

# Proposal for PhD Candidacy

Organization and elasticity of membranes containing pentameric-ligand  
gated ion channels

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# 1 Aims

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand gated ion channels (pLGICs) found throughout the central and peripheral nervous system. Function of these neurotransmitter receptors is very sensitive to composition of the surrounding lipid membrane. However, the mechanisms of interaction between nAChR and its lipid environment are poorly understood. Research has shown a requirement for cholesterol in reconstitution mixtures of nAChR and other neuronal pLGICs, but other potentially essential lipids abundant in native membranes (particularly n-3 polyunsaturated fatty acids or PUFAs) have not been investigated. For my PhD research I propose the following:

- 1. Aim 1: Coarse-grained simulations of multiple subtypes of mammalian pLGICs in quasiphysiological membranes** We observe nAChR partitioning into n-3 polyunsaturated fatty acid (PUFA) rich domains with the n-3 PUFA DHA-PE as nAChR's primary boundary lipid. The concentration of n-3 lipids is much lower in membranes, such as *Xenopus* oocytes, commonly used in electrophysiology experiments, than in native membranes. I hypothesize adding small concentrations of n-3 is likely to restore the native boundary lipids. I will model various n-3 supplemented quasi-physiological membranes (such as oocytes) to predict those likely to provide a native local environment within the non-native membrane.
- 2. Aim 2: Investigation of the relative importance of pLGIC sequence vs shape in determining preferred lipid domain** This can be tested by comparing effects on partitioning profiles upon mutation of lipid facing residues versus adjustments in membrane lipid composition. If the effect of the protein's sequence is measured to be greater than its shape, it is likely that pLGICs will display significant variation in partitioning behavior and annular lipid preferences. If the reverse is observed, it is likely that overall pLGIC shape and relative flexibility of domains drives partitioning, and thus all pLGICs may have similar partitioning behavior.
- 3. Aim 3: Development and release of a user-friendly VMD plugin for measuring elastic parameters of heterogenous membranes** While multiple individuals have developed scripts to determine the fluctuation spectrum of membranes, there is no universal tool computational chemists and biophysicists can use. I will develop a tool to measure elastic parameters and fluctuation spectra within the convenient scripting environment of the VMD software, which will alleviate the daunting nature of solving for the fluctuation spectrum and related moduli. This tool will assist us in optimizing lipid selections for modeled neuronal membranes; I can adjust lipid species and lipid concentrations to mimic elastic properties of neuronal membranes.

## 2 Introduction

The nicotinic acetylcholine receptor (nAChR) is an excitatory pentameric ligand gated ion channel (pLGIC) commonly found in the post synaptic membrane, neuromuscular junction (NMJ) [1, 2] and the *Torpedo* electric organ [3, 4]. nAChR is found at concentrations around  $10^4 \mu\text{m}^{-2}$  within the NMJ membrane [5].

The pLGIC super family has been shown to play roles in cognition [6], inflammation [7–9], addiction [9], chronic pain [10] and numerous diseases including: Alzheimer’s Disease, spinal muscular atrophy, and neurological autoimmune disease [11–15]. nAChR plays a major role in excitation of the central and peripheral nervous system and binds agonists such as nicotine, acetylcholine and general anesthetics in multiple sites [16–19].

nAChR is highly lipid sensitive and is functionally dependent on cholesterol and anionic lipids when reconstituted into a membrane [20–27]. Lacking native-like concentrations of cholesterol or an abundance of anionic lipids in reconstituted membranes does not inhibit ligand-nAChR binding, but prevents gating and conformational changes [28–30], impeding ion flux through the pore [20–27, 31]. Previous research suggests cholesterol may be bound within the inter- and intra-subunits of the transmembrane domain (TMD) [32]; and cholesterol has been hypothesized and recently found bound within the  $\gamma$ -Aminobutyric acid receptors (GABAARs) TMD [33, 34].

nAChR’s native membranes (*Torpedo*, synaptic)[1–4], are enriched in phosphoethanolamine (PE) and polyunsaturated fatty acids (PUFAs) when compared to *Xenopus* oocyte[35], and a generalized mammalian cell [36]. Membranes composed of saturated-, sphingo-lipids, cholesterol, and unsaturated lipids tend to de-mixing into separate domains. Saturated- sphingo-lipids and cholesterol form a liquid ordered phase ( $l_o$ ), while unsaturated lipids are likely to form a liquid disordered phase ( $l_{do}$ ) [37, 38]. Domain formation has been studied both experimentally and computationally in model membranes [36, 39–42], showing de-mixing of lipids with saturated fatty acids and unsaturated fatty acids [43, 44].

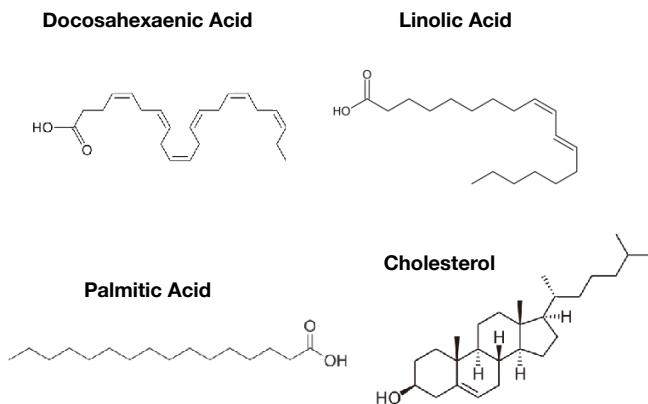


Figure 1: Chemical structures of lipids used in simulations.

Post-synaptic membranes in particular have distinctive lipid compositions, with elevated levels of n-3 PUFAs, cholesterol, and the phospholipid head group phosphoethanolamine (PE), and low abundance of monounsaturated acyl chains and sphingomyelin. [1, 2]. This composition is closer to the electric organs of electrogenic fish, like *Torpedo* [3, 4] than to typical mammalian cell membranes.

Deviations from neuronal lipid concentration can occur due to aging and health related disorders, including

disorders for which nAChRs are also implicated. Supplementing medication for schizophrenia with the n-3 PUFA eicosapentaenoic acid (EPA) has shown to improve schizophrenia symptoms in some patient populations [45–47] while a role for nicotine in reducing symptoms of schizophrenia is well established [48]. Imbalance in membrane lipids may lead to a number of health issues such as diabetes and Alzheimer’s disease, and may alter pathway signaling leading to lipidosis [49–51].

nAChR’s functional dependency on cholesterol has suggested that nAChR partitions into ordered cholesterol enriched domains [20, 21, 52–54],  $l_o$  domains (also known as lipid rafts). Bermdez et al [52] showed nAChR partitioned equally into  $l_o$  and  $l_{do}$  domains in model membranes of Chol:POPC:SM 1:1:1. Expanding on [52], Perillo et al [53] showed using the previous composition but inducing asymmetric membrane compositions, allowed nAChR to partition into the  $l_o$  domain.

### 3 Preliminary Data

Through coarse grained (CG) molecular dynamics simulations, we have analyzed nAChR-lipid interactions within quasi-native membranes using the structure of the nAChR from the electric organ of the Torpedo electric ray derived by Nigel Unwin using cryo-EM in 2005 [55]. The coarse grained model allows for significantly larger systems to be constructed than using atomistic models. Running simulations over  $\mu$ s allows CG lipids to diffuse enough for observation of domain formation or protein-induced lipid sorting.

We use the PUFAs Docosahexaenoic acid (22:6  $n - 3$ ) (DHA) and Linoleic acid (18:2  $n - 6$ ) (LA). Nearly all the  $n - 3$  PUFAs in both synaptic and *Torpedo*’s electric organ have been determined to be DHA [1–4]. LA was a useful test fatty acid; Risselada et al [42] showed it a usable PUFA for domain formation using Martini [56].

Our simulations show embedded nAChR consistently partitions into the  $l_{do}$  phase. This research explores nAChR’s partitioning behavior, boundary lipid affinity, and deep non-annular lipid-protein interactions termed embedding within domain forming membranes. It is our understanding this is the first study applying coarse grained molecular dynamics to nAChR.

#### 3.1 Methods

##### 3.1.1 System Composition

All simulations were built with Martini’s `martinize.py` to coarse grain the PDB structure and `insane.py` to embed the protein within a coarse grained membrane [56]. nAChR coordinates came from PDB 2BG9 derived by Unwin et al. 2005 [55]. The saturated lipids used are Dipalmitoylphosphatidylcholine (DPPC), Dipalmitoylphosphatidylethanolamine(DPPE). The PUFAs used were Didocosahexaenoylphosphatidylethanolamine (DHA-PE), Didocosahexaenoylphosphatidylcholine (DHA-PC), Dilinoleoylphosphatidylcholine (DLiPC), and Dilinoleoylphosphatidylethanolamine (DLiPE). Cholesterol (Chol) was the only sterol used.

Simulated membranes were composed of a saturated lipid, a PUFA, and Chol.

The lipids used are Dipalmitoylphosphatidylcholine (DPPC), Dipalmitoylphosphatidylethanolamine(DPPE), Didocosahexaenoylphosphatidylethanolamine (DHA-PE), Didocosahexaenoylphosphatidylcholine (DHA-PC), Dilinoleoylphosphatidylcholine (DLiPC), Dilinoleoylphosphatidylethanolamine (DLiPE) and cholesterol (Chol).

### 3.1.2 Simulations

Molecular dynamics were carried out using the Martini 2.2 force field [56] and Gromacs 5.0.6 [57]. Energy minimization was performed at 0.001  $ps$  using 10000 steps. However most energy minimizations finished within  $\sim 1700$  to 3000 steps. Molecular dynamics were run using a time step 0.025  $ps$  for 2  $\mu s$ . Simulations used NPT ensembles. We used Berendsen thermostat with an isotropic pressure couple. The reference temperature was set to 323 Kelvin with temperature coupling constant set to 1  $ps$ . The system's compressibility is set to  $3e^{-5} \text{ bar}^{-1}$  with a pressure coupling constant set to 3.0  $ps$ . All systems were run using van der Waals (vdW) and Electrostatics in shifted form with a dielectric constant of  $\epsilon_r = 15$ . vdW cutoff lengths were between 0.9 and 1.2 nm, with electrostatic cutoff length at 1.2 nm.

Secondary structures were determined by Martini's Martinize [56] protein coarse grainer routine. Secondary structures were maintained in simulations by Gromacs[57]. Tertiary structures were initially maintained using Gromacs [57] with a spring constant of 1000  $kJ \cdot mol^{-1}$ . Later elastic restraints based on Martini's [56] ElNeDyn algorithm [58] were implemented to maintain tertiary structure while allowing the protein to favorably interact with its environment. Elastic restraints used a spring constant 750  $kJ \cdot mol^{-1}$  using cutoff lengths between 1.0 nm to 1.5 nm. Root-mean-squared-displacement (RMSD) of the backbone was about 2.5 Å.

The majority of simulations have a box size between  $22x22x20 \text{ nm}^3$  and  $25x25x25 \text{ nm}^3$  and were run with Gromacs 5.0.6 [57]. Systems on the membrane size of  $75x75x35 \text{ nm}^3$  were run with Gromacs 5.1.2 [57].

### 3.1.3 Analysis

Extent of domain formation within the membrane was tracked by

$$M_{A,B} = \frac{\langle \eta_{A,B} \rangle}{x_B} - 1 \quad (1)$$

where  $\eta_{A,B}$  is the fraction of the 6 nearest neighbors around a given molecule of type A, that are of type B, and the average is over time and all molecules of type A. For a random mixture,  $\langle \eta_{A,B} \rangle = x_B$ , where  $x_B$  is the fraction of overall bulk lipids that are of type B.  $M_{A,B} > 0$  indicates demixing while  $M_{A,B} < 0$  indicates mixing.

Extent of receptor partitioning within the  $l_o$  or  $l_{do}$  domain was tracked by counting the number  $b_{\text{sat}}$  of saturated boundary lipids and comparing with the expectation for a random mixture, via the order parameter  $Q_{\text{sat}}$ :

$$Q_{\text{sat}} \equiv \frac{1}{x_{\text{sat}}} \left\langle \frac{b_{\text{sat}}}{b_{\text{tot}}} \right\rangle - 1, \quad (2)$$

where  $b_{\text{tot}}$  is the total number of lipids in the boundary region and  $x_{\text{sat}}$  is the fraction of overall bulk lipids that are saturated phospholipids.  $Q_{\text{sat}} < 0$  indicates depletion of saturated lipids among boundary lipids, as expected for partitioning into an  $l_{do}$  phase, while  $Q_{\text{sat}} > 0$  indicates enrichment and likely partitioning into an  $l_o$  phase. Each frame,  $b_{\text{tot}}$  and  $b_{\text{sat}}$  were calculated by counting the number of total and saturated lipids, respectively, for which the phosphate bead fell within a distance of 10 Å to 35 Å from the M2 helices, projected onto the membrane plane.

Two-dimensional distribution of a specific lipid of species  $B$  around the protein was calculated using both Cartesian

and Polar bins:

$$\rho_{B,i} = \left\langle \frac{n_{B,i}}{A_i} \right\rangle \quad (3)$$

where  $n_{B,i}$  is the number of lipid species  $B$  found within a given bin $_i$  and  $A_i$  is the area of a bin. In the case of Cartesian bins,  $A = \Delta x \Delta y$  where the bin widths  $\Delta x = \Delta y = 10\text{\AA}$ , while for Polar bins,  $A_i = r_i \Delta r \Delta \theta$  where  $r_i$  is the projected distance of the bin center from the protein center,  $\Delta r = 10\text{\AA}$  and  $\Delta \theta = \frac{\pi}{5}$  radians.

## 3.2 Results

### 3.2.1 Spontaneous association with cholesterol in binary membranes

In simulations of a single nAChR in membranes containing DPPC and 0-40% cholesterol, measurements (trajectories shown in Fig 2A) of  $Q_{\text{sat}}$  indicated moderate depletion of DPPC among nAChR boundary lipids (Figure 2B lower). Two-dimensional heat maps of the local cholesterol density reveal that at low concentrations of cholesterol, this depletion is almost entirely within “embedded” or non-annular lipids that are at least partially buried within the protein bundle (Figure 2C) as predicted in [32].

Saturation of such non-annular sites is apparent in the plateau in  $Q_{\text{sat}}$  between 10 and 20% cholesterol, which is remarkably consistent with the concentration of cholesterol typically required in reconstitution mixtures to restore native function. [20, 59, 60] Intriguingly, all simulations consistently yielded four general regions of differential embedded cholesterol density : a high density region ( $\alpha - / \gamma +$  interface and center of the  $\alpha$  subunit), a low/background density region ( $\alpha - / \beta +$  interface and center of  $\beta$  subunit) on the opposite face of the TMD, and two regions of intermediate density that separated them. In the absence of nAChR, DPPC/CHOL lipid bilayers are randomly mixed, so this result indicates an intrinsic cholesterol amphipathy.

At higher concentrations of cholesterol, cholesterol was also enriched in “annular” sites, particularly at the high affinity and low affinity face of the TMD. This enrichment actually extended many lipidation shells off the high affinity face, indicating nAChR was not just sorting lipids, but even inducing organization of the membrane. Enrichment far from the TMD is expected to be particularly sensitive to finite size effects, although there is no clear reason for why these effects would consistently have a dependence on protein orientation.

### 3.2.2 Domain formation in lipid bilayers containing PUFAs

Addition of phospholipids with unsaturated acyl chains to systems containing a saturated lipid and cholesterol is well-established to induce domain formation, and polyunsaturated phospholipids make these domains more well-defined[43]. Here we track, non-random mixing (including domain formation) via  $M_{A,B}$ . Enrichment of lipid species  $B$  among the nearest neighbors of lipid species  $A$  is quantified by  $M_{A,B}$  (see equation 1), with values close to 0 indicating random mixing, and positive and negative values indicating enrichment or depletion of species  $B$  around species  $A$ , respectively. Positive values of  $M_{A,B}$  close to 1 indicate substantial demixing.

Addition of PUFAs to DPPC/CHOL bilayers in the absence of nAChRs did induce phase separation, as indicated by the symbols in Figure 4. Adding a single nAChR did not change values for  $M_{A,B}$ ; i.e. the observed domains resulted from the lipid composition and were not dependent upon the presence of the protein. Across systems (Figure 4A), maximum values of  $M_{DHA,DHA}$  approached 5, and were significantly reduced (to less than 0.5) when

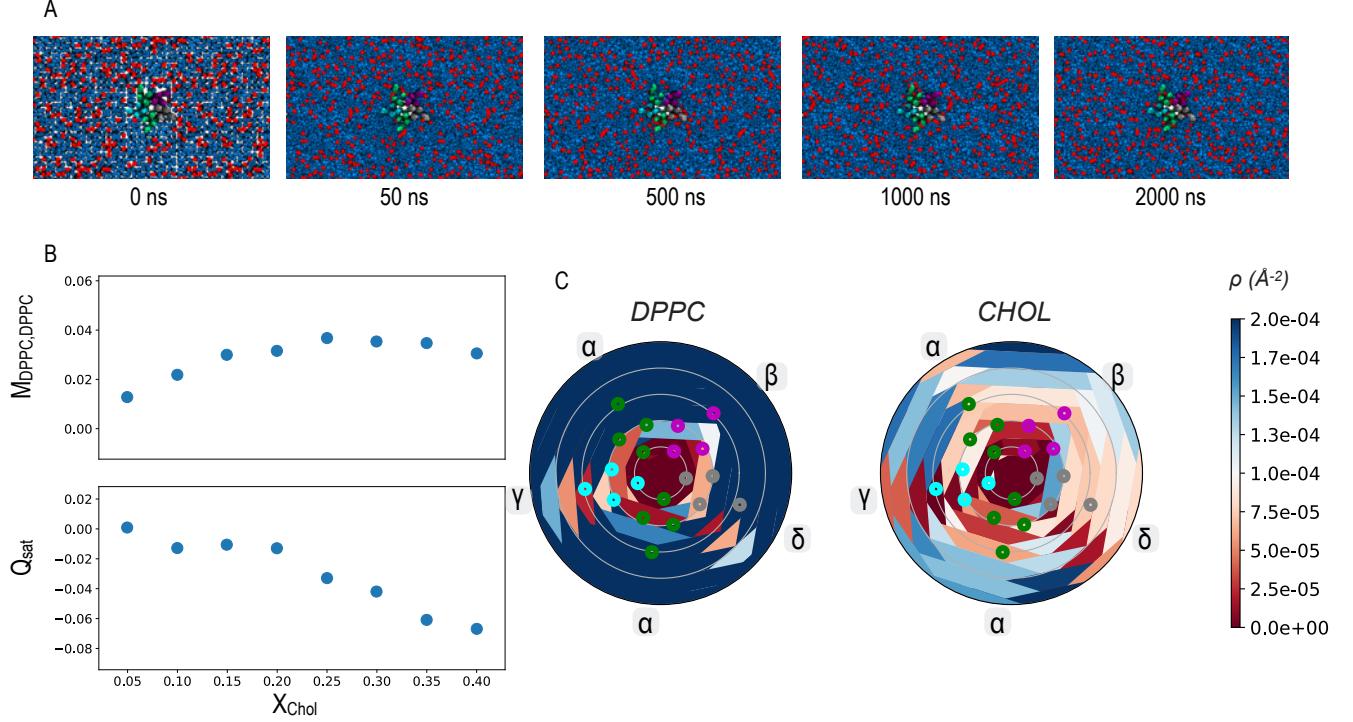


Figure 2: Lipid sorting by nAChR in binary mixtures of DPPC and CHOL. A: Representative frames from a simulated trajectory of a single nAChR, colored by subunit ( $\alpha$ :green,  $\beta$ :purple,  $\delta$ :gray,  $\gamma$ :cyan) in a 4:1 DPPC:Chol. B:  $M_{DPPC,DPPC}$  and  $Q_{sat}$  as defined in Eq 1 and 2, measured across nAChR:DPPC:CHOL mixtures (as in A) but with varying fractions of cholesterol. C: Average two-dimensional density of DPPC or cholesterol, up to 50 Å from the protein center, for the system shown in A. Protein helices are represented by hollow circles colored according to subunit. Blue indicates high density and red indicates low density.

DHA chains were replaced with LA chains. This result is consistent with that of [43] where a significant increase in miscibility temperature was observed upon supplementation of plasma membranes with  $n - 3$  lipids.

As shown in Figure 4a,  $M_{DHA,DHA}$  actually increases as the molar fraction of DHA-PE is reduced from the membrane, implying increased self-association of DHA at reduced concentrations of DHA, with even the lowest concentrations of DHA that we used here seemingly higher than the critical miscibility concentration.  $M_{DHA,DHA}$  is not sensitive to the ratio of cholesterol vs saturated lipid, at least over the compositions simulated. Relative to systems containing DHA,  $M_{LA,LA}$  implies miscibility for systems containing LA, with small amounts of domain formation sensitive to the CHOL:DPPC ratio; an apparent maximum for  $M_{LA,LA}$  occurs near 20% LA, 20% Cholesterol, and 60% DPPC.

Similarly, as shown in Figure 5, these results were insensitive to headgroup (PE or PC) for the compositions simulated. This is consistent with previous work, [36, 42, 61, 62], indicating domain formation in ternary mixtures to be primarily dependent on differences in acyl chain unsaturation and relatively insensitive to head group.

The well-defined boundaries between domains that were found in systems containing DHA-PE are also observed in systems containing DHA-PC. Shorter acyl chains and greater saturation did not promote well defined domains as seen using DHA (see Figure 3A). DHA is a relatively long chained n-3 fatty acid making it highly flexible. DHA has been shown to stabilize  $l_{do}$  domain formation [43, 44]. It may be the case running our simulations for longer time

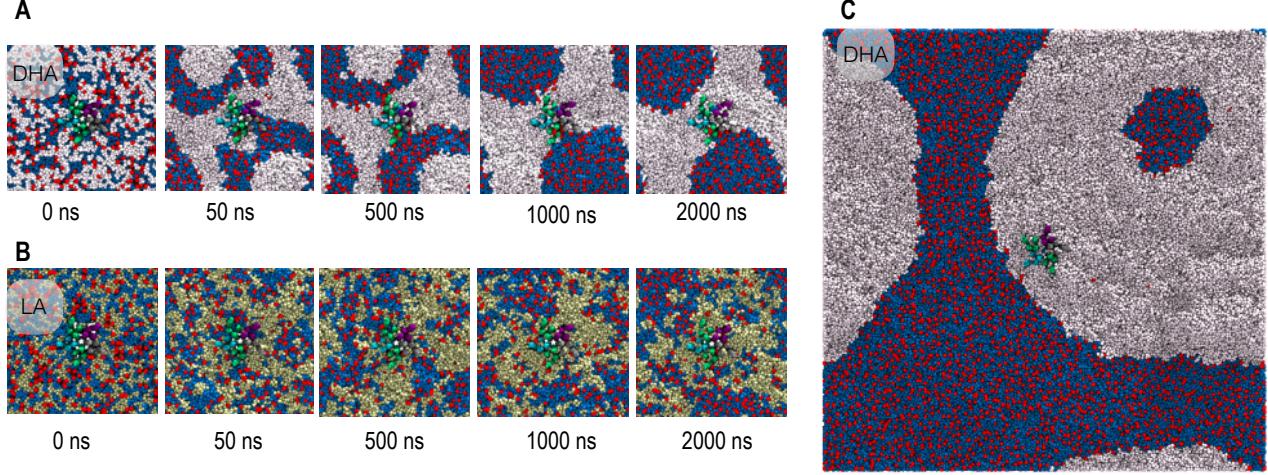


Figure 3: Extra-cellular view of nAChR in mixtures of 2:2:1 DPPC:PUFA:Chol, where A and B show systems comprised of the PUFAs DHA and LA respectively. C shows final image in a 4000 ns trajectory. A: Systems of di-DHA-PE, DPPC, and Chol. B: Systems of DLiPC, DPPC, and Chol de-mixing. C: System with large membrane of. Larger systems show nAChR partitioning parallel to A. Subunits are colored:  $\alpha$ : green,  $\beta$ : purple,  $\delta$ : gray,  $\gamma$ : cyan. Lipids are colored: Chol: red, DPPC: blue, di-DHA-PE: white, DLiPC: tan.

would produce well defined domains for any DLiPC/PE [42].

### 3.2.3 nAChR Partitioning Preference

For all lipid compositions tested, nAChR partitioned into the  $l_{do}$  phase if one was present, and as shown below, boundary lipids were enriched for unsaturated lipids even in the absence of domain formation. This includes all tested concentrations of the ternary mixtures of DPPC, DHA-PE, and Chol (Figure 3A), DPPE, DHA-PC (not shown) ,and Chol, and DPPC, DLiPC, and Chol (Figure 3B).These results are shown over either zwitterionic head group (PC and/or PE). A comparison of PC and PE head groups with C16:0 and DHA acyl chain preference is shown in Figure 5. In all four figures nAChR resides in  $l_{do}$  phases, reinforcing its dependency on acyl chains.

This result was quantified using a metric notated  $Q_{sat}$  and defined in equation 2; negative and positive  $Q_{sat}$  indicate depletion and enrichment DPPC among boundary lipids, respectively.  $Q_{sat}$  approaching 1 or -1 corresponds to partitioning within a well-defined  $l_o$  or  $l_{do}$  phase, respectively. (Figure 4B).In all systems studied here,  $Q_{sat} < 0$ , but in systems containing longer,  $n - 3$  chains,  $Q_{sat}$  was much closer to -1 than in systems containing shorter,  $n - 6$  chains. From  $Q_{sat}$  alone it is not clear whether this difference reflects a significantly higher affinity of nAChR for  $n - 3$  DHA than  $n - 6$  LA or simply the more well-defined  $l_{do}$  phase formed by DHA compared to LA. We did consistently measure  $Q_{sat} < 0$  regardless of restraints place on the protein or box size (Figure 4C).

Trends for  $Q_{sat}$  with changing fractions of unsaturated lipid and cholesterol were highly sensitive to whether  $n - 3$  or  $n - 6$  lipids were used. DPPC has such low affinity relative to  $n - 3$  lipids for most sites on the nAChR that  $b_{sat}/b_{tot} \leq 10\%$  regardless of  $x_{DPPC}$ . Intermediate amounts of  $n - 3$  unsaturated lipids (between 30 and 40%) further depletes DPPC from boundary lipids, even over a wider range of cholesterol ratios.

Figure 4B shows boundary lipids are highly dependent on species of PUFA and cholesterol. Figure 4B with DHA

demonstrates an approximately constant  $Q_{\text{sat}}$  at cholesterol concentrations between  $\sim 0.05\%$  to  $\sim 25\%$ , maintaining  $\sim$  constant DPPC concentration.  $Q_{\text{sat}}$  using the PUFA LA, still has a cholesterol dependence, however LA's affinity to mix with  $l_o$  lipids maintains much higher values of  $Q_{\text{sat}}$ .  $Q_{\text{sat}}$  values appear to be maximum in systems with near native  $x_{\text{Chol}}$ .

Observed partitioning into the liquid-disordered phase is inconsistent with some experimental interpretations using model membranes [53, 54], which suggest nAChR has no partitioning preference in symmetric model membranes. These experiments used only monounsaturated acyl chains, and may have had less well-defined domains. It is also not established that the detergent resistant membrane (DRM) method is powerful enough to distinguish between proteins with no partitioning preference vs proteins that persistently partition to one side of a boundary.

### 3.2.4 nAChR Preference for Domain Interface Including PUFAs

With the inclusion of PUFAs, our simulations have consistently shown nAChR partitions into the  $l_{\text{do}}$  phase. Interestingly, nAChR is recurrently in close or direct contact with the  $l_o$  phase, imaged in Figure 3A and C, Figure 5, Figure 6, and Figure 7.

To test whether the proximity of the nAChR to the interface was simply due to finite size effects, we increased the membrane area to  $\sim 75 \times 75 \text{ nm}^2$ . As shown in Figure 3C, nAChR partitions to the domain boundary even in the larger system. This is consistent with the results of [63], who recently showed partitioning of nAChR near a domain boundary using cryo-EM of nAChR in Torpedo electric organ membranes.

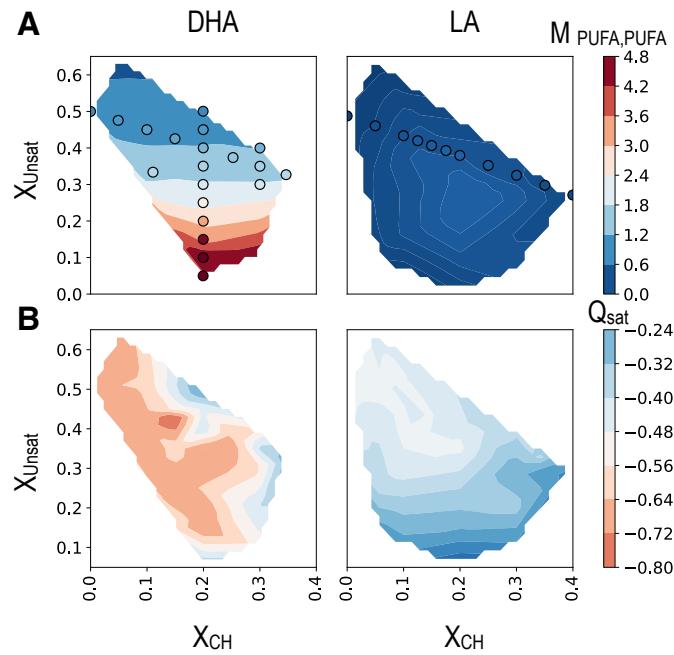


Figure 4: Quantitative analysis of bulk membrane mixing and nAChR boundary lipid composition. A:  $M_{\text{PUFA},\text{PUFA}}$ , derived in eq 1. Circles represent mixing of membrane only systems. B:  $Q_{\text{sat}}$ , derived in eq 2.

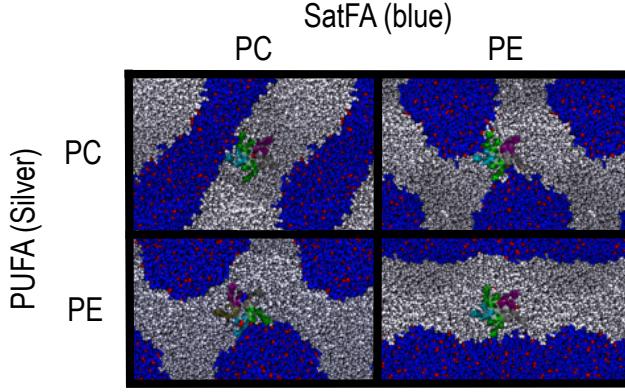


Figure 5: Comparison of nAChR partitioning based on lipid headgroups (PC and PE). All images are final snap shot of  $2\mu s$  simulations at ratio Sat:PUFA:Cholesterol 2:2:1. Rows show PUFAs with PC or PE head group. Columns are saturated lipids with PC or PE head group.

### 3.2.5 Subunit Preference

To evaluate whether different faces of the nAChR heteromer preferred to face different domains, we measured the average 2D density of lipids around the nAChR (Figures 2 C and 6). Comparing these figures 2 C and 6 shows subunits showing preference for cholesterol (Figure 2 C) have a preference for PUFAs (Figure 6).

nAChR shows a preference for  $l_{do}$  domain between the  $\alpha/\delta$  and  $\beta/\alpha$  subunits, in ternary systems, is observed having greater preference for cholesterol in binary systems. This preference was not significant in the systems with the shorter, less flexible PUFAs.

### 3.2.6 nAChR and Embedded Lipids

Cholesterol has been hypothesized in past computational experiments [32, 33] and recently found in [34], to embed within the inter- and intra-subunits of nAChR and other pLGICs. Docking has been used in previous computational research to determine optimal cholesterol binding domains [32, 33], but coarse grained systems have shown to be a novel method to simulate non-annular lipid binding without the need to dock lipids within proteins. While our simulations show cholesterol embedding within gaps of the 2BG9 cryo-EM structure [55], consistent with [32] (see Figure 7 sub-figure), PUFAs have greater occupation of the TMD structure (Figure 7).

## 4 Aims of the PhD Proposal

### 4.1 Aim 1: Coarse-grained simulations of multiple subtypes of mammalian pLGICs in quasi-physiological membranes

We observe nAChR partitioning into n-3 polyunsaturated fatty acid (PUFA) rich domains with the n-3 PUFA DHA-PE as nAChR's primary boundary lipid. The concentration of n-3 lipids is much lower in membranes, such as

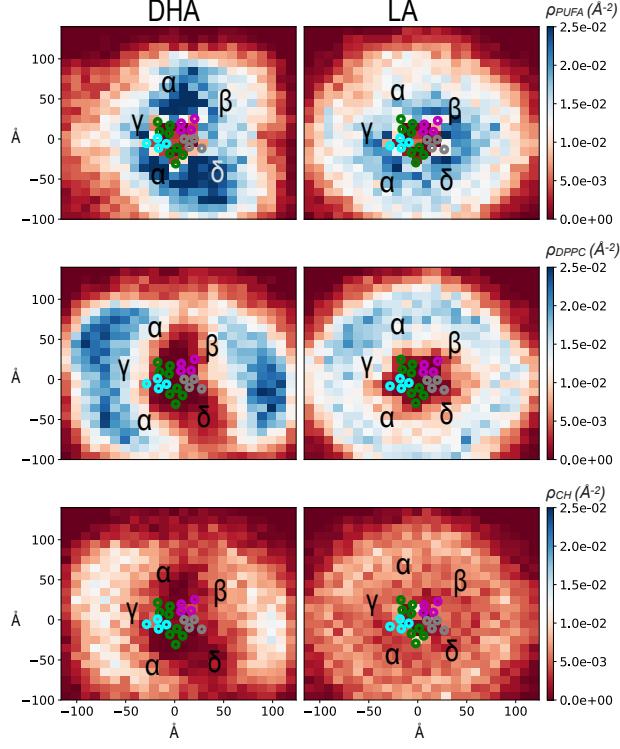


Figure 6: Heat maps depicting lipid densities averaged over three replicas at ratio DPPC:PUFA:CHOL 2:2:1.  $\rho_a$  is derived is derived in eq 3. Systems can be referred to Figure 3A and B.

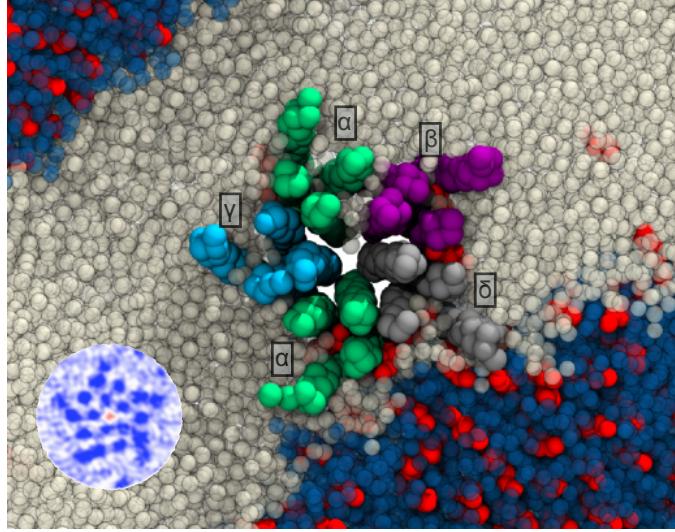


Figure 7: Embedded lipids in the nAChR. Main image: Representative frame from equilibrated simulation of nAChR in 2:2:1 DPPC:DHA-PE:CHOL. Backbone beads of the TMD helices are colored by subunit; side-chain beads are not shown. Both DHA-PE (white) and cholesterol (red) equilibrate to embedded sites in the subunit center and subunit interfaces. Inset : Cryo-EM density of nAChR from [64] as rendered in [65]; dark blue indicates high density, white is medium density, and red is low density. Lipid and protein subunits are colored as in Fig 3.

Xenopus oocytes, commonly used in electrophysiology experiments, than in native membranes. I hypothesize adding small concentrations of n-3 is likely to restore the native boundary lipids. I will model various n-3 supplemented

Lipid Comparison Table					
Saturation/Head Group	Torpedo	Synapse	Xenopus Oocyte	Soybean	Mammal
n-0	59	52	46	19	53
n-9	14	15	22	30	20
n-7	< 1	< 1	14	NA	13
PUFA	28	33	17	51	14
— n-3	— 19	— 18	— 6	— 15	— 4
— n-6	— 9	— 15	— 11	— 36	— 10
PC	43	43	36	27	27
PE	32	36	22	25	16
PS	13	12	5	NA	5
SM	8	4	26	NA	14
PI	4	3	7	28	2
PA	< 1	0	0	20	1
Other	6	2	4	0	5
Chol Mol Frac	32	39	21	0	30

Table 1: Generalized Table of Lipid Types Found within Torpedo and Synaptic Membranes: Data collected and averaged for synaptic membranes [1, 2] and for Torpedo membranes[3, 4], *Xenopus* Oocytes [35], soybean [66] and average mammalian [36]

quasi-physiological membranes (such as oocytes) to predict those likely to provide a native local environment within the non-native membrane.

pLGICs have complex gating behavior and structural requirements, and nAChRs are some of the most complex pLGICs. One of the most poorly understood components of nAChR is its unpredictable functional sensitivity to slight changes in its lipid environment, a property shared to a lesser extent by other pLGICs [60, 67].

Considerable experimental effort [20–25, 27] was expended, primarily in the 1980s and 1990s, to understand the underlying mechanism of nAChR lipid sensitivity, including identifying the likelihood of specific boundary lipids. Experimental studies focused primarily on cholesterol, which were required in native membranes (20-40% of lipid composition) to support native levels of ion flux in purified and reconstituted nAChR [20, 25]. Further experiments showed that while cholesterol could be depleted from the bulk membrane, a second pool of cholesterol could not be removed from nAChR-containing membranes by depletion [68]. However, results were inconclusive regarding whether cholesterol was sufficient to restore nAChR function. Interestingly, soybean lipids (which are also high in n-3 PUFAs) [66, 69, 70] are more effective at restoring ion flux than cholesterol alone [71].

The working assumption in the time of most of those experiments was that the membrane was randomly mixed in the absence of protein, although lipid sorting by proteins was considered likely; there was little evidence available then that cholesterol by itself can induce non-random mixing and even domain formation in just a ternary lipid mixture. We are now also aware of the critical role of acyl chain unsaturation in this process, but previous experiments focused primarily on the role of cholesterol and phospholipid headgroup without including the lipids with n-3 PUFA chains that are so abundant in both the fish electric organ and the postsynaptic membrane.

It is still unknown what factors determine the lipids interacting directly with pLGICs , leading to a substantial source of uncertainty in present-day experiments and introducing a divergence between simulations and experiments that cannot be reasonably estimated. Ionic flux of reconstituted neuronal  $\alpha 3\beta 4$ nAChR expressed in Xenopus oocytes is less than 50% of those expressed in mouse-fibroblasts, with neither consistently reproducing native behavior [20–

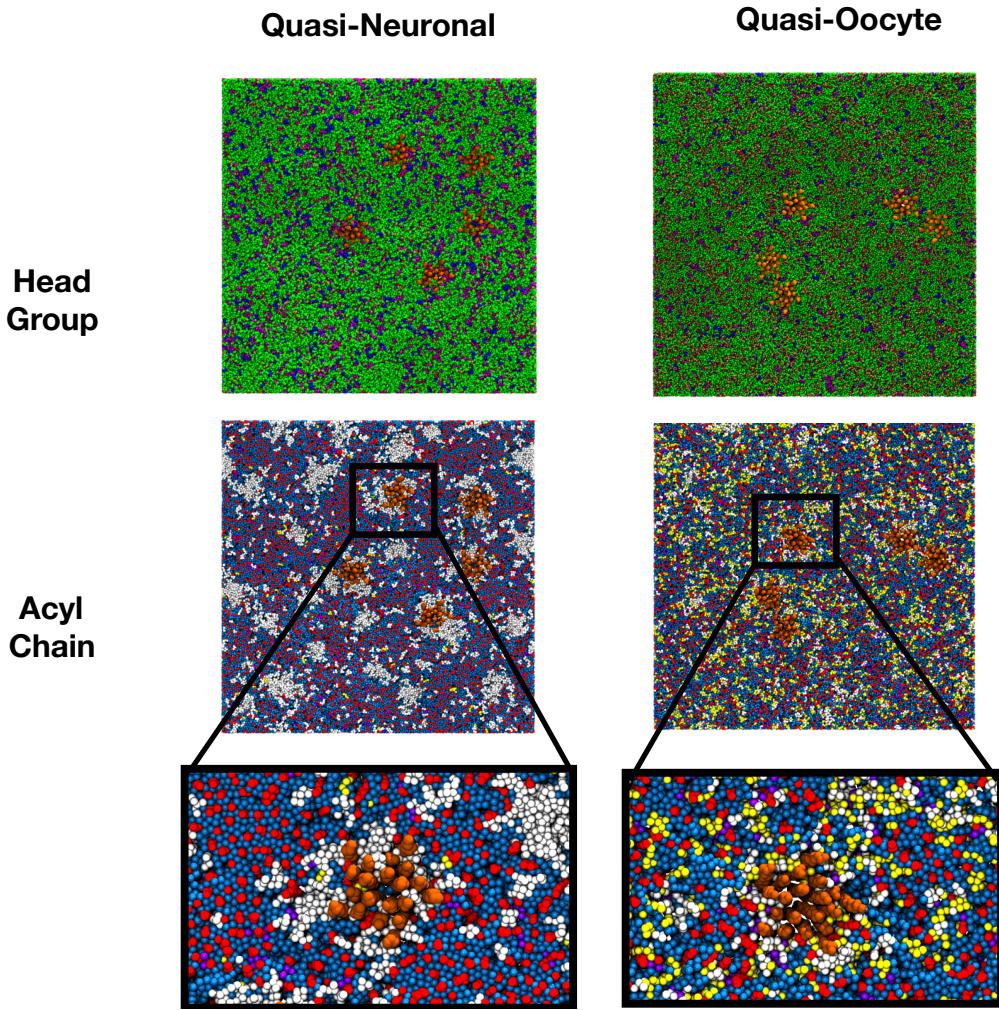


Figure 8: Comparison of nAChR lipid sorting in quasi-Neuronal and quasi-Xenopus oocyte membranes, colored according to either phospholipid head-group or location of acyl chain unsaturation, from coarse-grained MD simulations. nAChR transmembrane domain is shown in surface representation, colored by subunit. Top row: PE (purple), PC (green), SM (tan), PS (blue), cholesterol (red). Bottom Row: n-3 (white), n-6 (yellow), n-9 (purple), saturated (blue), cholesterol (red). Composition of quasi-Neuronal and quasi-Oocyte membranes reflects all species that are sufficiently abundant to yield at least 2 molecules in a membrane of the simulated size. Both systems are fun for  $\sim 1.5 \mu s$

27]. Contrasts in membrane lipids may contribute significantly to these differences, and specific lipid incorporation may bypass the need for microtransplantation of entire sections of neuronal membranes [67] into oocytes to achieve native function.

A large number of experiments, ranging from the straightforward to the particularly sophisticated, have been carried out to investigate the mechanisms underlying cholesterol modulation of pLGICs. The proposed studies involve investigation of lipid interactions with nAChR via coarse grained molecular dynamics. Numerous simulations of nAChR and other pLGICs with atomic resolution, powerful methods for investigating direct interactions of receptors with small molecules, are also reported in the literature. In the absence of realistic estimates for the protein-local lipid composition, most such simulations embed the receptor in a model membrane composed of DOPC or POPC, with occasional inclusion of cholesterol.

My preliminary studies, which use CG simulations capable of equilibrating a quasi-native membrane, indicate that

nAChR has a surprisingly strong preference for n-3 PUFAs (Figures 8 and 4B) as boundary lipids. Although these lipids are abundant in most native nAChR membranes, including the electric organ, they had not been included in experiments (except via an abundance in soybean lipids). This surprising observation, if true, offers possible explanations for limited success of many previous experiments.

It further suggests that regardless of the bulk membrane composition, nAChR functions natively in a homogeneous local environment of n-3 PUFAs. Our proposal for reproducing native boundary lipids within an oocyte relies on both this simplicity and the large difference in abundance of n-3 PUFAs between the oocyte and the neuron, which suggests there is a qualitative difference in lipid environment. Microtransplantation of neuronal cell membranes into oocytes [67] has been carried out and shown to improve ion flux through nAChRs embedded in oocyte membranes, but I will run calculations to inform an approach which restricts supplementation to a few species of preferred boundary lipid, and would substantially improves experimental control. These predictions will be tested by a collaborator of Dr. Brannigan's, Dr. John Baenziger at University of Ottawa.

I propose a series of simulations characterizing the boundary lipids surrounding nAChR embedded within a modified oocyte membrane, with the aim of finding the modifications which will reproduce native boundary lipids.

The proposed simulations will involve quasi-Oocyte lipid membranes and supplementing them with boundary lipids found in neuronal membranes. I will, first perform literature searches prudent to neuronal/synaptic, *Xenopus* oocyte, and *Torpedo* electric organ membrane compositions. If detailed compositions are not available, I will construct an acyl chain to acyl chain randomizer, to cover all potential lipid species. Constructing a spread sheet (Microsoft Excel) or program (in python), compare and select appropriate Martini equivalent lipids, based on acyl chain length and saturation. Having built a complementary selection of lipids, I will implement automation to allow for easy lipid to membrane supplementation. As there are various n-3 PUFAs, I will construct various series to test which neuronal/*Torpede* n-3 lipid species best assist with native like nAChR boundary domains.

It is possible (especially if elastic effects are essential) that increasing the number of receptors or the system size will modify these distributions. It is important then, to increase the number of receptors (aiming at around ten), enforcing realistic leaflet asymmetry, and incorporating the newer  $\alpha 4\beta 2$  nAChR structure [72].

Currently, I envision an initial analysis of these simulations by calculating membrane mixing, boundary lipid composition, and lipid-protein non-annular binding. Due to the variety of lipid species involved, calculations will be grouped into two sets: lipid head group (i.e. PC, PE, PS...), and acyl chain saturation (i.e. sat, n-1, n-2, n-3, n-6). Preliminary simulations show n-3 dominate the boundary lipids when lipids with n-3 acyl chains are increased by concentrations as low as ~5%. These initial simulations, quasi-oocytes with DHA increased to 15%, show DHA to make the dominant boundary lipid. Figure 8 shows two of these simulations, using five proteins in quasi-neuronal and quasi-oocyte membranes, and composition asymmetry (albeit mammalian concentration asymmetry). While I have focused on DHA, both n-3 PUFAs, Eicosapentaenoic acid (EPA) and  $\alpha$ -Linolenic acid (ALA), should also be considered for oocyte modulation.

Once a prediction has been developed for supplementation that would preserve boundary lipids, it will be shared with an experimental collaborator of Dr Brannigan's, Dr. John Baenziger, to be tested for improved nAChR function. If differences in boundary lipids are not observed, an enrichment protocol will be predicted for shifting the membrane viscoelastic properties to that of the native system.

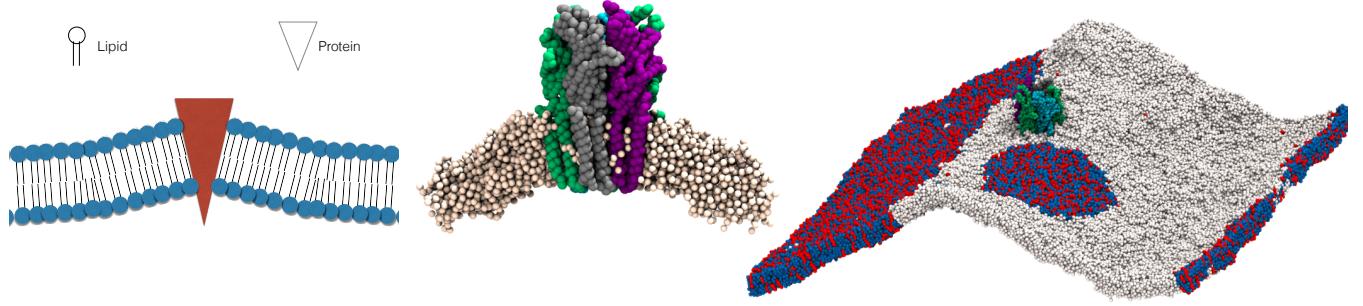


Figure 9: Possible role of membrane flexibility in determining nAChR partitioning. (A) A schematic representation of predicted membrane deformation around a cone-shaped protein, as described in Goulian et al, [74] in which the protein imposes constraints on the slope of the membrane at the interface with the protein (B) Cross-sectional cut of simulation-frame showing membrane deformation around nAChR embedded in an  $l_{do}$  phase composed of DHA-PE (white). (C) Phase-boundary partitioning in a larger membrane. nAChR is localized at the interface between the  $l_{do}$  phase composed of DHA-PE and the  $l_o$  phase composed of DPPC (blue) and cholesterol (red). Provided the membrane is sufficiently large, partitioning at the boundary permits tangential interaction with the cholesterol-rich  $l_o$  domain while the flexible  $l_{do}$  domain still absorbs the energetic cost of deformation.

#### 4.2 Aim 2: Investigation of the relative importance of pLGIC sequence vs shape in determining preferred lipid domain

This can be tested by comparing effects on partitioning profiles upon mutation of lipid facing residues versus adjustments in membrane lipid composition. If the effect of the protein’s sequence is measured to be greater than its shape, it is likely that pLGICs will display significant variation in partitioning behavior and annular lipid preferences. If the reverse is observed, it is likely that overall pLGIC shape and relative flexibility of domains drives partitioning, and thus all pLGICs may have similar partitioning behavior

Neuronal signaling relies heavily on transmembrane proteins such as ion channels and receptors, which are embedded in membranes with distinctive lipid compositions. The multitude of PUFA’s within nAChR native membranes does not necessarily discount them; in fact they may be critical to functionality. Such lipid dependence offers the organism numerous possibilities for lipid based regulation [73].

Separation of cholesterol and saturated phospholipids from unsaturated phospholipids, into liquid-ordered  $l_o$  (“raft”) and liquid-disordered  $l_{do}$  domains respectively, is detected even in simple ternary lipid mixtures. Increasing both cholesterol concentration and acyl chain unsaturation, as in neuronal membranes, increases the propensity of the membrane to form sharply-defined domains relative to other mammalian membranes.

As one example, neurotransmitter receptors must cluster at high density for efficient neurotransmission, and effects of lipid composition on membrane organization may serve an important modulatory role for an organism’s ion channel functionality. The high density of nAChR clusters found in the postsynaptic membrane of the mature neuromuscular junction ( $10^4 \mu\text{m}^{-2}$ ) is well-established to be stabilized by dimerization of nAChRs via binding of the cytoplasmic peripheral membrane protein rapsyn [75]. This process is also sensitive to membrane composition, particularly cholesterol. It has been frequently hypothesized [76, 77] that initial stages of clustering may require clustering via lipid domains, but experiments investigating whether nAChRs partition into lipid domains have been inconclusive [52, 78].

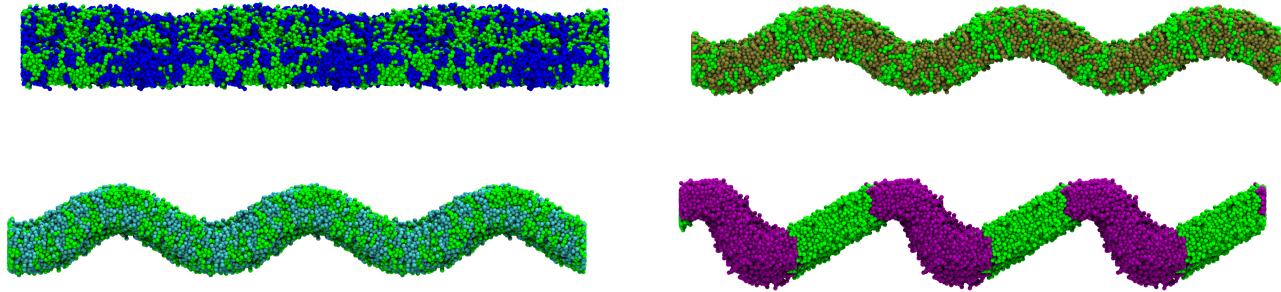


Figure 10: Binary, randomly-mixed membranes with increasing thermal undulations and decreasing bending modulus  $k_c$ . We predict that the more flexible membranes (lower  $k_c$ ) will accommodate a cone shaped protein with reduced energetic costs. The bending modulus  $k_c$  can be measured from the thermal undulation spectrum, as well as additional methods appropriate for small membranes. Aim 3 proposes implementation of these methods into a plugin for the widely used analysis-software VMD. For the purpose of this image, saturated lipids are green, while unsaturated lipids are brown, cyan, and purple.

Such experiments have focused primarily on detecting partitioning into liquid-ordered ( $l_o$ ) domains, which is often detected differently than partitioning into  $l_{do}$  domains; in my preliminary simulations we observe partitioning of nAChRs into the  $l_{do}$  domain (Figure 8). Partitioning into  $l_{do}$  domains would also cluster receptors and be cholesterol dependent, but would be a less effective mechanism in low cholesterol membranes, such as oocytes.

Our observation that nAChR partitions into  $l_{do}$  domains was surprising because these domains are very low in cholesterol; the simplest hypothesis to explain cholesterol-sensitivity of nAChR function and oligomerization was that nAChR would partition to the cholesterol rich  $l_o$  “raft” phase, although experimental data has been inconclusive.

Direct visualization, using computational microscopy, of the  $l_{do}$  domain around nAChR in the MD simulation trajectories revealed a deformation of the membrane around the cone-shape of the TMD (Figure 9). This type of deformation was predicted for a general cone shape protein two decades earlier, based on analytical elasticity theories of protein-induced membrane deformations [74, 79] but has not, to our knowledge, been used to predict partitioning preferences of proteins. According to these theories, the energetic penalty for the membrane deformation should increase with the bending rigidity  $k_c$ , so a significantly more flexible  $l_{do}$  phase would be the natural preferred phase for a single receptor.

My approach for this aim is to better determine the role of membrane flexibility in pLGICs partitioning; this has not previously been considered in simulation or experimental design, but it will provide essential insight into whether domain preferences are likely to be more sensitive to pLGIC sequence or to differences in domain rigidity.

If partitioning of pLGICs within domain-forming membranes is driven by membrane elasticity and the requirement for a flexible membrane around the cone-shaped protein, partitioning will be strongly sensitive to changes in lipid composition that affect flexibility or spontaneous curvature of  $l_o$  and/or  $l_{do}$  phases, and only weakly sensitive to pLGIC sequence, since pLGICs are structurally conserved. For example neuromuscular nAChR and GABA(A) receptor $\alpha 1\beta 3\gamma 2$   $\alpha$  subunit’s TMD share  $\sim 20\%$  identity [80], however it is unclear why either partition similarly. If partitioning is instead driven by specific interactions with lipids, it will be far more sensitive to pLGIC sequence,

particularly the presence of bulky versus small residues in the TMD.

My approach will involve first choosing purposeful pLGIC (GABA(A) receptor, glycine receptor, 5HT-3 receptor, and prokaryotic pLGICs such as GLIC or ELIC). Next, using multiple mutations to nAChR M1, M3, and M4 helices, to sequences of these other pLGICs. Lastly, adjust relative membrane elasticity parameters by e.g. increasing or decreasing membrane asymmetry or increasing or decreasing chain length. The effects of modifications on differences in elastic parameters will be quantified using the approach developed in Aim 3.

### 4.3 Aim 3: Development and release of a user-friendly VMD plugin for measuring elastic parameters of heterogenous membranes

While multiple individuals have developed scripts to determine the fluctuation spectrum of membranes, there is no universal tool computational chemists and biophysicists can use. I will develop a tool to measure elastic parameters and fluctuation spectra within the convenient scripting environment of the VMD software, which will alleviate the daunting nature of solving for the fluctuation spectrum and related moduli. This tool will assist us in optimizing lipid selections for modeled neuronal membranes; I can adjust lipid species and lipid concentrations to mimic elastic properties of neuronal membranes.

This package could be of significant use to both biochemists and biophysicists. It would allow them to easily predict the elasticity properties of a membrane composed of non-specific lipids. Combined with VMD's convenient scripting environment, this alleviates the daunting nature of solving for the fluctuation spectrum and related moduli, and promotes consideration of elastic effects in mechanisms by relying on the Monge Gauge  $h(r) = z$ , where  $z$  is a deviation in membrane height from 0.

Numerous computational methods with increasing sophistication for measuring elastic properties of membranes have been developed by physicists and physical chemists [81–85]. However, most are developed using in-house code, and none are integrated into a widely-used, flexible analysis package such as VMD [86].

I will develop a user-friendly VMD plugin that can measure elastic properties for generalized heterogeneous lipid bilayers, allowing comparison among different lipid compositions relying on measurement techniques in the convenient scriptable VMD environment. It will allow those with a need to quantify membrane flexibility, but without the requisite skills in lower-level programming or background in soft-condensed matter physics, to carry out reliable and straightforward calculations. It will also link the elasticity calculations to the extensive abilities of VMD for analyzing molecular interactions.

I envision the proposed plugin will be usable from both a GUI and VMD's tk terminal, and will provide the option to measure the bending modulus, stretching modulus, tilt modulus, equilibrium area per molecule, and monolayer spontaneous curvature, based on fluctuation spectrum methods [82, 85] and molecular fluctuations [81, 83, 84]. Calculations will be performed on a trajectory loaded through VMDs extensive trajectory format libraries, making analysis convenient for users of NAMD, GROMACS, LAMMPS, HOOMD, or any MD software that outputs in one of the many VMD-readable formats.

The analysis code will have the option to specify starting and ending frames, as well as how to approximate the membrane surface and/or lipid tilt angles simply based on VMD-based atom selections, greatly simplifying the required code. The plugin will serve as a wrapper to C code that performs fast Fourier transforms and spectral

analysis. Data will be output to a an ASCII file that can be easily manipulated in a scientific programing language of the user's choice (i.e. Python, Matlab).

Data will be collected by binning over a membrane with adjustable bin steps ( $dx$  and  $dy$ ), and averaging the membrane hight ( $z$ ) over the bin. Setting  $z$  to  $h(r)$  (where  $r = r(x, y)$ ) and performing a Fourier transform on  $h(r)$  results in  $\tilde{h}(q)$ ,

$$\begin{aligned}\tilde{h}(q) &= \frac{1}{L} \int dr (h(r) e^{-iqr}) \\ h(r) &= \frac{1}{L} \sum \tilde{h}(q) e^{iqr}\end{aligned}\tag{4}$$

where  $q$  is the spacial frequency. Allowing  $F$  to be the Helfrich Hamiltonian

$$F = k_c(H - 2C_0)^2 + k_G K,\tag{5}$$

where,  $k_c$  is the bending modulus,  $H$  is the mean curvature,  $k_G$  is the Guasian modulus, and  $K$  is the Gausian curvature. When applied to a closed membrane  $C_0, k_G, K$  drop out, and the equation reduces to

$$F = k_c H^2,\tag{6}$$

where  $H$  can be expressed as

$$H = |\nabla^2 h(r)|.\tag{7}$$

We can then determine the fluctuation spectrum,  $S(q)$ , which [85] defined as

$$S(q) = \langle |\tilde{h}(q)|^2 \rangle.\tag{8}$$

According to Helfrich elasticity of membranes,[87] at long wavelengths the spectrum should obey approximate to

$$S(q) \sim \frac{k_B T}{k_c q^4}\tag{9}$$

Where  $k_B$  is Boltzmann's constant,  $T$  is temperature, and  $k_c$  is the bending modulus.

We would work with VMD developers to incorporate the plugin into the official VMD distribution, and provide support to the VMD user's community, as well as necessary updates and improvements.

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