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In-Silico characterization of SCN9A: A protein that mediates voltage-dependent sodium ion permeability of excitable membranes

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Abstract

Sodium channel Nav 1.7, *SCN9A* gene encodes the SCN9A protein which is associated with either pain insensitivity typically with loss-of-function mutations, and chronic pain disorders typical with gain-of-function mutations. In the present study, we tried to characterize the SCN9A structure and predict its interactions with different proteins using different on-line tools. Protscale server predicted an unstable protein structure for SCN9A based on several parameters including accessibility, hydrophilicity, mutability, refractivity bulkiness, flexibility and polarity. Signal Server predicted no signal peptide in SCN9A. No acetylation site was present in SCN9A: as predicted by Net Acet. Different threonine, serine and tyrosine specific phosphorylation sites were predicted in SCN9A at different positions. Kinases like PKA and PKC were shown to be involved in phosphorylation of SCN9A. We also predicted different physiochemical parameters for SCN9A. The study also predicted that SCN9A might interact with other members of sodium channel family including SCN2A, SCN3A, SCN5A, SCN8A, SCN10A, SCN11A, SCN1B, SCN2B, SCN4B ABCB1 and CALM2 proteins. These results might be helpful to understand disease pathogenesis and molecular mechanisms of different inherited disorders of nervous system including congenital sensitivity to pain caused by *SCN9A* mutations.

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Introduction

The broad class of autosomal recessive neurological diseases known as hereditary sensory and autonomic neuropathies (HSAN) includes congenital insensitivity to pain (CIP; MIM 243000). Patients with CIP are unable to feel pain and sometimes heat. There are some cases reported in which patients are unable to smell (anosmia) (Cox *et al*., 2006).

Mutations in several genes have been identified as causes of CIP, including *PRDM12* on 9q34 (HSAN8; MIM 616488), *NTRK1* on 1q23 (CIPA; MIM 256800), and *SCN11A* on 3p22 (HSAN7; MIM 615548), with *NTRK1* specific to pain insensitivity with anhydrosis (www.omim.org). HSAN type IID is an autosomal recessive form of CIP, and is caused by loss-of-function mutations in the gene *SCN9A*, located on 2q24.3 having 26 exons (Yang *et al*., 2004: Michiels *et al*., 2005).

The sodium channel proteins consist of four domains and linked to intracellular linkers. Each domain has six transmembrane helical structures (de Lera Ruiz M and Kraus RL., 2015). The *SCNA*, is broad family of sodium channels named from *SCN1A-SCN11A*. *It has been evolved from* archetypal potassium channel with quadruplication. *SCN9A* has expression profiles at higher rates in dorsal root ganglion (DRG) neurons along with sympathetic ganglion neurons (Rush *et al*., 2006).

It has also been reported that evolutionary pressure is responsible for different types of variants in SCN9A gene. The protons are main factors for excitation and inhibition of nociception (St. John Smith *et al.,* 2011). Efforts are made to understand the pathogenesis of pain disorders caused by SCN9A mutants. The protein product was utilized in the present study to find out some physical and structural characteristics to find some link towards heterogeneity of the mutations spectrum and how they cause different diseases of nervous system.

Materials and methods

Retrieval of the protein primary sequence and prediction of different structural parameters for SCN9A.

The primary sequence for SCN9A protein was retrieved from UCSC human genome browser (hg19) https://genome.ucsc.edu/cgi-bin/hgGateway. This sequence was used as input to predict accessibility, hydrophilicity, mutability, refractivity bulkiness, flexibility and polarity of SCN9A. Within expasy platform (www.web.expasy.org/protscale) protscale Server with all default parameters except normalization of the scale was used. SignaI P-4.1 (www.cbs.dtu.dk/services/SignalP) was used to predict the signal peptide in SCN9A protein based on artificial neural network including cleavage sites. Phosphorylation sites for SCN9A were predicted by using Netphos 3.1 server (www.cbs.dtu.dk/services/ NetPhos) with default parametrs and SCN9A kinase-specific phosphorylation sites were also predicted by using Net Phos K 1.0 server (www.cbs.dtu.dk/services /NetPhosK). Net Acet web server (www.cbs.dtu.dk /services/NetAcet), Net CGlyc web server (www.cbs. dtu.dk/services/Net CGlyc) and Net OGlyc and Net NGlyc web server (www.cbs.dtu.dk/ services) were used to predict Acetylation, Mannosylation and Glycosylation sites for SCN9A respectively.

Prediction of physiochemical parameters for SCN9A

Protparam Server (www.web.expasy. org/protparam) was used to validate the physicochemical parameters including amino acid composition, molecular weight, instability index and estimated half-life of SCN9A. Different bioinformatic tools including GOR4 (Garnier *et al*., 1996) and JPred3 (Cole *et al*., 2008). were used to predict secondary structure of SCN9A primary amino acid sequence.

Prediction of SCN9A protein-protein interactions

STRING platform was used to predict the interaction of SCN9A with different proteins. The methodology used to characterize the SCN9A protein provided in the Fig. 1.

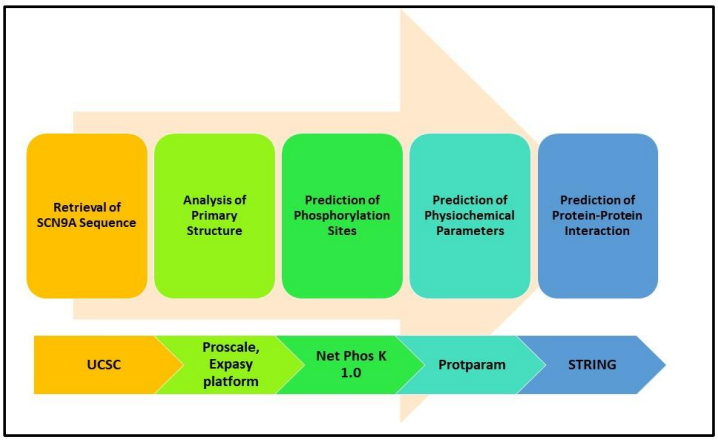

Fig. 1. Illustrating the methods used to characterize the SCN9A (Upper panel) and related software (lower panel).

Results

Primary Sequence Analysis of SCN9A

Primary Sequence Analysis of SCN9A protein is 1977 amino acid long and given below. >uc010fpl.3 (SCN9A) length= 1977.

Protscale server predicted results for accessibility, hydrophilicity, mutability, refractivity bulkiness, flexibility and polarity of SCN9A protein. The score values predict the probability of each parameter like lower values mean less probability and higher values mean more probability of parameters for SCN9A. The accessibility values were between 0.215 at aa position 1428 and 1.00 at aa positions 40 and 41. Janin score values were between 0.132 at position 483 aa and 0.881 at position 384 aa showing the free energy of transfer from inside to outside of a globular protein. Zimmerman score values were predicted between 0.957 at aa position 41 and 0.003 at aa position 864. These values predict the polarity: the dipole-dipole intermolecular interactions between negatively and positively charged residues. The relative mutability scores give the probability about amino acid over short evolutionary time frame and outcome was between 0.957 at aa position 41 and 0.003 at aa position 864. The average flexibility index of SCN9A was measured to show the flexibility of the protein with values between 0.907 at aa position 563 and 0.236 at aa position. Bulkiness: ratio of the side chain volume to the length of an amino acid, might affecting the native structure of protein was predicted for SCN9A with values 0.959 at aa position 384 and 0.358 at aa position 1716. All these results are illustrated in the Fig. 2 A, B, C, D, E and F. The amino acid sequences from N to C terminal are drawn on X-axis and scores subtracted from different algorithms are drawn on Y-axis.

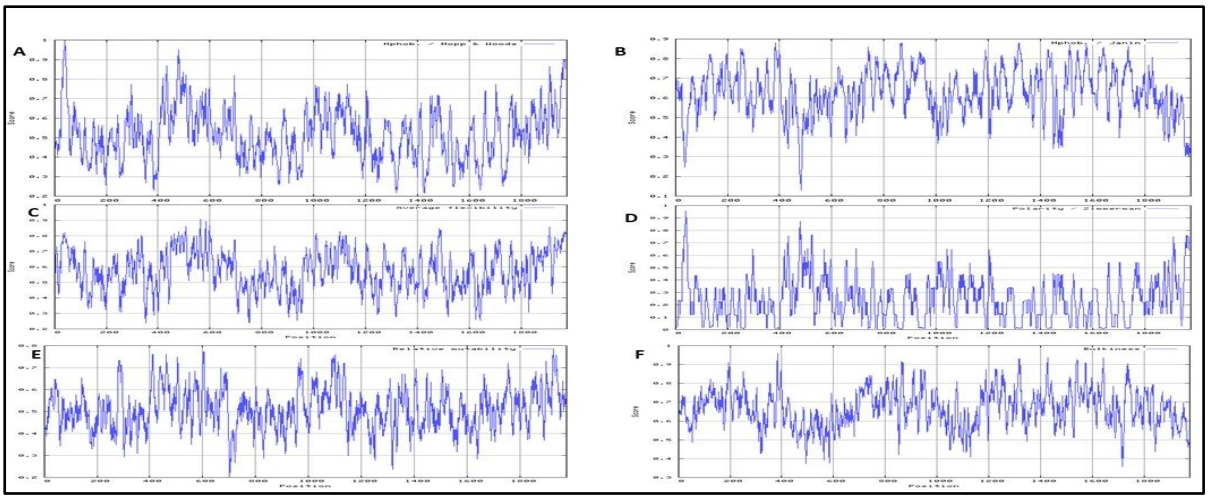

Fig. 2. Predicted hydrophilicity, accessibility, polarity, mutability, flexibility and bulkiness for SCN9A. X-axis: N-to C-terminal amino acid sequence Y-axis: scores from each algorithm. A: hydrophilicity, B: accessibility, C: flexibility, E: relative mutability, D: polarity and F: bulkiness.

No signal peptide was predicted in SCN9A and results were presented in Fig. 3A. Different scores like raw cleavage site score denoted by C, signal peptide score denoted by S and combined cleavage site score denoted by Y were used to predict the signal peptide. Signal peptide presence can be predicted by D score i.e. average of mean score value of S and max value of Y-score, for SCN9A it was 0.5 so no signal peptide was predicted. 24 transmembrane domains were predicted for SCN9A and shown in Fig. 3B.

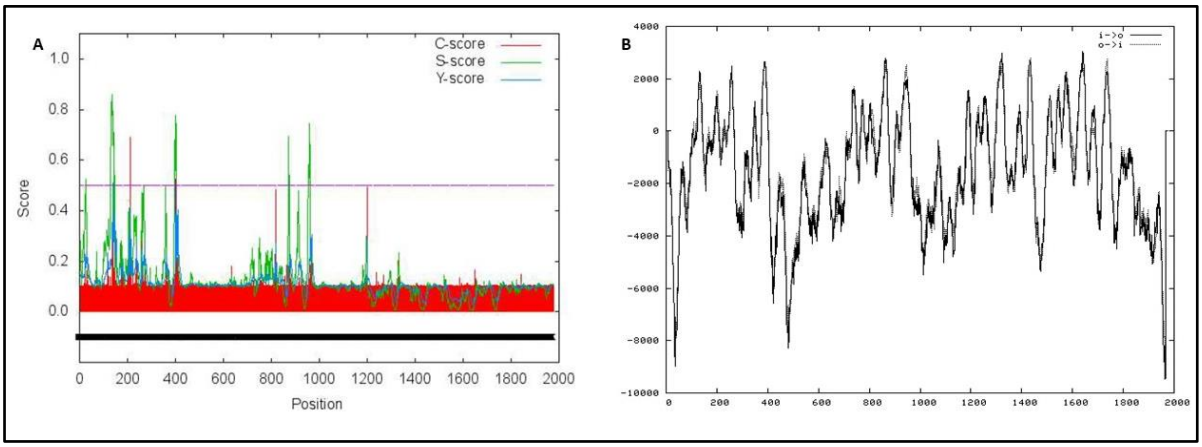

Fig. 3. A: Predicted no signal peptide in SCN9A by SignalP server. B: showing different 24 transmembrane domains. X-axis: N-to C-terminal amino acid sequence Y-axis: scores.

Post translation modification sites

Net Acet web server predicted no acetylation site in SCN9A with score value 0.461 while cut off value was 0.5. One mannosylation site for W was present in SCN9A predicted by using Net CGlyc web server with 0.505 score value at position 797. No O linked glycosylation site was predicted in the SCN9A. Phosphorylation sites for different threonine, serine and tyrosine residues were predicted and provided in the table 1 and Fig. 4A by using Netphos 3.1 server. Protparam server predicted different physiological parameters provided in the table 2.

Table 1, Different phosphorylation sites predicted for S, T and Y residues in SCN9A predicted by using Net Phos K 1.0 server.

Serine Phosphorylation Sites	Amino acid positions
PKA	19, 50, 119, 126, 246, 257, 279, 453, 513, 530, 542, 549, 550, 587, 599, 600, 660, 661, 680, 683, 802, 851, 1010, 1073, 1112, 1153, 1258, 1291, 1309, 1387, 1419, 1434, 1509, 1557, 1629,
CKII	50, 51, 343, 295, 461, 489, 490, 501, 503, 566, 568, 576, 665, 686, 688, 1063, 1085, 1096, 1097, 1099, 1137, 1181, 1737, 1753, 1764, 1842, 1850, 1930,
p38MAPK	110, 113, 535, 538, 608, 716, 1943
cdk5	110, 113, 535, 608, 1931, 1943,
PKG	119, 453, 472, 513, 550, 599, 600, 660, 1010, 1291,
CKI	129, 295, 462, 466, 469, 553, 556, 576, 1096, 1099, 1939, 1943,
RSK	119, 513, 550, 587, 600, 660, 661, 680, 1153,
PKC	241, 257, 390, 466, 469, 470, 489, 490, 538, 542, 545, 549, 553, 556, 583, 587, 661, 825, 851, 1004, 1054, 1067, 1161, 1479, 1699, 1902, 1972, 1975
DNAPK	279, 603, 1073, 1351, 1366,
cdc2	459, 461, 462, 469, 501, 503, 530, 542, 545, 599, 600, 683, 837, 958, 1099, 1104, 1114, 1258, 1270, 1529, 1862,
ATM	603,
PKB	680,

Serine Phosphorylation Sites	Amino acid positions
GSK3	1943
Threonine Phosphorylation Sites	Amino acid positions
PKG	87,
PKC	96, 138, 160, 215, 224, 252, 370, 465, 552, 893, 982, 994, 1021, 1077, 1073, 1210, 1223, 1261, 1280, 1393, 1548, 1562, 1590, 1835, 1871, 1951
p38MAPK	103, 531,
CKI	158, 285, 1266, 1672,
CKII	292, 806, 920, 966, 1021, 1760, 1963
DNAPK	359, 1031, 1783,
cdk5	531,
RSK	531,
cdc2	773, 1941
PKA	1210, 1398,
ATM	1783
Tyrosine Phosphorylation Sites	Amino acid positions
SRC	82, 1103, 1204, 1772
EGFR	339, 405, 447, 1204, 1744,
INSR	790, 1103, 1337, 1470, 1866, 1958
CKI	158, 285, 1266, 1672,
CKII	292, 806, 920, 966, 1021, 1760, 1963
DNAPK	359, 1031, 1783,
cdk5	531,
RSK	531,
cdc2	773, 1941
PKA	1210, 1398,
ATM	1783

Table 2. Different physiochemical parameters with obtained values from Protparam Software.

Parameter	Value
No. of aa	1977
Molecular Weight	225197.21
Theoretical P1	6.82
Total no. of negatively charged residues	228 (Asp+Glu)
Total no. of positively charged residues	225 (Arg+Lys)
Atomic composition	C=10207, H=15964, N=2612, O=2906 and S=107
Formula	$C_{10207}H_{15964}N_{2612}O_{2906}S_{107}$
Total no. of atoms	31796
Estimated half life	in mammals: 30 hours (in retinulocytes)
	>20 hours in yeast (*in-vivo*)
	>10 hours in *E. coli* (*in-vivo*)
Instability Index	44.78 (predicted as unstable protein)
Aliphatic index	93.23
Grand Average of Hydropathicity (GRAVY)	-0.027

Secondary structure prediction

Different on-line soft wares like JPred3 and GOR4 were used to predict the secondary structure of SCN9A, some soft-wares were having amino acid sequence limitations so we divided the amino acid primary structure to two domains. In supplementary Fig. 1A amino acid from 1 to 1303 were shown while in supplementary Fig. 1B amino acid 1304 to 1977 were shown with numerical values not aa position. Similar results were obtained from all soft-wares and provided in supplementary Fig. 1.

The secondary structure results predicted SCN9A as structured protein and from Protein Data Bank three-dimensional structure of SCN9A was viewed and it also confirmed our predicted results.

Prediction of protein-protein interactions

To predict the interactions of SCN9A with other cellular proteins STRING software was used and gave interesting results. It is found that SCN9A interacts with other sodium channel family members like SCN2A, SCN3A, SCN5A, SCN8A, SCN10A, SCN11A, SCN1B, SCN2B, SCN4B ABCB1 and CALM2 as illustrated in Fig. 4B.

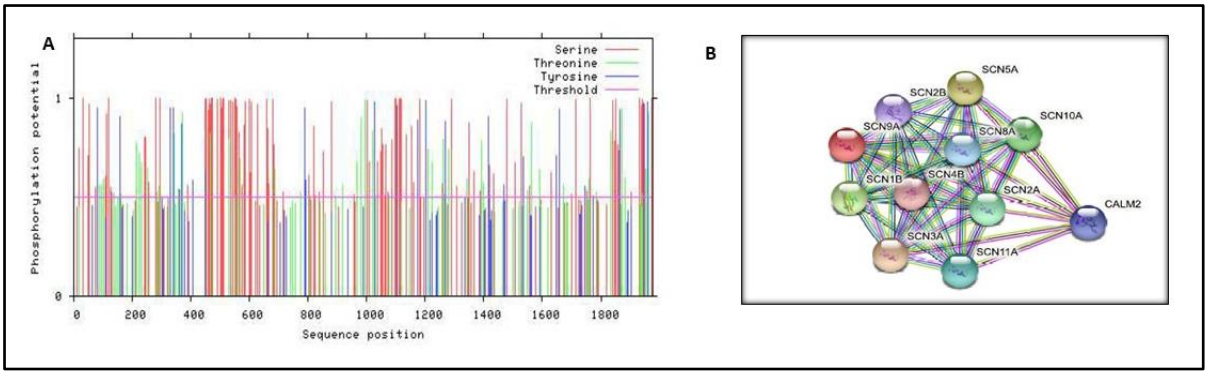

Fig. 4. A: Predicted phosphorylation sites in SCN9A, X-axis: N-to C-terminal amino acid sequence Y-axis: scores. B: protein-protein Interactions results of SCN9A.

Discussion

SCN9A codes for the alpha subunit of $Na_V1.7$, which plays a central role in nociception signaling. Dorsal root ganglion and sympathetic ganglion neurons have high expression of sodium channels and is an essential part of the peripheral nervous system (Raymond *et al.*, 2004). *SCN9A* has 26 exons and encodes a 1977 amino acid protein. In different human variant databases, it is evident that *SCN9A* is highly polymorphic gene with more than 130 coding variants and according to the Human Genome Mutation Database (www.hgmd.cf.ac.uk), 73 missense mutations of *SCN9A* have been reported about pain syndromes, either pain hypo- or hyper-sensitivity (Raymond *et al.*, 2004). In one of the study related to the peripheral neuropathies, morphometric analysis of the sural nerve shows a reduction of A delta fibers, which results in causing these neuropathies (Dyck *et al.*, 1983).

SCN9A protein comprises of 1977 amino acids and Protscale server values for accessibility, hydrophilicity, mutability, refractivity bulkiness, flexibility and polarity predicted SCN9A an unstable protein. Protparam also predicted SCN9A an unstable protein with instability index score of 44.78 and GRAVY score of -0.027.

In SCN9A there was no signal peptide present and no acetylation site was predicted. One Mannosylation and no O linked glycosylation sites were predicted in SCN9A.

Secondary structure showed presence of alpha helices, coils and beta sheets that were confirmed from tertiary structure from PDB (www.ebi.ac.uk/pdbe/entry/pdb/5EK0). SCN9A was also predicted to interact with SCN2A, SCN3A, SCN5A, SCN8A, SCN10A, SCN11A, SCN1B, SCN2B, SCN4B ABCB1 and CALM2. *SCN2A* mutations involved in epilepsy syndromes and autism spectrum disorder (ASD) (Raymond *et al.*, 2004) (Striano *et al.*, 2006). *SCN5A* involved in Brugada syndrome (Keller *et al.*, 2006). *SCN8A* is involved in cerebellar atrophy, ataxia, and mental retardation (Trudeau *et al.*, 2006), *SCN10A*, *SCN11A* and *SCN9A* are all involved in pain-related disorders (Baker MD and Wood JN 2001). SCN3A is voltage-gated sodium channel which is connected to smaller auxiliary subunits (Chen *et al.*, 2000).

SCN1B, SCN2B and *SCN4B* encode different beta subunits of sodium channels. *SCN1B* gene mutations are involved in different neurological and cardiac

disorders like epileptic seizures, Brugda syndrome 5 and cardiac conduction defects (www.omim. org/entry/600235). ABCB1 is member of ATP binding cassette superfamily proteins and termed as multiple drug resistance protein 1. and CALM2 (calmodulin 2) is responsible for cardiac arrest and has some function in long QT syndrome mice models (Juan *et al.,* 2014). All these proteins are shown to interact with SCN9A and might predict the involvement of different pathways in pathogenesis of congenital insensitivity to pain.

Regular follow up of the affected patients, plays an important role in avoiding complications. The abnormalities present in pain insensitivity syndromes provide vision into the complex functional nature of pain perception. In serious conditions of bone injuries and joint fractures, it is necessary to focus more on the prevention. Generally, efficient strategy against this rarely occurring disease is to educate the people and provide good training to the patients (Zhang *et al.*, 2014).

Functional studies of SCN9A are a focus for improvement in treatment strategy for Congenital Insensitivity to Pain (Raymond *et al.*, 2004) and may provide therapeutic insights for the development of new analgesics. An opiate antagonist known as Naloxone has been found to be reported for initiating the painful stimuli (Dehen *et al.*, 1977). Treatment for this disease is very challenging. Development of antagonist therapy is preferable and efficient to produce analgesia, as compared to agonist. The Nav 1.7 channel has been found to be a target for analgesic development and thus has been of interest to the pharmaceutical industry. Now Nav 1.7 is in the major interests of many big pharmaceutical companies as a key target for treating pain syndromes (Goldberg *et al.*, 2012). The inability to feel pain because of the absence of Nav 1.7 in mice and humans is significantly reversed by the opioid antagonist naloxone. Our results support to improve the therapeutic targets and develop efficient strategies to overcome the disease.

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Conflict of interest

All authors declared no conflict of interest.

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