Rare, pathogenic variants in *WNK3* kinase cause X-linked intellectual disability

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ABSTRACT

Purpose: The with-no-lysine (K) kinase WNK3 (PRKWNK3) has been implicated in the development and function of the brain via its regulation of the cation-Cl^- cotransporters, but the role of WNK3 in human development is unknown.

Method: We ascertained exome or genome sequence from individuals with rare familial or sporadic forms of intellectual disability (ID).

Results: We identified a total of six different maternally-inherited, hemizygous, three loss-of-function (LoF) or three pathogenic missense variants (p.Pro204Arg, p.Leu300Ser, p.Glu607Val) in *WNK3* in 14 male individuals from six unrelated families. Affected individuals had ID with the variable presence of epilepsy and structural brain defects. *WNK3* variants co-segregated with the disease in the three different families with multiple affected individuals. This included one large family previously diagnosed with X-linked Prieto syndrome. *WNK3* pathogenic missense variants localize to the catalytic domain and impede the inhibitory phosphorylation of the neuronal-specific Cl^- cotransporter KCC2 at threonine 1007, a site critically regulated during the development of synaptic inhibition.

Conclusions: Pathogenic *WNK3* variants cause a rare form of human X-linked ID with variable epilepsy and structural brain abnormalities and implicate impaired phospho-regulation of KCC2 as a pathogenic mechanism.

Keywords: WNK kinase, WNK3, SPAK, NKCC1, KCC2, GABA, neurodevelopmental disease, Prieto syndrome, speech delay, X-linked intellectual disability, exome sequencing.

INTRODUCTION

γ-aminobutyric acid (GABA) generates inhibitory currents in the adult brain but excitatory currents in the developing brain, which are important for neuronal proliferation, migration, and synaptogenesis.[1] This differential GABA response is a result of developmental changes in the intraneuronal concentration of chloride [Cl^-] determined by the *SLC12A* family cation-Cl^- cotransporters (CCCs) NKCC1 and KCC2.[2] A progressive postnatal increase in KCC2-dependent Cl^- efflux and simultaneous decrease in NKCC1-mediated Cl^- influx reduces $[Cl^-]_i$ such that Cl^--permeable $GABA_A$ receptor ($GABA_AR$) activation triggers Cl^- influx, hyperpolarization, and synaptic inhibition.[2] This developmental "switch" in NKCC1/KCC2 activity underlies the GABA excitatory-inhibitory transition that is critical for normal brain maturation and function.[3]

The *with* *no* lysine (*K*) (WNK) serine-threonine protein kinases (WNK1-4) are master regulators of NKCC1, KCC2, and other *SLC12A* family CCCs.[4] WNK3, a Cl^- and cell volume-sensitive kinase,[5] is by far the most highly expressed WNK kinase during early brain development, where it co-localizes with NKCC1 and KCC2 and $GABA_ARs$ in hippocampal, cerebellar, and cortical neurons in mice.[5] Through phosphorylation of its downstream kinase substrate SPS1-related proline/alanine rich kinase (SPAK)[6] and physical interaction with other WNKs and the CCCs, WNK3 potently stimulates NKCC1 but inhibits KCC2.[5] Depletion of WNK3 or loss of WNK3 kinase activity has the opposite effect, inhibiting NKCC1 but stimulating KCC2 by decreasing transporter phosphorylation.[5] In mice, WNK-regulated changes in KCC2 phosphorylation contribute to the GABA excitatory-inhibitory transition.[7]

In humans, dominant or recessive variants in *SLC12A2* (NKCC1), *SLC12A5* (KCC2), and other CCCs cause monogenic neurological disorders variably featuring epilepsy, neurodevelopmental delay, and structural brain defects.[8-10] Human variants in the KCCs disrupt critical regulatory sites of phosphorylation, including those mediated by *WNK3*.[4,8,11] Heterozygous variants in *WNK1* and *WNK4*, isoforms of which are kidney-specific, cause a rare Mendelian form of salt-sensitive hypertension (OMIM: 614992 and 614991) due to impaired phosphorylation of NCC and NKCC2.[12] Variants in the neuronal-specific isoform of *WNK1* cause an autosomal recessive congenital pain insensitivity (OMIM: 201300),[13] and its depletion in mice causes decreased KCC2 phosphorylation in the spinal cord.[14] These results show that the WNK-SPAK-CCC pathway is critical for human physiology and disease; however, to date, no pathogenic variants in *WNK3* have been found to cause a monogenic human disorder.

Through international data sharing, we have identified multiple maternally-inherited, hemizygous, loss-of-function (LoF) and pathogenic missense variants in *WNK3* in individuals with sporadic and familial forms of intellectual disability (ID). We showed that *WNK3* transcripts are highly expressed in the mid-gestational human brain and down-regulated with the emergence of the KCC2-dependent GABA-excitatory-inhibitory shift. In cultured cells, *WNK3* pathogenic missense variants lead to WNK3 degradation and impaired regulatory phosphorylation of KCC2.

METHODS

Families and subjects

We obtained written informed consent from each affected individual or his guardian and available family members prior to inclusion in genetic research in accordance with the

respective human ethics committees of each participating institution. All participants were assessed by at least one expert clinical geneticist from each respective participating center.

Exome sequencing and variant validation

Candidate variants were identified by whole exome or genome sequencing performed from DNA from probands in a research or diagnostic settings. Segregation analysis was performed by Sanger or exome sequencing (**Table S2**). Contact among participating teams was aided by the web-based tools GeneMatcher.[15]

Biophysical modeling

The amino acid sequence for human WNK3 was obtained from UNIPROT, accession number Q9BYP7. WNK3 structure was downloaded from the PDB (PDB id 5O2C). The structure accounted for ~21% of the protein from residue 123-500 (377 residues), which comprises most of the catalytic domain. The two missense variants located in the catalytic domain, c.611C>G p.(Pro204Arg) and c.899T>C p.(Leu300Ser), were modelled and the free energy of change calculated (ΔΔG) in silico using the mutagenesis program in the MolSoft ICM-Pro suite v3.8-7c (www.molsoft.com).

Cell culture and transfections

HEK293T (human embryonic kidney 293) cells were cultured on 10-cm-diameter dishes in DMEM supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For transfection experiments, HEK293T cells were transfected with 20 µl of 1 mg/ml polyethylenimine (Polysciences) and 5-10 µg of plasmid DNA as described previously [6]. At 36 hours post-transfection, cells were lysed in 0.3 ml of ice-cold lysis buffer/dish with lysis buffer, containing 50 mM Tris/HCl, pH 7.5, 1 mM

EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (w/v) NP-40, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and protease inhibitors (1 tablet per 50 ml). Lysates were clarified by centrifugation at 4°C for 15 minutes at 26,000 g and the supernatants were frozen in aliquots in liquid nitrogen and stored at -80°C. Protein concentrations were determined using the Bradford method.

Immunoblotting

Lysate protein samples were prepared with 1X-NuPAGE LDS sample buffer (Invitrogen), containing 1% (v/v) 2-mercaptoethanol, and subjected to immunoblot and immunoprecipitation as previously described.[6] Protein samples (15 µg) were boiled in sample buffer for 5 min, resolved by 7.5% sodium dodecyl sulfate polyacrylamide-gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane. Membranes were incubated for 30 min with TBST (Tris-buffered saline, 0.05% Tween-20) containing 5% (w/v) skim milk. Blots were then washed six times with TBST and incubated for 1 hour at room temperature with secondary HRP-conjugated antibodies diluted 5000-fold in 5% (w/v) skim milk in in TBS-Tween buffer (TTBS, containing Tris/HCl, pH 7.5, 0.15 M NaCl and 0.2% (v/v) Tween-20). After repeating the washing steps, signals were detected with enhanced chemiluminescence reagent. Antibodies detecting KCC2 phospho-threonine (Thr)1007 (corresponding to KCC3A phospho-Thr1048 (1 µg/ml, S961C) and WNK3 (1 µg/ml, S156C), were from The Division of Signal Transduction Therapy Unit at the University of Dundee. The pan-KCC2 antibody (1 µg/ml, NeuroMab clone N1/12) was from NeuroMab. GAPDH (1:5000 dilution, 60004-1-Ig) was from Proteintech Euro. Horseradish peroxidase-coupled secondary antibodies for immunoblotting were from Pierce. Immunoblots were developed using ChemiDoc™ Imaging Systems (Bio-Rad). Figures were generated using Photoshop/Illustrator (Adobe). Band densities were measured with ImageJ. For

phospho-antibody immunoprecipitation, KCC2 isoform was immunoprecipitated from indicated cell extracts. 2 mg of the indicated clarified cell extract was mixed with 15 µg of the indicated phospho-specific KCC2 antibody conjugated to 15 µl of protein-G–Sepharose, in the added presence of 20 µg of the dephosphorylated form of the phosphopeptide antigen and incubated 2 hours at 4°C with gentle shaking. Immunoprecipitates were washed three times with 1 ml of lysis buffer containing 0.15 M NaCl and twice with 1 ml of buffer A, containing 50 mM Tris/HCl, pH7.5 and 0.1 mM EGTA. Bound proteins were eluted with 1x LDS sample buffer.

Immunofluorescence

HEK293T cells were transfected with constructs encoding an empty vector or the indicated wild type (WT) or mutant constructs (for the three missense variants identified in the study) with a N-terminal FLAG epitope tagged WNK3. 36 hours post-transfection, cells were fixed with 4% paraformaldehyde, incubated at room temperature for 30 min and treated with buffer containing 0.1 M glycine in PBS (pH 7.4) and 0.3% Triton X-100. Fixed cells were washed with PBS for 5 minutes, and then were permeabilized by incubation with 0.25% TRITON™ X-100 (Catalog Number T9284), in PBS for 5 minutes. Cells were washed thrice with PBS for 5 minutes, and blocked by incubation with 10% bovine serum albumin (Catalog Number A9647), in PBS (10% BSA/PBS) for 30 minutes at 37°C. Cells were incubated with Monoclonal ANTI-FLAG M2 (Catalog Number F1804) diluted in the range of 1:500 to 1:2,000 in 3% BSA/PBS for 2 hours at 37 °C. Cells were washed with PBS for 5 minutes for three times, and incubated with the secondary antibody, Anti-Mouse IgG- FITC (Catalog Number F9137), at a 1:1,000 dilution in 3% BSA/PBS for 45 minutes at 37 °C. Cells were washed thrice with PBS for 5 minutes. Coverslips with cells side-down were mounted on glass slides using a small drop of mounting medium such as polyvinyl alcohol for semi-

permanent mounting. For fluorescence imaging, Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to a MRC1024 confocal scanning laser equipment (Bio-Rad, Richmond, CA), was used.

Gene expression profiling

The Developmental Transcriptome data set from BrainSpan (www.brainspan.org) [16] was used to create expression profiles for *WNK3* and *SLC12A5*. Expression trajectories for all genes were visualized with curves defined by the smoothed conditional means of expression values (normalized $\log_2(\text{RPKM} + 1)$) over time (days) using the geom_smooth function from the ggplot2 package in R. Expression profiles were plotted both as overall expression over time and as expression over time stratified by six brain regions as defined by Kang *et. al.*[16] (amygdala, cerebellum, diencephalon, forebrain, hippocampus, and neocortex). For each gene's overall expression profile, Spearman's rank correlation coefficients (Spearman's ρ) were used to test the correlation between expression and time using the cor.test function with "spearman" as the defined method argument in R.

RESULTS

Rare, pathogenic *WNK3* variants in sporadic and familial X-linked intellectual disability

We identified six rare single nucleotide variants (SNVs) in *WNK3* (in eight individuals from six unrelated families with neurodevelopmental disorders via datasharing among a group of international investigators studying ID and other neurodevelopmental disorders (see Methods and **Tables 1 and 2**)). Six probands were referred for clinical genetic testing for ID, developmental delay (DD), autism spectrum disorder, or neuropsychiatric symptoms. Eight individuals were identified through segregation analysis of families characterized by multi-generational ID (**Table 2; Table S1**), including a large family previously diagnosed with

Prieto syndrome (OMIM: 309610).[17] Linkage analyses in this family had linked the phenotype with a 9 cM locus at Xp11.3-Xp11.22.[18] Genome sequencing performed in the proband (individual 7) in this family identified the rare catalytic domain *WNK3* missense variant p.(Leu300Ser) in the linkage interval. This variant co-segregated with the disease phenotype over three generations in six male individuals (individuals 6 to 11 in Family 5; **Table 2**) and five asymptomatic heterozygous mothers.

All of the 14 affected males carrying rare hemizygous *WNK3* variants had inherited their variant from a healthy mother (**Table 1, Figure S1**). All *WNK3* variants co-segregated with the disease phenotype in individuals available for analysis from families with multiple affected members (**Figure S1**). Taking into account the affected and unaffected male individuals tested in each family, the probability that the observed variant-affected status data occurred by chance is $N = (1/2)^{16} = 1.5 \times 10^{-5}$. This probability is in favor of a strong pathogenicity of the *WNK3* variants, since it is well below the threshold of $N \leq 1/8$ defined by the American College of Medical Genetics and Genomics (ACMG) for variants segregating in multiple families.

Details of the identified *WNK3* variants and the phenotypes of the affected individuals with them are listed in Tables 1, 2 and S1. *WNK3* is intolerant to LoF variation (pLI = 1.00, o/e = 0.09 (0.05 - 0.19) according to gnomAD v2.1.1) and missense variation (missense-Z = 2.55, o/e = 0.71 (0.66 - 0.77).[19] All six *WNK3* variants found in affected subjects are rare, since they are absent in known variant databases (gnomAD v2.1.1, NHLBI GO Exome Sequencing Project (ESP), ClinVar, dbSNP). Three of them are predicted LoF: NM_001002838.3:c.538-2A>G p.(Asp180Valfs*5), c.721C>T p.(Arg241*) and c.1089+1G>A p.(Gly364Ilefs*10). p.(Arg241*) introduces a premature stop codon in exon 4/23. Mini-gene assay showed

[c.1089+1G>A] disrupted a canonical splice site and the use of an alternative donor splice site located four nucleotides downstream (c.1089+5), leading to a frameshift and the inclusion of a premature stop codon [p.(Gly364Ilefs*10)] (**Figure S4**). RNA analyses in individual 1 indicated that variant c.538-2A>G induces skipping of exon 3 that leads also to frameshifting and premature stop codon [p.(Asp180Valfs*5)] (**Figure S5**). All three LoF variants truncate WNK3 within the catalytic domain and are expected to lead to nonsense-mediated decay (NMD). Missense variants p.(Pro204Arg), p.(Leu300Ser), and p.(Glu607Val) are predicted to be likely pathogenic referring to CADD score (CADD ≥ 24) and bioinformatics analyses listed by MobiDetails (**Table 1**). p.(Pro204Arg) and p.(Leu300Ser) impact highly-conserved amino-acid residues in the catalytic domain that are designated intolerant to substitutions by Metadome and Missense Tolerance Ratio (MTR) and modeled to significantly impact the biophysical structure (**Table 1,** Figure 1b-d, and **Figure S3**). p.(Glu607Val) affects an amino acid residue distal to the kinase domain that is conserved across vertebrates (**Figure 1a**).

Affected individuals exhibit DD/ID and variable epilepsy, craniofacial and brain anomalies

Main clinical features of individuals with *WNK3* variants are summarized in **Table 2**. Additional details are provided in **Table S1**. In all families, tested mothers of affected male individuals were asymptomatic heterozygotes (**Figure S1**). The most frequently reported features included ID and DD (14/14). 10/13 (77%) individuals displayed mild dysmorphic facial features, but no unifying facial gestalt was evident across all cases, and features differed between individuals with Prieto syndrome (6/13) and individuals from other families (7/13) (**Table S1**). 5/13 (38%) individuals had behavioral or neuropsychiatric symptoms, including attention deficit hyperactivity disorder (x2), autistic features (x1) and auto-

aggressiveness (x1). 6/13 (46%) individuals exhibited mild microcephaly, with head circumference between -2SD and -2.4SD. 5/13 (38%) individuals had epilepsy. Brain magnetic resonance imaging for most available individuals (7/10; 70%) exhibited variable structural brain abnormalities (**Table 2**). For example, individual 2 and his brother (individual 3) from family 2 both showed polymicrogyria (**Figure S6**).

WNK3 missense variants impact WNK3 expression and KCC2

Transcriptome profiling of bulk RNAseq data of the normal human brain from BrainSpan (www.brainspan.org)[16] showed that *WNK3* is most highly expressed in the prenatal period and undergoes a significant decrease in expression during the transition from the prenatal to postnatal periods (Spearman's $\rho = -0.85$, $p < 2.2 \times 10^{-16}$) (**Figure S2**). This profile is consistent with previous work in the developing mouse brain and parallels the developmental shift of GABA from excitatory to inhibitory that arises from relative increase in KCC2 to NKCC1 activity.[5]

WNK3 is a Cl^--sensitive kinase[5] that exists in a complex with other WNK kinases, SPAK kinase, and KCC2[20-23]. It regulates the phosphorylation of KCC2 at Thr1007, which is important in tuning the strength of GABA inhibition by antagonizing KCC2-mediated Cl^- efflux during development[6,11,24,25]. To test the functional impact of the identified *WNK3* missense likely pathogenic variants, we expressed WT and variant WNK3 constructs in cells (see **Methods**) and examined the total expression of WNK3 and the total expression and phosphorylation state of KCC2, using specific antibodies (**Figure 2;** see **Methods**).[6,11,24] Wester-blotting assays indicated that p.(Pro204Arg), p.(Leu300Ser), and p.(Glu607Val) all resulted in decreased WNK3 expression and KCC2 threonine 1007 phosphorylation without altering KCC2 total protein expression. Immunocytochemistry confirmed the WNK3-

associated X-linked ID variants (Pro204Arg), p.(Leu300Ser), and p.(Glu607Val) resulted in decreased expression of WNK3 compared to wild type (**Figure 2c**).

DISCUSSION

We provide evidence that rare pathogenic variants in *WNK3* lead to X-linked ID. *WNK3* variants co-segregated with the phenotype in families with multiple affected male individuals, including a previously described large family with X-linked Prieto syndrome characterized by ID and subcortical cerebral atrophy.[17] Missense pathogenic variants decrease WNK3 expression and impair a critical regulatory phosphorylation event of KCC2. These genetic and functional findings support the pathogenicity of the identified *WNK3* variants and suggest a loss-of-function mechanism.

All individuals with *WNK3* variants exhibit DD or ID, variably accompanied by epilepsy, microcephaly, minor facial anomalies, and variable structural brain defects. The full spectrum of clinical and radiographic phenotypes resulting from *WNK3* variants will be more clearly defined as additional *WNK3* variant probands are identified, and detailed brain imaging becomes available for all affected individuals. Interestingly, an association has been reported between changes in *WNK3* expression and human epilepsy[26] and human schizophrenia.[27] In addition, two brothers with autism spectrum disorder (ASD), ID, and facial anomalies have been reported with an Xp11.2 microdeletion including *WNK3*.[28] Sequencing-based studies of large cohorts have also suggested *WNK3* as a candidate for neurodevelopmental disorders, including ASD and ID[29,30] which is consistent with the higher expression of Wnk3 in the developing mouse brain and with the observations made after experimental disruption of the KCC2-dependent GABA excitatory-inhibitory transition in animals[31,32] and humans[27,33-36]. We corroborated these results by finding *WNK3* transcripts are highly expressed in the mid-

gestational human brain but are down-regulated with the emergence of the KCC2-dependent GABA excitatory-inhibitory shift (**Figure S5**).

The three substitutions p.(Asp180Valfs*5), p.(Arg241*), and p.(Gly364Ilefs*10) truncate WNK3 prematurely and likely have a LoF effect due to either nonsense-mediated mRNA decay (NMD) or proteasomal degradation of truncated protein variants. p.(Pro204Arg), p.(Leu300Ser), and p.(Glu607Val) all resulted in decreased WNK3 expression and KCC2 threonine 1007 phosphorylation without altering KCC2 total expression. This is consistent with previous *in vitro* experiments showing that depletion of WNK3 or dominant-negative, kinase dead versions of WNK3 disrupt the regulated phosphorylation and activity of KCC2[5], the regulation of which is required for normal brain development and the emergence of GABA synaptic inhibition.[7,37] Furthermore, a recent study using cultured embryonic hippocampal neurons showed that knock-down of *WNK3* induces a decrease of KCC2 phosphorylated at threonine 1007, which confirms our observations.[38] Interestingly, the authors also reported morphological abnormalities in *WNK3*-knocked-down neurons.

Future work will study the impact of the variants reported here on KCC2 function and the GABA excitatory-inhibitory transition in *in vivo* model systems engineered with *WNK3* human variants. Moreover, variation in *WNK3* might impact other WNK3 targets, such as NKCC1 and KCC3,[11] calcium channels TRPV5 and TRPV6,[39] and neuronal mRNA splicing factor RBFOX1.[40] Whether the WNK3 variants detected herein alter these other processes is currently unknown.

DATA AVAILABILITY

All data are available upon request.

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WEB RESOURCES

BrainSpan, www.brainspan.org

Combined Annotation Dependent Depletion (CADD), https://cadd.gs.washington.edu/

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/

GeneMatcher, https://genematcher.org/

ggplot2 package in R, http://public.ebookcentral.proquest.com/choice/publicfullrecord.aspx?p=511468

GTEx portal, https://gtexportal.org/home/gene/WNK3

gnomAD, http://gnomad.broadinstitute.org/

Metadome, https://stuart.radboudumc.nl/metadome/

Missense Tolerance Ratio Gene Viewer, http://mtr-viewer.mdhs.unimelb.edu.au/

MobiDetails, https://mobidetails.iurc.montp.inserm.fr/MD/

MolSoft ICM-Pro suite v3.8-7c, www.molsoft.com

OMIM, http://www.omim.org/

Protein Data Bank, https://www.rcsb.org/

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ETHICS DECLARATION

Written informed consent was obtained for use of medical history, genetic testing report, and photograph (if applicable), as approved by the Institutional Review Board of the University Hospital Center (CHU) of Nantes.

DECLARATION OF INTERESTS

E.T., K.M., K.R., I.M.W., K.G.M., and L.R. are employees of GeneDx, Inc. K.R. is a shareholder of OPKO Health, Inc. The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from clinical genetic testing conducted at Baylor Genetics Laboratories.

FIGURE TITLES AND LEGENDS

Figure 1. Localization of the *WNK3* variants in the secondary (a, b) and tertiary structures (c, d). **a.**, Almost all of the variants (5/6) are in the catalytic domain. **b.,** The variants p.(Pro204Arg) and p.(Leu300Ser) affect amino-acid residues P204 and L300 conserved across species including invertebrates, whereas the residue E607 affected by variant p.(Glu607Val) is conserved across vertebrates only. **c.,** Amino-acid residue Pro204 (P204) is positioned at the tip of a loop between H2 helix and β2 strand. The cyclic side chain of P204 enables the rigidification of the loop, which in turn enables the switch back and maintains the correct spatial positioning of the secondary structural elements. A shift to residue Arg204 (R204; p.(Pro204Arg)) will make this loop more flexible. Additionally, the positively charged guanidinium side chain of Arg204 is also likely to make additional interactions and thereby further destabilizing the region. The ΔΔG of the variation from Pro to Arg is 1.83. **d.**, Amino acid residue Leu300 (L300) is positioned within the core of the protein and is surrounded by hydrophobic side chains of Phe158 (F158), Leu178 (L178), Leu183 (L183), Phe191 (F191), Leu225 (L225), Leu297 (L297), Met301 (M301) and Ala306 (A306). Introduction of a polar hydroxyl group side chain within a hydrophobic environment is not tolerated. The ΔΔG of the mutational change from Leu to Ser in this hydrophobic pocket is 2.39.

Figure 2. Impact of *WNK3* variants on WNK3 expression and KCC2 phosphorylation. **a.**, HEK293T cells were transfected with constructs encoding an empty vector (EV) or the indicated wild type (WT) or variant constructs of N-terminal FLAG epitope tagged WNK3. 36 hours after transfection, cell lysates were subjected to SDS-PAGE and immunobloted (IB) with the indicated antibodies. **b.**, Bar graphs summary of the ratios of phosphorylated target signal to total target intensity (e.g. KCC2 pThr1007/ total KCC2), and total target to GAPDH (e.g. WNK3/GAPDH) (mean ± SD, n=3). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, n.s., not significant. **c.,** Immunofluorescent staining of WNK3 and its missense variants. HEK293T cells grown on slides were transfected with constructs encoding an empty vector (EV) or the indicated wild type (WT) or variant constructs of N-terminal FLAG epitope tagged WNK3. 36 hours after transfection, cells were immediately subjected to immunofluorescence (IF) analysis with a Flag-specific polyclonal antibody.

TABLES

Table 1. Main characteristics of the X-linked *WNK3* variants identified in the affected male individuals included in the study

Variant	Chromosomal localization ChrX(GRCh37)	cDNA change	Protein change*	Variant public database**	CADD Phred score (v1.6)	PolyPhen -2	Metadome	MobiDetails***	Number of individuals (family)
V1	g.54337726T>C	c.538-2A>G	p.(Asp180Valfs*5)	Absent	34	N/A	N/A	23078	1 (F1)
V2	g.54337651G>C	c.611C>G	p.(Pro204Arg)	Absent	26.1	D	HI	50199	3 (F2)
V3	g.54335738G>A	c.721C>T	p.(Arg241*)	Absent	37	N/A	HI	50201	1 (F3)
V4	g.54335560A>G	c.899T>C	p.(Leu300Ser)	Absent	26	D	I	50202	6 (F4)
V5	g.54334354C>T	c.1089+1G>A	p.(Gly364Ilefs*10)	Absent	35	NA	N/A	50203	2 (F5)
V6	g.54319634T>A	c.1820A>T	p.(Glu607Val)	Absent	24.2	B	N	50204	1 (F6)

Nomenclature HGVS V2.0 according to mRNA reference sequence NM_001002838.3 for variants V2-V4 and V6 and sequence reference NC_000023.11(NM_001002838.3) for spice site variants V1 and V2. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.

N/A= not applicable; SIFT: D= deleterious, T= tolerated; PolyPhen-2: D= probably_damaging, PD= possibly damaging, B= benign; HI= highly inolerant; I= intolerant; SI= slightly intolerant; F#=Family #

*nomenclature determined according to functional assays (Figures S4 and S5)

***gnomAD v2.1.1, NHLBI GO Exome Sequencing Project (ESP), ClinVar, dbSNP

**To access the predictions for variant XXXXX, follow the link: https://mobidetails.iurc.montp.inserm.fr/MD/api/variant/XXXXX/browser/

Table 2. Clinical features of affected male individuals with *WNK3* variants.

										Total
Individual (Family)	1 (F1)	2 (F2)	3 (F2)	4 (F2)	5 (F3)	6 to 11 (F4)	12 (F5)	13 (F5)	14 (F6)	**14 M**
Variant*	V1	V2	V2	V2	V3	V4	V5	V5	V6	**6 variants**
Developmental delay or ID	yes	yes	yes	yes	yes	yes (x6)	yes	yes	yes	**14/14**
Speech delay	yes	yes	ND	ND	no	yes (x6)	yes	yes	yes	**11/12**
Hypotonia	no	yes	yes	ND	no	yes (x4)	no	no	yes	**7/13**
Seizures	no	yes	yes	ND	no	yes (x2)	no	no	yes	**5/13**
Anomalies in brain imaging	no	yes	yes	ND	ND	yes (x4)	ND	ND	yes	**7/10**
Behavioural / neuropsychiatric symptoms	no	ND	yes	yes	yes, ADHD	no	yes, ADHD	yes	no	**5/13**
Facial abnormalities	yes	yes	yes	ND	no	yes (x6)	yes	no	no	**10/13**
Microcephaly	yes (-2 SD)	no	no	ND	yes (-2.4 SD)	yes (x1) (-2 SD)	yes (-2 SD)	yes (-2 SD)	yes (-2.2 SD)	**6/13**

*Full variant name is indicated in Table 1.

M: male individuals; ASD: autism spectrum disorder; ADHD: attention deficit hyperactivity disorder; ND: not determined

SUPPLEMENTAL INFORMATION

2 tables and 6 figures

Figure S1. Pedigrees of six families with *WNK3* variants. Affected and unaffected individuals are indicated respectively by black and pale grey squares or circles. When sequencing was done, the genotype (using MT for variant allele and WT for wild type allele) is indicated below the symbol of the individual. The number of each affected individual participating in the study is indicated by numbers written in red and bold larger fonts. VAF means variant allele fraction.

Figure S2: Expression profiles of *WNK3* and *SLC12A5* (encoding KCC2) from BrainSpan (www.brainspan.org) Developmental Transcriptome[1]. **a.**, Overall expression profiles of *WNK3* and *SLC12A5* are plotted as smoothed conditional means of expression over time. *WNK3* demonstrates decreased expression over time, while *SLC12A5* demonstrates increased expression over time. **b.**, Expression profiles of *WNK3* and *SLC12A5* are plotted as smoothed conditional means of expression over time stratified by regions.

Figure S3. Tolerance predictions of the amino acid residues of WNK3 (NP_065973.2) affected by the variants reported in the study. a., MetaDome web tool (PMID: 31116477) indicates heterogeneous tolerance of WNK3 regions, inducing a classification of the residues of interest from highly intolerant to highly tolerant to missense variants. The catalytic domain is globally the most intolerant region to missense variants. **b.**, Analysis by Missense Tolerance Ratio (MTR; v1) suggests that variants affecting intolerant residues 204, 210 and 300 in the catalytic domain are expected to bear a severe effect. Horizontal lines show gene-specific MTR percentiles 5th (in green), 25th (in yellow), 50th (in black), and neutrality (in blue; MTR = 1.0) MTR calculated using WES component of gnomAD v2.0

Figure S4. Assessment of the effect on splicing of variant c.1089+1G>A by mini-gene assay. To evaluate the impact on splicing of the NM_001002838.3(*WNK3*):c.1089+1G>A variant identified in individuals 12 and 13 (Family 5), in the absence of available biological samples, we performed splicing mini-gene reporter assays using the pCAS2 vector based on a previously described protocol[1]. This functional assay is based on the comparative analysis of the splicing pattern of wild-type and mutant. Mini-gene constructs of the exon 12 reveal two different transcripts. The wild-type construct (E5-c.1089+1G) highlights the transcript 1 (415 base pairs (bp)) corresponding to the physiological splicing of the exon 5. However mutant construct (E5-c.1089+1A) presents predominantly the transcript 2, four bp longer that transcript 1. This is due to the recruitment of an alternative donor site in exon, 5 located 4 bp 3'-downstream to the physiological splice site. This results in a reading frame shift and the occurrence of a premature stop codon p.(Gly364Ilefs*10). DNA variants are described according to the nomenclature established by the Human Genome Variation Society. Nomenclature HGVS V2.0 is defined according to *WNK3* mRNA reference sequence NM_001002838.3. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.

Figure S5. Assessment of the effect on splicing of variant c.538-2A>G by RNA analyses. To evaluate the impact on splicing of the NM_001002838.3(*WNK3*):c.538-2A>G variant (intron 2), we performed a compared analysis of RNAs isolated from peripheral blood samples collected in individual 1 and in a healthy control having a wild-type WNK3 sequence. For both affected and healthy individuals, we did a nested-PCR amplification of the region containing the exon junctions predicted to be affected by the splice site according to prediction programs, as shown in the screenshot from Alamut software (Sophia Genetics)

(**a**). We thus designed two forward primers in exon 2 (E02F and E02F2) pairing two reverse primers located in exon 4 (E02R and E02R2) and sequenced the PCR products by Sanger sequencing (**b**). Whereas normal splicing in the control was objectified by the presence of exon 3 sequence, skipping of exon 3 was confirmed in individual 1 by a direct junction between exons 2 and 4 leading to a frameshift and a premature stop codon five codons downstream [p.(Asp180Valfs*5)].

DNA variants are described according to the nomenclature established by the Human Genome Variation Society. Nomenclature HGVS V2.0 is defined according to *WNK3* mRNA reference sequence NM_001002838.3. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.

Figure S6. Brain imaging of affected brothers from Family 2 [variant NM_001002838.3(WNK3):c.611C>G p.(Pro204Arg)]. MRI scans from individuals 2 and 3 (Family 2). **a.**, MRI of individual 2, imaged at 5 years of age. Axial image from 3D T1 MRI sequence demonstrates areas of perisylvian polymicrogyria (white arrows) as well as multiple foci of bilateral periventricular grey matter heterotopia (black arrows); **b.**, MRI of individual 3 at 19 months of age. Sagittal image from 3D T1 sequence showing areas of frontal and perisylvian polymicrogyria (arrows).

Table S1. Detailed molecular and clinical characteristics of affected individuals with *WNK3* pathogenic variants. Nomenclature HGVS V2.0 according to mRNA reference sequence NM_020922.4. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1. nd: not determined; SD: standard deviation; ASD: autism spectrum disorder; ADHD: attention deficit hyperactivity disorder; PFO: Patent Foramen Ovale.

Table S2. Sequencing and genotyping methodology for individuals 1-16. WES: whole-exome sequencing. N/A: not applicable.

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