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Effects of neurosteroids on a model membrane including cholesterol: A micropipette aspiration study

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ABSTRACT

Amphiphilic molecules supposed to affect membrane protein activity could strongly interact also with the lipid component of the membrane itself. Neurosteroids are amphiphilic molecules that bind to plasma membrane receptors of cells in the central nervous system but their effect on membrane is still under debate. For this reason it is interesting to investigate their effects on pure lipid bilayers as model systems. Using the micropipette aspiration technique (MAT), here we studied the effects of a neurosteroid, allopregnanolone (3α,5α-tetrahydroprogesterone or Allo) and of one of its isoforms, isoallopregnanolone (3β,5α-tetrahydroprogesterone or isoAllo), on the physical properties of pure lipid bilayers composed by DOPC/bSM/chol. Allo is a well-known positive allosteric modulator of $GABA_A$ receptor activity while isoAllo acts as a non-competitive functional antagonist of Allo modulation. We found that Allo, when applied at nanomolar concentrations (50–200 nM) to a lipid bilayer model system including cholesterol, induces an increase of the lipid bilayer area and a decrease of the mechanical parameters. Conversely, isoAllo, decreases the lipid bilayer area and, when applied, at the same nanomolar concentrations, it does not affect significantly its mechanical parameters. We characterized the kinetics of Allo uptake by the lipid bilayer and we also discussed its aspects in relation to the slow kinetics of Allo gating effects on $GABA_A$ receptors. The overall results presented here show that a correlation exists between the modulation of Allo and isoAllo of $GABA_A$ receptor activity and their effects on a lipid bilayer model system containing cholesterol.

1. Introduction

An increasing number of works in biophysics and in cell biology are nowadays devoted to the relevant role that lipid components of the biological membrane can have on the activity of membrane proteins, by both specific [1] and non-specific mechanisms that consider the bilayer as a continuum [2–4]. The non-specific roles of lipids are related to both their mechanical properties that are relevant in the conformational transitions of membrane proteins (mechanical spring constants of the bilayers and lateral pressure profile) and to the lateral heterogeneity of membranes. The latter aspect represents an issue that is not new [5,6], but it has received a strong burst after the so called "*raft* hypothesis" was introduced in the biological community [7] and it is far from being resolved. In fact, previous hypothesis on the basic role of the lipid bilayer in affecting the membrane protein activity relied mainly on evidence from model systems. In the first introduced models of the biological membrane, the lateral heterogeneity in the organization of the membrane regarded mainly the possibility of phase segregation or the presence of different phase domains such as in the case of solid ordered domains (S_o, also gel phase) coexisting with the liquid disordered phase (L_d, also liquid crystalline). The raft hypothesis shifted the attention to the possibility of another phase separation in biological membranes. The new idea is related to the possibility of a phase separation between two liquid domains in the biological membrane, the liquid ordered (L_o) phase and the L_d one [8]. In this case, relevant roles are played by the presence of sterols such as cholesterol in the membrane and by their preferred interactions with specific lipids. Many membrane proteins seem to have the tendency to segregate into one of the two phases and the segregation of different proteins in the same domain is considered as the basic mechanism by which many signaling pathways could be activated [8–10]. In this scenario, any mechanism able to produce an alteration of the bilayer phase properties could be relevant for the normal operation of a biological membrane [11]. However, the detection of phase separation between different liquid phases in biological membranes remains still elusive [12]. The elusive character of these domains

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in biological membranes is now ascribed to their small lateral dimensions (in the order of a few tens of nanometers) and to their dynamic aspects (lifetime in the order of milliseconds) [13]. However, the *raft* hypothesis stimulated a plethora of studies trying to elucidate if specific membrane proteins could be considered raft-associated. In cases where this association is possible, it can be concluded that any modification of the bilayer affecting the thermodynamics of raft domains can also affect protein function.

A very interesting breakthrough was the realization that specific thermodynamic properties could play important roles in the organization and consequently in the activity of a biological membrane. In particular, the proximity, in physiological conditions, of the biological membrane to a critical point or to several critical points emerged as an intriguing possibility in this research field [14,15]. Around physiological temperature, the membrane is thought to be, due to its lipid composition, just above its critical condition [16]. In this situation, fluctuations in lipid composition could explain both the small lateral dimensions of the domains and their dynamic organization. Moreover, it was demonstrated that lipid bilayer model systems composed of three different lipid types, one low-melting lipid type, one high-melting lipid type and cholesterol, could be considered representative of much more complex bilayers in eukaryotic cells [17] and useful information could be obtained studying these model systems.

The role of thermodynamics in the behavior of a membrane brings about considerations also on the possibility that a dopant in the bilayer could alter the thermodynamics of the system and impair the regular formation of domains. This aspect could be particularly relevant in the case of proximity of the membrane to a critical point or in general to a miscibility border.

Accordingly, the longstanding discussion on the interactions between drugs and lipids in the membrane [18] was enriched with a new viewpoint related to the thermodynamical aspects of lipid bilayers near critical points [19]. In fact, the simple insertion of a drug could change the bilayer position in the phase diagram affecting the domain organization as a consequence of a changed distance from the miscibility border and could consequently affect the activity of membrane proteins. Even if a mechanistic view of the process leading from the presence of the drug in the membrane to changes in its functions is not completely clear, it is evident that further studies of the interaction of drugs with membranes deserve great attention. This is particularly true for highly lipophilic drugs such as some anesthetics and neurosteroids.

In this work we studied the effects of neurosteroids on the physical properties of a lipid bilayer composed by DOPC, bSM and cholesterol. To do this, we exploited the micropipette aspiration technique (MAT), concentrating on giant unilamellar vesicle (GUV) model systems. The neurosteroids that we concentrated on are allopregnanolone (in the following Allo), an endogenous highly lipophilic molecule [20], known to modulate $GABA_A$ receptor activity [21,22] and one of its isoform isoallopregnanolone (in the following isoAllo). In particular, Allo potentiates GABA-evoked currents mediated by $GABA_A$ receptor activation at low nanomolar concentrations and is able by itself to activate the $GABA_A$ receptor at higher concentrations [23]. Many studies report on the possible interaction of Allo with the lipid bilayer [24], although a well-established understanding on this aspect is still lacking. For example, studies on the gating behavior of Allo found that the activation of $GABA_A$ receptors occurred with a slow kinetics and this behavior was hypothesized to derive from the slow accumulation of the neurosteroid in the plasma membrane [23]. However, the possible effects of Allo on the mechanical parameters characterizing the membrane have not yet been considered, neglecting the possible contribution of lipid bilayer properties on the behavior of the receptors. Instead, isoAllo is known to be a non-competitive antagonist of Allo with regard to $GABA_A$ receptors [25]. Thus, the two isoforms represent a very interesting test case to investigate if their different pharmacological properties at the level of a membrane protein could be somehow also related to differential effects on the lipid components of the membrane. In the past, the different effects of Allo and isoAllo on lipid bilayers have already been considered, albeit at higher concentrations than what we used in the present work [26,27]. Considering that the neurosteroid concentrations that we used are functionally relevant for the $GABA_A$ receptor, investigating their effect on pure lipid bilayers is worthwhile.

Here, besides measuring the kinetics of Allo and isoAllo uptake by a DOPC/bSM/cholesterol pseudo-ternary lipid bilayer ("pseudo-ternary" refers to the fact that bSM is already a mixture, even if largely composed by 18:0 acyl chains) in the form of GUVs by the MAT [28,29], we also studied their effects on the mechanical properties of the bilayer. We found that at nanomolar concentrations Allo produces an increase of the overall lipid bilayer area which is coupled to a decrease of the bilayer mechanical parameters. The kinetics of Allo uptake and the obtained time constant have been found to be in the same order of magnitude as the time constant observed for the gating effect of Allo on the $GABA_A$ receptors at similar concentrations. At variance with Allo, we found that isoAllo produced a decrease of the lipid bilayer area and a negligible variation of the mechanical properties. The obtained results are also interpreted on the basis of what is obtained when a supported lipid bilayer of the same lipid composition is studied by atomic force microscopy (AFM) as a function of Allo and isoAllo concentrations to which it is exposed [30]. Finally, we discuss the effects of the neurosteroid on the lipid bilayer properties in light of a general mechanism of action of lipophilic molecules on biological membranes [18].

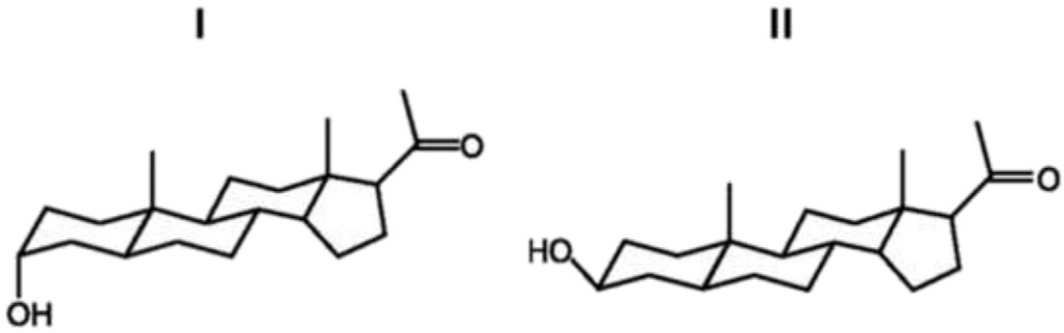
2. Material and methods

2.1. Lipids

Lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (Brain, Porcine) (bSM) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, USA) and were used without further purification. Specific lipid mixtures were prepared by mixing chloroform lipid solutions in the desired amount (the proportions used in this work are molar proportions). Allopregnanolone was purchased from Sigma-Aldrich and isoallopregnanolone was a generous gift from Dr A. Guidotti (see ref. [21] for details on isoAllo) (see Scheme 1 for their structure).

2.2. GUV preparation

GUVs were prepared by the electroformation method [31] with minor modifications. Briefly, lipid mixtures were suspended in chloroform and small drops (2–3 μL, 0.2 mg/mL total lipid) of the lipid mixture were deposited on two opposing Pt wires inside a PTFE chamber. Phospholipid compositions in GUVs are expressed as mole ratios: e.g., DOPC/SM/Chol (1:1:1) denotes an equimolar ternary mixture. Chloroform was removed initially by exposing the Pt wires to a nitrogen flux and then by using a vacuum chamber (10^{-2} mbar) for 2 h. Two Pt wires were then connected to a wave form generator to produce a sinusoidal voltage potential difference. The PTFE chamber was then filled with a 100–200 mM sucrose solution and sealed using glass coverslips and vacuum grease. The applied electroformation protocol was as follows: (1) 10 Hz, 3.0 V_{p-p} for 45 min; (2) 5 Hz, 2.5 V_{p-p} for 20 min; and (3) 2 Hz, 1.5 V_{p-p} for 15–20 min. As the final step we applied a square wave at 5 Hz in order to promote vesicle detachment from the wires. After formation, GUVs were gently extracted from the PTFE chamber and resuspended in a 95–250 mM glucose solution. This procedure assures an increased contrast in Differential Interference Contrast (DIC) images acquired with an inverted optical microscope (Olympus IX70) and a conserved internal volume, at least on a short time scale (a few minutes).



Scheme 1. Structure of the two neurosteroids: I) allopregnanolone; II) isoallopregnanolone.

2.3. Micropipette aspiration set-up

Microaspiration was performed using pulled glass capillaries with a cylindrical shape and an internal diameter in the order of 10–15 μm. Pipettes were tip-polished to ensure good membrane–pipette contact and pretreated with BSA (10 mg/mL^{-1}) to avoid adhesion between glass and lipid bilayers. Each pipette was then connected to a pneumatic pressure transducer (Lorenz MPCU-3) to apply pressure differences between the internal side of the pipette and the external solution at the same height with a sensitivity of 1 mm H_2O. The pressure difference was applied by controlling the air pressure on top of a cylindrical tube containing the same external solution and initially kept at the right height to assure an initial negligible pressure difference. The vesicles were manipulated inside a home-made glass chamber. In response to pressure differences (between the internal pipette and the region just outside the pipette), the vesicle is aspirated into the pipette and the progressive membrane deformation (projection) can be measured as a function of the applied pressure difference or as a function of time at constant applied pressure. To study the kinetics of the interaction of the molecules with a lipid bilayer a fast perfusion system would be required (the measurement time should start with an already established constant concentration of the neurosteroid). To circumvent this problem we assembled a cell with two chambers (Fig. 1a) [32,33]. The first chamber contains the vesicles in glucose solution while the second chamber contains the glucose solution plus a defined concentration of the neurosteroid at issue. A vesicle is grabbed by the micropipette and is then inserted inside a larger pipette filled with the same glucose solution (Fig. 1). All the chamber system is then moved and the vesicle inside the big pipette is brought inside the chamber with the neurosteroid. The large pipette is then removed and this marks the start for measuring the uptake kinetics. In some cases, to study the kinetics of the release from the bilayer, at the end of the uptake step the vesicle is again included in the pipette and taken back to the first chamber. The removal of the large pipette marks the start of the desorption kinetics.

This micropipette set-up was also used to measure the mechanical properties of the lipid bilayer and the effects of the neurosteroids on them. Briefly, the applied pressure difference when a liposome is sucked by the micropipette can be converted to lateral tension in the lipid bilayer once the outer vesicle diameter and the internal diameter of the pipette are known. The conversion is made by the Laplace equation

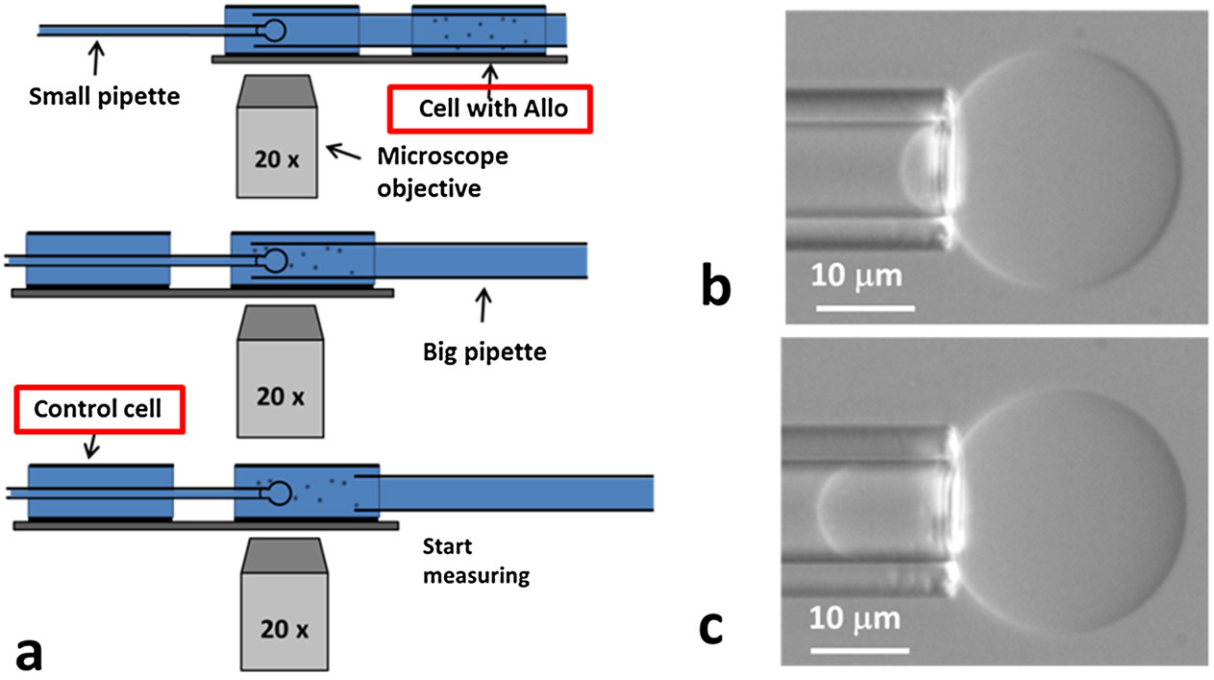


Fig. 1. a) Scheme of the set-up used to measure the kinetics of neurosteroid uptake by the liposomes. b) Example for the case of uptake from a 100 nM Allo solution: DIC microscopy image of the initial configuration of the liposome. c) Configuration of the liposome after 10 min.

according to Eq. (1)

$$\tau = \frac{\Delta P}{2}\left(\frac{r_p}{1-\frac{r_p}{R_v}}\right) \quad (1)$$

where τ is the lateral tension in the bilayer (in N/m), r_p is the internal diameter of the micropipette, ΔP is the pressure difference and R_v is the external vesicle radius. If the length of the bilayer projection inside the micropipette L is measured as a function of time or as a function of the applied lateral tension to the lipid bilayer it is possible to measure the relative increase of the bilayer area α as reported in Eq. (2)

$$\alpha = \frac{A-A_0}{A_0} = \frac{2\pi r_p}{A_0}\Delta L\left(1-\frac{r_p}{R_v}\right). \quad (2)$$

The applied lateral tension and the relative area variation are linked by the Helfrich constitutive (Eq. (3))

$$\alpha = \frac{k_B T}{8\pi k_c}\ln\frac{\tau}{\tau_0} + \frac{K_{App}}{\tau} \quad (3)$$

where k_B is the Boltzmann constant, k_c is the bilayer bending modulus, τ_0 is the reference value for each vesicle and K_{App} is the apparent stretching constant. The K_{App} value obtained by Eq. (3) is defined as the "apparent constant" because it includes also sub-visible fluctuations of the lipid bilayer at high applied tension. At low applied tension the first term of Eq. (3) dominates and it can be used to derive the bending constant from a plot of the natural logarithm of the lateral tension as a function of the relative deformation. However, sub-visible contributions to the projected area coming from the suppression of undulations with very small amplitudes are present also in the high-tension region. These contributions are particularly relevant for small values of the bending constant and can be taken into consideration by a procedure described in Supporting information. After that correction, a stretching constant K_s can be obtained. Other details for the measurements by micropipette aspiration are reported in Supporting information.

When the values of the stretching constant as a function of the Allo or isoAllo concentrations are reported, they refer to the behavior of the same vesicle. Accordingly, the first tension ramps are obtained up to a limited value of the tension (always in the region dominated by stretching deformation) in order to preserve bilayer integrity.

The experimental errors were estimated on the basis of the measurement procedure for the geometrical characteristics of the liposomes. The error is mainly associated with the pixelation in the acquired digital image. We assumed for the geometrical parameters an error corresponding to one pixel (±1 pixel) and for the applied pressure difference an error of ±0.5 mm H_2O. We then propagated the error in the calculation of the different parameters.

3. Results and discussion

In this study we mainly concentrated on the DOPC/bSM/Chol 1:1:1 lipid mixture. This combination is considered a canonical mixture representing a good model for the study of domain separation in ternary lipid mixtures. The mixture is considered a "pseudo-ternary mixture" because bSM is a blend in itself, even if largely (50%) composed by an 18:0 fatty acid chain. In any case, many investigations on its phase behavior have been performed and are reported in the literature [34,35]. As we did not perform an in-depth analysis of the phase diagram for the mixture at issue, our considerations on this aspect will mainly be based on the phase diagram reported by Petruzielo et al. [36] whose schematic representation is shown in Fig. 2 to help discussion and interpretation of our data. On the basis of the reported phase diagram, at room temperature, the particular composition that we chose should

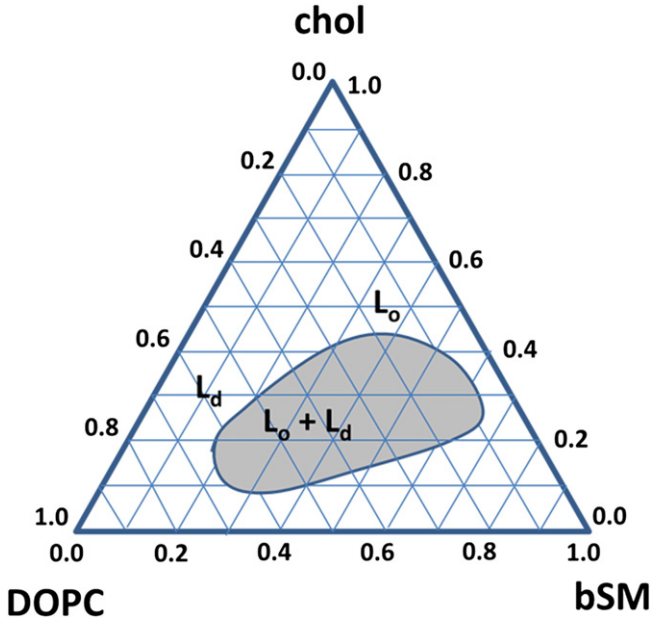


Fig. 2. Schematic phase diagram for the ternary lipid mixture DOPC/bSM/chol based on the data from ref. [36]. The darker area corresponds to the ($L_d + L_o$) phase coexistence region. The phase diagram represents a slice at about 25 °C of the complete phase diagram.

be inside the two-phase coexistence region but very near to the miscibility border through a critical point [37]. Moreover, considering the phase diagram with fixed 1:1 DOPC\bSM ratio as a function of cholesterol content and focussing on the high cholesterol concentration region, where the 1:1:1 mixture is to be positioned, the miscibility border as a function of cholesterol concentration is very steep [35]. This situation corresponds to the possibility that, with a very small variation in cholesterol concentration, it is possible to greatly affect the phase state of the lipid bilayer. At the same time, even a small variation in cholesterol concentration, which can result from uneven compositions of different vesicles, could produce different behaviors of the GUVs. The situation is particularly interesting when a component that could compete with cholesterol in the interaction with other lipids, such as sphingomyelin, is uptaken by the lipid bilayer.

As a first step, we performed the analysis of Allo uptake by vesicles for concentrations corresponding to the range where modulation of the $GABA_A$ receptor activity is found in electrophysiological measurements. These values span a range from a few nM to hundreds of nM. It has been reported that Allo has a high partitioning coefficient in the lipid bilayer [20]. In such conditions, a nM concentration in the bulk solution can be transformed into a μM one inside the lipid bilayer. To measure the kinetics of Allo uptake a liposome without visible internal vesicles was sucked by a micropipette using a very small pressure difference (typically corresponding to a tension of ≤1 mN/m). The procedure described in Fig. 1 and in the Material and methods section is then executed. In Fig. 3a a series of experiments measuring the relative area variation of the vesicles as a function of time is reported.

The average value for the time constant of Allo uptake by the lipid bilayer is 55 ± 25 s. The overall time constant that we measured could also include phenomena different from simple molecule uptake, such as transfer of the molecules from one leaflet to the other and transfer inside the GUV. The obtained value is consistent with the slow activation kinetics observed for Allo on the $GABA_A$ receptors and with the accumulation of fluorescently labeled neurosteroids in the plasma membrane [23]. It is evident that the range of measured time constant values is very broad for a given Allo concentration. This result could be related to vesicles with slightly different lipid compositions as it is typically found for GUVs produced by electroformation and including lipid mixtures. Analogously, our results by AFM [30] show that some lipid

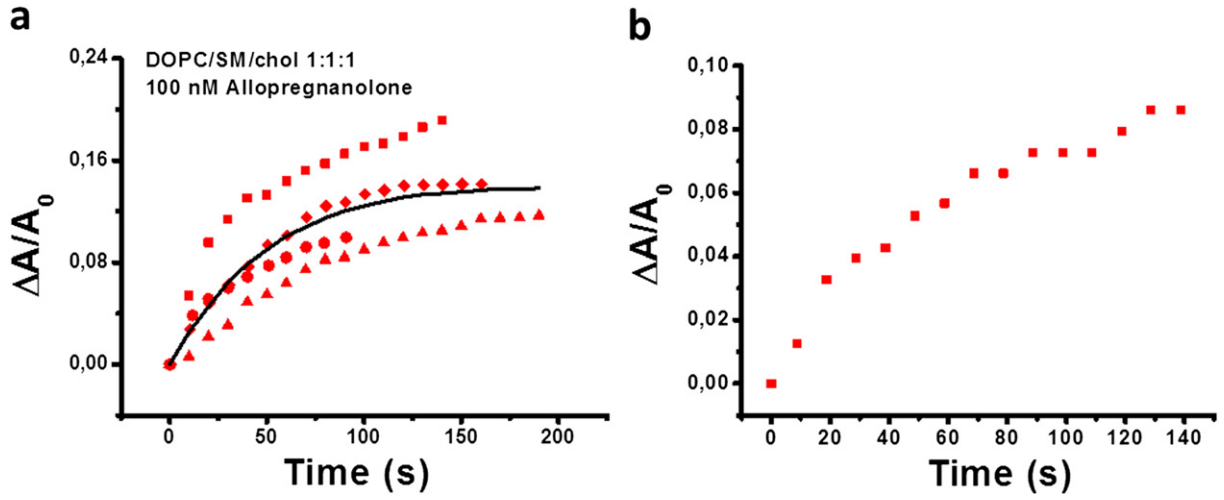


Fig. 3. Representative relative area variations for DOPC/bSM/chol 1:1:1: a) DOPC/bSM/chol 1:1:1 GUVs are moved from a chamber without to a chamber with a 100 nM Allo concentration. The different scattered traces correspond to different experiments, whereas the continuous line represents the average behavior resulting from 7 experiments; b) a DOPC/bSM/chol 1:1:1 liposome grabbed by a micropipette is exposed to a 100 μm/s flux of a solution containing 100 nM allopregnanolone and placed directly in front of the GUV (see Supporting information for details).

bilayer patches produced after vesicle fusion on a solid support, even if from small unilamellar vesicles (SUVs), present phase separation whereas other patches show a homogeneous phase. In the specific case of the presence of phase separation, AFM data show also that the relative proportion of L_o and L_d domains is broadly distributed in the different lipid patches. A similar result is obtained by epifluorescence microscopy (see Supporting information). Moreover, Allo uptake shifts the relative distribution in favor of the L_d phase [30]. It is interesting to note that other studies found that the insertion of exogenous molecules in lipid bilayers in the $L_o + L_d$ coexistence region leads to a modification of the relative proportions of the two liquid phases [37,38]. In addition, the shift amount depends on the lipid bilayer patch under observation. These considerations suggest a contribution from the phase distribution change to the increase of the lipid bilayer area upon Allo uptake by GUVs and they could also explain the broad distribution in the area variation values that we observed in the micropipette aspiration experiments (Fig. 3a). From a physiological point of view this behavior implies that the effect of neurosteroids on membranes depends on their specific lipid composition. We also measured the kinetics of the neurosteroid uptake by exposing a GUV grabbed by the micropipette to a flux of a solution containing an equal neurosteroid concentration produced by a larger pipette positioned just in front of the GUV (see also Supporting information). This experimental set-up allows verifying whether diffusion-limited phenomena are relevant or not for the uptake kinetics [39]. As shown in Fig. 3b, the characteristic time observed for this second experimental set-up is very similar to that observed in Fig. 3a. This behavior suggests that diffusion to the bilayer surface is not the rate-limiting step for the neurosteroid incorporation. We also measured the desorption process of Allo from lipid bilayers (Fig. 4a) and we found that it occurred typically with a time constant similar to that of the uptake process, even if the desorption process was better described using more than one characteristic time as it has been found for the fluorescence decay of fluorescently labeled neurosteroids [40]. In some cases the desorption process was slower than the corresponding uptake one and the liposome area did not go back to the original area value. The latter behavior could be related to (*i*) a small residual amount of the neurosteroid inside the lipid bilayer or (*ii*) to an induced volume variation of the vesicle besides its area variation. Exploiting electrophysiological studies, the fact that the plasma membrane could act as a neurosteroid reservoir has already been proposed, explaining in this way the long tail of potentiation effects when the aqueous solution is extensively washed [40]. In some cases (about 20% out of a total of 20 experiments), immediately after the exposure of the vesicle to Allo solution, we observed an initial decrease of the bilayer projection inside the micropipette followed by the usual increase (Fig. 4b). The projection retraction could also be due to a volume increase of the vesicle induced by

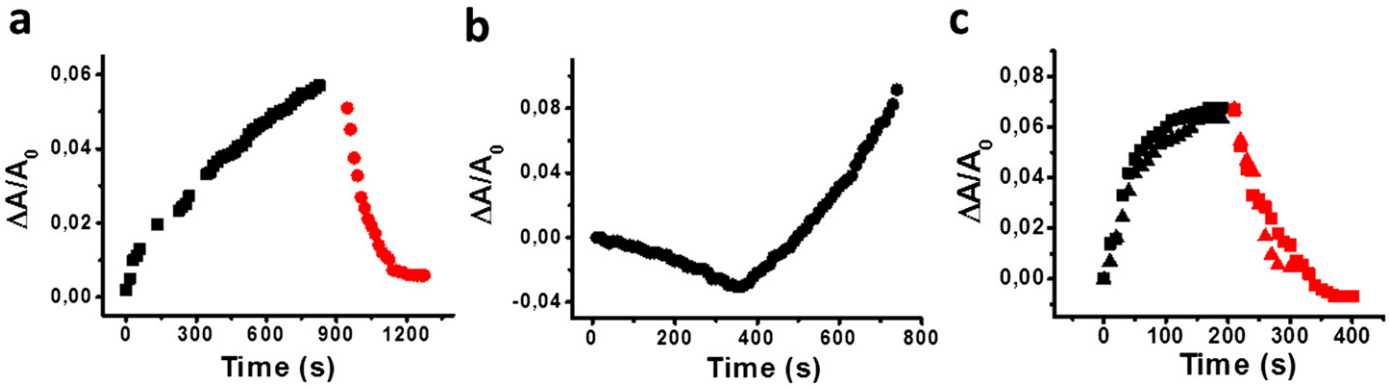


Fig. 4. Example of different behaviors for DOPC/bSM/chol 1:1:1 and DOPC GUVs. a) Kinetics of Allo uptake (followed by desorption) from a 100 nM solution in the case of a DOPC/bSM/chol 1:1:1 vesicle. The desorption was studied by taking back the vesicle in the original solution without Allo. b) Example of a case in which the DOPC/bSM/chol 1:1:1 vesicle, immediately after having been exposed to an Allo solution, undergoes an area decrease followed by the usual increase. This behavior was observed in about 20% of the 20 experiments that we performed. c) Example of the uptake and desorption processes for DOPC vesicles exposed to a 100 nM Allo concentration. In this case, the DOPC vesicle is in a uniform phase (L_d).

the formation of pores leading to a water-associated glucose flux inside the vesicle. However, we excluded this possibility because it is unlikely that, once formed, pores are closed by an increase of the same molecules that created them. The variability in the results obtained prompted us to study the effect of Allo uptake by a lipid bilayer which does not include cholesterol and which is in a homogeneous phase at room temperature. Hence, we chose to work with DOPC vesicles which have a phase transition from the L_d to S_o phase at −5 °C. Exposing DOPC vesicles to a 100 nM Allo solution produced the kinetics patterns shown in Fig. 4c, where we also measured the desorption process. In the case of DOPC vesicles we never observed a retraction of the projection inside the micropipette. Moreover, the relative area variations are very similar in different measurements. In light of these evidences, we think that the variable results obtained in the case of DOPC/bSM/chol vesicles are mainly due to the presence of different phases in the bilayer because of the specific lipid mixture and to the possible different distribution of the bilayer between the two phases.

When measuring the relative area variation ($\Delta A/A_0$), we kept the lipid bilayer tension at the smallest possible value that allowed stability of the liposome. This is useful to reduce the possible role of mechanical property variations, as a consequence of Allo uptake, in the uptake kinetics as deduced from the projection length variation [41,42]. This effect might be a consequence of mechanically induced different deformations at constant applied tension. To further clarify the effect of Allo uptake on the lipid bilayer [43], we measured the mechanical properties of the bilayers in equilibrium with defined Allo concentrations. However, these measurements could be affected by a dependence of the Allo uptake on the vesicle lateral tension. To investigate this aspect, we performed uptake kinetic experiments in which, after stabilization of the area variation as a consequence of Allo incorporation in the bilayer, we executed a jump in the tension applied to the bilayer. After the jump, the relative area variation obviously increases, but it took more than 200 s to reach a new equilibrium condition (see Supporting information). If a tension jump is applied in the absence of the neurosteroid, the new equilibrium value for the relative area variation is obtained in few seconds. This behavior suggests that the amount of Allo uptaken by the lipid bilayer depends on the tension applied to the bilayer, increasing with it. Accordingly, the comparisons between different kinetic experiments have been performed in the condition of similar applied tensions to the bilayer (~1 mN/m). At this point, it could be interesting to investigate the molecular details of the interaction between the neurosteroids and the lipids present in the bilayer. It would be probably helpful to perform molecular dynamics (MD) simulations in order to have a better picture of these interactions. Otherwise, it would be useful to exploit fluorescently labeled neurosteroids to follow their diffusion inside the bilayer. For example, it would allow establishment if the partition coefficient for Allo is the same for both the liquid phases present in these lipid bilayers.

It is well known that, to a greater or a lesser extent, the interaction of exogenous molecules with the lipid bilayer can affect its mechanical properties [41,42,44,45]. The MAT provides access to the measurement of both the bending constant and the stretching constant of a lipid bilayer [28,46–48]. A typical plot of the tension as a function of relative area deformation for a DOPC/bSM/chol 1:1:1 vesicle is reported in Fig. 5 together with the variation of the mechanical properties of the same vesicle in the presence of different Allo concentrations. In this case, the initial K_{App} is on the order of 490 mN/m. The effect of bending fluctuations on the relative area fluctuations is typically negligible in lipid mixtures including cholesterol [49,50]. Usually, when the contribution of thermal undulations is important, they remain present also in the high tension region which is used to measure the apparent stretching constant. To this aim, a procedure to remove the contribution from undulation suppression at high applied tension has been developed [51]. After exposing the same GUV to a 100 nM Allo solution, we again measured its mechanical properties. The plot in Fig. 5a shows that the mechanical properties of the bilayer have been strongly modified by Allo uptake. The value of K_{App} is now equal to 220 mN/m but the presence of a contribution from the bending stiffness of the bilayer for low applied tensions (bending constant $K_b = 0.6 \times 10^{-19}$ J for 100 and 200 nM Allo) is now evident. This evidence prompted us to calculate the effective stretching constant in the presence of Allo (see Supporting information for details). The obtained value is 340 mN/m, which is still lower than the value in the absence of Allo, meaning that the system becomes more deformable due to the presence of the neurosteroid. We then doubled the Allo concentration (200 nM) and we measured the mechanical parameters also in this case. Fig. 5a shows that in the 200 nM case the bending constant and the stretching constant are not significantly altered with respect to the 100 nM case. Measurements on other vesicles (see Supporting

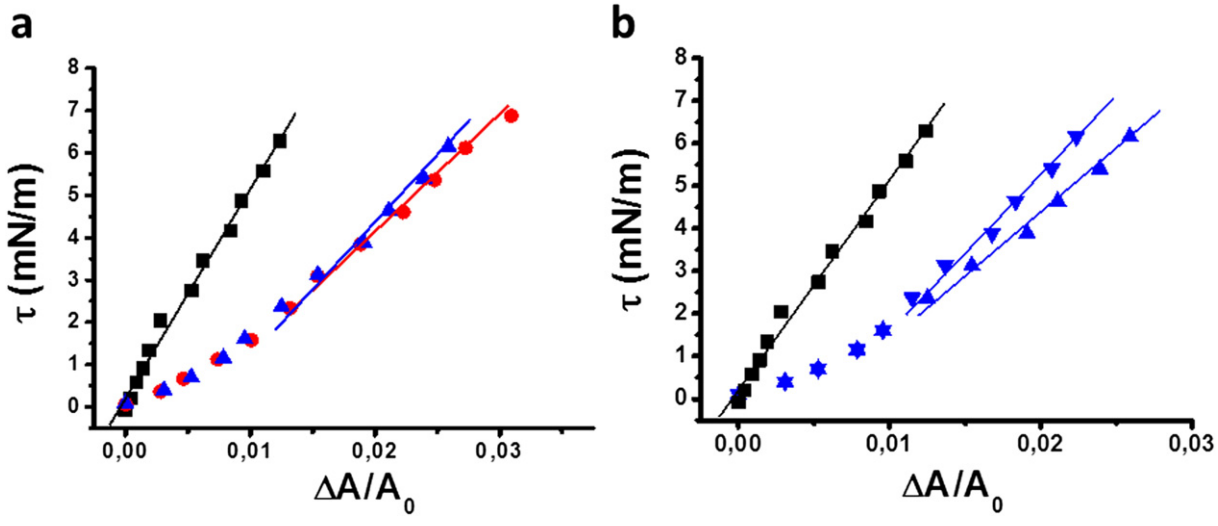


Fig. 5. Effect of Allo on the mechanical properties of GUVs. a) The black squares report the behavior of a DOPC/bSM/chol 1:1:1 vesicle as the applied tension is increased. The black line is the linear fit to obtain the apparent stretching constant. The red circles correspond to the mechanical characterization in a 100 nM Allo solution whereas the blue triangles to the case of 200 nM Allo solution. b) The black squares represent the same data as in a). The stars are related to the portion of the plot used to measure the bending constant of the bilayer (low tension region) and are the same points as in a) for the 200 nM Allo concentration case. The up-pointing triangles represent the same data as in a) for the high tension region. The down-pointing blue triangles represent the values of the high tension region after subtraction of the contributions coming from the bilayer bending modulus as specified in Supporting information. The apparent stretching constant of 490 mN/m changes to a value of 340 mN/m.

information) confirmed that the effect of Allo on the mechanical properties saturates at an Allo concentration of about 100/150 nM. Considering all the experiments that we performed (n = 5), the stretching constant for 1:1:1 DOPC/bSM/chol GUVs changes from 390 ± 100 mN/m to a value of 250 ± 100 mN/m when exposed to 200 nM Allo.

We previously found that the amount of neurosteroids incorporated in the bilayer depends on the tension applied to the lipid bilayer (see Supporting information). In principle, this behavior together with the effect of Allo on the mechanical parameters, could lead to non-linear trends due to the fact that by increasing the tension there could be also an increase of the Allo concentration inside the bilayer affecting consequently the mechanical properties. However, it is possible that at concentrations higher than 100 nM we are close to the maximal effect on the mechanical properties. The data from Fig. 5 allow us also to get an idea of the possible effect of mechanical parameters of the vesicles in the experiments dealing with the uptake kinetics. Indeed, considering a constant tension between 0.5 mN/m and 1 mN/m, we can establish that Allo uptake for concentrations up to 200 nM could contribute less than 1% to the overall relative area variation.

Hitherto, the obtained results show that the lipid bilayer mechanical properties are directly affected by the presence of Allo. This effect seems to reach saturation for nanomolar concentrations of the neurosteroid in the bathing solution. It is also interesting to note that changes in the properties of the lipid bilayer could affect the distribution of membrane proteins and promote different protein conformations. For instance, it has already been reported that the $GABA_A$ receptor activity depends on the physical properties of the hosting bilayer [52]. In fact, Søgaard et al. demonstrated that docosahexaenoic acid (DHA) increased the affinity between the receptor and the agonist (muscimol) and affected also receptor desensitization kinetics. In the same set of experiments the authors showed that DHA decreased the bilayer stiffness as measured by the gramicidin channel activity and concluded that the $GABA_A$ receptor can be affected by a change in the elasticity of the lipid bilayer. Accordingly, the effect that we observed on the bilayer mechanical properties produced by Allo should not be neglected when analyzing its effect on the $GABA_A$ receptor activity. In particular, it is possible that neurosteroids accumulated inside the bilayer progressively affect the mechanical properties of the membrane and consequently the activity of the receptor protein.

To better understand the variation of the mechanical properties, we studied the effect of Allo at the same concentrations on a pure DOPC GUV. In this case we found that K_{App} was practically unaffected by the uptake of Allo (see Supporting information). The mechanical property variations which are reported in the literature when exogenous molecules interact with lipid bilayers similar to DOPC are typically obtained for much higher concentrations than the ones used in our work [44]. Considering the comparison between DOPC and DOPC/bSM/chol GUVs, we can speculate that the variation in the stretching constant for the latter type of GUVs could be due to the variation of the relative proportion of L_d and L_o domains, as suggested by our AFM investigation [30]. Indeed, it has been demonstrated that the L_o phase has different mechanical properties compared to the L_d phase [53]. If the relative proportion of the two phases changes, the overall mechanical properties of the vesicles should also vary. In this sense, the above cited AFM investigation on this model system established that Allo favors a relative increase of the L_d phase [30]. Other possible explanations for the effect of Allo on the mechanical properties of DOPC/bSM/chol GUVs could be related to the effect of the molecule on the order inside the lipid bilayer. It is also important to consider that, even in the case of DOPC/bSM/chol 1:1:1 GUVs, in some rare cases, the addition of Allo did not change the lipid bilayer mechanical properties (see Supporting information). This behavior could be rationalized by considering cases in which the vesicle is in the homogeneous phase and the small quantity of Allo is not able to significantly affect the phase state and, consequently, the mechanical parameters.

Again, further interpretations for the decreased mechanical properties as Allo interacts with the bilayer, could be provided by MD simulations that, for example, unravel important details such as the specific interactions of Allo or in general of exogenous molecules with the lipids [54].

Very subtle changes in the structure of a biologically active molecule can give rise to a very different pharmacological activity. Even in the case of neurosteroids, small structural differences in the molecules, such as those brought about by enantiomers or in general by isoforms, produce different modulatory effects on the $GABA_A$ receptor. For molecules which are not enantiomers and which have different functional effects on membrane receptors it can be expected that they act differently also on pure lipid bilayers. For example, it has been reported that isoAllo, a 3β epimer of Allo (see Scheme 1), acts as a functional antagonist of Allo [25] and previous studies reported a different effect on the lipid bilayer, even if the experiments were performed at much higher concentrations than those used in this study [26,27].

Here we investigated if the two isoforms, Allo and isoAllo, at nanomolar concentrations, induce different effects on pure lipid bilayers composed by DOPC/bSM/chol 1:1:1. Interestingly, as shown in Fig. 6a, we found that liposomes in the presence of 100 nM isoAllo undergo a lipid area reduction. The decay time constant for the lipid area has a feature of about 120 ± 40 s, suggesting a slower kinetics with respect to the case of Allo uptake. Moreover, the absolute value of the relative area variation is smaller in the case of isoAllo. The area decrease could be interpreted as a condensation of the lipid bilayer induced by the presence of isoAllo. Remarkably, the observed phenomenon is consistent with what has been found by our AFM investigation in which isoAllo has been noticed to decrease the overall area of lipid bilayer patches and to induce an increase of the liquid ordered fraction of the bilayer [30]. Moreover, the stretching constant of the DOPC/bSM/chol vesicle exposed to an isoAllo solution of varying concentration does not change significantly (Fig. 6b) up to 400 nM. A possible interpretation for the lack of concentration dependence of the mechanical properties in the case of isoAllo could be related to a smaller partition coefficient with respect to Allo or to the fact that the area increase by isoAllo uptake could be compensated by an increase of the order in the bilayer. Even in this case, MD simulations could provide useful insight into this behavior.

Summarizing the results of the present investigation on the effects of Allo and its isoform isoAllo on pure lipid bilayers composed by DOPC/bSM/chol (1:1:1), we found opposite results in the two cases. Exposing lipid bilayers to Allo typically induces an increase in their area and a decrease in mechanical properties. This behavior could be due to incorporation of the exogenous molecule inside the lipid bilayer and, for a lipid bilayer in the phase coexistence condition including L_o and L_d regions, to an increase of the fraction of the L_d phase. Conversely, the exposure of pure lipid bilayers to isoAllo produces a small decrease of the lipid bilayer area while the mechanical properties are practically unchanged. This behavior is likely due to a condensation of the bilayer induced by the exogenous molecules. Allo and isoAllo have structures that are very similar to that of cholesterol and it is possible that their position inside the bilayer competes with that of cholesterol. As a consequence, their insertion in the bilayer could alter the effect produced by cholesterol on the phase state of the bilayer. Indeed, the phase state is extremely sensitive to the cholesterol concentration due to its specific interactions with other lipid components such as sphingomyelin [55] and particularly in the case of the chosen lipid mixture, which should be near to a critical point. A variation of the phase state of the membrane could affect the distribution of receptor proteins in the membrane if they preferentially partition within a specific phase. In the specific case of the $GABA_A$ receptor it has been found that it preferentially partitions in lipid rafts [56]. Moreover, it has also been hypothesized that cholesterol might have a specific binding site on the $GABA_A$ receptor modulating its activity [57]. Within the lipid raft hypothesis, it has been proposed that the nanoscale and dynamic features of the domains interpreted as lipid rafts could be due to fluctuations of the lipid bilayer above but in the proximity of a critical point [14]. Accordingly, any chemical components

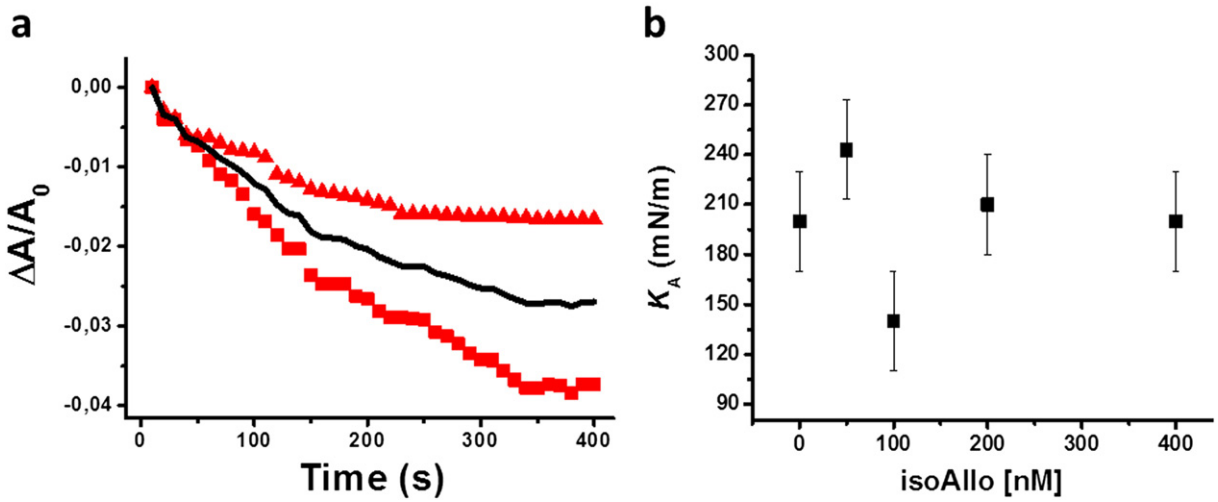


Fig. 6. a) The scattered points represent two traces for the relative area variation of a DOPC/bSM/chol 1:1:1 liposome exposed to a 100 nM isoAllo solution. The continuous line represents the averaged behavior. b) Stretching constant values for a DOPC/bSM/chol 1:1:1 vesicle as a function of the isoAllo concentration to which it is exposed.

in the lipid bilayer able to shift the phase state of the membrane will also affect the lipid raft dynamics as a consequence of the variation of its distance from the critical point. Ligand-gated ion channels which are associated with lipid rafts will be affected in their gating behavior as a consequence of alteration of the lipid raft lateral scale dimensions and characteristic lifetimes.

Single channel analysis of the $GABA_A$ receptor activity showed that the typical dwell times for opening and closure events of the single channel are affected by neurosteroids [58]. In particular, an increased activity of $GABA_A$ receptors after neurosteroid application can be explained by an increase of the mean open time of the channel and their opening frequency [21]. However, even if it is well accepted that the neurosteroid binding site for its "modulatory action" is located at the interface between the receptor and the membrane [59], the "agonist" binding site has not been identified yet. Some aspects of neurosteroid behavior are well described by a single binding site whereas other aspects require more than one binding site [60]. Our group recently demonstrated that the dwell times of a potassium channel (KcsA) can be modulated by a change in the phase of the lipid bilayer hosting the channel [4]. A similar effect, characterized by low specificity, might be at play also in the case of the activity of neurosteroids, besides specifically recognizing events between the neurosteroid and the receptor. From this perspective, the variation of the mechanical properties that we measure when a lipid bilayer is exposed to Allo could have an influence on the behavior of membrane proteins by changing rate constants for their conformational transitions. The possible effect of anesthetics and also neurosteroids on the lipid bilayer and, as a secondary effect, on membrane proteins, is one aspect of a long lasting debate in which the other aspect is related to a more specific effect of the drugs on the involved membrane proteins. When analyzing the kinetics of the mechanism of action of a drug that strongly partitions in a lipid bilayer and affects lipid bilayer mechanical properties, it is important to consider these aspects and also the diffusion inside the bilayer necessary to reach the site of interaction with the receptor protein [61]. The usual justification for excluding these contributions comes from the fact that in the specific case of enantiomers of a drug they have different effects on membrane proteins, whereas their effects on the bilayer are supposed to be similar, like it should be if colligative properties are considered [18]. Nevertheless, especially in the presence of cholesterol in the membrane, the lipid bilayer cannot be considered an achiral environment, and recent reports have shown that the effects of chiral molecules on the bilayer are different for the enantiomers [62]. In the case of isomers like Allo and isoAllo, the different effect on the membrane is clearly evident and understandable and it is particularly interesting when related to their different effect on the $GABA_A$ membrane receptor. Also in the case of enantiomers, it cannot be excluded that some properties of the lipid bilayers which are not experimentally measurable at the moment, such as the lateral pressure, could be affected differently by them or have different effects on membrane proteins [63].

4. Conclusions

Our studies evidenced that two neurosteroids, well known to have different interactions with the $GABA_A$ receptor protein, interact also differently with the pure lipid bilayer. We think that the influence of neurosteroids on the lipid bilayer should not be neglected, even if a specific interaction site has been identified on the receptor. In fact, their insertion in the lipid bilayer can profoundly affect membrane protein activity as a secondary effect resulting from a variation in the phase of the lipid bilayer, a change in its mechanical properties or an alteration of the lateral pressure profile inside the bilayer. In all cases, the lipid bilayer could change its properties helping to stabilize specific configurations for the channel, as, for example, in the case of the effect of polyunsaturated fatty acids and lysolipids stabilizing subconductances in the MscL channel [64,65]. Taking into consideration these effects could help reconciling different unresolved aspects of neurosteroid effects.

Conflict of interest

All the authors declare that they don't have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.01.017.

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