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Effect of neurosteroids on a model lipid bilayer including cholesterol: An Atomic Force Microscopy study

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ABSTRACT

Amphiphilic molecules which have a biological effect on specific membrane proteins, could also affect lipid bilayer properties possibly resulting in a modulation of the overall membrane behavior. In light of this consideration, it is important to study the possible effects of amphiphilic molecule of pharmacological interest on model systems which recapitulate some of the main properties of the biological plasma membranes. In this work we studied the effect of a neurosteroid, Allopregnanolone (3α,5α-tetrahydroprogesterone or Allo), on a model bilayer composed by the ternary lipid mixture DOPC/bSM/chol. We chose ternary mixtures which present, at room temperature, a phase coexistence of liquid ordered (L_o) and liquid disordered (L_d) domains and which reside near to a critical point. We found that Allo, which is able to strongly partition in the lipid bilayer, induces a marked increase in the bilayer area and modifies the relative proportion of the two phases favoring the L_d phase. We also found that the neurosteroid shifts the miscibility temperature to higher values in a way similarly to what happens when the cholesterol concentration is decreased. Interestingly, an isoform of Allo, isoAllopregnanolone (3β,5α-tetrahydroprogesterone or isoAllo), known to inhibit the effects of Allo on $GABA_A$ receptors, has an opposite effect on the bilayer properties.

1. Introduction

Many commercially available drugs are directed to membrane proteins. Membrane proteins are strongly coupled to the lipid component of the biological membrane both by specific chemical interactions [1] and by longer range, aspecific physical interactions [2]. The most commonly accepted mechanism of drug action relies on specific and saturable interactions between the exogenous molecules and membrane proteins. This mechanism has been demonstrated in many cases and the binding sites for many ligands and their receptors have been clearly identified [3,4]. A well accepted evidence for the specificity of the interaction between drugs and proteins lies in the different pharmacological properties of the enantiomeric forms of a drug [5]. These differences are typically related to the chiral structure of proteins and to the specific docking of the drugs to the proteins. Nevertheless, the effect of a drug on a membrane protein cannot be studied independently of the presence of the lipid bilayer whereas it is possible to study the effect of drugs on pure lipid systems. The kinetics of the drug/membrane protein interaction is typically described by schemes in which conformational changes of proteins with characteristic rate constants are involved. In many cases, several conformational states are introduced to reproduce theoretically the observed kinetic behavior, even if the introduced states are often difficult to identify experimentally. However, conformational states of membrane proteins can be affected also by the changing properties of the hosting lipid bilayer. Properties of a lipid bilayer could vary both as a consequence of changes in environmental parameters such as pH and temperature, but also, and probably in a more effective way, by modifications of the lipid bilayer components. Drugs, besides interacting specifically with membrane proteins, can also adsorb to the lipid bilayer affecting its properties hence favoring, from an energetic point of view, a specific protein conformation. Drugs/lipid bilayer interaction is considered as an adsorption process when the drug molecules remain confined at the lipid bilayer/water interface, whereas can be considered as an uptake process when the molecules are able to get inside the bilayer. Anyway, the rate constants for conformational changes could depend on the amount of drug interacting with the lipid bilayer. Many investigations demonstrated that lipophilic drugs affect the physical properties of lipid bilayers favoring for example the open or closed conformation of ion channels [6,7]. For example, it has been shown that the efficacy of amphiphilic molecules in activating mechanosensitive channels depends on their lipid solubility and has typically slow kinetics [8].

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Besides these aspects, the up to date view of the biological membrane behavior involves lateral heterogeneity in the organization of lipids and proteins. Specific domains with a lateral extension in the order of tens of nanometers could be present in the membrane [9]. Many proteins could preferentially partition in these domains and the partitioning could affect their interactions and their activation or deactivation of signaling pathways [10,11]. The membrane organization in nanometer-sized lipid aggregates could be related to its thermodynamic conditions [12] considering also the non-equilibrium situation of a biological cell membrane. The interaction of drugs with the membrane could in turn affect its thermodynamics and alter the bilayer organization [13]. These changes could have strong effects on the activity of membrane proteins which preferentially associate with specific domains. Accordingly, it would be very important to study how the insertion of exogenous molecules in a lipid bilayer affects its thermodynamics.

Dealing with the possible role of drugs in affecting the functional activities of biological membranes by an aspecific mechanism, one of the most studied effects is the interplay between anesthetics and lipid bilayers [14–18]. The longstanding debate between the specific effects of anesthetics on membrane proteins and their indirect action, mediated by changes they may produce in the lipid bilayer properties, has its roots in the Meyer–Overton rule. This rule states that the activity of anesthetics is strongly related to their partition coefficient in lipid bilayers. Among the mechanisms related to aspecific interactions, the lateral pressure profile change across the lipid bilayer has been proposed as a way to affect efficiently the equilibrium distribution of the membrane proteins in their different conformational states and the rate constants for their transitions [19]. Moreover, recent studies on model bilayers showed that the interaction of anesthetics with lipid bilayers affect their thermodynamic state by changing the relative distribution between different phases [20–22] or by changing the temperature of the bilayer at which a separation in two liquid phases occurs [13]. Another interesting class of molecules whose interactions with lipid bilayers could be relevant for their activity is exemplified by neurosteroids [NSs] [23] which are endogenous molecules able to modulate the activity of ion channels and relevant in the propagation of electrical signals in the nervous system [24]. Their action is typically explained by an allosteric interaction with a membrane protein, i.e., the $GABA_A$ receptor Cl channel, that, affecting the receptor conformational states, produces variation in the channel open time that ultimately results in a changed activity [25,26]. Their activity is usually detected at very low concentrations (in the nM range) and this aspect initially suggested a highly specific interaction with the corresponding membrane proteins [27]. Evidences for a specific NS binding site on $GABA_A$ receptor were provided by several groups. Using site-directed mutagenesis, single residues in the receptor protein that influence NS regulation of GABA receptor have been identified [28–30]. However, NSs are strongly lipophilic and, according to their structure, in some cases they have a partition coefficient which can produce, from a nM concentration in aqueous solution, a μM concentration inside the lipid bilayer [31]. In this case, a highly specific docking mechanism would not be required to explain their effect at a very low solution concentration. Moreover, it has been found that their docking site to the membrane protein could be located in their intramembraneous portion [27]. Accordingly, the effects of NSs could be also related to their partition and diffusion inside the lipid bilayer. It is also well known that, at high concentrations, NSs which modulate the activity of $GABA_A$ receptors can, independently from the GABA presence, activate the receptors [32]. This direct gating effect of the NS has typically slow kinetics which has been connected to its accumulation in the lipid bilayer [33]. The gating kinetics could be attributed both to the required increase of NS concentration inside the bilayer to bind the channels and to the increasing modification of the lipid bilayer properties as the NS concentration inside the bilayer increases.

In this work we studied the effect of two NSs, Allo and one of its isoforms, isoAllo, on a ternary lipid model-membrane containing a natural sphingomyelin extract. We concentrated on the DOPC/bSM/chol mixture with different lipid proportions. bSM is a mixture per se, even if it is mainly composed (~50%) by a 18:0 fatty acid chain. Accordingly, the mixture will be considered as a "pseudo-ternary lipid mixture". This lipid combination is considered a very representative case for the studies of phase behavior of ternary lipid bilayers [34]. One of its main characteristics is related to the fact that the typically used 1:1:1 molar mixture is, at room temperature, very near to a critical point. In fact, the presence of critical points in the thermodynamics of biological membranes is nowadays considered one of the possible explanations for the existence of small and dynamic domains usually called "lipid rafts" [12]. Here, to study the effects of Allo and of its isoform isoAllo we exploited Atomic Force Microscopy (AFM) after forming Supported Lipid Bilayers (SLBs) of the specific lipid composition. While Allo potentiates $GABA_A$ receptor activity in the presence of GABA and, at high concentrations, directly gates the $GABA_A$ receptor channel [25,35], isoAllo is devoid of modulatory activity but acts as a non-competitive antagonist of Allo [36]. In particular, isoAllo decreases the effect of potentiation by Allo and the effect is particularly evident at high GABA concentrations. Herein, we focussed on the modification of the thermodynamic state of the bilayer as the concentration of the NSs in the imaging chamber increased, measuring the relative proportions of different phases which might be present in the bilayer. We also used a temperature-controlled stage to investigate the behavior of the lipid bilayer as a function of temperature.

2. Materials and methods

2.1. Lipid bilayer formation and neurosteroid injection

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), Sphingomyelin (Brain, Porcine) (bSM) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, USA) and used without further purification. Allopregnanolone was purchased from Sigma-Aldrich and isoallopregnanolone was a kind gift from Dr A. Guidotti (refer to [25] for details on this molecule). Specific lipid mixtures were prepared by mixing chloroform lipid solutions in the desired molar amount. Chloroform was then evaporated under a flow of nitrogen while being heated in a water bath at 50 °C. Thereafter, the sample was kept under vacuum (10^{-2} mbar) for at least 2 hours in order to remove the remaining chloroform. Then lipids were rehydrated in a buffer solution (150 mM KCl, 8 mM Hepes, pH 7) to obtain a lipid concentration of 0.12/0.06 mg/ml. The sample was sonicated at room temperature for 15 min resulting in a homogenous lipid suspension.

SLBs were prepared by the vesicle fusion technique [37–39]. Briefly, immediately after sonication of the lipid suspension, 70–100 μl of the suspension was deposited onto a freshly cleaved mica sheet (SPI Supplies/Structure Probe, Inc., USA) fixed on a PTFE disc attached to a metal disc. The lipid suspension was incubated for 15 min at a temperature above 40 °C and then the sample subjected to extensive rinsing with the imaging buffer. The sample was then slowly cooled to 25°C.

Small amounts of the NSs (10^{-6} M concentration in the same buffer used for AFM imaging, diluted from a 10^{-2} M DMSO solution) were injected in the imaging chamber in order to reach the desired final concentration. After each injection, we waited for about 10–15 min in order to reach an equilibrium condition for the bilayer before acquiring images. It is important to stress that control experiments on lipid bilayer patches prepared in the same conditions but not exposed to NSs conserved good time stability in their properties. All the reported variations after the insertion of the exogenous molecules are therefore ascribable in large part to their effect on the bilayers.

2.2. Atomic Force Microscopy

Atomic Force Microscopy imaging was performed with a Bioscope I microscope equipped with a Nanoscope IIIA controller (Veeco

Metrology, USA). We used a temperature-controlled stage based on a circulating water bath on which we could mount the Bioscope head. The sample temperature was continuously monitored by a digital thermometer Fluke 16 (Fluke, Italy) equipped with a small K-thermocouple probe (Thermocoax GmbH, Germany) in direct contact with the imaging buffer. The stability of the temperature is assured within ±0.3 °C of the specified temperature value. Triangular silicon nitride cantilevers (Bruker DNP-S) with nominal spring constant of 0.32 N/m were used for tapping-mode imaging whereas cantilevers with a nominal spring constant of 0.06 N/m and 0.24 N/m were used for Atomic Force Spectroscopy (AFS). The elastic constants used to convert deflections into forces were determined by the thermal noise method [40]. All the images presented in this work have been obtained in tapping-mode AFM. However, to confirm that the information we retrieved from this technique regarding the height difference among different domains in the bilayer were not affected by imaging artifacts, we conducted comparative experiments by AFS identifying the contact point of the AFM tip with the bilayer and the shared reference given by the position of the underlying mica substrate. This analysis is reported in Supporting information and confirmed the good accuracy of the values obtained by tapping-mode imaging. Images have been analyzed using the WSXM [41] and the ImageJ NIH softwares. The height difference between the domains was obtained from the distance between the maxima corresponding to the distribution of the pixel heights in each image as specified in the Supporting information.

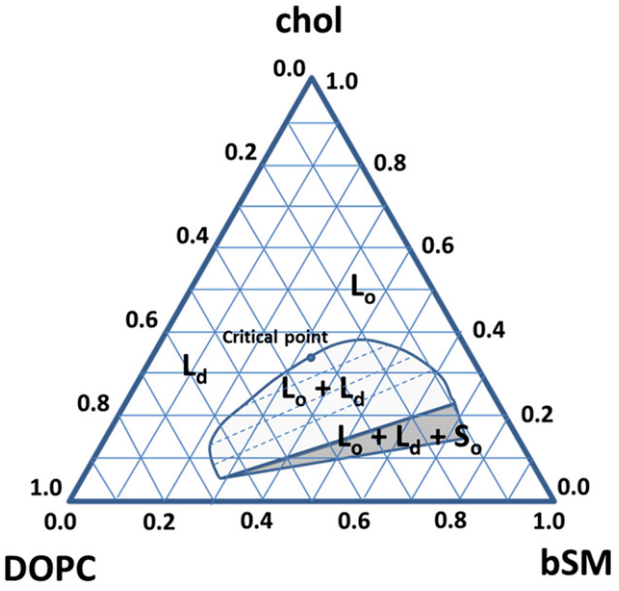

Fig. 1. Phase diagram for the ternary lipid mixture DOPC/bSM/chol based on Ref. [48]. Two regions of phase coexistence are highlighted: the Liquid Disordered/Liquid Ordered phase coexistence region ($L_d + L_o$) and the triangular three phase coexistence region in which also a Solid Ordered phase is present ($L_d + L_o + S_o$). The phase diagram represents a slice at about 25 °C of the complete phase diagram. The straight dashed lines inside the liquid–liquid phase coexistence regions represent schematically the tie-lines.

3. Results and discussion

Lipid bilayers containing a low T_m lipid, a high T_m lipid, and cholesterol show coexisting phases which correspond to different distributions and organizations of the lipids [42]. In particular, the interaction of cholesterol with specific lipids, such as sphingomyelin, can produce the appearance of domains in which the lipid acyl chains are tightly packed while preserving the high lateral mobility that is typical of a liquid phase [43]. This behavior gives rise to liquid–liquid phase separation whose occurrence depends strongly on the type of lipids and on their relative amount as exemplified by phase diagrams of ternary lipid mixtures. The two liquid phases are known as Liquid-disordered (L_d) and Liquid-ordered (L_o) phase. The L_d phase is similar to the well-known phase obtained at high temperature also in the case of lipid bilayers composed by only one type of lipids and is characterized by a low order in the configuration of the acyl chains and by a high lateral mobility of the lipids. The L_o phase is characterized by a higher degree of order coming from the condensing effect of cholesterol with a preserved lateral mobility and by an enrichment of the higher melting temperature lipid component [44,45]. Typically, the phase diagrams for these ternary lipid mixtures are represented in terms of the Gibbs triangle resulting from slices at a defined temperature of the overall isobaric mixture behavior. Their determination relies on the use of microscopy techniques such as Fluorescence Microscopy [45] or AFM [46], or on spectroscopic techniques such as NMR or FRET [47,48]. It is important to stress that, in the context of the lipid raft hypothesis, the raft domains somehow recapitulate the behavior of liquid-ordered domains in lipid bilayer model systems.

A scheme of the phase diagram for the DOPC/bSM/chol mixture, at a temperature of 25 °C, is reported in Fig. 1. The scheme is taken from a work by Petruzielo et al. [48]. It is important to stress that in ternary phase diagrams, as in general for lipid bilayers, phase separation does depend also on lateral pressure [49]. This is particularly relevant for SLBs whose formation could lead to lateral pressure variation [50] at variance with what happens for unsupported lipid bilayers. For example, it has been reported [51] that for lipid bilayer islands on solid supports the value of break-through force could be different if the central region of the patches or the regions along the perimeter are considered. This behavior points to the possibility of gradients in the lateral pressure in supported lipid bilayers. In fact the break-through force value can be considered an indication of the lateral packing in lipid bilayers, being higher for more densely packed membranes. For big membrane patches on a solid support the overall area is relatively more important than the perimeter regions and, on average, lateral pressure will be higher than in small patches. In this case, small patch phase behavior resembles closely that of vesicles in solution. Accordingly, in this work we mainly concentrated on small membrane patches. Moreover, using the Micropipette Aspiration technique it has been recently shown that the application of an increasing lateral pressure to Giant Unilamellar Vesicles (GUVs) affects the miscibility transition temperature of the bilayer [52,53]. Accordingly, lipid bilayer membranes of the same lipid composition and temperature but subjected to different lateral pressure, could exhibit a different phase organization.

The Gibbs triangle in Fig. 1 highlights the presence of two regions of phase separation. The first one is the $L_d + L_o$ phase coexistence region in which liquid domains belonging to two different phases are present. Due to the different structures of the bilayer in the two phases, with an enrichment of sphingomyelin and cholesterol in the liquid ordered one, the different phases will provide a different thickness to the bilayer. Thickness difference is the observable that allows AFM to infer the presence of different phases. However, the clear identification of the two phases would require an analysis of their lateral diffusion coefficient, which is not possible by AFM, and the assessment of the equilibrium condition of the bilayer. As to the first aspect, experimental investigations coupling different techniques allowed to establish the reliability of what is generally observed by AFM in terms of L_o and L_d phases [54,55]. The second region of phase separation is characterized by the simultaneous presence of the L_o, the L_d and the well-known solid ordered phases (S_o). This last region is generally difficult to identify in ternary lipid mixtures, both by fluorescence microscopy and AFM. In the latter case the difficulties arise from the small height difference of S_o with respect to the liquid ordered regions.

For the lipid composition 1:1:1, the bilayer, at 25 °C, should be in the liquid–liquid coexistence region. Fig. 2a reports a typical AFM image of the SLBs obtained for this composition. It is evident that some regions of the bilayer are in the phase coexistence state, with domains having a height difference of about 1 nm, whereas other large bilayer regions show a homogeneous structure. One possible explanation for this

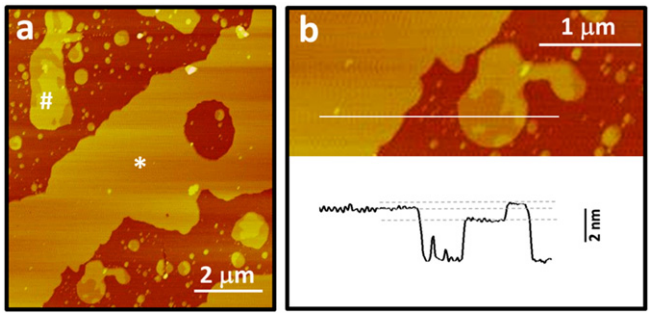

Fig. 2. AFM images of a DOPC/bSM/chol 1:1:1 supported lipid bilayer at 25 °C. a) The image shows homogeneous lipid bilayer patches (*) and smaller lipid bilayers in a phase coexistence state (#). b) Height difference between the homogeneous bilayer and the domains in a patch with phase coexistence. The height of the homogeneous bilayer is intermediate between the two domains in the small patch. The line section refers to the white line on the image.

behavior could be the different lipid composition of the bilayer patches resulting from intrinsic inhomogeneity in composition of the vesicles which gave rise to them [56]. However, we always found that the homogeneous phase is related to the larger patches on the substrate, whereas smaller patches are always found in the phase coexistence state. Fig. 2b shows the interesting analysis of the domain heights. The homogeneous bilayer has a height which is intermediate between those of the L_d and L_o domains in the small adjacent patch. This is exactly the behavior which is expected for a transition between a phase separated region and a nearby homogeneous phase. We suggest that the homogeneous patches are related to an increased lateral pressure that shifts the bilayer from a phase coexistence region to a homogenous region favoring the more condensed phase. Obviously, the different mechanical properties of the different phases could contribute to the height difference measured by AFM while scanning the sample at constant force. In the Supporting information we report a force spectroscopy technique analysis of the deformations induced by the tip while pressing on the different phases. A variation in the applied force by ~2 nN results in a variation in height difference of about 0.3/0.4 nm. As a consequence, the different applied forces cannot affect too much the measured height difference.

After bilayer formation, we perfuse the imaging chamber with increasing Allo concentrations. We typically use concentration steps in the order of 50–100 nM. Fig. 3 shows what happens to the bilayer after Allo perfusion. The immediate effect is represented by a strong increase of the lateral area occupied by the lipid bilayer. When we focus the attention on small patches such as those shown in the lower panel (sequence d, e, f) of Fig. 3, we find that the area increase is typically 15–20%. This behavior is consistent with what was found in the case of GUVs of the same lipid composition exposed to a solution containing Allo and studied by the Micropipette Aspiration Technique in which Allo uptake induces a global area increase of the bilayer [57]. Even if the two model systems, GUVs and SLBs, have different characteristics, in some cases they can be prepared so to provide similar results dealing with their phase organization, as shown in a recent report [58]. Moreover it has been reported that a SLB undergoes the same relative area variation of an unsupported lipid bilayer when it crosses its main phase transition temperature [59]. Accordingly, a similar behavior between a SLB and a GUV provides support to the interpretation of changes observed to a SLB.

Besides area increase, Allo induces also a reorganization of the lipid domains. It is important to point out that, in the absence of Allo, under similar imaging conditions, the domains are rather immobile. The reorganization is characterized by a relative size increase of the regions in the L_d phase. It is to be stressed that the lipid mixture at issue is very near to a critical point for this temperature and, accordingly, the relative distribution between the different phases is near 50% for each phase. An average estimation for the change in the relative presence of the domains shows that the L_d phase relative increase is (10 ± 4)% after reaching 300 nM Allo concentration. Table 1 summarizes the results showing the relative proportions of the different phases and the total area variation (see Supporting information for further details). At the level of the area of single domains, the insertion of Allo produces a small size increase also of the Lo domains. This behavior could be the result of the fact that Allo produces a global change of the bilayer state, as observed also for the introduction of trace amounts of fluorescent lipids in ternary lipid mixtures [60]. Fig. 3g shows the analysis of the height difference between the domains when the NS is added to the lipid bilayer. The presence of Allo leads to an increase of the height difference between the domains. This behavior is consistent with an increased composition difference for the domains and would correspond to a shift in the phase diagram of Fig. 1 towards a region of decreased cholesterol amount on the basis of the reported positive slope of the tie-lines in the two-liquid phase coexistence region [48]. Moreover, the height difference variation results by a variation of both the L_o and the L_d phases with respect to the mica substrate. In fact, the L_o phase thickness

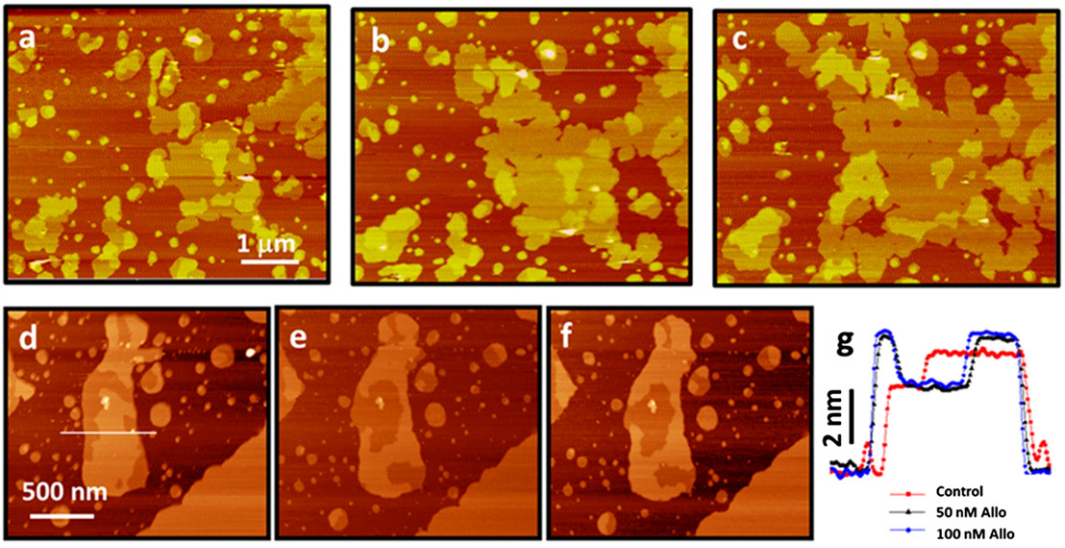

Fig. 3. Effect of Allo on the lipid bilayer. a) Lipid bilayer patches composed by the lipid mixture DOPC/bSM/chol 1:1:1 in the absence of Allo; b) The same sample area as in a) after the injection of 50 nM Allo; c) The same sample area after the injection of 100 nM Allo; d–f) Sequence of images at higher magnification representing the structure of a lipid bilayer patch presenting a phase coexistence state: d) control (no Allo); e) 50 nM Allo; f) 100 nM Allo. g) Cross sections corresponding to the image sequence d–f) (white line in d)).

Table 1
Effect of Allo and isoAllo on the relative proportion of L_o and L_d domains. The Table reports the average initial situation and the average situation at 300 nM Allo or isoAllo considering 12 lipid bilayer patches in each case. The last column represents the average variation of the total area of the membrane patches calculated on a set of 12 patches.

		Initial	300 nM	Average area variation
Allo	L_o	(43 ± 5)%	(33 ± 4)%	+(24 ± 5)%
	L_d	(57 ± 5)%	(67 ± 4)%	
iso-Allo	L_o	(50 ± 3)%	(52 ± 3)%	−(14 ± 3)%
	L_d	(50 ± 3)%	(48 ± 3)%	

increases after the introduction of Allo while the L_d phase slightly decreases. This behavior once again supports the idea that Allo induces a global transformation in the lipid bilayer. Considering these observations, the effect of Allo on this lipid bilayer is equivalent to decreasing the cholesterol content or reducing the interaction between cholesterol and bSM. In this context, it is interesting to note that the effect of Allo on the $GABA_A$ receptor has been found related to the amount of cholesterol in cells [61,62]. In particular, it has been demonstrated that the effect of steroidal GABA potentiators was reduced by increasing cholesterol amount in cells. At the same time the amount of cholesterol in cells is also able to modulate the GABA potency, with a reduction of the agonist potency after cholesterol depletion [63]. The results we obtained in our study suggest that NS could compete with cholesterol inside the bilayer and could alter its effect and its interaction with high melting temperature lipids.

As stated above, the hypothesis of the presence of a critical point in a biological membrane is nowadays considered very appealing because it could explain peculiar behaviors of these thermodynamic systems such as the existence of small and fluctuating domains [12]. From a biological point of view it is probable that a single critical point might represent a too specific situation to have functional relevance, but it must be stressed that multidimensional order parameters and several critical points might be present in these models [64]. Therefore we decided to

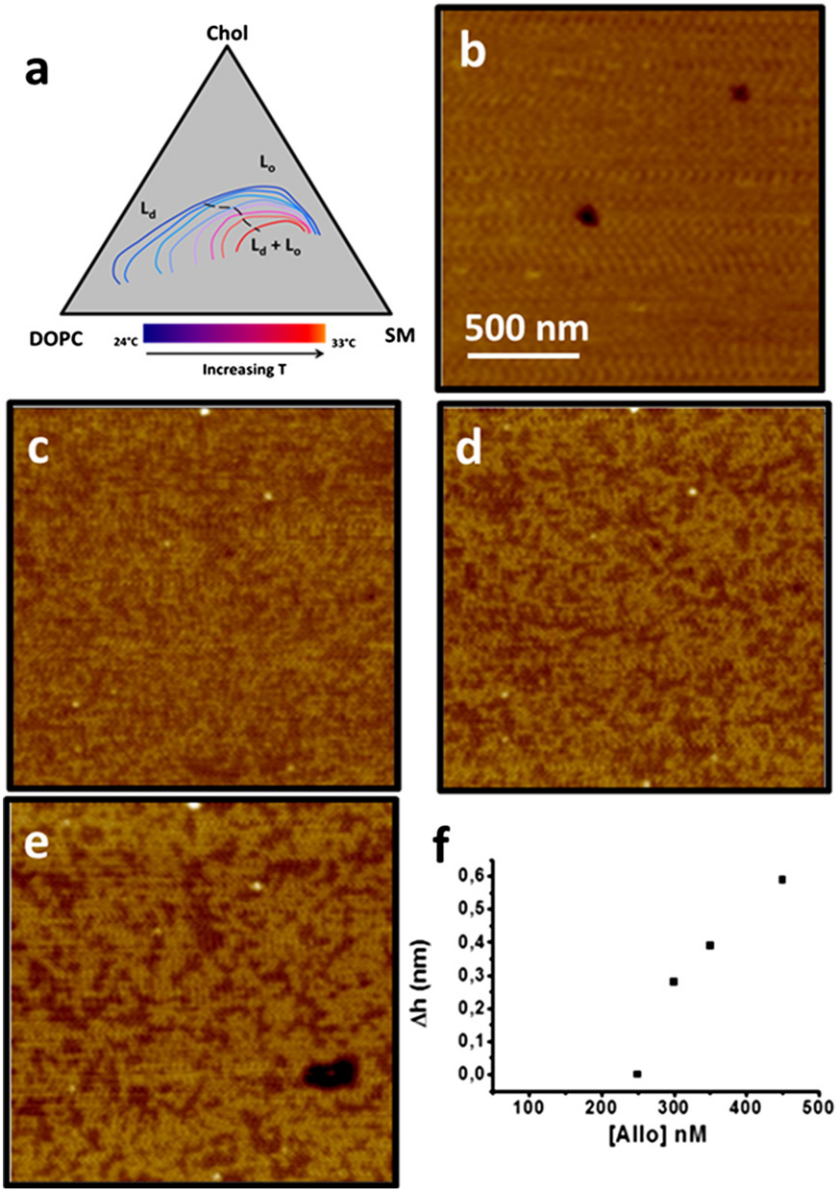

Fig. 4. Effect of Allo on the miscibility border of the DOPC/bSM/chol 1:1:1 bilayer. a) Schematic representation of the phase diagram as a function of temperature [based on Ref. 46]. The continuous lines represent the upper border of the miscibility region for different temperatures, whereas the dashed line shows the movements of the critical points as a result of temperature variation. As the temperature increases, the miscibility border shifts to lower regions of the Gibbs triangle. b) DOPC/bSM/chol 1:1:1 bilayer at 27 °C in a homogeneous phase. c–e) the same bilayer as in b) after the addition of c) 250 nM Allo, d) 350 nM Allo and e) 450 nM Allo. f) Height difference between the phases formed after Allo addition as a function of its concentration.

study the behavior of the lipid mixture at issue near to its critical point. The identification of critical points in similar lipid mixtures has been performed by fluorescence microscopy [42] as well as by AFM [46]. Here we increased the temperature of the imaging chamber until we completely removed the phase coexistence. We did not measure the parameters which are required to ascertain the real critical position of the lipid bilayer because we were mainly interested in the miscibility transition temperature. Increasing the temperature, the miscibility border in the pseudo-ternary phase diagram changes (see the scheme reported in Fig. 4a which has been reproduced as a sketch from ref [46]). A bilayer, initially in the phase coexistence region, is driven to a homogeneous phase by increasing temperature. Approaching the miscibility border near to a critical point involves the presence of domains with very similar composition and a very low line tension favoring the formation of small domains. Those domains are nevertheless visible by AFM. Outside the phase coexistence region and near to a critical point, compositional fluctuations should play a dominant role. Due to the limited time resolution of AFM, these fluctuations could result unattainable to the imaging capabilities of AFM or, at least, AFM images cannot be considered as valuable snapshots of the bilayer state. After getting a homogeneous bilayer, as shown in Fig. 4b, we added Allo to the lipid bilayer. The progressive addition of the NS leads to the reappearance of very small domains, as shown in Fig. 4c that corresponds to 250 nM Allo concentration. Upon further increase in the concentration of the exogenous molecule, the different domains in the bilayer progressively increase their height difference, according to an increasing composition difference between the domains (Fig. 4d–e). A plot of the height difference between the two different phases as a function of Allo concentration is reported in Fig. 4f. If, at a constant Allo concentration (in this case 450 nM), we decrease the temperature, we again observe an increase in the height difference (Fig. 5a–d). This behavior suggests that Allo addition is equivalent to what would happen by decreasing the temperature for the lipid bilayer (like in the *freezing point depression* case). It is interesting to note that the application of the NS to a lipid bilayer initially near to a critical point maintains a situation compatible with the neighborhood to a similar point, as manifested by the formation of small and fluctuating domains. This behavior is also confirmed by the fact that, after the insertion of Allo, the relative fraction of the two phases is again near to 50%.

Fig. 5e reports the height difference between domains for a pure lipid bilayer and a lipid bilayer exposed to 400 nM Allo concentration as a function of the temperature. For the pure lipid bilayer, the height difference approaches a value of 0 nm for a temperature of 30 °C as expected for a critical point at such temperature. The height difference can be represented by the function $\alpha\, IT_c - Tl^{\beta}$ with a value for β of 0.27. The expected value for a critical point in a 2D Ising system should be 0.125 whereas it should be 0.325 for a 3D Ising system. A value corresponding to a 2D Ising system has been obtained in the case of critical behavior in GUVs [65], whereas a value compatible with a 3D Ising system has been obtained on multilamellar systems [66]. A previous investigation on supported lipid bilayers obtained a value compatible with the 3D Ising case [46]. Here we obtained a figure that is not compatible with either of the two models. This could be due to many reasons, including the fact that the specific case of supported lipid bilayers could be a system subjected to constraints due to the presence of the substrate. Other reasons could be related to the fact that our system is not exactly near to a critical point and the predicted values are rigorously valid only for a system approaching very nearly the critical point. It is also to stress that our fits rely on a very small number of data points and data nearer to the critical point could largely affect the fit. It is however interesting to note that in some cases we observed a behavior which can be described by a feature for β of 0.33, probably connected with a bilayer very near to a critical condition (see Supporting information).

Fig. 5e shows also the behavior of a lipid bilayer exposed to 400 nM Allo concentration as a function of temperature. It is evident that the critical temperature of the bilayer increased by almost 5 °C. In that condition, the value obtained for β is 0.26, which is very similar to that obtained for a pure bilayer. These results clearly show that Allo has an effect on the miscibility transition temperature for this "pseudo-ternary mixture". To provide further support to this statement, we studied by epifluorescence microscopy on GUVs the effect of Allo concerning the miscibility temperature. The results (together with the detailed methods) are reported in the Supporting information (Figure S5). By measuring the fraction of vesicles in the phase separation region as a function of temperature in the absence and in the presence of Allo, we were able to support the effect we observed by AFM.

We also investigated the behavior for a lipid mixture that is not expected to reside near to the line of critical points. In this case we chose a lipid mixture composed by DOPC/bSM/chol in the molar proportions 0.42:0.33:0.25 (other cases are reported in the Supporting information). At 25 °C the bilayer presents a phase coexistence region which can be removed by increasing the temperature up to 30 °C (Fig. 6a). In this case we did not observe the appearance of small domains at variance with the previous case. Once the bilayer was in the homogeneous phase we inserted Allo. Fig. 6b shows the behavior of the bilayer after the insertion of the NS. It is clear that the NS took the bilayer back to the phase coexistence region with the formation of larger domains. The progressive addition of Allo leads also to the appearance of

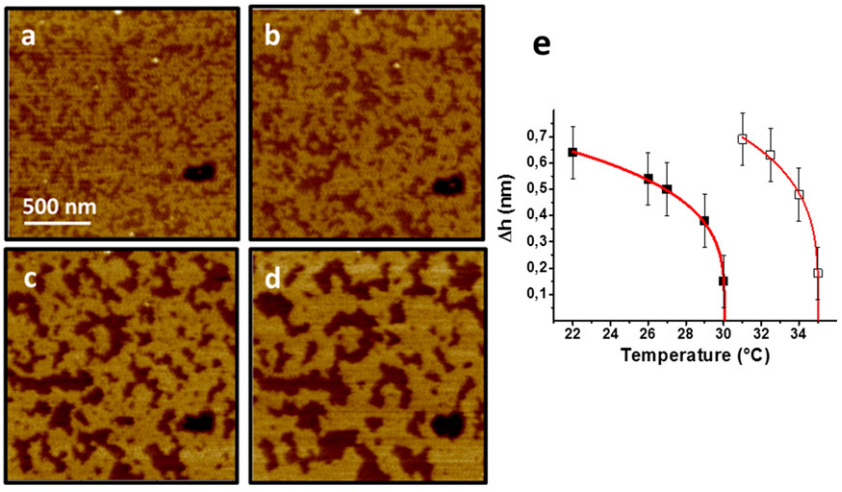

Fig. 5. Effect of the temperature in the presence of 450 nM Allo: a) 27 °C; b) 25.8 °C; c) 24.5 °C; d) 23.3 °C. e) Height difference between the L_o and L_d domains in the case of a pure DOPC/bSM/chol 1:1:1 supported lipid bilayer (filled squares) and in the case of the same bilayer exposed to a 400 nM Allo solution (open squares). The data have been fitted with the function $IT - T_c{}^{\beta}$. The obtained values for β are 0.28 for a pure lipid bilayer and 0.26 for a bilayer in the presence of Allo.

putatively solid domains, as it can be seen from Fig. 6c, d which corresponds to 400 nM Allo. Indeed, the L_o domain on the right and lower portion of Fig. 6c shows the presence of a region with a slightly larger thickness. The identification of S_o regions by AFM in ternary lipid mixtures can be elusive due to the very small height difference with respect to L_o domains. Fig. 6d shows an averaged line section of the domain in Fig. 6c whereas Fig. 6e shows the image acquired by the phase signal of tapping-mode AFM on the same region of Fig. 6c. The phase signal produces a visible contrast when the tip of the AFM is interacting with regions of different mechanical properties [67,68]. The variation of the phase contrast on the higher domain in Fig. 6e could correspond to the presence of a S_o region that is mechanically more rigid than the L_o phase.

In the debate on the possible effects of drugs on biological membranes a point that is usually considered crucial to distinguish between a specific effect on membrane proteins and an aspecific action on the lipid bilayer physical properties is the difference in stereoisomers' activity [69,70]. More generally, if colligative properties are considered, enantiomers of the same molecule should not produce different effects on an achiral lipid bilayer. Accordingly, if two enantiomers change differently the activity of a membrane protein, such as in the case of the two enantiomers of Allo on the $GABA_A$ receptor [71], the mechanistic explanation should foresee a specific interaction of the drug with the membrane protein. However, it is not trivial to study the complete effect of a molecule on a lipid bilayer, because it could affect properties which are not easy to study experimentally, such as the lateral pressure profile inside the bilayer [16], a property still waiting for an appropriate experimental technique able to provide its measurement. In general terms, it is important to stress that the lipid bilayer cannot be considered an achiral environment, especially if cholesterol is one of the components [72]. Dealing with the phase organization of lipid bilayers, it is important to study if isomers of the drugs could differently alter the phase state of the bilayer inducing the prevalence of one phase over other coexisting phases.

In this work we considered also the effect of isoAllopregnanolone, a 3β isoform of Allo differing only for the spatial orientation of an OH group, on a lipid bilayer of the same composition. It is important to stress that isoAllo is not a mirror image of Allo, and it is reasonable to expect a different interaction with a lipid bilayer [73,74]. However, given that the functional effect of isoAllo is that of inhibiting the activity of Allo in a non-competitive way [36,75], it is interesting to study if the two molecules have also different and somehow opposite effects on the lipid bilayer. Fig. 7 shows the effects of the perfusion of isoAllo on a DOPC/bSM/chol 1:1:1 supported bilayer. It is evident that in this case the insertion of this NS in the bilayer induces a decrease of the overall lipid bilayer area. Moreover, an analysis of the relative proportion of the two phases shows that the addition of isoAllo induces an increase of the L_o fraction over the L_d one. The variation of the L_o fraction for a 300 nM isoAllo concentration is $(2 \pm 1)\%$. Table 1 reports a comparison between the effects of the same concentration of the two molecules on the L_o and L_d and average area variation of the here considered ternary lipid mixture. Interestingly, the interaction of GABAergic phenols on lipid bilayers has been recently studied by 1H NMR [76] and it has been found that these molecules strongly interact with the lipid bilayer partitioning near to the polar group and the first part of the acyl chains. At the same time, these molecules favor a tighter packing of the lipid bilayer. It could be that the insertion of isoAllo in the DOPC/bSM/chol mixture induces a decreased repulsive force between the lipids involved in the L_o phase and a decrease of the overall membrane area.

Considering the results obtained in the present study, we could speculate that the antagonistic effect of isoAllo on Allo could also be related to an opposite effect on the lipid bilayer.

We also investigated the effect of isoAllo when the lipid bilayer is just outside the phase coexistence region. In this case we found that, up to 400 nM isoAllo concentration, no effect was evident from the AFM images. This is at variance with what happens in the case of Allo which stabilizes the phase separation of the bilayer. In the case of isoAllo it could be that the addition of the exogenous molecule shifts the system to the homogeneous phase.

In order to get an idea of the effect of the NSs on the global acyl chain order in the bilayer we followed by transmission FT-IR spectroscopy the position of the absorption maximum due to the symmetric $-CH_2$ stretching mode as a function of temperature and in the presence of Allo or isoAllo. This absorption mode is in fact considered representative of the acyl chain order in the bilayer, moving to higher wavenumbers if the disorder increases [77]. The results and the detailed methods are reported in Supporting information. Briefly, the insertion of Allo induces a marked variation in the peak maximum in the direction of an increased

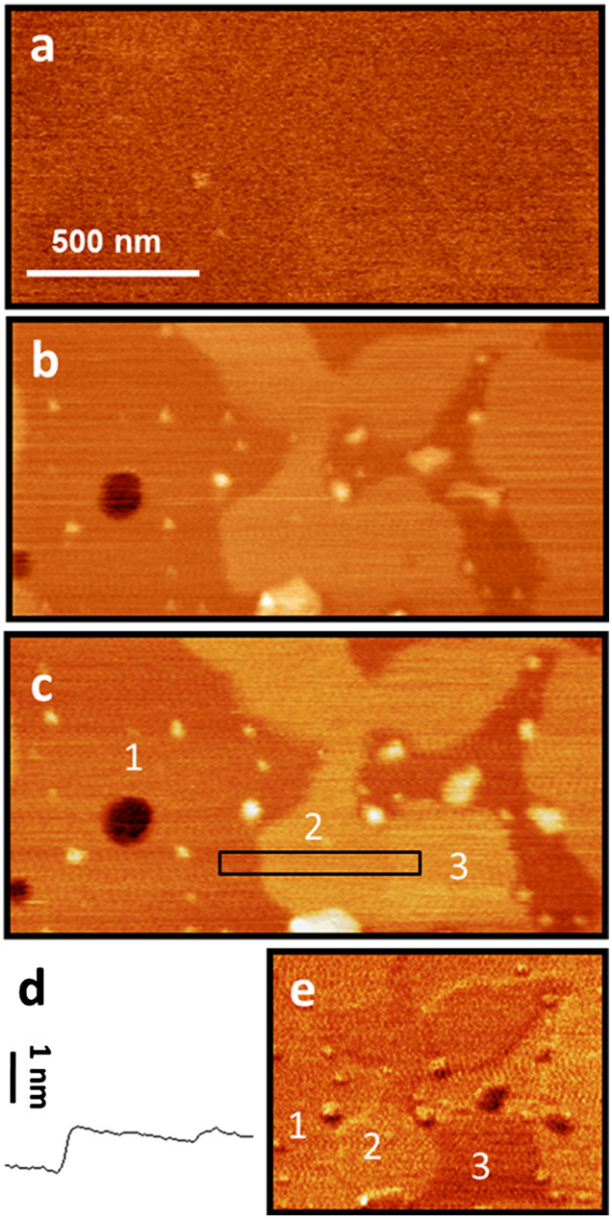

Fig. 6. Effect of Allo on a DOPC/bSM/chol 0.42:0.33:0.25 supported lipid bilayer. a) AFM image of a lipid bilayer in the absence of Allo at 30 °C. b) The same lipid bilayer as in a) with 200 nM Allo. c) The same lipid bilayer region as in b) at 400 nM Allo. The higher domain of the bilayer in the bottom part of the picture (see the white line) shows two height levels as shown in d). The higher level domain could be interpreted as a region in the solid ordered phase. Accordingly, the numbers refers to the different domains: 1) L_d; 2) L_o; 3) S_o. d) Average line section of the region highlighted in c) within the highlighted rectangle. e) Phase signal image of the same region as in c). The numbering has the same meaning as in c) The contrast on domain 3 could be related to the presence of a solid ordered phase.

disorder, in accordance with AFM experiments, whereas, in the case of isoAllo, the spectra are little affected with a small shift towards an increased order (Figure S8). Obviously, in these experimental conditions we cannot distinguish between the effects on the separate phases, but we gain an insight into the global effect of the NSs on bilayer order.

A recent investigation on the effect of liquid anesthetics on Giant Plasma Membrane Vesicles (GPMVs) demonstrated that these hydrophobic molecules are able to decrease the miscibility temperature of the membranes [13]. This fact implies that the effect of liquid anesthetics resembles that of a temperature increase. At the same time, the interaction of the liquid anesthetics with the lipid bilayer did not suppress the critical behavior of the membrane. In the present work we found that the interaction with a lipid bilayer of a NS whose activity modulates the function of the $GABA_A$ receptor produces an effect similar to what would happen by decreasing the temperature or, equivalently, by decreasing the cholesterol amount in the bilayer. However, it is to be considered that the effects on the lipid bilayers could also depend on the concentration of the exogenous molecule. Here we used physiologically relevant concentrations, which are known to modulate $GABA_A$ receptor function, whereas Gray et al. [13] studied the effect of concentrations in the μM/mM range. Even in our case, it seems that the critical behavior of the chosen lipid mixture is preserved after Allo interacted with the bilayer. Many reports highlighted the importance of the interaction of Allo with the lipid bilayer in order to fully understand its action on the $GABA_A$ receptor. We showed that Allo, even at very low concentrations (starting from 50 nM), significantly increases the bilayer area and, in case of L_d–L_o phase coexistence, increases the fraction of the L_d phase. In addition, our Micropipette Aspiration experiments show that the interaction of Allo with a bilayer with the same lipid composition as the one used in this work induces an increase of the membrane area and a decrease of its mechanical properties [57]. Considering the possibility that a biological membrane could be in a thermodynamical state above and near to its critical point, we can also speculate that Allo could change the miscibility border of the membrane phase diagram and could consequently affect the organization of lipid domains such as those considered in the lipid raft hypothesis. In fact, 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 its critical point. Accordingly, any chemical components in the lipid bilayer which is able to shift the critical point will also affect the lipid rafts dynamics at constant temperature. Lipid rafts have been shown to play important roles in the function of ligand-gated ion channels. Ligand-gated ion channels associated with lipid rafts will be affected in their behavior as a consequence of alteration of the lipid raft lateral scale dimensions and characteristic life times. In this context, in some reports it has been suggested that the $GABA_A$ receptor is localized in lipid rafts [78] and, according to the specific drug, the interactions with the receptors occur inside or outside the lipid raft [79]. If lipid rafts spatial and time characteristics are altered, the activity of the $GABA_A$ receptor will be affected and also its sensitivity to drugs. The activity of the $GABA_A$ receptor has been demonstrated to depend on the cholesterol concentration, whose amount in a bilayer significantly alters its thermodynamic state. Furthermore, the effect of allosteric modulators of the $GABA_A$ receptors, such as Allo, changes depending on the cholesterol concentration in the membrane. For example, we found that the effect of Allo and isoAllo on the phase distribution in the bilayer strongly depends on the phase situation of the bilayer, hence on the cholesterol amount, being much more evident when there is a strong unbalance between the two phases fractional occupancy (see Supporting information).

Moreover, we showed that an isoform of Allo, which is reported to inhibit functionally Allo effects, acts on the lipid bilayer in an opposite way with respect to Allo. In fact, isoAllo induces a condensation of the lipid bilayer and a relative increase of the L_o fraction. The effect of isoAllo when compared to that elicited by the same concentrations of Allo is smaller, probably due to a different partitioning in the membrane. It is important to note that in Micropipette Aspiration experiments [57] the relative area variation for the bilayer is also smaller in the case of isoAllo than of Allo.

4. Conclusions

Lipid membrane properties are strongly dependent on their composition. In general, the different behaviors of Allo and isoAllo highlighted in our work suggest that the effects of a drug on a lipid bilayer could represent a mechanism able to modulate the activity of membrane proteins even when these proteins are activated by specific interactions with the drug. In fact, considering the two hypothesis on the

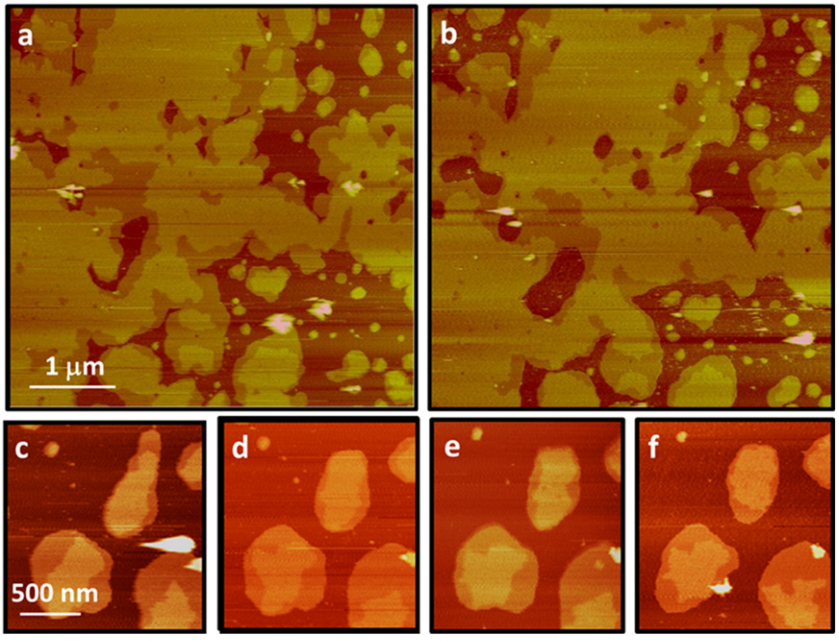

Fig. 7. Effect of isoAllo on DOPC/bSM/chol 1:1:1 supported bilayer. a–b) overall view of bilayer patches before a) and with 300 nM isoAllo in the imaging chamber b). c–f) sequence of images of lipid bilayer patches in the phase coexistence region as the concentration of isoAllo is increased: c) no isoAllo; d) 50 nM; e) 100 nM; f) 300 nM.

mechanism of action of lipophilic drugs, the aspecific effect mediated by the lipid bilayer interaction and the specific effect on a ligand-gated receptor, one cannot rule out the possible coexistence of the two mechanisms given the reciprocal, strong influence of lipids and proteins. Even if specific sites of interaction between NSs and the $GABA_A$ receptor have been identified, it is possible that the presence of these compounds in the lipid bilayer influences the rate constants for membrane protein conformational changes. The latter effect could be mediated by mechanisms such as the variation in the lateral pressure profile or in the time and spatial characteristics for lipid heterogeneities in the membrane.

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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.2015.01.002.

References

[1] A.M. Powl, J.M. East, A.G. Lee, Importance of direct interactions with lipids for the function of the mechanosensitive channel MscL, Biochemistry 47 (2008) 12175–12184.
[2] R. Phillips, T. Ursell, P. Wiggins, P. Sens, Emerging roles for lipids in shaping membrane-protein function, Nature 459 (2009) 379–385.
[3] T. Kenakin, M. Williams, Defining and characterizing drug/compound function, Biochem. Pharmacol. 87 (2014) 40–63.
[4] H. Nury, C. Van Renterghem, Y. Weng, A. Tran, M. Baaden, V. Dufresne, J.P. Changeux, J.M. Sonner, M. Delarue, P.J. Corringer, X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel, Nature 469 (2011) 428–431.
[5] S.W. Smith, Chiral toxicology: it's the same thing…only different, Toxicol. Sci. 110 (2009) 4–30.
[6] J.A. Lundbaek, R.E. Koeppe, O.S. Andersen, Amphiphile regulation of ion channel function by changes in the bilayer spring constant, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 15427–15430.
[7] J.A. Lundbaek, S.A. Collingwood, H.I. Ingólfsson, R. Kapoor, O.S. Andersen, Lipid bilayer regulation of membrane protein function: gramicidin channels as molecular force probes, J. R. Soc. Interface 7 (2010) 373–395.
[8] B. Martinac, J. Adler, C. Kung, Mechanosensitive ion channels of E. coli activated by amphipaths, Nature 348 (1990) 261–263.
[9] D.M. Engelman, Membranes are more mosaic than fluid, Nature 438 (2005) 578–580.
[10] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science 327 (2010) 46–50.
[11] I. Levental, D. Lingwood, M. Grzybek, U. Coskun, K. Simons, Palmitoylation regulates raft affinity for the majority of integral raft proteins, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 22050–22054.
[12] S.L. Veatch, P. Cicuta, P. Sengupta, A. Honerkamp-Smith, D. Holowka, B. Baird, Critical fluctuations in plasma membrane vesicles, ACS Chem. Biol. 3 (2008) 287–293.
[13] E. Gray, J. Karslake, B.B. Machta, S.L. Veatch, Liquid general anesthetics lower critical temperatures in plasma membrane vesicles, Biophys. J. 105 (2013) 2751–2759.
[14] A.S. Janoff, M.J. Pringle, K.W. Miller, Correlation of general anesthetic potency with solubility in membranes, Biochim. Biophys. Acta 649 (1) (1981) 125–128.
[15] R.S. Cantor, Breaking the Meyer–Overton rule: predicted effects of varying stiffness and interfacial activity on the intrinsic potency of anesthetics, Biophys. J. 80 (5) (2001) 2284–2297.
[16] R.S. Cantor, The lateral pressure profile in membranes: a physical mechanism of general anesthesia, Biochemistry 36 (1997) 2339–2344.
[17] H.C. Hemmings Jr., M.H. Akabas, P.A. Goldstein, J.R. Trudell, B.A. Orser, N.L. Harrison, Emerging molecular mechanisms of general anesthetic action, Trends Pharmacol. Sci. 26 (2005) 503–510.
[18] B.W. Urban, M. Bleckwenn, M. Barann, Interactions of anesthetics with their targets: non-specific, specific or both? Pharmacol. Ther. 111 (2006) 729–770.
[19] J.M. Sonner, R.S. Cantor, Molecular mechanisms of drug action: an emerging view, Annu. Rev. Biophys. 42 (2013) 143–167.
[20] M. Weinrich, D.L. Worcester, Xenon and other volatile anesthetics change domain structure in model lipid raft membranes, J. Phys. Chem. B 117 (2013) 16141–16147.
[21] M. Weinrich, H. Nanda, D.L. Worcester, C.F. Majkrzak, B.B. Maranville, S.M. Bezrukov, Halothane changes the domain structure of a binary lipid membrane, Langmuir 28 (2012) 4723–4728.
[22] N. Puff, C. Watanabe, M. Seigneuret, M.I. Angelova, G. Staneva, L_o/L_d phase coexistence modulation induced by GM1, Biochim. Biophys. Acta 1838 (2014) 2105–2114.
[23] M. Chisari, L.N. Eisenman, D.F. Covey, S. Mennerick, C.F. Zorumski, The sticky issue of neurosteroids and $GABA_A$ receptors, Trends Neurosci. 33 (2010) 299–306.
[24] D. Belleli, J.J. Lambert, Neurosteroids: endogenous regulators of the GABAA receptor, Nat. Rev. Neurosci. 6 (2005) 565–575.
[25] G. Puja, M.R. Santi, S. Vicini, D.B. Pritchett, R.H. Purdy, S.M. Paul, P.H. Seeburg, E. Costa, Neurosteroids act on recombinant human GABAA receptors, Neuron 4 (1990) 759–765.
[26] J.M. Mienville, S. Vicini, Pregnenolone sulfate antagonizes $GABA_A$ receptor-mediated currents via a reduction of channel opening frequency, Brain Res. 489 (1989) 190–194.
[27] G. Akk, D.F. Covey, A.S. Evers, J.H. Steinbach, C.F. Zorumski, S. Mennerick, The influence of the membrane on neurosteroid actions at $GABA_A$ receptors, Psychoneuroendocrinology 34S (2009) S59–S66.
[28] S. Mennerick, Y. He, X. Jiang, B.D. Manion, M. Wang, A. Shute, A. Benz, A.S. Evers, D.F. Covey, C.F. Zorumski, Selective antagonism of 5α-reduced neurosteroid effect at $GABA_A$ receptors, Mol. Pharm. 65 (2004) 1191–1197.
[29] A.M. Hosie, M.E. Wilkins, T.G. Smart, Neurosteroid binding sites on GABA(A) receptors, Pharmacol. Ther. 116 (2004) 7–19.
[30] A.M. Hosie, L. Clarke, H. da Silva, T.G. Smart, Conserved site for neurosteroid modulation of GABA A receptors, Neuropharmacology 56 (2009) 149–154.
[31] M. Chisari, L.N. Eisenman, K. Krishnan, A.K. Bandyopadhyaya, C. Wang, A. Taylor, A. Benz, D.F. Covey, C.F. Zorumski, S. Mennerick, The influence of neuroactive steroid lipophilicity on $GABA_A$ receptor modulation: evidence for a low-affinity interaction, J. Neurophysiol. 102 (2009) 1254–1264.
[32] M.D. Majewska, Neurosteroids: endogenous bimodal modulators of the $GABA_A$ receptor. Mechanism of action and physiological significance, Prog. Neurobiol. 38 (1992) 379–395.
[33] H.J. Shu, L.N. Eisenman, D. Jinadasa, D.F. Covey, C.F. Zorumski, S. Mennerick, Slow actions of neuroactive steroids at $GABA_A$ receptors, J. Neurosci. 24 (2004) 6667–6675.
[34] S.L. Veatch, S.L. Keller, A closer look at the canonical raft mixture in model membrane studies, Biophys. J. 84 (2003) 725–726.
[35] H. Callachan, G.A. Cottrell, N.Y. Hather, J.J. Lambert, J.M. Nooney, J.A. Peters, Modulation of the $GABA_A$ receptor by progesterone metabolites, Proc. R. Soc. Lond. B Biol. Sci. 231 (1987) 359–369.
[36] M. Wang, Y. He, L.N. Eisenman, C. Fields, C.-M. Zheng, J. Mathews, A. Benz, T. Fu, E. Zorumski, J.H. Steinbach, D.F. Covey, C.F. Zorumski, S. Mennerick, 3β-hydroxypregnane steroids are pregnenolone sulfate-like $GABA_A$ receptor antagonists, J. Neurosci. 22 (2002) 3366–3375.
[37] L.K. Tamm, H.M. McConnell, Supported phospholipid bilayers, Biophys. J. 47 (1985) 105–113.
[38] H.M. McConnell, T.H. Watts, R.M. Weis, A.A. Brian, Supported planar membranes in studies of cell–cell recognition, Biochim. Biophys. Acta 864 (1986) 95.
[39] H.M. Seeger, G. Marino, A. Alessandrini, P. Facci, Effect of physical parameters on the main phase transition of supported lipid bilayers, Biophys. J. 97 (2009) 1067–1076.
[40] S.M. Cook, T.E. Schäffer, K.M. Lang, Practical implementation of dynamic methods for measuring atomic force microscope cantilever spring constants, Nanotechnology 17 (2006) 2135–2145.
[41] I. Horcas, R. Fernández, J.M. Gómez-Rodríguez, J. Colchero, J. Gómez-Herrero, A.M. Baro, WSxM: a software for scanning probe microscopy and a tool for nanotechnology, Rev. Sci. Instrum. 78 (2007) 013705.
[42] S.L. Veatch, S.L. Keller, Seeing spots: complex phase behavior in simple membranes, Biochim. Biophys. Acta 1746 (2005) 172–185.
[43] S.L. Veatch, S.L. Keller, Miscibility phase diagrams of giant vesicles containing sphingomyelin, Phys. Rev. Lett. 94 (2005) 148101.
[44] O.G. Mouritsen, The liquid order come of age, Biochim. Biophys. Acta 1798 (2010) 1286–1288.
[45] L.A. Bagatolli, Membrane domains and their relevance to the organization of the biological membranes, Compr. Biophys. 5 (2012) 16–36.
[46] S.D. Connell, G. Heath, P.D. Olmsted, A. Kisil, Critical point fluctuations in supported lipid membranes, Faraday Discuss. 161 (2013) 91–111.
[47] S.L. Veatch, I.V. Polozov, K. Gawrisch, S.L. Keller, Liquid domains in vesicles investigated by NMR and fluorescence microscopy, Biophys. J. 86 (2004) 2910–2922.
[48] R.S. Petruzielo, F.A. Heberle, P. Drazba, J. Katsaras, G.W. Feigenson, Phase behavior and domain size in sphingomyelin-containing lipid bilayers, Biochim. Biophys. Acta 1828 (2013) 1302–13013.
[49] T. Hamada, Y. Kishimoto, T. Nagasaki, M. Takagi, Lateral phase separation in tense membranes, Soft Matter 7 (2011) 9061.
[50] M. Fuhrmans, M. Muller, Mechanisms of vesicle spreading on surfaces: coarse-grained simulations, Langmuir 29 (2013) 4335–4349.
[51] M.R. Nussio, G. Oncins, I. Ridelis, E. Szili, J.G. Shapter, F. Sanz, N.H. Voelcker, Nanomechanical characterization of phospholipid bilayer islands on flat and porous substrates: a force spectroscopy study, J. Phys. Chem. B 113 (2009) 10339–10347.
[52] T. Portet, S.E. Gordon, S.L. Keller, Increasing membrane tension decreases miscibility temperatures; an experimental demonstration via micropipette aspiration, Biophys. J. 103 (2012) L35–L37.
[53] D. Chen, M.M. Santore, Large effect of membrane tension on the fluid–solid phase transitions of two-component phosphatidylcholine vesicles, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 179–184.
[54] J.E. Shaw, R.F. Epand, R.M. Epand, Z. Li, R. Bittman, C.M. Yip, Correlated fluorescence-Atomic Force Microscopy of membrane domains: structure of fluorescence probes determines lipid localization, Biophys. J. 90 (2006) 2170–2178.
[55] S. Chiantia, J. Ries, N. Kahya, P. Schwille, Combined AFM and two-focus SFCS study of raft-exhibiting model membranes, Chemphyschem 7 (2006) 2409–2418.
[56] J. Zhao, J. Wu, H. Shao, F. Kong, N. Jain, G. Hunt, G. Feigenson, Phase studies of model biomembranes: macroscopic coexistence of $L_\alpha + L_\beta$, with light induced coexistence of $L_\alpha + L_o$ phases, Biochim. Biophys. Acta 1768 (2007) 2777–2786.
[57] D. Balleza, M. Sacchi, G. Vena, D. Galloni, G. Puia, P. Facci, A. Alessandrini, Effects of neurosteroids on a model membrane including cholesterol: A Micropipette Aspiration study, Biochim. Biophys. Acta 1848 (2015) 1268–1276.

[58] T. Bhatia, P. Husen, J.H. Ipsen, L.A. Bagatolli, A.C. Simonsen, Fluid domain patterns in free-standing membranes captured on a solid support, Biochim. Biophys. Acta 1838 (2014) 2503–2510.
[59] M. Gedig, S. Faiss, A. Janshoff, Melting and interdigitation of microstructured solid supported membranes quantified by imaging ellipsometry, Biointerphases 3 (2008) FA51.
[60] S.L. Veatch, S.S. Leung, R.E. Hancock, J.L. Thewalt, Fluorescent probes alter miscibility phase boundaries in ternary vesicles, J. Phys. Chem. B 111 (2007) 502–504.
[61] T. Sooksawate, M.A. Simmonds, Influence of membrane cholesterol on modulation of the $GABA_A$ receptor by neuroactive steroids and other potentiators, Br. J. Pharmacol. 134 (2001) 1303–1311.
[62] T. Sooksawate, M.A. Simmonds, Increased membrane cholesterol reduces the potentiation of $GABA_A$ currents by neurosteroids in dissociated hippocampal neurons, Neuropharmacology 37 (1998) 1103–1110.
[63] T. Sooksawate, M.A. Simmonds, Effects of membrane cholesterol on the sensitivity of the $GABA_A$ receptor to GABA in acutely dissociated rat hippocampal neurons, Neuropharmacology 40 (2001) 178–184.
[64] L.S. Hirst, P. Uppamoochikkal, L. Chai, Phase separation and critical phenomena in biomimetic ternary lipid mixtures, Liq. Cryst. 38 (2011) 1735–1747.
[65] A.R. Honerkamp-Smith, P. Cicuta, M.D. Collins, S.L. Veatch, M. den Nijs, M. Schick, S.L. Keller, Line tensions, correlation lengths, and critical exponents in lipid membranes near critical points, Biophys. J. 95 (2008) 236–246.
[66] J.H. Davis, M.L. Schmidt, Critical behavior in DOPC/DPPC/cholesterol mixtures: Static 2H NMR line shapes near the critical point, Biophys. J. 106 (2014) 1970–1978.
[67] A. Alessandrini, P. Facci, Atomic force microscope: a versatile tool in biophysics, Meas. Sci. Technol. 16 (2005) R65–R92.
[68] A. Alessandrini, P. Facci, Phase transitions in supported lipid bilayers studied by AFM, Soft Matter 10 (2014) 7145–7164.
[69] L.L. Wittmer, Y. Hu, M. Kalkbrenner, A.S. Evers, C.F. Zorumski, D.F. Covey, Enantioselectivity of steroid-induced gamma-aminobutyric acidA receptor modulation and anesthesia, Mol. Pharmacol. 50 (1996) 1581–1586.
[70] K.R. Nilsson, C.F. Zorumski, D.F. Covey, Neurosteroid analogues. 6. The synthesis and $GABA_A$ receptor pharmacology of enantiomers of dehydroepiandrosterone sulfate, pregnenolone sulfate, and (3α,5β)-3-hydroxypregnan-20-one sulfate, J. Med. Chem. 41 (1998) 2604–2613.
[71] D.F. Covey, D. Nathan, M. Kalkbrenner, K.R. Nilsson, C.F. Zorumski, A.S. Evers, Enantioselectivity of pregnanolone-induced γ-aminobutyric acid receptor modulation and anesthesia, J. Pharmacol. Exp. Ther. 293 (2000) 1009–1116.
[72] S. Lalitha, A.S. Kumara, K.J. Stine, D.F. Covey, Chirality in Membranes: First Evidence that Enantioselective Interactions Between Cholesterol and Cell Membrane Lipids Can Be a Determinant of Membrane Physical Properties, J. Supramol. Chem. 1 (2001) 53–61.
[73] A. Makriyannis, C.M. DiMeglio, S.W. Fesik, Anesthetic steroid mobility in model membrane preparations as examined by high-resolution 1H and 2H NMR spectroscopy, J. Med. Chem. 34 (1991) 1700–1703.
[74] A. Makriyannis, D.J. Siminovitch, S.K. Das Gupta, R.G. Griffin, Studies on the interaction of anesthetic steroids with phosphatidylcholine using 2H and 13C solid state NMR, Biochim. Biophys. Acta 859 (1986) 49–55.
[75] T. Bäckström, G. Wahlström, K. Wahlström, D. Zhu, M.D. Wang, Isoallopregnanolone; an antagonist to the anesthetic effect of allopregnanolone in male rats, Eur. J. Pharmacol. 512 (2005) 15–21.
[76] G.N. Reiner, L.F. Fraceto, E. de Paula, M.A. Perillo, D.A. García, Effects of gabaergic phenols on phospholipid bilayers as evaluated by 1H NMR, J. Biomater. Nanobiotechnol. 4 (2013) 28–34.
[77] C. Nicolini, J. Kraineva, M. Khurana, N. Periasamy, S.S. Funari, R. Winter, Temperature and pressure effects on structural and conformational properties of POPC/SM/cholesterol model raft mixtures—a FT-IR, SAXS, DSC, PPC and Laurdan fluorescence spectroscopy study, Biochim. Biophys. Acta 1758 (2006) 248–258.
[78] X. Li, D.R. Serwanski, C.P. Miralles, B.A. Bahr, A.L. De Blas, Two pools of Triton X-100-insoluble $GABA_A$ receptors are present in the brain, one associated to lipid rafts and another one to the post-synaptic GABAergic complex, J. Neurochem. 102 (4) (2007) 1329–1345.
[79] C. Nothdurfter, S. Tanasic, B. Di Benedetto, M. Uhr, E.M. Wagner, K.E. Gilling, C.G. Parsons, T. Rein, F. Holsboer, R. Rupprecht, G. Rammes, Lipid raft integrity affects $GABA_A$ receptor, but not NMDA receptor modulation by psychopharmacological compounds, Int. J. Neuropsychopharmacol. 16 (2013) 1361–1371.