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Selective Photostimulation of Genetically ChARGed Neurons

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Summary

To permit direct functional analyses of neural circuits, we have developed a method for stimulating groups of genetically designated neurons optically. Coexpression of the *Drosophila* photoreceptor genes encoding arrestin-2, rhodopsin (formed by liganding opsin with retinal), and the α subunit of the cognate heterotrimeric G protein—an explosive combination we term "chARGe"—sensitizes generalist vertebrate neurons to light. Illumination of a mixed population of neurons elicits action potentials selectively and cell-autonomously in its genetically chARGed members. In contrast to bath-applied photostimulants or caged neurotransmitters, which act indiscriminately throughout the illuminated volume, chARGe localizes the responsiveness to light. Distributed activity may thus be fed directly into a circumscribed population of neurons in intact tissue, irrespective of the spatial arrangement of its elements.

Introduction

Reconstitution of biological function with pure agents–catalysis by purified enzymes, rescue of mutant phenotypes by isolated genes—offers compelling insights into causality and mechanism. The instances in neuroscience where artificial stimulation of neurons has elicited sensations or movements (Penfield and Rasmussen, 1950) or influenced decisions (Salzman et al., 1990) are no exception: they demonstrate most vividly and directly the fundamental link between mental activity and its neuronal substrate. Due to the technical difficulty of stimulating functionally circumscribed but anatomically dispersed groups of neurons, however, most artificial stimuli are directed at single, isolated neurons or ill-defined clusters rather than the precisely delineated, coherent ensembles thought to be important for nervous system function. The ability to feed synthetic activity directly into such ensembles would provide a powerful tool for mapping functional connections and determining the response characteristics of circuits and systems, as well as for unveiling behaviorally relevant information carried in distributed neural representations.

Here we describe a general method for stimulating functionally circumscribed ensembles of neurons in intact tissue, in virtually any three-dimensional arrangement or anatomical location. The method uses a broadly transmitted optical signal that can be decoded and transduced into electrical activity by only a subset of all illuminated neurons. The "receiver" of the optical signal is encoded in DNA, and the responsive subset of neurons can therefore be restricted genetically (Crick, 1999; Zemelman and Miesenböck, 2001) to certain cell types (through cell-type specific promoters) or circuit elements (through viral vectors that spread through synaptic contacts). Localizing the susceptibility to stimulation is an inversion of the logic of existing stimulation methods, which, whether electrical (Pine, 1980; Regehr et al., 1989; Kovacs, 1994; Fromherz and Stett, 1995; Colicos et al., 2001) or photochemical (Farber and Grinvald, 1983; Callaway and Katz, 1993; Dalva and Katz, 1994; Denk, 1994; Pettit et al., 1997; Matsuzaki et al., 2001), must narrowly localize the stimulus to avoid indiscriminate responses. Since sensitivity to light is built into each target neuron, advance knowledge of its spatial coordinates is unnecessary. Large numbers of neurons can be addressed simultaneously and precisely, without undesirable cross-talk to neighboring cells that are functionally distinct.

Results and Discussion

Because the photoreceptors of vertebrate and invertebrate eyes are naturally equipped with genetically encoded "receivers" that allow them to respond to light, our search for sensitizing components that could be transplanted to nonphotoreceptor cells concentrated on them. Phototransduction in invertebrates (Montell, 1999; Hardie and Raghu, 2001) differs in two fundamental respects from that in vertebrates (Stryer, 1991; Burns and Baylor, 2001): the origin and polarity of the photoreceptor current, and the mechanism that regenerates the light-sensitive chromophore, 11-*cis* retinal, from the bleached all-*trans* isomer. The first difference is due to the activation of distinct classes of heterotrimeric G proteins by photoexcited metarhodopsins. Vertebrate metarhodopsins signal through transducin to cGMP phosphodiesterase. As cGMP is consumed, cGMP-gated cation channels carrying a depolarizing dark current close, and the photoreceptor hyperpolarizes (Stryer, 1991; Burns and Baylor, 2001). Invertebrate metarhodopsins, in contrast, couple to a member of the $G_{q/11}$ class of heterotrimeric G proteins (Lee et al., 1990), which activates phospholipase C (PLC) (Inoue et al., 1985; Bloomquist et al., 1988; Neer, 1995). In a poorly understood mechanism, a product of PLC—inositol-1,4,5-triphosphate (IP_3), diacylglycerol, or an indirect metabolite—opens cation channels in the plasma membrane (Montell and Rubin, 1989; Hardie and Minke, 1992; Phillips et al., 1992; Hardie and Raghu, 2001), and the photoreceptor depolarizes (Hardie, 1991; Ranganathan et al., 1991).

Binding of arrestin inactivates metarhodopsin (Stryer, 1991; Byk et al., 1993; Montell, 1999; Burns and Baylor, 2001) and initiates the biochemical cycle that regenerates 11-*cis* retinal. The vertebrate cycle consists of a

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series of enzymatically catalyzed transformations of the free chromophore, which is expelled from the sterically strained metarhodopsin-arrestin complex (Wald, 1968; Rando, 1992). The invertebrate photopigment, in contrast, is a bistable device that does not require the dissociation of retinal from metarhodopsin; it relies on photochemistry rather than enzyme catalysis to regenerate the light-sensitive chromophore (Hillman et al., 1983; Kiselev and Subramaniam, 1994; Ranganathan and Stevens, 1995; Kiselev and Subramaniam, 1997). Absorption of a second photon, of lower energy than that required for the transition that activates visual transduction, isomerizes bound all-*trans* to 11-*cis* retinal and releases the regenerated rhodopsin from its complex with arrestin (Byk et al., 1993; Ranganathan and Stevens, 1995).

The Minimal "chARGe"

The simplicity of its retinal cycle should enable invertebrate phototransduction to function outside a specialized photoreceptor environment. Expressed ectopically, the invertebrate—but not the vertebrate—transduction machinery could serve as a light-controlled source of depolarizing current to stimulate electrical activity in excitable cells, or of intracellular Ca^{2+} to activate Ca^{2+}-dependent processes such as neurotransmitter release. In a first test of this possibility, and in an effort to delineate the minimal set of transduction components necessary for sensitizing a nonphotoreceptor cell to light, *Xenopus* oocytes were programmed with pools of mRNAs encoding combinations of ten proteins with genetically or biochemically defined roles in *Drosophila* phototransduction, the most thoroughly characterized invertebrate system (Montell, 1999; Hardie and Raghu, 2001). The ten candidate proteins included NinaE, the blue-sensitive opsin of R1–R6 photoreceptors (O'Tousa et al., 1985; Zuker et al., 1985); NinaA, a peptidyl-prolyl *cis-trans* isomerase implicated in folding and intracellular transport of NinaE (Shieh et al., 1989); the major arrestin isoform, arrestin-2 (Hyde et al., 1990; LeVine et al., 1990; Yamada et al., 1990); the α, β, and γ subunits of the cognate heterotrimeric G protein (Lee et al., 1990; Yarfitz et al., 1991; Schulz et al., 1999); NorpA, an eye-specific PLC (Bloomquist et al., 1988); the "light-activated" cation channels TRP and TRPL (Montell and Rubin, 1989; Hardie and Minke, 1992; Phillips et al., 1992); and InaD, a multivalent adaptor thought to collect transduction components into discrete signaling units (Shieh and Niemeyer, 1995; Huber et al., 1996; Tsunoda et al., 1997; Scott and Zuker, 1998).

Unliganded NinaE in the membrane of voltage-clamped oocytes was reconstituted in the dark with 40 μM synthetic all-*trans* retinal, to form a metarhodopsin-like intermediate that could be further photoconverted (Hillman et al., 1983; Kiselev and Subramaniam, 1994; Ranganathan and Stevens, 1995) to rhodopsin. Since the reconstituted molecule incorporated a heterologous chromophore with unknown spectral properties—the retinal of vertebrates, which is available commercially, rather than the 3-hydroxyretinal of flies (Vogt and Kirschfeld, 1984), which is not—white light, expected to contain all spectral components necessary to drive the hybrid rhodopsin through its retinal cycle, was used as the stimulus.

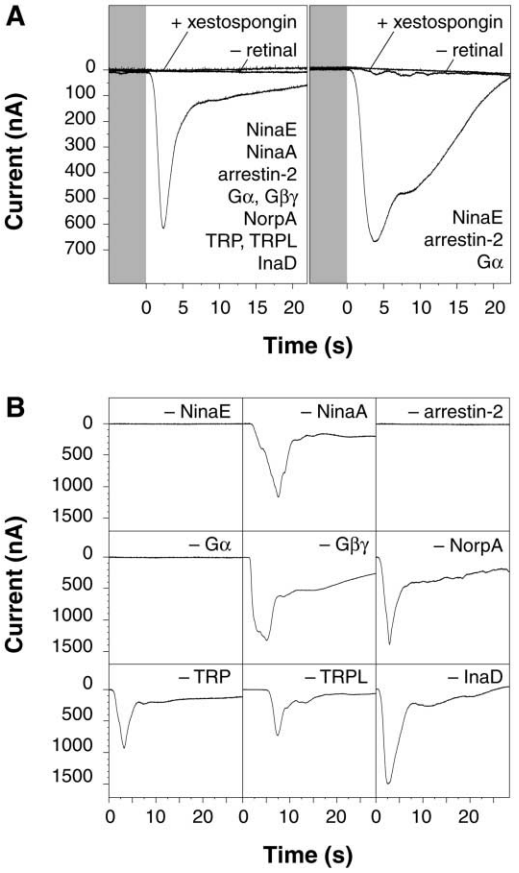

Figure 1. Reconstitution of *Drosophila* Phototransduction in *Xenopus* Oocytes: Delineation of the Minimal ChARGe

Membrane potentials were clamped to −80 mV and transmembrane currents recorded during periods of darkness (shaded backgrounds) and white illumination (white backgrounds). Inward currents are displayed as downward deflections from baseline; the zero time point is defined as the onset of the light stimulus. Oocytes were programmed to express: ([A], left) a "complete" set of ten phototransduction proteins, consisting of NinaE, NinaA, arrestin-2, Gα, Gβγ, NorpA, TRP, TRPL, and InaD; ([A], right) the minimal chARGe, consisting of NinaE, arrestin-2, and Gα; or (B) "complete" sets deficient in the indicated single components. Although formally the product of two genes, the obligate Gβγ heterodimer is treated as a single entity.

Illumination of oocytes expressing the full complement of ten photoreceptor proteins (expression of NinaE, arrestin-2, Gα, NorpA, TRP, and InaD was confirmed by immunoblotting of oocyte extracts [data not shown]; expression of NinaA, Gβ, Gγ, and TRPL was not tested) evoked positive currents with latencies to peak of 2.29 to 6.74 s and amplitudes of several hundred nA (n = 15 oocytes) (Figure 1A). Responses required reconstitution of NinaE with retinal (n = 9 oocytes) (Figure 1A) and were inhibited by 20 μM xestospongin C (n = 5 oocytes), an antagonist of Ca^{2+} release from IP_3-sensitive stores (Gafni et al., 1997) (Figure 1A).

The omission of any one of three mRNA species—those encoding NinaE, arrestin-2, and the G protein α subunit, respectively—abolished the photocurrent; deficiencies in other mRNA species had no effect (Figure 1B). The combination of the three essential phototrans-

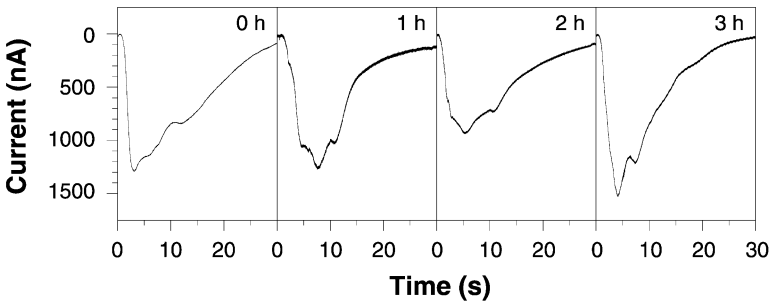

Figure 2. Stability of ChARGe after Reconstitution of NinaE with Retinal

Oocytes were programmed to express the minimal chARGe, consisting of NinaE, arrestin-2, and Gα, and exposed to 40 μM synthetic all-*trans* retinal for 15 min. At the end of the retinal load, the retinal-containing solution was displaced by a minimum of 10 chamber volumes of retinal-free extracellular recording solution, and the chamber was perfused with retinal-free recording solution throughout the remainder of the experiment. Light-evoked transmembrane currents were recorded at −80 mV after the indicated intervals following the retinal load. Inward currents are displayed as downward deflections from baseline; the zero time point is defined as the onset of the light stimulus.

duction components alone was also sufficient to support the full amplitude of a photocurrent with identical pharmacological sensitivities (n = 15 oocytes) (Figure 1A). Rhodopsin and its immediate interacting partners (Ranganathan and Stevens, 1995; Montell, 1999), arrestin-2 and $G_q\alpha$, thus constitute the minimal light-triggered "chARGe." Arrestin-2 presumably assists the conversion of metarhodopsin to rhodopsin (Kiselev and Subramaniam, 1994; Ranganathan and Stevens, 1995), while $G_q\alpha$ (which must assemble with an endogenous $G\beta\gamma$ dimer for function [Neer, 1995; Bohm et al., 1997]) couples activated NinaE to downstream effectors supplied by the oocyte.

The strict dependence on two auxiliary components, arrestin-2 and $G_q\alpha$, distinguishes NinaE from vertebrate rhodopsin, which by itself is able to trigger small photocurrents in oocytes (Khorana et al., 1988; Knox et al., 1993). While the two auxiliary components are essential for the function of NinaE, they in addition each contribute a unique and important feature to the mechanism of chARGe. $G_q\alpha$ provides a comparatively loss-less interface between the light-activated receptor and its effectors, resulting in photocurrents that exceed those elicited by vertebrate rhodopsin in the same system (Khorana et al., 1988; Knox et al., 1993) by 10- to 100-fold. Arrestin-2, through its ability to stabilize the metarhodopsin form of NinaE (Kiselev and Subramaniam, 1994, 1997), allows the molecule to be photoconverted back to rhodopsin. In the presence of arrestin-2, the photocycle of NinaE thus forms a closed loop that can regenerate the light-sensitive chromophore autonomously. The vertebrate rhodopsin, in contrast, depends on a steady supply of exogenous 11-*cis* retinal to replace the bleached all-*trans* isomer (Wald, 1968; Khorana et al., 1988; Rando, 1992; Knox et al., 1993).

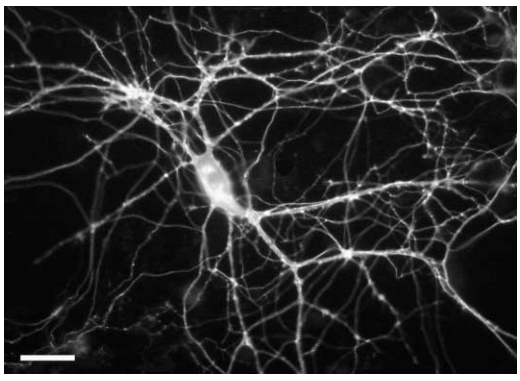

Figure 3. Identification of a ChARGed Hippocampal Neuron in Primary Culture

ChARGed neurons express a membrane-bound version of EGFP (Moriyoshi et al., 1996) that permits their identification during live microscopy. Scale bar, 20 μm.

The Requirement for Retinal

Although its photocycle can operate autonomously once functional, chARGe requires an initial dose of retinal to reconstitute rhodopsin from empty, unliganded NinaE (Figure 1A). Binding of retinal to opsin leads to the formation of a covalent Schiff base (Wald, 1968) between the chromophore and lysine-319 in the seventh transmembrane segment of NinaE (O'Tousa et al., 1985; Zuker et al., 1985). As a result, the initially reversible receptor-ligand equilibrium governed by mass action gives way to a stable association that is independent of the concentration of free ligand. For most practical purposes, a single saturating bolus of retinal should therefore be able to satisfy the chromophore requirement of NinaE permanently. Indeed, NinaE reconstituted with 40 μM synthetic all-*trans* retinal for 15 min remained functional for hours in the absence of exogenous retinal, with no attenuation of the photocurrent over time (n = 8 oocytes) (Figure 2).

Photostimulation of ChARGed Neurons

To examine its ability to transduce an optical stimulus into neuronal activity, the minimal chARGe (NinaE, arrestin-2, and $G_q\alpha$) was expressed in hippocampal neurons in primary culture. ChARGed neurons, identified by a cotransfected GFP marker (Figure 3), were indistinguishable in differential interference contrast (DIC) from their untransfected counterparts and had identical resting membrane potentials, synaptic potentials, and rheobases (see below). Neither chARGed nor untransfected neurons in retinal-treated cultures showed signs of damage or death after exposure to stimulating light (data not shown).

For intracellular recordings of light-evoked electrical activity, neurons were placed under whole-cell current clamp before reconstitution of NinaE with retinal. The membrane potential of chARGed neurons in the dark showed occasional excitatory postsynaptic potentials (EPSPs; Figure 4A, inset), which exceedingly rarely summed to threshold (e.g., Figure 4C). Exposure to white

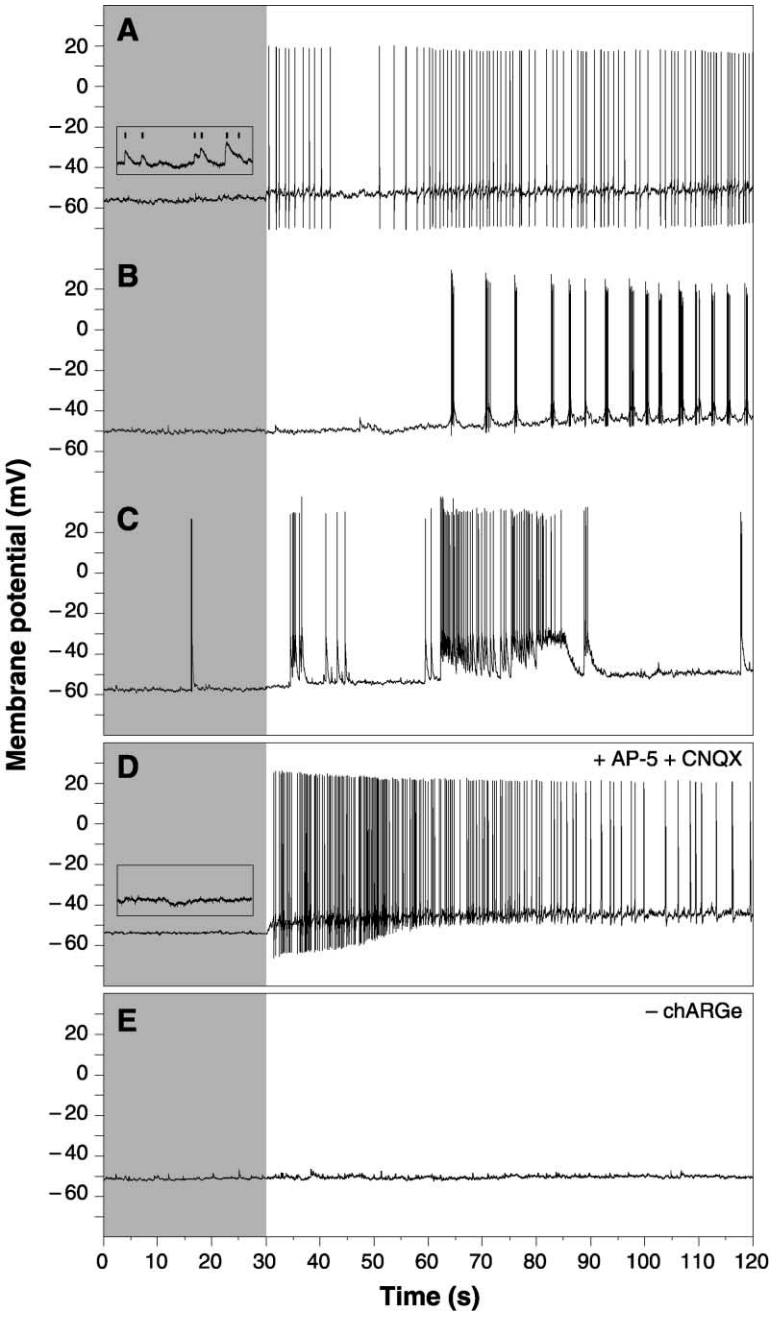

Figure 4. Photostimulation of ChARGed Hippocampal Neurons

Periods of darkness and white illumination are indicated by shaded and white backgrounds, respectively.

(A–D) Membrane potential records of four chARGed neurons, under conditions where excitatory synaptic transmission is intact (A–C) or blocked (D), reveal 86 (A), 86 (B), 59 (C), and 157 (D) light-evoked action potentials and spike latencies of 0.476 (A), 34.094 (B), 4.566 (C), and 1.330 s (D). Enlarged membrane potential traces (insets in [A] and [D]) show EPSPs ([A], vertical marks) and their elimination by 50 μM AP-5 and 10 μM CNQX (D). Boxes surrounding the enlarged traces extend 5 mV vertically and 1 s horizontally.

(E) An untransfected neuron incubated with retinal does not generate light-evoked action potentials but receives EPSPs at an increased frequency during illumination (2.08 EPSPs s^{-1} during illumination versus 0.87 EPSPs s^{-1} in the dark). The increase in EPSP frequency indicates light-triggered activity in chARGed neurons that are presynaptic to the recorded, untransfected cell.

light increased the frequency of action potentials dramatically (Figures 4A–4D and Figure 5). Peak firing frequencies of 7.5 Hz (evaluated in sliding 2 s windows), equal to those generated by the direct injection of 200–250 pA of sustained depolarizing current into transfected and untransfected neurons, appeared after latency periods of a few hundred ms to several tens of s ($n = 18$ neurons). In all likelihood, much of the cell-to-cell variation in light-triggered spike rates and spike latencies (Figures 4A–4D and Figure 5) was a consequence of the stochastic nature of transfection, which is expected to cause the absolute or relative levels of the three transiently expressed chARGe constituents (and, therefore, the cells' responsiveness to light) to fluctuate unpredictably from neuron to neuron. Stable integration of chARGe transgenes into the genomes of transgenic animals will reduce this variability.

While cell-to-cell variation in gene dosage made comparisons at the population level difficult in the current experimental setting, a series of repeated measurements on individual chARGed neurons ($n = 7$, Figure 5) demonstrated clear relationships between the electrical response elicited in each of these neurons and the dose and energy of the photons incident on it. Action potentials increased in frequency at higher light intensities (Figure 5A). At comparable intensities, photons needed to carry energies corresponding to visible wavelengths shorter than 600 nm to be effective (Figure 5A). The

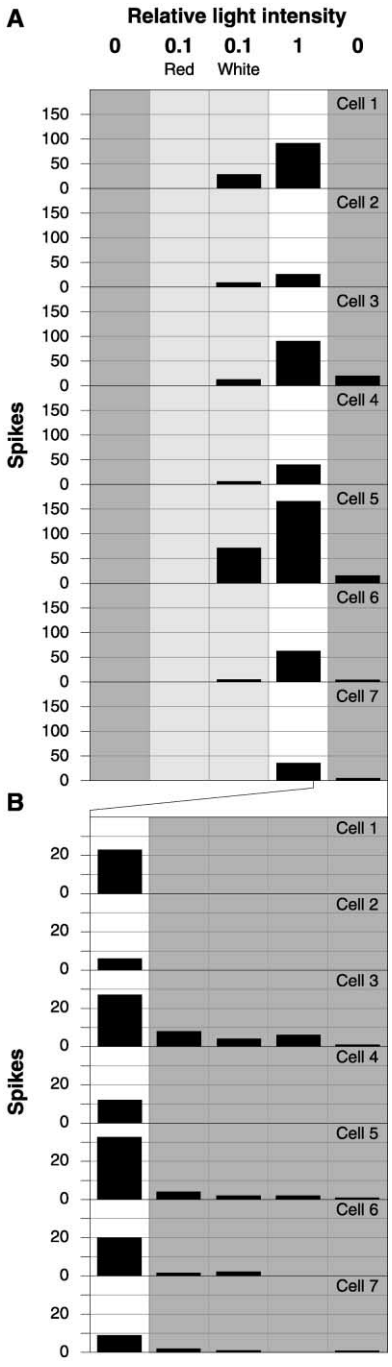

Figure 5. Intensity and Wavelength Dependence of Photostimulation

(A) Histograms of spike counts in seven neurons during consecutive 90 s episodes of darkness (relative light intensity = 0), red illumination (relative light intensity ~0.1), white illumination at relative intensities of 0.1 and 1, and darkness. A relative intensity of 1 corresponds to an optical power of 1.8 mW at the specimen. White light contains the wavelength band from 400 to 700 nm, red light the band from 605 to 645 nm (HQ625/40, Chroma), with a concomitant reduction in optical power to 0.15 mW (relative light intensity ~0.1). A neutral density filter (UVND 1.0, Chroma) was used to attenuate the white spectrum to a comparable relative intensity of 0.1.

(B) Histograms of spike counts in the seven neurons displayed in (A), showing the transition from light to darkness at higher temporal resolution. The five time bins cover the final 22.5 s of white illumination at relative intensity 1 and the subsequent 90 s dark period.

band of effective wavelengths (400–600 nm; Figure 5A) contains the absorption peak of native NinaE (430–550 nm; Hardie, 1983; Zuker et al., 1988).

Neuronal firing patterns under continuous illumination fell into two broad classes that echo the alternate operational modes accessible to many central neurons (McCormick et al., 1985; Llinás, 1988; Koch, 1999). Neurons in the first class (n = 14) fired stand-alone action potentials with large after-hyperpolarizations (Figures 4A and 4D). Spikes occurred in "random" sequences with almost exponentially distributed interspike intervals (n = 11 neurons; Figure 4A and Figure 6) or in the form of extended trains whose frequencies adapted (n = 3 neurons; Figure 4D). Action potentials of neurons in the second class (n = 4) were always superimposed on slower depolarizing potentials; they either clustered in stereotyped high-frequency bursts of four to nine spikes (n = 2 neurons; Figure 4B), or in irregularly timed epochs of variable duration and discharge intensity (n = 2 neurons; Figure 4C).

The observed electrical activity could be due to synaptic or autaptic (recurrent) excitatory inputs activated by an IP_3-induced rise in presynaptic Ca^{2+}. Alternatively, spikes could be initiated in a cell-autonomous manner. To distinguish between these mechanisms, glutamate receptors mediating excitatory synaptic transmission were blocked by 50 μM d,l-2-amino-5-phosphonovaleric acid (AP-5) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (n = 5 neurons). As expected, EPSPs vanished from the membrane potential record (Figure 4D, inset), but light-induced action potentials persisted (Figure 4D). Consistent with a cell-autonomous mechanism of spike initiation, only genetically chARGed neurons, but not untransfected neurons in the same cultures (n = 12), which are expected to receive similar synaptic input, fired light-evoked action potentials (Figure 4E).

Alternating periods of light and darkness caused alternating episodes of electrical activity and quiescence (Figures 5 and 6). Action potentials often appeared and disappeared with lag periods after the light stimulus was applied (Figures 4B, 4C, and 6B) and removed (Figure 5B and Figure 6C). The tight temporal coupling between stimulus and response that characterizes the native photoreceptor (Hardie, 1991; Ranganathan et al., 1991; Scott and Zuker, 1998) thus seems relaxed in a chARGed neuron driven by only the minimal phototransduction machinery. Augmentation of the chARGe core with additional catalytic, structural or regulatory components (Scott and Zuker, 1997, 1998; Montell, 1999), or fine adjustment of its stoichiometry (Ranganathan and Stevens, 1995), may be necessary to speed its response kinetics to photoreceptor timescales and may eventually afford accurate control over spike times and firing frequencies.

Prospects

The principle illustrated by chARGe promises to relieve serious impediments to neurobiological discovery, in many systems and experimental circumstances. We highlight only two examples. In vivo, sensory interfaces have provided the sole portals through which distributed inputs could be supplied to neural circuits. Because these inputs are repeatedly reformatted in intervening

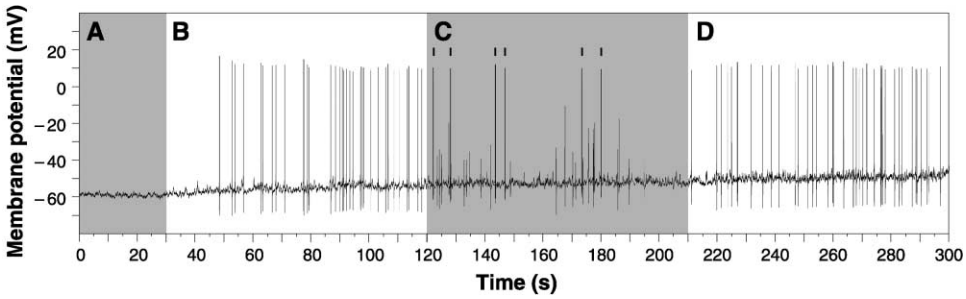

Figure 6. A ChARGed Hippocampal Neuron during Alternating Periods of Darkness and Illumination

Periods of darkness and white illumination are indicated by shaded and white backgrounds, respectively. The membrane potential record reveals 0 (A), 33 (B), 6 (C), and 40 (D) action potentials during the four consecutive episodes and "on" or "off" spike latencies of 18.091 ([B], "on"), 58.860 ([C], "off"), and 1.251 s ([D], "on"). Activity during the dark period after the first stimulus consists of six full-scale spikes that invert the polarity of the membrane ([C], vertical marks) and a number of lower-amplitude "spikelets." Spikelets, possibly dendritic action potentials triggered by residual chARGe activity, are seen in 67% of neurons (n = 18) and in these cases constitute the most prominent form of spillover activity after a stimulus. They disappear during repeated illumination (D).

processing stages, the exact signals reaching a neuron or circuit at some synaptic distance from the sensory surface are often obscure. Attempts in psychophysics to solve this problem by "operationally 'skipping' the peripheral processes and stimulating some central location" (Julesz, 1971) have remained limited to sensory systems and constrained in their inability to connect the observed phenomena back to their cellular foundation. ChARGe removes these constraints. Synthetic neural signals can now be inserted with high precision into virtually any location in any neural pathway of a transgenic animal and the behavioral or developmental consequences of such interventions explored.

Equally powerful applications are possible in vitro. By their very nature, explanted neural tissues such as cortical or hippocampal slices are stripped of all external connections, and no controlled way has existed to probe their circuitry with distributed inputs. Broad illumination of slices obtained from the brains of transgenic animals can now elicit population activity in a precisely defined class of chARGed neurons, or even in multiple classes independently that are each chARGed with a distinct spectral variant (Hardie, 1983; Montell, 1999) of NinaE. The spread of these synthetic test patterns and the transformations imposed on them by neural circuits can be traced in optical or multielectrode recordings, to reveal the underlying functional architectures and computational principles.

Experimental Procedures

Phototransduction Components

The coding regions (GenBank accession numbers in parentheses) of *ninaE* (K02315), *ninaA* (M62398), *arrestin-2* (M32141), $G\alpha$ (*dgq*; M58016), $G\beta$ (*gbe*; M76593), $G\gamma$ (AJ250440), *norpA* (J03138), *trp* (M34394), *trpl* (M88185), and *inaD* (U15803) were amplified by PCR from the *Drosophila* head cDNA library GH (Berkeley *Drosophila* Genome Project) and ligated to pXES43, a derivative of pGEMHE (Liman et al., 1992). Capped transcripts for injection into *Xenopus* oocytes were synthesized directly from these templates after linearization (MEGAscript T7, Ambion). Plasmids for expression in neurons (pChARGe-1 and pChARGe-2) were based on the pCI-neo backbone (Promega). pChARGe-1 carried *ninaE* and $G\alpha$ under CMV- and SV40-control, respectively; pChARGE-2 contained sequences encoding EGFP (with a 20-amino acid N-terminal GAP-43 tag that confers plasma membrane association [Moriyoshi et al., 1996]) and arrestin-2 -in the equivalent expression cassettes.

Synthetic all-*trans* retinal (Sigma) was diluted, from a 100 mM stock in DMSO, to 40 μM in the appropriate extracellular recording solution (see below), and allowed to bind to NinaE for 15 min in the dark. A mechanically shuttered (Uniblitz, Vincent Associates) Lambda DG-4 rapid wavelength changer (Sutter Instruments) delivered $<$2 mW of stimulating light without ultraviolet and infrared (E400LP, Chroma; Hot Mirror, Newport) components through 20$\times$/0.4 (oocytes) or 40$\times$/0.8 W (neurons) Zeiss Achroplan objectives. In some experiments, a short preillumination with red light (E600LP plus HQ625/40, both from Chroma) preceded the white light stimulus. Light source and shutter were controlled through pClamp 8.0 (Axon Instruments) and MetaFluor 4.5 (Universal Imaging).

Xenopus Oocytes

Stage VI oocytes were microinjected with the specified mRNA mixtures, which were adjusted to keep the doses of individual messages constant at 2–4 fmol/oocyte. Photocurrents were recorded at -80 mV with a two-electrode voltage-clamp amplifier (Axoclamp-2B, Axon Instruments) 2–4 days after mRNA injection. Electrodes (2–3 MΩ) were filled with 3 M KCl; the extracellular recording solution (Barth's Saline) contained: 87.5 mM NaCl, 2 mM KCl, 2.4 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.2). Signals were externally amplified (CyberAmp 380, Axon Instruments) before digitization at 100 Hz (Digidata 1200, Axon Instruments). To block IP_3-sensitive Ca^{2+} stores where indicated, 20 μM xestospongin C (Gafni et al., 1997; Calbiochem) was present throughout the experiment, beginning with the retinal load.

Rat Hippocampal Neurons

Hippocampal neurons obtained from E19 rats were grown in dissociated cultures (Yuste et al., 2000) and transfected with a calcium phosphate precipitate (pH 7.08) formed from a 1:1 mixture of CsCl-banded pChARGe-1 and pChARGe-2. Neurons were exposed to 4.2 μg/cm^2 of precipitated DNA for 20 min. Transfections were done on day 8 after plating, recordings on days 6–10 after transfection. Neurons were identified under DIC and epifluorescence illumination (to distinguish transfected from untransfected cells; Figure 3) and placed under whole-cell current clamp before reconstitution of NinaE with retinal. Patch pipettes ($\sim$2.5 MΩ) contained, in mM, 120 K-gluconate, 10 KCl, 5 ATP, 0.3 GTP, and 10 K-HEPES (pH 7.2). The extracellular recording solution contained: 119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, 25 mM Na-HEPES (pH 7.4). Membrane potentials were recorded with an Axoclamp-2B amplifier (Axon Instruments) in bridge mode and digitized at 5 kHz (Digidata 1200, Axon Instruments).

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