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Article

Unusual Robustness of Neurotransmitter Vesicle Membranes against Serotonin-Induced Perturbations

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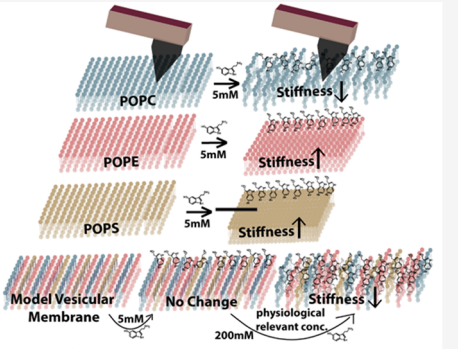
ABSTRACT: Nature confines hundreds of millimolar of amphiphilic neurotransmitters, such as serotonin, in synaptic vesicles. This appears to be a puzzle, as the mechanical properties of lipid bilayer membranes of individual major polar lipid constituents of synaptic vesicles [phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS)] are significantly affected by serotonin, sometimes even at few millimolar concentrations. These properties are measured by atomic force microscopy, and their results are corroborated by molecular dynamics simulations. Complementary ^{2}H solid-state NMR measurements also show that the lipid acyl chain order parameters are strongly affected by serotonin. The resolution of the puzzle lies in the remarkably different properties displayed by the mixture of these lipids, at molar ratios mimicking those of natural vesicles (PC:PE:PS:Cholesterol = 3:5:2:5). Bilayers constituting of these lipids are minimally perturbed by serotonin, and show only a graded response at physiological concentrations (>100 mM). Significantly, the cholesterol (up to 33% molar ratio) plays only a minor role in dictating these mechanical perturbations, with PC:PE:PS:Cholesterol = 3:5:2:5 and 3:5:2:0 showing similar perturbations. We infer that nature uses an emergent mechanical property of a specific mixture of lipids, all individually vulnerable to serotonin, to appropriately respond to physiological serotonin levels.



opens role of 5-HT-vesicular membrane interactions in vesicular processes

1. INTRODUCTION

In chemical neurotransmission, neurons communicate with each other through the exocytotic release and receptor-mediated sensing of neurotransmitter molecules. Exocytosis involves neurotransmitter-filled vesicles fusing with (or opening a pore through) the presynaptic membrane. This requires considerable remodelling of the membrane and is expected to depend on the mechanical properties of the lipid bilayer.[1] On the other hand, some neurotransmitters such as serotonin are known to be amphiphilic, and can strongly reduce the mechanical stability of membranes.[2−5] The vesicular membrane must therefore optimize between two somewhat contradictory requirements: it has to be stable enough to contain a high level of neurotransmitters,[6,7] but it has to be sufficiently fluid to allow for bending and pore opening required for exocytosis. The neurotransmitter vesicles accomplish this feat despite containing hundreds of millimolar of neurotransmitters, but the design principle underlying their optimal mechanical stability is not well understood. Here we address this issue.

Neurotransmitters such as serotonin, dopamine, melatonin, and norepinephrine bind lipid bilayer membranes with a high partition coefficient, penetrate the membrane, and modulate the membrane properties.[2,4,5,8−13] We have previously shown that serotonin (5-HT) can modulate the breakthrough force (F^*, measured by indenting a membrane with an AFM tip) and the ^{2}H NMR order parameter of a model membrane PPC111 (with the composition POPC:POPG:Cholesterol = 1:1:1, in molar ratio) significantly at just 5 mM concentration.[2,3] However, the neuronal serotonergic vesicles contain >250 mM serotonin.[6,7] So this raises the question: how do these serotonergic vesicles remain stable despite containing such a high amount of serotonin? We hypothesize that the specific lipid composition of the membrane has a significant role to play in this stability. However, if the vesicular membrane is too stiff (i.e., has a very high F^*), exocytosis may be hampered. So, we further hypothesize that the mechanical properties of the vesicles are tuned to respond at a physiological (>100 mM) range of serotonin concentrations

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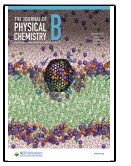

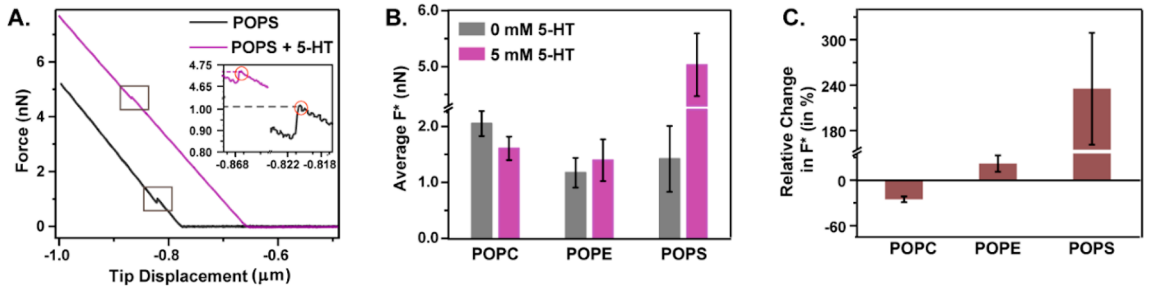

Figure 1. Effect of serotonin on the mechanical properties of membranes of pure lipids (POPC, POPS, POPE) present in physiologically relevant vesicular composition probed by AFM force indentation study. (A) Representative force vs tip displacement curve of POPS (gray) and POPS + 5 mM serotonin (light pink). The part of the traces marked by a square box is shown in the inset. The force corresponding to the kink in the trace (shown in the red circle) is known as the breakthrough force (F^*) (inset). (B) Representative average F^* was obtained by AFM force indentation on the POPC, POPE, and POPS, in the absence (gray) and presence (light pink) of 5 mM serotonin. (C) Relative change in the average F^* of POPC (N = 6), POPE (N = 13), and POPS (N = 9) (from left to right, red) bilayers in the presence of 5 mM serotonin. Negative change means F^* decreases by serotonin and *vice versa*. N is the total number of sets. Each set in AFM includes ∼600 force traces. Error bars represent SD (for Figure 1B) and SEM (for Figure 1C). All experiments are carried out in physiological salt conditions.

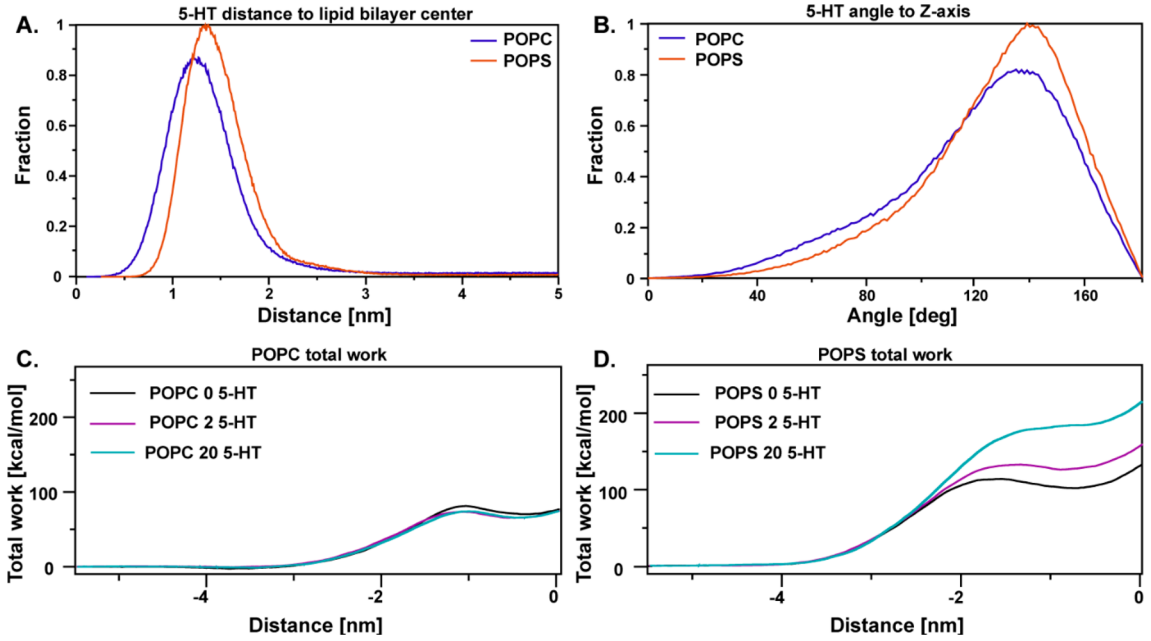

Figure 2. (A) Normalized fraction of snapshots in which serotonin molecules are at a given distance to the lipid bilayer center. (B) Normalized histogram of the angles of 20 serotonin molecules to the Z-axis of the periodic box during the MD simulation (90° indicates that serotonin is oriented parallel to the plane of the lipid bilayer). (C,D) The average work required to push a C720 fullerene through lipid bilayers. Here, the total work is the work obtained for the entire interval, and distance is measured from the center of the bilayer.

(unlike PPC111), so that exocytosis of mature vesicles is facilitated.

Nature likely achieves this mechanical tuning by using an appropriate composition of lipids in the vesicular membrane. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and cholesterol (Chol) occur in the molar ratio of 3:5:2:5 (henceforth denoted as PC/PE/PS/Chol/3/5/2/5), and these four components represent >90% of the lipid composition of the vesicular membrane.[14−16] We ask whether it is the individual components, or their specific mixture, that confers the desirable properties. Our main experimental tools in this study were AFM, which measures changes in F^*, and solid-state NMR, which measures changes in the order parameters of acyl chains. We also performed MD simulations to provide a molecular level understanding of the system. We studied how the individual major lipid components (POPC, POPE, and POPS), and their mixture in the natural ratio respond to physiological concentrations of serotonin. These results were compared with a non-physiological generic mixture of zwitterionic and negatively charged lipids (PPC111) so that any specific effects of the physiological composition could become apparent.

Of course, there are several possible caveats in limiting our studies to these few lipid species. First, other lipid components can be significant for the function of the vesicles. Membrane heterogeneity can also play a role[17] and so can membrane curvature.[18] We also do not take into account the effect of membrane proteins, which can make up to 50% of the mass fraction of the vesicles and can strongly influence membrane trafficking.[19,20] However, we focused here on the role of the lipids only, and asked if the lipids themselves provide an answer to the mechanical perturbation question. Therefore, we have not included the proteins in this study. Also, the acyl chains in the vesicles can vary for any given headgroup, but we

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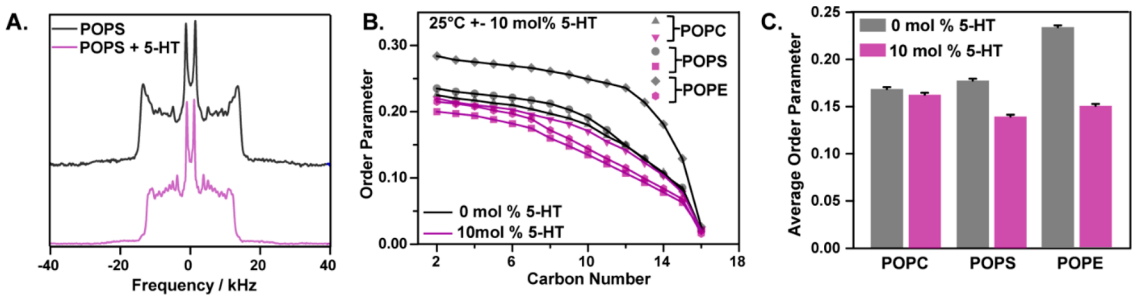

Figure 3. Effect of 10 mol % serotonin on single-component membranes (POPC, POPS, POPE) at 25 °C, measured by ^{2}H NMR. (A) ^{2}H NMR spectra of deuterated POPS-d_{31} membranes in the absence (gray) and presence of 10 mol % serotonin (light pink). (B) NMR order parameter along the lipid acyl chains and (C) the average ^{2}H NMR order parameter of POPC, POPS and POPE membranes.

have represented all acyl chains by PO (palmitoyl-oleoyl) in our experiments. Within the limits of these approximations, our results show how nature may be able to handle the high levels of serotonin in neurotransmitter vesicles.

2. RESULTS

2.1. Role of Individual Lipid Components. *2.1.1. Effect of Serotonin on the Breakthrough Force of Single-Component Lipid Membranes.* We first measured the serotonin-induced changes in the membranes composed of each of the individual components of synaptic vesicles. In the AFM force indentation technique, an AFM tip ruptures the supported membranes locally, allowing us to quantify the force required to break through the membrane. This force is defined as the breakthrough force (F^*) (Figure 1A, also see the inset). Since the initial event of exocytosis involves local pore formation, F^* provides a measure that is relevant in the present context. The representative average F^* of POPC bilayers in the absence and presence of 5 mM serotonin are shown in Figure 1B. While the average F^* varies with the membrane preparation, the relative change upon serotonin incubation is a much more robust quantity. There is a (25.2 ± 3.8) % decrease in the average F^* of POPC membranes (Figure 1C). Thus, POPC membranes are rather sensitive at even 5 mM serotonin concentration. For the POPS bilayer, we measure a 235 ± 74% increase in average F^* at 5 mM serotonin concentration. For POPE, there is a 21.8 ± 10.7% increase (Figure 1C). Overall, 5 mM of serotonin causes substantial perturbations of all the lipid bilayers. This is in qualitative agreement with previous studies with other lipid compositions.[2] Here, interestingly, both the direction and the magnitude of the perturbation are observed to be very different for individual lipid components.

2.1.2. Molecular Dynamics (MD) Simulations to Understand Molecular-Level Changes. We performed MD simulations to understand the molecular level changes which give rise to the opposite mechanical perturbations of POPC and POPS membranes caused by serotonin. The simulation time of 6000 ns was found to be sufficient to reach convergence for the lipid bilayer system with serotonin (see the Supporting Information (SI), Section 5, Figure S4A). The results show that serotonin is mostly localized near the lipid−water interface and inserted mostly into the hydrophilic part of both membranes (Figure 2A). Notably, serotonin interacts with the hydrophobic segments of the lipids more often for POPC than for POPS membranes (Figure 2A).

The analysis of the angular distribution between the molecular axis of serotonin (section 3 and Figure S3 of the SI) and the lipid bilayer normal (Z-axis) showed broadly distributed orientations of serotonin in both the membranes, with the charged group of serotonin mostly oriented at 135° to the plane of the hydrophilic part of the membranes (Figure 2B). Interestingly, serotonin molecules displayed a larger preference for a more ordered orientation in the case of the POPS lipid bilayer (Figure 2B). The position and angle distribution of serotonin are therefore somewhat different for POPC and POPS, and can in principle cause different effects.

We then performed a steered molecular dynamics (SMD) simulation to mimic the AFM force indentation experiment. A hydrophobic particle (a fullerene, C720) was inserted into the membrane and the work required to do so was computed. The analysis of the SMD simulation (8 trajectories per system, each of 250 ns, giving 2000 ns in total) showed that serotonin specifically increased the work required to push the C720 fullerene particle through the POPS membrane (Figure 2D). In contrast, a small decrease was observed for the POPC membrane (Figure 2C). The increase of work mainly arises from pushing the fullerene through the POPS bilayer (along the Z-axis), and not from the initial deformations of the membrane. Figure 2C,D shows the total work done for the POPC and POPS bilayers, and Figure S2A,B shows the work obtained for the distance of 1 Å. The difference between the total work for POPS lipid bilayer without serotonin and with 20 serotonin molecules is on average (69.8 ± 17.4)%, (88.9 ± 25.5)%, and (67.5 ± 18.9)% for distances of 12.5, 7.25, and 0 Å between the centers of C720 and POPS lipid bilayer, respectively. These correspond to the critical distances, in which the front part of the fullerene is in the middle of the lipid bilayer, fullerene is fully embedded into the lipid bilayer and the center of the fullerene is in the middle of the lipid bilayer, respectively. For POPC, analogical values are equal to −(10.0 ± 17.2)%, −(9.9 ± 17.5)%, and −(3.7 ± 7.2)% respectively. These values are lower than the experimental findings, but are in qualitative agreement with our F^* measurements.

2.1.3. Effect of Serotonin on the NMR Order Parameter of Single-Component Lipid Membranes. We also investigated the membrane properties using ^{2}H solid-state NMR, and measured the acyl chain order parameters for POPC, POPS and POPE respectively. In ^{2}H NMR experiments, the residual quadrupolar coupling is dependent on the time-averaged orientational fluctuations of C−^{2}H bond vectors and from the residual quadrupolar splitting, one can obtain the orientational order parameters.[21,22] In general, the higher the order parameter value of the lipid chains, the smaller is the amplitude of motion of the chain methylene groups and the more ordered is the membrane. The solid-state NMR study of

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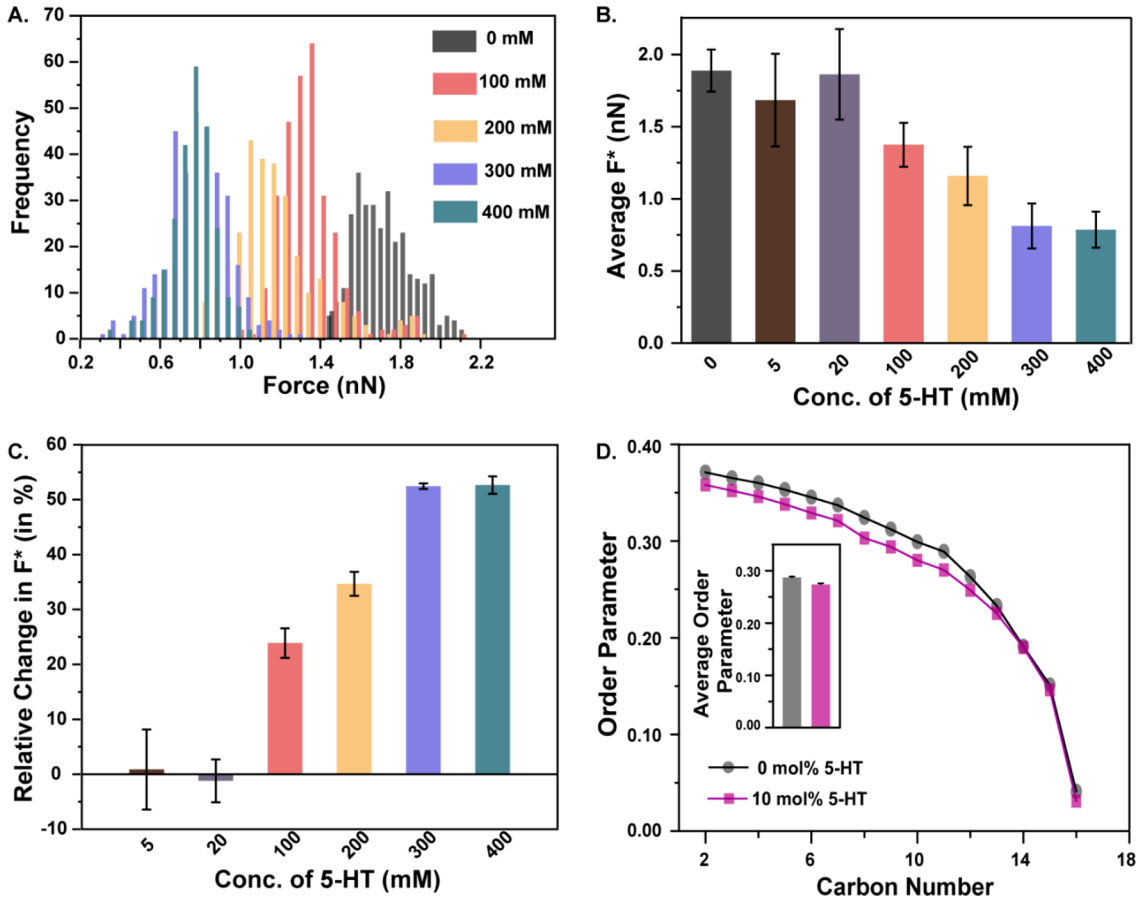

Figure 4. Effect of serotonin on the mechanical properties of physiologically relevant vesicular membrane in the presence of (5−400) mM serotonin probed by AFM force indentation study and in the presence of 10 mol % serotonin by ^{2}H NMR. (A) Representative force histogram of F^* is obtained by AFM force indentation on the PC/PE/PS/Chol/3/5/2/5 bilayer in the absence (gray) and presence of 100 mM (red), 200 mM (yellow), 300 mM (blue), and 400 mM (cyan) serotonin, respectively. (B) Representative average F^* values obtained by AFM force indentation on a PC/PE/PS/Chol/3/5/2/5 membrane in the absence (black) and presence of 5−400 mM serotonin, respectively. (C) Relative decrease in the average value of F^* of PC/PE/PS/Chol/3/5/2/5 bilayers in the presence of various serotonin concentrations (N = 3−4). N is the total number of sets. Each AFM set includes ∼600 force traces. Error bars represent SD (for Figure 4B) and SEM (for Figure 4C). All experiments are carried out in physiological salt conditions. (D) Effect of serotonin on the ^{2}H NMR order parameters of POPC-d_{31} in the vesicular membrane PC/PE/PS/Chol/3/5/2/5 in the absence (gray) and presence of 10 mol % serotonin (light pink). The inset shows the average lipid chain order parameters.

POPC membranes in the absence and presence of 10 mol % serotonin showed that serotonin decreases the average NMR order parameter of the acyl chains of the POPC membranes by a small but significant amount (3.6%, Figure 3B and C). This decrease qualitatively correlates with the AFM force indentation measurements. However, the average NMR order parameter also decreased for the POPS (21.3%, Figure 3A−C) and more substantially for POPE (35.6%, Figure 3B and C) membranes. So, for POPE and POPS, the NMR measurements do not correlate with the AFM F^* measurements. However, overall, the order parameter measurements also show that serotonin causes significant perturbations of all the individual lipid components of the synaptic vesicles.

2.2. Effect of Serotonin on the Physiologically Relevant Mixed Lipid Composition. If the mechanical properties for a mixture are additive in nature, then this would suggest that there would be a major decrease in the order parameters for any mixture of these components. Therefore, we ask how a mixture of these lipids mimicking the vesicular membrane composition responds to serotonin. Here we characterize the mechanical properties (F^*) and the NMR order parameters of the PC/PE/PS/Chol/3/5/2/5 membrane in the presence of serotonin.

2.2.1. Effect of Serotonin on the Breakthrough Force of Mixed Lipid Membrane. We observe only a (−0.9 ± 7.3)% and a (1.2 ± 3.9)% change in the average F^* of the PC/PE/PS/Chol/3/5/2/5 membrane (Figure 4B and C) at serotonin concentrations of 5 and 20 mM, respectively. These negligible changes are in complete contrast with the results obtained from single-component lipid membranes at 5 mM serotonin concentration.

We then measured the F^* at concentrations of serotonin which are 1 to 2 orders of magnitude higher, approaching those observed in neuronal vesicles. We determined the distribution of F^*s of PC/PE/PS/Chol/3/5/2/5 membrane in the absence (Figure 4A, gray) and in the presence of 100 mM (Figure 4A, red), 200 mM (Figure 4A, yellow), 300 mM (Figure 4A, blue), and 400 mM (Figure 4A, cyan) serotonin, respectively. F^* shows a gradual decrease with increasing serotonin concentrations from 100 mM to 300 mM, and no further change above 300 mM. The representative average F^* at each concentration of serotonin is shown in Figure 4B from one such measurement. There is a (23.9 ± 2.7)%, (34.7 ±

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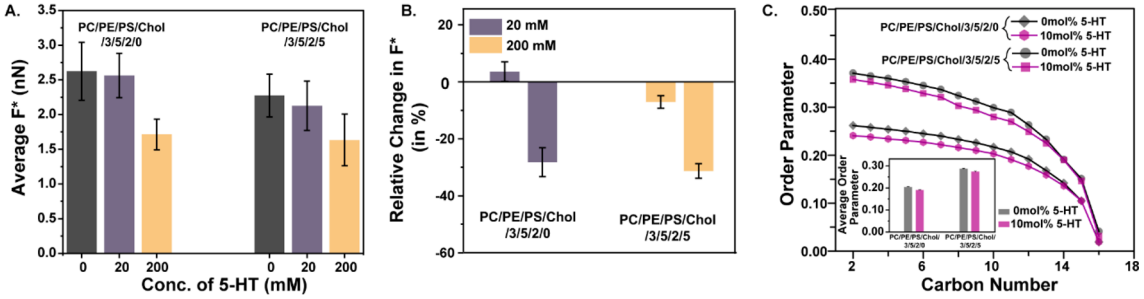

Figure 5. Role of cholesterol in serotonin-induced mechanical perturbations of vesicular membranes, measured by AFM force indentation and ^{2}H NMR. (A) Representative average F^* for PC/PE/PS/Chol/3/5/2/0 and PC/PE/PS/Chol/3/5/2/5 membranes in the absence (gray) and presence of 20 mM (purple) and 200 mM (yellow) serotonin, for one set of measurement. (B) Relative change (positive means increase and negative means decrease) in the average value of F^* of PC/PE/PS/Chol/3/5/2/0 (N = 5) and PC/PE/PS/Chol/3/5/2/5 (N = 4) membranes in the presence of 20 mM (purple) and 200 mM (yellow) serotonin. N = number of sets, and each set includes ~600 force traces. Error bars represent the SD (for Figure 5A) and SEM (for Figure 5B). All experiments are carried out in physiological salt conditions. (C) Effect of serotonin on the ^{2}H NMR order parameters of POPC-d_{31} in vesicular membranes at varying cholesterol molar ratios in the absence and presence of 10 mol % serotonin. Data are shown for two lipid compositions with varying cholesterol concentrations of PC/PE/PS/Chol/3/5/2/0 (0%) and PC/PE/PS/Chol/3/5/2/5 (33%) membranes in the absence and presence of 10 mol % serotonin. The inset shows the average lipid chain order parameters for POPC-d_{31} in the various preparations.

2.2)%, (52.5 ± 0.5)%, and (52.7 ± 1.6)% decrease in the average F^* of the PC/PE/PS/Chol/3/5/2/5 membrane induced by serotonin at the concentration of 100 mM, 200 mM, 300 mM and 400 mM, respectively (Figure 4C). Thus, in the mixed lipid membrane mimicking the composition of the natural vesicles, the sensitivity of the mechanical stiffness to serotonin is tuned down by one to two orders of magnitude.

2.2.2. Effect of Serotonin on the NMR Order Parameter of Mixed Lipid Membrane. We also perform solid-state ^{2}H NMR experiments to measure the orientational order parameter of each C−^{2}H group of the lipid chains of the PC/PE/PS/Chol/3/5/2/5 membrane in the absence and in the presence of 10 mol % serotonin (Figure 4D). The 10 mol % serotonin in NMR experiments is chosen as it is similar to the concentration of serotonin that is present in the membrane of the vesicles incubated with 200 mM serotonin used for AFM F^* experiments. This is measured using serotonin fluorescence after dialysis of the sample, as described in the SI, Section 4. The order parameter of the membrane along the lipid acyl chains for the deuterated POPC decreases only slightly in the presence of 10 mol % serotonin (Figure 4D). The slight decrease in the NMR order parameter shows that the PC/PE/PS/Chol/3/5/2/5 mixed membrane is very stable in the presence of a high concentration of serotonin, despite each of its components, taken separately, showing moderate to drastic reduction of the order parameter.

2.3. Role of Cholesterol in Serotonin−Vesicular Membrane Interactions. It can be argued that this resilience against high serotonin concentration is conferred by cholesterol, which has not been taken into account so far in our study. Cholesterol is an essential ingredient of mammalian lipid membranes (it is present at about 33 mol % level in vesicular membranes) and is known to strongly affect their mechanical properties.[23,24] So we now probe how the relative molar ratio of cholesterol (keeping the ratio of the other components constant) affects the response of the membrane to serotonin.

For our measurements of the F^*, we chose the membrane compositions PC/PE/PS/Chol/3/5/2/0 (zero cholesterol) and PC/PE/PS/Chol/3/5/2/5 (33% cholesterol), and measured the changes induced by 20 and 200 mM serotonin. The representative average F^* in the absence and presence of 20 and 200 mM serotonin are shown in Figure 5A. In the presence of 20 mM serotonin, there is a (3.5 ± 3.5)% increase in the average F^* of the PC/PE/PS/Chol/3/5/2/0, but a (7.0 ± 2.2)% decrease for PC/PE/PS/Chol/3/5/2/5 (Figure 5B, purple). Thus, there is no significant change in the mechanical stiffness of both membranes in the presence of 20 mM serotonin. In the presence of 200 mM serotonin, there is a (28.2 ± 5.1)% and (31.3 ± 2.6)% decrease in the average F^* of the PC/PE/PS/Chol/3/5/2/0 and PC/PE/PS/Chol/3/5/2/5, respectively (Figure 5B, yellow). Thus, irrespective of cholesterol, the change in F^* is similar. We do note that the absolute values of the F^* of PC/PE/PS/Chol/3/5/2/x decrease by 14 ± 8% when x changes from 0 to 5, somewhat contrary to our expectations.

We verify this observation with ^{2}H NMR experiments (Figure 5C). The average order parameter for 0 and 33% cholesterol in the PC/PE/PS/Chol membrane decreases by 0.014 and 0.013 units respectively in the presence of 10 mol % serotonin (Figure 5C). The difference is within the sensitivity of the technique. This shows that cholesterol does not affect the degree of perturbation of acyl chains by serotonin.

3. DISCUSSION

In this work, we explored the impact of the very high serotonin levels (>100 mM) on lipid membranes of specific lipid compositions. Serotonin has been shown to significantly alter the membrane properties of some lipid bilayers at much lower concentrations[2,3] than what is found inside the synaptic vesicles. Our work probes whether the lipid composition of the neurotransmitter vesicles, which is rather different from that of the plasma membrane or the other organelles, has a role to play in tackling this challenge. Our main experimental tools were AFM, which measured changes in the breakthrough force, and solid state NMR, which measured changes in the order parameters of acyl chains. We also performed MD simulations to have a molecular level understanding of the system.

We find that the mechanical stiffness of each major lipid component of the neurotransmitter vesicles (PC, PS, and PE lipids) are altered significantly in the presence of even 5 mM of serotonin (Figure 1). This is in qualitative agreement with previous studies with different lipid compositions.[2] Interestingly, serotonin has different effects on the F^* of the individual

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lipids. The F^* decreases for POPC, while it increases for POPS and POPE (Figure 1C). This may suggest that a mixture of these at the right proportion may be able to resist the mechanical perturbation at high serotonin levels, canceling each other's contributions. In contrast to the F^* measurements, NMR order parameter measurements show a decrease for all the three lipids in the presence of 10 mol % serotonin (Figure 3). The decrease is very significant for POPE and POPS membranes (Figure 3C). We note that the changes induced by serotonin in the NMR order parameter of POPE and POPS do not correlate with the F^* changes observed in the AFM measurements. F^* is likely determined by a larger spatial region of the membrane, which can be different from the response of NMR order parameter measurements that manifest the very local order of the lipid acyl chains. Indeed, this lack of correlation has been observed between several different membrane probes which probe the membrane at different temporal and spatial scales.[25]

We explored the origin of the difference of the mechanical response of two of the lipids (POPC and POPS) to serotonin using MD simulations. The breakthrough force (F^*) measurements were modeled by driving a hydrophobic particle (a fullerene, C720) through the lipid bilayer. A fullerene particle was chosen for these simulations because it is a successfully used model to study the mechanical properties of lipid bilayers and related force-field parameters for fullerene can be easily obtained as the molecule consists of only carbon atoms.[26] It is important to note that due to computational limitations the simulations cannot account for differences in the exact size, geometry and chemical property between the AFM tip and the fullerene molecule. Additionally, the spring constant, the time scale of indentation and the approach speed are also different between experiments and simulations. These are the crucial factors governing the magnitude of F^*. So, a quasi-spherical fullerene particle, smaller than the thickness of the lipid bilayer, would not provide a quantitative representation of the AFM tip. However, the work required to penetrate the bilayer should provide reliable differences in mechanical properties of the lipid bilayer and it can be expected to at least qualitatively show if and why there could be a substantial difference between different lipids in their response to serotonin as observed in the AFM experiment. In the simulations, we find that higher work is needed to insert the fullerene into the POPS membrane in the presence of serotonin (Figure 2D). Such an increase in work is not observed (and in fact a small decrease is observed) for the POPC membrane (Figure 2C). The increase in work done suggested that POPS membranes become stiffer in the presence of serotonin. This is in qualitative agreement with the F^* measurements. The origin of this effect seems to come from two factors. Serotonin appears to enter deeper into the POPC membrane, and the tilt angle of serotonin with respect to the membrane normal appears to be higher for POPC compared to POPS (Figure 2A,B). The lipid water interface of a biological membrane is a highly specialized and complex molecular assembly where various physical interactions contribute to the packing and lateral organization of the molecules constituting this layer.[27−32] Accordingly, the localization of a small molecule in this broad interface is subject to a complicated balance of forces which results in the specific orientation and average localization of the respected molecule that is described by a minimum in the free energy.[33−35] These interactions determine the impact of the small molecule on the surrounding lipids. Physical forces that contribute to this free energy include Coulomb, charge−dipole, dipole−dipole, and cation−pi (π), interactions. Furthermore, hydrogen bonding will play a role as both serotonin and the lipid segments represent hydrogen bond donors and acceptors. Finally, aromatic compounds prefer a hydrophobic environment due to the hydrophobic effect and also other entropic properties contribute to the minimum in free energy of a given molecule in the interface of a membrane of specific composition.

The MD simulations provide a clearer picture of this interface, and shows the molecular origin of the different effects that serotonin has on the POPC and POPS bilayers. It suggests that the higher mechanical stability of the POPS bilayer with serotonin molecules arises from the formation of a layer by three types of charged molecules: serotonin, Na^+ ions, and charged serine headgroups of POPS. This denser layer requires more force for breaking, contrary to the POPS without serotonin [Figure S5B (POPS bilayer with serotonin) vs Figure S5A (POPS bilayer without serotonin)]. Serotonin does not induce such a dense layer for POPC as it is uncharged [Figure S5D (POPC bilayer with serotonin) vs Figure S5C (POPC bilayer without serotonin)]. This is most likely connected to the electrostatic interaction of the lipid head groups of POPS and serotonin. We, therefore, see that there is a molecular basis for lipids with different headgroups to respond differently to serotonin, and that POPS can indeed offer higher resistance to mechanical indentation when incubated with serotonin.

These MD simulation results suggest that the synergistic action of the individual components of a biological membrane with serotonin can be responsible for the minimal perturbation of the membrane structure at biologically high serotonin concentration. However, a detailed theoretical treatment and quantification of the complex interaction of serotonin with phospholipids is still needed to gain a fuller understanding of this phenomenon.

As the individual lipid membranes are significantly perturbed by serotonin. However, when we perform the AFM force indentation and NMR order parameter measurements of the vesicular membranes with near-natural composition (PC/PE/PS/Chol/3/5/2/5) in the presence of serotonin, their behavior appears to be completely different. We find no change in the average F^* of PC/PE/PS/Chol/3/5/2/5 membrane in the presence of 5 and 20 mM serotonin (Figure 4C), which is in sharp contrast to the results obtained from the individual lipid components. When we performed the F^* measurements in the presence of 100−400 mM serotonin concentrations, we find that the average F^* only gradually decreases in this concentration range with a decrease of ∼50% at 300 mM, and no further decrease beyond that (Figure 4C). This makes it robust to high concentrations of serotonin, and shows some change only in the concentration ranges found inside the synaptic vesicles. We probed whether this could be a generic effect of any such mixture, or whether it was specific to the natural composition of the vesicular membrane. We studied a mixture of a zwitterionic and a negatively charged lipid, qualitatively similar to the PC/PE/PS/Chol/3/5/2/5 membrane composition used earlier, using a model membrane POPC/POPG/Chol in the molar ratio of 1:1:1 (PPC111). This contains POPG (which is not common in mammalian cells) instead of POPS as the negatively charged lipid. We previously showed that 5 mM serotonin could lower the F^* of PPC111 by about 50%,[2] but had not tested the behavior at

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higher serotonin concentrations. However, our current efforts to measure the F^* of the PPC111 membrane in the presence of 200 mM serotonin failed, as we found that the membrane became unstable, and provided no clear peaks in the force−displacement curves. Thus, the effect seems to be specific for vesicular lipid composition.

The NMR study also shows that the average ^{2}H NMR order parameter of POPC-d_{31} in PC/PE/PS/Chol/3/5/2/5 membrane is much more resistant to 10 mol % serotonin, compared to the pure lipids. There is small decrease in NMR order parameter in the presence of 10 mol % serotonin (Figure 4D). This is an even more remarkable result compared to the F^* results, as serotonin made all the individual lipid components (especially POPE and POPS) reduce their order drastically. Yet, their mixture is perturbed to a much smaller extent. In fact, the slight decrease in the NMR order parameter indicates that the PC/PE/PS/Chol/3/5/2/5 membrane is tuned to respond to serotonin, but only at high physiological concentrations. This is the most significant finding of our study.

We also found that the changes in the cholesterol content do not substantially change the F^* and order of the vesicle-mimicking membranes to serotonin (Figure 5). Thus, the robustness against high serotonin concentrations arises from the specific mixture of the other major lipids. We can only speculate that somatic and volume exocytosis, which is more common for monoamines, benefits from the coupling of membrane mechanical properties with that of intravesicular neurotransmitter content.

Our findings suggest that membrane perturbation observed for the individual lipid components and non-physiological lipid mixtures (PPC111) is not completely abolished in physiological lipid mixtures, but the sensitivity is tuned down so that a similar degree of response is observed only at 50× higher serotonin concentrations. This response does not seem to depend on the cholesterol content of vesicular membranes. This happens to be exactly the range of physiological serotonin concentration inside the vesicles.[6] This tuning may help the membrane to remain mechanically stable at high serotonin concentration, yet respond to differences in concentrations that would occur between a partially filled vesicle versus a mature serotonin-filled vesicle.

4. CONCLUSIONS

The amphiphilic nature of serotonin provides the driving force for membrane binding which modulates membrane properties already at low serotonin concentrations of a few mM. Remarkably, the lipid composition in synaptic vesicles is very different from that of other organelles in terms of the types of lipids and their relative molar ratios.[36−40] The biological meaning of these alterations and their functional roles remain unknown. It is known that cells carefully maintain the homeostasis of lipid composition in each type of membrane.[41] It is possible that the individual lipid species are required to play specific roles. It is also possible that the mechanical parameters of the membranes are an emergent property of the lipid mixture, not apparent in the individual components. The evolution of membranes had to account for its physicochemical environment,[42] and the need to contain amphiphilic molecules possibly played a role in determining the lipid composition of the vesicular membranes. What is often not appreciated is that the extraordinarily high concentration of small membrane-bound lipophilic molecules causes strong mechanical perturbations of the membrane, and nature has to tackle this challenge. This is reflected in the instability of a simple mixture of cholesterol, zwitterionic and negatively charged lipids at high serotonin concentrations. However, nature uses similar lipids to yield a much more robust response. This is highlighted by our work. Our study suggests that the lipid composition of synaptic vesicles has evolved in order to minimize the destructive effect of high concentrations of serotonin.

It may be questioned whether the F^* measurements have any correlation to physiological membrane fusion. The fusion of two membranes involves the bending of the membrane locally, transient exposure of the hydrophobic parts of the lipids, and finally the merger of two membranes. Thus, there is a significant energy barrier to this procedure. In the physiological SNARE-mediated membrane fusion, this energy is supplied by SNARE protein complex formation, which is driven by ATP consumption.[43,44] In the AFM force indentation, it is the AFM tip which locally and transiently bends the membrane and creates a pore to expose the hydrophobic part of the membrane. So, we believe that the process of indentation by AFM tip has a reasonable correspondence with the SNARE-mediated membrane fusion. Indeed, the interaction of monoamine neurotransmitters with the vesicular membrane has been shown to alter the release kinetics of vesicles.[45] Our results provide a possible explanation for this effect in terms of the mechanical properties. The reduction of mechanical stiffness of the membrane at physiological serotonin concentrations may be a biological necessity. This may allow the cell to preferentially perform exocytosis[46,47] of the mature serotonin-filled vesicles compared to that of partially filled vesicles.

The composition of vesicular lipids has likely evolved according to the constraints imposed by mechanical requirements to handle high neurotransmitter concentrations. Our study reveals a crucial connection between the physical chemistry of lipids and the process of serotonin neurotransmission that has not been recognized so far.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcb.2c07464.

Description of materials and methods; preparation of supported lipid bilayers and small unilamellar vesicles; experimental details and the analysis procedure to obtain AFM breakthrough forces of the membranes; sample preparation and experimental details to perform ^{2}H NMR of the membranes; analysis details to calculate NMR order parameters; simulation and computation details; computed work to indent fullerene molecule through the membranes, diagram to show the definition of angle of serotonin molecule with the lipid bilayer, description of quantifying serotonin to lipid molar ratios in membrane vesicles, the plots showing the convergence of simulations (PDF)

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Notes

The authors declare no competing financial interest.

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