Bioluminescence calcium imaging of network dynamics and their cholinergic modulation in slices of cerebral cortex from male rats

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ABSTRACT (219 words)

The activity of neuronal ensembles was monitored in neocortical slices **from male rats** using wide-field bioluminescence imaging of a calcium sensor formed with the fusion of green fluorescent protein and aequorin (GA) and expressed through viral transfer. GA expression was restricted to pyramidal neurons and did not conspicuously alter neuronal morphology or neocortical cytoarchitecture. Removal of extracellular magnesium or addition of GABA receptor antagonists triggered epileptiform flashes of variable amplitude and spatial extent, indicating that the excitatory and inhibitory networks were functionally preserved in GA-expressing slices. We found that agonists of muscarinic acetylcholine receptors largely increased the peak bioluminescence response to local electrical stimulation in layer I or white matter, and gave rise to a slowly-decaying response persisting for tens of seconds. The peak increase involved layers II/III and V and did not result in marked alteration of response spatial properties. The persistent response involved essentially layer V and followed the time-course of the muscarinic afterdischarge depolarizing plateau in layer V pyramidal cells. This plateau potential triggered spike firing in layer V, but not layer II/III pyramidal cells, and was accompanied by recurrent synaptic excitation in layer V. Our results indicate that wide-field imaging of GA bioluminescence is well-suited to monitor local and global network activity patterns, involving different mechanisms of intracellular calcium increase, and occurring on various time-scales.

SIGNIFICANCE STATEMENT (100 words)

Bioluminescence imaging attracts growing interest due to its high signal/noise ratio, minimal invasiveness, and to the increasing variety of genetically-encoded bioluminescent sensors. We used an aequorin-based calcium sensor to evaluate the performance of wide-field bioluminescence imaging in monitoring neuronal ensemble activities in slices of cerebral cortex. We found that bioluminescence imaging allowed establishing spatiotemporal maps of network activities evoked by diverse stimulation paradigms, and observed a good correspondence between bioluminescent signals of neuronal populations and electrophysiological responses of single neurons. We conclude that bioluminescence imaging is a valuable technology to monitor neural network activity on different spatial and temporal scales.

INTRODUCTION (717 words)

Genetically-encoded calcium (Ca^{2+}) sensors are widely used to image in real time the activities of single neurons or neuronal assemblies with fluorescence or bioluminescence microscopy techniques (Pérez Koldenkova and Nagai 2013, Ni et al. 2018). Aequorin is a natural bioluminescent sensor formed with the protein apoaequorin (luciferase) and the prosthetic group coelenterazine (luciferin), which emits blue light upon Ca^{2+} binding (Shimomura et al. 1962). The formation of the aequorin complex is slow, whereas the bioluminescence reaction occurs as a flash whose intensity increases with Ca^{2+} concentration (Hastings et al. 1969, Shimomura and Johnson 1970, Shimomura and Johnson 1975, Tricoire et al. 2006). The GFP-aequorin (GA) fusion protein allows fluorescence labeling and bioluminescence Ca^{2+} imaging from single cells to whole animals (Baubet et al. 2000, Rogers et al. 2005, Martin et al. 2007, Rogers et al. 2007, Drobac et al. 2010, Naumann et al. 2010, Knafo et al. 2017). Bioluminescence resonance energy transfer between aequorin and GFP shifts GA light emission to the green, and further to the red upon fusion of aequorin with other fluorescent proteins (Baubet et al. 2000, Curie et al. 2007, Bakayan et al. 2011). GA minimally interferes with cellular processes (Martin et al. 2007, Rogers et al. 2007, Drobac et al. 2010, Naumann et al. 2010), and behaves as a low-affinity sensor for the detection of fast Ca^{2+} transients in single neurons (Drobac et al. 2010). Aequorin has been historically the first indicator used for the study of intracellular Ca^{2+} signals (Ridgway and Ashley 1967), but it is now largely superseded by fluorescent sensors or bioluminescent sensors with improved brightness (Saito et al. 2012, Suzuki et al. 2016, Hossain et al. 2018) that provide superior spatial and temporal imaging resolution. Nonetheless, the virtual absence of background signal and large dynamic range of aequorin-based sensors (Allen et al. 1977, Drobac et al. 2010) make them useful to test the performance of bioluminescence imaging in monitoring the dynamics of neural networks using wide-field microscopy.

The effects of acetylcholine (ACh) on the neural network of the neocortex participate in arousal, sensory-motor information processing, attention and working memory (Sarter and Parikh 2005, Hasselmo 2006). Cholinergic fibers have a widespread distribution over the entire neocortex and throughout the six cortical layers (Mechawar et al. 2000). The neocortical network comprises glutamatergic pyramidal neurons that form the majority of cortical neurons (Peters and Jones 1984), and local GABAergic interneurons exhibiting diverse morphological, functional and molecular properties (Ascoli et al. 2008). ACh decreases the amplitude of intracortical excitatory and inhibitory synaptic events (Vidal and Changeux 1993, Gil et al. 1997, Kimura and Baughman 1997), whereas it generally excites neocortical neurons through somatodendritic nicotinic and muscarinic receptors, with the notable exception of layer IV spiny neurons (Eggermann and Feldmeyer 2009). The cholinergic response of pyramidal cells is layer-dependent, but predominantly consists in a depolarization, accompanied by a long-lasting increase in excitability (McCormick and Prince

1985, Gullledge and Stuart 2005, Gullledge et al. 2009, Hedrick and Waters 2015, Hay et al. 2016) notably associated with an afterdischarge depolarizing plateau potential (ADP, Andrade 1991, Haj-Dahmane and Andrade 1998, Egorov et al. 2002, Gullledge et al. 2009). The cholinergic activation of pyramidal cells is counterbalanced by phasic and/or tonic excitation of diverse inhibitory interneuron types by ACh (Kawaguchi 1997, Xiang et al. 1998, Porter et al. 1999, Christophe et al. 2002, Gullledge et al. 2007, Arroyo et al. 2012, Bennett et al. 2012). Hence, ACh elicits diverse time- and cell type-dependent effects on the neocortical network, whose outcome at the level of neuronal ensemble dynamics has been the subject of limited study (Kimura et al. 1999).

In the present study, we monitored neocortical network dynamics using bioluminescence imaging in rat brain slices expressing GA through a recombinant sindbis virus. We first verified that GA, expressed mainly in layers II/III and V pyramidal neurons, was able to report bioelectrical activities at the level of neuronal assemblies, and allowed the imaging of typical network activities elicited by low magnesium concentration or GABAergic antagonists. We next studied the modulation of network responses to local electrical stimulation by low concentrations of the cholinergic agonist carbachol. We found that carbachol increased the amplitude and duration of network responses and that the spatiotemporal dynamics of the bioluminescence signal followed the time-course of electrophysiological activities in layers II/III and V pyramidal cells.

MATERIALS AND METHODS

Recombinant Sindbis viral vector for GA expression

The GA sequence (Genbank™ accession number: EF212028) has been described elsewhere (Drobac et al. 2010) and was similar to that of the G5A fusion protein reported previously (Baubet et al, 2000). GA was inserted into the plasmid pSinRep5 (Invitrogen, Carlsbad, CA, USA) between SphI and Apal sites. The resulting plasmid and the helper plasmid pDH26S (Invitrogen) were linearized with PacI and XhoI, respectively; and *in vitro* transcribed into capped RNA using the Megascript SP6 kit (Ambion, Huntingdon, UK). Sindbis virus production was performed as described (Drobac et al. 2010). BHK-21 cells (ATCC # CCL-10) were electroporated (20×10^6 cells/ml, 950 μ F, 230 V) with recombinant and helper viral RNAs using the Gene Pulser II electroporation system (Bio-Rad Laboratories, Hercules, CA, USA) and grown 24 h at 37°C, 5% CO₂ in DMEM containing 5% fetal calf serum before collecting the cell supernatant containing viral particles. The recombinant virus titer (1.8×10^8 ip/ml) was determined after counting fluorescent BHK cells infected using serial dilution of the virus stock.

Preparation and infection of brain slices

Rats were housed in a temperature-controlled environment on a 12-h normal light/dark cycle. Food and water were provided *ad libitum*. All experiments were carried out in accordance to European Commission guidelines and French legislation (2010/63/UE) and animal protocols have been approved by the local ethics committee (Ce5/2014/ 00189). Male Wistar rats (13-16 days old, Janvier, Le Genest-St-Isle France) were killed by decapitation, brains were quickly immersed in cold (~4°C) oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 26 NaHCO₃, 20 glucose, 5 Na pyruvate. ACSF was supplemented with the low affinity glutamate receptor antagonist kynurenic acid (1 mM) for brain slice preparation in order to minimize excitotoxicity. Parasagittal sections (300 μ m-thick) of cerebral sensorimotor cortex were prepared as described previously (Cauli et al, 1997) using a Leica VT 1000S vibratome (Leica Microsystems GmbH, Wetzlar, Germany) and kept at room temperature for 30 min. Viral transduction and aequorin reconstitution was performed essentially as described (Drobac et al. 2010, Tricoire and Lambolez 2014). Slices were transferred onto a Millicell CM-membrane (Millipore, Billerica, MA, USA), and pre-equilibrated with culture medium (50% MEM, 50% HBSS, glucose 6.5 g/l, penicillin-streptomycin 100 U/ml) for 10 minutes at 32°C, 5% CO₂. Five microliters of the viral solution (9×10^5 ip/slice) was added dropwise onto the neocortex as described. Slices were incubated overnight at 32°C, 5% CO₂. Next, aequorin was reconstituted by adding coelenterazine (Lux Biotech, United Kingdom; stored at -80°C at 1.25 mM in ethanol) to the culture medium (final concentration, 10 μ M) and incubating slices for 3 h at 32°C. Prior to bioluminescence recording, slices were equilibrated in ACSF for 1 h at room temperature.

Bioluminescence imaging

Imaging experiments were performed essentially as described (Tricoire and Lambolez 2014).

Slices were transferred to the recording chamber and constantly perfused with ACSF (2 ml/min) at room temperature. Prior to data collection, slices were visualized by epifluorescence with an upright microscope BX51WI (Olympus, Tokyo, Japan) equipped with 10X (NA=0.3) and 20X (NA=0.95) water immersion objectives to select the recording field. Fluorescence images were acquired using an intensified CCD camera (ICCD225; 576x768 pixels; Photek Ltd., St Leonards on Sea, UK) in “bright field” mode connected to the C-port of the microscope. Aequorin light recordings were performed in complete darkness without emission filter with the same camera in “bining slice” mode at a frame rate of 25 images per second. Data were collected, stored and visualized with the IFS32 software (Photek). Electrical stimulations (burst of 5 steps at 10 Hz; current 0.5 mA-1.5 mA; duration of one step: 200 μ s; total duration: 500 ms) were delivered with a concentric bipolar electrode (diameter 100 μ m, model Rhodes SNEX-100, Phymep, France) with a stimulation isolation unit (DS3, Digitimer, UK) driven by a signal generator (Model 33120A, Hewlett Packard, Palo Alto, CA, USA).

Analysis of bioluminescence data

Analyses were performed with routines written in Matlab 7 (The Mathworks, Natick, MA, USA) and Visilog 6.2 (Noesis, Les Ulis, France). **Matlab procedures are available upon request to the corresponding authors.** Values of light intensity are given in counts (cnt) or count per second (cps).

The duration of a response to a single electrical stimulation was calculated as the time between the peak of the light response and the end of the response. The end of the response was the time when the light signal is not significantly superior (one-tailed t-test, $p=0.01$) to the mean signal measured during 10 s just before the stimulation. To average light responses from different experiments, individual responses were normalized to the baseline and to the peak amplitude of the response.

Density maps: To examine the spatial distribution of bioluminescence, density maps were calculated by convolving the original image with an 18-by-18 matrix of ones. In these density maps, each pixel value of the map is the mean intensity of the surrounding 18x18 pixels values in the original image. The barycenters of whole field light responses were calculated from the density maps of bioluminescence. Background was subtracted from the density map using a threshold calculated from light emitted during the 5 s before the stimulation. This threshold was chosen as the mean light intensity per pixel + 1.96 x standard deviation (corresponding to $P \leq 0.05$). The coordinates of the barycenter were determined from the bioluminescence image weighted by the light intensity of each pixel. Next, the barycenter was plotted on a model slice through a rotation and a translation whose reference elements, determined from the fluorescence image, were: the border between layer I and layer II/III (providing the rotation angle) and the point on this border vertical to the stimulation electrode (providing the alignment center).

Regions of analysis (RA): in order to analyze the spatiotemporal distribution of bioluminescence intensity, the field of observation was divided into an 18x24 array of RAs (size of one RA: 32x32 pixels).

Cluster analysis: an unsupervised cluster analysis was used to classify RAs according to their light response to single stimulations, without *a priori* knowledge of the number of groups. This was performed by combining 9 parameters describing the temporal evolution of each RA response. These parameters were: (i) The light emitted during seven successive 2.8 s intervals, during and after the stimulation (7 values) (ii) Light signal measured on the 5.6 s before the stimulation (1 value) (iii) the maximal value of light intensity during the light response (1 value). After standardizing the data by centering and reducing all values, cluster analysis was performed using squared Euclidean distances and Ward's method linkage rules (Ward 1963).

Electrophysiological recordings

Acute non-transduced neocortical slices, prepared as described above, were allowed to equilibrate in oxygenated ACSF at room temperature for at least 1 h before recordings. Next, slices were transferred into the recording chamber and continuously superfused at 2 ml/min with oxygenated ACSF at room temperature. Patch pipettes (3-5 MΩ) were pulled from borosilicate glass capillaries (Hilgenberg GmbH, Malsfeld, Germany) using a Narishige vertical micropipette puller (Tokyo, Japan). Electrodes were filled with internal solution containing (in mM) 144 K-gluconate, 3 MgCl₂, 0.5 EGTA, 4 Na₂-ATP and 0.4 Na₂-GTP and 10 HEPES. Biocytin (2 mg/ml; Sigma, St Louis, Missouri) was added for *post hoc* morphological processing of each recorded cell. The pH was adjusted to 7.2 and osmolarity to 285/295 mOsm. Neocortical pyramidal neurons were selected under infrared video microscopy. Whole-cell recordings were made with a patch-clamp amplifier (Multiclamp 700B, Molecular devices Corp, Sunnyvale, CA, USA) connected to a Digidata 1322A interface board (Molecular devices). Signals were amplified and collected using the data acquisition software PClamp 9.2 (Molecular devices). Resting membrane potential was measured just after passing in whole-cell configuration, and only cells with a resting membrane potential more hyperpolarized than -50 mV were analyzed. Membrane potentials were not corrected for junction potential (-11 mV in our experimental conditions). Cells were maintained at a holding potential of -60 mV by continuous current injection, and their firing behavior was tested by applying depolarizing 800 ms current pulses of increasing amplitudes (50 pA increment between steps from 0 pA to 400 pA). Action potential discharges were recorded by using the I-clamp mode of the amplifier. The signals were filtered at 5 kHz, digitized at 10 kHz, and analyzed off-line with Clampfit 9 software (Molecular devices).

To assay the effect of cholinergic agonists on the response to electrical stimulation, the stimulation electrode was positioned on layer I and stimulation consisted in the same train as described above. The amplitude of the afterdepolarization (ADP) was measured as the difference between the potential value before the stimulation and the mean potential measured on a 4 s interval, starting 2 s after the end of the stimulation. ADPs exhibiting spike

firing were excluded from the analysis. For voltage clamp recordings of excitatory postsynaptic currents, the holding potential of the recorded neurons was adjusted to -68 mV, corresponding to the reversion potential of inhibitory chloride currents in our experimental conditions. The series resistances were not compensated but were monitored throughout the experiments. Postsynaptic currents were analyzed off-line with Minianalysis 6 software (Synaptosoft, Decatur, GA, USA). Events < 7 pA were excluded from the measurements.

Immunohistochemistry

Slices were fixed overnight at 4°C in 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde. Next, slices were washed with PBS 6x10min and permeabilized with 0.2% gelatin/0.25% triton X-100 in 0.1 M PBS (GT-PBS) for 3x10 min. Slices were then incubated overnight at room temperature with primary antibody in GT-PBS. Primary antibodies used were rabbit anti-calbindin (1/10000, Swant, Bellinzona, Switzerland), rabbit anti-calretinin (1/5000, Swant) or mouse anti-parvalbumin (1/10000, Sigma), mouse anti-NeuN (1/200, Chemicon, Temecula, CA) and rabbit anti-GFAP (1/500, Dako, Glostrup, Denmark). After washes in 0.05% triton/PBS, slices were incubated for 2 h at room temperature with Cy3 conjugated anti-mouse or anti-rabbit IgG (1/2000, Jackson ImmunoResearch Inc, West Grove, PA, USA) in GT-PBS. After washes in 0.1 M PBS, slices were mounted in vectashield (Vector Labs, Burlingame, CA, USA). **Information on antibodies is given in Supplementary Table 1.** Images were acquired with a Leica DM IRBE SP1 confocal laser scanning microscope. Excitation was set at 488 nm (GFP) or 568 nm (Cy3/Alexafluor 568). Fluorescence was collected between 500 and 550 nm (GFP), or between 585 and 685 nm (Cy3/Alexafluor 568). Images were analyzed using **the cell counter plugin of ImageJ 1.32 software (National Institute of Health).** **Counting was performed upon visual inspection of confocal images and no procedure was implemented to reduce count bias.**

Drugs

Na (L)-glutamate, (+)-muscarine (10 μ M), carbachol (CCH, 20 μ M), (D,L)-2-amino-5-phosphonovaleric acid (APV, 50 μ M), D-APV (20 μ M), , bicuculline methiodide (10 μ M), SR95531 (gabazin, 100 nM) were purchased from Sigma; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μ M) from Tocris Cookson (Bristol, UK), and tetrodotoxin (TTX, 1 μ M) from Latoxan (Valence, France). Drug stock solutions were prepared in distilled water and stored at -80°C. On the day of use, drugs were diluted in ACSF to their working concentration. All drugs were applied through the bath perfusion.

Statistical analyses

A total of 57 rats were used in the present study, with between 2 and 4 animals for each recording condition, and a maximum of 3 cortical slices per animal. All raw data are available upon request to the corresponding authors. Statistical analyses were performed using Graphpad prism 6 software (La Jolla, CA, USA). Results are given as mean $\pm$ standard error

of the mean, as well as [$\pm$ standard deviation]. Error bars in figures represent standard error of the mean. For comparison of amplitude in Table 1, data were first submitted to a D'Agostino-Pearson omnibus normality test to assess whether data distribute according to a Gaussian law. If data passed the test with p-value under 0.05, comparison with control was performed using a one sample t-test. Otherwise, a Wilcoxon signed-rank test was used. For comparison of lateral extension in Table 1, Data were submitted to a one way ANOVA using a Geisser-Greenhouse correction to take into account possible violation of sphericity. Lateral extensions were then compared to control using a Holm-Sidak's multiple comparisons test. A full report of statistical analyses is given in supplementary Table 2

RESULTS

Expression of GA in neocortical slices

After overnight incubation of neocortical acute slices with the recombinant SIN-GA virus, GA expression was visualized using GFP fluorescence. Cells expressing GA (GA+) were localized preferentially in layers II-III and V and most exhibited the typical morphology of pyramidal neurons (Figure 1), consistent with earlier reports (Lendvai et al., 2000; Drobac et al., 2010). GA+ pyramidal cells were labelled throughout the entire somatodendritic compartment and showed no gross morphological alteration, as described (Drobac et al., 2010). All GA+ cells were immunoreactive for the neuronal marker NeuN and none of them were immunoreactive for the glial marker GFAP (Figure 1). Furthermore, only few GA+ cells were immunopositive for calcium-binding proteins parvalbumin (3%, n=5 out of 164 GA+ cells examined), calretinin (4%, n=14 out of 371) or calbindin (12%, n=45 out of 390; Figure 1), which are classically used as markers of GABAergic interneuron populations (Kubota et al, 1994; Cauli et al, 1997). Conversely, only a minority of cells immunopositive for these calcium-binding proteins expressed GA (Figure 1). These results indicate that the neocortical cytoarchitecture was essentially preserved in GA-expressing slices and that most GA+ neurons were pyramidal neurons.

Bioluminescence imaging of neocortical network activities

We previously showed that the present paradigm of GA expression does not result in conspicuous modification of electrophysiological properties and that GA bioluminescence reports calcium transients associated with action potential firing in single pyramidal neurons (Drobac et al. 2010). Here, we tested GA as a reporter of neuronal ensemble activities. After reconstitution of the aequorin moiety with coelenterazine, a field of GA-expressing neurons was selected under GFP epifluorescence prior to luminescence recordings (see methods). In absence of stimulation, the basal signal measured 20X objective at individual GA+ neuron somata (0.06 ± 0.03 [± 0.1] cps, n=10), or on the whole observation field (137 ± 8 [± 22] cps, including the dark noise of our setup, 50 cps; n=8 slices) was low. This is consistent with the low spontaneous activity commonly observed in neocortical slices (Holmgren et al., 2003) and the extremely low background luminescence of aequorin in absence of calcium (Allen et al. 1977). Bath application of glutamate (10-50 μ M) elicited bioluminescence peaks that involved most GA+ neurons, as judged from the almost perfect match between the image of luminescence responses and the distribution of GA fluorescence (Figure 2). The trace of the light signal measured at the soma of one of these GA+ neurons illustrates the increase of the light response with increasing glutamate concentration, and the low background of GA bioluminescence recordings (Figure 2). Similar results were obtained on 7 other slices. Responses to glutamate persisted in the presence of TTX (1 μ M), but were abolished by further addition of the NMDA receptor antagonist D-APV (20 μ M, n=4; not shown). Hence,

GA bioluminescence allows the imaging of intracellular calcium variations triggered by the opening of glutamate receptor-channels in neuronal ensembles. We used a 10X objective for all imaging experiments described in the rest of this study (size of the imaging field, 1080x1440 μm). We next tested the ability of GA bioluminescence imaging to monitor neocortical network activities elicited by the removal of extracellular magnesium (n=3 slices), which is known to induce episodes of synchronized network activity that rely on NMDA receptor activation (Thomson and West 1986; Hegstad et al. 1989, Silva et al. 1991, Flint and Connors 1996, Wu et al. 1999; Kawaguchi 2001). Light peaks appeared on the trace of whole-field recording about 5 minutes after the removal of magnesium (Figure 3). These peaks became more frequent with time in magnesium-free perfusion, and disappeared rapidly upon return to control conditions. In order to analyze the spatiotemporal distribution of these activities, the field of observation was divided into an 18x24 array of regions of analysis (RA, size: 60 μm x60 μm , see Figure 3). A 3D-plot of RA activities (Figure 3) shows that light peaks resulted from episodes of synchronized activities involving several RAs and that network active regions changed from one light peak to another. Bioluminescence images show examples of network activity patterns observed during synchronized activities. We also examined the effect of bicuculline, an antagonist of GABA_A receptors known to induce episodes of synchronized activities (Gutnick et al., 1982; Connors, 1984). Bath application of bicuculline (10 μM) elicited light peaks of large amplitude, which involved synchronized activation of most RAs and slowly decreased during bicuculline wash-out (Figure 3). Similar results were obtained with bicuculline on two other slices, and upon application of gabazine (100 nM, n=4, not shown), another antagonist of GABA_A receptors. These effects are consistent with previous electrophysiological and imaging reports (Gutnick et al., 1982; Connors, 1984; Wu and al, 1999; Kawaguchi, 2001). Similar also to earlier observations (Gloveli et al. 1995, Köhling et al. 2003), we found that, upon prolonged incubation of some slices in magnesium-free conditions (n=2), epileptiform activities progressed into slowly propagating waves of intense bioluminescence (Suppl. Figure S1) reminiscent of cortical spreading depression. These results confirm the integrity of excitatory and inhibitory networks observed upon histological examination of GA-expressing neocortical slices, and indicate that GA bioluminescence allows wide-field imaging of neuronal network dynamics with a high signal-to-background ratio during extended periods of time.

Cholinergic agonists increase the amplitude and duration of network responses

We next investigated the effects of cholinergic modulation on responses of the cortical network evoked by transient electrical stimulation. Local electrical stimulation of neocortical slices was performed in layer I (stim-L1) or in the white matter (stim-WM). Each stimulus consisted in a 500 ms train of 200 μs current pulses. For both stim-L1 and stim-WM, light responses spread vertically across all layers (see example in Suppl. Figure S2) and rapidly decayed at the end of the stimulus. Response amplitude increased with the frequency (Figure S2) and intensity (not shown) of the current pulses. Responses were abolished in the

presence of voltage-gated channel blockers (TTX, 1 μ M, n=3 or Ni^{2+} , 2 mM, n=3, Figure S2) and were reduced to 43 ± 5 [± 16] % (stim-L1, n=11 slices) and to 41 ± 3 [± 10] % (stim-WM, n=10 slices; see example in Figure 4) of control in the presence of glutamate receptor antagonists (CNQX 10 μ M and APV 50 μ M). These results indicate that light responses to electrical stimulation result from intracellular calcium changes associated with both excitatory synaptic transmission and activation of voltage-gated channels. In the rest of the study, the frequency of current pulses was set at 10 Hz and the intensity adjusted to a value yielding stable responses.

Application of the cholinergic agonist carbachol (CCH, 20 μ M) elicited a sustained luminescence increase of small amplitude (see example in Figure 4A), consistent with its modest effects on intracellular $[\text{Ca}^{2+}]$ of pyramidal neurons in absence of spiking activity (Egorov et al, 2003). However, CCH largely increased the amplitude and duration of whole-field responses to electrical stimulation (Figure 4). In the presence of CCH, response amplitude was increased to 402 ± 56 [± 177] % of control for stim-WM (n=10 slices) and to 630 ± 114 [± 395] % of control for stim-L1 (n=12 slices). Moreover, response duration increased from 0.77 ± 0.03 [± 0.10] s and 0.63 ± 0.03 [± 0.10] s in control condition, and to 31 ± 3 [± 9.6] s and 24 ± 2 [± 6.9] s in the presence of CCH, for stim-WM and stim-L1, respectively. For both kinds of stimulation, responses in the presence of CCH exhibited an initial fast decaying phase followed by a slow decrease of the light signal (Figure 4). The similarity of CCH effects on stim-WM and stim-L1 responses suggests that they reflect intrinsic dynamics of the neocortical network. CCH effects were essentially mimicked by muscarine (10 μ M, Figure 4), which increased the amplitude of responses to 263 ± 40 [± 98] % of control for stim-WM (n=6 slices) and to 493 ± 78 [± 234] % of control for stim-L1 (n=9 slices). In the presence of muscarine, response durations were 26.8 ± 2.4 [± 5.9] s and 28.5 ± 2.5 [± 7.5] s for stim-WM and stim-L1, respectively. These responses also exhibited biphasic decay. These results indicate that, in our experimental conditions, most of the effects of CCH were mediated by muscarinic acetylcholine receptors. The CCH-induced increase of response amplitude was also observed in the presence of CNQX-APV and reached 458 ± 67 [± 150] % and 619 ± 95 [± 212] % of the CNQX-APV control for stim-WM (n=5 slices) and for stim-L1 (n=5), respectively (Figure 4). In the presence of CCH and CNQX-APV, responses were longer than in control conditions (12.7 ± 1.2 [± 2.7] s for stim-WM, n=5 slices; 11.8 ± 1.2 [± 2.7] s for stim-L1, n=5), but shorter than with CCH alone (see above), suggesting that excitatory synaptic transmission contributed to CCH effects on response duration. These results indicate that cholinergic effects on bioluminescence network responses involved both the intrinsic properties of individual pyramidal neurons and intracortical excitatory transmission.

Spatiotemporal dynamics of network responses in the presence of CCH

The CCH effect on the spatiotemporal dynamics of network responses was analyzed using maps of bioluminescence activities integrated over 5 s time-windows (see Methods). Figure 5 shows an example of this analysis performed on the experiment using stim-WM illustrated in Figure 4A. In control conditions, the response spread vertically across layers and was limited to the [0-5s] time-window including the stimulation. In the presence of CCH, the response during the [0-5s] interval had similar vertical extent, but larger horizontal extent than in control conditions. Strikingly, the response largely outlasted the [0-5s] interval and persisted essentially in layer V throughout the subsequent time-windows. In CCH-CNQX-APV conditions, the spatial extent of the response was roughly similar to control, but activities persisted in layer V beyond the [0-5s] time-window. Nonetheless, the persistent activity in layer V vanished more rapidly than in CCH conditions. Essentially similar results were obtained in 9 other slices upon stim-WM, and in 12 slices upon stim-L1 (see example in Suppl. Figure S3).

The redistribution of bioluminescent activities observed during the course of cortical network responses in the presence of CCH, suggests that whole field responses resulted from the summation of individual neuron activities exhibiting different temporal profiles. To explore this point, we used an unsupervised cluster analysis to classify individual RAs of the imaging field on the basis of their activity pattern (see Methods). Figure 5 shows the tree diagram of cluster analysis applied to the individual responses of the 432 RAs of the imaging field (second stim-WM stimulation in the presence of CCH shown in Figure 4A). The analysis suggested 3 clusters of RAs. Cluster 1 was composed of 21 RAs, each exhibiting persistent activity. Their mean response, which was essentially tonic with relatively small early phase, had a duration similar to that of the whole-field response (figure 5). In contrast, cluster 2, which comprised 112 RAs, exhibited an essentially phasic mean response corresponding to the peak of the whole-field response. The third cluster contained 299 RAs showing no or low light response to the stimulation. The summation of light signals of clusters 1 and 2 closely matched the whole-field response. As illustrated in figure 5, all RAs from cluster 1 were located in layer V and most of their activity occurred after the stimulation. In contrast, RAs from cluster 2 distributed across layers II-V and exhibited most of their activity during the early phase of the whole-field response. This suggests that among pyramidal neurons contributing to the bioluminescent response, the majority exhibited a phasic activity responsible for most of the response peak and were distributed in both layers II/III and V. Conversely, a minor fraction of these neurons formed a distinct population restricted to layer V, which exhibited a tonic activity whose time-course followed that of the persistent response.

Spatial properties of the early response

CCH increased the intensity of the whole-field [0-500 ms] response evoked during electrical stimulation as compared to control conditions (Table 1). As exemplified in Figure 6, this

increase in light intensity was associated with an increased number of active RAs, and thus of spatial extent. The lateral (cortical horizontal) extent of [0-500 ms] responses was examined by plotting the sum of intensities of vertical RA columns as a function of horizontal distance (Figure 6). The shape of these horizontal profiles of light intensities was quantified by measuring the amplitude of the distribution peak ($A_{\max}$), the width of the peak at half amplitude ($W_{1/2}$), as well as the width of the CCH peak measured at the level of the half amplitude of the control peak (W^{control}). Mean values of $A_{\max}$, $W_{1/2}$ and W^{control} obtained for stim-WM (n=10 slices) and stim-L1 (n=12 slices) experiments are given in Table 1 and plotted in Figure 6. CCH increased both the maximal amplitude ($A_{\max}$ was 357 ± 87 [± 275] % and 254 ± 68 [± 236] % of control for stim-WM and stim-L1, respectively) and the lateral extent (W^{control} was 211 % and 219 % of control $W_{1/2}$ for stim-WM and stim-L1, respectively) of the [0-500 ms] response. Nonetheless, the shape of the distribution peak was not markedly altered by CCH: in the presence of CCH, $W_{1/2}$ was 112 % and 137 % of control for stim-WM and stim-L1, respectively (Figure 6). We next estimated the lateral precision of [0-500 ms] responses by calculating the barycenter of light emitting regions during this interval (see Methods), and measuring the distance between barycenters and the vertical of the stimulation electrode (Table 1). This distance was 51 ± 5 [± 16] μm in control and 41 ± 9 [± 29] μm in CCH conditions for stim-L1, and was 62 ± 17 [± 59] μm in control and 85 ± 22 [± 76] μm in CCH conditions for stim-WM, consistent with CCH having no significant effect on the lateral precision of the response. During the [500ms-1s] interval following stimulation, the amplitude and lateral extent of network responses decayed in control conditions. Conversely, in the presence of CCH, the amplitude of responses generally increased during the [500ms-1s] interval (Table 1, see example in Figure 6A), whereas $W_{1/2}$ and W^{control} , as well as the distance between barycenters and the vertical of the stimulation electrode, remained roughly stable (Table 1). These results suggest that CCH enhanced early responses of the cortical network without markedly altering their spatial shape and lateral precision.

Network responses persist in layer V in the presence of CCH

The spatiotemporal dynamics of network activity during responses in CCH condition was also analyzed by calculating barycenters of whole-field light emission during the stimulus [0-500ms], post-stimulus [500ms-1s] and late [5-20s] intervals (see Methods). Barycenters of each tested slice were then plotted on a model slice using the borders between layers determined from GA fluorescence images as a reference (see Methods). Figure 6 shows that most [0-500ms] and [500ms-1s] barycenters were located close to the vertical of the stimulation electrode, as stated above. A shift of individual barycenters occurred from the [0-500ms] to the [500ms-1s] interval, suggesting a redistribution of network activities in the post-stimulus period. However, the direction of this shift differed from slice to slice and did not appear to depend on the stim-L1 or stim-WM paradigm. In contrast, most barycenters clearly shifted to layer V from the [500ms-1s] to the [5-20s] interval, confirming that persistent network responses primarily occurred in layer V in both stim-L1 and stim-WM experiments.

The shift of activities towards layer V from the early [0-1s] to the persistent [5-20s] response was assessed by calculating the ratio of layer V over layer II/III light emission normalized to their respective GFP fluorescence intensity, which reflects the density of GA-expressing neurons. Figure 6 shows that the [0-1s] response to stim-L1 involved almost equally layer II/III and V in both control and CCH conditions (ratio values, 1.1 ± 0.2 [± 0.7] and 1.5 ± 0.2 [± 0.7], respectively, n=12 slices), whereas [5-20s] activity redistributed to layer V (ratio, 3.1 ± 0.5 [± 1.7]). The early response to stim-WM preferentially involved layer V in both control and CCH conditions (ratio values, 2.6 ± 0.5 [± 1.6] and 2.8 ± 0.5 [± 1.6], respectively, n=10 slices). Nonetheless, network activity also redistributed to layer V during the persistent phase, as judged from the increased ratio value (5.1 ± 1.1 [± 3.5]). Hence, the CCH-dependent persistent response involved primarily the layer V cortical network.

The persistent response is driven by the muscarinic afterdischarge depolarization of layer V pyramidal cells

The above results suggest that activities of layer V pyramidal neurons are critically involved in persistent bioluminescence responses observed in the presence of cholinergic agonists. The modulation of electrophysiological responses of layer V pyramidal neurons to stim-L1 by CCH (n=5) and muscarine (n=4) was thus examined in non-transduced acute slices using whole cell patch-clamp recordings (Figure 7). Electrical stimulation (same as above: 10 Hz, 0.5 s) was performed every minute before and during cholinergic agonist application that lasted for at least 5 minutes. In control conditions, the response consisted in a train of action potentials, which mimicked the stimulation pattern and ceased at the end of the stimulus. Application of CCH induced a sustained depolarization of small amplitude (8.0 ± 1.6 [± 3.6] mV). No change in spike frequency was observed during stimulation in CCH as compared to control conditions. This suggests that the CCH-induced increase of the early bioluminescence response relates primarily to the reported enhancement of spike-evoked calcium transients in pyramidal cells by muscarinic agonists (Yamamoto et al. 2000, Larkum et al. 2003). In the presence of CCH, an afterdischarge depolarization (ADP) followed electrical stimulation in 5 out of 5 cells tested (Figure 7). This ADP lasted for 31.0 ± 3.2 [± 7.2] s and its mean amplitude was 5.2 ± 1.0 [± 2.2] mV (see Methods). During the course of CCH application, long-lasting spike firing appeared on top of the ADP (n=5 out of 5 cells, Figure 7). The decay of the ADP amplitude was close to that of the persistent bioluminescence response obtained in GA-expressing slices in the presence of CCH (Figure 7). Similar results were obtained in the presence of muscarine. In these latter conditions, the ADP amplitude was 4.5 ± 0.6 [± 1.2] mV (n=4) and its decay was close to that of the persistent bioluminescence response in muscarine (Figure 7).

Because electrical stimulation in the presence of CCH failed to evoke a persistent bioluminescence response in layer II/III (see Figures 5 and 6), we also examined electrophysiological responses of layer II/III pyramidal cells (n=5, not shown). CCH effects on

layer II/III cells (tonic depolarization: 3.9 ± 0.6 [± 1.3] mV, ADP amplitude: 4.0 ± 0.4 [± 0.9] mV, ADP duration: 26.8 ± 5.6 [± 12.5] s) did not significantly differ from those observed in layer V, except for the complete absence of spike firing surmounting the ADP in layer II/III cells. The different responsiveness of layer II/III and layer V pyramidal cells may relate to the reportedly lower resting membrane potential (-75 ± 0.8 [± 1.8] mV and -63.8 ± 1.5 [± 3.4] mV in our sample of layer II/III and layer V cells, respectively), and lesser cholinergic facilitation of spike firing of layer II/III pyramidal neurons (Gulledge et al. 2009). The absence of ADP-driven spike firing in layer II/III cells is a likely explanation for the faster decay of the bioluminescence response in layer II/III than in layer V.

Finally, the correlation of recurrent excitatory transmission to the time-course of bioluminescence responses was tested using voltage-clamp recordings of excitatory postsynaptic currents in layer V pyramidal cells (EPSC, see Methods). In control conditions, no effect of the stimulation was observed on the EPSC frequency (see example in Figure 7). The application of CCH elicited a sustained increase in EPSC frequency. In the presence of CCH, electrical stimulation elicited a further increase of EPSC frequency to 288 ± 38 [± 76] % of the CCH baseline ($n=4$, see example in Figure 7). The decay of the EPSC frequency after stimulation closely followed that of the persistent bioluminescence response (Figure 7). Similar results were obtained in the presence of muscarine. The EPSC frequency, which increased to 746 ± 267 [± 534] % of the muscarine baseline following electrical stimulation ($n=4$, see example in Figure 7), decayed in parallel with the persistent bioluminescence response (Figure 7).

These results suggest that persistent bioluminescence responses observed in the presence of muscarinic agonists reflect the ADP-driven long-lasting spike firing of layer V pyramidal neurons and the associated recurrent excitatory transmission within the layer V network.

DISCUSSION (2068 words)

We used the bioluminescent calcium sensor GA to image the activity of neuronal ensembles in neocortical slices. GA expression was restricted to pyramidal neurons and did not conspicuously alter neocortical neuronal morphologies and cytoarchitecture. Imaging of stereotypical activities evoked by glutamate receptor stimulation or blockade of GABAergic inhibition indicated that the network was also functionally preserved in GA-expressing slices. We found that cholinergic agonists increased the amplitude and duration of network bioluminescence responses to local electrical stimulation. The amplitude increase involved layers II/III and V but did not result in marked alteration of spatial shape. The duration increase involved primarily layer V and followed the time-course of the ADP and EPSC increase evoked by the stimulation in layer V pyramidal neurons. **This study was carried out using only male samples and its outreach is thus limited by possible gender-related differences.**

GA expression in acute neocortical slices

Sindbis viral transduction essentially targeted GA expression to pyramids, as described (Lendvai et al., 2000; Drobac et al., 2010). Indeed, most cells exhibited the typical morphology of pyramidal neurons, and GA expression was generally low in layer IV, which is dominated by spiny stellate and star pyramidal excitatory neurons (Lund 1984, Staiger et al. 2004). Moreover, only limited co-localization was observed between GA and the interneuronal markers calcium binding proteins. The immunodetection of calbindin in 12% of GA+ cells is consistent with the expression of this marker in a subpopulation of pyramidal neurons (Kubota et al. 1994). Our previous (Drobac 2010) and current observations thus show that the morphology of pyramidal cells and the cortical cytoarchitecture are essentially preserved in GA-expressing slices. Hence, whole field bioluminescence signals recorded in this study report the activities of supragranular and infragranular pyramidal neurons in a morphologically preserved network.

GA bioluminescence reports the dynamics of cortical neuronal ensembles

Whole field bioluminescence recordings showed that virtually all GA+ neurons were responsive to bulk stimulation by pulses of exogenous glutamate, consistent with earlier observations of their responsiveness to single-cell electrical stimulation (Drobac et al. 2010). Responses to glutamate persisted in the presence of TTX, but were prevented by further addition of the NMDAR antagonist APV, showing that GA reports calcium entry through glutamate receptor-channels. Global network stimulation via removal of extracellular magnesium, or via blockade of GABAergic inhibition, elicited peaks of synchronized activity, as described (Gutnick et al. 1982; Connors 1984, Thomson and West 1986; Hegstad et al.

1989; Silva et al. 1991; Flint and Connors 1996; Wu et al. 1999; Kawaguchi 2001). Bioluminescence peaks obtained in magnesium-free conditions were generally associated with spatial patterns of activity that were more discrete than in the presence of GABAergic antagonists, consistent with unbalanced spread of excitation in these latter conditions. Upon prolonged incubation in magnesium-free or GABAergic antagonist conditions, epileptiform activities progressed into slowly propagating waves of bioluminescence reminiscent of cortical spreading depression, which reportedly occur in cortical slices under similar conditions (Gloveli et al. 1995, Köhling et al. 2003). We also found that bioluminescence responses elicited by local electrical stimulation were abolished by voltage-gated channel blockers but partially hampered by glutamate receptor antagonists, suggesting these responses resulted from both direct stimulation of neuronal processes and glutamatergic synaptic transmission. These observations attest to the functional integrity of excitatory and inhibitory networks in GA-expressing neocortical slices, and indicate that GA allows the wide-field imaging of local and global network activity patterns, involving different mechanisms of intracellular calcium increase, and occurring on various time-scales.

Cholinergic agonists increase the peak amplitude of bioluminescence responses to electrical stimulation

Local electrical stimulation in layer I or white matter elicited brief bioluminescence responses that involved both supra- and infragranular pyramidal neurons. We found that the peak amplitude and duration of these responses largely increased in the continuous presence of low CCH concentration. These effects were mimicked by muscarine and thus primarily mediated by muscarinic receptors, consistent with the desensitization properties of nicotinic receptors and their lower affinity for CCH. It is known that muscarinic agonists strongly potentiate the amplitude and duration of intracellular calcium increases associated with action potentials in layers II/III and V pyramidal cells, via the facilitation of calcium-induced calcium release (Yamamoto et al. 2000, Larkum et al. 2003). Several observations indicate that the cholinergic enhancement of the early [0-1s] bioluminescence response amplitude depends primarily on such potentiation of intracellular calcium signaling, rather than on enhanced electrophysiological activity. Indeed, response amplitudes in the presence of CNQX-APV were similarly enhanced by cholinergic agonists, pointing to the involvement of intrinsic properties of individual neurons and ruling out a major contribution of recurrent synaptic excitation. Furthermore, the number of action potentials recorded in pyramidal cells during the [0-500ms] stimulation interval was not increased by cholinergic agonists. Although the muscarinic ADP of pyramidal cells was present during the subsequent [500ms-1s] interval, a major contribution of this conductance to the increased bioluminescence intensity is unlikely since the relative contribution of layers II/III and V to the [0-1s] response was similar in control and CCH conditions. Consistent also with a modest enhancement of electrophysiological activities, CCH did not markedly change the spatial properties of [0-1s] responses, as judged from horizontal profiles of light intensities, lateral and vertical position

of barycenters, and relative contribution of layers II/III and V to the response. Our results thus suggest that the increase of bioluminescence [0-1s] responses by cholinergic agonists reflects primarily the muscarinic potentiation of intracellular calcium signaling in pyramidal cells (Yamamoto et al. 2000, Larkum et al. 2003) rather than an increase in network electrophysiological activity.

Cholinergic agonists prolong cortical responses to electrical stimulation

Following the peak of the whole-field response in the presence of cholinergic agonists, the overall light emission rapidly decayed but network activity persisted in layer V. Likewise, individual RA analyses revealed that the majority of active regions distributed across layers II-V and exhibited a phasic response essentially limited to the peak of the whole-field response. Conversely, a minority of regions localized in layer V exhibited a tonic response that accounted for the slowly decaying component of the whole-field response. This spatial shift was assessed by the migration of light emission barycenters and the increase of layer V over layer II/III light emission ratio after the peak of the whole-field response. The duration of the persistent response (above 20 s) largely outlasted that reported for calcium-induced calcium release events (~1 s; Yamamoto et al. 2000, Larkum et al. 2003), suggesting that the persistent bioluminescence reflected slowly decaying electrophysiological activities restricted to layer V. Indeed, electrophysiological recordings revealed that our stimulation paradigm elicited a long-lasting muscarinic ADP that triggered spike firing in layer V pyramidal cells, consistent with previous reports (Andrade 1991, Haj-Dahmane and Andrade 1998, Gullledge et al. 2009). Our results suggest that the persistent bioluminescence response of the layer V network was driven by the muscarinic ADP of layer V pyramidal cells, whose time course was similar to that of bioluminescence. In contrast, bioluminescence did not persist after the peak response in layer II/III, where pyramidal cell exhibited an ADP that did not result in spike firing, consistent with earlier observations (Gullledge et al. 2009). Electrical stimulation in the presence of cholinergic agonists also evoked a long-lasting EPSC increase in layer V that decayed in parallel with the ADP and the persistent bioluminescence. It is likely that this EPSC increase, fueled by ADP-dependent spiking in pyramidal cells, reflected recurrent excitation between layer V pyramidal cells that in turn contributed to the sustained response.

Beyond the contribution of pyramidal cells directly monitored through GA bioluminescence, other neuronal types of the layer V network are presumably involved in the persistent responses. Among cortical inhibitory neuron types, somatostatin-expressing interneurons are most prominently excited by muscarinic agonists and exhibit a muscarinic ADP similar to pyramidal cells, whereas fast-spiking interneurons have the lowest sensitivity to cholinergic agonists (Kawaguchi 1997, Xiang et al. 1998, Porter et al. 1999, Gullledge et al. 2007, Fanselow et al. 2008). Somatostatin interneurons target distal dendrites of pyramidal cells, are recruited supralinearly by neighboring pyramids, and inhibit fast-spiking interneurons, which exert powerful perisomatic inhibition on pyramidal cell firing (Kapfer et al. 2007,

Cottam et al. 2013, Pfeffer et al. 2013, Xu et al. 2013, Jiang et al. 2015). Muscarinic effects on somatostatin interneurons may thus contribute to the observed cortical network dynamics in at least two ways. Recurrent dendritic inhibition of pyramidal cells may curtail the initial response to electrical stimulation, which may explain the slower decay of the initial peak response observed in the presence of CCH in CNQX-APV conditions as compared to control conditions (see Figures 4C, 5A and S3). In addition, inhibition of fast-spiking interneurons may dampen recurrent somatic inhibition of pyramidal cells, thereby facilitating the CCH-dependent persistence of layer V network activities.

Bioluminescence imaging

The relative advantages and limitations of chemical versus genetically-encoded, and of bioluminescent versus fluorescent indicators have been extensively discussed earlier (see for example Brini et al. 1999, Pérez Koldenkova and Nagai 2013, Smith et al. 2018). In the present study, the selective transduction of pyramidal neurons with GA provided an obvious advantage over bulk loading of a fluorescent chemical dye in terms of signal specificity, signal/noise ratio, phototoxicity and photobleaching. As for any genetically-encoded sensor, selective targeting of GA to other neural types can be achieved in relevant Cre-driver mouse lines using Cre-dependent adenoviral vectors or transgenic mice (Agulhon et al. 2005, Rogers et al. 2007).

The peculiar properties of aequorin among other luciferase/luciferin reaction complexes (Wilson and Hastings 1998) have important consequences on its use as a biosensor.

1) The formation of the apoaequorin-coelenterazine complex is slow, being reportedly optimal *in cellulo* after 2 hours incubation (Brini et al. 1999, but 30 min suffice, see Agulhon et al. 2007) with coelenterazine concentrations (5-10 μ M) below cellular toxicity threshold ($\sim$ 20 μ M, Dubuisson et al. 2000, Shakhmin et al. 2016). Aequorin is thus generally reconstituted prior to the imaging experiment, although Ca²⁺-dependent bioluminescence is readily detectable *in vivo* 15 min after coelenterazine i.v. injection and reaches a plateau after 45 min (Rogers et al. 2007). The reconstituted aequorin pool is sufficient to image tissue slices for 95 minutes (present study, see also Agulhon et al. 2007), and to record GA bioluminescence signals for 10 hours *in vivo* (Rogers et al. 2007) without recharging aequorin. In contrast, other luciferases, notably the one used in the Nano-lantern-based series of sensors, have much faster turn-over rates, resulting in bioluminescence intensities orders of magnitude higher than achieved with aequorin-based sensors (Saito et al. 2012, Suzuki et al. 2016). While these bright sensors tremendously improve the spatiotemporal resolution and depth of imaging, their requirement for the continuous presence of coelenterazine during imaging sessions (20-40 μ M, Saito et al. 2012, Suzuki et al. 2016) is a significant drawback, because of coelenterazine toxicity and instability in the presence of oxygen.

2) The aequorin complex is very stable below Ca^{2+} threshold ($0.1 \mu\text{M}$), and aequorin bioluminescence intensity varies by several orders of magnitude in the $[0.1 \mu\text{M} - 1 \text{mM}]$ Ca^{2+} concentration range (Allen et al. 1977). As a consequence, GA bioluminescence is virtually devoid of background signal and has high dynamic range (as exemplified in Figs. 2-4), which compare favorably with those of Nano-lantern-based Ca^{2+} sensors (Saito et al. 2012, Suzuki et al. 2016). This endows GA bioluminescence imaging with high contrast, making discrete events clearly detectable on whole-field signals, and enabling the acquisition of whole field 2D data at video rate in a very compact format through the encoding of only active pixel coordinates (for comparison, the 90 minutes imaging performed for the experiment illustrated in Figure 4 generated a 3.5 Mo data file, whereas an experiment of same duration, acquisition frequency, pixel number (576×768), 8 bits encoding, would generate a 58 Go image file).

Further limitations of the present work concerning the use of electrical stimulation and the low spatial resolution should be reasonably simple to address in future studies. Indeed, the use of optogenetic actuators is readily compatible with bioluminescence imaging (Saito et al. 2012) to stimulate specific corticopetal pathways (e.g. Hay et al. 2016, Hay et al. 2018), and EMCCD cameras with 1 M pixels, >90 % sensitivity in the visible spectrum, and frame rates >100 Hz are now commercially available for low-light-level imaging.

Despite these limitations, the present study demonstrates a good correspondence between the calcium-dependent GA bioluminescence of neocortical pyramidal cell populations and their electrophysiological and calcium signaling properties. We conclude that wide-field bioluminescence imaging is well-suited to monitor the dynamics of neuronal ensembles.

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FIGURE LEGENDS

Figure 1. Expression of GA in neocortical slices. Confocal reconstructions of neocortical slices expressing the GA sensor following viral transduction. Cells expressing GA were labelled using anti-GFP immunohistochemistry (green). **(A-C)** GA-expressing cells were preferentially localized in layers II/III and V and exhibited the typical morphology of pyramidal neurons. Neocortical layers are indicated. **(D-H)** Dual immunostaining revealed that all GA-expressing cells were positive for the neuronal marker NeuN, and negative for glial fibrillary acidic protein (GFAP). Co-localization of GA with interneuron markers was only marginal for parvalbumin (PV) and calretinin (CR), but more substantial for calbindin (CaB), which is also expressed in a subpopulation of pyramidal neurons. Scale bars: 100 μm (A), 20 μm (B-C), 50 μm (D-H).

Figure 2. Glutamate-induced bioluminescence response of a GA-expressing neuronal population. **(A)** GFP fluorescence image of a neocortical slice expressing GA. **(B)** Luminescence image of the same slice integrated over three successive bath applications of glutamate with increasing concentration (see panel D). **(C)** Superimposition of GA fluorescence (green) and luminescence images (red). Note the good match between the two images. **(D)** Light emission measured at the level of the GA-expressing layer II/III neuron indicated by an arrowhead in (B). Note the low basal luminescence in the absence of glutamate. Scale bar: 50 μm .

Figure 3. Bioluminescence imaging of synchronized activities in the neocortical network. Analyses of bioluminescence spatiotemporal dynamics evoked by the removal of extracellular magnesium (A-C) or the application of bicuculline (Bic, D-F) in GA-expressing neocortical slices (greyscale GFP fluorescence images in C and F). **(A,D)** Whole field bioluminescence signals. **(B,E)** The spatiotemporal variations of bioluminescence intensity were represented as 3D-plots: regions of analysis (RA) numbered from left to right and from top to bottom of the slice (as indicated on the greyscale image in C) were plotted along the y-axis, time was plotted along the x-axis, and light intensity was coded in greyscale. Vertical lines on the 3D-plots showed that light peaks resulted from episodes of synchronized activities involving several RAs. Correspondence of the 3D-plots with some of the peaks on whole field bioluminescence traces shown in (A,D) is indicated by arrows. **(C,F)** GFP fluorescence images of GA-expressing slices (greyscale), and bioluminescence images of the same slices at times corresponding to peaks on whole field bioluminescence traces in (B,E), as indicated by arrows. Note that whole field luminescence peaks corresponded to episodes of local activity that involved different RAs (C) or of more generalized activity involving most RAs in both layers II/III and V (F). Scale bars: 30 s (B), 60 s (E), 100 μm (C and F).

Figure 4. Cholinergic agonists increase the amplitude and duration of network responses to electrical stimulation. (A) GFP fluorescence image (greyscale, scale bar 100 μ m) of a GA-expressing neocortical slice and luminescence image of the same slice integrated over the total duration of whole field luminescence traces shown in lower panels. Upper and lower traces are from the same recording of the slice shown in upper panels. The asterisk shows the position of the stimulation electrode in the white matter (upper panels) and arrowheads show the time of electrical stimulation (lower panels). Note the increase in amplitude and duration of whole field responses in the presence of CCH in both control and CNQX+APV conditions. (B) Summary of CCH and muscarine (M) effects on responses to electrical stimulation in layer I (Stim L1) or white matter (Stim WM) in control and CNQX+APV conditions. (C) Normalized and superimposed traces of mean luminescence responses obtained in indicated conditions. Note that cholinergic agonists induced the appearance of a slowly decaying response component that largely outlasted the initial peak response. The overall response was shorter, with a broader peak and more monotonous decay in CCH+CNQX+APV than in CCH conditions.

Figure 5. Cholinergic modulation of spatiotemporal network dynamics elicited by electrical stimulation. (A) GFP fluorescence image (greyscale, asterisk shows stimulation electrode position), whole field luminescence traces, and color-coded maps of bioluminescence emission integrated over indicated time-windows before and after stimulation (Stim) were obtained from the same GA expressing slice. Note the persistent activity in layer V in the presence of CCH. (B) *Upper panel:* cluster analysis of individual RAs based on the temporal profile of their luminescence response to a single electrical stimulation in the presence of CCH (tree diagram); and bioluminescence emission of each RA integrated over successive 2.8 s time-windows (color-coded time-plot). *Lower panels:* mean luminescence traces of RAs from clusters 1 and 2. Cluster 3 comprised n=299 RAs showing low light response to electrical stimulation. Superimposition of the sum of clusters 1+2 luminescence signal with the trace of the whole field response. (C) Position of selected cluster 1 and 2 RAs on the GFP fluorescence image of the GA-expressing slice (upper panels), luminescence traces of these individual RAs (middle panels), and of their summation (lower panels). *RAs illustrated are those exhibiting the highest light integral during the time-window of analysis.* Stimulation time is indicated by a blue bar. Scale bars: 100 μ m (A and C).

Figure 6. CCH effects on the spatial distribution of network responses to electrical stimulation. (A) Examples of whole field luminescence traces and corresponding and color-coded maps of bioluminescence emission showing the [0-500ms] response during electrical stimulation (black bars under traces represent the pattern of train stimulation) in layer I

(asterisk indicates the electrode position). Note that the response immediately decayed after the end of the stimulus in control condition, whereas the peak response outlasted the stimulus in CCH condition, and was followed by a slowly decaying component. **(B)** Plots of the sum of light intensities of vertical RA columns as a function of cortical horizontal distance obtained for the [0-500ms] responses shown in (A). The shape of these horizontal profiles of light intensity was quantified by measuring the amplitude of the distribution peak ($A_{\max}$), its width at half-amplitude ($W_{1/2}$), and the width of the CCH peak measured at the level of control peak half-amplitude (W^{control}). Note that the distribution peaks in control and CCH conditions have different amplitudes but similar $W_{1/2}$. **(C-E)** Analyses of responses to Stim-WM and Stim-L1 ($n=10$ and 12 slices, respectively). **(C)** Plot of mean values of $A_{\max}$, $W_{1/2}$, and W^{control} obtained for the [0-500 ms] responses. **(D)** Positions of the barycenters of whole-field light responses (colored dots) relative to the vertical of the stimulation electrode (dashed/dotted lines) and to layers II/III and V (separated by the horizontal dashed line). Barycenters of rapidly decaying control responses were calculated only for the [0-500 ms], whereas barycenters of long-lasting CCH responses were also calculated for subsequent time windows as indicated. Note the migration of barycenters to layer V between the [500ms-1s] and [5-20s] intervals in CCH conditions. **(E)** Ratios of layer V over layer II/III light emission normalized to their respective GFP fluorescence intensity.

Figure 7. Cholinergic modulation of electrophysiological responses of layer V pyramidal cells to electrical stimulation. Patch-clamp recordings of layer V pyramidal neurons from non-transduced acute slices. **(A)** Current clamp recording of a cell's responses to Stim-L1 in control condition, and at the beginning (left panel) or later during the course of CCH bath application (middle panel). In control condition, the trace returned to baseline after the train of action potentials elicited by electrical stimulation. In the presence of CCH, the cell was depolarized and the spike train was followed by a long-lasting ADP, which was surmounted by action potentials in subsequent sweeps. The graph shows the comparison between the mean decay of the ADP ($n=5$ cells) and the mean decay of the persistent bioluminescence response (Lumi, from Figure 4C). **(B)** Same as in (A) except that muscarine was used in place of CCH ($n=4$ cells). **(C)** Traces show EPSCs recorded in voltage clamp mode from the same cell before and after Stim-L1 (marked by large deflections) in indicated conditions. CCH application elicited an increase in EPSC frequency, and expanded traces illustrate the further increase in EPSC frequency induced by stimulation in the presence of CCH. The graph shows the comparison between the mean decay of the stimulation-induced EPSC increase ($n=4$ cells) and the mean decay of the persistent bioluminescence response. **(D)** Same as in (C) except that muscarine was used in place of CCH.

Supplementary Figure 1. Bioluminescence waves in the neocortical network. Analyses of bioluminescence spatiotemporal dynamics evoked by the removal of extracellular

magnesium. **(A)** GFP fluorescence image (greyscale, scale bar 100 μm) of a GA-expressing neocortical slice and luminescence image of the same slice integrated over the total duration of the luminescence trace shown in **(B)**. **(B)** Whole field luminescence recording showing epileptiform peak activities followed by long-lasting bursts of intense bioluminescence analyzed in panels **(C-D)** and **(E)** as indicated. Note the logarithmic scale of light intensity. **(C)** Expanded view of the trace of first luminescence burst (trace) and bioluminescence images corresponding to luminescence peaks as indicated by numbers above the trace. Note the wavelike propagation of light-emitting regions from left to right of the cortical slice during the burst. **(D)** 3D-plot of spatiotemporal variations of luminescence intensity with the 18x24 RAs plotted along the y-axis, time along the x-axis (scale bar 20 s), and light intensity coded in greyscale. Note the smooth downward shift of active RAs with time on the first half of the plot corresponding to the left-to-right propagation of the luminescent wave. **(E)** Same analysis as in **(D)** for the second light burst shown in **(B)**.

Supplementary Figure 2. Bioluminescence responses to electrical stimulation. **(A)** *Upper panels:* GFP fluorescence image (scale bar 100 μm) of a GA-expressing neocortical slice with the tip of the stimulation electrode positioned on layer I, and luminescence image of the same slice integrated over the total duration of the luminescence trace shown in lower left panel. *Lower panels:* Whole field bioluminescence recording of responses to series of 3 stimulation (500 ms train of 200 μs and 0.3 mA current pulses) with increasing pulse frequency, and plot of the peak amplitude of these responses. **(B)** Luminescence responses to electrical stimulation (arrowheads) were abolished in the presence of blockers of voltage-gated sodium channels (TTX) or calcium channels (Ni^{2+}).

Supplementary Figure 3. Cholinergic modulation of spatiotemporal network dynamics elicited by electrical stimulation in layer I. GFP fluorescence image (greyscale, asterisk shows stimulation electrode position), whole field luminescence traces, and color-coded maps of bioluminescence emission integrated over indicated time-windows before and after stimulation (Stim) were obtained from the same GA expressing slice. Note the persistent activity in layer V in the presence of CCH.

Table 1

Spatial properties of bioluminescence responses to electrical stimulation

	Stim WM (n=10 slices)		Stim L1 (n=12 slices)	
	During stimulation [0-500ms]	After stimulation [500ms-1s]	During stimulation [0-500ms]	After stimulation [500ms-1s]
Lateral extension (μm)				
$W_{1/2}$ (CTRL)	459 $\pm$ 44[$\pm$ 139]	272 $\pm$ 16[$\pm$ 51]	357 $\pm$ 16[$\pm$ 55]	220 $\pm$ 18[$\pm$ 62]
$W_{1/2}$ (CCH)	516 $\pm$ 67[$\pm$ 212]	576 $\pm$ 92[$\pm$ 291]*	488 $\pm$ 39[$\pm$ 135]*	490 $\pm$ 30[$\pm$ 104]*
W^{control} (CCH)	967 $\pm$ 133[$\pm$ 421]*	1022 $\pm$ 118[$\pm$ 373]*	781 $\pm$ 89[$\pm$ 308]*	816 $\pm$ 99[$\pm$ 343]*
Amplitude (%control)				
A_{max} (CCH)	357 $\pm$ 87[$\pm$ 275]*	978 $\pm$ 242[$\pm$ 765]*	254 $\pm$ 68[$\pm$ 236]*	1035 $\pm$ 184[$\pm$ 637]*
Lateral distance from electrode (μm)				
Barycenter CTRL	62 $\pm$ 17[$\pm$ 54]	81 $\pm$ 20[$\pm$ 63]	51 $\pm$ 5[$\pm$ 17]	49 $\pm$ 9[$\pm$ 31]
Barycenter CCH	85 $\pm$ 22[$\pm$ 70]	83 $\pm$ 23[$\pm$ 73]	41 $\pm$ 9[$\pm$ 31]	51 $\pm$ 12[$\pm$ 42]

* Difference from control
 $p < 0.05$ see statistical
report in supplementary
table 2

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

Supplementary table 1: Antibody reporting

antibody	supplier	Cat#	clonality	Host species	Antigen detail	RRID	dilution
Calbindin	Swant	CB38	polyclonal	rabbit	recombinant rat calbindin D-28k	AB_2721225	1:10000
Calretinin	Swant	7697	polyclonal	rabbit	recombinant human calretinin containing a 6-his tag at the N-terminal	AB_2619710	1:5000
Parvalbumin	Sigma	P3088	monoclonal, IgG1 clone PARV-19	mouse	Purified frog muscle parvalbumin	AB_477329	1:10000
NeuN	Chemicon	MAB377	Monoclonal, IgG1 Clone A60	mouse	Purified cell nuclei from mouse brain	AB_2298772	1:200
GFAP	Agilent	Z0334	polyclonal	rabbit	GFAP isolated from cow spinal cord.	AB_10013382	1:500
Cy3 conjugated anti-mouse	Jackson immuno research	115-165-062	Polyclonal	goat		AB_2338685	1:2000
Cy3 conjugated anti-rabbit	Jackson immuno research	111-165-144	Polyclonal	goat		AB_2338006	1:2000

Supplementary table 2

Reporting of statistical analyses of table 1

	Stim WM (n=10 slices)		Stim L1 (n=12 slices)	
	During stimulation [0-500ms]	After stimulation [500ms-1s]	During stimulation [0-500ms]	After stimulation [500ms-1s]
Lateral extension (μm)				
$W_{1/2}$ (CTRL)	-	-	-	-
$W_{1/2}$ (CCH)	n.s.	t(9)=3.176, p=0.0112	t(11)=3.735, p=0.0033	t(11)=8.845, p<0.0001
W^{control} (CCH)	t(9)=3.895, p=0.0073	t(9)=6.102, p=0.0004	t(11)=5.160, p=0.0006	t(11)=5.646, p=0.0001
ANOVA	F (1.269, 11.42) = 13.58, P = 0.0022	F (1.829, 16.46) = 24.44, P < 0.0001	F (1.242, 13.66) = 23.11, P = 0.0002	F (1.077, 11.84) = 27.73, P = 0.0002
Amplitude (%control)				
A_{max} (CCH)	t(9)=2.944, p=0.0164	W=74, p=0.0015	W=55, p=0.002	t(11)=5.091, p=0.0003
n.s.: non significant, p>0.05				

scale bar: A, 100 μ m, D, 20 s

Fig S1

Fig S2

A

B

Fig S3