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Remote Control of Neuronal Signaling

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Abstract—A significant challenge for neuroscientists is to determine how both electrical and chemical signals affect the activity of cells and circuits and how the nervous system subsequently translates that activity into behavior. Remote, bidirectional manipulation of those signals with high spatiotemporal precision is an ideal approach to addressing that challenge. Neuroscientists have recently developed a diverse set of tools that permit such experimental manipulation with varying degrees of spatial, temporal, and directional control. These tools use light, peptides, and small molecules to primarily activate ion channels and G protein-coupled receptors (GPCRs) that in turn activate or inhibit neuronal firing. By monitoring the electrophysiological, biochemical, and behavioral effects of such activation/inhibition, researchers can better understand the links between brain activity and behavior. Here, we review the tools that are avail-

able for this type of experimentation. We describe the development of the tools and highlight exciting *in vivo* data. We focus primarily on designer GPCRs (receptors activated solely by synthetic ligands, designer receptors exclusively activated by designer drugs) and microbial opsins (e.g., channelrhodopsin-2, halorhodopsin, *Volvox carteri* channelrhodopsin) but also describe other novel techniques that use orthogonal receptors, caged ligands, allosteric modulators, and other approaches. These tools differ in the direction of their effect (activation/inhibition, hyperpolarization/depolarization), their onset and offset kinetics (milliseconds/minutes/hours), the degree of spatial resolution they afford, and their invasiveness. Although none of these tools is perfect, each has advantages and disadvantages, which we describe, and they are all still works in progress. We conclude with suggestions for improving upon the existing tools.

I. Introduction

Over the past few decades, our understanding of neurobiology and of the links between brain function and behavior has increased exponentially. For example, we know now how light can be transduced from a photon into a chemical signal that the brain can interpret (Sung and Chuang, 2010). We understand the specific cellular loss that contributes to Parkinson's disease (PD¹) (Przedborski, 2005). In addition, we know how the cranial nerves and descending spinal tracts stimulate the neuromuscular junction to elicit muscle contraction and movement. Despite these advances, knowledge of how neuronal signals encode complex behaviors remains

vague and incomplete. Addictive drugs, for instance, induce dopamine release in the striatum, although the population of cells and the specific signaling pathways within those cells that modulate drug seeking and reward are incompletely identified. The brains of patients with psychosis show evidence of decreased cortical inhibition, but how such lowering of inhibitory interneuron activity relates to the symptoms of schizophrenia is unknown. In addition, we do not understand, for example, how and to what extent specific interneuron populations (e.g., parvalbumin- or calretinin-expressing) might be involved in the pathogenesis of diseases such as schizophrenia.

¹Abbreviations: β_2 -AR, β_2 -adrenergic receptor; 5-HT, 5-hydroxytryptamine (serotonin); A3A, A3 adenosine receptor; AAV, adeno-associated virus; ACh, acetylcholine; AlstR, allatostatin receptor; Arch, *Halorubrum sodomense* archaerhodopsin-3; ATR, all-trans retinal; CA, cornu ammonis; CaMKII α , calcium/calmodulin kinase II α ; ChETA, channelrhodopsin-2 E123T accelerated; ChR2, channelrhodopsin-2; CNO, clozapine-*n*-oxide; CNS, central nervous system; DBS, deep brain stimulation; dox, doxycycline; DREADD, designer receptor exclusively activated by designer drug; eNpHR, enhanced halorhodopsin; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; EYFP, enhanced yellow fluorescent protein; FKBP, FK506 binding protein; floxed, flanked by *loxP* sites; GFAP, glial fibrillary acidic protein; GIRK, G protein-coupled inwardly rectifying potassium channel; GPCR, G protein-coupled receptor; h, human; hM3Dq, human M₃ muscarinic cholinergic G_q-coupled DREADD; hM4Di, human M₄ muscarinic cholinergic G_i-coupled DREADD; IRES, internal ribosomal entry site; KOR, κ opioid receptor; L-158,870, 1-(3,4-dihydroxyphenyl)-3-methyl-1-butanone; LFP, local field potential; Mac, *Leptosphaeria maculans* opsin; MC4, melanocortin-4; MIST, molecule for inhibition of synaptic transmission; NpHR, *Natronomonas pharaonis* halorhodopsin; P, postnatal day (e.g., P28); PA-CNO, photoactivatable clozapine-*n*-oxide; PD, Parkinson's disease; PIP₂, phosphatidylinositol-4,5-bisphosphate; PKA, protein kinase A; PLC, phospholipase C; RASSL, receptor activated solely by synthetic ligand; rM3/β1Ds, rat M₃ muscarinic/β₁ adrenergic G_q-coupled DREADD; RO4, vertebrate rhodopsin 4; rtTA, reverse tetracycline transactivator; tet, tetracycline; THIQ, tetrahydroisoquinoline; TRE, tetracycline response element; TREC, therapeutic receptor-effector complex; TRPM, transient receptor potential melastatin; TRPV, transient receptor potential vanilloid; tTA, tetracycline transactivator; UK-14304, 5-bromo-6-(2-imidazolyl-2-ylamino)quinoxaline; VChR1, *Volvox carteri* channelrhodopsin-1.

Elucidating the cellular and molecular substrates of behavior remains a “grand challenge” for neuroscientists and for the neuroscience field in general. In the past, neuroscientists have administered a variety of drugs to laboratory animals and have observed how those drugs have subsequently affected behavior. Thus, any conclusions regarding the links between drug action, drug targets, and behavioral outcomes were indirect and equivocal. Mouse genetic technology has permitted study of loss- or gain-of-function phenotypes but does not allow for control of receptors or pathways over a range of activity levels, with finely tuned temporal resolution, or for bidirectional or reversible control of receptor activity. To conclusively define the neuronal pathways and networks involved in behaviors—and by extension, disruption or abnormalities of behavior—it would be useful to have a tool that enables the direct association between neuronal events (e.g., membrane hyper- or depolarization, action potential firing, neurotransmitter release) or activation of a specific receptor and second-messenger-induced signaling pathway and a behavioral outcome. In addition, such a tool should be suitable for *in vivo* use in an awake, freely mobile animal. Researchers have developed a variety of techniques that aim to achieve that goal, with varying degrees of success. In this article, we review those tools, describing

their development and technical aspects as well as their applications, with a particular focus on the central nervous system (CNS). Finally, we identify persistent weaknesses in the currently available technology and suggest ways to combine or improve upon the various techniques to address those weaknesses.

II. Overview of the Tools and Their Technical Considerations

A variety of tools is now available for remote, user-defined control of neuronal activity. From these, two classes of technology have emerged at the forefront of tools for controlling neuronal signaling. First, a class of designer GPCRs that selectively responds to small molecule ligands enables manipulation of G_q , G_i , and G_s protein signal transduction pathways. Receptors activated solely by synthetic ligand (RASSLs) and designer receptors exclusively activated by designer drug (DREADDs) constitute the first and second generations, respectively, of orthologous GPCR-ligand pairs (for review, see Conklin et al., 2008; Pei et al., 2008; Nichols and Roth, 2009; Dong et al., 2010a). Second, several microbial light-gated receptors (opsins) functionally express in mammalian tissue and, in response to light, can alter cell membrane potential to either hyperpolarize or depolarize neurons and to control precisely the intensity, frequency, and pattern of neuronal firing. As we describe below, individual opsins respond to specific wavelengths of light so that bi-directional modulation of a single cell is feasible in an intact animal. In addition to these two approaches to manipulating neuronal signaling, several non-native or peptide receptors, such as the TRPV1 ligand-gated ion channel and the *Drosophila melanogaster* allatostatin receptor (AlstR), have successfully provided control over neuronal activity. Ideally, each of these tools would confer a high degree of temporal, spatial, and directional control over neuronal signaling—a goal they all achieve to varying degrees.

A. Transgenic Systems and Approaches to Spatial Control

Spatial control enables a researcher to selectively modulate a particular cell type (e.g., parvalbumin-expressing interneurons) or a particular anatomical region (e.g., hippocampal CA1 pyramidal neurons). Each tool described in this review requires expression of an exogenous protein or receptor, either for light (opsins) or for small molecules (RASSLs, DREADDs, TRPV1, etc.). Controlling either where these receptors express or the availability of their cognate ligands (i.e., photons or small molecules) confers the necessary spatial resolution for defining neuronal pathways.

To date, researchers have either developed transgenic mouse lines or have locally infused viruses with cell type-specific promoters to achieve the necessary exogenous receptor expression (Fig. 1). For both techniques,

tissue-specific promoters convey a large degree of spatial specificity. For example, the parvalbumin, calcium/calmodulin kinase II α (CaMKII α), and glial fibrillary astrocytic (GFAP) promoters drive transgene expression in basket cell interneurons, forebrain pyramidal neurons, and astrocytes, respectively (Mayford et al., 1996; Hippenmeyer et al., 2005; Sweger et al., 2007). These promoters can drive transgene expression directly, as in the case of traditional transgenic mice, or indirectly through tet-on/off or Cre-flox systems (Fig. 1). The indirect systems are advantageous because they are inducible expression systems; the tet system has some advantages in that it is also reversible. In this latter system, a tissue-specific promoter drives expression of a transcription factor, either the tetracycline transactivator (tTA) or the reverse tetracycline transactivator (rtTA) (Fig. 1A, top). A tetracycline response element (TRE) promoter then drives expression of the transgene of interest (Fig. 1A, bottom). The tTA or rtTA transcription factor is required to initiate transcription at the TRE promoter, thus the tissue-specific promoter driving tTA or rtTA expression determines where TRE is active. The antibiotic tetracycline (tet) or its analog doxycycline (dox) regulates activity of tTA and rtTA. Tetracycline inhibits the former transcription factor to prevent transgene expression (tet-off; Fig. 1A, left), whereas it activates the latter transcription factor to enable transgene expression (tet-on; Fig. 1A, right). Expression in the tet system is thereby reversible, in that administering or removing tet/dox will turn expression on or off. To control the timing of receptor expression in vivo, mice consume either food or water that contains dox. This system is particularly useful when exogenous receptor expression per se induces pathologic conditions (e.g., Ro1; see section IV.A.2), because the timing of dox administration determines the timing and duration of receptor expression and thus controls the induction of the pathologic condition.

The Cre-flox system functions in a similar manner, requiring two transgenes. A tissue-specific promoter controls expression of Cre recombinase, an enzyme from bacteriophage that recombines DNA at specific recognition sequences termed *loxP* sites (Fig. 1B, top left). Cre recombinase typically excises DNA that is flanked by *loxP* sites (or “floxed”). Most often, floxed-stop constructs are knocked-in via homologous recombination to a housekeeping locus such as *Rosa26* (Fig. 1B, bottom left). In the presence of Cre, the stop signal is excised, and the *Rosa26* promoter drives transgene expression (Fig. 1B, right). This technique is cell-type-specific because Cre-mediated recombination occurs only in cells expressing Cre, which are those in which the tissue-specific promoter is active. This system is useful in that it is spatially regulated and inducible, but it is not reversible. Once recombination occurs, the user has no control over the timing of transgene expression; the

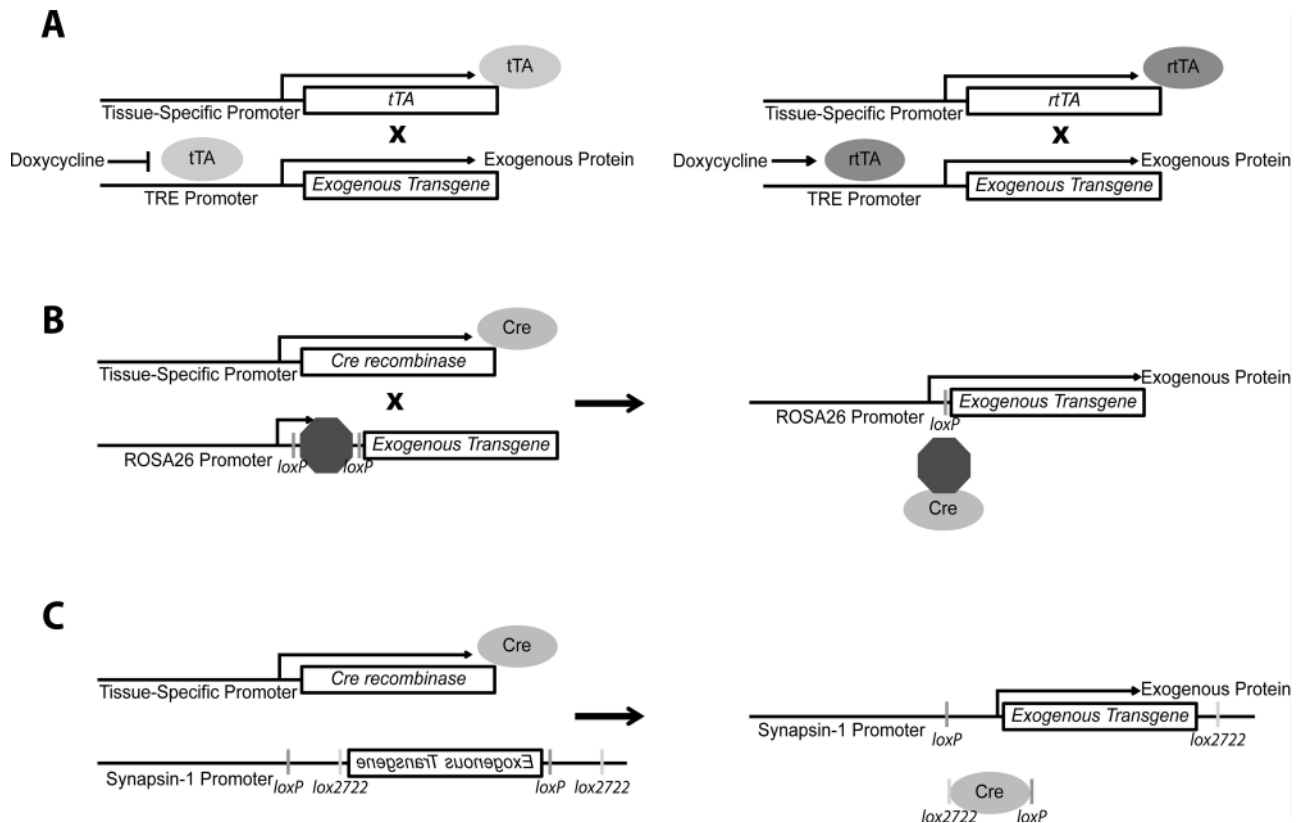


FIG. 1. Genetic approaches to achieving spatially regulated transgene expression. In the tet-regulated expression system (A), a tissue specific-promoter drives expression of the *tTA* (tet-off, left) or *rtTA* (tet-on, right) transgene to produce the *tTA* or *rtTA* transcription factor, respectively, in a driver mouse line. That line is crossed to a responsive mouse line in which the TRE promoter drives expression of the exogenous transgene of interest. The *tTA* or *rtTA* transcription factor binds to the promoter to initiate transcription of the endogenous transgene. In the tet-off system, dox inhibits *tTA* binding to the TRE promoter. In the tet-on system, doxycycline is required for *rtTA* binding to the promoter. In the Cre-flox system (B), a tissue-specific promoter controls expression of Cre recombinase in a driver line. In the responsive line, *loxP* recognition sequences flank a stop sequence 5' of the exogenous transgene of interest. Cre recombinase excises the stop site to allow the ubiquitous Rosa26 promoter to drive transgene expression. For virally mediated recombination (C), virus is infused into the Cre driver line. The virus carries a plasmid with a doubly floxed, inverted open reading frame. Cre mediates recombination at one of the two sets of recognition sites to cause inversion of the open reading frame and to allow the synapsin-1 promoter to drive transgene expression in neurons. The remaining *loxP* sites are incompatible, thereby preventing re-inversion.

Rosa26 promoter will continue to drive expression of the transgene.

Viral transgene delivery is advantageous in that it does not require the development of a novel transgenic mouse line and is thus less time-consuming. Virally mediated delivery is an effective method of expressing exogenous genes in rats and even primates—species in which traditional transgenic approaches are unfeasible. Viruses can directly encode the transgene of interest downstream of a tissue-specific promoter, or they can supply a component of the Cre-flox system. Several groups have developed viral constructs that are now widely used for this purpose. Alternatively called flip excision (Schnütgen et al., 2003; Atasoy et al., 2008) or doubly floxed, inverted open reading frame (Cardin et al., 2009, 2010) constructs, these viruses encode a transgene that is doubly floxed by variants of the canonical *loxP* site so that Cre does not excise the transgene but instead nonreversibly inverts it (Fig. 1C). The transgene is cloned into the construct in a 3'-to-5' orientation relative to the promoter (Fig. 1C, bottom left), and Cre recombinates the DNA so that the transgene is in a 5'-to-3'

orientation and transcription and translation of the transgene can proceed (Fig. 1C, right). This viral construct is locally infused into particular brain regions of a Cre mouse driver line to yield tissue-specific expression. This approach yields the same result as the traditional Cre-flox double-transgenic approach but requires neither the development nor the maintenance of novel transgenic lines, allows for temporal control over the recombination process, and provides additional spatial control as viral diffusion is limited so the location of viral infusion contributes to the spatial resolution.

B. Temporal and Directional Control

In addition to a high degree of spatial resolution, temporal resolution and directional modulation of signaling are important characteristics of tools for remotely controlling neuronal signaling. Temporal control enables one to determine when a receptor or pathway is active or inactive and for what duration. Temporal resolution can range from milliseconds (e.g., opsins) to minutes (e.g., TRPV1) to hours (e.g., DREADDs) and includes both “onset” kinetics (time between the experimental manipulation and the initiation

of signaling) and “offset” kinetics (time between the initiation of signaling and the termination of signaling). Directional control refers to the net effect of the tool on neuronal activity (i.e., activation versus inhibition); bidirectional modulation is ideal. Determining the effect of both turning on and turning off the same cell population would yield the most information regarding the role of that cell in a particular network or behavior.

Temporal resolution is critical for directly linking activation of particular targets or cell types to consequent molecular, cellular, electrophysiological, and behavioral changes. Here, we use the term “temporal resolution” to describe the temporal characteristics of receptor activity—how long it takes for the experimenter to effect a change and how long those changes last. Several techniques confer temporal control. Microbial opsins are light receptors that respond rapidly (within milliseconds) to photons of a particular wavelength. Although the duration of activity varies for each opsin, both the onset and offset kinetics are fast relative to other techniques, as we describe in detail below. On the other hand, DREADDs and RASSLs respond to small-molecule ligands, signal through G proteins, and induce responses that can last many hours (Alexander et al., 2009). The pharmacokinetic properties of the individual ligands and the particular route of administration determine how quickly neuronal signaling changes, but for systemically administered (e.g., oral administration, subcutaneous or intraperitoneal injection) small molecules, responses generally begin within 5 to 15 min after drug administration. Local administration (i.e., stereotaxic infusion) of the ligand increases the temporal resolution of the techniques requiring small molecule ligands, but responses still occur on the order of minutes. Additional techniques such as using photoactivatable or caged ligands yield a higher degree of temporal control but that control still does not approach that of the opsins.

Finally, directional control over system activity is important. The techniques described herein elicit neuronal depolarization and hyperpolarization, firing, and silencing. It is beneficial to bidirectionally modulate the same cell or population of cells to more thoroughly characterize the effects of activating particular pathways. Both designer GPCRs (through G_i versus G_q or G_s signaling partners) and opsins (through anion versus cation selectivity) can inhibit and activate neuronal firing. However, only some of these tools permit near-simultaneous bidirectional manipulation of the same cells in vivo.

C. In Vivo Considerations

In this review, we focus on in vivo applications of the techniques, which require a few special considerations above and beyond those for an in vitro system. First, the complexity of the nervous system and its similarity to that of humans varies by species. Invertebrate models (e.g., *D. melanogaster*, *Caenorhabditis elegans*) are rel-

atively simple and enable the complete dissection of particular signaling networks, but those networks do not necessarily translate to vertebrate nervous systems. In contrast, vertebrate models (e.g., mice, rats) have more in common with humans, and although it is much more complicated to map networks in vertebrate brains, understanding those networks is more likely to increase knowledge of the human nervous system.

Second, genetic versatility varies by species according to the length of gestation and mating tendencies. The *D. melanogaster*, *C. elegans*, and *Danio rerio* (zebrafish) systems are highly conducive to genetic modulation. In fact, Zhu et al. (2009) adapted the tet-on/off system to zebrafish. Among the vertebrate models, mice afford the greatest degree of genetic manipulation because they have a short gestation, and transgenic, knockin, and knockout mice are now standard tools. To express exogenous genes in rats and primates, viral delivery methods are currently necessary.

Third, the route, feasibility, and precision of local delivery of virus, light, and small-molecule ligands differ across species. The larger the brain region, the easier it is to precisely target a particular population of cells. Thus, stereotaxic surgery is more precise in rats than in mice, for example. In *D. melanogaster*, the cuticle diffuses the light needed to activate the opsins, thereby introducing an additional technical difficulty. Aerosolized compounds are useful for *D. melanogaster* studies; for example, scented compounds are useful as attractive or aversive stimuli. In rodents, aversive and attractive stimuli are distinguishable in self-administration paradigms. In addition, metabolic processes differ across species. For example, clozapine-*n*-oxide (CNO), the ligand for DREADDs, undergoes extensive back-metabolism to clozapine in humans (Chang et al., 1998) but not in mice (Guettier et al., 2009). Thus, the particular animal model determines the ideal formulation of a ligand.

Fourth, the invasiveness of the tools varies by technology. The optical approaches all require precise delivery of high-intensity light. In vertebrates, experimenters must surgically implant fiber-optics or other devices to deliver that light. On the other hand, the RASSL and DREADD ligands are systemically bioavailable; thus, their delivery is less invasive than delivery of light. Moreover, noninvasive approaches are more conducive to facile studies on awake, freely mobile animals, which more reliably reflect native in vivo signaling. Thus, noninvasive approaches are optimal, especially when considering translating these techniques to humans.

Finally, because these techniques require significant spatial resolution, visualization of the exogenous receptors is critical for verifying their localization. Fluorescent or epitope tags are particularly useful in vivo for the localization of transgene expression. Epitope tags such as hemagglutinin tags are small and generally do not alter receptor expression, trafficking, or signaling, and antibodies against the epitopes are readily available.

Fluorescent tags (directly fused to the receptor) are easier to detect and can localize receptors during live imaging, but these tags sometimes affect receptor trafficking and signaling; endoplasmic reticulum (ER) export sequences and signal peptides improve trafficking (Zhao et al., 2008; Dong et al., 2010a; Gradinaru et al., 2010). Alternatively, internal ribosomal entry sequences (IRESs) enable expression of bicistronic constructs so that the same cells express both the receptor and the fluorescent molecule as distinct proteins (for review, see Martínez-Salas, 1999). This approach allows for facile identification of cells that express the transgene but does not inform about the level of receptor expression or the subcellular localization of that expression. Another disadvantage of using IRESs is that expression of the gene downstream of the IRES is significantly less than expression of the upstream gene (Douin et al., 2004). Although differences in expression are permissible when the downstream gene is merely a reporter, such differences become problematic when the activity of the downstream gene is important. For example, if one wishes to express proteins that induce opposite changes in membrane potential (e.g., channelrhodopsin-2 and halorhodopsin; see section V) and to compare their effects on neuronal activity, ideally they should express at equal levels (Han et al., 2009). Conveniently, foot-and-mouth-disease virus and other Picornaviridae express a short sequence of 18 residues that promotes ribosomal skipping between a glycine and a proline residue, resulting in proportional translation of two separate peptides from a single mRNA molecule (Ryan et al., 1991; Ryan and Drew, 1994). Several groups have adapted this approach—variously termed a 2A sequence, self-cleaving peptide, or *cis*-acting hydrolase element—for expression of multiple transgenes, including distinct opsins (Tang et al., 2009; Zimmermann et al., 2009).

Clearly, a variety of species-specific differences affect the utility of the tools for particular applications. Thus far, the techniques we review have been employed *in vivo* in *D. melanogaster*, *C. elegans*, zebrafish, mice, rats, and nonhuman primates.

III. Early Approaches

In one of the first attempts at “remote control” of neuronal signaling, and, in fact, coining that phrase (Wisden et al., 2009), Zemelman et al. (2003) used photocaged capsaicin and ATP to activate the TRPV1 vanilloid nociceptive receptor and the P2X₂ purinergic receptor, respectively. Since then, several other groups have used TRPV1 (and, less notably, P2X₂) to manipulate neuronal events, as we describe below. In addition, neuroscientists have used caged ligands, dominant-negative presynaptic proteins, light-gated ionotropic glutamate receptors, and ligand-gated heterologous chloride channels, among others, to manipulate neuronal activity.

Here, we describe these techniques and some of their advantages and shortcomings (Table 1).

A. Activation

The TRPV1 vanilloid receptor is a ligand-gated, non-selective cation channel. TRPV1 is normally expressed in nociceptive (pain-sensing) neurons of the peripheral nervous system and causes membrane depolarization in the presence of its ligand, capsaicin (Caterina et al., 1997). Zemelman et al. (2003) first showed in cultured hippocampal neurons that they could use light to uncage a caged capsaicin derivative (4,5-dimethoxy-2-nitrobenzyl-capsaicin), thereby generating action potentials in a reliable and temporally precise manner. Depolarization occurred approximately 5 s after a 1-s light pulse, lasted for 2 to 3 s, and did not attenuate with multiple light pulses. No off-target effects of the caged ligand were evident. To our knowledge, this demonstration was the first of precise experimental control over the on and off kinetics of neuronal activity. Subsequently, Lima and Miesenböck (2005) used TRPV1 and P2X₂, along with their respective photocaged ligands, to control behavior and map neural circuits in *D. melanogaster*. In an extreme example, they decapitated flies and successfully made the headless bodies fly.

Arenkiel et al. (2008) subsequently used TRPV1 to achieve remote control of neuronal signaling in vertebrates, although they did not photocage capsaicin. Because the TRPV1 channel is not normally present in the mouse brain, and capsaicin is highly selective for it, this system provides high specificity. However, capsaicin activates peripheral pain receptors and is not blood-brain-barrier-permeant, so their approach required local infusion of capsaicin via an indwelling cannula. Using the Cre-flox system, Arenkiel et al. (2008) expressed TRPV1 throughout the brain using a *nestin-Cre* driver line. In acutely prepared slices, application of capsaicin triggered action potentials in TRPV1-expressing cells (identified by a bicistronic enhanced cyan fluorescent protein reporter). *In vivo*, anesthetized mice, the authors infused capsaicin directly into the dorsal cortex and recorded the resultant high-frequency action potentials. The spiking activity was dose-dependent (EC₅₀, 500 nM) and lasted for several seconds. Finally, they infused 500-nM capsaicin into awake, freely moving mice and elicited stereotypies between 5 and 15 min after infusion. At that concentration, capsaicin had no damaging effects, but doses of 5 to 10 μM were excitotoxic.

Another group compared TRPV1 with its family member TRPM8, a menthol-gated, nonselective cation channel important for sensing temperature. It is noteworthy that Zemelman et al. (2003) had used TRPM8 in their initial description of remote control of neuronal activity. Crawford et al. (2009) determined that TRPM8 generated depolarizing currents similar to those of TRPV1, but this did not result in excitotoxicity. In addition, expression of TRPV1, but not TRPM8, altered baseline

TABLE 1
Methods of activating and silencing neuronal activity

Tool	Approach	References
Activation ChARGe	Consists of <i>D. melanogaster</i> Arrestin, Rhodopsin, and G protein; application of light activates rhodopsin and initiates a G protein signaling cascade that results in action potential firing; binding of arrestin to activated rhodopsin stops the signaling cascade; frequency and timing of action potential firing are inconsistent and unpredictable; not feasible for in vivo applications	Zemelman et al., 2002
FMRF-amide channel	Phe-Met-Arg-Phe-amide (FMRFamide)-gated sodium channel from <i>Helix aspersa</i> (<i>HaFaNaC</i>) is activated by FMRFamide; mammals express homologous acid-sensing ion channels (ASICs) but not the ligand FMRFamide; although FMRFamide activates <i>HaFaNaC</i> at lower concentrations than ASICs, extent of cross-reactivity is unknown, as is ability of endogenous mammalian amidated peptides to activate <i>HaFaNaC</i>	Schanuel et al., 2008
Caged glutamate	Variety of caged glutamate compounds enable neuronal activation with fast kinetics and single-cell spatial resolution	Canepari et al., 2001; Matsuzaki et al., 2001; Nikolenko et al., 2007; Fino et al., 2009
Light-gated glutamate receptor	A photo-isomerizing glutamate molecule is covalently attached to the outer surface of the binding pocket of an ionotropic glutamate receptor; application of light causes isomerization, brings the glutamate into position in the binding pocket, and opens the cation-selective channel	Volgraf et al., 2006; Gorostiza et al., 2007; Szobota et al., 2007; Volgraf et al., 2007; Wang et al., 2007
Silencing Ivermectin-gated chloride channel	System relies on expression of a modified glutamate- and ivermectin-gated chloride channel from <i>C. elegans</i> , <i>GluCl$\alpha$$\beta$</i> ; point mutations reduce sensitivity to glutamate; slow onset (4–6 h) and time to peak (12 h) of silencing and prolonged recovery (4 days) with systemic administration; cells must express both α and β subunits (which allows for additional spatial resolution through intersectional expression strategies)	Li et al., 2002; Slimko et al., 2002; Slimko and Lester, 2003; Lerchner et al., 2007
<i>Shibire</i> ^{ts1}	Temperature-sensitive <i>D. melanogaster</i> dynamin mutant gene <i>shibire</i> ^{ts1} ; expression is induced by changes in temperature; expression causes reversible paralysis due to depletion of synaptic vesicles at nerve terminals; specific to <i>D. melanogaster</i>	Kitamoto, 2001, 2002; Kasuya et al., 2009
Tetanus toxin light chain	Tetanus toxin light chain TNT cleaves the synaptic vesicle protein VAMP2/syntaxin; TNT expression is inducible; slow recovery (14 days) occurs with VAMP2 resynthesis	Yamamoto et al., 2003; Yu et al., 2004; Kobayashi et al., 2008; Nakashiba et al., 2008
Kir2.1	Strongly rectifying potassium channel; no cytotoxic potassium leak; channel is constitutively active so reversibility is only achieved through transcriptional control	Nitabach et al., 2002; Yu et al., 2004
Membrane-tethered toxins	Peptide neurotoxins specific for various ion channels can be tethered to the membrane to inactivate ion channels; only reversible through transcriptional regulation	Ibañez-Tallon et al., 2004; Auer and Ibañez-Tallon, 2010; Auer et al., 2010; Stürzebecher et al., 2010
5-HT _{1A}	8-OH-DPAT is a systemically administrable ligand that selectively activates the G _i -coupled 5-HT _{1A} receptor to activate GIRK and hyperpolarize neuronal membranes; requires 5-HT _{1A} (-/-) background	Tsetsenis et al., 2007
GABA _A -Zolpidem	Positive allosteric GABA _A agonists bind benzodiazepine site at interface of α and γ_2 subunits; an F77I mutation of γ_2 abolishes zolpidem binding; conditional expression of wild-type γ_2 on a γ_2 ^{F77I} background enables selective neuronal silencing with zolpidem; the F77I mutation also abolishes binding of an inverse allosteric agonist, suggesting this same method could be used for neuronal activation	Cope et al., 2004, 2005; Ogris et al., 2004; Wulff et al., 2007; Wisden et al., 2009

8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin.

neurotransmission in the absence of agonist. Moreover, prior evidence had shown that TRPV1 is endogenously expressed in the central nervous system whereas TRPM8 is not, which raises the possibility of additional effects of capsaicin on neuronal firing independent of exogenous TRPV1 (Tóth et al., 2005; Cristino et al., 2008; Gibson et al., 2008). Therefore, Crawford et al. (2009) reasoned that TRPM8 is a better tool than TRPV1 for activating neuronal activity.

B. Silencing

More so than neuronal activation, neuronal silencing—and reversible silencing in particular—has re-

mained a challenge to neuroscientists. Knockout and lesion models, although in wide use, are both irreversible, and targeting particular cells is difficult or impossible with those approaches. Dissatisfied with the selectivity that the then-current generation of tools offered, Lechner et al. (2002) expressed in cultured mammalian neurons the *D. melanogaster* AlstR, which couples to G_{i/o} to modulate GIRK channel activity and silence neuronal activity (Birgül et al., 1999). The AlstR ligand allatostatin is a short peptide that is a selective and highly potent agonist for the *D. melanogaster* receptor; in addition, the cognate ligands of related mammalian receptors (e.g., somatostatin and opioid receptors) are inactive at AlstR.

Therefore, the AlstR-allatostatin system offers desirable two-way selectivity. Gosgnach et al. (2006) expressed AlstR in mice in V1 motor neurons in an attempt to dissect the role of the central pattern generator in triggering locomotion. Activating AlstR-expressing neurons in acutely prepared spinal cord slices decreased neuronal excitability, whereas activating AlstR in an isolated, intact spinal cord lengthened the electrophysiological interval corresponding to the step-cycle period—a finding that mimics the effects of developmental defects of V1 neurons. In rats, ferrets, and monkeys expressing AlstR, local application of high-nanomolar concentrations of allatostatin inhibited both spontaneous and evoked neuronal activity (Tan et al., 2006). Neuronal inhibition occurred within minutes of allatostatin application, and neurons usually recovered within minutes of washout with saline. Moreover, allatostatin repeatedly silenced neuronal activity without desensitization. In addition, expression and activation of AlstR in rat neurons that express glutamate and somatostatin in the pre-Bötzinger complex, a region of the ventrolateral medulla involved in respiratory drive, prevented respiratory movements and reduced breathing, suggesting that these somatostatin neurons are necessary for normal breathing rhythm (Tan et al., 2008).

These effects of allatostatin were highly specific: even at concentrations several orders of magnitude above the effective dose, allatostatin had no effect on wild-type tissues. The AlstR-allatostatin system is thus selective, reversible, and potent. The disadvantages of this system are that allatostatin must be locally applied via invasive techniques; its ability to reach its target (through infusion and diffusion) might limit its activity; and the temporal control over its activity depends on the ease of washing in or washing out the ligand. Because allatostatin is a small peptide, systemic administration is not feasible; it would likely undergo enzymatic degradation or would not cross the blood-brain barrier.

Also in search of an inducible and reversible method of neuronal silencing, Karpova et al. (2005) developed “molecules for inactivation of synaptic transmission” (MISTs) that prevent neurotransmitter release and the subsequent spread of action potentials. These MISTs are modified synaptic proteins that disrupt the synaptic vesicle cycle when they form homo- or heterodimers in the presence of small-molecule “dimerizers.” The authors fused synaptic proteins (i.e., VAMP2/synaptobrevin, synaptophysin) to FK506 binding protein (FKBP). In the presence of an FKBP ligand, the fusion protein dimerizes (chemical induction of dimerization) and sequesters the presynaptic proteins, thereby inhibiting normal neurotransmitter release. *In vitro*, application of dimerizer compounds to neurons expressing MISTs elicited a rapid decrease (50–100%) in monosynaptic EPSPs; this inhibition was highly selective for neurons expressing MISTs. *In vivo*, Karpova et al. (2005) expressed MISTs in cerebellar Purkinje neurons and then

injected dimerizer compounds into the lateral ventricles. They found that they could selectively disrupt performance in the Rotorod task. The inhibition lasted up to 36 h after injection and was reversible. The half-life of the dimerizer compound in blood is approximately 5 h, but the authors hypothesized that the compound is more stable when bound to FKBP. Although MISTs are certainly an innovative approach for remote control of neuronal signaling, the temporal control is insufficient for many applications, and the permeability of blood-brain barrier to the dimerizers is unknown. Whether MISTs affect cellular processes other than neurotransmitter release via “off-target” actions is also unknown.

The approaches described herein have their advantages, but none of them permits noninvasive remote control or achieves millisecond time scale resolution. In addition, these systems do not enable bidirectional modulation. Therefore, two techniques have emerged in the quest for methods of experimental manipulation of neuronal signaling: orthogonal GPCR-ligand pairs and microbial opsins. The first technique provides noninvasive control, whereas the second provides exquisite temporal control.

IV. Designer G Protein-Coupled Receptors

G protein-coupled receptor pathways (Fig. 2) are involved in a multitude of CNS disorders, and remotely activating and/or inhibiting select GPCR pathways both illuminates disease processes and identifies potential avenues of treatment. Unlike microbial opsins, which

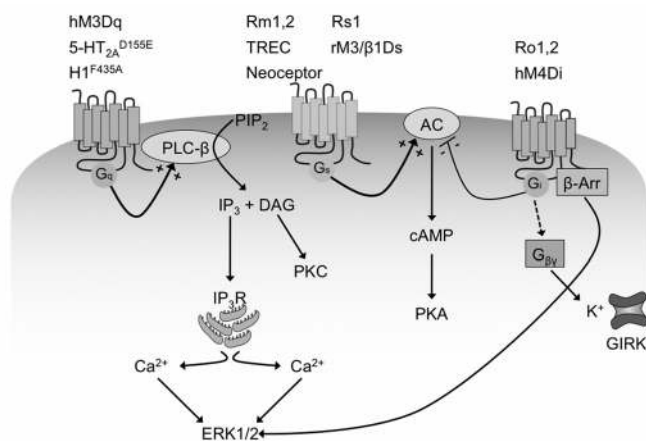


FIG. 2. Activation of second-messenger cascades by designer G protein-coupled receptors. The RASSLs and DREADDs activate G_q -, G_s -, and G_i -coupled GPCRs. Activation of G_q activates PLC- β to stimulate PIP_2 hydrolysis into inositol-trisphosphate (IP_3) and diacylglycerol (DAG), then DAG activates PKC, and IP_3 activates the IP_3 receptor (IP_3R) to cause calcium release from the ER, which causes ERK1/2 activation. Activation of G_s activates adenylyl cyclase (AC) to promote cAMP formation and subsequent PKA activation, whereas activation of G_i inhibits AC activity, cAMP formation, and PKA activation. The $G\beta\gamma$ subunit of G_i -coupled GPCRs opens GIRK to allow hyperpolarizing potassium flux. Finally, G protein-independent signaling occurs through β -arrestin, which activates ERK1/2.

are not activated *in vivo* by endogenous compounds or by ambient light and thus are functionally silent at baseline, a variety of endogenous ligands modulate native GPCR signaling *in vivo*, and GPCRs can display ligand-independent (i.e., basal, constitutive) activity (for review, see, Seifert and Wenzel-Seifert, 2002; Smit et al., 2007). Moreover, drug-like exogenous ligands are not selective enough, and they do not provide adequate spatial regulation for probing specific pathways *in vivo*. Therefore, over the past 2 decades, researchers have worked to develop highly selective orthologous ligand-receptor pairs that would convey a high degree of spatiotemporal control over GPCR signaling networks *in vivo*. This work has been reviewed previously (Conklin et al., 2008; Pei et al., 2008; Nichols and Roth, 2009; Dong et al., 2010a).

Efforts to selectively activate GPCRs began with the work of Strader et al. (1991) to mutagenize the β_2 -adrenergic receptor (β_2 -AR). The Strader group identified Asp113 as a critical residue for ligand binding to the β_2 -AR (Strader et al., 1987, 1988, 1989) and mutated Asp113 to serine to interrupt ionic interactions in the binding pocket. Although isoproterenol and the endogenous agonists epinephrine and norepinephrine activated β_2 -AR^{D113S} weakly and with low potency, catechol esters and ketones, which are inactive at the wild-type β_2 -AR, were agonists at the mutant receptor. Unfortunately, although it was a full agonist, the most potent ligand at β_2 -AR^{D113S} had an EC₅₀ of 40 μ M, which is still insufficient for *in vivo* applications. The authors presciently proposed rational design of drugs and genetically engineered receptors as a new therapeutic approach (Strader et al., 1991).

Since this first effort at rational design of a GPCR, mutations to more than a dozen native GPCRs have yielded a large family of selectively activated designer receptors. Most of these receptors fall into two classes: the first-generation RASSLs, which were developed using rational design, and the second-generation DREADDs, which were evolved through directed molecular evolution in yeast. We describe here the development, characteristics, and applications of these two groups of receptors.

A. First-Generation Receptors Activated Solely by Synthetic Ligands

1. Development. After Strader's progress, the next advancements in the field of designer GPCRs came from Coward et al. (1998), which took the same rational design approach as Strader et al. (1991) (i.e., mutagenizing key residues for native ligand binding) and applied it to the human κ opioid receptor (hKOR). It is noteworthy that peptide ligands bind to the extracellular loops, whereas small drug-like molecules bind to transmembrane regions of peptide receptors. Therefore, Coward et al. (1998) predicted it to be feasible to eliminate native ligand binding to hKOR without decreasing the affinity

of small molecules for the receptor. Indeed, by exchanging the second extracellular loop of hKOR with that of the δ opioid receptor, Coward et al. (1998) successfully reduced the binding affinity of the endogenous peptide dynorphin A by 200-fold (from 0.06 to 14.6 nM) yet maintained the affinity of synthetic small molecule agonists such as spiradoline and bremazocine. This novel receptor, Ro1, faithfully couples to G_i to inhibit adenylyl cyclase, and the potency of the native and small molecule ligands parallels their respective binding affinities. In addition, Coward et al. (1998) generated a second RASSL (Ro2) with an E297Q mutation of Ro1. This mutation replaces a negatively charged residue implicated in ligand binding (glutamate) with a hydrophobic residue (tryptophan) at the top of the sixth transmembrane region of hKOR. The affinity of several endogenous opioids for Ro2 was further decreased compared with Ro1 (e.g., the K_i of dynorphin A at Ro2 was 124.5 nM, a nearly 2000-fold decrease in affinity compared with wild-type hKOR). Moreover, in response to spiradoline, Ro2 induced cellular proliferation in a pertussis toxin-sensitive manner. Although dynorphin A elicited no detectable response in Ro2-expressing cells at concentrations up to 1 μ M, spiradoline was equipotent at hKOR and at Ro2 (EC₅₀, 4.4 and 5.5 nM, respectively). It is noteworthy that the basal proliferation rate was not different in spiradoline-treated and -untreated Ro2-expressing cells, suggesting that Ro2 does not signal constitutively in this cellular context. Additional RASSLs derived from Ro1 and Ro2 that contain fluorescent tags or have altered internalization and/or desensitization properties now exist (Searce-Levie et al., 2001, 2005; Pei et al., 2008).

The article by Coward et al. (1998) set the groundwork for the rational design of an entire class of engineered receptors. The authors had proposed biogenic amine—in particular serotonin—receptors as RASSL templates and had further suggested the benefit of developing RASSLs that signal through each of the canonical G protein signaling pathways. Indeed, Kristiansen et al., (2000) soon reported that mutation to glutamate of the highly conserved Asp155 residue of the serotonin 2A receptor (5-HT_{2A}), a G_q-coupled receptor, greatly decreased the potency of serotonin (5-HT) but increased the potency of gramine, a 5-HT analog that is inactive at the native 5-HT_{2A} receptor. In addition, the 5-HT_{2A}^{D155E} receptor had reduced basal activity compared with the wild-type receptor. Bruysters et al. (2005) also developed a G_q-coupled RASSL that they engineered from the H1 histamine receptor. An F435A mutation reduced the affinity and potency (25- and 200-fold, respectively) of histamine yet increased the affinity and potency of the synthetic phenylhistamines (54- and 2600-fold, respectively).

Srinivasan et al. (2003, 2007) developed the first RASSLs to signal through G_s. They introduced into the melanocortin-4 (MC4) receptor naturally occurring mu-

tations that abolish activity of the cognate ligand α -melanocyte-stimulating hormone. The resulting RASSLs, Rm1 and Rm2, are active in response to low (2–3) nanomolar concentrations of the synthetic ligand tetrahydroisoquinoline (THIQ) and differ primarily in their levels of basal signaling. It is noteworthy that THIQ is a more potent agonist at the wild-type MC4 receptor than is α -melanocyte-stimulating hormone (EC_{50} , 0.8 and 30.3 nM, respectively), so it is not selective for Rm1 or Rm2.

Around the same time, Claeysen et al. (2003) introduced into the G_s -coupled mouse 5-HT₄ serotonin receptor a D100A mutation that abolished the efficacy of 5-HT at the receptor without eliminating the activity of synthetic ligands. It is noteworthy that two of the synthetic ligands that were antagonists at the wild-type receptor were noted as agonists at the D100A receptor. In a subsequent article, they introduced the same D100A mutation into the human 5-HT_{4B} serotonin receptor to generate Rs1 (Chang et al., 2007). Like the mouse homolog, this receptor was insensitive to 5-HT, but a variety of synthetic ligands had full- or partial-agonist activity, and some ligands demonstrated functional selectivity (Urban et al., 2007) by directing coupling selectively to G_s or jointly to G_s and G_q . Further mutations to Rs1 or chimeras of Rs1 and the 5-HT_{2C} or 5-HT_{1A} serotonin receptors generated receptors that differed in their specificity of coupling to G_s , G_q , and G_i or that had varied levels of constitutive activity. Thus, using a single receptor template, Claeysen et al. (2003) generated a family of RASSLs that signal through the three primary G proteins.

In addition to these RASSLs, Pauwels and Colpaert (2000) generated α_{2A} -adrenergic receptors (α_{2A} -AR) with S200A or S204A mutations. Epinephrine had reduced potency at both mutant receptors and a 70% decrease in efficacy at the S204A mutant, and synthetic imidazoline derivatives, including 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline (UK-14304), had improved potency at both mutant receptors (Pauwels and Colpaert, 2000; Pauwels, 2003). Nonetheless, the reduction in potency of epinephrine (45- and 251-fold for the S200A and S204A mutations, respectively) is insufficient for *in vivo* selectivity.

We have described a variety of mutant receptors (coupling to the G_q , G_i , and G_s signaling pathways) and corresponding synthetic ligands that convey some level of experimental control over cellular activity (Fig. 2). Most of these ligand-receptor systems are useful only for *in vitro* applications because of their insufficient selectivity, but researchers have developed transgenic mice expressing two of these receptors, Ro1 and Rs1.

2. *In Vivo* Applications. The first application of RASSLs in an *in vivo* setting came from Redfern et al. (1999), who developed transgenic mice that expressed Ro1 in the heart under control of the α -myosin heavy chain promoter in a tet-off manner. Eleven days after

weaning adult mice from dox, 90% of atrial myocytes and 50% of ventricular myocytes expressed Ro1. As expected on the basis of data from other G_i -coupled receptors present in cardiomyocytes (e.g., M₂ muscarinic, A₁ adenosine), spiradoline induced a dose-dependent decrease in heart rate selectively in Ro1-expressing mice; at 5 mg/kg spiradoline *i.p.*, heart rate decreased by an average of 55% in approximately 26 s. Thus, the onset kinetics of spiradoline's peripheral effects are relatively fast. Heart rate gradually returned to near baseline over the course of two h. In addition, Redfern et al. (1999) showed that Ro1 desensitizes to spiradoline; repeated injections every 4 h elicited progressively weaker responses.

Surprisingly, once mice had been had not been receiving dox for 3 weeks, they began to die, and mortality was virtually 100% by 16 weeks (Redfern et al., 2000). Physiological and histological studies revealed that Ro1 expression per se (in the absence of agonist) induced a lethal dilated cardiomyopathy. Ventricular conduction delays (indicated by a wide QRS complex on electrocardiogram), enlarged ventricular size and decreased ventricular myocardial thickness, myocyte disarray, collagen deposition, reduced myocardial force and rate of contraction/relaxation, anasarca, and labored breathing—all characteristic of cardiomyopathy and/or indicative of systolic dysfunction—were present only in mice no longer receiving dox (Redfern et al., 2000; Baker et al., 2001). It is noteworthy that a single injection of pertussis toxin restored sinus rhythm for more than 3 days, confirming that the arrhythmias were due to G_i -coupled signaling. In addition, mRNA microarray studies revealed changes in ventricular expression of components of the G_i signaling pathway: GIRK gene expression decreased by 50%, whereas expression of adenylyl cyclase 7, PKA, and PKA regulatory proteins all increased. Thus, the heart tissue seemed to be trying to compensate for excessive G_i signaling by altering gene expression.

These data are critical for evaluating RASSLs as an *in vivo* neurobiological tool. On the one hand, the pertussis toxin sensitivity and the alterations in expression of genes associated with G_i signaling suggest that Ro1 signals selectively through G_i *in vivo*, as does hKOR. The complete penetrance of dilated cardiomyopathy in transgenic mice no longer receiving dox suggests, however, that Ro1 is signaling in the absence of spiradoline or another synthetic ligand. Whether that activity indicates constitutive activity or an insufficient reduction in the efficacy and/or potency of dynorphins is unclear. McCloskey et al. (2008) attempted to answer this question by administering the KOR antagonist nor-binaltorphimine. The antagonist did not reverse the contractile dysfunction, thus that dysfunction does not stem from acute Ro1 signaling; it is possible, however, that the myocardial changes are irreversible and that adminis-

tration of an antagonist after they develop cannot mitigate the accumulated effects of excess G_i signaling.

Other studies have revealed severe pathologic conditions stemming from Ro1 expression; thus, the baseline phenotype is not specific to the cardiac system. Ro1 expression in osteoblasts induces trabecular osteopenia (Peng et al., 2008) and in astrocytes induces hydrocephalus (Sweger et al., 2007). For osteoblast expression, the mouse 2.3- α type 1 collagen promoter drives expression of tTA, thus double-transgenic mice carrying both the tTA and Ro1 transgenes express Ro1 protein selectively in osteoblasts in a tet-off manner. Few Ro1-expressing mice survive to weaning, and those that do survive are severely runted, weighing less than 50% that of sex-matched littermate control mice. Double-transgenic embryos are normal and occur in Mendelian ratios. At birth, Ro1-expressing mice have reduced mineralization of bones and altered bone structure, and most die within 2 h from respiratory failure. In particular, trabecular bone formation is decreased. Double-transgenic mice that are maintained on a dox-containing diet until P28 develop reductions in trabecular bone volume after dox has been withdrawn from the diet.

Pathologic conditions can develop from Ro1 expression in the central nervous system as well. Sweger et al. (2007) expressed Ro1 in the tet-off system under control of the human GFAP promoter and on a KOR(-/-) background. Maintenance of mice on a dox regimen elicited no detectable phenotype, and Ro1 protein expression was undetectable by Western blot. In the absence of dox, Ro1 expression resulted in fatal hydrocephalus. All Ro1-expressing mice that were never exposed to dox developed macrocephalus by P15 and severe hydrocephalus by 12 weeks; half of the transgenic mice died by 12 weeks. If mice were exposed to dox until weaning, they developed hydrocephalus once dox was withdrawn, indicating that the phenotype is not developmentally regulated. The hydrocephalus was characterized by grossly enlarged ventricles (particularly the lateral ventricles), reduced thickness of the cerebral cortex, disruption and displacement of other brain structures, hemorrhage, altered white tract tissue, denudation of the ependymal lining of the lateral ventricles, and occlusion of the aqueduct of Sylvius. Expression of GFAP, which indicates reactive glia, and phosphorylated ERK, which is a downstream mediator of G_i signaling, both increased in Ro1-expressing animals. These Ro1-expressing mice represent a new in vivo model of hydrocephalus. The model is particularly useful because it occurs with 100% penetrance, and it does not require administration of any toxin or exogenous agent. In addition, one can remotely control the age of onset of hydrocephalus by administering or withholding dox and thereby can determine the role of development on hydrocephalus and its manifestations.

Thus far, Ro1 expression in transgenic mice has not induced a detectable baseline phenotype in only one cell

type: taste receptors. Zhao et al. (2003) used Ro1 to investigate the sensory underpinnings of taste. They first demonstrated that specific GPCRs in the T1R class of taste receptors detect distinct classes of tastants (sweet or umami). It is noteworthy that different populations of taste cells express the sweet or umami receptors. To determine whether it is activation of the particular receptors for sweet/umami or whether it is the sweet/umami receptor-expressing cells that encode taste, Zhao et al. (2003) expressed Ro1 selectively in sweet-sensing cells under control of a tet-on system. Transgenic mice receiving dox, but not animals not receiving dox or wild-type animals, were attracted to spiradoline. In a two-bottle preference assay, Ro1-expressing animals preferred a spiradoline-containing solution at concentrations as low as 10 nM; preference for the spiradoline solution increased in a concentration-dependent manner. That a nontaste receptor can elicit the sensation of sweet taste suggests that specific pathways, and not specific receptors, encode taste. In support of that hypothesis, Ro1 expression in bitter taste cells of transgenic mice induced behavioral aversion to low-nanomolar concentrations of spiradoline (Mueller et al., 2005). Accordingly, mice expressing a native "bitter" receptor in sweet taste cells exhibit a strong attraction to bitter compounds. The same receptor can convey sweet and bitter taste, depending on where it expresses. These studies demonstrate that taste cells, and not taste receptors, encode the individual taste modalities (sweet, salty, sour, bitter, umami).

Although most in vivo work with RASSLs has used Ro1, a few studies have reported on Rs1 expression and function in vivo. Although Ro1 expression in osteoblasts *decreases* bone mass, as we described above, Rs1 expression in osteoblasts *increases* bone mass (Hsiao et al., 2008, 2010a,b). Here, we have a nice example of bidirectional modulation of the signaling and phenotype for the same cell type (although activation and silencing of osteoblast signaling was not achieved in the same animal). As one might expect, inhibiting and activating adenyl cyclase, via G_i and G_s , respectively, have opposing effects on bone structure and growth. It is noteworthy that unlike Ro1 expression in cardiomyocytes and astrocytes, the timing of Rs1 expression and signaling in osteoblasts is critical for the development of pathologic conditions; Rs1 induction after P28 does not alter trabecular bone mass, which suggests a role for prepubertal bone growth in osteosclerosis (Hsiao et al., 2008). Significantly, induction of Rs1 expression in mice at P28, followed by continuous or intermittent exposure to the Rs1 agonist RS67333 from P70 to P140, greatly increased bone formation (Hsiao et al., 2010b), indicating that adult bone tissue is still sensitive to G_s -mediated signaling.

As these in vivo data show, RASSLs provide new models of human pathologic conditions, including cardiomyopathy, hydrocephalus, osteoporosis, and osteosclerosis. These models are useful for investigating the

signaling abnormalities that contribute to these disorders, but the lack of external control over the timing or magnitude of RASSL signaling limits their utility for probing the signaling pathways behind complex behaviors.

B. Second-Generation Receptors Activated Solely by Synthetic Ligand: Designer Receptors Exclusively Activated by Designer Drugs

Whereas the first generation of orthologous ligand-GPCR pairs represented a significant advancement in tools for manipulating neuronal signaling pathways, both the *in vitro* and *in vivo* data reveal potential shortcomings of RASSLs. First, although the receptors are activated solely by synthetic ligands, the synthetic ligands do not solely activate the designer receptors. For example, spiradoline has only 200-fold higher potency at Ro1 than at hKOR (Coward et al., 1998), and THIQ is more potent at MC4 than at Rm1 or Rm2 (Srinivasan et al., 2007). Therefore, to selectively activate only the RASSL and not the native receptor *in vivo*, one must either use a knockout background, which introduces a variety of confounds into the experimental design, or one must locally infuse the synthetic ligand to a tissue or region that expresses only the RASSL and not the wild-type receptor. The latter option eliminates one of the advantages of this system (i.e., its noninvasiveness). Second, *in vivo* use of RASSLs (Ro1 and Rs1) has resulted in fatal baseline (in the absence of agonist) pathologic conditions in multiple tissues. Whether these pathologic conditions result from constitutive activity of RASSLs or the local concentration of endogenous ligand is sufficient to activate RASSLs is unclear; however, the baseline pathologic conditions stem from up-regulated signaling of GPCR pathways.

Given these limitations of first-generation RASSLs, we sought to develop a new generation of designer receptors that would meet all the criteria of first-generation RASSLs [i.e., no endogenous ligand would activate the receptors, a synthetic ligand with favorable pharmacokinetics would activate the receptors with affinity suitable for *in vivo* use (low nanomolar), and the receptors would couple to each of the canonical G proteins] with the following additional stipulations: 1) the receptor would have no or minimal baseline activity; and 2) the synthetic ligand would be inert (i.e., active only at the designer receptor and not at any endogenous target). In this section, we summarize our progress toward this goal.

1. Development. The first designer receptor that displayed two-way selectivity was actually a rationally mutated β_2 -AR. With a goal of developing a tool suitable for gene therapy, Small et al. (2001) developed what they termed a therapeutic receptor-effector complex (TREC). They mutagenized the binding pocket and several regulatory residues of the β_2 -AR and fused the C terminus of the receptor to a modified G_s subunit. A nonbiogenic

amine [1-(3,4-dihydroxyphenyl)-3-methyl-1-butanone (L-158,870)] that Strader et al. (1991) had originally identified as being inactive at the wild-type β_2 -AR fully activated their mutant β_2 -AR with low micromolar potency, and endogenous β_2 -AR agonists were inactive at the novel receptor. Thus, they had achieved the two-way selectivity necessary for a TREC and a DREADD. However, they did not screen L-158,870 for activity at other endogenous targets and, although the TREC was an improvement upon the work from the Strader lab, the potency of L-158,870 for the designer receptor was still relatively low (EC_{50} , 7 μ M) and not ideal for *in vivo* applications.

Neoreceptors and neoligands represent additional tools with two-way selectivity (Jacobson et al., 2001, 2005, 2007; Gao et al., 2006). Neoreceptors are modified adenosine receptors, and their cognate ligands are modified nucleotides. Jacobson et al. (2001, 2007) employed rational design to generate an H272E mutation in the seventh transmembrane domain of the A3 adenosine receptor (A3A) that impaired affinity of native A3A ligands, including adenosine. In addition, they added an amino group to the ribose moiety of adenosine to yield 3'-amino-3'-deoxyadenosine, a novel nucleotide with 7-fold higher potency for A3A^{H272E} than for wild-type A3A. Additional adenosine derivatives yielded more selective neoligands (Gao et al., 2006). A similar approach generated a neoreceptor/neoligand pair for the A2 adenosine receptor (A2A). The A2A neoligand has 161-fold higher potency at the neoreceptor A2A^{Q89D} than at the wild-type A2A (Jacobson et al., 2005). As with the TREC, neoreceptors/neoligands were an advancement in selective modulation of a mutant receptor but were still not well suited for *in vivo* use.

We took a novel approach to creating designer receptors that exhibit the desired properties, namely, directed molecular evolution (Dong et al., 2010b). Our approach relied not on rational design—generating receptors with deliberate mutations at residues critical for ligand binding—but instead on random mutagenesis via error-prone polymerase chain reaction. Investigators have successfully applied this approach to generate proteins with specific enzymatic or catalytic activities, for instance (Yuan et al., 2005). For our purposes, we first chose a designer ligand, clozapine-*n*-oxide, known to be inert at endogenous targets (Weiner et al., 2004; Armbruster et al., 2007) and to be highly bioavailable and blood-brain-barrier-permeant in both humans and mice (Bender et al., 1994; Chang et al., 1998). Given its structural similarity to clozapine, which is a weak partial agonist at muscarinic receptors (Davies et al., 2005), we predicted that introducing only a few mutations to that receptor family could yield receptors that are sensitive to CNO (Armbruster et al., 2007). The muscarinic cholinergic receptor family includes five receptors: the M_1 , M_3 , and M_5 receptors couple to G_q , and the M_2 and M_4 receptors couple to G_i . We chose the G_q -coupled human

M3 (hM3) muscarinic receptor, for which clozapine has moderate affinity and weak potency, as our template receptor; used error-prone polymerase chain reaction to generate a library of mutants; and transformed the library into yeast. We used a strain of *Saccharomyces cerevisiae* in which heterologously expressed GPCRs couple to the pheromone signaling pathway, and activation of that pathway drives transcription and expression of essential nutrients, thus enabling growth on selective media (Pausch et al., 1998; Erlenbach et al., 2001; Dong et al., 2010b). We developed three successive generations of mutant receptor libraries; we screened each for growth in response to 10 μ M clozapine, 1 μ M CNO, or 5 nM CNO; and we selected mutants that exhibited potent growth profiles in response to CNO but not in response to ACh or in the absence of ligand (for a detailed protocol, see Armbruster et al., 2007; Dong et al., 2010b).

As predicted, introducing just two mutations rendered hM3 insensitive to its native ligand, ACh, and highly sensitive to the designer ligand, CNO. It is noteworthy that these two mutations (Y149C and A239G; Fig. 3A), which were necessary and sufficient to generate a DREADD, were at highly conserved residues. Mutating the analogous residues of other muscarinic family members generated a family of DREADDs that couple to G_i and G_q G proteins, as do the native muscarinic receptors. In smooth muscle cells, the G_q -coupled hM3 DREADD (hM3Dq) potently and selectively stimulates inositol phosphate hydrolysis (Fig. 3B), calcium release [in cultured neurons as well (Alexander et al., 2009)], and ERK1/2 activation in response to CNO, whereas the G_i -coupled human M4 DREADD (hM4Di; derived from the M4 muscarinic receptor) stimulates calcium release (in a pertussis toxin-sensitive manner) and ERK1/2 activation, inhibits forskolin-induced cAMP formation, and, in cultured neurons, activates GIRK, thereby causing hyperpolarization and inhibition of basal action potential firing (Armbruster et al., 2007). As Fig. 3B shows, these cellular responses occur only after treat-

ment with CNO; they are notably absent after treatment with ACh or carbachol. In addition, the receptors exhibit no apparent baseline activity.

Because none of the native muscarinic receptors couples to G_s , Guettier et al. (2009) generated a chimera of the rat M3 receptor in which they introduced the Y149C and A239G mutations and exchanged the second and third intracellular loops for those of the G_s -coupled turkey β_1 -AR (Fig. 3A). Clozapine-*n*-oxide maintains its potency and efficacy at the chimeric receptor, and the receptor couples selectively to G_s to stimulate adenylyl cyclase, thus yielding a G_s -coupled DREADD (rM3/ β 1Ds) (Guettier et al., 2009). We and others have now validated DREADDs that couple to the canonical G_q , G_i , and G_s signaling pathways. In vitro, these receptors have served as tools for investigating allosterism (Nawaratne et al., 2008) and GPCR dimerization (Alvarez-Curto et al., 2010). Each of these receptors also functions in vivo, as we describe next.

2. In Vivo Applications. For a proof-of-concept study, we used the CaMKII α promoter to drive expression of hemagglutinin-tagged hM3Dq in the forebrain of transgenic mice (Alexander et al., 2009). Given the pathological baseline phenotypes that Ro1 and Rs1 induced, we used the tet-off system so that we could control hM3Dq expression as well as its activity. We had evolved DREADDs to be devoid of constitutive activity, and they were in vitro, but we did not know whether that would also hold true in vivo. It is noteworthy that Ro1 exhibited no basal activity in cell culture (Coward et al., 1998) but clearly has high basal activity in vivo. Double-transgenic mice expressed hM3Dq on the apical dendrites of cortical pyramidal neurons and in the cell bodies and processes of hippocampal CA1 and CA2 pyramidal neurons. Bath application of CNO to acutely prepared brain slices depolarized CA1 pyramidal neurons in a PLC-dependent manner and induced bursting-type action potential firing when tetrodotoxin was withheld from the bath. We used multielectrode arrays to measure local field poten-

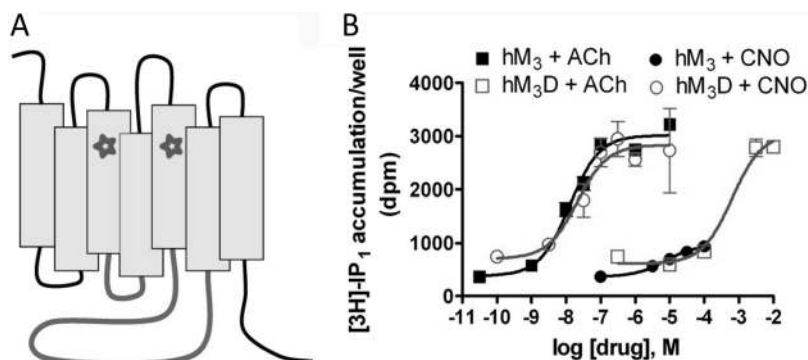


FIG. 3. DREADDs are mutant muscarinic receptors. A, DREADDs are formed by point mutations in the third and fifth transmembrane regions of muscarinic receptors (stars; Y149C and A239G in hM₃). In addition, the G_s -coupled DREADD contains the second and third intracellular loops of the β_1 -AR in place of those of the M₃ muscarinic receptor (gray loops). B, in human pulmonary artery smooth muscle cells, the hM3Dq receptor (hM₃D) is selectively activated by CNO but not by ACh, resulting in PIP₂ hydrolysis. Conversely, the wild-type M₃ muscarinic receptor (hM₃) is potently activated by ACh but not by CNO. [Adapted from Armbruster BN, Li X, Pausch MH, Herlitze S, and Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci USA* 104:5163–5168. Copyright © 2007 National Academy of Sciences, USA.]

tials (LFPs) in the CA1 hippocampal region in awake, mobile mice. We administered CNO intraperitoneally, and LFP changes were evident within 15 min. We observed a dose-dependent increase in the power of the γ band (20–100 Hz) on LFP spectrograms. γ Rhythm, which is associated with information encoding, storage, and recall, results from synchronous, regenerative firing of parvalbumin-expressing basket-cell interneurons. Indeed, we observed an increase in the interneuron firing rate during the period of γ rhythm. We knew from our slice recordings that CNO directly depolarizes pyramidal cells, and we demonstrated through immunohistochemical staining that hM3Dq expression is excluded from parvalbumin-expressing cells. Thus, we reasoned that CNO was activating glutamatergic pyramidal neurons that were in turn activating interneurons (Alexander et al., 2009).

We detected electrophysiological changes in double-transgenic mice at a minimum dose of 0.1 mg/kg CNO. At that dose, we also detected an increase in stereotypic behavior, and at 0.3 mg/kg CNO, hM3Dq mice were hyperlocomotive. Doses of 0.5 mg/kg and higher elicited behavioral and electrophysiological seizures, status epilepticus, and death. The behavioral and electrophysiological effects of CNO lasted for approximately 10 h. However, in mice, CNO clears from plasma within 2 h, with no back-metabolism to clozapine (Guettier et al., 2009); thus, we suspect that the long-lasting effects stem from regenerative neuronal firing and not directly from activation of hM3Dq by CNO.

Mice on a dox-containing diet did not respond (behaviorally or electrophysiologically) to CNO. Critically, when we raised mice on a dox-free diet, we detected no baseline pathologic condition or phenotype, indicating that hM3Dq has no significant constitutive activity and that an inducible expression system is unnecessary. Therefore, floxed DREADD mice would be useful new tools because they would allow researchers to profit from the plethora of Cre driver lines, and no pathologic condition should result from life-long DREADD expression. This first in vivo report of DREADD function validates its worth as a neurobiological tool.

Outside of the CNS, hM3Dq also functions in vivo. Guettier et al. (2009) expressed either hM3Dq or rM3/ β 1Ds in pancreatic β -cells and successfully modulated blood glucose levels and insulin secretion with CNO. When they administered CNO to obese, insulin-resistant mice expressing hM3Dq, glucose tolerance and β -cell mass both increased.

Ferguson et al. (2011) validated hM4Di function in vivo. They performed several proof-of-concept studies. First, in acutely prepared slices from rats infected with virus transducing hM4Di, application of CNO decreased striatal neuron excitability. Specifically, CNO mediated potassium conductance to hyperpolarize striatal neurons and decrease input resistance. It is noteworthy

that no change in input resistance occurred in the absence of CNO; no basal effect of hM4Di expression was evident. Second, administration of CNO to rats expressing hM4Di in the ventral tegmental area blocked the dopamine release that normally follows a food reward. Third, Ferguson et al. demonstrated inhibition with CNO of amphetamine-induced c-fos expression. Together, these studies validate the function of hM4Di for neuronal silencing in vivo. Next, Ferguson et al. (2011) applied DREADD technology to the investigation of the neurobiological activity that contributes to addiction. Using viral constructs with promoters specific for the direct and indirect striatal pathways, they expressed hM4Di in rat brains and determined the effects of CNO on behavioral sensitization to amphetamine. They determined that hM4Di-mediated silencing of direct and indirect pathway neurons inhibited and facilitated behavioral sensitization, respectively.

No published in vivo data on rM3/ β 1Ds expression in the brain currently exist, although we have verified the system in vivo (M. Farrell and B. L. Roth, manuscript in preparation). Many additional reports of DREADD use in vivo are appearing, including the bidirectional control of feeding behavior (Krashes et al., 2011).

V. Optical Approaches

In 2003, researchers cloned channelrhodopsin-2 (ChR2), a cation channel structurally similar to vertebrate rhodopsin that opens in response to blue light to allow Na^+ to flow into a cell. Nagel et al. (2003) cloned ChR2 from the green alga *Chlamydomonas reinhardtii* and then demonstrated through functional expression, both in *Xenopus laevis* oocytes and in mammalian cells, that ChR2 is a directly light-gated, cation-selective ion channel that opens rapidly upon stimulation with light to allow cation flux. Neuroscientists readily appreciated the potential utility of ChR2 as a neurobiological tool: photostimulation is a reliable and reproducible approach to induce neuronal spiking; upon activation of ChR2, current flows within milliseconds; and vertebrates already express the necessary cofactor, all-*trans* retinal (ATR), that would enable the translation of this technology to in vivo applications (Li et al., 2005; Herlitze and Landmesser, 2007). Indeed, in vivo studies based on activation of ChR2 were quickly forthcoming (see Table 2). Researchers also recognized that ion pumps or channels that either depolarize or hyperpolarize neurons in response to wavelengths of light distinct from those that activate ChR2 would have tremendous value as in vivo tools. Thus, several groups subsequently sought—and identified—rhodopsin-like proteins with those characteristics (Li et al., 2005; Han and Boyden, 2007; Zhang et al., 2007b, 2008) (Fig. 4). Below, we describe the “optogenetic” approach that combines light-activated receptors, or “opsins,” for temporal control with

TABLE 2
In vivo applications of optogenetics

Opsin	Expression Pattern	Principal Findings	Reference
Mice ChR2	Spinal neurons in and around phrenic motor pool	Photostimulation rescues diaphragmatic respiratory motor activity in mice with cervical spinal cord injuries and induces plastic and adaptive changes to enable motor activity in the absence of light	Alilain et al., 2008
	Layer 2/3 pyramidal neurons in somatosensory cortex	Mice learned to detect optical stimulation and to make decisions based on the presence or absence of the resultant cortical activity	Huber et al., 2008
	Pyramidal cells of lateral amygdala	Optical stimulation of lateral amygdala is sufficient to induce fear learning; mice learned to freeze in response to a tone that was paired with photoactivation	Johansen et al., 2010
	Dopaminergic neurons in ventral tegmental area	Phasic stimulation was sufficient to drive behavioral conditioning and elicited dopamine transients	Tsai et al., 2009
	Cortical interneurons	Developed PINP: can use ChR2 to “tag” neurons and monitor their activity	Lima et al., 2009
	Cortical and thalamic pyramidal neurons	Neuronal activation induces local blood oxygenation level-dependent signals on fMRI; optogenetic fMRI demonstrates causal effects—can visualize and map downstream neural activity	Lee et al., 2010
	Astrocytes in brainstem chemoreceptor areas	Activating astrocytes activated the chemoreceptor neurons in an ATP-dependent manner and caused a respiratory response, thus demonstrating a role of glia in a physiological reflex	Gourine et al., 2010
	Neuronal subsets throughout brain	Mapped spatial distribution of neural circuits in cortex	Wang et al., 2007
	Hypocretin neurons	Directly activating hypocretin neurons via ChR2 increased probability of awakening from slow-wave or REM sleep	Adamantidis et al., 2007
	Prefrontal cortex	Stimulation of ChR2 has antidepressant-like effects in mice previously exposed to chronic social defeat stress	Covington et al., 2010
Optin-receptor chimeras	Central amygdala	Two simultaneous papers mapped the microcircuitry required for conditioned fear	Ciocchi et al., 2010; Haubensak et al., 2010
	Nucleus accumbens	Opto- β_1 AR induced conditioned place preference, but activation of ChR2 or opto- β_2 AR did not	Airan et al., 2009
<i>Drosophila</i> ChR2	Abnormal chemosensory jump 6 neurons	These neurons are required for startle response and mediate escape behavior; stimulation of cells with ChR2 elicited escape in response to light	Zimmermann et al., 2009
	Olfactory receptor neurons in larvae	Stimulation caused illusion of attractive odor with associated crawling toward the stimulus	Bellmann et al., 2010
	Subset of Rohon-Beard and trigeminal somatosensory neurons	Inducing a single action potential generates escape behavior; first demonstration of electrical activation of single cells in unrestrained zebrafish; determined that endogenous ATR is sufficient	Douglass et al., 2008
Zebrafish NpHR, eNpHR, ChR2	Throughout brain	Localized swim command circuitry to hindbrain region; activating eNpHR in this region caused larvae to stop moving and lose coordination, while activating ChR2 elicited swimming behavior	Arrenberg et al., 2009
	NpHR, ChR2	Mapped eye moments that follow visual stimulus; ChR2 activation can restore saccades in a genetic mutant that does not normally exhibit them; determined that saccade circuit in zebrafish is similar to mammalian burst generator	Schoonheim et al., 2010
	ChR2	Adapted tet system and viral gene delivery to zebrafish; ChR2 activation induced forward and backward swimming	Zhu et al., 2009

PINP, photostimulation-assisted identification of neuronal populations; fMRI, functional magnetic resonance imaging; ATR, all-*trans* retinol.

genetic approaches to spatial control and now constitutes an established technique for direct depolarization and hyperpolarization of neurons *in vivo*.

A. Depolarizing Opsins

1. *Channelrhodopsin-2*. Boyden et al. (2005) published the first study that used ChR2 to alter neuronal

firing. They used a lentiviral approach to express ChR2 in cultured rat hippocampal neurons. It is noteworthy that the receptor trafficked to the cell membrane, expression was stable for several weeks, and expression did not alter baseline membrane potential or resistance, which suggested that ChR2 does not have basal activity and is non-toxic to neurons. Illumination of the cultures with blue

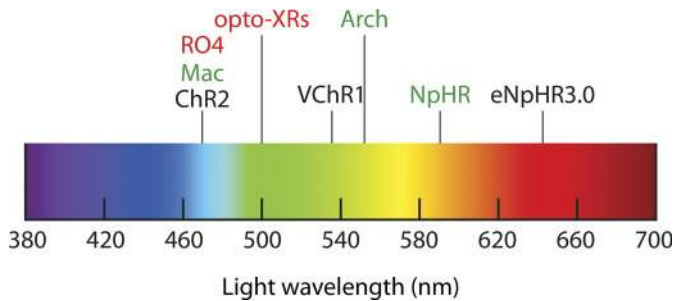


FIG. 4. Activation of optical tools by distinct wavelengths of light. Spectrum depicts the distinct wavelengths and corresponding colors of light that maximally activate each of the various microbial opsins. Opsins labeled in black text depolarize neuronal membranes; those labeled in green hyperpolarize membranes; and those labeled in red couple to G protein.

light (450–490 nm) induced large and rapid depolarizing currents, whereas illumination with longer wavelengths (490–510 nm) induced much smaller currents. Only neurons expressing ChR2 responded to the light stimulus. Continuous illumination evoked a rapid and predictable initial spike; thereafter, no spiking pattern was evident. Pulsed light, however, evoked reliable spike trains.

Concurrently, Li et al. (2005) used ChR2 and vertebrate rat rhodopsin 4 (RO4)—a light-activated, G_i -coupled GPCR that therefore silences neuronal firing (see section V.C.1)—to bidirectionally modulate the electrical activity of cultured hippocampal neurons and of intact embryonic chick spinal cords. This study independently replicated the results of Boyden et al. (2005), verifying that ChR2 rapidly and reliably depolarizes neurons and elicits action potentials. This study was also the first to demonstrate bi-directional control of neuronal firing with opsins. In particular, this study was important because it showed that light penetration through tissue does not interfere with opsin activation; light successfully activated opsins in intact chick embryos through a window in the eggshell.

Simultaneously, Nagel et al. (2005) also validated ChR2 as a tool for controlling cellular activity. They expressed the channel in muscle cells of *C. elegans*, raised the animals on an ATR-containing diet, and demonstrated light-induced muscle cell contraction in the whole animal. The contractions caused the animals to shrink and to lay eggs, and muscles relaxed within 1 s of the light turning off. It is noteworthy that photocurrents did not occur in animals raised without ATR. Next, Nagel et al. (2005) used ChR2 to depolarize mechanosensory neurons and elicited withdrawal reflexes in ATR-treated animals, affirming the function of this tool for remote control of neuronal activity *in vivo*.

2. *Volvox carteri* Channelrhodopsin-1. *V. carteri* expresses a photosensitive, cation-conducting channelrhodopsin (VChR1) that responds maximally to 589-nm light (Zhang et al., 2008). This excitation spectrum is red-shifted from that of ChR2, most likely because of electrostatic potential differences of the ATR-Schiff base

complex in the opsin active site (Kloppmann et al., 2005; Zhang et al., 2008). Zhang et al. (2008) found that light wavelengths of 406 and 589 nm are selective for ChR2 and VChR1, respectively, meaning that one could selectively activate these receptors within a single neuronal population. Another difference between the two cation channels is that VChR1 deactivates with a decay constant of 133 ms, which is notably slower than the 12 ms that ChR2 requires; they both activate within several milliseconds. However, the two opsins share many other properties: they are both nonselective cation channels; expression of neither opsin is toxic to cells or alters membrane integrity; and supplementation with ATR is unnecessary in mammalian neurons.

3. Channelrhodopsin-2 E123T Accelerated. Despite its wide use, several problems with ChR2 have persisted: high (and sometimes even low) levels of ChR2 expression can result in extraneous spiking in response to a single light pulse; ChR2 cannot sufficiently drive firing rates into the γ range; and high spike rates elicit plateau potentials. Hypothesizing that a decrease in the deactivation time of ChR2 could resolve all three of these problems, Gunaydin et al. (2010) used structural data from bacteriorhodopsin to identify point mutations in ChR2 that might accelerate the off-kinetics of channel activity. In particular, a ChR2^{E123T} mutant demonstrated faster peak recovery, increased steady state-to-peak current ratio, faster flash-to-peak current time, and offset kinetics that were nearly twice as fast (5.2 versus 9.8 ms) as those of wild-type ChR2. The mutant, which the authors refer to as ChETA (for ChR2 E123T Accelerated) was functional *in vitro* both in cultured hippocampal neurons and in acutely prepared brain slices from a mouse expressing ChETA in cortical interneurons. In the latter condition in response to 2-ms light pulses, ChETA evoked neuronal spiking more reliably than wild-type ChR2; each light pulse resulted in a single action potential, even at frequencies as high as 200 Hz.

B. Hyperpolarizing Opsins

1. Halorhodopsin. The necessity of developing complementary tools to directly hyperpolarize neurons is self-evident. Han and Boyden (2007) and Zhang et al. (2007b) initially described the use of halorhodopsin (NpHR), a light-activated chloride channel from *Natronomonas pharaonis* that responds to yellow light (580 nm), to silence neuronal firing. Like ChR2, NpHR silences with extremely fast kinetics. In addition, after exposure to continuous light, NpHR remains active for several minutes, allowing sustained neuronal silencing. Because the wavelengths of light that activate ChR2 and NpHR are distinct, one can express the two receptors in the same cells to bidirectionally modulate their activity. Han and Boyden (2007) demonstrated that alternating pulses of yellow and blue light drive hyperpolarizations and depolarizations, respectively, in cultured neurons expressing both ChR2 and NpHR.

Zhang et al. (2007b) expressed both ChR2 and NpHR in cholinergic motor neurons or muscle cells of *C. elegans* and demonstrated that NpHR activation could block ChR2-induced muscle contraction.

One problem with ectopic NpHR expression is that high levels of surface expression are necessary to optimally silence neuronal firing, but the channel accumulates in the ER (Gradinaru et al., 2008; Zhao et al., 2008). To overcome this obstacle, Gradinaru et al. (2008) identified an N-terminal signal peptide and a C-terminal ER export sequence that increase trafficking to the cell membrane and decrease channel retention in the ER. Moreover, these N- and C-terminal modifications increase the peak photocurrent that this enhanced NpHR (eNpHR or eNpHR2.0) could generate. Further modifications (i.e., addition of the trafficking signal from the Kir2.1 potassium channel) to eNpHR generated eNpHR3.0 (Gradinaru et al., 2010), which has improved cell-membrane expression, particularly in the processes; coincident with the improved expression are a 20-fold increase in NpHR photocurrents and sensitivity to far-red (630–680 nm) light. It is noteworthy that this last improvement permits more selective activation of ChR2 and eNpHR3.0 in the same cells, albeit with moderately (40%) reduced peak photocurrents compared with expression of only one opsin.

2. *Halorubrum sodomense* Archaeorhodopsin-3 and *Leptosphaeria maculans* Opsin. The NpHR pore is permeable to chloride ions, and light-activated chloride pumps inactivate for long periods of time (tens of minutes). However, light-activated outward proton pumps spontaneously reactivate within seconds. Therefore, Chow et al. (2010) mined the plant, archaeobacterial, bacterial, and fungal opsins for light-sensitive hyperpolarization and identified two outward proton pumps, archaeorhodopsin-3 (Arch) from *H. sodomense* and Mac from the fungus *L. maculans* (Waschuk et al., 2005), that enable neuronal silencing. In response to yellow light (peak photoactivation at ~550 nm), Arch pumps protons out of the cell within 10 ms with minimal effects on intracellular pH, and silencing of neuronal spiking occurs with 900 pA of current. Continuous light causes the current to decay, and the pump rapidly inactivates within 20 ms of the removal of light and then recovers—all within seconds. It is noteworthy that Arch-generated current did not saturate at high light intensities, as does NpHR-generated current, and Arch-induced silencing was of greater magnitude than that of NpHR; the authors speculate that Arch could silence 10-fold more brain tissue than NpHR. In vivo, Arch rapidly (with latency near 0 ms) reduces neuronal firing, with maximal silencing of 90%. The baseline firing rate recovers within a few hundred milliseconds. Like Arch, Mac also hyperpolarizes neurons, but does so in response to blue-green light, a feature that is rare among chloride pumps. Independent silencing of distinct neuronal populations is now possible with blue and red light. Indeed, the

authors used 630- and 470-nm light to selectively silence NpHR- and Mac-expressing neurons, respectively.

C. G Protein-Coupled Opsins

1. Vertebrate Rhodopsin. Li et al., 2005 first used light to activate GPCR pathways, expressing RO4 in cultured hippocampal neurons and in intact embryonic chick spinal cord neurons. They determined that RO4 functions both presynaptically (to inhibit voltage-gated calcium channels and to modulate neurotransmitter release and paired-pulse facilitation) and postsynaptically (to modulate the activity of GIRK). Therefore, RO4 allows one to study G protein signaling and the effects of neuronal silencing on a millisecond time scale. The same group has also cloned the C-terminal tail of the G_i-coupled 5-HT_{1A} serotonin receptor onto RO4 (Oh et al., 2010). The resultant chimeric protein, Rh-CT_{5HT1A}, traffics and signals like the native 5-HT_{1A} receptor and rescues 5-HT_{1A}-mediated signaling in neurons or tissue from 5-HT_{1A} knockout mice. These data evidence the ability of opsins to substitute for native mammalian receptors, which should provide new opportunities for the functional dissection of neural circuits.

2. Opsin-Receptor Chimeras. To modulate the same intracellular biochemical pathways as GPCRs, but with the time resolution of optical techniques, Airan et al. (2009) created a family of opsin-GPCR fusion proteins that are light-activated and couple to G proteins. They exchanged the intracellular portions of rhodopsin, which couples to G_t, with those of the α_1 - or β_2 -ARs to direct coupling to G_q or G_s, respectively. Airan et al. (2009) validated the selectivity of opsin-receptor chimeras for the appropriate G_q or G_s-coupled intracellular pathways. Whereas ligand-activated GPCRs can adopt a variety of active conformational states, thus leading to ligand functional selectivity (Urban et al., 2007), light-activated opsin-receptor chimeras most likely adopt a unique conformation in response to light (Airan et al., 2009). Hence, opsin-receptor chimeras offer less functional versatility but greater temporal resolution than designer GPCRs.

D. Advances in Light Delivery, Penetration, and Manipulation

Optogenetics can be a technically complicated approach for in vivo manipulation of neuronal activity, depending on the species, target tissue, and other experimental variables. For example, targeting deep brain areas (e.g., raphe nuclei) or diffuse neuronal populations (e.g., all parvalbumin-positive neurons throughout the forebrain) is difficult. Recently, technology for such photostimulation has greatly improved, incorporating flexible fiber optics (Aravanis et al., 2007), two-photon imaging (Mohanty et al., 2008; Rickgauer and Tank, 2009; Andrasfalvy et al., 2010), and/or improved spatial resolution (Schoenenberger et al., 2008; Andrasfalvy et al., 2010). Although a detailed description of these advances

is beyond the scope of this review, a few advances are highlighted here.

One problem with the basic optogenetic approach is that single-cell activation, even *in vitro*, has not been possible on an ultrafast time scale (i.e., <1 ms), in part because ChR2 has a low channel conductance, requiring activation of many channels to sufficiently depolarize a neuron. With standard one-photon imaging, enough channels are not activated with single-cell spatial resolution. Two-photon imaging techniques have improved the spatial resolution of optogenetic control of neuronal activity, but some of the time resolution is lost because the laser must scan a large area to activate enough channels to cause depolarization above threshold. Andrasfalvy et al. (2010) developed a technique, which they refer to as temporally focused laser pulses, that enables simultaneous excitation of many channels on single neurons or in individual neuronal compartments (e.g., dendrites, presynaptic terminals). Their approach uses multiple brief (0.1-ms) light pulses, with extremely fast (within 0.2 ms) movement of the light focus within a 100- μm field. This technique is effective *in vitro* on acute brain slices.

Another challenge in optogenetics is the simultaneous stimulation and recording of neural activity. To overcome this challenge, some groups have glued electrodes to the optical fibers so that both stimulation and recording from the same location are possible (Gradinaru et al., 2007), whereas others have developed and employed “optrodes,” dual-modality devices that simultaneously illuminate and record from neurons (Zhang et al., 2009a). In addition, simultaneous stimulation of scattered cell populations is difficult. High-intensity light-emitting diode (i.e., LED) arrays, optical prosthetics, and grids can provide multiple points of illumination or illumination to deep structures (Zhang et al., 2007a; Bernstein et al., 2008; Grossman et al., 2010). Technical advances will undoubtedly continue to improve the *in vivo* and translational applications of optogenetics.

E. *In Vivo* Applications

Optogenetic strategies are now widely used for *in vivo* investigation of neuronal circuits and will soon become *de rigueur* tools for circuitry-based dissection of neuronal function. Various groups have published data on optogenetic applications in zebrafish, *D. melanogaster*, and *C. elegans*, and a few recent articles have described applications in nonhuman primates (see Table 2). The majority of the *in vivo* data, however, comes from rodents. Table 2 describes the principal findings of many of these *in vivo* studies, and we highlight below some of the most exciting reports.

One *in vivo* application of optogenetics has been in PD research. Opsins provide a method of directly testing some of the existing hypotheses regarding the mechanisms of action of various therapies. For example, in classic basal ganglia circuitry, “direct” and “indirect”

pathway stimulation facilitates and inhibits movement, respectively, and activating D₁ receptors on direct medium spiny neurons should be therapeutic. In the past, directly activating direct pathway neurons without off-target effects has been difficult. Moreover, high-frequency deep brain stimulation (DBS) is a treatment for refractory PD, but its therapeutic mechanism is unclear. To investigate this circuitry, Kravitz et al. (2010) expressed ChR2 in D₁ (direct) or D₂ (indirect) dopamine receptor-expressing medium spiny neurons and determined that activation of D₁ neurons facilitates locomotion and decreases freezing, whereas activation of D₂ neurons inhibits locomotion and increases freezing behavior. Moreover, they showed in 6-hydroxydopamine-lesioned mice, an animal model of PD, that activation with light of the direct pathway completely rescues the PD phenotype. This article was the first to directly demonstrate a causal role of D₁-expressing neurons in ameliorating PD symptoms. In addition, with the use of 6-hydroxydopamine-lesioned mice, Gradinaru et al. (2009) used optogenetics to dissect the various subcomponents of the basal ganglia circuitry to determine the location of action important for the therapeutic effects of DBS. They found no positive changes in locomotor symptoms from either optical inhibition (via eNpHR), high-frequency optical stimulation (via ChR2) of substantia nigral excitatory neurons, or optical activation (via ChR2) of substantia nigral astrocytes and subsequent inhibition of substantia nigral neuron firing. However, when they targeted ChR2 to afferent terminals in the substantia nigra, high-frequency stimulation improved PD symptoms for the duration of the stimulation. It is noteworthy that as soon as the light pulses were discontinued, locomotion immediately returned to its prestimulation level, and low-frequency stimulation of the same afferent fibers worsened PD symptoms. These findings demonstrate well the translational potential of optogenetics and highlight the complexity of the system particularly with regard to the light pulse protocol.

One of the most promising translational applications of opsins thus far is to the restoration of vision after retinal degeneration. A variety of groups have expressed opsins in the retina of several species (mice, rats, marmosets) in photoreceptors, retinal ganglion cells, and on- or off-bipolar cells (Lin et al., 2008; Ivanova and Pan, 2009; Tomita et al., 2009, 2010; Zhang et al., 2009b; Busskamp et al., 2010; Ivanova et al., 2010a,b; Thyagarajan et al., 2010). Busskamp et al. (2010) expressed an eNpHR-EYFP fusion protein in photoreceptors using the human rhodopsin, human red opsin, and mouse cone arrestin-3 promoters. They administered eNpHR-EYFP AAV to mice with either slow or fast forms of retinal degeneration. Transgene expression in cone cells lasted for more than 8 months with no evidence of toxicity. Expression of eNpHR-EYFP and subsequent application of light resulted in large and sustained photocurrents that were faster than those in wild-type cones. In addi-

tion, the cones successfully transmitted current to downstream ganglion cells, and spatial processing features (e.g., lateral inhibition, directional selectivity for motion) remained intact. In light-dark box and optomotor reflex tests, eNpHR-expressing mice performed better than control mice, indicating that eNpHR-mediated photocurrent can modulate visually evoked behaviors and suggesting that higher-order sensory processing remains intact. Finally, Busskamp et al. (2010) treated human ex vivo retinal explants with AAV or lentivirus, successfully expressed eNpHR in human photoreceptors, and induced photocurrents and photovoltages via eNpHR. The authors propose optogenetics as a novel therapeutic approach to treating blind patients who retain cone cell bodies in the central region of the retina.

Other groups have also attempted to rescue blindness in animal models of retinal degeneration. Tomita et al. (2010) expressed Chr2 in retinal ganglion cells of genetically blind adult rats and observed visually evoked potentials and optomotor responses, whereas Zhang et al. (2009b) expressed NpHR or NpHR and Chr2 in inner retinal cells in mice with retinal degeneration and restored off or on-off responses, respectively.

In simultaneously published reports, Sohal et al. (2009) and Cardin et al. (2009) employed optogenetic strategies to investigate the molecular underpinnings of γ rhythm. Both studies used doubly-floxed, inverted open reading frame AAV constructs to target opsins to the forebrain. Sohal et al. (2009) expressed eNpHR-EYFP in parvalbumin-expressing interneurons and Chr2-EYFP in CaMKII α -expressing pyramidal neurons of the frontal cortex. Application of blue light elicited γ oscillations that were phase-locked to the flashes of blue light, whereas yellow light inhibited, but did not eliminate, γ rhythm; yellow light did not affect non- γ LFPs. Sohal et al. (2009) also showed in vitro in acutely prepared slices that direct activation of interneurons with Chr2 resulted in parvalbumin-interneuron firing, γ rhythm (even after nonrhythmic illumination), and inhibition of pyramidal neurons.

Cardin et al. (2009) targeted Chr2-mCherry to parvalbumin-expressing interneurons or CaMKII α -expressing pyramidal neurons in the barrel cortex of adult mice, a region that processes sensory input from the vibrissae (whiskers). In vivo, 1-ms light pulses to the barrel cortex of mice with interneuron Chr2 expression induced inhibitory postsynaptic potentials in pyramidal cells and inhibited the normal pyramidal response to vibrissae deflection; the reversal potentials implicated GABA $_A$ -mediated chloride conductance at the interneuron-pyramidal neuron synapse. To probe γ rhythms in particular, they stimulated at 20 to 80 Hz the barrel cortex in mice with interneuron Chr2 expression and observed an increase in the corresponding γ band on the LFP. On the other hand, an 8-Hz stimulation to mice with pyramidal Chr2 expression increased the power of the θ band on the LFP. It is noteworthy that light-induced γ rhythm

was inhibited by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or *N*-methyl-D-aspartate receptor blockade, suggesting that both the GABAergic interneurons and the glutamatergic pyramidal neurons are necessary to evoke γ rhythms.

The studies we have summarized are just a few examples of the emerging body of literature regarding in vivo applications of optogenetics. The rapidly growing number of reports validates the optogenetic approach and demonstrates that the technical aspects of photostimulation in vivo, although challenging, are not insurmountable.

VI. Conclusions

We have described diverse approaches to experimentally manipulating neuronal signaling. The approaches rely on light, small molecules, peptides, toxins, and other compounds to alter neuronal activity at the levels of neurotransmitter release, membrane potential and electrical excitability, postsynaptic receptors, and second messenger pathways. Although each of these approaches has its advantages and disadvantages (some of which we discuss below), the techniques are largely complementary. Ideally, researchers will soon begin to combine multiple approaches to modulating signaling to determine the interplay between the targeted pathways.

A. Advantages and Disadvantages of the Approaches

1. *Designer G Protein-Coupled Receptors.* The RASSLs and DREADDs, unlike most of the other techniques, directly target G protein signaling pathways and are likely to have extraordinarily diverse effects. These effects range from altering membrane excitability (e.g., G $\beta\gamma$ -mediated opening of GIRK, G $_q$ -mediated closing of PIP $_2$ -gated KCNQ channels) to inducing gene expression (e.g., G $_q$ induction of *c-fos* expression). On the one hand, such a broad spectrum of effects sometimes obscures the precise mechanisms underlying altered neuronal activity. On the other hand, most of the intracellular signaling pathways are well defined, and the induction of such varied effects increases the versatility of this tool.

Direct, noninvasive remote control of GPCR activity is particularly important for translational applications. The majority of drugs approved by the U.S. Food and Drug Administration act through GPCRs, and most prescribed drugs are available as oral formulations. Because many of these techniques for controlling neuronal signaling have as a long-term goal clinical use in humans, in general designer GPCRs are perhaps the most feasible as therapeutics (except for certain treatments, such as DBS).

One undefined aspect of DREADD/RASSL function is their ability to signal via G protein-independent mechanisms (i.e., β -arrestin). As researchers gain an understanding of the importance of β -arrestinergic signaling, functional selectivity becomes particularly relevant (Ur-

ban et al., 2007). Functional selectivity refers to both the ability of a ligand, acting at a given receptor, to preferentially activate different intracellular signal transduction pathways (biased agonism) and more broadly, the ability of a ligand, acting at a given receptor, to have different effects depending on the cell type and available intracellular signaling machinery. For example, both Chang et al. (1998) and Claeysen et al. (2003) described functional selectivity of 5-HT₄^{D100A} receptor ligands. The latter reported that ligands could be antagonists at the wild-type 5-HT₄ receptor but agonists at the D100A receptor, whereas the former group reported that various ligands could couple directly only to G_s or to both G_s and G_q signaling pathways.

Indeed, designer GPCRs provide an ideal method of investigating such differential signaling. First, we can express them in distinct tissues or cell types to determine how activating the same G protein can have different effects, depending on its signaling partners. For example, we hypothesized that the underlying mechanism of CNO-induced seizures in mice expressing hM3Dq in the hippocampus is reduced stability of the membrane potential as a result of activation of the G_q-PLC β -PIP₂ hydrolysis pathway and subsequent closing of PIP₂-gated KCNQ outwardly rectifying potassium channels (Alexander et al., 2009). In other neuronal populations or even subcellular compartments that do not express KCNQ channels, however, activation of the same G_q-PLC β -PIP₂ hydrolysis pathway might effect different alterations in neuronal function. Thus, we should continue to express these receptors in diverse cell types to determine the differential roles of G protein-mediated signaling.

Furthermore, native GPCRs express in distinct subcellular compartments, and their downstream effects depend on local signaling machinery and scaffolds. The postsynaptic density is a nice example of a subcellular compartment that mediates signaling of postsynaptic receptors. Using trafficking signals and signal peptides similar to those already in use for optogenetics (Gradinaru et al., 2007; Gradinaru et al., 2010), we are currently targeting DREADDs to specific subcellular compartments to modulate different intracellular pathways and to improve our ability to study functional selectivity (Dong et al., 2010a). Developing such tools would greatly further our ability to remotely modulate neuronal signaling.

Returning to β -arrestinergic signaling, we do not know the extent to which mutant GPCRs mimic the signaling properties of their wild-type templates. With regard to rM3/ β 1Ds in particular, it would be interesting to know the extent to which it signals through β -arrestin because the β -arrestinergic signaling properties of the β -adrenergic receptors are so well defined, and the second and third intracellular loops of rM3/ β 1Ds are those of the β ₁-AR. Work is under way in our lab to answer this question. Then, we hope to develop CNO analogs or

additional DREADD ligands that exhibit a range of efficacies for both G protein-dependent and -independent signaling. In addition, we can generate DREADDs with mutations or deletions of their intracellular loops that affect their ability to signal via these two mechanisms. Such avenues of research are particularly important given a recent report that the clinical efficacy of antipsychotic drug action might depend on G protein-independent signaling and that ligands that show a bias toward β -arrestinergic signaling might have high potential as antipsychotic treatments (Masri et al., 2008).

With regard to additional DREADD ligands, one disadvantage of second-generation designer GPCRs is that thus far, CNO is their only agonist. Therefore, one cannot express multiple DREADDs (e.g., hM3Dq and hM4Di) in the same cell or animal to bidirectionally modulate the same pathways, as is possible with optogenetics. On the other hand, first-generation designer receptors (RASSLs) are based on multiple native receptors, so different ligands activate the receptors; thus, the same mouse can express, for example, Rs1 and Ro1 in the same cell populations. With a bit of luck, other DREADDs that respond to novel ligands will soon be available to allow such bidirectional modulation within the same animal.

Another problematic aspect of DREADDs is that whereas CNO is an ideal ligand in mice—it is orally bioavailable, blood-brain-barrier-permeant, and does not undergo back-metabolism to clozapine, which has activity at more than 50 neuronal targets—back-metabolism in humans is significant and would prevent the transfer of DREADD technology to humans. Therefore, a nonmetabolized CNO derivative or a novel DREADD ligand is required before DREADDs can move into higher-order animals for preclinical testing. Alternatively, CNO analogs with altered pharmacokinetics (i.e., biodistribution) might permit the translation of DREADD technology to human therapeutics.

Finally, and probably the biggest drawback of designer GPCRs, particularly in comparison with opsins, is the time resolution they afford. Designer GPCRs activate over the course of tens of minutes. Many of the ligands are orally bioavailable and can penetrate the blood-brain barrier, their use thus enabling noninvasive remote control of neuronal signaling. Some users have directly infused the ligands into the brain, thereby reducing the time required to activate the receptors and further increasing the spatial resolution of the technology, but the time resolution still does not approach that of the opsins, and the benefit of a noninvasive approach is lost. Another method of improving the time resolution of CNO, for example, would be to develop a photoactivatable CNO derivative (PA-CNO). One could then treat a live animal with PA-CNO, allow enough time for absorption and distribution, and then use focal light to activate CNO in particular brain regions. This approach would increase both the time and spatial resolution of designer

GPCR technology. However, PA-CNO would no longer provide a noninvasive or nontechnical method of controlling neuronal signaling because an invasive approach would be necessary to introduce the light stimulus necessary to activate the PA-CNO.

On the one hand, GPCRs will never permit the time resolution of opsins. On the other hand, they might not need to. Signaling through GPCRs is notably slower than that through ion channels, but is no less important for normal cellular function. Although ion channels serve to rapidly transmit encoded information, GPCRs serve to amplify and diversify that transmission. Therefore, these two tools truly are complementary and equally valuable. Moreover, as we mentioned previously, most approved drugs act through GPCRs, so altering cellular function over a longer period clearly can be therapeutic.

2. Opsins. The numerous advantages of optogenetics are evident from the plethora of *in vivo* studies already reported in multiple species (see Table 2). Optogenetics, more than any other technology reviewed in this article, enables ultrafast millisecond time scale dissection of neural circuitry. Only optical approaches can replicate the kind of endogenous oscillatory activity seen in neurons. Although this ability is paramount for high-frequency DBS to alleviate PD symptoms (Gradinaru et al., 2009), whether such a high degree of temporal control is necessary for other applications is unclear. For example, some endogenous substances (e.g., hormones) travel a significant distance from their site of synthesis and release to their site of action, where they alter transcription. Extreme temporal precision with regard to such pathways is likely unnecessary.

Another advantage of the optical approaches is that they act via a simple mechanism: opening of a channel or pump to allow ion flow. In contrast to GPCRs, which directly activate G_{α} , $G\beta\gamma$, and β -arrestin, each with its own downstream effects, altering ion flow and therefore membrane potential is the only direct action of opsins. This well defined mechanism of action simplifies data interpretation and analysis. Some intracellular signaling pathways are accessible through vertebrate rhodopsin, melanopsin (Lin et al., 2008), and the opsin-receptor chimeras, but these approaches are unlikely to enable investigations into biased agonism or other forms of functional selectivity.

Finally, the optogenetic approach is the most specific. In mammals, the only light-sensitive endogenous receptors are in the retina, so photostimulation of the brain induces only opsin-mediated effects. Moreover, although light scatters as it penetrates tissue, there are no pharmacokinetic concerns with light as a ligand as there are for CNO and other metabolized compounds. In addition, some of the newer technical developments enable illumination of individual neuronal compartments (e.g., dendrites), providing an incredible degree of spatial resolution. With DREADDs/RASSLs, such spatial resolution

is possible only through photocaging of ligands or through targeting of receptor expression to those compartments. In the latter case, activation of individual neurons is possible but technically challenging.

Photostimulation presents a few challenges, particularly in conjunction with recording of electrical activity. First, light that is too strong or is applied for too long can cause tissue damage, abnormal neuronal activity, and excitotoxicity (Cardin et al., 2010). One can minimize these effects by using lower light intensity or shorter pulses instead of continuous illumination, but between the light scatter and minimizing the light intensity, it might be difficult to sufficiently target deep brain structures without using a stereotaxic approach to photostimulation. In addition, high levels of opsin expression are typically necessary to generate sufficient current; trafficking signals and other modifications improve surface expression and even maximal current flow to lessen this problem. Finally, electrical artifacts are common sequelae of the light source illuminating metal recording electrodes. In a recent protocol article, Cardin et al. (2010) describe several approaches to minimizing such artifacts, including using opaque glass electrodes, minimizing exposed metal, and using thin wire stereotrodes adjusted so that minimal light directly reaches them.

B. Combining the Systems

No one has yet combined optical and GPCR-based approaches to remote control of neuronal signaling. Such combinations of approaches would answer some of the questions about whether the precise timing and pattern of electrical activity or the intracellular second-messenger pathways are more important in certain contexts. The role of γ rhythm in cortical inhibition and psychosis presents a nice example of how these techniques can work together, because both hM3Dq and ChR2 can stimulate γ rhythm (Alexander et al., 2009; Cardin et al., 2009; Sohal et al., 2009). It would be interesting to use the viral self-cleaving 2A peptide sequence to equally express both hM3Dq and ChR2 or hM4Di and eNpHR under the parvalbumin or CaMKII α promoters and to compare the effects of electrical versus pharmacological stimulation and inhibition of each cell type. Then, one could induce γ rhythm electrically and pharmacologically in a mouse model of psychosis (e.g., phencyclidine-induced hyperlocomotion) and determine the direct effects of induction or inhibition of γ rhythm on psychotic behaviors and whether the two types of stimulation have differential effects on those behaviors. In this way, one could truly identify different signaling components that are necessary and/or sufficient for generating γ rhythm and improving psychotic symptoms.

The TRPV1 approach provides another opportunity for combining two techniques. Early studies used photocaged capsaicin to provide high spatiotemporal resolution (Zemelman et al., 2003; Lima and Miesenböck,

2005); several years later, Arenkiel et al. (2008) elicited stereotypies with uncaged capsaicin. The stereotypies occurred within 5 to 15 min after capsaicin infusion. Using caged capsaicin in vivo would improve the temporal precision of this approach. Local infusion of capsaicin is already invasive and technically more demanding than peripheral drug administration, so adding photostimulation to the experimental design should not be prohibitively challenging.

C. Concluding Thoughts

Techniques for experimental manipulation of neuronal signaling have improved phenomenally in the last decade, and we now have the ability to directly alter neuronal activity via several different mechanisms. Researchers in other fields are taking similar approaches to remotely modifying particular intracellular activities such as calcium channel activity [via a MIST-like dimerization approach (Yang et al., 2007)], kinase activity, and others. In a short time, researchers might be able to remotely control the level and/or direction of activity of many cellular proteins and functions. A DREADD-like receptor tyrosine kinase or a chimera of an opsin and a receptor tyrosine kinase would be a nice addition to the receptor toolbox.

We have described the translational potential of these techniques. In particular, the retina is well suited to clinical applications because the vitreous humor is easily accessible for viral injections, and viral gene therapy for blindness has already shown success in clinical trials. The main obstacle to translation of these techniques to human therapy, however, remains the ability to safely, reliably, and stably express an exogenous protein in human tissue. Until such gene therapy is routine, it will be difficult to fully realize the clinical potential of these techniques. Nonetheless, currently they are excellent tools for improving our understanding of the biological basis of behavior.

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