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First Experimental Evidence of Dopamine Interactions with Negatively Charged Model Biomembranes

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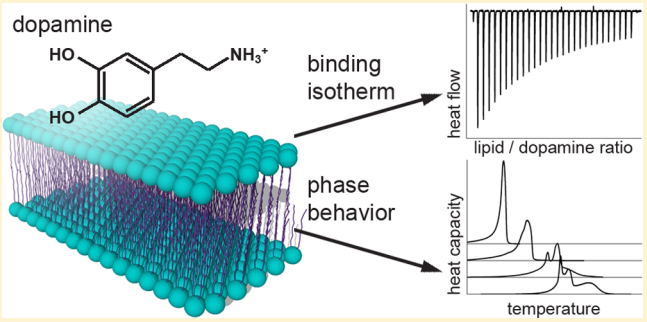
Supporting Information

ABSTRACT: Dopamine is essential for receptor-related signal transduction in mammalian central and peripheral nervous systems. Weak interactions between the neurotransmitter and neuronal membranes have been suggested to modulate synaptic transmission; however, binding forces between dopamine and neuronal membranes have not yet been quantitatively described. Herein, for the first time, we have explained the nature of dopamine interactions with model lipid membranes assembled from neutral 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), negatively charged 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), and the mixture of these two lipids using isothermal titration calorimetry and differential scanning calorimetry. Dopamine binding to anionic membranes is a thermodynamically favored process with negative enthalpy and positive entropy, quantitatively described by the mole ratio partition coefficient, *K*. *K* increases with membrane charge to reach its maximal value, 705.4 ± 60.4 M^{-1}, for membrane composed from pure DMPG. The contribution of hydrophobic effects to the binding process is expressed by the intrinsic partition coefficient, K^0. The value of K^0 = 74.7 ± 6.4 M^{-1} for dopamine/DMPG interactions clearly indicates that hydrophobic effects are 10 times weaker than electrostatic forces in this system. The presence of dopamine decreases the main transition temperature of DMPG, but no similar effect has been observed for DMPC. Basing on these results, we propose a simple electrostatic model of dopamine interactions with anionic membranes with the hydrophobic contribution expressed by K^0. We suggest that dopamine interacts superficially with phospholipid membranes without penetrating into the bilayer hydrocarbon core. The model is physiologically important, since neuronal membranes contain a large (even 20%) fraction of anionic lipids.



KEYWORDS: *Dopamine, neurotransmitter, membrane, interaction, microcalorimetry, anesthetic*

Dopamine (DA) acts as neurotransmitter in mammalian central and peripheral nervous systems. A crucial role of DA in the transmission of nervous stimuli was suggested for the first time by Carlsson.[1] Even a slight deregulation of DA activity may lead to pathological events,[1] and several neurological diseases are associated with abnormalities in DA signaling; depleted levels of DA underlie Parkinson's disease and attention deficit hyperactivity disorder, whereas DA hyperfunctional state may lead to schizophrenia.[1,2]

The dopamine neuron system is one of the best known and best mapped neurotransmitter systems in the brain.[3] DA participates in a variety of motor and mental functions of the organism, for example, complex motor behavior, emotional and motivational aspects of behavior, cognitive functions, and neuroendocrine regulation.[1,3] A mechanism of signal transduction mediated by DA, known as "slow synaptic transmission",[2] proceeds via the specific binding of DA to two major classes of receptors, D1 and D2, which triggers the biochemical cascade involving generation of secondary messengers, activation of distinct classes of protein kinases, and phosphorylation of proteins, serving as downstream physiological effectors.[1,2] Cantor predicted that the neurotransmitters can modulate synaptic transmission by affecting receptor conformational equilibria in a manner similar to anesthetics.[4] The suggested mechanism of anesthetic activity is nonspecific: neurotransmitters can alter conformations of all receptors present in the synaptic membrane.[4] The hypothesis of the possible anesthetic-like activity of neurotransmitters is strongly supported by the results of the whole-cell electrophysiology experiment showing that acetylcholine, glycine, and γ-aminobutyric acid (GABA) modulate the function of receptors for which they are not the native agonist.[5]

It is still largely uncertain if anesthetics influence the function of membrane-embedded proteins directly[6] or indirectly by interacting with lipid membranes.[7,8] Heimburg and Jackson[7] interpreted the activity of anesthetics as a result of their ability

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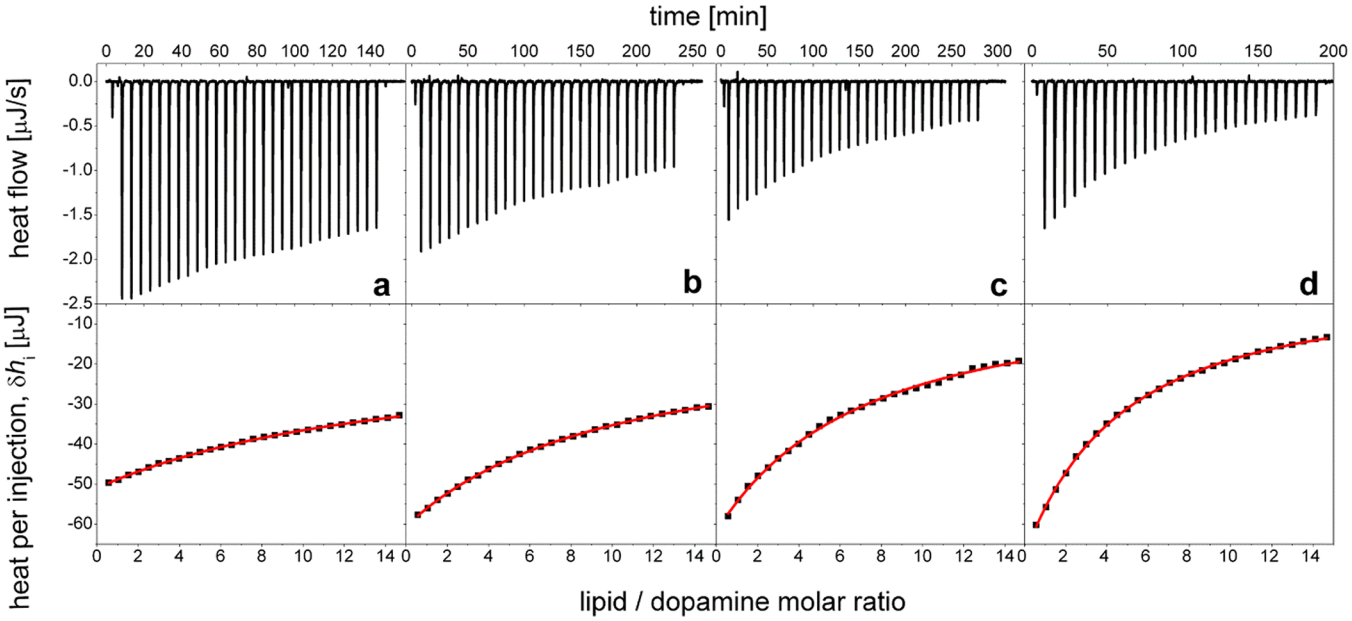

Figure 1. ITC plots of DA solution titrated with extruded LUVs assembled by DMPC and DMPG at molar ratios: (a) 3:1, (b) 1:1, (c) 1:3, and (d) pure DMPG. Experiments were performed at 37 °C in 50 mM phosphate buffer (pH 7.2). Upper panels: heat flow versus time for consecutive injections of 10 μL aliquots of 20 mM LUVs into a solution of 0.3 mM DA. Corresponding integrated heats per injection δh_i plotted versus lipid/DA molar ratio are presented in lower panels. Solid red lines correspond to the best fitting of experimental points with eq 6. See the text for details and Table 1 for parameters of fitting.

to decrease phase transition temperature of the lipid membrane (a concentration-dependent phenomenon similar to "freezing point depression").[7] This phenomenon plays a key role in anesthesia, since nerve pulses are accompanied by phase transitions of the neuronal membrane.[9] Cantor[8,10] hypothesized that the lateral pressure in the membrane is particularly sensitive to the presence of anesthetics and even a relatively small variation of the lateral pressure induced by anesthetics can change the activity of key membrane proteins (presumably postsynaptic ligand-gated ion channels).[8,10] A similar mechanism is possible for neurotransmitters at concentration sufficient to alter membrane properties and to modulate receptor conformational equilibria.[4]

Neurotransmitters are unevenly distributed in the neuronal tissue, and their concentration reaches an exceptionally high level inside the synaptic vesicles (250 mM for acetylcholine[4] or 270 mM for serotonin[11]). The concentration of neurotransmitters in the synaptic cleft is very high after the fusion of vesicles with presynaptic membranes (ca. 25 mM).[5] Such concentration should ensure a significant partitioning of neurotransmitters into synaptic membranes if the partition coefficients and rates of diffusion are sufficiently high. Data on neurotransmitter/membrane partitioning are limited in the current literature to three experimental reports[12−14] about interactions of lipid membranes with selected neurotransmitters (serotonin,[12,13] glycine, acetylcholine, glutamate, and GABA).[14] Seeger et al.[12] reported that serotonin interacts with neutral membranes and decreases both membrane phase transition temperature and maximum relaxation time. Very recently, Peters et al. have shown that this interaction has a dominant electrostatic component because it depends on a salt bridge between serotonin amine group and lipid phosphate.[13] Wang et al.[14] reported that zwitterionic (glycine, GABA) and cationic (acetylcholine) neurotransmitters are attracted by negatively charged membranes while glutamate anion does not interact with them. The interactions of neurotransmitters with lipids might affect the process of neuronal transmission via the anesthetic-like mechanism.[4] To our surprise, despite the extended knowledge on the physiological role of dopamine, the current view on the thermodynamics of DA interactions with biomembranes is far from complete, thus, the goal of this work is to obtain a quantitative description of DA interactions with model phospholipid membranes.

Beside playing a crucial role in the transmission of nervous stimuli, dopamine might behave as an endogenous phenolic antioxidant protecting neuronal tissue from effects of oxidative stress. Among phenols, catechols are the most effective radical scavengers due to an extra stabilization of phenoxyl radical by intramolecular hydrogen bond,[15,16] and antiradical properties of catecholamines were reported in several works including the kinetic[17,18] and theoretical studies.[19] The results of in vitro experiments on cell lines confirm the antioxidant activity of DA. Low concentration of DA exerts partial but long-term protection of neuronal cell lines,[20] reduces the level of intracellular reactive oxygen species (ROS),[21] and prevents apoptosis.[21,22] Liu and Mori claimed that catecholamines act as intrinsic system of the brain, protecting the neurons from oxidative damage.[23] On the other side, products of DA oxidation are cytotoxic: exposition of cell lines to DA at concentrations higher than 50 μM causes increased ROS production[21] and activation of pro-apoptotic caspase-3,[24] processes attributed to toxicity of DA oxidation products (semiquinones and quinones).[25]

Both cytoprotective and cytotoxic activities of DA are related to its ability to decrease or trigger oxidative stress in the neuronal tissue.[21] Since lipids of cellular and subcellular membranes are the primary targets for ROS,[26,27] the antioxidant activity of DA may be related to DA/lipid interactions. The effectiveness of other phenolic antioxidants have already been correlated with their ability to form external

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Table 1. Thermodynamic Parameters of the Interactions between DA and DMPG/DMPC Vesicles (x_{DMPG} = molar fraction of DMPG in the vesicle) Determined by Fitting Experimental ITC Data with eq 6[a]

x_{DMPG}	K [M^{-1}]	$\Delta H_d^{w\to b}$ [kJ/mol]	$\Delta G_d^{0,w\to b}$ [kJ/mol]	$T\Delta S_d^{w\to b}$ [kJ/mol]	$\Delta S_d^{w\to b}$ [J/K mol]
0.00	nd[b]	nd[b]	nd[b]	nd[b]	nd[b]
0.25	142.0 ± 16.6	−7.6 ± 0.6	−23.1 ± 0.3	15.6 ± 0.4	50.1 ± 1.4
0.50	302.3 ± 27.2	−6.5 ± 0.5	−25.1 ± 0.2	18.6 ± 0.5	59.8 ± 1.6
0.75	440.1 ± 52.3	−4.9 ± 0.5	−26.0 ± 0.3	21.1 ± 0.2	68.1 ± 0.8
0.90	571.6 ± 42.6	−4.0 ± 0.3	−26.7 ± 0.2	22.7 ± 0.3	73.1 ± 1.1
1.00	705.4 ± 60.4	−3.5 ± 0.4	−27.3 ± 0.2	23.8 ± 0.4	76.6 ± 1.1

[a]ITC experiments were performed at 37°C in 50 mM phosphate buffer, pH 7.2, K = mole ratio partition coefficient, $\Delta H_d^{w\to b}$ = molar enthalpy, $\Delta G_d^{0,w\to b}$ = molar standard free energy,and $\Delta S_d^{w\to b}$= molar entropy of DA transfer from water to the membrane. All numbers represent the average values obtained from series of measurements with calculated standard deviations. Full data are presented in Table S1 in the Supporting Information. [b]Binding not detected by ITC.

H-bonds (a phenomenon called the Kinetic Solvent Effect)[16] and with their ability to interact/penetrate lipid membranes.[28] The mechanism of antioxidant effects of DA has not yet been examined in regard to DA interactions with lipid membranes.

It is known that DA alters the cell ultrastructure and organization of actin cytoskeleton,[29,30] but, to our surprise, the only publication on DA/biomembrane interactions we have found was a molecular dynamics study assisted by a few measurements of surface pressure of lipid monolayer containing DA, a paper published very recently by Orłowski et al.[31] Therefore, we decided to carry out a series of experiments in order to obtain thermodynamic description of DA/membrane interactions. According to our knowledge, this is the first experimental study in this field.

Phospholipid liposomes are commonly applied as a lipid bilayer model of biomembranes.[32] Our experiments were performed on phospholipid liposomes with negative charge mimicking the synaptic membranes, vide infra. We applied isothermal titration calorimetry (ITC) to obtain thermodynamic parameters of binding process and differential scanning calorimetry (DSC) to characterize the thermotropic phase behavior of lipid mesostructures in the presence of dopamine.[33] On the basis of the results, a simple model of DA/membrane interactions is proposed.

RESULTS

Thermodynamic Parameters of Dopamine Binding to Lipid Membranes. ITC experiments were performed on LUVs assembled by two lipids having the same hydrophobic chains but different headgroups: neutral DMPC and anionic DMPG. At 37 °C DMPC and DMPG vesicles perfectly mimic the phase behavior of natural biomembranes in their fluid phase.[9] LUVs were obtained by extrusion of multilamellar vesicles assembled by pure DMPC, pure DMPG, or mixed lipids (DMPC and DMPG at molar ratios 3:1, 1:1, 1:3, and 1:9). Each injection of liposomal lipids into DA solution resulted in a sharp exothermic peak of heat flow, as demonstrated in Figure 1 (upper panels). After baseline subtraction, each peak of heat flow was integrated to obtain the heat of a single injection, δh_i (Figure 1, lower panels). The observed thermal effect of each injection is caused by DA transfer from the aqueous phase into the membrane to reach partition equilibrium. Therefore, the total DA concentration in the ITC vessel, C_d, is a sum of DA bound to the membrane, C_d^b, and free DA in the solution, C_d^f:

$$C_d = C_d^b + C_d^f \tag{1}$$

The equilibrium between lipid-bound DA and free DA can be described by the mole ratio partition coefficient K[33,34] (also denoted in literature as binding constant[35]):

$$K = \frac{C_d^b}{C_d^f C_L} \tag{2}$$

where C_L is the total concentration of lipid accessible for DA present in the aqueous phase. This model is appropriate for description of unspecific partitioning of some amphiphiles.[33] The combination of eqs 1 and 2 gives:[33,34]

$$C_d^b = C_d \frac{KC_L}{1 + KC_L} \tag{3}$$

After derivation of eq 3, any change in the concentration of bound DA, δC_d^b, can be calculated for gradually increasing C_L:[34]

$$\delta C_d^b = C_d \frac{K}{(1 + KC_L)^2} \delta C_L \tag{4}$$

where $C_L = i\delta C_L$ and i is the number of injections.

Each injection of lipid suspension causes thermal effect associated with the heat of mixing and the heat of dilution (both effects denoted together as Q_{dil}) and the heat of solute/membrane interactions.[34,35] During a series of consecutive injections, the amount of DA available for binding is decreasing; thus, the magnitude of the heat evolved after each lipid injection diminishes to reach a final value Q_{dil}. Assuming that after each injection δn_d^b moles of DA are transferred from water (w) to the membrane (b) and this transfer is accompanied by the molar enthalpy change, $\Delta H_d^{w\to b}$, the total heat released during a single ith injection can be expressed as:[34,36]

$$\delta h_i = \Delta H_d^{w\to b} \delta n_d^b + Q_{dil} = \Delta H_d^{w\to b} V_{cell} \delta C_d^b + Q_{dil} \tag{5}$$

where V_{cell} is the volume of calorimetric cell. Combination of eq 5 with eq 4 gives the expression:

$$\delta h_i = \Delta H_d^{w\to b} V_{cell} C_d \frac{K}{(1 + iK\delta C_L)^2} \delta C_L + Q_{dil} \tag{6}$$

where $C_L \to C_L^0$ (total lipid concentration) for solutes that can easily translocate between membrane layers.[33,34] Charged DA is a hydrophilic species[37] and should interact with the outer layer of lipid membrane, as it was generally predicted by Cantor[4] and confirmed by MD simulations.[31] For such solutes $C_L = \gamma C_L^0$, where γ is the asymmetry parameter. For LUVs, the outer lipid layer corresponds to 50% of the total lipid and the asymmetry parameter is 0.5.[33−35]

Detectable decrease of the heat evolved after consecutive injections (suggesting that DA does interact with lipid vesicles) was observed only for negatively charged DMPG or mixed DMPC/DMPG liposomes, while the interactions of DA with neutral DMPC liposomes were too weak to be detected by ITC. As presented in Figure 1, the experimental points perfectly agree with theoretical curves predicted by our model of partitioning. For negatively charged liposomes, we determined K and $\Delta H_d^{w\to b}$ by fitting parameters of eq 6 to experimental points with assumption that $C_L = 0.5C_L^0$ and Q_{dil} = constant. The parameters K and $\Delta H_d^{w\to b}$ collected in Table 1 strongly depend on the charge of the membrane, suggesting a dominant role of electrostatic component in DA/membrane interactions. The partition coefficient for DA/neat DMPG vesicles, $K = (0.7 \pm 0.1) \times 10^3$ M^{-1} (see Table 1) is in a reasonable agreement with 1.0×10^3 M^{-1} determined by Rolandi et al. for GABA interacting with phosphatidylserine (PS).[38]

The standard free energy of water/membrane partitioning of DA, $\Delta G_d^{0,w\to b}$, and the molar entropy of DA transfer from water into membrane, $\Delta S_d^{w\to b}$, were calculated from equation:

$$\Delta G_d^{0,w\to b} = -RT \ln(55.5K) = \Delta H_d^{w\to b} - T\Delta S_d^{w\to b} \quad (7)$$

where the factor 55.5 corresponds to the molar concentration of water as a solvent[33,34] (for calculated parameters see Table 1).

Values of mole ratio partition coefficient K were calculated from eq 2; however, the DA concentration in a bulk solution, C_d^f, is supposed to be underestimated because cationic DA is attracted by anionic membrane and excessively accumulates above the lipid surface. The local concentration of DA in the proximity of the negatively charged surface, denoted as C_d^{if}, can be calculated from the Boltzmann relation:[33,35]

$$C_d^{if} = C_d^f \exp\left(-\frac{z_s e_0 N_A \psi_0}{RT}\right) \quad (8)$$

where z_s, e_0, N_A, and ψ_0 denote, respectively: charge of DA cation ($z_s = 1$), elementary charge, Avogadro's number, and membrane surface potential.[33,35] For the DMPC bilayer, the surface potential is 0 while for DMPG $\psi_0(0) = -60$ mV.[39] For mixed DMPG/DMPC liposomes we assumed that ψ_0 depends linearly on the molar fraction x_{DMPG}, and is not affected by DA absorbed on the surface (for calculated values see Table 2). When C_d^f is replaced by C_d^{if}, eq 2 gives the intrinsic partition coefficient (also denoted in literature as surface partition coefficient or intrinsic binding constant), K^0:[35]

$$K^0 = \frac{C_d^b}{C_d^{if} C_L} \quad (9)$$

K^0 is related to K by equation:[33]

$$K = K^0 \exp\left(-\frac{z_s e_0 N_A \psi_0}{RT}\right) \quad (10)$$

Values of K^0 calculated for different x_{DMPG} are largely charge-independent (Table 2) with the average $\langle K^0 \rangle = (82.3 \pm 9.5)$ M^{-1}.

Phase Behavior of the Lipid/Dopamine Systems. The effect of DA on the thermotropic behavior of DMPC/DMPG LUV suspensions was determined with DSC on the basis of temperature-dependent isobaric heat capacities.[33] DA is extremely sensitive to light and oxygen, and its spontaneous oxidation to melanin could disrupt the long DSC experiments (performed at scanning rate 0.05 K/min). Roginsky et al.[40] showed that ascorbic acid (AA) effectively inhibits DA oxidation, and this observation was confirmed in cell culture medium;[24] therefore, we decided to carry out our DSC experiments in the presence of AA. Temperature-dependent molar heat capacities, C_p, recorded for buffered suspensions of DMPC and DMPG LUVs with and without AA are presented in Figures 2 and 3, where the peaks indicate a heat uptake during the phase transition from ordered (gel) to disordered (fluid) phase.[33] A careful comparison of thermal behavior of LUVs in the presence and the absence of AA (curves a and b in Figures 2 and 3) as well as a comparison of the transition temperatures, T_m, and the molar enthalpies of phase transition, ΔH, for these systems (Table 3) indicate that 1 mM AA did not cause any shift in the phase transition of DMPC or DMPG and did not change the transition parameters, giving a clear demonstration that AA has no impact on thermal behavior of

Table 2. Mole Ratio Partition Coefficients (K) Determined by ITC and Intrinsic Partition Coefficients (K^0) Calculated from eq 10 for DA Interacting with Vesicles Assembled from Pure DMPC, Mixed DMPG/DMPC (at different molar fraction of DMPG, x_{DMPG}), or pure DMPG[a]

x_{DMPG}	ψ_0 [mV]	K [M^{-1}]	K^0 [M^{-1}]
0.00	0.000	nd[b]	nd[b]
0.25	−0.015	142.0 ± 16.6	81.0 ± 9.5
0.50	−0.030	302.3 ± 27.2	98.4 ± 8.9
0.75	−0.045	440.1 ± 52.3	81.7 ± 9.7
0.90	−0.054	571.6 ± 42.6	75.8 ± 5.7
1.00	−0.060	705.4 ± 60.4	74.7 ± 6.4

[a]Experiments were performed at 37°C in 50 mM phosphate buffer, pH 7.2. The values of membrane surface potential ψ_0 were calculated assuming linear dependence of ψ_0 on molar fraction of DMPG. All numbers represent the average values obtained from series of measurements with calculated standard deviations. Full data are presented in Table S2 in the Supporting Information. [b]Binding not detected by ITC.

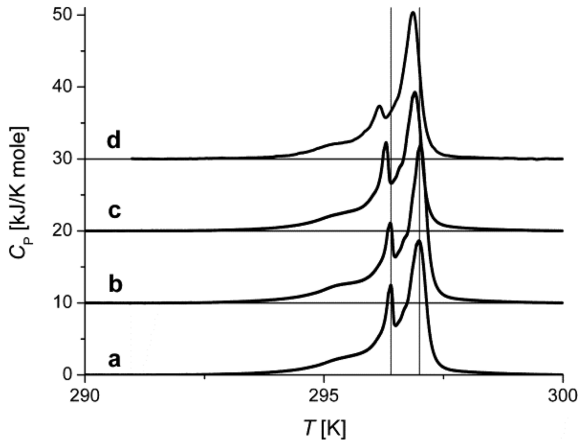

Figure 2. DSC cooling curves of DMPC LUVs containing DA and AA: (a) 0.0 mM DA, 0.0 mM AA (control I); (b) 0.0 mM DA, 1.0 mM AA (control II); (c) 1.0 mM DA, 1.0 mM AA; (d) 10.0 mM DA, 1.0 mM AA. Curves are referred to the calorimetric scans after equilibration. Two lines are entered into the plots at 296.4 and 297.0 K, respectively, as guiding lines. Experiments were performed in 50 mM phosphate buffer, pH 7.2.

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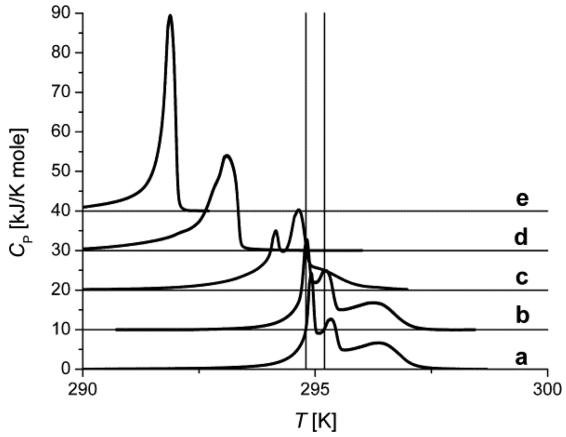

Figure 3. DSC cooling curves of DMPG LUVs containing DA and AA: (a) 0.0 mM DA, 0.0 mM AA (control I); (b) 0.0 mM DA, 1.0 mM AA (control II); (c) 1.0 mM DA, 1.0 mM AA; (d) 5.0 mM DA, 1.0 mM AA; (e) 10.0 mM DA, 1.0 mM AA. Curves are referred to the calorimetric scans after equilibration. Two lines are entered into the plots at 294.8 and 295.2 K, respectively, as guiding lines.

DMPC and DMPG LUVs and that AA does not interfere with DA/lipid interactions. Therefore, all subsequent experiments monitoring DA impact on phase behavior of DMPC and DMPG LUVs were performed in the presence of 1 mM AA and compared with systems containing LUVs and 1 mM AA as controls.

The presence of 1 mM DA has no effect on thermal properties of neutral LUVs composed from DMPC (Figure 2, curve c), whereas 10 mM DA leads only to a slight decrease in both T_m and ΔH of DMPC LUVs (Figure 2, curve d, Table 3). In contrast to DMPC, the thermal behavior of DMPG vesicles is different: the presence of DA considerably shifts the phase transition of DMPG toward lower temperatures. However, the total molar enthalpy of DMPG transition remains almost unaltered by DA (see Table 3).

An asymmetric broadening of transition peaks observed for DMPG in the presence of 5 and 10 mM DA (Figure 3d and e) might be caused by differences in DA accumulation in lipid bilayer before and after phase transition, with the fluid phase attracting more DA than the gel phase. Similar phenomenon, observed for octanol interacting with DPPC vesicles,[7] was described and discussed by Heimburg, who proposed a thermodynamic equation predicting the transition temperature (see the Supporting Information for details).

DISCUSSION

Interactions between dopamine and lipid membranes are crucial for modulation of the synaptic nerve transmission. The results of our calorimetric experiments provide a general picture of DA affinity for lipid bilayers and represent the first quantitative description of thermodynamics of DA/membrane interactions. Although the average physiological concentration of DA is ca. 0.1 μM,[41] neurotransmitters are unevenly distributed in neuronal tissue and their local concentration might be as high as 270 mM.[11] Thus, our calorimetric study fits within the broad range of DA concentrations found in real biological systems.

Cantor predicted that neurotransmitters are present in zwitterionic or charged headgroup region at the aqueous interface of biomembranes, without penetrating into the membrane core[4] and this prediction was confirmed by the results of molecular dynamics simulations for glycine, GABA, and acetylcholine.[14] At physiological pH, DA exists mainly as a cation (positive charge on protonated nitrogen).[37] Recent computational studies[31] indicate that the lipid headgroup region is the most probable site for the localization of DA and its precursor, L-3,4-dihydroxyphenylalanine (L-DOPA). Basing on these results, we suppose that DA/membrane interactions will depend on structure and charge of lipid headgroups.

Our study was performed with two lipids having the same hydrophobic chain but different headgroups. We chose DMPC as a model lipid, because phosphatidylcholines (PCs) are the most abundant class of lipids present in mammalian membranes, constituting ca. 50% of all cellular lipids.[42] DMPC has been used in numerous experiments as an accurate model for initial studies of lipid interactions.[14,39,43] Although preferentially localized in the inner (cytosolic) leaflet, as much as 20% of lipids in synaptic membranes are anionic;[5] thus, we used negatively charged DMPG in order to obtain a negatively charged model of synaptic membrane. Although phosphatidylglycerol (PG) is less frequently found in synapses than phosphatidylserine (PS),[14,42,44,45] we preferred PG (with monoanionic headgroup) over PS (with two negative and one positive charge in headgroup) as a model of negatively charged membrane. It has been shown that there are no sizable differences between the affinity of neurotransmitters (acetylcholine and glycine) toward PG and PS,[14] suggesting a dominant role of surface charge in neurotransmitter binding. In our artificial model, phospholipids were equally distributed between inner and outer layer of the membrane. It should be pointed out that such distribution differs from lipid distribution in real biological membranes, for example, PS (the most abundant anionic phospholipid, that constitutes up to 15% of

Table 3. Thermodynamic Parameters of the Main Transition of DMPC and DMPG LUVs in the Presence of DA at Various Molar Concentrations, C_d[a]

		DMPC		DMPG	
C_A [mM]	C_d [mM]	T_m [K]	ΔH [kJ/mol]	T_m [K]	ΔH [kJ/mol]
0.0	0.0	296.8 ± 0.1	17.5 ± 0.4	295.2 ± 0.2	21.5 ± 0.2
1.0	0.0	296.7 ± 0.1	17.5 ± 0.7	295.1 ± 0.1	21.5 ± 0.8
1.0	1.0	296.7 ± 0.1	17.4 ± 0.1	294.5 ± 0.1	21.2 ± 1.4
1.0	5.0			292.8 ± 0.1	21.2 ± 0.3
1.0	10.0	296.4 ± 0.2	16.2 ± 0.5	291.8 ± 0.1	20.7 ± 0.3

[a]AA at concentration C_A was used to prevent DA oxidation. Experiments were performed in 50 mM phosphate buffer, pH 7.2. T_m = temperature of transition and ΔH = molar enthalpy of transition. All numbers represent the average values obtained from series of measurements with calculated standard deviations. Full data are presented in Tables S3 and S4 in the Supporting Information.

all lipids present in the cell),[46] is preferentially located in the inner (cytosolic) leaflet of cell membranes.[46,47] The possible biological importance of dopamine interactions with cytosolic side of the lipid membranes will be discussed at greater length below.

ITC was applied to obtain thermodynamic parameters of DA binding to phospholipid vesicles and the ITC measurements were carried out on LUVs assembled by pure DMPC, pure DMPG or mixed DMPC/DMPG. Exemplary ITC plots obtained for DMPC/DMPG LUVs titrated into DA solution are presented in Figure 1 and thermodynamic parameters of binding: K, $\Delta H_d^{w\to b}$, $\Delta G_d^{0,w\to b}$, and $\Delta S_d^{w\to b}$ are listed in Table 1. The model applied by us for the interpretation of ITC data for mixed vesicles did not allow to distinguish between DA bound to PC or PG headgroups and we obtained "effective" thermodynamic parameters. Similar attitude was employed by Mertins and Dimova to analyze ITC data for interactions between positively charged chitosan and PC/PG vesicles.[48]

We detected DA binding only for negatively charged liposomes (assembled by pure DMPG or by mixed DMPC/DMPG). For the membrane composed by pure zwitterionic DMPC, the negative charge of the phosphate is partially reduced by a positive charge of the choline group[39] and the interaction with DA is too weak to be detected by ITC. Incorporation of 25% of anionic DMPG into DMPC membrane is sufficient to attract DA and such effect is clearly demonstrated by a binding isotherm presented in Figure 1a. It is consistent with observation made by Orłowski et al.[31] that DA affects the surface pressure of negatively charged monolayer (containing 18.75% of PS), but does not change the surface pressure of the monolayer composed from pure PC.

The affinity of DA to model DMPC/DMPG membrane strongly depends on negative charge of the membrane (see Table 1), suggesting a dominant electrostatic component of these interactions. In order to quantitatively describe the interactions between DA and lipid membranes with one universal parameter independent of membrane charge, we introduced the intrinsic partition coefficient K^0. Values of K^0 calculated for various DMPC/DMPG molar ratios are presented in Table 2 and their average value is (82.3 ± 9.5) M^{-1}. The K^0 parameter corresponds to hydrophobic effects and is largely independent of membrane charge.[35] As a result, we obtained a quantitative analysis of DA binding isotherms as a combination of electrostatic attraction of DA to membrane surface and nonpolar interactions (as K^0). We suppose that DA should also interact with neutral DMPC with partition coefficient $K = K^0$ but these interactions are probably too weak to be detected by ITC.

DA/membrane binding is characterized by the negative enthalpy $\Delta H_d^{w\to b}$, in agreement with other exothermic binding processes driven by electrostatic forces.[49] The negative values of $\Delta G_d^{0,w\to b}$ determined for all charged lipid vesicles (see Table 1) indicate that the binding process is energetically favored and probably stabilizes vesicles, due to shielding of the membrane charge by cationic DA accumulated at the water/membrane interface. The entropic contribution to standard free energy is substantial for all studied systems and the positive values of $T\,\Delta S_d^{w\to b}$ are due to release of counterions from the water/membrane interface as a consequence of DA binding process.

The impact of DA on the lipid phase behavior was evaluated with DSC. The presence of DA has negligible effect on the phase transition of LUVs assembled by neutral DMPC (calorimetric curves are presented in Figure 2 with parameters of phase transition collected in Table 3). Unlike for DMPC, DA considerably shifts the phase transition of DMPG toward lower temperatures, but the total molar enthalpy of DMPG transition remains almost unaltered (see Figure 3 for calorimetric curves and Table 3 for relevant parameters of phase transition). Such behavior, a decrease of the phase transition temperature (T_m) accompanied by a very small and negligible variation of enthalpy of transition, suggests that DA interacting with DMPG behaves as an "interstitial impurity",[50] that is, interacts superficially with lipid membranes without penetrating into bilayer hydrocarbon core. Similar behavior has been reported for GABA[38] and a derivative of tyrosine,[51] with more detailed description of DMPC/DMPG vesicles containing lidocaine, an anesthetic with aromatic ring and two amine groups.[43] Incorporation of lidocaine into lipid headgroups results in lateral membrane expansion and higher disorder of hydrocarbon chains.[43] We suppose that the same mechanism may also be valid for DA.

The results of our experiments clearly demonstrate that positively charged (protonated) nitrogen atom of dopamine is electrostatically attracted by lipid headgroup anions. This conclusion agrees with the molecular dynamics study showing H-bonding as the main driving force of DA association with interfacial region of the membrane.[31] Electrostatic forces were reported for cationic (acetylcholine,[14] serotonin[13]) and zwitterionic (glycine,[14] GABA[14,38,52]) neurotransmitters and also for linear monoamines[39] interacting with the lipid membranes but up to now, have not been described for DA.

There are several possible consequences of our study. Interactions of DA with lipid headgroup region of negatively charged membranes can affect lipid packing and can cause a redistribution of lateral stresses from the membrane interior to aqueous interface. It has been reported[4] that even small changes in the lateral pressure can lead to conformational changes and desensitization of membrane receptors. Superficial interaction of DA with membranes is also consistent with prediction of Ueda and Yoshida,[53] who suggested that anesthetics reside in the microenvironment with dielectric constant about 10 (for example, at water/membrane interface). DA dependent decrease of the transition temperature is in line with thermodynamic description of anesthesia proposed by Heimburg and Jackson,[7] and similar phenomenon has already been described for another neurotransmitter, serotonin.[12,13] Presumably, DA is able to act in the same way and the anesthetic-like activity of DA depends on DA interactions with the outer leaflet of neuronal membrane in the synaptic cleft.[4,5] Following its release from synaptic vesicles, DA might modulate the conformation of receptors embedded in the synaptic membrane (including receptors for which dopamine is not a native agonist). Thus, to evaluate the anesthetic effect of DA, some further studies are needed to check the ability of DA: (i) to interact with the membrane with lipid composition similar to the composition of outer leaflet of synaptic membrane (e.g., with some gangliosides as the sources of negative charge)[54,55] and (ii) to alter the activity of nondopaminergic receptors.

Another possible consequence of electrostatic interactions between DA and cellular membranes is a diminished amount of "free" DA available for synaptic transmission. Indeed, some neurological diseases are accompanied by higher concentration of anionic phospholipids like PS in the affected region of the brain (as reported in schizophrenia[56] and Parkinson disease[57]). Unfortunately, the available studies report an average lipid composition of the membranes, regardless of differences

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between cytosolic and noncytosolic (luminal) leaflets.[56,57] We demonstrated that negatively charged membranes attract more DA and these interactions can cause reduced availability of the neurotransmitter. This phenomenon might contribute to the progress of neurodegenerative diseases.[31] Association of DA with neuronal membrane can also diminish the effectiveness of L-DOPA therapy, which is still a gold standard in the treatment of Parkinson disease.[1]

The electrostatic interactions between cytosolic DA and anionic lipids located in the endofacial leaflet of lipid membranes can increase DA availability for proteins embedded in the membranes (similar phenomenon was shown for endofacial PS recruiting cytosolic proteins and thus contributing to the subcellular targeting of these proteins[58]). Three of membrane-embedded proteins: vesicular monoamine transporter 2 (VMAT2) and monoamine oxidases (MAO-A and MAO-B), are especially important for DA trafficking.[59] VMAT2 docked in the membrane of synaptic vesicles is crucial for DA transport from cytosol (where the neurotransmitter is synthesized) into the vesicles. Synaptic vesicles are enriched in PS which constitutes ca. 12% of their lipids.[45] We suggest that electrostatic attraction of DA toward cytosolic leaflet of synaptic vesicles can favor DA loading into the vesicles. DA not loaded into the vesicles is deaminated by MAO,[59] enzymes embedded in the cytosolic mitochondrial membrane;[60,61] thus, DA interactions with anionic lipids in mitochondrial membrane (cardiolipin, PS, PG, and phosphatidic acid)[42] can accelerate DA metabolism by MAO.[31]

Our experimental study gave the evidence of interactions between DA and negatively charged lipid membranes, and we believe that quantitative description of these interactions will contribute to the current discussion on the possible anesthetic and antioxidant activities of DA. Moreover, the possible consequences of DA association with neuronal membranes for DA metabolism and the progress of neurological diseases should be considered.

METHODS

Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (99%, powder, Avanti Polar Lipids), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (sodium salt) (DMPG) (99%, powder, Avanti Polar Lipids), dopamine hydrochloride (DA) (98.5%, powder, Sigma-Aldrich), ascorbic acid (AA) (99%, powder, Sigma-Aldrich), sodium phosphate monobasic (>98%, Sigma-Aldrich), and sodium hydroxide (99%, Merck) were used without further purification.

Preparation of Large Unilamellar Vesicles. Large unilamellar vesicles (LUVs) prepared from pure DMPC, pure DMPG, and mixed lipids (DMPC and DMPG at molar ratios 3:1, 1:1, 1:3 and 1:9) were obtained from multilamellar vesicles (MLVs) by a standard extrusion procedure[62] performed in a small volume extrusion apparatus.[63] For one-component liposomes (DMPC or DMPG), the weighed amount of lipid was dispersed with degassed (N_2) warm (ca. 30 °C) 50 mM phosphate buffer (pH 7.2) to a lipid concentration 7 mM (for DSC experiments) or 20 mM (for ITC experiments). Obtained suspensions were shaken vigorously in a Thermomixer for 30 min at 37 °C (temperature above that of gel to liquid-crystalline phase transition), 1400 rpm. For mixed (two-component) liposomes, lipids were dissolved in chloroform (DMPC) or in chloroform/ethanol (v/v 1/1) (DMPG), and these organic stock solutions of lipids were mixed in appropriate proportions to obtain a desired molar ratio of DMPC:DMPG. Solution was transferred to a pear-shaped glass flask. Chloroform was evaporated in a vacuum rotary evaporator, and the flask was kept overnight under vacuum to remove traces of solvents. Subsequently, the thin film of lipid was suspended in an appropriate amount of warm 50 mM phosphate buffer (pH 7.2) to give a final lipid concentration 20 mM (by intensive vortexing at temperature above the lipid phase transition). In order to obtain LUVs from MLVs, suspensions of MLVs were extruded at least 21 times through a Nucleopore polycarbonate membrane (Whatman, pore diameter of 100 nm) in Avanti Mini-Extruder (Avanti Polar Lipids Inc.). Vesicles prepared by this procedure have a narrow size distribution and are almost exclusively unilamellar.[62,63]

Isothermal Titration Calorimetry. A VP-ITC calorimeter from MicroCal Inc. and NanoITC from TA Instruments were used, and titrations were performed for unilamellar vesicles assembled from pure or mixed lipids (DMPC and DMPG) at temperature 37 °C. Small aliquots of 20 mM LUVs were injected into the measuring cell (volume 1.403 mL for VP-ITC and 0.95 mL for NanoITC) containing 0.3 mM DA solution in 50 mM phosphate buffer of pH 7.2. Approximately 31 injections were made: first injection of 2 μL (duration 2 s) and consecutive injections of 10 μL (duration 10 s) with 600 s equilibration time. This spacing ensures that the sample was equilibrated before the next injection.[33] Heat corresponding to the first small injection was not included in data evaluation. Experiments were repeated three to six times. Data analysis was performed in Origin 7.0 as described previously.[36]

Differential Scanning Calorimetry. DSC measurements were performed using a Nano DSC instrument from TA Instruments and a Nano II DSC instrument (model 6100) from Calorimetric Science Corp. The scanning rate employed was 0.05 °C/min. The measurements were performed at temperature range 15−30 °C at the 3 bar excess pressure in the presence or absence of AA and DA with a reference cell filled with 50 mM phosphate buffer of pH 7.2. Volume of the sample cell was 0.330 mL (for Nano II DSC) and 0.359 mL (for Nano DSC). For each sample, after the preincubation of the sample for 10 min at 30 °C, at least four calorimetric scans (alternate cooling and heating scans: two downscans, cooling scans; and two upscans, heating scans) were performed to check the stability of the system and the reproducibility of DMPC phase behavior. Downscans and upscans were reproducible; thus, the samples reached equilibrium during each experiment.[33] Experiments were repeated at least twice (usually three to four times). The analysis of DSC data was performed in Origin 7.0. Enthaplies were calculated from the peak areas using the template written in Origin 7.0.

ASSOCIATED CONTENT

Supporting Information

Complete data obtained by ITC measurements together with thermodynamic parameters of DA/membrane interactions and intrinsic partition coefficients for DA/membrane interactions; complete data obtained by DSC measurements together with thermodynamic parameters of the main transition of lipids in the presence of DA; theoretically predicted shifts in lipid transition temperatures in the presence of DA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

G.L. conceived the project. K.J.-P. and G.L. designed the experiments. K.J-P. performed the experiments and analyzed the data. K.J.-P. and G.L. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AA, ascorbic acid; DA, dopamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; GABA, γ-aminobutyric acid; L-DOPA, L-3,4-dihydroxyphenylalanine; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PS, phosphatidylserine

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