British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01507.x
www.brjpharmacol.org

REVIEW

HCN channelopathies: pathophysiology in genetic epilepsy and therapeutic implications

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Keywords
HCN1; HCN2; HCN4; idiopathic generalized epilepsy; febrile seizures; lamotrigine

Received
1 December 2010
Revised
14 April 2011
Accepted
13 May 2011

Hyperpolarization-activated cyclic nucleotide-gated channels (HCN) can act as pacemakers in the brain making them strong candidates for driving aberrant hypersynchronous network activity seen in epilepsy. Transcriptional changes in HCN channels occur in several animal models of epilepsy. However, only recently have genetic studies demonstrated sequence variation in *HCN1* and *HCN2* genes associated with human epilepsy. These include a triple proline deletion in HCN2 that increases channel function and occurs more often in patients with febrile seizure syndromes. Other *HCNx* gene variants have been described in idiopathic generalized epilepsy although the functional consequence of these remains unclear. In this review we explore potential cellular and network mechanisms involving HCN channels in the genetic epilepsies. We suggest how new genetic sequencing technology, medium-throughput functional assays and the ability to develop syndrome-specific animal models will provide a more comprehensive understanding of how I_h contributes to pathogenic mechanisms underlying human genetic epilepsy. We also discuss what is known about the pharmacological manipulation of HCN channels in the context of epilepsy and how this may help future efforts in developing HCN-channel-based therapy.

Abbreviations

FS, febrile seizures; GAERS, generalized Absence Epilepsy Rat from Strasburg; GGE, genetic generalized epilepsy; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; HCN2 KO, HCN2 knockout; IGE, idiopathic generalized epilepsy; I_h, current mediated by HCN; SWD, spike-and-wave discharge; TRIP8b, TPR-containing Rab8b interacting protein; WAG/Rij, Wistar Albino Glaxo rats, bred in Rijswijk

Introduction

Epilepsy, with a lifetime prevalence of 3%, is a common and serious neurological disorder with significant associated morbidity and mortality. Approximately a third of sufferers do not respond satisfactorily to current treatments. Understanding the molecular basis of epilepsy is key to the development of new approaches to therapy that have improved efficacy and tolerability. Hyperpolarization-activated cyclic nucleotide-gated channels (HCN) are one candidate group of ion channels that has received particular attention in recent years. There are four known HCN channels encoded by *HCN1*, *HCN2*, *HCN3* & *HCN4* (Ludwig *et al.*, 1998; Santoro and Tibbs, 1999), with each having distinct kinetic and voltage characteristics as well as cAMP sensitivity (reviewed in Biel *et al.*, 2009). HCN channels are expressed in both neurons and myocytes (Biel *et al.*, 2009). In the heart they conduct I_f which is critical for the pacemaker function underlying cardiac rhythm (reviewed in Mangoni and Nargeot, 2008). A pacemaker role is thought to be important for the analogous I_h current in the brain. Epilepsy is a group of neurological disorders characterized by the occurrence of recurrent spontaneous seizures, the basis of which is hypersynchronous network activity, typically driven by a pacemaker process. It is, therefore, not surprising that evidence for a role of HCN in both acquired and genetic epilepsies is accumulating (Chen *et al.*, 2001a; Brewster *et al.*, 2002; Bender *et al.*, 2003; Ludwig *et al.*,

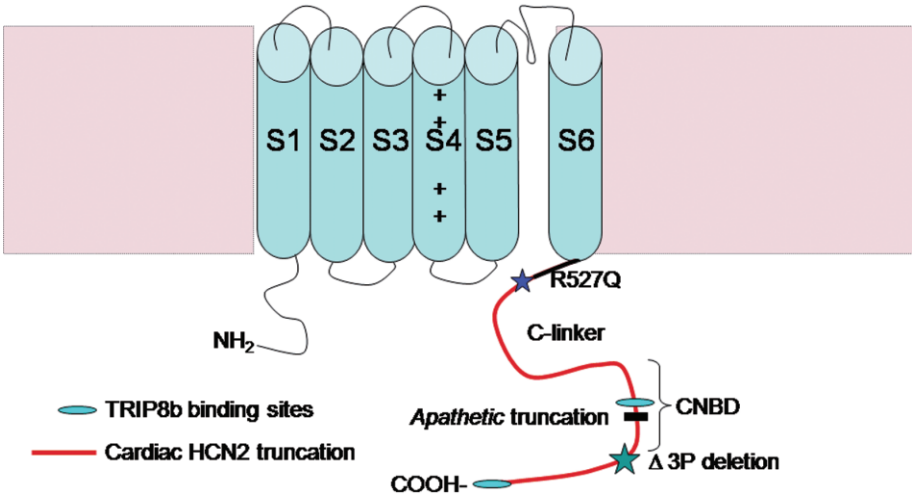

Figure 1

Cartoon of the HCN2 channel illustrating epilepsy associated amino acid variation, proposed TRIP8b binding sites and predicted proteolytic truncation in the C-terminus of the cardiac channel.

2003; Strauss *et al.*, 2004; Brewster *et al.*, 2005; Budde *et al.*, 2005; Kuisle *et al.*, 2006; Jung *et al.*, 2007; Kole *et al.*, 2007; Bender and Baram, 2008; Dyhrfjeld-Johnsen *et al.*, 2008; Powell *et al.*, 2008; Richichi *et al.*, 2008; Adams *et al.*, 2009; Huang *et al.*, 2009, Jung *et al.*, 2010). In this review we will explore the role of HCN channels in the genetic epilepsies, focusing on both human and animal studies and highlighting their potential therapeutic implications.

Genetic epilepsies

Epilepsy syndromes are broadly divided into focal (partial) and generalized syndromes. This largely empirical distinction is based on the idea that focal seizures are generated in a local unilateral network whereas generalized seizures are generated within bilateral networks. While there is some evidence for a genetic component in focal epilepsies there is much more compelling evidence in generalized epilepsies. Idiopathic generalized epilepsies (IGE) are a family of related syndromes that accounts for the majority of generalized epilepsy cases. IGE has been recently renamed Genetic Generalized Epilepsy (or GGE) to highlight the importance of genetic mechanisms (Berg *et al.*, 2010). GGE seizure types include absence seizures, myoclonic seizures and tonic–clonic seizures. A pattern of generalized spike-and-wave discharge (SWD) on EEG is characteristic of all GGEs (Loiseau *et al.*, 1990). The GGEs display complex inheritance patterns where the contribution of multiple 'susceptibility alleles' are required for disease expression (Dibbens *et al.*, 2007). Another common seizure disorder is febrile seizures (FS), a disorder which affects approximately 3% of all children. FS occurs during fever and is usually self-limiting (Verity *et al.*, 1985). FS also have a strong genetic based aetiology. Until recently, direct genetic evidence linking dysfunction in HCN channels to epilepsy in humans has been elusive.

Do HCN variants contribute to human epilepsy?

Mutation analysis has revealed sequence variation in *HCN1* and *HCN2* associated with epilepsy (Tang *et al.*, 2008; Dibbens *et al.*, 2010). Tang *et al.* (2008) described rare variants in both *HCN1* and *HCN2* that were associated with GGE. In one sporadic case they found an A881T mutation in HCN1 and a non-segregating R527Q mutation in HCN2 found in a small GGE family (Figure 1). Neither of these variants was found in unrelated controls. Our own study found population variation in both *HCN1* and *HCN2* (Dibbens *et al.*, 2010). Of particular interest was the observation that the triple proline deletion variant in HCN2 (719–721ΔP; $HCN2_{delPPP}$) was found in ~2.4% of patients with FS syndromes but only 0.2% in unaffected controls as would be expected for a susceptibility gene (Figure 1). This included patients with Genetic Epilepsy with Febrile Seizures plus ($GEFS^{+}$) that is a sub-syndrome of GGE. Despite enrichment in the epilepsy population statistical confirmation was not possible for any of the *HCN* variants. Functional validation can be used to infer pathogenicity of variants, complementing the genetic aspect of a study. A panel of biological assays for different protein families are going to ultimately be needed to test the spectrum variants likely to emerge from future epilepsy genetic studies. For ion channel proteins, electrophysiological assays are accepted gold standards.

Function analysis of HCN2 variants associated with epilepsy

The functional impact of changes associated with susceptibility genes are by definition likely to be subtle creating a detection problem. This is a particular problem with ion channel recordings that historically have relied on traditional labour-

intensive manual electrophysiological methods. The inherent data variability and inability to record sufficient experimental numbers using these manual methods is a significant limitation (Thomas *et al.*, 2009). For example, functional analysis using traditional electrophysiological methods failed to detect significant differences in the HCN2 (R527Q) variant, although trend level changes in the current voltage relationship were noted (Tang *et al.*, 2008). Our laboratory has developed a medium-throughput robotic electrophysiological assay for the HCN2 channel. In this assay automated two-electrode voltage clamp recordings are made from *Xenopus* oocytes expressing a channel of interest. Large sample sizes can be achieved in relatively short periods (10–100 per day, depending on protocol) that would take weeks to months to achieve using traditional electrophysiological approaches. This is important as power analysis determined that sample numbers >100 were required to pick a ~25% change in maximal conductance. Using the automated method we were able to demonstrate that currents generated by the mutant $HCN2_{delPPP}$ were 30% larger than those of controls (Dibbens *et al.*, 2010).

Potential mechanisms of excitability for a gain-of-function epilepsy variant

Predicting the impact of a change in HCN function on network excitability is complex due to the multifaceted role that I_h can play within a neuron (Dube *et al.*, 2007; Dyhrfjeld-Johnsen *et al.*, 2009). Both experimental data and computer simulations suggest that increased I_h is a pathological mechanism in epilepsy. A persistent increase in I_h is observed following induced FS which correlate with increased neuronal firing (Chen *et al.*, 2001a; Brewster *et al.*, 2002; Dyhrfjeld-Johnsen *et al.*, 2008). Computer simulation suggests that this increased neuronal excitability is predominantly due to the resulting depolarized resting membrane potential taking the neuron closer to the firing potential (Dyhrfjeld-Johnsen *et al.*, 2008, Dyhrfjeld-Johnsen *et al.*, 2009). As the $HCN2_{delPPP}$ mutation results in an increase in I_h a similar mechanism may underlie an increase in neuronal excitability in patients harbouring this mutation. It is important to note that the *in vitro* study is not exhaustive and several levels of experimentation, ultimately in an *in vivo* situation, will be required to establish that this mutation increases the likelihood of seizures. Animal studies highlight the complexity of attributing causation to functional change with decreases in I_h also able to contribute to network excitability, as we discuss in the next section.

HCN and animal models of genetic epilepsy

The most compelling experimental evidence linking reduced HCN function to epilepsy has been found using the HCN2 knockout (HCN2 KO) mouse (Ludwig *et al.*, 2003). This mouse displays bilateral synchronous SWD that are the hallmark of absence epilepsy, a common seizure type in GGE. A similar SWD phenotype was seen in the *apathetic* mouse, that harbours a spontaneous *Hcn2* truncation (Chung *et al.*, 2009). Thalmocortical (TC) neurons from the HCN2 KO mouse were significantly more hyperpolarized relative to controls consistent with a reduction in I_h and displayed a more prominent burst firing phenotype (Ludwig *et al.*, 2003). The more hyperpolarized resting membrane potential in the HCN2 KO TC neurons would increase the availability of T-type Ca^{2+} channels possibly explaining the increased burst firing that is a hallmark of firing patterns during SWDs (Crunelli and Leresche, 2002).

Investigations in two well-characterized rat models of absence seizures, the Generalized Absence Epilepsy Rat from Strasburg (GAERS) and Wistar Albino Glaxo rats, bred in Rijswijk (WAG/Rij) models, have also implicated changes in HCN as part of the pathogenic mechanism underlying epilepsy. Recordings from L2-3 pyramidal neurons showed a significant reduction in I_h and a slowing in activation kinetics in the WAG/Rij model (Strauss *et al.*, 2004). HCN1 channel protein levels were reduced, but levels of the slower activating HCN channels (HCN2–4) remained constant, explaining the slower current activation seen in these models. At resting membrane potentials I_h also contributes to membrane leak, especially in the dendritic arbour where HCN1 channel expression is the highest. Strauss *et al.* (2004) show an increase in the temporal summation of synaptic input and facilitation of dendritic burst firing, and propose that these neuronal properties contribute to network excitability in the WAG/Rij models. Kole *et al.* (2007) extended these findings by recording from L5 pyramidal neurons in WAG/Rij rats and show a similar reduction in HCN1 channel expression. They suggest that this HCN1 loss of function would increase the appearance of back-propagating action potentials in the distal dendrites potentially increasing excitability. Kole *et al.* (2007) also show that L5 pyramids of the WAG/Rij were hyperpolarized, had increased synaptic summation and burst firing as in L2-3 pyramids.

While it is clear that the absolute amount of I_h is an important factor in determining neuronal excitability, studies by Budde *et al.* (2005) show that the relative sensitivity of this current to cAMP may also contribute to pathogenicity in epilepsy. Their studies suggest that the cAMP sensitivity of I_h in WAG/Rij TC neurons was reduced as a result of an increase in the cAMP resistant HCN1 channel. Because termination of TC burst firing is thought to be dependent on cAMP activation of I_h (Luthi and McCormick, 1998, Luthi and McCormick, 1999), a loss of this TC neuron property would be expected to increase seizure susceptibility. A similar pathogenic mechanism may occur in GAERS (Kuisle *et al.*, 2006). Again, an increased expression of HCN1 with no change in HCN2 and HCN4 was observed in the thalamus. This also translated into a reduction in cAMP sensitivity of I_h recorded from TC neurons (Kuisle *et al.*, 2006). Interestingly, a compensatory mechanism developed in epileptic rats that was independent of HCN channel expression but maintained I_h during TC activity sufficient to terminate bursting.

Environmental influences on HCN expression

HCN channels are plastic with expression and function changing in response to a variety of environmental stimuli.

Epilepsy-based influences on HCN expression include prolonged FS in immature rats that cause regional and isoform-specific changes (Chen *et al.*, 2001a; Brewster *et al.*, 2002). Similarly, pro-convulsant challenges and electrical kindling can alter HCN expression (Shah *et al.*, 2004; Powell *et al.*, 2008; Richichi *et al.*, 2008; Adams *et al.*, 2009; Huang *et al.*, 2009). Even subtle insults such as maternal stress can result in long-term alteration in HCN expression which correlates with altered seizure susceptibility in the WAG/Rij model (Schridde *et al.*, 2006). This suggests that altered I_h needs to be considered as a potential pathological mechanism even in the absence of any mutation in *HCN* coding sequence.

I_h and early development

Network activity in the immature brain is critical to normal development defining neuron number, location and connectivity (Ben-Ari, 2001). We have demonstrated that the early expression of a human mutation in a $GABA_A$ receptor can determine seizure susceptibility in adulthood (Chiu *et al.*, 2008). I_h is necessary for normal network pacing in the immature brain (Agmon and Wells, 2003; Adams *et al.*, 2009). HCN variants could therefore alter this function thus changing network properties into adulthood. This potentially implicates HCN isoforms that predominate in early brain development, in particular HCN4 (Brewster *et al.*, 2007).

Auxiliary subunits and HCN channels

One recent development is the discovery of 'HCN auxiliary' proteins that can modulate both the biophysical properties and trafficking of HCN channels. TPR-containing Rab8b interacting protein [TRIP8b, also known as Pex5p-related protein (PEX5Rp) or H-channel interacting protein 1 (HIP1)], is a brain-specific protein which binds to Rab8b, a member of the Rab family of small GTPase proteins important for vesicle trafficking (Chen *et al.*, 2001b). Santoro *et al.* (2004) discovered a direct protein–protein interaction between TRIP8b and all HCN channels through a conserved sequence in their C-terminal tail (Figure 1). In this early study the co-expression of TRIP8b and HCN1 resulted in a significant reduction in I_h (Santoro *et al.*, 2004). Two independent studies recently extended this finding to show that HCN1 channel modulation by TRIP8b is splice-variant specific (Lewis *et al.*, 2009; Santoro *et al.*, 2009). Also, that through a splice-variant independent mechanism, TRIP8b left-shifts the voltage dependence of activation resulting in reduced function at physiological membrane potentials (Lewis *et al.*, 2009; Santoro *et al.*, 2009). Interestingly the TRIP8b isoforms that are most abundant in the brain are predicted to enhance HCN channel surface expression (Lewis *et al.*, 2009; Santoro *et al.*, 2009). In addition to evidence that TRIP8b and HCN1 channels co-localize in neurons (Santoro *et al.*, 2009), another recent study demonstrated that TRIP8b stoichiometrically co-purifies with native brain HCN channels, strongly implicating TRIP8b as the auxiliary subunit of HCN channels in brain (Zolles *et al.*, 2009). Together these results suggest that TRIP8b is an important modulator of I_h in neurons and could potentially be implicated in the pathogenic mechanism underlying epilepsy. For instance, in the kainic acid model of acquired epilepsy the co-immunoprecipitation of TRIP8b with HCN1 was reduced 28 days post status (Shin *et al.*, 2008). HCN1 channel membrane expression and function was also reduced at this time point, consistent with a loss of the trafficking role of TRIP8b for HCN1 (Shin *et al.*, 2008). Absolute TRIP8b protein levels do not change and this effect is specific to HCN1 raising questions as to the underlying molecular mechanisms. Nevertheless, these results provide a strong hint that changes in the TRIP8b-HCN interaction may alter neuronal excitability in epileptic states.

There are no published reports of altered HCN1–TRIP8b interaction in human or animal models of genetic epilepsy. However, the above data have clear implications for studies of genetic epilepsy. Firstly, HCN variants may alter the TRIP8b–HCN protein interaction, and those variants localized in or near the conserved HCN C-terminal tail region, the region critical for TRIP8b binding, should be tested in co-expression assays. Secondly, TRIP8b should be considered a strong candidate gene for genetic epilepsies.

Second messenger modulators of I_h

There are several second messengers that modulate I_h, including the well-established cAMP, intracellular protons and phosphatidylinositol-4,5-phosphate (PIP2) (Biel *et al.*, 2009). There is also evidence for a variety of kinases and lipids modulating HCN channel function (Biel *et al.*, 2009). Changes in any of these second messengers could therefore result in altered I_h and thus neuronal function in epilepsy. There is some precedence for this in the pilocarpine animal model of acquired epilepsy. Jung *et al.* (2010) report enhanced phosphatase calcineurin activity and reduced p38 mitogen-activated protein kinase activity in the CA1 in the acquired epilepsy model (Jung *et al.*, 2010). They propose that changes in these second messengers reduce HCN1 channel activity potentially contributing to seizure genesis. These mechanisms also need to be considered when researching the genetic epilepsies.

HCN channels as drug targets for treating genetic epilepsy

A benefit of being a validated human epilepsy gene is that the gene in question is likely to serve as a good antiepileptic drug target (Reid *et al.*, 2010). Functional changes seen in the $HCN2_{delPPP}$ variant justifies the investigation of this channel as a potential drug target with the prediction that selective HCN2 blockers could be antiepileptic (Dibbens *et al.*, 2010). This is in contrast to that predicted from animal studies. As discussed, down-regulation of HCN2 channel function (absolute or relative to HCN1) is proposed as part of the cellular basis of seizures in the HCN2 KO mouse and in the two rat models of genetic epilepsy (Ludwig *et al.*, 2003; Budde *et al.*, 2005; Kuisle *et al.*, 2006; Chung *et al.*, 2009). It is important

to note that rodent models of genetic epilepsy have their limitations. For example, in the case of the HCN2 KO, adaptive changes, even in channels or biochemical processes independent of HCN2, could contribute to epilepsy. These adaptive processes are likely to be different (or absent) for mutations that change channel properties as opposed to those caused by complete loss of channel protein. Although providing important information on mechanisms underlying absence seizures, how these relate to seizure generation in humans is yet to be determined. Therefore, despite the obvious challenges ahead, a full understanding of how HCN channel dysfunction contributes to epilepsy will need to come from human genetic studies.

Recent advances in genetic screening technologies are starting to make this a possibility. For example, the Ion Channel Project at the Baylor College of Medicine employs large scale gene sequencing and mutation analysis to test the coding sequences of channel genes in individual epilepsy patients. A number of non-synonymous HCN variants are published as part of this programme (http://www.hgsc.bcm.tmc.edu/ionchannel-snpList.xsp). Functional studies of human channels in heterologous expression systems are likely to form the basis of initial validation. This will help build a broader understanding of the variation in HCN channel function and how this relates to epilepsy. One hope of these large screening exercises is that we will be able to cluster the effects of HCN mutations into functional classes with a prediction that there will be fewer classes than mutations. This will help direct animal studies allowing one or two knock-in models to be developed based on a functional class. Animal models based on human epilepsy mutations are already helping to identify neuronal and network mechanisms underlying seizure genesis (Klaassen *et al.*, 2006; Ogiwara *et al.*, 2007; Teper *et al.*, 2007; Tan *et al.*, 2008; Wimmer *et al.*, 2010). These 'syndrome-specific' animal models will complement human genetic studies resulting in a better understanding of how HCN channels influence seizure susceptibility and how this may be exploited for drug discovery.

Antiepileptic drugs that may act through I_h

Some antieplileptic drugs have been reported to act through an I_h-mediated mechanism. Poolos and colleagues demonstrated in CA1 pyramidal neurons that lamotrigine positively shifted the activation of I_h by approximately 10 mV, effectively resulting in a gain-of-function (Poolos *et al.*, 2002). Lamotrigine reduces action potential firing in dendrites, as a result of increasing I_h, providing one potential mechanism for its antiepileptic action. Lamotrigine also increases I_h in CA1 interneurons although through a mechanism that did not involve changes in activation (Peng *et al.*, 2010). In this case, the depolarizing effects of lamotrigine increase inhibitory neuron firing thereby increasing inhibition of post-synaptic pyramidal neurons and providing another plausible antiepileptic action of lamotrigine. Further, using an *in vitro* model we earlier demonstrated that lamotrigine decreases the duration of synchronized burst activity in CA1 through an I_h-mediated mechanism (Adams *et al.*, 2009), another potential anti-epileptic action. In addition to lamotrigine other non-specific blockers of I_h can also be found amongst the anti-epileptic drugs. For example, gabapentin increases I_h in CA1 pyramidal neurons (Surges *et al.*, 2003) and acetazolamide, an inhibitor of carbonic anhydrase that has been used to treat epilepsy, can increase I_h through intracellular alkalinization (Munsch and Pape, 1999).

Drugs with HCN subtype selectivity are likely to be a requirement for a successful therapy. Recent studies have shown that several current drugs are relatively selective for HCN1 over other HCNs. Propofol, a short-acting hypnotic agent, that can be used in status epilepticus, inhibits HCN1 channels (Cacheaux *et al.*, 2005; Chen *et al.*, 2005; Lyashchenko *et al.*, 2007). Interestingly, both ketamine (hypnotic) and isoflurane (anaesthetic) also inhibit HCN1 channels specifically (Chen *et al.*, 2009a,b). These studies suggest that the development of HCNx selective channel antagonists and agonists is possible.

I_h: cardiac versus brain

HCN2 and HCN4 are the predominant cardiac subtypes underlying I_h (often referred to as I_f in the heart) (Biel *et al.*, 2009). One prediction is that the HCN2 variants discovered in epilepsy (Dibbens *et al.*, 2010) should alter both heart and brain function but no cardiac abnormalities were reported. While it is possible that the cardiac effects may have been subtle and overlooked, an alternative is that proteolytic processing of cardiac expressed HCN2 truncates a large portion of the C-terminus (Ye and Nerbonne, 2009). This truncation could either remove the mutated residue or alter the impact of remaining mutations in the now truncated C-terminal tail (Figure 1). Interestingly, both HCN2 variants described in epilepsy fall close to or beyond the predicted truncation site on the C-terminus tail (Figure 1). Secondly, from a drug discovery perspective, agents that act selectively on full length HCN2 channels and spare cardiac truncated channels may avoid cardiac complications.

Conclusion

There is substantial evidence implicating changes in HCN channel function as part of the underlying genesis of certain epilepsies. In particular, recent genetic studies have suggested that increased HCN2 channel function may contribute to GGE and FS syndromes. Further genetic and functional analysis of the *HCNs* in various epilepsy syndromes will be critical for reliably linking HCN dysfunction to network excitability. Ongoing drug discovery around subtype-specific agonists and antagonist is certainly justified. Efforts should also be made to develop agents that act selectively on the full-length brain HCN2 channels, while sparing the truncated version found in heart. Similarly, as the TRIP8b protein is brain specific, pharmacological manipulation of the interaction with HCN channels would be expected to have limited cardiac impact. This approach would have the additional advantage of not being limited

to the HCN2 channel. The development of selective HCN pharmacology is not only likely to benefit epilepsy research and therapy but may help in the understanding of a range of other physiological and disease mechanisms linked to I_h.

Acknowledgements

We would like to acknowledge the help of Chantel Trager. This study was supported by the National Health and Medical Research Council of Australia (Grant numbers 400121 & 628520). CAR was also supported by an Australian Future Fellowship, Australian Research Council (#FT0990628) and SP by an NHMRC Research Fellowship

Conflict of interest

The authors declare no conflict of interest.

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