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REVIEW ARTICLE

Non-canonical Molecular Targets for Novel Analgesics: Intracellular Calcium and HCN Channels

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Abstract: Pain is a prevalent biopsychosocial condition that poses a significant challenge to healthcare providers, contributes substantially to a disability, and is a major economic burden worldwide. An overreliance on opioid analgesics, which primarily target the μ-opioid receptor, has caused devastating morbidity and mortality in the form of misuse and overdose-related death. Thus, novel analgesic medications are needed that can effectively treat pain and provide an alternative to opioids. A variety of cellular ion channels contribute to nociception, the response of the sensory nervous system to a noxious stimulus that commonly leads to pain. Ion channels involved in nociception may provide a suitable target for pharmacologic modulation to achieve pain relief. This narrative review summarizes the evidence for two ion channels that merit consideration as targets for non-opioid pain medications: ryanodine receptors (RyRs), which are intracellular calcium channels, and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which belong to the superfamily of voltage-gated K^+ channels. The role of these channels in nociception and neuropathic pain is discussed and suitability as targets for novel analgesics and antihyperalgesics is considered.

Keywords: Neuropathic pain, drug development, HCN, ryanodine receptor, analgesic, antihyperalgesic.

1. INTRODUCTION

The treatment of pain is a frequent challenge to healthcare providers and a substantial medical and economic burden worldwide. In the United States (U.S.), 20% of adults have chronic pain, including 78% with high-impact pain that causes limitations to daily activities or work on most days [1]. These rates are consistent with estimates in other regions of the world, including Europe [2], China [3], and developing nations [4]. The total cost in medical care and lost productivity in the U.S. is estimated to be at least $560 billion each year [5, 6]. Pain accounts for over 20% of visits to the emergency department [7], while back and joint pain may account for over half of visits to healthcare providers to address chronic conditions [8]. A common cause of pain is surgery. Up to 12% of patients may experience severe to extreme pain following surgery, while over half report at least moderate pain [9]. Unfortunately, each increase of 10% in the proportion of time postoperatively with severe pain is associated with a 30% increased risk for developing chronic post-surgical pain [10]. Thus, both acute and chronic (which includes neuropathic) pains are common conditions that require substantial resources from the medical community to address.

A historical and persistent overreliance on opioids to treat pain has led to tragically high rates of morbidity and mortality in the U.S. and worldwide. In 2017, over 46,000 deaths were caused by opioid overdose in the U.S., and nearly a third of these fatalities were from prescription drugs [11]. Pain prescription misuse is the second most common form of illicit drug use in the U.S., with ~12% of the population aged 12 years and older reporting misuse of any prescription pain reliever in 2018 (Table 1.98B from [12]). The prevalence of opioid misuse has been driven by extraordinarily high prescribing rates of opioid-based analgesics. Although decreasing in number since 2012, there were over 168 million prescriptions for opioids in the U.S. in 2018, which translates to 51 prescriptions per every 100 persons (www.cdc.gov/drugoverdose/maps/rxrate-maps.html). For adults presented to an emergency department for pain, approximately 35-45% of visits will include a prescription for an opioid, depending on the patient's age. Prescription opioids have, in turn, led to rising rates of abuse of non-prescription drugs, such as heroin and fentanyl (www.cdc.gov/drugoverdose/data.heroin.html; www.cdc.gov/drugoverdose/data.prescribing/prescribing-practices.html). Worldwide, opioids are used by 58 million people, cause 66% of drug use disorder-related deaths, and account for half the disability-adjusted life year loss from drug abuse [13].

These issues have led to recent guidelines from the U.S. Centers for Disease Control and Prevention to limit the use of opioids [14]. The current emphasis on avoidance or mini-

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mization of opioid use, however, has led to wariness among providers to care for patients prescribed these medications, which may lead to conversion to illicit substitutes and failure to address other medical conditions [15, 16]. A multifaceted, aggressive strategy is needed to confront the opiate abuse crisis, and a chief component is the development of non-opiate pharmacologic therapies for pain [17]. This scientific goal is included in the mission of the Helping to End Addiction Long-term^{SM} (HEAL) Initiative, a multi-agency program to confront the opioid crisis led by the National Institutes of Health (NIH).

Pain is defined as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" [18]. It is a complex biopsychosocial experience [19] often triggered by nociception, the biological activity of the sensory nervous system in response to a noxious stimulus [18]. Nociception involves peripheral sensory neurons, the spinal cord, and the brain, with various ion channels mediating the transmission and processing of the nociceptive signal [20]. Opioids primarily target the μ-opioid receptor, but various other cellular proteins could be successfully modulated to achieve analgesia [21]. Pain can be broadly categorized into either acute or chronic pain, with chronic pain defined as pain that persists or recurs for more than three months and chronic pain can be further classified into multiple subtypes and/or conditions [22]. These subtypes are relevant because they may predict the efficacy of pharmacologic therapies, such as use of gabapentinoids for neuropathic pain [14].

The focus of this review is intentionally limited to two distinct ion channel families – ryanodine receptors (RyRs) and hyperpolarization-activated cyclic nucleotide (HCN) regulated ion channels and their potential as molecular targets for the development of novel therapeutics for the treatment of acute and chronic (neuropathic) pain. This focus is intended to cogently summarize existing knowledge as well as to provide a critical assessment of that information. It is worth noting, however, that numerous alternative targets have proposed these indications (beyond the commonly discussed voltage gated sodium and calcium channels [23]), including nicotinic acetylcholine receptors (nAChRs) [24, 25], Transient Receptor Potential (TRP) channels [26, 27], calcitonin gene-related peptide *(*CGRP) receptors [28, 29], cannabinoid receptors and related regulatory pathways [30-33], and neurotensin receptors [34-36], and the interested reader is directed to literature for further information in those areas. The basic neurobiology of RyRs and HCN channels is discussed, emphasizing their role in nociception and potential suitability as pharmacologic targets as analgesics for the treatment of acute pain and use of antihyperalgesics for the treatment of neuropathic pain.

2. INTRACELLULAR CALCIUM CHANNELS

2.1. Overview

Ryanodine receptors (RyRs) are intracellular channels that allow the efflux of calcium from the lumen of the sarco/endoplasmic reticulum to the cytoplasm. RyRs are homotetramers, exceeding 2000 kDa in size, with a mushroom-shaped quaternary structure, in which the stalk spans the ER membrane and cap protrudes into the cytoplasm [37]. There are three isoforms of RyRs, with ~65% homology, distinguished by their localization and method of channel opening. RyR1s are mechanically coupled to L-type, dihydropyridine receptor (DHPR) calcium channels ($Ca_V1.1$), such that depolarization-induced opening of DHPRs causes opening of RyR1s [38-40]. For RyR2 and RyR3, changes in cytosolic and SR/ER lumenal calcium are responsible for triggering channel opening [41, 42]. In skeletal and cardiac muscle, RyR1 and RyR2, respectively, mediate calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (SR), a process by which elevations in cytosolic calcium cause efflux of calcium from SR stores [43]. CICR enables excitation-contraction coupling by calcium binding to troponin to shift tropomyosin from actin, allowing myosin to bind to actin [44]. Several diseases have been definitively linked to inheritable mutations in RyR1 and RyR2, including malignant hyperthermia and central core disease, causing skeletal muscle pathologies, and catecholaminergic polymorphic ventricular tachycardia, which is associated with potentially fatal cardiac arrhythmias [45, 46].

The roles of RyRs in neurons are not as well understood [47]. Though originally designated as skeletal, cardiac, and brain isoforms, RyR1, RyR2 and RyR3, respectively, are all expressed in the central nervous system. In the brain, the isoforms display region-specific and developmental differences in their expression, but, overall, RyR2 predominates [48-50]. RyRs have been implicated in learning and memory [51] and in various CNS pathologies, such as Alzheimer's disease [52], post-traumatic stress disorder [53], and seizures [54].

2.2. Dorsal Root Ganglia and Spinal Cord Mechanisms

Of particular relevance to the topics of nociception and pharmacologic targets of analgesics are the dorsal root ganglia (DRG) and dorsal horn of the spinal cord [55]. The DRG contain sensory neurons that relay peripheral nociceptive signals to the central nervous system [55]. Thus, slices and cultures containing neurons from the dorsal horn and DRG are an important model to study mechanisms of pain transduction. Although the precise localization of RyRs in the spinal cord and sensory neurons is not well studied, RyR1 and RyR2 are likely to be present in both the anterior [56] and dorsal horns [57] of the spinal cord. In neurons of the DRG, RyR3 appears to predominate [58, 59], although mRNA of all three isoforms is present at approximately equal amounts, and RyR2 and RyR3 protein expression is increased in DRGs in response to spinal cord injury [60].

Despite limited evidence of their specific distribution, studies have demonstrated the relevance of RyRs to calcium signaling in DRG and the spinal cord and implicated their relevance to nociception. For example, in primary cultures of DRG neurons, caffeine, a RyR agonist, increased cytosolic calcium as measured by fura-2-based microfluorimetry,

although primarily in somata as opposed to processes [61]. Notably, the ability for caffeine to repeatedly elicit an increase in cytosolic calcium was dependent on whether the neuron had been previously depolarized, such that depolarization may have "charged" the ER to allow subsequent release. Thus, RyRs may be involved in magnifying calcium transients in the soma of DRG in a use-dependent manner. In slices of the lumbar spinal cord studied with patch-clamping and two-photon calcium imaging, back-propagating action potentials (APs) evoked increases in calcium in the somatic cytosol and nucleus of lamina I neurons, with a contribution in these compartments by CICR from RyRs [62]. The authors speculated that augmentation of cytosolic calcium by CICR in the somatic cytosol and nucleus may have a role in affecting gene transcriptions based on neuronal activity.

RyRs have been shown to contribute to the induction of long-term potentiation (LTP) in synapses of the spinal cord dorsal horn. LTP is a form of neuronal plasticity in which a conditioning stimulus leads to a sustained increase in synaptic efficacy, and it serves as a molecular model for hyperalgesia and chronic pain [63]. Low-frequency stimulation of the sciatic nerve in rats caused potentiation of C-fiber-mediated excitatory post-synaptic potentials (EPSPs) which could be blocked by dantrolene, a RyR receptor antagonist [64]. Similarly, inhibition of RyR by ryanodine (which causes inhibition of RyRs at high micromolar doses but potentiation at 1 μM and lower) and dantrolene prevented LTP in the lumbar dorsal horn following tetanic of Lissauer's tract in rat spinal cord slices [65]. Furthermore, LTP of C-fiber-evoked field EPSPs was prevented *in vivo* following tetanic stimulation of the sciatic nerve by intrathecally administered ryanodine [65, 66] in a dose-dependent manner and by dantrolene [66]. These results were behaviorally confirmed in experiments in which dantrolene, applied intrathecally prior to tetanic sciatic stimulation, prevented lowering the mechanical nociceptive threshold to the paw withdrawal test [66]. RyRs may also be involved in the development of hyperalgesia associated with diabetes. In streptozotocin-induced diabetic mice, tail-flick latencies were lowered compared to control mice. Inhibiting RyRs by intrathecal administration of ryanodine attenuated thermal hyperalgesia, whereas thapsigargin, a sarco(endo)plasmic reticulum ATPase (SERCA) inhibitor preventing calcium uptake into the ER, had the opposite effect [67]. Thus, by regulation of cytosolic calcium, RyRs may contribute to diabetes-associated pain.

RyRs have an important role in hyperalgesic priming, a process of sustained, excessive responsiveness of nociceptive neurons following a noxious stimulus to future insults mediated by increased PKCε activity [68]. Hyperalgesic priming is a molecular model for the transition from acute to chronic pain [68]. In rats, local RyR potentiation by injection of ryanodine into the paw lowered the mechanical nociceptive threshold measured with the paw withdrawal test after several days [69]. PKCε lowered the mechanical threshold to pain by increasing cytosolic calcium mediated by RyRs, which in turn activated α calmodulin kinase II (α-CaMKII) [70]. Interestingly, the ability of RyR potentiation to lower the mechanical nociceptive threshold in rats exhibits sexual dimorphism. The dose of ryanodine necessary to cause hyperalgesic priming was substantially lower in female rats, and knockdown of estrogen receptor α, but not β, reduced the priming effect [69, 71]. Inositol 1,4,5-triphosphate receptors (IP_3Rs) appear necessary to enable RyR-mediated hyperalgesic priming, as pharmacologic inhibition of IP_3R prevented priming [72]. Furthermore, IP_3R priming was dependent on both RyR and SERCA channels [72]. Thus, a positive feedback loop of calcium efflux from the ER, causing sustained elevation of cytosolic calcium, is an important step to cause hyperalgesia in rodent models.

RyRs may also be involved in hyperalgesia caused by inflammation. For instance, bradykinin, an inflammatory peptide involved in pain signaling, increased excitability in a subpopulation of neurons in primary cultures from the DRG, an effect that was substantially attenuated by inhibition of RyRs by ryanodine [73]. In neurons from the trigeminal ganglia, bradykinin agonism caused an acute rise in cytosolic calcium, which was reduced by dantrolene but not xestospongin C [74]. Those results, however, are challenged by experiments with DRG neurons in which caffeine and bradykinin did not overlap in their ability to elicit an increase in cytosolic calcium, suggesting these two agents caused an increase in calcium from separate pools [61].

While most studies have indicated that RyR potentiation mediates LTP and hyperalgesia, some results indicate the converse is true. For instance, in one study, RyR potentiation prevented PKCε activation through CaMKII inhibitory feedback, and local RyR injection prevented β-adrenergic mediated mechanical hyperalgesia in rats [75]. The authors of this study proposed intracellular signaling mechanisms in which the cell signaling "history" and, specifically, the degree of calmodulin-dependent kinase II (CaMKII) activation, determined if a stimulus would be sensitizing or desensitizing. In this context, RyR agonism could activate CaMKII by increasing cytosolic calcium concentrations, preventing PKCε-mediated hyperalgesia. In experiments examining the role of the imidazoline receptor (I_2R) in mechanical pain, inhibition of RyRs attenuated the antinociceptive effect of I_2R agonism [76]. Thus, while most studies have found that RyRs mediate hyperalgesia, RyRs may be involved in competing, calcium-dependent intracellular signaling pathways, and their role in pain processing is possibly context-dependent.

2.3. RyR Mechanisms in the Brain

RyRs may also be relevant to pain transduction in the brain. In thalamocortical neurons expressing primarily RyR2 and RyR3 isoforms, pharmacologic inhibition of RyRs increased tonic firing and increased behavioral pain responses to stimuli modeling chronic inflammatory pain but not acute mechanical or thermal pain; the converse responses were obtained with RyR potentiation [77]. Pharmacologic modulation of ER Ca^{2+} stores by intraventricular injection of various ER-targeted drugs altered behavioral responses to thermal pain in mice. Specifically, antagonism of RyRs and IP_3Rs decreased the nociceptive threshold and potentiation

of RyRs increased the threshold [78]. RyR inhibition was also able to inhibit the antinociceptive effect of physostigmine, an anticholinergic medication. In mice, intraventricular injection of antisense oligonucleotides against RyR isoforms decreased antinociception by physostigmine in response to thermal and mechanical pain [79]. This effect was isoform-specific, as knockdown by intraventricular injection of antisense oligonucleotides to RyR1 and RyR3, but not RyR2, reduced the effect of physostigmine [80].

2.4. Pharmacologic Considerations for RyRs

Drugs currently identified as analgesics may rely on the modulation of intracellular calcium stores to exert their effects. Consistent with the bulk of evidence demonstrating that RyR inhibition decreases or prevents hyperalgesia, inhibition of RyRs generally augments the analgesic effects of established pain medications, while the converse is true for RyR potentiation. For example, opiate-mediated antinociception is modulated by intracellular calcium concentrations [81]. Potentiation of RyRs inhibits the analgesic effect of morphine on thermal pain in mice [81] whereas inhibition of RyRs can partially reverse the development of morphine tolerance [82, 83]. Similarly, a decrease in thermal nociception by intracerebral injection of ryanodine to block RyRs was observed in the treatment of mice with trans-resveratrol [84]. RyRs may also have a role in opioid-induced hyperalgesia. Administration followed by abrupt washout of the potent opioid remifentanil caused LTP of C-fiber-evoked EPSPs in the spinal cord dorsal horn following low-frequency stimulation of the dorsal root, but inhibition of RyRs with dantrolene prevented LTP due to opioid withdrawal [85]. These studies suggest that a key pathway contributing to analgesia is the modulation of cytoplasmic calcium by RyRs.

Only one medication used in humans, dantrolene, specifically targets RyRs, and it is approved as a RyR antagonist to treat spasticity as well as malignant hyperthermia (MH) [86]. In off-label use, patients with MH-related myalgias or other musculoskeletal symptoms may experience improvement of cramping and pain with consistent administration of oral dantrolene [87, 88]. These studies demonstrate that long-term dantrolene treatment is well tolerated, indicating that RyR antagonism may be a safe, reasonable pharmacologic target for novel analgesics. As RyR channelopathies may be an underdiagnosed cause of myopathy with recurrent myalgia [88-91], dantrolene could have a limited role in the treatment of myopathic pain outside use for MH. Whether inhibition of RyRs, such as with dantrolene, ameliorate symptoms associated with other forms of myopathy, such as Duchenne muscular dystrophy, has yielded inconsistent results in limited studies [92-95].

Importantly, dantrolene inhibits RyR1 and RyR3 isoforms but not RyR2 [96, 97], the isoform which predominates in the heart and brain. Thus, it is unclear whether drugs that inhibit RyR2 would have similar tolerability to long-term oral dantrolene; however, rodent models of RyR2 knockdown exhibit cardiac and neurologic dysfunction. For example, in an inducible, cardiomyocyte-specific RyR2 knockout mouse, a decrease of RyR2 protein by 50% was associated with bradycardia, intermittent tachyarrhythmias, cardiomyopathy, and early demise, possibly due to sudden cardiac death [98]. Hippocampal knockdown of RyR2 by injection of antisense oligodeoxynucleotides into the CA1 region exhibit worsened performance in a previously trained spatial memory task [99]. Thus, it is possible that drugs inhibiting RyR2 may cause cardiac or CNS toxicity precluding their clinical use in human patients. Unfortunately, there are no pharmacologic agents that exhibit specificity for a single isoform [100]. Moreover, the evidence, as summarized above, does not clearly implicate one isoform as predominantly involved in nociception or hyperalgesia. A more complete understanding of isoform-specific effects of RyRs in pain processing is an important limitation to the further development of pharmacologic strategies for novel pain medications.

Though most of the preclinical studies indicate that RyR inhibition produces antinociception or reverses hyperalgesia, several drugs with analgesic effects in use currently demonstrate RyR agonism, which is incongruous with the previously discussed preclinical studies. For instance, a Cochrane systematic review found that caffeine, an established agonist of RyRs [86], was an effective adjuvant for several painful conditions at a safe, standard dose when added to conventional analgesic medications [101]. Anesthetic medications used for surgery and other painful procedures that have analgesic effects, including volatile anesthetics [102, 103] and aminoamide local anesthetics [104], have been shown to potentiate RyRs [105, 106]. However, because each of these drugs likely exerts its effects through other receptors [107-109], it is unclear what role RyR potentiation may have on their antinociceptive actions, so these examples do not invalidate that RyR inhibition is important for analgesia.

3. HCN CHANNELS

3.1. Overview

Hyperpolarization-activated cyclic nucleotide (HCN)-regulated channels are a four-isoform family of channels that belong to the K_V superfamily [110, 111], assemble as homo- and hetero-tetramers (with only HCN2 + HCN3 disfavored [112]), and are present throughout the nervous system [113, 114]. These channels are the molecular basis of the "pacemaker" current I_h (in neurons) [115, 116] and the "funny" current I_f (in cardiac tissues) [117, 118]. The cryo-EM HCN1 structure demonstrates four-fold symmetry around a central ion conduction pathway; binding of cAMP rotates cytoplasmic domains such that opening of the inner helical gate is the favored conformation [119]. Channel function is regulated by a number of modulators, including cyclic nucleotides (most prominently cAMP), phosphatidylinositol 4,5-bisphosphate (PIP_2), tetratricopeptide repeat-containing Rab8b-interacting protein (TRiP8b) (which also regulates cell-surface trafficking - [120-122]), H^+, MiRP1, Filamin A, and various tyrosine kinases (Src, p38-MAPK) [123-126]. HCN channel expression (either mRNA or protein) in human and rodent sensory neurons is similar (but not identical)

[127-132], and their presence in DRG neurons have made them an attractive target for drug development for treating pain [115, 133, 134].

Although I_h controls fundamental aspects of neuronal electrophysiology, most notably regulation of resting membrane potential, temporal summation, and subthreshold oscillatory electrical activity [135], suggesting that HCN channels could play an important role in normal sensory transduction, their role in pathologic neuropathic pain is more clearly evident. Following nerve injury, HCN channel expression and I_h increase in HCN1/2-rich sensory neurons [136-141]; the increase in expression is more pronounced for HCN1 than for HCN2 [141], and HCN subunit trafficking is altered [136, 141, 142]. The increase in expression and current is accompanied by increased cellular hyperexcitability [136, 138, 139, 143], and both I_h and excitability are inhibited by the pan-isoform HCN channel blocker ZD7288 [136, 137, 141, 144, 145]. In rodents, blockade of HCN channels with either of the pan-isoform blockers ZD7288 or ivabradine relieves peripheral painful neuropathy [136, 140, 144, 146, 147]. Non-selective blockade HCN blockade, however, is likely to produce undesirable cardiac effects (most notably bradycardia) due to the key role HCN4 and HCN2 play in establishing normal sinus rhythm [117, 148-150]. Indeed, the non-selective HCN inhibitor ivabradine (Corlanor®, Procoralan®) produces sinus bradycardia and is approved for use in patients "to reduce the risk of hospitalization for worsening heart failure in patients with stable, symptomatic chronic heart failure with center ventricular ejection fraction ≤ 35%, who are in sinus rhythm with resting heart rate ≥ 70 beats *per* minute, and either is on maximally tolerated doses of β blockers or have a contraindication to β blocker use."

In the following sections, we will consider the current evidence for and against the targeting of specific HCN channels for the treatment of peripheral neuropathic pain. The focus on *peripheral* neuropathic pain stems from the facts that: 1) HCN channels are widely, but variably, expressed in the human brain (Allen Institute Human Brain Project: http://human.brain-map.org/microarray/search - search terms: HCN1, HCN2, HCN3, HCN4) and inhibition of HCN isoforms present on central neurons may result in unacceptable neuropsychological side-effects, and 2) inhibition of HCN channels on peripheral, but not central, neurons demonstrates antihyperalgesic efficacy in a rat spinal nerve ligation model of neuropathic pain [136]. It is possible, though, that inhibition of HCN channels on central neurons at the spinal cord level may be relevant to other forms of chronic pain as intrathecal administration of ZD7288 in a neonatal colon irritation model of irritable bowel syndrome provides pain relief [151]. Those data cannot address, however, whether the pain relief results from inhibition of I_h in presynaptic or postsynaptic neurons as HCN channels are located presynaptically on axon terminals at numerous synapses [152-159], including those between primary afferents and principal neurons in the spinal cord dorsal horn [160-162].

3.2. Insights from HCN Gene Deletion and Other *In Vivo* Studies

3.2.1. HCN1

Hcn1 contributes to cold, and to a smaller degree, heat perception [163]. Following peripheral nerve injury (partial sciatic nerve ligation), mice rapidly develop cold allodynia, which is Hcn1-dependent [164]. Cold allodynia is an important feature of chemotherapy-induced peripheral neuropathy (CIPN [165], commonly seen with platinum-based compounds [166, 167]. Painful neuropathy also occurs with other commonly administered chemotherapeutics, including those in the vinca alkaloid and taxane families [168-172]. Oxaliplatin and paclitaxel administration results in a significant increase in *Hcn1* gene [173] or mRNA [174] expression in rodents. Correspondingly, DRG neurons from paclitaxel-treated rats are hyperexcitable (demonstrated by increased spontaneous AP firing and a lower rheobase) compared to DRG neurons from untreated animals [173], consistent with an increase in HCN channel expression. At the behavioral level, oxaliplatin produces dose-dependent cold allodynia in mice that is significantly relieved by the pan-HCN channel blocker ivabradine [147, 174]. These results, coupled with the increase in *Hcn1* gene/mRNA expression, strongly implicate HCN1 channels as an important therapeutic target for the treatment of CIPN.

Relevant to this discussion is the fact that HCN1-selective inhibitors have been identified; these include the intravenous general anesthetic 2,6-di-isopropylphenol [175, 176], the non-anesthetic congener 2,6-di-*tert*-butylphenol [177, 178], 2-ethoxy-N-((1-(4-isopropylpiperazin-1-yl) cyclohexyl) methyl)benzamide [179], and MEL57A ((R)-6 in Melchiorre *et al*. 2010 - [180]]: (*R*) *N*,*N*-bis-[(Z)-4-(7,8-dimethoxy-2-oxo-1,3-dihydrobenzo[d]azepin-3-yl) but-2-enyl]-2-(3,4-dimethoxyphenyl)-propanamine) [180, 181]. *In vivo*, MEL57A relieves oxaliplatin-induced mechanical hyperalgesia [182] and cold allodynia [183], and both 2-ethoxy-N-((1-(4-isopropylpiperazin-1-yl)cyclohexyl) methyl) benzamide [179] and the alkylphenols [177, 178] relieve nerve injury-induced hyperalgesia. Importantly, the bradycardia-inducing ED_{50} of 2-ethoxy-N-((1-(4-isopropylpiperazin-1-yl)cyclohexyl)methyl)benzamide is 4-fold higher than its antihyperalgesic ED_{50} (25 *vs*. 6 mg/kg) [179]. Similarly, MEL57A has no effect on heart rate [182], consistent with the proposition that within the HCN family, HCN4, and possibly HCN2, are the primary drivers of cardiac function [184, 185]. Thus, there is strong preclinical evidence supporting the ongoing development of HCN1-selective inhibitors for the treatment of peripheral neuropathic pain.

3.2.2. HCN2

A convincing role for Hcn2 has been demonstrated in the development of painful neuropathy. Using the promoter for $Na_V1.8$ as a driver for expression, an *Hcn2* conditional knockout mouse line was generated in which the loss of HCN2 expression was restricted to a subset of small-diameter

nociceptive primary sensory neurons [186]. $Na_V1.8$-$Hcn2^{-/-}$ mice were phenotypically normal, with preserved motor function and normal pain thresholds at baseline. In these mice, thermal hypersensitivity induced by injection of formalin into the hindpaw foot pad was markedly reduced and abolished in response to injection of prostaglandin E2 (PGE_2); following sciatic nerve chronic constriction (CCI, *i.e.*, the Bennet model, a common model of peripheral nerve injury-induced neuropathic pain [187]), mechanical and thermal hyperalgesia and cold allodynia responses were indistinguishable from those seen in sham-operated f/f$Hcn2^{+/+}$ and $Na_V1.8$-$Hcn2^{-/-}$ animals. Collectively, these results demonstrate that HCN2 is required for the initiation of both inflammatory and neuropathic pain. Tsantoulas *et al*. demonstrated in both streptozocin-induced and db/db diabetic mouse models that cAMP-mediated upregulation of HCN2 function (rather than changes in HCN2 expression *per se*) underlies the development of diabetic neuropathy [188].

There is a significant interest in developing HCN2 selective inhibitors for the treatment of neuropathic pain [189]. Indeed, in March 2019, King's College London and The Wellcome Trust entered into a licensing agreement with Merck & Co., Inc. (Kenilworth, NJ, USA) for up to $340 million in development and sales milestones, as well as royalties, if an HCN2-selective therapeutic was approved for clinical use (https://www.kcl.ac.uk/news/pioneering-pain-research-leads-to-landmark-deal). At present, however, no potent, highly selective, HCN2 blocker has been described [180, 190]. Of concern for this approach is the clear expression of HCN2 protein in the human heart (atria and ventricles) under normal and pathologic (ischemic cardiomyopathy) conditions [191], which correlates with the presence of a robust cAMP-sensitive I_f current in human cardiomyocytes [192]; coupled with the observation that global deletion of HCN2 results in sinus dysrhythmia [148], these data suggest that simply targeting HCN2 may result in an unacceptable cardiovascular risk profile.

3.3.3. HCN3

$Hcn3^{-/-}$ mice are viable, fertile, and have no overt physical abnormalities; they do, however, have an increase in the T-wave amplitude of the electrocardiogram that arises from acceleration of the late repolarization phase in epicardial myocytes [193], and their ability to process contextual (fear and neutral) information is impaired [194]. Although Hcn3 is present in mouse DRG neurons at relatively low levels [195, 196], global *Hcn3* deletion produced only a modest decrease in mechanical pinprick hypersensitivity in a partial sciatic nerve ligation model, and had no effect on acute or inflammatory pain [197]. These results would suggest that HCN3 has little, if any role, as an antihyperalgesic target. If, however, changes in channel expression and/or function are a reactive process in response to injury, these results may undervalue the potential role of HCN3. By way of example, streptozocin-induced hyperglycemia results in a significant increase in Hcn3 protein in nodose ganglia A- and C- fiber neurons (along with an increase in Hcn1 in A-fiber neurons and an increase in Hcn2 in A- and C-fiber neurons) [198], suggesting that it could play a more important role than the gene deletion studies would suggest. As with mice, HCN3 expression in human DRG neurons is significantly less than that for HCN1 and HCN2 (Table **1** [128]), making it an unlikely candidate for therapeutic targeting absent compelling data demonstrating pronounced upregulation in any neuropathic condition in humans.

Table 1. HCN mRNA transcript levels in human dorsal root ganglia.

-	n	Transcripts Per Million [median (25%, 75%)]	Comparison	P†
HCN1	21	63.2 (55.3, 80.9)	HCN1 *vs* HCN4	< 0.001
HCN2	21	30.7 (23.5, 50)	HCN1 *vs* HCN3	< 0.001
HCN3	21	5.8 (4.6, 7.7)	HCN1 *vs* HCN2	0.177
HCN4	21	2.2 (1.4, 2.8)	HCN2 *vs* HCN4	< 0.001
	-	-	HCN2 *vs* HCN3	< 0.005
-	-	-	HCN3 *vs* HCN4	0.066

†One-way ANOVA with Tukey Test for *post-hoc* comparison.
Data from Supplementary Table 2, North *et al*. 2019 [128].

3.3.4. HCN4

Constitutive deletion of *Hcn4* is embryonic lethal [199]. Using a Cre-LoxP strategy to produce a conditional Hcn4 knockout, Herrmann *et al*. demonstrated that: 1) Hcn4 was responsible for a significant fraction of I_f in sinoatrial atrial node cells, 2) the residual Hcn4-independent current activated with a slow time course and a $V_{1/2}$ that was markedly right-shifted by the membrane-permeable cAMP analog, 8-Br-cAMP, and 3) acceleration of heart rate in response to β-adrenergic stimulation was preserved in *Hcn4* null mice [150]; the latter two observations are consistent with HCN2 expression being responsible for the residual current given that cAMP regulation of HCN2 gating is more pronounced than for HCN1 [200]. The role of HCN4 in mediating neuropathic pain has not been studied using this paradigm; however, HCN4 expression (protein or mRNA) in rodent [201, 202] and human [127, 128] sensory neurons is extremely low (Table **1**), suggesting that targeting of the isoform with an HCN4-selective inhibitor [181] will not result in a meaningful therapeutic for the treatment of neuropathic pain. Supporting this hypothesis is the observation that the HCN4-selective inhibitor EC18 [181] does not relieve cold allodynia in mice [183]. There may be some promise for HCN4-selective inhibitors with regards to treatment for seizure disorders [203], but here, too, the critical contribution of HCN4 to cardiac I_f and normal sinus rhythm [150] may present a fundamental obstacle with respect to future development.

3.4. Novel Drug Development – Why Targeting HCN1 Matters

While both HCN2 and HCN1 are present in mouse DRG neurons, HCN2 appears to be the more predominant isoform (at least based on mRNA levels) [132]. There is strong evidence that HCN2 is a key driver in the development of neuropathic pain in rodents [186, 188]. Those studies also de-

monstrate that the cellular hyperexcitability seen in DRG neurons results from increases in intracellular cAMP, which strongly facilitates the gating of HCN2, but not HCN1 [200, 204-208]. This is in contrast to what is seen in human pluripotent stem cell (hPSC)-derived sensory neurons (which closely resemble native DRG neurons with respect to the expression of ion channel genes), where the observed I_h current displays fast activation time constants and is insensitive to modulation by the adenylyl cyclase activator forskolin [127], suggesting that HCN1, not HCN2, is the primary isoform present in human sensory neurons. This interpretation is consistent with the following observations:

[i] HCN1 is present in human DRG at the gene [129] and mRNA [132] level.

[ii] HCN1 mRNA is present in a larger proportion of human DRG neurons than is HCN2 (94.4% *vs*. 44.0%, respectively); this is in contrast to mice, where HCN2 mRNA-positive neurons are seen at the same level as those that are HCN1 mRNA-positive (71.5% and 70.9%, respectively) [132].

[iii] HCN1 may be more robustly expressed than HCN2 in human sensory neurons, where the relative RNA expression (as measured in transcripts *per* million, TPM) is HCN1 > HCN2 >> HCN3 > HCN4 (Table 1 [128]).

As noted by Shiers and colleagues, "these marked species differences may have important implications for the role of different HCN isoforms in pain states between mouse models and human patients" [132]. Consequently, despite the compelling data from mice linking HCN2 channels to the initiation of neuropathic pain, there is a strong rationale for the development of HCN1-selective therapeutics for treating neuropathic pain in humans.

Further support for the development of HCN1-selective inhibitors comes from the observation that alkylphenols inhibit HCN1 gating [175-177, 209] rather than simply blocking the pore [210] as do ivabradine [211] and ZD7288 [212, 213]. Within this class, 2,6-di-isopropylphenol (propofol) inhibits HCN1 homotetramers and HCN1 + HCN2 heterotetramers with equal efficacy [176]. Thus, it is conceivable that a potent alkylphenol-derived HCN1-selective inverse agonist would be an excellent antihyperalgesic given that it would block two relevant populations of HCN channels, HCN1 homotetramers and HCN1 + HCN2 heterotetramers. Such a proposal has biologic plausibility as heterologously expressed HCN1 and HCN2 channels freely co-assemble to form functional heterotetramers [200], and the same phenomenon appears to be true for natively expressed HCN channels in hippocampal neurons [214], and of direct relevance here, in trigeminal [163] and DRG [215] sensory neurons. Like 2,6-di-isopropylphenol, 2,6-di-*tert*-butylphenol selectively inhibits HCN1 channel gating [177, 209], but unlike 2,6-di-isopropylphenol, it is neither a positive allosteric modulator of $GABA_A$ receptors nor does it act as a general anesthetic [177, 178, 216, 217]. It does, however, relieve neuropathic pain in partial sciatic nerve ligation [177] and chronic constriction injury [178] mouse models. In addition to clear efficacy as antihyperalgesics in different animal models across different labs (which speaks to reproducibility), HCN1-selective inhibitors appear to have an excellent cardiovascular safety profile [179, 182]. Whether 2,6-di-*tert*-butylphenol and congeners thereof are equieffective as inverse agonists for HCN1 + HCN2 heterotetramers as they are for HCN1 homotetramers remains to be determined, but if they are, they offer the potential of being an important new class of therapeutics for the treatment of neuropathic pain.

CONCLUSION

Pain is a prevalent medical condition that causes suffering and disability [19] demanding efficacious pharmacologic therapies. Unfortunately, over-prescription of opioid analgesics has led to a widespread crisis of misuse and overdose-related deaths [11, 13]. Moreover, opioids are not superior to other analgesics for certain common pain conditions, such as low back pain [14, 218] and neuropathic pain [219-223]. Thus, novel analgesics are urgently needed to provide pain relief and minimize disability without the attendant risks of opioids [17]. A strategy for the identification and development of novel analgesics is to target ion channels involved in nociception [17], including the "low-hanging" targets that control peripheral neuronal hyperexcitability [224]. Both *in vitro* and *in vivo* studies indicate a possible role of RyRs in nociception and hyperalgesia at the level of the DRG, spinal cord, and brain. However, much remains to be resolved to clarify if RyRs are an appropriate pharmacologic target for pain therapy, such as which isoforms contribute most to nociception, where along the nervous system transduction pathway is the best target, and what subtypes of pain may be most amenable to RyR modulation. Because RyRs are ubiquitously expressed and have a fundamental role in maintaining cytosolic and ER calcium concentrations in multiple cell types, it will also be critical to evaluate, in appropriate animal models, if RyR modulation can treat pain without causing serious adverse effects. However, the tolerability of long-term oral dantrolene for patients with MH and myalgia is encouraging in that its adverse effect profile would not preclude *a priori* specific RyR antagonists as a pharmacologic therapeutic. A more extensive literature supports the role of HCN channels in pain, particularly in neuropathic pain, highlighting their suitability, especially that of HCN1 and 2, as therapeutic targets. Given their role as cardiac pacemakers, however, novel analgesics targeting HCNs will need to be evaluated carefully for potential to cause dysrhythmias, among other potential toxicities.

Due to the tremendous need for efficacious pain treatments without the potential for addiction and overdose, the rational selection of therapeutic targets for the development of novel analgesics and antihyperalgesics holds significant promise. Studies have implicated both RyRs and HCN channels in nociception and hyperalgesia. Further research will be required to understand whether pharmacologic modulation of either of these families of ion channels could safely provide pain relief in humans.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

PAG is a co-inventor on patents related to the development of novel alkylphenols for the treatment of neuropathic pain and serves on the Scientific Advisory Board for Akelos, Inc., a research-based biotechnology company that has secured a licensing agreement for the use of those patents.

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