Supplemental Figure 1. Models predicting the location of residue 575 in rNCC or 576 in flNCC. A) Model of the putative α-helix formed by TM segment 11 of rNCC. Serine 575 (red number) lies in the hydrophilic face of the helix; B) According to the hydropathy analysis, serine 575 of rNCC lies approximately in the middle of this TM segment (2); C) Structural model of the central hydrophobic region of rNCC generated by the FUGUE server. Top view from the extracellular side of the plasma membrane showing that TM segments 1-10 form a core structure (orange) similar to the one observed in ApcT and LeuT, outside of which lies TM segments 11 and 12 (magenta). The residue corresponding to serine 575 in rNCC or cysteine 576 in flNCC is depicted in cyan. D) Side view from the membrane of the model shown in panel C. The protein is depicted with the extracellular side facing upward.

We used two different computational programs that allowed us to recognize distant homologues by sequence-structure comparison. Models obtained from the FUGUE server (7) for the 12 transmembrane helix segments showed a ZSCORE of 10.2 for rNCC and 9.7 for flNCC, which is above the recommended cutoff value of 6.0 (99% confidence). The best models were constructed using the transmembranal region of the Protein Data Bank (PDB) coordinates 3GIA as template, which corresponds to the proton-coupled broad-specificity amino acid transporter ApcT (AAB98602). ApcT is a bacterial protein belonging to the superfamily of APC transporters (amino acids, polyamine, organocation) that comprises several families of solute carriers, in which SLC12 family is included, and functions in the proton-coupled transport of a wide range of amino acids (6). The Phyre server (4) found structural homology with the transmembrane region of the PDB 2QJU coordinates, corresponding to the bacterial leucine transporter LeuT (NP_214423), a

bacterial homologue of eukaryotic Na^+:Cl^--dependent neurotransmitter transporter in the solute carrier family SLC6 (1; 3; 8). Models from this server displayed an estimated precision of 100% (E-value 3.7e-10 for rNCC and 1.1e-12 for flNCC).

The crystal structures of LeuT and ApcT reveal a remarkable structural similarity between them (5). They both have 12 TM segments, intracellular amino and carboxyl-termini, and exhibit an internal structural repeat in the first ten transmembrane helices. Segments 1 to 5 and 6 to 10 share a similar conformation, and these two penta-helical repeats form a cylindrical barrel: the core structure of the transporters which contains the ion translocation pathway. TMs 11 and 12 reside on the outside of this core (6; 8). Structural homology is not restricted to general fold features as reported by Shaffer and coworkers (6). The resemblance between very distantly related transporters supports the possibility that a structural similarity may also exist between these transporters and NCC, and support the model construction. In fact, NCC is more closely related to ApcT than LeuT since they both belong to the APC superfamily (5; 6). Superposition of 320 alpha carbons of the TM regions of ApcT and LeuT displays a large root mean square deviation (RMSD) of 22.8 Å. Nevertheless, superposition of rNCC models produced by FUGUE and Phyre servers show a RMSD of 6.0 Å (364 alpha-carbon atoms, not considering loops, not modeled regions and chain termini). A similar superposition of flNCC models produced a RMSD of only 4.9 Å (356 alpha-carbon atoms). These low RMSD values suggest convergence of the models despite the fact that the servers used different algorithms for homology detection and two templates that display a large RMSD between them.

We are aware of the limitations of generating models based on structural data of distantly related proteins, but the observed similarity between LeuT and ApcT suggests that expecting a similar structure in NCC is sound. This is supported by a recent thorough

analysis of the structural conservation between sodium transporters (5). The structural models for rNCC and flNCC have, as expected, a similar organization to LeuT and ApcT. The model suggests that the first ten TM segments form part of a core structure, and TM11 and 12 lie outside (colored magenta). We are aware that the models constructed cannot be used to predict the exact position of the serine/cysteine residue, and that they can only give us information about the general disposition of the TM segments. So the cysteine in this position could be forming an interaction either with another residue within the same polypeptide chain or located in the second monomer of the NCC homodimer.

Reference List

1. **Chen NH, Reith ME and Quick MW**. Synaptic uptake and beyond: the sodium- and chloride-dependent neurotransmitter transporter family SLC6. *Pflugers Arch* 447: 519-531, 2004.
2. **Gamba G, Saltzberg S N, Lombardi M, Miyanoshita A, Lytton J, Hediger M A, Brenner B M and Hebert S C**. Primary structure and functional expression of a cDNA encoding the thiazide-sensitive, electroneutral sodium-chloride cotransporter. *Proc Natl Acad Sci USA* 90: 2749-2753, 1993.
3. **Hediger MA, Romero MF, Peng JB, Rolfs A, Takanaga H and Bruford EA**. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. *Pflugers Arch* 447: 465-468, 2004.
4. **Kelley LA and Sternberg MJ**. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* 4: 363-371, 2009.

5. **Krishnamurthy H, Piscitelli CL and Gouaux E**. Unlocking the molecular secrets of sodium-coupled transporters. *Nature* 459: 347-355, 2009.
6. **Shaffer PL, Goehring A, Shankaranarayanan A and Gouaux E**. Structure and mechanism of a Na+-independent amino acid transporter. *Science* 325: 1010-1014, 2009.
7. **Shi J, Blundell TL and Mizuguchi K**. FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. *J Mol Biol* 310: 243-257, 2001.
8. **Yamashita A, Singh SK, Kawate T, Jin Y and Gouaux E**. Crystal structure of a bacterial homologue of Na+/Cl--dependent neurotransmitter transporters. *Nature* 437: 215-223, 2005.

Supplemental Figure 1