Published in final edited form as:

Pflugers Arch. 2016 March ; 468(3): 503–511. doi:10.1007/s00424-015-1783-4.

Chloride Requirement for Monoamine Transporters

Louis J De Felice

Physiology and Biophysics, Virginia Commonwealth University, School of Medicine, Richmond VA 23298, 804 980 5902, louis.defelice@vcuhealth.org

Abstract

This review focuses on the Cl^- requirement for dopamine, serotonin, and norepinephrine (DA, 5-HT, and NE) transport and induced current via the transporters for these transmitters, DAT, SERT, and NET. Indirect evidence exists for the passage of Cl^- ions through monoamine transporters; however, direct evidence is sparse. An unanswered question is why in some preparations, notably native neurons, it appears that Cl^- ions carry the current through DAT, whereas in heterologous expression systems Na^+ ions carry the current often referred to as the uncoupled current. It is suggested that different functional states in monoamine transporters represent conformational states that carry dominantly Cl^- or Na^+ . Structures of monoamine transporters contribute enormously to structure-function relationships; however, thus far no structural features support the functionally relevant ionic currents that are known to exist in monoamine transporters.

Keywords

Transporters; Dopamine; Serotonin; Norepinephrine; Chloride; Sodium; Channels

Introduction

In a seminal article published in 1977, Gary Rudnick used plasma membrane vesicles isolated from human platelets to study how electrical potential and Na^+ gradients influenced 5-HT uptake via hSERT (62). These data provided direct evidence for Na^+ coupled 5-HT transport via hSERT, and they made the additional point that transport is absolutely dependent on external Na^+ and Cl^- . Subsequent research by Rudnick and colleagues reinforced the notion that monoamine transporters in general share a functional dependence on Na^+ and Cl^- . Ion dependence was measured by replacing NaCl with either LiCl or Na-isethionate and using titrated DA (for DAT and NET) and titrated 5-HT (for SERT) to measure uptake in model preparations. DA, and 5-HT transport as a function of ion concentration were well described by 1st order functions of Na^+ and Cl^- in all cases except DAT, whose dependence on Na^+ was sigmoidal (Figure 1). The conclusion reached was that 1 Na^+ and 1 Cl^- are needed per transmitter for SERT and NET, but 2 Na^+ and 1 Cl^- for DAT transport. Whereas K_M values for Na^+ were similar among the three transporters, the K_M for Cl^- varied markedly and was much lower for NET (28). This work resolved into what became the standard model for monoamine transporters, in which ionic gradients are coupled to monoamines in fixed ratios to drive neurotransmitters against their own gradient (27–34, 63, 64).

To this day the fixed stoichiometry, alternating access model of neurotransmitter transport prevails. For SERT the standard model took an extreme form in which the influx of 5-HT together with Na^+ and Cl^- in a 1:1:1 ratio is coupled to the efflux of one K^+ ion, rendering SERT electroneutral (66). SERT is not electroneutral, yet the electroneutral model for SERT and electrogenic models for DAT and NET have dominated the field for decades (67, 85). The standard model for monoamine transporters, which includes the Cl^- ion in virtually all publications and textbooks, leaves many questions unanswered. In this review we address primarily one aspect: do Cl^- ions actually permeate monoamine transporters, and if so is Cl^- coupled in fixed stoichiometry to transmitter uptake, or does Cl^- flow in an uncoupled channel mode as in glutamate transporters? The subject of neurotransmitter transport mechanisms and their role in synaptic transmission is quite broad and some topics are not covered here. I refer readers to previous reviews in this area (3, 7, 19, 43, 48, 65, 78, 79).

Text Figure

To begin, it may be interesting to note the relative size of the Cl^- ion compared to other ions and to dopamine, the principle monoamine neurotransmitter addressed in this review. See Text Figure: Space filling models of DA, Na^+ , Ca^{++} , K^+ , and Cl^- ions on the same scale. The Cl^- ion has a Pauling radius of 1.81 Angstrom. The benzene ring has an approximate radius of 2 Angstroms. *The Nature of the Chemical Bond*, Linus Pauling, 3rd ed. 1960, Cornell University Press.

Literature Survey

Recent structural models for monoamine transporters have bolstered the fixed stoichiometry, alternating access model with one substrate-binding site, one Cl^- binding site, and one or two Na^+ binding sites (41, 50, 51, 58, 93). The homology model used for monoamine transporters is the bacterial analogue, LeuT, which does not require the Cl^- ion for transport though a Cl^- binding site exists in LeuT. A structure for the *Drosophila* dopamine transporter, dDAT also has Na^+ and Cl^- binding sites (50, 51, 90).

Monoamine transporters

Figure 1 is from an early paper by Gu et al. that neurotransmitter transport through monoamine transporters depends on Na^+ and Cl^- ions (28). Na^+ dependence is sigmoidal only for DAT, whereas all other dependences are hyperbolic. Fits to the data assume a requirement for 2 Na^+ and 1 Cl^- ion for DA transport, and 1 Na^+ and 1 Cl^- ion in all other cases. Although the Hill coefficient is commonly used to estimate the stoichiometry of ligands to receptors for functional effect, the Hill equation does not reliably verify unique reaction schemes. Instead the Hill coefficient measures cooperativity among multiple binding sites (92). Nevertheless, it is remarkable that available structures for Na-dependent

transport show 2 Na⁺ binding sites (9, 40, 74, 89, 90) in apparent agreement with the ligand-receptor theory used by Gu et al. for Na⁺-transporter interactions in DAT.

Amino acid transporters

As an aside, note that excitatory amino acid transporters have been known for over 20 years to embody an authentic Cl⁻ channel, in which the protein responsible for Na⁺ coupled glutamate transport can operate both as a neurotransmitter carrier and as a Cl⁻ ion channel (4, 23, 38, 42, 86–88). Recently, Fahlke and colleagues have combined molecular simulations with a structural model of the glutamate transporter and electrophysiology (94, 95) to show how Na⁺ coupled, secondary active transporters can function normally with practical effect as both traditional carriers and as ion channels (46). Mammalian glutamate transporters thus transport the neurotransmitter through Na⁺ coupling but also conducts Cl⁻ ions through a separate pathway. The popular model for monoamine transporters, LeuT, has a canonical binding pocket for leucine and two adjacent Na⁺ binding sites. In WT LeuT, transport is Cl⁻ independent. A negative amino acid mutation near either Na⁺ binding site in GAT-1 renders GABA flux Cl⁻ independent. Equivalent mutations in LeuT render substrate binding and uptake Cl⁻ dependent, and reciprocal mutations in hDAT also result in Cl⁻ independent transport. Thus the Cl⁻ ion, or a negative charge on a residue near a Na⁺ binding site, are required during binding and uptake. This has been attributed to counterbalancing the charge of the co-transported Na⁺ ions (96). Unlike glutamate transporters, the monoamine transporters do not share the well-supported ion channel Cl⁻ pathway that has been demonstrated for amino acid transporters.

Dopamine transporters

In 2008 Galli and colleagues published a comprehensive analysis of hDAT including its dependence on the Cl⁻ ion (22). DA transport requires extracellular Cl⁻, which is indirectly supported by the structural models for transport and the functional dependence on Cl⁻ (24, 96). Functional interpretations of these structural models propose that Cl⁻ balances Na⁺ binding and leads to subsequent DA binding. The question remains open as to whether Cl⁻ actually moves through the transporter or whether it is merely regulates transport. In a version of the hypothesized hDAT stoichiometry, Galli and colleagues show that extracellular and intracellular Cl⁻ ions modulate transport, but they cast doubt on the standard model and Cl⁻ involvement in DAT mediated transport. Erreger et al. (22) propose a kinetic model to explain the observed fast transient and steady state response to AMPH, in which external Cl⁻ binds to the empty transporter followed by Na⁺ and AMPH binding. The fully loaded carrier transports AMPH, Na⁺, and Cl⁻ to the inside; if Cl⁻ remains bound to DAT it returns the transporter and renews the cycle. In this model, an empty carrier does not return.

Figure 2 from Ingram et al. makes a strong case that ionic currents through DAT in dopaminergic neurons are large enough to affect excitability. In these neuronal DA transporters, but not in heterologous hDAT expression systems (60), the current carrying ion appears to be Cl⁻ (36), which holds true when amphetamine is the substrate instead of DA. As regards the involvement of Cl⁻, these data are consistent with experiments on the *C. elegans* DA transporter (14). In both cases I(V) curves reveal that in the absence or presence

of 10 μM external DA, the reversal potential of the DA-induced current occurs near the expected Cl^- reversal potential.

Figure 3 is an analysis of DA and an amphetamine (AMPH) enantiomer that induces currents through hDAT expressed in *Xenopus laevis* oocytes. **(A)** DA or the enantiomer *R*(-)-AMPH induce a peak current after application; note, however, that both of these induced currents display a lazy return, which is not attributable to perfusion artifact. However, after removing *S*(+)-AMPH, the *S*(+)-AMPH induced current persists though at a lower level compared with the peak. The transporter appears to be locked in an open state in the absence of external *S*(+)-AMPH. Subsequent experiments supported the notion that *S*(+)-AMPH remains inside the cell to interact with the transporter (60). Cocaine blocks both the peak current and the persistent current. However, rather than returning the current to baseline, cocaine induces an apparent outward current because it blocks an endogenous hDAT leak, whose presence is independent of substrate (76). **(B)** Baseline subtracted *I*(*V*) curves for DA, *S*(+)-AMPH peak, and *S*(+)-AMPH persistent current. The *S*(+)-AMPH peak *I*(*V*) is shifted to the left compared with the DA peak. The *S*(+)-AMPH persistent current *I*(*V*) is not only smaller but shifted to the left compared with peak currents, consistent with the absence of *S*(+)-AMPH and presence of Cl^- ions flowing through hDAT (60). Although these interpretations do not prove Cl^- involvement, the data point to different functional states of hDAT with distinct *I*(*V*) characteristics and raise the possibility of separate pathways for Na^+ and Cl^- ions through DAT depending on the state of the transporter.

Zn⁺⁺ effect

Pifl et al. have studied how Zn^{++} regulates DAT. Low μM Zn^{++} decreases DA uptake in DAT transfected HEK293 cells but stimulates DA uptake in SK-N-MC cells. Phorbol-12-myristate-13-acetate, an activator of pKC, attenuated Zn^{++} inhibition in HEK cells and increased stimulation in SK-N-MC cells. Low external Cl^- switched Zn^{++} induced uptake stimulation to inhibition, whereas depolarization switched inhibition to stimulation. Thus both membrane potential and Cl^- regulate Zn^{++} modulation of DAT (53–55, 57).

DA release

Using rapid flow rates, Stigas et al. investigated depolarization-induced DA release from preloaded ^3H -DA rat striatal synaptosomes (75). In the low μM range, external DA releases the preloaded transmitter via a Ca^{++} independent but Cl^- dependent mechanism. Bupropion (also known as Wellbutrin, is an atypical antidepressant) and nomifensine (a NE and DA uptake inhibitor) block DA-induced DA release. Bupropion itself also releases DA, and nomifensine blocks this release, implicating DAT in DA-induced DA release. Depolarization also releases DA and release is Ca^{++} dependent, but depolarization induced DA release is Cl^- and nomifensine insensitive. DA induced and depolarization induced DA release are additive. Curiously, *reducing* external Cl^- *increases* DA release, and this release is blocked by nomifensine but is barely affected by the absence of external Na^+ . One conclusion is that two DA release mechanisms exist: DA or bupropion induced released are DAT dependent, but depolarization induced release is not. This conclusion is partially supported by the differential dependence of release on Cl^- . Unpublished data from Joel

Schwartz (Fig. 34) shows low external Cl^- concentrations increase substrate binding to hNET (see Figure 5 and the discussion below).

Substituting external Cl^- with isethionate increases spontaneous efflux of DA from rabbit striatal slices preloaded with ^3H -DA, and reducing external Ca^{++} increased low- Cl^- induced DA efflux. Depleting vesicular DA with reserpine resulted in the same inverse relationship between external Cl^- and DA efflux, and nomifensine and other DAT blockers increased this efflux in reserpine treated preparations. Low Cl^- also inhibited initial DA uptake rates. Thus, low Cl^- produces DAT dependent, non-exocytotic DA efflux. DAT blockers on the other hand are unaffected by low Cl^- (21). However, although Na^+ inhibits the substrates octopamine or tyramine, Cl^- reverses this inhibition (44).

It is thus proposed that DAT not only mediates DA uptake but also its efflux. DAT mediated DA efflux has an apparent DA affinity that is $300\times$ lower than for uptake. Increasing external DA or AMPH, or lowering external Na^+ or Cl^- , increases efflux (37). hDAT and hNET have similar functional profiles, but symmetric changes in their N- or C-terminals reveals Cl^- dependent transport linked to the C-terminal of hNET; swapping C-terminals modified Na^+ Hill values, which are close to $n = 2$ for hNET and hDAT. The N-terminal supports differences between uptake dependence on Na^+ and Cl^- , but the C-terminal plays the major role in Cl^- and Na^+ ion dependence (81, 82). DA release via DAT or NET loaded with metabolically stable ^3H 1-methyl-4-phenylpyridinium shows that DA, NE, or AMPH induced release is modulated in low external Na^+ or Cl^- . In low Na^+ (DAT: 10 mM; NET: 5 mM), no substrate could induce substrate release, contrary to Itokawa et al. (37). In low Cl^- (DAT: 3 mM; NET: 2 mM), all substrates were able to stimulate release, but release was similar in both transporters at low Na^+ or Cl^- concentrations (56). Summarizing, little evidence exists that the Cl^- gradient is energetically coupled to the buildup of a DA, 5-HT, or NE gradient. If the regulatory role of Cl^- ions for transport were correct, structural models suggest fixed Cl^- binding, but not necessarily authentic Cl^- flux.

β -Phenylethylamine

A plethora of papers using a variety of techniques provide indirect evidence for Cl^- permeability through monoamine transporters. β -Phenylethylamine (β PEA) is a trace amine found in the mammalian CNS and has been suggested as a neurotransmitter that mimics the effect of AMPH. β PEA activates DAT but, relevant to this review, it rapidly activates large amine-gated Cl^- channels, LGC-55. In *C. elegans* AMPH potentiates β PEA effects on LGC-55 in vitro and in vivo (13, 68). The possibility arises that Cl^- currents - apparently through DAT - may be an indirect effect on separate bona fide Cl^- channels. In DAT transfected *Xenopus* oocytes, Li^+ substituting for Na^+ induces $10\times$ larger substrate-induced currents, and mutating Na^+ coordinating sites suggests that Li^+ interacts with Na2 rather than the Na1 binding. Cl^- regulates the Li^+ leak, further suggesting that Li^+ lowers Na2 affinity, because DAT mutations that reduce Na2 affinity increase Na^+ permeability above Li^+ permeability, suggesting a functional connection between bound Cl^- and the Na2 site (8).

α -synuclein

In a heterologous expression system, α -synuclein forms a stable complex with DAT. In whole cell patch recordings, DAT-mediated currents reveal intracellular α -synuclein stimulates a Na^+ independent but Cl^- sensitive current that is blocked by GBR12935. A fluorescent substrate, 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP^+) (70, 72, 73) may be used to monitor real-time DAT function; ASP^+ data show that α -synuclein decreases the rate and amplitude of DAT uptake but does not decrease initial substrate binding at the plasma membrane. Thus DAT/ α -synuclein interactions result in Na^+ insensitive, Cl^- sensitive inward currents through DAT, albeit with decreased uptake (80).

Serotonin transporter

dSERT has considerable amino acid sequence identity with the mammalian transporters, SERT (51%), NET (47%), or DAT (47%). Transient expression in mammalian cells shows that 5-HT uptake in dSERT does not depend on extracellular Cl^- , but increasing extracellular Cl^- facilitates Na^+ dependent uptake. Antidepressants are 3–300 $\times$ less potent on dSERT than on hSERT, but mazindol has 30 $\times$ greater potency for dSERT. Cocaine or RTI-55 inhibited dSERT mediated 5HT uptake at 500 nM but 10 μM DA, NE, or other amines did not inhibit transport (20).

Henry et al. combined transporter mutations with molecular modeling to uncover a single residue in TMD 1 that couples Cl^- binding to Na^+ dependent neurotransmitter uptake (35). I(V) curves of hSERT leak currents, revealed by the SERT antagonist, RTI-55 and N101A mutants, show that leak currents are independent of Cl^- ions. In hSERT, Cl^- binding is essential for Na^+ -coupled 5-HT transport. Asn101 mutants have dramatically reduced uptake; however, they do not require Cl^- for 5-HT transport. Cl^- substitution with acetate, methanesulfonate, or gluconate correlate with their Stokes radii and anion selectivity, consistent with previously proposed Cl^- -binding sites that predicted Asn101 extends into the substrate-binding pocket. Interaction of Asn101 with Asn368 and indirectly with Ser336 through the NA1 site suggested an interaction between TM1 and TM6 in ion coupled substrate flux, and consequently that Cl^- removal would be less disruptive in Asn101 mutants. In bacterial, insect, and mammalian family members, replacing hSERT Asn101 with His, Ala, Cys, Gly, Thr, Ser, or Asp (6) suggest this residue as critical in coordination of Na^+ , Cl^- , and substrate. The 7 $\times$ increase in Na^+ flux in hSERT Asn101, and interactions with Syntaxin 1a with the N terminus of SERT (14, 59) may increase both leak and 5-HT induced currents. Thus the Asn residue (or analogous residues) in TM1 provides Cl^- coupling or regulation in SLC6 transporters that use Na^+ gradients for uphill transport and likely determine both Na^+ coupled transport as well as channel modes of neurotransmitter transport

Cl^- ion permeation

Among the internal ions, the dynamic changes in Cl^- concentrations are the least well studied, which is in part due to lack of reliable probes for Cl^- ions. Ingram et al. added a palmitoylation sequence to the Cl^- indicator YFPQS, that partitions the sensor to the plasma membrane (called mbYFPQS) Figure 4: In cultured midbrain neurons, Ingram and colleagues found a subpopulation of neurons that increased mbYFPQS fluorescence in

response to amphetamine that was blocked by the DAT inhibitor, GBR12909, indicating a rise in Cl^- inside axons and dendrites (91). Ingram et al. had previously demonstrated that DAT substrates generate an uncoupled Cl^- current associated with dopaminergic neurons in rat midbrain (36). DATs are expressed not only at synapses but also in axons, cell bodies, and dendrites of dopaminergic neurons. Furthermore, mbYFPQS fluorescence was AMPH concentration dependent in both the soma and in the processes of responding neurons (91).

Cl^- ions and substrate binding

Figure 5 makes use of a fluorescent substrate, ASP^+ (4-(4-(dimethylamino)styryl)-N-methylpyridinium) with micromolar potency for the human norepinephrine transporter, hNET (71–73). ASP^+ interactions distinguish binding from transport and provide temporal and spatial resolution to describe real-time kinetics of ASP^+ binding and uptake. ASP^+ accumulation is Na^+ , Cl^- , cocaine, and desipramine sensitive and temperature dependent. ASP^+ enters the cell and competes with NE uptake. Schwartz et al. demonstrated that NETs are efficient buffers for substrate, with binding rates exceeding transport rates by 100 $\times$. Although transport depends on Na^+ and Cl^- , binding is nearly independent of Na^+ and actually increases in low Cl^- .

Structures and Simulations

Recently Gouaux and coworkers have solved co-crystals of neurotransmitter or psychostimulants with a functional dDAT structure. Structures of dDAT bound to DA, a DA analogue, S(+)-AMPH, S(+)-METH, cocaine, or cocaine analogues revealed that all compounds bind to a central site near bound Na^+ and Cl^- ions. dDAT is thus studied in complex with both substrates and inhibitors. The DA-dDAT structure identifies the catechol ring and amine hydrogen bonds as closing extracellular gates, leading to an occluded state. S(+)-AMPH and S(+)-METH are without the hydroxyl groups present on DA implying hydrophobic interactions with specific residues. These same hydroxyl groups figure prominently in functional differences among DA, R(-)-AMPH, and S(+)-AMPH (45, 60, 83). The inhibitor-bound structures of dDAT indicate that the binding pocket accommodates various compounds by orientation of TM6 and certain side chains (90). However, the study adds little to the existing knowledge of Na^+ or Cl^- binding sites. Another recent paper uses molecular dynamics and a homology model to study DA reuptake via hDAT in atomic level, microsecond, and accelerated simulations (16). In this model external DA binding closes extracellular gates and stabilizes an occluded intermediate state near the binding pocket. Disruption of the pocket enlarges the cavity near bound Na^+ and Cl^- ions. Na^+ dislocation reorganizes salt bridges at the inner hDAT surface and tilts exposed helices, enabling Na^+ , Cl^- , and DA release. However, the configuration switch that completes transport requires forced protonation of D79. While providing some insight into the function of specific residues in hDAT, the support drawn from this simulation for fixed stoichiometry, alternating access transport falls short. (49)

Conclusion

Do Cl^- ions actually permeate monoamine transporters, and if so is Cl^- coupled in fixed stoichiometry to transmitter transport? Data rarely support a direct Cl^- pathway through

monoamine transporters, though review articles assume such a pathway, e.g., (84). Electrophysiological data from neurons, however, provide strong indirect evidence for the passage of Cl^- ions through dopamine transporters (15, 36). Curiously, DATs in neurons appear to support Cl^- currents, whereas DATs in heterologous expression systems appear to conduct Na^+ ions (25, 26, 60), although Cl^- ions may be involved. In particular the 'molecular stent hypothesis' proposes a persistent Cl^- conductance. These data have been challenged as a *Xenopus* oocyte artifact (69); however, the primary observation - a persistent leak after exposure to S(+)AMPH - exists not only in the oocyte expression systems but also in mammalian cells, and R(-)AMPH does not show the same effect. Thus AMPH lipofelicity as proposed in (69) is not a plausible explanation (17).

Existing data thus leads one ask whether Cl^- pathways exist alongside Na^+ pathways beyond their co-existence in the alternating access model. But how could this be? As a possible explanation consider, that DAT and SERT have distinct functional states (47, 60) that may represent different ion-dominant pathways. Functional models with fixed stoichiometric and perfectly coupled transport must be incomplete considering the amply verified reports of leak currents in monoamine transporters (76). Nevertheless, whereas structural data provide a rationale for coupled transport, they provide no evidence as yet for the existence of uncoupled Na^+ or Cl^- pathways in transporters. What then is the nature of monoamine transport mechanisms if leak currents, non-stoichiometric currents, and uncoupled channel-like currents exist? Alternative theories based on flux coupling and frictional models account not only for currents too large to explain by the standard model, but also the observation of single transporter events (1, 2, 10, 15, 19, 26, 52, 77). Finally, we note that channel-like currents through monoamine transporters may help explain the action of amphetamine-like drugs and synthetic cathinones on dopamine and serotonin transporters (5, 11, 12, 18, 39, 61, 67).

REFERENCES

1. Adams SV, DeFelice LJ. Flux coupling in the human serotonin transporter. *Biophys J.* 2002; 83:3268–3282. [PubMed: 12496095]
2. Adams SV, DeFelice LJ. Ionic currents in the human serotonin transporter reveal inconsistencies in the alternating access hypothesis. *Biophys J.* 2003; 85:1548–1559. [PubMed: 12944272]
3. Amara SG. Neurotransmitter transporters: new insights into structure, function and pharmacology. *Rev Bras Biol.* 1996; 56(Su 1 Pt 1):5–19. [PubMed: 9394486]
4. Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J Neurosci.* 1994; 14:5559–5569. [PubMed: 7521911]
5. Baumann MH, Clark RD, Rothman RB. Locomotor stimulation produced by 3,4-methylenedioxymethamphetamine (MDMA) is correlated with dialysate levels of serotonin and dopamine in rat brain. *Pharmacol Biochem Behav.* 2008; 90:208–217. [PubMed: 18403002]
6. Beuming T, Shi L, Javitch JA, Weinstein H. A comprehensive structure-based alignment of prokaryotic and eukaryotic neurotransmitter/ Na^+ symporters (NSS) aids in the use of the LeuT structure to probe NSS structure and function. *Mol Pharmacol.* 2006; 70:1630–1642. [PubMed: 16880288]
7. Blakely RD, DeFelice LJ. All aglow about presynaptic receptor regulation of neurotransmitter transporters. *Mol Pharmacol.* 2007; 71:1206–1208. [PubMed: 17329498]

8. Borre L, Andreassen TF, Shi L, Weinstein H, Gether U. The second sodium site in the dopamine transporter controls cation permeation and is regulated by chloride. *J Biol Chem*. 2014; 289:25764–25773. [PubMed: 25063810]
9. Boudker O, Ryan RM, Yernool D, Shimamoto K, Gouaux E. Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. *Nature*. 2007; 445:387–393. [PubMed: 17230192]
10. Bruns D, Engert F, Lux HD. A fast activating presynaptic reuptake current during serotonergic transmission in identified neurons of *Hirudo*. *Neuron*. 1993; 10:559–572. [PubMed: 8386524]
11. Cameron KN, Kolanos R, Solis E, Glennon RA, De Felice LJ. Bath salts components mephedrone and methylenedioxypyrovalerone (MDPV) act synergistically at the human dopamine transporter. *Br J Pharmacol*. 2013b; 168:1750–1757. [PubMed: 23170765]
12. Cameron KN, Kolanos R, Verkariya R, De Felice L, Glennon RA. Mephedrone and methylenedioxypyrovalerone (MDPV), major constituents of "bath salts," produce opposite effects at the human dopamine transporter. *Psychopharmacology (Berl)*. 2013a; 227:493–499. [PubMed: 23371489]
13. Carvelli L. Amphetamine activates/potentiates a ligand-gated ion channel. *Channels (Austin)*. 2014; 8:294–295. [PubMed: 25478621]
14. Carvelli L, Blakely RD, DeFelice LJ. Dopamine transporter/syntaxin 1A interactions regulate transporter channel activity and dopaminergic synaptic transmission. *Proc Natl Acad Sci U S A*. 2008; 105:14192–14197. [PubMed: 18768815]
15. Carvelli L, McDonald PW, Blakely RD, DeFelice LJ. Dopamine transporters depolarize neurons by a channel mechanism. *Proc Natl Acad Sci U S A*. 2004; 101:16046–16051. [PubMed: 15520385]
16. Cheng M, Bahar I. Molecular Mechanism of Dopamine Transport by Human Dopamine Transporter. *Structure*. 2015
17. De Felice LJ, Cameron KN. Comments on 'A quantitative model of amphetamine action on the serotonin transporter', by Sandtner et al., *Br J Pharmacol* 171: 1007–1018. *Br J Pharmacol*. 2015; 172:4772–4774. [PubMed: 24824549]
18. De Felice LJ, Glennon RA, Negus SS. Synthetic cathinones: chemical phylogeny, physiology, and neuropharmacology. *Life Sci*. 2014; 97:20–26. [PubMed: 24231923]
19. DeFelice LJ, Goswami T. Transporters as channels. *Annu Rev Physiol*. 2007; 69:87–112. [PubMed: 17059369]
20. Demchyshyn LL, Pristupa ZB, Sugamori KS, Barker EL, Blakely RD, Wolfgang WJ, Forte MA, Niznik HB. Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*. 1994; 91:5158–5162. [PubMed: 8197200]
21. Diliberto PA, Jeffs RA, Cubeddu LX. Effects of low extracellular chloride on dopamine release and the dopamine transporter. *J Pharmacol Exp Ther*. 1989; 248:644–653. [PubMed: 2521901]
22. Erreger K, Grewer C, Javitch JA, Galli A. Currents in response to rapid concentration jumps of amphetamine uncover novel aspects of human dopamine transporter function. *J Neurosci*. 2008; 28:976–989. [PubMed: 18216205]
23. Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature*. 1995; 375:599–603. [PubMed: 7791878]
24. Forrest LR, Tavoulari S, Zhang YW, Rudnick G, Honig B. Identification of a chloride ion binding site in Na⁺/Cl⁻-dependent transporters. *Proc Natl Acad Sci U S A*. 2007; 104:12761–12766. [PubMed: 17652169]
25. Fraser R, Chen Y, Guptaroy B, Luderman KD, Stokes SL, Beg A, DeFelice LJ, Gnegy ME. An N-terminal threonine mutation produces an efflux-favorable, sodium-primed conformation of the human dopamine transporter. *Mol Pharmacol*. 2014; 86:76–85. [PubMed: 24753048]
26. Galli A, Blakely RD, DeFelice LJ. Patch-clamp and amperometric recordings from norepinephrine transporters: channel activity and voltage-dependent uptake. *Proc Natl Acad Sci U S A*. 1998; 95:13260–13265. [PubMed: 9789076]

27. Gu H, Caplan MJ, Rudnick G. Cloned catecholamine transporters expressed in polarized epithelial cells: sorting, drug sensitivity, and ion-coupling stoichiometry. *Adv Pharmacol.* 1998; 42:175–179. [PubMed: 9327872]
28. Gu H, Wall SC, Rudnick G. Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence. *J Biol Chem.* 1994; 269:7124–7130. [PubMed: 8125921]
29. Gu HH, Ahn J, Caplan MJ, Blakely RD, Levey AI, Rudnick G. Cell-specific sorting of biogenic amine transporters expressed in epithelial cells. *J Biol Chem.* 1996; 271:18100–18106. [PubMed: 8663573]
30. Gu HH, Wall S, Rudnick G. Ion coupling stoichiometry for the norepinephrine transporter in membrane vesicles from stably transfected cells. *J Biol Chem.* 1996; 271:6911–6916. [PubMed: 8636118]
31. Gu HH, Wu X, Giros B, Caron MG, Caplan MJ, Rudnick G. The NH(2)-terminus of Norepinephrine Transporter Contains a Basolateral Localization Signal for Epithelial Cells. *Mol Biol Cell.* 2001; 12:3797–3807. [PubMed: 11739781]
32. Gu HH, Wu X, Han DD. Conserved serine residues in serotonin transporter contribute to high-affinity cocaine binding. *Biochemical and biophysical research communications.* 2006; 343:1179–1185. [PubMed: 16580636]
33. Gu XF, Azmitia EC. Integrative transporter-mediated release from cytoplasmic and vesicular 5-hydroxytryptamine stores in cultured neurons. *Eur J Pharmacol.* 1993; 235:51–57. [PubMed: 8100196]
34. Gu Y, Shrivastava IH, Amara SG, Bahar I. Molecular simulations elucidate the substrate translocation pathway in a glutamate transporter. *Proc Natl Acad Sci U S A.* 2009; 106:2589–2594. [PubMed: 19202063]
35. Henry LK, Iwamoto H, Field JR, Kaufmann K, Dawson ES, Jacobs MT, Adams C, Felts B, Zdravkovic I, Armstrong V, Combs S, Solis E, Rudnick G, Noskov SY, DeFelice LJ, Meiler J, Blakely RD. A conserved asparagine residue in transmembrane segment 1 (TM1) of serotonin transporter dictates chloride-coupled neurotransmitter transport. *J Biol Chem.* 2011; 286:30823–30836. [PubMed: 21730057]
36. Ingram SL, Prasad BM, Amara SG. Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons. *Nat Neurosci.* 2002; 5:971–978. [PubMed: 12352983]
37. Itokawa M, Lin Z, Uhl GR. Dopamine efflux via wild-type and mutant dopamine transporters: alanine substitution for proline-572 enhances efflux and reduces dependence on extracellular dopamine, sodium and chloride concentrations. *Brain Res Mol Brain Res.* 2002; 108:71–80. [PubMed: 12480180]
38. Kavanaugh MP, Arriza JL, North RA, Amara SG. Electrogenic uptake of gamma-aminobutyric acid by a cloned transporter expressed in *Xenopus* oocytes. *J Biol Chem.* 1992; 267:22007–22009. [PubMed: 1429551]
39. Kehr J, Ichinose F, Yoshitake S, et al. Mephedrone, compared with MDMA (ecstasy) and amphetamine, rapidly increases both dopamine and 5-HT levels in nucleus accumbens of awake rats. *Br J Pharmacol.* 2011; 164:1949–1958. [PubMed: 21615721]
40. Krishnamurthy B, Chee J, Jhala G, Fynch S, Graham KL, Santamaria P, Morahan G, Allison J, Izon D, Thomas HE, Kay TW. Complete Diabetes Protection Despite Delayed Thymic Tolerance in NOD8.3 TCR Transgenic Mice Due to Antigen-Induced Extrathymic Deletion of T Cells. *Diabetes.* 2012; 61:425–435. [PubMed: 22190647]
41. Krishnamurthy H, Gouaux E. X-ray structures of LeuT in substrate-free outward-open and apo inward-open states. *Nature.* 2012; 481:469–474. [PubMed: 22230955]
42. Larsson HP, Picaud SA, Werblin FS, Lecar H. Noise analysis of the glutamate-activated current in photoreceptors. *Biophys J.* 1996; 70:733–742. [PubMed: 8789090]
43. Lester HA, Cao Y, Mager S. Listening to neurotransmitter transporters. *Neuron.* 1996; 17:807–810. [PubMed: 8938113]
44. Li LB, Reith ME. Interaction of Na⁺, K⁺, and Cl[–] with the binding of amphetamine, octopamine, and tyramine to the human dopamine transporter. *J Neurochem.* 2000; 74:1538–1552. [PubMed: 10737611]

45. LJ DF, KN C. Comments on "A Quantitative Model of Amphetamine Action on the Serotonin Transporter, by Sandtner et al. (2013). *Br J Pharmacol*. 2014
46. Machtens JP, Kortzak D, Lansche C, Leinenweber A, Kilian P, Begemann B, Zachariae U, Ewers D, de Groot BL, Briones R, Fahlke C. Mechanisms of anion conduction by coupled glutamate transporters. *Cell*. 2015; 160:542–553. [PubMed: 25635461]
47. Mager S, Min C, Henry DJ, Chavkin C, Hoffman BJ, Davidson N, Lester HA. Conducting states of a mammalian serotonin transporter. *Neuron*. 1994; 12:845–859. [PubMed: 8161456]
48. Naftalin, RJ.; De Felice, LJ. *Transporters and Co-Transporters in Theory and Practice*. Academic Press; 2012.
49. Pedersen AV, Andreassen TF, Loland CJ. A conserved salt bridge between transmembrane segments 1 and 10 constitutes an extracellular gate in the dopamine transporter. *J Biol Chem*. 2014; 289:35003–35014. [PubMed: 25339174]
50. Penmatsa A, Wang K, Gouaux E. X-ray structure of dopamine transporter elucidates antidepressant mechanism. *Nature*. 2013; 503:85–90. [PubMed: 24037379]
51. Penmatsa A, Wang KH, Gouaux E. X-ray structures of *Drosophila* dopamine transporter in complex with nisoxetine and reboxetine. *Nat Struct Mol Biol*. 2015; 22:506–508. [PubMed: 25961798]
52. Petersen CI, DeFelice LJ. Ionic interactions in the *Drosophila* serotonin transporter identify it as a serotonin channel. *Nat Neurosci*. 1999; 2:605–610.
53. Pifl C, Agneter E, Drobny H, Reither H, Singer EA. Induction by low Na⁺ or Cl[–] of cocaine sensitive carrier-mediated efflux of amines from cells transfected with the cloned human catecholamine transporters. *Br J Pharmacol*. 1997; 121:205–212. [PubMed: 9154329]
54. Pifl C, Drobny H, Reither H, Hornykiewicz O, Singer EA. Mechanism of the dopamine-releasing actions of amphetamine and cocaine: plasmalemmal dopamine transporter versus vesicular monoamine transporter. *Mol Pharmacol*. 1995; 47:368–373. [PubMed: 7870046]
55. Pifl C, Rebernik P, Kattinger A, Reither H. Zn²⁺ modulates currents generated by the dopamine transporter: parallel effects on amphetamine-induced charge transfer and release. *Neuropharmacology*. 2004; 46:223–231. [PubMed: 14680760]
56. Pifl C, Singer EA. Ion dependence of carrier-mediated release in dopamine or norepinephrine transporter-transfected cells questions the hypothesis of facilitated exchange diffusion. *Mol Pharmacol*. 1999; 56:1047–1054. [PubMed: 10531412]
57. Pifl C, Wolf A, Rebernik P, Reither H, Berger ML. Zinc regulates the dopamine transporter in a membrane potential and chloride dependent manner. *Neuropharmacology*. 2009; 56:531–540. [PubMed: 19000913]
58. Piscitelli CL, Krishnamurthy H, Gouaux E. Neurotransmitter/sodium symporter orthologue LeuT has a single high-affinity substrate site. *Nature*. 2010; 468:1129–1132. [PubMed: 21179170]
59. Quick MW. Regulating the conducting states of a mammalian serotonin transporter. *Neuron*. 2003; 40:537–549. [PubMed: 14642278]
60. Rodriguez-Menchaca AA, Solis E Jr, Cameron K, De Felice LJ. S(+)-amphetamine induces a persistent leak in the human dopamine transporter: molecular stent hypothesis. *Br J Pharmacol*. 2012; 165:2749–2757. [PubMed: 22014068]
61. Ruchala I, Cabra V, Solis E Jr, Glennon RA, De Felice LJ, Eltit JM. Electrical coupling between the human serotonin transporter and voltage-gated Ca(2+) channels. *Cell Calcium*. 2014; 56:25–33. [PubMed: 24854234]
62. Rudnick G. Active transport of 5-hydroxytryptamine by plasma membrane vesicles isolated from human blood platelets. *JBiolChem*. 1977; 252:2170–2174.
63. Rudnick G. Bioenergetics of neurotransmitter transport. *J Bioenerg Biomembr*. 1998; 30:173–185. [PubMed: 9672239]
64. Rudnick G. Ion-coupled neurotransmitter transport: thermodynamic vs. kinetic determinations of stoichiometry. *Methods Enzymol*. 1998; 296:233–247. [PubMed: 9779452]
65. Rudnick G. Serotonin transporters--structure and function. *J Membr Biol*. 2006; 213:101–110. [PubMed: 17417703]
66. Rudnick G, Nelson PJ. Platelet 5-hydroxytryptamine transport, an electroneutral mechanism coupled to potassium. *Biochemistry*. 1978; 17:4739–4742. [PubMed: 728383]

67. Rudnick G, Wall SC. The molecular mechanism of "ecstasy" [3,4-methylenedioxy-methamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. *Proc Natl Acad Sci U S A*. 1992; 89:1817–1821. [PubMed: 1347426]
68. Safratowich BD, Hossain M, Bianchi L, Carvelli L. Amphetamine potentiates the effects of beta-phenylethylamine through activation of an amine-gated chloride channel. *J Neurosci*. 2014; 34:4686–4691. [PubMed: 24672014]
69. Sandtner W, Schmid D, Schicker K, Gerstbrein K, Koenig X, Mayer FP, Boehm S, Freissmuth M, Sitte HH. A quantitative model of amphetamine action on the 5-HT transporter. *Br J Pharmacol*. 2014; 171:1007–1018. [PubMed: 24251585]
70. Schwartz J, Piston D, DeFelice L. Molecular Microfluorometry: Converting Fluorescence Units into Absolute Concentrations to Study Binding and Transport in Norepinephrine Transporters. *Handb Exp Pharmacol*. 2006; 175:23–57. [PubMed: 16722229]
71. Schwartz JW. Dissertation, Vanderbilt Graduate School. 2004. Norepinephrine Transporter Kinetics and Stoichiometry by Microfluorometry; p. p135Fig. 135
72. Schwartz JW, Blakely RD, DeFelice LJ. Binding and transport in norepinephrine transporters. Real-time, spatially resolved analysis in single cells using a fluorescent substrate. *J Biol Chem*. 2003; 278:9768–9777. [PubMed: 12499385]
73. Schwartz JW, Novarino G, Piston DW, DeFelice LJ. Substrate binding stoichiometry and kinetics of the norepinephrine transporter. *J Biol Chem*. 2005; 280:19177–19184. [PubMed: 15757904]
74. Singh SK, Yamashita A, Gouaux E. Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. *Nature*. 2007; 448:952–956. [PubMed: 17687333]
75. Sitges M, Reyes A, Chiu LM. Dopamine transporter mediated release of dopamine: role of chloride. *Journal of neuroscience research*. 1994; 39:11–22. [PubMed: 7807588]
76. Sonders MS, Zhu SJ, Zahniser NR, Kavanaugh MP, Amara SG. Multiple ionic conductances of the human dopamine transporter: the actions of dopamine and psychostimulants. *J Neurosci*. 1997; 17:960–974. [PubMed: 8994051]
77. Su A, Mager S, Mayo SL, Lester HA. A multi-substrate single-file model for ion-coupled transporters. *BiophysJ*. 1996; 70:762–777. [PubMed: 8789093]
78. Sulzer D. How Addictive Drugs Disrupt Presynaptic Dopamine Neurotransmission. *Neuron*. 2012; 69:628–649. [PubMed: 21338876]
79. Sulzer D, Galli A. Dopamine transport currents are promoted from curiosity to physiology. *Trends Neurosci*. 2003; 26:173–176. [PubMed: 12689764]
80. Swant J, Goodwin JS, North A, Ali AA, Gamble-George J, Chirwa S, Khoshbouei H. alpha-Synuclein stimulates a dopamine transporter-dependent chloride current and modulates the activity of the transporter. *J Biol Chem*. 2011; 286:43933–43943. [PubMed: 21990355]
81. Syringas M, Janin F, Giros B, Costentin J, Bonnet JJ. Involvement of the NH2 terminal domain of catecholamine transporters in the Na(2+) and Cl(-)-dependence of a [3H]-dopamine uptake. *Br J Pharmacol*. 2001; 133:387–394. [PubMed: 11375255]
82. Syringas M, Janin F, Mezghanni S, Giros B, Costentin J, Bonnet JJ. Structural domains of chimeric dopamine-noradrenaline human transporters involved in the Na(+)- and Cl(-)-dependence of dopamine transport. *Mol Pharmacol*. 2000; 58:1404–1411. [PubMed: 11093780]
83. Tang QY, Kolanos R, LJ DF, RG G. Structural Analysis of Dopamine- and Amphetamine-Induced Depolarization Currents in the Human Dopamine Transporter. *ACS Chemical Neuroscience*. 2015 <http://dx.doi.org/10.1021/cn500282f>.
84. Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. *Nat Rev Neurosci*. 2003; 4:13–25. [PubMed: 12511858]
85. Torres GE, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation and function. *Nat Rev Neurosci*. 2003; 4:13–25. [PubMed: 12511858]
86. Vandenberg RJ, Arriza JL, Amara SG, Kavanaugh MP. Constitutive ion fluxes and substrate binding domains of human glutamate transporters. *J Biol Chem*. 1995; 270:17668–17671. [PubMed: 7629063]
87. Wadiche JJ, Amara SG, Kavanaugh MP. Ion fluxes associated with excitatory amino acid transport. *Neuron*. 1995; 15:721–728. [PubMed: 7546750]

88. Wadiche JI, Arriza JL, Amara SG, Kavanaugh MP. Kinetics of a human glutamate transporter. *Neuron*. 1995; 14:1019–1027. [PubMed: 7748550]
89. Wang H, Gouaux E. Substrate binds in the S1 site of the F253A mutant of LeuT, a neurotransmitter sodium symporter homologue. *EMBO Rep*. 2012; 13:861–866. [PubMed: 22836580]
90. Wang KH, Penmatsa A, Gouaux E. Neurotransmitter and psychostimulant recognition by the dopamine transporter. *Nature*. 2015; 521:322–327. [PubMed: 25970245]
91. Watts SD, Suchland KL, Amara SG, Ingram SL. A sensitive membrane-targeted biosensor for monitoring changes in intracellular chloride in neuronal processes. *PLoS One*. 2012; 7:e35373. [PubMed: 22506078]
92. Weiss JN. The Hill equation revisited: uses and misuses. *Faseb J*. 1997; 11:835–841. [PubMed: 9285481]
93. Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E. Crystal structure of a bacterial homologue of Na⁺/Cl[−]-dependent neurotransmitter transporters. *Nature*. 2005; 437:215–223. [PubMed: 16041361]
94. Yernool D, Boudker O, Folta-Stogniew E, Gouaux E. Trimeric subunit stoichiometry of the glutamate transporters from *Bacillus caldotenax* and *Bacillus stearothermophilus*. *Biochemistry*. 2003; 42:12981–12988. [PubMed: 14596613]
95. Yernool D, Boudker O, Jin Y, Gouaux E. Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. *Nature*. 2004; 431:811–818. [PubMed: 15483603]
96. Zomot E, Bendahan A, Quick M, Zhao Y, Javitch JA, Kanner BI. Mechanism of chloride interaction with neurotransmitter:sodium symporters. *Nature*. 2007; 449:726–730. [PubMed: 17704762]

Figure 1. Ion dependance of monoamine transport

Gu et al measured the the dependance of transport on Na⁺ and Cl⁻ ions (2 minute incubations and 100 μ M cocaine to subtract background). Solid lines are non-liner regressions assuming 2 Na⁺ ions with equal affinities are required for DA transport, and 1Na⁺ and 1 Cl⁻ ion required for all other cases. The LLC-PK1 cells used in these experiments, in which DAT, NET, and SERT were expressed, do not normally express monoamine transporters (28).

Figure 2. Substrate-induced currents depend on Cl^- ions

Left: I(V) curves in the absence and presence of 10 μM external DA with CsCl internal solution. The reversal of the DA-induced current occurs at -10 mV). **Right)** Reversal potentials of DA-induced currents as a function of extracellular Cl^- , substituting the impermeable anion, methanesulfonate, for Cl^- . The slope of the line is 48mV/decade. K-gluconate is the internal solution. Data is mean $\pm$ s.e.m. and the number of cells for each data point is indicated. Adapted from (36).

Figure 3. DA and AMPH induced currents through hDAT expressed in *Xenopus laevis* oocytes (A) At -60 mV, $10\text{ }\mu\text{M}$ DA or $10\text{ }\mu\text{M}$ R(-)AMPH induced a peak current on application but return to baseline albeit somewhat slowly after removing external DA or R(-)AMPH. S(+)-AMPH, however, has a prominent persistent current (or 'shelf' current) after its removal. I.e., rather than a lazy return to baseline the transporter appears to be locked in an open state when challenged with S(+)-AMPH. Cocaine ($10\text{ }\mu\text{M}$) blocks the initial peak current and the persistent current in all cases; rather than returning to baseline, cocaine appears to induce an outward current, which is actually the block of an endogenous hDAT

leak that is independent of substrate. (B) Baseline subtracted I(V) curves for DA, S(+)-AMPH peak and S(+)-AMPH shelf. The S(+)-AMPH peak I(V) shifts to the left compared with the DA peak, consistent with the conductance of Na⁺ and S(+)-AMPH ions through hDAT. The S(+)-AMPH shelf I(V) shifts even further to the left, consistent with the absence of S(+)-AMPH and presence of Cl⁻ ions flowing through hDAT (60).

Figure 4.

Fluorescence images of cultured midbrain neurons expressing mbYFPQS. Single Z-stack images (1 μm) were taken 3 days post-transfection at mid-soma level. Fluorescence in processes in non-membrane bound cytYFPQS-expressing cell is difficult to observe. The intensity of fluorescence is proportional to Cl^- . Adapted from (91).

Figure 5. Cl⁻ removal increases ASP⁺ binding

hNET transfected HEK cells were exposed to 2 μM of ASP⁺ for 1 sec with 120 μM Na⁺ replacing NaCl and 100 μM Cl⁻ (left panel) and Cl⁻ free (right panel). Bar code represent low (blue) to high (red) ASP⁺ binding. Adapted from (71)