# Review of: "Structure-guided unlocking of NaX reveals a non-selective tetrodotoxin-sensitive cation channel"

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In the human genome, ten genes encode distinct Na channels, that is, nine voltage-gated channels ($Na_v1.1$–$Na_v1.9$) and one atypical Na channel, which has been designated as $Na_X$ because it was thought to be voltage-independent and also because of its previously unknown physiological function. Although most Na channel types are expressed in multiple tissues, they are often classified by the tissue in which they are most abundantly expressed. $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$ are highly expressed in the central nervous system (CNS). $Na_v1.5$ is highly expressed in the heart and has also been reported to be present in limbic regions of the brain. $Na_v1.4$ is expressed in adult skeletal muscle. $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ are highly expressed in the peripheral nervous system (PNS)**(DOI: 10.2174/187152708784083830)**..

$Na_V$ channels consist of one α-subunit (260 kDa) that is comprised of four homologous domains termed DI-DIV, each of which contains six transmembrane helices called S1-S6 and a hairpin-like P-loop located between S5 and S6. The short linkers connecting S5 and S6 form the outer narrow mouth of the pore and the selectivity filter, while the inner wider pore is formed by the S5 and S6 helices. The S4 segments in each domain contain positively charged amino acids that form the voltage-gated sensors, which act as gating charges and move across the membrane to trigger channel activation in response to membrane depolarization. The short cytoplasmic intracellular loop connecting homologous domains III and IV acts as the inactivation gate, which bends back into the channel and blocks the pore from the inside during membrane depolarization. The inactivation gate contains a three-amino-acid cluster (isoleucine, phenylalanine, and methionine; IFM) that helps stabilize the inactivation particle.

In addition to the major α-subunit, most voltage-gated Na channels have one or more regulatory β-subunits. At least four types of β-subunits have been identified (β1-β4). In general, the auxiliary β-subunits are 22-36 kDa proteins with a single transmembrane-spanning segment, a long glycosylated extracellular N-terminus containing an immunoglobulin-like motif, and a short intracellular C-terminus domain. β-subunits play an important role in the cell surface expression of Na channels and the regulation of their gating kinetics. They are widely expressed in the CNS, PNS, and heart.

$Na_X$ is an atypical Na channel whose gating activity is Na concentration-dependent but not voltage-dependent**(DOI: 10.1038/379257a0)**. It is expressed in many tissues, including the uterus, heart, CNS, PNS, and skeletal muscle. It plays an important role in salt homeostasis by sensing extracellular Na concentrations in the CNS **(DOI: 10.1523/JNEUROSCI.20-20-07743.2000)**..

In the CNS, the $Na_X$ channel is expressed in the circumventricular organs (CVO) and is the specific Na receptor for sensing extracellular Na concentrations **(DOI: 10.1038/nn0602-856)**. Other studies carried out in the laboratory of the late Dr. Didier Mouginot at Université Laval showed that it is expressed in median preoptic nucleus (MnPO) neurons and plays

an important role in sensing Na concentration fluctuations in the cerebrospinal fluid (CSF) **(DOI: 10.1523/JNEUROSCI.3720-03.2004)**. Transgenic mice that do not express $Na_X$ are unable to control salt intake, suggesting that this channel regulates salt-intake behaviour **(DOI: 10.1523/JNEUROSCI.20-20-07743.2000).**

It has been reported that this channel is tetrodotoxin (TTX)-resistant. TTX is known to bloc $Na_V$ channels. As $Na_X$ has not been expressed in heterologous expression systems, including *Xenopus* oocytes and several mammalian cell lines, unlike $Na_V$ channels, it does not appear to encode functional Na channels with typical activation and inactivation kinetics. The lack of Na currents is likely due to sequence differences in three functionally important regions. First, compared with other voltage-gated Na channels, $Na_X$ is characterized by fewer positive charges in the S4 segment, which acts as a voltage-gated sensor. Second, the IFM inactivation particle is replaced with IFI. Third, the selectivity filter motif (aspartate, glutamate, asparagine, and alanine (DENA)) is different from the DEKA motif (aspartate, glutamate, lysine, and alanine), which is the selectivity filter locus of voltage-gated Na channels. More importantly, this channel has never been adequately characterized but is clearly a leak channel similar to NALCN channels and has to be biophysically characterized as such.

Noland et al. used CryoEM to investigate the structure of the human $Na_X$ channel complexed with the β3-subunit in a 200 mM Na solution at 3.2Å resolution **(DOI: 10.1038/s41467-022-28984-4)**. The authors did not explain why they chose the β3-subunit rather than the β1-subunit. As expected, the construct used for the CryoEM investigation did not generate the fast-gating kinetics of a typical Na channel nor did it generate leak currents. None of the drugs that are known to activate voltage-gated Na channels, including veratridine, aconitine, and sea anemone toxin (ATX-II), induced currents when $Na_X$ was expressed in *Xenopus* oocytes. In addition, extracellular Na concentrations up to 190 mM did not generate any currents when the channel was expressed in Neuro-2a cells.

The structure shows that the voltage-sensor like domains (VSLD) are organized in a domain-swap fashion. The β3-subunit has a large extracellular structure with a helical transmembrane region that interacts with VSLD3. However, the C-terminal region is not well resolved. The overall structure is very similar to several recently resolved $Na_V$ structures, with several structural deviations that distinguish this channel from $Na_V$ channels **(DOI: 10.1016/j.cell.2019.11.041)**. Since the S4 segments lack positive charges in several domains and in key regions of S4, it is not clear why the authors did not consider investigating the possibility that the voltage-sensor domains could accommodate gating pore currents. Is there water in the voltage-sensor domains? If the voltage sensor is “wetter,” this may mean that Na ions can permeate through the gating pore.

Like $Na_V$ channels, the outer mouth of the pore is considered a selectivity filter even though no Na binding site or an inner pore formed by the S6 segments of each domain (DIS6-DIVS6) were detected. The DIVS6 phenylalanine, which contributes to the binding site for local anaesthetics in $Na_V$ channels, has been replaced with a tryptophan. Like $Na_V$ and $Ca_V$ structures, $Na_X$ has four lateral fenestrations that may accommodate lipids and drugs. One of the surprising findings, for an ion channel, is the presence of four lipids (3 phospholipids and a cholesterol) in the inner pore that seals the S6 gate. The presence of lipids in the pore is not related to the presence of lipids during nanodisc reconstitution given that the authors investigated this issue.

In $Na_V$ channels, the DIII-DIV linker acts as a lid. The IFM particle triggers pore closure through allosteric interactions. In

$Na_X$, the IFM is replaced by IFI, and mutating IFI with QQQ did not generate a current. However, mutating the docking sites, including the cytoplasmic linkers connecting S4-S5 in DIII and the cytoplasmic end of the S6 in DIII, resulted in robust currents, suggesting that the DIII-DIV linker probably restricts S6-dilatation and is responsible for the non-conductive property of the channel construct used in this study. Mutating three key residues (F724Q-I1189T-T1492T; or QTT) resulted in robust currents when expressed in *Xenopus* oocytes, while a single mutation of these residues failed to generate currents. The authors suggest that the QTT triple mutation induces wetting of the pore and unlocks the permeation pathway. Why the wild-type $Na_X$ channel does not generate currents when expressed in heterologous expression systems is still an open question. Does the channel require an interacting protein to be functional? Chimera channels in which one or several domains from $Na_V1.7$, a channel with known sD structure and $Na_X$ were swapped did not exhibit fast activating and inactivating currents but resulted in robust leak currents when $Na_V1.7$/DIII was part of the chimera.

In $Na_V$ channels, the narrowest part of the pore acts as the selectivity filter, which is composed of four amino acids (the DEKA locus). In $Na_X$, the DEKA selectivity filter locus is replaced by DENA in which the lysine is replaced by asparagine and may result in a wider selectivity filter compared, for example, to $Na_V1.7$ as the structure suggests. Since QTT generated robust currents, the authors used this triple mutation to investigate the selectivity of the $Na_X$ channel and its pharmacology. Their results showed that $Na_X$ is a cation ion channel and that it is impermeable to the large organic cation N-methyl-D-glucamine (NMDG), most likely due to its size. Divalent ions such as $Ca^{2+}$, $Zn^{2+}$, and $Co^{2+}$ blocked $Na_X$ currents. The trivalent ion $Gd^{3+}$ also inhibited $Na_X$ currents. Unexpectedly, TTX inhibited $Na_X$ currents unlike in *in vitro* studies. Other $Na_V$ blockers such as lidocaine, quinidine, and loperamide also inhibited $Na_X$ currents.

This study has helped demystify the $Na_X$ structure. However, further studies are warranted to elucidate the permeability and physiological role of this channel and to develop drugs that target the channel.