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WNK1/HSN2 kinase inhibition ameliorates neuropathic pain by reducing maladaptive KCC2 inhibitory phosphorylation and restoring GABA inhibition

Kristopher T. Kahle^{1,2,†,*}, Jean-François Schmouth^{3,4,†}, Valérie Lavastre^{3,4,†}, Alban Latremoliere⁵, Jinwei Zhang⁶, Nick Andrews⁵, Janet Laganière^{3,4}, Daniel Rochefort^{3,4}, Pascale Hince³, Geneviève Castonguay³, Rébecca Gaudet³, Josiane C.S. Mapplebeck⁷, Susana G. Sotocinal⁷, JingJing Duan¹, Catherine Ward⁵, Arjun R. Khanna², Jeffrey S. Mogil⁷, Patrick A. Dion^{3,4}, Clifford J. Woolf⁵, Perrine Inquimbert⁸, and Guy A. Rouleau^{3,4,*}

¹Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston (MA) 02115, USA ²Department of Neurosurgery, Boston Children's Hospital, Boston (MA), 02124 USA ³Montreal Neurological Institute and Hospital, McGill University, Montréal (QC), H3A 2B4, Canada ⁴Department of Neurology and Neurosurgery, McGill University, Montréal (QC), Canada ⁵F.M. Kirby Neurobiology Center, Boston Children's Hospital and Department of Neurobiology, Harvard Medical School, Boston (MA), 02115, USA ⁶MRC Protein Phosphorylation and Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland ⁷Department of Psychology and Centre for Research on Pain, McGill University, Montréal (QC), H3A 0G1, Canada ⁸Institut des Neurosciences Cellulaires et Integratives, UPR 3212 Centre National de la Recherche Scientifique, Strasbourg, France; Université de Strasbourg, 5 rue Blaise Pascal, F-67084, Strasbourg, France

Abstract

WNK1/HSN2 kinase, mutated in a Mendelian form of congenital pain insensitivity, contributes to a maladaptive decrease in KCC2 cotransporter activity and a loss of GABA inhibition in the spared nerve injury (SNI) model of neuropathic pain by increasing KCC2 inhibitory phosphorylation at Thr906/Thr1007. Antagonizing WNK1/HSN2 signaling reduces SNI-induced cold allodynia and mechanical hyperalgesia, decreases up-regulated KCC2 Thr906/Thr1007 phosphorylation, and normalizes pathological GABA depolarizations of injured spinal cord lamina

*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, guy.rouleau@mcgill.ca; Kristopher T. Kahle M.D., Ph.D., Phone: +1-203-737-2096, Fax: +1-203-785-2044, 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

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.

II neurons. These data collectively provide novel 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) resulting from loss of KCC2 cotransporter (*SLC12A5*) function that leads to Cl⁻ accumulation in dorsal horn (DH) post-synaptic neurons (3). A compelling potential analgesic strategy is to restore ionotropic GABAergic inhibition by enhancing dorsal horn KCC2 activity (4). However, the specific mechanisms responsible for the injury-induced impairment of KCC2-dependent Cl⁻-extrusion are poorly understood, and KCC2 activators are only beginning to be discovered (4).

The study of rare monogenic diseases can lead to the unbiased identification of key regulatory genes in complex pathways with relevance for more common forms of disease. 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 the spinal dorsal horn (DH), dorsal root ganglia (DRG), and peripheral nerves (5). Mutations in the alternatively-spliced “*HSN2*” exon 10 of *PRKWNK1*, encoding WNK1 kinase, cause HSANII (6). Interestingly, intronic deletions in the same *PRKWNK1* gene, which cause over-expression of a *WNK1* isoform lacking the *HSN2* 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 NCC cotransporter, a renal-specific cation-Cl⁻-cotransporter (CCC) relative of KCC2 (8).

WNK1/*HSN2* localizes to 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. Recently, WNK1-dependent inhibitory phosphorylation of KCC2 was shown to maintain the depolarizing action of GABA in the developing mouse brain (10). In immature neurons, WNK1 inhibition triggered a hyperpolarizing shift in GABA activity by reducing KCC2 Thr906/Thr1007 phosphorylation and enhancing KCC2-mediated Cl⁻ extrusion. However, whether WNK1/*HSN2* regulates KCC2 in the spinal cord is unknown. To begin to elucidate these questions, we generated the first *Wnk1/Hsn2* knockout mouse model and investigated the development of neuropathic pain after spared nerve injury and inflammatory pain.

Results & Discussion

Wnk1/Hsn2 knockout does not produce significant neurologic deficit

We utilized cre recombinase technology to generate the first knockout mouse model of the *Wnk1/Hsn2* isoform by specifically targeting the *Hsn2* exon (Fig 1a). Homozygote animals harbouring the *Wnk1/Hsn2*-floxed allele (*Wnk1^{flox/flox}*) were crossed to the pCX-NLS-Cre general deleter strain, and the resulting heterozygote animals (*Wnk1^{Hsn2/+}*) were crossed together to generate both mutant (*Wnk1^{Hsn2/Hsn2}*) and wild-type (*Wnk1^{+/+}*) animals (Suppl Fig 1). This resulted in specific depletion of the *Wnk1/Hsn2* isoform, as revealed by the lack of *Wnk1/Hsn2* transcripts (Fig 1b and Suppl Table 1) and WNK1/HSN2 protein (Fig 2) in *Wnk1^{Hsn2/Hsn2}* but not *Wnk1^{+/+}* mice. *Wnk1^{Hsn2/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 sural sensory nerves, revealed normal axonal distribution and morphology in *Wnk1^{Hsn2/Hsn2}* mice (Fig 1c and Suppl Fig 2 and Suppl Table 2).

Wnk1^{Hsn2/Hsn2} mice performed similarly to *Wnk1^{+/+}* mice in the SHIRPA test of general neurobehavioural function, the elevated plus maze and light dark box test (assessing neophobia/anxiety), and the holeboard test (assessing spontaneous motor activity and exploration) (Suppl Table 3). *Wnk1^{Hsn2/Hsn2}* male mice exhibited a significantly increased latency to tail withdrawal at both 47°C (** $P < 0.05$) and 49°C (* $P < 0.01$) (Fig 1d), but otherwise *Wnk1^{Hsn2/Hsn2}* mice exhibited similar response thresholds compared to *Wnk1^{+/+}* mice in tests assessing noxious thermal and mechanical sensitivity (Table 1). Global deletion of *Wnk1/Hsn2* therefore does not phenocopy the sensory neuropathy seen in HSANII patients, which is due to truncating mutations that leave intact the N-terminal kinase domain (6). Indeed, *Wnk1/Hsn2* knockout produces no significant neurological effects outside of a mild, sex-dependent loss of 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 speculated WNK1/HSN2 might play a role in chronic pain hypersensitivity after nerve injury or inflammation. We compared the neurobehavioural responses of *Wnk1^{Hsn2/Hsn2}* and *Wnk1^{+/+}* mice in the complete Freund's adjuvant (CFA) model of inflammatory pain (11) and the spared nerve injury (SNI) model of chronic neuropathic pain (12). *Wnk1^{Hsn2/Hsn2}* mice at both early and delayed time points (up to 21 days) exhibited significantly attenuated responses to the evaporation of acetone, and von Frey filaments, indicating decreased cold hyperalgesia and mechanical allodynia, respectively, in the SNI model ($P < 0.05$; Fig. 1e and 1f). In contrast, *Wnk1^{Hsn2/Hsn2}* mice performed similarly to *Wnk1^{+/+}* mice in the CFA model in tests measuring mechanical (von Frey) and thermal (Hargreaves) nociception (Table 1). Therefore, *Wnk1/Hsn2* knockout reduces pain hypersensitivity associated with nerve injury but not inflammation.

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

WNK kinases are master regulators of the CCCs (13), stimulating NKCC1 and inhibiting KCC2 via SPAK kinase-dependent phosphorylation at a homologous motif in each transporter (NKCC1^{Thr202/Thr207/Thr212}; KCC2^{Thr906/Thr1007}) (14, 15). Dephosphorylation of KCC2 at these sites stimulates KCC2 activity (14, 15) to significantly higher levels, capable of eliciting hyperpolarizing GABA_A responses even in neurons with an extremely negative resting membrane potentials (16). We examined the phosphorylation status (P) of NKCC1^{Thr202/Thr207/Thr212} and KCC2^{Thr906/Thr1007} in spinal cords from naïve and SNI-treated *Wnk1*^{Hsn2^{-/-}Hsn2^{-/-} and *Wnk1*^{+/+} mice (Fig 2a and 2b). The low basal levels of P-KCC2^{Thr906/Thr1007} seen in *Wnk1*^{+/+} mice were significantly reduced in naïve *Wnk1*^{Hsn2^{-/-}Hsn2^{-/-} mice ($P < 0.001$). SNI led to a significant ~3-fold increase in P-KCC2^{Thr906/Thr1007} in *Wnk1*^{+/+} mice ($P < 0.001$), that was markedly reduced in *Wnk1*^{Hsn2^{-/-}Hsn2^{-/-} mice to levels approximating those seen in naïve *Wnk1*^{+/+} mice ($P < 0.002$). Interestingly, the levels of WNK1/HSN2 were also increased by SNI ($P < 0.001$). In contrast, P-NKCC1^{Thr202/Thr207/Thr212} phosphorylation and SPAK expression were unchanged by WNK1/HSN2 knockout in either basal conditions or after SNI treatment (Fig 2a and 2b).}}}

Genetic or pharmacologic knockdown of Wnk1/Hsn2 restores SNI-induced E_{GABA} depolarization in lamina II neurons

Impaired KCC2-dependent Cl⁻ extrusion results in depolarizing GABA-evoked responses in rat spinal nociceptive pathways modified after SNI (3, 4). We performed electrophysiology in spinal cord slices derived from naïve mice, sham-operated controls, or those treated in the SNI model in the presence of vehicle or STOCK1S-50699 (17), a specific inhibitor of WNK signaling that activates the KCCs by reducing KCC inhibitory phosphorylation (15) (Fig 2c–2e). We found that E_{GABA} in lamina II neurons from SNI mice was significantly more depolarized than in lamina II neurons from naïve mice or sham-operated controls ($P < 0.05$). In SNI treated animals, E_{GABA} was restored by slice incubation with 10 μM STOCK1S-50699 for 30 to 45 minutes prior to recordings ($P < 0.01$), reflecting an increase in neuronal Cl⁻ extrusion capacity. Similarly, in lamina II neurons derived from *Wnk1*^{Hsn2^{-/-}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).}

Our results suggest WNK1/HSN2 kinase contributes to a maladaptive decrease in KCC2 activity and GABA inhibition associated with chronic pain hypersensitivity after nerve injury due to an up-regulation of KCC2 inhibitory phosphorylation at Thr906/Thr1007. Kinases are highly-druggable molecules that have been successfully targeted in cancer but infrequently in neuroscience (21). The discrete localization of WNK1/HSN2, its importance for human physiology, and the beneficial impact of its genetic inhibition on neuropathic pain behavior without producing other major neurological phenotypes, suggests WNK1/HSN2 kinase is a compelling pharmacotherapeutic 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 (Suppl Table 1). The three separate fragments were assembled using the SLIC method, and cloned in a modified pDELBOY-3X vector (Addgene plasmid 13440)(22–25). 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 (Fig. 1a). The construct also contained a phosphoglycerate kinase (PGK) promoter-driven neomycin resistance cassette flanked by two *flp* 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-Slf*) 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 × 10⁶ 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 (Suppl Table 1). Clones passing this first screening process were further confirmed by Southern blot analyses (Fig. 1a and Suppl Fig 1). 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 with the following primers (Suppl Table 1). The *Hsn2* exon was removed by crossing homozygote animals harbouring the *Wnk1/Hsn2*-floxed allele (*Wnk1^{fllox/fllox}*) to animals expressing the cre recombinase under the activity of the chicken β -actin gene (general-deleter, pCX-NLS-Cre animals, kindly provided by Dr. Nagy laboratory, University of Toronto, Toronto, ON, Canada). The resulting animals contained an intact allele of the *Hsn2* exon and a recombined allele (*Wnk1^{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^{Hsn2/+}*; X/X, *Wnk1^{Hsn2/+}*) that yielded both knockout (*Wnk1^{Hsn2/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^{Hsn2/ 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 SuperScript™ III Reverse Transcriptase (18080-044) (Invitrogen). PCR was performed with 5 µl of first-strand cDNA using primers pairs listed in Suppl Table 1 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 per group) from the lumbar 4 (L4) spinal section as well as sural nerves (n=5–6 per group) were dissected and post-fixed overnight. After several washes, the tissues were osmified 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, ventral roots as well as sural nerves.

General behavior phenotyping tests

SHIRPA—The SHIRPA test was performed according to the method described by Rogers et al (2001) (26).

Elevated plus-maze—The elevated plus maze 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 (Noldus, NL). 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 levels of open arm exploratory activity have greater neophobia.

Light-dark box—The Light 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 levels of white side exploratory activity have greater neophobia.

Holeboard—The hole-board test was used to assess mouse exploratory behavior in a novel environment. The apparatus was a Perspex 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 (e.g., walking and running) and fine (e.g., 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 behavioural experiments were performed with handlers blinded to groups. *Wnk1^{Hsn2} 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 and 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 (3 months for SNI) 5–11 months old when the study began. All nociceptive assays executed in this study have been fully described in detail in the literature (27–31). Tests measuring mechanical (von Frey) and thermal (Hargreave's) nociception stimuli were utilized in the Freund's adjuvant (CFA) model of inflammatory pain as described (11). For neuropathic pain mechanical allodynia (von Frey filaments test) (32) and cold hypersensitivity (evaporation of a drop of acetone) (33) 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, incision was sutured and mice were allowed to recover on heated pads before being returned to their home cage.

Statistical analyses

Statistical data were analysed 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 analysed using factorial ANOVA for genotype and sex effects. All chronic tests (SNI mechanical sensitivity, CFA mechanical and thermal sensitivity) were analysed 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 $\pm$ 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-Thr⁹⁹¹ [residues 975–989 of human KCC3a phosphorylated at Thr⁹⁹¹, SAYTYER(T)LMMEQRSRR, corresponding to residues of rat KCC2 phosphorylated at Thr⁹⁰⁶, SAYTYEK(T)LMMEQRSRR]; KCC2a phospho-Thr⁹⁰⁶ [residues 975–989 of human KCC3a phosphorylated at Thr⁹⁹¹, 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 Thr¹⁰⁰⁶, TDPEKVHLTW(T)KDKSV]. NKCC1 total antibody [residues 1–288 of human NKCC1]; NKCC1 phospho-Thr²⁰³/Thr²⁰⁷/Thr²¹² antibody [residues 198–217 of human NKCC1 phosphorylated at Thr²⁰³, Thr²⁰⁷ and Thr²¹², HYYDYD(T)HTN(T)YYLR(T)FGHNT]; SPAK-total antibody [full-length GST-tagged human SPAK protein]; SPAK/OSR1 (S-motif) phospho-Ser³⁷³/Ser³²⁵ antibody [367–379 of human SPAK, RRVPGS(S)GHLHKT, which is highly similar to residues 319–331 of human OSR1 in which the sequence is RRVPGS(S)GRLHKT]; HSN2 total antibody [full-length human 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.

Phospho-antibody immunoprecipitations

KCCs phosphorylated at the KCC2 Thr⁹⁰⁶ and Thr¹⁰⁰⁷ equivalent residue were immunoprecipitated from clarified spinal cord lysates. The phospho-antibody was coupled with 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 dephosphopeptide 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 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 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 phosphospecific sheep antibodies was performed with the addition of 10 µg/mL of the dephosphopeptide antigen used to raise the antibody. The blots were then washed six times with TTBS and incubated for 1 hour at RT 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 recording were performed in 300 µm transverse slices of lumbar segment L4 to L6 as previously described (4, 34, 35). For experiment involving SNI mice, all recordings were performed in ipsilateral side of the nerve injury. Recordings were performed at room temperature in standard 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 MgCl₂ (pH 7.3). To measure E_{GABA} under a Cl⁻ load, 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 experiments, slices were incubated in STOCK1S-50699 (10 µM for 30–45 minutes) before recordings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One Sentence Summary

Wnk1/Hsn2 kinase inhibition ameliorates neuropathic pain behavior after peripheral nerve injury by reducing maladaptive KCC2 inhibitory phosphorylation and normalizing depolarizing GABA signaling.

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) Schematic representation of the vector designed to generate the *Wnk1/Hsn2*^{fllox} allele. Mouse genomic DNA, spanning exons 7 to 12 of the *Hsn2* targeting construct, is represented. The targeting construct, containing *loxP* sites (arrows) in introns 9 and 10 is also depicted. The pGK Neo cassette is closely flanked by *frt* sequences (X) recognized by FLPe recombinase. Within genomic DNA, the *Hsn2* exon of *Wnk1/Hsn2* is between exon 8b

and exon 11 of *Wnk1*. The targeting vector introduces a PGK neo cassette; downstream of this the *Hsn2* exon is flanked by *loxP* recombination sites recognized by cre recombinase. Restriction sites; K, KpnI, X, XhoI, Xb, XbaI, B, BamHI, E, EcoRI, S, Sall, N, NotI. Schematic to scale.

(B) Complete excision of the *Hsn2* exon in neuronal tissues of *Wnk1^{Hsn2/ Hsn2}* mice. RT-PCR amplification between exon 8 and *Hsn2* from brain, cerebellum, spinal cord and liver. Based on the primer locations depicted in the schematic diagram representing *Wnk1* DNA, the *Hsn2* exon from the *Wnk1/Hsn2* isoform was present in neuronal tissues of *Wnk1^{+/+}* mice with bands at 146 and 404 bps, but absent in *Wnk1^{Hsn2/ Hsn2}* mice (absence of amplification product). The two different amplification products obtained from neuronal tissues of *Wnk1^{+/+}* animals represent two different isoforms originating from the presence or absence of the alternatively spliced 8b exon. Equal loading of amplified PCR product was confirmed using primers specific for the RNA polymerase encoding gene (Suppl Table 1).

(C) *Wnk1^{Hsn2/ Hsn2}* mice exhibit normal axonal distribution and morphology. 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^{Hsn2/ Hsn2}* mice (scale = 10 mm).

(D) *Wnk1^{Hsn2/ Hsn2}* mice exhibit only a mild, sex-dependent loss of distal thermal sensitivity without other apparent neurological deficits. Male *Wnk1^{Hsn2/ Hsn2}* mice displayed a significant longer latency to withdraw their tail at either 47°C and 49°C when compared to *Wnk1^{+/+}* animals (Bonferroni ** $P < 0.05$, * $P < 0.01$); female *Wnk1^{Hsn2/ Hsn2}* mice did not display significant longer latency to withdraw their tail at both temperatures. Tail-withdrawal test was performed on 8 *Wnk1^{+/+}* and 7 *Wnk1^{Hsn2/ Hsn2}* males, and 8 *Wnk1^{+/+}* and 10 *Wnk1^{Hsn2/ Hsn2}* females inclusively. Two-way ANOVA was performed on test results. Error bars represent the mean $\pm$ SEM). *Wnk1^{Hsn2/ Hsn2}* mice performed similarly to *Wnk1^{+/+}* mice in all other tests assessing general central nervous system function, including SHIRPA tests (basic neurological screen), elevated plus maze and Light Dark Box test (assessing neophobia/anxiety), and holeboard test (spontaneous motor activity and exploration test) (see Suppl Table 3). *Wnk1^{Hsn2/ Hsn2}* mice also responded similarly to *Wnk1^{+/+}* mice in all other nociception tests (see Table 1).

(E) Decreased development of cold allodynia in *Wnk1^{Hsn2/ Hsn2}* mice in the spared nerve injury (SNI) model of neuropathic pain. Response to evaporation of acetone was tested on 7–8 mice per group (*Wnk1^{Hsn2/ Hsn2}*, *Wnk1^{+/+}*). Significant lower mean responses to acetone in *Wnk1^{Hsn2/ Hsn2}* mice were observed at day 7 and onward (Bonferroni * $P < 0.01$). Mixed model ANOVA 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.

(F) Decreased development of mechanical hypersensitivity in *Wnk1^{Hsn2/ Hsn2}* mice in the SNI model. Response to mechanical von Frey stimulation was tested on 7–8 mice per group (*Wnk1^{Hsn2/ Hsn2}*, *Wnk1^{+/+}*). *Wnk1^{Hsn2/ Hsn2}* mice had significantly higher threshold responses to noxious mechanical stimulation at day 7 and onward (Bonferroni * $P < 0.01$). Mixed model ANOVA 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.

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 immunoprecipitation (IP) with the indicated KCC2 total-, and Thr906 and Thr1007 phospho-antibodies as described in Methods. Immunoprecipitates were then immunoblotted with the indicated specific KCC2 antibodies. The lysates were also subjected to immunoblot analysis with the indicated total and

phospho-specific antibodies. The Western blots illustrate results obtained in two separate experiments. Molecular masses are indicated in kDa on the left-hand side of the figure.

(B) Graphic representations of western blots quantification results obtained in (a). Quantification was performed as described in Methods. The quantification (ratio calculation) is based on (phospho KCC2)/(total KCC2), or HSN2/ERK1. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns - non significant. Statistical analysis was performed using unpaired t-test ($n=3$, all values represent the mean $\pm$ SEM).

(C) Currents recorded in spinal cord lamina II neurons from naïve and SNI-treated (1 week) adult mice as described in Methods in response to 30 ms. GABA (1mM) puffs in the presence of a Cl^- load (29mM). Slices were incubated for 30 to 45 minutes with vehicle control or STOCK 1S-50699 (10 μM) prior to recordings.

(D) IV relationship for GABA normalized currents ($I/I_{80\text{mV}}$) 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 as above ($n=14$).

(E) E_{GABA} measured in lamina II spinal cord neurons from naïve mice under control conditions ($n=13$) or after treatment with the WNK kinase signaling inhibitor 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 $\pm$ S.E.M. Two-way analysis of variance (ANOVA), with factors surgery and treatment (Bonferroni ** $P < 0.05$, * $P < 0.01$)

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

Table 1

Nociception test summary of *Wnk1*^{+/+} and *Wnk1* *Hsn2*^Δ *Hsn2* mice. Statistical results from seven nociception tests performed on *Wnk1* *Hsn2*^Δ *Hsn2* and *Wnk1*^{+/+} animals are presented. Column one presents the name of the test, column two highlights if the test reached significance, and column three presents the associated *P*-value.

Nociception Test	Results	<i>P</i> -values
Tail clip	NS	sex and genotype; F (1, 18) = 0.45 <i>P</i> = 0.51 sex; F (1, 18) = 1.49, <i>P</i> = 0.24 genotype; F (1, 18) = 0.65, <i>P</i> = 0.43
Hargreave	NS	15% female (<i>P</i> = 0.06) 15% male (<i>P</i> = 0.73) 20% female (<i>P</i> = 0.31) 20% male (<i>P</i> = 0.73)
Hotplate	NS	50°C female (<i>P</i> = 0.86) 50°C male (<i>P</i> = 0.35) 53°C female (<i>P</i> = 0.20) 53°C male (<i>P</i> = 0.56)
Manual von Frey	NS	female (<i>P</i> = 0.71) male (<i>P</i> = 0.36)
Formalin	NS	early female (<i>P</i> = 0.82) early male (<i>P</i> = 1.00) late female (<i>P</i> = 0.82) late male (<i>P</i> = 0.32)
CFA-von Frey	NS	sex and genotype; F (7, 10) = 1.39 <i>P</i> = 0.33 sex; F (7, 10) = 1.76, <i>P</i> = 0.23 genotype; F (7, 10) = 1.23, <i>P</i> = 0.41
CFA-Hargreave	NS	sex and genotype; F (7, 10) = 1.68 <i>P</i> = 0.25 sex; F (7, 10) = 1.15, <i>P</i> = 0.44 genotype; F (7, 10) = 1.43, <i>P</i> = 0.33

*
NS, Non-significant