Accumulation of an Antidepressant in Vesiculogenic Membranes of Yeast Cells Triggers Autophagy

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Abstract

Many antidepressants are cationic amphipaths, which spontaneously accumulate in natural or reconstituted membranes in the absence of their specific protein targets. However, the clinical relevance of cellular membrane accumulation by antidepressants in the human brain is unknown and hotly debated. Here we take a novel, evolutionarily informed approach to studying the effects of the selective-serotonin reuptake inhibitor sertraline/Zoloft® on cell physiology in the model eukaryote Saccharomyces cerevisiae (budding yeast), which lacks a serotonin transporter entirely. We biochemically and pharmacologically characterized cellular uptake and subcellular distribution of radiolabeled sertraline, and in parallel performed a quantitative ultrastructural analysis of organellar membrane homeostasis in untreated vs. sertraline-treated cells. These experiments have revealed that sertraline enters yeast cells and then reshapes vesiculogenic membranes by a complex process. Internalization of the neutral species proceeds by simple diffusion, is accelerated by proton motive forces generated by the vacuolar H+-ATPase, but is counteracted by energy-dependent xenobiotic efflux pumps. At equilibrium, a small fraction (10–15%) of reprotonated sertraline is soluble while the bulk (90–85%) partitions into organellar membranes by adsorption to interfacial anionic sites or by intercalation into the hydrophobic phase of the bilayer. Asymmetric accumulation of sertraline in vesiculogenic membranes leads to local membrane curvature stresses that trigger an adaptive autophagic response. In mutants with altered clathrin function, this adaptive response is associated with increased lipid droplet formation. Our data not only support the notion of a serotonin transporter-independent component of antidepressant function, but also enable a conceptual framework for characterizing the physiological states associated with chronic but not acute antidepressant administration in a model eukaryote.

Introduction

Cationic amphiphilic/amphipathic drugs (CAD) represent a subset of Food and Drug Administration (FDA) approved compounds that promiscuously interact with both proteinaceous and non-proteinaceous targets, the latter being cellular membranes [1,2]. CAD association with cellular membranes depends on an ionizable amine that is positively charged at physiological pH and a lipophilic polycyclic scaffold, but does not depend on stereochemistry, as in the peculiar case of the antidepressant sertraline/Zoloft® moonlighting as a fungicide [3]. The primary protein target of sertraline is thought to be the human serotonin transporter (hSERT), which localizes to synaptic clefts and recycles the monoamine neurotransmitter serotonin after each burst of neurotransmission. According to the monoamine hypothesis of depression, antidepressants like sertraline bind hSERT and acutely block reuptake of serotonin in the brain [4]. However, a latency period whose molecular basis is unknown precedes the emergence of the actual antidepressant effect in humans, and in rodent behavioral models of depression, suggesting that antidepressants exert additional effects at targets besides hSERT. Given the well known and wide-ranging effects of CAD on cellular membrane homeostasis in the absence of specific proteins targets [5,6], the clinical relevance of antidepressant accumulation in neuronal cell membranes has been vigorously debated. For example, there is evidence that supports the existence of serotonin transporter-independent components of antidepressant function in vertebrate cellular models [7], some of which appears to involve membrane accumulation by antidepressants [8,9]. Yet a comprehensive model of antidepressant function that accounts for all drug-target interactions in the human brain has so far been elusive.

The goal of the present study is to begin developing and validating a comprehensive model of complex antidepressant function in humans. The first step in this arduous process is to reconcile two pharmacological perspectives that have historically dominated conventional thinking about CAD activity in cells lacking specific integral membrane protein targets. On the one hand, a molecular view of drug-membrane interactions derives from the seminal work of Singer and Sheetz on amphipath-induced morphological transformations of freshly isolated human erythrocytes, a cell-based model system superior to reconstituted liposomes but still lacking endomembranes. Singer and Sheetz proposed the bilayer couple/balance model, which states that a charged amphipath preferentially accumulates at equilibrium in the leaflet (monolayer) exhibiting the opposite net charge [10]. A
disparity in inter-leaflet surface area of less than 1% resulting from asymmetric partitioning by charged amphipaths can be readily observed as dramatic macroscopic changes in the topology of the erythrocyte plasma membrane. On the other hand, a physiological view was developed around the same time by Christian de Duve and colleagues, and is called lysosomotropism, or "ion trapping." Lysosomotropism is defined as the concentrative capacity of acidic organelles to trap protonated weak bases within, and cannot be modeled by red blood cells [11]. Lysosomotropism has been documented in various mammalian cell lines and in whole organisms treated with CAD.

Here we build on an effort begun in our previous study of sertraline-induced "overdose" [12], in which we demonstrated that the model eukaryote Saccharomyces cerevisiae (budding yeast) is an ideal experimental system in which to combine the biophysical insights of the bilayer couple model with the physiological insights of lysosomotropism. In that study, we reported the isolation and genetic characterization of sertraline overdose-resistant mutants (sertrR) with altered clathrin function or reduced vacuolar H+-ATPase complex activity. Others have also shown that yeast is amenable to studying cellular membrane accumulation by CAD [13–15]. However, a caveat of our previous study is that selection for sertrR mutants required supra-therapeutic (~10^{-5} M) drug concentrations. Here we applied techniques of classical pharmacology to yeast, which enabled us to measure membrane concentration by radiolabeled sertraline – hereafter [3H]sertraline – at clinically relevant (~10^{-9} M) concentrations. We conclude the present study by proposing an evolutionarily informed model of antidepressant function that may provide a molecular basis for neurotrophism induced by chronic treatment with antidepressants in rodent models of human depression, and by extension the therapeutic lag observed in patients taking antidepressants.

Results

Two thermodynamic drivers of sertraline entry into yeast cells

We treated wildtype BY4716 cells (hereafter "wildtype") with [3H]sertraline, which we obtained by custom synthesis (see Materials and Methods). We report a total [3H]sertraline cellular accumulation (B_{max}) equal to 0.019 pico moles (pmol) per 10^7 cells (+/- 0.0014 SEM), and a half-maximal [3H]sertraline cellular uptake rate equal to 3.1 minutes (+/- 0.97 SEM) (Fig. 1A). These data are consistent with the lysosomotropic mechanism originally described de Duve and colleagues [11]. Briefly, as the pH of the extracellular medium increases, the deprotonation of sertraline is favored; the ratio of neutral to cationic species reaches unity at the pKa of sertraline. Neutral sertraline is membrane-permeable while charged sertraline is not. Therefore, more [3H]sertraline is internalized by cells growing in alkaline media compared to acidic media. Several classical studies showed that cellular uptake and accumulation of radiolabeled tricyclic antidepressants by primary neurons and fibroblast cell lines is lysosomotropic and Na+-independent [16,17].

However, [3H]sertraline cellular uptake is only partially dependent on proton motive forces generated by vacuolar H+-ATPase complexes (V-ATPases) [18], which can be specifically inhibited by the macrolide antibiotic bafilomycin A (BAF). Pretreatment of wildtype cells with BAF for 30 minutes resulted in a 65% reduction in [3H]sertraline cellular accumulation compared to the control condition, while treatment of wildtype cells with BAF 30 minutes after exposure to [3H]sertraline resulted in reduced [3H]sertraline cellular accumulation that was 57% of the control amount (Fig. 1B). We performed four controls in order to demonstrate the specificity of V-ATPase-dependent proton motive forces. First, a day1,2,3A triple mutant, which exhibits constitutive vacuolar hyper-acidification [19], significantly hyper-accumulates [3H]sertraline while the ywa9 mutant, which exhibits constitutive vacuolar alkalinization, hypo-accumulates [3H]sertraline (Fig. 1C). Second, the effects of BAF on [3H]sertraline accumulation are completely abolished in a ywa9 (YCL003V-A) mutant, which normally encodes a subunit e of the V0 subunit of the V-ATPase complex (Fig. 1B). Third, before and after treatments of wildtype cells with oligomycin, a specific chemical inhibitor of the F1-F0 mitochondrial ATPase, actually resulted in slightly increased [3H]sertraline accumulation (Fig. 1B). Fourth, FCCP, a non-specific proton ionophore, phenocopies the effects BAF but co-administration of these two agents does not exhibit additivity (Fig. 1B). Interestingly, single cells overdose in the presence of sertraline in a stochastic manner (Fig. 1D). Thus, at the population level and at the level of single cells, the cellular uptake of [3H]sertraline appears to be non-uniform; a fraction of internalized sertraline is "ion trapped," while the remainder is associated with cellular membrane sites.

Next we measured [3H]sertraline cellular uptake and accumulation in response to several environmental perturbations that affect cellular membrane function globally. First, we examined the effect of low temperature, as low temperature promotes the liquid crystalline-gel transition of membranes, i.e., decreases membrane fluidity. Membrane fluidity has been shown to be a determinant of local anesthetic partitioning into reconstituted liposomes [20]. The initial rate of [3H]sertraline cellular uptake by wildtype cells is four times slower at 0°C versus 25°C; after 60 minutes, cells incubated at 0°C accumulate 45% of the total [3H]sertraline taken up by isogenic cells incubated at 25°C (Fig. 2A). To rule out that low temperature mediates this dampening effect through cessation of vesicle-mediated transport, we also measured [3H]sertraline cellular accumulation by a sec18" mutant, which is conditionally unable to perform membrane-membrane fusion reactions after temperature up-shift [21]. We observed no significant differences between sec18" and its wildtype reference after a short (25 minute) or long (60 minute) incubation at the non-permissive temperature (Fig. 2B). Next, we tested whether [3H]sertraline cellular uptake and accumulation depends on energy. We pretreated wildtype cells with a cellular ATP depleting cocktail containing 10 mM sodium azide and 10 mM 2-deoxy-D-glucose. Total [3H]sertraline cellular accumulation was increased 3.4-fold in the presence of energy poisons (Fig. 2C). We interpret this result to mean that energy-dependent xenobiotic efflux pumps constitutively extrude [3H]sertraline from the cell. Thus, the association of [3H]sertraline with cellular membranes appears to depend on bulk physical properties of the bilayer.

Sertraline permeates vesiculogenic membranes

We characterized the subcellular distribution of [3H]sertraline in wildtype cells by biochemical fractionation experiments. As shown in Figure 3A, over 80% of intracellular [3H]sertraline sediments in the P_{10,000} fraction following osmotic lysis; that percentage climbs to 90% after mechanical lysis (Fig. 3B). After accounting for the trace amount of [3H]sertraline present in the P_{100,000} microsomal fraction, only 10–15% of intracellular [3H]sertraline appears to be truly soluble, demonstrating that at equilibrium the vast majority of [3H]sertraline stably partitions into cellular membranes, presumably as a neutral species. To verify that [3H]sertraline partitioned into cellular membranes we performed the same experiment but in the presence of the nonionic detergent Triton X-100. Triton X-100 completely solubilizes P_{10,000}-associated [3H]sertraline (P<0.0001, ANOVA;
However, detergent-sensitive cellular membrane binding sites may not be chemically uniform. We reasoned that we could distinguish between at least two types of membrane association – adsorption (topical) versus intercalation (deep) – on the basis of chemical extractability of [3H]sertraline from the P10,000 fraction. The results of this analysis are presented in Figure 3B. Chemical agents that disrupt electrostatic interactions (e.g., 0.5 M Tris) either have no or little solubilizing effect on membrane-associated [3H]sertraline, while chemical agents that disrupt hydrophobic interactions, including the mild detergent digitonin, solubilize membrane-associated [3H]sertraline to varying degrees. Proteolytic digestion of surface-exposed membrane proteins has a modest solubilizing effect, ruling out membrane proteins as essential for amphipath-membrane association. Interestingly, excess (100 μM) ''cold'' sertraline only has a modest solubilizing effect, which is consistent with the notion that sertraline is buried in the hydrophobic phase of the bilayer at equilibrium.

A P10,000 fraction is thought to be enriched in large organelles like vacuoles and mitochondria at the expense of small organelles like Golgi and ER. As an initial attempt to biochemically purify sertraline-associated cellular membranes, we performed analytical density-gradient centrifugation on a P100,000 fraction after a single-step centrifugation of lysates from wildtype cells. A single peak spanning two fractions of intermediate density (refractive index in the range 1.37–1.38) contains on average \( \frac{70}{60} \)%, of membrane-associated [3H]sertraline, with \( \frac{60}{70} \)% concentrated in a single fraction (fraction 6) (Fig. 3C). We screened these gradient fractions against a panel of antibodies specific for markers residing in different subcellular compartments, and densitometry plots are shown for each marker in Figure 3D. Although our gradient had limited resolving power at fractions 6–7, we observed the strongest co-localization of markers specific for ER and vacuolar membrane markers, as well as the vacuolar resident enzyme carboxypeptidase, with the [3H]sertraline peak in fraction 6, but we did not observe co-localization with a plasma membrane marker (data not shown). We observed less albeit still significant co-localization of Golgi and endosomal membrane markers with [3H]sertraline, though these markers themselves peak in the slightly denser fraction 7. Although the mitochondrial marker porin is also present in fractions 6–7, we showed above that disrupting proton motive forces with oligomycin actually increased [3H]sertraline accumulation (Fig. 1B), so while association with mitochondrial cannot be ruled out it appears coincidental.
Pharmacological characterization of mutants with altered sertraline sensitivity

We reasoned that comparison of measurements of $[^{3}H]$sertraline cellular uptake and accumulation in a panel of mutant strains with altered sertraline cellular response would allow us to develop a cell biological model of cellular membrane accumulation by sertraline. The wildtype strain serves as a reference for three previously described de novo sert$^R$ mutants: vma9 (described above), sec62 (YDR329C) and che1 (YGR206C) [12]. ACY769 is a wildtype prototrophic strain derived from S288c (hereafter “prototroph”), which serves as a reference for four sertraline-hypersensitive (sert$^{HS}$) homozygous gene deletion mutants involved in clathrin coat formation: af11Δ, cdc50Δ, sec62Δ and sac1Δ. ARF1 (YDL192W) encodes a small GTPase that is thought to be a master regulator of vesiculogenesis at internal membranes. DRS2 (YAL026C) encodes an aminophospholipid flippase that localizes to Golgi and endosomal membranes, and CDC50 (YCR094W) encodes its regulatory subunit. SAC1 (YKL212W) encodes a lipid phosphatase with specific activity against phosphatidylinositol 4-phosphate (PIP), and localizes to the endoplasmic reticulum (ER) and Golgi. Together these sert$^R$ and sert$^{HS}$ mutants comprise a “vesiculogenesis” module centered around ARF1 and PIP, as deletion of ARF1 is synthetically lethal with loss of SWA2, DRS2 or CDC50 [22,23].

Table 1: Total $[^{3}H]$sertraline cellular accumulations ($B_{max}$) for each of the mutants appear in Table 1.

Unlike the vma9 mutant or BAF-treated wildtype cells, the che1 mutant and to a lesser extent the sec62 mutant, unexpectedly hyper-accumulate $[^{3}H]$sertraline. Therefore it may be more appropriate to classify che1 and sec62 mutants as “sertraline-tolerant.” This hyper-accumulation is distinct from that exhibited by af11, cdc50, as both che1 and sec62 have a constitutive vacuolar acidification defect that phenocopies V-ATPase deficiency [12]. Controlling for cell number and cell size, we observed that two other mutants hyper-accumulate $[^{3}H]$sertraline: sac1Δ and af11Δ, sec62Δ and cdc50Δ mutants are indistinguishable from the control condition. These results indicate that there are at least two qualitatively distinct phases of the cellular response to sertraline: the first phase affects acute sertraline uptake, while the second phase, which is typified by sec62Δ, has no effect on acute sertraline uptake, and so instead must be involved in the adaptation to chronic sertraline exposure.

To explore this phenomenon more rigorously, we measured the kinetics of $[^{3}H]$sertraline cellular uptake as a function of substrate concentration. Kinetic experiments with both wildtype references and mutants with altered sertraline cellular response indicate a single non-saturable component of $[^{3}H]$sertraline cellular uptake, i.e., internalization by simple diffusion (Fig. 4A–B). We estimate $V_{max}$ to be 0.62 pmol $[^{3}H]$sertraline/10^7 cells/min, which translates to ~1000 sertraline molecules per cell per minute subject, of course, to individual differences between clones. We estimate the $K_m$ of sertraline to be 71.5 nM, which represents high-affinity binding but less than the affinity exhibited by it for the human serotonin transporter [24]. The panel of mutants exhibited a range of acute $[^{3}H]$sertraline cellular uptake rates. Neither vma9 nor sec62Δ mutants show a measurable difference in the rate of $[^{3}H]$sertraline cellular uptake compared to wildtype. However, as expected che1, sec62, af11Δ and sac1Δ take up more $[^{3}H]$sertraline per unit time, in the order sac1Δ>che1>af11Δ>sec62Δ. Acute sertraline hyper-accumulation appears to have two different molecular bases, as indicated by the results of screening the mutants on a secondary phenotype of BAF-induced release of internalized $[^{3}H]$sertraline (Fig. 4C–D).

Among the sert$^R$ mutants, sec62 is more resistant to the effects of BAF than wildtype, while che1 is unchanged compared to wildtype.
indicating that one component of sertraline’s cellular membrane association may be complexly regulated by the rate of clathrin assembly and disassembly. The \textit{sac1} \textit{D} mutant appears to accumulate sertraline by a completely distinct mechanism. \textit{sac1} \textit{D} cells exhibit a near 20\% reduction in the fraction of [3H]sertraline retained after inhibition of V-ATPase complexes compared to wildtype (Fig. 4D). This result suggests that another component of sertraline’s cellular membrane association may be regulated by the levels of the phosphoinositide PIP. Phosphoinositides have been shown to constitute anionic binding sites for CAD at the interfacial region of the bilayer in addition to the phosphate groups forming the backbone of glycerophospholipids [25]. We also observed that cell growth rate in the presence of overdose concentrations of sertraline is not correlated with the rate of nanomolar [3H]sertraline cellular uptake, as clearly demonstrated by a comparison between the sert\textit{HS} mutants \textit{sac1} \textit{D} and \textit{drs2} \textit{D} (Fig. 4E–F). This result demonstrates that phospholipid asymmetry, as opposed to membrane anionicity per se, is a key component of the adaptive physiological response to chronic cellular membrane accumulation by sertraline, as was originally proposed by Huestis and colleagues in experiments on erythrocytes [26].

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Subcellular fractionation demonstrates [3H]sertraline accumulation in vesiculogenic membranes. (A) The percent [3H]sertraline detected in soluble ("S") versus pellet ("P") fractions following sequential centrifugations (10,000 \( \times \) g and 100,000 \( \times \) g) in the absence (white columns) or presence of 0.5\% Triton X-100 detergent (hatches). (B) The ratio of soluble (yellow) to P\(_{100,000}\)-associated (blue) [3H]sertraline following extraction with a diverse panel of chemical agents. * \( P<0.05\), ANOVA; ** \( P<0.0001\), ANOVA. (C) Distribution of [3H]sertraline across ten equal volume fractions following Optiprep gradient separation of total organellar membranes (P\(_{100,000}\)) from wildtype BY4716 cells treated with [3H]sertraline for one hour at 25\°C. The refractive index is plotted (blue slashed line) on the left y-axis. (D) Densitometry plots of six organellar markers distributed across the ten gradient fractions. Error bars indicate SEM. Means were generated from three independent biological replicates. doi:10.1371/journal.pone.0034024.g003
\end{figure}

Ultrastructural analysis of autophagy induction and membrane quality control

The pharmacological and biochemical experiments described above localize sertraline to vesiculogenic membranes. We therefore performed an ultrastructural examination of organellar membrane homeostasis on yeast cells exposed to 60 \( \mu \)M sertraline for 45 minutes, which constitutes a sub-lethal chronic treatment (Fig. S1). If the bilayer couple model holds, then exogenous cationic amphipaths will favor the more negatively charged leaflet of organellar membranes. In the specific case of sertraline, we expected to find examples of damaging local curvature stress in membranes comprising the vesicular transport pathway. Initial support for this hypothesis was found in the observation that micromolar sertraline treatment has three noteworthy effects on vacuole homeostasis, which serves as a quantitative cell biological read out. First, we observed a decrease in the number vacuoles per cell in wildtype cells treated with 60 \( \mu \)M sertraline (Fig. 5A). The percentage of vacuole-less cells increases (36\% vs. 12\%) as does the percentage of cells containing one large consolidated vacuole (49\% vs. 29\%). In fact, loss of vacuoles was observed in all strains treated with 60 \( \mu \)M sertraline (Fig. 5B–H). Second, the steady-state distributions of vacuoles per cell are increased relative to wildtype...
in three mutants: *cht1*, *swa2* and *arf1D*. Interestingly, the vacuoles of the *sertr* mutant *arf1D* exhibits polygonal and tubulated morphologies [Fig. 5F, inset], while the vacuoles of the *sertr* mutants *cht1* and *swa2* cells are more likely to have regions of thickened bilayer [Fig. 5D, inset]. The seminal study by Lieber et al documented membrane thickening in erythrocytes bathed in the phenothiazine chlorpromazine [27]. Third, we observed a sharp increase in the percentage of electron-lucent wildtype vacuoles (24% vs 97%) [Fig. 5A, inset], and comparable increases were observed across all the strains [Fig. 5, red insets].

We reasoned that the distribution of vacuoles per cell would allow us to test the hypothesis that sertraline hyper-accumulation is caused by an increase in the surface-area-to-volume ratio of vacuoles or other V-ATPase-acidified organelles of the vesicular transport pathway. The steady-state number of vacuoles per cell separates the four sertraline hyper-accumulating mutants into two unequal groups. Group One is *arf1D*, *cht1*, and *swa2*, three genes that interact physically and genetically [22]. Group Two is *sertr*; *sertr* cells accumulate the phosphoinositide PIP, which recruits and activates *ARF1* and presumably stimulates vesiculogenesis. Changes in the number or surface-area-to-volume ratio of organelles may explain the hyper-accumulation phenotype of Group One mutants, but does not appear to explain the hyper-accumulation phenotype of *sertr* cells, which have fewer vacuoles per cell at steady state. Also, the vacuolar membranes of *sertr* cells appear to have an aberrant crenated morphology [Fig. 5F, inset].

The spike in electron lucency combined with a reduction in the total number of vacuoles per cell is accompanied by the appearance of double membrane-bound autophagosomes, a tell-tale sign of autophagy. A typical sertraline-treated wildtype cell after five minutes of exposure to 60 μM sertraline is illustrative (Fig. 6A). Changes in gross vacular morphology, electron lucency, and fine vacuolar membrane structure are typical of sertraline-treated cells at all time points, and a magnified view of a representative vacuole highlights the salient changes (Fig. 6B, C). The aberrant multilamellar membranous structure marked by an asterisk in Figure 6A is clearly encased in an autophagosome (Fig. 6C). Quantification of autophagosomes like those appearing in Figure 6C–D in wide-field images corroborates the single-cell snapshots: untreated wildtype cells contain on average 0.11

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**Table 1.** Total [3H]sertraline cellular accumulation (Bmax) as a function of pH. Bmax is defined as pmol/10⁷ cells/hour.

|          | Bmax | Bmax 95% CI | r²   |
|----------|------|-------------|------|
| **pH 6.0** |      |             |      |
| BY4716   | 0.00558 (+/−0.000532) | 0.00448 to 0.00669 | 0.593 |
| chc1     | 0.0199 (+/−0.0288)** | 0.0139 to 0.0259 | 0.403 |
| swa2     | 0.0118 (+/−0.00127)** | 0.00919 to 0.0144 | 0.457 |
| vma9     | -    | -           | -    |
| ACY769   | 0.0109 (+/−0.000767) | 0.00933 to 0.0124 | 0.522 |
| drs2.Δ   | 0.0166 (+/−0.00805)** | 0.0149 to 0.0183 | 0.881 |
| cdc50.Δ  | 0.0183 (+/−0.0114)** | 0.0159 to 0.0206 | 0.844 |
| arf1.Δ   | 0.0168 (+/−0.00223)** | 0.0122 to 0.0215 | 0.462 |
| sac1.Δ   | 0.0399 (+/−0.00250)** | 0.0343 to 0.0447 | 0.826 |
| **pH 6.5** |      |             |      |
| BY4716   | 0.0189 (+/−0.00140) | 0.0161 to 0.0217 | 0.575 |
| chc1     | 0.0363 (+/−0.00091)** | 0.0240 to 0.0486 | 0.333 |
| swa2     | 0.0196 (+/−0.00245)** | 0.0145 to 0.0247 | 0.499 |
| vma9     | 0.0142 (+/−0.000111) | 0.0119 to 0.0165 | 0.750 |
| ACY769   | 0.0326 (+/−0.00136) | 0.0299 to 0.0353 | 0.864 |
| drs2.Δ   | 0.0401 (+/−0.00224)** | 0.0355 to 0.0447 | 0.873 |
| cdc50.Δ  | 0.0410 (+/−0.00295)** | 0.0348 to 0.0471 | 0.835 |
| arf1.Δ   | 0.0747 (+/−0.00688)** | 0.0605 to 0.0890 | 0.750 |
| sac1.Δ   | 0.0896 (+/−0.00857)** | 0.0718 to 0.107 | 0.685 |
| **pH 7.0** |      |             |      |
| BY4716   | 0.0292 (+/−0.000217) | 0.0248 to 0.0337 | 0.826 |
| chc1     | 0.0750 (+/−0.00633)** | 0.0619 to 0.0882 | 0.682 |
| swa2     | 0.0797 (+/−0.00533)** | 0.0686 to 0.0907 | 0.847 |
| vma9     | -    | -           | -    |
| ACY769   | 0.0639 (+/−0.00203) | 0.0599 to 0.0680 | 0.925 |
| drs2.Δ   | 0.0724 (+/−0.00460)** | 0.0628 to 0.0819 | 0.900 |
| cdc50.Δ  | 0.0717 (+/−0.00516)** | 0.0610 to 0.0824 | 0.860 |
| arf1.Δ   | 0.128 (+/−0.0159)** | 0.0946 to 0.161 | 0.715 |
| sac1.Δ   | 0.246 (+/−0.0148)** | 0.215 to 0.277 | 0.877 |

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autophagosomes, while sertraline-treated cells contain 0.48, an increase of 4.2-fold. Multilamellar structures encapsulated by autophagosomes were observed in wildtype cells at all time points.

Interestingly, similar membranous structures have been observed in brain tissues of rodents treated with the illicit psychoactive CAD 3,4-Methylenedioxymethamphetamine (MDMA) [28]. Two par-
Figure 5. Vacuolar number, morphology and contents assessed from transmission electron micrographs of untreated and sertraline-treated cells. (A) BY4716; (B) vma9; (C) swa2; (D) chc1; (E) prototroph; (F) sac1Δ; (G) dss2Δ; (H) arf1Δ. For each strain, the distribution of vacuoles per cell is shown for a population of untreated cells (black columns) and 60 μM sertraline-treated cells (red columns). Insets contain a representative vacuole from the untreated (black outline) and 60 μM sertraline-treated (red outline) populations.

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ticularly striking examples are highlighted. In Figure 6D, black arrows mark the sites where two unilamellar vesicles, one of which contains a densely packed membranous whorl, are clearly discerned from the outer membrane of the autophagosome. And in Figure 6E, lamellar plumes of membrane form a Medusa-like structure trapped inside a large electron-lucent vacuole.

If autophagy is triggered by sertraline accumulating in organelar membranes, one would expect to observe ultrastructural evidence of membrane curvature stress throughout the vesicular transport pathway, not just in vacuolar membranes. In this instance, a representative wildtype cell treated with 60 μM sertraline for 45 minutes is illustrative (Fig. 7A). The structure marked by an asterisk is a shown at higher magnification to be a dilated cisterna, possibly a Golgi stack or an autophagosomal precursor, with circular or crescent morphology (Fig. 7B). Similar structures were observed in other sertraline-treated cells at this time point, including an example of a dilated cisterna with clearly thickened membranes on the convex surface. (Fig. 7C). Comparison of an untreated aff1Δ cell to a sertraline-treated aff1Δ cell reveals several localized exaggerated regions of membrane expansion in comparable organelar structures (Fig. 7D–E).

Finally, two mutants, chc1 and swa2, exhibited a unique ultrastructural phenotype that may be involved in adaptive cellular pathways that degrade and regenerate cellular membranes infiltrated by exogenous cationic amphipaths. At steady state, chc1 and swa2 mutants exhibit a vacuolar expansion/fragmentation phenotype that is “normalized” after 60 μM sertraline treatment for 45 minutes insofar as the number of vacuoles per cell decreases (Fig. 5C–D). However, these vacuoles exhibit a non-random distribution of osmiophilic luminal contents and appear to contain undigested vesicular compartments (Fig. 8B). Interestingly, the number of lipid droplets, which are storage depots for neutral lipids (e.g., triglycerides) and marked by red asterisks (Fig. 8B), increased after sertraline treatment in wildtype and sertR and sertHS mutant strains by an unknown mechanism (Fig. 8C). This increase is most pronounced in sertraline-treated chc1 and swa2 cells; untreated chc1 cells have a mean lipid droplet count equal to 0.32, while sertraline-treated chc1 cells have a mean lipid droplet count equal to 2.2. By contrast, untreated wildtype cells have a mean lipid droplet count equal to 0.31, while sertraline-treated wildtype cells have a lipid droplet count equal to 0.55. One explanation for this result is that lipid droplets form during adaptation of yeast cells to secretory pathway stress, as has been suggested by others [29].

Discussion

We presented evidence that supports an evolutionarily informed, cell biological explanatory model of cellular membrane accumulation by sertraline in a simple eukaryote with an intact secretory pathway. Explication of our model begins with the passive internalization of neutral sertraline molecules at the plasma membrane. Lysosomotropism acts as an amplifier of simple diffusion, and suggests a mechanism whereby sertraline may distribute non-uniformly throughout vertebrate tissues as a result of tissue-specific activity or regulation of V-ATPase-dependent acidification [30]. The internalization of sertraline appears to follow an entry route that is orthogonal to ATP-dependent, SEC gene-requiring internalization of larger arylic cationic amphipaths (e.g., lysophospholipids), i.e., does not depend on the endocytic pathway [31]. However, passively internalized sertraline appears to be a potent substrate for ATP-dependent efflux pumps. In fact, efflux may explain the paradoxical observation that at physiological pH, sertraline is predicted to be over 99% ionized yet only a minority fraction (10–15%) is observed to be soluble at equilibrium. Our interpretation is that the ionized pool of

Figure 6. A time course of sertraline treatment reveals early and persistent induction of autophagy and the appearance of aberrant multilamellar structures. (A) Representative 60 μM sertraline-treated wildtype BY4716 cell after 5 minutes of sertraline treatment. Magnification is 6,300× (scale bar = 0.5 micron). The lone vacuole of the cell in (A) is shown at higher magnification (16,000×; scale bar = 0.1 micron) in panel (B). The black asterisk denotes an autophagosome that has encapsulated a membranous whorl, which is shown at higher magnification (25,000×; scale bar = 0.1 micron) in (C). The black arrows in (D) mark the telltale double membrane of an autophagosome at higher magnification (25,000×; scale bar = 0.1 micron). An elaborated multilamellar structure from a BY4716 cell treated with 60 μM sertraline for 20 minutes is shown at higher magnification (16,000×; scale bar = 0.1 micron) in (E).

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sertraline is actively depleted both by ATP-dependent efflux and by sequestration in cellular membranes as a neutral species.

Despite our best efforts to separate vesiculogenic membranes into discrete organelles by density-gradient centrifugation, parsimony dictates that sertraline asymmetrically accumulates in the membranes of all V-ATPase-acidified organelles, presumably in proportion to local lysosomotropic driving forces. Specifically, sertraline associates with organellar membranes by two mechanisms: adsorption to solvent-exposed anionic sites, and intercalation into the bulk hydrophobic phase of the bilayer. A two-state, weakly binding and strongly binding model has been proposed for local anesthetic association with reconstituted liposomes [32], and we argue that adsorptive binding by sertraline in yeast cells may be mediated by electrostatic interactions, while intercalative binding by sertraline in yeast cells may be mediated by lipophilic interactions. However, several uncertainties remain in part because in vivo experiments on CAD in living cells are unlike experiments on CAD in reconstituted liposomes, in which the ionization state of CAD can be experimentally manipulated. Therefore, we conclude that sertraline-membrane association is a composite of more than one binding interaction, a conclusion supported by molecular dynamic simulations [33,34].

The central finding of our study is that at micromolar doses, cellular membrane accumulation by sertraline induced curvature stresses throughout the organelles of the vesicular transport pathway. This membrane curvature stress triggers an autophagy-dependent membrane quality control response that appears to be enhanced in mutants with altered clathrin function. Although we have not provided biochemical evidence of autophagy induction, our ultrastructural approach revealed unambiguous induction of autophagy. We argue that autophagy mitigates cationic amphipath accumulation in cellular membranes. This interpretation is supported by a recent study that documented induction of autophagy by antidepressants in mammalian neuronal cell lines [35]. Autophagy may be one of several buffering systems that evolved to preserve cellular membrane homeostasis in the face of endogenous (or exogenous) charged amphipath accumulation, but when sertraline-induced membrane curvature stresses become too punishing at high or sustained doses, cytotoxicity ensues [36]. However, the cell-physiological effects of sub-lethal doses of sertraline may not be deleterious. We previously showed that low micromolar sertraline partially rescues the constitutive growth defect of yeast mutants with altered clathrin function [12]. If these buffering systems are defective due to genetic (e.g., clathrin dysregulation) and/or environmental stressors – resulting in a maladaptive homeostatic set point – sub-lethal accumulation of amphipath may normalize this set point. A “trophic” or cytoprotective effect might ensue given the ancient coupling between membrane transport and cell growth [37]. A similar argument was proffered by researchers explaining the cytotoxic effects of the phenothiazine antipsychotic drug chlorpromazine in yeast cells [13]. In conclusion, our model supports the notion of a serotonin transporter-independent component of antidepressant pharmacology in humans, and appears to buttress the neurotrophic hypothesis of depression [38]. It is tempting to speculate that amphipath accumulation may, in specific contexts, provide a trophic signal through direct modulation of the physical properties of cellular membranes, perhaps resulting in vesicle formation, thereby exploiting (or “short-circuiting”) the aforementioned coupling between membrane transport and cell growth. Such a...
mechanism would be expected to unfold over long time scales given the vigilance of those buffering systems, and the large effective volume of cellular membranes of the mammalian brain.

Materials and Methods

Yeast strains and culture conditions

Standard growth conditions wereYPD media (1% yeast extract, 2% peptone and 2% dextrose) buffered with 10 mM HEPES to the desired pH. Some experiments with prototrophic strains were carried out in HEPES-buffered minimal media (yeast nitrogen base containing ammonium sulfate, 2% dextrose). BY4716, BY4742 or ACY769 were employed as wildtype reference strains as appropriate, and are all derived from S288c. sertR mutant strains were previously described [12]. sertHS deletion strains were derived from ACY769 and belong to a prototrophic homozygous deletion collection generated by D. Hess (Santa Clara University) and A. Caudy (University of Toronto). The dnf1Δ dnf2Δ dnf3Δ mutant was kindly provided by T. Graham (Vanderbilt). The sec18ts mutant was kindly provided by W. Prinz (NIH).

Chemical compounds

Sertraline hydrochloride (Sigma-Aldrich) was resuspended in dimethyl sulfoxide (DMSO) to a final concentration of 25 mg/mL (~73 mM), and 100 μL aliquots were stored in glass vials at −20°C until use and subjected to a maximum of one freeze/thaw cycle. Sertraline [N-methyl-3H] hydrochloride (American Radiolabeled Chemicals, Inc.) is 99% pure by HPLC, has a specific activity of 80 Ci/mmol, and was kept at a stock concentration of 1 mCi/mL in ethanol.

Pharmacological/biochemical assays and analysis

Overnight cultures were diluted in fresh pH-buffered YPD medium and incubated at 30°C till log phase. Cell number and cell size were determined by the Coulter counter method. For uptake and accumulation experiments, 0.25 μCi [3H]sertraline (American Radiolabeled Chemicals) was added to 2 mL yeast culture aliquot (all 0-minute time point aliquots contained 1 μM sodium azide and sodium fluoride). Cells were collected on Durapore® PVDF filters (Millipore) by passing through a filter unit, and washed with 18 ml ice cold pH-buffered YPD. Filters were incubated with 300 μL cold lysis buffer (acetonitrile:methanol:water, 40:40:20) at −20°C for 10 minutes. Cell pellets were collected by pipetting and transferred to scintillation vials containing 4 mL Cytoscint fluid (Fisher Scientific, Inc.). Counts were obtained using a Perkin Elmer Tri-Carb 2800TR liquid scintillation analyzer. For kinetic experiments, cells were incubated with specified concentrations of [3H]sertraline for 2 minutes, then collected on filters and washed with ice cold YPD containing 10 μM unlabeled sertraline [39]. Subcellular fractionation was carried out as follows. Cells were sphereplasted following Zymolyase (Zymo Research) digestion at 30°C for 1 hour according to manufacturer’s protocol. Spheroplasts were osmotically lysed in lysis buffer (50 mM Tris-HCl, 0.8 M sorbitol). The resulting lysate was centrifuged at 400 rcf for 10 minutes, yielding a post-nuclear lysate. An initial spin at 10,000 rcf for 10 minutes yielded P10,000 (enriched in large organellar membranes) and S10,000 fractions; S10,000 was spun at 100,000 rcf for 50 minutes, yielding a microsomal (P100,000) and a true soluble fraction (S100,000). All pellets were resuspended in equal volumes of the same buffer as the corresponding supernatant fractions. For experiments involving density-gradient centrifugation, the 10,000 rcf pellet obtained during fractionation was resuspended in 1 mL 35% Optiprep solution (Sigma Aldrich), on top of which

Figure 8. The chc1 mutant exhibits unique adaptation to chronic sertraline exposure. (A) A representative untreated chc1 cell contains five electron-dense vacuoles (10,000×; scale bar = 0.5 micron). (B) A representative sertraline-treated chc1 cell contains a single vacuole and four lipid droplets marked by red asterisks (10,000×; scale bar = 0.5 micron). (C) Quantification of lipid droplet formation in untreated and sertraline-treated cells. The “−” column correspond to lipid droplet counts performed on untreated cells. The “+” column correspond to lipid droplet counts performed on cells treated with 60 μM sertraline for 45 minutes. The strain identities are: BY4716 (black); chc1Δ (red); swa2Δ (green); vmad9 (magenta); arflΔ (orange); sds2A (yellow); sac1Δ (blue). doi:10.1371/journal.pone.0034024.g008
from wide fields as previously described [12]. Wide-field cell counts for the 45-minute dataset were as follows: BY4716 (untreated n = 216, sertraline-treated n = 193); prototroph (untreated n = 247, sertraline-treated n = 184); ahr1 (untreated n = 234, sertraline-treated n = 202); stn2 (untreated n = 165, sertraline-treated n = 160); van9 (untreated n = 212, sertraline-treated n = 160); sar1 (untreated n = 219, sertraline-treated n = 151); dhs2 (untreated n = 220, sertraline-treated n = 150); aip1 (untreated n = 194, sertraline-treated n = 133).

Supporting information
Figure S1 Growth rate of BY4716 cells as a function of sertraline concentration. Cells at optical density (OD600) equal to 1.0 were used as the initial inoculum.

Methods S1 Detailed protocol describing “ROTO” (Reduced Osmium tetroxide Thiocarbohydrydrase - reduced Osmium) transmission electron microscopy. (DOCX)

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Author Contributions
Conceived and designed the experiments: EOP. Performed the experiments: JC DK. Analyzed the data: EP JC DK. Contributed reagents/materials/analysis tools: SL. Wrote the paper: EOP.

References
1. Seeman P (1972) The membrane actions of anesthetics and tranquilizers. Pharmacol Rev 24: 583–655.
2. Goldstein DB (1984) The effects of drugs on membrane fluidity. Annu Rev Pharmacol Toxicol 24: 43–64.
3. Oliver TJ, Pryde GP, Perros D, Parkinson M, Young T (2003) Antifungal activity of selective sterol requisite inhibitors attributed to non-specific cytotoxicity. Journal of Antimicrobial Chemotherapy 51: 1045–1047.
4. Thompson BJ, Jessen T, Henry LK, Field JR, Gamble KL, et al. (2011) Transgenic elimination of high-affinity antidepressant and cocaine sensitivity in the presynaptic serotonin transporter. Proc Natl Acad Sci USA 108: 3783–3789.
5. Duman RS, Heninger GR, Nestler EJ (1997) A molecular and cellular theory of the molecular mechanism of drug-erythrocyte interactions. Proc Natl Acad Sci USA 94: 7717–7724.
6. Sturton RG, Brindley DJ (1977) Factors controlling the activities of phosphatidate phosphohydrolase and phosphatidate cytidylyltransferase. The effects of chlorpromazine, demethylchamipramine, cinchocaine, norfluoxetine, meptrypamine and magnumion. Biochem J 162: 25–32.
7. Duman RS, Heninger GR, Nestler EJ (1997) A molecular and cellular theory of the molecular mechanism of drug-erythrocyte interactions. Proc Natl Acad Sci USA 94: 7717–7724.
8. Stowe SL, Rauenstein MM (2010) Chronic treatment with escitalopram but not R-citalopram translocates Galpha(s) from lipid raft domains and potentiates the presynaptic serotonin transporter. Proc Natl Acad Sci USA 108: 3783–3789.
9. Brett CL, Kallay L, Hua Z, Green R, Chyou A, et al. (2011) Genome-wide materials/analysis tools: SL. Wrote the paper: EOP.

Transmission electron microscopy and analysis
We based our protocol on a membrane-preserving procedure previously described [40]. Our step-by-step protocol is available as Methods S1. 10 mL of fresh YPD media were inoculated with cells from an overnight culture and allowed to double several times to a maximum OD600 of ~0.5. For drug treatments, cells were treated with 60 μM sertraline for 5, 10, 20 and 45 minutes. Multicell and single-cell fields were collected. Quantification of ultrastructural phenotypes was performed on a dataset compiled with ImageJ (GraphPad Software, Inc.).
27. Lieber MR, Lange Y, Weinstein RS, Steck TL (1984) Interaction of chlorpromazine with the human erythrocyte membrane. J Biol Chem 259: 9225–9234.

28. Formai F, Gesi M, Lenzi P, Ferrucci M, Pellegri A, et al. (2002) Striatal postsynaptic ultrastructural alterations following methylenedioxymethamphetamine administration. Ann N Y Acad Sci 965: 381–398.

29. Gaspar ML, Jesch SA, Viswanatha R, Antosh AL, Brown WJ, et al. (2006) A block in endoplasmic reticulum-to-Golgi trafficking inhibits phospholipid synthesis and induces neutral lipid accumulation. J Biol Chem 281: 25735–25751.

30. Daniel WA (2003) Mechanisms of cellular distribution of psychotropic drugs. Significance for drug action and interactions. Prog Neuropsychopharmacol Biol Psychiatry 27: 65–73.

31. Kean LS, Fuller RS, Nichols JW (1993) Retrograde lipid traffic in yeast: identification of two distinct pathways for internalization of fluorescent-labeled phosphatidylcholine from the plasma membrane. J Cell Biol 123: 1403–1419.

32. Boulanger Y, Scherker S, Leitch LC, Smith RC (1980) Multiple binding sites for local anesthetics in membranes: characterization of the sites and their equilibria by deuterium NMR of specifically deuterated procaine and tetracaine. Can J Biochem 58: 986–995.

33. Pickholz M, Oliveira ON, Skaf MS (2007) Interactions of chlorpromazine with phospholipid monolayers: effects of the ionization state of the drug. Biophys Chem 125: 425–434.

34. Jerabek H, Pabot G, Rappolt M, Stockner T (2010) Membrane-mediated effect on ion channels by the anesthetic drug ketamine. J Am Chem Soc 132: 7960–7967.

35. Zschocke J, Zimmermann N, Berning B, Ganal V, Holsboer F, et al. (2011) Antidepressant drugs diversely affect autophagy pathways in astrocytes and neurons: dissociation from cholesterol homeostasis. Neuropsychopharmacology 36: 1754–1768.

36. Peropadre A, Freire PF, Herrero O, Perez Martin JM, Hazen MJ (2011) Cellular responses associated with dibucaine-induced phospholipidosis. Chem Res Toxicol 24: 185–192.

37. Noxick P, Schekman R (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. Proc Natl Acad Sci USA 76: 1858–1862.

38. Li N, Lee B, Liu R, Banasr M, Dwyer JM, et al. (2010) mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science 329: 959–964.

39. Boiron P, Drouhet E, Dupont B, Improvisi L (1987) Entry of ketoconazole into Candida albicans. Antimicrob Agents Chemother 31: 244–248.

40. Banta LM, Robinson JS, Klionsky DJ, Emr SD (1988) Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. The Journal of Cell Biology 107: 1369–1383.