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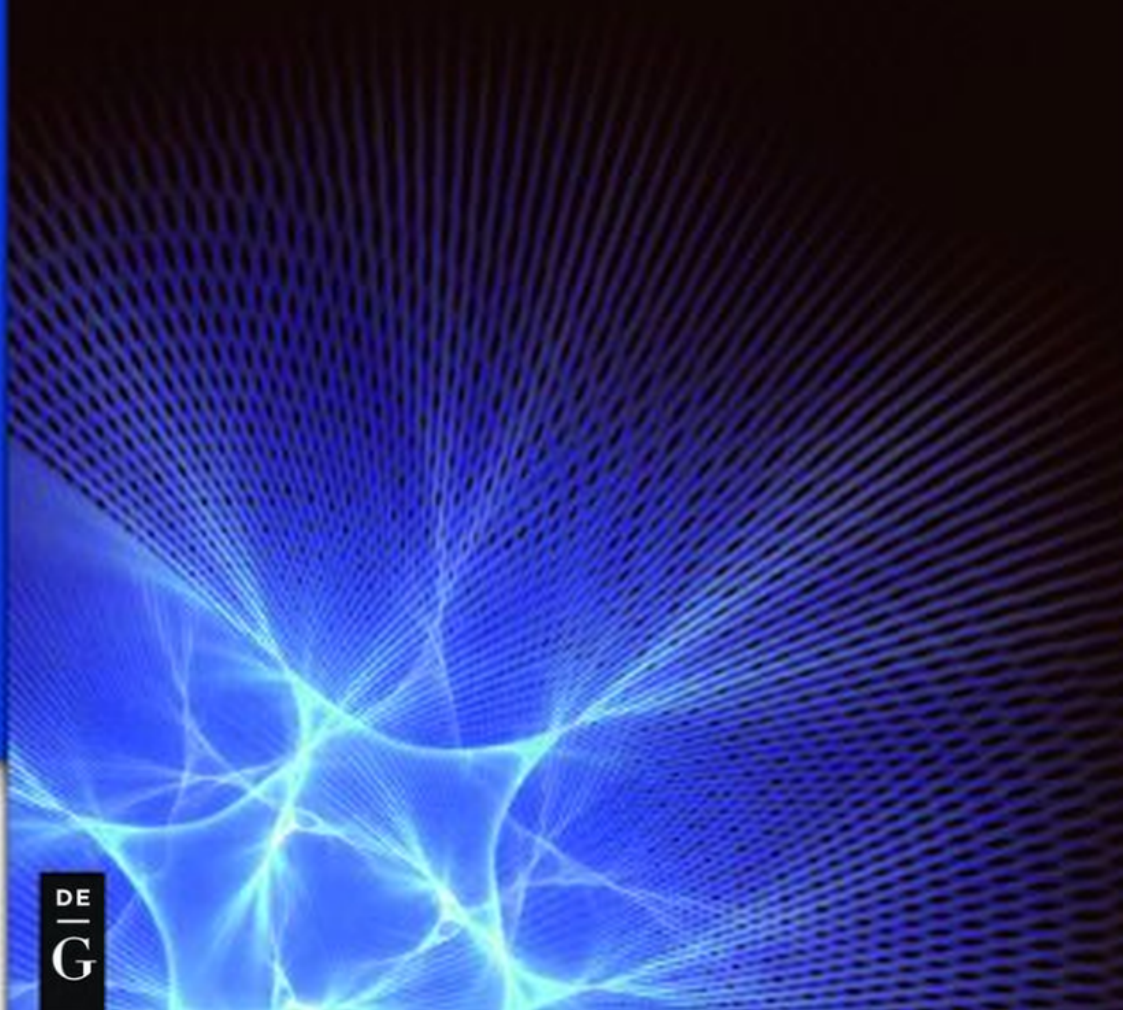

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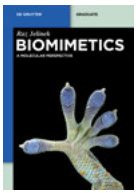

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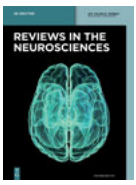

Reviews in the Neurosciences
Editor-in-Chief: Joseph P. Huston
ISSN 0334-1763

Optogenetics

Edited by Peter Hegemann and Stephan Sigrist

DE GRUYTER

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ISBN 978-3-11-027071-6
e-ISBN 978-3-11-027072-3
ISSN 2196-4769
e-ISSN 2196-4777

Photographs (Appendix) have been kindly provided by Denise Stewin.

Library of Congress Cataloging-in-Publication Data
A CIP catalog record for this book has been applied for at the Library of Congress.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.dnb.de.

Typesetting: PTP-Berlin Protago-TEX-Production GmbH, Berlin
Printing and binding: Hubert & Co. GmbH & Co. KG, Göttingen
♾ Printed on acid-free paper
Printed in Germany

www.degruyter.com

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Content

Introduction

When I was asked by my colleague and member of the Dahlem Conferences Scientific Advisory Board, Robert Bittl, in 2010 to organize a Dahlem conference about optogenetics, I was extremely reluctant and skeptical about the purpose of such a conference. There are already too many conferences, and we are presenting similar data about our research on many occasions and locations around the world. Moreover, the Dahlem conferences are unstructured in the sense that there is no fixed program or schedule, the number of attendants is limited to 40 and there is no big audience listening to what the participants have to say. Even worse, there are no talks and very little chance to present any of the latest research. What should encourage the best researchers in a certain field to come to such a conference or workshop and to be locked up for a few of days despite the extremely tight schedules they already have in most cases?

The idea of the Dahlem Conferences is to discuss challenges and potential risks of a novel technology, traditionally during five days in a closed venue, and not to present data. It is anticipated that the participants know the state of the art prior to the meeting. Why optogenetics? Optogenetics is a new technology that combines genetics with the latest optical technology to study neuronal networks on different scales of space and time. This technology developed very rapidly, from zero at the year 2002 to a widely accepted research field 10 years later. It has now reached a level where it is even considered for clinical applications. This rapid development convinced the Scientific Advisory Board members of the Dahlem Conferences to bring researchers of the optogenetics field together to discuss future perspectives of the technology.

Prior to the conference, Karl Deisseroth, Stephan Sigrist, Uwe Heinemann, Thomas Oertner, Zhuohu Pan, and Sabine Schleiermacher identified candidate subtopics that were later used in the initial discussion groups, before the participants mixed and reassembled during the following days. A number of participants had sent discussion manuscripts with provocative questions and considerations, and all participants were asked to send in "seed questions" that they wanted to be discussed during the workshop. The idea originally brought up by Stefan Sigrist was extremely useful, and we collected some 140 "seeds" as starting material for the conference. The topics that we selected during the pre-conference stage are the following:

1. *Optogenetic tools*, chaired by Karl Deisseroth (Stanford), Roger Tsien (San Diego), and myself. As optogenetics is a comparatively young discipline, many of its tools are currently under investigation and active development. Light-gated protein switches (*i.e.*, photoreceptors), with improved or entirely new molecular function, enable enhanced light control over cellular processes, and expand the scope of optogenetics. Several lines of research are pursued to address the need for additional and streamlined optogenetic tools. Firstly, multiple research groups try to obtain a description at the molecular level of the structure, function, and signaling mechanism of photoreceptors. Insights into these properties

allow rational improvement of proteinaceous light switches. For example, several channelrhodopsin variants, with differing spectral sensitivities and photocurrent kinetics, have been produced. Secondly, genome databases are sifted for previously unknown light-regulated proteins and enzymes. These new light switches permit optogenetic control in ways complementary to existing approaches, if they possess molecular functions different from other optogenetic tools. For example, recently several light-activated adenyl cyclases have been discovered that perform their enzymatic activity in a light-regulated manner. Thirdly, the repertoire of natural photoreceptors has recently been expanded by the design of synthetic photoreceptors. Inspired by natural systems, custom-made light switches allow light control over yet other cellular processes. In the most striking demonstration to date of synthetic photoreceptors, the motility of fibroblasts has been controlled by blue or red light, via a small light-activated GTPase, the Rac1 protein. In an ideal scenario, any arbitrary protein activity could be subjected under light control; if this can be accomplished, metabolism, signaling networks, and the behavior of cells and organisms could be manipulated in precise ways with only minimal perturbation of other processes.

2. *Application in cellular systems and lower model organisms*, chaired by Stephan Sigrist (Berlin), Alexander Gottschalk (Frankfurt), and Erik Jorgensen (Utah). Two kinds of devices address complementary needs for the research with lower model organisms: light-driven actuators control electrochemical signals, while light-emitting sensors report them. When actuators are expressed in genetically defined neurons in the intact animal, previously unattainable insight into the organization of neural circuits, the regulation of their collective dynamics, and the causal relationships between cellular activity patterns and behavior can be achieved. Animal model systems, which combine high optical transparency with easy and efficient genetics, are particularly effective in further progressing these aspects of optogenetics. The nematode *Caenorhabditis elegans*, with a comparatively simple nervous system, is clearly suitable for optogenetics, *e.g.*, neurotransmission has been analyzed with high temporal precision in a neurotransmitter-selective manner. The fruitfly *Drosophila* affords similar advantages, although it resembles a significantly higher level of complexity. Quite a few tools for remotely activating neural circuits by light in *Drosophila* have become available as well. As for vertebrate systems, the translucent brain of zebrafish (*Danio rerio*) offers superior experimental conditions for optogenetic approaches *in vivo*. Enhancer and gene-trapping approaches have generated many Gal4 driver lines in which the expression of UAS-linked effectors can be targeted to subpopulations of neurons. Local photoactivation of genetically targeted light-activated channels or pumps, such as channelrhodopsin and halorhodopsin, or channels chemically modified with photoswitchable agents, such as LiGluR, have uncovered novel functions for specific areas and cell types in zebrafish behavior. Despite widespread and growing use, very little work has been done to characterize exactly how optoge-

netic tools affect activity in model system neurons. We discussed these aspects in addition to new exciting examples of optogenetic tools for circuit analysis of model systems.

3. *Mapping neuronal networks*, chaired by Thomas Oertner (Hamburg). Recent progress in optics, genetics, and chemistry has provided new tools for the morphological dissection and functional analysis of neuronal networks, both *in vitro* and *in vivo*. Not only can light-controlled actuators of neuronal activity, *e.g.*, channelrhodopsin, be activated with millisecond precision, but this activation can also be performed in a targeted, cell-specific manner. Alternatively, the activity of distinct neurons can be blocked by ion pumps, *e.g.*, halorhodopsins, or by the use of recently designed K-selective ionotropic glutamate receptors. The specificity in the optical control of the activity of neuronal networks can be enhanced by various ways of targeting the light specifically to individual neurons by new scanning devices. Of particular promise is two-photon microscopy for neuron-specific activation, which grants access to deeper tissue layers. With these approaches, the control of activity can be exerted at various levels of neuronal circuits, ranging from neuronal subcompartments, such as axons and dendritic spines, up to entire classes of neurons within a circuit; for example, all or specific GABAergic inhibitory interneurons. The range of conceivable applications is enormous and includes the identification of synapses within the networks that control synaptic plasticity, the study of how neurons are connected to each other to control defined behaviours *in vivo*, or the determination of basic mechanisms of default circuitries in the brain, such as those underlying the central pattern generators (CPGs) which generate periodic motor commands for rhythmic movements.
4. *Clinical application*, chaired by Uwe Heinemann (Berlin), and Luis de Lecea (Stanford). Optogenetic methods have already been applied to study circuits and symptoms relevant to narcolepsy, blindness, depression, fear, anxiety, addiction, schizophrenia, autism, Parkinson's disease, and epilepsy. Moreover, the potential of the technology to fundamentally advance our understanding of neural circuit dysfunction is enormous. This session covered clinical applications of optogenetics, including efforts dedicated to understanding disease circuitry in animal models, and efforts focused on direct clinical translation. Topics in the latter category included applications to deep brain stimulation, peripheral nerve stimulation, and motor prosthetics. Topics in the former category were motivated by the fact that a most fundamental impact of optogenetics need not arise from direct introduction of opsins into human tissue, but rather from use as a research tool to obtain insights into complex tissue function, as has already been the case for Parkinson's disease. Many opportunities exist in both categories. Due to technological limitations in probing intact neural circuits with cellular precision, our current understanding of brain disorders does not do full justice to the brain as a high-speed cellular circuit. Rather than conceptualizing the brain as a mix of neurotransmitters, ideally we would be able to move toward a circuit-engineering

approach, in which devastating symptoms of disease are understood to causally result from specific spatiotemporal patterns of aberrant circuit activity relating to specific neuronal populations. But technology has been lacking for the requisite high-speed, targeted, causal control of intact neural circuit function, and this challenge extends to basic neuroscience and other biological systems as well. Optogenetics now provides a means to address this challenge.

5. *Restoration of vision and hearing*, chaired by Zhuohua Pan (Detroit) and Botond Roska (Basel). Retinitis pigmentosa (RP) refers to a diverse group of progressive, hereditary diseases, leading to incurable blindness, and affecting two million people worldwide. There is no general cure for RP, but several approaches that offer some degree of treatment in some forms of RP are in clinical trials and others are on the horizon. Gene replacement shows great promise if the disease is caused by the lack of function of the mutated gene, which mostly occurs in recessive forms of RP. Progress in replacing mutated RPE65 in the retinal pigment epithelium in Leber congenital amaurosis not only offers hope for patients of this disease, but also shows promise for other gene-replacement strategies by demonstrating the safety and efficacy of adeno-associated viral vectors for gene therapy in the human eye. Gene replacement can only be envisioned if the cell type expressing the gene is still alive and therefore, in the case of the most common rod-specific genes, early diagnosis and gene therapy in childhood might be necessary. When the mutation creates a "toxic" protein or the gene is too large to fit the viral vectors authorized in clinical trials, this approach is limited. Nevertheless, in the cases when it is feasible and unlike other approaches documented below, gene replacement may provide a real cure for a group of patients. Secondly, approaches to decrease the speed of degeneration of photoreceptors attempt to slow down the progression of the disease. This approach is feasible until visual function is preserved. Thirdly, a number of approaches attempt to restore photosensitivity without interfering with the intrinsic progress of the disease by creating new photosensors and couple them into the remaining retinal circuitry. Patients who are legally blind are the key target population of these approaches. Three different approaches in this group are the implantation of differentiated or undifferentiated photoreceptors, electronic retinal implants, and optogenetic approaches. The symposium introduced, contrasted, and debated the different approaches to restore photosensitivity to animal models of Retinitis pigmentosa and to human patients. Current clinical and preclinical trials were discussed in terms of safety, efficacy, and impact on society.

Conclusions and final considerations: the key issue of the optogenetic technology is its cell specificity, but at the same time, this is also its major limitation. Neuroscientists might apply optogenetic approaches to cure, or at least alleviate, diseases in the near future, and the first trials will probably be carried out within the next two years for retinal prosthesis or Parkinson's disease. But optogenetics is limited

to those brain diseases that localize to a clearly defined area of the brain. These diseases are extremely rare, whereas most brain disorders are of a much higher level of complexity, involving many cells distributed over a large area of the brain. Not only is the causality of these malfunctions unknown, but they are also out of reach for any optogenetic applications. Optogenetics is certainly an innovative technology and of great analytical value in the context of many diseases, but at present we should be humble about the potential as a therapeutic technology to cure brain malfunction by any means. This will only become true for a very small number of diseases, based on defects of single genes with very local activity of the gene products. Last, but not least, ethical questions should be constantly discussed – from early experiments on mammals, to non-human primates, and to eventual applications in humans.

I personally was extremely amazed about how the discussion developed during the progress of the conference, about the precision with which key issues crystallized during these days, the cross-border discussions that developed between *tool makers* and *appliers*, and the careful consideration of potential application, including ethical perspectives. Finally, I was enlightened that most of the organization and bureaucracy that we experience in our usual conferences is not necessarily needed; thus, fruitful discussions were not unduly hampered and went into the depth required to address questions that really matter for the promotion of a new field.

Finally, I thank my colleagues who worked with me on the planning of the conference, especially Karl Deisseroth, Uwe Heinemann, Andreas Möglich, Zuohuo Pan, Sabine Schleiermacher, Stephan Sigrist, and several others that sent in suggestions and discussion manuscripts. I am also indebted to the three graduate students, Elena Knoche, Franziska Schneider, and Stephanie Wegener, who meticulously recorded the main ideas and outcomes of the sessions and provided these to the authors that you, as the reader, will find in this book. Last, but not least, I thank Michael Brückner, a person quite invisible during the conference, but who ran the organization smoothly, did all the logistics, the financing, and everything that made the conference enjoyable.

I hope that this conference helped to develop the field of optogenetics in a direction where it brings insight into the organization of neuronal networks, where it uncovers origins of brain diseases, and where it might even help to develop curative strategies which make the life of patients more enjoyable.

Berlin, April 2013 *Peter Hegemann*

Keith Moffat, Feng Zhang, Klaus Hahn, Andreas Möglich

1 The biophysics and engineering of signaling photoreceptors

1.1 Photoreceptors

Image formation, vision, and certain developmental and behavioral processes in diverse organisms are naturally sensitive to light. The primary event is absorption of a photon by a photoreceptor protein comprised of at least two units: a photosensor which absorbs light and an effector whose light-dependent activity ultimately elicits a physiological response. (Other units may be present, *e.g.*, those that confer specific intermolecular interactions, but these two units are essential). Since the common constituents of organisms (amino acids and proteins, ribonucleic acids, lipids, carbohydrates, small metabolites) do not absorb the wavelengths significantly present in sunlight, absorption by the photosensor typically occurs in a covalently or non-covalently bound, small organic moiety known as a chromophore. Retinal, flavin nucleotides, and bilin are common examples of chromophores (Figure 1.1). A quite different example is offered by UV-sensitive photoreceptors exemplified by UVR8 [1] where the "chromophore" is believed to be a cluster of tryptophan side chains which naturally absorb in the near-UV region of the spectrum.

When photoreceptors are classified by the chemical nature of their chromophore and the photochemistry that follows photon absorption, they fall into seven distinct classes [2]: UV receptors; photoactive yellow protein and relatives [PYP]; light-oxygen-voltage [LOV]; sensors of blue light utilizing FAD [BLUF]; cryptochromes; rhodopsins; and phytochromes (Figure 1.2). To these may be added cyanobacteriochromes [3, 4]. The term "distinct classes" is loosely defined. Quite different chromophores and photochemistry are found in LOV domains (flavins) and PYP-like molecules (*p*-coumaric acid), yet the photosensor proteins that contain these two distinct chromophores are structurally related. Each forms a subclass of Per-ARNT-Sim domains, which are widely distributed in signaling proteins more generally [5, 6].

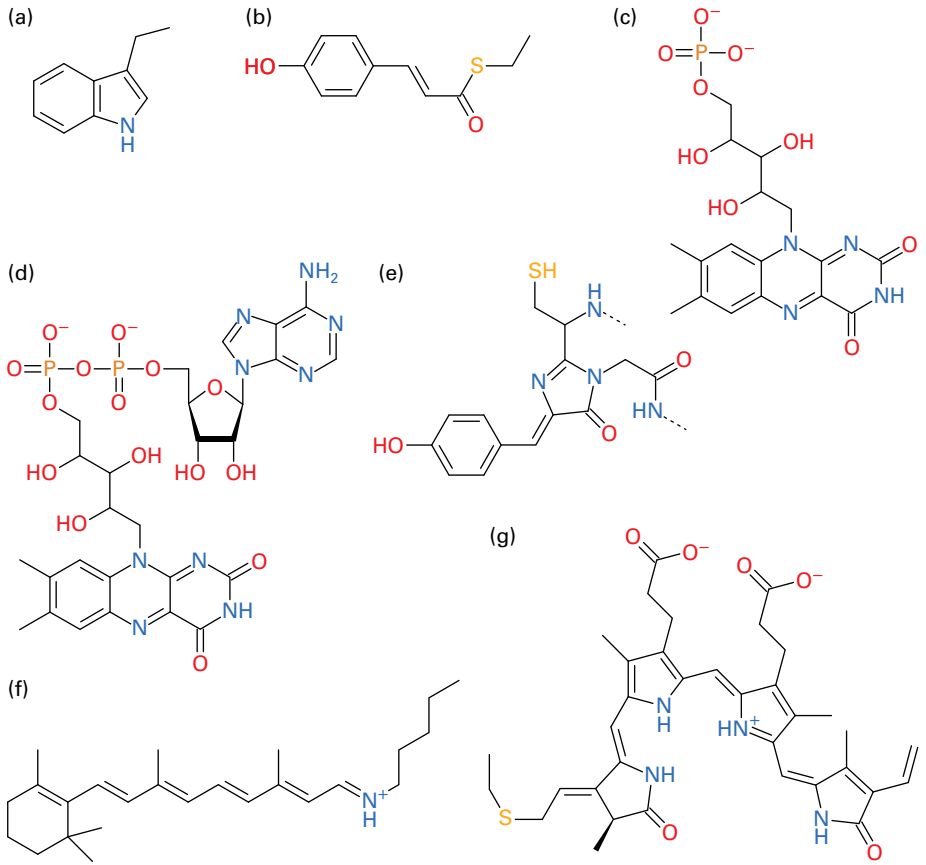

Figure 1.1: Chromophores of photoreceptors. Each photoreceptor harbors an aromatic chromophore molecule that absorbs electromagnetic radiation in the near-UV, visible or near-infrared ranges. As chromophores, plant UV photoreceptors use tryptophan side chains (a); photoactive yellow protein uses *p*-coumaric acid (b); LOV photoreceptors use flavin mononucleotide (c) or dinucleotide (d); cryptochromes and BLUF domains use flavin adenine dinucleotide (d); rhodopsins use retinal (f); and phytochromes use linear tetrapyrroles such as biliverdin (g). In jellyfish fluorescent proteins, the heterocyclic chromophore is formed autocatalytically from three amino acid side chains (e).

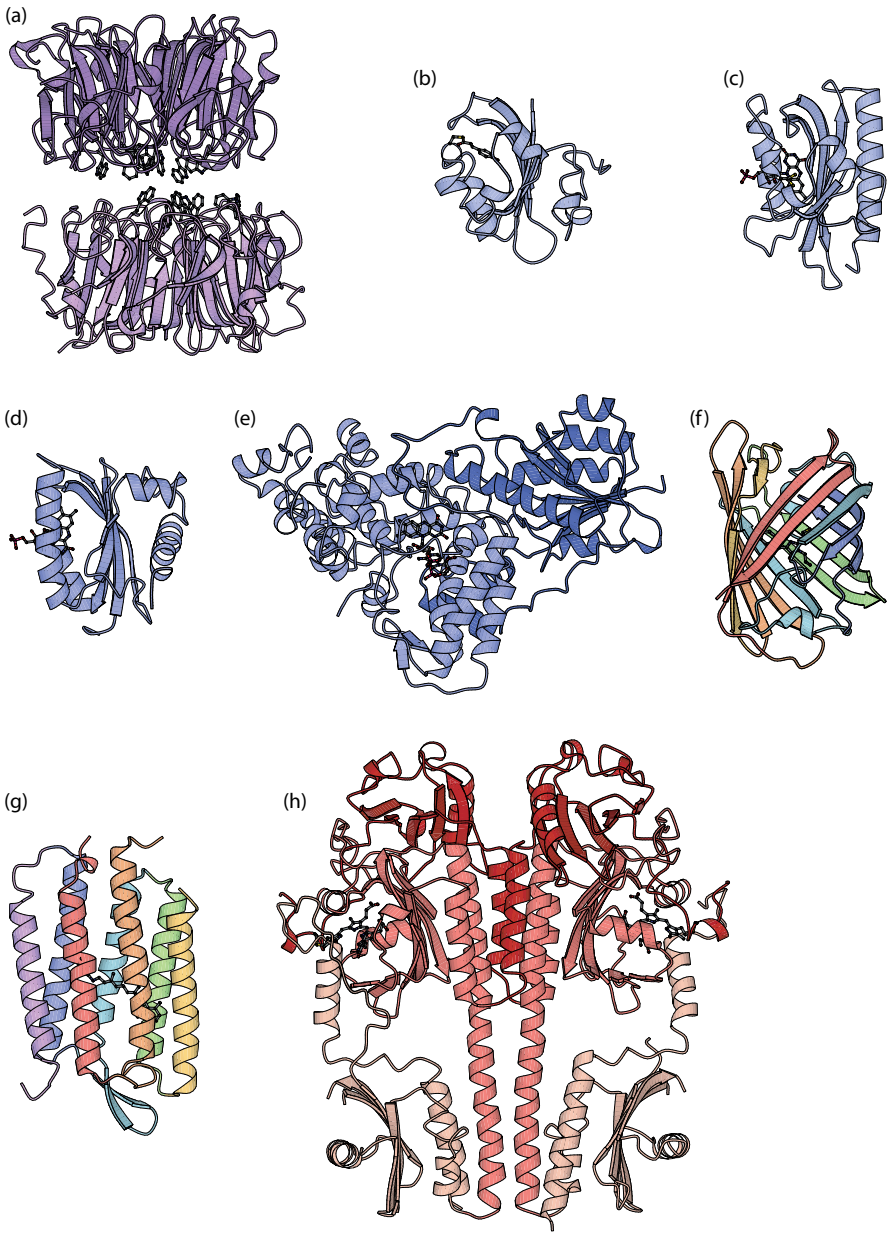

Figure 1.2: Architecture of photoreceptors. Three-dimensional folds of representative members of the different photoreceptor families where the color is meant to indicate which colors of light can be absorbed by a given photoreceptor. (a) *Arabidopsis thaliana* UVR8 (PDB code 4D9S; [7]). (b) *Halorhodospira halophila* photoactive yellow protein (1MWZ; [8]). (c) *Avena sativa* phototropin 1 LOV2 domain (2V0U; [9]). (d) *Rhodobacter sphaeroides* AppA BLUF (2BYC; [10]). (e) *Drosophila melanogaster* cryptochrome (4GU5; [11]). (f) *Echinophyllia sp.* Dronpa (2IE2; [12]). (g) *Halobacterium salinarum* bacteriorhodopsin (1M0L; [13]). (h) *Pseudomonas aeruginosa* bacteriophytochrome (3C2W; [14]).

1.1.1 Novel photoreceptors

It is likely that other classes of photoreceptors remain to be discovered in addition to those noted above. The process for photoreceptor discovery typically originates in identifying a novel, light-dependent process in one, often little studied, organism. Its photochemical action spectrum is obtained, the cell type housing the putative photoreceptor is located, the candidate photoreceptor is purified (often challenging, since its abundance may be very low) and chemically characterized, and its photochemistry *in vitro* matched with that of the biological process *in vivo*. From its protein and gene sequences, related examples in other organisms are quickly found. A recent example is the discovery and characterization of a light-modulated adenylyl cyclase in a marine bacterium [15, 16]. To qualify as an authentic signaling photoreceptor, direct evidence that a particular biological process in that organism is modulated by light absorbed by the candidate photoreceptor must be sought. Sequence similarity is powerful in initial identification but does not substitute for direct demonstration!

Since photons readily traverse membranes, most photoreceptors such as LOV proteins or phytochromes are cytoplasmic, soluble proteins, which allows light to directly regulate an intracellular process. In contrast, rhodopsin-based photoreceptors, *e.g.*, visual rhodopsins, channelrhodopsins or sensory rhodopsins, are integral membrane proteins in which light alters an activity of the protein intrinsic to its location in the membrane, such as its ability to act as a channel or ion pump. Many of the more widely studied chemoreceptors are also integral membrane proteins that respond to extracellular chemical signals which cannot traverse the cell membrane. An interesting question is the extent to which there are parallels in general mechanisms of signal transduction between chemoreceptors and photoreceptors [6].

The key feature of signaling photoreceptors is that absorption of a photon produces a change in a specific biological activity, either directly in the photoreceptor molecule itself, or more usually, in a spatially distant downstream component such as a metabolic enzyme, kinase or transcription factor; light serves as a *specific source of information*. In contrast, light-driven electron transfer processes in photosynthesis generate a change in membrane potential that ultimately drives many biological processes; light serves as a *general source of energy*. Optogenetics is based on genetically encoded, light-dependent control of a biological activity [17]. Thus, we concentrate here on the features of those natural and engineered signaling photoreceptors that exhibit this control.

1.1.2 Biophysics of photoreceptors and signal transduction

Absorption of a photon excites the chromophore to higher electronic and vibrational energy levels; internal conversion on the picosecond time scale rapidly dissipates energy and thus returns the chromophore to the lowest vibrational level of the first

electronically excited state, S_1. Generally, three competing processes can occur from the S_1 state: signal generation, radiative de-excitation (fluorescence) or non-radiative de-excitation (internal conversion). Arguably due to selection pressure during evolution, signaling photoreceptors possess respectably high quantum yields for signal generation in the range of 0.05–0.6 and correspondingly low quantum yields for the competing de-excitation processes. As fluorescence and internal conversion commonly occur on the nanosecond timescale, a high quantum yield for signal generation requires that the initial reaction leading to signal generation occur even faster, *e.g.*, *Z*/*E* bond isomerization in phytochromes occurs on the picosecond timescale [18]. Conversely, a light-sensitive, fluorescent molecule such as GFP has a high quantum yield for fluorescence and a low quantum yield for signal generation. These quantum yields can be purposefully manipulated, which may enable signaling photoreceptors and fluorescent proteins to be interconverted [19]. For example, replacement of a key cysteine residue in LOV domains by alanine abolishes normal photochemistry and signal generation but enhances fluorescence [17, 20]; and site-specific mutagenesis of residues in the chromophore-binding pocket of bacteriophytochromes can enhance their quantum yield for fluorescence in the red / far-red spectral region, in efforts to develop imaging tools applicable to mammalian tissue [19, 21]. Conversely, a naturally highly fluorescent protein such as Dronpa can be modified to serve as a light-dependent signaling molecule while retaining significant fluorescence [22]. Understanding the molecular bases for signaling and fluorescence quantum yields and how they can be manipulated is an active research area [23].

The chemical nature of the chromophore determines its photochemistry, both the wavelengths where it absorbs and the nature of the structural changes it undergoes. For example, a larger spatial extent of electron delocalization (as in the extended, linear tetrapyrrole of bilin chromophores of plant phytochromes and bacteriophytochromes) promotes absorption further in the red (Figure 1.1). Both isomerization (as in retinal, bilins and the *p*-coumaric acid chromophore of PYP) and electron transfer occur extremely rapidly on the femtosecond to picosecond timescale and are usually reversible. In contrast, formation of a covalent bond (as in LOV photoreceptors, between the FMN chromophore and a nearby Cys side chain) is slower, typically on the microsecond timescale, but once formed the bond is often highly stable. Some photoreceptors, *e.g.*, PYP, recover the dark, ground state rapidly in ~1 s via a purely thermal process. In other cases, the signaling state either does not thermally revert to the ground state or does so exceedingly slowly [24], *e.g.*, the covalent bond ruptures and LOV domains revert to the ground state in 10–10000 s [25]. In certain photoreceptor classes, *e.g.*, phytochromes, the ground state can also be recovered in an active, light-driven manner via absorption of a second photon of different wavelength. Together, these photochemical properties control the nature and lifetime of the signaling state.

The control of activity in signaling photoreceptors is normally thought to have its origin in a change in structure but this need not be so. The minimum requirement for

generation and transduction of a signal is that the affinity between two components of the system be sensitive to light. That is, the signal generated by light is essentially thermodynamic in nature. The affinity can be between one part of the photoreceptor and another part of the same photoreceptor, *i.e.*, an intramolecular affinity, in which absorption of a photon leads to a structural change in the photoreceptor itself. Atoms move and/or their dynamics changes. Alternatively, the affinity can be between the photoreceptor and another, non-covalently-bound molecule, *i.e.*, an intermolecular affinity, in which absorption of a photon leads to a change in their dissociation equilibrium constant. Photoreceptors are considered to exist in thermodynamic equilibrium between two states of high biological activity, denoted R, and low biological activity, denoted T (Figure 1.3) [6, 26]. Some of each state R and T is present in both the light and the dark, with light shifting the equilibrium to favor one or the other state. Within this model, the dynamic range of light regulation, *i.e.*, the difference in biological activity between dark and light conditions, is determined by the intrinsic activities of the R and T states, and the equilibrium between them in the dark (K_{dark}) and the light (K_{light}).

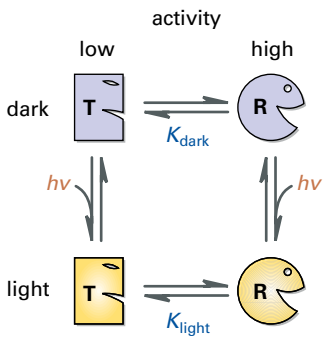

Figure 1.3: Thermodynamics of photoreception. A photoreceptor is assumed to populate at least two functional states, one denoted R of higher biological activity, and one denoted T of lower biological activity. Light absorption alters the relative stabilities of R and T, and thus shifts the equilibrium between them from K_{dark} in the dark to K_{light} in the light. Figure modified after [27].

1.2 Engineering of photoreceptors

For an excellent overview of optogenetics as a whole, see the set of articles in the January 2011 issue of Nature Methods, which designates "Optogenetics" as the "Nature Method of the Year 2010" [28, 29]. The engineering of signaling photoreceptors is reviewed by Möglich and Moffat [27].

Most biological processes are completely insensitive to light. Evidently, evolution has not found a selective advantage in conferring (or retaining) sensitivity to light on

these processes, in any organism yet studied. It follows that if optogenetics is restricted to deploying only natural signaling photoreceptors, the range of processes and biological reactions that can potentially be controlled by light is quite limited. As many articles in this volume attest, huge progress has been made by relying entirely on the light-gated ion cation channel channelrhodopsin [30], its variants and ion pumps, and by restricting the biological processes under study to those in the nervous system, at levels from molecular to the whole organism. However, if we wish to expand the reach of optogenetics beyond its foundations there are two obvious questions. Can any biological process be rendered sensitive to light? And, how should we go about it? We assert that the answer to the first question is "probably yes"; and that one effective answer to the second question is "use a biologically-inspired approach".

1.2.1 Approaches to designing light-regulated biological processes

Consider the overall approach, since that generates some modest confidence in the positive answer to the first question. The goal is to confer regulation on at least one step of a biological process by a novel stimulus, light. In principle, regulation may be achieved by either a biological approach or a chemical approach. We choose the former, biological approach for three main reasons. First, the broad usefulness as a tool of the archetypal, genetically-encoded, light-sensitive protein, GFP, and its variants gives confidence that genetic encoding of a protein "tag" to control activity is both feasible and will have desirable properties. Second, the ability to base designs on the structure and properties of natural signaling photoreceptors, rather than having to design the system from the ground up, greatly aids the design process. Third, the longer-range goal of using the engineered photoreceptors *in vivo* requires that they be cleanly available in specific cells and tissues under controlled conditions. Again modeling the approach on GFP, this goal is readily achieved by cell-specific expression. Fluorescent proteins such as GFP are covalently fused to the N- or C-terminus of the target protein to form an imaging tag, and the fusion must not interfere with the localization or properties of the target molecule. As discussed below, many engineered signaling photoreceptors are also based on fusion, which in contrast to GFP fusion is deliberately designed to control a fundamental property of the target, its biological activity. Design of signaling photoreceptors thus extrapolates from a large base of successful experience with fluorescent proteins. *De novo* protein design is advancing [31], but is not yet at the stage where a new protein with desired properties – in the present case, sensitivity of a specified biological activity to light – could be created from first principles. However, design that modifies a property of an existing protein, *e.g.*, replacing its regulation by oxygen with regulation by blue light [32], is indeed feasible. *In vivo* studies require the delivery problem to be effectively addressed. Genetic encoding and controlled expression or degradation of an engineered photoreceptor in a specific cell type is often readily achieved, thus enabling spatiotemporal control

of the photoreceptor's concentration. Chromophore incorporation is often spontaneous or aided by co-expression of enzymes that synthesize the chromophore, *e.g.*, heme oxygenase catalyzes the production of bilins from readily bioavailable heme.

The chemical approach involving the synthesis, delivery and light-dependent activation or inactivation of small molecules with the desired properties (an approach known as optochemical genetics [33]) is discussed in Chapter 3 of this book.

Regulation in response to stimuli is universal in biology and offers enormous diversity. If no general principles existed, then attempts to engineer or design regulation by light in an intelligent manner would have to be approached on a protein-by-protein basis. Such an approach would necessarily be on a large scale: synthesize a very large library of variants of the protein whose activity is to be regulated by light; screen this library to identify that very small fraction of variants whose activity exhibits, even in a limited way, regulation by light; then seek to improve this regulation by rounds of mutagenesis while retaining the desired photochemistry and overall activity. Since the size of any candidate library is astronomically large, some rational approach to the synthesis of this initial library is still called for. Fortunately, some principles of regulation by light are emerging from study of the sequence, structure and properties of natural signaling photoreceptors (Text Box 1.1), which provide the basis for a rational approach. However, we caution that the scope of the systems to which these principles apply remains to be established.

Text Box 1.1: Design principles of natural signaling photoreceptors. (Adapted from Table 2 of [27]. See also [38].)

1 **Modular architecture:** sensor and effector functions are located in different proteins or protein domains (exceptions: certain rhodopsins, cryptochromes, the UVR8 photoreceptor).

2 **Domain fusion:** evolution of modular photoreceptors appears to have occurred by domain fusion that links photosensor and effector domains. Fusion may also integrate multiple photosensor domains, *e.g.*, in *Rhodospirillum centenum* Ppr [34] and plant phototropins [35], or chemosensor domains [36]; or add "interaction" domains, *e.g.*, PAS domains in plant phytochromes; or add downstream regulatory domains, *e.g.*, the receiver domain in certain bacteriophytochromes [18].

3 **Domain arrangement:** covalent linkage of sensor and effector domains usually places the sensor N-terminal to the effector, occasionally C-terminal, but never inserted into the effector.

4 **Mix and match:** a single type of sensor domain is found linked to many types of effector domains; and conversely, a single effector type is found linked to many types of sensor domains.

5 **Helical linkers:** linkers between domains play important roles in signal transduction and are often α-helical. These helices have been designated "signaling helices" [37] and are usually found at the N- and C-termini of sensor domains. Examples: bacteriophytochromes, PYP, and LOV and BLUF domains.

Most of these principles derive from examination of gene or protein sequences. However, the existence and nature of compact input / sensor domains that contain the chromophore, linkers between domains that are often α-helical in structure and

distinct output / effector domains that contain the activity are derived largely from X-ray crystallographic studies. Principles 1, 2, and 3 establish domain fusion; principle 4 suggests that three-dimensional, structure-specific interactions between sensor and effector domains are uncommon; and principle 5 suggests that the linkers which keep the sensor and effector domains from direct contact and transmit the signal from the chromophore in the sensor domain to the active site in the effector domain are α-helical. On the relevant length scale of a few nanometers to a few tens of nanometers, stable α-helices are essentially rigid rods [39] that can readily transmit longitudinal, transverse or rotational signals input at one end. However, we have only limited knowledge of the structures of intact, full-length signaling photoreceptors in either their "dark" or "light" forms and our knowledge of the structures of representative effector domains in both "active" and "inactive" forms is limited. Hence, our understanding of the molecular details of signal transduction from photosensor to effector remains modest. Moreover, we caution that the existence of α–helical linkers is largely based on sequences and structures of prokaryotic provenance. Proteins fold and refold, *i.e.*, change their structure in response to stimuli in the same general manner in proteins from all organisms, but exactly how refolding is deployed in signal transduction may well significantly differ in prokaryotic and eukaryotic systems. For example, LOV domains were originally discovered in phototropin in higher plants where they confer blue-light-dependent control of a serine / threonine kinase, and also form the sensor component of bacterial two-component systems where they control a histidine kinase that is entirely different in structure [40]. Control by a single class of sensor domain may be exercised in very different ways.

But there must be more to it than that; caution is called for. In at least two examples of engineered, LOV-based photoreceptors, a variant of the Trp repressor denoted LOV-TAP [26] and a variant of the small GTPase denoted PA-Rac1 [41], two quite different molecular mechanisms of sensitivity to light appear to hold: mutually exclusive folding of the sensor and effector domains in LOV-TAP, and direct inhibition of substrate binding to the effector domain by the sensor domain via steric interference in PA-Rac1. Both approaches employ the identical LOV domain yet rely on different mechanisms, which strikingly illustrates that structural and dynamic changes induced by light absorption in the photosensor can be tapped in different and ingenious ways to accomplish regulation of effector activity. Although both examples successfully confer regulation by light, a difficulty is that they are based on the specific properties of the system and may not be readily extended to other systems. A potentially more general approach is based on the fact that biological activity is often modulated by assembly of multimers, or by intracellular spatial localization [38]. For example, a monomer may be inactive and its homodimer, active; or activity may require localization and binding of a desired protein to a membrane; or in the classical example of signal transduction, the ligand-binding activity of tetrameric hemoglobin is quite different from that of its constituent, monomeric α- and β-chains. If the dissociation equilibrium constant between the components can be modulated by light, then regulation

by light is directly achieved. Signaling photoreceptors are often dimeric and exhibit a light-dependent monomer-dimer equilibrium that can be exploited to modulate the activity of an attached effector [42, 43, 44, 45, 46, 47]. Design strategies relying on light-mediated protein association appear particularly versatile: the exact sequence and properties of the linker between photosensor and effector are not as critical and extensive screening of linker variants is often not necessary. Strikingly, recent studies have shown that certain variants of the fluorescent protein Dronpa also undergo light-dependent association. This property can be used to produce light-dependent association with the membrane, or to "cage" the activity of proteins such as Cdc42 inserted into the linker between Dronpa monomers [22]. As Dronpa undergoes autocatalytic maturation to its fluorescent form, no endogenous or exogenous chromophore is required; moreover, Dronpa retains its fluorescence characteristics and therefore offers both imaging / localization and control of activity within one molecule.

Biologists seek simple and versatile approaches that can control the activity of many different proteins, and do not require extensive protein engineering different for every target. Recent attempts to target new proteins with the approaches described above indicate that each will have its place, and it remains to be seen whether one method will be more versatile than another. It is appealing to think that controlling membrane translocation via light-induced heterodimerization will be simple, but extension to other membrane proteins has not always proven straightforward. Protein geometries at the membrane affect both heterodimerization and subsequent biological interactions, with sometimes competing requirements. By contrast, use of light-induced heterodimerization has proven relatively straightforward even when both dimerizing proteins are incorporated into a single chain fused to the target protein, as in the applications of Dronpa described above. In the case of PA-Rac1, substantial engineering of linkers was required, but once this had been accomplished there was hope that the design could be readily applied to the many other similar GTPases. Unfortunately, for this particular protein family, adventitious LOV-GTPase interactions played an important role in PA-Rac1, making it difficult to generalize [48]. However, this need not be true for other protein families. Single-chain designs will likely have an important role simply because of their more compact nature and because many important proteins cannot be controlled by translocation to the membrane or by intermolecular dimerization. Recent studies also indicate that engineered domains inserted in conserved sites in protein families will be able to provide allosteric regulation of active sites [49], in contrast to the distribution of sensor domains in natural photoreceptors noted above.

An important aspect of protein control involves the interplay between the endogenous concentration of the targeted protein, and the susceptibility of different signaling pathways to changes in the concentration of a specific active species. As discussed above, photoreceptors exist in equilibrium between low-activity (T) and high-activity (R) states (*cf*. Figure 1.3), and the relatively small amount of active species R present even in the dark can have profound biological effects. This will depend on the expression level of the photo-modulated construct relative to endogenous material, and the

sensitivity of the signaling network to the active species. It can be challenging to find a "sweet spot" where the photoreceptor is expressed at an appropriate level, generating an inconsequential amount of active species in the dark, but producing sufficient active material to generate a phenotype in the light. The differences in the lit and dark equilibria, K_{dark} and K_{light}, will be a very important characteristic as new tools are built for sensitive and/or low-abundance pathways.

Different photo-responsive domains will have complementary advantages and disadvantages. For example, the phytochromes / PIF system provides the ultimate in spatial and temporal control, as different wavelengths are used to turn the system on and off [42, 44]. In single cells, precise boundaries between active and inactive species can be produced, with light of a different color on each side. However, in animals, where light diffracts through deep tissue, it may be more practical to use systems based on LOV, cryptochromes or Dronpa, which can be controlled simply by turning the light on and off. In particular, photoreceptors that use naturally occurring chromophores, *e.g.*, flavin nucleotides, will be easier to use. The slow photocycle kinetics of the LOV system, which to considerable extent can be tuned by mutagenesis [50], limits the maximally attainable time resolution. Here, the phytochrome system provides important advantages when studying precise kinetic control.

1.3 Case study – transcriptional control in cells by light

As discussed above, diverse plant and microbial species employ light-regulated dimerization domains to control protein interactions. Several of these systems have been employed to achieve light-regulated interactions of target proteins, *e.g.*, on the basis of phytochrome [42, 44] and cryptochrome [45, 51] domains. Common to all systems, the modular architecture of photoreceptors is exploited such that the light-sensitive photosensor domains are decoupled from their natural effector domains and connected to a different effector domain of interest [42, 44, 45, 52, 53]. In particular, light-regulated protein dimerization enables the generation of light-inducible two-hybrid transcription factors for control of gene expression inside living cells. In these systems, light is used to trigger the recruitment of a transcription effector domain to a DNA-binding domain (*e.g.*, the DNA binding domain from Gal4). These systems have been applied in a variety of cell types including yeast [42] and mammalian cells [44, 45]. While both phytochrome and cryptochrome domains have been successfully engineered as light-sensitive transcription factors, it is worth noting that phytochrome-based solutions require the addition of exogenous phycocyanobilin chromophores in order to function in non-plant cells. Both phytochrome and cryptochrome system are able to mediate reversible transcription modulation.

Beyond the currently realized approaches, combining phytochrome and cryptochrome dimerization domains with programmable DNA-binding proteins such as zinc finger proteins (ZFPs) [54] and transcription activator-like effectors (TALEs) [55, 56]

has the potential to enable modulation of transcription at the level of the endogenous genome (Figure 1.4) [57]. Of decisive advantage, ZFPs and TALEs can be customized to target the promoter region of specific genes or other unique locations in eukaryotic genomes. A variety of transcriptional effectors (*e.g.*, activators, repressors, and chromatin modifying enzymes) can be fused to the interacting proteins of cryptochromes and phytochromes and recruited to specific loci in the genome in a light-dependent fashion. These systems extend the capability of the conventional optogenetics toolbox by allowing researchers to perturb gene expression in addition to manipulating cellular electrical and biochemical activities.

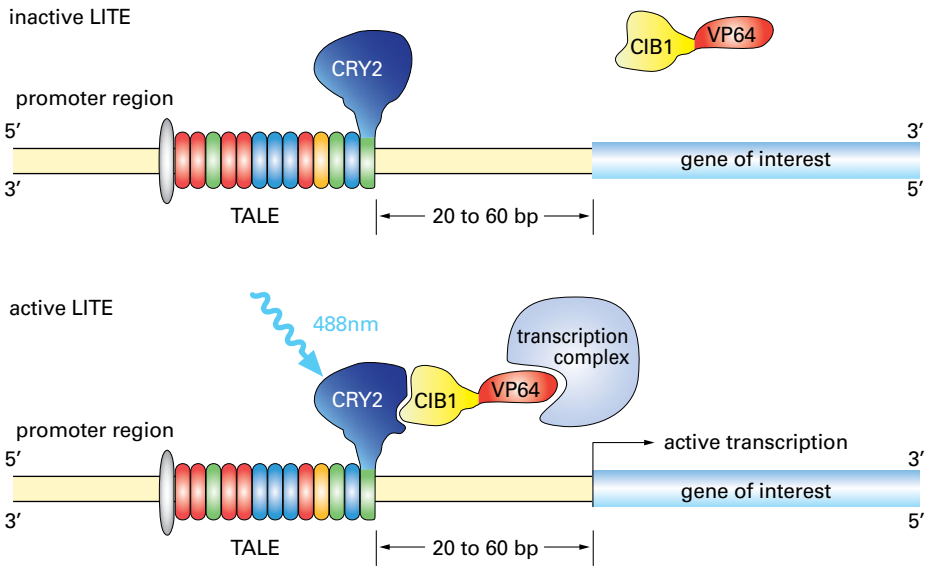

Figure 1.4: Optogenetic control over transcription. In the LITE system, a cryptochrome photosensor (Cry2) is directed to an arbitrary target DNA locus via conjugation to sequence-specific TALE proteins [57]. Upon blue-light-absorption, Cry2 reversibly associates with its effector CIB1. Via ligation to CIB1, the strong transcriptional activator VP64 is recruited to desired DNA loci and can thus turn on the expression of arbitrary genes of interest in light-controlled fashion.

1.4 Conclusion

Decades of research into the photochemical, structural and mechanistic characteristics of natural signaling photoreceptors have now provided protein engineers and biologists with a broad array of building blocks, and many more remain to be discovered. The engineering of photoreceptors is very much in its infancy. In the spirit of provoking discussion and with any luck, suggesting experimental areas to explore,

we end by posing a number of open challenges to biophysicists and biochemists (Text Box 1.2). We emphasize, however, that the utility of optogenetic approaches is likely to be largely *in vivo*, which places particular emphasis on challenge 5 below. It will be exciting to see what creative and completely unexpected designs will belie any predictions we can make today. Will we – or when will we – reach the biomedical stage of "Take a photon, not a pill!" [27]?

Text Box 1.2: Open challenges in the engineering of photoreceptors.

1 **Signal transduction mechanisms**. How general are mechanisms of light-dependent signal transduction at the molecular level? Are there only a small number, say <10, of truly distinct mechanisms? If there are in fact a very large number, what approaches are likely to be most effective in the design and engineering of photoreceptors?

2 **Design strategies**. Should the basis for engineered photoreceptors be based on light-regulated protein association, direct contacts between the photosensor and effector (as in the PA-Rac1 system, or engineered allosteric regulation? How will different designs meet the challenges of controlling diverse signaling pathways? Can we develop universal designs that are suitable for proteins with activity that is cytosolic, rather than restricted to the membrane? How do different protein families and structural types lend themselves to different designs? To what extent can design approaches successfully applied to one system be "recycled" to other systems? Can we rationally design photoreceptors at the drawing board with a high (ideally 100 %) success rate? Is there a need for selecting engineered photoreceptors or optimizing their properties by screening strategies, *e.g.*, by directed evolution? What role can rapidly maturing, computational protein-engineering methods play at the design stage?

3 **Selection of photosensors and effectors**. Given a biological question one wishes to address by optogenetics, how should the most suitable sensor and effector modules be identified? If an activity is associated with an effector domain of known structure, what biophysical and biological factors bear on selection of a photosensor domain? Addressing this selection is intimately linked to the choice of design rationale, *e.g.*, a strategy based on intermolecular dimerization; steric interference via conformational changes or intramolecular dimerization; allosteric control; or other approaches.

4 **Engineering/Fine-tuning of photoreceptors.** Can natural sensor domains readily be further engineered, in particular with regard to eventual biological applications? One goal would be to modify their photochemistry, *e.g.*, their extinction coefficients, absorption spectra, quantum yield, dynamic range, suitability for two-photon excitation, rate of reversion to the ground state or reversibility (on/off kinetics). Another goal would be to modify the protein itself, *e.g.*, its solubility, efficiency of expression in the desired cell type or tissue, lack of immunogenicity and toxicity, robustness, or propensity for specific degradation. Can we adjust the light-dependent equilibria between active and inactive photoreceptor forms such that light absorption produces a large difference in their relative concentrations and a high dynamic range of light regulation is achieved (*cf.* Figure 1.3)? Yet another goal would be to modify the chromophore, *e.g.*, to alter substituents on the pyrrole rings of bilin in bacteriophytochromes, or to restrict conformational changes between adjacent rings.

5 **Optogenetic deployment.** Engineered photoreceptors are intended to be expressed in an active (and photoactive) state in a particular cell type in a specified tissue and organism. How should this be achieved? Is the chromophore readily bioavailable in many or all tissue types, *e.g.*, FMN, or can it be supplied by enhanced, endogenous synthesis, *e.g.*, bilins, or must it be supplied

exogenously, *e.g.*, the *p*-coumaric acid chromophore of PYP? How can the biochemistry and biophysics of purified photoreceptors be related to their *in vivo* behavior?

6 **Do non-proteinaceous photoreceptors exist?** All known natural photoreceptors are protein-based. Can optogenetic approaches be devised to render the properties of biomolecules other than polypeptides light-sensitive? There appears to be no chemical reason why, for example, an RNA-based, light-dependent riboswitch could not be engineered, or a light-sensitive RNA aptamer be selected. These might offer effective control of RNA-based biological processes.

Acknowledgements

We acknowledge participants of the Dahlem conference for stimulating discussions, much of which we have tried to incorporate into this article. In particular, we thank Franziska Schneider for providing her conference notes.

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2 Current challenges in optogenetics

2.1 Introduction

Studying intact systems with simultaneous local precision and global scope is a fundamental challenge in biology. Part of a solution may arise from optogenetics: the combination of genetic and optical methods to achieve gain- or loss-of-function of temporally-defined events in specific cells within intact living tissue or organisms. Such precise causal control within the functioning intact system can be achieved via introduction of genes that confer to cells both light-detection capability and specific effector function. For example, microbial opsin genes can be expressed in neurons to mediate millisecond-precision and reliable control of action potential firing in response to light pulses [1, 2, 3], and indeed this approach has now been used to control neuronal activity in a wide range of animals and systems resulting in insights into fundamental aspects of physiology, as well as insights into dysfunction and possible treatments for pathological states [4]. Many other strategies for optical control (besides the microbial opsin gene approach) may be applied as well [5, 6, 7, 8]. Yet despite the diversity of approaches, rapid growth of the field, and wide scope of applications, fundamental challenges remain to be addressed in the basic technology development of optogenetics. Here we review these challenges, as well as the opportunities at hand; aspects of the figures and text here are from recent reviews [1, 4, 9, 10].

2.2 Background: current functionality of tools

Diverse and elegant mechanisms have evolved to enable organisms to harvest light for survival functions (Figure 2.1). For example, opsin genes encode 7-transmembrane (TM) proteins that (when bound to the small organic chromophore all-*trans* retinal) constitute light-sensitive rhodopsins, which are found across all kingdoms of life. Many prokaryotes employ these proteins to control proton gradients and to maintain membrane potential and ionic homeostasis and many motile microorganisms have evolved opsin-based photoreceptors to modulate flagellar motors and thereby direct phototaxis toward environments with optimal light intensities for photosynthesis. Owing to their structural simplicity (both light-sensing and effector domains are encoded within a single gene) and fast kinetics, microbial rhodopsins can be treated as precise and modular photosensitization components for introduction into non-light sensitive cells to enable rapid optical control of specific cellular processes [1]. Alternatively, the light receptor can be a small organic molecule that is introduced into the biological system, with or without a designed binding protein as effector and

many other non-opsin classes of naturally-occurring protein have been explored as well, including flavin chromophore-utilizing light-activated enzymes such as adenylyl cyclases, as well as engineered systems in which light-sensation modules become physically linked to effector modules [5, 6, 7, 8].

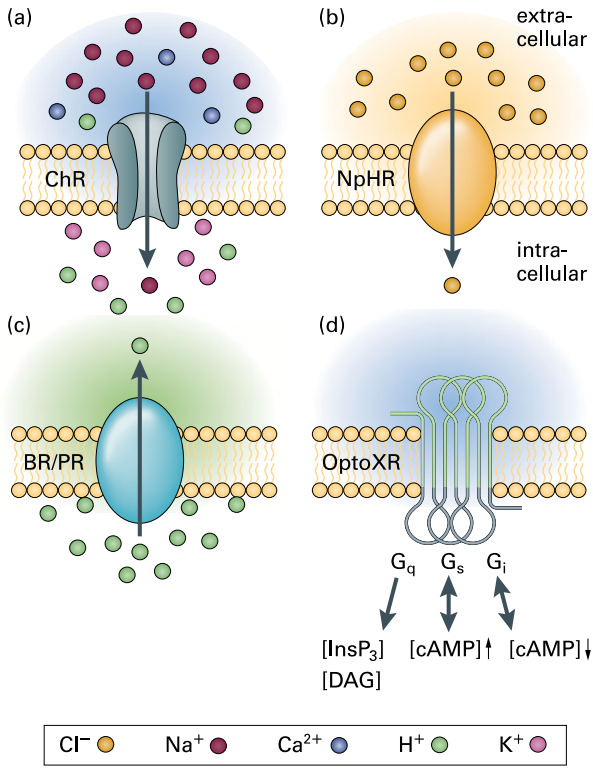


Figure 2.1: Single-component optogenetic tool categories. Four major classes of opsin commonly used in optogenetics experiments, each encompassing light sensation and effector function within a single gene, include: (a) channelrhodopsins (ChR), which are light-activated cation channels that give rise to inward (excitatory) currents under physiological conditions; (b) halorhodopsins (NpHR shown), which are inhibitory (outward-current) chloride pumps; (c) bacteriorhodopsins and proteorhodopsins (BR/PR), proton pumps that tend to be inhibitory and include archaerhodopsins; and (d) optoXRs, which modulate secondary messenger signaling pathways. Adapted from Zalocusky and Deisseroth [9].

The experimental potential of optogenetics has triggered a surge of genome prospecting and molecular engineering to expand the repertoire of tools and generate new functionality, in turn catalyzing further mechanistic studies of microbial proteins [10]. High-resolution crystal structures are now available for most of the major photoreceptor modules, most recently including channelrhodopsin (ChR) [11]; this infor-

mation has been important not only for enhancing understanding of the biophysical mechanism, but also for guiding optogenetics in the generation of variants with novel function related to spectrum, selectivity, and kinetics. For example, ChR variants have been engineered with shorter or longer open-state lifetimes, shifted absorption spectra, reduced desensitization, increased expression, and increased photocurrent magnitude [1, 12]. Likewise, high-resolution crystal-structural insights have been used to help guide the assembly of light-sensitive modules together with effector modules into artificial proteins, thereby creating parallel information-streams capable of carrying optogenetic control signals for modulation purposes [5].

This diversity of optogenetic tool function will be important for making significant headway in our understanding both of normal brain function and of dysfunctional processes in neuropsychiatric disease (for example, many disease states may relate to impaired interaction of multiple distinct cell- or projection-types, pointing to the experimental value of achieving multiple-color excitation and multiple-color inhibition optogenetically within the same living mammalian brain for neuropsychiatry research [4, 9]). It is encouraging that optogenetic interventions have now provided a precise foothold for the study of both normal function and brain disease states. However, major areas of optogenetic tool advancement are nevertheless required in the future, as detailed next.

2.3 Unsolved problems and open questions: technology from cell biology, optics, and behavior

One group of technological challenges to be addressed in optogenetics lies within the natural domain of metazoan biology. First, development of guided subcellular trafficking will be important. Membrane trafficking strategies have already improved expression of opsins at the membrane [13], but further exploration in this area may produce targeting strategies that allow selective optogenetic tool expression in subcellular compartments such as dendrites, somata or axon terminals. Indeed, while efforts have been made in this regard, achieving truly robust (near 100 %) exclusion of heterologously expressed optogenetic proteins from axons would prevent undesired optical drive of axons of passage during illumination of a transduced brain region. While the expression of optogenetic tools in axons is one of the most useful features of this approach in allowing “projection-targeting”-based recruitment of cells defined only by selective illumination and projection pattern [1, 4, 9], this effect also confounds certain kinds of functional mapping procedures that employ optogenetics.

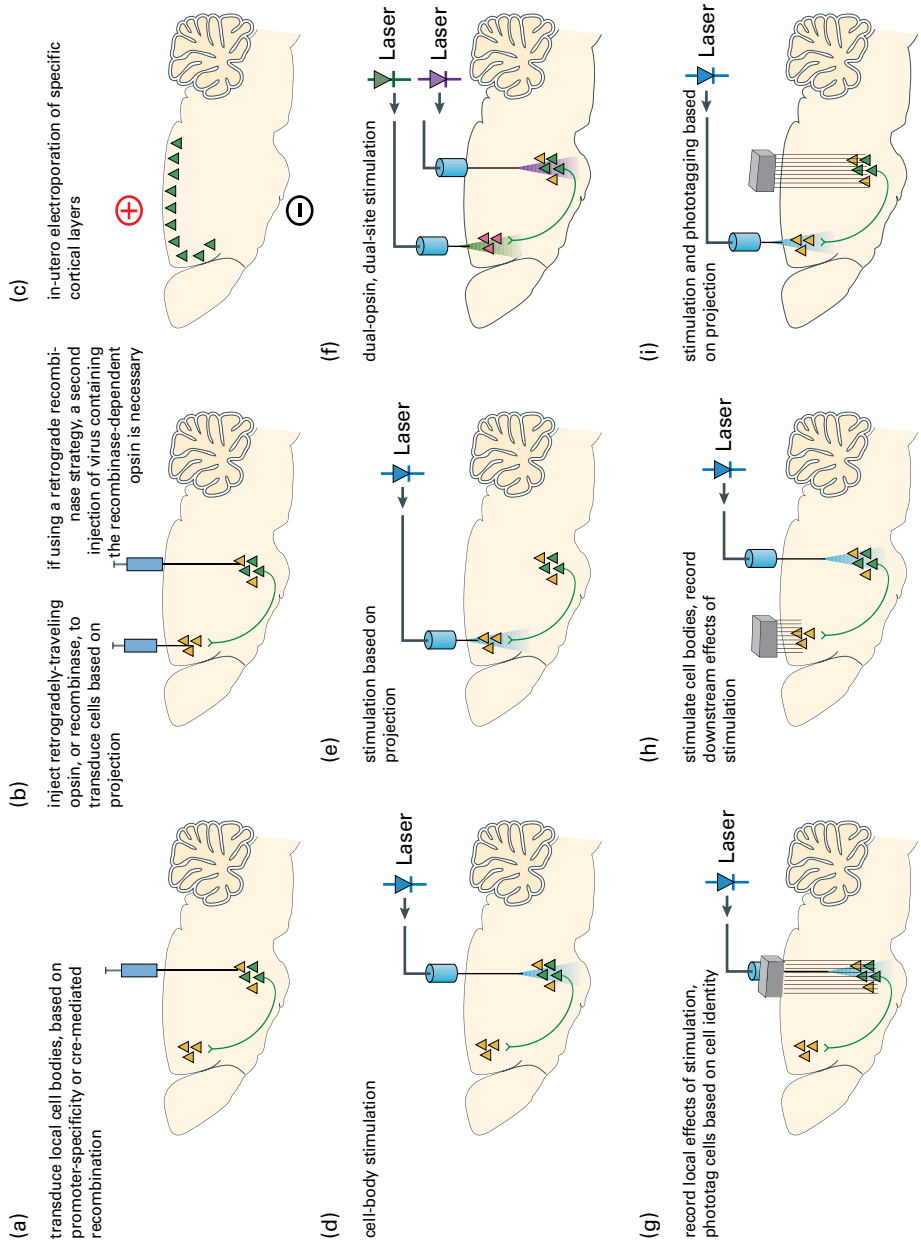
Second, it would be valuable to develop a robust and versatile optical (non-pharmacological) strategy to (when desired) prevent the propagation of optogenetically elicited action potentials in the antidromic direction or along axon collaterals during projection targeting experiments. Again, sometimes this antidromic drive is desired, but in other cases it is not (wherein the experimenter seeks to allow only general-

izable, selective excitation of spatially-defined projections, and wishes to not take advantage of the existing capability to recruit cells defined by projection [1]).

Third, improved high-speed volumetric (3D) light delivery strategies with single-cell resolution would be of great value, so that populations of cells even within intact mammalian brain tissue could be recruited optogenetically with any required extent of synchrony or asynchrony. For example, optogenetics applications *in vivo* to questions in mammalian circuit dynamics and behavior have typically involved synchronous optogenetic control of entire genetically targeted cell populations over millimeter-scale spatial domains; for example, in studies of sleep-wake transitions, Parkinsonian circuitry, gamma rhythms, feeding behavior, olfaction, aggression, and memory consolidation. Yet methods for guiding spatial delivery of multiple wavelengths of light excitation in 3D volumes could allow much improved precision and complexity in optogenetic modulation, taking the next step beyond the single-photon guided-light strategies which have already been used even in mammalian tissue for applications such as highly refined optogenetic circuit mapping and dissection of anxiety circuitry. Optogenetic two-photon illumination could provide a distinct means to manipulate single or multiple genetically and spatially targeted cells with high temporal resolution over sustained intervals and within intact tissue volumes, in order to delineate and define components that work in concert to generate circuit dynamics or behavior. One pioneering two-photon study was able to overcome the low single channel conductance of ChR2 and produce action potentials in cultured neurons using complex scan patterns in order to open sufficient channels on individual neurons. Two-photon optogenetic manipulation of spatially and genetically defined cells within intact tissue volumes with simpler (standard) raster scanning would further broaden the reach of this approach to many laboratories worldwide. Two other reports of neuron activation in slice preparations with optogenetics relied on elegant hardware innovations and larger focal spots of laser illumination to overcome the modest conductance of individual channels. Other non-scanning methods such as SLM and light field microscopy could allow myriad opportunities to probe the temporal mechanisms by which population codes are set up and employed in neural

Figure 2.2: Optogenetic targeting and experimental design suitable for any vertebrate species including mouse, rat, and primate. Panels (a)–(c) illustrate strategies for transducing the cell population of interest with an opsin. These include (a) transduction of cell bodies via viral injection, (b) single or dual-virus retrograde strategies for projection-specific opsin expression, and (c) in-utero electroporation for cortical layer specific expression. Panels (d)–(f) illustrate possible configurations for optical stimulation, including (d) illumination at the site of transduced cell bodies, (e) illumination of downstream projections, (f) illuminating multiple distinct populations of cells at the same or different locations, which can express opsins sensitive to different wavelengths of light. Panels (g)–(i) illustrate combinations of electrical recording with optical stimulation. Possible configurations include: (g) recording at the site of optical stimulation, (h) recording downstream of optical stimulation, (i) recording at transduced cell bodies, while stimulating downstream projections. Adapted from Fenno and Deisseroth [4] and Zalocusky and Deisseroth [9].

circuit function. Two recent reports have made headway in developing two-photon raster scanning and SLM based methods for versatile optogenetic control in intact tissue or *in vivo* in mammals [14, 15].



Fourth, robust extension of optogenetic tool-targeting strategies to non–genetically tractable species or cell types will be enormously helpful. The generation of Cre-driver rats has been important, and projection-targeting provides an independent step forward. But improved intersectional targeting strategies will also be crucial since few relevant cell types can be specified by only a single descriptor such as cell body location, projection target, or activity of one promoter / enhancer region. Design and validation of optogenetic tool-carrying viruses or other vectors that depend on multiple recombinases (for example, with Boolean AND or other logical gates) will be essential, and improved methods to selectively *exclude* optogenetic tool expression in cells with a given genetic identity will also be useful. Finally, true retrograde and anterograde wiring-based strategies (*i.e.*, targeting cells that project to a particular region, or cells that receive projections from a particular region) would greatly enhance the flexibility of optogenetic control, both in mice and in other species; such strategies exist but are not always robust or well-tolerated [4, 9] (Figure 2.2).

Fifth, it would be immensely valuable to develop methods to rapidly and efficiently extract brainwide wiring (connectomic) patterns, or at least projection patterns, from optogenetically driven cells that had been shown to have a known and quantified impact on behavior in the very same animal. And sixth, it would be of great value to rapidly and efficiently extract the brainwide elicited-activity patterns arising from optogenetic control of a targeted population. This can be achieved to some extent with ofMRI (optogenetic functional magnetic resonance imaging), an optogenetic method that enables unbiased global assessment of the neural circuits upstream and downstream of focal stimulation. However, fMRI methods generally suffer from poor spatial and temporal resolution. In general, improved integration of optogenetic control with readouts will be important – whether behavioral, electrophysiological, or imaging. Moreover, closing the loop so that neural activity or behavioral readouts can feed back and control the inputs played in via optogenetics will be of great interest – as will the development of computational methods to begin "reverse engineering" the studied circuitry by identifying the underlying transformations of information carried out in the tissue.

Addressing the above technological challenges, all squarely in the domain of modern neuroscience, will help provide experimental leverage that may lead to key insights into neural circuit function and dysfunction, and that would be difficult or impossible to establish by other means.

2.4 Unsolved problems and open questions: genomics and biophysics

Another group of technological challenges to be addressed in optogenetics falls more into the natural domain of microbial biologists and protein biophysicists (although of course many laboratories and investigators span the metazoan and the microbial realms).

First, the ongoing identification of additional genomically identified tools (via searching databases, broad-based next-generation sequencing efforts, and ecological genome mining) will profoundly improve our ability to perturb and understand biological systems [10, 12]. Many thousands of new light-sensitive modules will be accessible in this way. For example, even though known opsins already span most of the visual spectrum and a very broad kinetic space, it is very likely that new kinds of light sensitivity, kinetic properties, and even ion selectivity will emerge. One important goal is moving into the infrared, which will achieve 1) deeper light-penetration at a given irradiance value; 2) reduced scattering for improved resolution; and 3) provision of an additional control channel. Infrared actuation has already been achieved for certain non-opsin-based optogenetic approaches but may encounter physics-based limitations for retinal-based photoreceptors.

Second, engineering of these known or new tools for narrowed (as well as shifted) action spectrum would enable more clean separation of control channels. For example, engineering of blue-shifted hyperpolarizing opsins with narrower activation wavelength spectra could ultimately allow for enhanced combinatorial neuronal inhibition experiments within scattering mammalian tissue volumes. While action spectrum peaks for existing tools span the visible spectrum and beyond, the broad shoulders of relevant action spectra may prevent use of more than 2–3 channels of control at once, unless spectra can be narrowed. Such efforts might involve mutations that prevent access of the photocycle to specific states or intermediates that have shifted absorbance properties. This class of engineering will be facilitated by structure-based insights into photocycles; for example, to understand the ChR photocycle in more detail, further studies beyond the current closed-state structure [11] (Figure 2.3), including that of open and intermediate photocycle states, are clearly needed. These efforts may also lead to the generation of mutants with novel kinetic properties [16].

Third, engineering the light-sensors of optogenetics for higher quantum efficiency, greater light sensitivity, and/or increased biological effect (*e.g.*, current) elicited per optogenetic-protein molecule, would be of substantial value in allowing the use of lower irradiances for targeting a given tissue volume or depth, which may be important in minimizing photodamage, heating, or power use / deposition constraints [1, 12, 17]. While for opsins, many orders-of-magnitude increased light-sensitivity can be achieved with the bistable or step-function approach, this comes at a kinetic cost (slowing down the deactivation after light-off [16]).

Fourth, developing a potent electrically-inhibitory optogenetic channel (rather than a pump) would be of immense value. Current hyperpolarizing tools are pumps rather than channels, and therefore do not provide shunting or input resistance changes (and also can only move one ion per photon); as a result these optogenetic tools are not nearly as effective as the channels or native inhibitory receptors, especially in projection-targeting experiments wherein the goal is to intercept action potentials in axons. Achieving this goal would also rapidly enable the generation of

a hyperpolarizing SFO or bistable optogenetic tool [1, 4, 16] that would allow sustained inhibition of neurons without requiring constant illumination. New structural knowledge of the ChR cation-conducting pathway and pore vestibules may facilitate construction of ChR variants with potassium selectivity for this purpose, as well as improved photocurrents, light-sensitivity, and kinetic properties [11].

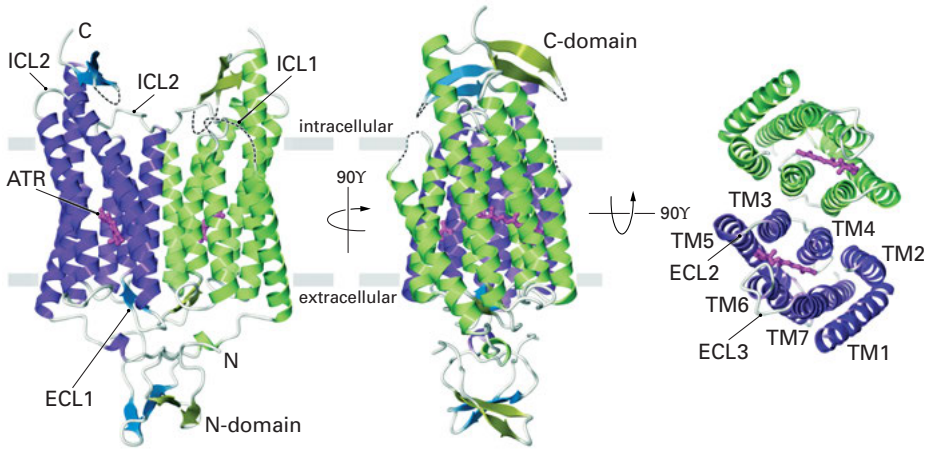


Figure 2.3: Channelrhodopsin crystal structure. This structure is of C1C2, a chimera between ChR1 and ChR2, consisting of the N-domain, the seven transmembrane helices (TM) connected by extracellular loops (ECL) and intracellular loops (ICL), and a truncated C domain. All-*trans*-retinal (ATR) is colored pink. This high-resolution structure [11] includes description of the environment around the retinal-binding pocket, which will enable optimized design of red- and blue-shifted ChR variants. In addition, structure of the cation-conducting pathway may facilitate construction of ChR variants with improved photocurrents, photosensitivity, cation selectivity, and kinetics. Already, structure-guided mutagenesis has resulted in some degree of K^+ selectivity, which could be useful to suppress neural activity. Further structural studies, including determination of crystal structures in intermediate states, are clearly needed which will help enable the principled design of ChR variants with new properties, and which in turn will accelerate both applications of optogenetics to intact-systems biology, and basic mechanistic understanding of these remarkable photoreceptor proteins. Adapted with permission from Kato *et al.* [11].

Fifth, we have cautioned that powerful and prolonged light delivery can cause heating effects that could, in principle, alter neural activity even in non-expressing cells, and we have provided quantitative estimates of the magnitude of this effect [1]. This potential caveat can be addressed by maintaining moderate-intensity or pulsed-light protocols, and by including experimental cohorts in which no opsin is expressed but all other manipulations are performed in the target animals, including (if relevant) surgery, viral transduction, hardware implantation, and light delivery [1]. Similar controls are useful for identifying and/or correcting for confounds linked to any perception of the light by the animal's sensory systems. Moreover, overexpres-

sion of any foreign protein could cause altered structure, function, or survival of host cells, and opsins provide no exception to this rule. However, optogenetic methods do intrinsically provide a powerful means to control for such effects by allowing light-on and light-off assessment of physiology or behavior in each experimental subject to ensure normal baseline behavior in the same animal at virtually the same time, and overexpression of control proteins in parallel experimental animals allows the experimenter to ensure that light effects are not observed only because the animal or tissue is in an unusual state imposed by opsin expression independent of optical activation (Figure 2.4). Fluorescent proteins (XFPs) are most often employed as this control protein, since opsins are often expressed as XFP fusions; ongoing work is focused on developing photocurrent-null opsins for improved experimental control purposes. Such truly "dead" optogenetic tool mutants with expression and targeting properties comparable to active tools, but with no light-induced effector function, would be useful as controls to ensure that effects seen are specifically due to optical recruitment of opsins in targeted cells, but it will be important to ensure that the photocurrents are truly zero even under high membrane expression levels *in vivo*. Knowledge of pore structure (and pump mechanisms) may facilitate the generation of such tools [11].

Sixth, in addition to light-sensitive pumps and channels, continued expansion of optically-recruited biochemical signaling will be important, with increasing attention to strategies for modular and easily programmable signaling pathway recruitment, improved specificity, expanded spectral responsivity bands, and adaptation to additional classes of native chromophores (such as flavins, biliverdins, and the like [8]). The optoXR family of light-activated 7TM neurotransmitter / neuromodulator receptors will see addition of novel tools based on chimeras between vertebrate rhodopsins and both well-known and orphan GPCRs [7]. Also, light-sensitive domains are being added to an increasing number of receptor and even intracellular signaling proteins, so that optogenetics will expand to occupy the full breadth of cell signaling, far beyond the study of neural activity [6].

2.5 Conclusion

In summary, continued investigation from the microbial and biophysical side into ecological diversity, high-resolution structures, photocycle properties, and functional phylogenetics of light-sensitive protein modules will enable the discovery and engineering of new and improved classes of optogenetic control. Moreover, investigation from the neuroscience side into targeting, trafficking, selective spatiotemporal properties of illumination, precise circuit-element recruitment, and diverse readout engineering and analysis, will fundamentally advance the scope and precision of resulting insights into complex intact biological systems (Figure 2.4). Existing methods represent only the tip of the iceberg in terms of what may be ultimately achieved, in maximally enabling the principled design and application of optogenetics.

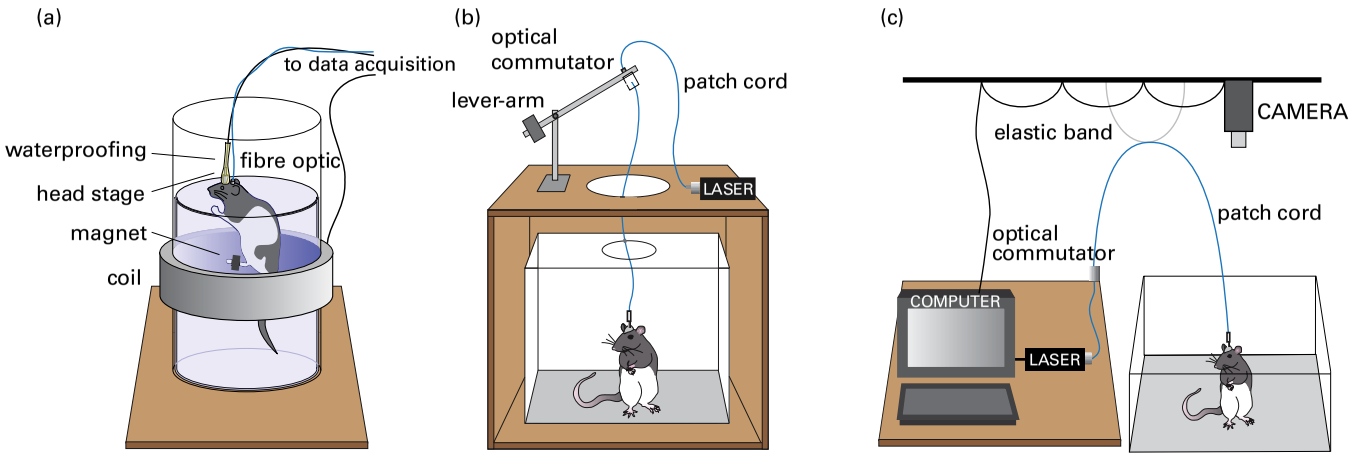


Figure 2.4: Integrating optogenetics with behavior. Diverse behavioral rigs can be outfitted for optogenetic experimentation. (a) The forced swim test has been automated with magnetic induction-based detection of kicks combined with optogenetic stimulation and electrical recording. (b) Operant behavior can also be combined with optogenetics. The chamber itself is modified to accommodate the entry of fiber optics and recording wires, and the stimulation/recording assembly is kept out of the reach of the rodent with a counter-weighted lever arm. (c) Optogenetic manipulations can also be combined with behavior in open fields or large mazes. In these experiments, an elastic band, rather than a lever arm, is used to support stimulation / recording equipment. Video recording combined with custom or commercially available software can be used to synchronize optical stimulation with behavior. Adapted from Zalocusky and Deisseroth [9].

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3 Challenges and opportunities for optochemical genetics

"The trick then is not to use the clumsy and inefficient techniques of classical organic chemistry by themselves but to make use of Nature's tools."

Francis Crick, 1999 [1]

3.1 Introduction

The transmembrane proteins that underlie neural processing are now known at a level of detail that has greatly increased our understanding of these sophisticated molecular machines. Starting with MacKinnon's seminal structure of a potassium channel, several voltage-gated ion channels and ionotropic receptors have been revealed with atomic resolution (Figure 3.1) [2, 3, 4, 5, 6]. This has been complemented by structures of G-protein coupled receptors, adding opsins and metabotropic receptors to the ever-increasing repertoire of transmembrane proteins elucidated with structural biology [7, 8, 9, 10]. As a consequence of this structural revolution and recent advances in pharmacology, Nature's molecular machines can now be manipulated with relative ease. This can be done, for instance, via synthetic on-off switches or tuning elements that are attached to the signaling protein of interest to allow for its orthogonal control with non-natural input signals. Amongst these signals, light is particularly useful, since it is unmatched in terms of temporal and spatial precision and techniques for the delivery and control of light are highly developed.

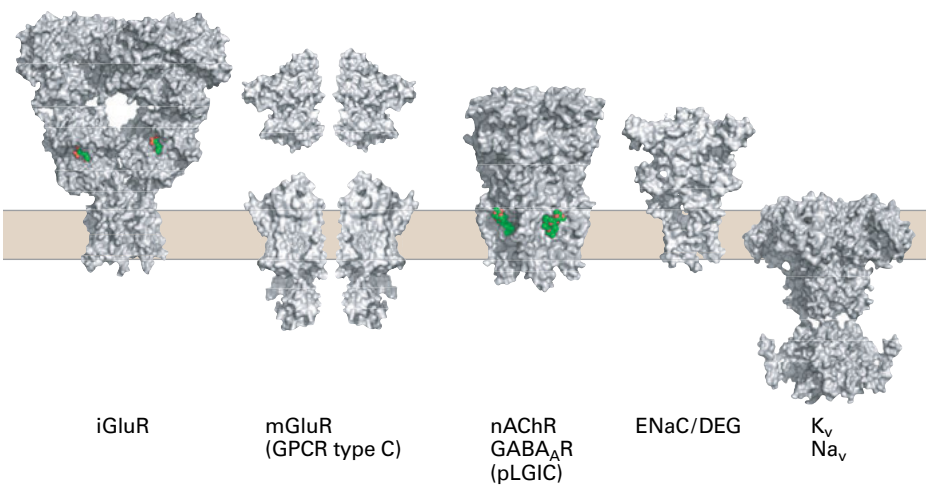


Figure 3.1: A selection of transmembrane receptors that have been characterized in atomic detail.

The resulting semisynthetic photoreceptors are particularly useful for applications in neuroscience since they can be used to control neuronal firing patterns and to mimic or block synaptic signals. This kind of approach was proposed as a method of choice for unraveling neural systems by Francis Crick in his 1999 Kuffler lecture [1] – a challenge that was met shortly thereafter giving rise to a rapidly developing field, now termed "optogenetics". Like their naturally light-sensitive counterparts, the opsin channels, pumps and GPCRs, semisynthetic photoreceptors work in animals and can be applied to the restoration of vision, the optical control of touch sensation and the dissection of neural circuits underlying behavior. The integration of synthetic photoswitches into mammalian receptor proteins has the unique advantage of enabling native signals to be optically controlled, thereby providing an additional ability to elucidate the physiological mechanisms by which the regulation of excitability and synaptic transmission controls circuits and behavior. The new functional dimension of chemistry to this field led us to term the approach "Optochemical Genetics (OCG)" [11].

3.2 Photosensitizing receptors

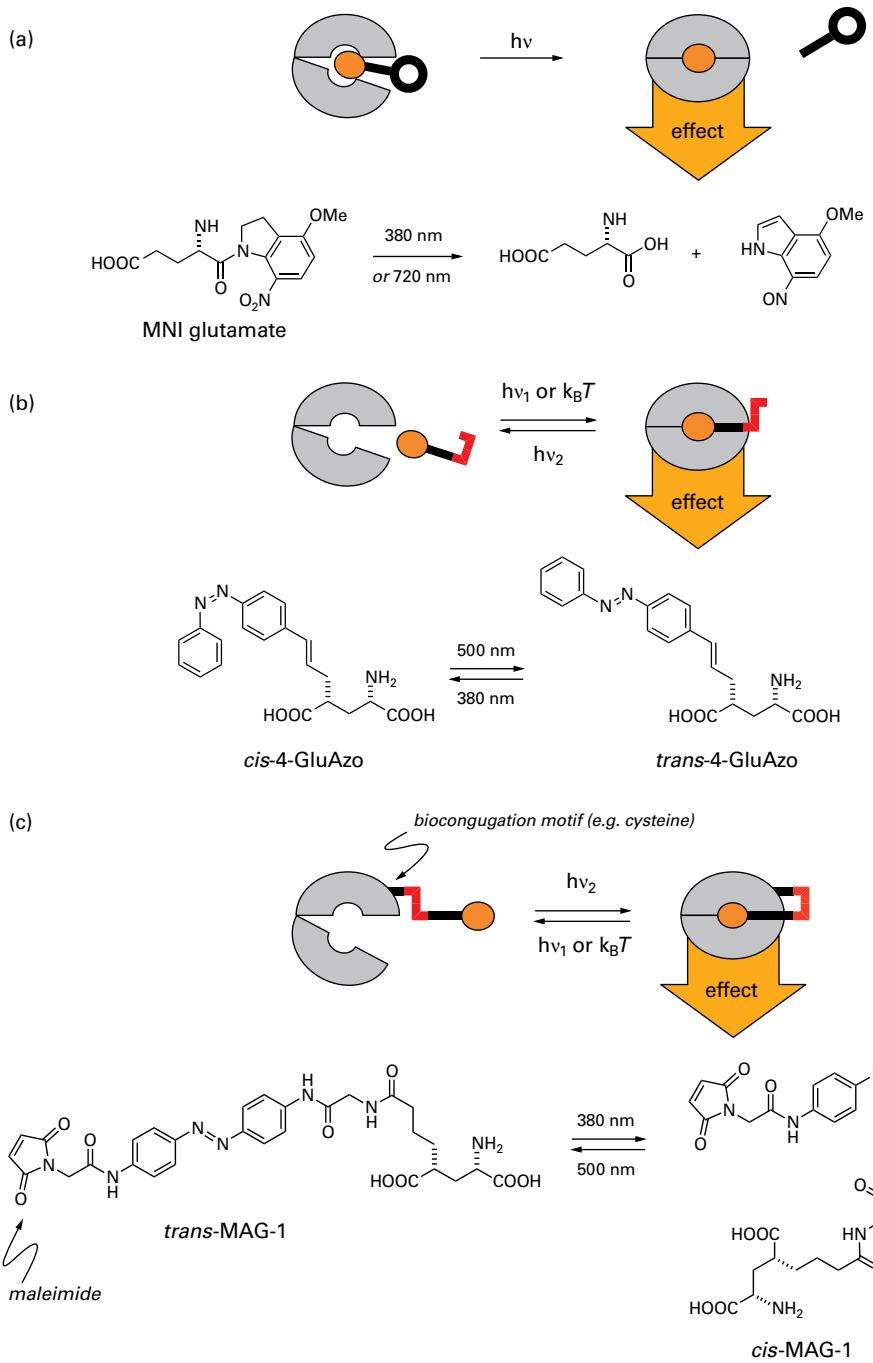
Three general chemical strategies have emerged to directly or indirectly endow receptors and channels with light-sensitivity. The simplest and oldest approach employs so-called caged ligands (CL) [12] (Figure 3.2A). Here, a ligand is endowed with a protecting group (the "cage") that masks a functional group crucial to the ligand-receptor interaction and thus renders it ineffective. Photochemical cleavage of this protecting group then sets the active ligand free and triggers the desired biological effect. While this approach has been employed with much success, especially in neuroscience (*e.g.*, in the form of caged glutamate), there are certain functional disadvantages associated with caged ligands. Uncaging is a unidirectional process and it is difficult, if not impossible, to "stuff the beast back into the cage". Unless one is interested in tonic effects, one is limited to uncaging in a small volume with two-photon illumination to enable diffusion, uptake, and/or enzymatic destruction to mimic the physiological rise and fall in synaptic concentration. In addition, uncaging produces byproducts, *i.e.*, the remnants of the protecting group, which can be toxic.

Some of these shortcomings can be overcome with a second approach that we call the photochromic ligand (PCL) approach (Figure 3.2B) [11]. Herein, the ligand carries a photoswitchable side-chain that can be switched between two (or more) configurations but is not cleaved off upon irradiation. As the photoswitch toggles between different states, the efficacy of the ligand changes, triggering the desired biological effect in a reversible fashion. The PCL can even be an agonist in one form and an antagonist in the other. PCLs have all the advantages of small-molecule drugs, including their ease of application and fast tissue distribution. As with drugs, selectivity between

receptor subtypes can be a challenge, but this can often be overcome through systematic variation of the molecule.

Of course, there are situations where receptor-subtype selectivity and cellular targeting is highly desirable. In this case, a third approach, which we call the photoswitched tethered ligand (PTL) approach, can be employed (Figure 3.2C) [11]. Here, the ligand is *covalently* attached to its receptor in a site-directed manner through a tether that contains a photoswitch. As the photoswitch toggles between extended and bent forms, the local concentration, position and/or efficacy of the ligand changes, thereby activating, antagonizing or modulating an allosteric domain, or blocking an effector domain of the signaling protein in a reversible fashion. Importantly, PTL-gated proteins can be genetically encoded, since the point of attachment can be an engineered cysteine residue or any other encodable chemical motif that allows for specific bioconjugation. Since the PTL is covalently tethered, its local concentration at the site of attachment is very high in the active form of the photoswitch, which means that the affinity of the ligand is not a major concern. In fact, low-affinity ligands have the advantage of ensuring that photoswitching can rapidly remove the ligand from the binding site.

The CL and the PCL strategies are akin to "chemical genetics" [13]. Chemical genetics attempts to address every protein target with a selective small-molecule ligand. Although such pharmacological control can have a rapid onset once the chemical reaches its target, diffusion barriers often slow the onset of drug action considerably and make washout difficult, especially *in vivo*. This limitation is overcome by allowing for long equilibration times to get the caged molecule into place and then rapidly optically controlling its function in the CL and PCL approaches. The PTL approach is essentially a variant of optogenetics, since it combines a genetically encodable receptor with light to precisely control neural activity. As opposed to conventional optogenetics with opsins, in which the retinal photoswitch is biologically available in vertebrates, the synthetic PTL is not endogenously produced, but needs to be supplied by a chemist. This poses the additional burdens of synthesis and delivery, while providing the advantage of controlling native channels and receptors and providing the elegant negative control where the genetic component is expressed, but it is left inert to light, because the photoswitch is withheld. For therapeutic applications, the PCL approach has the appeal of avoiding the need to implement gene therapy, while the PTL approach shares with conventional optogenetics the advantage of genetically constrained cell-specific targeting. It is also extremely useful in the functional dissection of closely related receptor subtypes, since selectivity is achieved through covalent attachment to genetically engineered isoforms and high affinity ligands are not required (Figure 3.3) [14, 15].


(a)
hν
effect
NH
OMe
HOOC
O
N
O2N
MNI glutamate
380 nm
or 720 nm
NH
HOOC
OH
O
+
OMe
HN
ON
(b)
hν1 or kBT
hν2
effect
N=N
NH2
HOOC
COOH
500 nm
380 nm
cis-4-GluAzo
trans-4-GluAzo
(c)
biocongugation motif (e.g. cysteine)
hν2
hν1 or kBT
effect
trans-MAG-1
maleimide
380 nm
500 nm
cis-MAG-1

◂ **Figure 3.2:** Strategies for photosensitization. (a) The caged ligand approach (**CL**); (b) the Photochromic Ligand Approach (**PCL**) with an azobenzene switch; and (c) the Photoswitched Tethered Ligand Approach (**PTL**) using an azobenzene switch. A corresponding glutamate derivative is shown in each case.

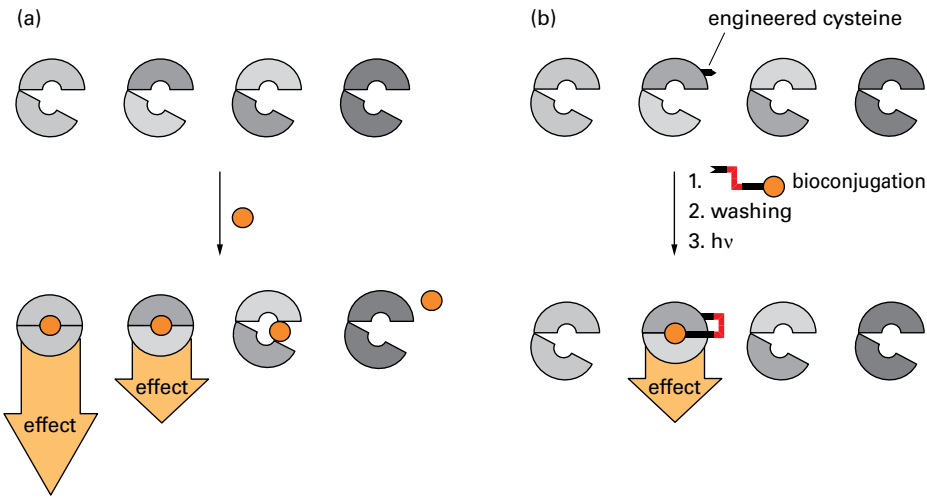


Figure 3.3: The PTL approach to selective pharmacology. (a) Selectivity between receptor subtypes is difficult to achieve. (b) In OCG, selectivity can be achieved through genetic engineering of a bioconjugation site. In addition, the photoactivatable ligand can have low affinity to the receptor subtype.

3.3 PCL and PTL development and applications

Optochemical genetics had an early forerunner with Erlanger's and Lester's PCLs and PTLs for neuromuscular nicotinic acetylcholine receptors [16, 17, 18, 19], but only came of age in 2004 when we introduced the synthetic azobenzene-regulated potassium channel (SPARK) [20]. Incidentally, this was the second system that could be used to control action potentials in neurons following Miesenböck's pioneering "ChARGe" system [21]. Although SPARK was overshadowed shortly after its introduction by the advent of channel- and halorhodopsins [22, 23, 24, 25, 26], OCG has undergone a rapid development, providing alternative ways to optically control neural activity and a singular approach for the remote control of native synaptic signals. Since 2004, PTLs have been developed for a number of channels and receptors, including voltage-gated potassium channels (*e.g.*, SPARK) [14, 20, 27], two-pore-domain potassium channels [15], kainate receptors (LiGluR) [28, 29, 30, 31, 32, 33], potassium-selective glutamate receptors (HyLighter) [34], metabotropic glutamate

receptors (LimGluR) [35], $GABA_A$-receptors [36], and neuronal nicotinic acetylcholine receptors (LinAChR) [37]. Conversely, photochromic ligands (PCLs) are available for K_v-channel [38, 39, 40, 41, 42], Na_v-channels [42], Ca_v-channels [42], kainate receptors [43, 44, 45], AMPA-receptors [46], NMDA-receptors [47], $GABA_A$-receptors [36, 48], and neuromuscular nicotinic acetylcholine receptors [16, 17, 18, 19]. A photochromic agonist for AMPA receptors, ATA [46], and its effect on mouse layer 2/3 cortical neurons is shown in Figure 3.4.

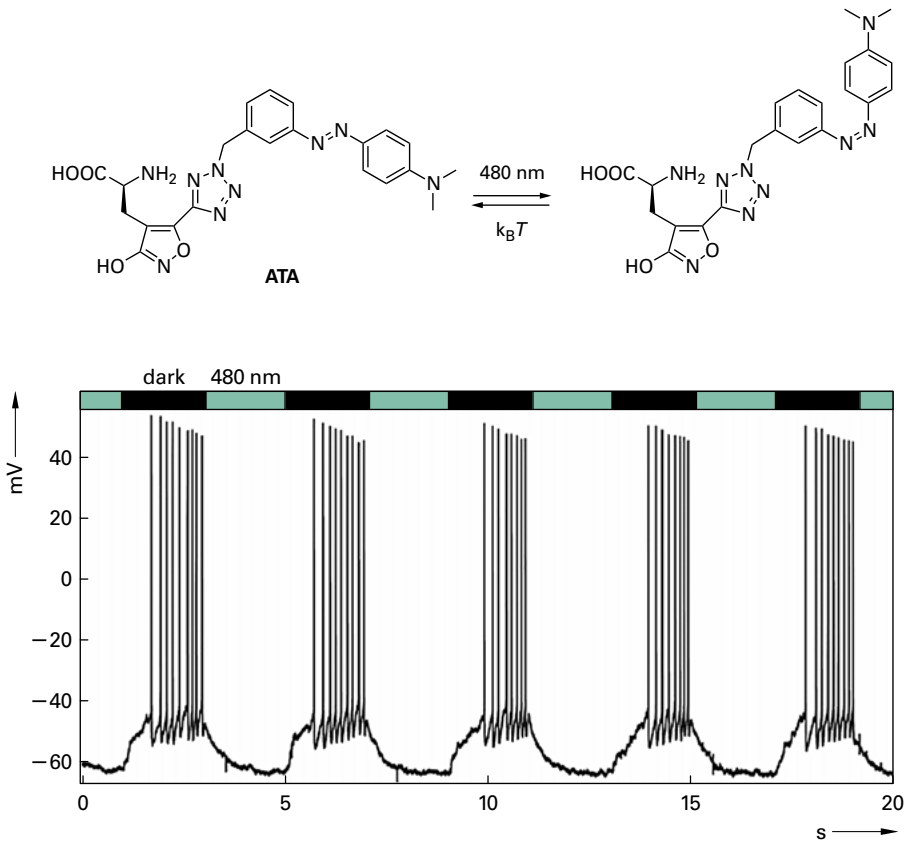


Figure 3.4: ATA, a photochromic agonist of AMPA-receptors, as an example of a PCL that can control mouse cortical neurons.

Synthetic photoswitches of both types work well in dissociated hippocampal and Purkinje neurons; cortical, hippocampal and cerebellar slices; intact dorsal root ganglia; flat-mounted retinas; and *in vivo* in the zebrafish central nervous system and the rodent eye. In terms of its applications to biological and clinical problems, the optochemical approach has been used to selectively block specific potassium chan-

nels and dissect their functional role [14], elucidate the role of auxiliary channel subunits [15], control calcium exocytosis [49], analyze the spinal circuitry of zebrafish larvae and control their escape behavior [29, 50], control heart rate in the leech *Hirudo medicinalis* [38], restore visual responses in blind mice [51, 52], and optically control pain sensation in rodents [42].

3.4 Advantages and disadvantages of PCLs and PTLs

When compared with the conventional tools of optogenetics, *i.e.*, rhodopsins, halorhodopsins and channelrhodopsins, PCLs and PTLs show certain functional advantages and disadvantages. PTLs constitute two-component systems that require synthetic compounds, few, if any, of which are presently commercially available. The currently used reactive maleimides have to penetrate tissues to find their protein targets, something that occurs readily in larval zebrafish and the rodent eye, but waits to be demonstrated in the mammalian brain. Selectivity is not a major concern since accessible surface cysteines are rare and the PTLs undergo affinity labeling, concentrating them at the intended sites. However, the instability of the maleimides in the sera of target organisms potentially poses problems, which could be overcome, for instance with SNAP-tags [53] or other genetically encoded bioconjugation motifs. On the other hand, the currently used maleimides PTLs operate on native receptors that are only modified by the substitution of a single cysteine with little or no effect on protein expression or function. Because the receptors are full length, they are expected to undergo their normal protein interactions and, therefore, be targeted precisely in the neuron and maintain native signaling specificity.

PCLs represent one-component systems that lack the selectivity and precision provided by genetic targeting but they are as easy to apply as regular drugs and molecular probes. Their usefulness in dissecting neural circuitry might be somewhat limited but their medical impact could be significant, since they do not require genetic manipulation (and ultimately human gene therapy) to function. Indeed, first applications in vision restoration and pain management have already surfaced. Like caged ligands (and PTLs), they require a commercial source to gain in popularity.

In principle, the action spectra of PCLs and PTLs could cover a much wider range than the opsins, since they are made by organic synthesis and their photophysical and functional features are not limited by biosynthetic pathways. It should be possible, for instance, to extend the action spectra into the deep red or near infrared range, as originally proposed [1]. It should also be possible to develop synthetic photoswitches with much higher extinction coefficients or useful two-photon cross sections. Both bistable and fast-relaxing photoswitches, which mirror the development of step-function opsins [54] and very fast relaxing optogenetic tools, such as ChETA [55], have been explored but need to be investigated more systematically. Singlet-oxygen production via intersystem crossing does not seem to be a big concern with azo-

benzenes, which are currently the most popular photoswitches in OCG. Other types of photoswitches, however, should be explored.

3.5 Conclusion

In summary, OCG adds an extra dimension to optogenetics, further increasing our ability to control neuronal activity with light. Like conventional optogenetics, it benefits from the huge advances that have been made in light delivery and viral transfection. Despite the comparatively small number of laboratories that have joined the effort to date, OCG has enjoyed rapid growth and has already made the transition from proof of concept to applications in neuroscience and preclinical investigations. Given the enormous advances in structural biology mentioned in the introductory paragraph and recent progress in the design and synthesis of photoswitches, it is likely that this pace is going to increase. OCG employs the techniques of classical organic chemistry, which were apparently held in so little esteem by Francis Crick. However, as suggested in his lecture [1], it does not use these techniques *by themselves* but rather *in conjunction with* Nature's magnificent tools, benefiting from advances in both areas. As such, it has enabled the optical control of neural systems and entire animals, just as a leash is able to (inefficiently) control the behavior of a dog, a sophisticated being only slightly modified through genetic manipulation (Figure 3.5).

Figure 3.5: An attempt to control Paula, a complex system, with a clumsy and inefficient technique.

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Thomas Knöpfel

4 Optogenetic imaging of neural circuit dynamics using voltage-sensitive fluorescent proteins: potential, challenges and perspectives

4.1 Introduction

Like other optogenetic tools, protein engineered voltage indicators can be targeted to preselected genetically defined classes of neurons. Voltage imaging using genetically encoded voltage indicators, therefore, overcomes the lack of cell type specificity inherent to classical voltage imaging techniques. Here I take the position that *voltage-sensitive fluorescent proteins (VSFPs)* open an entirely new window to the investigation of neuronal circuits and provide a bridge between the functions of circuits and behavior.

4.2 The biological problem

The biological function of muscles, glands or even more complex organs, like the kidney, can be reasonably well explained by the properties of their cellular elements. Direct extrapolation from cellular function to the function of the organ in the context of the intact organisms is much more difficult, and perhaps impossible, in the case of the mammalian brain. One reason for this complication results from the diversity of brain cell classes and the complexity of the functional connectivity between neurons. For this reason, approaches that provide an experimental bridge between the level of single cells and intact system functions are of essential importance in brain sciences. The combination of gene manipulation and light-based technologies has the potential to provide the foundations for such a bridge. Here I use the term "optogenetic approach" in reference to the observation and control of groups of cells that express light-activated (*e.g.*, photocurrent generating) or light-emitting (*e.g.*, fluorescent) indicator proteins. The "optogenetic toolbox", hence, has two main compartments; light-driven actuators control electrical or chemical signals, while light-emitting indicators sense and report these signals.

4.3 The large scale challenge of circuit neurosciences

Behavior arises from the electrical activities of neurons (fueled and maintained by biochemical reactions). For any slightly complex behavior, the number of neurons that are involved in its generation is very large (even if the activity of a single neuron

can show a significant impact on behavior, a large number of neurons are needed to initiate and execute the behavior).

Based on the conjecture that behavior only emerges from the activities of very large numbers of neurons (referred to as the large-scale integration problem, (for example, [1]), a (full) record of neuronal activity across the whole intact brain embedded in a behaving animal would be required to (fully) decipher (in principle) the link between neuronal action potentials and behavior [2]. Verification of what may be inferred from the correlation between *spike patterns* and behavior could be aided by interventional strategies using optogenetic control tools [3] or computer simulations (once the brain has been fully dissected and reconstructed *in silico* [4]).

Unfortunately, a full record of neuronal activity across the whole intact brain in a behaving mammalian is far beyond current technical reach. Therefore, to advance practically, we need to compromise with respect to the number of neurons, size of the observation field / volume, and temporal resolution when recording neuronal activities.

4.4 The current approach to the large-scale integration problem

The most widely applied and successful approach to "linking spike patterns and behavior" is provided by two photon (2p) calcium imaging *in vivo* at single cell level resolution [5] and by microelectrode array recordings [6].

The limitations (compromises) of these approaches entail [7]:

- For 2p calcium imaging, the areas / volumes of observation are limited to a diameter <300 µm and typically to much less than ~500 neurons.
- For microelectrode arrays, the observation area can be larger but the density of individually recorded neurons is smaller so that the total number of neurons also usually remains below ~500.
- Both 2p calcium imaging and microelectrode arrays record only spikes; in the case of 2p calcium imaging temporal resolution is low.

Traditional methods for the monitoring of the activities of very large numbers of cells are local field potential recordings, electroencephalography (EEG), electrocorticography (ECoG), and magnetoencephalography (MEG). These recording techniques sacrifice single cell resolution in favor of population signals that represent the activities of very large numbers of neurons. Their caveat is that they are blind to *cellular diversity*. They can therefore not provide a solid link between cellular and circuit mechanisms.

4.5 Large-scale recordings of neuronal activities using optogenetic approaches

Since optogenetic (genetically encoded) indicators (either for voltage or calcium) can be targeted to specific cell classes, they allow for cell class specific population recordings [7]. This feature allows the imaging of very large numbers of neurons, while cell class specific targeting of the indicators minimizes the loss of information that would otherwise result by averaging over heterogeneous cell populations. In principle, several cell classes (*e.g.*, excitatory and inhibitory cells) might be monitored simultaneously (using indicators of different colors) with high spatial and temporal resolution. Moreover, genetically encoded indicators facilitate chronic recordings (sampling data over multiple sessions spaced by days or weeks). This not only enables the investigation of slowly developing, experience-dependent changes in activity patterns that may be related to behavioral changes, but also increases the number of cells that can be sampled in high resolution experiments from a single animal.

4.6 Genetically encoded voltage indicators: state of development and application

Voltage indicators have one major disadvantage and several advantages, when compared to calcium indicators. Their main disadvantage is the low signal-to-noise ratio that is generally obtained with optical voltage imaging (this is true for both low molecular weight classical voltage-sensitive dyes and genetically encoded voltage indicators). Part of the signal-to-noise problem arises from the fast sampling rate employed in voltage imaging, but changes in fluorescence associated with action potentials are also small, even for the best voltage indicators, when compared to calcium indicators. The main advantages of voltage indicators are their responsiveness to subthreshold activities (including membrane hyperpolarization), and their ability to resolve faster signals. Accordingly, voltage imaging can provide information about both the input (synaptic potentials) and the output (action potentials) of neurons and hence, offers access to neuronal computation, rather than to coding (representations) only.

With this motivation, and to complement what can be achieved with calcium indicator-based imaging, my lab and other labs have invested considerable efforts in the development of Voltage-Sensitive Fluorescent Proteins (VSFPs) [8, 9]. Figure 4.1 depicts the main features of their design, along with sample recordings obtained from neurons in culture and acute brain slice.

A detailed description of VSFPs is found elsewhere [10]. The basic engineering idea of VSFPs is to obtain an optical readout of voltage by coupling an isolated voltage-sensing domain (derived from a naturally occurring voltage-dependent membrane protein) to a FRET pair of fluorescent proteins or a single fluorescent

protein [11]. This concept, along with the identification of a suitable voltage sensing domain derived from *Ciona intestinalis* [12], has been followed up by several laboratories, resulting in probes of different names, but comparable features (an overview is found in [8]). A totally different class of genetically encoded voltage indicators are based on microbial opsins used in a "reverse mode" [13, 14]. The current limitation of these opsin-based voltage indicators is their low fluorescence quantum yield (resulting in low brightness) and their low effective excitability using 2p fluorescence excitation.

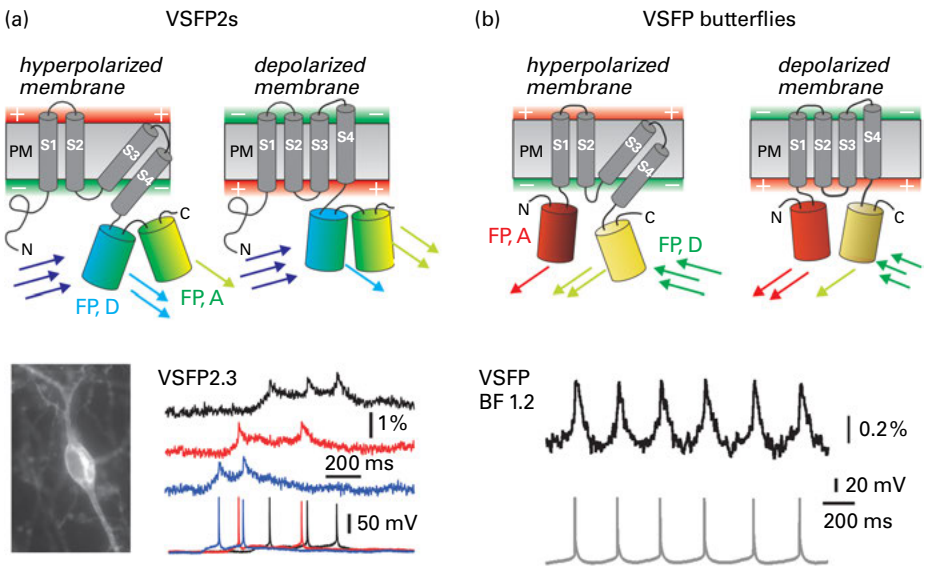

Figure 4.1: VSFP design and example recordings. (a) Upper panel: Schematic of FRET (Förster resonance energy transfer) -based voltage-sensitive probes of the VSFP2 family. The voltage-sensor domain, consisting of four segments (S1–S4) crossing the plasma membrane (PM), is fused to a pair of fluorescent proteins (FP, D: FRET donor; FP, A: FRET acceptor). A change in membrane potential (e.g. from hyperpolarization to depolarization) induces a rearrangement of the two fluorescent proteins that is optically reported as a change in the ratio of donor and acceptor fluorescence. Arrows indicate excitation and emission light at wavelenghts associated with color. Lower panels: Example recording from cultured hippocampal cells showing spontaneous action potential firing. The three sweeps of optical recordings shown in black, red and blue color correspond to the superimposed microelectrode recording traces of same color. (b) Upper panel: FRET-based voltage sensitive probes of the VSFP-Butterfly family, where the voltage-sensor domain is sandwiched between two fluorescent proteins. Lower panel: Example recording from a layer 2/3 pyramidal cell in an acute slice preparation. The upper trace shows the fluorescence ratio signal and the lower tarce shows the corresponding intracellular recording. Action potentials were induced by a constant current injection and traces are single sweeps. Adapted from Akemann et al. 2010 and 2012.

In a proof-of-principle study, we have shown that VSFP2s (Figure 4.1a) and VSFP-Butterflies (Figure 4.1b) enable the imaging of sensory evoked responses from genetically-defined neurons in living mice [15, 16]. VSFPs can be genetically targeted to specific cell populations, and allow for chronic recordings over weeks and months, both of which is very difficult to achieve with classical *voltage-sensitive dye imaging*. Thus, VSFP imaging provides the spatial resolution of optical imaging and, in contrast to calcium imaging techniques, provides sufficient sensitivity to subthreshold synaptic activation at the temporal resolution that is required for the analysis of neuronal computations.

Our best-performing variant in a new series of red-shifted VSFPs, VSFP-Butterfly-1.2 (Figure 4.1b), exhibits markedly enhanced localization to neuronal membranes after long-term expression *in vivo*, as well as a large amplitude voltage report, resolving action potentials from individual neurons in single sweeps, and enabling the imaging of subthreshold membrane oscillations [16].

Imaging of very large pools of genetically specified classes of neurons reveal their synchronized and coordinated activities. The example shown in Figure 4.2 illustrates how the high spatial and temporal resolution of VSFP-based voltage imaging allows us to study the temporal organization of activities in multiple cortical areas, in responses to sensory inputs and their initiation of motor programs.

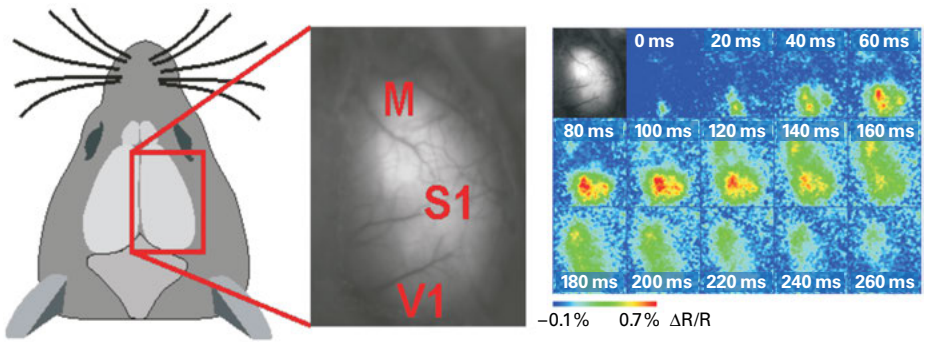

Figure 4.2: Voltage imaging in living mice. Spread of neuronal information from sensory to motor areas. VSFP-butterfly 1.2 (Akemann et al., 2012) was expressed over a large cortical area including motor cortex (M), primary somato-sensory cortex (S1) and primary visual cortex (V1). A light flash (t=0) triggered a response in primary visual cortex and higher visual areas from where it spread over a large portion of the hemisphere, triggering a response in the motor cortex.

In this example experiment, a light flash triggered a response in the primary visual cortex, which subsequently propagated over the cortical hemisphere to activate circuits in the motor cortex. The illustrated experiment was performed in a head fixed anaesthetized mouse, but similar experimental settings are possible for awake animals implanted with a chronic optical window to the brain [16].

4.7 Unsolved methodological / technical challenges

- Genetically encoded voltage indicators with excitation / emission at far-red / infra-red wavelengths are needed to improve imaging from deeper brain structures, and to facilitate combinations with blue light-activated photocurrent-generating opsins. In addition to fluorescence, bioluminescence might be considered for recordings of (slow) signals from very deep brain structures [17].
- Optical instrumentation that allows experimenters to both zoom in to microscopic levels (field of view <500 μm) and zoom out to the mesoscopic (millimeters) recording fields. Increased depth resolution would be immensely valuable. A possible approach to achieve this is 2p excitation via temporal focusing / light patterning techniques [18].
- Development of novel analytical approaches that take advantage of the full data content obtained with large-scale voltage imaging.
- Development of (genetic) methods to target indicator expression to specific pools based on their function in the circuit (*e.g.*, cells with the same stimulus response tuning, cells that can trigger the same behavioral program).

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Gero Miesenböck

5 Why optogenetic "control" is not (yet) control

From the beginning, the term "control" has been applied to optogenetic manipulations of biological systems [1, 2, 3, 4, 5, 6]. As I have been responsible for introducing this practice [1, 2, 4, 7], it is perhaps appropriate that I finally ask whether it is justified. Would an engineer recognize as a form of "control" what a neuroscientist does when she expresses a light-responsive protein in a neuron in order to influence the cell's electrical activity? In this short opinion piece, I argue that the answer is likely to be negative. I also suggest that aligning optogenetic "control" better with the concept of control, as it is understood in engineering, sets a worthwhile agenda for the future.

A controller "*senses the operation of a system, compares it against the desired behavior, computes corrective actions based on a model of the system's response to external inputs and actuates the system to effect the desired change. The basic feedback loop of sensing, computation and actuation is the central concept in control*" [8]. Sensing without feedback is merely measurement; actuation without an ability to predict and sense the consequences of actuation is merely perturbation. By this standard, most optogenetic "control" experiments reported to date have produced perturbations, not control.

Failure to exert effective control is unavoidable whenever the basic feedback loop of sensing, computation, and actuation is interrupted. The break can occur at any stage of the loop, beginning with an inability to sense the system's behavior and, consequently, the impact of actuation. This is a commonly encountered situation in experiments on the nervous system, for a multitude of reasons. Some neurons, including those in invertebrates like *Caenorhabditis elegans* and *Drosophila*, are too small to make electrophysiological recordings routine, particularly in a behavioral setting. Where recordings are possible, creating access for the electrode, (for example, by removing a piece of chitinous cuticle or bony skull), also alters the optical path traveled by the stimulating photons. A carefully calibrated light response under recording conditions may thus have very little bearing on the responses of the same neuron in the intact, behaving animal. Actuators responding to red-shifted optical control signals [9] that penetrate tissue more easily promise to improve this situation.

Confounds also arise from other sources. For instance, the impact of the same photocurrent will be different on a neuron in the low-conductance state *in vitro* or under anesthesia than in the high-conductance state of wakefulness. The accuracy of extrapolations from one state to the other is again limited. Given their amplitudes and kinetics, the currents generated by optogenetic actuators may drive reliable membrane potential changes under conditions of relative quiescence *in vitro*, but the forces they produce may be outweighed by synaptic currents under naturalistic conditions. When the experimental goal is to generate precise spiking patterns, as is

required for deciphering neural codes, there is therefore no alternative to combining optogenetic actuation with simultaneous sensing of neuronal activity; in the absence of direct measurement, there is no guarantee that actuation does indeed have the intended effect, unless the forces exerted by the actuators are overwhelmingly large. Although this is generally not the case, the vast majority of optogenetic actuation is still done blindly.

In favorable circumstances, extracellular recording devices, such as optrodes [10] or diode probes [11], allow the activity of a handful of neurons to be sensed simultaneously with optogenetic interventions. However, these devices sample only a small, random fraction of cells, which may or may not include some of the actuated neurons. This situation illustrates an important general problem in control: observability [8]. The problem is best framed as a question: do the sensors provide enough information to reconstruct the state of the system?

Naively, one might think that only a complete microscopic description, with numerous sensors providing real-time data on the state of every neuronal element, would be sufficiently informative to characterize the behavior of a neural system. Electrophysiology, with its severe restrictions on the number of sites that can be surveyed, is fundamentally incapable of furnishing such an extensive microscopic description, but optical imaging of voltage or calcium dynamics with high temporal and spatial resolution might. Despite recent progress in the development and use of optical and optogenetic sensors [12, 13, 14, 15, 16], however, the prospect of capturing the rapid dynamics of extended neuronal ensembles in three dimensions remains dim. This is not only because of performance limitations imposed on the sensors by their absolute brightness, response amplitudes, kinetics, specificity of labeling, and interference with cell physiology [17], but also because of the difficulty of extracting and quantifying short-lived fluorescence signals in parallel from many spatially resolved locations in scattering tissue [17, 18].

In addition to the technical hurdles associated with its acquisition, the large volume of data needed for a microscopic description of a neural system carries a significant analytical overhead that makes the design and execution of algorithms for real-time control cumbersome. Observers using meso- or macroscopic variables reduce this overhead considerably. A mesoscopic description might be based on a variable that reflects not the activity of every individual neuron, but rather a collective property of a neuronal population, such as the phase relationship between field potential recordings obtained at different sites, or the rhythmicity of a single neuron's output. A macroscopic description might ignore neuronal activity altogether and take into account only the behavior of the experimental animal, such as its preference for one of two alternatives in a choice task. Where optogenetic "control" has approached the engineering standard of actuation based on sensing, the sensed parameters have invariably been meso- or macroscopic descriptors of the system's behavior [19, 20, 21]. These efforts have been remarkably successful, for reasons that are unlikely to be merely practical. If nervous systems have indeed evolved to generate rapid, integrated

sensorimotor control, rather than explicit internal representations at successive perceptual, cognitive, and motor stages [22], then action-contingent interference with signals in sensorimotor feedback loops may turn out to be a particularly opportune form of optogenetic intervention.

The concept of observability has a control-theoretic counterpart or “dual”: the concept of reachability [8]. As in the case of observability, the problem of reachability can be cast into the form of a question: is it possible, given a particular set of actuators, to drive the system into a specific state? In what follows, I somewhat artificially divide the analysis of reachability into three separate domains: the performance characteristics of the actuators themselves; the interplay of actuation with the intrinsic dynamics of the actuated system; and the question of actuator placement.

To begin, imagine an optogenetic analog of a voltage-clamp experiment on a single neuron. Keeping the membrane potential of the neuron at its setpoint requires, first and foremost, an ability to sense perturbations due to the opening and closing of membrane conductances, and second, a capacity to counteract these perturbations. Assume for the purpose of this toy example that the problem of sensing has been solved (as it actually might easily have been by impaling the single neuron with an intracellular electrode). The arsenal of corrective actions available via the suite of actuators expressed by the neuron will then determine whether the setpoint of stable membrane potential is reachable under a particular perturbation. If the neuron is equipped only with actuators capable of injecting depolarizing currents, as was true in the early days of optogenetic “control” [1, 2, 4, 5], synaptic inputs that drive the membrane potential toward action potential threshold cannot be opposed, and the system becomes unstable if the depolarization is sufficiently large. A similar loss of control occurs when the corrective force exerted by an actuator is too weak to offset a perturbation, as is the case when the depolarizing current flowing through a passive conductance overpowers the hyperpolarizing actuator current generated by a pump [23, 24]. Effective optogenetic control of a single neuron’s membrane potential thus depends on a capacity to elicit bi-directional corrective actions with adequate, tunable force and short time delays. There is room for significant improvement of actuator performance on all of these fronts [25, 26]: replacement of weak actively transporting actuators (ion pumps) with strong passively conducting ones (ion channels); enlargement of single-channel conductances; tuning of optical responses (separation, bandwidth, and peak wavelength of absorption spectra; quantum yield of photochemical processes); and adjustment of response kinetics.

To see how intrinsic properties of the actuated system affect reachability, consider another experimental goal: that of causing the single neuron to fire an irregular sequence of action potentials. This control problem differs from that of clamping the membrane potential at a predetermined resting value. Instead of antagonizing each perturbation with a matching counterforce (a form of feedback control), a good controller will now use the intrinsic dynamics of the neuron to produce the action potential waveform and merely act to recruit the spike generator when an action potential is

required but keep it off at all other times (a form of feedforward control). This simple example illustrates that reachability reflects the entwined dynamics of actuation and the actuated system. No control policy exists in this setting that could double the amplitude of an action potential, or release a spike during the refractory period of the neuron. Because we understand the dynamics of single neurons so relatively well, we instantly realize that these control goals are not reachable, and make no attempt to attain them.

The same, however, is not true for neural systems whose dynamics we do not understand well – that is, the overwhelming majority of systems composed of more than two cells. Without quantitative models equivalent to those of Hodgkin–Huxley or FitzHugh–Nagumo to guide our intuition, what is to say that the patterns of population activity we seek to impose are actually reachable? Would we know it if they were not, given that it is generally impossible to observe the impact of actuation on all targeted cells? Behavioral evidence indicates that reachability can be a concern already under rather elementary forms of optogenetic actuation. For instance, the synchronous activation of a small group of neurons is sufficient to trigger the courtship behavior of male flies, but attempting to activate the same group of neurons as part of a larger set of cells leads to a suppression of the behavior [27]. Apparently, intrinsic interactions among the actuated neurons in the large set preclude the activation of the smaller subset. In other words, the state in which the small subset of neurons is active as part of the larger set is not reachable.

This elementary example provides a sobering perspective on efforts to control behavior by prescribing detailed patterns of neuronal activity. The main difficulty may not arise from the technical challenge of delivering detailed, cell-specific optical control signals at multiple wavelengths simultaneously to many identified neurons in a three-dimensional volume of tissue (although this challenge is, of course, formidable). Fundamentally more important is the fact that designed behavior is an instance of feedforward control: To precompute the inputs that will cause the system to respond in the desired way, a model of the system's dynamics must be at hand. Without such a model, there is no rational way of designing a controller, or even of determining whether a particular state is reachable.

In the absence of rational, model-based approaches, could a controller perhaps be found by brute-force searches for input patterns that elicit the desired behavior? The idea of optogenetic screens may seem absurd at first, given how large the space of possibilities is. However, it is quite conceivable that neural systems occupy only a thin slice of all theoretically possible states: constrained dynamics is the hallmark of robustness [28]. Rather than having to specify the exact state of every neuron in detail, it may be possible to control a robust system with relatively imprecise inputs. Just as the injection of a broad range of depolarizing currents gives rise to the same, precisely reproducible action potential waveform in a single cell, it is likely that a range of multicellular input patterns will evoke the same, precisely reproducible form of population activity. The surprising effectiveness of crude optogenetic actuation in

generating coordinated behavior suggests that this is indeed the case. Robustness, in other words, is the flip side of limited reachability.

Reachability, of course, also depends on the ability to place actuators where they are needed. If a cell cannot be genetically programmed to produce an actuator protein, its activity can at best be influenced indirectly. Unfortunately, regulating the expression of genetic material is itself a control problem with many reachability quandaries [7, 25]. The currently available inputs for synthesizing expression patterns are the transcription rates of endogenous promoter/enhancer sequences, the somatic locations of neurons, and the regions targeted by their projections. A limited set of Boolean operators also exist for intersecting two or three of these inputs. Needless to say, these genetic control mechanisms are far too coarse to make every desirable expression pattern reachable. Echoing the situation in optogenetic control, the most significant impediment to progress in the design of gene expression patterns is the lack of a principled, quantitative understanding of the dynamics of the natural system we aim to control.

Acknowledgments

Work in my laboratory is supported by the Wellcome Trust, the Gatsby Charitable Foundation, the Medical Research Council, the National Institutes of Health, and the Oxford Martin School.

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6 Optogenetic actuation, inhibition, modulation and readout for neuronal networks generating behavior in the nematode *Caenorhabditis elegans*

6.1 Introduction – the nematode as a genetic model in systems neurosciencesystems neuroscience

Elucidating the mechanisms by which nervous systems process information and generate behavior is among the fundamental problems of biology. The complexity of our brain and plasticity of our behaviors make it challenging to understand even simple human actions in terms of molecular mechanisms and neural activity. However the molecular machines and operational features of our neural circuits are often found in invertebrates, so that studying flies and worms provides an effective way to gain insights into our nervous system.

Caenorhabditis elegans offers special opportunities to study behavior. Each of the 302 neurons in its nervous system can be identified and imaged in live animals [1, 2], and manipulated transgenically using specific promoters or promoter combinations [3, 4, 5, 6]. The chemical synapses and gap junctions made by every neuron are known from electron micrograph reconstruction [1]. Importantly, forward genetics can be used to identify molecules that modulate *C. elegans*' behavior. Forward genetic dissection of behavior is powerful because it requires no prior knowledge. It allows molecules to be identified regardless of *in vivo* concentration, and focuses attention on genes that are functionally important. The identity and expression patterns of these molecules then provide entry points to study the molecular mechanisms and neural circuits controlling the behavior.

Genetics does not provide the temporal resolution required to study neural circuit function directly. However, neural activity can be monitored using genetically encoded sensors for Ca^{2+} (*e.g.*, GCaMP and cameleon) [7, 8, 9, 10] and voltage (*e.g.*, mermaid, arclight or VSFP-Butterfly) [11, 12, 13]. In *C. elegans*, imaging studies have focused largely on single neurons in immobilized animals [14]. However, it is now becoming possible to image the activity of single neurons in freely moving animals, and of multiple neurons in three dimensions. Additionally, increasingly sophisticated hardware allows precise spatial control of neural activity in freely moving *C. elegans*, using light activated channels and pumps (see Section 6.2). From a reductionist perspective, the worm model is very exciting because it has the potential to reveal how neural circuits work in enormous detail. This potential has fostered collaborations between physicists, engineers, and neuroscientists. Here we try to convey some of the excitement in this fast moving field.

6.2 Imaging of neural activity in the nematode

6.2.1 Genetically encoded Ca^{2+} indicators (GECIs)

C. elegans has played a key role in the development of optogenetic methods for monitoring the activities of neurons and other excitable cells *in vivo*. The primary tools for optogenetic recordings, in worms and in other organisms, are the genetically-encoded calcium indicators (GECIs). GECIs are engineered derivatives of fluorescent proteins with emission properties dependent on intracellular calcium levels. The feasibility of using GECIs to image calcium in transgenic animals was first demonstrated in *C. elegans*, with the FRET-based ratiometric indicator yellow cameleon [8] used to detect calcium transients associated with pharyngeal muscle activity as well as depolarization-evoked calcium influx in single neurons [15]. *C. elegans* is extremely well-suited to GECI-based optical recordings because of its small and transparent body, through which single-photon illumination can easily penetrate, and its small and well-characterized nervous system, which allows straightforward targeting of GECI transgenes to single or groups of identified neurons. GECIs have been used to record activity from a wide range of *C. elegans* neurons, including sensory receptors, interneurons and motor neurons [16, 17, 18, 19].

The development of GECIs with improved speed and sensitivity has increased the utility of these indicators for neuroimaging in *C. elegans*. While there has been significant improvement in ratiometric indicators, optimization has been particularly notable for non-ratiometric indicators such as GCaMP [9]. GCaMPs, which have been widely used in *C. elegans* [20], are circularly-permuted fluorescent proteins that undergo a large increase in emission intensity upon calcium binding, due to conformational changes that reduce the access of quenching solvents to the chromophore [21]. Successive rounds of mutagenesis, informed by structural knowledge, have led to orders of magnitude of improvement in signal-to-noise ratio and off-kinetics [22, 23]. Despite these significant advantages, a potential disadvantage of non-ratiometric indicators is their vulnerability to motion artifacts that may occur in behaving animals. While the reciprocal change in the emission intensities of FRET donor and recipient fluorophores provides an intrinsic control for such artifacts, non-ratiometric indicators must often be used together with, or in parallel to, a non-calcium sensitive fluorescent protein control [24].

6.2.2 Imaging populations of neurons in immobilized animals

Ultimately, understanding how the nervous system works requires us to simultaneously monitor neural activity in populations of neurons. More than half the 302 neurons of an adult *C. elegans* hermaphrodite have their cell bodies located in the head, in a volume of ~80 (x) × 60 (y) × 30 (z) microns [1]. With a 63× objective, all

these head neurons can be imaged in the same field of view, albeit at different focal planes. Optogenetic imaging requires that the target neuron or neurons be kept in the microscopic field of view. The standard solution to this problem is to immobilize the subject animal, typically by gluing it to an agarose pad in a recording chamber. This also makes it possible to apply a precisely defined stimulus to the worm (either a natural stimulus to sensory neurons, like mechanical, chemical, or thermal stimuli [17, 25–31], or by optogenetic actuators – see Sections 6.3 and 6.4); however, immobilization can interfere with the execution of behavior or even the properties of sensory circuits. One way this problem has been surmounted is using microfluidic devices, which immobilize animals more gently than glues and in some cases allow enough freedom of movement for behavior to be assayed [32, 33]. Because *C. elegans* is transparent and thin, imaging across the entire depth of the animal is possible, and optical sectioning methods (*e.g.*, using a confocal microscope) allow fluorescence emitted from different neuronal layers to be collected separately. The high capture rates possible with multi-beam scanning confocal microscopes, such as the spinning disc confocal, can achieve the required high frame rate capture. These approaches are limited by the light budget coming from the specimen, the camera speed, and fluorophore bleaching, rather than the confocal hardware. The efficiency of the latest generation of Ca^{2+} sensors make it feasible to use spinning disc confocal imaging to record the activity of populations of *C. elegans* neurons in immobilized animals. For some neuron groups, confocal sectioning does not provide sufficient resolution to separate closely apposed neurons. Use of Ca^{2+} indicators with different spectral properties (*e.g.*, combining R-GECO [34] or RCaMP [35] and GCaMP) may provide a way around this difficulty. Ultimately, the goal must be to image neural populations in freely moving animals. Although daunting, this is becoming possible in *C. elegans*.

6.2.3 Imaging neural activity in freely moving animals

The transparency of *C. elegans*, combined with improvements in automated tracking microscopy affords the possibility, at least in principle, of monitoring neural activity in behaving animals. Monitoring neural activity in freely behaving animals permits neurons and neural circuits to be linked to behavior. Unlike experiments carried out with immobilized or sedated animals, studying freely moving animals allows temporal relationships between neural activities and behavioral outputs to be established; it enables feedback from motor activity to be identified; and it permits the succession of neural activities that underlie behavioral sequences to be elucidated.

Imaging neural activity in freely moving *C. elegans* presents challenges. Unrestrained animals can move quickly, and enough light has to be collected in a brief time interval to obtain reliable signals. High magnification objectives with high numerical aperture (NA) are optimal for light collection and resolving power. However, they

provide a small field of view and have a shallow depth of field. Their use requires ways to efficiently re-center the moving animal to keep it in the field of view. Several methods have been designed that use feedback loops to rapidly re-center XY motorized stages [19, 36–39]. An alternative solution is to reduce the animal's locomotory activity; for example, by putting it on partially dry, sticky substrates. Shallow microfluidic devices make it possible to minimize movements in the Z axis, by slightly squeezing the animal onto its substrate. An alternative solution to the focus problem involves imaging in 3D, capturing different focal planes by moving either the objective or the stage [40].

Imaging neural activity at low magnification (10× or lower) reduces these difficulties. Low NA lenses have large depth of field. The trade-off is poorer light collection and resolving power. The large dynamic range of the latest generation of Ca^{2+} sensors (*e.g.*, YC3.60 and GCaMP3 and higher) [7, 41] provides sufficiently large signals, such that they can be used with low magnification lenses in favorable circumstances; that is when it is possible to drive strong expression of the sensor without perturbing neural properties and when the neural response is strong.

6.2.4 Other genetically encoded indicators of neuronal function

Although GECIs have been the most widely used optogenetic indicators, genetically encoded sensors to monitor other aspects of neuronal function are also desirable. Sensors of presynaptic activity, such as synaptophluorin [42], have been successfully used in *C. elegans*, but their relatively low sensitivity has made them more suitable for measuring neural activity over time than for dynamic neuronal recording [43]. Recently, a genetically-encoded glutamate sensor has been described that can detect synaptic glutamate signaling in intact worms [44]. Similar sensors for other neurotransmitters would clearly be very useful. Finally, the holy grail of optogenetic indicators would be a voltage sensor that could directly measure membrane potential, with sensitivity comparable to the present generation of GECIs. A number of promising candidate molecules have recently been generated [11, 12, 45], though thus far none has been successfully used in *C. elegans*.

6.3 Optogenetic tools established in the nematode

6.3.1 Channelrhodopsin (ChR2) and ChR variants with different functional properties for photodepolarization

C. elegans was the first animal in which Channelrhodopsin-2 (ChR2) and Halorhodopsin (NpHR) were expressed and utilized as optogenetic tools [46, 47]. Muscle contractions or relaxations were the first proof of light-evoked de- or hyper-polarization in

this system, which strictly depended on the presence of the obligate chromophore all-*trans* retinal (ATR). This was confirmed by directly measuring photocurrents in *C. elegans* muscle. In addition, several neuron types were probed using these tools, namely mechanosensory neurons, photoactivation of which evoked an escape reflex, as well as cholinergic motorneurons, in which photoactivation or inhibition evoked downstream muscle contraction or relaxation. The two proteins could also be concomitantly used for independent control of two cell types [47].

Several ChR variants with different functional properties and thus, applicability, could be established in the meantime for use in nematodes. These include C128X mutants, also called "step-function opsins", with a slowed-down photocycle, which allow for photostimulation at low light intensities and for prolonged activation in the minute (or, with repetitive stimuli, hour) time range [48]. The ChR2 variant H134R; T159C exhibits higher expression levels and largely increased photocurrents, thus evoking strong effects at low light intensities, and was combined with the spectrally red-shifted chimeric variant C1V1-ET/ET (a fusion of ChR1s from *Chlamydomonas* and *Volvox*), to achieve independent two-color photostimulation of two different cell types in the same animal [49].

6.3.2 Halorhodopsin and light-triggered proton pumps for photohyperpolarization

Halorhodopsin is a yellow-light-driven Cl^- pump that mediates photoinhibition in *C. elegans* [47]. However, NpHR shows poor membrane insertion in this system, so most of the synthesized protein never reaches the plasma membrane and for successful application, "strong" promoters are needed. Trafficking signals used in mammalian cells to improve this were, unfortunately, not recognized in *C. elegans* [50, 51]. Thus, as an alternative, outward directed H^+ pumps from *Leptosphaeria maculans* ("Mac") and Archaerhodopsin3 ("Arch") from *Halorubrum sodomense* were also introduced as hyperpolarizing optogenetic tools [51, 52]. These work very efficiently and respond to blue-green wavelengths, thus allowing inhibition either independent of, or concomitant with, ChR2 mediated activation.

6.3.3 Photoactivated Adenylyl Cyclase (PAC) for phototriggered cAMP-dependent effects that facilitate neuronal transmission

A qualitatively and quantitatively different type of neuronal stimulation can be achieved with photoactivated adenylyl cyclase (PACα) from *Euglena* [53]. This is a protein containing a BLUF domain (blue light sensor using flavin) and an adenylyl cyclase domain. The protein generates cAMP from ATP in a light-dependent fashion, and thus permits it to trigger processes that depend on this second messenger, most prominently to stimulate protein kinase A (PKA) activity. In neurons, PKA activity

appears to increase the rate of priming of synaptic vesicles (SVs), thus more SVs can be released upon neuronal activation. Accordingly, PAC activity accentuates intrinsic activity patterns, but does not override them, like the strong stimulation achieved via ChR2. In *C. elegans* cholinergic neurons, PACα activation led to exaggerated locomotion and an increase in the rate of miniature post-synaptic currents (mPSCs) [54]. However, as PKA has a multitude of downstream targets, it is not clear which other effects may be evoked by PACα photoactivation. PACα from *Euglena* has a comparably high dark activity, thus expression levels need to be carefully adjusted to avoid basal cAMP elevation that leads to compensatory effects. As an alternative, PAC from *Beggiatoa* has been introduced [55, 56], which has very low dark activity and is highly light sensitive due to a slower photocycle, and several labs are using this now in *C. elegans*.

6.3.4 Other optogenetic approaches

Other optogenetic approaches, *e.g.*, optochemical genetics, where endogenous ion channels or receptors are rendered light sensitive by modifying them with covalently tethered, photo-isomerizable ligands [57, 58], have not been described yet for *C. elegans*. Yet, this approach provides an additional route to triggering neuronal activity, and importantly, allows stimulating individual receptors at the post-synapse. This differs from ChR2-mediated pre-synaptic stimulation, which also spreads to other synapses innervated by the same neuron and may have further effects. This methodology requires carefully designed photoswitchable ligands and strategically positioned cysteines in the respective ion channel, to which the ligands are covalently linked.

An approach to trigger GPCR pathways using optogenetics has been described, in which visual rhodopsin is modified in its intracellular loops that bind and activate heterotrimeric G-proteins. When the respective loops from α- or β-adrenergic receptors were transferred, they mediated coupling to G_s or G_q proteins [59]. This has not been used for *C. elegans* yet, however, along these lines, mammalian rhodopsin and melanopsin were expressed in *C. elegans* motor neurons and coupled to $G_{i/o}$ or G_q pathways, respectively, affecting locomotion behavior [60].

6.3.5 Stimulation of single neurons by optogenetics in freely behaving *C. elegans*

To specifically influence neurons by optogenetics, one can generate animals that express the optogenetic tool in just the neuron of interest, and thus use straightforward whole-field illumination, as only the transgenic neuron will be activated. This can be achieved by expressing ChR2 or other optogenetic tools via conditional expression. Using two promoters whose expression patterns overlap in just the cell of inter-

est, a recombinase is expressed from one promoter to activate an otherwise silent construct for expression of ChR2 from the other promoter [4, 5, 6].

Often, however, single-cell expression is difficult to achieve, such that one ends up working with transgenic animals that express the respective tool not only in the neuron of interest. In such cases, one can restrict the light delivery to just the cell of interest, sparing other cells. Microscope systems have been developed that allow selective illumination of distinct neurons (or rather, body regions) in freely behaving animals. One such system uses an LCD video beamer to project a segmented binary image of the animal onto the body region of the animal that contains the respective neuron(s) of interest [61, 62]. The animal is tracked using a computer-controlled x-y translational stage, and the system is updated at a maximum frequency of 25 Hz, thus ensuring that the respective neuron is faithfully kept in the light. The system can transmit light of different colors and intensity via its three independent light paths, and it was used to analyze circuits for nociception [51, 63]. A second system with basically similar properties was developed (the software is faster; it updates at a maximum frequency of 50 Hz), and uses a digital mirror device (DMD) to reflect light from an expanded laser beam onto the specimen, to generate the light geometries needed for cell specific illumination [64]. This system was used to analyze the influence of proprioception during locomotion [18]. A third system described for selective illumination uses a spatial light modulator (liquid crystal on silicon chip), however this system does not track the animal automatically and thus the animal's movement was restricted in a microfluidic arena. This system was used to analyze the function of O_2 sensing neurons [65].

A recent, remarkable approach, that further developed the tracking and selective illumination systems described above, realized the generation of a virtual environment by using dynamic optogenetic feedback, depending on the automatically detected behavior of the animal [39]. First, the authors observed by Ca^{2+} imaging the activity of AIY interneurons, which act downstream of chemosensory neurons, in animals navigating in an odorant gradient. Then they used animals expressing ChR2 in AIY neurons to mimic the inferred activity of AIY in a virtual odorant gradient, while the animals crawled on a plate without any actual odorants. The tracking system registered the movement of the nose of the animal within the artificial gradient and increased or decreased the light intensity accordingly, when the animal turned its nose towards or away from the virtual chemoattractant source. An earlier attempt towards the goal of generating virtual environments using optogenetics was to "simulate" a region of aversive chemicals, *e.g.*, high osmolar solution. This was done by photoactivating the aversive chemosensory neuron ASH whenever the animals' nose entered the area of the sham aversive chemical [37].

6.4 Examples for optogenetic applications in *C. elegans*

6.4.1 Optical control of synaptic transmission at the neuromuscular junction and between neurons

Chemical synaptic transmission in *C. elegans* is generally believed to be graded. Thus, it can be conveniently studied using optogenetic methods, as one can stimulate neurons precisely, and cell-type specifically, with a light stimulus (in contrast to electrical stimulation), and the extent of neurotransmitter release can be finely tuned by the light intensity used. As readout, one can quantify evoked behavioral effects, *e.g.*, body contraction following stimulation of cholinergic motor neurons, or record photoevoked postsynaptic currents. Stimulation can be repeatedly done, allowing one to study plasticity. To analyze postsynaptic transmitter receptors, optogenetics allows stimulating transmitter release at synaptic sites only and in native amounts, in contrast to the frequently used pipette-application.

The first optogenetic studies of synaptic transmission were concerned with the neuromuscular junction (NMJ). At this synapse, muscle cells are innervated by cholinergic and GABAergic motor neurons. Liewald *et al.* [66] and Liu *et al.* [67] analyzed transmission from cholinergic and from GABAergic neurons using ChR2-mediated photostimulation. Graded transmission could thus be confirmed for these neurons [67, 68]. A number of pre- and postsynaptic mutants were analyzed, and phenotypes observed by optogenetic stimulation could be compared to those evoked electrically [66]. Postsynaptic ACh receptors at the NMJ were also investigated using optogenetics [67, 69], as well as a $GABA_B$ receptor [68]. Furthermore, PACα was used to photoevoke increased synaptic transmission; however, not by depolarizing the neurons, rather, by increasing the rate at which transmitter vesicles would fuse with the plasma membrane in response to depolarization, as well as increasing the amplitude of postsynaptic currents per release event, indicating higher transmitter content [54]. Additionally, two papers probed the role of gap junctions in the neuromuscular system [70, 71].

Interneuronal synaptic transmission has also been analyzed by optogenetics in *C. elegans*. Previously, such analyses were restricted to neuron-neuron synapses involving sensory neurons, to which a natural stimulus was presented, and the postsynaptic response was recorded. Photo-electrophysiology can be used to analyze interneuronal transmission without a natural stimulus, thus making "central" synapses accessible. Thus far, two different synapses were analyzed by optogenetics, namely between the thermosensory AFD neuron and the interneuron, AIY [72], as well as between the polymodal nocisensory neuron ASH and the premotor-interneuron AVA [73]. In both synapses it was found that transmission was graded, *i.e.*, transmitter release increased proportionally with increasing light intensity. This emphasizes that results obtained from synaptic transmission experiments using optogenetics will depend also on the amount of ChR2 expressed at the respective synapse, and that alterations in ChR2's peak current during prolonged or repeated stimulation have to be taken into account [66, 67].

6.4.2 Optical control of neural network activity in the generation of behavior

In addition to work on the chemotaxis circuit [39], described in Section 6.4, several other neuronal networks and their roles in behavior have been analyzed by optogenetics in *C. elegans*. The first neurons photostimulated were the touch receptor neurons (TRNs) [46]. Photostimulation caused escape behavior, and behavioral habituation was observed upon repeated optical stimulation, just as in mechanically evoked behavior. This could be achieved independently of the MEC-4/MEC-10 mechanoreceptor channel, emphasizing that habituation is not merely due to desensitization of this channel. The TRNs were subject to more detailed work using optogenetics, in which single TRN contribution to behavior was analyzed using patterned illumination [62, 64].

The TRNs evoke backward locomotion by influencing signaling in the premotor interneurons AVA, AVD and AVE, which control A-type motor neurons for backward locomotion, while signaling to the interneurons AVB and PVC mediates forward locomotion through activation of B-type motor neurons [74, 75, 76]. The locomotion premotor interneuron circuits, and other neurons involved in the control of forward versus backward locomotion, were subject of several optogenetic studies. The generally accepted role of the AVA and PVC interneurons to control backward and forward locomotion, respectively, have been confirmed by direct photostimulation or photoinhibition [4, 51, 62, 63]. Other cells through which backward locomotion can be influenced by optogenetics are the RIM interneuron [77], as well as a second circuit acting in parallel to the "classic" AVA B-type motor neuron circuit for backward locomotion, which involves inhibitory signaling from AIB to RIM [38]. How locomotion itself is evoked and influenced by motor neurons and how proprioception leads to propagation of the locomotory wave of contraction and relaxation, and influences the body posture, has been subject to an elegant study using optogenetics, imaging and carefully designed microfluidic devices, to manipulate parts of the animal's body, and of the nervous system [18].

Locomotion is also controlled by other sensory neurons. Aversive mechanical or chemical sensory perception, which can also be interpreted as nociception, leads to rapid escape responses. Here, the polymodal neuron ASH (and modulation of its output), as well as the harsh touch receptors FLP and PVD, were analyzed using optogenetics [37, 63, 77, 78]. For PVD, optogenetics were further used to identify and study genes acting within the nociceptor, downstream of the primary nocisensor molecules, for encoding and/or transmission of these signals to downstream circuits [63].

Further sensory modalities that were addressed by optogenetic manipulation (and other methods) are the sensory circuits for oxygen and CO_2 [65, 79], and for temperature [80], as well as circuits for mechanosensation used during male mating behavior [81].

6.5 Future challenges

6.5.1 Closed-loop optogenetic control and optical feedback from behavior and individual neurons

To fully understand how a neuronal circuit generates behavior, one would need to achieve true optogenetic *control* over a neuronal circuit, rather than optogenetic *perturbation*, as is currently the case. *Control* would require an "optical voltage clamp", which would allow suppressing all intrinsic activity, and instead imposing the activity from outside, using optical signals. This type of manipulation should be coupled with a non-invasive but fast feedback, such that activity patterns and the extent of signals could be monitored, and corrections or new control paradigms be imposed accordingly by the optogenetic manipulation, in a closed-loop. Furthermore, in this way, it would be possible to stimulate or inhibit particular cells and determine the effect on other neurons in the circuitry. What exactly do we mean by "closed loop optogenetics", and how could this be achieved?

In a first approximation, we can treat neurons in *C. elegans* as simple entities that receive excitatory or inhibitory input, and accordingly release more or less transmitter themselves, at all of their synapses, and/or influence other cells by gap junction electrical coupling. Control over membrane potential can be achieved using light-gated ion channels or pumps, like ChR2 or NpHR, Mac and Arch3. Some of these can be independently controlled by light of different wavelengths; the most spectrally separated are ChR2 and NpHR. If these proteins are co-expressed, and if the mediated photocurrents are strong enough, one could, in principle, very precisely regulate the membrane potential of that neuron using these two optogenetic tools. The neuronal membrane, of course, is not unaffected by such exogenously induced currents – rather, voltage gated Ca^{2+}- and K^{+}-channels (it is generally accepted that there are no voltage-gated Na^{+}-channels in *C. elegans*) will respond to this by currents of their own, which will affect the membrane potential. In order to compensate for this, one would have to be able to very quickly measure (at sub-millisecond timescales) the membrane potential, and induce compensatory light-gated currents accordingly. In a behaving animal, non-immobilized and non-dissected, this can only be realized with a fast optical voltage sensor, *e.g.*, genetically encoded, whose excitation and emission spectra would have to be far removed from the ChR2 and NpHR spectra, such that the optical readout of membrane potential does not *per se* interfere with the optogenetic actuators. Above all, the behavior itself also needs to be tracked and interpreted, such that feedback to the optically controlled neuron can be computed and integrated into the stimulus regime. This would require that training of the software needs to occur, such that for a given inferred activity, it can be predicted which behavioral outcome this may have. Similar to the work by Kocabas *et al.* [39], recording membrane voltage from the neuron of interest during spontaneous behavior may first be used to learn which natural activity patterns are associated with which type of behavior. A summary of these points is shown in Figure 6.1.

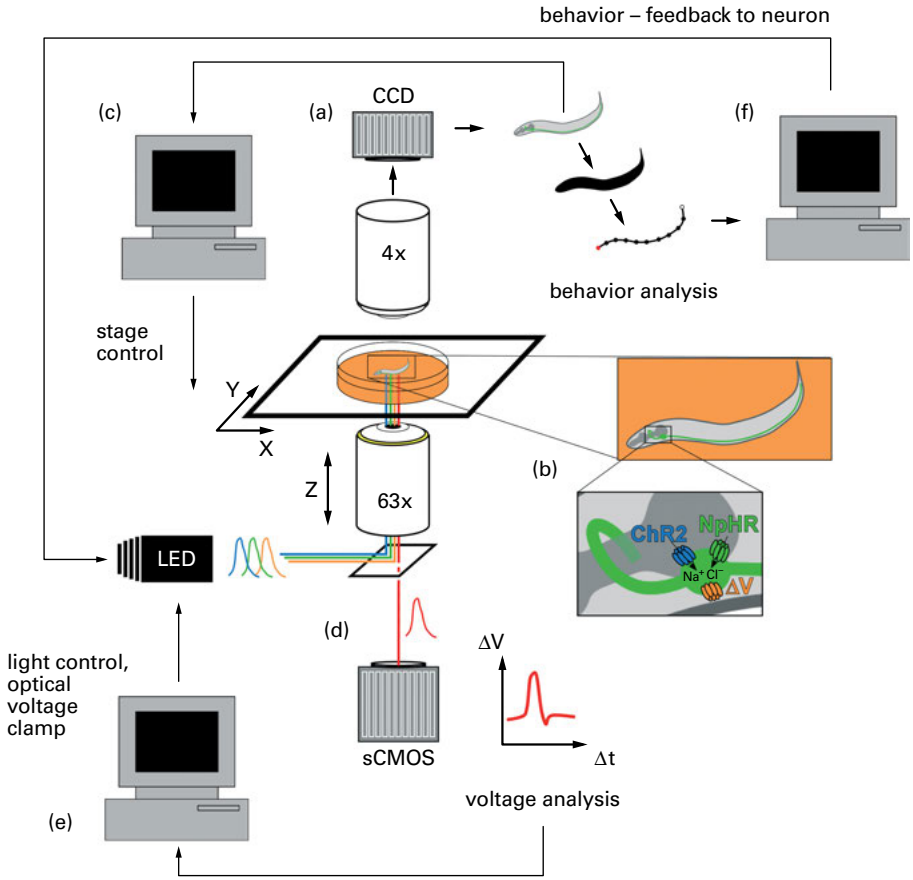

Figure 6.1: How closed-loop optogenetics could work. (a), (b) An animal expressing an optogenetic actuator (ChR2) and inhibitor (NpHR), as well as an optical voltage sensor (ΔV) is allowed to crawl on a culture dish. (c) The position of the animal is monitored by a low magnification objective and used to steer an XY translational stage, to follow the animal. (d) A high NA, high resolution objective is used to image voltage signals, and to guide light to the optogenetic actuators. This objective can be used to quickly scan through different focal planes, to image several neurons. (e) Voltage signals are used to quickly compute a feedback of light signals to the optogenetic actuators, to keep the neuron membrane potential at the desired membrane voltage. (f) Behavior can be analyzed on-line, to provide feedback signals to the actuators that would allow true optogenetic control of the behavior.

To truly recapitulate natural behavior this way, one likely has to incorporate information and control of several neurons simultaneously, a major challenge for both the molecular biology to generate the respective transgenic animal, but also for microscopy hardware, readout and light delivery techniques, tracking and most importantly, for software development that allows fast computing and rapid dynamic feedback to the light actuators in each of the neurons involved.

6.5.2 Requirements for integrated optogenetics in the nematode

What do we need to work out for a closed loop all-optical system for control of animal behavior?

Optogenetic actuators and inhibitors for optical voltage clamp and combination with GECIs:
Here, we may already have a good set of tools that allow us to achieve this type of control, particularly ChR2 and NpHR, whose action spectra are nicely distinct. These tools have been fused into a tandem protein [82], which allows for precise control of the relative expression levels, and reduces uncertainties in the extent of effects of illumination with each light wavelength, due to otherwise variable expression levels of either protein. Unfortunately, there is significant overlap in the action spectra of optogenetic modifiers and indicators; thus, recording neural activity with indicators such as GCaMP or cameleon can lead to inappropriate activation of channelrhodopsin in the same animal. To some extent this problem can be overcome by using patterned illumination and sophisticated optics to separately excite the neurons whose activities are modified and monitored [77]. However, in moving animals, or in circuits where the neurons are closely packed, this approach has limitations. Another option is to shift the activation spectrum of either the modifier or activator, typically to a longer wavelength. Some progress has been made on this front, with the development of red-shifted indicators such as RCaMP [35, 63] and R-GECO [34]. However, the signal-to-noise ratio of RCaMP is relatively low, and R-GECO is susceptible to photoconversion, which could lead to artifacts when used together with channelrhodopsin [35]. Thus, improved red-shifted indicators with better spectral separation and better sensitivity are clearly desirable.

Optical measure of membrane voltage:
This is a much more challenging problem, as currently existing genetically encoded voltage sensors provide rather small signals and are slow, and thus far have not been widely used in *C. elegans*. Since they generally do not produce action potentials, *C. elegans* neurons also do not exhibit very large voltage changes, making their resolution by voltage indicators even more challenging. The excitation and emission spectra of the currently available voltage sensors are also not compatible with independently using ChR2 and NpHR in the same cells, as the sensors are based on fluorescent proteins requiring excitation in the same spectral range [12, 83–85]. An exception may be the recently described finding that retinal in proteins like Arch3 or proteorhodopsin can act as a red-fluorescent sensor for membrane voltage [45, 86]. However, the fluorescence of retinal is extremely dim, such that it is not conceivable that this tool will produce sufficient signal in the tiny *C. elegans* neurons. One way to possibly increase the fluorescence, and to shift the spectrum to the far red, could be retinal analogs with altered π electron system; such chromophores can be simply

provided to *C. elegans* with the bacterial diet. They would, however, also be affecting the actuator rhodopsins (ChR2 and NpHR), unless they would be specifically incorporated in Arch and not the other proteins.

Integrated behavioral tracking, voltage imaging and on-line analysis software:
This is not a trivial task either, as the computations required to detect a worm, follow it, determine its outline, segmenting it to guide the light delivery, imaging voltage signals and interpreting them, and the interpretation of a particular behavior are time consuming. At the same time, these computations need to be fast, such that the system can update itself with at least a frequency of 25 Hz, 50 Hz or even faster. The hardware to achieve this may exist already, as several imaging and tracking systems have been developed; they may have to be wed in a fruitful way. Light delivery in at least two color channels is needed, and a third color channel needs to be used for voltage sensor excitation and imaging. Integrating all information will likely require novel and ingenious algorithms, and extremely fast computers.

Optics, microscopes, cameras:
Neurons of *C. elegans* are not found in the same focal plane, at least if they are to be imaged at somewhat high resolution, and high NA objectives are needed for imaging, particularly if the fluorescent signals are small. The field of view of such objectives is too small to image the whole animal and to track behavior at the same time, thus two optical systems are required, one at high and one at low magnification [37]. To image and to deliver light to neurons in different focal planes, fast scanning in the third dimension (z) will be required, generating an additional challenge. Fast cameras with high resolution are available now (sCMOS cameras), that allow high frame rates and can capture larger fields of view, allowing one to image bigger portions of the animal. They produce high volumes of image data, the handling of which requires large memory and powerful computers.

An all-optical imaging, tracking, and actuation system will be further complicated by the biology to be studied; additional, unknown neurons will influence the behaviors controlled via the chosen set of optically targeted neurons. This may involve physical synaptic connections that can be probed, and since they are mapped, the neurons potentially influencing the cells under study are known [1]. However, the "wireless network", *i.e.*, remote signaling by neuromodulators and neuropeptides, is much more difficult to monitor and to control, and thus the systems we laid out here will still be only marginally able to recapitulate signaling and network activity occurring during natural behavior.

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7 Putting genetics into optogenetics: knocking out proteins with light

7.1 Introduction

The disruption of gene function has been a central method for understanding biological processes for over a century. However, there are three major weaknesses to classical genetic analysis: pleiotropic mutations, strain lethals, and cell lethals. First, many genetic knockouts affect multiple tissues, which can complicate the interpretation of phenotypes for any particular cell. These pleiotropies may arise from non-autonomous effects from other tissues or broad nonspecific defects, such as general ill health. Second, some genes are mutated to lethality; that is, the mutants die during development, making it difficult to study the role of a gene at later time points. Third, some genes are required for the viability of a cell; for example, genes involved in basic cellular functions, like transcription and translation. A cell with such a mutation will die even as a mosaic in an otherwise wild-type animal. The most common methods to circumvent these drawbacks of traditional genetic analysis are mRNA destabilization by RNA interference, or inducible DNA mutations; for example, by using Cre/Lox recombination. These methods allow the study of gene function later in development in specific tissues. However, the temporal resolution of these methods is limited by the half-life of the protein of interest. Ideally, an experimenter would be able to instantaneously remove a protein from any cell of choice. Here we review methods for the induction of protein degradation, and speculate about the potential use of light to stimulate protein degradation.

7.2 Protein degradation

Methods for inducing protein degradation should satisfy multiple criteria to maximize their utility. First, the method should be rapidly inducible so that acute phenotypes can be studied. Second, protein levels should be reduced as close as possible to that of a genetic null. Third, the technique should be applicable to a broad range of protein substrates and model organisms. This list of requirements is ambitious and current technologies for inducible protein degradation are not yet ideal.

Perhaps the simplest and most direct method for disrupting the function of a protein would be to have a protease that is highly specific for the protein of interest, to simply cut the protein and inactivate it. The classic examples for such proteases are the clostridial neurotoxins [1, 2, 3]; these toxins are made by bacteria from the genus *Clostridium*, and are among the deadliest toxins known. These toxins are highly specific proteases for particular SNARE proteins that function in the release of neu-

rotransmitters at the synapse. They bind with extremely high specificity and cleave the target protein (Figure 7.1A). However, nature has not been so generous to provide a broad-range of specific proteases, so their use is limited. One could imagine engineering such proteases for new targets, but so far this approach has not been pursued.

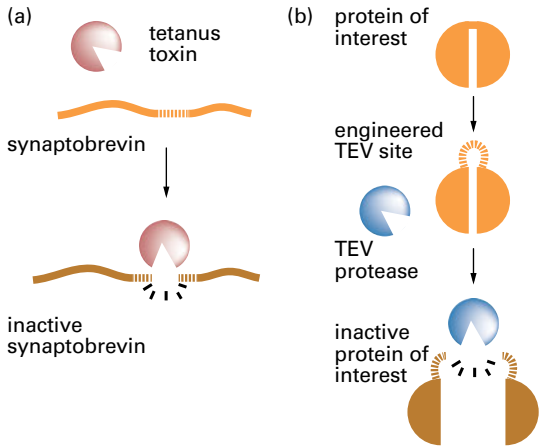


Figure 7.1: Sequence-specific protease destruction of proteins. (a) Tetanus toxin. Clostridial neurotoxins are made by bacteria that cleave specific proteins that function in neurotransmitter release. The tetanus toxin cleaves the SNARE protein synaptobrevin. (b) TEV protease cleavage. Tobacco Etch Virus (TEV) proteases cleave at a specific seven amino acid sequence. This sequence can be inserted into sequence and cleave the protein to disrupt function.

An alternative to developing target-specific proteases is to modify the target to be sensitive to a pre-existing protease (Figure 7.1B). For example, one could insert the seven amino target sequence for the Tobacco Etch Virus (TEV) protease into a gene. Transgenics that express this modified protein can then be targeted by expression of the TEV protease [4]. A problem with this method is that it does not generate a knockout, but rather two fragments of the target protein. These fragments can bind other proteins acting in the pathway and eliminate their function as well, thereby generating a more severe phenotype than the simple loss of the target protein. Alternatively, the fragments may have novel targets and act in a 'neomorphic' fashion on other molecular pathways.

A more interpretable result can be achieved not just by cleaving the protein, but instead by fully degrading it. Inducible protein degradation systems (or degrons) use the conserved ubiquitin–proteasome pathway to quickly degrade specific proteins. The ubiquitin pathway begins with the transfer of ubiquitin from the E1 ubiquitin-activating enzyme to the E2 ubiquitin-conjugating enzyme (Figure 7.2A). The E3 ubiquitin ligase recognizes both the target protein and the E2 conjugating enzyme and transfers the ubiquitin to a lysine in the target protein. At least three more ubiquitins must be added to the first ubiquitin modification to generate a poly-ubiquitinated substrate that is tar-

geted for degradation by the proteasome [5]. E3 ubiquitin ligases can be divided into two molecular classes based on conserved domains: HECT and RING [6, 7].

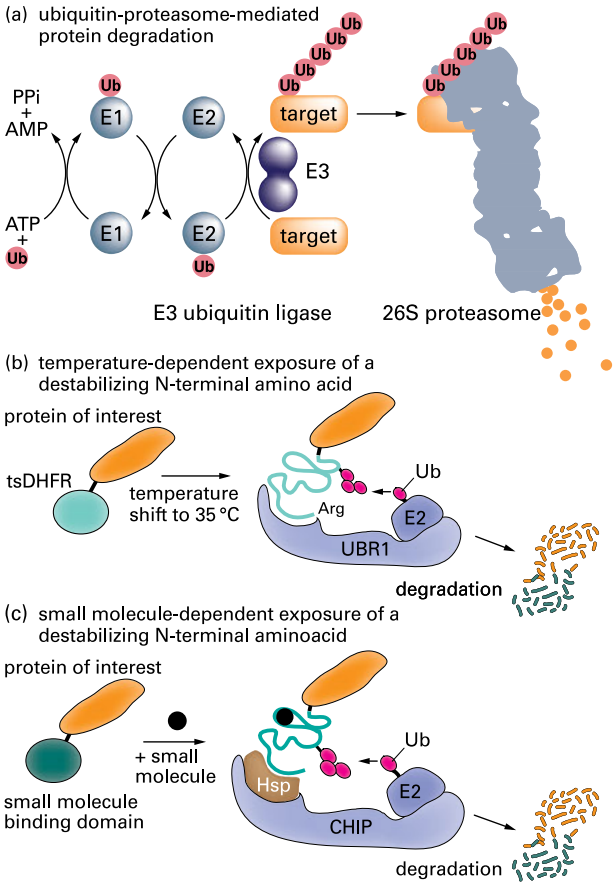


Figure 7.2: Ubiquitin-proteasome protein degradation. (a) The canonical ubiquitin-proteasome pathway. The E1-ubiquitin activating enzyme transfers ubiquitin to the E2 ubiquitin-conjugating enzyme. There are many E3 ubiquitin ligases and they add ubiquitin to specific substrates. Polyubiquitinated proteins are degraded by the proteasome. (b) Heat-inducible degron. The sequence for a temperature-sensitive dihydrofolate reductase (DHFR) sequence can be attached to the N-terminus of a protein. This sequence has an arginine at the N-terminus, which destabilizes proteins, but in this case the arginine is buried in the folded DHFR structure. A shift to 35 °C induces a conformational change in the tsDHFR, which exposes the N-terminal Arg. The E3 ubiquitin ligase, UBR1, binds the Arg-DHFR, recruits E2 Ub-conjugase, and promotes the poly-ubiquitination and degradation of the DHFR and the attached protein of interest. (c) Small molecule inducible degron. The FKBP domain undergoes a conformational change upon small molecule binding that exposes a degron. We presume that this results in binding by chaperones (Hsp) and recognition by the E3 ubiquitin ligase CHIP. The E3 ligase recruits E2 Ub-conjugase, which attaches a ubiquitin chain on the degradation domain, resulting in degradation of this domain and the protein of interest.

The RING finger E3 ligase family has been used to destabilize specific proteins [8]. These proteasome-dependent degrons differ in their method for inducing interactions between the E3 ubiquitin ligase complex and the target protein. The protein can be destabilized by: amino acids at the N-terminus of the protein that promote degradation; a sequence that can be unfolded by temperature; a sequence that can unfolded by a small molecule; or by binding the target protein to F-box proteins in the E3 ligase. We discuss each of these approaches below.

The UBR1 E3 ligase acts on proteins with destabilizing N-terminal amino acids according to the N-end rule [9]. Varshavsky and coworkers observed that the half-life of a protein is determined by the identity of its N-terminal amino acid and accessible lysine residues, now known as the N-end rule [10]. They placed the coding sequence for ubiquitin at the 5' end of a lacI linker sequence followed by the β-galactosidase (β-gal) gene. A yeast deubiquitinating protease rapidly cleaved off the ubiquitin, and a new N-terminal amino acid was exposed. The half-life of β-gal radically changed, depending on the identity of the N-terminal residue. For example, Met and Val N-termini resulted in a β-gal half-life of greater than 20 hours, whereas Arg and Phe N-termini showed a half-life of approximately 3 minutes [11]. Varshavsky and coworkers found that degradation was dependent on lysines found in the lacI linker that had been placed at the N-terminus of β-gal [12]. β-gal degradation relies on the UBR1 protein, which is an E3 ubiquitin ligase that recognizes destabilizing N-terminal residues and recruits an E2 ubiquitin conjugase to add poly-ubiquitin chains to the protein. These poly-ubiquitin chains mark the β-gal protein for degradation by the proteasome.

This unstable LacI sequence can be attached to a protein of interest to generate an unstable version of the protein [13]. Proteins tagged with this sequence are unstable and are degraded in about three minutes. However, this degron is not inducible. To generate a heat-inducible degron, Dohmen *et al*. screened for a variant of dihydrofolate reductase in which the N-terminus is only exposed at high temperatures (tsDHFR)[14]. The N-terminal acid in this sequence is a destabilizing arginine (Figure 7.2B). When the tsDHFR degron was fused to a protein of interest, the target protein was rapidly degraded at the non-permissive temperature [14]. The tsDHFR degron has been used to degrade proteins in yeast [7, 15] and at the *Drosophila* neuromuscular junction [16]. There are two limitations to this technology. First, the degron only destabilizes proteins at 35 °C, which limits its utility to organisms that can survive at this temperature. Second, the degron only works as an N-terminal fusion, which restricts its application to proteins that are functional with large N-terminal extensions.

An alternative to induction of the degron by elevated temperature is induction by small molecules or drugs (Figure 7.2C). The Wandless lab screened for DHFR and FK506-binding protein (FKBP) domains that are stabilized or destabilized in the presence of a small molecule [17, 18]. The destabilized domains can be fused to either end of a target protein, and addition of the small molecule either stabilizes the protein, or leads to target protein degradation. However, degrons of this type suffer from one major deficiency – they are slow. The time to degradation is reported to be greater than 4 hr

[17, 18, 19, 20]. This is most likely due to the mechanism of degradation. The destabilizing domain becomes unfolded when the molecule is removed [18, 19, 21, 22], or even more usefully becomes unfolded when the small molecule is added [17]. The unfolded protein is then likely to be recognized by chaperone proteins, which attempt to stabilize and refold it. In this model, the chaperone and target protein complex are bound by CHIP, which is a U-box E3 ligase, structurally related to the RING finger ligases [23]. The CHIP E3 ligase recruits the E2 Ub-conjugase, which poly-ubiquitinates the target protein and results in its degradation. CHIP recruitment is likely the limiting step; the unfolded protein can go through multiple rounds of attempted refolding by the chaperone before it is finally degraded [24]. If essential proteins are degraded slowly, the cell will undergo a prolonged and perhaps highly pleiotropic cell death. If researchers wish to study essential genes, a degron must be fast – a strength of the tsDHFR system, which can be achieved by more direct coupling to proteasome degradation.

The Cullin-RING complex ubiquitin ligases are particularly adaptable for the direct targeting of the ubiquitin-proteasome machinery to a protein substrate [8, 23, 25]. F-box proteins from E3 ligase complexes can be modified to act as protein-specific degrons [8]. The F-box domain protein binds the target protein and recruits the Cullin-RING complex, also called the SCF complex for its conserved constituents Skp1, Cullin, and F-box, which ubiquitinates the target (Figure 7.3A) [26]. Importantly, the F-box recruits all the required machinery for degradation; targeting a protein of interest to an F-box results in degradation within ~1 hr in yeast and mammalian cells [26]. For example, a 35aa segment from the human papilloma virus (HPV) binds the oncogenic retinoblastoma RB protein; this segment was fused to a Cullin-RING complex F-box protein. The HPV-F-box fusion protein caused the degradation of the retinoblastoma protein. In another example, an F-box was designed to specifically target and degrade β-catenin in trans [27]. The APC protein from the canonical Wnt signaling pathway, binds to β-catenin. Su *et al*. fused this binding domain to an F-box to make a chimeric F-box protein. They expressed this chimera in colorectal cancer cells, which targeted free β-catenin for degradation [27].

These methods are specific to a single target and are not inducible, but demonstrate that researchers need only recruit the F-box to a protein target to induce degradation. A more broadly applicable method is to use antibody fragments fused to the F-box protein to recruit the target to the Cullin-RING complex. For example, the Affolter group fused the coding sequence for a single-domain antibody targeted to GFP to an F-box [28]. When expressed in cells containing a GFP-tagged target protein, the anti-GFP domain binds the GFP moiety of the target protein and the Cullin-RING complex induces degradation of the protein (Figure 7.3A). This design is convenient for multiple reasons: first, GFP fusion constructions already exist for many proteins, second, the degradation is measurable by loss of fluorescence, and third, tissue-specific degradation is possible by expressing the antibody-F-box chimera in specific cells. Importantly, it has been shown that this degron works *in vivo* in *Drosophila melanogaster* larvae [28]. The method, however, is not yet inducible.

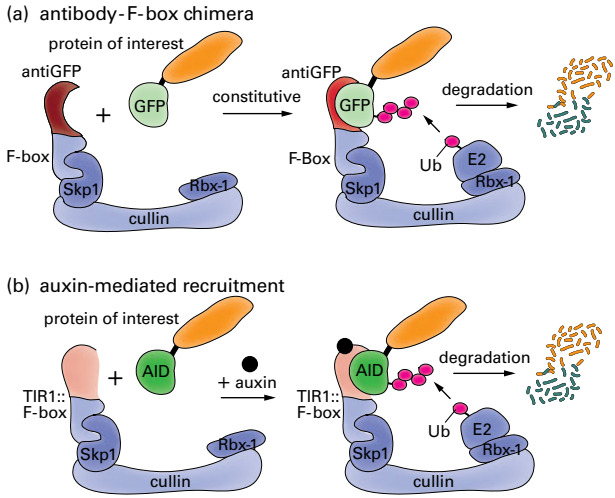


Figure 7.3: Cullin-RING ubiquitin ligases. Protein degradation is often mediated by interactions between F-box containing proteins and their specific substrates. (a) Antibody-driven recruitment. F box-antiGFP fusion protein binds to the GFP-tagged target protein. The F-box protein recruits the bound protein to the E3 ubiquitin ligase. The target protein is polyubiquitinated and degraded by the 26S proteasome. (b) Auxin-mediated recruitment. TIR1 normally targets transcriptional repressors for degradation via the Cullin-RING ubiquitin ligase. This interaction depends on the plant hormone auxin. The auxin-inducible degron (AID) can be fused to a target protein. When auxin is added TIR1 binds the domain and targets the protein of interest for degradation.

One F-box in particular is well-suited for small molecule induction of protein degradation. The F-box protein TIR1 (transport inhibitor response-1) degrades particular plant transcriptional repressor proteins in response to binding the plant hormone auxin [29, 30, 31, 32]. TIR1 only binds its target protein when the auxin is present, but on the other hand, it is not species specific, since it can interact with the E3 ligase protein Skp1 from yeast to humans (Figure 7.3B). Nishimura *et al.* fused the TIR1 targeting domain of the plant transcriptional repressors to a protein of interest and expressed it in transfected cells. They then expressed the F-box TIR1 in these cells and found that when they applied auxin that the target protein was degraded within an hour [33]. Importantly, auxin hormones are biologically silent in non-plant cells so that they are unlikely to have off-target effects. While this tool has been effectively used in yeast and cell culture from many organisms [34, 35, 36], it remains to be seen if this degron will be useful in more complex organisms.

The methods discussed so far are temporally regulated by either elevation of temperature (for example, tsDHFR is stable at 23 °C, but unstable at 35 °C), or small molecule induction. Most organisms cannot easily tolerate such temperature shifts, or cannot easily take up large concentrations of small molecules. Thus, these methods are largely limited to microorganisms or cell culture. Nor are these methods fast or

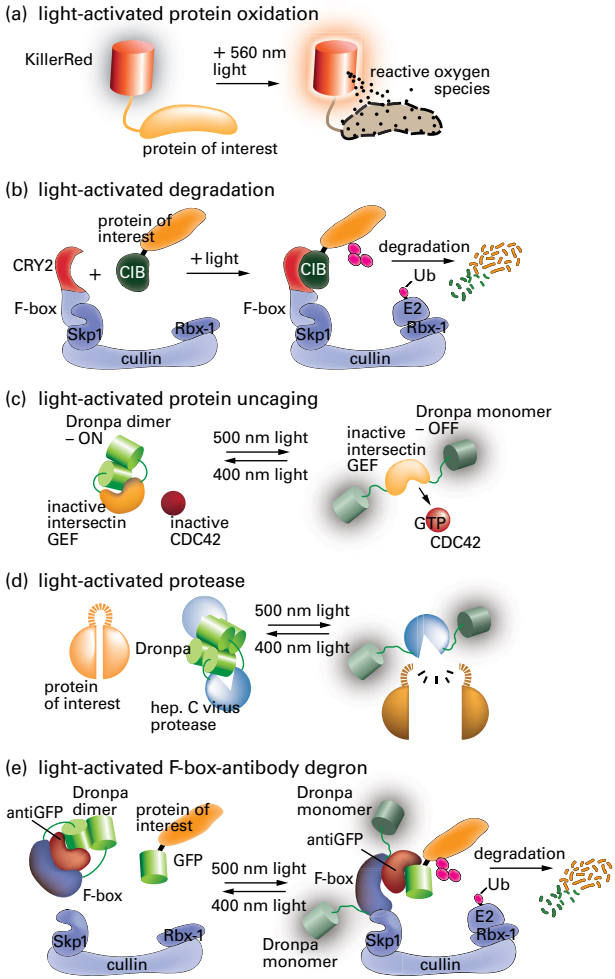
limited to specific tissues. Light-inducible methods of protein degradation could increase induction speed and degrade proteins locally.

7.3 Light stimulation

Investigators can deactivate proteins with light using photo-reactive organic dyes. This technique, chromophore-assisted light inactivation (CALI), uses dyes such as malachite green or fluorescein (FALI) [37, 38]. Stimulation of the chromophore generates short-lived reactive oxygen species, which can oxidize and inactivate proteins within a limited diameter. More recently, the fluorophore KillerRed provides an example of how light can be used to destroy proteins (Figure 7.4A). KillerRed is a red fluorescent GFP-like protein that releases reactive oxygen species when stimulated by green light. KillerRed can be fused to a protein of interest and then stimulation by light will generate reactive oxygen species, which destroy the protein, with spatial and temporal control [39]. A problem with protein inactivation using reactive oxygen species is that it is not specific and will damage proteins complexed with, or near the target protein. The radius of damage has been estimated to range from 3 nm to 50 nm [40]; to put these values into perspective, GFP has a diameter of 2.5 nm. In addition, KillerRed is cytotoxic and can also be used to induce cell death. Experimenters using KillerRed to target specific proteins should interpret results with caution due to nonspecificity and cytoxicity. A more precise method for disrupting protein function is needed; light controlled protein-protein interactions may be the solution.

There are a number of strategies for light-induced protein-protein interactions [41]. One could imagine generating a light-inducible degron using light-activated dimerization domains, in this case a gift – once again – from plants. The cryptochrome 2 (CRY2) protein from *Arabidopsis* dimerizes to the CIB1 protein when exposed to blue light [42] and this interaction occurs rapidly (within 1 second) even in mammalian cells [43]. One could fuse CRY2 to an F-box protein and fuse CIB1 to the target protein. Stimulation with blue light would cause these proteins to dimerize, and the target protein would be polyubiquitinated and degraded (Figure 7.4B). This technique may require optimization of the proteins in eukaryotes living at temperatures below 30 °C. At these temperatures, the CRY2 and CIB1 irreversibly dimerize after light stimulation [44]. However, any optimization may be worth the effort; the sub-second dimerization time of CRY2–CIB1 is orders of magnitude faster than heat shock or small molecule methods of induction.

A novel method to block protein function by light stimulation uses the conformational changes in a fluorescent protein to activate or inactivate the protein [45]. Specifically, Michael Lin's lab used the dimerization properties of the fluorescent protein Dronpa. Dronpa is fluorescently active as a dimer. Intense stimulation with 500 nm light causes Dronpa to dissociates into monomers that are in a dark state, that is they are no longer fluorescently active. The monomers will redimerize when stimulated by



400 nm light. Lin and coworkers fused Dronpa monomers to each terminus of the CDC42 GEF domain of intersectin (Figure 7.4C). These monomers could form a dimer by bringing the N- and C- termini together. This 'caged' conformation of the protein is inactive. Stimulation by 500 nm light caused the Dronpa dimer to fall apart into monomers. The uncaged protein was now functional, and enzymatic activity was observed. Thus, light could be used to switch on and off the activity of a protein directly. The disadvantage of this method is that each protein must be carefully engineered and tested to see if Dronpa dimerization will inactivate the protein, which might not be possible for some proteins. Moreover, many proteins will not tolerate the attachment of a fluorescent protein to both ends.

Figure 7.4: Controlling protein activity with light. (a) Light-activated protein oxidation. KillerRed is a fluorescent protein that emits reactive oxygen species when illuminated. Proteins in the neighborhood of KillerRed will become oxidized and nonfunctional. (b) Light-activated protein ubiquitin-mediated degradation. The CRY2 cryptochrome binds the CIB protein when stimulated by blue light. If CRY2 were fused to an F-box protein and CIB were fused to the protein of interest, then light stimulation would recruit the protein to the E3 ligase. The protein of interest would be ubiquitinated and degraded by the proteasome. (c) Light-activated protein uncaging. Dronpa is switched to a dark or OFF state by 500nm light and is switched to a fluorescent or ON state by 400 nm light. The OFF state of an engineered tandem dimer (145K-145N) is monomeric, and forms a dimer in the ON state. These Dronpa monomers were fused to the termini of the CDC42 GEF intersectin. Stimulation using 500 nm light stimulation caused the Dronpa dimer dissociate, and thereby uncaged the GEF. (d) Light-activated protease. Dronpa monomers were fused to both termini of the Hepatitis C Virus protease. The wild-type Dronpa monomers form tetramers that occulude the activity of the protease. 500 nm light stimulation causes monomerization and uncages the protease, which can now cleave the target sequence. In this imaginary example the protease cleavage sequence is inserted into a protein and cleavage generates an inactive protein. (e) Light-activated F-box-antibody degron. Dronpa monomers, fused to an antiGFP F-box, dimerize and cage the antibody recognition domain. When 500 nm light is applied, the Dronpa dimer falls apart into monomers, exposing the antibody binding surface. antiGFP then recruits the GFP-tagged target protein to the E3 ligase. In this specific example, the antiGFP antibody would need to be specific for the jellyfish-derived GFP, and not crossreact with the coral-derived Dronpa. The target protein is then polyubiquitinated and degraded by the 26S proteasome.

Lin and colleagues generalized the process by using Dronpa dimerization to inactivate a protease (Figure 7.4D) [45]. They used the hepatitis C protease since it exhibits no toxicity in mammalian cells. The protease target sequence was placed between a membrane tether and mCherry. Stimulation with 490 nm light switched Dronpa fluorescence off and caused the fluorescent proteins to adopt a monomeric configuration and activated the protease. Cleavage was monitored by measuring cytosolic mCherry, which peaked 60 minutes after activation. This approach was not designed to produce a protein knockout but rather to demonstrate that a protease could be activated. However, one could easily imagine combining this approach with that described above for the TEV protease. In short, the TEV protease target sequence, or the hepatitis C target sequence, could be engineered into a protein, such that cleavage would yield two nonfunctional targets and permanently inactivate the protein. On the other hand, this method would share some of the same disadvantages described above; specifically, cleavage would not mimic the loss of a protein but rather generate dominant negative protein fragments.

Alternatively, Dronpa dimerization could be combined with degron technology. For example, the anti-GFP F-box (see Figure 7.4E) can be fused to Dronpa monomers on either terminus. In the dark state, Dronpa dimers could either shield the anti-GFP antibody from recognizing its epitope, or Dronpa dimerization may prevent the F-box domain from binding the E3 ligase complex. Application of 500 nm light would dissociate Dronpa into monomers and restore an ‘open’ conformation of the anti-GFP F-box chimera. Anti-GFP could then bind the GFP-tagged substrate and the F-box could

recruit the E3 ligase complex and degrade the target protein. Additionally, one could apply 400 nm light to re-dimerize the Dronpa monomers, and inactivate the degron and reverse the phenotype. This method is worth developing because it has many advantages over current systems. First, light is the fastest potential method for the induction of changes in protein function. Second, light can be non-invasively applied to many organisms, with perhaps the exception of deep tissue in mammals.

Acute protein knockouts could allow the study of embryonic lethal and pleiotropic genes. Currently, degrons are limited by their speed of induction and applicability across systems. Light induction may be the solution. Optical control may allow rapid and cell-specific degradation of proteins. These methods could bypass some of the inherent weaknesses of the DNA mutations used in genetic analysis and disrupt proteins directly and on a rapid time scale. These methods could lead to a revolutionary tool for perturbation studies in biology.

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André Fiala

8 Optogenetic approaches in behavioral neuroscience

8.1 Introduction

Discovering the principles of how sensory stimuli, internally generated motivations, and learned experiences are integrated to produce behavioral actions in animals and humans, represents a fundamental challenge in neuroscience. One approach to uncover proximate, causal determinants of a particular behavior is based on the structural and functional dissection of neuronal circuits. In recent years, more and more techniques have been invented to manipulate individual elements of neuronal circuits in order to observe the effects of the interference on behavior, often with the help of transgene expression [1]. Among the variety of approaches to functionally dissect neuronal circuits, optogenetic and thermogenetic tools have raised a high degree of attention, and have already contributed to this field of research substantially [2]. The advantages of these tools are evident. First, in contrast to classical electrophysiological or pharmacological techniques, the neurons under investigation can be selected and targeted, based on a common genetic identity and, potentially, common function. Second, the neurons under investigation can be manipulated simply through illumination, which facilitates studies on freely moving, behaving animals. Although several optogenetic approaches have been described in recent years (reviewed in [3]), a breakthrough in optogenetics came with the characterization of the microbial, light-gated cation channel "channelrhodopsin-2" (ChR2) [4, 5], and the light-sensitive chloride pump "halorhodopsin" [6, 7], as tools to depolarize or hyperpolarize neuronal membranes through illumination (reviewed by [2, 3, 8, 9]). Since the first descriptions of these tools, a variety of variants of light-sensitive microbial opsins have been designed or discovered with genetic modifications that change or improve biophysical parameters of those molecules, *e.g.*, excitation wavelength, light-sensitivity, time constants of opening and closing, or conductance [9]. Since in relatively small animals, a rather complete illumination of brain tissue can be achieved without crude physical surgeries, behavioral observations can be conducted simultaneously with optogenetic manipulation of neuronal activity in intact organisms. For larger animals, *e.g.*, rodents, devices have been engineered that allow for delivering light into the brain without restraining the animal [10, 11, 12]. But which insights can actually be gained by means of optogenetic interventions, with regard to the mechanisms underlying behavior? At first glance, transiently bypassing the inputs to neuronal circuits and directly manipulating centrally located neurons at high temporal resolution might enable one to "remote-control" the activity of specific elements of a neuronal circuitry. However, being able to affect neuronal circuits in a controlled way and, thereby, to influence the behavior of animals, is of course not synonymous to under-

standing the circuitry or the neuronal determinants of behavior. Deciphering how neuronal circuits and their elementary constituents contribute to behavior requires, besides an anatomical and physiological characterization of the connectivity, tests to ascertain whether they are necessary and sufficient to drive the behavior of interest. Here, I would like to exemplify how modern optogenetic approaches can substantially facilitate addressing these questions. This is illustrated by means of examples from relatively small animals that provide distinct advantages as model organisms, when compared to mammals.

8.2 Approaches to dissect neuronal circuits: determining physiological correlations, requirement and sufficiency of neurons

The question of how neuronal circuits function and causally control behavior can be experimentally and theoretically subdivided. First, the interconnections of neurons within neuronal circuits must be described anatomically, and the physiological, dynamic properties of neurons in *correlation* with a particular behavior or with behavior-eliciting stimuli must be characterized. State-of-the-art microscopy techniques, *e.g.*, two-photon microscopy, in combination with genetic techniques [1] to target fluorescent marker proteins and functional sensor proteins [13] to specific cells, represent a rapidly advancing field. Second, one can aim at determining which neuronal circuits or constituents of a circuitry are *required* for eliciting or modulating a particular behavior, and third which are *sufficient* to do so. The determination of the requirement of a neuronal structure or function can be accomplished by *disruptive alterations* of neuronal circuits. Mechanically or chemically induced ablations of cells and tissue, but also pharmacological interventions at the molecular level and genetic alterations through mutations that disrupt neuronal function, fall into this category. All of these approaches are rather non-specific with respect to spatial and temporal precision. Hyperpolarizing stimulation electrodes have sometimes been used to silence neurons with less “collateral damage”. However, electrophysiological techniques usually preclude simultaneous behavioral tests and are, in most cases, restricted to immobile nervous system preparations. Transgenic approaches, *e.g.*, the ectopic expression of a potassium channel [14] or a toxin that disrupts synaptic transmitter release [15] can be used in intact animals, but the effects of the transgene are often not reversible. An example for an exception is the reversible, temperature-dependent block of synaptic transmitter release by ectopic expression of the dominant negative temperature-sensitive *shibire* protein in *Drosophila* [16]. The description of DNA-endoded light-sensitive chloride pumps (halorhodopsin) [5, 6] or proton pumps [17] has, however, marked a breakthrough because hyperpolarization of neurons can be achieved rapidly and reversibly in intact animals with unprecedented temporal precision. Conversely, determining the sufficiency of a neuron or

a group of neurons for a particular behavior can be assessed by *artificial mimicry* of its natural activity. This approach requires to bypass the "natural" input to a neuron and to directly activate it artificially. Of course, depolarizing stimulation electrodes have also provided the method of choice for many years, again with the disadvantage of being, in most cases, restricted to non-moving nervous system preparations. The description of ChR2 [4, 5] offered a solution to circumvent these problems. The two criteria of sufficiency and necessity neither exclude nor imply each other, as neurons can be sufficient, but not required, for eliciting a particular behavior, or vice versa. Combining the three approaches of correlative observation, test for sufficiency, and test for necessity, might enable one to determine causative roles of neuronal circuits and its constituents for behavior.

8.3 Optogenetic analysis of simple stimulus-response-connections

Very early concepts about the organization of animal behavior have focused on stimulus-response-connections, and in fact, simple reflexes represent cases in which researchers have come close to a characterization of the neuronal basis underlying behavior. Escape, startle, or withdrawal reflexes, by which animals seek protection from harmful stimuli or predators, are well suited for these studies, because they are typically very reliably and unambiguously executed at a certain stimulus threshold. Classical examples for studies on neuronal circuits underlying these stimulus-response connections and their experience-dependent modulation are the escape reflex in crayfish [18], the shadow reflex of barnacles [19], and the gill withdrawal reflex in *Aplysia* [20]. In an attempt to describe the principles of neuronal circuits mediating these types of stimulus-response connections, the concept of the "command neuron" or "decision neuron" has been formulated [21, 22]. Command neurons have been defined as those neurons whose activity is both necessary and sufficient for initiating the reflexive behavior [22]. The above-mentioned "classical" animal models used for such neuroethological studies have been chosen because their nervous systems, or preparations of them, are amenable to electrophysiological recording and stimulation. However, testing whether a neuron is sufficient or required to initiate the reflexive behavior is inherently difficult, and only correlates of behavior (*e.g.*, action potential trains in motor nerves or EPSPs recorded from motorneurons) can be monitored in nervous system preparations. This is one major reason why genetically tractable model organisms, in which transgenes can be stably expressed in specific groups of neurons, *e.g.*, encoding for optogenetic tools, provide a significant step forward. Here, invertebrate or small vertebrate animals, *e.g., Caenorhabditis elegans*, *Drosophila melanogaster*, or the zebrafish, *Danio rerio*, offer distinct advantages over mammals [23, 24, 25, 26, 27, 28, 29]. They all have in common that, first, the entire animal and its nervous system can be illuminated at once, and com-

plicated insertions of light guides into the brain are not required. Second, the sheer number of neurons, and thereby, the complexity of neuronal circuits in invertebrates or small vertebrates, is magnitudes lower than that of rodents or other mammals. Distinct functions can sometimes be assigned to very few, or even individual, identifiable cells. Shortly after the first description of ChR2, this tool has been used *in vivo* to elicit reflexive behaviors by optogenetically activating sensory neurons, *e.g.*, in *C. elegans* [30], larval [31] and adult *Drosophila* [32], as well as zebrafish [33], which demonstrated the feasibility of the approach. Subsequently, more complex, stereotypic behaviors have been investigated optogenetically, sometimes with different optogenetic approaches. In zebrafish, a command neuron controlling a central pattern generator has been investigated using the modified, light-gated glutamate receptor LiGluR [34]. Here, optogenetic activation of the so-called Kolmer–Agduhr cell drives swim-like rhythmic movements, whereas genetic silencing of these cells using tetanus toxin resulted in a reduced frequency of swim movements [35]. In *Drosophila*, neurons that trigger a central pattern generator that controls wing beat movements associated with the fly's typical courtship song has been characterized using an optogenetic technique [36]. Here, an ATP-dependent cation channel has been expressed that is opened by light-dependent uncaging of caged ATP injected into the animal. In *C. elegans*, optogenetic interference has helped to clarify neuronal circuits that produce rhythmic locomotion patterns [37]. Another type of relatively simple behaviors represents taxis movements guided by gradients of sensory stimuli. An example of how optogenetics can characterize the way in which sensory neurons guide taxis movements in *Drosophila* larvae has been reported by Bellman *et al.* [38]. *Drosophila* larvae are attracted by most odorants, and repelled only by very few. Bellmann *et al.* addressed the question of which olfactory sensory neurons (the larva has 21 per hemisphere) induce appetitive, positive taxis movements, and which ones induce repulsive, negative taxis locomotion. This question is difficult to address using natural odors, because most odorants activate overlapping subsets of olfactory sensory neurons, to various degrees. Therefore, ChR2 was expressed in distinct types of olfactory sensory cells, and odor stimuli were mimicked by blue light. The attraction towards the light, or the repulsion away from the light, was indicative whether the sensory neuron expressing ChR2 induces attractive or repulsive taxis behavior. These examples demonstrate how optogenetic tools can be used to address the question of whether distinct neurons are required and/or whether they are sufficient to elicit a particular stereotypical, reflexive behavior. The advance in comparison to more classical approaches relies on the specificity by which neurons can be targeted genetically, and by the fact that behavior can be observed simultaneously with the neuronal manipulation. This applies also for investigations of experience-dependent changes in behavior, *i.e.*, learning and memory.

8.4 Optogenetic and thermogenetic analysis of modulatory neurons: artificial mimicry of relevance

Associative learning relies on the temporal contiguity of a stimulus with a salient, relevant stimulus. In the case of classical conditioning [39], a relatively neutral stimulus is temporally paired with a rewarding or punitive stimulus. As a consequence, the animal learns to assign relevance to the formerly neutral stimulus, and adjusts its behavior in response to it accordingly. If one aims to analyze which neurons mediate the relevant, reinforcing information induced by the rewarding or punitive stimulus, one can again subdivide the question into which neurons are sufficient and which ones are required to do so. In fact, this question has also been exceedingly studied at the hand of "classical" model organisms and learning paradigms, *e.g.*, the experience-dependent modulation of the gill-withdrawal reflex in *Aplysia* [40], or olfactory conditioning of the proboscis extension reflex in the honeybee [41]. In both cases, pharmacological and electrophysiological techniques were applied to test which neurons and modulatory transmitters mediate the reinforcing information during training [40, 41]. In the course of these extensive studies, it has been revealed that the reinforcing information evoked by the salient stimulus is mediated by neurons releasing biogenic amines as transmitters. In the case of the gill withdrawal reflex in *Aplysia*, the salient aversive stimulus, an electric shock on the tail of the animal causes release of serotonin which facilitates the reflex circuit [42, 43, 44]. In the case of the honeybee, a sugar stimulus causes release of octopamine from an identified neuron, whose electrophysiological activation can substitute for a rewarding sugar stimulus during olfactory learning [45, 46]. Of course, these experiments had to be performed on immobile preparations, with the neurons or the entire brains exposed. Based on these "classical" experiments, equivalent questions have recently been addressed in *Drosophila*, which now offers the possibility to activate or silence neurons in intact, behaving animals [23]. Both larval and adult fruit flies can be trained to avoid an odor that has been temporally paired with a punishment. In the case of adult flies, an electric shock is typically used as punishment [47]; in the case of larval flies, an unpleasantly high salt solution is used [48]. Conversely, the animals can learn that an odor predicts reward, if it occurs simultaneously with sugar [48, 49]. A large body of experiments has demonstrated that biogenic amines, and the respective neurons releasing it, are involved in associative learning [reviewed in 50]. In particular, a seminal study by Schwärzel *et al.* [51], has clearly demonstrated that a distinct population of neurons that release dopamine is required for aversive odor learning. Conversely, a mutation that disrupts the synthesis of the transmitter octopamine impairs appetitive odor learning. The hypothesis that the respective neurons are also sufficient to mediate reinforcing effects during the learning process was first optogenetically tested in *Drosophila* larvae [52] (Fig. 8.1).

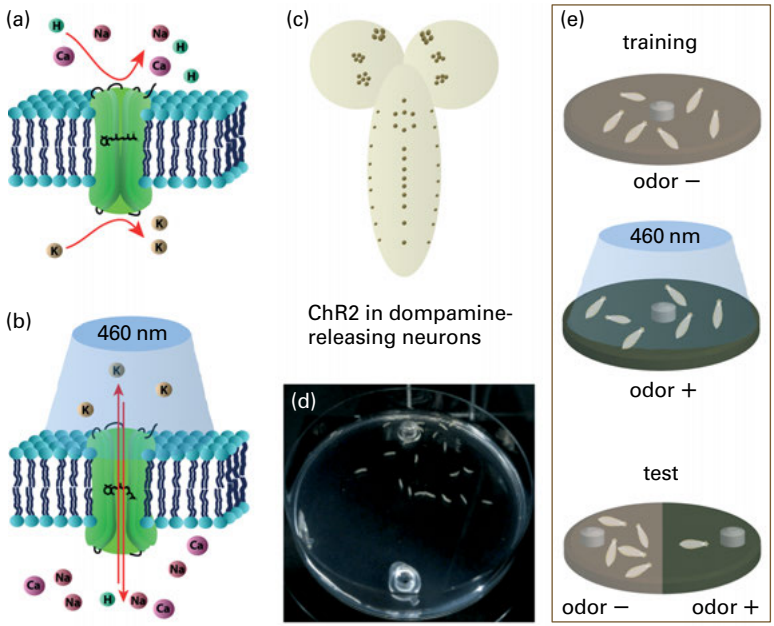

Figure 8.1: Substitution of a punitive stimulus through optogenetic activation of a set of dopaminergic neurons triggers associative learning in *Drosophila* larvae. A) Schematic illustration of the light-sensitive cation channel "channelrhodopsin-2" (ChR2). B) Absorption of light at ~ 480 nm causes a conformational change that in turn results in the opening of the channel. If Ch-R2 is expressed in neurons membrane depolarization is induced upon illumination. C) Schematic depiction of the distribution of dopamine releasing neurons in the nervous system of *Drosophila* larvae. D) Olfactory choice behavior in a classical olfactory conditioning paradigm: Larvae are placed between a trained and a control odor and are allowed to distribute freely across the plate and to choose between the two odors. E) In a modified, optogenetic classical conditioning paradigm groups of animals are placed in a dish half-filled with agarose in a dark box. One odor (odor-) is applied in darkness; a second odor (odor+) is applied simultaneously with the optogenetic activation of dopaminergic neurons. In a subsequent test situation the animals can chose between the two odors. If the activation of the particular subset of dopaminergic neurons is temporally paired with one odor (odor +) an aversive memory for that smell is induced [52].

In transgenic animals, ChR2 was expressed in a subset of dopaminergic neurons in the larval brain. When the animals were exposed to an odor and dopaminergic neurons depolarized through illumination, the animals acquired an aversive memory for that odor. Conversely, when ChR2 was expressed in octopaminergic neurons, and those cells were optogenetically activated in coincidence with the application of an odor, the animals acquired an appetitive memory for that odor [52]. The approach to optogenetically induce learning has also been conceptually transferred to associative learning in adult *Drosophila*, although a different tool was used [53]. But also in this case, optogenetic activation of a subset of dopaminergic neurons, simultaneously with an odor, caused the formation of an aversive odor memory. Using a thermoge-

netic technique, *i.e.*, through expression of the temperature-sensitive cation channel dTRPA1 [54] by which neurons can be depolarized dependent on the temperature, the neuronal subsets mediating aversive and appetitive reinforcing information, have been further disentangled [55, 56, 57, 58]. In conclusion, optogenetics and similar techniques, *e.g.* thermogenetic tools, are helpful methods to address the question about sufficiency of neurons in the context of learning and memory.

8.5 Conclusion

The invention of optogenetic tools has driven behavioral neurobiology substantially forward. For clarifying the role of neuronal circuits, or parts of it, in behavior one needs to test whether they are required, and whether they are sufficient, to drive the behavior under investigation. In the past, experimental tests of these questions, in most cases, involved interventions into the nervous tissue, and so precluded a simultaneous readout of behavior. Optogenetic tools, in combination with transgenic animals, help to circumvent these problems and allow one to manipulate neuronal activity simultaneously with behavioral observations. In this regard, invertebrates or small vertebrate animals offer particular technical advantages.

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9 Combining genetic targeting and optical stimulation for circuit dissection in the zebrafish nervous system

9.1 Introduction

The larval zebrafish offers unique opportunities for optogenetic investigations of neural circuits and behavior due to both optical and genetic accessibility. Here, we discuss optogenetic tools available in zebrafish and identify the technical obstacles that need to be overcome to take the fullest advantage of this system. A major focus of this review will be on the insights, gained through optogenetic analysis, into the contribution of defined circuits and cell types to zebrafish behavior.

9.2 Zebrafish neuroscience: Genetics + Optics + Behavior

Optogenetics provides a powerful approach for dissecting neural circuits and their contributions to behavior [1]. The larval zebrafish is a superb system, not only in the field of neuroscience in general [2], but also in the field of *in vivo* optogenetics, for several reasons. First, zebrafish embryos and larvae are optically transparent, and thus ideal for optical stimulation *in vivo*. Second, the size of the entire larval zebrafish brain is small; at five days post fertilization, it is maximally 400 µm wide, less than 500 µm thick, and 1.3 mm long [2]. Thus, the entire brain of this vertebrate is about the size of a single ocular dominance column in cat visual cortex or a few barrels in the rodent somatosensory cortex. The small size, together with its translucency, enables imaging of the entire depth of the brain by two-photon microscopy *in vivo*, while retaining the ability to resolve single cells. This is particularly advantageous for investigating response dynamics across neurons *in vivo*. Third, excellent genetic tools are available in zebrafish. It is possible to express optogenetic sensors or actuators in genetically-identifiable cell types using transgenic zebrafish, generated by promoter-specific or enhancer/gene trap techniques. Finally, larval zebrafish exhibit a rich repertoire of behaviors (Table 9.1). Already at larval stages, basic sensory functions, such as vision, auditory and vestibular senses, and somatosensation, elicit appropriate motor responses. For example, zebrafish larvae display different types of visually-evoked behavior, including compensatory oculomotor and locomotor responses to whole-field motion, pursuit of prey, and collision avoidance [3]. Together, a unique combination of experimental advantages makes larval zebrafish eminently suitable for optogenetic approaches.

Table 9.1: List of behaviors in larval zebrafish (modified from Nevin *et al*. [3])

Behavior	Description
Visual startle	Fast start swimming after changes in ambient light levels
Photomotor response	Muscle contractions in response to bright light
Visual background adaptation	Neuro-endocrine response of pigment cells to ambient light levels
Circadian photoentrainment	Responses in locomotor activity to the natural light-cycle (sleep/wakefulness)
Phototaxis	Swimming and turning toward a light source
Rheotaxis	Aligning body axis against constant water flow; dependent on lateral line and vision
Optokinetic response	Slow eye movements following moving visual stimulus; punctuated by saccades
Optomotor response	Swimming and turning in the direction of moving visual stimulus
Vestibulo-ocular reflex	Compensatory eye movements during head movement; dependent on otolith and tangential nucleus neurons
Escape response	Rapid swimming and turning away from acoustic, tactile or visual stimulus
Visual avoidance	Swimming away from large moving objects
Prey capture	Complex behavior involving turns, tracking swims and fast capture swims in pursuit of small prey

9.3 Genetic targeting of optogenetic proteins to specific neurons

Optogenetic studies often require reproducible targeting of effector proteins to genetically-identifiable populations of neurons. In zebrafish, this can be achieved by generating stable transgenic lines, using a gene-specific promoter/enhancer element or a recombineered bacterial artificial chromosome (BAC), all of which can be efficiently integrated into the zebrafish genome by Tol2 transposition [4]. In addition, two-component expression systems such as Gal4/UAS, Cre/loxP, and Tet systems have also been adapted to zebrafish. Among them, the Gal4/UAS system has been most widely used, and hundreds of Gal4 enhancer- and gene- trap lines have been generated by several groups and made publicly available for the community (www.zfin.org) [5, 6].

One major challenge of transgene expression systems is that Gal4 expression patterns in transgenic zebrafish are often less specific than desired, and include multiple regions and types of neurons. Intersectional strategies, such as the combination of Gal4 with the Gal80 repressor or split Gal4, are promising approaches to label more specific subpopulations of neurons. In addition, the combination of different two-component systems could conceivably refine the expression patterns of the optogenetic effectors in space and time. Application of sophisticated genetic techniques should further expand the possibilities for precise targeting in zebrafish.

9.4 Optical stimulation in behaving zebrafish

In zebrafish, optical stimulations of optogenetic effectors are often conducted in semi-restrained preparations, in which zebrafish larvae are immobilized in agarose, and part of the agarose is removed to free the tail or the eyes, for monitoring behavior with a high-speed camera. This preparation provides a convenient means to spatially restrict the illumination volume to a defined region of the brain, while the animal is performing behavior. Importantly, due to the transparency and small size of the larval zebrafish brain, most brain areas can be efficiently photostimulated without any invasive surgery. Naumann *et al.* introduced a method to record activity of genetically targeted neurons in unrestrained zebrafish larvae using the bioluminescent reporter Aequorin [7]. A method for optogenetic manipulation of neurons in freely swimming zebrafish, however, has yet to be developed.

Several options for the optical stimulation of optogenetic actuators have been used in zebrafish, including optic fibers, single photon, and two-photon illuminations [8]. Wyart *et al.* used patterned light stimulation based on a digital micromirror device (DMD) to photostimulate a small number of neurons on only one side of the spinal cord [9]. This DMD-based method will be useful in the future for illuminating multiple regions in a desired spatial pattern and temporal sequence. Two-photon stimulation offers advantages over DMDs in terms of the precise axial (z-axis) resolution for light delivery, although it is still technically challenging, due to the small excitation volume obtained with two-photon illumination and the small photocurrent that optogenetic actuators can generate. Several groups are working to refine the two-photon illumination and/or develop new spatial light modulation methods tailored to the zebrafish preparation [10].

9.5 Annotating behavioral functions of genetically-identified neurons by optogenetics

Application of optogenetic techniques in zebrafish has started to elucidate functions of genetically-identified neurons in the intact nervous system [11]. In our view, this approach has, so far, been the major contribution of zebrafish to neuroscience. Below we summarize several key discoveries of recent years (Figure 9.1).

9.5.1 Spinal cord neurons (Rohon–Beard and Kolmer–Agduhr cells)

Rohon–Beard (RB) neurons are mechanosensory neurons in the spinal cord that have been implicated in touch-evoked escape behavior. Two studies optogenetically activated RB neurons following genetic targeting of either the light-gated glutamate receptor (LiGluR) [12] or channelrhodopsin2 (ChR2) [13] to RB neurons. Interestingly,

single spikes evoked by photostimulation of ChR2 in single RB neurons were sufficient to elicit escape responses [13]. In proof of principle experiments with LiGluR, Szobota *et al.* showed that the bilateral activation of RB neurons causes paralysis in fish larvae [12]. Wyart *et al.* took further advantage of specific Gal4 lines and investigated the contribution of other types of neurons in the spinal cord [9]. When a specific class of GABAergic, cerebrospinal fluid contacting neurons, the Kolmer–Agduhr (KA) cells, was activated with LiGluR, zebrafish larvae displayed symmetrical tail beats that resembled forward swimming. This behavior was distinct in tail beat amplitude and frequency from the RB-induced escape swim. It was also distinct from the short tail flicks induced by activation of spinal motoneurons. This work identified KA cells as an important spinal input to the central pattern generator circuitry during locomotion.

9.5.2 Hindbrain motor command neurons

Halorhodopsin (eNpHR) and ChR2 have been used successfully in zebrafish to dissect the contributions of neurons in the hindbrain to premotor circuits directly linked to eye or tail movements. These studies identified non-overlapping populations of: a) command neurons for forward swimming in the caudal-most hindbrain; b) saccade generator neurons (burst neurons) in rhombomere 5; and c) oculomotor integrator neurons in rhombomere 7/8 [11, 14]. These studies are revealing the highly conserved premotor networks in the vertebrate brainstem and promise to yield new insights into motor control.

9.5.3 Tangential neurons in the vestibular system

Vestibular signals generated by the head or body movements evoke compensatory eye movements, called vestibulo-ocular reflex (VOR), to stabilize gaze. In larval zebrafish, vestibular information detected in the otolith organ of the inner ear is sent to a group of neurons in the hindbrain, the tangential neurons, which then project to motor/premotor areas, such as ocular motoneurons and the reticulospinal network. By optically labeling tangential neurons using photoactivatable GFP followed by targeted laser ablation of the same population, Bianco *et al.* showed that the tangential neurons are responsible for rotational VOR [15]. This study demonstrated for the first time the functional role of the tangential neurons in processing vestibular signals in guiding the VOR.

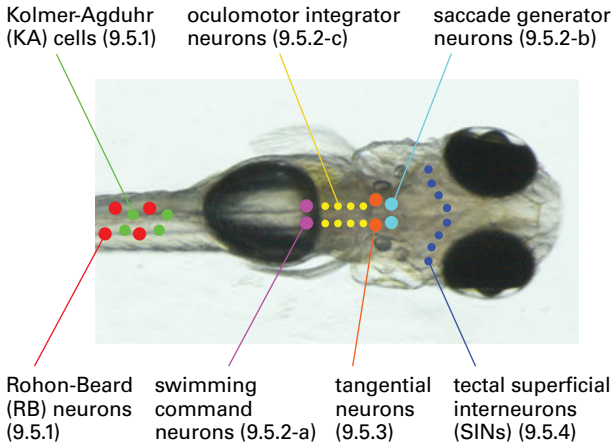

Figure 9.1: Optogenetic annotation of neuronal functions in larval zebrafish. The corresponding sections in the text are given in parentheses.

9.5.4 Size filtering neurons in the tectum

Several genetically-encoded calcium indicators, including GCaMP, have been employed in zebrafish [11]. Del Bene *et al*. imaged neural activity in the optic tectum with GCaMP3 to investigate a characteristic response property of the deeply situated tectal neurons – their tuning to small, moving objects in visual space [16]. To understand the neural basis of this size selectivity, this study focused on a genetically-identified population of GABAergic interneurons (superficial inhibitory neurons, SINs) located in the superficial layer of the tectum. Strikingly, SINs preferentially responded to large visual stimuli. Ablation of SINs using the photosensitizer KillerRed abolished the small-spot selectivity of deep tectal neurons. Thus, the combination of optophysiology and optogenetic ablation in the zebrafish tectum uncovered a candidate neural circuit with a specific function in the processing of visual information.

9.5.5 Whole-brain calcium imaging of motor adaptation at single-cell resolution

A recent study using two-photon calcium imaging revealed a brain-wide activity map during motor adaptation in larval zebrafish [17]. Ahrens *et al*. imaged a large population of neurons in a transgenic fish expressing GCaMP2 driven by a panneuronal promoter, while the animal navigated in a virtual environment. Visual feedback was fictively altered in response to attempts by the immobilized fish to change its motor output. With this "closed-loop" configuration, the authors identified neural populations that were activated during specific phases of adaptive locomotion. This study

showcases the power of *in vivo* whole-brain imaging in larval zebrafish to study motor adaptation.

9.6 Future directions

In the near future, technical advances can be expected in the following areas:
- Refined targeting of transgenes (*e.g.*, intersectional expression systems)
- Improved resolution of optical stimulation (*e.g.*, spatial light modulation)
- Non-invasive, "touch-free" optogenetics in freely swimming zebrafish larvae
- Simultaneous optogenetic manipulation, calcium imaging and behavioral recording for functional circuit mapping.

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10 Optogenetic analysis of mammalian neural circuits

10.1 Introduction

The identification, characterization and development of optogenetic probes, described extensively in the previous chapters, have provided us with a remarkable set of tools for probing brain function. The resulting "optogenetic revolution" has captured the attention of a whole generation of scientists working at different levels of nervous system function. The power of the optogenetic approach is particularly appealing for those working at the levels of neural circuits and neural systems in the mammalian brain. This is because circuits and systems pose special challenges – and offer remarkable opportunities – for experimenters seeking to understand the functional organization of the mammalian brain. Neural circuits and systems are at the interface between the cellular and molecular levels of analysis and higher-level functions such as behavior and cognition; thus, any mechanistic understanding of brain function must embrace the level of the circuit as an essential bridging element. However, not only are mammalian circuits incredibly complex, consisting of diverse cell types with elaborate morphologies linked by intricate webs of synaptic connections; but activity patterns in neural circuits during behavior take place on the millisecond timescale and engage thousands to millions of neurons. It is partly because of

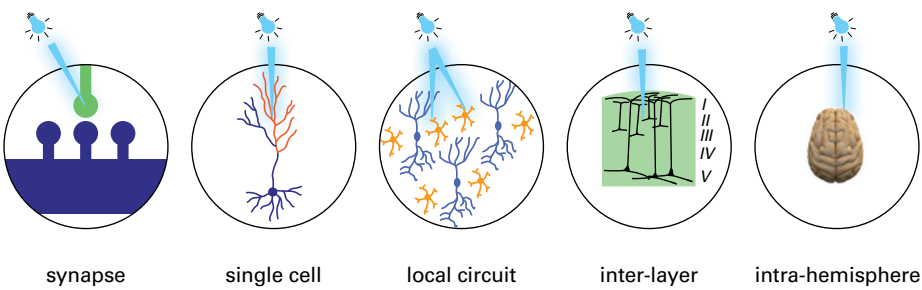

Figure 10.1: Levels of optogenetic manipulation in mammalian neural circuits. A schematic illustration of the different levels at which optogenetics can be used to probe function in neural circuits. From left to right: investigating the function of single synapses; probing dendritic excitability, and determining the subcellular mapping of synaptic inputs; activating or inactivating specific groups of neurons within a neural circuit; activating particular layers in cortical structures; investigating information transmission between the two hemispheres.

this complexity that optogenetics holds such promise, because it offers the possibility of precisely targeted interventions both in space and time. Figure 10.1 illustrates the various levels at which optogenetic strategies can be used to manipulate function within mammalian neural circuits. In this chapter we will discuss how optogenetics has helped us to address fundamental questions at each of these levels, and also outline some of the challenges that remain, both for interpreting existing experimental data, and in designing new probes and approaches for maximizing the power of optogenetic intervention.

10.2 Optogenetic approaches to probe integrative properties at the cellular level

All neurons in the central nervous system integrate thousands, to tens of thousands, of synaptic inputs to form an all-or-none action potential output that is the key unit of information transfer between neurons. The integration of complex temporal and spatial input patterns arising from this multitude of excitatory, inhibitory and modulatory synaptic contacts is arguably the most important characteristic of neuronal function in the context of a network. Nevertheless, the principles governing the input-output transformation performed by neurons are far from understood. The following examples provide key areas where optogenetic approaches may prove particularly useful to unravel fundamental principles of dendritic integration.

10.2.1 Excitatory signal integration at dendrites

When considering the comparatively simple case of dendritic integration of excitatory input in isolation, the spatial organization of inputs (*i.e.*, clustered versus distributed), their strength, and their distance to the soma are crucial factors in determining their efficacy in generating neuronal action potential outputs [1]. Moreover, the dendrites act as non-linear integrators. The active conductances, which are dependent on voltage-dependent Na^+ channels, Ca^{2+} channels, K^+ channels, hyperpolarization-activated cyclic nucleotide-gated (HCN, I_h) channels, and N-methyl-D-aspartate (NMDA) receptor channels, contribute to the generation of back-propagating action potentials, the focal elevation of intradendritic Ca^{2+}, the shaping and boosting of synaptic potentials and membrane resonance [1]. Many of these channels, and thereby dendritic integration, are strongly modified by neuromodulators such as, *e.g.*, acetylcholine or dopamine. A quantitative assessment of dendritic integration that addresses its complex features requires the stimulation of specific synaptic inputs with precise spatial and temporal control. Multiphoton uncaging of glutamate has been used for this purpose and has provided insights into fundamental rules of excitatory input integration in dendrites [2, 3, 4]. However, this

approach suffers from the shortcoming that: i) stimulation of excitatory synapses with multiphoton uncaging techniques may differ in some important aspects from their activation by synaptically released glutamate; and ii) multiphoton uncaging does not allow one to distinguish excitatory inputs arising from different presynaptic neuronal populations. Optogenetic approaches may be useful to overcome these difficulties. Firstly, it is feasible to activate individual presynaptic terminals expressing ChR2 by targeted scanning or patterned illumination, opening the possibility to use synaptically released glutamate to generate input patterns onto dendrites [5, 6]. Secondly, and perhaps more importantly, these approaches can be combined with genetic targeting strategies that confine ChR2 to defined populations of input axons [5]. This will in the future be an important approach to disentangle the function of different types of excitatory synapses, and how they interact.

10.2.2 Control of excitatory signal integration by inhibition or neuromodulation

The modulation of excitatory signal integration by modulatory and inhibitory inputs is complex, and many key issues regarding the interaction of these different input forms have remained unclear. This is primarily due to the difficulties inherent in achieving a precise spatial and temporal control of both excitatory and inhibitory / modulatory inputs on neuronal dendrites in the complex micro-environment of native brain tissue, while simultaneously recording from the stimulated neuron. Stimulation of defined inhibitory neuron types can be achieved with optogenetic techniques, and can be combined with synaptic stimulation or iontophoresis techniques to activate excitatory inputs [7]. A particularly interesting development will be the advent of caged glutamate compounds that do not interact with GABAergic transmission, this would allow combining multiphoton uncaging and optogenetic approaches. A further important development will be the generation of optogenetic tools that allow independent stimulation of two neuron types at different wavelengths [8]. This would allow for an all-optical stimulation experiment that addresses the interaction of excitatory, inhibitory or modulatory input systems. The technologies to perform multicolor activation of different neuron types in parallel are, in principle, available (*i.e.*, using DMD technology) [9, 10, 11]. In contrast, there is a need for well-tolerated red-shifted opsins for neuronal activation. While proof-of-concept experiments have been published with red-shifted mutants [8, 12, 13], the “therapeutic window” of the published mutants is still very small: Too little expression will prevent reliable spike induction, while high expression levels compromise cell survival, at least in mammalian neurons. Especially for chronic and *in vivo* experiments, where expression levels vary greatly from cell to cell, it will be absolutely necessary to develop well tolerated mutants.

Text Box 10.1: Examples of key fields for further development of optogenetic tools required in order to address the neurobiological questions proposed in the text.

- *Development of combinations of spectrally separated actuators and sensors:* ChRs have a very broad excitation spectrum, and most CFP, GFP, or YFP related fluorescent reporters will be activated during ChR activation. Even when combined with red fluorescent labels or indicators (green excitation), simultaneous ChR2 activation is substantial. This problem can be partially avoided in two-photon microscopy, in which activation of ChR occurs [14], but is usually modest. Nevertheless, development of compatible combinations of actuators and sensors would be highly desirable. In the future, we might see stimulation tools with inbuilt reporters of calcium concentration or voltage [15], to provide feedback about successful optical spike induction.
- *Development of combinations of spectrally separated actuators:* It would be highly desirable to independently manipulate more than one neuron type. Combinations of spectrally separate actuators would allow to manipulate two genetically defined input systems, or to manipulate pre- and postsynaptic neurons in an all-optical manner. Various red-shifted ChR variants have been reported [8, 12, 16], but have to be optimized with respect to the amount of red-shift, the toxicity and the photocurrent magnitude in mammals. A further elegant direction is the design of fusion constructs that combine depolarizing and hyperpolarizing actuators for dual-wavelength control of membrane potential [17]. Such arrangements could also be used to restrict the spectral width of the depolarization window.
- *Development of ChR2 variants that allow generation of physiological presynaptic action potentials:* Due to the relatively slow closing of even the fastest ChR variants (~6 ms), light-induced APs are typically broader than "natural" (EPSP-triggered) APs. The resulting increased calcium influx and increased release probability can be beneficial, as it results in extremely reproducible amplitudes of postsynaptic responses [18]. This is often desirable, *e.g.*, to provide a stable baseline for LTP experiments. For experiments where an unperturbed release probability is crucial, however, the slow closing of ChRs can be problematic. Asking for ChRs that close in 1–2 ms, however, is probably not realistic, as such mutants would deliver small photocurrents [19]. Instead, one has to restrict ChR activation to the soma and dendrite of the stimulated cells, thus triggering a normal, forward-propagating AP in the axon. This can be achieved by dendritic targeting of ChR [20], or by focused somatic illumination that spares axons and presynaptic terminals from direct light. As ChRs are also distributed to the endosomal membrane, Ca^{2+} release from intracellular Ca^{2+} store may be evoked upon irradiation. This background Ca^{2+} elevation could modify the biochemical milieu regulating the transmitter release through binding to the high-affinity Ca^{2+}-binding proteins in the cytoplasm. To prevent this, ChRs impermeable to Ca^{2+} should be targeted to the plasma membrane.
- *Development of tools for multiphoton excitation of ChRs:* Reliable induction of action potentials in individual neurons in living tissue by two-photon excitation of ChR2 would be desirable to probe the connectivity of large cell ensembles when combined with imaging (see Figure 10.3B). This is possible using optimized laser-scanning modes [14]. Newly developed opsins are now emerging that allow effective suprathreshold two-photon activation under *in vivo* conditions [21].
- *Development of novel tools for long-term inhibition of neurons*. Last, but not least, there is no optogenetic tool to reliably prevent neurons from spiking for extended periods of time (see Figure 10.3C). Hyperpolarizing pumps (halorhodopsin, arch) require constant illumination at relatively high intensities [22, 23]; and are likely to be not suitable for chronic inactivation of large neuronal populations. The ideal tool would be a channel conductive for K^+ or Cl^-, preferably with bistable characteristics. An inhibitory light-gated channel would be of special interest for circuit analysis in awake, behaving animals.

10.2.3 Long-term analysis of synaptic function

A particularly interesting opportunity afforded by optical manipulation and imaging techniques is that these approaches can be applied over several days to weeks. Electrophysiological approaches with a similar resolution are typically limited to a few hours of recording time. Clearly, plastic and homeostatic changes at synapses take place at much longer timescales, and the key mechanisms regulating synaptic stability over longer time periods are not well understood. To investigate the properties of individual synapses over long time scales, a promising approach is optogenetic stimulation, which allows re-activating defined sets of presynaptic neurons over many days, a feat that is not possible with other techniques. These tools have been combined with functional imaging of the postsynaptic neuron, allowing not only stimulation with light, but also readouts of postsynaptic responses of individual synapses in a quantitative fashion (Figure 10.2). By postsynaptic calcium imaging, it is possible to distinguish between mere contacts ("potential synapses") and bona fide functional synapses [18]. Moreover, as stated above, optogenetic induction of presynaptic APs triggers the natural transmitter release machinery. Thus, it is suitable to assess presynaptic function and plasticity, along with postsynaptic properties. As most forms of long term plasticity affect both pre- and postsynaptic function, it is desirable to measure both release probability and potency of the postsynapse at the same time.

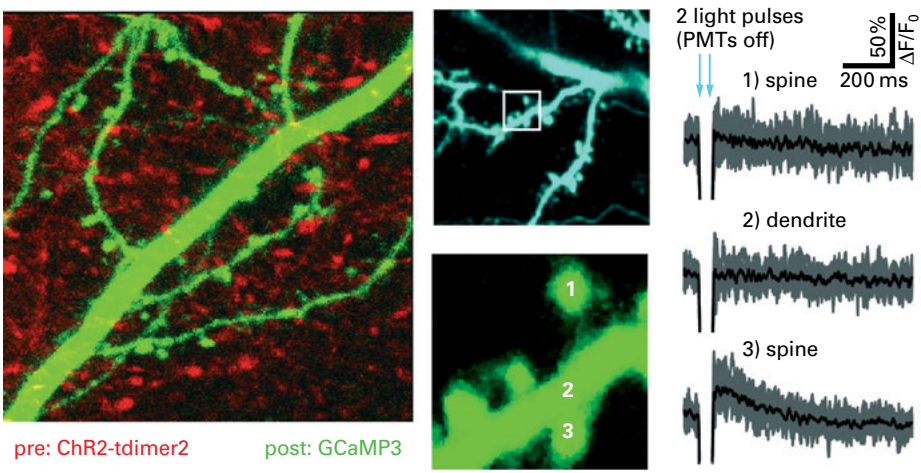

Figure 10.2: Example of all-optical analysis of synaptic function. Presynaptic cells express ChR2 (ET/TC) along with a dimeric red fluorescent protein (tdimer2). Postsynaptic CA1 pyramidal cells express the genetically encoded calcium indicator GCaMP3 and CFP. A pair of blue light pulses leads to increased fluorescence in spines functionally connected to ChR2-expressing presynaptic terminals (spine #3). Line scans are used to sample spine calcium at 500 Hz [24].

Despite the great promise of optogenetics combined with high-resolution physiology, as well as all-optical approaches, there are several technological developments that need to be addressed to further improve the utility of these tools (see Text Box 10.1).

10.3 Circuits and systems level

Moving up the hierarchy of neural complexity, optogenetic tools now enable novel approaches to tackle the complexity at the level of local neural circuits and global brain systems (see Figure 10.1). Similar to the synaptic and single-cell level described in the previous section, the goal is to uncover principles of neural computation at these higher levels. On the one hand, we consider "microcircuits", which are loosely defined as subdivisions of particular brain areas (*e.g.*, in thalamus or cortex) with prominent, local connectivity. For extensive structures such as the neocortex, the idea is that particular local connectivity patterns exist forming "canonical microcircuits" as computational units that repeat themselves over the entire structure [25, 26]. Understanding the basic operational principles of such functional units remains a major challenge of neuroscience. Typically, microcircuits extend several hundred micrometers and contain a dense meshwork of several thousand interconnected neurons of different types, both excitatory and inhibitory. The neuronal networks are intermingled with glial networks and linked to the vascular system, supporting basic regulatory, homeostatic, and metabolic functions. Defining clear boundaries of microcircuits is often difficult, as they show strong interconnections with many other brain areas, including specific long-range projections and more diffuse neuromodulatory pathways. On the systems level, microcircuits are thus embedded within larger-scale "macrocircuits", and the functional organization of inputs and outputs of many microcircuits still remains elusive. In the end, therefore, microcircuit function cannot be understood in isolation, but needs to be studied in the context of the entire working brain, and during specific behaviors. With their *in vivo* applicability and exquisite specificity, optogenetics offers multiple options to functionally dissect neural circuitry both on the micro- and macro-level and to gain fundamental insights about how neural network dynamics governs behavior.

To learn about microcircuit function, there are two questions: what experiments are required and how can optogenetics help? A first approach to dissect microcircuit function is to comprehensively read out *in vivo* activity in dense neuronal networks, and to basically "watch" ongoing neural dynamics (Figure 10.3A). The theoretical goal would be to measure "every spike in every neuron" [27], ideally under relevant behavioral conditions, and to obtain a complete map of spatiotemporal activity within a given microcircuit, *e.g.*, a cortical column. For imaging with genetically encoded calcium- or voltage indicators (for review see [28, 29]), this ambitious goal requires high sensitivity with single-spike detection, spatial sampling of large numbers of

neurons in 3D volumes, as well as high temporal precision in the millisecond range. Despite significant recent progress, further advances are still required. For example, a whole palette of sensitive genetically encoded calcium indicators (GECIs) is now available for monitoring spiking activity *in vivo*, with some of the newest generation GECIs – including GCaMPs [30, 31], cameleons [32], and others – starting to outperform the best synthetic small-molecule calcium indicators. While for excitatory pyramidal neurons, reliable detection of calcium transients evoked by single action potentials is now in reach [33, 34], calcium influx per spike in fast-spiking GABAergic interneurons is too small to be reliably detected at present [35]. In addition to GECIs, novel voltage-sensitive fluorescent proteins (VSFPs) with improved signal-to-noise ratio may soon become applicable for *in vivo* single-cell spike detection in networks [36, 37]. These would have the additional advantage of reporting subthreshold membrane potential dynamics. Importantly, long-term expression of GECIs or VSFPs, combined with chronic two-photon imaging, allows monitoring of activity patterns in the same local network over weeks to months, enabling studies of fundamental aspects of neural circuit stability and plasticity [38, 39]. Furthermore, high-resolution imaging studies have become possible in awake, behaving animals trained to tolerate head fixation [40, 41, 42]. Here, movement artifacts in the imaging data can pose an extra challenge, and single-spike sensitivity under behavioral conditions still needs to be demonstrated.

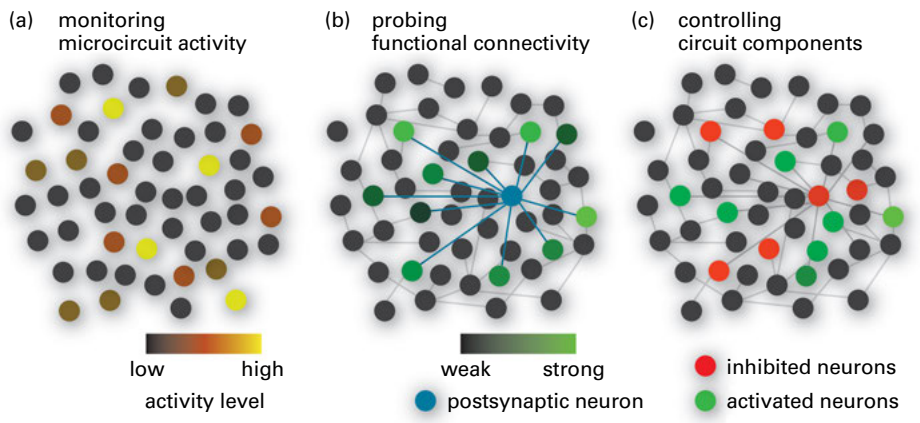

Figure 10.3: Schematic of several application modes of optogenetic tools for dissecting neural circuit function. (a) Sensitive activity indicators such as GECIs or VFSPs in principle allow comprehensive monitoring of network activity. (b) Single-cell activation by two-photon activation of opsins, which is now possible, allows mapping of functional connections converging on a particular neuron. (c) Activation or inhibition of specific subsets of neurons (simultaneously or in a temporal pattern) permits direct testing of the functional role of this neuronal ensemble within the circuit.

Most recent GECI imaging studies were performed on rather small-sized networks of a few tens to hundreds of neurons and with moderate acquisition rates between 1–10 Hz. Clearly, temporally precise measurements from larger networks are desirable, especially as they would allow for investigation of rules for network reconfigurations based on spike-timing dependent plasticity (STDP) within typical time windows of a few ten milliseconds [43]. Currently, large-scale 2D or 3D population imaging from several thousand neurons would be possible only at rather slow rates (<1 Hz). On the other hand, very high acquisition rates (up to kilohertz) have been achieved for groups of tens of neurons with high-speed *in vivo* microscopes [44, 45], demonstrating for synthetic calcium indicators spike train reconstruction with near-millisecond precision [44]. Establishing similar measurements with GECIs in behaving animals, with a reasonable compromise between network size and speed, is a prime current goal. A fundamental limiting factor is the total photon count, determined by fluorescence photon flux, detection efficiency, and integration time. Besides optimization of detection efficiency, further improvements in indicator brightness and optimization of protein expression are thus desirable. Additional obstacles that have to be overcome are the limited imaging depth and incomplete discrimination of cellular sub-types. GECI variants with red-shifted excitation and emission spectra [46, 47], combined with long-wavelength, high-peak power pulsed laser light illumination, should help to extend depth penetration. Endoscopic approaches, however, may remain the only option to access very deep circuits [48]. For cell-type identification, several methods are available, including genetic means (viral or transgenic) for cell-type specific protein expression [49, 50], as well as post hoc immunostaining of the neurons previously studied *in vivo* [51, 52]. In summary, optogenetic monitoring of *in vivo* microcircuit activity from thousands of neurons seems feasible, which should help to characterize the heterogeneous distribution of activity within local networks and find sparse neuronal subsets with salient dynamic activation features [53].

Comprehensive optical readouts of neuronal population activity is a first major goal, but will not in itself lead to a mechanistic understanding of microcircuit function. A further important step is to relate network activation patterns to the underlying wiring diagram. Novel large-volume electron microscopic anatomical reconstruction techniques now can provide detailed post hoc information on connectivity in tissue blocks investigated *in vivo* [54]. Nonetheless, a pure anatomical “connectome” will not be sufficient, because the strengths of synaptic connections, which largely determine the actual signal flow through a circuit, are variable and underlie continual modulation [55]. Therefore, a “functional connectome” is required, constituting a map of who is actually talking to whom and how loud, given a particular brain state or behavioral context. Building upon previous work using two-photon uncaging of caged neurotransmitters [56, 57], the use of opsins for specific light control of microcircuit components seems ideal for performing connectivity mapping (Figure 10.3B). However, reliable induction of action potentials in individual neurons in living tissue by two-photon excitation of ChR2 turned out to be challenging due to intrin-

sic low efficacy of two-photon induced ionic currents. As a work-around solution, several groups devised special laser-scanning modes to optimize the pattern of laser illumination on the cell for achieving cumulatively strong enough currents. These approaches included spiral scanning patterns in the cell [14], light sculpting [10], and patterned light stimulation using spatial light modulators combined with temporal focusing for excitation confinement to the focal plane [11]; for reviews see [58, 59]. In a recent breakthrough, newly developed opsins (C1V1 variants) were introduced with customized properties for effective suprathreshold two-photon activation of neurons, using standard laser scanning schemes and even under *in vivo* conditions [21]. These new tools fully open the field for mapping of the functional connections converging onto a particular neuron by addressing the surrounding pool of neurons one-by-one with two-photon optogenetics [60]. Moreover, simultaneous activation of specific subsets of neurons within the local circuit is possible using scanless, patterned light excitation approaches [11, 21]. For the future, we envision all-optical circuit mapping approaches, in which neurons, in addition to a light-activatable protein, also express a voltage indicator, so that postsynaptic activation of many neurons can be probed optically, allowing rapid measurement of a functional connectivity matrix. Such methods will have to await voltage indicators capable of reporting small-amplitude postsynaptic potential changes. Alternatively, the postsynaptic neuron could also be filled with a sensitive GECI so that optogenetic activation of presynaptic neurons, or neuronal pools, would allow the determination of the distribution of synaptic inputs over the dendritic tree, which would be highly relevant for understanding dendritic integration. Even further, long-range projecting axonal pathways could be made to express an opsin so that the subcellular organization of their excitatory inputs impinging on target neurons is revealed *in vivo*, as has been studied for cortical neurons in brain slices [5].

Besides the mapping of functional connectivity, precise optical control of single neurons, or a specific subset of neurons, could be used to address a number of key questions regarding *in vivo* circuit dynamics. One example for this strategy are studies designed to reveal the specific functions of the various GABAergic interneuron subsystems [61, 62, 63, 64]. It would be important to add to these studies the systematic inhibition of key neuronal populations *in vivo*, to assess their role in ensemble activity and behavior (see Text Box 10.1, Figure 10.3C). While very useful to assess the capability of a genetically defined cell type to affect the activity of the neuronal ensemble as a whole, these studies have usually employed a wide-spread light-based activation both for *in vitro* studies using wide-field illumination, and *in vivo* studies using implanted light fibers. In fact, in most of these studies, the number of light-activated neurons is not precisely known. This issue is not merely a quantitative one, since quantitative differences in the level of activation of modulatory systems may lead to qualitative differences in the ways information is routed in complex neuronal networks. Thus, it would be highly desirable to: i) precisely determine the level of optogenetic activation [65]; and ii) titrate it to a level similar to endogenous levels of activity (see Figure 10.4).

This would go a long way to determining how much activity is really required in order to trigger a particular ensemble activity or behavior. An alternative approach would obviously be to restrict opsin expression to small groups or even single neurons [66] of a particular cell type.

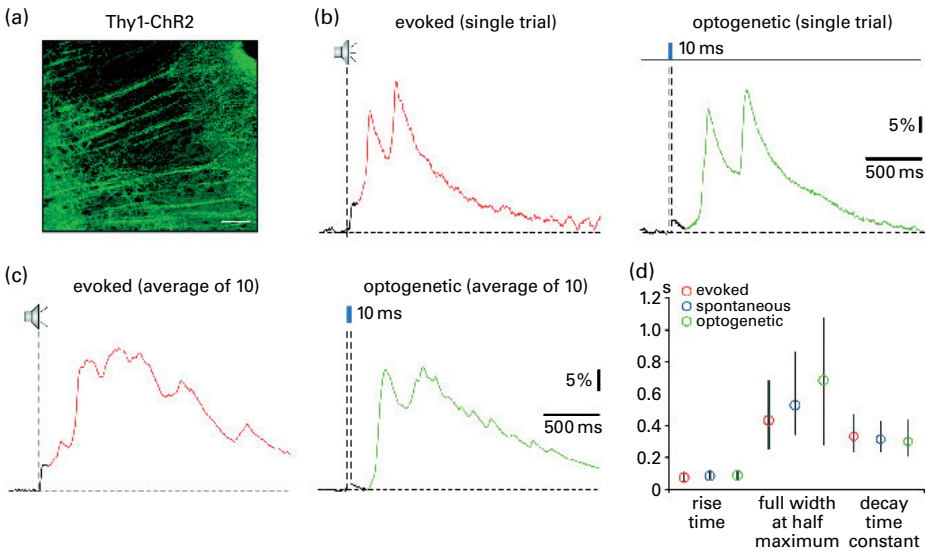

Figure 10.4: Calibration of optogenetically induced network calcium transients with sensory-evoked transients. (a) shows the expression of channelrhodopsin-2 (ChR2) in layer 5 pyramidal neurons of the ChR2-Thy1 transgenic mouse line. Scale bar = 100 µm. (b) and (c) show the single response (b) and average of 10 responses (c) to noise (left panels) and light stimulation (right panels). (d) shows the rise times, full widths at half-maximum, and decay time constants of sound-evoked slow network calcium transients (red, n = 253 events from 10 animals), spontaneous network calcium transients (blue, n = 213 events from 10 animals) and optogenetically initiated network calcium transients (green, n = 159 events from 5 animals). Note the similarity of the three types of event. From Grienberger *et al*. [67].

In addition to the dissection of circuit function using a combination of comprehensive readouts of population activity, and controlled activation of single neurons or specific neuronal sub-ensembles, light-based actuators and sensors used in combination will be of great value in the study of circuit plasticity. Similar to the importance of these approaches in understanding long term stability and plasticity of individual synapses, such techniques can be used to probe circuit activation patterns over time. Very little is known about how stable these activity patterns are under steady-state conditions and how comprehensively they adapt to changing behavioral requirements. Chronic imaging of GECIs has been used to track circuit dynamics longitudinally before, during, and after an experimental manipulation [39, 41]. The same experimental

protocol is applicable to mouse models of brain injuries or diseases (*e.g.*, stroke or Alzheimer's disease) to reveal impairments in circuit function during disease progression. Adding to these studies optogenetic actuators to study the impact of defined neuron types on evolving networks will provide further considerable impetus to our knowledge of the mechanisms of plasticity and homeostasis on the circuit level. It should also be mentioned that the ability to control neuronal firing with millisecond precision may be an important tool to study the role of spike-timing-dependent forms of plasticity in the overall plasticity and stability of networks. Finally, targeting opsin expression to non-neuronal cell types, such as astrocytes or microglia, will allow addressing their impact on circuit dynamics and plasticity.

So far, these considerations were focused on the local architecture of neuronal circuits. However, as stated earlier, microcircuits are embedded in larger-scale macrocircuits, such as brain region sets involved in visual processing, sensorimotor integration, or reward-based learning. A first step to address this larger scale integration is to measure activity in multiple systems at once. Wide-spread GECI or VFSP expression – especially in transgenic mouse lines [49, 50] provide new opportunities for monitoring macrocircuit dynamics. For example, ongoing and evoked activity in the neocortical sheet, spanning nearly a whole hemisphere, can be imaged with a fast camera [38, 68]. Alternatively, fiber-optic recording can be used for readouts of regional activity, even from deep brain regions [34, 69]. Taking advantage of selective GECI expression in defined neuronal subsets, such bulk fluorescence recordings can be highly informative about behavioral-related activity in specific interregional pathways [69]. A particular promising avenue for understanding "functional macroconnectivity" is the combination of fiber-optic recording, multichannel LFP recording or *in vivo* patch-clamp recording with high-resolution imaging in multiple brain regions. On the stimulation side, fiber-optic illumination is the most widely applied technique for optogenetic control of brain regions, including nuclei from which neuromodulatory pathways originate (see also next section). Typically, one or two regions are controlled, but multi-region control is feasible and may be increasingly employed to examine the crosstalk between different brain regions. Finally, both fiber-optic stimulation and recording are easily combined with fMRI [70, 71, 72], opening novel avenues to place local and specific measurements in the context of the global brain activity map.

In summary, optogenetic readouts and control of neural circuit components, on both micro- and macrocircuit level, provide great opportunities to functionally dissect circuit function. With these tools at hand, we now need to formulate a theoretical framework that generates hypotheses about the computational roles of specific circuit components which are then testable *in vivo* and during behavior.

10.4 Optogenetics and behavior: testing causal relationships in freely moving animals

Perhaps the most attractive feature of light-based actuators is that they allow us to penetrate into the mesoscale organization of brain circuits and systems with unprecedented cellular specificity. As reviewed by Deisseroth *et al.* (in Chapter 2), opsins expressed in genetically defined neuron types allow us to perform experiments linking their activity modulation to behavior (Figure 10.5). The targeting strategies for these approaches have been extensively reviewed elsewhere, but have utilized various strategies for promoter driven cell-specific expression. In addition, the use of projection based targeting strategies allows direct light-based stimulation to specific neuronal populations, based on the anatomy of their projections.

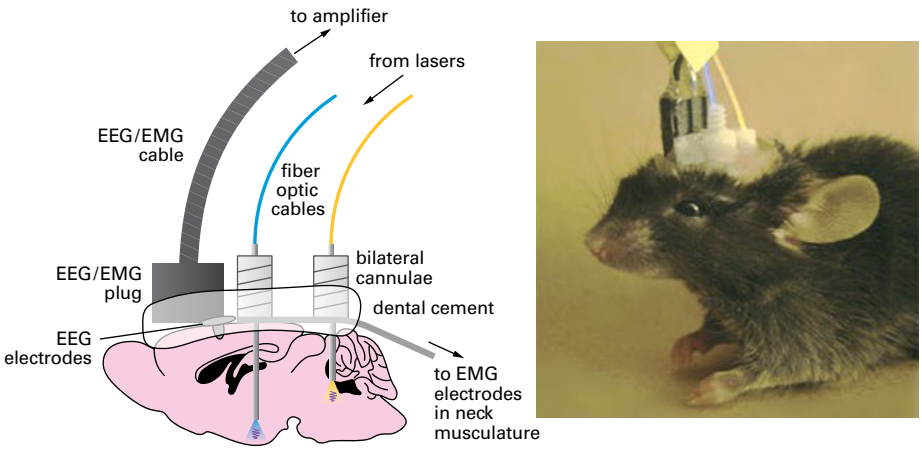

Figure 10.5: Schematic of an optogenetic preparation in freely behaving animals. This setup was used to interrogate the functional connectivity between a neuromodulator (hypocretinhypocretin/orexin system) and an effector system (noradrenergic neurons in the locus coeruelus) [73].

In vivo optogenetics has allowed detailed descriptions of hitherto poorly understood complex circuits such as the mesocorticolimbic brain reward pathway [74, 75, 76, 77, 78, 79]. These approaches have also been applied to the study of other modulatory systems in the brain, such as monoamines [80, 81] or peptide neurotransmitter systems such as the hypocretin/orexin system [82, 83]. Also, optogenetics has allowed us to raise new questions about the organization and dynamics of neuronal ensembles in memory engrams. For instance, it is now possible to ask the question of how many neurons constitute an engram of stable memories [84]. Alternatively, targeting specific subtypes of neurons, such as adult-born granule cells, allows us to ask what their role is in specific memory tasks [85]. A further important area of *in vivo* research has

been the investigation of brain-wide neuronal oscillations and their role in memory processes. *In vivo* optogenetics has already allowed us to address some mechanisms governing the initiation and maintenance of sleep spindles, gamma activity and delta waves [86]. Undoubtedly, more details about the role of local circuitry and neuromodulators will clarify the function of particular frequency bands on behavior.

As stated in the previous section, these approaches will clearly benefit from refinement, for instance regarding a precise quantitative evaluation of the optogenetic stimulus. Also at the level of behavior, a combination of optical actuators with readouts of neuronal activity is necessary. In this respect, the use of genetically encoded calcium and voltage sensitive probes may allow real time feedback control over optogenetic control of cell types and systems. This seems feasible, as recently a closed loop feedback system has been designed to provide on-demand inhibition of epileptic seizures of different types [87, 88]. Moreover, to truly understand how behavior of intact animals is modified by optogenetic approaches, it will be necessary to add to the observation of altered behavior the observation of the dynamics on the intermediate level of micro- and macrocircuits. This will allow us to better understand what functional modifications in the more extended neuronal system are caused by optogenetic stimulation. To this end, the rapid development of capabilities for large-scale optical imaging of neuronal networks *in vivo* detailed in the previous section will be invaluable.

In summary, optogenetics holds great promise to unravel the function of micro- and macro-networks in the intact brain and their role for complex behavior. The future will see both technological developments allowing more precise and multimodal modulation of neuronal networks. A major avenue for future development will be to couple optogenetics with multiple optical and electrophysiological readout modalities *in vivo* in order to understand the impact of these perturbations on different levels of neuronal complexity.

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Viviana Gradinaru

11 Optogenetics to benefit human health: opportunities and challenges

11.1 Introduction

Optogenetics has greatly impacted neuroscience research [1, 2] and has potential for clinical applications as well [3, 4, 5]. By carefully considering the opportunities and challenges, we can speed up the progress for clinical optogenetics.

11.2 Opportunities for translational applications

Indications that could benefit from optogenetics are primarily those arising from deficits in the central and the peripheral nervous systems (CNS and PNS). Potential tractable indications include Parkinson's disease, depression, anxiety, or addiction in the CNS, and pain or locomotion control in the PNS. PNS indications could be especially tractable in spinal cord injured patients to restore, for example, locomotion or micturition, or improve vision in disorders of the retina [4]. Applications outside the nervous system that also capitalize on the capability of optogenetics to modulate electrically excitable cells include cardiac or skeletal muscle control [6, 7]. With additional tool development (especially optogenetic control of biochemical pathways via light-controlled protein-protein or protein-DNA interaction, or by developing opsins permeable to, for example, calcium), non-electrically excitable tissues could become viable candidates for optogenetics as well.

There are many potential ways to use optogenetics for therapeutical purposes; here we highlight three of them where optogenetics can be used as a therapy by itself, or where optogenetic approaches could lead to drug discovery for known and novel targets in the nervous system and beyond. First, one could envision a combination product of opsin and light device to be used in humans; this could, for example, be an alternative to electrical deep-brain-stimulator-like implants (Figure 11.1) that are currently successfully used for some motor and mood disorders (*e.g.*, Parkinson's disease, obsessive compulsive disorder, even depression) [8]. Because electrical stimulation is non-specific, a stimulation modality such as optogenetics, that has high network element specificity and leaves bystanders unaffected, could have fewer side effects and greater efficacy. Although electrical deep brain stimulation has been greatly optimized for disorders such as Parkinson's disease, for other disorders, especially mood and cognitive disorders, optogenetics could provide a much-needed alternative. Second, optogenetics could be used as a platform for drug screening, either *in vitro* or *in vivo*. For *in vitro* drug screening one could design cell lines (Figure 11.2) [9]

to screen for modulators of voltage-gated ion-channels (for example, drugs that affect L-type calcium channel function could be used for cardiac or CNS indications), with the added temporal and cost advantage of all-optical control of membrane voltage and channel functional readout, as compared with traditional methods that use, for example, high potassium solutions to change membrane voltage. For *in vivo* drug screening one could create animal models of disease using optogenetics and search for candidates that can reverse the behavioral deficits. Third, optogenetics can be used to identify specific novel circuits that are involved in ameliorating a disease and then find associated molecular markers, especially drug-able markers.

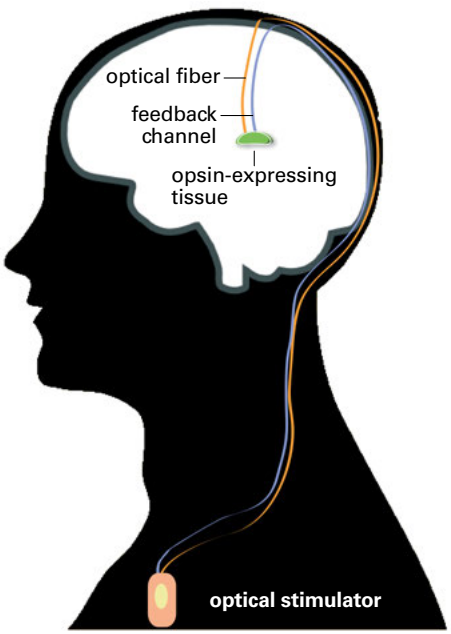

Figure 11.1: Optical Implant for Specific Modulation of Brain Activity. A deep brain stimulator based on light: the benefit is, when combined with optogenetics, specificity (can be for cell types, projection pattern, or other common denominators). The main challenge remains safe delivery of the opsins. A feedback line can collect electrophysiological or imaging data and could serve 2 goals: to detect opsin levels to aid in controlled expression of the opsin and also to instruct the stimulation parameters (courtesy of Bin Yang and Viviana Gradinaru).

Although a less explored possibility, optogenetics could positively influence neurodegenerating cells by regulating the cellular milieu to, for example, control excessive glutamate release or elevate levels of protective growth factors (both of these methods showed promise in the case of dopaminergic neurons in the *substantia nigra pars compacta* and their projections, which are affected in Parkinson's disease). With

appropriate further development, optogenetics could also assist in some cancer indications by developing tools that can trigger apoptotic events in fast dividing cells (genetically targeted) at the tumor site (light targeted).

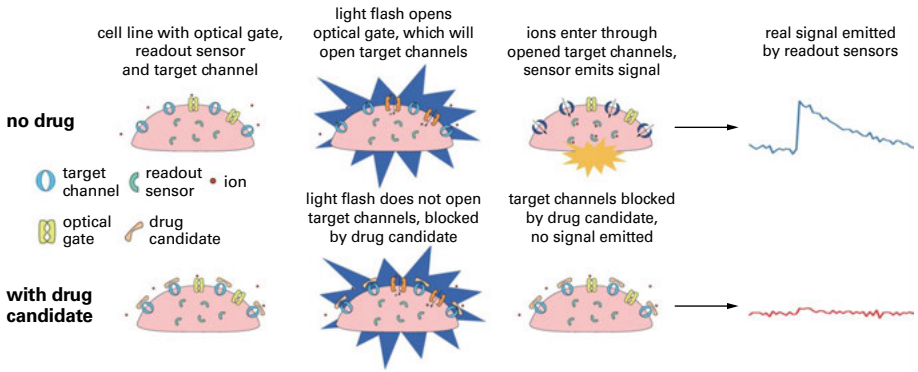

Figure 11.2: Optogenetics for Drug Screening. Optogenetics-based cell lines can facilitate drug screening via: (1) light-mediated control of membrane voltage via the light-sensitive Channelrhodopsin-2 (ChR-2); (2) optical readout of ion channel activity via genetically-encoded fluorescence readout; (3) modular architecture that allows easy incorporation of different ion channel targets. Example data of calcium traces shows that the method can be used to screen for ion channel modulators (courtesy of Feng Zhang and Viviana Gradinaru).

11.3 Safety challenges

Although clinical optogenetics for drug discovery could be applied immediately, with fewer downstream regulatory barriers, optogenetics as a device, and opsin therapeutic packages, might need to overcome significant challenges. Potentially, the biggest challenges are opsin delivery and opsin tolerability, because any therapy has to be, first and foremost, safe. Opsin delivery will be associated with all the challenges that gene therapy has, although viral delivery methods via adeno-associated viruses (AAVs of different serotypes) have been extensively used now in clinics, and ongoing parallel developments in gene therapy can facilitate clinical optogenetic applications. Opsin tolerability will have to be tested extensively to insure that the opsin does not trigger the immune system (especially important for non-CNS applications) and that opsins do not compromise the integrity of the membrane in which they are expressed (even in the absence of light, the opsin itself could occupy space needed for endogenous proteins). For peripheral applications, it is especially important that the opsin traffics along the nerve, towards the innervated tissue. The light device design and placement will also require significant engineering and prototyping in the clinical setting. One needs to consider potential challenges with placement (for example, in

the PNS the device could physically hinder nerves and cause them to malfunction), light penetration, or heating.

11.4 Need for feedback

An optogenetic therapy that relies on opsin expression in the living human tissue would greatly benefit from the availability or real-time feedback on opsin and light impact to the tissue – this can take the form of simultaneous local or distal electrophysiological and/or optical readouts. One potential development would be feedback-based optogenetics, where the opsin genes turn themselves off once the membrane expression reaches an effective level, or once an electrode can detect adequate function to prevent crowding endogenous receptors and disrupt natural function. This can be aided by a built-in regulatory sequence on the vector that can be acted upon with a drug. Inducible systems have been used successfully in basic research where, for example, doxycycline can be used to either turn on or turn off expression of certain proteins.

11.5 Conclusion

Because the foundation for any optogenetics discovery or therapy is the opsins themselves, there is a need for ongoing opsin development. We now need to be prepared and anticipate the needs of the future: opsins responsive to infrared light for better, safer light penetration; an inhibitory opsin in the form of a channel, *e.g.*, one selective for potassium – the physiological ion for neuronal inhibition (non-existent), rather than a pump (existing but not physiological due to use of chloride or proton pumping for inhibition [10]); an opsin selective to calcium to modulate biochemical pathways. A parallel example is the generation of multicolor fluorescent proteins – the many available variants (GFP, RFP, mCherry, tdTomato, BFP, eYFP, *etc.*) are now ubiquitous tools in biology. Further development of the basic optogenetics toolbox will aid both basic science and facilitate translational applications.

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12 Optogenetic tools for controlling neural activity: molecules and hardware

12.1 Overview

The brain is composed of an incredible diversity of cells that differ in molecular composition, morphology, and electrophysiological properties, as well as in how they change in different neurological and psychiatric disorders. These cells exhibit millisecond-timescale subthreshold and suprathreshold electrical activities, as they integrate information received from upstream cells (via chemical messengers that trigger electrical signals) and generate output signals (via chemical release) to communicate with downstream targets. Ideally it would be possible to activate and silence the electrical activity of different sets of cells, with millisecond timescale precision, in order to assess how they contribute to neural computations and brain functions: activating cells can reveal how strong the connections are between the activated cells and observed downstream cells, and indicate what neural or behavioral functions the activated cells are sufficient to initiate (or sustain), whereas silencing cells can reveal their necessity in either initiating or sustaining specific neural and behavioral functions. Because different cell types are intermeshed with one another, purely chemical and electrical means of stimulating and silencing neurons cannot be fully cell-specific, and furthermore chemical means are limited to slow perturbations of brain activity. Optogenetic tools, which make the electrical activity of defined neurons sensitive to being activated or silenced by light, address this problem. In this chapter, we give a brief overview of the molecular and hardware tools that make up the optogenetic toolset in widespread use in neuroscience, and discuss future directions for this toolset.

12.2 Molecular tools for sensitizing neural functions to light

Microbial opsins are light-driven ion pumps and light-gated ion channels, seven-transmembrane proteins that normally serve photosensory and photosynthetic roles in a diversity of cells and organisms, including Archaea, bacteria, fungi, and algae. Microbial opsins bind at a defined lysine residue all-trans-retinal, which serves as the light-capture component, and which isomerizes upon receiving light of wavelength defined not only by the intrinsic properties of all-trans retinal but the molecular environment. The first set of opsins to be discovered were the bacteriorhodopsins, light-driven outward proton pumps from Archaea that are used to drive ATP production [1, 2]. Proteins related to bacteriorhodopsins have been found in many other species,

ranging from bacteria to fungi [3, 4]. Another set of opsins, the halorhodopsins, light-driven inward chloride pumps, were also found in Archaea, where they also play a role in energy production in halophilic species that live in high salt concentration environments [5, 6, 7, 8, 9]. In the early 2000s, channelrhodopsins, light-driven nonspecific inward cation channels (which pass H^+, Na^+, Ca^{2+}, and K^+ into cells), were discovered in flagella-bearing green algae, where they play a role in supporting photosensation-driven motility [10, 11]. Almost since the beginning of the field, attempts were made to express these opsins in heterologous expression systems, cell types other than the ones in which they were discovered, and thus might be regarded as early optogenetics experiments, initially *Escherichia coli*, to facilitate spectral characterization of bacteriorhodopsin mutants [12, 13], and later in eukaryotic cells such as oocytes, yeast cells, and human embryonic kidney (HEK) cells [14, 15, 16, 17], to facilitate electrophysiological characterization of opsins. Perhaps one of the first papers attempting to control a defined physiological function in a heterologous cell type was a paper in which the mitochondria of yeast were genetically targeted with bacteriorhodopsin [18], which resulted in yeast that required less glucose when they were illuminated (perhaps because the mitochondria were now photoactive). Other papers that pointed towards the use of opsins as tools, included a paper that highlighted the *Natronomonas pharaonis* halorhodopsin as one that, despite its halophile source, had excellent chloride transport properties at low chloride concentrations such as those found in mammalian brain [19], and a study transporting the *Drosophila* phototransduction cascade into cultured mammalian neurons, enabling the neurons to be activated by light [20], although the kinetics were slow. Finally, the paper reporting the discovery of channelrhodopsin-2 (ChR2) demonstrated its expression and function in HEK cells, and commented on its potential utility as a tool in biology [11].

Our group, and several other groups whose writings are featured in this book, working collaboratively and independently, demonstrated that specific opsin classes could be effectively expressed in neurons, and used to control them. In 2005, we showed that ChR2 could be expressed in mammalian neurons and used to mediate the activation of neurons with millisecond-timescale precision pulses of blue light, sufficient to result in single-action-potential precision with light irradiances similar to those used to image green fluorescent protein (GFP) [21]. In 2007, we and others showed that the *N. pharaonis* halorhodopsin could be expressed in neurons and used to mediate the hyperpolarization of neurons with orange light, to a degree sufficient to quiet neural activity [22, 23]. And in 2010, we showed that bacteriorhodopsins, especially of the archaerhodopsin class, but also including fungal opsins, could be used to mediate the hyperpolarization of neurons with green light [24, 25]. In the time since, these three classes of optogenetic neural control molecule have come to be commonly used throughout neuroscience, in species ranging from *Caenorhabditis elegans* [26] to primate [27], to study how neurons contribute to neural computations [28, 29], and they have even been considered as candidates for translational medicine as well [30].

The spread of these technologies has been facilitated by the development of powerful and easy to use transgenic mice [31, 32, 33, 34], simple-to-implant optical fibers coupled to LEDs or lasers [35, 36, 37], and the existence of microscope-mountable LEDs and lamps for opsin stimulation.

Currently, much activity is focusing both on the discovery of novel optogenetic tool classes, which can be used to control specific kinds of signaling in neurons and other kinds of cells, as well as the improvement of the microbial opsin class towards better performance for scientific applications. Focusing on the latter topic, there has been significant activity working on slowing down [38, 39] and speeding up [40] the post-illumination off-kinetics of channelrhodopsins, which has the effect of making the opsins effectively more light sensitive and less light sensitive respectively (in the sense that a slower-to-inactivate channel will pass more charge than a fast-to-inactivate channel, per photon) [41]. An interesting frontier is to know whether it is possible to, say, make opsins simultaneously significantly faster and significantly more light sensitive, to result in "all around optimized" opsins that can both mediate very fast neural events, and also require low light powers. There has also been significant activity aimed at discovering and engineering color-shifted opsins [24, 42, 43], important both because red-shifted opsins can be actuated with redder colors of light that penetrate better into tissue, and also because opsins with different spectral peaks could in principle be driven by different colors of light (although the fact is that all opsins can to some extent be driven by blue light, as a result of the nature of retinal itself). Work aimed at developing tools that enable perturbation of the ion compositions of cells, *e.g.*, the enhanced-calcium-permeability channelrhodopsin CatCh [44], or the use of halorhodopsins to alter the GABA reversal potential of neurons by pumping large amounts of chloride into cells [45], also point to an interesting area of future development. For example, by expressing a light-gated proton channel or pump in synaptic vesicles or mitochondria, and then illuminating them, one might achieve control over synaptic vesicle content or neuronal metabolism. Altering the trafficking of opsins, *e.g.*, aiming opsin expression towards cellular compartments such as axons, dendrites, spines, and other regions of neurons [46, 47, 48, 49], and increasing trafficking of opsins to the membrane overall [24, 50–52], may also open up new frontiers in perturbing cellular computations occurring in specific parts of cells. It has also been observed that intracellular expression of opsins, presumably on membranes of organelles hosting intracellular calcium stores, can mediate calcium signaling in cells even in the presence of zero external calcium, indicative of ion-specific effects of opsins when expressed and activated intracellularly [53], and pointing to other potential uses within cells. Finally, although not an optogenetics innovation *per se*, good promoters and related genetic handles that allow unique and specific targeting of different cell types of the brain, perhaps assisted by novel technologies that enable molecular characterization and classification of neurons at single cell level [54], are needed in order to enable finer and more cell specific optogenetic control of neurons in the brain.

There is excitement about clinical uses of optogenetics, notably for disorders like retinitis pigmentosa, a mutation-caused disease that results in photoreceptor loss blindness, which can in animal models be treated by genetic targeting of a microbial opsin to a class of spared cells in the retina [55, 56, 57, 58]. For such a treatment to be viable, of course, it is critical that a fully optimized opsin – with carefully chosen amplitude of effect, light-sensitivity, kinetics, dynamic range, and ion selectivity – be chosen and utilized, since unlike the scientific application case, one cannot simply pick and choose opsins according to varying scientific goals – a single enduring treatment scenario would be needed. It is also important to carefully choose a defined cellular target for optogenetic intervention, both to take advantage of the cell-type precision of optogenetics, and to result in an effective treatment with maximal efficacy and minimum side effects, key needs in the spaces of neurology and psychiatry [30]. Also importantly, safety studies must be carefully contemplated, since optogenetic interventions, which would require a gene therapy event, may be expected to operate for years or decades. Thus, the human cellular and organismal tolerance for microbial opsin expression, including the possibility of any immune response, must be considered. Long-term toxicity of optogenetic tools should also be explored, perhaps by using standard safety and biodistribution assays, but applied over clinically relevant timescales [27, 58]. Recent experiments which successfully demonstrate perturbation of primate behavior through optogenetic activation or silencing of neurons are reassuring [59, 60], but more detailed molecular and cellular assessments, as well as longer-term studies, are essential.

12.3 Hardware for delivery of light into intact brain circuits

In freely moving animals, the use of optical fibers to mediate optogenetic activation of neurons has become commonplace [35, 37], and recently wirelessly powered and controlled devices have emerged [36], as well as multisite targeting devices comprising arrays of optical fibers targeted at different sites in a circuit [28, 29, 61, 62]. *In vitro*, a diversity of standard light sources such as LEDs, lasers, and lamps, coupled to microscopes (and made temporally controlled, if needed for slowly modulated light sources like lamps, through standard shutters, galvanometers or digital micromirror devices), can be used. Both single-photon [31, 63–65] and multi-photon [66, 67, 68, 69, 70] activation of optogenetic tool-expressing neurons have enabled activation of defined pathways in circuits, as well as defined cells.

Future directions for scientific applications include the creation of nanofabricated and microfabricated structures, for example made out of many parallel waveguides to guide light to deep targets, that enable the delivery of light to thousands to millions of cells distributed throughout intact neural circuits [71, 72], as well as integration of such tools with scalable neural recording and imaging methodologies [73, 74]. Technologies that allow patterned stimulation to address greater numbers of

cells than currently possible, perhaps using newer generations of spatial light modulation strategy, might be of particular relevance. For clinical applications, it is key to have low-power, biocompatible, and long-lasting implants, which perhaps build off of existing insights from electrical neural implants such as those used for deep-brain stimulation [30].

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13 *In vivo* application of optogenetics in rodents

13.1 Introduction

Optogenetics allows functional interrogation of genetically identified neuronal circuits with unprecedented spatial and temporal precision. *In vivo* application of optogenetic methods has allowed us a much deeper understanding of the basic circuits underlying complex behaviors. Here we summarize how optogenetics has advanced our knowledge on neuronal connectivity, and opened new possibilities in the study of neurological and neuropsychiatric disorders.

13.2 Sleep / wake regulation

Sleep / wake disturbances are tightly associated with many psychiatric disorders, such as depression, addiction and anxiety disorders [1]. Alterations between arousal states involve complex interactions between activity in populations of neurons that promote arousal and those that promote sleep [2]. The use of optogenetics lead to great progress in the study of two neuronal populations: the Hypocretin-expressing neurons and the noradrenergic locus coeruleus (LC) neurons (Figure 13.1-a). The Hypocretins (Hcrt1 and Hcrt2; also known as orexins) are a pair of neuroexcitatory peptides, exclusively produced by a cluster of neurons in the lateral hypothalamus [3]. These neurons have a pivotal role in the stabilization and maintenance of wakefulness. In the first *in vivo* application of optogenetics in behaving animals, Adamantidis *et al.* [4] targeted ChR2 into Hcrt-expressing neurons, and showed that direct optical stimulation of these neurons during both NREM and REM sleep increased the probability of awakening in the following 20–30 seconds. This induction was frequency-dependent; only a stimulating pattern of 5–30 Hz increased awakening probability, whereas a 1 Hz stimulation pattern did not. The arousal inducing effect of Hcrt-expressing neurons does not overcome homeostatic processes; Hcrt-mediated sleep-to-wake transitions were blocked by sleep pressure caused by sleep deprivation [5]. Optogenetic silencing of Hcrt neurons induced sleep during the light phase, but not during the dark phase [6]. These findings were further validated [7] using a newly developed pharmacogenetic technology (DREADD's) [8] that allows the modulation of neural activity with temporal resolution of several hours.

The second central neuronal population in sleep-wake circuitry studied using optogenetics is the LC noradrenergic neurons. Optogenetic stimulation of these neurons caused immediate sleep-to-wake transition from both NREM and REM sleep [9] (Figure 13.1B). As opposed to Hcrt neurons for which awakening occurred ~30 seconds following stimulation, stimulating LC neurons lead to an awakening

event in less than 5 seconds from the initiation of the stimulation. Photostimulating LC neurons during wakefulness increased locomotor activity and total wake time, while photoinhibition decreased the duration of wake episodes but did not block sleep-to-wake transitions [9]. Interestingly, high-frequency stimulation of the LC neurons caused reversible behavioral arrests, resembling those seen in individuals suffering from neuropsychiatric disorders [9]. These results demonstrate that noradrenergic LC neurons activity is sufficient to promote wakefulness from sleep and general locomotor arousal, but is not necessary for animals to wake from sleep. It has been recently shown that the effects of Hcrt neurons on sleep-to-wake transitions are dependent on noradrenergic LC neurons [10]. Photoinhibiting LC neurons during Hcrt stimulation blocked Hcrt-mediated sleep-to-wake transitions, whereas photostimulating LC neurons during Hcrt stimulation increased the probability of sleep-to-wake transitions [10]. Additional studies are needed to determine whether there are other neuronal populations necessary for the arousing effects of Hcrt neurons. There is somewhat contradicting evidence regarding histamine neurons [11], and future studies could clarify this issue.

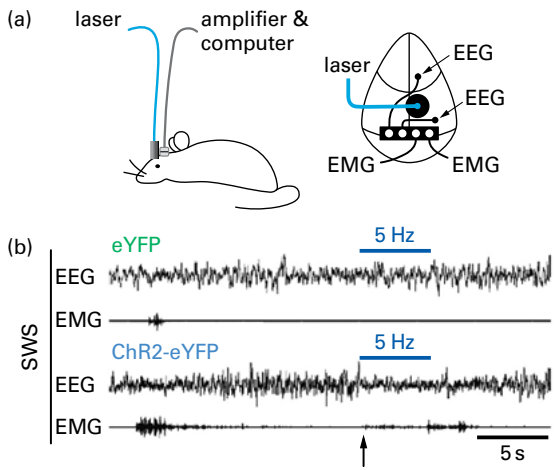

Figure 13.1: Optogenetic interrogation of the sleep-wake circuitry. (a) Schematic representation of the behavioral set up used for in vivo photostimulation along with polysomnographic recording in mice. Magnification shows the EEG / EMG connector used for sleep recording and the cannula guide used for light delivery through an optical fiber. (b) In vivo optogenetic stimulation of noradrenergic neurons in the locus coeruleus elicits immediate sleep-to-wake transitions. EEG / EMG traces of mice transduced with eYFP or ChR2-eYFP virus. 5 Hz stimulations do not cause any effect on the EEG / EMG in eYFP but immediately induces waking in ChR2 transduced animals.

Optogenetic methods offer vast new opportunities in sleep / wake research, and deciphering the underlying neuronal network would allow manipulating this circuit in

sleep-associated psychiatric disorders. For example, it is now possible to assess the relative importance of specific features of sleep to cognitive functions. Sleep continuity is disrupted in many psychiatric disorders, which are also frequently accompanied by memory deficits. Rolls *et al.* [12] used optogenetics to fragment sleep in mice without effecting its total duration or intensity. The authors photostimulated Hcrt-expressing neurons during the first hours of the inactive phase following learning of a novel object, and found that sleep fragmentation hampered memory consolidation. Furthermore, they identified a minimum length of uninterrupted sleep required for proper memory consolidation. In addition to subcortical structures of the reticular activating system, thalamocortical systems are known to generate oscillations of cortical excitability associated with sleep / wake patterns. In particular, spindles, 8–12 Hz oscillations that accompany NREM sleep, have also been linked to memory consolidation processes. A few groups have now used optogenetics to manipulate neurons in the reticular thalamus, which results in the generation of spindles [13]. Combining manipulations of spindles and sleep continuity will allow us to decipher the actual role of these features in cognitive function and disease.

13.3 Addiction

The prolonged exposure to drugs of abuse or alcohol induces persistent neuronal adaptations in the reward-seeking pathways leading in many occasions to addictive disorders. Optogenetics has importantly contributed to dissect the neuronal pathways related with reward seeking, and to identify the adaptations that take place in these circuits after the exposure to drugs of abuse [14, 15, 16] (Figure 13.2). Two highly interconnected brain regions play critical roles in mediating reward: the ventral tegmental area (VTA) and the nucleus accumbens (NAc). The VTA is a heterogeneous brain structure that contains different neuronal populations, which include dopaminergic, GABAergic and glutamatergic cells. Dopamine (DA) neurons in the VTA are the main effectors of reward. These DA neurons fire constantly at a tonic rate, and when they fire phasically they induce reward. Voltammetry studies showed that optogenetic stimulation of VTA DA neurons mirror natural patterns of DA release in the striatum [17]. This allowed extensive optogenetic studies on the role of VTA DA neurons in reward. Phasic, but not tonic, optogenetic stimulation of DA neurons in the VTA induced conditioned place preference [18], and self-stimulation in both mice [19, 20] and rats [21]. VTA DA neurons co-release glutamate together with DA, and optogenetic stimulation of these neurons elicits glutamatergic EPSCs in the NAc. Glutamate release cannot directly account for the typical reward-related responses of NAc neurons, but may modulate the long-term plasticity of cortical and limbic inputs that lead to addiction [22]. The VTA also contains GABAergic neurons that synapse directly onto DA neurons regulating their activity. It has been shown that activation of VTA GABAergic neurons *in vivo* suppresses the activity of neighboring DA neurons, and

disrupts reward consummatory behavior. Cohen and colleagues [23] showed that DA neurons are sensitive to reward outcome whereas GABA neurons in the VTA are sensitive to the predicting cues. These studies suggest that the interplay between VTA DA and VTA GABA neurons can control the initiation and termination of reward-related behaviors, and encode prediction error discount.

Outputs from the lateral VTA, especially those activated by laterodorsal tegmentum neurons are integrated in the NAc, and mediate reward [24]. More than 90% of the neuronal population in the NAc are GABAergic medium spiny neurons. As shown by optogenetic studies, they specifically target VTA GABAergic neurons, but not VTA DA neurons [25]. Medium spiny neurons are classified into two populations depending on the DA receptor they express; D1 or D2. Optogenetic studies provide evidence for an opposite role of these two pathways in reward-related behaviors. Optogenetic stimulation of D1 receptor-expressing (D1R) neurons induced persistent reinforcement, whereas stimulating D2 receptor-expressing (D2R) neurons induced transient punishment in operant and place conditioning tasks [26]. The NAc also contains cholinergic interneurons which constitute less than 1% of the local population. Nevertheless, the activation of cholinergic receptors in the NAc can strongly influence medium spiny neurons. Optogenetic stimulation of cholinergic interneurons in the NAc inhibited the firing of medium spiny neurons [27], and induced DA release in this region [28].

In addition to the local innervation and the afferents from the VTA, the NAc receives glutamatergic inputs from the amygdala, the prefrontal cortex, the hippocampus and the thalamus [29]. Optogenetic studies explored the role of glutamatergic projections from the prefrontal cortex and the amygdala to the NAc in reward-seeking behavior. Interestingly, mice self-stimulated the basolateral amygdala, but not the prefrontal cortex glutamatergic afferents to the NAc. In addition, silencing basolateral amygdala afferents to the NAc reduced cue-reward associations [30]. Altogether, these data suggest that DA release from VTA neurons and glutamatergic projections from the basolateral amygdala activates the D1R neurons and facilitates reward seeking, while GABAergic external inputs from the VTA and local interneurons (cholinergic and D2R neurons) may inhibit the D1R neurons and turn down reward-seeking behavior.

The reward-seeking pathways experience neuronal adaptations after repeated exposure to drugs of abuse, often leading to addictive disorders. Several optogenetic studies show how the chronic administration of cocaine dysregulates the reward circuitry inducing responses that are not observed in naive subjects. Optogenetic activation of D1R neurons in the NAc had no effect on the locomotor activity of naïve mice, whereas it enhanced locomotor activity in mice repeatedly treated with cocaine [31]. Also, optogenetic stimulation of NAc D1R or D2R medium spiny neurons alone was unable to induce any type of place conditioning. However, optogenetic stimulation of D1R neurons, combined with a subthreshold dose of cocaine, induced conditioned place preference. On the contrary, the effectiveness of cocaine inducing place preference at an active dose was reduced when D2R neurons were optogenetically activated [31]. Similarly, activation or inhibition of NAc cholinergic interneurons had no evident

behavioral effects in naïve mice. However, while the optogenetic activation of NAc cholinergic interneurons could not induce place conditioning, the optogenetic inhibition of these neurons significantly reduced the efficiency of cocaine-induced conditioned place preference [32].

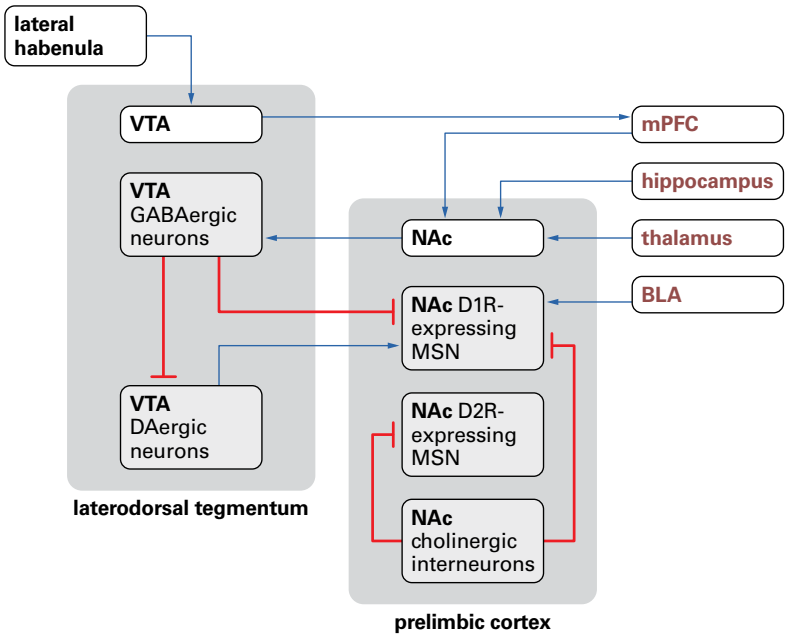

Figure 13.2: Optogenetic interrogation of the reward circuitry. Model of excitatory (in blue) and inhibitory (in red) interactions between different neuronal populations across brain structures mediating reward, based on optogenetic studies. VTA, ventral tegmented area; GABA, gamma-aminobutyric acid; DA, Dopamine; NAc, nucleus accumbens; D1 / 2R, D receptor expressing neurons; MSN, medium spiny neurons; mPFC, medial prefrontal cortex; BLA, basolateral amygdala.

Glutamatergic inputs to the NAc, especially those coming from the prelimbic cortex, play a crucial role in the plasticity induced by repeated cocaine administration. Optogenetic studies show that stimulation of infralimbic cortex inputs to the NAc reverses long-term potentiation in NAc D1R neurons and behavioral sensitization induced by cocaine [33]. Also, inhibition of prelimbic cortex to NAc afferents blocks cocaine- and cue-induced reinstatement of cocaine-seeking [34].

Remarkably, inhibition of the reward circuit may also induce aversion. Optogenetic activation of GABA neurons in the VTA inhibited DA neurons and induces conditioned place aversion, and aversive stimuli increased the firing rate in these GABAergic neurons [35]. In addition, optogenetic activation of neurons in the lateral habenula, which mainly project to the medial VTA, inhibits those VTA neurons and

induces conditioned place aversion [24]. These seemingly contradictory results (both activation and inhibition of VTA neurons inducing aversion) may be due to differences in activation patterns or recruitment of different DA neuronal ensembles. At any rate, these results indicate that circuits that convey aversive and reward pathways are strongly related.

13.4 Fear, anxiety and depression

An exaggerated or prolonged exposure to conditions that induce fear or anxiety is the major cause of psychiatric disorders such as generalized anxiety disorder, post-traumatic stress disorder, and depression. Using traditional techniques, a basic description of the fear circuit has been already delineated; however, optogenetics now allows a deeper understanding of the functional anatomy of the neuronal populations involved in fear and anxiety. Traumatic events generate robust and persistent memories. Both humans and animals learn that specific sensory cues or conditioned stimuli (CS) predict aversive events or unconditioned stimulus (US), by a form of associative learning called fear conditioning. The amygdala is a critical site for fear conditioning, and it is divided into different nuclei connected by highly organized circuits. The lateral amygdala (LA) integrates CS and US, and induces associative plasticity [36]. Supporting this, when optogenetic activation of pyramidal neurons in the LA is paired together with an auditory sensory cue, it induces fear conditioning, in a similar way that an aversive stimuli does [37]. The LA projects directly and indirectly to the central nucleus of the amygdala (CE). While the LA integrates CS and US, the CE controls the elicitation of the conditioned response (CR). The CE is divided into two subnuclei: the lateral division of the CE (CEl) and the medial division of the CE (CEm), which contains a highly organized microcircuitry of GABAergic inhibitory neurons. Optogenetic studies show that direct projections from the LA activate neurons in the CEl [38]. Also, the CEl transmits to the CEm, and the CEm transmits the information to other effector sites outside the amygdala. These studies also suggest that the CEl contains two populations that show opposite responses to the presentation of the CS after fear conditioning. CEl “on” neurons are activated with the presentation of the CS, whereas CEl “off” neurons are inactivated with the presentation of the CS. CEl “on” neurons modulate the activity of CEl “off” neurons, and CEl “off” neurons modulate the activity of CEl “on” and CEm neurons. Therefore, the presentation of the CS activates CEl “on” neurons, which inactivate CEl “off” neurons. Decreased activity of CEl “off” neurons disinhibits the CEm and induces freezing [39, 40]. Interestingly, the majority of CEl “off” neurons expresses oxytocin receptor [39], and a recent study showed that the optogenetic stimulation of oxytocinergic axons in the CE attenuates fear conditioning, and that the cell bodies of these oxytocinergic neurons are most likely located within the magnocellular subpopulation of the paraventricular nucleus of the hypothalamus (PVN) [41].

Brain regions that convey CS are also influenced directly by US. Studies using optogenetics show that the auditory cortex is activated by aversive US. Foot-shock activates cholinergic neurons in the basal forebrain that subsequently activate GABAergic interneurons in layer 1 of the auditory cortex [42]. Then, layer 1 interneurons inhibit parvalbumin expressing GABAergic interneurons in layer 2/3 of the auditory cortex, which at the same time inhibit pyramidal neurons of layer 2/3. Therefore, presentation of aversive stimuli induces a disinhibition in the auditory cortex [42]. Disinhibition of pyramidal neurons by aversive stimuli might also occur in the visual cortex, indicating that aversive stimuli might influence the regions integrating CS for fear conditioning through pathways alternative to the amygdala [42].

Fear conditioning has a strong memory component, and the hippocampus plays an important role in the consolidation of fear-related memories. A very elegant study used novel optogenetic tools to express ChR2 in dentate gyrus neurons that were active during fear conditioning. Optogenetic reactivation of these neurons in a new context was sufficient to induce freezing. This indicate that the activation of a specific ensemble of cells involved in memory encoding is sufficient to retrieve fear memories [43]. In another study, Goshen and colleagues [44] showed that, contrary to the prevailing view, the hippocampus is required for the recall of fear memories at long times (~1 month) after conditioning, but that this requirement can only be revealed if optogenetic inhibition is carried out on a short time-scale (~5 min), presumably preventing the recruitment of compensating mechanisms.

Some of the circuits mentioned above not only control fear but also anxiety. Optogenetic studies have shown that glutamatergic projections from the basolateral amygdala (BLA) to the CE play an important role in anxiety. Optical activation of glutamatergic projections from the BLA to the CEl decreases anxiety, whereas optical inhibition of these projections increases anxiety [38]. GABAergic projections from the CE to the bed nucleus of the stria terminalis (BNST) have been hypothesized to play a critical role in the control of anxiety. There is still no behavioral evidence that optogenetic activation of this pathway controls anxiety; however, the optical activation of GABAergic neurons in the CE induced inhibitory currents in the BNST [45].

Depression is among the most disabling medical disorders and poses a serious public health concern [46]. Many patients suffer from treatment-resistant depression, for which the only effective treatment to date is deep brain stimulation (DBS). Although DBS is used to treat different psychiatric and neurodegenerative diseases, it was until recently unknown what is the mechanism underlying its therapeutic benefits (*i.e.*, whether they stem from the stimulation or the suppression of neuronal activity, and whether this is taking place in the local brain region or in other brain regions). Deisseroth and colleagues have showed that the direct targets of DBS in the subthalamic nucleus (examined for Parkinson's disease) are not local cell bodies but afferent axons probably arising from different regions [47]. Kumar *et al.* further demonstrated that stimulating descending projection neurons of the prefrontal cortex (PFC, layer V neurons) in chronically stressed mice modulated oscillatory activity across limbic

networks, induced limbic beta synchrony, and reduced anxiety-related behavior [48]. Evidence suggests that in both humans suffering from treatment-resistant depression, and in chronically socially-defeated mice, there is reduced neuronal activity in the PFC, measured by the expression of immediate early genes [49]. There is somewhat contradicting evidence regarding the effects of optogenetic stimulation of PFC neurons on anxiety-related behaviors, and additional studies are needed to clarify this issue. Covington *et al.* found that optogenetic stimulation of mPFC neurons, in chronically socially-defeated mice, restores normal social interaction and sucrose preference, but not anxiety-related behaviors [49]. By contrast, Kumar *et al.* found that chronic photostimulation of PFC neurons reduces anxiety-related behavior [48]

13.5 Autism and schizophrenia

Social dysfunction is a common symptom in many psychiatric diseases [50]. Although human social behavior is much richer than that of typical rodent model organisms, a wide range of social behaviors can be studied using laboratory animals. Recently, the neuronal circuits underlying social behavior have started to be dissected using newly developed optogenetic tools. It has been hypothesized that behavioral deficits associated with psychiatric disorders, such as autism and schizophrenia, arise from elevation in the cellular balance of excitation and inhibition (E/I balance) within neuronal microcircuits [51, 52]. This hypothesis was tested by optogenetically elevating the E/I balance in the medial-prefrontal cortex using a step-function opsin (SSFO), together with red-shifted opsins (C1V1) [52]. Increased excitation in excitatory pyramidal neurons (but not inhibitory), lead to social and cognitive dysfunctioning which are similar to those seen in autism [52]. Cortical gamma oscillations are an indicator of enhanced information processing, which is highly affected in schizophrenic patients [53]. Recently, GABAergic inhibitory neurons that express parvalbumin as their calcium binding protein have been shown to have a causal role in the generation of gamma activity [54, 55]. Additional studies are expected to advance our understanding on the contribution of local or large-scale cellular imbalances to information processing.

13.6 Aggression

Another aspect of social living for which there is an enormous negative impact in our society is aggression, but not much is known about its neurobiological bases. A neuronal population in the ventrolateral aspect of the ventromedial hypothalamus, which is a region previously shown to be activated during both aggression and mating, has been optogenetically targeted [56]. The authors revealed overlapping but distinct neuronal subpopulations involved in aggression and mating. Optogenetic activation of

these neurons, but not electrical, induced aggression while pharmacogenetic silencing inhibited aggression. Interestingly, neurons that were activated during aggression were inhibited during mating [56].

13.7 Breathing

Spinal cord or brainstem injuries can lead to paralysis, and in severe cases, to an inability to breathe. Nevertheless, the neural circuitry underlying the control of respiration is not fully understood. Phox2b-expressing neurons in the retrotrapezoid nucleus (RTN) were hypothesized to function as central respiratory chemoreceptors modulating ventilation in mammals, yet it was particularly impossible until recently to explicitly demonstrate this. Guyenet and colleagues used optogenetic tools to causally demonstrate that these neurons play an important role in central respiratory chemoreception [57, 58]; in both anesthetized and awake rats, photostimulating Phox2b-expressing neurons increases breathing. The same group has used optogenetic tools to demonstrate that serotonergic neurons in the raphe obscurus (RO) potentiate the central respiratory chemoreflex, but do not have a central respiratory chemoreceptor function [59].

13.8 Seizures

Epilepsy affects about 1% of the population, yet effective treatments are lacking. Current antiepileptic therapies are often unfeasible or unsuccessful, and many patients continue to experience seizures. Recently, different groups have started to elucidate the mechanisms responsible for seizures using optogenetic tools, and developed new technologies to monitor, detect, and block seizures in real-time. Kullmann and colleagues assessed the efficacy of optogenetic control of seizures in a tetanus toxin-induced rat model of epilepsy [60]. Pyramidal cells in the cortex were transduced with halorhodopsin, and photoinhibition of the neurons decreased electrical seizure activity. Continuous inhibition of action potential generation using a mammalian potassium channel (Kv1.1) that normally regulates both neuronal excitability and neurotransmitter release, provided long-term protection from seizures. Furthermore, overexpression of Kv1.1 channels for several weeks in animals with established epilepsy had eliminated seizures completely. Paz *et al.* [61] assessed which regions of the brain are involved in post-stroke seizures, using a photothrombotic model of focal cortical stroke; in which epilepsy occurs around 1 month after stroke. This form of epilepsy was associated with damage to the thalamus; thalamocortical neurons connected to the injured epileptic cortex became hyperexcitable. The authors succeeded to detect the initiation of seizure within two seconds from their onset, and by photoinhibiting neurons in the thalamus, they interrupted seizure generation, preventing

its spread throughout the thalamocortical network. Krook-Magnuson *et al.* [62] used a temporal lobe mouse model of epilepsy, and demonstrated that both activation of inhibitory neurons and inhibition of excitatory neurons in the hippocampus following the onset of seizures, succeeded to terminate the seizures.

Taken together, these studies show that seizures can be detected and terminated by modulating specific cell populations in a spatially restricted manner, and open promising possibilities for future therapies for patients with epilepsy.

13.9 Conclusion

One of the central hallmarks of psychiatric and neurological disorders is altered function in the communication between neuronal circuits. Using optogenetics, it is now possible to study normal neuronal circuit function and dysfunction. Optogenetic studies have already contributed to a better understanding of the neural circuits affected in many disorders. New branches of optogenetics, which include cellular probing of signaling mechanisms and optical readout of neuronal activity, are rapidly emerging, and may set the stage for precise closed-circuit control and therapeutic intervention in human disease.

Acknowledgments

L.d.L. is supported by National Institutes of Health Grants MH83702, MH87592, and DA21880 and the Klarman Family Foundation. A.E.R is supported by a postdoctoral fellowship from the Edmond and Lily Safra Center for Brain Sciences (ELSC).

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V. Sturm

14 Potential of optogenetics in deep brain stimulation

14.1 DBS history and indications

Half a century ago, deep brain stimulation (DBS) was introduced by Hosobuchi *et al.* [1] and Mazars [2] in the treatment of chronic and medically intractable pain. Benabid *et al.* [3] was the first to use this technique to treat tremor in Parkinson's disease. Over thin electrodes, permanently implanted in specific in unspecific nuclei of the thalamic pain-processing system, the ventral intermediate (VIM) nucleus of the thalamus, the subthalamic nucleus (STN), and different nuclei of the basal ganglia, weak electrical impulses are administered temporally or permanently to brain structures, the pathological activity of which would interfere with physiological impulse flow in dependent circuits. The aim of DBS at high frequencies (usually around 130 Hz) is either to suppress this neuronal activity by causing a depolarization block or to replace the symptom-causing discharge pattern by the pattern of DBS [4].

Unexpectedly beneficial therapeutic results of DBS, with a low rate of side effects, soon rendered the previously used ablative stereotactic methods obsolete.

In the last decade, DBS has successfully been used to treat severe psychiatric disorders like obsessive compulsive disorder [5], Tourette's syndrome [6], unipolar depression [7, 8], and recently, alcohol addiction [9], Parkinson-dementia [10], and even autism related self-injurious behavior [11].

14.2 Electrical DBS: advantages and drawbacks

Electrical DBS has major advantages over ablative procedures:

1. DBS does not damage the targeted central nervous structures (neurons and axons), even if permanently administered over many years.
2. The procedure is fully reversible. If side effects occur, the impulse generators (IPGs) can simply be reprogrammed, or if necessary, be switched off.
3. The treatment can be adjusted to changing clinical necessities at any time by reprogramming. Stimulation parameters – and sites – can easily be changed.
4. Advanced image-guided stereotactic localization and trajectory planning programs, along with highly precise stereotactic operation devices, allow accurate and safe implantation of DBS electrodes into the chosen targets. Battery-driven or rechargeable impulse generators, which are usually implanted in a subcutaneous pocket over the breast muscle in a similar manner to cardiac pacemakers, are connected to the DBS electrodes with thin wires placed subcutaneously, allowing

the application of a wide range of short and weak electrical impulses, usually at frequencies around 130 Hz.

Based on the experience with neuromodulation through DBS, which has been used worldwide in about 90 000 patients so far, it is fair to label DBS a little invasive, but highly efficient procedure.

Nevertheless, electrical DBS has a major drawback, the nonspecificity of its mode of action:

1. Lack of electrophysiological specificity.
   Stimulation at low frequencies (up to 20–30 Hz) yields activation; stimulation at higher frequencies (over 60 Hz) yields inhibition of cell somata, most probably by inducing a depolarization block [4], but activation of axons occurs in both frequency ranges. The thicker the axonal myelin sheet, the lower the threshold is for activation. Thus, pure activation of the targeted central nervous tissue cannot be achieved, but instead, a mostly unpredictable mixed pattern of inhibition of cell somata and activation of axons, which can yield opposite effects, depending on circuitry.
2. Lack of biological specificity.
   Depending on width and amplitude of the mostly rectangular electrical impulses, as well as the impedance of the affected tissue, the generated electrical field has a spherical to oval shape with a diameter up to 4–5 mm. Within this space, different types of neurons are affected in the same way.
   Each of the currently addressed target areas contains multiple neurons with different morphological and biochemical characteristics. For example, a mix of glutamatergic, *i.e.*, activating, GABAergic, *i.e.*, inhibiting projection neurons, and GABAergic interneurons, all are affected by DBS in the same way. Thus, electrical DBS can simultaneously inhibit glutamatergic projection and GABAergic interneurons, which under physiological conditions counteract each other, and would give a net yield of less inhibition than desired. With electrical stimulation, it is impossible to selectively block pathologically activating projection neurons, or to selectively activate GABAergic interneurons.

14.3 Potential of optogenetic stimulation

Optogenetic stimulation has the potential to overcome the drawbacks of electrical DBS, as described above.

1. Light with different wave lengths can selectively depolarize, *i.e.*, activate, or inhibit all types of transfected neurons (somata and axons). This enables one to overcome the undesirable mix of effects caused by inhibition of cell somata and simultaneous activation of axons by high frequency electrical stimulation. Thus, optogenetic DBS could precisely mimic the effects of tissue-ablation, which was the first, but so far not achievable, aim of DBS.

2. The choice of specific promoters creates the potential for cell-specific viral transfection, making only the targeted class of neurons, *e.g.*, glutamatergic projection neurons or GABAergic interneurons, sensitive for photo-activation or inhibition. Preliminary data hold promise that this goal seems achievable [12], but a large body of further experimental work will be needed to translate this principle into clinical practice.

If the mentioned drawbacks of electrical DBS could be overcome by optogenetics, the therapeutic options of DBS in a wide variety of neurological and psychiatric disorders, currently considered therapy-resistant, would improve dramatically. New options to specifically address the pathogenesis of these disorders could become feasible.

To achieve this goal, four major problems of optogenetic DBS have to be addressed and overcome.

1. Since optogenetic treatment requires viral transfection which must be sharply restricted to the target area, safe viral carrier systems have to be developed which have no capacity for systemic transfection, toxicity, or to induce tumor growth.
2. Stable and long lasting expression of the Rhodopsins by the transfected neurons must be guaranteed.
3. To enable selective and homogeneous transfection of the target area, stereotactic infusion-systems have to be developed which enable, *e.g.*, convection enhanced delivery of the carrier-systems by using micropumps and solutions, which are detectable by MRI.
4. Last, but not least, the problem of the short penetration range of blue and yellow light in tissue must be overcome. One solution could be to place multiple optical fibers into the target area, which are stereotactically implanted, and similarly to conventional DBS systems, subcutaneously connected to the optogenetic impulse generator.

14.4 Conclusion

Major biological and technical difficulties of electrical DBS can principally be overcome by optogenetics which holds promise to create new dimensions in DBS, one of the most rapidly growing fields in neurological and psychiatric therapy.

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15 Optogenetic approaches for vision restoration

15.1 Introduction

Vision begins in the retina when rod and cone photoreceptor cells respond to light and convert light signals into electrical signals that are conducted through second- and third-order retinal neurons, also referred to as inner retinal neurons, to the brain (see Text Box 15.1). The severe loss of photoreceptor cells in many inherited and acquired retinal degenerative diseases, such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD), could result in partial or complete blindness. RP consists of a group of inherited diseases that damage photoreceptor cells and affect about 1.5 million people worldwide. AMD is the leading cause of blindness in people older than 60 years of age and results from the progressive deterioration of the photoreceptor cells in the macula, which is near, or at the center, of the retina. Once photoreceptor cells have been lost, the only retina-based approaches that could potentially restore vision are reintroducing photoreceptor cells or restoring the retinal light responses. Potential techniques include transplanting normal photoreceptor cells or progenitor / stem cells, using retinal implant devices (see Chapter 16) that provide a direct electrical stimulation to the surviving retinal neurons, and more recently, optogenetics. Optogenetic approaches will be the focus of this chapter.

Text box 15.1: Retina anatomy and visual information processing in the retina

The retina is a layered structure that lines the inner posterior wall of the eyeball. The retina is not only the site of initiating phototransduction, which is the function of photoreceptor cells, but also plays an important role in processing visual information before it is transmitted to the brain. Visual signals are processed in the retina through multiple parallel pathways, such as the ON and OFF pathways, the scotopic (rod) and photopic (cone) pathways, edge detecting, motion and directional selectivity, and color coding (Figure 15.1). All photoreceptor cells hyperpolarize in response to increases in light. Bipolar cells (second-order retinal neurons) and ganglion cells (third-order retinal neurons) are divided into ON and OFF cells that depolarize and hyperpolarize, respectively, in response to increases in light. For photopic vision, visual signals from the cones are either passed from ON cone bipolar cells to ON ganglion cells or from OFF cone bipolar cells to OFF ganglion cells. For scotopic vision, signals from the rods are transmitted through a unique rod pathway from rod bipolar cells to AII amacrine cells (both are ON cells). The signal is then transferred to the ON pathway through gap junctions and to OFF cone bipolar cells through sign-reversed glycinergic synapses. The segregation of visual signals into ON and OFF pathways is believed to be important in enhancing the sensitivity to contrast. Additionally, horizontal cells and amacrine cells generate inhibitory lateral interactions that mediate the formation of a center-surround antagonistic receptive field in bipolar cells and retinal ganglion cells. The center-surround antagonistic receptive field is an essential feature of visual information processing in the retina and is believed to enhance contours and provide better visual acuity.

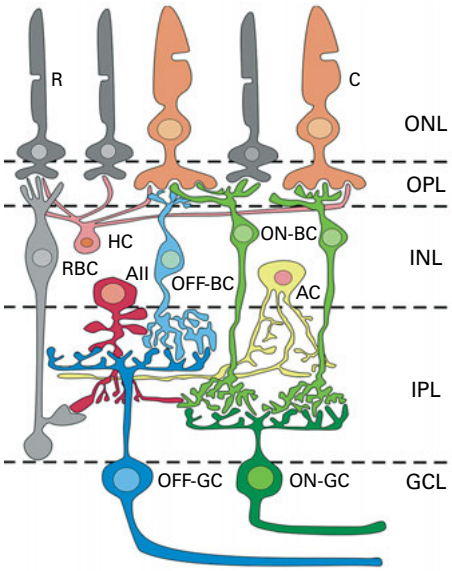

Figure 15.1: A schematic diagram illustrating the retinal rod/cone and ON/OFF pathways. ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. R: rod photoreceptor cell; C: cone photoreceptor cell; RBC: rod bipolar cell; ON-CB: ON cone bipolar cell; OFF-BC: OFF cone bipolar cell; HC: horizontal cell; AII: AII amacrine cell; AC: amacrine cell; ON-GC: ON ganglion cell; OFF-GC: OFF ganglion cell.

15.2 Proof-of-concept studies

The optogenetic strategy is based on the fact that a significant number of inner retinal neurons survive long after the death of photoreceptor cells. Thus, an attractive strategy for restoring vision would be to genetically convert the surviving inner retinal neurons directly into photosensitive cells. The feasibility of this strategy, however, requires a simple genetically encoded light sensor. The discovery of light-gated channelrhodopsins [1, 2], especially channelrhodopsin-2 (ChR2), immediately made them appealing candidates for this application.

The first study that investigated whether ChR2 expression in the retina is able to restore light sensitivity was conducted in *rd1/rd1* mice, a mouse model for RP, using recombinant adeno-associated virus (rAAV) vector delivered by intravitreal injection [3]. The study showed that robust and stable expression of ChR2 can be achieved in retinal third-order neurons in *rd1/rd1* mice *in vivo* (Figure 15.2). This study also demonstrated that the expression of ChR2 restored retinal light sensitivity, ON light responses, and visually evoked potentials in the visual cortex without an exogenous supply of the all-trans retinal chromophore (Figure 15.3). Using similar rAAV vectors to deliver ChR2, the restoration of visually evoked potentials

and visually guided behaviors induced by optomotor responses has been reported in Royal College of Surgeons (RCS) rats, a rat model for inherited retinal degeneration [4, 5]. Furthermore, the OFF light responses in the retina were restored with the expression of halorhodopsin [6]. Limited by a lack of retinal cell-specific promoters, these early studies could only achieve non-selective expression of ChR2 in the retina, predominantly in third-order neurons. More recently, the targeted expression of ChR2 in ON-type retinal bipolar cells using an mGluR6 promoter and *in vivo* electroporation or viral-mediated subretinal delivery restored the visual function in *rd1/rd1* mice and other photoreceptor-degenerated mouse models [7, 8]. Furthermore, the viral-mediated expression of halorhodopsin in the surviving light-insensitive cone photoreceptors restored additional retinal signal processing features, such as ON, OFF, and ON-OFF light responses, lateral inhibition, and directional selectivity [9]. It has also been reported that visual function can be restored by expressing melanopsin, an endogenous photopigment found in a small population of intrinsic photo-sensitive retinal ganglion cells [10], and by using chemical-based photoswitches that are attached to either endogenous potassium channels or engineered glutamate receptors [11, 12].

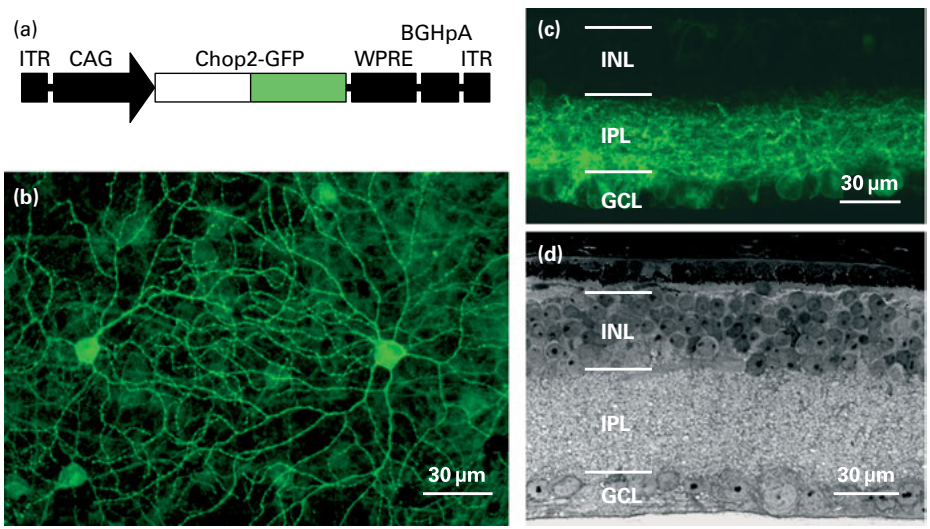

Figure 15.2: rAAV-mediated expression of ChR2-GFP in inner retinal neurons in *rd1/rd1* mice *in vivo*. (a) rAAV-CAG-ChR2-GFP-WPRE expression cassette. CAG: a hybrid CMV enhancer / chicken β-actin promoter. WPRE: woodchuck post-transcriptional regulatory element. BGHpA: a bovine growth hormone polyadenylation sequence. ChR2-GFP fluorescence viewed in a flat retinal whole-mount (b) and a retinal vertical section (c). (d) Light microscope image of a semithin vertical retinal section from *rd1/rd1* mice. ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. (From Bi *et al.* [13], with permission from Elsevier).

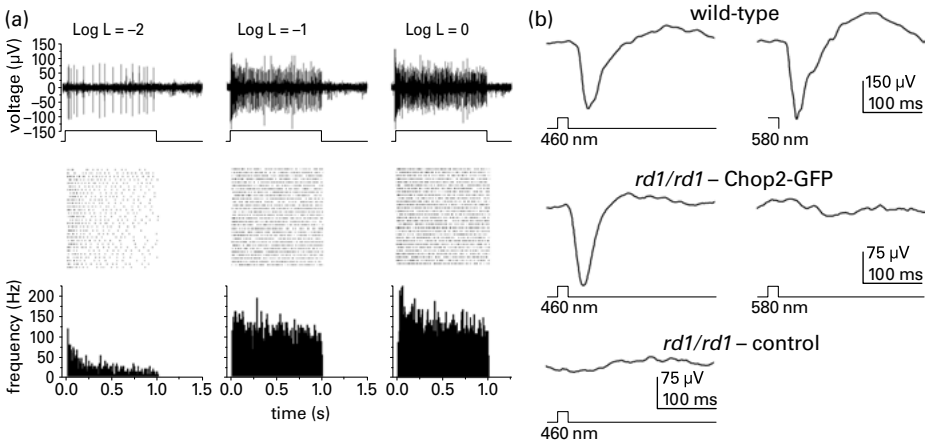

Figure 15.3: Electrophysiological recordings of ChR2-mediated light responses from retinal ganglion cells by multi-electrode array recordings and visual evoked potentials (VEPs) from primary visual cortex in *rd1/rd1* mice. (a) Top panel: Sample light-evoked spikes recorded from a single electrode to three incremental light intensities. Middle panel: The raster plots of 30 consecutive light-elicited spikes. Bottom panel: The averaged spike rate histograms. (b) Top panel: VEPs recorded from a wild-type mouse. The responses were observed both to the wavelengths of 460 and 580 nm. Middle panel: VEPs recorded from an *rd1/rd1* mouse injected with ChR2-GFP viral vectors. The responses were elicited only by light at the wavelength of 460 nm but not at the wavelength of 580 nm. Bottom panel: No detectable VEPs were observed from *rd1/rd1* mice injected with viral vectors carrying GFP alone. (From Bi *et al.* [13], with permission from Elsevier).

Follow-up studies have shown that the stable expression of functional ChR2 in the retinal neurons of rodents using a single administration of viral vectors can last the entire lifespan of the animal. The expression of wild-type ChR2 does not appear to produce any neurotoxic or harmful immunological responses *in vivo* [14, 15]. The virus-mediated expression of ChR2 and physiological responses have also been evaluated in non-human primates, although the transduction efficiency is much lower [16]. Thus, the proof-of-concept studies for using optogenetic approaches to restore vision have been convincingly demonstrated in animal models. However, further studies are needed for moving this treatment strategy into clinical applications. These studies should include developing better optogenetic tools, improving the efficiency of viral-mediated gene delivery, and developing retinal cell type-specific targeting.

15.3 Light sensors

Currently, the optogenetic light sensors that have been used for vision restoration include microbial rhodopsins, such as ChRs and their derivatives [3, 4, 5, 7, 8], halorhodopsin [6, 9], melanopsin [10], and chemical-based photoswitches [11, 12].

The use of microbial rhodopsins, especially ChRs, as light sensors for vision restoration has several advantages. They are genetically encoded membrane channels or pumps that are permeable to physiological ions, have fast kinetics, and use endogenously available chromophore. Initially, a major hurdle with using microbial rhodopsins was their low operational light sensitivity in their expressing cells. For example, the light intensity required for activating wild-type ChR2-expressing retinal neurons is at least 4 log units higher than the light intensity required to activate cone photoreceptors. Recently, a number of ChR mutants and variants have been reported that show a substantial increase in the operational light sensitivity in their expressing cells [17, 18, 19, 20, 21]. For almost all of the reported ChR mutants and variants, however, the increase in the operational light sensitivity was shown to be correlated with a decrease in the kinetics or an increase in the OFF rate [20, 21]. Thus, when using these more operational light-sensitive ChR mutants for vision restoration, the trade-off between light sensitivity and temporal dynamics must be considered. Further development of ChR2 with higher operational light sensitivity but retaining fast kinetics is certainly needed. Yet, with the discovery of these more operational light-sensitive ChR mutants and considering that the visual system may function with a relatively slow light sensor, the low operational light sensitivity for ChRs might not represent a major hurdle for vision restoration. Furthermore, the low sensitivity may be partially compensated for by an extra ocular imaging device, which would likely be required for the optogenetic-based retinal prosthesis.

Currently, very few hyperpolarizing light sensors have been developed. It has been reported that cells expressing ArchT, a light-driven outward proton pump, exhibit larger currents than that of halorhodopsin [22]. Its expression and function in retinal neurons, however, remains unknown. The development of K^+ selective ChR variants would be especially valuable.

Optogenetic approaches, especially using microbial rhodopsins, could also potentially restore color vision. The human retina contains three different cone opsins with peak spectral sensitivities at 437, 533, and 564 nm. With the recent reports of red-shifted ChR variants [20, 21], optogenetic tools for restoring color vision may already exist. Major challenges for restoring color vision would be the development of retinal cell type and circuit-specific targeting (see Section 15.5).

Melanopsin is a photopigment that is expressed in a small population of intrinsic photosensitive retinal ganglion cells. It is mainly involved in regulating the circadian rhythm, pupillary light reflex, and other non-visual responses to light. One of the major advantages of using melanopsin as a light sensor is that it is an endogenous photopigment protein in the retina. Thus, there would be no biocompatibility concern in expressing melanopsin in retinal neurons. Additionally, the activation of melanopsin to open cation channels is coupled with a G-protein-coupled amplifying intracellular signaling cascade. Therefore, its operational light sensitivity is much higher, approximately two log units higher, than wild-type ChR2. Furthermore, it has been reported that all retinal ganglion cells endogenously express the downstream signal-

ing pathway required to produce the light-evoked responses. Therefore, like microbial rhodopsins, only the introduction of the melanopsin gene is required to render light sensitivity to retinal ganglion cells. The major disadvantage of using melanopsin, however, is its slow kinetics. The melanopsin-mediated light response can last for many seconds after the light stimulus has terminated. Therefore, the expression of melanopsin would lack temporal dynamics, and while blind mice that ectopically express melanopsin were able to distinguish light from dark, they did not show signs of pattern recognition [10]. Modifying the signaling pathway involved with melanopsin to speed up the kinetics may be a possible solution.

Chemical-based photoswitches are created by covalently linking photoisomerizable molecules to either endogenous membrane channels, such as K^+ channels, or ectopically expressed receptors, such as ionotropic glutamate receptors [11, 12]. The operational sensitivity of these light sensors was similar to that of wild-type ChR2. One of the main disadvantages of these light sensors is that they require a continuous supply of the photoisomerizable molecules. In addition, the molecules that are currently used require using UV light, but red-shifted variants may be developed in the future. The long-term neurotoxicity of these molecules to retinal neurons remains to be determined.

Currently, microbial rhodopsins, especially the ChRs, are considered to be the most favorable candidates for the optogenetic approaches of vision restoration. However, it should be mentioned that the long-term safety of expressing microbial rhodopsins in the retina have only been conducted for wild-type ChR2 in animal models. Similar studies will need to be conducted for the modified ChR variants as well as other microbial rhodopsins. Furthermore, the neurotoxic effects associated with, and the immunological responses to, the expression of microbial rhodopsins in humans will need to be evaluated.|

15.4 rAAV-mediated retinal gene delivery

Recombinant adeno-associated virus (rAAV) vectors are considered to be the most promising vehicle for therapeutic gene delivery in the retina because of their nonpathogenic and nonimmunogenic properties towards the host, efficient transduction rate in both dividing and non-dividing cells, and broad cell and tissue tropisms. rAAV vectors have already been used in several clinical trials, including for the treatment of retinal degenerated diseases [23]. Therefore, using rAAV vectors for the therapeutic delivery of optogenetic tools to retinal neurons is a rational approach. Subretinal viral injections are commonly used to transfect photoreceptor cells. Intravitreal viral injections are more preferable for delivering transgenes to inner retinal neurons because they should result in a broad expression and cause less operational damage to the retina. In general, however, the transduction efficiency with intravitreal injection in distal retinal neurons, photoreceptors and bipolar

cells, is poor. The low transduction efficiency might be partially due to a physical barrier that affects the diffusion of viral vectors to reach these distal retinal neurons. In primates, the transduction efficiency with intravitreal injection is poor even in the third-order retinal neurons [16]. It has been suggested that the inner limiting membrane (ILM) is a major barrier that prevents intravitreal gene delivery in the primate retina [16, 24] and this scenario will most likely also be the case in humans. Therefore, further studies to improve the transduction efficiency are required for retinal gene therapy in humans. In mice, rAAV vectors with capsid mutations have substantially higher transduction efficiencies in distal retinal neurons [25]. Furthermore, it has been shown that a mild digestion of the ILM with a nonspecific protease increases the transduction efficiency of several rAAV serotypes in rats [24]. Removing the ILM by mechanical peeling at the time of virus vector injection may also be a viable solution.

One major limitation for using the rAAV system is that only a small genome (<4.8 kb) can be packaged. This limitation restricts the size of the promoters and regulatory components that can be used for retinal cell type-specific targeting. Furthermore, the rAAV vector genomes mainly exist in the host cells as circular episomes. Although the retina is a post-mitotic quiescent tissue, the long-term stability of rAAV-mediated expression in retinal neurons remains to be studied.

15.5 Retinal cell-type specific targeting

Regarding the implementation of the optogenetic approaches for vision restoration, it is unknown whether the ubiquitous expression of a depolarizing or hyperpolarizing light sensor in inner retinal neurons to convert all of these neurons to ON or OFF cells would sufficiently restore adequate vision. Clinical trial results for device implants that indiscriminately deliver a depolarizing current stimulation to all the inner retinal neurons have suggested that useful vision could be restored. However, it is commonly thought that the targeted expression of depolarizing and hyperpolarizing light sensors in retinal ON and OFF cells, respectively, would better mimic the intrinsic visual processing and may produce better results for restoring vision. If this hypothesis is true, then methods that target the expression of depolarizing or hyperpolarizing light sensor to specific retinal cell types or subcellular compartments will be required.

Regarding retinal cell specific targeting, there are a number of possible approaches, with advantages and disadvantages. In general, a major advantage of targeting the more distal retinal neurons, such as the surviving cone photoreceptors and bipolar cells, is that these cells may utilize the remaining retinal circuitries and thereby partially retain the intrinsic retinal processing pathways [9]. Targeting the distal retinal neurons may increase the operational light sensitivity and spatial resolution, because of the signal convergence from photoreceptor cells to bipolar and

ganglion cells. This would be a particular advantage for targeting the surviving cone photoreceptors because only cone photoreceptors are present in the fovea, which is the area of the retina with the highest spatial acuity. In addition, targeting ChRs to the distal neurons in the rod pathway, such as rod bipolar cells and AII amacrine cells, could potentially restore the ON and OFF responses in the downstream retinal ganglion cells. A major disadvantage for targeting the more distal retinal neurons, however, is the concern over the condition of the diseased retinas resulting from the retinal remodeling caused by the death of photoreceptor cells. It has been shown that during retinal degeneration, the inner retinal neurons and circuits undergo progressive and time-dependent remodeling [26]. The severity in the remodeling varies between the different degenerative diseases, but the distal retinal neurons appear to be the most affected. On the other hand, studies in RP animal models have shown that retinal ganglion cells appear to be the most resistant to remodeling [27]. Therefore, for many cases of advanced retinal degeneration, targeting the retinal ganglion cells could be the only option for retina-based optogenetic therapies. Therefore, developing multiple targeting approaches would be needed to treat the blindness caused by different degenerative diseases.

Restoring the ON and OFF pathways by directly expressing optogenetic tools to ganglion cells will require the targeted expression of depolarizing light sensors in ON ganglion cells, and hyperpolarizing light sensors in OFF ganglion cells. Furthermore, the differential expression of ON and OFF light sensors in the proximal dendrites / soma and distal dendrites of retinal ganglion cells using subcellular targeting motifs, can be used to create center-surround receptive fields in retinal ganglion cells [28].

Further studies will be needed to identify and develop retinal cell type specific promoters that can be used in effective delivery vehicles, such as the rAAV vector, to express light sensors in a specific retinal cell type(s). Currently, only photoreceptor cell-specific opsin promoters and ON bipolar cell-specific mGluR6 promoters have been characterized. Opsin promoters constructed in the rAAV vector have been used to successfully target halorhodopsin to cone photoreceptors [9]. A short mGluR6 promoter has been identified that can drive transgene expression in ON type retinal bipolar cells using electroporation. However, the use of this short promoter in the rAAV vector for targeted expression in ON type retinal bipolar cells has only been reported through subretinal virus injection [8]. Cell type-specific promoters for many other inner retinal neurons remain unknown. Specifically, ON and OFF ganglion cell-specific promoters have not been identified. The identification and development of cell-specific promoters and regulatory components for retinal cell-specific targeting will be one of the most challenging tasks in the development of optogenetic therapy.

15.6 Summary

- Restoring lost vision to the blind may be one of the most promising clinical applications for optogenetics.
- Proof-of-concept studies have already been conducted in animal models.
- Further developments of optogenetic tools that can better fit the needs for restoring vision are required.
- The viral-mediated gene delivery and transduction efficiency in the human retina need to be improved.
- The long-term stability and safety of expressing optogenetic tools in the human retina need to be evaluated.
- The development of retinal cell-specific targeting will be one of the most challenging tasks.

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16 Restoration of vision – the various approaches

16.1 Introduction

This position paper discusses the various approaches for restoration of vision in retinal dystrophies and dysfunction disorders that are presently under development. There are at least three categories of visual loss that need different approaches of restoration:

Category 1: All neurons are still present, at least for a long time, but they are not functional as one of the constituents, *e.g.*, the phototransduction process, is not working properly. Only this particular defect needs to be repaired to restore function.

Category 2: The degenerative process is progressing from the beginning, changing morphology and function of photoreceptors, sometimes in a process that lasts several decades. The general health of the affected cell groups has to be the focus of treatment strategies to slow down or stop degeneration.

Category 3: Photoreceptor cells have been lost completely. In this case substitution of cells and/or their function is the only possible remedy.

16.2 The various conditions to be treated

Type 1: Hereditary retinal diseases where photoreceptors are *slowly* dying, *i.e.*, becoming functionally and morphologically altered, but maintaining useful function for 3–5 decades of life (*e.g.*, retinitis pigmentosa; RP). *Treatment options*: gene therapy, neuroprotective agents.

Type 2: Congenital retinal dysfunction is due to single defects in the phototransduction cascade or the visual cycle like in achromatopsia, other channelopathies, or RetGC1 mutations, where cones and rods are initially intact. The retina may look quite normal for several decades, yet neither rods nor cones function. No proper neuronal network is cortically formed in these early forms and thereby the therapeutic outcome is presumably limited. *Treatment option*: turning on the "light switch" by gene therapy.

Type 3: The genetic defect induces degeneration of photoreceptors that become functionally and morphologically altered, and ultimately die. However, they may maintain useful function over several decades of life. This is the group of rod-cone-dystrophies also called retinitis pigmentosa (RP), and of cone-rod-dystrophies. *Treatment options*: gene therapy, neuroprotective agents, optogenetics.

Type 4: Rods and cones are almost completely lost early in life, as in some forms of Leber's congenital amaurosis (LCA) and early onset severe retinal dystrophies (EOSRD), with blindness occurring after several years in some cases. *Treatment*

options: optogenetics of inner retina cells, stem cells: in cases where some vision was possible during the first decade of life, electronic implants (epiretinal or subretinal) may help.

Type 5: Acquired forms of retinal blindness and with preserved inner retina. *Treatment options*: electronic retinal implants, stem cells.

Type 6: Retina and/or optic nerve fibers destroyed (*e.g.*, glaucoma, diabetic retinopathy, retinal detachment). *Treatment option*: cortical implants.

Type 7: Cortical destruction (*e.g.*, stroke, trauma). *Treatment option*: multi-electrode arrays addressing tongue sensors.

General treatment options: training measures and devices to improve utilization of various sensory inputs, as well as multiple rehabilitation measures.

16.3 State of the various restorative approaches

16.3.1 Neuroprotection

16.3.1.1 Encapsulated cell technology (ECT)

It has been shown that various growth factors (CNTF, ciliary nerve growth factor, BNDF, brain derived neurotrophic factor, and others) can slow down or revive photoreceptor function.

Neurotech Inc. [1] has developed a capsule filled with cells that produce CNTF. This capsule is inserted into the vitreous and releases CNTF for many months without being affected by immunological mechanisms, as the capsule has pores for releasing the growth factor that are too small to allow immune competent cells to enter the capsule. This approach is in phase II and III studies in retinitis pigmentosa and age-related macular degeneration (see Figure 16.1).

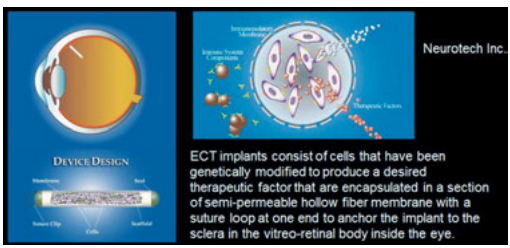

Figure 16.1: Encapsulated cell technology implants. It has been shown in Ocular Coherence Tomography (OCT) that the outer nuclear layer (ONL) thickness increases upon treatment, and by histology that the nuclei increase in size. However, it has not been shown unequivocally that rod or cone outer segments increase in length *i.e.*, get back to better morphology. Some proteins are up regulated, and some are down regulated. Further research is necessary to establish this treatment. (Images from the Neurotech Inc. website [1]).

16.3.1.2 Electrostimulation

It has been shown in preclinical [2] and clinical studies that ocular electrostimulation via corneal electrodes (Figure 16.2) may improve vision in hereditary retinal degenerations. There is data that corneal electrostimulation with 20 Hz biphasic pulses in retinitis pigmentosa patients slows down loss of visual field area in patients with retinitis pigmentosa (prospective investigator masked study of Schatz *et al.* [3]). It has been shown in various species in preclinical studies that this type of electrostimulation releases *endogenous* growth factors (BNDF, CNTF, and FGF) which may prolong survival of neurons. Presently, devices (including CE mark) are available (*e.g.*, OkuStim by Okuvision GmbH) for patients to stimulate both eyes with weak electrical currents. Long-term data are missing at present.

Figure 16.2: Ocular electrostimulation via corneal electrodes. (From Naycheva *et al.* [4])

The number of patients treated in this series was limited (24 in 3 groups, including a control group). Further results from the ongoing trials are required to appreciate the effectiveness of this method in the various forms of retinal degeneration.

16.3.1.3 Visual Cycle modulators

Orally administered synthetic retinoids are used to replace 11-cis-retinal, a key biochemical component of the visual retinoid cycle in cases of 11-cis-retinal deficiency (RPE65 deficiency and LRAT deficiency, both associated with severe and early onset retinal degeneration). It has been shown in dogs [5] that the biochemical defect in the retinoid cycle (RPE65 or LRAT deficiency) can be corrected by synthetic retinoids. Clinical studies have shown promising results already [6]. There is an increase in light sensitivity after prolonged dark adaptation that is, however, not useful in everyday life, as it immediately disappears once the patient gets to natural surroundings. Further studies will evaluate the benefit for cone vision.

16.3.1.4 Gene replacement therapy

There are numerous animal and human studies [7, 8, 9, 10, 11, 12] where viral or non-viral vectors are used to restore insufficient or lacking gene function as a result of pathogenic mutations, especially attractive for treatment in recessively inherited retinal degenerations (average prevalence approximately 1:5 000). The goal is to stop or prevent degenerative processes, by restoring regular physiological function of the affected tissue by postnatal supplementation of the normal gene in sufficient amounts and at the right place. The eye is particularly suited for gene therapy because of its small size and self contained structure. The availability of advanced tools for non-invasive clinical analyses improves specificity and sensitivity of assessing therapeutic effects. Several groups (see [13]) have performed clinical studies for phase I trials in human patients with RPE65 deficiency. Some studies have moved forward already to phase III. Currently, further clinical trials that target other genes such as REP-1 (Choroideremia), ABCA4 (Stargardt disease) or Usher 1b Syndrome are on the way, and more are being planned, *e.g.*, in Tübingen CNGA3 (achromatopsia). Most commonly adeno-associated viruses (AAV) are used that have been tested already extensively in long term expression studies. Presently no major severe adverse effects have been reported in clinical studies.

However, the results in human RPE65 trials are much less promising than in the dog, mainly limited to positive effects on light sensitivity and some effects on visual behavior. Yet, to date, no ERG recovery has been observed in humans, in contrast to the dog. Differences between preclinical and clinical studies may be due to missense versus stop mutations and the degree of retinal atrophy at the time of treatment. There are reports that degenerative processes, albeit slowed down, may continue to go on [14].

Achromatopsia: fMRI data suggest that rods are wired to brain areas that are usually stimulated by cones. OCT data show small abnormalities in the fovea. Therefore, although gene therapy is very promising in animal studies, it may be less effective in humans. However, in principle, gene therapy should function better in achromatopsia than in early onset severe cone dystrophies. More work will be needed to increase efficiency of this approach, and the degree of atrophy and miswiring in the optic pathways may limit treatment success.

16.3.1.5 Stem cell approaches

In recent years diverse cell populations were used for retinal transplantation experiments, *e.g.*, retinal stem cells, Müller glia cell derived cells or mesenchymal stem cells in the retina. It was demonstrated that transplanted cells differentiated and survived for long time periods in host retinae, and that some donor cells integrated into ganglion cell layers, plexiform layers or nuclear layers [15, 16, 17, 18, 19]. Although these experiments show promising results, functional replacement of photoreceptors could not be demonstrated in these studies on a larger scale. Evidence for differentiation of

the transplanted cells into “true” photoreceptors and functional integration into host retinae were only rarely reported.

Induced pluripotent stem cells, iPS cells or iPSCs, are artificially derived from adult somatic cells – by inducing a "forced" expression of specific genes. Induced pluripotent cells have been made from adult stomach, liver, skin, and blood cells. Induced pluripotent stem cells are similar to natural pluripotent stem cells, such as embryonic stem (ES) cells in potency and differentiability, but the full extent of their relation to natural pluripotent stem cells is still being assessed extensively (*e.g.*, by Shinya Yamanaka, Kyoto University and James Thomson, University of Wisconsin-Madison).

iPSCs are an important advance in stem cell research, as they may allow researchers to obtain pluripotent stem cells, which are important in research and potentially have therapeutic uses, without the controversial use of embryonic cells. Depending on the methods used, reprogramming of adult cells to obtain iPSCs may pose significant risks that could limit their use in humans. Approaches are underway to overcome these limitations by enrichment of stem cell derived photoreceptors by cell surface markers and manipulations of the host retina for enhanced integration of stem cell derived photoreceptors. At present, stem cell research is still at its infancy as to clinical applications.

16.3.1.6 Optogenetic approaches

Optogenetic approaches try to restitute light sensitivity in remaining neurons of the inner retina. Proof of principle has been established in animal experiments but light sensitivity is still several orders of magnitude lower than that of cones. Therefore, there is presently no chance to restitute light sensitivity without amplification of light. This can be done by a camera and goggles that allow the projection of an extremely bright image onto the retina, or by subretinal light emitting diode (LED) arrays that are positioned next to opsin-containing bipolar cells, *e.g.*, in an arrangement similar to that shown in Figure 16.3 (electrodes being replaced by LEDs). Moreover toxicity and long term stability have not been analyzed sufficiently to justify clinical studies at this time although surprising progress has been made in animal models (see Chapter 15 in this book). Still major developmental steps will be necessary prior to any clinical application.

16.3.1.7 Electronic retinal prosthesis

About 15 years ago, several groups worldwide began to develop various concepts by which the function of degenerated photoreceptors is replaced by electronic devices [21, 22, 23, 24]. Nowadays essentially three concepts are being pursued:

1. *Epiretinal electrode arrays* (bluish items in Figure 16.3) that are controlled by a camera outside the body and a computer that translates the video image into

pulses that are sent directly to the retinal output cells, *i.e.*, the ganglion cells and their nerve fibers.

2. The *subretinal approach* (yellowish items in Figure 16.3), aiming at replacing the photoreceptors by photodiodes at the input side of the degenerated retina, and stimulating bipolar cells, as photoreceptors would normally do; this approach utilizes the natural processing network of the inner retina, and places the stimulation electrodes at the place of the degenerated photoreceptors.
3. The *suprachoroidal approach* (greenish items in Figure 16.3) that is similar to the subretinal approach except that surgery is easier, as the electrode arrays are implanted from the scleral side. However due to the position *under* the choroid and the larger distance from the neuroretina spatial resolution is very low [25].

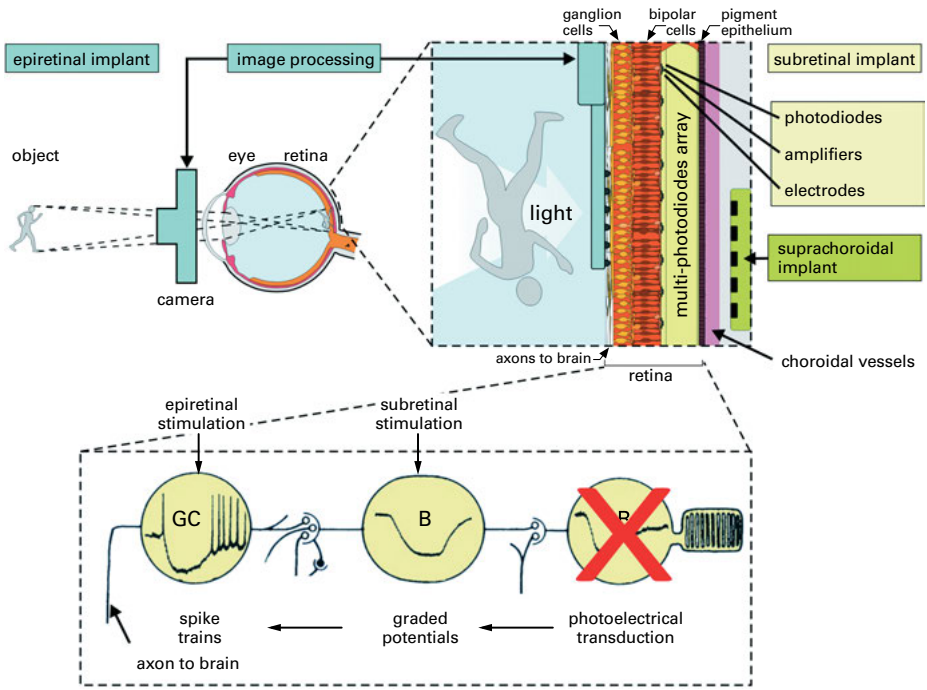

Figure 16.3: Three principal approaches to retinal implants to replace photoreceptor function: epiretinal (bluish), subretinal (yellowish) and suprachoroidal (greenish). (From Zrenner [20].)

All three approaches have the problem of providing power and control signals through transdermal inductive coils that deliver energy and signals via intraocular cables to the stimulating epi- or subretinal electrodes, a problem that limits the numbers of electrodes, addressed serially or in parallel, thereby limiting spatial resolution of artificial vision. In addition, complex surgical procedures are necessary for the cabling and

inductive coils to energize the electrode arrays. Moreover, waterproof encapsulation of intra- or paraocular electronic circuits with very thin material, due to limited space, is necessary, and heat production by electronic circuits has to be properly dealt with.

Presently only two systems are available to patients, the ARGUS II (Second Sight, Sylmar USA, CE mark recently approved) and the Alpha-IMS (Retina Implant AG, Reutlingen, Germany, CE mark pending). A wirebound pilot study has started in Australia in three patients [26]. Many other groups are also working hard to develop implants for artificial vision in blind people [27].

The US-based Second Sight's ARGUS II video camera system and its processor that addresses 60 *epiretinal* electrodes has been applied in 30 subjects in a clinical trial [24] and has received the CE mark in Europe and FDA approval in USA for end-stage retinitis pigmentosa. Besides the limitation of a relatively low number of electrodes, and therefore, limited spatial resolution of the ARGUS II system, the image receiver is located outside the body on the goggle frame and therefore, lacks the relation to eye movement and gaze.

The *subretinal implant* Alpha IMS from Retina Implant AG, Germany, with 1500 photodiodes and 70 µm spacing, has the highest pixel density available for patients so far [23]. To date, it has been implanted subretinally in 36 patients, and has allowed some patients to again read words, to recognize facial expressions and to find a knife, spoon, cup, plate, or a beer glass on a table [28]. The natural micro-saccades of the eye that involuntarily and constantly move the retina (and the chip) with a 3 Hz jitter across the visible objects, allow natural refreshing of the perceived image while with extraocularly mounted camera systems of other approaches, image refreshing can only be achieved by head nodding or head-shaking, in blind people (see recent review [27], Figure 16.4).

An example of a *subretinal* device (Retina Implant AG, Reutlingen, Germany) is shown in Figure 16.4. Each of the 1500 subfoveal photodiodes within an 11 × 11 degree field controls an amplifier that, depending on the strength of the light, ejects currents onto bipolar cells via an electrode [29]. Power and control signals are supplied in the new version by inductivity via a subdermal retroauricular coil from which a subdermal cable leads to the eye ball. In the meantime, a multicenter trial (Oxford, Hongkong, London, Tübingen) with a wireless version of the implant Alpha-IMS (Retina Implant AG, Tübingen, Germany) has been started. The new wireless device has been implanted in Tübingen in 9 patients since 2010 (average age 46y ±7.9; 5 males, 4 females). Function was tested with 4 procedures:

(1) Monitor-based standardized tests with controlled conditions for testing light perception threshold, light localization and movement [30], as well as grating acuity and Landolt C-rings (2 or 4 alternative forced choice); (2) Recognition tasks at a table setting with table ware and geometric objects; (3) Reading of letters; (4) Outdoor activity reported by the patients. All test results were controlled with power switched off.

As published by Stingl *et al.* [28], the chip was at the desired subfoveal position in all 9 patients, except two, where it was slightly parafoveal. Proper chip function was proven by measuring chip output via electroretinography. All patients were able to

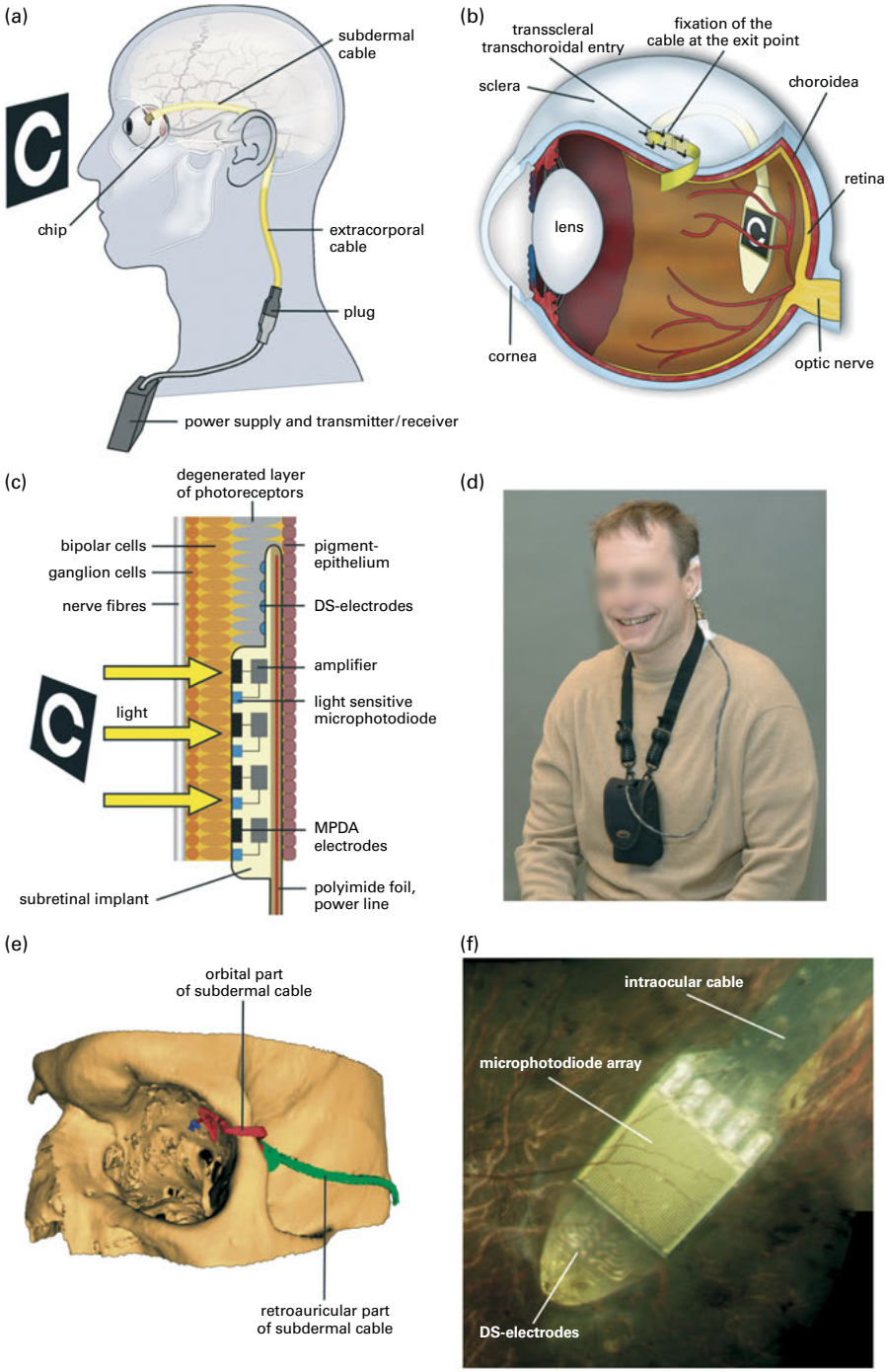

Figure 16.4: Subretinal electronic chips (from [23]).

perform the function tests, except one, who experienced loss of inner retina function after surgery. The following results were obtained: light perception: 9/9, light localization: 8/9; motion recognition 5/9; grating resolution 8/9 (up to 3.3 cycles/degree); Landolt C rings 3/9 (up to 0.036); recognition of geometric objects 8/9; recognition of objects in table setup 8/9; Letter reading 4/9; clock hands reading 3/9; grey scale differentiation 6/9; improved outdoor mobility and activity 5/9.

Patients reported numerous beneficial visual experiences in daily life, with regained recognition of unknown objects, recognition of facial or clothing characteristics, moving objects in nature and traffic, improved self-sustaining actions (recognition of doors, door handles), recognition of small objects (glasses, telephone, stapler, washing basin, even dice and numbers of dots on dice), and improved mobility. This example shows that the wireless Alpha-IMS implant is able to restore useful visual abilities to blind RP-patients.

16.3.2 Cortical prosthesis

Following the early work of G. Brindley in the late 1960s, several groups attempted to stimulate the primary visual cortex, using fine wire electrodes that are inserted into the area V1, for the purpose of restoring vision in blind patients [22, 31]. A major limitation of this work is the development of an image processing system to convert an electronic image captured by a camera into a real-time data stream for stimulation of the implanted electrodes in a way that can be “understood” by the cortical neurons. Flexible arrays with penetrating electrodes have been developed (Utah Electrode Array by Richard Normann) that have been tested in various animals including monkeys [31]. It is, however, not yet clear to which extent such approaches can provide useful vision.

16.3.3 Tongue stimulators

For the past ten years, researchers at the University of Wisconsin [32] have been developing a device that delivers spatially structured input to the tongue via a matrix of electrodes worn inside the mouth. Using a camera, a computer and the input device, individuals who have been blind for their whole life are able to use this relatively simple and non-invasive device to recognize basic patterns (BrainPort-Technology by WIBCAB). A one-year study with 75 patients is underway.

In much the same way that people can use their fingertips to read Braille letters, people can recognize simple special patterns using comfortable electrical stimulation of the tongue, allowing subjects to identify simple geometric patterns such as circles, squares and triangles. Due to the electrotactile stimulation, the electrical stimuli on the tongue feels like a tickle or vibration, and application is not painful.

16.4 The current situation

1. Optogenetic approaches to date have only limited applicability in RP patients with well-maintained inner retinal neurons and otherwise intact visual system and constitute only one of several possibilities. There are still major problems to generate high light sensitivity without major limitations of temporal resolution and vice versa. Presently, there is no indication that light amplifying technically demanding goggles can be avoided by this approach.
2. There is a multitude of different blinding retinal diseases which need various types of treatments depending on age of onset and age at treatment *i.e.*, stage of degeneration, *e.g.*, gene therapy, neuroprotection, electrostimulation, artificial vision implants, tongue stimulators, and possibly optogenetics and stem cell approaches in the future, depending on the extent and localization of the defect.
3. Not much is known about toxicity, long-term applicability, safety and efficacy of the optogenetic approach. It will likely take a decade or more until a product will be available for patients, similar to the experiences made with other therapy developments in the field.
4. The spectral sensitivity of the light sensitive channels added by optogenetics into the cytoplasmatic membrane of inner retinal cells is, in most cases, not directly related to the hard-wired sensitivity of the various color channels in the retina and the brain. This could result in a disturbing false color image ("chromatic pointilistic chaos") in human vision approach that could make it difficult to identify line segments, chromatically coherent areas, *etc.* which constitute an image. The problem could be possibly solved by developing promoters that specifically address the various chromatic channels and provide the particular opsin with the appropriate spectral sensitivity.

16.5 Open Questions

1. Nature has developed specific photoreceptor cells, *i.e.*, rods and 3 types of cones, with stacks of thousands of disks and with large amounts of specific photosensitive molecules per receptor cell. Will it ever be possible with the present optogenetic approaches to gain enough sensitivity (without hampering temporal resolution) to allow vision under regular illumination without complex technical aids? If external artificial light amplification aids are necessary, might the necessary strength of light get close to the maximum permissible exposure and act destructively on optogenetically engineered structures?
2. Given the problem of low sensitivity of light sensitive channels in the optogenetic approach, would a hybrid between subretinal implants, consisting of thousands of photodiodes (instead of electrodes as in subretinal electronic implants), allow us to achieve spatially ordered vision, as subretinal photodiode arrays are in

very close contact with optogenetically treated bipolar cells and therefore only few photons might be necessary for excitation? Might spectrally programmable LEDs close to bipolar cells allow for adjusting "true color" perception, difficult to achieve?

3. Is it really necessary to separate ON- from OFF-channels, given the fact that patients who have OFF-channels only (complete congenital stationary night blindness cCSNB) have sufficient vision (with slightly lowered contrast)? This question is also based on the observation that vision is possible with stroboscopic stimulation where ON- and OFF-channels are stimulated quasi-simultaneously by micro second light pulses.

16.6 Conclusion

Almost all treatment options discussed in this chapter were not imaginable twenty years ago. The multitude of approaches developed during the last two decades, and presently under investigation in many laboratories and clinics worldwide, gives justified hope that during the next decade effective therapies will become available to many patients with degenerative retinal diseases.

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2. Retinal Imaging of Subjects Implanted With Ciliary Neurotrophic Factor (CNTF)-Releasing Encapsulated Cell Implant for Early-stage Retinitis Pigmentosa. ClinicalTrials.gov Identifier: NCT01530659
3. A Study of an Encapsulated Cell Technology (ECT) Implant for Patients With Atrophic Macular Degeneration. ClinicalTrials.gov Identifier: NCT00447954
4. Repeated Treatments of QLT091001 in Subjects With Leber Congenital Amaurosis or Retinitis Pigmentosa (Extension of Study RET IRD 01). ClinicalTrials.gov Identifier: NCT01521793
5. Oral QLT091001 in Retinitis Pigmentosa (RP) Subjects With an Autosomal Dominant Mutation in Retinal Pigment Epithelial 65 Protein (RPE65). ClinicalTrials.gov Identifier: NCT01543906
6. Phase I/IIa Study of StarGen in Patients With Stargardt Macular Degeneration. ClinicalTrials.gov Identifier: NCT01367444
7. Study of UshStat in Patients With Retinitis Pigmentosa Associated With Usher Syndrome Type 1B. ClinicalTrials.gov Identifier: NCT01505062
8. Safety and Efficacy of Subretinal Implants for Partial Restoration of Vision in Blind Patients
9. ClinicalTrials.gov Identifier: NCT01024803

Tobias Moser

17 Optogenetic approaches to cochlear prosthetics for hearing restoration

17.1 Background and state of the art

The WHO estimated that in 2005 there were 278 million people in the world with disabling hearing impairment [1]. So far, a causal treatment is not available for its most common form: sensorineural hearing impairment. Therefore, hearing aids and auditory prostheses represent the only means to restore auditory function in most hearing impaired subjects. Cochlear implants (CIs) bypass the dysfunctional sensory organ of Corti in the cochlea via direct electric stimulation of spiral ganglion neurons (SGNs). CIs enable open speech comprehension in the majority of deaf or profoundly hearing impaired users [2, 3, 4, 5]. However, the quality of hearing with electrical CIs is low *e.g.*, in terms of speech comprehension in noisy background and music appreciation. This is largely attributed to the wide-spread current around an electrode contact (schematized in Figure 17.1, see [6]), which leads to channel crosstalk [7] and limits the number of useful frequency channels to less than ten [8]. Frequency resolution can be improved using multipolar stimulation, at the expense of higher power consumption [9, 10], or by intraneural electrodes [11]. Information coding by CIs is also limited with respect to sound intensity: the dynamic range of their output is typically below 10 dB [4, 12]. Increasing the frequency and intensity resolution of auditory coding with CIs, is an important objective for improving the quality of hearing and speech comprehension. Optical stimulation is expected to dramatically increase the frequency resolution of CI coding, because light enables spatially confined activation of SGNs, and therefore promises to overcome the limitations of current CIs (Figure 17.1).

In addition, activation of smaller populations of neurons will also enhance the dynamic range of coding by varying recruitment of neighboring channels. Finally, optical stimulation may be controlled in a closed loop when combined with electric recording of neural activity. Important technological advances in optoelectronics now enable tailored optical stimulation. Light emitting diodes (LEDs) [13] have reached a power efficiency of more than 50% [14] and LEDs in the tens of micrometers (μLEDs) can be fabricated, bonded and prepared for collimation of the emitted light [15]. Vertical cavity self-mixing laser diodes for different light colors, microfabricated waveguide arrays, as well as thin-film flexible electronics on shape memory polymer substrates [16], have become available.

Currently, two alternative strategies to develop a new generation of CIs based on optical stimulation of SGNs are pursued. Richter *et al.* have pioneered infrared neural stimulation of SGNs. They have characterized and refined the method for a few years now and have achieved a frequency resolution of coding that was compa-

rable to normal hearing [17]. Pulsed infrared light changes the surface charge on the neuronal membrane and thereby triggers action potentials [18]. However, the energy requirement per channel and stimulation pulse seems to exceed that used in clinical implants by 2–3 orders of magnitude [17], and some controversy remains about the leading mechanism for cochlear stimulation by strong laser pulses [19].

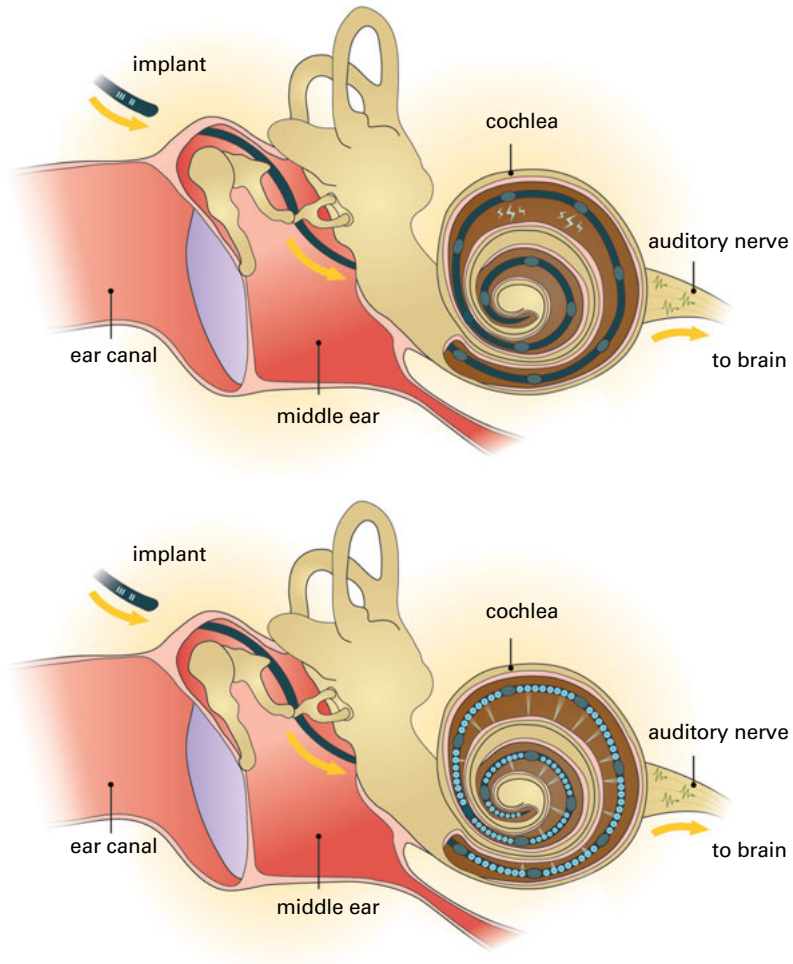

Figure 17.1: Electrical versus optical stimulation of cochlear spiral ganglion neurons. **Top:** in electrical CI usually 8–22 electrodes are used to stimulate SGN by charge-neutral biphasic stimuli in a monopolar configuration. Current spread leads to activation of a large population of neurons along the tonotopic axis thereby limiting the frequency resolution and dynamic range of electrical coding. **Bottom:** optical stimulation promises spatially confined activation of SGNs allowing for a higher number of independent stimulation channels and, thereby, improving frequency and intensity resolution.

Optical stimulation of SGNs expressing microbial light-sensitive channelrhodopsins (reviewed in [20, 21]), pioneered by our laboratory, has: i) lower power requirements; ii) thereby permitting a broader choice of technological solutions for optical coding (*e.g.*, including LEDs); and iii) offers molecular optimization of the SGN photoresponse. So far, there is no published work on cochlear optogenetics, but several groups have now started to work on this approach. Companies have recognized the potential of optical stimulation for fundamentally improved clinical hearing restoration with increased frequency and intensity resolution of CI coding. In addition to its potential for clinical prosthetics, cochlear optogenetics will become a useful tool for auditory neuroscience. Cochlear optogenetics will likely serve for studies of the auditory system's development (*e.g.*, role of pre-sensory activity in the assembly of tonotopy), function (*e.g.*, cochlear amplification, sound encoding and localization, higher central auditory processing) and dysfunction (*e.g.*, tinnitus). Here I focus on the optogenetic stimulation of SGNs, provide a brief summary of the current research, and an outline for future work towards optogenetic restoration of hearing.

17.2 Current research on cochlear optogenetics

Recent work on transgenic mice and rats, as well as on virus-injected mice expressing ChR2, or its variant CatCh, in SGNs in a collaborative effort led by our laboratory, has proven the concept of cochlear optogenetics at the single neuron and neuronal population levels (Figure 17.2).

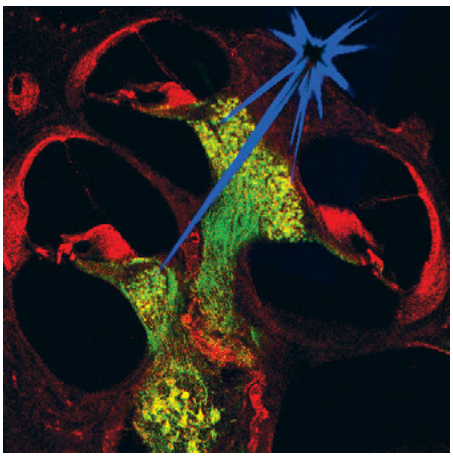

Figure 17.2: Expression of channelrhodopsin in spiral ganglion neurons. Confocal image of a cochlear cryosection immunolabeled for GFP (green) and labeled by phalloidin-rhodamin (red). GFP-immunoreactive neurons express ChR2-YFP fusion protein and are housed in the center of the cochlear, the so-called modiolus. Blue star motivates the blue light activation of the neuron to be achieved by an optical implant in reality.

We demonstrated specific activation of the auditory pathway, restoration of auditory systems responses in deaf mice, and AAV-mediated viral transfer of optogenes. Cochlear optogenetics drove spiking in single SGNs expressing CatCh with low jitter up to at least 60 Hz, as well as in single inferior colliculus neurons. We succeeded to use transcochlear (via a fenestration of the bony cochlear capsule) and intracochlear stimulation strategies employing µLEDs and fiber-coupled lasers. Moreover, we characterized morphology of mouse and rat cochleae by X-ray tomography. We developed fully implantable µLED stimulators for behavioral experiments and established chronic fiber stimulation. Together with our collaborators we are working on establishing optical stimulation using flexible µLED arrays (with Schwarz *et al.*, Fraunhofer IAF, Freiburg, Germany) and waveguide arrays (with *Voit et al.*, Dallas, USA).

17.2.1 Current and future work on cochlear optogenetics aims at

1. Efficient, long-term and safe postnatal viral transduction of auditory neurons in rodents and other species, eventually in primates.
2. Identifying / generating channelrhodopsins with properties most appropriate for optogenetic restoration of hearing.
3. Technological development of active (flexible, safely sealed and durable, as well as power-efficient emitter arrays, *e.g.*, with µ-LED) and passive (waveguide arrays) multichannel optical implants and studies of insertion, orientation and illumination.
4. Development of electronic control architecture and coding strategies for multichannel optical stimulation.
5. Detailed physiological characterization of optogenetic cochlear stimulation *in vitro* and *in vivo* at several levels of the auditory pathway, with the foremost goals of addressing the temporal bandwidth and reliability of optogenetic responses of spiral ganglion neurons and quantifying the spread of neural excitation in the cochlea (as an estimate for the frequency resolution of optical coding).
6. Behavioral experiments to characterize perception of optical stimulation in comparison to acoustic and/or electrical stimulation in rodents, and eventually, in primates.
7. Feasibility and safety studies for long-term optogenetic stimulation of the cochlea in larger animals, potentially including non-human primates.

17.3 Potential and risks of cochlear optogenetics for auditory prosthetics

Cochlear optogenetics has the advantage of working in a relatively well-understood sensory system and in the framework of successful neural prosthetic rehabilitation of hearing impaired subjects. Different from the use of optogenetics for restoration

of vision, where the alternative treatment modalities so far had only modest success, cochlear optogenetics, in order to be translated into a clinical application, must benchmark with and outperform the widely used electrical stimulation of SGNs by CIs. Moreover, the development of cochlear optogenetics occurs in parallel to that of infrared cochlear stimulation, which does not require genetic manipulation of neurons, but has higher power demands.

We argue that clinical trials become feasible once research on animals has been demonstrated cochlear optogenetics to achieve:

1. a frequency resolution of at least 1/3 of an octave
2. at least dozens of independent stimulation channels
3. reliable spiking of cochlear neurons for stimulation of at least 100 Hz for strong stimuli
4. reliable, efficient and safe optical cochlear implants with a per channel power consumption not exceeding that of electrical implants by more than an order of magnitude and that remain operational *in situ* for at least 1 year in rodents and non-human primates
5. a demonstration of safety of viral transduction and optical stimulation of SGNs (opsin expression limited to the manipulated ear and ideally to SGNs; lack of neural loss, scar formation, immune response and tumor genesis) over the time course of at least 1 year in rodents and non-human primates
6. stable expression of the optogenes for at least 1 year in rodents and non-human primates.

We anticipate that the development of efficient and safe cochlear optogenetics will benefit from parallel research efforts. We expect further rapid progress with identifying or generating longer wavelength, more light-sensitive, potentially higher conductance and rapidly gating channelrhodopsins. Moreover, safety studies and other findings of the highly advanced work on visual restoration using AAV-mediated viral gene transfer into the eye will greatly benefit efforts towards translation of cochlear optogenetics into the clinic. Finally, clinical trials on AAV-mediated gene transfer into the cochlea as well as synergistic efforts towards the development of optical stimulation architecture for infrared and optogenetic stimulation of the cochlea will be help advancing cochlear optogenetics.

In summary, cochlear optogenetics is an exciting interdisciplinary research topic with great potential for basic research and translation into clinic application.

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Sabine Schleiermacher

18 History in the making: the ethics of optogenetics

Optogenetics is a newly emerging, interdisciplinary research area, bringing together scientists from a wide range of disciplines, including biologists, chemists, medical clinicians and psychiatrists. Rapid advances in methods, processes and discoveries in the broader field of genetics are now also being applied in this area. The promise is that optogenetic techniques will allow research of cerebral function that is significantly more direct, precise and carries less risk of side effects than conventional methods, along with intervention into deep recesses of the brain. Utopias include improving the function of impaired brain areas, controlling the brain by means of external technology, and boosting its capabilities [1].

The new research approach and its ethical implications have raised similar questions to those that arose during the emergence of neurosurgery and psychopharmacological or genetic interventions. In fact, the questions resemble those stemming from the development and application of medical applications and practices, and have been answered to a large extent by national and international policy [2].

For example, general standards for research involving humans are specified by the World Medical Association (WMA) in the Declaration of Helsinki (DoH) [3]. The latter are echoed by Council of Europe's Convention on Human Rights and Biomedicine, whose guidelines are binding, at least for the states that have signed the treaty [4].

As part of the Nuremberg Doctors Trial in 1946/47, which tried physicians for their brutal experiments on humans during National Socialism, the judges of the US Military Tribunal formulated the Nuremberg Code, a set of ten principles serving as prerequisites for experiments involving humans [5] (Figure 18.1).

In reaction to the crimes of physicians that came to the light, representatives from a range of medical organizations organized themselves into the World Medical Association (WMA) in 1946 [6] (Figure 18.1). The WMA's 1964 Declaration of Helsinki is based on the Nuremberg Code of 1947, and has been revised several times over the following 40-plus years. By being based on the 1948 Universal Declaration of Human Rights, the much more recent convention of the Council of Europe convention positions itself within the same historical context. However, removed from the events of WWII and with much broader scope, it was not drafted from the perspective of medical practice, but instead as a regulatory framework for biomedical research and applications in as many European states and their various legal systems as possible [7].

The "Ethical Principles for Medical Research on Humans" in the WMA Declaration of Helsinki addresses physicians, along with "other participants in medical research involving human subjects." "Research on identifiable human material or data" is explicitly included in the DoH's scope [8]. After specifying the intended addresses, the document declares the physician's duty to "promote and safeguard

The Nuremberg Code (1947)

The judgment by the war crimes tribunal at Nuremberg laid down 10 standards to which physicians must conform when carrying out experiments on human subjects.

PERMISSIBLE MEDICAL EXPERIMENTS

The great weight of the evidence before us to effect that certain types of medical experiments on human beings, when kept within reasonably well-defined bounds, conform to the ethics of the medical profession generally. The protagonists of the practice of human experimentation justify their views on the basis that such experiments yield results for the good of society that are unprocurable by other methods or means of study. All agree, however, that certain basic principles must be observed in order to satisfy moral, ethical and legal concepts:

1. The voluntary consent of the human subject is absolutely essential. This means that the person involved should have legal capacity to give consent; should be so situated as to be able to exercise free power of choice, without the intervention of any element of force, fraud, deceit, duress, overreaching, or other ulterior form of constraint or coercion; and should have sufficient knowledge and comprehension of the elements of the subject matter involved as to enable him to make an understanding and enlightened decision. This latter element requires that before the acceptance of an affirmative decision by the experimental subject there should be made known to him the nature, duration, and purpose of the experiment; the method and means by which it is to be conducted; all inconveniences and hazards reasonably to be expected; and the effects upon his health or person which may possibly come from his participation in the experiment. The duty and responsibility for ascertaining the quality of the consent rests upon each individual who initiates, directs, or engages in the experiment. It is a personal duty and responsibility which may not be delegated to another with impunity.

2. The experiment should be such as to yield fruitful results for the good of society, unprocurable by other methods or means of study, and not random and unnecessary in nature.

3. The experiment should be so designed and based on the results of animal experimentation and a knowledge of the natural history of the disease or other problem under study that the anticipated results justify the performance of the experiment.

4. The experiment should be so conducted as to avoid all unnecessary physical and mental suffering and injury.

5. No experiment should be conducted where there is an a priori reason to believe that death or disabling injury will occur; except, perhaps, in those experiments where the experimental physicians also serve as subjects.

6. The degree of risk to be taken should never exceed that determined by the humanitarian importance of the problem to be solved by the experiment.

7. Proper preparations should be made and adequate facilities provided to protect the experimental subject against even remote possibilities of injury, disability or death.

8. The experiment should be conducted only by scientifically qualified persons. The highest degree of skill and care should be required through all stages of the experiment of those who conduct or engage in the experiment.

9. During the course of the experiment the human subject should be at liberty to bring the experiment to an end if he has reached the physical or mental state where continuation of the experiment seems to him to be impossible.

10. During the course of the experiment the scientist in charge must be prepared to terminate the experiment at any stage, if he has probable cause to believe, in the exercise of the good faith, superior skill and careful judgment required of him, that a continuation of the experiment is likely to result in injury, disability, or death to the experimental subject.

Taken from Mitscherlich A, Mielke F. *Doctors of infamy: the story of the Nazi medical crimes*. New York: Schuman, 1949: xxiii-xxv.

Declaration of Helsinki (1964)

Recommendations guiding physicians in biomedical research involving human subjects

Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975, and the 35th World Medical Assembly, Venice, Italy, October 1983

INTRODUCTION

It is the mission of the physician to safeguard the health of the people. His or her knowledge and conscience are dedicated to the fulfilment of this mission.

The Declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient."

The purpose of biomedical research involving human subjects must be to improve diagnostic, therapeutic and prophylactic procedures and the understanding of the aetiology and pathogenesis of disease.

In current medical practice most diagnostic, therapeutic or prophylactic procedures involve hazards. This applies especially to biomedical research.

Medical progress is based on research which ultimately must rest in part on experimentation involving human subjects. In the field of biomedical research a fundamental distinction must be recognised between medical research in which the aim is essentially diagnostic or therapeutic for a patient, and medical research the essential object of which is purely scientific and without implying direct diagnostic or therapeutic value to the person subjected to the research.

Figure 18.1: The Nuremberg Code / Declaration of Helsinki. Kindly provided by BMJ Publishing Group Ltd.

Special caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research must be respected.

Because it is essential that the results of laboratory experiments be applied to human beings to further scientific knowledge and to help suffering humanity, the World Medical Association has prepared the following recommendations as a guide to every physician in biomedical research involving human subjects. They should be kept under review in the future. It must be stressed that the standards as drafted are only a guide to physicians all over the world. Physicians are not relieved from criminal, civil and ethical responsibilities under the law of their own countries.

I. BASIC PRINCIPLES

1. Biomedical research involving human subjects must conform to generally accepted scientific principles and should be based on adequately performed laboratory and animal experimentation and on a thorough knowledge of the scientific literature.

2. The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol which should be transmitted to a specially appointed independent committee for consideration, comment and guidance.

3. Biomedical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given his or her consent.

4. Biomedical research involving human subjects cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the subject.

5. Every biomedical research project involving human subjects should be preceded by careful assessment of predictable risks in comparison with foreseeable benefits to the subject or to others. Concern for the interests of the subject must always prevail over the interests of science and society.

6. The right of the research subject to safeguard his or her integrity must always be respected. Every precaution should be taken to respect the privacy of the subject and to minimize the impact of the study on the subject's physical and mental integrity and on the personality of the subject.

7. Physicians should abstain from engaging in research projects involving human subjects unless they are satisfied that the hazards involved are believed to be predictable. Physicians should cease any investigation if the hazards are found to outweigh the potential benefits.

8. In publication of the results of his or her research, the physician is obliged to preserve the accuracy of the results. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

9. In any research on human beings, each potential subject must be adequately informed of the aims, methods, anticipated benefits and potential hazards of the study and the discomfort it may entail. He or she should be informed that he or she is at liberty to abstain from participation in the study and that he or she is free to withdraw his or her consent to participation at any time. The physician should then obtain the subject's freely given informed consent, preferably in writing.

10. When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship to him or her or may consent under duress. In that case the informed consent should be obtained by a physician who is not engaged in the investigation and who is completely independent of this official relationship.

11. In case of legal incompetence, informed consent should be obtained from the legal guardian in accordance with national legislation. Where physical or mental incapacity makes it impossible to obtain informed consent, or when the subject is a minor, permission from the responsible relative replaces that of the subject in accordance with national legislation. Whenever the minor child is in fact able to give a consent, the minor's consent must be obtained in addition to the consent of the minor's legal guardian.

12. The research protocol should always contain a statement of the ethical considerations involved and should indicate that the principles enunciated in the present declaration are complied with.

II. MEDICAL RESEARCH COMBINED WITH PROFESSIONAL CARE (CLINICAL RESEARCH)

1. In the treatment of the sick person, the physician must be free to use a new diagnostic and therapeutic measure, if in his or her judgement it offers hope of saving life, re-establishing health or alleviating suffering.

2. The potential benefits, hazards and discomfort of a new method should be weighed against the advantages of the best current diagnostic and therapeutic methods.

3. In any medical study, every patient—including those of a control group, if any—should be assured of the best proven diagnostic and therapeutic method.

4. The refusal of the patient to participate in a study must never interfere with the physician-patient relationship.

5. If the physician considers it essential not to obtain informed consent, the specific reasons for this proposal should be stated in the experimental protocol for transmission to the independent committee (1, 2).

6. The physician can combine medical research with professional care, the objective being the acquisition of new medical knowledge, only to the extent that medical research is justified by its potential diagnostic or therapeutic value for the patient.

III. NON-THERAPEUTIC BIOMEDICAL RESEARCH INVOLVING HUMAN SUBJECTS (NON-CLINICAL BIOMEDICAL RESEARCH)

1. In the purely scientific application of medical research carried out on a human being, it is the duty of the physician to remain the protector of the life and health of that person on whom biomedical research is being carried out.

2. The subjects should be volunteers—either healthy persons or patients for whom the experimental design is not related to the patient's illness.

3. The investigator or the investigating team should discontinue the research if in his/her or their judgment it may, if continued, be harmful to the individual.

4. In research on man, the interest of science and society should never take precedence over considerations related to the well-being of the subject.

Figure 18.1: (continued) The Nuremberg Code / Declaration of Helsinki.

the health of patients" and to "act in the patient's best interest". Any necessary medical research on humans must take as its goal to "understand the causes, development and effects of diseases and improve preventive, diagnostic and therapeutic interventions". Proven procedures were also to be "evaluated continually" to reconfirm their "safety, effectiveness, efficiency, accessibility and quality." However, the "wellbeing of the individual research subject must take precedence over all other interests." The DoH expressly notes the "special protection" required by particularly vulnerable research populations, such as those, "who cannot give or refuse consent for themselves". Earlier versions also noted the "economically and medically disadvantaged"; however, this provision was dropped in the 2008 revision of the document [9]. Research involving human subjects must "conform to generally accepted scientific principles", which should be "based on a thorough knowledge of the scientific literature, other relevant sources of information, and adequate laboratory and, as appropriate, animal experimentation." Furthermore, the DoH requires that, firstly, monitoring information, including both positive results and adverse events, be published in detail; secondly, beyond the parameters of the study itself, any direct or indirect funding be declared; and finally, the involvement of an ethic committee. As part of their application to the ethics committee, researchers are required to submit a "statement of the ethical considerations" involved in their proposed study and to demonstrate how the study has been designed to conform to the ethical principles specified in the DoH [10]. Medical researchers are obliged to evaluate a study's potential benefits on a wider level against possible risks for the study subjects throughout the lead-up to and during the experiments, and must terminate the study as soon as the risks outweigh the benefits or when "conclusive proof of positive and beneficial results" has been obtained. Once the researcher has confirmed that the study subject has "understood" the obligatory explanation of "aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail", he or she must obtain and document the subject's freely-given consent (commonly known as 'informed consent'). A central tenet is that the "physicians ... in medical research ... protect the life, health, dignity, integrity, right to self-determination, privacy ... of research subjects" and that "(t)he responsibility for the protection of research subjects must always rest with the physician or other health care professional and never the research subjects, even though they have given consent."

The spirit of the DoH has been summarized in Beauchamp and Childress' authoritative work on modern bioethical decision-making into the following four principles: Autonomy, nonmaleficence, beneficence and justice [11].

In the Federal Republic of Germany, all ethical decision-making must accord with Article 1 of the German Basic Law, which guarantees the inviolability of human dignity; even this tenet seems to have come under threat in recent times. The latter article was also formulated in response to the crimes against humanity perpetrated during the National Socialist era in Germany. This inviolability of human dignity

has since been understood within prevailing legal philosophy as an objective legal norm [12]. The Declaration of Helsinki names human dignity as its central tenet.

The Declaration of Helsinki, and particularly the European Convention on Bioethics, have attracted much criticism, including the contradictory charges that, on the one hand, they provide too much freedom for research involving humans, and on the other, that their stringency hampers medical research.

Maintaining a human subject's autonomy and right to self-determination is the primary priority in any experimental treatment. The subject must be provided with all available information in a way that can be understood. The individual must provide their consent freely; that is, consciously and unaffected by external influence. This also implies that the medical researcher not take advantage of the hierarchical relationship between expert and layman to shift this responsibility to the patient. Contractual agreements and regulations do not absolve researchers of responsibility. All stakeholders in a medical study have to consider both the limits and conditions of this freedom to choose and the interests of all directly and indirectly involved parties.

Optogenetic interventions that cause for irreversible changes in the brain go far beyond the purely physical into the realm of personality. The latter can be considered incongruous with the DoH stipulation that any medical studies "minimize the impact" on the "physical, mental and social integrity" of the subject to safeguard the subject's "integrity".

Optogenetics appears so promising that its application, including areas in which deep brain stimulation (DBS) has been applied to date, is considered as extremely attractive due to its precision. However, DBS has yet to be established as beneficial, effective, and safe in the short-term, let alone the long-term, by basic research across a variety of disciplines. Confirmed assessment of such existing methods is needed before the risks of new, irreversible interventions can be tested, even if the latter are currently only performed as part of research projects. This also holds true for its other application areas.

To understand the Declaration of Helsinki, and the Federal Republic of Germany's Basic Law, one has to consider the concrete historical situation to which they were drafted as a response. The authors of these documents were aware of this context. Against the background of their experience of the brutal disregard of human dignity and the reduction of so many individuals to mere objects, both by definition and in practice, law- and policy-makers were unanimous in what they rejected. Today, we need to keep this context in mind. These are the utopias of National Socialism, which were used to transform widely established ethical standards, along with the possibilities available to scientists in the pursuit of their research interests. Only by remembering this, can the intention of the authors of both documents be properly understood. Similarly, we have to consider the current sociohistorical context of any ethical framework that informs today's decisions and actions. Today, too, the question arises as to the utopias underlying the development of the above applications

and with which they are associated. The demand that risk and benefit be evaluated depends in no small part on this.

Prior to requesting a patient's consent to a particular treatment, clinician researchers have to perform a risk-benefit analysis. Medical experts must be aware that informed consent to a treatment can only be sought from a patient if basic research on the treatment is conclusive. At the same time, patients have to be able to rely on the fact that only evaluated applications are being offered to them. This risk-benefit analysis imposes a particularly heavy responsibility on the medical researcher, as the risks and disadvantages for the subject, on the one hand, and the potential benefit, within the context of the utopia, can vary. When the Declaration explicitly states that the "wellbeing of the individual research subject must take precedence over all other interests", the utopias envisaged by medical experts themselves can affect their decisions. This is why such utopias have to be elucidated, along with the sociohistorical context, in which such risk-benefit analyses are performed.

Examples of application-utopias in modern medical research and their "Zeitgeist" were provided by geneticists, physicians and biochemists, including five Nobel prize winners, at the 1962 CIBA symposium "Man and His Future", at which "the eugenics of old merged with the new, laying bare the red thread from eugenics and social engineering to 'modern-day genetics'" [13]. Here, Nobel Prize winner Joshua Lederberg, commented that "most geneticists" were "deeply concerned over the status and prospects of the human genotype" [14]. He continued by stating that "(t)he facts of human reproduction are all gloomy" and suggested that "the creative possibilities of genetic improvement" should not be ignored. Lederberg promoted "euphenics, the manipulation of human development" and saw, for example, regulating "the size of the human brain" as a viable possibility. He considered eugenics and euphenics "the biological counterpart to education". Later, he expanded his vision to include the "breeding of subhuman individuals, crossbreeds of humans and apes, for special tasks" and the cloning of "already living humans" [15]. In the 1970s, he proposed "prenatal genetic diagnosis combined with selective abortion" as the "most important immediate measure" and envisaged "selective replacement of defective genes". From today's perspective, Lederberg's ideas would be considered similar to the National Socialist utopia of the optimized human.

These questions have been widely debated over the 50 years that have passed since the symposium, and have led to measures such as legal guidelines, international conventions such as on bioethics, as discussed above, and the establishment of ethics commissions. However, researchers are neither absolved of their responsibility by guidelines and regulations, nor can they shift this responsibility to others, such as patients or commissions. Clinical researchers always work within a sociohistorical context, in which, and for which, they research and develop their ideas. Researchers need to reflect on the role and interactions of their work in this context and with a Zeitgeist. They must question themselves and remain accountable for the motives underlying their actions, and their responsibility to third-parties and their interests.

The ambivalence of the possibilities and consequences of research makes discussing treatment utopias necessary, and testing whether the associated social utopias are desirable and intended. In this context, it seems sensible to not only name the possible applications of medical research as standard practice, but also those that are by no means desired and also, how the latter can be excluded. Here, we can turn to the intentions of those whose concrete historical experience prompted them to clearly name exclusions. A human being, researcher and patient alike, cannot be reduced to a mechanistic stimulus-reaction automatism comprising a collection of biochemical processes, but is instead a social being, in interaction with others, whose inviolable dignity must be safeguarded. Thus, any individual can only be considered within a context of a complex and systemic set of relationships. For this reason, medical research cannot be left to the scientific community alone. Instead, calls for support and critique by outside expertise, such as those of political scientists, sociologists, historians and practitioners, to reflect on research in the interplay between social implications, which cannot be individualized or reduced to the relationship between doctor and patient, and to come to common solutions. All participants, researchers and external experts alike, must be open to ongoing, transparent and public debate about the paths and future implications of research, from the very earliest stages of research studies.

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Henrik Walter, Sabine Müller

19 Optogenetics as a new therapeutic tool in medicine? A view from the principles of biomedical ethics

19.1 Principles of optogenetics

Optogenetics is a new and rapidly developing field of research that is now widely used in laboratories worldwide and delivers new insights into the functioning of neural circuits [1, 2]. It is based on the idea to use naturally occurring or chemically modified molecules that are reactive to light (opsins) in order to excite or inhibit particular kinds of neurons. Using standard genetic tools (genetic transfection via viral vectors), opsins can be transferred into specifically targeted neurons in which they are expressed and integrated into the cell membranes. By exposing the targeted cells to light of a certain wavelength, the opsins change their confirmation, causing ion flux across the lipid membranes of cells. The ion flux changes the membrane voltage potential of the cell, resulting in either depolarization (activation by blue light), or hyperpolarization (inhibition by yellow light). Thus, light can be used as an on/off switch for excitable cells. With the help of optical fibers or miniaturized LEDs, this can be done *in vivo, i.e.*, in living animals. Importantly, as the stimulation is not elicited by electric currents or magnetic fields, it is possible to record changes in membrane potentials simultaneously. Thus, optogenetics provides an effective tool to manipulate and record brain activity *in vivo*, thus enabling the causal assessment of the roles that different sets of neurons play within specific neural circuits. This is a major step in understanding how different sets of neurons contribute to computational and behavioral functions of the living brain. Besides its role in basic research, optogenetics is also recently discussed as a tool to improve neural function in patients, particularly as a tool for neuroprosthetics, *e.g.*, for constructing artificial retinas for patients with degenerative retina disease, or for improving deep brain stimulation for patients in neurology or psychiatry.

Naturally, the possibility to use on-off switches for treating human diseases excites curiosity and interest, as well as hopes and fears. On the one hand, optogenetics may be seen, in particular by medical experts treating and by patients suffering from devastating diseases, as a novel, exciting and promising therapeutic option. On the other hand, there may be concerns and fears that this approach, combining genetics and brain stimulation, might have serious, even uncontrollable side-effects. Furthermore, some people will have fundamental concerns with technologies that could enable someone to switch on and off psychological effects via neurotechnological devices. Similar concerns have been formulated against most novel techniques that have been introduced to measure or manipulate the human mind via measuring and manipulat-

ing the human brain [3, 4, 5, 6]. Some of this fundamental criticism is based on drawing historical analogies, particularly to the psychosurgery of the middle of last century. In this contribution, we will not discuss these principal concerns against interventions into the human brain. Rather, we want to provide an ethical analysis based on the principles of biomedical ethics [7], since this ethics is a world-wide acknowledged, sophisticated method to deal with concrete biomedical issues. Given the fact that applications of optogenetics are becoming a real option of medicine, we are convinced that it is worthwhile to start with a bioethical analysis of those applications of optogenetics which seem to be the most promising candidates for the near future.

19.2 Principles of biomedical ethics

In contrast to philosophical ethics which is characterized by a multitude of quite different theories, in medical ethics something like a gold standard exists: namely Tom L. Beauchamp and James F. Childress' principle-based ethics. Their book "Principles of Biomedical Ethics" was first published in 1977, and then developed further, up to the seventh edition in 2013 [7]. Worldwide, medical students study, at least cursorily, the principles of biomedical ethics. Doubtlessly, this opus is the most influential position in medical ethics, particularly in the Anglo-American world. Nevertheless, this ethical approach has been met with criticism since its first publication. Since the authors have reflected the critical arguments, and responded to many concerns in each (respectively) next edition, their work is not only very influential, but also on top of the debate.

Beauchamp and Childress' ethics argues mostly on the level of so-called medium principles, which are positioned between meta-ethical principles and detailed moral positions concerning special issues. The ethics of principles is based on four ethical principles: respect for the patient's autonomy, nonmaleficence, beneficence, and justice. The restriction to these more or less consensual medium principles makes this approach very useful both in clinical praxis and in research contexts, since it helps to avoid fruitless debates on last principles. But although this strategy is a pragmatic way out, it is no general solution for fundamental ethical dissent, and therefore its theoretical and practical adequacy as an ethical approach has been broadly discussed (overview: [8]).

For concrete ethical problems in medicine and health care, the four principles have to be applied, interpreted, and weighed against each other. The principles may conflict in certain situations; *e.g.*, the principle of respect for the patient's autonomy and the principle of nonmaleficence conflict in the question as to whether physician-assisted suicide should be allowed. Beauchamp and Childress stress that, in general, no principle ranks higher than others; in concrete cases, their best balance has to be found. An algorithm for that does not exist; therefore, for concrete cases, different ethical recommendations are often deduced by different authors.

In favor of applying the ethics of principles to emerging medical technologies, speaks firstly that it allows for balancing benefits, risks, and adverse effects of new interventions; secondly, its emphasis on the respect for the patient's autonomy and the procedure of informed consent, which is particularly important in case of therapy options that still lack empirical knowledge and medical experience. Finally, the principle of justice is relevant for the ethical evaluation of emerging medical technologies, since some ethical issues result from conflicts of interests. Patients' interests can be at odds with interests of their relatives, of providers of medical services, of producers of devices or drugs, or of research and education. Particularly in research domains which are characterized by an unavoidable mix of industry and academia, there are multiple sources of conflict, including sources of funding, intellectual property exchange, and reimbursement specific to the conduct of research and practice [9]. Since many innovative therapies are extremely expensive, they have to be justified in the rationing health care debate; therefore, this context has to be also considered in an ethical investigation of this issue. On the one hand, the request for maximum therapy could be driven by research or profit interests; on the other side, the claim for therapy limitations might be motivated by an interest in cost reduction. Beauchamp and Childress' approach is convenient to deal with these issues, too.

Although optogenetics is a rapidly emerging medical technology, which will raise many ethical issues, we find parallels for all of them in other fields of medical research, namely in nanomedicine [10, 11, 12, 13] and deep brain stimulation [9, 14, 15]. We and other authors have applied the ethics of principles to discuss concrete ethical issues of these fields, since the main ethical issues are firstly, the assessment of risk and benefit, and secondly, the capability of autonomy of the patients.

Nevertheless, this approach has conceptual limitations, so that a number of ethical issues cannot be adequately analyzed within this concept. This is also valid for some, but not all, applications of optogenetics, as we will show in the following.

In this contribution we use the principles of medical ethics to evaluate potential new clinical applications in the emerging field of optogenetics. We focus on possible applications of optogenetics for the following three diseases: (1) retinitis pigmentosa in formerly seeing patients (ophthalmology); (2) drug-resistant Parkinsonism (neurology); and (3) treatment-resistant depression (psychiatry).

Retinitis pigmentosa is a group of inherited, degenerative eye diseases that causes severe vision impairment, and often blindness, with a highly variable course. In many cases, the disease causing mutation occurs in genes expressed in the rod photoreceptors leading to their degeneration. The idea for an application of optogenetics for treating retinitis pigmentosa is explained by Busskamp and Roska as follows [16, 17]. First, one has to transfect specific cell types within the retina with the opsin genes (via a viral vector) to make them more light sensitive in order to improve vision. This implies that viable cells are still left. The main methodological challenges are how to target the right cells and how to make them light sensitive enough. In contrast to the natural rods and cones in the retina, optogenetically equipped cells need high light

intensity and do not adapt to the mean illumination. Therefore, an external visual device, mounted on goggles, will be needed that regulates the ambient light intensity and illuminates the retina in a given, but adjustable, intensity range. Furthermore, to avoid strong pupil constrictions that would limit the amount of light projected onto the retina, optogenetic tools that are activated at red-shifted wavelengths would be ideal. These are also preferable in order to avoid light-induced tissue damage. Apart from retinitis pigmentosa, any blindness with remaining retinal circuitry is a possible application for optogenetics.

Parkinson disease is a degenerative neuropsychiatric disorder with severe motor dysfunctions (tremor, rigor, akinesia, and dyskinesia), as well as cognitive, affective and behavioral symptoms, including depression (prevalence 70%), anxiety (69%), apathy (48%), irritability (47%), and executive impairment (41%) [18]. Standard medical treatments with the dopamine precursor levodopa and dopamine agonists are only symptomatic, do not stop disease progression, and lead to more and more unwanted side effects, since, with disease progression, the drug dosage has to be increased. Among the unwanted side-effects are on-off phenomena due to pharmacokinetics, levodopa-induced dyskinesia, and non-motor symptoms, such as mood and anxiety fluctuations, psychosis, and impulse control disorders [19]. Because of these shortcomings of medical PD treatment, deep brain stimulation (DBS) has been developed as a treatment option for advanced Parkinsonism. DBS has, contrary to older ablative neurosurgical procedures, the advantage of nearby reversibility and adaptability. It has impressive beneficial effects on motor functions, and additionally, sometimes reduces depression, apathy, and anxiety. Nevertheless, in a minority of patients, it has severe mental side effects, both in the spectrum of apathy and depression, as well as in the spectrum of hypomania and impulse control disorders [20].

Depression is a field in which DBS is a promising new approach. Depression is a frequent disease with the core symptoms of depressed mood, apathy, and anhedonia, as well as other symptoms including hopelessness and suicidality. Normally, it can be treated successfully with psychotherapy or pharmacotherapy, or is self-limited. However, there are a considerable number of patients who are severely and chronically depressed despite multiple trials of psychopharmacotherapy, psychotherapy, and even electroconvulsive therapy. In the first studies in which deep brain stimulation was used to treat such treatment-resistant patients, in about 50% of the patients, the depression was significantly improved [21]. As of now, the use of optogenetics with deep brain stimulation has not been worked out in detail. The main idea behind it is that it would be much more efficient to stimulate certain cell types by light within the neural circuits relevant for movement or affect, than to stimulate permanently with a current of 100 Hz frequency [22]. Nevertheless, as this combination of DBS and optogenetics is the next logical step, we will discuss it as an example of thinking-in-advance in biomedical ethics.

The general ethical tension for applied optogenetics can be summarized as follows: there is a new and potentially helpful option for treating severe diseases.

However, there are unmet challenges related to the safety (genetic transfection, effects of brain manipulation on the personality and behavior), the clinical efficiency, adverse effects, and to broader ethical issues (*e.g.*, societal consequences of the development of these options). We will now discuss these three potential applications, with respect to the principles of biomedical ethics.

19.2.1 Respect for the patient's autonomy

Two conditions are essential for autonomy: first, liberty (independence from controlling influences); and second, agency (capacity for intentional action) [7]. Beauchamp and Childress analyze autonomous action in terms of normal persons who act (1) intentionally, (2) with understanding, and (3) without controlling influences that determine their action [7]. The principle of autonomy emphasizes the independence of individuals against (medical) authorities and further coercive or manipulative influences. The principle of respect for the patient's autonomy demands from physicians to respect the patients' autonomous decisions about given therapies and to bring their autonomy forward. In medical practice, the demand for informed consent is the most important demand based on this principle. This does not mean that physicians just offer therapies to patients, and patients choose whether they accept or refuse certain therapies. Rather, it means that physicians should supply patients with sufficient, relevant, and understandable information, in order to enable the patients' informed decision. Furthermore, in the case of patients who suffer from a substantial loss of autonomy, *e.g.*, because of a brain disorder, the autonomy principle also demands for improving the capabilities for autonomous decision-making, *e.g.*, by improving the relevant brain functions. An example of the latter is a cortisone therapy for patients suffering from elevated brain pressure due to a tumor and accumulated cerebrospinal fluid, so that they lose their intellectual capabilities, or even their consciousness. This is often reversible under cortisone therapy, so that the patients then will become able to give informed consent to further therapies. Also, therapies which slow down the growth of malignant brain tumors offer the chance to save or restore the patient's autonomy. That is even valid for patients who have deficits in understanding the therapy, and are not capable of giving informed consent [13].

The obligations to respect autonomy do not extend to persons who cannot act in a sufficiently autonomous manner because they are immature, incapacitated, ignorant, coerced, or exploited. Examples of patients who cannot be rendered autonomous are infants, irrationally suicidal individuals, and drug-dependent persons [7]. For such patients, the principles of beneficence and nonmaleficence have to be adopted.

The main questions for applications of optogenetics in medicine are twofold. The first is the question of autonomy, as a prerequisite of informed consent. This is not problematic in the case of retinitis pigmentosa, as for this disease, effects on central brain functions are neither known nor expectable. In contrast, Parkinsonism and

depression can reduce the capability for autonomy, since these disorders can impair neurocognitive functions which are essential for the ability to give fully informed consent. Severe depression may impair cognitive abilities, and thus undermine the ability to understand medical information; but more importantly, severe depression undermines the will to live and the self-evaluation, and makes patients pessimistic and hopeless. Patients who do not mind whether they live or die, or who even hope to die by an intervention, or who think that they are so worthless that they should be taken as research objects, are not able to give informed consent to an intervention that bears severe risks. This is particularly true if the risks cannot yet be judged rationally because there are no data on safety. Nevertheless, severe depression is not generally seen as excluding the ability for informed consent; this ability has to be assessed individually. Parkinson disease is mostly accompanied by depression, and cognitive function deteriorates in its later stages, leading to a syndrome of dementia. Nevertheless, the ability for giving informed consent is not denied, generally, for Parkinson patients, but has to be assessed individually.

The second point with respect to autonomy is often neglected in discussions focusing on risks. Are we allowed to exclude patients from a possible therapeutic option? Put differently, isn't there a duty to perform clinical trials, if the tools exist which promise benefit for patients who desperately need help? The principle of respect for autonomy also means that we should respect autonomous decisions for new and risky treatments, even if many medical experts might be skeptical. From our experience, this second point depends clearly on cultural factors. Whereas it is often the first question in Anglo-American discussions about innovative high-tech therapies, it tends to be neglected in European and particularly, in German contexts. As it is related to the principle of beneficence, we will discuss it also there.

19.2.2 Nonmaleficence

The principle of nonmaleficence is the modern version of the Hippocratian principle "Above all, do no harm" (*primum non nocere*). In short, this principle says: One ought not to inflict evil or harm. The principle of nonmaleficence supports several moral rules which are *prima facie*, not absolute, namely: Do not kill; do not cause pain or suffering; do not incapacitate; do not cause offense; do not deprive others of the goods of life [7].

In our view, for medical applications of optogenetics, three major issues of nonmaleficence have to be considered. The first relates to genetic transfection. One big problem is how we can be sure to transfect only those cells that are relevant for the therapeutic effects. Since adeno-associated viral vectors (AAV) have already been used in clinical trials for gene replacement, it is highly probable that they will be used as a method of first choice in applied optogenetics. The cell specificity depends on several factors, including the promoter that drives the optogene, as well as the

"serotype", that is the outer coat of the virus that determines its capacity to enter particular cell types. Another factor is the injection site, *i.e.*, where the virus that is supposed to bring the gene to the target cell is injected. Clearly, the risk of side effects depends on all of these factors. However, one central issue is crucial: Will the injection of AAV lead to immunoreactions? Here, ophthalmology seems to have an advantage compared to brain injection necessary in neurology and psychiatry: the so-called subretinal space seems to be immunologically privileged, since injections of agents seem to provoke no immunoreactivity there. For the brain, this issue has not been explored. Importantly, research in rodents or smaller organisms does not help here, as the immune system is different in primates. So, it seems indispensable that if we want to use optogenetics in the human brain, safety studies in non-human primates will have to be performed first. Again, in ophthalmology more evidence exists, as gene therapy has been used there for five years [23, 24, 25].

There are additional safety issues which are not related to immunoreactions. (1) Do opsins in the required concentration have any toxic effects? (2) Will the opsins that are integrated into the membrane disturb regular membrane functions; in particular, if a large amount of opsins are expressed? (3) Do activated opsins have any systemic, non-intended, but harmful effects? This is of particular relevance in the brain, which is much more complex and multifunctional than the retina.

In general, the question can be asked: how much knowledge we need before we are going into clinical trials? Before optogenetics will be allowed to be applied to the human brain, more research is needed, probably with primates. Whether this can be justified ethically, is a matter of ethical debate which cannot be decided here. As in other medical applications, it is also clear that first clinical trials will be made only when a certain amount of safety is given, and if there are conditions for which no other efficient treatments are available. As deep brain stimulation is a highly effective therapy with lesser risks, there will have to be a substantial amount of information before it can be justified for the development of optogenetical brain stimulation in humans.

19.2.3 Beneficence

The beneficence principle is the modern expression of the Hippocratian principle "The wellbeing of the patient is the highest law" (*Salus aegroti suprema lex*).

Attending to the patients' welfare embodies medicine's goal, rationale, and justification. The principle of beneficence means the moral obligation to act for the benefit of others. That implies to prevent evil or harm, to remove evil or harm, and to do or promote good. The principle of beneficence supports an array of *prima facie* rules of obligation, including the following: protect and defend the rights of others, prevent harm from occurring to others, remove conditions that will cause harm to others, help persons with disabilities, rescue persons in danger [7].

Generally, risks and adverse effects can be justified according to the principle of beneficence, if their benefit for the patient would override their harm. Benefit can consist in improvements of the quality of life and/or the prolongation of the (quality-adjusted) life expectancy. To be beneficial, a given therapy has to fulfill the following conditions: (1) clinical relevant effectiveness; (2) sustainability of the effect; and (3) non-existence of a less noxious therapy.

Although it is difficult to evaluate at the moment the beneficence of the different applications of optogenetics, at least the following can be stated. For all three examples discussed, it is unclear whether optogenetical methods would show a clinically relevant effectiveness. In this respect, it is of particular interest that first experiments in primates with optogenetical stimulation in the sensorimotor domain showed clear excitatory and inhibitory effects, as measured by electrical recordings; however, no [26], or only very modest, behavioral effects [27] were demonstrated. Future research has to investigate the reasons for this effect. Accordingly, the sustainability of the effects is unclear, too. Finally, for Parkinsonism and severe depression, less noxious therapies do exist. For retinitis pigmentosa, there is no cure, but there are alternative therapies, such as electrical implants, which have been shown to work. This means that the justification for developing optogenetics applications would have to make it at least plausible that a clear benefit, compared to existing therapies, can be expected.

19.2.4 Justice

The principle of justice reads formally: Equals must be treated equally, and unequals must be treated unequally. In this formal form, the principle lacks all substance, since it neither identifies particular respects in which equals ought to be treated equally, nor provide criteria for determining whether different individuals are, in fact, equals. Principles that specify the relevant characteristics for equal treatment are material principles. One important example is the principle of need, which declares that essential social resources, including health care, should be distributed according to the need. Alternative material principles of justice are the principle of free-market distribution [7].

In medical ethics, the principle of justice concerns the distribution of scarce health resources and the fair distribution of burdens and risks, particularly in medical research. What does this mean for our examples? One aspect of a just allocation of resources is the incidence of a certain condition. The more persons are concerned by a given disease, the higher investigations for the research for therapies can be justified. Parkinsonism and severe depression (even severe and treatment-resistant forms) are much more frequent than progressive retinitis pigmentosa. A second aspect of a just resource allocation is the severity of the patients' need: diseases that shorten life-expectancy remarkably, as well as diseases that cause significant suffering, justify

more resources than less severe diseases. A third aspect of allocation justice is the principle of responsibility: according to that, the society should give more resources for diseases for which the patients are not responsible themselves. But this aspect is highly controversial, particularly since the responsibility for most diseases is unknown. A fourth aspect is the age of the typically concerned patients: diseases which shorten the life expectancy of children take away more life chances than diseases which affect only old persons. Apart from all justice considerations, pragmatic reasons have to be considered. By way of example, it may be preferable to focus on less frequent or less severe, but more manageable, disorders in which applications are more promising; this could serve in the long run the goal of developing therapies for more frequent or more severe disorders.

Table 19.1: Overview of the ethics of principles applied to the three chosen examples.

	Opthalmology (Retinitis pigmentosa in formerly seeing patients)	**Neurology (Parkinsonism)**	**Psychiatry (treatment resistant depression)**
Autonomy	++ Patients able to consent even in severe cases	+ Most patients able to consent	(+) Informed consent possible, but problematic
Non-maleficence	++ Immunoreactivity low No direct path to the brain	– Nearly no data on immunoreactivity	– Nearly no data on immunoreactivity
Beneficence	++ Electrical implant works but combination with optogenetics promises better resolution Strong effects, cones good for integrating opsins	(+) DBS is effective (about 70,000 patients) Effects in primate brain small	(+) DBS works (<100 patients) Effects in primate brain small
Justice	+ Moderate incidence Typically old patients	++ High incidence Typically old patients	++ High incidence Younger and elder patients

19.3 Conclusion

If we try to gain an overview what the ethics of principles has to say to the three chosen examples (see Table 19.1 for a rough overview), it clearly seems that the application of optogenetics for retinitis pigmentosa is ethically more justified and less problematic than the applications in neurology and psychiatry.

Obviously, there are many more general ethical questions that should be investigated, but these go beyond the approach of the ethics of principles. An example is the question of whether potential applications of optogenetics in neurology and psychiatry imply the risk of unwanted personality changes. This concern stems from observations in the practice of deep brain stimulation, both in neurology and in psychiatry, where unwanted personality changes occur in a small fraction of patients [15, 20, 28]. Because of the possibility of an external control, the development of optogenetics also raises the fundamental concern that it may allow for mind control. Although this concern will not be relevant before effective applications of optogenetics for treating the brain have been developed, we are convinced that we should think in advance about this potential threat.

We want to conclude with a few further concrete recommendations. Since at present, the risk of uncontrolled gene transfer (viral transfection) cannot be specified, but might be at least theoretically severe, research on this risk is required. This research should ideally be done by institutions without conflicts of interests, *i.e.*, which are independent from the pharmaceutical enterprises and the hospitals which develop optogenetics. Furthermore, we think that it should be mandatory to register all clinical interventions and clinical trials in applied optogenetics, since otherwise a publication bias will inevitably occur. Such a bias implies a faulty evaluation of therapies by scientists, clinicians, and patients, and, as in deep brain stimulation, it might otherwise lead to unnecessary clinical trials [29].

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Appendix

Dahlem-Conference (Berlin, September 2–5, 2012): “Optogenetics. Challenges and Perspectives.”

The photographs have been kindly provided by Denise Stewin.

Figure A.1: Group photograph 1

Figure A.2: Group photograph 2

Figure A.3: Mapping neuronal networks. Yawo, Beck, Lecea, Konnerth, Helmchen, Häusser, Oertner, Knöpfel

Figure A.4: Application in cellular systems and lower model organisms. Baier, Fiala, Evanko, Miesenböck, Sigrist, Gottschalk, Schäfer, de Bono, Jörgensen

Figure A.5: Optogenetic Tools. Deisseroth, Tsien, Möglich, Schneider, Hegemann, Schlichting, Moffat, Bamberg, Zhang, Trauner, Nagel

Figure A.6: Clinical application, Restoration of vision and hearing. Schleiermacher, Zrenner, Granidaru, Sahel, Roska, Walter, Lorenz, Heinemann, Pan, Böhning

Figure A.7: Herwig Baier

Figure A.8: Heinz Beck

Figure A.9: Mario de Bono

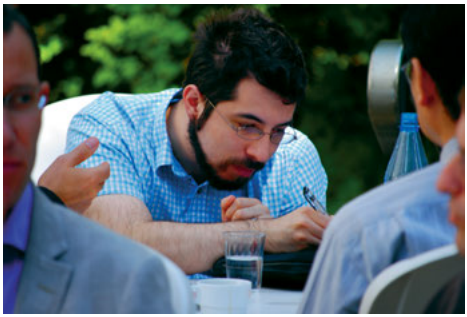

Figure A.10: Ed Boyden

Figure A.11: Karl Deisseroth

Figure A.12: Alexander Gottschalk

Figure A.13: Viviana Gradinaru

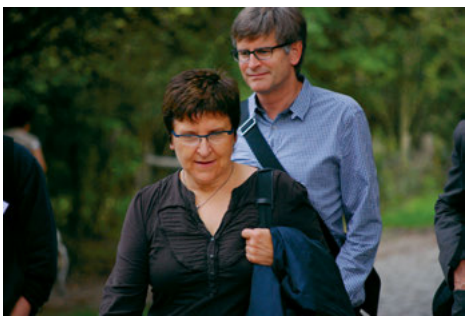

Figure A.14: Birgit Lorenz and Michael Häusser

Figure A.15: Peter Hegemann

Figure A.16: Uwe Heinemann

Figure A.17: Fritjof Helmchen

Figure A.18: Erik Jorgensen

Figure A.19: Benjamin Kaupp, Heinz Beck, Arthur Konnerth, Miss Heinemann

Figure A.20: Arthur Konnerth

Figure A.21: Gero Miesenböck

Figure A.22: Keith Moffat

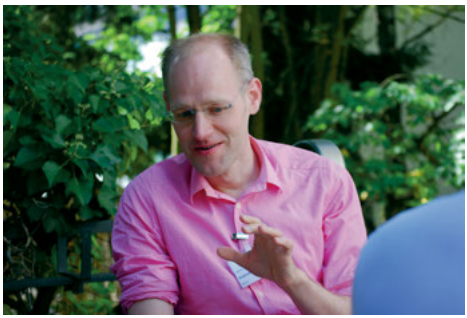

Figure A.23: Andreas Möglich

Figure A.24: Tobias Moser

Figure A.25: Thomas G. Oertner

Figure A.26: Georg Nagel (left), André Fiala (right)

Figure A.27: Zhuo-Hua Pan

Figure A.28: Botond Roska

Figure A.29: José-Alain Sahel

Figure A.30: Wiliam Schafer

Figure A.31: Stephan Sigrist

Figure A.32: Dirk Trauner

Figure A.33: Henrik Walter

Figure A.34: Hiromu Yawo

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