Supporting Online Material for

HCN2 Ion Channels Play a Central Role in Inflammatory and Neuropathic Pain

Edward C. Emery, Gareth T. Young, Esther M. Berrocoso, Lubin Chen, Peter A. McNaughton*

*To whom correspondence should be addressed. E-mail: pam42@cam.ac.uk

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This PDF file includes:

Methods

Generation of mice

Mice in which the HCN2 gene had been flanked with loxP sites (floxed) were created by homologous recombination by Lexicon Pharmaceuticals Inc, and were made available to us by MSD Pharmaceutical, Newhouse, Scotland. Exons two and three of the HCN2 gene, which code for the first five of the six transmembrane regions, were flanked with LoxP sites (floxed HCN2, Fig. S1A). A phosphoglycerate kinase (PGK) promoter-driven neomycin resistance (neoR) cassette was inserted within the intronic region between exons three and four, upstream of the second LoxP site, and was used for ES cell selection. The neoR cassette was flanked with flippase recognition target (FRT) sites to allow for the subsequent excision by pairing with flippase-expressing mice after homologous recombination. The insertion of the LoxP sites was confirmed by PCR using primers targeted to the sites of insertion (Fig. S1B). Floxed HCN2 mice were used for future pairings to generate either global or tissue-specific HCN2 knock-out mice. All mice were C57/BL6 background.

Global HCN2$^{-/-}$

Globally deficient HCN2 mice (HCN2$^{-/-}$) were generated by pairing homozygous floxed HCN2 (f/fHCN2) mice with mice in which globally expression of Cre was driven by the cytomegalovirus (CMV) promoter[S1]. The CMV-driven Cre excised HCN2 in the germ line, and was subsequently bred out of the strain. The global HCN2 deletion causes a severe phenotype and these mice seldom live beyond six weeks[S2]. Global HCN2$^{-/-}$ mice were therefore obtained by pairing heterozygotes (HCN2$^{+/-}$), which produced HCN2$^{+/+}$, HCN2$^{+/-}$ and HCN2$^{-/-}$ in Mendelian ratios. The excision of exons two and three from the HCN2 locus was verified using PCR (Fig. S1B). Three primers were used to determine the genotype of the global HCN2$^{-/-}$ mice (Fig. S1A). The common forward primer (AAGCCTTCTCTGCGGTCTGG, primer A) is complementary to an intronic sequence upstream of exon two. The first reverse primer (AGAACCTGGAGAGAGGCAGG, primer B) binds within exon two, and detects the unexcised gene. The second reverse primer (GCTGGCAGGCATCAGATACC, primer C) is complementary to an intronic sequence downstream of exon three, 2788 bp downstream of primer A. Primers A and B give a product of 415 bp for the presence of an HCN2 gene, while primers A and C give a product of 800 bp in the presence of a HCN2 gene deletion (Fig. S1B).

Na$_V$1.8- HCN2$^{-/-}$

The generation of mice where HCN2 deletion was targeted to the Na$_V$1.8 expressing population of neurons was achieved by crossing heterozygous floxed HCN2 (f/-HCN2) mice with mice that were homozygous for Cre driven by the Na$_V$1.8 promoter (Na$_V$1.8^{Cre}/Na$_V$1.8^{Cre}). These mice were generously provided by Prof. John Wood, UCL, London[S3]. From the F1 generation, mice that were Na$_V$1.8^{Cre}/Na$_V$1.8^{WT}-f/-HCN2 were backcrossed with the parental f/-HCN2 line in order to generate F2 mice that were Na$_V$1.8^{Cre}/Na$_V$1.8^{WT}-f/fHCN2 as well as mice that were Na$_V$1.8^{WT}/Na$_V$1.8^{WT}-f/fHCN2. Mice with these genotypes were paired to give tissue-specific Na$_V$1.8^{Cre}/Na$_V$1.8^{WT}-f/fHCN2 (referred to as Na$_V$1.8-HCN2$^{-/-}$) and Na$_V$1.8^{WT}/Na$_V$1.8^{WT}-f/fHCN2 (referred to as f/f-HCN2) in equal numbers; no deviation from Mendelian ratios was observed. The f/f-HCN2$^{+/+}$ floxed mice were used as wild-type littermate controls for the Na$_V$1.8-HCN2$^{-/-}$ mice. The presence of the targeted deletion of HCN2 within the DRG neuronal population, and its absence elsewhere in the CNS, was confirmed using PCR (Fig. S1B). The genotype of each animal was confirmed by PCR before and after use. Primers A and B (see above) were used to determine the presence of a LoxP site upstream of exon two. This would result in either an unfloxed 415 bp or floxed 486 bp product being produced (Fig. S1A). The Na$_V$1.8 promoter-driven Cre gene was detected by a common upstream forward primer (TGTAGATGGACTGCAGAGGATGGA, primer D) and two

reverse primers (Fig. S1A, B). Primers D and E (AAATGTTGCTGGATAGTTTTTACTGCC) give a product of 420 bp for the presence of $Na_V1.8^{Cre}$; primers D and F (TTACCCGGTGTGTGCTGTAGAAAG) give a product of 460 bp for the presence of $Na_V1.8^{WT}$.

ROSA26, YFP; $Na_v1.8$, Cre
Homozygotic ROSA26-YFP mice were generously provided by Drs. Sandovici and Constancia. These were crossed with heterozygotic $Na_v1.8$/Cre mice resulting in offspring expressing the YFP reporter gene in $Na_v1.8$ positive cells in a 50/50 ratio. Offspring were genotyped for the presence of the Cre recombinase gene using primers D and E as outlined above. DRG neurons from these offspring were dissociated as outlined below and imaged by confocal microscopy.

PCR
Brain tissue, spinal cord tissue or DRGs were placed in 5µl DNA releaser and heated for 5min at 75°C and 2min at 96°C. DNA samples were diluted by adding distilled water to a final volume of 50µl. The PCR protocol was 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 2 minutes 30 seconds, repeat 40 cycles. PCR product was mixed with loading buffer and run on an agarose gel with 1kb DNA marker at 80V for 30 minutes.

Western blot analysis
DRGs, spinal cord or brain tissue were placed into ice cold RIPA buffer and triturated using 19G, 21G and 26G needles. The sample solution was placed on a rotator for 2 hours at 4°C, and centrifuged at 12,000 rpm for 20 minutes. Supernatant was stored at -80°C. For Western blot 50-100 µg total protein was loaded onto each lane. Protein extract was mixed in 1:1 ratio with loading buffer and heated at 50-65ºC for 5 min and run on SDS-PAGE. Before use, PVDF membrane was activated in methanol for 1-2 minutes and equilibrated in ice cold transfer buffer for 5 minutes. Proteins were transferred onto polyvinylidene fluoride (PVDF) transfer membrane using semi-dry transfer method at 12V for 30 minutes. After transfer, the membrane was placed in blocking buffer (5% w/v Non Fat Milk in PBST) for 60 minutes and then incubated with anti-rabbit HCN2 primary antibody (1:400, in 5% milk in PBST) at 4°C overnight. After primary incubation, the blot was washed 3×10 minutes in PBST before being incubated with anti-rabbit IgG (NA934VS;) HRP-conjugated secondary antibody (1: 10,000, in 5% milk in PBST) at RT for 60 minutes. The membrane was then washed in PBST and visualised using ECL Plus Kit (GE Healthcare, USA).

Behaviour

All mice used for behavioral experiments were aged between two and five months. For both the thermal (Hargreaves) and mechanical (von Frey) tests, all mice were exposed to the experimental observation chamber for at least three hours per day for three days before the start of the experiment to allow for habituation. In the case of the formalin test, mice were exposed to the chambers at least two hours before the start of the experiment. All animals were kept in a temperature controlled environment (21°C ± 1°C) on a normal 12-hour light/dark cycle (light 07:00-19:00) and had food and water available *ad libitum*. All experimenters were blinded to the genotype or injection and were only unblinded once the study had finished. For testing the effects of ZD7288, injections were given i.p. at 10ml/kg in saline 30 mins prior to injection of inflammatory stimuli. Each experimental group contained 8-11 animals of mixed sex. All licensed procedures were granted by the Home Office and adhered to the legislation set out in the Animals (Scientific Procedures) Act 1986.

Surgery
Chronic constriction injury (CCI) was used as a model of neuropathic pain as previously described[S4]. Each animal received oral Baytril (2 ml in 250 ml water) one day before and three days

after surgery. On the day of surgery animals were given subcutaneous Baytril (80 µl; 1:10) and Rimadyl (20 µl; 1:10). Anaesthesia was induced using 5% isoflurane and maintained at 2.5% (V/V O_2 2 $l.min^{-1}$). The level of anaesthesia was assessed using tail pinches. The left hind leg was shaved, cleaned using iodine and a small incision was made. The sciatic nerve was exposed by blunt preparation of connective tissue at the mid-thigh level, proximal to the sciatic trifurcation. Three non-absorbable sterile surgical sutures (0.1 mm) were loosely tied around the sciatic nerve, 1-1.5 mm apart. For the sham treated animals, the sciatic nerve was exposed as described above; however, no ligation was performed. The skin was sutured and the anaesthesia discontinued. The animal was transferred to a recovery cage until it showed signs of locomotive competence and a reproducible righting reflex.

Formalin test

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 31G needle. The time the animal spent licking, biting or lifting the injected paw was recorded in five minute intervals. The experiment continued for 60 minutes, at which time the animals were euthanised. Response patterns of the early (0-10 minutes) and the late phase (15-60 minutes) were analysed as previously described[S5].

Thermal threshold

The thermal threshold for paw withdrawal was assessed using a plantar aesthesiometer. The withdrawal latency was measured using a fixed infra-red thermal stimulus of 50 (units of infra-red intensity) with a cut-off latency of 15 seconds to prevent damage. To assess inflammatory heat hyperalgesia, PGE_2 (1 µg/10 µl) or saline (10 µl) was injected into the left hind paw of each animal. Inflammation-induced oedema of the injected paw was assessed using a digital micrometer to measure paw diameter (plantar-dorsal) after PGE_2 or saline injection. The paw withdrawal latency was assessed at 30, 60 and 120 minutes and 24 hours after injection. Paw withdrawal latency of animals that had undergone CCI was tested at 2, 7, 14 and 21 days after surgery.

Carrageenan

Lambda carrageenan (2% 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 31G needle. The thermal threshold for paw withdrawal was measured as above (*see thermal threshold)* at 30, 60, 90, 120, 150, 180 minutes and 24 hours after Carrageenan injection.

Mechanical threshold

The mechanical threshold for paw withdrawal was assessed using a dynamic plantar aesthesiometer. A maximum force of 5 g was applied over a 10 second ramp, after which the force remained constant at 5 g for 30 seconds. To assess inflammatory hyperalgesia, PGE_2 (1 µg/10 µl) or saline (10 µl) was injected into the left hind paw of each animal and paw withdrawal latency was assessed at 30, 60 and 120 minutes and 24 hours after injection. Paw withdrawal threshold in animals that had undergone CCI was tested 2, 7, 14 and 21 days after surgery.

Cold and hot plate

Sensitivity to a non-noxious cold stimulus was assessed using a Hot/Cold plate. Animals that had undergone CCI injury (see above) were assessed for cold plate sensitivity by placing them on a platform cooled to 5°C for a five minute period. The number of paw lifts ipsilateral and contralateral to the site of injury was counted and the difference plotted as a function of time. Animals were assessed at 2, 7, 14 and 21 days post surgery. For testing sensitivity to noxious heat, the device was set to a habituation temperature of 45°C and each animal was left on the hotplate platform for two minutes, after which they would be placed back into their cage for a 30 minute recovery period. Each animal was then returned to the hotplate platform, where the time taken for

independent front and rear paw lifting to be observed was recorded separately at temperatures of 52.5, 55.5 and 58.5°C. The maximum exposure time to the hotplate platform was limited to 30 seconds per session to prevent any harm to the animal.

Rotarod
Mice were trained over three successive days on an accelerating rotarod (4-40 r.p.m. over 120 seconds). On the fourth day the endurance of the mice on the rod was assessed. Mice were tested at 30, 60 and 120 minutes either directly or after administration of ZD 7288 or saline.

Cell culture

Primary cell culture
Dorsal root ganglion (DRG) neurons were isolated from either neonatal (<P10) or 4-6 weeks old $HCN2^{+/+}$, $HCN2^{-/-}$, f/f$HCN2^{+/+}$ or $Na_V1.8$-$HCN2^{-/-}$ C57/BL6 mice. All mice were euthanised by cervical dislocation followed by decapitation. The dorsal side of the mouse was cut using a scalpel to reveal the spinal column. The dorsal side of the spinal column was cut away using dissecting scissors and the spinal cord removed. DRGs were dissected from the entire length of the spinal column and placed directly into cooled PBS ($0Ca^{2+}$, $0Mg^{2+}$) as a cleaning step. The DRGs were transferred into pre-incubated Dulbecco's Modified Eagle Medium, supplemented with L-glutamine (1%), glucose (4.5 g/l), sodium pyruvate (110 mg/l) and 1% penicillin-streptomycin (DMEM), and containing collagenase (2.5 mg/ml) for one hour (37°C, 5% CO_2). DRGs were centrifuged at 1,000 r.p.m for three minutes, supernatant discarded and resuspended in 2 ml DMEM supplemented with 10% foetal bovine serum (FBS), mechanically triturated and washed through a 100 μm Falcon Filter. The filtered solution was centrifuged at 1,000 r.p.m. for 10 minutes and the pellet resuspended in 200 μL of fresh pre-incubated DMEM (+FBS) supplemented with 50 ng/ml nerve growth factor (NGF). Cells were plated onto 35 mm Petri dishes pre-coated with poly-L-lysine (1 mg/ml) and laminin (1 mg/ml) and supplemented with additional DMEM (+FBS, +NGF) after one hour. Cells were incubated (37°C, 5% CO_2) for 15-50 hours before use. Neurons were classified based on soma diameter: small <20 μm; medium 20-30 μm; large >30 μm[6].

HEK cell culture
HEK293 cells were transiently co-transfected with cDNAs encoding mouse HCN2 (mHCN2) and GFP using calcium phosphate. HEK293 cells were cultured in DMEM (+FBS) and three hours before transfection, the cell medium was replaced with fresh DMEM (not containing FBS). mHCN2 (500 ng) and GFP (500 ng) cDNA was added to 100 μl $CaCl_2$ (250 mM) and the solution was mixed. This solution was then added drop-wise into a Hanks Buffered Saline (100 μl) containing HEPES (50 mM), NaCl (280 mM) and Na_2HPO_4 (1.4 mM), while stirring vigorously. Once mixed, the combined solution (200 μl) was immediately added drop-wise to the HEK cell cultures. The cells were then left to incubate (37°C, 5% CO_2) for 6-8 hours, and cells were reseeded onto 35 mm dishes with fresh DMEM (+FBS). After transfection, cells were cultured at 37°C and 5% CO_2 for 24-48 hours before use. Cells showing GFP fluorescence were used for electrophysiology.

DRG neuron transfection
DRG neurons were isolated from neonatal pups as outlined above, transfection of murine HCN2 into neurons was carried out in a Nucleofector® II Device according to manufacturer's instructions (Small Cell Number). Plasmid DNA was transfected at a ratio of 10:1 (HCN2:GFP) for recovery experiments or with GFP alone in control experiments, 1μg of plasmid DNA was used in both conditions. Successfully transfected DRG neurons were identified by GFP fluorescence prior to patch-clamp recordings.

Electrophysiology

Solutions

All experiments were carried out using extracellular solution containing 140 mM NaCl, 4 mM KCL, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 5 mM glucose; pH was adjusted to 7.4 with NaOH (5 M) and osmolarity was between 300 and 310 mOsm. The intracellular solution contained 140 mM KCl, 1.6 mM $MgCl_2$, 2.5 mM MgATP, 0.5 mM NaGTP, 2 mM EGTA and 10 mM HEPES; pH was adjusted to 7.3 and osmolarity was between 300-310 mOsm. Drugs used were forskolin (FSK; 50 mM stock in DMSO), PGE_2 (10 mM stock in DMSO), ZD7288 (100 mM stock in water) and CsCl (5M stock in water), diluted in extracellular solution on the day of the experiment. All drugs were bath applied using a gravity-driven perfusion system.

Whole cell patch clamp

Whole cell patch clamp recordings were performed using an Axopatch 200B patch-clamp amplifier. Patch pipettes (GB150F-8P) were pulled using a P-97 horizontal micropipette puller (Sutter Instruments, USA). All pipettes were fire polished before use with a Microforge 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 75-90% with a lag time of 10 μs. Cells were held at -60 mV in the whole cell configuration. Current-clamp protocols were performed using I-Clamp fast mode. Junction potentials were calculated and corrected offline by -4.3 mV for all recordings[S7]. Whole cell recordings were low-pass Bessel filtered at 10 KHz and sampled at 20 KHz. Data were acquired and analysed using pClamp9/10 software. All experiments were performed at 21-23°C. All recordings were made from cells 1-2 days after plating/transfection.

Protocol

Whole cell patch clamp recordings were undertaken on cells that carried an HCN current (I_h). The presence of I_h in cells was verified by the presence of a "voltage-sag" equal to, or greater than 10% of the total voltage deflection upon a hyperpolarising current injection, which elicited a membrane voltage of > -100 mV[S6]. Quantification of the voltage-sag (V-sag) was performed by a series of five hyperpolarising current injections in 30 pA increments to elicit a membrane voltage "V_{min}" of -100 to -130 mV (a range at which I_h is maximal). The V-sag ratio (Fig. 1) was calculated by dividing the membrane voltage at steady state (V_{ss}) by the minimum membrane voltage after current injection (V_{min}). Action potential firing was investigated by injecting a series of depolarising current pulses (0-80 pA in 5 pA increments; 1-5 second duration). Effect on action potential frequency of injected current (Fig. 1D-F) was calculated by fitting a linear regression.

The voltage-clamp mode was used to investigate the rate and voltage-dependence of channel activation. An initial holding potential of -60 mV was used on all cells and a series of hyperpolarising and depolarising pulses ranging from -130 to -40 mV in 10 mV increments was applied to the cells for five seconds, followed by a final fixed hyperpolarisation step to -130 mV for three seconds. Between pulses cells were held at -60 mV for 8 seconds. CsCl (5 mM) was applied to subtract contaminating currents (Cs-subtraction) [S6]. The rate of channel activation was determined by fitting a double exponential function of the following form to Cs-subtracted traces:

$$\boldsymbol{I_h(t) = A_f e^{(-t/\tau_f)} + A_s e^{(-t/\tau_s)} + C}$$

Where $I_h(t)$ represents the amplitude of the current at time t, A_f and A_s represent the initial current-clamp amplitude coefficients for the fast (τ_f) and slow (τ_s) time constants of channel activation, respectively, and C represents the current amplitude reached at steady-state. The potential at which

half the maximal current was evoked ($V_{½}$) was determined from the tail current elicited upon the final hyperpolarising step to -130 mV. Activation curves were fitted with a Boltzmann equation of the following form:

$$\frac{I_t}{I_{t(max)}} = (1 + exp^{\left(\frac{V_m - V_{½}}{k}\right)})^{-1}$$

Where V_m is the membrane potential of the pre-pulse, $V_{½}$ is the membrane potential at half maximal Ih activation, k is the slope factor, I_t is the current amplitude of the tail current recorded for a given pre-pulse and $I_{t(max)}$ is the maximum current amplitude of the tail current.

Data analysis

All data presented are mean ± SEM. All relevant results were analysed using STATISTICA 8.0, using either paired or unpaired Student's *t*-test, one-way or two-way ANOVA with or without repeated measures, as appropriate. All further post-hoc analysis was carried out using a Tukey post-hoc test. Non-parametric testing was performed on any study where n<9. p<0.05 was considered significant.

References

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Supplementary Fig. 1

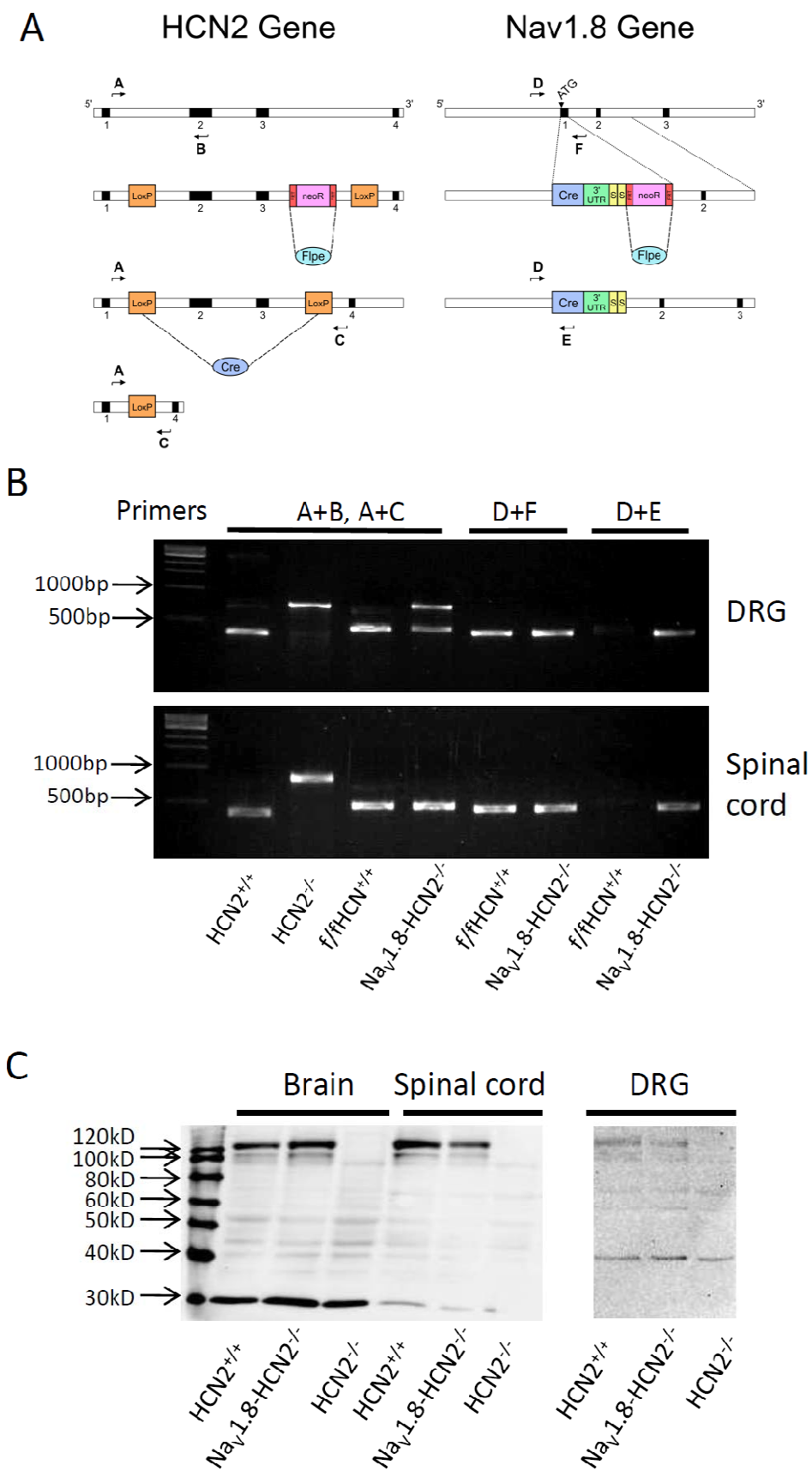

Fig. S1. Global deletion of HCN2 and $Na_V1.8$-specific deletion of HCN2 in DRG neurons. A. From top: diagram of partial HNC2 and NaV1.8 genomic DNA with exons in black; insertion by homologous recombination of constructs and neomycin selection cassettes; genomic sequence post-excision of neoR by flippase (Flpe) expression; and HCN2 gene structure post-excision by Cre. Positions of primers used for PCR in B, together with sizes of products, are as follows: A, B for intact HCN2 (415 bp in WT or 486 bp with LoxP site inserted), A, C for excised HCN2 (800 bp), D, F for intact $Na_V1.8$ (460 bp) and D, E for Cre (420 bp). B. PCR of genomic DNA from DRG and spinal cord of WT, $HCN2^{-/-}$, f/fHCN2 and $Na_V1.8$-$HCN2^{-/-}$ as shown. In first four lanes primer pairs A+B and A+C were amplified separately and the products mixed before running on the gel. Note that the Cre-excised HCN2 appears only in DRG. Similar results to spinal cord obtained for brain. C. Western blot showing that HCN2 is present in brain, spinal cord and DRG of WT and $Na_V1.8$-$HCN2^{-/-}$ but is absent in $HCN2^{-/-}$. Cre-driven excision of HCN2 does not produce any product as shown by the absence of lower MWt bands in $HCN2^{-/-}$. Results for DRGs taken from separate Western. Note strong non-specific bands at 40 kDa in brain and 30kDa in DRG.

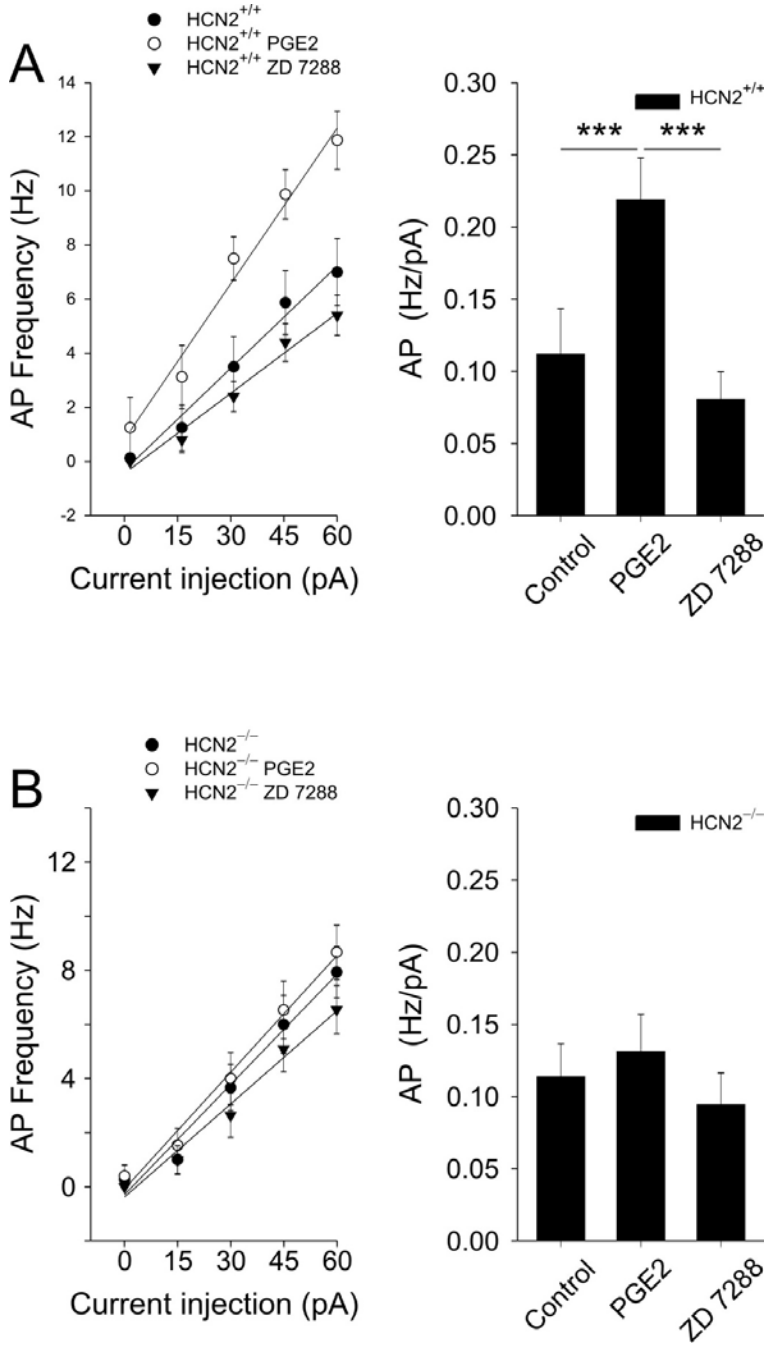

Fig. S2. PGE_2 promotes firing of action potentials in WT but not $HCN2^{-/-}$ small DRG neurons (each n = 8). A. PGE_2 (50 μM) increases frequency of action potentials elicited by a 1-second current pulse in I_h-positive neurons, and the increase is reversed by the I_h blocker ZD7288. Bar graph to right shows mean increase of AP frequency per pA injected current after PGE2 (p = 0.0005) and PGE2 + ZD7288 (p = 0.0002).. B. In neurons from $HCN2^{-/-}$ mice the rate of firing in response to a constant-current pulse is similar but PGE_2 has no effect on action potential frequency. Significance values calculated using Wilcoxon matched pairs test.

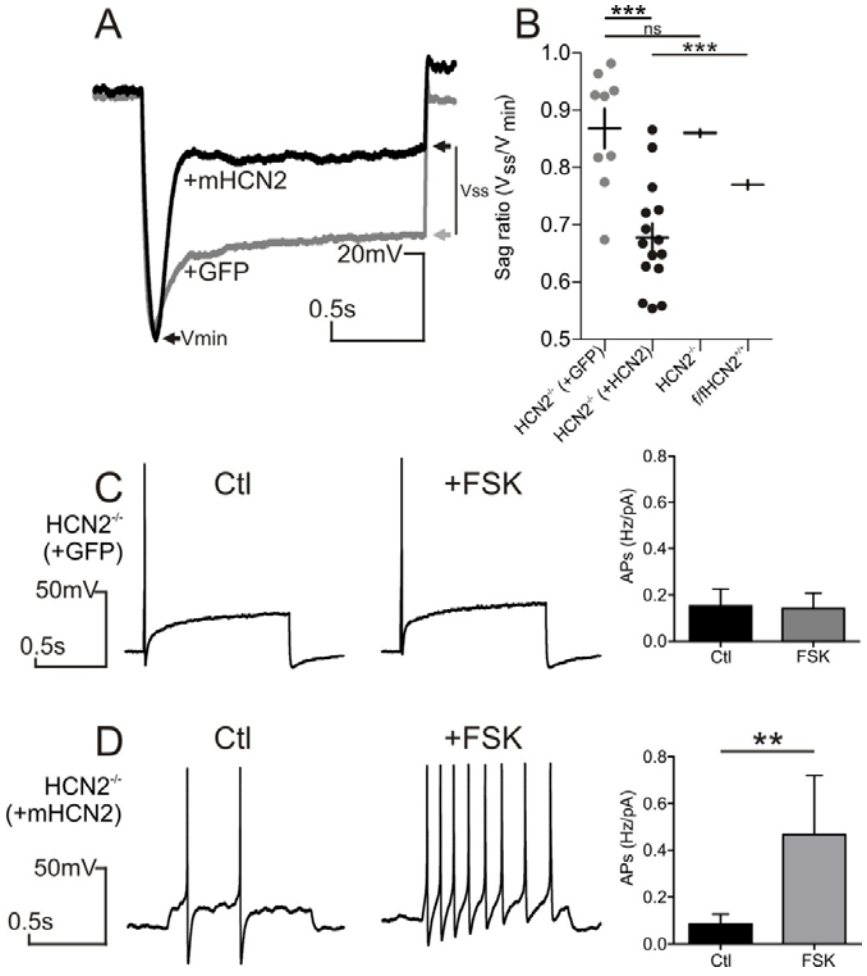

Fig. S3. Action potential sensitisation can be recovered by transfection of the mHCN2 subunit into HCN2$^{-/-}$ DRG neurons. A. Typical membrane potential traces from HCN2$^{-/-}$ neurons transfected with either mHCN2 plus GFP or with GFP alone in response to hyperpolarising constant-current injection. Successfully transfected neurons identified from GFP fluorescence. B. Scatter plot of Vsag ratio (V_{ss}/V_{min}, see Fig. 1) from HCN2$^{-/-}$ neurons transfected with mHCN2 or GFP alone shows that HCN2 transfection increased I_h (mean Vsag ratios: mHCN2, 0.68 ± 0.02, n = 15; GFP, 0.87 ± 0.03, n = 9; p = 0.001). Last two points (taken from Fig 1) show comparable data for HCN2$^{-/-}$, to demonstrate that transfection with GFP alone did not affect I_h, and for f/fHCN2$^{+/+}$, to show that transfection of HCN2 "over recovers" I_h. C. Left: typical traces showing action potentials elicited by depolarising current injection in control and forskolin treated GFP-transfected HCN2$^{-/-}$ neurons. Right: No significant increase in mean AP firing in response to FSK was observed, as with untransfected HCN2$^{-/-}$ neurons (c.f. Fig. 1D, E; control: 0.15 ± 0.07 Hz/pA, FSK: 0.14 ± 0.07 Hz/pA, n = 6, p = 0.63). D. Similar experiment with HCN2$^{-/-}$ neurons transfected with mHCN2. A strong HCN2-mediated increase in AP frequency was evoked by FSK (control: 0.09 ± 0.04 Hz/pA FSK: 0.47 ± 0.25 Hz/pA, n = 10, p = 0.004).

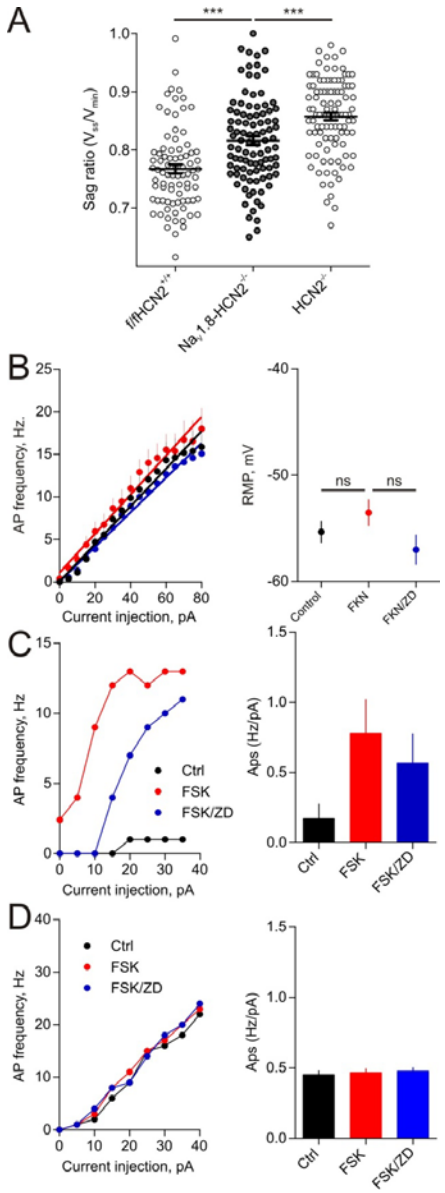

Fig. S4. $Na_v1.8$-Cre driven knockout of HCN2 reduces I_h and removes sensitisation of action potential firing from a sub-population of DRG neurons. A. V-sag ratios show that $Na_v1.8$-$HCN2^{-/-}$ DRG neurons (centre) have on average an I_h amplitude intermediate between $HCN2^{+/+}$ and $HCN2^{-/-}$ ($f/fHCN2^{+/+}$ = 0.76 ± 0.01, n = 82, $Na_v1.8$-$HCN2^{-/-}$ = 0.82 ± 0.01, n = 96, $HCN2^{-/-}$ = 0.86 ± 0.01, n = 102, p = <0.0001). B-D. Action potential firing frequency as a function of injected current in $Na_v1.8$-$HCN2^{-/-}$ DRG neurons - control (black), forskolin (red) and forskolin/ZD-7228 (blue). B. Left panel: overall there was no significant difference between the three conditions (n = 16), but in a subpopulation sensitisation was observed (6 out of 16 cells). Right panel: no significant difference was observed in RMP between the three conditions. C. Example of a FSK-sensitive $Na_v1.8$-$HCN2^{-/-}$ neuron. D. Example of a FSK-insensitive $Na_v1.8$-$HCN2^{-/-}$ neuron.

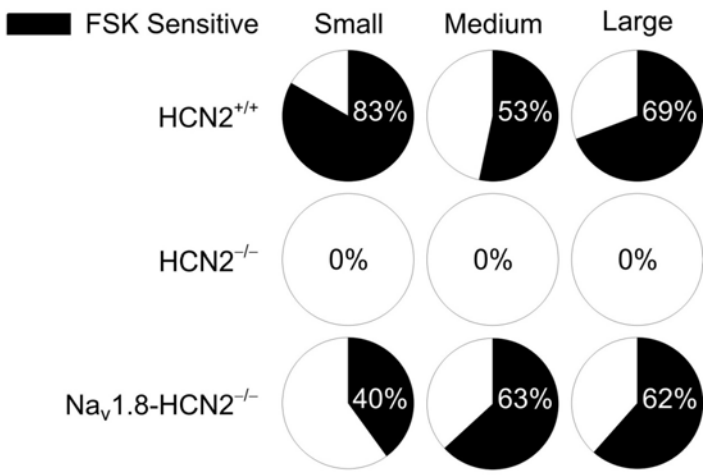

Fig. S5. Proportion of neurons in which shifts in $V_{½}$ of I_h were observed in response to application of forskolin (for examples of original data see Fig. 2C-F). Top row: in most $HCN2^{+/+}$ neurons across all size classes a shift in $V_{½}$ was observed and therefore these neurons are likely to express HCN2 (NB in all large and many medium-sized neurons I_h was dominated by a fast-relaxing I_h attributable to HCN1 – see Fig 2A - and therefore these neurons also express HCN1[14]). Middle row: in $HCN2^{-/-}$ neurons no shift in $V_{½}$ was observed in any size class, showing that deletion of HCN2 abolishes all forskolin sensitivity. Bottom row: in neurons from $Na_v1.8\text{-}HCN2^{-/-}$ mice forskolin sensitivity of $V_{½}$ was removed in around 50% of small neurons but was unaffected in medium and large neurons.

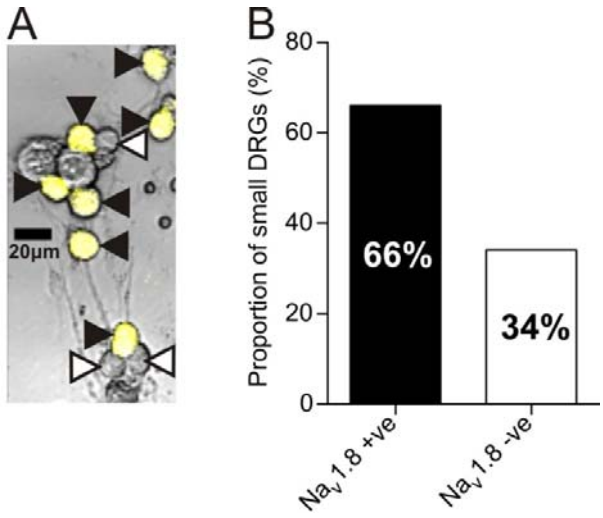

Fig. S6. **$Na_v1.8$ drives Cre expression in a subset of small DRG neurons.** $Na_v1.8$-Cre mice were crossed with the Cre reporter mouse line ROSA26-YFP in which expression of YFP is suppressed by a floxed transcriptional stop signal upstream of the reporter gene[44]. $Na_v1.8$ driven Cre expression excises the stop signal and therefore causes expression of YFP. A. Representative image from $Na_v1.8$-R26/YFP DRG neurons. YFP expression was observed in a majority of small neurons (dia < 20µm, solid arrowheads) but a substantial minority was YFP negative (open arrowheads). B Quantification of $Na_v1.8$ positive (66%, n = 273) and negative (34%, n = 140) small DRG neurons.

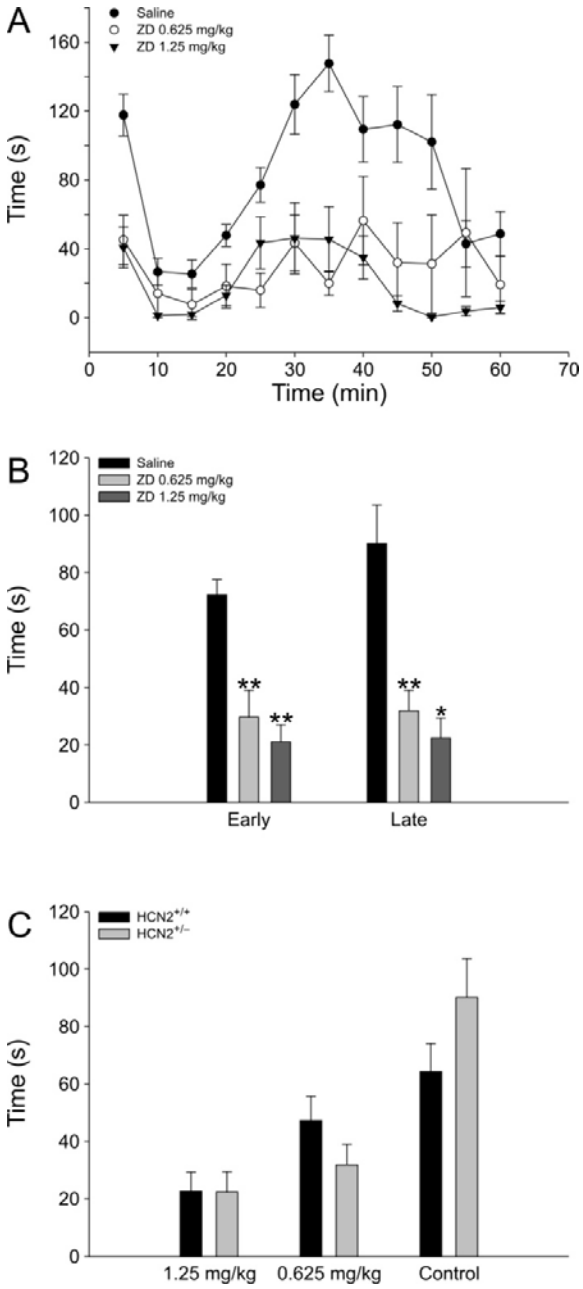

Fig. S7. A. HCN2$^{+/-}$ heterozygous animals behave similarly to wild-type following injection of 4% formalin, and pain behaviour is suppressed by ZD7288 in a similar manner (n = 6-7). B Summary data on effect of ZD7288 on early (p = 0.0073 for 0.625 mg/kg and p=0.0053 for 1.25 mg/kg) and late phase (p = 0.0073 for 0.625 mg/kg and p = 0.0124 for 1.25 mg/kg) behavior in HCN2$^{+/-}$. C. Comparison between suppression of late phase pain behavior by ZD7288 in WT and HCN2$^{+/-}$. Significance values calculated using Mann-Whitney U test.

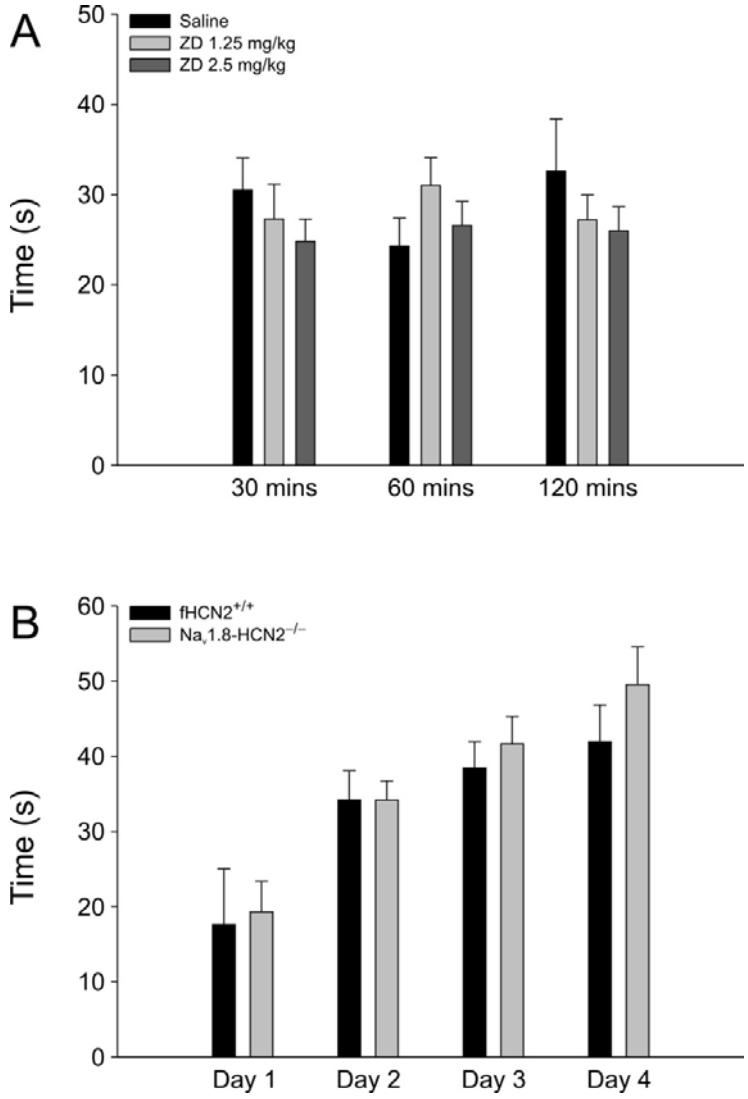

Fig. S8. Motor coordination on rotarod test is unaffected by ZD7288 or by $Na_V1.8$-driven HCN2 excision. A. Effect of ZD7288. Ordinate gives time to falling off accelerating rotarod for WT mice; abscissa gives time post-injection of ZD7288 (doses as shown). B. Effect of $Na_V1.8$-driven HCN2 excision. Ordinate gives time to falling off accelerating rotarod; abscissa gives day of training. In both A and B n = 10 for each group.

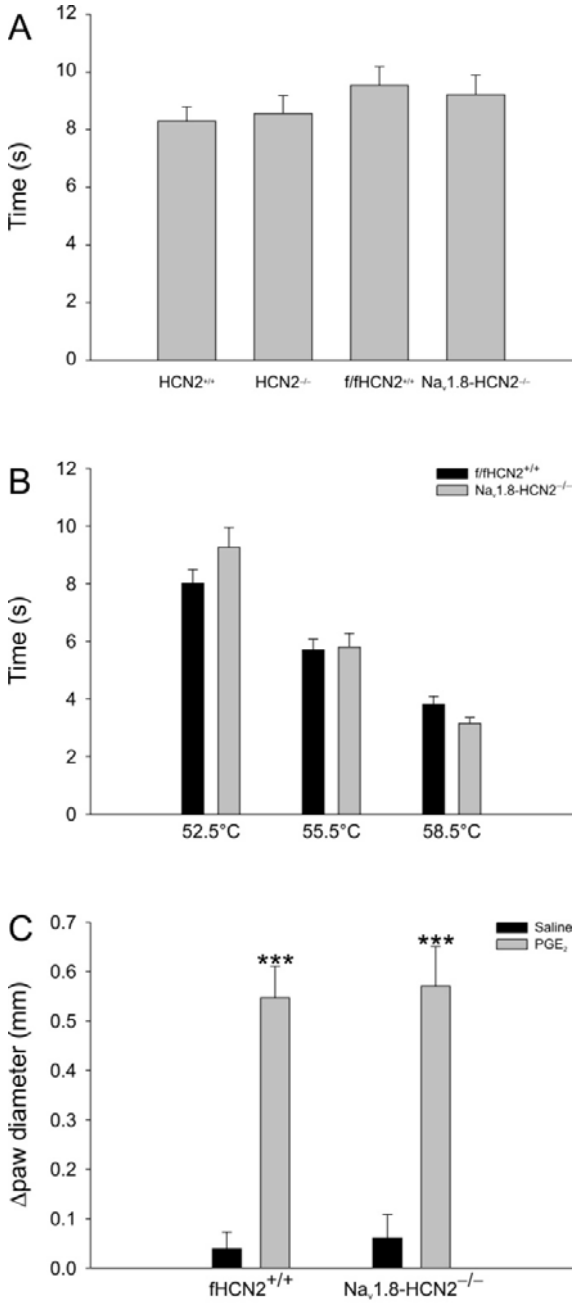

Fig. S9. A. Thermal threshold (Hargreaves test) is unaffected by global excision of HCN2 ($HCN2^{-/-}$), by floxing HCN2 ($f/fHCN2^{+/+}$) or by $Na_V1.8$-driven excision of HCN2 ($Na_V1.8$-$HCN2^{-/-}$) (n = 5-11). B. Reaction time on hot plate test is unaffected by $Na_V1.8$-driven excision of HCN2 (n = 10). C. Paw swelling following injection of PGE2 is unaffected by $Na_V1.8$-driven excision of HCN2 (n = 11; p = 0.0002 for $f/fHCN2^{+/+}$ and p = 0.0002 for NaV1.8-$HCN2^{-/-}$). Significance values calculated using Student's unpaired t-test.

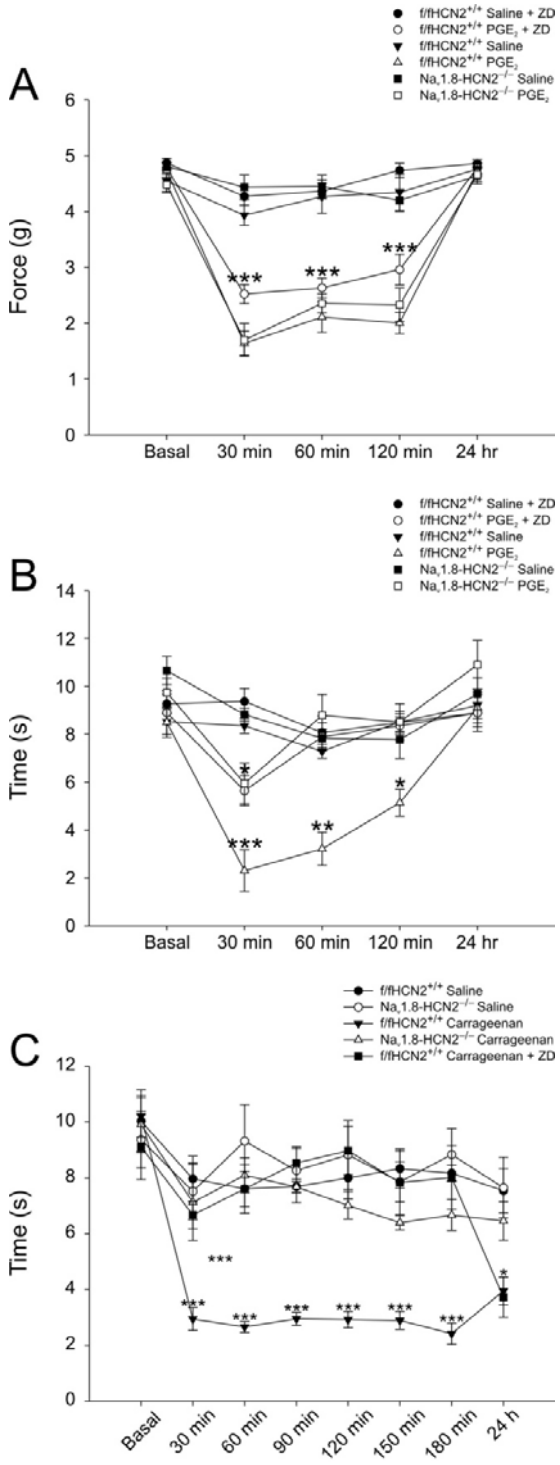

Fig. S10. Inflammatory thermal hyperalgesia depends on HCN2. Data as in Fig 3C-D but showing control groups individually. A: experiments shown in Fig. 3E. B: experiments shown in Fig. 3C. C: experiments shown in Fig. 3D.

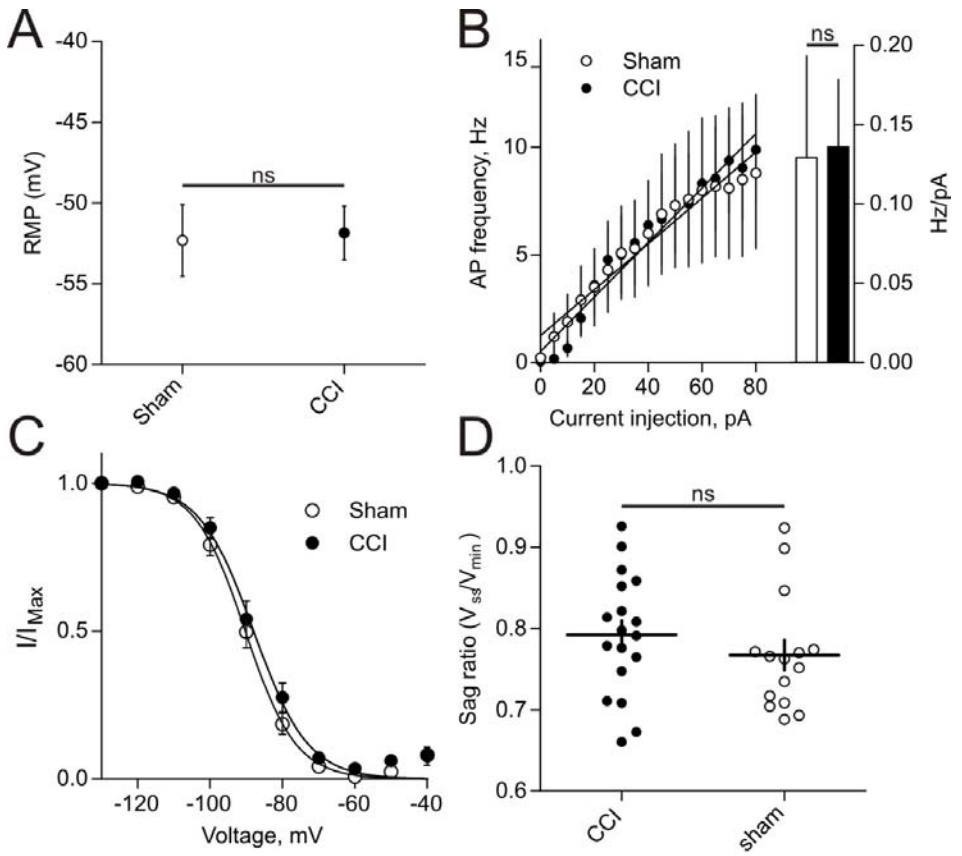

Fig. S11. I_h is normal in small neurons removed following chronic constriction injury. Small DRG neurons (<20μm dia) from mice which had undergone chronic constriction injury or sham operation were examined using patch-clamp electrophysiology. Recordings were performed on mice 2-14 days post operation, and all recordings were performed acutely (4-24 hours *in vitro*) from ipsilateral L4-L5 DRG neurons. No systematic changes in any parameters were observed with time after either CCI nerve lesion or sham operation so all data was averaged over the entire time course. A. Resting membrane potential is unaffected by CCI (CCI: -51.85 mV, n = 20; sham = -52.32mV, n = 19; p = 0.867). B. Action potential firing rate is unaffected by CCI (CCI: 0.14 ± 0.04 Hz/pA, n = 18; sham = 0.13 ± 0.06 Hz/pA, n = 12; p = 0.953). C. Voltage-dependence of I_h is unchanged (CCI: $V_{½}$ = -87.92 ± 0.50mV, n = 12; sham: $V_{½}$ = -90.32 ± 0.5mV, n = 11; p = 0.284) D. I_h maximum current measured from V_{sag} ratio is unaffected (CCI: mean V_{sag} = 0.79 ± 0.02, n = 18; sham: mean V_{sag} = 0.78 ± 0.02, n = 15; p = 0.335).

Additional reference cited in Fig. S6 legend:

44. S. Srinivas *et al*., *BMC.Dev.Biol.* **1**, 4 (2001).