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Review

Bridging the Gaps between Synapses, Circuits, and Behavior

Pamela M. England[1,*]
[1]Departments of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94158, USA
*Correspondence: england@picasso.ucsf.edu
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The decade of the brain may have come and gone, but the final frontier, cracking the neuronal code, still lies ahead. Today, new technologies that allow precise spatiotemporal remote control over the activity of genetically defined populations of neurons within intact neural circuits are providing a means of obtaining a functional wiring diagram of the mammalian brain, bringing us one step closer to understanding precisely how neuronal activity codes for perception, thought, emotion, and action. These technologies and the design principles underlying them are reviewed herein.

Introduction

In 1990, the U.S. Congress designated the 1990s the "Decade of the Brain," and then-sitting president George H.W. Bush proclaimed that "a new era of discovery is dawning in brain research." In the ensuing years, our understanding of the mammalian brain greatly advanced. At the level of individual neurons, tremendous progress was made identifying the molecular mechanisms and components underlying signaling within and between neurons (Sudhof and Malenka, 2008). At the same time, functional imaging technologies were used to correlate the activity of specific brain regions with particular brain functions (Haber and Knutson, 2010; Maguire, 2001). Despite these advances, the relationship between the activity of individual neurons and higher brain function remained poorly understood. Today, new tools are being developed that will build a bridge between the activity of individual neurons and the behavior of whole animals by providing remote control over the functional neuronal circuitry that lies between the two.

These bridging technologies rely on a combination of genetic engineering, to target functionally circumscribed populations of neurons, and light and/or chemicals, to rapidly control the activity of those neurons. Genetic strategies for targeting specific neurons have recently been reviewed elsewhere (Bernard et al., 2009; Luan and White, 2007; Luo et al., 2008). Briefly, DNA sequences (promoters) unique to the desired population of neurons are used to drive the expression of exogenous proteins in those neurons. Inspired by key control points in neuronal signaling, these exogenous proteins are designed to place the neuronal circuitry governing behavior under the control of exogenous light and/or chemicals. These control points, and several of the technologies developed to rapidly and reversibly manipulate them, are described in the following sections.

Key Control Points in Neuronal Signaling

A typical neuron is comprised of a cell body (soma), many short processes called dendrites that receive information from other neighboring cells, and one long process with many branches, called the axon, that transmits information to other neurons (Figure 1). Signaling along and between neurons depends critically on the potential of the membrane, the release of neurotransmitter, and the subsequent activation of postsynaptic receptors.

Membrane Potential

All neurons maintain a voltage difference across the plasma membrane known as the membrane potential. This membrane typically rests at –70 mV, meaning the voltage inside the cell is 70 mV more negative than the outside of the cell. This polarization is accomplished by ion channels and transporters that move Na^+ ions out of the cell and K^+ ions into the cell (creating a concentration gradient for these ions). Excitatory and inhibitory inputs to a neuron cause the membrane potential to rise (depolarize) or fall (hyperpolarize), respectively. If enough depolarization accumulates to bring the membrane potential to approximately –55 mV (threshold potential), a pulse of electricity called the action potential is passed from the cell body, along the axon, to the end of the neuron. At the biophysical level, action potentials result from the coordinated opening and closing of voltage-gated Na^+ and K^+ channels. When the membrane potential crosses the threshold potential, voltage-gated Na^+ channels open, Na^+ rushes down its concentration gradient into the cell, and the membrane potential abruptly shoots upward, typically reaching a peak at approximately +40 mV. The opening of Na^+ channels is followed by the opening of voltage-gated K^+ channels that permit the exit of K^+ out of the cell. Sodium channels close at the peak of the action potential, while K^+ continues to leave the cell. The continued efflux of K^+ briefly hyperpolarizes the cell (–80 mV) before the K^+ channels close to permit the cell to return to the resting membrane potential (–70 mV). The size and shape of the action potential does not vary, regardless of the magnitude of the stimulus that caused it. Rather, axonal information contained in the stimulus intensity is coded in the frequency of successive action potentials, which can range from about 1 to 100 per second.

Neurotransmitter Release

Axonal signals arriving at the synapse in the form of action potentials are transformed by the synapse into a chemical signal. When the action potential reaches the end of the axon (nerve terminal), the associated change in membrane potential induces the opening of voltage-gated calcium channels. The subsequent influx of Ca^{+2} ions into the cell causes vesicles containing

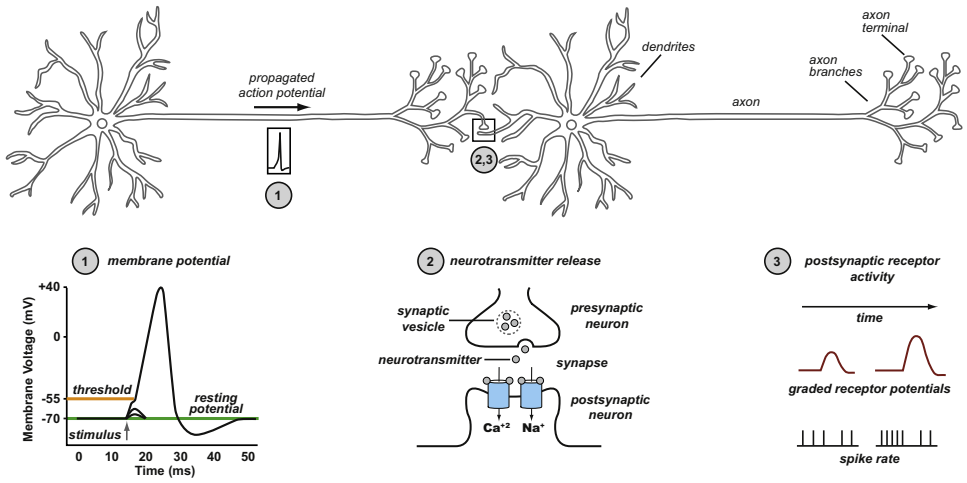

Figure 1. Cartoon Depicting Synaptic Transmission in a Typical Neuron and the Three Control Points Used to Manipulate This Signaling
Action potentials are generated in the presynaptic neuron when the membrane potential is depolarized to a threshold potential. At the nerve terminal, this electrical signal drives the release of neurotransmitter from synaptic vesicles. Binding of the neurotransmitter to ligand-gated ion channels on the postsynaptic membrane induces graded postsynaptic receptor potentials that may sum to depolarize the membrane to its threshold value and, thereby, generate action potentials at various frequencies.

neurotransmitter (synaptic vesicles) to fuse with the cell membrane and release neurotransmitter into the synaptic cleft, the space between the axon terminal and the dendrite of another neuron.

Postsynaptic Receptor Activity

The frequency of action potentials arriving at the nerve terminal determines the concentration of neurotransmitter in the synaptic cleft, and postsynaptic neurotransmitter-gated receptors compute this information. Neurotransmitters released by the axon terminal rapidly diffuse (0.3 to 1.0 ms) across the synaptic cleft to the postsynaptic membrane. Subsequent opening of neurotransmitter-gated receptors allows the flow of ions into the cell, which produces a small change in the membrane potential called a postsynaptic potential. Glutamate, the principal excitatory neurotransmitter in the mammalian brain, causes postsynaptic AMPA receptors to open, and the resulting influx of sodium ions into the cell depolarizes the membrane and produces an excitatory postsynaptic potential (EPSP). GABA, the principal inhibitory neurotransmitter causes postsynaptic $GABA_A$ receptors to open, and the resulting flow of Cl^- ions into the cell hyperpolarizes the membrane and produces an inhibitory postsynaptic potential (IPSP). Each axon has multiple branches, forming synapses with many dendrites, and each dendrite may receive inputs from multiple axons. The summed activity of postsynaptic receptors across multiple synapses may cause the membrane to reach threshold and fire an action potential, depending on the nature (EPSP versus IPSP) and sequence of postsynaptic events.

The following sections discuss how these three main control points (membrane potential, neurotransmitter release, postsynaptic receptor activity), combined with the selective activation of genetically targeted exogenous proteins using light and/or chemicals (Figure 2), are being used to provide precise control over neuronal activity. The technologies discussed are limited to those that have proven to provide reversible control over the behavior of intact organisms.

Controlling Membrane Potential

Various portable systems for reversibly controlling the membrane potential of neurons in vivo have been developed. Some strategies rely on components of metabotropic signaling cascades to stimulate or inhibit the firing of action potentials, whereas others use ion channels and pumps that are directly gated by light and/or chemicals. These approaches are discussed below.

chARGe/Retinal

One of the first portable systems developed to provide rapid, reversible remote control over membrane potential used three components of the fly visual system to transduce an optical stimulus into neural activity (Zemelman et al., 2002). The three components—arrestin, rhodopsin (opsin complexed with retinal), and the alpha subunit of cognate heterotrimeric G-protein—comprise what is termed the chARGe system. In this system, the light-gated generation of membrane depolarization begins with the absorption of light by rhodopsin (430–550 nm absorption peak), which triggers the 11-*cis* to all-*trans* photoisomerization of bound retinal. The resulting activated rhodopsin catalyzes the exchange of GDP for GTP on the G-protein alpha subunit, which then dissociates and activates phospholipase C. This leads to the opening of two classes of endogenous calcium-permeable channels, the transient receptor potential (TRP) and TRP-like (TRPL) channels, which depolarize the membrane. Binding of arrestin inactivates the rhodopsin, and absorption of lower energy light (~580 nm) isomerizes bound all-*trans* retinal to 11-*cis* retinal and releases the regenerated rhodopsin from its complex with arrestin.

Ectopic expression of the chARGe system in cultured hippocampal neurons, with alternating periods of light and darkness, produced alternating episodes of electrical activity (peak firing frequency of 7.5 Hz) and quiescence. However, action potentials appeared and disappeared with unpredictable frequencies and lag periods (hundreds of milliseconds to tens of seconds) after the light stimulus was applied and removed. The normally tight

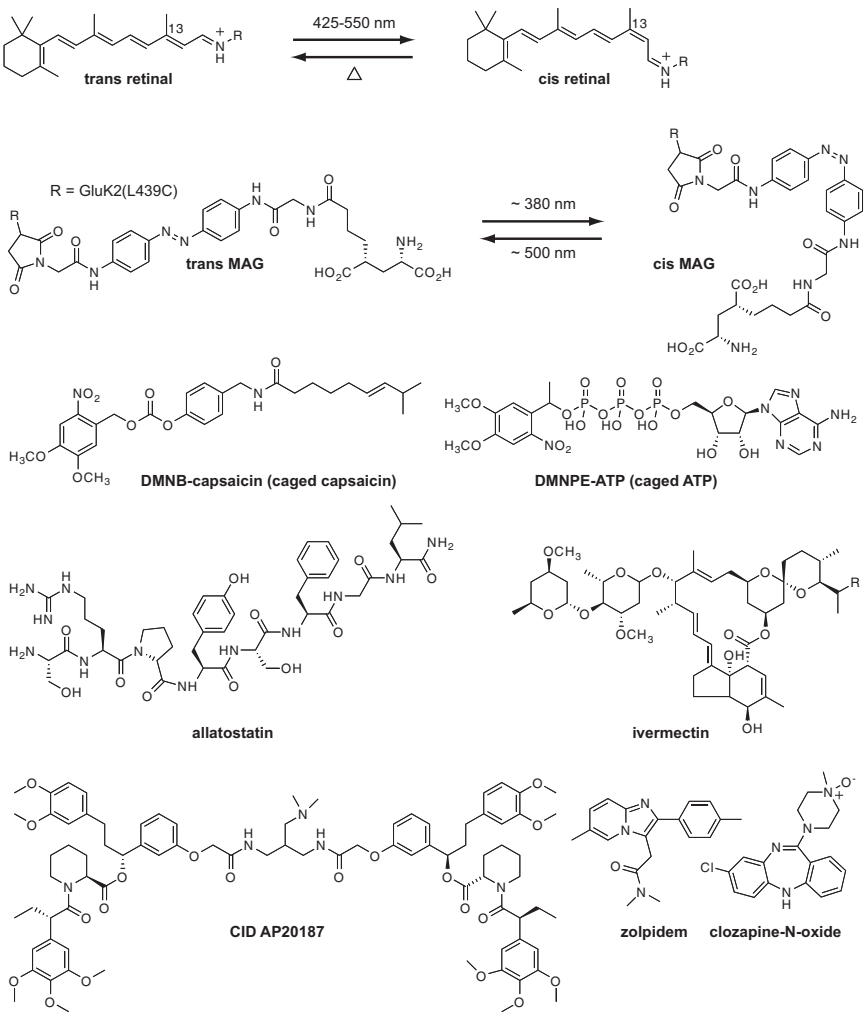

Figure 2. Structures of the Chemicals Used in Combination with Ectopically Expressed Proteins to Manipulate the Activity of Neurons

The light-activated isomerization of all-*trans* retinal to *cis* retinal is used to gate the cation channel ChR2 and the anion pump NpHR. Structure shown is the Schiff's base formed between retinal and a lysine residue in the protein. The light-gated isomerization of MAG, a cysteine reactive azobenzene-tethered glutamate analog, is used to gate the glutamate-gated ion channel GluK2. The light-activated uncaging of caged ATP and caged capsaicin are used to gate $P2X_2$ and capsaicin (TRPV1) receptors, respectively. The octapeptide allatostatin activates the allatostatin receptor. Ivermectin selectively activates a glutamate-gated chloride channel. The synthetic ligand AP20187 induces the homodimerization of proteins containing the FKBP ligand-binding domain. AL, allatostatin; ATP, adenosine-5′-triphosphate; CID, chemical inducer of dimerization; CNO, clozapine-N-oxide; DMNB-capsaicin, 4,5-dimethoxy-2-nitrobenzyl-capsaicin; DMNPE-ATP, P^3-[1-(4,5-dimethoxy-2-nitrophenyl)ethyl]-ATP; IVM, ivermectin; MAG, cysteine-reactive maleimide, photoisomerizable azobenze, glutamate-based agonist; MIST, molecular systems for inactivation of synaptic transmission.

coupling between stimulus intensity and response frequency characteristic of the native *Drosophila* photoreceptor system is apparently relaxed in the transplanted three-component chARGe system. Although this system would not prove to be of practical value in intact animals, it nonetheless demonstrated that synthetic neuronal signals could be inserted into neurons and introduced the concept of achieving specificity by controlling susceptibility to a stimulus, rather than the stimulus itself.

$P2X_2$/ATP

Following the development of the chARGe system, it was recognized that using ionotropic receptors for selective control over designated populations of neurons would overcome many of the limitations associated with using metabotropic receptors. Instead of relying on a metabotropic cascade to trigger the activity of an ion channel and transduce the signal, the excitatory stimulus could be directly transduced by an orthogonal ion channel, thus accelerating the response kinetics. Two ion channels used for this purpose are the ATP-gated $P2X_2$ receptor and the capsaicin-gated TRPV1 receptor (Claridge-Chang et al., 2009; Lima and Miesenbock, 2005; Miesenbock and Kevrekidis, 2005; Zemelman et al., 2003). Although these nonselective cation channels are primarily expressed in the peripheral nervous system of mammals, they are also present in the central nervous system, somewhat limiting the utility of these systems in such animals.

Heterologous expression of either $P2X_2$ or TRPV1 channels in cultured hippocampal neurons, followed by application of the cognate agonist, led to brief bursts of action potentials at frequencies as high as 40 Hz (Zemelman et al., 2003). The current amplitudes and firing frequencies could be tuned by varying the concentration of agonist, though dose-response relationships were complicated. The high firing rates were not sustainable over extended periods of time, however; high-frequency activity was followed by long-lasting plateaus, during which the membrane remained depolarized but action potentials were absent. These limitations associated with pharmacologically activating the receptors were overcome by delivering pulses of agonist using caged ATP and caged capsaicin combined with flashes of light. In particular, ATP and capsaicin were photochemically liberated from DMNPE-ATP and DMNB-capsaicin with near-UV wavelength light. Flashes (1 s) of unfocused light successfully triggered the firing of action potentials at frequencies of 15–40 Hz. The onset of activity lagged behind the optical stimulus (~5 s with TRPV1, ~1 s with $P2X_2$), and lasted slightly longer than the light exposure (~2.6 s with TRPV1, 2.4 s with $P2X_2$). Additional studies have further characterized the ability of TRPV1 and a related channel, TRPM8, to drive action potentials in cultured neurons (Crawford et al., 2009).

The $P2X_2$/ATP system was subsequently introduced into the *Drosophila* central nervous system (which lacks purinoceptors) where it was used to evoke action potentials and drive behaviors in the intact fly (Claridge-Chang et al., 2009; Lima and Miesenbock, 2005). Specifically, $P2X_2$ receptors were ectopically

expressed in small subsets of central neurons (representing just ~0.05% of the total CNS population), and caged ATP was microinjected through the eye into the brain. Brief illumination (150–250 ms) drove escape movements, including flying. The efficacy of photostimulation declined with a half-life of ~75 min after the injection of caged ATP, reflecting the clearance of the caged compound from the CNS. Ectopic expression of TRPV1 in nociceptor neurons of *Caenorhabditis elegans* combined with capsaicin application was used to elicit "synthetic" avoidance behaviors that are lacking in wild-type animals (Tobin et al., 2002).

ChR2/Retinal

The most widely used system thus far developed for remotely controlling membrane potential combines a light sensor and an ionic conductance within one protein (Boyden et al., 2005). The protein is a light-gated cation channel called channel rhodopsin 2 (ChR2) derived from the green alga *Chlamydomonas reinhardtii* (Nagel et al., 2003; Sineshchekov et al., 2002; Suzuki et al., 2003). The photosensor is a molecule of all-*trans* retinal bound to the protein core. Illumination with blue (~470 nm) light causes the retinal to isomerize, inducing the channel to open. The resulting influx of sodium ions into the cell depolarizes the membrane. The ability of ChR2 to drive action potentials was initially characterized in cultured hippocampal neurons (Boyden et al., 2005). Photostimulation drove defined trains of action potentials with frequencies ranging as high as 30 Hz, or subthreshold synaptic events, depending on the light intensity. The delay between the light flash and the beginning of the photocurrent is <50 μs and, although the channel inactivates with sustained light, it rapidly recovers in the dark (tau ~5 s) due to the reisomerization of *cis*-retinal to the all-*trans* ground state. Thus, multiple pulses of blue light, interspersed by periods of darkness, could be used to drive action potentials at physiological frequencies with millisecond temporal resolution. A drawback to ChR2 is that the number of channels that needs to be localized to the plasma membrane in order to successfully drive action potentials is rather high (100,000 to 1 million) owing to the low conductance (~50 femtosiemans) of the channel.

ChR2 has been used in vivo in several organisms, including zebrafish (Douglass et al., 2008), worms (Nagel et al., 2005), flies (Schroll et al., 2006), mice (Bi et al., 2006; Petreanu et al., 2007; Wang et al., 2007), rats (Abbott et al., 2009), and monkeys (Han et al., 2009), to investigate how brain states and behaviors are generated by neural circuits. Notably, the endogenous expression of retinal in mammals eliminates the need to introduce the ligand exogenously. In addition to ChR2, several other microbial opsin-based tools are being developed to interrogate the organization and function of neuronal circuits, including a yellow-light-sensitive cation channel (VchR1) (Zhang et al., 2008), a yellow-light-sensitive proton pump (Arch) (Chow et al., 2010), a blue-light-sensitive proton pump (Mac) (Chow et al., 2010), and a blue-light-sensitive chloride pump (NphR, vide infra) (Zhang et al., 2007). Several reviews of these opsins are available (Gradinaru et al., 2007; Gunaydin et al., 2010; Zhang et al., 2010).

GluCl/IVM

Paralleling the development of portable remote-controlled systems for exciting neuronal activity has been the development of systems for reversibly silencing the activity of specific neurons. One strategy developed for this purpose uses pharmacological manipulation of a glutamate-gated chloride channel (GluCl) derived from *Caenorhabditis elegans* (Lerchner et al., 2007; Slimko and Lester, 2003; Slimko et al., 2002). Glutamate-gated chloride channels are found in several invertebrate phyla, but not in mammals, and are the targets for various antiparasitic drugs, such as ivermectin (IVM). Thus, targeted expression of GluCl in mammalian neurons, combined with pharmacological activation with IVM, could be used to hyperpolarize neurons and, thereby, inhibit the generation of action potentials. Indeed, in hippocampal neurons expressing GluCl, robust chloride currents were induced in response to IVM, and the membrane potential was fixed at values ranging between –50 and –65 mV. The time to activate the chloride current was dose dependent (5–500 nM IVM), with the fastest time constant (6 s) observed at the highest concentration. The silencing effect of IVM reversed following washout of the drug, but required several hours (~8 hr).

The GluCl/IVM system later proved to be an effective strategy for manipulating the behavior of awake-behaving mice (Lerchner et al., 2007). The two subunits of GluCl receptor (α and β) were expressed in the mouse striatum, and IVM was introduced intraperitoneally. Behavioral effects were observed 4 hr after a single i.p. injection of IVM and reached maximal levels by 12 hr. The effects were almost fully reversed by 4 days and multiple cycles of silencing and recovery in a single animal were demonstrated. Recently a modified human alpha1 glycine receptor (GlyR) was reported as an improved ivermectin-gated silencing receptor (Lynagh and Lynch, 2010). Like GluCl, gating of the glycine receptor by ivermectin produces an inward chloride flux that hyperpolarizes the membrane. The crucial improvement is that the sensitivity of the receptor to glycine, an endogenous neurotransmitter, was eliminated though the introduction of a single point mutation (F207A). The sensitivity of GlyR to ivermectin was rendered similar to that of GluCl through the introduction of a second point mutation (A288G) in the receptor.

AlstR/AL

Another system developed for silencing neuronal activity in genetically defined populations of neurons uses the allatostatin (AlstR) receptor, a G protein-coupled receptor from *Drosophila*, and its cognate ligand allatostatin (AL), an octapeptide (Birgul et al., 1999; Lechner et al., 2002; Luo et al., 2008; Tan et al., 2006; Wehr et al., 2009). Although the AlstR is structurally related to the mammalian somatostatin/galanin/opiod receptor family, there is no similarity between AL and mammalian peptides such as somatostatin, galanin, or enkephalins. Thus, the activity of the AlstR can be solely controlled by AL, which has a very high apparent affinity (EC_{50} of 55 pM) for the receptor. Stimulation of the AlstR leads to activation of G protein-gated inwardly rectifying potassium channels (GIRKs) at subthreshold voltages, via free G-protein βγ subunits. The resulting efflux of K^+ ions hyperpolarizes the membrane, thus preventing the firing of action potentials. The AlstR/AL receptor/ligand system has been used to silence activity of cortical neurons quickly and reversibly. In cortical slices from ferrets ectopically expressing the AlstR, application of AL inhibited the firing of action potentials within minutes, and the effect reversed within 15 min upon washout of the ligand (Lechner et al., 2002).

The utility of the AlstR/AL system was subsequently demonstrated in vivo in cortical and thalamic neurons of mice, rats, ferrets, and monkeys (Tan et al., 2006; Wehr et al., 2009). AL

was applied to anesthetized animals directly onto the cortical surface (exposed by removing a portion of the skull). Neurons inactivated within seconds or minutes (depending on the preparation) upon application of AL and recovered within minutes of saline washout.

RASSL/CNO

Another approach for controlling the activity of neurons uses G protein-coupled receptors that have been reengineered such that they are exclusively activated by synthetic ligands possessing no biological activity (Conklin et al., 2008). In particular, a family of muscarinic acetylcholine receptors (mAChRs) that are potently activated by the pharmacologically inert compound clozapine-N-oxide (CNO), but not by the native ligand acetylcholine (ACh), were developed using directed molecular evolution (Alexander et al., 2009; Armbruster et al., 2007). Double mutation of the human M3 receptor (Y149C$^{3.33}$ and A239G$^{5.46}$) yielded a GPCR that is potently (~20–30 nM) activated by CNO, with a sever reduction (>40,000-fold) in ACh potency compared with wild-type receptors. Activation with CNO of similarly mutated human M4 receptors, which couple to $G_{i/o}$ and can induce the activation of GIRKs, was shown to silence cultured hippocampal neurons (Armbruster et al., 2007).

In transgenic mice expressing the mutated M3 receptors in forebrain principal neurons, peripheral administration of CNO (which readily crosses the blood–brain barrier) produced changes in the activity of hippocampal neurons and in the behavior of whole animals. Specifically, activation of these M3 receptors, which couple to G_q and stimulate phospholipase C, increased the firing rate of hippocampal interneurons and, in hippocampal slices isolated from these animals, increased the firing rate of excitatory neurons (CA1 pyramidal cells). At low doses, CNO increased the activity (locomotion, episodes of beam breaks) of the mice, and at higher doses evoked limbic seizures.

NpHR/Retinal

Halorhodopsin (NpHR) is a light-gated chloride pump from the archaebacterium *Natronomonas pharaonis* (Kalaidzidis et al., 1998; Schobert and Lanyi, 1982). This seven-transmembrane protein, which has homology to the light-driven proton pump bacteriorhodopsin, uses all-*trans* retinal as the photosensor. Illumination with yellow (~570 nm) light causes the retinal to isomerize, activating the pump. The resulting influx of chloride ions into the cell hyperpolarizes the membrane, thus suppressing the firing of action potentials. NpHR silencing has been demonstrated electrophysiologically in cultured neurons (Zhang et al., 2007; Zhao et al., 2008). Illumination with yellow light induced rapid currents on the millisecond timescale (~26 ms delay from light onset to 50% of the hyperpolarization peak). The pump current exhibited a linear voltage dependence and was active across the entire physiological voltage range.

NpHR has also been successfully used in vivo to paralyze worms (Zhang et al., 2007) and to control the swimming of zebrafish (Arrenberg et al., 2009). Used in combination with ChR2 (vide supra), NpHR enables multiple-color optical activation, silencing, and desynchronization of neural activity (Zhang et al., 2007).

Controlling Neurotransmitter Release

Two systems have been developed for inhibiting neurotransmitter release in genetically specified populations of neurons in vivo. One method uses the inducible expression of tetanus toxin light chain (TNT), which cleaves VAMP2/synaptobrevin, a synaptic vesicle protein required for the release of neurotransmitters (Kobayashi et al., 2008; Nakashiba et al., 2008). Although this system can be used in vivo, the transcriptional induction has a relatively slow onset of 14 days, and recovery only occurs when the cell resynthesizes VAMP2/synaptobrevin (Nakashiba et al., 2008). A second approach uses a small molecule-induced crosslinking of genetically modified forms of VAMP2/synaptobrevin to rapidly and reversibly inhibit neurotransmission and is described below.

MIST/CID

The dimerization of proteins is a general control mechanism in biological systems, which contributes to the activation of cell membrane receptors, transcription factors, vesicle fusion proteins, and other classes of proteins. In the 1990s, Schreiber and colleagues developed cell-permeant synthetic ligands to reversibly control the intracellular dimerization of modified intracellular proteins (Rosen and Schreiber, 1992; Schreiber, 1991). MIST, an acronym for molecular systems for inactivation of synaptic transmission, uses this approach to enforce nonproductive interactions between vesicle proteins (Karpova et al., 2005; Tervo and Karpova, 2007). By sequestering essential components of the neurotransmitter release machinery in this way, the release of neurotransmitter is inhibited and the communication between neurons is silenced. The method involves fusing the ligand-binding domain of the immuophilin FKBP to the intracellular domain of the target protein. Introduction of a dimeric immunophilin ligand (e.g., AP20187), a so-called chemical inducer of dimerization (CID), then enforces the homodimerization of that target protein. Heterodimerization can also be accomplished using an additional immunophilin ligand-binding domain. This approach was used to homodimerize the synaptic vesicle protein VAMP2/synaptobrevin. Expression of the VAMP2/synaptobrevin-FKBP binding domain fusion in hippocampal neurons, combined with application of the CID produced rapid, specific, and reversible inhibition of synaptic transmission. Postsynaptic currents were inhibited on the minute timescale and the level of block remained constant after several hours in the presence of the dimerizer. Inactivation was completely reversed after 1 hr in medium lacking the CID.

The MIST system was further tested in vivo using transgenic mice expressing the VAMP/synaptobrevin-FKBP fusion in Purkinje neurons. Introduction of the CID by direct injection into the brain led to deficits in motor learning and balance that reversed within 48 hr. A disadvantage of this approach is that the postsynaptic targets of the manipulated presynaptic neurons must already be known in order to validate silencing electrophysiologically.

Controlling Postsynaptic Receptors

Three approaches have been developed for controlling the activity of postsynaptic receptors in genetically specified neurons in vivo. One method uses the expression of peptide neurotoxins tethered to the surface of the neuron to inhibit target receptors (Auer et al., 2010; Ibanez-Tallon et al., 2004; Stürzebecher et al., 2010). However, as this system is controlled at the level of transcription, it is effectively irreversible. A second system uses the expression of genetically modified receptors

that are activated and inactivated through the light-induced isomerization of a tethered agonist. The third approach uses the expression of $GABA_A$ receptors that are uniquely sensitive to the agonist zolpidem in targeted neurons. The latter two approaches are discussed in further detail below.

LiGluR/MAG

LiGluR is a cation-selective glutamate-gated ion channel engineered to respond to light through a covalently attached analog of glutamate containing a photoisomerizable azobenzene moiety (Gorostiza and Isacoff, 2008; Szobota et al., 2007; Volgraf et al., 2006). An early version of an optically controlled ligand-gated ion channel was reported in 1980 in the form of a nicotinic acetylcholine receptor that could be photoactivated via a tethered choline analog (Lester et al., 1980). In both cases, light controls the *cis*/*trans* isomerization of the azobenzene tether and thus the binding and unbinding of the agonist to the receptor. LiGluR is generated by attaching the thiophilic photoswitchable agonist MAG (cysteine-reactive maleimide, photoisomerizable azobenze, glutamate-based agonist) to an engineered cysteine residue (L439C) located near the ligand-binding domain of GluK2 (a kainite-type glutamate-gated channel). The *cis* conformation of the azobenzene, which positions the tethered agonist within the ligand-binding domain, is favored at 380 nm, whereas the *trans* conformation, which directs the agonist away from the binding site, is favored at 500 nm. Azobenzenes also thermally isomerize to the lower energy *trans* state in the dark (*cis* state $t_{1/2}$ = 17.65 min). This thermal bistability of the MAG allows sustained activation following a brief pulse of light, which may be advantageous in behavioral experiments. In cultured hippocampal neurons expressing LiGluR and treated with MAG, brief (1–5 ms) pulses of 374-nm light triggered reproducible patterns of action potentials at frequencies up to 50 Hz that persisted for minutes in the dark until extinguished by a short pulse of 488-nm light. The amplitude of the responses could also be reduced to induce EPSP-like depolarizations that mimic synaptically evoked EPSPs by attenuating illumination intensity. These subthreshold depolarizations were reliably evoked at frequencies as high as 100 Hz.

The LiGluR system has successfully been used in vivo to control the behavior of zebrafish. GluK2(L439C) was introduced into sensory neurons in zebrafish larvae, and the receptor was labeled with MAG upon incubation of the larvae (125 μM MAG in 5% DMSO) for 30 min. Illumination of the larvae with low-intensity 365-nm light for 15 min using a handheld UV lamp inhibited the animal's touch response, an escape response evoked by mechanical pressure. The touch response was restored by illumination with 488-nm light for 30 s. When more intense UV light was used, the touch response was reversibly blocked by 10 s of illumination. A drawback to this approach is that a reactive extracellular cysteine is required to attach the photoisomerizable ligand to the receptor. In most cases this cysteine will need to be engineered into the receptor, and the appropriate location for activating the receptor will have to be identified by trial and error. The design of LiGluR was guided by extensive structure-activity relationship analyses that had been performed on ionic glutamate receptor agonists and an X-ray structure of the ligand-binding domain of GluK2 in complex with the agonist (2S,4R)-4-methyl glutamate. An unexplored drawback of this method is the cross-reactivity of the ligand with cysteine residues on off-target proteins, potentially altering the functioning of those proteins.

$GABA_A$(F77I)/Zolpidem

Another strategy for manipulating the propagation of impulses within neuronal circuits is to control the activity of postsynaptic $GABA_A$ receptors. These ligand-gated chloride channels are present on virtually all neurons in the mammalian brain and when activated prevent membrane depolarization. $GABA_A$ receptors are pentameric structures most commonly comprised of two α and two β subunits, of which there are several variants, and a single γ subunit (γ2). The γ2 subunit confers sensitivity to zolpidem, an allosteric modulator that increases the function of $GABA_A$ receptors. When Phe77 of the γ2 subunit is mutated to isoleucine, the receptors function normally with respect to activation by GABA, but they are no longer modulated by zolpidem (Buhr et al., 1997; Wingrove et al., 1997). Pharmacological control over genetically defined populations of neurons could, therefore, be obtained by first generating knockin mice in which the wild-type (Phe77) γ2 subunit is universally replaced by the zolpidem-insensitive (Ile77) γ2 subunit, and then selectively putting the wild-type subunit back into targeted neuronal populations (Cope et al., 2004). The power of this method was first demonstrated by expressing the γ2F771I subunit in the cerebellum of transgenic mice (Wulff et al., 2007). In these mice, zolpidem, which effectively crosses the blood–brain barrier, rapidly induced significant motor deficits and revealed how interneurons regulate cerebellum-dependent behavior.

The Ideal System and Future Directions

The next decade of brain research will likely see improved iterations of existing systems, as well as the development of new strategies, for controlling the activity of neurons. A number of features are important to consider when choosing an existing system, or designing a new one.

Genetic Encoding

Every region of the brain contains complex circuits made up of many different kinds of neurons. Deciphering the connectivity and dynamics of those circuits requires being able to target each type of neuron individually. Methods that are genetic in nature allow targeting of functionally circumscribed, rather than anatomically defined, populations of neurons. Systems that rely on the expression of only one transgene simplify the creation of genetically modified cells, tissues, and organisms, and obviate the need to balance the relative expression levels of more than one gene. However, it is unlikely that unique promoters for every neuron subtype will be identified. Therefore, approaches requiring the expression of multiple proteins (e.g., the GluCl/IVM system), and that use multiple promoters with partially overlapping specificities, may ultimately be more effective at targeting specific types of neurons.

Orthogonality

The nervous system is incredibly plastic, and as such it is important that addition of the exogenous protein itself does not perturb the normal functioning of neurons. This may be an issue with the LiGluR/MAG and GluCl/IVM systems, as these receptors can be activated by endogenous glutamate. However, the efficacy of glutamate at the GluCl receptor was reduced by greater than 6-fold by introducing a single point mutation in the glutamate-binding site (Li et al., 2002). The use of the $P2X_2$/ATP and

TRPV1/capsaicin systems may be problematic in mammals as these receptors are expressed on some neurons in the brain.

Implementablity

The reversible systems developed thus far rely on the addition of light and/or a chemical to control the activity of targeted neurons. Delivering these reagents to the brain of an intact animal can be challenging. For example, introducing light deep into a mammalian brain (e.g., mouse, rat) requires surgically implanting optical waveguides into the animal. The delivery of light into other commonly studied organisms is comparatively straightforward. For example, activation of the $P2X_2$/caged ATP and the LiGluR/MAG systems in flies and transparent zebrafish, respectively, is accomplished noninvasively by shining light of the appropriate wavelength on the intact animal. Introducing chemicals into the mammalian brain can also be challenging, owing to the restricted permeability of the blood–brain barrier, and require invasive procedures. It is worth noting the accessibility of chemicals to widely distributed populations of neurons will be greater than that of light, due to scattering in tissue (see below).

The wavelength of light is another factor to consider. The shorter the wavelength of light is, the greater the scattering is in tissue and the closer the light output will have to be to the targeted neurons. In addition, illumination with short wavelength (e.g., ultraviolet) light is damaging to cells over prolonged periods of time. The ChR2/retinal and NpHR/retinal systems are both activated by long-wavelength light (i.e., blue, yellow). The LiGluR/MAG system uses UV light to stimulate activity and blue light to withdraw the tethered agonist from the receptor. However, once activated by a brief pulse of light, the channel remains on for minutes in the dark, thus enabling depolarizations and trains of action potentials to be evoked with minimal exposure to UV light. In the $P2X_2$/ATP and TRPV1/capsaicin systems, the ligands can be activated using either single photon or two-photon uncaging (Geissler et al., 2003; Zhao et al., 2006). Photosensitive caging groups have recently been reviewed (Ellis-Davies, 2007; Mayer and Heckel, 2006).

Introducing chemicals into the brain of an organism can also be challenging, owing to the restricted permeability of the blood–brain barrier. The most important advantage of ChR2 and NpHR is that the photosensor retinal is endogenously expressed in mammals. In the GluCl/IVM system, although IVM poorly penetrates the blood–brain barrier, administering IVM systemically (i.e., i.p. injection) results in an effective concentration in the mammalian brain. In the $GABA_A$/Zolpidem and RASSL/CNO systems, both ligands effectively cross the blood–brain barrier following systemic administration. In the other systems, the ligand either does not cross the blood–brain barrier (e.g., the octapeptide in the AlstR/AL system, caged ATP in the $P2X_2$/ATP system, the glutamate-azobenzene agonist in the LiGluR/MAG system) or has not been tested (e.g., the chemical dimerizer in the MIST/CID system).

Time Course and Reversibility

Deciphering the temporal coding of neural systems requires a system that is capable of mimicking normal physiological patterns of spike activity and synaptic events—that is, the system must have fast temporal kinetics and fine temporal resolution. The temporal resolution of light is generally superior to pharmacological activation. The LiGluR/MAG and ChR2/retinal systems are ~1000× faster than the other systems and can be used to drive physiological patterns of neuronal activity. If exquisite temporal control over the response is not essential, pharmacological stimulation is a powerful alternative.

In conclusion, tools that offer the capacity to control the function of genetically delineated neurons are expected to provide insight into the connectivity of neural circuits and the relationship between the activity of those circuits and behavioral content. Along with similar approaches being developed to provide remote control over intracellular signaling cascades (Airan et al., 2009; Conklin et al., 2008; Pei et al., 2008), these tools may ultimately be used to restore information in circuits corrupted by disease or injury.

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