REVIEW ARTICLE

Optogenetics and photopharmacology in pain research and therapeutics

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ABSTRACT

Pain afflicts billions of people worldwide, who suffer especially from long-term chronic pain. This gruelling condition affects the nervous system at all levels: from the brain to the spinal cord, the Dorsal Root Ganglia and the peripheral fibres innervating the skin. The nature of the different molecular and cellular components of the somatosensory modalities, as well as the complexity of the peripheral and central circuitry are yet poorly understood. Light-based techniques such as optogenetics, in concert with the recent advances in single-cell genetic profiling, can help to elucidate the role of diverse neuronal sub-populations in the encoding of different sensory and painful stimuli by switching these neurons on and off via optically active proteins, namely opsins. Recently, photopharmacology has emerged from the efforts made to advance optogenetics. The introduction of azo-benzene-based light-sensitive molecular switches has been applied to a plethora of molecular targets, from ion channels and receptors to transporters, enzymes and many more, some of which are paramount for pain research and therapy.

In this review, we summarise the past and ongoing research in the fields of optogenetics and photopharmacology and we discuss the use of light-based techniques for the investigation of acute and chronic pain physiology, besides their potential for future therapeutic use to improve pain treatment.

Keywords: Optogenetics · Photopharmacology · Pain · Phototherapy

Introduction

Pain, according to the International Association for the Study of Pain, is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (1). It is a condition that torments more than 1.5 billion people globally, who suffer especially from long-term chronic pain (2). Chronic pain indeed affects an estimated 20% of adults in Europe and U.S., and the current available treatments produce limited reliefs and moderate to severe side effects (2,3).

In contrast to many neurological disorders, pain affects the nervous system at all levels: from brain regions to spinal cord, Dorsal Root Ganglia (DRGs) and peripheral fibres that innervate the skin and the organs (4). Noxious sensation is mediated through the transmission of sensory inputs from the periphery to the spinal cord via modality-specific afferents that reside in the DRGs and discriminate between the different tissue damaging stimuli (4,5). Furthermore, the different nature of pain sensations (mechanical, thermal, chemical) is also dependent on the integration of the sensory inputs in the dorsal horn of the spinal cord, and abnormalities at any level lead to several pathological conditions, including chronic pain (6-9).

Albeit in the last few years technological advances have shed new light on the different molecular and cellular components of painful sensation, the precise circuitry, as well as the changes that occur in pathological conditions, remain not fully understood.

Genetic profiling of single neurons in the peripheral and central nervous systems has allowed the distinction of different sub-populations of sensory neurons based on specific molecular and cellular markers and may serve as a catalogue of the molecular and chemical bases of somatic sensation and pain (10).

Recently, the development and use of light-based approaches that aim to modulate these neurons and dissect the role of each sub-population in the encoding

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of the different painful stimuli grew exponentially. Optogenetics offers powerful genetic tools to analyse the function of these distinct cellular circuits (11), while photopharmacology is focused on the modulation of channels and receptors that are differentially expressed throughout the nervous system and paramount for pain input transmission with precise spatial and temporal resolution (12,13).

This article aims to review the recent literature on light-based techniques and their applications for research on acute and chronic pain physiology.

Origin and development of light-based pharmacological approaches

Optogenetics and photopharmacology are techniques that enable precise spatial and temporal control of the activity of specific sub-population of neurons. Optogenetics involves the use of genetically encoded light-sensitive ion channels whose sensitivity is dependent on chromophores of natural origin, such as retinal or flavins, in order to modulate cellular activity within specific cell types (14). Photopharmacology, on the other hand, adopts entirely synthetic photoswitches, that are exogenous and need to be specifically delivered to control the function of native biological targets (15,16). Such compounds need to have the capability to undergo a conformational change upon the delivery of a light stimulus and the physiological activities of these two forms must differ (17).

The development of both optogenetics and photopharmacology is inevitably linked: the first step in the development of optogenetics was the discovery by Stoeckenius and Oesterhelt, in 1971, of the light-sensitive ion channel bacteriorhodopsin. Bacteriorhodopsin is a proton pump driven by green light (maximum activation at 568 nm wavelength) that is used for photosynthesis in archaeon *Halobacterium halobium* (18). Six years later, in 1977, halorhodopsin (HR), an inhibitory, yellow light-sensitive chloride channel was discovered by Matsuno-Yagi and Mukohata (19). However, optogenetics as biotechnology was not established until 2002, when Hegemann and Nagel discovered in green algae the channelrhodopsin (ChR), an excitatory cation channel activated by blue light (20). Concurrently, in a paper published in 2002, Miesenbock showed that light could be used as a tool to stimulate action potential discharge in genetically localised neuron subpopulations (21). Later, in 2005, it was then demonstrated by the same group that light-driven activation of diverse circuits in the brain had a direct effect on animal behaviour in *Drosophila melanogaster* (22). In 2004 Kramer, Trauner and Isacoff applied a chemical optogenetic approach to render voltage-gated potassium channels responsive to light and thus controlling the on-off activity of neurons in culture (23). In 2005, ChR was then used to evoke action potentials in mammalian neurons (11) and from 2007 scientists started to use optogenetics as a tool in live, freely-moving animals (24). Successively, from 2012 onwards, a series of important advancements were made in this field: firstly, the design of red-shifted opsins allowed to use red light wavelengths to reduce scattering in tissues and improve both the efficiency and the spatial depth of the excitation (25,26). Secondly, in 2014, Berndt and colleagues engineered an inhibitory isoform of channelrhodopsin-2 (ChR2), capable of conducting chloride anions instead of monovalent cations (27). Thirdly, extremely relevant for the purpose of this review was the development in 2016 of a bi-stable variant, step-waveform inhibitory channelrhodopsin (SwiChR): this isoform is capable of long-lasting activation upon a brief exposure to blue light and deactivates promptly when illuminated with red light (28,29). Besides ion channels, the continuous improvement of the optogenetic tools has brought to the engineering of chimeric light-sensitive G Protein Coupled Receptors (GPCR) called OptoXRs, that are capable, upon light exposure, of activating the intracellular signalling pathways as efficiently as their endogenous versions (30). Moreover, other components of subcellular signalling have been made light-sensitive: enzymes such as photoactivated adenylyl cyclase, light-oxygen-voltage sensors that facilitate protein-protein interactions, and finally gene expression factors such as photoactivatable Cre recombinase (14,31). These advancements greatly expand the complexity of intracellular modulation beyond the simple on-off switch of the first rhodopsin-based opsins (32).

Photopharmacology originated as an effort to provide more reliable tools to optogenetics and in the last few years has grown noticeably due to its applicability in living systems and its role in complementing the conventional optogenetic techniques. The first breakthrough in this area dated as early as the 1960s, when Erlanger and Nachmansohn investigated azobenzene-based inhibitors of acetylcholinesterase (33,34). However, it was only back in 2012 that Trauner and Kramer matured the idea of developing drugs containing synthetic light-switching molecules. The molecule they synthesized, specifically, was a diethylamine-azobenzene-quaternary ammonium able to replicate the light switching function of opsins by blocking the cell potassium-ion channels when activated by light and unblocking the channels in the dark (35,36). Since then, chemistry in couple with biology have offered a wide variety of synthetic photoswitches with highly convertible properties targeted to ion channels, GPCRs, transporters, enzymes, cytoskeleton proteins and lipids, just to name some (15,37).

Designing probes for light-based research and therapy

Optogenetics

Optogenetics, as mentioned before, is a technique that mainly exploits light-sensitive ion channels, the so-called opsins, to modulate neuronal activity with high spatial and temporal resolution (38). Excitatory opsins, like ChR2, are cation selective channels that cause cation influx and photo-controlled neuron depolarisation when illuminated at blue wavelengths (**Figure 1A**) (11,20,39).

Inhibitory opsins, like Archaeorhodopsin (Arch) or HR, provoke either proton efflux or chloride influx respectively to drive an outward photocurrent that generates hyperpolarisation and promptly inhibits neuronal activity (**Figure 1B**) (40-42). In recent years, the endeavour in genome screening and molecular engineering to expand the optogenetics toolbox has generated faster recovery variant for high-speed imaging (43,44), red-shifted opsins to improve the depth of the light penetration (45,46), and bi-stable opsin variants to induce long-lasting changes in neuronal activity. These latter variants are particularly interesting from a therapeutic point of view, since their capability to induce chronic effects with minimal light delivery would reduce both the need for constant light treatment and the risk of long-term phototoxicity (47).

Moreover, recent works focused on the modulation of intracellular signalling cascades with the engineering of photo-activatable cell-surfaced GPCRs for adrenergic, serotoninergic, dopaminergic, adenosine, glutamate (metabotropic) and μ-opioid receptors (30,48-52). These new OptoXR probes, as they are called, generate the same signalling cascade as the endogenous receptors, whilst they can be triggered with a spatio-temporal precision that is not achievable with traditional pharmacological approaches, thus bringing great advantage in the study of relevant targets in defined regions of the body (**Figure 1C**). This level of precise spatio-temporal control, particularly in the case of μ-opioid receptors, is fundamental in dissecting the opioid contribution in peripheral and central nociceptive circuits (53).

The investigation of somatosensation and pain with optogenetics goes unavoidably in pair with the possibility to deliver the opsins to defined neuronal sub-populations in the central and peripheral nervous systems. Two approaches have

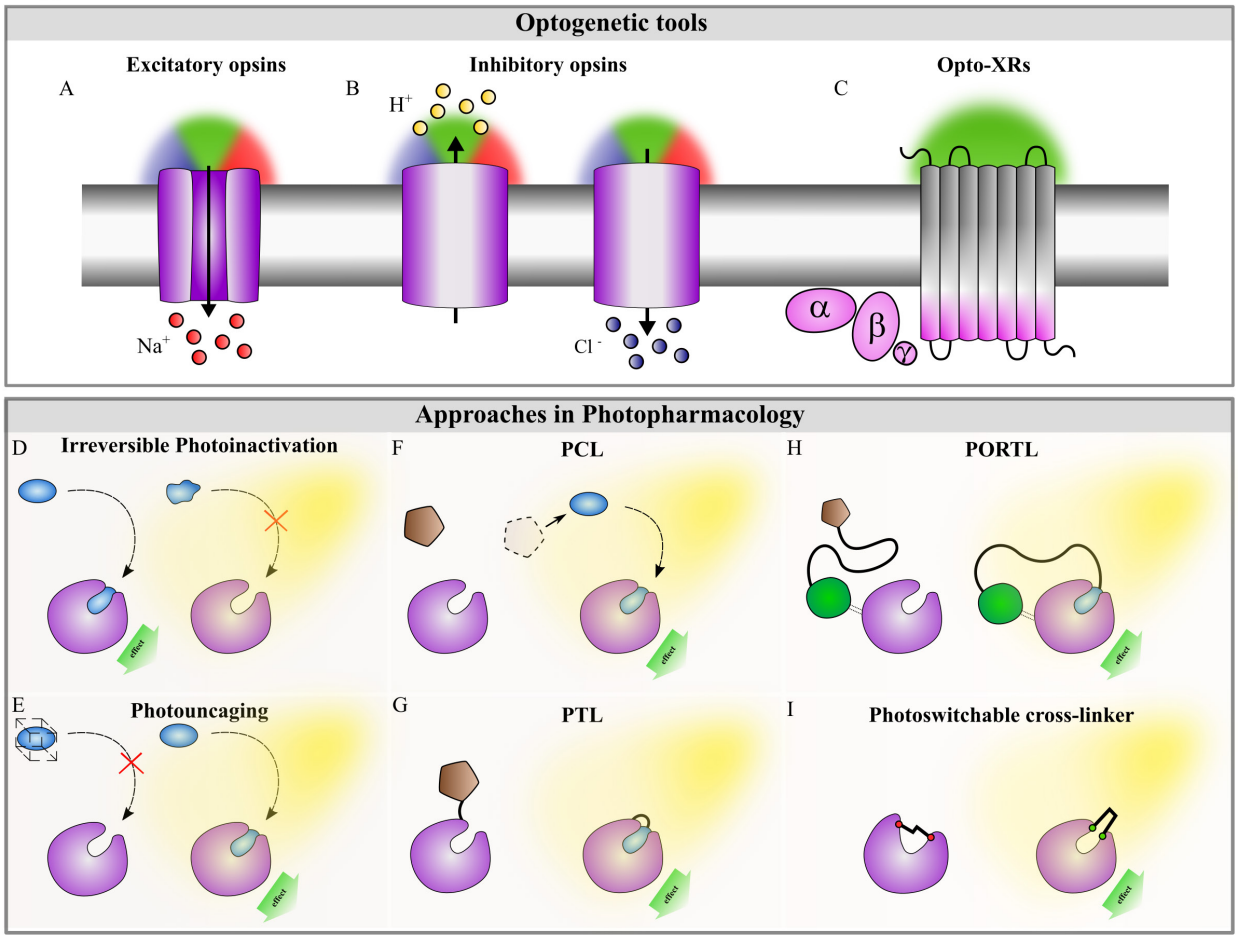

Figure 1. Optogenetic toolbox and Photopharmacological approaches. (A-C) Principal opsins used as optogenetic tools are illustrated. Arrows indicate direction of ion flux. **(A)** Excitatory opsins (*ChR2*) are non-specific cation channels that depolarize neurons when stimulated by light. **(B)** Inhibitory opsins elicit either chloride influx (*iC1C2*, *SwiChR, ChloC, HR*, *Jaws*) or proton efflux (*Arch*) to silence the neuronal activity when illuminated by light. **(C)** Chimeric light-sensitive G-protein coupled receptors (*optoXRs*), consisting of the extracellular and transmembrane domains of light-sensitive rhodopsins with the intracellular regions of a GPCR of interest. **(D-I)** Diverse Photopharmacological approaches are illustrated. Red crosses indicate the switch to the inactive conformation of the photomodulators. **(D)** Irreversible photoinactivation. **(E)** Irreversible photoactivation (*photouncaging*). **(F)** Reversible photoactivation/ inactivation using a PhotoChromic diffusible Ligand (PCL) that upon irradiation switches between an inactive (brown pentagon) and an active (blue ellipse) form, modulating the activity of the target. **(G)** Photo-switchable (closely) Tethered Ligand (PTL) - the photoswitch is, in this case, covalently bound to the target and in close proximity of it. **(H)** Photo-switchable Orthogonal Remotely Tethered Ligand (PORTL) - As for the PTL, the photoswitch is connected to the target but is not in proximity of it. **(I)** Photo-switchable cross-linker - the photoswitch is conjugated on both sides to the target and usually prevents the activity of the target in one of its conformations.

been the most utilised in tackling this issue: viral vectors and opsin-expressing transgenic mice (54-56). The specificity of the viral transgene delivery can be obtained mainly via incorporation of endogenous promoters (57) or recombinase-dependent expression (58,59). Given the experimental problems that can arise with the former method, as well as its partial lack of specificity, the most widely used method for opsin gene delivery is the Cre/Lox-P mediated recombination and conditional expression of transgenes delivered by Adeno-Associated Viruses (AAVs) (60-62). These viruses are injected locally into transgenic mice in which the Cre recombinase expression is restricted to specific neuronal sub-populations (62-64). Conversely, crossing Cre-expressing mice with opsin-expressing lines gives yet another possibility to manipulate molecularly defined sets of neuronal and non-neuronal cells (65-67). These strategies are very advantageous in the study of large cell populations, that however can still comprise heterogeneous sub-populations with different functions within them. Thus, a novel approach called INTRSECT that uses multiple recombinase steps to further refine the specificity of selected subpopulations offers new advantages and great prospect for the study of neuronal circuits underlying specific roles in somatosensation and pain at all levels in the nervous system (68-71).

Photopharmacology

One of the main principles at the basis of photopharmacology is the ability to modulate the pharmacokinetic or pharmacodynamic properties of synthetic molecules by using light. This can be achieved, in most of the cases, with the alteration of a functional group of the drug with a photolysable element (72-74). The main benefit of using this technique is that it permits to reduce the off-target and systemic side effects and to decrease the drug resistance in comparison to a standard drug delivery method (15). Photopharmacological tools have been previously applied to study cancer, diabetes, microbial infections and neurology (15,16,75-81). The importance of this method derives from the fact that potentially every kind of molecule, even with very different range of sizes, can be optically-controlled and thus allowing a fine temporal and spatial control over intracellular or extracellular targets (82).

The effect that the light exerts on its target can be classified into two modalities: reversible and irreversible; each of them have been employed in biology (83).

Irreversible photoinactivation is realized when a freely diffusible compound is irreversibly modified by irradiation and has been mainly used to probe the functional role of a biological target (**Figure 1D**) (84). Also, caged compounds belong to this first category of molecules: they can only be activated once and the chemical strategy approach to gain photocontrol of a target by using these molecules is called *photouncaging*. Technically, a photocage is a chemical group that converts the energy of a photon into energy that is then used to disrupt a chemical bond, strategically placed in a position in which it can modulate the activity of a bioactive molecule (74). Irradiation promotes a reaction that causes the removal of the photocage, triggering the release of the biologically active molecule, switching on (or off) the targeted process (**Figure 1E**) (85). To date, this is the most broadly used photopharmacological approach, and several new photocages continue to appear (73,74,86,87). The other approaches worth mentioning are the recent development of the so-called Photobody (87), that uses the specificity of an antibody fragment to selectively bind and modulate the activity of the desired target, and the family of BODIPY-derived photocages (86); the latter are caged compounds that can be activated with the highest known wavelengths of light through a mechanism that involves a single-photon-release.

As mentioned before, the major drawback of this technique is that the photouncaging process is irreversible and allows to control the properties of a pharmacological compound just once.

Reversible photoswitches, on the basis of the position relative to their target, can be classified into those that interact with their targets through noncovalent interactions (photo-chromic ligands - PCLs) and the ones in which the formation of a covalent bond is involved for the connection to the target (photo-switchable tethered ligands - PTLs, photo-switchable orthogonal remotely tethered ligands - PORTLs). There is also another class of reversible photoswitches, called cross-linkers, that rely on the aid of bioconjugation motifs at both sides of an optically active molecule.

PCLs are freely diffusible molecules in which the irradiation triggers the switch between two different isomeric conformations. As already mentioned, the switch into two different isoforms confers each of them different affinity and/or efficacy, diverse pharmacodynamics properties and may also affect the pharmacokinetics properties. (**Figure 1F**) (13).

A second class of reversible photoswitches includes ligands that are covalently attached to the target through a connection that can be either through a native or an engineered residue. Major advantages of this approach include the ability to accelerate the response by increasing the local concentration of the switches, the ability of the ligand to remain in the proximity of the target and the loss of the need for reapplication of the drug. On the other hand, this approach requires genetic encoding for its full applicability (88).

As mentioned before, tethered ligands can be sub-classified into **(1)** Photo-switchable Tethered Ligands (PTLs) and **(2)** Photo-switchable Orthogonal Remotely Tethered Ligands (PORTLs), depending on the length of the covalent attachment with respect to the ligand binding site.

In respect of PTLs, the photoswitch is attached close to the binding site and the tether is mainly constituted by the photoswitch itself. The switch between the different isomers mainly modifies the concentration of the pharmacophore in the near proximity of the target. They are ideally built as if in one configuration the ligand is physically impeded to reach the binding site while in the other it can exert its function. It requires small bioconjugation molecules, like cysteines (**Figure 1G**).

Conversely, in a PORTL, the tether is much longer, bringing the photoswitch far from the binding site. In this way, the light-induced conformational change affects the efficacy of the tethered ligand rather than its local concentration near the target (**Figure 1H**) (89).

Another class is constituted by light-responsive cross-linkers in which the photoswitch is attached by a covalent bond on both its ends to the target. This method requires the presence of two conjugation motifs on the biomolecule. Upon irradiation, the photoswitch modifies its conformation, triggering then a change in the activity and conformation of the target itself (**Figure 1I**) (13,16,75).

Further considerations on designing photoswitches

An ideal photoswitch must fulfil several requirements to be used in an in vivo model: it should have favourable pharmacokinetics and should be metabolically stable in a given environment. Phototoxicity is an important parameter to bear in mind and, in addition, the photoswitch should have useful photophysical properties, such as high absorbance and quantum yields, and useful thermal relaxation rates (13). A wide range of photoswitches have been used in the last few years but one of the most encouraging one, in terms of its properties, is the reversible molecule called azobenzene. Azobenzene is constituted by a diazo bond (***N*==*N***) that is linked to two phenyl rings. It can adopt the *trans*- or *cis*- conformation: in the former, the phenyl rings are on the opposite sides while in the latter, they are on the same side. UV light triggers the swap between the two isomers of which the *trans*- one is thermodynamically more stable. This process is reversible and can be inverted using heat or by using visible light irradiation (13,35,36,90).

Biological targets in pain research

Pain is an extremely intricate disease which can progress into severe conditions. The effective treatment of pain often lacks the desired level of efficacy, tolerability and target specificity. Optogenetics in the last two decades had a pivotal role in the investigation of pain physiology both in the central and peripheral nervous systems (84). Photopharmacology emerged in the recent years as a potential new approach to be applied in pain research and treatment (91). In this section, we pass into exam all the development in pain research and the potential biological targets that have been unravelled with the aid of these approaches.

Within few years of demonstrating optical control of neuronal cells via ChR2, optogenetic probes were applied *in vivo*, together with surgically implanted optical fibres, to control and study different neural circuits within the brain. This idea has been recently implemented also in the investigation of the central circuits of both the sensory and the affective components of nociception (92,93). In the cortico-limbic networks, the BasoLateral Amygdala (BLA) has been revealed to have a prominent role in the encoding of the 'unpleasantness' of pain (94). The sensory information from the BLA is transmitted to the medial PreFrontal Cortex (mPFC). ChR2 injection in the BLA revealed direct connectivity that was input-specific, and the stimulation of these neurons in rodent models of chronic pain revealed increased feed-forward inhibition by mPFC GABAergic neurons (95). Moreover, the activation of the parvalbumin-positive GABAergic interneurons of the mPFC exacerbated pain responses after peripheral nerve injury, and conversely their inhibition alleviated these responses (96). These data reveal that persistent chronic pain states, provoked by peripheral nerve injuries, lead to a selective activation of BLA inputs on specific mPFC GABAergic interneurons, that in turn inhibit projection neurons in the ventro-lateral PeriAqueductal Gray area (vlPAG): this alteration produces a serial dysfunction of the inhibitory tone of the circuit itself, reducing the strength of serotoninergic and noradrenergic descending pathways involved in pain modulation (**Figure 2A**) (96,97).

Anatomical and physiological evidence has been collected to demonstrate the presence of a circuit between ParaBrachial Nucleus (PBN) and the Central nucleus of the Amygdala (CeA) and its role in the affective dimension of pain (98-100). Excitatory synapses within this circuit are potentiated in various chronic pain models (99,101-103), and direct excitation of CeA neurons with ChR2 induced visceral hyperalgesia after bladder distension (102). Moreover, the investigation of the mechanisms involved in neuropathic pain revealed the presence of a complex modulation (both excitation and inhibition) of the neurons within this circuit, based both on specific molecular identity of the neurons and on their location within different sub-regions of the CeA (**Figure 2A**) (103). Together, these results offer a minor but precise overview of some of the complexity of the circuits that process both the sensory and affective component of pain within the brain, and how paramount is optogenetics to elucidate the role of single projections and specific neuronal subpopulations in the central processing of nociceptive information.

On the other hand, pain perception, as well as the processing of pain information, starts from the periphery, with the nociceptive stimuli travelling through a plethora of sub-populations of sensory neurons in the DRGs to the substantia gelatinosa (laminae I and II) of the dorsal horn (**Figure 2B**) (5,104). A recent article identified 11 neuronal sub-populations by single-cell RNA sequencing, highlighting the complexity of the peripheral coding of multi-modal somatosensation (10). Optogenetics is therefore extremely useful in dissecting the role of these different populations in the coding of sensory and nociceptive inputs (105).

The first peripheral neurons targeted with ChR2 excitatory opsin were Mas-related G-protein coupled receptor member D (Mrgprd)-positive nociceptive neurons: their photo-stimulation revealed the circuitry of their connections to most known classes of lamina II spinal cord neurons (106). Light-dependent activation of Advillin-positive, Transient Receptor Potential Vanilloid 1 (TRPV1)-positive and NaV1.8-positive neurons selectively expressing ChR2 elicited strong nociceptive behaviours, which could be blocked by analgesics administration, indicating that a direct activation of these neuronal sub-populations is

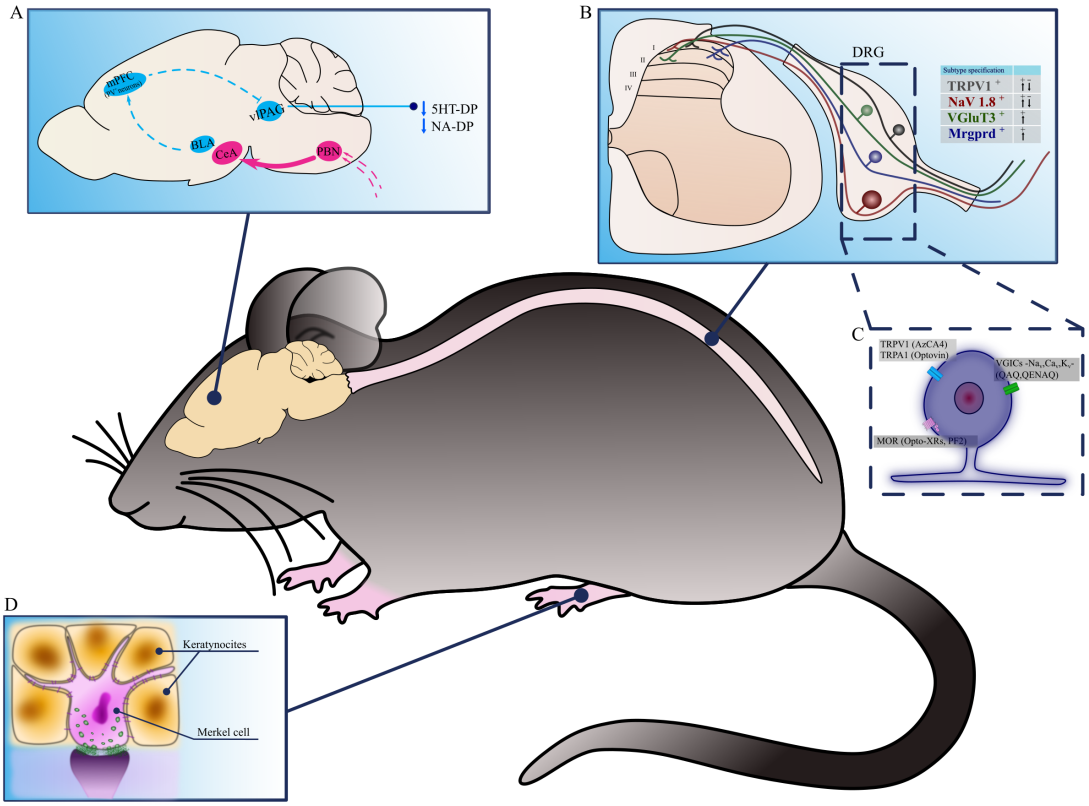

Figure 2. Biological targets of optogenetics and photopharmacology in pain research. Cartoon illustrating some of the targets of interest in the Central and Peripheral Nervous Systems, discussed in this review. **(A)** Overview of some neural circuits of pain within the brain. In light blue it is shown the pathway involving the basolateral amygdala (*BLA*), the medial prefrontal cortex (*mPFC*) and the ventro-lateral PeriAqueductal Gray area (*vlPAG*). The use of optogenetic tools has demonstrated that the modification of the inhibitory tone circuit deeply affects pain modulation during persistent chronic pain states, induced by peripheral nerve injuries. In pink it is shown the circuit between the ParaBrachial Nucleus (*PBN*) and the Central nucleus of the Amygdala (*CeA*) that has a prominent role in the affective dimension of pain. Optogenetics has begun to unravel the profound complexity of this circuit and of the specific sub-populations of neurons involved. **(B)** Diverse sub-populations of sensory neurons in the DRGs form connections at different levels of the substantia gelatinosa (laminae I and II) of the dorsal horn. A list of sub-populations of nociceptive neurons that have a crucial role in pain perception and have been investigated by using optogenetic tools are also shown in the picture (*Mrgprd*$^+$, *TRPV1*$^+$, *NaV1.8*$^+$, *VGLUT3*$^+$). **(C)** Enlargement of a representative neuron in the DRG, showing the main molecular targets involved in nociception that can be currently targeted by specific photo-controllable drugs (*TRP channels in light blue, VGICs in green and MOR in light pink*). **(D)** Optogenetic tools have also been used to elicit responses in peripheral mechano-sensitive cells (*Merkel cells and keratinocytes*) in the epidermis to investigate the role of non-neuronal cells in the perception of innocuous and painful mechanical stimuli.

sufficient to elicit a painful response (62,63,65,107–109). Moreover, prolonged activation of NaV1.8-positive and TRPV1-positive neurons caused a hypersensitivity that lasted long after the stimulus was removed (110,111). Interestingly, the selective activation of the Vescicular GLUtammate Transporter type 3 (VGLUT3)-positive primary afferents elicited only very mild nocifensive behaviours but exacerbated nociceptive responses in a model of chemotherapy-induced neuropathic pain through the Transient Receptor Potential Melastatin 8 (TRPM8) ion channel (112). Conversely, inhibition of the same neuronal populations (NaV1.8-positive, TRPV1-positive) with Arch or HR optogenetic probes alleviates pain behaviours in naïve mice together with murine models of inflammatory and neuropathic pain (**Figure 2B**) (62,111,113). These results are particularly important in pioneering the use of light as an analgesic, opening to the possibility of the use of optogenetics to treat chronic pain. Furthermore, the combination of optogenetic and chemogenetic techniques refines the selection and classification of neuronal sub-populations that have not been specifically genetically identified yet: the use of resiniferatoxin to ablate TRPV1-positive fibres in a transgenic mice expressing ChR2 in Calcitonin related polypeptide 1 (Calca)-positive neurons has brought to the identification of a novel, specific population of High-Threshold MechanoReceptors (HTMR) with unique endings that can be activated by the pulling of a single hair (114).

Furthermore, optogenetic manipulations are not restricted to neuronal cells: in several recent studies peripheral mechano-sensitive cells in the epidermis have been infected to express excitatory and inhibitory opsins. Activation of Merkel cells and keratinocytes is sufficient to elicit action potential discharge in different types of primary afferents, whereas silencing of these cells decreases the spiking of peripheral sensory neurons in response to natural stimuli, as well as ATP release and

nocifensive responses to mechanical painful stimuli (**Figure 2D**) (67,115,116).

Despite revolving mainly around the on/off modulation of whole cell populations, optogenetics has been a keystone in the study of pain circuits, and together with other genetic, electrophysiological and molecular techniques led to the discovery of many important molecular targets for the modulation of pain perception. A more advanced, photopharmacological approach can then be exploited to increase the complexity and capability of research to devise novel approaches to pain modulation and analgesia that can then be translated into therapeutics. To date, only few photo-switchable regulators of nociception have been developed and even less have been described in an *in vivo* system (13,91). In terms of potential targets involved in the pain pathways, one of the most obvious classes is represented by ion channels. However, of the 215 ion channels that exist in the human genome, with 85 ion channels that have been linked to nociception, only a minor number has been successfully targeted for pain research (117).

TRPV1 is a Ca^{2+} permeant non-selective cation channel expressed in various subset of populations of primary afferent neurons and with a well-established role in nociception (118,119). To date, optical control of TRPV1 has been investigated and the result is the development of several azo-capsaicin derivatives (AzCAs). These molecules are photo-switchable agonists of TRPV1 channels, they are fairly inactive in the dark and are activated upon irradiation with UV-A light (120). Among these, *cis*-AzCA4 (121) has been shown to be one of the most effective in activating TRPV1 and to possess a reversible action. In addition, *in vivo* tests demonstrated a TRPV1-mediated hyperalgesia exerted after the application of this compound (**Figure 2C**) (16,120,122).

A photo-switchable compound (Optovin) that reversibly activates another member of the TRP channel family, Transient Receptor Potential Ankyrin 1 (TRPA1), has also been developed so far (123,124). This molecule has been used to modulate TRPA1b channels in zebrafish (**Figure 2C**) (125). Recently, photo-switchable diacylglyerols have also been used to optically-tune the activity of TRPC2, TRPC6 (126) and TRPC3 (127).

GABA-A receptors are chloride-selective pentameric ligand gated ion channels activated by Gamma Ammino-Butirric Acid (GABA). In post-synaptic neurons, GABA receptors trigger a decrease of action potential firing upon their activation. Given that, GABA-A receptors have been investigated as potential target for the development of anaesthetics (128,129). Photo-compounds that act on GABA-A receptors have been synthetized resembling the structure of Propofol, a lipophilic anaesthetic agent that acts through potentiation of GABA-induced currents (128). These compounds operated as allosteric modulators, potentiating GABA currents in the dark and being inactivated upon application of light. Additional Azo-benzene derivatives of propofol were produced (AP1-16) and among these, AP2 showed anaesthetic activity in an *in vivo* animal model in albino *Xenopus laevis* tadpoles (128). Also, the so-called LiGABAR, that is a genetically modified light-controlled GABA receptor, has been developed, so far, by using tethered photopharmacology (130). The resulting design of a transgenic line of mice constitutively expressing LiGABAR, facilitated the development of higher efficient new PTLs (PAG-1C) and finally allowed to control the activity of cortical neurons in mice by using the light (131).

Voltage-gated ion channels (VGICs) play an essential role in the generation of action potentials and in synaptic transmission and represent a privileged target of photopharmacology. They have also been fundamental for the development of the field (132). The photo-switchable azobenzene derivative QAQ is structurally composed of two azo-linked quaternary amines and, together with its derivative QX-314, has been developed on the basis of lidocaine, a local anaesthetic that blocks VGICs (133,134). These compounds are blockers of KV, NaV, and CaV channels and, importantly, are membrane-impermeable and thus they need to be transported into the cell via TRPV1 channels or P2X receptors, allowing the selective targeting of TRPV1 expressing cells for the optical control of nociception. These molecules have been used, in addition to capsaicin, to selectively block TRPV1-positive nociceptors (135,136). So far, a QAQ derivative has also been developed, namely QENAQ, that is controlled by using visible light. This compound allows to photo-control the pain signalling without issues deriving from invasiveness and with high specificity and fast kinetics (**Figure 2C**) (137). Another compound (fotocaine) based on azologisation of the local anaesthetic fomocaine has been also developed. Neurophysiological application of this compound has opened up the way to test its applicability as a potential analgesic (135,136).

μ-opioid receptors are GPCRs that activate inhibitory G-proteins. They assemble as homo- and hetero-dimeric complexes and scaffold a variety of proteins. GPCRs are potentially involved in all physiological processes in eukaryotic organisms, including acute and chronic pain (91). Indeed, most of the potent analgesics currently in use act through the μ-opioid receptor. Moreover, they belong to the class A (Rhodopsin-like family) of GPCRs and thus they have been an exclusively amenable class of proteins for the development of phototunable compounds. For these reasons, photo-switchable opioids have been under thorough investigation in the last few years. The usage of such compounds, as possible photo-analgesics, may enable the optical-control of μ-opioid receptors. The first compound that has been developed was an azobenzene derivative of the synthetic μ-opioid receptor agonist Fentanyl (photofentanyl-2 or PF2) (**Figure 2C**) (138). The development of this compound generated interest in a potential future use of photo-analgesics (16,139).

Photopharmacology is constantly growing and its usage to control nociception is an emerging but interesting field. New compounds are frequently synthetized in order to get accurate control of novel targets (ionotropic glutamate receptors (37,140), metabotropic glutamate receptors (141,142), adrenergic receptors, muscarinic acetylcholine

receptors, dopamine, histamine, serotonin receptors, calcium and potassium channels and a number of transporters and pumps (12,13,75)).

Advancements in light delivery methods

Optogenetics and photopharmacology have the great potential to dissect the somatosensory circuitry and the key molecular players involved in pain biology and pathobiology (143,144). However, one of the major limitations of these approaches, particularly in behavioural experiments, is the complexity to deliver light especially to neurons in the spinal cord and in the periphery in freely behaving mice (143). Brain imaging and optogenetics in awake rodents with chronic optic fibre implants is currently well established and can be used also in combination with electrophysiology to optically stimulate and record, at the same time, from different neuronal circuits *in vivo* (**Figure 3A, B**) (94,144). Imaging peripheral tissues however poses major technical difficulties in the absence of a solid structure like the skull, that can help to stabilise the implants. The first attempts involved peripheral light delivery to the hind paws by implementing optical fibres or Light-Emitting Diode (LED) arrays in cages to target opsin-expressing afferents for behavioural and place aversion tests (**Figure 3G**) (63,65,110). To overcome the limitations of this approach, and in the effort to target more central structures like the spinal cord, tethered optical fibres have been adapted for peripheral nervous system stimulation. Laser-driven optical fibres have been implanted chronically in the epidural space of the spinal cord, allowing for direct modulation of opsin-expressing peripheral sensory neurons innervating the dorsal horn of the spinal cord, as well as interneurons in the substantia gelatinosa (**Figure 3C**) (113,145). Another similar approach involves the use of a nerve cuff that surrounds the peripheral nerve: the light stimulation is provided by an optical fibre tethered to the skull and delivered subcutaneously to reach the implanted cuff (**Figure 3E**) (146). These new technologies have propelled the use of optogenetics to investigate peripheral nociception. However, these implants are still dependent on an apparatus that is partially fixed to the skull, hindering the free movement of the animals. Wireless implantable LED devices for the stimulation of superficial areas in the brain, spinal cord and peripheral tissue have seen a great popularity in the last years. Different laboratories have used similar approaches for the construction of miniaturised probes that utilise microscale LEDs to allow light stimulation in freely behaving rodents (107,109,110,147–149). These implants utilise inductive coupling to remotely power the μLEDs, eliminating the need for batteries and circuits and dramatically reducing the dimension of the implants themselves, that can be as small as 10 mm^3 and weight as less as 20 mg (**Figure 3D, F, H**) (110). The most recent versions of these devices use near-field power coupling and radio frequencies transmission to power and activate the LEDs, as well as softer and more durable encapsulation of the microcircuits, strategies that reduce both the fabrication cost and the technical expertise necessary to produce such devices (109,148,149). Further technological improvements of these wireless approaches will make the simultaneous stimulation and recording of responses possible, as it has been demonstrated in the central nervous system (150), and will help to render a more complete picture of the somatosensory coding of multi-modal stimuli in freely moving animals.

Therapeutic potential and challenges of light-based pharmacology

The possibility to achieve a high spatial and temporal resolution in controlling the signalling of defined neuronal populations throughout the nervous system opens the path towards the development of more effective therapies for disease and pain treatment. Pain management and chronic pain treatment, as stated before, are fundamental problems that are poorly addressed by current treatments and often burdened by unwanted side effects.

Optogenetic and photopharmacological tools employ a spatially defined beam of light as stimulus to elicit a response in the desired target. It is exactly this spatial definition that may be a very effective way to modulate chronic pain in suffering patients (151). The obvious targets to exploit are the numerous ion channels that are expressed centrally and peripherally and are involved in nociception: photo-controllable drugs have been designed to modulate TRPV1, TRPA1, μ-opioid, GABA-A and metabotropic glutamate receptors (118,120,123,128,137,139,152,153). Photochemical and optogenetic controllers of opioid signalling harbour the most promise in delivering peripheral analgesia without involving central circuits linked to addiction (53,139). Another interesting approach is the use of photo-reversible local anaesthetics that target TRPV1-positive nociceptors (QAQ and QENAQ) and have been effective in controlling pain signalling in behaving rodents (134,137). Moreover, well-established light-based techniques now exist for bidirectional control of primary afferents via transdermal stimulation: these techniques could potentially harbour a future of non-invasive, implant-free optogenetic control of chronic pain disorders (147). A fascinating, similarly non-invasive use of light-based therapy is the prolonged exposure of patients to specific light wavelengths to treat pain and anxiety; this kind of therapy has already been used to control depression in chronic pain and disease suffering patients (154,155), and has been recently associated with profound, opioid-dependent peripheral and central anti-nociception in naïve and neuropathic pain suffering rodents (156).

Despite harbouring great promise, several hurdles have still to be overcome in order to deliver a safe and effective therapy for pain management. The two principal issues in the implementation of light-based therapies are the genetic delivery of the opsins or photo-switches to their targets and the delivery of light to inaccessible organs like the brain and the spinal cord. As stated before, the development of wireless light delivery methods using μLEDs, that are miniaturised, injectable and programmable,

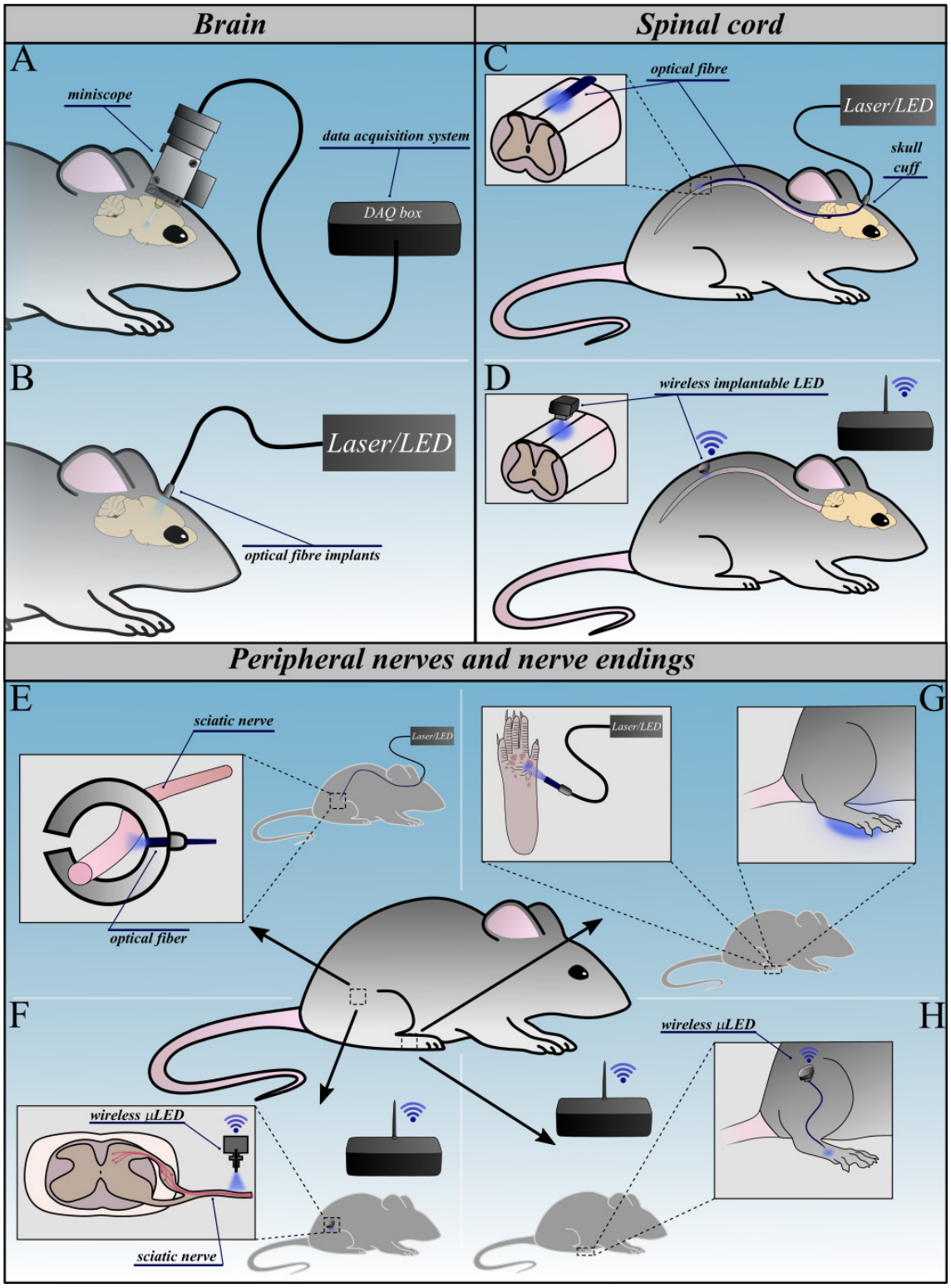

Figure 3. Past, present and future approaches for light delivery within the nervous system. Cartoon illustrating all the different approaches used to deliver light to different regions within the Central and Peripheral Nervous Systems, discussed in this review. **(A, B)** Light delivery approaches for brain imaging. **(A)** Head-mounted microscope system. **(B)** Skull-implanted cuff with an optical fibre cannula; **(C, D)** Light delivery techniques used in the spinal cord imaging. **(C)** Skull cuff with implanted epidural flexible light-emitting diode (LED). **(D)** Wirelessly powered µLED device for stimulation of spinal afferents or spinal cord neurons. **(E, F)** Light delivery approaches for Peripheral Nerves. **(E)** The sciatic nerve is represented, as an example. Fiber-optic coupled nerve cuffs are implanted subcutaneously and connected to the skull. **(F)** Small, wireless µLED devices can directly deliver light to the nerve. **(G, H)** Nerve endings light delivery techniques. **(G)** Transdermal illumination of sensory nerve endings through an external source of light. In the picture two alternative methods are represented (enlargements). **(H)** µLEDs implanted subcutaneously for wireless light-delivery to the area of interests.

is becoming more and more effective, and these devices allow efficient remote photocontrol with minimal tissue damage (107,109,147,157,158). Concomitant light and drug delivery is currently being explored via a combination between light-emitting and microfluidic devices (159).

Gene therapy is the principal tool to successfully and safely deliver photo-controllable molecules to patients. The use of viral vectors has already been effective in the peripheral delivery of transgenes to patients, albeit most studies addressing chronic pain involve direct production and release of analgesic molecules, like GABA or opioid agonists (160,161). AAV vectors are currently used to express ChR2 in retinal ganglion cells of patients, and Herpes Simplex Virus vectors have been used to successfully deliver gene products in humans through intradermal injections (162,163).

Other current limitations of light-based approaches for therapy are the safeness as well as the transient nature of the expression of opsins and photoswitches. Maximal expression of AAV-delivered proteins takes a few weeks, after which the level decreases: routine administration may solve this problem maintaining optimal expression levels. Delivery of the newly engineered bi-stable opsins may partially solve the problem by eliciting long-lasting changes in neuronal activity following low light stimulation (47). Moreover, continuous increase in clinical trials that employ virally mediated gene therapy will boost the improvement of safer vectors for therapeutic treatment, reducing, therefore, the potential occurrence of immune responses.

Thus, despite the critical issues stated before, light-based approaches already represent a powerful and fundamental tool in the study of pain physiology and pathology. Future technological, as well as biological improvements will help to surmount their current obstacles making them a promising candidate for the development of novel therapies in the challenging field of pain management.

Conclusion and future remarks

Light-based pharmacology and genetics have undergone great development in the past two decades. Researchers from various fields recognise the impact that the implementation of these techniques has on their research, as well as the great clinical potential of these approaches, and new interesting targets and applications are emerging at a swift rate. The further development of more and more specific photo-switchable molecules and optogenetic probes, coupled with the advancements in gene therapy and engineering of non-invasive tools to visualise and manipulate their functions *in-situ*, may enable selective and powerful therapeutic interventions and will continue to refine the research on complex neuronal circuits and functions. Finally, the high temporal resolution and cell specificity allowed by these techniques offer great potential for the development of phototherapy as a routinary, powerful and personalised approach to pain treatment that could overcome the limitation of conventional pharmacology.

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Conflict of interest
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