Optogenetics: 10 years of microbial opsins in neuroscience

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Abstract

Over the past 10 years, the development and convergence of microbial opsin engineering, modular genetic methods for cell-type targeting and optical strategies for guiding light through tissue have enabled versatile optical control of defined cells in living systems, defining modern optogenetics. Despite widespread recognition of the importance of spatiotemporally precise causal control over cellular signaling, for nearly the first half (2005–2009) of this 10-year period, as optogenetics was being created, there were difficulties in implementation, few publications and limited biological findings. In contrast, the ensuing years have witnessed a substantial acceleration in the application domain, with the publication of thousands of discoveries and insights into the function of nervous systems and beyond. This Historical Commentary reflects on the scientific landscape of this decade-long transition.

Optogenetics is the combination of genetic and optical methods to cause or inhibit well-defined events in specific cells of living tissue and behaving animals[1]. This technology, as employed today to study the neural circuit underpinnings of behavior, most commonly involves three core features: (i) microbial opsins, members of an ancient, but uniquely well-suited, gene family adapted from evolutionarily distant organisms such as algae and archaebacteria, with each gene encoding a distinct protein that directly elicits electrical current across cellular membranes in response to light, (ii) general methods for targeting sufficiently strong and specific opsin gene expression to well-defined cellular elements in the brain, and (iii) general methods for guiding sufficiently strong and precisely timed light to specific brain regions, cells or parts of cells while the experimental subject carries out behaviors of interest.

None of these three components was enabled for general optogenetic discovery in neuroscience 10 years ago[2], or even when we suggested the new word to describe this emerging process a year later[3], but each has scientific origins dating back decades. The cross-integration that enabled optogenetics over the past 10 years only became possible under the right conditions. Each component also continues to rapidly evolve for greater precision and complexity. Indeed, these fundamental elements of optogenetics are increasingly recruiting and bringing to bear more branches of science and engineering,

ranging from computational tools for system identification to automated optical readouts of behavior and neural activity to high-content anatomical data extraction methods for discovering structural and wiring relationships[4].

The most recent technological developments, along with experimental guidelines, challenges and limitations, have already been reviewed in detail this year[4]. In the present Historical Commentary, I focus on the optogenetic transition itself over the past 10 years, from scientific conditions surrounding the early work to the major discoveries that have arisen with application of this technology over the same time period, all set in the context of the development of three converging disciplines that could hardly be more disparate in origin and tradition.

Developing and assembling the components of optogenetics

The first of these three components has been part of the fabric of biochemistry and physiology for many decades: the microbial opsin genes and the microbial rhodopsin proteins they encode[5], a family of molecules (Fig. 1a) functionally completely distinct from (and unrelated in primary sequence to) the better-known rhodopsins that mediate phototransduction in the vertebrate eye[6]. Indeed, the initial evidence from Oesterhelt and Stoeckenius, beginning in 1971, that microbial organisms might also produce and use rhodopsin-like proteins[7] was surprising and intriguing. Instead of coupling to intracellular second-messenger cascades to indirectly influence ion channels, like their vertebrate counterparts, these microbial proteins for the most part directly transduce photons into electrical current (Fig. 1a,b). This molecular feat provoked intense curiosity and has inspired thousands of investigations over the decades[5]. Indeed, within a few years, this discovery had given birth to a vibrant community that produced a steady output of about 100 papers annually for four decades extending to the present day, spanning genomic, functional and structural investigations into the photocycles and mechanisms of these microbial proteins, and defining part of the textbook training of biologists[8].

Three branches of this family tree have found utility in optogenetics: the bacteriorho-dopsins, the halorhodopsins and the channel-rhodopsins (Fig. 1a). The naturally occurring bacteriorhodopsins (the first-discovered members of this family, which pump protons out of the cell) and halorhodopsins (which pump chloride ions into the cell) are typically inhibitory in neural systems, as both of these types of hyperpolarizing current make it harder for neurons to fire action potentials; in contrast, the naturally occurring channel-rhodopsins for the most part allow positively charged ions to flow freely through the opsin pore and so tend to be depolarizing and excit-atory[5] (Fig. 1a). This pattern held for many years until the high-resolution crystal structure of channelrhodopsin[9] allowed structure-guided engineering of the opsin channel pore (Fig. 1c)[10] to create inhibitory chloride-conducting channels[10,11] in 2014, followed by identification of a natural chloride-conducting channelrhodopsin[12,13] in 2015. Over the years, more variants in these protein families have been discovered in nature (or engineered in the laboratory) to have faster kinetics, bistable properties, altered ion conductances and shifted color-response properties; this diversity is now leveraged to powerful effect in optogenetic experimentation.

The key functional properties of these proteins were widely known for decades, and many investigators had sought to create strategies for controlling neurons with light. So why did it take time to develop and apply methods for placing these proteins into different classes of neurons in behaving animals? As mentioned above, the development of optogenetics was a biological three-body problem in which it was hard to resolve (or, even more importantly, to motivate attempts to resolve) any one of the three challenges without first addressing the other components. For example, microbial rhodopsin photocurrents were predicted to be exceedingly small, suggesting a difficult path forward even if efficient delivery and incorporation of the all-*trans* retinal chromophore were possible in adult non-retinal brain tissue, and even in the event of safe and correct trafficking of these evolutionarily remote proteins to the surface membrane of complex metazoan neurons. For these weak membrane conductance regulators to work, high gene-expression and light-intensity levels would have to be attained in living nervous systems while simultaneously attaining cell-type specificity and minimizing cellular toxicity. All of this would have to be achieved even though neurons were well known to be highly vulnerable to (and often damaged or destroyed by) overexpression of membrane proteins, as well as sensitive to side effects of heat and light. Motivating dedicated effort to exploration of microbial opsin-based optical control was difficult in the face of these multiple unsolved problems, and the dimmest initial sparks of hope would turn out to mean a great deal.

Outside neuroscience, several examples of functional heterologous expression of opsins for light-activated ion flow had been published in non-neural isolated-cell systems for microbial opsins[14–16] (beginning[14] in the early 1990s) or vertebrate opsins[17] (beginning in the late 1980s), although neuroscience or behavior applications were not suggested. It is not known how many investigators actually did attempt to transduce microbial opsins into neurons before 2005, but even in this one step, among the many steps required for optogenetics, much can go wrong[18] (Fig. 1d). Meanwhile, over the years leading up to 2005, several other strategies for optical control of targeted neurons, involving multiple simultaneously delivered metazoan genes or coordinated delivery of both a metazoan gene and a light-sensitive synthesized chemical, were devised[19–23], perhaps by their very elegance reducing enthusiasm for another approach based entirely on a family of far more foreign microbial proteins that would seem much less likely to work.

Worthiness for allocation of time and effort was not a trivial consideration, as my laboratory group in 2004 had limited material resources; moreover, there was still a great deal of self-doubt, with the realization that many more steps of equal or greater magnitude and risk would be needed to reach even the most basic initial goal. However, once neural membrane expression with appreciable light-activated functionality of a microbial opsin had been seen (Fig. 2a) and it first became possible to report "I think it worked," the landscape changed from speculation to action. The ensuing 2 years indeed saw action on many levels: constructing and concentrating the crucial expression vectors for stable, well-tolerated expression (Fig. 2b), testing real-time readouts using electrophysiology and behavior *in vitro* (Fig. 2b,c) and then, crucially, *in vivo*; and design (Fig. 2d) and implementation (Fig. 2e) of neural interfaces for *in vivo* light delivery and behavior[2,3,24–26]. Many investigators were discussing the possibilities and working on related efforts, and although a number of papers

were published in 2005 and 2006 from pioneering laboratories around the world[2,3,27–30], it was not for two more difficult years that the journey to mammalian behavioral control was ultimately completed[25,26] (Fig. 3a,b and Supplementary Video 1), finally allowing confidence that the microbial opsin approach was going to be generally useful.

Some issues that had seemed formidable for *in vivo* work turned out to be readily addressed. For example, invertebrates could make use of dietary all-*trans* retinal as the chromophore[28,31–33], immature and developing vertebrate nervous systems and the retina required no supplementation[27,29,30], and the adult mammalian brain required no exogenous retinal or other component[3,24–26]. With these discoveries, along with the long-known single-gene logic of microbial opsin transduction of light into ion flow[7,8,14], single componency for optogenetics had been achieved—as with green fluorescent protein (GFP), a property crucial for utility. But still much more challenging discovery and development were required, as evidenced by the fact that broad adoption of optogenetics with microbial opsins did not occur until 2009. The transition that followed was enabled only with the convergence of optics and genetics components of optogenetics, the second and third fields that would have to come together with microbial opsin genes. Between 2004 and 2009, discovery along these key dimensions proceeded rapidly.

Only a few new biological findings using microbial opsin optogenetics had emerged by early 2007, chiefly from small, optically accessible invertebrate systems. For example, in *Drosophila* larvae, the effects of activity in distinct neuromodulator (dopaminergic versus octopaminergic/tyraminergic) systems had been tested in classical odorant conditioning[31], and a role for nociceptive neurons in defensive behaviors protecting larvae from wasps had been defined[32]. In addition, opsin-expressing mammalian brain surfaces had been acutely exposed and illuminated with spots of light to drive and map responses[3,24,34–36]. However, control of mammalian behavior had not been achieved, and indeed the intact brain seemed largely inaccessible to optogenetics. There was therefore a compelling need to safely, focally and flexibly deliver visible light via a neural interface deep into the brain of a freely behaving mammal at a high-intensity: ~100 mW mm^{-2} at the interface output, ~100× greater than needed at the opsin-expressing cells themselves because of expected scattering losses over the effective brain volume[25] and much more intense than needed for imaging. LEDs at the time were underpowered for coupling to optical fibers, and so necessity rapidly drove development of optogenetic interfaces based on laser diode–coupled fiber optics[25,26]. Among other features, including heat isolation and activity feedback, these interfaces crucially also registered virus injection to illumination site (Fig. 2d,e), which opened a new realm of experimental possibilities for targeted control and readout during behavior.

By mid-2007 (Fig. 3a,b) it was possible[26] to selectively target a microbial opsin gene with high specificity and penetrance to a defined population of neurons deep in the brain of adult mice (in this case, hypocretin/orexin neurons in the hypothalamus), to play in a broad range of spike patterns through an optical fiber to those cells, to collect simultaneous multimodal system readouts during freely moving behavior (in this case, describing sleep/wake status via electroencephalography (EEG) and electromyography), and to demonstrate a causal role for defined activity patterns in specific brain cells in a natural behavior (in this case, sleep-wake transitions)[26]. It is worth noting that, although optogenetic control of behavior through

the intact skull[37] or a cra-niotomy[38] using LEDs directly apposed to the cranium was reported later that year, this approach is rarely taken today, as LEDs generate substantial local heating, the light delivered in this way is not as readily targeted to defined circuit elements and, in contrast, the fiber-optic method also allows imaging light to be collected back through the same interface to report on local neural activity.

This example also illustrates how genetic targeting of circuit components had to advance in a fundamental way that was adapted to the high expression levels needed for the microbial opsins. In these first 5 years, the field accordingly saw development of versatile, high-titer cell-targeting opsin viruses[3,24–26,39–42] and the creation of the initial broadly expressed[35,36] and specific[43] transgenic opsin mouse lines. These advances were complemented by the initial reports of *in vivo* behavioral effects resulting from genetic targeting of cells in the worm (for excitation[28] and inhibition[33]), fly[31], fish[44] and rodent[25,26]. Cell-type targeting of opsin genes was not only achieved with genetics, however. It was the fiber-optic hardware method that enabled what is now the one of most widely used and generalizable approaches for targeting cells in behaving animals on the basis of anatomy or wiring. Termed projection targeting, this involves the use of the fiber-optic neural interface directed to axonal projections of the opsin-expressing cells to recruit cells defined by wiring for control in behavior[4,45].

Dovetailing with this genetics and optics development in those first few years were advances in opsin genomics and engineering. In a different sort of reverse translation situated entirely in the basic science realm, new opportunities in optogenetics were in turn now driving the basic study of microbial opsins, including the development of new structural models, photocycle analyses, and opsin gene discovery with modern genomic methods. This field was thriving before opto-genetics; for example, two of the sparks that ultimately led to optogenetics arose from the identification of rhodopsins in microbes[7] by Stoeckenius and Oesterhelt in 1971 and the identification of rhodopsin-regulated currents in *Chlamydomonas*[46] by Hegemann and Harz in 1991. But optogenetics returned the favor, providing a new spark to the field of microbial opsin biology. Very much relevant to the early optical hardware development occurring at the same time, the new proteins discovered and engineered in these first few years were able to take advantage of red-shifted light[47], bistable 'step-function' photocurrents that could be stably turned on with a pulse of one wavelength of light and off with another[48,49], much higher cellular light sensitivity[48,49], and higher safe expression levels *in vivo* (further contributing to improved cellular light sensitivity) based on membrane trafficking[50,51] and chimerization[52,53] innovations. These opsin-engineering advances thus guided and defined constraints for initial engineering of the optical hardware elements (Fig. 2d,e).

In this way, the microbial opsins, *in vivo* optics and expression-targeting genetics finally came together as a mutually interdependent and complementary whole. Although genetic targeting for optogenetics has also advanced further for multiple-feature targeting in the second half of the decade[54] (Fig. 3c,d), even by 2009 it was already possible to achieve cell-type targeting not just for isolated tractable cases, but also in a generalizable fashion, with recombinase-dependent viruses[39–42] that were strong and specific enough to be used to control behavior and with projection-targeting behavioral control enabled by the fiber-optic

interface that began to move beyond the need for genetic targeting reagents[45]. This experimental and conceptual framework for optogenetics allowed adoption, application and discovery by investigators around the world.

Discoveries with optogenetics

Optogenetic methods have now enabled acquisition of insights into a broad range of questions in behavior, physiology and pathology, spanning domains of sensation, cognition and action. Although many of these studies have been conducted in mammals (typically rats and mice), optogenetic methods have also become a standard resource for scientific communities studying neural circuit foundations of behavior in invertebrates. Beyond simple observation of evoked electrical activity, numerous fascinating biological findings on regulation of complex behavioral states have been revealed in the nematode (for example, ref. 55) and the fruit fly (for example, ref. 56), while more gradually, scientific insights from optogenetics in species such as the songbird (for example, ref. 57), the zebrafish (for example, ref. 58) and the nonhuman primate (for example, ref. 59) have also emerged. The capabilities that have come along with the emergence of single-component optogenetics as a standard research tool displaying speed, simplicity and versatility were cited for justifying, in part, the timing and scale of national-scale neuroscience research initiatives launched in 2013, including the BRAIN initia-tive[60]. Although the full scope of the findings that have resulted in the field can no longer be reviewed in detail, it is interesting to take note of key examples in the different categories of investigation that have emerged.

Broadly speaking, optogenetic methods have now illuminated the causal role of defined cell types and projections in natural as well as disease-related physiology and behaviors, ranging from the most basic homeostasis to advanced cognitive functions. For example, optogenetic methods have now been used to illuminate the causal neuronal underpinnings of movement regulation[43,61], including the identification of surprising bottom-up circuit mechanisms by which the spinal cord and cerebellum regulate forebrain control of skilled and voluntary movements[62–64]. Patterns of activity arising from genetically and anatomically defined cells have been identified, even when these cells are intermixed with other cell types, that specifically drive or inhibit the most fundamental of organismal functions: hunger, thirst and energy balance[65–69]; respiration[70,71]; and arousal, sleep and circadian rhythm[26,72–75]. And the transmission of primary sensory information to the brain has also been studied extensively with optogenetics, including in the domains of olfactory[76–78], auditory[79], visual[80,81] and tactile[82,83] processing.

Many studies have employed optogenetics to discover and map the pathways along which such information flows in the brain, including analysis of physical circuit connectivity itself [84,85], tagging cells defined by type or connectivity for use in other analyses[86], and integrating with fMRI or PET imaging to generate brain-wide maps of activity patterns recruited by defined neural cells or projections[87,88]. Optogenetic methods have also yielded insight into the dynamics of information transmission in neural circuits more generally—for example, regarding the modulation of activity by oscillatory rhythms[41,89–97], the causal significance of phase timing within the theta rhythm[98], the influence of gamma rhythmicity on information propagation[41,89,90] and (dovetailing with parallel insights from genetic

interventions[99,100]) the real-time regulation of information flow via the dynamic balance of excitation and inhibition[90,101–110].

Circuit activity patterns have also been identified, using microbial opsin genes expressed in conditional viruses and targeted with the fiber-optic interface, that control and modulate many motivated behaviors. Social behavior, which places intense demand on circuitry across the brain involving sensation, motivation, reward, cognition and memory, has been studied under a range of conditions, leading to identification of cells and projections that modulate same-sex social patterns[90,111], as well as parental[112], mating-related[113,114], aggression-related[113–116] and defensive[115,116] interactions. Studies of tradeoffs between active and passive coping in response to challenge[117] and tradeoffs between avoiding risk and seeking reward[118] have also proven readily accessible to optogenetic identification of the cells and circuits involved. Indeed, insights have been derived into the causal circuit underpinnings of reward itself[42,119–124], as well as into the circuit implementations of fear and anxiety[125–135]. Optogenetics in these cases has enabled the delineation of different cell types that, even though juxtaposed and intertwined, can have fundamentally oppositional roles in these complex behaviors. Principles of brain functional organization have emerged along the way; for example, revealing the potency and adaptability of brain-wide connections, defined by origin and target and so not previously accessible to investigators for specific control, in the precise regulation of behaviors and behavioral states[136]. And long-term action and electrical patterns have been illuminated as well, with resulting insights into the cell activity–specific causation of stable brain states underlying behavior[137].

Beyond the dynamics of brain and behavioral states, the mechanisms underlying information storage in the brain have also been illuminated by optogenetic methods[138–140]. One of the most exciting developments in recent years has been the testing of long-held and much-debated models of neural information representation. Use of clever activity-guided expression of microbial opsin genes has led to causal identification of sparse and distributed population representations (engrams)[139–142] underlying memory states and mediating linkages among features of these memory states. Learning itself has been studied with optogenetics in ventral striatum and amygdala[143,144], as well as in the hippocampus[138], and inputs to the hippocampal formation have been studied in some detail with regard to causal roles in learning, navigation and information flow[145–147]. Finally, precisely timed activity patterns in diverse well-defined cell types, projections and populations have been causally implicated in synaptic plasticity[148,149], wiring and microcircuit plasticity[150,151], and in long-timescale devel-opmental[152,153] and adaptive changes such as activity-dependent myelination[154] and adult neurogenesis[155,156].

Many discoveries have also emerged regarding the neural circuitry of symptoms related to disease states; indeed, the precise circuit-level mechanisms and causal tissue-level manifestations of neuropsychiatric disease have been mysterious for many of the same reasons that made optogenetics useful for basic neuroscience. For example, optogenetic methods have been applied to study the cellular activity underpinnings of seizure propagation and termination[157–159]; these studies and those that followed have included some of the first closed-loop approaches in behaving animals (Fig. 4), as well as the surprising identification of highly focal sites for immediate seizure termination remote from

the initiation site[158]. The latter finding is still being explored, but, if the percolation of activity imbalance through the brain occurs not generally, but instead chiefly through surprisingly well-defined and separated nodes where highly effective point control (as with a fiber optic or electrode; Fig. 4) may be exerted, the implications are substantial not just for understanding epilepsy but for understanding the brain as a dynamical system.

Also on the neurological front, optogenetic investigations have led to the determination of the cells and pathways that promote or inhibit normal and parkinsonian movement patterns[45,61]. The latter study included the initial specific and causal demonstration of the oppositional roles of the striatal direct and indirect pathways in action initiation and suppression, respectively, under either healthy or parkinsonian conditions[61]—in this case, resolving a longstanding question that had not been susceptible to direct testing, with clear implications for understanding the disease but also for understanding adaptive movement regulation.

A full discussion of whether or not optogenetics will be directly applied in a therapeutic sense (such as through direct viral approaches[160] or introduction of light-sensitive cells[161]) is beyond the scope of this Historical Commentary. Laboratory-derived circuit-level insights into maladaptive behavior are important in the scientific sense, however, and will in turn feed back and enhance the basic study of natural circuits and processes that go awry in disease states. Optogenetics has been used to identify neoplastic and degenerative processes such as activity-dependent double-strand DNA breaks in neurons[162], amyloid-β pathology augmentation by chronic moderate activity in Alzheimer's model mice[163], altered glioma growth mediated by activity-driven neuroligin-3 s ecretion[164] and altered striatal inhibition in Huntington's disease mouse models[165], all tested and modulated by precisely initiated activity in defined cell populations expressing microbial opsin genes. Regenerative processes have been studied as well, including restoration of light responses in retinitis pigmentosa via halorhodopsin[166], altered activity-dependent myelination of long-range projections from neocortex[154] and recovery and rewiring processes after stroke[167,168].

Despite the seemingly intractable brainwide complexity of addictive drug effects and our clinical difficulty in treating these disorders pharmacologically, optogenetic methods have been employed for the discovery of cells and synapses that can powerfully and specifically control the expression of cocaine-related behaviors[119,120,169–171]. For example, optogenetic methods were used to resolve a controversy on the causal role of a small (~1%) population of cholinergic cells in the nucleus accumbens that controls acute cocaine condi-tioning[119]. Additionally, a surprising series of papers revealed the potency of focally initiated control from the frontal cortex over long-term cocaine-initiated behavioral patterns[169–171], with potential relevance for understanding how stable patterns of behavior can be put in place and specifically modulated in a top-down manner (as also seen in a pair of papers on optogenetic modulation of corticostriatal control over obsessive-compulsive behavior mechanisms[172,173]).

Psychiatric diseases in general have been illuminated[136] through the determination of mechanisms by which features that contribute to behavioral states, such as those of altered motivation, reward, social interaction and anxiety, are recruited, assembled and coordinated

across the brain. Each of these symptom domains is shared, though with different qualities and in different combinations, across diverse clinical conditions[117,128–130,133]. When a projection is targeted, symptom domain– specific behavioral consequences can manifest in a manner that is more specific and/or potent than when activity is modulated in either the originating structure or the target structure of this projection alone[4], as in the transition from active-coping to passive-coping behavioral strategies in the context of insuperable challenge[117]. In particular, it was found that a specific long-range projection from prefrontal cortex to the dorsal raphe was able to promote (when optogenetically resolved and excited) or suppress (when optogenetically resolved and inhibited) swimming and climbing behavior in an automated forced-swim test[117], with implications for understanding the assessment and assignment of cost/effort relationships that may vary with affective state in health and disease. Less specific, absent, or opposite-direction effects were seen with control of the projection target, the projection origin, or a differently targeted projection to habenula, respectively[117]. A common theme in such optogenetic studies is the identification of defined cell populations (or projections arising from these cells) with mechanistic contributions to individual symptom domains, revealing causal mapping with explanatory power for both adaptive and maladaptive states. Findings from the clinically inspired side, together with those from the basic science side, have resulted in both the emergence of these broad concepts as well as the resolution of longstanding questions.

Current experimental considerations and challenges

As with any scientific method, there are important technical limitations and potential confounding factors, known to practitioners and addressed over the years[4,37,136,174] and discussed here for completeness. Rigorous optogenetics requires careful consideration of (i) controls to account for effects of the experimental system itself, (ii) definition of the stimulus pattern delivered, and (iii) definition of the directly modulated tissue element. These are general considerations for any intervention in biology, although optogenetics affords new opportunities for both specificity and means of validating specificity.

First, accounting for the experimental system requires considering the influence of light delivery in itself and of the opsin gene in itself. Regarding the light delivery, the flexible fiber-optic interface is comparable to, though smaller and lighter than, the electrical interfaces that have been a staple of freely moving systems neurobiology for decades[25,26,174]. This optical interface further enables the two new capabilities of projection-targeting for behavioral control[45,121,128] and readout of activity in cells and projections during behavior[4,111]. Nevertheless, the careful behaviorist must carry out baseline behavioral testing with the interface in place to validate the task for each cohort and each experimental subject. It is also standard practice for behavioral experiments to include no-opsin arms, in which animals receive every other aspect of the preparation, including viral transduction and light delivery; these controls address many theoretical concerns, ranging from toxic side effects of the surgery to distracting or heating effects of the light itself[174]. Notably, it would be much harder to build in such controls for electrical stimulation, providing the same energy in such a way that the tissue could not respond functionally. Optogenetic experimentation both requires and makes possible such controls.

Regarding the opsin gene expression itself, as with any foreign or native gene (especially those encoding membrane proteins), genes for optical actuation and for readout of structure and function bring a risk of possible toxic effects in the setting of high, long-term expression. This was addressable[45,50,51], and now every major class of overexpressed microbial opsin (proton pumps, chloride pumps and channels) in mammals has been shown to benefit from the provision of mammalian membrane trafficking signals[18,45,50,51,90] to facilitate efficient movement through protein production pathways to the cell surface membrane. Avoiding long-term expression with certain viruses (for example, rabies and herpesviruses) or with certain promoter/enhancer combinations that are too strong (such as CMV-based promoters in many settings[174]) is also important, and optogenetics experiments should include, where practical, histological and/or electrophysiological validation of cell health, as well as baseline comparisons with non-opsin-expressing cells or animals. Of course, in most cases, each animal can also serve as its own light-off/light-on/light-off control to verify that the experiment is truly reading out an effect of actuation rather than of preparation or changes over time—controls that are harder to achieve in other approaches such as lesion studies.

Second, defining the stimulus pattern delivered is of substantial importance for fully capitalizing on the capabilities of optogenetic control. Light pulse patterns can be mapped across a broad range from undetectable to pathological in outcome while reading out the behavior and physiology of interest (for example, ref. 26) or can be tuned to match various distinct patterns observed from recording studies (for example, ref. 42). Moreover, when optogenetics is used as a circuit-level tool (as is usually the case)—for example, to study the influence of one population of cells on another population in a different brain region—the train of action potentials optogenetically elicited in the presynaptic cell population is created by native voltage-gated sodium channels and propagates as a spike to the axon terminal, where it will be susceptible to the same mechanisms of presynaptic plasticity (for example, via releasable vesicle pool dynamics) and post-synaptic plasticity (for example, via receptor internalization and phosphorylation) as native action potential trains with similar rates and timing. Of course, optogenetics may encounter more challenges for within-cell studies, where the region studied is also the region illuminated and so nonphysiological distribution of light-activated channels and abnormal relative timing of voltage changes across the cell could cause artifactual patterns of intracellular plasticity and cell signaling; hence, this approach is usually not recommended.

Relative timing of activity among the cells of the targeted population is also of interest, although experimental data describing such relationships are not usually available in a meaningful form. However, much like the amplitude of population activity, relative synchrony of the population is also titratable with optogenetics. At one extreme, subthreshold modulation of excitability within the population (without directly driving spikes) is readily achieved using bistable step-function optogenetic tools[48,49,90] or with weak steady light delivery to conventional microbial opsin–expressing cells; in these cases, spike timing is asynchronous and depends on each cell's native synaptic input pattern[48,90]. At the other extreme, synchrony to the level that can be observed *in vivo* during local field potential or EEG oscillations can be achieved with very intense but brief light flashes,

although (just as with native synaptic drive) variation in cell and synapse history will inevitably still give rise to variation in spike latency. As capabilities in the field move toward independent control of multiple single cells in a population (Fig. 5; recently achieved with two-photon illumination-optimized opsins[175] and spatial light targeting[176] during behavior[177,178]) and to optogenetic control of sparse, distributed ensembles of cells defined by activity history[139,142], relative intrapopulation timing will become an increasingly interesting avenue of experimentation. Experimental leverage provided by optogenetics in this domain extends to facilitating the use of native activity patterns detected in the very same animal by optical or electrical means[177–180], wherein these activity patterns can then be recapitulated with optical control. For example, the initial neural demonstrations of red light excitation and inhibition with microbial opsins[18,90] have begun to enable integration of optogenetic control with blue light–responsive, genetically encoded Ca^{2+} indicators[4,177,178], even through the same optical fiber interface[4]. Further optogenetic experimental leverage regarding definition of the activity pattern provided arises from avoiding interpopulation synchrony confounds to which electrical stimulation is susceptible, such as inadvertently driving nontargeted fibers of passage or afferent fibers to a region[136].

Third, defining and validating the directly modulated tissue element is crucial— attainable in optogenetics to a level not previously possible but therefore all the more in need of proper experimental design and interpretation[4]. First and most fundamentally, any strategy to target a cell type or population—by genetics, anatomy, guided light or other means—must be validated in the same preparation wherein the behavior or physiology is conducted, especially since promoters and driver lines may not have specificity properties that translate exactly across precise subregions or developmental stages spanning different laboratories, animals and scientific questions[4]. To address this issue, expression of opsins is tracked (via GFP or related fusions, or staining for coexpressed tags) and correlated with endogenous cell type markers (in turn labeled or described with antibodies, transgenic fluorescent labels, anatomy and morphology). Sparing of nontargeted populations (specificity) is of course even more important than successful access to the targeted population (penetrance) and must be quantified.

Targeting based on anatomy rather than (or in addition to) genetics is also widely practiced in optogenetics, and it requires similar care with validation; some approaches involve various forms of retrograde tracing for targeting actuator expression to neurons that project to the injected brain area, while other strategies involve anterograde targeting[136]. One version of the latter approach, called projection targeting, involves optogenetic tool expression in an upstream neuronal population followed by selection of an output-defined subset of these neurons by restricting light delivery to target brain regions where light-sensitive axons defined by projection origin and target will reside, in a location that distinguishes the pathway *in vivo* during behavior[128]. Excitation of opsin-containing axons can lead to anti-dromic spikes spreading to somata and axon collaterals across the brain[45]; with the field's movement toward increasingly realistic input of activity patterns, fully recruiting a wiring-defined cell type as a functional unit becomes increasingly preferred, since isolated excitation of one axonal collateral alone is unlikely to occur naturally. However, for those cases where it may be experimentally desired to isolate a specific

collateral, inhibition is valuable for remaining local in direct effect[128,136], and with excitation, pharmacological activity blockade at the cell body can provide more specificity if necessary. Excitation and inhibition are both widely used forms of projection targeting for testing sufficiency and necessity of wiring-defined cells; as noted above, improved resolution of behavioral effects is often seen as compared with simple regional targeting[4,117,136].

The future of optogenetics and companion technologies

Optogenetics as a field has in some cases driven and advanced technological developments that later became useful for non-optogenetic approaches; for example, the same fiber-optic-based neural interfaces developed for optogenetic control are now also used to record natural activity signals[111], the membrane trafficking signals used to help opsins express safely at high levels have turned out to be useful for other classes of heterologous protein expression[179] and the recombinase-dependent viruses originally developed for targeting microbial opsins[39–42] are now in use for targeting diverse genes in a general-purpose fashion[69,111]. But optogenetic technology is also now able to leverage technological advances arising from other fields. For example, two-photon microscopy helped enable temporally precise optogenetic control of single cells in living mammalian brains (Fig. 5)[175–178], and liquid crystal–based holography allowed the use of spatial light modulators to control multiple individually defined cells[176]—both long-held goals not possible in behaving animals with electrical or pharmacological methods.

The fullest imaginable promise of this approach—controlling separately all of the cells in a mammalian brain with diffraction-limited spatial and temporal resolution during behavior—is not likely to be realizable, in part owing to fundamental physical challenges including light scattering and power deposition requirements associated with targeting large numbers of individually specified cells. But an important conclusion from the optogenetic memory engram studies[139] has been that not only optics, but also genetics, can be used to achieve control of multiple individual cells in optogenetics; of course, powerful intersections of the two are also possible and are certain to be part of the future of optogenetics. When working together with opsin-targeting genetic methods, light-guidance optical methods now enable cells of interest to be readily controlled in a general way to study physiology and behavior in freely behaving animals, and development of new classes of optics, light sources and computational methods will continue to open up new avenues for optogenetic control[4].

Employing data streams for use in optogenetic experiments that were collected with non-optogenetic technologies will also become increasingly common, and indeed crucial[136,180]. It is of substantial value to deliver patterns of activity with optogenetics that are guided by native patterns of activity, whether at the level of cells, projections, ensembles, type-defined populations or combinations thereof[4]. Recent advances in readout methods for neural activity (both in the biological sensor and the hardware domains)[177–180] and closed-loop methods for detecting and feeding back this activity information in real time as desired for activity-guided control[4] have provided avenues for new kinds of experiments that substantially augment the utility of opto-genetics[4] (Fig. 5).

Though electrophysiological readouts were available and used during optogenetic control of behavior even in 2007 (ref. 26), early studies were nevertheless all open-loop in nature. But the halfway point of the decade in 2009 also marked the advent of closed-loop opto-genetics[41] (Fig. 4); in this study, feedback was used to guide stimulation in a principled way as parvalbumin interneurons were driven in a manner triggered by observed pyramidal neuron spikes to achieve, and study the causal consequences of, well-defined circuit-level feedback-type inhibition[41]. Such precise timing of optogenetic control relative to neural activity events has become one of the fastest-growing methods in the field[4], and among the most important; for example, optogenetic interventions have been found to exert different effects depending both on endogenous oscillation phase and on sensory information availability[98]. However, increasing the speed and precision of feedback in closed-loop control, conditional on brain and behavioral state, will be an important engineering challenge for years to come, proceeding hand in hand with the development of new technologies for detecting neural activity (as with genetically encoded fluorescent sensors) and behavioral activity (as with machine vision methods), as well as with the development of computational tools for extracting these meaningful readout motifs in real time[4].

Like the advances that led to these new cellular-resolution activity data streams, recent advances in anatomical methods resulting in the availability of local and brainwide wiring data sets now confer interpretability and power on a broad range of other experiments—for example, in terms of identifying the components observed to have specific activity patterns during behavior[136]. Linking those structural and anatomical data sets with knowledge of the causal impact of the cells and ensembles involved is now in principle possible. Each aspect of acquisition, storage, analysis, morphing and registration of these large data sets is no small feat, which in turn is driving advances in data sharing, cluster computing and cross-validation of manual and automated tools for data set segmentation and annotation.

The drive to develop optogenetic tools has also advanced, and will continue to advance, the study of the microbial opsins themselves. For example, understanding of the mysterious light-activated pore of channelrhodopsin was enabled in the course of a long-term effort to create inhibitory channelrhodopsins, by first obtaining the high-resolution crystal structure (in this case of a high-expressing chimeric channelrhodopsin engineered for this purpose, C1C2)[9] followed by structure-guided pore engineering[10] to convert the protein from a cation channel into a chloride channel[10,11]; along with subsequent discovery of naturally occurring chloride channels[12,13], this body of work on the one hand has broadened the inhibitory channel toolkit and on the other has advanced understanding of the pore. Likewise, discovery of the red-shifted channelrhodopsin VChR1 (ref. 47), which gave rise to the initial red light-activated channelrhodopsin C1V1 (ref. 90), led to both deeper understanding of the spectral diversity of channelrhodopsins and new technological capability for *in vivo* single-cell two-photon control and integration with genetically encoded activity-imaging readouts[175–178].

The discovery and engineering of new single-component optogenetic tools proceeds on many fronts not limited to the microbial opsins, as with the engineering of potassium channels using a light-sensing LOV (naturally occurring light-oxygen-voltage protein) domain[181]. Indeed, the development of optogenetics with microbial opsins also created tools

that turned out to be useful in non-microbial opsin strategies, and this process is likely to continue (Fig. 6). Beginning in 2009, single-component optogenetics in neurons moved beyond the microbial opsins while still taking advantage of the newly assembled optogenetic toolkit. First came all-in-one approaches for creating light-activated regulators of defined intracellular molecular signaling pathways. These strategies initially provided for recruitment of heterotrimeric G proteins, small GTPases, cyclic nucleotides or direct transcriptional regulators. The first of these to be tested were the optoXRs[182]: single-protein chimeras between G protein– coupled rhodopsins for light responsivity and G protein– coupled signaling receptors for signaling specificity[183], designed and tested for expression in neurons of behaving ani-mals[182]. The initial optoXRs were mammalian expression– optimized light-activated a_2 adrenergic receptors and b_1 adrenergic receptors for control of behavior[182]; subsequent work by many groups extended the optoXR toolkit to the m-opioid receptor[184,185], the ade-nosine 2A receptor[186], the 5HT1A receptor[187], the metabotropic glutamate type 6 receptor[188] and the dopamine type 1 receptor[111], with *in vivo* control and new behavioral findings typically achieved using the very same fiberoptic interface and recombinase-dependent viral targeting that had been developed for microbial opsins[174,182]. Just as with chemo-genetics[189], in which overexpressed GPCRs are driven with synthetic ligands, the optoXR approach could rightly be criticized for driving G protein–mediated signaling pathways at levels that might be either too weak or too strong relative to native pathway recruitment mechanisms. In principle, the rapid actuation and reversibility of the optoXR approach could facilitate parametric mapping from weak to strong, as with microbial opsin optogenetics; with development of the right concomitant readouts for signaling pathway recruitment and activity modulation, as occurred with electrical control, biochemical optogenetics may move toward activity-guided and closed-loop control to attain a new level of precision.

After the single-component optoXR approach was shown to modulate behavior in a behaviorally closed-loop real-time place preference task by recruiting heterotrimeric G-protein pathways[182], single-component control of a small GTPase (Rac1) pathway was achieved later in 2009 using a LOV domain to create conformationally altered protein-protein interactions and allow activation of the small GTPases by recruitment to the plasma membrane[190]. Subsequent identification of microbial light-activated nucleotide cyclases followed[191,192], and direct light-activated regulation of transcriptional and epigenetic state was achieved with cryptochrome-derived tools called LITEs[193] (Fig. 6a).

Though not conducted with microbial opsins, these subsequent single-component optogenetic strategies now complement microbial opsin-based optogenetics in several important ways. First, as single-component methods, these reagents also deliver temporally precise and reproducible effects that capitalize on the genetic-targeting and fiberoptic-interface tools developed for the microbial opsin approach (for example, ref. 111; Fig. 6). Second, the slower timescale of these tools, along with the previously identified step-function bistable variants of the microbial opsins, provides functionality on longer timescales that complement the fast action of the typical microbial opsins. Third, even though the microbial opsins have demonstrated their utility in non-neuronal contexts ranging from stem cells[161] to cardiac cells[194] to astroglia[45,70], the biochemical single-component

optogenetic approaches began in 2009 to truly open the door to light-mediated control of any cell. The central concept of optogenetics, optically providing or deleting well-defined and precisely timed events in targeted cell types of complex functioning biological systems, is useful throughout biology, and the second half of the last decade has witnessed the increasing growth of non-neuronal and nonelectrical single-component optogenetics (Fig. 6).

Other candidate interventional methods are now sometimes contrasted directly: for example, as optogenetics without light[195] or optogenetics without genetics[196,197]. Nonoptical genetically targeted routes to control will continue to be explored for other modalities of information delivery—including thermal, ultrasonic and magnetic, as they have for chemical[189]—and nongenetic interventional methods will continue to be explored by accessing cell types for control not by exogenous gene expression but by targeting surface proteins[197]. These efforts are intriguing, and they will be pursued further in efforts to improve speed, signal-to-noise ratio, compatibility with free behavior, and minimal invasiveness. One major class of such efforts involves thermal activation, in which nanoparticles that serve as antennas for visible-light, magnetic or radio-frequency energy are used as sources for local heating, which is converted into neural activity using genetically delivered heat-responsive ion channels such as those of the TRPV1 family (Fig. 6b,c). These methods, though innovative, face challenges associated with nanoparticle diffusion properties in dense tissue, extremely high energy requirements that can be many orders of magnitude greater than for microbial opsins, substantial basal or unstimulated activity, and challenges associated with multicomponency (including speed relative to neural coding and dynamics, temporal precision and targetability). Yet even when performance in one or another of these important parameters falls short, such additional strategies will always be of value in complementing the optogenetic approach by adding orthogonality for multidimensional combinatorial control, where limits are encountered regarding the number of control channels available with light alone due to the broad action spectra of the opsins[4,5].

Reflecting on the past 10 years brings up a final point, always worth making in the context of optogenetics, regarding a concept that could be conveyed more widely to the public: the essential value of exploratory basic science research, even for investigators, institutions and funding agencies primarily interested in health and translational research. It seems unlikely that the initial experiments described here would have been fundable, as such, by typical grant programs focusing on a disease state, on a translational question, or even on solidly justified basic science. Though clinical and commercial applications are not addressed here, the advances brought by microbial opsin-based optogenetics may inform the pathophysiology and treatment of neurological and psychiatric disease states, as well as other clinical conditions, in addition to the broad basic science discoveries described above that have from the beginning constituted the core motivation of optogenetics. In this way, progress over the last ten years (Fig. 7) has revealed not only much about the brain, but also something about the scientific process.

It is sometimes noticed that the thrill of patch clamping, of gaining access and listening directly to the inner workings of a living, processing, responding neuron, never fully habituates for electrophysiologists—over any number of years or neurons. With optogenetics, a similar connection is felt, as investigators see the subject's behavior and

physiology change in real time while specific cells and projections are controlled—a connection still thrilling after all these years. The connection with light links experimenter and subject, but also in some sense spans the tree of life: microbial DNA has yet again returned to eukaryotic cells, a recurrent and curious theme of life on earth over billions of years, to provide another symbiosis, this one scientific. Such relationships coevolve and persist once formed, and though biology as a whole in the coming years will continue to move in unexpected directions, the ancient microbial opsins now seem inextricably part of our journey.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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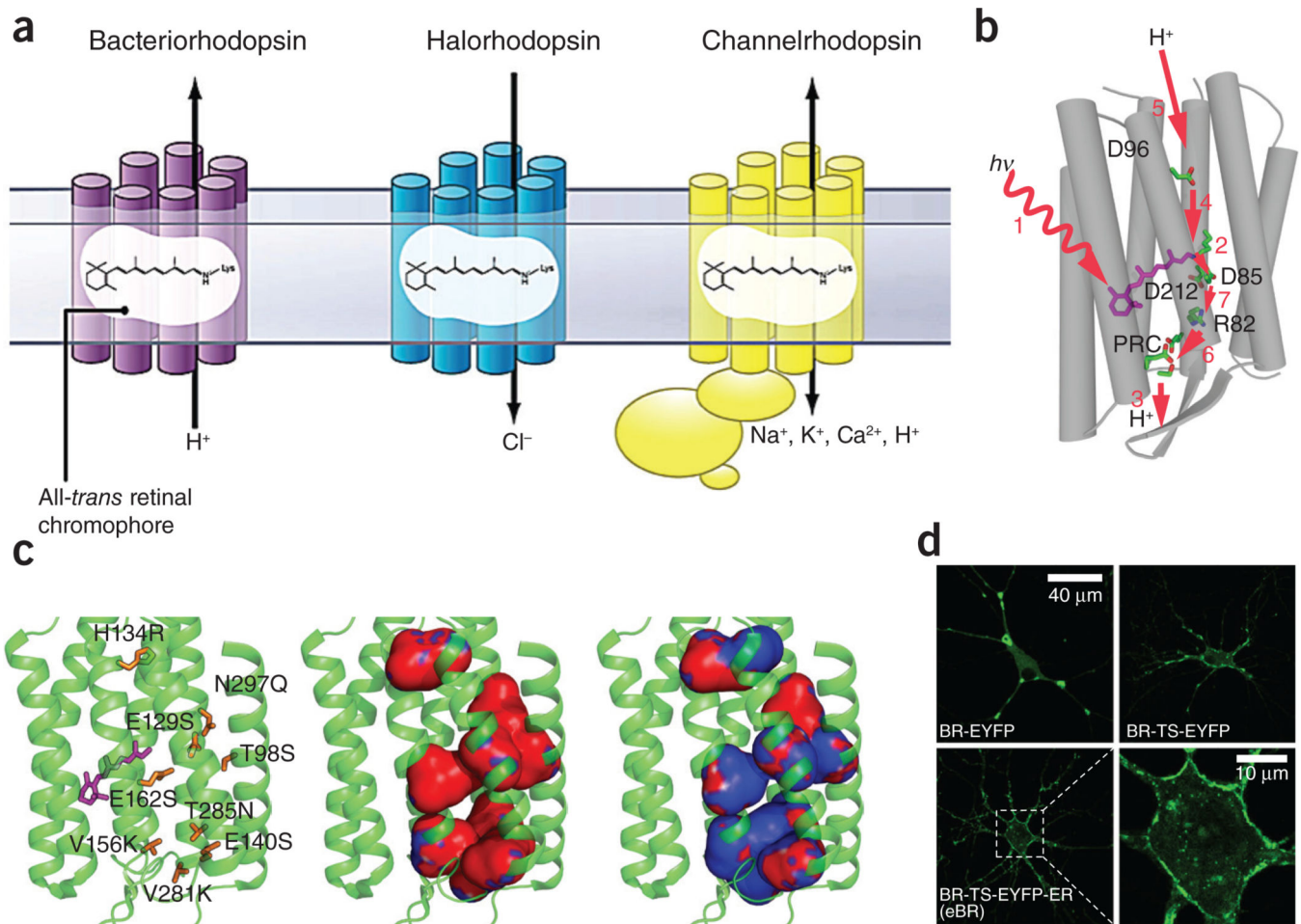
Figure 1.

The biochemical foundations of the study of microbial light-activated proteins. (**a**) The three major classes of microbial proteins used for single-component optogenetics (adapted from ref. 5, Elsevier). (**b**) Light-activated transmembrane current mechanism of the proton pump bacteriorhodopsin (BR)[5]. Photon (*hv*) absorption initiates a conformational switch, leading to discontinuous proton transfers involving Asp85, Asp96, Asp212, Arg82 and the proton release complex (PRC), and net charge movement across the membrane. The core concept of single-component light-activated transmembrane ion conductance had become textbook material by the 1980s[8] (reproduced from ref. 5, Elsevier). (**c**) Elucidation of channel-type conductance. The channelrhodopsin crystal structure[9] revealed positioning of transmembrane helices (green), the binding pocket of *all-trans* retina (purple), and angstrom-scale positioning of residues lining the pore (left). In the course of testing the pore model, structure-guided mutagenesis[10] of the residues in orange (left) shifted expected pore electrostatics from largely negative (red, center) to largely positive (blue, right) and switched ion selectivity from cation to anion (chloride) conductance[13]. (**d**) All three classes of microbia opsin-derived proteins suffer to some degree from formation of aggregations within metazoan host cells[18,50,51], but in all cases this can be addressed with membrane trafficking motifs borrowed from mammalian channels[18,50,51,90]. Shown: original BR fused to enhanced yellow fluorescent protein (EYFP); upper left depicts accumulations seen with

wild-type BR expression in mammalian neurons, upper right shows the effect on surface membrane expression of adding a neurite targeting motif (TS), and the lower row shows the effect of combined TS and ER (endoplasmic reticulum export) motif provision (reproduced from ref. 18, Elsevier).
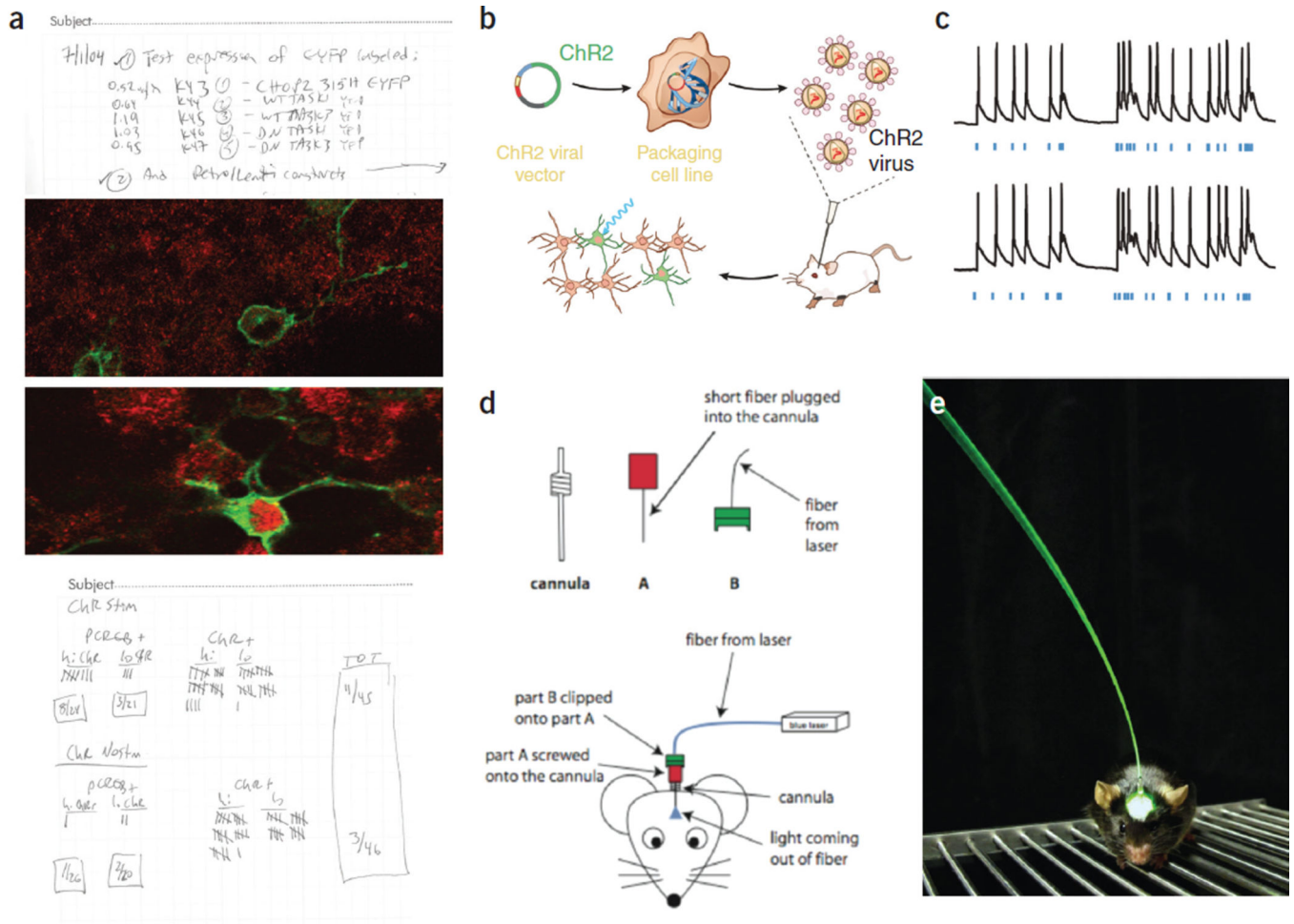
Figure 2.

Putting the pieces together. (**a**) Top, original notebook page from my laboratory's first microbial opsin experiment. Several channel clones were tested in parallel to build strategies for neuronal control, including the highest-risk opsin expression; constructs and plasmid concentrations that I used to transduce the neurons on 1 July 2004 are recorded at top left, including a channelrhodopsin (clone K43) and wild-type (WT) or dominant-negative (DN) TASK1/TASK3 potassium channels (K44-47). Host neurons were differentiated from adult-derived mammalian CNS progenitors. Center: neuronal expression, localization and light activation; green, subcellular distribution of fluorescent protein fused to channelrhodopsin in the ~10-µm-diameter neuronal somata and proxima dendrites. After carrying out illumination for optogenetic photostimulation and the CREB Ser-133 phosphorylation (red) assay for reporting activation (14 July 2004), activation was noted as a neuron-by-neuron tally (bottom; z-test, $\chi^2 = 9.0634$, d.f. = 3; $P = 0.028$) and neurons were transduced for the next steps. (**b**) The next steps included design of electrophysiological, imaging and behavioral readouts; design and introduction of high-titer opsin virus; and focal illumination of transduced brain regions (workflow of planned steps shown). (**c**) Stable, well-tolerated, reproducible control across experiments, crucia for optogenetics, was enabled with high-titer opsin viruses. Electrophysiological readout shows spikes from two cultured neurons

receiving the same light pattern (adapted from ref. 2, Springer Nature). (**d**) Initial engineering sketch of the fiber-optic neural interface for spatially registering viral transduction with focal high-intensity illumination (drawings courtesy F. Zhang, Stanford). (**e**) Instantiation of the interface that ultimately allowed depth-targeted control[26] and observation[4,111] of population-level activity in cells and projections of freely moving mammals (photo courtesy I. Goshen, Stanford). Supplementary Video 1 shows initial mammalian behavioral control.
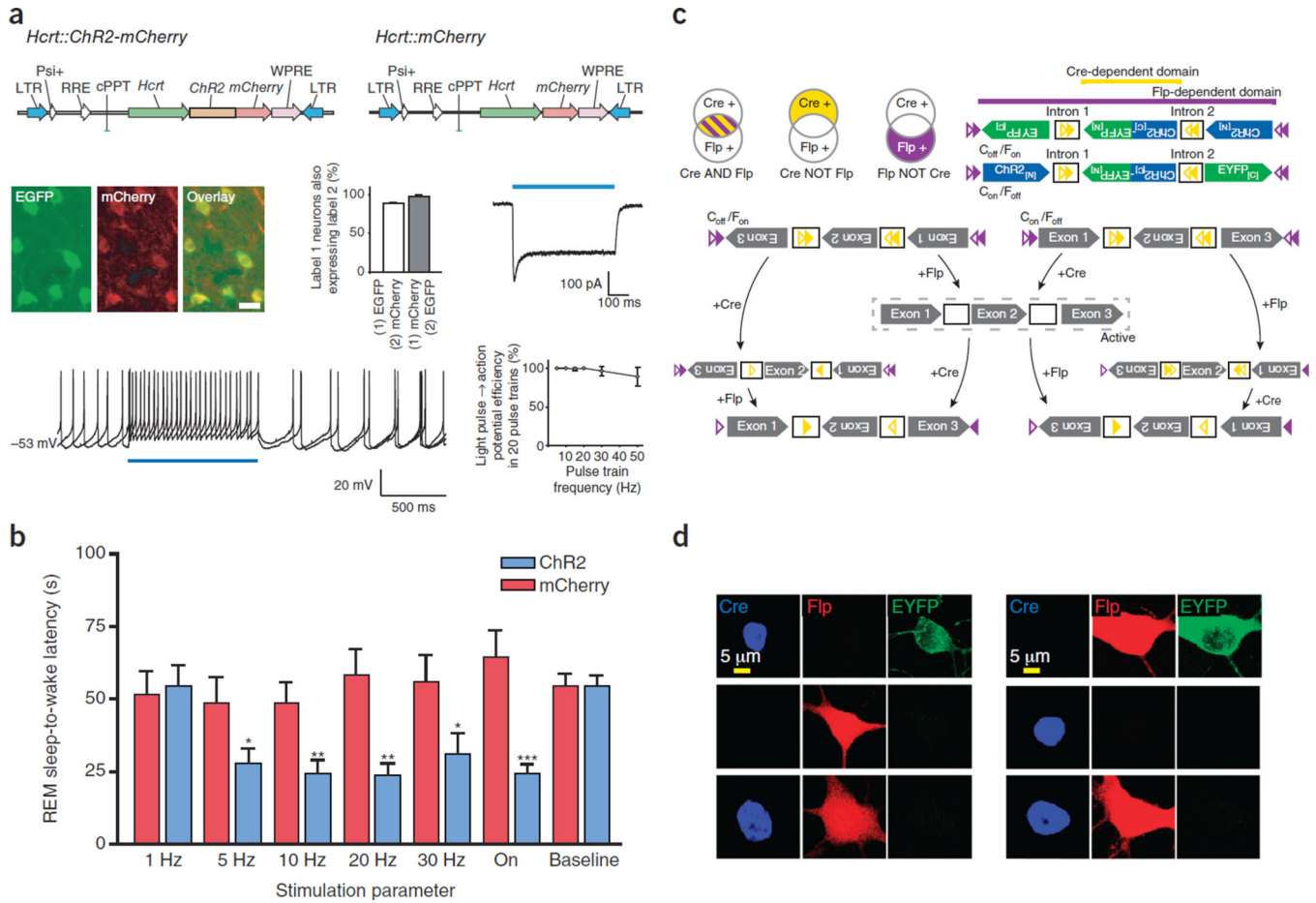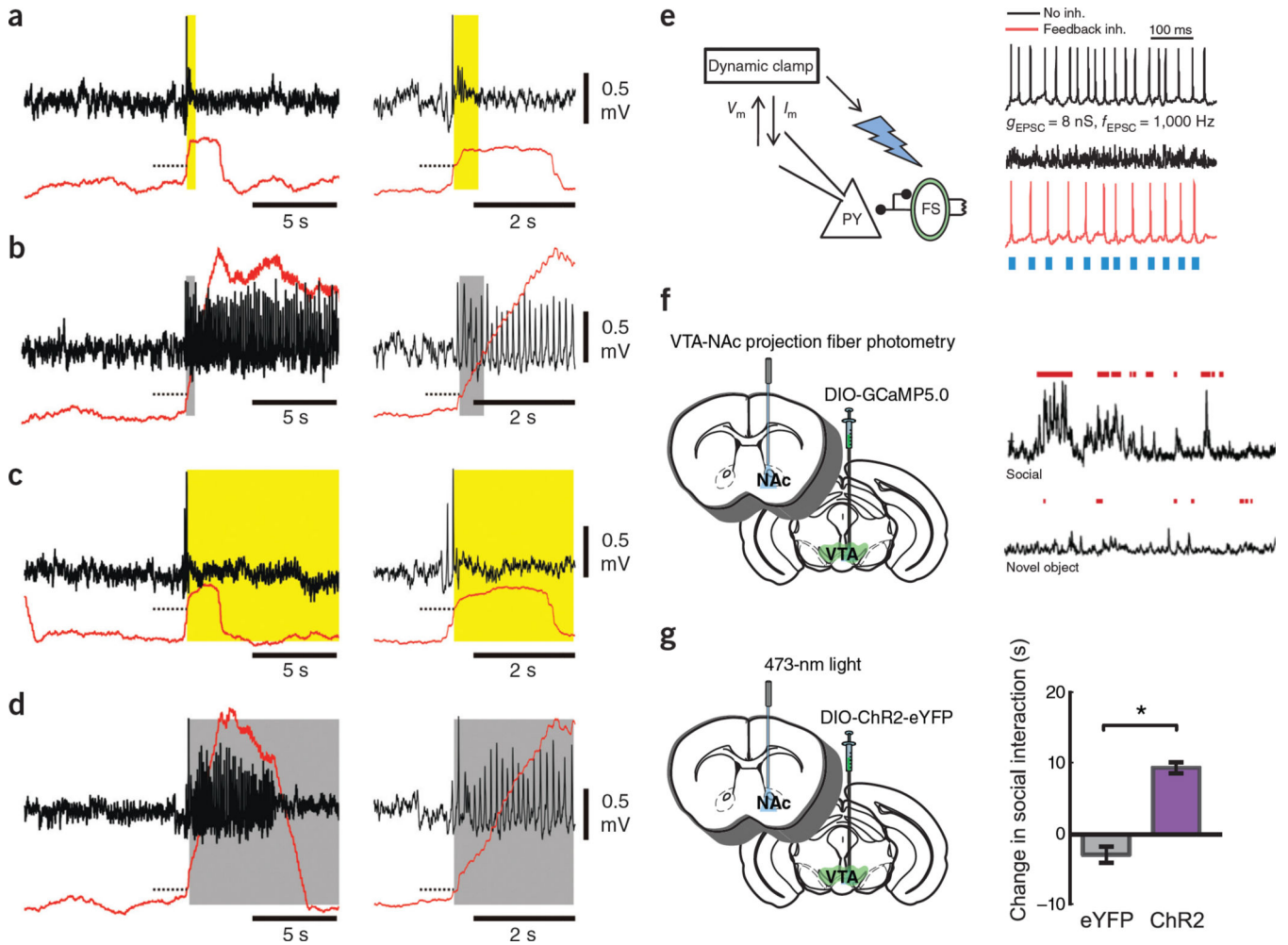
Figure 3.

Progress in genetically guided intervention for optogenetics. (**a**) Top, initial cell-type targeting for optogenetics in behaving mammals, based on a 3.1-kb hypocretin (*Hcrt*) promoter fragment in lentivirus; control vector without opsin gene at right[26]. LTR, long terminal repeats; RRE, Rev-responsive element; WPRE, woodchuck post-transcriptional regulatory element; ChR2, channelrhodopsin-2; cPPT, central polypurine tract; Psi+, *cis*-acting packaging sequence; mCherry, a red fluorescent protein. Middle, specificity, penetrance and efficacy of expression in Hcrt neurons (green); ChR2–mCherry fusion (red) shown in mouse lateral hypothalamus (scale bar, 20 μm); right, photocurrent in hypothalamic slice. Bottom, neurons firing action potentials upon illumination; two sweeps superimposed. Error bars, s.e.m. (**b**) Dose-response of light flash effects; in experiment corresponding to **a**, latencies of wake transitions are shown from rapid eye motion (REM) sleep after a single 10-s photostimulation bout at different frequencies (15-ms light pulses; **a,b** adapted from ref. 26, Springer Nature). Error bars, s.e.m. (**c**) Mammalian genetic targeting for optogenetics: AND or NOT opsin-expression logic conditional on multiple recombinase-expression-defined genetic features using single adeno-associated viral vectors[54]. Top left, schematics representing selection of target populations of cells expressing (or not) the recombinases Cre or Flp, which are often used to create animal lines with cell-type-targeted recombinase expression patterns. Top right, mechanism of targeting

based on recombination followed by removal of recombination sites in introns[54]. (**d**) Neurons transfected with combinations of Cre (blue), Flp (red) and the vectors that implement Cre AND NOT Flp (C_{on}/F_{off}) or Flp AND NOT Cre (C_{off}/F_{on}). ChR2-YFP is only expressed in cells marked by one or the other, but not both, recombinases (**c,d** adapted from ref. 54, Springer Nature).
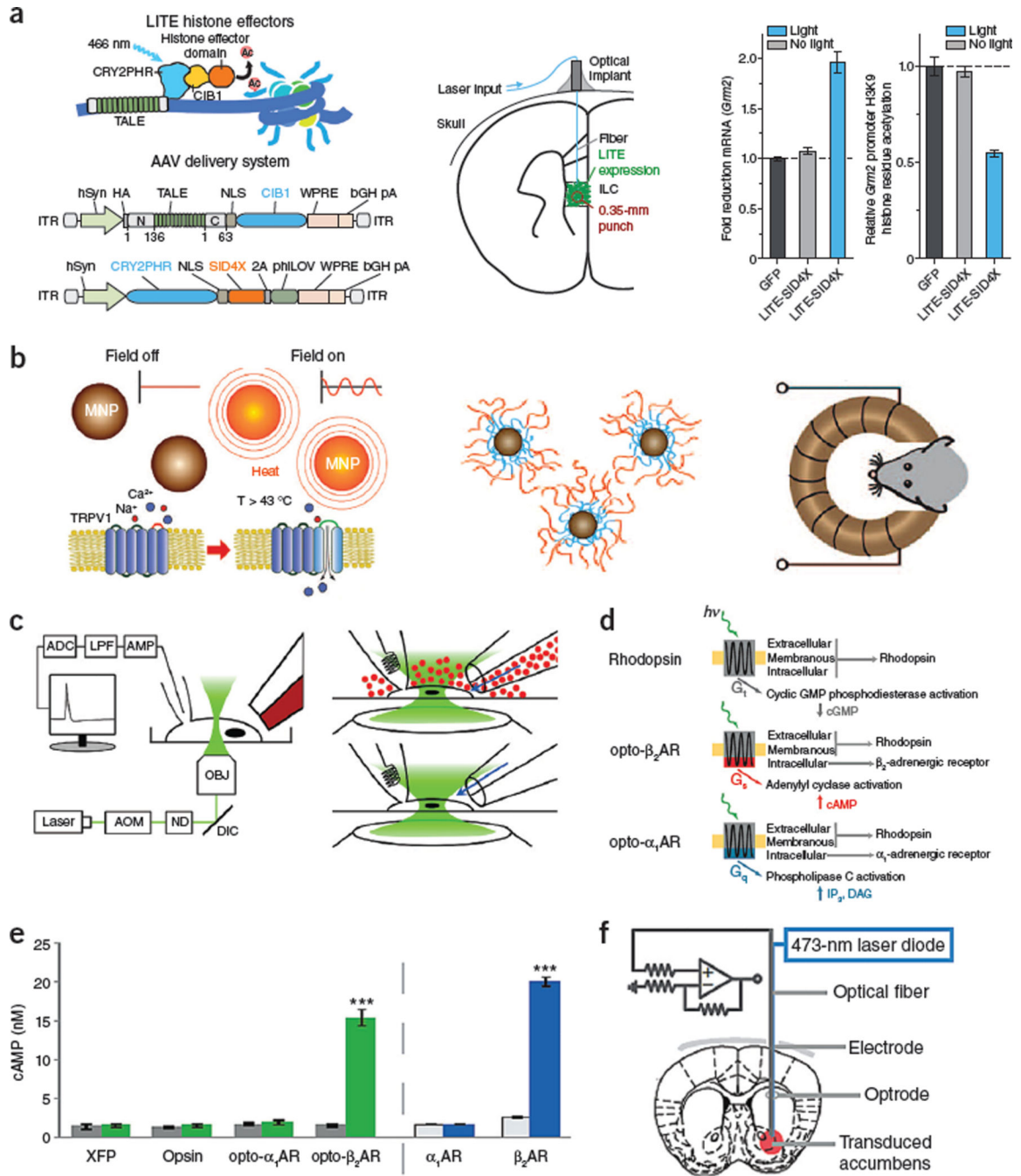
Figure 4.

Progress in activity-guided intervention for optogenetics. (**a**–**d**) Closed-loop targeting of thalamocortical neurons in epileptic cortex (reproduced from ref. 158, Springer Nature). Yellow light terminates seizures defined by EEG and behavior, detected and interrupted in real time with closed-loop optogenetic inhibition using eNpHR3.0, an engineered inhibitory halorhodopsin (**a,c**). Without yellow light, native epileptic events follow an unmodified time course (**b,d**). Thalamocortical activity was thus, surprisingly, identified as necessary for poststroke epileptic events in this context. (**e**) Optogenetic closed-loop control in 2009. Optogenetic stimulation of ChR2-expressing inhibitory (FS) neurons was made conditional on spike detection in pyramidal (PY) neurons, implementing feed-forward inhibition under experimenter control (adapted from ref. 41, Springer Nature). Black and red traces show action potentials in PY cells without or with, respectively, use of closed-loop optogenetic excitation of FS cells. (**f**) Closed-loop, all-optical control could be implemented using deep brain fluorescence (for example, GCaMP) detection of genetically specified activity signals, via techniques such as fiber photometry (shown here detecting in real time the activity of ventral tegmental area (VTA) dopamine neuron projections to the nucleus accumbens (NAc) during appetitive social interaction)[111]. Red bars indicate interaction episodes (social or object). (**g**) With considerable potential for closed-loop control of projection dynamics, the

same fiber-optic interface[25,26] can be used not only to observe activity in defined deep-brain projections, but also to control activity in deep-brain projections, as shown here modulating social behavior (**f,g** adapted from ref. 111, Elsevier). These specified methods for activity-guided opsin expression[139].
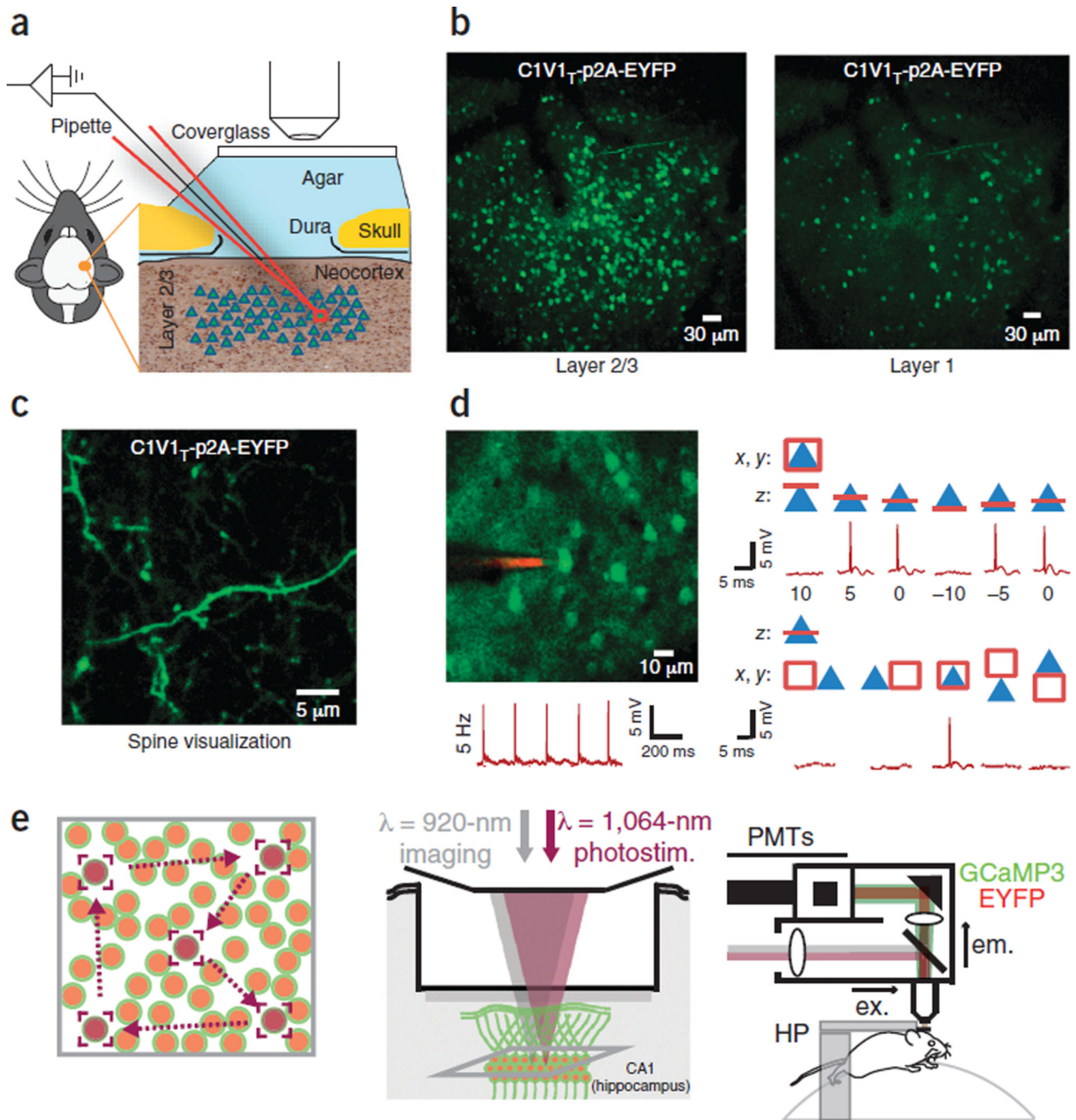
Figure 5.
Progress in spatially guided intervention for optogenetics: beyond cell subpopulation or projection targeting. (**a–d**) Initial *in vivo* two-photon, single-cell-resolution optogenetics with guided light: optogenetic control of spiking in adult mice (adapted from ref. 175, Springer Nature). (**a**) Experimental setup targeting superficial layer 2/3 somatosensory neurons with C1V1 (ref. 175). (**b,c**) Transduced neurons in somatosensory cortex shown at low (**b**) and high (**c**) magnification with cell-filling fluorophore version of the opsin virus used to facilitate cell identification, imaging and control[175]. (**d**) Left, layer 2/3 pyramidal

cells transduced with C1V1 under loose patch conditions (note red dye-filled patch electrode). Lower left, trace showing 5-Hz control of spiking with 1,040-nm raster-scanning illumination. Right, axial (upper) and lateral (lower) single-cell resolution of two-photon optogenetic spiking control *in vivo*. Blue triangles indicate pyramidal neurons and red boxes illustrate region-of-interest raster-scan positioning; traces show spiking occurring only while scanning within the cell. (**e**) An example of single-cell targeting with optogenetics using temporally focused two-photon control of C1V1, with single-cell resolution optical feedback, in a virtual reality environment in behaving mice (adapted from ref. 177, Springer Nature); for spatial light modulator use together with C1V1, see refs. 175,176,178. Target neurons are illuminated in turn via temporally focused soma-sized spots (<15 μm). Left, light path; center, lasers used; right, behavioral preparation[177]. PMTs, photomultiplier tubes; ex., excitation; em., emission; H P, headplate.

Figure 6.

Variations on the theme. (**a**) Optogenetic control of histone acetylation and gene expression. The LITE system[193] uses light-responsive cryptochromes, not microbial opsins, and has been adapted to create histone effectors targeted to specific genomic sequences using TALE reagents (left). TALE, transcription activator–like effector; CRY2PHR, cryptochrome-2 photolyase homology region; CIB1, CRY2 interacting partner. The same laser diode/fiber-optic optogenetic interface developed for microbial opsins (middle) can be used to control this optogenetic system *in vivo* (right) (adapted from ref. 193, Springer Nature). ILC,

infralimbic cortex; SID4X, four concatenated mSin3 domains for histone effector control. (**b**) Left, multicomponent transduction of magnetic signals into neural activity (left), via introduced nanoparticles (MNP) and TRPV1 temperature-sensitive ion channel genes. Middle, nanoparticles with polyacrylic acid coating (blue) and polyethylene glycol chains (orange). Right, the magnetic field exposure system. (Adapted from ref. 195, AAAS). (**c**) Multicomponent transduction of optical signals into neural activity via nanoparticles directly targeted to cells (which, as in **b**, must be distributed through tissue); light intensities required are many orders of magnitude greater than those used in microbial opsin optogenetics (adapted from ref. 196, Elsevier). (**d**) Single-component control of defined biochemical pathways in neurons by the optoXR method[182]. IP$_3$, inositol trisphosphate; DAG, diacylglycerol. (**e**) Biochemical validation of signaling specificity[182]; optoβ$_2$AR recruits the cAMP pathway in response to light (green bars) to a level comparable to that of direct pharmacological agonism of the original G protein–coupled receptor (blue bars), while not recruiting separate pathways[182]; in contrast, other optoXRs recruit distinct signaling mechanisms[111,184–188]. (**f**) Optrode methodology for activity recording[182]. As with the LITE system shown in **a**, biochemical systems continue to exploit the neural interfaces for electrophysiology[182] and for behavior[111] that were developed for microbial opsin optogenetics[26,25,37] (**d**–**f** adapted from ref. 182, Springer Nature).
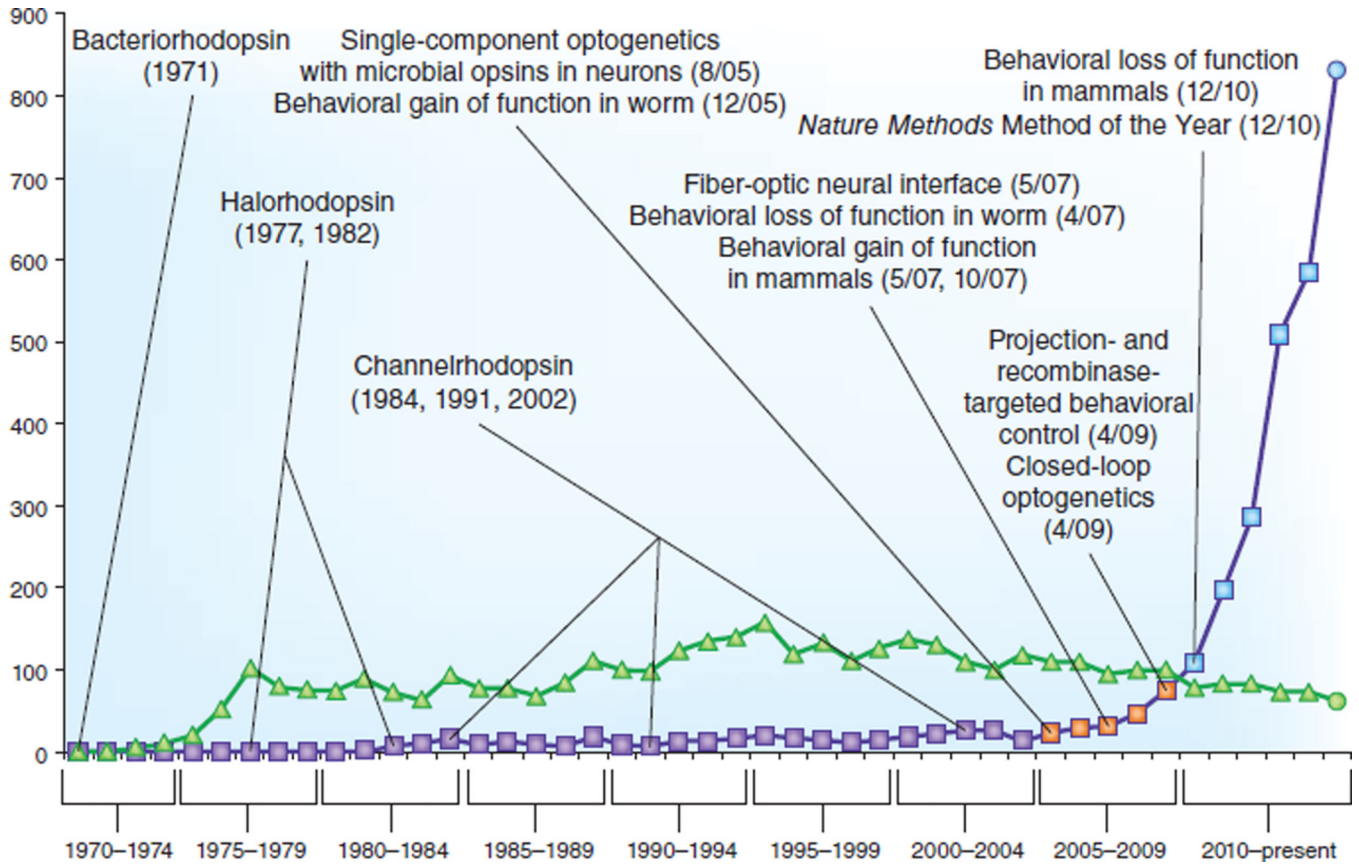
Figure 7.
Publication timeline for microbial opsins and optogenetics over 45 years. Trajectory of the number of papers per year searchable in PubMed by bacteriorhodopsin (triangles); the second trajectory (squares) shows papers searchable by keywords encompassing all other related efforts: halorhodopsin, channelrhodopsin or variations of optogenetics. Note steady progress of the groundbreaking bacteriorhodopsin literature, not surpassed by the rest of the field until 2010. Key pioneering papers relevant to bacteriorhodopsin[7], halorhodopsin[198,199] and channelrhodopsin[15,46,200] are indicated. Publication counts: PubMed search on 1 July 2015. The first 5 years of single-component optogenetics are shown in orange, during which time few papers were published, and the second 5 years (to the present) shown in blue. Circular symbols for 2015 represent linear extrapolation based on the first 6 months.