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

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protein (*CREBdn*), which inhibits cAMP-induced transcription activation (*31*). Expression of either transgene specifically in LN(v)s obliterated their ability to adjust dendrite length under different light-dark conditions (fig. S9). Calcium imaging further revealed that the expression of *PKAmc* or *CREBdn* eliminated changes of LN(v)s' light responses produced by different light-dark conditions (fig. S10). Thus, the cAMP pathway regulates both structural and functional plasticity of LN(v)s.

Our screen for mutants with defective LN(v) dendritic plasticity also identified *babos-1*, a mutant with a P-element insertion near the transcriptional start site of CG3624, a previously uncharacterized immunoglobulin domain–containing cell surface protein (*28*). The LN(v) dendrite length of *babos-1* mutant larvae was comparable to controls in LD and LL but has no compensatory increase in DD (Fig. 4C). Similar phenotypes were found in larvae expressing an RNAi transgene targeting CG3624 in LN(v)s (Fig. 4C). Moreover, flies carrying a hypomorphic allele of CG3624, $CG3624^{[KG05061]}$ (fig. S11), also showed defective light-induced dendritic plasticity, which was fully rescued by expressing the UAS-CG3624 transgene specifically in LN(v)s (Fig. 4D). Thus, the function of this immunoglobulin domain–containing protein in LN(v)s is important for the dendrite expansion in constant darkness.

Bioinformatic analyses suggest that CG3624 is a cell surface protein containing an N-terminal signal peptide, extracellular immunoglobulin domains followed by a transmembrane helix, and a short C-terminal cytoplasmic tail (*32*). CG3624 is widely expressed in the nervous system throughout development (Flybase and BDGP database). Its specific requirement for the adjustment of LN(v)s' dendrite length in constant darkness suggests that elevation or reduction of sensory inputs likely invokes separate mechanisms for compensatory modifications of central neuronal dendrites.

A functioning nervous system must have the capacity for adaptive modifications while maintaining circuit stability. Our study of the *Drosophila* larval visual circuit reveals large-scale, bidirectional structural adaptations in dendritic arbors invoked by different sensory exposure. Whereas the circuit remains functional with modified outputs, this type of homeostatic compensation may modify larval light sensitivity according to its exposure during development and could facilitate adaption of fly larvae toward altered light conditions, such as seasonal changes. Our observations also suggest shared molecular machinery between homeostasis and the Hebbian plasticity with respect to the cAMP pathway (*2*, *4*) and indicate the feasibility of genetic studies of experience-dependent neuronal plasticity in *Drosophila*.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/333/6048/1458/DC1
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Figs. S1 to S11
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HCN2 Ion Channels Play a Central Role in Inflammatory and Neuropathic Pain

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The rate of action potential firing in nociceptors is a major determinant of the intensity of pain. Possible modulators of action potential firing include the HCN ion channels, which generate an inward current, I_h, after hyperpolarization of the membrane. We found that genetic deletion of HCN2 removed the cyclic adenosine monophosphate (cAMP)–sensitive component of I_h and abolished action potential firing caused by an elevation of cAMP in nociceptors. Mice in which HCN2 was specifically deleted in nociceptors expressing $Na_V1.8$ had normal pain thresholds, but inflammation did not cause hyperalgesia to heat stimuli. After a nerve lesion, these mice showed no neuropathic pain in response to thermal or mechanical stimuli. Neuropathic pain is therefore initiated by HCN2-driven action potential firing in $Na_V1.8$-expressing nociceptors.

The HCN ion channel family comprises four isoforms, HCN1 to HCN4, which carry an inward current called I_h (also I_q or I_f) (*1*, *2*). I_h is activated by hyperpolarization in the range of membrane potentials between –60 and –90 mV, and the activation of this inward current is proposed to be a major driver of the pacemaker potential in cardiac muscle (*3*, *4*). The dependence of the activation of HCN2 and HCN4 on the cell membrane potential is shifted in the positive direction when cyclic adenosine monophosphate (cAMP) binds to a domain in the C-terminal tail, but HCN1 and HCN3 are relatively insensitive to cAMP (*1*, *2*). I_h is important in driving repetitive firing in some CNS neurons (*5*–*8*) and also in primary nociceptive neurons (*9*–*15*).

HCN1 and HCN2 are the isoforms most strongly expressed in primary somatosensory neurons (*12*, *15*–*20*). Large non-nociceptive sensory neurons express a fast, cAMP-insensitive I_h attributable mainly to HCN1 (*14*, *15*, *19*, *21*). HCN1 is not functionally expressed in small neurons, the majority of which are nociceptors, apart from in a small subpopulation of cold-sensitive neurons (*14*, *22*). Consistent with this, deletion of HCN1 has little effect on most modalities of pain (*14*). In the majority of small neurons, I_h has slower kinetics and is sensitive to intracellular cAMP,

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which suggests that it is mediated mainly by HCN2 (*14*, *15*).

The Cre-LoxP system was used to disrupt the gene encoding HCN2. Exons 2 and 3, coding for five of the six transmembrane segments, were flanked with LoxP sites (f/fHCN2$^{+/+}$; fig. S1A). Mice carrying the targeted allele were then mated with CMV-Cre universal deleter mice to generate HCN2 knockout mice (global HCN2$^{-/-}$). As was found with a different HCN2 deletion (*6*), homozygous HCN2$^{-/-}$ mice were ataxic and seldom lived beyond 6 weeks. Colonies of heterozygous HCN2$^{+/-}$ mice, which are phenotypically normal (fig. S7), were therefore maintained and produced wild-type, HCN2$^{+/-}$, and HCN2$^{-/-}$ mice in Mendelian ratios.

In view of the adverse phenotype of global HCN2$^{-/-}$ mice, we used mice expressing Cre driven by the promoter of the voltage-dependent sodium channel isoform $Na_V1.8$, which is expressed only in nociceptive primary sensory neurons (*23*, *24*). Relative to wild-type mice, $Na_V1.8$-HCN2$^{-/-}$ mice were phenotypically normal, had similar heat thresholds and performance on the rotarod test of motor function, and showed similar paw swelling in response to prostaglandin E2 (PGE_2) injection (figs. S8 and S9). An advantage of both the global and the targeted breeding strategies is that global HCN2$^{-/-}$ and $Na_V1.8$-HCN2$^{-/-}$ mice could be compared with wild-type littermates (or with f/fHCN2$^{+/+}$ mice, which are effectively wild type) in electrophysiological and behavioral experiments.

The amplitude of I_h in small isolated dorsal root ganglion (DRG) neurons was assessed from the amplitude of the rebound in membrane voltage after a hyperpolarizing current pulse (Fig. 1A). In global HCN2$^{-/-}$ neurons, I_h was significantly smaller than in f/fHCN2$^{+/+}$ neurons but was not abolished (Fig. 1, A and B), showing that another HCN isoform must also be expressed. There was, however, a major difference in the neuronal response to elevation of intracellular cAMP. Forskolin (FSK), which elevates cAMP by directly activating adenylate cyclase, caused a membrane depolarization and an ongoing firing that was blocked by the nonselective HCN antagonist ZD7288 in wild-type neurons. In HCN2$^{-/-}$ neurons, FSK had no effect (Fig. 1, C and E). FSK and PGE_2, which acts on the EP4 receptor to activate adenylate cyclase, both significantly accelerated action potential firing in response to current injection in wild-type but not in HCN2$^{-/-}$ neurons (Fig. 1, D and E, and fig. S2). The sensitivity to FSK of the firing rate in HCN2$^{-/-}$ neurons could be restored by transfection of HCN2 (fig. S3).

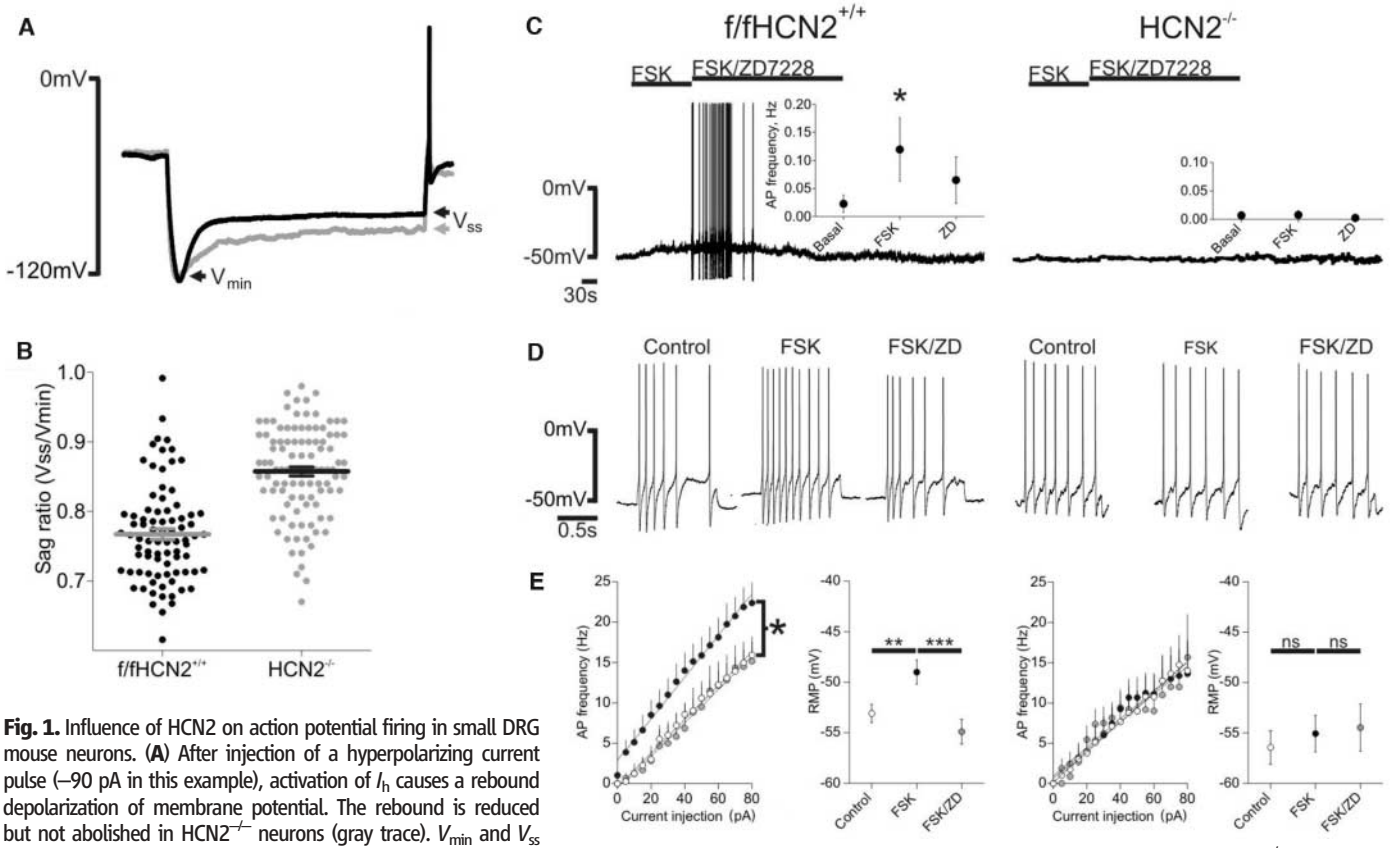

Fig. 1. Influence of HCN2 on action potential firing in small DRG mouse neurons. (**A**) After injection of a hyperpolarizing current pulse (–90 pA in this example), activation of I_h causes a rebound depolarization of membrane potential. The rebound is reduced but not abolished in HCN2$^{-/-}$ neurons (gray trace). V_{min} and V_{ss} denote minimum and steady-state voltage after current pulse, respectively. (**B**) Ratios V_{ss}/V_{min} in neurons from f/fHCN2$^{+/+}$ (black) and HCN2$^{-/-}$ mice (gray). Bars show means ± SEM (f/fHCN2$^{+/+}$, 0.77 ± 0.01, n = 82; HCN2$^{-/-}$, 0.86 ± 0.01, n = 102; $P < 0.0001$). (**C** to **E**) Vertical columns of data from neurons isolated from f/fHCN2$^{+/+}$ mice (left) and from HCN2$^{-/-}$ littermates (right). (C) Left: Activation of adenylate cyclase by application of forskolin (FSK, 50 μM) causes a depolarization of resting membrane potential [RMP, details in (E)] and activates maintained low-frequency firing of action potentials in neurons from f/fHCN2$^{+/+}$ mice. Both depolarization and firing are abolished by application of the HCN antagonist ZD7288 (for means, see inset graph: control, 0.02 ± 0.02 Hz; FSK, 0.12 ± 0.06 Hz; FSK/ZD, 0.07 ± 0.04 Hz; n = 18, control compared to FSK, P = 0.02; control compared to FSK/ZD, $P > 0.05$). Right: Membrane depolarization and discharge of action potentials are not seen in neurons from HCN2$^{-/-}$ mice (control, 0.01 ± 0.01 Hz; FSK, 0.01 ± 0.01 Hz; FSK/ZD, 0.001 ± 0.001 Hz; n = 10, $P > 0.05$). (D) Left: Action potential firing elicited by a constant-current pulse (30 pA) in neurons from f/fHCN2$^{+/+}$ mice is enhanced by FSK. Application of ZD7288 returned firing rate to a value not significantly different from control. Right: In HCN2$^{-/-}$ neurons, FSK has no effect on action potential frequency elicited by constant-current pulse. (E) Far left: Action potential firing as a function of injected current in f/fHCN2$^{+/+}$ neurons in experiments as shown in (D). Open symbols, control; solid symbols, FSK; gray symbols, FSK/ZD7288. Mean firing rates, 0.182 ± 0.034 Hz/pA before FSK, 0.379 ± 0.071 Hz/pA after FSK (P = 0.019, n = 8) and 0.149 ± 0.066 Hz/pA in ZD7288 ($P > 0.05$ compared to control). Middle left: RMP (control, –53.1 ± 0.9 mV; FSK, –49.0 ± 1.2 mV, P = 0.01; FSK/ZD, –54.9 ± 1.2 mV; n = 22, P = 0.001 compared to FSK). Middle right and far right: Mean firing rates in HCN2$^{-/-}$ neurons (control, 0.19 ± 0.07 Hz/pA; FSK, 0.21 ± 0.04 Hz/pA; FSK/ZD, 0.20 ± 0.05 Hz/pA; n = 9, $P > 0.05$) and RMP (control, –56.4 ± 1.7 mV; FSK, –55.1 ± 1.8 mV; FSK/ZD, –54.5 ± 2.3 mV; n = 15, $P > 0.05$). Diameter of all neurons, <20 μm.

In voltage-clamped HCN2$^{-/-}$ neurons, I_h was still present (Fig. 2A), but the activation rate after a hyperpolarizing voltage step was significantly faster than in wild-type neurons (Fig. 2, A and B). An increase in cAMP caused a depolarizing shift in the voltage dependence of activation of I_h in wild-type or f/fHCN2$^{+/+}$ neurons (Fig. 2, C and D), which was completely absent in global HCN2$^{-/-}$ neurons (Fig. 2E). These results agree with those obtained with current clamp in showing that HCN2 is expressed in small DRG neurons together with a cAMP-insensitive HCN isoform with a slightly faster time constant of relaxation, which is almost certainly HCN3 because the time constant of relaxation is slow relative to HCN1, the only other cAMP-insensitive isoform (Fig. 2, A and B), and because I_h in small neurons is unaffected by deletion of HCN1 (*14*).

Small neurons from $Na_V1.8$-HCN2$^{-/-}$ mice could be classified into a "wild-type" population (10 of 22 cells), in which the voltage activation curve shifted positive after an increase in cAMP, and a "HCN2$^{-/-}$" population (12 of 22 cells, 55%) in which there was no effect of cAMP on voltage activation. Many medium (diameter 20 to 30 μm) and large (diameter >30 μm) cells also exhibited shifts in the I_h activation curve, consistent with expression of HCN2, but the proportions of these were unaffected by the $Na_V1.8$-driven HCN2 deletion (fig. S5). In experiments on the effect of FSK on action potential firing, there was also evidence for two populations of FSK-sensitive and -insensitive cells (fig. S4, FSK-insensitive ~63%). In experiments with a Cre-reporter mouse, yellow fluorescent protein (YFP) expression was seen in 66% of small neurons (fig. S6). Taken together, these results are consistent with deletion of HCN2 by $Na_V1.8$-Cre expression in ~60% of small DRG neurons.

The effects of HCN2 deletion on action potential firing in small sensory neurons, the majority of which are nociceptors, suggest that HCN2 may be important in inflammatory pain, where the firing of action potentials in response to a noxious stimulus is enhanced by inflammatory mediators such as PGE_2. We initially used the formalin model, in which injection of 4% formalin into a hindpaw causes two phases of pain behavior: an initial phase due to direct activation of nociceptors, followed by a later phase due to release of inflammatory mediators (*25*–*27*). The nonselective HCN blocker ZD7288 reduced pain behavior caused by injection of 4% formalin (*28*), with a particularly marked effect on the late phase

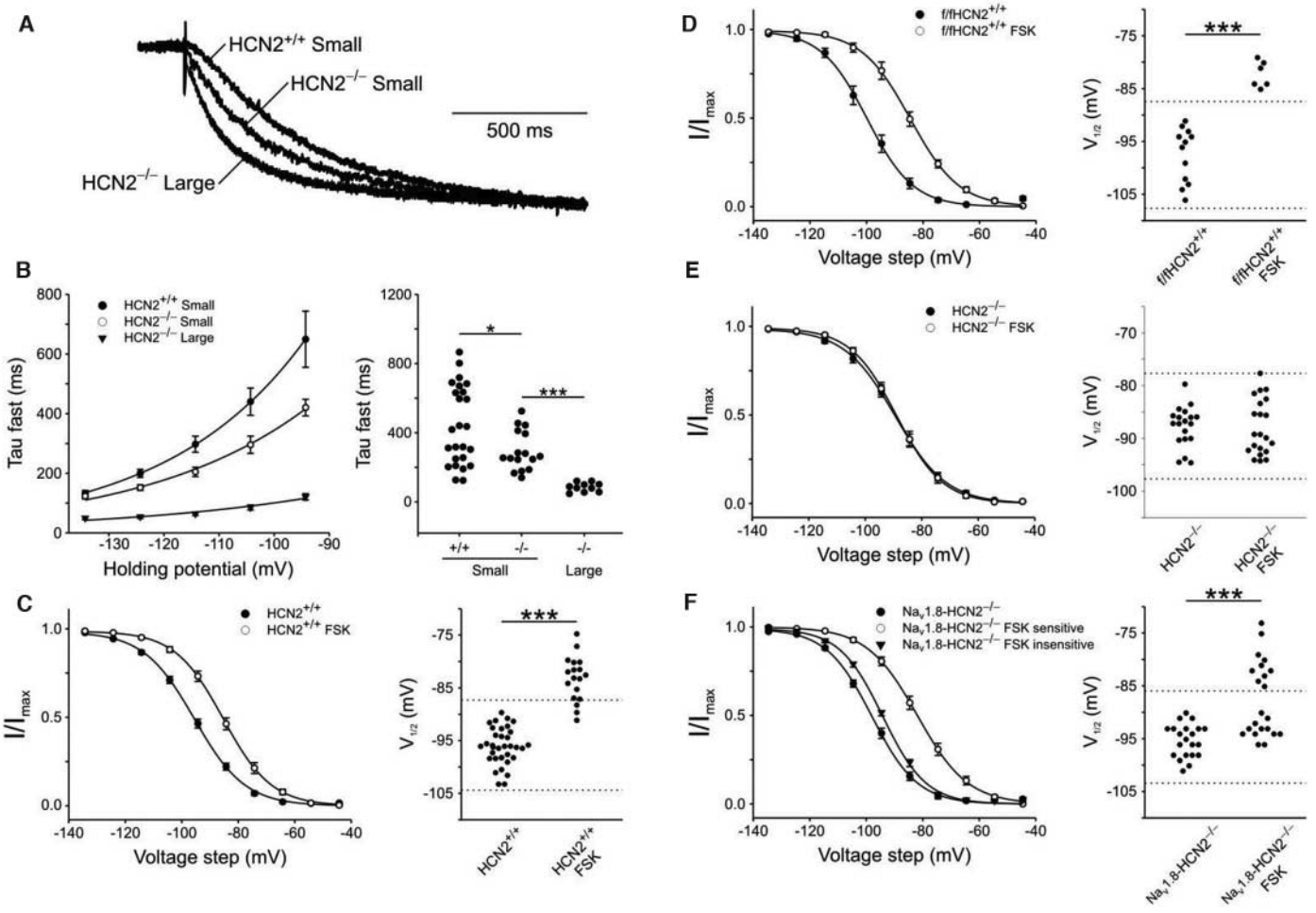

Fig. 2. Electrophysiological characteristics of voltage-clamped small DRG neurons (diameter <20 μm). (**A**) Relaxation rate of I_h in small DRG neurons after voltage pulse from −60 mV to −100 mV is slower in wild-type than in HCN2$^{-/-}$ neurons. Relaxation rate in large neurons (diameter >30 μm) is significantly faster. (**B**) Left: Time constants of fast component of current relaxation as a function of membrane potential from experiments similar to those in (A). Right: Scatterplot of time constants of individual cells at −100 mV (difference between small wild-type and HCN2$^{-/-}$ neurons significant, P = 0.0245, n = 16 to 24). Data in (A) and (B) were obtained using the Cs subtraction technique (see supporting online material). (**C**) Left: In neurons from wild-type mice, elevation of cAMP using forskolin (FSK, 50 μM) causes a shift in voltage dependence of the activation curve for I_h (shift in half-activation voltage $\Delta V_{½}$ = 12.3 ± 1.3 mV; P = 0.0001, n = 18 to 42). Right: Scatterplot of all values of $V_{½}$. Dotted lines show 5% two-sided confidence limits for control data. (**D**) Voltage shift observed in small neurons from f/fHCN2 mice was similar to the wild type ($\Delta V_{½}$ = 15.6 ± 2.0 mV; P = 0.0002, n = 6 to 13). (**E**) Voltage dependence of I_h in neurons from HCN2$^{-/-}$ mice was unaffected by elevation of cAMP ($\Delta V_{½}$ = 0.9 ± 1.7 mV; n = 19 to 22). (**F**) Voltage dependence of I_h in neurons from $Na_V1.8$-HCN2$^{-/-}$ mice fell into two distinct groups: In 12 neurons, no shift in $V_{½}$ was seen upon elevation of cAMP by FSK ($\Delta V_{½}$ = 1.2 ± 1.1 mV, inverted triangles); in 10 neurons, a shift in $V_{½}$ similar to that in wild-type neurons was observed ($\Delta V_{½}$ = 14.1 ± 1.5 mV, open circles; P = 0.0001). Significance values calculated using Student's unpaired t test or Mann-Whitney U test if n < 9.

(Fig. 3A). In $Na_V1.8$-$HCN2^{-/-}$ mice, the late phase was attenuated by ~50% (Fig. 3B). Formalin is a nonspecific stimulus that engages multiple pain and nonpain pathways (*27*), so we sought more specific stimuli. PGE_2 injection into the paws of wild-type animals caused a prominent heat and mechanical hyperalgesia (Fig. 3, C and E). PGE_2-induced thermal hyperalgesia was abolished in wild-type mice injected with the nonspecific HCN blocker ZD7288 and in $Na_V1.8$-$HCN2^{-/-}$ mice (Fig. 3C). After injection of carrageenan, which causes a more long-lasting inflammation, thermal hyperalgesia was also completely abolished by $Na_V1.8$-driven deletion of HCN2 or by pharmacological block with ZD7288 (Fig. 3D). In contrast, mechanical hyperalgesia was unaffected either by $Na_V1.8$-specific HCN2 deletion or by ZD7288 (Fig. 3E). Note that the pre-injection pain thresholds of $Na_V1.8$-$HCN2^{-/-}$ mice to both heat and mechanical stimuli are indistinguishable from those of wild-type mice.

Neuropathic pain is a long-lasting pain state caused by nerve injury and is poorly treated by current pharmaceuticals. In animal models of neuropathic pain, maintained firing is observed in C-fibers (*29*, *30*), suggesting a possible involvement of repetitive activity driven by HCN2. We therefore examined the effect of $Na_V1.8$-specific HCN2 deletion in the chronic constriction injury (CCI) neuropathic pain model. We tested for thermal hyperalgesia, mechanical hyperalgesia, and cold allodynia, all of which are substantially enhanced in neuropathic pain. Hyperalgesia to thermal and mechanical stimuli was prominent after lesion in f/fHCN2$^{+/+}$ animals and persisted unchanged for at least 3 weeks. In $Na_V1.8$-$HCN2^{-/-}$ mice, however, both heat and mechanical thresholds were indistinguishable from sham-operated animals (Fig. 4, A and B). Cold allodynia was

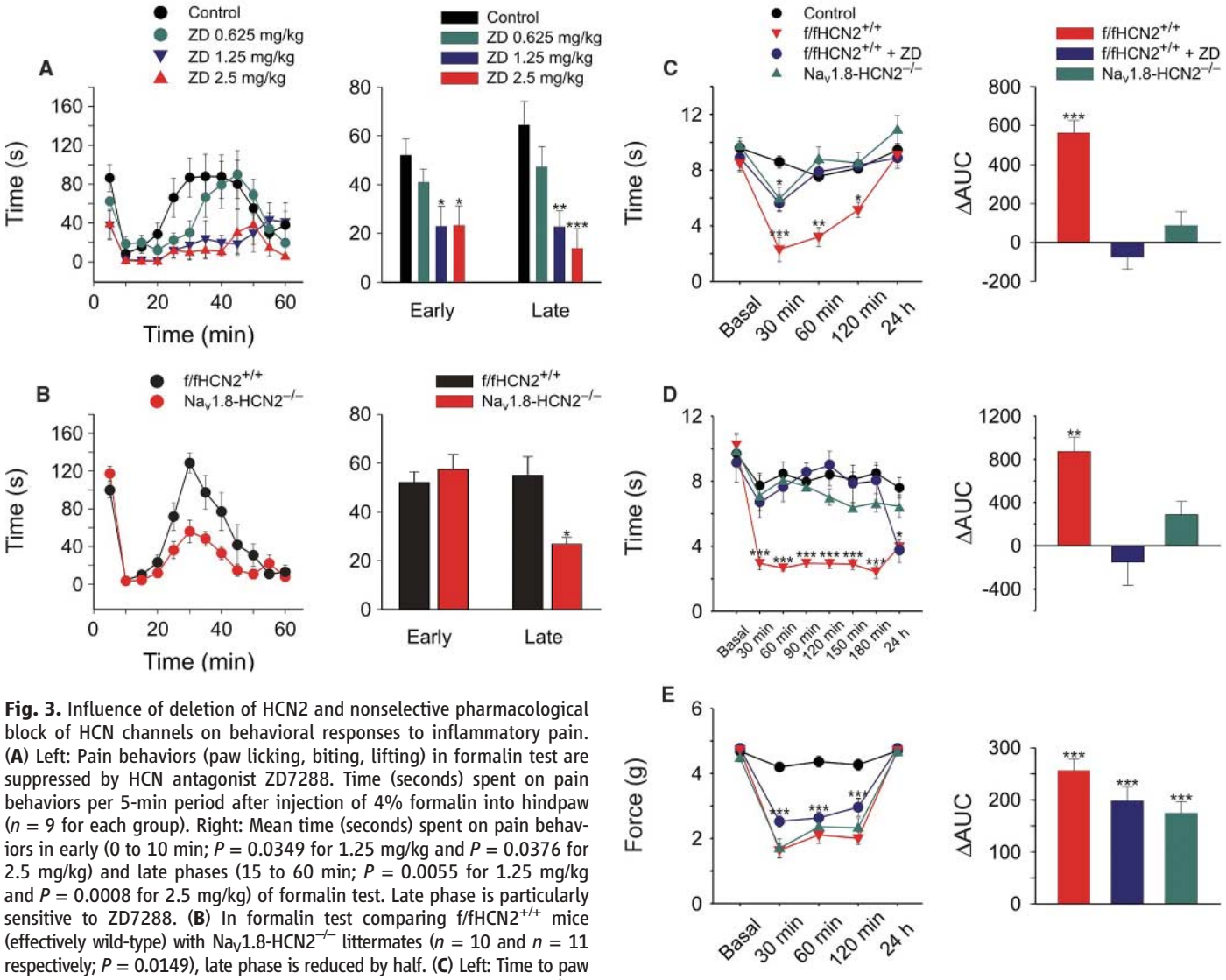

Fig. 3. Influence of deletion of HCN2 and nonselective pharmacological block of HCN channels on behavioral responses to inflammatory pain. (**A**) Left: Pain behaviors (paw licking, biting, lifting) in formalin test are suppressed by HCN antagonist ZD7288. Time (seconds) spent on pain behaviors per 5-min period after injection of 4% formalin into hindpaw (n = 9 for each group). Right: Mean time (seconds) spent on pain behaviors in early (0 to 10 min; P = 0.0349 for 1.25 mg/kg and P = 0.0376 for 2.5 mg/kg) and late phases (15 to 60 min; P = 0.0055 for 1.25 mg/kg and P = 0.0008 for 2.5 mg/kg) of formalin test. Late phase is particularly sensitive to ZD7288. (**B**) In formalin test comparing f/fHCN2$^{+/+}$ mice (effectively wild-type) with $Na_V1.8$-$HCN2^{-/-}$ littermates (n = 10 and n = 11 respectively; P = 0.0149), late phase is reduced by half. (**C**) Left: Time to paw withdrawal in response to radiant heat stimulus after injection of PGE_2 (n = 11 for each group). Concentration of ZD7288, 2.5 mg/kg. Each test group had its own control group of littermates injected with saline; withdrawal latency as a function of time was similar for all control groups. For purposes of illustration, data from all controls are averaged (black line), but all statistical tests were performed between data from the test and appropriate control group (see fig. S10). Right: Area under curve (ΔAUC) for difference between each test and control group (integral between 0 and 120 min; difference from control significant only for f/fHCN2$^{+/+}$, P = 0.0001). (**D**) Left: Similar experiment to (C) in which carrageenan, which causes a more long-lasting hyperalgesia, was injected into one paw. Note that the effect of the bolus intraperitoneal injection of ZD7288 wears off after 24 hours. Right: ΔAUC between 1 and 180 min significantly different from control only for f/fHCN2$^{+/+}$ (P = 0.0097, n = 6 to 10). (**E**) Threshold mechanical force for paw withdrawal (von Frey test) after injection of PGE_2 (n = 10 for each group). Mechanical hyperalgesia not abolished in $Na_V1.8$-$HCN2^{-/-}$ mice, nor in mice injected with ZD7288. Right: ΔAUC between 0 and 120 min; all test groups significantly different from control, P = 0.0001 for f/fHCN2$^{+/+}$ + ZD, P = 0.0002 for f/fHCN2$^{+/+}$, P = 0.0001 for $Na_V1.8$-$HCN2^{-/-}$. Significance values for all panels calculated using Student's unpaired t test or Mann-Whitney U test if n < 9.

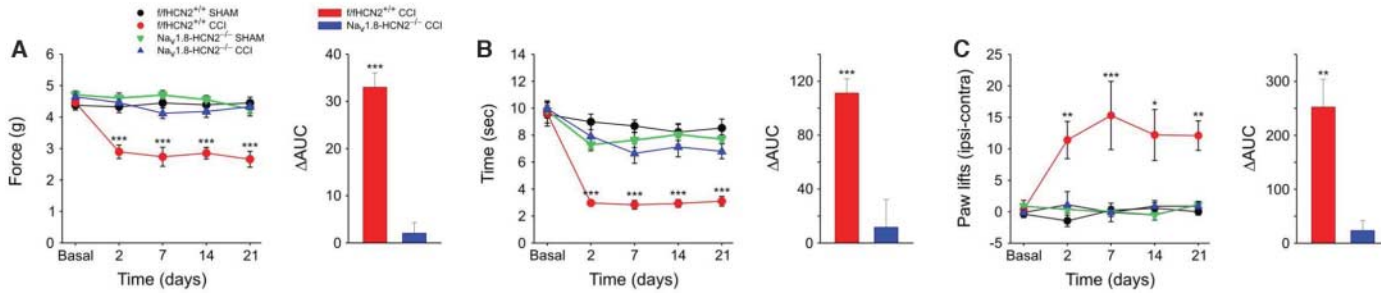

Fig. 4. Pain behaviors after induction of neuropathic pain by chronic constriction injury (CCI) to sciatic nerve. Animals were paired littermates that were either f/fHCN2$^{+/+}$ (effectively wild type) or Na$_V$1.8-HCN2$^{-/-}$; n = 10 animals in each group. (**A**) Left: Force at which withdrawal occurs in response to increasing mechanical stimulus. Right: Integral from 0 to 21 days of difference between each CCI test and the appropriate sham-operated control (ΔAUC, area under curve; difference significant, P = 0.0002, between f/fHCN2$^{+/+}$ and sham only). (**B**) Withdrawal time to radiant thermal stimulus and ΔAUC (P = 0.0005) as in (A). (**C**) Cold allodynia on 5°C plate. Ordinate shows difference between number of paw lifts ipsilateral and contralateral to lesioned nerve in 5-min test period. ΔAUC (P = 0.0026) as in (A) and (B). All significance values calculated using Student's unpaired t test.

tested by placing the mice on a cold plate at 5°C and comparing the number of paw lifts ipsilateral and contralateral to the nerve lesion. Wild-type mice showed a prominent allodynia on the lesioned side, but this cold allodynia was absent in the Na$_V$1.8-specific HCN2$^{-/-}$ mice (Fig. 4C).

Our results show that removal of HCN2 in the Na$_V$1.8-expressing subpopulation of small afferent neurons abolishes the effects of PGE_2, both in enhancing action potential firing in isolated neurons and in producing inflammatory thermal hyperalgesia in vivo. PGE_2 is well established to be the downstream target of the nonsteroidal anti-inflammatory (NSAID) family of analgesics, and our results now show that HCN2 is in turn an important target of PGE_2. Interestingly, inflammatory mechanical hyperalgesia was unaffected either by genetic deletion of HCN2 or by nonselective pharmacological block of HCN channels, perhaps because PGE_2 has a local effect on mechanically gated ion channels independent of its effect on HCN2. Work in several labs has suggested that nociceptors detecting mechanical pain form a separate group from those detecting heat pain (*31*–*33*).

Deleting HCN2 from the Na$_V$1.8-expressing subpopulation of sensory neurons completely abolished neuropathic pain measured using three separate indices, even though HCN2 was not deleted in all small neurons (Fig. 2F and fig. S5); hence, only the Na$_V$1.8-expressing population is critical for initiating neuropathic pain. These findings implicate action potential firing, elicited by HCN2 in the Na$_V$1.8-expressing population of nociceptive afferents, as the originating event in neuropathic pain. Nonspecific pharmacological block of HCN ion channels also abolishes neuropathic pain (*11*, *12*, *21*, *28*). Down-regulating the expression of Na$_V$1.8 (*34*, *35*) or pharmacological block of Na$_V$1.8 (*36*, *37*) partially relieve neuropathic pain; the less than complete relief could be explained by the presence of other voltage-activated Na channels in the Na$_V$1.8-expressing afferent fiber population (*38*). Neuropathic pain is not relieved by knockout of Na$_V$1.8 (*39*) or specific embryonic killing of Na$_V$1.8-expressing nociceptors (*31*) in adult mice, which appears to contradict our results but could be explained by compensatory up-regulation of other Na channels when Na$_V$1.8 is knocked out (*38*) or rewiring of connections in the spinal cord after embryonic elimination of Na$_V$1.8-expressing nociceptors (*33*).

We propose that an inflammatory mediator, released at the site of nerve injury, acts to modify the voltage dependence of activation of HCN2 in nociceptive afferents expressing Na$_V$1.8, thus promoting repetitive firing of action potentials and initiating neuropathic pain. There is no long-lasting change in the electrical properties [resting membrane potential (RMP), I_h, action potential firing] of isolated neurons from nerve-injured mice (fig. S11), consistent with a short-acting mediator. Neuropathic pain is reduced by deletion of adenylate cyclase 5 (*40*) and also by inhibition of COX2 (*41*, *42*) or by deletion of mPGES-1 (*43*), two enzymes that are responsible for the biosynthesis of PGE_2. These observations suggest that the proposed mediator may be PGE_2. The work reported here suggests that HCN2-selective blockers may have value as analgesics to combat both inflammatory and neuropathic pain.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/333/6048/1462/DC1
Materials and Methods
Figs. S1 to S11
Reference (*44*)

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