KCC2-dependent Steady-state Intracellular Chloride Concentration and pH in Cortical Layer 2/3 Neurons of Anesthetized and Awake Mice

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INTRODUCTION

Neuronal intracellular Cl⁻ concentration ([Cl⁻]) influences a wide range of processes such as neuronal inhibition, membrane potential dynamics, intracellular pH (pHᵢ) or cell volume. Up to date, neuronal [Cl⁻] has predominantly been studied in model systems of reduced complexity. Here, we implemented the genetically encoded ratiometric Cl⁻ indicator Superclomeleon (SCLM) to estimate the steady-state [Cl⁻] in cortical neurons from anesthetized and awake mice using 2-photon microscopy. Additionally, we implemented superecliptic pHluorin (SE-pHluorin) as a ratiometric sensor to estimate the intracellular steady-state pH (pHᵢ) of mouse cortical neurons in vivo. We estimated an average resting [Cl⁻] of 6 ± 2 mM with no evidence of subcellular gradients in the proximal somato-dendritic domain and an average somatic pHᵢ of 7.1 ± 0.2. Neither [Cl⁻] nor pHᵢ were affected by isoflurane anesthesia. We deleted the cation-Cl⁻ co-transporter KCC2 in single identified neurons of adult mice and found an increase of [Cl⁻] to approximately 26 ± 8 mM, demonstrating that under in vivo conditions KCC2 produces low [Cl⁻] in adult mouse neurons. In summary, neurons of the brain of awake adult mice exhibit a low and evenly distributed [Cl⁻] in the proximal somato-dendritic compartment that is independent of anesthesia and requires KCC2 expression for its maintenance.

Keywords: neuronal, intracellular, chloride, pH, in vivo, ratiometric imaging

Neuronal [Cl⁻], is of crucial importance for processes such as inhibitory neurotransmission, maintenance of the resting membrane potential, regulation of intracellular pH (pHᵢ) and cell volume (Kaila et al., 2014; Doyon et al., 2016). This work is an attempt to gain insight into mammalian neuronal [Cl⁻] in vivo and thus contribute to the current knowledge in this field, which stems mainly from in vitro experiments. Although invaluable ground breaking work about neuronal [Cl⁻], has been produced in vitro (Delpire, 2000; Ben-Ari, 2002; Kaila et al., 2014), many factors inherent to routine in vitro preparations such as brain slices may influence [Cl⁻], regulation (Dzhala et al., 2012). Recent pioneering work achieved in vivo measurements of bulk tissue Cl⁻ dynamics (Wimmer et al., 2015; Berglund et al., 2016; Wells et al., 2016), but presently we know
very little about the \textit{in vivo} steady-state neuronal $[\text{Cl}^-]_i$ and the factors affecting it (Bregestovski et al., 2009; Arosio and Ratto, 2014; Kaila et al., 2014; Doyon et al., 2016). Clomeleon was the first genetically encoded sensor to measure intracellular $\text{Cl}^-$ and consists of a CFP-YFP pair in which the YFP FRET-acceptor is the $\text{Cl}^-$-sensitive variant Topaz (Kuner and Augustine, 2000). However, the original version of Clomeleon has a $\text{Cl}^-$ affinity of $\sim 160$ mM that is out of the expected physiological neuronal $[\text{Cl}^-]_i$ and a limited signal to noise ratio (Grimley et al., 2013). This led to the development of a second generation of promising genetically encoded $\text{Cl}^-$ indicators with improved affinity and signal to noise ratio that could be used for \textit{in vivo} applications, such as ClopHensor (Arosio et al., 2010) and Superclomeleon (SCLM; Grimley et al., 2013).

The affinity of most $\text{Cl}^-$ sensitive fluorescent proteins for $\text{Cl}^-$ is highly dependent on $pH$ (Arosio et al., 2010; Grimley et al., 2013; Arosio and Ratto, 2014), which demands monitoring the cellular $pH$ to validate the $\text{Cl}^-$ readings made with these indicators. The design of ClopHensor-based sensors allows for simultaneous ratiometric $pH$ and $\text{Cl}^-$ measurements, but at the expense of being an excitation and emission ratiometric sensor. This hampers the \textit{in vivo} implementation of ClopHensor-based sensors as its different excitation and emission wavelengths might be differentially scattered throughout the tissue at increasing imaging depths. While the present work was under review, a study by Sulis Sato et al. (2017) reported simultaneous \textit{in vivo} optical estimations of neuronal $\text{Cl}^-$ and $pH$ in anesthetized mice using an optimized version of ClopHensor. The strong depth-dependent differential light scattering of the different wavelengths was compensated for by an offline correction algorithm. SCLM is not exempt of differential depth-dependent light scattering, but as it is an emission only ratiometric sensor instead, it might produce measurements less influenced by light scattering. This is at the expense of the need to monitor $pH_i$ by other means.

Although neuronal $pH$ has been studied \textit{in vivo} using techniques such as nuclear magnetic resonance (Vorstrup et al., 1989), up to date mammalian neuronal $pH_i$ has not been studied with cellular resolution \textit{in vivo}. The use of a genetically encoded ratiometric fluorescent indicator is again a promising alternative to achieve this. Recently, the implementation of superecliptic (SE) pHluorin (Miesenböck et al., 1998) as an emission ratiometric $pH$ indicator to monitor neuronal $pH_i$ has been reported in flies (Rossano et al., 2013). This suggests a strategy combining ratiometric SCLM and superecliptic pHluorin (SE-pHluorin) recordings as a promising alternative to study neuronal steady-state $\text{Cl}^-$ and $pH$ \textit{in vivo} using 2-photon microscopy.

Thus, to contribute to the on-going \textit{in vivo} implementation of $\text{Cl}^-$ imaging we established \textit{in vivo} 2-photon microscopy in mice expressing SCLM or SE-pHluorin and explored the strengths and caveats of this ratiometric \textit{in vivo} imaging approach. To gain insight into the factors affecting neuronal intracellular steady-state $\text{Cl}^-$ concentration, we compared $[\text{Cl}^-]_i$ and $pH_i$ in anesthetized and awake mice as well as in single neurons lacking the cation-$\text{Cl}^-$ co-transporter KCC2 (Delpée, 2000; Ben-Ari, 2002; Kaila et al., 2014). These results and approach will contribute to future studies of the role of $\text{Cl}^-$ regulation in pathophysiological conditions such as epilepsy or schizophrenia \textit{in vivo} (Kaila et al., 2014; Sullivan et al., 2015).

\section*{MATERIALS AND METHODS}

\subsection*{Ethics Statement}

This study was carried out in accordance with the European Communities Council Directive (86/609/EEC) to minimize animal pain or discomfort. All experiments were conducted following the German animal welfare guidelines specified in the TierSchG. The local animal care and use committee (Regierungspräsidium Karlsruhe of the state Baden-Württemberg) gave approval for the study under the number G183/15.

\subsection*{Animals}

Experiments were performed on wild type (WT) or homozygous knock-in mice bearing floxed KCC2 alleles. The mouse strains used were C57Bl6N/CR and KCC2\textsubscript{lox/lox} (Seja et al., 2012; Gödde et al., 2016). The latter mouse line was provided by Prof. Dr. Thomas Jensch (FMP/MDC, Berlin). All animals were 3–4 months of age by completion of data collection. Mice were housed up to three per cage and kept on a 12/12 h light/dark cycle. Food and water was available \textit{ad libitum} except while the animals were restrained for imaging. Mice of either sex were used.

\subsection*{Genetically Encoded Indicators}

The SCLM variant (Grimley et al., 2013) of Clomeleon (Kuner and Augustine, 2000) was used for non-invasive imaging of $[\text{Cl}^-]_i$ in layer 2/3 (L2/3) pyramidal cells of motor cortex. SCLM consists of the $\text{Cl}^-$-insensitive Cerulean variant of green fluorescent protein connected by a linker to the $\text{Cl}^-$-sensitive Topaz variant. The SE-pHluorin (Miesenböck et al., 1998) was used for $pH_i$ estimations. The Cerulean-Venus tandem pair and EGFp were used as variants less sensitive to $\text{Cl}^-$ and $pH$ of SCLM and SE-pHluorin respectively, to evaluate imaging depth dependent scattering effects on ratiometric imaging. In all cases, expression in L2/3 pyramidal cells of motor cortex was driven by the Synapsin minimal promoter using a Cre-dependent DIO cassette. Plasmids encoding SCLM, SE-pHluorin, Venus and Cerulean were kind gifts of Prof. Dr. George Augustine (DukeNUS Medical School), Prof. Dr. Gero Miesenböck (University of Oxford), Dr. Atsushi Miyawaki (RIKEN) and Prof. Dr. Dave Piston (Vanderbilt University), respectively.

\subsection*{Calibration Curves}

All calibrations were done \textit{in vitro}, as \textit{in vivo} calibration is not feasible because of the lack of control of local conditions. Calibration curves were constructed as described elsewhere (Grimley et al., 2013). Briefly, $[\text{Cl}^-]_i$ and $pH_i$ in cultured hippocampal neurons expressing SCLM or SE-pHluorin were matched to those of the extracellular saline by treatment with ionophores: 10 $\mu$M nigericin and 5 $\mu$M tributyltin acetate (Alfa Aesar). Cells were perfused in the presence of ionophores.
with calibration solutions of different [Cl\(^-\)] or pH at a rate of 2 ml/min. High-[Cl\(^-\)] calibration solution contained 105 mM KCl, 48 mM NaCl, 10 mM HEPES, 20 mM D(+)-glucose, 2 mM Na-EGTA and 4 mM MgCl\(_2\). Cl\(^-\) free calibration solution was composed of 10 mM HEPES, 20 mM D(+)-glucose, 48 mM Na-gluconate, 105 mM K-glucuronate, 2 mM Na-EGTA, and 4 mM Mg(gluconate).\(_2\). Intermediate [Cl\(^-\)] solutions were prepared by mixing these two solutions. The KF solution used to saturate SCLM contained 10 mM HEPES, 20 mM D(+)-glucose, 48 mM NaF, 105 mM KF, 2 mM Na-EGTA and 4 mM Mg(gluconate).\(_2\). For SCLM calibration curves, all salines were adjusted to pH 7.10 or pH 7.45. For SE-pHluorin calibration curves a calibration solution of 5 mM Cl\(^-\) was titrated to different pH values. Image acquisition started after 10 min. of incubation with the first calibration solution containing the ionophores and subsequent incubations were 6 min. each. Only a maximum of four incubations were performed per coverslip to avoid deterioration of the culture. SCLM calibration curves were constructed by fitting scatter plots of FRET ratios vs. [Cl\(^-\)] to the equation \[ [\text{Cl}^-] = \frac{R_{\text{dipFRET}} - R_{\text{max}}}{R_{\text{max}} - R_{\text{min}}} \times \frac{C_{\text{F}}}{C_{\text{B}}} \] where \(R_{\text{dipFRET}}\) is the apparent dissociation constant at a given pH, R is the YFP/CFP emission ratio, \(R_{\text{max}}\) and \(R_{\text{min}}\) are the maximum and minimum ratios, \(C_{\text{F}}\) and \(C_{\text{B}}\) are the absolute CFP fluorescence values in the Cl\(^-\) free and fully bound state respectively (Grimaldi et al., 2013). Average \(R_{\text{max}}\), \(R_{\text{min}}\), \(C_{\text{F}}\) and \(C_{\text{B}}\) were determined experimentally for the fitting equation. SE-pHluorin calibration curves were constructed by fitting scatter plots of 530 nm/480 nm emission ratios to the Boltzmann equation (Rossano et al., 2013): \[ \text{pH} = \frac{R_{\text{min}} + (R_{\text{max}} - R_{\text{min}})/(1 + \exp((V50 - R)/n))}{R} \] where R is the 530 nm/480 nm emission ratio, \(R_{\text{max}}\) and \(R_{\text{min}}\) are the maximum and minimum ratios, V50 is the half maximum pH value and n is the slope.

### Viral Vector Expression

Adeno-associated viral particles (AAV2/1-2 serotype) driving Cre recombinase expression, Cre-dependent or -independent expression of SCLM, SE-pHluorin, Cerulean-Venus or EGFP, all under the control of the Synapsin minimal promoter, were prepared for stereotaxic injections (Schwenger and Kuner, 2010). To achieve sparse but bright labeling of neurons that permitted the identification of each cell’s projections, we co-injected AAVs encoding the Cre-dependent fluorescent proteins/sensors together with highly diluted (1/1000 to 1/5000) AAVs encoding nucleus-targeted Cre recombinase. Thus, due to the likelihood of the multiplicity of infection, very sparse but brightly labeled neurons could be imaged resolving their individual projections.

### Stereotaxic Injection

For injection and craniectomy, mice were anesthetized by i.p. injection of a mixture of 40 \(\mu\)l fentanyl (1 mg/ml; Janssen), 160 \(\mu\)l midazolam (5 mg/ml; Hameln) and 60 \(\mu\)l medetomidin (1 mg/ml; Pfizer), dosed in 3.1 \(\mu\)l/g body weight, placed in a stereotaxic headholder (Kopf) and a circular craniectomy (~7 mm) centered at bregma was made with a dental drill. The dura was carefully removed over the area for later injection with fine forceps cautiously avoiding damaging blood vessels. AAV particles were injected at L2/3 M1 motor cortex using the following coordinates: in mm from the center of the craniectomy, considered bregma (x; y) = (1; 0). M1 cortex injections were performed using glass pipettes lowered to a depth of 300 \(\mu\)m to target L2/3. AAVs were injected using a syringe at a rate of ~1 \(\mu\)l/h. Volumes of virus of ~0.2 \(\mu\)l were injected per spot. All mutant mice used for virus injection were homozygous for the floxed KCC2 allele. A round 6 mm diameter number 0 coverslip (cranial window) disinfected with 70% ethanol and a custom made round plastic holder crown surrounding it for head fixation were cemented to the skull using dental acrylic (Hager and Werken). We targeted L2/3 of M1 cortex due to the accessibility of this cortical layer for 2P imaging and the convenience of the location over M1 cortex for hosting a 6 mm round cranial window, which allowed for multiple AAV injection sites thus refining our technique to reduce the number of animals needed for our experiments in conformity with our ethics statement. The skin wound around the window was also closed with dental acrylic. Mice received an i.p. mixture of 120 \(\mu\)l naloxon (0.4 mg/ml; Inresa), 800 \(\mu\)l flumazenil (0.1 mg/ml; Fresenius Kabi) and 60 \(\mu\)l antipamezole (5 mg/ml; Pfizer) dosed in 6.2 \(\mu\)l/g body weight at the end of surgery to antagonize the anesthesia mix. Mice were given carprofen (Rimady, 5 mg/kg; Pfizer) as an analgesic immediately before surgery. Mice were single housed after surgery and typically imaged at least 28 days after virus injection and window implantation.

**Two-Photon (2P) Imaging**

2P imaging was done on a TriM Scope II microscope (LaVision BioTec GmbH) equipped with a pulsed Ti:Sapphire laser (Chameleon; Coherent). The light source was tuned to 840 nm for SCLM and Cerulean-Venus measurements or 810 nm for SE-pHluorin and EGFP measurements. Imaging was performed with a 25×, 1.1 numerical aperture, apochromatic, 2 mm working distance, water immersion objective (Nikon, MRD77225) and emitted fluorescence was split with a 495 nm laser (Chameleon; Coherent). Fluorescence emission was collected with low-noise high-sensitivity photomultiplier tubes (PMTs; Hamamatsu, H7422-40-LV 5M).

**In Vivo 2P Imaging**

For the imaging sessions of cranial window implanted mice, anesthesia was induced with 6% isoflurane (Baxter) in O$_2$ and maintained at 0.8%–1%. The anesthetized mouse was then head fixed on top of a rotating disc treadmill by a custom made head holder designed to fix a plastic holder crown surrounding the cranial window. This device was mounted onto the intravital stage of the 2P microscope. Fluorescence imaging in the anesthetized state was performed using line scanning. Typically, 16 bit frames of 491 \(\mu\)m$^2$ and 1024 px$^2$ resolution were acquired at a frame rate of 0.6 Hz and at 2 \(\mu\)m z steps. A full 3D stack spanning typically ~300–350 \(\mu\)m in depth took ~5–6 min. to acquire. Anesthetized imaging sessions lasted no longer than ~30 min. After the imaging session in the anesthetized state, the animal was allowed to
recover from anesthesia while still being head fixed at the microscope’s stage (usually it took the mice 2–3 min to show clear signs of being awake such as to start whisking, blinking and walking). Throughout the imaging session the animal’s breathing and behavior was monitored by means of an infra-red camera mounted to the stage while keeping the stage in the dark. After 5 min from withdrawal of isoflurane, awake imaging was performed under the aforementioned settings. Awake sessions (since the withdrawal of anesthesia) lasted no longer than ~15 min. After this the imaging session ended and the animal was returned to its cage.

**In Vitro 2P Imaging**

Hippocampal cultures were prepared from E16 rats according to established techniques (Kuner and Augustine, 2000). Infection of neuronal cultures with AAVs delivering the different expression constructs was performed on (DIV) 7–8. After virus infection and 4–7 day incubation for fluorescent protein expression, coverslips with cultured cells were transferred to a custom made perfusion chamber with temperature control mounted on the intravital stage of a trimscope microscope (LaVision) equipped with a pulsed Ti:Sapphire laser (Chameleon; Coherent). All recordings were conducted at 35 ± 2°C. Imaging was performed under the previously described settings. Except for calibration curves, hippocampal cultures were imaged in artificial cerebrospinal fluid (ACSF) gassed with carbogen (125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1.25 NaH₂PO₄, 1 mM MgCl₂, 2 mM CaCl₂, 25 mM D(+) glucose, pH = 7.4). All chemicals were purchased from Sigma-Aldrich, except otherwise noted.

**2P Imaging of Ratiometric Sensors**

In a typical imaging session, the fluorescence emission collected at the shorter wavelength channel (cyan) was much weaker than the one from the longer wavelength channel (yellow/green). Hence, excitation laser power settings were optimized for cyan emission while avoiding saturation of the yellow/green emission channel, a compromise inherent to the dynamic range of ratiometric sensors based on CFP and YFP FRET pairs. To avoid “divided by background” ratio artifacts, we only analyzed regions of interest (ROIs) that exceeded the emission intensity of background twofold. For the sake of consistency, we only analyzed fluorescence ratios from cells with L2/3 pyramidal morphology, defined by the depth of the soma in the imaged volume, its size, shape and the presence of a marked apical dendrite (for simplicity referred to as pyramidal cells).

**Analysis of Imaging Data**

Image analysis was performed using Fiji (Schindelin et al., 2012) and data analysis and statistics were performed using Prism 7 software (GraphPad). ROIs spanning the cell somas, dendrites, dendritic spines or a cell-free background area were determined manually. Mean fluorescence intensity from each ROI were subtracted with the mean fluorescence intensity from the corresponding background area and background corrected fluorescence intensity ratios were calculated. Due to the low intensity emission registered for Cerulean and SE-pFluorin at 480 nm, only ROIs in which this fluorescence intensity was two-fold to that of the background were considered for further analysis.

**Statistical Analyses**

Normality was tested using Kolmogorov-Smirnov, D’Agostino-Pearson and Shapiro-Wilk tests. For paired samples with normally distributed frequencies, statistical significance was evaluated using paired t-tests. Wilcoxon tests were used for pairwise comparisons in which one or both samples were not normally distributed. For unpaired comparisons between highly variable samples, the whole frequency distributions were compared using Kolmogorov-Smirnov tests. Statistical differences to a theoretical value was evaluated using Wilcoxon signed rank tests. Variances of samples with different sizes were compared with Levene’s test. Correlation was evaluated using the Pearson correlation coefficient. A p < 0.05 was considered significant.

**RESULTS**

**In Vivo Imaging of SCLM-expressing Layer 2/3 Cortical Pyramidal Neurons in Anesthetized Mice**

To explore the [Cl−], of neurons in the intact brain, we sparsely targeted SCLM expression to L2/3 of M1 cortex (Figure 1A), typically obtaining 1–20 neurons expressing SCLM in the entire mouse brain (see “Materials and Methods” section for details). Using this criterion 31 pyramidal cells were analyzed in dual color 3D stacks obtained from 5 head-fixed anesthetized WT mice. The Topaz component of SCLM gave rise to high fluorescence intensities even at depths of ~300 μm from the brain surface (Figures 1A,B), yet Cerulean fluorescence was lower. At low [Cl−]ᵢ, Topaz effectively acts as an acceptor for fluorescence resonance energy transfer from Cerulean, hence limiting the overall brightness of Cerulean fluorescence emission. To illustrate the signal strength over background of each emission channel rather than the intensity differences between the channels, both channels were clipped for display (Figure 1). In most neurons analyzed, a section of the apical dendrite of L2/3 pyramidal cells, proximal to the soma, consistently produced bright fluorescence in both emission channels that allowed reliable analysis. Regions with signal to background ratios of less than twofold were excluded for calculating the 540 nm/480 nm emission ratio (SCLM ratio), with high ratios representing low [Cl−]ᵢ (Figure 1B).

A cell by cell pairwise comparison between the average SCLM ratio measured from a ROI spanning the soma or the apical dendrite of the 31 L2/3 pyramidal cells revealed no significant difference (p = 0.3096, paired t-test; Figure 1B), suggesting that intracellular Cl− levels are rather homogeneous throughout these two subcellular compartments. Distal dendritic and spine [Cl−]ᵢ, could be imaged in some neurons (Figure 1C), nevertheless the lower signal to background ratio in these structures precluded a systematic analysis. Additionally, these few distal spiny dendritic stretches could not be traced back to...
In summary, in vivo SCLM imaging with cellular resolution is feasible within cellular compartments that produce sufficiently high fluorescence intensity in the Cerulean emission channel at illumination intensities that do not saturate the Topaz channel, i.e., somata and thick dendrites of neurons. Within the proximal somato-dendritic domain spanning ~100 µm, [Cl\(^-\)]\(_i\) would be evenly distributed with no detectable intracellular gradients in [Cl\(^-\)].

Depth-Dependent Differential Scattering of Cyan and Yellow Fluorescence?

An intrinsic issue of ratiometric sensors is that their different emission wavelengths could be differentially scattered by the tissue, hence photons of different wavelengths emitted by SCLM traveling through different amounts of brain tissue at different imaging depths could produce depth-dependent artificial ratios. To address this problem, we designed an environmentally insensitive SCLM variant by replacing the Cl\(^-\) and pH sensitive Topaz yellow fluorescent protein with the reduced environmental sensitivity YFP variant Venus (Nagai et al., 2002). The pK\(_a\) value of Venus (6, Nagai et al., 2002) is slightly lower than that of SCLM (6.4, Grimley et al., 2013) and the K\(_d\) for Cl\(^-\) of Venus (10 M, Nagai et al., 2002) is strongly increased. Thus, Venus can be considered insensitive to Cl\(^-\), yet slightly sensitive to pH. Cerulean is insensitive to pH in the range tested (pK\(_a\) = 4.7, Shaner et al., 2005) and its emission...
is not influenced by Cl⁻ (Kuner and Augustine, 2000; Grimley et al., 2013). Hence, we replaced Topaz in SCLM with Venus, obtaining Cerulean-Venus. Thus, depth-dependent ratio changes would indicate differential scattering, while an invariant ratio would argue for depth-independence.

We sparsely expressed Cerulean-Venus in L2/3 pyramidal neurons of the motor cortex, under the same settings used with SCLM. We then recorded emission ratios at somas and apical dendrites at different imaging depths and included the surface of the brain in the image stack to estimate the depth of the somas (Figure 2A). In vivo 2P 3D stacks obtained from six head fixed anesthetized WT mice revealed that average somatic Cerulean-Venus ratios did not significantly correlate with the depth of the soma centroid (Pearson $r = -0.01256$, $p = 0.9296$, $n = 52$ cells; Figures 2A,B). To further assess the influence of light scattering in our measurements, we studied the emission ratio along the longitudinal axis of the neurons from the base of the soma towards the surface of the cortex along the apical dendrite until the most superficial position possible (Figure 2A, right panel). While individual cells had rather different ratios, we could not find systematic shifts of the ratio along the dendritic tree (Figure 2C). We evaluated the correlation of the emission ratio vs. the distance along the longitudinal axis for each cell and found both positive and negative correlations (Figure 2D). The expected effect of scattering should manifest itself in only one sense of correlation, likely negative in this case due to higher scattering of shorter wavelengths. When analyzing this correlation in a cell by cell basis we found that 67% of the cells ($n = 52$) displayed Pearson correlation coefficients between $-0.3$ and $0.3$, which could be considered weak or no correlation. Only in 26% of the cells, we observed coefficients lower than $-0.3$ and in 7% higher than 0.3. Thus, we conclude that differential depth-dependent scattering, within the range covered by our experiments, does not contaminate the ratiometric readout.

Similar to the ratios recorded with SCLM, Cerulean-Venus ratios displayed a pronounced dispersion when comparing different cells (Figure 2C). The observation that the ratios obtained with the environmentally much less sensitive
Cerulean-Venus and SCLM are dispersed in a similar fashion, suggests that the observed dispersion in SCLM ratios is not due to variation in intracellular \([\text{Cl}^-]\), between cells. This point will be further elaborated below.

**In Vivo Ratiometric Imaging of SE-pHluorin-expressing Layer 2/3 Cortical Pyramidal Neurons**

SCLM presents a sensitivity to pH that cannot be ignored as pH significantly affects the \(K_d\) of Cl\(^-\) for SCLM (Grimley et al., 2013). To determine the neuronal \(pH_i\), we imaged L2/3 neurons of the motor cortex expressing SE-pHluorin. SE-pHluorin has been successfully used as a ratiometric pH indicator in vitro using excitation and emission wavelengths similar to those used for SCLM (Rossano et al., 2013), facilitating the implementation of this sensor for ratiometric pH estimations through minimal modifications to our method. SE-pHluorin was excited at 810 nm and 530 nm and 480 nm emission filters were used to calculate the 530 nm/480 nm ratio as a measure of \(pH_i\) (see “Materials and Methods” section).

To determine the average \(pH_i\) of the imaged cells, we constructed a calibration curve in SE-pHluorin-expressing hippocampal neuronal cultures by clamping \(pH_i\) to the extracellular saline by means of ionophore treatment at close to physiological temperature (35 ± 2°C). We fitted the data to the Boltzmann equation \((r^2 = 0.7009)\) obtaining a best fit \(V_{50}\) value of 7.49 ± 0.05 (Figure 3A).

We sparsely expressed SE-pHluorin in superficial layers of motor cortex. Image stacks obtained from anesthetized mice revealed a similar compromise between the high intensity 530 nm emission and low intensity 480 nm emission as reported above for SCLM. In this case, the 480 nm emission at all processes including apical dendrites was too low to produce reliable ratios (double 480 nm emission than background fluorescence without saturating 530 nm emission). Consequently, we considered pyramidal cells having an oval/piriform soma with an extension representing the proximal portion of an apical dendrite. Thus, this in vivo imaging approach deep in intact brain tissue is limited to the somata of neurons and cannot be used to assess \(pH_i\) in small compartments (Figure 3B).

Using the best fit parameters we calculated the \(pH_i\) corresponding to the average ratios recorded in vivo at the somas of 27 L2/3 pyramidal neurons from three mice. We found \(pH_i\) values ranging from 6.5 to 7.5 with a mean of 7.1 ± 0.2 (mean ± 95% C.I., Figure 3C).

To rule out ratio artifacts due to imaging depth and differential scattering of the emission wavelengths, we repeated our experiments employing EGFP, which has reduced pH sensitivity (\(P_{K_a} = 6\), Shaner et al., 2005). We co-injected three mice with AAVs encoding Cre-dependent EGFP together with highly diluted Cre AAVs for bright and sparse labeling and repeated our measurements at L2/3 neurons using the same settings as with SE-pHluorin. We recorded somatic average emission ratios as a function of imaging depth. 3D stacks obtained from in vivo imaging sessions from three head fixed anesthetized WT mice revealed that the 530 nm/480 nm emission ratio of EGFP is not significantly correlated to the depth of the recorded soma in the tissue (Figure 3D). Hence, depth-dependent scattering in the emission wavelengths of SE-pHluorin is unlikely to affect our in vivo ratiometric pH estimations.

Interestingly, the ratios recorded with SE-pHluorin, displayed a pronounced dispersion that was significantly greater than those recorded with EGFP (for average somatic ratios, standard deviation: EGFP = 1.8, \(n = 29\) cells; SE-pHluorin = 2.5, \(n = 27\) cells; \(p = 0.0010\), Levene’s test). This suggests that the observed dispersion in SE-pHluorin ratios is indicating an actual variation in \(pH_i\) between cells in vivo.

**Calibration of SCLM and in Vivo \([\text{Cl}^-]_i\) Estimation**

Ideally, \(pH_i\) and \([\text{Cl}^-]_i\) should be simultaneously determined in the same cellular compartment. However, SE-pHluorin imaging...
of neuronal pHi is not compatible with simultaneous SCLM recordings. This means that it is not possible to calculate \([\text{Cl}^-]_i\) in a cell by cell basis from each cell’s SCLM ratio at the corresponding pHi. Hence, to estimate the average neuronal steady-state \([\text{Cl}^-]_i\) we constructed a calibration curve by clamping the \([\text{Cl}^-]_i\) and pHi of SCLM-expressing hippocampal neurons to the extracellular saline through treatment with ionophores at a fixed pH corresponding to the average pHi estimated \textit{in vivo} (pHi = 7.1, Figure 4A). To match physiological conditions we constructed this curve at near physiological temperature (35 ± 2°C). In these conditions our best fit value of \(K_d\) for \([\text{Cl}^-]_i\) was 13.6 ± 1.5 mM (\(r^2 = 0.6972\), Figure 4B). Using the best fit calibration curve, we estimated that the \([\text{Cl}^-]_i\) corresponding to the average SCLM ratio recorded \textit{in vivo} at the somas of L2/3 pyramidal neurons of the motor cortex was 5 ± 2 mM in the anesthetized state (mean ± 95% C.I.).

**Other Factors Affecting Ratiometric Emission Measurements and Error Estimates Associated with pH Changes**

As mentioned above, the observed variability in our ratiometric measurements could be explained by irregularities in the optical properties of the tissue (e.g., presence of blood vessels) at different parts of the volume imaged. To explore this possibility we compared the Cerulean-Venus emission ratios obtained \textit{in vivo} from L2/3 cortical neurons to the ones observed \textit{in vitro} in hippocampal neuronal cultures expressing Cerulean-Venus. Thus, neuronal cultures should be significantly less influenced by uneven optical properties as the cells would be more exposed than they would when located deep in intact brain tissue. We observed that the dispersion of Cerulean-Venus expressing cells \textit{in vitro} and \textit{in vivo} did not differ significantly (for average somatic ratios, standard deviation: \textit{in vivo} = 2.4, \(n = 52\) cells; \textit{in vitro} = 3.5, \(n = 18\) cells; \(p = 0.1406\), Levene’s test), indicating that the variability in our ratiometric measurements was not caused by tissue inhomogeneity (Figure 5A), leaving pHi differences between cells as a possible factor underlying this heterogeneity. To explore this further, we treated hippocampal cultures expressing Cerulean-Venus or SCLM with ionophores to manipulate their pHi at a fixed \([\text{Cl}^-]_i\) of 5 mM and compared their ratios to our \textit{in vivo} datasets (Figure 5B). To compare the variability in the ratios from these \textit{in vitro} recordings at different pHi to our \textit{in vivo} datasets we evaluated the ratio difference from the population mean ratio (signed ratio deviation, dRatio) at pHi 7 (\textit{in vitro} dataset) or 7.1 (\textit{in vivo} dataset). We compared the span of the best fit curve and the 95% confidence band from the Cerulean-Venus or SCLM dRatio as a function of pHi, within the range of pHi estimated \textit{in vivo} (6.5–7.5), to the ratio deviation obtained from \textit{in vivo} measurements. In doing so, we observed that the pH sensitivity of Cerulean-Venus or SCLM could account for practically all of the variability observed \textit{in vivo} (Figures 5C,D).

**FIGURE 4** | Calibration of SCLM. (A) Representative images showing the \textit{in vitro} SCLM ratio of hippocampal cultures in the presence of ionophores at different \([\text{Cl}^-]_i\) and a fixed pH of 7.1. To avoid deterioration of the cultures due to ionophore treatment, a maximum of four consecutive solutions were applied per coverslip. (B) Calibration curve constructed from ratiometric imaging of SCLM-expressing hippocampal cultures in the presence of ionophores at a fixed pH of 7.1. Mean and SEM values are plotted. \(F = 153\) mM fluoride solution.
Other factors affecting ratiometric emission measurements. (A) Ratio difference from the population mean ratio at pH 7–7.1 (signed deviation, \(dR\)) evaluated for Cerulean-Venus expressing L2/3 pyramidal neurons from M1 cortex (in vivo) or hippocampal cultures (in vitro, ACSF). (B) Representative images showing the in vitro Cerulean-Venus ratio of hippocampal cultures at different pH and a fixed [Cl\(^-\)] of 5 mM (first 3 panels) and the in vivo Cerulean-Venus ratio variability (fourth panel). (C) Red: Cerulean-Venus \(dR\) evaluated from hippocampal cultures in the presence of ionophores and different pH values. Mean \(dR\) and best fit linear regression plus 95% confidence band are plotted. Blue: \(dR\) distribution of Cerulean-Venus expressing L2/3 pyramidal neurons from M1 cortex in vivo. Segmented vertical lines: range of somatic pH measured in vivo. (D) As in (C) but considering SCLM. (E) Calculated [Cl\(^-\)] values from the SCLM signals obtained in the experiment shown in (D) at the in vivo pH range (6.5–7.5) using the calibration curve from Figure 4 (constructed at pH 7.1).

In Vivo Imaging of SCLM and SE-pHluorin-expressing Layer 2/3 Cortical Pyramidal Neurons in Awake Mice

The anesthetized condition generally involves increased GABAergic inhibition (Maciver, 2014). This could influence [Cl\(^-\)], producing estimates that are not representative of the physiological awake state. We imaged SCLM-expressing neurons after initial isofluorane anesthesia in the awake state (Figure 6A). We performed again a cell by cell pairwise comparison between the average somatic SCLM ratio and the average apical dendritic ratio of 24 L2/3 pyramidal cells from five mice and observed no significant difference (\(p = 0.5330\), Wilcoxon signed rank test; Figure 6B), suggesting that our previous observation about [Cl\(^-\)], being homogeneous throughout these two subcellular compartments is not related to the anesthetized condition. Imaging in the awake state did not reveal significant differences in the distribution of somatic SCLM ratios (\(p = 0.5330\), Wilcoxon signed rank test; Figure 6C), indicating that the steady-state neuronal [Cl\(^-\)] is not affected by isofluorane anesthesia and that SCLM ratios measured under anesthesia are representative of the awake state. A cell by cell pairwise comparison between the average somatic SCLM ratio recorded from 24 identified L2/3 pyramidal cells from five mice in the anesthetized and awake state showed no significant difference (\(p = 0.6949\), paired t-test; Figure 6D), further supporting this notion.

To estimate [Cl\(^-\)] in the awake state using a calibration curve, we first studied pH\(_i\). Hence, we imaged SE-pHluorin in the awake state (Figure 6E) and found that the pH\(_i\) distribution is not significantly different to the one obtained under isofluorane anesthesia (\(p = 0.2750\), Kolmogorov-Smirnov; Figure 6F). This result suggests that imaging SE-pHluorin under isofluorane anesthesia also does not produce artifactual measures of steady-state pH\(_i\). A cell by cell pairwise comparison between the average somatic pH recorded from 11 identified L2/3 pyramidal cells from three mice in the anesthetized and awake state showed no significant difference (\(p = 0.4822\), paired t-test; Figure 6G).

More importantly, these results suggest that the previous observation about the steady-state SCLM ratio being unaffected by anesthesia is truly reflecting a lack of effect on [Cl\(^-\)]. Using our best fit calibration curve (Figure 4), we estimated the [Cl\(^-\)] using the average somatic SCLM ratio recorded in vivo in the awake state to be 6 ± 2 mM (mean ± 95% C.I.).

To conclude, we did not find differences in [Cl\(^-\)] or pH\(_i\) in cortical neurons of isofluorane anesthetized vs. awake mice, suggesting that steady-state [Cl\(^-\)], or pH\(_i\) are not affected by isofluorane anesthesia.

In Vivo Effect of KCC2 Deletion on the Steady-state [Cl\(^-\)] of Adult Layer 2/3 Cortex Neurons

As a proof of principle of our in vivo imaging approach and to explore the factors affecting the steady-state neuronal intracellular Cl\(^-\) in vivo, we co-injected AAVs encoding...
Cre-dependent SCLM together with highly diluted AAVs encoding Cre recombinase into L2/3 of M1 cortex in adult KCC2\textsuperscript{lox/lox} conditional KO mice (Seja et al., 2012; Gödde et al., 2016). Thus, expression of Cre recombinase will yield SCLM expression and abolish expression of KCC2. These mice were imaged at least 4 weeks after virus injection, which widely exceeds the reported KCC2 protein turnover time (Rivera et al., 2004). As a control for $\left[\text{Cl}^{-}\right]_i$ estimations, we injected AAVs encoding Cre-independent SCLM to assess $\left[\text{Cl}^{-}\right]_i$ in neurons of KCC2\textsuperscript{lox/lox} mice without inducing the KO (from now on Ctrl cells). Additionally, to validate these measurements we determined the pH\textsubscript{i} in Ctrl cells and KCC2 KO cells of KCC2\textsuperscript{lox/lox} mice using SE-pHluorin and following the aforementioned approach ($\pm$ Cre). The expression of Cre-independent SCLM and SE-pHluorin under the Synapsin promoter in vivo did not permit to distinguish pyramidal cell morphology as clearly as our Cre-dependent dilution approach, as the concomitant labeling of the tissue is much denser. Therefore, in these cases cells with clear pyramidal neuron morphology were carefully selected for further analysis, thus discarding the great majority of labeled cells.

In vivo imaging of SE-pHluorin labeled KCC2 KO and Ctrl cells from six KCC2\textsuperscript{lox/lox} mice showed that the KO did not affect the pH\textsubscript{i} distribution (Anesthetized: $p = 0.0990$; Awake: $p = 0.2980$; Kolmogorov-Smirnov; Figure 7A). Again, we confirmed in the KCC2\textsuperscript{lox/lox} genetic background, that isoflurane anesthesia does not affect the steady-state neuronal pH\textsubscript{i} distribution (+Cre: $p = 0.8180$; −Cre: $p = 0.2710$; Kolmogorov-Smirnov; Figure 7A).

In vivo imaging of SCLM-labeled KCC2 KO cells from six mice showed significantly different average somatic SCLM ratio distributions than Ctrl (−Cre) cells from three mice (Anesthetized: $p < 0.0001$; Awake: $p < 0.0001$; Kolmogorov-Smirnov; Figures 7B,C). Once again, we observed no significant effect of isoflurane anesthesia in the SCLM ratio distributions from KCC2\textsuperscript{lox/lox} mice, further supporting that isoflurane does not affect the distribution of steady-state neuronal $\left[\text{Cl}^{-}\right]_i$ (+Cre: $p = 0.6650$; −Cre: $p = 0.8600$; Kolmogorov-Smirnov;...
**DISCUSSION**

Our study reports non-invasive *in vivo* steady-state $[\text{Cl}^-]$, and pH$_i$ estimations in L2/3 cortical neurons of anesthetized and awake mice. Independently of anesthesia, we estimated a native steady-state neuronal $[\text{Cl}^-]$, of 6 ± 2 mM which is consistent with numerous *in vitro* reports (Berglund et al., 2008; Bregestovski et al., 2009; Raimondo et al., 2013). According to the Nernst equation, a 6 mM steady-state neuronal $[\text{Cl}^-]$, paired with the expected extracellular $[\text{Cl}^-]$ (145 mM; Raimondo et al., 2015) would produce an $E_{\text{Cl}^-}$ of $\sim(-85 \text{ mV})$ at 37°C, which is compatible with the expected values of healthy adult neurons (Doyon et al., 2016). If the Goldman equation is used to calculate the respective GABA$_A$ R $E_{\text{rev}}$, using the reported $[\text{Cl}^-]$, and the expected $[\text{HCO}_3^-]_i$ (15 mM) at the reported average pH$_i$, paired with the expected extracellular $[\text{Cl}^-]$ (145 mM) and $[\text{HCO}_3^-]$ (24 mM) concentrations (Raimondo et al., 2015) and a 4:1 permeability ratio for these two anions (Bormann et al., 1987), an $E_{\text{rev}}$ of $\sim(-73 \text{ mV})$ is obtained, consistent with previously reported values (Kaila et al., 1993; Lee et al., 2015). This GABA$_A$ $E_{\text{rev}}$ could support hyperpolarizing inhibition, although the driving force at typical resting membrane potentials would be rather small. Therefore, $\text{Cl}^-$ conductances will tend to produce shunting inhibition *in vivo*. Hence our results demonstrate that somatic steady-state $[\text{Cl}^-]$, determined *in vitro* can, in principle, be extrapolated to the native state found in the awake brain.

**Figure 7B**. The observed lack of change in the pH$_i$ distribution (Figure 7A) confirms that the observed changes in the SCLM ratio distribution induced by deleting KCC2 (Figure 7B) are caused by changes in the steady-state neuronal $[\text{Cl}^-]$. Altogether, these results point out that the KO of KCC2 produces a shift towards lower SCLM ratios, which represent higher neuronal $[\text{Cl}^-]$, and that this effect is discernible *in vivo* using SCLM.

To calculate an estimate of the change in the average neuronal steady-state $[\text{Cl}^-]_i$, we constructed an *in vitro* calibration curve as previously described. We chose to produce this calibration curve at pH 7.45 because this value was not significantly different to the mean pH$_i$ recorded in KCC2 KO cells from KCC2$^{lox/lox}$ mice in both anesthetized or awake conditions (Anesthetized: $p = 0.2163$; Awake: $p = 0.1941$; Wilcoxon signed rank test). Thus, we used this single calibration curve produced at pH 7.45 to calculate estimates of the steady-state $[\text{Cl}^-]_i$, from the mean SCLM ratios recorded *in vivo* at the somas of KCC2 KO cells. This calibration curve showed a higher maximum ratio and $K_d$ than the one produced at pH 7.1 (Figure 7D). The resulting steady-state $[\text{Cl}^-]_i$, estimation produced by KCC2 KO was 24.2 ± 8 mM in the anesthetized state and 25.7 ± 8 mM in the awake state (mean ± 95% C.I.). In summary, KCC2 deletion produced a higher neuronal $[\text{Cl}^-]_i$, demonstrating a requirement of KCC2 for low $[\text{Cl}^-]_i$ in the adult mouse brain. Furthermore, these results indicate that effects in the magnitude of the changes reported here can be detected by *in vivo* SCLM imaging.
In this study we did not find evidence of differences in neuronal [Cl\(^-\)] along the soma to proximal regions of the apical dendrite of cortical L2/3 pyramidal neurons. This is in contrast to in vitro reports studying other cell types such as cultured hippocampal or spinal cord neurons and retinal bipolar cells where clear somato-dendritic gradients were detected (Kuner and Augustine, 2000; Duebel et al., 2006; Waseem et al., 2010). Thus, our study suggests that within these subcellular compartments GABAergic inputs would be homogeneously inhibitory. The work of Waseem et al. (2010) in cultured neurons reports a stark gradient in [Cl\(^-\)], between the soma and proximal dendritic compartment within a ∼20 µm distance. Nevertheless, this observation can be influenced by the maturation stage of the cultured cells, limited neuron-glia interactions and other uncontrolled factors inherent to this in vitro preparation such as abnormal neuronal activity, which can affect cation-Cl\(^-\) co-transporter trafficking and activation (Kaila et al., 2014). In this respect our study undertakes the effort to keep the intact native conditions of brain tissue as close as possible to the physiological state to avoid these issues. Under these conditions, we did not find evidence of a [Cl\(^-\)] gradient at the proximal portion (up to ∼100 µm away from the soma) of the apical dendrite of L2/3 pyramidal neurons.

We found a steady-state pH\(_i\) of 7.1 ± 0.2 at the neuronal soma which is consistent with in vitro reports (Caspers and Speckmann, 1972; Raimondo et al., 2012). Our in vivo estimations of neuronal pH\(_i\) suggest that individual L2/3 pyramidal neurons differ in pH\(_i\) across a large range, also consistent with in vitro reports (Schwiening and Boron, 1994; Ruffin et al., 2014) and a recent in vivo report (Sulis Sato et al., 2017). Neuronal activity can dynamically affect pH\(_i\) (Chesler and Kaila, 1992). Thus it is possible that cells with different activity levels would underlie the observed range in pH\(_i\). We could not systematically explore the occurrence of gradients in pH\(_i\) along the somato-dendritic axis, which is necessary to validate our result suggesting homogeneous [Cl\(^-\)], along this compartment. Nevertheless, the occurrence of simultaneous [Cl\(^-\)] and pH\(_i\) gradients producing perfectly opposing effects on SCLM ratios is highly unlikely. This is supported by our observation that somato-dendritic pH gradients could not be detected with the Cl\(^-\)-insensitive but slightly pH-sensitive Cerulean-Venus protein that otherwise reported cell-to-cell differences in pH. In conclusion we found that under native conditions of the awake adult brain, pH\(_i\) differs between cells by a large margin.

We provide in vivo evidence of the essential role of KC\(\text{C2}\) in producing low neuronal [Cl\(^-\)] in adult mouse neurons (Kaila et al., 2014; Doyon et al., 2016). In doing so, we show that despite the limitations of the SCLM/SE-pHluorin based in vivo imaging approach, it could resolve changes in neuronal [Cl\(^-\)] that would be compatible to the ones expected during the developmental shift in [Cl\(^-\)] (Ben-Ari, 2002; Kaila et al., 2014). Thus, a plausible future perspective of this work would be to explore the developmental [Cl\(^-\)] shift in different genetically defined neuronal populations. The KO of KC\(\text{C2}\) is expected to impact neuronal activity as it is evidenced in reports using pharmacological inhibitors of KC\(\text{C2}\) in primary neuronal cultures, acute brain slices and in vivo (Sivakumaran et al., 2015). In consequence, this change in neuronal activity could affect pH\(_i\) (Chesler and Kaila, 1992). Nevertheless, the sparse AAV-driven Cre recombinase expression in KC\(\text{C2}\)lox/lox mice affects only a small subset of neurons in the cortex in contrast to the global network effect of pharmacological KC\(\text{C2}\) inhibitors which leads to epileptiform activity. Hence, even though the Cl\(^-\) driven synaptic inputs to KC\(\text{C2}\) KO neurons would likely cause excitation, the overall inputs to the neuron would not be as drastically enhanced as in an epileptiform event, consistent with the observed lack of effect of the KC\(\text{C2}\) KO on pH\(_i\). Nonetheless, further controls of neuronal activity in KC\(\text{C2}\) KO and Ctrl cells should be produced to fully address this point in future research.

Finally, our study provides a foundation for future in vivo research employing Cl\(^-\) and pH imaging in healthy and diseased tissue. While this article was under review, Sulis Sato et al. (2017) published an in vivo simultaneous Cl\(^-\) and pH imaging study using ClopHensor. In the following paragraphs we briefly compare the results produced by both sensors and discuss their strengths and caveats. In vivo 2P imaging of SCLM or SE-pHluorin showed a modest subcellular resolution. The main factor limiting this is the restricted 2P illumination settings needed to excite SCLM without saturating the Topaz emission channel, still obtaining reliable signal to noise levels in the Cerulean channel. Shorter 2P excitation wavelengths (800 nm) did not significantly improve this (data not shown). Alternatively, changing the relative sensitivity of the PMTs or replacing Cerulean for the higher quantum yield variant Teal, dramatically reduced the dynamic range of SCLM, thus practically abolishing the ability to resolve different [Cl\(^-\)]; (data not shown). Hence, the settings reported here represent the best compromise we found, still allowing us to explore the subcellular steady-state [Cl\(^-\)] between the soma and apical dendrite of pyramidal neurons. Sulis Sato et al. (2017) did not explore subcellular differences in [Cl\(^-\)] and pH\(_i\) due to better signal-to-noise levels at the soma compared to the dendrites, implying a similar limitation of ClopHensor.

The approach we describe to produce ratiometric Cl\(^-\) and pH estimations is not affected by isoflurane anesthesia. The anesthetized condition generally involves increased GABAergic inhibition (Maciver, 2014) and can have profound effects on breathing which could potentially alter cellular pH homeostasis (Massey et al., 2015). In the case of isoflurane, it has been reported to significantly enhance GABAergic inhibition in rat hippocampal slices and to mask the chemosensitivity of pH/CO\(_2\) sensitive serotonergic neurons of the medulla (Maciver, 2014; Massey et al., 2015). Despite these known effects of isoflurane, in vivo optical steady-state estimations of [Cl\(^-\)] and pH\(_i\) in L2/3 pyramidal neurons using SCLM and SE-pHluorin are not significantly affected by this anesthetic. The study of Sulis Sato et al. (2017) found similar [Cl\(^-\)] and pH\(_i\) under urethane anesthesia.

Our approach was also not affected by light scattering caused by tissue depth or uneven optical properties. The use of ratiometric sensors such as SCLM deep in scattering tissue is expected to be hampered by differential scattering of two spectrally different fluorescence emissions. This study was started...
with such expectation in mind and fluorescence life time imaging (FLIM) of the SCLM donor (Cerulean) was employed to obtain a depth-independent readout. However, the changes in life time as a function of [Cl$^{-}$] in in vitro calibration curves were too small in order to resolve changes of [Cl$^{-}$] in the physiologically relevant range (data not shown). The emission ratio of an environmentally less sensitive construct, Cerulean-Venus, did not significantly change up to a depth of approximately 350–400 µm. Therefore, motor cortex, and possibly brain tissue in general, appears to scatter emission light in the range from 480 nm to 540 nm in a manner so similar that changes in the ratio as a function of depth were not evident, suggesting that this spectral range can be used for ratiometric recordings without applying complicated depth-dependent corrections. This is reported to be fundamentally different when ratiometric indicators using the green-red range of the emission spectrum are employed, such as ClopHensor, as brain tissue shows a strong depth-dependent differential scattering for this range of the spectrum (Sulis Sato et al., 2017). On the other hand excitation light of different wavelengths can be differentially scattered as a function of imaging depth producing “distortions” on the absorption spectra of fluorescent proteins (Sulis Sato et al., 2017). Nevertheless, our ratiometric imaging approach required only one excitation wavelength, making it relatively less prone to be affected by this issue in comparison to ClopHensor (Sulis Sato et al., 2017). Again, this is supported by the observed lack of correlation between the imaging depth and the recorded ratios for Cerulean-Venus or EGFP expressing neurons in vivo.

Additionally the presence of blood vessels and fiber bundles can make the optical properties of brain tissue inhomogeneous, which can also cause scattering artifacts. By comparing the Cerulean-Venus ratios recorded in vivo to the ones recorded in hippocampal cultures we demonstrated that our ratiometric approach is not significantly affected by this factor. Altogether, we provide evidence supporting that ratiometric imaging in the emission range from 480 nm to 540 nm is robust against these inherent scattering-related issues of ratiometric imaging in intact brain tissue, an insight that will be relevant for in vivo applications of a multitude of genetically encoded indicators employing the CFP-YFP FRET pair.

The use of SCLM for intracellular Cl$^{-}$ estimations needs validation due to its pH sensitivity. Yet, simultaneous recordings of pH$_i$ and [Cl$^{-}$]$_i$ using this approach are technically not yet possible. The observed diverse range of neuronal pH$_i$ makes SCLM readouts quite variable between individual cells. This is a major limitation in SCLM imaging that demands a compatible method to simultaneously measure pH$_i$ and achieve calculations of [Cl$^{-}$]$_i$ for individual cells. Additionally, as neuronal activity also affects pH$_i$ (Chesler and Kaila, 1992), this also stresses the need of simultaneous pH$_i$ readouts to assess dynamic neuronal Cl$^{-}$ changes using SCLM.

In conclusion, this report contributes to the study of neuronal [Cl$^{-}$]$_i$ and pH$_i$ by providing estimations as close to the native state as practically possible. These results and approach represent a step forward towards the understanding of essential neuronal processes involving [Cl$^{-}$], in the intact organism and aid in the quest to relieve pathophysiological conditions such as epilepsy or schizophrenia (Kaila et al., 2014; Sullivan et al., 2015).

AUTHOR CONTRIBUTIONS

JCB, JK and TK designed research, reviewed and edited the manuscript. MK designed and constructed expression plasmids. JCB and JK performed imaging experiments. JCB performed data analysis and prepared figures. JCB and TK wrote the manuscript.

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