University of Dundee

Inhibition of the kinase WNK1/HSN2 ameliorates neuropathic pain by restoring GABA inhibition

Kahle, Kristopher T; Schmouth, Jean-François; Lavastre, Valérie; Latremoliere, Alban; Zhang, Jinwei; Andrews, Nick; Omura, Takao; Laganière, Janet; Rochefort, Daniel; Hince, Pascale; Castonguay, Geneviève; Gaudet, Rébecca; Mapplebeck, Josiane C S; Sotocinal, Susana G; Duan, JingJing; Ward, Catherine; Khanna, Arjun R; Mogil, Jeffrey S; Dion, Patrick A; Woolf, Clifford J; Inquimbert, Perrine; Rouleau, Guy A

Research Article

One-sentence summary: Mice lacking the HSN2 form of the WNK1 kinase are protected from neuropathic pain due to nerve injury.

Overline (20 characters max): Pain

Rotator title: "WNK"ing out pain
Rotator teaser: Blocking the HSN2 form of the kinase WNK1 reduces neuropathic pain

Editor's Summary:
"WNK"ing out pain

Mutations in the HSN2 exon present in the nervous system specific isoform of the serine-threonine kinase WNK1 cause a Mendelian form of congenital insensitivity to pain, termed hereditary sensory and autonomic neuropathy type IIA (HSANII). HSANII affects the peripheral and spinal nerves and results in loss of touch, temperature, and pain sensation. Kahle *et al.* generated transgenic mice specifically lacking this alternatively-spliced variant of WNK1, which is highly expressed in the spinal cord's dorsal horn, the gateway for pain processing from the periphery to the brainstem. These mice exhibited no gross neurological defects and did not exhibit symptoms of HSANII, likely because mutations in HSANII patients generate a truncated form of the kinase retaining an intact catalytic domain. The HSN2-deficient mice were protected from pain hypersensitivity in a model of neuropathic pain resulting from peripheral nerve injury, but not in an inflammatory pain model. Mechanistically, HSN2-deficient mice had less maladaptive inhibitory phosphorylation of the K^+-Cl^- cotransporter KCC2 in spinal cord, thereby resetting of the inhibitory response to GABA. Thus, by alleviating GABA "disinhibition", a known major contributor to neuropathic pain, drugs that inhibit HSN2 might be able to reduce injury-induced neuropathic pain, a current unmet need for many patients.

WNK1/HSN2 kinase inhibition ameliorates neuropathic pain by restoring GABA inhibition

Kristopher T. Kahle[1,2][†][*][#], Jean-François Schmouth[3,4][†], Valérie Lavastre[3,4][†], Alban Latremoliere[5], Jinwei Zhang[6], Nick Andrews[5], Janet Laganière[3,4], Daniel Rochefort[3,4], Pascale Hince[3], Geneviève Castonguay[3], Rébecca Gaudet[3], Josiane C.S. Mapplebeck[7], Susana G. Sotocinal[7], JingJing Duan[1], Catherine Ward[5], Arjun R. Khanna[2], Jeffrey S. Mogil[7], Patrick A. Dion[3,4], Clifford J. Woolf[5], Perrine Inquimbert[8], and Guy A. Rouleau[3,4][*]

Affiliations:

[1] Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston (MA) 02115, USA.

[2] Department of Neurosurgery, Boston Children's Hospital, Boston (MA), 02124 USA

[3] Montreal Neurological Institute and Hospital, McGill University, Montréal (QC), H3A 2B4, Canada.

[4] Department of Neurology and Neurosurgery, McGill University, Montréal (QC), Canada.

[5] F.M. Kirby Neurobiology Center, Boston Children's Hospital and Department of Neurobiology, Harvard Medical School, Boston (MA), 02115, USA.

[6] MRC Protein Phosphorylation and Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland.

[7] Department of Psychology and Centre for Research on Pain, McGill University, Montréal (QC), H3A 0G1, Canada.

[8] Institut des Neurosciences Cellulaires et Integratives, UPR 3212 Centre National de la Recherche Scientifique, Strasbourg, France; Universite de Strasbourg, 5 rue Blaise Pascal, F-67084, Strasbourg, France

*To whom correspondence should be addressed:

Guy A. Rouleau M.D., Ph.D., F.R.C.P.C.

Phone: +1-514-398-2690

Fax: +1-514-398-8248

E-mail: guy.rouleau@mcgill.ca

Kristopher T. Kahle M.D., Ph.D.

Phone: +1-203-737-2096

Fax: +1-203-785-2044

E-mail: kristopher.kahle@yale.edu

† These authors contributed equally to this work.

#Current affiliation:

Departments of Neurosurgery, Pediatrics, and Cellular and Molecular Physiology, Yale School of Medicine, New Haven (CT) 06511 USA

Abstract

HSN2 is a nervous system-predominant exon of of the WNK1 kinase (HSN2-containing splice variant referred to as "WNK1/HSN2") and is mutated in an autosomal recessive, inherited form of congenital pain insensitivity. We created a knockout mouse specifically lacking the HSN2 exon of WNK1. Although these mice had normal spinal neuron and peripheral sensory neuron morphology and distribution, the mice were less susceptible to hypersensitivity to cold and mechanical stimuli after peripheral nerve injury. In contrast, thermal and mechanical nociceptive responses were similar to control mice in an inflammation-induced pain model. In the nerve injury model of neuropathic pain, WNK1/HSN2 contributed to a maladaptive decrease in the activity of the K^+-Cl^- cotransporter KCC2 by increasing its inhibitory phosphorylation at Thr^{906} and Thr^{1007}, resulting in an associated loss of GABA-mediated inhibition of spinal pain-transmitting nerves. Electrophysiological analysis showed that WNK1/HSN2 shifted the concentration of Cl^- such that GABA signaling resulted in depolarization (increased neuronal activity) rather than hyperpolarization (decreased neuronal activity) in mouse spinal nerves. Consistent with the results in the WNK1/HSN2-deficient mice, pharmacologically antagonizing WNK1/HSN2 normalized pathological GABA depolarization of injured spinal cord lamina II neurons. These data provide mechanistic insight into, and a compelling therapeutic target for, neuropathic pain after nerve injury.

Introduction

Chronic neuropathic pain after peripheral nerve injury is common, debilitating, often resistant to treatment, and an economic burden on society (*1*). Novel therapeutic strategies and druggable targets based on disease pathogenesis are needed. One contributor to neuropathic pain after nerve injury is the central disinhibition of $GABA_A$ receptor-dependent spinal nociceptive pathways (*2*) that can result from loss of function of the K^+-Cl^- cotransporter KCC2 (encoded by *SLC12A5*), which leads to Cl^- accumulation in dorsal horn (DH) postsynaptic neurons (*3*). A compelling potential analgesic strategy is to restore ionotropic GABAergic inhibition by enhancing KCC2 activity in DH neurons (*4*). However, the specific mechanisms responsible for the injury-induced impairment of KCC2-dependent Cl^- extrusion are poorly understood, and pharmacologic KCC2 activators are only beginning to be discovered (*4*).

The study of rare monogenic diseases can identify key regulatory genes in complex pathways, and these genes may also be relevant to more common forms of diseases with similar symptoms. Hereditary sensory and autonomic neuropathy type IIA (HSANII; OMIM #201300) is an autosomal recessive ulcero-mutilating neuropathy characterized by the progressive reduction of pain, temperature, and touch sensation, and neurodegeneration in DH and dorsal root ganglia (DRG) neurons of the spinal cord and neurodegeneration of peripheral nerves (*5*). *PRKWNK1*, encoding the kinase WNK1, is alternatively spliced with one isoform containing a nervous system-specific exon called *HSN2* (*6*). Mutations in the *HSN2* exon cause HSANII (*6*). Intronic deletions in *PRKWNK1*, which cause overexpression of a *WNK1* isoform lacking the *HSN2*-specific exon in the kidney, result in pseudohypoaldosteronism type 2C (PHA2C; OMIM #614492) (*7*), an autosomal dominant form of Cl^--sensitive hypertension resulting from WNK1-dependent constitutive phosphorylation and activation of the Na^+, Cl^- cotransporter NCC, a renal-specific cation-Cl^- cotransporter (CCC) related to KCC2 (*8*).

The HSN2 variant of WNK1 (referred to hereafter as WNK1/HSN2) is highly expressed in the DH, DRG, and peripheral nerves (*9*), but the normal function, downstream targets, and pathogenic mechanism by which mutations in WNK1/HSN2 cause disease are unknown. WNK1-dependent inhibitory phosphorylation of KCC2 maintains the depolarizing action of GABA in the developing mouse brain (*10*). In immature neurons, WNK1 inhibition triggers a hyperpolarizing shift in GABA activity by reducing KCC2 Thr^{906} and Thr^{1007} phosphorylation and enhancing KCC2-mediated Cl^- extrusion. However, whether WNK1/HSN2 regulates KCC2 in the spinal cord is unknown. To begin to elucidate the role of WNK1/HSN2 *in vivo*, we generated a *Wnk1/Hsn2*-knockout mouse and investigated the development of neuropathic pain in the spared nerve injury (SNI) pain model and the complete Freund's adjuvant (CFA) model of inflammatory pain.

Results

Wnk1$^{\Delta Hsn2/\Delta Hsn2}$ mice have normal spinal and sensory nerve morphology and no gross neurological deficits

We utilized Cre recombinase technology to generate a knockout mouse model of the *Wnk1*/*Hsn2* isoform by specifically targeting the *Hsn2* exon (**Figure 1A**). Homozygous animals with the *Wnk1/Hsn2*-floxed allele (*Wnk1$^{flox/flox}$*) were crossed to the pCX-NLS-Cre general deletor strain, and the resulting heterozygote animals (*Wnk1$^{\Delta Hsn2/+}$*) were crossed together to generate both mutant (*Wnk1$^{\Delta Hsn2/\Delta Hsn2}$*) and wild-type (*Wnk1$^{+/+}$*) animals. This resulted in specific depletion of the *Wnk1*/*Hsn2* isoform, as revealed by the lack of *Wnk1*/*Hsn2* transcripts in *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* but not *Wnk1$^{+/+}$* mice **(Fig. 1B)**. The HSN2 form of WNK1 can be detected by Western blotting with an isoform-specific antibody, which showed that HSN2 was lacking from knockout mouse cerebrum, cerebellum, spinal cord, and liver (**Fig. 1B).** *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* mice exhibited no gross anatomical abnormalities, including ulcerative mutilations in either upper or lower limbs, after up to 80 weeks of observation. Histological examination of small and large nerve fibers in lumbar (L4) dorsal and ventral spinal roots and of sural sensory nerves revealed normal axonal distribution and morphology in *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* mice (**Fig. 1C and Table 1 and Fig S1**).

Wnk1$^{\Delta Hsn2/\Delta Hsn2}$ mice performed similarly to *Wnk1$^{+/+}$* mice in the SHIRPA protocol, a standardized, 3-stage battery of tests of general behavioral and neurological function (*11*), the elevated plus maze and light dark box test (assessing fear of new places and anxiety), and the hole board test (assessing spontaneous motor activity and exploration) (**Table 2)**. To monitor nociceptive responses, we measured sensitivity to hot and cold using multiple assays, tactile sensitivity to pressure, and sensitivity to chemical irritants or inflammatory stimuli. *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* male mice exhibited a significantly increased latency to tail withdrawal at both 47°C and 49°C (**Fig. 1D**), but otherwise *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* mice exhibited similar response thresholds compared to *Wnk1$^{+/+}$* mice in tests assessing noxious thermal and mechanical sensitivity (**Table 2**). Global deletion of *Wnk1/Hsn2* in mice, therefore, did not phenocopy the sensory neuropathy seen in HSANII patients, which is due to truncating mutations that leave the N-terminal kinase domain intact (*6*). Indeed, *Wnk1/Hsn2* knockout produced no significant

neurological effects in these assays other of a mild, male-specific reduction in distal thermal sensitivity.

Wnk1/Hsn2-knockout reduces pain hypersensitivity associated with nerve injury

Given the HSANII phenotype and localization of WNK1/HSN2 (*6, 9*), we hypothesized that WNK1/HSN2 had a role in chronic pain hypersensitivity after nerve injury or in response to inflammation. We compared the neurobehavioral responses of $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ and $Wnk1^{+/+}$ mice in two different pain models. We used the spared nerve injury (SNI) model of chronic neuropathic pain by ligating the tibial and common peroneal nerves (*12*), and the Complete Freund's Adjuvant (CFA) model of inflammatory pain by injecting CFA into the hind paw to trigger inflammation. To assess sensitivity to cold, we measured the duration of hind paw flicking in response to the sensation induced by the application and evaporation of acetone to the foot pad; to assess the sensitivity to heat, we measured the time to move the paw away from a high energy beam of light (the Hargreaves assay); and to assess sensitivity to mechanical stimuli, we used the von Frey assay of poking the paw with a filament and recording the force required to elicit a withdrawal response. Because SNI animals do not show heat allodynia (*12*), we compared the sensitivity to cold in the SNI pain model, sensitivity to heat in the CFA pain model, and mechanical allodynia in both models. In the SNI pain model, $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice at both early and delayed time points (up to 21 days) exhibited significantly attenuated responses to the evaporation of acetone and pressure measured with von Frey filaments, indicating decreased cold hyperalgesia and mechanical allodynia, respectively (**Fig. 1D**). In contrast, in the CFA pain model, $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice performed similarly to $Wnk1^{+/+}$ mice in tests measuring mechanical (von Frey) and thermal (Hargreaves) nociception (**Table 3**). Therefore, *Wnk1/Hsn2* knockout reduced pain hypersensitivity associated with nerve injury but not hypersensitivity resulting from inflammation.

Knockout of Wnk/Hsn2 prevents SNI-induced inhibitory phosphorylation of KCC2

The WNK family of kinases stimulate the downstream SPS1-related proline/alanine-rich kinase (SPAK) or the SPAK analogue, oxidative stress responsive kinase-1 (OSR1), to regulate the CCCs (*13*), stimulating the Na^+, K^+, Cl^- cotransporter NKCC1 and inhibiting KCC2, through phosphorylation at multiple residues in a homologous motif in each transporter (NKCC1$^{Thr202/Thr207/Thr212}$; KCC2$^{Thr906/Thr1007}$) (*14, 15*). Dephosphorylation of KCC2 at these sites

significantly stimulates KCC2 activity (*14, 15*), such that activation of the GABA$_A$ Cl$^-$-conducting channel elicits hyperpolarization even in neurons with extremely negative resting membrane potentials (*16*). To determine if lack of WNK1-dependent phosphorylation of CCCs contributed to the decreased pain hypersensitivity in the *Wnk1*$^{ΔHsn2/ΔHsn2}$ mice, we examined the phosphorylation status of NKCC1$^{Thr202/Thr207/Thr212}$ and KCC2$^{Thr906/Thr1007}$ in spinal cords from naïve and SNI-subjected mice (**Fig. 2A and 2B**). We observed a low amount of phosphorylation of KCC2 at Thr906 and a higher amount of phosphorylation at Thr1007 in the *Wnk1*$^{+/+}$ animals under control conditions, and the phosphorylation at these two sites in the naïve *Wnk1*$^{ΔHsn2/ΔHsn2}$ mice was significantly less than that of the naïve *Wnk1*$^{+/+}$ mice ($P < 0.001$). SNI significantly increased phosphorylation at both sites in KCC2 in mice of both genotypes; however, the increase in the phosphorylation status in the *Wnk1*$^{ΔHsn2/ΔHsn2}$ mice resulted in an amount of phosphorylation that was similar to those seen in naïve *Wnk1*$^{+/+}$ mice.

Interestingly, in the *Wnk1*$^{+/+}$ mice the amount of WNK1/HSN2 was also increased by SNI. In contrast, phosphorylation of NKCC1$^{Thr202/Thr207/Thr212}$ and the abundance of SPAK and OSR1 were unchanged by WNK1/HSN2 knockout in either naïve mice or after SNI (**Fig. 2A and 2B**). We did not detect differences under any condition in the phosphorylation of SPAK at Ser373, a WNK phosphorylation target in the non-catalyitic C-terminal domain with an unclear role in SPAK regulation (*17*), indicating that the degree of SPAK Ser373 phosphorylation is independent of WNK1/HSN2 abundance. We quantified the amount of the kinase ERK1 as a negative control, because this kinase has not been implicated as a regulator of KCC2 or as a component of the WNK pathway.

Genetic knockout or pharmacologic inhibition of Wnk1/Hsn2 restores SNI-induced GABA-mediated depolarization in lamina II neurons

Impaired KCC2-dependent Cl$^-$ extrusion results in depolarizing GABA-evoked responses in rat spinal nociceptive pathways after SNI (*3, 4*). We performed electrophysiology of lamina II neurons in spinal cord slices derived from naïve mice, sham-operated controls, or those subjected to the SNI pain model in the presence of vehicle or STOCK1S-50699 (*18*), a specific inhibitor of WNK signaling that activates the KCCs by reducing KCC inhibitory phosphorylation (*15*) (**Fig. 2C-2E**). To detect the activity of KCC2, we delivered Cl$^-$ through the patch pipette and measured the response of the neurons to GABA. If KCC2 is active, the excess Cl$^-$ is extruded and GABA

promotes hyperpolarization; if KCC2 activity is inhibited, the intracellular Cl⁻ remains high and the apparent reversal potential for the GABA-induced current (E_{GABA}) shifts to a less negative value. Thus, by measuring the apparent E_{GABA} under these Cl⁻ challenge conditions, we can indirectly monitor KCC2 activity (*19*). We found that E_{GABA} in lamina II neurons from SNI mice was significantly less negative than in lamina II neurons from naïve mice or sham-operated controls. In mice subjected to SNI, E_{GABA} was restored to more negative values by slice incubation with STOCK1S-50699 for 30 to 45 minutes prior to recording, reflecting an increase in neuronal Cl⁻ extrusion capacity. Similarly, in lamina II neurons derived from $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice, E_{GABA} was not altered after SNI (**Fig. 2F**). STOCK1S-50699 had no significant effect on E_{GABA} in naïve animals (**Fig. 2E**).

Discussion

Here, we report the generation of a *Wnk1/Hsn2*-knockout mouse inspired by the mutation in the HSN2 exon that causes HSANII. Global deletion of *Wnk1/Hsn2* in mice did not produce the phenotype of HSANII patients. Whereas patients with HSANII exhibit a progressive neuropathy characterized by the progressive reduction of pain, temperature, and touch sensation, and neuronal degeneration (*6*), $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice lacked any neurological defects except for a mild male-specific reduced distal thermal sensitivity. The difference between these phenotypes may be due to the nature of HSN2 mutation in either case. The mutations that cause HSANII result in a truncated protein that retains the N-terminal kinase domain (*6*), which may produce a kinase lacking critical regulatory regions and with altered substrate specificity. In contrast, the $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ do not produce the nervous system-specific WNK1/HSN2 isoform, but otherwise produce fully functional WNK1 expressed in other tissues. Thus, this knockout model is useful for exploring the specific functions of this isoform without compromising other WNK1 functions.

Our results suggested that WNK1/HSN2 contributes to a maladaptive decrease in KCC2 activity and loss of GABA-mediated inhibition of neuronal activity that is associated with chronic pain hypersensitivity after nerve injury. Furthermore, our data indicated that the reduction in KCC2 activity in response to nerve injury resulted from an increase in WNK1/HSN2-dependent inhibitory phosphorylation of KCC2 at Thr⁹⁰⁶ and Thr¹⁰⁰⁷. Kinases are highly-druggable molecules that have been successfully targeted in cancer but have been

infrequently targeted for neurological diseases (*20*). The nervous system-specific expression of WNK1/HSN2, its importance for human physiology as evidenced by the diseases associated with its mutation, and the beneficial impact of its genetic inhibition on neuropathic pain behavior without producing other major neurological phenotypes in mice, suggests that WNK1/HSN2 is a compelling therapeutic target worthy of future investigation.

Materials and Methods

Generation of floxed Hsn2 and knockout mice

The *Hsn2*-containing genomic regions and its neighbouring exons (8b, 11) were obtained by PCR amplification of three separate fragments from total genomic DNA of 129S1/SvImJ origin (**table S2**). The three separate fragments were assembled using the SLIC method, and cloned in a modified pDELBOY-3X vector (Addgene plasmid 13440) (*21-24*). The construct included a 5' and 3' homology arms, allowing homologous recombination within the *Wnk1* gene, as well as the *Hsn2* exon flanked with two *loxP* sites. The construct also contained a phosphoglycerate kinase (PGK) promoter-driven neomycin resistance cassette flanked by two *frt* sites within the 5' homology arm that was used as a positive selection marker, and a PGK-driven diphtheria gene used as a negative selection marker that lay downstream of the 3' homology arm (**fig. 1A**). All construct and DNA fragments were sequenced verified at each step and checked for correct orientation prior to electroporation in embryonic stem cells (ESCs).

The targeting vector was electroporated in R1 ($+^{Kitl-SlJ}$) ESCs using a Gene Pulser Xcell electroporator (BioRad, Mississauga, Canada). Briefly, 25 µg of vector DNA were linearized using the unique NotI restriction site and electroporated in 10 to 20 X 10^6 ESCs with the following conditions: ionic strength, low; pulse length, 960 µF; voltage, 250 V at room temperature. The cells were grown in double selection media and proper recombinant clones were first verified by PCR screening (**table S1**). Clones passing this first screening process were further confirmed by Southern blot analyses (**fig. S2**). Using this procedure, one correctly targeted ESC clone was recovered from 300 candidates. The correctly targeted clone was microinjected into C57BL/6J mouse blastocysts from which eight resulting male chimeras from the same clone were obtained that showed germline transmission. Three resulting strains (351, 352, 355) were backcrossed for two to three generations to mice on the C57BL/6J-background (N2-3) and kept for histological and nociception characterization.

Mice were screened for carrying a *loxP*-flanked *Hsn2* allele using PCR genotyping (see table **S1** for primers). The *Hsn2* exon was removed by crossing homozygote animals harboring the *Wnk1/Hsn2*-floxed allele (*Wnk1$^{flox/flox}$*) to animals expressing the Cre recombinase under the activity of the chicken *β*-actin gene (general-deletor, pCX-NLS-Cre animals). The resulting animals contained an intact allele of the *Hsn2* exon and a recombined allele (*Wnk1$^{\Delta Hsn2/+}$*). The cre recombinase transgene was outbred and experimental animals were generated by subsequent mating of two heterozygotes animals for the *Hsn2* recombined allele (X/Y, *Wnk1$^{\Delta Hsn2/+}$*; X/X, *Wnk1$^{\Delta Hsn2/+}$*) that yielded both knockout (*Wnk1$^{\Delta Hsn2/\Delta Hsn2}$*) and wild-type animals (*Wnk1$^{+/+}$*). All mice were maintained on a standard light cycle of 12 hours on and 12 hours off and had food and water *ad libitum*. This study was conducted in compliance with the ethic committees at the Centre Hospitalier de l'Université de Montréal, the McGill University, and was approved by the Boston Children's Hospital Animal Care and Use Committee. All experiments were performed in a blinded fashion in a quiet room (temperature 22±1°C) from 9 AM to 6 PM.

RNA extractions and RT-PCR

RNA extractions from different neuronal and non-neuronal tissues (brain, cerebellum, spinal cord, liver, kidney) of *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* and *Wnk1$^{+/+}$* animals were performed using TRIzol® Reagent (Invitrogen by Life technologies, Cat. No.15596-018). Reverse transcription was performed with 1 μg of total RNA per reaction mix using SuperScriptTM III Reverse Transcriptase (18080-044) (Invitrogen). PCR was performed with 5 μl of first-strand cDNA using primers pairs listed in **table S1** and Taq DNA Polymerase (Qiagen). A thermocycler was used with the following parameters: a nucleic acid denaturation/reverse transcriptase inactivation step (94°C, 4 min) followed by 35 cycles of denaturation (94°C, 1 min) and annealing (55°C, 40 sec) and primer extension (72°C, 50 sec) followed by final extension incubation (72°C, 10 min). For each amplification, an internal control (RNA polymerase) was co-amplified.

Tissue preparation and histological analyses

Mice from the strain 352, between 10 and 13 months of age were anaesthetized with ketamine (100 mg/ml) and xylazine (20 mg/ml) prior to perfusion with saline solution and 3% glutaraldehyde. The dorsal and ventral spinal roots (n=3-4 mice per group) from the lumbar 4 (L4) spinal section, as well as sural nerves (n=5-6 mice per group) were dissected and post-fixed overnight. After several washes, the tissues were osmicated with 2% osmium tetroxide

(Canemco), dehydrated, and embedded with Epon [TAAB 812 resin (45%), Distilled Dodecenyl Succinic Anhydride (DDSA) (35%), nadic methyl anhydride (18%), Tri-(dimethylaminomethyl) phenol (DMP30) (2%)] (Canemco). The tissues were cut (5 µm thick) and mounted on glass slides. Sections were stained with toluidine blue and observed under light microscopy. The number of axons was counted and classified into 1 µm groups for each type of nerves. Axon diameters were calculated at magnification 320 X from surface areas using the particle analysis function in Image J (http://imagej.nih.gov/ij/). The total numbers of axons for each type of nerves were counted, averaging 600, 530, and 2,100 axons for dorsal roots, ventral roots, and sural nerves, respectively.

General behavior phenotyping tests

SHIRPA

The SHIRPA test was performed according to the method described by Rogers *et al*. (1997) (*11*). Briefly, SHIRPA is a 3-stage protocol designed to identify neurologic and behavioral phenotypic abnormalities in mice. The first stage provides a general behavioral observation profile, during which the tester observes undisturbed mice for any abnormal behaviors or movements. The second stage includes a host of behavioral tests that generate quantitative phenotypic data, including tests of locomotor activity, food and water intake, balance and coordination, and analgesia, along with histological and biochemical analysis of tissues sampled from test mice. Finally, the third stage adds more detailed testing based on known or potential modes of neurologic disease, including tests of anxiety, learning and memory, prepulse inhibition, and others.

Elevated plus maze

The elevated plus maze (Noldus, NL) was constructed of black plastic with 2 "open" arms with no walls and 2 "closed" arms with walls (30 × 5 cm) extending out opposite from each other from a central platform (decision zone) to create a "plus" shape. The arms were raised 85 cm above the floor. At the start of a trial, a mouse was placed on the center platform of the maze, facing a closed arm, and allowed to explore the apparatus for 5 minutes. The maze was cleaned between subjects with a weak ethanol solution. A computer-assisted video-tracking system (Ethovision XT 9.0, Noldus, NL) recorded the total time spent exploring the different regions of the maze [open arms, center (decision zone) and closed arms]. The percent time spent in open

arms (defined as when all 4 paws were placed on the open arm) was used as a surrogate measure of exploratory behavior in a novel environment; mice with lower amounts of open arm exploratory activity have greater neophobia (fear of new stimuli).

Light-dark box

The light and dark box was a plastic box divided into two compartments and connected by an aperture in the divider separating the two compartments. One compartment was constructed of white plastic and was 66% of the box and the other compartment was made of black plastic and formed 33% of the box. There was also a lid on top of the black compartment. A computer-assisted video-tracking system (Ethovision XT 9.0, Noldus, NL) recorded the total time spent exploring the different regions of the box. The percent time spent in the white side of the box (defined as when all 4 paws were placed in the white side of the box) was used as a surrogate measure of exploratory behavior in a novel environment; mice with lower amounts of white side exploratory activity have greater neophobia.

Hole board

The hole-board test was used to assess mouse exploratory behavior in a novel environment. The apparatus was an acrylic box with a floor and 4 walls (40 cm × 40 cm × 30 cm). The plastic floor contained 9 holes evenly spaced apart that were 2 cm deep. Mice were placed in the box for 15 minutes. Gross (for example, walking and running) and fine (for example, active grooming but not moving from one position) motor activity pertaining to location in the box and proximity to the 9 holes was recorded through interruption of infrared beams located in the walls of the arena (for horizontal activity and fine movements) and in the holes to measure exploratory head dips.

Nociception tests

All behavioral experiments were performed with handlers blinded to groups. $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ and $Wnk1^{+/+}$ mice from three different strains backcrossed onto the C57BL/6J-background were used for nociception analyses (strain 351, 352, 355) in two different centers (McGill and Boston Children's Hospital). The animals were tested simultaneously over the same day, tested in multiple litters, and assessed in the same cohort of animal (n=7-10 per genotype for each group). All mice were acclimated to their novel environment for 3 months prior to study.

All mice were 5–11 months old when the study began with the exception of mice undergoing SNI, which was performed at 3 months. All nociceptive assays executed in this study have been fully described in detail in the literature (*25-29*). Tests measuring mechanical (von Frey) and thermal (Hargreave's) nociception stimuli were utilized in the CFA model of inflammatory pain as described (*30*). For neuropathic pain, mechanical allodynia (von Frey filaments test) (*31*) and cold hypersensitivity [evaporation of a drop of acetone (*32*)] were assessed before and up to 21 days after peripheral nerve injury (SNI).

Surgeries

SNI surgery was performed under 3% induction/2% maintenance with isoflurane on adult mice. The tibial and common peroneal branches of the sciatic nerve were tightly ligated with a 5.0 silk suture and transected distally, while the sural nerve was left intact as previously described (*12*). After injury, the incision was sutured and mice were allowed to recover on heated pads before being returned to their home cages.

Statistical analyses

Statistical data were analyzed using STATISTICA© 10 (StatSoft, Inc., Tulsa, OK, USA) and SPSS. Data were initially examined using Shapiro-Wilk test for normal distribution. Data that did not fit a normal distribution underwent non-parametric analysis, whereas data that were normally distributed were subjected to parametric analysis. Axon diameter, Hargreaves plantar, hotplate Hargreaves, hot plate, von Frey, and formalin data were not normally distributed and, therefore, underwent non-parametric analyses (Kruskall-Wallis ANOVA). Tail-clip data and tail-withdrawal data were analyzed using factorial ANOVA for genotype and sex effects. All chronic tests (SNI mechanical sensitivity, CFA mechanical and thermal sensitivity) were analyzed using mix model ANOVA. *Post hoc* tests with Bonferroni correction were performed for between and within-subject comparisons when appropriate. All data are reported as mean values ± standard error of the mean (SEM).

Buffers

Buffer A contained 50 mM Tris/HCl, pH7.5 and 0.1mM EGTA. Lysis buffer was 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (w/v) Triton-100, 0.27 M sucrose, 0.1% (v/v)

2-mercaptoethanol and protease inhibitors (complete protease inhibitor cocktail tablets, Roche, 1 tablet per 50 mL). TBS-Tween buffer (TTBS) was Tris/HCl, pH 7.5, 0.15 M NaCl and 0.2% (v/v) Tween-20. SDS sample buffer was 1X NuPAGE LDS sample buffer (Invitrogen), containing 1% (v/v) 2-mercaptoethanol.

Antibodies

Antibodies used for Western Blots included antibodies raised in sheep and affinity-purified on the appropriate antigen by the Division of Signal Transduction Therapy Unit at the University of Dundee: KCC2a total antibody [residues 1-119 of human KCC2a]; KCC3a phospho-Thr^{991} [residues 975-989 of human KCC3a phosphorylated at Thr^{991}, SAYTYER(T)LMMEQRSRR, corresponding to residues of rat KCC2 phosphorylated at Thr^{906}, SAYTYEK(T)LMMEQRSRR]; KCC2a phospho-Thr^{906} [residues 975-989 of human KCC3a phosphorylated at Thr^{991}, SAYTYER(T)LMMEQRSRR]; KCC3a phospho-Thr$^{1039/1048}$ [residues 1032-1046 or 1041-1055 of human KCC3a phosphorylated at Thr$^{1039/1048}$, CYQEKVHM(T)WTKDKYM, corresponding to residues of rat KCC2 phosphorylated at Thr^{1006}, TDPEKVHLTW(T)KDKSV]. NKCC1 total antibody [residues 1-288 of human NKCC1]; NKCC1 phospho-Thr^{203}/Thr^{207}/Thr^{212} antibody [residues 198-217 of human NKCC1 phosphorylated at Thr^{203}, Thr^{207} and Thr^{212}, HYYYD(T)HTN(T)YYLR(T)FGHNT]; SPAK total antibody [full-length GST-tagged human SPAK protein]; SPAK/OSR1 (S-motif) phospho-Ser^{373}/Ser^{325} antibody [367–379 of human SPAK, RRVPGS(S)GHLHKT, which is similar to residues 319–331 of human OSR1 in which the sequence is RRVPGS(S)GRLHKT]; HSN2 total antibody [full-length human WNK1/HSN2 protein]; ERK1 total antibody [full-length human ERK1 protein]. KCC2 total antibody [residues 932-1043 of rat KCC2] was purchased from NeuroMab. Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce. IgG used in control immunoprecipitation experiments was affinity-purified from pre-immune serum using Protein G-Sepharose.

Phosphorylation-specific antibody immunoprecipitations

KCCs phosphorylated at the KCC2 Thr^{906} and Thr^{1007} equivalent residue were immunoprecipitated from clarified spinal cord lysates. The phosphorylation-specific antibody was coupled to protein-G–Sepharose at a ratio of 1 mg of antibody per 1 mL of beads. A total of 2 mg of clarified spinal cord lysate were incubated with 15 µg of antibody conjugated to 15 µL

of protein-G–Sepharose in the presence of 20 µg/mL of the corresponding dephosphorylated peptide dissolved in buffer. Incubation was carried for 2 hours at 4°C with gentle agitation, and the immunoprecipitates were washed three times with 1 mL of lysis buffer containing 0.15 M NaCl and twice with 1 mL of buffer A. Bound proteins were eluted with 1X lithium dodecyl sulfate (LDS) sample buffer.

Immunoblotting

Spinal cord lysates (15 µg) in SDS sample buffer were subjected to electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated for 30 min with Tris-buffered salt solution with Tween (TTBS) containing 5% (w/v) skimmed milk. The membranes were then immunoblotted in 5% (w/v) skimmed milk in TTBS with the indicated primary antibodies overnight at 4°C. Sheep antibodies were used at a concentration of 1-2 µg/ml. The incubation with phosphorylation-specific sheep antibodies was performed with the addition of 10 µg/mL of the dephosphorylated peptide antigen used to raise the antibody. The blots were then washed six times with TTBS and incubated for 1 hour at room temperature with secondary HRP-conjugated antibodies diluted 5000-fold in 5% (w/v) skimmed milk in TTBS. After repeating the washing steps, the signal was detected with the enhanced chemiluminescence reagent. Immunoblots were developed using a film automatic processor (SRX-101; Konica Minolta Medical) and films were scanned with a 600-dpi resolution on a scanner (PowerLook 1000; UMAX). Figures were generated using Photoshop/Illustrator (Adobe).

Electrophysiological recordings in spinal neurons

All electrophysiology experimental procedures were conducted in conformity with the recommendations of the European Union directive on animal experimentation (2012/63/EU). Whole-cell patch-clamp recordings were performed in 300 μm transverse slices of lumbar segment L4 to L6 as previously described (*4, 33, 34*). For experiments involving SNI mice, all recordings were performed in the ipsilateral side of the nerve injury. Recordings were performed at room temperature in standard artificial cerebrospinal fluid (ACSF). Data were filtered at 10 kHz and acquired using pClamp 10 (molecular device). Borosilicate patch pipettes (4-6 MΩ) were filled with (composition in mM): 120 K-methanesulfonate, 25 KCl, 10 HEPES, 2 $MgCl_2$ (pH 7.3). To measure E_{GABA} under a Cl^- challenge, neurons in lamina II were recorded while

GABA (1 mM) was puffed locally for 30 ms. Experimental E_{GABA} was extrapolated from GABA I-V curve relationship. For STOCK1S-50699 (Molport, LV) experiments, slices were incubated in STOCK1S-50699 (10 μM for 30-45 minutes) before recordings.

Supplementary Materials

Fig. S1. Nerve size quantification of the L4 ventral and dorsal spinal roots, and sural sensory nerves, of *Wnk1*$^{+/+}$ and *Wnk1*$^{\Delta Hsn2/\Delta Hsn2}$ mice.

Fig. S2. Southern blot analyses revealed correctly targeted ESC clones using *Wnk1*-specific 5′ and 3′ probes.

Table S1. PCR primers used.

Table S2. Performance of *Wnk1*$^{+/+}$ and *Wnk1*$^{\Delta Hsn2/\Delta Hsn2}$ mice in assays testing general central nervous system function.

Author contributions

KTK, JFS, VL, PI, and GAR conceived and designed the study.

KTK, JFS, VL, AL, JZ, NA, JL, DR, PH, GC, RG, JCSM, SGS, JD, CW, PAD, and PI performed the experiments.

KTK, JFS, VL, JZ, PAD, CJW, PI, and GAR analyzed the data.

KTK, JFS, VL, JZ, ARK, PAD, PI, and GAR prepared the manuscript.

Competing interests

The authors declare they have no competing financial interests.

Data and materials availability

XXXX

Fig. 1. *Wnk1/Hsn2*-knockout in mice results in a mild sex-dependent loss of distal thermal sensitivity and reduces chronic pain hypersensitivity after peripheral nerve injury.

(A) Recombination scheme and **(B)** Western blot analysis confirming that HSN2 is not detected from knockout mouse brain (Br), cerebellum (Cer), spinal cord (SC), and liver (Li). Genomic analysis was performed by RT-PCR with primers specific for between exon 8 and *Hsn2*. WNK1 protein was analyzed in XXXd with an antibody that recognizes the HSN2 isoform specifically.

(C) $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice exhibit normal axonal distribution and morphology. (Left) Histological transverse sections of ventral and dorsal spinal roots of lumbar 4 (L4) as well as sural nerves of 11 month-old $Wnk1^{+/+}$ and $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice (scale = 10 mm). (Right) Quantification of nerve diameter and distribution of nerve fibers in $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice and $Wnk1^{+/+}$ mice is shown. The Kruskal-Wallis H test was performed on 3-6 mice of each genotype. Error bars represent standard error of the mean. See table S1 for statistical analysis. No statistically signficant differences were detected.

(D) $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice exhibit only a mild, sex-dependent loss of distal thermal sensitivity without other apparent neurological deficits. Latency of females and males for tail withdrawal from either 47°C or 49°C water. Tail-withdrawal test was performed on 8 $Wnk1^{+/+}$ and 7 $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ males, and 8 $Wnk1^{+/+}$ and 10 $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ females. Error bars represent the mean ± SEM, **$P < 0.05$, *$P < 0.01$, Two-way ANOVA with Bonferroni post test.

(E) Response to acetone evaporation as a measure of cold allodynia. Duration of paw flicking response to evaporation of acetone was tested on 7-8 mice per group ($Wnk1^{\Delta Hsn2/\Delta Hsn2}$, $Wnk1^{+/+}$).

(F) Response to von Frey stimulation as a measure of mechanical hypersensitivity. Withdrawal threshold to von Frey hairs of varying diameters that apply a range of forces to the paw (measured in grams) was tested on 7-8 mice per group ($Wnk1^{\Delta Hsn2/\Delta Hsn2}$, $Wnk1^{+/+}$). Mixed model ANOVA with Bonferroni post-hoc testing was performed on test results with between factor variable being genotype and within factor variable being time (days). Error bars represent the mean $\pm$ SEM. $*P < 0.01$.

Fig. 2. Antagonizing spinal *Wnk1/Hsn2* kinase signaling decreases maladaptive KCC2 inhibitory phosphorylation and normalizes depolarizing GABA-evoked responses after nerve injury.

(A) Spinal cord homogenates were subjected to Western blot analysis of either immunoprecipitates or lysates with the indicated antibodies. Proteins or phosphorylated proteins recognized by the antibodies that were used for immunoprecipitation are indicated with IP, proteins recognized by antibodies that were used for Western blotting are indicated with IB. The WNK1/HSN2 antibody is specific for this isoform. The Western blots illustrate results obtained in two separate experiments.

(B) Quantification of indicated Western blot data from results shown in A. The quantification (ratio calculation) is based on (phospho KCC2) / (total KCC2), or HSN2/ERK1. Statistical analysis was performed using unpaired t-test (n=3, all values represent the mean $\pm$ SEM, ***, p<0.001; **, p<0.01; *, p<0.05).

(C) Representative currents recorded in spinal cord lamina II neurons from naive and SNI-subjected (1 week) adult mice in response to 30 ms puffs of GABA (1 mM) in the presence of a Cl$^-$ load (29 mM), introduced through the patch pipette. Slices were incubated for 30 to 45 minutes with vehicle control or STOCK 1S-50699 (10 μM) prior to recording.

(D) Current-voltage (IV) relationship for GABA normalized currents ($I/-I_{80mV}$) obtained from naïve mice under control conditions (n=13) or after SNI treatment (1 week), in the presence of vehicle control (n=9) or STOCK1S-50699 (n=14). Conditions as described in C.

(E) Apparent E_{GABA} measured in lamina II spinal cord neurons from naïve mice under control conditions (n=13) or after treatment with STOCK1S-50699 (n=6). Also shown are E_{GABA}

measurements in sham-operated mice (1 week) under control conditions (n=6) or after treatment with STOCK1S-50699 (n=5), and in SNI (1 week) under control conditions (n=9) or after STOCK1S-50699 treatment (n=14). Data are presented as mean ± S.E.M. Two-way analysis of variance (ANOVA), with factors surgery and treatment (Bonferroni **$P < 0.05$, *$P < 0.01$). XX

(**F**) Apparent E_{GABA} measured in lamina II neurons from $Wnk1^{+/+}$ and $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ mice under control or SNI (1 week) conditions. Data are presented as mean ± S.E.M. Unpaired t-test; **$P < 0.05$.

(**G**) Statistical analysis of the differences in KCC2 phosphorylation between $Wnk1^{+/+}$ and $Wnk1^{\Delta Hsn2/\Delta Hsn2}$ under control and SNI conditions and of differences in apparent E_{GABA} between $Wnk1^{+/+}$ mice under control, sham-operated, and SNI conditions in the presence and absence of STOCK1S-50699.

Table 1. Nerve size quantification summary of *Wnk1$^{+/+}$* and *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* mice. Statistical results associated with the nerve size calculation from both *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* and *Wnk1$^{+/+}$* animals are presented. Column one to three present results from the ventral L4, dorsal L4, and sural nerve with their respective *P*-values.

Ventral L4		Dorsal L4		Sural nerve	
Diameters (µm)	*P*-value	Diameters (µm)	*P*-value	Diameters (µm)	*P*-value
0 to 1	1.00	0 to 1	0.59	0 to 1	0.17
1 to 2	0.48	1 to 2	1.00	1 to 2	0.31
2 to 3	0.29	2 to 3	1.00	2 to 3	0.85
3 to 4	0.16	3 to 4	0.72	3 to 4	0.41
4 to 5	0.72	4 to 5	0.72	4 to 5	0.27
5 to 6	0.37	5 to 6	0.72	5 to 6	0.23
6 to 7	0.29	6 to 7	0.86	6 to 7	0.22
7 to 8	0.29	7 to 8	1.00	7 to 8	0.55
>8	0.48	>8	0.37	>8	0.36

Table 2. Performance of *Wnk1$^{+/+}$* and *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* mice in assays testing general central nervous system function. Four of each genotype were assessed in each test.

Test	Metric	*P*-values
SHIRPA	Combined score	0.5039
Elevated plus maze	% time on open arms	0.8394
	Total distance traveled	0.4002
Light and dark box	Time in light side	0.064
Holeboard	Total distance traveled	0.4236
	Holes poked in center	0.5414
	Holes poked in periphery	0.5021

Table 3. Nociception test summary for *Wnk1$^{+/+}$* and *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* mice. Statistical results from seven nociception tests performed on *Wnk1$^{\Delta Hsn2/\Delta Hsn2}$* and *Wnk1$^{+/+}$* animals are presented. CFA nociception tests were performed XX after CFA injection into the hind paw. Differences at day 21 after SNI in nociception tests are shown. NS, nonsignificant

Pain model	Nociception Test	P-values
none	Tail clip	sex and genotype; F (1, 18) = 0.45 P = 0.51
		sex; F (1, 18) = 1.49, P = 0.24
		genotype; F (1, 18) = 0.65, P = 0.43
none	Hargreave	15% female (P = 0.06)
		15% male (P = 0.73)
		20% female (P = 0.31)
		20% male (P = 0.73)
none	Hotplate	50°C female (P = 0.86)
		50°C male (P = 0.35)
		53°C female (P = 0.20)
		53°C male (P = 0.56)
none	von Frey	female (P = 0.71)
		male (P = 0.36)
none	Formalin	early female (P = 0.82)
		early male (P = 1.00)
		late female (P = 0.82)
		late male (P = 0.32)
CFA	von Frey	sex and genotype; F (7, 10) = 1.39 P = 0.33
		sex; F (7, 10) = 1.76, P = 0.23
		genotype; F (7, 10) = 1.23, P = 0.41
CFA	Hargreave	sex and genotype; F (7, 10) = 1.68 P = 0.25
		sex; F (7, 10) = 1.15, P = 0.44
		genotype; F (7, 10) = 1.43, P = 0.33
SNI	acetone	
SNI	Von Frey	

References

1. C. Toth, J. Lander, S. Wiebe, The prevalence and impact of chronic pain with neuropathic pain symptoms in the general population. *Pain Med* **10**, 918-929 (2009).

2. C. A. von Hehn, R. Baron, C. J. Woolf, Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron* **73**, 638-652 (2012).

3. J. A. Coull, D. Boudreau, K. Bachand, S. A. Prescott, F. Nault, A. Sik, P. De Koninck, Y. De Koninck, Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* **424**, 938-942 (2003).

4. M. Gagnon, M. J. Bergeron, G. Lavertu, A. Castonguay, S. Tripathy, R. P. Bonin, J. Perez-Sanchez, D. Boudreau, B. Wang, L. Dumas, I. Valade, K. Bachand, M. Jacob-Wagner, C. Tardif, I. Kianicka, P. Isenring, G. Attardo, J. A. Coull, Y. De Koninck, Chloride extrusion enhancers as novel therapeutics for neurological diseases. *Nat Med* **19**, 1524-1528 (2013).

5. I. Kurth, in *GeneReviews(R),* R. A. Pagon, M. P. Adam, H. H. Ardinger, S. E. Wallace, A. Amemiya, L. J. H. Bean, T. D. Bird, C. R. Dolan, C. T. Fong, R. J. H. Smith, K. Stephens, Eds. (Seattle (WA), 1993).

6. M. Shekarabi, N. Girard, J. B. Riviere, P. Dion, M. Houle, A. Toulouse, R. G. Lafreniere, F. Vercauteren, P. Hince, J. Laganiere, D. Rochefort, L. Faivre, M. Samuels, G. A. Rouleau, Mutations in the nervous system--specific HSN2 exon of WNK1 cause hereditary sensory neuropathy type II. *J Clin Invest* **118**, 2496-2505 (2008).

7. F. H. Wilson, S. Disse-Nicodeme, K. A. Choate, K. Ishikawa, C. Nelson-Williams, I. Desitter, M. Gunel, D. V. Milford, G. W. Lipkin, J. M. Achard, M. P. Feely, B. Dussol, Y. Berland, R. J. Unwin, H. Mayan, D. B. Simon, Z. Farfel, X. Jeunemaitre, R. P. Lifton, Human hypertension caused by mutations in WNK kinases. *Science* **293**, 1107-1112 (2001).

8. K. T. Kahle, A. M. Ring, R. P. Lifton, Molecular physiology of the WNK kinases. *Annu Rev Physiol* **70**, 329-355 (2008).

9. M. Shekarabi, R. G. Lafreniere, R. Gaudet, J. Laganiere, M. M. Marcinkiewicz, P. A. Dion, G. A. Rouleau, Comparative analysis of the expression profile of Wnk1 and Wnk1/Hsn2 splice variants in developing and adult mouse tissues. *PloS one* **8**, e57807 (2013).

10. P. Friedel, K. T. Kahle, J. Zhang, N. Hertz, L. I. Pisella, E. Buhler, F. Schaller, J. Duan, A. R. Khanna, P. N. Bishop, WNK1-regulated inhibitory phosphorylation of the KCC2 cotransporter maintains the depolarizing action of GABA in immature neurons. *Science Signaling* **8**, ra65-ra65 (2015).

11. D. C. Rogers, E. M. Fisher, S. D. Brown, J. Peters, A. J. Hunter, J. E. Martin, Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mammalian genome : official journal of the International Mammalian Genome Society* **8**, 711-713 (1997).

12.   I. Decosterd, C. J. Woolf, Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* **87**, 149-158 (2000).

13.   D. R. Alessi, J. Zhang, A. Khanna, T. Hochdorfer, Y. Shang, K. T. Kahle, The WNK-SPAK/OSR1 pathway: master regulator of cation-chloride cotransporters. *Sci Signal* **7**, re3 (2014).

14.   J. Rinehart, Y. D. Maksimova, J. E. Tanis, K. L. Stone, C. A. Hodson, J. Zhang, M. Risinger, W. Pan, D. Wu, C. M. Colangelo, B. Forbush, C. H. Joiner, E. E. Gulcicek, P. G. Gallagher, R. P. Lifton, Sites of regulated phosphorylation that control K-Cl cotransporter activity. *Cell* **138**, 525-536 (2009).

15.   P. de Los Heros, D. R. Alessi, R. Gourlay, D. G. Campbell, M. Deak, T. J. Macartney, K. T. Kahle, J. Zhang, The WNK-regulated SPAK/OSR1 kinases directly phosphorylate and inhibit the K+-Cl- co-transporters. *The Biochemical journal* **458**, 559-573 (2014).

16.   S. Titz, E. M. Sammler, S. G. Hormuzdi, Could tuning of the inhibitory tone involve graded changes in neuronal chloride transport? *Neuropharmacology* **95**, 321-331 (2015).

17.   Alberto C. Vitari, M. Deak, Nick A. Morrice, Dario R. Alessi, The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. *Biochemical Journal* **391**, 17-24 (2005).

18.   T. Mori, E. Kikuchi, Y. Watanabe, S. Fujii, M. Ishigami-Yuasa, H. Kagechika, E. Sohara, T. Rai, S. Sasaki, S. Uchida, Chemical library screening for WNK signalling inhibitors using fluorescence correlation spectroscopy. *The Biochemical journal* **455**, 339-345 (2013).

19.   W. Jarolimek, A. Lewen, U. Misgeld, A furosemide-sensitive K+-Cl- cotransporter counteracts intracellular Cl- accumulation and depletion in cultured rat midbrain neurons. *J Neurosci* **19**, 4695-4704 (1999).

20.   P. Cohen, D. R. Alessi, Kinase drug discovery--what's next in the field? *ACS Chem Biol* **8**, 96-104 (2013).

21.   D. J. Rossi, A. Londesborough, N. Korsisaari, A. Pihlak, E. Lehtonen, M. Henkemeyer, T. P. Makela, Inability to enter S phase and defective RNA polymerase II CTD phosphorylation in mice lacking Mat1. *Embo J* **20**, 2844-2856 (2001).

22.   N. Korsisaari, D. J. Rossi, A. Paetau, P. Charnay, M. Henkemeyer, T. P. Makela, Conditional ablation of the Mat1 subunit of TFIIH in Schwann cells provides evidence that Mat1 is not required for general transcription. *J Cell Sci* **115**, 4275-4284 (2002).

23.   M. Z. Li, S. J. Elledge, Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nat Methods* **4**, 251-256 (2007).

24.   P. Soriano, The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. *Development* **124**, 2691-2700 (1997).

25.   J. S. Mogil, J. Ritchie, S. G. Sotocinal, S. B. Smith, S. Croteau, D. J. Levitin, A. K. Naumova, Screening for pain phenotypes: analysis of three congenic mouse strains on a battery of nine nociceptive assays. *Pain* **126**, 24-34 (2006).

26.   J. S. Mogil, M. L. Chanda, The case for the inclusion of female subjects in basic science studies of pain. *Pain* **117**, 1-5 (2005).

27.   C. L. Steinmiller, A. M. Young, Pharmacological selectivity of CTAP in a warm water tail-withdrawal antinociception assay in rats. *Psychopharmacology* **195**, 497-507 (2008).

28.   R. Y. Walder, R. Radhakrishnan, L. Loo, L. A. Rasmussen, D. P. Mohapatra, S. P. Wilson, K. A. Sluka, TRPV1 is important for mechanical and heat sensitivity in uninjured animals and development of heat hypersensitivity after muscle inflammation. *Pain* **153**, 1664-1672 (2012).

29.   K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**, 77-88 (1988).

30.   P. Honore, S. D. Rogers, M. J. Schwei, J. L. Salak-Johnson, N. M. Luger, M. C. Sabino, D. R. Clohisy, P. W. Mantyh, Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. *Neuroscience* **98**, 585-598 (2000).

31.   M. Costigan, A. Moss, A. Latremoliere, C. Johnston, M. Verma-Gandhu, T. A. Herbert, L. Barrett, G. J. Brenner, D. Vardeh, C. J. Woolf, M. Fitzgerald, T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. *J Neurosci* **29**, 14415-14422 (2009).

32.   K. J. Kim, Y. W. Yoon, J. M. Chung, Comparison of three rodent neuropathic pain models. *Exp Brain Res* **113**, 200-206 (1997).

33.   P. Inquimbert, K. Bartels, O. B. Babaniyi, L. B. Barrett, I. Tegeder, J. Scholz, Peripheral nerve injury produces a sustained shift in the balance between glutamate release and uptake in the dorsal horn of the spinal cord. *Pain* **153**, 2422-2431 (2012).

34.   M. Cordero-Erausquin, J. A. M. Coull, D. Boudreau, M. Rolland, Y. D. Koninck, Differential Maturation of GABA Action and Anion Reversal Potential in Spinal Lamina I Neurons: Impact of Chloride Extrusion Capacity. *The Journal of Neuroscience* **25**, 9613-9623 (2005).