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

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Abbreviations

CSF – Cerebrospinal fluid
hCSF – Human cerebrospinal fluid
aCSF – Artificial cerebrospinal fluid
CA – Cornu ammonis
CaSR – Calcium sensing receptor
NALCN – Sodium leakchannel non-selective
RMP – Resting membrane potential

EPSP – Excitatory postsynaptic potential
IPSP – Inhibitory postsynaptic potential
EPSC – Excitatory postsynaptic current
IPSC – Inhibitory postsynaptic current
AMPAR - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
GABAR - γ-Aminobutyric acid receptor
CNQX – 6-Cyano-nitroquinoxaline-2,3-dione
LTP – Long-term potentiation
PTX – Picrotoxin
PPR –Paired pulse ratio

Abstract

It is well known that the extracellular concentration of calcium affects neuronal excitability and synaptic transmission. It is less well known what the physiological concentration of extracellular calcium is in the brain. In electrophysiological brain slice experiments, the artificial cerebrospinal fluid (aCSF) traditionally contains relatively high concentrations (2-4 mM) of calcium to support synaptic transmission and suppress neuronal excitability.
Using an ion-selective electrode, we determined the fraction of ionized calcium in healthy human cerebrospinal fluid (hCSF) to about 1.0 mM of a total concentration of 1.2 mM (86%). We then compared the effects of this physiological concentration of calcium with the commonly used 2 mM on neuronal excitability, synaptic transmission and long-term potentiation (LTP) using patch-clamp- and extracellular recordings in the CA1 region in acute slices of rat hippocampus.

Increasing the total extracellular calcium concentration from 1.2 to 2 mM decreased spontaneous action potential firing, induced a depolarization of the threshold and increased rate of both depolarization and repolarization of the action potential. Evoked synaptic transmission was approximately doubled with a balanced effect between inhibition and excitation. LTP induced with high-frequency stimulation during blockade of GABAergic inhibition was negligible at 1.2 mM calcium, whereas a prominent LTP was observed at 2 or 4 mM calcium.
In conclusion, an increase from the physiological 1.2 mM to 2 mM calcium in the aCSF has striking effects on neuronal excitability, synaptic transmission and the induction of LTP.

Introduction

In brain extracellular fluid, the concentration of ionized (physiologically active) calcium is an important parameter regulating the excitability of central neurons (Frankenhaeuser & Hodgkin, 1957). Yet, the concentration of ionized calcium in normal cerebrospinal fluid (CSF) is not well established. Since the 1950s, ionized calcium in CSF has been reported to account for between 50–96% of the total concentration in different studies (Goldstein, Romoff, Bogin, & Massry, 1979; Pedersen, 1971; Robertson & Marshall, 1981; Schaer, 1974). As in serum, calcium ions in CSF exist in three forms: ionized (Ca^{2+}), protein-bound and complexed with other ions. These fractions together make up the total calcium concentration (Franklin C. McLean, 1934). The main binder of calcium in plasma is albumin. However, the concentration of albumin and other proteins are about 200 times lower in CSF compared to plasma (M. B. Segal, 1993). Moreover, it is not clear how large the protein-bound fraction of calcium is in CSF. The complex-bound fraction of calcium in hCSF could possibly be larger than the protein-bound fraction since at least bicarbonate exists in a concentration comparable to the concentration in plasma. Bicarbonate is the main pH-buffering agent in hCSF, and it is present at a much higher concentration than any other inorganic molecules capable of binding calcium. Therefore, one can assume that bicarbonate plays a major part in binding calcium in hCSF (Schaer, 1974). Citrate and other intermediates in the Krebs cycle exist in about the same concentrations in hCSF as in plasma (Hoffmann et al., 1993). Lactate does not have the same binding capacity as citrate but dominate the organic anion content of hCSF in terms of concentration. The formation of complex-bound calcium is pH-dependent (Toffaletti & Abrams, 1989), since protons compete with calcium ions for binding sites on organic/inorganic anions.

The influence of extracellular calcium on the excitability of neurons is diverse. The extracellular concentration of calcium potently regulates both synaptic (Dodge & Rahamimoff, 1967) and intrinsic (Frankenhaeuser & Hodgkin, 1957) properties of the neuron. Through a set of incompletely understood interactions with the neuronal membrane, calcium ions are thought to regulate the intrinsic excitability of neurons via a process known as charge screening (Hille, 1968; McLaughlin, Szabo, & Eisenman, 1971). Although this hypothesis is still used to explain the actions of divalent cations on membrane excitability, direct

experimental evidence of the underlying mechanisms has been scarce and alternative mechanisms, including a direct pore blocking action of the calcium ion om voltage-gated sodium channels, has been proposed (Armstrong, 1999; Armstrong & Cota, 1990, 1991, 1999).

The effects of calcium on synaptic excitability are somewhat better understood. Calcium is well known to positively regulate the probability of transmitter release (Dodge & Rahamimoff, 1967). In addition to this prominent release-promoting effect, due to an increased driving force of calcium entering the cell, extracellular calcium also negatively modulates evoked transmitter release, and positively modulates spontaneous transmitter release, via activation of the calcium sensing receptor (CaSR) (Phillips, Harnett, Chen, & Smith, 2008; Vyleta & Smith, 2011). Moreover, increasing extracellular calcium also indirectly decreases evoked synaptic transmission via a reduction of neuronal and axonal excitability, as described above. Thus, the net effect of a change in extracellular calcium is not easily predicted and it is unclear if excitatory and inhibitory synapses are equally affected, or if changes in the calcium concentration alters the balance between synaptic inhibition and excitation in the brain.

The induction of long-term potentiation (LTP) requires calcium influx through NMDA receptors (Lynch, Larson, Kelso, Barrionuevo, & Schottler, 1983; Malenka, Lancaster, & Zucker, 1992). Most, if not all, *in vitro* studies of LTP has been performed with calcium concentrations at, or above, 2 mM. Early *in vitro* studies of LTP showed that it was very difficult to induce LTP when the extracellular calcium concentration was lower than 2 mM (Dunwiddie & Lynch, 1979; Muller & Lynch, 1989), but if this difficulty persists when LTP induction is facilitated by blocking GABAergic inhibition has not been tested.

In this study we determine the concentration of ionized calcium in human cerebrospinal fluid (hCSF), and to characterize the effects of using physiological (~1.2 mM), as compared to standard (2 mM), aCSF calcium on intrinsic neuronal excitability, synaptic transmission, paired-pulse plasticity and long-term potentiation in CA1 pyramidal cells of the rat.

Methods

Human and artificial cerebrospinal fluid

Human cerebrospinal fluid was collected by lumbar puncture from healthy volunteers (20-51 years old). The samples were immediately centrifuged to separate the cells from the medium

and frozen to -80°C. The samples were thawed twice, first at the end of the sampling period in order to pool them and to take samples for analysis of total concentrations of electrolytes (sodium, potassium, calcium, magnesium and chloride) and glucose. The second thawing occurred when the hCSF was used for electrophysiological measurements. Glucose and electrolyte concentrations were determined by board-certified laboratory technicians at the Clinical Chemistry Laboratory at Sahlgrenska University Hospital using accredited methods with inter-assay coefficients of variation (CVs) below 2%. Sodium, potassium and chloride were measured using ion selective electrodes. Total calcium and magnesium concentrations were measured using colorimetric *o*-cresolphthalein and chlorophosphonazo III methods, respectively. Glucose concentration was measured using the hexokinase method.

The aCSF was mixed to match the hCSF with respect to the major ions (sodium, potassium and chloride). Small adjustments were made in order to balance the salts added. The content of the aCSF was (in mM): 124.2 NaCl, 2.79 KCl, 1.14 $MgCl_2$, 23 $NaHCO_3$, 0.4 NaH_2PO_4, 1.18 $CaCl_2$ and 3.66 glucose. For comparison of ionized fraction, a standard aCSF was also used, containing (in mM): 129 NaCl, 2.98 KCl, 1.14 $MgCl_2$, 26 $NaHCO_3$, 1.24 NaH_2PO_4, 1.18 $CaCl_2$ and 10 glucose (aCSF* in Fig. 1). In the last aCSF that was included, we used the matched aCSF with addition of 1.7 mM lactate, 0.3 mM citrate and 20mg/100mL albumin (aCSF** in Fig. 1).

Measurements of ionized calcium

The concentration of ionized calcium was measured with an ion-selective electrode (PerfectION ™ Combination Calcium Electrode, Mettler Toledo). The electrode was initially calibrated with a solution containing the same ionic content as hCSF (calcium excluded) to minimize the impact from possible interferences. Measurements were carried out in a standard aCSF, a matched aCSF, an aCSF with additional chelators and hCSF. Temperature and pH was simultaneously measured and the samples were continually stirred and bubbled (95 % O_2 and 5% CO_2 (AGA Gas AB, Lidingö, Sweden). After every series of measurements the electrode was tested in a solution of known concentration and if the value had drifted more than 3% the series was discarded and the electrode recalibrated.

Animals and slice preparation

Experiments were performed on hippocampal slices from 17 - 30 (mixed sex) day old Wistar rats. Animals were anaesthetized using Isofluran (Baxter Medical, Kista, Sweden) and decapitated. All animals were raised in our animal facility in compliance with ethical guidelines and approved by the animal ethics committee of Gothenburg, ethical permit 29-2014.

The brain was extracted and placed in a cooled (1-3°C) solution containing (in mM); 219 glycerol, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $CaCl_2$, 7 $MgCl_2$, 26 $NaHCO_3$ and 11 D-glucose under continuous supply of carboxygen (95% O_2, 5% CO_2). Parasagittal slices 300-400 µm thick from the dorsal hippocampus was cut with a vibratome and placed in a bath of aCSF (room temperature) containing (in mM): 129 NaCl, 20 $NaHCO_3$, 1.29 NaH_2PO_4, 3 KCl, 4 $MgCl_2$, 2 $CaCl_2$, 0.5 ascorbic acid, 3 myo-inositol, 4 L,D-lactic acid and 10 D-glucose under continuous supply of carboxygen (95% O_2, 5% CO_2) and allowed to rest for at least one hour. For a subset of the experiments the slices rested in a special recovery solution (32°C) for the first 15 minutes after cutting containing (in mM): 92 N-methyl-D-glucamine, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 2 thiourea, 5 ascorbate, 3 pyruvate, 0.5 $CaCl_2$, 8 $MgCl_2$, 25 glucose.

Whole cell recordings

Whole cell recordings were performed on pyramidal neurons of the CA1 area as previously described (Riebe & Hanse, 2012). In short, slices were submerged in the matched aCSF described above with the exception that the concentration of calcium varied as described in results. The aCSF was continuously supplied with carboxygen (95% O_2, 5% CO_2). A differential interference contrast microscopy (Nikon E600FN) together with a CCD camera (Sony XC-73CE) was used to visually identify the cells and to visualize the recording electrode connected to the neuron via a borosilicate glass micropipette (resistance 3-6 MΩ). The micropipette was filled with an intracellular solution containing (in mM); 127 K-gluconate, 8 KCl, 10 HEPES, 15 phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP (pH ~7.3 and osmolality 280–300 mOsm). The experiments with evoked potentials used a pipette solution containing (in mM): 130 Cs-methanesulfonate, 2 NaCl, 20 HEPES, 0.6 EGTA, 5 QX-314, 4 Mg-ATP, and 0.4 GTP (pH ~7.3 and osmolality 280–300 mOsm). The data was collected with a sampling frequency of 10 kHz and filtered at 3 kHz by an EPC-9 amplifier (HEKA Elektronik). After opening, the cell was allowed to rest for 5 minutes before recordings started. Series resistance was monitored using a 20 ms 10 mV hyperpolarizing pulse. The series resistance was not allowed to exceed 20 MΩ in whole-cell recordings, or to change

more than 20% during an experiment, otherwise the experiment was discarded. All whole-cell recordings were carried out at room temperature.

Recordings of spontaneous activity, input-output and action potential properties was carried out in current clamp. Recordings started in the matched aCSF with 2 mM calcium. First, a stepwise increasing current was injected in the cell (se Fig 3A, inset), then no current was injected and spontaneous activity was recorded for 3-6 minutes, depending on the stability of the recording. Next, the calcium concentration of the aCSF was decreased to 1 mM and after 10 minutes of wash in the protocol was repeated. Lastly, the calcium concentration was returned to 2 mM and the protocol was repeated a third time in order to control for effect over time.

Recordings of evoked and spontaneous postsynaptic currents was carried out in voltage clamp. Cells were patched in stratum pyramidale as described above and clamped at -70 mV for recordings of α-amino-3-hydroxy-5-methyl-4-Isoxazolepropionic acid receptor (AMPAR) mediated excitatory postsynaptic currents (EPSCs) and at 0 mV for recordings of γ-aminobutyric acid receptor (GABAR) mediated inhibitory postsynaptic currents (IPSCs). Schaffer collateral/commissural afferents were stimulated using 0.2 ms biphasic (negative / positive) constant current pulses (5–60 µA; STG 1002, Multi-Channel Systems, Reutlingen, Germany) delivered through an insulated tungsten microelectrode (resistance ~ 0.3-0.5 MΩ). Stimulation electrodes were positioned in the stratum radiatum, at least 100 µm from the recorded cell, and synaptic inputs received test pulse stimulation every 5 s.
After patching a cell and an initial recovery period of 10 min, experiments were initiated by recording evoked AMPAR EPSCs at -70 mV in aCSF containing either 1.2 mM or 2 mM calcium. Spontaneous EPSCs were then recorded for 5 min before repeating the measurements of evoked and spontaneous GABAR inhibitory postsynaptic currents (IPSCs) at 0 mV. The calcium concentration was then changed to 1.2 or 2 mM depending on the starting concentration and, following a recovery period of 5 min, subsequently the recording procedure of AMPAR EPSCs and GABAR IPSCs was repeated. Paired-pulse ratio was measured by means of two pulses spaced 5 ms apart and dividing the second pulse by the first. The amplitude of the second pulse was calculated by subtracting an extrapolation of the initial pulse generated by fitting a curvilinear regression. The number of experiments that were initiated in 1.2 mM calcium was matched to those initiated in 2 mM calcium. Evoked

and spontaneous responses were analyzed off-line using custom-made IGOR Pro (WaveMetrics, Lake Oswego, OR, USA) software.

Extracellular field recordings

During the extracellular field recordings the perfusion solution contained (in mM): 124 NaCl, 3 KCl, 1.2-4 $CaCl_2$, 1-4 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 D-glucose and 0.1 picrotoxin (PTX). All recordings were performed at a temperature of 30-32˚C under continuous supply of carboxygen (95% O_2, 5% CO_2). Slices were allowed to recover for 20 min once transferred to the recording chamber. Extracellular field recordings were made by means of a glass micropipette filled with 1 M NaCl (resistance ~2 MΩ) placed in the stratum radiatum. Data was sampled at 10 kHz using a Multiclamp 700B amplifier and converted digitally by means of a Digidata 1440A analog/digital converter (Axon Instruments Inc, USA) and used unfiltered to measure the slope of the field EPSP (fEPSP).

Electrical stimulation of Schaffer collateral afferents in stratum radiatum was applied to two inputs alternately at 0.2 Hz. Stimulation consisted of 200 µs constant current pulses generated by a stimulator (Model DS3, Digitimer Ltd, Letchworth Garden City, UK) and delivered through an insulated tungsten microelectrode (resistance ~0.5 MΩ). Stimulation intensity was set such that there were no signs of action potential activity on the fEPSP. Slopes for the fEPSP were determined as the k-value of a linear regression over eight data points. Following a baseline recorded at 0.2 Hz for 10 min, we induced LTP by five trains of 20 impulses at 100 Hz, in one of the two inputs and recorded for another 40 min. The second input was used as a control and recordings which drifted by more than 10 % in the control input were omitted.

Statistical analysis

All statistics were performed with GraphPad Prism 7.03 (GraphPad Software Inc, La Jolla, USA). Statistical tests and sample sizes are indicated for each figure in the figure legend and p-values are stated for each experiment in the results. Data is expressed as means ± SEM and where applicable individual data points are shown. Because of confines in sample size data is assumed to be normally distributed.

Results

Ionized calcium in normal cerebrospinal fluid

We first measured total concentrations of Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+} in the hCSF pool from healthy volunteers (see Table 1). The total concentration of calcium in hCSF was 1.18 ± 0.03 mM (n = 10, Fig. 1B). Using a calcium-selective electrode, measurements of ionized calcium were taken at pH 7.34–7.38 under continuous supply of carboxygen (95% O_2, 5% CO_2). We measured the concentration of ionized calcium to 1.00 ± 0.01 mM at room temperature (n = 10, Fig. 1A). In separate measurements from the same hCSF pool at 37°C ionized calcium was similar to that measured at room temperature (1.04 ± 0.01 mM, pH 7.26–7.34, n = 10, not shown). Based on measured concentrations of total and ionized calcium, the fraction of ionized calcium in hCSF was calculated to 85.6 ± 0.8%. Finally, we examined the sensitivity of ionized calcium to pH in a number of aCSF solutions, among which the measured pH varied between 7.3–7.5 (Fig. 1 A, C). The fraction of ionized calcium in aCSF was found to correlate well with pH (Fig. 1D).

Bicarbonate is largely responsible for binding calcium ions in cerebrospinal fluid

To investigate possible chelators of calcium in hCSF, we created an aCSF with addition of three substances we considered as possible chelators; albumin, citrate and lactate. Albumin and lactate are major anions in the hCSF. Citrate exist in much lower concentration but is a known strong binder of calcium (Franklin C. McLean, 1934). In order to determine if these three chelators produced an aCSF (aCSF** in Fig. 1) with a more physiological calcium binding capacity we measured ionized calcium and pH in this aCSF. We also investigated an aCSF with slightly elevated concentration of bicarbonate (aCSF* in Fig 1) to examine the importance of calcium binding to bicarbonate. The results show that the aCSF with lactate, citrate and albumin binds more calcium than hCSF does (78.8 ± 2.6 % free calcium compared to 85.6 ± 0.8% in hCSF, Fig. 1A). In the aCSF with extra bicarbonate, 86.1 ± 1.3 % of the calcium is in its free form (Fig. 1A). We conclude that bicarbonate is likely to be a major binder of calcium also in hCSF and there is no need to add any other anion to the aCSF from a calcium-binding perspective.

Extracellular calcium depresses excitability via a depolarization of the threshold

It is a well-known fact that extracellular calcium depresses neuronal excitability and several hypotheses have been presented to explain the mechanism(s) behind this effect (Jones & Smith, 2016). Using whole-cell recordings, we examined how intrinsic excitability in CA1 pyramidal neurons was affected by a change from an aCSF with high calcium concentration

(2 mM) to an aCSF with physiological calcium concentration (1.2 mM). We choose to compare these two concentrations since 2 mM is most widely used in the field of *in vitro* electrophysiology and 1.2 mM is close to the physiological concentration as measured in this study.

We first recorded spontaneous firing in hippocampal pyramidal neurons (Fig 2). As expected, a reduction of extracellular calcium from 2 to 1.2 mM increased the spontaneous firing rate dramatically from 1.2 ± 0.3 to 2.3 ± 0.2 Hz ($p < 0.01$). This increase was fully reversible when returning to the high calcium aCSF (1.0 ± 0.3 Hz, Fig. 2B-D). The data in Fig. 2D is shown normalized to the spontaneous firing frequency in high calcium aCSF and shows an increase to 235.1 ± 33.5 % in 1.2 mM calcium and a return to 72.3 ± 20.0 % when the 2 mM calcium aCSF was brought back.

Next, we asked how a change from high to physiological calcium affects the input/output function of the pyramidal neurons by using stepwise increasing current injections (800 ms, see Fig. 3A, inset) and record the resultant frequency of action potentials (Fig. 3A-D). The current-frequency plot in Fig. 3A displays a clear left-shift when extracellular calcium is decreased, corresponding to an increased excitability (more firing for a given current injection). The data is normalized to the maximum firing frequency of each cell. The gain (the slope between 25 and 75% of maximal frequency) of the F/I-plot is not affected by the change in calcium (0.090 ± 0.006 Hz/pA in 2 mM calcium aCSF, 0.089 ± 0.007 Hz/pA in 1.2 calcium aCSF, $p = 0.73$, Fig. 3E), nor is the maximal firing frequency (22.9 ± 1.05 Hz in 2 mM calcium aCSF and 23.3 ± 1.21 Hz in 1.2 mM calcium aCSF, $p = 0.67$, Fig. 3F). However, there seems to be an effect over time concerning the maximal firing frequency since there is a significant drop in last recordings. This effect over time can potentially mask an effect on maximal firing frequency in the recordings in physiological calcium. The rheobase (the smallest injected current that produced at least one action potential) in contrast, was significantly decreased in the lower calcium concentration, from 112.5 ± 6.4 pA in 2 mM Ca^{2+} to 75 ± 6.7 pA in 1.2 mM Ca^{2+} ($p < 0.0001$, Fig 3G) and his effect was fully reversible (116 ± 11.66 pA).

Extracellular calcium increases de- and repolarization rate of the action potential

A decreased rheobase suggests a decreased threshold for the action potential. To examine this, we selected the first action potential in the train triggered by an 800 ms depolarization at the smallest injected current that triggered action potential both in high and physiological calcium (Fig. 4A). We then created phase-plane plots from these action potentials by plotting the derivative (the rate of change) of the membrane potential (e.g. re- and depolarization velocity) against the membrane potential (Fig. 4B). These plots visualize some aspects of the action potential very clearly (e.g. re- and depolarization, amplitude and threshold).

The threshold (defined as the potential when the depolarization velocity exceeded 10 mV/ms) hyperpolarized from -47.8 ± 0.6 mV in aCSF with 2 mM calcium to -51.8 ± 0.9 mV in aCSF with 1.2 mM calcium ($p < 0.0001$, Fig. 4G).

The maximal rate of depolarization was significantly lower in 1.2 mM calcium, 216.8 ± 19.0 mV/ms compared to 252.1 ± 23.9 mV/ms in aCSF with 2 mM calcium ($p < 0.0001$, Fig. 4D). The maximal repolarization velocity displayed the same pattern with the rate increasing from -49.56 ± 1.91 mV/ms in 1.2 calcium aCSF to -53.13 ± 2.28 mV/ms in 2 mM calcium aCSF ($p < 0.05$, Fig 4C). These changes in de- and hyperpolarization were accompanied by a decrease in the amplitude of the action potential in the physiological concentration of calcium from 102.2 ± 1.8 mV in 1.2 mM calcium to 96.5 ± 1.4 mV in 2 mM calcium ($p < 0.0001$, Fig. 4E). There was, however, no significant difference in the width of the action potential (defined as the width at the midpoint between the threshold and the maximal amplitude) which was 1.56 ± 0.06 ms in 2 mM calcium and 1.58 ± 0.06 ms in 1.2 mM calcium ($p = 0.15$, Fig. 4F).

Evoked synaptic transmission

Next, we investigated the effect of a change in extracellular calcium from 1.2 to 2 mM on evoked synaptic currents in CA1 pyramidal neurons. A tungsten stimulation electrode was placed in the stratum radiatum to stimulate the Schaffer collaterals. CA1 neurons were voltage clamped and held at -70 mV (the approximate reversal potential for $GABA_A$Rs) to record synaptic AMPAR-currents, or at 0 mV (the approximate reversal potential for AMPARs) to record synaptic $GABA_A$R-currents (Fig. 5A). We found that the synaptic currents at -70 mV increased by 229 ± 95% from 68.6 ± 14.6 pA in 1.2 mM calcium to 168.1 ± 34.2 pA in 2 mM calcium ($p < 0.05$, Fig. 5D). At 0 mV the synaptic current increased by 283 ± 181 % from 212.2 ± 36.8 pA in 1.2 mM calcium to 467.3 ± 77.3 pA in 2 mM calcium ($p < 0.01$, Fig. 5C). In 1.2 mM calcium, the synaptic currents recorded at 0 mV were 309 % larger than the

synaptic currents at -70mV, and when using 2 mM calcium this relative difference was 277 %.

This data seems to suggest that inhibitory and excitatory synapses are about equally affected by changes in the extracellular concentration of calcium, but this conclusion is potentially confounded by the fact that the inhibition to the CA1 pyramidal cells via the Schaffer collaterals comes both via monosynaptic and disynaptic pathways. The disynaptic inhibition is mediated by local inhibitory interneurons that receive excitatory input from the Schaffer collaterals. It is therefore possible that the inhibitory input to CA1 pyramidal neurons could decrease with a decrease in calcium even if only the excitatory synapses are affected. To investigate this we added 6-cyano-nitroquinoxaline-2,3-dione (CNQX), an AMPA receptor antagonist to the extracellular medium at the end of the experimental protocol and recorded GABAergic currents again. CNQX will remove the disynaptic component and leave only the purely GABAergic monosynaptic inhibition. After the addition of CNQX, 37 ± 15 % of the synaptically evoked GABAergic current remained, indicating a mixture of mono- and disynaptic inhibitory input with slightly more disynaptic. Graph 5F shows the ratio of mono- and disynaptic inhibition versus the effect of lowering extracellular calcium. If extracellular calcium only affects excitatory synapses (and thus only the disynaptic part of the inhibition), one expects a clear correlation between the effect of higher calcium and the relative proportion of disynaptic inhibition. No such correlation was found ($R^2 = 0.124$, $p = 0.44$, Fig 5F).

In order to investigate the impact of calcium concentration on the presynaptic release probability, we recorded evoked events 50 ms apart at both -70 mV and 0 mV. The ratio of the amplitude of the second stimulus (adjusted to baseline) to the first stimulus, i.e. the paired pulse ratio (PPR), revealed a consistently larger PPR in 1.2 mM calcium aCSF compared to 2 mM calcium aCSF at -70 mV (2.08 ± 0.10 and 1.65 ± 0.14 , respectively, $p < 0.05$, Fig. 5G) and 0 mV (2.45 ± 0.36 and 1.75 ± 0.18, respectively, $p < 0.05$, Fig. 5H), consistent with an increased release probability at both glutamatergic and GABAergic synapses at elevated calcium. Fig. 5I shows ratio between the PPR in 1.2 mM and 2 mM calcium for AMPA EPSCs (left bar) and GABA IPSCs (right bar).

Spontaneous synaptic transmission

We also tested whether a change in the concentration of extracellular calcium affects the frequency of spontaneous EPSCs and/or IPSCs. Without any stimulation, we recorded such spontaneous currents in voltage clamp. We saw no difference in spontaneous activity depending on the calcium concentration for either AMPAR-mediated currents, EPSCs (0.52 ± 0.08 Hz in 1.2 mM calcium aCSF and 0.53 ± 0.08 Hz in 2 mM calcium aCSF, $p = 0.56$), or $GABA_AR$-mediated currents, IPSCs (4.10 ± 0.51 Hz in 1.2 mM calcium aCSF and 3.91 ± 0.38 Hz in 2 mM calcium aCSF, $p = 0.69$) (Fig 6A-B). We also analyzed the amplitude of the post-synaptic currents but could not detect any changes due to the shift in calcium concentration (sEPSC amplitude 9.7 ± 0.7 pA in 1.2 mM Ca^{2+} and 10.6 ± 0.9 pA in 2 mM Ca^{2+}, $p = 0.28$, sIPSC amplitude 19.4 ± 1.6 pA in 1.2 mM Ca^{2+} and 20.8 ± 2.3 pA in 2 mM Ca^{2+}, $p = 0.23$, Fig 6A-B).

Long-term potentiation

Lastly, we determined if aCSF with a physiological concentration of calcium could support LTP. By stimulation of Schaffer collaterals and recording field potentials in the CA1 region evoked, we attempted to induce LTP with five trains each with 20 pulses at 100 Hz (Fig 7A). We started out by inducing LTP using an aCSF containing 4 mM calcium, 4 mM magnesium and picrotoxin (PTX, a $GABA_AR$ antagonist), an aCSF commonly used in many electrophysiological field recordings. In this aCSF, LTP induction produced a clear potentiation (1.55 ± 0.07, $p < 0.0001$) when averaged over 6 min at 22 min after LTP-induction (Fig 7A-B). Next, we tried an aCSF containing 2 mM calcium and 1 mM magnesium with PTX, which supported a LTP of the same magnitude (1.50 ± 0.15, $p < 0.05$) as the aCSF containing 4 mM calcium and 4 mM magnesium (Fig 7A-B). Knowing that these concentrations of calcium were sufficient to induce LTP in the presence of PTX, we tried to induce LTP at the physiological calcium concentration (i.e. 1.2 mM calcium and 1 mM magnesium). Despite the presence of PTX we were unable to induce a significant LTP (0.99 ± 0.18, $p = 0.96$) measured over a 6 min period at 22 min after LTP-induction (Fig 7A-B). A one-way ANOVA confirmed that 4 mM (51.4 %, $p < 0.05$) and 2 mM (56.5 %; $p < 0.05$) calcium produced a significantly larger LTP compared to 1.2 mM.

Discussion

We found that the fraction of ionized calcium was 86% in hCSF and 87% in the matched aCSF, corresponding to 1.0 mM ionized calcium and a total extracellular calcium

concentration of 1.2 mM. Since *in vitro* brain slice experiments commonly use 2mM calcium, or more, we examined the sensitivity of several physiological processes to a change from 1.2 mM to 2 mM calcium. Our results indicate a high sensitivity to calcium in this concentration range. Thus, we observed a more than two-fold reduction in spontaneous action potential firing rate, an increase of the action potential threshold by 4 mV, a two-fold increase of evoked glutamateric and GABAergic synaptic transmission, and a qualitative shift in the induction of LTP.

Ionized calcium in hCSF

The very close match of the fraction of ionized calcium in the hCSF (86%) and in the matched aCSF (87%, with no added lactate, citrate or albumin) indicates that bicarbonate is responsible for the majority of the calcium-binding capacity in the hCSF. We believe that our measurements of ionized (~1 mM) and total (~1.2 mM) calcium concentration in the hCSF reasonably well reflect the concentrations in the brain extracellular fluid, which communicates freely with the CSF. Calcium concentrations has been reported to be consistent between extracellular fluid and CSF (Heinemann, Lux, & Gutnick, 1977), and also between lumbar and ventricular CSF (Hunter & Smith, 1960). A strength in our study is that we collected CSF from healthy volunteers, and not from patients. It should, however, be noted that the CSF in our study was collected from awake and alert persons during daytime, and somewhat different results regarding ion concentrations might have been obtained if the CSF had been collected during night time (Ding et al., 2016). The concentrations of calcium and magnesium in our healthy hCSF do correspond well with what Ding et al. (2016) found in awake mice, but we measure a considerably lower concentration of potassium (2.79 mM, compared to 4.00 mM in the study of Ding et al. (2016)). The different species might contribute to this difference, but it is also possible that the micro dialysis method used by Ding et al. (2016) may cause some tissue damage (Chefer, Thompson, Zapata, & Shippenberg, 2009; Nesbitt, Jaquins-Gerstl, Skoda, Wipf, & Michael, 2013; Westerink & Cremers, 2007), which in turn can cause an increase in extracellular potassium via direct release from damaged cells or, at a later stage reactive gliosis and altered potassium uptake in astrocytes (Bordey, Lyons, Hablitz, & Sontheimer, 2001; Olsen et al., 2015). Variation in ion concentrations of human CSF during day and night and its possible role in control of sleep and wakefulness need further investigation.

Excitability

Although the mechanism is debated (Jones & Smith, 2016), the effect of extracellular calcium concentration on neuronal excitability is well established and shown in many species (Frankenhaeuser & Hodgkin, 1957; Su, Alroy, Kirson, & Yaari, 2001; Wang, Wang, Cottrell, & Kass, 2004; Woodhull, 1973; Xiong, Lu, & MacDonald, 1997) and regions of the brain (Anderson et al., 2013; Ma et al., 2012; Su et al., 2001). Our major findings when changing from a total calcium concentration of 1.2 to 2 mM were a more than two-fold reduction in spontaneous action potential frequency, a clear leftward shift of the current-frequency relation (with no change of the gain), an about 4 mV increase of the action potential threshold and a somewhat faster depolarization and repolarization velocity of the action potential. We did not observe any change of the membrane potential. The depolarizing shift of the threshold as well as the increased velocity and amplitude of the action potential indicate that voltage-gated sodium channels are more difficult to activate, and to desensitize, when extracellular calcium is increased. Our findings are thus broadly consistent with several proposed mechanisms for the effect of extracellular calcium on neuronal excitability, including charge-screening, pore block of voltage-gated sodium channels and activation of calcium-activated potassium channels (M. Segal, 2018). More in-depth studies will be necessary to delineate the relative importance of these mechanisms to changes in excitability in the physiological range of extracellular calcium.

Synaptic transmission

A decrease in the concentration of extracellular calcium may affect evoked synaptic transmission in at least three different ways. First, it may lower the release probability by reducing the driving force for calcium in to the synaptic terminal (Dodge & Rahamimoff, 1967). Second, it may increase release probability by decreasing the negative feedback exerted by extracellular calcium acting on the G-protein coupled calcium sensor (Phillips et al., 2008). Third, it may increase the number of activated synapses by increasing the excitability of the axons (Frankenhaeuser & Hodgkin, 1957). When changing from 1.2 to 2 mM extracellular calcium, we found an about two-fold increase of both the evoked glutamatergic and GABAergic synaptic transmission. Also, the relative effect on the paired-pulse ratio for glutamate and GABA synapses was very similar. We did not, however, examine the effect of extracellular calcium on the response to longer trains of high-frequency stimulation. Nevertheless, our results on evoked synaptic transmission indicate that the

balance between synaptic excitation and inhibition is preserved in this concentration range of calcium.

In contrast to the evoked synaptic transmission, we did not find any change in either the amplitude, or the frequency, of spontaneous glutamatergic or of spontaneous GABAergic PSCs (Fig. 6). Spontaneous PSCs contain a mixture of action potential dependent and action potential independent PSCs, which may respond differently to an increased extracellular calcium concentration. The action potential dependent sPSCs may decrease in frequency since spontaneous action potential firing is decreasing (cf. Fig. 1D), whereas action potential independent sPSCs are expected to increase their frequency via an effect from the G-protein coupled calcium sensor (Phillips et al., 2008). The net effect of extracellular calcium on spontaneous PSCs will thus depend on the relative contribution of action potential dependent versus independent PSCs in the preparation, and we did not examine that in our study. Nevertheless, our finding of a comparable effect on spontaneous glutamatergic and GABAergic PSCs is consistent with a preserved balance between synaptic excitation and inhibition when changing the extracellular concentration of calcium.

Long-term potentiation

For almost 50 years, LTP has been extensively studied in brain slices, but the importance of the concentration of extracellular calcium for the induction of LTP has not been highlighted. Early studies showed that it was very difficult, if not impossible, to induce LTP when the extracellular calcium concentration was around 1 mM (Dunwiddie & Lynch, 1979; Muller & Lynch, 1989). We tested whether that difficulty could be overcome by facilitating the induction of LTP by blocking GABAergic inhibition (Wigstrom & Gustafsson, 1983). Somewhat surprisingly, we did not observe any LTP at the physiological concentration of 1.2 mM calcium during blockade of GABAergic inhibition, although LTP was readily induced at 2 and 4 mM calcium under the same conditions. Such a qualitative effect on the induction of LTP between 1.2 and 2 mM is surprising since although the driving force for calcium influx through the NMDA receptors is increased, it is expected to be increased by only some 7 % (according to Nernst equation). Moreover, an increase in extracellular calcium will decrease neuronal excitability, which likely will impede the induction of LTP. Although the explanation for the difficulty to induce LTP at 1.2 mM in the slice is obscure, and will require further examination, it can be contrasted to the fact that LTP can be induced *in vivo* (Bliss &

Gardner-Medwin, 1973; Namgung, Valcourt, & Routtenberg, 1995) where the concentration of extracellular calcium likely is in the 1.2 mM range. Conceivably, modulatory substances facilitating LTP exist in the extracellular fluid *in vivo*, at least during waking, that are missing in aCSFs used for *in vitro* studies.

In this study, we have determined the physiological concentration of ionized calcium in hCSF. We have compared this concentration with a higher concentration, commonly used in the field of neurophysiological research in terms of intrinsic excitability, synaptic transmission and LTP. Since our results show that even rather a modest increase from the physiological concentration of calcium alters several fundamental neuronal properties quite substantially, and since the mechanisms of action for extracellular calcium, in most cases, are not firmly established, further research in this area is warranted.

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