

The WNK-SPAK/OSR1 pathway master regulator of cation-chloride-cotransporters

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Abstract

The WNK-SPAK/OSR1 kinase complex is composed of the kinases WNK (with no lysine) and SPAK (SPS1-related proline/alanine-rich kinase) or the SPAK homolog OSR1 (oxidative-stress responsive kinase 1). All 3 kinases exist in same complex; SPAK/OSR1 are related to one another, and have redundant functions; WNKs are a separate kinase family, and operate upstream of both SPAK and OSR1 hence WNK-SPAK/OSR1 (same issue for title – should be listed as “WNK-SPAK/OSR1 to capture this nuance in nomenclature) The WNK family senses changes in intracellular Cl^- concentration, extracellular osmolarity, and cell volume and transduces this information to sodium (Na^+), potassium (K^+), and chloride (Cl^-) cotransporters [collectively referred to a CCCs (cation-chloride transporters)] and ion channels to maintain cellular and organismal homeostasis and affect cellular morphology and behavior. Several genes encoding proteins in this pathway are mutated in human disease and the cotransporters are targets of commonly utilized drugs. WNKs stimulate the kinases SPAK and OSR1, which directly phosphorylate and stimulate Cl^- -importing, Na^+ -driven CCCs or inhibit the Cl^- -extruding K^+ -driven CCCs. These coordinated and reciprocal actions on the CCCs, triggered by an interaction between RFXV/I motifs within the WNKs and CCCs and a conserved C-terminal (CCT) docking domain in SPAK and OSR1, pinpoint a potentially druggable node that could be more effective than targeting the cotransporters directly. The INTERACTION between the WNK and SPAK/OSR1 via the CCT-RFxV/I is the druggable node – the best drug available for inhibition of this pathway in fact disrupts the INTERACTION. In the kidney, WNK-SPAK/OSR1 inhibition decreases epithelial NaCl reabsorption and K^+ secretion to lower blood pressure while maintaining serum K^+ . In neurons, WNK-SPAK/OSR1 inhibition could facilitate Cl^- extrusion I put “could” because action of WNK-SPAK pathway in kidney much more known than in neurons, though compelling data exists for this in neuronal culture systems and heterologous expression systems studying neuron-specific transporters; also, I know of several papers including my own either in press or in submission that will make this emphasis timely. IMPORTANTLY, As negatively charged Cl^- ions exit through activated KCC2 , and are inhibited from entry via NKCC1 , the level of intracellular Cl^- is lowered; therefore, upon GABA binding to its GABAR (which is a Cl^- permeable ion channel), Cl^- rushes in, and membrane potential is hyperpolarized and promotes GABAergic inhibition; the opposite occurs in immature or diseased neurons, where elevated levels of Cl^- cause Cl^- to run out of cells upon GABA binding to GABA_ARs, which causes negative charge to leave cell, membrane depolarization, and excitation.... and promote GABAergic inhibition. Such drugs could have efficacy as K^+ -sparing blood pressure-lowering agents in essential hypertension, nonaddictive analgesics in neuropathic pain, and mediators of ionotropic inhibition in diseases associated with neuronal hyperactivity, such as epilepsy, spasticity, and schizophrenia.

Physiology and Regulation of Cation-Chloride Cotransporters

Protein kinases have become an important class of drug targets, particularly in the field of oncology (1). In the past decade, more than 20 different drugs targeting kinases have been approved for clinical use for the treatment of various types of cancer (2). However, the use of kinase inhibitors in other human diseases, including those with cardiovascular, renal, neurological, and psychiatric phenotypes, have lagged behind despite promising kinase targets identified by genetic studies in humans and model organisms (2).

The electroneutral cation-Cl⁻ cotransporters (CCCs) of the *SLC12A* gene family are secondary-active plasmalemmal ion transporters that utilize electrochemically-favorable cellular gradients of Na⁺ or K⁺, established by primary active transport mediated by the ouabain-sensitive Na⁺/K⁺-ATPase, to transport Cl⁻ (and K⁺) into or out of cells. Two branches of CCCs exist: the Cl⁻-importing, Na⁺-driven CCCs (NCC, NKCC1, and NKCC2; collectively referred to as N[K]CCs), and the Cl⁻-exporting, K⁺-driven CCCs (KCC1-4; collectively referred to as KCCs) (3). These evolutionarily-conserved transporters are among the most important mediators of ion transport in multicellular organisms (4), and their knockout in worms, flies, and mice demonstrate their necessity throughout the animal kingdom for proper function and survival (5) (Fig. 1).

CCCs are critically important for human physiology. CCCs are targets of commonly utilized drugs, for example, furosemide (also known as Lasix) and bumetanide (also known as Bumex) inhibit NKCC2 and NKCC1, and thiazides inhibit NCC (Fig. 1). Three different CCCs are mutated in autosomal recessive disorders. Gitelman syndrome can be caused by a loss-of-function mutation in NCC (6) and Bartter syndrome can be by a loss-of-function mutation in NKCC2 (7); both are characterized by hypotension and hypokalemic alkalosis due to imbalances in renal electrolyte handling. Andermann syndrome can be caused by a mutation in KCC3 and is characterized by seizures, motor and sensory neuropathies, and agenesis of the corpus callosum due to a lack of KCC3 function in developing neurons (8). Alterations in CCC protein abundance or functional regulation, including phosphorylation, have also been demonstrated in numerous animal models of diseases with renal or neurological phenotypes (9).

In many different cell types, the balance of Cl⁻ import through the N[K]CCs and Cl⁻ export through the KCCs sets the intracellular concentration of Cl⁻ ([Cl⁻]_i). This has important implications for several core physiological processes, including transepithelial solute and water transport, cell volume regulation, and neuronal excitability (4). Accordingly, altered CCC function contributes to NaCl-sensitive hypertension (due to altered epithelial transport in the distal nephron), cytotoxic edema following cerebral ischemia (in part due to altered regulation of cell volume), and seizures and neuropathic pain (due to a reduction or loss of GABAergic inhibition, a phenomenon called "ionotropic disinhibition") (9). In these disease states, the altered function of the CCCs impairs Cl⁻ homeostasis to alter cellular structure and function.

Early physiological studies using radiotracer flux assays and relatively nonspecific kinase and phosphatase inhibitors illustrated a powerful mechanism that coordinately but reciprocally regulates the N[K]CCs and the KCCs in cells by a system of serine-threonine protein kinases and phosphatases (10-19). Cell shrinkage, intracellular Cl⁻ depletion, and experimental inhibition of protein phosphatase activity, promotes NCC, NKCC1, and NKCC2 activity, but inhibits the KCCs, by increasing transporter serine-threonine phosphorylation. Cell swelling and intracellular Cl⁻ accumulation have the opposite effects (Fig. 2). OK This inverse regulation of Na⁺- and K⁺-driven CCCs by the same signals and likely the same kinase-phosphatase pathway ensures that cellular Cl⁻ influx and efflux are tightly coordinated, and needless ATP expenditure is avoided. The importance of this phosphoregulatory mechanism is exemplified by its evolutionary conservation from worms to humans (20).

The identity of the molecular signaling complex that controls these events remained unknown until the combined work of molecular genetics (21-23), physiology (24-32), and biochemistry (33, 34) revealed that WNKs exert their physiological effects by phosphorylating and activating two downstream serine/threonine protein kinases that are highly related to each other by sequence homology, SPAK OK[SPS1-related proline/alanine-rich kinase; also known as STK39 (serine threonine kinase 39)] and OSR1 (oxidative stress-responsive kinase 1) (33, 34). SPAK and OSR1 are functionally-redundant kinases in experimental systems, often exist in the same kinase complex, and operate as central components in a switch mechanism that, through phosphorylation at critical N- or C-terminal residues within the transporters, stimulates the N[K]CCs and inhibits the KCCs (35). Inhibiting these phosphorylation events or promoting dephosphorylation “flips the switch” of this regulatory module to inhibit the N[K]CCs but activate KCCs (36). The elucidation of this switch mechanism, coupled with the development of the specific inhibitor STOCK1S-50699 (37) that prevents activation of SPAK/OSR1 thereby activating KCCs and inhibiting N[K]CCs, has set the stage for a next phase of exploration in which modulation of the CCCs by inhibition of the WNK-SPAK/OSR1 pathway for therapeutic benefit.

Here, we review the molecular genetic discovery, biochemical mechanisms, and physiological functions of the WNK-SPAK/OSR1-CCC pathway; provide the rationale of why and how targeting the WNK-SPAK/OSR1 complex might be beneficial in several human diseases characterized by a shared pathologic signature of abnormal Cl⁻ homeostasis; and explain why this approach may be particularly efficacious relative to existing drugs or strategies that target specific CCCs. Research on the WNK-SPAK/OSR1 kinases exemplifies how genetics and biochemistry can synergistically be used to identify and target critical regulatory components within complex homeostatic networks and to exploit idiosyncrasies of kinase structure and unique mechanisms of catalytic activation to develop specific protein kinase inhibitors with therapeutic potential.

Discovery and characterization of the WNK-SPAK/OSR1-CCC pathway

Identifying a connection between WNK and CCCs

In 2001, Lifton and colleagues utilized positional cloning to find that mutations in *WNK1* or *WNK4*, two previously uncharacterized genes (21), caused pseudohypoaldosteronism type II [(PHAII)Correct – is hypo; Online Mendelian Inheritance in Man (OMIM) no. 145260; also known as Gordon's Syndrome], a rare autosomal recessive form of thiazide-sensitive and NaCl-sensitive hypertension that is also characterized by high concentrations of serum K⁺ (hyperkalemia). This discovery opened an exciting new field in renal physiology, defining a hitherto unrecognized signaling pathway responsible for the coordination of two aldosterone-controlled processes (NaCl reabsorption and K⁺ secretion)XXXsecretion in the classical renal physiology sense: from blood to urine via transepithelial transportXXX in the kidney's distal nephron to regulate blood pressure and electrolyte homeostasis in humans (38). The WNKs were subsequently shown to regulate the phosphorylation and activities of two CCCs in the kidney, NCC in the distal convoluted tubule, KCC4 and NKCC2 in the thick ascending limb (27, 39), in concert with the renal outer medullar potassium channel (ROMK) (40) and the amiloride-sensitive epithelial Na⁺ channel (ENaC) in the distal tubule and collecting duct (41). The characterization of the mechanism of action of the WNKs on the CCCs in heterologous expression systems like oocytes and mouse models solved a long-standing paradox in human physiology by revealing how aldosterone, via differential regulation of WNK-SPAK/OSR1 activities in the presence or absence of angiotensin II, can affect both NaCl reabsorption and K⁺ secretion, and preferentially shunt the distal nephron into discrete activity states in which one of these functions is favored at the expense of the other to combat physiological perturbation and restore homeostasis (42, 43) (Fig. 1).

Like the N[K]CCs, the KCCs are controlled by serine-threonine phosphorylation (Fig. 2) (36, 44, 45). Early experiments suggested the WNKs were likely key functional regulators (46-48). Two Thr residues in the C-terminal cytoplasmic domain, which are conserved in all KCC isoforms, termed Site-1 (Thr⁹⁰⁶ in KCC2; Thr⁹⁹¹ in KCC3) and Site-2 (Thr¹⁰⁰⁷ in KCC2; Thr¹⁰⁴⁸ in KCC3), play a critical role in controlling the activity of the KCCs and are regulated by phosphorylation by the WNKs alone XXXWNK1 regulates site 1, but not SPAK/OSR1; site 2 regulated by WNK1-SPAK/OSR1, hence "alone or in combination" XXXor in combination with SPAK/OSR1. Hypotonic high K⁺ conditions, which activate KCCs and inhibit the N[K]CCs, induce a rapid and robust dephosphorylation of Site-1 and Site-2 (36). Dual mutation of Site-1 and Site-2 to Ala in the KCCs, which prevents their phosphorylation, results in constitutively-active KCCs with >25-fold activity compared to wild-type KCCs in similar conditions (49). Knockdown of WNK1 in XXX HEK293 cells partially suppresses the phosphorylation of Site 1 in KCC3 49). Overexpression of WNK isoforms inhibits the KCCs, whereas overexpression of dominant-negative WNK3 stimulates KCC activity (30, 46, 47, 50, 51).

Thus, WNK activity regulates the activity of N[K]CCs and KCCs through a phosphorylation-dependent mechanism.

Characterizing WNK activation mechanisms and downstream targets

Biochemical experiments subsequently clarified the molecular mechanism by which the WNKs and their downstream kinase substrates, SPAK and OSR1, both can do it, functionally redundant phosphorylate and stimulate N[K]CC activity (Fig. 3) (33, 34). WNK isoforms stimulate the kinase activity of SPAK/OSR1 by phosphorylating a conserved Thr residue (SPAK Thr²³³, OSR1 Thr¹⁸⁵) within the SPAK/OSR1 catalytic T-loop motif (24). Mouse protein-25 (MO25) interacts with both SPAK and OSR1 to enhance their catalytic activities over 100-fold using in vitro CATCh tide assays (52). A unique correct, not in any other kinases, hence druggability, conserved C-terminal (CCT) docking domain within SPAK/OSR1 binds RFXV/I, which means either/or motifs in the N-terminus of NCC, NKCC1, and NKCC2 (53, 54). The CCT domain in SPAK/OSR1 is also required for binding to and activation by WNKs, which also possess RFXV/I motifs (55). Following hypertonic or hypotonic low-Cl⁻ conditions, WNKs and then SPAK/OSR1 are rapidly activated and SPAK/OSR1 phosphorylate WNKs, which are the direct phosphorylators of NKCC1, a cluster of conserved Thr residues in the N-terminal cytoplasmic domain of the N[K]CCs (53). This mechanism of CCC phosphorylation and activation is conserved for NCC, NKCC1, and NKCC2. OK

MO25 α and MO25 β are closely related and functionally redundant scaffolding proteins, collectively referred to as MO25 α/β , that stimulate the activity of SPAK and OSR1 (Fig. 3) (36). In the presence of a MO25 subunit, SPAK/OSR1, promote inhibition of all KCC isoforms by directly phosphorylating Site-2 in HEK 293 and cells (36). Inhibiting SPAK/OSR1 activation suppresses KCC Site-2 phosphorylation to a greater extent than it suppresses Site-1 phosphorylation (36). SPAK/OSR1 are required for KCC Site-2 phosphorylation, because phosphorylation of all KCC isoforms at Site-2 is abolished in embryonic stem cells lacking SPAK/OSR1 kinase activity. Cells with engineered point mutations silencing catalytic function of both SPAK and OSR1. Furthermore, these SPAK/OSR1-deficient cells have increased basal activity of Cl⁻-dependent, furosemide-sensitive ⁸⁶Rb⁺ flux, consistent with KCC activation (36).

Together, these data reveal that WNK-regulated SPAK/OSR1 directly phosphorylate NKCC1 and KCC2, promoting their stimulation and inhibition, respectively. For the N[K]CCs, WNK isoforms bind, phosphorylate, and activate SPAK/OSR1, which in turn bind and activate the N[K]CCs by directly phosphorylating a tripartite cluster of N-terminal conserved Thr residues in each cotransporter. For the KCCs, the mechanism is more complex and not yet completely defined, but the current data suggest a model by which the WNKs bind, phosphorylate, and activate SPAK/OSR1, which in turn binds and partially inhibits the KCCs by directly phosphorylating Site-2. WNKs also regulate Site-1 phosphorylation, likely through a yet-to-be-identified kinase (Fig. 3).

How are WNKs regulated? In response to a reduction in [Cl⁻]_i, exposure of cells to hyperosmotic conditions, or these stimuli can separately activate WNK function. A reduction in cell volume, WNK isoforms are activated following autophosphorylation of their T-loop residue (Ser³⁸² in WNK1) (56-58). How WNK isoforms sense these conditions is poorly understood. However, a potential

breakthrough in our understanding of how WNKs might sense chloride has emerged from the analysis of the crystal structure of the kinase domain of WNK1, revealing that it directly bound to a chloride ion (59). Biochemical and mutational data suggested that Cl⁻ binding to this site inhibits autophosphorylation of WNK1, thereby inhibiting kinase activity (59). These results, therefore, suggest that the catalytic domains of WNKs function as direct Cl⁻ sensors and that in low [Cl⁻]_i, OK the dissociation of Cl⁻ from the kinase domain of WNKs results in their activation. Although this is an attractive model, further work is needed to validate this model in an in-vivo setting.

Identifying upstream regulators of WNK activity

Whole-exome sequencing experiments by the Lifton (60) and Jeunemaitre (61) groups have identified mutations in the ubiquitin E3 ligase components Cullin-3 (CUL3) or Kelch-like 3 (KLHL3) in families with PHAII that do not have mutations in WNK-encoding genes. Subsequent experiments revealed CUL3 and KLHL3 form a heterodimeric complex, with CUL3 mediating the ubiquitylation of substrates and KLHL3 functioning as the substrate-recognition moiety (Fig. 3) (62-64). WNKs contain XXX, a degron recognized by KLHL3 (XX). The CUL3-KLHL3 complex interacts with and ubiquitylates WNK isoforms; most PHAII-causing KLHL3 mutations inhibit binding to either WNK isoforms or CUL3 (62). Consistent with this, CUL3-KLHL3 complexes containing disease-associated mutations failed to ubiquitylate WNK1 in vitro (60, 62-65). Refs reordered.

Genetic data supporting a role for WNK-SPAK/OSR1 in essential hypertension

One quarter of adults in Western societies have increased blood pressure (that is hypertension), which is a major risk factor for ischemic and hemorrhagic stroke, congestive heart failure, and end stage renal disease (66). Hypertension is a tremendous burden on the budgets of healthcare systems worldwide; more than \$130 billion was spent on the treatment of this condition in 2010 (66). Although lifestyle changes can sometimes ameliorate hypertension, most patients require drugs to lower blood pressure. However, many patients on multidrug regimens with currently available agents (for example, thiazides, Ca²⁺-channel blockers, angiotensin-converting enzyme inhibitors, and loop diuretics) have poorly controlled disease or suffer from side effects of the drugs, such as orthostatic hypotension and K⁺ wasting. The treatment of hypertension is, therefore, an area of continuing clinical need, and the development of potent drugs with fewer side effects would be useful.

In the kidney, the WNK-SPAK/OSR1-mediated activation of NCC and NKCC2, which together mediate ~25% of renal salt reabsorption, is critical to maintain extracellular volume, which in turn influences blood pressure and electrolyte homeostasis. NCC is inhibited by thiazides, and NKCC2 is inhibited by furosemide (Fig. 1) – these two drugs are some of the most common agents used in the treatment of hypertension and edematous states (XX)XXXthis is so common I don't think we need citation; it's like saying Tylenol is used for headacheXXX. Furthermore, the importance of the WNK-

SPAK/OSR1-CCC pathway for renal physiology is exemplified by both human and mouse genetics.

Starting with the upstream regulators, loss-of-function mutations in *KLHL3* and *CUL3*, XXXall these mutations in are humans!XXX genes encoding negative regulators of WNK1 and WNK4, cause PHAI by increasing WNK1 and WNK4 abundance (61-65, 67-70). The KLHL3 degron binding site in WNK4 encompasses residues that are mutated in PHAI XXXGordon's syndrome synonymous with PHAI. PHAI disease-causing WNK4[D564A] and WNK4[Q565E] mutations suppress the interaction with KLHL3 (62, 71). Moreover, the WNK4[D564A]-knockin mice display increased abundance of WNK4 (53). A KLHL3[R528H]-knockin mouse, which mimics the most frequent KLHL3 mutation associated with PHAI in humans, display a marked PHAI phenotype, including increased blood pressure and increased abundance of WNK1 and WNK4 (XX)(72).

In mice, gain-of-function mutations in *WNK1* and *WNK4* result in increased phosphorylation and activation of NCC and NKCC2, which causes hypertension in this mouse model of human PHAI (73-76). In distal nephron cells, WNK4 inhibits epithelia sodium channels (ENaC) (77), decreased ENaC abundance compensates for the increased NCC activity following inactivation of the kidney-specific isoform of WNK1 and prevents hypertension in mouse models (78).

Genome-wide association studies of systolic and diastolic blood pressure reveal a strong association with common variants of *SPAK* and blood pressure variation (79, 80). *SPAK*-knockout mice exhibit reduced NCC activation (81) and knockin mice expressing *SPAK* or *OSR1* mutants that cannot be activated by WNK isoforms exhibit reduced NCC- and NKCC2-activating phosphorylation, hypotension, and are resistant to hypertension when crossed to transgenic knockin mice bearing a PHAI-causing mutant WNK4 (82, 83).

Loss-of-function mutations in *NCC* and *NKCC2* cause hypotension in humans with Gitelman's syndrome and Bartter's type 1 syndrome, respectively (6, 7). Rare heterozygous mutations in *NCC* and *NKCC2* alter renal NaCl handling and contribute to blood pressure variation and susceptibility to hypertension in the general population (84). A mutation in *NCC* okat a residue (T60M) that abolishes the critical WNK-regulated *SPAK*-*OSR1* activating phosphorylation event causes Gitelman's syndrome in Asians (85, 86).

Together, these genetic data suggest that inhibition of the WNK-*SPAK*/*OSR1* pathway might yield a new opportunity to develop improved antihypertensives. WNK-*SPAK*/*OSR1* inhibitors are likely to have increased efficacy over either thiazides or furosemide alone, because they would simultaneously inhibit both NKCC2 and NCC activity. Additionally, WNK-*SPAK*/*OSR1* inhibitors would likely spare K⁺, which would reduce blood pressure without the side effect of hypokalemia that is commonly associated with thiazides and loop diuretics (87). How can the WNK-*SPAK*/*OSR1* pathway be targeted to treat hypertension?

Strategies of WNK-SPAK/OSR1 inhibition

Direct inhibition of the kinase activity of WNKs

One approach to WNK-SPAK/OSR1 inhibition might be to exploit the atypical position of the catalytic lysine residue in the WNKs, which is unique compared with all other kinases in the human proteome (Fig. 4A)xxxagreed, we emailed the PDB #sXXX. This feature could be potentially utilized to develop WNK-specific ATP-competitive inhibitors. However, there are four different WNKs encoded by separate genes and the proteins have highly similar kinase domains. Furthermore, each of the *WNK* genes encodes alternatively-spliced isoforms with discrete spatial and temporal expression profiles. For example, WNK1 and a renal-specific isoform of WNK1, lacking the kinase domain, bind one another, and this interaction is affects NCC activity (22, 88). Some reports suggest that WNK4 inhibits WNK1 in some contexts (89), suggesting that WNK4 inhibition might stimulate renal NaCl reabsorption and increase blood pressure. In contrast, reports characterizing WNK4-knockout mice suggest that WNK4 is the key regulator of NCC phosphorylation in the kidney (74, 76). Harnessing the tissue-specific localization of WNK isoforms will be critical in the development of drugs that exert specific effects on the kidney (or other target tissues, such as the central nervous system) without interfering with WNK activity in the other tissues in which it is present and plays a vital role in cellular ionic homeostasis. Presently, it is not clear which isoform(s) of WNKs would need to be inhibited to treat hypertension.

Inhibition of the kinase activity of SPAK/OSR1

An alternative, and potentially more straightforward approach, would be to target SPAK or OSR1, which are likely to function redundantly in the regulation of NCC and NKCC2. The SPAK and OSR1 kinase domains are ~90% identical; therefore, drugs inhibiting both isoforms could be developed. A dual SPAK/OSR1 inhibitor would likely be more efficacious at blood pressure reduction over current agents that target either NCC or NKCC2 alone, because SPAK/OSR1 inhibition would coordinately reduce the activities of both NCC and NKCC2, as well as other substrates of these kinases (for example, other WNK-associated ion channels like ENaC) that are important for renal physiology.

Inhibiting the interaction between WNKs and SPAK/OSR1

Because hypertension is a chronic, mostly asymptomatic condition, it will be important to develop WNK or SPAK/OSR1 inhibitors that are sufficiently selective that do not cause intolerable side effects by inhibiting other signaling components. The strategy of targeting the ATP-binding site of the SPAK/OSR1 or WNK kinases raises concern regarding the ability to develop sufficiently selective inhibitors that do not suppress other kinases. The development of STOCK1S-50699 has introduced the possibility of developing inhibitors of SPAK/OSR1 signaling by targeting the CCT domain rather than the kinase domain. Crystallographic analysis demonstrates that the CCT domain adopts a unique fold not found in other proteins and possesses a pocket that forms a network of interactions with the conserved RFXV/I residues on WNKs and substrates!xxxgreat idea!xxx(Fig. 4B) (90). A compound that binds to this structurally distinct CCT domain

pocket and thus blocks the interaction with the RFXI/V motif could display high selectivity and not interfere with other signaling pathways.

The Uchida group has exploited this biochemical information and performed high-throughput screening of > 17,000 chemical compounds with fluorescent correlation spectroscopy to discover inhibitors that disrupt the WNK(RFXV/I)-SPAK/OSR1(CCT) interaction (37). This screen identified STOCK1S-50699 and STOCK2S-26016. In vitro, both compounds inhibit binding of the CCT domain to RFXV motifs; in cellular studies, only STOCK1S-50699 suppresses SPAK/OSR1 and NKCC1 phosphorylation induced by hypotonic low chloride conditions (37). Neither STOCK1S-50699 nor STOCK2S-26016 inhibited the activity of 139 different protein kinases tested (37). Further experiments are required to study the pharmacokinetics and pharmacodynamics of STOCK1S-50699 to establish whether it could be tested in preclinical animal models. However, these initial studies offer encouragement that targeting the CCT domain could lead to the development of a novel class of antihypertensive drugs. In addition to inhibiting the activity of NCC and NKCC2, while concurrently sparing renal K⁺ wasting, WNK-SPAK/OSR1 inhibition may elicit antihypertensive effects by decreasing NKCC1-mediated vasoconstriction in blood vessels (81). Such an action would offer synergistic effects on both renal and extrarenal targets for blood pressure reduction.

Targeting KLHL3-CUL3, upstream regulators of WNK

The WNK-SPAK/OSR1 kinase cascade could also be inhibited indirectly by targeting their upstream regulators. The crystal structure of the KLHL3 kelch domain in complex with the degron motif of WNK4 reveals that the degron motif forms an intricate web of interactions with conserved residues on the surface of the kelch domain β -propeller (71) (Fig. 4C). xxxyes that would be great!xxxMany of the disease-causing mutations in either WNK4 or KLHL3 inhibit binding by disrupting critical interface contacts, and are thus likely to result in reduced ubiquitylation and increased abundance of WNK isoforms (71). Indeed, KLHL3[R528H]-knockin mice, in which Arg⁵²⁸ that makes critical interactions with the WNK4 degron motif is mutated, display a marked PHAll phenotype that includes increased blood pressure and increased abundance of WNK1 and WNK4 isoforms (XX) (72). These data, therefore, point towards CUL3 and KLHL3 mutations resulting in inappropriate activation of the WNK-SPAK/OSR1 kinase cascade and hence causing hypertension through excess activity of the WNK=SPAK.OSR1-CCC pathway. Therefore, it would be interesting to explore whether it would be possible to identify compounds that promote binding of KLHL3 to either CUL3 or WNK1 and WNK4 to lower blood pressure by stimulating ubiquitylation and degradation of WNKs.

Achieving tissue-specific effects by enhancing this upstream negative regulation could be difficult. WNK2 and WNK3, WNK isoforms that function in the brain, could also be substrates of KLHL3-CUL3 E3 ligase, because the KLHL3-binding domain of WNK4 is conserved in all WNK isoforms (71). Moreover, when coupled with CUL3, KLHL2, which shares greater than 90% homology with KLHL3, could function as an E3 ligase for all WNK isoforms (XX)(71). Thus, KLHL2 or KLHL3 may regulate WNKs in tissues outside the kidney. Consistent with this, the crystal structure of KLHL2 bound to the WNK4

degron has also been elucidated, indicating that KLHL2 can interact with this WNK isoform in a manner similar to that of KLHL3 with WNK4 (71).

Inhibition of MO25, a key SPAK/OSR1 regulator

Mouse protein-25 (MO25) alpha and beta isoforms were originally identified as critical scaffolding subunits required to bind to the STRAD pseudokinase and stabilize it in a conformation that can activate the LKB1 tumor suppressor kinase (91). Further studies demonstrated that MO25 isoforms have roles in binding to several STE20 family kinases, such as SPAK and OSR1 kinases, and induce their kinase activity for approximately 100-fold, and dramatically enhancing their ability to phosphorylate the ion cotransporters NKCC1, NKCC2 and NCC (52), whereas for MST3, MST4 and YSK1 involved in controlling development and morphogenesis, MO25 isoforms also stimulate their kinase activity 3- to 4-fold based on in vitro kinase assays (52, 92, 93). However, the regulatory mechanism of MO25-mediated kinase activation is not fully understood. Structural studies of MO25 alpha-STK25 and MO25 alpha-MST3 reveal that MO25 binds to and activates STE20 family kinases or STRAD pseudokinase through a unified structural mechanism, featuring an active conformation of the α C helix and A-loop stabilized by MO25, represent a transition and intermediate state and a fully activated state, respectively (94). Therefore, compounds interfere with MO25 isoforms binding to SPAK and OSR1 would reduce the activity of these kinases and could potentially represent a strategy for reducing blood pressure. However, it would be important that these compounds not affect regulation of other STE20 kinases or STRAD pseudokinase controlled by MO25 isoforms.

Targeting WNK-SPAK/OSR1 in neurological diseases associated with GABAergic xxxsynonomous terminologyxxx, would use GABAergicXXXdisinhibition

GABA is main inhibitory neurotransmitter in the adult central nervous system, exerting its fast synaptic hyperpolarizing effect through the activation of ligand-gated, Cl⁻-permeable, GABA_A receptors (GABA_ARs). However, in immature neurons, GABA_AR-mediated responses are depolarizing and even excitatory, which is important for neuronal proliferation, migration, and synaptogenesis (95, 96). A developmental increase in the activity of the Cl⁻-extruding KCC2 relative to Cl⁻-importing NKCC1 reduces the concentration of [Cl⁻]_i of neurons to amounts that favor GABA_AR-mediated hyperpolarization and inhibition in the mature central nervous system. KCC2 deficiency in worms (97, 98), flies (99, 100), and mice (101) results in neuronal network hyperexcitability. In humans, pathological KCC2 functional downregulation and NKCC1 upregulation, which increases neuronal [Cl⁻]_i to facilitate GABA_AR-mediated depolarization, has been demonstrated or implicated in the pathogenesis of several neurological disorders, including various subtypes of epilepsy, posttraumatic spasticity, neuropathic pain from peripheral nerve injury, and autism (102) (Fig. 4).ok

Despite the role of impaired GABAergic inhibition in these conditions, existing GABA_AR modulators, such as benzodiazepines xxxyes, I am sure of this, major side effectsxxx or barbiturates, are rarely successfully used for treatment because of their narrow therapeutic window and unwanted side effects, such as sedation or motor impairment.

Furthermore, in some situations, such as neonatal seizures, these drugs have paradoxical depolarizing and excitatory effects due to the increased concentrations of $[Cl^-]_i$ in immature or injured neurons. Given these limitations of targeting GABA_AR directly, a promising “indirect” approach to anti-epileptic, analgesic, or anti-spasmodic drug development might be the modulation of neuronal Cl^- gradients through NKCC1 inhibition and KCC2 activation (103). Targeting these CCCs would not affect neuronal excitability directly, but would reduce neuronal excitability by lowering $[Cl^-]_i$ to restore endogenous inhibition. This approach would likely yield more specificity xxxyes, GABA affects both GABA_A and GABA_B receptors, as do GABAergic drugs, but GABA_A receptors are more relevant for the pathologies I am discussing herexxx and a more favorable side effect profile than existing GABAergic agents. although drugs exist to inhibit the N[K]CCs, these drugs generally have poor penetration into the central nervous system (104). However, prodrug forms of bumetanide that have improved BBB permeability show therapeutic potential (105). Large-scale screening efforts have identified drugs that inhibit (106) or activate (107) KCC2. Because the CCCs work in concert with one another to achieve homeostasis, it is unknown whether functional compensation may occur through other CCC family members. For example, populations of post-synaptic cortical neurons have NKCC1, KCC2, and KCC3. The optimal drug would be one that targets the influx and efflux CCCs simultaneously, yet with opposite effects – thereby synergistically achieving net Cl^- influx or efflux (9) (Fig. 4).xxxok

Indeed, genetic or pharmacological inhibition of WNK-SPAK/OSR1 activity would promote cotransporter dephosphorylation, inhibiting NKCC1 and activating KCC2, with anticipated net effects of enhancing cellular Cl^- extrusion. In neurons, enhancing cellular Cl^- extrusion would facilitate GABA_AR-mediated hyperpolarization and thus inhibit neuronal activity. We, therefore, postulate that regulation of NKCC1 and KCC2 by WNK-SPAK/OSR1-mediated phosphorylation may be a key determinant of the molecular mechanism underlying the paradoxical depolarizing (excitatory) effect of GABA in hyperexcitable disease states. Thus, inhibition of WNK-SPAK/OSR1 in these states might provide a means of enhancing Cl^- extrusion and facilitating inhibition through combined inhibitory and stimulatory effects on NKCC1 and KCC2, respectively. These efforts may prove useful for multiple syndromes that are associated with depolarizing responses to GABA and altered Cl^- homeostasis (108-114). Genetic mutation that inhibits the kinase activity of WNK3, which is abundantly expressed in the developing brainxxxyes, is an effective means of concurrently inhibiting NKCC1 activity and stimulating KCC2 activity in heterologous expression system like oocytes (46, 47).

Mutations in a nervous system-specific isoform of WNK1, HSN2xxx, no, the isoform is termed HSN2, hence the accepted nomenclature “WNK1/HSN2”, have been found in a disease with symptoms that include congenital pain insensitivity, hereditary sensory and autonomic neuropathy type II (HSANII) (115). The gene encoding HSN2 is highly expressed in the dorsal horn of the spinal cord, a key site of expression of the gene encoding KCC2. In the spinal cord dorsal horn, normally inhibitory GABAergic interneurons synapse with both presynaptic (primary sensory) and postsynaptic (lamina

l) dorsal horn neurons to modulate afferent input. In neuropathic pain states, such as those induced by peripheral nerve injury, GABA disinhibition due to a pathological decrease in KCC2 activity and the resulting increase in $[Cl^-]_i$ causes hyperpolarizing GABA synaptic currents to become depolarizing, which lowers the threshold for A-fiber-mediated transmission to central nociceptive lamina I neurons (116, 117). In this situation, a positive modulator of KCC2 would be expected to provide effective therapy by restoring neuronal anion gradients and GABAergic inhibition (107). Indeed, drugs that enhance KCC2 activity have been developed and exhibit efficacy in cell models and in animal models of neuropathic pain. Targeting WNK-SPAK/OSR1 in this context might also be valuable. The gene encoding HSN2 is also highly expressed in the dorsal root ganglion, where the genes encoding NKCC1 and KCC3, (but not the one encoding KCC2) are highly expressed, and these cotransporters are responsible for the unusually high $[Cl^-]_i$ in these primary sensory neurons and GABA-stimulated depolarization (118). Data supporting these hypotheses are emerging and targeting the WNK-SPAK/OSR1-CCC pathway in these types of neuronal hyperexcitability disorders will be rich areas of future investigation.

Conclusion and future directions

The importance of coordinating cellular Cl^- influx and efflux in renal epithelia and neurons is well known (4 [ENREF 4](#), 119). The finding that SPAK/OSR1 phosphorylate and thereby trigger activation of the Na^+ -driven, Cl^- -influx CCCs (NKCC1, NKCC2, and NCC) and also phosphorylate and inhibit K^+ -driven, Cl^- -efflux CCCs (KCC1, KCC2, KCC3, and KCC4) helps explain how the CCCs are reciprocally and coordinately controlled to achieve homeostasis in multiple tissues. The WNK-SPAK/OSR1-CCC pathway is critically important for normal human physiology, making it an attractive target for drug development. Analysis of humans and mice with mutations in this pathway has illustrated the potential benefits of targeting this pathway in human diseases. One specific mechanism for interfering with this pathway is by the targeting of the CCT domain within SPAK/OSR1, which interferes with the activation of SPAK/OSR1 by WNK. A disease amenable to inhibition of the WNK-SPAK/OSR1 pathway would include essential hypertension, one of the most common diseases of the industrialized world. In addition, given the recent enthusiasm for the discovery of KCC2 activators that enhance neuronal Cl^- extrusion in diseases with GABAergic disinhibition, exploring the effects of WNK-SPAK/OSR1 inhibition in ameliorating seizures, neuropathic pain, spasticity, and other diseases associated with neuronal excitability is compelling. WNK-SPAK/OSR1 inhibition not only enhances cellular Cl^- extrusion by concurrently inhibiting NKCC1-mediated Cl^- influx through NKCC1 and activating KCC-mediated Cl^- efflux through the KCCs, but the WNK-SPAK/OSR1 cascade also serves as both the sensor and transducer of Cl^- perturbation. Therefore, targeting these kinases might also prevent inhibition of feedback on other CCCs or molecules that aim to equilibrate ion gradients, offering a coordinated, multivalent, and sustained effect.

Figure 1. WNKs regulate NCC and NKCC2 through the kinases SPAK and OSR1 to achieve blood pressure and K⁺ homeostasis humans. WNK1 and WNK4 are abundant in the kidney. Inhibition of WNKs in the kidney is predicted to elicit a K⁺-sparing, anti-hypertensive effect by reducing the reabsorption of NaCl by NCC in the distal collecting and connecting tubules (DCT/CNT) and by NKCC2 in the thick ascending limb (TAL). Red asterisks depict nodes in the signaling pathway where inhibition would be expected to decrease blood pressure. The blue asterisk depicts a node where stimulation would be expected to decrease blood pressure. Mendelian diseases labeled in blue are those resulting from mutation of the indicated gene in humans. PHAI1 = pseudohypoaldosteronism type II. CNT = connecting tubule. TAL = thick ascending limb. STOCK1S-50699, a recently-developed WNK-SPAK/OSR1 inhibitor.

Figure 2. The N[K]CCs and KCCs are reciprocally regulated by reversible serine-threonine phosphorylation. Hypotonic low Cl⁻ conditions or a reduction in cell volume (not shown) lead to phosphorylation and activation of the N(K)CCs and phosphorylation and inhibition of the KCCs to promote Cl⁻ and water influx through N[K]CCs. If intracellular Cl⁻ become too high or cell volume increases, the cotransporters become dephosphorylated because their upstream kinases are inhibited and Cl⁻ and water efflux through KCCs occurs to restore ion and osmotic homeostasis. Cl⁻ and water are indicated by the blue fill.

Figure 3. Domains and sites important for regulation of and signaling through the WNK-SPAK/OSR1 pathway. Proteins with slashes indicate that multiple isoforms have the same properties. For SPAK/OSR1, the residue numbering above the protein represents SPAK and the residue numbering below represents OSR1. Kinase X refers to a yet-unidentified kinase that is regulated by WNKs and mediates the direct phosphorylation and inhibition of site 1 on the KCCs. Rbx and Nedd8 are part of the ubiquitin ligase complex. E1 and E2 represent the two enzymes involved in transfer of ubiquitin (Ub) onto itself to form polyubiquitin chains. STOCK15-5069 is a small molecule inhibitor that blocks the interaction between SPAK/OSR1 and WNK by binding to the CCT domain.

Figure 4. Structural insights into the WNK-SPAK/OSR1 pathway. (A) The kinase domain of WNK showing the unusually positioned Lys in the catalytic site. Based on PDB XXX. (B) The CCT domain bound to an XXX peptide from WNKX. Based on PDB XXX. (C) The kelch domain of KLHL3 bound to the WNKX degron motif. Based on PDB XXX.

Figure 5. A strategy to facilitate neuronal Cl⁻ extrusion by inhibiting the WNK-SPAK/OSR1 pathway. Top shows that switch in the abundance of NKCC and KCC2 that occurs during postnatal development. This switch converts the GABAergic signal from depolarizing to hyperpolarizing. Left middle shows that in developing neurons and in some diseased neurons, [Cl⁻]_i (blue fill) is increased due to high NKCC1 activity, low

KCC2 activity, or both. Activation of GABA_AR results in Cl⁻ efflux, depolarization and excitation.. Right middle shows that in healthy mature neurons, [Cl⁻]_i because KCC2 activity predominates and GABA_AR activation results in Cl⁻ influx and hyperpolarization.

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