Hereditary sensory and autonomic neuropathy type 2 is a rare autosomal recessive pathology presenting with early onset peripheral sensory defects. It arises from mutations affecting a specific isoform of the WNK1 kinase (with-no-lysine protein kinase 1) termed WNK1/HSN2. The role of WNK1 in the nervous system is not well understood. In our recent paper, we examined the effect of a pathological loss-of-function of the Wnk1/Hsn2 isoform on the development of the peripheral nervous system of the zebrafish embryo. Upon Wnk1/Hsn2 silencing using antisense morpholino oligonucleotides, we observed defects in the development of the sensory peripheral lateral line (PLL). Phenotypical embryos were also found to overexpress RNA for potassium-chloride cotransporter 2 (KCC2), a downstream target of WNK1 phosphorylation. Injection of recombinant mRNA for active KCC2, but not for inactive mutant KCC2-C568A, replicated the PLL defects observed in wnk1/hsn2 deficient animals, suggesting an essential role for WNK1/HSN2 in KCC2 regulation.

Hereditary sensory and autonomic neuropathy type 2 (HSANII) is an early onset peripheral neuropathy characterized by lack of sensory perception for all modalities in the distal limbs. Early evidence from sural nerve biopsies of HSANII patients showed a lower number of sensory fibers but no sign of neurodegeneration, leading to the hypothesis that this pathology has a developmental origin. Many mutations have been identified in HSANII patients, most of which are found in the alternatively spliced “HSN2” exon of the WNK1 gene (with-no-lysine protein kinase 1), coding for a ubiquitously expressed serine/threonine kinase. The WNK1/HSN2 isoform has a marked expression in the nervous system and a specific axonal localization at the level of the neuron. HSANII-linked mutations are located within the HSN2 exon and lead to a truncated protein isoform, which is non-functional. As WNK kinases (four family members) are known to regulate the activity of potassium-chloride cotransporters of the KCC family (coded by SLC12 genes) by phosphorylation, our recent paper focused on the role of protein isoform WNK1/HSN2 and its interaction with KCC2 (Potassium-chloride cotransporter member 5, SLC12A5 or KCC2 gene) in HSANII pathogenesis.

The posterior lateral line (PLL) of the zebrafish embryo appeared to be an appropriate model for studying the role of Wnk1/Hsn2 in peripheral nerve development. Indeed, we observed expression of Wnk1/Hsn2 and Kcc2 in the neuromasts of the PLL, a peripheral mechanosensory system formed when a group of progenitor cells (the primordium) migrates from the cephalic placodes along the midline of the tail and deposits bundles of cells, leaving behind the PLL ganglion. The progenitor bundles then differentiate into functional sensory organs named neuromasts, using Delta/Notch signaling to specify differentiation into either support cells or sensory hair cells. The posterior lateral line nerve, trailing the migration of the primordium, will innervate the neuromasts, completing a basic functional PLL by 72 h post-fertilization (hpf).

Using antisense oligonucleotide technology, we silenced the expression of the Wnk1/Hsn2 isoform in embryos to mimic
the pathogenic loss of function in HSANII and observed a defect in the development of the PLL. The knockdown embryos had fewer neuromast organs, which were themselves composed of fewer cells. The PLL progenitor primordium was also of a smaller size in absence of Wnk1/Hsn2, despite being constituted of normal sized and adequately organized cells. These defects could be partially rescued by the injection of a recombinant human WNK1 mRNA, but not by injection of mRNA coding for a short variant mimicking the truncation of the protein resulting from HSNAII mutations, even though both constructs contained the full HSN2 exon.

Previous studies showed that WNK1 is responsible for phosphorylating sites that control the cotransporter activity in all KCC family members, probably by inhibiting surface expression at the cell membrane. It was found that phosphorylation by WNK1 is essential for deactivation of the KCCs and that it occurs in immature neurons but is absent in adult neurons. This is consistent with the expression pattern described in the zebrafish embryo, where kcc2 is increasingly expressed as neurons mature.

KCC2 was also reported as having a rapid turnover rate at the cell membrane, suggesting it has the capacity to respond quickly to changes in the environment. Additionally, KCC2 activation has been shown to be influenced by neuronal activity, neuronal, and oxidative stress, and to regulate transcription and expression levels of the co-transporter, a phenomenon possibly dependent on phosphorylation.

We therefore looked at the expression levels of the zebrafish KCC2 ortholog kcc2 (gene slc12a5) and found an overexpression at the mRNA level in Wnk1/Hsn2 knockdown embryos at 72hpf, when the developmental phenotype is visible, but also at 24hpf, when the primordium has just begun its migration. To test whether the PLL defects observed were due to KCC2 overexpression, we performed a controlled overexpression by injection of recombinant KCC2 mRNA in wild-type zebrafish eggs, producing embryos phenocopying Wnk1/Hsn2 silencing. These results suggest that KCC2 overexpression might be downstream of WNK1/HSN2 in HSANII pathogenesis. To further validate this new pathogenic pathway, we performed a double knockdown experiment where sub-optimal doses of AMOs were used in combination, to partially knockdown the expression of both Wnk1/Hsn2 and Kcc2. While each AMO injected by itself produced no discernible phenotype, the additive effect of the co-injection phenocopied the PLL defects observed upon Wnk1/Hsn2 silencing.

To determine if the defects observed in the PLL development following KCC2 mRNA overexpression were due to the cotransporter’s function at the membrane, we injected recombinant mRNA for an inactive KCC2 point mutant, KCC2-C568A. This dominant negative mutant was shown to lead to disrupted chloride extruding function and to be incapable of being expressed at the cell membrane due to impaired interaction with structural protein 4.1N, responsible for linking the cotransporter with the cytoskeleton. We observed that an overexpression of KCC2-C568A mRNA mimicked the overexpression of wild-type KCC2 mRNA and the knockdown of Wnk1/Hsn2 by producing embryos with the previously described PLL defects. We therefore propose that WNK1 mutations linked with HSANII function via an overexpression of KCC2 mRNA, and that the cotransporter can act independently of its chloride-extruding function to interfere with proper development of the peripheral sensory system (Fig. 1). This is consistent with recent reports that KCC2 in developing rat brain is found in a larger proportion in the cytoplasm, rather than being associated with the plasma membrane, supporting a role independent of its chloride-extruding function.

The expression of this neural-specific cotransporter is responsible for the developmental GABAergic response switch. Effectively, in the mature brain, GABA is the main inhibitory neurotransmitter, but it is excitatory during development. In immature neurons, KCC2 is absent from the membrane, but antagonistic cotransporter NKCC1 is active, leading to a higher intracellular chloride concentration. The activation of KCC2 takes place when neurons undergo maturation and
Once it is located at the membrane, causes the efflux of K-Cl from the cell, leading to a reduced intracellular chloride concentration. This results in a hyperpolarizing (inhibitory) response to GABA and glycine, where it was excitatory during early development. The presence and activation of KCC2 is therefore narrowly controlled in time as proper neurogenesis depends on its chloride extrusion function. In the zebrafish embryo, neurogenesis in the spinal cord has been shown to be regulated by Kcc2, where a precocious overexpression of the cotransporter led to fewer neurons, stalling progenitors before differentiation.10 At the level of the PLL, we found that neuromast hair cells loaded with a Ca2+ indicator did not respond to glycine application but evoked Ca2+ transients upon glutamate application, suggesting that these cells are viable but that the presence of Kcc2, responsible for lowering intracellular chloride levels, made them unresponsive to glycine. On the contrary, progenitor cells composing the PLL primordium responded to the application of glycine. These results suggest that the PLL progenitors, like the ones found in the spinal cord10 have a high intracellular chloride concentration and support the hypothesis that Kcc2 expression at the level of the neuromasts might have a role in their differentiation.

Although phosphorylation studies were not feasible in our model, it would be of great interest to understand the interaction between KCC2 and WNK1. It was previously suggested that regulation of KCC2 activation by the WNK kinases might be a direct interaction, or via the inactivation of downstream phosphatases, but it might also implicate the SPAK/OSR1 pathway, as is the case for phosphorylation of antagonist cotransporter NKCC1.16 However, it was reported that the expression of a mutant form of WNK3 lacking a specific SPAK binding site (WNK3-F1337A) led to loss of activation for NKCC2 but retained inhibition of KCC4 activity, suggesting the latter to be independent of SPAK/OSR1 interaction.17 Additionally, knockdown of SPAK and OSR1 did not affect phosphorylation of sites T991 and T1048 of KCC3, while knockdown of WNK1, but not other WNK family members, reduced it.1 These results suggest that while the regulation of KCC and NKCC are both dependent on WNK1 activity, only NKCCs are phosphorylated via downstream kinases SPAK/OSR1. As our in vivo model did not permit verification of the ratio of plasmalemmal and cytosolic KCC2 expression or its phosphorylation state in either localization, further study needs to be done to uncover more of the pathogenic pathway leading to HSANII-linked developmental deficits.

Recent studies describe the presence of the WNK1/HSN2 isoform outside of the nervous system, where it was thought to be confined.5,18 While presence of this isoform in non-neuronal tissue, like kidneys and testis might not be explained by our HSANII pathogenesis model, presence in satellite cells, which are glial cells of the peripheral nervous system,19 could also support the involvement of KCC2 in HSANII pathogenesis. Indeed, KCC2 was found to be expressed at the level of the nodal segments of peripheral nerve axons and in Schwann cells,17 and to colocalize with transport vesicles15 indicating an overlap with the localization of the WNK1/HSN2 isoform. In fact, early reports showed that this isoform has a particular sub-localization within neurons when compared with other WNK1 isoforms: it is also expressed at the level of the axons while others are restricted to the cell body.3 As KIF1a (coding for kinesin family member 3) mutations have been found in HSANII patients,20 it is tempting to speculate that the WNK1/HSN2 isoform has other functions that are essential to the proper development of peripheral sensory neurons. Indeed, the KIF1a kinesin, a motor protein responsible for anterograde axonal transport of synaptic vesicles, was reported to interact with a domain encoded by the HSN2 exon.9 As WNK1/HSN2 and Kif1a colocalize at the level of the axon, we could hypothesize that this specific interaction affects the function of the motor protein, whether it be via phosphorylation, which has been shown to influence both cargo binding and motor domain stability, or through other mechanisms.21,22 In the context of WNK1/HSN2 mutations, we could conjecture that the few peripheral sensory neurons that would develop following pathogenic changes in KCC2 activity and expression during early development would do so improperly, perhaps due to deficient axonal transport. The resulting alterations, possibly in distribution of specific cargo at the nodes of Ranvier, could then interfere with their function.

While our model is a first insight into the mechanisms underlying HSANII pathophysiology, much work remains to be done to uncover the functional contribution of the WNK1/HSN2 kinase isoform to health and disease as it has proven to have an essential role in proper development of the peripheral sensory system.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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