1. Introduction
The implementation of optogenetic tools to manipulate neuronal activity with light is transforming our ability to investigate neural systems. By expressing light-sensitive proteins, or opsins, in genetically defined neuronal populations, optogenetic approaches permit new experimental questions that span from the specific properties of defined synaptic connections to their roles in complex behaviors. In this review, we focus on opsins for manipulating neuronal activity and discuss their biophysical properties, delivery strategies, and how these techniques have been adapted to unravel somatosensory circuits.

2. Opsin features
Light-gated ion flux was first identified in extremophile bacteria, but 3 decades passed until this phenomenon was exploited to activate mammalian neurons with light using channelrhodopsin-2 (ChR2). The majority of opsins used for fast optical control are light-gated ion channels and pumps isolated from diverse microorganisms, and these proteins exhibit a vast array of biophysical properties and light sensitivities. Excitatory opsins, such as ChR2, are cation selective, light-gated chloride channels for prolonged optical inhibition, and gate inward photocurrents that depolarize neurons when illuminated by a variety of light wavelengths. The most widely used inhibitory opsins are pumps that mediate chloride influx or proton efflux to generate outward photocurrents and rapidly silence neuronal firing.

Currently available opsins are the result of genome screening and molecular engineering strategies to expand the optogenetic toolbox for diverse applications. These approaches have generated faster excitatory variants for reliable high-speed manipulations, and red-shifted opsins for improved light penetration, and bistable step-function mutations to trigger long-lasting changes in activity. Recently, using the crystal structure of channelrhodopsin, rationale-based protein engineering strategies transformed excitatory opsins into chloride channels for prolonged optical inhibition.

In addition to rapid control of ion flux, molecular engineering strategies have developed optical approaches for manipulating intracellular signaling cascades of cell-surface G-protein-coupled receptors (GPCRs). Endowing light sensitivity to GPCRs was achieved by splicing the intracellular loops of GPCRs of interest to the extracellular and transmembrane domains of the light-sensitive GPCR rhodopsin. This chimeric approach has generated photoactivatable adrenergic, serotonergic, -opioid, dopamine, adenosine, and metabotropic glutamate receptors. Different optoXRs can couple to their endogenous G-protein-mediated intracellular signaling cascades to modulate second messenger systems. Of potential clinical relevance, an optically activated -opioid receptor was recently demonstrated to engage the same signaling cascades as native receptors, including G-mediated inhibition of CAMP, and activation of inwardly rectifying potassium channels. Triggering GPCR activation with light offers spatiotemporal precision currently impossible with traditional pharmacological agents or chemogenetic manipulations. By mimicking the signaling systems of endogenous receptors in a physiologically relevant manner, optoXRs may be advantageous for studying the roles of these important therapeutic targets in precisely defined regions of the body.

3. Using optogenetics in the nervous system
Two primary strategies have been used to deliver opsins into the nervous system: viral vectors and opsin-expressing transgenic mice. Expression specificity with viral transgene delivery can be generated by natural tropism, incorporation of endogenous promoters, or recombinase-dependent expression. The first 2 approaches have limitations with cell-type specificity and variable or non-specific transgene expression, potentially leading to problems in experimental interpretation. The most common viral expression method relies on Cre/LoxP-mediated recombination and conditional expression of transgenes delivered by adenoviral vectors. These viruses must be injected into transgenic animals where Cre-recombinase expression is restricted to genetically defined cell types. Crossing Cre driver mice with genetically encoded opsin lines can enable specific photo-manipulation of molecularly defined neurons. This strategy is advantageous for investigating large neuronal populations; however, targeting of pathway-specific projections with viral...
strategies is lost (Figs. 2C and D). Although Cre driver lines grant genetic access to molecularly defined cell types, they may still comprise heterogeneous populations with distinct functions. New strategies using intersectional genetic techniques, which uses multiple recombinase steps to enable genetic specificity, offer tremendous potential for investigating neuronal subpopulations. By targeting different recombinase enzymes to distinct but partially overlapping cellular populations, transgene expression can be more precisely refined. Opsins are now being implemented with these new genetic approaches to investigate neural circuits.
Figure 2. Opsin expression strategies and light delivery approaches. (A) Viral vector strategies for delivering opsins in vivo. Transgene delivery most commonly uses injections of adeno-associated viral vectors, although Herpes simplex virus and lentivirus can also be used. Specificity can be achieved by using a promoter of interest to restrict expression to specific cell types (pCell, top); however, this approach can lead to weak or nonspecific opsin expression. Cre-recombinase-dependent vectors using flip-excision (FLEX) or double-inverted orientation (DIO) targeting approaches (bottom). An inverted opsin gene (inactive) is flanked with 2 nonhomologous recombination sites (loxP and lox2272). In the presence of Cre-recombinase, the opsin is excised and flipped into a functional orientation. Because specificity is generated through tissue-specific expression of Cre-recombinase, strong ubiquitous promoters (pUB) can be used; however, these viruses must be injected in Cre-expressing transgenic animals (Cre database, http://www.gensat.org/cre.jsp). (B) Cartoon illustrating focal transgene expression (yellow) from a non-Cre-dependent construct in a population of cells near the injection site (left). Similar example illustrating viral delivery of a FLEX/DIO vector into a transgenic animal with Cre-recombinase, depicted here with triangles (right). (C) Transgenic approach for expressing opsins in vivo. Opsin constructs can be introduced into genetic loci to generate transgenic animals for conditional expression. In this approach, opsin genes are preceded by multiple stop codons to prevent expression. These stop codons are flanked by loxP sites, which are excised in the presence of Cre-recombinase, generating strong cell type- and region-specific expression. (D) Crossing transgenic opsin lines with Cre driver mice can grant optogenetic access to large neuronal populations (left). Further genetic refinement can be achieved using intersectional genetic approaches such as INTRSECT (right). Here, cellular specificity is controlled by using multiple recombination events with different enzymes. One group of genetically defined cells is depicted in green, whereas a separate population is red. Yellow triangles represent the targeted subpopulation of cells that exhibit genetic features of both of groups. (E) Different brain regions can be anatomically targeted by implanting optical fibers into cannulas affixed to the skull. Light is frequently delivered through a laser or light-emitting diode (LED) light source; however, mice must be tethered to optical cables during behavioral experiments. (F) Optical fibers can be used to illuminate external tissues with light from a laser or LED light source. Although animals are untethered, access to deeper tissues is not possible, and uniform light delivery can be problematic. (G) Opsins expressed in peripheral tissues of the paw can be stimulated by LED arrays placed in the floor of behavioral chambers, allowing for untethered movement during experiments. This approach can easily incorporate different stimulation wavelengths. (H, I) Wireless implantable LED devices for stimulating superficial areas in the brain, spinal cord, and peripheral tissues. Reproduced from Ref. 68 with permission. (J) Image of a freely moving animal with a LED device implanted in the hindpaw. Wireless activation is achieved via radio frequency waves generated by a resonant cavity below the chamber. This couples electromagnetic energy to the mouse, which is harvested by the implant. Reproduced from Ref. 68 with permission. (K, L) Flexible wireless µLED devices designed to directly interface with the sciatic nerve (K) or be threaded into the epidural space of the spinal column to spinal cord (L) for optogenetic stimulation. Reproduced from Ref. 122 with permission. (M) Image of a freely moving mouse with a flexible device implanted in the spinal column. Wireless activation is achieved by an external radio frequency antenna that directly powers the µLED device. Reproduced from Ref. 122 with permission. Adaptations are themselves works protected by copyright. So in order to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
3.1. Illuminating pain circuits in the brain

Within 2 years of demonstrating optical control of cultured neurons with ChR2, optogenetic approaches were applied in vivo, using surgically implanted optical fibers in cannulas affixed to the skull. This approach has been used to interrogate a variety of neural circuits in the brain and has recently been applied to studying components of the pain neuraxis.

Initial studies using optogenetics to understand higher-order nociceptive processing targeted brain circuits involved in regulating the sensory and affective aspects of pain. In corticolimbic networks, sensory information from the basolateral amygdala (BLA) is routed to the medial prefrontal cortex (mPFC). Viral expression of ChR2 in ascending BLA projections to the mPFC revealed input-specific connectivity. Synaptic efficacy depended on both the laminar location and postsynaptic target of mPFC neurons in a region-specific manner, highlighting the complexities of these circuits. In rodent models of chronic pain, selective photostimulation of BLA inputs onto pyramidal neurons in the mPFC revealed increased feed-forward inhibition by local GABAergic neurons. Similarly, projections from the mPFC returning processed nociceptive information to the limbic system are also suppressed after peripheral nerve injury by elevated interneuron activity. This suggests that persistent pain states can lead to widespread dysfunction in the inhibitory tone of this circuit. Enhancing the activity of parvalbumin neurons GABAergic interneurons, possibly through synapse-specific alterations in endocannabinoid signaling. Human brain imaging experiments have revealed enhanced activity in the prefrontal cortex of patients with chronic pain, which may reflect the increased excitability of local interneurons observed in rodents. Alternatively, elevated activity in human prefrontal cortex may represent a long-term maladaptive response to the acute suppression of these projections by local interneurons, possibly through synapse-specific alterations in endocannabinoid signaling.

A major target region of these cortical projections is the central nucleus of the amygdala (CeA), which plays a key role in regulating the emotional components of sensory stimuli. The CeA, pain processing and plasticity are localized, and selective optogenetic stimulation of ChR2 in the right CeA induced visceral hyperalgesia in response to bladder distention. Nociceptive information is also relayed to the CeA via projections from the lateral parabrachial nucleus in the brainstem. Viral expression of ChR2 in projections from the lateral parabrachial nucleus revealed direct monosynaptic glutamatergic inputs onto CeA neurons, which are increased by inflammatory pain. In the nucleus accumbens (NAC), cortical inputs converge and are integrated with signals from midbrain dopaminergic neurons. Optogenetic stimulation of cortical projections to NAC had antinociceptive effects and alleviated negative affective behaviors in a model of neuropathic pain. At the synaptic level, persistent pain produced input-specific changes in synaptic connectivity to indirect pathway spiny projection neurons of the NAC shell and increased their excitability. Dampered excitability of indirect pathway spiny projection neurons alleviated tactile allodynia, whereas enhancing their activity increased mechanical hypersensitivity. Persistent pain can lead to numerous synapse-specific and network-wide changes in activity that influences the processing of nociceptive information. These initial optogenetic investigations of corticolimbic connections are a small snapshot of the central circuits involved in pain processing. Additional cell- and region-specific manipulations of these projections are needed to develop a clearer picture of how these connections are altered during the transition from acute to chronic pain.

Monoaminergic neurons are powerful modulators of nociceptive information; however, their broad-reaching projections to both pronociceptive and antinociceptive cell types have made their distinct roles in pain processing difficult to pin down. Noradrenergic (NA) neurons in the locus coeruleus project throughout the brain and spinal cord and are thought to be predominantly antinociceptive. However, optogenetic stimulation of LC NA neurons caused either pronociceptive or antinociceptive behavioral responses to thermal pain stimuli. Subsequent anatomical investigation of viral expression and fiber optic placement revealed that pronociceptive behavior resulted from neurons located dorsally within the LC, whereas ventral NA neurons were antinociceptive. The rostral ventral medulla (RVM) contains another family of neuromodulatory neurons in the brainstem that send descending serotonergic projections to the spinal cord. However, serotonin has both excitatory and inhibitory effects in the dorsal horn, and electrical stimulation of the RVM produced both pronociceptive and antinociceptive behaviors. These disparate observations have made it difficult to establish precise roles for these neurons in nociceptive processing. By targeting the serotonergic system in transgenic mice with ChR2 restricted to tryptophan hydroxylase-2 neurons, optical stimulation of the RVM produced robust hypersensitivity. Repeated stimulation led to hypersensitivity that lasted over 2 weeks, suggesting that these connections are plastic. Whether discrete subpopulations of serotonergic neurons engage distinct nociceptive behaviors can now be addressed with refined genetic targeting approaches and optical stimulation strategies.

4. Optical investigation of peripheral pain systems

Optogenetic experiments venturing outside the brain have focused on the transmission of nociceptive information from sensory neurons of dorsal root ganglia to the spinal cord. Despite initial difficulties in delivering light to peripheral structures, optogenetic stimulation was rapidly implemented for studying these systems in vitro. The first transgenic mouse to express ChR2 in the peripheral nervous system, targeted Mrgprd+ polymodal nociceptive neurons. Importantly, opsins were efficiently trafficked to both peripheral nerve endings in the skin and central terminals in the spinal cord. Photostimulation of these molecularly defined terminals revealed that Mrgprd+ neurons form synaptic connections with all known classes of spinal cord neurons in lamina II. A similar approach was also used to dissect contributions of opioid and GABAergic receptors in synaptic transmission onto spinal neurons by distinct subtypes of primary afferents. Activation of presynaptic δ-opioid receptors did not affect synaptic responses, in contrast to the µ-opioid receptor agonist DAMGO, which preferentially inhibited C-fibers innervating lamina I over lamina II. Presynaptic GABAergic activation depressed transmission from all fiber types, demonstrating clear input-specific modulation of sensory transmission. Optogenetic approaches to
studying synaptic specificity are not restricted to nociceptive afferents. Selective expression of ChR2 in GABAergic interneurons in the spinal cord revealed a critical role for presynaptic inhibition of proprioceptive axons to execute smooth movements.49 These studies highlight the unique advantages to using optical approaches in delineating neural circuit connectivity.133

Optogenetic targeting of different types of primary afferent fibers may also be useful in dissecting their contributions to pain behaviors. Illumination of the hindpaw of transgenic mice expressing ChR2 in Na1.8 neurons caused robust nociceptive responses and place aversion, both of which could be blocked with analgesics.40 Real-time place aversion was seen by selectively stimulating ChR2-expressing nociceptors in TrpV1Cre mice.108,122 Conversely, pain behaviors are attenuated in transgenic mice expressing the inhibitory opsin archaerhodopsin.39 Optical manipulations of pain behaviors have also been achieved in nontransgenic animals using AAV vectors to transduce peripheral fibers.68 More relevant clinically, viral delivery of the inhibitory opsins halorhodopsin68 or archaerhodopsin19,85 to peripheral sensory neurons enabled light-dependent blunting of behavioral responses to thermal and mechanical stimulation.68,85 Optical inhibition also reversed mechanical and thermal hypersensitivity in models of neuropathic pain.19,68 suggesting a possible therapeutic option for using optogenetics in treating chronic pain. These manipulations of somatosensory signaling are not limited to neurons that comprise these circuits. Illumination of the epidermis in transgenic mice expressing ChR2 or inhibitory opsins in Merkel cells and keratinocytes, bidirectionally influenced the activity of innervating sensory neurons.12,99 Although these approaches have great potential to dissect somatosensory circuits, behavioral studies have largely been restricted to tethered fiber optic implantation in the brain or nontargeted illumination of peripheral tissues (Figs. 2E and F).

5. Novel approaches to light delivery

Recent engineering advancements have generated breakthroughs in light delivery solutions. In the brain, chronic fiber optic implants are straightforward and effective5,5,123,152 (Fig. 2E). However, these approaches require tethered operations that can hamper behavior experiments and limit chronic stimulation paradigms. Advances in optoelectronic interfaces led to untethered light-emitting diode (LED) devices that can be secured to the skull and powered wirelessly154 or by battery.167 Further refinement of this approach has miniaturized designs and replaced optical fibers with microscale LEDs that can be injected into the brain,76,102,158 permitting focal control of illumination through independent LEDs.4,76,158 These engineering advances allow for more precise stimulation, particularly for anatomically distinct subpopulations of neurons.

In contrast to optogenetic manipulations in the brain, approaches to photostimulation of sensory fibers and spinal circuits have been extremely limited until recently. Initial approaches relied on illumination of the hindpaw in restrained animals73 or the exposed sciatic nerve in anesthetized transgenic mice.90 Illumination via fiber optic cables permitted basic reflexive assays of thermal and mechanical pain but required simultaneous illumination of the hindpaw during stimulation40,68,85 (Fig. 2F).

The aversive nature of peripheral optogenetic manipulations was cleverly explored using behavioral chambers with multicolored illumination through the floor (Fig. 2G). This consisted of a blue light “stimulation” zone and an orange/red “neutral” area. When placed in these chambers, ChR2-expressing mice exhibited aversion to the blue light zone.40,68 This approach limits photostimulation to cutaneous fibers in the paw and does not allow manipulation of sensory afferents projecting to other areas of the body or nociceptive circuits in the spinal cord. Additionally, illumination of these chambers may trigger off-target behavioral effects, particularly as they relate to stress, anxiety, and affective components of pain.64,84,118,119 Potential solutions to these confounds have recently been developed, using implantable light delivery interfaces.

The first implantable light delivery approach in peripheral tissues consisted on an “optical cuff” surrounding the sciatic nerve. Light was delivered through an optical cable tethered to the skull and tunneled subcutaneously, where it was reflected by an encapsulated aluminum sheet.142 Illumination of ChR2-expressing motor neurons elicited activity from those innervated leg muscles. To access opsin-expressing neurons and sensory afferents in the spinal cord, fiber optic cables were threaded into the epidural space of the spinal column, allowing for activation of either ChR2 or archaerhodopsin.20 Extending these approaches, 2 recent reports have demonstrated fully implantable and wireless devices to stimulate peripheral sensory axons and neurons in the spinal cord in freely moving mice109,122 (Figs. 2H–M). One version of these implantable devices can be constructed using standard equipment, and their energy-harvesting properties allow reliable activation throughout the body (Figs. 2H–J). Wireless functionality was provided by a resonant chamber that coupled electromagnetic energy from lattice in the floor directly to the mouse, which was harvested by the device.109,161 Arenas were designed to accommodate these chambers; however, their current dimensions may limit their application with existing behavioral equipment.109 Although broadly applicable to implantation near a wide variety of biological structures, device rigidity may preclude access to deeper brain and spinal cord neurons.

Another untethered, implantable approach for light delivery consisted of a flexible device with stretchable miniaturized antennas to harvest energy through capacitive coupling (Figs. 2K–M). This permitted direct LED activation by radio frequency transmission, operation throughout 3-dimensional spaces, and implementation with most existing behavioral equipment.122 Both of these wireless, implantable designs effectively demonstrated behavioral aversion from wireless activation of nociceptive neurons in peripheral tissues and in the spinal cord, greatly expanding experimental flexibility for investigating these circuits in vivo. Instructions for fabricating these wireless devices by independent laboratories are available102,109,122; however, continued development and technology refinements should lead to more widespread availability. Future implementation of these and other multimodal devices77,78 will help to develop a more complete picture of somatosensory processing in freely moving animals.

6. Important experimental considerations

Implementing optogenetic techniques grants unprecedented access to neurons and the circuits they comprise. However, as with any technology, limitations exist.42 Although temporal control of activity is a primary strength of optogenetics, many studies report single frequency trains of stimulation. Ideally, frequencies should be tuned around normal physiological firing rates, and “dose-response” data can be very informative.52 Synchronous neuronal stimulation may also generate non-physiological phase-locked firing patterns, in addition to possibly activating plasticity mechanisms. This could lead to inaccurate
interpretations of their function within a network or the unintended engagement of downstream targets. 62,120

The locus of photostimulation is also an important consideration. Activation of ChR2 in axon terminals directly gates presynaptic calcium channels, potentially affecting normal transmitter release. 135 Sustained illumination of archaerhodopsin-expressing presynaptic terminals for silencing experiments directly stimulated Ca2+ influx and spontaneous vesicle release. 98 Ionic equilibrium potentials must also be considered when designing optogenetic experiments. Activation of the chloride pump NpHR shifted the GABAAR reversal potential, resulting in excitatory GABA-mediated currents and rebound firing. 136 This has important behavioral consequences for studying chronic pain conditions that alter chloride gradients, resulting in excitation rather than inhibition. 37,42 Careful experimental planning can mitigate these known confounds. However, other limitations are bound to exist, and caution should be exercised when interpreting optogenetic data.

7. Clinical applications

The enormous potential in using light to control signaling of defined cell types throughout the nervous system not only provides researchers with precise tools to dissect these neural networks, but also could potentially open doors to novel approaches in treating human diseases. Phase I/II clinical trials are now underway using intraocular injections of AAV vectors to express ChR2 in the retina to restore sight to blind patients suffering from retinitis pigmentosa (ClinicalTrials.gov). In the somatosensory system, peripheral sensory fibers represent an obvious target for optogenetic therapies in treating chronic pain, where dampening excitability with inhibitory opsins should provide analgesia. Viral vectors have already been used for peripheral delivery of transgenes to patients in clinical trials. 80 We have confirmed functional expression of these tools in human sensory neurons, taking advantage of the natural tropism of herpes simplex viral vectors (Fig. 1E). A major hurdle for implementing optogenetics in different brain regions will be targeting the appropriate neuronal populations in humans. 136 Across different neural circuits, simple ON/OFF control may not be desirable or effective. The vast majority of clinically available drugs target various GPCRs, raising the possibility that manipulating endogenous signaling pathways with optoXRs may have therapeutic potential. For example, activation of optically sensitive μ-opioid receptors could be restricted to desired targets, such as spinal pain-processing circuits, while avoiding reward centers in the brain that are activated by opioid medications. 137

Light delivery strategies will likely require custom implementations to stimulate the targeted region of the nervous system. Because of the improved tissue penetration by longer wavelengths of light, red-shifted opsins have permitted transcranial stimulation. 138 Further spectral refinements could permit fewer invasive methods for light delivery. In combination with these strategies, advances in wireless device technology may offer potential for future optogenetic clinical trials. 17,80

8. Conclusion

The implementation of optogenetics offers remarkable potential for understanding complex circuits underlying sensory processing. The development of noninvasive tools to probe neural circuits, coupled with engineering advancements to visualize and manipulate their functions, are rapidly advancing this front. Future breakthroughs may enable highly selective therapeutic interventions and will continue to transform our ability to interrogate complex physiological systems.

Conflicts of interest
R. W. Gereau is a co-founder and stockholder of Neurolux Systems. The remaining authors do not have a conflict of interest.

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