Emerging role of *WNK1* in pathologic central nervous system signaling

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ABSTRACT

WNK1 (with no lysine (K)) is a widely expressed serine/threonine protein kinase. The role of this kinase was first described in the kidney where it dynamically controls ion channels that regulate changes in cell volume. *WNK1*, through intermediates oxidative stress-responsive kinase-1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK), phosphorylates the inwardly directed Na^+-$K+$-Cl^--cotransporter 1 (NKCC1) and the outwardly directed K^+-Cl^--cotransporter 2 (KCC2), activating and deactivating these channels, respectively. *WNK1*, NKCC1 and KCC2 are also expressed in the central nervous system (CNS). Growing evidence implicates *WNK1* playing a critical role in pathologic nervous system signaling where changes in intracellular ion concentration in response to γ-aminobutyric-acid (GABA) can activate otherwise silent pathways. This review will focus on current research about *WNK1*, its downstream effectors and role in GABA signaling. Future perspectives include investigating *WNK1* expression in the CNS after spinal cord injury (SCI), where altered neuronal signaling could underlie pathological states such as neuropathic pain (NP).

KEYWORDS: NKCC1, KCC2, GABA, Neuropathic Pain, Spinal Cord Injury

Introduction

The NKCC1 and KCC2 Channels

The human form of the Na^+-$K+$-Cl^--cotransporter 1 (NKCC1) channel is located on chromosome 5q.23.2 and is expressed by the SLC12a2 gene. This cotransporter is a 1212 amino acid protein with a molecular weight of 132 kDa and 12 transmembrane domains. The NKCC1 channel transports 1 Na^+:1 K^+:2 Cl^- into the cell.[1] This channel is highly expressed on the apical surface of mammalian neurons in the mature central nervous system[2–4] and dorsal root ganglion (DRG) sensory neurons in the peripheral nervous system.[3,5]

The K^+-Cl^--cotransporter 2 (KCC2) protein, also expressed by the SLC12 gene,[6] contains 1116 amino acids, 12 transmembrane domains and has a molecular mass of 123.6 kDa. It transports potassium and chloride out of the cell in 1:1 stoichiometry.[7] The channel is neuronal specific[8,9] and is found primarily in dendritic spines of inhibitory synapses in the dorsal horn of the spinal cord.[9,10]

The SLC12 channels may play a role in epilepsy and pathological excitability. Bumetanide (BU), a NKCC1 blocker, suppresses seizures and attenuates electrographic activity in neonatal rats, *in vivo*.[11] Similarly, mice lacking KCC2 channels frequently seize and die shortly after birth.[9,12] Three hours of epileptic-like neuronal stress decreases KCC2 mRNA expression in rat hippocampal slices.[13]

NKCC1 and KCC2 are co-expressed in specific neurons.[14,15] After contusion spinal cord injury (cSCI), NKCC1 and KCC2 channel expression are increased and decreased, respectively.[16] Phosphorylation activates NKCC1 but inhibits KCC2, whereas dephosphorylation activates KCC2 and deactivates NKCC1.[4,17–20]

GABA, Chloride and the CNS

Central nervous system (CNS) excitability and behavior is dynamically regulated by variations in intracellular ion concentration.[21–23] Changes in $[Cl^-]_i$ govern the response to the neurotransmitter γ-aminobutyric-acid (GABA). In GABA-ergic stimulated neurons, Cl^-_{in} will occur if $[Cl^-]_i$ is below E_{Cl^-}, increasing the probability of hyperpolarization. Conversely if $[Cl^-]_i$ is above E_{Cl}-, GABA stimulation will result in Cl^-_{out}, driving the V_m towards E_{Cl}-, and potential depolarization.[6,22,24] Rat hippocampal slices with downregulated KCC2 channels show reduced Cl^- extrusion.[25] In KCC2 knockout (K0) mice motorneurons, stimulation with GABA results in depolarization whereas wild-type (WT) neurons hyperpolarize under identical stimulation.[9] Spatial-temporal changes in $[Cl^-]_i$ modify GABA-ergic responses in retinal bipolar cells.[14] Early postnatal GABA induced depolarization[25] may be due to increased accumulation of $[Cl^-]_i$ through increased NKCC1 channel expression[26,27] and activity.[28] As the CNS matures, NKCC1 channel expression is decreased[26,29] and KCC2 channel expression is increased;[29,30] this could contribute to the changes in $[Cl^-]_i$[27–29] and subsequent switch of GABA from an excitatory to inhibitory neurotransmitter in development.[13,24,27,29–31]

WNK Family

WNK1 is a serine/threonine protein kinase that is activated by phosphorylation and was first described in 2000 by Xu *et al.* as a 2126 amino acid long protein with a molecular weight of about 230 kDa. *WNK1* is named so (with no lysine (K)) because it lacks a catalytic lysine found in subdomain II of most of the other protein kinases.[32] The *WNK1* gene is under the control of at least 3 different promoter regions. This allows for tissue specific distribution. Alterative splicing and polyadenylation sites can achieve further differentiation.[33] The *WNK1* kinase is found among other places, in cell bodies of DRG neurons.[34]

Most of the research on the WNK kinases has been done in the kidney and their role in governing blood pressure. Pseudo-hypoaldosteronism type II (PHAII) is an autosomal dominant disorder where patients present with hypertension and hyperkalemia. Rats with mutations in *WNK1* intron 1, mimic PHAII and show a five-fold increase in *WNK1* expression. Thus *WNK1* appears to play a role in this disease by either increasing the reabsorption of potassium and other ions, or by inhibiting their secretion or excretion.[35]

The WNK family is upstream activators of the NKCC1 and KCC2 channels. Hypertonic stress increases *WNK1* activity.[36] *WNK1* has been shown to phosphorylate and activate oxidative stress-responsive kinase-1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK, or PASK or STK39).[6,37-41] SPAK and OSR1 share

amino acid sequence homology in their N-terminal catalytic domain (96%) and C-terminal regulatory domain (67%).[41] SPAK and OSR1 phosphorylate three residues on the NKCC1 channel.[7,17,38,40–42] Hyperosmotic stress increases NKCC1 phosphorylation[17,38] and K^+ uptake by this channel.[43] *WNK1* induced phosphorylation of OSR1 activates this kinase to phosphorylate its NKCC1 substrates in HeLa cells *in vitro*. HeLa cells injected with *WNK1* siRNA exhibit reduced NKCC1 activity.[37] MDCKII cells overexpressing *WNK1* show increased chloride permeability, *in vivo*.[44] WNK4 phosphorylates SPAK at sites homologous to those phosphorylated by *WNK1*.[39,43] In *Xenopus laevis* oocytes, coexpression of both WNK4 and SPAK increases NKCC1 channel activation and desensitizes the channel to osmotic conditions. Coexpression of WNK4 and SPAK results in downregulation of KCC2, regardless of osmotic environmental conditions.[43] Expression of WNK3 phosphorylates NKCC1 regardless of the osmotic state of the environment. WNK3 increases Cl^- influx via NKCC1 and decreases Cl^- efflux via the KCC2 channel.[17]

Thus *WNK1*, WNK3 and WNK4 behave like volume sensitive kinases that control SLC12 family members. However WNK3 can regulate the NKCC1 and KCC2 transporters alone, where as *WNK1*/4 require OSR1 and SPAK coexpression; suggesting a separate mechanism for the different kinases. Perhaps WNK3 works by inhibiting phosphatases and thus increasing the phosphorylation state of SCL12 family channels; a different mechanism could exist for *WNK1*/4: they phosphorylate OSR1 and SPAK which go onto phosphorylate NKCC1 and KCC2.[45]

The WNK family and its biological cascade play an important role in the nervous system. *WNK1* knockdown C17.2 cells show altered morphology, slower motility and reduced invasive ability; suggesting *WNK1*'s role in proliferation, migration and differentiation in neural development.[46] SPAK and OSR1 are expressed in adult neurons of the spinal cord, DRG and brain.[47–49] SPAK or OSR1 knockdown mice show about a 50% reduction of spinal cord NKCC1 channel activity,[49] and knockdown of both kinases is additive.[4] WNK3 is highly expressed in the nervous system and appears to be important in neuronal development: absent in mice on postnatal day 10, but becoming highly expressed by postnatal day

21. This might suggest a role of WNK3 in switching from normal GABA excitation in prenatal life, to GABA inhibition in adulthood.[8] Post-mortem analysis of human brain specimens shows schizophrenics have increased WNK3 expression in the dorsolateral prefrontal cortex, an area known to have altered synchrony in diseased patients.[50] Additionally, perhaps the WNK family's ability to dynamically regulate Cl^- channels plays a role in the circadian variation of $[Cl^-]_i$[21,22] and GABA transmission that occurs in the suprachiasmatic nucleus that controls sleep wake cycles.[22,51]

Hereditary sensory and autonomic neuropathy Type 2 (HSAN2) is a recessive disorder associated with loss of sensitivity.[52] A mutated alternatively spliced exon of the *WNK1* gene that selectively occurs in nervous tissues called HSN2 is involved in HSAN2.[34,52] Specific isoforms of *WNK1* have been characterized to be organ specific.[53] HSN2 is found primarily in the spinal cord, but is also present in the DRG and sciatic nerve of adult mice. Within the spinal cord, HSN2 is more predominant in the dorsal roots compared to the ventral roots. It is also highly expressed in the laminae II and III, dorsolateral funiculus and lateral funiculus that contain ascending sensory fibers.[34] Interestingly, twenty-five human carriers of the defective exon were shown to have lower warm and cool detection thresholds. It was hypothesized that one truncated copy of the *WNK1*/HSN2 gene results in an increase in membrane excitability lowering detection threshold; however in homozygous HSN2 isoform carriers that have HSAN2, the increased excitability may lead to excitotoxicity leading to decreased sensation.[52]

Pain Perception

Modulation and/or modification of the nervous system can lead to hyperalgesia (noxious stimuli eliciting a greater than normal pain response) or allodynia (stimuli that normally do not produce pain begin to do so). Recent pain theories propose lose of inhibition (disinhibition) as being crucial for the development of chronic pain.[54] There are two types of afferent fibers in the spinal cord: Aβ-fibers that perceive tactile sensations, and Aδ- and C-fibers involved in nociception. A presynaptic link exists between these two fibers[55,56] that contains a GABA-ergic interneuron.[57] Under normal conditions mechanically stimulated Aβ fibers, acting via the GABA interneuron, will cause pri-

mary afferent depolarization (PAD) of the nociceptive terminals; thus shunting pain perception via a presynaptic inhibition mechanism. Following injury, an increased afferent barrage from the Aδ- and C-fibers converges onto the GABA-ergic spinal interneurons that mediate the presynaptic link between mechano and nociceptive receptors. Thus when the Aδ-fibers are now stimulated, the increased excitability of the interneuron produces a much more intense PAD capable of producing spike activity. This results in antidromically conducted dorsal root reflexes (DRR)[58] and produce secondary hyperalgesia or allodynia.[55,56]

Role of NKCC1 and KCC2 in Pain

DRG NKCC1 KO mice show increased thermal pain thresholds. Mutant cells hyperpolarize and WT cells depolarize to identical stimuli, and mutant cells lack the $GABA_aR$-mediated anion outward flux current.[5] Blocking NKCC1 channels lowers $[Cl^-]_i$ accumulation after vagal motorneuron axonotomies.[15] Elevations in $[Cl^-]_i$ after rat sciatic nerve axonotomies is attributable to phosphorylation of the NKCC1 channel.[59] Additionally, axonotomies increase DRG NKCC1 phosphorylation.[4] NKCC1 KO mice have reduced Aβ-fiber mediated touch evoked hyperalgesia following intradermal capsaicin injections, a known method to induce allodynia.[60] Intracolonic injection of capsaicin increases dorsal spinal NKCC1 phosphorylation within 10 minutes of injection and membrane mobilization 90-180 minutes after instillation. Total NKCC1 mRNA levels do not change.[61] Intrathecal (IT) injections of BU have antinociceptive properties for hindpaw formalin injection models, a known method to induce acute pain.[62] IT injections of BU also attenuates intracolonic capsaicin injection induced referred abdominal allodynia after its establishment.[63] Recently, it was shown that IT BU injections reduces dorsal horn and nociceptive specific signaling after intraplantar capsaicin injections.[64]

Adult male Sprague-Dawley rats after cSCI show increased NKCC1 channel and decreased KCC2 channel expression 2-14 days post cSCI at the injury epicenter. Injured rats develop thermal hyperalgesia (TH) 21-42 days post cSCI. Administration of BU increases noxious thermal paw withdrawal latency time, signaling decreased TH. NKCC1 and KCC2 expression did not change in sham control animals in this experiment. This suggests the role of

NKCC1 and KCC2 in the role of development and maintenance of cSCI induced NP.[16] Inflammatory mediators induce phosphorylation of DRG NKCC1 channels and increases $[Cl^-]_i$ within one hour, and increases NKCC1 expression and decreases KCC2 channel expression within three hours, in vitro.[65]

Hemisection spinal cord injury (SCI) decreases KCC2 expression in the dorsal horn that correlates with $\geq$ twelve-week mechanical allodynia. This type of injury also results in a positive shift in $GABA_i$ that changes prior inhibitory post-synpatic potentials to long lasting excitatory post-synaptic potentials in laminae I dorsal horn neurons.[10] cSCI rats show a 84% reduction in ventral horn KCC2 channel expression 7-45 days after injury, and continuously decreased expression into 4-5 month post-injury chronic phases.[66] Spinal cord KCC2 protein levels are decreased in rats with painful diabetic neuropathy.[67] IT injections of anti-sense KCC2 oligodeoxynucleotides or a KCC2 channel blocker decreases mechanical and thermal nociceptive thresholds in injured and uninjured animals.[68,69] Rat hindpaw formalin injection models show reduced KCC2 immunoreactivity in lamina I and II of L5, although total KCC2 mRNA is unchanged.[70] Mice given subcutaneous injections of formalin show reduced KCC2 channel expression in the medullary horn that is associated with pain behaviors.[69] Peripheral inflammation induced by hindpaw injections of complete Freund's adjuvant reduces dorsal horn KCC2 channel expression and thermal nociceptive thresholds.[71] Cuff-induced injuries of the rat sciatic nerve results in reduced expression of the KCC2 channel, and reverses GABA response polarity to excitatory in lamina I neurons, in vitro.[68] In rat vagal motorneurons, in vivo axonotomies result in decreased expression of KCC2 mRNA. Subsequent accumulation of $[Cl^-]_i$ is directly attributable to new GABA induced excitation.[15]

Role of GABA in Pain

GABA receptors are found in primary afferent terminals and interneurons in laminae I-IV in the spinal cord dorsal horn,[72,73] which is the main site of Aδ- and C-fiber afferent termination and nociceptive signaling. GABA-ergic interneurons are important in spinal nociceptive processing and nociceptive attenuation.[74,75] Elevation of $[Cl^-]_i$ can lead to GABA-ergic hypersensitivity by reversing both E_{Cl^-} and the normal inhibitory action of GABA.[24] Lamina

I GABA-ergic interneurons become more excitable with depolarizing membrane potentials, larger spike heights, increased firing frequencies and increased incidence of spontaneous plateau potentials after SCI.[76] The GABA antagonist bicuculine alleviates formalin induced tactile allodynia in rats with painful diabetic neuropathy.[67] Administration of complete Freund's adjuvant into the rat hindpaw reverses spinal $GABA_a$ signaling. Muscimol (a $GABA_a$ receptor agonist) increases and gabazine (a $GABA_a$ antagonist) decreases nociceptive thresholds in naïve rats, where as after inflammation muscimol decreases and gabazine increases nociceptive thresholds.[77] In vitro scraping injuries to hypothalamic neurons changes their electrophysiological properties: depolarizing chloride reversal potentials that result in GABA induced excitation.[78]

Future Perspectives

In summary, WNK1 phosphorylates SPAK and OSR1, which go onto phosphorylate NKCC1 and KCC2, activating and deactivating these channels, respectively. Subsequent accumulation of $[Cl^-]_i$ could reverse GABA polarity in dorsal horn spinal interneurons. Altered WNK1 expression could be important in post-injury neuronal signaling.

SCI is a devastating[79] and costly injury with an estimated 12,000 new cases reported within the US each year.[80] Anywhere between 25.5-96.2% of people develop chronic pain after their injury.[81-83] Neuropathic pain (NP) can occur from altered processing in the central nervous system in the absence of peripheral nerve damage.[84] PAD and presynaptic inhibition could be modified by changes in WNK1 activity and/or expression, and subsequent changes in NKCC1 and KCC2 channel activity after cSCI to alter nociceptive sensory processing in the spinal cord. Altering these channels would change $[Cl^-]_i$ and result in a larger potential shift when $GABA_a$ receptor channels open. This could lead to PAD changing to DRR and/or increased GABA activity of interneurons mediating PAD; ultimately leading to heightened excitability that would translate into hyperalgesia and allodynia (Fig. 1). Future electrophysiological studies could help understand post-injury changes in spinal circuitry.

The hyperosmotic induced WNK1 and NKCC1 activation, and KCC2 deactivation previously reported in the renal system[17,37,39,44] could be similar to the in-

flammatory response elicited in the nervous system after injury. Vasodilatation and subsequent invasion from neutrophils, monocytes, T and B lymphocytes; and cytokine secretion from astrocytes, microglial cells, endothelial cells and leukocytes[85] could possibly increase extracellular osmolarity and activate WNK1, which has previously been described as a volume sensitive kinase.[17,37,39,44] NKCC1 and KCC2 expression is increased and decreased, respectively, at a CNS injury center.[16] NKCC1 phosphorylation stimulates peripheral nerve regrowth after axonotomy.[59] Perhaps during injury GABA induced depolarizations, because of altered chloride homeostasis, are induced in an attempt to revert neurons back to a state of developmental flexibility needed for sprouting and retargeting.[86] As a consequence, NP develops.

SLC12 channel phosphorylation precedes changed channel expression in nervous system injury models.[65] And although NKCC1 phosphorylation has been shown to increase membrane mobilization,[61] an exact role of WNK1 and increased SLC12 channel expression has not been described. Future studies directed at studying the consequences of altered WNK1 expression in the CNS will be important in understanding the various roles of this kinase.

Abbreviations

WNK1: with no lysine (K) kinase I; SPAK: STE20/SPS1-related proline/alanine-rich kinase; OSR1: oxidative stress-responsive kinase-1; NKCC1: Na^+-$K+$-Cl^--cotransporter I; KCC2: K^+-Cl^--cotransporter 2; GABA: γ-aminobutyric-acid; SCI: spinal cord injury; NP: neuropathic pain; DRG: dorsal root ganglion; BU: bumetanide; cSCI: contusion spinal cord injury; $[Cl^-]_i$: intracellular chloride; $Cl^-_{in/out}$: chloride current in/out; E_{Cl^-}: chloride equilibrium potential; V_m: membrane potential; KO: knock-out; WT: wild-type; CNS: central nervous system; Pseudohypoaldosteronism type II: PHAII; HSAN2: hereditary sensory and autonomic neuropathy type 2; PAD: primary afferent depolarization; DRR: dorsal root reflexes; IT: intrathecal; TH: thermal hyperalgesia; $GABA_i$: GABA induced current.
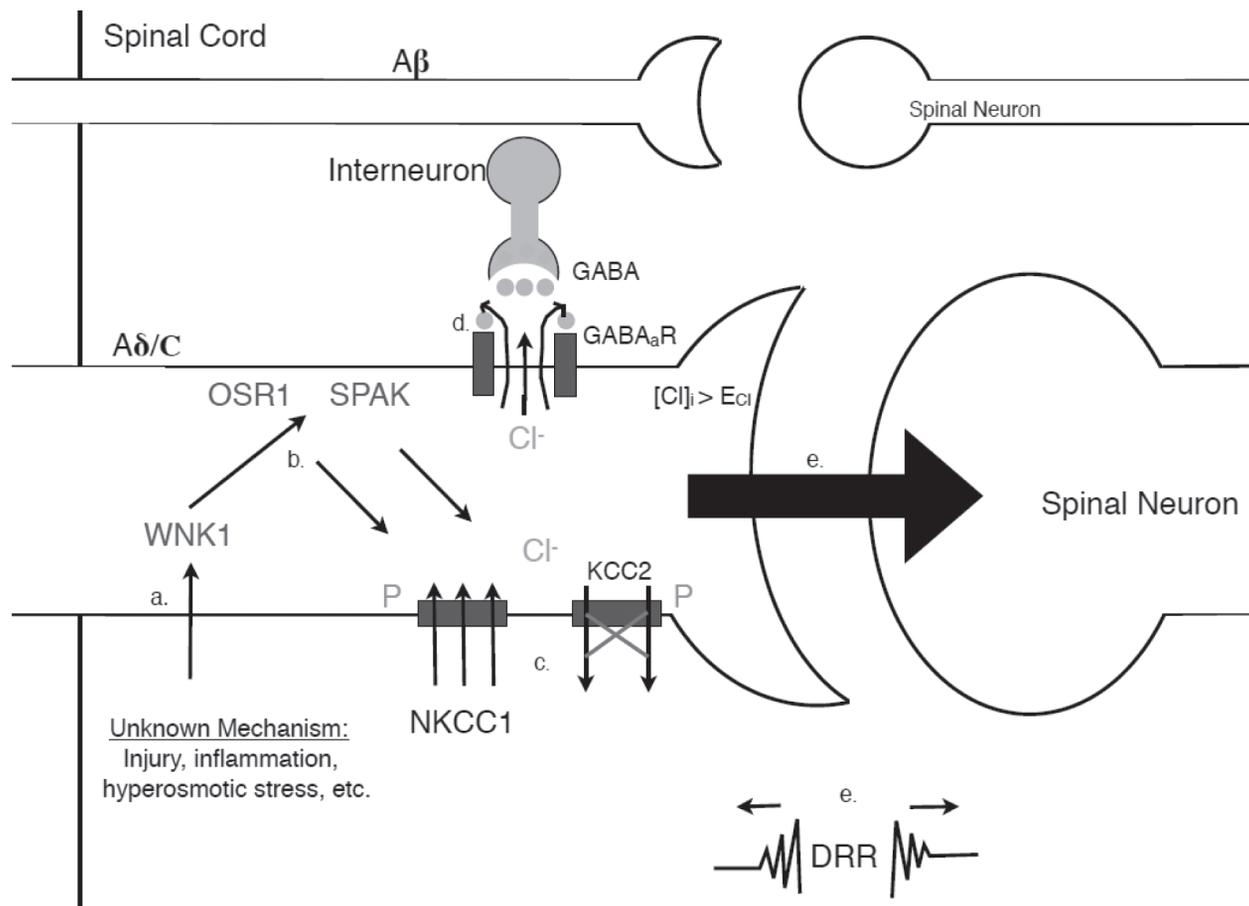
Acknowledgments

Fig. 1: Hypothetical role of *WNK1* in pathologic spinal cord signaling. In normal spinal cord signaling tactile information is processed by Aβ-fibers, and a presynaptically linked GABA-ergic interneuron causes PAD of nociceptive pathways. However, an unknown mechanism such as injury will **a.** cause phosphorylation of *WNK1* which, **b.** phosphorylates OSR1 and SPAK which, **c.** phosphorylates the NKCC1 and KCC2 channels, activating and deactivating these channels, respectively. This leads to $[Cl^-]_i > E_{Cl^-}$, **d.** reversed GABA signaling, and **e.** activation of otherwise silent nociceptive pathways and antidromically conducted DRR, leading to hyperalgesia or allodynia.

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