Neuromodulation of hippocampal single cell- and network activity by human cerebrospinal fluid

Andreas Björefeldt

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UNIVERSITY OF GOTHENBURG

Department of Physiology
Institute of Neuroscience and Physiology
Sahlgrenska academy at University of Gothenburg

Cover illustration: Fast-spiking hippocampal CA1 interneuron adapted from Bjorefeldt et al., 2015 (paper II).

Somewhere, something incredible is waiting to be known.

Carl Sagan

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Göteborg, Sweden

ABSTRACT

Neuromodulation is a key process determining the function of central neurons. The brain extracellular fluid contains numerous neuromodulatory substances (neuromodulators), but how they collective influence neuronal activity *in vivo* is not known. This thesis work attempts to shed light on this issue by examining the neuromodulatory influence of human cerebrospinal fluid (hCSF) on neurons in rat and mouse hippocampal brain slices, using a matched artificial cerebrospinal fluid (aCSF, devoid of neuromodulators) as control. The methodology comprises intracellular and extracellular recording techniques and, to lesser extent, biochemical and histological procedures. In **paper I** we examine the effect of hCSF on CA1 pyramidal cells. We find that hCSF induces *in vivo*-like properties in these neurons, powerfully boosting spontaneous action potential firing, depolarizing the resting membrane potential and lowering the action potential threshold. **In paper II** we record from GABAergic fast-spiking and non-fast-spiking interneurons in the CA1 hippocampus and show that hCSF excites both types of neurons through mechanisms involving reductions in afterhyperpolarization amplitudes and action potential threshold. Finally, in **paper III**, we show that hCSF induces spontaneous network gamma oscillations in the CA3 stratum pyramidale, via a cholinergic mechanism, and enhances electrical theta resonance in CA1 pyramidal cells through potentiation of two separate voltage-gated conductances. Taken together, the findings in this thesis project suggest that neuromodulators in brain extracellular fluid significantly contribute in shaping neuronal activity *in vivo*.

CONTENTS

PREFACE

List of papers

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

I. Bjorefeldt A, Andreasson U, Daborg J, Riebe I, Wasling P, Zetterberg H, Hanse E. Human cerebrospinal fluid increases the excitability of pyramidal neurons in the *in vitro* brain slice. J Physiol. 2015; 20: 121-133.

II. Bjorefeldt A, Wasling P, Zetterberg H, Hanse E. Neuromodulation of fast-spiking and non-fast-spiking hippocampal CA1 interneurons by human cerebrospinal fluid. Submitted manuscript.

III. Bjorefeldt A, Kurudenkandy R, Hanse E, Fisahn A. Human cerebrospinal fluid promotes spontaneous gamma oscillations in the hippocampus *in vitro*. In manuscript.

Abbreviations

aCSF	Artificial cerebrospinal fluid
AHP	Afterhyperpolarization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential
APHW	Action potential half width
CA1	Cornu ammonis region 1
CA3	Cornu ammonis region 3
CNS	Central nervous system
CSF	Cerebrospinal fluid
DG	Dentate gyrus
EC	Entorhinal cortex
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
fEPSP	Field excitatory postsynaptic potential
FS	Fast-spiking
GABA	γ-aminobutyric acid
GTPγS	Guanosine 5´- [γ-thio] triphosphate
hCSF	Human cerebrospinal fluid
I_h	H-current
I_m	M-current
IPSC	Inhibitory postsynaptic current

IPSP	Inhibitory postsynaptic potential
ISF	Interstitial fluid
LFP	Local field potential
NFS	Non-fast-spiking
NPH	Normal pressure hydrocephalus
PPR	Paired-pulse ratio
R_{in}	Input resistance
sEPSP/C	Spontaneous excitatory postsynaptic potential/current
sIPSP/C	Spontaneous inhibitory postsynaptic potential/current
V_m	Membrane potential
V_{rest}	Resting membrane potential

Definitions in short

Cerebrospinal fluid

A clear transparent fluid that bathes neurons of the central nervous system

Excitability

The probability of action potential discharge in a neuron

Neuromodulation

The process by which specific chemical substances regulate the excitability of neurons

Gamma oscillations

Fast (30-80 Hz) network oscillations arising from synchronized neuronal activity

INTRODUCTION

The hippocampus

The hippocampus is a medial temporal lobe structure involved in the encoding of declarative memory and spatial processing [1, 2]. It is arguably the most well studied area of the mammalian brain and its scrutiny has provided countless insights as to how neurons and neural networks function in health and disease.

The hippocampus is part of a larger medial temporal lobe region known as the hippocampal formation, consisting of the entorhinal cortex (EC), the dentate gyrus (DG), the hippocampus proper (areas CA1, CA2 and CA3) and the subiculum (Fig. 1). These individual regions are interconnected in a tri-synaptic loop where the EC serves as both input and output area. In layer II of the EC, pyramidal cells send their axons to the DG via the medial and lateral perforant pathways, where they provide excitatory input onto granule cell dendrites. The granule cells project forward to pyramidal cells in area CA3 of the hippocampus proper via axons called mossy fibers. CA3 pyramidal cells in turn send their axons, known as Schaffer collaterals, to pyramidal cells in area CA1, which project back to layers V/VI of the EC via

the subiculum. The hippocampal formation is also extrinsically connected to a number of subcortical areas, as well as to the contralateral hippocampal region via commissural fibers.
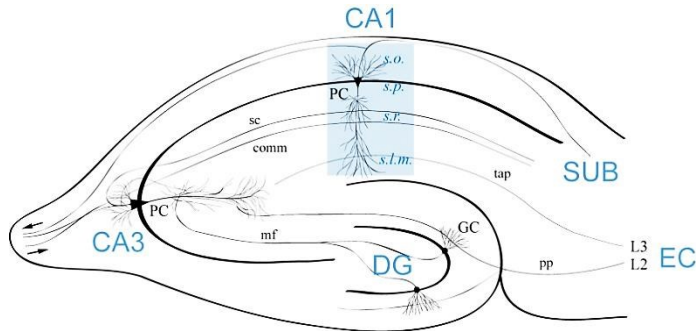
Figure 1. *Anatomy of the hippocampal brain slice. EC; entorhinal cortex, DG; dentate gyrus, CA1; cornu ammonis region 1, CA3; cornu ammonis region 3, SUB; subiculum, GC; granule cell, PC; pyramidal cell, pp; perforant path, tap; temporoammonic path, mf; mossy fiber, sc; schaffer collateral, comm; commissural fiber, s.o.; stratum oriens, s.p.; stratum pyramidale, s.r.; stratum radiatum, s.l.m; stratum lacunose moleculare, L2/L3; layer 2/3 of EC.*

Two main types of neurons populate the hippocampal formation, excitatory principal cells (pyramidal cells and granule cells) and inhibitory interneurons. In the hippocampus proper, approximately 90% of neurons are glutamatergic pyramidal cells [3], while the remaining population is made up of various types of GABAergic interneurons [3, 4]. With respect to the pyramidal cell population, GABAergic interneurons are considerably more diverse and differ in their general morphology, axonal projections, electrophysiological properties and expression of neuropeptides and calcium-binding proteins. In the CA1 hippocampus, over 20 different types of interneurons have been characterized [4, 5]. Among the prominent subtypes of interneurons are fast-spiking basket cells that frequently express the calcium binding protein

parvalbumin. These interneurons form powerful perisomatic inhibitory synapses onto pyramidal cells that effectively control their firing [6, 7].

Early indications that the medial temporal lobe was critically involved in the encoding of declarative memory came from observations made by Scoville and Milner in the 1950s [8]. Scoville and Milner studied the patient H.M. who had undergone surgery to remove both medial temporal lobes as a result of severe intractable epilepsy. Following the medial temporal lobe resections, H.M. was unable to form new declarative memories (anterograde amnesia) and could not remember events that took place up to a year prior to his surgery (retrograde amnesia). H.M. was, however, able to access memories that had been formed earlier than a year prior to his surgery, and also showed intact motor skill learning. These observations suggested (i) that structures in the medial temporal lobe are essential to the formation of new memories (ii) that the medial temporal lobe is not the sole final storage site for declarative memories and (iii) that multiple memory systems must exist that deals with the encoding of specific types of memories. Although the full implications were not apparent at the time, the findings with patient H.M. boosted interest in the question of where and how different types of memories are encoded, stored and retrieved. Over the ensuing decades, the development of experimental animal models, as well as further assessment of amnesic patients, eventually identified the hippocampal formation as crucial in declarative memory function [9, 10].

In the 1970s, O'Keefe and Dostrovsky discovered that the firing patterns of some principal cells in the rat hippocampus were closely linked to the animal's position in space [11]. The cells, known as 'place cells', fired whenever the animal entered a specific location in its environment (the place field). This finding suggested that a major function of the hippocampal

formation was to process information regarding an animal's position in space. Since the discovery of place cells, several additional types of 'spatial cells' have been identified, including head direction cells [12] and grid cells [13], each dealing with a specific aspect of spatial navigation.

It is currently debated whether the hippocampal formation is mainly concerned with declarative memory functions or the processing of spatial information [14-16]. Although there is considerable evidence to support both views, neither can alone fully account for what the hippocampus does. Recent work has focused on how these opposing views can be reconciled to yield a better understanding of hippocampal function [14, 15].

Cerebrospinal fluid

Cerebrospinal fluid (*liquor cerebrospinalis*, CSF) is a clear transparent extracellular fluid that occupies the brain's ventricular system and subarachnoid space. The CSF has long been considered to be produced by the choroid plexa of the four brain ventricles, and to flow unidirectionally along the ventriculo-cisternal axis until it reaches the subarachnoid space and is absorbed by arachnoid villi in the venous sinuses [17, 18]. However, this view is now being increasingly challenged as more recent work in CSF physiology has provided little support for the classical hypothesis. As a result, an alternative hypothesis based on current experimental data has recently emerged [19, 20]. According to this hypothesis, CSF production and absorption mainly takes place across blood vessels all over the central nervous system (CNS), driven by hydrostatic and osmotic forces across the capillary walls [17, 19]. The hypothesis further suggests that the movement of CSF inside the brain is bidirectional and driven by arterial pulsations

coupled to the systolic/diastolic phases of the cardiac cycle [19, 20], with no significant unidirectional flow (circulation) occurring along the ventriculo-cisternal axis.

A major function of CSF appears to be to provide neurons and glia of the CNS with a chemical environment well suited to promote their function and survival. Brains surrounded by CSF are in fact a well preserved feature in animals [21], suggesting that the CSF system may have played an important role over the course of brain evolution. The CSF has long been recognized to (i) protect the brain from injury during physical impact to the head, to (ii) distribute vital nutrients to neurons and glia within the CNS and (iii) to remove metabolic waste [18, 22]. More recent research has suggested an important role of CSF in providing neural progenitor cells with a specialized proliferative environment during the embryonic developmental stage [23].

The CSF communicates freely with the interstitial fluid (ISF) of the brain parenchyma [24-26] and its composition is thought to reflect the biochemical environment of CNS neurons. CSF sampling is thus often used for diagnostic and prognostic purposes in patients with various neurological disorders [27]. The normal protein content in CSF is $\leq 1\%$ of that in blood serum [28], the major CSF protein being albumin. Additional constituents include various amino acids, peptides, lipids, sugars, neurotrophic factors and neuromodulatory substances [29-31].

Neuronal excitability

The excitability of a neuron is typically defined as the probability that it will produce an action potential (AP). A wide range of factors influence this probability in a given neuron, such as neuromodulation, plasticity,

connectivity (the balance and relative strength of excitatory and inhibitory synaptic input), membrane receptor/ion channel expression, intra- and extracellular electrolyte concentrations, the expression of ion pumps and transporters, glial cell activity, temperature, pH and osmolality.

Neurons express a diverse set of ionic membrane conductances that collectively determine their functional properties. Voltage-gated Na^+, K^+ and Ca^{2+}-channels importantly shape intrinsic properties of the neuron, such as the AP threshold and waveform, afterhyperpolarization (AHP) and resting membrane potential. Their expression also shapes synaptic integration in dendrites and controls the firing pattern of the cell. Voltage-gated ion channels distributed in the dendrites, soma and axon thus influence the intrinsic excitability of neurons.

At synapses, the strength of synaptic transmission is largely determined by the expression of a series of voltage- and ligand-gated ion channels in the pre- and postsynaptic membrane. The signaling strength of a synapse fundamentally depends on the three quantal parameters n (the number of individual release sites), p (the probability of transmitter release) and q (the quantal size) [32]. Ligand-gated glutamate and GABA receptors in the postsynaptic membrane determine the quantal size at excitatory and inhibitory synapses, respectively, whereas voltage-gated Ca^{2+} and K^+ channels, together with G-protein coupled modulatory receptors, regulate presynaptic release probability.

Neurons can be viewed as threshold detectors that produce AP output in response to a critical (threshold) level of membrane depolarization generated by synaptic inputs. This is known as the transfer function (or input-output function) and critically depends on the neuron's excitability (intrinsic and synaptic) at a given point in time. Neuronal input-output function is often

studied as a frequency-current (F-I) relationship in whole-cell current clamp recordings, where APs are evoked by depolarizing current injection (Fig. 2).

Two critical measures of neuronal responsiveness to input is the rheobase (minimum current injection needed to evoke one or more APs) and gain (slope) of the F-I curve (Fig. 2A). Changes in rheobase (threshold modulation) alter the responsiveness of the neuron to low amplitude input (Fig. 2B), whereas gain modulation changes a neuron's sensitivity to input across a wider range of input amplitudes (Fig. 2C).

It is apparent that neuronal function *in vivo* differs from that of neurons studied in *in vitro* brain slices [33]. Neurons in brain slices are often quiescent in the sense that the rarely fire spontaneously. However, *in vivo* neurons operate under a continuous synaptic bombardment, a 'high-conductance state', and show more depolarized resting membrane potentials and higher levels of spontaneous firing [33, 34]. The strong synaptic drive (or synaptic 'noise') onto postsynaptic neurons produces fluctuating membrane potentials and has been shown to decrease neuronal gain [33, 35]. Fig. 2D shows how the introduction of synaptic noise impacts the transfer function of a stereotypical quiescent neuron.

The observed differences in neuronal function *in vivo* compared to *in vitro* are often attributed to the difference in synaptic connectivity. However, the seemingly higher excitability of neurons *in vivo* may, as outlined above, have additional explanations.
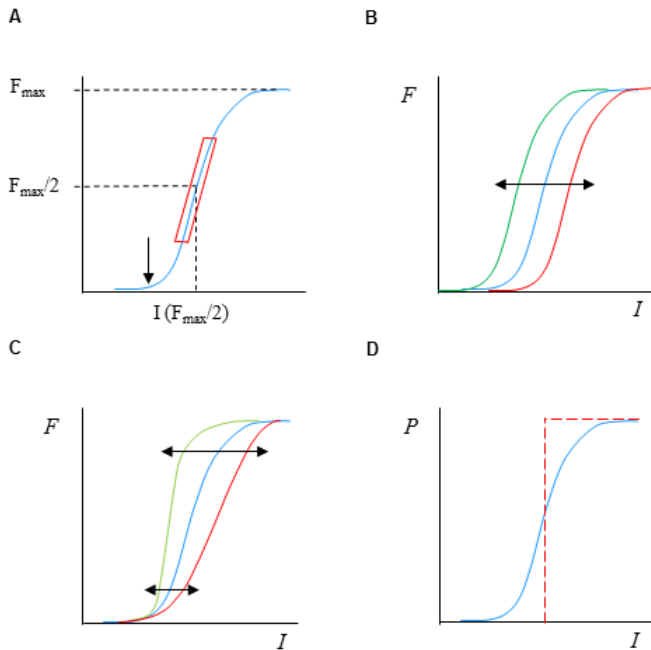
Figure 2. *Properties of neuronal input-output function. (A) Commonly measured parameters in frequency-current (F-I) plots. Rheobase (minimum current injection needed to evoke one or more APs, black arrow) gain (slope, red area,) firing frequency at half of maximum current injection (I F_{max}/2) and maximum firing frequency (F_{max}) are indicated. (B) Threshold modulation resulting in left- or right shift of the F-I curve and corresponding decrease (green) or increase (red) in rheobase. (C) Gain modulation leading to increased (green) or decreased (red) slope of the F-I relationship. (D) Firing probability of a quiescent neuron in response to increasing levels of depolarizing current in absence (red dotted line) and presence (blue) of synaptic noise.*

Neuromodulation

Neuromodulation is the process by which neuromodulatory substances (also known as neuromodulators) regulate the excitability of neurons. It is a fundamental feature of neural networks that increases the functional complexity and controls the network's operational state [36, 37]. In order to have such effects, neuromodulators specifically act to regulate the function

(as well as expression) of intrinsic and synaptic membrane conductances. This is commonly achieved via activation of specific G-protein coupled receptors that directly, or via second messenger systems, target ion channel function [37, 38]. The modulation may alter properties such as maximum conductance, open probability or voltage-dependence of an ion channel. A given neuromodulator may regulate the function of multiple types of ion channels, and a particular ion channel can be targeted by several different neuromodulators.

The effect of classical transmitters such as acetylcholine, noradrenaline and histamine has been studied extensively in a variety of neurons and brain areas. A typical example is the cholinergic modulation of neurons in hippocampal brain slices where acetylcholine (ACh) acts to increase the intrinsic excitability of CA1 pyramidal cells via activation of muscarinic (G-protein coupled) ACh receptors (mAChRs). Activation of mAChRs leads to the inhibition of certain calcium-activated K^+-channels, resulting in a reduction in AHP amplitude and an increased AP output [39]. A similar neuromodulatory effect is seen with noradrenaline and histamine [40]. ACh also modulates synaptic excitability at glutamatergic CA3-CA1 synapses via activation of presynaptic mAChRs, which decreases presynaptic release probability and reduces synaptic signaling strength [41].

The nature of neuromodulation can be either intrinsic or extrinsic depending on where and how the particular neuromodulator is released. Intrinsic modulation occurs in the case where the source of the neuromodulator is a cell residing within the target circuit, whereas extrinsic modulation occurs when the source is external to the target circuit [36]. The effect of a neuromodulator can be highly local, e.g. confined to a single synapse, or broadcasted widely via non-synaptic (volume) transmission. In the latter case,

its action often depends on the distribution and affinity of the specific receptor in the downstream neuronal population. Neuromodulation can also be temporally diverse, occurring at multiple time scales simultaneously from milliseconds to hours [42].

The number and diversity of neuromodulators found in the brain is immense (examples given in Table 1), ranging from different monoamines and neuropeptides to glial transmitters, hormones and cytokines. Much has been learnt about their influence on neuronal activity from molecular level to circuit function. However, whereas most studies have focused on the action of individual neuromodulators in various systems, neuromodulation *in vivo* is likely to involve multiple substances acting simultaneously. How their collective influence, and interactions, impact neuronal function is one aspect of neuromodulation that has yet to be revealed.

Table 1. *Examples of common neuromodulators, of different classes, found in the brain.*

Biogenic amines + Acetylcholine	Amino acids	Neurosteroids	Purines	Neuropeptides	Gases	Cytokines
Noradrenaline	Glutamate	Cortisol	ATP	Cholecystokinin	Nitric oxide	TNF-α
Histamine	GABA	Progesterone	Adenosine	Oxytocin	Carbon monoxide	IL-1β
Serotonin	Glycine	Estrogen		Vasopressin		
Dopamine	Aspartate			Orexin		
Acetylcholine				Substance P		

Gamma oscillations

Rhythmic neuronal activity, also known as neuronal oscillations, is observed across many brain areas at frequencies ranging from zero to several hundred hertz [43-47]. Gamma oscillations (30-80 Hz) are fast network rhythms associated with higher cognitive functions such as sensory binding [48],

memory [49], visual attention [50] and consciousness [51]. In the hippocampus, they typically occur superimposed on slower theta oscillations (4-10 Hz) in animals engaged in exploratory behavior [52]. The firing of neurons with respect to the gamma cycle is thought to provide a plausible mechanism for information coding in neuronal networks [53, 54].

Gamma oscillations are critically dependent on fast synaptic inhibition [55, 56]. In the hippocampus and neocortex, phasic inhibition is provided by a heterogeneous population of GABAergic interneurons with different functional properties. Several lines of evidence suggest that FS interneurons are especially important in the generation and maintenance of gamma rhythms. For example, these cells fire phase-locked to gamma oscillations both *in vivo* [57] and *in vitro* [58] and display electrical membrane resonance at gamma frequencies [59]. It has also been shown that optogenetic activation of FS interneurons induces gamma oscillations in the rat cortex *in vivo* [60]. A number of properties make these interneurons particularly well suited to synchronize AP firing of pyramidal cells. By targeting the soma and proximal dendrites their synapses exert powerful influence over action potential firing in pyramidal cells. Moreover, FS interneurons are electrically coupled via dendritic gap junctions [6, 61] that allow rapid and efficient spread of membrane potential changes among interconnected cells and increases the probability of them firing synchronously [62]. FS interneurons also innervate themselves via strong autaptic synapses that have been shown to additionally contribute to synchronize AP firing [63, 64].

Two mechanistically different models are used to explain the generation of gamma oscillations (Fig. 3), differing in their dependence on inhibitory and excitatory synaptic transmission. In the ING (interneuron network gamma) model, mutual inhibition between interneurons in an interconnected network

is alone sufficient to establish gamma rhythmicity. This is supported by studies in *in vitro* brain slices showing that gamma oscillations, induced from tonic excitation of interneurons, are not abolished by blocking excitatory synaptic transmission [65, 66]. The minimum requirement for gamma oscillations thus appears to be: (i) tonic driven firing, (ii) mutual synaptic connectivity among interneurons in a network and (iii) a postsynaptic conductance with a decay constant of ~30 milliseconds (such as the $GABA_A$ receptor-mediated conductance). In the PING (pyramidal cell-interneuron network gamma) model, pyramidal cell activity drives the recruitment of FS interneurons that in turn pace firing in the pyramidal cell population via feedback inhibition. *In vivo* recordings show that pyramidal cells fire synchronously at lower frequencies during gamma oscillations and that their activity precedes FS interneuron firing [67], consistent with a PING mechanism. Further evidence supporting the involvement of phasic excitation includes the observation that selective ablation of AMPA receptors on FS interneurons weakens gamma oscillations *in vitro* [68].

Network gamma oscillations can be induced in acute hippocampal brain slices by application of a depolarizing agent, which have proved to be a valuable model system for studying their mechanisms *in vitro*. Commonly used depolarizing agents include kainate [69], carbachol [70] an metabotropic glutamate receptor agonist [71, 72]. Depending on the induction method, the evoked oscillations differ in their dependence on phasic excitation and inhibition. For example, kainate-induced gamma oscillations in CA3 stratum pyramidale are entirely blocked by the $GABA_A$ receptor antagonist bicuculline, but unaffected by AMPA receptor blockers [66]. Carbachol-induced gamma oscillations are likewise blocked by $GABA_A$ receptor antagonists, but notably also inhibited by AMPA receptor antagonists [70, 73]. Because hippocampal network oscillations are known to be promoted by

acetylcholine *in vivo* [74, 75], brain slice oscillations induced by cholinergic agonists may more accurately model properties of gamma rhythms in intact brains.
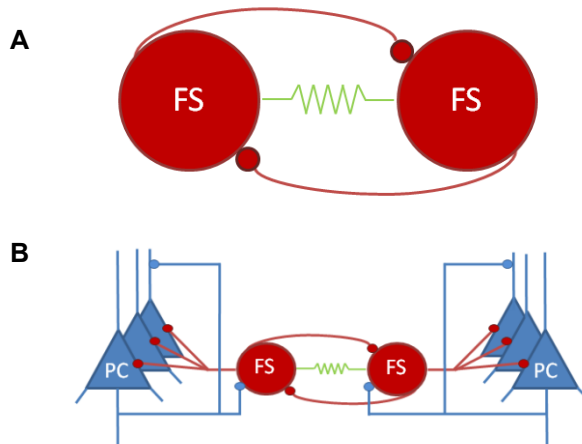
Figure 3. Two different working models of gamma oscillations. *(A)* ING model. In an interconnected network of fast-spiking (FS) interneurons reciprocal inhibition can be sufficient to generate gamma oscillations. *(B)* PING model. Pyramidal cells drive excitation of FS interneurons that, via feedback inhibition, pace action potential output in the population. Electrical gap junctions (green) contribute in synchronizing AP firing among FS interneurons.

AIMS

How neuromodulators distributed in brain extracellular fluid collectively impact the activity of central neurons *in vivo* is not known. The general aim of this thesis work was to elaborate on this issue by examining the effect of human CSF (hCSF) on hippocampal cellular- and network activity. The following specific questions were addressed:

    I.   How does hCSF affect intrinsic and synaptic properties
                of excitatory pyramidal cells? (paper I)

   II.   Are GABAergic interneurons modulated by hCSF?
                (paper II)

III.   Does hCSF influence oscillatory network activity?
                (paper III)

Methodological considerations

The purpose of this section is to reflect on practical and theoretical aspects of the experimental methodology used in the papers comprising this thesis. Detailed information on specific methodological procedures associated with each study can be found in the methods section of papers I, II and III.

The hippocampal brain slice preparation as experimental model

Electrophysiological studies in acute brain slices from the rodent hippocampus were first initiated in the early 1970s [76]. The hippocampal brain slice preparation has since then remained a major experimental model in neuroscience to study molecular, cellular and network mechanisms *in vitro*. There are a number of reasons for the wide experimental use of this preparation. To begin with, the intrinsic lamellar organization of the hippocampus [77] allows for functional examination of preserved connections within the hippocampal circuitry. Moreover, as compared to most other *in vitro* approaches (e.g. neuronal cultures), the preserved neuronal architecture within the brain slice offers a higher degree of transferability of experimental data. Another major advantage of the brain slice preparation is the level of experimental manipulation it offers the

researcher. In absence of an intact blood-brain barrier, the extracellular environment of brain slice neurons can be easily modified by introducing drugs and other chemicals to the perfusion, allowing neuronal function to be studied at multiple levels.

In terms of limitations, the preparation of hippocampal brain slices is associated with significant loss of neuronal input, as the tissue is cut and isolated from the brain. In addition, the cutting procedure itself causes severe trauma to the tissue and may induce inflammatory responses whose impact on neuronal activity in the slice is often not considered. Another notable limitation when studying neuronal function in brain slices (or neuronal cultures) is the fact that neurons are kept in artificial extracellular fluid. Thus, effects of organic substances, such as neuromodulators and neurotrophic factors, found in brain extracellular fluid *in vivo* are not appreciated.

The whole-cell patch clamp method

The patch clamp technique, first introduced by Neher and Sakmann in 1976 [78], allows small voltages and currents (resulting from the movement of ions through channels in the neuronal membrane) to be recorded from individual cells. A glass micropipette (tip diameter ~ 1-2 μm) containing a silver chloride electrode is filled with an intracellular solution and positioned against the somatic membrane of a single neuron. A slight negative suction is then applied to the micropipette, forcing the neuronal membrane up the tip of the micropipette and creating a high resistance seal (giga seal). To obtain a whole-cell patch clamp configuration, the neuronal membrane is subsequently ruptured by applying a brief voltage pulse (zap) to the membrane patch. This procedure establishes a direct contact between the cell

interior and the intracellular solution of the micropipette, allowing electric current and voltage to be recorded from, as well as applied to, the cell.

A major advantage with the whole-cell patch clamp technique is the ability to study neuronal function under conditions where most intrinsic and synaptic membrane conductances are maintained, and thus able to interact with one another. This allows experimenters to study how a particular neuron transforms input to output under various experimental conditions, and to examine the influence of individual channels and receptors in this process. Drugs and chemicals can also be applied to the intracellular micropipette solution to examine multiple intracellular parameters, such as specific signaling pathways, second messenger systems and protein transport. Moreover, cell morphology can be studied in real time or post hoc if a fluorescent dye is added to the intracellular micropipette solution and distributed throughout the cell interior. Multiple neurons can also be patched simultaneously to sample morphological and functional parameters of their mutual connections.

Although the fluid bridge between the cell interior and the micropipette (intracellular solution) is necessary to record neuronal activity, and provides control over the intracellular chemical environment, this is also a major drawback of the whole-cell patch clamp method. Since the volume of the intracellular solution in the micropipette is typically much larger than that of the cytosol (cell interior), critical intracellular constituents of the cell are "washed out" (replaced) by the micropipette solution in a time-dependent manner after access to the cell is obtained. As a consequence, over the course of an experiment the researcher may observe a rundown effect on a particular parameter over time, e.g. the amplitude of a membrane current or general excitability of the recorded neuron.

Some technical pitfalls and errors are also associated with whole-cell patch clamping. Effective voltage clamping of a cell rests on the assumption that the series resistance is at least two orders of magnitude lower than the input resistance, and that the cell being patched is spherical. Under such circumstances, the command voltage (delivered at the pipette tip) is expected to approximately equal the membrane voltage. However, because neurons are in fact highly aspherical cells and series resistance is often only one order of magnitude lower than the input resistance in typical recordings, voltage clamping becomes less effective. One consequence of this is poor "space clamping", i.e. distal parts of the membrane will be poorly voltage clamped as the potential drops significantly over the axial resistance provided by dendrites and axons. The other consequence is voltage (point) clamp error due to voltage drop across the series resistance. This error can, however, be reduced through the process of series resistance compensation. Yet another problematic factor during whole-cell recordings can be pipette offset (DC) drift. This means that the initial pipette potential relative to ground changes over time, leading to "false" depolarization or hyperpolarization of the neuronal membrane depending on the polarity of drift.

Extracellular recordings

Current flow across the membranes of neurons creates transient electrical field potentials that can be registered by placing a recording electrode in the extracellular medium. The field potential signal derives from the sum of all current flow occurring across the membranes of nearby neurons and thus consists of multiple neuronal events distributed over the entire membrane. Because synaptic events, i.e. excitatory and inhibitory postsynaptic potentials, are relatively slow but frequent they are often considered the most

important contributors to the field potential [79]. A field potential can either be evoked by electric stimulation of afferent axons (as in paper I) or result from spontaneous activity in a neuronal population. In brain slices, spontaneous neuronal activity is normally sparse but can be increased by modifying the chemical composition of the extracellular fluid. This is typically done in *in vitro* brain slice investigations of oscillatory neuronal activity, where network excitability is increased by application of a chemical agent to yield spontaneous local field potential (LFP) oscillations (see paper III). An advantage with extracellular recordings is that they are typically less technically challenging and time consuming compared to patch clamp techniques.

Artificial and human cerebrospinal fluid

In the present thesis, artificial CSF (aCSF) was used throughout as control when studying the neuromodulatory effects of human CSF (hCSF) on various aspects of neuronal activity. However, other factors than neuromodulation, such as electrolyte distribution, glucose content, pH, osmolality and temperature can affect the excitability on hippocampal neurons if differing between artificial and human CSF. To minimize the risk of such factors (artefacts) influencing the experimental outcome, effort was made to carefully match electrolyte concentrations (Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+}), pH, osmolality, glucose and temperature as closely as possible in each experiment. This was done by repeatedly measuring these variables in every batch of hCSF and then designing an aCSF based on those variables.

In the case of Ca^{2+} and Mg^{2+}, a fraction of these ions (with respect to total concentration) is thought to be complex-bound to organic anions in hCSF. Thus, the ionized (biologically active) concentration will be somewhat lower

than the measured total concentration. Because the exact percentage of ionized Ca^{2+} and Mg^{2+} in hCSF is not well established, varying between 50-90% in studies [80, 81], obtaining highly matched concentrations of these ions between aCSF and hCSF is not trivial. Furthermore, the Ca^{2+}/Mg^{2+}-binding capacity of artificial and hCSF could potentially differ.

An additional component that critically influences neuronal activity and viability in the brain slice is tissue oxygenation [82]. The solubility and retention of oxygen in artificial and hCSF could, if differing sufficiently, potentially impact recordings.

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Paper I

In the first paper we show that hCSF strongly increases the excitability of pyramidal cells in hippocampal (and neocortical) brain slices. As compared to a matched aCSF, hCSF powerfully increased spontaneous firing and depolarized V_{rest} in CA1 pyramidal cells (**Fig. 1A-C**). When current-clamped at -70 mV, hCSF increased the sEPSP frequency onto these cells, indicating increased spontaneous firing also in CA3 pyramidal cells, as well the membrane potential coefficient of variation (**Fig. 1D-G**). This was accompanied by a ~20% decrease in R_{in}, suggesting an hCSF-mediated increase of inward current.

Frequency-current (F-I) plots showed that hCSF lowered the AP threshold by ~5 mV in CA1 pyramidal cells (**Fig. 2 A-C**), reduced the rheobase and caused a left-shift in the input-output relationship (**Fig. 2G-J**). No effect was observed on the slope (gain). Control experiments confirmed that there was no increase in excitability over time in whole-cell recordings. Rather, there was tendency towards rundown of AP frequency over time (**Fig. 2I**).

We found the above effects of hCSF (except for R_{in}) to be dependent on G-protein coupled receptor signaling, as excitability-increasing effects were occluded when GTPγS (a non-hydrolysable GTP analogue) was intracellularly applied through the patch pipette (**Fig. 3**). This strongly suggests that hCSF effects were highly specific and resulted from neuromodulation of multiple intrinsic conductances. A change in AP threshold can occur through various modulatory mechanisms, e.g. via targeting of voltage-gated calcium or sodium channels in the axon initial segment [83, 84].

In voltage-clamp recordings we found that hCSF strongly increased the frequency, but not amplitude, of AMPA receptor-mediated sEPSCs onto CA1 pyramidal cells (**Fig. 4A-C**). This was not accompanied by an increased frequency of GABA$_A$ receptor-mediated sIPSCs. In fact, we found a decreased sIPSC amplitude in hCSF (**Fig. 4D-F**). This suggests that hCSF differentially affects glutamatergic and GABAergic neurons, promoting spontaneous excitatory, but inhibitory, synaptic transmission onto CA1 pyramidal cells.

We proceeded to examine synaptic effects of hCSF by recording evoked fEPSPs at CA3-CA1 synapses in stratum radiatum. A powerful potentiation of fEPSP magnitude was seen in hCSF, which was accompanied by a small but significant increase in the fiber volley (**Fig. 5A-B**). A significant decrease in the paired-pulse ratio indicated that hCSF potentiated glutamatergic synaptic transmission through an increase in release probability (**Fig. 5C**). When dialyzed hCSF (devoid of all substances ≤ 8 kDa in size) was introduced following a 15 minute recording period in control hCSF, the fEPSP magnitude decreased substantially while the paired-pulse ratio was reversed to baseline (**Fig. 5D**, **E**). This indicates that small organic

substances, such as peptides or monoamines, were responsible for the effects of hCSF, consistent with the biochemical profile of most neuromodulators.

We conclude that hCSF induces *in vivo*-like properties in hippocampal and neocortical pyramidal cells that likely results from neuromodulation of multiple intrinsic and synaptic conductances. The findings may help explain observed differences in activity of neurons *in vivo* and *in vitro* [33].

Paper II

In this study we explore the influence of hCSF on the activity of GABAergic CA1 hippocampal interneurons. Over 20 distinct types of interneurons have been documented in this area [4, 5]. We found that resident interneurons whose somata bordered stratum pyramidale/stratum oriens showed either (FS) fast-spiking or non-fast-spiking (NFS) phenotype, and were functionally classified accordingly (**Fig. 1** and **Table 1**). Since recordings in this study were performed at higher temperature (32-34°C), we chose to also reexamine the previously described effects of hCSF on CA1 pyramidal cells (recorded at room temperature in (paper I).

In current-clamp recordings at V_{rest} (I=0), hCSF increased spontaneous AP firing in both FS and NFS interneurons, without significantly affecting V_{rest} itself (**Fig. 2**). This finding appeared inconsistent with the previous result that sIPSC frequency in CA1 pyramidal cells was not increased by hCSF (in paper I, **Fig. 4D-F**). This could be explained by evidence suggesting that miniature IPSCs (mIPSCs), rather than AP-dependent IPSCs, constitute the major component of sIPSCs onto CA1 pyramidal cells [85]. In addition, it is possible that spontaneous AP firing is decreased in other types of GABAergic interneurons innervating CA1 pyramidal cells.

We next examined whether changes in spontaneous AP properties could underlie the increased spontaneous firing of FS and NFS interneurons in hCSF (**Fig. 3**). We found that hCSF decreased fAHP amplitude in FS interneurons (**Fig. 3A**, **F**), and mAHP amplitude in NFS interneurons (**Fig. 3G**, **M**), providing a plausible mechanism for increased spontaneous firing. Several neuromodulators (e.g. noradrenaline, histamine and serotonin) are known to regulate firing by targeting conductances that contribute to the AHP, including I_{AHP}, I_h, I_m and I_{NaP} [40, 86-89].

As seen in **Fig. 4**, hCSF increased the frequency of sEPSPs in current-clamp recordings at -70 mV, proving an additional mechanism that could contribute to the increased spontaneous firing in FS and NFS interneurons. This effect was more notable in FS interneurons, suggesting that they may be preferentially recruited by CA1 and CA3 pyramidal cells (cf. **Fig. 4A**, **B** and **C**, **D**).

In F-I experiments hCSF lowered the rheobase and left-shifted the input-output function in both FS and NFS interneurons (**Fig. 5A-C**, **E-G**). In FS cells, this was accompanied by a significant decrease in input-output gain (**Fig. 5B**). hCSF lowered the AP threshold in both FS and NFS interneurons in these experiments (**Fig. 5D**, **H**). At V_{rest}, however, hCSF only lowered the threshold of spontaneous APs in NFS interneurons (cf. **Fig. 3B**, **C** and **H**, **I**). This could relate to the differences in V_{rest} observed for FS and NFS interneurons (**Fig. 2**). Considering a mechanism of threshold regulation involving hCSF potentiation of I_h [90], a stronger activation of this current would be expected at a more hyperpolarized V_{rest}. This could in turn lead to a stronger activation of subthreshold I_{NaP} [91] and an increased rate of depolarization.

In experiments where sinusoidal current waveforms were injected at theta (5 Hz) frequency, hCSF increased the responsiveness (AP output) of both FS and NFS interneurons (**Fig. 6A**, **B** and **E**, **F**). At gamma (40 Hz) frequencies, FS interneurons were more prone to fire than NFS interneurons (**Fig. 6C**, **D**). These results extend the finding that hCSF modulates the input-output function of FS and NFS interneurons to include more physiologically relevant forms of input stimulus.

In the case of CA1 pyramidal cells, we confirm our previous findings at room temperature and show that hCSF strongly increases their excitability (**Fig. 8**). We add the finding that hCSF reduces the AHP amplitude (**Fig. 8D**, **I**) and increased responsiveness to sinusoidal current waves in theta and gamma frequency range also in CA1 pyramidal cells. One inconsistency with respect to paper I was that we did not observe a decreased R_{in} in of CA1 pyramidal cells in hCSF at 32-34°C.

We conclude that hCSF potently enhances the excitability of both FS and NFS hippocampal CA1 interneurons, increasing their responsiveness to excitatory input. An even stronger excitation was observed in CA1 pyramidal cells, suggesting that hCSF would promote network oscillatory activity through efficient recruitment of FS interneurons, in turn synchronizing the firing of both pyramidal cells and other interneurons [7, 92].

Paper III

In paper III we studied the influence of hCSF on fast network oscillations in CA3 stratum pyramidale. We first show that hCSF strongly increases the power of kainate-induced gamma oscillations in the (**Fig**. **1a-c**). Several

different mechanisms contribute to field oscillations power. The balance of excitatory and inhibitory synaptic transmission, number of participating neurons and the degree of AP synchronization in the population can regulate gamma power in the hippocampal slice [55, 70, 93]. It is likely that the excitability-increasing effect of hCSF on both interneurons and pyramidal cells (papers I and II) results in increased numbers of neurons participating in the gamma oscillation.

Next we show that hCSF is alone sufficient to generate spontaneous gamma oscillations in the brain slice (**Fig. 2a-c**). This strongly suggests that hCSF increases the degree of AP synchronization in the CA3 network. Tonic excitation of CA3 pyramidal cells by hCSF could in theory be sufficient to induce spontaneous gamma via enhanced recruitment of FS interneurons that in turn synchronize the pyramidal cell population [7, 56]. However, additional mechanisms could contribute to enhance synchrony, such as modulation of ionic conductances involved in the regulation of AP timing in pyramidal cells, e.g. I_h (h-current) and I_m (m-current) [93-95]. The power of hCSF-induced gamma oscillations varied in between experiments but was consistently lower than typically seen with 100 nM kainate [66, 96]. It can however be questioned to what extent the field CA3 gamma power obtained with typical induction protocols *in vitro* is physiologically relevant.

We proceeded to test whether muscarinic acetylcholine (ACh) receptor (mAChR) activation was required in hCSF-induced gamma oscillations. Application of atropine (5 μM) entirely abolished the spontaneous gamma oscillations (**Fig. 3a-c**), suggesting a crucial role of mAChRs. This result raises the question of whether hCSF-induced gamma oscillations may simply be a weaker form of cholinergic induction, or if other neuromodulators or factors in hCSF are required to induce this activity.

In whole-cell recordings from CA1 pyramidal cells we found that hCSF enhanced two forms of electrical theta resonance known to be mediated by I_h (H-resonance, **Fig**. **4**) and I_m (M-resonance, **Fig**. **5**) at hyperpolarized and depolarized membrane potentials, respectively [97]. hCSF increased the strength and frequency of both H-resonance (**Fig**. **4c**, **d**) and M-resonance (**Fig**. **5c**, **d**). Voltage-clamp recordings further indicated that hCSF caused potentiation of I_h (**Fig**. **4g**, **i**) and I_m (**Fig**. **5g**, **h**), which likely explains the effects on theta resonance. This conclusion is consistent with previous work showing that resonance frequency and strength increases with increased activation of the resonating conductance [97, 98]. We found no evidence of gamma frequency resonance in pyramidal cells (**Fig**. **4e**, **f** and **Fig. 5e, f**), in accordance with previous studies [59, 99] and consistent with intrinsic membrane properties of these cells.

We conclude that hCSF promotes fast oscillatory activity in the hippocampus involving a cholinergic mAChR-dependent mechanism, and that potentiation of I_h and I_m in pyramidal cells provide plausible mechanisms contributing to this effect. The enhancement of pyramidal cell theta resonance suggests that hCSF may also support theta rhythmicity, which should be addressed in future work. Our findings that hCSF induces spontaneous gamma oscillations in hippocampal brain slices opens up the possibility to study mechanisms of these oscillations under conditions of higher physiological relevance.

GENERAL DISCUSSION

Significance

This thesis work describes how ambient neuromodulators in brain extracellular fluid are likely to collectively influence neuronal activity *in vivo* at cellular and network level. Large efforts in neuroscience are currently being aimed at constructing highly accurate models of neuronal circuits, incorporating extensive amounts of experimental anatomical and physiological data, to advance our current understanding of the brain. Our findings could prove an important piece in this puzzle, providing a link between *in vivo* and *in vitro* experimental electrophysiological data.

Identity and mechanism of action of neuromodulators

Taken together, the findings in this thesis support the involvement of multiple neuromodulators and mechanisms of action in the effects of hCSF on hippocampal neuronal activity. The single most likely candidate to mediate these neuromodulatory effects is arguably acetylcholine (ACh). The reasoning behind this conclusion is the following: (1) Spontaneous network activity (gamma oscillations) induced by hCSF was abolished by application of the mAChR antagonist atropine (paper III). (2) ACh is known to reliably depolarize hippocampal pyramidal cells [88, 100] while effects on interneurons are heterogeneous [101, 102]. This could explain why CA1 pyramidal cells are (on average) depolarized by hCSF but not interneurons (paper II). (3) hCSF reduced the mAHP amplitude in both CA1 pyramidal cells and interneurons (paper II). mAChR activation reduces the mAHP in both hippocampal pyramidal cells [103, 104] and interneurons [105]. (4) ACh could explain the lowered AP threshold in hCSF in both pyramidal cells and interneurons. Pharmacological block of I_h (h-current) increases the AP threshold in CA1 pyramidal cells [106] and FS interneurons of the dentate gyrus [90], inversely indicating that potentiation of I_h could lower the threshold in the cells. Because hCSF appears to potentiate I_h (paper III), and such potentiation has been demonstrated by mAChR activation [88, 89], this is a plausible mechanism for the lowered AP threshold. In addition, cholinergic potentiation of I_h may also explain the above mentioned reduction in AHP amplitude caused by hCSF. (5) Anatomical and physiological evidence suggest that ACh acts mainly through volume transmission *in vivo* [102, 107, 108]. This makes it plausible that ACh would withstand degradation relatively well and thus remain biologically active in hCSF.

Although there is good evidence to suggest a key role of ACh in effects of hCSF, a number of observations are inconsistent with cholinergic modulation. For example, our findings in paper III suggest that hCSF, in addition to I_h, also potentiated I_m (m-current) in CA1 pyramidal cells. However, one of the most well recognized effects of mAChR activation in CA1 pyramidal cells is inhibition of I_m (thereof the name m-current) [87, 109]. Moreover, we observed substantial potentiation of evoked glutamatergic transmission at CA3-CA1 synapses by hCSF (paper I), that appeared to result from an increased presynaptic release probability at these synapses. In contrast, presynaptic mAChR activation has been shown to reduce release probability at these synapses [41, 110]. Cholinergic modulation is also known to increase cellular R_{in} [100, 111], which is inconsistent with our observations in papers I and II.

In addition to ACh, several other classical transmitters (e.g. dopamine, serotonin, noradrenaline and histamine) negatively modulate the AHP and other intrinsic properties of CA1 pyramidal cells [40, 86, 112], and could contribute to the effects of hCSF. A potential involvement of neuropeptides should also be considered. In fact, the neuropeptide somatostatin is one of few documented positive modulators of I_m in pyramidal cells [113]. Several neuropeptides appear to act by enhancing the effects of other neuromodulators such as ACh [114, 115]. They could thus play a permissive role in the effects of hCSF, as ACh may not be present at sufficient concentration to significantly modulate neuronal activity. Many neuropeptides are, however, themselves capable of strongly exciting hippocampal neurons. For example, cholecystokinin and vasoactive intestinal polypeptide both depolarize CA1 pyramidal cells and increase their excitability [116, 117], in agreement with effects of hCSF.

Origin of neuromodulators

It is evident from this thesis work that neuromodulators in hCSF shape multiple aspects of hippocampal neuronal activity. But where do these substances in hCSF originate from? The traditional subcortical "diffuse" neuromodulatory systems of biogenic amines and acetylcholine (Table 1) innervate large areas of the brain and are prominent in the neocortex and hippocampus [118, 119]. In the case of ACh, cholinergic projections from the nucleus basalis and medial septum/diagonal band of Broca provide the main source of release in the neocortex and hippocampus, respectively [120]. Subcortical neuromodulatory systems are thought to operate largely via volume transmission, and could therefore distribute and persist in hCSF. The cortical levels of biogenic amines and acetylcholine are high during wakefulness [118, 119, 121] and may thus strongly influence the neuromodulatory composition of CSF during daytime, which is also when CSF samples were taken from patients and healthy volunteers.

Another possible source of CSF neuromodulators could be the CSF-contacting neurons located in periventricular brain areas. These neurons have varicose neuromodulatory fibers that terminate directly in ventricular and subarachnoid CSF [122, 123], suggesting that they may be specialized to convey neuromodulatory messages via CSF volume transmission.

Some neuromodulators in CSF could also derive from outside of the CNS and be transported to the brain via the circulation. Although the blood-brain and blood-CSF barriers largely restrict the movement of blood-borne substances into the CNS, there is evidence that specific transport mechanisms exist for certain neuromodulators that may facilitate their entry into the CSF

[24, 124]. In addition, the choroid plexa are known to secrete a number of neuroactive substances into ventricular CSF [124, 125].

Practical and theoretical considerations regarding hCSF

The use of hCSF throughout this thesis work has posed some practical and theoretical questions. For example, could effects of CSF on neurons be species dependent? Would rat CSF influence hippocampal neurons similarly to hCSF? We attempted to address this question in the first paper by sampling CSF from cisterna magna of rats. Unfortunately, the small extractable CSF volume each rat (~150 μl) did not allow for proper electrophysiological experimentation. However, there are multiple reasons to expect that rat CSF neuromodulators would similarly boost neuronal activity in the brain slice.

Another relevant question is whether the sampling location may influence the effect of hCSF on neuronal activity, i.e. does the neuromodulatory composition of hCSF differ between brain and spinal compartments? The hCSF used in this thesis project was consistently sampled from the lumbar subarachnoid space, and could in theory differ somewhat from cerebral hCSF sampled e.g. intraventricularly. It is also possible that certain neuromodulators could have been degraded or inactivated prior to the time of experimentation, e.g. from freezing and thawing of hCSF samples.

Implications for brain slice electrophysiology

Electrophysiological recordings from *in vitro* brain slice preparations have provided invaluable insight to neuronal function over the last decades. One notable limitation, however, in this experimental approach is that neurons are kept in an artificial extracellular environment. Our findings with hCSF have shown how 'lack of neuromodulation' (i.e. use of aCSF) influences neuronal activity in hippocampal brain slices. First, use of aCSF (of typical composition) reduces both intrinsic and synaptic excitability of neurons and contribute, in addition to the reduced synaptic connectivity causes by slicing, to neuronal quiescence. Second, the excitation/inhibition (E/I) ratio of synaptic transmission is lowered by use of aCSF, which likely contribute to reduce spontaneous firing in pyramidal cells. Third, our measurements of electrolyte concentrations in hCSF show that extracellular Ca^{2+} levels are low, the measured total concentration ranging from 1.1-1.2 mM. However, most studies typically use 2 mM Ca^{2+} in their aCSF. Modifying extracellular Ca^{2+} will affect several neuronal properties, most notable presynaptic release probability [126] and intrinsic membrane excitability [127]. Lowering Ca^{2+} will negatively modulate release probability but increase intrinsic excitability and spontaneous firing in neurons.

CONCLUSIONS

I.   hCSF strongly increases the excitability of hippocampal and neocortical pyramidal cells. CA1 pyramidal cells in hCSF show *in vivo*-like functional properties.

II.   hCSF increases the responsiveness of both fast-spiking and non-fast-spiking GABAergic CA1 interneurons to excitatory input and enhances their spontaneous firing.

III.   hCSF promotes fast spontaneous (gamma) network activity in CA3 stratum pyramidale via a mAChR-dependent mechanism and enhance electrical resonance behavior in CA1 pyramidal cells via positive modulation of I_h and I_m.

Our findings with hCSF suggest that ambient neuromodulators in brain extracellular fluid promote spontaneous activity in cortical networks and significantly contribute in shaping neuronal function *in vivo*.

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