UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

Maestría y Doctorado en Ciencias Bioquímicas

Alpha synuclein expression in sarcolemma and transverse tubules from muscle fibers during aging

TESIS

QUE PARA OPTAR POR EL GRADO DE:
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Presente

Los miembros del Subcomité Académico en reunión ordinaria del día **22 de marzo** del presente año, conocieron su solicitud de asignación de **JURADO DE EXAMEN** para optar por el grado de **Maestro en Ciencias**, con la réplica de la tesis **"Alpha synuclein expression in sarcolemma and transverse tubule from muscle fibers during aging"**, dirigida por el/la Dr(a). **Ortega Aguilar Alicia.**

De su análisis se acordó nombrar el siguiente jurado integrado por los doctores:

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Sin otro particular por el momento, aprovecho la ocasión para enviarle un cordial saludo.

A t e n t a m e n t e
"POR MI RAZA, HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd. Mx., a 22 de marzo de 2021
COORDINADORA

Dra. Claudia Lydia Treviño Santa Cruz

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Contents

ABSTRACT

Significance: Alpha-synuclein (⟨-SN) is a 140 aa protein which has been shown to exist as a cytoplasmic natively disordered monomer that can interact with membranes. ⟨-SN has been extensively studied due to its association with neurodegenerative diseases such as Parkinson's disease and Lewy body dementia. These diseases are characterized by a progressive loss of neurons with intracytoplasmic proteinaceous aggregates that are detection positive for α-SN. One of the principal factors which lead to synucleinopathies is aging. Increasing evidence shows that α-SN is expressed in cells of most tissues. In skeletal muscle α-SN is present during development, in adulthood and in aging. The association between α-SN in skeletal muscle and aging remains to be evaluated, in particular distribution and interaction in surface membranes. Understanding the interaction of α-SN with lipid membranes and the conformational properties of its bound state in vivo can help identify what promotes transition from physiological to pathological condition during aging.

Aims: To characterize the presence, organization, and distribution of α-SN in sarcolemma and transverse tubule membranes, we isolated membranes from skeletal muscle of young adult and aging rats. Membranes were isolated from 3 and 24-month-old rats and ⟨-SN was detected by western blot analysis. The distribution of α-SN in membranes of detergent resistant domains (lipid rafts) and non-detergent resistant domain from sarcolemma and transverse tubules is analyzed after fractionation of the sample treated with Triton x-100.

Results: α-SN is expressed in surface membranes (sarcolemma and transverse tubules) of skeletal muscle. In young adult, ⟨-SN is present in sarcolemma as several homo-oligomeric species in all cases. The predominant components observed correspond to a tetramer and pentamer forms of α-SN in a ratio of 2:1 respectively. In addition, aging influences the expression of α-SN in sarcolemma, increasing its expression with age. Most notably, the tetramer form of α-SN is increased more than other oligomer sizes with aging, 52.6% in SL. The expression of α-SN in transverse tubule membranes is significantly less as compared to sarcolemma. However, aging

also changes the expression pattern of ⟨-SN in transverse tubules, increasing expression of the tetramer.

Conclusion: Finally, our results show that under healthy conditions α-SN is expressed in skeletal muscle surface membranes as oligomers and tends to be obtained in the non-detergent resistant membranes in standard purification protocol of membranes treated with Triton X-100.

INTRODUCTION

Skeletal muscle

In adult mammalian vertebrates, skeletal muscle (SM) represents approximately 40% of total body mass. This tissue has evolved to produce contractions and is mostly known as the tissue responsible for movement. SM contraction initiates due to an external stimulus from voluntary nerve impulses. Through attachments to bone, that articulate with each other, skeletal muscle can produce coordinated activities such as walking, sustaining body posture, and manipulating objects.

SM is a tissue with high plasticity, able to adapt to various environmental demands. These adaptations include muscle fiber cross sectional area and shifts in mitochondrial volume, which ultimately alter the physiological capabilities of skeletal muscle. Non-pathological aging is an unavoidable process that occurs as part of the normal development of mammals. During aging, there are physiological changes in all organ systems. In SM, aging produces several changes, both physically and functionally. A distinct characteristic seen in older people is the presence of skeletal muscle weakness and atrophy, producing a loss of lean body mass with aging known as sarcopenia [1]. In humans, the aging process leads to muscle mass and strength loss, beginning at 40 years old and steadily declining with age [2]. There is a significant acceleration of the rate of decline in muscle mass after the age of 65 years [3]. Since skeletal muscle is the principal reservoir of amino

acids in whole-body metabolism that sustains protein synthesis in other tissues, the limited muscle mass seen with sarcopenia often produces a weakened response to stress and illness in the elderly [4].

Muscle fiber anatomy

Skeletal muscle is composed of bundles of muscle cells known as muscle fibers or myofibers. Myofibers are formed by the fusion of myoblasts during embryonic development. As a result, the myofiber is a multinucleated, syncytial, cylindrical structure that is relatively large compared to other cells. A typical myofiber can have a diameter of around 50-100 μm and can be several millimeters to centimeters long [5]. This length can be attributed to the fact that most myofibers connect to bones in the limbs at both ends through tendons, a type of connective tissue composed mainly of collagen fibers. The nuclei of muscle fibers are distributed evenly along the length and are in the periphery of the fiber. The nuclei are anchored closely to the internal surface of the plasma membrane. Myofiber cytoplasm is occupied by the contractile machinery which is composed of protein filaments of actin and myosin arranged longitudinally along the myofiber into a repeating structure known as sarcomeres (Figure 1.1). In addition, the myofiber contains a network of interweaving membranes that can be resolved into two distinct intracellular membranes: the transverse tubules (TT) and the sarcoplasmic reticulum (SR). Both the TT and SR are specialized organelles of the myofiber that ensure the simultaneous release of calcium, necessary for contraction to occur. TTs are invaginations of the myofiber cell membrane known as sarcolemma (SL) that conduct the action potential of the SL to internal areas of the myofiber transversally as well as longitudinally ensuring a simultaneous contraction of the fiber. The SR is

a specialized endoplasmic reticulum of striated muscle. It forms an internal membrane system of cisterns along the myofiber, this structure has relevant functions as the main site of calcium storage. SR calcium release coupled to membrane depolarization triggers contraction in a mechanism known as excitation-contraction coupling. The action of the SR calcium transport ATPase (SERCA) membrane enzyme is responsible for calcium reuptake into the SR during muscle relaxation.

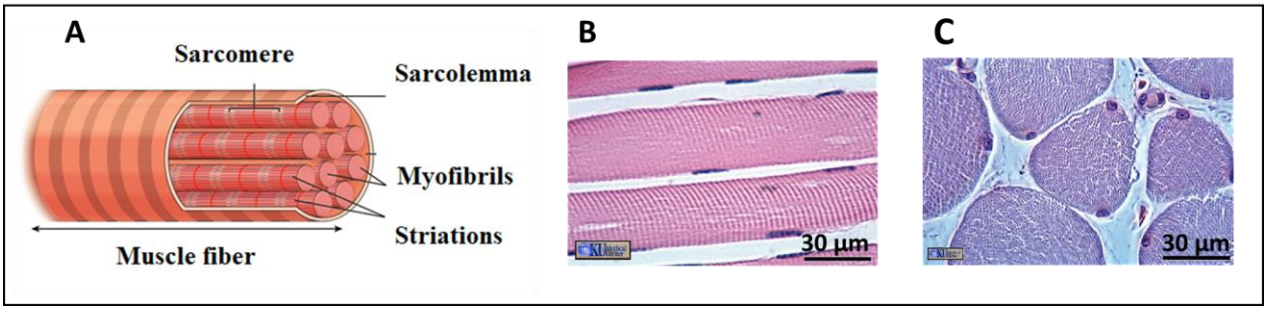

Figure 1.1. (A) A skeletal muscle fiber is surrounded by a plasma membrane called SL. Interior of fiber is mostly composed of myofibrils, cylindrical structures that lie parallel to fiber and contain the main contractile proteins. Repeating structure of sarcomeres is responsible for visible striations in muscle fiber. (B) Longitudinal section of skeletal muscle fibers with characteristic striations visible. Dark inclusions located in the periphery of fiber are nuclei. (C) Transverse section of skeletal muscle fiber with myofibrils visible as mosaic pattern in interior of muscle fiber. [6-7]

Excitation-contraction coupling

The excitation-contraction coupling of skeletal muscle is a complex physiological process that converts an action potential into force generation. Muscle contraction begins with a nerve impulse from a motor neuron axon. The impulse reaches the motor axon terminal and causes acetylcholine release from the motor axon terminal. The acetylcholine interacts with acetylcholine receptors located on the myofiber cell membrane. This interaction in turn initiates an action potential in the myofiber which spreads across the cell membrane and into the interior of the myofiber through the TTs. The action potential in the TTs induces calcium release from the tightly

associated SR due to a protein-protein interaction of the T-tubule voltage sensing dihydropyridine receptor (DHPR) and the calcium release channel Ryanodine Receptor (RyR) located in the SR. Calcium release from SR increases cytoplasmic calcium concentration from approximately 10^{-7} to 10^{-5} M [8]. Calcium release into the myoplasm ultimately results in actomyosin interaction. Contraction of the skeletal muscle is brought about through the action of actin and myosin filaments sliding past one another. Myosin head groups contain ATPase hydrolytic activity. In addition, the myosin head group can bind to actin, forming cross-bridges between the two filaments. Current models suggest that the binding of ATP to myosin causes dissociation from actin filaments. The hydrolysis of ATP then causes a conformational change that displaces the myosin head to a further position on the actin filament. Hydrolyzed ATP by-products are released, ADP and Pi, returning the myosin head to its original orientation. Successive ATP hydrolysis slides the actin filaments across the thick filament [8]

Sarcolemma and Transverse tubule system

The plasma membrane is a fundamental cellular structure composed of a lipid bilayer. In eukaryotes, it can be as thin as 3-5nm depending on the lipid composition and partitioning. Functionally, it defines the physical limit of a cell, actively transports substrates into and out of the cell interior, and anchors several cytoskeleton proteins [9]. The eukaryotic plasma membrane has a lipid composition that differs from other cell membranes. However, all cellular membranes contain glycerophospholipids, containing one of a variety of head groups attached via glycerol to two fatty acyl chains, which contain either saturated or unsaturated chains varying in length [10]. Most eukaryotic cells contain two additional classes of lipids in cell membrane bilayers: sterols and sphingolipids (Figure 1.2). The sphingolipid backbone is a ceramide that has either a choline

head group such as in sphingomyelin or an array of carbohydrates such as in glycosphingolipids. Unlike most phospholipids, sphingolipids tend to have long and saturated hydrocarbon chains [10].

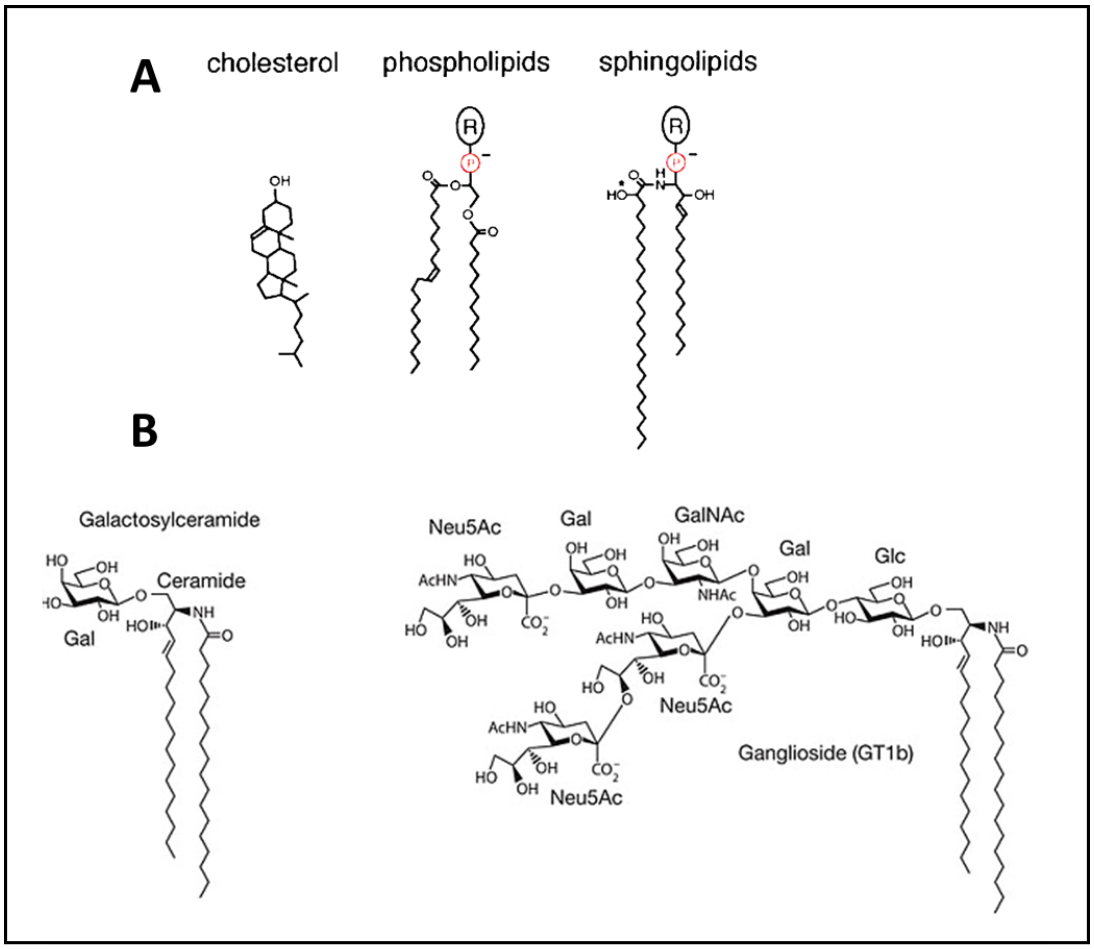

Figure 1.2. Lipid structures. (A) Structure of the major lipid classes of eukaryotic cells. Cholesterol is based on a four-ring structure and is present in animal cells. Phospholipids are based on diacylglycerol and carry acyl chains of 16-18 carbon atoms with one chain containing a double bond. The head group (R) can be neutral or basic. The sphingolipids are based on ceramide. In mammals the head group is either choline or in the case of glycosphingolipids, the phosphate is replaced with glucose and can be further elaborated to make a variety of glycosphingolipids. The acyl chain is saturated and has a length of 16-26 carbons. (B) Structure of representative glycosphingolipids [11].

Cholesterol is the main sterol found in the biological membranes of vertebrates. Both cholesterol and sphingolipids have a low concentration in internal cell membranes and are mainly

found in external membranes such as the plasma membrane. However, cholesterol is synthesized in the endoplasmic reticulum and sphingolipids are synthesized mainly in the Golgi [12].

Cholesterol is highly concentrated in external membranes in its unsterified form (free of long chain fatty acids that bind to the cholesterol hydroxyl group) and affects the biophysical properties of lipid bilayers. Lipid bilayers exist in a fluid "liquid" state above their respective melting temperature. The fluid structure of bilayers is altered by the presence of high levels of cholesterol [13]. The rigid planar structure of the cholesterol molecule influences strongly the acyl chains to become closely packed and the bilayer to be thickened. Cholesterol is arranged perpendicular to the bilayer and organizes the membrane in a directed manner. Therefore, the acyl chains in the membrane cannot deform to allow movement across the membrane but can still move laterally past each other. The cholesterol rich regions of the membrane become ordered and is termed a liquid ordered region, in contrast to the liquid disordered state of the membrane with lower proportions of cholesterol. Reduced membrane permeability is caused by the partial immobilization of phospholipid molecules by cholesterol interaction. By decreasing mobility of phospholipid molecules, cholesterol makes the lipid bilayer more rigid in this region, decreasing permeability of the bilayer in those regions. When a high cholesterol content is segregate in a small area, a liquid ordered, and liquid disordered phase can exist within a single bilayer, such as in the plasma membrane. The formation of liquid orders phases in a bilayer are promoted by lipids that contain saturated acyl chains with cholesterol. As mentioned previously, saturation is more common in sphingolipids, which are also mainly located in external membranes, specifically the outer leaflet.

The excitation/conduction of the electrical signal in SL is due to the presence of ion conduction pathways and channels that are integrated into the lipid bilayer. The SL forms regular

interspersed invaginations continuous with the SL throughout the length of the muscle fiber known as TT. They are arranged perpendicular to the surface membrane and penetrate the interior of the muscle fiber (Figure 1.3) with an opening diameter of about 5 nm. Muscle fibers are relatively large, with a diameter of approximately 100 µm. TT functions physiologically to transmit the depolarization of SL into the interior of the muscle fiber, here the intracellular membrane system promotes simultaneous contraction of the fiber [9].

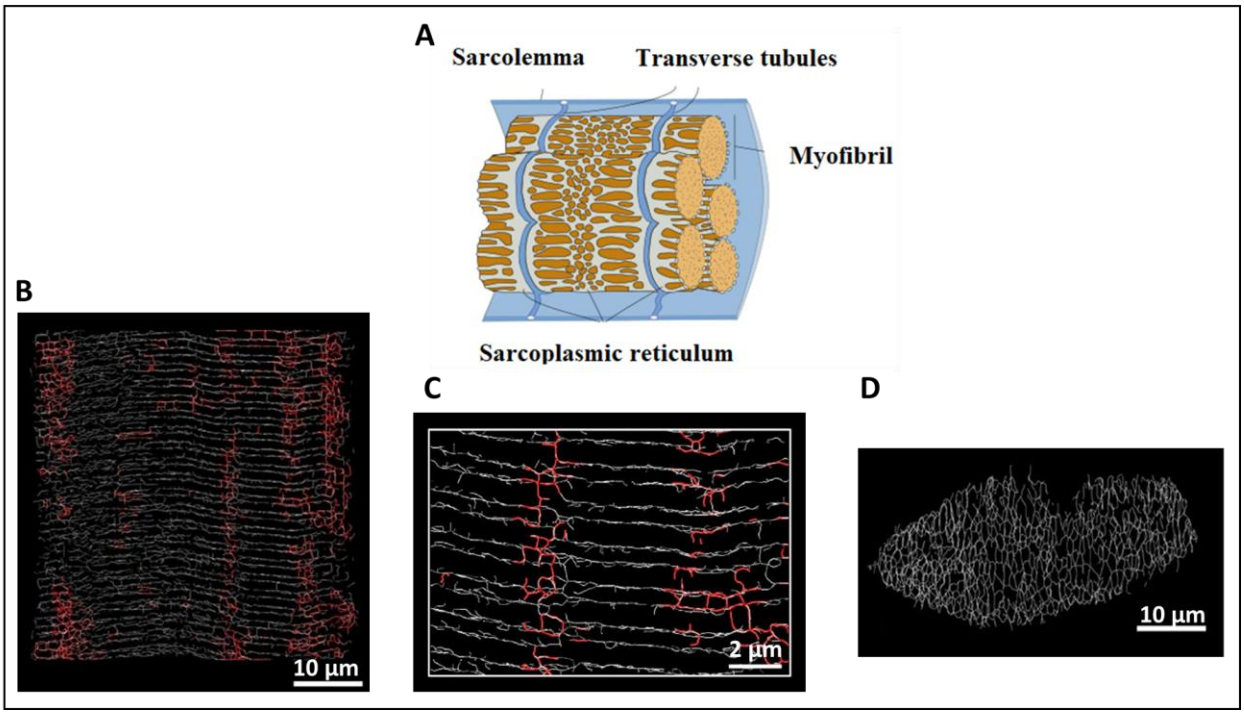

Figure 1.3. (A) Diagram of membrane systems in skeletal muscle fiber showing SL and TT as continuous membranes. (B) Longitudinal view of a human 3D TT system. TT colored white is the predominate perpendicularly arrange tubules while those colored red are longitudinal. (C) Magnified view of image in B. (D) Transverse view of the human TT system illustrating the network formed by the tubules that penetrate the muscle fiber [14-15].

Even though the TT system is an extension of the SL, it differs in its protein and lipid composition. The overall electrophoretic protein pattern of SL, TT and even SR are dissimilar [16]. This indicates that these membrane systems contain a different protein population. Previous studies

demonstrate that SL and TTs differ in their lipid composition, most significantly in relation to total cholesterol content [16-17]. In normal SL/ TT systems, there is a dramatic increase in cholesterol content in TT with respect to SL. By standardizing against the protein content measured in mg, the total cholesterol content of TT and SL can be compared, having 0.903 μmol/mg and 0.217 μmol/mg respectively. Approximately half of the phospholipids in SL correspond to phosphatidylcholine, with lesser contributions of other glycerophospholipids such as phosphatidylethanolamine, and sphingolipids. TT contains less phosphatidylcholine with respect to SL, and a higher contribution of sphingolipids to the total phospholipid composition of the membrane, approximately 14% [17], (Figure 1.4).

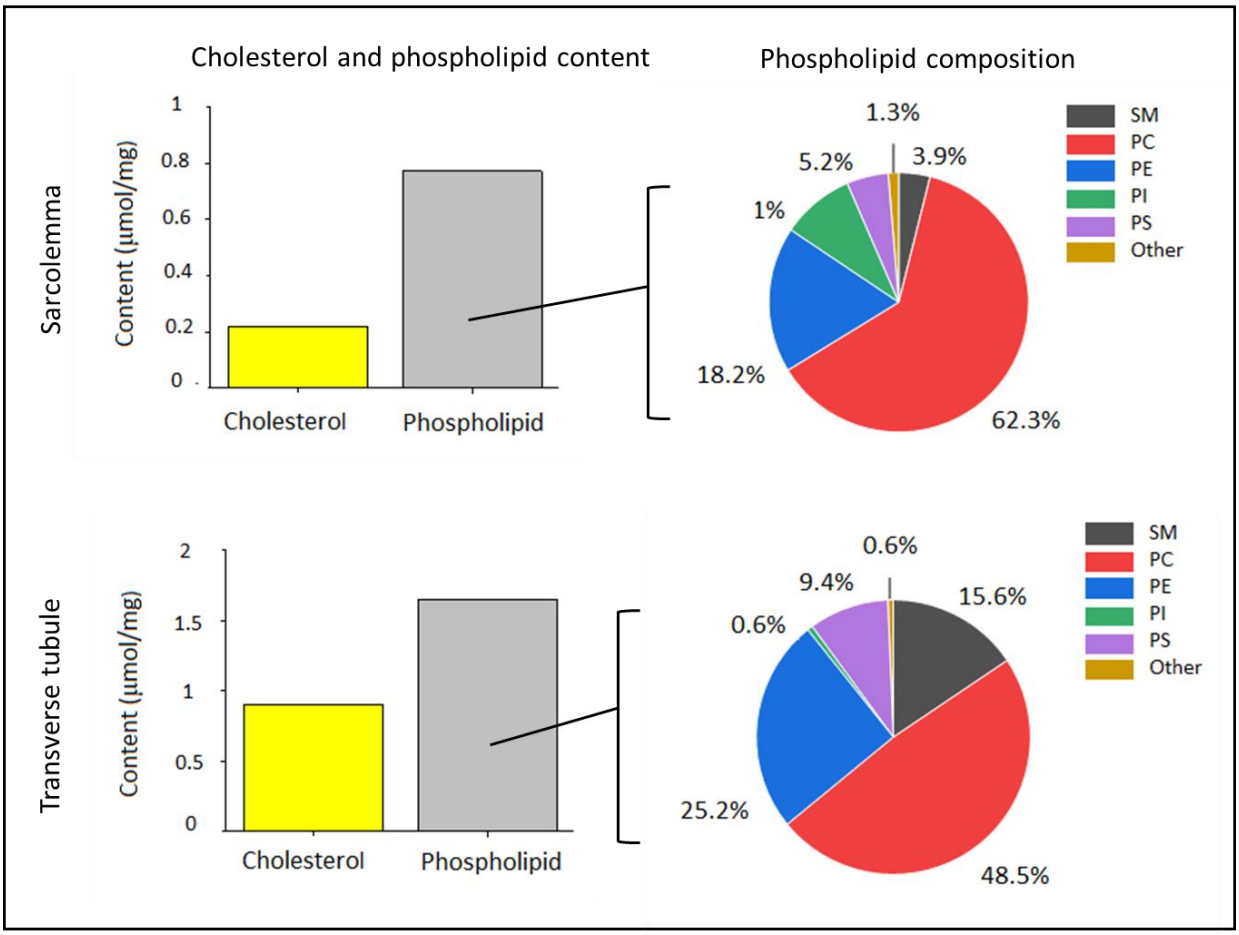

Figure 1.4. Cholesterol content and phospholipid composition of SL and TT from skeletal muscle. SM: sphingomyelin, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine. Data obtained from [17].

Lipids are not randomly distributed along biological membranes [10]. The organized distribution of lipids in cellular membranes leads to asymmetry. The internal leaflet of superficial membranes like plasma membranes are composed mainly of charged phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine, while the outer leaflet is composed of sphingolipids and phosphatidylcholine. The compositional asymmetry of the plasma membrane does not correspond to differential lipid synthesis or degradation in the membrane leaflets [18]. Therefore, the asymmetry found in the membrane is formed and maintained by cellular

mechanisms after lipid synthesis to counterbalance randomization. The lipid distribution found in the membrane bilayer is a result of continuous inward and outward movements of lipids between leaflets. Lipids can cross biological membranes by various mechanisms [18].

i) Spontaneous diffusion occurs when lipid movement occurs between membrane leaflets. The rate of diffusion is due to the biophysical properties of the lipid in movement and the membrane.

ii) Another ATP-independent lipid movement is due to the presence of proteins in the membrane which facilitate the bidirectional movement of lipids between the leaflets known as flippases. As a result, flippases allow for rapid redistribution of newly synthesized lipids which cannot rapidly diffuse between the membrane leaflets but are unable to accumulate a given lipid in one leaflet [18].

iii) Through ATP-hydrolysis, translocases promote unidirectional lipid movement against a gradient in the membrane. This activity therefore can maintain permanent lipid asymmetry with respect to a specific lipid either in the cytoplasmic or exoplasmic leaflet. Since plasma membrane asymmetry is maintained by ATP driven processes, it is essential for normal cell function.

In fact, if cells fail to maintain lipid asymmetry in the plasma membrane, the presence of aminophospholipids such as phosphatidylserine and phosphatidylethanolamine which are normally restricted to the inner membrane appear at the cell surface, and serves as a signal for macrophage recognition in apoptotic cells, promoting phagocytosis of the dying cell [19].

Cholesterol is present in both leaflets of the plasma membrane. Cholesterol has a specific structure, a small polar group, a rigid steroid ring, and a flexible chain. The polar section of cholesterol is in the single hydroxyl group. The non-polar region of cholesterol displays an asymmetric structure with two faces referred to as α and β. The α face is planar, in contrast to the β face which has a rough face due to the presence of two methyl groups and a terminal isooctyl chain. The structure of cholesterol can form specific lipid-lipid and lipid-protein interactions leading to the formation of domains [20]. Sphingolipids contain a complementary steric structure to cholesterol, as a result, cholesterol tends to preferentially interact with sphingolipids forming sphingolipid/cholesterol clusters in the plane of the membrane [21]. This favorable interaction is attributed to the saturated acyl chain in sphingolipids together with the trans-unsaturated sphingosine backbone such as in Galactosylceramide, which would allow maximal van der Waals interactions with cholesterol [22], (Figure 1.5). The lipid composition determines membrane properties, and cholesterol plays a major role in this determination especially in the formation of separated phases and domains in the lipid bilayer.

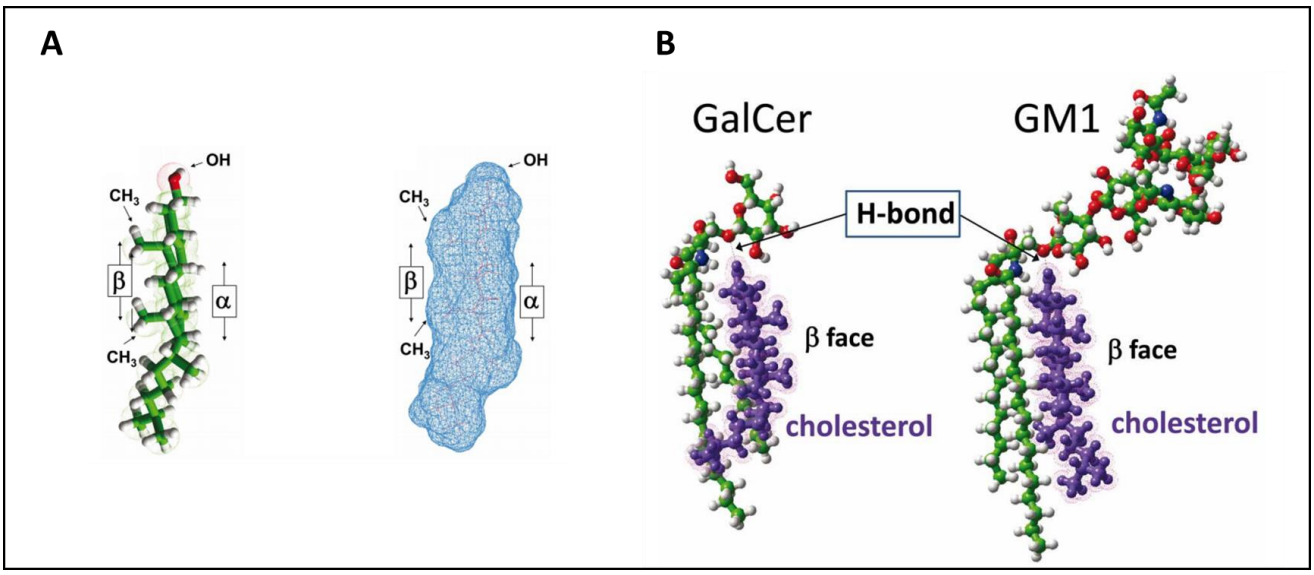

Figure 1.5. Structural properties of cholesterol and sphingolipids. (A) Asymmetric structure of cholesterol due to distribution of aliphatic groups. Smoother side is referred to as the α face and the rough side is referred to as β face. Cholesterol structure illustrated in a tube model and molecular surface model. (B) Molecular dynamic simulation of cholesterol and sphingolipid interaction. In the GalCer-cholesterol interaction, the OH of cholesterol participates in a hydrogen bond with the oxygen atom of the glycosidic linkage between galactose and ceramide. A similar hydrogen bond is formed between GM1-cholesterol interaction. The other interactions between cholesterol and sphingolipids are through van der Waals interactions between the non-polar ceramide and the α face of cholesterol [22].

Additionally, cholesterol plays a role in the functionality of skeletal muscle contraction. The skeletal muscle SL is adapted to resist the consequences of muscle fiber shortening during contraction. The Dystrophin-glycoprotein complex (DGC) is embedded in SL and is composed of several proteins including dystrophin (DP), dystrobrevin (DB), syntrophin (SP), dystroglycans (α- and β-DG), and sarcoglycans (α-, β-, δ-, and γ-SG). These proteins transmit lateral force produced during contraction to the SL and extracellular matrix [23]. The association of these sub-SL complexes to the Z-disk of the sarcomere found in skeletal muscle are known as costameres (Figure 1.6). Costameres are critical components of muscle morphology, several myopathies develop as a result of this connection being altered [24].

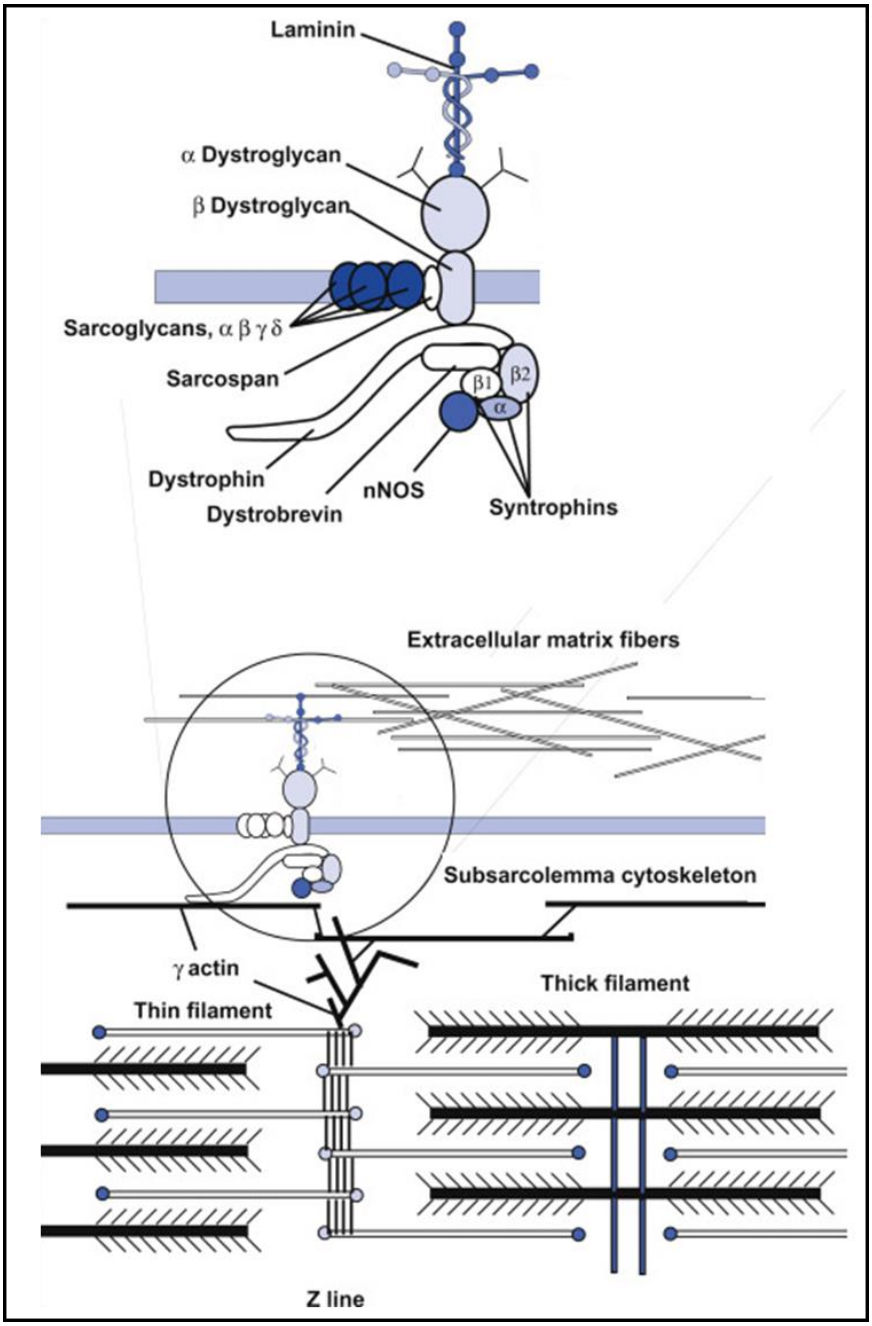

Figure 1.6. Arrangement of proteins in costameres of skeletal muscle. The dystrophin-glycoprotein complex (DGC) is made up of cytosolic, transmembrane, and extracellular proteins that link the contractile filaments to the extracellular matrix though cytoskeletal filaments. In the DGC, Z-disks are linked to dystrophin though actin filaments. Dystrophin also connects to β dystroglycan, a transmembrane protein. β dystroglycan forms a heterodimer with α dystroglycan, which binds to laminin. Laminin in turn binds to collagen and other extracellular matrix proteins [25].

Costameric proteins are found in cholesterol rich membrane fractions. Cholesterol depletion of SL removes β-DG from the microdomain, interfering with the β-DG/dystrophin interaction (Figure 1.7). There is a related impairment of mechanical activity of skeletal muscle, decreasing the absolute force of contraction when cholesterol is depleted (Figure 1.8).

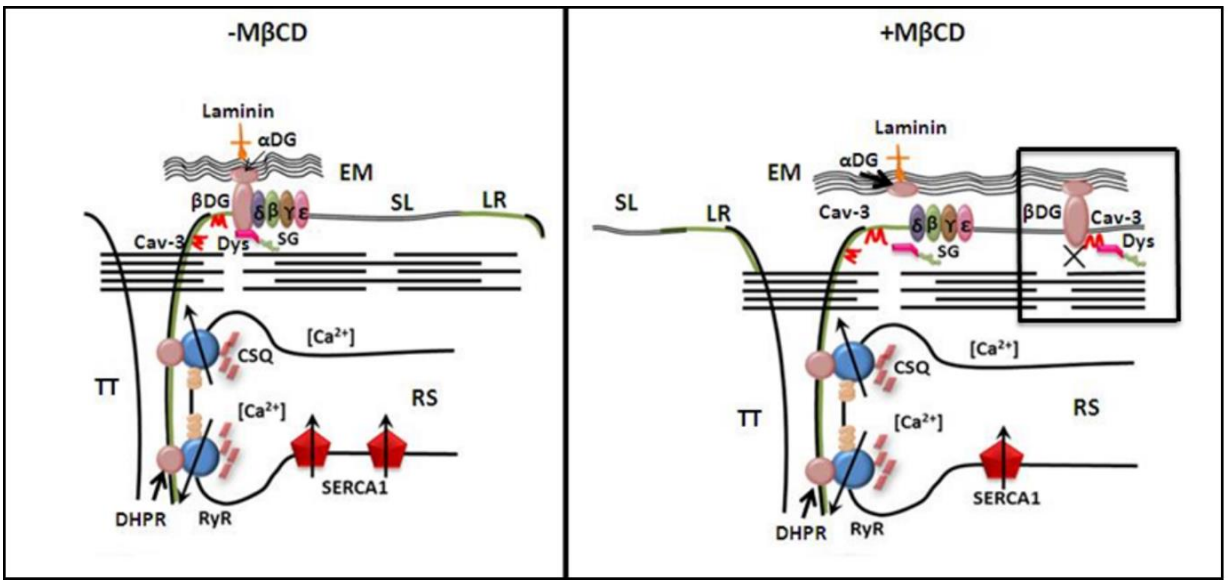

Figure 1.7. Model depicting the effect of membrane cholesterol depletion by incubation with Methyl-β-cyclodextrin (MβCD) on the distribution of β-DG. The opening of the TT-membranes are enriched in cholesterol, GM1, Cav-3 and β-DG. The diagram illustrated diminished contact between β-DG and dystrophin in the presence of β-mercaptoethanol. SERCA1, and RyR function normally .SL, SL; SR SR; EM extracellular matrix; LR, lipid raft; Dys, dystrophin; SG, sarcoglycan; CSQ, calsequestrin [26].

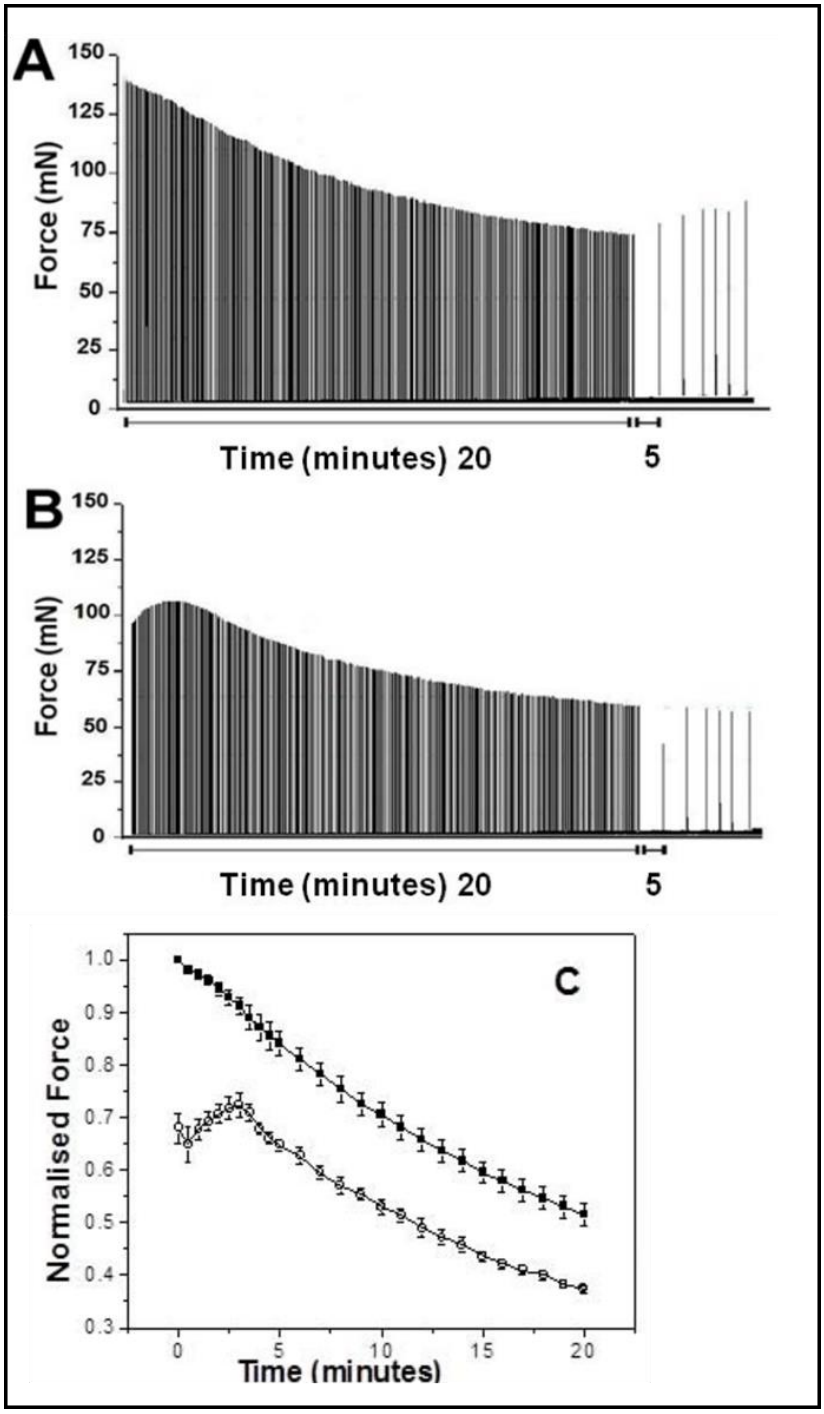

Figure 1.8. The effect of cholesterol depletion on fatigue development with a single twitch train stimulation protocol. (A)Representative experiment in control extensor digitorum (EDL) muscle and (B) representative experiment in EDL muscle incubated with β-mercaptoethanol. (C) Normalized tension in control EDL muscle (●) and EDL incubated with β-mercaptoethanol (○) [26].

Aging in skeletal muscle

Aging is part of normal physiological development of most animals. The aging process is characterized by deterioration in physiological functions and metabolic processes. During normal aging, post-mitotic tissues such as skeletal muscle have been associated with a progressive loss in number of cells. The reduction in total number of cells therefore leads to an accelerated decline in function. In skeletal muscle, the loss of skeletal muscle mass with age is defined as sarcopenia. The loss in muscle mass is attributed to a decrease in muscle fiber cross sectional area and a loss of muscle fiber number.

There are several intrinsic alterations during aging in skeletal muscle that are associated with the overall condition of muscle weakness and loss [27]. Muscles of aged animals show myofiber atrophy [28], in part related to a decrease in protein synthesis [29]. In addition, preclinical evidence shows an age dependent increase in myocyte apoptosis which agrees with old-rat animal models that undergo acute apoptosis during aging [30]. An age-related decline in autophagic degradation has been observed as well. Autophagy is a vital cellular process where intracellular components of the cell are degraded within lysosomes [31]. Interestingly, insufficient autophagy has been linked to age-related accumulation of cellular components such as non-degradable lysosome-bound lipofuscin, protein aggregates and mitochondrial damage [32].

The decrease in muscle mass with age can be attributed to a decrease in nutrient transport to the interior of the muscle fiber. Notably, during aging, there is a decrease in the expression levels of amino acid transporters in muscle fibers mainly by a decrease in LAT2 (L- type amino acid transporter 2) expression in TT membrane [33]. This correlates with the hypotrophy observed in muscle fiber size since a reduction in amino acid availability would reduce protein synthesis in

the fiber. As a result, the muscle fiber is in a weakened state and is unable to maintain appropriate protein levels to sustain muscle fiber contractions (Figure 1.9).

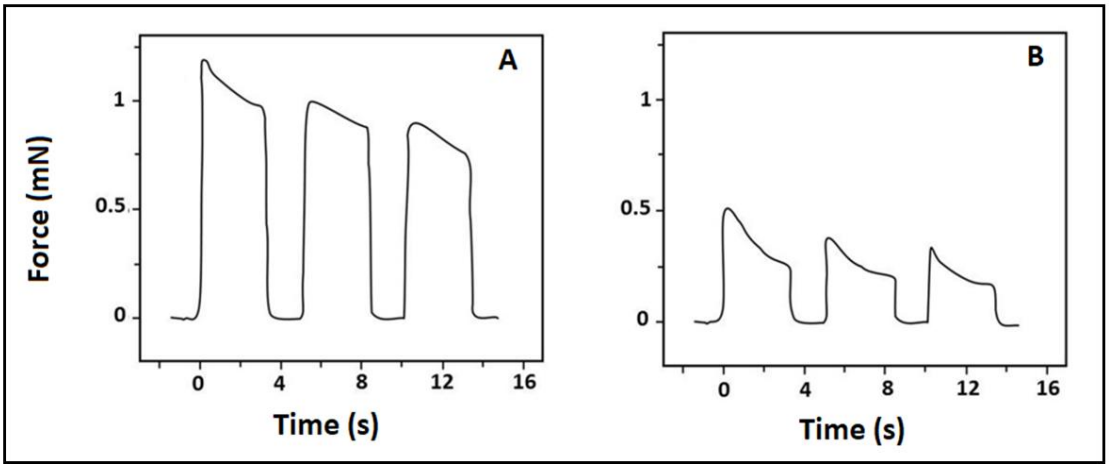

Figure 1.9. Representative tetanic stimulation of extensor digitorum longus (EDL) muscle isolated from young (A) and old (B) rat. Contraction force and resistance to fatigue during tetanic stimulation is reduced 50% in old EDL muscle [33].

In this weakened state, the muscle fiber is more likely to suffer damage to the integrity of the fiber, initiating cell processes that lead to muscle fiber loss. Skeletal muscle fibers tend to employ non-apoptotic mechanisms when muscle fiber cells are lost. Apoptosis is an important regulatory process that occurs during normal development and in the progression of some diseases, ultimately leading to death of a cell. Apoptosis is induced by two alternative signaling routes: in one route, eternal factors bind to the membrane death receptors outside the cell and in the second route internal cellular events lead to the release of cell death molecules. In fact, it appears that mature skeletal muscle is usually precluded from initiating apoptosis in that it upregulates

expression of survival proteins, inhibitors of apoptosis initiating proteins, and simultaneously expresses low levels of apoptosis mediators like apoptotic proteases activating factor 1 [34]. Since muscles are exposed to continuous perturbations throughout life including disruption of SL after intense contractions, it would be dangerous if cell death is triggered under these conditions rather than other cell processes like repair. Additionally, once apoptosis is initiated, the process is irreversible, and the cell is committed to cell death. In the unique structure of muscle fibers, which are a multinucleated syncytial cell structure, this would mean that there would be a massive propagation of the apoptosis pathway throughout the length of the fiber, causing complete loss of the fiber. This scenario is not beneficial for the muscle fiber and so it makes sense that the muscle fiber is resistant to apoptosis pathways. Therefore, the loss in muscle fiber seen with age is not necessarily a product of apoptosis mechanisms in the cell. Instead, it seems that the weakened state of muscle fiber, due to other factors such as nutritional status, is the factor that promotes cell death under these conditions, the muscle fiber is likely to undergo structural damage caused by normal muscle activity and is unable to be repaired. If not repaired, then there is invasion of the fiber by immune system phagocytes that degrade damaged fiber areas. Taken together, this suggests that the loss of fibers seen with aging is caused mainly by necrotic factors in the case of muscle fibers.

Skeletal muscle membrane and aging

Aging has a deleterious effect on the membrane systems of skeletal muscle cells. This can be seen by the reduced enzymatic activity of critical membrane bound proteins required for muscle relaxation. When compared to SERCA activity isolated from the SR of young rats, SERCA activity

from old rats is reduced approximately by 40% after a 30-minute reaction time (Figure 1.10) [33]. The PMCA (plasma membrane Ca^{2+} ATPases) in skeletal muscle participates in the regulation of cytosolic calcium, necessary for relaxation after muscle contraction. In skeletal muscle, the PMCA is distributed in the TT [35]. The activity of PMCA in TT of old rats is reduced by 85% when compared to PMCA of young rats (Figure 1.10). During aging, both calcium regulators, located in SR and TT, have a significant reduction in activity. These changes in activity therefore contribute to the reduced functionality of skeletal muscle with aging. The cause for reduced activity in both systems are not clear, but it is possible that changes in the lipid environment are likely to alter the normal function of these membrane inserted proteins.

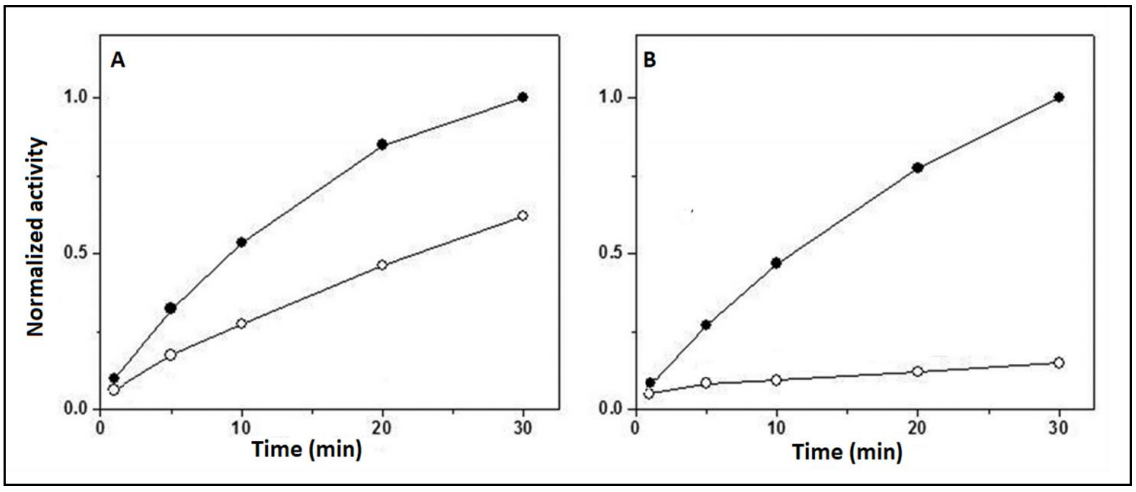

Figure 1.10. Hydrolytic activity of SERCA (A) and PMCA (B). SR and TT isolated from fast skeletal muscle. Young rat (●), old rat (○) [33].

Investigations into the lipid composition of skeletal muscle with respect to aging has revealed some interesting changes. The overall lipid identity of muscle was assayed in rats with the ages of 10 and 25 months [36]. This investigation revealed that comparison between these age

groups with respect to the levels of major phospholipid groups did not reveal significant changes during aging (Table 1).

Phosphatidylcholine	27.2 ± 0.7	24.8 ± 0.8
Phosphatidylethanolamine	13.2 ± 0.5	10.9 ± 0.4
Phosphatidylserine/inositol	3.0 ± 0.2	3.2 ± 0.2
Sphingomyelin	1.1 ± 0.1	1.3 ± 0.2

Table 1. Phospholipid composition of lipid extracts prepared from muscles of mature rats. Values are in nmol lipid/ mg dry weight and represented as mean ± SEM. Adapted from [36].

Taking this information into account the membrane composition and therefore physicochemical properties of skeletal muscle membrane remains unchanged with age. However, a more detailed investigation into the fatty acid acyl chain saturations of the phospholipids in skeletal muscle with aging showed some changes with age. By assessing the poly-unsaturation of total lipids in muscle membranes, an overall reduction in the percentage of poly-unsaturated fatty acid chains is seen with older animals, 29 months old, as compared to young ,11 months old [37]. This suggests that skeletal muscle membrane tends to decrease poly unsaturated phospholipids with age. Therefore, the proportion of saturated to unsaturated phospholipids is altered in skeletal muscle membranes with age. A change in this ratio would affect the fluidity of the membrane. Poly- unsaturated lipids tend to make the plasma membrane more fluid due to the presence of double bonds which add a bend to the carbon chain. The double bonds add rigidity to the lipid molecule since double bonds are unable to freely turn. Membranes that have a high proportion of

poly-unsaturated lipids are more fluid because the lipids are unable to pack closely together. On the other hand, saturated lipids can interact closely together, reducing the fluidity of the membrane.

Additionally, cholesterol, the other major lipid molecule in membranes, is also altered with aging in skeletal muscle membranes. Several investigations have shown that cholesterol levels are increased with age in skeletal muscle membranes isolated from rats [38]. These reports indicate that as compared to young rats, the level of cholesterol increases anywhere from 20-46%, depending on the specific muscle used. Cholesterol also affects the fluidity of membranes depending on its concentration. Increased cholesterol levels tend to reduce fluidity and promote ordering in membranes.

To test the fluidity of skeletal muscle membranes with age, 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization measurements were performed in isolated membranes from skeletal muscle [37]. The hydrophobic nature of DPH molecules promotes the incorporation into lipid membranes. DPH molecules have restricted movement in highly ordered membranes, as a result the fluorescence intensity is high. On the other hand, in more fluid membranes, the DPH molecule is free to rotate, so fluorescence emission is lower. When the polarization of DPH is measured in skeletal muscle membrane, it is increased with age (Figure 1.11). An increase in DPH polarization indicates a decrease in membrane fluidity and order. As a result, there are clear changes that occur in skeletal muscle membranes with age, ultimately leading to a more rigid membrane with decreased fluidity and increased ordering of the lipids when compared to younger rats (Figure 1.11).

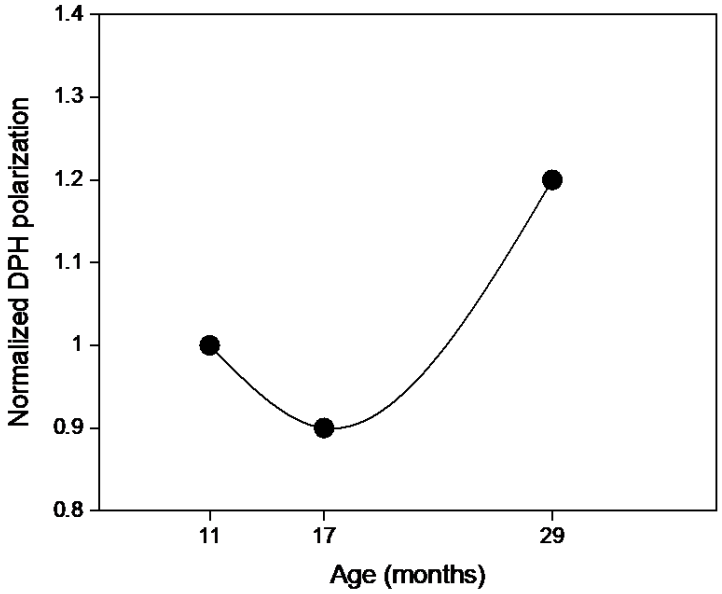

Figure 1.11. Normalized membrane fluidity of SL with age. Membrane fluidity was determined by 1, 6-diphenyl-1,3,5-hexatriene (DPH). A decrease in DPH polarization indicates an increase in membrane fluidity. Data adapted from [37].

Lipid rafts

The lipid raft model proposes that cholesterol and sphingolipids located in the outer membrane leaflet of plasma membrane tend to cluster into liquid ordered domains, where they are enriched [39]. Lipid rafts in plasma membrane have been defined as highly dynamic microdomains with an approximate area size of 10-200 nm. The lipid raft biophysical properties due to enrichment in cholesterol and sphingolipids tend to compartmentalize molecules within the lipid raft. For example, membrane proteins either integral or peripheral associated proteins, would either reside in or be excluded from lipid rafts dependent on the partitioning due to physical properties of the lipid raft. The formation on lipid rafts in the exoplasmic leaflet of plasma membrane is driven by the tight packing and ordering of long saturated achy chains of sphingolipids with cholesterol interactions. Investigations into lipid rafts reveal that the formation

of ordered microdomains in the exoplasmic leaflet influences the connected cytoplasmic leaflet. The extended acyl chain conformation of saturated lipids in the exoplasmic leaflet promotes the ordering and packaging of the cytoplasmic lipids by interactions that occur in the acyl chains. Additionally, cholesterol forms dimers that traverse both leaflets, further concentrating cholesterol in the connected cytoplasmic area. As a result, the connected cytoplasmic leaflet also contains significant liquid ordered structure via the exoplasmic leaflet even though sphingolipids are not abundant (Figure 1.12).

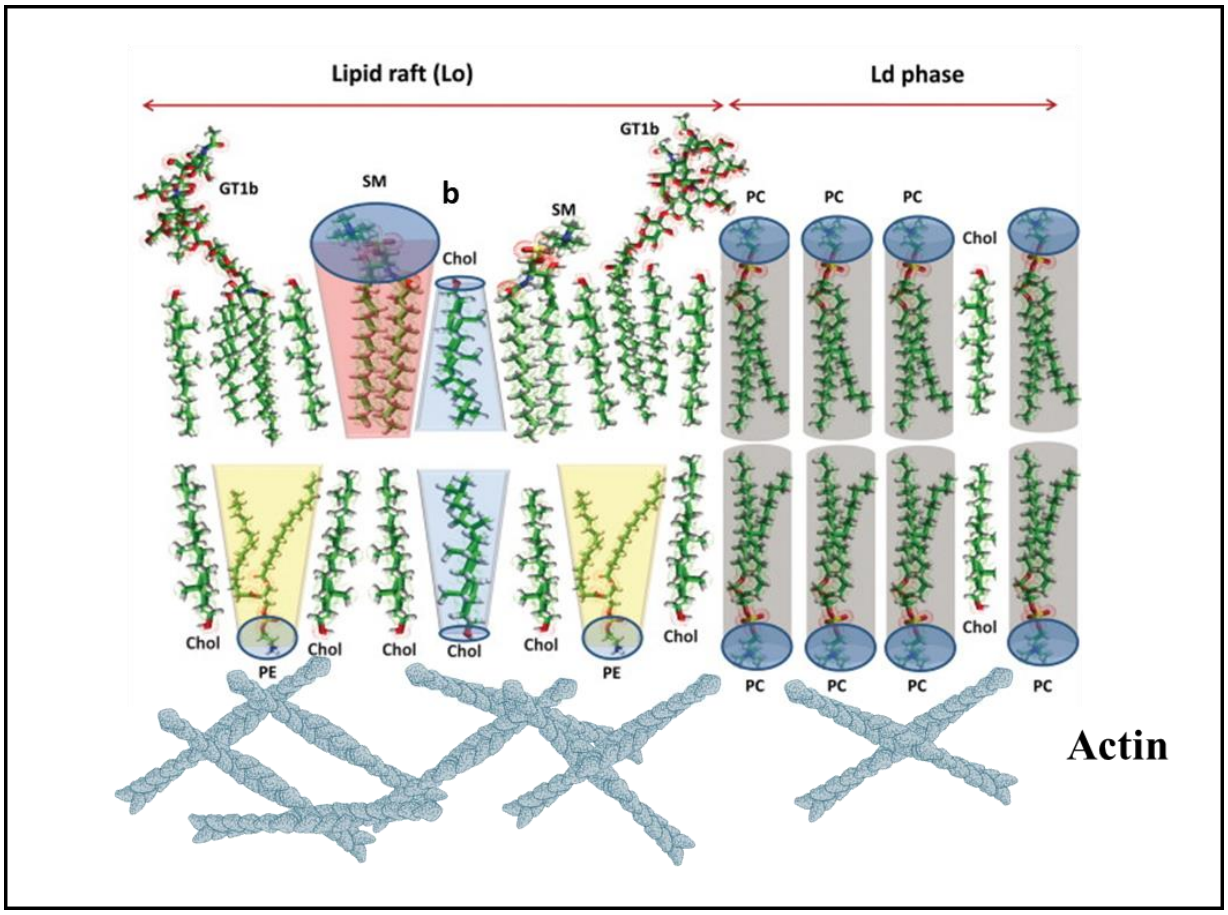

Figure 1.12. General plasma membrane model with lipid raft domain. These are plasma membrane structures enriched in saturated lipids and cholesterol. Lipid organization of plasma membrane with lipid raft based on shape of membrane lipids and composition. The lipid raft domain is liquid ordered (Lo) and are enriched in cholesterol. Sphingolipids (sphingomyelin (SM) and glycosphingolipids such as GT1b tend to localize in the exofacial leaflet. Cholesterol shape ensures cohesion of micro-domain by filling voids between asymmetric lipids like SM. PC: phosphatidylcholine, PE: phosphatidylethanolamine, Chol: cholesterol. Modified from [40].

A question that arises in the lipid organization of plasma membrane is how the different phases are prevented from coalescing into two large regions of rat and non-raft membrane domains. More recent investigations show that the cytoskeleton, an actin meshwork that comes into proximity in the membrane bilayer participates in the separation of lipid phases [41].

These domains can be isolated using nonionic detergents. By doing so, the cholesterol rich domains tend to resist solubilization by detergent and so detergent resistant membranes can be

easily isolated biochemically in a sucrose gradient and tend to float to the top of the centrifuge tube. The remaining membranes are located at the bottom of the tube and are solubilized by the detergent and are referred to as non-detergent resistant membranes. Direct experimental evidence using fluorescence microscopy to track sphingolipid enriched domains in live fibroblasts demonstrated that depolymerizing the cortical actin cytoskeleton interrupted the sphingolipid microdomains [42]. The maintenance of lipid microdomains is influenced by the cytoskeleton and acts as an actin "fence" to prevent coalescence of the lipid phases.

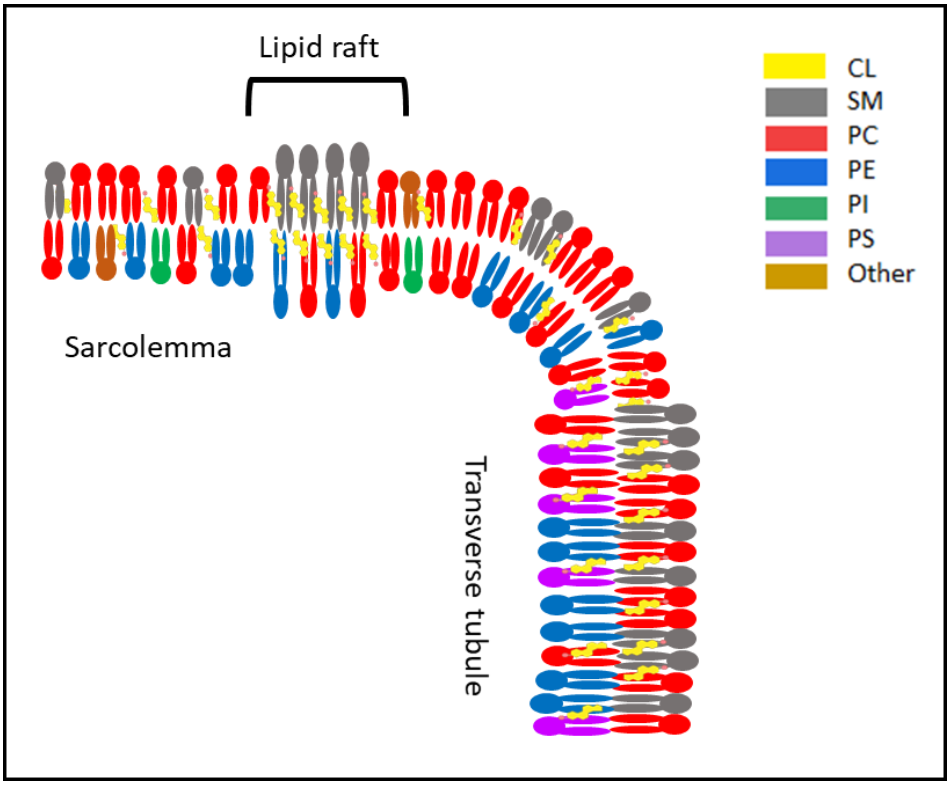

Figure 1.13. Model of SL and TT from skeletal muscle. Colors represent major phospholipids. CL: cholesterol, SM: sphingomyelin, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine. Model demonstrates the continuous nature of SL and TT membrane systems; however, the phospholipid composition is different. TT membranes tend to thicker due to high cholesterol content.

Alpha synuclein

Alpha synuclein (α-SN) belongs to a vertebrate specific closely related protein family known as synuclein proteins, [43], where beta synucleins are more closely related to α-SN than to gamma synucleins. The synuclein proteins are relatively small proteins, with a calculated molar mass close to 12-14 kDa. The amino terminal region of the synuclein protein family members is conserved, while the C-terminal is diverse in size as well as in sequence [44]. Originally, the synucleins were found in brain tissues and so were predicted to participate in brain function. Still, the functions of synuclein proteins have remained elusive. Knockdown genetic models of mice which lack both alpha and beta synucleins does not alter normal brain functions or decrease survival [45]. On the other hand, live cell imaging of yeast cells overexpressing α-SN showed localization in the plasma membrane and accumulations in vesicles [46]. Taken together, these studies suggest the synuclein proteins do not display an obvious biological function, however there is strong evidence that an interaction with membrane lipids exists.

α-SN has been extensively studied when compared to the other two members due to its association with neurodegenerative diseases such as Parkinson's disease. These diseases are characterized by a progressive loss of neurons with characteristic intracytoplasmic proteinaceous aggregates that are positive for α-SN, known as Lewy bodies. Recent research suggests that the Lewy pathology seen in brains afflicted with Parkinson's disease also consists of a considerable amount of lipid material such as fragmented membranes, organelles, and vesicles [47]. The α-SN present in these inclusions has an altered native structure, adopting an antiparallel beta sheet structure. Several α-SN containing antiparallel beta sheet structure polymerize into amyloid-like fibrils. The aggregation of α-SN into beta amyloid fibrils is thought to be one of the initial changes that promote the formation of Lewy bodies in cells. This is due to α-SN's ability to form amyloid

fibrils *in vitro* under heat and agitation. *In vivo*, α-SN amyloid fibrils have been reported in Lewy bodies and is one of the characteristic features of Parkinson's disease. However, this observation comes into question given that recent groups have found that Lewy body morphology is significantly varied at a nano-scale level and fibrils composed of α-SN are not always present in the Lewy body [47].

Alpha synuclein expression in tissues

Even though α-SN is shown to coincide with Lewy body inclusions in neurodegenerative diseases, its expression is not restricted to neuro cells. Northern blot analysis shows that under normal physiological conditions α-SN is expressed in a variety of tissues including brain, heart, placenta, liver, skeletal muscle, kidney, and the hematopoietic cell line [48-49]. Other groups have observed α-SN protein in different human and rat tissues such as kidney liver, lung, heart, and brain [50]. Taken together, these results suggest that α-SN is almost ubiquitously expressed in normal cells.

Alpha synuclein structure

There are three main regions in the α-SN protein. The amino terminal of α-SN consists of amino acid residues (1-60) and has an amphipathic nature due to the identity of its amino acid residues. The NAC (non-amyloid component) (61-95), has a high number of hydrophobic amino acid residues, believed to be necessary for the initiation process of aggregation into fibrils. The carboxyl terminus (96-140) has an acidic nature and is characterized as a disordered region of the protein and is the most variable region among the 3 known synucleins[51].

The presence of 7 segments of imperfect 11 residue repeats reminiscent of the amphipathic alpha helical domains present in apolipoproteins, suggested that α-SN has a lipid binding property [52], (Figure 1.14). Since then, several groups have reported an interaction of α-SN with membrane lipids accompanied by a conformational change from a disordered structure in solution, to alpha helix structure upon membrane binding [53-54]. These conformational studies reveal that α-SN has a dynamic structure, readily changing its conformation depending on its environment.

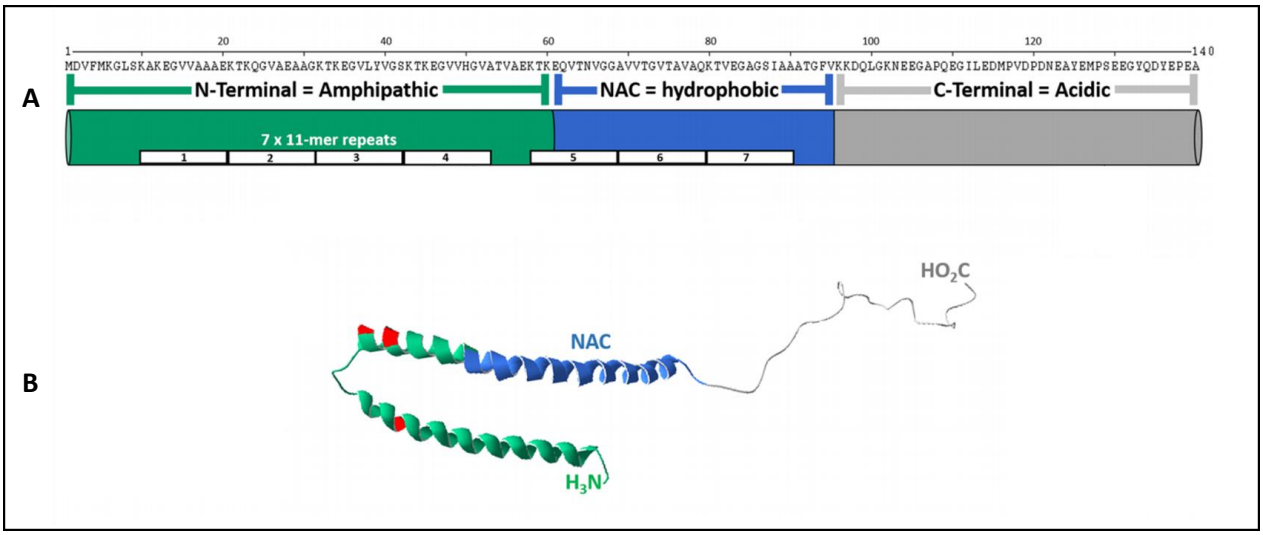

Figure 1.14 Structure of α-SN. (a) Primary structure of α-SN.The N-terminal residues are labelled in green, the non-amyloid component residues are blue and C-terminal residues are grey. The 7 imperfect 11 residue repeats are labelled in white. (b) Molecular model of a crystal structure of micelle bound human α-SN [55].

Interaction of alpha synuclein with membranes

Details about the binding between α-SN and lipid membranes has been well characterized *in vitro* [56-57]. However, the mechanisms by which lipids influence the aggregation of α-SN into non-native beta amyloid structures *in vivo* are still unknown. In the last two decades, a wide range of biological and biophysical studies have been conducted, aimed to elucidate the relationship between the interaction of α-SN with lipids and the propensity of the protein to oligomerize or

aggregate [58-59]. For example, changes in specific lipid levels such as fatty acids, sphingolipids, cholesterol etc. have been associated with the degree of solubility and aggregation of the protein in different cellular models and animal tissues [60]. It is not yet clear how changes in lipid levels or chemical properties induce the aggregation of αSN, whether through a direct interaction with the protein or through the influence of other cellular processes.

Alpha synuclein as a multimeric protein

α-SN was quickly characterized as a monomeric, disordered protein present in the cytoplasm of neuron cells soon after its discovery. Many of its initial characterization utilized α-SN overexpression in bacterial systems [61]. More recently however, studies involving α-SN in mammalian cells found that it exists as a stable helix rich tetramer in cultured and red blood cells [62]. In addition, endogenous α-SN from different cell types including primary neurons was shown to exist mainly as a 60 kDa tetramer alongside other multimeric forms as well [63]. Therefore, α-SN exists in the cell as either a monomer or a variety of multimers of different molecular weights (Figure 1.15). Detection of endogenous α-SN multimers is highly dependent on interaction with lipids; this implies that α-SN multimers are conformations of membrane bound α-SN. In the mature erythrocyte, a relatively simple cell model composed of a plasma membrane and cytosol containing no intracellular organelles, 90% of α-SN exists in the cytosol and 10% associated to the plasma membrane. Because the mature erythrocyte can no longer synthesize new protein, this suggests that there is a dynamic equilibrium between membrane bound and membrane-free states in the cell. Taken together, the previous findings indicate that there is a relevant multimeric form of α-SN in mammalian cells.

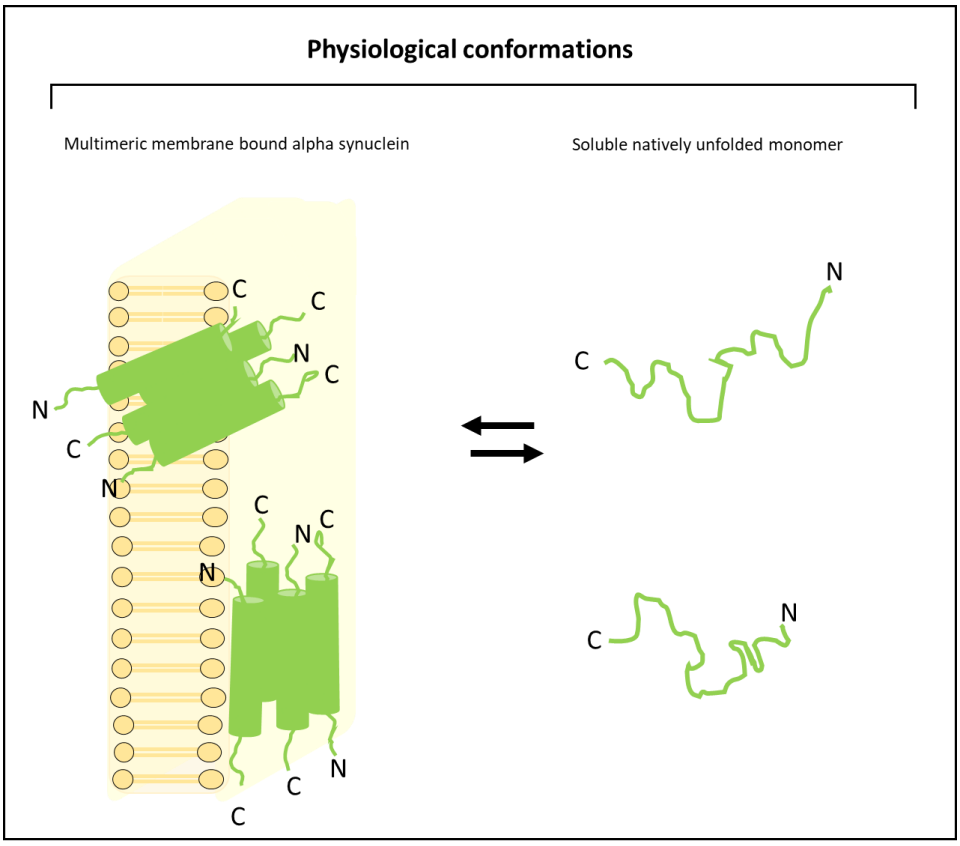

Figure 1.15. Schematic of α-SN dynamic conformations. Soluble α-SN is a natively unstructured monomeric protein. When interacting with membranes, α-SN undergoes conformational changes and adopts a mostly alpha helix structure and has multimeric conformations.

Possible functions of α-SN

The nearly ubiquitous expression of α-SN in vertebrate tissues suggests a physiological function that is performed by most cells. As mentioned previously, α-SN adopts secondary helical structure when interacting with lipid membranes. Taking this into consideration, it is likely that α-SN function is related to their interaction with lipids of biological membranes. There is increasing evidence for a functional involvement of α-SN in lipid membranes. Upon membrane interaction, atomic force microscopy images reveal that α-SN binding can induce lipid interdigitation. The

interdigitation of lipids in a membrane causes a lateral expansion of the membrane and ultimately the membrane becomes thinner [64]. These lipid movements caused by α-SN interaction ultimately create changes that remodel the overall structure of the membrane. Remodeling of cellular membranes is a critical process that occurs in bilayer membranes due to environmental forces or active cell processes such as endocytosis and exocytosis, or the formation and flattening of membrane invaginations/evaginations [65]. Additionally, α-SN can form lipid assemblies. These nano-sized particles are complexes formed by the interaction between one or more α-SN proteins with lipid membranes as well as fatty acids, forming round to discoidal shaped particles [66]. These nanoparticles have shapes and dimensions that are similar to the apolipoprotein that help solubilize and transport lipids in the blood. This information suggests an apolipoprotein-like role for α-SN as a lipid carrying protein. Since α-SN has a higher binding affinity for negatively charged lipids, it thus possible that α-SN may serve as a temporary store for negatively charged lipids and traffic lipids between different biological membranes (Figure 1.16). This possible physiological role is more consistent with α-SN expression pattern in vertebrate tissues including skeletal muscle since all cells require proteins to actively remodel and transport lipids between lipid bilayers.

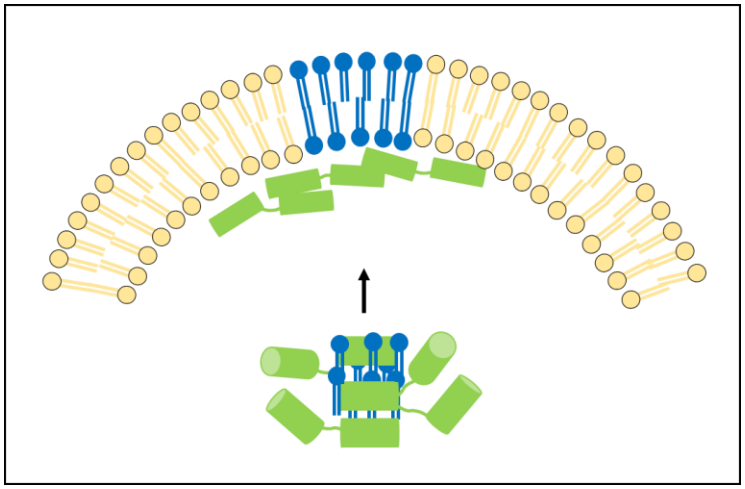

Figure 1.16. Schematic of a possible physiological role of α-SN as a carrier of negatively charged lipids. The α-SN lipoprotein particles is composed of α-SN (green) surrounding negatively charged lipids (blue). Interaction with membrane deposits negatively charged lipids into the lipid bilayer.

JUSTIFICACION

α-SN is expressed in most cells and tissues. In skeletal muscle the status of α-SN remains to be evaluated especially with respect to surface membranes. In addition, the role of α-SN during normal physiological aging remains to be understood. Since skeletal muscle is the most abundant tissue in mammals and is mainly affected by aging, it would be crucial to investigate if there is any association between α-SN expression in skeletal muscle surface membranes during aging.

HYPOTHESIS

α-SN interaction with lipids suggests a stable population of α-SN associated to the plasma membrane. Evidence of transport across cell membranes can be seen by the presence of α-SN secretion into extracellular fluids in vivo [67]. During aging, α-SN mRNA expression in other tissues such as brain decreases, while α-SN protein expression is maintained at high levels.

The α-SN protein expression increases on surface membranes (SL and TT) in discrete regions of aging muscle fibers.

OBJECTIVES

General

To characterize the presence, organization, and membrane partitioning of α-SN in SL and TT membranes isolated from skeletal muscle of young adult and old rats.

Individual

1. Isolate SL and TT from skeletal muscle of young and old rats.
2. Isolate detergent resistant membranes (Triton X-100) from purified SL and TT membranes.
3. Compare expression level of α-SN in plasma membranes in muscle fiber and brain from young (3 months rats.
4. Determine the presence of α-SN in SL and TT in young (3 months) and old rats (24months).
5. Determine the segregation of α-SN in membranes of SL and TT in detergent resistant fractions from young (3 months).

METHODOLOGICAL STRATEGY

Animals.

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the United States, as well as by the guide approved in Mexico by the Ethics Committee of the School of Medicine of the National Autonomous University of Mexico (UNAM) (NOM-062-ZOO1999).

Isolation of microsomes containing brain plasma membrane.

Whole brain expression of synuclein was used as a reference to compare the expression of α-SN in skeletal muscle since it is the tissue most widely associated with α-SN expression. Whole brain plasma membranes were isolated using a modified protocol described by [68]. Isolated fresh or frozen brains were first minced and then homogenized with a glass-Teflon homogenizer by hand with 20 strokes in 15mM Tris-HCl, 320mM sucrose buffer, pH 7.8 and centrifuged three times to discard nuclei and other debris at 3000 x g for 10 minutes at 4°C. The collected supernatants were layered over an 850mM, Tris-HCl 15mM, pH 7.8 sucrose cushion and centrifuged at 70,000 x g, at 4°C. The interface between 320 mM and 850 mM sucrose buffers corresponds to the myelin cloud and was discarded. To eliminate mitochondria and possible residual myelin, the remaining sample was centrifuged for 30 minutes at 20,000 x g, 4 °C. The supernatant was recovered and centrifuged for 1 hour at 100,000 x g, 4 °C to sediment the microsomes.

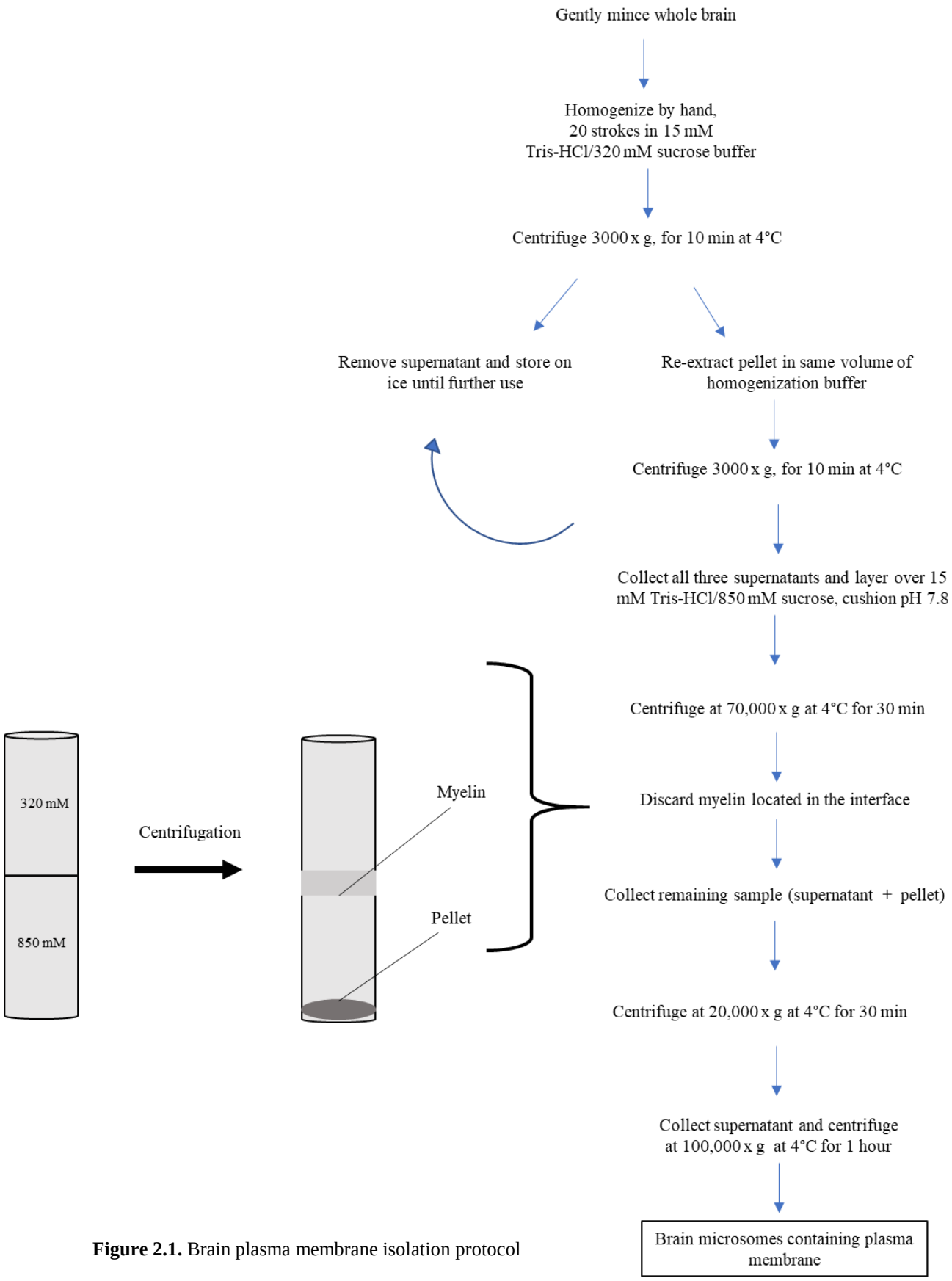

Figure 2.1. Brain plasma membrane isolation protocol

Isolation of sarcolemma

Male Wistar rats weighing 240 to 280 g were euthanized by cervical dislocation; the fast skeletal muscle (forelimbs, hindlimbs, and back muscles) was isolated and used to obtain SL by differential centrifugation and a discontinuous sucrose gradient with modifications of the methods used as previously described [69]. Cleaned muscle was homogenized with a polytron for 10 seconds, followed by a 10 second rest; this procedure was repeated two times. The membrane was isolated in buffered medium containing 20 mM Tris-malate and 100mM KCl, pH 7.0. The homogenate was centrifuged at 12,000 x g for 20 minutes at 4°C. The supernatant was collected and filtered through four layers of gauze. Solid KCl was added to a final concentration of 0.6 M, and the sample was incubated for 1 hour with continuous stirring on ice. The homogenate was then centrifuged at 140,000 x g for 40 min at 4°C. To eliminate excess KCl, the precipitated fraction was suspended in a solution containing 20 mM Tris-malate and 100 mM KCl, pH 7.0, and thereafter transferred to a sucrose gradient of 23%, 26%, 29% and 25% w/v and centrifuged at 75,000 x g for 16 hours. The 23/26% interface which corresponds to SL as determined previously [26]. This fraction in the gradient was collected separately and suspended in a solution of 20 mM Tris-malate and 100 mM KCl and centrifuged at 140,000 g for 40 minutes. The precipitate was collected and suspended once again in 20 mM Tris-malate and used in the isolation of lipid rafts

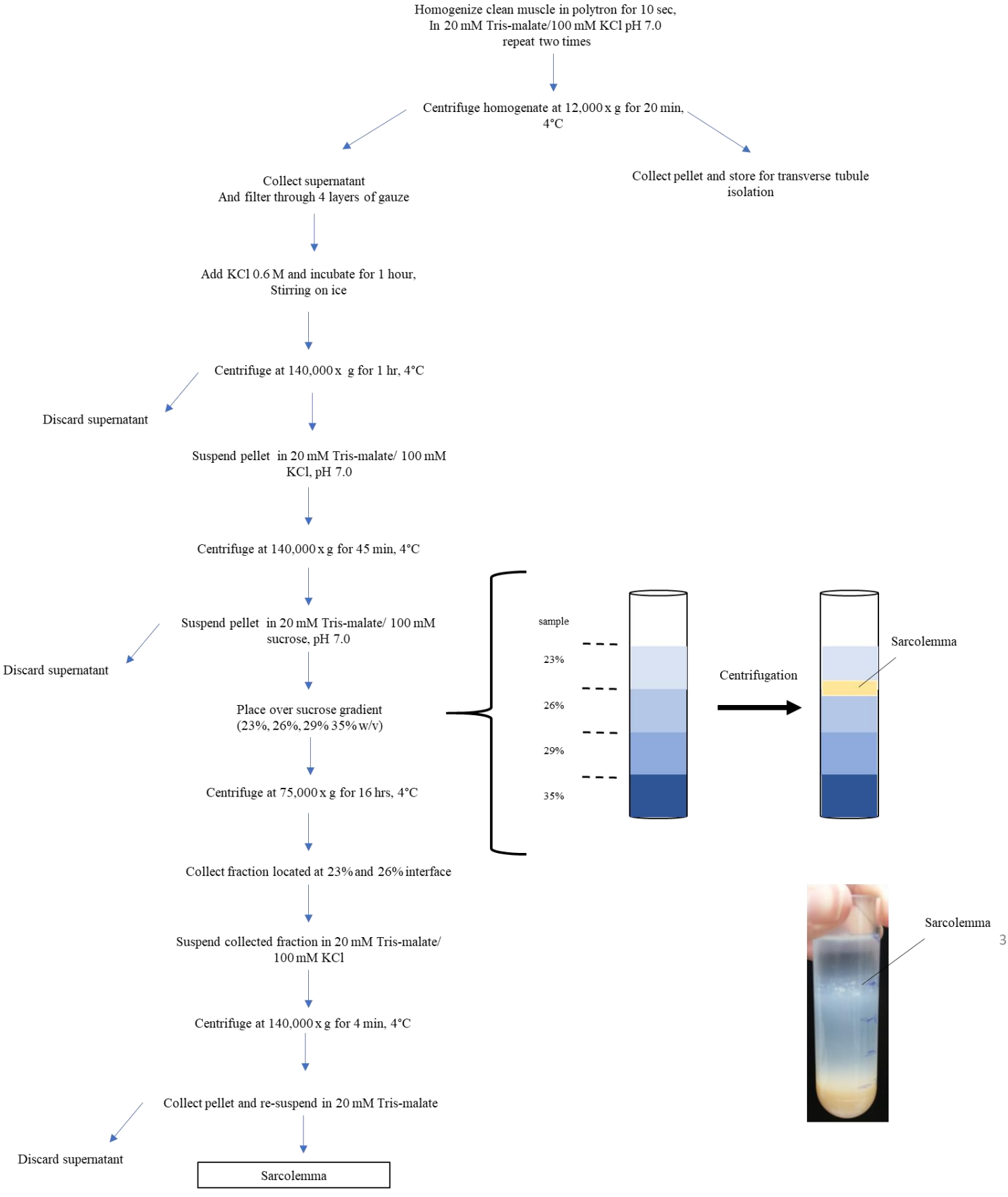

Figure 2.2. Sarcolemma isolation protocol

Isolation of transverse tubules

The TT membrane was obtained from fast skeletal muscles (forelimbs, hindlimbs and back muscles). TT microsomes were obtained by differential centrifugation and placed on a discontinuous sucrose gradient of 25, 27.5, 30, and 35% (w/v) and centrifuged at 75,000 x g for 16 h. The membranes isolated from the sucrose gradient at the 25–27.5% interphase was considered as TT membranes

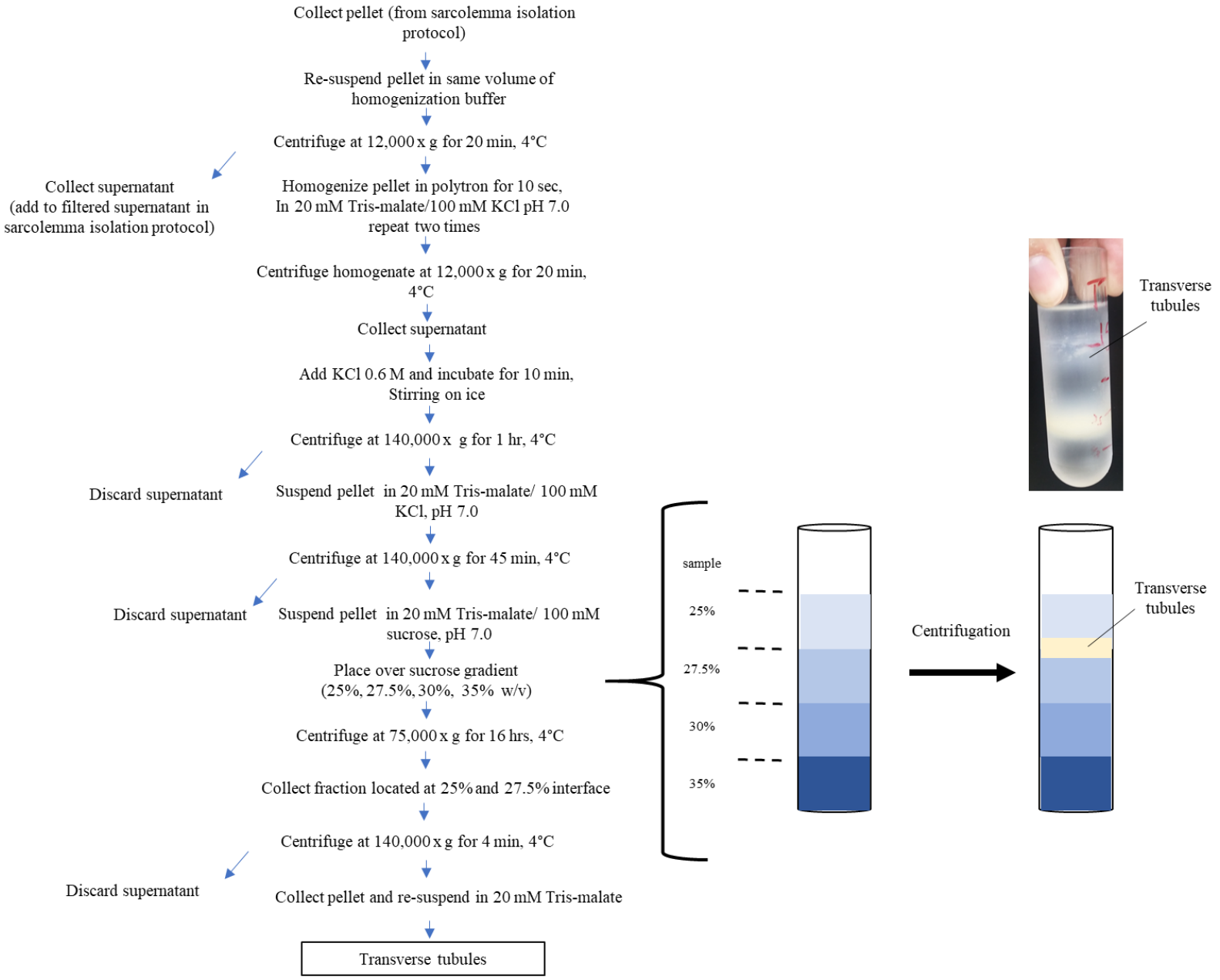

Figure 2.3. Transverse tubule isolation protocol

Isolation of detergent-resistant membrane (DRM)

Briefly, aliquots of 0.1 mg/ml of total protein isolated from SL or TT were incubated in 1% Triton X-100 for 30 minutes at 4°C and then diluted in an equal volume of an 80% sucrose solution. A discontinuous gradient formed by the addition of 30% and 5% sucrose solutions was then centrifuged for 18 h at 200,000 ×g, at 4 °C. Nine fractions were recollected from the top to the bottom where fraction 1 (F1) corresponds to the top of the tube and fraction 9 (F9) the bottom.

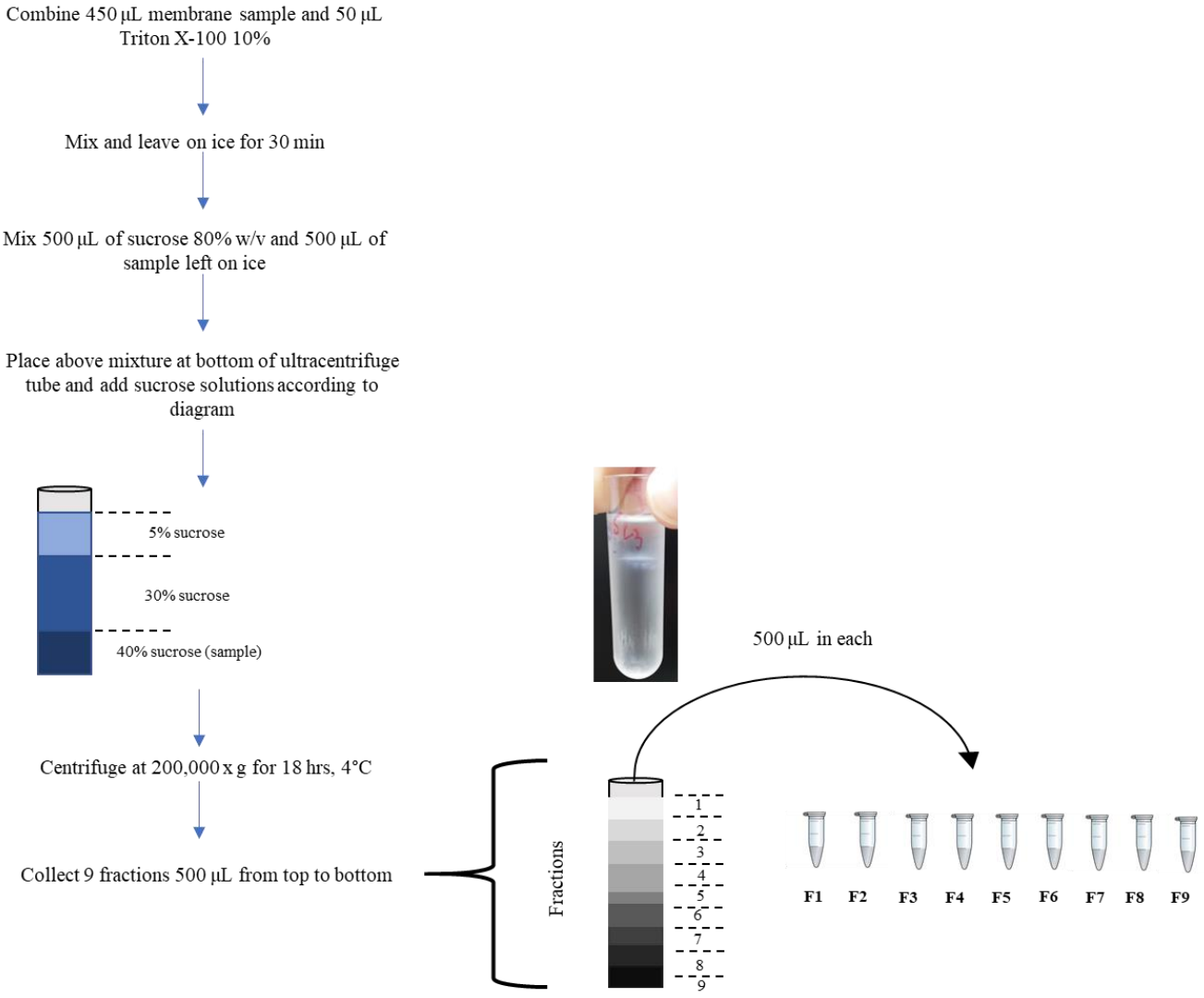

Figure 2.4. Lipid raft isolation protocol

Immunoblots and densitometry

Fraction samples were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. Membranes for Western blot were blocked at least 30 minutes with 5% non-fat dry milk (Bio-Rad) and incubated with the anti-alpha-synuclein primary antibody (1:500, monoclonal, Santa Cruz) for 45 minutes. After washing and incubation with the corresponding peroxidase-labelled secondary antibody (1:2000, Santa Cruz) for 45 minutes, blots were developed using the C-DiGit Blot scanner (LI-COR Biosciences). Densitometric analysis of each Western blots were performed by using the Image studio software (Li-COR Biosciences). Quantification is with absolute values taken from a defined area surrounding bands. The same total area for quantification is used for all bands. The densitometric values of the bands were corrected for the background by subtracting the non-reactive part of the membrane for that transfer.

RESULTS

Expression of alpha synuclein in plasma membrane of neuron and skeletal muscle fiber from young rats (3 months).

To investigate the presence of α-SN in SL, rat brain plasma membrane was used to compare the expression levels. The α-SN has been reported to be highly expressed in neuronal tissues. Isolated rat brain plasma membrane and SL from young rats was used in a western blot analysis against α-SN. The amount of rat brain plasma membrane or SL used was standardized against total protein content, 6 μg of total membrane protein was calculated and placed in each well. The relative expression of α-SN bands obtained via densitometry analysis from Western blots is displayed in the bar graph in Figure 3.1. Image A and B show a representative Western blot of rat brain plasma membrane (n = 1) and SL (n = 3), respectively. Results show multiple positive bands for α-SN at different molecular weights. However, the high molecular weight bands are not as abundant as the two bands seen in the representative image. For this reason, analysis in expression is focused on the two most abundant bands observed. Interestingly, the most abundant band corresponds to 55 kDa and is true in both tissues. The expression level in SL appears to be increased in SL when compared to brain plasma membrane. In the most abundant band, 55 kDa, there is an approximate 27.3% increase in α-SN expression in SL as compared to brain plasma membrane.

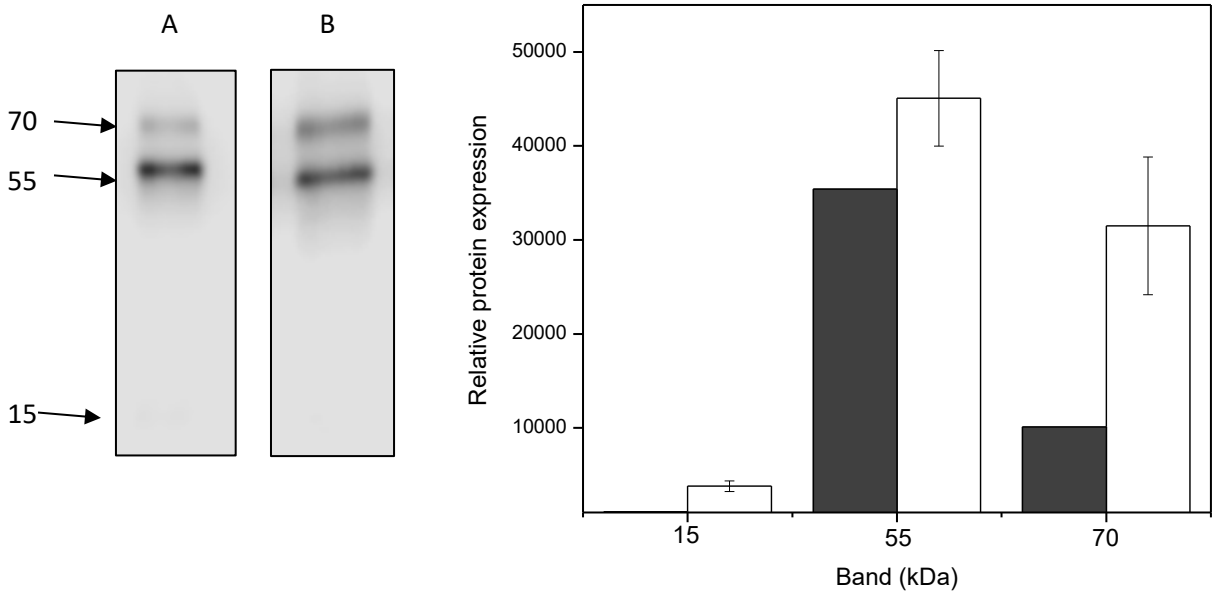

Figure 3.1. Expression of α-SN in plasma membrane of neuron and skeletal muscle fiber from young rats (3 months). The relative protein expression of α-SN is depicted in the bar graph for young neuron plasma membrane (dark grey bars), (n=1) and young SL (white bars), (n=3). Western blot A is a representative image of neuron plasma membrane from young rats against α-SN, B is a representative image of SL from young rats against α-SN.

Expression of alpha synuclein in sarcolemma of skeletal muscle fiber from young (3 months) and old (24 months) rats.

The expression levels of α-SN in SL from young and old rats were explored. Isolated SL from young and old rats were used in three independent Western blot analyses each to determine α-SN expression and compare changes with aging. The amount of membrane used was

standardized to total protein content, using 6 µg of total membrane protein per lane in Western blot. The expression level of α-SN in SL from young and old rats is shown in the bar graph in figure 3.2. Image A and B are representative images of Western blots against α-SN in young and old SL, respectively. Once again, results show multiple immuno-reactive bands to α-SN. The expression level of α-SN increases with age. In the most abundant band, 55 kDa, there is a 52.6% increase in α-SN expression with age.

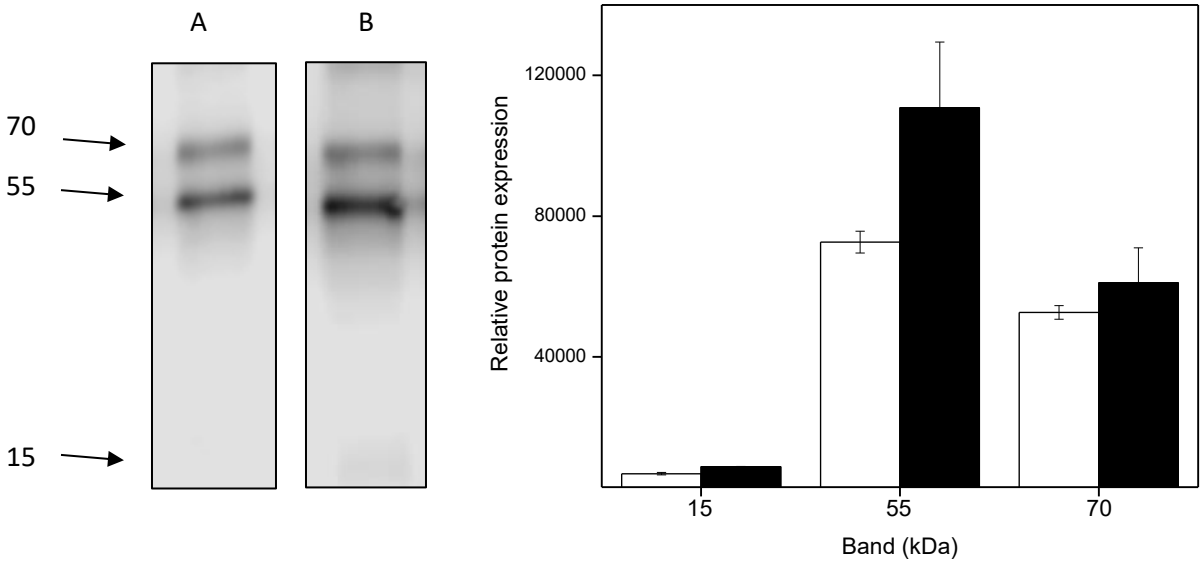

Figure 3.2. The relative protein expression of α-SN is depicted in the bar graph for young SL (white bars), (n=3) and old SL (black bars), (n=3). Western blot A is a representative image of SL from young rats against α-SN, B is a representative image of SL from old rats against α-SN

Expression of alpha synuclein in transverse tubule of skeletal muscle fiber from young (3 months) and old (24 months) rats.

Next, the presence and expression level of α-SN was evaluated in TT. Since transverse tubules in skeletal muscle fibers are superficial cell membranes that are continuous with SL and are also in contact with the extracellular/intracellular environment, it is important to determine the presence and expression levels of α-SN. Isolated TT from young and old rats were used in three independent Western blot analyses each to determine α-SN expression and compare changes with aging. The amount of membrane used was standardized to total protein content, using 6 μg of total membrane protein per lane in Western blot. The expression level of α-SN in TT from young and old rats is shown in the bar graph in figure 3.3. Image A and B are representative images of Western blots against α-SN in young and old TT, respectively. In the case of TT, it appears that α-SN expression increases with age, markedly so in the 55 kDa α-SN positive band. In the most abundant band, 55 kDa, there is a 22-fold increase in α-SN expression with age.

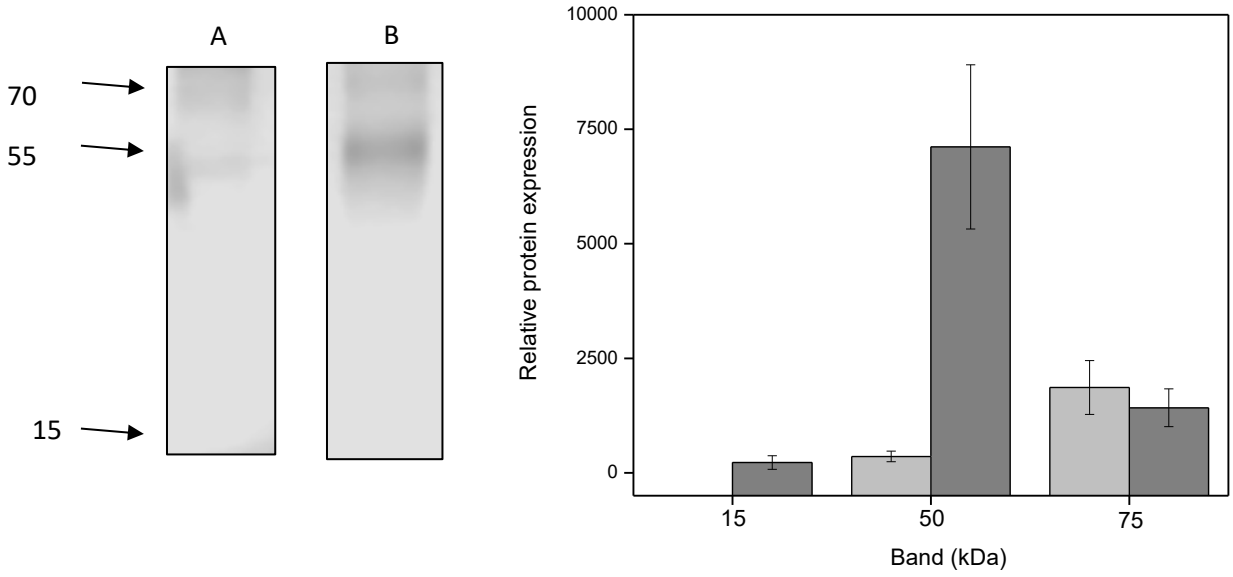

Figure 3.3. The relative protein expression of α-SN is depicted in the bar graph for young TT (light grey), (n=3) and old TT (grey), (n=3). Western blot A is a representative image of TT from young rats against α-SN, B is a representative image of TT from old rats against α-SN.

Distribution of alpha synuclein in lipid rafts of membranes obtained from skeletal muscle fibers of young (3 months) rats

Purified SL isolated from young rats was treated with 1% of Triton X-100 for 30 minutes on ice and then subjected to sucrose density gradient centrifugations. Fractions were then collected from the top of the gradient and labeled F1-F9. The distribution of α-SN in SL was detected by Western blot analysis using appropriate antibodies in figure 2.4. Representative image shows how α-SN is located in a discrete region of the membrane. Lower fractions such as F6-F9 are representative of sections of the membrane which are non-detergent resistant. In SL α-SN is in

the non-detergent resistant fractions. This means that α-SN is preferentially associated to regions of the membrane that do not contain saturated lipids.

Figure 2.4. Representative Western blot using antibody against α-SN of lipid rafts from SL obtained from young rats. A total of 2 µg per fraction was used so that there was an equal amount of total protein per well. (F1-F4) contain detergent resistant membranes and (F5-F9) are non-detergent resistant membranes. Mw = molecular weight marker.

DISCUSSION

When expression of α-SN in SL is compared to expression in plasma membranes obtained from whole brain, the apparent levels seem to be similar for the major bands observed (Figure 3.1). This puts into question the idea that α-SN is exclusively expressed in neuronal tissues. Another common misconception regarding α-SN is that it can be expressed in other tissues, but neuronal tissues have the highest expression of α-SN. Figure 3.1 demonstrates that this is not necessarily true in the case of brain plasma membrane and SL since there are similar expression levels.

The α-SN multimers were detected in neuron plasma membrane and SL from young animals (Figure 3.1). This means that in these biological membrane systems, α-SN exists mostly as multimeric species and not as a monomer. Also, the presence of α-SN in neuron plasma membrane has been documented before, however, this is the first time that α-SN multimers are seen in SL directly. Many reports claim that aggregation of α-SN into oligomers has cytotoxic effects by altering cell membrane integrity or protein homeostasis. Interestingly, this study reveals that α-SN multimers are expressed in normal, non-transgenic, healthy young animals throughout adulthood. Therefore, it is likely that α-SN multimers are not cytotoxic elements by themselves. In fact, previous research demonstrates that α-SN exists in the blood in normal, healthy human patients as a tetramer, indicating that under normal conditions α-SN can exist as multimers without causing harm [62]. In addition, α-SN is present during erythropoiesis and in the mature erythrocyte as well. The mature erythrocyte is a simple cell composed of a cell membrane and no internal cytosolic organelles. If α-SN multimers were cytotoxic, it would surely harm and disrupt the function of mature erythrocytes, which is not the case. In this study, both membrane sources contain α-SN multimers forms in young healthy animals. This immediately brings into question previous research which demonstrates that multimeric associations of α-SN lead to cytotoxic effects through the formation of pore-like complexes that disrupt cell homeostasis. Molecular dynamic models reveal that α-SN associates to cell membranes and can form pore-like complexes by the addition of 8-11 α-SN monomers forming an annular pore-like structure. Our data shows that α-SN does form multimers on neurolemma and skeletal muscle cell membranes. However, it seems unlikely that the multimers observed have pore-like structures, especially in the case of young control animals. This is because the presence of pore-like structures in excitable cells such as those found in brain and skeletal muscle would eventually deregulate the ion homeostasis and

therefore the physiological action of these systems. Experimental evidence against cytotoxic pore forming α-SN can be seen when α-SN homo-tetramers are incubated with liposomes. The liposome's permeability for potassium, sodium and calcium does not change [70]. Also, a dye release assay was performed with membranes of differing composition and it was revealed that a 20 μM (equivalent monomer concentration) of α-SN does not produce permeabilization of membranes [71]. If α-SN were able to form pore-like structures in the membrane than dye would be detected in the outside of membrane structures. Furthermore, cell viability of neuronal tissue culture with α-SN tetramers is unchanged, proving that the α-SN tetramer has no membrane disrupting effects when applied extracellularly [70].

Additionally, figure 3.1 demonstrates the expression pattern of α-SN in bran and skeletal muscle plasma membranes. In both cases, the 55 kDa α-SN positive band has the highest expression level. Given that the molecular weight of monomeric α-SN is reported to be approximately 14kDa, the 55 kDa α-SN positive band is most likely a homo-tetramer of α-SN. This means that the most abundant species of α-SN corresponds to the tetramer in both brain and skeletal muscle plasma membranes. A higher molecular weight band is seen in both brain and skeletal muscle plasma membrane with an approximate weight of 70 kDa and is likely a homo-pentamer of α-SN. In this band however, there is a significant difference in expression with respect to brain and skeletal muscle plasma membrane. There is an approximate threefold increase in the 70 kDa homo pentamer expression in SL with respect to brain plasma membrane. Since α-SN is dependent on its environment to adopt an ordered structure, it is likely that the difference can be caused by the lipid environment in brain and skeletal muscle plasma membrane. Cell membranes are complex assemblies of lipids and proteins, where a typical plasma membrane contains hundreds of different lipids that are incorporated into the membrane [10]. The composition of

lipids varies significantly between different cells and tissue types. As a result, it is possible that the different lipid compositions in SL can promote the assembly and stabilization of the α-SN pentamer, more so than brain plasma membrane.

The next logical question to ask is why the tetramer of α-SN is the most abundant multimeric form in both membrane sources. The stability of the α-SN tetramer has been evaluated using all-atom molecular dynamic simulations at 300 ^{o}K and higher temperatures, 500 ^{o}K. The α-SN tetramer is stable at both temperatures. Stability of this assembly arises from the presence of a hydrophobic core at the center of the tetramer. Nonpolar residues pertaining to an α-helix chain are the main associations in the hydrophobic interactions between α-SN subunits in the tetramer. In addition, the lysine residues in the KTKEGV repeat motif located on the amino terminus of α-SN form salt bridges with negatively charged residues such as aspartic acid or glutamic acid. Both hydrophobic and salt bridge formation are the forces that promote and maintain the tetramer assembly of α-SN [72]. Similar forces may be maintaining a stable association in the α-SN pentamer. However, due to the increase in one subunit, the force of attraction may be reduced, and therefore the association of the pentamer is not as stable as the tetramer and is therefore less abundant.

Young and old SL were isolated and probed for the presence and expression pattern of α-SN (figure 3.2). Both young and old SL express α-SN mainly as a multimeric protein. In SL, α-SN exists predominantly as a tetramer corresponding to the 55 kDa band and pentamer corresponding to the 70 kDa band. Taking into consideration the monomeric α-SN as 1, the proportion expressed by monomer: tetramer: pentamer for young SL is 1:11:8, while for old SL the expression proportion is 1:13:7. Aging alters the expression pattern of α-SN as can be seen in the bar graph in figure 3.2. There is an approximately 52.6% increase in the expression of the α-

SN tetramer in SL with aging, while the α-SN pentamer expression remains approximately the same as the monomer in SL. This suggests that aging in SL promotes the formation of the α-SN tetramer.

What about the α-SN tetramer leads to its increased formation when compared to other related multimers? The formation of the other major synuclein conformations, monomer and pentamer, remain similar with aging. As a result, it is unlikely that an increase in translation synthesis of α-SN is the cause of the increase because there would be an overall increase in the other conformations as well. Additionally, the overall protein synthesis of proteins is reduced with age in human and rats in a variety of *in vitro* and tissue studies [73]. The other remodeling process in protein turnover is degradation, leading to changes in expression by either increasing or decreasing the breakdown of proteins. There are two main systems for the degradation of intracellular proteins: the proteasomal and lysosomal pathways. Our results show that α-SN is membrane bound protein and therefore changes in its degradation rate should be a consequence of the lysosomal pathway. This is because invaginations of the plasma membrane are fused with lysosomes to be degraded by the proteolytic machinery within. Evidence suggests that aging leads to suppression of the lysosomal degradation pathway [74]. Therefore, a possible mechanism for the tetramer increase in expression in the SL of old rats can be caused by a decrease in its lysosomal degradation. Given that the other α-SN conformations in old SL have a similar expression pattern as control, it seems that the α-SN tetramer is more resistant to degradation. This could in part be due to its increased conformational stability with respect to other related multimers or because α-SN is somehow able to escape the lysosomal degradation pathway.

Figure 3.3 demonstrates the expression of α-SN in TT isolated from young and old rats. In TT membranes isolated from control rats, the most abundant form of α-SN is the tetramer and

pentamer, as in the case of SL. This further proves that α-SN mostly exists as multimers when in contact with biological membranes. However, the presence is far lower in TT membranes than in SL. Only considering the α-SN tetramer, there is an over 200-fold decrease in the relative expression of α-SN in TT with respect to SL, in young rats. TT membranes isolated from aged rats have a similar expression level of α-SN with one exception, the α-SN tetramer. The bar graph in figure 3.3 shows there is 22-fold increase in the detection of tetramric α-SN from aged rats when compared to SL from young rats. Just as in SL, the α-SN tetrameric detection is increased in membranes from aging rats. It is important to note however that even at its detection, the tetramer in aged TT, it is still far lower than what is present in SL membranes. This suggests that α-SN preferentially associates with SL rather than TT membranes. This is interesting because SL and TT membranes are both surface level membranes and are continuous with each other. Additionally, TT composes approximately 60% of the cell surface, one would expect that α-SN would be found more in TT fraction due to its increased surface area. Since the opposite is true, it appears that TT membranes somehow exclude α-SN more than SL. As mentioned previously, the main difference between the SL and TT membrane systems is lipid composition. A noticeable difference is the cholesterol content, where TT contain approximately double the concentration of cholesterol than SL when measured with respect to phospholipid concentration [75]. Taken all together, it is likely that cholesterol could be the factor which tends to exclude α-SN from the TT membrane. The high cholesterol and sphingolipid content present in the TT membrane endow it with a rigid lipid environment, caused by the association between sphingolipid and cholesterol, where geometric compatible association between these molecules is thermodynamically favored and so tend to form associations in the membrane. These associations are highly ordered and structured when compared to the rest of the membrane composed mainly of phospholipids. As a

result, the association between cholesterol and sphingolipids in a lipid bilayer tend to form a liquid ordered phase, and thus has physical properties that differ from other parts of the membrane. It is possible that α-SN is unable to penetrate effectively into rigid membranes that contain high cholesterol/sphingolipid content. But, given its presence in SL, α-SN can associate and bind to fluid disordered membranes. Experimental evidence with oligomeric α-SN and artificial membrane vesicles shows that α-SN can bind to membranes composed of anionic lipids with a liquid disordered phase.

The distribution of α-SN into detergent resistant membranes and non-detergent resistant membranes in SL isolated form control rats can be seen in figure 3.4. The technique used here in SL has been used previously to isolate detergent resistant membranes with Triton x-100. Under these conditions, the detergent resistant membranes tend to float to lower density regions of the ultracentrifuge tube when ultracentrifugation is complete. Therefore, the detergent resistant membranes are in the upper fractions of the ultracentrifuge tube, in this case corresponding to fractions F1-F5. The membrane that remains in the lower fractions is therefore non-detergent resistant membranes. In figure 3.4 α-SN tends to be in fractions F7-F9 corresponding to the non-detergent resistant membranes in SL of control rats. The tetramer and pentamer are discretely visible in the whole membrane. In this figure it is possible to see that the tetramer and pentamer are not the only multimeric conformations of α-SN present. Beginning at around 80 kDa there are α-SN positive bands that increase in molecular weight up to 150 kDa which is the highest molecular weight marker used in this gel. What is interesting to note is that the pentamer and tetramer are more abundant and clearly separate from one another, there is no streaking or intermediate weights. In addition, this discrete jump in α-SN bands can be seen from the pentamer to the higher molecular weight α-SN band around 80 kDa. From the 80 kDa α-SN band and above

there is some streaking visible. The presence of these high molecular weight α-SN bands demonstrates that α-SN can exist in other multimeric conformations other than tetramer and pentamer when interacting with biological membranes. This has been observed previously in whole brain cell membranes of mice, where multimeric conformations of α-SN are present [76]. Together, these experiments show that α-SN preferentially forms multimer with biological membranes.

Previously, α-SN has been described as a lipid raft located protein. This is due to a report that demonstrated the presence α-SN in lipid raft fractions from two different cell types, HeLa and neurons isolated from mice [77]. Here, α-SN is reported to colocalize with known lipid raft markers in permeabilized HeLa cells that are incubated with external recombinantly expressed α-SN. First, the α-SN detected in this experiment is not endogenous to the cell, the cell medium is incubated with purified α-SN obtained from bacteria that have been modified to produce α-SN. It is possible that the amount of α-SN incubated with the cells is not reflective of the amount that exists in a normal cell and this fact may alter its distribution in the cell. Additionally, in the merged images shown to demonstrate α-SN and lipid raft marker co-localization, there are significant areas where α-SNs does not colocalize, which brings into question if it is truly located exclusively in lipid rafts. Using a similar extraction method as in this study, membrane fractions were obtained from HeLa cells transfected with α-SN to observe distribution in the plasma membrane. They show α-SN fractionates into the detergent resistant membranes, and therefore conclude that α-SN is located exclusively in lipid rafts. However, examination of this result shows α-SN is present in mostly all fractions, including the lower fraction corresponding to the non-detergent resistant membranes. Therefore, it is not accurate to describe α-SN as a protein that is localized in lipid rafts exclusively, clearly it is present in all fractions. Additionally, the fact that α-SN is in almost

all fractions can be a result of α-SN overexpression in these cells. This is because the cells utilized during the fractionation method are transfected HeLa cells that utilize promoters that increase the expression of α-SN in the cell. The overexpression of α-SN can be causing excess α-SN to be present in almost all membrane fractions due to its increased concentration in the cell. However, under normal expressing conditions α-SNs does not seem to fractionate with detergent resistant membranes. Therefore, it is not likely that α-SN is a lipid raft located protein under normal cell conditions. Mouse brain cell membranes were also utilized to observe whether α-SN fractionates into lipid raft associated membranes. A similar result is observed where there is presence of α-SN in most of the fractions including non-detergent resistant membranes. In fact, the majority of α-SN fractionates into the non-detergent resistant membranes seen by band optical density and size. The presence of α-SN in upper fraction can be due to a similar effect as seen in the case of transfected HeLa cells. The mice utilized to isolate brain cell membranes are transgenic animals overexpressing wild type human α-SN. As a result, this data is not indicative of what is occurring under normal conditions. It is important to clarify this because this article is often cited in scientific literature as evidence for α-SN existing as a lipid raft associated protein. The above evidence indicates that the biological models used in this study are likely responsible for the localization of α-SN in upper fractions. The authors in this study describe α-SN as a lipid raft associated protein due to its location in upper fractions. This has caused other authors to continue to refer to α-SN as a lipid raft associated protein, but a look at the experimental data shows that it is present in almost all fractions. According to these very same results, it is not accurate to describe α-SN as a lipid raft protein.

It is likely that α-SN exists outside of lipid raft membranes given the number of experimental work with α-SN and membranes indicating α-SN binds to a variety of phospholipids

such as 1-palmitoyl-2-oleoylphosphatidic acid (POPA), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) in small unilamellar vesicles [58], but also binds to large unilamellar vesicles of POPS and POPC phospholipids [78]. There are contradictory reports regarding α-SN lipid binding. Some authors report that α-SN is only able to bind to negatively charged lipid membranes while others report that it binds to neutral lipid membranes. These conflicting reports are likely due to differences in methodology and the method used to ultimately detect binding. However, there is experimental evidence for binding to neutral and negatively charged phospholipids in both cases. This is important because the mammalian plasma membrane cytoplasmic leaflet is significantly composed of neutral and negatively charged phospholipids such as phosphatidylcholine and phosphatidylserine [79]. α-SN is synthesized intracellularly so it must eventually interact with the cytoplasmic leaflet of the plasma membrane. It has been previously reported that a population of α-SN exists localized in the plasma membrane, thus the interaction must be at least initially with the lipids of the cytoplasmic leaflet [80]. The composition of the cytoplasmic leaflet is different from the exoplasmic leaflet. The exoplasmic leaflet is mainly composed of saturated lipids, sphingolipids, and sterols [79]. It is these lipids that also preferentially interact to form lipid rafts. Since these lipids are found in the exoplasmic leaflet, it is less likely for the intracellularly produced α-SN to interact and bind to these lipids than the cytoplasmic leaflet lipids. Because the cytoplasmic leaflet lipids do not form the interactions necessary to form lipid rafts, it is unlikely that α-SN is a lipid raft associated protein.

Interesting to note is the presence of multimeric species under the denaturing conditions of SDS-PAGE. Usually, these multimeric bands in the SDS-PAGE would be thought to involve a covalent bond, since the SDS detergent is unable to separate covalently bonded proteins. However,

in this experiment, all samples are incubated with β-mercaptoethanol (commonly used reducing agent in SDS-PAGE) and boiled at 100 ºC by 5 minutes before loading into the gel to eliminate covalently attached proteins. As a result, it is unlikely that the observed high molecular weight bands are due to covalently attached α-SN monomers. This is not an unheard-of phenomenon for membrane proteins. For example, the major histocompatibility complex (MHC) class II is a heterodimer membrane protein composed of an alpha and beta subunit that are not attached covalently, yet it displays SDS resistance in SDS-PAGE [81]. This has also been observed in phospholamban, a membrane integrated protein located in SR. SDS-PAGE shows that it can be detected as a monomer or in several multimeric states, from pentamer to dimers [82]. An explanation for the resistance to SDS denaturation is that the detergent is unable to interrupt the preexisting hydrophobic protein-protein interactions that bind the α-SN monomers into multimeric configurations. It has previously been reported that SDS binding to membrane spanning proteins tends to stabilize alpha helix secondary structure [83]. In other words, the SDS molecules substitute the lipid-protein interactions that occur normally when the transmembrane portion is in the membrane, maintaining secondary structure. As mentioned previously, the N-terminal region has been proven to be involved in lipid membrane binding and in the region that adopts α-helix secondary structure. Additionally, the N-terminal region contains KTKEGV repeat motifs. This region has been shown to be involved in the multimerization of α-SN [84]. Directed mutations in the repeat motifs causes a shift in the conformation, from multimers to monomers. It is likely that α-SN exists integrated into the plasma membrane as multimers in part by associations between the α-helix adopted once in the presence of lipid membranes. Therefore, mutations that occur in the N-terminal portion tend to interrupt the association between the α-SN monomers. In this scenario,

the SDS detergent is unable to interrupt the hydrophobic interaction between α-SN multimers so it appears as a multimeric band even on an SDS denaturing gel.

Previous reports describe α-SN as a peripheral membrane protein. These are membrane associated proteins that can reversibly bind to the surface of a lipid membrane mainly through electrostatic interactions [85]. α-SN is traditionally described as a peripheral membrane protein that adopts amphipathic helix structure in the N terminal region, residues 1-60 on the membrane surface, where hydrophobic residues are facing the lipid membrane and hydrophilic residues of the helix are in contact with the solvent. It is thought that α-SN can form an amphiphilic helix in this region because of the presence of imperfect repeats KTKEGV coding for amphipathic helices a motif found in other lipid binding proteins. This similar sequence motifs have been observed in the apolipoproteins, protein known to reversibly bind to phospholipids [86]. However, evidence of α-SN as a membrane penetrating protein has previously been shown. Molecular dynamic simulations reveal that α-SN can penetrate the cell membrane rapidly. Within 4ns of simulation α-SN can penetrate up to 2/3 of the membrane and within 9 ns can fully penetrate the lipid bilayer. The molecular dynamic model of α-SN insertion into lipid bilayer reveals that membrane penetration promotes the incorporation of additional α-SN monomers into the complex [87]. These studies provide a molecular explanation for the incorporation and multimerization of α-SN in a lipid membrane. *In vivo* studies suggest that α-SN can exist embedded into the hydrophobic core of the lipid membrane. Membrane fractions isolated from rat brain were used to test the involvement of electrostatic force in the interaction between α-SN and a biological membrane. To do this, membrane fractions were incubated in different salt concentrations, and were then isolated and probed for remaining α-SN. This experiment showed that α-SN remained bound to membranes throughout the ionic strength range tested [88]. If α-SN were a peripheral membrane protein, the

expected result would be α-SN presence in the supernatant. This salt wash experiment shows that α-SN interaction with membranes is of a hydrophobic nature since hydrophobic interactions are not interrupted by changes in the ionic strength of the solvent. Our results also indicate that α-SN interaction with biological membranes is mainly through hydrophobic interactions. As part of the SL isolation protocol, homogenized skeletal muscle is incubated with 0.6 M KCl to extract and eliminate the major contractile proteins. Under these conditions, any membrane associated protein that are bound by electrostatic forces would be displaced from the membrane into the solvent. However, our results show that α-SN remains incorporated into SL and is detected in the SL preparations by western blot analysis.

The hydrophobic interactions between α-SN and lipid membranes could only occur in the hydrophobic core of the membrane along the hydrocarbon chain of lipids. Therefore, α-SN can penetrate the membrane, traverse the hydrophilic head groups and have hydrophobic interactions in the core of the lipid membrane. This scenario implies that α-SN is at least partially inserted into the lipid bilayer. *In vivo* evidence of lipid bilayer insertion can be seen using a protease protection assay. The assay is based on the inaccessibility of proteases to membrane integrated proteins. Protein regions displayed on the surface of membranes are degraded by proteases, while regions in the lipid membrane resist degradation [89]. Tryptic digestion of α-SN in the presence of small unilamellar vesicles produces different results depending on the lipid composition [90]. In the presence of pure phosphatidylcholine vesicles, α-SN is completely degraded by trypsin digestion within ten minutes. Vesicles composed of a mixture of phosphatidylcholine and phosphatidylserine resist tryptic digestion. Tris-tricine gel stained with Coomassie Blue shows that under these conditions an approximately 6-8 kDa fragment is produced while a population of α-SN is not digested and appears as whole α-SN. Western blot analysis using an antibody to the N-

terminal 24 residues of α-SN reveal that the 6-8 kDa fragment produced is derived from the N-terminus of α-SN [90]. Collectively, these results suggest that the 6-8 kDa α-SN fragment produced from trypsin digestion is protected from protease activity and therefore is inserted into the lipid vesicles. During interaction with lipid membranes, the N-terminal portion corresponding to this fragment inserts into the lipid membrane and the remainder of the protein is accessible to protease activity. Additionally, the fact that whole α-SN can still be seen with the mixture of phosphatidylcholine and phosphatidylserine and trypsin suggests that at least a population α-SN can cross lipid membranes as a whole protein. A portion of whole α-SN is still observable even after 30 minutes of trypsin digestion when in the presence of small unilamellar vesicles composed of phosphatidylcholine and phosphatidylserine. An explanation for this is that whole α-SN can traverse membranes of the small unilamellar vesicle and are located inside, protected from protease activity. Support for α-SN translocation has been observed previously. A study showed that fluorescently tagged α-SN is transported internally into a variety of cells, including HeLa, neuronal, hematopoietic, and Chinese hamster ovary cells [44]. A confocal analysis of the fluorescently tagged α-SN revealed that α-SN begins to appear inside cells within 5 minutes of incubation. Flow cytometric analysis of cells without and with α-SN incubation corroborate the confocal microscopic observations. Since the translocation of α-SN into cells is not cell type specific this also suggests that α-SN does not require a specific cell receptor for transport into cells. Interestingly, the same study discovered that translocation into cells requires one or more of the repeat sequence motifs located in the amino terminal of α-SN [44]. By creating a series of amino terminally truncated α-SN lacking repeat sequence motifs and fusion proteins containing the repeat sequence motifs, it was confirmed that transduction efficiency into cells either decreases or increases depending on the presence of repeat sequence motifs. The uptake of α-SN into cells was

independent of receptor-mediated endocytosis, demonstrated by cellular uptake of α-SN at 4 °C, an established inhibitor of receptor mediated endocytosis. The internalization of α-SN was also insensitive to general endocytosis inhibitors. These features of α-SN prove that it is behaving as a cell penetrating protein.

α-SN is notable for its role as a pathological element in neurodegenerative diseases. Throughout the years, several studies demonstrate that when α-SN is expressed in cells, there is oligomerization of α-SN, leading to proteinaceous aggregates in the cytoplasm and ultimately cell death. At the membrane level, other studies demonstrate *in vivo* and *in silico* that α-SN forms oligomeric associations in the membrane, forming a pore like structure that disrupts the ion homeostasis in cells leading to cytotoxicity. Increasing studies seem to suggest that the oligomeric association of α-SN are the initiating factor in the development of degenerative diseases, acting as a seed that promotes the misfolding of α-SN into pathological fiber structures. However, these results are not in agreement with what is seen in SL and TT. It is important to note that many other experiments evaluating α-SN toxicity *in vivo* utilize transgenic organisms, including cell lines and mammalian models such as mice. These transgenic organisms express either a mutated humanized α-SN known to be involved in the development of familial Parkinson's disease or express wild type human α-SN at significantly higher levels than occurs endogenously in a normal cell. These experimental models are pushed to overexpress α-SN and likely cause cell stress by overloading protein homeostasis. Evidence suggests that α-SN expression in transgenic mice produces a chronic unfolded protein response. Activation of this pathway promotes cell death [91]. These transgenic animal and cell models do not characterize what happens in a healthy organism, they are more indicative of what happens in a cell with α-SN overexpression and not necessarily what happens in neurodegenerative disease such as Parkinson's. This is evidenced by the fact that

transgenic animal models do not display other characteristics of neurodegenerative disease such as loss of dopaminergic neurons and the behavioral changes seen in people afflicted with disease. As a result, these models fail to reproduce what happens physiologically. This suggests that the role of α-SN in degenerative diseases is still poorly understood and the accumulation of α-SN in cells is probably not the leading cause in neurodegeneration.

α-SN is described as a cytosolic protein that is intrinsically disordered. However, several reports indicate that α-SN can interact with a variety of lipids. *In vitro*, reports demonstrate that α-SN can bind exclusively to negative lipids, while others suggest neutral lipids. In vivo however it is clear that α-SN can interact with different biological membranes such as the plasma membrane, intracellular vesicles, Golgi apparatus and nuclear outer membrane [92]. Intracellular biological membranes and plasma membranes of cells differ in their lipid composition. As a result, the association of α-SN to a biological membrane seems to be non-specific. The presence of α-SN in cytosol and cell membranes is indicative of its dynamic nature. Not only is α-SN dynamic with respect to its cellular localization, but also in its conformational state. α-SN displays a range of possible conformational states each composed of associations of α-SN subunits such as dimers, trimers, tetramer, etc.. In this case, α-SN is found in the skeletal muscle membrane as multimers. Interesting to note is the comparatively small amount of monomeric α-SN in SL and TT. This information indicates that α-SN forms multimers upon membrane association, which has been seen before in mouse brain. Western blot analysis reveals multimeric membrane-bound α-SN in mouse brain homogenates, while cytosolic fractions of mouse brain show α-SN exists mostly as a monomeric protein [93]. As a result, α-SN can bind to lipids in membrane and adopt a specific structure that promotes the assembly of higher order multimers. Additionally, these associations in the membrane are highly stable, demonstrated by the resistance to SDS solubilization.

The reason behind this change in conformation assemblies upon membrane association remains unclear. However, under normal conditions α-SN exists mostly as multimeric assemblies in the cell plasma membrane and as a free monomer in solution. Disruption in the population of membrane bound of α-SN is thought to be one of the changes involved in the development of pathogenic aggregation.

This study revealed that α-SN exists as multimeric complexes in the non-raft domains of SL. Combining this fact with other information provided by studies regarding the transport of α-SN an interesting hypothetical model of skeletal muscle as a source of α-SN during aging arises. Even though the exact mechanism is still unknown, the transport of α-SN across cell membranes is possible [67]. This means that α-SN exits SL and enters the extracellular environment and becomes incorporated into biological fluids. Here it is possible for α-SN to be carried in the bloodstream and eventually reach the blood-brain barrier. It is established that α-SN crosses the blood-brain barrier in the blood-to-brain direction [94]. The SL lipid composition with age changes to produce a more rigid and ordered plasma membrane, resembling the ordered stable structure of lipid rafts. This study reveals α-SN is present in the non-raft domains of SL. Given this information, it is possible that the biophysical changes in SL with age promote the dissociation of α-SN from SL. As a result, the equilibrium that normally exists between membrane-associated and membrane-free α-SN during younger stages in life is shifted towards membrane-free. This could potentially increase the concentration of extracellular α-SN. The effect could be very significant especially considering the amount of muscle mass that exists in the vertebrate body.

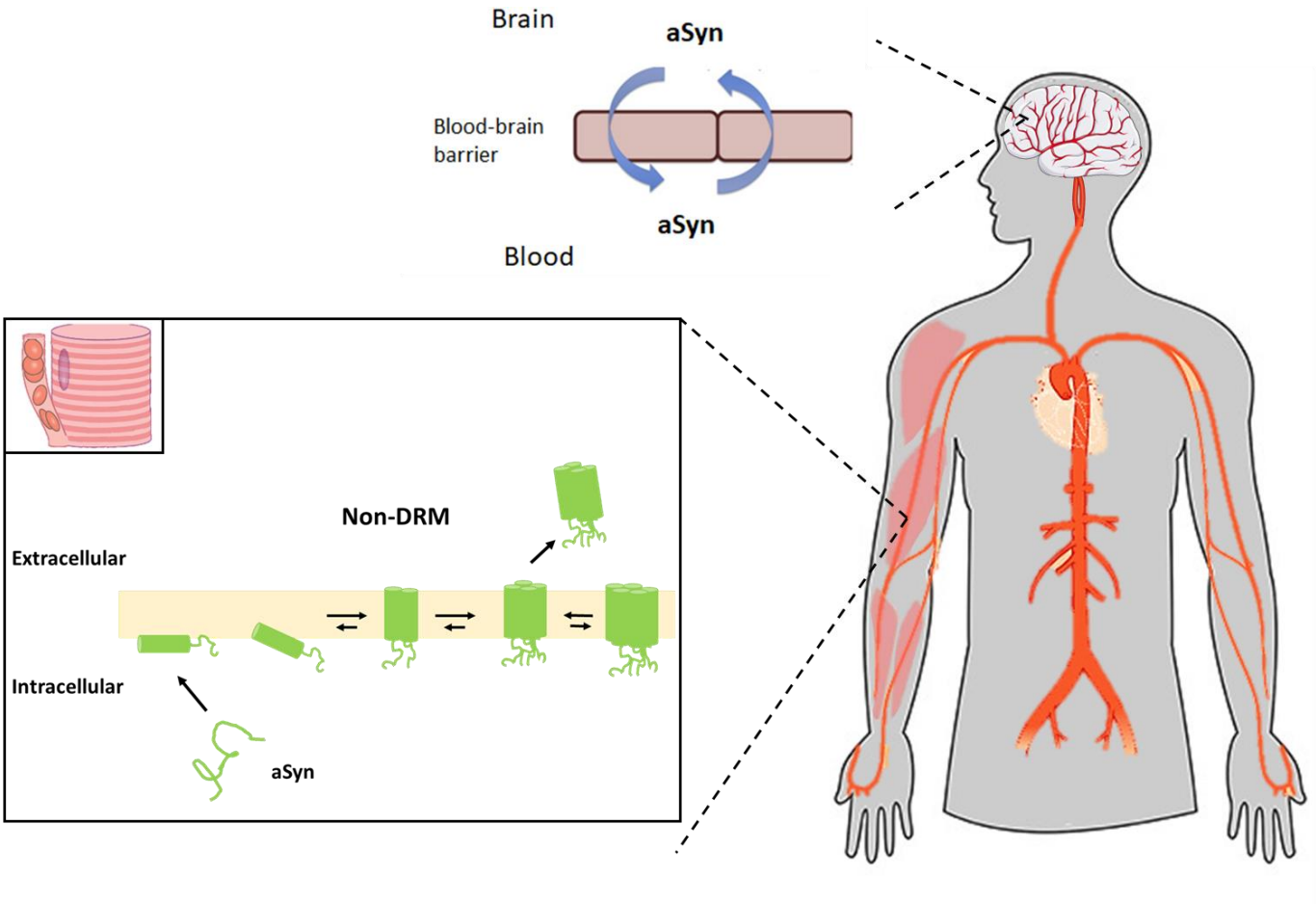

Figure 4. Proposed mechanism of skeletal muscle as a possible source of α-SN during aging. Diagram in inset demonstrates the SL of skeletal muscle. A-SN exists intracellularly in the cytosol as an intrinsically disordered protein. Upon membrane association to non -lipid raft domains, α-SN adopts mainly alpha helix secondary structure and insertion into membrane bilayer begins. Once embedded in membrane, α-SN can form association with other α-SN monomers forming multimers. Several multimeric species exist, but tetramers and pentamers are among the most abundant in membranes, tetramer is the most abundant species though. Previous evidence suggests α-SN is able to traverse the membrane and be exported into extracellular environment. Once located extracellularly, α-SN can travel through bloodstream and ultimately cross the blood brain barrier.

CONCLUDING REMARKS

It is very evident that α-SN is expressed in the surface membranes (SL and TT) of skeletal muscle. In membranes from young rats, α-SN is present in SL membranes as several oligomeric species. Given its monomer molecular weight of 14 kDa, the bands observed would correspond to a homo-tetramer (approximately 55 kDa) and a homo-pentamer (approximately 70 kDa). This is not unusual since several previous groups have reported oligomeric forms of α-SN when isolated from mammalian tissues. In addition, aging seems to influence the expression of α-SN in SL membranes, increasing its expression with age. Most notably, the band at 55 kDa which corresponds to the homo-tetramer of α-SN is visibly more detected than the other form of α-SN with aging. A-SN is also associated to TT membranes; however, the amount present is significantly lower than in SL membranes. However, aging also changes the pattern of α-SN in TT, increasing the detection especially for the homo-tetramer form. Finally, demonstrates that α-SN tends to be distributed into the non-detergent resistant membranes. The hypothesis of this work is proven since α-SN in the surface membranes of myofibers and protein expression level increase with age.

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