated by altered voltage-dependence of voltage-gated sodium channels. *Elife,* 10.

MCLAUGHLIN, S. G., SZABO, G. & EISENMAN, G. 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J Gen Physiol,* 58**,** 667-87.

MEECH, R. W. 1972. Intracellular calcium injection causes increased potassium conductance in Aplysia nerve cells. *Comp Biochem Physiol A Comp Physiol,* 42**,** 493-9.

MIYAZAKI, I. & ASANUMA, M. 2016. Serotonin 1A Receptors on Astrocytes as a Potential Target for the Treatment of Parkinson's Disease. *Curr Med Chem,* 23**,** 686-700.

MOCZYDLOWSKI, E., ALVAREZ, O., VERGARA, C. & LATORRE, R. 1985. Effect of phospholipid surface charge on the conductance and gating of a Ca2+-activated K+ channel in planar lipid bilayers. *J Membr Biol,* 83**,** 273-82.

MOLLEMAN, A. 2003. *Patch clamping: an introductory guide to patch clamp electrophysiology*, John Wiley & Sons.

MUHEIM, C. M., SPINNLER, A., SARTORIUS, T., DURR, R., HUBER, R., KABAGEMA, C., RUTH, P. & BROWN, S. A. 2019. Dynamic- and Frequency-Specific Regulation of Sleep Oscillations by Cortical Potassium Channels. *Curr Biol*.

NEHER, E. & SAKMANN, B. 1976. Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature,* 260**,** 799-802.

NESKE, G. T. 2017. Sleepy Circuits in Vigilant Mice? A Slow Cortical Oscillation Occurring during Multiple Arousal States. *J Neurosci,* 37**,** 7294-7296.

NICHOLSON, C., BRUGGENCATE, G. T., STEINBERG, R. & STOCKLE, H. 1977. Calcium modulation in brain extracellular microenvironment demonstrated with ion-selective micropipette. *Proc Natl Acad Sci U S A,* 74**,** 1287-90.

OLSSON, M., ARLIG, J., HEDNER, J., BLENNOW, K. & ZETTERBERG, H. 2019. Repeated lumbar punctures within 3 days may affect CSF biomarker levels. *Fluids Barriers CNS,* 16**,** 37.

ORESKOVIĆ, D. & KLARICA, M. 2010. The formation of cerebrospinal fluid: nearly a hundred years of interpretations and misinterpretations. *Brain Res Rev,* 64**,** 241-62.

PAPOUIN, T., DUNPHY, J. M., TOLMAN, M., DINELEY, K. T. & HAYDON, P. G. 2017. Septal Cholinergic Neuromodulation Tunes the Astrocyte-Dependent Gating of Hippocampal NMDA Receptors to Wakefulness. *Neuron,* 94**,** 840-854 e7.

PASCUAL, O., CASPER, K. B., KUBERA, C., ZHANG, J., REVILLA-SANCHEZ, R., SUL, J. Y., TAKANO, H., MOSS, S. J., MCCARTHY, K. & HAYDON, P. G. 2005. Astrocytic purinergic signaling coordinates synaptic networks. *Science,* 310**,** 113-6.

PEDERSEN, K. O. 1971. On the presence of calcium complexes in aqueous bicarbonate solutions. *Scand J Clin Lab Invest,* 27**,** 9-13.

PEYRON, C., TIGHE, D. K., VAN DEN POL, A. N., DE LECEA, L., HELLER, H. C., SUTCLIFFE, J. G. & KILDUFF, T. S. 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci,* 18**,** 9996-10015.

PHILLIPS, C. G., HARNETT, M. T., CHEN, W. & SMITH, S. M. 2008. Calcium-sensing receptor activation depresses synaptic transmission. *J Neurosci,* 28**,** 12062-70.

RASMUSSEN, R., JENSEN, M. H. & HELTBERG, M. L. 2017. Chaotic Dynamics Mediate Brain State Transitions, Driven by Changes in Extracellular Ion Concentrations. *Cell Syst,* 5**,** 591-603 e4.

RECIO-PINTO, E., THORNHILL, W. B., DUCH, D. S., LEVINSON, S. R. & URBAN, B. W. 1990. Neuraminidase treatment modifies the function of electroplax sodium channels in planar lipid bilayers. *Neuron,* 5**,** 675-84.

ROBERTSON, W. G. & MARSHALL, R. W. 1981. Ionized calcium in body fluids. *Crit Rev Clin Lab Sci,* 15**,** 85-125.

ROSHCHIN, M. V., IERUSALIMSKY, V. N., BALABAN, P. M. & NIKITIN, E. S. 2020. Ca(2+)-activated KCa3.1 potassium channels contribute to the slow afterhyperpolarization in L5 neocortical pyramidal neurons. *Sci Rep,* 10**,** 14484.

RUAT, M. & TRAIFFORT, E. 2013. Roles of the calcium sensing receptor in the central nervous system. *Best Pract Res Clin Endocrinol Metab,* 27**,** 429-42.

SAKURAI, T., AMEMIYA, A., ISHII, M., MATSUZAKI, I., CHEMELLI, R. M., TANAKA, H., WILLIAMS, S. C., RICHARDSON, J. A., KOZLOWSKI, G. P., WILSON, S., ARCH, J. R., BUCKINGHAM, R. E., HAYNES, A. C., CARR, S. A., ANNAN, R. S., MCNULTY, D. E., LIU, W. S., TERRETT, J. A., ELSHOURBAGY, N. A., BERGSMA, D. J. & YANAGISAWA, M. 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell,* 92**,** 573-85.

SAKURAI, T., NAGATA, R., YAMANAKA, A., KAWAMURA, H., TSUJINO, N., MURAKI, Y., KAGEYAMA, H., KUNITA, S., TAKAHASHI, S., GOTO, K., KOYAMA, Y., SHIODA, S. & YANAGISAWA, M. 2005. Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. *Neuron,* 46**,** 297-308.

SANCHEZ-VIVES, M. V. & MCCORMICK, D. A. 2000. Cellular and network mechanisms of rhythmic recurrent activity in neocortex. *Nat Neurosci,* 3**,** 1027-34.

SANDSTROM, D. 2014. Insight into putative NALCN *PLoS One (Unpublished results),* https://journals.plos.org/plosone/article/comment?id=10.1371/annotation/c2983d2f-0c75-4b1c-9274-5ded264b39d9.

SAPER, C. B., CHOU, T. C. & SCAMMELL, T. E. 2001. The sleep switch: hypothalamic control of sleep and wakefulness. *Trends Neurosci,* 24**,** 726-31.

SCHAER, H. 1974. Decrease in ionized calcium by bicarbonate in physiological solutions. *Pflugers Arch,* 347**,** 249-54.

SEGAL, M. 2018. Calcium stores regulate excitability in cultured rat hippocampal neurons. *J Neurophysiol,* 120**,** 2694-2705.

SEGAL, M. B. 1993. Extracellular and cerebrospinal fluids. *J Inherit Metab Dis,* 16**,** 617-38.

SENATORE, A., MONTEIL, A., VAN MINNEN, J., SMIT, A. B. & SPAFFORD, J. D. 2013. NALCN ion channels have alternative selectivity filters resembling calcium channels or sodium channels. *PLoS One,* 8**,** e55088.

SENATORE, A. & SPAFFORD, J. D. 2013. A uniquely adaptable pore is consistent with NALCN being an ion sensor. *Channels (Austin),* 7**,** 60-8.

SHERIN, J. E., ELMQUIST, J. K., TORREALBA, F. & SAPER, C. B. 1998. Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus of the rat. *J Neurosci,* 18**,** 4705-21.

SHERIN, J. E., SHIROMANI, P. J., MCCARLEY, R. W. & SAPER, C. B. 1996. Activation of ventrolateral preoptic neurons during sleep. *Science,* 271**,** 216-9.

SMITH, S. M., BERGSMAN, J. B., HARATA, N. C., SCHELLER, R. H. & TSIEN, R. W. 2004. Recordings from single neocortical nerve terminals reveal a nonselective cation channel activated by decreases in extracellular calcium. *Neuron,* 41**,** 243-56.

SMITH, S. M., CHEN, W., VYLETA, N. P., WILLIAMS, C., LEE, C. H., PHILLIPS, C. & ANDRESEN, M. C. 2012. Calcium regulation of spontaneous and asynchronous neurotransmitter release. *Cell Calcium,* 52**,** 226-33.

SOJA, P. J., PANG, W., TAEPAVARAPRUK, N. & MCERLANE, S. A. 2001. Spontaneous Spike Activity of Spinoreticular Tract Neurons During Sleep and Wakefulness. *Sleep,* 24**,** 18-25.

STEINBERG, E. A., WAFFORD, K. A., BRICKLEY, S. G., FRANKS, N. P. & WISDEN, W. 2015. The role of K(2)p channels in anaesthesia and sleep. *Pflugers Arch,* 467**,** 907-16.

STEININGER, T. L., ALAM, M. N., GONG, H., SZYMUSIAK, R. & MCGINTY, D. 1999. Sleep-waking discharge of neurons in the posterior lateral hypothalamus of the albino rat. *Brain Res,* 840**,** 138-47.

STERIADE, M., MCCORMICK, D. A. & SEJNOWSKI, T. J. 1993. Thalamocortical oscillations in the sleeping and aroused brain. *Science,* 262**,** 679-85.

STORM, J. F. 1989. An after-hyperpolarization of medium duration in rat hippocampal pyramidal cells. *J Physiol,* 409**,** 171-90.

THANNICKAL, T. C., MOORE, R. Y., NIENHUIS, R., RAMANATHAN, L., GULYANI, S., ALDRICH, M.,

CORNFORD, M. & SIEGEL, J. M. 2000. Reduced number of hypocretin neurons in human narcolepsy. *Neuron*, 27**,** 469-74.

TING, J. T., LEE, B. R., CHONG, P., SOLER-LLAVINA, G., COBBS, C., KOCH, C., ZENG, H. & LEIN, E. 2018. Preparation of Acute Brain Slices Using an Optimized N-Methyl-D-glucamine Protective Recovery Method. *J Vis Exp*.

TOBLER, I., BORBELY, A. A., SCHWYZER, M. & FONTANA, A. 1984. Interleukin-1 derived from astrocytes enhances slow wave activity in sleep EEG of the rat. *Eur J Pharmacol*, 104**,** 191-2.

TRAYNELIS, S. F. & DINGLEDINE, R. 1988. Potassium-induced spontaneous electrographic seizures in the rat hippocampal slice. *J Neurophysiol*, 59**,** 259-76.

TURNER, R. W., BAIMBRIDGE, K. G. & MILLER, J. J. 1982. Calcium-induced long-term potentiation in the hippocampus. *Neuroscience*, 7**,** 1411-6.

VYSOTSKAYA, Z. V., MOSS, C. R., 2ND, GILBERT, C. A., GABRIEL, S. A. & GU, Q. 2014. Modulation of BK channel activities by calcium-sensing receptor in rat bronchopulmonary sensory neurons. *Respir Physiol Neurobiol*, 203**,** 35-44.

WOODHULL, A. M. 1973. Ionic blockage of sodium channels in nerve. *J Gen Physiol*, 61**,** 687-708.

WOTTON, C. A., CROSS, C. D. & BEKAR, L. K. 2020. Serotonin, norepinephrine, and acetylcholine differentially affect astrocytic potassium clearance to modulate somatosensory signaling in male mice. *J Neurosci Res*, 98**,** 964-977.

XU, J., ZHANG, X., QIAN, Q., WANG, Y., DONG, H., LI, N., QIAN, Y. & JIN, W. 2018. Histamine upregulates the expression of histamine receptors and increases the neuroprotective effect of astrocytes. *J Neuroinflammation*, 15**,** 41.

YANG, J., ELLINOR, P. T., SATHER, W. A., ZHANG, J. F. & TSIEN, R. W. 1993. Molecular determinants of Ca2+ selectivity and ion permeation in L-type Ca2+ channels. *Nature*, 366**,** 158-61.

YANG, J., MCBRIDE, S., MAK, D. O., VARDI, N., PALCZEWSKI, K., HAESELEER, F. & FOSKETT, J. K. 2002. Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor Ca(2+) release channels. *Proc Natl Acad Sci U S A*, 99**,** 7711-6.

ZHOU, W. & JONES, S. W. 1995. Surface charge and calcium channel saturation in bullfrog sympathetic neurons. *J Gen Physiol*, 105**,** 441-62.

APPENDIX

Article I

Article II

Article III