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

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120 # ABSTRACT

121

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

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

127 **Results:** We identified a total of six different maternally-inherited, hemizygous, three loss-
128 of-function (LoF) or three pathogenic missense variants (p.Pro204Arg, p.Leu300Ser,
129 p.Glu607Val) in *WNK3* in 14 male individuals from six unrelated families. Affected
130 individuals had ID with the variable presence of epilepsy and structural brain defects. *WNK3*
131 variants co-segregated with the disease in the three different families with multiple affected
132 individuals. This included one large family previously diagnosed with X-linked Prieto
133 syndrome. *WNK3* pathogenic missense variants localize to the catalytic domain and impede
134 the inhibitory phosphorylation of the neuronal-specific Cl^- cotransporter KCC2 at threonine
135 1007, a site critically regulated during the development of synaptic inhibition.

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

139

140 **Keywords:** WNK kinase, WNK3, SPAK, NKCC1, KCC2, GABA, neurodevelopmental
141 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 *w*ith *n*o 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]

167 In humans, dominant or recessive variants in *SLC12A2* (NKCC1), *SLC12A5* (KCC2), and
168 other CCCs cause monogenic neurological disorders variably featuring epilepsy,
169 neurodevelopmental delay, and structural brain defects.[8-10] Human variants in the KCCs
170 disrupt critical regulatory sites of phosphorylation, including those mediated by *WNK3*.[4,8,11]
171 Heterozygous variants in *WNK1* and *WNK4*, isoforms of which are kidney-specific, cause a
172 rare Mendelian form of salt-sensitive hypertension (OMIM: 614492 and 614491) due to
173 impaired phosphorylation of NCC and NKCC2.[12] Variants in the neuronal-specific isoform of
174 *WNK1* cause an autosomal recessive congenital pain insensitivity (OMIM: 201300),[13] and its
175 depletion in mice causes decreased KCC2 phosphorylation in the spinal cord.[14] These results
176 show that the WNK-SPAK-CCC pathway is critical for human physiology and disease;
177 however, to date, no pathogenic variants in *WNK3* have been found to cause a monogenic
178 human disorder.

179

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

187

188 **METHODS**

189 **Families and subjects**

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

192 respective human ethics committees of each participating institution. All participants were
193 assessed by at least one expert clinical geneticist from each respective participating center.
194
195 **Exome sequencing and variant validation**
196 Candidate variants were identified by whole exome or genome sequencing performed from
197 DNA from probands in a research or diagnostic settings. Segregation analysis was performed
198 by Sanger or exome sequencing (**Table S2**). Contact between participating teams was aided
199 by the web-based tool GeneMatcher.[15]
200
201 **Biophysical modeling**
202 The amino acid sequence for human WNK3 was obtained from UNIPROT, accession number
203 Q9BYP7. WNK3 structure was downloaded from the PDB (PDB id 5O2C). The structure
204 accounted for ~21% of the protein from residue 123-500 (377 residues), which comprises
205 most of the catalytic domain. The two missense variants located in the catalytic domain,
206 c.611C>G p.(Pro204Arg) and c.899T>C p.(Leu300Ser), were modelled and the free energy
207 of change calculated (ΔΔG) in silico using the mutagenesis program in the MolSoft ICM-Pro
208 suite v3.8-7c (www.molsoft.com).
209
210 **Cell culture and transfections**
211 HEK293T (human embryonic kidney 293) cells were cultured on 10-cm-diameter dishes in
212 DMEM supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 U/ml
213 penicillin and 0.1 mg/ml streptomycin. For transfection experiments, HEK293T cells were
214 transfected with 20 μl of 1 mg/ml polyethylenimine (Polysciences) and 5-10 μg of plasmid
215 DNA as described previously [6]. At 36 hours post-transfection, cells were lysed in 0.3 ml of
216 ice-cold lysis buffer/dish with lysis buffer, containing 50 mM Tris/HCl, pH 7.5, 1 mM

217 EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium
218 orthovanadate, 1% (w/v) NP-40, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and protease
219 inhibitors (1 tablet per 50 ml). Lysates were clarified by centrifugation at 4°C for 15 minutes
220 at 26,000 g and the supernatants were frozen in aliquots in liquid nitrogen and stored at -
221 80°C. Protein concentrations were determined using the Bradford method.

222

223 **Immunoblotting**

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

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

250

251 **Immunofluorescence**

252 HEK293T cells were transfected with constructs encoding an empty vector or the indicated
253 wild type (WT) or mutant constructs (for the three missense variants identified in the study)
254 with a N-terminal FLAG epitope tagged WNK3. 36 hours post-transfection, cells were fixed
255 with 4% paraformaldehyde, incubated at room temperature for 30 min and treated with buffer
256 containing 0.1 M glycine in PBS (pH 7.4) and 0.3% Triton X-100. Fixed cells were washed
257 with PBS for 5 minutes, and then were permeabilized by incubation with 0.25% TRITON™
258 X-100 (Catalog Number T9284), in PBS for 5 minutes. Cells were washed thrice with PBS
259 for 5 minutes, and blocked by incubation with 10% bovine serum albumin (Catalog Number
260 A9647), in PBS (10% BSA/PBS) for 30 minutes at 37°C. Cells were incubated with
261 Monoclonal ANTI-FLAG M2 (Catalog Number F1804) diluted in the range of 1:500 to
262 1:2,000 in 3% BSA/PBS for 2 hours at 37 °C. Cells were washed with PBS for 5 minutes for
263 three times, and incubated with the secondary antibody, Anti-Mouse IgG- FITC (Catalog
264 Number F9137), at a 1:1,000 dilution in 3% BSA/PBS for 45 minutes at 37 °C. Cells were
265 washed thrice with PBS for 5 minutes. Coverslips with cells side-down were mounted on
266 glass slides using a small drop of mounting medium such as polyvinyl alcohol for semi-

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

270

271 **Statistical analysis**

272 Statistical analyses were conducted with GraphPad Prism (GraphPad software 9). For
273 analysis of Western blot, relative band densities were measured with ImageJ. The mean ± SD
274 values were results of analysis of three independent (n=3) experiments. Non-parametric test
275 was used to ensure the relative band densities were normally distributed. The one-way
276 ANOVA or non-parametric Mann–Whitney tests were employed to examine the statistical
277 significance of the differences between groups of data, otherwise indicated.

278

279 **Gene expression profiling**

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

290

291 # RESULTS

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

293 We identified six rare single nucleotide variants (SNVs) in *WNK3* (in 14 individuals from six
294 unrelated families with neurodevelopmental disorders via datasharing among a group of
295 international investigators studying ID and other neurodevelopmental disorders (see Methods
296 and **Tables 1 and 2**)). Six probands were referred for clinical genetic testing for ID,
297 developmental delay (DD), autism spectrum disorder, or neuropsychiatric symptoms. Eight
298 individuals were identified through segregation analysis of families characterized by multi-
299 generational ID (**Table 2; Table S1**), including a large family previously diagnosed with
300 Prieto syndrome (OMIM: 309610).[17] Linkage analyses in this family had linked the
301 phenotype with a 9 cM locus at Xp11.3-Xp11.22.[18] Genome sequencing performed in the
302 proband (individual 7) in this family identified the rare catalytic domain *WNK3* missense
303 variant p.(Leu300Ser) in the linkage interval. This variant co-segregated with the disease
304 phenotype over three generations in six male individuals (individuals 6 to 11 in Family 5;
305 **Table 2**) and five asymptomatic heterozygous mothers.

306

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

316

317 Details of the identified *WNK3* variants and the phenotypes of the affected individuals with
318 them are listed in Tables 1, 2 and S1. *WNK3* is intolerant to LoF variation (pLI = 1.00, o/e =
319 0.09 (0.05 - 0.19) according to gnomAD v2.1.1) and missense variation (missense-Z = 2.55,
320 o/e = 0.71 (0.66 - 0.77)).[19] All six *WNK3* variants found in affected subjects are rare, since
321 they are absent in known variant databases (gnomAD v2.1.1, NHLBI GO Exome Sequencing
322 Project (ESP), dbSNP); we deposited them in ClinVar under accession numbers
323 SCV002107163 to SCV002107168 (Tables 1 and S1). Three of them are predicted LoF:
324 NM_001002838.3:c.538-2A>G p.(Asp180Valfs*5), c.721C>T p.(Arg241*) and
325 c.1089+1G>A p.(Gly364Ilefs*10). p.(Arg241*) introduces a premature stop codon in exon
326 4/23. Mini-gene assay showed [c.1089+1G>A] disrupted a canonical splice site and the use
327 of an alternative donor splice site located four nucleotides downstream (c.1089+5), leading to
328 a frameshift and the inclusion of a premature stop codon [p.(Gly364Ilefs*10)] (**Figure S4**).
329 RNA analyses in individual 1 indicated that variant c.538-2A>G induces skipping of exon 3
330 that leads also to frameshifting and premature stop codon [p.(Asp180Valfs*5)] (**Figure S5**).
331 All three LoF variants affect the catalytic domain of WNK3 and are expected to lead to either
332 production of non-functional truncated proteins, or degradation of variant transcripts by
333 nonsense-mediated decay (NMD). This last hypothesis could not be verified, given that
334 Individual 1's mRNAs were extracted from blood collected in PAXGene® blood tubes
335 (Ozyme) that are known to partially inhibit NMD. Missense variants p.(Pro204Arg),
336 p.(Leu300Ser), and p.(Glu607Val) are predicted to be likely pathogenic referring to CADD
337 score (CADD $\geq$ 24) and bioinformatics analyses listed by MobiDetails (**Table 1**).
338 p.(Pro204Arg) and p.(Leu300Ser) impact highly-conserved amino-acid residues in the
339 catalytic domain that are designated intolerant to substitutions by Metadome and Missense
340 Tolerance Ratio (MTR) and modeled to significantly impact the biophysical structure (**Table**
341 **1,** Figure 1b-d, and **Figure S3**). Even if p.(Pro204Arg) is absent in variant database, as

342 mentioned above, it is worth noting that another variant affecting also amino acid (aa) residue
343 204, p.(Pro204Thr), is reported in a heterozygous male from the neurological subset of
344 gnomAD v2.1.1. database. We have no clues as to the possible neurodevelopmental nature of
345 the individual's symptoms. However, the presence of this variant, which is unambiguously
346 predicted pathogenic (see
347 https://mobidetails.iurc.montp.inserm.fr/MD/api/variant/89924/browser/), and which was also
348 reported in lung adenocarcinoma (COSM6187822), would stress the functional importance of
349 aa 204. The third missense variant, p.(Glu607Val), affects an amino acid residue distal to the
350 kinase domain that is conserved across vertebrates (**Figure 1a**).
351

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

354 Main clinical features of individuals with *WNK3* variants are summarized in **Table 2**.
355 Additional details are provided in **Table S1**. In all families, tested mothers of affected male
356 individuals were asymptomatic heterozygotes (**Figure S1**). The most frequently reported
357 features included ID and DD (14/14). 10/13 (77%) individuals displayed mild dysmorphic
358 facial features, but no unifying facial gestalt was evident across all cases, and features
359 differed between individuals with Prieto syndrome (6/13) and individuals from other families
360 (7/13) (**Table S1**). 5/13 (38%) individuals had behavioral or neuropsychiatric symptoms,
361 including attention deficit hyperactivity disorder (x2), autistic features (x1) and auto-
362 aggressiveness (x1). 6/13 (46%) individuals exhibited mild microcephaly, with head
363 circumference between -2SD and -2.4SD. 5/13 (38%) individuals had epilepsy. Brain
364 magnetic resonance imaging for most available individuals (7/10; 70%) exhibited variable
365 structural brain abnormalities (**Table 2**). For example, individual 2 and his brother (individual
366 3) from family 2 both showed polymicrogyria (**Figure S6**).

367

368 ***WNK3* missense variants impact WNK3 expression and KCC2**

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

376

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

389

390 # DISCUSSION

391 We provide evidence that rare pathogenic variants in *WNK3* lead to X-linked ID. *WNK3*
392 variants co-segregated with the phenotype in families with multiple affected male individuals,
393 including a previously described large family with X-linked Prieto syndrome characterized
394 by ID and subcortical cerebral atrophy.[17] Missense pathogenic variants decrease WNK3
395 expression and impair a critical regulatory phosphorylation event of KCC2. These genetic
396 and functional findings support the pathogenicity of the identified *WNK3* variants and suggest
397 a loss-of-function mechanism.
398
399 All individuals with *WNK3* variants exhibit DD or ID, variably accompanied by epilepsy,
400 microcephaly, minor facial anomalies, and variable structural brain defects. The full spectrum
401 of clinical and radiographic phenotypes resulting from *WNK3* variants will be more clearly
402 defined as additional *WNK3* variant probands are identified, and detailed brain imaging
403 becomes available for all affected individuals. Interestingly, an association has been reported
404 between changes in *WNK3* expression and human epilepsy[26] and human schizophrenia.[27] In
405 addition, two brothers with autism spectrum disorder (ASD), ID, and facial anomalies have
406 been reported with an Xp11.2 microdeletion including *WNK3*.[28] Sequencing-based studies of
407 large cohorts have also suggested *WNK3* as a candidate for neurodevelopmental disorders,
408 including ASD and ID[29,30] which is consistent with the higher expression of Wnk3 in the
409 developing mouse brain and with the observations made after experimental disruption of the
410 KCC2-dependent GABA excitatory-inhibitory transition in animals[31,32] and humans[27,33-36].
411 We corroborated these results by finding *WNK3* transcripts are highly expressed in the mid-
412 gestational human brain but are down-regulated with the emergence of the KCC2-dependent
413 GABA excitatory-inhibitory shift (**Figure S2**).
414

415 The three substitutions p.(Asp180Valfs*5), p.(Arg241*), and p.(Gly364Ilefs*10) truncate
416 WNK3 prematurely and likely have a LoF effect due to either nonsense-mediated mRNA
417 decay (NMD) or proteasomal degradation of truncated protein variants. p.(Pro204Arg),
418 p.(Leu300Ser), and p.(Glu607Val) all resulted in decreased WNK3 expression and KCC2
419 threonine 1007 phosphorylation without altering KCC2 total expression. This is consistent
420 with previous *in vitro* experiments showing that depletion of WNK3 or dominant-negative,
421 kinase dead versions of WNK3 disrupt the regulated phosphorylation and activity of KCC2[5],
422 the regulation of which is required for normal brain development and the emergence of
423 GABA synaptic inhibition.[7,37] Furthermore, a recent study using cultured embryonic
424 hippocampal neurons showed that knock-down of *WNK3* induces a decrease of KCC2
425 phosphorylated at threonine 1007, which confirms our observations.[38] Interestingly, the
426 authors also reported morphological abnormalities in *WNK3*-knocked-down neurons.
427
428 Future work will study the impact of the variants reported here on KCC2 function and the
429 GABA excitatory-inhibitory transition in *in vivo* model systems engineered with *WNK3*
430 human variants. Moreover, variation in *WNK3* might impact other WNK3 targets, such as
431 NKCC1 and KCC3,[11] calcium channels TRPV5 and TRPV6,[39] and neuronal mRNA splicing
432 factor RBFOX1.[40] Whether the WNK3 variants detected herein alter these other processes is
433 currently unknown.
434

435 DATA AVAILABILITY

436 All data are available upon request. The sequence variants in *WNK3* (NM_004656.3) reported
437 in the paper have been deposited in ClinVar database. Their respective accession numbers
438 (SCV002107163 to SCV002107168) are indicated in Tables 1 and S1.
439

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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/

465 ggplot2 package in R,
466 http://public.ebookcentral.proquest.com/choice/publicfullrecord.aspx?p=511468
467 GTEx portal, https://gtexportal.org/home/gene/WNK3
468 gnomAD, http://gnomad.broadinstitute.org/
469 Metadome, https://stuart.radboudumc.nl/metadome/
470 Missense Tolerance Ratio Gene Viewer, http://mtr-viewer.mdhs.unimelb.edu.au/
471 MobiDetails, https://mobidetails.iurc.montp.inserm.fr/MD/
472 MolSoft ICM-Pro suite v3.8-7c, www.molsoft.com
473 OMIM, http://www.omim.org/
474 Protein Data Bank, https://www.rcsb.org/
475

476 **AUTHOR INFORMATION**

477 Conceptualization: SK, BI, KTK, JZ
478 Data curation: All authors
479 Methodology: SK, BI, KTK, JZ
480 Writing – original draft: SK, BI, KTK, JZ
481 Writing – review & editing: All authors
482

483 **ETHICS DECLARATION**

484 Written informed consent was obtained for use of medical history, genetic testing report, and
485 imaging (if applicable), as approved by the Institutional Review Board of the University
486 Hospital Center (CHU) of Nantes.
487

488 **DECLARATION OF INTERESTS**

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

493

494 # FIGURE TITLES AND LEGENDS

495

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

513

514 **Figure 2. Impact of *WNK3* variants on WNK3 expression and KCC2 phosphorylation.**
515 **a.**, HEK293T cells were transfected with constructs encoding an empty vector (EV) or the
516 indicated wild type (WT) or variant constructs of N-terminal FLAG epitope tagged WNK3.
517 36 hours after transfection, cell lysates were subjected to SDS-PAGE and immunoblotted
518 (IB) with the indicated antibodies. **b.**, Bar graphs summary of the ratios of phosphorylated
519 target signal to total target intensity (e.g. KCC2 pThr1007/ total KCC2), and total target to
520 GAPDH (e.g. KCC2/GAPDH, WNK3/GAPDH) (mean ± SD, n=3). *, p<0.05; **, p<0.01;
521 ***, p<0.001, n.s., not significant. **c.,** Immunofluorescent staining of WNK3 and its missense
522 variants. HEK293T cells grown on slides were transfected with constructs encoding an empty
523 vector (EV) or the indicated wild type (WT) or variant constructs of N-terminal FLAG
524 epitope tagged WNK3. 36 hours after transfection, cells were immediately subjected to
525 immunofluorescence (IF) analysis with a Flag-specific polyclonal antibody.

526 # TABLES

527

528 **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***	**Accession number in ClinVar**	**CADD Phred score (v1.6)**	**PolyPhen -2**	**Metadome**	**MobiDetails****	**Number of individuals (family)**
V1	g.54337726T>C	c.538-2A>G	p.(Asp180Valfs*5)	SCV002107163	34	N/A	N/A	23078	1 (F1)
V2	g.54337651G>C	c.611C>G	p.(Pro204Arg)	SCV002107164	26.1	D	HI	50199	3 (F2)
V3	g.54335738G>A	c.721C>T	p.(Arg241*)	SCV002107165	37	N/A	HI	50201	1 (F3)
V4	g.54335560A>G	c.899T>C	p.(Leu300Ser)	SCV002107166	26	D	I	50202	6 (F4)
V5	g.54334354C>T	c.1089+1G>A	p.(Gly364Ilefs*10)	SCV002107167	35	NA	N/A	50203	2 (F5)
V6	g.54319634T>A	c.1820A>T	p.(Glu607Val)	SCV002107168	24.2	B	N	50204	1 (F6)

529 Nomenclature HGVS V2.0 according to mRNA reference sequence NM_001002838.3 for variants V2-V4 and V6 and sequence reference
530 NC_000023.10(NM_001002838.3) for splice site variants V1 and V5. Nucleotide numbering uses +1 as the A of the ATG translation initiation
531 codon in the reference sequence, with the initiation codon as codon 1.
532 N/A= not applicable; SIFT: D= deleterious, T= tolerated; PolyPhen-2: D= probably_damaging, PD= possibly damaging, B= benign; HI= highly
533 intolerant; I= intolerant; SI= slightly intolerant; F#=Family #
534 *nomenclature determined according to functional assays for V1 and V5 (Figures S4 and S5)
535 **To access the predictions for variant XXXXX, follow the link: https://mobidetails.iurc.montp.inserm.fr/MD/api/variant/XXXXX/browser/

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

538

										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**

539 *Full variant name is indicated in Table 1.

540 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)

593 (**a**). We thus designed two forward primers in exon 2 (E02F and E02F2) pairing two reverse
594 primers located in exon 4 (E02R and E02R2) and sequenced the PCR products by Sanger
595 sequencing (**b**). Whereas normal splicing in the control was objectified by the presence of
596 exon 3 sequence, skipping of exon 3 was confirmed in individual 1 by a direct junction
597 between exons 2 and 4 leading to a frameshift and a premature stop codon five codons
598 downstream [p.(Asp180Valfs*5)].
599 DNA variants are described according to the nomenclature established by the Human
600 Genome Variation Society. Nomenclature HGVS V2.0 is defined according to *WNK3* mRNA
601 reference sequence NM_001002838.3. Nucleotide numbering uses +1 as the A of the ATG
602 translation initiation codon in the reference sequence, with the initiation codon as codon 1.
603
604 **Figure S6. Brain imaging of affected brothers from Family 2 [variant**
605 **NM_001002838.3(WNK3):c.611C>G p.(Pro204Arg)].** MRI scans from individuals 2 and 3
606 (Family 2). **a.**, MRI of individual 2, imaged at 5 years of age. Axial image from 3D T1 MRI
607 sequence demonstrates areas of perisylvian polymicrogyria (white arrows) as well as multiple
608 foci of bilateral periventricular grey matter heterotopia (black arrows); **b.**, MRI of individual
609 3 at 19 months of age. Sagittal image from 3D T1 sequence showing areas of frontal and
610 perisylvian polymicrogyria (arrows).
611
612 **Table S1. Detailed molecular and clinical characteristics of affected individuals with**
613 ***WNK3* pathogenic variants.** Nomenclature HGVS V2.0 according to mRNA reference
614 sequence NM_020922.4. Nucleotide numbering uses +1 as the A of the ATG translation
615 initiation codon in the reference sequence, with the initiation codon as codon 1. nd: not
616 determined; SD: standard deviation; ASD: autism spectrum disorder; ADHD: attention deficit
617 hyperactivity disorder; PFO: Patent Foramen Ovale.

618

619 **Table S2. Sequencing and genotyping methodology for individuals 1-16**. WES: whole-

620 exome sequencing. N/A: not applicable.

622 # REFERENCES

623 1. Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E. The GABA excitatory/inhibitory shift
624 in brain maturation and neurological disorders. The Neuroscientist : a review journal
625 bringing neurobiology, neurology and psychiatry. 2012;18(5):467-486.

626 2. Rivera C, Voipio J, Payne JA, et al. The K+/Cl- co-transporter KCC2 renders GABA
627 hyperpolarizing during neuronal maturation. Nature. 1999;397(6716):251-255.

628 3. Spitzer NC. Electrical activity in early neuronal development. Nature.
629 2006;444(7120):707-712.

630 4. Shekarabi M, Zhang J, Khanna AR, Ellison DH, Delpire E, Kahle KT. WNK Kinase
631 Signaling in Ion Homeostasis and Human Disease. Cell metabolism. 2017;25(2):285-
632 299.

633 5. Kahle KT, Rinehart J, de Los Heros P, et al. WNK3 modulates transport of Cl- in and
634 out of cells: implications for control of cell volume and neuronal excitability.
635 Proceedings of the National Academy of Sciences of the United States of America.
636 2005;102(46):16783-16788.

637 6. de Los Heros P, Alessi DR, Gourlay R, et al. The WNK-regulated SPAK/OSR1
638 kinases directly phosphorylate and inhibit the K+-Cl- co-transporters. The
639 Biochemical journal. 2014;458(3):559-573.

640 7. Heubl M, Zhang J, Pressey JC, et al. GABAA receptor dependent synaptic inhibition
641 rapidly tunes KCC2 activity via the Cl(-)-sensitive WNK1 kinase. Nature
642 communications. 2017;8(1):1776.

643 8. Kahle KT, Flores B, Bharucha-Goebel D, et al. Peripheral motor neuropathy is
644 associated with defective kinase regulation of the KCC3 cotransporter. Science
645 signaling. 2016;9(439):ra77.

646 9. Stodberg T, McTague A, Ruiz AJ, et al. Mutations in SLC12A5 in epilepsy of infancy
647 with migrating focal seizures. Nature communications. 2015;6:8038.
648 10. Wongsaengsak S, Vidmar AP, Addala A, et al. A novel SLC12A1 gene mutation
649 associated with hyperparathyroidism, hypercalcemia, nephrogenic diabetes insipidus,
650 and nephrocalcinosis in four patients. Bone. 2017;97:121-125.
651 11. Zhang J, Gao G, Begum G, et al. Functional kinomics establishes a critical node of
652 volume-sensitive cation-Cl(-) cotransporter regulation in the mammalian brain.
653 Scientific reports. 2016;6:35986.
654 12. Kahle KT, Wilson FH, Leng Q, et al. WNK4 regulates the balance between renal
655 NaCl reabsorption and K+ secretion. Nat Genet. 2003;35(4):372-376.
656 13. Lafreniere RG, MacDonald MLE, Dube M-P, et al. Identification of a novel gene
657 (HSN2) causing hereditary sensory and autonomic neuropathy type II through the
658 study of Canadian genetic isolates. Am J Hum Genet 2004;74:1064-1073.
659 14. Friedel P, Kahle KT, Zhang J, et al. WNK1-regulated inhibitory phosphorylation of
660 the KCC2 cotransporter maintains the depolarizing action of GABA in immature
661 neurons. Science signaling. 2015;8(383):ra65.
662 15. Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for
663 connecting investigators with an interest in the same gene. Human mutation.
664 2015;36(10):928-930.
665 16. Kang HJ, Kawasawa YI, Cheng F, et al. Spatio-temporal transcriptome of the human
666 brain. Nature. 2011;478(7370):483-489.
667 17. Prieto F, Badia L, Mulas F, Monfort A, Mora F. X-linked dysmorphic syndrome with
668 mental retardation. Clinical genetics. 1987;32(5):326-334.
669 18. Martinez F, Prieto F, Gal A. Refined localization of the Prieto-syndrome locus.
670 American journal of medical genetics. 1996;64(1):82.

671 19. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum
672 quantified from variation in 141,456 humans. Nature. 2020;581(7809):434-443.
673 20. Kahle KT, Rinehart J, Lifton RP. Phosphoregulation of the Na-K-2Cl and K-Cl
674 cotransporters by the WNK kinases. Biochimica et biophysica acta.
675 2010;1802(12):1150-1158.
676 21. Piechotta K, Lu J, Delpire E. Cation chloride cotransporters interact with the stress-
677 related kinases Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress
678 response 1 (OSR1). The Journal of biological chemistry. 2002;277(52):50812-50819.
679 22. Plotkin MD, Snyder EY, Hebert SC, Delpire E. Expression of the Na-K-2Cl
680 cotransporter is developmentally regulated in postnatal rat brains: a possible
681 mechanism underlying GABA's excitatory role in immature brain. Journal of
682 neurobiology. 1997;33(6):781-795.
683 23. Vitari AC, Thastrup J, Rafiqi FH, et al. Functional interactions of the SPAK/OSR1
684 kinases with their upstream activator WNK1 and downstream substrate NKCC1. The
685 Biochemical journal. 2006;397(1):223-231.
686 24. Zhang J, Bhuiyan MIH, Zhang T, et al. Modulation of brain cation-Cl(-) cotransport
687 via the SPAK kinase inhibitor ZT-1a. Nature communications. 2020;11(1):78.
688 25. Alessi DR, Zhang J, Khanna A, Hochdorfer T, Shang Y, Kahle KT. The WNK-
689 SPAK/OSR1 pathway: master regulator of cation-chloride cotransporters. Science
690 signaling. 2014;7(334):re3.
691 26. Jeong KH, Kim SH, Choi YH, Cho I, Kim WJ. Increased expression of WNK3 in
692 dispersed granule cells in hippocampal sclerosis of mesial temporal lobe epilepsy
693 patients. Epilepsy research. 2018;147:58-61.

694 27. Arion D, Lewis DA. Altered expression of regulators of the cortical chloride
695 transporters NKCC1 and KCC2 in schizophrenia. Archives of general psychiatry.
696 2011;68(1):21-31.
697 28. Qiao Y, Liu X, Harvard C, et al. Autism-associated familial microdeletion of
698 Xp11.22. Clinical genetics. 2008;74(2):134-144.
699 29. Grozeva D, Carss K, Spasic-Boskovic O, et al. Targeted Next-Generation Sequencing
700 Analysis of 1,000 Individuals with Intellectual Disability. Human mutation.
701 2015;36(12):1197-1204.
702 30. Piton A, Gauthier J, Hamdan FF, et al. Systematic resequencing of X-chromosome
703 synaptic genes in autism spectrum disorder and schizophrenia. Molecular psychiatry.
704 2011;16(8):867-880.
705 31. Dzhala VI, Talos DM, Sdrulla DA, et al. NKCC1 transporter facilitates seizures in the
706 developing brain. Nature medicine. 2005;11(11):1205-1213.
707 32. He Q, Nomura T, Xu J, Contractor A. The developmental switch in GABA polarity is
708 delayed in fragile X mice. The Journal of neuroscience : the official journal of the
709 Society for Neuroscience. 2014;34(2):446-450.
710 33. Cellot G, Cherubini E. GABAergic signaling as therapeutic target for autism spectrum
711 disorders. Frontiers in pediatrics. 2014;2:70.
712 34. Deidda G, Bozarth IF, Cancedda L. Modulation of GABAergic transmission in
713 development and neurodevelopmental disorders: investigating physiology and
714 pathology to gain therapeutic perspectives. Frontiers in cellular neuroscience.
715 2014;8:119.
716 35. Kaila K, Price TJ, Payne JA, Puskarjov M, Voipio J. Cation-chloride cotransporters in
717 neuronal development, plasticity and disease. Nature reviews Neuroscience.
718 2014;15(10):637-654.

719 36. Tang X, Kim J, Zhou L, et al. KCC2 rescues functional deficits in human neurons
720 derived from patients with Rett syndrome. Proceedings of the National Academy of
721 Sciences of the United States of America. 2016;113(3):751-756.
722 37. Watanabe M, Zhang J, Mansuri MS, et al. Developmentally regulated KCC2
723 phosphorylation is essential for dynamic GABA-mediated inhibition and survival.
724 Science signaling. 2019;12(603).
725 38. Lim WM, Chin EWM, Tang BL, Chen T, Goh ELK. WNK3 Maintains the
726 GABAergic Inhibitory Tone, Synaptic Excitation and Neuronal Excitability via
727 Regulation of KCC2 Cotransporter in Mature Neurons. Front Mol Neurosci.
728 2021;14:762142-762142.
729 39. Zhang W, Na T, Peng JB. WNK3 positively regulates epithelial calcium channels
730 TRPV5 and TRPV6 via a kinase-dependent pathway. American journal of physiology
731 Renal physiology. 2008;295(5):F1472-1484.
732 40. Lee AY, Chen W, Stippec S, et al. Protein kinase WNK3 regulates the neuronal
733 splicing factor Fox-1. Proceedings of the National Academy of Sciences of the United
734 States of America. 2012;109(42):16841-16846.
735