WNK1/HSN2 Regulates Neural Development via GSK3 β

Masahiro Shimizu

Tokyo Medical and Dental University (TMDU)

Hiroshi Shibuya (✉ shibuya.mcb@mri.tmd.ac.jp)

Tokyo Medical and Dental University (TMDU)

Research Article

Keywords:

Posted Date: February 28th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1383513/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

With no lysine kinase 1 (WNK1) phosphorylates and activates STE20/SPS1-related proline-alanine-rich protein kinase (SPAK) and oxidative stress responsive kinase 1 (OSR1) to regulate ion homeostasis in the kidney. Mutations in *WNK1* result in dysregulation of the WNK1-SPAK/OSR1 pathway and cause pseudohypoaldosteronism type II (PHAII), a form of hypertension. WNK1 is also involved in the autosomal dominant neuropathy, hereditary sensory and autonomic neuropathy type II (HSANII). Mutations in a neural-specific splice variant of *WNK1* (HSN2) cause HSANII. However, the mechanisms underlying HSN2 regulation in neurons and effects of HSN2 mutants remain unclear. Here, we found that HSN2 regulated neural development through OSR1 activation and glycogen synthase kinase 3 β (GSK3 β). Moreover, HSN2-OSR1 and HSN2-GSK3 β signalling induced expression of *LIM homeobox 8* (*Lhx8*), which is a key regulator of cholinergic neural function. The HSN2-OSR1/GSK3 β -LHX8 pathway is therefore important for neural development. Consistently, HSN2 mutants reported in HSANII patients suppressed SPAK and OSR1 activation and LHX8 induction. Interestingly, HSN2 mutants also suppressed neural development by preventing interaction of between wild-type HSN2 and GSK3 β . These results indicate that HSN2 mutants cause dysregulation of neural development via GSK3 β in the HSN2 and/or WNK1 pathways.

Introduction

The with no lysine (WNK) family of serine/threonine protein kinases is conserved in many species^{1,2}. There are four mammalian WNK family members, WNK1–4, and mutations in *WNK1* and *WNK4* cause a hereditary form of hypertension, pseudohypoaldosteronism type II (PHAII)³. WNK1 and WNK4 phosphorylate and activate SPAK and OSR1 kinases, which subsequently induces activation of ion co-transporters, such as NKCC1, NKCC2 and NCC in the kidney^{4–7}. Moreover, *Wnk4*^{D561A} knockin mice exhibit phenotypes similar to PHAII⁸. These data indicate that dysregulation of the WNK-SPAK/OSR1 pathway causes unbalanced ion homeostasis and results in hypertension with hyperkalaemia in PHAII patients.

A neuron-specific *WNK1* splice variant, called HSN2, is expressed in the brain, dorsal root ganglia and sciatic nerve^{9,10}. Mutations in a neural specific exon of *HSN2* cause the hereditary neuropathy, hereditary sensory and autonomic neuropathy type II (HSANII)^{9,11–15}. HSANII is an autosomal dominant neuropathy characterized by sensory dysfunction, such as loss of pain, touch and temperature sensation¹⁶ and limb abnormalities, such as finger deformity and Charcot's joint¹⁵. Knockdown of *HSN2* enhances K⁺-Cl[−] cotransporter 2 (KCC2) expression and leads to abnormal neuromast development in zebrafish embryos¹⁷. Moreover, a knockout mouse lacking the *Hsn2* exon of *Wnk1* showed reduced pain hypersensitivity and restored γ -aminobutyric acid (GABA)-mediated depolarization after nerve injury¹⁸. Although these reports indicate that HSN2 has an essential neural function, the precise mechanism by which HSN2 mutants cause HSANII is still unclear.

LIM homeobox 8 (LHX8) is a LIM homeobox transcription factor and a key regulator of cholinergic neural function¹⁹. LHX8 is also involved in the determination of cholinergic or GABAergic cell fate²⁰. Furthermore, LHX8 is required for cholinergic neurons in the ventral forebrain^{21,22}. Consistent with these observations, we have reported that WNK1 and WNK4 induce LHX8 through OSR1 and GSK3 β and that this signalling pathway is important for cholinergic neural specification^{23,24}. These findings indicate that not only WNK1 but also HSN2 regulates neural development via LHX8 induced by OSR1 and GSK3 β .

In this study, we analysed HSN2 functions in the OSR1-LHX8 pathway. We found that HSN2 was involved in the phosphorylation of SPAK and OSR1 and induced *Lhx8* expression to modulate neural specification identically to WNK1. We also demonstrated that HSN2 mutants suppressed OSR1 activation, *Lhx8* expression and effects of GSK3 β on neural specification. These results reveal a novel role of HSN2 in neural development and help to elucidate the pathogenic mechanism of HSNII.

Materials And Methods

Cell culture and treatment

HEK293T and Neuro2A cells were cultured in DMEM (Gibco, Waltham, MA, USA) containing 10% foetal bovine serum (FBS). For neuronal differentiation, Neuro2A cells were treated with serum-free DMEM containing 100 ng/ml nerve growth factor (NGF) for 24 h. Images of neurite outgrowth from Neuro2A cells were obtained using Axioscope microscopes (Carl Zeiss, Germany).

Antibodies

Polyclonal antibodies, Anti-DDDDK, anti-Myc and anti-HA were obtained from Medical & Biological Laboratories (Japan). Monoclonal antibody, anti-GAPDH (5A12), was purchased from Wako (Japan). Anti-OSR1 and anti-phospho-OSR1 antibodies were prepared as previously described⁶. These antibodies were used for immunoblotting at 1/1000. Anti-Myc (9B11) monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, USA) and anti-Flag (M2) monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA), and these antibodies were used for immunoprecipitation at 1/500.

Transfection of expression vectors and siRNA

The coding sequence of the human *HSN2* gene (NM_001184985) was cloned into the pRK5 vector with different molecular tags (Myc and HA). *HSN2* mutants (2743delA⁹ and 3237_3238insT¹⁴) were also cloned into pRK5. The sequence of these constructs was confirmed using a 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Other plasmids used were: HA-WNK1, Myc-WNK1, HA-WNK4, Flag-SPAK, Flag-OSR1, Flag-mOsr1^{K46M} and Flag-GSK3 β in pRK5. Cultured cells were transfected with these constructs using polyethyleneimine (Polysciences, Warrington, PA, USA). We also performed reverse transfection of siRNA using the TransIT-X2 Dynamic Delivery System (Mirus Bio, Madison, WI, USA). siRNA mouse target sequences were: *Wnk1* 5'-GAUAGGGUGUCCUAAUUA-3', *Wnk4* 5'-

CUACUCCGAGUGUCAGAAUGC-3', *Osr1* 5'-UAAAUUCAAAUCGAAUAUCAU-3', *Lhx8* 5'-AGAAUAAGCCAUUUCUUCCTT-3' and *Gsk3 β* 5'-UAUAAUUUGGGUUCAUUUCUC-3'.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). Double-strand cDNA was prepared from total RNA using oligonucleotide (dT), random primers and Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). *Gapdh* was used to normalize the cDNA samples. The mouse sequences of the primer pairs for PCR were as follows: *Wnk1* 5'-AGAGGATGGCTCAGGTAGTCCACAC-3' and 5'-AACACACAGCTGCCCAGGAGCAGAG-3', *Wnk4* 5'-AAGCTCTGGCTGCGCATGGAGGATG-3' and 5'-GGATCGAGGTCTCCGTCGAAGAGTC-3', *Osr1* 5'-TGGCCGTCTCCATAAGACAGAGGAC-3' and 5'-TATCCGAGCCTTCAACACCAGATGC-3', *Gsk3 β* 5'-GCAGCAAGGTAACCACAGTAGTGGC-3' and 5'-TGGTGCCCTGTAGTACCGAGAACAG-3', *Lhx8* 5'-GACCCAGCTGCCAATAAGTCATACC-3' and 5'-GACACACACTCGAGCCAACTATCTC-3', *Chat* 5'-CAGTGCATGCAACACCTGGTACCTG-3' and 5'-GAACAGATCACCTCACTGAGACGG-3', *Gad1* 5'-CATCTTCCACTCCTTCGCTGCAAC-3' and 5'-CAGTCAACCAGGATCTGCTCCAGAG-3', *Gapdh* 5'-GCCATCACTGCCACCCAGAAGACTG-3' and 5'-CATGAGGTCCACCACCCTGTTGCTG-3'.

Quantitative real-time PCR

Quantitative PCR was performed using a 7300 Real-Time PCR Cycler (Applied Biosystems) and THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). The sequences of the primer pairs for mouse *Lhx8*, *Chat*, *Gad1* and *Gapdh* were described previously^{25–27}. *Gapdh* was used to normalize the cDNA samples.

Immunoprecipitation and immunoblot analysis

Cells were lysed with TNE buffer [10 mM Tris-HCl (pH 7.4), 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail cOmplete (Roche, Switzerland)]. Lysates were pre-cleared with Protein A/G PLUS-agarose (Santa Cruz Biotechnology, Dallas, TX, USA) and immunoprecipitated with the indicated antibodies. For immunoblot analysis, cell lysates or immunoprecipitates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Merck Millipore, Germany). The membranes were probed with primary antibodies, followed by incubation with horseradish peroxidase-conjugated mouse or rabbit immunoglobulin G (GE Healthcare) and visualized using Immobilon Western (Merck Millipore). The protein bands were digitalized using the image analyser LAS-4000 Mini (Fujifilm, Japan).

Statistical analysis

All experiments were independently repeated at least three times. Data are presented as the mean $\pm$ standard deviation (SD). Statistical analysis was performed with Student's t-test using Microsoft Excel (Microsoft, DC, USA). $P < 0.05$ was considered statistically significant.

Results

Wild-type but not mutant HSN2 binds to WNK1, WNK4, SPAK, OSR1 and HSN2.

HSN2 is a neural specific isoform of *WNK1*, and mutations in *HSN2* cause the autosomal recessive neuropathy, HSANII⁹. *WNK1* can form complexes with *WNK1* and *WNK4* to activate downstream effectors^{28,29}. To analyse whether *HSN2* binds to *WNK1* and *WNK4*, we transiently expressed HA-tagged *WNK1* and *HSN2* with Myc-tagged *WNK1* or *WNK4* in HEK293T cells. Cell extracts were immunoprecipitated with a Myc antibody and immunoprecipitates were subjected to immunoblotting. As shown in Fig. 1A and B, *HSN2* bound similarly to *WNK1* and *WNK4*. In HSANII patients, mutations have been identified in the neural-specific alternatively spliced exon of *HSN2*, including a 1-bp deletion (2743delA⁹, referred to as *HSN2-delA*) and a 1-bp insertion (3237_3238insT¹⁴, referred to as *HSN2-insT*). These mutations cause a frameshift and result in truncated *HSN2* proteins at amino acid 916 and 1080, respectively. We also checked the binding of these *HSN2* mutants to *WNK1* and *WNK4* and found that they could not interact with *WNK1* or *WNK4* (Fig. 1A and B). *WNK1* interacts with and phosphorylates the STE20 kinases, SPAK and OSR1, to activate the downstream pathway^{5,6}; therefore, we analysed the binding of *HSN2* and its mutants to SPAK and OSR1. We found that *HSN2* and *WNK1* bound similarly to SPAK and OSR1, but the binding of *HSN2* mutants to SPAK and OSR1 was weak compared with that of *WNK1* and *HSN2* (Fig. 1C and D). Interestingly, *HSN2* also bound to itself, but *HSN2-delA* and *HSN2-insT* could not bind to wild-type *HSN2* (Fig. 1E). These results indicate that *HSN2-delA* and *HSN2-insT* mutants are unable to promote activation of downstream signalling because the *HSN2* mutants could not interact with *HSN2*, *WNK1*, *WNK4* or SPAK/OSR1.

HSN2 mutants, *HSN2-delA* and *HSN2-insT*, exhibit loss of neural development function.

We have previously reported that *WNK1* and *WNK4* are involved in induction of neural marker genes and neurite elongation in mouse neuroblastoma Neuro2A cells^{23,24}. *HSN2* may, therefore, have similar functions in neural development and *HSN2* mutants may exhibit loss of function. To examine whether *HSN2* is involved in neural development, we transiently expressed *HSN2* in Neuro2A cells and observed that *HSN2* induced neurite elongation identically to *WNK1* (Fig. 2A upper panel). We then examined the effect of *HSN2* mutants on neural development. As shown in Fig. 2A, *HSN2-delA* and *HSN2-insT* mutants could not induce neurite elongation. Next, we checked the effects of wild-type and mutant *HSN2* on neural differentiation induced by nerve growth factor (NGF). *HSN2* constructs were transfected into Neuro2A cells and 24 h later cells were treated with serum-free medium containing 100 ng/ml NGF for 24 h. This treatment induced neurite elongation, and the expression of *WNK1* or *HSN2* enhanced this induction (Fig. 2A lower panel). In contrast, the expression of *HSN2-delA* or *HSN2-insT* suppressed neurite elongation in response to NGF-containing medium (Fig. 2A lower panel). We also analysed the expression of neural marker genes, *Lhx8* and *Choline acetyltransferase (ChAT)* for cholinergic neurons and *Glutamine acid decarboxylase 1 (Gad1)* for GABAergic neurons. qPCR analysis indicated that NGF treatment greatly induced the expression of *Lhx8*, *ChAT* and *Gad1*, and that exogenous expression of *WNK1* or *HSN2* induced *Lhx8* and *ChAT* expression (Fig. 2B). *WNK1* and *HSN2* enhanced NGF-mediated expression of *Lhx8* and *ChAT*, but reduced that of *Gad1*. In contrast, expression of *HSN2-delA* and *HSN2-insT*

insT mutants decreased *Lhx8* and *ChAT* expression and increased *Gad1* expression (Fig. 2B). These results indicate that WNK1 and HSN2 are involved in cholinergic neural specification, but that HSN2-delA and HSN2-insT mutants fail in this activity. Interestingly, HSN2 mutants inhibited NGF-independent WNK1- and HSN2-mediated neurite elongation (Fig. 2C), suggesting that these mutants have a dominant negative effect on wild-type WNK1 and/or HSN2.

As shown in Fig. 2D, *siWnk1* and *siWnk4* treatment suppressed NGF-containing serum-free medium-mediated neurite elongation in Neuro2A cells. Moreover, knockdown of *Wnk1* and *Wnk4* suppressed *Lhx8* and *ChAT* expression, and induced *Gad1* expression (Fig. 2E), indicating that WNK proteins are required for neural differentiation of Neuro2A cells, as previously reported^{23,24}. We next examined whether expression of HSN2 constructs could rescue these effects. We found that expression of WNK1 or HSN2 rescued neurite elongation and expression of neural marker genes (Fig. 2D and E). In contrast, these rescue effects were not observed with HSN2-delA or HSN2-insT mutants (Fig. 2D and E). These data indicate that HSN2 mutants fail to exert the normal functions of WNK1/HSN2 in neural development.

NGF activates SPAK/OSR1 through WNK1 and HSN2.

To analyse whether HSN2 regulates activation of SPAK and OSR1 in neural differentiation, we checked phosphorylation of SPAK and OSR1 in Neuro2A cells treated with NGF-containing serum-free medium. Phosphorylation of SPAK and OSR1 was observed at 10 to 15 minutes after treatment with NGF-containing medium (Fig. 3A). In contrast, knockdown of either *Wnk1* or *Wnk4* suppressed the phosphorylation of SPAK and OSR1 by NGF (Fig. 3B). Moreover, knockdown of both *Wnk1* and *Wnk4* completely abolished SPAK and OSR1 phosphorylation (Fig. 3B), indicating that NGF activates the WNK-SPAK/OSR1 pathway in Neuro2A cells. We next analysed the effect of HSN2 constructs on the phosphorylation of SPAK and OSR1 mediated by NGF. We found that HSN2 expression increased the level of SPAK and OSR1 phosphorylation identically to that following WNK1 expression (Fig. 3C). By contrast, the expression of HSN2-delA and HSN2-insT mutants suppressed the NGF-induced phosphorylation of SPAK and OSR1 (Fig. 3C). These results indicate that HSN2 mutants cannot activate SPAK and OSR1 and work in a dominant negative manner. These data are consistent with the neurite elongation and neural marker gene expression results (Fig. 1A–C).

We previously demonstrated that the WNK1-OSR1 pathway is involved in neural specification²³. To confirm whether NGF-induced neural development is mediated by the HSN2-OSR1 pathway, we examined the effect of *Osr1* knockdown on neural development. As expected, WNK1 expression enhanced neurite outgrowth induced by NGF treatment, and knockdown of *Osr1* inhibited this elongation (Fig. 3D). Similar results were obtained with HSN2 expression and knockdown of *Osr1* (Fig. 3D). In contrast, expression of HSN2 mutants suppressed NGF-induced neurite elongation, and knockdown of *Osr1* completely inhibited neurite outgrowth (Fig. 3D). RT-PCR and qPCR analysis showed that NGF-induced *Lhx8* and *ChAT* expression was completely suppressed by *Osr1* knockdown even though WNK1 or HSN2 was expressed in Neuro2A cells (Fig. 3E). Moreover, although NGF-induced *Gad1* expression was reduced by WNK1 or HSN2 expression, knockdown of *Osr1* rescued this reduction (Fig. 3E). We also examined the effect of a

kinase-negative form of OSR1 (OSR1^{K46M}), and similar results were obtained to those with *Osr1* knockdown (Fig. 3F and G). These results demonstrate that NGF-induced neural development via WNK1 and HSN2 require OSR1 activity.

The HSN2-OSR1-LHX8 pathway is important for NGF-induced neural development.

LHX8 is a key regulator of cholinergic neural function and is involved in the determination of cholinergic and GABAergic cell fate^{19,20}. We previously demonstrated that the WNK1-OSR1 pathway is important for *Lhx8* expression²³. To determine whether *Lhx8* expression is mediated by the HSN2-OSR1 pathway, we examined the effects of HSN2 constructs on Neuro2A cells in the absence of NGF stimulation. We confirmed that expression of WNK1 or HSN2 mediated *Lhx8* induction, and that knockdown of *Osr1* suppressed this induction (Fig. 4A). We also examined the effect of a kinase-negative OSR1^{K46M} mutant on *Lhx8* expression, and found that OSR1^{K46M} also suppressed *Lhx8* expression induced by WNK1 or HSN2 expression (Fig. 4B). In contrast, HSN2-delA and HSN2-insT did not induce *Lhx8* expression (Fig. 4A and B). We next analysed whether HSN2-induced neurite outgrowth and neural specification in Neuro2A cells is mediated by LHX8. *Lhx8* knockdown inhibited not only WNK1- but also HSN2-induced neurite outgrowth (Fig. 4C). Furthermore, although the expression of HSN2 caused an increase in *ChAT* expression and a decrease in *Gad1* expression, knockdown of *Lhx8* suppressed *ChAT* expression and enhanced *Gad1* expression by the expression of HSN2 (Fig. 4D). These results indicate that HSN2-induced neural differentiation, including neurite elongation and expression of cholinergic neuron marker genes, was mediated by LHX8 through OSR1 activity.

HSN2 mutants suppress neural development via GSK3 β .

GSK3 β is a positive effector of WNK signalling in neural development²⁴. To confirm whether HSN2 mediates NGF-induced neural specification through GSK3 β , we examined the effect of *Gsk3 β* knockdown on neural development of Neuro2A cells. As shown in Fig. 5A, WNK1- and HSN2-mediated neurite elongation was suppressed by *siGsk3 β* treatment. Moreover, HSN2 mutants suppressed NGF-induced neurite elongation, and knockdown of *Gsk3 β* completely inhibited neurite outgrowth (Fig. 5A). We also analysed expression of neural marker genes in Neuro2A cells and found that *Gsk3 β* knockdown suppressed NGF-induced *Lhx8* and *ChAT* expression, even though WNK1 or HSN2 was expressed (Fig. 5B). Furthermore, although WNK1 or HSN2 expression decreased NGF-induced *Gad1* expression, knockdown of *Gsk3 β* rescued this reduction (Fig. 5B). These results demonstrate that NGF-induced neural development via WNK1 and HSN2 requires not only OSR1 activity but also GSK3 β .

We next analysed the interaction between GSK3 β and HSN2, and found that HSN2 interacted more strongly with GSK3 β than with WNK1 (Fig. 5C). Interestingly, HSN2 mutants associated more robustly with GSK3 β than with wild-type HSN2 (Fig. 5D). From these results, we predicted that HSN2 mutants might suppress the binding of WNK1 and HSN2 to GSK3 β by covering the binding sites of GSK3 β . To confirm this hypothesis, we transiently expressed Flag-GSK3 β and HA-WNK1 or HA-HSN2 with or without Myc-HSN2-delA and Myc-HSN2-insT mutants in HEK293T cells and then immunoprecipitated cell extracts

with a Flag antibody. The binding of WNK1 and HSN2 to GSK3 β was inhibited by expression of each HSN2 mutant (Fig. 5E and F), indicating that HSN2 mutants suppress the function of GSK3 β in the WNK1/HSN2 pathway by preventing the interaction. We then examined the effect of HSN2 mutants on GSK3 β function in neural development. As shown in Fig. 5G, GSK3 β expression weakly induced neurite elongation of Neuro2A cells and WNK1 and HSN2 enhanced this induction under treatment with vehicle or NGF. In contrast, HSN2 mutants inhibited the GSK3 β -induced neurite elongation (Fig. 5G). Consistent with these results, RT-PCR and qPCR analysis showed that GSK3 β elevated NGF-induced *Lhx8* and *ChAT* expression and decreased *Gad1* expression (Fig. 5H). In addition, although wild-type HSN2 enhanced the expression of *Lhx8* and *ChAT*, HSN2 mutants suppressed the induction of these genes and facilitated *Gad1* expression (Fig. 5H). These results indicate that HSN2 mutants suppress the neural development function of GSK3 β by preventing the interaction of WNK1 and HSN2 with GSK3 β .

Discussion

HSANII is a hereditary neuropathy that is characterized by sensory dysfunction, including loss of pain, touch and temperature sensation¹⁶. HSANII is caused by mutations in a neuron specific exon of *HSN2*, which is a neural-specific splice variant of the *WNK1* gene. These mutations include HSN2-delA and HSN2-insT^{9,14}. Although HSN2 is predicted to have an essential function in neurons, the precise mechanism by which HSN2 mutants cause HSANII is still unclear. In this study, we made constructs of HSN2, including HSN2-delA and HSN2-insT mutants, and analysed their effects on the WNK/HSN2 pathway and neural development using Neuro2A cells. WNK1 forms complexes with WNK1 and WNK4 to promote activation of itself and to activate the downstream effectors, SPAK and OSR1^{28,29}. We showed that HSN2 bound to WNK1 and WNK4 (Fig. 1A and B), and formed a complex with itself (Fig. 1E). These results indicate that HSN2 is also activated by forming a homodimer and auto-phosphorylation. We also observed that NGF induced SPAK and OSR1 phosphorylation and that the phosphorylation levels were enhanced by HSN2 expression (Fig. 3C), indicating that HSN2 activates the SPAK/OSR1 pathway identically to WNK1. We previously reported the involvement of the WNK1-OSR1 pathway in neural specification²³. Therefore, we postulated that the HSN2-OSR1 pathway is also involved in regulating neural development. As expected, knockdown of *Osr1* suppressed HSN2-mediated neural elongation and expression of the cholinergic neural markers, *Lhx8* and *ChAT* (Fig. 3D and E). In addition, kinase-negative OSR1^{K46M} expression also inhibited HSN2-mediated neurite elongation and *Lhx8* and *ChAT* expression (Fig. 3F and G). These results indicate that activation of OSR1 mediated by HSN2 is required for cholinergic neural specification. In contrast to HSN2, HSN2-delA and HSN2-insT mutants did not interact with WNK1, WNK4 or HSN2, and weakly bound to SPAK and OSR1 compared with wild-type HSN2 (Fig. 1). Additionally, we showed that expression of HSN2-delA and HSN2-insT mutants suppressed NGF-induced phosphorylation of SPAK and OSR1 (Fig. 3C). These data indicate that HSN2 mutants are not able to activate the SPAK/OSR1 pathway even though they include the kinase domain. One study has reported that the C-terminal domain of WNK isoforms is important for interaction and auto-phosphorylation of WNK kinases²⁹, suggesting that the C-terminal domain of the WNK1 isoform, HSN2, is required for its self-activation. Most mutations in *HSN2* occur in the neural-specific *HSN2* exon and cause

deletion of subsequent domains, for example, HSN2-delA and HSN2-insT¹⁴. We therefore consider that the C-terminal domain of HSN2 is required for the activation of SPAK and OSR1, and that activation of the HSN2-SPAK/OSR1 pathway is involved in the pathological mechanism of HSANII.

We showed that HSN2 regulates cholinergic neural specification through *Lhx8* induction in Neuro2A cells (Fig. 4D). In addition, OSR1 activation was required for HSN2-mediated *Lhx8* expression (Fig. 4A and B). These results indicate that the HSN2-OSR1-LHX8 pathway is important for cholinergic neural specification. In contrast, HSN2-delA and HSN2-insT mutants, which are observed in HSANII patients, could not induce activation of SPAK and OSR1, or expression of *Lhx8* (Fig. 3C and Fig. 4A and B), indicating that HSANII might be caused by dysregulation of neural specification via HSN2 mutant-mediated suppression of the HSN2-OSR1-LHX8 pathway. However, concerning the pathological mechanism of HSANII, knockdown of *HSN2* in the zebrafish embryo enhances expression of ion co-transporter, KCC2, and leads to abnormal neuromast development¹⁷. Moreover, *HSN2*-deficient mice display increased KCC2 activity and decreased levels of Cl⁻ in nerves¹⁸. These reports suggest that the regulation of KCC2 and ion-homeostasis by HSN2 is important for normal neural development. Here, we showed that the SPAK/OSR1 pathway was similarly activated by HSN2 and WNK1. Because WNK1-mediated activation of SPAK/OSR1 regulates the concentration of Cl⁻ ions in neurons through KCC2³⁰, the HSN2-SPAK/OSR1 pathway might regulate not only *Lhx8* expression but also ion concentrations in neurons. Therefore, *HSN2* mutation can dysregulate neural specification and ion-concentrations in neurons, and might cause loss of pain, touch and temperature sensation in HSANII patients.

Previous reports provide strong evidence that GSK3 β has essential roles in neurodevelopmental processes^{31,32}. We have also reported GSK3 β to be a positive downstream regulator in the WNK-SPAK/OSR1 pathway, which regulates neural development²⁴. Here, we found that GSK3 β also has a positive effect on the HSN2 pathway in Neuro2A cells (Fig. 5A and B). In contrast, HSN2-delA and HSN2-insT mutants suppressed GSK3 β -mediated neurite elongation and specification (Fig. 5G and H). Interestingly, HSN2 mutants interacted more strongly with GSK3 β than with wild-type HSN2 and prevented the binding of WNK1 and HSN2 to GSK3 β (Fig. 5D–F). These results indicate that HSN2 mutants suppress GSK3 β in neural development by inhibiting its binding to WNK1 and HSN2. Conditional knockout mice of both GSK3 α and GSK3 β isoforms in neural progenitors has shown that GSK3s are essential for cortical neurogenesis³³; however, GSK3 α -S21A/GSK3 β -S29A double knock-in mice show that phosphorylation of GSK3s at their N-terminal serine residues is not necessary for neural polarization, including neurite outgrowth³⁴. In support of these findings, we reported that GSK3 β is a downstream effector of the WNK-SPAK/OSR1 pathway in the regulation of neural specification, but neither WNK1 nor OSR1 kinases can phosphorylate GSK3 β ²⁴. Therefore, formation of GSK3 β , WNK1/HSN2 and/or SPAK/OSR1 complexes has essential roles in neural development and HSN2 mutants might cause HSANII by inhibiting formation of these complexes. However, further studies are needed to identify the precise mechanism of HSANII pathology.

In conclusion, our results demonstrate a critical role for HSN2 and suppressive effects of HSN2 mutants on the functions of GSK3 β in neural development. These findings may be beneficial for understanding the pathogenesis of HSANII.

Declarations

Acknowledgments

We thank Atsushi Sato for providing the basis of this study. We also thank Jeremy Allen, PhD, from Edanz (<https://jp.edanz.com/ac>) for editing a draft of this manuscript. This work was supported by Grants-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (MEXT/JSPS KAKENHI Grant Number JP19K16782(M.S.), and JP21K06165(H.S.)) and Nanken-Kyoten, Tokyo Medical and Dental University (H.S.).

Data availability

The data are available from the corresponding author upon request.

Author contributions

M.S. and H.S. designed the study, analysed the data, discussed the data and wrote the manuscript. M.S. performed all of the experiments.

Competing interests statement

The authors declare no competing interests.

References

1. Xu, B. E. *et al.* WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *J. Biol. Chem.* **275**, 16795–16801 (2000).
2. Veríssimo, F. & Jordan, P. WNK kinases, a novel protein kinase subfamily in multi-cellular organisms. *Oncogene* **20**, 5562–5569 (2001).
3. Wilson, F. H. *et al.* Human hypertension caused by mutations in WNK kinases. *Science* (80-.). **293**, 1107–1112 (2001).
4. Piechotta, K., Lu, J. & Delpire, E. Cation chloride cotransporters interact with the stress-related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1). *J. Biol. Chem.* **277**, 50812–50819 (2002).
5. Vitari, A. C., Deak, M., Morrice, N. A. & Alessi, D. R. The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. *Biochem. J.* **391**, 17–24 (2005).

6. Moriguchi, T. *et al.* WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *J. Biol. Chem.* **280**, 42685–42693 (2005).
7. Gagnon, K. B. E., England, R. & Delpire, E. Volume sensitivity of cation-Cl⁻ cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. *Am. J. Physiol. - Cell Physiol.* **290**, 134–142 (2006).
8. Yang, S. Sen *et al.* Molecular Pathogenesis of Pseudohypoaldosteronism Type II: Generation and Analysis of a Wnk4D561A/+ Knockin Mouse Model. *Cell Metab.* **5**, 331–344 (2007).
9. Shekarabi, M. *et al.* Mutations in the nervous system-specific HSN2 exon of WNK1 cause hereditary sensory neuropathy type II. *J. Clin. Invest.* **118**, 2496–2505 (2008).
10. Vidal-Petiot, E. *et al.* A new methodology for quantification of alternatively spliced exons reveals a highly tissue-specific expression pattern of WNK1 isoforms. *PLoS One* **7**, 1–9 (2012).
11. Potulska-Chromik, A., Kabzińska, D., Lipowska, M., Kostera-Pruszczyk, A. & Kochański, A. A novel homozygous mutation in the WNK1/HSN2 gene causing: Hereditary sensory neuropathy type 2. *Acta Biochim. Pol.* **59**, 413–415 (2012).
12. Davidson, G. L. *et al.* Frequency of mutations in the genes associated with hereditary sensory and autonomic neuropathy in a UK cohort. *J. Neurol.* **259**, 1673–1685 (2012).
13. De Filette, J. *et al.* Polyneuropathy in a young Belgian patient: A novel heterozygous mutation in the WNK1/HSN2 gene. *Neurol. Genet.* **2**, 1–3 (2016).
14. Yuan, J. H. *et al.* WNK1/HSN2 founder mutation in patients with hereditary sensory and autonomic neuropathy: A Japanese cohort study. *Clin. Genet.* **92**, 659–663 (2017).
15. Rahmani, B. *et al.* A novel nonsense mutation in WNK1/HSN2 associated with sensory neuropathy and limb destruction in four siblings of a large Iranian pedigree. *BMC Neurol.* **18**, 1–8 (2018).
16. Axelrod, F. B. & Gold-Von Simson, G. Hereditary sensory and autonomic neuropathies: Types II, III, and IV. *Orphanet J. Rare Dis.* **2**, 1–12 (2007).
17. Bercier, V. *et al.* WNK1/HSN2 Mutation in Human Peripheral Neuropathy Deregulates KCC2 Expression and Posterior Lateral Line Development in Zebrafish (*Danio rerio*). *PLoS Genet.* **9**, (2013).
18. Kahle, K. T. *et al.* Inhibition of the kinase WNK1/HSN2 ameliorates neuropathic pain by restoring GABA inhibition. *Sci. Signal.* **9**, 1–9 (2016).
19. Tomioka, T. *et al.* LIM homeobox 8 (Lhx8) is a key regulator of the cholinergic neuronal function via a tropomyosin receptor kinase A (TrkA)-mediated positive feedback loop. *J. Biol. Chem.* **289**, 1000–1010 (2014).
20. Manabe, T. *et al.* L3/Lhx8 is involved in the determination of cholinergic or GABAergic cell fate. *J. Neurochem.* **94**, 723–730 (2005).
21. Zhao, Y. *et al.* The LIM-homeobox gene Lhx8 is required for the development of many cholinergic neurons in the mouse forebrain. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 9005–9010 (2003).
22. Mori, T. *et al.* The LIM homeobox gene, L3/Lhx8, is necessary for proper development of basal forebrain cholinergic neurons. *Eur. J. Neurosci.* **19**, 3129–3141 (2004).

23. Sato, A. & Shibuya, H. WNK Signaling Is Involved in Neural Development via Lhx8/Awh Expression. *PLoS One* **8**, e55301 (2013).
24. Sato, A. & Shibuya, H. Glycogen synthase kinase 3 β functions as a positive effector in the WNK signaling pathway. *PLoS One* **13**, 1–11 (2018).
25. Zhang, Y., Cardell, L. O. & Adner, M. IL-1 β induces murine airway 5-HT_{2A} receptor hyperresponsiveness via a non-transcriptional MAPK-dependent mechanism. *Respir. Res.* **8**, 1–12 (2007).
26. Huang, H. S. *et al.* Prefrontal dysfunction in schizophrenia involves mixed-lineage leukemia 1-regulated histone methylation at GABAergic gene promoters. *J. Neurosci.* **27**, 11254–11262 (2007).
27. Xu, B. *et al.* Proliferating cell nuclear antigen (PCNA) regulates primordial follicle assembly by promoting apoptosis of oocytes in fetal and neonatal mouse ovaries. *PLoS One* **6**, (2011).
28. Yang, C. L., Zhu, X., Wang, Z., Subramanya, A. R. & Ellison, D. H. Mechanisms of WNK1 and WNK4 interaction in the regulation of thiazide-sensitive NaCl cotransport. *J. Clin. Invest.* **115**, 1379–1387 (2005).
29. Thastrup, J. O. *et al.* SPAK/OSR1 regulate NKCC1 and WNK activity: Analysis of WNK isoform interactions and activation by T-loop trans-autophosphorylation. *Biochem. J.* **441**, 325–337 (2012).
30. Friedel, P. *et al.* WNK1-regulated inhibitory phosphorylation of the KCC2 cotransporter maintains the depolarizing action of GABA in immature neurons. *Sci. Signal.* **8**, 23–26 (2015).
31. Hur, E. M. & Zhou, F. Q. GSK3 signalling in neural development. *Nat. Rev. Neurosci.* **11**, 539–551 (2010).
32. Jaworski, T., Banach-Kasper, E. & Gralec, K. GSK-3 β at the intersection of neuronal plasticity and neurodegeneration. *Neural Plast.* **2019**, (2019).
33. Kim, W. Y. *et al.* GSK-3 is a master regulator of neural progenitor homeostasis. *Nat. Neurosci.* **12**, 1390–1397 (2009).
34. Gärtner, A., Huang, X. & Hall, A. Neuronal polarity is regulated by glycogen synthase kinase-3 (GSK-3 β) independently of Akt/PKB serine phosphorylation. *J. Cell Sci.* **119**, 3927–3934 (2006).

Figures

Figure 1

HSN2, but not HSN2 mutants, binds to WNK1, WNK4, SPAK, OSR1 and HSN2. (A–E) Interaction between WNK1 (A), WNK4 (B), SPAK (C), OSR1 (D) or HSN2 (E) and WNK1, HSN2, HSN2-delA and HSN2-insT was analysed in HEK293T cells by immunoprecipitation assays. Cells were transiently transfected with indicated vectors and 48 h later lysates were harvested and immunoprecipitated with an anti-Myc antibody for 4 h. Immunoprecipitates were subjected to immunoblotting with the indicated antibodies.

Figure 2

HSN2 mutants, HSN2-delA and HSN2-insT, exhibit loss of neural development function. (A) Neurite outgrowth from Neuro2A cells treated with NGF (100 ng/ml) or vehicle-containing FBS-free medium for 24 h. Cells were transiently transfected with pRK5, HA-WNK1, HA-HSN2, HA-HSN2-delA or HA-HSN2-insT vectors 24 h prior to treatment. (B) RT-PCR and qPCR analysis of mRNA levels of neural marker genes, *Lhx8*, *Chat* and *Gad1*, in Neuro2A cells indicated in A. The value of each gene in empty vector-transfected, vehicle-treated cells was set to 1 and the value in empty vector-transfected, NGF-treated cells was set to 100. (C) Neurite elongation of Neuro2A cells 24 h after transfection of HA-WNK1 or HA-HSN2 with pRK5, HA-HSN2-delA or HA-HSN2-insT vectors. (D) Neurite outgrowth of Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for 24 h. Cells were transiently transfected with control siRNA (siCTL) or *siWnk1* and *siWnk4* 24 h prior to treatment. (E) RT-PCR and qPCR analysis of mRNA levels of *Lhx8*, *Chat* and *Gad1* in Neuro2A cells indicated in C. The value of each gene from vehicle-

treated cells under siCTL treatment and from NGF-treated cells under siCTL treatment was set to 1 and 100, respectively.

Figure 3

NGF activates SPAK/OSR1 through WNK1 and HSN2. (A) Immunoblot analysis of phosphorylated SPAK and OSR1 in Neuro2A cells treated with NGF (100 ng/ml) or vehicle-containing FBS-free medium for 0–60

minutes. (B) Phosphorylation of SPAK and OSR1 in Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for the indicated times. Cells were transfected with control siRNA (siCTL) or *siWnk1* and *siWnk4* 24 h prior to the treatment. (C) Phosphorylation of SPAK and OSR1 in Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for the indicated times. Cells were transiently transfected with pRK5, HA-WNK1, HA-HSN2, HA-HSN2-delA or HA-HSN2-insT vectors 24 h prior to the treatment. (D) Neurite outgrowth of Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for 24 h. Cells were transiently transfected with siCTL or *siOsr1* and indicated vectors 24 h prior to the treatment. (E) RT-PCR and qPCR analysis of mRNA levels of *Lhx8*, *ChAT* and *Gad1* in Neuro2A cells indicated in D. The value of each gene from vehicle-treated cells under siCTL treatment and from NGF-treated cells under siCTL treatment was set to 1 and 100, respectively. (F) Neurite outgrowth of Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for 24 h. Cells were transiently transfected with empty vector or kinase-negative OSR1 (OSR1^{K46M}) 24 h prior to treatment. (G) RT-PCR and qPCR analysis of mRNA levels of *Lhx8*, *ChAT* and *Gad1* in Neuro2A cells indicated in F. The value of each gene from empty vector-transfected, vehicle-treated cells and from empty vector-transfected, NGF-treated cells was set to 1 and 100, respectively.

Figure 4

The HSN2-OSR1-LHX8 pathway is important for NGF-induced neural development. (A and B) RT-PCR and qPCR analysis of *Lhx8* mRNA levels in Neuro2A cells 24 h after transfection with pRK5, HA-WNK1, HA-HSN2, HA-HSN2-delA or HA-HSN2-insT vectors and control siRNA (siCTL) or *siOsr1* (A) and Flag-OSR1^{K46M} (B). **P < 0.01. (C) Neurite outgrowth of Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for 24 h. Cells were transiently transfected with siCTL or *siLhx8* and indicated vectors 24 h prior to treatment. (D) RT-PCR and qPCR analysis of mRNA levels of *Lhx8*, *Chat* and *Gad1* in Neuro2A cells indicated in C. The value of each gene from vehicle-treated cells under siCTL treatment and from NGF-treated cells under siCTL treatment was set to 1 and 100, respectively.

Figure 5

HSN2 mutants suppress GSK3β functions in neural development. (A) Neurite outgrowth of Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for 24 h. Cells were transiently transfected with control siRNA (siCTL) or *siGsk3β* and indicated vectors 24 h prior to treatment. (B) RT-PCR and qPCR analysis of mRNA levels of *Lhx8*, *Chat* and *Gad1* in Neuro2A cells indicated in A. The value of each gene from vehicle-treated cells under siCTL treatment and from NGF-treated cells under siCTL treatment was

set to 1 and 100, respectively. (C and D) Binding analysis of WNK1 and HSN2 to GSK3 β (C), and of HSN2-delA and HSN2-insT mutants to GSK3 β (D). Cells were transiently transfected with indicated vectors and 48 h later lysates were immunoprecipitated with a Flag antibody for 4 h. Immunoprecipitates were subjected to immunoblotting assays with the indicated antibodies. (E and F) Interaction between WNK1 (E) or HSN2 (F) and GSK3 β with and without HSN2-delA and HSN2-insT mutants was analysed in HEK293T cells by immunoprecipitation assays. Cells were transiently transfected with indicated vectors and 48 h later lysates were immunoprecipitated with a Flag antibody for 4 h. Immunoprecipitates were subjected to immunoblotting assays with the indicated antibodies. (G) Neurite elongation of Neuro2A cells treated with 100 ng/ml NGF-containing FBS-free medium for 24 h. Cells were transiently transfected with the indicated vectors 24 h prior to treatment. (H) RT-PCR and qPCR analysis of mRNA levels of *Lhx8*, *ChAT* and *Gad1* in Neuro2A cells indicated in G. The value of each gene from control vector-transfected and vehicle- or NGF-treated cells was set to 1 and 100, respectively.