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Microscopic interactions of melatonin, serotonin and tryptophan with zwitterionic phospholipid membranes

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Abstract: The interactions at the atomic level between small molecules and the main components of cellular plasma membranes are crucial to elucidate the mechanisms allowing the entrance of such small species inside the cell. We have performed molecular dynamics and metadynamics simulations of tryptophan, serotonin and melatonin at the interface of zwitterionic phospholipid bilayers. In this work we will review recent computer simulation developments and report microscopic properties such as the area per lipid and thickness of the membranes, atomic radial distribution functions, angular orientations and free energy landscapes of small molecule binding to the membrane. Cholesterol affects the behavior of the small molecules, which are mainly buried in the interfacial regions. We have observed a competition between the binding of small molecules to phospholipids and cholesterol through lipidic hydrogen-bonds. Free energy barriers associated to translational and orientational changes of melatonin have been found to be between 10-20 kJ/mol for distances of 1 nm between melatonin and the center of the membrane. Corresponding barriers for tryptophan and serotonin obtained from reversible work methods are of the order of 10 kJ/mol and reveal strong hydrogen bonding between such species and specific phospholipid sites. Diffusion of tryptophan and melatonin is of the order of $10^{-7}$ cm$^2$/s for the cholesterol-free and cholesterol-rich setups.

Keywords: melatonin; serotonin; tryptophan; phospholipid membrane.

1. Introduction

The cell membrane plays a central role in the control of the exchange of key elements (nutrients, wastes, drugs and heat as the most relevant) between the exterior of a cell and its cytoplasm. Lipids, proteins and cholesterol (CHOL) are among the main components of human cell membranes. Phospholipids are usually formed by two leaflets of amphiphilic lipids divided into a hydrophilic head and one or two hydrophobic tails. Such lipids can self-assemble by hydrophobicity[1,2]. Lipid bilayer membranes formed by dipalmitoylphosphatidylcholine (DPPC, $C_{40}H_{80}NO_4P$) and dimyristoylphosphatidylcholine (DMPC, $C_{36}H_{72}NO_8P$) are of great interest to computational studies[3–6] because of the abundance of experimental data[7–11] and their usability as model systems[12,13]. In the last decades, cell membrane systems have been extensively studied on their association with drugs and small molecules[14–18]. Among these, we will focus our attention to three species deeply related, belonging to the indole group: melatonin (MEL, $C_{13}H_{16}N_2O_2$), its precursor serotonin (SER, $C_{10}H_{12}N_2O$) and its precursor tryptophan (TRP, $C_{11}H_{12}N_2O_2$), given their interest as pharmaceutical compounds with a wide variety of applications, in particular a large number related to sleep disorders[19–21]. The relationships between the three components have been reviewed a significant number of times in different contexts[22–25]. The sequence of transformations...
Melatonin is a neurohormone produced in the pineal gland, first isolated in 1958 by Lerner et al. [29] after it was identified in bovine pineal extracts. There is evidence that MEL can regulate circadian rhythms [30] and mood, induce sleep [31] and contribute in protecting the organism from Alzheimer disease [32], having become one of most studied hormones in relation to its affectation to the human body [33–36]. MEL plays a role in aging processes, being mainly protective of oxidative stress and damage [37] and it is also related to skin pigmentation and to DNA repair systems. Hence, MEL has become a very good candidate for treating several dermatoses associated with substantial oxidative damage, by means of the increase of intracutaneous melatonin production as well as by exogenous application and intake [37–39]. MEL has a significant effect on decreasing cholesterol absorption, causing great reduction of the concentration of cholesterol in membrane bilayers and in the liver [40]. It is able to cross most physiological barriers, such as the blood-brain barrier [41, 42] so that it may help to control brain function [43] and it has also interesting immunotherapeutic potential in both viral and bacterial infections [44]. MEL has been also related to the protection of the organism from carcinogenesis and neurodegenerative disorders [32, 45]. Recently, the use of MEL to attenuate the effects of the severe acute respiratory syndrome (Covid-19 or SARS-CoV-2) has been under debate [46], since melatonin is a well known antiinflammatory agent and it could be protective against viral pathogens. A comprehensive description of its functions has been summarised [47–49]. A the microscopical level, several works have analysed the structure and interactions of MEL with phospholipid membranes [50, 51]. Both experiments and simulations suggest that small solutes such as TRP and MEL are bound to the phosphate and carbonyl regions of phospholipid species [52–55]. Recent studies indicate that cellular permeation rates in the pineal gland are of the order of 1.7 μm/s and that they can occur by pure diffusion under high temperatures and pressures [56]. However, other studies found that the active action of glucose transporters are required for the entrance of MEL inside cancer cells [57] allowing MEL to help inhibiting tumor growth [58]. The safety of MEL in humans has been addressed. Andersen et al. [59] reported that in animal and human studies the short term use of MEL is safe, even in extreme doses. After long-term treatments, there have been reported only mild side effects, none of them being dangerous for human health. Experimental and computational work on mixtures of CHOL and MEL at phosphatidylcholine membranes have analysed the joint effects of the two species [60, 61].

The precursors of melatonin, serotonin and tryptophan, have been thoroughly studied from long ago. SER (also known as 5-hydroxytryptamine or 5-HT) is a biogenic amine most noted for its role as a neurotransmitter, mainly produced by enterochromaffin cells in the gut and also by neurons of the brain stem [62]. It was first isolated and characterised in 1948 by Rapport et al. [63]. Serotonin was quickly identified in many tissues including brain, lung, kidney, platelets and in the gastrointestinal tract. It is thought to be a contributor to the regulation of human mood and happiness [64]. It has been also suggested that SER also regulates the connectivity of the brain [65]. As the third small molecule to describe here, TRP is an α-aminoacid used in the biosynthesis of proteins. Tryptophan contains an α-amino group, an α-carboxylic acid group and a side chain indole, making it a nonpolar aromatic aminoacid. TRP is essential in humans and it is also a precursor to the vitamin B3 and it is commonly used to treat insomnia and sleep disorders like apnea [66, 67]. TRP can act as a building block in protein biosynthesis, while proteins perform a vast array of functions within organisms, such as catalysing metabolic reactions, replicating DNA, responding to stimuli, providing structure to cells and organisms, and transporting molecules from one location to another.

Our main aim here is to review and study at atomic detail the interactions and binding mechanisms between melatonin, serotonin and tryptophan with the cell membrane, modelled as a mixture of phospholipids and cholesterol, in aqueous ionic solution. We have employed two types
of computational methods, molecular dynamics (MD) and well tempered metadynamics (WTM). MD is a classical simulation tool able to generate a bundle of Newtonian trajectories, one for each single particle of the system, at the atomic level. As atoms interact through pairwise force fields, their trajectories (composed of positions and linear momenta of all particles) are deployed and stored at regular time intervals, in order to be analysed using tools from Statistical Mechanics[68]. MD is a versatile method able to successfully reproduce a large number of microscopic properties of a wide variety of systems, from simple atomic liquids such as argon[69] to molecular liquids as water[70,71], aqueous solutions at interfaces[72–76] up to complex biophysical systems like DNA[77–79] or model cell membranes[6,80–84]. To handle the problem of computing free energy landscapes in multidimensional systems, different classes of methods have been proposed, such as quantum mechanics/molecular mechanics[85], transition path sampling[86–92], adaptive biasing force[93], umbrella sampling methods[94,95], density functional theory molecular dynamics[96] or calculations of potentials of mean force[97] based on reversible work methods[98]. In this work we have employed reversible work and WTM, a method able to efficiently explore free energy surfaces of complex systems using multiple reaction coordinates what has been revealed to be very successful[99] for a wide variety of complex systems[100–104]. The technical characteristics of all simulations are reported in Section 3.

2. Results and discussion

2.1. Structural properties of the membranes

The first group of properties to be analysed are the structural characteristics of the membranes and the local distributions of atomic species. To do so, we have sketched the detailed atomic structures of the three small molecules, the two phospholipids composing the membranes (DMPC, DPPC) and CHOL in Fig.1. There, the highlighted sites of TRP are the zwitterions ‘H1’, sharing a positive charge between the three hydrogens bound to ‘N1’; ‘H2’, bound to ‘N2’ and the zwitterions ‘O1’ and ‘O2’, bound to ‘C1’ and sharing a negative charge. In SER, we highlight ‘H1’, ‘H4’ and ‘O’ and for MEL we will keep special attention into ‘O1’, ‘O2’, ‘H15’ and ‘H16’. Finally, the sites ‘O1’ and ‘O2’ sharing the negative charge and oxygen atoms ‘O6’, ‘O8’ will be considered for DPPC and DMPC and the hydroxilic OH pair for CHOL.

![Atomic structures of melatonin, serotonin, tryptophan, DPPC, DMPC and cholesterol.](image)

Figure 1. Atomic structures of melatonin, serotonin, tryptophan, DPPC, DMPC and cholesterol. Backbone hydrogens not shown explicitly. The highlighted labels will be referred in the text.

A common test in computer simulations of cell membranes is the comparison of the area per lipid and thickness of the membrane with experimental data from scattering density profiles[105]. We have monitored the surface area per lipid $A$ considering the total surface along the $XY$ plane (plane parallel
to the bilayer surface) divided by the number of lipids $N_l$ plus the number of cholesterol molecules $N_{chol}$ in one lamellar layer\cite{106} as defined in Eq. 1:

$$A = \frac{L_x \times L_y}{N_l + N_{chol}},$$  \hspace{1cm} (1)

where $L_x$ and $L_y$ are the length of the simulation box along $X$-axis and $Y$-axis, respectively. $Z$-axis is the (instantaneous) normal direction to the surface of the bilayer, set along plane $XY$. Fluctuations in the thickness of the membrane are related to the effect of cholesterol on the rigidity of the membrane and its capability to allow the passing of species in and out the cell. In this work, we defined the thickness $\Delta z$ as the distance between the phosphate groups of the lipids at the two sides of the membrane. The area per lipid and thickness along the last 500 ns of each simulation have been computed (see Fig. 2) and the average values are reported in Table 1.

| Small molecule and cholesterol percentage | Phospholipid species | $A$ (nm$^2$) | $\Delta z$ (nm) |
|-------------------------------------------|----------------------|--------------|-----------------|
| TRP-0%                                    | DPPC                 | 0.614 (0.008) | 3.97 (0.05)     |
| TRP-30%                                   | DPPC                 | 0.408 (0.002) | 4.89 (0.04)     |
| TRP-50%                                   | DPPC                 | 0.401 (0.002) | 4.78 (0.03)     |
| SER-0%                                    | DPPC                 | 0.613 (0.015) | 3.83 (0.05)     |
| MEL-0%                                    | DMPC                 | 0.618 (0.005) | 3.49 (0.06)     |
| MEL-30%                                   | DMPC                 | 0.421 (0.007) | 4.43 (0.03)     |
| MEL-50%                                   | DMPC                 | 0.402 (0.008) | 4.47 (0.03)     |

Area per lipid decreases as cholesterol concentration increases: this is a well known trend, as will see below. We obtained a value of around 0.61 nm$^2$ for a cholesterol-free system and smaller values down to 0.40-0.42 nm$^2$ when cholesterol has been incorporated. In all cases, the area per lipid is practically independent of the small molecule imbedded in the membrane and it has little influence of the main type of phospholipid. These results are in excellent agreement with experimental data\cite{109,110} where the value for pure DMPC is of about 0.6 nm$^2$ at 303 K. Further, and according to Nagle et al.\cite{1} values of $A$ of pure DMPC membranes can be obtained from multiple methods (neutron scattering, X-ray and NMR) and have been reported to be between 0.59 and 0.62 nm$^2$ at the liquid crystal phase. In the case of DPPC, the best estimations were of between 0.48 and 0.52 nm$^2$ in the gel phase (293 K) and 0.64 nm$^2$ in the liquid phase. These results are also in overall good agreement with other computational data in a wide variety of thermodynamical conditions\cite{11,109,111–113}, where the values for pure DPPC ranged between 0.50 and 0.63 nm$^2$ and the trend of decreasing areas for increasing cholesterol percentages was clearly reported. The huge change produced at 30% cholesterol concentration is consistent with the fact that phosphatidylcholine membranes experience a phase transition liquid disordered (cholesterol-free system) to liquid ordered phase (systems of cholesterol 30% and 50%)\cite{114,115}.
The thickness of the membranes are in good agreement with those reported by Kucerka et al. [110] by means of X-ray and neutron scattering. The reported value was of 3.67 nm at 303 K for the DMPC membrane at 0% cholesterol. From the results reported in Table 1, we get values around 3.5-4 nm for pure bilayers and of 4.4-4.9 nm when cholesterol is considered. We observe a tendency to larger bilayer thickness for rising cholesterol concentration. Given the reduction of the area per lipid at high cholesterol percentages, we can conclude that cholesterol favors the compression of the structure of the bilayer membrane. This feature can increase the rigidity of the membrane and, by extending the tails of the lipids, give larger bilayer thickness. Such increase of the rigidity of the membrane was observed from both experimental and computational sides [60, 61] in MEL-CHOL mixtures nearby phosphatidylcholine bilayers. According to these studies, the effect of MEL reducing the thickness of the membrane and enhancing its fluidity was partially compensated by the condensating effect of cholesterol.

2.2. Preferential localisations of the small molecules at the interfaces of phospholipid membranes: Atomic radial distribution functions

Each of the three small molecules considered in the present work has been simulated for long MD trajectories of hundreds of nanoseconds. We have monitored their positions and velocities and obtained structural, energetic and dynamical information. In this section, we will focus our attention on the local structure of the probes when embedded in the membrane. As a general fact, we have observed that all three selected species show a strong tendency to be continuously adsorbed at the interface of the membrane during long periods of the order of 10 ns. In the remaining time, the small molecules move away to be solvated by the ionic solution surrounding the membrane. As an example, we report in Fig. 3 the evolution in time (window of 60 ns) of the position of the center of mass of melatonin when adsorbed at a DMPC-cholesterol membrane.
Figure 3. Z-axis location of the center of mass of MEL in a DMPC lipid membrane with different cholesterol contents as a function of simulation time. The green dashed line indicates the geometrical center of the bilayer membrane. Data partially taken from Ref. [108].

In Fig. 3 we can observe that the influence of cholesterol is of paramount importance: when the concentration of cholesterol in the membrane reaches 50% of all lipids, MEL can easily shift between the interface of the membrane and the solvating aqueous ionic solution, but at lower concentrations the small molecule is likely inside the membrane during the whole time span considered. This indicates that moderate changes of cholesterol concentration may induce some specific organic probes to retreat from the inside of cellular membranes to the outer regions and remain outside the cell. This might have strong implications in melatonin deliver. The relationships between MEL and CHOL and their interactions have been studied since long time ago[40,60,61] but the knowledge of their effects are still quite elusive.

Since the best way to investigate atom-atom local structures is the computation of radial distribution functions (RDF), we have computed a series of specific RDF in order to have an overview for TRP and MEL. We define the RDF for an atomic pair composed by particles ’1’ and ’2’ as \( g_{12}(r) \) and it is given by:

\[
g_{12}(r) = \frac{V}{4N_2 \pi r^2} \frac{\langle n_2(r) \rangle}{\Delta r},
\]

where \( n_2(r) \) is the number of atoms of species ’2’ surrounding a given atom of species ’1’ inside a spherical shell of width \( \Delta r \). \( V \) stands for the total volume and \( N_2 \) is the total number of particles of species ’2’. In the case of TRP, we have considered the partial RDF reported in Fig. 4, whereas for MEL we will analyse the RDF presented in Fig. 5.
From the data reported in Fig. 4, we can observe that TRP stays bound to the inner part of the membrane during long periods of time, according to the time scale of our simulations, in good agreement with results indicating that in a cholesterol-free DOPC bilayer membrane TRP is preferentially located in the interfacial region [55]. In this work, we have observed that the average continuous lifetime of TRP at the interface of the DPPC bilayer is of the order of 10 ns (data not shown). From Fig. 4, hydrogen bond (HB) connections between sites ‘H1’ and ‘H2’ of TRP and DPPC sites ‘O2’ and ‘O8’ (labels according Fig. 1) have been found. HB are very short, since the maxima of the $g(r)$ related to ‘H1’ hydrogens in TRP are located around 1.7 Å for ‘O2’ and around 1.75 Å for ‘O8’ of DPPC. So, the presence of cholesterol reduces ‘H1-O2’ binding but enhances the ‘H1-O8’ one. As a general fact, the presence of cholesterol increases the length of HB but also making such bonds stronger. This indicates that the influence of the cholesterol in the TRP-DPPC binding is a major effect. Interestingly, ‘H2’-DPPC binding was observed in all analysed setups, but with maxima found at larger distances (1.9-2.0 Å). Again, the presence of cholesterol showed a major influence on the characteristics of hydrogen bonding.
The structural results for MEL are reported in Fig. 5. All RDF show fluctuating profiles, especially at distances larger than \( r = 3 \) Å and beyond (higher order coordination shells). There is a clear first coordination shell in all cases, located around 1.8-2.0 Å, due to HB between MEL and the remaining species, such as in the TRP case. The largest maximum of all RDF is the one for MEL-CHOL association (not shown here), centered at 1.9 Å when the concentration of cholesterol is of 30%. Interactions of MEL-CHOL in DPPC bilayers were reported by Choi et al.[61], but at finite melatonin concentration. In the remaining cases, HB lengths are around 1.9 Å and were between both ‘H15’ and ‘H16’ of MEL and DMPC sites ‘O1’ (or ‘O2’, since both sites are sharing the negative charge of the zwitterion). In a similar fashion, MEL can also form HB between both ‘H15’ and ‘H16’ with the DMPC’s sites ‘O6’ (or ‘O8’) for all three percentages of cholesterol. The present findings are in good agreement with experimental data from Severcan et al. [50] obtained by Fourier transform infrared spectroscopy, who observed hydrogen bonding connections between the N-H group of the furanose ring of MEL (‘H16’ in this work) and the carbonyl (C=O) and phosphate (PO\(_4\)) groups in DPPC membranes. Our results indicate HB between ‘H15’ and ‘H16’ of MEL with DMPC’s phosphate group (‘O1’) as well as with the more internal carbonyl groups (‘O6’ and ‘O8’). The previously unobserved hydrogen bonds of ‘H15’ with the two well known acceptor groups in phosphatidylycholines indicated above are the responsible of the absorption of MEL into the membrane deeper than TRP, with the two selected donors (‘H15’ and ‘H16’) together with the ‘H15-O Chol.’ bridges. These findings are in excellent agreement with results reported by Drolle et al.[60] by means of small angle neutron diffraction and MD simulations.

### 2.3. Orientational distributions of melatonin

Several previous studies have shown that the orientations of drugs on membranes significantly impact their function in cells [116–120]. In the present work, we have computed the principal orientations of MEL through the definition of three different dihedral angles. We have observed...
that in all cases two preferential orientations arise, since the averaged angular distributions of MEL are centered around two well defined angular values, that we call "folded" and "extended" configurations of MEL, found at all cholesterol concentrations. The dihedral angle which has a better distribution regarding its fluctuations around mean values is the angle $\Psi$ represented in Fig. 6.

![Figure 6](image)

Figure 6. Two principal configurations of MEL: folded (left) and extended (right), indicated by the dihedral (torsional) angle $\Psi$. The atoms forming the melatonin molecule are: carbon (cyan), oxygen (red), hydrogen (white) and nitrogen (blue).

The torsional angle considered here is related to the nitrogen atom labelled ‘N1’ in Fig. 1, namely the nitrogen chemically bound to the hydrogen labelled ‘H15’. For this angle $\Psi$, after analysing 100 ns of equilibrated trajectories (production runs), we found averaged values corresponding to $81 \pm 10^\circ$, (folded) and $170 \pm 23^\circ$, (extended), as it is shown in Fig. 7. Further, from the distributions reported there we can observe that $\Psi$ is neatly defined and it reaches nearly the same mean value regardless of the concentration of cholesterol of the system. Interestingly, we find that the extended configuration of MEL is most favored in the case of the highest concentration 50% (green triangles), what suggests that introducing cholesterol into the system could help MEL change from its folded to its extended configuration more easily through hydrogen-bonding between MEL-DMPC and MEL-cholesterol.

In addition, according to this $\Psi$ is an excellent candidate for being used as a collective variable in metadynamics calculations[121,122] of free energy landscapes for MEL binding in biomembranes, as we will report in section 2.4.
Figure 7. Angular distributions for the selected dihedral angle $\Psi$ as a function of simulation time. Percentages of cholesterol are: 0% (black circles), 30% (red squares), and 50% (green triangles). Dashed lines indicate average values and are a guide for the eye.

2.4. Free energy profiles of small molecules and free energy hypersurfaces of melatonin binding

Once we have established preferential locations and angular distributions of the small molecules, if assuming some of these coordinates as good candidates for collective variables, we are ready to use the WTM technique in order to obtain precise, quantitative values of the free energy barriers that need to be surmounted by the small molecules to move throughout the system, mainly exchanging positions between the interfacial regions and the bulk like aqueous regions of the system. Computationally speaking, WTM is a very expensive method which requires very long trajectories so that the target subsystem, i.e. the small molecule, can move in the full configurational space, visiting regions of low energy with high probability and also regions of high energy, very unlikely to be accessed. In this work, we will complement WTM with a much simpler technique, based in the knowledge of RDF described above, namely the computation of the reversible work needed for the target to move between selected regions, indexed by one dimensional coordinate, such as a radial distance $r$. The theory has been nicely described in chapter 7 of Ref.[123]. It states that we can obtain $W_{12}(r)$ i.e. the reversible work (sometimes also known as potential of mean force, PMF) required to move two tagged particles from infinite separation to a relative separation $r$ from:

$$W_{12}(r) = -\frac{1}{\beta} \ln g_{12}(r),$$

where $\beta = 1/(k_B T)$ is the Boltzmann factor, $k_B$ the Boltzmann constant and $T$ is the temperature. $W_{12}(r)$ can be understood as the relative Helmholtz (canonical ensemble) or Gibbs (isothermal-isobaric ensemble) free energy associated to atomic pairing. The $W(r)$ found for the three small molecules are reported in Fig.8 and the quantitative estimations of the main energy barriers are reported in Table 2.
Reversible work calculations can give us a only rough approach to the size of real barriers, since it is based in the use of the interparticle distance \( r \) as the only reaction coordinate, which is known to produce some underestimation\[97\]. However, given that accurate reaction coordinates are usually unknown, very hard to obtain and multidimensional, \( W(r) \) is a reasonable way to estimate the order of magnitude of the free energy barriers. Data reported in Table 2 and in Fig.8, reveal us that the highest barrier corresponds to the pairing of ‘H1’ of TRP with ‘O2’ of DPPC. We have found that all small molecules are able to establish HB with ‘O2’ and also with the site ‘O8’ of DPPC, the latter being located deeper in the membrane (see Fig.1). Conversely, we did not find bindings between ‘H15’ site of MEL and ‘O2’ site of DPPC. The position of maxima of the first barrier are mostly centered around 2.45 Å for small molecule–‘O2’ binding, whereas barriers of ligand ‘H4’ of serotonin associated to the ‘O8’ sites are centered around 2.75 Å. In the case of SER, only a first minimum is clearly found, what indicates that SER is normally bound to the plasma membrane and it does not move to the solvent bulk.

The binding of ‘O2’ in DPPC to TRP is located at 1.75 Å, corresponding to the first minimum of the PMF between TRP and DPPC, which is of the order of the typical HB distance in water. Nevertheless, stable positions for ‘O8’ sites of DPPC are found between 1.7 and 2 Å, a remarkable wider distance. We can compare these values with the barrier for TRP (attached to a polyoleucine \( \alpha \)-helix) inside a DPPC membrane of 12 kJ/mol\[52\] or the barrier of the order of 16 kJ/mol found for TRP in a dioleoylphosphatidylcholine bilayer membrane\[55\]. Finally, the agreement of the barriers reported in

**Figure 8.** Reversible work \( W(r) \) (in \( k_B T \)) for DPPC-small molecules: TRP, SER and MEL. In the present system \( 1 k_B T \sim 2.7 \text{ kJ/mol} \). Hydrogen and oxygens of DPPC are indicated with same labels as described in Fig.1. Data partially taken from Ref. [98].
the present work (Table 2) with other neurotransmitters such as glycine, acetylcholine or glutamate, of around 2-5 kJ/mol when located close to the lipid glycerol backbone[124], is also quite remarkable.

Table 2. Free energy barriers $\Delta F$ (in kJ/mol) for the binding of small molecules to DPPC.

| Probe (Active site) | O2-DPPC | O8-DPPC |
|---------------------|---------|---------|
| H1 TRP              | 11.29   | 7.53    |
| H2 TRP              | 8.02    | 4.18    |
| H1 SER              | 7.95    | 6.83    |
| H4 SER              | 7.45    | 7.87    |
| H15 MEL             | -       | 4.85    |
| H16 MEL             | 8.03    | 1.97    |

One way of getting much more precise free energy estimations is through methods operating with multidimensional reaction coordinates. As we will explain in section 3.2, one of best methods is well tempered metadynamics., though it is a very expensive computational tool. As an specific example, we have applied WTM to the calculation of the hypersurface of free energy for the system composed by MEL and DMPC, at the three cholesterol concentrations of 0, 30 and 50 % described in section 3.1. The WTM specifications have been reported with full details in section3.2. In order to compute the three sets of two dimensional (2D) well tempered metadynamics simulations, we need to define several specific collective variables (CV) able to meaningfully describe characteristic configurations of MEL.

The results from Fig. A2 give us an indication of the convergence of WTM. To achieve this goal, we had to run trajectories of 1400 ns. These trajectories followed from the MD production runs employed to obtain structural and dynamical information.

The resulting 2D free energy surfaces (FES) of MEL bound to DMPC membranes are shown in Fig.9 and correspond to Gibbs free energy calculations. Each state has been indexed by two CV: (1) the $z$ distance between the center of mass of MEL and the center of the membrane ($z = 0$); (2) the torsional angle $\Psi$ defined and analysed in section 2.3. The inspection of Fig.9 show that regions with clear minima are present in the FES in all cases. The main features are the global minima of the FES located between $z \in [0.7, 3]$ nm and around two distinctive values for the dihedral angle, namely those around $|\Psi| \sim [70, 180^\circ]$. Such orientations are in excellent agreement with the average values of $\Psi$ obtained from ordinary MD simulations (see Fig. 7) that correspond to the two "folded" and "extended" geometries of melatonin previously reported.

The 2D free energy landscapes reveal that the most favorable stable states of melatonin binding to the membrane (basins A,B,C,D) correspond to $z$-distances around 0.8 nm at the cholesterol-free system, whereas such distance tends to increase significantly around to 1.3 nm for the 30 % cholesterol concentration and up to 2.3 nm when cholesterol reaches 50 %. As a general fact, the 2D surfaces shown in Fig.9 correspond to contour plots with values referred to a global zero. The zero of each 2D plot has been set at the highest free energy value of all, in our case corresponding to locations at the computed maxima of the coordinate $z$.

According to the CV1, MEL is preferentially located at the interface of the DMPC-cholesterol bilayer (regions with $0.8 < z < 3.0$ nm). Locations of MEL outside the interface and far enough of lipid headgroups ($z > 4.3$ nm) show very larger free energies and cannot be considered as stable states of the system. Those regions will be considered as the "bulk", i.e. the region containing the electrolyte solution surrounding the membrane. Considering the information revealed by CV2, we can distinguish two sets of minima: (1) For $|\Psi| = 67^\circ$ (basins B and C) and (2) for $|\Psi| = 180^\circ$ (basins A, and D. These minima are related to the two preferential configurations of MEL close to a DMPC-cholesterol bilayer (folded, extended) indicated above around 80 and 170$^\circ$ (see section 2.3).
Figure 9. 2D free energy landscapes $F(\Psi, z)$ (in kJ/mol) in the cholesterol-free case. Four stable state basins (A,B,C,D) are indicated.

We collected data extracted from Fig.9 in order to estimate the main free energy barriers for the main configurational changes on MEL in the quantitative side. The values are reported in Table 3.

Table 3. Free energy barriers $\Delta F$ (in kJ/mol) for the main transitions of a DMPC-bound MEL. Folded to extended corresponds to transitions between basins A and B or between C and D. Internal regions correspond to $z \sim 0$.

| Cholesterol percentage | Folded-extended | Interface-bulk | Interface to internal regions |
|------------------------|-----------------|----------------|-----------------------------|
| 0 %                    | 18.8            | 25.3           | 40.2                        |
| 30 %                   | 19.7            | 14.1           | 50.7                        |
| 50 %                   | 17.6            | 9.1            | 55.5                        |

Our findings have revealed a rather wide range of absolute free energies, in good agreement with the range reported by Jämbeck and Lyubartsev[125] for small molecules (ibuprofen, aspirin, and diclofenac) at the surroundings of lipid bilayers, of the order of free energy ranges up to 70 kJ/mol.
and barriers around 40 kJ/mol. For the sake of comparison, we should remark that the barriers of 2-10 kJ/mol reported in Table 2 obtained from PMF of Fig. 8 were related to the formation and breaking of HB, when the small molecules were located inside the interfacial region, regardless of its orientation. However, free energy barriers of orientational changes or those related to large displacements of MEL to the center of the membrane or to the extracellular bulk are much larger. For instance (see Table 3), the free energy required to exchange between folded and extended MEL configurations is very stable around 15-20 kJ/mol for all cholesterol concentrations. Florio et al. [126] using a combination of several fluorescence and spectroscopic techniques, found the conformational preferences of an isolated MEL molecule under molecular beams. These authors found MEL three \textit{trans} and two \textit{cis} conformers showing free energy gaps of about 12.5 kJ/mol, in quantitative agreement with the values reported here for orientational changes.

However, the barrier to be surmounted by MEL to move from the interface of the membrane to the extracellular fluid is strongly dependent of cholesterol concentration. We observed that it decreases with larger amounts of cholesterol, between 25 kJ/mol at the cholesterol-free case to around 10 kJ/mol for the 50% concentration. Finally, the probability for MEL to access the central, hydrophobic regions of the membrane is scarce, since it will require to surpass free energy barriers of more than 40 kJ/mol. This will make very difficult to observe transmembrane crossings in the simulated scale of 1 \( \mu \)s. In a recent work by Wang and coworkers[127], small solutes such as glycerol, caffeine, isopropanol, or ethosuximide were simulated nearby a model cell membrane. These authors found that, in order to observe transmembrane crossings of such small solutes in the time length of a simulation at the atomic level of description, they needed to run trajectories of 10 \( \mu \)s at low temperatures (310 to 330 K) or, alternatively, raise the temperatures to more than 400 K (for simulation times of 1 \( \mu \)s). In our case, we did not record any transition of MEL between the two sides of the DMPC membrane along the simulated trajectory.

2.5. Diffusion coefficients of small molecules: tryptophan and melatonin

Microscopic translational dynamics of tryptophan and melatonin has been considered. We have evaluated the mean square displacement (MSD) of the carbon 'C2' in TRP (see Fig. 1) and of the center of mass of MEL. From the long time slopes of both MSD, we obtained the corresponding self diffusion coefficients \( D \) through Einstein formula of Brownian motion:

\[
D = \lim_{t \to \infty} \frac{\langle |\mathbf{r}_i(t) - \mathbf{r}_i(0)|^2 \rangle}{2d\Delta t},
\]

where \( \mathbf{r}_i(t) \) is the instantaneous position of particle \( i \). In this general procedure the spatial dimension of the diffusion regions \( d \) is considered. TRP and MEL showed lateral like diffusion (\( d = 2 \)). The results have been summarised in Table 4.

**Table 4.** Self diffusion coefficients \( D \) (in \( 10^{-7} \) cm\(^2\)/s) of TRP and MEL in systems with different cholesterol percentages. Estimated errors in parenthesis.

| Small molecule | 0% CHOL | 30% CHOL | 50% CHOL |
|---------------|---------|----------|----------|
| TRP           | 3.48(0.80) | 2.91(0.35) | 14.0(0.2) |
| MEL           | 1.1(0.4)   | 3.9(0.6)  | 4.1(0.9)  |

The main finding is that self-diffusion coefficients \( D \) for TRP (Table 4) are between 3-14 \( \times 10^{-7} \) cm\(^2\)/s which, even within the same order of magnitude, are significantly larger than those of the diffusion of DMPC molecules[6] (0.6 \( \times 10^{-7} \) in absence of cholesterol). The main trend is that \( D \) increases for rising cholesterol concentrations. Overall, we find that the mobility of TRP is significantly higher than that of DMPC. Nevertheless, the effect of temperature is remarkable here, since at complementary
simulations at 310 K, TRP diffusion was of about $2 \times 10^{-7}$, i.e. significantly slower given the gel like state of the membrane in such a case.

In the case of MEL, $D$ also shows a tendency to increase when cholesterol is mixed with DMPC, regardless of its concentration. In Table 4, at 30% cholesterol the value of $D$ for MEL is six times larger than the value of $D$ of DMPC molecules in pure DMPC bilayer membrane systems [6]. This fact would suggest that its mechanisms of diffusion may be similar to those of an individual particle (such as in Fickian diffusion) and qualitatively different of those of lipids, whose diffusion was observed to occur in a sort of collective way, associated in local groups of a few units (around 5-10 units) [6].

3. Methods

3.1. Molecular dynamics

We have performed seven independent series of MD simulations for TRP, SER and MEL in different environments (DMPC, DPPC and different concentrations of CHOL, namely: 0%, 30% and 50% for TRP and MEL, whereas for SER only the cholesterol-free membrane was simulated. Each system contains a total of 204 lipid and/or cholesterol molecules fully solvated by $\sim 5,000-10,000$ TIP3P water molecules and 17-21 sodium chloride pairs at the human body concentration (0.15 M), yielding a system size of about 40,000-60,000 atoms. The characteristics of all simulations are summarised in Table 5.

| Phospholipids | Small molecule | Waters | Total length (ns) | Temperature (K) | Ion pairs |
|---------------|----------------|--------|-------------------|----------------|-----------|
| 204 DPPC TRP  | 4962           | 800    | 323.15            | 17 Na$^+$ + 17 Cl$^-$ |
| 204 DPPC SER  | 4962           | 800    | 323.15            | 17 Na$^+$ + 17 Cl$^-$ |
| 204 DMPC MEL  | 10250          | 800    | 303.15            | 21 Na$^+$ + 21 Cl$^-$ |

Our MD inputs were created with the CHARMM-GUI web-based tool [128]. All systems were simulated at the isobaric-isothermal ensemble. i.e. at constant number of particles (N), pressure (P) and temperature (T) conditions, with equilibration periods for all simulations of more than 200 ns. After equilibration, we recorded statistically meaningful trajectories of more than 600 ns. A typical size of the system was of $80 \times 80 \times 81$ Å. The simulation time step was of 2 fs in all cases. Given its ability to reproduce area per lipid of DMPC and DPPC in excellent agreement with experimental data, the CHARMM36 force field [129,130] was used. All bonds involving hydrogens were fixed to constant length, allowing fluctuations of bond distances and all sorts of angles for the remaining atoms. Van der Waals interactions were cut off at 12 Å with a smooth switching function starting at 10 Å. Long ranged electrostatic forces were taken into account by means of the particle mesh Ewald method [131], with a grid space of about 1 Å and updated every time step. Periodic boundary conditions were considered in each spatial direction.

3.2. Well tempered Metadynamics

As we pointed out above, obtaining free energy profiles and estimating the height of the main barriers between stable states is a very difficult task in condensed matter systems [132]. In the present work, we have presented in Section 2.4 two possible pathways to do the job: (1) using a direct method based in the reversible work theorem, but knowing that it is, at its best, a first approach to the real barriers and (2) employing a more sophisticated tool called “metadynamics” that, given a well chosen set of a few reaction coordinates (the collective variables), is able to provide a much more exact picture of the free energy hypersurface. The method was initially proposed by Huber et al. [133] and Grubmüller [134] and later on developed by Laio and Parrinello [99,121] as a method to explore
multidimensional free energy surfaces as a function of a \textit{a priori} unknown CV. Given some deficiencies of the original method, well tempered metadynamics\cite{122,135} was introduced. In the present work we have run 1.4 $\mu$s well-tempered metadynamics simulations in order to get Gibbs free energies of the binding states of MEL at phospholipid membrane surfaces made by DMPC lipids and CHOL in sodium chloride aqueous solution. Starting from the long trajectories generated by unbiased MD simulations for MEL-DMPC, we could make a reliable guess of two potentially appropriate CV. All the metadynamics simulations were carried out by means of the PLUMED2 plugin\cite{136,137} within the joint GROMACS/2018.3-plumed tool and were performed in the NPT ensemble. The particular details of the WTM simulations are reported in Table 6. Usual periodic boundary conditions in all directions of space were considered.

| Parameter                                    | 0%     | 30%    | 50%    |
|----------------------------------------------|--------|--------|--------|
| Gaussian width of CV1 [nm]                   | 0.30   | 0.30   | 0.25   |
| Gaussian width of CV2 [degrees]              | 20     | 20     | 20     |
| Starting (Gaussian) hill [kJ/mol]            | 1.0    | 1.0    | 1.0    |
| Deposition stride [ps]                        | 1      | 1      | 1      |
| Bias factor                                  | 10     | 10     | 20     |
| Simulation time [ns]                          | 1100   | 1400   | 1400   |

4. Conclusions

The interactions of some small molecules with human cells are undoubtedly a relevant field of research. In particular, the hormone melatonin has an important role in the treatment of a wide variety of diseases and problems related to sleep. It works as a regulator of circadian rhythms and as an antioxidative. Further, its precursor serotonin is a neurotransmitter playing a key role in a variety of physiological processes and in the regulation of mood and cognitive learning. Serotonin is synthesised by the body from its precursor, the essential aminoacid tryptophan. Tryptophan is a zwitterion, with a protonated amino group (NH$_3^+$) and a deprotonated carboxylic acid (COO$^-$) and it is used as an antidepressant. In the present work, we are reviewing a series of MD and WTM simulations of different lipid bilayer membranes in an aqueous ionic solution of NaCl with embedded small molecules. The calculations have been performed using the CHARMM36 force field. Among them, cholesterol at two concentrations (30% and 50%) has been considered together with the cholesterol-free reference systems in order to explore the influence of CHOL concentrations on the properties of the small molecules.

In a preliminary study on the adsorption of tryptophan at a DPPC bilayer membrane at 310.15 K (gel phase)\cite{138}, we observed a strong first coordination shell for TRP-water and TRP-DPPC pairs. In this study we focussed in the liquid phase and found relevant changes in local structure and dynamics of TRP only for cholesterol concentrations above 30%. TRP-DPPC binding involved coordination shells for the different oxygen sites of DPPC able to associate (‘O2’ and ‘O8’) versus the two tagged hydrogens (‘H1’ and ‘H2’) in TRP. Also, the distribution functions of TRP-CHOL revealed very stable hydrogen bonding. TRP is able to establish strong interactions with all solvating particles (water, DPPC and CHOL) including a sort of double bridge between DPPC and cholesterol species. Typical HB distances have been found to be around 1.7-2.0 Å, in good agreement with experimental data\cite{139}. Finally, self diffusion coefficients of TRP are of the order of $10^{-7}$ cm$^2$/s, being strongly dependent of cholesterol’s concentration.

In the case of melatonin, we have simulated its behavior when embedded in a cholesterol rich DMPC membrane at 303 K and 1 atm. Our interest was firstly focused on the local structure and angular distributions of MEL. In a similar fashion as in the case of TRP, strong hydrogen bonds between MEL-DMPC and MEL-CHOL have been found. The most important structures of MEL have been observed for two angular configurations: “folded” and “extended”. Using a particular dihedral
angle ($\Psi$) we observed two preferential values. The angle $\Psi$ was revealed to potentially work as a reliable reaction coordinate, since two neat angular distributions around $\sim 81^\circ$ and $\sim 170^\circ$ were clearly distinguished. We also observed that introducing cholesterol into the system can favor MEL to exchange between extended and folded configurations. Again, the self diffusion coefficient of MEL was found to be of the order of $10^{-7}$ cm$^2$/s, although in this case with a very mild dependence on cholesterol’s concentration.

Free energy barriers for serotonin, melatonin and tryptophan at 323.15 K and 1 atm have been analysed using the reversible work theorem, which provides us a simple way to estimate the height of the barriers related to the interatomic distances. These features will be directly related to the formation and breaking of hydrogen-bonds. We have found marked first and second coordination shells corresponding to two minima of the PMF, with energy barriers for TRP-DPPC of the order of 10 kJ/mol. Most remarkable have been the binding between hydrogen ‘H1’ of TRP and oxygens ‘O2’ and ‘O8’ of DPPC. In the case of serotonin, we have found it to be a molecule strongly anchored at the membrane unlike to be solvated by water. Interestingly, melatonin has revealed to be able to interact both with water and DPPC, still showing moderately strong free energy barriers. In order to get more precise information, we have conducted well-tempered metadynamics simulations, and obtained 2D free energy landscapes for MEL binding to the DMPC-CHOL membranes. Two CVs have been considered: a dihedral angle $\Psi$ and the distance $z$ between the center of mass of MEL and the center of the lipid bilayer (set at $z = 0$). From our results, we have found that MEL is usually bound to the external side of the membrane, at distances $z \sim 1$ - 2 nm and in two main configurations with $\Psi = 70^\circ$ (folded) and $180^\circ$ (extended), with an energetic cost for the exchange between the two conformations of about 15-20 kJ/mol. After CHOL is introduced into the system, it pushes MEL to escape outside the interfacial region of the membrane and to move away until it is fully solvated by the aqueous ionic solution. The energetic cost for MEL to leave the interface of the membrane towards the water bulk ($z$-distances around 4 nm), has been estimated at $\sim 10$-25 kJ/mol. A very uncommon situation in our simulations was that of MEL accessing the center of the membrane, an energetically expensive process (free energy barriers of 40 kJ/mol). We believe that the findings presented in this work could be of practical use in two ways: (1) for the design of new reaction coordinates in similar systems of small molecules of biochemical interest such as amino acids, neurotransmitters, drugs or hormones and (2) from a more general perspective, to contribute the unveiling of the microscopic interactions of small molecules with cell membranes and to the key role played by cholesterol in the properties of such molecules. All this can lead to advances in the research of new pharmaceutical compounds and to a better understanding of the currently available ones.

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**Abbreviations**

The following abbreviations are used in this manuscript:
Appendix A. Supporting information

Appendix A.1. Convergence of MD simulations

Here we can see how full convergence is obtained for the two relevant RDF, related to TRP-water and TRP-DPPC binding. After 50 ns (initial setup + full equilibration) and 100 ns (50 ns of production) the results are qualitatively similar but not fully converged yet, but when we extend to 120 ns, the results are virtually the same as those of 100 ns. Remarkably, the TRP-water structures quickly converged, because of the large amount of statistics (+5000 waters, stable hydration shells of TRP). This show that 100 ns is a reasonable length to achieve fully equilibrated results in the present case.

Figure A1. Convergence of MD simulations through RDF profiles as a function of simulation time for the production run of TRP in the DPPC and CHOL model membrane.
Appendix A.2. Convergence of WTM simulations

Finally, to further evaluate the convergence of the metadynamics simulations, we reported the time cumulative average of 1D free energy profiles as defined in a previous work (see formula S2 in Ref.[140]), i.e. averaging the two leaflets and projecting onto (integrating out) the alternative CV in a range larger than 1 microsecond in all cases. We have taken the case of 30% concentration of CHOL as an example. From the results of Figure A2, we can see that after long cumulative time lengths, the differences between a profile and the one immediately before are very small (up to 2.5 kJ/mol) and lead us to fully converged free energies for the two CV considered in the present study.

Figure A2. Time cumulative free energy profiles at the 30 % cholesterol system. Bottom: CV1, top: CV2.

References

1. Nagle, J.F.; Tristram-Nagle, S. Structure of lipid bilayers. Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes 2000, 1469, 159–195.
2. Mouritsen, O.G. Life-as a matter of fat; Springer, 2005.
3. Bassolino-Klimas, D.; Alper, H.E.; Stouch, T.R. Solute diffusion in lipid bilayer membranes: an atomic level study by molecular dynamics simulation. Biochemistry 1993, 32, 12624–12637.
4. Berneche, S.; Nina, M.; Roux, B. Molecular dynamics simulation of melittin in a dimyristoylphosphatidylcholine bilayer membrane. Biophysical journal 1998, 75, 1603–1618.
5. Högberg, C.J.; Lyubarov, A.P. A molecular dynamics investigation of the influence of hydration and temperature on structural and dynamical properties of a dimyristoylphosphatidylcholine bilayer. The Journal of Physical Chemistry B 2006, 110, 14326–14336.
6. Yang, J.; Calero, C.; Martí, J. Diffusion and spectroscopy of water and lipids in fully hydrated dimyristoylphosphatidylcholine bilayer membranes. The Journal of chemical physics 2014, 140, 03B606_1.
7. Mabrey, S.; Sturtevant, J.M. Investigation of phase transitions of lipids and lipid mixtures by sensitivity
differential scanning calorimetry. *Proceedings of the National Academy of Sciences* 1976, 73, 3862–3866.
8. Almeida, P.F.; Vaz, W.L.; Thompson, T. Lateral diffusion in the liquid phases of
dimyristoylphosphatidylcholine/cholesterol lipid bilayers: a free volume analysis. *Biochemistry* 1992, 31, 6739–6747.
9. Clerc, S.G.; Thompson, T.E. Permeability of dimyristoyl phosphatidylcholine/dipalmitoyl
phosphatidylcholine bilayer membranes with coexisting gel and liquid-crystalline phases. *Biophysical
journal* 1995, 68, 2333–2341.
10. Kučerka, N.; Kiselev, M.A.; Balgavý, P. Determination of bilayer thickness and lipid surface area
in unilamellar dimyristoylphosphatidylcholine vesicles from small-angle neutron scattering curves: a
comparison of evaluation methods. *European Biophysics Journal* 2004, 33, 328–334.
11. Edholm, O.; Nagle, J.F. Areas of molecules in membranes consisting of mixtures. *Biophysical Journal* 2005,
89, 1827–1832.
12. Vaz, W.L.; Clegg, R.M.; Hallmann, D. Translational diffusion of lipids in liquid crystalline phase
phosphatidylcholine multibilayers. A comparison of experiment with theory. *Biochemistry* 1985,
24, 781–786.
13. Tocanne, J.F.; Teissié, J. Ionization of phospholipids and phospholipid-supported interfacial lateral diffusion
of protons in membrane model systems. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes* 1990,
1031, 111–142.
14. McLaughlin, S.; Murray, D. Plasma membrane phosphoinositide organization by protein electrostatics.
*Nature* 2005, 438, 605–611.
15. Ingólfssson, H.I.; Melo, M.N.; Van Eerden, F.J.; Arnarez, C.; Lopez, C.A.; Wassenaar, T.A.; Periole, X.;
De Vries, A.H.; Tieleman, D.P.; Marrink, S.J. Lipid organization of the plasma membrane. *Journal of the american chemical society* 2014, 136, 14554–14559.
16. Zhang, Y.; Chen, X.; Gueydan, C.; Han, J. Plasma membrane changes during programmed cell deaths. *Cell research* 2018, 28, 9–21.
17. Krapf, D. Compartmentalization of the plasma membrane. *Current opinion in cell biology* 2018, 53, 15–21.
18. Zhang, J.; Jin, R.; Jiang, D.; Chen, H.Y. Electrochemiluminescence-based capacitance microscopy for
label-free imaging of antigens on the cellular plasma membrane. *Journal of the American Chemical Society* 2019, 141, 10294–10299.
19. Wyatt, R.; Kupfer, D.; Sjoerdma, A.; Engelma, K.; Fram, D.; Snyder, F. Effects of L-tryptophan (a natural
sedative) on human sleep. *The lancet* 1970, 296, 842–846.
20. Spinweber, C.L. L-tryptophan administered to chronic sleep-onset insomniacs: late-appearing reduction of
sleep latency. *Psychopharmacology* 1986, 90, 151–155.
21. Jouvet, M. Sleep and serotonin: an unfinished story. *Neuropsychopharmacology* 1999, 21, 245–275.
22. Słomińsk; A.; Semak, I.; Pisarchik, A.; Siewinmac, T.; Szczesniewski, A.; Wortsman, J. Conversion of
L-tryptophan to serotonin and melatonin in human melanoma cells. *FEBS letters* 2002, 511, 102–106.
23. Wang, L.; Erlandsen, H.; Haavik, J.; Knappskog, P.M.; Stevens, R.C. Three-dimensional structure of human
tryptophan hydroxylation and its implications for the biosynthesis of the neurotransmitters serotonin and
melatonin. *Biochemistry* 2002, 41, 12569–12574.
24. Paredes, S.D.; Barriga, C.; Reiter, R.J.; Rodríguez, A.B. Assessment of the potential role of tryptophan as
the precursor of serotonin and melatonin for the aged sleep-wake cycle and immune function: Streptopelia
risoria as a model. *International Journal of Tryptophan Research* 2009, 2, IJTR–S1129.
25. Huether, G.; Kochen, W.; Simat, T.J.; Steinhart, H. *Tryptophan, serotonin, and melatonin: Basic aspects and
applications*; Vol. 467, Springer Science & Business Media, 2012.
26. Yu, H.S. *Melatonin in the eye: functional implications*; Vol. 365, CRC Press, Boca Raton, FL, 1993.
27. Mockus, S.M.; Vrana, K.E. Advances in the molecular characterization of tryptophan hydroxylation. *Journal of Molecular Neuroscience* 1998, 10, 163–179.
28. Kema, I.P.; de Vries, E.G.; Muskiet, F.A. Clinical chemistry of serotonin and metabolites. *Journal of Chromatography B: Biomedical Sciences and Applications* 2000, 747, 33–48.
29. Lerner, A.; Case, J.; Takahashi, Y.; Lee, T.; Mori, W. Isolation of melatonin, a pineal factor that lights
melanocytes. *J Am Chem Soc* 1958, 80, 2057–2058.
30. Mousavi, S.S.; Shohrati, M.; Vahedi, E.; Abdollahpour-Alitappeh, M.; Panahi, Y. Effect of melatonin administration on sleep quality in sulfur mustard exposed patients with sleep disorders. *Iranian journal of pharmaceutical research* 2018, 17, 136.

31. Savoca, A.; Manca, D. Physiologically-based pharmacokinetic simulations in pharmacotherapy: selection of the optimal administration route for exogenous melatonin. *ADMET and DMPK* 2019, 7, 44–59.

32. Kostoglou-Athanassiou, I. Therapeutic applications of melatonin. *Therapeutic advances in endocrinology and metabolism* 2013, 4, 13–24.

33. Cutolo, M.; Sulli, A.; Pizzorni, C.; Secchi, M.E.; Soldano, S.; Seriolo, B.; Straub, R.H.; Otsa, K.; Maestroni, G.J. Circadian rhythms: glucocorticoids and arthritis. *Annals of the New York Academy of Sciences* 2006, 1069, 289–299.

34. Forrest, C.M.; Mackay, G.M.; Stoy, N.; Stone, T.W.; Darlington, L.G. Inflammatory status and kynurenine metabolism in rheumatoid arthritis treated with melatonin. *British journal of clinical pharmacology* 2007, 64, 517–526.

35. Bang, J.; Chang, H.W.; Jung, H.R.; Cho, C.H.; Hur, J.A.; Lee, S.I.; Choi, T.H.; Kim, S.H.; Ha, E. Melatonin attenuates clock gene Cryptochrome1, which may aggravates mouse anti-type II collagen antibody-induced arthritis. *Rheumatology international* 2012, 32, 379–385.

36. Huang, C.C.; Chiou, C.H.; Liu, S.C.; Hu, S.L.; Su, C.M.; Tsai, C.H.; Tang, C.H. Melatonin attenuates TNF-α and IL-1β expression in synovial fibroblasts and diminishes cartilage degradation: Implications for the treatment of rheumatoid arthritis. *Journal of pineal research* 2019, 66, e12560.

37. Rusanova, I.; Martínez-Ruiz, L.; Florido, J.; Rodríguez-Santana, C.; Guerra-Libero, A.; Acuña-Castroviejo, D.; Escames, G. Protective effects of melatonin on the skin: Future perspectives. *International journal of molecular sciences* 2019, 20, 4948.

38. Slominski, A.T.; Zmijewski, M.A.; Semak, I.; Kim, T.K.; Janjetovic, Z.; Slominski, R.M.; Zmijewski, J.W. Melatonin, mitochondria, and the skin. *Cellular and molecular life sciences* 2017, 74, 3913–3925.

39. Slominski, A.T.; Hardeland, R.; Zmijewski, M.A.; Slominski, R.M.; Reiter, R.J.; Paus, R. Melatonin: a cutaneous perspective on its production, metabolism, and functions. *Journal of Investigative Dermatology* 2018, 138, 490–499.

40. Hussain, S.A.R. Effect of melatonin on cholesterol absorption in rats. *Journal of pineal research* 2007, 42, 267–271.

41. Costa, E.; Lopes, R. Lamy–Freund, MT, Permeability of pure lipid bilayers to melatonin. *Expert opinion on investigational drugs* 2015, 17, 273–281.

42. Bongiorno, D.; Ceraulo, L.; Ferrugia, M.; Filizzola, F.; Ruggirello, A.; Liveri, V.T. Localization and interactions of melatonin in dry cholesterol/lecithin mixed reversed micelles used as cell membrane models. *Journal of pineal research* 2005, 38, 292–298.

43. Acuña-Castroviejo, D.; Escames, G.; Macías, M.; Muñoz Hoyos, A.; Carballo Molina, A.; Arauzo, M.; Montes, R.; Vives, F. Minireview: cell protective role of melatonin in the brain. *Journal of pineal research* 1995, 19, 57–63.

44. Maestroni, G.J. The immunotherapeutic potential of melatonin. *Expert opinion on investigational drugs* 2001, 10, 467–476.

45. Dies, H.; Toppozini, L.; Rheinstädter, M.C. The interaction between amyloid-β peptides and anionic lipid membranes containing cholesterol and melatonin. *PLoS One* 2014, 9, e99124.

46. Zhang, R.; Wang, X.; Ni, L.; Di, X.; Ma, B.; Niu, S.; Liu, C.; Reiter, R.J. COVID-19: Melatonin as a potential adjuvant treatment. *Life sciences* 2020, 250, 117583.

47. Pandi-Perumal, S.R.; Srinivasan, V.; Maestroni, G.; Cardinale, D.; Poeggeler, B.; Hardeland, R. Melatonin: nature’s most versatile biological signal? *The FEBS journal* 2006, 273, 2813–2838.

48. Hardeland, R.; Cardinale, D.P.; Srinivasan, V.; Spence, D.W.; Brown, G.M.; Pandi-Perumal, S.R. Melatonin—A pleiotropic, orchestrating regulator molecule. *Progress in neurobiology* 2011, 93, 350–384.

49. Tan, D.X.; Manchester, L.C.; Esteban-Zubero, E.; Zhou, Z.; Reiter, R.J. Melatonin as a potent and inducible endogenous antioxidant: synthesis and metabolism. *Molecules* 2015, 20, 18886–18906.

50. Severcan, F.; Sahin, I.; Kazanci, N. Melatonin strongly interacts with zwitterionic model membranes—evidence from Fourier transform infrared spectroscopy and differential scanning calorimetry. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 2005, 1668, 215–222.
76. Rodriguez, J.; Elola, M.D.; Martí, J.; Laria, D. Surface behavior of aprotic mixtures: dimethyl sulfoxide/acetonitrile. The Journal of Physical Chemistry C 2017, 121, 14618–14627.
77. MacKerell Jr, A.D.; Banavali, N.K. All-atom empirical force field for nucleic acids: II. Application to molecular dynamics simulations of DNA and RNA in solution. Journal of computational chemistry 2000, 21, 105–120.
78. Ponomarev, S.Y.; Thayer, K.M.; Beveridge, D.L. Ion motions in molecular dynamics simulations on DNA. Proceedings of the National Academy of Sciences 2004, 101, 14771–14775.
79. Johnson, R.R.; Johnson, A.C.; Klein, M.L. Probing the structure of DNA- carbon nanotube hybrids with molecular dynamics. Nano letters 2008, 8, 69–75.
80. Van der Ploeg, P.; Berendsen, H. Molecular dynamics simulation of a bilayer membrane. The Journal of Chemical Physics 1982, 76, 3271–3276.
81. Egberts, E.; Marrink, S.J.; Berendsen, H.J. Molecular dynamics simulation of a phospholipid membrane. European biophysics journal 1994, 22, 423–436.
82. Berkowitz, M.L. Detailed molecular dynamics simulations of model biological membranes containing cholesterol. Biochimica et Biophysica Acta (BBA)-Biomembranes 2009, 1788, 86–96.
83. Gedeon, P.C.; Indarte, M.; Surratt, C.K.; Madura, J.D. Molecular dynamics of leucine and dopamine transporter proteins in a model cell membrane lipid bilayer. Proteins: Structure, Function, and Bioinformatics 2010, 78, 797–811.
84. Venable, R.M.; Kramer, A.; Pastor, R.W. Molecular dynamics simulations of membrane permeability. Chemical reviews 2019, 119, 5954–5997.
85. Senn H.M., T.W. QM/MM methods for biological systems. In: Atomistic approaches in modern biology; Springer-Verlag Berlin Heidelberg, 2006; pp. 173–290.
86. Martí, J.; Csajka, F.S.; Chandler, D. Stochastic transition pathways in the aqueous sodium chloride dissociation process. Chemical Physics Letters 2000, 328, 169–176.
87. Martí, J.; Csajka, F. The aqueous solvation of sodium chloride: A Monte Carlo transition path sampling study. The Journal of Chemical Physics 2000, 113, 1154–1161.
88. Geissler, P.L.; Dellago, C.; Chandler, D.; Hutter, J.; Parrinello, M. Autoionization in liquid water. Science 2001, 291, 2121–2124.
89. Martí, J. Transition path sampling study of the local molecular structure in the aqueous solvation of sodium chloride. Molecular Simulation 2001, 27, 169–185.
90. Dellago, C.; Bolhuis, P.G.; Geissler, P.L. Transition path sampling. Advances in Chemical Physics 2002, 123, 1–78.
91. Martí, J.; Csajka, F.S. Transition path sampling study of flip-flop transitions in model lipid bilayer membranes. Physical Review E 2004, 69, 061918.
92. Dellago, C.; Bolhuis, P.G. Transition path sampling simulations of biological systems. In Atomistic Approaches in Modern Biology; Springer, 2006; pp. 291–317.
93. Henin, J.; Fiorin, G.; Chipot, C.; Klein, M.L. Exploring multidimensional free energy landscapes using time-dependent biases on collective variables. Journal of Chemical Theory and Computation 2009, 6, 35–47.
94. Mezei, M. Adaptive umbrella sampling: Self-consistent determination of the non-Boltzmann bias. Journal of Computational Physics 1987, 68, 237–248.
95. Bartels, C.; Karplus, M. Multidimensional adaptive umbrella sampling: Applications to main chain and side chain peptide conformations. Journal of Computational Chemistry 1997, 18, 1450–1462.
96. Calero, C.; Martí, J.; Guàrdia, E.; Masia, M. Characterization of the methane–graphene hydrophobic interaction in aqueous solution from ab initio simulations. Journal of Chemical Theory and Computation 2013, 9, 5070–5075.
97. Trzesniak, D.; Kunz, A.P.E.; van Gunsteren, W.F. A comparison of methods to compute the potential of mean force. ChemPhysChem 2007, 8, 162–169.
98. Lu, H.; Martí, J. Binding free energies of small-molecules in phospholipid membranes: Aminoacids, serotonin and melatonin. Chemical Physics Letters 2018, 712, 190–195.
99. Barducci, A.; Bonomi, M.; Parrinello, M. Metadynamics. Wiley Interdisciplinary Reviews: Computational Molecular Science 2011, 1, 826–843.
100. Bussi, G.; Gervasio, F.L.; Laio, A.; Parrinello, M. Free-energy landscape for β hairpin folding from combined parallel tempering and metadynamics. Journal of the American Chemical Society 2006, 128, 13435–13441.
101. Deighan, M.; Bonomi, M.; Pfaendtner, J. Efficient simulation of explicitly solvated proteins in the well-tempered ensemble. *Journal of Chemical Theory and Computation* 2012, 8, 2189–2192.

102. Palmer, J.C.; Car, R.; Debenedetti, P.G. The liquid–liquid transition in supercooled ST2 water: a comparison between umbrella sampling and well-tempered metadynamics. *Faraday Discussions* 2013, 167, 77–94.

103. Haldar, S.; Kührová, P.; Banáš, P.; Spiwok, V.; Sponer, J.; Hobza, P.; Otyepka, M. Insights into stability and folding of GNRA and UNCG tetraloops revealed by microsecond molecular dynamics and well-tempered metadynamics. *Journal of Chemical Theory and Computation* 2015, 11, 3866–3877.

104. Marti, J. Free-energy surfaces of ionic adsorption in cholesterol-free and cholesterol-rich phospholipid membranes. *Molecular Simulation* 2018, 44, 1136–1146.

105. Poger, D.; Mark, A.E. On the validation of molecular dynamics simulations of saturated and cis-monounsaturated phosphatidylcholine lipid bilayers: a comparison with experiment. *Journal of Chemical Theory and Computation* 2010, 6, 325–336.

106. Pandey, P.R.; Roy, S. Headgroup mediated water insertion into the DPPC bilayer: a molecular dynamics study. *J. Phys. Chem. B* 2011, 115, 3155–3163.

107. Lu, H.; Martí, J. Effects of cholesterol on the binding of the precursor neurotransmitter tryptophan to zwitterionic membranes. *The Journal of chemical physics* 2018, 149, 164906.

108. Lu, H.; Martí, J. Binding and dynamics of melatonin at the interface of phosphatidylcholine-cholesterol membranes. *PloS one* 2019, 14, e0224624.

109. Prakash, P.; Zhou, Y.; Liang, H.; Hancock, J.F.; Gorfe, A.A. Oncogenic K-Ras binds to an anionic membrane in two distinct orientations: a molecular dynamics analysis. *Biophysical journal* 2019, 116, 179–183.
123. Chandler, D. *Introduction to Modern Statistical Mechanics*; Vol. 40, Oxford University Press, Oxford, UK, 1987.

124. Peters, G.H.; Werge, M.; Elf-Lind, M.N.; Madsen, J.J.; Velardez, G.F.; Westh, P. Interaction of neurotransmitters with a phospholipid bilayer: A molecular dynamics study. *Chemistry and physics of lipids* 2014, 184, 7–17.

125. Jämbeck, J.P.; Lyubartsev, A.P. Exploring the free energy landscape of solutes embedded in lipid bilayers. *The journal of physical chemistry letters* 2013, 4, 1781–1787.

126. Florio, G.M.; Christie, R.A.; Jordan, K.D.; Zwier, T.S. Conformational preferences of jet-cooled melatonin: Probing trans-and cis-amide regions of the potential energy surface. *Journal of the American Chemical Society* 2002, 124, 10236–10247.

127. Wang, Y.; Gallagher, E.; Jorgensen, C.; Troendle, E.P.; Hu, D.; Searson, P.C.; Ulmschneider, M.B. An experimentally validated approach to calculate the blood-brain barrier permeability of small molecules. *Scientific reports* 2019, 9, 1–11.

128. Jo, S.; Kim, T.; Iyer, V.G.; Im, W. CHARMM-GUI: a web-based graphical user interface for CHARMM. *Journal of computational chemistry* 2008, 29, 1859–1865.

129. Klauda, J.B.; Venable, R.M.; Freites, J.A.; O’Connor, J.W.; Tobias, D.J.; Mondragon-Ramirez, C.; Vorobyov, I.; MacKerell Jr, A.D.; Pastor, R.W. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. *The journal of physical chemistry B* 2010, 114, 7830–7843.

130. Lim, J.B.; Rogaski, B.; Klauda, J.B. Update of the cholesterol force field parameters in CHARMM. *The Journal of Physical Chemistry B* 2012, 116, 203–210.

131. Essmann, U.; Perera, L.; Berkowitz, M.L.; Darden, T.; Lee, H.; Pedersen, L.G. A smooth particle mesh Ewald method. *The Journal of chemical physics* 1995, 103, 8577–8593.

132. Ytreberg, F.M.; Swendsen, R.H.; Zuckerman, D.M. Comparison of free energy methods for molecular systems. *The Journal of chemical physics* 2006, 125, 184114.

133. Huber, T.; Torda, A.E.; Van Gunsteren, W.F. Local elevation: a method for improving the searching properties of molecular dynamics simulation. *Journal of computer-aided molecular design* 1994, 8, 695–708.

134. Grubmüller, H. Predicting slow structural transitions in macromolecular systems: Conformational flooding. *Physical Review E* 1995, 52, 2893.

135. Bonomi, M.; Parrinello, M. Enhanced sampling in the well-tempered ensemble. *Physical review letters* 2010, 104, 190601.

136. Bonomi, M.; Branduardi, D.; Bussi, G.; Camilloni, C.; Provasi, D.; Raiteri, P.; Donadio, D.; Marinelli, F.; Pietrucci, F.; Broglia, R.A.; others. PLUMED: A portable plugin for free-energy calculations with molecular dynamics. *Computer Physics Communications* 2009, 180, 1961–1972.

137. Tribello, G.A.; Bonomi, M.; Branduardi, D.; Camilloni, C.; Bussi, G. PLUMED 2: New feathers for an old bird. *Computer Physics Communications* 2014, 185, 604–613.

138. Martí, J.; Lu, H. Molecular dynamics of di-palmitoyl-phosphatidyl-choline biomembranes in ionic solution: adsorption of the precursor neurotransmitter tryptophan. *Procedia computer science* 2017, 108, 1242–1250.

139. Liu, H.; Zhang, H.; Jin, B. Fluorescence of tryptophan in aqueous solution. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 2013, 106, 54–59.

140. Lu, H.; Martí, J. Long-lasting Salt Bridges Provide the Anchoring Mechanism of Oncogenic Kirsten Rat Sarcoma Proteins at Cell Membranes. *The Journal of Physical Chemistry Letters* 2020, 11, 9938–9945.

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