

Title

Changing channels in pain and epilepsy: Exploiting ion channel gene therapy for disorders of neuronal hyperexcitability

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Highlights

- Millions of chronic pain and epilepsy sufferers remain resistant to pharmacotherapy.
- Both disorders are characterised by neuronal hyperexcitability.
- Ion channel gene therapy has emerged as a tool to counteract such hyperexcitability.
- Optogenetics, chemogenetics, and manipulating endogenous channels all show promise.
- We review the key experimental successes and translational challenges of each approach.

Abstract

Chronic pain and epilepsy together affect hundreds of millions of people worldwide. While traditional pharmacotherapy provides essential relief to the majority of patients, a large proportion remains resistant, and surgical intervention is only possible for a select few. As both disorders are characterised by neuronal hyperexcitability, manipulating the expression of the most direct modulators of excitability – ion channels – represents an attractive common treatment strategy. A number of viral gene therapy approaches have been explored to achieve this. These range from the up- or down-regulation of channels that control excitability endogenously, to the delivery of exogenous channels that permit manipulation of excitability via optical or chemical means. In this review we highlight the key experimental successes of each approach and discuss the challenges facing their clinical translation.

1. Introduction

Chronic pain and epilepsy represent major health concerns, together affecting hundreds of millions of people worldwide at a cost to the global economy of hundreds of billions of dollars [1,2]. In Europe, it is estimated that 0.6-0.7% of the general population suffers from epilepsy [3], and up to 20% will experience chronic pain at some point in their lives [4]. Despite the impressive array of drugs available to treat these disorders, a significant proportion of patients remains resistant to traditional pharmacotherapy; 25% of epilepsies are refractory in nature [5], and as many as two-thirds of chronic pain sufferers are dissatisfied with treatment efficacy [6]. Refractoriness is not the only concern. Many antiepileptic and analgesic drugs elicit a range of unpleasant side effects that place restrictive limits on dosing [7,8], and opioid agents used to treat more severe forms of chronic pain are at high risk of abuse [9]. Because surgical intervention is only possible in a small number of cases [10,11], there is a pressing need to develop therapeutic alternatives.

One such alternative is gene therapy, which involves the long-term introduction, overexpression or knockdown of particular genes for therapeutic purposes. In most cases these manipulations take advantage of the natural infection and genome-editing properties of viruses. Viral vectors are engineered to encode the therapeutic genes or gene-editing constructs under the control of promoter elements that target their expression to specific cell types. As such, gene therapy theoretically provides what traditional pharmacotherapy cannot: a long-lasting intervention delivered to a pre-determined population of target cells. Though plagued by a number of early setbacks [12–14], gene therapy now displays considerable promise for the treatment of a number of neurological disorders [15,16].

Neuronal hyperexcitability is a common feature of epilepsy and chronic pain. In epilepsy, hyperexcitability emerges from a range of pathological alterations that shift the excitation-inhibition balance within neuronal networks, leading to the generation of spontaneous, recurrent seizures [17–19]. These alterations include (among others) the death of GABAergic inhibitory interneurons and loss of GABAergic synaptic terminals [20,21], the strengthening of excitatory synaptic transmission by axonal sprouting [22,23], changes in the release and/or re-uptake of excitatory or inhibitory neurotransmitters [24,25], and changes in the type, number, distribution and activation properties of particular ion channels or neurotransmitter receptors [26–28]. Neuronal hyperexcitability in chronic pain can also have multiple underlying causes. These are often grouped according to the type of chronic pain they produce. “Nociceptive” or “inflammatory” chronic pain arises from persistent activation and immunoinflammatory sensitisation of primary nociceptive afferents during ongoing tissue injury [29,30]. In “neuropathic” chronic pain, the culprit is damage to nociceptive neural pathways; accompanying changes in gene expression can lead to ectopic action potential (AP) generation that long outlasts the original nerve injury [31,32]. In both types of chronic pain, initial increases in excitability may be maintained and even amplified by maladaptive strengthening of transmission across central synapses in the ascending nociceptive pathway, a phenomenon termed central sensitisation [33,34].

Despite their immensely different aetiologies, because chronic pain and epilepsy are both characterised by neuronal hyperexcitability, and because the mechanisms underlying excitability are conserved across a broad range of neurons, any manipulation capable of dampening neuronal firing might prove therapeutically efficacious in the treatment of both disorders. As the most direct modulators of cellular excitability, ion channels represent a particularly attractive target in this regard. Guided by extensive functional data detailing the biophysical characteristics of different ion channels, a number of viral gene therapy approaches to reducing pathological hyperexcitability have been explored. These range from the knockdown or overexpression of ion channels that regulate neuroexcitability endogenously, to the delivery of exogenous channels that permit manipulation of excitability via optical or chemical means. In this review we discuss the different ion channel gene therapy approaches that show promise in the treatment of chronic pain and/or epilepsy. We consider the translational hurdles facing each, as well as the difficulties associated with clinical progression of gene therapy in general.

2. Optogenetics

The development of optogenetics over the past decade has revolutionised many branches of basic neuroscience research [35]. The technique employs a set of microbial ion channels and pumps that activate upon stimulation by particular wavelengths of light. After transgenic expression within neurons, these type I opsins permit optical modulation of membrane depolarisation or hyperpolarisation with temporal precision on the order of milliseconds. When combined with the spatial specificity created by placing opsins under the control of cell type specific promoters, this generates a system capable of exquisite on-demand regulation of excitability and firing in a genetically defined population of neurons. In this section we review the therapeutic potential of optogenetics for the treatment of chronic pain and epilepsy.

2.1. Epilepsy

Epileptiform activity is believed to arise from an excitation-inhibition imbalance within neuronal networks. This balance can be restored by either downregulating excitation or upregulating inhibition.

2.1.1. Upregulating inhibition

Upregulation of inhibition can be achieved by optical stimulation of GABAergic inhibitory interneurons expressing algae-derived channelrhodopsin-2 (ChR2). Activation of this non-selective cation channel with 450-490nm blue light produces marked neuronal depolarisation and AP firing [36,37].

ChR2-mediated stimulation of interneurons was recently exploited for therapeutic effect in a rodent model of temporal lobe epilepsy (TLE). Mice expressing ChR2 exclusively within parvalbumin-positive (PV+) interneurons were generated by crossing animals expressing Cre under the PV promoter with those carrying Cre-dependent ChR2. In the resulting PV-ChR2 offspring, *in vivo* optical stimulation of the hippocampal formation significantly attenuated established seizures induced by intrahippocampal injection of kainic acid; almost 60% of seizures detected online via automated electroencephalographic (EEG) analysis were stopped within 5 seconds of ChR2 activation, and average seizure duration fell by more than 40% [38]. Although opsin delivery via selective breeding is obviously not feasible in a clinical setting, this study provided proof-of-principle that optogenetic stimulation of GABAergic interneurons could attenuate seizures *in vivo*.

Stimulation of just one genetically defined neuronal population, the perisomatic-targeting PV+ interneurons, was sufficient to reliably suppress seizures. This result has inevitably fuelled debate over which interneuronal subtype should be stimulated for optimal anti-seizure efficacy. PV+ interneurons are known to play important roles in subduing and synchronising the axonal output of CA3 hippocampal pyramidal neurons [39], yet recent recordings from CA1 have suggested dendritic-targeting somatostatin-positive (SST+) interneurons may have an even stronger influence on pyramidal cell AP generation [40]. Work from Ledri and colleagues supports an approach based on global activation of interneurons. In acute hippocampal slices from mice transgenically expressing ChR2, optical silencing of chemically-induced epileptiform activity was more effective when the opsin was expressed throughout GABAergic interneurons than when it was expressed specifically within PV+ or SST+ subpopulations [41].

2.1.2. Downregulating excitation

Downregulation of excitation can be achieved by optical stimulation of excitatory glutamatergic neurons expressing halobacteria-derived halorhodopsins (HRs). These chloride pumps are preferentially activated by orange/yellow light with a wavelength ~590nm. When transgenically expressed within neurons, such activation elicits membrane hyperpolarisation capable of suppressing the generation of APs [42].

The anti-epileptic potential of HR-mediated neuronal silencing has been investigated by a number of groups. In early *ex vivo* work by Tønnesen and colleagues, mouse pups received

intrahippocampal injections of a lentivirus encoding an HR under the control of the glutamatergic neuron-specific calcium/calmodulin-dependent protein kinase II α (CaMKII α) promoter. In hippocampal organotypic slice cultures prepared from these animals, optical stimulation significantly attenuated epileptiform bursting induced by high frequency stimulus trains or the GABA_A receptor (GABA_AR) blocker picrotoxin [43].

These promising *ex vivo* findings set the stage for more recent work demonstrating HR-mediated seizure suppression *in vivo*. In a breeding strategy similar to that used to deliver ChR2 to PV+ interneurons, mice expressing HR exclusively within excitatory principal neurons were generated by crossing animals expressing Cre under the CaMKII α promoter with those carrying Cre-dependent HR. In the CaMKII α -HR offspring, optical stimulation of the hippocampal formation again successfully attenuated established seizures elicited by intrahippocampal kainic acid. Nearly 60% of seizures detected online were stopped within 1 second of HR activation, and average seizure duration fell by 70% [38].

As for Cre-dependent delivery of ChR2 to interneurons, the weakness of this approach from a translational perspective is its reliance on selective breeding to express HR exclusively within glutamatergic neurons. Should optogenetics progress to the clinic, opsins will need to be delivered using viral vectors. In work by our group, injection of an HR-encoding lentivirus into layer V of the rat motor cortex produced preferential expression of the opsin within excitatory principal neurons. Subsequent optical stimulation of these neurons in a tetanus toxin model of chronic focal neocortical epilepsy significantly diminished established epileptiform activity in the absence of behavioural side effects [44].

The relatively selective expression of HR in excitatory neurons was unexpected given that the transgene was placed under the control of a non-cell type specific cytomegalovirus (CMV) promoter. For translation to the clinic, a more sensible approach would guarantee glutamatergic neuron-specific opsin expression with the use of a CaMKII α promoter. The therapeutic potential of such a construct has recently been demonstrated in a rat model of thalamocortical epilepsy [45]. CaMKII α promoter-driven HR was delivered to excitatory neurons of the ventrobasal thalamus using a serotype-5 adeno-associated viral vector (AAV5). Optical stimulation of transduced thalamocortical neurons reliably interrupted cortical seizures established after photothrombotic induction of stroke.

Intracellular chloride accumulation is a frequent concern in the field of HR-based optogenetics. Chloride accumulation can shift the reversal potential of GABA_AR-mediated inhibitory postsynaptic currents, causing them to become depolarising [46]. For the treatment of chronic pain and epilepsy such a shift would be particularly problematic, as optical stimulation would begin to exacerbate rather than suppress neuronal hyperexcitability. This is especially true for disorders in which chloride ion homeostasis may already be adversely disrupted, such as TLE [47]. Although not all HR variants elicit excessive intracellular chloride accumulation [43], the issue can be avoided by modifying the optogenetic strategy. The use of pulsed rather than continuous optical stimulation, for example, would allow endogenous chloride extrusion pumps (e.g. KCC2) more time to counteract chloride influx [48]. Alternatively, HR might be exchanged for a hyperpolarising opsin that pumps protons rather than chloride ions, such as Arch or ArchT [49,50]. Such proton pumps have yet to be utilised for *in vivo* seizure suppression.

2.2. Chronic pain

The transmission of nociceptive signals from the periphery to the spinal cord is mediated by small-diameter dorsal root ganglion (DRG) neurons. So named for the location of their somata, these pseudounipolar neurons have a bifurcating axon that projects in one direction to the periphery and in another to the dorsal horn of the spinal cord. Hyperexcitability within nociceptive afferents is a critical feature of many forms of inflammatory and neuropathic chronic pain [32,51]. Consequently, optogenetic silencing of these neurons represents a potentially efficacious alternative to classical analgesic intervention. Although optogenetic techniques have been used to modulate the firing of nociceptive neurons *in vitro*, the feasibility of *in vivo* therapeutic silencing has long been doubted due to the misconception that DRG neurons would need to be optically stimulated along their entire

length. As this can be more than a metre in some adult humans, the technical difficulties of total illumination are self-evident.

However, recent work in rodents suggests these concerns may have been unfounded. Using an AAV6 vector encoding HR under the neuron-specific synapsin promoter, Iyer and colleagues expressed the inhibitory opsin within DRG neurons of the mouse hindlimb. Although viral transduction was not specific for nociceptive neurons, subsequent optical stimulation produced an acute analgesic effect, and completely reversed the mechanical and thermal hypersensitivity experienced in a chronic sciatic nerve constriction model of neuropathic pain [52]. An acute analgesic effect has also been achieved with optogenetic inhibition of DRG neurons expressing the light-sensitive proton pump ArchT [53]. In an attempt to selectively transduce nociceptive afferents, an AAV5 vector was designed in which the ArchT transgene was placed under the control of a transient receptor potential cation channel subfamily V member 1 (TRPV1) promoter; TRPV1 is a non-selective cation channel preferentially expressed within nociceptive DRG neurons [54]. Optical stimulation of afferents transduced by direct intraganglion injection of the AAV5 vector resulted in significant increases in the thresholds for acute thermal and mechanical pain. A very promising aspect of these two studies was that optical stimuli were delivered *transdermally*, by shining light on the hindpaw corresponding to the injected/ligated nerve or ganglion. Techniques such as this, which eliminate the need for surgical implantation of stimulation devices, will obviously have a tremendous impact on the speed with which optogenetic therapy can progress to the clinic.

In the work described above analgesic effects were elicited via optogenetic inhibition of neurons within the *ascending* nociceptive neural pathway. Endogenously, pain perception is heavily modified in both a pro- and anti-nociceptive manner by *descending* projections emanating from various regions of the brainstem [55–57]. Recent work by Hickey et al. investigated whether optogenetic stimulation of one such region, the locus coeruleus (LC), could attenuate peripheral nociception [58]. A lentiviral vector encoding ChR2 under the control of a catecholaminergic-specific PRS promoter was used to target expression of the opsin transgene to pontospinal noradrenergic neurons of the LC. In lightly anaesthetised rats, optical stimulation of these neurons in the ventral, but not dorsal, portion of the LC significantly increased withdrawal thresholds to a thermal nociceptive stimulus applied to hindpaw. Although this study did not investigate analgesic efficacy in a model of chronic pain, and experiments were performed on anaesthetised rather than freely behaving animals, it nevertheless represents an exciting proof-of-concept for modulating pain perception via optogenetic stimulation of central brain regions. In the future this may prove therapeutically useful in more global chronic pain conditions that are not amenable to treatment by local suppression of DRG neuron excitability.

2.3. Translating optogenetics to the clinic

The future for optogenetic therapy of disorders of neuronal hyperexcitability looks bright. Despite encouraging experimental advances however, several translational hurdles must still be overcome.

The first of these is a technical issue concerning the miniaturisation of stimulation devices. The power expenditures required for optical neuromodulation are vast; stimulation of an equivalent volume of brain tissue by more archaic electrical means can use as little as one-thousandth of the energy [59]. Creating a fully integrated, implantable device that can generate continuous optical signals for a therapeutically useful length of time will therefore require considerable battery power, and as battery power increases, so must device size. These devices need not be implanted at the site of stimulation; subcutaneous placement will suffice if narrow fibre optic cables are used to transmit light signals to opsin-expressing neurons. But this does not eliminate the need for miniaturisation. Indeed the size of implanted equipment is likely to be an important factor determining acceptance of optogenetic techniques into the clinic, by patients and clinicians alike.

The miniaturisation of optical stimulation devices will be driven predominantly by technical advances in the fields of bioengineering and power storage [60]. The molecular engineering of opsins will also contribute. One such innovative approach exploits single amino acid substitutions to alter opsin deactivation kinetics. The resulting molecules, termed step-function opsins (SFOs),

display prolonged open states that long outlast the activating stimulus [61,62]. From a translational perspective these SFOs represent an impressive energy-saving tool, capable of dramatically reducing stimulation device size by permitting long-term (tens of minutes) modulation of neuronal excitability with short-term (tens of milliseconds) optical illumination.

In the case of epilepsy, an important requirement of optogenetic devices used clinically will be the ability to simultaneously detect and suppress seizures. Such “closed-loop” systems have already been trialled *in vivo* with notable success [38,45,63]. However, their clinical translation will be hampered by a number of issues. First amongst these is the selection of an appropriate seizure detection algorithm. Deconstructing EEG traces to reliably and automatically detect epileptiform activity still represents a central challenge in epilepsy research [64,65], and was recently the subject of a contest jointly sponsored by NINDS, the American Epilepsy Society and the Epilepsy Foundation (<http://ieeg.org>). In a closed-loop system, compromises between the speed and accuracy of seizure detection will inevitably have to be made, and selecting a suitable approach for the clinic can only be guided by extensive *in vivo* testing. One exciting prospect is the emergence of seizure *prediction* algorithms for pre-emptive ictal silencing. Although these have classically suffered from poor reliability, promising recent advances have been made [66].

Unfortunately the creation of closed-loop systems will only exacerbate the issue of device miniaturisation. Current approaches employ tethered set-ups that connect EEG electrodes to external amplifiers and computers. Such tethering is obviously not feasible in a clinical setting, and significant technological advances will be necessary if seizure detection and suppression capabilities are to be combined into a fully integrated, implantable system.

Perhaps the greatest hurdle facing clinical translation of optogenetics is the poor penetration of visible light through brain tissue due to photon scattering and absorption [67]. Gradinaru and colleagues, for example, report that light power sufficient to activate an enhanced HR is only present up to 1.5mm from the tip of an optical fibre with a 30mW output [68]. Although such shallow penetration might suffice in rodent disease models, producing therapeutic efficacy in humans with brains many times larger will require optical stimulation of much greater tissue volumes [48].

Increasing the strength and/or number of optical stimuli seems like the most straightforward solution to this problem. However, the requisite increases in device size are undesirable, and local heating effects from sustained stimulation with powers in excess of $100\text{mW}/\text{mm}^2$ can lead to irreversible tissue damage [69]. An increase in tissue damage is also likely to accompany the implantation of multiple optic fibres, particularly if they are targeted to deep brain regions. Moreover, there is a risk that optical stimulation will be compromised if fibre tips are encased by connective tissue or shifted out of position after surgery [59]; the probability of such outcomes evidently increases with an increasing number of fibres.

To overcome the issue of poor light penetration the molecular engineering of opsins may again prove critical. By rendering opsins more sensitive to optical activation at their preferred wavelengths, weaker signals further from a stimulating source are able to modulate neuronal excitability [70]. A more innovative approach increases the sensitivity of opsins to longer wavelengths of light, creating so-called red-shifted opsins (RSOs) [71,72]. As light of longer wavelength has greater tissue penetration [73], RSOs also permit modulation of excitability in neurons much further from an optical source, opening optogenetic stimulation up to deep and diffuse brain structures for which implantation of optical fibres may be deemed too invasive. In addition, RSOs also offer the tantalising prospect of non-invasive *transcranial* optogenetics; as discussed above, proof-of-principle for *transdermal* optogenetic modulation of DRG neuron firing has already been demonstrated [52,53,74]. And finally, RSOs could permit bimodal suppression of neuronal excitability. Expressing within inhibitory neurons a red-shifted ChR2 with a wavelength preference matching that of an excitatory neuron-expressed HR would allow coincident excitatory inhibition and inhibitory excitation by a single-wavelength optical stimulus [69]. It remains to be seen whether such an approach could be optimised to generate therapeutic efficacy superior to the single-opsin convention.

The many technical challenges associated with *in vivo* optogenetic modulation of neuroexcitability means successful clinical translation is still several years away. However, the

essential role optogenetics now plays in many branches of basic neuroscience research will continue to drive rapid advances in the technology of stimulation devices [60] and the molecular engineering of opsins [72,75], both of which will have considerable translational impact.

3. Chemogenetics

The ability to modulate neuronal excitability via on-demand activation of ion channels is not an exclusive property of optogenetics. Chemogenetic techniques achieve a similar feat using transgenic expression of channels gated by ligands. As administration of these ligands *in vivo* is typically performed systemically, the chemogenetic approach avoids the complications of optogenetics arising from the need for local light delivery. Invasive surgery apart from injection of the viral vector is not necessary, and deep and diffuse neural structures can be targeted.

It should be noted that chemogenetic tools are not restricted to ion channels; a number of G protein-coupled receptors (GPCRs) have been developed that modulate neuronal excitability indirectly via activation of intracellular second messenger cascades [76–78]. Although these GPCRs have recently demonstrated impressive anti-seizure efficacy [79], they fall beyond the scope of this review.

3.1. Chloride channels

Ligand-gated chloride channels permit chemogenetic suppression of neuronal excitability. The glutamate-gated chloride channel from *Caenorhabditis elegans*, GluCl, has proven particularly popular. A heteropentamer composed of α and β subunits, GluCl is activated by ivermectin (IVM), a widely used and orally available anti-parasite drug [80]. Since the early demonstration of its ability to silence neurons [81], GluCl has been optimised for use in mammalian cells [82,83]. One important modification has been the introduction of a single amino acid substitution that dramatically reduces the channel's sensitivity to glutamate [83]. By ensuring GluCl can only be activated by exogenously delivered IVM, this mutation confers upon the channel the property of orthogonality. Orthogonality, the lack of endogenous interaction partners for either the receptor or its cognate ligand, is essential for limiting off-target effects during chemogenetic (and optogenetic) stimulation.

Transgenic expression of GluCl allows regulation of neuronal excitability *in vivo* in an IVM-dependent manner. In the first example of behavioural modification using ion channel chemogenetics, mice received unilateral striatal injections of AAV2 vectors encoding α and β GluCl subunits. When IVM was systemically administered via intraperitoneal injection, the resulting suppression of dopaminergic neurotransmission initiated amphetamine-induced rotational behaviour. This effect emerged within 4 hours of IVM delivery and was completely reversed 4 days later [84].

Although behavioural modification using GluCl/IVM has been achieved by numerous groups since [85–87], the therapeutic potential of the system for treatment of chronic pain and epilepsy has yet to be evaluated. In addition to its protracted on/off kinetics, the GluCl/IVM system is limited by the high doses of IVM required to achieve consistent neuronal silencing [88]. As IVM is known to activate a range of endogenous central nervous system (CNS) receptors at high concentrations [89–92], this dosing limitation could easily compromise the orthogonality of the system. Such concerns have recently been assuaged by the design of modified GluCl channels with greater sensitivity to IVM [93].

One “chemogenetic” chloride channel that has demonstrated therapeutic potential is the human glycine receptor (hGlyR). Although endogenous expression of hGlyRs within the CNS precludes any intervention targeted to the brain or spinal cord, the receptor is absent from primary sensory neurons, suggesting it could be used to suppress DRG neuroexcitability for the treatment of chronic pain. Employing just such an approach, Goss and colleagues delivered the hGlyR $\alpha 1$ subunit to sensory afferents of the rat hindlimb using subcutaneous hindpaw injections of a herpes simplex virus (HSV) vector [94]. Although viral transduction was not specific for nociceptive DRG neurons, subsequent local application of glycine generated profound analgesia in two separate models of

inflammatory chronic pain. The system also demonstrated therapeutic efficacy in a model of visceral inflammatory pain. After injection of the hGlyR α 1-encoding HSV vector directly into the bladder wall, intravenous glycine administration reversed chemically induced bladder hypercontractility.

3.2. The capsaicin receptor

As demonstrated by the Chr2-based optogenetic strategies described above, chemogenetic *enhancement* of excitability within the CNS may prove therapeutically beneficial in the treatment of chronic pain and epilepsy. For neuronal activation, the best-characterised chemogenetic ion channel is the TRPV1 receptor. As mentioned above, TRPV1 is a non-selective cation channel expressed predominantly within nociceptive DRG neurons. An essential nociceptor, the channel is gated by heat, protons and capsaicin, an active component of chilli peppers that serves as the cognate ligand in chemogenetic applications [54].

Although the TRPV1/capsaicin system has been used to successfully enhance neuronal activity both *in vitro* [95] and *in vivo* [96], it is unsuitable for clinical use for a number of reasons [88]. First, orthogonality is compromised by endogenous TRPV1 expression within the mammalian brain [97], and activation of these receptors by endocannabinoid neuromodulators [98]. Second, transgenic TRPV1 expression has been shown to alter aspects of intrinsic neuronal excitability (e.g. resting membrane potential) in the absence of capsaicin activation [95]. Third, the calcium permeability of TRPV1 receptors means high doses of capsaicin can lead to neuronal excitotoxicity [96]. And fourth, administration of capsaicin to central regions could only be performed by intracranial infusion, as systemic delivery would activate peripheral TRPV1 receptors causing intense pain.

Thus, while the TRPV1/capsaicin system may continue to play an important role in chemogenetic neuronal activation in a research setting [99], the approach does not at present display therapeutic potential for the treatment of chronic pain or epilepsy.

3.3. Engineered channels and ligands

Engineered ligand-gated ion channels (eLGICs) represent an exciting technical advance in the field of ion channel chemogenetics. The ligand-binding domain (LBD) of the α 7 nicotinic acetylcholine receptor (nAChR) behaves as an *independent actuator module* in the cys-loop family of ionotropic receptors [100]. This means it can be combined with the ion pore domain (IPD) of any cys-loop receptor to create a chimeric channel gated by ACh. In recent work by Magnus and colleagues, α 7 LBDs were mutated to yield pharmacologically selective actuator molecules (PSAMs) that are unresponsive to ACh but selectively gated by synthetic ligands termed pharmacologically selective effector molecules (PSEMs) [101]. These PSAM domains were combined with distinct cys-loop receptor IPDs permeable to calcium ions, cations or chloride ions, creating a toolkit of orthogonal ligand-gated channels with the ability to depolarise or hyperpolarise neurons in which they were expressed. Since their creation, these eLGICs have been used by a number of groups *in vivo* to chemogenetically modify rodent behaviour [102–105]. As expected given their bespoke design, the PSAM/PSEM systems display unfailing orthogonality, and relatively rapid on/off kinetics have been reported (effect onset and brain clearance within one hour of systemic PSEM administration) [101].

Despite such encouraging early results, the therapeutic potential of these engineered channels for the treatment of chronic pain and epilepsy has yet to be explored. Nevertheless, they will clearly form the foundation of rapid advances in the development of ion channel chemogenetics for both basic neuroscience research and clinical intervention. The cys-loop family of ligand-gated channels comprises no less than 43 vertebrate subunits [106], all with diverse ion selectivity and conductance properties. This is a vast library from which *any* IPD could theoretically be selected to create an orthogonal channel with properties ideally suited to a researcher or patient's particular needs.

3.4. Translating chemogenetics to the clinic

While direct evidence for the therapeutic potential of ion channel chemogenetics in the treatment of chronic pain and epilepsy is still lacking, the approach is clinically attractive. Like optogenetics, chemogenetics permits on-demand, graded modulation of neuronal excitability in a genetically defined population of cells. Unlike its optical cousin however, chemogenetics does not rely on local delivery of an activating stimulus; ligands can be administered systemically (often orally), allowing deep and diffuse neural structures to be targeted without invasive implantation of any stimulation device.

One important weakness of the chemogenetic approach is its temporal imprecision [107]. In stark contrast to the millisecond precision of optogenetics, chemogenetic modulation of neuronal excitability can display onset latencies of tens of minutes to hours depending on the system used, and there is often a significant delay between the termination of ligand delivery and the return of excitability to baseline. For the treatment of chronic pain or epilepsy characterised by persistent seizures, such temporal imprecision could be acceptable, but this is unlikely to be the case for epileptic conditions in which seizures present sporadically, and patients do not have sufficient warning to pre-emptively administer an activating ligand. Ictal episodes that are preceded by premonitory auras or cluster at predictable times (e.g., in catamenial epilepsy) might be more amenable to chemogenetic intervention [79].

One way that onset latencies might be reduced is through the use of subcutaneous pumps that deliver activating ligands intravenously. Although these would require surgical implantation, they would completely abolish temporal delays arising from orogastric absorption of orally delivered agonists. Pumps could also potentially be incorporated into closed-loop devices that dispense ligands automatically upon detection of pathological hyperexcitability [79].

4. Overexpression or knockdown of endogenous ion channels

The greatest hurdles facing clinical translation of optogenetic and chemogenetic techniques stem from their need to deliver exogenous agents, light or ligands, to modulate neuronal excitability. These hurdles can be avoided by employing a gene therapy strategy that constitutively modifies the expression of endogenous ion channels. Although the absence of any activating stimulus removes the capacity to attenuate seizures or pain on-demand, this approach is attractive for its relative simplicity and the variety of potential targets.

4.1. Modulating intrinsic neuronal excitability

Modulating *intrinsic* excitability represents the most direct route by which neuronal firing can be manipulated for therapeutic means. Altering the expression of sodium and potassium channels allows modification of intrinsic excitability without any effect on calcium-dependent signalling.

4.1.1. Overexpressing potassium channels

As essential suppressors of intrinsic excitability, potassium channels can be overexpressed to therapeutically subdue the activity of neurons, or to silence them entirely.

For analgesic purposes, research along this avenue has focussed on the inwardly rectifying potassium channel 2.1 ($K_{ir}2.1$). $K_{ir}2.1$ overexpression has been shown to dampen excitability in several neuronal subtypes both *in vitro* [108,109] and *in vivo* [110]. Adenoviral delivery of the $K_{ir}2.1$ gene to rat DRG neurons significantly reduced the hyperexcitability emerging from chronic ganglion compression [111]. When the viral vector was administered immediately after the compression insult, the development of mechanical hyperalgesia in this model of neuropathic pain was partially prevented. This finding suggests $K_{ir}2.1$ overexpression could be used clinically in a preventative context, to minimise the risk of developing chronic pain after peripheral nerve injury. Delayed

induction of $K_{ir}2.1$ overexpression did not significantly influence pain behaviour, suggesting the approach cannot provide analgesic relief *after* a chronic pain state has already been established. This lack of effect may be the result of irreversible central sensitisation processes that maintain upregulated nociceptive signalling irrespective of subsequent changes in primary afferent firing.

For the suppression of neuronal hyperexcitability in epilepsy, two types of potassium channel have been tested *in vivo*. In work by our group, established neocortical seizures in a tetanus toxin rat model of chronic refractory epilepsy were progressively suppressed by lentiviral delivery of a human voltage-gated potassium channel subfamily A member 1 ($K_v1.1$) gene [44]. A more recent study has demonstrated the anticonvulsant efficacy of transgenic introduction of a 2-pore domain potassium leak channel. Intrahippocampal injection of an AAV5 vector encoding a constitutively active TWIK-related potassium (TREK) channel led to a significant reduction in the duration of acute seizures elicited by lithium pilocarpine [112]. In both these studies, transgene expression was observed predominantly within excitatory neurons despite the use of non-cell type specific promoters. As with any gene therapy approach, it is essential that potassium channels are overexpressed in the correct neuronal subtypes; high levels of expression within GABAergic interneurons for example could amplify pathological hyperexcitability.

4.1.2. Knocking down voltage-gated sodium channels

Voltage-gated sodium channels (Na_v s) are important enhancers of intrinsic excitability, making Na_v knockdown an attractive approach in the therapeutic attenuation of neuronal activity.

Chronic pain seems particularly amenable to gene therapy approaches based on Na_v knockdown; Na_v upregulation contributes heavily to the increases in DRG neuron excitability seen in neuropathic [113,114] and inflammatory [115,116] pain disorders, and many clinically prescribed analgesic drugs function by Na_v antagonism [117].

To avoid unwanted side effects arising from global Na_v knockdown within somatosensory afferents, therapeutic strategies have focussed on Na_v isoforms expressed predominantly within nociceptive neurons. $Na_v1.7$, encoded by *SCN9A*, is one such isoform. Abundantly and preferentially expressed within nociceptive DRG neurons [118], $Na_v1.7$ mutations are associated with a range of genetic pain disorders [119,120]. In 2005 Yeomans and colleagues investigated the therapeutic potential of DRG $Na_v1.7$ knockdown for the treatment of inflammatory chronic pain [121]. Transgenic expression of an HSV vector-delivered *SCN9A* antisense sequence completely prevented the emergence of thermal hyperalgesia after hindpaw injection of the immunopotentiator complete Freund's adjuvant (CFA). $Na_v1.7$ knockdown in DRG neurons has also proven therapeutically efficacious in a rat model of painful diabetic neuropathy (PDN). HSV vector delivery of a Na_v -targeting microRNA completely reversed the increases in DRG $Na_v1.7$ expression that followed induction of diabetes with streptozotocin, and in doing so significantly reduced PDN-associated cold allodynia and thermal and mechanical hyperalgesia [122].

$Na_v1.7$ is not the only isoform whose knockdown may provide long-lasting protection from chronic pain; $Na_v1.3$, encoded by *SCN3A*, is another promising candidate. Although normally expressed at very low levels in adult neurons [123], $Na_v1.3$ expression is dramatically upregulated following nerve injury [124], when the isoform is thought to play an important role in the emergence of neuronal hyperexcitability [125]. In recent work from the group of Stephen Waxman, two $Na_v1.3$ -targeted short hairpin RNAs (shRNAs) were packaged into separate AAV2 vectors injected directly into the DRG of rats with partial ligation of the sciatic nerve. The resulting $Na_v1.3$ knockdown led to partial attenuation of mechanical allodynia in this model of neuropathic pain; both shRNAs were equally efficacious [126].

These studies demonstrate the therapeutic potential of isoform-specific DRG Na_v knockdown for the treatment of inflammatory and neuropathic chronic pain. Although sodium channels are strongly implicated in epileptogenesis, and several front line anti-epileptic drugs selectively inhibit these channels [127,128], Na_v knockdown has yet to be investigated for its

therapeutic effect in models of epilepsy. This may be due to difficulties in selecting an appropriate Na_v target, or concerns about potential off-target effects on physiological network activity.

4.2. Modulating synaptic excitability

Modulating *intrinsic* excitability is not the only way by which neuronal firing can be manipulated. Within neuronal networks, activity can also be modified by varying the degree of excitatory or inhibitory synaptic input. One way to achieve this is via up- or down-regulating the expression of excitatory or inhibitory neurotransmitter receptors. From a gene therapy perspective, the most popular targets in this regard have been NMDA and GABA_A receptors.

4.2.1. Knocking down NMDA receptors

NMDA receptors (NMDARs) play a critical role in excitatory glutamatergic synaptic transmission within the CNS. Receptor activation results in a non-selective cation flux that underlies the slow component of the fast excitatory post-synaptic current [129]. Increases in post-synaptic calcium following NMDAR activation are known to be crucial for activity-dependent strengthening of synaptic transmission [130], and this synaptic plasticity is often essential for long-term enhancement of network excitability. Perhaps unsurprisingly therefore, a number of studies have explored the therapeutic potential of NMDAR knockdown for the treatment of disorders of neuronal hyperexcitability.

Early work by Doring and colleagues took an unusual approach, investigating whether functional NMDAR knockdown could be achieved by eliciting a humoral immune response against the receptor's obligatory NR1 subunit [131]. Transduction of intestinal M cells using a perorally administered NR1-encoding AAV was used to generate this response. Surprisingly, the resulting anti-NR1 antibodies seemed to protect sensitised animals against seizures induced by systemic kainic acid. Moreover, the relative impermeability of the non-seizure-compromised blood-brain barrier (BBB) to the antibodies ensured NMDAR blockade was minimal under resting physiological conditions. Despite these encouraging experimental outcomes, this approach is probably unfeasible from a translational perspective. The risk of side effects arising from global NR1 knockdown is significant, and anti-NR1 antibodies are now thought to be pathogenic in their own right [132].

The therapeutic potential of NMDAR knockdown using more direct gene-silencing constructs has also been investigated. AAV-mediated delivery of a CMV-driven NR1 antisense sequence was shown to significantly increase thresholds for the electrical induction of focal seizures in the rat temporal cortex [133]. Similar anticonvulsant efficacy has been achieved with shRNA-mediated NR1 knockdown. Intrahippocampal injection of an AAV1/2 chimera encoding an NR1-targeted shRNA under the control of the non-cell type specific U6 promoter protected rats against seizures induced by local kainic acid administration [134]. However, such protection came at the expense of impaired hippocampal-dependent learning and compromised neurogenesis. Whether these adverse effects will render central NMDAR knockdown clinically untenable remains to be seen.

NMDAR knockdown outside the brain may display a more forgiving side effect profile, and several studies have investigated this approach for its analgesic potential [135–138]. The first synapse in the ascending nociceptive neural pathway, between DRG primary afferents and neurons of the spinal cord dorsal horn, has proven a particularly popular target; NMDAR-dependent strengthening at this synapse can underlie certain forms of central sensitisation [139]. In work by Garraway and colleagues, an AAV vector encoding an NR1-targeted shRNA was delivered directly to the dorsal horn of mice by intraparenchymal injection. The resulting NR1 knockdown had no effect on the perception of acute nociceptive stimuli, but did prevent the emergence of mechanical allodynia after CFA-mediated induction of inflammatory chronic pain [140]. Similar findings were obtained in rats with chronic pain induced by the inflammatory agent formalin [141]. These studies suggest that inhibiting central sensitisation by knocking down

NMDARs at the DRG neuron-dorsal horn synapse may prove therapeutically efficacious in preventing the emergence of chronic pain after peripheral tissue damage.

4.2.2. Overexpressing GABA_A receptors

GABA_ARs play an essential role in inhibitory synaptic transmission within the CNS. Overexpressing this chloride-permeable ionotropic receptor thus has the potential to therapeutically suppress local network excitability. Within the dentate gyri of pilocarpine rat models of TLE, there are significant reductions in the expression of GABA_AR α 1 subunits accompanied by significant increases in levels of α 4 [142,143]. In an innovative attempt to reverse this α 1 deficit, Raol and colleagues designed an AAV2 vector that encoded the α 1 gene (*GABRA1*) under the control of the α 4 gene (*GABRA4*) promoter. When this vector was injected directly into epileptic dentate gyri, the enhanced α 1 expression not only attenuated acute pilocarpine-induced seizures, but also decreased by 60% the number of rats that went on to develop established epilepsy [144]. It should be noted that a significant proportion of AAV2- α 1-treated rats experienced behavioural abnormalities such as excessive sedation and weight loss. This suggests that, as with NMDAR knockdown, the clinical translation of GABA_AR overexpression may be hindered by adverse side effects.

4.2.3. Knocking down TRPV1 receptors

Although the knockdown of TRPV1 receptors within DRG neurons strictly represents a modification of neither *intrinsic* nor *synaptic* excitability, the approach has recently been used for mild analgesic effect in a mouse model of neuropathic pain [145]. A TRPV1-targeting shRNA was packaged into an AAV9 vector and administered via intrathecal injection to mice with partial ligation of the sciatic nerve. TRPV1 knockdown resulted in a slight but significant increase in the hindpaw withdrawal threshold to a 50°C thermal stimulus. However, the fact that no significant changes were observed in withdrawal thresholds for a thermal stimulus 5°C hotter, an acetone-induced noxious cold stimulus, or a focal mechanical stimulus suggests this approach may struggle in its clinical translation.

4.3. Translating overexpression or knockdown of endogenous ion channels to the clinic

Gene therapy approaches based on overexpression or knockdown of endogenous ion channels will likely progress to the clinic much more rapidly than optogenetic or chemogenetic methods. Technical hurdles stemming from the need to deliver activating light or chemical stimuli do not exist, and the burden of pre-clinical safety and tolerability testing will probably be significantly lighter for human genes than those derived from distant species [146].

Nevertheless these approaches still face a number of translational hurdles. Of particular concern is the constitutive nature of the therapy, which could prove problematic for several reasons. First, constitutive manipulation of neuronal excitability is more likely to be counteracted by homeostatic compensatory mechanisms than a therapeutic intervention delivered transiently and intermittently [147]. Such compensation could adversely influence excitability in other parts of the network, or simply abolish the beneficial effects of transgene expression, generating the need for repeat vector administrations that become progressively less efficacious. The latter concern might be assuaged by the delivery of transgenes specifically designed to resist compensatory downregulation [112].

Second, constitutive manipulations will suppress neuronal excitability continuously, whether pathological activity is present or not. The aim of these interventions is to abolish disease-related hyperexcitability, but the silencing of physiologically salient neuronal communication could generate unwanted treatment side effects. This is less of a concern for chronic pain or epilepsies characterised by persistent seizures, such as *epilepsia partialis continua*, where ongoing suppression of excitability is

therapeutically necessary [148]. In the vast majority of epileptic conditions however this is not the case, and seizures present sporadically separated by long periods of interictal activity. Unfortunately the effects of ion channel gene therapy on interictal activity are often ignored, and appropriately titrating a given treatment to minimise the silencing of normal neuronal function can be challenging.

Finally, should constitutive modulation of neuronal excitability produce unwanted side effects, the intervention cannot simply be “switched off”, as is achieved in optogenetics and chemogenetics by removal of the activating stimulus. Such irreversibility in the face of adverse treatment outcomes will necessitate careful subject selection in the early phases of human testing. For epilepsy, patients with seizure foci deemed suitable for surgical resection would be ideal, as transduced tissue could be immediately excised if undesirable side effects were to arise [44,146].

5. General considerations for the creation and translation of efficacious gene therapy

The studies presented above highlight the great variety of transgenes used to manipulate ion channel expression in the therapeutic attenuation of neuronal hyperexcitability (summarised in Figure and Table). Selecting an appropriate transgene though is only the first step in producing a clinically viable gene therapy. To ensure expression is achieved in the desired target neurons at levels sufficient to counteract pathological firing, all in the absence of adverse side effects, the promoter and viral vector employed for transgene delivery must be carefully considered. In this section we review the different promoters and vectors that have been used in studies of gene therapy for chronic pain and epilepsy. We then discuss the regulatory, manufacturing and socioeconomic hurdles facing clinical translation of gene therapy in general. A comprehensive discussion of vector design, testing and approval is beyond the scope of this article; more detailed accounts can be found in several excellent recent reviews [15,146,149–152].

5.1. Viral vectors

Viral vectors vary considerably in their suitability for different therapeutic applications. In studies investigating ion channel gene therapy for chronic pain and epilepsy, four have so far been used: lentiviral, AAV, HSV and, to a lesser extent, adenoviral vectors.

5.1.1. Adenoviral vectors

Adenoviral vectors have proven a popular delivery strategy in several gene therapy applications, particularly those targeting cancer and liver disease. The vectors have a transgene packaging capacity of ~8kb (can be increased to >35kb in so-called “gutless” vectors), and can efficiently transduce post-mitotic neurons to support high levels of transgene expression. Despite this, adenoviruses are rarely selected for transgene delivery in the treatment of neurological disorders [149]; indeed just one of the studies presented above employs an adenoviral vector [111].

The most likely reasons for this are the vectors’ tendency to support only short-term transgene expression and their significant immunogenic potential. The seriousness of this immunogenicity was made clear in an early clinical trial of enzyme replacement therapy for the non-fatal disease ornithine transcarbamylase (OTC) deficiency. 18-year-old Jesse Gelsinger suffered an acute immunoinflammatory response after intravenous delivery of an OTC-encoding adenoviral vector, and died from multiple organ failure 4 days later [12]. Although adenoviral vectors can be engineered to reduce their immunogenic potential [153], and the risks of an acute systemic response are greatly diminished by delivery directly into the immune-privileged CNS, this early trial continues to bias vector selection for neurological gene therapy against adenoviruses.

5.1.2. AAV vectors

AAVs are the most common vector in clinical trials of gene therapy for neurological disease [149]. Their popularity is reflected in the large number of studies that have employed AAV-mediated transgene delivery in preclinical testing of gene therapy for chronic pain and epilepsy [45,52,53,112,126,131,133,134,140,141,144,145]. Like adenoviral vectors AAVs are capable of transducing post-mitotic neurons, but can support transgene expression of much longer duration (up to 8 years in non-human primates (NHPs) [154]). Moreover, AAVs are not associated with any human diseases and although they can elicit both humoral and cell-mediated immune responses, these tend to result simply in vector elimination rather than acute systemic reactions [155].

AAV vectors do have limitations however. First amongst these is their relatively small transgene packaging capacity (~4.5kb), which may preclude delivery of therapeutic constructs containing large promoter elements and/or ion channel genes. Another disadvantage is their occasional propensity for genomic integration. Although the vast majority (~99%) of AAV-delivered DNA persists in extrachromosomal episomes, a small proportion of vectors integrate their DNA into the genome of the host cell [156,157]. Such integration is concerning as it can lead to a phenomenon known as insertional mutagenesis, where potentially catastrophic mutations arise from genomic insertions within or near the coding regions of actively transcribed genes. While the large proportion of non-coding DNA in the human genome makes insertional mutagenesis unlikely, the consequences of ignoring the possibility can be disastrous. In a 2002 gene therapy trial, retroviral delivery of a γ c chain transgene to patient-derived CD34+ bone marrow cells was initially successful in treating X-linked severe combined immunodeficiency (X-SCID) [158]. A subset of patients soon developed a leukaemia-like condition however, the cause of which was traced to transcriptional disruption arising from transgenic integration near the LMO-2 proto-oncogene [13]. No such adverse outcomes have arisen from human gene therapy trials employing AAV vectors, but oncogenic insertional mutagenesis after AAV-mediated transgene delivery has been observed in rodents [159].

AAVs comprise 12 identified human and NHP serotypes, and over 100 isolated variants with unestablished serology [160]. Such variety, which stems from structural differences in the protein shell (capsid) of the virus, has provided a range of vectors with diverse properties suiting different gene therapy applications. One property that varies considerably among AAV serotypes is viral tropism – the specific cells or tissues a virus will preferentially, or exclusively, transduce. In the CNS for example, AAV8 and AAV9 serotypes only infect neurons [161], while AAV5 vectors have been reported to transduce both neurons and astrocytes [162]. Selection of an appropriate serotype can therefore play an important role in determining the cell type specificity of transgene expression. Viral pseudotyping, in which one viral genome is packaged into the wild-type or modified capsid of a second with preferable tropism, has proven valuable in the design of recombinant vectors with transduction properties specifically suited to a researcher's particular needs [160,163].

Serotype variety has also kept AAV vectors at the forefront of strategies aimed at solving the so-called “volume obstacle” of gene therapy translation [48]. Briefly alluded to above, this term refers to the increases in viral transduction volume that may be necessary if therapeutically efficacious vector delivery in rodents is to be successfully translated to humans with brains many times larger. Increasing the spread of viral transduction through CNS tissue will be especially important for treating more global neurological pathologies, including generalised epilepsies where small, spatially restricted foci of hyperexcitability do not exist.

A number of different delivery strategies have exploited AAV serotype variety to achieve such increases in viral transduction volume [15]. These include the use of serotypes that spread further from the site of single stereotaxic injections (e.g. AAV9 and AAV10) [164]; the delivery of serotypes amenable to transport along neuronal pathways (e.g. AAV8 and AAV9) into brain nuclei with divergent connectivity [165,166]; and vector injection directly into cerebrospinal fluid spaces (e.g. AAV4 and AAV5) [162]. Another innovative approach involves intravenous administration of serotypes that are BBB-permeable, such as AAV9 [167]. With respect to

epilepsy, a very exciting advance has been the combination of multiple capsid serotypes into chimeric AAV vectors that selectively cross the seizure-compromised BBB [168].

5.1.3. Lentiviral vectors

Lentiviral vectors are also able to support long-lasting transgene expression within post-mitotic neurons when appropriately pseudotyped. They are currently the second most popular vector for CNS gene therapy [149], and have been employed by a number of studies investigating such treatments for epilepsy and pain [43,44,58]. Relative to AAV vectors their advantages include a larger transgene capacity (~9kb) and a virtually non-existent immunogenic profile [169].

As with all members of the retrovirus family, lentiviruses naturally integrate their DNA into the host cell's genome, meaning the risk of insertional mutagenesis with these vectors is relatively high. This risk is amplified by the fact that several lentiviral subtypes, such as HIV-1, display an integration location bias for transcriptionally active genes [170]. Fortunately safety concerns regarding genomic insertion have been largely mitigated by the design of therapeutically efficacious integration-deficient lentiviral vectors [171].

5.1.4. HSV vectors

HSV vectors are non-integrating, highly infectious viruses that preferentially transduce neurons. They display a vast transgene capacity (>100kb), meaning almost any therapeutic construct, or combination of constructs, can be delivered in a single vector. Despite these advantageous features, very few clinical trials for CNS gene therapy have employed HSV vector delivery strategies [149], perhaps because of their frequent inability to support long-term transgene expression (*but see 'Promoters' section below*).

One application for which HSV vectors have proven very popular is transgene delivery to DRG neurons [94,121,122]. The unique infection cycle of wild-type HSV explains why. HSV released from infected skin or mucosal membranes invades the peripheral nerve endings of DRG neurons, where the virus is retrogradely transported along the entire length of the peripheral axon branch to the soma [172]. From a gene therapy perspective this retrograde transport is ideal, as it abolishes the need to deliver vectors by direct injection into relatively inaccessible ganglia. Instead, vectors can be administered peripherally via intradermal inoculation, ensuring only those afferents innervating the painful dermatome(s) are transduced.

The retrograde transport of HSV vectors could be utilised centrally to increase the spread of viral transduction through brain tissue [173,174]. Such increases might contribute to a solution for the aforementioned "volume obstacle" of gene therapy translation.

5.2. Promoters

An important decision in the design of gene therapies for epilepsy and chronic pain is whether to suppress pathological hyperexcitability directly by quelling the activity of excitatory principal neurons, or indirectly by boosting the firing of inhibitory interneurons. Whichever approach is chosen, it is vital that the transgene be expressed exclusively within the correct target neurons; accidental suppression of firing within inhibitory interneurons for example would exacerbate rather than attenuate network hyperexcitability. Although the choice of vector can determine cell type specificity up to a point (e.g. in the distinction between glia and neurons), reliably expressing a transgene within a predetermined neuronal subtype almost always requires the use of cell type specific promoter elements. For excitatory glutamatergic neurons the element of choice is the CaMKII α promoter, while for inhibitory GABAergic neurons glutamate decarboxylase (isoform 65 or 67) promoters are the most popular. Subpopulations of GABAergic interneuron may also be selectively targeted using, for example, PV or SST promoters.

The importance of driving transgene expression using cell type specific promoters was

demonstrated in early work by Haberman and colleagues [133]. To suppress seizure-inducing hyperexcitability via NMDAR knockdown, they used two independent non-cell type specific promoters to drive expression of an NR1 antisense sequence within neurons of the rat temporal cortex. When transgene expression was driven using a CMV promoter, there was an increase in the threshold for induction of focal seizures by electrical stimulation. When the transgene was placed under the control of a promoter suppressed by the presence of tetracycline (the Tet-off promoter) however, seizure induction thresholds were significantly reduced. Immunohistochemical analysis demonstrated that these opposite effects likely arose from NR1 antisense expression within distinct neuronal populations: excitatory principal neurons with the CMV promoter and inhibitory interneurons with the Tet-off construct.

This result highlights the inherent unpredictability of driving transgene expression with strong non-cell type specific promoters. But because many commercial viral vectors are supplied with such elements, this strategy is often employed [44,133,134]. Even if cell type specific expression can be achieved with a non-selective promoter in rodent models however, it should not be assumed such specificity will be replicated in humans. As a case in point, lentiviral delivery of a CMV-driven transgene to the rat motor cortex leads to preferential expression within excitatory principal neurons [44], yet a similar vector-promoter combination delivered to the motor or visual cortices of NHPs elicits transgenic expression almost exclusively within glial cells [164]. This difference highlights the importance of thoroughly characterising the cell type specificity of transgene expression in multiple animal models *before* a therapeutic vector progresses to clinical trials.

Promoters also play an important role in determining the longevity of transgene expression. For most gene therapy applications long-term, even indefinite, expression is preferable. The advantages of such longevity are clear; the viral vector need only be delivered once, increasing patient compliance and reducing the risk of eliciting an immune response against repeat administrations. In reality though long-term transgene expression is often difficult to achieve. One reason for this is promoter silencing. Although this phenomenon can occur with a number of therapeutic vectors, those based on HSV are particularly susceptible [175]. Even strong constitutively active promoters such as CMV are silenced within a few weeks of neuronal transduction [176–178]. HSV latency-active promoter 2 (LAP2) sequences may offer a solution to the problem. In wild-type HSV, the LAP2 element escapes silencing while the virus lies dormant in infected neurons [179]. Vectors containing LAP2 promoters have been shown to support CNS transgene expression lasting several months [180,181], and it is thought they might be combined with cell type specific promoters to achieve similar longevity in genetically identified neuronal populations [150].

Despite the general preference for constitutive transgene expression, the ability to terminate transcription in the event of adverse side effects would be extremely attractive from a clinical perspective. Such termination can be achieved with inducible promoters. In these systems, exogenous pharmacological agents are delivered to either activate or suppress promoter-driven transcription [182]. Although inducible promoters have been incorporated into vectors for gene therapy of chronic pain and epilepsy [111,133], their progression to the clinic will be restricted by the need for additional toxicology and tolerability testing of pharmacological components.

5.3. Regulatory, manufacturing and socioeconomic translational hurdles

1990 saw the first human clinical trial for gene therapy [183]. 22 years and hundreds of trials later, alipogene tiparvovec (AAV1-LPL^{S447X}; Glybera) became the first such treatment to achieve regulatory approval in the Western world [184,185]. This extremely low conversion rate reflects the difficulties faced in translating experimentally promising gene therapies to the clinic. In this section we review these difficulties and offer tentative suggestions for how they might be more successfully overcome.

Perhaps the greatest challenge to gene therapy translation comes from regulatory agencies.

In Europe and the USA, gene therapy is subject to the same regulatory controls as traditional pharmacotherapy [186]. As such, viral vector production must comply with good manufacturing practice (GMP) guidelines, and therapeutic efficacy must be determined in multi-phase human clinical trials. The academic research facilities in which most gene therapy vectors are designed rarely have the infrastructure, financial resources or expertise necessary to meet these demands. There therefore exists a strong argument for the creation of publically subsidised or privately funded consortia that might shoulder the responsibility [187].

Whether these calls for greater financial collaboration are heard or not, regulations could certainly be relaxed to allow consenting patients faster access to promising but as-yet-unapproved gene therapies. Although extensive analyses of efficacy, toxicology and tolerability have their benefits, for many patients suffering from diseases refractory to traditional treatments gene therapy represents a last resort, and regulatory restrictions should take this into account if individuals are willing to accept the inherent risks of an incompletely tested therapeutic agent [186]. With respect to toxicology and tolerability, academics responsible for vector design could speed up translation by employing vector backbones that have already been characterised [188].

It is reasonable to assume that the clinical translation of gene therapy might benefit from greater pharmaceutical involvement in vector production and testing. Thus far contribution from these companies has been minimal, possibly due to concerns regarding the small financial returns on offer. Viral vector production would necessitate infrastructural updates to manufacturing and storage facilities, and the cost of these would be difficult to recoup from sales of biotherapies designed to be administered just once to (initially) small populations of patients. Moreover, pharmaceutical companies are unlikely to willingly sacrifice profit by introducing market competition for their highest-earning pharmacological agents.

An additional concern for gene therapy translation is public opinion of the approach. Adverse outcomes from early clinical trials [12,13] continue to cast a shadow in the minds of patients and clinicians alike. From the clinician's perspective, the perceived complexity of genetic approaches relative to established pharmacotherapy may initially limit clinical usage. This complexity issue will be particularly relevant for combination therapies such as chemogenetics and optogenetics [59].

Despite the many translational hurdles gene therapy faces, the technique has recovered from early setbacks to demonstrate genuine promise for the treatment of a wide variety of disorders. The growing list of successful human trials [16] will continue to assuage regulatory, financial and social concerns surrounding the approach, generating momentum for the translation of many more therapeutic vectors.

6. Conclusions and perspectives

Chronic pain and epilepsy are ideally suited to therapeutic interventions targeted at suppressing neuronal hyperexcitability. The manipulation of ion channel expression using viral gene therapy represents one such intervention that is already progressing from proof-of-principle research to the clinic.

From a translational perspective the most straightforward ion channel targets are those that regulate excitability endogenously. While optogenetic and chemogenetic approaches display promise in an experimental setting, the added complexity of activation by exogenous stimuli will inevitably slow their clinical development. Conversely, for the genetic manipulation of endogenous protein expression there have already been numerous successful clinical trials for various disorders and one approved treatment. To identify similar gene therapy strategies capable of treating chronic pain and epilepsy, further preclinical testing will be necessary to guide rational selection of appropriate transgenes. So far these have consisted primarily of ion channel cDNAs or shRNAs targeting ion channel genes. However, exciting recent advances in gene-editing technologies such as the CRISPR-Cas9 system could soon open the door to an entirely novel set of transgenic tools

capable of not only upregulating or downregulating ion channel expression, but also repairing disease-causing channel mutations.

To speed up clinical translation, therapeutic transgenes should be packaged into vectors already characterised for toxicology and tolerability within human patients. As the gold standard for delivery of exogenous genetic material, viral vectors have formed the central focus of this review. It will be interesting to see whether emerging technologies such as nanoparticles can overcome the established bias against non-viral delivery strategies. Whatever strategy is used, the performance of therapeutic vectors within clinical trials for chronic pain and epilepsy will depend heavily on the degree to which regulatory, financial and social concerns surrounding gene therapy are addressed, and ideally, relaxed.

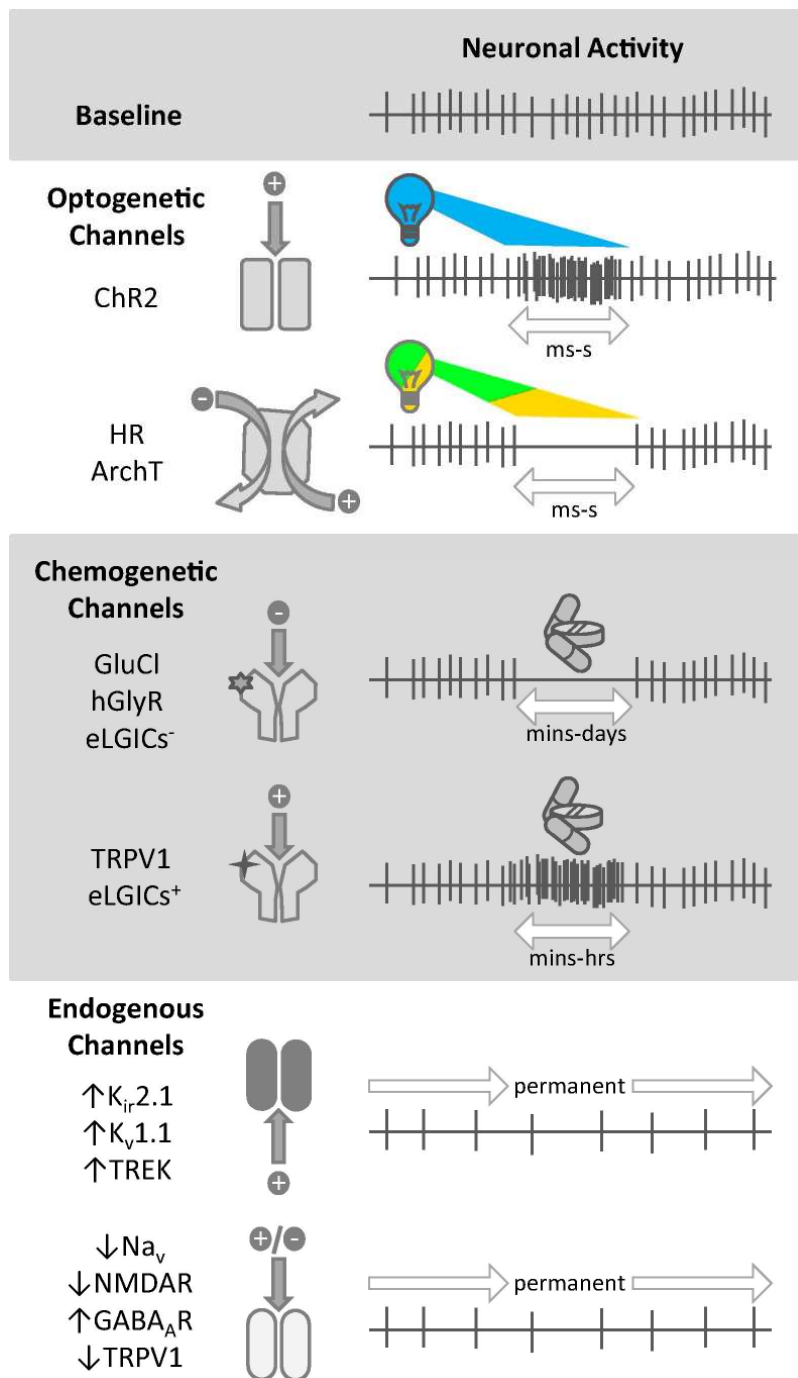


Figure: Schematic of the different gene therapy approaches used to alter neuronal excitability via the manipulation of ion channel expression. The activity of a hypothetical neuron is represented on the right as vertical spikes along a baseline. Optogenetic channels permit light-mediated enhancement (ChR2) or suppression (HR, ArchT) of neuronal firing with a stimulus duration on the order of millisecond/seconds. Chemogenetic channels also permit bidirectional modulation of spiking. Neuronal firing is dampened by chloride ion influx through GluCl, hGlyR, and eLGICs⁻ channels, and increased by cation flow through TRPV1 and eLGICs⁺ channels. In both cases the channels are activated by pharmacological stimuli with effect durations on the order of minutes to days. The expression of endogenous ion channels can be manipulated to suppress neuronal excitability by increasing potassium ion efflux (K_{ir}2.1, K_v1.1, TREK), reducing cation influx (Na_v, NMDAR, TRPV1), or increasing chloride influx (GABA_AR). In all cases the effects are long lasting and most will likely be considered permanent in a clinical setting. As indicated by the small number of remaining spikes, these approaches would ideally spare a small amount of neuronal activity.

	Channel	Effect on Neuronal Firing	Anti-epileptic Potential		Analgesic Potential		Key References
			Feasible?	Tested?	Feasible?	Tested?	
Optogenetic Channels	ChR2	∧	Y	Y	Y	Y	Krook-Magnuson <i>et al.</i> (2013) [38] Ledri <i>et al.</i> (2014) [41] Hickey <i>et al.</i> (2014) [58]
	HR	V	Y	Y	Y	Y	Tønnesen <i>et al.</i> (2009) [43] Krook-Magnuson <i>et al.</i> (2013) [38] Wykes <i>et al.</i> (2012) [44] Paz <i>et al.</i> (2013) [45] Iyer <i>et al.</i> (2014) [52]
	ArchT	V	Y	N	Y	Y	Li <i>et al.</i> (2015) [53]
Chemogenetic Channels	GluCl	V	Y	N	Y	N	<i>n/a</i>
	hGlyR	V	N	N	Y	Y	Goss <i>et al.</i> (2011) [94]
	eLGICs ⁻	V	Y	N	Y	N	<i>n/a</i>
	TRPV1	∧	N	N	N	N	<i>n/a</i>
	eLGICs ⁺	∧	Y	N	Y	N	<i>n/a</i>
Endogenous Channels	K _{ir} 2.1 (↑)	V	Y	N	Y	Y	Ma <i>et al.</i> (2010) [111]
	K _v 1.1 (↑)	V	Y	Y	Y	N	Wykes <i>et al.</i> (2012) [44]
	TREK (↑)	V	Y	Y	Y	N	Dey <i>et al.</i> (2014) [112]
	Na _v (↓)	V	Y	N	Y	Y	Yeomans <i>et al.</i> (2005) [121] Chattopadhyay <i>et al.</i> (2012) [122] Samad <i>et al.</i> (2013) [126]
	NMDAR (↓)	V	Y*	Y	Y	Y	During <i>et al.</i> (2000) [131] Haberman <i>et al.</i> (2002) [133] Kalev-Zylinska <i>et al.</i> (2009) [134] Garraway <i>et al.</i> (2007) [140] Garraway <i>et al.</i> (2009) [141]
	GABA _A R (↑)	V	Y*	Y	Y	N	Raol <i>et al.</i> (2006) [144]
	TRPV1 (↓)	V	N	N	Y	Y	Hirai <i>et al.</i> (2014) [145]

Table: Summary of the different ion channels investigated for gene therapy of chronic pain and epilepsy. For endogenous channels, arrows refer to the direction of the manipulation (↑ or ↓ for up- or down-regulation of expression, respectively). ∧ and V represent the predicted effect of a given manipulation on neuronal firing (enhancement or suppression, respectively). Y (yes) and N (no) denote whether a given channel has been (Tested?) or could be (Feasible?) effectively utilised in gene therapy for epilepsy and/or pain. Theoretically promising manipulations that have not yet been tested are highlighted in grey. *denotes adverse side effects that may limit the therapeutic potential of central NMDAR knockdown or GABA_AR overexpression.

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