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Highlights

- Recapitulation of the anticonvulsant properties of ivabradine, a broad spectrum HCN channel blocker, in a PTZ proconvulsant assay
- Ivabradine can also reduce thermogenic seizure susceptibility
- HCN Isoform preferring channel blockers differentially impact seizure susceptibility
- Our data motivates a need to develop CNS penetrant HCN isoform-specific drugs to test on seizure susceptibility

Abstract:

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels have been implicated in the pathogenesis of epilepsy and consequently as targets for anticonvulsant drugs. Consistent with this, broad-spectrum block of HCN-mediated current (I_h) reduces seizure susceptibility in a variety of epilepsy models. However, HCN channel isoforms have distinct biophysical characteristics and

anatomical expression suggesting that they may play different roles in setting neuronal excitability. Here we confirm that the broad-spectrum blocker ivabradine is effective at reducing seizure susceptibility in the s.c.PTZ seizure assay and extend this, showing efficacy of this drug in a thermogenic assay that models febrile seizures. Ivabradine is also effective at reducing thermogenic seizures in the Scn1a mouse model of Dravet syndrome in which febrile seizures are a feature. HCN isoform-preferring drugs were tested in the s.c.PTZ seizure assay. We confirm that the HCN4-preferring drug, EC18, is efficacious in reducing seizure susceptibility. Conversely, the HCN2/1-preferring drug, MEL55A, increased seizure susceptibility in the s.c.PTZ seizure assay. MEL57A, an HCN1-preferring drug, had no effect on seizure susceptibility. Mouse pharmacokinetic studies (for MEL55A and MEL57A) and screening against additional ion channels have not been thoroughly investigated on the HCN isoform-preferring compounds. Our results need to be considered in this light. Nevertheless, these data suggest that HCN isoform-selective block can have a differential impact on seizure susceptibility. This motivates the need to develop more HCN isoform-selective compounds to better explore this idea.

Abbreviations: ANZCCART: Australian and New Zealand Council for the Care of Animals in Research and Teaching, DMSO: Dimethyl sulfoxide, EEG: Electroencephalogram, FDA: Food and Drug Administration, GABA: Gamma aminobutyric acid, HCN: Hyperpolarization-activated cyclic nucleotide-gated, i.p.: Intraperitoneal, I_h: Hyperpolarisation activated current, MES: Maximal Electroshock, NHMRC: National Health and Medical Research Council, PCR: Polymerase chain reaction, PK: Pharmacokinetic, PTZ: Pentylenetetrazole, s.c.: Subcutaneous, s.c.PTZ: Subcutaneous PTZ seizure assay

Keywords: Hyperpolarization-activated Cyclic Nucleotide-gated Channels, Ion Channels, HCN Channel Block, Anticonvulsant, Drug Target, Neuronal Excitability.

1. Introduction

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels carry a non-selective cation conductance, I_h, that controls a range of neuronal functions including setting resting membrane potential and modulating the integration of dendritic synaptic input (Bender and Baram, 2008; Biel et al., 2009). Four genes (*HCN1-4*) encode distinct HCN channel isoforms that are expressed in a brain region and neuronal compartment-specific manner (Notomi and Shigemoto, 2004; Oyrer et al., 2019; Santoro and Tibbs, 1999). HCN channels have been strongly implicated in the pathogenesis of epilepsy (Benarroch, 2013; Bender et al., 2003; DiFrancesco and DiFrancesco, 2015; Reid et al., 2012). Transcriptional changes in HCN channels occur in both acquired and genetic rodent models of epilepsy (Powell et al., 2008; Strauss et al., 2004), although the relationship between changes in function and excitability is complex (Brennan et al., 2016). Genetic variations in *HCN1*, *HCN2* and *HCN4* are also associated with epilepsy, but again the relationship is complex with both gain- and loss-of-function variants described in several clinical studies (Becker et al., 2017; Bonzanni et al., 2018; Campostrini et al., 2018; Dibbens et al., 2010; Li et al., 2018; Marini et al., 2018; Nava et al., 2014). These data suggest that HCN channels are important modulators of neuronal excitability in epilepsy and potentially targets for anticonvulsant medication.

Many clinically available drugs alter the biophysical properties of the HCN channel class, including neuroactive drugs such as: lidocaine, ketamine, and propofol (Gao et al., 2018; Ramírez et al., 2018). For instance, lidocaine, a local anaesthetic, inhibits HCN1, HCN2, and HCN4 channel currents (Meng et al., 2011). The analgesic and anaesthetic ketamine has also been reported to inhibit HCN1, HCN2, and HCN4 channels (Chen et al., 2009; Luo et al., 2019; Ramírez et al., 2018; Xing et al., 2017; Zhang et al., 2016). Furthermore, the sedative propofol has been demonstrated to act on HCN channels, with inherent selectivity toward HCN1 (Cacheaux et al., 2005; Chen et al., 2005; Lyashchenko et al., 2007). Pre-clinical studies have shown that propofol also inhibits I_h in mouse neocortical and hippocampal pyramidal neurons, which is postulated to contribute to its anaesthetic effect

(Cacheaux et al., 2005; Chen et al., 2005; Funahashi et al., 2001; Higuchi et al., 2003; Ying et al., 2006).

From an epilepsy perspective, two clinically available anticonvulsant drugs, lamotrigine and gabapentin, have been shown to have effects on I_h and HCN channels. Lamotrigine is an anticonvulsant drug that is thought to act mainly as a dependant sodium channel blocker (Huang et al., 2016; Lehnhoff et al., 2019). Lamotrigine is an HCN channel agonist and is known to enhance I_h in rodent cortical and hippocampal pyramidal neurons (Berger and Lüscher, 2004; Omrani et al., 2015; Poolos et al., 2002). Gabapentin interacts with the α2δ subunit of the Ca^{2+} channel, leading to reduced voltage-gated Ca^{2+} channel trafficking to the membrane surface of glutamatergic synapses (Fink et al., 2002). Among other targets, gabapentin has been shown to cause a shift in the voltage of activation of HCN4 channels in *Xenopus* oocyte expression studies (Tae et al., 2017). However, given that gabapentin and lamotrigine act on multiple ion channels it is difficult to attribute the antiseizure effects of these drugs solely to changes in HCN channel function (Postea and Biel, 2011; Quintero, 2017).

Several broad-spectrum I_h blockers with greater pharmacological specificity such as caesium chloride (CsCl), ZD7288 and ivabradine have also demonstrated anticonvulsant activity in pre-clinical seizure models. CsCl and ZD7288 increase the threshold to stimulus-evoked paroxysmal discharges in the rabbit hippocampal CA1 region (Kitayama et al., 2003). ZD7288-mediated I_h block has been shown to prevent formation of epileptiform discharges in rat hippocampal CA1 and CA3 regions (Xiong and Stringer, 1999). Furthermore, CsCl and ZD7288 decrease the severity and frequency of spontaneous seizures in the seizure-sensitive Mongolian gerbil (Matsuda et al., 2008). The FDA approved drug, ivabradine, is used clinically for the treatment of chronic stable angina and chronic heart failure (Ide et al., 2019). Ivabradine reduces kainic acid-induced seizures in rats (Mansour and Ibrahim, 2015) and increases the threshold of maximal electroshock seizures in mice (Luszczki et al., 2013). In addition, ivabradine has shown anticonvulsive effects in the subcutaneous PTZ (s.c.PTZ) seizure assay

in mice (Cavalcante et al., 2019). The antiseizure effects of ivabradine have also been studied in conjunction with other classical antiepileptic drugs such as lacosamide, pregabalin, lamotrigine, and topiramate within the mouse maximal electroshock (MES) and tonic-clonic seizure models, although determining efficacy is complicated by drug-drug interactions (Sawicka et al., 2017a; Sawicka et al., 2017b).

In general, these data support the involvement of HCN channels in epilepsy and suggest that HCN channels may indeed be viable antiepileptic drug targets. Here we extend our understanding of the antiseizure capabilities of the broad-spectrum blocker ivabradine within the thermogenic seizure assay, a model of febrile seizures (Reid et al., 2013; Wimmer et al., 2010). This includes testing the impact of ivabradine on thermogenic seizures in a mouse model of Dravet syndrome. We also test the antiseizure impact of ivabradine and additional compounds with greater HCN isoform-selectivity in the s.c.PTZ seizure assay.

2. Methods

All experiments were approved by the Animal Ethics Committee at the Florey Institute of Neuroscience and Mental Health, comply with the ARRIVE guidelines, and performed in accordance with the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

2.1. Experimental Animals

C57BL/6J post-weaning (P21-28) male mice ordered from the Animal Resources Centre (WA, Australia) were used for all behavioural experiments. Scn1a mice containing a knock-in of a nonsense mutation (R1407X) on a 129S1/SvImJ background were crossed with C57BL/6J background mice to produce the experimental cohort of mice as described in Richards et al. (2018). The mixed background mice modelled have spontaneous seizures that model Dravet syndrome (Ogiwara et al., 2007). Tail samples were taken at P7 and genotyping was carried out by TransnetYX (TN, USA). Heterozygous

Scn1a $^{RX/+}$ (P18-P21) mice on a C57BL/6J/129S1/SvImJ mixed background were used for experiments with a ratio of male to female mice approximately 1:1. Mice were housed at the animal facility located at the Florey Neuroscience Institute in standard 15x30x12cm cages, and maintained under natural dark and light cycles, with access to a standard dry pellet diet along with normal tap water. To minimise the suffering of animals, mice were monitored daily and were rapidly killed by cervical dislocation (an ANZCCART approved method) at the experimental endpoint.

2.2. Preparation of Drugs and Solutions

Normal saline solution was prepared by dissolving NaCl (Sigma Aldrich, cat. no. S5886) in purified MilliQ water to make a final concentration of 0.9% w/v. Pentylenetetrazole (Sigma Aldrich, cat. no. P6500) was dissolved in saline (0.9% w/v) to prepare a 200 mg/ml injectable solution. Ivabradine hydrochloride (Sigma Aldrich, cat. no. SML0281) was dissolved in MilliQ water to make a stock solution of 10 mg/ml then diluted to make two injectable solutions with a concentration of 1 mg/ml or 2 mg/ml. Retigabine (Sigma Aldrich, cat. no. SML0325) was dissolved in Dimethyl sulfoxide (DMSO) (Sigma Aldrich, cat. no. D8418) to make a stock solution 10 mg/ml then diluted to 0.5 mg/ml for injections. The HCN channel block compounds EC18, MEL55A and MEL57A (all in hydrochloride salt form) (Melchiorre et al., 2010; Romanelli et al., 2019), were provided by Prof. Romanelli (Florence, Italy). EC18 and MEL55A were dissolved in MilliQ water to make a stock solution with a concentration of 10 mg/ml. MEL57A was dissolved in DMSO to make a 10 mg/kg injectable solution.

2.3. Behavioural and Seizure Testing

Prior to all behavioural experiments, mice were acclimatized for one hour in a dimly lit behavioural room. Time to maximal hindlimb extension seizures was measured and used as the most robust seizure endpoint for all seizure assays (Chiu et al., 2008).

2.3.1. Subcutaneous PTZ Seizure Assay

Mice were weighed, then injected intraperitoneally with either the control solution (normal saline (0.9% w/v) or DMSO) to establish a control threshold, or the experimental drug at the desired dose. After 30 minutes, two mice from each cohort were injected subcutaneously with PTZ at a convulsant dose of 100 mg/kg and monitored contemporaneously in a transparent glass cage. Mice were culled immediately after the hindlimb extension seizure or after 40 minutes if they remained seizure-free.

2.3.2. Thermogenic Seizure Assay

Mice were injected intraperitoneally with either the experimental drug or normal saline (0.9% w/v) 30 minutes prior to being placed in a thermally controlled chamber set to 42 degrees Celsius. This temperature induces tonic-clonic seizures in mice, as a model of febrile seizures (Reid et al., 2013). Animals were monitored contemporaneously similar to s.c.PTZ seizure testing and culled immediately after the first observed seizure or 20 minutes after entering the chamber if they remained seizure-free.

2.4. Statistical Analysis

The total number of subjects used for each experiment was expressed as N. A P-value less than 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla California, USA). For both the s.c.PTZ and thermogenic seizure assays, data was plotted as Kaplan-Meier plots and analysed using the Log-rank (Mantel-Cox) Test; a hypothesis test used to compare survival times between samples.

3. Results

3.1. Scn1a $^{RX/+}$ mouse seizure susceptibility is reduced by ivabradine

We tested the impact of the broad-spectrum HCN channel blocker, ivabradine, on thermogenic seizure susceptibility in wildtype C57BL/6J mice. Ivabradine (10 mg/kg, i.p.) increased time to thermally-induced tonic-clonic seizures (Fig. 1A, Table S1). We extended our testing to the Scn1a $^{RX/+}$ mouse model of Dravet syndrome that recapitulates the febrile seizure susceptibility noted in patients (Richards et al., 2018). Ivabradine (10 mg/kg, i.p.) significantly right-shifted the latency to thermogenic seizures consistent with a reduction in seizure susceptibility in these mice (Fig. 1B, Table S2). Similarly, ivabradine at a higher dose (20 mg/kg, i.p.) also significantly decreased seizure susceptibility in the Scn1a R1407X Dravet mouse model (Table S3). Ivabradine (10 mg/kg, i.p.) was as effective as the antiepileptic drug retigabine (5 mg/kg, i.p.) against thermogenic seizures in the Dravet mouse model (Fig. 1B, Table S2).

3.2. Broad-spectrum HCN channel block reduced seizure susceptibility in the s.c.PTZ seizure assay

We assessed the impact of ivabradine on s.c.PTZ-induced seizures in male C57BL/6J wildtype mice. Consistent with previous studies (Cavalcante et al., 2019; Mansour and Ibrahim, 2015; Sawicka et al., 2017a; Sawicka et al., 2017b), ivabradine (10 mg/kg, i.p.) reduced seizure susceptibility, increasing the latency to s.c.PTZ-induced maximal tonic hindlimb extension seizures (Fig. 2, Table S1).

3.3. The impact of isoform-preferring HCN channel blockers on seizure susceptibility in the s.c.PTZ seizure assay

We assessed the anticonvulsant effects of three compounds with relative HCN4, HCN2, or HCN1 isoform-preferring blocking properties. EC18 is a compound that is preferentially selective for HCN4 channels, with reduced selectivity for HCN1 channels and HCN2 channels (Del Lungo et al., 2012; Postea and Biel, 2011). EC18 at 5 mg/kg had a protective effect against s.c.PTZ-induced seizures in

male wildtype mice, producing a right shift in latency to hindlimb extension seizures consistent with previously published data at 10 mg/kg (Kharouf et al., 2020) (Fig. 3A, Table S4). In contrast, the preferential HCN1 channel blocker MEL57A (Del Lungo et al., 2012) had no significant effect on seizure susceptibility in the s.c.PTZ seizure assay (Fig. 3B, Table S5). Interestingly, MEL55A, a HCN2/1-preferring channel blocker (Dini et al., 2018; Postea and Biel, 2011) exacerbated seizure susceptibility by significantly reducing the time to hindlimb extension seizures in the s.c.PTZ seizure assay (Fig. 3C, Table S6).

4. Discussion

HCN channels have long been implicated in the pathogenic mechanisms underlying epilepsy and consequently considered potential anticonvulsant targets (Benarroch, 2013; DiFrancesco and DiFrancesco, 2015; Reid et al., 2012). Several previous studies have reported the anticonvulsant properties of the broad-spectrum HCN channel blocker, ivabradine (Cavalcante et al., 2019; Luszczki et al., 2013; Mansour and Ibrahim, 2015). Here we confirm the anticonvulsant properties of ivabradine in the s.c.PTZ seizure assay. Additionally, we demonstrate that ivabradine is effective at reducing thermogenic seizure susceptibility in wildtype mice and in the Scn1a R1407X mouse model of Dravet syndrome (Cao et al., 2012). The anticonvulsant properties of EC18, a HCN4 isoform-preferring drug, highlights the HCN4 channel as a potential anticonvulsant target (Kharouf et al., 2020). In contrast, the HCN2/1 isoform-preferring drug, MEL55A, caused an increase in seizure susceptibility. While the preferential HCN1 channel blocker MEL57A had no effect on seizure susceptibility. Together these data further highlight the HCN channel class as a potential anticonvulsant target and suggest that isoform-selective drugs may be required for optimal efficacy.

Several studies support the premise that broad-spectrum HCN channel block reduces seizure susceptibility in animal models. These include the efficacy of HCN channel blockers in s.c.PTZ and kainate seizure models, as well as the MES model of epilepsy (Cavalcante et al., 2019; Luszczki et al., 2013; Mansour and Ibrahim, 2015). Furthermore, other broad-spectrum I_h blockers reduce the rate of spontaneous seizures in the seizure-sensitive Mongolian gerbil (Matsuda et al., 2008) and increased the threshold to stimulus-evoked paroxysmal discharges in the rabbit hippocampal CA1 region (Kitayama et al., 2003). Here we have extended this to include efficacy of ivabradine in a thermogenic assay that models febrile seizures. However, broad-spectrum HCN block is not effective across all proconvulsant animal models. For example, Cavalcante and colleagues show that ivabradine was not effective in the pilocarpine model of temporal lobe epilepsy (Cavalcante et al., 2019). Furthermore, ivabradine affects the potency of other anticonvulsants when administered

(Sawicka et al., 2017a; Sawicka et al., 2017b). It is also important to note that FDA approved ivabradine crosses the blood brain barrier poorly (Savelieva and Camm, 2008), discouraging testing broad-spectrum HCN block in humans. Based on this, it is clearly important to extend testing of broad-spectrum I_h block to other models of epilepsy. This should include models that exhibit spontaneous focal and in particular generalised seizures where HCN channels have been specifically implicated (Crunelli and Leresche, 1991, 2002).

Although our data suggests that isoform-selective HCN channel blockers may be preferable to broad-spectrum block in reducing seizure susceptibility, these data do need to be considered with care. Firstly, whilst pharmacokinetic data in mice is available for EC18 (Kharouf et al., 2020), pharmacokinetic studies have not yet been carried out for MEL55A and MEL57A. Whilst we know that EC18 is brain penetrant, it is possible that the lack of anticonvulsant efficacy of MEL57A is due to poor brain penetration given its physico-chemical properties (MW: 737.9 Da; hydrophilicity (ClogP): 5.92) are not ideal for CNS bioavailability. Secondly, MEL55A, MEL57A and EC18 have not been screened against a range of other ion channel targets. EC18, for example, is known to also block K^+ channels (Romanelli et al., 2019). This said, we have confidence that EC18 is acting through HCN4 channels given that its antiseizure activity is blunted in a conditional HCN4 channel knockout mouse (Kharouf et al., 2020). However, we cannot rule out the fact that the impact of MEL55A on seizure susceptibility may be due to off-target effects. Thus, this present study motivates the need to develop novel compounds with greater isoform-selectivity and suitable pharmacokinetics to further explore the role different HCN channel isoforms play in setting seizure susceptibility.

The thalamus is thought to be critical in seizure generalisation (Blumenfeld, 2005; Stafstrom, 1998). HCN2 and HCN4 channels are present at high levels in the thalamus with a significant proportion of thalamocortical neurons co-expressing both subtypes (Oyrer et al., 2019; Santoro et al., 2000). Furthermore, there is evidence from preclinical studies using knock down models that HCN2 and HCN4 channels form heterotetramers (Hammelmann et al., 2019). It is therefore somewhat

surprising that the HCN2/1-preferring drug, MEL55A, exacerbates seizure susceptibility while the HCN4-preferring drug, EC18, is protective. However, this was consistent with results from HCN knockout mouse studies. Both engineered and spontaneous HCN2 knockout mice display classical spike-and-wave discharges during electroencephalogram (EEG) recordings, a hallmark of generalised seizures (Chung et al., 2009; Ludwig et al., 2003). Furthermore, the apathetic mouse model which lacks the HCN2 subunit is more susceptible to a proconvulsant challenge (Chung et al., 2009). Similarly, viral-vector-mediated targeted deletion in mice indicate that thalamic deletion of HCN2 results in spike-and-wave discharges (Hammelmann et al., 2019). In contrast, conditional brain and thalamic targeted knockout of HCN4 channels does not cause spike-and-wave discharges in mice (Hammelmann et al., 2019; Kharouf et al., 2020; Zobeiri et al., 2019). These data strongly support the idea that HCN2 and HCN4 channels are playing distinct roles within the brain. For example, in thalamocortical neurons HCN4 channels are highly localised in the soma whereas HCN2 channels show greater expression in dendritic spines (Abbas et al., 2006). Moreover, a subset of cells within the thalamus only express HCN2 and not HCN4 mRNA, and similar expression patterns are also seen in other brain regions (Oyrer et al., 2019). Importantly, how HCN channels impact brain excitability is not likely to be limited to the thalamus. HCN2 channels are more widely expressed across the brain, including the cortex which is implicated in the initiation of spike-and-wave discharges (Polack et al., 2007). Collectively, these data suggest that drugs targeting either HCN4 or HCN2 channels will have distinct impact on brain excitability, the basis of which needs additional investigation.

We also show that HCN isoform-preferring drugs can both increase and decrease seizure susceptibility. This is in contrast with the clear anticonvulsant properties of broad-spectrum HCN channel block and difficult to reconcile. It is possible that native HCN channels that associate with auxiliary subunits, including Trip8b, may differ in their response to broad-spectrum blockers when compared with that observed in heterologous expression systems (Santoro et al., 2011). Alternatively, it is possible that the functional impact of HCN4 channel block dominates HCN2 channel block during seizures to provide protection. Both these ideas need further investigation.

The central side-effects of HCN isoform-preferring drugs is not easy to predict. We know that systemic broad-spectrum HCN channel block is well tolerated, with ivabradine causing minimal side effects such as bradycardia and a retinal phenomenon known as phosphenes in humans (Bocchi and Salemi, 2019). Consistent with this, ivabradine does not alter motor performance, long term memory, or skeletal muscle strength in mice (Sawicka et al., 2017a). Furthermore, brain HCN4 knockout in mice is well tolerated (Kharouf et al., 2020). In contrast, brain HCN2 and HCN1 knockout is poorly tolerated (Herrmann et al., 2007) with HCN2 knockout mice suffering spontaneous seizures and ataxia (Ludwig et al., 2003), and HCN1 knockout mice displaying deficits in motor learning (Nolan et al., 2004; Nolan et al., 2003). Additional isoform-selective and brain penetrant pharmacological tools are required to fully explore the central side-effects of blocking the different HCN isoforms.

In summary, we provide additional evidence that broad-spectrum HCN channel block protects against seizures. The use of HCN isoform-selective drugs suggest HCN2 channel block may be proconvulsant whilst, as previously reported, HCN4 channel block is anticonvulsant. However, this data provides no clear indication of how HCN1 channel block alters seizures susceptibility. Our results motivate a need to develop a range of brain penetrant HCN isoform-specific drugs to better explore the relationship between brain excitability and HCN channel block.

Figures

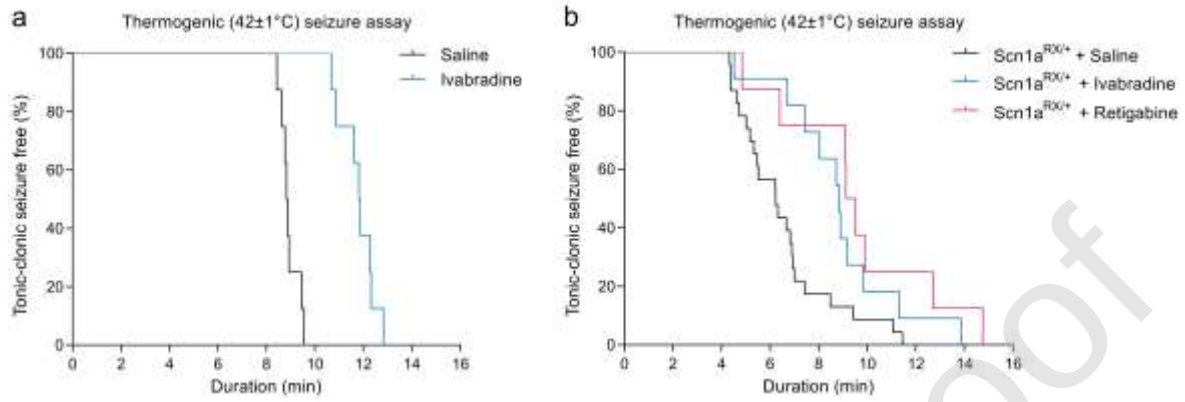

Fig. 1. Effect of Ivabradine and Retigabine on thermogenic seizure susceptibility. (a) Effect of ivabradine (10 mg/kg, i.p.) in C57BL/6J mice on the thermally-induced febrile seizure susceptibility 30 minutes post injection (Saline n=8, ivabradine n=8, $p<0.0001$). (b) Effects of ivabradine (10 mg/kg, i.p.) and retigabine (5 mg/kg, i.p.) on the thermally-induced febrile seizure latency of Scn1a $^{RX/+}$ mice 30 minutes post injection (Scn1a $^{RX/+}$ + saline n=23, Scn1a $^{RX/+}$ + ivabradine n=11, Scn1a $^{RX/+}$ + retigabine n=8). Saline vs ivabradine ($p=0.020$); Saline vs retigabine ($p=0.013$); Retigabine vs ivabradine ($p=0.344$).

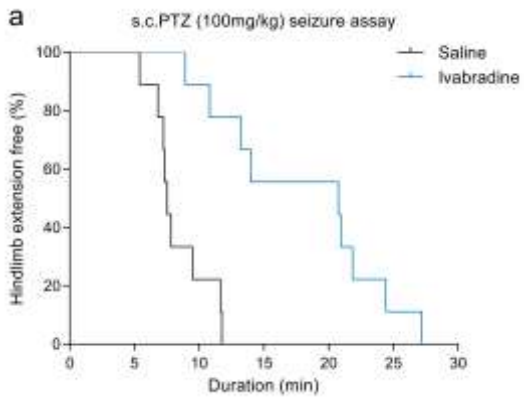

Fig. 2. Effect of the broad-spectrum HCN channel blocker ivabradine on s.c.PTZ seizure susceptibility in C57BL/6J mice. (a) Effect of ivabradine (10 mg/kg, i.p.) in C57BL/6J mice on s.c.PTZ-induced hindlimb extension seizure susceptibility 30 minutes post injection (Saline n=9, Ivabradine n=9, p=0.0003).

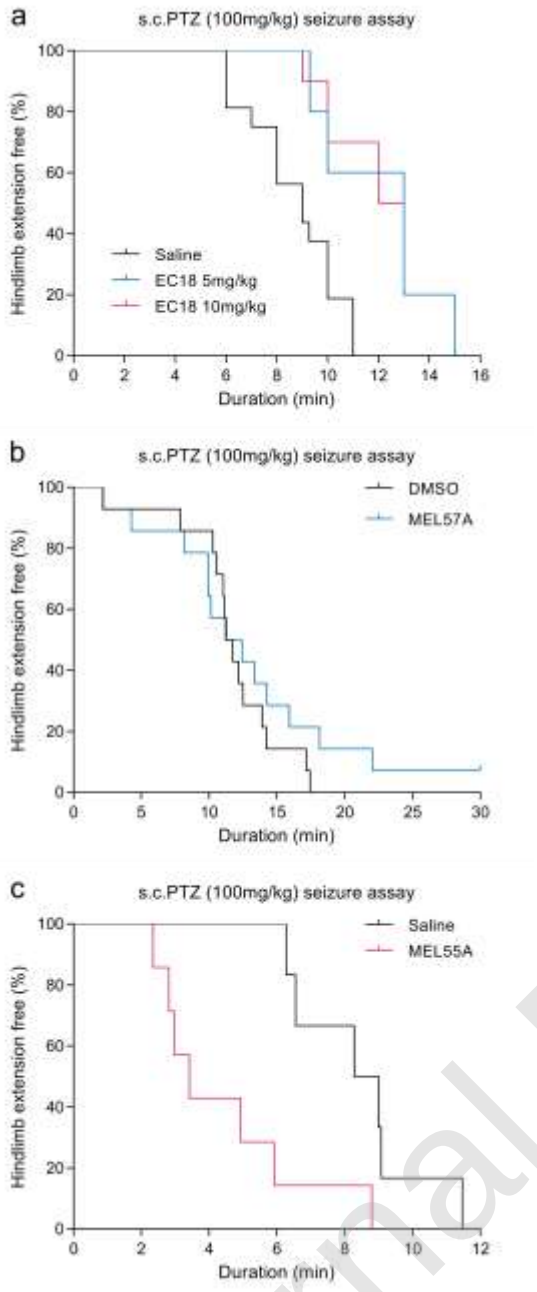

Fig. 3. Effect of isoform-preferring HCN channel blockers on s.c.PTZ seizure susceptibility in C57BL/6J mice.

(a) Effect of HCN4-preferring blocker EC18 (5 and 10 mg/kg, i.p.) in C57BL/6J mice on s.c.PTZ-induced hindlimb extension seizure susceptibility 30 minutes post injection (Saline n=16, EC18 5 mg/kg n=5, EC18 10 mg/kg n=10). Saline vs EC18 5 mg/kg (p=0.011); Saline vs EC18 10 mg/kg (p=0.0002); EC18 5 mg/kg vs EC18 10 mg/kg (p=0.948). (b) Effect of HCN1-preferring blocker MEL57A (10 mg/kg, i.p.) in C57BL/6J mice on s.c.PTZ-induced hindlimb extension seizure susceptibility 30 minutes post injection (DMSO n=14, MEL57A n=14, p=0.883). (c) Effect of HCN2/1-preferring blocker MEL55A (10 mg/kg, i.p.) in C57BL/6J mice on s.c.PTZ-induced hindlimb extension seizure susceptibility 30 minutes post injection (Saline n=6, MEL57A n=7, p=0.008).

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