Microglia, phosphatidylserine, and synapse loss: an investigation of the “vulnerable” synapse

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This PDF contains only the literature review of my thesis. The proteomic analysis is yet to be published and has been omitted.

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NB: Greyed-out sections are omitted from this PDF

1. Abbreviations

Aβ	amyloid-β
AD	Alzheimer's disease
APAF1	apoptotic protease-activating factor 1
APP	amyloid precursor protein
BBB	blood-brain barrier
BCSFB	blood-cerebrospinal fluid barrier
CDR	Clinical Dementia Rating Scale
CERAD	Consortium to Establish a Registry for Alzheimer's disease
CNS	central nervous system
CP	choroid plexus
CPEC	choroid plexus epithelial cell
CR3	complement component 3 receptor
C1q	complement component 1q
C3	complement component 3
dLGN	dorsal lateral geniculate nucleus
DRG	dorsal root ganglion
EB	Evans blue
ePS	externalised phosphatidylserine
ETC	electron transport chain
FACS	fluorescence-activated cell sorting
FcγR	fc gamma receptor
fMRI	functional magnetic resonance imaging
GPR56	G protein-coupled receptor 56

HC	healthy control
HPLC	high performance liquid chromatography
IgG2c	immunoglobulin gamma 2c
Ighg2c	immunoglobulin heavy constant gamma 2C
iTRAQ	isobaric tags for relative and absolute quantitation
kDa	kilodalton
KO	knock-out
LTP	long term potentiation
MCL	Markov clustering
MEF	mouse embryonic fibroblasts
MMSE	Mini-Mental Status Examination
mo	months-old
mPTP	mitochondrial permeability transition pore
MS	mass spectrometry
nLC	nano-scale liquid chromatography
oAβ	oligomeric amyloid-β
P	postnatal day
PPI	protein-protein interaction
PS	phosphatidylserine
PSD	postsynaptic density
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TMEM16C	transmembrane protein 16C
TMT	tandem mass tag

TR	targeted replacement
TREM2	triggering receptor expressed on myeloid cells 2
WT	wild-type
2D-DIGE	two-dimensional difference gel electrophoresis

2. Abstract

Research over the last two decades has demonstrated that the brain and the innate immune system are intimately connected. Synapse loss – a phenomenon common to a diverse range of scenarios, from developmental circuit refinement to neurodegenerative disease – is one striking manifestation of this interaction. Microglia, the major tissue-resident macrophages of the brain, and the classical complement cascade, an innate immune pathway that aids in the clearance of unwanted material, have been implicated in mediating synapse loss; however, key questions remain unanswered. What makes synapses vulnerable to loss? Are specific synapses targeted for elimination? Are the synapse removal mechanisms preserved from physiology to pathology? In this thesis, I review recent work implicating complement and microglia as mediators of synapse loss and examine the proteomic signature of synapses vulnerable to loss. In doing so, I attempt to address the unanswered questions from a synaptocentric perspective.

3. Introduction

The elimination of synapses from neurons is essential for normal brain development (Katz and Shatz 1996). However, in certain pathological conditions this process is altered. Alzheimer's disease (AD) is perhaps the most stark example of this, where synapses are aberrantly lost early in the disease process and this pathological loss correlates strongly with cognitive decline (Davies *et al.* 1987; Dekosky and Scheff 1990; Terry *et al.* 1991; Scheff *et al.* 2006; Scheff *et al.* 2007; Scheff *et al.* 2011). Understanding why synapses are lost may lead to the development of new therapeutics to fight neurodegenerative disease.

In this thesis, I attempt to answer two key questions: What makes synapses vulnerable to loss? Are certain synapses specifically targeted for elimination? To answer these questions, I first discuss some of the key papers that have contributed to our understanding of synapse loss in the developing brain, before moving on to the most important discoveries regarding synapse loss in the AD brain. This leads me to propose that synapses that have externalised the membrane lipid phosphatidylserine may be specifically targeted for elimination. Furthermore, I suggest that this externalisation may be downstream of synaptic mitochondrial dysfunction, hence dysfunctional mitochondria may be a core feature making synapses vulnerable to loss. Finally, I analyse the proteome of synapses vulnerable to loss and find that proteins essential for mitochondrial function are altered in these "vulnerable" synapses.

3.1 Developmental synaptic pruning

During the development of the nervous systems, an excess of synaptic connections form between neurons. Removal of these superfluous synapses, a process termed 'synaptic pruning', is necessary to sculpt and refine neuronal circuity (Katz and Shatz 1996). Complement proteins are innate immune molecules that become activated in cascades and assist immune cells in the clearance of unwanted cells and debris (Ricklin *et al.* 2010). Complement component 1q (C1q) initiates the classical complement cascade by binding unwanted material. Downstream in this cascade complement component 3 (C3) becomes cleaved, which leads to removal of the C1q-bound material in one of two ways. Activated C3 fragments can promote further complement activation that terminates in the formation of the membrane attack complex, a transmembrane channel that promotes cell lysis. Alternatively, C3 fragments can act as opsonins, binding to the cell or debris surface and stimulating phagocytosis of the material upon interaction with macrophage C3 receptors (CR3). In this latter pathway, C1q deposition leads to phagocytosis of the material on which it is deposited, hence can be thought of as an 'eat-me' signal. Microglia, the tissue-resident, CR3-expressing macrophages of the brain, have been implicated in pruning synapses in a complement-dependent manner (Stevens *et al.* 2007; Schafer *et al.* 2012; Scott-Hewitt *et al.* 2020).

3.1.1 Microglia, complement, and TREM2

Microglia are a major source of C1q in the mouse brain (Fonseca *et al.* 2017). In the developing murine dorsal lateral geniculate nucleus (dLGN), C1q and C3 are upregulated and colocalise with synaptic proteins during periods of synaptic pruning, and genetic deletion of *C1q*, *C3*, or *CR3* attenuates synapse loss (Stevens *et al.* 2007; Schafer *et al.* 2012). It is

important to note that the colocalisation of two proteins does not necessarily mean one is binding to the other. Nonetheless, coupled with the known role of C1q as an 'eat-me' signal that binds unwanted material, these findings suggests that C1q may 'tag' synapses for elimination via the classical complement cascade. Furthermore, as *CR3* deletion attenuated synapse loss and microglia are the major CR3-expressing macrophages of the brain parenchyma, these findings suggest microglia may play an important role in synaptic pruning. Indeed, numerous studies implicate microglia in mediating developmental pruning by engulfing synaptic material (Paolicelli *et al.* 2011; Schafer *et al.* 2012; Filipello *et al.* 2018; Li *et al.* 2020; Scott-Hewitt *et al.* 2020). The standard approach used by these studies to measure *in vivo* synaptic engulfment is fluorescence microscopy to identify fluorescently labelled synaptic proteins within, or 'engulfed' by, fluorescently labelled microglia. However, an important consideration when interpreting such studies is what a synaptic protein 'engulfed' by a microglial cell actually represents. Has the microglial cell internalised synaptic material, or is the material just tightly enwrapped by microglial processes (Weinhard *et al.* 2018)? If the synaptic protein is inside the microglia, how did it get there? Do microglia phagocytose whole synapses, or gently 'nibble' them by trogocytosis (Weinhard *et al.* 2018)? Do they remove functional synapses from live neurons, or simply clear up synaptic debris? No study has clearly elucidated exactly how microglia are involved in synapse loss, hence these questions will be important to answer in future work.

Triggering receptor expressed on myeloid cells 2 (TREM2) is another myeloid receptor that appears to mediate developmental synaptic pruning (Filipello *et al.* 2018). Relative to wild-type (WT) mice, postnatal day (P) 18-20 *Trem2* knock-out (KO) mice exhibit an increase in the density of pre- and postsynaptic proteins in the hippocampus, and microglia from this

region engulf significantly less postsynaptic protein. This suggests that loss of TREM2 impairs microglia-mediated synaptic pruning. As this was a global knockout mouse model, these experiments cannot reveal whether the phenotype was due to loss of TREM2 from brain-resident microglia or from peripherally recruited myeloid cells. However, *in vitro*, *Trem2* KO primary microglia engulf significantly fewer apoptotic membranes than WT microglia. Furthermore, co-culturing WT primary hippocampal neurons with *Trem2* KO primary microglia does not lead to synapse loss, while culturing these neurons with WT microglia does. Coupled with the fact that TREM2 is primarily expressed by microglia in the brain (Saunders *et al.* 2018), these data suggest that it is microglial TREM2 that is required for normal developmental synapse loss, and that microglial TREM2 may facilitate the microglial engulfment of synapses.

3.1.2 Phosphatidylserine and CD47

Microglia-mediated synapse loss in development is highly regulated and spatially restricted, whereby synapses are lost from specific brain regions during restricted temporal windows (Stevens *et al.* 2007; Paolicelli *et al.* 2011; Schafer *et al.* 2012). However, what causes specific synapses to be eliminated while others are spared is not known. One mechanism employed by the immune system to eliminate specific cells is the recognition by macrophages of 'eat-me' and 'don't eat-me' signals expressed on the cell's surface. For example, apoptosis, a form of programmed cell death, has an essential role in triggering the removal of damaged or dying cells by the immune system (Lemke 2019). Cell-surface 'eat-me' signals, such as phosphatidylserine (PS; Nagata *et al.* 2016), promote phagocytosis of the apoptotic cell, while cell-surface 'don't eat-me' signals, for instance CD47 (Barclay and Van den Berg 2014), inhibit phagocytosis. PS is a membrane phospholipid which can be

reorganised within the plasma membrane. In healthy cells, almost all PS is localised to the inner, cytoplasm-facing leaflet. In apoptotic cells, PS is translocated to the outer-leaflet, and this externalised PS (ePS) can then be recognised by macrophage receptors to promote phagocytosis (Lemke 2019). Interestingly, PS is a ligand for C1q (Païdassi *et al.* 2008) and TREM2 (Wang *et al.* 2015; Shirotani *et al.* 2019), and is externalised on C1q-bound synaptosomes of WT mice (Györffy *et al.* 2018). As mentioned above, C1q and TREM2 are required for normal developmental synaptic pruning, but what they might bind to on a synapse is not known. An intriguing possibility is that, similar to its role on apoptotic cells, ePS acts as an 'eat-me' signal targeting specific synapses for elimination, perhaps being recognised by C1q or TREM2.

Recent work supports a role for synaptic ePS in synapse loss. In mice, PS is externalised on synapses *in vivo* and colocalises with C1q during periods of synaptic pruning in the retinogeniculate system (Scott-Hewitt *et al.* 2020). Furthermore, neuronal terminals with ePS were found engulfed within lysosomal compartments of microglia, and the extent of engulfment increased during periods of synaptic pruning. Genetic deletion of *C1q* attenuated the microglial engulfment of ePS+ material and increased the proportion of synapses externalising PS *in vivo*, while the addition of a PS-binding protein to conceal ePS prevented microglia-mediated synapse loss *in vitro*. It is unclear whether C1q and PS simply colocalise on the same synapse, or whether C1q directly binds to ePS on the synapse. Nonetheless, these findings implicate synaptic ePS in C1q-dependent synapse loss. A similar study confirmed a number of these findings and identified G protein-coupled receptor 56 (GPR56) as an additional PS receptor (Li *et al.* 2020). Furthermore, they found that microglia-specific deletion of *GPR56* attenuated developmental synapse loss and the

microglial engulfment of ePS+ neuronal material. While future work should aim to block synaptic PS externalisation and confirm the attenuation of synapse loss *in vivo*, these findings suggest synaptic ePS may act as an 'eat-me' signal during microglia-mediated synaptic pruning. Microglia express a number of other receptors that can recognise ePS (Lemke 2019), raising the question of whether these receptors also play a role in synapse loss.

CD47 is a cell-surface 'don't eat-me' signal known to protect viable cells from phagocytosis (Barclay and Van den Berg 2014). In the developing murine retinogeniculate system, less active synapses are preferentially eliminated (Schafer *et al.* 2012). CD47, expressed by neurons of the developing dLGN, preferentially colocalises with active synapses and the level of CD47 increases during periods of synaptic pruning (Lehrman *et al.* 2018). Furthermore, genetic deletion of *Cd47* leads to a decrease in the number of synapses, an increase in microglial engulfment of neuronal material, and abolishes the preferential elimination of less active synapses. These findings suggest that loss of CD47 leads to excessive microglia-mediated synaptic pruning. SIRPα is the phagocytic receptor for CD47 and is highly expressed by microglia during periods of synaptic pruning (Lehrman *et al.* 2018). Genetic deletion of *Sirpα* phenocopies that of *Cd47*, leading to a decrease in the number of synapses and an increase in the microglial engulfment of neuronal material. Additionally, *in vitro*, when fed synaptosomes from WT mice and *Cd47* knock-out (*Cd47* KO) mice, WT primary microglia preferentially engulfed *Cd47* KO synaptosomes. Therefore, in direct contrast to PS, CD47 appears to function as a 'don't eat-me' signal that protects synapses from microglial engulfment.

3.1.3 Summary

The recent discovery of these synaptic 'eat-me' and 'don't eat-me' signals is beginning to illuminate how specific synapses might be targeted for elimination; it may be the ratio of 'eat-me' to 'don't eat-me' signals, as well as the relative levels of their receptors, that will determine whether or not a synapse is eliminated. As PS and CD47 govern phagocytosis of disparate cells of the body (Lemke 2019), it seems plausible that they might also be conserved mediators of synapse elimination from development to adulthood, and from health to disease.

It is important to note that mechanisms not expanded on here – such as a potential role for the neuronally-secreted chemokine fractalkine as a chemotactic 'find-me' signal that recruits microglia to synapses (Paolicelli *et al.* 2011; Zhan *et al.* 2014), and a role for astrocytes in directly engulfing synaptic material (Chung *et al.* 2013; Chung *et al.* 2015; Chung *et al.* 2016; Jay *et al.* 2019) and promoting the microglial engulfment of synapses (Vainchtein *et al.* 2018) – have also been implicated in mediating synaptic pruning. The limited scope of this thesis prevents an in-depth discussion of these findings; nonetheless, an intriguing question is whether these represent redundant mechanisms for eliminating synapses, or whether different mechanisms drive synapse loss in different contexts, at different ages, and across different brain regions. As C1q has been implicated in mediating synapse loss in multiple animal models (Stevens *et al.* 2007; Hong *et al.* 2016; Lui *et al.* 2016; Dejanovic *et al.* 2018; Figueiredo *et al.* 2019; Vukojicic *et al.* 2019; Scott-Hewitt *et al.* 2020), I focus on C1q-dependent mechanisms of synapse loss in this thesis.

3.2 Pathological synapse loss

While developmental synaptic pruning is necessary, aberrant pruning may underlie cognitive and behavioural defects in a range of pathological conditions, including viral infection, schizophrenia, autism, spinal muscular atrophy, frontotemporal dementia, and AD (Hong *et al.* 2016; Lui *et al.* 2016; Sekar *et al.* 2016; Vasek *et al.* 2016; Filipello *et al.* 2018; Figueiredo *et al.* 2019; Sellgren *et al.* 2019; Vukojicic *et al.* 2019). The ubiquity of pathological synapse loss across a spectrum of central nervous system (CNS) diseases indicates that it may be a common hallmark of neurological defects, demonstrating the importance of elucidating the mechanisms that drive it. AD is the most common form of dementia, with early synapse loss being a well-studied phenomenon that correlates strongly with cognitive decline (Davies *et al.* 1987; Dekosky and Scheff 1990; Terry *et al.* 1991; Scheff *et al.* 2006; Scheff *et al.* 2007; Scheff *et al.* 2011). The stark and early synapse loss observed in AD makes it an ideal model for investigating pathological synapse loss. As with developmental pruning, a number of important questions arise: What drives pathological synapse loss? Are specific synapses targeted for elimination? Is pathological synapse loss aberrant reactivation of a developmental program? These questions deserve particular attention, as their answers may drive the discovery of life-changing therapeutics. Indeed, current AD therapies targeting the end stage of the disease have consistently failed clinical trials (Yiannopoulou *et al.* 2019). Targeting early pathological phenomena such as synapse loss, occurring *before* irreversible neuronal death, may prove more successful.

3.2.1 Phosphatidylserine externalisation and pathological synapse loss

Synapse loss is one of the earliest pathological events in AD, preceding overt neuronal death (Selkoe 2002). This synapse loss is region-specific, with the hippocampus displaying a selective vulnerability to synapse loss in both human (Davies *et al.* 1987; Scheff *et al.* 2006; Scheff *et al.* 2007; Scheff *et al.* 2011) and murine (Hong *et al.* 2016) forms of the disease. AD is also characterised by the accumulation of two key protein pathologies: intracellular neurofibrillary tangles, consisting of hyperphosphorylated tau; and extracellular amyloid-β (Aβ) plaques, consisting primarily of the misfolded Aβ peptide – a peptide fragment produced from the cleavage of the amyloid precursor protein (APP; Ittner and Götz 2011). The Aβ peptide has hydrophobic residues that facilitate its aggregation into multimers (Jarrett *et al.* 1993; Pike *et al.* 1993; Snyder *et al.* 1994). The soluble, oligomeric form of Aβ (oAβ) accumulates early, before the formation of insoluble plaques, and is thought to be the primary synaptotoxic species (Lambert *et al.* 1998; Walsh *et al.* 2002; Wang *et al.* 2002; Lacor *et al.* 2004; Shankar *et al.* 2008; Koffie *et al.* 2009; Zempel *et al.* 2010; Jin *et al.* 2011; Li *et al.* 2011; Selkoe and Hardy 2016). Indeed, intracerebroventricular injection of oAβ leads to hippocampal synapse loss in WT rats (Freir *et al.* 2011) and mice (Hong *et al.* 2016).

As with development, synapse loss in mouse models of AD appears to be microglia- and complement-dependent. In mouse models of Aβ pathology, C1q is upregulated in brain regions vulnerable to synapse loss, there is an increase in the colocalisation of C1q and C3 with postsynaptic proteins, and genetic deletion of *C1qa*, *C3* or *CR3* prevents synapse loss. Furthermore, microglia engulf synaptic proteins in a CR3-dependent manner (Hong *et al.* 2016). In mouse models of tau pathology, C1q colocalises with postsynaptic proteins and injecting C1q-blocking antibodies into the hippocampus of these mice attenuates the loss

and microglial engulfment of pre- and postsynaptic proteins (Dejanovic *et al.* 2018). C1q, therefore, may bind to synapses vulnerable to loss and is required for their elimination. However, what C1q might bind to on an AD synapse is not known. Interestingly, relative to WT mice, the extent of PS externalisation is increased on hippocampal synaptosomes from 3-month-old AD mice, an age at which these mice exhibit an increase in synapse loss (D'Amelio *et al.* 2011). It is important to confirm whether or not ePS is increased on synapses of AD brains *in vivo*; however, ePS is a ligand for C1q (Païdassi *et al.* 2008), colocalises with C1q on synapses (Györffy *et al.* 2018; Scott-Hewitt *et al.* 2020), and appears to regulate C1q-dependent synapse loss in development (Scott-Hewitt *et al.* 2020). I propose that PS will also mediate C1q-dependent synapse loss in AD, whereby ePS+ synapses will be specifically targeted for elimination.

It is important to note that most studies discussed thus far utilised rodent models to investigate mechanisms of synapse loss, and these models do not faithfully represent the human condition. For example, most rodent models exhibit *either* Aβ *or* tau pathology, hence will not recapitulate any synergistic or interacting effects of these proteins that may occur in the human brain. Additionally, many models overexpress transgenes at non-physiological levels (Hall and Roberson 2012). For example, many Aβ models overexpress human APP and APP processing transgenes, leading to a pattern of Aβ accumulation that does not reflect the human condition. Furthermore, the APP protein itself has important physiological roles and can be cleaved into a number of different peptide fragments besides Aβ (van der Kant and Goldstein 2015). Altering the levels of these proteins due to transgene overexpression may disrupt neurophysiology in ways that do not represent human AD. Therefore, it is not clear how relevant the findings from studies using rodents are to the

human condition. However, human genetic data implicates common variation in complement and microglial genes as risk factors for AD (Jansen *et al.* 2019; Kunkle *et al.* 2019). Additionally, in human AD brains, mRNA expression of complement pathway genes is upregulated relative to healthy control (HC) brains, and the level of C1q protein is increased in postsynaptic densities (PSD) isolated from AD brains (Dejanovic *et al.* 2018). It seems plausible that microglia might also mediate complement-dependent synapse loss in the human AD brain.

3.2.2 Caspase-3 activation and phosphatidylserine externalisation

As mentioned above, PS is an 'eat-me' signal that promotes the phagocytosis of apoptotic cells when it becomes externalised on the surface of these cells (Lemke 2019). I have proposed that, as appears to be the case in the developing brain, the externalisation of PS on AD synapses will lead to the elimination of these specific synapses. If this hypothesis is correct, to answer the question 'What makes synapses vulnerable to loss?' it is important to identify what events lead to the externalisation of PS on AD synapses. On apoptotic cells, the location of PS is dependent on the relative activity of flippases and scramblases – enzymes that translocate PS between the two leaflets of the plasma membrane (Nagata *et al.* 2016). Flippase activity promotes PS internalisation, while scramblase activity promotes PS externalisation. In healthy cells, flippases are active and this ensures that almost all PS is internalised to the inner-leaflet. On apoptotic cells, the protease caspase-3 is cleaved to its active form, and this active caspase-3 can cleave flippases and scramblases (Jun Suzuki *et al.* 2013; Segawa *et al.* 2014; Suzuki *et al.* 2014). This irreversibly activates scramblases and inactivates flippases, driving an increase in the number of PS molecules externalised to the outer-leaflet. Interestingly, apoptotic cascades involving caspase-3 activation can occur

locally in synapses and dendrites (Mattson *et al.* 1998a) and incubation of synaptosomes or dendrites with the Aβ peptide can activate these cascades (Mattson *et al.* 1998b). Indeed, the level of cleaved caspase-3 is increased in postsynaptic densities from post-mortem human AD brains (Louneva *et al.* 2008). Furthermore, the increase in ePS on hippocampal synaptosomes from AD mice occurs alongside an increase in cleaved caspase-3 (D'Amelio *et al.* 2011). Pathological caspase-3 activation due to focal apoptotic cascades in AD synapses could be one event leading to PS externalisation. Apoptosis also triggers the redistribution of cell-surface CD47, such that its ability to act as a 'don't eat-me' signal to macrophages is impaired (Gardai *et al.* 2005; Lv *et al.* 2015). Focal activation of apoptotic cascades in AD synapses could drive a spatially-restricted redistribution of both 'eat-me' and 'don't eat-me' signals, such as the externalisation of PS and removal of CD47, promoting engulfment of these synapses by microglia.

It is worth noting that caspase-3 activation has been associated with dendritic spine loss in different contexts, providing further support for a role of increased caspase-3 activation in pathological synapse loss. For example, in the dendrites of cultured mouse neurons, local activation of caspase-3 as a result of focal mitochondrial damage leads to spine loss, while pharmacological inhibition of caspase-3 prevents this spine loss (Ertürk *et al.* 2014). Additionally, caspase-3 knock-out mice exhibit increased spine density on hippocampal neurons, and this is observed at both 7 weeks and 3 months of age (Ertürk *et al.* 2014). In an AD context, the level of caspase-3 activity is positively correlated with the extent of spine loss in organotypic slice cultures containing Aβ-producing neurons (Park *et al.* 2020). Furthermore, there is an increase in caspase-3 activity in the dendritic spines of hippocampal neurons at the onset of synapse loss and memory decline in AD mice

(D'Amelio *et al.* 2011). While mostly correlational, these findings support a role for caspase-3 activation in the loss of dendritic spines.

3.2.3 Mitochondrial dysfunction as a common trigger of phosphatidylserine externalisation: caspase-3 activation, ATP depletion, oxidative stress, and Ca^{2+} dyshomeostasis

The apoptosome is a caspase-activating enzyme and crucial mediator of the intrinsic, mitochondrial pathway of apoptosis (Riedl and Salvesen 2007). In this pathway, when mitochondria become 'overloaded' by some insult, such as an excessive increase in mitochondrial Ca^{2+} or reactive oxygen species (ROS), the mitochondrial permeability transition is stimulated (Bernardi *et al.* 2015). The mitochondrial permeability transition leads to the release of cytochrome c through mitochondrial permeability transition pores, and cytosolic cytochrome c then complexes with apoptotic protease-activating factor 1 (APAF1) to form the apoptosome (Riedl and Salvesen 2007). Interestingly, the increase in cleaved caspase-3 levels in hippocampal synaptosomes from AD mice is apoptosome-dependent, whereby cleaved caspase-3 was not detected in synaptosomes from AD mice lacking the *Apaf1* gene (D'Amelio *et al.* 2011). This implicates mitochondrial dysfunction in the activation of caspase-3 in AD synapses. Furthermore, the proteomic signature of a C1q-bound synaptosome relative to a non-C1q-bound synaptosome from AD mice is characterised by the altered expression of primarily mitochondrial proteins (Györffy *et al.* 2020), suggesting C1q may preferentially bind synapses with dysfunctional mitochondria. Mitochondria become dysfunctional in the early stages of AD (Chakravorty *et al.* 2019) and Aβ, the protein that forms synaptotoxic oligomers in early AD, accumulates within synaptic mitochondria of AD mice (Du *et al.* 2008; Hansson Petersen *et al.* 2008; Du *et al.* 2010). As

outlined below, mitochondrial dysfunction may be a common event upstream of synaptic PS externalisation by triggering events that modulate the activity of flippases and scramblases, such as an increase in caspase-3 activity, Ca^{2+} levels, ROS, or a drop in ATP levels.

The primary role of synaptic mitochondria is to supply the synapse with ATP. However, mitochondrial ATP synthase activity is impaired in human and mouse AD brains (Terni *et al.* 2010; Cha *et al.* 2015; Beck *et al.* 2016), particularly in synaptic mitochondria (Beck *et al.* 2016). Congruently, when comparing their ability to synthesise ATP *in vitro*, synaptic mitochondria from pre-plaque AD mice synthesise significantly less ATP than those from WT mice (Beck *et al.* 2016). Flippase activity is driven by the hydrolysis of ATP (Zachowski *et al.* 1989; Tang *et al.* 1996; Pomorski *et al.* 2002; Yabas *et al.* 2011; Segawa *et al.* 2016), hence a fall in ATP levels could inactivate flippases and trigger PS externalisation. Indeed, in dorsal root ganglion (DRG) explant cultures from WT mice, impairing mitochondrial ATP production by inhibiting the mitochondrial ATP synthase leads to PS externalisation on axons, while supplementing degenerating axons with NAD^+ prevents a drop in ATP levels and significantly attenuates PS externalisation (Shacham-Silverberg *et al.* 2018). AD synapses with dysfunctional mitochondria will become starved of ATP and it seems plausible that, as is the case with neurons, PS will become externalised on these ATP-depleted synapses.

Mitochondria are an important source and target of ROS (Zorov *et al.* 2014). Importantly, synaptic mitochondria from AD mice exhibit signs of increased oxidative stress relative to those from WT mice, as measured by increased levels of 4-hydroxynonenal and hydrogen peroxide (Du *et al.* 2010). Furthermore, there is an increase in ROS levels in synaptosomes from pre-plaque AD mice relative to WT mice (Ahmad *et al.* 2017). An increase in ROS

production can activate scramblases (Schreiber *et al.* 2018), hence oxidative stress may drive PS externalisation. Indeed, recent work on AD mice exhibiting tau pathology is in line with this proposal. Incubating cultured DRG neurons from these mice with antioxidants reduces the extent of neuronal PS externalisation, while addition of the pro-oxidant arsenite induces PS externalisation (Brelstaff *et al.* 2018). Extrapolating from these findings in neurons, it seems plausible that PS would also become externalised on oxidatively stressed AD synapses.

Mitochondria are important buffers of Ca^{2+} following synaptic activity (Billups and Forsythe 2002; Pivovarova *et al.* 2002), but the ability of mitochondria to buffer Ca^{2+} is impaired in hippocampal neurons from AD mice (Lee *et al.* 2012). Furthermore, the frequency of Ca^{2+} transients is aberrantly increased in hippocampal neurons of pre-plaque AD mice (Busche *et al.* 2012) and in WT mice after application of oAβ to the brain (Busche *et al.* 2012; Arbel-Ornath *et al.* 2017; Zott *et al.* 2019). Flippases can be reversibly inhibited by increased levels of intracellular Ca^{2+} (Segawa *et al.* 2016) while scramblases can be reversibly activated (Suzuki *et al.* 2010; J. Suzuki *et al.* 2013). Furthermore, transmembrane protein 16C (TMEM16C), one of the Ca^{2+}-activated scramblases identified by Suzuki *et al* (2013), is highly expressed in hippocampal neurons (Saunders *et al.* 2018). Therefore, the increase in Ca^{2+} transients in hippocampal synapses could activate scramblases, driving the externalisation of PS.

3.2.5 Summary

Taken together, these findings show that a number of factors that can lead to PS externalisation – an increase in caspase-3 activity, Ca^{2+} levels, ROS, or a drop in ATP levels – may all be features of AD synapses. These changes could synergise to drive the increase in ePS identified on synaptosomes from AD mice. As synaptic PS externalisation appears to act as an 'eat-me' signal in microglia- and C1q-dependent synapse loss in development, I hypothesise that the increase in PS externalisation promotes microglia- and C1q-dependent synapse loss in AD. Furthermore, the cell-surface distribution of PS and CD47 is regulated by the internal state of the cell or synapse on which they are expressed – CD47 preferentially localises with more active synapses and is redistributed on apoptotic cells, while PS is externalised on oxidatively stressed, ATP-depleted, and apoptotic neurons. Therefore, an increase in synaptic ePS or a decrease in CD47 may act as a 'damage' or 'dysfunction' signal when recognised by microglial receptors, leading to the elimination of these specific synapses. In this context, synapse loss could be seen as a mechanism for specifically removing dysfunctional synapses, dependent on the microglial integration of synaptic cell-surface signals. Compiling the evidence discussed above, I have outlined a possible synapse loss pathway in Figure 1, whereby mitochondrial dysfunction is a core event making synapses vulnerable to loss. To further investigate what makes synapses vulnerable to loss and to address some of the unanswered questions, I analysed the proteomic signature of synapses vulnerable to loss.

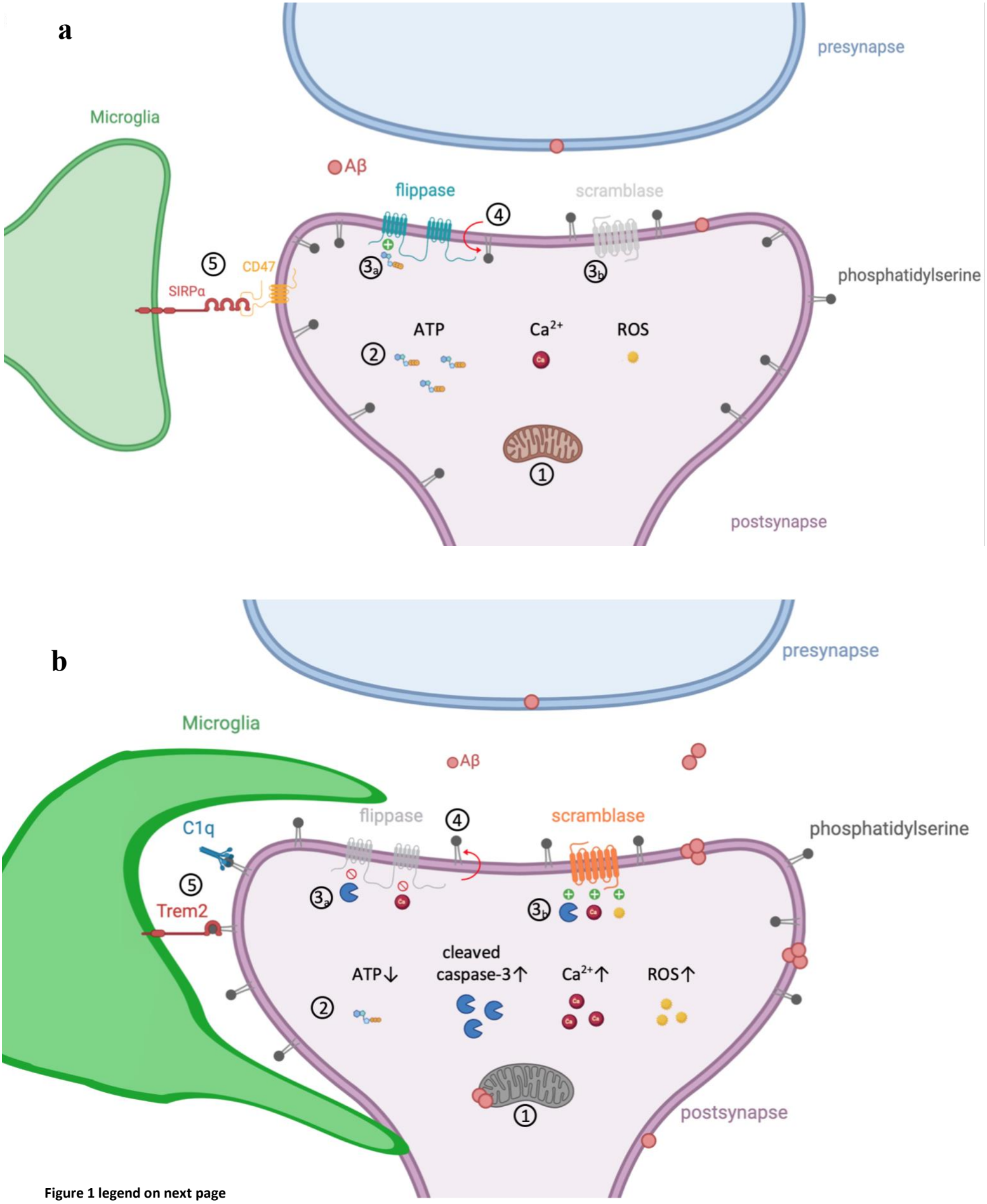

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Figure 1. Proposed mechanism targeting 'dysfunctional' AD synapses for elimination

a, A non-diseased synapse is shown. **(1)** Synaptic mitochondria are healthy and functional. **(2)** Synapses containing healthy mitochondria have an abundant supply of ATP and the levels of Ca^{2+} and ROS are maintained within a healthy range. **(3a)** The abundant supply of ATP ensures that flippases activity is high. **(3b)** Low levels of Ca^{2+}, ROS and cleaved caspase-3 ensure that scramblase activity is low. **(4)** Active flippases translocate PS from the outer- to the inner-leaflet of the plasma membrane which, coupled with the low scramblase activity, ensures that most PS molecules are internalised. **(5)** Healthy synapses may express CD47 on their cell-surface which acts as a 'don't eat-me' signal to microglia when detected by the SIRPα receptor. Coupled with the fact that microglia do not recognise synaptic ePS, these synapses are not engulfed. **b,** A diseased synapse is shown. **(1)** Synaptic mitochondria are dysfunctional in AD synapses. **(2)** The ability of mitochondria from AD synapses to produce ATP is impaired, leading to a decrease in synaptic ATP. There is an apoptosome-dependent increase in cleaved caspase-3 in AD synapses, occurring at the onset of memory decline and synapse loss. The ability of mitochondria from AD synapses to buffer Ca^{2+} is impaired and the frequency of Ca^{2+} transients is aberrantly increased – these will lead to increased levels of synaptic Ca^{2+}. Synaptic mitochondria from AD brains exhibit signs of increased oxidative stress, and there is an increase in ROS levels in synaptosomes from AD mice. **(3a)** Flippases are ATP-dependent, hence a fall in ATP levels may inactivate flippases. Furthermore, cleaved caspase-3 can permanently inactivate flippases, while Ca^{2+} can transiently inactivate flippases. These events may all synergise to impair flippase activity. **(3b)** Cleaved caspase-3, Ca^{2+}, and ROS have all been shown to activate scramblases, hence the increase in these factors in AD synapses may synergise to increase scramblase activity. **(4)** Active scramblases translocate PS from the inner- to the outer-leaflet of the plasma membrane which, coupled with the low flippase activity, may lead to the increase in ePS observed on synaptosomes from AD mice. **(5)** The synaptic ePS may be recognised by microglial receptors (such as TREM2) and/or be bound by C1q. Synapses undergoing synaptic apoptosis may no longer have functional CD47 molecules expressed on their cell-surface. An increase in 'eat-me' signals (ePS) coupled with a decrease in 'don't eat-me' signals (CD47) may drive microglia to engulf these specific synapses. In other words, synapses containing dysfunctional mitochondria may express the cell-surface signature of an 'edible' synapse.

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