Supplementary Material

Congenital insensitivity to pain: a novel mutation affecting a U12-type intron causes multiple aberrant splicing of SCN9A

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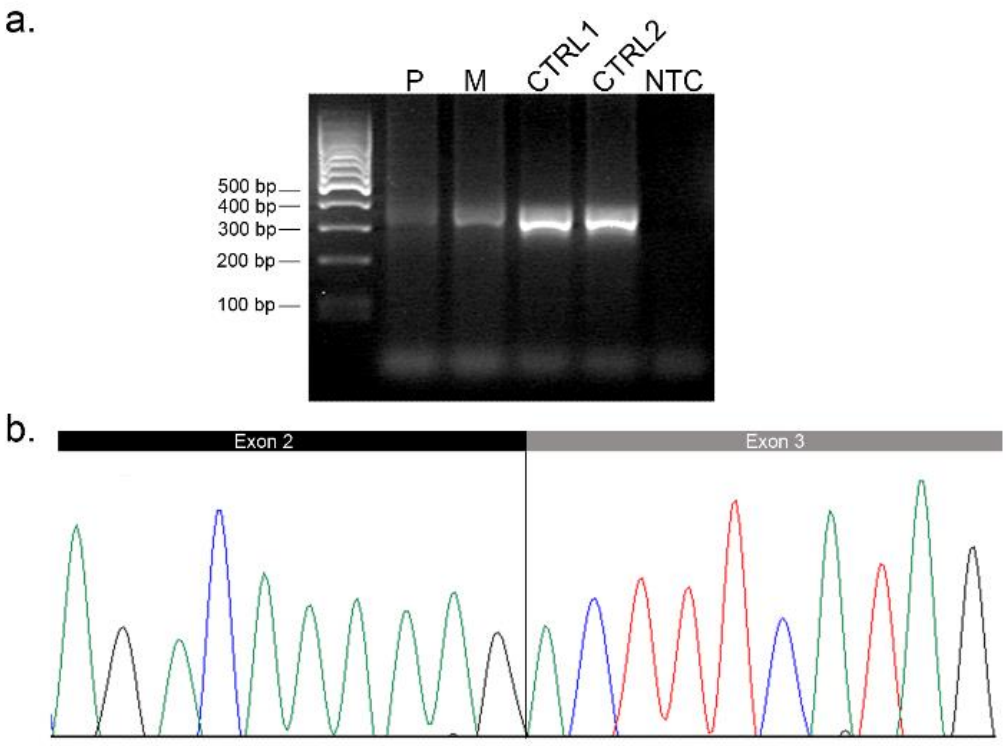

Figure S1. Investigation of residual amount of wild-type transcript in the proband. WT-selective primers, precluding the amplification of the two aberrant transcripts, have been designed to avoid quantitative bias in PCR amplification. (**a**) Agarose gel shows a very weak signal corresponding to the WT allele in the proband and in the mother, whereas it is well represented in the control samples. P: Proband; M: Mother; CTRL: healty controls; NTC: No Template Control. (**b**) Sanger sequencing of the proband wild-type allele, cropped-out from agarose gel, confirmed the expected nucleotide sequence.

Table S1. Quantitative RT-PCR data on SCN9A transcript from skin and from blood. HCs: healthy controls; GAPDH, housekeeping gene; NTC: No Template Control.

Skin		
Sample	**Target**	**Average Ct**
HC-1	GAPDH	28,92
HC-1	SCN9A	36,09
HC-2	GAPDH	28,3
HC-2	SCN9A	36,83
mother	GAPDH	28,15
mother	SCN9A	38,66
proband	GAPDH	28,03
proband	SCN9A	39,61
NTC	GAPDH	Undetermined

Blood		
Sample	**Target**	**Average Ct**
HC-1	GAPDH	21,25
HC-1	SCN9A	30,6
HC-2	GAPDH	25,52
HC-2	SCN9A	33,39
HC-3	GAPDH	25,26
HC-3	SCN9A	31,94
HC-4	GAPDH	25,21
HC-4	SCN9A	33,53
Proband	GAPDH	23,17
Proband	SCN9A	35,83
NTC	GAPDH	Undetermined
NTC	SCN9A	Undetermined

Supplementary Methods

Splice sites predictions

Comparison of Splice sites prediction between WT allele and the mutated c.377+7T>G with NetGene2 World Wide Web Server considering the genomic region from exon 2 to 4. The tool recognizes positions 310 and 5299 as the canonical DONORS for intron 2 and 4 respectively, but fails to identify the natural splice DONOR for intron 3 and its ACCEPTOR. The tool identifies as acceptor only the consensus of *SCN9A* isoform ENST00000303354.6 in position 5204.

The tool does not highlight any difference between the WT and the Mutated sequence; only the molecular analysis revealed that the consensus in position 4660 represents the strongest DONOR, resulting in a cryptic intronic donor site. The best ACCEPTOR consensus for this donor is recognized by the tool in position 4733 in both the WT and the Mutated sequence, but transcript molecular analysis revealed that, in presence of c.377+7T>G substitution, the stronger ACCEPTOR is position 4539.

NetGene2 World Wide Web Server:

Donor splice sites, direct strand, WT allele

pos 5'->3'	phase	strand	confidence	5' exon intron 3'	
310	**0**	**+**	**1.00**	**AGACAAAAAG^GTGAGTTTAT**	H
333	2	+	0.31	GACTTCAGTG^GTCAGTTTCT	
602	0	+	0.44	CATTGTGCAG^GTTAGTTACA	
1972	2	+	0.32	TTGAAGTCAG^GTAGCGTGAT	
2268	1	+	0.34	CACATCCCTT^GTAAGTTGGA	
2559	2	+	0.45	CAAGTATAAG^GTAACTTGAT	
3008	2	+	0.34	GGGACTACAG^GTACCCGCCA	
3818	0	+	0.64	CCTGAATACG^GTGAGAAATG	
4120	0	+	0.34	AATTACCGTG^GTTAGTAAGT	
4124	1	+	0.54	ACCGTGGTTA^GTAAGTGAGG	
4660	1	+	0.71	**ATGTAGAAAG^GTAAACTGCT**	
5299	0	+	0.96	**AAAATGTCGA^GTAAGTGGGT**	H
5303	1	+	0.70	TGTCGAGTAA^GTGGGTATAA	
5307	2	+	0.24	GAGTAAGTGG^GTATAAGTAC	
6017	2	+	0.67	TTGTTTTTGC^GTAAGTACTT	

Acceptor splice sites, direct strand, WT allele

pos 5'->3'	phase	strand	confidence	5' intron exon 3'
105	0	+	0.43	CACAAAACAG^TCTCTTGCCC
717	2	+	0.33	CCCACCACAG^TCCCTAGAGT
1199	0	+	0.16	TCCTCTCCAG^CACCTGTTGT
1602	2	+	0.27	GGTGTTTTAG^ACATGAAGTC
1830	2	+	0.56	TTTTTCTCAG^GTTTGTCAAA
2008	2	+	0.43	TTTGGCTTAG^GATTGACTTG
2071	2	+	0.43	TTTTTTCCAG^TTCTGTGAAG
2081	1	+	0.07	TTCTGTGAAG^AAAGTCATTG
2767	2	+	0.53	ATTTTTCTAG^GAAGTTCCTT
3462	0	+	0.27	TTACCACCAG^GTCCCTCCCC
3682	1	+	0.16	TTGTATTTAG^ATAGAACTGA
4516	0	+	0.17	TAACAAATAG^CTCAATTTTT
4530	2	+	0.19	**ATTTTTAAAG^TTACTATGAA**

4541	1	+	0.19	TACTATGAAG^AAGTGGACTT
4544	1	+	0.19	TATGAAGAAG^TGGACTTGGA
4555	0	+	0.18	GGACTTGGAG^TTCATTGGCC
4733	0	+	0.94	**CCTCCTGCAG^ACTTTCATAG**
4813	0	+	0.33	CTCCTTTCAG^TCCTCTAAGA
5121	1	+	0.49	TTTTTCTTAG^GAAAAGTTGT
5204	2	+	0.14	**TGATTCTAAG^CTACCTTATT**
5217	0	+	0.19	CCTTATTCAG^CATGCTCATC
5367	1	+	0.56	TTCCTGCCAG^GAAGTTACTG

Donor splice sites, direct strand, c.377+7T>G allele

pos 5'->3'	phase	strand	confidence	5' exon intron 3'	
310	**0**	**+**	**1.00**	**AGACAAAAAG^GTGAGTTTAT**	**H**
333	2	+	0.31	GACTTCAGTG^GTCAGTTTCT	
602	0	+	0.44	CATTGTGCAG^GTTAGTTACA	
1972	2	+	0.32	TTGAAGTCAG^GTAGCGTGAT	
2268	1	+	0.34	CACATCCCTT^GTAAGTTGGA	
2559	2	+	0.45	CAAGTATAAG^GTAACTTGAT	
3008	2	+	0.34	GGGACTACAG^GTACCCGCCA	
3818	0	+	0.64	CCTGAATACG^GTGAGAAATG	
4120	0	+	0.34	AATTACCGTG^GTTAGTAAGT	
4124	1	+	0.54	ACCGTGGTTA^GTAAGTGAGG	
4660	**1**	**+**	**0.71**	**ATGTAGAAAG^GTAAACTGCT**	

Acceptor splice sites, direct strand, c.377+7T>G allele

pos 5'->3'	phase	strand	confidence	5' intron exon 3'
105	0	+	0.43	CACAAAACAG^TCTCTTGCCC
717	2	+	0.33	CCCACCACAG^TCCCTAGAGT
1199	0	+	0.16	TCCTCTCCAG^CACCTGTTGT
1602	2	+	0.27	GGTGTTTTAG^ACATGAAGTC
1830	2	+	0.56	TTTTTCTCAG^GTTTGTCAAA
2008	2	+	0.43	TTTGGCTTAG^GATTGACTTG
2071	2	+	0.43	TTTTTTCCAG^TTCTGTGAAG
2081	1	+	0.07	TTCTGTGAAG^AAAGTCATTG
2767	2	+	0.53	ATTTTTCTAG^GAAGTTCCTT
3462	0	+	0.27	TTACCACCAG^GTCCCTCCCC
3682	1	+	0.16	TTGTATTTAG^ATAGAACTGA
4516	0	+	0.17	TAACAAATAG^CTCAATTTTT
4530	**2**	**+**	**0.19**	**ATTTTTAAAG^TTACTATGAA**
4541	1	+	0.19	TACTATGAAG^AAGTGGACTT
4544	1	+	0.19	TATGAAGAAG^TGGACTTGGA
4555	0	+	0.18	GGACTTGGAG^TTCATTGGCC
4733	**0**	**+**	**0.95**	**CCTCCTGCAG^ACTTTCATAG**
4813	0	+	0.43	CTCCTTTCAG^TCCTCTAAGA
5121	1	+	0.49	TTTTTCTTAG^GAAAAGTTGT

CUTOFF values used for confidence:

Highly confident donor sites (H): 95.0 %
Nearly all true donor sites: 50.0 %

Highly confident acceptor sites (H): 95.0 %
Nearly all true acceptor sites: 20.0 %

The splicing prediction analysis with other on-line free tools Splice Site Prediction by Neural Network (FruitFly) and Alternative Splice Site Predictor (ASSP), gave the same results.

The genomic sequence of *SCN9A* (RefSeq NCBI NM_002977; Ensembl ENST00000409672.1) is reported below, beginning from the START codon in exon2, showing the exonic regions in blue capital letters, and introns in black lower case. Splicing consensus sequences are highlighted in grey.
Underlined nucleotides represent the retained intronic region (129 bp from intron 2 and 4bp from intron 3), caused by the mutation c.377+7T>G, in the aberrant transcript, named as SK3_INT2_ENST354 in the main text.

SCN9A gene sequence (Ensembl ENST00000409672.1)

ATGGCAATGTTGCCTCCCCCAGGACCTCAGAGCTTTGTCCATTTCACAAAACAGTCTCTTGCCCTCATTGAACAACGCAT
TGCTGAAAGAAAATCAAAGGAACCCAAAGAAGAAAAGAAAGATGATGATGAAGAAGCCCCAAAGCCAAGCAGTGACTTGG
AAGCTGGCAAACAGCTGCCCTTCATCTATGGGGACATTCCTCCCGGCATGGTGTCAGAGCCCCTGGAGGACTTGGACCCC
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taaattgagattagtgaaatttagtgaataaccatatcatgaccagaatgttaaattaaatatatatatatatatatata
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tactatgaagaagtggacttggagttcattggccaaggtgacattgatagatgcgttgatgacattggaaccacattgct
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Catatcct**t**ttaaaaatgattacatccagtggcactttatggtgtaatttttgctattttattcaaatatagatgttata
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tgaaaatctcaggcagcacagtgttccaataacagcaaacctctggggtctgatttttcca

Comparison between the SCN9A transcript isoforms

Alignment, made by Clustal W Multiple Sequence Aligner, of the two transcript isoforms of SCN9A, including the region between exon 2 and exon 5, reveals the presence of 3 supernumerary nucleotides in position c.378-380. ENST00000409672.1 transcript is annotated in NCBI with the Reference Sequence: NM_002977.3 and is classified as reference standard in the RefSeqGene project. Transcript ENST00000303354.6 is not reported in NCBI. The adjacent exons are alternately colored in blue and black letters.

Notably, the splice-junction between exon 3 and exon 4 is 1-bp shifted in the two transcripts.

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ENST00000409672.1  ACATTCCTCCCGGCATGGTGTCAGAGCCCCTGGAGGACTTGGACCCCTACTATGCAGACA  253
ENST00000303354.6  ACATTCCTCCCGGCATGGTGTCAGAGCCCCTGGAGGACTTGGACCCCTACTATGCAGACA  253
                   ************************************************************

ENST00000409672.1  AAAAGACTTTCATAGTATTGAACAAAGGGAAAACAATCTTCCGTTTCAATGCCACACCTG  313
ENST00000303354.6  AAAAGACTTTCATAGTATTGAACAAAGGGAAAACAATCTTCCGTTTCAATGCCACACCTG  313
                   ************************************************************

ENST00000409672.1  CTTTATATATGCTTTCTCCTTTCAGTCCTCTAAGAAGAATATCTATTAAGATTTTAGTAC  373
ENST00000303354.6  CTTTATATATGCTTTCTCCTTTCAGTCCTCTAAGAAGAATATCTATTAAGATTTTAGTAC  373
                   ************************************************************

ENST00000409672.1  ACTC---CTTATTCAGCATGCTCATCATGTGCACTATTCTGACAAACTGCATATTTATGA  430
ENST00000303354.6  ACTCTACCTTATTCAGCATGCTCATCATGTGCACTATTCTGACAAACTGCATATTTATGA  433
                   ****   ***************************************************

ENST00000409672.1  CCATGAATAACCCACCGGACTGGACCAAAAATGTCGAGTACACTTTTACTGGAATATATA  490
ENST00000303354.6  CCATGAATAACCCACCGGACTGGACCAAAAATGTCGAGTACACTTTTACTGGAATATATA  493
                   ************************************************************

ENST00000409672.1  CTTTTGAATCACTTGTAAAAATCCTTGCAAGAGGCTTCTGTGTAGGAGAATTCACTTTTC  550
ENST00000303354.6  CTTTTGAATCACTTGTAAAAATCCTTGCAAGAGGCTTCTGTGTAGGAGAATTCACTTTTC  553
                   ************************************************************

ENST00000409672.1  TTCGTGACCCGTGGAACTGGCTGGATTTTGTCGTCATTGTTTTTGCGTATTTAACAGAAT  610
ENST00000303354.6  TTCGTGACCCGTGGAACTGGCTGGATTTTGTCGTCATTGTTTTTGCGTATTTAACAGAAT  613
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Translation predictions by Expasy

Wild-type aminoacidic sequence from the first Met (exon 2) till exon 4:

MAMLPPPGPQSFVHFTKQSLALIEQRIAERKSKEPKEEKKDDDEEAPKPSSDLEAGKQLPFIYGDIPPGMVSEPL
EDLDPYYADKKTFIVLNKGKTIFRFNATPALYMLSPFSPLRRISIKILVHSLFSMLIMCTILTNCIFMTMNNPPD
WTKNV[…]

Predicted consequence on translation for the transcript SK3_INT2_ENST354: Intron 2 partial retention + exon 3 skipping + Exon 4 ENST00000303354.6

ATGGCAATGTTGCCTCCCCCAGGACCTCAGAGCTTTGTCCATTTCACAAAACAGTCTCTTGCCCTCATTGAACAA
CGCATTGCTGAAAGAAAATCAAAGGAACCCAAAGAAGAAAAGAAAGATGATGATGAAGAAGCCCCAAAGCCAAGC
AGTGACTTGGAAGCTGGCAAACAGCTGCCCTTCATCTATGGGGACATTCCTCCCGGCATGGTGTCAGAGCCCCTG
GAGGACTTGGACCCCTACTATGCAGACAAAAAG|ttactatgaagaagtggacttggagttcattggccaaggtg
acattgatagatgcgttgatgacattggaaccacattgctggagtctgatggcagtgtttcttcaatataacact
gccatgtaaaaag|CTACCTTATTCAGCATGCTCATCATGTGCACTATTCTGACAAACTGCATATTTATGACCAT
GAATAACCCACCGGACTGGACCAAAAATGTCGA

Predicted Consequence: p.Lys86fs2Stop

MAMLPPPGPQSFVHFTKQSLALIEQRIAERKSKEPKEEKKDDDEEAPKPSSDLEAGKQLPFIYGDIPPGMVSEPL
EDLDPYYADKKLL**Stop**RSGLGVHWPRStopHStopStopMRStopStopHWNHIAGVStopWQCFFNITLPCKK
LPYSACSSCALFStopQTAYLStopPStopITHRTGPKMS

Predicted consequence on translation of the transcript SK3_ENST354: Exon 3 skipping + Exon 4 ENST00000303354.6

ATGGCAATGTTGCCTCCCCCAGGACCTCAGAGCTTTGTCCATTTCACAAAACAGTCTCTTGCCCTCATTGAACAA
CGCATTGCTGAAAGAAAATCAAAGGAACCCAAAGAAGAAAAGAAAGATGATGATGAAGAAGCCCCAAAGCCAAGC
AGTGACTTGGAAGCTGGCAAACAGCTGCCCTTCATCTATGGGGACATTCCTCCCGGCATGGTGTCAGAGCCCCTG
GAGGACTTGGACCCCTACTATGCAGACAAAAAG|CTACCTTATTCAGCATGCTCATCATGTGCACTATTCTGACA
AACTGCATATTTATGACCATGAATAACCCACCGGACTGGACCAAAAATGTCGA

Predicted Consequence: p.Lys86fs12Stop

MAMLPPPGPQSFVHFTKQSLALIEQRIAERKSKEPKEEKKDDDEEAPKPSSDLEAGKQLPFIYGDIPPGMVSEPL
EDLDPYYADKKLPYSACSSCALF**Stop**QTAYLStopPStopITHRTGPKMS

Web resources

Ensembl: *http://grch37.ensembl.org/Homo_sapiens/*

NetGene2: *http://www.cbs.dtu.dk/services/NetGene2/*

FruitFly: *https://www.fruitfly.org/seq_tools/splice.html*

ASSP: *http://wangcomputing.com/assp/*

Clustal W: *https://www.genome.jp/tools-bin/clustalw*

ExPASy: *https://web.expasy.org/translate/*