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## New insights into the physiology and pathophysiology of the atypical sodium leak channel NALCN

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**New insights into the physiology and pathophysiology of the atypical sodium leak channel NALCN**

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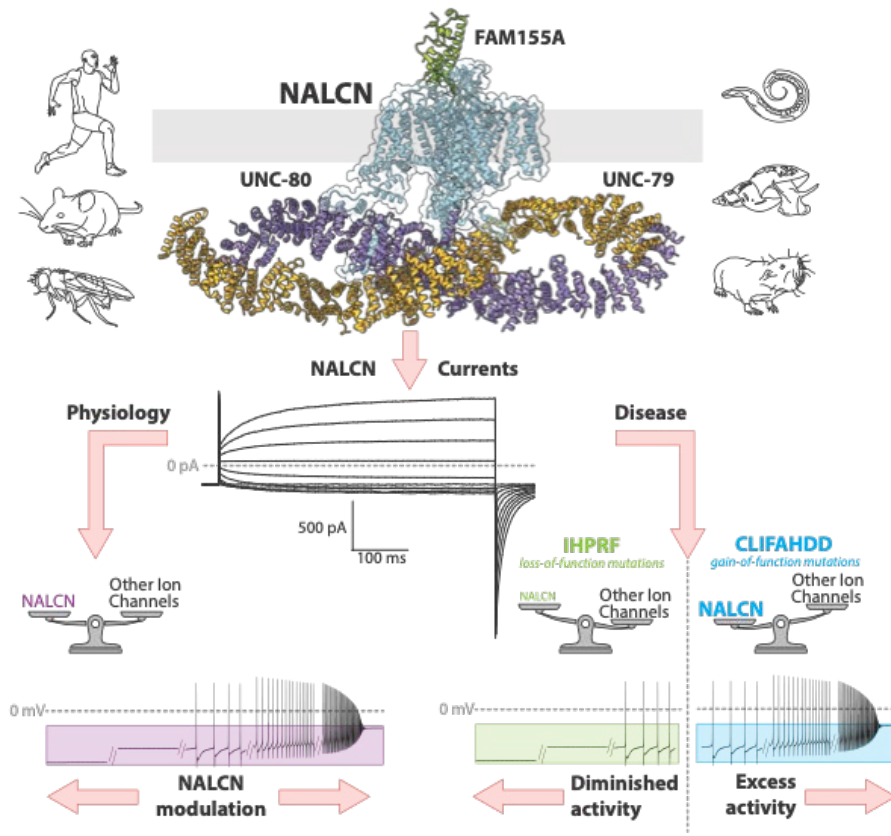
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**Graphical Abstract**



**CLINICAL HIGHLIGHTS**

This article reviews our current knowledge on the sodium leak channel NALCN, an important player of cell excitability. NALCN is highly conserved across species and is involved in fundamental physiological functions such as respiratory rhythm, circadian rhythm, sleep and locomotion. NALCN dysfunction, especially in patients carrying genetic mutations, results in severe disease states including serious respiratory defects, failure to thrive, hypotonia, developmental and cognitive delay, ataxia, epilepsy and early death. The recent development of animal and cellular models for the study of NALCN-related diseases now provides tools to understand their etiology and pave the way to the identification of novel therapeutic opportunities.

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39 Cell excitability and its modulation by hormones and neurotransmitters involve the  
40 concerted action of a large repertoire of membrane proteins, especially ion channels.  
41 Unique complements of co-expressed ion channels are exquisitely balanced against each  
42 other in different excitable cell types, establishing distinct electrical properties that are  
43 tailored for diverse physiological contributions, and dysfunction of any component may  
44 induce a disease state. A crucial parameter controlling cell excitability is the resting  
45 membrane potential (RMP) set by extra- and intra-cellular concentrations of ions, mainly  
46 Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup>, and their passive permeation across the cell membrane through leak ion  
47 channels. Indeed, dysregulation of RMP causes significant effects on cellular excitability.  
48 This review describes the molecular and physiological properties of the Na<sup>+</sup> leak channel  
49 NALCN, which associates with its accessory subunits UNC-79, UNC-80, and NLF-  
50 1/FAM155 to conduct depolarizing background Na<sup>+</sup> currents in various excitable cell  
51 types, especially neurons. Studies of animal models clearly demonstrate that NALCN  
52 contributes to fundamental physiological processes in the nervous system including the  
53 control of respiratory rhythm, circadian rhythm, sleep and locomotor behavior.  
54 Furthermore, dysfunction of NALCN and its subunits is associated with severe  
55 pathological states in humans. The critical involvement of NALCN in physiology is now  
56 well established, but its study has been hampered by the lack of specific drugs that can  
57 block or agonize NALCN currents *in vitro* and *in vivo*. Molecular tools and animal models  
58 are now available to accelerate our understanding of how NALCN contributes to key  
59 physiological functions, and the development of novel therapies for NALCN  
60 channelopathies.

61  
62  
63 *Keywords: Sodium leak channel, NALCN, Resting membrane potential, Cell excitability,*  
64 *Fundamental physiological functions, Human disease, Phylogeny and evolution*

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## Abbreviations

125		
126		
127	2-APB:	2-aminoethoxydiphenylborane
128	AAV:	Adeno-Associated Virus
129	Ach:	Acetylcholine
130	AD:	Alzheimer disease
131	AITC:	Allyl isothiocyanate
132	AP:	Action potential
133	AS:	Association study
134	AVP:	Arginine vasopressin
135	BASIC:	Bile acid-sensitive ion channel
136	BLAST:	Basic Local Alignment Search Tool
137	BPAP:	Bilevel positive airway pressure
138	CaM:	Calmodulin
139	CaSR:	Calcium-sensing receptor
140	Ca <sub>v</sub> :	Voltage-gated Ca <sup>2+</sup> channel
141	CCI:	Chronic constriction injury
142	CCS:	Case control study
143	CFA:	Complete Freund's adjuvant
144	CLIFAHDD:	Contractures of the Limbs and Face, Hypotonia, and Developmental Delay
145	CNV:	Copy number variation
146	CP96345:	(2S,3S)-cis-2-(Diphenylmethyl)-N-[(2-methoxyphenyl)methyl]-1
147		azabicyclo[2.2.2]octan-3-amine
148	CPAP:	Continuous positive airway pressure
149	CPG:	Central pattern generator
150	CRD:	Cysteine-rich domain
151	Cryo-EM:	Cryo-electron microscopy
152	CTD:	Carboxyterminal domain
153	DA:	Dopamine
154	DD:	Constant darkness
155	dbcAMP:	dibutyryl cAMP
156	DMH:	Dorsomedial hypothalamic nucleus
157	DN:	Dorsal neuron
158	DNV:	<i>De novo</i> variation
159	Do:	Day-old
160	DpMe:	Deep mesencephalic nucleus
161	DRG:	Dorsal root ganglia
162	E2:	Estrogen
163	EEG:	Electroencephalogram
164	eGFP	Enhanced green fluorescent protein
165	ELISA:	Enzyme-linked immunosorbent assay
166	EMG:	Electromyogram
167	ENaC:	Epithelial Na <sup>+</sup> channel
168	ENU:	Ethylnitrosourea
169	ER:	Estrogen receptor
170	ERE:	Estrogen responsive element
171	E <sub>rev</sub> :	Reversal potential
172	FBAT:	Family-based association test
173	FGF21:	Fibroblast growth factor 21

174	FFA:	Flufenamic acid	
175	FRET:	Fluorescence resonance energy transfer	
176	GABA:	$\gamma$ -aminobutyric acid	
177	GDP $\beta$ -S:	Guanosine-5'-( $\beta$ -thio)-diphosphate	
178	GDV:	Gastric dilatation-volvulus	
179	GHK:	Goldman, Hodgkin, Katz	
180	GI:	Gastrointestinal	
181	GPCR:	G proteins-coupled receptor	
182	GTP $\gamma$ S:	Guanosine 5'-[ $\gamma$ -thio]triphosphate	
183	GWAS:	Genome-wide association study	
184	GWM:	Genome-wide methylation	
185	HAM:	Haplotype association mapping	
186	HCN:	Hyperpolarization-activated cation channel	
187	HP:	Holding potential	
188	ICC:	Interstitial cell of Cajal	
189	IA:	Integrative analysis	
190	IHPRF:	Infantile Hypotonia with Psychomotor Retardation and characteristic	
191		Facies	
192	I/V:	Intensity/Voltage	
193	K <sub>2P</sub> :	Two-pore K <sup>+</sup> channel	
194	KI:	Knockin	
195	K <sub>ir</sub> :	Inward rectifier K <sup>+</sup> channel	
196	K <sub>v</sub> :	Voltage-gated K <sup>+</sup> channel	
197	KO:	Knockout	
198	L703606:	(2S,3S)-2-Benzhydryl-N-((2-iodophenyl)methyl)-1 azabicyclo(2.2.2)octan	
199		3-amine	
200	LA:	Linkage analysis	
201	LD:	12 hr Light:12 hr dark conditions	
202	LepRb:	Long form of the leptin receptor	
203	LFP:	Local field potential	
204	LN:	Lateral neuron	
205	lncRNA:	Long non-coding RNA	
206	LPN:	Lateral posterior neuron	
207	M3R:	Muscarinic M3 receptor	
208	Maropitant:	(2S,3S)-2-Benzhydryl-N-(5- <i>tert</i> -butyl-2-methoxybenzyl)	quinuclidin-3-
209		amine	
210	MCA:	Multiple correlation analysis	
211	MeSa:	Methyl salicylate	
212	Mo:	Month-old	
213	MSMC:	Myometrial smooth muscle cell	
214	mTLE:	mesial temporal lobe epilepsy	
215	mV:	milli Volts	
216	NALCN:	Na <sup>+</sup> leak channel	
217	Nav:	Voltage-gated Na <sup>+</sup> channel	
218	NK1:	Tachykinin 1	
219	NREM:	Non-rapid-eye-movement sleep	
220	ND:	Not determined	
221	NI:	Not indicated	
222	NP:	Non-pregnant	

223	NT:	Neurotensin
224	NTS:	Tractus solitary tract
225	P4:	Progesterone
226	PB:	Parabrachial
227	PBA:	Pathways-based analysis
228	PD:	Parkinson disease
229	PIP2:	Phosphatidylinositol-4,5-bisphosphate
230	PLA:	Proximity ligation assays
231	PP:	Post-partum
232	PR:	Progesterone receptor
233	PRE:	Progesterone responsive element
234	PreBötC:	PreBötzinger complex
235	PRL:	Prolactin
236	QTL:	Quantitative trait loci
237	REM:	Rapid-eye-movement sleep
238	RPeD1:	Right pedal dorsal 1
239	RMP:	Resting membrane potential
240	RTN:	Retrotrapezoid nucleus
241	rVRG:	Rostral ventral respiratory group
242	SF:	Selectivity filter
243	SFK:	Src family kinase
244	SNP:	Single nucleotide polymorphism
245	SNr:	Substantia nigra pars reticulata
246	SP:	Substance P
247	SPN:	Spino-parabrachial neuron
248	TRP:	Transient receptor potential
249	TTX:	Tetrodotoxin
250	Vm:	Membrane potential
251	VSD	Voltage-sensor domain
252	WB:	Western blotting
253	WES:	Whole exome sequencing
254	WGS:	Whole genome sequencing
255	WT:	Wild-type
256	Yo:	Year-old

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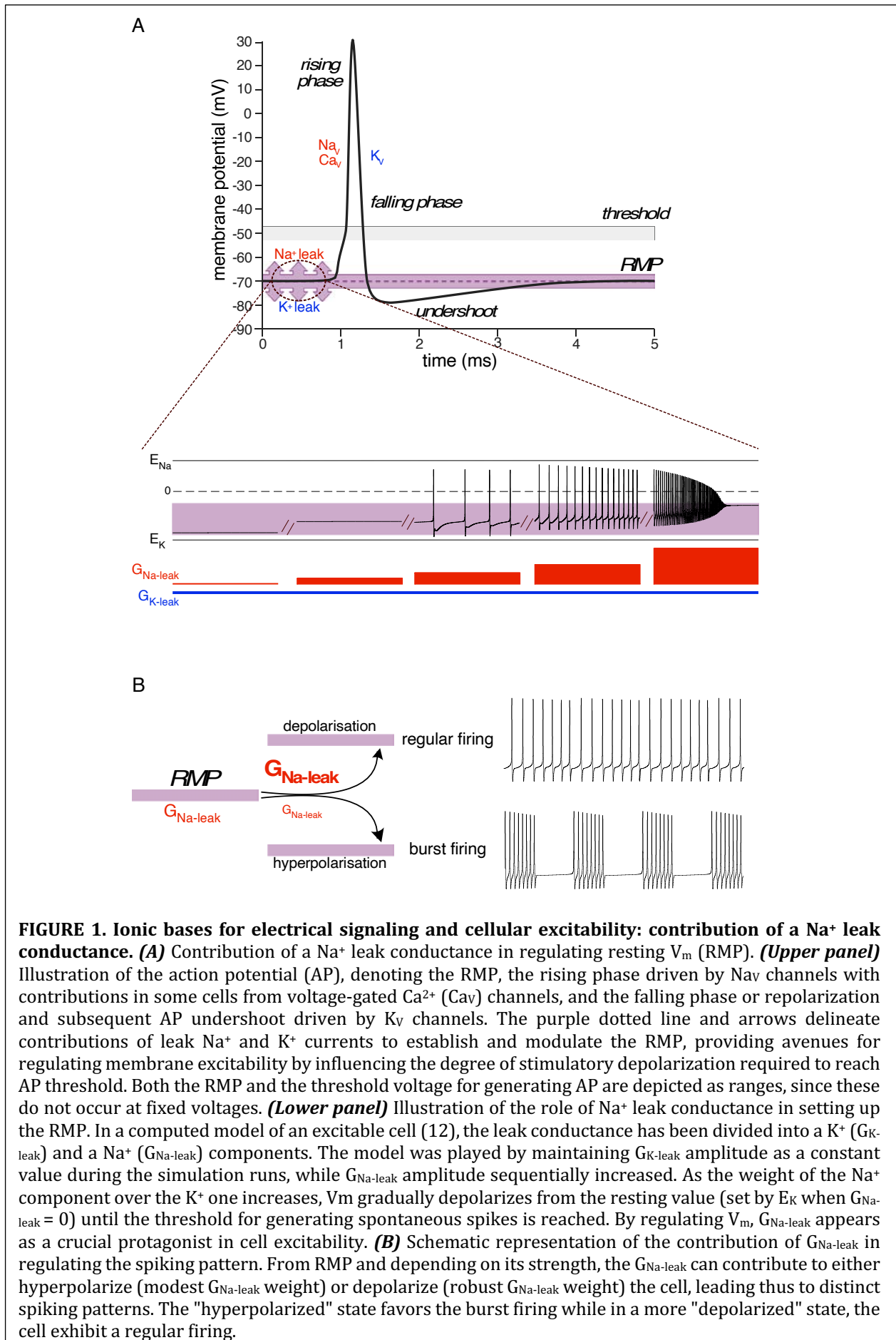
## I. INTRODUCTION

Since its molecular identification roughly 25 years ago by Perez-Reyes and colleagues (1), the sodium leak channel NALCN has emerged as an important regulator of cellular excitability. NALCN is a four domain Pore-loop channel homologous to voltage-gated sodium and calcium channels which conducts depolarizing  $\text{Na}^+$  leak currents in neurons and recombinant systems (2).

The first experimental and quantitative description of a  $\text{Na}^+$  leak current was provided by Hodgkin and Katz over 70 years ago (3), when they estimated that the  $\text{Na}^+$  permeability ( $P_{\text{Na}}$ ) across the squid giant axon membrane is, at rest, only 4% relative to that of  $\text{K}^+$  (*i.e.*,  $P_{\text{Na}}/P_{\text{K}} = 0.04$ ). Sub-threshold  $\text{Na}^+$  leak currents have since been reported in a multitude of neurons and other cell types from both vertebrates and invertebrates. Physiologically, beyond providing an offset current that counters  $\text{K}^+$  leak to establish a more depolarized RMP,  $\text{Na}^+$  leak currents serve numerous important functions. First, by varying the baseline expression of  $\text{Na}^+$  leak currents, animal cells can establish heterogeneous resting  $V_m$ , as in different neurons types as well as other excitable and non-excitable cell types (4, 5). By depolarizing RMP to voltages near action potential (AP) threshold,  $\text{Na}^+$  leak currents also contribute to neuronal pacemaking, for example by providing a depolarizing drive between APs that brings  $V_m$  to threshold sooner, increasing frequency (6). A schematic representation of the general contribution of the  $\text{Na}^+$  leak conductance in excitable cells is shown in **FIGURE 1**. As  $G_{\text{Na-leak}}$  amplitude increases,  $V_m$  depolarizes and the frequency of AP discharge increases (**FIGURE 1A**). From resting  $V_m$ , APs can fire either phasically or tonically, and subtle changes of a few millivolts can orchestrate significant changes in the spiking pattern. One of the most important changes is likely the switch from a regular tonic discharge mode to a rhythmic bursting activity (**Figure 1B**), which can serve to regulate neurological processes such as locomotion, breathing, and hormone secretion.

Like  $\text{K}^+$  leak channels (7, 8), dynamic modulation of  $\text{Na}^+$  leak currents through cellular signaling allows modulation of RMP and cellular excitability. An example of this is observed in bag cell neurons in the central nervous system of the sea slug *Aplysia californica*, which secrete neuropeptides that initiate egg laying behavior (9). Presynaptic stimulation of these neurons activates a cation leak current that depolarizes the  $V_m$  resulting in a prolonged bout of excitation and a burst of APs, triggering exocytosis of the egg laying neuropeptides at neurohemal interfaces (*i.e.*, blood-brain interfaces) (10, 11). Similar depolarizing leak currents subject to neuromodulation have been reported in neurons, endocrine, and neuroendocrine cells of mammals and other vertebrates (12, 13). Another general role for leak channels, especially those that are non-selective, is to contribute to neuronal input resistance, an intrinsic feature that determines how electrical signals propagate through membranous cellular structures like dendrites and axons (14).





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**FIGURE 1. Ionic bases for electrical signaling and cellular excitability: contribution of a  $Na^+$  leak conductance.** (A) Contribution of a  $Na^+$  leak conductance in regulating resting  $V_m$  (RMP). (Upper panel) Illustration of the action potential (AP), denoting the RMP, the rising phase driven by  $Na_v$  channels with contributions in some cells from voltage-gated  $Ca^{2+}$  ( $Ca_v$ ) channels, and the falling phase or repolarization and subsequent AP undershoot driven by  $K_v$  channels. The purple dotted line and arrows delineate contributions of leak  $Na^+$  and  $K^+$  currents to establish and modulate the RMP, providing avenues for regulating membrane excitability by influencing the degree of stimulatory depolarization required to reach AP threshold. Both the RMP and the threshold voltage for generating AP are depicted as ranges, since these do not occur at fixed voltages. (Lower panel) Illustration of the role of  $Na^+$  leak conductance in setting up the RMP. In a computed model of an excitable cell (12), the leak conductance has been divided into a  $K^+$  ( $G_{K-leak}$ ) and a  $Na^+$  ( $G_{Na-leak}$ ) components. The model was played by maintaining  $G_{K-leak}$  amplitude as a constant value during the simulation runs, while  $G_{Na-leak}$  amplitude sequentially increased. As the weight of the  $Na^+$  component over the  $K^+$  one increases,  $V_m$  gradually depolarizes from the resting value (set by  $E_K$  when  $G_{Na-leak} = 0$ ) until the threshold for generating spontaneous spikes is reached. By regulating  $V_m$ ,  $G_{Na-leak}$  appears as a crucial protagonist in cell excitability. (B) Schematic representation of the contribution of  $G_{Na-leak}$  in regulating the spiking pattern. From RMP and depending on its strength, the  $G_{Na-leak}$  can contribute to either hyperpolarize (modest  $G_{Na-leak}$  weight) or depolarize (robust  $G_{Na-leak}$  weight) the cell, leading thus to distinct spiking patterns. The "hyperpolarized" state favors the burst firing while in a more "depolarized" state, the cell exhibit a regular firing.

343 Electrophysiological studies have reported a diversity of Na<sup>+</sup> leak conductances in  
 344 neurons and other excitable cell types, attributable to different ion channel types capable  
 345 of producing sub-threshold Na<sup>+</sup> leak currents. The molecular counterparts of these Na<sup>+</sup>  
 346 leak conductances include Na<sub>v</sub> channels (through different mechanisms) (15, 16),  
 347 hyperpolarization-activated cation (HCN) channels (17), Transient Receptor Potential  
 348 (TRP) channels (12), Epithelial Na<sup>+</sup> Channels (ENaC channels), Bile Acid-Sensitive Ion  
 349 Channels (BASIC channels) (18), and finally, the Na<sup>+</sup> leak channel NALCN (19).

350

351 In this review, we aim to provide a detailed and up-to-date presentation of the current  
 352 knowledge on NALCN, expanding on previous reviews published over 9 years ago by Ren,  
 353 2011 (13), Lu & Feng 2012 (20) and most recently by ourselves (Cochet-Bissuel *et al*, 2014  
 354 (19)). Since 2014, numerous important studies have emerged that increase our  
 355 understanding of NALCN, including its protein structure in complex with its ancillary  
 356 subunits UNC-79, UNC-80, and FAM155/NLF-1, its unique ion-conducting properties in  
 357 recombinant expression systems, its modulation by various cell signaling pathways, and  
 358 its contribution to physiology and various pathologies in humans. Hence, below we seek  
 359 to highlight these advances, and integrate them into the general understanding of NALCN  
 360 channel function and dysfunction. Some important milestones from the first identification  
 361 of the *nalcn* allele (*i.e.*, *narrow abdomen*) in *D. melanogaster* in 1934 to the identification  
 362 of the cryogenic electron microscopy (Cryo-EM) structure of the NALCN channelosome is  
 363 shown in Figure 2.

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## 365 II. THE NALCN CHANNELOSOME

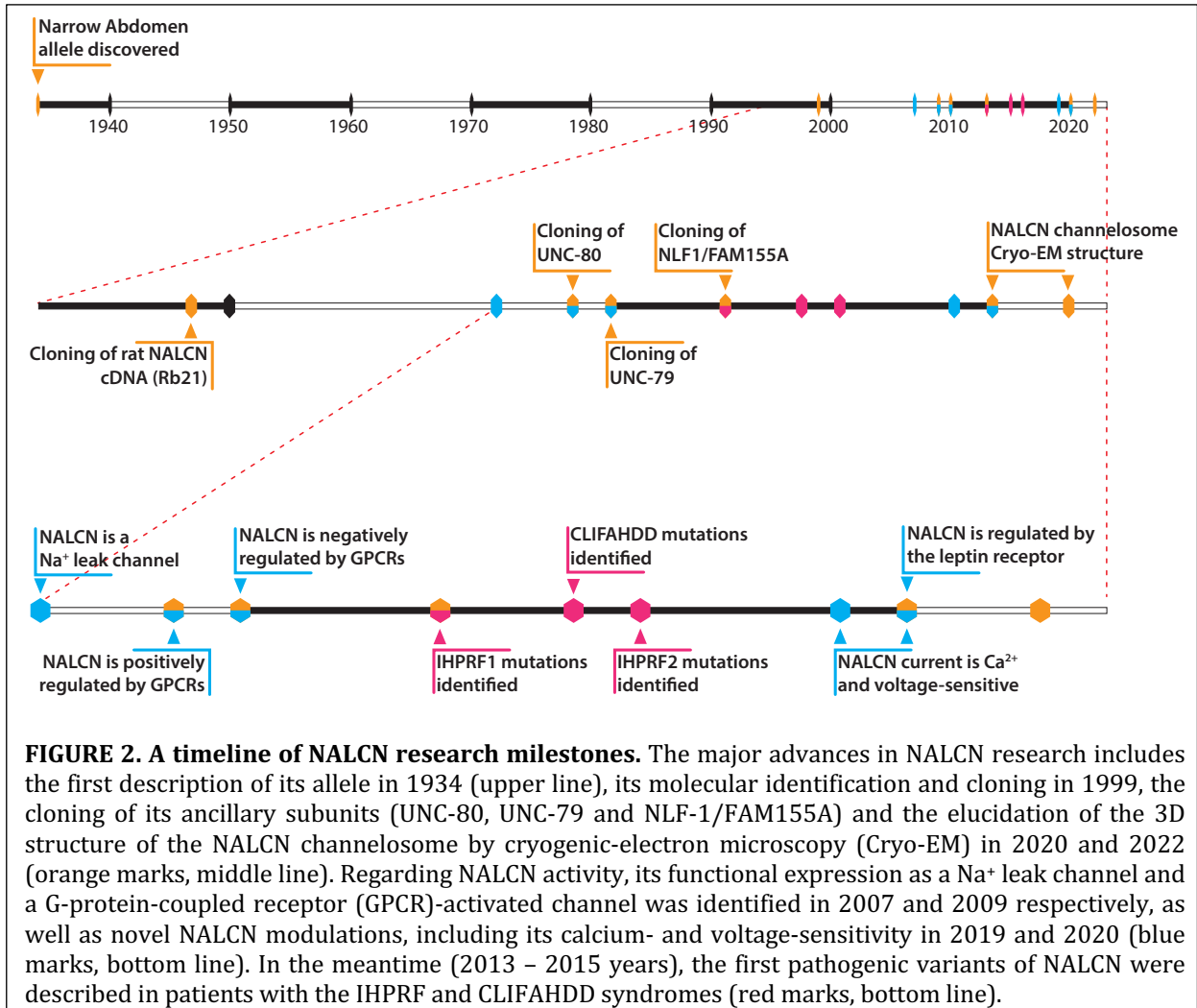
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### 367 1- Identification and structure of the Na<sup>+</sup> Leak Channel NALCN

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369 After the initial discovery of a subset of ion channel genes and corresponding protein  
 370 structures (*e.g.*, Na<sub>v</sub> and K<sub>v</sub> channels), sequencing of various genomes in the late 1990s  
 371 and early 2000s (21), as well as next-generation mRNA sequencing, ushered in an era of  
 372 accelerated ion channel discovery. For example, identification of a human expressed  
 373 sequence tag transcript encoding a portion of a novel voltage-gated calcium (Ca<sub>v</sub>) channel  
 374 led to the discovery that Ca<sub>v</sub>3 or T-type channels give rise to neuronal low-voltage  
 375 activated Ca<sup>2+</sup> currents (22). Similarly, the discovery that HCN channels give rise to  
 376 hyperpolarization-activated (*a.k.a.* funny currents or I<sub>f</sub>) began with the sequence  
 377 identification of ion channel genes that resembled K<sub>v</sub> channels but had distinct structural  
 378 features (23, 24). During this era, several other putative ion channel-related genes were  
 379 identified with unknown functions or known current counterparts in native cells. This  
 380 was the case for NALCN (for *Na<sup>+</sup> Leak Channel*; formerly named Rb21 or VGCNL1 in  
 381 mammals), whose gene sequence was first reported in 1999 (1). In this study, the  
 382 program Basic Local Alignment Search Tool (BLAST) (25) was used to search the NCBI  
 383 GenBank database, using known ion channel protein sequences as query, to identify 2  
 384 novel genes related to Na<sub>v</sub> and Ca<sub>v</sub> channels present within the *C. elegans* genome. These  
 385 were referred to as C27f2.2 (acc NP\_498054; now referred to as *nca-2*) and C11d2.6 (acc  
 386 NP\_741413; formerly referred to as *unc-77* and now referred to as *nca-1*). A rat homolog  
 387 cDNA was then cloned from a rat brain complementary DNA (cDNA) library (acc.  
 388 Q6Q760.1; **FIGURE 3A**) (1). By homology with Na<sub>v</sub> and Ca<sub>v</sub> channel α<sub>1</sub> subunits (26), the  
 389 pore-forming subunit of NALCN was similarly composed of four homologous repeat  
 390 domains, referred to as Domains I to IV, each containing six membrane spanning helices  
 391 or segments (*i.e.*, segments 1 to 6 or S1 to S6; **FIGURE 3B**). Segments 1 to 4 make of the

392 voltage-sensor domain (VSD), while segments 5 and 6 make up the pore-domain, with  
 393 each set projecting a large pore-loop (P-loop) structure into the extracellular  
 394 environment, shared with all members of the P-loop superfamily of ion channels which  
 395 includes,  $Ca_v$  channels,  $Na_v$  channels,  $K_v$  channels, HCN channels, and TRP channels  
 396 (FIGURE 3B).  
 397



410 Interestingly, the S4 segments within the VSDs of the NALCN protein contain less  
 411 positively-charged lysine/arginine amino acids compared to the other channels,  
 412 especially in Domain IV (FIGURE 3C). This is notable because these residues are critical  
 413 for voltage-sensing. In addition, four key amino acids in the P-loops that form the ion  
 414 selectivity filter (SF) motif resemble  $Ca_v$  channels in having glutamate (E) residues in  
 415 Domains I, II, and IV, but also  $Na_v$  channels in having a lysine (K) in domain III (*i.e.*,  
 416  $NALCN=EEKE$ ;  $Ca_v=EEEE/EEDD$ ;  $Na_v=DEKA$ ; FIGURE 3A). The 3D cryo-electron  
 417 microscopy (Cryo-EM) structure of NALCN, in complex with its ancillary subunit NLF-  
 418 1/FAM155A subunit (*see below*), confirmed an overall similar architecture to  $Na_v$  and  $Ca_v$   
 419 channels despite some unique features (PDB: 6XIW (27); PDB: 7CM3 (28); PDB: 7CU3  
 420 (29); FIGURE 4A,B). In contrast to  $Na_v$  and  $Ca_v$  channels, the S4-S5 linkers that surround  
 421 the S6-bundle give rise to an asymmetrical assembly (27, 29). The Domain I S5-helix  
 422 extends in the cytoplasm by 3 additional  $\alpha$ -helical turns leaving a shorter loop connecting  
 423 to the Domain I S1-S4 VSD (27, 28). The Domain IV S5-helix is 1 turn shorter than expected

424 from Nav and Cav channels, displacing the distal end of the Domain IV S4-S5 linker alpha  
 425 helix towards the extracellular side. These observations suggest key differences in how  
 426 the VSDs are functionally coupled to the S5-S6 pore module in NALCN compared to Nav  
 427 and Cav channels. This is supported by functional data indicating that it is mostly the VSDs  
 428 of DI and DII that contribute to NALCN voltage sensitivity (*see below*)(30). These  
 429 structural studies also identified 4 disulfide bonds on the extracellular P-loops of NALCN  
 430 (28), which play important roles in maintaining the structure of the P-loops. Additional  
 431 possible extracellular post-translational modifications of perceived importance were also  
 432 identified. This included 3 glycosylation sites (p.N210, p.N216, p.N1064)(28), all with  
 433 possible implications for NALCN structure and stability. The SF appears slightly narrower  
 434 and more electronegative than those found in Nav, but similar to those in Cav channels (27,  
 435 28). In analogy to Nav and Cav channels, the SF side chains of p.E280, p.E554 and p.K1115  
 436 point towards the center of the pore, while p.E1389 exhibits 2 alternative conformations  
 437 (29). The 3D structure of NALCN was only elucidated in a closed conformation, likely  
 438 reflecting a low opening probability that was observed experimentally through single  
 439 channel recordings (27-29, 31). The closed helix bundle crossing of the S6 helices from  
 440 DI-DIV forms the channel gate (28). Both the DII-III and DIII-IV linkers interact with the  
 441 carboxyterminal domain (CTD)(27-29), which consists of at least 5 to 6 short helices (C1-  
 442 C6). Among these, C1-C4 form an EF-hand-like domain that packs below the III-IV linkers  
 443 (28, 29). GST-pull down experiments further revealed an interaction between a fragment  
 444 of the DII-DIII linker (aminoacids 751-765) and the CTD of NALCN (aminoacids 1455-  
 445 1571)(29). Both NALCN structure and sequence suggest the absence of a fast inactivation  
 446 motif, such as the IFM motif present in Nav channels (27).

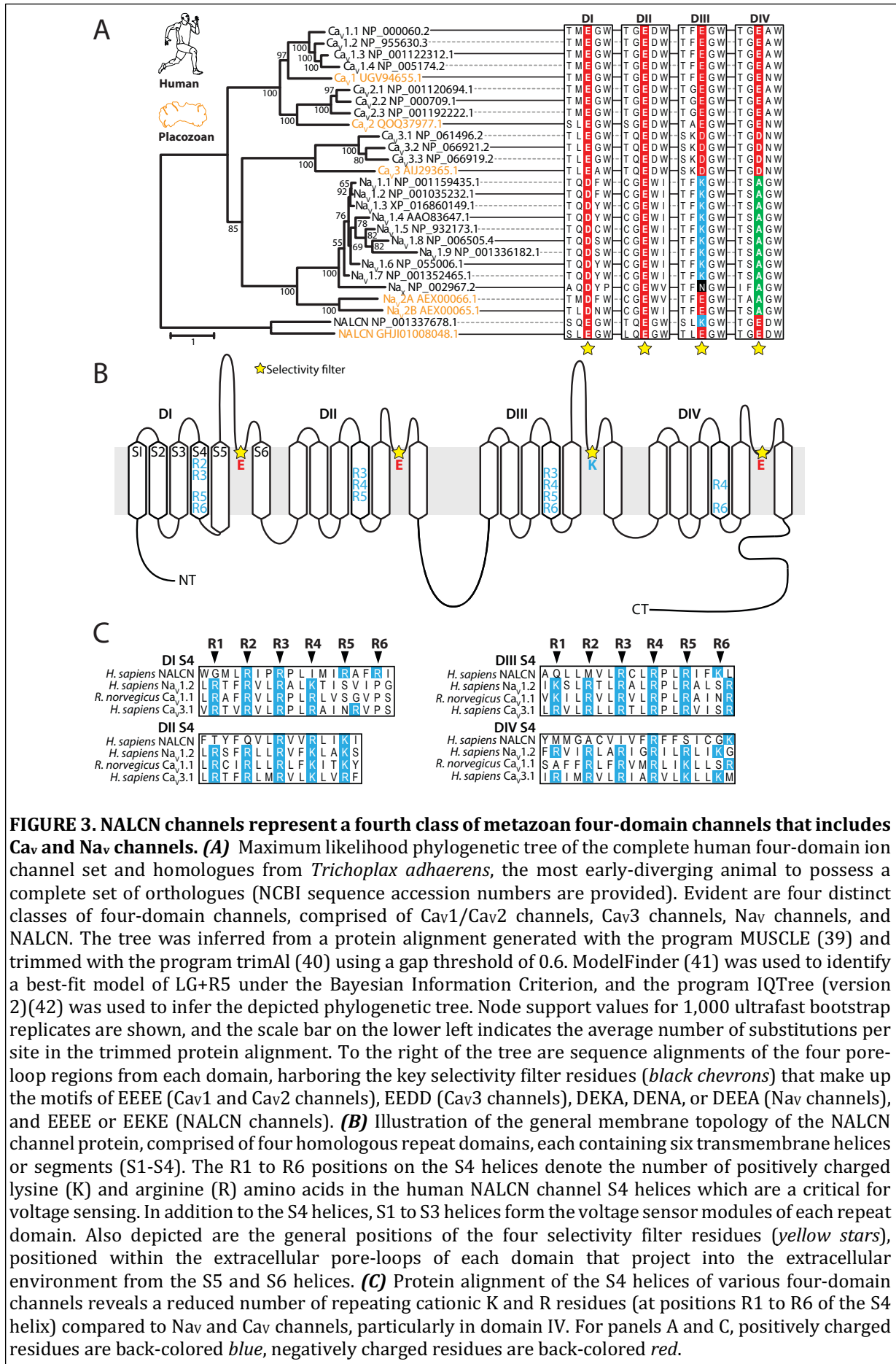
## 447 448 **2- NALCN belongs to a protein complex that includes at least 4 subunits**

### 449 450 *Identification of NALCN ancillary subunits in invertebrates.*

451  
452 Many ion channels from the superfamily of voltage-gated and related cation channels  
 453 require ancillary subunits to function *in vitro* and *in vivo* (26). This is also the case for  
 454 NALCN, with first insights coming from studies in *C. elegans* which resulted in the  
 455 identification of 3 NALCN subunits referred to as UNC-79 (for uncoordinated-79), UNC-  
 456 80 (uncoordinated-80), and NLF-1/FAM155A (NALCN localization factor)(32-35).  
 457 Specifically, a genetic screen aimed at identifying proteins that act downstream of  
 458 Phosphatidylinositol-4,5-bisphosphate (PIP2) during synaptic vesicles endocytosis  
 459 revealed that three alleles of the *unc-80* gene could suppress locomotor deficits caused by  
 460 mutation of synaptojanin (*unc-26*), a key regulator of vesicle recycling at the presynaptic  
 461 terminal (32). *Unc-26* mutant animals exhibited a severe uncoordinated locomotor  
 462 activity and an increased number of synaptic vesicle number due to a recycling defect.  
 463 These phenotypes were rescued when *unc-26* mutants were crossed with *unc-80* mutants.  
 464 In this study, the locomotor behavior defects of *unc-80* mutant animals were similar to  
 465 *nca-1:nca-2* double mutants (*C. elegans* harbors two gene copies of NALCN, NCA-1 and  
 466 NCA-2), with further investigation showing reduced expression of NCA-1 (~40%) and  
 467 NCA-2 (~48%) in axons of *unc-80* animals, but not in the cell bodies of motoneurons and  
 468 nerve rings compared to control animals. Taken together, these results suggested that  
 469 UNC-80 is required for NCA-1 and NCA-2 neuronal localization. UNC-80 is a large protein  
 470 with at least two isoforms and no homologous domains and motifs that could suggest its  
 471 function. The use of the GFP under the control of the *unc-80* promoter further revealed  
 472 that *unc-80* is broadly expressed in the nervous system in both acetylcholine and GABA

473 motoneurons (32). This study also identified another gene referred to as *unc-79* with a  
474 suppressor effect in the uncoordinated phenotype of synaptojanin mutants like *unc-80*  
475 and *nca-1:nca-2* mutants (32). UNC-79 resembles UNC-80 in being a large protein with no  
476 described domains or motifs that could suggest function. Notably, the expression of the  
477 NCA-1 protein in *C. elegans*, and its orthologue Narrow Abdomen (NA) *a.k.a* Dm $\alpha$ 1U in *D.*  
478 *melanogaster*, was attenuated below detection limits in UNC-79 mutants as determined  
479 *via* both Western blotting and immunocytochemistry (33). The NCA-1/NA disappearance  
480 did not correlate with the expression of the corresponding mRNAs, altogether indicating  
481 that UNC-79 regulates NALCN expression through a post-translational mechanism in both  
482 of these invertebrate species (33). Interestingly, the use of reporter constructs with GFP  
483 linked to the promoter regions of *unc-79*, *nca-1*, or *nca-2* showed that, although each  
484 reporter shows conspicuous (but not exclusive) expression in the nervous system, the  
485 pattern of *unc-79* does not coincide with that of *nca-1* or *nca-2* or with their sum (33).  
486 Nonetheless, the requirement of UNC-79 and UNC-80 for protein expression and  
487 localization of NCA-1 in cells was then confirmed using immunocytochemistry by Yeh et  
488 *al*, 2008, who found that UNC-79, UNC-80, and NCA-1 facilitate each other's colocalization  
489 along axons (34). Here, NCA-1 staining was eliminated or greatly reduced in multiple *unc-*  
490 *80* mutant strains, and further rescue experiments indicated that UNC-80 is both  
491 necessary and sufficient to localize NCA-1 along axons. Conversely, UNC-80 localization  
492 along axons depended on the presence of NCA-1 and/or NCA-2 (34). A strongly reduced  
493 NCA-1 and UNC-80 signal was also observed in *unc-79* animals while a reduction of UNC-  
494 79 expression/localization was observed in both *unc-80* and *nca-1:nca-2* animals (34, 35).  
495 This interdependent regulatory relationship between NALCN, UNC-79, and UNC-80 is also  
496 apparent in *D. melanogaster* (36). In addition, co-immunoprecipitation experiments from  
497 *D. melanogaster* head extracts indicated that NALCN (NA), UNC-79 and UNC-80 belong to  
498 the same protein complex (36).  
499

500 The fourth subunit of the NALCN/NCA-1/NCA-2/NA channelosome to be  
501 identified was FAM155A, also in *C. elegans*. It was named *NALCN Localization Factor-1* or  
502 *NLF-1* (35). NLF-1 was identified through a genetic suppressor screen for altered  
503 locomotor behavior of *nca-1* gain-of-function mutants. In this study, premotor  
504 interneurons of *nlf-1* mutants exhibited a drastically reduced Na<sup>+</sup> leak current and a more  
505 negative RMP, attributable to a partial loss of NCA-1/NCA-2 function (35, 37).  
506 Immunostaining of both endogenous NLF-1 as well as an NLF-1::GFP transgene both  
507 indicated that NLF-1 is expressed in most if not all neurons and was first described as an  
508 ER resident protein that specifically promotes axon delivery of the NCA channel as well  
509 as of UNC-79 and UNC-80 (35, 37). Indeed, the protein expression and axonal localization  
510 of NCA-1 and NCA-2 was abolished in *nlf-1* animals similarly to what is observed in *unc-*  
511 *79* and *unc-80* animals (32, 34, 35, 37). The axonal localization of NCA-1 and NCA-2 was  
512 restored by acute expression of NLF-1. In addition, endogenous UNC-79 levels were  
513 significantly decreased in *nlf-1* mutants (35). Notably, decreased expression of a Na<sup>+</sup> leak  
514 conductance concomitant with altered electrical activity has also been observed in *D.*  
515 *melanogaster* neurons upon *nlf-1* knockdown, attributable to dysregulation of  
516 NA/Dm $\alpha$ 1U activity (38).  
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550 *NALCN ancillary subunits are conserved in mammals.*

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552 The relevance of the identified subunits mentioned above was confirmed in  
 553 mammals via co-immunoprecipitation experiments using protein lysates from both  
 554 transfected HEK-293T cells and mouse brain tissue (30, 43, 44). Notably, UNC-79 was first  
 555 described to form a complex with NALCN *via* its interaction with UNC-80 but not NALCN  
 556 in the mouse brain (44). However, more recent structural studies reveal that both UNC-  
 557 79 and UNC-80 directly interact with NALCN, although the former with considerably  
 558 fewer contacts perhaps accounting for its inability to coprecipitate with the channel (31,  
 559 45, 46). Contrasting with observations made in *C. elegans* and *D. melanogaster*, the  
 560 expression levels of NALCN are not appreciably disrupted in mice harboring UNC-79 or  
 561 UNC-80 knockout mutations while UNC-79 knockout mutations decrease the expression  
 562 levels of UNC-80 (44, 47, 48). UNC-79 is also detected in brain extracts from *Unc-80*  
 563 knockout mice (49). This indicates that there are differences between mammals and  
 564 invertebrates with respect to the noted interdependent nature of subunit expression.  
 565 Functionally, the Na<sup>+</sup> background conductance is strongly reduced in hippocampal  
 566 neurons from *Unc-80* knockout mice and restored by overexpression of UNC-80, but not  
 567 UNC-79, suggesting that UNC-80 is more critical for the proper functional expression of  
 568 NALCN. Indeed, there is no obvious difference in amplitude of the Na<sup>+</sup> background  
 569 conductance between wild-type and *Unc-79*<sup>-/-</sup> hippocampal neurons (44), despite the  
 570 observation that both UNC-79 and UNC-80 appear to be specific ancillary subunits of  
 571 NALCN since affinity-depletion of NALCN from mouse brain extracts also fully depletes  
 572 the UNC-79 and UNC-80 proteins (49). Altogether, although there may be some  
 573 differences between mammals and invertebrates with respect to the interplay between  
 574 NALCN subunits, the strong interdependence of NALCN, UNC-80, and UNC-79 appears to  
 575 be conserved.

576

577 Accordingly, UNC-80 promotes NALCN expression at the plasma membrane by  
 578 approximately threefold when both proteins are co-expressed in HEK-293T cells (49). Co-  
 579 expression experiments of NALCN along with UNC-80 in HEK-293T cells followed by  
 580 electrophysiological recordings and co-immunoprecipitation revealed that the N-  
 581 terminal part of UNC-80 (*i.e.*, amino acids 300 to 1,700) is sufficient for the interaction  
 582 with the channel, since its deletion abrogates the interaction along with NALCN activity  
 583 (49). Of note however, a separate study reported that deletion of the 733 first amino-acids  
 584 of UNC-80 does not impact NALCN currents *in vitro* (30). UNC-80 with deletions of C-  
 585 terminal residues up until residue 2,554 is fully functional and residues 1,701 to 2,387  
 586 are essential for the generation of a NALCN current *in vitro* (49). Similar deletion  
 587 experiments also delineated an UNC-79-interacting domain (amino acids 2,758 to 2,947)  
 588 on UNC-80. The binding of UNC-79 to UNC-80 was proposed to relieve a soma-retention  
 589 of the complex. Indeed, a soma-retention region was identified within residues 2,387 to  
 590 2,657 of UNC-80, which presumably becomes masked *via* interactions with UNC-79 at a  
 591 more C-terminal location.

592

593 With respect to NLF-1, two proteins referred to as FAM155A (human acc.  
 594 NP\_001073865.1; 458 amino acids) and FAM155B (human acc. NP\_056501.2; 472 amino  
 595 acids) were identified in mammals for their sequence homology to the invertebrate  
 596 subunit. Evidence that these are true orthologues comes from observations that the  
 597 mammalian NLF-1/FAM155A protein is able to functionally substitute for NLF-1 in *C.*  
 598 *elegans* neurons, and its knockdown in primary mouse cortical neurons strongly

599 decreases a Na<sup>+</sup> leak conductance (35). In COS7 cells, ectopically-expressed NLF-1 and  
 600 NLF-1/FAM155A both exhibit ER localization, and both co-immunoprecipitate with  
 601 NALCN (35). It was therefore suggested that NLF-1 and NLF-1/FAM155A have a  
 602 conserved function by acting as chaperones to facilitate NALCN folding and delivery to  
 603 the plasma membrane. Both NLF-1 and NLF-1/FAM155A exhibit a tail-anchored, type I  
 604 ER membrane topology, and a membrane yeast two-hybrid system showed the  
 605 interaction between NALCN and NLF-1/FAM155A involves the Domain II S5 to S6 pore  
 606 module of the channel subunit (35).

607  
 608 In mammals, NLF-1/FAM155A co-immunoprecipitates with NALCN when co-  
 609 expressed in HEK-293T cells and can be substituted by NLF-1/FAM155B, a mammalian  
 610 paralogue of NLF-1/FAM155A, to produce functional NALCN channels (*see below*)(30).  
 611 However, unlike in *C. elegans* where NLF-1 is thought to be restricted to the ER, FAM155A,  
 612 along with UNC-79, UNC-80, and NALCN, were shown to traffic to the cell surface when  
 613 expressed alone in HEK-293T cells *via* cell-surface biotinylation assays. These new  
 614 findings prompt further investigation to determine the subcellular localization of NLF-1  
 615 in mammals, where NLF-1/FAM155A (458 amino acids) is predicted to contain at least  
 616 one transmembrane segment and a conserved cysteine-rich domain (CRD)(35, 50).

617  
 618 *Structure of the NALCN channelosome in mammals.*

619  
 620 Recent cryo-electron microscopy (Cryo-EM) structures of NALCN in complex with  
 621 NLF-1/FAM155A, and subsequently of the entire NALCN channelosome complex (*i.e.*,  
 622 NALCN, NLF-1/FAM155A, UNC-79 and UNC-80) revealed that UNC-79 and UNC-80 do not  
 623 impose notable structural changes in the NALCN-NLF-1/FAM155A subcomplex (**FIGURE**  
 624 **3A**)(31, 45, 46). However, the intracellular linker regions of NALCN show extensive  
 625 ordering when associated with UNC-79 and UNC-80, which is not apparent in the NALCN-  
 626 NLF-1/FAM155A subcomplex (31, 45, 46). UNC-79 and UNC-80 form a massive,  
 627 intertwined, intracellular assembly that hangs beneath the voltage sensor domains  
 628 (VSDs) from Domains I to III of NALCN. Below the DIV VSD, the C-terminal domain (CTD)  
 629 of NALCN is embraced by calmodulin (CaM), which itself is docked against UNC-80 near  
 630 the midpoint of the intracellular assembly. A small portion of the NALCN DI-DII linker  
 631 contacts a membrane proximal surface of UNC-79, while two discrete regions from the  
 632 DII-DIII linker form an elaborate clamp-like interaction with UNC-80. Systematic  
 633 substitution of intracellular regions from Na<sub>v</sub>1.4 into NALCN revealed an absolute  
 634 requirement for the DI-DII and DII-DIII linkers for NALCN channelosome activity *in vitro*  
 635 (31). The identified channelosome structure also provided first structural insights into  
 636 UNC-79 and UNC-80. These 2 proteins are well-folded intracellular globular proteins that  
 637 form a head-to-tail super helical assembly, resembling an infinity sign. Both proteins  
 638 appear to belong to the HEAT repeat superfamily, in bearing tandem repeat structural  
 639 motifs composed of two  $\alpha$ -helices linked by a short loop, although some of the repeats in  
 640 both UNC-79 and UNC-80 display motifs comprised of 3  $\alpha$ -helices, as observed in the  
 641 related armadillo repeat proteins. UNC-79 and UNC-80 contain 32 and 31 HEAT repeats  
 642 respectively. Notably, UNC-79 and UNC-80 are reported to lack significant sequence  
 643 identity, however both proteins share a similar structural architecture. UNC-80 also  
 644 exhibits a ubiquitin-like domain (1,983 to 2,076), while both UNC-79 and UNC-80 bear 3  
 645 tandem importin- $\alpha$ 3 motifs. 3 large interacting regions between UNC-79 and UNC-80  
 646 were identified, referred to as the N<sub>UNC-79</sub>-C<sub>UNC-80</sub> interface (N-C), the Crossover Interface,  
 647 and the C<sub>UNC-79</sub>-N<sub>UNC-80</sub> (C-N) interface. The presence of the three extensive and conserved



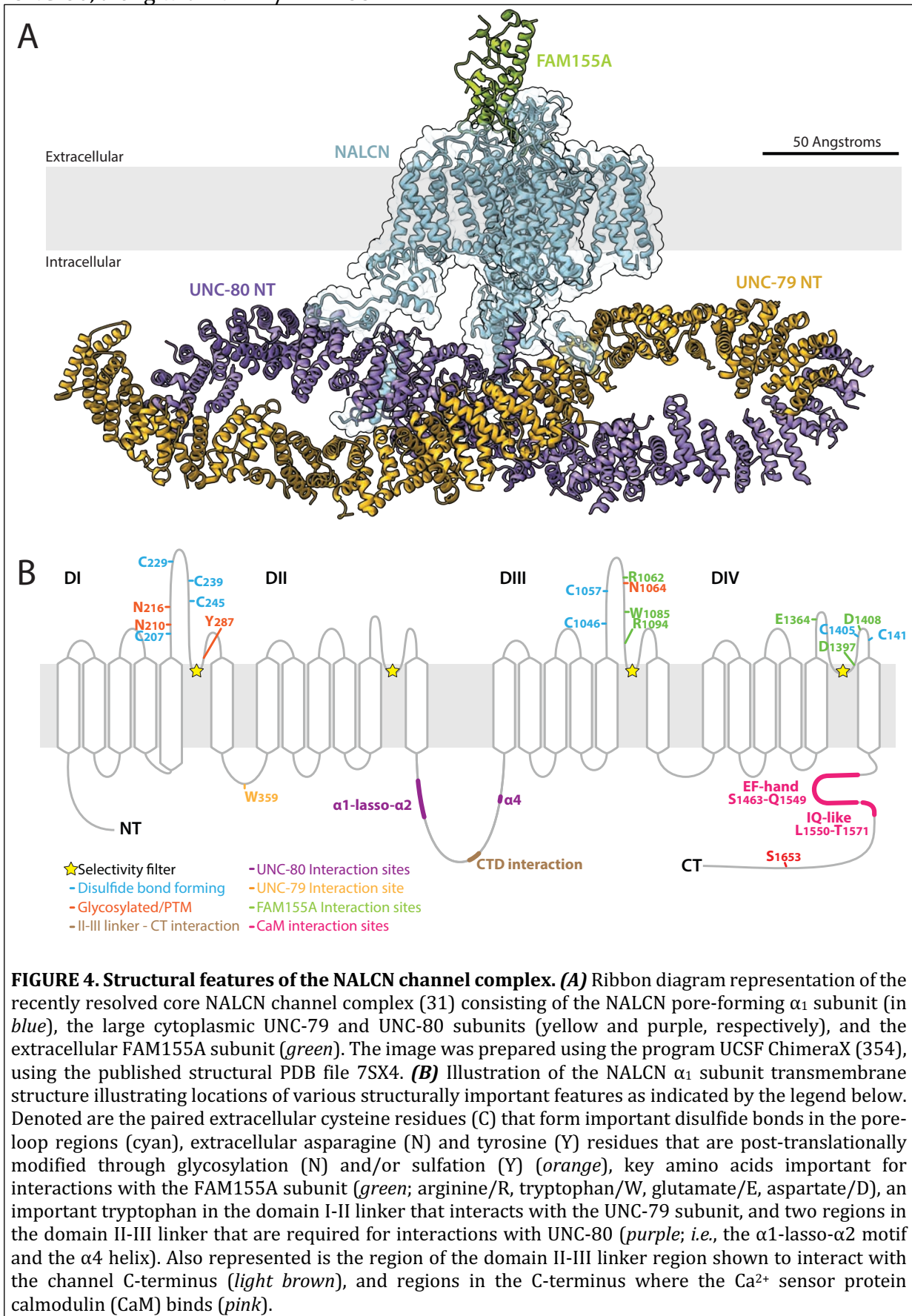
648 UNC-79-UNC-80 interaction interfaces suggests that these subunits form a  
 649 phylogenetically conserved subcomplex. *In vitro* channelosome function tolerates  
 650 deletion from the C-terminal region of UNC-79 ( $\Delta$ 2,401-2,635) and the N-terminal region  
 651 of UNC-80 ( $\Delta$ 1-733), indicating that portions the C-N interface are dispensable, at least  
 652 for functional expression in *Xenopus laevis* oocytes (30).  
 653

654 Cryo-EM studies also showed that NLF-1/FAM155A contains 2 lobes, the N-lobe and  
 655 the C-lobe, which are stabilized by 6 disulfide bonds (27-29). The N-lobe consists of 3  
 656 short  $\beta$  strands and 2 short  $\alpha$  helices (H1-H2), while the C-lobe contains 4  $\alpha$  helices (H3-  
 657 H6). Part of this extracellular domain fold shows similarity to the structure of the frizzled-  
 658 like cysteine-rich domain (CRD) of the receptor tyrosine kinase MuSK. The C-lobe of NLF-  
 659 1/FAM155A interacts with NALCN. A sugar moiety linked to p.N1064 in the NALCN  
 660 domain III P-loop appears to contribute to the interaction by potentially forming a  
 661 hydrogen bond with residue p.E350 on NLF-1/FAM155A. The globular CRD formed by  
 662 NLF-1/FAM155A is anchored onto the DIV pore module and forms extensive interactions  
 663 with P-loops in domains I, III, and IV (28, 29). The interaction between NLF-1/FAM155A  
 664 and NALCN involves the highly conserved NALCN P-loop residues  
 665 p.W1085/p.R1062/p.R1094 located in domain III, a p.D1408 residue in domain IV, and  
 666 p.D1397 and p.E1364 in domain IV. In vertebrates, NLF-1/FAM155A has 2 predicted  
 667 transmembrane helices that may anchor the CRD in proximity to NALCN, and creates a  
 668 sealed dome-like structure that walls off most extracellular access pathways to the SF (27-  
 669 29). As a consequence and in contrast to  $Na_v$  and  $Ca_v$  channels, NALCN does not show  
 670 lateral fenestrations in its pore module that could provide an access route for drugs to  
 671 enter into the central cavity for block in the closed state (27). One glycosylation site on  
 672 NLF-1/FAM155A (p.N217) is detected (28), however the functional role of glycosylation  
 673 on NLF-1/FAM155A is currently unknown and needs to be investigated. The functional  
 674 relevance of having NLF-1 paralogues in mammals, FAM155A and FAM155B, is also  
 675 unknown. Of note, while the *NLF-1/FAM155A* mRNA is mainly expressed in various  
 676 regions of the brain as well as in the pituitary gland, the *NLF-1/FAM155B* mRNA is mainly  
 677 expressed in heart and thyroid gland in humans (<https://www.gtexportal.org>).  
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### 679 3- NALCN and its subunits are conserved in most animals

680  
 681 Here, we provide an updated survey of NALCN channel complex homologues present  
 682 within the genomes and transcriptomes of several species from a range of animal phyla  
 683 (**Figure 4A**). Consistent with previous reports (51, 52), it appears that humans and other  
 684 vertebrates (phylum Chordata) possess single-copy genes for NALCN, UNC-79, and UNC-  
 685 80, and two NLF-1/FAM155 paralogues, NLF-1/FAM155A and NLF-1/FAM155B (**Figure**  
 686 **4B**). The early-diverging chordate *Ciona intestinalis*, an invertebrate tunicate (sea squirt),  
 687 only possesses a single NLF-1/FAM155 homologue. Generally, protostome and  
 688 deuterostome invertebrates similarly possess single copy genes for all four NALCN  
 689 subunits. A well-known exception is the nematode worm *Caenorhabditis elegans*, which  
 690 appears to have duplicated NALCN to give rise to the paralogues NCA-1 and NCA-2 (33).  
 691 Given the absence of duplicated NALCN genes in early-diverging nematodes such as the  
 692 parasitic species *Trichinella pseudospirallis*, this duplication likely occurred within a  
 693 subclade of the nematode phylum. Similar within-phylum variability is evident for the  
 694 Platyhelminthes, where the non-parasitic (free-living) flatworm *Macrostomum lignano*  
 695 duplicated NALCN and NLF-1/FAM155 to give rise to two copies each, while the parasitic  
 696 species *Schistosoma mansoni* retained single copies of all subunits. The fellow spiralian,

697 and freshwater rotifer *Rotaria socialis* is unique amongst examined species in duplicating  
 698 UNC-80, along with NLF-1/FAM155.



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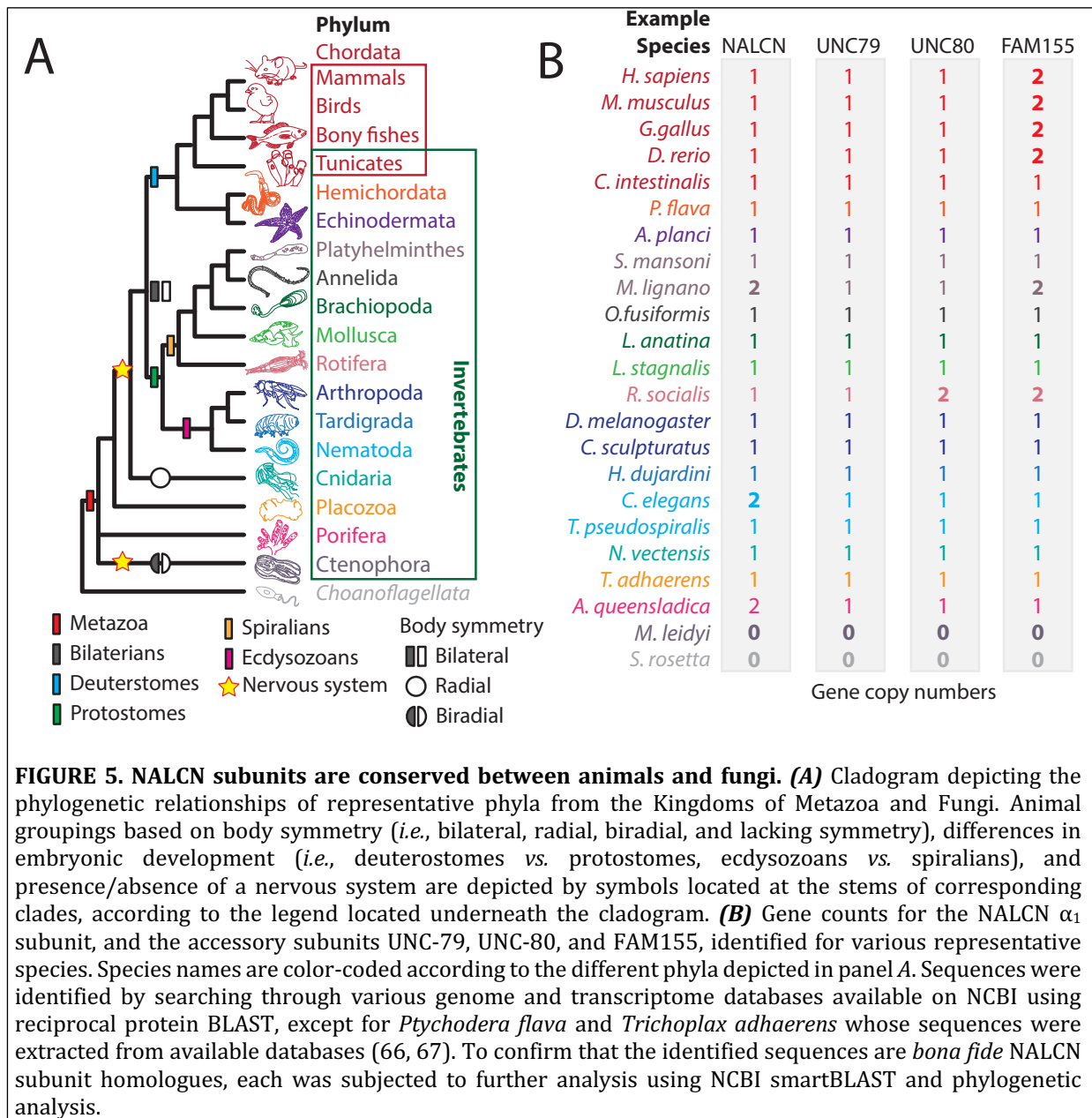
716 Previously, the non-bilaterian invertebrate *Nematostella vectensis* (sea anemone,  
 717 phylum Cnidaria which includes jellyfish and corals), as well as the Great Barrier Reef  
 718 sponge *Amphimedon queenslandica* (phylum Porifera), were reported to possess 2  
 719 NALCN channel genes (51, 52). However, only a single homologue is evident in the most  
 720 recent and complete *N. vectensis* genome assembly (53). Instead, the 2 *A. queenslandica*  
 721 paralogues are significantly different in their sequence, and hence represent *bona fide*  
 722 paralogues that likely emerged through duplication. That the NALCN subunits duplicated  
 723 independently in *M. lignano*, *C. elegans*, and *A. queenslandica* is suggested by their  
 724 respective monophyletic relationships on a protein phylogenetic tree (**Figure 5**). Like *N.*  
 725 *vectensis*, the other non-bilaterian species *Trichoplax adhaerens* (phylum Placozoa) also  
 726 possesses single copy genes for all NALCN subunits. Perhaps most remarkable within the  
 727 Metazoa is the complete absence of NALCN subunits in the gene data available for  
 728 ctenophores, including the well-studied species *Mnemiopsis leidyi* (**Figure 5B**). Recent  
 729 phylogenomic studies strongly support either sponges or ctenophores as the most  
 730 divergent animal lineage (54, 55), suggesting that either ctenophores lost the channel  
 731 complex (if sponges are the most divergent), or it evolved after ctenophores diverged in  
 732 the common ancestor of Porifera, Placozoa, Cnidaria, and Bilateria (**Figure 5A**).

#### 734 4- Distribution and cellular localization of NALCN

736 To date, our understanding of the cellular (and subcellular) co-expression of NALCN and  
 737 its subunits is incomplete and requires further investigation, especially in mammals. In  
 738 rodent and human, northern and dot blot analysis highlighted that *NALCN* mRNA is  
 739 expressed in all brain regions, as well as the heart, pituitary, and adrenal glands (1, 56). A  
 740 similar expression pattern was described by RT-qPCR in the pond snail *Lymnea stagnalis*  
 741 that express *NALCN* mainly in the central nervous system but also in the heart, secretory  
 742 organs, and the buccal musculature (51). Of note, this latter study also reported  
 743 localization of *NALCN* mRNA at axons/neurites, suggesting that translation of NALCN  
 744 could occur outside the cell body through a process referred to as local protein synthesis.  
 745 In mice, *NALCN* mRNA is abundantly expressed in neurons  
 746 (<http://www.mousebrain.org>)(57), while in humans, the temporal expression of *NALCN*  
 747 mRNA in different parts of the brain closely parallels the expression of synapse, dendrite,  
 748 and myelination development genes (<http://hbatlas.org/>)(58).

750 In mammals, the development of antibodies that can reliably detect NALCN in tissues  
 751 has recently paved the way for determining its cellular and subcellular distribution,  
 752 permitting confirmation that the channel protein is expressed in proximal dendritic  
 753 compartments of rodent nigral dopaminergic neurons as well as in dorsal root ganglia  
 754 neurons, dorsal spinal cord neurons and in a human cell line *via* immunocytochemistry  
 755 (59-63). Furthermore, the NALCN ancillary subunit UNC-80 localizes to dendrites in  
 756 mouse cultured hippocampal neurons when overexpressed *in vitro* (49), implying that  
 757 NALCN and its other subunits might also be localized to dendrites. In *C. elegans*, the use of  
 758 both GFP-tagged endogenous proteins and immunostaining revealed that the two NALCN  
 759 channel paralogues, NCA-1 and NCA-2, both localize along axons and are enriched at  
 760 nonsynaptic regions (32, 34). By contrast, the *D. melanogaster* NA/Dm $\alpha$ 1U channel  
 761 protein is mainly concentrated in the neuropil region of the brain which is enriched in  
 762 synapses, but not areas occupied primarily by cell bodies or axon tracts (64). Another  
 763 study reported expression of NA/Dm $\alpha$ 1U in the cell body and terminals of pacemaker  
 764 ventral lateral neurons which play a central role in regulating circadian rhythm (65). In *L.*

765 *stagnalis*, the NALCN homolog of appears to be enriched in neurites (acc.  
 766 AGC13754.1)(51).  
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 770 **FIGURE 5. NALCN subunits are conserved between animals and fungi.** (A) Cladogram depicting the  
 771 phylogenetic relationships of representative phyla from the Kingdoms of Metazoa and Fungi. Animal  
 772 groupings based on body symmetry (*i.e.*, bilateral, radial, biradial, and lacking symmetry), differences in  
 773 embryonic development (*i.e.*, deuterostomes vs. protostomes, ecdysozoans vs. spiralians), and  
 774 presence/absence of a nervous system are depicted by symbols located at the stems of corresponding  
 775 clades, according to the legend located underneath the cladogram. (B) Gene counts for the NALCN  $\alpha_1$   
 776 subunit, and the accessory subunits UNC-79, UNC-80, and FAM155, identified for various representative  
 777 species. Species names are color-coded according to the different phyla depicted in panel A. Sequences were  
 778 identified by searching through various genome and transcriptome databases available on NCBI using  
 779 reciprocal protein BLAST, except for *Ptychodera flava* and *Trichoplax adhaerens* whose sequences were  
 780 extracted from available databases (66, 67). To confirm that the identified sequences are *bona fide* NALCN  
 781 subunit homologues, each was subjected to further analysis using NCBI smartBLAST and phylogenetic  
 782 analysis.

783  
 784 **5- NALCN currents in native cells**  
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786 The first study describing the functional properties of NALCN as a TTX-resistant Na<sup>+</sup> leak  
 787 channel in neurons was provided by Lu *et al*, in 2007 (2). In this pioneer study, Dejian Ren  
 788 and colleagues generated a knockout mouse model by removing the first coding exon of  
 789 the *Nalcn* gene. Primary hippocampal neurons from knockout mice were found to be  
 790 hyperpolarized with a RMP ~10 mV lower than wild-type neurons. Furthermore, patch-  
 791 clamp recordings of wild type hippocampal neurons where external Na<sup>+</sup> was replaced  
 792 with the impermeant large inorganic cation NMDG<sup>+</sup>, in the presence of TTX and Cs<sup>+</sup> which  
 793 block Na<sub>v</sub> and HCN channels respectively, revealed the disappearance of a pronounced  
 794 Na<sup>+</sup> background conductance. Instead, this effect was not evident in neurons from  
 795 knockout mice, strongly suggesting that NALCN is responsible for this leak current.

796 Furthermore, both the RMP and the Na<sup>+</sup> background conductance were restored by  
 797 overexpressing NALCN in a knockout background, producing a more depolarized RMP  
 798 and the re-appearance of a Na<sup>+</sup> background conductance compared to control neurons.  
 799 The relative contributions of I<sub>NALCN</sub>, I<sub>h</sub> and I<sub>Nav</sub> to the total background Na<sup>+</sup> current was  
 800 estimated to be ~70%, ~10%, and ~20% respectively in hippocampal neurons (2). As a  
 801 consequence of the *Nalcn* knockout, neuronal electrical activity is almost fully abolished  
 802 as demonstrated by electrophysiological recordings from the C4 nerve root in the spinal  
 803 cord. That the major contribution of NALCN in regulating the RMP and the firing rate of  
 804 neurons is by conducting background Na<sup>+</sup> leak currents was also confirmed in  
 805 suprachiasmatic nucleus neurons (38), GABAergic substantia nigra pars reticulata (SNr)  
 806 neurons (68), neurons in the deep mesencephalic nucleus (69), CO<sub>2</sub>/H<sup>+</sup>-sensitive neurons  
 807 of the retrotrapezoid nucleus (34, 70), glutamatergic pre-Bötzinger Complex neurons  
 808 (34), spino-parabrachial neurons (71), dopamine neurons from the substantia nigra (72),  
 809 type-2 neurons in the nucleus of the solitary tract expressing the leptin receptor (73),  
 810 dorsal root ganglia neurons and dorsal spinal cord neurons (61, 62). In addition, the  
 811 depolarizing function of NALCN is clearly conserved in neurons from invertebrates such  
 812 as posterior dorsal neurons 1 (DN1p) neurons from *D. melanogaster* (38), pacemaker  
 813 RPeD1 neurons from *L. stagnalis* (74), and premotor interneurons from *C. elegans* (35,  
 814 75). The contribution of NALCN in regulating the RMP and electrical activity was also  
 815 extended to an endocrine anterior pituitary cell model (76). **TABLE 1** summarizes studies  
 816 describing NALCN-related currents from native cells, and **Figure 7** (*see below*) illustrates  
 817 several of these electrical activity modulations described here in association with the  
 818 physiological roles of NALCN (*see* 'NALCN & Physiology' section).

**TABLE 1: Endogenous NALCN currents in native cells**

Species	Cell Type	RMP change	Method	References
<i>M. musculus</i>	Hippocampal neurons	≈ -10 mV NI NI ≈ -5 mV	Knockout  Knockdown	<a href="#">Lu et al, 2007 (2)</a> <a href="#">Lu et al, 2009 (43)</a> <a href="#">Lu et al, 2010 (44)</a> <a href="#">Ou et al, 2020 (77)</a>
<i>M. musculus</i>	Ventral tegmental area neurons	NI	Knockout	<a href="#">Lu et al, 2009 (43)</a>
<i>M. musculus</i>	Pancreatic b-MIN6 cells	NI NI	Knockdown Overexpression	<a href="#">Swayne et al, 2009 (56)</a>
<i>M. musculus</i>	Interstitial cells of Cajal	NI	Knockout	<a href="#">Kim et al, 2012 (78)</a>
<i>M. musculus</i>	Cortical neurons	NI	Knockdown FAM155A	<a href="#">Xie et al, 2013 (35)</a>
<i>M. musculus</i>	SCN pacemaker neurons	≈ -11,4 mV	Knockout	<a href="#">Flourakis et al, 2015 (38)</a>
<i>H. Sapiens</i>	Myometrial smooth muscle cells	NI	Knockdown	<a href="#">Reinl et al, 2015 (79)</a>
<i>M. musculus</i>	Retrotrapezoid neurons	NI ≈ -9,12 mV NI	Knockdown Knockout Knockdown	<a href="#">Shi et al, 2016 (70)</a> <a href="#">Yeh et al, 2017 (80)</a> <a href="#">Yang et al, 2020 (81)</a>
<i>M. musculus</i>	Deep Mesencephalic neurons	≈ +7,8 mV	Gain-of-function mutation	<a href="#">Funato et al, 2016 (69)</a>
<i>M. musculus</i>	Substantia nigra pars reticulata neurons	NI	Knockout	<a href="#">Lutas et al, 2016 (68)</a>
<i>M. musculus</i>	Glutamatergic preBötC neurons	≈ -5,34 mV	Knockout	<a href="#">Yeh et al, 2017 (80)</a>

<i>M. musculus</i>	Spino-parabrachial neurons	≈ -15,7 mV	Knockout	<a href="#">Ford et al, 2018 (71)</a>
<i>M. musculus</i>	Dopamine neurons from the substantia nigra	NI	Knockout	<a href="#">Philippart &amp; Khaliq, 2018 (72)</a>
		NI	Pharmacological inhibition L-703,606 (10 μM)	<a href="#">Um et al, 2021 (82)</a> <a href="#">Hahn et al, 2023 (59)</a>
<i>M. musculus</i>	Granule cells of the dentate gyrus	NI	Pharmacological inhibition L-703,606 (10 μM)	<a href="#">Gonzalez et al, 2023 (83)</a>
<i>M. musculus</i>	Type-1 and type-2 leptin receptor-expressing neurons in the nucleus of the solitary tract	NI	Knockout	<a href="#">Do et al, 2020 (73)</a>
<i>R. Norvegicus</i>	Pituitary GH3 cells	≈ -5.3 mV ≈ +11.7 mV	Knockdown Overexpression	<a href="#">Impheng et al, 2021 (76)</a>
<i>R. Norvegicus</i>	Dorsal spinal cord neurons	NI	Knockdown	<a href="#">Zhang et al, 2021 (61)</a> <a href="#">Li et al, 2021 (62)</a>
<i>R. Norvegicus</i>	DRG neurons	NI	Knockdown	<a href="#">Zhang et al, 2021 (61)</a> <a href="#">Li et al, 2021 (62)</a>
<i>Bos Taurus</i>	Chromaffin cells	NI	Pharmacological inhibition CP96345 (20-50 μM)	<a href="#">Yang et al, 2022 (84)</a>
<i>L. stagnalis</i>	RPed1 neurons	NI	Knockdown	<a href="#">Lu &amp; Feng, 2011 (74)</a>
<i>C. elegans</i>	Premotor interneurons	≈ -6 mV	Knockout FAM155A Knockout NALCN	<a href="#">Xie et al, 2013 (35)</a> <a href="#">Gao et al, 2015 (75)</a>
<i>D. Melanogaster</i>	DN1p neurons	NI	Knockout	<a href="#">Flourakis et al, 2015 (38)</a>

821 **Abbreviations: NI, not indicated**

## 822 **6- Functional expression of NALCN in recombinant systems**

823  
824  
825 A rigorous understanding of NALCN function/dysfunction through the study of its  
826 biophysical and pharmacological properties requires the ability to express it as a  
827 functional channel complex *in vitro* in cell lines such as HEK-293T (85-87). Several  
828 configurations and recombinant systems were used to achieve functional expression of  
829 NALCN (**TABLE 2**). In addition to the first demonstration that NALCN encodes a Na<sup>+</sup>  
830 background conductance in hippocampal neurons, Lu et al, 2007, also reported the first  
831 functional expression of NALCN in a recombinant system (2). Transient expression of rat  
832 NALCN alone in HEK-293T cells resulted in the expression of a voltage-independent leak  
833 current with a linear current-voltage (I/V) relationship within the range of -80 to +80 mV,  
834 and a reversal potential (E<sub>rev</sub>) of 0 mV. No voltage-dependent inactivation was observed  
835 (2). In this study, NALCN was permeable to cations with a selectivity sequence of P<sub>Na</sub>(1.3)  
836 > P<sub>K</sub>(1.2) > P<sub>Cs</sub>(1) > P<sub>Ca</sub>(0.5). Interestingly, a mutation in the selectivity filter to create an  
837 EEKA sequence instead of an EEKE sequence in the wild-type NALCN resulted in a  
838 selectivity sequence of P<sub>Na</sub>(1.3) = P<sub>K</sub>(1.3) > P<sub>Cs</sub>(1) > P<sub>Ca</sub>(0.1), indicating a major role of the  
839 fourth glutamate in Ca<sup>2+</sup> permeability. Instead, no current was detected when the  
840 selectivity filter was modified to resemble high voltage-activated Cav channels, with an

841 EEEE configuration. In this study, NALCN currents were blocked by Cd<sup>2+</sup> (IC<sub>50</sub> = 0.15 mM),  
 842 Co<sup>2+</sup> (IC<sub>50</sub> = 0.26 mM), Gd<sup>3+</sup> (IC<sub>50</sub> = 1.4 μM), but not by Ni<sup>2+</sup> (1 mM) and La<sup>3+</sup> (100 μM).  
 843 NALCN was also found to be insensitive to TTX (10 μM), but completely blocked by 1 mM  
 844 verapamil, a Ca<sub>v</sub> channel blocker. Of interest, the fourth glutamate of the selectivity filter  
 845 was implicated in the sensitivity of NALCN to Gd<sup>3+</sup> block. Although an enhanced leak  
 846 current was also reported by other groups, when NALCN was expressed alone in HEK-  
 847 293T cells (77, 88, 89), a debate has remained for some time regarding how to achieve  
 848 functional expression of NALCN in this cell line without co-expressing its subunits NLF-  
 849 1/FAM155, UNC-79 and UNC-80, as NALCN current measured in the absence of its  
 850 accessory subunits could not be reproduced in other studies (1, 51, 56, 90, 91).

851  
 852 **TABLE 2: Functional expression of NALCN in recombinant system**

Cell type	Current type	Method/ Subunits expressed	References
HEK-293T	Leak	NALCN	<a href="#">Lu et al, 2007 (2)</a> <a href="#">Lu et al, 2009 (43)</a> <a href="#">Egenbrod et al, 2019 (88)</a> <a href="#">Hahn et al, 2020 (89)</a> <a href="#">Ou et al, 2020 (77)</a>
HEK-293T	Substance P-activated	NALCN, UNC-80, TACR1	<a href="#">Lu et al, 2009 (43)</a>
HEK-293T	Leak	NALCN, UNC-80, SrcY529F	<a href="#">Lu et al, 2009 (43)</a> <a href="#">Lu et al, 2010 (44)</a> <a href="#">Funato et al, 2016 (69)</a> <a href="#">Lee et al, 2019 (92)</a>
HEK-293T	Leak	NALCN, UNC-80, Src activator*	<a href="#">Stray-Pedersen et al, 2016 (93)</a> <a href="#">Wie et al, 2020 (49)</a>
HEK-293T	Leak	NALCN, UNC-79, UNC-80, NLF-1/FAM155A (or B)	<a href="#">Chua et al, 2020 (30)</a> <a href="#">Kschonsak et al, 2020 (27)</a> <a href="#">Kang et al, 2020 (29)</a> <a href="#">Xie et al, 2020 (28)</a> <a href="#">Kschonsak et al, 2022 (31)</a> <a href="#">Zhou et al, 2022 (46)</a> <a href="#">Kang &amp; Chen, 2022 (45)</a>
HEK-293T	Leak	NALCN, NLF-1/FAM155A	<a href="#">Xie et al, 2020 (28)</a>
HEK-293T	Acetylcholine-activated	NALCN, M3R	<a href="#">Swayne et al, 2009 (56)</a>
	Carbachol-activated		<a href="#">Hahn et al, 2020 (89)</a>
MIN6	Acetylcholine-activated	NALCN**	<a href="#">Swayne et al, 2009 (56)</a>
<i>Xenopus</i> Oocyte	Acetylcholine-activated	NALCN, M3R	<a href="#">Swayne et al, 2009 (56)</a>
SH-SY5Y	Leak	NALCN	<a href="#">Lu et al, 2010 (44)</a>
NG108-15	Leak	NALCN, NLF-1/FAM155A***	<a href="#">Bouasse et al, 2019 (94)</a>
GH3	Leak	NALCN****	<a href="#">Impheng et al, 2021 (76)</a>
<i>Xenopus</i> Oocyte	Leak	NALCN, UNC-79, UNC-80, NLF-1/FAM155A (or B)	<a href="#">Chua et al, 2020 (30)</a> <a href="#">Kschonsak et al, 2020 (27)</a> <a href="#">Kschonsak et al, 2022 (31)</a>

853 \* Introduced in the Patch pipette; \*\* MIN6 cells endogenously express *Unc-79* and *Unc-80* mRNAs  
 854 (not known for *Nlf-1/Fam155A* and *Nlf-1/Fam155B*)(63); \*\*\* NG108-15 cells endogenously express  
 855 *Unc-79* and *Unc-80* but not *Nlf-1/Fam155A* mRNAs (not known for *Nlf-1/Fam155B*)(92); \*\*\*\* GH3  
 856 cells endogenously express *Unc-79*, *Unc-80* and *Nlf-1/Fam155A* mRNAs (not known for *Nlf-1/Fam155B*)(80).  
 857  
 858

859 Another configuration was proposed to achieve the functional expression of  
 860 NALCN in HEK-293T cells. Here, the functional expression of NALCN in HEK-293T cells  
 861 required co-expression with UNC-80 and a constitutively active form of the src kinase (*i.e.*,  
 862 a p.Y529F mutant (43). This kinase is involved in NALCN activation by G-protein-coupled  
 863 receptors (*see below*)(43, 56). This configuration, which was successfully reproduced in  
 864 other studies (44, 69, 92), also gave rise to a voltage-independent cationic current with a  
 865 linear I/V relationship, corroborated by loss of inward currents when external Na<sup>+</sup> and K<sup>+</sup>  
 866 were replaced with the impermeant cation NMDG<sup>+</sup>. Furthermore, inclusion of a peptide  
 867 in the patch pipette that activates src was found to regulate the NALCN-UNC-80 currents  
 868 *in vitro* (44, 49, 93).

870 More recently, a study showed that functional expression of NALCN in HEK-293T  
 871 cells and *Xenopus* oocytes requires co-expression with UNC-79, UNC-80 and either NLF-  
 872 1/FAM155A or NLF-1/FAM155B (30). This configuration revealed some novel and  
 873 important functional features of the NALCN channelosome. First, contrasting with  
 874 previous studies performed in HEK-293T cells, the Na<sup>+</sup> leak current generated by NALCN  
 875 exhibited some voltage-dependence, as was previously described in a neuronal cell line  
 876 (*see below*; **FIGURE 6**)(94). This voltage-dependence of NALCN current involves most if  
 877 not all the basic residues located in the S4 helices of Domains I and II, which are conserved  
 878 with the voltage-sensor residues of Cav and Nav channels. Second, and contrasting with  
 879 data from Lu *et al*, 2007, suggesting NALCN conducts Ca<sup>2+</sup> and is negatively regulated by  
 880 extracellular Ca<sup>2+</sup> through the calcium-sensing receptor (2, 44), marked Ca<sup>2+</sup> block of  
 881 monovalent cation currents though the pore was revealed by site-directed mutagenesis  
 882 (30). This block was not specific to Ca<sup>2+</sup> but generalizable to other divalent cations such  
 883 as Ba<sup>2+</sup> and Mg<sup>2+</sup>. In sum, in the presence of ancillary subunits NALCN is permeable to  
 884 small monovalent cations and is potently blocked by divalent cations such as Ca<sup>2+</sup> through  
 885 a direct pore-blocking mechanism. This latter study determined a permeability sequence  
 886 for NALCN of P<sub>Na</sub> ≈ P<sub>Li</sub> > P<sub>K</sub> > P<sub>Cs</sub>. This sequence changed to P<sub>Na</sub> ≈ P<sub>Li</sub> ≈ P<sub>K</sub> > P<sub>Cs</sub> when the  
 887 selectivity filter of the wild-type NALCN EEKE was mutated to DEKA to resemble Nav  
 888 channels. Mutation p.E1389A slightly lowered the P<sub>Na</sub>/P<sub>K</sub> and increased the P<sub>Na</sub>/P<sub>Cs</sub> ratio,  
 889 while p.D1390A had no effect on P<sub>Na</sub>/P<sub>K</sub> but markedly increased P<sub>Na</sub>/P<sub>Cs</sub> (29). In addition  
 890 to extracellular Ca<sup>2+</sup>, NALCN current was also inhibited by Mg<sup>2+</sup> and Ba<sup>2+</sup>, but less potently  
 891 at a concentration of 1 mM (83 ± 10%, 59 ± 11%, and 37 ± 11% inhibition respectively).  
 892 The substitution of eight charged side chains in the selectivity filter of NALCN with alanine  
 893 showed that inhibition by divalent cations occurs through an interaction with negatively  
 894 charged residues within the pore. Indeed, SF residue mutations p.E280A (DI) and p.E554A  
 895 (DII), as well as an adjacent p.D1390A mutation in DIV, showed drastically reduced Ca<sup>2+</sup>  
 896 block; p.D558A, p.E1119A, and p.E1389A showed moderately reduced sensitivity, while  
 897 p.D561A and p.K1115A showed wild type-like sensitivity. Like the original description of  
 898 NALCN in the absence of ancillary subunits (2), NALCN channelosome currents are not  
 899 sensitive to TTX (10 μM). Also similar is the block of NALCN currents by Gd<sup>3+</sup> (10 μM)  
 900 and verapamil (1 mM). However, the src kinase inhibitors PP1 (5 μM) and SU6656 (1 μM)  
 901 do not have noticeable effects on recorded currents. A lack of inhibition by PP1 of the  
 902 basal leak current mediated by NALCN was also described in primary dopaminergic  
 903 neurons, altogether suggesting that the leak activity of NALCN does not depend on basal  
 904 activity of the src kinase (89). The functional expression of NALCN co-expressed with  
 905 UNC-79, UNC-80 and NLF-1/FAM155A in HEK-293T cells was subsequently confirmed in  
 906 numerous other studies (27-29, 31, 45, 46). Notably however, expression of just NALCN  
 907 with NLF-1/FAM155A is also reported to produce a functional channel, but with more



908 depolarized activation properties (28). Cell-attached recordings of the complete NALCN  
909 channelosome transfected in HEK-293T cells revealed a single-channel conductance of  
910  $27.2 \pm 1.1$  pS with a reversal potential of  $5.7 \pm 1.1$  mV and a low single-channel open  
911 probability ( $P_0$ ) of roughly 0.04 (31).

912  
913 The functional expression of NALCN has also permitted a better understanding of  
914 its voltage-sensitivity (29). Specifically, wild-type NALCN exhibits responses to voltage  
915 pulses from holding potential of 0 mV, but mutation of positive residues in the S4 helix of  
916 Domain I (*i.e.*, p.R146Q + p.R152Q, or R3 + R5) abrogates voltage sensitivity. A similar  
917 observation was made in Domain II, where mutation of S4 residues R3 (p.R481) together  
918 with one of R4 (p.R484) or R5 (p.K487) render NALCN unresponsive to voltage changes.  
919 Instead, neutralizing mutations of all gating charges in the Domain III S4 helix (p.R989Q  
920 + p.R992Q + p.R995Q), or mutation of one of the two gating charges in the Domain IV S4  
921 helix (*i.e.*, p.R1310), does not have any effect on voltage sensitivity, indicating that only  
922 Domains I and II are required for voltage sensitivity (30). With these recent studies,  
923 requirement of the complete channelosome (*i.e.*, NALCN, NLF-1/FAM155, UNC-79 and  
924 UNC-80 subunits) to achieve functional expression of NALCN channels in HEK-293T is  
925 now well supported, revealing the unique electrophysiological properties of NALCN.  
926 **FIGURE 6**, presents the inward background current  $\text{Na}^+$  current that is recorded in the -  
927 80 mV / 0 mV range of the  $V_m$  (**FIGURE 6A-C**), and the marked slow deactivation current  
928 observed when cells are repolarized (**FIGURE 6A**) or hyperpolarized (**FIGURE 6B**). The  
929 current-voltage and conductance-voltage relationships of this NALCN current reveal its  
930 voltage-dependence, with a reversal potential ( $E_{\text{Rev}}$ ) near + 10mV (**FIGURE 6C-D**).

931  
932 Prior to observations of NALCN channelosome currents made in HEK-293T cells  
933 and *Xenopus* oocytes, a separate study reported the use of the rodent neuronal cell line  
934 NG108-15 to functionally express the channel complex indeed providing first evidence  
935 that NALCN currents can be voltage-dependent and blocked by extracellular  $\text{Ca}^{2+}$  ions  
936 (94). NG108-15 cells are a neuroblastoma line that can be differentiated into cholinergic  
937 neuron-like cells *via* reduction of serum levels in the culture medium and addition of  
938 dibutyryl cAMP (dbcAMP) and dexamethasone for 4 to 5 days (95, 96), allowing the  
939 functional expression of ion channels in a neuron-like environment. Bouasse et al, 2019,  
940 found that differentiated NG108-15 cells express both *Unc-79* and *Unc-80* but not *Nalcn*  
941 and *Nlf-1/Fam155a* at the mRNA level as assayed by RT-qPCR (94). Accordingly, transient  
942 transfection of NALCN and NLF-1/FAM155A in these cells resulted in a  $\text{Na}^+$  background  
943 conductance with properties consistent with those later reported in HEK-293T cells and  
944 *Xenopus* oocytes, including block of inward currents by replacement of external  $\text{Na}^+$  with  
945 NMDG<sup>+</sup>, insensitivity to TTX (10  $\mu\text{M}$ ), and sensitivity to  $\text{Gd}^{3+}$  block (10  $\mu\text{M}$ ) (**FIGURE 6E**).

946  
947 The functional expression of NALCN was also reported in the mouse pancreatic  $\beta$   
948 cell line MIN6, stemming from observations that *Nalcn*, *Unc-79*, and *Unc-80* are  
949 endogenously co-expressed in this cell type (56). Through combined knockdown and  
950 overexpression experiments, NALCN was shown to conduct an acetylcholine-activated  
951  $\text{Na}^+$  current activated by the muscarinic M3 receptor (M3R), lacking a baseline leak  
952 current component in the absence of receptor activation. This current was completely  
953 abolished by the replacement of extracellular  $\text{Na}^+$  by NMDG<sup>+</sup>, and reduced by  $\approx 50\%$  by  
954 replacement of  $\text{Na}^+$  with  $\text{Li}^+$ . Consistent with the studies describe above, the current was  
955 also sensitive to  $\text{Gd}^{3+}$  (10  $\mu\text{M}$ ) and resistant to TTX (2  $\mu\text{M}$ ). Furthermore, the current had  
956 a linear I/V relationship within the -100 mV to -25 mV range, with a reversal potential  $E_{\text{Rev}}$

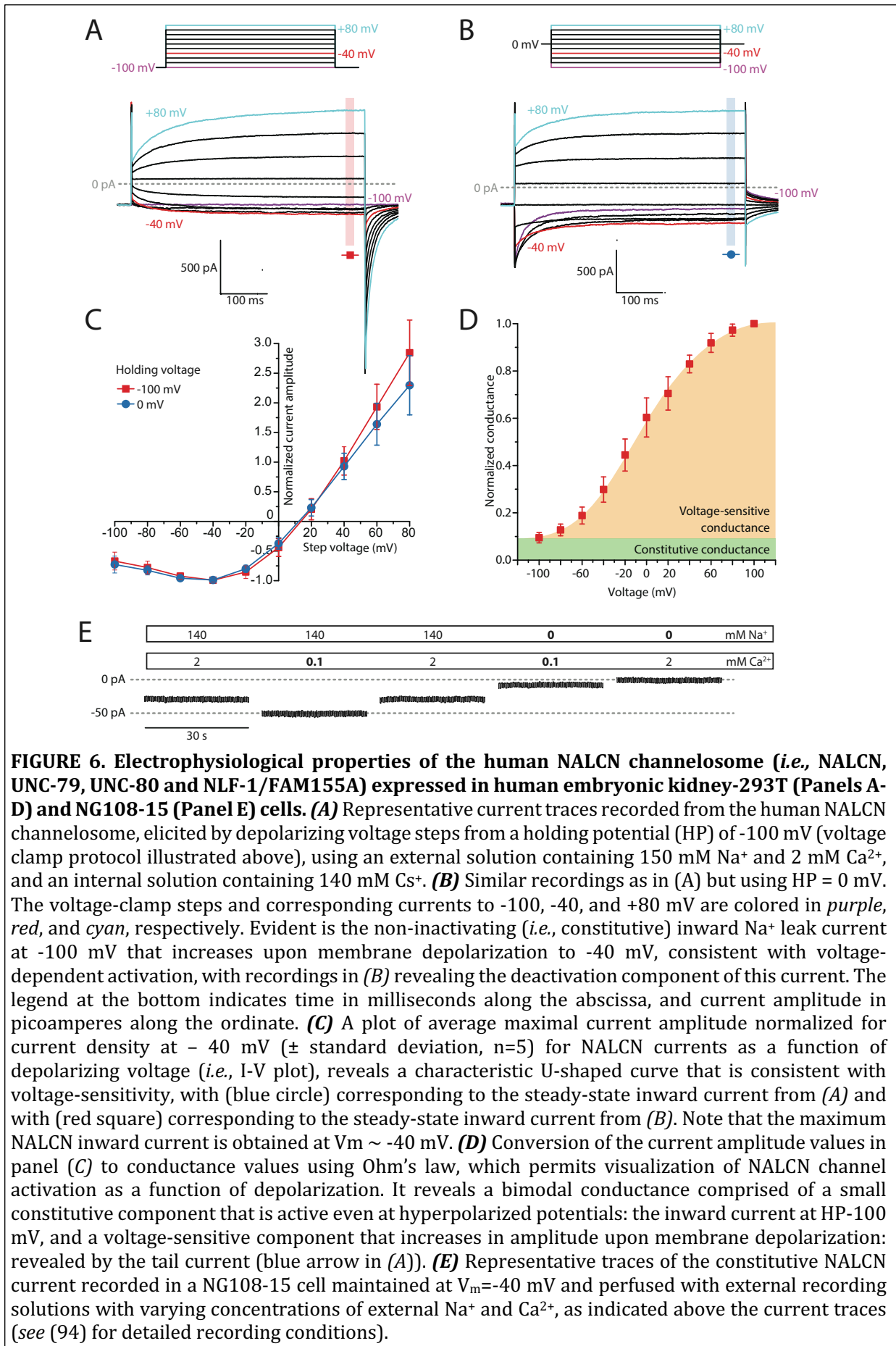
957 of roughly -23 mV. In this study, noise analysis was used to estimate a single-channel  
 958 conductance of ~27 pS, which notably is similar to the one reported for the Na<sup>+</sup> leak  
 959 current recorded in HEK-293T cells (27). This acetylcholine-activated current could be  
 960 recapitulated by co-expressing NALCN and M3R in both HEK-293T cells and *Xenopus*  
 961 oocytes (56, 89). Functional expression of NALCN was also successful in another  
 962 endocrine cell line: the rat pituitary somatotroph GH3 cell line that endogenously  
 963 expresses *Nalcn*, *Unc-79*, *Unc-80* and *Nlf-1/Fam155a* at the mRNA level (76). In this cell  
 964 type, a combination of knockdown and overexpression approaches showed that NALCN  
 965 generates a Ca<sup>2+</sup>-sensitive Na<sup>+</sup> leak conductance with a linear I/V relationship within the  
 966 -110 mV to -30 mV range, and notably, *Nalcn* knockdown significantly reduced prolactin  
 967 secretion. Lastly, NALCN was also described as a leptin-activated current in type-1  
 968 neurons in the nucleus of the solitary tract expressing the leptin receptor (73).

969  
 970 Overall, various *in vitro* studies using different subunits and cell lines (*i.e.*, HEK-293T,  
 971 *Xenopus* oocytes, NG108-15, MIN6, and GH3) have revealed a core set of functional  
 972 features of the NALCN current, but also some notable differences. Altogether, most studies  
 973 agree that NALCN conducts a cation (mainly Na<sup>+</sup>) current that is TTX-resistant and Gd<sup>3+</sup>-  
 974 sensitive. Given that some laboratories have recorded NALCN currents in HEK-293T cells  
 975 by expressing it without its ancillary subunits (2, 43, 77, 88, 89), while others have not (1,  
 976 51, 56, 90, 91), questions remain about the requirement of these subunits for NALCN  
 977 function *in vitro*. Perhaps, some of this discrepancy is attributable to variation in the  
 978 repertoire of expressed genes affected by culture conditions, passage number, and sub-  
 979 clone types (87). For example, Swayne *et al*, 2009, were unable to detect endogenous  
 980 expression of *NALCN* in HEK-293T cells, whereas Hahn *et al*, 2020, reported its expression  
 981 (56, 89). Indeed, we cannot exclude that HEK-293T cells cultured in some laboratories  
 982 endogenously express genes required for NALCN functional expression not occurring in  
 983 HEK-293T cells from other laboratories.

## 984 7- Pharmacology

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 987 To date, no pharmacological compound has been discovered that selectively and  
 988 specifically modulate NALCN activity *in vitro* or *in vivo*. Future discovery of such  
 989 compounds is of interest not only in basic research, but also in human therapy where the  
 990 ability to modulate NALCN activity appears clinically relevant (*e.g.*, pain, cancer,  
 991 CLIFAHDD syndrome). NALCN's insensitivity to TTX is attributed to the absence of key  
 992 residues present in Na<sub>v</sub> channels that are associated with TTX binding (28). NALCN is also  
 993 mildly sensitive to the Na<sub>v</sub> channel blocker Lamotrigine (300 μM), as well as the Ca<sub>v</sub>  
 994 channel blockers Nifedipine (100 μM), Diltiazem (1 mM), Verapamil (1 mM), D-600 (1  
 995 mM), and mibefradil (100 μM) (2, 27, 97-99). No effects however are observed for several  
 996 other known Ca<sub>v</sub> and Na<sub>v</sub> channel blockers, toxins, and other selected compounds at  
 997 relevant concentrations (27). There is evidence that NALCN current is inhibited by 2-  
 998 aminoethoxydiphenylborane (2-APB, 100 μM)(27, 68, 100) and flufenamic acid (FFA, 100  
 999 μM)(68), but not by the TRPC inhibitor SKF-96365 (100 μM)(68). However, 2-APB is  
 1000 pharmacologically promiscuous in that it also positively modulates K<sub>ir</sub> and K<sub>2P</sub> K<sup>+</sup>  
 1001 channels, and negatively modulates store-operated channels and TRP channels (100).  
 1002 Indeed, 10 μM 2-APB has only a marginal effect on substance P (SP)-mediated  
 1003 depolarization of Interstitial cells of Cajal attributed to NALCN (78). Similarly, FFA  
 1004 modulates the activity of a large panel of ion channels (101).

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1032 A recent study reported that N-benzhydryl quinuclidine compounds originally  
 1033 developed as tachykinin receptor-1 antagonists are potent inhibitors of NALCN in a src  
 1034 kinase-independent manner (89). These compounds, referred to as L703606, CP96345  
 1035 and Maropitant, inhibit NALCN currents in both HEK-293T cells and acutely isolated  
 1036 mouse nigral dopaminergic neurons with IC<sub>50</sub> values in the 16 to 20 μM range. In contrast,  
 1037 no effect is observed for recombinant TRPC channels, and concentrations of L703606  
 1038 below 10 μM do not exert effects on Na<sub>v</sub>1.5, Ca<sub>v</sub>1.2 and Ca<sub>v</sub>2.1 currents. Notably, this  
 1039 particular compound (10 μM) was recently used to implicate NALCN in electrical activity  
 1040 of mouse SNc dopaminergic neurons and granule cells of the dentate gyrus (59, 82, 83),  
 1041 and in cell proliferation and migration of lung cancer A549 cells (102). However, this  
 1042 latter observation is confounded by the fact that this cell line also expresses the tachykinin  
 1043 receptor-1 which as noted is sensitive to L703606 (103). Lastly, CP96345 (20 μM & 50  
 1044 μM) was used to implicate NALCN in the Na<sup>+</sup> background conductance observed in  
 1045 myometrial smooth muscle cells and bovine chromaffin cells respectively (84, 104).

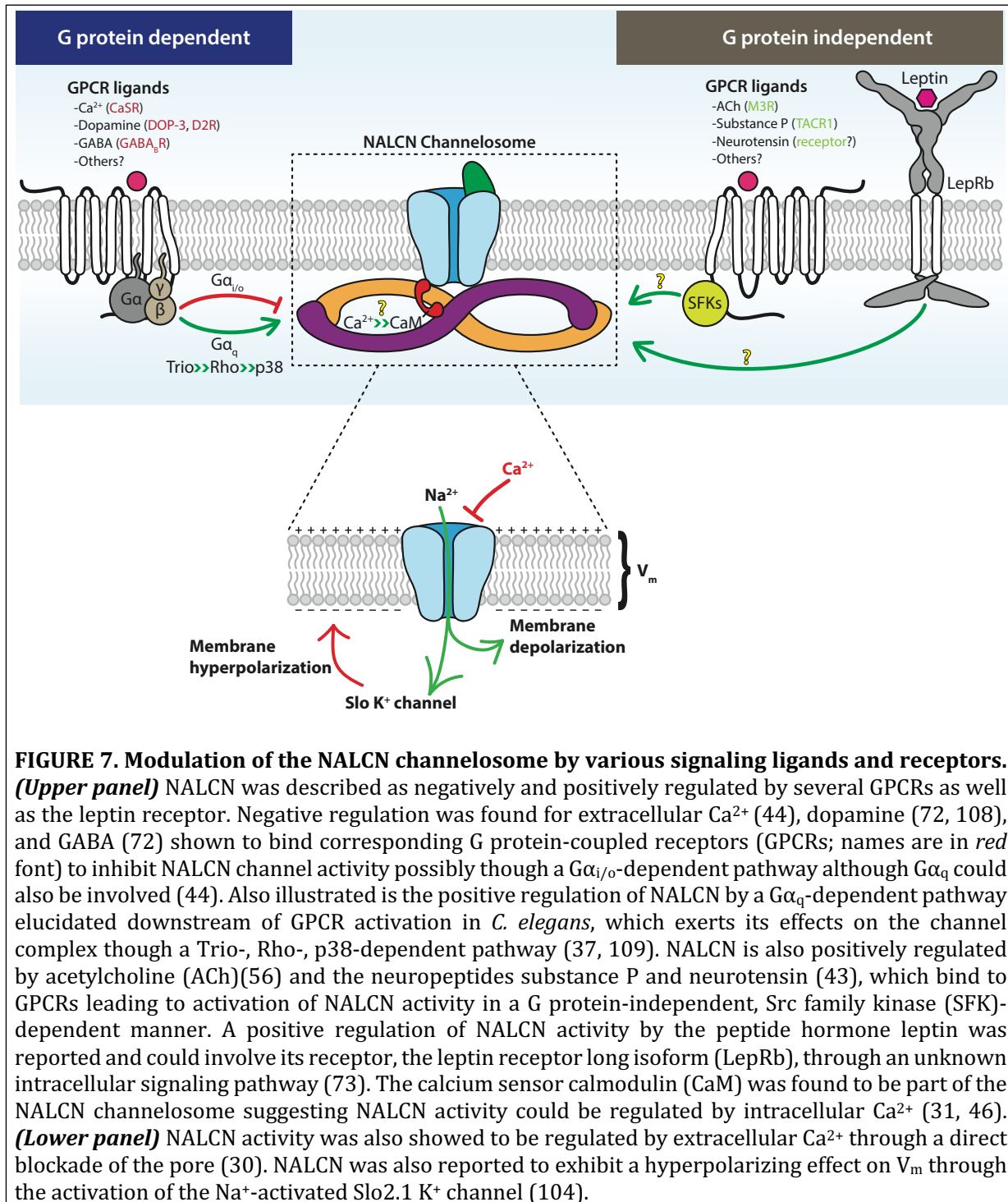
### 1046 **8- Regulation of NALCN by GPCRs**

1047  
 1048  
 1049 The regulation of NALCN activity through activation of G protein-coupled receptors  
 1050 (GPCRs) has emerged as an important pathway used for hormones and neurotransmitters  
 1051 to modulate cell excitability. Indeed, several studies have revealed that NALCN function is  
 1052 both positively and negatively regulated by GPCRs (**FIGURE 7**).

#### 1053 *Positive modulation of NALCN by GPCRs:*

1054  
 1055  
 1056 Almost simultaneously, Swayne *et al*, 2009, and Lu *et al*, 2009, found that NALCN is  
 1057 positively modulated by the GPCR ligands acetylcholine, substance P, and neurotensin in  
 1058 MIN6 pancreatic β cells and hippocampal and VTA neurons (43, 56). This positive  
 1059 modulation involves the muscarinic M3 receptor (M3R) for acetylcholine and the  
 1060 Tachykinin 1 (NK1) receptor for substance P, while the specific identity of the neurotensin  
 1061 receptor subtype remains to be determined. Interestingly, the positive modulation of  
 1062 NALCN by GPCRs occurred through a G protein-independent but src kinase-dependent  
 1063 pathway (43, 56). Here, application of src inhibitors such as PP1 and SU6656 inhibited  
 1064 the positive modulation, while inclusion of GDP-β-S and GTP-γ-S in the patch pipette,  
 1065 which lock G proteins in an active and inactive state respectively, had no effect. Of note,  
 1066 the involvement of src in the full activation of NALCN by M3R following acetylcholine  
 1067 application was recently challenged in HEK-293T cells transfected with both NALCN and  
 1068 M3R, where the acetylcholine-activated NALCN current was only partially inhibited by  
 1069 PP1 (89). Nonetheless, co-immunoprecipitation experiments from transfected HEK-293T  
 1070 cells revealed that M3R and NALCN belonged to the same protein complex involving the  
 1071 intracellular I-II loop of NALCN and the intracellular I3 loop and carboxyterminal region  
 1072 of the M3R (56). Since the I-II loop of NALCN forms key contacts with the UNC-79-UNC-  
 1073 80 dimer (31, 46), it is unclear whether this interaction is direct, or mediated by the UNC  
 1074 subunits acting as scaffolding proteins. Nonetheless, this interaction seems to be required  
 1075 for the positive modulation of NALCN by acetylcholine, since overexpression of these  
 1076 intracellular fragments fused to enhanced green fluorescent protein (eGFP) in MIN6 cells  
 1077 inhibits acetylcholine-evoked NALCN currents. Consistent with the notion that the UNC  
 1078 proteins might act to scaffold NALCN with other proteins, co-immunoprecipitation  
 1079 experiments revealed that UNC-80 can recruit the src kinase into the NALCN  
 1080 channelosome, presumably to facilitate phosphorylation of both NALCN and UNC-80 (43,

1081 105). However, the src kinase phosphorylation sites on NALCN and UNC-80 remain to be  
 1082 identified. The positive regulation of NALCN by acetylcholine and substance P was also  
 1083 described in interstitial cells of Cajal, substantia nigra pars reticulata neurons, and spino-  
 1084 parabrachial neurons (68, 71, 78). One may hypothesize that positive regulation of  
 1085 NALCN by these and other GPCRs might occur in other neurons and cell types as well. For  
 1086 example, G protein-independent activation of a cationic conductance by glutamate and  
 1087 acetylcholine through GPCRs is observed in CA3 pyramidal neurons and ventricular  
 1088 myocytes respectively (106, 107), where perhaps NALCN is viable molecular counterpart  
 1089 of this cationic conductance.  
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1110 Positive G-protein-dependent modulation (**FIGURE 7**) of NALCN homologs NCA-1  
 1111 and NCA-2 has also been reported in *C. elegans* (37, 109). This modulation is mediated by  
 1112 the Gαq protein via a downstream phospholipase C (PLC)-independent signaling cascade  
 1113 that sequentially involves UNC-73, RHO-1, and SEK-1 in cholinergic neurons. These 3  
 1114 proteins are the respective homologues of the mammalian RhoGEF Trio protein, the RHO  
 1115 proteins, and the MAPKK protein of the p38 MAP kinase pathway. Indeed, a forward  
 1116 genetic screen identified both NCA-1 and NCA-2 as downstream targets of the Gαq-Rho  
 1117 pathway. Ethylnitrosourea (ENU) mutagenesis to screen for suppressors of the  
 1118 hyperactive locomotion and looping posture of a constitutively activated Gαq mutant led  
 1119 to the identification of 10 recessive mutants: 6 for *unc-80*, 3 for *unc-79* and 1 for *nlf-*  
 1120 *1/fam155a*. Indeed, the loopy waveform locomotion of Gαq mutant animals was abolished  
 1121 in these 10 recessive mutants. This aberrant behavior was also suppressed by crossing  
 1122 the constitutively activated Gαq mutant with a *nca-1:nca-2* double mutant, thus  
 1123 confirming the requirement of the complete NALCN channelosome. Of note, a *nca-1*  
 1124 mutant on its own partially suppressed the locomotor phenotype of the constitutively  
 1125 activated Gαq mutant whereas a *nca-2* mutant did not. This suggests that although *nca-1*  
 1126 and *nca-2* redundantly contribute to normal worm locomotion, channels containing the  
 1127 NCA-1 pore-forming subunit have a larger role in transducing Gαq signals than NCA-2  
 1128 channels. The loopy waveform behavior of a constitutively activated mutant of Rho that  
 1129 acts downstream of Gαq was also either fully suppressed in *unc-79*, *unc-80*, *nca-1:nca-2*  
 1130 mutants, or partially suppressed in *nlf-1* mutants (37). The forward genetic screen  
 1131 mentioned above also identified the kinase SEK-1 as a suppressor of the locomotor  
 1132 phenotype of the constitutively activated Gαq mutant (109). Since SEK-1 is part of the p38  
 1133 MAP kinase pathway, this result was extended to mutants of other members of this  
 1134 pathway: the adaptor protein TIR-1, the MAPKKK NSY-1, and the MAPKs PMK-1 and PMK-  
 1135 2. However, this was not the case for the transcription factor ATF-7. Mutations in the SEK-  
 1136 1 p38 MAPK pathway also suppressed some aspects of the locomotor defects in *nca-1*  
 1137 gain-of-function mutant animals. One may hypothesize the activation of this pathway  
 1138 would result in NCA-1 channelosome phosphorylation, serving to modulate NALCN  
 1139 activity, cell localization, and/or protein expression. However, the relevance of this  
 1140 regulation on NCA-1 and NCA-2 currents remains to be determined, and whether a similar  
 1141 process occurs in mammals also remains to be explored, but is not without precedent  
 1142 since direct phosphorylation of the Na<sub>v</sub>1.6 and Na<sub>v</sub>1.8 by p38 is known to regulate the  
 1143 properties of these particular channels (110, 111).

1144  
 1145 *Negative modulation of NALCN by GPCRs:*

1146  
 1147 In addition to positive modulation of NALCN by some GPCRs, negative regulation by other  
 1148 GPCRs is also described (**FIGURE 7**). The first report of negative regulation involved the  
 1149 Ca<sup>2+</sup>-sensing receptor CaSR (44). A decrease in external [Ca<sup>2+</sup>] usually excites neurons and  
 1150 also activates depolarizing, nonselective cation currents in cell bodies and nerve  
 1151 terminals (112-119). Such regulation of neuronal excitability by diminished external  
 1152 [Ca<sup>2+</sup>] is apparent in primary hippocampal neurons from wild-type mice but not from  
 1153 *Nalcn* knockout mice (44). This response was partially restored by transfecting NALCN  
 1154 cDNA back into neurons of *Nalcn* knockout mice. Furthermore, the current amplitude of  
 1155 NALCN was dependent on external [Ca<sup>2+</sup>] in both hippocampal neurons and transfected  
 1156 SH-SY5Y cells. Deletion mutants indicated that NALCN modulation by external Ca<sup>2+</sup> ions  
 1157 required molecular determinants located in the last residues of the carboxy terminus of  
 1158 NALCN. This modulation required the UNC-79 subunit given that it was absent in *UNC79-*

1159 +/- hippocampal neurons. Following cell dialysis with a pipette solution containing GTP $\gamma$ S,  
 1160 lowering external [Ca<sup>2+</sup>] no longer increased the background Na<sup>+</sup> current, suggesting that  
 1161 activation of G proteins by GTP- $\gamma$ -S suppresses the low Ca<sup>2+</sup> activation of NALCN.  
 1162 Conversely, cell dialysis with GDP- $\beta$ -S increased a holding current which was not  
 1163 impacted by external [Ca<sup>2+</sup>], a process which did not occur in neurons from *Nalcn*  
 1164 knockout mice. In addition, transfection of wild-type hippocampal neurons with an active  
 1165 form of the G $\alpha$ q protein (*i.e.*, p.Q209L) disrupted the regulation of the Na<sup>+</sup> background  
 1166 conductance by external [Ca<sup>2+</sup>]. This positive regulation of NALCN by decreased external  
 1167 [Ca<sup>2+</sup>] can be recapitulated in HEK-293T cells by co-expressing NALCN, UNC-80, a  
 1168 constitutively active form of the src kinase (*i.e.*, SRC-p.Y529F), and CaSR. A more recent  
 1169 study confirmed the negative regulation of NALCN by CaSR and further showed that CaSR-  
 1170 mediated NALCN inhibition involves phosphorylation of NALCN by PKC at residue  
 1171 p.S1652 (92), requiring pre-methylation of residue p.R1653 by the methyltransferase  
 1172 PRMT7. The negative regulation of NALCN by external [Ca<sup>2+</sup>] was also confirmed in D2  
 1173 dopamine neurons (72), NG108-15 cells (94), GH3 cells (76), and HEK-293T cells (30), as  
 1174 well as in RPeD1 neurons from *L. stagnalis* (74). However, the CaSR-mediated regulation  
 1175 of NALCN by low external [Ca<sup>2+</sup>] was recently challenged (30), as noted above. Specifically,  
 1176 external [Ca<sup>2+</sup>] will directly block the NALCN pore when co-expressed with UNC-79; UNC-  
 1177 80 and NLF-1/FAM155A in HEK-293T cells in the absence of co-expressed CaSR.  
 1178

1179 In addition to CaSR, negative modulation of NALCN by the D2 dopamine receptor and  
 1180 the GABA<sub>B</sub> receptor through a G protein-dependent pathway was described in dopamine  
 1181 neurons (72). Whether this regulation similarly involves phosphorylation of NALCN by  
 1182 PKC is not known. A constitutively active inhibitory G-protein signaling pathway that  
 1183 suppresses intrinsic excitability of granule cells of the dentate gyrus, attributed to the  
 1184 combined activation GIRK channels and suppression of NALCN channels is also reported  
 1185 (83). Lastly, negative regulation of NALCN by the GABA<sub>B</sub> receptor may be cell type-  
 1186 dependent since there is no effect on the holding current of spino-parabrachial neurons  
 1187 that express NALCN when the GABA<sub>B</sub> receptor agonist R-baclofen (100 mM) is applied in  
 1188 presence of Cs<sup>+</sup> (71).  
 1189

1190 Negative regulation of NALCN by GPCRs is also apparent in invertebrates. In *C. elegans*,  
 1191 dopamine and the D2-like dopamine receptor DOP-3 negatively modulate NCA-1 and  
 1192 NCA-2 in premotor interneurons (108). This negative modulation involves activation of  
 1193 GOA-1, a G $\alpha$ o protein. However, inhibition of NCA-1 and NCA-2 is not direct but rather *via*  
 1194 the inhibition of the G $\alpha$ q-dependent activation of NALCN described above.  
 1195

## 1196 9- Other regulations

### 1197 *Metabolic regulation of NALCN:*

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 1199  
 1200 Several studies have reported links between metabolism and NALCN function. GABAergic  
 1201 neurons from the substantia nigra pars reticulata (SNr) exhibit spontaneous firing at a  
 1202 relatively high frequency (~30 spikes per second)(120-123). In order to explore a role for  
 1203 NALCN in this spontaneous activity, Lutas *et al*, 2016, generated conditional knockout  
 1204 mice where *Nalcn* expression could be disrupted by injection of a viral vector (*i.e.*, AAV9),  
 1205 which drives Cre recombinase expression selectively in neurons *via* the human syntaxin  
 1206 promoter. This study revealed that NALCN deficient neurons have impaired spontaneous  
 1207 electrical activity, and additionally, impaired modulation of firing by glycolytic inhibition

1208 and muscarinic receptor activation. Indeed, in wild-type SNr neurons, inhibition of  
 1209 glycolysis with iodoacetic acid (1 mM) in the presence of the mitochondrial fuel beta-  
 1210 hydroxybutyrate (bHB) slowed the firing rate. In contrast, *Nalcn* knockout neurons  
 1211 showed only a modest change in firing rate after inhibition of glycolysis. Altogether, it is  
 1212 apparent that NALCN activity is regulated by glycolysis and cholinergic signaling in SNr  
 1213 neurons, the latter resembling modulation of NALCN in MIN6 cells by muscarinic  
 1214 acetylcholine receptors (56).

1215

1216 In addition to a modulation of NALCN by glycolysis, leptin, an adipocytokine that  
 1217 regulates neural circuits in the brain that regulate appetite, positively regulates NALCN  
 1218 current in type-1 neurons in the nucleus of the solitary tract *via* the leptin receptor (73).  
 1219 This receptor is a cytokine-related receptor which when activated by leptin binding  
 1220 activates various signaling pathways including Janus tyrosine kinase 2 (JAK2)/STAT3, src  
 1221 homology-2-containing protein tyrosine phosphatase 2 (SHP2)/growth factor receptor-  
 1222 bound protein 2 (Grb2)/mitogen-activated protein kinase (MAPK), forkhead box protein  
 1223 O1 (FoxO1), 5' adenosine monophosphate-activated protein kinase (AMPK), and others  
 1224 (124). Whether leptin activates NALCN through activation of the leptin receptor and the  
 1225 exact molecular pathway(s) involved remain to be determined.

1226

#### 1227 *Potential regulation of NALCN by PIP2*

1228

1229 In *C. elegans*, mutations eliminating both NCA-1 and NCA-2 (*i.e.*, *nca-1:nca-2* double  
 1230 mutant) or their ancillary subunits (*unc-79* or *unc-80*) suppress the phenotypes of mutant  
 1231 animals bearing a lesion in the PIP2 phosphatase synaptojanin gene (*unc-26*), as well as  
 1232 phenotypes caused by overexpression of the Type 1 PIP kinase gene *ppk-1* (32). Since the  
 1233 abnormal phenotypes caused by excess PIP2 are exasperated in the presence of NALCN,  
 1234 and genetic disruption of NALCN counters this effect, it seems possible that this signaling  
 1235 lipid directly regulates NALCN activity in a positive manner, and that this contributes to  
 1236 the observed phenotypes. Of note, the Cryo-EM structure of the channel revealed that the  
 1237 pore region in domain II has a binding site for lipids, presumably phospholipids,  
 1238 suggesting a capacity for fine-tuning NALCN activity by lipids (28). However, since PIP2  
 1239 is known to modulate the activity of numerous other ion channels and transporters (125)  
 1240 the observed phenotypic interaction between NALCN and PIP2 signaling could be at the  
 1241 level of electrophysiology. That is, overexpression of PIP2 might tip the balance of  
 1242 excitation by modulating ion channels and/or transporters that work in parallel with  
 1243 NALCN, such that loss of NALCN activity restores this imbalance.

1244

#### 1245 *Potential regulation of NALCN by cytoplasmic Ca<sup>2+</sup>*

1246

1247 In addition to its regulation by extracellular Ca<sup>2+</sup>, two recent studies highlighted the  
 1248 possibility that NALCN is also regulated by intracellular Ca<sup>2+</sup> (31, 46). Indeed, the recent  
 1249 elucidation of the Cryo-EM structure of the NALCN channelosome revealed that the Ca<sup>2+</sup>  
 1250 sensor protein calmodulin (CaM) binds to the NALCN carboxy-terminus. This raises the  
 1251 possibility intracellular Ca<sup>2+</sup> and CaM can modulate NALCN, as described for several other  
 1252 ion channels including Cav and Nav channels (126). However, the physiological relevance  
 1253 of this elucidated interaction remains to be determined.

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### 1257 III. NALCN & PHYSIOLOGY

1258

1259 The expression of NALCN in a wide range of tissues and organs and in a variety of species  
 1260 suggests the channel contributes to many physiological functions, both in mammals and  
 1261 non-mammals. The early forward genetic screen studies that identified the NALCN  
 1262 channelosome in *C. elegans*, as well as *D. melanogaster* (**TABLE 3**)(19), also provided first  
 1263 insights into the physiological roles of NALCN and its subunits long before NALCN was  
 1264 identified as a channel that generates a Na<sup>+</sup> leak current that contributes to the regulation  
 1265 of cellular excitability. During the last decade, the continued development of animal  
 1266 models to study NALCN physiology, especially mice, was instrumental to uncover  
 1267 physiological roles of NALCN (**TABLE 3**). Indeed, the combined studies of NALCN function  
 1268 in mammals and invertebrates point to a conserved function for the channel in regulating  
 1269 the excitability of various cell types, especially neurons. For example, in mammals,  
 1270 changes in NALCN expression result in substantial changes in neuronal spiking, as  
 1271 illustrated in **FIGURE 8** for regions in the central nervous system, as well as non-neuronal  
 1272 excitable cells in peripheral organs. In this section, we summarized the current knowledge  
 1273 of how the NALCN channelosome contributes to the physiology of living organisms, from  
 1274 invertebrates to mammals (**FIGURE 9**).

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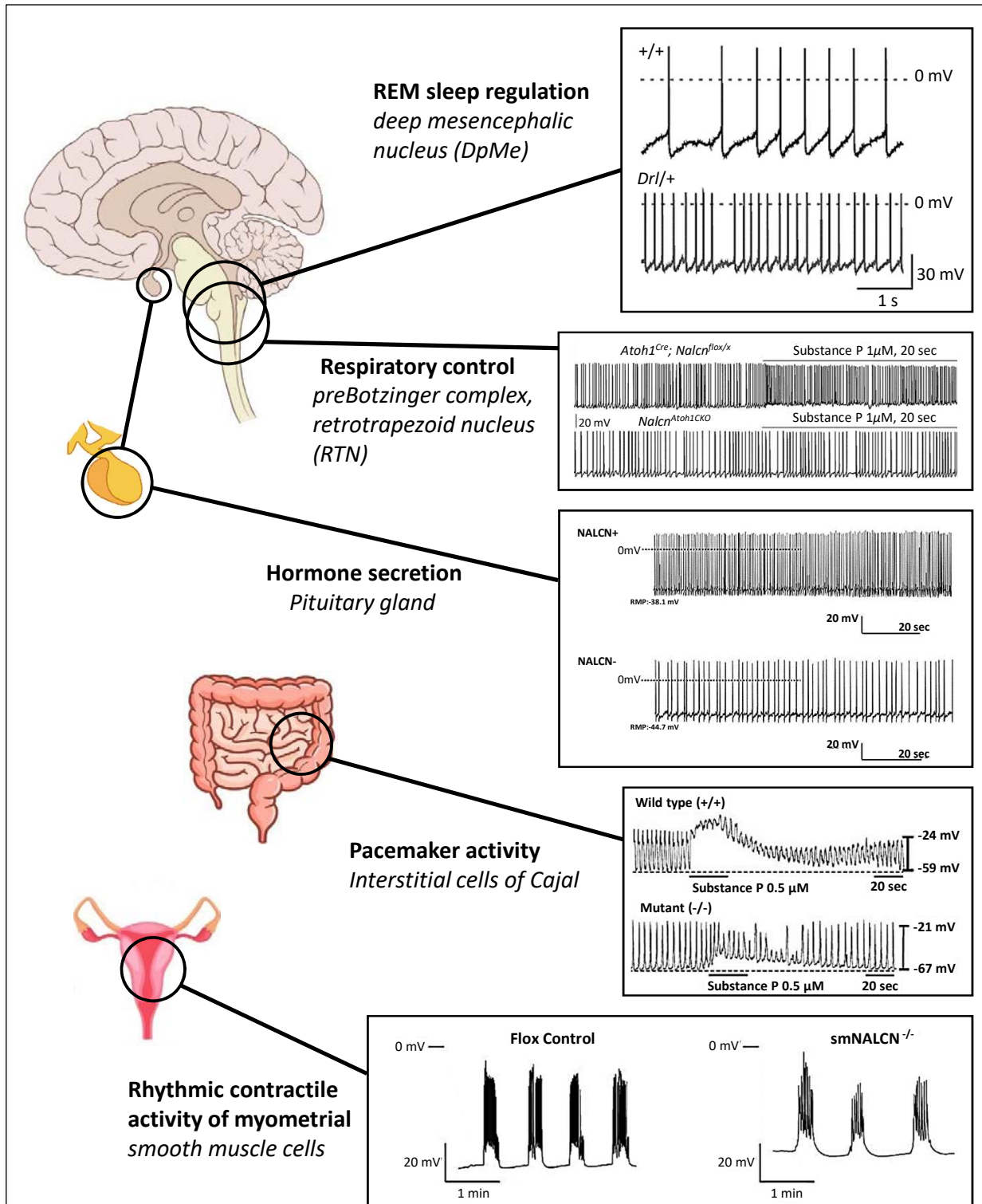
#### 1276 1- Locomotor behavior

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1278 Several studies from *C. elegans* showed that locomotor alteration is induced by NALCN  
 1279 channelosome dysfunction in a rhythmic network of neurons known as a central pattern  
 1280 generator (CPG), which includes motor neurons and premotor interneurons located in the  
 1281 ventral nerve cord (127, 128). Indeed, null mutations of both *NALCN* orthologs *nca-1* and  
 1282 *nca-2* (*i.e.*, *nca-1:nca-2*), as well as ancillary subunits *unc-79*, *unc-80* and *nlf-1*, cause a  
 1283 “fainter” phenotype (*i.e.*, frequent halting in locomotion)(32-34, 129-131). Mutant  
 1284 animals fail to sustain sinusoidal locomotion and succumb to long periods of halting. A  
 1285 similar phenotype was observed in *D. melanogaster*, referred to as “hesitant walking”,  
 1286 caused by null mutations of *na/Dmα1U* (132, 133). Conversely, in *C. elegans* semi-  
 1287 dominant gain-of-function mutations of *nca-1* impose a “coiler” phenotype, characterized  
 1288 by uncoordinated and exaggerated body bends during both spontaneous and stimulated  
 1289 locomotion (34). This phenotype is also apparent in *C. elegans* strains in which CLIFAHDD  
 1290 gain-of-function mutations are emulated in NCA-1 (134)(*see the NALCN & human diseases*  
 1291 *section*). As noted previously, NCA-1/2 as well as UNC-80 are axonally localized and  
 1292 expressed in both excitatory cholinergic and inhibitory GABAergic motor neurons, as well  
 1293 as premotor interneurons (32, 34). Functional studies revealed that loss of NCA-1 and  
 1294 NCA-2 activity leads to a more negative RMP and reduced synaptic transmission at  
 1295 GABAergic, cholinergic, and serotonergic neuromuscular junctions (34, 35, 135).  
 1296 Conversely, animals carrying gain-of-function mutations of *nca-1* have aberrant synaptic  
 1297 activity (34). *In vivo* Ca<sup>2+</sup> imaging experiments further indicated that while Ca<sup>2+</sup> influx in  
 1298 the cell bodies of egg-laying motoneurons is unaffected by altered NCA-1/2 activity,  
 1299 synaptic Ca<sup>2+</sup> transients are significantly reduced in *nca-1:nca-2* loss-of-function mutants  
 1300 and increased in *nca-1* gain-of-function mutants (34). In addition, rescue experiments  
 1301 suggest that reduced premotor interneuron network activity, instead of motoneuron  
 1302 dysfunction, is the primary cause of the frequent halting exhibited by fainters (35, 75).  
 1303 Taken together, these results suggest that NCA-1 and NCA-2 are required for the  
 1304 persistent motor circuit activity that sustains locomotion, mainly through premotor  
 1305 interneurons. Whether NALCN function in locomotory CPGs is conserved in vertebrate

1306 species remains to be investigated, with a potential link in the locomotory deficits found  
1307 in patients with the NALCN channelopathies IHPRF1 and CLIFAHDD (*see the NALCN &*  
1308 *human diseases* section). A role for NALCN in the *C. elegans* reversal behavior has also  
1309 been observed in the avoidance response to methyl salicylate (MeSa) (131). Here, a  
1310 forward genetic screen to identify mutants with defective response to MeSa led to the  
1311 identification of defective alleles for *unc-79* and *unc-80*. This result was extended to *nca-*  
1312 *1:nca-2* and *nlf-1* mutants. Of note, all these mutants exhibit wild type-like responses to  
1313 repelling odorants 1-octanol and 2-nonanone, and attractive odorants diacetyl and  
1314 benzaldehyde, suggesting these mutants have normal odorant responses. Neuron-specific  
1315 transgene rescue experiments of UNC-80 in *unc-80* mutants, combined with neuron-  
1316 specific knockdown of *unc-80* in wild-type animals, revealed that NALCN's role in  
1317 regulating reversal behavior induced by MeSa is specific to the command interneurons  
1318 AVA and AVE, as well as the guidepost neuron AVG (131).  
1319

1320 In mammals, a mouse strain carrying a heterozygous loss-of-function mutation of  
1321 *Unc-79* exhibits moderate hyperactivity in the form of increased locomotor behavior (47).  
1322 This mouse strain was identified in a forward genetic screen using ENU to mutate and  
1323 identify loci that influence locomotor behavior in a quantitative manner (136). This led to  
1324 the identification of a c.G5469T mutation in the coding sequence of UNC-79 (reference  
1325 sequence NM\_001081017.2) which results in a p.E1292X conversion (47). This allele is  
1326 referred to as *Lightweight*. No UNC-79 protein is detectable via Western blotting in  
1327 homozygous animals, and Northern blot analysis reveals a decrease in mRNA expression  
1328 suggesting the occurrence of nonsense-mediated decay. Like homozygous knockout  
1329 animals of *Nalcn*, *Unc-79* and *Unc-80*, homozygous *Lightweight* mice die shortly after birth  
1330 (2, 49, 136, 137). Thus, the hyperactivity phenotype noted above has not been assessed  
1331 in adult, homozygous null mutants of NALCN or its subunits. However, the recent  
1332 development of mice strains bearing conditional knockout alleles for *Nalcn*, as well as  
1333 adeno-associated virus-mediated knockdown approaches, will undoubtedly circumvent  
1334 the observed premature death for null alleles (38, 61, 62, 70, 72, 73, 77, 80, 81).  
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**FIGURE 8. Examples of NALCN contribution in tissue function: link with the modulation of RMP and pacemaker activities. (Upper panel)** Change in cell excitability in deep mesencephalic nucleus (DpMe) in the *dreamless* mouse model carrying a gain-of-function mutation in *Nalcn*. Note the DpMe neuron depolarization and the ensuing increased spiking discharges (from (69), Figure 5 panel f). **(Mid-upper panel)** NALCN expression in the brainstem neurons of the preBotzinger complex and retrotrapezoid nucleus (RTN) regulates rhythmic activity, RMP and response to substance P in RTN neurons (from (80), Figure 2 panel E). **(Middle panel)** NALCN modulates the RMP and AP firing frequency in the GH3 cell line, which models pituitary endocrine cells (from (76), Figure 2 panel A). **(Mid-lower panel)** NALCN in the interstitial cells of Cajal (ICCs) is involved in the electrophysiological response to substance P, by modulating the pacemaking activity. This effect is lost in *Nalcn* knockout (from (78), Figure 7 panel B).

**TABLE 3: The NALCN channelosome in Physiology**

Physiological function	Subunit	Species	Reference
Locomotor behavior	<i>unc-79</i>	<i>C. elegans</i>	Sedensky & Meneely, 1987 (129) Jospin et al, 2007 (32) Humphrey et al, 2007 (33) Yeh et al, 2008 (34) Pierce-Shimomura et al, 2008 (130) Zhou et al, 2020 (131)
	<i>unc-80</i>	<i>C. elegans</i>	Sedensky & Meneely, 1987 (129) Jospin et al, 2007 (32) Yeh et al, 2008 (34) Pierce-Shimomura et al, 2008 (130) Zhou et al, 2020 (131)
	<i>nca-1/nca-2</i> (NALCN orthologues)	<i>C. elegans</i>	Jospin et al, 2007 (32) Humphrey et al, 2007 (33) Yeh et al, 2008 (34) Pierce-Shimomura et al, 2008 (130) Bouhours et al, 2011 (135) Xie et al, 2013 (35) Gao et al, 2015 (75) Zhou et al, 2020 (131)
	<i>nlf-1</i> (FAM155A/B orthologue)	<i>C. elegans</i>	Xie et al, 2013 (35) Gao et al, 2015 (75) Zhou et al, 2020 (131)
	<i>na</i> (NALCN orthologue)	<i>D. melanogaster</i>	Krishnan & Nash, 1990 (132) Mir et al, 1997 (139) Guan et al, 2000 (133) Humphrey et al, 2007 (33)
	<i>unc-79</i>	<i>D. melanogaster</i>	Humphrey et al, 2007 (33)
Respiratory rhythm	<i>Unc-79</i>	<i>M. musculus</i>	Specia et al, 2010 (47)
	<i>Nalcn</i>	<i>M. musculus</i>	Lu et al, 2007 (2) Shi et al, 2016 (70) Yeh et al, 2017 (80) Yang et al, 2020 (81) Do et al, 2020 (73)
	<i>Unc-80</i>	<i>M. musculus</i>	Wie et al, 2020 (49)
Circadian Rhythm/Photic control of locomotion	<i>U-type</i> (NALCN orthologue)	<i>L. stagnalis</i>	Lu & Feng, 2011 (74) Lu et al, 2016 (140)
	<i>na</i> (NALCN orthologue)	<i>D. melanogaster</i>	Nash et al, 2002 (64) Lear et al, 2005 (65) Lear et al, 2013 (36) Ghezzi et al, 2014 (141) Flourakis et al, 2015 (38)

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	<i>unc-79</i>	<i>D. melanogaster</i>	Lear et al, 2013 (36) Murakami et al, 2021 (142)	
	<i>unc-80</i>	<i>D. melanogaster</i>	Lear et al, 2013 (36)	
	<i>nlf-1 (FAM155A/B orthologue)</i>	<i>D. melanogaster</i>	Ghezzi et al, 2014 (141) Flourakis et al, 2015 (38)	
	<i>Nalcn?</i> *	<i>M. musculus</i>	Flourakis et al, 2015 (38)	
<b>Sleep/Rest activity behavior</b>	<i>Nalcn</i>	<i>M. musculus</i>	Funato et al, 2016 (69)	
	<i>na (NALCN orthologue)</i>	<i>D. melanogaster</i>	Joiner et al, 2013 (143)	
	<i>unc-79</i>	<i>D. melanogaster</i>	Murakami et al, 2021 (142)	
	<i>nca-1 (NALCN orthologues)</i>	<i>C. elegans</i>	Huang et al, 2018 (144)	
<b>Myometrial activity during labor</b>	<i>unc-79</i>	<i>C. elegans</i>	Huang et al, 2018 (144)	
	<i>Nalcn</i>	<i>M. musculus</i>	Reinl et al, 2015 (79) Reinl et al, 2018 (138) Amazu et al, 2020 (60) Ferreira et al, 2021 (104)	
	<i>na (NALCN orthologue)</i>	<i>D. melanogaster</i>	Krishnan & Nash, 1990 (132)	
<b>Gastrointestinal motility</b>	<i>Nalcn</i>	<i>M. musculus</i>	Kim et al, 2012 (78)	
<b>Morphology</b>	<i>na (NALCN orthologue)</i>	<i>D. melanogaster</i>	Krishnan & Nash, 1990 (132) Mir et al, 1997 (139) Nash et al, 2002 (64) Humphrey et al, 2007 (33)	
	<i>unc-79</i>	<i>D. melanogaster</i>	Humphrey et al, 2007 (33)	
<b>Pain</b>	Neuropathic pain	<i>Nalcn</i>	<i>M. musculus</i> & <i>R. norvegicus</i>	Zhang et al, 2021 (61)
	Inflammatory pain	<i>Nalcn</i>	<i>R. norvegicus</i>	Li et al, 2021 (62)
	Chemical nociception	<i>Nalcn</i>	<i>C. h. pretoriae</i>	Eigenbrod et al, 2019 (88)
	Thermonociception	<i>nca-1/nca-2 (NALCN orthologues)</i>	<i>C. elegans</i>	Saro et al, 2020 (145)
<b>Osmoregulation</b>	<i>Nalcn</i>	<i>M. musculus</i>	Sinke et al, 2011 (146)	
<b>Sensitivity to ethanol and volatile anesthetics</b>	Volatile anesthetics	<i>Unc-79</i>	<i>M. musculus</i>	Specia et al, 2010 (47)
		<i>Nalcn</i>	<i>M. musculus</i>	Ou et al, 2020 (77) Yang et al, 2020 (81)
		<i>unc-79</i>	<i>C. elegans</i>	Morgan & Cascorbi, 1985 (147) Sedensky & Meneely, 1987 (129) Morgan et al, 1988 (148) Morgan et al, 1990 (149)

NALCN in physiology and pathophysiology

Ethanol	<i>nca-1/nca-2</i> (NALCN orthologues)	<i>C. elegans</i>	Morgan & Sedensky, 1995 (150) Humphrey et al, 2007 (33)  Humphrey et al, 2007 (33)
	<i>unc-80</i>	<i>C. elegans</i>	Sedensky & Meneely, 1987 (129) Morgan et al, 1988 (148) Morgan et al, 1990 (149)
	<i>na</i> (NALCN orthologue)	<i>D. melanogaster</i>	Krishnan & Nash, 1990 (132) Nash et al, 1991 (151) Campbell & Nash, 1994 (152) Leibovitch et al, 1995 (153) Mir et al, 1997 (139) Nishikawa & Kidoroko, 1999 (154) Guan et al, 2000 (133) van Swinderen, 2006 (155) Humphrey et al, 2007 (33)
	<i>unc-79</i>	<i>D. melanogaster</i>	Humphrey et al, 2007 (33)
	<i>Unc-79</i>	<i>M. musculus</i>	Specia et al, 2010 (47)
	<i>Nalcn</i>	<i>M. musculus</i>	Wu et al, 2021 (156)
	<i>unc-79</i>	<i>C. elegans</i>	Morgan & Sedensky, 1995 (150) Specia et al, 2010 (47)
	<i>unc-80</i>	<i>C. elegans</i>	Specia et al, 2010 (47)
	<i>nca-1/nca-2</i> (NALCN orthologues)	<i>C. elegans</i>	Specia et al, 2010 (47) Davies et al, 2012 (157)
	<b>Metabolism</b>	<i>Unc-79</i>	<i>M. musculus</i>
<b>Social clustering</b>	<i>na</i> (NALCN orthologue)	<i>D. melanogaster</i>	Burg et al, 2013 (158) Ghezzi et al, 2014 (141)
	<i>nlf-1</i> (FAM155A/B orthologue)	<i>D. melanogaster</i>	Ghezzi et al, 2014 (141)
<b>Others</b>			
Swallowing	<i>Nalcn, Unc-80</i>	<i>R. Norvergicus</i>	Li et al, 2021 (159)
Cell proliferation	<i>Fam155A</i>	<i>H. sapiens</i>	Lu et al, 2019 (160)
Adaptation to environment	<i>Nalcn</i>	<i>G. gallus</i>	Gu et al, 2020 (161)
Breeding intensification	<i>Nalcn</i>	<i>S. s. domesticus</i>	Kolosov et al, 2022 (162)
Male reproduction	<i>Nacn</i>	<i>Anas platyrhynchos domesticus</i> × <i>Cairina</i>	Yang et al, 2022 (163)

		<i>moschata</i> <i>domestica</i>	
--	--	-------------------------------------	--

1356 \* The *Nalcn* current was found to exhibit a circadian-dependent expression in neurons from the  
 1357 suprachiasmatic nucleus in mouse but a role in the circadian-dependent locomotor activity remains  
 1358 to be demonstrated.  
 1359

## 1360 2- Respiratory rhythm

1361  
 1362 Mice bearing genetic lesions of the *Nalcn* gene die within 24 hours after birth from  
 1363 respiratory defects. These mutant animals exhibit a severely disrupted respiratory  
 1364 rhythm characterized by a regular rhythm interrupted by periods of apnea (2). Breathing  
 1365 by NALCN null mice is characterized by apnea for ~5 seconds followed by a burst of  
 1366 breathing for ~5 seconds at a rate of ~5 bouts of apnea per minute. The cause of this, at  
 1367 least in part, is attributable to a complete loss of electrical activity from the fourth cervical  
 1368 nerve root (C4) that innervates the diaphragm and discharges rhythmic electrical signals  
 1369 that regulate breathing. However, since *Nalcn* is widely expressed in the nervous system,  
 1370 a global reduction of excitability in mutant animals may lead to the disruption in  
 1371 respiration. Indeed, both the rhythm and neuromodulatory responses of breathing are  
 1372 controlled by brainstem neurons in the preBötzing complex (preBötC) and the  
 1373 retrotrapezoid nucleus (RTN)(164-166). Neurons in the preBötC show characteristics of  
 1374 a CPG that generates inspiratory movements through a glutamatergic subpopulation of  
 1375 neurons which are functionally coupled with glutamatergic chemosensory neurons such  
 1376 as the RTN neurons. Here, targeted knockdown of *Nalcn* in the CO<sub>2</sub>/H<sup>+</sup>-sensitive neurons  
 1377 of the mouse RTN (70) demonstrated that *Nalcn* contributes to a background Na<sup>+</sup> current  
 1378 in these neurons, and is required for their activation by the neuropeptide substance P but  
 1379 not serotonin. Further, RTN-specific depletion of *Nalcn in vivo* constrained CO<sub>2</sub>-induced  
 1380 activation of RTN neurons as inferred from activity-dependent *c-fos* expression after  
 1381 exposure to 8% CO<sub>2</sub>, and also blunted the stimulation of the respiratory responses to CO<sub>2</sub>  
 1382 (70, 81). In another study, specific depletion of *Nalcn* in mouse glutamatergic, GABAergic,  
 1383 cholinergic, serotonergic, and glycinergic neurons was performed using a Cre/lox  
 1384 approach (80). In glutamatergic and GABAergic neuron knockout mice, this resulted in  
 1385 full lethality (*i.e.*, 100%) and partial lethality (*i.e.*, 13.2%) within 24h after birth,  
 1386 respectively. Furthermore, while specific depletion of *Nalcn* in glutamatergic RTN  
 1387 neurons did not result in lethality, it did so (partially) in glutamatergic neurons of the  
 1388 preBötC with 32.6% neonatal lethality attributed to respiratory defects. 28.6% of the  
 1389 knockout newborns exhibited a significantly greater frequency of apnea than other  
 1390 newborns. Of note, it was hypothesized that the surviving knockout newborns might  
 1391 receive compensatory respiratory activity from other glutamatergic neurons (80). Patch-  
 1392 clamp recordings on glutamatergic RTN and preBötC neurons revealed a hyperpolarized  
 1393 RMP, a lower firing frequency, and a loss of excitatory modulation by substance P. Taken  
 1394 together, these data demonstrate an important involvement of NALCN in respiratory  
 1395 rhythm generation and its modulation by substance P. Of note, contrasting with results  
 1396 from Shi *et al*, 2016 (70) and Yang *et al*, 2020 (81) mentioned above, the stimulation of  
 1397 the *in vivo* respiratory responses to an increase in CO<sub>2</sub> is not impacted in mice bearing a  
 1398 *Nalcn* knockout in RTN neurons (80), a discrepancy that remains to be explained. In RTN  
 1399 neurons, NALCN is also required for the ventilatory response to CO<sub>2</sub> during anesthesia  
 1400 (81). Interestingly, like *Nalcn*, both *Unc-80* and *Unc-79* knock-out mice die shortly after  
 1401 birth (49, 137). Whereas *Unc-80* knockout animals also suffer from respiratory defects,  
 1402 the origin of mortality observed for *Unc-79* knockout mice is currently unknown.  
 1403

1404 Strengthening the role of NALCN in breathing function is the finding that NALCN  
 1405 channels can be activated by leptin. Leptin is a pleiotropic hormone produced by  
 1406 adipocytes. It is a master regulator of metabolism and, interestingly, of many other  
 1407 biological functions including breathing (167). A recent study identified NALCN as a major  
 1408 player in the leptin-mediated link between breathing and metabolism (73). In mice, leptin  
 1409 activates NALCN to depolarize a subgroup of LepRb (*i.e.*, long form of the leptin receptor)-  
 1410 expressing neurons of the tractus solitarius tract (NTS), distinguished by their expression  
 1411 of the glutamate transporter vGluT2 and the neuropeptide galanin. Selective knockout of  
 1412 *Nalcn* in mouse LepRb neurons causes an increase in spontaneous apneas and breathing  
 1413 irregularity as well as a decrease in CO<sub>2</sub> chemosensitivity (73). In addition, while wild-  
 1414 type mice fed with a high-fat diet for 3 weeks maintain constant minute ventilation,  
 1415 LepRb-selective *Nalcn* knockout mice show a marked depression in ventilation. LepRb  
 1416 NTS neurons are classified into two electrophysiologically distinct populations according  
 1417 to their somatic size referred to as type-1 (smaller neurons) and type-2 (larger neurons).  
 1418 Single-cell transcriptomics revealed a different molecular profile between these two  
 1419 populations of neurons. Leptin is able to depolarize type-1 NTS neurons (which uniquely  
 1420 express vGluT2 and galanin) but not type-2. Leptin-mediated depolarization is lost in  
 1421 LepRb-selective *Nalcn* knockout mice. Interestingly, type-2 neurons from these knockout  
 1422 mice have hyperpolarized resting potentials and increased membrane resistance  
 1423 consistent with depleted leak channels at the cell membrane. These results demonstrated  
 1424 that NALCN is tightly regulated by leptin in type-1 neurons while it conducts a Na<sup>+</sup>  
 1425 background conductance that facilitates spontaneous firing in type-2 neurons. The  
 1426 molecular underpinnings for this observed difference in NALCN function remain to be  
 1427 elucidated.

1428  
 1429 The importance of NALCN in regulating the respiratory rhythm appears to be  
 1430 conserved across species, as demonstrated by two studies using the pond snail *L. stagnalis*  
 1431 as an animal model (74, 140). The aerial respiration of *L. stagnalis* is controlled by a  
 1432 simple well-described respiratory CPG network consisting of three large identified  
 1433 neurons, including one intrinsic pacemaker neuron, the right pedal dorsal 1 (RPeD1), that  
 1434 initiates rCPG rhythmic activity, as well as the VD4 and IP3I neurons (168). The  
 1435 pacemaker neuron RPeD1 exhibits rhythmic activity characterized by intermittent action  
 1436 potential bursts. This rhythmic activity is fully inhibited upon RNA knockdown of NALCN  
 1437 (referred to as the U-type channel) (74). A computational model of RPeD1 activity based  
 1438 on recording measurements of the various conductances expressed in these neurons  
 1439 illustrated the high sensitivity of this neuron to changes in the Na<sup>+</sup> leak current (140).  
 1440 Lastly, *in vivo*, RNA knockdown of NALCN resulted in a reduced opening of the  
 1441 pneumostome (*i.e.*, breathing pore) reflecting reduced breathing activity (74).

1442

### 1443 3- Photic control of locomotion

1444

1445 In addition to rhythmic locomotor activity and respiration, NALCN is also involved in the  
 1446 photic control of locomotion in *D. melanogaster* (36, 38, 64, 65). Hypomorphic mutants of  
 1447 NALCN in *D. melanogaster* referred to as *Dmα1U<sup>na</sup>*, *Dmα1U<sup>har38</sup>* and *Dmα1U<sup>har85</sup>*, exhibit  
 1448 most of their activity at night in contrast to wild-type animals that exhibit most of their  
 1449 activity during the daytime (64). A strong decrease in the rhythmic amplitude of behavior  
 1450 is also observed in *Dmα1U<sup>na</sup>* mutant animals compared to wild-type animals when flies  
 1451 were put in a constant darkness (*i.e.*, DD). Western-blotting experiments of brain extracts  
 1452 obtained at different times within a 24-hour period did not reveal alterations in



1453 NA/Dm $\alpha$ 1U protein levels suggesting that expression of the channel protein is not under  
1454 circadian control, a finding which was confirmed at the mRNA level (169). Since there was  
1455 no difference in activity between wild-type and mutant animals maintained in DD, it  
1456 seems as though aberrant motor responses to photic input, despite normal clock function,  
1457 is a feature of NA/Dm $\alpha$ 1U mutants (64). In agreement, another study reported that neural  
1458 *na/Dm $\alpha$ 1U* RNAi knockdown induces a robust mutant phenotype characterized by a  
1459 significant reduction of morning activity as well as reduced anticipation of the “lights-off”  
1460 transition compared to control animals (141). Notably, an inverted day/night behavioral  
1461 pattern does not occur in knocked down flies, but significant reductions in activity are  
1462 observed during both “lights-on” and “lights-off” transitions. These observations are  
1463 phenocopied by *nlf-1* knockdown (141). There are several populations of pacemaker and  
1464 clock-expressing neurons identified in *D. melanogaster*: the lateral neurons (LN<sub>d</sub>, l-LN<sub>v</sub>, s-  
1465 LN<sub>v</sub>), the Dorsal Neurons (DN<sub>1</sub>, DN<sub>2</sub>, DN<sub>3</sub>), and the three cells in the posterior lateral brain  
1466 (lateral posterior neurons; LPN)(170). Notably, *na/Dm $\alpha$ 1U* is expressed in a subset of  
1467 these neurons (65). During a 12 hr light:12 hr dark conditions (*i.e.*, LD), *Dm $\alpha$ 1U<sup>na</sup>* mutants  
1468 exhibit an altered evening anticipation, timing of morning anticipation, and response to  
1469 lights on. Further genetic rescue experiments showed that *na/Dm $\alpha$ 1U* expression in all  
1470 lateral neuron groups and a subset of dorsal neurons is sufficient to promote evening  
1471 anticipation but fails to fully rescue the morning anticipation phenotype, indicating that  
1472 additional dorsal neuron expression may be required for wild-type morning behavior. It  
1473 was concluded that *na/Dm $\alpha$ 1U* expression within lateral neurons and a broad group of  
1474 dorsal neurons is required for complete wild-type behavior in LD conditions. As noted  
1475 above, *na/Dm $\alpha$ 1U* likely acts outside of the core molecular clock since the circadian  
1476 expression of the PERIOD protein is not different in a *Dm $\alpha$ 1U<sup>na</sup>* background compared to  
1477 wild-type animals.

1478  
1479 Loss-of-function mutants for the *unc-79* and *unc-80* subunits also displayed severe  
1480 defects in circadian locomotor rhythmicity in both LD and DD conditions which are  
1481 indistinguishable from *Dm $\alpha$ 1U<sup>na</sup>* mutant phenotypes (36). Like *na/Dm $\alpha$ 1U*, these defects  
1482 can be rescued by expression of the subunits in pacemaker neurons. Also similar, neither  
1483 *unc-79* nor *unc-80* display rhythmic expression at least in DN1p pacemaker neurons (38).  
1484 Nonetheless, patch-clamp recordings revealed that both DN1p and large-LN<sub>v</sub> neurons  
1485 from *Dm $\alpha$ 1U<sup>na</sup>* mutants have hyperpolarized membrane potentials and are  
1486 electrophysiologically silent compared to wild-type DN1p neurons (38). Furthermore,  
1487 these neurons no longer exhibit circadian regulation of membrane excitability and RMP,  
1488 associated with *na/Dm $\alpha$ 1U* function. A possible mechanism for altered NALCN activity,  
1489 without changes in channel protein expression could involve regulation by the NFL-1  
1490 subunit. Specifically, expression of *nlf-1/fam155* was found to be under circadian control,  
1491 and the CLOCK protein, a core component of the circadian transcriptional feedback loop,  
1492 binds the genetic locus of *nlf-1* in a rhythmic manner (171). *In vivo* knockdown of *nlf-*  
1493 *1/fam155* phenocopies cell excitability defects of DN1p neurons observed in *Dm $\alpha$ 1U<sup>na</sup>*  
1494 mutants. Indeed, Flourakis *et al*, 2015, found dramatic reductions in rhythmic strength in  
1495 DD and reduced anticipation of “lights-on” and “lights-off” transitions under LD  
1496 conditions (38). Conversely, *nlf-1/fam155* overexpression depolarized the membrane,  
1497 elevated the firing rate and cellular excitability, and resulted in increased NA/Dm $\alpha$ 1U  
1498 current density. It was concluded that *nlf-1/fam155* is rhythmic and mediates the  
1499 rhythmic activity of *na/Dm $\alpha$ 1U*, with the following model proposed: during the  
1500 morning/day, the Na<sup>+</sup> leak current mediated by NA/Dm $\alpha$ 1U is elevated while resting K<sup>+</sup>  
1501 currents are reduced, depolarizing cells and promoting sustained firing rates. During the

1502 evening/night, the Na<sup>+</sup> leak current is low and resting K<sup>+</sup> currents are elevated, thus  
 1503 hyperpolarizing the cell to decrease firing rate. The clock-controlled transcript *nlf-*  
 1504 *1/Fam155a* drives the rhythm of the NA/Dmα1U current, linking the core clock to ion  
 1505 channel activity. Notably, one study has shown contrasting results to the ones described  
 1506 above. Ghezzi *et al*, 2014, who used RNA extracted from whole *Drosophila* heads, failed to  
 1507 observe changes in the mRNA expression of *nlf-1* according to the circadian rhythm (141).  
 1508 Perhaps, this inconsistency is attributable to differences in cell/tissue sampling, where  
 1509 circadian expression of *nlf-1* is restricted to a specific subset of neurons. Nonetheless, this  
 1510 same study reported only weak effects on circadian (evening) behavior after knockdown  
 1511 of *nlf-1/fam155* knockdown on evening behavior (141).  
 1512

1513 Not yet determined is the mechanism by which the differentially expressed NLF-  
 1514 1/FAM155 subunit regulates NA/Dmα1U channel activity. Indeed, in *D. melanogaster*, the  
 1515 temperature-inducible knockdown of *na/Dmα1U*, *nlf-1/fam155* and *unc-79* showed that  
 1516 proteins produced during the development are stable and functional at least 5-7 days at  
 1517 the adult stage (172). The half-life of NA/Dmα1U is estimated at ~29 days. This pool of  
 1518 proteins is sufficient for rhythmic behavior in adults. This suggests that the complex is  
 1519 highly stable and it is tempting to speculate that the NALCN channelosome cycles between  
 1520 endocytic compartments and the plasma membrane through a NLF-1/FAM155-  
 1521 dependent mechanism during circadian cycles. However, it is difficult to reconcile the  
 1522 observed rhythmic expression of *Nlf-1/Fam155a* (38), with the noted observation that  
 1523 the NALCN channelosome, including the NLF-1/FAM155A subunit, is expressed early  
 1524 during development and endures well into adulthood to regulate circadian function.  
 1525

1526 A role for *Nalcn* in circadian rhythm-dependent locomotor activity in mammals has not  
 1527 been thoroughly established. However, as discussed below, NALCN contributes to sleep  
 1528 behavior in mice with implications for circadian behavior. Furthermore,  
 1529 electrophysiological recordings from organotypic slices from wild-type and CaMKIIα-  
 1530 Cre;*NALCN*<sup>fx/fx</sup> mice demonstrated that the NALCN current is under control of the  
 1531 circadian clock in suprachiasmatic pacemaker neurons (SCN) in the hypothalamus (38).  
 1532 However, this finding was recently challenged. Indeed, Yang *et al*, recently described that  
 1533 Na<sup>+</sup> leak current amplitudes are similar in daytime and nighttime SCN neurons and  
 1534 proposed a mechanism by which *Nalcn* modulates daily rhythms in the rates of  
 1535 spontaneous action potential firing of SCN neurons as a consequence of rhythmic changes  
 1536 in subthreshold K<sup>+</sup> currents (173). In this context, whether oscillations in NALCN activity  
 1537 regulated by the NLF-1/FAM155 subunit occurred in mammals as documented in *D.*  
 1538 *melanogaster*, is not clear. Whether *Nalcn* also contributes to circadian locomotory  
 1539 behavior, remains to be determined.  
 1540

#### 1541 4- Sleep/Rest-activity behavior

1542  
 1543 In mammals, *Nalcn* mRNA is highly expressed in several brainstem nuclei involved in REM  
 1544 sleep regulation, such as the ventrolateral periaqueductal grey, deep mesencephalic  
 1545 nucleus and sublaterodorsal nucleus, and its function has been implicated in sleep (69).  
 1546 Mammalian sleep is a swiftly reversible state of decreased metabolism, responsiveness  
 1547 and, motor activity, which is broadly categorized into two states: a non-rapid-eye-  
 1548 movement sleep (NREM sleep also referred to as deep sleep) and rapid-eye-movement  
 1549 sleep (REM sleep also known as paradoxical sleep or dream sleep)(174, 175). Both states  
 1550 are defined by characteristic activity of electroencephalogram (EEG) and electromyogram

1551 (EMG). A large forward-genetic screen in mice (*i.e.*, >8000 animals) aiming to identify  
 1552 genes involved in sleep was carried out using ENU to introduce random point mutations.  
 1553 This approach led to the identification of two groups of mice with variations in sleep  
 1554 patterns (69). One of these, dubbed *Dreamless*, showed a reduction in the total time spent  
 1555 in REM sleep (~44%), and in the duration of REM bouts. Subsequent genome sequencing  
 1556 revealed that the mutation in *Dreamless* mice affected *Nalcn* by inducing a missense  
 1557 mutation of p.N315K, within the 6th transmembrane segment of Domain I of NALCN.  
 1558 Functional expression of p.N315K NALCN in HEK-293T cells showed a gain-of-function  
 1559 effect, while deep mesencephalic nucleus (DpMe) neurons, which are REM-inhibiting  
 1560 neurons, exhibited a depolarized RMP and an increased action potential firing frequency  
 1561 (FIGURE 8)(69). *Dreamless* mice have normal circadian period lengths but exhibit a  
 1562 greatly reduced amplitude of behavioral circadian rhythms under DD. A marked  
 1563 reduction in the REM sleep time of *Dreamless* mice is also apparent under DD. Spectral  
 1564 analysis of EEG data showed a decrease in the theta-range power during both NREM sleep  
 1565 and REM sleep and an increase in low-frequency power during the wake state and REM  
 1566 sleep in *Dreamless* mice, suggesting that NALCN may regulate various oscillations in the  
 1567 brain.

1568  
 1569 A role for NALCN in regulating rest-activity behavior is also documented in  
 1570 invertebrates (FIGURE 9). In *D. melanogaster*, both *Dm $\alpha$ 1U<sup>na</sup>* and *unc-79* mutants exhibit  
 1571 highly fragmented bouts of waking and rest-activity behavior (143). A more recent study  
 1572 in this species identified a wake-promoting role for *unc-79* (142). Here, a genetic screen  
 1573 to identify genetic regulators of both rest-activity behavior and metabolic function  
 1574 delineated several candidate genes including *unc-79*. Flies with ubiquitous knockdown or  
 1575 knockout of *unc-79* exhibit significantly more rest-activity than control flies and  
 1576 resistance to rest suppression induced by starvation, suggesting that *unc-79* is required  
 1577 for metabolic regulation of rest behavior. These phenotypes do not likely depend on  
 1578 NALCN function in circadian activity as *unc-79* knockdown in circadian neurons does not  
 1579 affect sleep and starvation resistance. By contrast, a specific knockdown in the mushroom  
 1580 body, a brain region associated with sleep regulation as well as olfactory learning and  
 1581 memory, largely phenocopies ubiquitous knockdown or knockout of *unc-79*.  
 1582 Interestingly, knockdown of *na/Dm $\alpha$ 1U* or *unc-80* throughout the mushroom body does  
 1583 not increase rest-activity behavior duration or disrupt starvation-induced rest  
 1584 suppression, and has little effect on starvation resistance. Together, these findings raise  
 1585 the possibility that *unc-79* functions independently of its canonical complex with *unc-80*  
 1586 and *na/Dm $\alpha$ 1U* to regulate rest-activity behavior and starvation resistances.

1587  
 1588 Another study reported a role for NCA-1 in regulating rest-activity behavior in *C.*  
 1589 *elegans* (144). Rest bouts during L4-to-Adult (L4/A) lethargus are almost fully abolished  
 1590 in animals carrying a gain-of-function *nca-1* mutation. However, animals carrying loss-of-  
 1591 function mutations for both *nca-1* and *nca-2* spend normal amounts of time in rest bouts  
 1592 during L4/A lethargus compared to wild-type animals, and rest/motion bout timing and  
 1593 duration are also normal. *Unc-79* loss-of-function animals exhibit a small increase in total  
 1594 rest time while *unc-80* loss-of-function animals do not. However, loss-of-function alleles  
 1595 of *nca-1/nca-2*, *unc-79* and *unc-80* cause increased latency to respond to an arousing blue  
 1596 light stimulus during bouts of rest. Instead, no changes are evident in L4/A lethargus rest  
 1597 quantity, or arousal thresholds for rest or motion bouts in *nlf-1* loss-of-functions animals.

1598  
 1599

## 5- Myometrial activity during labor

Beyond roles in neurons, the NALCN channelosome is also described as an important player in the rhythmic contraction of the uterus during labor (60, 79, 104, 138). This rhythmic activity is under the control of myometrial smooth muscle cells (MSMCs). Before the labor, MSCMs exhibit a RMP of -75 mV and this potential becomes depolarized to -50 mV during labor (176). In this context, MSMCs exhibit a phasic and regenerative depolarization and repolarization of the membrane resulting in rhythmic spontaneous contractions (177). RNA sequencing and specific non-quantitative RT-PCR data from human myometrial tissues revealed the expression of the NALCN channelosome suggesting it could play a role in the rhythmic contraction of the uterus (79, 178). In mouse, the *Nalcn* mRNA is detected in the uterus of non-pregnant animals and all pregnant stages without any significant differences in expression levels (138). However, Western blotting revealed that NALCN levels in the uterus decrease in mid-pregnancy (P14, P18, P19) compared to early pregnancy (P7, P10), and then increase during labor and remain high in the early postpartum period (138). It is not clear why there is no difference in RNA level between P7 to P19 while the protein expression level decreases, perhaps involving post-translational regulation of NALCN expression. *Nalcn* expression at the transcriptional level is be modulated by both progesterone (P4), a proquiescent hormone and estrogen (E2), a procontractile hormone (60, 179). Indeed, using the human myometrial HM6ERMS2 cell line as a cell model, quantitative RT-PCR revealed a 2.3-fold decrease in *NALCN* mRNA expression when cells were treated with E2 while a 5.6-fold increase was observed in cells treated with P4+E2 (60). The use of specific estrogen receptor (ER) and progesterone receptor (PR) antagonists showed these receptors are involved in the transcriptional regulation of *NALCN* induced by the E2 and P4 hormones, respectively. At the protein level, Western blotting experiments confirmed the negative regulation of NALCN expression by E2. However, there was no significant upregulation of NALCN in cells exposed to both P4+E2 compared to control cells, although the negative regulation of E2 was lost. The positive regulation of *NALCN* mRNA transcription by progesterone was found to involve three progesterone responsive elements (PRE) identified in the *NALCN* promoter. Notably however, negative regulation of *NALCN* mRNA expression does not involve an identified estrogen responsive element (ERE) in the *NALCN* promoter since mutations of this element did not impact the inhibition of *NALCN* transcription by E4. This suggests that E2 and ER $\alpha$  work through a separate mechanism to modulate *NALCN* transcription. Of note, the identified ERE sequence overlaps with one of the PRE sequences. For an unknown reason, all these data contrast with Western blotting experiments of mouse uterus at different stages, which instead suggest that NALCN protein levels are downregulated by P4 and upregulated by E2 (138). A temporal analysis of NALCN expression and hormone concentrations would be of interest to explain the species-specific differences in hormonal regulation of NALCN expression.

At the functional level, a Na<sup>+</sup> leak current with sensitivity to Gd<sup>3+</sup> in the micromolar range is reported in MCSCs from pregnant rats, the human myometrial HM6ERMS2 cell line, as well as freshly isolated and cultured MCSCs from pregnant women (60, 79, 180). Knockdown of *NALCN* in human MCSCs indicated the leak Na<sup>+</sup> current is, at least in part (~50%), attributable to NALCN (79). Acetylcholine and substance P, which are known to positively modulate NALCN, also increase uterine contractility (181, 182). The relevance of NALCN being involved in parturition was explored in a mouse model in which *Nalcn* expression was specifically deleted in smooth muscle cells using the Cre/lox system

1649 (smMHC<sup>Cre-eGFP</sup> x NALCN<sup>fx/fx</sup>)(138). SmNALCN<sup>-/-</sup> and smNALCN<sup>+/-</sup> mice experience a  
 1650 higher rate of abnormal labor (*i.e.*, prolonged labor, delayed labor, dysfunctional  
 1651 parturition) of ~40 and ~60%, respectively compared to control animals with only ~20%.  
 1652 In addition, these mice produce smaller litter sizes and their labor has a longer duration.  
 1653 Further, smNALCN<sup>-/-</sup> mice have larger pups than control animals. An examination of  
 1654 myometrial electrical activity at P19 revealed no difference neither in burst interval or  
 1655 spike density but a significant decrease in burst duration and the number of spikes/burst  
 1656 in smNALCN<sup>-/-</sup> mice. It was concluded that NALCN is important for sustaining burst  
 1657 duration in the myometrium and is necessary for successful delivery (138). The  
 1658 involvement of NALCN in reproduction appears to be of importance also in *D.*  
 1659 *melanogaster*, as loss-of-function Dm $\alpha$ 1U<sup>har38</sup> and Dm $\alpha$ 1U<sup>85</sup> animals produce  
 1660 considerably fewer offspring than wild-type flies (132).

1661  
 1662 A more recent study identified a functional coupling between the Na<sup>+</sup>-activated K<sup>+</sup>  
 1663 channel SLO2.1 and NALCN for the regulation of human MSMCs membrane potential,  
 1664 excitability and uterine contractility (104). Indeed, the use of two non-specific inhibitors  
 1665 of NALCN (*i.e.*, 10  $\mu$ M Gd<sup>3+</sup> and 50  $\mu$ M CP96345,) uncovered that Na<sup>+</sup> influx into the  
 1666 cytoplasm of myometrial smooth muscle cells, working as a cytoplasmic messenger,  
 1667 activates SLO2.1 to induce K<sup>+</sup> efflux resulting in hyperpolarization of the membrane.  
 1668 Conversely, a decrease in this SLO2.1/NALCN functional coupling induces a membrane  
 1669 depolarization which triggers Ca<sup>2+</sup> through Cav channels to promote contractions. The  
 1670 functional coupling between the SLO2.1 and NALCN likely involves the co-inclusion of the  
 1671 two channels in the same protein complex, as revealed by proximity ligation assays (PLA)  
 1672 in both human MSMCs and the related cell line hTERT. However, the existence of a  
 1673 physical complex remains to be confirmed with other approaches such as co-  
 1674 immunoprecipitation and FRET/BRET studies. Notably, SLO2.1 is also known to form a  
 1675 functional complex with Na<sub>v</sub> channels in neurons (183, 184). In sum, the proposed model  
 1676 for the role of NALCN in MSMC contractility is the following: rather than acting to  
 1677 depolarize RMP, NALCN causes hyperpolarization of myometrial smooth muscle cells  
 1678 through its functional interaction with SLO2.1. Consistent with this idea, NALCN conducts  
 1679 about 50% of the Na<sup>+</sup> leak current at the membrane potential in human MSMCs (79). If  
 1680 NALCN was involved in setting pacemaker activity, cells lacking NALCN should have a  
 1681 disrupted inter-burst action potential frequency. Instead, MSMCs from NALCN knockout  
 1682 mice (at day 19 of pregnancy) only show a significant reduction in burst duration, but no  
 1683 significant effect on inter-burst frequency, indicating the channel does not contribute to  
 1684 pacemaking in these cells (**FIGURE 8**)(138). Clearly, an interesting prospect that should  
 1685 be investigated is whether SLO2.1 and NALCN are also functionally coupled in the nervous  
 1686 system. Taken together, these data suggest that during quiescence, progesterone serves  
 1687 to induce NALCN expression and activity. As a result, Na<sup>+</sup> current through NALCN  
 1688 activates SLO2.1, leading to membrane hyperpolarization (**FIGURE 7**). In these  
 1689 conditions, L-type voltage-gated Ca<sup>2+</sup> channels are closed and uterine contractions do not  
 1690 occur. Instead, in the contractile state, NALCN expression is inhibited by estrogen leading  
 1691 to a decrease in SLO2.1 activity, depolarizing the membrane resulting in activation of L-  
 1692 type Cav channels that drive uterine contractility.

## 1693 1694 **6- Pacemaker activity in the intestine**

1695  
 1696 NALCN is involved in regulating the pacemaking activity of Interstitial cells of Cajal  
 1697 (ICCs). ICCs are pacemaking cells in gastrointestinal (GI) muscles that generate rhythmic

1698 oscillations of the RMP known as slow waves (185). In mice, the pacemaker activity of  
 1699 ICCs is modulated by substance P (SP) through the activation of a non-specific cation  
 1700 channel (78, 186, 187). Here, SP induces a concentration-dependent depolarization of the  
 1701 membrane that is prevented by application of SP receptor antagonists (78), one of which,  
 1702 notably, is also a potent blocker of NALCN (*i.e.*, L703706; *see section above on NALCN*  
 1703 *pharmacology*) (89). In this study, NALCN expression was detected at both the RNA and  
 1704 protein levels in ICCs (78). The SP-dependent depolarization was prevented by  
 1705 application of external  $Gd^{3+}$  or by decreasing extracellular  $Na^+$ . Application of the  
 1706 constitutive G-protein antagonist GDP- $\beta$ -S and the agonist GTP- $\gamma$ -S did not affect SP-  
 1707 induced depolarizations, while bath application of the phosphotyrosine kinase inhibitor  
 1708 genistein or PP1 inhibited them. Conversely, intracellular dialysis of a Src Family Kinase  
 1709 (SFK) was found to mimic the SP-induced depolarization, as did activation of the  
 1710 neurotensin (NT) receptor (**FIGURE 7**)(78). Taken together, these data strongly suggest  
 1711 that NALCN is involved in the SP- and NT-induced depolarization in ICCs. ICCs of *Nalcn*  
 1712 knockout mice exhibit attenuated SP-induced depolarization (~75%), and an almost fully  
 1713 abolished inward current at -60 mV. The remaining contribution to the SP-induced  
 1714 depolarization (~25%) is attributed to TRPC channels. The physiological relevance of  
 1715 these findings remains to be confirmed in animal models in which NALCN expression can  
 1716 be selectively manipulated in ICCs, for example by crossing the available *Nalcn*<sup>fx/fx</sup> allele  
 1717 with a c-Kit<sup>CreERT2</sup> knock-in allele to specifically target ICCs (188).

1718  
 1719 In light of the implication that the NALCN channelosome contributes to digestive  
 1720 system physiology, a genome wide association study (GWAS) was done in dogs, combined  
 1721 with whole genome sequencing (WGS) of a subset of individuals, identifying a possible  
 1722 association between *NALCN* and canine gastric dilatation-volvulus (GDV)(189). GDV is  
 1723 characterized by rotation of the stomach around its axis, trapping air within the gastric  
 1724 lumen and increasing intragastric pressure. Dogs with GDV exhibits abnormalities in both  
 1725 gastrointestinal and gastric motilities (190).

1726

## 1727 7- Pain

1728

1729 Several studies have identified a role for NALCN in pain signaling and sensory processing  
 1730 in both mammals and invertebrates (61, 62, 71, 88, 145, 191).

1731

### 1732 *Ascending pain signaling*

1733

1734 A large-scale screen to search for genes that enriched in the dorsal spinal cord compared  
 1735 to the ventral cord in rat using DNA microarray analysis revealed that *Nalcn* mRNA is 6.9-  
 1736 fold more expressed in the dorsal spinal cord (191). This result was confirmed by  
 1737 quantitative RT-PCR, although the fold increase was lower than observed in the DNA  
 1738 microarray analysis. Accordingly, NALCN is described as an important player in the  
 1739 excitability of spino-parabrachial neurons (SPN)(71), which convey nociceptive signals  
 1740 from the dorsal horn to multiple brain regions. This includes the parabrachial (PB)  
 1741 nucleus, a cell grouping that surrounds the superior cerebellar peduncles in the  
 1742 dorsolateral pons acting as a sensory hub for pain signaling (192). The firing of SPNs is  
 1743 strongly controlled by neuromodulators released from primary afferent C fibers,  
 1744 including SP. SPN neurons express a  $Gd^{3+}$ -sensitive  $Na^+$  leak current with biophysical  
 1745 properties consistent with those of NALCN (71). Furthermore, application of  $Gd^{3+}$   
 1746 significantly hyperpolarizes their membrane, decreasing their firing frequency, and

1747 increasing the threshold for excitation. NALCN function in this cell type was confirmed by  
 1748 disrupting its expression in the PB nucleus of the mouse brain. Additionally, application  
 1749 of the selective NK1R antagonist SR140333 blocked SP-evoked transient inward current  
 1750 in SPN cells, which like NALCN, was sensitive to block by  $Gd^{3+}$  but insensitive to GDP- $\beta$ -S.  
 1751 However, this current was inhibited by the src kinase inhibitor PP2, whereas NALCN is  
 1752 insensitive to the similar compound PP1 *in vitro* (see section above on NALCN  
 1753 pharmacology). Nonetheless, SP significantly depolarizes the membrane of SPNs and  
 1754 increases the firing frequency in wild type mice, but has no significant effect on the  
 1755 membrane potential of SPNs from NALCN knockout animals (71). Altogether, NALCN  
 1756 seems to be a crucial player in regulating the excitability of SPNs, implicating the channel  
 1757 in ascending pain signaling to the brain.

1758

### 1759 *Neuropathic pain*

1760

1761 A recent study found a significant increase in *Nalcn* mRNA, potentially through a cAMP-  
 1762 PKA-dependent pathway, after chronic constriction injury of the hind limb (CCI) in adult  
 1763 rats, both in dorsal root ganglia (DRG) neurons and the spinal cord (61).  
 1764 Immunocytochemistry revealed that the NALCN protein is widely expressed in nearly all  
 1765 DRG neurons. Specifically, NALCN is expressed in DRG neurons that co-labeled with  
 1766 neurofilament (NF200, A-fibers), transient receptor potential cation channel subfamily V  
 1767 member 1 (TRPV1, noxious receptor), isolectin B4 (IB4, non-peptidergic neurons), and  
 1768 peptidergic SP neurons. The percentage of DRG neurons that co-express NALCN and SP is  
 1769 greater after CCI. Broad NALCN protein expression is also evident in the spinal cord. An  
 1770 increase in a  $Gd^{3+}$ -sensitive and  $Na^+$ -dependent background conductance following CCI is  
 1771 documented in both DRG and spinal cord neurons. This current is potentiated by  
 1772 application of SP. A knockdown approach demonstrated that: (i) this current is mediated  
 1773 by NALCN, (ii) increased expression of NALCN contributes to the hyperactivity of both  
 1774 DRG and spinal cords neurons after CCI in rats, and (iii) *Nalcn* knockdown alleviates the  
 1775 mechanical allodynia and thermal hyperalgesia after CCI. *Nalcn* knockdown also reduces  
 1776 SP-induced pain behaviors in adult rats. Notably, the involvement of NALCN in the CCI-  
 1777 induced mechanical allodynia and thermal hyperalgesia appears to be conserved at least  
 1778 among rodents, being also observed in mice. Indeed, conditional knockout of *Nalcn*  
 1779 completely prevents mechanical allodynia and thermal hyperalgesia in adult mice. These  
 1780 results strongly suggest that NALCN contributes to the initiation and maintenance of  
 1781 neuronal sensitization in neuropathic pain. Thus, NALCN could represent a viable  
 1782 therapeutic target for the treatment neuropathic pain. This is especially relevant since  
 1783 effective treatment for neuropathic pain is currently unavailable because of an imprecise  
 1784 understanding of its etiology (193, 194). Indeed, general sensory functions of rats and  
 1785 mice are not altered by *Nalcn* knockdown/knockout, suggesting that targeting NALCN  
 1786 could be valuable to treat neuropathic pain without altering normal sensation. Lastly,  
 1787 transcriptomic data from Sun *et al*, 2002, showed that *Nalcn* mRNA is not upregulated in  
 1788 the dorsal horn of a chronic neuropathic pain model, suggesting that in this case, NALCN  
 1789 is not playing an active role in pain development (191).

1790

### 1791 *Inflammatory pain*

1792

1793 In addition to neuropathic pain, the same laboratory identified a role for NALCN in  
 1794 inflammatory pain (62). Specifically, the same *Nalcn* knockdown approach mentioned  
 1795 above was applied to a rat model of inflammatory pain induced by complete Freund's

1796 adjuvant (CFA) injection in the left footpad. A significant increase in *Nalcn* mRNA  
1797 expression, as well as SP, was detected in both DRG neurons and the spinal cord 2 hours  
1798 following CFA injection, lasting up to 8 hours (DRG) or 3 days (spinal cord). A significant  
1799 increase of NALCN protein was also observed, concurrent with an increase in the current  
1800 density of a  $Gd^{3+}$ -sensitive,  $Na^+$ -dependent background conductance in small- and  
1801 medium-sized DRG neurons from adult and neonatal animals. In contrast, increased  
1802 current density was not observed upon downregulation of *Nalcn* expression. As expected,  
1803 small- and medium-sized DRG neurons in both adult and neonatal CFA-animals exhibit a  
1804 hyperactive electrical activity (*i.e.*, a depolarized RMP, an increased excitation threshold,  
1805 and an increased action potential frequency), which is absent in siRNA-treated animals.  
1806 As expected, knockdown of *Nalcn* in the DRG neurons and the spinal cord reduces both  
1807 SP- and CFA-induced pain behaviors. Like neuropathic pain, this study suggests that  
1808 NALCN could be a therapeutic target to treat inflammatory pain.

1809

1810 *Chemical nociception*

1811

1812 In addition to neuropathic and inflammatory pain, NALCN is also proposed to be involved  
1813 in chemical nociception in the highveld mole-rat (88). This study aimed at characterizing  
1814 algogen/algiesiogenic-driven behaviors in several African rodent species using a  
1815 combination of techniques including next-generation RNA sequencing and *de novo*  
1816 transcriptome assembly, gene expression analysis, and functional experiments. One  
1817 species, the highveld mole-rat (*Cryptomys hottentotus pretoriae*) is completely insensitive  
1818 to allyl isothiocyanate (AITC). When comparing gene expression levels of this species to  
1819 others, only the *Nalcn* mRNA was found to be significantly up-regulated, specifically in the  
1820 spinal cord with levels more than six-fold higher than all other species. It was thus  
1821 postulated that the overexpression of NALCN in sensory neurons could contribute to AITC  
1822 insensitivity. In this context, the presence of increased numbers of NALCN channels at  
1823 nociceptor terminals would induce a depolarized membrane, preventing initiation of  
1824 action potential firing by TRPA1 by promoting the inactivation of  $Na_v$  channels (**FIGURE**  
1825 **9**). Consistent with this idea, pain behaviors of verapamil-treated highveld mole-rats were  
1826 increased to resemble those of all other rodents. However, since verapamil is not a NALCN  
1827 specific blocker, the use of specific inhibitors, when available, as well as molecular  
1828 techniques such as *in vivo*-mediated knockdown in sensory neurons, will be required to  
1829 definitively prove that NALCN is involved in chemical nociception in this species, as well  
1830 as other rodents and humans.

1831

1832 *Thermal nociception*

1833

1834 In addition to the role of NALCN in ascending nociceptive transmission highlighted by  
1835 Ford *et al*, 2018 (71), a role for NALCN in primary nociceptors has also been described. In  
1836 *C. elegans* FLP neurons, the NALCN orthologues NCA-1/NCA-2 were shown to contribute  
1837 to thermonociception (**FIGURE 9**)(145). FLP neurons are polymodal nociceptors capable  
1838 of detecting high temperatures and harsh touch (195). They exhibit an arborized  
1839 morphology like peripheral polymodal sensory neurons in vertebrates (196). Using null  
1840 mutants of ion channels, the authors of this study discovered that TRPV channels (OSM-9  
1841 and OCR-2), the  $Ca_v$  channels L-, N-, but not T-type (*i.e.*, EGL-19, UNC-2), as well as NCA-  
1842 1 and NCA-2, play a role in FLP activation. In addition, data suggested that NCA-1 controls  
1843 FLP thermal sensitivity, whereas NCA-2 plays a role in thermal sensitivity, sensory gain  
1844 maintenance, and signal kinetics during thermal stimuli termination (145). Of interest,



1845 *nca-2* animals who display slow kinetics of cytoplasmic  $[Ca^{2+}]$  decrease in FLP upon  
 1846 stimulus cessation also present a delayed decrease in reversal rate in the post-stimulus  
 1847 period. The mechanism involved in the NCA-1- and NCA-2-mediated FLP activation  
 1848 remains to be investigated. It is also of importance to determine whether this mechanism  
 1849 is conserved in mammals.

1850

## 1851 **8- Other functions**

1852

### 1853 **a) Metabolism/Weight/Hormone secretion**

1854

1855 Male and female *Lightweight* (*Lwt*)/+ and heterozygous *Unc-79* mice weigh less and are  
 1856 smaller than their wild-type littermates (47, 48). Monitoring food and water consumption  
 1857 for 19 days starting at post-natal day 80 (P80), followed by body composition analysis,  
 1858 revealed that *Lwt*/+ mice consume similar amounts of food and water, yet accumulate  
 1859 less fat than wild-type animals. Mutant specimens with similar weights as wild-type mice  
 1860 consumed significantly more food on a gram per kilogram basis, and on average the body  
 1861 of *Lwt*/+ animals consist of a higher proportion of lean mass and less body fat. These  
 1862 results suggest a higher metabolic rate, an increase in protein turnover, and/or a  
 1863 difference in energy usage compared to wild-type animals. Interestingly, a few *Unc-79*  
 1864 knockout pups can survive beyond 24 hours after birth, sometimes until P7. Although  
 1865 these pups are active soon after birth, after half a day, they appear weak and their  
 1866 movements decrease gradually presumably due to an observed inability to nurse and  
 1867 consume milk (137).

1868

1869 Supporting a role of the NALCN channelosome in metabolism, a genome-wide  
 1870 association study in human found an association between FAM155A and circulating  
 1871 fibroblast growth factor 21 (FGF21)(197). FGF21 is an evolutionarily conserved factor  
 1872 that plays important roles in metabolic homeostasis (198).

1873

1874 In the same vein, the expression of NALCN (either at the RNA and/or protein level)  
 1875 in endocrine/neuroendocrine tissues suggests that NALCN could contribute to hormone  
 1876 secretion (56, 76, 84, 199). At rest, a  $Na^+$  leak conductance which shares common  
 1877 biophysical properties with the NALCN current is present in endocrine cells of the  
 1878 anterior pituitary, pancreatic beta cells, and adrenomedullary chromaffin cells (12).  
 1879 Interestingly, this conductance mainly operates at resting membrane potential and thus  
 1880 contributes to regulate RMP and action potential discharges, which in turn trigger  
 1881 increases in cytosolic  $Ca^{2+}$  and exocytosis. It therefore seems reasonable to suggest that  
 1882 NALCN contributes to hormone secretion, a question that remains open for future  
 1883 investigation.

1884

### 1885 **b) Osmoregulation**

1886

1887 A role of NALCN in osmoregulation is also described (146). Haplotype association  
 1888 mapping (HAM) on aging groups of mice from 27 different strains was carried out to  
 1889 identify novel genes involved in the regulation of water homeostasis. Although this HAM  
 1890 analysis did not identify any peaks above the stringent significance threshold of  $\alpha = 0.05$ ,  
 1891 several peaks above the suggestive threshold were identified, with the strongest mapping  
 1892 to a genetic locus containing the distal part of the NALCN channel coding sequence. The  
 1893 involvement of NALCN in regulating levels of  $Na^+$  in blood serum was then confirmed in

1894 mice harboring a heterozygous *Nalcn* knockout allele (since homozygous *Nalcn* knockout  
 1895 mice die within 24 h after birth). At 12 weeks of age, heterozygous mice were found to  
 1896 display small but significantly higher serum Na<sup>+</sup> concentrations compared to wild-type  
 1897 mice (146). Supporting a role of NALCN in osmoregulation, a study aiming to investigate  
 1898 genomic regions associated with Na<sup>+</sup> intake, K<sup>+</sup> intake, and Na<sup>+</sup> -to- K<sup>+</sup> ratio measured  
 1899 from 24-h or half-day urine samples revealed a possible association between NALCN and  
 1900 potassium excretion in humans (200). How NALCN impacts serum Na<sup>+</sup> levels, as well as  
 1901 K<sup>+</sup> excretion, remains to be determined. A better understanding of the cell types that  
 1902 express *Nalcn*, combined with the recent development of conditional knockout mouse  
 1903 models will be useful in this context (38, 71-73, 80). This will allow the determination of  
 1904 whether NALCN plays a role in physiological processes that are known to influence ionic  
 1905 homeostasis. This includes, for example, the potential role of NALCN in regulating heart  
 1906 rhythm, in the neuronal arginine vasopressin (AVP) release and thirst sensation, and in  
 1907 adrenocortical aldosterone release.

### 1908 **c) Sensitivity to ethanol and volatile anesthetics**

1909 A large set of studies reported the involvement of NALCN in the sensitivity to volatile  
 1910 anesthetics in both *C. elegans* and *D. melanogaster* (33, 129, 132, 133, 139, 147-  
 1911 155)(TABLE 3; FIGURE 8). General anesthetics are known to depress fast excitatory and  
 1912 enhance fast inhibitory synaptic transmission mediated primarily by glutamate and  
 1913 GABA, respectively (201, 202). Several ion channels are proposed to contribute to the  
 1914 anesthetized state by inhaled anesthetics. This includes several types of K<sup>+</sup> channels (*e.g.*,  
 1915 K<sub>v</sub>, K<sub>2P</sub>), HCN, Na<sub>v</sub>, GABA-A, NMDA, nicotinic acetylcholine receptors and glycine receptors  
 1916 (201, 203-206). Stemming from combined observations that the NALCN channelosome is  
 1917 a crucial regulator of neuronal excitability, and is involved in anesthesia by inhaled  
 1918 anesthetics in *C. elegans* and *D. melanogaster* (*see below*), researchers sought to examine  
 1919 whether NALCN could also be involved in inhaled anesthetics responses in mammals (77).  
 1920 This work revealed that isoflurane enhances NALCN conductance at sub-anesthetic  
 1921 concentration and *Nalcn* knockdown reduces behavioral hyperactivity after isoflurane  
 1922 induction. In mouse CA3 hippocampal neurons that express NALCN, isoflurane at sub-  
 1923 anesthetic concentrations increases the firing rate and depolarizes the membrane while  
 1924 isoflurane at anesthetic concentrations decreases the firing rate and hyperpolarizes the  
 1925 neurons. Within both concentration ranges, a Na<sup>+</sup> leak current is increased at -60 mV. *In*  
 1926 *vivo* knockdown of *Nalcn* revealed that isoflurane-induced hyperactivity during  
 1927 anesthesia induction is reduced in NALCN knockdown animals compared to wild-type  
 1928 counterparts. Because isoflurane inhibits Na<sub>v</sub> channels at anesthetic concentration, but  
 1929 not at sub-anesthetic concentrations, it was concluded that isoflurane bidirectionally  
 1930 modulates the excitability of hippocampal CA3 pyramidal neurons, by either enhancing  
 1931 NALCN current to induce hyperexcitability, or by inhibiting Na<sub>v</sub> channels to induce  
 1932 hypoexcitability. The same laboratory that reported these findings also identified a  
 1933 NALCN-mediated increase in neuronal activity by isoflurane, as well as sevoflurane but  
 1934 not propofol in retrotrapezoid nucleus neurons (81). However, contrasting with CA3  
 1935 hippocampal neurons, isoflurane at anesthetic concentrations does not decrease the  
 1936 firing rate of RTN neurons (81). Importantly, isoflurane has no effect on NALCN current  
 1937 when expressed in HEK-293T cells (77), likely because these cells lack other factors  
 1938 involved in NALCN sensitivity to isoflurane. Indeed, it is possible that the configuration  
 1939 used for NALCN expression in these experiments (in HEK-293T cells without accessory  
 1940 subunits) may not have been suitable for detecting isoflurane effects on NALCN currents.

1943 Overall, this study suggested that NALCN is not involved in promoting the anesthetic state.  
 1944 This contrasts with published data showing that *Lightweight* heterozygous mice (Lwt/+)   
 1945 with decreased level of *Unc-79* are resistant to anesthesia with isoflurane (47). Indeed,   
 1946 there is no apparent alteration in minimal alveolar concentration in response to   
 1947 halothane, cyclopropane, or sevoflurane in Lwt/+ mice. However, there *is* a significant   
 1948 resistance to isoflurane-induced anesthesia relative to wild-type animals. Consistent with   
 1949 NALCN contributing to the response to volatile anesthetics, an identified patient with a *de*   
 1950 *novo* gain-of-function mutation on the *NALCN* gene (p.A319V) was found to exhibit high   
 1951 sensitivity to sevoflurane anesthesia at usual dosage with severe respiratory depression   
 1952 culminating in cardio-respiratory arrest (27, 207). Further work is now needed to clarify   
 1953 the role of the NALCN channelosome in volatile anesthetic-mediated effects on neurons   
 1954 in mammals.

1955  
 1956 Previous studies performed In *C. elegans* showed *unc-79* and *unc-80* mutants are   
 1957 either moderately resistant to ethanol-induced immobility, or to exhibit a wild-type   
 1958 sensitivity in liquid media (150). These results contrast with other studies showing a   
 1959 pronounced hypersensitivity to ethanol in *unc-79*, *unc-80*, *nca-1*, *nca-2* and *nca-1:nca-2*   
 1960 double mutants when compared to wild-type N2 animals (47, 157). In mammals, a highly   
 1961 significant increase in the sensitivity to the acute sedative effects of ethanol is apparent   
 1962 for Lwt/+ mice as measured using righting reflex tests (47), despite blood ethanol levels   
 1963 remaining similar to wild-type littermates. Unlike wild-type animals that exhibit   
 1964 significant locomotor activation following ethanol injection, no such activation is   
 1965 observed in Lwt/+ mice. Also, Lwt/+ mice exhibit a higher preference for ethanol relative   
 1966 to wild-type mice, but no alteration in taste sensitivity for either consumption or   
 1967 preference for saccharin- and quinin-containing solutions over water (47). Taken   
 1968 together, these data showed that Lwt/+ mice have acute hypersensitivity to and increased   
 1969 voluntary consumption of ethanol. The mechanisms involved in alcohol hypersensitivity   
 1970 and preference by loss-of-function UNC-79 mutations (and possibly other NALCN   
 1971 subunits) remain to be established. Interestingly, ethanol induces dopamine release in rat   
 1972 nucleus accumbens (Nac) and dopamine is known to negatively modulate the NALCN   
 1973 channelosome through the D2 dopaminergic receptor (D2R)(72, 208). One may postulate   
 1974 that ethanol decreases the electrical activity of D2R-expressing neurons, an effect which   
 1975 would be partly attenuated if NALCN is rendered unfunctional or diminished in its   
 1976 activity.

1977  
 1978 Since many studies have highlighted the importance of the Nac in regulating the   
 1979 process of drug-induced locomotor sensitization, such as methamphetamine and ethanol   
 1980 (209-212), a recent study examined the hypothesis that NALCN in NAc may contribute to   
 1981 the ethanol-induced acute stimulant responses and locomotor sensitization in mouse   
 1982 (156). In the NAc, *Nalcn* mRNA (as well as *Unc-79* but not *Unc-80* mRNA) and NALCN   
 1983 protein levels increase after ethanol-induced locomotor sensitization. *In vivo* knockdown   
 1984 of *Nalcn* demonstrated that decreased NALCN expression alleviates both ethanol-induced   
 1985 acute stimulant responses and locomotor sensitization. The mechanisms involved remain   
 1986 to be elucidated and are likely complex since the acute effect is probably due to a direct   
 1987 or indirect functional effects on the NALCN channelosome, while the effect on the   
 1988 locomotor sensitization is likely due to effects on *Nalcn* gene expression.

1989  
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 1991

1992 **d) Social clustering**

1993

1994 In *D. melanogaster*, mutation of *na/Dmα1U* impacts several complex behaviors including  
 1995 social clustering (*i.e.*, the distance maintained between individuals) (158). *Dmα1U<sup>har38</sup>*  
 1996 hypomorphic mutants exhibit a significantly reduced social space index in several  
 1997 behavioral tests, and effect that can be either fully or partially restored by expressing  
 1998 *na/Dmα1U* in cholinergic neurons and glutamatergic neurons, respectively. Reduced  
 1999 social clustering is also observed when *na/Dmα1U* and *nlf-1* are knocked down (141).

2000

2001 **e) Development and morphology**

2002

2003 Adult *D. melanogaster* specimens with the genotypes *Dmα1U<sup>na</sup>*, *Dmα1U<sup>har38</sup>*, and *Dmα1U<sup>85</sup>*  
 2004 have subtle morphological phenotypes: they are noticeably smaller and their abdomens  
 2005 are slenderer and more elongated compared to wild-type flies (*i.e.*, hence the name  
 2006 narrow abdomen), implicating NALCN in development (64, 132). Yet to be determined is  
 2007 whether the NALCN channelosome contributes to development and morphology in  
 2008 mammals. Since mouse pups harboring *Nalcn*, *Unc-79* and *Unc-80* knockout lesions  
 2009 appear physically normal at birth but die shortly afterwards, assessment of abnormal  
 2010 morphology has not been possible during postnatal development (2, 47, 49, 137).  
 2011 However, it is notable that patients with pathogenic variants of *NALCN/UNC-80* exhibit  
 2012 numerous morphological alterations (*see below*). To date, no morphological alterations  
 2013 associated with NALCN disruption have been reported for *C. elegans*.

2014

2015 **f) Miscellaneous**

2016

2017 Western blotting of protein lysates from the brainstem nucleus tractus solitarius of a rat  
 2018 model of dysphagia after stroke revealed increased expression of NALCN and UNC-80  
 2019 proteins (159), suggesting NALCN could be involved in swallowing.

2020

2021 A comparative transcriptomic study between human supernumerary teeth  
 2022 derived stem cells (SNTSCs) and normal dental pulp stem cells (DPSCs) highlighted a total  
 2023 of 12,861 differentially expressed genes, including *NLF-1/FAM155A* which was found to  
 2024 be more highly expressed in SNTSCs compared to DPSCs (160). Here, the proliferative  
 2025 capacity of SNTSCs was found to be inhibited by *FAM155A* knockdown, while cell  
 2026 migration was promoted. Thus, *NLF-1/FAM155A* might contribute to cell proliferation  
 2027 and migration.

2028

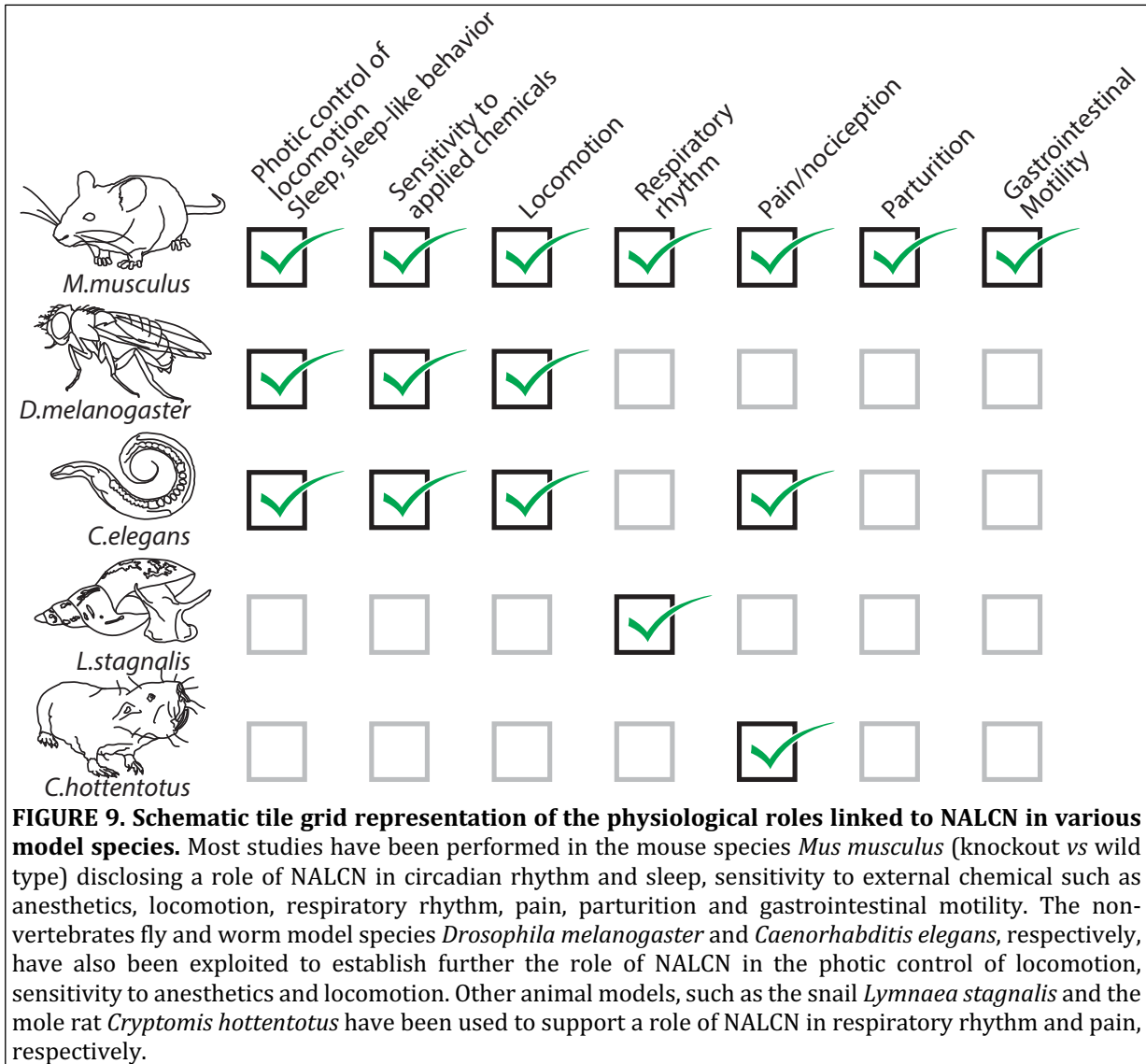
2029 A study investigating putative genome regions associated with adaptation to harsh  
 2030 and arid environments in native chickens revealed a putative involvement of *Nalcn* (161).  
 2031 Here, whole-genome sequencing of different chicken breeds living in different  
 2032 environmental conditions across China led to the identification of 161,322 SNPs located  
 2033 within coding regions of the chicken genome, including 46,063 non-synonymous SNPs,  
 2034 355 stop gain, 89 stop loss and 114,815 synonymous SNPs. SNP analysis highlighted the  
 2035 association of several genes in adaptation to harsh and arid environments including  
 2036 *NALCN*.

2037

2038 Another study investigating the genetic architecture of pigs subjected to breeding  
 2039 intensification (162) identified *Nalcn* as a possible locus involved in changes associated  
 2040 with the intensification of the selection process in pigs.

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More recently, a comparative transcriptome study aimed at identifying genes related to sexual differentiation and sterility of the sterile Mule duck compared to Jinding and Muscovi ducks revealed that *Nalcn* is part of a group of 8 genes involved in male sterility (163).



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To close this chapter on the various physiological functions of the NALCN channelosome, we provide a graphical abstract (**FIGURE 9**) that summarizes the main take home messages, namely that: i) NALCN is involved in many cell and body functions; ii) NALCN functions in several species, including mammals and non-mammals, point to conserved functions but also differences in contributions to physiology; and iii) there are still many gaps in our understanding of NALCN physiology in mammals and non-mammals especially, and additional roles will likely be discovered as new research tools are developed and implemented.

#### IV- THE NALCN CHANNELOSOME IN HUMAN DISEASE

In this section, we describe the studies linking NALCN and its subunits to various human diseases.

##### *General genomic features of NALCN subunit genes in humans.*

The five genes encoding NALCN subunits in humans are located on chromosomes 13q32.3-q33.1 (*NALCN*), 14q32.12 (*UNC-79*), 2q34 (*UNC-80*), 13q33.3 (*FAM155A*), and Xq13.1 (*FAM155B*) (**TABLE 4**). Interestingly, an intronic and probably non-coding transcript referred to as *FAM155A-IT1* is predicted within the *FAM155A* gene, arranged in the same orientation as *FAM155A*. Similarly, a long non-coding RNA (lncRNA) referred to as *NALCN-AS1* that partially overlaps with *NALCN* in an inverted orientation is also predicted. Exons #6 and #8 of *NALCN-AS1* overlap with exons #43 and #44 of *NALCN*, respectively, raising the possibility *NALCN-AS1* regulates *NALCN* mRNA expression. Of note, both *FAM155A-IT1* and *NALCN-AS1* appear to be unique to humans. A search in the GTex portal (<https://www.gtexportal.org>) indicates that *FAM155A-IT1* is mainly expressed in testis and at low levels in the pituitary gland and brain tissues. *NALCN-AS1* is mainly expressed in brain tissues, pituitary gland, bladder, and arteries. Since non-coding RNAs are important regulators of cell physiology and function, the relevance of both *FAM155A-IT1* and *NALCN-AS1* in regulating *FAM155A* and *NALCN* expression/function remains to be investigated (213). A search in the NCBI database (<https://www.ncbi.nlm.nih.gov>) also suggests the existence of alternative splicing events for *NALCN*, *UNC-79* and *UNC-80* (*not shown*). Many studies have described pathogenic variants of the NALCN channelosome in rare genetic diseases as well as possible association with many other diseases such as psychiatric diseases and cancer (**TABLES 5-11**).

**TABLE 4: Chromosomal location of the human NALCN channelosome-encoding genes**

Gene	Chromosomal location	Reference sequence*	Coordinates	Size	Number of identified exons (Reference sequence)
<i>NALCN</i> <i>Canlon</i> <i>BA430M15.1</i> <i>VGCNL1</i> <i>Rb21</i>	13q32.3-q33.1	<a href="#">NC 000013.11</a>	chr13:101,053,774-101,417,206	363,433 bases	44 (NM_052867.4)
<i>NALCN-AS1</i>	13q32.3	<a href="#">NC 000013.11</a>	chr13:100,708,312-101,066,090	357,779 bases	6 (NR_047687.1)
<i>UNC-79</i> <i>KIAA1409</i>	14q32.12	<a href="#">NC 000014.9</a>	chr14:93,333,182-93,710,473	377,292 bases	50 (NM_020818.5)
<i>UNC-80</i> <i>KIAA1843</i> <i>C2orf21</i>	2q34	<a href="#">NC 000002.12</a>	chr2:209,771,832-209,999,300	227,469 bases	66 (NM_001371986)
<i>NLF-1/FAM155B</i> <i>NALF1</i>	13q33.3	<a href="#">NC 000013.11</a>	chr13:107,163,510-107,867,496	703,987 bases	3 (NM_001080396.3)
<i>FAM155A-IT1</i> <i>NALF1-IT1</i>	13q33.3	<a href="#">NC 000013.11</a>	chr13:107,787,360-107,835,458	48,098 bases	2 (NR_046848.1)
<i>NLF-1/FAM155B</i> <i>NALF2</i>	Xq13.1	<a href="#">NC 000023.11</a>	chrX:69,504,326-69,532,508	28,183 bases	3 (NM_015686.3)

TMEM28					
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2099 \*Genome assembly GRCh38/hg38

2100

2101 **1- Infantile Hypotonia with Psychomotor Retardation and characteristic Facies**  
 2102 **1 and 2 (IHPRF1 and 2)**

2103

2104 In both humans and mice, individuals heterozygous for *UNC-80*, *UNC-79* and *NALCN* null  
 2105 mutations develop normally, are fertile, and do not have obvious abnormalities such as  
 2106 lethality and severe intellectual disability, suggesting that a reduction in gene dosage is  
 2107 phylogenetically tolerable. However, homozygous deleterious mutations of *NALCN* and  
 2108 *UNC-80* are described in patients with the IHPRF1 (OMIM #615419) and IHPRF2 (OMIM  
 2109 #616801) syndromes respectively (*see below*). Previous studies reported that patients  
 2110 with interstitial deletions of the long arm of chromosome 13 (referred to as the 13q  
 2111 syndrome) where *NALCN*, *NALCN-AS1* and *FAM155A* localize, have widely varying  
 2112 phenotypes (214-217), including severe malformations in the brain, heart, kidneys, lungs,  
 2113 other organ systems, and digits. Instead, other individuals are only mildly affected with  
 2114 minor dysmorphic features, developmental delay, and growth failure (218).

2115

2116 Microdeletions in a region of chromosome 2, where *UNC-80* resides along with  
 2117 several other genes, are associated with brain anomalies, mental retardation and  
 2118 dysmorphic features (219, 220). Since these regions underlie several genes, it is not  
 2119 possible to correlate *NALCN* function with these phenotypic defects. However, symptoms  
 2120 described below for *NALCN* and *UNC-80* loss-of-function pathogenic variants clearly  
 2121 suggest an involvement, since the observed defects in patients with deletions in  
 2122 chromosomes 13q and 2q highly resemble these *NALCN* channelopathies.

2123

2124 *NALCN* subunit mutations, IHPRF1 (OMIM #615419):

2125

2126 A first study described 2 siblings from a co-sanguineous family suffering from a severe  
 2127 developmental disorder (221) (*also see* (222) for previous descriptions of siblings). These  
 2128 siblings carried a recessive homozygous truncating mutation p.Q642X on *NALCN*. The  
 2129 disease was first referred to as an atypical form of Infantile Neuroaxonal Dystrophy and  
 2130 later renamed IHPRF1. Following this first report, several patients carrying mainly  
 2131 homozygous nonsense, but also missense mutations (*i.e.*, p.W1287L; p.P908L; p.R1094Q;  
 2132 p.F1427L; p.V1528I; p.V1400F; p.G1303D), were described with no specific hot spots  
 2133 regarding their location on the protein sequence (**TABLE 5; FIGURE 10**)(223-236).  
 2134 Nonsense mutations are predicted to result either in a mRNA decay process or the  
 2135 expression of a truncated and non-functional protein. The missense pathogenic variant  
 2136 p.W1287L was found to be loss-of-function when expressed both in the neuronal NG108-  
 2137 15 cell line and *Xenopus* oocytes (27, 94). The recessive mutation p.R1094Q occurs at the  
 2138 interface between *NALCN* and *FAM155A* and is also predicted to be loss-of-function (29).  
 2139 More than 43 IHPRF1 patients from 29 families are described to date. Unsurprisingly,  
 2140 patients exhibit a large panel of symptoms of variable severity (**TABLE 7**).

2141

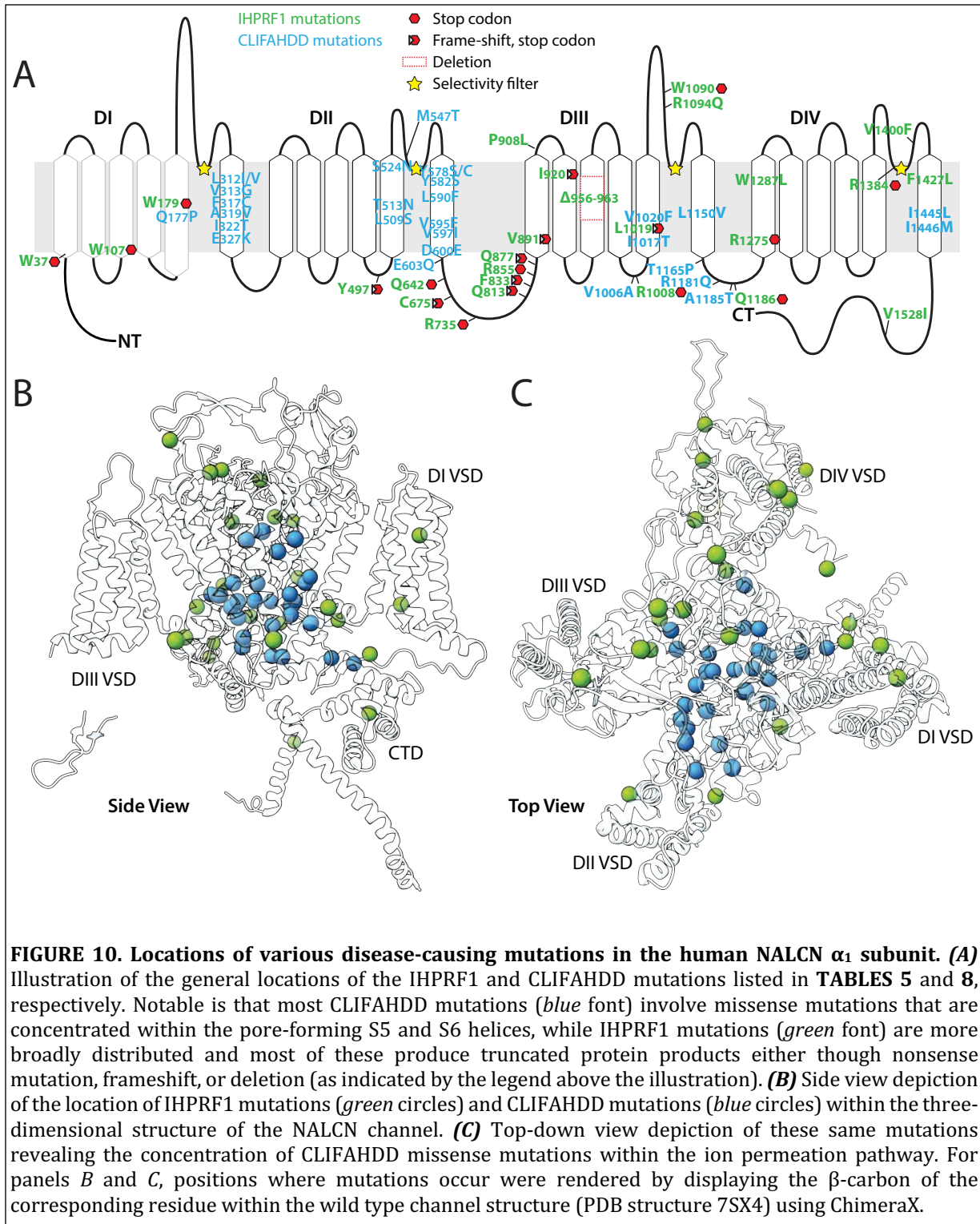
2142 *UNC-80* subunit mutations, IHPRF2 (OMIM #616801):

2143

2144 Autosomal recessive mutations in the *UNC-80* gene are also reported (39 patients from  
 2145 25 families; **TABLE 6**)(93, 229, 237-246). Like *NALCN*, both nonsense and missense  
 2146 mutations are described. Imposing the p.P1700S mutation on the mouse *UNC-80* cDNA  
 2147 causes a loss-of-function property to recorded currents when expressed in HEK-293T

2148 cells (93). Interestingly, the truncated p.L2586X mutant is functional when overexpressed  
2149 both in HEK-293T cells and mouse hippocampal neurons (49). However, while the wild-  
2150 type UNC-80 localizes to both soma and neuronal processes, the mouse UNC-80 variant  
2151 truncated at p.L2654X (*i.e.*, the analogous truncation of the human p.L2586X truncation  
2152 in mouse) is retained in the soma without detectable expression in axons and dendrites.  
2153 Consistent with findings for other *UNC-80* nonsense mutations, three nonsense mutations  
2154 predicted to be detrimental to UNC-80 folding or channelosome assembly (p.R51X,  
2155 p.R174X, and p.R2706X) also fail to evoke currents when co-expressed with NALCN,  
2156 FAM155A and UNC-79 in *Xenopus* oocytes. Three biallelic *UNC-80* missense mutations  
2157 (p.P1700S, p.V189M and p.R2536T) show wild-type-like function in *Xenopus* oocytes but  
2158 potentially impact UNC-79/UNC-80 subcomplex stability in neurons (31). A patient  
2159 appeared to have a similar but milder phenotype than those found in the individuals with  
2160 *UNC-80* null mutations (49). This patient exhibited a biallelic variations in the *UNC-80*  
2161 gene. In one allele, he inherited variations of c.1020G > T and c.1021C > T  
2162 (p.Q340\_P341delinsHS) from his mother. In the other, he inherited c.3883G > C  
2163 (p.E1295Q) from his father. The three mutated residues (p.Q340, p.P341, and p.E1295) in  
2164 this individual are highly conserved among deuterostome animals, from sea urchins to  
2165 fishes to humans. Rescue experiments in hippocampal neurons from *Unc-80* knockout  
2166 mice revealed that while the p.Q341H;P342S mutant (*i.e.*, the mouse homologue mutation  
2167 of the human p.Q340\_P341delinsHS mutation) generates little or no Na<sup>+</sup> leak current, the  
2168 p.E1296Q mutant (*i.e.*, the mouse homologue mutation of the human p.E1295Q mutation)  
2169 partially restores it. Indeed, *in vitro* NALCN channelosome current amplitudes are < 50%  
2170 compared to those of the wild-type complex. In addition, co-transfecting equal amounts  
2171 of both UNC-80 mutant cDNA constructs into *UNC-80* knockout neurons generates a Na<sup>+</sup>  
2172 leak current with amplitudes of only ~25% compared to wild-type UNC-80. This led Wie  
2173 *et al*, 2020, to conclude that while a reduction of 50% in *UNC-80* gene dosage is tolerated,  
2174 further reduction of UNC-80 function below 25% likely leads to severe phenotypes. This  
2175 finding unlikely reflects a difference in the stability of the protein since the wild-type UNC-  
2176 80 and the 2 mutants express at similar protein levels in HEK-293T cells. As for recessive  
2177 mutations of *NALCN*, patients with recessive *UNC-80* mutations exhibit a large panel of  
2178 symptoms of variable severity (**TABLE 7**).  
2179





**FIGURE 10. Locations of various disease-causing mutations in the human NALCN  $\alpha_1$  subunit.** (A) Illustration of the general locations of the IHPRF1 and CLIFAHDD mutations listed in TABLES 5 and 8, respectively. Notable is that most CLIFAHDD mutations (blue font) involve missense mutations that are concentrated within the pore-forming S5 and S6 helices, while IHPRF1 mutations (green font) are more broadly distributed and most of these produce truncated protein products either through nonsense mutation, frameshift, or deletion (as indicated by the legend above the illustration). (B) Side view depiction of the location of IHPRF1 mutations (green circles) and CLIFAHDD mutations (blue circles) within the three-dimensional structure of the NALCN channel. (C) Top-down view depiction of these same mutations revealing the concentration of CLIFAHDD missense mutations within the ion permeation pathway. For panels B and C, positions where mutations occur were rendered by displaying the  $\beta$ -carbon of the corresponding residue within the wild type channel structure (PDB structure 7SX4) using ChimeraX.

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**TABLE 5: Genetics of the IHPRF1 syndrome**

Mutation	Location	Patients	Gender (Older Age Reported)	Reference
c.1924C>T p.Q642X	DII-DIII loop	2 siblings	Female (21 yo) Male 18 (18 yo)	<a href="#">Koroglu et al, 2013 (221)</a>
c.1489delT p.Y497Tfs*21	Domain I	2 siblings  1 cousin	Male (7.1 yo) Male (4.4 yo)  Male (7.3 yo)	<a href="#">Al-Sayed et al, 2013 (223)</a>

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c.3860G>T p.W1287L	Domain IV	3 siblings	Female (17.6 yo) Female (16 yo) Female (9.4 yo)	<a href="#">Al-Sayed et al, 2013 (223)</a>
c.4197+1G>A (Parent 1)  c.2392C>T (Parent 2) p.R735X  Note: Patient also carries a mutation of ANO3 associated with Dystonia 24	Domain IV  DII-DIII loop	1	NI	<a href="#">Farwell et al, 2016 (224)</a>
c.G3390A (IVS29-1G > A)  p.W1090X	Domain III	3 siblings	Female (8 yo) Female (death at 5 yo) Male (death at 20 mo)	<a href="#">Gal et al, 2016 (225)</a>
c.1267-2A>G	nonsynonymous mutation at a splice-acceptor site in intron 11	1	Female (3.8 yo)	<a href="#">Takenouchi et al, 2018 (226)</a>
c.2022_2023delAT p. C675Lfs*23	DII-DIII loop (spliced exon 17)	1	Female (11 mo)	<a href="#">Takenouchi et al, 2018 (226)</a>
c.3823C>T (Mother) p.R1275X  c.2495_2496insTCATA (Father) p.F833Hfs*40  Note : mutations in TRAPPC9	Domain IV  DII-DIII loop	2 siblings	Male (22 yo) Female (20 yo)	<a href="#">Angius et al, 2018 (227)</a>
c.110G>A (Mother) p.W37X  c.2723C>T (Father) p.P908L  Note: mutations in ALMS1	N-terminus  Domain III	2 siblings	Female (7 yo) Male (8 yo)	<a href="#">Campbell et al, 2018 (236)</a>
c.3281G>A (Mother) p.R1094Q  c.2563C>T (Father) p.R855X	Domain III  Domain III	1	Female (10 yo)	<a href="#">Campbell et al, 2018 (236)</a>
c.3823C>T p.R1275X  Note: referred to as c.3910C>T, p.R1304X in the original article	Domain IV	1	Male (9 yo)	<a href="#">Bourque et al, 2018 (228)</a>
c.3022C>T (Mother) p.R1008X  c.2629delC (Father)	Domain III  DII-DIII loop	1	Female (11.7 yo)	<a href="#">Bramswig et al, 2018 (229)</a>

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p.Q877Nfs*16				
c.3022C>T p.R1008X	Domain III	1	NI	<a href="#">Abul-Husn et al, 2023 (235)</a>
c.3056dupT, p.L1019Ffs*30	Domain III	2 siblings	Male (7 yo) Female (3.10 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.4281C > A (Mother) p.F1427L	Domain IV	2 siblings	Male (4.6 yo) Male (3 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.4103 + 2T > C (Father) splice donor variant	Domain IV			
c.3556C > T p.Q1186X	DIII-DIV loop	1	Female (7 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.2435dupA p.E813Gfs*23	DII-DIII loop	1	Female (18.5 yo)  Note: One sibling died at the age of 6 years with the same clinical features but undiagnosed	<a href="#">Bramswig et al, 2018 (229)</a>
c.2889 + 3_6delAAGT (IVS25 + 3_6) p.V956_L- 963del	Domain III	1	Female (6.10 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.4150C > T p.R1384X	Domain IV	1	Male (5 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.321G > A p.W107X	Domain I	2 siblings	Female (4 yo) Male (NI)	<a href="#">Bramswig et al, 2018 (229)</a>
c.2758delA p.I920Lfs*7	Domain III	1	Female (3 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.2671delG, p.V891Sfs*2	Domain III	2 siblings	Male (death at 5 yo) Female (2.9 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.2671delG p.Val891Sfs*2	Domain III	1	Male (Death at 5.5 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.537delG p.W179X	Domain I	1	Female (3 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
rs767980482 (C>T) pV1528I  rs771656968 (C>A) pV1400F  Note: Patient also heterozygote for loss of function variants in <i>VPS13B</i> , associated with Cohen syndrome	C-terminus  Domain IV	1	Female (5 yo)	<a href="#">Carneiro et al, 2018 (230)</a>
c.2203C>T p.R735X	DII-DIII loop	2 siblings	Female (4 mo) Male (Death before the 3 yo)	<a href="#">Ope et al, 2020 (231)</a>
c.2563C>T p.R855X	DII-DIII loop	1	Female (9 yo)  Note: an older male sibling with similar abnormalities who has died undiagnosed at the age of 3.5 months.	<a href="#">Karimi et al, 2020 (232)</a>

c.3908C>T p.G1303D	Domain IV	1	Male (10 yo)	<a href="#">Khan et al, 2022 (233)</a>
c.1267-924_1434 + 2024del (Mother)	Deletion en- compassing exon 12	1	Male (3.2 yo)	<a href="#">Maselli et al, 2022 (234)</a>
c.3022C>T (Father)	NI			

Abbreviation: NI, not indicated

## 2- Congenital contractures of the Limbs and Face, Hypotonia and Developmental Delay (CLIFAHDD; OMIM #616226)

The description of CLIFAHDD, the severe developmental syndrome linked to *de novo* dominant mutations of *NALCN*, arose from the study by Chong et al 2015, of large series of children with congenital contractures of the limbs and face, hypotonia, and global developmental delay (247). Up to 40 patients with CLIFAHDD syndrome are now reported (**TABLE 8**)(134, 207, 248-261). Like the IHPRF1 and 2 patients, CLIFAHDD patients exhibit a large panel of symptoms of variable severity (**TABLE 9**). Most, but not all, of these mutations are located in the pore-forming region of *NALCN* (*i.e.*, transmembrane helices 5 and 6 of Domains I to IV) (**FIGURE 9**), suggesting they might exert their pathological functions by modulating *NALCN* gating directly.

A first study described the impact of the *de novo* pathogenic variant p.R1181Q found in a patient on the locomotor activity in *C. elegans* (256). Indeed, the pan-neuronal expression of *nca-1* carrying the corresponding mutation (*i.e.*, p.R1230Q) in a null *nca1/2* background (*i.e.*, *nca-1(gk9);nca-2(gk5)*) induced a coiling locomotion identical to that of the gain-of-function *nca-1(hp102)* or *nca-1(e625)* mutants. By contrast, the pan-neuronal expression of wild-type *nca-1* in the same background restored normal locomotor activity. It was concluded the p.R1230Q mutation of *nca-1* in *C. elegans* and by extrapolation, the p.R1181Q mutation in *NALCN* are gain-of-function ones. At the functional level, a study then reported that 2 mutations found in CLIFAHDD patients, p.L509S and p.Y578S, cause an increased current density compared to wild-type channels when transiently expressed in the neuronal cell line NG108-15 (94). These results were the first demonstration that pathogenic variants found in the CLIFAHDD syndrome are gain-of-function ones. The gain-of-function property of the p.L509S and p.Y578S variants were then confirmed following transient expression in HEK-293T cells (89). These findings were thereafter extended to a large panel of mutations found in patients following functional expression in *Xenopus* oocyte (27). Functional expression of the *NALCN* p.Y578S mutation in HEK-293T cells and single channel recordings revealed prolonged channel openings and an increase in open probability ( $P_0$ ), with no change in the unitary conductance (31). Structural mapping of 25 mutations reveals clusters of local interaction networks, both intra and interdomains (27). Indeed, the p.R1181Q variant identifies the DIII-DIV linker as a hotspot with a potential role in channel gating. Two mutations on the DIII-DIV linker, p.T1165P and p.R1181Q, may lead to changes in channel gating by affecting the local interactions of the III-IV linker with adjacent Domain IS6, the DII-DIII linker, Domain IVS6, and the CTD (28). p.R1181 of the DIII-DIV linker interacts with the main-chain carboxyl group of p.S1451, p.L1452 and p.Y1454 (29).

**TABLE 6: Genetics of the IHPRF2 syndrome**

Mutation	Patients	Gender (Older Age Reported)	Reference
c.151C>T p.R51X	2 related families  <i>Family 1</i> 2 siblings + 1 cousin  <i>Family 2</i> 4 siblings	aged between 9 months and 12 years  Female (5 yo) Female (NI) Male (NI)  Male (NI) Male (NI) Male (NI) Male (NI)	<a href="#">Perez et al, 2016 (237)</a>
c.5098C>T p.P1700S	1	Female (4 yo)	<a href="#">Stray-Pedersen et al, 2016 (93)</a>
c.6495G > A p.W2165X	1	Male (6 yo)	<a href="#">Kelesoglu et al, 2023 (246)</a>
c.7607G>C p.R2536T  Note : Predicted to give rise aberrant alternative splicing	1	Female (4 yo)	<a href="#">Stray-Pedersen et al, 2016 (93)</a>
c.7757T>A p.L2586X  c .2033delA p.N678Tfs*15	2 siblings	Female (15 yo) Female (9 yo)	<a href="#">Stray-Pedersen et al, 2016 (93)</a>
c.3793C>T p.R1265X	1	Male (6.5 yo)  Note: Two siblings with similar abnormalities died undiagnosed at the age of 4 yo and 6 yo respectively.	<a href="#">Shamseldin et al, 2016 (238)</a>
c.3793C>T p.R1265X	1	Male (2 yo)  Note: has older sister with similar abnormalities who died undiagnosed at 16 yo	<a href="#">Shamseldin et al, 2016 (238)</a>
c.1078C>T p.R360X	1	Female (7 yo)  Note: has younger brother (13 mo) with similar abnormalities	<a href="#">Shamseldin et al, 2016 (238)</a>
c.565G>A p.V189M	2 siblings	Female (4 yo) Female (8 yo)	<a href="#">Shamseldin et al, 2016 (238)</a>
c.2431C>T (Mother) p.R811X  c.3983-3_3994delinsA (Father) p.S1328Rfs*19	2 siblings	Female (13 yo) Female (Death at 7 yo)	<a href="#">Valkanas et al, 2016 (239)</a>

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c.8525G>A p.R2842Q	1	Female (13 yo)	<a href="#">Obeid et al, 2018 (240)</a>
c.8116C>T p.R2706X	1	Male (1 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.520C>T p.R174X	2 siblings	Male (3 yo) Female (1 yo)  Note: had 2 older siblings with similar abnormalities who died undiagnosed at 16 yo	<a href="#">Bramswig et al, 2018 (229)</a>
c.520C>T p.R174X	2 siblings	Female (NI) Female (NI)	<a href="#">Bramswig et al, 2018 (229)</a>
c.520C>T (Mother) p.R174X  c.2399delT (Father) p.L800Wfs*19	1	Male (4 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.1681_1682delAC p.T561Rfs*33	2 siblings	Male (10 yo.) Male (Death at 4 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.5671C>T p.R1891X	1	Female (Death at 2.7 yo)  Note: a similarly affected paternal cousin passed away at the age of 3 years	<a href="#">Bramswig et al, 2018 (229)</a>
c.8058+2T>G p.L2660Rfs*20	1	Male (4 yo)	<a href="#">Bramswig et al, 2018 (229)</a>
c.601-1G>A p.R174Qfs*58	2 siblings	Male (Death at 10 yo.) Male (Death at 9.6 yo)  Note: had another sibling with similar abnormalities who died undiagnosed at 1.6 yo	<a href="#">Bramswig et al, 2018 (229)</a>
c.3719G>A p.W1240X  c.4926_4937del p.Q1643_L1646del	2 siblings	Female (5.9 yo)  Note: had another sibling (Male) with similar abnormalities who died undiagnosed at 1 yo	<a href="#">He et al, 2018 (241)</a>
c.4963C>T p.R1655C  c.8385C>G p.Y2795X	1	Male (9.5 yo)	<a href="#">He et al, 2018 (241)</a>
Mutation not indicated	1	Male (8 yo)	<a href="#">Hong et al, 2018 (242)</a>
c.3226C > T p.R1076X  c.3205C > T p.R1069X	1	Female (Death at 3 yo)	<a href="#">Kuptanon et al, 2019 (243)</a>
Mutation not indicated	1	Male (3 yo)	<a href="#">Stenehjem et al, 2019 (244)</a>

c.5609-4G>A	1	Male (8 yo)	<a href="#">Tao et al, 2021 (245)</a>
Note: Predicted to give rise aberrant alternative splicing			

Abbreviation: NI, not indicated

### 3- Symptomatology of the IHPRF and CLIFAHDD syndromes

Mutations in *NALCN* and *UNC-80* are associated with IHPRF1, IHPRF2 and CLIFAHDD syndromes (*see above*). To the best of our knowledge, since the first publications in 2013 (221, 223), there are more than 100 cases of *NALCN/UNC-80*-related disorders reported in the literature (*see TABLES 5, 6 and 8*). However, despite the growing number of articles, the lack of homogeneity in the clinical data provided is a major obstacle towards establishing a complete clinical picture. *NALCN* is universally expressed in the brain, especially in neurons, but also in a variety of other organs/cell types (*e.g.*, heart, endocrine cells, intestine interstitial cells of Cajal, myometrial smooth muscle cells) and takes part in numerous physiological functions (**TABLE 3**). Therefore, diseases can lead to a broad spectrum of clinical manifestations. In general, *NALCN/UNC-80*-related disorders can be described as a static encephalopathy usually detected since the first months of life, leading to a severe global developmental delay and intellectual disability, in association with hypotonia, failure to thrive, dysmorphic facial features, and distal arthrogyriposis in CLIFAHDD syndrome, as the main clinical characteristics. Also, there are several other prominent manifestations reported in the literature, including central apneas and periodic breathing, epilepsy, hyperkinetic movement disorders, ataxia, sleep disturbances and constipation. All clinical manifestations are summarized in **TABLE 7** and **TABLE 9**.

**TABLE 7: Symptoms in patients with the IHPRF1 & 2 syndromes**

System reviewed	Clinical description	Reference
Perinatal problems	<ul style="list-style-type: none"> <li>- Intra-uterine growth retardation</li> <li>- Oligohydramnios</li> <li>- Need for hospitalization at birth</li> </ul>	<a href="#">Al-Sayed et al, 2013 (223)</a> <a href="#">Pérez et al, 2016 (237)</a> <a href="#">Valkanias et al, 2016 (239)</a> <a href="#">Takenouchi et al, 2018 (226)</a> <a href="#">Bramswig et al, 2018 (229)</a> <a href="#">Bourque et al, 2018 (228)</a> <a href="#">Campbell et al, 2018 (236)</a> <a href="#">Ope et al, 2020 (231)</a>
Growth, feeding difficulties and gastrointestinal disturbances	<ul style="list-style-type: none"> <li>- Poor sucking after birth</li> <li>- Feeding difficulties (not characterized)</li> <li>- Severe failure to thrive</li> <li>- Need for feeding tube</li> <li>- Severe constipation</li> </ul>	All authors reported feeding difficulties or failure to thrive. See tables 5 and 6 for the complete list of references.
Dysmorphic facial features	<ul style="list-style-type: none"> <li>- Frontal bossing</li> <li>- Secondary microcephaly</li> <li>- Triangular face</li> <li>- Convergent strabismus</li> <li>- Large, low set, posteriorly rotated ears</li> <li>- Mouth persistently opened, wide mouth</li> <li>- Thin and/or tented upper lip</li> <li>- Short and/or smooth philtrum</li> <li>- Hypotonic facies, only mild dysmorphism</li> </ul>	All authors reported some dysmorphic facial features. See tables 5 and 6 for the complete list of references.

Musculoskeletal features	<ul style="list-style-type: none"> <li>- Long thin fingers</li> <li>- Tapering of distal phalanx</li> <li>- Scoliosis</li> <li>- Small hands and/or feet</li> <li>- Joint hypermobility/lax joints</li> <li>- Joint contractures, developing progressively</li> </ul>	<p><a href="#">Koroglu et al, 2013 (221)</a>  <a href="#">Pérez et al, 2016 (237)</a>  <a href="#">Stray-Pedersen et al, 2016 (93)</a>  <a href="#">Valkanas et al, 2016 (239)</a>  <a href="#">Takenouchi et al, 2018 (226)</a>  <a href="#">Bramswig et al, 2018 (229)</a>  <a href="#">Campbell et al, 2018 (236)</a>  <a href="#">Obeid et al, 2018 (240)</a>  <a href="#">Karimi et al, 2020 (232)</a></p>
Developmental milestones, cognitive functioning, psychiatric disturbances	<ul style="list-style-type: none"> <li>- Absence of speech development</li> <li>- Severe persistent hypotonia after birth: most cannot sit or walk</li> <li>- Severe intellectual disability</li> <li>- Autistic features</li> <li>- Irritability/self-injury behaviors</li> <li>- Sociable, happy disposition</li> </ul>	<p>All authors reported developmental delay. See tables 5 and 6 for the complete list of references.</p>
Other neurological disturbances	<ul style="list-style-type: none"> <li>- Seizures, usually non-refractory when reported (interpret with caution)</li> <li>- Dystonic posture of limbs</li> <li>- Dyskinesias, mainly in upper limbs</li> <li>- Choreoathetoid movements</li> <li>- Stereotypic movements</li> </ul>	<p><a href="#">Al-Sayed et al, 2013 (223)</a>  <a href="#">Koroglu et al, 2013 (221)</a>  <a href="#">Pérez et al, 2016 (237)</a>  <a href="#">Stray-Pedersen et al, 2016 (93)</a>  <a href="#">Valkanas et al, 2016 (239)</a>  <a href="#">Gal et al, 2016 (225)</a>  <a href="#">Bramswig et al, 2018 (229)</a>  <a href="#">Obeid et al, 2018 (240)</a>  <a href="#">Angius et al, 2018 (227)</a>  <a href="#">Bourque et al, 2018 (228)</a>  <a href="#">Hong et al, 2018 (242)</a>  <a href="#">Kuptanon et al, 2019 (243)</a>  <a href="#">Khan et al, 2022 (233)</a>  <a href="#">Maselli et al, 2022 (234)</a>  <a href="#">Kelesoglu et al, 2023 (246)</a></p>
Respiratory disturbances	<ul style="list-style-type: none"> <li>- Central sleep apneas</li> <li>- Periodic breathing with frequent central apneas</li> </ul>	<p><a href="#">Stray-Pedersen et al, 2016 (93)</a>  <a href="#">Gal et al, 2016 (225)</a>  <a href="#">Bramswig et al, 2018 (229)</a>  <a href="#">Campbell et al, 2018 (236)</a>  <a href="#">Bourque et al, 2018 (228)</a>  <a href="#">Hong et al, 2018 (242)</a>  <a href="#">Karimi et al, 2020 (232)</a>  <a href="#">Maselli et al, 2022 (234)</a></p>
Sleep disturbances	<ul style="list-style-type: none"> <li>- Light sleep</li> <li>- Complete reversion of sleep-wake cycle</li> <li>- Periodic limb movements during sleep</li> <li>- Sleep disturbances not characterized</li> </ul>	<p><a href="#">Stray-Pedersen et al, 2016 (93)</a>  <a href="#">Takenouchi et al, 2018 (226)</a>  <a href="#">Campbell et al, 2018 (236)</a>  <a href="#">Bramswig et al, 2018 (229)</a>  <a href="#">Kuptanon et al, 2019 (243)</a>  <a href="#">Maselli et al, 2022 (234)</a></p>
Other	<ul style="list-style-type: none"> <li>- Increased tendency to infections</li> <li>- Small atrial defect in echocardiography</li> <li>- Recurrent increases of TSH</li> <li>- Hypothyroidism</li> </ul>	<p><a href="#">Stray-Pedersen et al, 2016 (93)</a>  <a href="#">Shamseldin et al, 2016 (238)</a>  <a href="#">Takenouchi et al, 2018 (226)</a>  <a href="#">Bramswig et al, 2018 (229)</a>  <a href="#">Stenhjen et al, 2019 (244)</a>  <a href="#">Karimi et al, 2020 (232)</a></p>

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Patient characteristics are summarized in **TABLES 5, 6** and **8**. Sex distribution seems to be very similar among the three syndromes, while the age at last evaluation is highly variable. Early death is reported in 5 cases of IHPRF1 and in 6 cases of CLIFAHDD, with the causes attributed to respiratory failure after pneumonia or sepsis in three cases (one



2266 associated with a status epilepticus), and withdrawal of life supporting measures in one  
 2267 severe case of CLIFAHDD. Byrne *et al*, 2023 also found a heterozygous variant in *NALCN*  
 2268 in a case of fetal/newborn death (259).

2269  
 2270 Most patients are born full term after an uneventful pregnancy. Oligohydramnios,  
 2271 polyhydramnios, and intrauterine growth retardation are reported in IHPRF1, IHPRF2  
 2272 and CLIFAHDD patients, respectively. Birth weight, height, and head circumference are  
 2273 within the norm in almost all cases. The need for a prolonged hospital stay after birth is  
 2274 not consistently reported, but some cases required hospitalization and respiratory  
 2275 support, usually after an emergency C-section for fetal distress (134, 207, 223, 228, 229,  
 2276 231, 236, 249, 252).

2277  
 2278 *Failure to thrive*

2279  
 2280 Failure to thrive is one of the most striking features in IHPRF1 and 2 syndromes, even  
 2281 despite adequate caloric intake provided by gastrostomy (225). This particular study  
 2282 suggested a role for *NALCN* in metabolic pathways involved in the pathogenesis of  
 2283 cachexia, as this is a common manifestation of pancreatic tumors, where mutations in  
 2284 *NALCN* are found (**TABLE 10**)(262). Feeding difficulties and poor sucking after birth are  
 2285 also very commonly reported, which mirrors observations made in *Unc-79* knockout mice  
 2286 (137). In some cases (5 patients with IHPRF1, 7 patients with IHPRF2 and 5 patients with  
 2287 CLIFAHDD), a feeding tube was necessary for adequate nutrition. The origin of these  
 2288 feeding difficulties remains unknown, and while endocrine function is described as  
 2289 normal in some cases, exocrine function has not been documented in the majority of  
 2290 patients with *NALCN/UNC-80*-related disorders, leaving a possible explanation to date  
 2291 unexplored.

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**TABLE 8: Genetics of the CLIFAHDD syndrome**

Mutation	Location	Gender (Older Age Reported)	Reference
c.191A>G p.Y64C	Domain IS1	NI	<a href="#">Pergande et al, 2020 (260)</a>
c.530A>C p.Q177P*	Domain IS5	Death at 6 months	<a href="#">Chong et al, 2015 (247)</a>
c.934C>A p.L312I*	Domain IS6	NI	<a href="#">Chong et al, 2015 (247)</a>
c.934C>G p.L312V*	Domain IS6	Female (4.6 yo)	<a href="#">Fukai et al, 2016 (248)</a>
c.938T>G p.V313G*	Domain IS6	Death at 6 years	<a href="#">Chong et al, 2015 (247)</a>
c.950T>G p.F317C*	Domain IS6	Female (Death at 9 mo)	<a href="#">Karakaya et al, 2016 (249)</a> <a href="#">Pergande et al, 2020 (260)</a>
c.956C>T p.A319V*	Domain IS6	Female (3 yo)	<a href="#">Lozic et al, 2016 (207)</a>
C.965T>C p.I322T	Domain IS6	Male (3 yo)	<a href="#">Sivaraman et al, 2016 (250)</a>
c.979G>A p.E327K*	Domain IS6	NI	<a href="#">Chong et al, 2015 (247)</a>
c.985A>G p.R329G	DI-DII loop	NI	<a href="#">Stark et al, 2016 (261)</a>

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c.1526T>C p.L509S*	Domain IIS5	NI	Chong et al, 2015 (247)
c.1534T>G p.F512V*	Domain IIS5	NI	Chong et al, 2015 (247)
c.1538C>A p.T513N*	Domain IIS5	NI	Chong et al, 2015 (247)
c.1571G>A p.S524N*	Domain IIS5	Male (Death at 10 mo)	Angius et al, 2019 (251)
c.1640T>C p.M547T	Domain II P loop	Male (3 yo)	Kumaki et al, 2022 (252)
c.1733A>C p.Y578S*	Domain IIS6	NI	Chong et al, 2015 (247)
c.1733A >G p.Y578C*	Domain IIS6	Male (17 yo)	Vivero et al, 2017 (253)
		Female (33 mo)	Vivero et al, 2017 (253)
c.1745A>C p.Y582S*	Domain IIS6	Male (9 mo)	Vivero et al, 2017 (253)
c.1768C>T p.L590F*	Domain IIS6	Death at 4 months	Chong et al, 2015 (247)
		Female (NI)	Bend et al, 2016 (134)
c.1783G>T p.V595F*	Domain IIS6	Female (Death at 3 mo)	Karakaya et al, 2016 (249) Pergande et al, 2020 (260)
c.1789G > A p.V597I*	Domain IIS6	Female (33 yo)	Wang et al, 2016 (254)
c.1800C>A p.D600E*	Domain IIS6	Female (Death at 3.5 mo)	Angius et al, 2019 (251)
c.1870G > C p.E603Q	Domain IIS6	Male (2 yo)	Winczewska-Wiktor et al, 2022 (255)
c.3017T>C p.V1006A*	Domain III ECL 4-5	NI	Chong et al, 2015 (247)
c.3050T>C p.I1017T*	Domain IIIS5	NI	Chong et al, 2015 (247)
c.3058G4T p.V1020F*	Domain IIIS5	Female (15 mo)	Fukai et al, 2016 (248)
c.3448C >G p.L1150V	Domain IIIS6	Female (13 yo)	Vivero et al, 2017 (253)
c.3493A>C p.T1165P*	DIII-DIV loop	NI	Chong et al, 2015 (247)
c.3542G>A p.R1181Q*	DIII-DIV loop	NI	Chong et al, 2015 (247)
		Female (7.5 yo)	Aoyagi et al, 2015 (256)
		Female (5 yo)	Fukai et al, 2016 (248)
c.3553G>A p.A1185T	DIII-DIV loop	NI	Vissers et al, 2017 (257)
c.4205C>T p.L1324F	Domain IVS4	fetal or newborn death	Byrne et al, 2023 (259)
c.4300A>G p.I1434V	Domain IVS6	Male (3 yo)	Liao et al, 2022 (258)
c.4333A>T p.I1445L	Domain IVS6	Male (Death at 4 mo)	Bramswig et al, 2018 (229)
c.4338T>G* p.I1446M	Domain IVS6	NI	Chong et al, 2015 (247)

2294

\* Functionally expressed in recombinant system, gain-of-function. Abbreviation: NI, not indicated

2295 *Dysmorphic features*

2296  
2297 Almost all patients have some dysmorphic facial features, which seem to be different  
2298 between IHPRF1/2 and CLIFAHDD patients. Most common facial features reported for  
2299 each syndrome are summarized in **TABLES 7** and **9**, but a variety of other features are  
2300 also described, such as down slanting palpebral fissures, fine hair, palpebral ptosis,  
2301 retrognathia or a high nasal bridge. Some individuals are just described as having  
2302 hypotonic facies with mild dysmorphism. Plagiocephaly and dolichocephaly are reported  
2303 in some cases of IHPRF1 and 2. Interestingly, postnatal microcephaly is common in  
2304 IHPRF1 and 2, whereas only five cases of CLIFAHDD documented this (248, 253).

2305  
2306 Distal arthrogryposis is a distinctive feature of CLIFAHDD syndrome, with  
2307 camptodactyly, ulnar deviation of wrist and/or fingers and clubfoot also reported in most  
2308 patients. Also, about half of the authors reported joint contractures in hips, knees or  
2309 elbows. In contrast, none of the cases of IHPRF1/2 have been diagnosed with  
2310 arthrogryposis, though there were a few cases of clubfoot and joint contractures in  
2311 IHPRF2 patients (237).

2312  
2313 *Developmental delay, intellectual disability and behavioral manifestations*

2314  
2315 All cases, regardless of the mutation, reported global developmental delay and intellectual  
2316 disability when this information was available. These are described as severe in almost  
2317 all cases of IHPRF1 and 2, with most patients being non-verbal and non-ambulatory. Data  
2318 for CLIFAHDD is more inconsistent in this regard. Nevertheless, a high number of patients  
2319 did not develop speech, but showed more variability in gross motor milestones.

2320  
2321 Behavioral problems are not always described, which constitutes a clear bias. Most  
2322 cases of CLIFAHDD do not provide sufficient information. Autistic features, irritability and  
2323 hypersensitivity to stimuli are reported mostly in IHPRF2 patients. Self-injury behaviors  
2324 are also reported (229, 239, 241), while other patients are described as being sociable  
2325 and having a happy disposition (93, 240, 241, 243, 246).

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2327

**TABLE 9: Symptoms for the CLIFAHDD syndrome**

System reviewed	Clinical description	Reference
Perinatal problems	<ul style="list-style-type: none"> <li>- Polyhydramnios</li> <li>- Oligohydramnios</li> <li>- Clubfoot detected before birth</li> <li>- Need for hospitalization at birth</li> <li>- Fetal or newborn death</li> </ul>	<ul style="list-style-type: none"> <li><a href="#">Karakaya et al, 2016 (249)</a></li> <li><a href="#">Bend et al, 2016 (134)</a></li> <li><a href="#">Sivaraman et al, 2016 (250)</a></li> <li><a href="#">Vivero et al, 2017 (253)</a></li> <li><a href="#">Pergande et al, 2020 (260)</a></li> <li><a href="#">Kumaki et al, 2022 (252)</a></li> <li><a href="#">Winczewska-Wiktor et al, 2022 (255)</a></li> <li><a href="#">Liao et al, 2022 (258)</a></li> <li><a href="#">Byrne et al, 2023 (259)</a></li> </ul>
Growth, feeding difficulties and gastrointestinal disturbances	<ul style="list-style-type: none"> <li>- Poor sucking after birth</li> <li>- Feeding difficulties (not characterized)</li> <li>- Failure to thrive</li> <li>- Need for feeding tube</li> <li>- Gastroesophageal reflux disease</li> <li>- Severe constipation</li> </ul>	<ul style="list-style-type: none"> <li><a href="#">Chong et al, 2015 (247)</a></li> <li><a href="#">Fukai et al, 2016 (248)</a></li> <li><a href="#">Bend et al, 2016 (134)</a></li> <li><a href="#">Lozic et al, 2016 (207)</a></li> <li><a href="#">Vivero et al, 2017 (253)</a></li> <li><a href="#">Bramswig et al, 2018 (229)</a></li> <li><a href="#">Kumaki et al, 2022 (252)</a></li> </ul>

		<a href="#">Winczewska-Wiktor et al, 2022 (255)</a>
Dysmorphic facial features	<ul style="list-style-type: none"> <li>- Divergent strabismus</li> <li>- Broad nasal bridge</li> <li>- Anteverted nasal tip, large nares</li> <li>- Short and/or long philtrum</li> <li>- Short columella</li> <li>- Micrognathia</li> <li>- Pursed lips, small mouth</li> <li>- Full cheeks, deep nasolabial folds</li> <li>- H-shaped dimpled chin</li> </ul>	All authors reported some dysmorphic facial features. See table 8 for the complete list of references.
Musculoskeletal features	<ul style="list-style-type: none"> <li>- Distal arthrogyposis</li> <li>- Camptodactyly</li> <li>- Ulnar deviation of wrist and/or fingers</li> <li>- Clubfoot</li> <li>- Calcaneovalgus deformity</li> <li>- Joint contractures (hips, knees, elbows)</li> <li>- Short neck</li> </ul>	All authors reported arthrogyposis. See table 8 for the complete list of references.
Developmental milestones, cognitive functioning, psychiatric disturbances	<ul style="list-style-type: none"> <li>- All authors reported developmental delay and intellectual disability, but data regarding severity is inconsistent; usually severe when reported.</li> <li>- Hyperactivity</li> </ul>	All authors. See table 8 for the complete list of references.
Other neurological disturbances	<ul style="list-style-type: none"> <li>- Seizures</li> <li>- Episodic ataxia</li> <li>- Slurred speech and gait ataxia</li> <li>- Stereotypic movements</li> </ul>	<a href="#">Chong et al, 2015 (247)</a> <a href="#">Aoyagi et al, 2015 (256)</a> <a href="#">Fukai et al, 2016 (248)</a> <a href="#">Wang et al, 2016 (254)</a> <a href="#">Vivero et al, 2017 (253)</a> <a href="#">Bramswig et al, 2018 (229)</a>
Respiratory disturbances	<ul style="list-style-type: none"> <li>- Central sleep apneas</li> <li>- Periodic breathing with frequent central apneas</li> <li>- Respiratory insufficiency</li> </ul>	<a href="#">Chong et al, 2015 (247)</a> <a href="#">Bend et al, 2016 (134)</a> <a href="#">Lozic et al, 2016 (207)</a> <a href="#">Stark et al, 2016 (261)</a> <a href="#">Vivero et al, 2017 (253)</a> <a href="#">Pergande et al, 2020 (260)</a> <a href="#">Kumaki et al, 2022 (252)</a> <a href="#">Winczewska-Wiktor et al, 2022 (255)</a>
Sleep disturbances	<ul style="list-style-type: none"> <li>- Complete reversion of sleep-wake cycle</li> </ul>	<a href="#">Lozic et al, 2016 (207)</a>
Other	<ul style="list-style-type: none"> <li>- Inguinal hernia</li> <li>- Increased tendency to infections</li> <li>- Small ventricular or atrial defect in echocardiography</li> <li>- Patent foramen ovale</li> <li>- Bilateral hydronephrosis</li> <li>- Episodes of severe dysautonomia</li> <li>- Excessive drooling</li> </ul>	<a href="#">Chong et al, 2015 (247)</a> <a href="#">Aoyagi et al, 2015 (256)</a> <a href="#">Karakaya et al, 2016 (249)</a> <a href="#">Lozic et al, 2016 (207)</a> <a href="#">Bend et al, 2016 (134)</a> <a href="#">Vivero et al, 2017 (253)</a> <a href="#">Pergande et al, 2020 (260)</a> <a href="#">Winczewska-Wiktor et al, 2022 (255)</a> <a href="#">Liao et al, 2022 (258)</a>
Sensitivity to volatile anesthetics	<ul style="list-style-type: none"> <li>- Sevoflurane sensitivity caused respiratory depression and cardiac arrest</li> </ul>	<a href="#">Lozic et al, 2016 (207)</a>

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2331 *Epilepsy and movement disorders*

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 2333 The NALCN channelosome regulates the RMP of neurons and their excitability, leading to  
 2334 the possibility of excitability disorders in NALCN syndromes, including epilepsy. Almost  
 2335 all authors reported presence/absence of seizures, which are more common in IHPRF1  
 2336 and 2 syndromes, affecting approximately half of the patients. Age of onset is very  
 2337 variable, ranging from the first months of life up to 13-year-old in IHPRF1, and up to 5-  
 2338 year-old in IHPRF2. Most cases present with bilateral tonic-clonic seizures, but authors  
 2339 also describe behavioral arrest, tonic, atonic and myoclonic seizures.  
 2340 Electroencephalogram (EEG) recordings could be simply reported as abnormal, or as  
 2341 showing generalized slowing, generalized spike and wave epileptiform activity, or, in  
 2342 some cases, multifocal epileptiform discharges.  
 2343

2344 Only 5 IHPRF patients are reported to have refractory epilepsy, out of a total of 32,  
 2345 although this is highly dependent on the moment of publication and can have changed  
 2346 over time; furthermore, in 10 cases this information is not available. Kelesoglu *et al* 2023  
 2347 reported a case with only febrile seizures at 7 months (246). Antiseizure medications do  
 2348 not seem to be selected specifically with *NALCN/UNC-80* mutations in mind, as they are  
 2349 those commonly used for a broad spectrum of epilepsies: valproic acid, levetiracetam,  
 2350 lamotrigine and phenobarbital, either in monotherapy or in combination. Also, Ope *et al*,  
 2351 2020, reported good seizure control with carbamazepine in one patient (231), while Gal  
 2352 *et al*, 2016, reported two cases treated with vigabatrin (225), one of whom died after a  
 2353 status epilepticus complicated with sepsis and respiratory failure. Ketogenic diet was  
 2354 successful in one case reported by Bramswig *et al*, 2018, but had no effect in the case of  
 2355 IHPRF2 reported by Hong *et al*, 2018 (229, 242).  
 2356

2357 Epilepsy is reported in five cases of CLIFAHDD, but there is insufficient data to  
 2358 document the type of seizures or response to treatment (247, 253).  
 2359

2360 Persistent hypotonia after birth is the main motor manifestation in all cases, and  
 2361 only a few authors report spastic paraparesis or hypertonia in upper or lower limbs (221,  
 2362 229, 238, 249, 253, 260). NALCN is also expressed in the basal ganglia, which could lead  
 2363 to the appearance of movement disorders (263). In this regard, several patients with  
 2364 IHPRF1 and 2 syndromes present with hyperkinetic disorders, such as choreoathetoid  
 2365 movements of limbs, dyskinesia, dystonia and stereotypes, although a specific description  
 2366 is usually lacking (**TABLE 7**). Magnetic Resonance Imaging (MRI) is reported as normal  
 2367 in approximately half of the patients with IHPRF1 and 2 syndromes, but Bramswig *et al*,  
 2368 2018, and Pérez *et al*, 2015, reported the finding of a thin corpus callosum in some  
 2369 patients, as did Karayaka *et al*, 2016, and Vivero *et al*, 2017, in three cases with CLIFAHDD  
 2370 (229, 237, 249, 253). As for CLIFAHDD, some authors describe a cerebellar syndrome with  
 2371 gait ataxia and slurred speech, which present as episodic ataxia in a case described by  
 2372 Aoyagi *et al*, 2015, but developed progressively during young adulthood in the patients  
 2373 reported by Wang *et al*, 2016, and Bramswig *et al*, 2018 (229, 254, 256). Interestingly,  
 2374 cerebellar atrophy is observed in the MRI of 11 CLIFAHDD patients (229, 247, 248, 252-  
 2375 254, 256). In addition, Aoyagi *et al*, 2015 and Kumaki *et al*, 2022, also documented a  
 2376 previous normal MRI, further suggesting that cerebellar involvement may develop  
 2377 progressively (252, 256).  
 2378  
 2379

2380 *Respiratory and sleep disturbances*

2381  
 2382 Chong *et al*, 2015, reported several cases of respiratory insufficiency in their series of 14  
 2383 patients with CLIFAHDD, but did not characterize the dysfunction (247). Since then,  
 2384 respiratory disturbances are reported more frequently, mostly in IHPRF1 and CLIFAHDD,  
 2385 and are characterized as periodic breathing patterns with frequent central apneas (228,  
 2386 236, 242, 260, 261). This periodic breathing in human patients is reminiscent to the  
 2387 pattern described in animal models. Indeed, Lu *et al*, 2007 showed pathological breathing  
 2388 patterns characterized by several seconds of apnea followed by short episodes of deep  
 2389 breaths in a knockout model of *Nalcn* in mice, which was predictive of death in the first  
 2390 24h of life (2). Treatment of the reported cases is based on oxygen supplementation at  
 2391 night, associated or not with positive airway pressure therapy. A tracheostomy is  
 2392 necessary in severe cases (134, 207, 252, 253). Hong *et al*, 2018, documented complete  
 2393 resolution of apneas after instauration of bilevel positive airway pressure (BPAP) in a  
 2394 patient with IHPRF2 (242). Winczewska-Wiktor *et al*, 2022, reported a case of CLIFAHDD  
 2395 with severe central apneas in which a myasthenic syndrome was suspected before the  
 2396 definitive diagnosis, which improved after empirical treatment with pyridostigmine  
 2397 bromide (255). However, continuous positive airway pressure (CPAP) and oxygen  
 2398 supplementation were used at the same time, which could have contributed to the clinical  
 2399 improvement.

2400  
 2401 In agreement with a role for the NALCN channelosome in regulating sleep, Lozic *et*  
 2402 *al*, 2016, Stray-Pedersen *et al*, 2016, Takenouchi *et al*, 2018, and Campbell *et al*, 2018,  
 2403 described a complete reversion in sleep-wake cycle in at least one patient for each one of  
 2404 the three syndromes (93, 207, 226, 236). Other authors also reported sleep disturbances,  
 2405 which seem to be common in these syndromes, but most did not characterize them.  
 2406 Maselli *et al*, 2022, noted a high index of periodic limb movements index in a case with  
 2407 IHPRF1 syndrome, in association with iron-deficiency anemia. This index remained high  
 2408 despite dietary treatment and improvement of sleep efficiency after initiation of BPAP,  
 2409 hypothesizing a possible genetic association (234).

2410  
 2411 *Constipation, dysautonomia and other manifestations*

2412  
 2413 *NALCN* is expressed in the interstitial cells of Cajal in the gastrointestinal tract, mediating  
 2414 the pacemaking of gastrointestinal functions (78). Almost all individuals with IHPRF1 and  
 2415 2 syndromes are reported to have constipation. This is less frequently described in  
 2416 individuals with CLIFAHDD, but in these patients, there is a larger proportion of  
 2417 gastroesophageal reflux disease, which could lead to frequent vomiting in severe cases.  
 2418 These findings suggest that there may be different patterns of involvement of interstitial  
 2419 cells of Cajal in the upper and lower intestine, depending on whether the mutation results  
 2420 in a loss- or gain-of-function. However, this should be interpreted with caution, as there  
 2421 are authors who do not report the presence of these symptoms, so it could correspond to  
 2422 a reporting bias. In a similar line, some authors reported patients with excessive drooling,  
 2423 mainly in CLIFAHDD syndrome (207, 232, 247, 255). Other authors reported frequent  
 2424 aspiration pneumonias or an inability to clear airway secretions (229, 244, 253), which  
 2425 suggests that excessive drooling might be more common, but it could be easily overlooked  
 2426 and thus underreported. Although sialorrhea could be a consequence of swallowing  
 2427 difficulties. *NALCN* is expressed in several glands (1, 56), but a possible role in the  
 2428 regulation of salivary secretions is yet to be explored.

2429 *NALCN* is also expressed in the dorsal motor nucleus of the vagus, linking it to  
 2430 autonomic control (264). However, are not taken into account respiratory, sleep, and  
 2431 gastrointestinal disturbances, only a few authors have reported other forms of  
 2432 dysautonomia. Karayaka et al, 2016, and Bend et al, 2016, reported two CLIFAHDD  
 2433 patients with severe episodes of apnea, hyperthermia, tachycardia (249), or bradycardia  
 2434 and hypertension (134). Frequent life-threatening episodes led to intubation in the case  
 2435 reported by Bend et al, 2016, but the patient worsened over time and life support was  
 2436 eventually withdrawn (134).

2437  
 2438 Lastly, it is noteworthy that cardiac and endocrine functioning are reported as normal  
 2439 in the majority of cases in which this information is available, however there are no  
 2440 systematic studies addressing the possibility of subclinical cardiac and endocrine  
 2441 involvement, which linked to the inability of these patients to communicate, rises the  
 2442 possibility of overlooking mild or evolving cardiac and endocrine pathology. Possible  
 2443 disturbances in other systems, like kidney or urological functioning, are not significantly  
 2444 reported, so there is insufficient information in this regard.

2445

#### 2446 **4- Therapies in the IHPRF and CLIFAHDD syndromes**

2447

2448 With the exception of the study mentioned above reporting a beneficial effect of a  
 2449 treatment with pyridostigmine bromide on severe central apneas observed in a  
 2450 CLIFAHDD patient (255), there are no efficient therapies for treating patients identified  
 2451 to date. However, the development of animal and cell models have paved the way for  
 2452 identifying pharmacological and gene therapy approaches. For example, the ability to  
 2453 functionally express the NALCN channelosome in recombinant systems such as *Xenopus*  
 2454 oocytes and HEK-293T cells led to the identification of molecules with inhibitory  
 2455 properties (2, 27, 89). One may hypothesize NALCN blockers could be used to treat  
 2456 patients with the gain-of-function CLIFAHDD syndrome. However, the currently known  
 2457 compounds (*i.e.*, 2-APB, Lamotrigine, Verapamil, and N-benzhydryl quinuclidine  
 2458 compounds) are low-affinity and non-specific NALCN blockers. It remains necessary to  
 2459 identify more specific NALCN channelosome blockers that could be of clinical relevance  
 2460 to treat patients with CLIFAHDD mutations. Both invertebrate (*i.e.*, *C. elegans* and *D.*  
 2461 *melanogaster*) and vertebrate (*i.e.*, *M. musculus*) animal models are now available (**TABLE**  
 2462 **10**). This includes loss- and gain-of-function alleles that may reasonably be considered as  
 2463 relevant to investigate novel therapeutics for the IHPRF and CLIFAHDD syndromes  
 2464 respectively. In keeping with this idea, a targeted drug screening aiming to identify drugs  
 2465 that correct locomotion deficits in a *C. elegans* gain-of-function *nca-1* mutant (*i.e.*, a  
 2466 CLIFAHDD model) (100) identified 2-ABP, nifedipine, nimodipine, flunarizine, and  
 2467 ethoxzolamide as significantly rescuers of abnormal movements in mutant animals.  
 2468 Whether these compounds are able to rescue the altered sleep phenotype observed in the  
 2469 *Dreamless* mouse, a gain-of-function (CLIFAHDD) mouse model (69), remains to be  
 2470 investigated. Conversely, drugs that block K<sup>+</sup> channels (*e.g.*, 4-AP, Quinine, Quinidine,  
 2471 loratadine) and gap junctions (*i.e.*, CBNX) significantly improve movement of *C. elegans*  
 2472 loss-of-function mutants for *nca-1:nca-2*, (*i.e.*, a IHPRF1 model), *unc-80* (*i.e.*, a IHPRF2  
 2473 model) and *unc-79* (265). The relevance of these compounds to rescue phenotypes  
 2474 observed in loss-of-function (IHPRF1/2) mouse models needs to be explored. Taken  
 2475 together, these data provide proof-of-principle that it is possible to pharmacologically  
 2476 modify NALCN function *in vivo* in order to correct (at least partially) phenotypes induced  
 2477 by functional deficiencies of the channel.

2478

**TABLE 10: Animal models to study NALCN channelosome-related diseases**

Specie	Genetic change	Functional effect (Disease)	References
<i>M. musculus</i>	Knockout of <i>Nalcn</i>	Loss-of-function (IHPRF1)	Lu et al, 2007 (2)
<i>M. musculus</i>	Conditional Knockout of <i>Nalcn</i>	Loss-of-function	Flourakis et al, 2015 (38) Philippart & Khaliq, 2018 (72) Zhang et al, 2021 (61) Do et al, 2020 (73)
<i>M. musculus</i>	Knockout of <i>Unc-79</i>	Loss-of-function (IHPRF ?)*	Nakayama et al, 2006 (137)
<i>M. musculus</i>	Hypomorphic mutation ( <i>Unc-79</i> )	Loss-of-function (IHPRF ?)*	Specia et al, 2010 (47)
<i>M. musculus</i>	Knockout of <i>Unc-80</i>	Loss-of-function (IHPRF2)	Wie et al, 2020 (49)
<i>M. musculus</i>	p.N315K	Gain-of-function (CLIFAHDD)	Funato et al, 2016 (69)
<i>C. elegans</i>	<i>nca-1;nca-2</i> (Random mutagenesis)	Loss-of function (IHPRF1)	Jospin et al, 2007 (32)
<i>C. elegans</i>	<i>unc-79</i>	Loss-of-function (IHPRF ?)*	Sedensky & Meneely, 1987 (129)
<i>C. elegans</i>	<i>unc-80</i>	Loss-of-function (IHPRF2)	Sedensky & Meneely, 1987 (129)
<i>C. elegans</i>	p.R403Q (NCA-1) p.A717V (NCA-1) (Random mutagenesis)	Gain-of-function (CLIFAHDD)	Yeh et al, 2008 (34)
<i>C. elegans</i>	Transgenesis with p.R1230Q (p.R1181Q in human)	Gain-of-function (CLIFAHDD)	Aoyagi et al, 2015 (256)
<i>C. elegans</i>	p.V359G (p.V313G) p.Y625S (p.Y578S) p.E373K (p.E327K) p.T560N (T513N) p.F559V (p.F512V) p.L556S (p.L509S) p.L637F (p.L590F) p.D647E (p.D600E) p.A643V (p.A596V) (knockin with CRISPR/Cas9)	Gain-of-function** Gain-of-function** Gain-of-function** Gain-of-function Gain-of-function Gain-of-function Gain-of-function Gain-of-function Gain-of-function (CLIFAHDD)	Bend et al, 2016 (134)
<i>D. melanogaster</i>	<i>na</i>	Loss-of function (IHPRF1)	Krishnan & Nash, 1990 (132)
<i>D. melanogaster</i>	<i>unc-79</i>	Loss-of function (IHPRF ?)*	Humphrey et al, 2007 (33)

\*Given the requirement of *UNC-79* in the NALCN channelosome function, loss-of-function animal models may be relevant as IHPRF models. \*\*First described as loss-of-function based on locomotor phenotype.

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Gene therapy approaches may also be relevant to treat patients with the IHPRF1/2 and CLIFAHDD syndromes. In this context, genome editing techniques combined with the use of viral vectors may be used to correct mutations found in patients *in vivo* (266). In the case of the CLIFAHDD syndrome, it is also conceivable to use *in vivo* AAV-based RNA interference strategies to partially reduce *NALCN* expression to restore NALCN current levels toward more physiological ranges. The development of such treatment strategies will require mouse models and other technologies such as brain organoids to provide proof-of-concept. In the context of NALCN loss-of-function, a study suggested that NALCN



2491 and TRPC3 maybe interchangeable in neurons opening possibilities for compensatory  
 2492 therapy by promoting or enhancing TRPC3 function (82). Specifically, midbrain  
 2493 dopaminergic (DA) neurons are slow pacemakers that maintain extracellular DA level, in  
 2494 which both TRPC3 and NALCN contribute equally to slow depolarization. However, in  
 2495 TRPC3 knockout mice, the lack of TRPC3 is compensated by NALCN through a  
 2496 transcription-dependent mechanism that remains uncharacterized. Perhaps, it would be  
 2497 possible to rescue the *in vivo* the loss-of-function of NALCN by expressing TRPC3 under  
 2498 the control of the NALCN promoter using an AAV-based approach.

2499

## 2500 **5- Syndromes linked to heterozygous variants of UNC-79 and NALCN**

2501

2502 A recent study reported 6 unrelated patients with heterozygous *de novo* truncating and  
 2503 presumed loss-of-function mutations in *UNC-79* responsible for a panel of severe  
 2504 neurological symptoms with a patient-dependent variability (48). This includes mild  
 2505 developmental delay affecting both motor and verbal skills, hypotonia, autistic features  
 2506 and epileptic seizures. Of note, the phenotypes described for *UNC79* variants seem milder  
 2507 than those of *IHPRF1* and 2, as all patients are ambulatory, and all but one case could  
 2508 communicate using words or short sentences. Autistic features, attention deficit and  
 2509 irritability are common, and epilepsy is described as one of the main prominent  
 2510 symptoms, but otherwise patients did not exhibit growth failure, gastrointestinal or major  
 2511 dysmorphic features. The seizure-like phenotype is recapitulated in *D. melanogaster* by  
 2512 knocking down *unc-79* expression both globally and specifically in neurons. In addition,  
 2513 *Unc-79* haploinsufficiency in mouse leads to deficiency in hippocampal-dependent  
 2514 learning and memory. In keeping with the idea that haploinsufficiency of the NALCN  
 2515 channelosome components may induce a disease state, another study reported 2 patients,  
 2516 a father and son, presenting with focal epilepsy. One of them exhibits a discrete atrophy  
 2517 of the left temporal lobe including the left hippocampus. A genetic panel performed in  
 2518 both patients showed an heterozygous deletion in *NALCN* that includes the exon #27  
 2519 (267). This exon encodes for most of the IIS5 transmembrane segment and its deletion is  
 2520 predicted to result in a loss-of-function phenotype. Curiously, epileptic seizures are not  
 2521 described in relatives of *IHPRF1* and *IHPRF2* patients with heterozygous predicted loss-  
 2522 of-function mutations in *NALCN* and *UNC-80* respectively. Indeed, these relatives are  
 2523 apparently healthy and are not described as having epilepsy.

2524

## 2525 **6- Other diseases**

2526

2527 In addition to rare genetic diseases, the NALCN channelosome components are implicated  
 2528 in other human diseases (**Table 11**). This chapter reviews several other human  
 2529 conditions in which the NALCN channelosome may be involved.

2530

### 2531 *Psychiatric disorders*

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2533 Psychiatric disorders differ from most of the other medical conditions in their intrinsic  
 2534 multifactorial etiology in which both genetic and experiential factors contribute to their  
 2535 development (268). Considering the crucial role of *NALCN* in regulating neuronal  
 2536 excitability, its association with psychiatric disorders is not surprising. Several  
 2537 association studies highlighted a possible role of *NALCN* in schizophrenia and/or bipolar  
 2538 disorders (269-274). However, a lack of association of *NALCN* with schizophrenia is also  
 2539 reported (275, 276). *NALCN* is also described as a potential susceptibility locus for

2540 alcoholism (277, 278). Supporting a role of the NALCN channelosome in alcoholic  
 2541 addiction, another study revealed a possible association of *UNC-79* with alcohol and  
 2542 nicotine dependence (279). Of note, a region of chromosome 2 that includes *UNC-80* is  
 2543 also found to be involved in alcoholic addiction (280, 281). This is consistent with the  
 2544 ethanol hypersensitivity of *C. elegans* specimens bearing null alleles for the NALCN  
 2545 channelosome (47, 150, 157). Furthermore, and as noted above, *Nalcn* in NAc may  
 2546 contribute to the ethanol-induced acute stimulant responses and locomotor sensitization  
 2547 in mouse, given that *Lwt/+* (*Unc-79* loss-of-function) mice exhibit an increase in both  
 2548 sensitivity to the acute sedative effects of ethanol, and in their appetitive for consumption  
 2549 of ethanol (47, 156).

2550 *UNC-79*, *UNC-80* and *NLF-1/FAM155A* have also been implicated in autism (282-  
 2551 284), while other studies have reported a chromosome 2 region that includes *UNC-80*  
 2552 which is involved in autism (285-287). A role of the NALCN channelosome in autism is  
 2553 supported by autistic features seen in patients suffering with CLIFAHDD and IHPRF  
 2554 syndromes (*see above*). Decreased expression of *UNC-80* is reported in the context of the  
 2555 neonatal opioid withdrawal syndrome (288). Children who develop this syndrome are at  
 2556 increased risk of attention-related disorders, lower IQ, and poorer performance in  
 2557 academic testing compared to their unexposed counterparts (289). A correlation between  
 2558 *Nalcn* and depression is reported in a mouse model (63). Indeed, reduced expression of  
 2559 *Nalcn* and subsequent alterations in electrical activity are observed in glutamatergic  
 2560 neurons of the ventral dentate gyrus in an inflammation-induced depression mouse  
 2561 model. Moreover, the overexpression of *Nalcn* in these neurons decreases the  
 2562 susceptibility of mice to inflammation-induced depression. *FAM155A*, *UNC-80* and  
 2563 *NALCN-AS1* are also putative susceptibility loci for anorexia nervosa, anxiety and  
 2564 attention-deficit hyperactivity disorder respectively (290-292), and *Lwt/+* mice also  
 2565 exhibit a hyperactive phenotype (47).  
 2566  
 2567

**TABLE 11: The NALCN channelosome in other diseases**

Disease	gene	Study Type	Reference
<b>Schizophrenia/Bipolar disorders</b>			
<i>Bipolar Disorder</i>	<i>NALCN</i>	GWAS	<a href="#">Baum et al, 2008a,b (269, 270)</a>
<i>Schizophrenia</i>	<i>NALCN</i>	PBA/GWAS	<a href="#">Askland et al, 2009 (271)</a>
<i>Bipolar Disorder</i>	<i>NALCN</i>	GWAS	<a href="#">Ollila et al, 2009 (272)</a>
<i>Bipolar Disorder + Schizophrenia</i>	<i>NALCN</i>	GWAS	<a href="#">Wang et al, 2010 (273)</a>
<i>Schizophrenia</i>	<i>NALCN</i>	CCS	<a href="#">Zhang et al, 2018 (274)</a>
<b>Anorexia Nervosa</b>	<i>NLF-1/FAM155A</i>	GWAS	<a href="#">Wang et al, 2011 (290)</a>
<b>Addiction</b>	<i>UNC-80</i> ?*	AS	<a href="#">Schuckit et al, 2001 (280)</a>
	<i>UNC-80</i> ?*	AS	<a href="#">Nurnberger et al, 2001(281)</a>
	<i>UNC-79</i>	GWAS	<a href="#">Lind et al, 2010 (279)</a>
	<i>NALCN</i>	GWAS	<a href="#">Wetherill et al, 2014 (277)</a>
	<i>NALCN</i>	CCS	<a href="#">Baronas et al, 2018 (278)</a>
<b>Attention-Deficit Hyperactivity Disorder</b>	<i>NLF-1/FAM155A</i> ?*	FBAT	<a href="#">Anney et al, 2008 (293)</a>
	<i>NALCN-AS1</i>	CCS	<a href="#">Kweon et al, 2018 (291)</a>
<b>Anxiety</b>	<i>UNC-80</i>	GWAS	<a href="#">Chu et al, 2021 (292)</a>

## NALCN in physiology and pathophysiology

<b>Autism</b>	<i>UNC-80 ?*</i> <i>UNC-80 ?*</i> <i>UNC-80</i> <i>UNC-80 ?*</i> <i>UNC-79</i> NLF-1/FAM155A	microdeletion microdeletion WES (nonsense mut) Microdeletion WES (DNV) ELISA from plasma (Down)	Brandau et al, 2008 (285) Rosenfeld et al, 2010 (286) Iossifov et al, 2012 (282) Jang et al, 2015 (287) Krupp et al, 2017 (283) Al-Mazidi et al, 2023 (284)
<b>Neonatal opioid withdrawal syndrome</b>	<i>UNC-80</i>	CNV (Decrease)	Radhakrishna et al, 2021 (288)
<b>Depression</b>	<i>Nalcn</i>	Mouse model	Wang et al, 2023 (63)
<b>Alzheimer Disease</b>	<i>UNC-80 ?*</i> <i>UNC-79 ?*</i> <i>UNC-79 ?*</i> <i>UNC-80</i>  <i>NALCN</i>	LA WGAS LA Differential editing level in hippocampus from patients WGAS	Scott et al, 2003 (294) Grupe et al, 2007 (295) Lee et al, 2008 (296) Khermesh et al, 2016 (297)  Prokopenko et al, 2021 (298)
<b>Ataxia/Dystonia</b>	<i>UNC-80</i>	CNV (Decrease)	Canet-Pons et al, 2021 (299)
<b>Cervical dystonia</b>	<i>NALCN</i>	GWAS	Mok et al, 2014 (300)
<b>Parkinson disease</b>	<i>NALCN</i>	AS	Yang et al, 2021 (301)
<b>Epilepsy</b>	<i>NALCN</i>  <i>UNC-80?*</i> <i>UNC-80?*</i>  <i>UNC-80</i> <i>UNC-80</i>	Change in alternative splicing in brain tissues from patients with mTLE  LA LA  WES (point mutation) Microdeletion	Heinzen et al, 2007 (302)   Ratnapriya et al, 2010 (303) Epicure Consortium et al, 2012 (304) Yang et al, 2017 (305) Westphal et al, 2018 (306)
<b>Restless legs syndrome</b>	<i>NALCN, NALCN-AS1</i>	AS	Balaban et al, 2012 (307)
<b>Polyglutamine disorders</b>	<i>NLF-1/FAM155A?</i>	Genome analysis	Whan et al, 2010 (308)
<b>Cancer</b>			
<i>Gastric, Intestinal, pancreatic adenocarcinomas</i>	<i>Nalcn</i>	Knockout in mouse models	Rahrmann et al, 2022 (309)
<i>Prostate cancer</i>	<i>NALCN</i>	knockdown/overexpression	Folcher et al, 2023 (310)
<i>Tumor-derived endothelial cells</i>	<i>NLF-1/FAM155A</i>	Exome Sequencing (Mut)	McGuire et al, 2012 (311)
<i>Pancreatic cancer</i>	<i>NALCN</i> <i>UNC-80</i>	CNV (Down) CNV (Down)	Biankin et al, 2012 (262) Hu et al, 2017 (312)
<i>Non-Small Cell lung cancer</i>	<i>NALCN</i>	GWAS	Lee et al, 2013 (313)
<i>Non-Small Cell lung cancer with brain metastasis</i>	<i>UNC-79</i>	CNV (Gain)	Kim et al, 2023 (102)
<i>Lung adenocarcinoma</i>	<i>NALCN</i>	CNV (Down)	He et al, 2023 (314)
<i>Lung squamous cell carcinoma</i>	<i>NALCN</i>	CNV (Down)	He et al, 2023 (314)

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<i>Ewing Sarcoma</i>	<i>UNC-80</i>	Deep Sequencing (Mut)	<a href="#">Agelopoulos et al, 2015 (315)</a>
<i>Hepatocellular Carcinoma</i>	<i>UNC-79</i> <i>NALCN</i> <i>NALCN</i>	WES (Mut) CNV (Down) CNV (Down)	<a href="#">Zhan et al, 2017 (316)</a> <a href="#">Chen et al, 2018 (317)</a> <a href="#">He et al, 2023 (314)</a>
<i>Neuroblastoma</i>	<i>NALCN, UNC-79</i>	WES (Mut)	<a href="#">Esposito et al, 2018 (318)</a>
<i>Clear Cell Renal Cell Carcinoma</i>	<i>NALCN-AS1</i> <i>NLF-1/FAM155A</i>  <i>NALCN</i>	CNV (Gain) GWM (hypermethylated) CNV (Down)	<a href="#">Wang et al, 2018 (319)</a> <a href="#">Kang et al, 2019 (320)</a>  <a href="#">He et al, 2023 (314)</a>
<i>Kidney renal papillary cell carcinoma</i>	<i>NALCN</i>	CNV (Down)	<a href="#">He et al, 2023 (314)</a>
<i>Papillary Thyroid Carcinoma</i>	<i>NLF-1/FAM155B</i> <i>NLF-1/FAM155B</i>	CNV (Down) GWM (hypermethylated)	<a href="#">Yu et al, 2019 (321)</a> <a href="#">Li et al, 2022 (322)</a>
<i>Glioma/Glioblastoma</i>	<i>NLF-1/FAM155A</i> <i>NALCN</i> <i>NALCN</i> <i>NALCN</i> <i>circNALCN</i>	CNV (Down) CNV (Down) CNV (Down) CNV (Down) CNV (Down)	<a href="#">Fontanillo et al, 2012 (323)</a> <a href="#">Wang et al, 2015 (324)</a> <a href="#">Zhang et al, 2020 (325)</a> <a href="#">He et al, 2023 (314)</a> <a href="#">Liu et al, 2021 (326)</a>
<i>Bladder</i>	<i>NALCN-AS1</i> <i>NALCN</i>	CNV (Down) CNV (Down)	<a href="#">Zhong et al, 2022 (327)</a> <a href="#">He et al, 2023 (314)</a>
<i>Colorectal</i>	<i>NALCN</i> <i>NALCN</i>	CNV (gain) CNV (Down)	<a href="#">Huang et al, 2023 (328)</a> <a href="#">He et al, 2023 (314)</a>
<i>Rectum adenocarcinoma</i>	<i>NALCN</i>	CNV (Down)	<a href="#">He et al, 2023 (314)</a>
<i>Breast invasive carcinoma</i>	<i>NALCN</i>	CNV (Down)	<a href="#">He et al, 2023 (314)</a>
<i>Cervical squamous cell carcinoma &amp; endocervical adenocarcinoma</i>	<i>NALCN</i>	CNV (Down)	<a href="#">He et al, 2023 (314)</a>
<i>Thyroid carcinoma</i>	<i>NALCN</i>	CNV (Down)	<a href="#">He et al, 2023 (314)</a>
<i>Uterine corpus endometrial carcinoma</i>	<i>NALCN</i>	CNV (Down)	<a href="#">He et al, 2023 (314)</a>
<i>Cholangio carcinoma</i>	<i>NALCN</i>	CNV (Gain)	<a href="#">He et al, 2023 (314)</a>
<i>Pheochromocytoma</i>	<i>NALCN</i>	CNV (Gain)	<a href="#">He et al, 2023 (314)</a>
<i>Paraganglioma</i>	<i>NALCN</i>	CNV (Gain)	<a href="#">He et al, 2023 (314)</a>
<i>Stomach adenocarcinoma</i>	<i>NALCN</i>	CNV (Gain)	<a href="#">He et al, 2023 (314)</a>
<b>Chemotherapy toxicity</b>	<i>NALCN</i>	QTL	<a href="#">King et al, 2014 (329)</a>
<b>Randall plaques</b>	<i>NALCN</i>	CNV (Down)	<a href="#">Taguchi et al, 2017 (330)</a>
<b>Coronary Heart disease</b>	<i>NALCN</i>	MCA	<a href="#">Yan et al, 2018 (331)</a>

<b>Congenital Heart Disease</b>	<i>NALCN</i>	CNV (Down)	<a href="#">Audain et al, 2021 (332)</a>
<b>Hypertension</b>	<i>NLF-1/FAM155A</i>	GWAS	<a href="#">Adeyemo et al, 2009 (333)</a>
<b>Obesity</b>	<i>NLF-1/FAM155A</i>	GWAS	<a href="#">Wilson et al, 2015 (334)</a>
<b>Primary Ovarian Insufficiency</b>	<i>NLF-1/FAM155A</i>	CNV (up)	<a href="#">Li et al, 2016 (335)</a>
<b>Diverticular disease and diverticulitis</b>	<i>NLF-1/FAM155A</i> <i>NLF-1/FAM155A</i> <i>NLF-1/FAM155A</i> <i>NLF-1/FAM155A</i>	GWAS GWAS GWAS GWAS	<a href="#">Sigurdsson et al, 2017 (336)</a> <a href="#">Maguire et al, 2018 (337)</a> <a href="#">Schafmayer et al, 2019 (338)</a> <a href="#">Reichert et al, 2020 (339)</a>
<b>Chiari malformation Type I</b>	<i>NLF-1/FAM155A</i>	AS	<a href="#">AvSar et al, 2020 (340)</a>
<b>Primary biliary cirrhosis</b>	<i>NLF-1/FAM155A</i>	GWAS	<a href="#">Hirschfield et al, 2009 (341)</a>
<b>13q syndrome</b>	<i>NALCN</i> <i>NLF-1/FAM155A</i>		<a href="#">Brown et al, 1995 (214)</a> <a href="#">Kirchhoff et al, 2009 (215)</a> <a href="#">Huang et al, 2012 (216)</a> <a href="#">Lalani et al, 2013 (217)</a>
<b>Brain anomalies</b>	<i>UNC-80?</i>	microdeletion	<a href="#">van Binsbergen et al, 2014 (220)</a>
<b>Mental retardation, dysmorphic features</b>	<i>UNC-80?</i>	microdeletion	<a href="#">Bisgaard et al, 2006 (219)</a>

2568 \* Other genes may be involved in the considered genomic region. Abbreviations: GWAS, genome wide  
2569 association study; PBA, pathways-based analysis; QTL, quantitative trait loci; CCS, case control study; AS,  
2570 association study; WES, whole exome sequencing; WGS, whole genome sequencing; CNV, copy number  
2571 variation; GWM, genome wide methylation; FBAT, family-based association test; MCA, multiple correlation  
2572 analysis; LA: linkage analysis; DNV, *de novo* variation; IA, integrative analysis; WB, western blotting; mTLE,  
2573 mesial temporal lobe epilepsy; ELISA: enzyme-linked immunosorbent assay.

2574

### 2575 *Neurodegenerative disorders*

2576

2577 Alzheimer's disease (AD) is a neurodegenerative disorder characterized by gradual  
2578 deterioration of cognitive function, memory, and inability to perform daily, social, or  
2579 occupational activities. Its etiology is associated with the accumulation of  $\beta$ -amyloid  
2580 peptides, phosphorylated tau protein, and neuro-inflammatory and oxidative processes  
2581 in the brain (342). Rare variants of several genes have been associated with AD (343).  
2582 This includes genome regions where *UNC-79* and *UNC-80* reside (294-296), however  
2583 these regions also bear other genes including *MAP2* on chromosome 2 which is associated  
2584 with AD (344). *UNC-80* also exhibits a change in gene editing levels in the hippocampus  
2585 from patients with AD (297). More recently, a genome-wide association study (GWAS)  
2586 identified *NALCN* as a potential susceptibility locus for AD (298). Electrophysiologically,  
2587 inhibitory interneurons from APdE9 mice, a model for AD, exhibit altered electrical  
2588 activity (345). Indeed, these neurons exhibit an inability to reliably spike, a higher RMP,  
2589 an enhanced depolarizability in response to applied stimulus, and smaller mean AP  
2590 amplitude as compared to those from control mice, all consistent with an increase in a  $\text{Na}^+$   
2591 background conductance. It is tempting to speculate that *NALCN* contributes to this  $\text{Na}^+$   
2592 background conductance although other proteins could be involved including amyloid  $\beta$   
2593 which can itself form a cation-permeable pore at the plasma membrane (346-348).

2594

2595 Another association study suggested *NALCN* could be a susceptibility locus for  
2596 Parkinson's disease (PD)(301). PD is a common neurodegenerative movement disorder  
2597 for which the etiology in most patients is unknown, but different genetic causes have been  
2598 identified (349). Notably, *NALCN* was also identified as a susceptibility locus for cervical  
2599 dystonia (300), and dystonia often precedes other motor symptoms in PD (350, 351).

2600 However, a lack of association between *NALCN* and dystonia is also reported in two  
 2601 studies (352, 353). Nonetheless, an involvement of *NALCN* in PD is supported by the fact  
 2602 that disturbances observed in patients with PD are similar to phenotypes reported in  
 2603 animal models for *NALCN* channelosome dysfunctions (263). Of note, a decrease in the  
 2604 copy number of *Unc-80* is reported in a mouse model for spinocerebellar ataxia type 2  
 2605 (299). Some patients with the CLIFAHDD syndrome also exhibited episodic ataxia (*see*  
 2606 *above* and **TABLE 9**). Since NLF-1/FAM155A contains polyglutamine repeats, it is  
 2607 possible it could be involved in polyglutamine disorders (308).

2608

### 2609 *Epilepsy*

2610

2611 Epilepsy is a brain condition characterized by the recurrence of unprovoked seizures and  
 2612 is one of the most common serious brain conditions, affecting over 70 million people  
 2613 worldwide. It is characterized by a lasting predisposition to generate spontaneous  
 2614 epileptic seizures and has numerous neurobiological, cognitive, and psychosocial  
 2615 consequences (354). A study aiming to identify disease-associated alternative splicing  
 2616 patterns in ion channel genes by comprehensively screening affected brain tissue  
 2617 collected from patients with mesial temporal lobe epilepsy revealed an altered alternative  
 2618 splicing pattern for *NALCN* (302). A genomic region on chromosome 2 associated with  
 2619 susceptibility to epilepsy which includes *UNC-80* was also described (303, 304). Point  
 2620 mutations within *UNC-80* in patients with early onset epileptic spasm with unknown  
 2621 reason are also reported (305). More recently, a microdeletion of a chromosomal region  
 2622 including the *UNC-80* gene (but also *LANCL1* and *MAP2*) was detected in a patient with  
 2623 epilepsy (305, 306). Several patients with the IHPRF and CLIFAHDD syndromes as well  
 2624 as with monoallelic predicted loss-of-function variants of *NALCN* and *UNC-79* also exhibit  
 2625 seizures (*see above* and **TABLES 7 & 9**). Taken together, these data strongly point the  
 2626 *NALCN* channelosome-encoding genes as susceptibility loci for epilepsy.

2627

### 2628 *Movement disorder*

2629

2630 An association study suggested is a susceptibility locus for the restless leg syndrome  
 2631 within 13q32.3-33.2, where *NALCN* and *NALCN-AS1* reside (307). This syndrome is a  
 2632 common neurological disorder affecting up to 15% of the general population and is  
 2633 categorized as a sleep-related movement disorder.

2634

### 2635 *Cancer*

2636

2637 A recent study demonstrated that *Nalcn* is a key regulator of cancer metastasis in mouse.  
 2638 Indeed, deletion of *Nalcn* from gastric, intestinal, or pancreatic adenocarcinomas in mice  
 2639 models does not alter tumor incidence, but markedly increases the number of circulating  
 2640 tumor cells and metastases (309). Conversely, *NALCN* is also described as promoting  
 2641 metastasis as a major initiator and regulator of Ca<sup>2+</sup> oscillations required for invadopodia  
 2642 formation in human prostate cancer cells (310). Several other studies also indicate a  
 2643 possible association of the *NALCN* channelosome with several types of cancers (**TABLE**  
 2644 **11**). This includes the report of somatic mutations within *NALCN*, *UNC-79* and *UNC-80* in  
 2645 pancreatic cancer, hepatocellular carcinoma, and Erwin sarcoma respectively (262, 315,  
 2646 316). Mutations within *NALCN* and *UNC-79* are also described in neuroblastoma (318). In  
 2647 addition, the COSMIC database that provides a catalogue of somatic mutations in cancer  
 2648 references a large number of mutations for *NALCN* channelosome-encoding genes in a

2649 large panel of cancer types (<https://cancer.sanger.ac.uk/cosmic>). Functional  
 2650 consequences of these mutations and a correlation between NALCN function and cancer  
 2651 severity have now to be explored. Copy number variations (CNV) are also reported, where  
 2652 *NALCN* is downregulated in hepatocellular carcinoma and glioma/glioblastoma (317, 324,  
 2653 325). *NALCN* expression is also differentially regulated in colorectal cancer cells where it  
 2654 could interact with endomucin to exert a carcinogenic role (328). A recent data mining  
 2655 analysis also revealed that *NALCN* expression is significantly downregulated in bladder  
 2656 urothelial carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma,  
 2657 endocervical adenocarcinoma, colon adenocarcinoma, glioblastoma multiforme, kidney  
 2658 renal clear cell carcinoma, kidney renal papillary cell carcinoma, liver hepatocellular  
 2659 carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, rectum adenocarcinoma,  
 2660 thyroid carcinoma, uterine corpus endometrial carcinoma, but upregulated in  
 2661 cholangiocarcinoma, pheochromocytoma and paraganglioma, stomach adenocarcinoma  
 2662 (314). Downregulation of *UNC-80*, *NLF-1/FAM155A* and *NLF-1/FAM155B* are described in  
 2663 pancreatic cancer, glioma/glioblastoma and Papillary Thyroid Carcinoma respectively  
 2664 (312, 321-323). Conversely, upregulation of *NALCN-AS1*, *NLF-1/FAM155A*, and *UNC-79* are  
 2665 reported in clear cell renal cell carcinoma, tumor-derived endothelial cells and non-small  
 2666 cell lung cancer with brain metastasis respectively (102, 311, 319). In this context, the  
 2667 *NALCN* channelosome could be involved in proliferation and migration of lung cancer  
 2668 A549 cells (102). Also, a downregulation of *NALCN-AS1* is correlated with survival in  
 2669 patients with bladder cancer (327). Interestingly, a study reported the existence of a  
 2670 circular RNA referred to as *circNALCN* which is derived from exons 2 to 7 of *NALCN* (326).  
 2671 This *CircNALCN* element is downregulated in glioma tissues and acts as a sponge for miR-  
 2672 493-3p, affecting the expression of PTEN to modulate glioma tumorigenesis and  
 2673 progression. In addition to all these findings, a genome-wide association study suggested  
 2674 a possible association between *NALCN* and non-small cell lung cancer and *NLF-1/FAM155A*  
 2675 was found to be hypermethylated in clear cell renal cell carcinoma (313, 320). Overall,  
 2676 these studies indicate a possible role of the *NALCN* channelosome in cancer. However,  
 2677 whether detected alterations within these genes reflect a cause or a consequence of cancer  
 2678 must be investigated. Of interest for cancer treatment, a study aiming to map quantitative  
 2679 trait loci affecting toxicity to chemotherapy in *D. melanogaster* highlighted *na/Dma1U* as  
 2680 impacting toxicity to carboplatin (329).

#### 2681 2682 *Miscellaneous*

2683  
 2684 In addition to studies mentioned above, the *NALCN* channelosome is also suspected to be  
 2685 involved in other disorders. Microarray analysis from renal papillary highlighted that a  
 2686 down regulation of *NALCN* is associated with calcium phosphate lesions in the renal  
 2687 papilla known as Randall's plaques (330). *NALCN* is also associated to coronary heart  
 2688 disease (multiple correlation analysis) and found to have a decrease expression level in  
 2689 congenital heart disease (331, 332). Genome-wide association studies also suggest *NLF-1/FAM155A*  
 2690 as a susceptibility locus in hypertension and obesity (333, 334). Indeed, *NLF-1/FAM155A*  
 2691 is associated with several other diseases including primary ovarian  
 2692 insufficiency, diverticular disease and diverticulitis, Chiari malformation of type I and  
 2693 primary biliary cirrhosis (335-341). However, another study did not find any association  
 2694 between *NLF-1/FAM155A* with either diverticular disease or diverticulitis (355).

2695  
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 2697

2698 **V- CONCLUDING REMARKS**

2699  
2700 *NALCN channel: from cloning to structure, physiological roles and diseases*

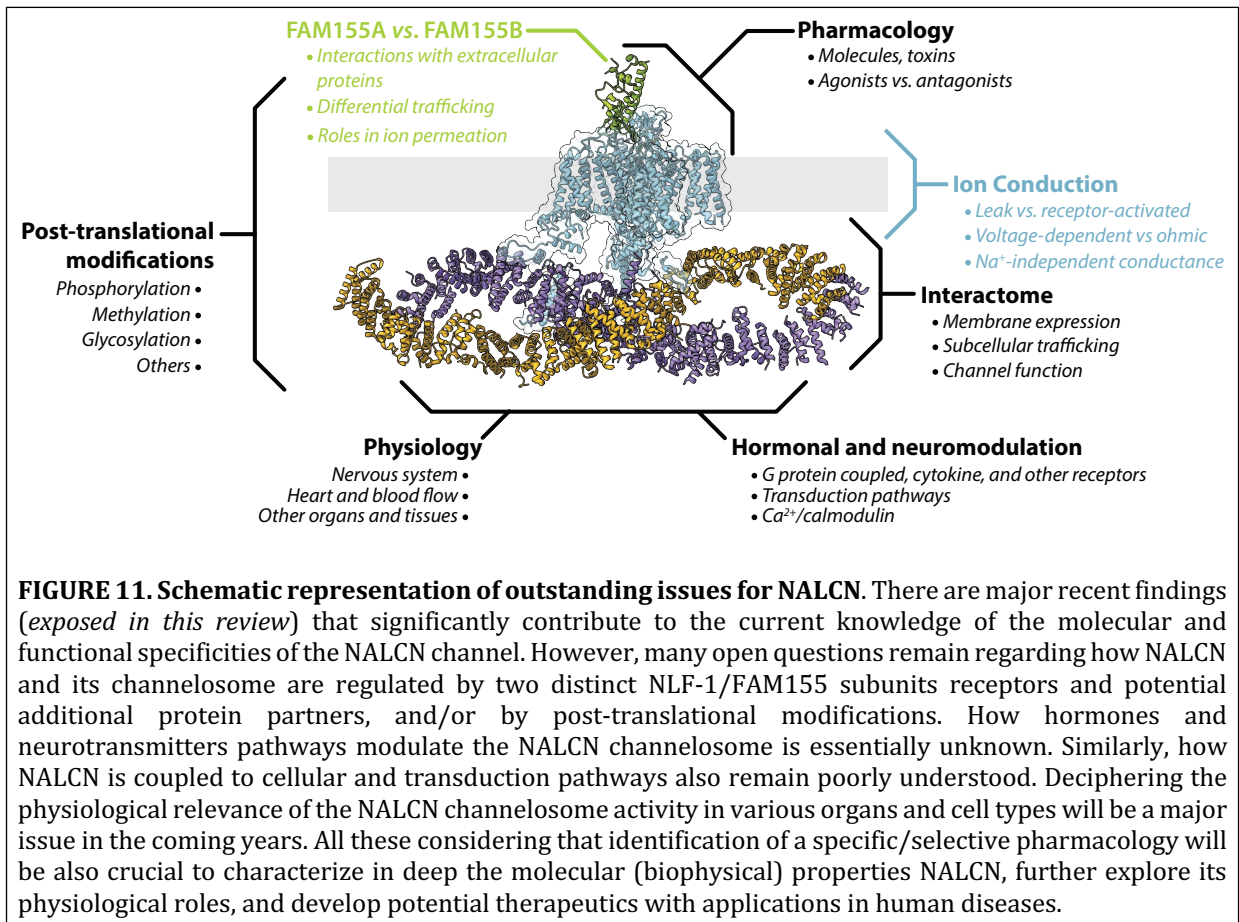
2701  
2702 While it took more than 8 years from its initial molecular cloning (1) to its first functional  
2703 description (2), NALCN is now well documented as a channel that critically regulates the  
2704 resting membrane potential and the electrical activity of neurons and other cell types.  
2705 Recent studies also established that NALCN channel requires a complex arrangement of 4  
2706 subunits (*NALCN*, *UNC-80*, *UNC-79* and *NLF1/ FAM155*) for its proper activity (31). Several  
2707 studies in *C. elegans* and *D. melanogaster* mutant animals had previously described  
2708 phenotypic features linked to NALCN deficiency, even before knowing its channel function  
2709 (*reviewed in* (356)), but the ground-breaking findings by Lu *et al*, 2007 (2) launched and  
2710 fostered investigations related to its physiology, functional regulation, and  
2711 pathophysiology. Molecular characterization of NALCN channelosome has also recently  
2712 benefited from the elucidation of its 3D Cryo-EM structure. Unfortunately, no selective  
2713 pharmacological modulator of NALCN, either inhibitor or activator, has been identified to  
2714 date. Such molecules will undoubtedly be valuable to better decipher the physiological  
2715 roles of NALCN, and eventually treat NALCN-related pathologies. This review highlights  
2716 how the use of various and complementary animal models, mainly *M. musculus*, *C. elegans*,  
2717 and *D. Melanogaster*, revealed the precise involvement of NALCN in key physiological  
2718 functions including respiratory and circadian rhythms, sleep, nociception,  
2719 gastrointestinal motility, parturition and locomotion (**TABLE 3**). These animal and cell  
2720 models led to the identification of pathways that regulate the NALCN channelosome both  
2721 at the transcriptional and functional levels (**TABLES 2 & 10**). Last but not least, genetic  
2722 diseases resulting from mutations within the NALCN channelosome-encoding genes have  
2723 been described as well as possible associations with other human diseases (**TABLES 5-9**;  
2724 **TABLES 12**), increasing our understanding of NALCN functions.

2725  
2726 Overall, an increasing number of studies point to major roles of the NALCN  
2727 channelosome in physiology, as well as its involvement in human diseases when  
2728 dysfunctional. Addressing some of the remaining questions in the near future (pictured  
2729 in **FIGURE 11**) will further increase our knowledge of how NALCN contributes to the  
2730 fundamental physiological process of cell excitability.

2731  
2732 *Perspectives offered by some unresolved issues*

2733  
2734 Most of the recent work identified how *NALCN* is important in many neurophysiological  
2735 processes. However, NALCN is also expressed in the heart, aorta and endocrine glands  
2736 where its physiological roles have not yet been described (56). Deciphering the  
2737 physiological roles of NALCN in non-neuronal tissues requires further investigation, and  
2738 may ultimately lead to important novel discoveries regarding NALCN functions. The  
2739 availability of animal models, especially mice in which *Nalcn* expression may be  
2740 modulated in a Cre-dependent manner now allows research that can fill gaps in our  
2741 knowledge. Other vertebrate animal models such as zebrafish could also be relevant for  
2742 the study of physiological function of the NALCN channelosome. Indeed, zebrafish with  
2743 predicted loss-of-function alleles for *nalcn*, *unc-79*, *unc-80* and *nlf-1/fam155a* (but not *nlf-*  
2744 *1/fam155b*) are now available (<https://zebrafish.org>).





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2756 NALCN regulates cellular excitability in the pituitary somatolactotroph GH3 cell  
2757 line (76), as well as the β-pancreatic MIN6 cell line (56), and is consequently involved in  
2758 hormone release. In MIN6 cells, NALCN behaves as an acetylcholine-activated Na<sup>+</sup> current  
2759 through activation of the M3 muscarinic receptor, without detectable leak channel  
2760 activity. Similarly, activation of NALCN by leptin, without a background/constitutive Na<sup>+</sup>  
2761 leak current, is described in leptin receptor-expressing neurons of type 1 in the nucleus  
2762 of the solitary tract (73). A hypothesis that has been put forward is that the leak activity  
2763 of NALCN could result from basal activity of src kinase (19). However, pharmacological  
2764 inhibition of this kinase does not affect the Na<sup>+</sup> leak current generated by NALCN when  
2765 expressed in *Xenopus* oocytes (30). Why NALCN acts as a leak channel in some cell types  
2766 and as a receptor-activated channel in others is yet not understood.

2767  
2768 Electrophysiological recordings in endocrine pituitary cells also revealed a  
2769 correlation between a Na<sup>+</sup>-independent conductance and the expression level of NALCN  
2770 (76). Whether NALCN conducts this Na<sup>+</sup>-independent conductance or impact on other  
2771 channels' function, as could be the case through allosteric modulation, is currently  
2772 unknown. NALCN current is also described to be both positively and negatively regulated  
2773 by few G proteins-coupled receptors (GPCRs). Whether other GPCRs or members of other  
2774 families of membrane receptors may modulate NALCN current and the underlying  
2775 mechanisms is a crucial question towards understanding NALCN physiology. The  
2776 possibility to functionally express NALCN in recombinant systems now allows for a  
2777 candidate-based approach to identify NALCN-coupled GPCRs.  
2778

2784 Several recent studies have identified a voltage-dependent component of the  
2785 NALCN current (30, 45, 94), contrasting early reports on NALCN current described as a  
2786 pure 'ohmic' leak current (2). This novel finding is supported by the non-linear current-  
2787 voltage relationship and the time-dependent decay in NALCN current amplitude,  
2788 measured during a test-pulse protocol, and attributable to a deactivation process (*see*  
2789 **FIGURE 6**). These findings should stimulate further studies of the gating mechanism for  
2790 NALCN. The voltage-dependent mode *versus* the ohmic mode of NALCN gating may  
2791 possibly depend on cellular environment, or on the complex organization and regulation  
2792 of the NALCN channelosome. It could also be affected by genetic mutations as reported in  
2793 Bouasse *et al* 2019 (94). The physiological relevance of a 'voltage-gated' NALCN activity  
2794 is not yet known but could likely impact NALCN's contributions not only to RMP, but also  
2795 membrane excitation.

2796  
2797 Of note, instead of a "depolarizing" role, the NALCN channelosome is also proposed  
2798 to have a "hyperpolarizing" function in myometrial cells through functional coupling with  
2799 SLO2.1, a Na<sup>+</sup>-activated K<sup>+</sup> channel (104). In this context, Na<sup>+</sup> ions entering the cell  
2800 through NALCN act as a second messenger that activate SLO2.1 causing a negative shift in  
2801 the membrane potential. This raises the possibility that this process also occurs in other  
2802 cell types including neurons, and possibly involves additional Na<sup>+</sup>-activated K<sup>+</sup> channels.

2803  
2804 Further characterization of the proteins that belong to the NALCN channelosome  
2805 and regulate its function is also needed, as well as the identification of post-translational  
2806 modifications. NALCN associates with at least UNC-79, UNC-80 and NLF-1/FAM155A (or  
2807 NLF-1/FAM155B) whose properties and roles are still poorly understood. Whether these  
2808 proteins have NALCN-independent functions and whether additional proteins contribute  
2809 to the NALCN complex are important questions that need to be addressed. Indeed, CaM  
2810 and Endomucin were recently proposed to also belong to the NALCN channelosome  
2811 complex (31, 46, 328). However, such biochemical studies of NALCN are hampered by the  
2812 lack of specific antibodies to immuno-precipitate NALCN (as well as UNC-79, UNC-80 and  
2813 NLF-1/FAM155A and NLF-1/FAM155B). Some new commercially available antibodies  
2814 that recognize native NALCN and the development of a GFP-tagged NALCN mouse model  
2815 (49) will make it possible to explore its interactome and post-translational modifications  
2816 in different tissues and cell types. These approaches might also provide clues as to why  
2817 NALCN behaves as leak channel in some cell types and not in others, why it exhibits a  
2818 voltage-sensitivity in some conditions and not in others, and whether the NALCN  
2819 channelosome could interact with other channels and receptors.

2820  
2821 With respect to NLF-1/FAM155, the physiological relevance of 2 interchangeable  
2822 NLF-1/FAM155 (A and B) paralogues is another unresolved question (**FIGURE 11**). Since  
2823 the 2 corresponding mRNA do not seem to be expressed in the same regions of the body  
2824 (<https://www.gtexportal.org>), a comparative proteomics study could indicate whether  
2825 these 2 proteins interact differently with other proteins. Considering the 3D structure of  
2826 the NALCN channelosome, it is also conceivable that NLF-1/FAM155A and NLF-  
2827 1/FAM155B proteins could differentially interact with extracellular matrix proteins  
2828 similarly to the  $\alpha_2\delta$ -1 subunit of Ca<sub>v</sub> channels (357).

2829  
2830 NALCN is critically missing selective pharmacological blockers. Some blockers of  
2831 NALCN channelosome function have been tentatively described, however these molecules  
2832 are not specific and exhibit a low affinity for NALCN. The identification of specific

2833 compounds able to activate or inhibit NALCN currents is an important objective not only  
 2834 for providing tools for physiological studies but also as potential therapeutic agents. This  
 2835 is especially true for the CLIFAHDD syndrome, which is induced by gain-of-function  
 2836 mutations in NALCN. Indeed, partial inhibition of gain-of-function pathogenic variants of  
 2837 NALCN in patients with the CLIFAHDD syndrome is predicted to have beneficial effects.  
 2838 The possibility to functionally express NALCN in cell lines such as HEK-293T cells paves  
 2839 the way for setting up of high throughput pharmacological screening assays, for example  
 2840 by using automated patch-clamp devices (358). The Cryo-EM structure of the NALCN  
 2841 channelosome also allows for simulation and machine learning strategies towards drug  
 2842 design (359).

2843  
 2844 Finally, an important and stimulating question to address is life expectancy of  
 2845 living organisms with NALCN channelosome dysfunction. Remarkably, NALCN  
 2846 channelosome dysfunctions do not affect life expectancy in both *C. elegans* and *D.*  
 2847 *melanogaster* while *Nalcn* and *Unc-80* knockout mice die within 24h after birth from  
 2848 respiratory defects. There is also a great variability in the severity of the syndromes in  
 2849 patients carrying different loss- and gain-of function mutations of *NALCN*, or loss-of-  
 2850 functions of *UNC-80* (TABLES 5,6,8). This probably reflects the different importance of  
 2851 NALCN channelosome function across species, or the existence of specific compensatory  
 2852 mechanisms in some species/individuals but not in others.

2853  
 2854 *Perspectives*

2855  
 2856 Research on NALCN has progressed significantly, and the importance of this channel in  
 2857 physiology and disease is clear. Though many questions remain unanswered, continued  
 2858 efforts and the development of new tools are set to increase our understanding of how  
 2859 NALCN contributes to cell excitability, a fundamental and finely balanced physiological  
 2860 process, and how its dysregulation and contributions to disease can be mitigated.

2861  
 2862  
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2881

2882 **DISCLOSURES**

2883

2884 The authors have no conflicts of interest, financial or otherwise, to disclose.

2885

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