

Antipsychotics Chlorpromazine and Clozapine Inhibit Membrane Fusion Through Direct Interaction with Lipid Bilayers

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Abstract

Schizophrenia is a multifactorial psychiatric disorder in which dysfunctional dopamine signaling plays a central role. Although antipsychotics (APs) are canonically thought to act through dopamine D2 receptor antagonism, differences in clinical efficacy among APs with similar receptor affinities, together with the superior effectiveness of clozapine (CLOZ) – despite its relatively low affinity for D2 receptors – suggest that additional mechanisms contribute to their therapeutic effects. Here, we investigated a membrane-dependent mechanism of AP action by examining their effects on model membrane fusion *in vitro* and vesicle delivery at the plasma membrane *in cellulo*. Three APs with distinct D2 receptor affinities – CLOZ, chlorpromazine (CPZ), and amisulpride (AMI) – were incorporated into liposomes mimicking synaptic membranes. Liposome fusion was assessed using lipid mixing assays, in which membrane merging was induced either by polyethylene glycol (PEG) or by membrane-reconstituted neuronal soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Vesicle delivery was quantified by measuring the cell surface expression of the fluorescent synaptic vesicle protein VAMP2-GFP in COS-7 cells. AP-membrane interactions were characterized using second-derivative absorption spectroscopy. CPZ and CLOZ, but not AMI, showed strong partitioning into lipid bilayers, inhibited both PEG- and SNARE-mediated fusion *in vitro*, and reduced vesicle exocytosis *in cellulo*. These findings support a membrane-dependent mechanism of action for lipophilic APs, arising from their direct interaction with lipid bilayers. This mechanism may complement canonical D2 receptor antagonism and contribute to the distinct clinical profiles of these drugs.

Introduction

Schizophrenia (SCZ) is a complex and heterogeneous mental illness resulting from the interplay of multiple environmental, genetic, neurodevelopmental, and immune factors (1–3). At the neurobiological level, dysfunction of dopaminergic transmission plays a central role (4). Dopamine is packaged into synaptic vesicles that fuse with the presynaptic plasma membrane, allowing dopamine to enter the synaptic cleft. Dopamine then binds to its receptors on the postsynaptic plasma membrane, initiating signal transduction.

Synaptic vesicle fusion is mediated by the assembly of the vesicular (v-) SNARE protein VAMP2, which resides in the synaptic vesicle membrane, and the target (t-) SNARE protein complex Stx1A/SNAP25, located in the presynaptic plasma membrane, to form a membrane-bridging trans-SNARE complex that drives close membrane apposition and fusion (5, 6). Synaptic vesicle fusion is also regulated by the lipid composition of the fusing membranes. Lipids can influence fusion either by modulating membrane fusogenicity (7) (e.g., curvature-inducing lipids such as phosphatidylethanolamine (PE) and plasmenylethanolamine (PlsmE)) or by regulating the local concentration of SNARE proteins within membrane domains where synaptic vesicles dock and fuse (8) (e.g., membrane-organizing lipids such as sphingomyelin (SM) and cholesterol (CHOL)).

In addition, neurotransmitters like dopamine can interact directly with lipid bilayers, suggesting a membrane-binding contribution to neurotransmission, where neurotransmitters first adhere to the postsynaptic membrane and then diffuse along its surface toward their receptor binding site (9).

Together, these observations suggest that changes in the lipid composition of synaptic membranes could significantly impact dopamine signaling, thereby contributing to SCZ pathophysiology. Interestingly, several research teams, including ours, have identified altered membrane lipid profiles in patients with SCZ (10–14), notably reductions in SM and PlsmE, which may affect the biophysical and fusion properties of the pre- and/or postsynaptic membranes.

Antipsychotics (APs) are used to treat SCZ symptoms and are believed to exert their therapeutic effects primarily by modulating dopamine signaling through antagonism of postsynaptic dopamine D2 receptors (15). A sufficient degree of D2 receptor blockade is indeed necessary to achieve efficacy in treating SCZ symptoms. Given the current dopamine hypothesis of SCZ, all AP compounds exhibiting comparable D2 receptor blockade activity should yield similar clinical responses. However, this is not observed in clinical practice, where two APs with the same affinity for D2 receptors may not be equally effective for a given patient. In addition, clozapine (CLOZ), despite its relatively low D2 receptor affinity, shows superior efficacy and is recommended for treatment-resistant patients (16, 17) (*i.e.*, when at least two different conventional APs have failed to improve symptoms). This indicates that additional mechanisms may contribute to the mode of action of APs in reducing SCZ symptoms.

Here, we hypothesize that the therapeutic effect of APs also involves their direct interaction with lipid membranes. Due to their lipophilic properties and planar geometry, AP drugs can intercalate into lipid bilayers and affect their biophysical properties (18–20). These modifications could occur at the pre- and/or postsynaptic membranes, influencing synaptic vesicle fusion and the dynamics of SNARE proteins and dopamine D2 receptors. This raises the interesting possibility that AP-membrane interactions could alleviate synaptic transmission dysfunctions mediated by lipid abnormalities observed in SCZ patients.

In this study, we explored a non-receptor, membrane-dependent mechanism of AP action by examining the effect of three APs – CLOZ, chlorpromazine (CPZ) and amisulpride (AMI) – on membrane fusion *in vitro* and *in cellulo*. These APs were selected for their distinct D2 receptor affinities: CLOZ (low), CPZ (moderate) and AMI (high), which correspond to their canonical mechanisms of action and largely guide their clinical use. *In vitro*, APs were incorporated into liposomes mimicking synaptic membranes at clinically relevant AP-to-lipid (AP/L) ratios, chosen to reflect their tendency to accumulate in brain tissue (21–23). Fusion was induced either by polyethylene glycol (PEG) using protein-free liposomes or by the synaptic SNARE proteins VAMP2 and Stx1A/SNAP25 reconstituted in two separate liposome populations, and monitored using a fluorescence resonance energy transfer (FRET)-based lipid mixing assay (24). *In cellulo*, fusion was assessed in COS-7 cells overexpressing VAMP2-GFP by immunolabeling surface-exposed VAMP2, as a measure of vesicle delivery at the plasma membrane (25). This combined approach allowed us to evaluate how APs modulate membrane fusion events and to

provide insight into a potential mechanism of action of APs that directly depends on their interaction with membranes and complements the canonical dopamine D2 receptor model.

Materials and methods

A detailed description of all materials and methods is provided in the Supplementary Information.

Results

Effect of CPZ, CLOZ, and AMI on PEG-mediated liposome fusion in vitro

The effect of CPZ, CLOZ, and AMI on membrane fusion was evaluated using a PEG-mediated FRET-based lipid mixing assay (Fig. 1A). Fusion experiments were performed at 37°C using liposomes composed of the main lipids found in synaptic membranes: phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) at 65, 10, and 25 mol%, respectively, while APs were incorporated at an AP/L molar ratio of 1/100. Overall, AP treatment significantly affected PEG-mediated liposome fusion events (Type III ANOVA, $F(3, 25.03) = 12.85$, $p = 2.81 \times 10^{-5}$). Fusion kinetics revealed a marked inhibition of PEG-mediated lipid mixing in the presence of CPZ, CLOZ, or AMI (Fig. 1B). After 90 minutes, mean fusion levels (expressed as normalized NBD fluorescence) were significantly reduced relative to the no-AP control, as determined by a linear mixed-effects model including AP treatment as a fixed effect and experiment (batch) as a random intercept. Estimated marginal means (EMMs, 95% CI) were 22.7% (18.4–27.0) for CPZ ($p = 4.1 \times 10^{-5}$; $n = 11$), 21.9% (17.5–26.2) for CLOZ ($p = 6.7 \times 10^{-6}$; $n = 11$), and 23.7% (19.1–28.3) for AMI ($p = 0.0026$; $n = 6$), compared to 28.8% (24.4–33.1) in the absence of APs (no-AP). Relative to the no-AP condition, these differences correspond to fusion inhibitions of approximately 21% for CPZ, 24% for CLOZ, and 18% for AMI. Control experiments in which PEG was replaced with buffer are provided in the Supplementary Information (Fig. S1).

We next assessed the effect of APs on liposome size, as changes in size across AP conditions could account for the observed differences in fusion activity, given that larger liposomes are less fusogenic (26, 27). Liposome size measurements were performed by multi-angle dynamic light scattering (MADLS) on both donor and acceptor liposomes in the absence or presence of APs (Fig. 1C). The results showed largely monodisperse and overlapping size distributions, with an average peak diameter of 65 nm across all conditions. More specifically, the peak diameters of acceptor and donor liposomes were respectively 65 ± 3 nm and 66 ± 2 nm under the no-AP condition, 61 ± 4 nm and 66 ± 4 nm in the presence of CPZ, 63 ± 4 nm and 68 ± 2 nm in the presence of CLOZ, and 61 ± 4 nm and 67 ± 5 nm in the presence of AMI ($n = 6$). No significant difference in liposome size was observed across conditions ($p > 0.05$).

Effect of CPZ, CLOZ, and AMI on SNARE-mediated liposome fusion in vitro

To determine whether the inhibitory effects of APs observed in the minimal protein-free PEG-mediated system were preserved in a more physiologically relevant context, synaptic SNARE-mediated fusion was

analyzed using proteoliposomes reconstituted with purified neuronal SNARE proteins at physiological lipid-to-protein ratios (28, 29) (Table 1 and Fig. S2). Fusion was monitored using the same FRET-based lipid mixing assay at a final lipid concentration of 1 mM, with acceptor t-SNARE liposomes containing Stx1A/SNAP25 and donor v-SNARE liposomes containing VAMP2 (Fig. 2A).

Table 1
Lipid-to-protein ratios for neuronal SNARE-reconstituted proteoliposomes determined by SDS-PAGE (see Supplementary Text and Fig. S2).

Liposome type	Proteins	No AP	CPZ	CLOZ	AMI
t-SNARE	Stx1A	217 ± 23	176 ± 25	210 ± 28	153
	SNAP25	220 ± 22	188 ± 14	225 ± 20	221
v-SNARE	VAMP2	93 ± 16	100 ± 19	136 ± 26	144

Under these conditions, AP treatment also significantly affected SNARE-mediated fusion events (Type III ANOVA, $F(3, 27.05) = 9.26$, $p = 2.22 \times 10^{-4}$), as determined by a linear mixed-effects model including AP treatment as a fixed effect and experiment (batch) as a random intercept. Fusion kinetics revealed a significant inhibition of SNARE-mediated lipid mixing in the presence of CPZ or CLOZ (Fig. 2B). After 90 minutes, EMMs for fusion levels (95% CI) reached 12.3% (8.9–15.8) for CPZ ($p = 8.0 \times 10^{-5}$; $n = 13$) and 12.4% (9.0–15.9) for CLOZ ($p = 1.2 \times 10^{-4}$; $n = 13$), compared to 15.3% (11.9–18.8) in the absence of APs. Relative to the no-AP condition, these differences correspond to fusion inhibitions of approximately 20% for CPZ and 19% for CLOZ. In contrast, AMI induced only a slight reduction in SNARE-mediated fusion, with an EMM of 13.3% (9.5–17.0), corresponding to approximately 13% fusion inhibition, which did not reach statistical significance ($p = 0.0539$; $n = 4$). Control experiments, in which SNARE proteins were omitted or trans-SNARE complex formation was prevented by pre-incubating t-SNARE liposomes with the cytosolic domain of VAMP2 (CDV), confirmed the SNARE dependence of the fusion signal (Fig. S3).

To exclude changes in liposome size as a contributing factor to fusion inhibition, proteoliposome size distributions were analyzed by MADLS under all AP conditions. As observed for protein-free liposomes, proteoliposomes displayed monodisperse and overlapping size distributions, with an average peak diameter of approximately 75 nm across all conditions and no statistically significant differences detected ($p > 0.05$; Fig. 2C). More specifically, the peak diameters of v- and t-SNARE liposomes were respectively 74 ± 3 nm and 72 ± 2 nm under the no-AP condition, 74 ± 3 nm and 74 ± 2 nm in the presence of CPZ, 74 ± 3 nm and 74 ± 2 nm in the presence of CLOZ, and 73 ± 3 nm and 77 ± 4 nm in the presence of AMI ($n = 6$ for the no-AP, CPZ, and CLOZ conditions; $n = 4$ for AMI). These results confirm that the AP-induced changes in fusion activity are not attributable to differences in proteoliposome size.

Effect of CPZ, CLOZ, and AMI on vesicle exocytosis in cellulo

Although reconstituted liposome systems allow precise dissection of membrane fusion mechanisms under highly controlled conditions (e.g., membrane lipid composition, lipid-to-protein ratios, and AP/L

ratios), they do not fully capture the regulatory complexity of cellular exocytosis. To determine whether the inhibitory effects of CPZ, CLOZ, and AMI observed in reconstituted systems extend to a more complex cellular context, we next examined vesicle delivery at the plasma membrane in COS-7 cells overexpressing VAMP2-GFP. We measured surface-exposed and total VAMP2-GFP signals by immunocytochemistry, with surface-exposed VAMP2 used as an index of vesicle-mediated delivery at the plasma membrane (Fig. 3A).

Compared to control cells not exposed to APs ($n = 4$ independent experiments; $N = 266$ cells), treatment with CPZ or CLOZ resulted in a significant reduction in surface-exposed VAMP2-GFP. CPZ reduced VAMP2-GFP surface exposure by 26% ($n = 4$ independent experiments; $N = 208$ cells; $p < 0.05$), while CLOZ induced a stronger reduction of 34% ($n = 4$ independent experiments; $N = 298$ cells; $p < 0.05$). No statistically significant difference was detected between CPZ- and CLOZ-treated cells ($p > 0.05$; data not shown). In contrast, AMI treatment did not significantly affect vesicle delivery. Only a slight 4% decrease in surface-exposed VAMP2-GFP was observed compared to control conditions ($n = 4$ independent experiments; $N = 359$ cells), which did not reach statistical significance ($p > 0.05$) (Fig. 3B). Importantly, the presence of VAMP2 at the plasma membrane reflects the balance between its delivery by exocytosis and its retrieval by endocytosis. Since CPZ and CLOZ also inhibit dynamin- and clathrin-mediated endocytosis (30), measuring surface-exposed VAMP2 likely underestimates the true inhibitory effect of these drugs on vesicle exocytosis.

Overall, these cellular data are consistent with the *in vitro* fusion assays, indicating that CPZ and CLOZ significantly impair vesicle fusion, whereas AMI displays minimal or no significant inhibitory effect under our experimental conditions. This apparent lack of effect of AMI suggests that differences in membrane affinity may underlie the distinct functional impacts of these APs.

Partition of CPZ, CLOZ, and AMI into PC:PS:PE liposome bilayers

To examine whether the effects of CPZ, CLOZ, and AMI observed in PEG- and SNARE-mediated liposome fusion assays *in vitro* and in the vesicle delivery assay *in cellulo* involve a mechanism dependent on AP interactions with the lipid bilayer structure, we quantified the membrane partitioning of the three APs in protein-free PC:PS:PE (65:10:25 mol%) liposomes using absorption spectroscopy and second-derivative analysis (31). Drugs were added to the liposomes at 30 μM in buffer supplemented with 1% (v/v) DMSO to ensure solubility of CLOZ.

We first measured the absorption spectra of the three APs in the absence of liposomes. CPZ exhibited absorption maxima at 255 nm and 310 nm, CLOZ at 235 nm, 258 nm, and 295 nm, and AMI at 233 nm and 283 nm (Fig. S4). For partition calculations, well-resolved absorption maxima sufficiently separated from the far-UV region (to avoid contributions from the buffer) and of adequate intensity for second-derivative analysis were selected: 255 nm for CPZ, 285 nm for CLOZ, and 283 nm for AMI.

In the presence of liposomes at various concentrations, CPZ and CLOZ exhibited progressive lipid concentration-dependent increases and red shifts in their absorption maxima, consistent with membrane insertion, whereas AMI showed no detectable effect, indicating negligible membrane partitioning under these experimental conditions (Fig. S5).

Second-derivative absorption spectra were then calculated for each AP at all lipid concentrations, and ΔD values, defined as the difference between the second-derivative intensity at a given lipid concentration and that measured in the absence of liposomes, were determined (see Fig. 4A for CPZ; for CLOZ and AMI, see Fig. S6). ΔD values were subsequently plotted as a function of lipid concentration and fitted using the following membrane partitioning equation:

$$\Delta D = (Kp \cdot \Delta D_{max} \cdot [L]) / ([W] + Kp \cdot [L])$$

where [L] and [W] are the molar concentrations of lipids and water, respectively.

This equation allows extraction of the molar partition coefficient (Kp) and the maximum derivative change (ΔD_{max}) (see Fig. 4B for CPZ; for CLOZ and AMI, see Fig. S6). The membrane fraction of each AP was then estimated from the ratio ΔD/ΔD_{max} as a function of lipid concentration (Fig. 4C).

Under these conditions, CPZ exhibited a molar partition coefficient $Kp = 1.51 \times 10^5$ ($\log Kp = 5.18 \pm 0.04$), whereas CLOZ displayed a $Kp = 1.32 \times 10^5$ ($\log Kp = 5.12 \pm 0.20$). At the lipid concentrations used in the *in vitro* fusion assays, CPZ exhibited membrane fractions of approximately 58% at 0.5 mM lipid (concentration used in the PEG-mediated fusion assay) and 71% at 1 mM lipid (concentration used in the SNARE-mediated fusion assay), while CLOZ reached approximately 54% and 68% under the same conditions, respectively (Table 2). Because no AP-induced red shift was detected for AMI, a Kp value could not be determined for this AP under our experimental conditions.

Table 2

Membrane partition coefficient parameters of CPZ and CLOZ at 30 μM in buffer supplemented with 1% (v/v) DMSO in the presence of PC:PS:PE (65:10:25 mol%) liposomes. 95% CI, 95% confidence interval.

Kp (95% CI)	CPZ 30 μM	CLOZ 30 μM
	151 004 (137 486 – 165 805)	132 369 (105 054–166 264)
ΔD _{max} (95% CI)	3.25 (2.82–3.88) x 10 ⁻³	2.68 (1.88–5.11) x 10 ⁻⁴
ΔD/ΔD _{max} (0.5 mM lipid)	58%	54%
ΔD/ΔD _{max} (1 mM lipid)	71%	68%

To assess the potential effect of DMSO on membrane partitioning, additional experiments were performed with CPZ at 10 μM or 30 μM in buffer without DMSO, matching the buffer conditions used in

the fusion assays. Under these conditions, CPZ exhibited a $K_p = 2.63 \times 10^5$ ($\log K_p = 5.42 \pm 0.14$) at 10 μM and a $K_p = 2.32 \times 10^5$ ($\log K_p = 5.37 \pm 0.10$) at 30 μM (Table S2). These partition coefficients correspond to membrane fractions of $\sim 70\%$ (at 0.5 mM lipid) and $\sim 79\%$ (at 1 mM lipid) at 10 μM CPZ, and $\sim 68\%$ (at 0.5 mM lipid) and $\sim 83\%$ (at 1 mM lipid) at 30 μM CPZ. These values are slightly higher than those obtained in the presence of 1% DMSO ($\log K_p = 5.18 \pm 0.04$), suggesting that DMSO could compete with the membrane for AP solubilization in the aqueous phase, thus reducing the apparent membrane-bound fraction. Nonetheless, both conditions yielded $\log K_p$ values well above 5, confirming strong membrane partitioning of CPZ regardless of the presence of DMSO.

Together, these data demonstrate that CPZ and CLOZ strongly partition into lipid bilayers at clinically relevant concentrations, supporting a mechanism of action that depends on their capacity to directly interact with membranes, a property that appears to be absent in AMI.

Discussion

The aim of this study was to investigate a potential mode of action for APs through their effects on lipid membranes, beyond their canonical effects on dopamine receptors. Using complementary *in vitro* and *in cellulo* approaches, we evaluated the impact of CPZ, CLOZ, and AMI on liposome fusion and vesicle exocytosis. Both CPZ and CLOZ induced similar reductions in PEG- and SNARE-mediated fusion ($\sim 20\%$), as well as in vesicular exocytosis in COS-7 cells ($\sim 30\%$), at clinically relevant concentrations, whereas AMI showed minimal effects. Interestingly, the inhibitory effect of AMI progressively diminished, becoming negligible as the experimental system's complexity increased and approached physiological conditions. Specifically, AMI-mediated inhibition was lower in SNARE-mediated fusion and lowest in the vesicle delivery assay. The similar magnitude of inhibition observed for CPZ and CLOZ in both PEG- and SNARE-mediated fusion suggests that the inhibition induced by CPZ and CLOZ primarily arises from lipid bilayer alterations rather than direct interference with the protein fusion machinery. Importantly, the observed fusion inhibition could not be attributed to changes in membrane curvature or protein surface density, which remained comparable across conditions. Together, these findings support a mechanism in which APs modulate membrane fusion by altering membrane physicochemical properties.

This interpretation is further supported by measurements of AP membrane partitioning using second-derivative absorption spectroscopy. CPZ and CLOZ displayed high and comparable partition coefficients in PC:PS:PE (65:10:25 mol%) liposomes, with $\log K_p$ exceeding 5, corresponding to 54–71% membrane insertion for lipid concentrations used in the fusion assays (Table 2). These values agree with published coefficients obtained using the same method in PC-containing liposomes ($\log K_p = 5.47$ with pure PC liposomes (31); $\log K_p = 5.32$ – 5.40 with PC:PE (70:30 mol%) liposomes and $\log K_p = 5.53$ – 5.58 with PC:PS (90:10 mol%) liposomes (32)). Despite relying on distinct membrane insertion mechanisms, both drugs displayed comparable membrane partition coefficients in our PC:PS:PE liposomes and exerted a similar degree of fusion inhibition ($\sim 20\%$). CPZ, which is $> 95\%$ protonated at pH 7.4 ($\text{pK}_a = 8.7$ (33)), partitions primarily through electrostatic interactions with anionic PS headgroups, whereas CLOZ, which is $\sim 50\%$ neutral at pH 7.4 ($\text{pK}_a = 7.41$ (34)), inserts predominantly through hydrophobic interactions of

its neutral form and exhibits weaker electrostatic interactions with PS (35, 36). This suggests that, regardless of how these APs intercalate into lipid bilayers, the resulting perturbation of membrane biophysical properties is sufficient to impair fusion to a similar extent under our experimental conditions. In contrast, AMI showed no detectable membrane partitioning, consistent with its lipophilicity, measured as the octanol/water partition coefficient $\log P_{(o/w)}$, being several orders of magnitude lower than those of CPZ and CLOZ ($\log P_{(o/w)} = 1.10\text{--}1.40$ for AMI vs. 5.34 for CPZ and 3.64 for CLOZ (34, 37)), and its negative distribution coefficient $\log D$ at physiological pH ($\log D(7.4) = -0.27$ (34)), rendering membrane insertion thermodynamically unfavorable. In fact, differential scanning calorimetry measurements of seven different APs, including CLOZ and AMI, interacting with PC:sphingomyelin:cholesterol (85:10:5 mol%) liposomes revealed a negative free energy change for all AP-membrane systems, indicating spontaneous bilayer insertion, except for the AMI-membrane system, which exhibited a positive free energy change (38). These differences in membrane partitioning provide an independent rationale for the observed differences in fusion inhibition among the three APs, and support a mode of action in which lipophilic APs modulate membrane fusion through direct interaction with lipid bilayers.

Several previous studies have reported the effects of CPZ and other APs on membrane fusion. At concentrations exceeding 50 μM , where it starts forming micelles (39), CPZ was shown to promote the transition from hemifusion to full fusion in hemagglutinin- and vesicular stomatitis virus glycoprotein G-mediated viral fusion (40–44), SNARE-mediated vacuole fusion (45, 46), as well as myomaker/myomerger-mediated cell-cell fusion (47). The proposed mechanism was CPZ's ability to generate positive curvature in the inner monolayers of lipid membranes forming the hemifusion diaphragm, thus facilitating fusion pore formation (44).

At lower concentrations (below 50 μM), CPZ – as well as the APs trifluoperazine and fluphenazine – was found to inhibit cell-cell fusion mediated by several viruses, including herpes viruses, canine distemper virus, Newcastle disease viruses, and Sendai virus (48). The authors hypothesized that these APs inhibit cell fusion by sequestering membrane sites that must remain unoccupied for membrane fusion to occur. Within the same concentration range, trifluoperazine, fluphenazine, and promazine were shown to inhibit fusion between Hepatitis C virus and liposomes *in vitro* and to increase the water permeability and fluidity of cholesterol-containing membranes, suggesting that they perturb local membrane heterogeneities associated with cholesterol-rich domains that are critical for interactions between the viral fusion peptide and the host cell membrane (49). In line with that study, we recently found that CPZ reduced fusion peptide-mediated liposome-liposome fusion *in vitro* and Spike-mediated cell-cell fusion *in situ*, and observed that adding CPZ had the same effect on fusion as depleting the membrane of cholesterol (50). This fusion-inhibitory effect was attributed to CPZ's ability to compete with cholesterol and alter lipid order, specifically by increasing the lipid bilayer thickness outside of cholesterol-rich domains and decreasing it within them (18, 19). By reducing the thickness mismatch between these two phases, CPZ eliminates the hydrophobic defects at domain boundaries where the fusion peptide typically inserts. Beyond direct effects on membrane fusion, CPZ and CLOZ were also reported to accumulate in

synaptic vesicles and inhibit evoked exocytosis; in that context, however, the reduction of synaptic transmission by these APs was attributed to sodium channel inhibition (51).

In the present study, the inhibitory effect of CPZ and CLOZ on PEG- and SNARE-mediated membrane fusion was observed only when these APs were added during liposome reconstitution, not when they were added to pre-formed liposomes at the start of the fusion assay (Fig. S7), suggesting that these compounds must reach the inner monolayers of the lipid membranes to exert their effect. Their mode of action in inhibiting PEG- and SNARE-mediated fusion would thus differ from that proposed for viral fusion, where they act on hydrophobic defects in the outer monolayers. SNARE-mediated fusion occurs through membrane-bridging SNARE complexes that assemble across opposing membranes as zipper-like structures, bringing them into close apposition and driving membrane deformation and merging through progressive assembly that extends into their transmembrane domains (6, 52–54). We propose that CPZ and CLOZ inhibit PEG- and SNARE-mediated membrane fusion by increasing lipid ordering and bilayer rigidity. This interpretation is consistent with the recent observation that CPZ and CLOZ both increase lipid ordering in fluid membranes similar to those used in the present study (36). Interestingly, lithium, used in the treatment of bipolar disorder, was recently shown to rigidify lipid bilayers through direct lipid binding, thereby affecting membrane deformation processes and increasing the energetic barrier to membrane fusion (55).

Beyond mechanistic considerations, our findings may have clinical relevance. First, they provide a potential biophysical rationale for the delayed therapeutic onset of CPZ and CLOZ. While both drugs rapidly occupy dopamine D2 receptors, full clinical efficacy requires several weeks of administration. Our results suggest that these highly lipophilic compounds must accumulate progressively within brain cell membranes before reaching a threshold necessary to affect vesicle fusion. Second, SCZ has been associated with altered red blood cell and brain membrane lipid composition, including enrichment in saturated fatty acids and sphingolipids, along with reductions in polyunsaturated fatty acids, PC, PS, PE, and plasmalogens (10, 12, 13, 56–60). Several of these lipid classes play critical roles in membrane fusion (e.g., cone-shaped lipids such as PE and plasmalogens). Such lipid abnormalities could intrinsically impair membrane fusion in the absence of APs and modulate the membrane partitioning and local efficacy of lipophilic drugs such as CPZ and CLOZ. Ultimately, these changes may influence both presynaptic fusion processes and postsynaptic lipid organization and receptor mobility.

Thus, interindividual variability in membrane lipid composition could contribute to the heterogeneous therapeutic responses observed in SCZ. Membrane-mediated effects of APs may therefore represent an additional non-receptor mechanism of pharmacological action. Overall, this study provides quantitative and mechanistic evidence supporting a membrane-dependent mode of action of lipophilic APs that complements their canonical receptor-based mechanisms and may contribute to their variable clinical outcomes.

Declarations

Conflict of Interest

The authors declare no conflict of interest.

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Figures

Figure 1

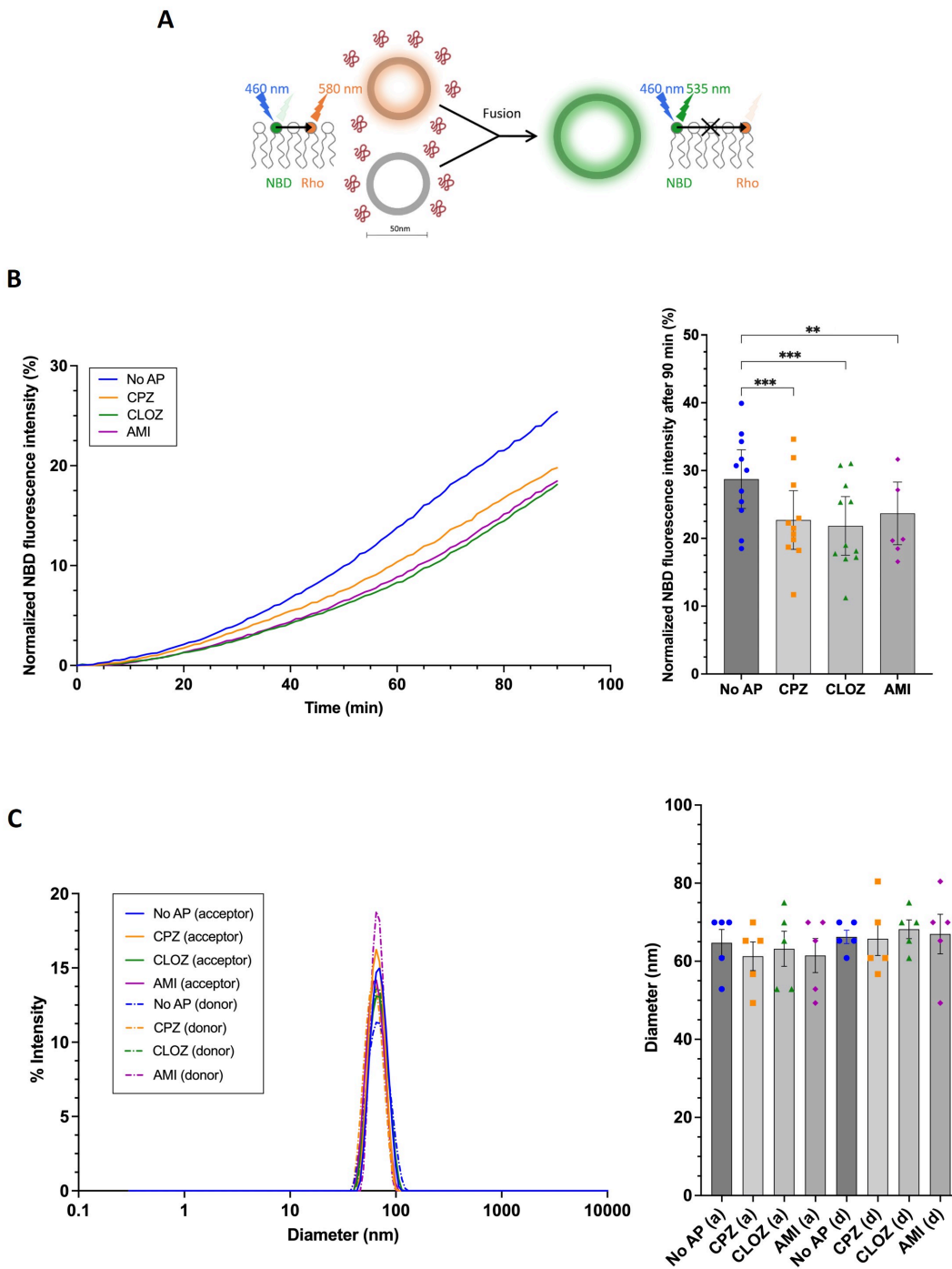


Figure 1

Influence of the antipsychotics CPZ, CLOZ, and AMI on PEG-mediated liposome fusion *in vitro*. (A) Schematic representation of the FRET-based lipid mixing assay used to monitor PEG-mediated liposome fusion, with PEG represented by orange balls. Fusion between donor liposomes labeled with NBD-PE (or NBD-PS) and Rho-PE, and unlabeled acceptor liposomes results in the dilution of the FRET pair and an increase in NBD fluorescence recorded at 535 nm. (B) Representative fusion kinetics (left panel) and

average liposome fusion percentages after 90 min (right panel) for liposomes composed of PC:PS:PE (65:10:25 mol%; 500 μ M final lipid concentration) in the absence or presence of CPZ, CLOZ, or AMI incorporated at an AP/L molar ratio of 1/100. Data (right panel) represent the estimated marginal means $\pm$ 95% confidence intervals from $n = 11$ (no-AP, CPZ, and CLOZ conditions) and $n = 6$ (AMI) independent reconstitution experiments. Statistical analysis was performed using linear mixed-effects models with AP treatment as a fixed effect and experiment (batch) as a random intercept. Fewer replicates were performed for the AMI condition; these were appropriately accounted for and incorporated into the model estimation. (C) *Left panel*: Effect of CPZ, CLOZ, and AMI (AP/L = 1/100) on liposome size distributions, as determined by multi-angle dynamic light scattering (MADLS). *Right panel*: Peak diameters in the absence or presence of APs. Error bars represent the SEM and each data point corresponds to an individual size measurement ($n = 6$ independent liposome reconstitutions). Labels (a) and (d) denote acceptor and donor liposomes, respectively.

Figure 2

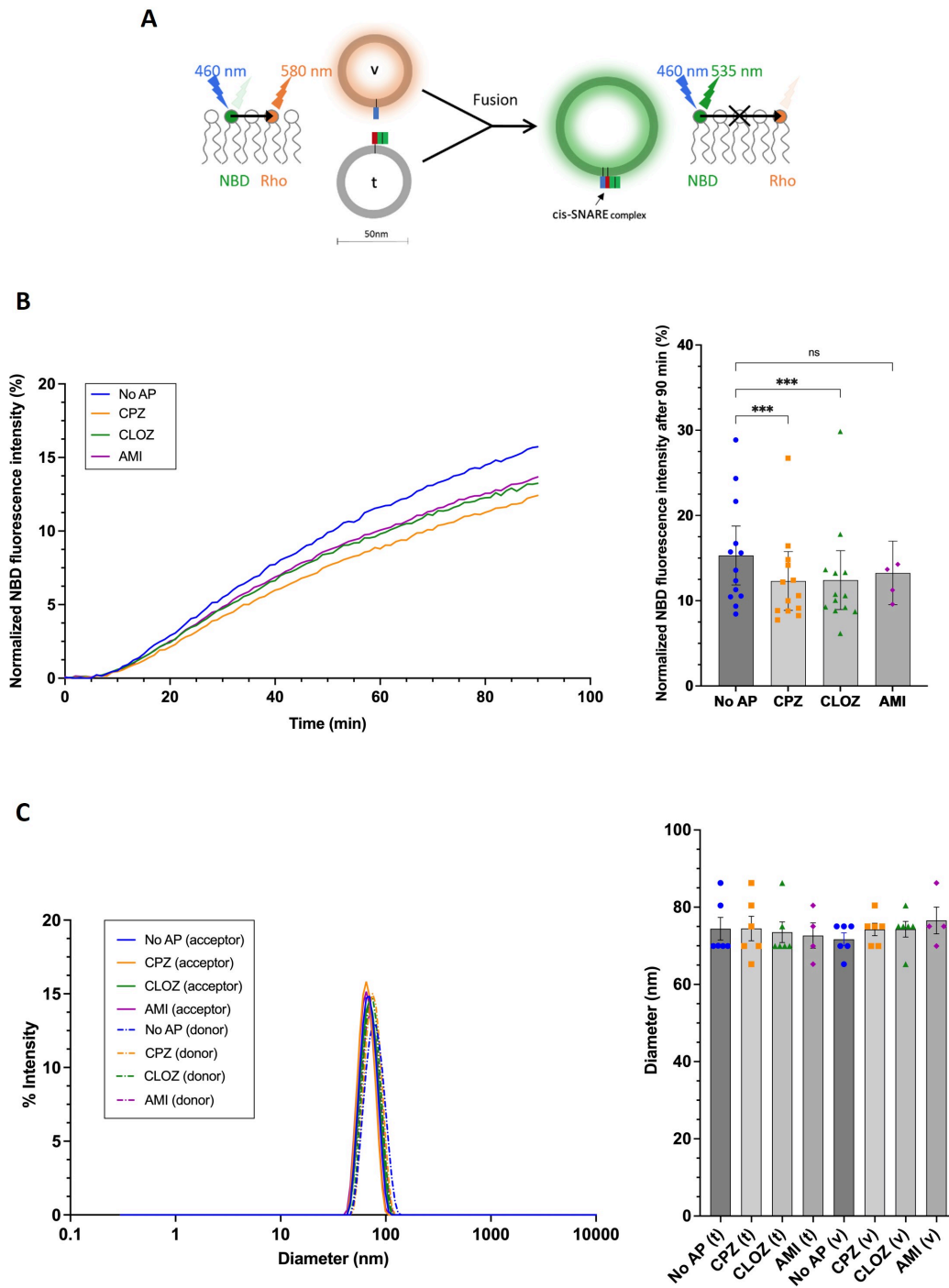


Figure 2

Influence of the antipsychotics CPZ, CLOZ, and AMI on SNARE-mediated liposome fusion *in vitro*. (A) Schematic representation of the FRET-based lipid mixing assay applied to SNARE-mediated membrane fusion using proteoliposomes reconstituted with purified neuronal SNARE proteins. Acceptor t-SNARE liposomes contain Stx1A (blue) and SNAP25 (green), whereas donor v-SNARE liposomes contain VAMP2 (red). SNARE-mediated membrane fusion results in the merging of proteoliposomes and the

formation of the cis-SNARE complex within the fused membrane. (B) Representative fusion kinetics (left panel) and average liposome fusion percentages after 90 min (right panel) for SNARE-reconstituted proteoliposomes composed of PC:PS:PE (65:10:25 mol%) at a final lipid concentration of 1 mM in the absence or presence of CPZ, CLOZ, or AMI, incorporated at an AP/L molar ratio of 1/100. Data (right panel) represent the estimated marginal means $\pm$ 95% confidence intervals from $n = 13$ (no-AP, CPZ, and CLOZ conditions) and $n = 4$ (AMI) independent experiments. Statistical analysis was performed using linear mixed-effects models (LMMs) with AP treatment as a fixed effect and experiment (batch) as a random intercept. Fewer replicates were performed for the AMI condition; these were appropriately accounted for and incorporated into the model estimation. (C) *Left panel*: Size distributions of SNARE-reconstituted proteoliposomes in the absence or presence of CPZ, CLOZ, or AMI (AP/L = 1/100), as determined by MADLS. *Right panel*: Peak diameters for each condition ($n = 6$ independent proteoliposome reconstitutions for the no-AP, CPZ, and CLOZ conditions; $n = 4$ for AMI). Error bars represent the SEM and each data point corresponds to an individual size measurement. Labels (v) and (t) denote v- and t-SNARE liposomes, respectively.

Figure 3

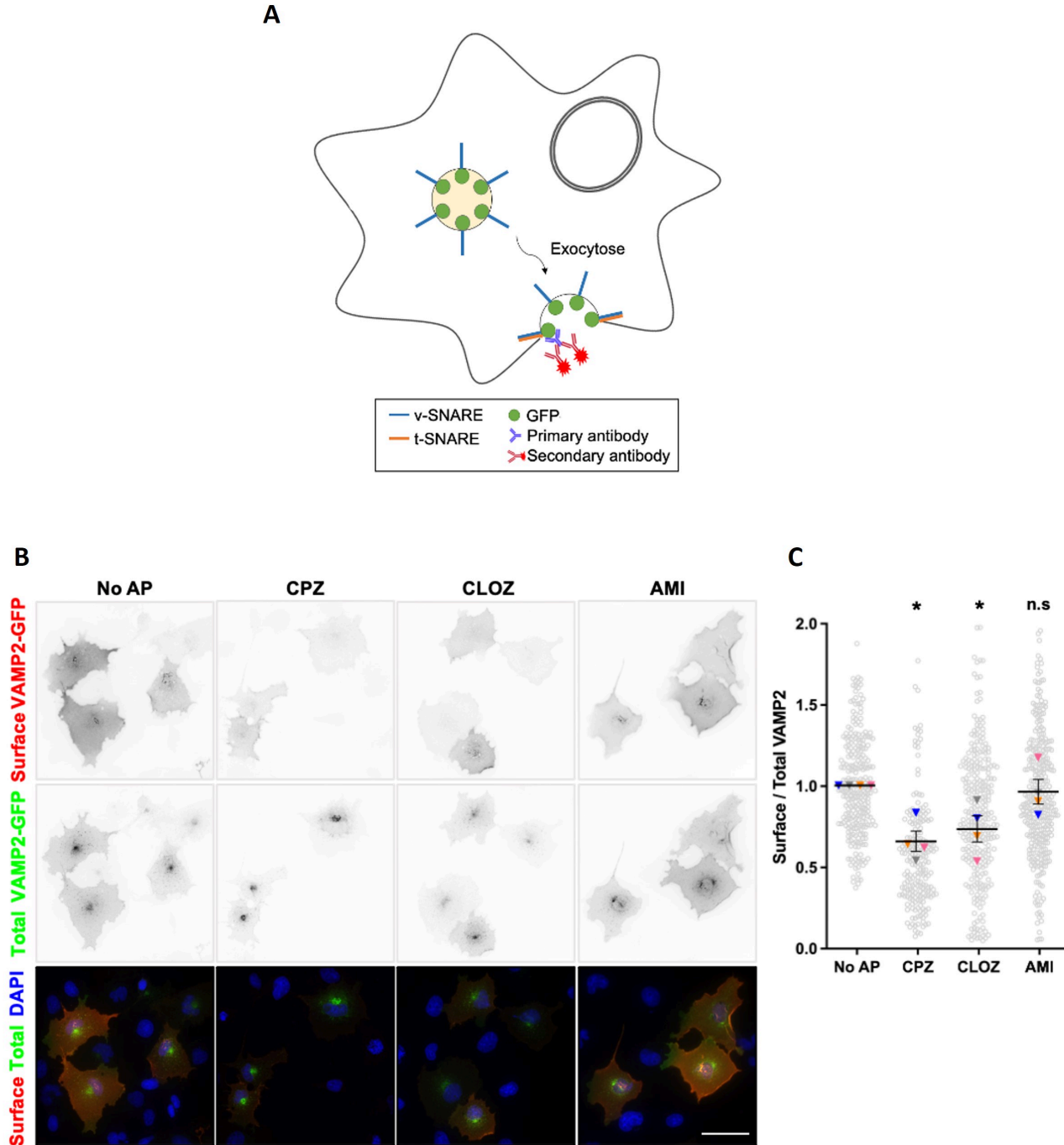


Figure 3

Effect of CPZ, CLOZ, and AMI on vesicle exocytosis in COS-7 cells. (A) Experimental design. COS-7 cells overexpressing VAMP2-GFP were treated overnight with 10 μ M CPZ, CLOZ, or AMI (from a DMSO stock solution). Surface VAMP2-GFP was labeled on ice using a mouse monoclonal anti-GFP antibody, followed by fixation with 4% PFA. Total VAMP2-GFP was then labeled after permeabilization using a rabbit polyclonal anti-GFP antibody, together with DAPI staining. (B) Surface and total VAMP2-GFP

signals were revealed with goat anti-mouse Cy3 and goat anti-rabbit Alexa 488 secondary antibodies, respectively. Images were acquired on a motorized Leica DMRA2 microscope (20x objective) coupled to a Hamamatsu ORCA-Flash4.0 LT CMOS camera. The scale bar is 50 μm . (C) Quantification was performed in ImageJ from image stacks. Integrated density for each channel was measured per cell, and the Surface/Total VAMP2-GFP ratio was calculated (no-AP, N = 266 cells; CPZ, N = 208 cells; CLOZ, N = 298 cells; AMI, N = 359 cells). Ratios were normalized to the mean of the control (DMSO condition; set to 1). Data are presented as superplots, with single-cell values in gray and experiment means in color (n = 4 independent experiments), and are reported as mean $\pm$ SEM. Statistical analysis was performed using paired Student's *t*-tests vs. DMSO (*: $p < 0.05$).

Figure 4

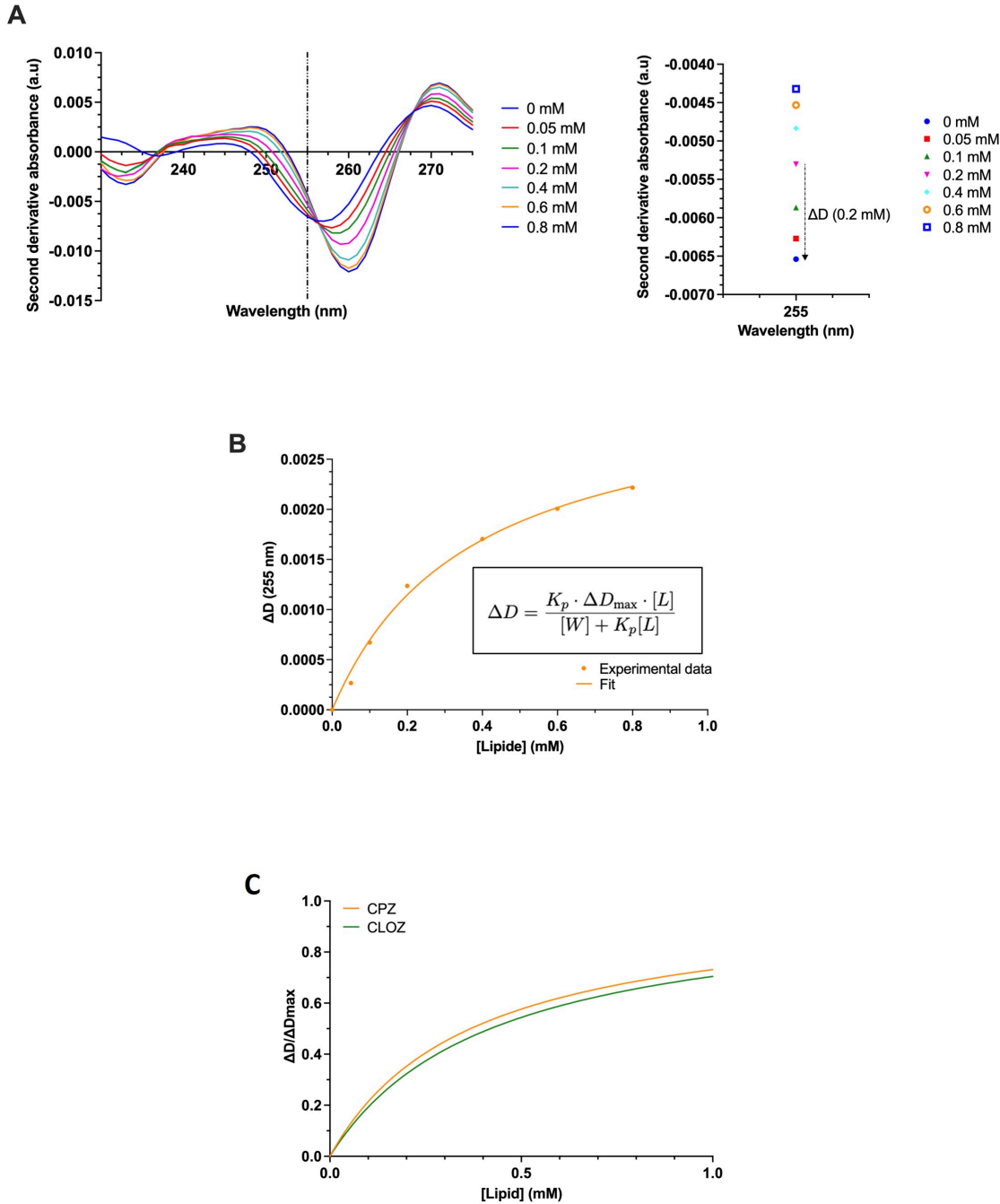


Figure 4

Quantification of AP membrane partitioning using second-derivative absorption spectroscopy. (A) Second-derivative absorption spectra of CPZ at increasing lipid concentrations (left panel). The vertical dashed line indicates the working wavelength (255 nm). ΔD values were calculated as the difference between the derivative intensity at a given lipid concentration and that measured in the absence of liposomes (right panel, example at 0.2 mM lipid). (B) ΔD values of CPZ at 255 nm plotted as a function

of lipid concentration and fitted using a membrane partitioning model (see text for details) to extract the molar partition coefficient (Kp) and the maximal derivative change (ΔD_{max}). (C) Estimated membrane fraction of CPZ and CLOZ at 30 μ M in buffer supplemented with 1% (v/v) DMSO in PC:PS:PE (65:10:25 mol%) liposomes, calculated as $\Delta D/\Delta D_{max}$.

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