Serotonin Transporter Function and Pharmacology Are Sensitive to Expression Level

EVIDENCE FOR AN ENDOGENOUS REGULATORY FACTOR*

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We express mammalian serotonin transporters (SERTs) in Xenopus oocytes by cRNA injection and measure 5-hydroxytryptamine (5-HT) transport and 5-HT-induced current at varying expression levels. Transport and current both increase sigmoidally with the amount of cRNA injected, but current requires ~5-fold more cRNA to elicit a half-maximal response. Western blots of SERT protein demonstrate that current, but not transport, correlates linearly with the amount of SERT on the plasma membrane. In oocytes co-injected with wild-type SERT and an inactive SERT mutant, transport is similar to SERT alone, but current is attenuated. The charge/transport ratio reports the differential sensitivity of transport and current to increasing SERT cRNA injection and mutant co-expression. Manipulations that alter the charge/transport ratio also perturb substrate and inhibitor recognition. 5-HT, d-amphetamine, cocaine, and paroxetine inhibit transport more potently at lower expression levels; however, 5-HT potency for induction of current is similar at high and low expression. Moreover, the apparent potency of cRNA for transport depends on 5-HT concentration. We postulate that SERT interacts allosterically with an endogenous factor of limited abundance to alter substrate and inhibitor potency and the balance of 5-HT transport and channel-like activity.

Serotonin transporters accumulate serotonin into cells following 5-HT1 release, thereby modulating serotonergic signaling and neurotransmission (1). 5-HT is implicated in a variety of behaviors, including mood, sleep, pain, appetite, aggression, and sexual behavior, and serotonin-selective reuptake inhibitors are useful for the treatment of human diseases (depression, obsessive-compulsive disorder, bulimia and eating disorders, alcoholism and panic disorders, and premenstrual dysphoric disorder) (2, 3). SERT is a receptor for psychostimulant drugs of abuse such as 3,4-methylenedioxymethamphetamine, amphetamine, and cocaine (4–6).

SERT (SLC6A4) is a member of the GAT/NET transporter family that encodes carriers for neurotransmitters, solutes, and amino acids that are similar in function (Na+, Cl−-dependent presynaptic) (7). SERT forms oligomeric complexes supported by FRET interactions (11), chemical cross-linking (12), and functional reconstitution following purification of a large protein complex (13, 14). Epitope-tagged rSERT proteins can be co-immunoprecipitated and co-expression of (2-aminoethyl)methanethiosulfonate-sensitive or -insensitive mutants alters (2-aminoethyl)methanethiosulfonate inhibition, indicating that inter-subunit interactions in the oligomeric complex modulate SERT function (15). Additional evidence for oligomeric complexes in GAT/NET transporters is suggested by expression of concatenated mouse SERT cDNA (16) and radiation inactivation of human dopamine transporter (17, 18).

Classically, GAT/NET transporters function with fixed ion-substrate stoichiometry (19, 20). However, 5-HT transport deviates from classical expectations in stably transfected HEK-293 cells (21). Biophysical studies show that hundreds of elementary charges can move through SERT, NET, and DAT for each neurotransmitter molecule (22–24). Transporter-mediated ion channel activity, evidenced by single channel currents or current fluctuations, is reported for GAT1, NET, and SERT (25–27). The ion dependence of 5-HT transport predicts electroneutral function (28, 29), yet SERT generates three phenomenologically distinct currents: constitutive current (also termed leak or slippage), 5-HT-induced steady-state currents, and a rapidly inactivating transient current (22, 23).

Large transporter-associated currents and channel-like activity are not universally observed for GAT/NET transporters. Fixed ion-substrate coupling is reported for rGAT1 (30, 31) and rat brain-specific proline transporter (32) under voltage clamp, suggesting that variable stoichiometry and excess current may depend on as yet unidentified factors. In vivo, serotonergic synapses of Hirudo manifest rapid, Na+-dependent presynaptic currents ascribed to SERT (33). In the face of a conserved sequence in the GAT/NET family, which would suggest similar structure and function, these apparent discrepancies in the literature regarding ion fluxes in excess of transmitter flux are puzzling. GAT/NET transporters are known to associate with proteins that alter their function (34–36), and heterologous
Expression systems could yield disparate results if they fail to reconstitute interactions between transporters and other regulatory proteins or cellular factors. Changes in 5-HT transport, 5-HT-induced current, and plasma membrane transporter density are associated with protein kinase C activation, SERT phosphorylation, and ligand occupancy (37, 38). Transporter activity may also be sensitive to allosteric interactions between subunits of an oligomeric SERT complex (15, 39, 40).

Here we measure 5-HT transport (Φ5-HT) and 5-HT-induced current (i5-HT) in Xenopus oocytes injected with cRNA encoding wild-type and mutant mammalian SERTs. We show that increasing the quantity of cRNA injected or co-injection with an inactive SERT mutant alters the balance of Φ5-HT to i5-HT, which is reported by a change in the charge/transport ratio (ρ). Expression of biotinylated surface hSERT protein correlates linearly with Φ5-HT but not Φ5-HT. In oocytes with high levels of SERT expression, the apparent affinity of the transporter for substrates and inhibitors is decreased. The data suggest that SERT interacts with an endogenous factor that modulates channel-like and 5-HT transport activities and alters ligand potency. We posit a model for interpreting how SERT expression affects SERT function and pharmacology. The model explains discrepancies regarding variable stoichiometry and transporter-mediated channel activity for SERT and other GAT/NET transporters.

EXPERIMENTAL PROCEDURES

Xenopus laevis Oocyte Isolation and cRNA Injection—The oocytes were isolated as described previously (23) and incubated in frog Ringer’s solution (96 mM NaCl, 2 mM KCl, 4 mM MgCl2, 0.6 mM CaCl2, 5 mM HEPES, pH 7.6, at 23 °C, 195–205 mOsm). cDNA constructs encoding either the human serotonin transporter (hSERT) in pOTV (gift of M. Sonders, Vollum Institute, Portland, OR), rSERT in pBS II SK, or either the human serotonin transporter (hSERT) in pOTV (gift of M. Sonders, Vollum Institute, Portland, OR), rSERT in pBS II SK, which is reported by a change in the charge/transport ratio (ρ). Expression of biotinylated surface hSERT protein correlates linearly with Φ5-HT but not Φ5-HT. In oocytes with high levels of SERT expression, the apparent affinity of the transporter for substrates and inhibitors is decreased. The data suggest that SERT interacts with an endogenous factor that modulates channel-like and 5-HT transport activities and alters ligand potency. We posit a model for interpreting how SERT expression affects SERT function and pharmacology. The model explains discrepancies regarding variable stoichiometry and transporter-mediated channel activity for SERT and other GAT/NET transporters.

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[3H]5-HT Transport Assays—The assays were initiated by addition of [3H]5-HT (12–30 nM) in a final volume of 200 or 500 μl and proceeded for the indicated time at 24 °C. For 5-HT saturation studies, [3H]5-HT for 1 or 2 min as indicated, and incorporated [3H] radioactivity was determined as described for 5-HT transport. Q3.5-HT was calculated from transport data assuming a valence of +1 e for 5-HT. The currents were base-line subtracted and corrected for off-line to determine the total 5-HT-induced net charge movement (Q3.5-HT) and p was calculated by dividing Q3.5-HT by Q3.5-HT, within the same oocyte. Specific 5-HT transport and charge movements were defined by subtracting responses seen in noninjected oocytes of the same batch from those in SERT cRNA-injected oocytes.

Western Blotting—Equal numbers of oocytes from each cRNA injection (typically 20–30) were washed with ice-cold frog Ringer’s solution and incubated with 1.0 mg/ml EZ-Link Sulfo-NHS-biotin (Pierce) in with ice-cold frog Ringer’s solution with gentle agitation for 60 min. The oocytes were incubated for 60 min in ice-cold frog Ringer’s solution containing 100 mM glycine, washed twice, and stored at −80 °C until further use (<1 month). After thawing on ice, the oocytes were solubilized by freezing (42). Briefly, the oocytes were incubated with lysis buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4, at 24 °C, 1 mM EDTA, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, and 250 μM phenylmethylsulfonyl fluoride, 20 μl/oocyte) for 10 min on ice. The extracts were triturated with a pipette until smooth, incubated for 15 min on ice, and centrifuged (15,000 × g for 15 min) to pellet insoluble yolk material. The supernatant was removed, and an aliquot was saved for determination of total protein using the BCA Reagent (Pierce). Immunopure immobilized Streptavidin beads (Pierce) were washed by centrifugation with lysis buffer (3 × 0.5 ml), and the final bead pellet was resuspended to ~2× bead volume with lysis buffer. The oocyte extracts were incubated with 50 μl of streptavidin bead slurry for 60 min at 24 °C with rocking and then centrifuged for 10 min at 15,000 × g at 4 °C. The supernatant (intracellular fraction) is saved, and the pellet (surface fraction) was washed twice with 1 ml of ice-cold lysis buffer. Streptavidin beads were incubated with 40 μl of 4× loading buffer (62.5 mM Tris–HCl, pH 7.0, 10% glycerol, 2% SDS, 0.05% 2-mercaptoethanol) for 30 min at 24 °C to elute biotinylated proteins, and the entire sample was loaded in a single lane for SDS-PAGE in 10% acrylamide slab gels. For intracellular fractions, we loaded 25 μg of total protein. The total extracts were transferred to Immobilon-P membranes (Millipore) overnight (4 °C) and three times with PBS-T (phosphate-buffered saline, 0.1% Tween 20, 0.5 g/ml nonfat dry milk powder) for 15 min at 24 °C. The blots were incubated for 60 min at 24 °C with anti-hSERT mouse monoclonal antibody ST51–2 (MAB Technologies) diluted 1:2,000 in PBS-T and then washed three times with PBS-T (15 min) before incubation with a conjugated AffiniPure goat anti-mouse antibody (Jackson ImmunoResearch) diluted 1:2,000 in PBS-T for 60 min at 24 °C. Immunoreactive proteins were detected using the Renaissance Western blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and Hyperfilm XL (Amersham Biosciences) per the manufacturer’s instructions. Exposed film was scanned with a Duoscan T1200 (Agfa) and quantified using Quantity One densitometry software (Bio-Rad). Multiple blot exposures of varying duration indicated that film responses are approximately linear and do not saturate in the range used to calculate SERT density.

Statistics and Curve Fitting—Reported values represent means ± S.E. unless otherwise indicated. Statistical significance was determined using Student’s nonpaired t test (Microcal Origin, Northampton, MA). Significant differences are indicated by one asterisk for p < 0.05 and two asterisks for p < 0.01. For curve fits, parameter values represent the means ± 95% confidence interval using the Levenberg-Marquardt nonlinear least squares algorithm (Origin). Parameter values are fixed where confidence intervals are not given.

RESULTS

Xenopus oocytes were injected with increasing amounts of SERT cRNA and assayed after a 2-day incubation (24 °C). Fig. 1A shows a representative Western blot of biotinylated oocyte proteins probed with the hSERT-specific monoclonal antibody ST51–2. hSERT immunoreactivity was absent in noninjected oocytes (lane 1 and 2) and depends on the amount of cRNA
batches (was measured in five different oocyte batches (2.5 min (n = 3) or 30 min (n = 2) assays (30 nm [H]5-HT). I-5-HT, (open squares, solid line) was measured in response to brief application of 5-HT (3.2 or 10 μM) in four different oocyte batches (n = 4–5 oocytes/cRNA injection) at −80 mV, pH 7.6. Surface SERT (solid triangles, dashed line) was determined from two different oocyte batches (n = 20–30 oocytes/cRNA injection). The data represent the means ± S.E., where indicated, of responses normalized to the maximal response from respective oocyte batches. Average maximal responses from all batches presented: PHTmax = 12.9 ± 4.5 nfmol/oocyte; I-5-HTmax = −3.84 ± 6.7 nA. The lines represent the fits to the Hill equation: PHT = EC50,cRNA = 0.13 ± 0.03 ng/oocyte; I-5-HT, EC50,cRNA = 3.42 ± 0.86 nA/oocyte; surface SERT, EC50,cRNA = 6.36 ± 0.81 nA/oocyte; nH was set to 1 for all fits.

Injected (lanes 3–8), indicating that ST 51–2 detects authentic hSERT protein. Several forms of hSERT were distinguished: a small band (~60 kDa) that is present only in the intracellular fraction, a broad band (70–100 kDa) that is typical of mature hSERT (43), and a larger band (~220 kDa) in both surface and intracellular fractions that may represent an SDS-resistant SERT protein complex. Despite the fact that surface (biotin +) lanes represent the entire biotinylated fraction and intracellular (biotin −) lanes are loaded with only 5% of the total extract, band densities were comparable (Fig. 1A, lanes 5–8). Therefore, −5% of SERT protein resided on the oocyte surface. We observed similar results in blots from two different oocyte batches. The relative densities of the mature surface hSERT (70–100 kDa) bands were averaged and plotted against the amount of cRNA injected (Fig. 1B).

To correlate hSERT surface expression with hSERT function, we measured 5-HT transport (PHT) and 5-HT-induced current (I5-HT) in the same batches of oocytes under conditions that are commonly used to study neurotransmitter transporters. Here PHT assays were conducted in low substrate concentration (30 nM [H]5-HT) over a short time (2.5 min) without voltage clamp, and I5-HT was elicited by rapid superfusion (10–15 s) of high substrate concentration (3.2 or 10 μM 5-HT) at −80 mV (Fig. 2 compares PHT and I5-HT under identical conditions). 5-HT-induced currents in noninjected oocytes were typically less than −5 nA (10 μM 5-HT, −80 mV). To discriminate 5-HT-induced current from current carried by 5-HT itself (labeled I5-HT by Galli et al. (23)), we employed the following nomenclature for the current induced by 5-HT:

I5-HT = [I5-HT] - [I(Control)].

PHT increased sigmoidally with the amount of hSERT cRNA injected, saturated above 5 ng/oocyte (Fig. 1B), and was well fit to the Hill equation (Fig. 1B, EC50,cRNA = 0.13 ± 0.03 ng/oocyte, nH = 1.0). Increasing cRNA also increased I5-HT, but the cRNA dependence of I5-HT was shifted rightward with respect to PHT. Surprisingly, mature surface hSERT protein correlated linearly with I5-HT, (R2 = 0.90), but not PHT, indicating that plasma membrane expression of SERT is selectively reported by current.

Because rSERT cRNA yielded smaller currents than hSERT, we cultured rSERT-injected oocytes for longer times at 18 °C to boost signal strength. At 18 °C, expression of both PHT and I5-HT was delayed compared with currents measured at room temperature (22–24 °C), but oocytes survived longer after cRNA injection (data not shown). We also increased uptake incubation time (60 min) in rSERT-injected oocytes cultured at 18 °C. Under these conditions, titration of rSERT cRNA yielded qualitatively similar results to Fig. 1B: cRNA more potently increased PHT than I5-HT (Hill fits: PHT, EC50,cRNA = 0.59 ± 0.02 ng/oocyte; I5-HT, EC50,cRNA = 20.0 ± 4.8 ng/oocyte; n = 4–6 oocytes from the same batch; data not shown). In the same
FIG. 3. Increasing 5-HT increases hSERT cRNA dependence of $\Phi_{\text{ transcripts but not } I_{\text{oHT}}.}$ The oocytes were injected with hSERT cRNA and cultured for 2 days at 24°C. $\Phi_{\text{ transcripts were measured during 2-min assays in the presence of 0.1 mM 5-HT (solid circles, solid line), 1.0 mM 5-HT (open circles, solid line), and 10.0 mM 5-HT (solid triangles, dotted line) in } n = 5-6$ oocytes from the same oocyte batch. The data were normalized to their respective maxima. The lines represent fits to the Hill equation: 1.0 $\pm 0.01$ ng/oocyte; 3.2 $\pm 0.04$ ng/oocyte; 32 $\pm 0.12$ ng/oocyte; $n_H$ was set to 1.3 for all fits; $n = 4-5$ oocytes from the same batch.

![Graph A](image1.png)

![Graph B](image2.png)

batch of rSERT-injected oocytes, both $I_{(5-HT)}$ and peak transient current correlated linearly with $I_{(5-HT)}$ at pH 7.6 ($R^2 = 0.99$ versus $I_{(5-HT)}$ and $R^2 = 0.87$ versus the transient current; data not shown). Therefore, the differential cRNA dependence of $\Phi_{\text{ transcripts and } I_{\text{oHT}}} was not unique to the particular SERT clone, its plasmid vector, or culture conditions (44).

To test whether the differential cRNA dependence of $\Phi_{\text{ transcripts and } I_{\text{oHT}}$ depends on voltage, we measured $I_{(5-HT)}$ and $I_{(5-HT)}$ simultaneously under voltage clamp at $-80$ mV in oocytes injected with increasing quantities of rSERT cRNA. To accurately measure currents in oocytes injected with low cRNA, we recorded $I_{(5-HT)}$ at pH 5.0. Fig. 2A shows that the change/transport ratio $\rho$ increased 4.3-fold as cRNA increased from 1.4 to 33.6 ng ($\rho = 6.7 \pm 1.6$ and 28.6 $\pm 3.1$, respectively). In contrast, the ratio of $I_{(5-HT)}$ at pH 5.0 to $I_{(5-HT)}$ at pH 7.6 was constant over the same range of cRNA (average $I_{(5-HT)}$ of $I_{(5-HT)}$ at pH 7.6 was $8.8 \pm 0.4$ for oocytes injected with 1.4–33.6 ng/oocyte; Fig. 2B). From these data we calculated that under physiological conditions ($-80$ mV, pH 7.6), $\rho$ varies between $-1$ and $-4$ over the range of cRNA tested. The validity of this calculation was reinforced by the observation that $\rho = 4.4$ when measured at pH 7.6 in a separate batch of oocytes injected with 8.3 ng/oocyte rSERT cRNA (see Fig. 5A). Thus, even when measured simultaneously under voltage clamp and in equal 5-HT concentrations, $\Phi_{\text{ transcripts and } I_{\text{oHT}}$ were differentially dependent on SERT expression level.

The unexpected expression level differences in 5-HT transport and current suggested that other properties of SERT might be sensitive to cRNA injection. We therefore examined 5-HT saturation kinetics in $\Phi_{\text{ transcripts by 35%}}.$ We therefore injected oocytes with rSERT cRNA either alone or together with cRNA encoding a transport-inactive rSERT mutant, D98G, and measured $\Phi_{\text{ transcripts and } I_{\text{oHT}}$, simultaneously at $-80$ mV (as in Fig. 2). Although D98G retained surface expression in mammalian cells, mutation of this conserved TM1 aspartate ablated transport (41). Fig. 5 shows that co-injection of rSERT with D98G attenuated $Q_{\text{oHT}}$ by 51% compared to 5-HT but not when 5-HT-induced current was measured.

That transport and current are differentially sensitive to SERT expression suggested that SERT may interact with a protein or cellular factor that modulates its function. One possibility is that $\rho$ is sensitive to intersubunit interactions in an oligomeric complex (11, 15). The structure of ion channels, most recently determined by x-ray crystallography for CIC chloride channels (45) and by freeze-fracture electron microscopy for glutamate transporters (46), further suggested that functional SERTs may be composed of more than one gene product. We therefore injected oocytes with rSERT cRNA either alone or together with cRNA encoding a transport-inactive rSERT mutant, D98G, and measured $\Phi_{\text{ transcripts and } I_{\text{oHT}}$, simultaneously at $-80$ mV (as in Fig. 2). Although D98G retained surface expression in mammalian cells, mutation of this conserved TM1 aspartate ablated transport (41). Fig. 5 shows that co-injection of rSERT with D98G attenuated $Q_{\text{oHT}}$ by 51% compared with oocytes injected with rSERT alone (8.3 ng/oocyte); however, $Q_{\text{oHT}}$ was unaffected (Fig. 5, A and B). D98G thus decreased $\rho$ by 35% compared with wild-type rSERT (Fig. 5C). To determine whether D98G altered ion permeation, we examined the voltage dependence of $I_{(5-HT)}$ in oocytes injected with rSERT cRNA either alone or with D98G cRNA. Fig. 6A shows raw currents...
during voltage ramps in the absence and presence of 10 μM 5-HT (pH 5.0) in an oocyte injected with rSERT cRNA alone (8.3 ng/oocyte). I_{5-HT}(V) is plotted as a function of membrane voltage in Fig. 6B. With rSERT cRNA alone, I_{5-HT}(V) exhibited the characteristic inward rectification seen in SERTs (22, 23) (Fig. 6B). The shape of I_{5-HT}(V) is not affected by extracellular acidification from pH 7.6 to 5.0 (data not shown). In oocytes injected with a mixture of rSERT + D98G cRNA (8.3 + 33.1 ng/oocyte, respectively), I_{5-HT} was attenuated relative to rSERT alone (8.3 ng/oocyte) at all membrane potentials tested.

**Fig. 4. Substrate and inhibitor potency for I_{5-HT} but not I_{cist} was sensitive to hSERT expression.** The oocytes were injected with 0.42 ng/oocyte (open symbols) or 21 ng/oocyte (solid symbols) hSERT cRNA and cultured for 2 days at 24 °C. Transport was measured in 2.5-min assays (30 nM ^3^H-5-HT plus indicated unlabeled 5-HT), and current was measured by brief (10–15 s) applications of 5-HT. A, I_{5-HT} in the presence of increasing concentrations of nonlabeled 5-HT (squares). Hill fits: 0.42 ng/oocyte (dashed line) I_{C50} = 1.6 ± 0.3 μM; 21 ng/oocyte (solid line) I_{C50} = 7.7 ± 0.9 μM; n_{H} was set to 1.0, n = 4–6 oocytes. B, I_{5-HT} in the presence of increasing concentrations of cocaine (triangles). Hill fits: 0.42 ng/oocyte (dashed line) I_{C50} = 1.9 ± 0.4 μM; 21 ng/oocyte (solid line) I_{C50} = 20 ± 5.2 μM; n = 4–6 oocytes. C, I_{5-HT} normalized to maximal current elicited by increasing concentrations of 5-HT at 80 mV, pH 7.6 (circles). Hill fits: 0.42 ng/oocyte (solid line) E_{C50} = 1.6 ± 0.3 μM, I_{C50} = 7.8 ± 0.9 nA, n = 5 oocytes; 21 ng/oocyte (dashed line) E_{C50} = 2.4 ± 0.4, I_{C50} = 20.4 ± 0.9 nA, n = 7 oocytes. For A–C experiments in a given panel were performed on oocytes from the same batch, and different baths were used for each panel; n_{H} was set to 1.0 for all Hill fits. D, pK_{i} for inhibition of I_{5-HT} by 5-HT (open squares, data from A) and cocaine (solid triangles, data from B), and pEC_{50} for 5-HT (open circles, data from C). pK_{i} values were determined from I_{C50} (76), assuming K_{m} = 1.8 μM (0.42 ng/oocyte) and K_{m} = 8.0 μM (0.42 ng/oocyte) (see “Results”).

**Fig. 5. Co-expression of rSERT with D98G alters charge/transport ratio ρ.** The oocytes were injected with increasing amounts of SERT cRNA (8.3 ng/oocyte) alone or together with D98G cRNA (33.1 ng/oocyte) and cultured for 8 days at 18 °C. Transport and current were measured during 2-min assays (~80 mV, 3.2 μM 5-HT). A, total charge movement (Q_{ch-5-HT}) obtained from integration of I_{5-HT}. B, charge movement caused by transport of 5-HT itself (Q_{ch-5-HT}) during I_{5-HT}. C, ratio of total charge movement to charge carried by 5-HT (ρ = Q_{ch-5-HT}/Q_{ch-5-HT}). For A–C, the data represent the means ± S.E. for oocytes of the same batch injected with: rSERT (8.3 ng, open bars; n = 6 oocytes) or rSERT + D98G (8.3 ng + 33.1 ng, solid bars; n = 5 oocytes).

**Fig. 6. Co-expression of rSERT and D98G does not alter I_{5-HT} voltage dependence.** Oocytes were injected with increasing amounts of SERT cRNA and cultured for 8 days at 18 °C. A, upper panel, voltage ramp command protocol; lower panel, representative raw currents at pH 5.0 in the absence (Control, gray line) and presence of 5-HT (10 μM, black line) in an oocyte injected with 8.3 ng of rSERT cRNA. The dashed line indicates zero current level. B, I_{5-HT}(V) at pH 5.0 from individual oocytes injected with either rSERT (8.3 ng, black line) or rSERT + D98G (8.3 ng + 33.1 ng, gray line). C, I_{5-HT}(V) normalized to current at ~120 mV, pH 5.0. The data represent the means ± S.D. from oocytes of the same batch injected with either rSERT (8.3 ng, closed triangles; n = 3 oocytes) or rSERT + D98G (8.3 ng + 33.1 ng, open circles; n = 4 oocytes).
SERT Expression Level Alters Its Function and Pharmacology

Plasma membrane expression of hSERT protein linearly correlated with \( I_{5-HT} \) (Fig. 1A). The amount of cRNA required to elicit \( \Phi_{5-HT} \) was, however, 5-fold lower than for \( I_{5-HT} \) (Fig. 1B). Because \( \Phi_{5-HT} \) and \( I_{5-HT} \) each required functional SERTs, it is surprising that they were not linearly correlated. The differential cRNA dependence was not explained by an inability to measure current at low cRNA. At pH 7.6 even below 1 ng of cRNA, \( I_{5-HT} \) was significantly greater than in noninjected oocytes. \( I_{5-HT} \) exhibited the same cRNA dependence at pH 5.0 as it did at 7.6, despite a nearly 10-fold increase in magnitude (Figs. 1B and 2B).

If increasing transporter number significantly depletes 5-HT in the bath, \( \Phi_{5-HT} \) might saturate because of limited substrate availability. We measured initial transport rates (47) within 2.5 min to minimize substrate depletion or accumulation. Increasing incubation time 30-fold, which should magnify bath depletion and intracellular buildup of 5-HT, did not change EC\(_{50}\) cRNA (Fig. 1B and data not shown). Even during long (60 min) transport assays with 33.6 ng of rsSERT cRNA, less than 5% of added \(^{3}H\)5-HT was accumulated inside the oocyte. Voltage could also affect \( \Phi_{5-HT} \) and \( I_{5-HT} \) differently to shift apparent cRNA potency. However, \( \rho \) was sensitive to the amount of injected cRNA even under constant voltage (Fig. 2A). Furthermore, all properties of SERT function were not sensitive to expression; for example, \( I_{5-HT} \) and \( \Phi_{5-HT} \) (Figs. 1B and 2B). Extrinsic chemical or electrical driving forces are therefore unlikely to account for the results shown in Figs. 1B and 2A. The amount of transporter protein on the plasma membrane likely altered an intrinsic property that depends on interactions of SERT with itself or with other factors. Such interactions were manifested as changes in the balance of 5-HT transport to 5-HT-induced current.

Channel-like activity in heterologous expression systems is generally ascribed to GAT/NET transporters because (a) transmitter-induced currents correlate with transport and radioligand binding in transfected or injected cells and are absent from naïve controls, (b) transport and current exhibit similar ion requirements and inhibitor sensitivities (see Refs. 20, 48, and 49 for reviews), and (c) channel events correlate with NE spikes patches containing hNET (50). For SERT we observed significant \( \Phi_{5-HT} \) even under conditions when \( I_{5-HT} \) was negligible but never the reverse, indicating that current was present only in cells with transport activity. Furthermore, \( I_{5-HT} \) was highly correlated with plasma membrane SERT protein (Fig. 1A). It is therefore unlikely that SERT currents were due to induction of endogenous channels (51); rather, \( I_{5-HT} \) was intrinsically associated with SERT function.

Nonclassical transporter properties are not unique to the GAT/NET family. EAAC1-mediated glutamate transport is biphasic with respect to the amount of injected cRNA (52), and Cl\(^−\) flux through NaPi-1 exhibits a different cRNA dependence than P\(_i\) transport (53). Large currents and variable stoichiometry common for GAT/NET and EAAT transporters (20, 48, 49) also appear in other transporters. Depending on Na\(^+\) concentration, sugar concentration, and membrane voltage, \( \rho \) may be either 1 or 2 for the SGLT1 Na\(^+/\)glucose co-transporter (54), and hormones modulate both glucose transport kinetics and ion stoichiometry in *Tilapia* intestinal brush-border membranes (55). Stoichiometry of the Na\(^+/\)HCO\(_3\) \(-\) transporter, NBC1, depends on the cellular host in which it is expressed (56).

Classical models predict \( \rho \) between 0 and 2 for GAT/NET transporters (19, 20, 48, 57). Experimentally, however, \( \rho \) is as high as 7 for hDAT (24) and rSERT (22) and 20–50 for *Drosophila* SERT (23, 58). In our experiments low SERT expression resulted in \( \rho = -7 \) at pH 5.0, and from Fig. 2B we calculated that \( \rho = -1 \) at pH 7.6. However, \( \rho \) increased over 4-fold as the expression level rose (Fig. 2A). Therefore, SERT behaved as a classical transporter at low expression level but became increasingly channel-like as the amount of surface SERT protein increased. Indeed, our results were consistent with reports of excess currents following injection of large quantities of cRNA (22–24, 47, 58).

The balance of current to transport was sensitive to coinjection of rsSERT with an inactive mutant, D98G (Fig. 5). If SERTs form mixed oligomers (11, 15), D98G may interact directly with wild-type SERT in an oligomeric complex to alter \( \rho \). Transmembrane-spanning segment I contained Asp\(^{98}\) and was implicated in substrate recognition, inhibitor binding, and 5-HT translocation (41, 59, 60); thus, mutations in this domain may be associated with an endogenous cellular factor. The amount of transporter protein on the plasma membrane likely altered an intrinsic property that depends on interactions of SERT with itself or with other factors. Such interactions were manifested as changes in the balance of 5-HT transport to 5-HT-induced current.
incomplete assemblies (61). EAAT3 glutamate transporters apparently form a pentameric structure, and the number of freeze fracture particles representing the multimer correlate linearly with the pre-steady-state current (46). In light of our findings, it would be interesting to know how EAAT3 transport and glutamate-induced current correlate with particle density and size.

The potency of SERT cRNA may reflect associations with molecules known to interact with GAT/NET transporters (34–36). For example, protein kinase C-mediated increase in surface localization of rGAT1 is lost at high expression, indicating that transporters interact with an endogenous factor that alters their subcellular trafficking (62). Substrates are also known to modulate GAT/NET transporter properties, regulation, and trafficking (37, 63, 64). For rGAT1, N-terminal amino acids and syntaxin 1A function coordinately to regulate GABA transport rate (35).

Although existing transporter models can account for large transporter-mediated ion fluxes (22, 23, 65, 66), they do not predict the expression level dependence of either ρ or ligand potency that we observed. To explain these new data, we propose the following: functional SERT is a ternary complex composed of the SERT oligomer (T), substrate (S), and an endogenous factor of limited abundance (X) (Fig. 7). The total number of oligomeric transporters on the plasma membrane is N = T + S + TX + TSX. In our experiments, we varied N by increasing cRNA injection, and we varied the S by addition of 5-HT. X was required for 5-HT transport and promoted transport over current when associated with T. A leftward shift in the apparent cRNA potency for Ψ5-HT therefore reported the functional association of X and T. Because TSX generated Ψ5-HT but little I5-HT, ρ = ~1. As N increased, X became limiting, Ψ5-HT reached a plateau, and TS generated I5-HT. Finally, X must govern substrate and inhibitor associations because ligands interact with higher potency at low N. This representation for transporters is reminiscent of the ternary complex model for G protein-coupled receptors (67), which describes how association with an interacting factor (G protein) can act as a molecular switch to regulate ligand affinity. Another parallel was found with ion channel β subunits, which translate cellular signals that modulate channel activity and localization (68).

Our model provides a framework for understanding functional complexity in SERT. Depending on expression level, SERT ranged from transporter-like (small charge movement accompanying 5-HT transport) to channel-like (large charge movement with 5-HT flux). The model thus provides an explanation for the surprising heterogeneity in currents associated with GABA transporter function (27). Apparent discrepancies (30, 31, 69, 70) are reconciled if different protocols alter the relative abundance of transporters and regulatory factors. We speculate that in the native environment, the balance of 5-HT transport to 5-HT-induced current will depend on the expression and localization of transporters and their associated factors. Indeed, large presynaptic 5-HT-induced currents are observed in native synapses (33). In vesicles and synaptosomes, 5-HT is more potent for uptake than in heterologous hosts (EC50 = 50–100 nm versus 0.5–1 μM), suggesting that expression of SERT alone may not recapitulate the native transport system (5, 7, 19, 57, 71–75). Transporter-associated currents may be tailored to neuronal activity and participate in shaping synaptic transmission if interactions between transporters and endogenous regulatory factors are dynamically regulated in the native environment.

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