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Spotlight on pain: optogenetic approaches for interrogating somatosensory circuits

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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 [41,57,106,121]. 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.

Opsin Features

Light-gated ion flux was first identified in extremophile bacteria [114,115], but three decades passed until this phenomenon was exploited to activate mammalian neurons with light using channelrhodopsin-2 (ChR2) [22,87,110,111]. 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 [46,162] (Fig. 1A,B). Excitatory opsins, like ChR2, are cation selective [110,111], and gate inward photocurrents that depolarize neurons when illuminated by a variety of light wavelengths [6,22,166] (Fig. 1A,D). The most widely used inhibitory opsins are pumps that mediate chloride influx [54,56,167] or proton efflux [34,83] to generate outward photocurrents and rapidly silence neuronal firing (Fig. 1B,E).

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Currently available opsins are the result of genome screening [34,53,78,149,156,165] and molecular engineering strategies [36,55,79,89,101,104,126,127,150,153] to expand the optogenetic toolbox for diverse applications. These approaches have generated faster excitatory variants for reliable high-speed manipulations [15,60,145], red-shifted opsins for improved light penetration [35,88], and bistable step-function mutations to trigger long-lasting changes in activity [9,16]. Recently, using the crystal structure of channelrhodopsin [74], rationale-based protein engineering strategies transformed excitatory opsins into chloride channels for prolonged optical inhibition [13,14,155,157].

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 [3,75]. This chimeric approach has generated photo-activatable adrenergic [3,75,138], serotonin [116], μ-opioid [10,137], dopamine [59], adenosine [86], and metabotropic glutamate receptors [159]. Different optoXRs can couple to their endogenous G-protein-mediated intracellular signaling cascades to modulate second messenger systems (Fig. 1C). Of potential clinical relevance, an optically activated μ-opioid receptor was recently demonstrated to engage the same signaling cascades as native receptors, including G_i-mediated inhibition of cAMP (Fig. 1C,F), and $G_{\beta/\gamma}$ activation of inwardly rectifying potassium channels [137]. Triggering GPCR activation with light offers spatiotemporal precision currently impossible with traditional pharmacological agents or chemogenetic manipulations [142]. 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.

Targeting Opsin Expression *in vivo*

Two primary strategies have been employed to deliver opsins into the nervous system: viral vectors and opsin-expressing transgenic mice [66,93,163,164]. Expression specificity with viral transgene delivery can be generated by natural tropism [17,164], incorporation of endogenous promoters [1,166], or recombinase-dependent expression [23,112] (Fig. 2A–D). The first two 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 adeno-associated viruses (AAVs) [7,28,134,139] (Fig. 2A). These viruses must be injected into transgenic animals where Cre-recombinase expression is restricted to genetically-defined cell types [51,52,61,144] (Fig. 2B). Crossing Cre driver mice with genetically encoded opsin lines can enable specific photo-manipulation of molecularly-defined cell-types [96,97]. This strategy is advantageous for investigating large neuronal populations; however, targeting of pathway-specific projections with viral strategies is lost (Fig. 2C,D). While Cre driver lines grant genetic access to molecularly-defined cell-types, they may still comprise heterogeneous populations with distinct functions. New strategies employing intersectional genetic techniques, which utilizes multiple recombinase steps to enable genetic specificity [44,95], offers tremendous

potential for investigating neuronal subpopulations [21,45,69,117,129,131,132] (Fig. 2D). 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 [47,95,132].

Illuminating Pain Circuits in the Brain

Within two years of demonstrating optical control of cultured neurons with ChR2 [22], optogenetic approaches were applied *in vivo,* utilizing surgically implanted optical fibers in cannulas affixed to the skull [1,5]. This approach has been used to interrogate a variety of neural circuits in the brain [94,140,141,146,147] and has recently been applied to studying components of the pain neuraxis [29].

Initial studies utilizing 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) [113]. 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 [33]. 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 [77]. Similarly, projections from the mPFC returning processed nociceptive information to the limbic system [72,113], are also suppressed following peripheral nerve injury by elevated interneuron activity [168]. This suggests that persistent pain states can lead to wide-spread dysfunction in the inhibitory tone of this circuit. Enhancing the activity of parvalbumin^{+} GABAergic interneurons in the mPFC, using optogenetic stimulation in transgenic mice, exacerbated both sensory and emotional pain behaviors in rodent models of chronic pain. In contrast, optogenetic silencing of this same neuronal population blunted these responses [168]. Increased polysynaptic inhibition from inputs arising in the BLA, was attributed to reduced mGlu5-mediated endocannabinoid signaling at these synapses [77]. Human brain imaging experiments have revealed enhanced activity in the PFC of patients with chronic pain [8], which may reflect the increased excitability of local interneurons observed in rodents. Alternatively, elevated activity in human PFC 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 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 [103]. In the CeA, pain processing and plasticity are lateralized [30,31,71], and selective optogenetic stimulation of ChR2 in the right CeA induced visceral hyperalgesia in response to bladder distension [38]. Nociceptive information is also relayed to the CeA via projections from the lateral parabrachial nucleus (LPB) in the brainstem. Viral expression of ChR2 in projections from the LPB revealed direct monosynaptic glutamatergic inputs onto CeA neurons, which are increased by inflammatory pain [143]. In the nucleus accumbens (NAc), cortical inputs converge and are integrated with signals from midbrain dopaminergic neurons. Optogenetic

stimulation of cortical projections to NAc exhibited anti-nociceptive effects and alleviated negative affective behaviors in a model of neuropathic pain [82]. At the synaptic level, persistent pain produced input-specific changes in synaptic connectivity to indirect pathway spiny projection neurons (iSPNs) of the NAc shell and increased their excitability [130]. Dampened excitability of iSPNs alleviated tactile allodynia, while enhancing their activity increased mechanical hypersensitivity [130]. 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 pro- and anti-nociceptive cell types has made their distinct roles in pain processing difficult to pin down [105,107,119]. Noradrenergic (NA) neurons in the locus coeruleus (LC) project throughout the brain and spinal cord [58,91,92] and are thought to be predominantly anti-nociceptive [124,125]. However, optogenetic stimulation of LC NA neurons caused either pro- or anti-nociceptive behavioral responses to thermal pain stimuli [63]. Subsequent anatomical investigation of viral expression and fiber optic placement revealed that pro-nociceptive behavior resulted from neurons located dorsally within the LC, while ventral NA neurons were anti-nociceptive [63]. The rostral ventral medulla (RVM) contains another family of neuromodulatory neurons in the brainstem that send descending serotonergic projections to the spinal cord [11,48,81]. However, serotonin has both excitatory and inhibitory effects in the dorsal horn, and electrical stimulation of the RVM produced both pro- and anti-nociceptive behaviors [25,48,100,160,170]. 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 [169], optical stimulation of the RVM produced robust hypersensitivity [24]. Repeated stimulation led to hypersensitivity that lasted over two weeks, suggesting that these connections are plastic [24]. Whether discrete subpopulations of serotonergic neurons engage distinct nociceptive behaviors can now be addressed with refined genetic targeting approaches and optical stimulation strategies [4,44].

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 (DRG) to the spinal cord. Despite initial difficulties in delivering light to peripheral structures, optogenetic stimulation was rapidly implemented for studying these systems *in vitro* [26]. The first transgenic mouse to express ChR2 in the peripheral nervous system, targeted *Mrgprd*$^{+}$ polymodal nociceptive neurons [151,171]. Importantly, opsins were efficiently trafficked to both peripheral nerve endings in skin and central terminals in spinal cord. Photostimulation of these molecularly defined terminals revealed that *Mrgprd*$^{+}$ neurons form synaptic connections with all know classes of spinal cord neurons in lamina II [151]. A similar approach was also used to dissect contributions of opioid and $GABA_B$ receptors in

presynaptic modulation of 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 [65]. Presynaptic $GABA_BR$ activation depressed transmission from all fiber types, demonstrating clear input-specific modulation of sensory transmission [43,65]. 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 utilizing 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 behavior. Illumination of the hindpaw of transgenic mice expressing ChR2 in $Na_v1.8^+$ neurons caused robust nociceptive responses and place aversion, both of which could be blocked with analgesics [40]. Real-time place aversion is also seen by selectively stimulating ChR2-expressing nociceptors in $TrpV1^{Cre}$ 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 halorodopsin [68] or archaerhodopsin [19,85] 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]. While these approaches have great potential to dissect somatosensory circuits, behavioral studies have largely been restricted to tethered fiber optic implantation in the brain or non-targeted illumination of peripheral tissues (Fig. 2E,F).

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 effective [2,5,123,152] (Fig. 2E). However, these approaches require tethered operation that can hamper behavior experiments and limit chronic stimulation paradigms. Advances in optoelectronic interfaces led to untethered LED devices that can be secured to the skull and powered wirelessly [154] or by battery [67]. 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 manipulation in 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 animals [73] 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 stimulation [40,68,85] (Fig. 2F). The aversive nature of peripheral optogenetic manipulations was cleverly explored using behavioral chambers with multi-colored 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 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 [148]. 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, two recent reports have demonstrated fully implantable and wireless devices to stimulate peripheral sensory axons and neurons in the spinal cord in freely moving mice [109,122] (Fig. 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 (Fig. 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]. Though 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 (Fig. 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 available [102,109,122]; however, continued development and technology refinements should lead to more widespread availability. Future implementation of these and other multimodal devices [27,70], will help to develop a more complete picture of somatosensory processing in freely moving animals.

Important Experimental Considerations

Implementing optogenetic techniques grants unprecedented access to neurons and the circuits they comprise. However, as with any technology, limitations exist [62]. While 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 [32]. 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 Ca^{2+} 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 $GABA_AR$ reversal potential, resulting in excitatory GABA-mediated currents and rebound firing [128]. 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.

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 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 [50]. We have confirmed functional expression of these tools in human sensory neurons, taking advantage of the natural tropism of herpes simplex viral (HSV) 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, like 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. Due to the improved tissue penetration by longer wavelengths of light, red-shifted opsins have permitted transcranial stimulation [35]. Further spectral refinements could permit less invasive methods for light delivery. In combination with these strategies, advances in wireless device technology may offer potential for future optogenetic clinical trials [18,80].

Conclusion

The implementation of optogenetics offers remarkable potential for understanding complex circuits underlying sensory processing. The development of non-invasive 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.

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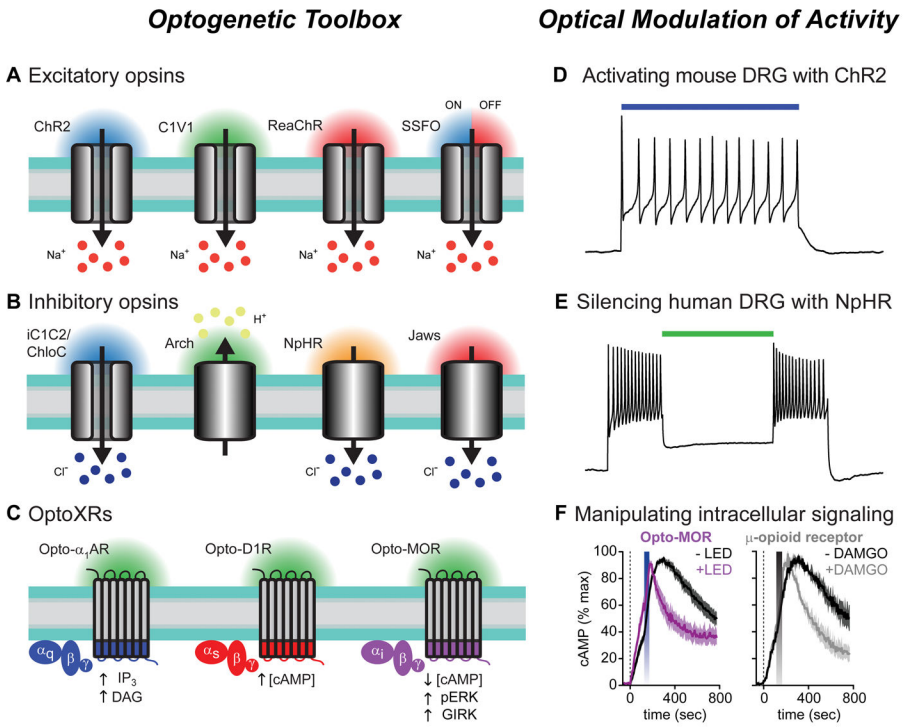

Figure 1. Optogenetic toolbox and functional properties

(A–C) Opsins are illustrated with their respective wavelengths of light activation. Arrows indicate direction of ion flux or effect on intracellular signaling pathways. **(A)** Excitatory opsins like ChR2 (far left) are nonspecific cation channels that depolarize neurons when stimulated with blue light. Interest in separately controlling different cell-types led to the development of opsins with shifted spectral properties, including the green light activatable C1V1 chimera (center left), and a red-light activatable ChR2 variant, ReaChR (center right). Stable step-function opsins (SSFOs) gate long-lasting photocurrents after brief pulses of blue light that can be rapidly terminated by red light (far right). Sodium ions flowing down their electrochemical gradient are depicted as the predominant charge carrier; however, these channels are also permeable to protons, potassium, and calcium ions.

(B) iC1C2 and ChloC (far left) are channelrhodopsin variants engineered to function as inhibitory chloride-conducting channels. These opsins silence neuronal activity when illuminated with blue light. Archaerhodopsin (Arch, center left) is a green-light activated proton pump that generates proton efflux to hyperpolarize neurons. Halorhodopsins, like NpHR (center right) and Jaws (far right), are chloride pumps activated by yellow and red light, respectively. Owing to the greater tissue penetration of red-shifted light, Jaws can silence activity *in vivo* in combination with non-invasive transcranial illumination with red light.

(C) Cartoon depicting optoXR strategy for optogenetic control of G-protein coupled receptor (GPCR) signaling. These chimeric proteins consist of the extracellular and transmembrane domains of light-sensitive rhodopsins with the intracellular regions of a GPCR of interest. Shown are Gq-coupled α1 adrenergic receptors (left, blue), Gs-coupled D1 dopamine receptors (middle, red) and Gi-coupled μ-opioid receptors (right, purple). The

intracellular signaling molecules engaged by optical stimulation with blue-green light are shown below each receptor.

(D) Voltage trace showing action potential firing in ChR2-expressing mouse DRG neurons during illumination with blue light (indicated by the colored bar). Modified from reference [122] with permission.

(E) Using green light to silence human DRG neurons expressing the inhibitory chloride pump NpHR. Primary cultures of human sensory neurons were infected with Herpes simplex virus carrying the inhibitory opsin NpHR. Voltage trace showing sustained action potential firing during depolarizing current injections, which was inhibited when neurons were illuminated with green light (colored bar).

(F) Summary graphs showing Gi-mediated inhibition of forskolin-induced cAMP production in cells expressing the light sensitive μ-opioid receptor chimera opto-MOR after blue LED stimulation (left graph, blue bar). The kinetics of cAMP inhibition were indistinguishable from cells expressing the wild-type μ-opioid receptor stimulated with the agonist DAMGO (right graph, gray bar). Modified from reference [137] with permission.

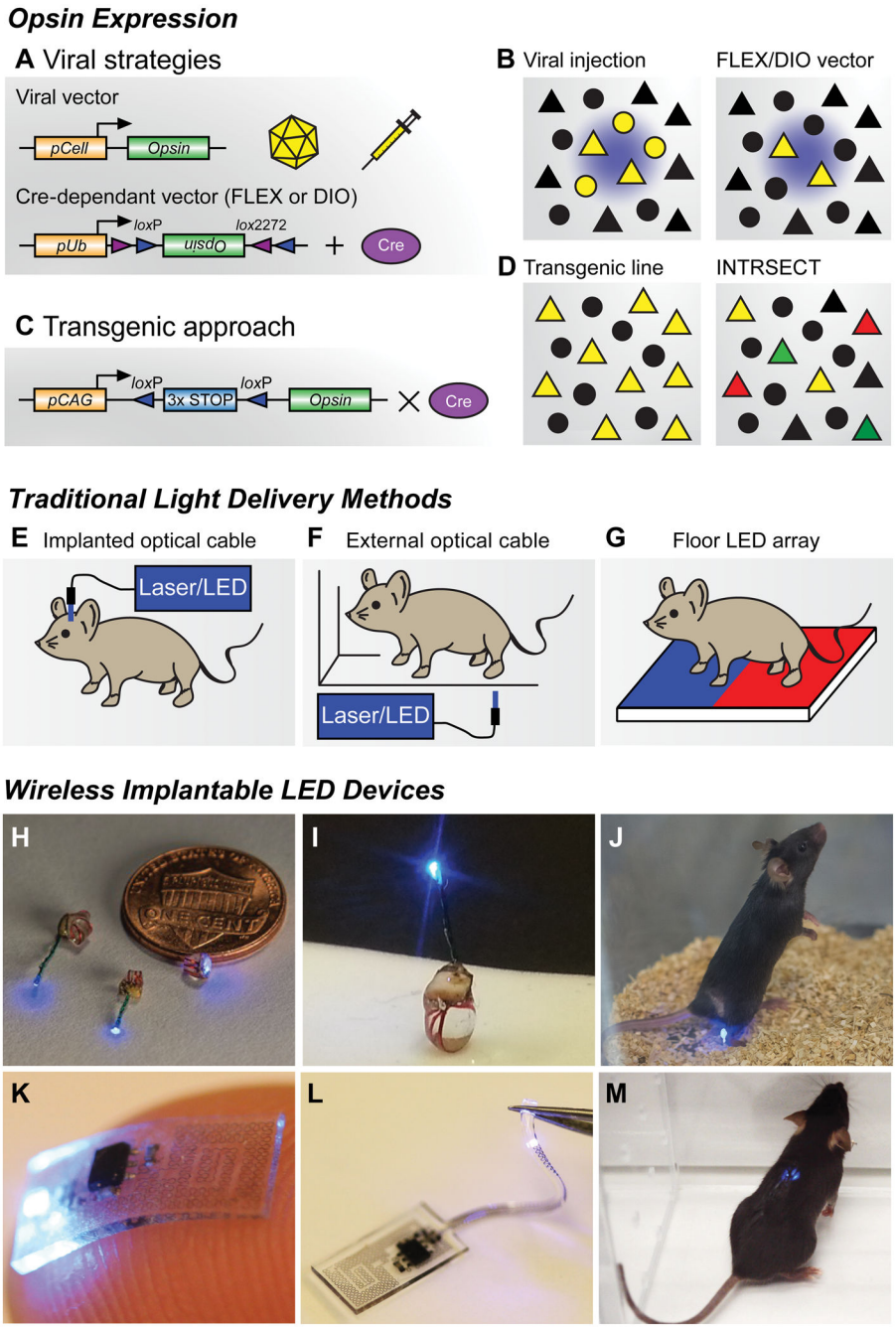

Figure 2. Opsin expression strategies and light delivery approaches

(A) Viral vector strategies for delivering opsins *in vivo*. Transgene delivery most commonly uses injections of adeno-associated viral (AAV) vectors, though 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 non-specific 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 two non-homologous 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 like 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, while 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 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. While 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 reference [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 reference [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 reference [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 reference [122] with permission.