

Biallelic Mutations in *UNC80* Cause Persistent Hypotonia, Encephalopathy, Growth Retardation, and Severe Intellectual Disability

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Ion channel proteins are required for both the establishment of resting membrane potentials and the generation of action potentials. Hundreds of mutations in genes encoding voltage-gated ion channels responsible for action potential generation have been found to cause severe neurological diseases. In contrast, the roles of voltage-independent “leak” channels, important for the establishment and maintenance of resting membrane potentials upon which action potentials are generated, are not well established in human disease. *UNC80* is a large component of the *NALCN* sodium-leak channel complex that regulates the basal excitability of the nervous system. Loss-of-function mutations of *NALCN* cause infantile hypotonia with psychomotor retardation and characteristic facies (IHPRF). We report four individuals from three unrelated families who have homozygous missense or compound heterozygous truncating mutations in *UNC80* and persistent hypotonia, encephalopathy, growth failure, and severe intellectual disability. Compared to control cells, HEK293T cells transfected with an expression plasmid containing the c.5098C>T (p.Pro1700Ser) *UNC80* mutation found in one individual showed markedly decreased *NALCN* channel currents. Our findings demonstrate the fundamental significance of *UNC80* and basal ionic conductance to human health.

Among mammalian neurons from different brain regions, there is considerable variability in basal excitability and resting membrane potentials (from approximately -85 mV to -50 mV) upon which action potentials are generated. Many mutations in voltage-gated ion channels responsible for the generation of action potentials have been found to cause severe diseases, including migraine, episodic muscle paralysis, ataxia, epilepsy, autism, and sudden cardiac death (see Jurkat-Rott et al., Kullmann and Waxman, and Catterall et al.^{1–3} for reviews). In contrast, the molecular mechanisms and the role of basal excitability in human diseases are not well understood.

The resting membrane potential is primarily determined by ion channels that are open and “leaky” at rest. The basal Na^+ leak is mainly established through the *NALCN* Na^+ -leak channel. In mouse hippocampal neurons, *NALCN* is responsible for $\sim 70\%$ of the total basal Na^+ leak.⁴ *NALCN* is a member of the 24-transmembrane domain (24-TM) ion channel superfamily, which includes

the ten voltage-gated Ca^{2+} channels and ten Na^+ channels.⁵ It forms a voltage-independent, Na^+ -permeable, non-selective, non-inactivating cation channel. *NALCN*'s unique properties allow it to generate background sodium-leak currents. The balance between Na^+ influx through *NALCN* and K^+ efflux through other channels is predicted to generate a large dynamic range of neuronal excitability. In mammalian brains, the *NALCN* complex also contains *UNC79* and *UNC80*, homologs of *C. elegans* *Unc-79* and *Unc-80*.^{6–8} In situ hybridization analysis, RNA sequencing (RNA-seq), and expression sequence-tag (EST) database searches suggest that *NALCN* (MIM: 611549), *UNC79*, and *UNC80* (MIM: 612636) are widely expressed in the brain.⁴ Both *UNC79* and *UNC80* are large proteins ($\sim 3,000$ amino acids) but do not have recognizable domains. *NALCN* and *UNC79* do not directly interact with each other; the three components are brought together by *UNC80*'s association with both *UNC79* and *NALCN*.^{6,7} *UNC80* is required for the channel's regulation

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Table 1. Clinical Characteristics of Individuals with *UNC80* Mutations

Subject	Family 1	Family 2	Family 3	
	IV.1	V.5	II.1	II.3
Age at last review	4 years	4 years	15 years	9 years
Gender	female	female	female	female
Country of parental origin	Iraq	Morocco	Norway	Norway
Parental consanguinity	+	+	–	–
Gestational age	41 weeks	41 weeks	38 weeks	40 weeks
Birth weight (percentile)	3,000 g (25 th)	3,158 g (50 th)	2,960 g (25 th)	3,070 g (25 th)
OFC at birth (percentile)	33 cm (25 th)	unavailable	32 cm (10 th)	35 cm (75 th)
Hypotonia first noted	birth	birth	birth	3 months
Persistent hypotonia	+	+	+	+
Feeding difficulties	+	+	–	+
Age at G-tube insertion (degree of dependency)	2 years (dependent)	3.5 years (dependent)	15 years (supplemental use)	10 months (completely dependent)
Current age	4 years	4 years	15 years	10 years
Current height (percentile)	72 cm (<<3 rd)	91.5 cm (<3 rd)	144 cm (<<3 rd)	125 cm (3 rd) ^a
Current weight (BMI)	7.4 kg (14.3)	12.9 kg (15.4)	40.2 kg (17)	23.5 kg (15.7) ^a
Current OFC (percentile)	43 cm (<<3 rd , or –4 SD)	47 cm (2 nd , or –1 SD)	51.5 cm (3 rd)	50.5 cm (10 th) ^a
Ophthalmology	punctate keratopathy, normal visual evoked potentials	alternating esotropia, normal visual evoked potentials	esotropia, normal vision	esotropia, normal vision, normal visual evoked potentials
Hypotonic facies	+	+	+	+
Small hands and feet	+	–	+	+
Severe global developmental delay and intellectual disability	+	+	+	+
Communication	no speech	no speech	no speech, babbles socially, expressive body language	no speech, babbles socially, expressive body language
Psychosocial	poor eye contact, minimal interest in surroundings	sociable, studies faces with interest	sociable, content, interested in surroundings	sociable, content, interested in surroundings
Gross motor	unable to sit, non-ambulatory	sits without support, non-ambulatory	sits with support, pulls to stand, walks with a walker	sits without support, stands and walks with support
Fine motor	transfers objects across midline	brings food to mouth, manipulates toys	brings food to mouth, manipulates toys	Manipulates objects
Muscle weakness	+, MRC grade 3/5	difficult to assess due to severe hypotonia	+, MRC grade 4/5	+, MRC grade 4/5
Seizures, age of onset	+, 3 months (none since 3 years old)	+, 3 years and 10 months	+, 6 months	+, 3 years
Seizure types	generalized tonic-clonic	generalized tonic-clonic	myoclonic, atonic, generalized tonic-clonic, atypical absences	atonic, atypical absences
Current anti-convulsive therapy	clonazepam	valproate, rivotril	valproate, lamotrigine, levetiracetam, vagal nerve stimulator	valproate
Severe constipation	+	+	+	+
Tactile aversion	–	–	+	+

(Continued on next page)

Table 1. Continued

Subject	Family 1	Family 2	Family 3	
	IV.1	V.5	II.1	II.3
Hypersensitivity for stimuli	–	+	+	+
Sleep disturbance	+, obstructive sleep apnea	+	–	–
Brain MRI	thin corpus callosum	no structural anomalies	no structural anomalies	no structural anomalies
EEG	encephalopathic background with occasional spike and wave discharges	encephalopathic background pattern with frequent multifocal discharges	encephalopathic background activity with frequent spike and wave discharges	encephalopathic background activity with frequent spike and wave discharges
Other	non-specific myopathic changes on muscle biopsy	not reported	mild scoliosis, euthyroid	milk protein intolerance in infancy, euthyroid
Mutation ^b	chr2:210783340C>T	chr2:210824431G>C	chr2:210832310T>A	chr2:210685105delA
<i>UNC80</i> (GenBank: NM_032504.1)	c.5098C>T	c.7607G>C	c.7757T>A	c.2033delA
Exon number	32	50	51	13
Predicted effect on protein	p.Pro1700Ser	p.Arg2536Thr, predicted to cause aberrant splicing	p. Leu2586*	p.Asn678Thrfs*15
Type of mutation	missense	missense	stop	frameshift insertion

Abbreviations are as follows: +, present; –, not present; OFC, occipital frontal circumference; MRC, Medical Research Council scale for muscle strength.

^aMeasurements for individual II.3 were taken at the last examination at the age of 9 years, rather than at the current age.

^bBased on UCSC Genome Browser GRCh37.

by extracellular Ca²⁺ through a G-protein-dependent pathway and by neuropeptides, such as substance P, through a G-protein-independent pathway that also requires the Src family of kinases.⁵

In worms and flies, mutations in *Nalcn*, *Unc-79*, and *Unc-80* lead to uncoordinated movements, abnormal circadian rhythms, and altered sensitivity to anesthetics.^{9–13} In mice, knocking out *Nalcn* or *Unc79* leads to severe apnea and neonatal lethality.^{4,5,7} In humans, inherited homozygous loss-of-function *NALCN* mutations cause infantile hypotonia with psychomotor retardation and characteristic facies (IHPRF [MIM: 615419]).^{14,15} De novo heterozygous *NALCN* mutations are also found in individuals with arthrogryposis, hypotonia, intellectual disability, and developmental delay.^{16,17}

Despite its widespread expression in the brain, the in vivo function of *UNC80* is largely unknown, and no mutations in mammals have been reported. We report the identification and characterization of *UNC80* mutations as the cause of severe neurological phenotypes in four female individuals from three unrelated families.

The four subjects have similar phenotypes characterized by severe hypotonia of neonatal onset and that persists until adolescence, motor delays, lack of independent ambulation, constipation, encephalopathy, seizures, absent speech, and severe intellectual disability. The clinical features and genetic results of all subjects are summarized in Table 1 and Figure 1. Research protocols were approved in each country via institutional research boards and regional ethics committees, in keeping with national guidelines and the principles laid out in the Declaration

of Helsinki. For the Norwegian family (F3), molecular analyses were performed in accordance with the Norwegian National Biotechnology Act. All parents provided written informed consent for study participation for themselves and their children, as well as for publication of clinical information, molecular findings, and photographs.

For family 1, the whole-exome sequencing (WES) library preparation, exon capture, and sequencing were performed at the Genome Québec Innovation Center (Montréal) as previously described.¹⁸ Genomic DNA was captured with the SureSelect Human 50 Mb All Exon V5 kit (Agilent Technologies). Sequencing was performed on an Illumina HiSeq 2000 (Illumina) with paired-end 100-bp reads. A mean coverage of 134× was obtained, and 97% of the bases were covered at more than 10×. WES trio average coverage was 55×. Read alignment, variant calling, and annotation were done with a pipeline based on the Burrows-Wheeler Aligner (BWA), SAMtools, ANNOVAR, and custom annotation scripts, and reads were aligned to the reference human genome (UCSC Genome Browser hg19).

For family 2, DNA capture was performed with the SeqCap EZ Exome v.3.0 (Roche Nimblegen). Sequencing was performed on an Illumina HiSeq 2000 (Illumina) with paired-end 100-bp reads. A mean coverage of 76× was obtained, and 94% of the bases were covered at more than 10×. Read alignment to the reference human genome (hg19) and variant calling were done with a pipeline based on BWA-MEM v.0.7 and the Genome Analysis Toolkit (GATK v.3.1-1-g07a4bf8). Variant annotation and prioritizing were done with Cartagenia NGS Bench (Cartagenia).

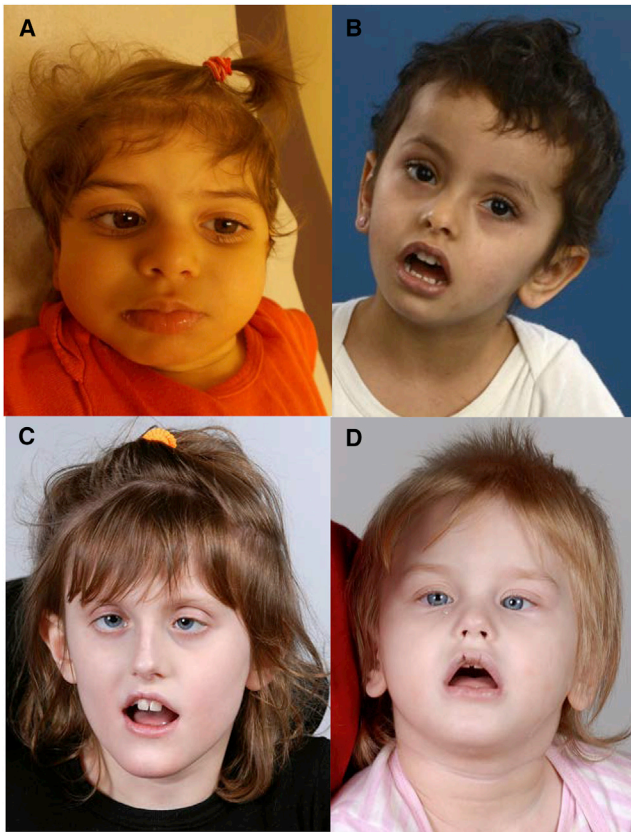


Figure 1. Clinical Photographs of All Four Subjects
 (A) Subject F1-IV.1 at the age of 4 years.
 (B) Subject F2-V.5 at the age of 3 years.
 (C) Subject F3-II.1 at the age of 9 years.
 (D) Subject F3-II.3 at the age of 3 years.

Four individuals from family 3 were tested and included in the Center for Mendelian Genomics project at the Baylor College of Medicine (BCM) in Houston, Texas. The

WES method used at the BCM-HGSC (Human Genome Sequencing Center) has previously been described.¹⁹ In brief, DNA was prepared for Illumina paired-end libraries, and capture was performed with the in-house-developed BCM-HGSC Core design and sequenced on the Illumina HiSeq 2500 platform (Illumina). Data produced was processed through the HGSC-developed Mercury pipeline, available in the cloud,²⁰ to produce variant call format files (.vcf) with the Atlas2 variant calling method.^{21–23} Variants were annotated with the in-house-developed “Cassandra”²⁴ annotation pipeline, based on ANNOVAR.²⁵ Variants of interest were selected on the basis of inheritance pattern, rarity, and evaluation of possible genotype-phenotype correlation by knowledge of gene function, pathway, expression pattern, and results from other model organisms. Alamut v.2.4.6 (Interactive Biosoftware) was used in evaluation of pathological relevance. Additional support was sought by application of computational prediction tools (PhyloP, SIFT, PolyPhen-2, LRT, and MutationTaster). All *UNC80* variants were confirmed by Sanger sequencing. Unaffected family members were also Sanger sequenced to confirm that the variant segregated with disease. Sanger sequencing was performed on genomic DNA from peripheral blood. Primers for Sanger sequencing were designed with Primer3 software and sequenced on an ABI 3730 sequencer (Applied Biosystems, Life Technologies), and sequence data was analyzed with SeqScape v.2.7 (Life Technologies) and 4Peaks.

Subject F1-IV.1 was found to be homozygous for a c.5098C>T missense variant (p.Pro1700Ser) in exon 32 of *UNC80* (GenBank: NM_032504.1). The variant is predicted to be deleterious and is present at a position completely conserved among all animals (Figure S3). The subject’s father was unable to be tested, but her mother was confirmed to be a carrier of the variant (Figure 2).

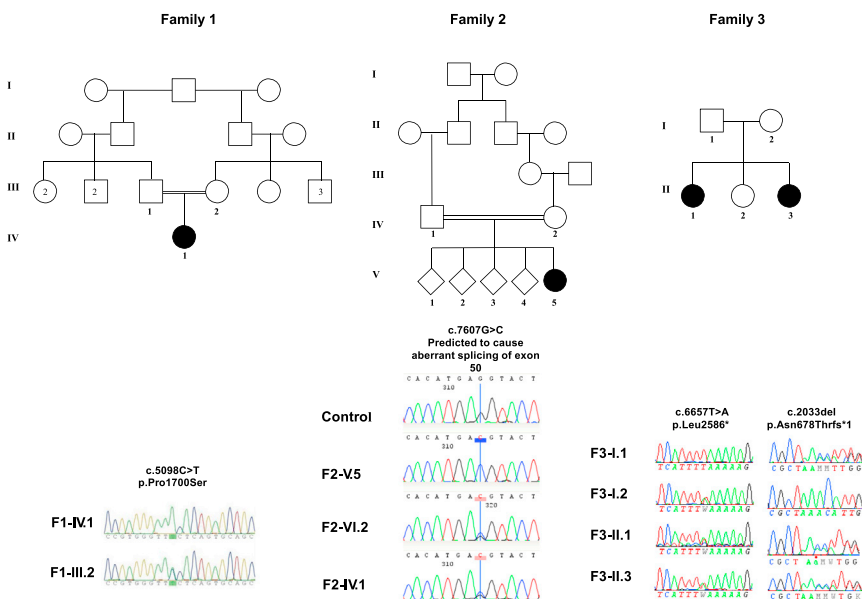


Figure 2. Pedigrees and Chromatograms from All Three Families
 Affected individuals are shaded in black.

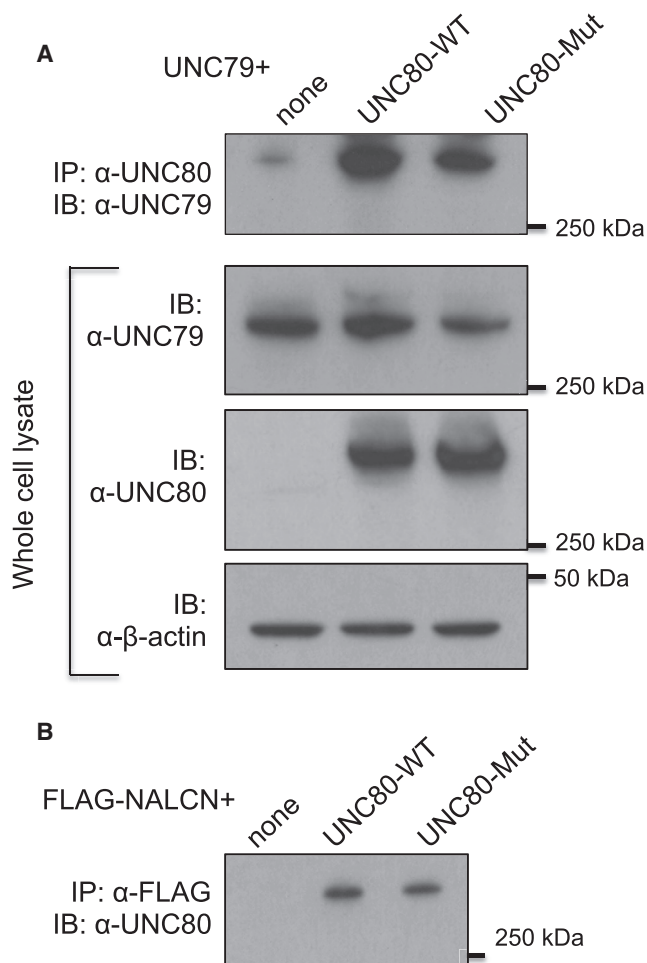


Figure 3. Variant *UNC80* Associates with *UNC79* and *NALCN*
 (A) Cells were transfected with *UNC79* alone or together with WT or variant *Unc80* as indicated in each lane. Immunoprecipitates (IP) with an anti-*UNC80* antibody were blotted (IB) with anti-*UNC79* to allow us to probe the association between *UNC79* and *UNC80* (upper panel). Total cell lysates (lower three panels) were blotted with anti-*UNC79* or anti-*UNC80* antibodies for comparison of protein amounts. Immunoblotting with anti- β -actin (lower panel) was used as a control for sample loading.
 (B) Cells were transfected with FLAG-tagged *NALCN* alone or together with the WT or variant *Unc80*, as indicated. Immunoprecipitates with anti-FLAG were blotted with anti-*UNC80* to allow us to probe the association between *UNC80* and *NALCN*. For immunoprecipitation, cells were solubilized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% PIC [Roche]) and immunoprecipitated with anti-*UNC80* (2 μ g/ml)⁷ or anti-FLAG (5 μ g/ml, no. F3165 [Sigma]). The immune complexes were analyzed by western blot with anti-*UNC80* (2.2 μ g/ml) or anti-*UNC79* (3 μ g/ml). Cell lysates (inputs) were also analyzed by western blot with anti-*UNC80* or anti- β -actin (1:1,000, no. 4970S [Cell Signaling]) as indicated.

Subject F2-V.5 had a homozygous missense variant in *UNC80*, c.7607G>C (p.Arg2536Thr). G7607 is the last nucleotide of exon 50. The c.7607G>C variant disrupts the “AG” exonic sequence commonly found in exon-intron junctions upstream of the conserved splice-donor sequence “GT.” The variant is predicted by five independent methods to affect splicing efficiency (Figure S4). Splicing studies of *UNC80* were not performed because

UNC80 is not expressed in peripheral leukocytes and no other tissue was available from this individual. The variant (p.Arg2536Thr) is also predicted to neutralize a charged residue highly conserved in all animals (Figure S5), suggesting that the variant disrupts protein function even in transcripts that are correctly spliced. Splicing analysis in neurons and functional characterization of the corresponding protein are required to determine the molecular consequence of this variant. Both healthy parents of subject F2-V.5 were heterozygous carriers, and all four healthy siblings were either heterozygous or bi-allelic wild-type (WT) at this nucleotide position (Figure 2). Subject F3-II.1 and subject F3-II.3 were both found to be compound heterozygous for the following *UNC80* variants: c.2033delA (p.Asn678Thrfs*15) and c.7757T>A (p.Leu2586*) (GenBank: NM_032504.1). Both parents were found to be heterozygous carriers of one of the variants (Figure 2). None of the variants were present in publically available databases (dbSNP, 1000 Genomes, and the ExAC Browser), and the c.5098C>T variant was found in the heterozygous state in one individual in the database of over 3,000 exomes at the Center for Mendelian Genomics.

The three mutations in subjects F2-V.5, F3-II.1, and F3-II.3 are predicted to lead to disruption (in F2-V.5, due to aberrant splicing) or truncation (in F3-II.1 and F3-II.3) of *UNC80* and are most likely loss-of-function mutations. To determine whether the c.5098C>T missense variant (p.Pro1700Ser) in subject F1-IV.1 affects expression and function of *UNC80*, we introduced the variant into mouse *Unc80* cDNA, which encodes a protein with 97% identity to the human *UNC80* and has been functionally characterized. The mouse *Unc80* cDNA (GenBank: FJ210934) was cloned into the EcoRI and NotI sites of vector pcDNA3.1. The p.Pro1769Ser variant (equivalent to human p.Pro1700Ser) was introduced with the Gibson assembly method. The sequences of the primers used for mutagenesis are as follows: 5'-GTTCGACCCTCCGTGGGTCtCTCAGTGCAGCGGGAGTG-3' (forward), 5'-CACTCCCGCTGCCTGAGAGACCCACGGAGGGTCAAC-3' (reverse). The mutation was confirmed with Sanger sequencing.

We transfected the WT and variant *Unc80* into human HEK293T cells (from ATCC, maintained in DMEM [GIBCO] supplemented with 10% FBS [Atlanta Biologicals] and 1 \times Glutamax [GIBCO]; transfection done with Lipofectamine 2000 as the transfection reagent). The *Unc80* variant yielded an amount of protein comparable to that yielded by the WT *Unc80* (Figure 3A, lower two panels), suggesting that this missense variant does not disrupt production of the protein. The variant protein also retained its ability to associate with *UNC79* (Figure 3A) and *NALCN* (Figure 3B), which was tested by co-immunoprecipitation.

We next used electrophysiological assays to test whether the p.Pro1769Ser (mouse equivalent of p.Pro1700Ser) variant affects the function of *UNC80*. A unique property of *UNC80* is its ability to scaffold Src kinase and to enhance

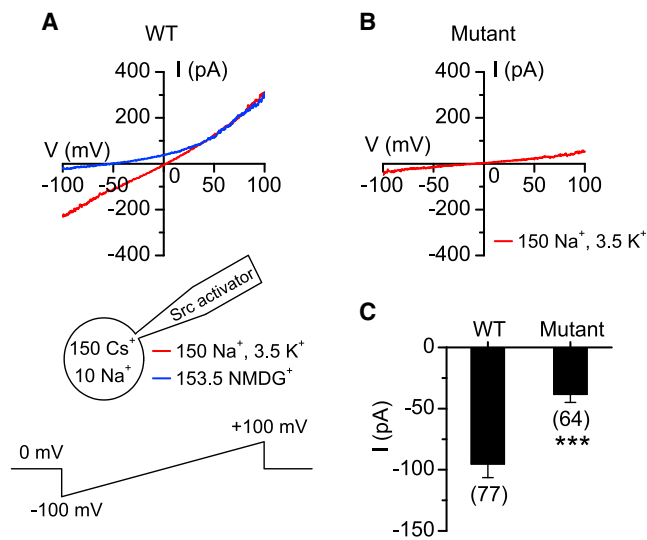


Figure 4. Altered Function in Variant *UNC80*

(A and B) Representative NALCN currents recorded in HEK293T cells co-transfected with *NALCN* and WT *Unc80* (A) or the mutant version of *Unc80* (B). Whole-cell currents were recorded with a voltage ramp from -100 mV to $+100$ mV in 1 s ($V_h = 0$ mV, illustrated in the lower panel of A). In cells with currents larger than 50 pA, NMDG was used to substitute Na^+ and K^+ to confirm that the cells had no non-specific leak, as demonstrated by the abolishment of inward currents with ion substitution.

(C) Summary of the current amplitudes recorded at -100 mV. Numbers of cells recorded are in parentheses. Data were presented as mean \pm SEM. * $p < 0.05$. *** $p < 0.001$. For patch clamp recordings, cells in a 35-mm dish ($\sim 90\%$ confluency) were transfected with 3 μg plasmids DNA with Lipofectamine 2000 for ~ 40 hr and plated onto polylysine-coated coverslips. Recordings were done 40 – 48 hr after transfection. Cells with GFP fluorescence intensity within the top 20% – 30% on each coverslip were selected for patch clamp recordings. Signal was acquired with an amplifier (Axopatch 200B or Multiclamp 700B) and a Digidata 1440A data acquisition system controlled by PClamp software (Molecular Device). The pipette solution contained 150 mM Cs, 120 mM Mes, 10 mM NaCl, 10 mM EGTA, 4 mM CaCl_2 , 0.3 mM Na_2GTP , 2 mM Mg-ATP, 10 mM HEPES and 2 μM Src family kinase activator (pH 7.4 ; from Santa Cruz). Bath solutions contained 150 mM NaCl, 3.5 mM KCl, 1 mM MgCl_2 , 1.2 mM CaCl_2 , 20 mM glucose, and 10 mM HEPES (pH 7.4). In the NMDG bath, Na^+ and K^+ were replaced by NMDG $^+$. Liquid junction potentials were corrected online.

NALCN currents through a Src-dependent pathway.^{6,8} We recorded *NALCN* currents from HEK293T cells that we co-transfected with *NALCN* cDNA (human isoform,⁷ in a pTracer vector expressing GFP under a separate promoter) and WT or variant *Unc80* by using patch clamp pipettes containing a peptide Src activator (Figure 4A, lower panel). Compared to those in cells transfected with WT *Unc80*, the sizes of the currents were significantly reduced in cells expressing the variant (Figures 4B and 4C).

In summary, our studies reveal a genetic cause for global developmental delay and neurological dysfunction and demonstrate the importance of *UNC80* in humans. From a motor perspective, all four individuals were significantly delayed in sitting; one individual was still unable to achieve this at the age of 4 years. None of the individuals were able to ambulate independently. These symptoms

are comparable with those observed in individuals with loss-of-function *NALCN* mutations.^{14,15} Unlike individuals with *NALCN* mutations, our four *UNC80* individuals show no true facial dysmorphism, although their faces are characterized by features related to severe hypotonia. All individuals in our study were described as encephalopathic, both clinically and on electroencephalogram (EEG) monitoring. Growth failure, severe constipation, and the feeding difficulties requiring G-tube insertion might be due, in part, to dysfunctional muscle coordination via neuronal compromise; however, standardized evaluation of swallowing function for all individuals would be required to confirm this. Subject F1-IV.1 also had severe sleep disturbance, including complete reversal of her sleep-wake cycle, which was resistant to treatment with melatonin. Subject F2-V.5 also had sleep disturbance, including difficulty falling asleep. In flies and mice, *NALCN* is essential for the depolarization of clock neurons in the morning and the maintenance of normal circadian rhythms.²⁶ It is possible that abnormal *UNC80* prevents activation of the *NALCN* complex and inhibits the ability of pacemaker neurons to maintain basal sleep-wake rhythm.

Compared to phenotypes associated with mutations found in known neuronal genes, the neurological phenotypes we describe in these four *UNC80* individuals are at the severe end of the spectrum. This underscores the pivotal importance of leak ion channel genes and the associated basal excitability in human health and disease.

Supplemental Data

Supplemental Data include additional case histories, five figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2015.11.004>.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, <http://browser.1000genomes.org>
4Peaks, <http://nucleobytes.com/4peaks>
dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>
ExAC Browser, <http://exac.broadinstitute.org/>
GenBank, <http://www.ncbi.nlm.nih.gov/genbank/>
GeneMatcher, <https://genematcher.org/>
MutationTaster, <http://www.mutationtaster.org/>
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>
OMIM, <http://www.omim.org/>
PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>
SIFT, <http://sift.bii.a-star.edu.sg/>
UCSC Genome Browser, <http://genome.ucsc.edu>

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