Hybrid Voltage Sensor Imaging from Genetically Targeted Neurons in Hippocampal and Spinal Cord Slices

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Running head: Voltage imaging from genetically targeted cells

Abstract

Genetically encoded optical voltage sensors function well in cultured cells, but their greatest promise, the study of intact neural circuits, remains unfulfilled. Here we tested genetically encoded probes in brain and spinal slices from transgenic mice using
the hybrid voltage sensor (hVOS) imaging method. hVOS is based on targeting green fluorescent protein (GFP) to the plasma membrane, and adding lipophilic, negatively-charged dipicrylamine (DPA) to quench fluorescence by resonance energy transfer as it translocates within the lipid bilayer during voltage changes. In hippocampal slices from transgenic mice expressing membrane-anchored GFP from a thy-1.2 promoter, and in spinal cord slices from mice expressing membrane-anchored GFP from an HB9 promoter, electrical stimulation in the presence of 0.5-2 \(\mu M\) DPA elicited fluorescence changes of 1-3%. Patch clamp recordings indicated that 2 \(\mu M\) DPA had no significant effect on neuronal excitability, while 4 \(\mu M\) DPA broadened action potentials and miniature synaptic currents slightly. Thus, hVOS imaging in transgenic mice introduces a general approach to the study of neural circuits.
Introduction

The elucidation of neural circuit function requires techniques for recording electrical activity from many neurons simultaneously in intact circuits in real time. Voltage imaging has the potential to provide this information. Most synthetic probes detect voltage by an electrochromic mechanism, which produces rapid, linear responses to voltage changes (Grinvald et al. 1988; Jin et al. 2002; Momose-Sato et al. 1999; Salzberg 1983; Wu et al. 1998). The sensitivity of these probes can surpass 20%/100 mV (Grinvald et al. 1983; Rohr and Salzberg 1994). However, synthetic voltage-sensitive dyes superimpose signals from many cells. Such signals from intact circuits cannot be resolved into components from distinct cell types. Genetically-encoded optical voltage sensors could potentially solve this problem (Knopfel et al. 2006). By exploiting advances in genetic targeting, one could express a probe in a selected population of cells, and study that population without interference from other cells.

Efforts to develop genetically-encoded voltage sensors follow two strategies. A green fluorescent protein (GFP) variant can be incorporated into a voltage-sensing protein such as an ion channel or phosphatase, so that conformational changes of the voltage-sensing protein propagate to the GFP. Some of these probes produce signals as large as 40%/100 mV, but respond too slowly to report action potentials with accuracy (Dimitrov et al. 2007; Guerrero et al. 2002; Siegel and Isacoff 1997; Tsutsui et al. 2008). One such probe responds in about 1 msec but gives small signals of about 0.5%/100 mV (Ataka and Pieribone 2002). A second strategy exploits fluorescence resonance energy transfer (FRET), building on a method in which a fluorescent tag on the cell surface is within range for FRET with a second charged fluorophore within the hydrophobic
membrane core (Gonzalez and Tsien 1995). Voltage changes move the charged fluorophore and alter the distance to the surface tag. This in turn alters intermolecular FRET so that the fluorescence from each molecule changes. This method has produced sensitive optical voltage probes, and has been developed into a genetically-encoded sensor by replacing the synthetic surface tag with a membrane-targeted GFP. The membrane soluble fluorophore was also replaced by dipicrylamine (DPA), a non-fluorescent absorber (Chanda et al. 2005). In this hybrid voltage sensor (hVOS) technique, negatively charged DPA quenched GFP fluorescence in a voltage dependent manner, and because DPA translocates across the membrane in 0.5 msec (Chanda et al. 2005), hVOS can follow action potentials.

Genetically-encoded voltage sensors have been tested in cultured cells but have not been used in intact circuits such as brain slices. This remains a fundamental challenge for genetically-encoded voltage probes, and raises the question of whether the approach is feasible. Here, we demonstrate the performance of hVOS in transgenic mice with GFP targeted to neurons in two different preparations, hippocampal slices from animals expressing membrane-targeted GFP from the thy-1.2 promoter (De Paola et al. 2003; Feng et al. 2000), and spinal cord slices from animals expressing membrane-targeted GFP from the HB9 promoter (Arber et al. 1999; Lee et al. 2004; Thaler et al. 1999). Furthermore, tests of DPA helped to clarify its pharmacological action. Thus, imaging voltage from genetically specified cell types is feasible and can only improve with the development of better probes.
Materials and Methods

Fluorescence imaging. hVOS imaging was performed with a CCD-SMQ camera (RedshirtImaging, Decatur GA) coupled to an Olympus BX-51 fluorescence microscope equipped with an Olympus U-N41017 GFP filter cube and an Olympus 20x objective (NA=0.95). All imaging experiments were performed in the presence of DPA (0.5 or 2 μM), which was added at least 10 minutes before imaging. Most recordings were made after slices had been bathing in DPA for much longer times. Slices were illuminated with a 75 W Xe Arc lamp (Optiquip, Highland Mills, NY). The CCD-SMQ camera has an 80x80 pixel chip and can acquire full frames at a rate of up to 2 kHz. Pixels can be combined to improve signal quality at the expense of spatial resolution. The camera was controlled through an interface by a PC running Neuroplex (the RedshirtImaging software provided with the camera). Optical signals were low-pass filtered with a 5th order Butterworth filter at 500 Hz for display of traces or 100 Hz for display of color maps. Data analysis was also performed with this software; individual traces were imported into Origin (OriginLab, Northampton, MA) for further analysis. Two-photon micrographs were taken with an Ultima system (Prairie Technology, Middleton, WI).

Slices. Hippocampal slices were prepared according to previously described methods (Chang and Jackson 2006), using artificial cerebrospinal fluid consisting of (in mM) 124 NaCl, 3 KCl, 26 NaCO3, 1.25 NaH2PO4, and 10 glucose (pH 7.3 when bubbled...
with 95% O₂-5% CO₂) for both slicing and subsequent recording. Slices 300 μm thick were sectioned from the hippocampus of adult thy-1.2-mGFP mice (De Paola et al. 2003) with a tissue slicer (HR2, Sigmann Elektronik, Germany). The thy-1.2 promoter drives expression in a subset of neurons (Feng et al. 2000). mGFP is GFP with the 41 amino acid MARCKS motif (Wiederkehr et al. 1997) appended at the N-terminus to achieve plasma membrane targeting.

Transverse spinal cord slices were prepared from HB9::fGFP mice, a new line generated with farnesyl-GFP (fGFP) under the control of the HB9 promoter. The HB9 promoter targets spinal motor neurons in mice (Arber et al. 1999; Thaler et al. 1999). Spinal cord slices (300 μm thick) from 2-4 day old mice were prepared following a published protocol (Demir et al. 2002). For imaging experiments slices were perfused with a solution consisting of (in mM) 128 NaCl, 4 KCl, 1.5 CaCl₂, 1 MgSO₄, 0.5 NaH₂PO₄, 21 NaCO₃, and 30 glucose (Hinckley and Ziskind-Conhaim 2006).

The HB9::fGFP transgenic mouse line was generated by pronuclear DNA microinjection into a CB6F1 (C57BL/6J x BALB/c) background at the Salk Institute Transgenic Core Facility. The DNA construct was made by placing the sequence for farnesylated-GFP (pEGFP-F, BD Biosciences Clontech 6074-1) under the control of the previously characterized 9kb HB9 motor neuron promoter (Lee et al. 2004; Thaler et al. 1999). PCR screening of 37 pups revealed 6 male and one female carrying GFP in their genomes. Test matings and whole mount visualization of GFP at E12.5 identified three male founders reliably transmitting the transgene to their progeny. Further analysis determined that embryos from all three lines reliably express farnesylated-GFP as expected from the HB9 promoter. A single line, 2844, was identified as having the
brightest fluorescence signal. The 2844 founder was crossed to C57BL/6J mice to establish the HB9::fGFP line used in these experiments.

**Patch clamp recording.** Patch clamp recordings were performed with an Axopatch 200B patch clamp amplifier (Molecular Devices, Sunnyvale, CA) using patch electrodes fabricated from borosilicate glass capillaries. Electrodes were filled with a solution consisting of (in mM) 130 K-gluconate, 4 EGTA, 5 HEPES, 5 phosphocreatine, 2 Mg-ATP, 0.3 Na-GTP (pH=7.3) and had resistances of 2-4 MΩ. During recordings cells were bathed in a solution consisting of (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 0.8 MgCl₂, 10 HEPES, 10 glucose (pH=7.4). Action potentials were evoked under current-clamp. Spontaneous EPSCs were recorded under voltage clamp at a holding potential of -70 mV. For spontaneous EPSC recording the bathing solution also contained 1 μM TTX to block Na⁺ channels, 50 μM AP-5 to block NMDA receptors, and 100 μM picrotoxin to block GABA_A receptors. Recordings in 2 or 4 μM DPA were made at least 10 minutes after the start of perfusion of recording solutions with DPA. For capacitive current recording, the pipette solution contained (in mM) 135 CsCl, 10 HEPES, 1 EGTA, 4 Mg-ATP, 0.4 Na-GTP, with pH adjusted to 7.3 by CsOH.

**Cell Culture.** Primary cultures of hippocampal neurons were prepared from E-18 embryonic rats (Banker and Cowan 1977). Hippocampi were dissected and digested with trypsin for 30 minutes followed by trituration. The cell suspension was plated on poly-D-lysine coated 12 mm cover slips in 24-well plates. Neurons were plated in Neurobasal-A Medium (600 μl/well) supplemented with B27 (2%) and 2 mM Glutamax (Gibco/Invitrogen, Carlsbad, CA). Neurons were cultured in a humidified 95% air/5% CO₂ incubator and usually used at 12-16 days in vitro.
PC12 cells were cultured in DMEM with 5% horse serum and 5% iron-supplemented calf serum in 10 cm plates. The cells were incubated at 37 °C in a humidified 90% air / 10% CO₂ atmosphere. Cells are replated to collagen-poly-D-lysine coated cover slips in 24-well plates 48 h before recording.

Statistical Analysis. The student’s t-test was used to evaluate the actions of DPA.

Results

Hippocampal slices were prepared from a mouse with a MARCKS plasma membrane targeting motif (Wiederkehr et al. 1997) fused to enhanced GFP (mGFP) under the control of the thy-1.2 promoter (De Paola et al. 2003). Thy-1 promoters drive expression in neurons, with specific patterns varying between founder lines (Feng et al. 2000). The animal used in the present study has been shown to label pyramidal cells, interneurons, and granule cells sparsely in the hippocampus (De Paola et al. 2003). The fluorescence in these slices is clearly visible with the camera used here for voltage imaging (see Methods) (Fig. 1A). A 2-photon micrograph indicated targeting of mGFP to the plasma membrane of cell somata and processes (Fig. 1E). This image also shows some fluorescence in the cytoplasm, and this may indicate that the MARCKS motif does not target protein efficiently to the plasma membrane. In the presence of 2 μM DPA (added at least 10 minutes prior to imaging) the fluorescence signals showed a rapid change in response to electrical stimulation (Fig. 1B-D). This optical signal develops in a few msec and then decays to baseline in about 20 msec. The time course of these signals resembles that seen with conventional voltage sensitive dyes in hippocampal slices (Chang and Jackson 2006; 2003; Grinvald et al. 1982; Momose-Sato et al. 1999), and thus is likely to reflect the combination of synaptic and action potentials that these dyes
reveal in populations of cells. The fluorescence decreases with membrane depolarization because positive voltage drives DPA from the outer surface to the inner surface of the cell membrane. mGFP resides at the inner surface, and the arrival of DPA quenches its fluorescence.

The optical signals evoked by electrical stimulation are visible at distances up to \(~150 \, \mu \text{m}\) from the stimulating electrode (Fig. 1C), indicating that the responses can propagate over considerable distances. The average magnitudes of fluorescence changes normalized to resting light intensity (ΔF/F) are presented for 4 experiments in Fig. 5A (this figure summarizes both hippocampal and spinal cord slice experiments). Fig. 5B presents the average signal-to-noise ratio for these hVOS signals.

To test the dependence of these signals on the activation of excitatory synapses, kynurenic acid (5 mM) was added. This broad spectrum glutamate receptor antagonist reduced optical signals by more than 50%, and this inhibition reversed upon return to control recording solution (N=4). Fig. 5C presents the average signal magnitudes before, during and after kynurenate application, normalized to the initial response magnitude.

To evaluate the spread of responses further, we generated maps of optical response amplitude (ΔF/F) encoded as color (Fig. 2). This map is based on signal amplitudes 5 msec after stimulation, and shows that responses are half maximal at distances of 50 - 100 \(\mu \text{m}\) from the site of stimulation (Fig. 2 - control). After applying kynurenic acid the spread of responses was reduced considerably (Fig. 2 - KA block), and after removal of kynurenic acid the spread of responses recovered (Fig. 2 - recovery). Fig. 2 makes the point that the magnitude of the block depends on the distance from the site of stimulation. Close to the stimulating electrode some of the fluorescence change reflects a
direct effect of the stimulus on membrane potential whereas at distances of ~ 50 μm a
larger fraction of the fluorescence change depends on glutamate receptor activation and is
blocked by the antagonist.

We then tested hVOS in spinal cord slices prepared from HB9::fGFP mice. These
mice express fGFP from an HB9 promoter. fGFP was used in the original hVOS study
(Chanda et al. 2005), and the HB9 promoter identifies motoneurons (Arber et al. 1999;
Lee et al. 2004; Thaler et al. 1999). fGFP::HB9 mice show labeling of motoneurons and a
small cluster of spinal interneurons (Hinckley et al. 2005). Spinal cord slices from 2-4
day old fGFP::HB9 mice show bright fluorescence in the ventral region of a slice where
motoneurons are located (Fig. 3A). As in hippocampal slices, a 2-photon microscope
image showed fluorescent label in or near the plasma membrane (Fig 3E). In the presence
of either 0.5 or 2 μM DPA (added at least 10 minutes prior to imaging), electrical
stimulation elicited a rapid fluorescence decrease consistent with depolarization (Fig. 3B-
D). The time course was similar to that of signals in rat spinal cord slices evoked by
dorsal root stimulation and detected with a synthetic absorbance voltage sensitive dye
(Ziskind-Conhaim and Redman 2005). The signals were visible at distances of up to 200
μm from the site of stimulation (Fig. 3C) and show a rapid time course (Fig. 3D) similar
to those in hippocampal slices (Fig. 1D). Tetrodotoxin reversibly blocked the electrically-
evoked fluorescence changes (Figs. 3D and 5D), indicating that these optical signals
depended on voltage-gated Na⁺ channels, as expected for action potentials (N=3). Maps
of response amplitude encoded as color provide a global view of how responses spread
through a spinal cord slice, and show how tetrodotoxin blocks this spread (Fig. 4).
With 2 μM [DPA] the signal magnitudes in spinal cord slices were somewhat larger than with 0.5 μM. Fig. 5A displays the fractional fluorescence changes normalized to resting light (ΔF/F) for the two concentrations. For the experiments reported here, these values range from 1-3%, and are larger than those seen with synthetic fluorescence or absorbance dyes in slices (typically 0.1-0.5%) (Jin et al. 2002; Momose-Sato et al. 1999; Wu et al. 1998). Fig. 5D presents the signal-to-noise ratios, which are generally smaller than those obtained with synthetic dyes.

Comparing the 2 μM DPA data between these two preparations, signals were noticeably higher for spinal cord slices than for hippocampal slices. Since both the preparation and membrane targeting motif differ between the two experiments, the difference is difficult to evaluate. However, the membrane targeting motif used in the spinal cord slice experiments (pEGFP-F vector from Clontech; see Methods) is only 20 amino acids whereas the MARCKS membrane targeting motif used in mGFP in the hippocampal slice experiments is 40 amino acids (De Paola et al. 2003). Shortening the membrane targeting motif of a probe improves its performance (Sjulson and Miesenbock 2008; Wang et al. 2008), and this may account for the different signal intensities between the two experiments. Comparison of Figs. 1E and 3E suggests that the farnesylated probe targets the plasma membrane better than the MARCKS probe and this also may contribute to the differences.

DPA induces increases in membrane capacitance, which can impair membrane excitability at concentrations above 5 μM (Chanda et al. 2005; DiFranco et al. 2007; Fernandez et al. 1983). It has also been reported that 2 μM DPA completely abolished electrically evoked voltage changes in Drosophila antenna lobe (Sjulson and Miesenbock September 2009).
To evaluate the concentration range over which DPA can be used without pharmacological or toxic effects on neurons we patch clamped cultured neurons and recorded action potentials evoked by current pulses in the presence of 0, 2, and 4 μM DPA. Evoked action potentials (Fig. 6A) and spontaneous action potentials (Fig. 6B) were readily recorded under all conditions tested. The amplitudes of evoked action potentials were unchanged by the addition of up to 4 μM DPA (Fig. 6C). The action potential decay time increased slightly, but the difference only became statistically significant at 4 μM (Fig. 6D).

We also recorded miniature excitatory postsynaptic currents (mEPSCs) from cultured neurons in 0, 2 and 4 μM DPA (Fig. 7A). The cumulative amplitude distributions were only slightly altered (Fig. 7B), and the mean amplitude was significantly different from controls only for 4 μM (Fig. 7C). The mEPSC rise times were indistinguishable for the three conditions tested (Fig. 7D), and the decay times were slightly increased by 4 μM DPA (Fig. 7E).

Because the pharmacological activity of DPA probably arises through its effect on membrane capacitance (DiFranco et al. 2007; Fernandez et al. 1983; Oberhauser and Fernandez 1995), we made measurements of cell capacitance in PC12 cells, where the round shape eliminates the problem of poor space clamp. Voltage steps produced a charging transient which could be integrated to estimate charge and thus capacitance. DPA produced increments in the charging transient (Fig. 8A), thus increasing membrane capacitance in a voltage dependent manner (Fig. 8B). The voltage dependence resembles a Boltzmann function as expected for the two-state nature of DPA movement within lipid bilayers (Oberhauser and Fernandez 1995). The capacitance change occurred
between -80 and 20 mV, and the increments due to DPA were 11% and 32% for 2 and 4 μM DPA, respectively (Fig. 8B). These results indicate that acute application of DPA has a weak pharmacological action at 4 μM.

Discussion

This study has demonstrated the feasibility of hVOS imaging from genetically targeted neurons in transgenic mice. The fluorescence changes evoked by electrical stimulation can be attributed to the activation of neurons in hippocampal slices and motoneurons and HB9 interneurons in spinal cord slices. Thus, the temporal-spatial patterns of activity revealed by these probes report the circuit activity of a genetically defined subset of neurons within an intact circuit. Recordings similar to these from panels of animals with different targeted cell types will provide important information about the timing of activation of different cell types during different forms of electrical activity. This technique thus can address an important general class of questions about neural circuitry.

The fluorescence images from the slices used in this study show some cell bodies (e.g., the bright spots in Fig. 1A), but also show a great deal of diffuse fluorescence, which presumably arises from processes. Because of the diffuse fluorescence, the evoked optical signals reported here cannot be assigned to specific cells. The generation of transgenic animals with fluorescence in sparser populations of cells will likely lead to preparations where single cells can be recognized more clearly and in these cases we can expect to resolve fluorescence changes from single cells.

Because the probes employed in the present study have not been optimized for hVOS, it is very encouraging that they performed well. ΔF/F and signal-to-noise ratio are
often used to evaluate the performance of optical probes and these values were presented in Figs. 5B and 5C. However, these quantities are very sensitive to experimental conditions, such as background light level and stimulus protocol. The signal-to-noise ratio also depends on illumination intensity, pixel size, recording bandwidth, and number of averages. This complicates the use of the values presented in Figs. 5B and 5C in quantitative comparisons with other probes. Nevertheless, these measurements were made under fairly typical conditions for voltage imaging experiments in brain slices, and the signals were generally good for optical voltage sensors. Thus, these results indicate that hVOS will be useful in voltage imaging experiments.

A number of probe properties under experimental control influence the sensitivity and signal-to-noise ratio in hVOS imaging, and a systematic study of different molecular variants promises significant improvement in the technique. Fluorescent proteins with different spectral characteristics should optimize FRET efficiency with DPA. Previous studies have suggested that fluorescent proteins with spectra shifted toward shorter wavelengths improve the quality of signals (DiFranco et al. 2007; Sjulson and Miesenbock 2008; Wang et al. 2008). Varying the amino acid sequence that anchors a fluorescent protein to the plasma membrane may improve membrane targeting as well as optimize the distance and orientation for FRET. However, making FRET so efficient that DPA can quench on either side of the membrane would reduce the effectiveness in hVOS. Other aspects of performance could be improved by finding a better membrane soluble synthetic molecule. A molecule that can be used at higher concentrations or that traverses a greater distance in response to changes in voltage may offer advantages over DPA.
The voltage dependent optical signals seen with hVOS must be weighed against the concern over possible toxicity of DPA. In 2 μM DPA action potentials and mEPSCs were indistinguishable from controls; in 4 μM DPA mEPSCs were slightly larger and both mEPSCs and spikes had slightly slower decay times. These results are virtually identical to those in a recent study in cultured neurons (Bradley et al. 2009). The longer decay times probably reflect increases in membrane capacitance (Fig. 8) (DiFranco et al. 2007; Fernandez et al. 1983; Oberhauser and Fernandez 1995). Our results indicate that 2 μM DPA will have very small effects on most forms of cellular excitability, but the absence of effects on action potentials and mEPSCs must interpreted with caution. Even though these wave forms in 2 μM DPA are indistinguishable from controls, action potential conduction velocity depends on membrane capacitance, scaling as \( C_{m}^{-0.5} \) (Hodgkin and Huxley 1952). The 11% increase in capacitance at 2 μM DPA will slow action potential conduction by 5%; the 32% increase at 4 μM DPA will slow action potential conduction by 15%. It should be noted that the theoretical dependence of conduction velocity on \( C_{m}^{-0.5} \) depends on a linear capacitance and instantaneous charge movement. The nonlinearity of the DPA-induced capacitance increase will alter the functional dependence of velocity on \( C_{m} \) in ways that are difficult to predict; the 0.5 msec DPA charging time will reduce the effect of DPA on velocity. DPA induced changes in action potential conduction velocity could generate subtle alterations in the timing and synchrony within intact neural circuits and these subtle actions will have to be borne in mind in future efforts to use hVOS to study neural circuits. In this regard it is encouraging that signals were seen with only 0.5 μM DPA (Fig. 3 and 5) and at this concentration action potential conduction velocity should not be significantly altered.
Our recordings of action potentials and mEPSCs are difficult to reconcile with a recent study in *Drosophila* antenna lobe, where 2 μM DPA completely blocked an electrically evoked signal (Sjulson and Miesenbock 2008). However, it has been noted recently that the use of the genetically-encoded probe synaptophluorin is of limited value in experiments with DPA (Bradley et al. 2009). This synaptic vesicle-targeted, pH-sensitive GFP variant generates an increase in fluorescence of synaptophluorin as it relocates to the extracellular surface of the plasma membrane during synaptic vesicle exocytosis. The authors found no change in synaptophluorin fluorescence in the presence of DPA and concluded that DPA inhibited electrical activity. However, DPA will quench synaptophysin fluorescence when it comes within range for FRET. When synaptophysin moves to the outer surface of the plasma membrane during exocytosis, DPA will very likely quench its fluorescence and thus block the ability of this probe to report electrical activity. The results of the present study, in which we have directly recorded electrical activity in the presence of DPA, are consistent with reports in different preparations (Bradley et al. 2009; Chanda et al. 2005; DiFranco et al. 2007). Thus, at concentrations of 2 μM or less DPA produces a small increment in membrane capacitance and does not significantly dampen changes in membrane potential. Thus, hVOS imaging can be performed with 2 μM DPA with little if any adverse effects on electrical activity.

With the success of hVOS in two transgenic mice, this work introduces a viable method for studying electrical activity in genetically targeted cells in intact neural circuits. Animals expressing membrane-targeted GFP constructs in other cell types are likely to yield optical signals comparable to those reported here, and with the prospect of
improving the fluorescent proteins, we can expect to create animals for which hVOS will
yield signals of even higher quality.

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Figure Legends

Fig. 1. hVOS signals in hippocampal slices from thy-1.2-mGFP mice ([DPA]=2 μM).

A. GFP fluorescence in a hippocampal slice as seen with the SMQ-CCD camera, taken
with full resolution (80 x 75 pixels) (image size 400 μm x 375 μm). The star indicates the
site of stimulation and the very faint shadow of the stimulating microelectrode is visible
above. B and C. Spatial spread of hVOS signals. B shows selected traces on a
background of gray shading to indicate resting light intensity. C shows the same traces
highlighted among a grid of all traces. Traces colors are arbitrary assignments by the
software. A stimulation pulse (250 μA, 2 msec) was applied 20 msec after the start of
data acquisition. The image combines 5 pixels into one superpixel to yield a 16 x 15
image. Frames were acquired at 2 kHz and displayed with high pass filtering at 6 Hz. All
traces were divided by resting light intensity and because illumination was weaker at the
periphery, dividing by the smaller numbers increased the noise. D. The evoked signals
from a superpixel from B/C shows reversible block by kynurenic acid (KA, 5 mM). A
control response, a response after perfusion with KA for 1 hour, and a response after
returning to control solution for 30 min illustrate the effect of reversible block of
glutamate receptors. DPA was present throughout. Similar results were obtained in 4
slices (see Fig. 3 for average response magnitude). E. A 2-photon micrograph taken of a
hippocampal slice from a thy-1.2-mGFP mouse shows membrane labeling of the cell
soma, fine processes, and dendritic spines.

Fig. 2. Maps of hVOS response amplitude (∆F/F) encoded as color illustrate the spread of
responses in a hippocampal slice from a thy-1.2-mGFP transgenic mouse (based on a
time point 5 msec after stimulation from the experiment used to make Figs. 1A-D).
Signals for all three maps were normalized to the largest response in the field of view of the control experiment (left) so that the maximum and minimum determine the range of the color scale indicated horizontally above. Kynurenic acid (KA block) blocked the responses (middle), which recovered after KA removal (right). The data were high pass filtered at 6 Hz and low pass filtered at 100 Hz. Note that the shadow of the stimulation electrode produces a small artifact along a trajectory slightly off vertical.

**Fig. 3.** hVOS signals in spinal cord slices from HB9::fGFP mice ([DPA]=0.5 μM). A. GFP fluorescence, as seen with the SMQ-CCD camera. The full resolution image has dimensions of 400 μm x 375 μm. The star indicates the site of stimulation. B and C. Spatial spread of hVOS signals as in Fig. 1. A stimulation pulse (250 μA, 2 msec) was applied 20 msec after the start of data acquisition. The image combines 5 pixels to give an image of 16 x 15 superpixels. Frames were acquired at 2 kHz and displayed with high pass filtering at 4.5 Hz. D. The evoked signals from a superpixel from B/C shows reversible blocked by tetrodotoxin (TTX, 1 μM). A control response, a response after perfusion with TTX for 1 hour, and a response after returning to control solution for 30 min illustrate the effect of blocking Na⁺ channels. Similar results were obtained in 3 slices. DPA was present throughout. E. A 2-photon micrograph taken of a spinal slice from an fGFP::HB9 mouse shows membrane labeling of the cell soma, fine processes, and dendritic spines.

**Fig 4.** Maps of response amplitude (ΔF/F) encoded as color illustrate the spread of responses in a spinal cord slice an HB9::fGFP transgenic mouse (based on the experiment used for Figs. 3A-D.). As in Fig. 2, signals were normalized to the maximum of the control experiment, and tetrodotoxin (TTX - middle) reversibly blocked the responses.

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(recovery - right). The data were high pass filtered at 4.5 Hz and low pass filtered at 100 Hz. Note that the stimulation electrode from above produces small artifacts along a trajectory slightly off vertical.

**Fig. 5.** Average responses and signal-to-noise ratios (S/N) for hVOS signals. **A.** Fractional fluorescence changes were normalized to the resting light to calculate $\Delta F/F$. **B.** S/N was taken as $\Delta F/F$ divided by the root mean square noise of a 30 msec segment of fluorescence corrected for linear drift. $\Delta F/F$ and S/N were determined for 10 trial averages from a 25 μm x 25 μm area at 500 Hz. **C.** Averages of $\Delta F/F$ for thy-1.2 transgenic mice before, during, and after 5 mM KA. Signals were normalized to the initial, control response (see Fig. 1D). **D.** Averages of $\Delta F/F$ for fGFP::HB9 mice before, during, and after application of 1 μM TTX, normalized to the initial control response (see Fig. 2D). Error bars indicate standard error of mean.

**Fig. 6.** Tests of DPA on action potentials in cultured hippocampal neurons. **A.** Action potentials in 0, 2 and 4 μM DPA evoked by 1 msec current pulses under current clamp. **B.** Spontaneous action potentials (in neurons current clamped and held above threshold). **C.** Action potential amplitudes were not significantly different between 0, 2, and 4 μM DPA. Control, 90.2 mV (n=10); 2 μM DPA 93.2 mV (n=11); 4 μM DPA (n=10), 86.9 mV. **D.** Decay times increased slightly as [DPA] increased. Control, 2.38 msec; 2 μM DPA, 2.55 msec; 4 μM DPA, 2.85 msec. * indicates a statistically significant difference compared to 0 DPA (P < 0.05 by the student’s t-test). Error bars indicate standard error of mean.

**Fig. 7.** DPA and miniature EPSCs in cultured rat hippocampal neurons under voltage clamp. **A.** mEPSCs in 0, 2, or 4 μM DPA recorded at a holding potential of -70 mV. **B.** The cumulative probability of mEPSC amplitude before and after DPA addition. **C.** DPA
increased the mEPSC amplitude, but only 4 μM produced a statistically significant increase of ~10%. D. The 10%-90% rise time was not significantly changed by 2 and 4 μM DPA. E. The time for 37% decay increased by ~15% in 4 μM DPA. For controls there were 1365 mEPSCs from 9 neurons; for 2 μM DPA there were 948 mEPSCs from 8 neurons; for 4 μM DPA there were 970 mEPSCs from 8 neurons. Error bars indicate standard error of mean.

Fig. 8. DPA increased membrane capacitance in PC12 cells. A. Capacitive current was elicited by steps from -80 to 0 mV. Voltage steps were applied before and after 2 μM or 4 μM DPA perfusion (7 cells for each concentration). B. Normalized cell capacitance as a function of membrane potential before and after DPA perfusion. Different voltage steps were applied, as in A, and the corresponding capacitance was calculated by dividing the integrated charge of the transient by the amplitude of the depolarization step. Cell membrane capacitance was normalized to the capacitance at 0 mV in zero DPA. At 0 mV, 2 μM and 4 μM DPA increased the capacitance by 11% and 32%, respectively. Error bars indicate standard error of mean.
Fig. 1

A. [Image of Figure 1A]

B. [Image of Figure 1B]

C. [Image of Figure 1C]

D. [Image of Figure 1D]

E. [Image of Figure 1E]

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Fig. 3

A.

B.

C.

D.

E.

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Fig. 4

Control  TTX block  recovery

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