Optogenetic Approaches to Study IEG Activation

Christine E. Gee, Oana M. Constantin, Andreas Franzelin, and Thomas G. Oertner

Abstract Immediate early genes (IEGs) are reliably activated by pathological events such as seizures, but the activity patterns that trigger IEG expression in individual neurons under physiological conditions are not well understood. Optogenetic stimulation allows controlling the activity of single neurons or groups of neurons. Different spike patterns can be imposed in channelrhodopsin-expressing neurons, while expression of photoactivated adenylyl cyclase (PACmn) allows to increase intracellular cyclic adenosine monophosphate (cAMP) in single neurons or populations of neurons. No microscope is required for these optogenetic stimulation experiments; an LED (light-emitting diode) light source within a cell culture incubator is sufficient. As application examples, we show that Fos expression in CA1 pyramidal cells is activated by synchronized excitatory input and that raising cAMP in a single neuron is not sufficient to induce Fos. We conclude that some IEGs are induced by network-level events rather than by autonomous activity of individual cells.

Keywords Spike rate · Channelrhodopsin · Signaling pathways · cAMP · CREB · cFos

1 Specificity and Precision of Neuronal Stimulation

To study the mechanism and timing of activity-dependent gene transcription, a strong stimulus is required to provide a well-timed onset of high neuronal activity. In cell or tissue culture systems, it is common to increase the concentration of extracellular K^+ ions to depolarize all cells simultaneously (Sheng & Greenberg, 1990; Greenberg et al., 1986). Blocking gamma-aminobutyric acid ($GABA_A$) receptors with bicuculline or picrotoxin or stimulating adenylyl cyclases with forskolin are

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other methods to generate high activity in brain tissue. In vivo, pentylenetetrazol (PTZ), pilocarpine, or kindling-induced seizures have been widely used. These stimulation methods are very reliable and have led to the discovery of the classic immediate early genes (IEGs) including Fos, Jun, Zif, and Arg3.1/Arc (Morgan & Curran, 1988; Dragunow et al., 1989; Worley et al., 1990; Link et al., 1995; Cole et al., 1989).

However, if the goal is to unravel the complex, potentially cell-type-specific regulation of specific IEGs, it is desirable to avoid stimulating all cell types within an organism or tissue simultaneously. The discovery of channelrhodopsins (ChRs) and photoactivatable enzymes such as bacterial adenylyl cyclases (Stierl et al., 2011) and photoactivatable CaMKII (Shibata et al., 2021) has led to the development of methods to optically depolarize and spike neurons or directly activate intracellular signaling pathways in individual neurons or in defined subpopulations of cells. Here we describe the general methodology we use to precisely drive neuronal spiking at defined frequencies using the channelrhodopsin ChrimsonR and the photoactivatable adenylyl cyclase PACmn to increase cyclic adenosine monophosphate (cAMP) and activate downstream signaling pathways in individual neurons or throughout the hippocampus.

Organotypic slice cultures of rodent hippocampus (Gee et al., 2017; Muller et al., 2003) are particularly suitable for optogenetic stimulation experiments. Neurons in slice cultures can be easily transfected with optogenetic tools. To transfect individual neurons, plasmids are diluted in a standard intracellular recording solution in patch pipettes with small tips (approximately 10–15 MΩ) and electroporated into the cells (Wiegert et al., 2017a). All neurons in a slice were virally transduced by applying ~200 nL of ~10^{12} vg/μL adeno-associated virus (AAV) to the top of the slice, and small clusters of neurons were transduced by local injection of the AAV suspension using a picospritzer (Wiegert et al., 2017b) (Fig. 1). In slice cultures, the ordered connectivity from the dentate gyrus via CA3 to CA1 is well preserved after several weeks in vitro, making it easy to selectively transduce presynaptic or postsynaptic cell populations and independently assess their role in IEG expression. Neuron-specific promoters (e.g., synapsin1, CaMKII) prevent glia cells from expressing the optogenetic tool. In addition, slice cultures are optically accessible and can be light-stimulated inside the incubator using high-power light-emitting diodes (LEDs) prior to assessing IEG expression (Schoenenberger et al., 2009).

We use high-power LEDs (Roschwege Star Red, 620 nm; Roschwege Star UV, 405 nm; Roschwege Star Blue, 475 nm) to stimulate slice cultures in the incubator (5% CO_2/37 °C). LED stimulation towers (Fig. 1a) were assembled with aspheric lenses using the Thorlabs 30 mm cage system, which happens to perfectly accommodate a 35 mm plastic culture dish. The LEDs are driven from outside the incubator using cables with waterproof connectors (TRU Components 1572446) that are fed through the back port of the incubator. To generate the pulse trains, we use a

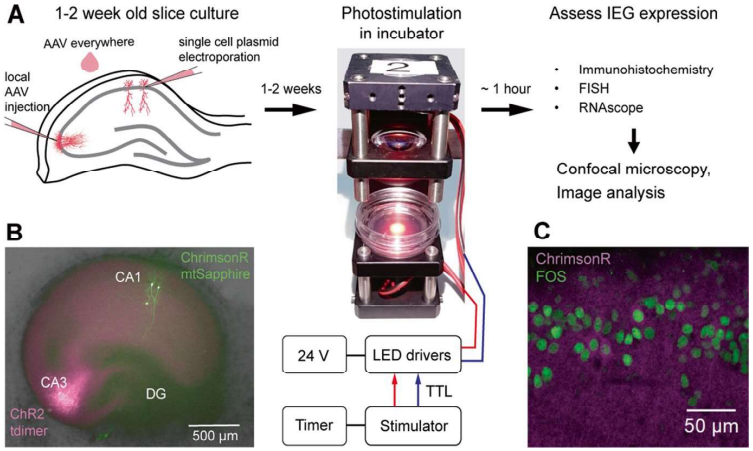

Fig. 1 Optogenetic experiments in the cell culture incubator. (**a**) Optogenetic tools are expressed via single-cell electroporation, by applying AAV to the surface of slice cultures or local AAV injection. Stimulation of neurons expressing the optogenetic tools is achieved inside the incubator using stimulation towers with two high-power LEDs (405 nm, 635 nm) controlled by external electronics. Depending on the method and IEG to be studied, the slice cultures are fixed 1 h after stimulation and further analyzed, e.g., by immunostaining for Fos. (**b**) Superimposed brightfield and fluorescence image of a live hippocampal slice culture with viral transfection in CA3 (ChR2-tdimer2) and three electroporated neurons in CA1 (ChrimsonR-tdTomato plus mtSapphire). (Courtesy of Bas von Bommel) (**c**) Details of CA1 in a stimulated slice showing ChrimsonR-tdTomato expression (magenta) after immunostaining against Fos (green). (Courtesy of Margarita Anisimova)

Grass S8800 dual-pulse digital stimulator or an A.M.P.I. Master 8 controlling two RECOM RCD-24-1.20 adjustable constant current drivers. A digital timer (Matsushita NAiS LT4H) disconnects the LED drivers from the power supply at the end of the stimulation time. Light intensity is calibrated by placing a Newport 918D-ST-UV sensor, connected to a 1936R power meter, in the plane of the cell culture behind a centered circular aperture (3.14 mm^2) while adjusting the LED current. We check and adjust the light intensity before stimulating each batch of cultures, as different opsins (and different times of expression) require different intensities (Anisimova et al., 2022). After stimulation, we wait 1 h to fix the slices in 4% paraformaldehyde and assess Fos expression with immunohistochemistry. We chose this time because in CA3 neurons, we observed that Fos expression peaks at around 1 h and has already declined substantially at 90 min. In CA1 the optimal time would be to fix the slices later than 90 min, but then we may fail to detect Fos expression, if it occurred, in CA3. We have good staining results using Synaptic Systems rat anti-Fos antibody (#226017) at 1:1000 dilution.

2 Controlling Neuronal Spiking with the Channelrhodopsin ChrimsonR

Channelrhodopsins (ChRs) are a family of directly light-gated ion channels that were first discovered in the green alga *Chlamydomonas* (Nagel et al., 2002). *Chlamydomonas* ChRs require blue light for activation, but several red-light-sensitive channels were later discovered in a large genetic screen (Klapoetke et al., 2014). Red light penetrates brain tissue better than blue light and does not carry the risk of phototoxic effects. Among the red-light-sensitive ChRs, ChrimsonR is distinguished by its fast closing kinetics, which allows the generation of single action potentials in neurons in response to short (1 ms) flashes of red light (Anisimova et al., 2022). To identify transfected neurons, ChrimsonR was fused to the red fluorescent protein tdTomato (Addgene plasmid #59171). For visualization, tdTomato can be excited with green light, but it does not absorb red light. To transduce neurons with the fusion construct, recombinant AAV was produced in-house at the UKE Vector Facility.

Figure 2 shows an example of a key result obtained after local injection of $AAV^{2/Rh10}$ synapsin-ChrimsonR-tdTomato (4×10^{13} vg/µL) using a picospritzer into CA3 of 2-week-old rat hippocampal slice cultures. In the evening, 9 days after the AAV injection, synaptic transmission blockers were added to the medium to prevent synaptic activation of spiking in the slice while the CA3 neurons were stimulated with red light. The next morning (10 days after transduction), the CA3 neurons were stimulated with a light intensity and duration that was previously determined to elicit single action potentials in ChrimsonR-expressing CA3 neurons (see Fig. 2 legend). Notably, there is an almost complete absence of Fos expression in the spiking CA3 neurons, whereas many neurons in CA1 express Fos (Fig. 2, *left*). In a

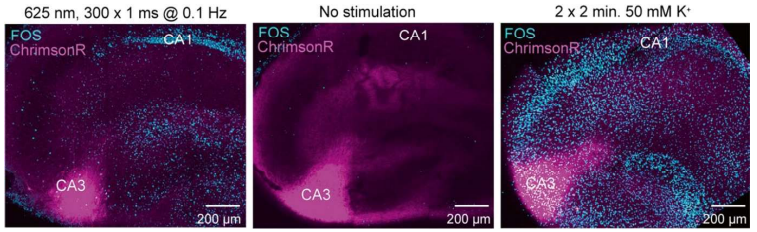

Fig. 2 Fos is expressed in postsynaptic rather than spiking neurons. The channelrhodopsin ChrimsonR was expressed in a subset of CA3 neurons (magenta). Fast synaptic transmission was inhibited with 6-Nitro-2,3-dioxo-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (10 µM), 2-Piperazinecarboxylic acid (CPPene) (1 µM), and picrotoxin 100 µM. *Left*: Stimulation of single action potentials with a 625 nm LED at 8 mW mm^{-2} at 0.1 Hz over 1 h induces Fos expression (cyan) in postsynaptic CA1 neurons, but not in the spiking presynaptic CA3 neurons. *Center*: Negative control. Transduced culture that received no light pulses. *Right*: Positive control. High K^+-treated slice that did not have synaptic transmission inhibited. All images are composites from multiple confocal stacks. The settings for the cyan (Fos) channel were identical during imaging and in all steps of figure production

similarly prepared slice that was not stimulated with light, there are only a few scattered Fos-expressing neurons (*center*). Clearly, CA3 neurons are also capable of expressing Fos, as treatment of a slice with high K^+ (in the absence of inhibitors) induces Fos in all regions of the slice (*right*). The key findings we have made using ChrimsonR to drive Fos expression are: (1) Synchronous spiking at 0.1 Hz is the optimal stimulus for Fos induction; 50 Hz spiking also induces Fos, but intermediate frequencies of 1–10 Hz are not effective. (2) Spiking per se does not induce Fos in a hippocampal neuron, although it may increase the propensity of that neuron to express Fos. (3) Fos is induced in neurons that are postsynaptic to the spiking neurons (Anisimova et al., 2023). Thus, the neurons expressing Fos during behavioral experiments are not necessarily spiking themselves at high frequency but are clearly important in the control network as they receive synchronized input. Our observations may provide an explanation for inconsistencies in the literature from studies that have attempted to correlate Fos expression with calcium signals recorded during behavior or after in vivo stimulation (Mahringer et al., 2022; Guzowski et al., 2001; Sheng et al., 1993).

In a previous study, we used a step-function opsin (ChR2-C128A) to trigger free-running high-frequency bursts in individual CA1 neurons (Schoenenberger et al., 2009). In many neurons, prolonged (40–80 s) high-frequency spiking (40–120 Hz) was sufficient to induce Fos expression. In vivo, however, bursts during place field activity are very short (2–5 action potentials) and do not exceed 40 Hz (O'Neill et al., 2006). During a sharp wave ripple, most participating pyramidal cells generate only a single action potential (Buzsáki, 2015). Thus, while it is possible to trigger Fos by long spike trains in the absence of synaptic input, prolonged high-frequency firing resulting from step-function opsin activation does not resemble any form of physiological activity. In contrast, using ChRs with fast kinetics to control the millisecond timing of each individual action potential enabled us to precisely reproduce firing patterns that were previously recorded during behavior (Rose et al., 2013) or have specific statistical properties, e.g., Poisson-distributed interspike intervals (Anisimova et al., 2023). To generate complex spike patterns, the LED drivers are controlled by software (Matlab or Labview) through a suitable input-output (I/O) device (e.g., NI USB-6001). We found that short bursts of 3 action potentials at 50 Hz trigger Fos expression in CA1 pyramidal cells when paired with single presynaptic spikes in CA3 neurons and repeated 300 times at 5 Hz (Anisimova et al., 2022). Why very low and very high, but not intermediate frequencies trigger Fos expression is not yet understood. Fos expression appears to be a network effect that requires synchronous activity in many neurons, transmitter release, and mGluR activation (Anisimova et al., 2023). There must be a mechanism that integrates synchronous events over long periods of time, a process that may involve intracellular signaling, cumulative epigenetic modifications, or even glutamate uptake and release from non-neuronal cell types. In the following section, we present an example how intracellular signaling can be probed with light-activated enzymes.

3 Using Photoactivatable Adenylyl Cyclase (PACmn) to Drive Fos Expression

Activity-dependent Fos expression is largely dependent on phosphorylation of cAMP-responsive element-binding protein (CREB) (Shaywitz & Greenberg, 1999). An important pathway leading to CREB phosphorylation begins with calcium or G-protein activation of adenylyl cyclases, which increases intracellular cAMP, activating cAMP-dependent protein kinase (PKA), which ultimately leads to CREB phosphorylation and Fos expression. Recent results suggest the need for an additional signaling pathway that requires synaptic activity before cAMP increases Fos.

Forskolin, a potent agonist of transmembrane adenylyl cyclases, induces Fos (Didier et al., 2018) (Fig. 3a). The membrane-targeted photoactivatable adenylyl cyclase (PACmn) increases intracellular cAMP when activated by ultraviolet to blue

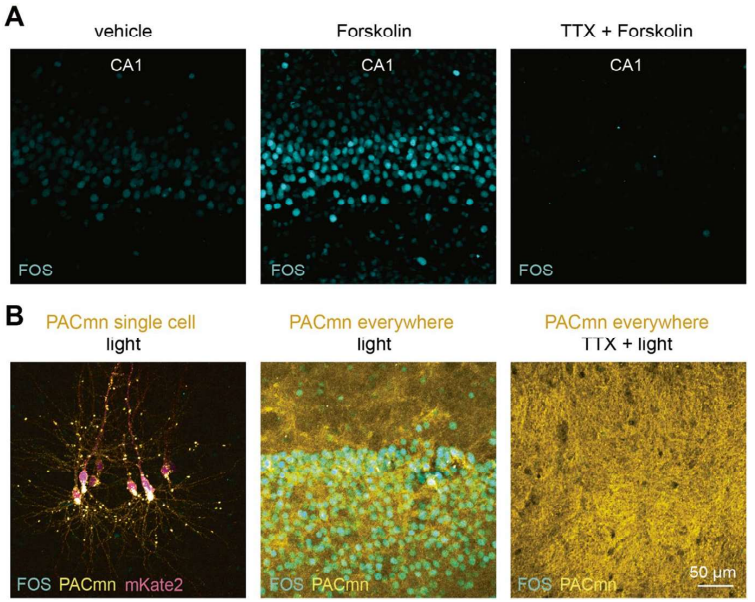

Fig. 3 Cyclic AMP-induced Fos requires network activity and is not cell-autonomous. (**a**) Pharmacological activation of endogenous adenylyl cyclases with forskolin triggers Fos expression (cyan) in CA1 (*center*), but not when electrical activity is blocked by tetrodotoxin (TTX) (*right*). (**b**) *Left*: Expression of PACmn in a few individually electroporated neurons (PACmn yellow/mKate2 magenta) and activation with light does not activate Fos (cyan). *Center*: Viral expression of PACmn (yellow) and activation with light lead to widespread expression of Fos (cyan). *Right*: When spiking is blocked with TTX, light activation of virally expressed PACmn (yellow) does not trigger Fos expression. Settings for the Fos channel (cyan) are identical in all images

light (Yang et al., 2021). In contrast to cytoplasmic photoactivatable adenylyl cyclases (Stierl et al., 2011), it has no measurable enzymatic activity in the dark. When PACmn is expressed in individual neurons and activated by blue light, no Fos is expressed (Fig. 3b), although intracellular cAMP increases and PKA is clearly activated (Yang et al., 2021). However, PACmn activation leads to Fos expression when it is expressed in all neurons in the slice, but only when neurons are capable of firing action potentials and synaptic transmission is intact (Fig. 3b). Interestingly, forskolin-induced Fos also requires an intact spiking network (Fig. 3a). Thus, a second signaling pathway, dependent on the release of an extracellular signaling molecule, is required and cAMP-PKA alone is not sufficient to induce Fos.

4 Advantage of Precision Stimulation for a Careful Dissection of IEG Activation Pathways

Optogenetic stimulation has been a key technology for dissecting the importance of specific neurons for cognition and behavior (Peron & Svoboda, 2011). We propose that the same set of well-characterized tools can also be applied to unravel the many molecular pathways thought to be involved in IEG activation. We present two examples of the type of experiments that convinced us that Fos induction cannot be understood as a cell-autonomous process. In the first example (Fig. 2), synchronous spiking of CA3 pyramidal cells at 0.1 Hz drives Fos expression in postsynaptic CA1 neurons, despite the fact that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), and $GABA_A$ receptors were blocked in these experiments. Pharmacological dissection showed that activation of mGluRs is absolutely required for Fos activation, even when triggered by high K^+ (Anisimova et al., 2023). Our interpretation is that released glutamate is sensed by metabotropic receptors and integrated by an unidentified mechanism that is able to trigger Fos expression. This explains why very little Fos expression was observed in the optogenetically driven CA3 pyramidal cells (Fig. 2). From the Fos signal alone, it would not be possible to localize the spiking neurons in the tissue, which has important implications for the interpretation of Fos expression patterns generated during learning experiments (Reijmers, 2009; Tonegawa et al., 2018).

In the second example (Fig. 3), global optogenetic cAMP elevation in all neurons recapitulates the strong Fos activation seen after forskolin stimulation, consistent with the idea of a signaling pathway leading from cAMP to Fos. However, elevation of cAMP in single neurons does not lead to Fos activation. Furthermore, Fos induction after global cAMP elevation is sensitive to tetrodotoxin (TTX), which is inconsistent with a cell-autonomous signaling pathway. Importantly, we observed that high cAMP leads to a small depolarization of neurons. Taken together, these results suggest that global cAMP elevation leads to increased spontaneous activity (spiking) in slice cultures, glutamate release, and subsequent Fos activation in

postsynaptic neurons, very similar to the indirect mechanism of channelrhodopsin-mediated Fos activation.

Fos activation requires electrical activity in the form of action potentials, but in presynaptic rather than in postsynaptic neurons. This raises an interesting question: which events trigger Fos expression in behaving animals? We speculate that highly synchronized activity, such as sharp wave ripples, perhaps in conjunction with neuromodulatory input, is the optimal signal to activate IEGs in vivo (Buzsáki, 2015). Replacing the sledgehammer of seizure-like activity with the precision of the optogenetic scalpel will force us to revise our theories about the physiological function of IEGs.

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