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Hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2) ion channels drive pain in mouse models of diabetic neuropathy

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Abstract

Diabetic patients frequently suffer from continuous pain that is poorly treated by currently available analgesics. Here we used mouse models of Type 1 and Type 2 diabetes to investigate a possible role for the hyperpolarization-activated cyclic nucleotide-gated 2 (HCN2) ion channels as drivers of diabetic pain. Blocking or genetically deleting HCN2 channels in small nociceptive neurons suppressed diabetes-associated mechanical allodynia and prevented neuronal activation of second-order neurons in the spinal cord in mice. In addition, we found that intracellular cyclic adenosine monophosphate (cAMP), a positive HCN2 modulator, is increased in somatosensory neurons in an animal model of painful diabetes. We propose that the increased intracellular cAMP drives diabetes-associated pain by facilitating HCN2 activation and consequently promoting repetitive firing in primary nociceptive nerve fibers. Our results suggest that HCN2 may be an analgesic target in the treatment of painful diabetic neuropathy.

Introduction

Diabetes causes a characteristic degeneration of peripheral sensory nerves, frequently associated with continuous pain. Painful diabetic neuropathy (PDN) affects approximately one in four diabetic patients and typically manifests itself as a range of unpleasant positive symptoms such as spontaneous pain, mechanical allodynia (a painful sensation caused by light touch) and paresthesias (tingling, shooting pain), as well as negative symptoms such as thermal hyposensitivity (1). Despite its high prevalence, the pathophysiology of PDN remains poorly understood. Both anatomical changes (demyelination, loss of epidermal nerve density) and functional changes (reduced nerve conduction velocity) are characteristic of PDN and of other 'die-back' neuropathies (1–4). The pathologies observed in the peripheral nervous system suggest that peripheral nerve damage is the driver of PDN, and

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Author contributions: CT and PAM planned the study and wrote the manuscript. CT carried out all in vivo experiments, immunohistochemistry and biochemistry. SL performed DRG culture and HCN2 electrophysiology (Fig. 7). SW and IM carried out cAMP ELISA and HCN2 immunohistochemistry, respectively. BV did patch-clamping (fig. S8).

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that the associated ongoing pain is likely to be due to repetitive discharge of action potentials in nociceptive (pain-sensitive) nerve fibers (5–7). However, the molecular basis of peripheral nociceptor hyperexcitability remains elusive.

Hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels have recently emerged as crucial determinants of nociceptive excitability (reviewed in 8, 9). HCN channels are unusual in that they are activated by hyperpolarization in the range -60 mV to -90 mV, in contrast to all other voltage-activated channels which are activated by depolarization. There are four HCN isoforms (HCN1-4) expressed in sensory neurons. More than half of small nociceptive neurons express HCN2 channels (10), whereas in large sensory neurons the fast HCN current (I_h) is mediated mainly by HCN1 (11, 12). HCN3 is widely expressed across dorsal root ganglion (DRG) neurons of all sizes (11), whereas HCN4, which has a key pacemaking role in the heart (13, 14), shows limited expression in somatosensory neurons (15, 16). Elevations of intracellular cAMP cause a strong shift in the voltage dependence of activation of HCN2 and HCN4 to more positive membrane voltages, causing an increase in the inward current carried by these channels at resting membrane voltage, whereas HCN1 and HCN3 are relatively insensitive to cAMP and so have less influence in modulating neuronal excitability (17). In nociceptive neurons, inflammatory mediators such as prostaglandin E2 (PGE2) and bradykinin activate adenylate cyclase via a G_s -protein-coupled pathway, thus causing a rise in intracellular cAMP, HCN2 activation, and increased spontaneous firing of action potentials (10).

A critical role for HCN2 in inflammatory pain and in the neuropathic pain caused by direct mechanical damage to sensory nerves has been demonstrated by the potent analgesic actions of specific HCN channel blockers and by targeted deletion of the *Hcn2* gene in nociceptive neurons in mice (10). Pharmacological inhibition of I_h prevents pain in chemotherapy-induced neuropathy (18), which also features a 'die-back' denervation pattern, and circumstantial evidence has hinted at an involvement of unidentified HCN family members in autonomic diabetic neuropathy (19, 20).

Here we expand our understanding of the critical role of HCN2 in chronic pain by showing that cAMP-mediated HCN2 activation in a mouse model of diabetic neuropathy can trigger repetitive activity in small nociceptive fibers, leading to central sensitization and ongoing pain. Pharmacological or genetic block of HCN2 activity exerts potent analgesic effects in animal models of both Type 1 and Type 2 diabetes.

Results

Streptozotocin treatment results in symptoms indicative of painful diabetic neuropathy

Diabetes was induced in wild-type (WT) mice by a single injection of streptozotocin (STZ). Selective accumulation of STZ in pancreatic islet β cells causes DNA alkylation, cell death, and consequent loss of insulin production, resulting in an elevation of blood glucose. Mice treated with STZ exhibited significantly increased blood glucose concentrations by day 7 compared to the pre-STZ baseline (Fig. 1A; 21.9 ± 2.1 mmol/L vs 8.4 ± 0.2 mmol/L, $P < 0.001$). STZ-treated mice remained hyperglycemic thereafter, whereas blood glucose concentration did not change in vehicle-treated mice (10.1 ± 0.7 mmol/L). The small

number of mice that did not exhibit hyperglycemia after STZ injection were excluded from analysis (see Table 1).

We examined whether the diabetic state was accompanied by altered pain sensation, similar to that encountered in human PDN. Diabetic mice showed a progressive hypersensitivity in response to mechanical stimulation (Fig. 1B). The mechanical pain threshold was significantly lower than baseline at 2 weeks, as indicated by the mean force required to elicit paw withdrawal to mechanical stimulation (4.1 ± 0.1 g vs 3.5 ± 0.1 g, $P=0.012$) and continued to decrease until 8 weeks (2.3 ± 0.1 g, $P<0.001$), representing a 44% increase in mechanical pain sensitivity (Fig. 1B). Mechanical hypersensitivity persisted for at least 18 weeks post-STZ (2.7 ± 0.1 g, $P<0.001$). In contrast, mechanical pain thresholds of vehicle-treated mice remained at baseline levels for the duration of the study (Fig. 1B). Testing with a thermal heat stimulus revealed thermal hyposensitivity 6-8 weeks after induction of diabetes, as indicated by increased latency of paw withdrawal (6 weeks, 12.0 ± 0.6 sec vs 9.2 ± 0.6 sec, $P=0.002$; 8 weeks, 12.7 ± 0.5 sec vs 9.4 ± 0.5 sec, $P<0.001$ compared to vehicle-treated mice) (Fig. 1C). The dual manifestation of mechanical hypersensitivity and thermal hyposensitivity mirrors the clinical presentation of diabetic neuropathy in humans. Activation of transient receptor potential ankyrin 1 (TRPA1) by STZ may be responsible for some of the manifestations of pain for 10 days after administration of STZ (21), and for this reason we carried out behavioral pain measurements at later time points (8-18 weeks post-STZ). Of note, Fig. 1B and C include all mice that developed diabetes, but for unknown reasons only 70-90% of these mice developed substantial pain hypersensitivity (see Methods and Table 1).

Blocking peripheral HCN channels reverses mechanical hypersensitivity

We next investigated whether HCN function is involved in the mechanical pain hypersensitivity in PDN. To address this, we selected mice with the most robust pain phenotypes at 8 weeks post-STZ and injected them intraperitoneally with ivabradine, which inhibits all four HCN isoforms with approximately equal potency (22). Note that ivabradine is excluded from the CNS (18), so its actions can be attributed to blockade of HCN ion channels in the periphery. A single injection of 2.5 mg/kg ivabradine increased diabetic pain thresholds (from 2.2 ± 0.2 g after STZ to 3.1 ± 0.2 g at 30 min, $P=0.006$ and 3.2 ± 0.2 g at 60 min after ivabradine injection, $P=0.002$) (Fig. 1D). This effect was also observed with higher ivabradine doses (5 mg/kg: from 2.3 ± 0.1 g after STZ to 3.2 ± 0.5 g at 30 min and 3.1 ± 0.3 g at 60 min after ivabradine injection, $P=0.018$ and $P=0.024$, respectively; 10 mg/kg: from 2.1 ± 0.1 g after STZ to 3.1 ± 0.3 g at 30 min and 3.3 ± 0.3 g at 60 min after ivabradine injection, $P=0.001$ and $P<0.001$, respectively). At all doses, the ivabradine-mediated analgesic effect on pain threshold was significant compared to the vehicle group at 60 min after ivabradine injection (2.5 mg/kg, $P=0.014$; 5 mg/kg, $P=0.016$; 10 mg/kg: $P=0.021$) and subsided after 120 min. Ivabradine had no effect on acute mechanical and thermal pain thresholds of control mice (Fig. 1D and fig. S1), confirming previous observations that HCN channels are not engaged during normal acute pain processing (10). In addition, thermal hypoesthesia in mice treated with STZ was unaltered by ivabradine (fig. S1), suggesting that thermal hyposensitivity is unrelated to changes in HCN ion channel function.

Because ivabradine did not completely restore normal pain sensitivity, we asked whether the analgesic effect could be enhanced by repeated administration of the drug. Two consecutive injections (5 mg/kg, separated by 2 hours) increased pain thresholds beyond those of a single administration, and a third injection completely restored thresholds to pre-diabetic levels (from 3.9 ± 0.2 g before STZ to 4.0 ± 0.2 g 30 min after ivabradine injection, 3.8 ± 0.3 g 60 min after ivabradine injection, and 3.7 ± 0.3 g 120 min after ivabradine injection; $P=0.789$, $P=0.843$, $P=0.960$, respectively) (Fig. 1E). Note that there is a small change in threshold following each saline injection, likely to be due to analgesia caused by the stress of handling and injection, but the analgesia caused by ivabradine injection is significantly greater, particularly after repeated injection.

Finally, we examined whether this cumulative effect could also be seen with a more clinically relevant, spaced-out dosing regimen. We treated the animals with ivabradine twice a day, 3 hours before and 3 hours after pain threshold measurements over 4 consecutive days. Mechanical thresholds on days 2-4 in mice treated with ivabradine were not different from those recorded before STZ injection, and progressively increased compared to saline-treated animals (day 2, $P=0.012$; day 3, $P=0.002$; day 4, $P=0.014$). The analgesia was fully reversible, and cessation of treatment for 4 days resulted in a drop of mechanical thresholds back to diabetic levels.

Because ivabradine inhibits all HCN isoforms, including HCN4 which is important for cardiac pacemaking, we investigated the effect of the drug on cardiac function using pulse oximetry. As expected, 5 mg/kg ivabradine caused a reduction in basal heart rate in control mice (29.2% at 30 min vs baseline, $P<0.001$) and a smaller reduction in diabetic mice (15.8% at 30 min, $P=0.009$ vs baseline, $P=0.001$ vs CTRL+IVA at 30 min; fig. S2). This lesser effect of ivabradine on diabetic cardiac function has also been documented in STZ rats (23). Ivabradine reduced respiratory rate (fig. S2B) in both control and diabetic mice, but no change was detected in oxygen saturation (fig. S2C) or pulse distention at 30 min post-injection (fig. S2D). There was no obvious effect on alertness or exploratory behavior as detected by visual observation. Put together, the pulse oximetry data suggest that an analgesic dose of ivabradine is well tolerated in both control and diabetic mice, causing bradycardia but no other adverse effects on cardiac function.

Peripheral HCN block is analgesic in Type 2 diabetes

Type 2 diabetes is the most common form of diabetes in humans; therefore, we tested the effect of HCN block in the db/db mouse model of Type 2 diabetes, in which mice homozygous for a leptin receptor mutation (db/db) become obese in adulthood and show hyperglycemia together with an array of diabetic symptoms (24). At 18 weeks of age, all db/db mice were obese and hyperglycemic compared to heterozygous age-matched controls (db/+) (body weight db/db: 48 ± 0.6 g; db/+: 29.3 ± 0.4 g; $P<0.001$; blood glucose db/db: 27 ± 1.3 mmol/L; db/+: 9.4 ± 0.4 mmol/L; $P<0.001$) (Fig. 2A). The db/db mice exhibited significantly lower mechanical pain thresholds (3.6 ± 0.2 g vs 4.5 ± 0.2 g, $P<0.001$) (Fig. 2B, C). Note that both the elevation in blood glucose and the mechanical hyperalgesia were less than those of Type 1 diabetic (STZ-injected) mice (compare Fig. 2 with Fig. 1A, B). A single ivabradine administration (5 mg/kg, i.p.) restored normal pain thresholds in db/db

mice (from 3.6 ± 0.2 g to 4.6 ± 0.2 g, $P < 0.001$ vs saline at 60 min), whereas saline injection had no significant effect on pain threshold ($P = 0.47$ at 60 min) compared to baseline (Fig. 2C).

Induction of spinal *c-Fos* in diabetic mice is reversed by HCN inhibition

We next sought a more direct way of demonstrating that enhanced excitability of peripheral nociceptive neurons is involved in diabetes-induced hyperalgesia. Activation of second-order neurons in the spinal cord by primary afferent input induces *c-Fos*, an immediate-early gene rapidly upregulated by neuronal activity (25). Therefore, C-FOS protein expression in neurons of the outer layers of the dorsal horn, where nociceptive fibers terminate, may be indicative of sustained peripheral nociceptive input and ongoing pain in diabetes.

Quantification of C-FOS immunoreactivity in lumbar spinal cord sections (laminae I-II) revealed an increased number of C-FOS positive neurons in the dorsal horn at 8 weeks after STZ administration, compared to controls (26.0 ± 2.6 vs 1.2 ± 0.2 neurons/section, $P < 0.001$) (Fig. 3). Although C-FOS activation was notably less compared to that induced by a strong noxious stimulus (fig. S3 and (26)), it is in agreement with previous studies in diabetes (27). Ivabradine treatment (three i.p. injections of 5 mg/kg every 2 hours, as in Fig. 1E) reduced diabetes-induced C-FOS activation 6 hours after the first injection (6.2 ± 0.4 neurons/section, $P < 0.001$ vs STZ). These data indicate that painful diabetic neuropathy increases activation of dorsal horn neurons, likely due to enhanced nociceptive input to the spinal cord, and that this activation can be reversed by pharmacological block of HCN in the periphery.

Genetic deletion of HCN2 in nociceptive neurons prevents development of diabetic pain

Ivabradine blocks all four HCN isoforms equally (22), so in order to decipher which HCN isoform causes hyperalgesia in diabetes we used a conditional knock-out mouse line in which HCN2 had been selectively deleted in nociceptive (Nav1.8-expressing) DRG neurons (HCN2 cKO) (10). STZ treatment of HCN2 cKO mice or their littermate controls (fHCN2) caused a similar hyperglycemia within one week (HCN2 cKO pre-STZ vs post-STZ, 7.8 ± 0.5 mmol/L vs 21.5 ± 1.4 mmol/L, $P < 0.001$; fHCN2 pre-STZ vs post-STZ, 8.1 ± 0.3 mmol/L vs 25.2 ± 3.4 , $P < 0.001$) (Fig. 4A). High blood glucose concentrations were also present at 8 weeks (HCN2 cKO, 40.4 ± 0.6 mmol/L; fHCN2, 34.2 ± 6.3 mmol/L, $P < 0.001$ vs pre-STZ) (Fig. 4A). There was no significant difference between genotypes at any time point (1 week, $P = 0.07$; 8 weeks, $P = 0.291$), indicating that both genotypes become diabetic to an equal extent.

We next studied the development of PDN in these transgenic mice. Diabetic fHCN2 mice, which are effectively wild-type, developed a progressive mechanical hypersensitivity, evident as a significant drop in mechanical thresholds compared to pre-diabetic baseline (2w, 3.7 ± 0.2 g; 4w, 3.4 ± 0.2 g; 6w, 3.1 ± 0.2 g; 8w, 3.2 ± 0.3 g), $P < 0.001$ vs BL (4.2 ± 0.1 g) (Fig. 4B). When we assessed the HCN2 cKO mice, however, we found no signs of mechanical hypersensitivity, despite the ongoing diabetes. Mechanical thresholds at 2, 4, 6, and 8 weeks post-STZ (4.2 ± 0.1 g, 4.2 ± 0.2 g, 4.3 ± 0.1 g and 4.4 ± 0.1 g, respectively) were not significantly different to pre-diabetic values (4.2 ± 0.1 g; $P = 0.987$, $P = 0.964$,

$P=0.623$, $P=0.737$, respectively) (Fig. 4B) and remained significantly higher than the corresponding values in the fHCN2 group (2w, $P=0.037$; 4-8w, $P<0.001$).

Using C-FOS immunoreactivity in the dorsal horn as a measure of nociceptive activity impinging on second-order neurons of the spinal cord, we observed an increase in the numbers of C-FOS positive neurons in diabetic fHCN2 mice, whereas C-FOS expression was greatly reduced in diabetic HCN2 cKO mice (15.6 ± 0.8 vs 1.4 ± 0.2 neurons/section, $P<0.001$) (Fig. 4C). Put together, these data suggest that genetic deletion of HCN2 in Nav1.8-positive nociceptors recapitulates the analgesia and suppression of peripheral nociceptive drive caused by pharmacological block of HCN channels, and therefore that HCN2 expressed in nociceptive neurons is necessary for diabetes-induced pain in the mouse STZ model.

Diabetes reduces plantar skin innervation

Intra-epidermal nerve fiber density is often used as a diagnostic criterion for peripheral neuropathy in human diabetic patients. We calculated intraepidermal nerve fiber density in plantar skin of the hindpaw by staining for PGP9.5, a pan-neuronal marker present in nerve terminals, and for collagen IV, which stains the basement membrane at the dermis-epidermis boundary. We also used DAPI stain to highlight the nuclei of basal layer cells of the basement membrane. With this combination of stains, nerve fibers crossing into the epidermis were clearly visualized (Fig. 5A). Quantification of intraepidermal nerve fiber density revealed a significant reduction in WT diabetic mice at 8 weeks post-STZ, compared to non-diabetic WT controls (26.8 ± 2.0 vs 44.2 ± 3.5 fibers/mm, respectively, $P=0.016$) and this reduction was also present 8 weeks post-STZ in both fHCN2 mice (effectively WT) and in HCN2 cKO mice (fHCN2, 25.0 ± 2.6 , $P=0.023$ vs control; HCN2 cKO, 28.1 ± 6.7 fibers/mm, $P=0.014$ vs control) (Fig. 5B). These results confirm the peripheral neuropathy in the STZ mouse model and demonstrate that the lack of a pain phenotype in the HCN2 cKO mice is not due to a change in the denervation pattern. To further characterize the identity of affected intraepidermal fibers, we stained for calcitonin gene-related peptide (CGRP), which identifies peptidergic fibers, including transient receptor potential cation channel subfamily V member 1 (TRPV1) positive fibers that sense noxious heat. Diabetes reduced intraepidermal CGRP⁺ fibers from 17.4 ± 0.8 to 12.5 ± 0.6 fibers/mm (Fig. 5C, $P=0.006$; bottom left graph). Because most TRPV1⁺ fibers in mouse co-express CGRP (28), this reduction is in agreement with the observed thermal hyposensitivity in STZ mice (Fig. 1C). Indeed, there was a significant inverse correlation between intraepidermal CGRP⁺ fiber density and heat pain thresholds in diabetic mice ($R^2=0.88$, $P=0.006$; bottom right graph).

Diabetes does not affect HCN2 expression in sensory neurons

The preceding experiments show that enhanced peripheral HCN2 function plays a critical role in pain and mechanical hypersensitivity in diabetes. To determine whether this is due to an upregulation of HCN2 expression in sensory neurons, we used immunohistochemistry to visualize HCN2 in lumbar DRG sections (Fig. 6A). In control animals, HCN2 immunoreactivity was detected in $73.2 \pm 4.5\%$ of all neurons, as revealed by co-localization with the pan-neuronal marker $\beta 3$ -tubulin (examples denoted by arrows). As previously reported, HCN2 was detected in a range of cell sizes, including most small diameter

neurons, the majority of which are nociceptive, as well as in larger neurons with a characteristic membrane ring staining (29). In diabetic animals at 8 weeks post-STZ, the percentage of HCN2-positive neurons was $72.5 \pm 7.0\%$, not significantly different from control ($P=0.934$) (Fig. 6B, left). In addition, the distribution of HCN2⁺ neurons amongst sub-classes of DRG neurons (small, medium, and large diameter classes) was also not different between control and diabetic ($P=0.116$; Fig. 6B, middle). When examining staining intensity in HCN2-positive neurons, we found no effect of diabetes amongst all neurons (control, 3.5 ± 0.2 A.U. vs diabetic, 3.9 ± 0.1 A.U., $P=0.102$) or amongst DRG sub-populations ($P=0.802$) (Fig. 6B, right). The specificity of the HCN2 antibody was confirmed by detection of HCN2 in small Nav1.8⁺ nociceptive DRG neurons of WT mice but not of HCN2 cKO mice, as well as by the complete lack of HCN2 immunoreactivity in DRG from global HCN2 knockout mice (Fig. 6C).

We investigated whether enhanced HCN2 function could be due to changes not reflected in the cell soma (such as axonal trafficking), by examining HCN2 expression in the sciatic nerve. The intensity of axonal HCN2 staining was similar between control and STZ mice at 8 weeks (2.6 ± 0.1 A.U. vs 2.6 ± 0.6 A.U., $P=0.871$), suggesting that there is no change in axonal HCN2 expression in diabetes (fig. S4A). We did not detect any HCN2 in epidermal nerve fibers of control mice, probably because the channel is present in low quantities in the very fine peripheral nerve endings (fig. S4B).

We also examined expression and regulation by diabetes of the other HCN isoforms in DRG neurons. HCN1 was predominantly localized in medium-large neurons (fig. S5), with no difference between control and diabetic conditions in overall proportion of HCN1⁺ neurons, distribution of HCN1⁺ neurons amongst size classes, or intensity of stain as a function of cell size. The HCN3 isoform was detected in a mixture of neurons of all sizes (fig. S6) and there was no effect of diabetes on either proportion of HCN3⁺ neurons or stain intensity as a function of size. HCN4 was found mainly in medium-large neurons (fig. S7), and again there was no effect of diabetes on any parameter. Overall, the immunohistological data show that diabetes does not induce upregulation of any HCN isoform at the protein level. Note that a previous study reported that diabetes increases HCN expression and currents in parasympathetic neurons of the nodose ganglion, suggesting that HCN channels may be differentially regulated in autonomic neuropathy (19).

Diabetes does not change electrophysiological properties of isolated nociceptive neurons

We next examined the in vitro electrophysiological properties of small DRG neurons (<20 μm diameter, predominantly nociceptive in nature (30)) from control and STZ mice, to determine whether diabetes alters HCN ion channel properties. In response to steady current injection, neurons from STZ and non-diabetic mice showed a similar increase in firing frequency with increasing injected current, reaching a similar plateau level (14.58 ± 1.87 spikes/sec vs 16.45 ± 2.99 spikes/sec, Fig. 7A&B). Exposure to 50 μM forskolin, which increases intracellular cyclic adenosine mono phosphate (cAMP) and shifts the activation curve of HCN2 to a more positive membrane voltage, increased firing frequency after current injection, from 13.51 ± 2.26 spikes/sec to 21.68 ± 2.72 spikes/sec (40 pA, $P<0.001$) and from 14.58 ± 1.87 spikes/sec to 24.0 ± 2.94 spikes/sec (50 pA, $P=0.009$). The cAMP-

dependent sensitization of neuronal firing was also reflected in a change in the pattern of firing, from phasic (steady current elicits one or a few spikes) to tonic (firing continues throughout current injection; see Fig. 7C and (31)). The proportion of phasic/tonic firing neurons was similar between STZ and non-diabetic mice at baseline (Fig. 7D, $P>0.05$) and cAMP augmented tonic firing ($P=0.043$ and $P=0.081$, respectively) with no significant difference between groups (Fig. 7D, $P=0.652$).

We used voltage clamp to compare maximum current densities in nociceptive neurons by fully activating HCN channels with a hyperpolarizing voltage step from -60 mV to -140 mV. Similar maximal current amplitudes were observed in STZ and control mice and in the presence or absence of forskolin, showing that maximum current density is not affected by diabetes nor by cAMP increase (Fig. 7E) ($P=0.545$). This result supports the immunohistological data that showed no upregulation of HCN2 expression in diabetic neuropathy.

We examined the effect of cAMP on the voltage dependence of HCN channel activation in small DRG neurons from STZ and non-diabetic mice. Forskolin caused a depolarizing shift in HCN half-activation voltage ($V_{1/2}$) in both STZ (+10.83 mV, $P<0.001$ vs pre-forskolin, -97.1 ± 1.6 mV) and control neurons (+12.36 mV, $P<0.001$ vs pre-forskolin, -99.0 ± 1.1 mV) (Fig. 7F). However, the magnitude of the shift was not significantly different between diabetic and control neurons ($P=0.779$). Finally, to investigate the possibility that the altered concentration of glucose in diabetes might affect the function of HCN channels, we compared I_h currents in neurons in extracellular solutions containing either low (5 mM) or high (25 mM) glucose. Glucose concentration had no effect on activation curves or current densities in either control or STZ neurons (fig. S8).

PDN is associated with increased cAMP in sensory neurons

The immunohistochemical and electrophysiological experiments above show that diabetes does not cause any change in expression of HCN2 nor in the electrophysiological properties of isolated nociceptive neurons. The marked effect of diabetic neuropathy on HCN2 function in vivo could instead be due to factors which are present in diabetes and potentiate HCN2 function, but are rapidly lost after neuronal isolation. The most likely way in which these unidentified factors might act is by causing an increase of intracellular cAMP concentration in nociceptive neurons, resulting in a potentiation of HCN2 activation through a positive shift in its activation curve on the voltage axis. We therefore examined the effect of diabetes on the cAMP content in whole DRG from control and diabetic mice. Analysis of DRG tissue from STZ-treated mice in which prominent mechanical hypersensitivity had been verified through behavioral tests showed that the cAMP content was increased more than 8-fold (non-diabetic, 3.1 ± 1.6 pmol/mg; STZ pain, 26.6 ± 10.3 pmol/mg of total protein; $P=0.014$). When samples from fully diabetic mice which did not exhibit substantial mechanical hypersensitivity were assessed (STZ no pain), cAMP concentrations were not significantly different from non-diabetic controls ($P=0.811$ vs non-diabetic) (Fig. 8A).

Apart from binding directly to HCN ion channels, cAMP can also activate the cAMP-dependent protein kinase A (PKA), potentially causing phosphorylation of other targets involved in pain transduction, such as Nav1.8 and TRPV1. We therefore investigated the

effect of the PKA inhibitor H-89 on diabetes-induced hyperalgesia, at doses found to be effective in reducing prostaglandin E2 (PGE2)-induced inflammatory pain (fig. S9) (32). Systemic treatment with 5 mg/kg H-89 had some effect in increasing mechanical pain thresholds 8 weeks after STZ ($P < 0.05$ vs post-STZ), but the magnitude of this effect was smaller compared to the effect of ivabradine ($P < 0.05$ ivabradine vs H-89 at any dose) (Fig. 8B).

Discussion

PDN is a typical ‘die-back’ neuropathy, in which unmyelinated C-fibers and thinly myelinated A δ nociceptive fibers retreat first (33, 34), and in which spontaneous firing develops in small fibers (5, 6, 35). The work reported here provides further support for the idea of spontaneous firing in small nociceptive nerve fibers, because in diabetic mice C-FOS, which is an index of nociceptive afferent activity (25), is induced in outer layers of the dorsal horn, where nociceptive afferents terminate. A critical role for the HCN2 ion channel in maintaining the firing in small-diameter afferent nerve fibers, and thus in promoting the pain of diabetes, is supported by: (1) the complete suppression of pain in mouse models of Type 1 and Type 2 diabetes by the HCN channel blocker ivabradine, which does not distinguish between HCN isoforms but is peripherally restricted (18), therefore localizing the site of HCN block to the peripheral nervous system; (2) the similar suppression of the pain associated with diabetes caused by deletion of HCN2 in the Na v 1.8-expressing population of nerve fibers, which are predominantly small unmyelinated C-fibers (36, 37); and (3) the reduction in C-FOS expression in the dorsal horn achieved both by blocking HCN channels with ivabradine and by genetically deleting HCN2 in Na v 1.8-expressing nerve fibers.

As many as 80% of diabetic patients with reduced small-fiber skin innervation, caused by nerve degeneration, also exhibit hyposensitivity to heat (3, 38, 39), which is consistent with the preferential expression of the heat-sensitive ion channel TRPV1 in small unmyelinated nerve fibers (40). Indeed, an inverse correlation between TRPV1⁺ fiber skin innervation and heat detection has been directly demonstrated in diabetic patients (41), and we also found an association between degeneration of peptidergic intraepidermal nerve fibers (which include TRPV1⁺ fibers) and increased heat thresholds in STZ-treated mice.

The painful sensation caused by light touch, known as mechanical allodynia, which is one of the most unpleasant features of PDN, is likely to be conveyed by large myelinated fibers (42). In agreement with this, preventing conduction in large myelinated fibers alleviates mechanical allodynia in PDN (43). How can spontaneous activity in small HCN2-expressing unmyelinated fibers enhance the sensation of mechanical allodynia, which is conveyed via large myelinated fibers? A probable explanation lies in the phenomenon of central sensitization, in which activation of small nociceptive nerve fibers causes a sensation of mechanical allodynia, conveyed via large fibers. Central sensitization is thought to be a result of neural plasticity in the dorsal horn, in which afferent mechanoreceptor input is switched from second-order neurons signaling innocuous light touch, to second-order neurons signaling a sensation of pain (44).

One possible explanation for increased firing in nociceptive fibers in the mouse model of diabetes could be an upregulation of HCN2 expression in sensory neurons, but both immunohistochemical and electrophysiological approaches indicate that HCN2 protein expression and maximal current density were not different in neurons isolated from diabetic and non-diabetic mice. In sensory neurons, however, HCN2 function can be potentiated by an increased intracellular cAMP, which we found to be elevated in DRG from the mouse model of diabetes. Interaction of cAMP with the cyclic nucleotide binding domain in the C-terminal region of HCN2 results in a depolarizing shift in the voltage dependence of activation, leading to an enhanced inward current carried by HCN2 and thus to augmented spontaneous and evoked firing (10). In agreement, raising cAMP concentrations with forskolin in vitro shifted the voltage dependence of I_h towards more positive membrane voltages and increased firing rates in small neurons both in the animal model of type 1 diabetes and in control animals, and previous work has shown that both the shift in I_h and the increased firing rate depend on HCN2 (10).

The mechanisms by which cAMP is elevated in diabetes have not been determined in the present study, but are unlikely to involve only inflammatory mediators released at a peripheral location where neurodegeneration takes place, because we found an increase in cAMP in DRG cell bodies, some distance from the site of neurodegeneration. In other pain states, cAMP can be increased by binding of PGE2 or other inflammatory mediators to GPCRs, such as the prostaglandin E2 receptor 4 (EP4), resulting in activation of adenylate cyclase and increased cAMP production (10). Several lines of evidence support the idea that diabetes is also associated with a similar pro-inflammatory phenotype. Diabetic neuropathy increases the amounts of inflammatory mediators in the DRG and sciatic nerve of rodents as well as in human blood (45–48). Administration of inhibitors of adenylate cyclase restores mechanical thresholds in diabetic rats, whereas cAMP analogs have the opposite effect (49). The expression of cyclooxygenase-2 (COX-2), which is upstream of PGE2, is increased in diabetic DRG (50) and in sciatic nerve (51), and bradykinin-induced PGE2 secretion is also potentiated by diabetes (52). In support of a sensitizing role of cAMP in sensory neurons, COX-2 inhibitors are analgesic in diabetic mouse models when applied peripherally but not intrathecally (53, 54) and COX-2 knock-out mice have attenuated signs of peripheral neuropathy after induction of diabetes (55). Other pro-inflammatory pathways may also be involved in activating adenylate cyclase and so increasing levels of cAMP.

Increased cAMP also activates PKA, which in turn phosphorylates other ion channels in the pain pathway, such as $Na_v1.8$ (56) and TRPV1 (57). Although an involvement of PKA-dependent sensitization of these channels in PDN is possible (58), the complete abolition of hypersensitivity either by an HCN blocker with few off-target actions (18) or by genetic deletion of HCN2 strongly suggests that HCN2 has the major influence in diabetes-induced hyperalgesia. This point is further substantiated by the much larger effect of ivabradine on diabetic mechanical pain thresholds compared to that of the PKA inhibitor H-89.

We propose that HCN2 sensitization, mediated by increased intracellular cAMP, mediates pain in diabetes both by causing spontaneous firing in nociceptive nerve fibers, due to HCN2 activation at the resting membrane potential, and by promoting higher spiking frequencies in response to a painful stimulus, due to a more rapid “pacemaker” depolarization rate between

action potentials. Central sensitization is then activated by the enhanced firing in small nociceptive afferents and causes mechanical allodynia, a painful sensation initiated by activation of large fibers by tactile stimuli. The occurrence of spontaneous firing in C-fibers in STZ-treated rodents has been directly demonstrated using microneurography (6). We found evidence supporting the idea of increased peripheral nociceptive drive in diabetic mice by using C-FOS staining. In addition, diabetes-induced enhancement of firing frequency in C polymodal nociceptors has been reported using a skin-nerve preparation (35).

Diabetic rats treated with the potent TRPV1 agonist resiniferatoxin, which is neurotoxic to the TRPV1-expressing subpopulation of C-fibers, maintain mechanical allodynia (59), an observation which is often cited as indicating that C-fibers are not involved in PDN. However, two major classes of C-fibers are unaffected by resiniferatoxin, namely nociceptive non-peptidergic C-fibers expressing the receptor MAS Related GPR Family Member D (MRGPRD), and low-threshold mechanoreceptors (60). Activity in these C-fibers may be able to drive central sensitization and so cause mechanical allodynia.

Patients with neuropathic pain exhibit a variety of positive symptoms, for example hyperalgesia and spontaneous pain, together with negative symptoms such as sensory loss, and different treatments may be appropriate depending on the symptoms displayed (61). In that context, our results may be particularly relevant for diabetic patients in which ongoing pain, likely to be driven by spontaneous activity in peripheral nerves, is a prominent feature. Microneurography studies in humans have shown that peripheral nerve activity is routinely present in neuropathic pain syndromes involving peripheral neuropathy (62–64), including diabetic neuropathy (65), and that the incidence of spontaneous activity is higher when accompanied by reports of ongoing pain (7). We note, however, that there may not be an invariant link between spontaneous activity in peripheral nerves and ongoing pain, as a number of studies have proposed that changes in CNS processing can also influence the pain of diabetic neuropathy. Potential CNS events affecting neuropathic pain include microglial activation in the spinal cord (66), reduced thalamic activity and communication with cortex (67), loss of gray matter in the thalamus and somatosensory cortex (68), and impaired descending modulation (69).

Intriguingly, we found that repeated treatment with ivabradine provided superior analgesia compared to single administration. Although the mechanism underlying this cumulative effect is not clear, it has been documented before (70) and may indicate the existence of a long-lived ivabradine metabolite, similar to the morphine metabolite morphine-6-glucuronide (M6G), which is 10-fold more potent than morphine itself (71). Ivabradine is already used clinically to treat angina pectoris via inhibition of HCN-dependent firing in cardiac pacemaker cells, which slows heart rate and reduces the oxygen demand of cardiac muscle. The established clinical experience and available pharmacological profiling means that ivabradine could be swiftly assessed in PDN trials. One consideration will be the bradycardic effect due to HCN4 inhibition in the sinoatrial node; nevertheless, a previous study of ivabradine for diabetes-associated coronary artery disease showed a good safety profile with no notable bradycardia, visual disturbances, or adverse effects on glucose metabolism (72). In addition, we demonstrate here that targeted genetic deletion of HCN2 in the periphery gives effective pain relief without detectable side effects. Therefore, the

discovery of CNS-excluded and HCN2-selective inhibitors is likely to provide marked analgesia in PDN, together with a safe pharmacological profile.

HCN2 channels are closed and generate no inward current over the normal range of the action potential, because their voltage range of activation is negative under non-pathological conditions (9). An important consequence of that is that blockade of HCN2 channels has no effect on baseline neuronal excitability, nor does it modulate pain thresholds under normal conditions. This is in contrast to the effects of blocking Na_v channels with local anesthetic, which produces total insensitivity to pain, or of human genetic deletion of Na_v1.7, which causes complete analgesia even under normal conditions, with consequent self-injury (73). We propose here that in pathological pain states, including PDN, HCN2 channels are recruited as initiators of spontaneous activity only when the voltage range of activation undergoes a positive shift after interaction of inflammatory mediators with G_s-coupled receptors and a consequent elevation of intracellular cAMP. Thus, HCN2 channels are an ideal analgesic target, because their blockade has an analgesic effect only under pathological conditions and should have no effect on normal nociception.

Materials and Methods

Study design

The main objective of this study was to test the effect of pharmacological or genetic inhibition of HCN2 on pain in diabetic mice. For behavioral assessment, animals were randomly chosen from multiple cages, provided they satisfied the inclusion criteria outlined in detail in the 'diabetes models' section below. Sample sizes for animal behavior, histology, biochemistry, and electrophysiology were chosen based on previous experience with these assays as the minimum number of independent observations expected to achieve statistical significance. All behavioral testing was conducted during the day in a quiet temperature-controlled room by an experimenter blinded to the identity of drug treatment and/or mouse genotype.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM), and group sizes are noted in each figure legend. All replicates refer to biological replicates. Statistical significance was determined with Prism (GraphPad Software) or SigmaPlot (Systat Software Inc.) using unpaired, equal variance, two-tailed Student's *t*-test, linear regression analysis, chi-square test, one-way analysis of variance (ANOVA), two-way ANOVA, or two-way ANOVA with repeated measures (RM) and Student–Newman–Keuls or Bonferroni tests (for post hoc analysis), as indicated in the corresponding figure legends. Significance is indicated by: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. A *p*-value of $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Blocking HCN2 ion channel activity in peripheral nociceptive neurons alleviates the pain hypersensitivity associated with diabetic neuropathy.

Figure 1. Ivabradine is analgesic in a mouse model of Type 1 diabetes

(A) Time course of blood glucose concentrations after a single injection of vehicle (VEH, blue) or STZ (red, 150 mg/kg, i.p.) in mice. BL: pre-injection baseline blood glucose. *** $p < 0.001$ vs BL; VEH, $n = 6$ mice; STZ, $n = 11$ at BL and $n = 9$ at 52 days, due to STZ-induced weight loss over the experimental time course (see Table 1). One-way repeated measurements (RM) ANOVA followed by Student–Newman–Keuls (SNK) post hoc; *** $P < 0.001$ vs BL; VEH, $n = 6$ mice; STZ, $n = 11$ at BL and $n = 9$ at 52 days, due to STZ-induced lethality over the experimental time-course. (B) Time course of sensitivity to mechanical stimulation, shown as the von Frey force threshold for withdrawal, in VEH or STZ treated mice; * $P < 0.05$, ** $P < 0.01$ vs baseline; VEH, $n = 6$; STZ, $n = 18$ (BL), $n = 12$ (8 weeks). (C) Time course of the thermal withdrawal latency in VEH or STZ treated mice; ** $P < 0.01$ vs vehicle; VEH, $n = 12$; STZ, $n = 13$ (baseline), $n = 10$ (8 weeks). (D) Time course of sensitivity to mechanical stimulation, shown as the von Frey force threshold for withdrawal in control (WT) or diabetic (STZ-injected) mice treated with different doses of ivabradine (IVA) or with vehicle. Dotted lines show time of injection of STZ and IVA; * $P < 0.05$ vs saline; # $P < 0.05$ vs post-STZ; +++ $P < 0.001$ vs baseline; 10mg/kg, $n = 8$; 2.5mg/kg, $n = 8$; 5mg/ml, $n = 6$; saline, $n = 14$ mice. (E) Effect of repeated injection of 5 mg/kg IVA on the von Frey force threshold in STZ mice. Dotted lines show the time of STZ (red) and IVA (blue) injection. * $P < 0.05$, *** $P < 0.001$ vs saline; ## $P < 0.01$, ### $P < 0.001$ vs post-STZ; +++ $P < 0.001$ vs BL; $n = 6$ /group. (F) Effect of two daily injections of 5 mg/kg IVA for 4 days on the von Frey force threshold in STZ mice. Dotted lines show the time of injection of STZ (red) and IVA (blue). IVA was injected twice daily over 4 days, 3 hours before and 3 hours after von Frey threshold testing. * $P < 0.05$, ** $P < 0.01$ vs saline; ## $P < 0.01$, ### $P < 0.001$ vs post-STZ; +++ $P < 0.001$ vs BL; $n = 6$ /group. Statistical analysis was performed using two-way repeated measures ANOVA followed by Student–Newman–Keuls post hoc tests.

Figure 2. Ivabradine is analgesic in a mouse model of Type 2 diabetes

(A) Blood glucose concentration in mice heterozygous (db/+, controls) and homozygous (db/db, model of type 2 diabetes) for the db mutation. Student's t-test; *** $P < 0.001$; db/db ($n = 12$ mice), db/+ ($n = 6$ mice). (B) Von Frey force threshold in control (db/+) or diabetic (db/db) mice; Student's t-test; *** $P < 0.001$; $n = 6$ mice/group. (C) Effect of 5 mg/kg IVA or vehicle i.p. injection on the von Frey force threshold in db/db mice. Dotted line shows the von Frey force threshold in control (db/+) mice. Two-way ANOVA followed by Bonferroni; *** $P < 0.001$ vs saline, $n = 14$ mice/group except 120 min, where $n = 6$ mice/group.

Figure 3. Diabetes-induced C-FOS expression in spinal dorsal horn neurons is reduced by ivabradine

(A) Representative images of the immunohistochemical analysis showing NeuN (left, red) and C-FOS (right, green) expression in spinal dorsal horn neurons in WT vehicle, STZ vehicle and STZ+IVA injected mice. Inset, higher magnification of dashed rectangles. Scale bars, 50 μm. Arrows indicate cells co-expressing NeuN and C-FOS. (B) Quantification of C-FOS-positive neurons in the three different experimental conditions depicted in (A). One-way ANOVA followed by Student–Newman–Keuls post hoc tests; *** $P < 0.001$; control, $n = 4$; STZ, $n = 3$; STZ+IVA, $n = 3$ mice.

Figure 4. HCN2 deletion from nociceptive neurons prevents development of pain and C-FOS induction in diabetic mice

(A) Blood glucose concentration in mice in which HCN2 has been genetically deleted in $\text{Na}_v1.8$ -expressing sensory neurons (HCN2 cKO), as well as in their littermates (fHCN2^{+/+}, effectively WT, used as control). Two-way repeated measures ANOVA followed by Student–Newman–Keuls post hoc test; *** $P < 0.001$ vs BL; $P > 0.05$ for fHCN2 vs HCN2 cKO; HCN2 cKO, $n = 12$; fHCN2, $n = 20$ mice. (B) Time course of the von Frey force threshold in fHCN2 and HCN2 cKO mice after STZ injection (indicated by dotted line). Two-way repeated measures ANOVA followed by Student–Newman–Keuls post hoc test; * $P < 0.05$, *** $P < 0.001$ vs fHCN2, ### $P < 0.05$ vs baseline; HCN2 cKO, $n = 17$ (baseline), $n = 10$ (8 weeks) mice; fHCN2, $n = 21$ (baseline), $n = 10$ (8 weeks) mice. (C) Representative immunohistochemical images showing NeuN (left, red) and C-FOS (right, green) expression in spinal dorsal horn neurons 8 weeks after STZ in fHCN2 and HCN2 cKO. The graph on the right shows quantification of C-FOS-positive neurons in fHCN2 and HCN2 cKO STZ-treated animals.

Student's t-test; *** $P < 0.001$; $n = 3$ mice per group. Inset, higher magnification of dashed rectangle. Scale bar, 50 μm ; arrows show neurons co-expressing NeuN and C-FOS.

Figure 5. Diabetes decreases intra-epidermal nerve fiber (IENF) density in the skin of the hindpaw in mice

(A) Representative skin section from a control mouse stained for PGP9.5 (marker of nerve fibers), collagen IV (marker of basement membrane separating dermis from epidermis), and DAPI (marker of cell nuclei). Merged signal is shown in the far-right panel. Multiple free nerve endings crossing into the epidermis are visible in the merged image (arrows). (B) Top, representative immunohistochemical images of skin innervation in WT control, WT diabetic (WT STZ), fHCN2^{+/+} (effectively WT) diabetic (fHCN STZ), and Na_v1.8-Cre/HCN2 KO (HCN2 cKO) mice. Bottom, quantification of IENF density. One-way ANOVA followed by Student–Newman–Keuls; * $P < 0.05$; $n = 3$ mice per group except HCN2 cKO STZ where $n = 6$. (C) Top, representative immunohistochemical skin images showing CGRP, collagen IV, and DAPI staining. Bottom left, quantification of CGRP⁺ fiber density in control and STZ mice. Student's t-test; ** $P < 0.01$; control, $n = 6$; STZ, $n = 3$ mice. Bottom right, correlation of CGRP⁺ fiber density and threshold for noxious heat in diabetic mice. Pearson's correlation test; ** $P < 0.01$; $n = 6$ mice. Scale bars, 40 μ m.

Figure 6. HCN2 expression in sensory neurons is not regulated by diabetes

(A) Representative immunohistochemical staining for the pan-neuronal marker β3tubulin (green), HCN2 (red), and the merged signal in transverse sections of lumbar DRG from control or diabetic (STZ) WT mice. Examples of HCN2-positive neurons are indicated by arrows. Scale bar = 40 μm. (B) Left, percentage of HCN2⁺ neurons in control and STZ mice (Student's t-test). Middle, distribution of HCN2⁺ neurons amongst DRG size classes (chi-square test). Right, intensity of HCN2 immunoreactivity as a function of neuronal size (linear regression analysis comparing slopes). Control (*n*=4 mice), STZ (*n*=4 mice). (C)

Representative immunohistochemical staining in transverse sections of lumbar DRG for $\text{Na}_v1.8^+$ (marker of small nociceptive DRG neurons, green), HCN2 (red), and the merged signal in WT, HCN2 cKO, and HCN2 gKO. Examples of $\text{Na}_v1.8^+$ neurons are indicated by arrows. Scale bar, 40 μm .

Figure 7. Diabetes does not affect electrical properties of isolated nociceptive neurons
 (A, B) Action potential firing frequency as a function of injected current (0-50 pA) in small DRG neurons (<20 μ m diameter) from STZ (A) and non-diabetic (B) mice ($n=5$ mice/group), in control conditions (control, ■ $n=36$; STZ, ▲ $n=29$) or exposed to 50 μ M forskolin (control, ● $n=16$; STZ, ▼ $n=16$). Only neurons showing a tonic type firing were analyzed (see panel C). Student's t-test after combining the numbers of spikes/s for each neuron at 40 and 50 pA current injection; *** $P<0.001$ for (A) and ** $P<0.01$ for (B). (C) Examples of the two firing patterns observed in small DRG neurons after 10 (left) and 50

(right) pA current injection. Cells showing only one spike or transient firing of a few spikes after 50 pA current injection were classified as strongly adapting or phasic (upper traces), whereas those showing a maintained train of action potentials were classified as tonic (lower traces). **(D)** Proportions of small DRG neurons from STZ and non-diabetic mice, classified as in C, in control conditions and after increase in cAMP by forskolin (50 μ M). Forskolin increased the ratio of tonic/phasic firing neurons (control, $P=0.043$; STZ, $P=0.081$), with no significant difference between the two groups (chi-squared test; $P>0.05$; cell numbers noted above graphs). **(E)** Maximum current densities in small DRG neurons from STZ and control mice, recorded using a hyperpolarizing voltage step from -60 mV to -140 mV for 1.5 seconds, without and with application of 50 μ M forskolin. One-way ANOVA; $P>0.05$; STZ, $n=39$; control, $n=37$ cells. **(F)** I_h activation curves in small DRG neurons. Left panel, curves from STZ mice in the absence (■, solid line, $n=32$) or presence (●, dotted line, $n=15$) of 50 μ mol/L forskolin. Right panel, curves from non-diabetic mice without (▲, solid line, $n=23$) or with (▼, dashed line, $n=16$) forskolin. A significant shift to a more depolarized voltage is observed in both cases (two-way ANOVA followed by Bonferroni; ** $P<0.001$) but there is no difference between STZ and control mice (two-way ANOVA; $P>0.05$).

Figure 8. Pain in diabetes is associated with increased cAMP in DRG

(A) Bar graph showing cAMP concentration in lumbar DRG in control (non-diabetic, $n=6$), STZ mice with no mechanical hyperalgesia ('STZ no pain', $n=5$) and STZ mice with mechanical pain hypersensitivity ('STZ pain', $n=4$). One-way ANOVA followed by Student–Newman–Keuls; $*P<0.05$. (B) Effect of systemic treatment with IVA or different doses of PKA inhibitor H-89 on von Frey threshold ($P<0.05$ IVA vs H-89 at any dose). $n=12$ /group; two-way RM ANOVA followed by Student–Newman–Keuls; $*P<0.05$; $**P<0.01$, H-89 5 mg/kg or IVA vs post-STZ; $##P<0.01$; $###P<0.001$ IVA vs saline; $+++P<0.001$ vs BL.

Table 1

WT mouse usage and percentages that developed diabetes and painful diabetic neuropathy

	Injected with STZ	Developed diabetes	Culled before 8 weeks	Pain hypersensitivity at 8 weeks
Cohort 1	18	15/18 (83%)	2	9/13 (69%)
Cohort 2	18	17/18 (94%)	0	13/17 (76%)
Cohort 3	20	16/20 (80%)	1	13/15 (87%)
Cohort 4	20	19/20 (95%)	0	16/19 (84%)
Total	76	67/76 (88%)	3	51/64 (80%)