Gene Therapy for Pain: Emerging Opportunities in Target-Rich Peripheral Nociceptors

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Abstract

With sweeping advances in targeting and manipulations of genome and transcriptome, medical biotechnology offers unprecedented selectivity and control of a wide variety of biological processes, forging new opportunities for therapeutic interventions. This perspective summarizes the state-of-the-art gene therapies enabled by recent innovations with an emphasis on the expanding portfolio of molecular targets that govern the activity and functions of primary sensory neurons, which might be exploited for the effective treatment of chronic pain.

Introduction

Our brains respond to mechanical, thermal, or chemical stimuli as painful when they reach injurious or life-threatening intensities. At the core of pain sensation lies a complex interplay of a consortium of ion channels in primary sensory neurons, that generate action potentials which convey signals from the periphery to the central nervous system (CNS).

Under physiological conditions, pain sensation is adaptive, but in a variety of pathological changes, it can take on a life of its own, with the generation of pain signals in the absence of noxious stimuli. As many as 20-45% of individuals in Europe and the United States are affected by some form of chronic pain [1,2]. Despite its prevalence and major research advances, therapeutic options remain limited. Commonly prescribed nonsteroidal anti-inflammatory drugs, glucocorticoids, or paracetamol are of partial efficacy. Opioid agonists used as first-line therapy for severe pain, while generally effective, come with major drawbacks, including dose-limiting adverse effects and addiction.

Considering the unmet medical needs, the specificity and efficacy of emerging gene therapy offer an attractive alternative for muting chronic pain. Recent research has revealed a cohort of targets within the electrogenisome - a set of molecules that confer and regulate electrical excitability of primary sensory neurons in dorsal root ganglion (DRG) and trigeminal ganglion (TG) [3] (Figure 1). With improving neuronal targeting and precision delivery methods, gene therapy offers unprecedented control over the transfer of vectors, with growing prospects for medical use. The clinical translation of gene therapy in the treatment of pain, however, comes with challenges imposed by biocompatibility and toxicity, low efficacy and stability of genetic material, off-target action with side effects, high costs and others. Nonetheless, with the arrival

of precision technologies, new opportunities have been recognized, offering tremendous prospects for the use of gene therapy in pain management.

In this article, we review progress in the pursuit of gene therapy for chronic pain, focusing on ion channels within the nociceptive electrogenisome [3], i.e. Na^+, K^+, HCN, TRP, ASIC and Ca^{2+} channels (Figure 1, A-C). We consider the expanding collection of therapeutic targets in experimental models and humans, discuss the advantages and limitations of current approaches, and reflect upon challenges and opportunities for future advances. With further improvements in targeting and delivery systems, the evolving gene therapy methods are anticipated to empower innovative treatments that may alter the landscape of pain medicine.

Genetic approaches in pain therapy

Recent breakthroughs in precision biotechnologies and molecular genetics tools have empowered unprecedented access and control over the genome and transcriptome of mammalian cells, with implications for nearly all facets of animal and human biology. These developments prompted revision of the conventional gene therapy model seeking substitution of dysfunctional genes [4-6] with new concepts and techniques that allow specific manipulations of the genome and transcriptome (Figure 2 A-D). RNA interference (iRNA), for instance, shows promise for therapy of chronic pain, and takes advantage of sequence-specific suppression of gene expression to interfere with protein translation, with several iRNA-based procedures approved for clinical use in other disease conditions [7]. Another approach, also potentially applicable for chronic pain, uses antisense oligonucleotides (ASOs) and acts at the transcriptome level to interfere with mRNA processing, leading to depletion of a protein of interest and suppression of its functions. Both iRNA and ASO-based interference, however, have

limitations in terms of efficiency and cell-tissue specificity. Despite improvements in gene delivery methods, targeting vectors of interest to a specific group of cells such as primary sensory neurons remain a challenge, hindering the translation of experimental therapies into clinical use.

Nobel-winning discoveries in gene editing have generated exciting opportunities for therapeutic intervention, enabling manipulations at the level of single or small groups of nucleic acids and regulatory elements, and offer the possibility of tuning the activity of selected groups of cells, including primary sensory neurons [8,9]. The CRISPR-Cas9 system, for instance, has allowed molecular amendments at the DNA level, with major translational prospects (Figure 2D). Considerable efforts are on the way to improving and broadening CRISPR systems for better efficacy and safety, including targeting regulatory sites or using catalytically inactive Cas9 enzyme (dead Cas9, also known as CRISPRi) with a repression domain, which allows transcriptional changes without interference with genome [10]. The precision of CRISPRi is superior as compared to RNAi and ASO, partly because, unlike interference methods targeting mRNA, dCas9 enables selective manipulations at transcriptional levels. More recent approaches, which take advantage of RNA targeting by Adenosine Deaminase that Act on RNA (ADARs) or catalytically inactive Cas13, also allow RNA editing, yielding transient and reversible modulation of protein expression with better safety [11,12]. Like DNA editing methods, emerging technologies targeting epigenetic mechanisms have shown considerable translational potential for therapy [13].

Most gene therapy systems, including CRISPR, rely on transgene delivery of transgenes using viral vectors (Figure 3), which come with risks of biological incompatibility, genomic stress and unwanted off-target effects. Given these challenges, more precise and effective targeting and delivery methods have been

investigated, including the use of stem cells, functionalized liposomes and immunologically neutral nanocarriers [4,14]. Customizing viral capsids with careful selection of the insertion site of vectors in the genome and self-inactivating properties, as well as the use of synthetic delivery systems is expected to minimize some of the adverse effects and enhance therapeutic outcomes.

Voltage-gated Na^+ channels

Of nine mammalian voltage-gated Na^+ channels ($Na_V1.1$-$Na_V1.9$), $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$ are preferentially expressed in primary sensory neurons, playing an essential role in chronic pain [15]. Most extensively studied, and most thoroughly validated as a human pain target, is $Na_V1.7$, with gain-of-function causing inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD) [16,17] while $Na_V1.7$ loss-of-function produces total insensitivity to pain, including painless fractures, burns and tooth extractions [18]. The key role of $Na_V1.7$ in the activity of peripheral nociceptors and several chronic pain conditions has made it an attractive target for gene therapy (Table 1).

Within primary sensory neurons, $Na_V1.7$ plays a role of a ‘threshold channel”, amplifying weak inputs and facilitating the generation of action potentials, thereby acting as a volume knob [19]. In animal studies, inflammatory pain has been linked with a rise in tetrodotoxin-sensitive current and $Na_V1.7$ overexpression in sensory neurons, while its deletion attenuated the inflammatory hyperalgesia [20]. Data from $Na_V1.7$ KO mice suggests its role in hypersensitivity to heat without effects on neuropathic pain and tactile allodynia [21,22], while transfection of gain-of-function mutant $Na_V1.7$ channels into DRG neurons lowers the firing threshold and increases their response to weak inputs [23]. In line with its role in chronic pain, $Na_V1.7$ repression in nociceptors with the

CRISPR-dCas9 system attenuated pain response in mice [24]. After intrathecal infusion of CRISPR-dCas9 AAV9, pain reaction was reduced in carrageenan-induced inflammatory, paclitaxel-induced neuropathic and BzATP-induced pain models. The deficiency of the $Na_V1.7$ channel also decreased tactile allodynia in neuropathy and reduced thermal hyperalgesia, without changing other sensory functions [24]. Overall, the evidence from preclinical studies advocates the importance of $Na_V1.7$ in inflammatory and neuropathic pain as well as heat hypersensitivity, with ameliorative effects of CRISPR-dCas9 implying $Na_V1.7$ as a viable gene therapy target (Table 1).

Like its forerunner, $Na_V1.8$ has been implicated in inflammatory pain, with $Na_V1.8$ KO mice displaying reduced or delayed carrageenan- and NGF-induced thermal hyperalgesia [25,26]. $Na_V1.8$ was also implicated in chronic visceral inflammatory pain, consistent with its enrichment in abdominal nociceptors [27]. Gain-of-function of $Na_V1.8$ leads to enhanced spontaneous activity, which is likely to contribute to inflammatory pain along with its expressional upregulation caused by proinflammatory mediators [28,29]. Finally, Nav1.8 gain-of-function mutations have been implicated in small-fiber neuropathy in human subjects [30]. Using the antisense approach, $Na_V1.8$ was shown to mediate pain sensitization after chemical irritation of the rat bladder [31]. Attenuation of mechanical allodynia and hyperalgesia with $Na_V1.8$ antisense oligodeoxynucleotide (ODN) implies its role in neuropathic pain [32,33] (Table 1). From the gene therapy perspective, $Na_V1.8$ thus also presents a potentially tractable target in thermal and visceral hyperalgesia and inflammatory pain.

Nav1.9 is the third major Na^+ channel implicated in pathological pain. It produces slow currents over a broad range of potentials, contributing to sustained nociceptive response [34]. Like $Na_V1.8$, $Na_V1.9$ downregulation with ASOs ameliorates mechanical pain in rodent models without adverse effects [35] (Table 1). However, a

study of antisense ODN-mediated knockdown failed to induce antinociceptive effects [36]. Of note, $Na_V1.9$ deficient mice show impaired inflammatory pain without neuropathic pain [37], while its upregulation is implicated in trigeminal neuralgia induced by infraorbital nerve constriction [38]. Gain-of-function mutations have been found to produce severe pain in human subjects [39] with dynamic clamp studies showing a direct role of $Na_V1.9$ in increasing the firing of human nociceptors [40]. The development of methods that target or knock down $Na_V1.9$ has been hindered by its low expression in heterologous systems. New experimental platforms for research of $Na_V1.9$ are being currently explored and may facilitate gene therapies targeting this channel.

In addition to the $Na_V1.7$-$Na_V1.9$ constitutively expressed in nociceptors, the Nav1.3 channel is up-regulated in DRG neurons with injured afferents [41,42]. Because of rapid repriming, this channel makes neurons hyperexcitable [43,44]. Accordingly, $Na_V1.3$ knockdown in DRG neurons attenuates tactile allodynia in diabetic rat models [45] as well as mechanical allodynia induced by nerve injury [46]. The results of genetic manipulation, however, remain controversial, with some suggesting that global or DRG-specific $Na_V1.3$ deletion does not alter pain response [47]. Nevertheless, the re-expression of $Na_V1.3$ channels in DRG neurons following nerve injury with enrichment in human neuromas [48] advocates its relevance as a potential target for genetic interventions.

Voltage-gated K^+ channels

Human potassium channels are divided into four sub-families: (1) voltage-gated K channels (K_V1-4) mediating delayed rectifier and fast inactivating currents; (2) Ca^{2+} activated K^+ channels of repolarization and afterhyperpolarization currents; (3) two transmembrane-domain inwardly rectifying K^+ channels (Kir), and (4) two pore-forming

K^+ channels (K2p) mediating leak currents [49]. Members of all four subfamilies are found in mammalian DRG neurons, with a variety of changes reported in chronic pain [50,51]. K^+ channel loss-of-function due to autoimmune response has been reported in neuromyotonia characterized by inflammation and pain [52] and Morvan syndrome displaying neuromyopathy [53]. KCNQ (K_V7.2 and K_V7.3) gain-of-function mutations [54,55], on the other hand, were shown to stabilize resting potential and attenuate pain by countering DRG neuron hyperexcitability caused by Na_V1.7 gain-of-function [56]. Finally, multiple single nucleotide polymorphisms (SNPs) of K_V9.1 predisposing to chronic pain have been reported [57]. Given the ubiquitous presence of K^+ channels in neurons and glial cells with importance in the action potential generation, it is not surprising that they have been implicated in multiple pain conditions with major relevance as a target to emerging gene therapies.

The unique coupling of S4 and S5 domains in the K_V1.1 subunit endows its mechanoreceptive properties [58], with genetic and pharmacological blockade inducing mechanical hypersensitivity without pain [59]. Its main partner, K_V1.2 has been implicated in chronic pain in rodent studies. A decrease in K_V1.2 mRNA and protein was observed in DRG following peripheral nerve injury [60], an effect mediated by upregulation of an antisense lncRNA that lowers K_V1.2 expression, leading to membrane hyperexcitability and sensitization [61] (Table 1). Inhibition of the lncRNA using antisense RNA spares K_V1.2 expression, attenuates pain response and counters injury-related downregulation of K_V1.2 in a spinal nerve ligation (SNL) model. Overexpression of K_V1.2 using AAV5 full-length K_V1.2 sense RNA rescues mRNA and protein in SNL and mitigates mechanical allodynia, as well as thermal and cold hyperalgesia [61]. Importantly, K_V1.2 sense RNA fragment or full-length K_V1.2 sense RNA did not alter basal nociception, capsaicin-induced pain, or locomotor function

[60,61]. K_V1.2 knockdown by siRNA, on the other hand, induces mechanical and thermal hypersensitivity [62]. Because K_V1.2 function is controlled by non-coding miR-137, K_V1.2-related nociceptive mechanisms could be also targeted using miRNA, reducing allodynia in rats subject to chronic constriction injury (CCI) [62]. Finally, epigenetic silencing of K_V1.2 by G9a (histone-lysine N-methyltransferase 2) was proven feasible, with relevance to the sensitization of peripheral afferents [63]. As clear from several examples of genetic interventions with *Shaker*-related channels leading to modulation of pain sensing mechanisms, gene therapy could be applied for adjustments of their adjustments with effects on chronic pain-related conditions.

K_V2.1 is also of major relevance to chronic pain (Table 1). With slow kinetics and high activation threshold, it plays a stabilizing role during prolonged firing, with itsdeficiency in DRG neurons caused by injury enhancing their firing activity [64]. Importantly, K_V2.1 is regulated by members of the K_V5, K_V6, K_V8, and K_V9 sub-families, i.e. “silent subunits” (K_VS) [65]. Given that K_VS promote hyperpolarization and accelerates the inactivation of K_V2.1-containing channels [66], genetic manipulations of K_VS expression might be useful for chronic pain management. Accordingly, injury-induced K_V9.1 downregulation was reported to reduce K_V2.1 activity and enhance neuronal excitability, leading to augmentation of pain response [50,67]. Likewise, deletion of K_V9.1 promoted the development of neuropathic pain [68], while siRNA-mediated inhibition of K_V9.1 in rats led to neuropathic pain [67]. Finally, mutations in K_V6.4 were shown to promote the excitability of TG neurons during human migraine attacks [69] as well as pain sensation during childbirth [70], with effects attributed to K_V2.1 deficiency. The available data, thus, support the need for additional study of K_VS targeting as a potential strategy for adjusting K_V2.1 activity.

Other K^+ channels implicated in pathological pain include TRAAK, TREK1, and TREK2 K2Ps, which act as mechano- and thermosensors, with their deletion altering mechanical, heat, and oxaliplatin-induced cold sensitivity [71,72]. This effect seems to be mediated by reduced rheobase and increased excitability of sensory neurons [73], with a decrease in TRESK/(k2p18)/KCNK18 mRNA caused by sciatic nerve transection lowering the threshold of pain to mechanical stimuli [74]. The contribution of Kir channels in chronic pain has been also reported, via alterations of microglial response to inflammation [75,76]. These effects are partly due to impairments of buffering extracellular K^+ during intense firing, with Kir4.1 KO mice showing depolarized membrane potentials and reduced K^+ uptake by astrocytes [77]. Finally, members of the Kir3 family (also known as GIRK1-4 proteins) appear to contribute to spinal and general analgesia, in part via coupling to G proteins linked to opioid receptors and related antinociceptive response [78]. While the results of functional studies support the involvement of Kir and K2p channels in the generation of pain response, their suitability as a target for gene therapy of chronic pain remains to be shown.

TRP: transient receptor potential channels

Based on sequence homology, TRP channels are grouped into six subfamilies: TRPA, TRPV, TRPM, TRPC, TRPP, and TRPML, which are expressed along the entire neural axis [79]. Since the discovery of TRP channels as capsaicin and heat receptors [80], major progress has been made in elucidating their structure and mechanisms, a number of endogenous and exogenous ligands identified [81,82]. Genetic aberrations in TRP channels are associated with an array of channelopathies affecting sensory functions, with polymorphisms of TRP genes influencing their prevalence and functions [83]. One of the best characterized TRP channelopathies, rs10166942, is known to lower

TRPM8 expression and reduce migraine incidence [84], while another TRPM8 mutation (p.Arg30Gln) occurs in patients with familial trigeminal neuralgia and enhances channel activation, thereby increasing TRP current amplitude and intracellular Ca^{2+} transients [85]. TRPV1 mutations, on the other hand, have been found in patients with severe, long-lasting pain following corneal refractory surgery, suggesting its role in chronic postoperative pain [86]. As evident from the emerging clinical reports as well as from preclinical studies, dysfunctional TRP channels not only cause a variety of pain-related conditions but also hold great promise as targets for pharmacotherapy and gene therapy of chronic pain (Table 1).

In addition to sensing peripheral noxious, chemical, and thermal stimuli, TRP channels also contribute to pain transmission at central terminals of sensory neurons. Activation of TRPV1 and TRPA1 in brain slices and intrathecal infusion of capsaicin or mustard oil results in increased release of glutamate and pain neuropeptides with the sensation of primary afferents [87,88]. It emerges that TRPV1 and TRPA1 are key players in pathological pain, with their stimulation causing burning, itching, piercing, pricking, and stinging sensation. TRPV1 is particularly well-studied with its integrator role of multiple painful stimuli reported. Compensatory overexpression of TRPV1 in response to nerve injury was shown to stimulate the release of pain mediators and overactive nociceptors [89,90]. Deletion studies of TRP genes also suggest their role in various aspects of nociception, with relevance to the chronic pain. *Trpv4* KO mice, for instance, display attenuated pain-induced behaviour in several tests, including visceral pain response [91] while pharmacological blockade of TRPC5 was found to prevent mechanical hypersensitivity and reduce tactile nociception, including pain sensation induced by skin incision, chemotherapy or complete Freund adjuvant (CFA) injection [92,93].

In animal studies, the role of TRPV1 in nociception has been validated by genetic deletion, depletion of TRPV1 transcripts with siRNA, antibody-induced channel blockade, as well as inhibition with selective antagonists [94-97]. Intrathecal administration of TRPV1 siRNA in mice attenuated capsaicin-induced visceral and neuropathic pain, whereas administration of TRPV1 ASOs reversed mechanical hypersensitivity in spinal nerve ligated rats [98,99]. These observations are of significant interest given that the antinociceptive effects induced by systemic pharmacological inhibitors of TRPV1 have major drawbacks. Injection of AAV6 encoding TRPV1 interfering peptide aptamer encompassing residues 735-772 of TRPV1 into DRG caused a reduction in Ca^{2+} currents and attenuation of cytoplasmic Ca^{2+} transients induced by capsaicin in neuropathic pain model of rat [100]. The same vector applied to rat DRG resulted in attenuation of the symptoms of traumatic nerve injury [100]. With improved delivery vectors and precise targeting, similar genetic interventions might provide effective means for the treatment of chronic pain, especially in conditions associated with heat hypersensitivity and neuropathy.

The gene therapy potential of TRPV1 shRNA was also shown in the orthodontic pain model [101]. In rats, TRPV1 play a key role in the intensification of orofacial pain, with protein and mRNA levels enhanced by tooth movement. Delivery of shRNA lowered the TRPV1 expression and eased the pain response, suggesting the viability of this approach for therapy of orthodontic pain [101]. For retro-axonal delivery of gene therapy, lentiviral targeting to TG neurons using an anti-p75 neurotrophin receptor (NTR) antibody was successfully utilized [97]. After demonstrating the mitigation of capsaicin response in cultured neurons, TRPV1 activity was suppressed in TG of rats, resulting in an antinociceptive effect. A recent report showcased the utility of CRISPR/Cas9 editing for inhibition of TRPV1 phosphorylation by protein kinase-C

(PKC) in inflammatory hyperalgesia [102], where the edition of PKC phosphorylation residue S801 by CRISPR/Cas9 reduced the pain caused by masseter muscle inflammation in mice, without blocking physiological TRPV1 functions [102].

Overall, the depletion of TRPV1 channels in TG and DRG nociceptors with shRNA, or editing of the regulatory sites by the CRISPR/Cas9 system emerges to present viable options for therapeutic interventions with mechanical allodynia, inflammatory pain and hypersensitivity caused by a traumatic injury. These encouraging results underscore the potential importance of TRP channels as targets for gene therapy.

ASIC: Acid Sensing Ion Channels

ASICs are expressed in CNS and peripheral sensory neurons, functioning as sensors of pH changes caused by ischemia, inflammation, trauma, and other pathological conditions. These channels include six members, ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4, which are encoded by four genes [103,104]. Functional ASIC consists of ASIC1a, ASIC2a, and ASIC3 subunits forming homo- or heterotrimers which are expressed in sensory neurons [105,106]. Of note, ASIC2b does not form H^+ gated channels on its own but can influence the properties of channels produced by other subunits [107,108], while ASIC4 regulates the trafficking of functional channels [108]. Because of differential proton-sensitivity, activation and inactivation kinetics, and pharmacological profiles [109], expressional differences and functional changes in ASICs could have wide-ranging effects on pain response.

Making and characterization of *Asic1*, *Asic2*, *Asic3* and *Asic4* KO mice show differences in pain-sensing not only in comparison with wildtype but also between various KO lines. While ample data supports the role of ASIC in nociceptive response,

the interpretation of results has been complicated by residual H^+ activated currents in DRG neurons of KO models [105,110]. Immunohistochemical studies show ample presence of ASIC1a, ASIC1b, ASIC2a and ASIC2 in the CNS of rodents [104,109]. ASIC1a appears as the most ubiquitous and important functional subunit in nociceptors, as evident from the results of biochemical and pharmacological tests [109,111]. Overall, although reports of ASIC1b subunit-containing channels support their key involvement in pathological pain, functional studies show a major redundancy of various ASIC1 and ASIC2 subunits, calling for an in-depth analysis with characterization of their specific role. The challenges imposed by the functional overlap and complex interactions of ASIC1 and ASIC2 in nociceptors complicate the analysis of their translational relevance to various aspects of pathological pain and their targeting with gene therapy.

Unlike ASIC1 and ASIC2 expressed uniformly in all neurons, ASIC3 is enriched in PNS and is the most prevalent ASIC subunit in the DRG [112], playing a key role in inflammatory pain. In DRG neurons, the expression of the *Asic3* gene increases after nerve injury, while its deletion shortens and attenuates mechanical and thermal hyperalgesia in neuropathic pain studies [113,114] (Table 1). The results of rat orthodontic pain studies show that *Asic3* expression is increased in TG neurons in the orthodontic force model, with its silencing by shRNA alleviating mechanical hyperalgesia [115]. ASIC3 suppression by intrathecally siRNA prevented CFA-induced heat hyperalgesia and flinching caused by acidified capsaicin, serotonin, or formalin [112]. In addition to attenuating inflammation and related nociceptive response, inhibition of ASIC3 was shown to eliminate secondary mechanical hyperalgesia of the paw of mice following joint and muscle inflammation. To verify the specific role of ASIC3 in pathological pain, its functionality has been rescued in *Asic3* null mice using a recombinant herpes

simplex virus (HSV) vector of full-length cDNA for the ASIC3 channel [116,117]. In the same vein, the re-expression of ASIC3 with a recombinant HSV vector in afferents of the gastrocnemius muscle restored mechanical hyperalgesia [118], implying that in secondary hyperalgesia, muscle afferents might be the primary site of ASIC3 effects. Another study used artificial miRNAs (miR-ASIC3) directed against the mouse *Asic3* gene to validate its role [116]. In CHO-K1 cells transfected with ASIC3 cDNA, miR-ASIC3 inhibited the expression of ASIC3 and lowered acidic H^+ evoked currents, without altering the ASIC1a channel [116]. After HSV delivery, miRNA-ASIC3 reduced paw and gastrocnemius muscle mechanical hyperalgesia in carrageenan-induced inflammation and suppressed the mRNA and protein expression in DRG and muscle. These findings agree with the reported effects of pharmacological ASIC antagonists, which reduced hyperalgesia associated with muscle inflammation evoked by carrageenan or exercise [111,119]. In CHO-K1 cells co-transfected with ASIC1a and ASIC3, selective depletion of ASIC3 by miR-ASIC3 lowered the amplitude of H^+ currents [116] implying that similar downregulation of ASIC3 in human afferents innervating muscle might counter the hyperalgesia with the inflammatory pain response.

Overall, unlike ASIC1 and ASIC2, ASIC3 subunit emerges as an important gene therapy target with major relevance to neuropathic and inflammatory pain as well as other pain-related conditions concerned with changes in tissue pH, including ischemia and cancer pain. Nonertheless, clinical translation of experimental observation in animal models has been obstructed by the scarcity of mechanistic data and conflicting reports. Also, it remains unclear how the results of ASIC3 studies in rodents, where it is predominantly expressed in the peripheral nervous system, relate to humans with more ubiquitously distributed ASIC3 subunits throughout the central and peripheral nervous system [119,120].

HCN: Hyperpolarization-activated, Cyclic Nucleotide-gated channels

Four members of the HCN family (HCN1-HCN4) belong to the superfamily of K_V and CNG channels activated by hyperpolarization and cAMP [121,122]. Upon activation, HCNs produce *I*h current driving membrane toward the resting potential. Selective permeability for Na^+ and K^+ with an open state at the rest of a neuron makes HCN channels an important regulator of membrane excitability, with their dysfunctions reported in neurological disorders such as epilepsy, cerebellar ataxia, Parkinson's and Alzheimer's diseases [123-125]. Studies of sensory neurons using pharmacological and genetic manipulations suggest that HCN1, 2, and 4 can contribute to chronic pain, while the involvement of HCN3 remains controversial [123].

Comparison of HCN channels in different groups of DRG neurons showed a notable heterogeneity, with HCN1 dominating in large mechanoceptors, while HCN2 is prevalent in medium and small neurons mediating nociception, chemoreception, and thermoreception [126,127]. HCN3 and HCN4, on the other hand, display low and nondiscriminatory expression in all DRG neurons[127]. Because HCN2 controls the firing of pain-sensing primary sensory neurons, it is viewed as an important therapeutic target in neuropathic and inflammatory pain. The results of preclinical studies vary, depending on the model and experimental design. For instance, in CFA-induced inflammatory pain, HCN2 deletion in nociceptors prevented mechanical but not thermal hyperalgesia [128], whereas, in another study, HCN2 KO prevented thermal but not mechanical hyperalgesia in prostaglandin E2 (PGE2) included inflammatory pain model [129]. Importantly, HCN2 deletion prevented both mechanical and thermal hyperalgesia in the neuropathic pain induced by CCI of the sciatic nerve. The loss of HCN2 in primary afferents attenuated the pain response induced in rodents by formalin

(licking, biting, paw lifting), which is thought to be due to the release of inflammatory mediators [129]. From the gene therapy perspective, differential expression of HCN1 and HCN2 channels in different groups of primary sensory neurons presents an opportunity for targeted modifications of their functions with the management of various forms of pathological pain. The results of molecular profiling of sensory neurons demonstrating several distinct types of cells [130] support the prospect of their targeting and functional manipulations with therapeutic outcome.

Difficulties in translational studies of HCN channels are largely due to the dual electro-chemical nature of channel activation, as well as their complex interplay. In small nociceptors, for instance, deletion of *Hcn2* was shown to abolish the voltage shift of the I_h current following cAMP elevation, whereas deletion of *Hcn3* did not alter this voltage shift [131], in agreement with the higher sensitivity of HCN2 to cAMP. On the other hand, while deletion of *Hcn3* had little effect on evoked firing in small DRG neurons, it enhances the excitability of medium-sized neurons, implying its potential role in specific aspects of pathological pain [131]. The involvement of HCN2 has been also explored in diabetic pain models [132], showing that its deficiency attenuates diabetes-associated mechanical allodynia and prevents the activation of secondary sensory neurons in the spinal cord. The same report demonstrated an increase in the intracellular cAMP in neurons in diabetic models, implying that the latter might regulate the level of pain by enhancing HCN2 activity. Importantly, neither neuropathic nor inflammatory pain are altered in *Hcn* null mice [133].

Despite the presence of HCN3 in all groups of DRG neurons and its alleged role in the excitability of medium size sensory cells, in functional tests, HCN3 deletion had little or no impact on inflammatory and neuropathic pain. There was no change also in the threshold of pain response to heat or mechanical stimuli in HCN3 deficient

mice, with no alteration in nociception found also in the inflammatory pain model [131]. Nonetheless, nerve-injured *Hcn3* null mice exhibited levels of mechanical allodynia and thermal hyperalgesia like the wildtype but with reduced mechanical hyperalgesia in response to pinprick [131]. In addition to differential effects in various nociceptive stimuli, HCN2 and HCN3 alterations also display differential effects on various phases of pain response. HCN2 deletion, for instance, does not affect the initial rapid phase of the nociceptive reaction to formalin injection (licking and biting of the injected paw) but lowers the slowly developing inflammatory pain response [129]. In summary, while the results of functional studies of HCN channels in primary sensory neurons advocate their relevance as gene therapy targets for the treatment of multiple aspects of chronic pain, the nondiscriminatory expression of HCN3 and HCN4 in primary sensory neurons with functional redundancy among various members impose a major challenge for selective manipulations of pain response. The intricacies of the biology of HNC channels along with complex neurobehavioral phenotypes of preclinical research models had undoubtedly contributed to the slow progress in their translational studies with regard to gene therapy of chronic pain.

Voltage-Gated Ca^{2+} Channels

Based on their activation voltage, Ca^{2+} channels are divided on (1) high-voltage activated (HVA) that include L- ($Ca_v1.1$-1.4), P/Q- ($Ca_v2.1$), N- ($Ca_v2.2$), and R-types ($Ca_v2.3$), and (2) low voltage-activated (LVA) T-type ($Ca_v3.1$-3.3) [134,135]. The most abundantly expressed in DRG neurons are N- and T-type, followed by $Ca_v1.2$ L-type channels [136,137]. R-type is absent in DRG while P/Q channels are expressed in trace amounts, regulating transmitter release from C and δ-fibers [138].

The nociceptive role of Ca^{2+} channels involves two mechanisms: (1) membrane depolarization and release of excitatory transmitters and (2) regulating Ca^{2+} activated K^+ currents, which control the membrane potential and firing activity of neurons [139]. Most of the data supporting the role of Ca^{2+} channels in pathological pain comes from pharmacological studies, with recent genetic evidence supporting their involvement in inflammatory and neuropathic pain. $Ca_V1.2$ knockdown in dorsal horn neurons using siRNA was shown to reverse nerve injury-related mechanical hypersensitivity [140] (Table 1). CCI of the sciatic nerve causes mechanical allodynia, partly attributed to the upregulation of α2δ-1 accessory subunit of $Ca_v1.2$ in nociceptors [141]. The latter was eliminated by anti-$Ca_v1.2$ siRNA or by $Ca_v1.2$ knockout in the dorsal horn, suggesting the potential utility of gene therapy for countering nociceptor sensitization and mechanical allodynia associated with L-type channel dysfunctions. Interestingly, the increase in $Ca_V1.2$ in the spinal cord after spinal nerve ligation, which correlates with hypersensitivity, is associated with downregulation of $Ca_V1.2$ and $Ca_V1.3$ in DRG neurons [142], implying a complex regulation of L-type channels expression and function.

Reduced inflammatory and neuropathic pain in N-type channel deficient mice is consistent with its role in pathological pain [143] with experimental axotomy causing significant changes in N-type channel expression in DRG neurons [144]. The therapeutic utility of genetic manipulations of $Ca_V2.2$ in pathological pain was shown by targeting collapsin response mediator protein 2 (CRMP-2), known to enhance synaptic transmission [145]. Using a short peptide designated as Ca2þ channel-binding domain 3 (CBD3), it was reported that disruption of CRMP-2 interactions with $Ca_V2.2$ leads to inhibition of N-type current with antinociceptive outcome [146]. These findings suggest that gene therapies targeting N-type channel in primary sensory neurons could preempt, and potentially reverse, neuropathic and inflammatory pain (Table 1).

While counterintuitive, blockade of HVA Ca^{2+} current can increase the excitability of neurons, via a reduction in Ca^{2+}-activated K^+ currents. Genetically encoded auxiliary $Ca_V\beta$-subunit-targeted nanobody (nb.F3) fused to the catalytic HECT domain of the E3 ubiquitin ligase Nedd4L was used to inhibit the activity of HVA Ca^{2+} currents [147]. Upon delivery by AAV9 into the hind paw of mice, Ca_V-aβlator expression in a subset of DRG neurons lowered HVA Ca^{2+} currents and enhanced the spontaneous IPSCs in dorsal horn sensory neurons. In addition to proof-of-concept for delivery of Ca_V-aβlator by AAV with the blockade of Ca^{2+} currents *in vivo*, this report showed that the approach can lead to alleviation of pain. Of note, the antinociceptive effects of Ca_V-aβlator expression in DRG neurons after nerve injury extend over all HVA channels [147,148]. Along with the described antinociceptive effects induced by AAV-targeted ABD3 peptide, these results suggest significant prospects of selective modulation of specific HVA Ca^{2+} subtype as well as a more general approach and interference with HVA in DRG neurons, with an antinociceptive outcome (Table 1).

Unlike HVA N-type, T-type Ca^{2+} influx is activated by weak depolarization, tuning the near-threshold excitability and neuronal response to weak inputs [138,139]. Ample data shows that T-type channels can regulate nociceptive response through interactions with presynaptic syntaxin 1A and SNAP25 proteins [149]. The density of T-type currents in DRG neurons is increased after peripheral nerve injury in rat models of diabetic neuropathy and in spinal cord injury [150,151]. While there are no known human mutations of $Ca_V3.2$ that produce a painful phenotype, it appears that conditional knockout of the T-type channel may attenuate mechanical allodynia linked to neuropathic pain [152]. Several mechanisms controlling $Ca_V3.2$ activity could be used for therapeutic targeting with the antinociceptive outcome. Disruption of $Ca_V3.2$ ubiquitination by the USP5 enzyme, for instance, was reported to cause allodynia

linked to neuropathic pain, whereas ubiquitination increases counter the mechanical hypersensitivity in inflammatory and neuropathic pain murine model [153]. Antinociceptive effects were also achieved with intrathecal $Ca_V3.2$ ASO which caused ~80% decrease in T-type currents in DRG neurons and attenuation of nociceptive responses in naïve and neuropathic rats [154], while similar interventions with $Ca_V3.1$ or $Ca_V3.3$ produced no effects [154]. Overall, the results of targeted manipulation of T-type channels support the viability of the therapeutic approach using genetic methods for achieving analgesia and their potential relevance to targeting various Ca^{2+} channels in primary sensory neurons, to achieve relief of various forms of pathological pain.

Future directions and prospects

Since the first proof-of-concept studies in the 1980s, gene therapy has made major strides in preclinical research and medical translation, delivering multiple life-changing therapies. Invigorated by the success of the Human Genome Project and technological innovations, over the recent decade, the focus of gene therapy has shifted from replacement of faulty genes to DNA and RNA editing and fine-tuning their activity, and transcriptome regulation, empowering unprecedented control and precision in manipulating of the coding and non-coding genome. With over 800 gene and cell therapy programs under development and over a dozen approved by the European Medicines Agency and US Food and Drug Administration, gene therapy has become a new reality, unveiling major opportunities for personalized medicine of a variety of diseases intractable to traditional treatments. PERS MED For more than 1.5 billion people worldwide affected by chronic pain, these developments signal the likely arrival of desperately needed relief.

The rapidly expanding knowledge of the neurobiology of chronic pain with the new data on differential roles of electrogenisome channels in pain mechanisms (Figure 4, Table 1) and the first successful advances in gene therapy of chronic pain in preclinical models marks the arrival of major research and translational opportunities. In the race for effective therapies for chronic pain, identification, cloning and sequencing of ion channels and sensors of noxious signals, and characterization of their roles in neuro-behavioural studies have been crucial milestones. As discussed throughout this article, these innovations hand in hand with clinical data PAIN BIOMARKERS from patients with familial pain conditions have uncovered the major relevance of specific members of the electrogenisome to traditional and emerging gene therapy techniques. Together with functional genome studies, cell reprogramming and new methods for regulation of transcriptomes, these developments have greatly expanded the molecular targets for therapeutic intervention with alleviation of chronic pain of various origin.

The swift pace of innovation in gene therapy of chronic pain exposed also major challenges, among which the immunogenicity of delivery systems, off-target actions of gene therapy with potential toxicity, biostability, and insertional mutagenesis with risks of carcinogenesis are most widely discussed. Research is underway to overcome these obstacles using optimized viral vectors and targeting approaches PERSONALIZED MED, as well as systems with higher transduction efficacy. The application of synthetic nanoparticles and targeting receptors and functionalized carriers, as well as optimizations of RNA-based therapies, among many others, are expected to address some of the challenges. Progress is also being made on the design of more informative clinical trials that meet regulatory and ethical considerations.

Given the expanding portfolio of therapeutic targets and the increasing number of gene therapy approaches, it is not unrealistic to suggest that there will be substantial progress toward more effective treatments for chronic pain in the foreseeable future. As it emerges from the innovative research and technological advances discussed in this article, we are on a glide path toward a new realm of pain medicine.

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Figure legends

Figure 1: Major molecular targets for gene therapy of chronic pain in primary sensory neurons. (A) Schematic representation of the electrogenisome with principal ion channels governing the excitability and generation of action potentials transmitting the pain signal. (B) Representative traces of repetitive firing activity of DRG sensory neuron and its changes by steady hyperpolarizing current, which leads to complete cessation of action potentials and unmasks subthreshold membrane oscillations, showing a highly complex voltage dynamics governing the activity of sensory neurons. Reproduced with permission [155]. (C) Illustrations of typical currents mediated by principal channels of elecrogenisome subject to modifications by gene therapy: Na^{+}, K^{+}, Ca^{+2}, TRPV1, HCN and ASIC currents.

Figure 2: Illustration of major gene therapy approaches used in preclinical models of chronic pain. Selected examples. (A) RNA interference: shRNA – short hairpin RNA; dsRNA – double-stranded RNA; siRNA – small interfering RNA; mRNA – messenger RNA, RISC – RNA-induced silencing complex. After cleavage of shRNA or dsRAN, a siRNA is formed, which reacts with mRNA to provide a platform for the RISC complex, to engage, slice and inactivate the mRNA, and block protein translation. (B) Antisense oligonucleotides (ASO), ssDAN - single-stranded DNA. ASO identifies and hybridizes in a complementary complex with DNA or RNA, attracting RNase H enzyme that cleaves and breakdowns the mRNA, inhibiting protein translation. (C) Gene

augmentation: cDNA – complementary DNA, ZNF – zinc finger nuclease. After cleavage of the target gene by ZFN, the cDNA of the wildtype gene gets inserted in the host genome encoding for a protein with functional enhancement. (D) CRISPR gene editing system: sgRNA – single guide RNA; NHEJ – non-homologous end joining; HRD – homologous recombination deficiency. By targeting Cas9 nuclease to a specific segment of the genome with sgRNA, contingent on protospacer adjacent motif (PAM) and matching sequences, the target DNA is cut at a specific site. This is followed by its editing and NHEJ or HRD repair, which could enable the addition or removal of DNA fragments, leading to changes in protein synthesis with functional alterations.

Figure 3: Summary of major characteristics of common viral vectors used in gene therapy of chronic pain. AV – adenovirus; ND – non-dividing; AAV – adeno-associated virus, LV – lentivirus, HSV-1 – herpes simplex virus – 1. BSL – biosafety level.

Figure 4: Neurobiological mechanisms underlying chronic pain in primary sensory neurons and related changes in their activity, presenting targets for gene therapy. (A) Schematic illustration of the spinal cord with dorsal root ganglion (DRG) comprising primary sensory neurons. Through their afferents, DRG neurons conduct a variety of sensory signals from peripheral receptors to the sensory nucleus of the dorsal horn of the spinal cord (SN). MN – motor neurons. (B) Drawing of an enlarged DRG with three main types of primary sensory neurons: large proprioceptive neurons with fast conducting afferents (blue), medium size mechanoreceptive sensory neurons with medium diameter afferents (brown) and small pain sensing sensory neurons with slow conducting thin afferents (red). (C) Mechanisms of chronic pain in peripheral sensory

neurons and underlying neurobiological processes leading to enhanced nociceptive signalling.

Table 1: Gene therapy targets in the electrgenisome of the primary sensory neurons, their involvements in major neurophysiological processes related to the generation of pain and potential therapy approaches used in preclinical studies.

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Authors contribution: SVO and SGW designed the review and carried out the literature review. SVO and SGW analysed the literature, drafted the manuscript and critically revised multiple versions. Both authors have read the approved the final, submission version of the manuscript to be published.

Competing interests: During the past twelve months SGW has served on the scientific advisory boards of OliPass Corp., Navega Therapeutics, and Medtronic, and has served as an advisor to Sangamo Therapeutics, Exicure, Alnylam Pharmaceuticals, Chromocell, Ionis Pharmaceuticals, and Replay Therapeutics.

Keywords: chronic pain; electrogenisome; sensory neurons; precision medicine; gene editing; CRISPRi-Cas9; ion channels; nociceptive signaling

Figure 1

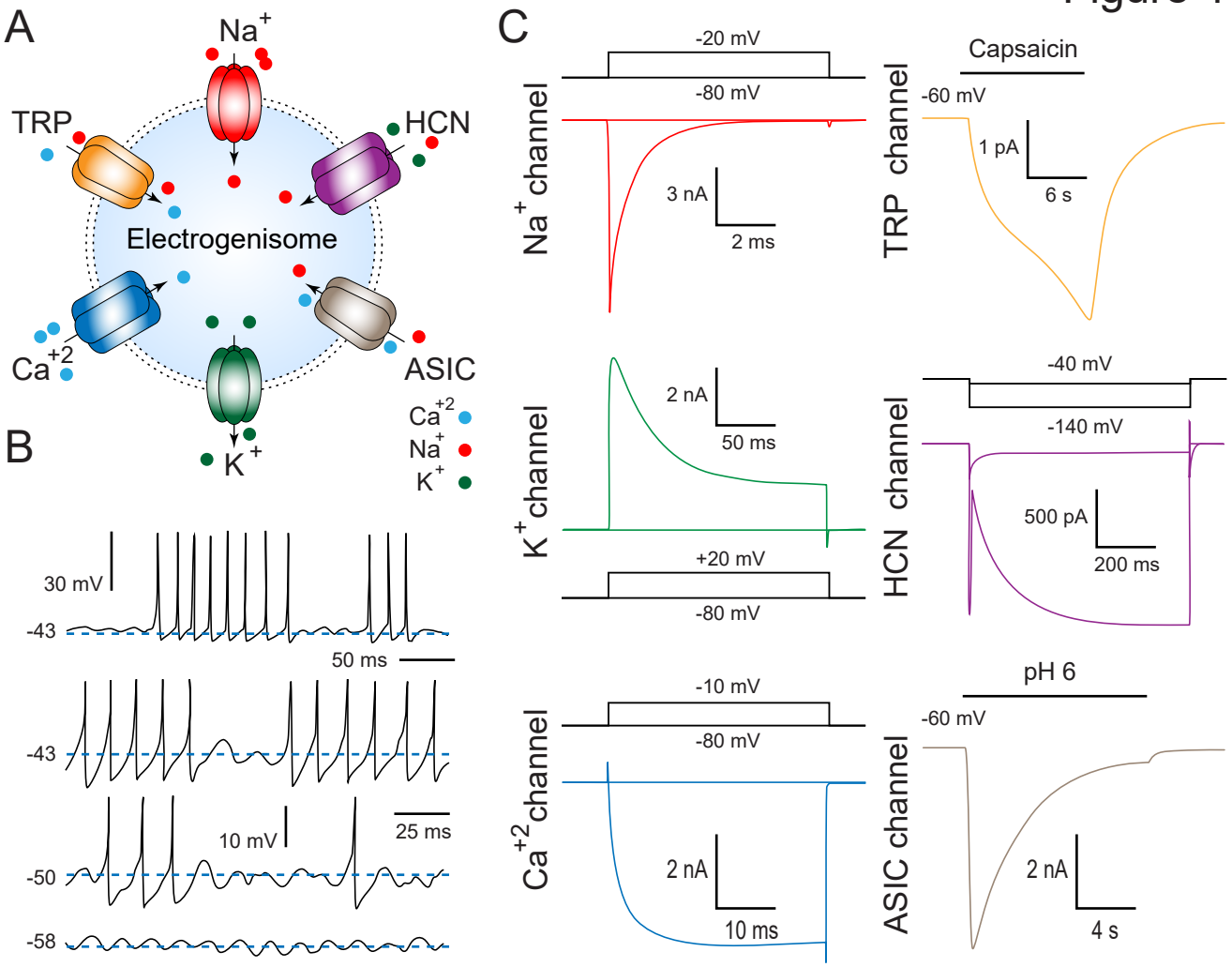
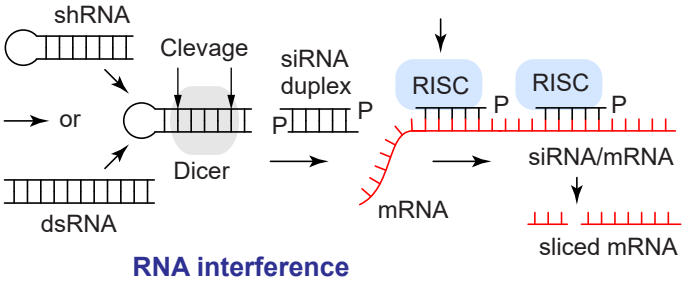

A
Na+
TRP
HCN
Electrogenisome
Ca+2
ASIC
K+
Ca+2
Na+
K+
B
30 mV
-43
50 ms
-43
10 mV
25 ms
-50
-58
C
-20 mV
-80 mV
Na+ channel
3 nA
2 ms
Capsaicin
-60 mV
TRP channel
1 pA
6 s
2 nA
50 ms
K+ channel
+20 mV
-80 mV
-40 mV
-140 mV
HCN channel
500 pA
200 ms
-10 mV
-80 mV
Ca+2 channel
2 nA
10 ms
pH 6
-60 mV
ASIC channel
2 nA
4 s

Figure 2

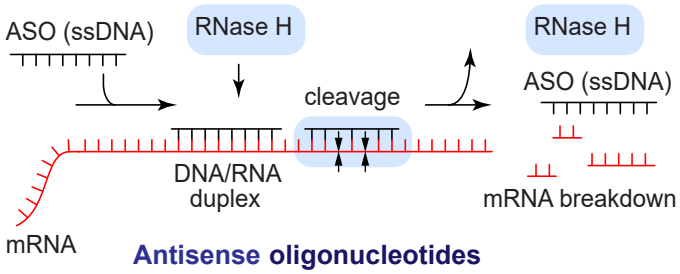
A



RNA interference

forms small RNA double strands to target specific genes and residues of protein expression in neurons

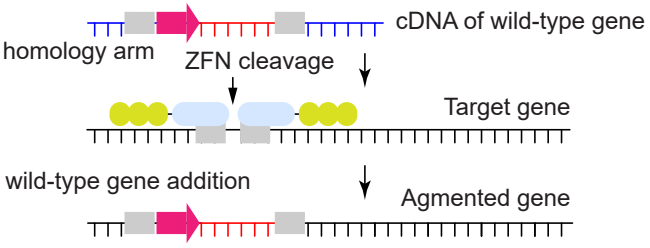
B



Antisense oligonucleotides

hybridize with specific nucleotide sequences of targeted mRNA or micro-RNA in neurons

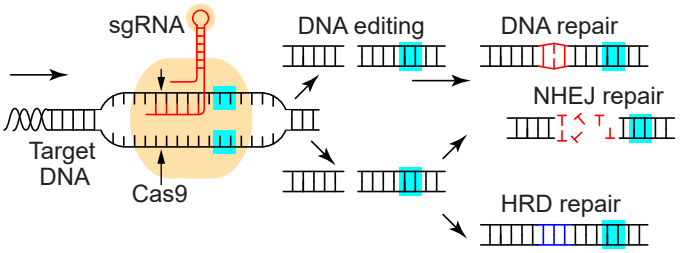
C



Gene augmentation

introdices the target gene into defective cells to compensate for impaired functions with gene products

D



CRISPR gene editing

modifies specific target genes in the genome or RNA or an organism to alter their activity in neurons

Figure 3

Common viral vectors used in gene therapy of chronic pain

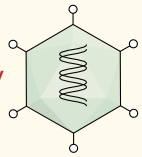
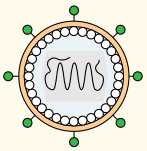
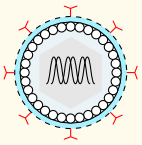
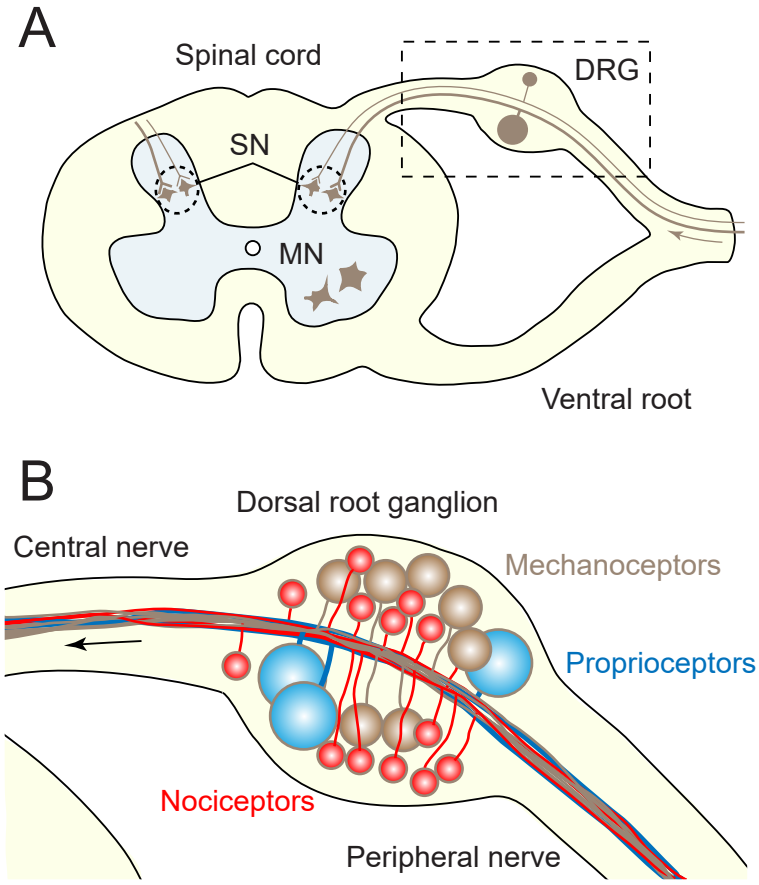
Vector	AV 	AAV 	LV 	HSV-1 
Size (nm)	~ 90-100	~ 25	~ 80-100	~ 80-100
Genome	dsDNA	ssDNA	ssRNA	dsDNA
Capacity (kB)	~ 8-36	~ 4.7	~ 8	~ 40
Transduction	D&ND	D&ND	D&ND	dsDNA
Transduction Efficiency	High	Moderate	Moderate	Moderate
Immunogenicity	High	Low-Moderate	Moderate-High	Low-Moderate
Integration	Non-Integrating	Integrating	Integrating	Non-Integrating
Expression	Days to Weeks	> 1 Year	Lifelong	> 1 Year
Biosafety	BSL-2	BSL-1	BSL-2	BSL-2

Figure 4



C **Mechanisms of chronic pain in sensory neurons and their effects**

Peripheral sensitization	Reduction in threshold and/or an increase in magnitude of responses of the peripheral endings of sensory nerve fibres and at their cell bodies in sensory ganglia. This can occur, e.g. in response to the inflammatory mediators at the site of tissue injury
Ectopic neuronal activity	Inappropriate spontaneous action potential activity in sensory fibres and/or cell bodies, in many cases due to misplaced or dysfunctional (mutant) channels
Gene dysregulation	Transcriptional changes or epigenetic alterations in sensory neurons causing dysregulation of channel expression and function setting off chronic pain after tissue damage or nerve injury
Presynaptic modulation	Pathologically enhanced neurotransmitter release from nociceptors after nerve injury causing synaptic plasticity or sprouting of sensory terminals via presynaptic dysregulation
Switch of sensory modality	Acquisition of new modalities by sensory neurons after injury, i.e. transmission of nociceptive signals by mechanoreceptors (mechanical allodynia) after injury