OLIGOMERIZATION OF NICOTINIC ACETYLCHOLINE RECEPTORS IN MEMBRANES WITH DHA-ENRICHED DOMAINS

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ABSTRACT OF THE THESIS

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The nicotinic acetylcholine receptor (nAChR) is an excitatory neurotransmitter receptor that mediates muscle functioning by forming nAChR-associated, lattice networks. At the neuromuscular junction (NMJ), synaptic and intracellular proteins, notably Agrin, MusK, and rapsyn, ultimately stabilize these highly dense networks. Interestingly, experimental evidence suggests that cholesterol-rich domains, known as lipid rafts, facilitate signaling among Agrin-Musk and rapsyn, and their presence is essential for healthy nAChR clustering. In spite of their importance, the structural and functional mechanisms of lipid domains are currently unknown. Alongside cholesterol, Docosahexaenoic acid omega-3 fatty acids (DHA-PUFAs) are prevalent at the NMJ, correlate with domain formation, and strongly promote neuronal health. In the present study, we computationally explored the role of DHA-PUFAs on nAChR clustering in the presence and absence of lipid domains. Within coarse-grained (CG) model membranes, nAChRs consistently partitioned into flexible, liquid-disordered domains; boundary lipids were rich in DHA-PUFAs regardless of the number of nAChR molecules, but preventing

domain formation also reduced the likelihood of these acyl chains aggregating around nAChR. Taken together, our findings suggest that by inducing domain formation in membranes, DHA plays a critical role in the early stages of nAChR oligomerization.

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
CG-MD	Coarse-grained Molecular Dynamics
CMS	Congenital Myasthenic Syndrome
Dok-7	Docking protein 7
DPPC	Di-palmitoyl-phosphatidylcholine
DHA-PE	Di-docosahexaenoyl phosphatidylethanolamine
DHA-PS	Di-docosahexaenoyl phosphatidylserine
ECD	Extracellular domain
ICD	Intracellular domain
l_d	Liquid-disordered
l_o	Liquid-ordered
LRP4	Low-density lipoprotein receptor-related protein 4
M1	Transmembrane α-helix 1
M2	Transmembrane α-helix 2
M3	Transmembrane α-helix 3
M4	Transmembrane α-helix 4
MuSK	Muscle-specific kinase
nAChR	Nicotinic acetylcholine receptor
NMJ	Neuromuscular Junction
pLGIC	Pentameric ligand-gated ion channel
Rapsyn	Receptor-associated protein of the synapse
TMD	Transmembrane domain

Chapter 1

Background information

1.1 Neuromuscular disorders

The motivation for my research is to better characterize and diagnose disorders at the neuromuscular junction (NMJ): the synapse separating a motor neuron from its target muscle fiber[17]. One of the most prevalent neuromuscular conditions, Myasthenia gravis, is an autoimmune disease in which antibodies bind to and destroy nicotinic acetylcholine receptors (nAChRs): transmembrane proteins critical for muscle movement[42, 69]. As a result, nAChRs are significantly reduced, which in turn, lead to symptoms such as muscle weakness[31, 46], ptosis (droopy eyelids)[38, 28], and diplopia (double vision)[70].

A separate class of genetic disorders, known as Congenital Myastheniac Syndrome (CMS), disrupt the development and functioning of the NMJ[52]. Typically, patients with CMS experience progressive fatigue, reduced mobility, and poor motor coordination [22, 18]. Since the 1970s, scientists have identified 20 CMS-related genes that alter the expression and/or activity of neuromuscular proteins, including nAChRs [20, 21, 19]. While drug therapy can be partially effective in treating neuromuscular diseases, scientists currently lack the physiological understanding of the NMJ, in order to fully alleviate patients' symptoms [13].

In an attempt to bridge the gaps between science and treatment, I study the membrane dynamics associated with healthy nAChR clustering: The organizational phenomenon underlying efficient muscle contraction[78]. In particular, I focus on the role of surrounding membrane lipids in mediating nAChR clustering. By using sophisticated computational tools, the present research can offer meaningful insights into human health and disease. Before elaborating on the details of my thesis, I will first provide descriptions of the neuromuscular junction, the nicotinic acetylcholine receptor, and protein-lipid interactions in native cell membranes. Taken together, this background information will provide sufficient context for understanding my research and its implications in treating neuromuscular disorders.

1.2 Signaling and development of the neuromuscular junction

The NMJ is the chemical synapse that facilitates muscle movement through a cascade of signaling proteins, including nAChR[56]. Within the postsynaptic membrane, nAChRs cluster in high densities (10,000 per μs^2) to properly activate the skeletal muscle[57, 6]. A series of experiments indicate that rapsyn and the Agrin-MuSK complex, a group of interactive synaptic proteins, are essential for nAChR clustering [24, 43, 10]. In the mature neuromuscular membrane, nAChRs are linked by the intracellular anchoring protein, rapsyn, which bridges receptors together at their bases [78]. By activating the Agrin-MuSK complex, the lipoprotein receptor-related protein 4 (LRP4) and docking-protein 7 (Dok-7) play important roles in rapsyn migration; without these mediating proteins, rapsyn remains primarily in the synapse, and nAChR networks are unable to form [35].

Communication at the NMJ involves a series of events between the presynaptic and postsynaptic cells: First, an electrical signal, known as an action potential, travels down the presynaptic neuron to trigger the release of neurotransmitter, acetylcholine (ACh),

at the axon terminal. ACh then diffuses across the synaptic cleft to bind nAChRs on the post-synaptic membrane, resulting in an excitatory postsynaptic potential (EPP). If sufficient, the EPP depolarizes the cell, leading to muscle contraction. After activation, the ion channel closes and ACh disassociates from its binding sites, where it is degraded by the regulatory enzyme, acetylcholinesterase (AChE) [56].

In early embryonic development, several motor neurons may contact a single synaptic site. As the NMJ matures, the number of contacts are reduced until only one motor neuron signals a muscle fiber. During later developmental stages, fetal nAChRs are replaced by their adult structure (in which the γ subunit is substituted for an ϵ subunit) [47]. Additionally, the membrane changes shape to support protein clustering, with nAChR-rapsyn complexes concentrated along the postsynaptic folds[61]. Neuromuscular diseases, especially CMS, can interfere with healthy NMJ development. For this reason, our studies focus on the fetal nAChR found at the NMJ and the Torpedo electric organ. In the next two sections, I will provide a brief description of nAChR classification and structure, followed by an in-depth review of the fetal nAChR, with an emphasis on its lipid sensitivities and functional properties.

1.3 Pentameric-ligand gated ion channels: nAChR

Pentameric ligand-gated ion channels (pLGIC) are a major class of transmembrane proteins that immediately respond to the binding of stimulating molecules, such as neurotransmitters, to give rise to rapid and effective signaling [60, 8, 54, 34]. In humans, pLGICs play significant roles in inflammation [50], addiction[11], chronic pain [73], and the onset of diseases, including Alzheimer's disease[51] and Spinal Muscular Atrophy[3]. The nicotinic acetylcholine receptor (nAChR) is one of the most well-studied, fundamental pLGICs for understanding human cognition, memory, and muscle contraction[30].

Structurally, nAChR is composed of five subunits; each subunit is composed of an extracellular domain for ligand binding, a transmembrane domain (TMD), and an intracellular domain (ICD). The TMD is the embedded portion of nAChR that interacts most often with surrounding membrane lipids; this region of nAChR is composed of four alpha-helices, M1-M4, with innermost, M2 helices closest to the ion pore[62]. The ICD, or cytoplasmic domain, is highly disordered, making it challenging to obtain information on its structure [48].

As a major neurotransmitter receptor, nAChR can be located in both the central and peripheral nervous systems, with various subunit combinations [1]. In vertebrates, 17 subunits have been identified and categorized by the following polypeptide chains; 10 alpha (α_1 - α_{10}), four beta (β_1 - β_4), one epsilon (ϵ), one gamma (γ), and one delta (δ). Regardless of its cellular environment, nAChRs must contain at least two alpha subunits for ACh binding. Outside of the neuromuscular junction, such as in brain and epithelial tissue, nAChRs can take on numerous subunit combinations. For instance, nAChR can adopt a homopentamer conformation of all α_7, α_8, α_9, or α_{10} subunits, or it can exist as a heteropentamer, of distinct subunits, such as α and β. In muscle cells, nAChR can adopt one of two heteropentamer arrangements: the fetal configuration $(\alpha_1, \beta_1, \delta, \alpha_1, \gamma)$, or the adult configuration $(\alpha_1, \beta_1, \delta, \alpha_1, \epsilon)$ As stated previously, we will focus on the fetal, or child-type, nAChR found at both the NMJ and the Torpedo electric organ. For the remainder of this thesis, I will refer to α_1 and β_1 as α and β (Please see Figure 1.1 below).

1.4 nAChR: neuromuscular structure, 2BG9

First resolved through cryo-electron microscopy[9], the muscle-derived nAChR (PDB id-2BG9)[68] is the most abundant nAChR at the NMJ in most vertebrates, including humans [1]. For nAChR to maintain functionality, it depends upon a highly specific

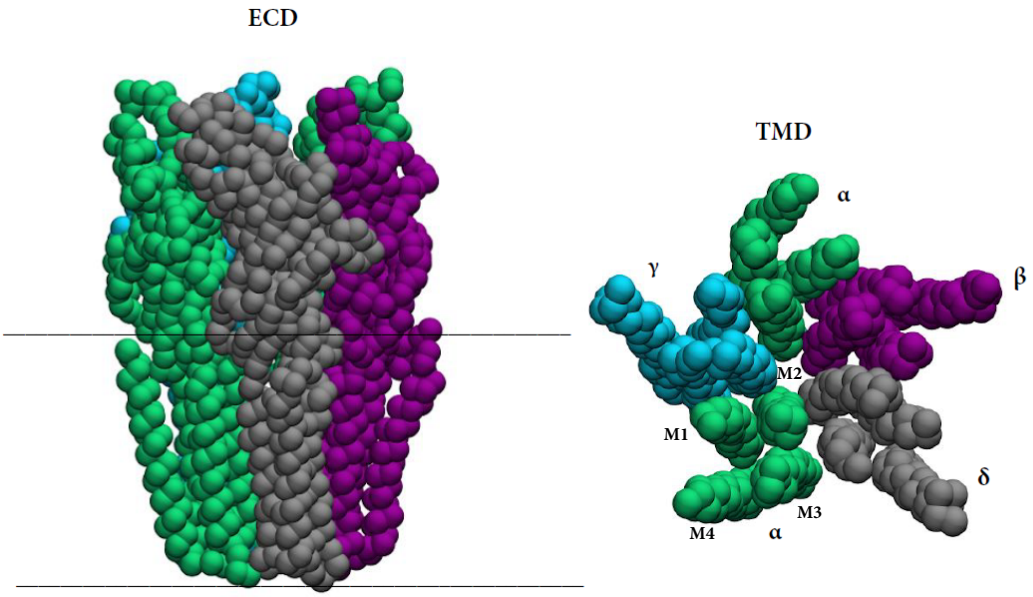


Figure 1.1: **Nicotinic acetylcholine receptor (nAChR) structure.** Modified neuromuscular nAChR, with atomic coordinates adopted from Unwin et al's cryo-EM structure, pdb-id: 2BG9. The protein is colored by subunit (α : $green, \beta$: $purple, \delta$: $gray, \gamma$: $cyan$), with its structural components highlighted above. The extracellular domain (ECD) is located above the bilayer and is critical for ligand-binding. The transmembrane domain (TMD) is positioned within the lipid bilayer and is made up of four alpha helices (depicted on the right). The outermost M4 helices closely interact with surrounding membrane lipids, while the M2 helices outline the ion pore; the M1 and M3 helices make up the body of the transmembrane domain. The intracellular domain is poorly resolved, and is not necessary for this study [68].

lipid environment. Experimentally, lipids influenced nAChR activity by stabilizing its open and closed states[12], channel gating properties[14], and position within the membrane[2]. It is important to understand how changes in lipid environment impact nAChR's structure and activity, given that lipids can change in response to aging and disease [74].

Over the past few decades, considerable progress has been made in uncovering lipid sensitivities associated with nAChR [4, 12]. In a majority of experiments, scientists have prioritized cholesterol over other membrane lipids due to its high concentration in

the native membrane [49, 40, 45, 53]. Early studies revealed that, when reconstituted into membrane mixtures, nAChR failed to conduct cations across the lipid bilayer unless cholesterol and anionic phospholipids were present [25, 66, 7, 26, 15]. In fact, according to several analyses, anionic lipids and cholesterol constitute approximately 40-45 % of the Torpedo membrane composition [29].

More recently, Brannigan et. al (2008) provided computational evidence for deeply embedded cholesterol in the transmembrane gaps of nAChR [5]. According to these simulations, nAChR subunits collapsed in the absence of embedded cholesterol. These results were made possible by Unwin and colleagues, whose advancements in electron microscopy revealed unoccupied gaps in the 2BG9 transmembrane domain [68].

Subsequent experiments confirmed the necessity of embedded cholesterol for proper nAChR conformation, ion conductance, and signaling. Based on this literature, Corrie and Baenziger (2013) proposed direct mechanisms for cholesterol-based regulation, including nAChR boundary lipid preferences [16]. In spite of this compelling evidence, experiments with liposomes revealed an important and counterintuitive phenomenon: removing cholesterol from the bulk membrane further improved functionality of nAChR [40, 76, 37]. Given these results, researchers began focusing on large-scale organizational principles, such as membrane elasticity and thickness, associated with the bulk membrane [41, 36].

Research on indirect lipid-protein interactions are especially important for understanding the dynamics of the NMJ[78, 11]. In particular, Zhu (2006) discovered that cholesterol-rich microdomains (i.e., lipid rafts) directly mediate communication between rapsyn and the Agrin-MuSk complex. After disrupting lipid raft formation, Zhu observed a significant loss nAChR-rapsyn clusters in-vitro [77]. In the same year, Willmann (2006) and Stetzkowski-Marden (2006) proposed that lipid rafts can further stabilize receptor-rapsyn networks and may even provide a localized environment for

nAChR [72, 65].

While this research links lipid rafts to NMJ organization, the structural and functional mechanisms of lipid domains are currently unknown.In the final background section, I will briefly describe membrane lipid properties and organization in biological systems.

1.5 Phospholipid properties and organization in cell membranes

The building blocks of a cell membrane are the phospholipids, which contain a polar headgroup and two non-polar fatty acid tails. The hydrophobic tails are hydrocarbon sequences and generally exhibit chain asymmetry, due to differences in molecular weight, degree of saturation, and chain length [58]. Domain formation can occur when the following conditions are satisfied: the membrane must contain a sufficient concentration of cholesterol, unsaturated fatty acids, and a molecule that interacts closely with cholesterol such as saturated fatty acids or sphingomyelin[23, 75]. Here, we construct model membranes with cholesterol, polyunsatuated fatty acids (PUFAs), and saturated fatty acids.

Saturated fatty acids are characterized by single bonds in their hydrocarbon sequences. Due to their molecular structure, saturated fatty acids prefer to pack tightly with other rigid molecules, such as cholesterol, forming a gel-like state; this gel-like structure is considered the liquid-ordered (l_o) phase. As a compressed microdomain within the membrane, l_o phases are commonly referred to as lipid rafts. According to some theories, lipid rafts can serve as potential scaffolding platforms for signaling molecules, including nAChR, rapsyn, and other peripheral proteins [41]. The unsaturated molecules, in contrast, separate from lipid rafts to form fluid, liquid disordered (l_d) domains [71].

Within the l_d domain, lipids can be classified based on their degree of unsaturation.

Monounsaturated fatty acids have one double bond in their hydrocarbon sequence. A double bond can produce a kink in the fatty acid chain, which creates free space within the bilayer, and allows additional flexibility between neighboring chains. By disrupting lipid packing, unsaturated fatty acids have low phase transition temperature. Polyunsaturated fatty acids (PUFAs) have multiple double bonds, with each double bond reducing its phase transition temperature by fifty degrees Celsius; as a result, these PUFAs are highly disordered and can induce domain formation in membranes [59, 33].

In neuromuscular membranes, intrinsic domain formation is dependent upon several lipid species, including the widely influential omega-3 PUFAs (n-3 PUFAs). One omega-3 in particular, Docosahexaenoic acid (DHA), is prevalent in the native neuromuscular membrane and is strongly associated with flexible and well-defined domains [67, 63]. Additionally, DHA is a major contributor to brain functioning, motor activity, and cardiac health; however, its specific effects on neuromuscular health are poorly understood [39, 71, 27].

Recently, Sharp et. al (2018)[64] computationally investigated nAChR-lipid interactions in mixtures containing PUFAs. By using coarse-grained simulations, they could effectively observe its partitioning behavior within the lipid domains. Contrary to expectations, nAChR consistently preferred a local lipid environment characterized by PUFAs, especially long-chained omega-3s, such as DHA. Interestingly, the protein also exhibited an orientation preference along the domain interface, with its alpha subunits frequently bordering cholesterol-enriched domains. While cholesterol occupied the transmembrane gaps of nAChR, PUFAs were even more likely to embed, regardless of their zwitterionic headgroup. Although these findings suggest that acyl chains primarily dictate lipid preferences, this study did not incorporate anionic headgroups in their membrane compositions. As mentioned previously, anionic headgroups were

required for nAChR to function properly.

The present study adopts a similar approach to Sharp et. al (2018), with a particular focus on nAChR-associated clustering. Through molecular dynamics simulations, we investigate nAChR lipid preferences and clustering behavior in membranes with and without domains. For this study, we tested four major hypotheses: 1) Membrane organization affects nAChR boundary lipid specificity: when PUFA chains are prevented from forming PUFA rich domains, their prevalence among nAChR boundary lipids will be significantly reduced. 2) Anionic headgroups will readily embed within the transmembrane helices of nAChR, relative to more prevalent zwitterionic headgroups. 3) Domain formation will indirectly facilitate the clustering of nAChRs, by inducing partitioning preferences and restricting diffusion within the membrane. 4) Within a dimer, we will observe sequence preferences in facing subunits.

Chapter 2

Methods

2.1 System setup

We constructed 48 coarse-grained (CG) molecular dynamics simulations containing 1-4 nAChRs, derived from the Torpedo electric organ[68]. One membrane had intrinsic domain formation, due to its fully saturated and fully unsaturated phospholipids: 3:3:3:1 Di-palmitoyl phosphatidylcholine (DPPC): Di-docosahexaenoyl phosphatidylethanolamine (DHA-PE) Cholesterol: Di-docosahexaenoyl phosphatidylserine (DHA-PS). In a second composition, domain formation was prevented by using a mixture of hybrid lipids, each with one DHA and one saturated tail: 3:3:3:1 1-palmitoyl-2-docosahexaenoyl-phosphatidylcholine (PUPC): 1-palmitoyl 2-docosahexaenoyl-phosphatidylethanolamine (PUPE): Cholesterol: 1-palmitoyl 2-docosahexaenoyl-phosphatidylserine (PUPS). [44]

The Martini CG force field allowed us to observe large-scale membrane interactions that are inaccessible through atomistic approaches (AA); such interactions include domain formation, protein partitioning, and protein clustering. By using a CG model, we could run simulations for longer length and time scales, with systems approaching equilibrium at approximately 2 μs. [44] We converted protein structures into coarse-grained models using the Martini script "martinize.py", mapping four non-hydrogen atoms to one CG interaction. We constructed and assembled our protein-bilayer systems using the Martini script, "insane.py", specifying a box size of 22x22x20nm^3, for one-protein

systems, and 44x44x22nm^3 for multiple-protein systems, respectively. [44]

2.2 Simulation details

Simulations were run using the Martini 2.2 force field parameters and the Gromacs 5.1.2 simulation package.[44, 55] Each simulation consisted of two phases: energy minimization and molecular dynamics. For each system, we ran two consecutive equilibrium simulations for 10,000 steps at a 0.001 ps time-step.

Harmonic restraints between backbone atoms were imposed to preserve nAChR conformation. More specifically, we applied an elastic force constant of 750 kJ/mol and set lower and upper bounds on the bond with lengths 0.9 nm and 1.6 nm, respectively[44, 55].

The molecular dynamics simulations ranged between 3 and 10 μs at a 0.025 ps time-step. Simulation temperature and pressure were kept constant at values of 323 K and a reference pressure of 1 bar. The isotropic pressure coupling compressibility constant was maintained at 3.0 $\times 10^{-5}$ bar^{-1} .

2.3 Analysis

We visualized and imaged all simulation results using the Visual Molecular Dynamics (VMD) program [32]. First, we quantified the degree of protein partitioning within a lipid domain by counting the number of b boundary lipids and comparing it with its expectation in a randomly mixed membrane. In the case of saturated lipids, the metric Q can be written as follows:

$$Q_{\mathrm{sat}} \equiv \frac{1}{x_{\mathrm{sat}}} \left\langle \frac{b_{\mathrm{sat}}}{b_{\mathrm{tot}}} \right\rangle \tag{2.1}$$

where b_{sat} is the number of saturated boundary lipids, b_{total} represents the total

number of boundary lipids, and x_{sat} represents the expected value for saturated boundary lipids, based on the initial, randomly-mixed composition. Similarly, we constructed two-dimensional distributions of boundary lipids over a series of polar bins. This order parameter is defined as follows:

$$\rho_{B,i} = \left\langle \frac{n_{B,i}}{A_i} \right\rangle \tag{2.2}$$

where $n_{B,i}$ is the number of lipid b found within bin$_i$ and A_i represents bin area.

Next, we directly compared the tendency for nAChR to self-dimerize in the presence or absence of lipid domains. For each simulation, we calculated the center of mass (COM) separating unique pairs of proteins, and computed the average pairwise distance per time-step. We calculated the average pairwise distance between all proteins in the system as:

$$\langle D \rangle = \frac{\sum D_{ij}}{N_p} \tag{2.3}$$

where D_{ij} is the distance between the ith protein and the jth protein, and N_p refers to the number of unique protein pairs.

Lastly, we calculated the percentage of time that a subunit pair faced one another with the following equation:

$$F_{x,y} = 100 \cdot \frac{n_{x,y}}{n_d} \tag{2.4}$$

Here, $n_{x,y}$ represents the number of frames in which subunits "x and y" are the closest and n_d is the total number of frames that the two proteins cluster together (satisfying a cutoff of 200 Å).

Chapter 3

Results

3.1 nAChR boundary lipid preferences

For simulations containing 2-4 nAChRs, we measured the fraction of annular lipids that occupy the outer perimeter of the protein. Using Equation 2.1, we constructed distributions of annular acyl chains across all 36 systems, with data collected from 2000 to 3000 ns trajectories, respectively. As shown in Figure 3.1, in domain-forming compositions DHA occupied the largest proportion of annular lipids (57 %) followed by cholesterol (28 %) and saturated lipids (15 %). For saturated and polyunsaturated lipids, the observed annular concentrations were significantly different than the values expected based on their starting compositions, with annular DHA higher than the bulk concentration and annular saturated less than the bulk concentration. In hybrid mixtures, nAChRs no longer exhibited acyl chain preferences, and acyl chain distributions better fit the expected annular ratios in a randomly mixed membrane (Please see Figure 3.1 for additional details).

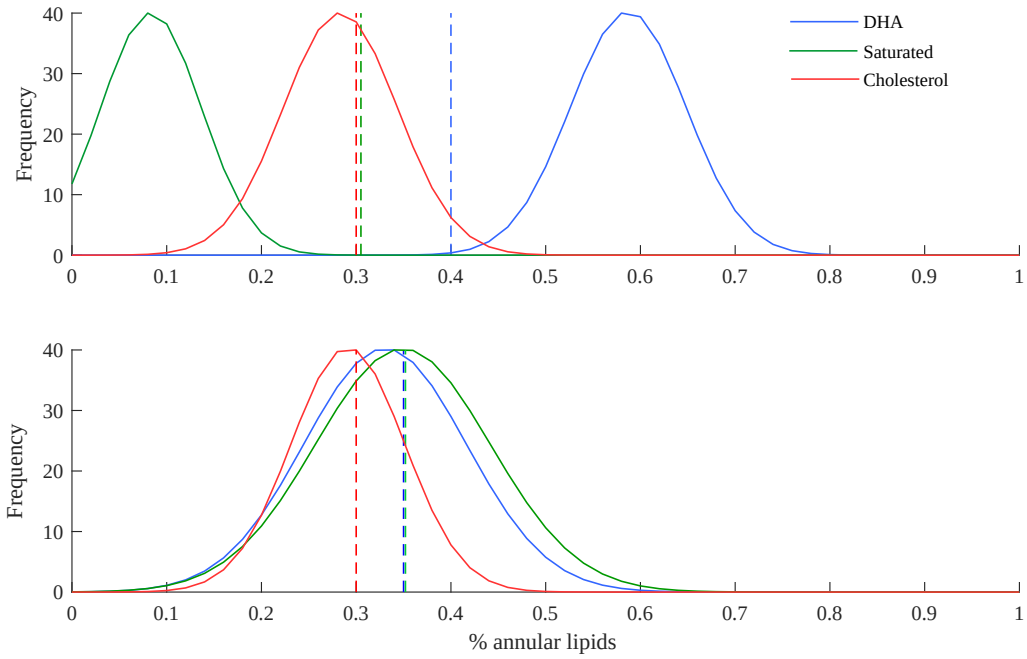
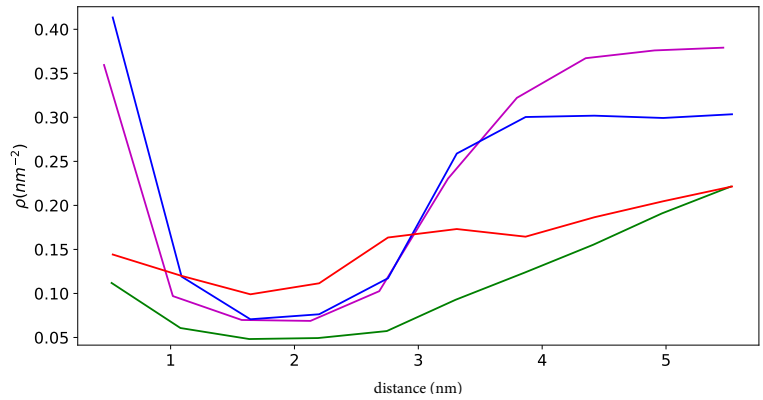


Figure 3.1: **nAChR annular lipid preferences.** Probability function showing the distribution of lipid concentrations in close proximity to nAChR (10-35 Å from the the center M2 helices). Top depicts membranes with domain formation. Bottom depicts membranes without domain formation. Distributions reflect aggregate data for systems containing two, three, and four nAChRs. Dashed lines represent expected boundary ratios for a randomly-mixed membrane, based on overall lipid composition.

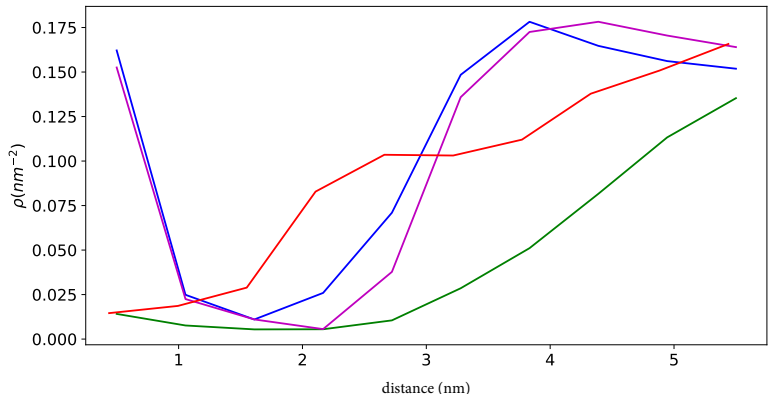
Next, using Equation 2.2, we calculated the average density of each lipid species within 5 nm from the center of nAChR; these data represented the average non-annular lipid concentrations buried within the transmembrane bundle. In Figure 3.2, we plotted densities based on headgroup, given that both compositions have idenitical headgroup concentrations (30:PC, 30:PE, 30:CHOL, 10:PS). All data were normalized to account for differences in headgroup concentrations, as well as membrane thicknesses.

In both domain-forming and hybrid membrane compositions, PS (blue) and PE

(magenta) phospholipids were predominately concentrated near the innermost and outermost transmembrane helices, located respectively approximately 1 nm and 4 nm from the center of mass of nAChR. However, in domain-forming membranes these lipids exhibited a greater affinity for M2 and M4. Meanwhile, cholesterol occupies only 15 % of non-annular sites in the presence of domains, and up to 12.5 % in the absence of domains, as seen in Figure 3.2; these values show cholesterol relatively depleted throughout the transmembrane bundle compared to its annular concentration shown in Figure 3.1. Across all systems, PC phospholipids were least likely to embed, with a maximum concentration of 10-12 % over the 5 nm range. Please see Figure 3.2 for more information.



(a) Domains



(b) No domains

Figure 3.2: **Nonannular binding of lipids based on headgroup.** Lipid densities within 5 nm of the nAChR COM (Top: Domain-forming, Bottom:Hybrid). Lipids were colored by headgroup in both compositions (PC: green, PE: purple: CHOL: red, PS: blue). All data were normalized to account for differences in headgroup concentrations, as well as membrane thicknesses.

3.2 nAChR clustering in the presence and absence of lipid domains

To better characterize the clustering nAChRs, we constructed two distributions of pairwise distances for each membrane composition, using equation 2.3 above. In figure 3.3, distributions reflect aggregate data averaged over 36 simulations from 2000 to 3000 ns. In domain-forming membranes, the majority of nAChR pairs were close together, with a mean distance around 125 Å. In contrast, for hybrid membranes, nAChRs were farther apart, averaging around 225 Å.

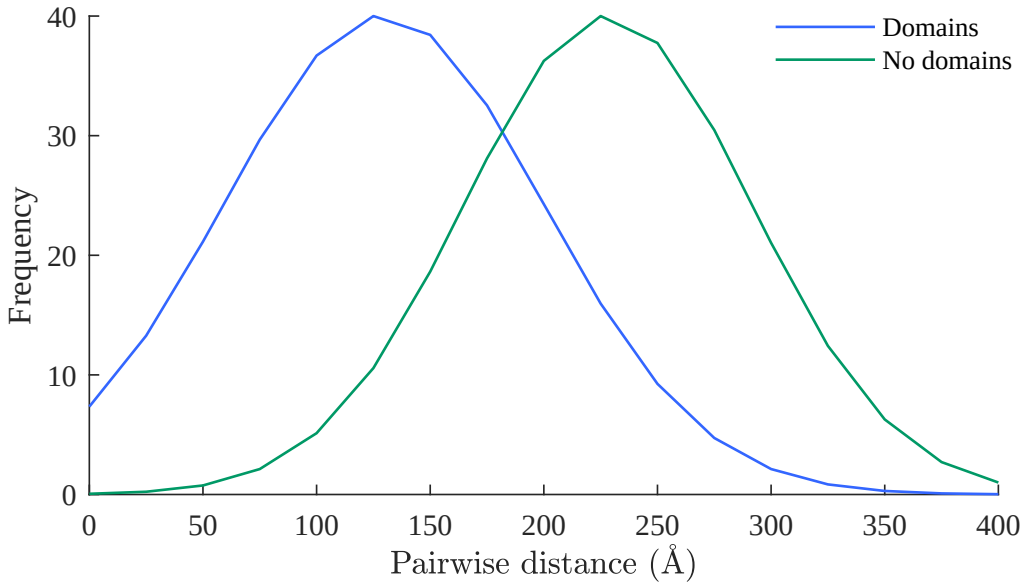


Figure 3.3: **Pairwise distance distribution across multi-protein systems.** Distribution of average pairwise distances across all 36 simulations. Data collection began at 2000 ns into each trajectory and ended at 3000 ns, respectively.

In the majority of domain-forming membranes, we observed the formation of nAChR dimers along domain interfaces. Along domains, a majority of pairwise distances fell within 100-150 Å by 3000 ns (see Figure 3.4). This trend persisted within and across individual simulations, regardless of protein concentration or the size and shape of

domains.

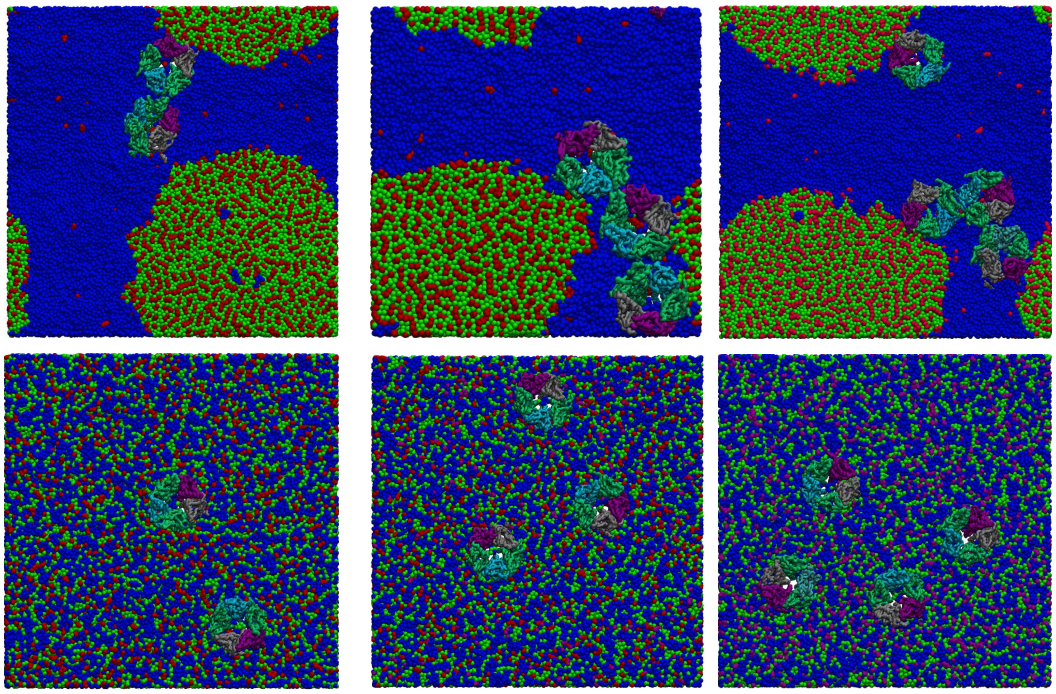


Figure 3.4: **Protein clustering in domain-forming (top row) and hybrid (bottom row) membranes.** nAChRs were colored by subunit ($\alpha : green, \beta : purple, \delta : gray, \gamma : cyan$), and lipids by acyl chain (DHA: blue, saturated: green, and cholesterol: red). Clustering of nAChRs on the borders of lipid rafts is visible in the domain-forming membranes.

3.3 Closest subunits across dimerizing proteins

Our final analysis calculated the distance between nAChR subunit pairs for dimerizing proteins, using equation 2.4, focusing on identifying the closest pair among the 15 possible subunit combinations. Among all 36 simulations, the nAChR α_δ subunit most frequently formed the closest pair, pairing with α_γ, δ, and γ subunits well above expectation (10–20 %) in both domain-forming and hybrid membranes. In domain-forming membranes, the most frequest closest subunit pairs were (α_δ, δ) and (α_δ, γ), each appearing in more than 20 % of simulations. In hybrid membranes, in addition to the α_δ subunits mentioned above, (α_γ, γ) were among the closest subunits at a level above expectation. On the other hand, the nAChR β subunits formed the fewest subunit pairs, falling well below the expectation value for each pair. Please refer to Figure 3.5 for a histogram of these data.

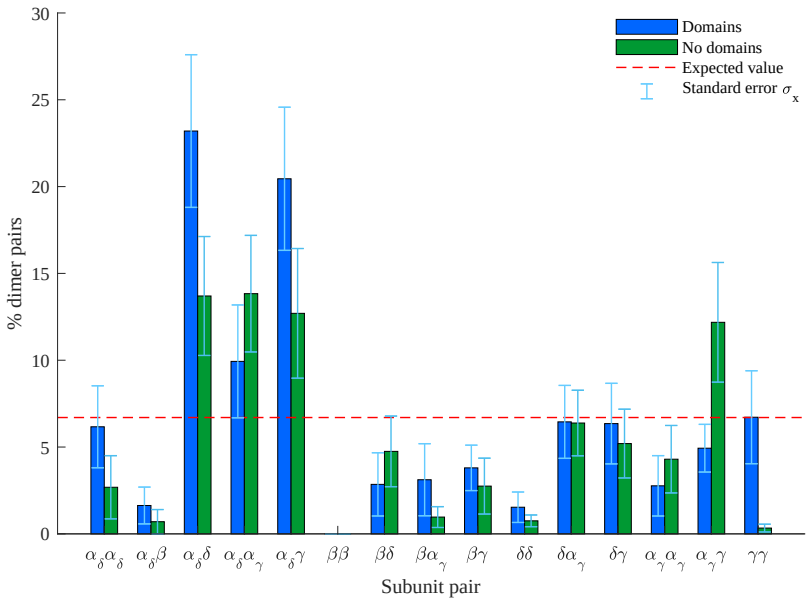


Figure 3.5: **Subunit pairs among dimerizing proteins.** Histogram showing the number of frames in which two subunits were closest together among dimerizing proteins (200 Å threshold).

Chapter 4

Discussion

Found primarily in fish oil, DHA has been generally associated with enhanced cognitive functioning, as well as benefits in cardiac health and disease prevention [39, 71, 27]. As stated previously, DHA is an omega-3 fatty acid found primarily in the brain, synapses, and the Torpedo electric organs, all of which are native membranes for human nAChR. While DHA is implicated in human health and disease, its interactions with critical membrane proteins, such as nAChR, have been largely overlooked in previous studies. Consistent with MD-CG simulations using one nAChR [64], multiple nAChRs continue to prefer the liquid-disordered phase containing long-chain omega-3 fatty acids. More specifically, nAChRs partitioned into DHA-enriched domains.

While it is well-established that cholesterol leads to a gain-of-function in nAChR, adding anionic lipids like POPA to a purely POPC or POPC:CHOL reconstitution mixture also results in significant gain-of-function, causing some researchers to suggest anionic lipids are required for functional conformations of nAChR [15]. If so, we might expect anionic lipids (such as the DHA-PS used here) to have a higher probability of being in the nAChR boundary than zwitterionic lipids. Instead, we find that anionic lipids and zwitterionic lipids (PE headgroups) had similar affinities for nAChR's TMD. It is important to emphasize that in domain-forming membranes, PS and PE phospholipids both had DHA acyl chains and their probabilities of embedding were much higher compared to those in hybrid mixtures (See Figure 3.2). While cholesterol embedded throughout the nAChR transmembrane bundle, DHA-PS and DHA-PE occupied these

non-annular sites in higher concentrations. This supports the hypothesis that, alongside cholesterol, PUFAs can provide additional support to nAChR structural and functional integrity, as hypothesized by Sharp et. al (2018) [64].

There are several potential explanations for the discrepancy between our results and those of previous experimentalists. To our knowledge, previous studies have not included PUFAs in their membrane mixtures, which may affect the interactions between lipids and the nAChR ion pore. [25, 66, 7, 26, 15]. It is possible that even in these experiments, PA and PC had equal affinities for nAChR, but PA stabilized the conducting state while PC did not. We would not be able to detect this in our simulations.

Our data show that by inducing domain formation in membranes, DHA leads to the clustering of nAChRs in the flexible, liquid-disordered phase and supports their boundary acyl chain preference for PUFAs (Figure 3.1). Without domain formation, since hybrid lipids only have one DHA chain each, DHA cannot aggregate around nAChRs to the same degree.

Pentameric ligand-gated ion channels, such as nAChR and GABA-A, have retained function for millions of years and are found in many species - from bacteria to humans; however, heteromers, which include γ or δ subunits, are found in higher-level organisms and between synapses [34]. In our simulations, δ and γ subunits promoted oligomerization of muscle-type nAChRs. This preference in subunit pairs may indicate an essential role for heteromerization in pLGIC oligomerization within the nervous system. Building on this work, we aim to expand the scope of our computational models, with special consideration to the individual effects of membrane elasticity, protein concentration, and pLGIC sequence on nAChR oligomerization.

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