Inflammatory and neuropathic pain are rapidly suppressed by peripheral block of hyperpolarisation-activated cyclic nucleotide-gated ion channels

Gareth T. Young,1,3 Edward C. Emery,2,3 Elizabeth R. Mooney,3 Christoforos Tsantoulas,3 Peter A. McNaughton

Department of Pharmacology, University of Cambridge, Cambridge, UK
Wolfson Centre for Age-Related Research, King’s College London, Guy’s Campus, London Bridge, London, UK

Keywords: Inflammatory pain Neuropathic pain Primary sensory neuron HCN channel Ion channel blocker

Abstract

Previous studies have shown that hyperpolarisation-activated cyclic nucleotide-gated (HCN)–2 ion channels regulate the firing frequency of nociceptive sensory neurons and thus play a central role in both inflammatory and neuropathic pain conditions. Here we use ivabradine, a clinically approved anti-anginal agent that blocks all HCN channel isoforms approximately equally, to investigate the effect on inflammatory and neuropathic pain of HCN ion channel block. We show that ivabradine does not have major off-target effects on a sample group of Na, Ca, and K ion channels, and that it is peripherally restricted because it is a substrate for the P-glycoprotein (PgP) multidrug transporter that is expressed in the blood–brain barrier. Its effects are therefore likely to be due to an action on HCN ion channels in peripheral sensory neurons. Using patch clamp electrophysiology, we found that ivabradine was a use-dependent blocker of native HCN channels expressed in small sensory neurons. Ivabradine suppressed the action potential firing that is induced in nociceptive neurons by elevation of intracellular cAMP. In the formalin model of inflammatory pain, ivabradine reduced pain behaviour only in the second (inflammatory) phase. In nerve injury and chemotherapy models of neuropathic pain, we observed rapid and effective analgesia as effective as that with gabapentin. We conclude that both inflammatory and neuropathic pain are rapidly inhibited by blocking HCN-dependent repetitive firing in peripheral nociceptive neurons.

1. Introduction

Many painful conditions are poorly treated by current therapies, and the drive to discover novel targets and therapies is consequently high. Therapeutic interventions directed at peripheral sensory neurons may offer an approach with fewer side effects than those directed at targets expressed in the central nervous system. The validity of approaches aimed at peripheral targets is controversial, however, because both inflammatory and neuropathic pain are known to be augmented by a poorly understood process of central sensitization [23,44], with the result that both centrally and peripherally acting drugs can deliver effective analgesia [4,22]. One major aim of the present study is to investigate whether a peripherally restricted intervention can alleviate inflammatory and neuropathic pain. Another unresolved question is whether central sensitization is a short-lasting state, maintained by continuous input from peripheral nociceptors, or whether it is long-lasting and persists independently of activity in peripheral nociceptors. A second aim was therefore to investigate whether long-established neuropathic pain can be rapidly reversed by a peripherally restricted intervention.

The family of hyperpolarisation-activated cyclic nucleotide-gated (HCN) ion channels has recently emerged as a potential analgesic target [9,14,15,24,25,29,39,43]. The HCN channel family comprises 4 members, HCN1 to HCN4. Of these, HCN1 and HCN2 are the dominant isoforms expressed in sensory neurons.
recorded from cultured neurons, we measured in small (<20-170 m) dendritic trees, with time in culture. Our cultures did not contain NT3, which has been previously shown to affect the expression of HCN1 and HCN2 in large neurons but to have little impact on expression levels in small neurons [1]. Table 1 shows that no significant changes in properties of Ih in small (<20-μm-diameter) DRG neurons were observed between 24 and 48 hours in culture.

2.3. Cell permeability

Caco-2 and MDCK (Madin-Darby canine kidney) cultured cell monolayers were used to assess directional transport of ivabradine. MDCK cells were stably transfected with the MDR1 gene which codes for the multidrug P-glycoprotein transporter (PGP). Cells were seeded at a density of 5 × 10⁵ cells onto the BD Falcon multiwell insert plate with microporous polyethylene terephthalate (PET) membrane and cultured for 21 days before the assay. Ivabradine (1% in dimethyl sulfoxide [DMSO]) was added to either the basal or the apical cell surface for 60 minutes at 37°C with shaking, and the rate of transport to the transepithelial compartment was measured with LCMS/MS (liquid chromatography/tandem mass spectrometry). The effect of inhibiting Pgp was tested by applying the specific inhibitor elacridar [2] to the apical cell surface. Transport assays were carried out by Argenta Inc (Spire Green, Harlow, UK).

2.4. Electrophysiology

2.4.1. Solutions

Manual patch clamp experiments were carried out using extracellular solution containing (in mmol/L) 140 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, and 5 glucose; pH was adjusted to 7.4 with NaOH, and osmolarity was 300 to 310 mOsm. The intracellular solution contained (in mmol/L) 140 KCl, 1.6 MgCl₂, 2.5 MgATP, 0.5 NaGTP, 2 EGTA, and 10 HEPES; pH was adjusted to 7.3, and osmolarity was 300 to 310 mOsm. Drugs used were forskolin (FSK, 50 mmol/L stock in DMSO (Sigma-Aldrich, St. Louis, MO) and ivabradine (iva, 50 mmol/L stock in DMSO; Avachen, San Antonio, TX) and were diluted in extracellular solution on the day of the experiment.

2.4.2. Whole-cell patch-clamp

Small DRG neurons <20 μm or less in diameter were selected for electrophysiological recording. Whole-cell patch-clamp recordings were performed using an Axopatch 200B patch-clamp amplifier. Patch pipettes were pulled using a P-97 horizontal micropipette puller (Sutter Instruments, USA). All pipettes were fire polished with a microforge before use and had resistances ranging between 3.5 and 5.5 MΩ. Pipette offset was corrected before seal formation. Once a giga-seal was formed between the pipette and the cell surface pipette capacitance transients were cancelled before achieving the whole cell configuration. Whole cell series resistance was compensated by 75% to 90% with a lag time of 10 μs. Cells were held at ~60 mV in the whole-cell configuration. Current-clamp protocols were performed using an I-Clamp fast mode. Junction potentials were calculated and corrected offline by ~4.3 mV for all recordings. Whole cell recordings were low-pass Bessel filtered at 10 KHz and sampled at 20 KHz. Data were acquired and analysed using pClamp 9/10 software.
Ih was measured in whole-cell patch-clamped mouse neurons by imposing a series of hyperpolarizing voltage pulses from a holding potential of –60 mV (detailed by Emery et al. [14]). \(V_{ih}\) is voltage at which \(I_h\) is half-activated both in control solution and after addition of forskolin (FSK, 50 μmol/L) to increase levels of intracellular cAMP. Change in \(V_{ih}\) after addition of FSK given as \(ΔV_{ih}\). Current density is amplitude of relaxation of \(I_h\) after voltage pulse from –40 mV to –130 mV, expressed relative to membrane surface area measured from cell capacitance. Values are given as mean ± standard error of the mean; number of cells are given in bottom row. There is no significant difference in any parameter between neonatal and adult neurons.

### Table 1
Comparison of properties of \(I_h\) in neonatal and adult mouse DRG neurons.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Large neurons (diameter &gt;30 μm)</th>
<th>Medium neurons (diameter 20–30 μm)</th>
<th>Small neurons (diameter &lt; 20 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{ih}) (mV)</td>
<td>Neontal</td>
<td>Adult</td>
<td>Neontal</td>
</tr>
<tr>
<td></td>
<td>-92.5 ± 0.6</td>
<td>-94.0 ± 1.5</td>
<td>-97.8 ± 2.0</td>
</tr>
<tr>
<td>(V_{ih}) (mV) + FSK</td>
<td>-83.9 ± 0.6</td>
<td>-86.3 ± 1.43</td>
<td>-85.1 ± 2.1</td>
</tr>
<tr>
<td>(ΔV_{ih}) (mV)</td>
<td>8.6 ± 0.85</td>
<td>7.7 ± 2.1</td>
<td>12.7 ± 2.9</td>
</tr>
<tr>
<td>Current density (pA/pF)</td>
<td>13.7 ± 3.9</td>
<td>15.2 ± 6.1</td>
<td>16.9 ± 2.9</td>
</tr>
<tr>
<td>No. of neurons</td>
<td>24</td>
<td>8</td>
<td>13</td>
</tr>
</tbody>
</table>

\(I_h\) was measured in whole-cell patch-clamped mouse neurons by imposing a series of hyperpolarizing voltage pulses from a holding potential of –60 mV (detailed by Emery et al. [14]). \(V_{ih}\) is voltage at which \(I_h\) is half-activated both in control solution and after addition of forskolin (FSK, 50 μmol/L) to increase levels of intracellular cAMP. Change in \(V_{ih}\) after addition of FSK given as \(ΔV_{ih}\). Current density is amplitude of relaxation of \(I_h\) after voltage pulse from –40 mV to –130 mV, expressed relative to membrane surface area measured from cell capacitance. Values are given as mean ± standard error of the mean; number of cells are given in bottom row. There is no significant difference in any parameter between neonatal and adult neurons.

### 2.4.3. Experimental protocol
The voltage-clamp mode was used to investigate the voltage-dependence of channel activation. An initial holding potential of –60 mV was used for all cells, and an activating step (–110 mV) was applied for 4 seconds. Between pulses, cells were held at –60 mV for 8 seconds. To assess inhibition of \(I_h\) by ivabradine, hyperpolarising steps from –60 to –110 mV were applied at intervals of 12.5 seconds, and the current magnitude averaged over 3 steps was compared before and after 425 seconds after application of ivabradine. To assess the state-dependent inhibition of ivabradine, blockade was quantified at 4 time points during a closed-channel protocol in which ivabradine was bath applied while the membrane potential was clamped at –60 mV, after which the voltage protocol continued as above. All cells were quantified at the same time points (A: final baseline step before ivabradine application, B: first sweep after resumption of activation protocol, C: halfway point to end of protocol, D: final activation step in protocol, see Fig. 1).

Quantification of the voltage-sag (V-sag) was performed by injecting a series of depolarising current pulses (0–40 pA in 10-pA increments; 1 second duration). A steady baseline of evoked firing was achieved and the train of action potentials was quantified. Resting potential was assessed via the patch amplifier just before protocols were run for each treatment group.

### 2.4.4. Automated patch clamp experiments
To assess off-target inhibition, ivabradine was tested at a concentration of 10 μmol/L against a panel of human ion channels expressed in HEK293 cells. An Ionworks Quattro automated patch clamp (Millipore Inc, St. Charles, MO) was used as described on the Millipore website (http://www.millipore.com/life_sciences/ld4/ldIon#tab1=3:tab2=1).

### 2.5. Behavioural tests

#### 2.5.1. Formalin test
Drugs or saline solution were injected intraperitoneally 30 minutes before the experiment began (see below). At time 0, formalin (4% in saline, 20 μL) was injected into the plantar surface of the left hind paw of each animal using a 50-μL Hamilton syringe and a 30-gauge needle. The time that the animal spent licking, biting, or lifting the injected paw was recorded in 5-minute intervals. The experiment continued for 60 minutes, at which time the animals were euthanised. Response patterns of the early phase (0–10 minutes) and late phase (15–60 minutes) were analysed as described elsewhere [14].

#### 2.5.2. Chronic constriction injury
Chronic constriction injury (CCI) was used as a model of neuropathic pain as described previously [14]. Briefly, after 4% isoflurane anaesthesia, the sciatic nerve was exposed and 3 nonabsorbable sterile surgical sutures (0.1 mm) were loosely tied around the sciatic nerve. For the sham-treated animals, the sciatic nerve was exposed but no ligation was performed.

#### 2.5.3. Oxaliplatin
Systemic administration of oxaliplatin was used to generate global neuropathy as described previously [11]. A single dose of 6 mg/kg oxaliplatin (Sigma-Aldrich, 6 mg dissolved in 10 mL saline solution) was administered by intraperitoneal injection.

#### 2.5.4. Heat threshold
The heat threshold was measured using a Hargreaves radiant thermal apparatus (Ugo Basile, Milan, Italy). A constant radiant stimulus was applied to the plantar surface of the hindpaw, and withdrawal time was measured, as previously detailed [14].

### Table 2
Comparison of parameters of \(I_h\) after 24 and 48 hours in vitro culture.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>24 Hours</th>
<th>48 Hours</th>
<th>P (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{ih}) (mV)</td>
<td>-97.04 ± 2.31 (n = 12)</td>
<td>-97.00 ± 0.96 (n = 13)</td>
<td>.99 (NS)</td>
</tr>
<tr>
<td>(V_{ih}) (mV) + FSK</td>
<td>-83.60 ± 2.17 (n = 7)</td>
<td>-86.09 ± 1.20 (n = 7)</td>
<td>.34 (NS)</td>
</tr>
<tr>
<td>(ΔV_{ih}) (mV)</td>
<td>13.44 ± 2.17</td>
<td>10.91 ± 1.20</td>
<td>.33 (NS)</td>
</tr>
<tr>
<td>Current density (pA/pF)</td>
<td>15.87 ± 1.36 (n = 12)</td>
<td>15.99 ± 1.55 (n = 13)</td>
<td>.72 (NS)</td>
</tr>
<tr>
<td>(t_s) (milliseconds)</td>
<td>179.65 ± 23.22 (n = 10)</td>
<td>192.83 ± 21.62 (n = 10)</td>
<td>.68 (NS)</td>
</tr>
</tbody>
</table>

NS, not significant.

\(I_h\) measured from small (≤20-μm-diameter) neonatal mouse dorsal root ganglion neurons. \(V_{ih}\) is time constant of fast phase of relaxation of \(I_h\) at –140 mV. Other parameters have the same meaning as in Table 1.
2.5.5. Mechanical threshold

The mechanical threshold for paw withdrawal was assessed using a dynamic plantar aesthesiometer (Ugo Basile, Milan, Italy). A force increasing to 5 g was applied over a 10-second ramp to the plantar surface of the hindpaw, after which the force remained constant at 5 g for 30 seconds as detailed elsewhere [14]. Paw withdrawal threshold in animals that had undergone CCI was tested before and 7, 14, 21, and 28 days after surgery, whereas for oxaliplatin-injected animals the threshold was tested before injection and on 4 subsequent days.

2.5.6. Dynamic cold plate

Sensitivity to noxious and non-noxious cold stimuli was assessed using a cold plate (Panlab/Harvard Apparatus, UK). Animals that had undergone systemic oxaliplatin administration were assessed for cold plate sensitivity by placing them on a platform at 20 °C, which decreased at a steady rate of 2 °C/minute to 0 °C over 10 minutes. Jumps (defined as both rear paws off the plate simultaneously) and the temperature at which they occurred were recorded and the number of jumps grouped into 2 °C bins. Animals were removed from the plate as soon as the temperature reached 0 °C to prevent tissue injury. Animals were assessed before and 4 days after oxaliplatin administration.

2.5.7. Rotarod

Mice were trained over 3 successive days on an accelerating rotarod (Ugo Basile, Milan, Italy; 4–40 rpm. over 120 seconds). On the fourth day, the time that the mice remained on the rotarod was assessed. Mice were tested before and 30, 60, and 120 minutes after administration of ivabradine, gabapentin, or saline solution.

2.5.8. Pulse oximetry

Mice were habituated in individual Perspex chambers for 1 to 2 hours for days and for >10 minutes immediately preceding each experiment. A collar transducer clip connected to a MouseOx pulse oximetry system (Starr Life Sciences, USA) was fitted to the animal and monitoring commenced. The heart rate trace (derived by an algorithm within the MouseOx system from the pulse-to-pulse fluctuation in arterial oxygen saturation) was monitored and recording commenced when the trace was stable. The trace was recorded for 5 minutes and the clip was removed. After intraperitoneal (IP) injection of ivabradine or saline solution, the mouse was returned to the chamber for 25 minutes, after which the collar clip was reattached and heart rate was recorded for a period of 27.5 to 32.5 minutes after the IP injection. The same mice were reused for different drugs and doses, with at least 24 hours between each dose.

2.5.9. Injection of drugs

Ivabradine (Avachen, San Antonio, TX) and gabapentin (a kind gift from Dr Mike Rigby) were diluted to the desired concentrations in saline solution and injected intraperitoneally (IP). Injection volumes were 10 μl/kg body weight. All experimenters were blinded to the identity and concentrations of injected drugs.

2.6. Statistical analysis

All datasets were tested for approximation to a normal distribution using Graphpad Prism software (D’Agostino’s test used where n > 7, Kolmogorov–Smirnov test used where n ≤ 7). All datasets except one were found to be normally distributed by these criteria and were analysed by parametric statistical tests (t test for single comparisons or one-way analysis of variance [ANOVA] with Bonferroni post-hoc correction for multiple comparisons). The one dataset that did not pass these criteria was analysed with a nonparametric test (Kruskal–Wallis test followed by Dunn’s multiple comparison test).

3. Results

3.1. Characteristics of Ih current carried by HCN channels

Table 1 outlines the properties of Ih, the current carried by HCN ion channels, in cultured DRG neurons of different sizes. Current densities and the shift in V1/2 caused by elevation of intracellular cAMP are similar in adult and neonatal neurons of all sizes. In both adult and neonatal neurons, the maximum current amplitude is largest in large-diameter neurons and smallest in small-diameter neurons, even when the difference in neuronal size is taken into account by normalising to cell capacitance, in agreement with studies of DRG neurons recorded using single-electrode voltage clamp in rats in vivo [17]. In small neurons, the large shift of V1/2 after elevation of cAMP is consistent with expression of HCN2 [29]. In large neurons, expression of HCN1 is shown by the rapid time course of relaxation of current, and the absence of this rapidly relaxing current component in neurons from HCN1-deleted animals [29]. In large neurons, a shift in V1/2 is observed in response to elevation of cAMP (Table 1), however, consistent with at least some expression of HCN2. The smaller size of this shift when compared with that in small neurons can be attributed to co-expression of cAMP-sensitive HCN2 with cAMP-insensitive HCN1. Immunocytochemical studies have also found evidence for expression of HCN2 in both small and large neurons [19,43].

3.2. Ivabradine selectively inhibits HCN ion channels

In intact rabbit sinoatrial cells, 10 μmol/L ivabradine has been found to have no detectable effect on T-type calcium currents (CaV3.x) and to have only a small effect on L-type calcium currents (CaV1.x) and potassium currents [16% and 18% inhibition, respectively [61]]. To extend these studies, we tested 10 μmol/L ivabradine against a standard panel of 7 ion channels (Table 3), most of which are expressed in DRG neurons (Kv4.3, members of the Kv7.x family, and Kc, 2.1 are expressed in DRG neurons [42], and CaV1.2 channels are also expressed in DRG neurons [16]). The largest inhibition was ~20% for Kc11.1 (hERG), with the cardiac sodium channel (NaV1.5) inhibited 14% and the L-type cardiac channel (CaV1.2) not detectably inhibited. Inhibition of potassium channels apart from hERG was small and probably within the variability of the assay. Block of the ion channels in Table 3 by ivabradine was therefore much less than block of HCN ion channels at the same concentration. An inhibition of hERG of 20% at 10 μmol/L [ivabradine] corresponds to an IC50 ~ 40 μmol/L for first-order inhibition, compared to IC50 ≈ 2 μmol/L for the HCN1-4 ion channel isoforms [37]. Ivabradine is therefore at least 20-fold more selective for HCN ion channels than the other ion channels tested here, which are a subset of those expressed in DRG neurons.

3.3. Ivabradine is actively transported by P-glycoprotein

Levels of ivabradine in the brain are less than 3% of the plasma concentration (submission by Servier Inc to EMEA, 2005, see pg. 11 in http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000597/WC500043587.pdf). To determine the reason for CNS exclusion of ivabradine, we tested the permeability of ivabradine across a Caco-2 polarised cell monolayer, which strongly expresses the P-glycoprotein (Pgp) multidrug-resistance transporter [33]. Flux ratios were highly asymmetrical, with a ratio of 12.2:1 between flux from the basal to the apical membrane compared to the flux in the reverse direction, suggestive of active extrusion across the apical membrane by Pgp (Table 4). To more directly assess the involvement of Pgp, we used an MDCK cell monolayer stably transfected with MDR1, the gene encoding Pgp. A strongly asymmetrical flux was also
observed in MDCK cells (flux ratio 10.3). The asymmetry was abol-
ished by the specific Pgp inhibitor elacridar (flux ratio 0.7) (Table 4).

The strong Pgp-dependent extrusion demonstrates that ivabradine is a substrate for Pgp transport, in support of the idea that low brain
penetration is due to active transport by Pgp.

### 3.4. Ivabradine inhibits native Ih in small DRG neurons

Inhibition of native Ih in dorsal root ganglion (DRG) neurons has
been reported for ZD7288 [5,45] but the action of ivabradine has not
previously been investigated. Whole-cell recordings of small
cultured DRG neurons were performed to examine the blocking
properties of ivabradine on Ih. Note that HCN1 is expressed in large
and medium-sized neurons but not in small neurons [29], whereas
HCN2 and HCN3 are expressed in small neurons (diameter
<20 μm) [14]. All neurons used in the present study were small,
and Ih is therefore likely to reflect the properties of HCN2/3. It is
possible that HCN2 and HCN3 may form heteromers with proper-
ties different from those of the individual homomeric channels, but
an investigation of this possibility was beyond the scope of the
present study. HCN2 is also expressed in some large neurons
[1,14]. The Na\(_{\text{a1.8}}\)-driven Cre construct used to delete HCN2 in this
and an earlier study from our laboratory [14] will therefore delete
HCN2 in both large and small neurons because Na\(_{\text{a1.8}}\) is expressed
in neurons across the whole size range [36].

In voltage-clamp mode, prolonged application of 30 μmol/L ivab-
radine slowly blocked hyperpolarisation-activated inward
currents elicited by repeated steps from −60 to −110 mV (Fig. 1A, upper
panels; mean ± standard error of the mean (SEM) current after 500 seconds ivabradine 0.05 ± 0.02 relative to control, n = 9). With repeated pulsing in current-clamp mode, a corresponding
block of the “voltage sag” was seen (Fig. 1A, lower panels; control V-sag = 0.81 ± 0.01; post-ivabradine V-sag = 0.96 ± 0.01, n = 14). Block of Ih as a function of ivabradine concen-
tration gave a value of IC\(_{50}\) = −1.4 μmol/L (Fig. 1B). Ivabradine has
been reported to have comparable potency at all recombinantly
expressed HCN channels (~2 μmol/L at HCN 1–HCN4) [37], and
the data presented here show that ivabradine acts with a similar
potency on native Ih in DRG neurons.

The slow block of neuronal Ih by ivabradine (Fig. 1C) could be
due to poor permeability across the neuronal cell membrane or
to a state-dependent block of open HCN channels. It has been
shown previously that ivabradine preferentially blocks open
HCN4 channels and closed HCN1 channels [8]. Ih was activated
by voltage steps from −60 to −110 mV (Fig. 1D), and ivabradine
was applied while HCN channels were maintained in the closed
state by clamping at −60 mV. No block was observed in the
absence of channel activation (current ratio B/A 0.92 ± 0.04, n = 7), but, on
resumption of voltage, pulsing progressive block resumed with a similar time course to that in Fig. 1C (current ratio D/A 0.20 ± 0.03 at end of protocol, n = 7). This experiment demon-
strates that permeability across the cell membrane is not a factor
in the slow block; block is therefore use dependent, and only open
Ih channels are blocked. Because ivabradine preferentially blocks
HCN1 channels in their closed state [8], these data support our pre-
vious findings [14,29] that Ih in small diameter neurons is com-
posed of HCN2 and HCN 3 and that HCN1 expression is restricted
to large-diameter DRG neurons.

Activation of HCN2 is critical for the generation of repetitive
firing by inflammatory mediators such as prostaglandin E\(_2\) (PGE\(_2\))
that elevate cAMP [14,29]. In agreement, we found that the
increase in action potential frequency induced by forskolin, which
directly activates adenylate cyclase and thus elevates cAMP, was
completely reversed by ivabradine (Fig. 1E, left). Firing in response
to depolarizing current pulses was found to be enhanced when
cAMP was elevated (Fig. 1E, right), and the increase was inhibited
by ivabradine (control cells 0.14 ± 0.07 Hz/pA, n = 6; forskolin-
treated 0.35 ± 0.06 Hz/pA, n = 6, P = .04, paired t test, compared to
test; forskolin/ivabradine-treated cells 0.17 ± 0.11 Hz/pA,
 n = 6, P > .05, paired t test, compared to control).

### 3.5. Ivabradine does not affect acute pain thresholds

We initially tested whether ivabradine had any effect on normal
thermal and mechanical pain thresholds in vivo. Withdrawal time
to a standard thermal stimulus (Hargreaves’ test) was 7.68 ± 1.46
seconds in saline-injected mice and 8.53 ± 1.27 seconds 30 min-
utes after injection with 5 mg/kg ivabradine. Withdrawal force to
a standard increasing mechanical stimulus was 3.37 ± 0.34 g in
saline-injected mice and 3.21 ± 0.32 g 30 minutes after injection
with 5 mg/kg ivabradine. There was no significant difference in
either case (P = .52 and .89 respectively; all values mean ± SEM, n = 6). The lack of effect of ivabradine on acute pain thresholds is
consistent with its lack of effect on first-phase pain behaviour in
the formalin test (see below).

### 3.6. Ivabradine is analgesic in a model of inflammatory pain

We and other groups have observed that HCN blockade can alle-
viate inflammatory pain [14,39], and we therefore examined the
analgesic action of ivabradine in inflammatory pain models
in vivo. We used the formalin model, in which the first phase of
pain behaviour is thought to be due to a direct action of formalin
on nociceptors [28] and the second phase to an indirect action of
inflammatory mediators on nociceptors [21]. The formalin test
was performed as previously described [14], with ivabradine or
saline control injected intraperitoneally 30 minutes before
formalin (Fig. 2A). A typical biphasic response was observed in
saline-injected animals, with an early phase of pain behaviour that
rapidly decayed followed by a prolonged secondary phase. Ivabra-
dine had no effect on the early nociceptive phase (0–10 minutes) at
any concentration (Fig. 2B). However a dose-dependent suppres-
sion of the inflammatory phase (15–60 minutes) was observed.
This experiment provides further evidence that HCN channels play
a role in pain associated with inflammation, and, coupled with the
patch-clamp data above, suggests that second-phase inflammatory
pain in the formalin test can be at least in part attributed to
enhanced HCN channel activity.

Ivabradine causes bradycardia (Fig. 7 below), raising the possi-
bility that the analgesia observed in the formalin test could be due
to an indirect effect of the suppression of heart rate, perhaps
attributable to a slowing of movement or reaction time caused

<table>
<thead>
<tr>
<th>Ion channel</th>
<th>% Inhibition ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(_{\text{a1.5}})</td>
<td>14.6 ± 2.1</td>
</tr>
<tr>
<td>Ca(_{\text{v1.2}})</td>
<td>−1.2 ± 3.0</td>
</tr>
<tr>
<td>K(_{\text{v1.5}})</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td>K(_{\text{v4.3}})</td>
<td>−11.1 ± 1.6</td>
</tr>
<tr>
<td>K(_{\text{v7.1}}) (KCNQ1)</td>
<td>−5.9 ± 1.4</td>
</tr>
<tr>
<td>K(_{\text{v11.1}}) (hERG)</td>
<td>20.5 ± 1.2</td>
</tr>
<tr>
<td>K(_{\text{v2.1}})</td>
<td>1.2 ± 2.3</td>
</tr>
</tbody>
</table>

HCN, hyperpolarisation-activated cyclic nucleotide-gated; SEM, standard error of the mean.

Ivabradine was tested at a concentration of 10 μmol/L in
0.33% dimethyl sulfoxide (DMSO) against a standard panel of 7 human ion channels (left-hand column). Percent inhibition is mean ± SEM (n = 8) expressed relative to mean change in current observed in parallel experiments with DMSO alone. (See Methods section for details.)
Ivabradine is actively transported by Pgp.

<table>
<thead>
<tr>
<th>Cell monolayer</th>
<th>Flux basal → apical (x 10⁻⁶ cm/s)</th>
<th>Flux apical → basal (x 10⁻⁶ cm/s)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>31.2</td>
<td>2.6</td>
<td>12.2</td>
</tr>
<tr>
<td>MDCK</td>
<td>28.6</td>
<td>2.8</td>
<td>10.3</td>
</tr>
<tr>
<td>MDCK+elacridar</td>
<td>15.9</td>
<td>22.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Fluxes of ivabradine from basal to apical membrane and from apical membrane to basal were measured in Caco-2 and MDCK cells ± inhibition of P-glycoprotein (Pgp) by apical application of 10 μmol/L elacridar. MDCK (Madin-Darby canine kidney) cells were stably transfected with the MDRI gene, which codes for Pgp, whereas Caco-2 cells show endogenous Pgp expression. Asymmetrical flux ratios in the 2 cell lines were similar, and the asymmetrical flux in MDCK cells was abolished by Pgp inhibition using elacridar.

by inadequate tissue perfusion. We therefore used the rotarod model to assess whether locomotor ability was affected in ivabradine-treated mice. Mice were given the highest concentration of ivabradine examined in the formalin test (10 mg/kg) and compared to saline-injected animals. No significant effect of ivabradine on motor agility was observed in any of the trials (Fig. 2C), suggesting that ivabradine is unlikely to cause a decrease in formalin-induced pain behaviours through an effect on motor performance.

### 3.7. HCN2 block accounts for analgesic effects of ivabradine in inflammatory pain

Previous work has shown that the HCN2 isoform plays an important role in both inflammatory and neuropathic pain [14], but the possibility that other isoforms may also contribute has not been investigated. We therefore compared the effect of ivabradine, which blocks all HCN isoforms equally [37], on pain behaviour in WT mice and in mice in which the HCN2 gene had been deleted selectively in neurons expressing NaV1.8 by the use of a Cre-lox system (cKO mice) [14]. None of these interventions had any significant effect on first-phase pain behaviour. In agreement with previous results, we found that deletion of HCN2 significantly reduced second-phase pain behaviour in the formalin test (Fig. 3). Ivabradine reduced second-phase pain behaviour to a level not significantly different from that seen in cKO mice. Treatment of cKO mice with ivabradine may have caused a small additional inhibition of second-phase pain behaviour (Fig. 3, last bar); but any such effect, if it existed, was small and did not reach statistical significance when compared with the effects of either NaV1.8-specific HCN2 deletion or ivabradine treatment alone. There is therefore no clear evidence for involvement of any HCN isoform apart from HCN2 in inflammatory pain.

### 3.8. Ivabradine is analgesic in models of neuropathic pain

Neuropathic pain is a chronic pain state associated with nerve damage. We have previously shown that genetic deletion of HCN2 ion channels from NaV1.8 expressing neurons prevents thermal hyperalgesia, mechanical hyperalgesia, and cold allodynia in a model of neuropathic pain [14]. These experiments showed that neuropathic pain failed to develop when HCN2 was deleted in nociceptive neurons, but they do not show whether block of HCN2 will be analgesic in the more clinically relevant situation of treatment after the development of neuropathic pain. We therefore tested whether ivabradine could act as an interventional therapy in an established neuropathic pain state. Gabapentin was used for comparison with ivabradine, as it is a widely used treatment for neuropathic pain. Gabapentin is not peripherally restricted, and a prominent side effect is sedation [7].

To induce neuropathic pain, we used the chronic constrictive nerve injury model and assessed mechanical hyperalgesia using a dynamic plantar aesthesiometer on days 7, 14, 21, and 28 post-surgery. Mechanical hyperalgesia was observed in all groups at all time points post-surgery (Fig. 4A). On assessment days, a baseline measurement of mechanical threshold was made before injection of saline solution, ivabradine, or gabapentin. Mechanical threshold was then re-assessed 30 minutes after treatment. In the post-treatment measurement, saline-injected animals exhibited mechanical withdrawal thresholds similar to pre-treatment levels. Ivabradine and gabapentin-injected animals exhibited much higher mechanical pain thresholds, approaching levels observed before surgery (Fig. 4A, B). The effects of treatment with ivabradine and gabapentin were not significantly different on any of the trial days (Fig. 4B).

Mechanical hyperalgesia and cold allodynia are often observed in patients undergoing chemotherapy with compounds such as oxaliplatin and cisplatin. In rodents, oxaliplatin also causes a generalised neuropathy [11]. After a single injection of oxaliplatin, mechanical pain thresholds were found to decrease progressively over 4 days, reflecting a developing neuropathy (Fig. 5). On day 4 mice were injected with saline, ivabradine, or gabapentin. Administration of ivabradine or gabapentin resulted in a significant analgesia, as shown by an increase in mechanical pain threshold.

Strikingly, ivabradine treatment completely reversed mechanical hyperalgesia, to the level observed before oxaliplatin treatment, whereas a small residual mechanical hyperalgesia was still observed in the gabapentin-treated animals (Fig. 5, right panel).

To assess cold allodynia in mice treated with oxaliplatin, a dynamic cold ramp decreasing slowly from 20°C to 0°C was used. The number of jumps in response to the cold ramp was collected into 2°C bins before and 4 days after oxaliplatin injection (Fig. 6). The number of jumps for saline-injected mice was significantly greater after oxaliplatin treatment than before (Fig. 6A, D). In particular, it was clear that after oxaliplatin injection, the mice were sensitive to temperatures in the non-noxious range (6°C–14°C). The data were separated into 3 segments of 0°C to 6°C (noxious cold), 6°C to 14°C (non-noxious cold), and 14°C to 20°C (non-noxious cool). The number of jumps after oxaliplatin administration was significantly different from control in the non-noxious cold range (Fig. 6A), consistent with the generation of cold allodynia, a hallmark of oxaliplatin-induced neuropathy in humans.

In mice treated with ivabradine, the behaviour over all temperature ranges was indistinguishable from that before treatment with oxaliplatin (Fig. 6B, D); in particular, there was no sign of increased jumping in the non-noxious cold range of temperatures. Gabapentin was also successful in reducing cold-induced jumping (Fig. 6C, D), although in this case there was also reduced jumping in response to noxious cold temperatures, which may be attributable to the well-known sedative effect of gabapentin [7].

### 3.9. Ivabradine and bradycardia

The heart rate of conscious, freely moving mice after administration of ivabradine was recorded using a pulse oximeter. As the ivabradine concentration was increased, the heart rate stabilised at approximately 60% of its pre-ivabradine level (Fig. 7) and was not further suppressed even at high doses of ivabradine (up to 50 mg/kg). The bradycardic IC₅₀ of ivabradine was 2.71 ± 1.2 mg/kg. To estimate the dose–response relation for pain suppression, the data from Fig. 2 are replotted in Fig. 7 and give an IC₅₀ of 2.03 ± 1.3 mg/kg. Ivabradine thus acts as an analgesic with a potency similar to its action as a bradycardic agent. HCN2-selective compounds will therefore be required to achieve a usable therapeutic window between analgesia and bradycardia.
4. Discussion

Inflammatory pain is initiated by tissue injury and normally resolves once the injury heals. Neuropathic pain is initiated by damage to peripheral nerves, whether direct (e.g., mechanical damage) or caused by metabolic insufficiency (e.g., in diabetic neuropathy). In both cases, pain is augmented by a poorly understood process of central sensitization, as a result of which even nonpainful stimuli, applied to undamaged tissue distant from the site of injury, are perceived as painful [23,32,44]. Analgesics applied either peripherally or intrathecally can reduce pain, which has led to a debate over the location of the analgesic target, with many analgesics such as nonsteroidal anti-inflammatory drugs (NSAIDs) thought to be acting at both locations [21]. Neuropathic pain continues long after the inflammation associated with damage to a peripheral nerve has apparently subsided, which has led to a view that neuropathic pain is sustained by a long-lived state of central sensitization, independent of action potential firing in nociceptors.

![Ivabradine is a blocker of native hyperpolarisation-activated cyclic nucleotide-gated (HCN) ion channels in small sensory neurons.](image)

- **Fig. 1.** Ivabradine is a blocker of native hyperpolarisation-activated cyclic nucleotide-gated (HCN) ion channels in small sensory neurons. (A) Upper panel: In voltage-clamp mode, $I_{h}$ was activated by a 4-second voltage-step from a holding potential of −60 mV to −110 mV. $I_{h}$ was completely abolished with bath application of 30 μmol/L ivabradine in the presence of repetitive voltage-clamp pulses to −110 mV (4-second pulses repeated every 12 seconds; ivabradine-treated current 0.05 ± 0.02 expressed as fraction of control, mean ± standard error of the mean [SEM], n = 9). Lower panel: In current-clamp mode, a prominent voltage sag was observed after a 1-second hyperpolarising current injection (−100 pA in this cell). $V_{max}$ was abolished with bath application of 30 μmol/L ivabradine in the presence of repetitive current pulses (1-second pulses repeated every 12 seconds; control $V_{max} = 0.81 ± 0.01$; ivabradine $V_{max} = 0.96 ± 0.01$, n = 14). (B) Concentration–response curve obtained in voltage-clamp experiments as in A. Each point represents the mean fractional inhibition (±SEM) of $I_{h}$ at the stated concentrations (number of cells tested given above each point). IC50 derived from fitted first-order function at −1.4 μmol/L. (C) Representative time course of current amplitudes demonstrating blockade of $I_{h}$ by ivabradine (30 μmol/L) during repetitive voltage clamp pulses as in A, with complete block occurring after 400 seconds. Inset shows representative traces from time points A to D as marked. (D) Representative trace showing that ivabradine (10 μmol/L) does not block closed channels. $I_{h}$ measured as in A with cessation of voltage pulses between points A and B. Mean value of current ratio B/A was 0.92 ± 0.04 (n = 7). Ratio C/A (open-channel block over the same period as B/A) was 0.37 ± 0.03 (n = 7). (E) Left panel: Representative action potentials induced by 30-pA current injection (control), after elevation of cAMP by addition of forskolin (FSK, 50 μmol/L) and after 30-μmol/L ivabradine application (FSK/Iva). Middle panel: frequency of evoked action potentials as a function of injected current. Right panel: Action potentials evoked per second per pA current injected over range of 0 to 40 pA. Control cells (0.14 ± 0.07 Hz/pA, n = 6, $P = .04$ paired t test) but not different from forskolin/ivabradine treated cells (0.17 ± 0.11 Hz/pA, n = 6, $P > .05$ paired t test). Resting membrane potential was significantly depolarised by FSK (control, $−57.3 ± 3.0$ mV, n = 6; FSK, $−46.9 ± 4.2$ mV, n = 6, $P = .04$, paired t test) and hyperpolarised again after ivabradine treatment ($−58.1 ± 5.7$ mV, n = 6, $P > .05$ compared to control, paired t test) consistent with increased inward $I_{h}$ at rest after elevation of cAMP by FSK.
Recent work shows, however, that electrical activity continues in nociceptive C-fibres of both rodents and humans even years after a nerve injury [13,35]. Recent evidence from our laboratory and others has shown that the HCN2 ion channel isoform drives both inflammatory and neuropathic pain [9,14,15,24,25,29,39,43]. One main objective of the present study was to further investigate the role of HCN ion channels in inflammatory and neuropathic pain. We used the HCN blocker ivabradine, which is a nonselective blocker of all 4 HCN isoforms [8,37] but does not appear to have significant off-target actions. Ivabradine also did not affect motor performance (Fig. 2C), suggesting that it does not affect general alertness or neural circuits involved in motor control, or muscle spindle afferents, which have been shown to express HCN2 [1], either directly or indirectly via bradycardia. We note, in addition, that side effects indicative of interference with the function of large sensory neurons (paraesthesias, problems with motor control) have not been previously reported for ivabradine (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000597/WC500043587.pdf).

We first studied the effects of ivabradine on the \( I_h \) current in small sensory neurons. The IC_{50} of ivabradine for \( I_h \) in small sensory neurons found here (~1.4 \mu mol; Fig. 1B) is similar to the value previously reported for \( I_h \) in cardiac tissue of ~2.8 \mu mol/L [6,37]. The onset of block in sensory neurons is slow because of preferential open-channel block by ivabradine (Fig. 1C, D). Previous work has described an open-channel block of HCN4 ion channels by ivabradine [8,37] but a block of closed HCN1 ion channels [8]. The lack of closed-channel block in small sensory neurons is consistent with other evidence showing that HCN1 is not expressed in small sensory neurons [14,29].

HCN2 modulates action potential firing in PGE_2- or forskolin-treated sensory neurons because elevated cAMP shifts the \( I_h \) activation curve to more positive membrane potentials [14,29]. Consistent with previous findings with ZD7288, we found that ivabradine was also able to reverse the increase in the excitability of small neurons, the majority of which are nociceptors, after sensitization of \( I_h \) with forskolin (Fig. 1E). Notably, the relation between injected current and action potential firing was similar to control after block of HCN channels with ivabradine, showing that HCN channels do not contribute to small-neuron excitability in the absence of inflammatory mediators (Fig. 1E). This result is consistent with the lack of effect of ivabradine on acute pain thresholds and on the first phase of the formalin test.

Rodent models of pain can mimic painful conditions experienced by patients. As a model of inflammatory pain, we used the formalin test, in which the initial acute phase of pain behaviour after plantar formalin injection is due to direct activation of
nociceptors, and the second phase to indirect activation of nociceptors by the release of inflammatory mediators. The lack of known off-target effects of ivabradine allowed us to assess more accurately the effect of HCN ion channel inhibition in behavioural models. In contrast to ZD7288, ivabradine had no effect on the initial phase of pain behaviour in the formalin model, but it attenuated (by \( \frac{1}{2} \)) the late, inflammation-driven phase in a way similar to that of ZD7288. The effect of ivabradine in the formalin test closely matches that of nociceptor-targeted deletion of the HCN2 gene, which causes a similar \( \frac{1}{2} \) reduction in late-phase pain behaviours without affecting the early acute nociceptive phase [14] (Fig. 3).

Do HCN ion channels other than HCN2 contribute to inflammatory pain? To address this question, we tested ivabradine in mice in which the HCN2 gene had been deleted in nociceptive neurons. In the formalin test, there was some suggestion of an additional second-phase analgesia beyond that produced by ivabradine or by HCN2 deletion alone (Fig. 3), but the change was not statistically significant, so there is no compelling evidence that block of HCN channels other than HCN2 contributes to the analgesic effect of ivabradine.

We have recently shown that targeted deletion of the HCN2 gene within the Na\(_{\text{v}}\)1.8-expressing population of DRG neurons completely prevents the onset of thermal and mechanical hyperalgesia after a nerve lesion [14]. These experiments do not, however, offer any insight into whether HCN2 ion channels are necessary for the maintenance of a neuropathic pain state. In mice subjected to a nerve lesion, ivabradine administration was found to abolish mechanical allodynia when administered up to 4 weeks postsurgery, a level of analgesia not significantly different from that obtained using gabapentin, a common clinical treatment for neuropathic pain (Fig. 4). This experiment demonstrates a role for peripheral HCN channels in the long-term maintenance of chronic neuropathic pain conditions. This result suggests that HCN2 in peripheral nociceptors continues to be modulated by inflammatory mediators long after nerve injury has occurred. In recordings from intact nerve fibres after nerve injury, maintained activity is observed in both injured A-fibres and in intact C-fibres [13,35]. Both cyclooxygenase 2 (COX2) and PGE\(_2\) levels remain elevated at the site of nerve injury for at least 18 months [18,26], and PGE\(_2\) is therefore a possible long-term contributor to neuropathic pain. In a clinical setting, however, neuropathic pain is notoriously insensitive to COX inhibitors, and other inflammatory mediators may therefore also contribute to the long-term maintenance of the neuropathic pain state [3].

We investigated the ability of ivabradine to reverse a systemic neuropathy induced by oxaliplatin, used in the treatment of cancer. A common dose-limiting side effect of oxaliplatin is the development of a painful peripheral neuropathy that manifests as a cold and mechanical hypersensitivity [38]. The underlying cause of chemotherapy-dependent neuropathic pain is largely unknown, although recent evidence has suggested a role for oxidative stress [12]. We found that ivabradine abolished mechanical allodynia in the oxaliplatin model, and in this respect was as at least as effective as gabapentin (Fig. 5). In mice treated with oxaliplatin, we observed cold allodynia in the normally nonnoxious range.
between 8°C and 15°C (Fig. 6A). Ivabradine abolished cold allostodynia in this temperature range without affecting sensitivity to normally noxious temperatures of 5°C and below (Fig. 6B). Gabapentin, by contrast, partially suppressed cold sensitivity in the non-noxious range but also inhibited sensitivity to noxious cold below 5°C, an effect that may be related to the sedative effect of gabapentin [7]. These results support the hypothesis that nociceptor HCN2 channels are involved in the neuropathic allostodynia induced by chemotherapy.

A major disadvantage of ivabradine as a potential analgesic is bradycardia caused by block of cardiac HCN4 ion channels. We investigated whether there might be any “therapeutic window” in which analgesia was produced by HCN2 block before the onset of bradycardia caused by block of HCN4 in the heart. It is perhaps...
not surprising, in view of the lack of selectivity of ivabradine for HCN channel isoforms, that the IC50 value for alleviation of inflammatory pain, ~2 mg/kg, was similar to the value of ~2.7 mg/kg for half-maximal inhibition of heart rate (Fig. 7B).

The results presented here show a significant role for peripheral HCN ion channels in regulating both inflammatory and neuropathic pain in mice. The clinically approved pan-HCN blocker ivabradine was analgesic in the formalin model of inflammatory pain, in a nerve injury neuropathic pain model, and in a systemic oxaliplatin neuropathic pain model. In both neuropathic pain models, the analgesic effect of ivabradine was not inferior to that of gabapentin, suggesting that HCN inhibition can provide the same level of analgesia as a common current treatment for neuropathic pain. Ivabradine was an effective analgesic even long after the initiation of neuropathic pain, showing that ongoing activity of HCN ion channels in peripheral nerve fibres is critical to the maintenance of the neuropathic pain state. The results described here suggest the testing of ivabradine as an analgesic in humans.

Conflict of interest statement

The authors declare that they have no conflict of interest in regard to this work.

Acknowledgements

The authors thank A. Charlton for technical assistance. This work was supported by the BBSRC (Biotechnology and Biological Sciences Research Council, UK) and by an MRC (Medical Research Council, UK) studentship (to E.R.M.).

References


