

Original Research

Imaging of Evoked Cortical Depolarizations Using Either ASAP2s, or chi-VSFP, or Di-4-Anepps, or Autofluorescence Optical Signals

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Abstract

Background: Population voltage imaging is used for studying brain physiology and brain circuits. Using a genetically encoded voltage indicator (GEVI), "VSFP" or "ASAP2s", or a voltage-sensitive dye, Di-4-Anepps, we conducted population voltage imaging in brain slices. The resulting optical signals, optical local field potentials (LFPs), were used to evaluate the performances of the 3 voltage indicators. **Methods**: In brain slices prepared from VSFP-transgenic or ASAP2s-transgenic mice, we performed multi-site optical imaging of evoked cortical depolarizations - compound excitatory postsynaptic potentials (cEPSPs). Optical signal amplitudes ($\Delta F/F$) and cEPSP decay rates (OFF rates) were compared using analysis of variance (ANOVA) followed by unpaired Student's *t* test (31–104 data points per voltage indicator). **Results**: The ASAP2s signal amplitude ($\Delta F/F$) was on average 3 times greater than Di-4-Anepps, and 7 times greater than VSFP. The optical cEPSP decay (OFF rate) was the slowest in Di-4-Anepps and fastest in ASAP2s. When ASAP2s expression was weak, we observed slow, label-free (autofluorescence, metabolic) optical signals mixed into the ASAP2s traces. Fast hyperpolarizations, that typically follow depolarizing cortical transients (afterhyperpolarizations), were prominent in ASAP2s but not present in the VSFP and Di-4-Anepps experiments. **Conclusions**: Experimental applications for ASAP2s may potentially include systems neuroscience studies that require voltage indicators with large signal amplitude ($\Delta F/F$), fast decay times (fast response time is needed for monitoring high frequency brain oscillations), and/or detection of brain patches in transiently hyperpolarized states (afterhyperpolarization).

Keywords: autofluorescence; cerebral cortex; excitatory postsynaptic potentials; temporal summation; paired pulse facilitation; photobleaching

1. Introduction

Functioning neurons generate electrical fields, which are best studied by electrodes [1]. However, voltage imaging can also be used to monitor neuronal electrical fields [2,3], especially when large areas are under investigation [4–8], or when the voltage imaging is done through an intact animal skull [9].

Genetically encoded voltage indicators (GEVI) have emerged as a valuable tool in experimental neuroscience [10–13]. The performance of newly developed GEVIs vary between laboratories, preparations, and applications [14,15]. Previously, we developed the GEVI, ASAP1 with a four-helix voltage-sensing domain. We mutated the positively charged residues in the fourth helix (S4) responsible for sensing the transmembrane electrical field. The resulting GEVI variant, ASAP2s, contained the R415Q mutation, which neutralizes one of the sensing charges in S4. ASAP2s showed an improved voltage responsiveness, slower offrate than ASAP1, and compatibility with two-photon imaging applications [16]. In the current study, we explore the properties of ASAP2s for population voltage imaging. Wide-field population voltage imaging methods lack cellular resolution. Mixed synaptic and action potentials arising from hundreds of dendrites and axons arrive onto the same optical detector. Such optical signal represents the "mean" response of many neurons. In population imaging experiments, the activity of many elements, such as desynchronized activations of hundreds of neurons, is mixed into one representative signal, a signal that represents a given population of cells projected onto the optical detector [17–22].

Using side-by-side measurements, including the same light source, optical path, detector, and stimulation paradigm, we compared three voltage indicators: two GEVIs (ASAP2s and VSFP) and one voltage-sensitive dye (Di-4-Anepps) [23,24]. Both GEVIs were congenitally expressed in cortical pyramidal neurons in transgenic mice, thus eliminating variables commonly associated with the quality of the adeno-associated virus (AAV) vectors or quality of intracranial injections.



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2. Materials and Methods

2.1 Animals

Brain slices were harvested from transgenic mice (age 25-90 days, both sexes) according to the institutionally approved animal protocol. All mice were housed in standard conditions with free access to food and water, in a 50% dark/light cycle. VSFP. The transgenic mouse line CaMK2A-tTA; tetO-chiVSFP was donated by Thomas Knopfel (Hong Kong Baptist University, Hong Kong, China). The chi-VSFP mice expressed chimeric voltage sensitive fluorescent protein (chi-VSFP) in all cortical pyramidal neurons [25]. ASAP. Generation of the Ai169-ASAP2s mouse line (Jax Lab #:031569, Bar Harbor, ME, USA) harboring TIT2L-ASAP2-ICL-tTA2, a Credependent ASAP2s gene at the TIGRE2.0 locus, has been previously described [26]. Initially, transgenic ES cells of a 129S6/SvEvTac x C57BL/6 F1 background were used to generate chimeric mice, which were bred to PhiC31expressing C57BL/6 transgenic mice (Jax Lab) to establish germline founders while removing a AttB/AttPflanked PGK-hygro-SV40polyA cassette in the transgene. These F1 founders were outcrossed to C56BL/6 three times, and F4 mice with TIT2L-ASAP2-ICL-tTA2 but lacking the PhiC31 gene were crossed to generate TIT2L-ASAP2-ICL-tTA2 homozygotes. These TIT2L-ASAP2-ICL-tTA2 homozygotes were then crossed with a Thy1-Cre mouse line (Jax Lab #006143) to generate mice expressing ASAP2s in all pyramidal cells, in all cortical layers. TIT2L-ASAP2-ICL-tTA2 homozygotes were also crossed to Cux2-CreERT2 mice in a C57BL/6 background (MMRC 032779, MMRRC at Univ. of California, Davis, CA, USA). Resulting double-hemizygous progeny were mated to each other and multiple double-homozygous Cux2-CreERT2 TIT2L-ASAP2-ICL-tTA2 were identified. These in turn were mated to each other to create a stable line in which tamoxifen induces ASAP2s expression in layer 2/3 cortical pyramidal neurons selectively. Two weeks prior to sacrifice and brain tissue collection, mice were fed a tamoxifen diet (TAM Diet (500, 2016), Teklad, ENVIGO, https://insights.inotivco.com/) for 2-7 consecutive days, ad libitum.

2.2 Immunohistochemistry

Following extraction, brains were fixed in 4% paraformaldehyde (PFA) for 24 h. After dehydration in rising sucrose concentrations (10, 20, and 30%) in 0.2 M phosphate buffer (PB), tissue was sliced using a cryostat and stored at -20 °C. Coronal brain sections (50 µm) were dehydrated in 0.01 M phosphate buffer saline (PBS) for 10 min and incubated in a blocking solution containing 10% bovine serum albumin (BSA) and 0.01% Triton-X 100 in 0.01 M PBS for 1 hour. Next, slices were incubated with a primary mouse anti- glial fibrillary acidic protein (GFAP) antibody (1:100, Aves Labs, Inc, Davis, CA, USA) or primary mouse anti-NeuN antibody (1:100, Merck Millipore, Burlington,

MA, USA) in 0.01 M PBS overnight at 4 °C. Slices were washed in PBS 3 times for 10 min and incubated with secondary goat anti-mouse AlexaFluor 555 antibody (1:200, Invitrogen, Waltham, MA, USA) for 2 hours. After washing in 0.01 M PBS 3 times for 10 min, slices were stained with nuclear counterstain Hoechst 3342 (1 μ g/mL) for 10 min, washed 4 times for 5 min in 0.01 M PBS and mounted using MOWIOL medium. Images of immunolabeled coronal brain slices were acquired using Keyence: BZ-X800 microscope (2x, 10x, and 20x lens; Itasca, IL, USA), DAPI (OP-87762), GFP (OP-87763), and TexasRed (OP-87765) filter set.

2.3 Synaptic Stimulation and Voltage Imaging

Following a deep anesthesia with isoflurane, mice of both sexes (ages P21-P56) were decapitated. Brains were extracted with the head immersed in ice-cold saline (artificial cerebrospinal fluid [ACSF] containing in mM: 125 NaCl, 26 NaHCO₃, 2.3 KCl, 1.26 KH₂PO₄, 2 CaCl₂ and 1 MgSO₄ and 10 glucose). Coronal slices (300 μ m) were cut from the frontoparietal cortex, incubated at 37 °C for 30 min, and then at room temperature. Acute brain slices were transferred to an Olympus BX51WI upright microscope $(10 \times \text{ objective}, 0.3 \text{ NA}; \text{Shinjuku-ku}, \text{Tokyo}, \text{Japan})$ and perfused with aerated (5% CO2/95% O2) saline. All experimental measurements were performed at 34 °C. Synaptic stimulation was achieved through a stimulus isolation unit (IsoFlex, A.M.P.I., Jerusalem, Israel). The stimulation electrodes (1.5 mm borosilicate glass with filament, resistance $\sim 2 \text{ M}\Omega$) were backfilled with saline. Triplets of synaptic shocks (1 ms duration, 135 nA) were delivered first at 120 ms inter-stimulus interval (ISI), 8.3 Hz (Train-1) and then, 1 second later, at 12 ms ISI, 83 Hz (Train-2). Optical trials, each containing two synaptic triplets, were 3 sec-long (3 sec of light exposure), with at least a 12 sec dark (no light exposure) period between two consecutive experimental sweeps. Optical signals were sampled at a 1.020 ms full-frame interval (~1 kHz frame rate) with a NeuroCCD camera (80×80 pixel configuration; RedShirtImaging, Decatur, GA, USA). Both GEVIs, ASAP2s, and chi-VSFP were excited using the same 470 nm light emitting diode, LED (pE, CoolLED, Andover, UK), and imaged using the same optical filter set: excitation: 480/40 nm; dichroic 510 nm, and emission: 535/50 nm. The light source for Di-4-Anepps was LED GYR 500-600 nm (pE, CoolLED, Andover, UK). The optical filter set for Di-4-Anepps included excitation 520/60 nm/dichroic 570 nm/emission 600LP.

2.4 Data Analysis

Optical traces were conditioned and analyzed in Neuroplex (RedShirtImaging). Bleach correction was performed by subtracting an exponential fit from the optical trace. Temporal averaging (n = 4 sweeps), spatial averaging (21 or 37 pixels), low-pass Gaussian filter with 100 Hz cutoff, and high-pass Tau filter (10) were also conducted. For

measurements of optical noise (root mean square, RMS) and signal-to-noise ratio (SNR), the low-pass filter was not used. Optical signal amplitude was measured as fractional change in light intensity ($\Delta F/F$). The 4th optical peak, caused by a triplet of synaptic inputs at 83 Hz (Train-2) was used to quantify amplitudes in section 3.3. The 1st optical peak, caused by a triplet of synaptic inputs at 8.3 Hz (Train-1), was used to quantify amplitudes in section 3.7. Decay rates of compound EPSPs (cEPSPs) were measured as fractions of the peak amplitude remaining 90 ms after the occurrence of the cEPSP peak. In this setting, a large fraction remaining 90 ms after the peak indicates slow decay of cEPSP. Lost light due to photobleaching was calculated by measuring the amplitude difference between two time points on the same trace (in mV); the two time points were selected at 125 ms and 2990 ms from the beginning of the optical sweep. Resting fluorescence intensity (RFI) was measured in the same region of interest (ROI) in which the photobleaching and GEVI signal amplitude were measured. RFI is expressed in arbitrary units (a.u.) after adjusting for the illumination intensity (light power at the object plane). Data organization, plotting, and statistical testing using analysis of variance (ANOVA) and unpaired Student's t-tests were done in Excel.

3. Results

Three strains of transgenic mice were used to prepare acute brain slices for electrophysiology optical imaging experiments. Wild type mice were used for experiments with autofluorescence optical signals and voltage-sensitive dye, Di-4-Anepps. Each brain slice (experimental groups varied in size from 31 to 104 brain slices) was stimulated in an identical manner and simultaneous multisite optical recordings were performed using the same experimental setup (light source, optical filters, dichroic mirror, objective lens, and camera). We present a comprehensive analysis of voltage indicator performances, and we point to both advantages (optical sensitivity, speed of response, afterhyperpolarization, spatial spread of voltage transiens) and disadvantages (signal decay time, photobleaching, autofluorescence optical signal bleed-through) attributed to each indicator.

3.1 Expression of ASAP2s in Cerebral Cortex

Crossing of the Thy1-Cre and ASAP2s_LoxP mouse lines achieved strong labeling of all cortical layers, with the exception of the L4 lamina. That is, in the ASAP2s channel (green), we found a darker horizontal band coinciding with cortical layer 4 (Fig. 1). This darker band was not caused by a lower density of cells, as both the nuclear stain, Hoechst (Fig. 1A₁), and the glial stain, GFAP (Fig. 1A₂), detected normal density of cellular elements (Fig. 1A₂, no abnormal features = none). Pyramidal neurons comprise the majority of nerve cells in neocortex (~80%). Overlapping dendritic trees appear as a "sea of light" with pyramidal cell bodies showing as "dark holes" (Fig. $1B_1-B_4$). This finding applies to both Thy1-ASAP2s (Fig. $1B_1-B_4$) and VSFP animals [27].

In tamoxifen-fed Cux2-CreER-ASAP2s mice, ASAP2s expression was restricted to L2/3 pyramidal cells (Fig. 2A). The ASAP2s-labeled axons of L2/3 pyramidal neurons could be traced in corpus callosum (CC), superior portions of neostriatum (Fig. 2A2, cortico-striatal projections, csp), and cortical L4 (Fig. 2B, L4). At higher magnifications, we were able to delineate cell bodies of L2/3 pyramidal cells (Fig. 2C). Unlike Thy1-ASAP2s, in Cux2-ASAP2s mice, the apical dendrites of L4, L5, and L6 pyramidal cells are not labeled, suggesting there was less background fluorescence. This allowed the cell bodies of L2/3 pyramids to stand out against the background (Fig. 2C), which was not the case in Thy1-ASAP2s or VSFP mice. In Thy1-ASAP2s and VSFP mice, fluorescent protein (GEVI) was in all neurites (dendrites and axons) of all pyramidal neurons, in all cortical layers, hence pyramidal cell bodies did not stand out against the background.

3.2 Interplay between ASAP2s and Label-Free Autofluorescence Metabolic Optical Signals

In brain slices collected from wild type mice, we performed optical recording sessions in the absence of GEVIs or dyes (Fig. $3A_1$). In Fig. 3A, we marked the beginning of the synaptic stimulation train by a gray dashed vertical line. In 24 out of 27 brain slices tested in this way, we observed synaptically-evoked optical signals in the green optical channel (Fig. 3A2, Autofluorescence Signal). The mean amplitude of synaptically-evoked autofluorescence optical signals, measured at the stimulation site (ROI-1) in layer 2/3, was 0.99 \pm 0.10 % Δ F/F (mean \pm s.e.m., n = 24). In 6 brain slices, following the autofluorescence signal recordings, we applied Di-4-Anepps (Fig. 3A₃). Without changing stimulation parameters or visual field, we then recorded synaptically-evoked Di-4-Anepps signals (Fig. 3A₄). We used Di-4-Anepps signals in the same brain slice to demonstrate that: [1] the brain slice was alive; [2] synapses were activated; and [3] cortex was depolarized. In these experiments, an average amplitude of synaptically-evoked autofluorescence optical signal before voltage-dye staining was 0.89 ± 0.28 % (n = 6). In the same ROI, the average amplitude of synaptically-evoked Di-4-Anepps optical signal (after staining) was 0.64 ± 0.08 % (n = 6). Autofluorescence optical signals (excitation 480/40 nm) and voltagesensitive dye signals (excitation 540 nm) were of an opposite polarity (Fig. $3A_1 - A_4, B_1 - B_4$). Note that optical traces are displayed with inverted polarity.

Two types of optical signals, autofluorescence and voltage sensitive dye (VSD), exhibited different temporal dynamics. In Fig. 3B, we marked the end of the synaptic stimulation train by a gray dashed vertical line. While the VSD signal begins to decay immediately at the end of



Fig. 1. Thy1-ASAP2s mice. ASAP2s is expressed in all pyramidal cells of all cortical layers. (A₁) A brain section (30 μ m) from a Thy1-ASAP2s mouse, stained with nuclear stain, Hoechst (10× lens, composite image). The granular layer is marked by two white lines. (A₂) Same brain section – expression of the glial marker GFAP. None – indicates a lack of interesting features in L4. (A₃) Expression of ASAP2s in the GFP optical channel. Fluorescence is weak in L4 – dark band. (A₄) White lines on the right-hand side delineate cortical layers. w.m., white matter. (B₁) A different brain section, nuclear stain (Thy1-ASAP2s animal, layer 2/3, 20× lens). (B₂) Neuronal marker NeuN. (B₃) Expression of ASAP2s. Because ASAP2s molecules are located in the membrane, and all neurons are labeled, the neuropile is "fused" and the neuronal cell bodies appear as dark holes. (B₄) A merge of three channels. Arrows marks two out of several NeuN-negative (non-neuronal) cells in this field of view (FOV). GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein.

the synaptic stimulation train (Fig. $3B_4$), the autofluorescence optical signal was still rising after the end of the stimulus train (Fig. $3B_2$), eventually achieving its peak (p) several hundred milliseconds after the end of the stimulus train (Fig. $3B_2$, arrow). A slow temporal dynamic of the autofluorescence signal (Fig. 3A₂), different from that of the VSD signal (Fig. $3A_4$), suggests that autofluorescence optical signal were not reporting membrane voltage [28]. Furthermore, the lateral propagation of the autofluorescence optical signal along the cortical lamina 2/3 (Fig. $3B_1,B_2$), was notably weaker than the propagation of the corresponding VSD signals in the same brain slice, n = 6 (Fig. 3B₃, B₄). At 550 µm away from the stimulation site, the autofluorescence optical transients were either undetectable or extremely small (Fig. 3B1,B2, ROI-4), while at same distance from the stimulation site, 550 μ m (Fig. 3B₃,B₄, ROI-4), the VSD signals were clearly distinguishable (n = 6).

The amplitudes of the autofluorescence optical signals tended to decrease with repetitive measurements, from *Trial-1* to *Trial-2*, for example (compare Fig. $3C_1$, ROI-1 versus Fig. $3C_2$, ROI-1). In the autofluorescence optical imaging, experimental trials (*Trial-1* to *Trial-3*) were separated by 60 sec. Each trial was an average of 4 sweeps, 12–25 sec apart. On average, the autofluorescence signal amplitude in the second trial was 88.4 + 2.2 % (n = 24) of the amplitude measured in the first trial (Fig. $3C_3$). On average, the signal amplitude in the third trial, *Trial-3*, was 87.9 + 4.3 % (n = 20) of the amplitude measured in the first trial, *Trial-1* (Fig. $3C_3$).

Both the autofluorescence and ASAP2s signals were recorded in the same optical channel (green fluorescent protein, GFP, excitation 480/40 nm), meaning there was a possibility of autofluorescence optical signals interfering (mixing) with the ASAP2s signals. By reducing the number of days that the Cux2-ASAP2s animals were fed the ta-



Fig. 2. Cux2_ASAP2s animal. ASAP2s is expressed in layer 2/3 pyramidal cells. (A₁) A brain slice (300 μ m-thick) from a tamoxifenfed Cux2-ASAP2s mouse, captured in Texas Red optical channel (2× lens). Vertical impressions were made by nylon strings of the slice-anchor, during optical recordings. (A₂) Same brain slice captured in GFP channel. Axon fibers of L2/3 pyramidal cells are detected in corpus callosum (CC), and in superior striatum (cortico-striatal projections, CSP). (A₃) Merge red (autofluorescence) and green (ASAP2s) channel. Cx, cortex; Str., striatum. Longitudinal impressions in brain tissue were made by an anchor used to hold brain slice during synaptic stimulation. Scale, 1 mm. (B) Pyramidal neurons in the superficial cortical layers (10× lens, composite image). Bright fluorescence in L2/3 is from dendrites of L2/3 pyramidal cells. A weak fluorescence in L4 is from axons of the L2/3 pyramidal cells. Scale, 200 µm. (C) Cell bodies of L2/3 pyramidal cells expressing ASAP2s (20× lens). Four out of many pyramids are label with arrows. Scale, 50 µm.

moxifen (TAM) diet, from 7 to 2-3 days, we were able to achieve weak expression of GEVI. In weakly expressing ASAP2s mice, we observed slow negative undulations superimposed onto fast voltage signals (n = 4 brain slices, Fig. 3D, black traces). To illustrate this point, we have included the autofluorescence optical signal from Fig. $3C_2$ onto the ASAP2s optical signal (Fig. 3D, thin and thick trace). In weakly-expressing ASAP2s mice, the autofluorescence optical signal influenced the ASAP2s optical signal (Fig. 3D). However, in Cux2-ASAP2s mice with strong expression of ASAP2s, the autofluorescence optical signal was eliminated by the strong baseline fluorescence of ASAP2s (resting fluorescence level). In other words, in strongly expressing ASAP2s brain slices, or strongly expressing VSFP brain slices, the autofluorescence optical signal was eclipsed by the strong GEVI signal (present study); or strong expression of a fluorescent calcium sensor [29]. This was obvious in the time windows ~400 ms after the end of the stimulation train, in which the negative peak of the autofluorescence optical signal was expected to deviate the ASAP2s optical signal (Fig. 3E). Instead, the slow, negative waves belonging to the autofluorescence optical signals were not seen in these ASAP2s recordings (n = 30 slices). In remote ROIs (Fig. 3E, ROI-4), where autofluorescence optical signal is virtually non-existent (thin trace), the ASAP2s optical signal showed a fast negative transient, akin to afterdepolarization (thick trace).

3.3 VSFP, Di-4-Anepps and ASAP2s, Side-by-Side Comparisons

Using an identical experimental paradigm (8.3 Hz and 83 Hz stimulation) and the same recording setup (same light source, objective lens, and camera), we compared the performances of 3 voltage indicators, two GEVIs (VSFP and ASAP2s), and one VSD (Di-4-Anepps). Three synaptic



Fig. 3. Optical signals of various origins evoked by identical stimuli. (A1) Autofluorescence in brain slice from wild-type mice before application of dye. The GFP optical channel filters are described in white font. (A2) Synaptically-evoked optical signals in an unstained slice - GFP channel. Each region of interest (ROI) is a spatial average of 37 pixels. Each trace is temporal average of 4 subsequent sweeps (20 sec inter-sweep interval). Vertical dashed lines mark onsets of the stimulus trains. (A₃) Same brain slice, same field of view (FOV) and stimulus, except a voltage sensitive dye, Di-4-Anepps, was injected into the bath. LED and Di-4-Anepps optical filter set described in white font. (A₄) Di-4-Anepps optical signals. (B₁) Autofluorescence (GFP channel) in brain slice from transgenic Cux2-ASAP2s mice, in which tamoxifen induction was unsuccessful. (B2) Synaptically-evoked autofluorescence optical signals. The peak of this label-free optical signal ("p") occurs >100 ms after the last stimulus pulse in train (vertical dashed line). (B₃) same brain slice after application of Di-4-Anepps. (B₄) The peak of the Di-4-Anepps optical signal ("p") coincides with the last stimulus pulse in the train. (C_1, C_2) Two subsequent experimental trials in the same FOV, same stimulus intensity. Amplitude of the autofluorescence optical signal declines in the later trial (Trial-2, lost amplitude). (C₃) Changes in the optical signal amplitude across 3 subsequent experimental trials (n = 24 recordings in 22 brain slices). (D) Optical signals obtained in Cux2 mice with a very weak expression of ASAP2s. Negative deflections in the ASAP2s optical signal (black) suggest an underlying autofluorescence optical signal (gray). In this and the following panes, thin black traces are autofluorescence optical signals from C₂, superimposed onto the ASAP2s optical signal (thick black trace). (E) Optical signal in brain slice from a Cux2-ASAP2s mouse in which expression of the ASAP2s gene was strong (tamoxifen, TAM diet, 7 days). Rectangles mark time windows in which an autofluorescence optical signal, if present, should inflict a negative deflection. ROI-4 is "too far away" from the stimulation site (ROI-1), for label-free (metabolic) optical signals to reach it, but not for the ASAP2s (voltage) optical signals. All optical signals in this figure are shown with inverted polarity.

transients separated by 120 ms (8.3 Hz) were clearly delineated by VSFP (Fig. 4A, 8.3 Hz), Di-4-Anepps (Fig. 4B, 8.3 Hz), and ASAP2s (Fig. 4C, 8.3 Hz). Three synaptic transients separated by 12 ms (83 Hz) were not delineated as the membrane voltage did not have enough time to repolarize between stimuli. Instead, the 83 Hz synaptic triplet appeared as a one complex voltage transient (summation) in recordings made by VSFP (Fig. 4A, 83 Hz), Di-4-Anepps (Fig. 4B, 83 Hz), or ASAP2s (Fig. 4C, 83 Hz). We used the peak of the 83 Hz event to quantify optical signal amplitudes $\Delta F/F$ in %. We found that the mean amplitude in VSFP, VSD, ASAP Cux2, and ASAP Thy1 recordings were 0.341 ± 0.007 % (n = 104), 0.786 ± 0.035 (n = 31), 2.809 ± 0.136 (n = 37), and 3.032 ± 0.121 % (n = 71), respectively (Fig. 4D). These data indicate that ASAP2s signals are on average 7-fold stronger than VSFP signals and 3-fold stronger than VSD signals when it came to synaptically-evoked compound EPSPs (extracellular stimuli delivered in Layer 2/3). An ANOVA was conducted to compare the effect of voltage indicator on the optical signal amplitude. Comparisons were made between experimental groups: VSFP, Di-4-Anepps, ASAP2s Cux2, and ASAP2s Thy1. There was a significant difference in optical signal amplitude (p < 0.01) for the 4 experimental groups, F (3, 239) = 309.72, $p = 4.95 \times 10^{-82}$. Post-hoc testing determined that VSFP was significantly smaller than VSD (p < 0.00001), VSD was significantly smaller than ASAP2s Cux2 (p < 0.000001), and VSD was significantly smaller than ASAP2s Thy1 (p < 0.000001). There was no statistically significant difference in optical signal amplitude between ASAP2s Cux2 versus ASAP2s Thy1 (p = 0.2534). Overall, these data indicate that in the context of evoked cEPSPs, the optical sensitivity of ASAP2s is the highest among the 3 indicators tested.

We selected the best traces from VSFP (n = 18), ASAP2s (n = 15), and Di-4-Anepps recordings (n = 14) and calculated optical noise (RMS) in 40 sampling points (40.8 ms duration), 10 ms prior to the onset of the stimulus. The average RMS in VSFP, ASAP2s, and Di-4-Anepps traces were remarkably similar: 0.0430 ± 0.0019 (n =18), 0.0437 ± 0.0020 (n = 15), and $0.0416 \pm 0.0031\% \Delta$ F/F (n = 14). The SNR was calculated as the peak signal amplitude divided by RMS. The average SNR in VSFP, ASAP2s, and Di-4-Anepps traces was 15.34 ± 0.85 (n = 18), 87.77 \pm 4.89 (n = 15), and 30.29 \pm 2.13 (n = 14). In the context of evoked cEPSPs, ASAP2s produced optical signals with the highest SNR. An indicator with less bleaching at a given standard excitation intensity could be illuminated with more intense light while keeping the available usable recording time. More intense light would increase the indicator's SNR [30]. One potential approach to address the relation between illumination intensity and SNR would be to normalize the calculated SNR by the bleaching time constant. However, this was not done in the present study.

Interestingly, the decay phases of the cEPSP showed different temporal dynamics between the 3 voltage indicators. We estimated the "rate of decay" by measuring the fraction of the optical signal (peak = 100%) remaining 90 ms after the onset of the extracellular stimulus (Fig. 4E, gray ball). We found that VSD optical signals showed the slowest decay. In the VSD recording mode, at 90 ms post stimulus, the optical signal repolarized down to only 26.16 \pm 1.65 % (n = 31) of its peak value. In the VSFP recording mode, the optical signal repolarized down to 18.05 ± 0.71 % (n = 104), while in the ASAP2s experiments the optical signals repolarized near-completely; 5.57 ± 0.8 % (n = 71) for Thy1 promoter and 1.11 ± 1.33 % (n = 37) for the Cux2 promoter (Fig. 4F). An ANOVA was conducted to compare the effect of GEVI on the decay phase of the cEPSP optical signal. Comparisons were made between experimental groups: Di-4-Anepps, VSFP, ASAP2s Thy1, and ASAP2s Cux2. There was a significant difference in optical signal decay rate at the p < 0.01level for the 4 experimental groups, F (3, 239) = 101.78, $p = 1.7 \times 10^{-42}$. Post-hoc testing determined that cEPSP decay phase in VSD group was significantly slower than in the VSFP group (p < 0.000001) and the ASAP2s Thy1 group ($p = 7.13 \times 10^{-23}$), and significantly slower than in the ASAP2s Cux2 group (p < 0.000001). The cEPSP decay phase in the VSFP group was significantly slower than in either ASAP2s_Thy1 (p < 0.000001) or ASAP2s_Cux2 (p < 0.00001) groups. There was a statistically significant difference between EPSP decay rates in optical recordings using ASAP2s Thy1 versus ASAP2s Cux2 (p = 0.0028). Overall, these data indicate that the repolarization phase of an evoked cEPSP was the slowest in the Di-4-Anepps group (Fig. 4). These data may also suggest that optical- local field potentials (LFP) decay-rate is inversely proportional to the number of circuit elements contributing to compound (population) optical signal. In Di-4-Anepps experiments, all membranes were labeled with fluorescent indicator, hence the slowest signal. In ASAP2s_Cux2 experiments, where ASAP2s expression is significantly weaker than in ASAP2s Thy1 experiments, the fewest membranes were labeled with fluorescent indicator, and hence the fastest EPSP decay rate. The difference in decay time could be attributed to various factors. It's essential to consider the GEVI or VSD's real-time dependence on the membrane potential change, the membrane components of the signal source, and autofluorescence's potential impact on ASAP2s and chi-VSFP.

3.4 Propagation of the Depolarization Wave

Using the same experimental paradigm (8.3 Hz) and recording setup, we evaluated the spread of synapticallyevoked depolarization along the L2/3 cortical lamina, in VSFP, Di-4-Anepps, and ASAP2s experiments. A glass stimulation electrode was positioned in L2/3, and evoked optical signals were recorded simultaneously in 5 regions



Fig. 4. Three voltage indicators. (A) Transgenic mice expressing VSFP in cortical pyramidal neurons. Surface of brain slice with stimulating electrode in L2/3. Stimulation was the same as in Fig. 3. Voltage indicator is VSFP. (B) Same as in A, except the subject was a wild-type mouse (Black 6) and the voltage indicator is voltage-sensitive dye, Di-4-Anepps. (C) Same as in A, except mouse was transgenic, expressing voltage indicator ASAP2s in pyramidal neurons (Thy1). (D) Signal amplitude at the stimulation site ROI 1. Four experimental groups based on the voltage indicator used. Each dot represents one measurement in one brain slice. VSFP n = 104, VSD n = 31, ASAP-Cux2 n = 37, ASAP-Thy1 n = 71. The ASAP2s signal amplitude was not affected by the type of transgenic mouse, Thy1-Cre or Cux2-CreER (no significant difference). (E) The signal decay dynamics was quantified at 90 ms after the stimulus pulse; and expressed as a percentage of the first peak amplitude. (F) Quantifications of the signal decay rate (explained in E) are plotted here as raster and bar graphs, where "bar" indicates mean \pm standard error of mean, SEM. *** p < 0.0001; # p > 0.05. Same number of experiments (n) as in panel D. GEVI, genetically encoded voltage indicator; VSFP, GEVI made by the T. Knopfel laboratory; VSD, voltage sensitive dye.

of interest, including the stimulation site (Fig. 5A₁, ROI-1). Typically, amplitude of optical signal decreased with distance from the stimulation site (Fig. 5A₂, from ROI-1 to ROI-5). In the VSFP group (n = 20), at ROI-5 (positioned 610 μ m away from the stimulation site ROI-1), the amplitude of the optical signal was on average 9.9 \pm 2.2% of the amplitude measured at the stimulation site (ROI-1). In the Di-4-Anepps group (n = 20) the ROI-5 amplitude was 19.6 \pm 2.3% of the ROI-1 amplitude. In the ASAP2s_Thy1 group (n = 20), the ROI-5 / ROI-1 ratio was 16.5 \pm 2.0%. At ROI-5, both Di-4-Anepps experiments and ASAP2s experiments received a larger fraction of the propagating voltage signal than the VSFP experiments (Fig. 5A–C). At ROI-5, unpaired *t*-tests determined statistically significant difference between VSFP versus Di-4-Anepps (p = 0.00444, Fig. 5B₃, asterisk) and between VSFP versus ASAP2s (p = 0.03446, Fig. 5C₃, asterisk). No significant difference was detected between the Di-4-Anepps and ASAP2s experimental groups (p = 0.3247). Overall, these data indicate that optical sensitivity of Di-4-Anepps and ASAP2s was better than that of VSFP, and thus more suitable for studying the spread of evoked cEPSPs through cortical neuropil.



Fig. 5. Spatial spread of synaptically evoked voltage transients assessed by three voltage indicators. (A₁) Transgenic mice expressing VSFP in cortical pyramidal neurons. Surface of brain slice with stimulating electrode in L2/3. A triplet of stimulus pulses was delivered at 120 ms interval (8.3 Hz). (A₂) Synaptically evoked cortical depolarizations were imaged in five ROIs simultaneously (ROI 1-5). (A₃) Signal amplitude at each ROI is normalized by the signal amplitude obtained at the stimulation site ROI 1. Each bar is a mean \pm sem of 20 measurements (20 brain slices, from 12 mice). (B₁–B₃) Same as in A, except this animal is a wild type, and voltage indicator is Di-4-Anepps (n = 20 brain slices, 9 mice). (C₁–C₃) Same as in A, except mouse is transgenic, expressing voltage indicator ASAP2s in pyramidal neurons (Thy1 promoter) (n = 20 brain slices, 7 mice). (A₃,B₃,C₃) Dashed line marks the best fit (linear), with R value displayed. The negative slope of the distance-dependent amplitude decay was slightly steeper for the VSFP data (–0.231) compared to the Di-4 data (–0.208), or the ASAP2s data (–0.216). The normalized ROI-5 amplitude was compared between three experimental groups: VSFP, Di-4 and ASAP (unpaired *t*-test). VSFP produced significantly smaller amplitudes at ROI-5 than the other two indicators did (*, *p* < 0.05).

3.5 Temporal Summation

Using the same experimental paradigm (8.3 Hz) and recording setup, we evaluated the temporal summation of synaptically-evoked depolarization in VSFP and ASAP2s experiments. A glass stimulation electrode was positioned in L2/3 and synaptically evoked optical signals were recorded at the stimulation site. We show 5 brain slices from the VSFP experimental groups (Fig. 6A) and 5 brain slices from the ASAP2s group (Fig. 6B). Interestingly, in the VSFP experimental series, the 3rd synaptic event often exhibited a higher amplitude than the 1st synaptic event

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(Fig. 6A, dashed horizontal line), reminiscent of temporal summation of synaptic potentials (paired pulse facilitation). On the contrary, in the ASAP2s experiments, the 3rd synaptic event often showed a smaller amplitude than the 1st synaptic event in the same optical trace (Fig. 6B, dashed horizontal line), reminiscent of synaptic depression or synaptic fatigue. Note that both VSFP and ASAP2s signals were recorded in the same optical channel in which we detect autofluorescence optical signals (GFP, excitation = 470 nm, emission = 510 nm). However, VSFP signals show facilitation (Fig. 6A) while the ASAP2s signals show depression, on the same experimental paradigm.



Fig. 6. Temporal dynamics of the two genetically encoded voltage indicator (GEVI) variants, VSFP and ASAP. (A) Three synaptic pulses were delivered into L2/3 at 120 ms interval (8.3 Hz). Five brain slices expressing VSFP – one trace per slice. Red arrow indicates that the 3rd peak is of a greater amplitude than the 1st peak. Black arrows emphasize that membrane voltage is always above the resting membrane potential (beige horizontal line). Each trace is an average of 12 trials. (B) Identical stimulus as in A. Five traces from 5 brain slices expressing ASAP2s. Red arrow indicates that the 3rd peak is of a lower amplitude than the 1st peak. Black arrow indicates voltages more negative than the resting potential. Each trace is an average of 3 trials. Note different amplitude scales in A versus B. (C₁) Five recordings from 5 brain slices in the VSFP group are shown with normal polarity and without a correction for bleaching. (C₂) Same as in C1 except, the mouse is ASAP2s. Note a 5-fold difference in the mV scales. (C₃) The average amount of lost light (in mV, per 3 second of light exposure) is plotted for 3 experimental groups. (C₄) Resting fluorescence intensity (RFI) for 3 groups analyzed in C₃. (C₅) Optical signal amplitudes for the data shown in C₃. (C₆) Each data point has two coordinates x and y; where "x" is RFI (arbitrary units) and "y" is signal amplitude (Δ F/F). Each data point is from a different brain slice. Inset: ASAP2s and VSFP traces on the same scale, amplitude = 1%, time = 50 ms. (D) Brain slice obtained from a VSFP transgenic mouse. Synaptically-evoked cortical depolarizations are first recorded in the green emission mode (d1), and then in the red emission mode (d2). Each trace is an average of 4 trials. Scale, 200 µm.

3.6 Apparent Cortical Hyperpolarization

Another difference between VSFP and ASAP2s was the absence or presence of a fast negative voltage transient following the evoked depolarizing voltage transient in cortical layer 2/3. This negative voltage transient is dubbed "apparent hyperpolarization" (Fig. 6B). First, we made sure that we conditioned optical traces in both experimental groups with an identical set of low-pass and highpass digital filters. In the VSFP experimental series (n = 104brain slices, 34 mice), on their return to the baseline (repolarization phase) the optical traces did not cross the baseline established before the arrival of the stimulus (Fig. 6A, baseline is marked by a beige horizontal line). VSFP traces approached the baseline, but never crossed (Fig. 6A, black arrow). Conversely, in the ASAP2s experiments (n = 30)brain slices, 10 animals), optical traces regularly crossed the baseline established before the arrival of the stimulus (Fig. 6B, purple horizontal line). The ASAP2s traces traversed the baseline after the 1st, 2nd, and 3rd synaptic event (Fig. 6B, black arrows). In brain slice experiments, the baseline established before stimulation was reminiscent of a resting membrane potential, and negative deflections in the ASAP2s optical signal (Fig. 6B, black arrows) are reminiscent of cortical hyperpolarizations in population voltage imaging experiments [31,32]. Fast hyperpolarizations in ASAP2s traces (Fig. 6B, black arrows) were not caused by underlying autofluorescence optical signals (Fig. $3C_1$), because weak autofluorescence signals have a slower dynamic (Fig. $3B_2$), autofluorescence signals are eclipsed by the high background GEVI fluorescence (Fig. 3E, ROI-1), and rapid negative voltage transients can be detected at distances from the stimulation electrode at which autofluorescence signals are non-existent (Fig. 3E, ROI-4). Optical traces displayed in Figs. 1,2,3,4,5 have been conditioned by subtracting an exponential function that mimics the exponential decline in RFI, known as "photobleaching". Several factors might cause a gradual shift (decline in optical signal), including heat drift in LED light sources, electron fluctuations in the CCD device over time, and photobleaching of the fluorophore. Next, we will address the bleaching of the two GEVI variants, VSFP and ASAP2s, using the same LED source and CCD camera.

3.7 Photobleaching and Dual Emission

The camera used in the current study (Neuro-CCD, RedShirtImaging) converted the incoming photon flux into voltage (current-to-voltage converter). The RFI (in mV) is the fluorescence level at the beginning of an optical recording sweep (e.g., time = 0 ms). In any type of a fluorescence measurement, RFI at the beginning is different from that recorded at the end of an optical sweep (e.g., time = 3000 ms), due to photobleaching. In the current study, the bleaching rate of a fluorescent indicator was quantified by measuring the amount of the RFI reduction (in mV) during a 3-second optical sweep (Fig. 6C1-C3, lost light). Contrary to other figures, in Fig. 6C1, C2, optical traces are displayed with normal polarity. We found that photobleaching in the VSFP recordings was approximately 10-fold slower compared to the photobleaching occurring in the ASAP2s experiments (Fig. $6C_1, C_2$). More specifically, the amount of *lost light* per 3 seconds in VSFP was 31.1 ± 1.3 mV (n = 28), where as in ASAP2s Thy1 and ASAP2s Cux2 experiments, the "lost light" was about 10-fold higher, 476.8 \pm $37.99 \text{ mV} (n = 33) \text{ and } 505.84 \pm 38.26 \text{ mV} (n = 31)$, respectively (Fig. $6C_3$). In VSFP experiments, the RFI measured at the beginning of the optical trace was 5.993 ± 0.568 a.u. (n = 28), which was notably higher than the RFI achieved in ASAP2s Thy1, 2.011 \pm 0.158 a.u. (n = 30). In the ASAP2s Cux2 experiments, the RFI was low due to weak

tamoxifen-induction of the ASAP2s expression, 0.319 \pm 0.046 a.u. (n = 31), (Fig. 6C₄). Next, using only the data used in panels C_3 and C_4 of Fig. 6, we quantified amplitudes of the first voltage transient (8.3 Hz train). These amplitude measurements were consistent with the amplitude measurements shown in Fig. 4D that were made on a larger experimental set (e.g., n = 104), and using the 4th voltage peak caused by an 83 Hz train (Fig. 4D). Despite a strong bleaching in ASAP2s (Fig. $6C_3$) and weaker fluorescence levels (Fig. $6C_4$), the ASAP2s optical signals consistently showed higher optical signal amplitudes ($\Delta F/F$) compared to the VSFP signals (Fig. $6C_5$). In Fig. $6C_6$ (inset), VSFP and ASAP2s traces are displayed on the same amplitude scale (1%, $\Delta F/F$). To illustrate the relation between RFI and the optical signal amplitude in the same ROI where the RFI was measured, we plotted an Amplitude vs RFI graph (Fig. $6C_6$). Each data point is the product of two coordinates: x-axis (RFI), and y-axis (Amplitude). The VSFP group showed strong RFI levels but weak optical signal amplitudes (Fig. $6C_6$, beige data points). The ASAP2s Cux2 group, in which expression of ASAP2s was induced by a 2 to 7-day tamoxifen diet, showed weak RFI, but a strong optical signal (Fig. $6C_6$, red data points). The ASAP2s Thy1 group, in which expression of ASAP2s was driven by Thy1 promoter, since the animal's conception, demonstrated stronger RFI levels than the ASAP2s_Cux2 group and strong optical signals (Fig. 6C₆, purple data points).

While ASAP2s emits only in the green channel, VSFP generates a dual emission in both green and red, stemming from the presence of two fluorophores in each VSFP indicator molecule [33]. When excited by 488 nm light, the voltage indicator VSFP glowed in green channel (emission 510-545 nm), but also in the red spectra (emission 578-625 nm) [27]. In the same brain slice (Fig. 6D), we recorded synaptically evoked optical signals first in the green (donor) and then in the red emission channel (acceptor). These recordings produced signals of opposite polarity (n = 6 brain slices). Invariably, the signal to noise ratio in the green emission channel (Fig. 6D, green emission) was better than in the red emission channel (n = 24 ROIs in 6 brain slices). The dual emission of VSFP (Fig. 6D) could be useful for in vivo experiments, in settings in which both channels, donor and acceptor, are recorded simultaneously [25]. Optical signals in the red channel could potentially be used to correct for mechanical artifacts, as well as for conditioning optical signals.

4. Discussion

4.1 Signal Polarity

The true polarity of VSFP and ASAP optical signals is shown in Fig. $6C_1,C_2$. All three indicators used in the current study (VSFP, ASAP2s, and Di-4-Anepps) decreased light with depolarization (negative optical signal). On the contrary, autofluorescence optical signals increased the intensity of emitted light (510 nm) with cortical depolarization (synaptic stimulation). In all figures of the current manuscript, except in Fig. $6C_1,C_2$, optical traces are shown with inverted polarity. We feel that in display (presentation), voltage imaging optical signals (VSFP, ASAP2s, and Di-4-Anepps) should increase with depolarization.

4.2 Population Imaging

Wide-field population imaging (macroscopic, mesoscopic) and fiber photometry lack single-cell resolution. Activity of many neurons is mixed into one representative signal; a signal that represents a given population of neurons [17–22,34–37]. In the current population imaging approach, single-cell resolution was lost due to dense neuron labeling with fluorescence indicators (Fig. 2A,B), indiscriminate expression of fluorescent indicator in all neuronal compartments (dendrites and axons, Fig. 1B₃), absence of axial sectioning (single-photon wide-field illumination), and low magnification + thick focal volume ($10 \times$ objective lens, one pixel covers ~ $20 \times 20 \ \mu m$ of the brain slice surface, Figs. 4,5).

4.3 Origin of GEVI Physiological Signal

In Figs. 3,4,5,6, all optical signals were evoked by synaptic stimulation, involving extracellular current pulse delivered in L2/3. Depolarization responses of many L2/3 pyramidal cells were projected to the same detector pixel, and for that reason the reported optical signals are similar to LFP, which are known to be dominated by synaptic potentials [38]. Population voltage imaging has a better spatial resolution than the LFP signal [17,38]. Electrical LFPs report extracellular current densities. When a depolarization wave passes under an LFP electrode, the LFP signal flips its polarity. In contrast, the GEVI method reports transmembrane voltage changes. In our GEVI recordings, a membrane depolarization is always with positive polarity, and membrane hyperpolarization is always negative, regardless of the voltage transient's propagation velocity or direction. One should also consider the possibility that this negative signal component (apparent hyperpolarization) may represent an activity-related alkalinization of extracellular space to which some GEVIs are sensitive [39,40].

4.4 Population GEVI Imaging for Understanding Brain Functions

Integration of fragmented sensory inputs is best studied by measuring neuronal voltages at multiple sites [41, 42]. In association cortices, integration of primary sensory information with other sensory modalities (touch, light, or sound) and quick comparisons with previously stored patterns (memory), lead to the generation of adequate motor actions. These operations engage both local and distributed region-wide computations [43] conducted using both rapid and slow changes in neuronal membrane voltage [44–46]. Only macroscopic GEVI voltage imaging (GEVI-produced optical LFP) can be used to achieve 4 cardinal requirements of a modern systems neuroscience:

(1) To record fast changing voltage transients (compound action potentials (APs) and EPSPs).

(2) To record slow changing voltage transients (longlasting depolarizations or hyperpolarizations).

(3) Record simultaneously at many sites.

(4) Record from an identified neuronal subtype (e.g., neocortical layer 2/3 pyramidal cells).

In departure from standard voltage-sensitive dye imaging [4,17], GEVI imaging offers three important advantages: (a) physiological signals restricted to a specific cell population of interest (e.g., neocortical layer 2/3 pyramidal cells); (b) GEVI transgenic mice do not require removal of dura or skull-bone - imaging through a thinned skull [43]; and (c) repeated imaging sessions in living mice over the time course of an experiment lasting days [47].

4.5 Autofluorescence Optical Signals

In the absence of any fluorescent labeling (no GEVI expression or dye application), autofluorescence optical signals (excitation 470 nm, emission 510 nm) reliably detect synaptically evoked "events" in cerebral cortex. Amplitudes of autofluorescence optical signals ($\Delta F/F$) were comparable to signals obtained with VSD in the same brain slice and ROI, but the time course of an autofluorescence optical signal was notably slower. In the autofluorescence imaging recordings, signal polarity was opposite to the VSD or the GEVI signal polarity. The autofluorescence signal dynamics was slow (signal amplitude continued to grow after the stimulus train has ended), and the signal-tonoise ratio (signal quality) was significantly worse than in the VSD or GEVI recordings (Fig. 3). Our data show that one does not need any indicator to evaluate the viability of a brain slice or determine the success of synaptic stimulations. In slices of poor health, or when stimulation current intensity was 20% of that used in Fig. 3, autofluorescence optical signals were weak or absent. Our experiments indicate that autofluorescence optical signals, which may be due to flavoprotein [48-50], only occur in viable brain slices and only with adequate synaptic stimulation. Interestingly, autofluorescence optical signals cannot be used for studying propagation of voltage waves through cortical parenchyma, as these signals decay quickly with distance (Fig. 3B₁,B₂), compared to VSD or GEVI optical signals, which regularly report cortical voltage transients at 550 µm distance from the stimulation site (Fig. $3B_3, B_4$).

4.6 GEVI Optical Signals

Both VSFP and ASAP2s produced optical signals that can be used for comparing amplitudes and time courses (rise time, decay time, and temporal summation) of the evoked voltage waveforms between experimental groups. For example, comparing an experimental group composed of Alzheimer's disease model mice versus an experimental group composed of their healthy littermates [51]. Both GEVI indicators, VSFP and ASAP2s, can be used for monitoring "*vertical*" propagation of evoked depolarizations between cortical layers (e.g., from L4 to L2/3), or "*horizontal*" propagation along one cortical lamina (e.g., along layer 2/3, Fig. 5). One important difference between the two GEVI indicators was temporal summation at the 120 ms stimulus interval (8.3 Hz). While the VSFP optical signals integrated photons and showed a gradual increase in the synaptic event amplitude at the end of a synaptic train (activity-dependent amplitude facilitation, Fig. 6A), the ASAP2s optical signals showed gradual declines (activity-dependent amplitude depression, Fig. 6B).

The half-width (duration at half amplitude) of the cEPSP voltage waveforms in ASAP2s and VSFP experimental series were similar (compare Fig. 4A vs Fig. 4C), suggesting that these two GEVI variants faithfully report cEPSP decay phases in cerebral cortex, while the voltage-sensitive dye Di-4-Anepps alters cortical cEPSP waveforms. Two factors may potentially account for a slightly slower decay phase in the Di-4-Anepps experimental series (Fig. 4B,E,F). First, ASAP2s and VSFP signals were restricted to excitatory pyramidal cells, while Di-4-Anepps indiscriminately labeled all neuronal and nonneuronal membranes. It is not clear how optical signals from interneurons, astrocytes, blood vessels, and other elements affect the cEPSP voltage waveforms. Second, Di-4-Anepps exerts pharmacological effects on neurons [52], therefore the slowing of the EPSP decay phase observed in the present study (Fig. 4B,E,F) may also be an undocumented pharmacological effect of this voltage-sensitive dye. However, to demonstrate a pharmacological effect, one should present the dye-induced effects (e.g., a change in the time course of the voltage transient) with electrophysiological methods, which was not done here.

5. Conclusions

All three voltage indicators evaluated in the present study (ASAP2s, VSFP, and Di-4-Anepps) have demonstrated versatility in population voltage imaging of synaptically-evoked cortical depolarizations, in vitro, in brain slices. Transgenic mouse lines carrying VSFP or ASAP2 offer major practical and conceptual advantages. Transgenic expression eliminates invasive and laborintensive intracranial injections of AAV vectors. A fast OFF dynamic of the GEVI variant, ASAP2s, renders this indicator the first choice for studying rapidly changing voltage fields in cerebral cortex (e.g., cortical oscillations). A strong resting fluorescence of VSFP, renders this indicator the first choice for intact-skull imaging (imaging through a thinned skull bone). Among three indicators tested, ASAP2s was the only one showing apparent cortical hyperpolarizations. In the same optical channel in which we recorded ASAP2s optical signals (excitation 470 nm, emission 510 nm), we also recorded autofluorescence optical



signals. These "negative" optical signals, autofluorescence optical signals, should not be confused for hyperpolarizing cortical events in ASAP2s traces. Autofluorescence optical signals have an opposite polarity and a slow dynamic compared to ASAP2s signals of evoked cEPSPs. When ASAP2s expression is low, like in Cux2_ASAP2s mice, the autofluorescence optical signals mix with the ASAP2s signals, and introduce slow wave artifacts. ASAP2s bleaches notably faster than VSFP. While VSFP produces optical signals in both green and red channel simultaneously, ASAP2s has no functional signal and no fluorescence in the red segment of the visible light. ASAP2s has approximately 7-fold greater signal amplitude (Δ F/F) than VSFP and 3-fold greater signal amplitude than Di-4-Anepps.

Abbreviations

ACSF, artificial cerebrospinal fluid; ASAP2s, GEVI made by the M.Z. Lin laboratory; Age P21, postnatal day 21; cEPSPs, compound excitatory postsynaptic potentials; Di-4-Anepps, a commercially available voltage-sensitive dye; ES cells, embryonic stem cells; F1, the first filial generation of animal offspring; GEVI, genetically encoded voltage indicator; Jax Lab, Jackson laboratory; LFP, local field potential; VSFP, GEVI made by the T. Knopfel laboratory; TAM Diet, tamoxifen diet; VSD, voltage sensitive dye.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Author Contributions

SDA and MZL designed the research study. BLB and LXL produced animals expressing GEVIs. KDM and SDA generated histological data. KDM, MHZ, BLB, OB, ZYE, and SDA performed optical recordings. KDM and SDA analyzed the data. KDM, SDA and MZL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Brain slices were obtained from mice according to the animal protocol (AP-200902-0526) approved by the UConn Health Institutional Animal Care and Use Committee (IACUC).

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Conflict of Interest

The authors declare no conflict of interest.

References

- Stoelzel CR, Bereshpolova Y, Gusev AG, Swadlow HA. The impact of an LGNd impulse on the awake visual cortex: synaptic dynamics and the sustained/transient distinction. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2008; 28: 5018–5028.
- [2] Antic SD, Empson RM, Knöpfel T. Voltage imaging to understand connections and functions of neuronal circuits. Journal of Neurophysiology. 2016; 116: 135–152.
- [3] Salzberg BM, Grinvald A, Cohen LB, Davila HV, Ross WN. Optical recording of neuronal activity in an invertebrate central nervous system: simultaneous monitoring of several neurons. Journal of Neurophysiology. 1977; 40: 1281–1291.
- [4] Orbach HS, Cohen LB, Grinvald A. Optical mapping of electrical activity in rat somatosensory and visual cortex. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 1985; 5: 1886–1895.
- [5] Tominaga T, Kajiwara R, Tominaga Y. Stable wide-field voltage imaging for observing neuronal plasticity at the neuronal network level. Biophysics and Physicobiology. 2023; 20: e200015.
- [6] Kajiwara R, Tominaga Y, Tominaga T. Network Plasticity Involved in the Spread of Neural Activity Within the Rhinal Cortices as Revealed by Voltage-Sensitive Dye Imaging in Mouse Brain Slices. Frontiers in Cellular Neuroscience. 2019; 13: 20.
- [7] Baker B, Gao X, Wolff BS, Jin L, Cohen LB, Bleau CX, et al. In Vivo Voltage-Sensitive Dye Imaging of Mammalian Cortex Using "Blue" Dyes. Cold Spring Harbor Protocols. 2015; 2015: 1000–1002.
- [8] Vitantonio D, Xu W, Geng X, Wolff BS, Takagaki K, Motamedi GK, *et al.* Emergence of dominant initiation sites for interictal spikes in rat neocortex. Journal of Neurophysiology. 2015; 114: 3315–3325.
- [9] Liang Y, Song C, Liu M, Gong P, Zhou C, Knöpfel T. Cortex-Wide Dynamics of Intrinsic Electrical Activities: Propagating Waves and Their Interactions. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2021; 41: 3665–3678.
- [10] St-Pierre F, Marshall JD, Yang Y, Gong Y, Schnitzer MJ, Lin MZ. High-fidelity optical reporting of neuronal electrical activity with an ultrafast fluorescent voltage sensor. Nature Neuroscience. 2014; 17: 884–889.
- [11] Storace D, Sepehri Rad M, Kang B, Cohen LB, Hughes T, Baker BJ. Toward Better Genetically Encoded Sensors of Membrane Potential. Trends in Neurosciences. 2016; 39: 277–289.
- [12] Siegel MS, Isacoff EY. A genetically encoded optical probe of membrane voltage. Neuron. 1997; 19: 735–741.
- [13] Baker BJ, Lee H, Pieribone VA, Cohen LB, Isacoff EY, Knopfel T, *et al.* Three fluorescent protein voltage sensors exhibit low

plasma membrane expression in mammalian cells. Journal of Neuroscience Methods. 2007; 161: 32–38.

- [14] Panzera LC, Hoppa MB. Genetically Encoded Voltage Indicators Are Illuminating Subcellular Physiology of the Axon. Frontiers in Cellular Neuroscience. 2019; 13: 52.
- [15] Milosevic MM, Jang J, McKimm EJ, Zhu MH, Antic SD. In Vitro Testing of Voltage Indicators: Archon1, ArcLightD, ASAP1, ASAP2s, ASAP3b, Bongwoori-Pos6, BeRST1, FlicR1, and Chi-VSFP-Butterfly. eNeuro. 2020; 7: ENEURO.0060–20.2020.
- [16] Chamberland S, Yang HH, Pan MM, Evans SW, Guan S, Chavarha M, *et al.* Fast two-photon imaging of subcellular voltage dynamics in neuronal tissue with genetically encoded indicators. eLife. 2017; 6: e25690.
- [17] Grinvald A, Petersen CCH. Imaging the Dynamics of Neocortical Population Activity in Behaving and Freely Moving Mammals. Advances in Experimental Medicine and Biology. 2015; 859: 273–296.
- [18] Baker B, Gao X, Wolff BS, Jin L, Cohen LB, Bleau CX, et al. Voltage-Sensitive Dye Imaging of Population Signals in Brain Slices. Cold Spring Harbor Protocols. 2015; 2015: 995–999.
- [19] Allen WE, Kauvar IV, Chen MZ, Richman EB, Yang SJ, Chan K, et al. Global Representations of Goal-Directed Behavior in Distinct Cell Types of Mouse Neocortex. Neuron. 2017; 94: 891–907.e6.
- [20] Pinto L, Rajan K, DePasquale B, Thiberge SY, Tank DW, Brody CD. Task-Dependent Changes in the Large-Scale Dynamics and Necessity of Cortical Regions. Neuron. 2019; 104: 810–824.e9.
- [21] Momose-Sato Y, Sato K. Voltage-sensitive dye recording of glossopharyngeal nerve-related synaptic networks in the embryonic mouse brainstem. IBRO Reports. 2019; 6: 176–184.
- [22] Storace DA, Cohen LB, Choi Y. Using Genetically Encoded Voltage Indicators (GEVIs) to Study the Input-Output Transformation of the Mammalian Olfactory Bulb. Frontiers in Cellular Neuroscience. 2019; 13: 342.
- [23] Loew LM, Cohen LB, Dix J, Fluhler EN, Montana V, Salama G, et al. A naphthyl analog of the aminostyryl pyridinium class of potentiometric membrane dyes shows consistent sensitivity in a variety of tissue, cell, and model membrane preparations. The Journal of Membrane Biology. 1992; 130: 1–10.
- [24] Fluhler E, Burnham VG, Loew LM. Spectra, membrane binding, and potentiometric responses of new charge shift probes. Biochemistry. 1985; 24: 5749–5755.
- [25] Song C, Piscopo DM, Niell CM, Knöpfel T. Cortical signatures of wakeful somatosensory processing. Scientific Reports. 2018; 8: 11977.
- [26] Daigle TL, Madisen L, Hage TA, Valley MT, Knoblich U, Larsen RS, *et al.* A Suite of Transgenic Driver and Reporter Mouse Lines with Enhanced Brain-Cell-Type Targeting and Functionality. Cell. 2018; 174: 465–480.e22.
- [27] Zhu MH, Jang J, Milosevic MM, Antic SD. Population imaging discrepancies between a genetically-encoded calcium indicator (GECI) versus a genetically-encoded voltage indicator (GEVI). Scientific Reports. 2021; 11: 5295.
- [28] Georgakoudi I, Quinn KP. Label-Free Optical Metabolic Imaging in Cells and Tissues. Annual Review of Biomedical Engineering. 2023; 25: 413–443.
- [29] Díez-García J, Matsushita S, Mutoh H, Nakai J, Ohkura M, Yokoyama J, *et al.* Activation of cerebellar parallel fibers monitored in transgenic mice expressing a fluorescent Ca2+ indicator protein. The European Journal of Neuroscience. 2005; 22: 627– 635.
- [30] Knöpfel T, Díez-García J, Akemann W. Optical probing of neuronal circuit dynamics: genetically encoded versus classical fluorescent sensors. Trends in Neurosciences. 2006; 29: 160–166.
- [31] Nakajima R, Baker BJ. Mapping of excitatory and inhibitory postsynaptic potentials of neuronal populations in hippocampal

slices using the GEVI, ArcLight. Journal of Physics D: Applied Physics. 2018; 51: 504003.

- [32] Canepari M, Willadt S, Zecevic D, Vogt KE. Imaging inhibitory synaptic potentials using voltage sensitive dyes. Biophysical Journal. 2010; 98: 2032–2040.
- [33] Mishina Y, Mutoh H, Song C, Knöpfel T. Exploration of genetically encoded voltage indicators based on a chimeric voltage sensing domain. Frontiers in Molecular Neuroscience. 2014; 7: 78.
- [34] Short SM, Wachowiak M. Temporal Dynamics of Inhalation-Linked Activity across Defined Subpopulations of Mouse Olfactory Bulb Neurons Imaged *In Vivo*. eNeuro. 2019; 6: ENEURO.0189–19.2019.
- [35] Adelsberger H, Garaschuk O, Konnerth A. Cortical calcium waves in resting newborn mice. Nature Neuroscience. 2005; 8: 988–990.
- [36] Patel AA, McAlinden N, Mathieson K, Sakata S. Simultaneous Electrophysiology and Fiber Photometry in Freely Behaving Mice. Frontiers in Neuroscience. 2020; 14: 148.
- [37] Marshall JD, Li JZ, Zhang Y, Gong Y, St-Pierre F, Lin MZ, et al. Cell-Type-Specific Optical Recording of Membrane Voltage Dynamics in Freely Moving Mice. Cell. 2016; 167: 1650– 1662.e15.
- [38] Buzsáki G, Anastassiou CA, Koch C. The origin of extracellular fields and currents–EEG, ECoG, LFP and spikes. Nature Reviews. Neuroscience. 2012; 13: 407–420.
- [39] Makani S, Chesler M. Rapid rise of extracellular pH evoked by neural activity is generated by the plasma membrane calcium ATPase. Journal of Neurophysiology. 2010; 103: 667–676.
- [40] Kang BE, Lee S, Baker BJ. Optical consequences of a genetically-encoded voltage indicator with a pH sensitive fluorescent protein. Neuroscience Research. 2019; 146: 13–21.
- [41] Hubel DH, Wiesel TN. Sequence regularity and geometry of orientation columns in the monkey striate cortex. The Journal of Comparative Neurology. 1974; 158: 267–293.
- [42] Blasdel GG, Salama G. Voltage-sensitive dyes reveal a modular

organization in monkey striate cortex. Nature. 1986; 321: 579–585.

- [43] Knöpfel T, Song C. Optical voltage imaging in neurons: moving from technology development to practical tool. Nature Reviews. Neuroscience. 2019; 20: 719–727.
- [44] Brecht M, Schneider M, Sakmann B, Margrie TW. Whisker movements evoked by stimulation of single pyramidal cells in rat motor cortex. Nature. 2004; 427: 704–710.
- [45] Xu NL, Harnett MT, Williams SR, Huber D, O'Connor DH, Svoboda K, *et al.* Nonlinear dendritic integration of sensory and motor input during an active sensing task. Nature. 2012; 492: 247–251.
- [46] Economo MN, Viswanathan S, Tasic B, Bas E, Winnubst J, Menon V, et al. Distinct descending motor cortex pathways and their roles in movement. Nature. 2018; 563: 79–84.
- [47] Grutzendler J, Kasthuri N, Gan WB. Long-term dendritic spine stability in the adult cortex. Nature. 2002; 420: 812–816.
- [48] Kunz WS, Gellerich FN. Quantification of the content of fluorescent flavoproteins in mitochondria from liver, kidney cortex, skeletal muscle, and brain. Biochemical Medicine and Metabolic Biology. 1993; 50: 103–110.
- [49] Shibuki K, Hishida R, Murakami H, Kudoh M, Kawaguchi T, Watanabe M, *et al.* Dynamic imaging of somatosensory cortical activity in the rat visualized by flavoprotein autofluorescence. The Journal of Physiology. 2003; 549: 919–927.
- [50] Uytingco CR, Puche AC, Munger SD. Using Intrinsic Flavoprotein and NAD(P)H Imaging to Map Functional Circuitry in the Main Olfactory Bulb. PloS One. 2016; 11: e0165342.
- [51] Zhu MH, Jogdand AH, Jang J, Nagella SC, Das B, Milosevic MM, *et al.* Evoked Cortical Depolarizations Before and After the Amyloid Plaque Accumulation: Voltage Imaging Study. Journal of Alzheimer's Disease: JAD. 2022; 88: 1443–1458.
- [52] Mennerick S, Chisari M, Shu HJ, Taylor A, Vasek M, Eisenman LN, *et al*. Diverse voltage-sensitive dyes modulate GABAA receptor function. The Journal of Neuroscience: the Official Journal of the Society for Neuroscience. 2010; 30: 2871–2879.