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# Negatively Charged Gangliosides Promote Membrane Association of Amphipathic Neurotransmitters

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## Abstract

Lipophilic neurotransmitters (NTs) such as dopamine are chemical messengers enabling neurotransmission by adhering onto the extracellular surface of a post-synaptic membrane in a synapse, followed by binding to their receptors. Previous studies have shown that the strength of the NT-membrane association is dependent on the lipid composition of the membrane. Negatively charged lipids such as phosphatidylserine, phosphatidylglycerol, and phosphatidic acid have been indicated to promote NT-membrane binding, however these anionic lipids reside almost exclusively in the intracellular leaflet of the post-synaptic membrane instead of the extracellular leaflet facing the synaptic cleft. Meanwhile, the extracellular leaflet is relatively rich in biologically relevant anionic gangliosides such as monosialotetrahexosylganglioside (GM1), yet the role of gangliosides in NT-membrane association is not clear. Here, we explored the role of GM1 in modulating the binding of dopamine and histamine (as amphipathic/cationic NTs) as well as acetylcholine (as a hydrophilic/cationic NT) with the post-synaptic membrane surface. Atomistic molecular dynamics simulations and free energy calculations indicated that GM1 fosters membrane association of histamine and dopamine. For acetylcholine, this effect was not observed. The *in silico* results suggest that gangliosides form a charge-based vestibule in front of the post-synaptic membrane, attracting amphipathic NTs to the vicinity of the membrane. Yet the results also stress the importance to understand the significance of the structural details of NTs, as exemplified by the GM1-acetylcholine interaction. In a larger context, the NT-membrane adherence, coupled to lateral diffusion in the membrane plane, is proposed to improve neurotransmission efficiency by advancing NT entry into the membrane-embedded ligand-binding sites.

## Key words

Dopamine, acetylcholine, histamine, neurotransmitter, neurotransmission, cholesterol, monosialotetrahexosylganglioside (GM1), molecular dynamics (MD), binding free energy

## Abbreviations

Neurotransmitter (NT); Alzheimer's disease (AD); molecular dynamics (MD); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); monosialotetrahexosylganglioside (GM1); cholesterol (CHOL); γ-aminobutyric acid (GABA)

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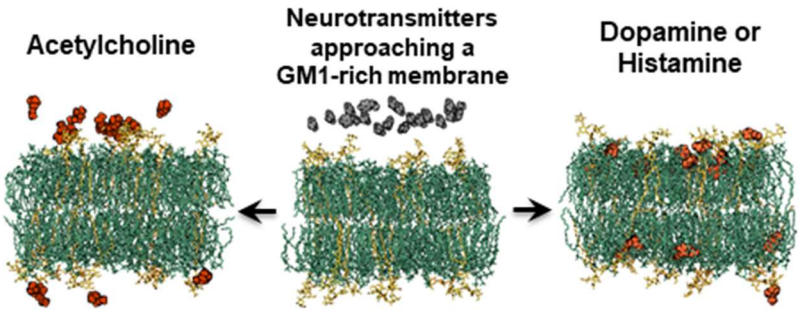
## Graphical abstract




## Highlights

- Anionic ganglioside GM1 facilitates membrane association of amphipathic histamine.
- GM1 promotes membrane association of amphipathic dopamine.
- GM1 does not promote membrane association of cationic acetylcholine despite its charged nature.
- Anionic gangliosides assist amphipathic neurotransmitters to enter membrane-buried binding sites.

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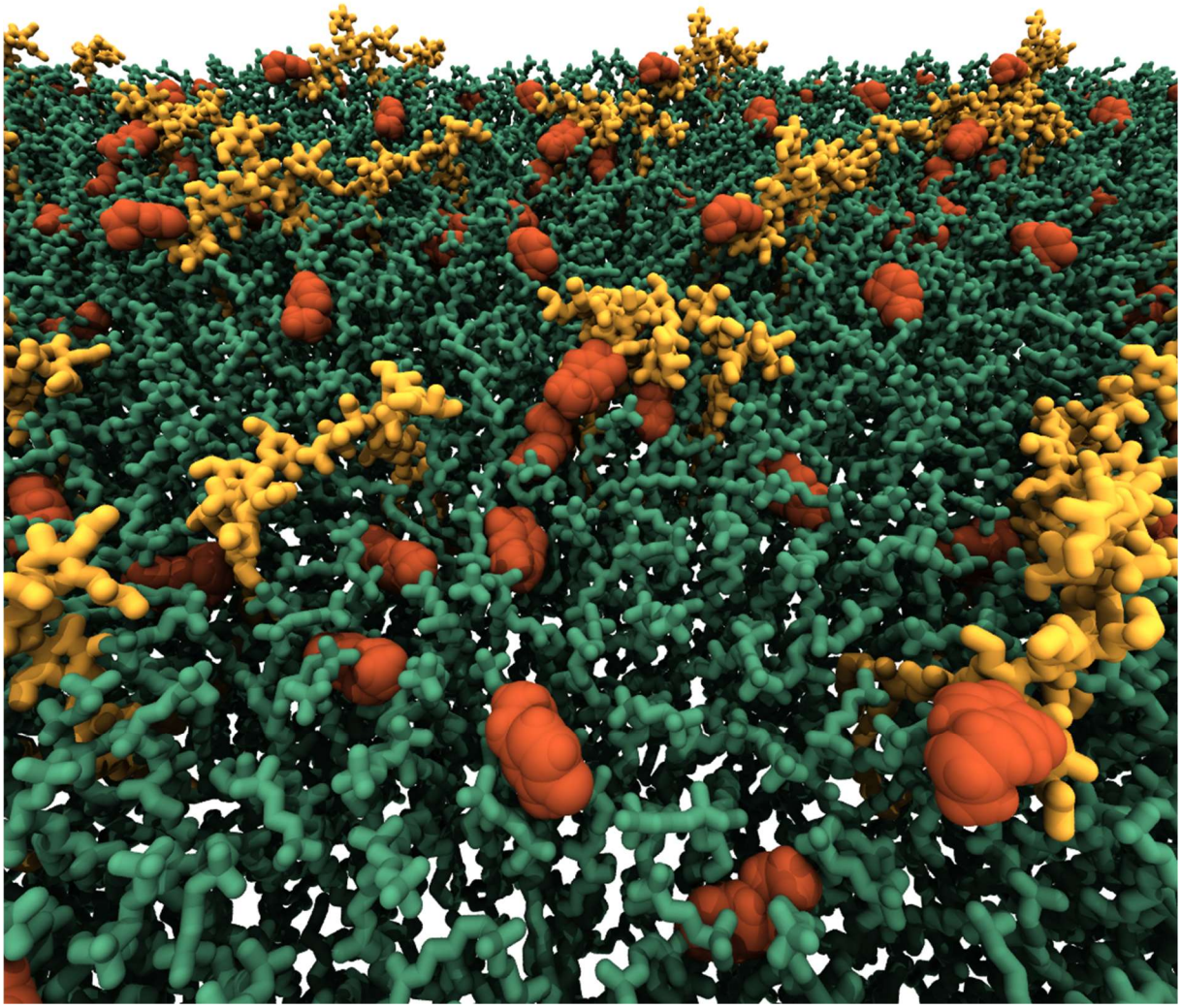
**Cover illustration**



## Introduction

Lipids form a major macromolecule class of vital importance and considerable functional and structural diversity. Lipids are not just passive components of membranes providing a physical barrier between the cellular or vesicular contents and the aqueous environment, but they also play significant roles in numerous biological processes (Simons 2016). Among others, lipids function as structural elements in macromolecular assemblies and integral components of membrane-embedded proteins (Róg and Vattulainen 2014; Hedger and Sansom 2016).

In the synaptic neurotransmission, specific lipids and membrane lipid compositions (MLCs) affect various molecular processes. First, the release of neurotransmitters (NTs) is coupled to synaptic vesicle fusion and fission with the pre-synaptic membrane. The required protein assembly is instigated by the aggregation of anionic phosphatidylinositols (Lauwers et al. 2016). Second, lipids participate in bilayer curvature changes related to membrane bending *via* cholesterol translocation or through changes in the size ratio between the lipid head groups and hydrocarbon chains controlled by phospholipases (Lauwers et al. 2016). Third, lipids are involved in the function of receptor proteins and signal transduction (Allen et al. 2007).

Lipidomics studies have revealed correlations between the MLC changes and pathologies (*e.g.,* Llorente et al. 2013; Pietilainen et al. 2011); for a recent review, see Yang et al. (2016). Lipids such as cholesterol, phosphatidylinositols, and phosphatidic acids are involved in the pathology of Alzheimer's disease (AD) (Di Paolo and Kim 2011; Astarita and Piomelli 2011). Based on the postmortem analyses, significant MLC changes are present in the AD affected brain tissue. Furthermore, these disease-specific MLC differences are detectable from the blood plasma within a 2-3 year window (Mapstone et al. 2014). MLC abnormalities of brain tissue and erythrocytes have been observed with schizophrenia patients (Puri 2016; Tessier et al. 2016; Wood and Holderman 2015; Vendramini et al. 2016; Schmitt et al. 2004). Furthermore, brain lipidome changes occur in stress (Oliveira et al. 2016; Miranda and Oliveira 2015) and under major depressive and anxiety disorders (Müller et al. 2016). Aberrant brain phospholipid metabolism takes place even with dyslexia and fatigue syndrome (Puri 2016).

The role of lipids in cellular processes is typically considered in relation to their ability to interact with proteins. This is because lipids modulate protein structure and activation *via* direct interactions (*e.g.,* Pöyry et al. 2013; Manna et al. 2016) or, less directly, by altering the physical properties of membranes that in turn modulate protein structure and activation (Róg and Vattulainen 2014). Moreover, studies have indicated that small molecules such as NTs and

drugs can influence protein structure indirectly by binding to the lipid bilayer (*e.g.*, Jerabek et al. 2010). For example, NTs have been suggested to affect protein conformations and produce anesthetic effects by adhering onto the membrane and altering membrane properties such as bilayer thickness (Cantor 2003).

In the context of neurotransmission, membranes have been suggested to affect synaptic receptor protein activation in a twofold manner (Postila et al. 2016). First, the NTs released from the pre-synaptic cell bind into their post-synaptic receptors either directly from the water phase or, second, their entry happens *via* the cell membrane. If the target receptor's binding site is extracellular, the NT does not adhere onto the membrane but instead it enters the site directly from the water phase. Meanwhile, if the receptor's binding site is membrane-buried, the NT first adheres onto the membrane surface and then migrates towards the receptor by lateral diffusion along the membrane plane. From the perspective of neurotransmission, the diffusion of NTs across the synaptic cleft is the slowest phase of the signaling process (Aguilar et al. 2017). Hence, the expedient NT-receptor association induced by the membrane-based sorting process involving membrane adherence/repulsion can be a crucial part of neurotransmission.

This division of neurotransmission into the membrane-independent and membrane-dependent mechanisms is supported by several studies. Atomistic molecular dynamics (MD) simulations and/or umbrella sampling-based membrane binding free energy calculations have identified a group of six non-peptidic NTs (dopamine, melatonin, adenosine, epinephrine, serotonin, norepinephrine) that preferentially adhere onto the post-synaptic membrane surface (Postila et al. 2016). The membrane association and lateral diffusion of these lipophilic NTs would be needed to facilitate efficient entry into the membrane-buried ligand-binding sites of G protein-coupled receptors. Meanwhile, a number of hydrophilic NTs (glutamate, aspartate, glycine, serine, acetylcholine, γ-aminobutyric acid or GABA) prefer the water phase to the membrane surface based on the simulations. The lack of membrane adhesion is suggested to promote the entry of these hydrophilic NTs into their receptors' extracellular ligand-binding sites.

Both experimental and simulation studies corroborate the above hypothesis regarding membrane-based sorting of NTs. Peptidic NT encephalin interacts strongly with lipid bilayers (Chandrasekhar et al. 2003); however, the membrane adherence of non-peptidic NTs dopamine (Orłowski et al. 2012; Jodko-Piorecka and Litwinienko 2013; Matam et al. 2016), serotonin (Peters et al. 2013), and melatonin (Drolle et al. 2013; Choi et al. 2014) are also well-documented. Glutamate and GABA interactions with an anionic lipid bilayer have been

observed in the presence of divalent calcium ions or in response to pH changes (Pérez-Isidoro and Ruiz-Suárez 2016). Hydrophilic or polar NTs glutamate, acetylcholine, GABA, and glycine do not prefer to adhere onto a phosphatidylcholine lipid bilayer in MD simulations, yet their interactions with bilayers have been detected using highly sensitive methods such as calorimetry and dialysis equilibrium experiments (Wang et al. 2011; Peters et al. 2014). The strength of membrane adherence of histamine depends on the MLC: it is weak with neutral lipids but strong in the presence of anionic lipids (Postila et al. 2016). Finally, hydrophilic/cationic acetylcholine has been found to partition to some extent onto membrane models containing anionic lipids (Postila et al. 2016; Pérez-Isidoro and Ruiz-Suárez 2016) but not onto membranes with neutral MLCs (Postila et al. 2016).

A well-balanced MLC is likely needed to maintain and fine-tune the biologically relevant order and function in the chemical synapse. For example, by alternating the anionic lipid content of membranes, it could be possible that acetylcholine either adheres onto the membrane or remains predominantly in the water phase. This minor mechanistic detail could explain how the positively charged NT can have both membrane-buried and extracellular ligand-binding sites, respectively, with the muscarinic and nicotinic acetylcholine receptors (Postila et al. 2016). Similarly, anionic lipids such as gangliosides have been proposed to facilitate histamine binding onto the post-synaptic membrane where its receptors' ligand-binding sites are buried – an effect that is not seen in MD simulations with a neutral post-synaptic leaflet model (Postila et al. 2016).

Gangliosides overall are exceptionally important in signaling and recognition. They constitute a large group of lipids with considerable variation in size and structure of the head group. GM1, in particular, is a common and important lipid species (for review, see Lingwood 2011, Manna et al. 2014). It plays a vital role as a membrane receptor for various bacterial toxins, lectins, myelin-associated glycoproteins, and Alzheimer's β amyloid peptide. GM1 also regulates the activity of tyrosine kinase receptors such as nerve growth factor receptors from the tropomyosin receptor kinase family, promoting receptor dimerization (Mutoh et al. 1995) and, thus, receptor activity. For this reason, GM1 promotes neuronal regeneration and therapeutic effects in Parkinson's disease (Schneider 1998).

Altogether, previous studies have highlighted the role of anionic lipids such as phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylglycerol (PG) in the dynamics of NTs. The problem is that these anionic lipids reside mostly in the intracellular

compartments rather than in the extracellular leaflet of the post-synaptic membrane (van Meer et al. 2008). On the other hand, NTs can interact with the extracellular leaflet that is relatively rich in anionic gangliosides.

In the present study, the aim was to explore the role of anionic gangliosides in the membrane association of positively charged NTs histamine, acetylcholine, and dopamine. By focusing on the neurologically relevant MLC, containing GM1, this study aims to determine whether the charge of gangliosides provides a sufficiently strong driving force to attract positive NTs to the post-synaptic membrane surface, and to clarify the importance of NT structure on GM1-NT interactions.

## Methods

Atomistic molecular dynamics (MD) simulations and free energy calculations based on umbrella sampling were performed for four systems, either with or without cholesterol (CHOL). The first system was CHOL-free and contained 202 (93 mol%) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) molecules, 14 (7 mol%) monosialotetrahexosylganglioside (GM1) molecules, and 20 dopamine NTs. The other three systems were CHOL-rich and contained 202 POPC (70 mol%), 14 GM1 (5 mol%), and 72 CHOL (25 mol%) molecules, and were used to investigate how 20 NTs (dopamine, histamine, or acetylcholine) interact with a CHOL- and GM1-rich membrane. The chemical structures of the molecules are shown in Fig. 1. The lipid bilayer models used in the simulations had a symmetric leaflet composition, which is different from asymmetric distributions often observed in biological membranes. Nevertheless, the lipid bilayer is thick enough (~3.5 nm) to prevent the opposing leaflet compositions from affecting the NT-lipid interactions on either side of the membrane.

The all-atom OPLS-AA force field lipid parameters (Maciejewski et al. 2014; Kulig et al. 2015; Kulig et al. 2016, Rog et al. 2016) were used for the lipids. NTs and ions were also parameterized based on the OPLS force field (Postila et al. 2016). For water, we employed the OPLS-compatible TIP3 water model (Jorgensen et al. 1983).

The initial equilibrated lipid bilayer configuration was taken from a previous study (Manna et al. 2017). The lipid bilayers were hydrated with 16,000-17,000 water molecules. NTs were added randomly to the water phase. Six $Cl^-$ anions were added to neutralize the systems. In the binding free energy studies, in which the free energy profile of membrane partitioning was studied for one NT at a time, 13 $Na^+$ cations were added to balance the net negative charge. It

has been shown previously that monovalent cations do not affect NT-lipid interactions (Mokkila et al. 2017) to a significant degree; accordingly, only counter ions we added into the systems. This setup is also justified by the methodological problems associated with non-polarizable ion parameters (Martinek et al. 2018).

The simulations were performed with the GROMACS 5.x simulation package (Abraham et al. 2015). The 3D periodic boundary conditions were imposed. A simulation time step of 2 fs was used. The LINCS algorithm (Hess et al. 1997) was employed to preserve the covalent bond length between the heavy atoms and the hydrogen atoms. The long-range electrostatic interactions were calculated by the Particle Mesh Ewald algorithm with a real space cutoff distance of 1 nm (Essmann et al. 1995). Likewise, the cutoff of 1 nm was also used for the Lennard-Jones potential, and the neighbor list was updated every 10 simulation steps. The simulation temperature was set to 310 K and was controlled by the Parrinello-Rahman algorithm (Parrinello and Rahman 1981). A pressure of 1 atm was maintained by the Nosé-Hoover algorithm (Hoover 1985; Nosé 1984). In addition, the semi-isotropic pressure scheme was used. Each system was simulated for 500 ns. The first 200 ns of the simulation trajectories were discarded to allow for equilibration of the NT-membrane interface. This was evaluated, for example, by looking at the number of NT-lipid hydrogen bonds.

The free energy calculations were performed with the replica exchange umbrella sampling (REUS) protocol (Sugita et al. 2000; Fukunishi et al. 2002) using GROMACS 4.6.7 coupled with PLUMED v2.1 (Bonomi et al. 2009; Bussi 2013; Tribello et al. 2014). The initial configurations were generated by pulling molecules along the bilayer normal direction using a harmonic potential with a force constant of 1000 kJ $mol^{-1}$ $nm^{-2}$ and a pull rate of 5 x $10^{-3}$ nm/s. A total of 48 windows, 0.1 nm spaced, were generated. The replicas were set to attempt the exchange every 500 steps during a period of 60 ns of REUS-MD calculations with a harmonic restraint force constant of 1000 kJ $mol^{-1}$ $nm^{-2}$ along the bilayer normal and an average exchange probability of 18% over the last 15 ns. The first 20 ns of each simulation was considered as equilibration and discarded from the actual free energy calculation. The convergence of the free energy profiles was evaluated by comparison of the reconstructed profiles every 10 ns.

Potential of mean forces were calculated by using a non-parametric variant weighted histogram analysis method (WHAM) (Bartels C. 2000) implemented by Enkavi at al. (Enkavi at al. 2017). The variable used in the calculation was the distance along the bilayer normal direction from the center of mass of the given NT to the center of mass of the membrane.

The statistical errors were estimated with the Bayesian block bootstrap approach (Hub et al. 2010, Moradi and Tajkhorshid 2014), using each independent simulation as a single block from 200 bootstrap samples. Each bootstrapped profile was shifted such that the point where the NT studied was completely in the water phase corresponded to 0 kJ/mol (a distance of 4.7 nm from the bilayer center). The error bars were estimated by calculating the standard deviation of the 200 aligned bootstrapped profiles.

## Results

Neurotransmitter (NT) molecules were inserted randomly into the water phase. During the simulations the NTs could either stay in the bulk water or diffuse to the lipid head group region depending on where their free energy is minimized. Notably, the NT-membrane association is usually not an on/off process but rather an equilibrium state. Accordingly, the simulations were extended to sufficiently long times to reach conditions, where the binding and unbinding processes of NTs with the membrane were in equilibrium. This was confirmed based on the number of intermolecular hydrogen bonds (H-bonds) between the NTs and the lipids (see below).

All three NTs (dopamine, histamine, and acetylcholine), whose membrane adherence is probed in this study, contain a positively charged group. The working hypothesis was that their net positive charge would assist the NTs in adhering onto the negatively charged sialic acid groups of GM1 that are directly exposed to the water phase above the lipid head group region.

Representative snapshots from the four simulated systems are shown in Fig. 1 for both the initial and final simulation configurations. To give a more complete and dynamic view into the binding processes, a movie showing the migration of the dopamine and acetylcholine around the membranes is included in the Supporting Information (SI).

Dopamine was observed to partition to the membrane-water interface in the simulations. This observation is consistent with the fact that dopamine is very lipophilic (with a log P value of -0.98 (see http://www.hmdb.ca/metabolites/HMDB00073#references)) and its aggregation onto the membrane surfaces has been reported in previous studies (Orłowski et al. 2012; Jodko-Piorecka and Litwinienko 2013; Postila et al. 2016; Matam et al. 2016). Similarly, histamine molecules also partitioned to the membrane-water interface to a large degree (log P of -0.70 (see http://www.hmdb.ca/metabolites/HMDB0000870)). The histamine-membrane association has previously been observed with both highly anionic intracellular and neutral PC-containing

membranes (Postila et al. 2016). In contrast, acetylcholine molecules were found to remain in the water phase in the simulations (predicted log P having a value of -4.2 (see http://www.chemicalize.org)), although a slight membrane preference has been reported with an intracellular membrane model (Postila et al. 2016).

To quantify the membrane partitioning of NTs more precisely, partial density profiles were calculated along the bilayer normal direction. The results (Fig. 2) indicate how the NTs, the phosphate groups of POPC, and the sialic acid of the GM1 head group are distributed in the membrane-water interface region.

For dopamine, the density profiles show strong preference for binding to the phospholipid head group region (Fig. 2a,b). Moreover, the region occupying sialic acid also contains a considerable concentration of the NT (Fig. 2a,b). In fact, the membrane adherence of dopamine is so complete that only a minor fraction of dopamine molecules remain in the bulk water phase. In contrast, acetylcholine is clearly more hydrophilic and prefers the water phase, though it also interacts with sialic acid of the GM1 head group, and to a lesser extent with the phosphate group of POPC (Fig. 2c). Histamine partitions in a similar manner to acetylcholine, the main differences being its preference for the phosphate group and stronger partitioning onto the membrane (Fig. 2d).

Next, the NT-membrane interactions were characterized by calculating the time development of the number of H-bonds between NTs and GM1, and between NTs and other lipids including CHOL (Fig. 3). The greatest number of H-bonds was formed between dopamine and POPC (Fig. 3a,b). The number of H-bonds between them is about three and the bonding happens essentially independently of CHOL. The number of H-bonds between dopamine and GM1 is ~0.4-0.5, and this is only weakly dependent on the presence of CHOL. Meanwhile, acetylcholine contains only one H-bond acceptor (the keto group) and thus the extent of H-bonding between acetylcholine and any lipid is negligible (Fig. 3c). Histamine, however, forms about two H-bonds with POPC and ~0.1 H-bonds with the anionic GM1 (Fig. 3d). The H-bonding analysis also reveals that H-bonding (or salt bridge formation) between the positively charged amine groups of NTs and the negatively charged carboxyl groups of GM1 sialic acids (salt bridging) is not in a significant role in the NT-membrane association.

In summary, the results of the H-bonding analysis are consistent with the picture given by partial density profiles that dopamine is strongly bound to the membrane, followed by histamine, while the interaction of acetylcholine with the membrane surface is much weaker.

The most appropriate way to quantify this phenomenon is to determine the free energies of NT binding to the membrane surface. Free energy profiles (Fig. 4) clearly indicate that dopamine prefers the membrane-water interface to the bulk water phase. The free energy difference between the CHOL-free lipid bilayer and bulk water is 21.3 ± 0.1 kJ/mol (red full line in Fig. 5). In the CHOL-rich membrane, the free energy difference increases to 23.0 ± 0.1 kJ/mol (red dashed line in Fig. 4), thus, CHOL slightly increases the binding strength of dopamine. This is likely due to CHOL-induced modulation of the GM1 head group conformation (Rissanen et al. 2017; Lingwood et al. 2011). The range of membrane interaction is more significant given that dopamine senses the attraction already a few nm above the membrane surface, and the attraction is associated with the GM1 head groups protruding deep into the water phase.

Acetylcholine is clearly not attracted towards the membrane (gray line in Fig. 4) as its free energy minimum lies in the water phase instead of the membrane surface. However, the free energy profile has a local minimum at a distance of ~2.15 nm from the bilayer center, corresponding to the position of the membrane's phosphate groups. This feature is in agreement with the density profile (Fig. 2c). The free energy cost of reaching this position from the water phase is small, about 10 kJ/mol (4 $k_BT$), which explains the shape of the partial density profile in Fig. 2c. The free energy minimum around 2.15 nm is too shallow to allow more than transient binding. Thus, it is unlikely to play a significant role in the dynamics of acetylcholine inside the synaptic cleft. As the distance from the bilayer center is decreased further, the density of dopamine is lowered down to zero at ~1.2 nm (Fig. 2c), matching the free energy cost that is ~20 kJ/mol (8 $k_BT$).

In agreement with the partial density profile and the H-bonding data, indicating aggregation of histamine at the membrane surface (green line in Fig. 4), the free energy minimum of histamine is located at the membrane-water interface at the depth of 8.6 ± 0.2 kJ/mol. This suggests that histamine binds to the membrane surface, but the binding affinity of histamine is lower than that of dopamine.

## Discussion

Previous studies have shown that amphipathic NTs, which possess both hydrophilic and lipophilic properties, partition to the membrane-water interface. The strength of this association is dependent on the presence of anionic lipids in the lipid bilayer (Orłowski et al. 2012; Jodko-

Piorecka and Litwinienko 2013; Matam et al. 2016; Postila et al. 2016; Pérez-Isidoro and Ruiz-Suárez 2016). This sort of NT-membrane adhesion has been reported also for zwitterionic NTs such as GABA and glycine (Pérez-Isidoro and Ruiz-Suárez 2016).

It is not surprising that anionic lipids contribute to NT binding, because the negatively charged head groups of anionic lipids should attract the positive groups of amphipathic NTs. Other structural features of NTs and lipids also regulate the strength of the association. For example, the size of the NT (*e.g.,* adenosine *vs.* glycine) affects its ability to find an optimal binding pose or favorable steric interactions at the membrane-water interface. NTs containing aromatic or hydrophobic ring systems pack better onto membrane surfaces (Postila et al. 2016). Moreover, the charge-nulling effect of divalent calcium cations aggregating onto a negatively charged membrane surface can reduce the affinity of amphipathic NTs such as dopamine towards the lipid head group region (Mokkila et al. 2017).

There is growing interest to understand how charged lipids influence NT-membrane dynamics. Experimental and *in silico* studies focusing on NT-membrane association have been performed using model membranes with MLCs containing anionic phospholipids PS, PG, and PA (Orłowski et al. 2012; Jodko-Piorecka and Litwinienko 2013; Matam et al. 2016; Postila et al. 2016; Pérez-Isidoro and Ruiz-Suárez 2016). These lipids are abundant in intracellular organelles such as the endoplasmic reticulum or in the inner leaflet of a cell membrane. They are also relevant in intracellular processes such as NT catabolism (for discussion, see Orlowski et al. 2012). However, these anionic lipids are present only in low amounts in the extracellular leaflet of the post-synaptic membrane or in the inner leaflets of pre-synaptic vesicles.

The only anionic lipid types known to be present in moderate amounts at the extracellular leaflet of the cell membrane are glycolipids, including gangliosides and sulfogalactosyl ceramides. The membrane concentration of glycolipids is small, typically of the order of a few mol%. However, in neural tissues their concentration can be higher, even about 30 mol% (Stoffel and Bosio 1997; Degroot et al. 2004). Furthermore, experimental measurements done so far cannot exclude the possibility that glycolipids could exist in higher concentrations in specific membrane domains surrounding, for example, synaptic receptors or NT transporters. Thus, it is possible that glycolipids are not uniformly distributed in the post-synaptic membrane and could exert much larger local effects on NT dynamics than what the average lipid concentrations suggest.

It was proposed in Postila et al. (2016) that elevated levels of glycolipids in the outer leaflet of the post-synaptic membrane could play a role especially in the dynamics of histamine and acetylcholine, and in their receptor entry processes. The ganglioside structure differs markedly from typical phospholipids: the head groups of gangliosides are considerably larger than those of typical phospholipids, and the negatively charged carboxylic groups of gangliosides are located in the third sugar units (Fig. 1). From this position, the carboxylate groups are protruding directly towards the water phase (Fig. 1).

The effect of glycolipids has not been considered in previous simulation studies that have focused on NT-membrane association, because the used membrane models were comprised of more general MLCs. However, recent progress in the lipid force field development (Lyubartsev and Rabinovich 2016) has rendered atomistic MD simulations involving even the most specific lipid species more and more possible. Based on this progress, in the present study we explored the role of GM1 as one of the most common charged ganglioside species (Fig. 1) in NT-membrane binding, using a combination of atomistic MD simulations and free energy calculations.

Histamine receptors belong to the G protein-coupled receptor family and, accordingly, they have membrane-buried ligand-binding sites. For this reason, the receptor entry of histamine should benefit from membrane adherence and the resulting lateral diffusion towards the membrane-buried binding site. In other words, membrane adhesion should bring histamine closer to its eventual binding site and lateral diffusion along the membrane plane should speed up the binding rate. In previous simulations with membrane models lacking glycolipids, histamine was not found to aggregate on the extracellular membrane with a neutral MLC (Postile et al. 2016). Instead, *vice versa*, the aggregation was observed on the intracellular leaflet containing the anionic lipid PS (Postile et al. 2016). Nevertheless, unlike in the case with anionic gangliosides, PS is not present in substantial amounts in the extracellular leaflet.

Importantly, the MD simulations reported in this article suggest that the presence of gangliosides in a lipid bilayer induces histamine aggregation onto the membrane surface. Density profiles show that histamine aggregates at the membrane-water interface, and there is also an elevated histamine density in the vicinity of the GM1-sialic acid region. This suggests that the negatively charged head group of GM1 attracts the positively charged histamine molecules to the vicinity of the membrane, but the eventual membrane binding takes place in the head group region of the phospholipids. The electrostatic interaction between the

glycolipids and the histamine molecules, therefore, helps to drag the NTs close enough to the membrane to render membrane binding and receptor entry efficient.

Similarly, compared to a pure PC bilayer, the presence of GM1 increases the affinity of dopamine for the lipid head group region. As a side remark, the binding of dopamine is further strengthened by CHOL, which is an important non-phospholipid component of the cell membrane. The positive effect of anionic lipids on dopamine adhesion onto the membrane has been shown in previous studies (Orlowski et al. 2012; Jodko-Piorecka and Litwinienko 2013; Postila et al. 2016). Here, the effect was demonstrated for the first time for GM1, which is a biologically relevant component of the extracellular leaflet of the post-synaptic membrane.

Interestingly, the dopamine-GM1 attraction begins at a distance of 3.0-4.2 nm from the bilayer center. This space is essentially bulk water except for the region housing the GM1 head groups that protrude into the water phase. Already in this region, the free energy of dopamine is ~7 kJ/mol lower compared to the value calculated in the actual bulk water phase far away from the membrane (Fig. 4). This finding suggests that GM1 acts as an anionic antenna, attracting cationic dopamine molecules towards the membrane. Furthermore, this hypothesis is supported by the time evolution of the number of H-bonds between dopamine and GM1 (Fig. 3). It shows a higher number of NT-membrane H-bonding in the beginning of the simulation (Movie in SI), when dopamine molecules reside mostly in the water phase.

Similarly to histamine, acetylcholine partitions preferably onto membranes containing the anionic lipids PS (Postila et al. 2016, Wang et al. 2014), PG (Wang et al. 2014) or PA (Pérez-Isidoro and Ruiz-Suárez 2016). Because acetylcholine binds to both the extracellular and membrane-buried ligand-binding sites of the nicotinic and muscarinic acetylcholine receptors, respectively, it has been suggested that the divergent binding is controlled by the levels of anionic glycolipids in the synaptic membrane (Postila et al. 2016). Accordingly, the positively charged acetylcholine would adhere more strongly to specific ganglioside-containing membranes or membrane domains hosting G protein-coupled muscarinic receptors. The opposite could hold true for those synapses that house ion channel forming nicotinic acetylcholine receptors.

However, in contrast to histamine and dopamine, the present simulations indicate that the presence of gangliosides does not promote acetylcholine aggregation onto the membrane surface. The mere presence of GM1 does not seem to be sufficient to foster membrane adhesion of acetylcholine. This behavior may be related to the MLC. Alternatively, acetylcholine may

function in a different manner than histamine and dopamine at the membrane-water interface. For instance, acetylcholine differs markedly from the other positively charged NTs regarding the way it is removed from the synaptic cleft after neuronal signaling winds down: while histamine and dopamine removal relies on specific transporters that move the NTs directly across the membrane utilizing cation-symport (Focke et al. 2013), acetylcholine has to be catalyzed by acetylcholinesterase before the reaction products (acetate and choline) can move across the membrane. This view is supported by the chemical structure of acetylcholine (Fig. 1), which is distinctly different from those of histamine and dopamine: while histamine and dopamine share the amine group, acetylcholine is characterized by choline. It is likely that this structural difference is the underlying cause for the different binding behavior observed for acetylcholine.

Further, free energy data (Fig. 4) suggest that the differences in chemical structures between acetylcholine compared with dopamine and histamine change the enthalpic contribution of free energy; the entropic contributions are not expected to be very different, since the sizes and conformations of these three NTs are highly similar. A more detailed consideration of the membrane-NT interaction modes in this context remains to be done in future studies, however.

In summary, the *in silico* results indicate that anionic ganglioside lipids, GM1 in particular, promote the membrane adhesion of both dopamine and histamine. A similar effect was not observed for acetylcholine. In previous studies, anionic lipids have been shown to promote NT-membrane association (Orłowski et al. 2012; Jodko-Piorecka and Litwinienko 2013; Matam et al. 2016; Postila et al. 2016; Pérez-Isidoro and Ruiz-Suárez 2016). However, this is the first study to explore this effect with neurologically relevant gangliosides (Stoffel and Bosio 1997; Degroot et al. 2004). The results corroborate the previous hypothesis that gangliosides assist positively charged NTs in membrane adherence needed for efficient entry into their receptors' membrane-buried ligand-binding sites (Postila et al. 2016). The gangliosides present in the extracellular leaflet, protruding deep into the water phase, are suggested to form a charge-based vestibule that attracts (or repels) NTs with positively charged groups. With histamine and dopamine, the negative charge of the gangliosides assist them to aggregate on the membrane-water interface and, hence, promote their entry into their membrane-embedded receptors.

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**AUTHOR CONTRIBUTIONS:** HJ prepared, executed and analyzed the MD simulation trajectories, and participated in the design of the experiments and in the writing of the manuscript; PAP, SR, and FL participated in the analysis of MD simulation data, design of the studies, supervision, and the manuscript writing; IV and TR designed and supervised the project and wrote the manuscript.

**SUPPORTING MATERIALS:**

**Video 1.** Aggregation of dopamine at the POPC-CHOL-GM1 lipid bilayer interface.

**Video 2.** Acetylcholine behavior next to the POPC-CHOL-GM1 lipid bilayer.

# Figure Legend

**Figure 1. Simulated systems and structures of molecules considered in this study.** Top: snapshots of the initial (0 ns) and final configurations (500 ns) of the simulated systems. Color coding: POPC (green), CHOL (orange), GM1 (red), NT (blue). Bottom: chemical structures of lipids and NTs considered in this study. Bottom right corner: snapshots of GM1 (yellow) with the carboxylic group shown in the CPK representation (purple); PC lipids are shown in green.

**Figure 2. Partial density profiles of neurotransmitters in the simulated lipid bilayers**: a) dopamine (black line) in a membrane containing POPC and GM1; b-d) dopamine (red line), acetylcholine (gray line), and histamine (cyan line) in a membrane containing POPC, GM1, and CHOL. The densities of phosphorous (P) atoms of POPC (blue line) and sialic acid of the GM1 (green line) head groups are shown separately. The center of mass of a lipid bilayer is in the origin on the *x* axis. The vertical dotted lines identify the maxima of density of the phosphate groups.

**Figure 3. Time evolution of the number of hydrogen bonds between neurotransmitters and lipids per neurotransmitter molecule**. a) Dopamine in the membrane model containing POPC and GM1; b-d) dopamine, acetylcholine, or histamine in the membrane models containing POPC, GM1, and CHOL. The results highlight H-bonding between phospholipids and NTs (black line), GM1 and NTs (blue lines), and between the carboxyl group ($COO^-$) of GM1 and the amine ($NH_3^+$) groups of the NTs (green lines).

**Figure 4. Free energy profiles of the neurotransmitters translocating from the water phase to the lipid bilayer.** The center of mass of the bilayer is at 0 nm. Vertical dashed lines show the approximate region of the membrane-water interface.

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