THE ROLE OF VACUOLE MEMBRANE PROTEIN-1 IN NEURAL CALCIUM HOMEOSTASIS AND SYNAPTIC PLASTICITY

WANG MENGMENG

UNIVERSITI SAINS MALAYSIA

2024

THE ROLE OF VACUOLE MEMBRANE PROTEIN-1 IN NEURAL CALCIUM HOMEOSTASIS AND SYNAPTIC PLASTICITY

by

WANG MENGMENG

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

August 2024

ACKNOWLEDGEMENT

Everything happens for a reason! Initially, pursuing a Ph.D. degree was not my academic ambition. But it is me who chose this challenging path, knowing that it would not be easy, yet underestimating the true extent of its difficulty. Looking back, I realize it wasn't an unachievable task for anyone; rather, it required a commitment to sacrifice time and focus, coupled with qualities like patience, optimism, objectivity, and, most importantly, critical thinking. All of these are the achievements I've nurtured and reaped over these five years, with the papers merely a readout of them.

My deepest gratitude goes to Dr. Tan Jun Jie, a true mentor who guided me through my academic journey hand in hand, imparting techniques, and academic skills, and shaping my work ethic; Prof. Lu Cheng Biao, who is exceptionally kind, knowledgeable, and supportive of my academic endeavors. Dr. Michael Ling provided important advice at a critical phase of my research.

Several lecturers contributed significantly to my progress. Dr. Li Song Ji helped me establish the stereotaxic microinjection protocol. Dr. Yin Ya Ling and her master's student, Yue Rui Zhu, helped me troubleshoot calcium imaging and primary neuron culture. Dr. Li Han generously supplied SH-SY5Y cells. Prof. Han Fa Bin provided fibroblast-derived hiPSCs. During a challenging period in my in vivo work, Dr. Wang Ya Li provided crucial encouragement and support, perhaps unbeknownst to her. Special appreciation goes to international Ph.D. students and lecturers Saliah and Samar, who generously assisted me and offered guidance on navigating the challenges of a Ph.D. in my first semester as a newcomer.

I extend my gratitude to my colleagues, particularly the brilliant and kind-hearted Fatin Fazi Rina and Yu Yue Xin, who are a source of warmth for me in this quiet

village. Special thanks to Li Jun Mei and Yu Bin, who assisted me with behavior test work and offered support in various aspects. Hu Qin Lin and Ye Zhen Li played a crucial role in the preliminary experiment. Additionally, I am thankful for the support from undergraduate students, including Ren Xin Yin, who helped with Western blot work in vitro, and Han Yu Yuan, who provided valuable assistance with various animal-related tasks.

Beyond the lab, this Ph.D. journey wouldn't have been possible without the financial support of my family over these five years. Lastly, Qin Ying Hao, whose presence and company relieved some spiritual pressure on me.

And thanks to the unnominated supporters whose unconscious contribution helped this work go well!

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

±	plus-minus (used to indicate a range or uncertainty)
-	minus
>	more than
<	less than
°C	degrees celsius
%	percent
cm^2	square centimeter
α	alpha
g	gram
β	beta

LIST OF ABBREVIATIONS

α-syn	alpha-synuclein
Aβ	amyloid-β
AAV	adeno-associated virus
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's diseases
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
α-syn	alpha-synuclein
ATF6α	activating transcription factor 6α
ATG	autophagy-related gene
BARHL1	barH like homeobox 1
BCA	bicinchoninic acid assay
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenic protein
BSA	bovine serum albumin
Ca^{2+}	calcium
CaM	calcium-bound calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CA	cornu ammonis
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CP-AMPARs	calcium permeableα-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors
CRAC	calcium-release-activated calcium
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium

EN1	engrailed homeobox 1
EGFP	enhanced green fluorescent protein
EPSP	excitatory postsynaptic potential
FACS	fluorescence-activated cell sorting
FAD	familial Alzheimer's disease
FBS	fetal bovine serum
GDNF	glial cell line-derived neurotrophic factor
GRP78	Glucose-regulated protein
GSK3β	glycogen synthase kinase-3 beta
HFS	high-frequency stimulation
HBSS	Hank’s buffered salt solution
hiPSCs	human induced pluripotent stem cells
IRE1α	inositol-requiring enzyme-1α
ITPRs	inositol 1,4,5-trisphosphate receptors
LTP	long-term potentiation
LTD	long-term depression
MCU	mitochondrial calcium uniporter
Mg^{2+}	magnesium
MOI	multiplicity of infection
mGluRs	metabotropic glutamate receptors
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA	N-methyl-D-aspartate
OFT	open field test
p-CaMKII	phosphorylated calcium/calmodulin-dependent protein kinase II
ORAI1	calcium release-activated calcium channel protein 1
PD	Parkinson’s Disease
PERK	protein kinase R-like endoplasmic reticulum kinase

PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLN	phospholamban
PSD	postsynaptic density
ROCK	Rho-associated protein kinase
PSD-95	postsynaptic density protein 95
RA	retinoic acid
RyRs	ryanodine receptors
SAM	sterile α-motif
SDS	Sodium dodecyl sulphate
SERCA	sarco/endoplasmic reticulum calcium ATPase
SHH	sonic hedgehog
shRNA	short hairpin RNA
SLN	sarcolipin
SOCE	store-operated calcium entry
SR	sarcoplasmic reticulum
STIM	stromal interaction molecules
TGFβ	transforming growth factor-beta
TH	tyrosine hydroxylase-positive
TG	thapsigargin
TRPC1	transient receptor potential canonical 1
UPR	unfolded protein response
VMP1	vacuole membrane protein 1
VGCC	voltage-gated calcium channels
WB	Western blot
Kd	dissociation constant

kDa	kilodalton
L	liter
mA	milliampere
MΩ	megaohm (unit of electrical resistance)
mg	milligram
mM	millimolar
ml	milliliter
mm	millimeter
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer
nM	nanomolar
nm	nanometer
V	voltage

LIST OF APPENDICES

PERANAN PROTEIN MEMBRAN VAKUOL-1 DALAM HOMEOSTASIS KALSIUM NEURONAL DAN PLASTISITI SINAPTIK

ABSTRAK

Gangguan dinamika kalsium intraselular telah diketahui menjejaskan keplastikan sinaptik dan fungsi kognitif. Sehingga kini, kesan protein membran vakuola 1 (VMP1) pada homeostasis kalsium intraselular neuron, keplastikan sinaptik, dan fungsi kognitif masih belum diterokai. Kajian ini bertujuan untuk mengkaji kesan VMP1 terhadap peraturan kalsium neuron secara in vitro dan in vivo, serta hubungannya dengan keplastikan sinaptik dan fungsi kognitif dalam vivo. Ekspresi VMP1 telah diturunkan dalam sel neuroblastoma SH-SY5Y dan kesan kemasukan kalsium yang dikendalikan oleh simpanan (SOCE) serta saluran kalsium dinilai menggunakan analisis Western blot. Fungsi in vivo VMP1 dalam hippocampus dicapai pada tikus C57/BL6 menggunakan virus yang berkaitan dengan adeno. Kesan ekspresi berlebihan dan downregulation VMP1 pada saluran kalsium, fungsi kognitif haiwan, potensi jangka panjang (LTP), neuron dan ketumpatan dendritik, serta protein yang berkaitan dengan sinaptik telah dinilai. Kajian in vitro menunjukkan bahawa ketukan VMP1 mengurangkan SOCE ($p<0.0001$), protein saluran potensi reseptor sementara kanonik (TRPC1), dan protein saluran kalsium diaktifkan kalsium 1 (Orai1) dalam sel neuroblastoma SH-SY5Y ($n=4$, $P<0.01$). Penurunan ekspresi VMP1 dalam hippocampus tikus menyebabkan penurunan ungkapan TRPC1 ($n=4$, $P<0.01$). Sebaliknya, overexpression VMP1 menyebabkan peningkatan ungkapan TRPC1 ($n=4$, $P<0.01$) dan penurunan tahap STIM2 ($n=3$, $P<0.05$). Disfungsi kognitif yang ketara juga diperhatikan pada tikus dengan overexpression VMP1 dalam hippocampus, yang ditambah pula dengan pengurangan ketara dalam LTP, jumlah neuron, dan ketumpatan

dendritik, serta penurunan protein kalsium/calmodulin kinase II (p-CaMKII) ($n=3$, $p<0.05$) dan subunit GluR2 reseptor AMPA ($n=3$, $p<0.01$). Sebaliknya, ketukan VMP1 menyebabkan peningkatan p-CaMKII ($n=3$, $p<0.05$) dan GluR2 ($n=3$, $p<0.01$), tetapi tiada perubahan ketara dalam fungsi kognitif, LTP, jumlah neuron, dan ketumpatan dendritik. Untuk mewujudkan neuron manusia yang sihat untuk kajian mekanistik lanjut mengenai VMP1, sel stem pluripoten teraruh manusia (hiPSCs) yang diprogram semula dari darah tali pusat telah digunakan. Dengan pengoptimuman perencatan glycogen synthase kinase 3β dan isyarat sonic hedgehog, 99.05% ± 0.22% βIII-tubulin dan tyrosine hydroxylase menyatakan neuron dopaminergik dihasilkan ($n=4$). Protokol pembezaan yang dioptimumkan ini diulangi menggunakan hiPSCs yang berasal dari fibroblast, dan transduksi yang berjaya menggunakan lentivirus terbukti daripada progenitor hiPSCs-dopaminergik yang telah dibekukan ($n=1$). Kesimpulannya, kajian ini mendapati bahawa VMP1 adalah penting untuk homeostasis kalsium neuron. Penggandaan VMP1 dalam hippocampus tikus menyebabkan fungsi kognitif terjejas, pengurangan potensi jangka panjang (LTP), dan kehilangan neuron serta duri neuron. Penyelidikan lanjut adalah penting untuk meneroka peranan patologi VMP1 dalam penyakit neurodegeneratif. Selain itu, kami telah menghasilkan dan menubuhkan stor neuron yang berasal dari hiPSCs untuk kajian lanjut. Menggunakan neuron hiPSCs yang sihat dan yang berasal dari pesakit untuk memodelkan penyakit neurodegeneratif akan memperluas pemahaman kita tentang mekanisme molekul VMP1 dan memudahkan strategi perubatan peribadi.

THE ROLE OF VACUOLE MEMBRANE PROTEIN-1 IN NEURAL CALCIUM HOMEOSTASIS AND SYNAPTIC PLASTICITY

ABSTRACT

Disruption of the intracellular calcium (Ca^{2+}) dynamics is previously known to affect synaptic plasticity and cognitive function. To date, the effects of vacuole membrane protein 1 (VMP1) on neuronal intracellular calcium homeostasis, synaptic plasticity, and cognitive function remain unexplored. This study aimed to examine the effects of VMP1 on neuronal calcium regulation in vitro and in vivo, and its relationship with synaptic plasticity and cognitive function in vivo. VMP1 expression was knocked down in SH-SY5Y neuroblastoma cells and the effects on store-operated calcium entry (SOCE) and calcium channels were assessed using western blot. The in vivo gain-and-loss function of hippocampal VMP1 was achieved in C57/BL6 mice using the adeno-associated virus. The effects of VMP1 overexpression and downregulation on calcium channels, animal cognitive functions, long-term potentiation (LTP), neuron dendritic spine density, and synaptic-related proteins were evaluated. In vitro study showed that VMP1 knockdown reduced SOCE ($p<0.0001$), transient receptor potential canonical (TRPC1), and calcium release-activated calcium channel protein 1 (Orai1) in the SH-SY5Y neuroblastoma cells (n=4, $P<0.01$). Hippocampal VMP1 knockdown in mice led to decreased TRPC1 expression (n=4, $P<0.01$). VMP1 overexpression elevated TRPC1 expression (n=4, $P<0.01$) and reduced stromal interaction molecule 2 (STIM2) level (n=3, $P<0.05$). Notable cognitive dysfunction was also observed in hippocampal VMP1 overexpressed mice, which coupled with a significant reduction in long-term potentiation (LTP), neuron, dendritic spine loss and downregulations of phosphorylated calcium/calmodulin-

dependent protein kinase II (p-CaMKII) (n=3, $p<0.05$) and GluR2 subunit of AMPA receptor (n=3, $p<0.01$). On the other hand, VMP1 knockdown led to upregulation of p-CaMKII (n=3, $p<0.05$) and GluR2 (n=3, $p<0.01$), but no significant alteration in the cognitive function, LTP, neuron number, and dendritic spine density was observed. To enable the study of VMP1 pathogenesis in patient-derived neurons, hiPSCs-derived from skin fibroblast and cord blood were used to derive dopaminergic neurons. With stepwise optimization of the glycogen synthase kinase 3β inhibition and sonic hedgehog signaling, 99.05% ± 0.22% βIII-tubulin and tyrosine hydroxylase expressing dopaminergic neurons were generated (n=4). Cryopreserved dopaminergic progenitors were thawed and transducted using lentivirus (n=1). In conclusions, this study found that VMP1 is essential for neuronal calcium homeostasis. Overexpression of VMP1 in the mouse hippocampus led to impaired cognitive function, reduced long-term potentiation, and neuron and spine loss. Additionally, this study generated and established hiPSCs-derived neuron stores for further study. Healthy and patient-specific hIPSCs can be differentiated into dopaminergic neuron and served as a cell model for studying the role of VMP1 in the pathogenesis of neurodegenerative diseases.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Vacuole Membrane Protein 1 (VMP1), initially identified in acute pancreatitis (Dusetti et al., 2002), is an endoplasmic reticulum (ER) -localized transmembrane protein involved in autophagy (Ropolo et al., 2007, Itakura and Mizushima, 2010, Kishi-Itakura et al., 2014, Tabara and Escalante, 2016). An initial report suggested a role for VMP1 in promoting sarco/endoplasmic reticulum calcium (Ca^{2+}) ATPase (SERCA) pumps activity (Zhao et al., 2017), followed by several recent research have presented diverse findings on VMP1's involvement in calcium regulation or Store-operated calcium Entry (SOCE) (Li et al., 2023, Liu et al., 2023, Zack et al., 2023).

The regulation of calcium dynamics within neuronal cells is crucial for synaptic plasticity, learning, and memory. SOCE is a key mechanism in maintaining calcium homeostasis, initiated by a decrease in ER calcium levels and occurring at specialized ER-plasma membrane contact sites(Majewski and Kuznicki, 2015). Dysregulation of SOCE in neurons has been linked to synaptic instability, memory deficits, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Serwach and Gruszczynska-Biegala, 2020). Despite the importance of calcium regulation in neural functions, the specific role of VMP1 in neural cells remains inadequately explored.

1.2 Research Gap and Problem Statement

Calcium dynamics are crucial for synaptic plasticity, learning, and memory in neuronal cells. While VMP1 is known to influence calcium dynamics in non-central nervous system (CNS) cells, its role in neural cell calcium regulation and its impact on synaptic plasticity and cognitive function are underexplored. This study aims to

fill this research gap by elucidating the mechanisms through which VMP1 affects SOCE and exploring its implications for neuronal function.

1.3 Rationale

The potential findings of this research could significantly advance our understanding of calcium dynamics in neuronal physiology and pathology, ultimately leading to the development of novel therapeutic strategies for neurological disorders and cognitive enhancement.

1.4 Hypothesis

VMP1 affects calcium levels in the ER and SOCE, thereby influencing synaptic plasticity and cognitive function.

1.5 Research Objectives

Main objective: To study the role of VMP1 in calcium regulation and synaptic plasticity.

Specific objectives:

1: To investigate the effects of VMP1 knockdown on calcium regulation of SY-SH5Y neuroblastoma cells.

2. To examine the effects of gain-and-loss of mice hippocampal VMP1 functions on synaptic plasticity and cognitive functions.

3. To optimize the differentiation of dopaminergic neurons from human induced pluripotent stem cells for establishing the VMP1 KD cell model.

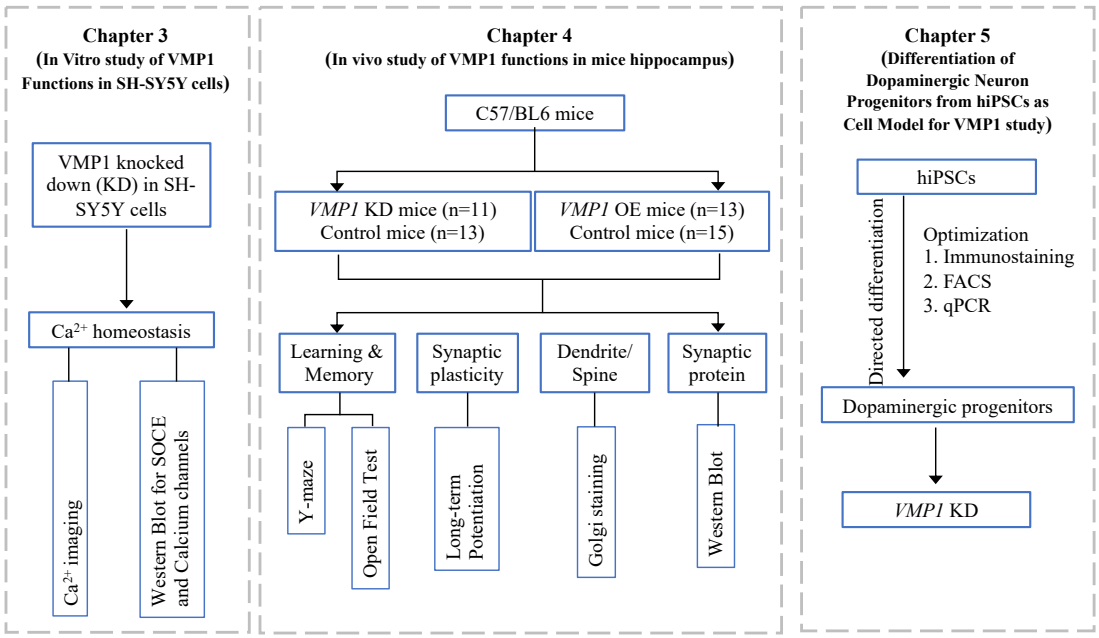

Figure 1.1 Experimental flow chart.
The overall design of the research study in this thesis. hiPSCs, human induced pluripotent stem cells; FACS, fluorescence-activated cell sorting; qPCR, quantitative real-time polymerase chain reaction.

CHAPTER 2

LITERATURE REVIEW

Calcium (Ca^{2+}) is a critical intracellular element involved in multiple functions in neuronal cells, such as neurogenesis, neurotransmission, and synaptic plasticity.

At physiology state, the cytosolic free calcium levels are maintained at a relatively low range (50-100 nanomolar, nM), contrasting with higher levels in the extracellular space (2 millimolar, mM) or the intracellular calcium store such as the endoplasmic reticulum (ER) (100–800 micromolar, μM) (Berridge et al., 2003, Schwaller, 2010, Berridge et al., 2000). During the firing of a neuronal action potential, cytosolic calcium can surge by 10-1000 times (Sabatini et al., 2002). This elevated calcium level governs neuronal and synaptic activity through the modulation of neurotransmission and dendritic spine density.

Maintaining precise control of cytosolic calcium concentration is important for neuronal function and viability. Several neuropathologies arise from mutations or disorders in calcium handling proteins, such as presenilin, which was found as an ER calcium leak channel (Schrank et al., 2020, Cheung et al., 2008, Tu et al., 2006). Calcium dysfunction is also a characteristic of aging neurons (Schrank et al., 2020). Disturbances in calcium signaling have been identified as early events that precede the onset of classic Alzheimer's diseases (AD), such as neurofibrillary tangles and amyloid beta plaques (Smith et al., 2005).

Expansive across the cellular terrain, ER encompassing both pre- and postsynaptic regions, specifically within synaptic terminals and dendritic spines, is critical for neuron function.

2.1 Vacuole Membrane Protein 1

Vacuole Membrane Protein 1VMP1 (also known as TMEM49) is an ER localized transmembrane protein with six hydrophobic regions, which was first reported in acute pancreatitis (Dusetti et al., 2002). Following studies discovered that it was conserved in most eukaryotes except in the fungal lineage (Calvo-Garrido et al., 2014). Both starvation and rapamycin treatment induce VMP1 expression. VMP1 interacts with an autophagy initiation protein Beclin 1 through its hydrophilic C-terminal region, termed as autophagy-related gene (ATG) domain (Ropolo et al., 2007). VMP1 carrying mutant ATG domain loses interaction with Beclin 1, fails to recruit microtubule-associated protein-1 light chain 3 (LC3), and blocks autophagy initiation (Ropolo et al., 2007). VMP1 transduced HeLa cells and 293T cells showed multiple autophagic features, like cup-shaped isolated membrane structures, double-membrane autophagosome-like structures, as well as single-membrane autolysosome-like structures, and increased LC3-II levels. While these LC3 punctate structures diminished in VMP1-siRNA cells (Ropolo et al., 2007). Thus, it demonstrated that VMP1 is crucial for autophagosome development. Abnormal large and non-functional autophagosomes were found in VMP1 mutant Dictyostelium cells (Calvo-Garrido and Escalante, 2010), VMP1 downregulated *C. elegans* (Tian et al., 2010) and mammalian cells (Itakura and Mizushima, 2010). VMP1 overexpression in Cos 7 cells induced vacuole formation and cell death (Dusetti et al., 2002). Further studies verified that VMP1 is not essential for the formation of immature isolation membranes but is important for their elongation or maturation (Tian et al., 2010, Calvo-Garrido et al., 2014, Kishi-Itakura et al., 2014).

Detailed examination revealed that the LC3 labeled isolated membrane was closely surrounded with ER and failed to leave ER to form mature autophagosome in VMP1 deficit cells (Zhao et al., 2017). Mechanically, VMP1 affects isolated membrane and

ER contacts by regulating SERCA activity. Specifically, VMP1 interacts with SERCA, PLN (phospholamban), and SLN (sarcolipin) and suppresses the formation of the inhibitory complex between SERCA and PLN/SLN, promoting SERCA activity but does not affect protein levels of SERCA2. In VMP1-deficient cells, the interaction between SERCA and PLN/SLN is enhanced, leading to SERCA inactivation, albeit no sustained global changes in cytosolic calcium levels (Zhao et al., 2017). They speculated that cytosolic calcium homeostasis in VMP1-deficient cells is maintained through compensatory mechanisms. A recent study reported depleted ER calcium store but increased intracellular calcium under ATP stimulation. RNA sequencing showing decreased SERCAl, and increased ORAI1, RYR, and CAMK1 expression in VMP1 KO THP-1 cells, a human acute monocytic leukemia cell line which is used to study macrophage and microglial cells (Zack et al., 2023). Hence, additional studies are required to elucidate the precise regulatory mechanisms of VMP1 in calcium signaling.

As calcium holds a crucial position within excitable neurons, the regulatory role of VMP1 in calcium signaling and its impact on neurological disorders is an area of greatest interest. There has been limited research on the role of VMP1 in neuronal contexts. Stratum dopaminergic neurons with VMP1 knockdown in mice showed motor dysfunction and a-synuclein accumulation, suggesting the importance of VMP1 in neuronal health (Wang et al., 2021). VMP1 was also found to decrease in peripheral blood samples from Parkinson's disease patients (Al-Nusaif et al., 2022). These observations raise the question of whether VMP1's role in calcium regulation could contribute to neurodegenerative processes.

In addition, VMP1's impact spans across various cellular functions, revealing its crucial role in multiple processes. Depletion of VMP1 not only disrupts ER structure, lipid droplets, and mitochondria morphology but also impairs ER-mitochondria contact

sites (Tabara and Escalante, 2016) and ER-to-Golgi trafficking pathway (Bard et al., 2006), which may affect protein secretion and transport between subcellular organelle. Moreover, VMP1 plays a pivotal role in lipid metabolism by facilitating the release of lipoproteins from the ER membrane into the lumen, which in turn affects processes such as lipid biogenesis. Accordingly, VMP1 levels are reduced in human non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (Jiang et al., 2022). Notably, upregulated VMP1 levels were found in breast cancer (Park et al., 2023) and lung cancer (Zhou et al., 2011), underlining its relevance in cancer progression.

2.2 SH-SY5Y cell line

SH-SY5Y cells, a subline of SK-N-SH cells derived from a neuroblastoma patient in the 1970s, are widely used to study neurodegenerative diseases (Xicoy et al., 2017). These cells exhibit a homogeneous and neuroblast-like (N) phenotype, possessing numerous biochemical and functional characteristics of neurons, including enzyme activity for tyrosine and dopamine-β-hydroxylases, norepinephrine uptake, and the expression of neurofilament proteins (Kovalevich and Langford, 2013). They also express various receptors such as opioid, muscarinic, and nerve growth factor receptors. SH-SY5Y cells can proliferate continuously, maintaining an undifferentiated, neuroblast-like morphology and expressing immature neuronal markers (Appendix A). SH-SY5Y cells can differentiate into mature neuron-like cells when treated with agents such as retinoic acid, phorbol ester, brain-derived neurotrophic factor (BDNF), dibutyryl cyclic AMP (dcAMP), purine, or staurosporine (Kovalevich and Langford, 2013, Xicoy et al., 2017). These features make the SH-SY5Y cell line a reliable and valuable model for a wide range of neural related studies.

2.3 Calcium regulation in ER

ER calcium fluxes are regulated by two mechanisms: store-operated calcium entry (SOCE), which controls calcium influx, and calcium-induced calcium release (CICR), which governs calcium efflux channels (Majewski and Kuznicki, 2015, Verkhratsky and Shmigol, 1996).

2.3.1 Store-operated calcium entry (SOCE)

SOCE is critical for sustaining heightened ER calcium levels and proper ER function, like protein synthesis, folding, and ER-mediated transport functions. SOCE is a ubiquitous calcium entry pathway that is activated in response to the depletion of ER calcium stores (Figure 2.1). Originally identified in electrically non-excitable cells, the presence and functionality of SOCE have later also been confirmed in excitable cells such as neurons and muscle cells. In neurons, SOCE plays a prominent role, as seen in hippocampal (Bouron, 2000) and cortical neurons (Yoo et al., 2000). Correct regulation of neural SOCE is necessary for the stability of postsynaptic contacts to preserve memory formation. Of note, molecular components of SOCE are possible targets for therapeutic interventions.

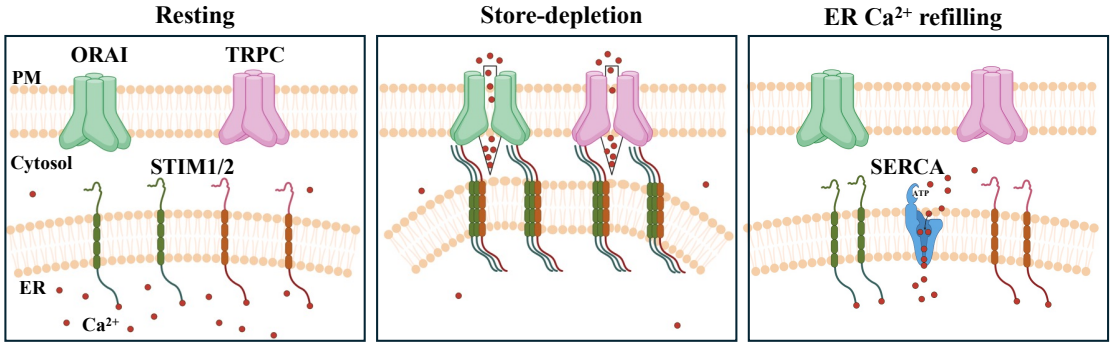

Figure 2.1 Mechanism of SOCE mediated by STIM and ORAI/TRPC proteins. Store-operated calcium entry (SOCE) primarily relies on calcium sensor stromal interaction molecules (STIM1/2) on the ER membrane, calcium-release-activated calcium (CRAC) channels, such as calcium release-activated calcium channel protein (ORAI), and transient receptor potential canonical (TRPC) channels on the plasma membrane (PM). In the resting state, when ER calcium levels are sufficient, STIMs are diffusely distributed within the ER and bind to ER calcium. Upon ER calcium store depletion, calcium-free STIMs undergo a conformational shift and self-aggregation, allowing STIM to bind and activate ORAI or TRPC channels, inducing calcium influx, known as SOCE. As cytosolic calcium rises, calcium-bound calmodulin (CaM) interacts with STIM, causing it to detach from ORAI/TRPC, resulting in channel closure and reduced STIM aggregation. ER calcium-ATPase (SERCA) helps pump calcium back to the ER and refill ER calcium stores. Figure created with Biorender.com.

2.3.1(a) Stromal interaction molecules (STIM)

Two STIM isoforms, STIM1 and STIM2, function as calcium sensors positioned in the ER membrane, with STIM1 additionally found in the plasma membrane (PM) (Kraft, 2015). STIM1 and STIM2 feature conserved structural domains, including a transmembrane segment, helix–loop–helix structural EF-hand, which is a calcium-binding domain, sterile α-motif (SAM) domain, and coiled-coil regions (Soboloff et al., 2012, Stathopulos and Ikura, 2013). The EF-hand and SAM domain in STIM serve as sensors for calcium levels within the luminal compartment of the ER (Roos et al., 2005, Liou et al., 2005, Stathopulos et al., 2008, Yuan et al., 2009). The STIM1 EF-hand point mutant led to the pre-formation of puncta near the PM without causing actual ER calcium depletion, rendering it unresponsive to the actual calcium levels in the ER (Baba et al., 2006). STIM2, a mammalian homolog of STIM1, differs in its C-terminal region, resulting in distinct calcium-binding properties (Williams et al., 2001, Soboloff et al., 2006a). EF-hand domain of STIM1 binds calcium with a

dissociation constant (Kd) of about 250 μM, while the Kd of STIM2 is about 500 μM (Zheng et al., 2008, Berna-Erro et al., 2017). This makes STIM2 more sensitive to minor ER calcium changes, allowing it to remain partially active at resting ER calcium levels. In contrast, STIM1 initiates calcium influx during substantial ER depletion, initiating SOCE in response to external stimuli (Brandman et al., 2007). Additionally, STIM2 exhibits slower oligomerization kinetics and weaker ORAI binding properties than STIM1, which may suggest a crucial restraint against excessive SOCE (Stathopulos et al., 2009, Wang et al., 2014, Bird et al., 2009).

While STIM2 predominates in primary hippocampal neurons, its presence extends to both soma and dendrites. In contrast, STIM1 is primarily confined to the soma (Berna-Erro et al., 2009, Skibinska-Kijek et al., 2009). As a consequence, STIM2 regulates SOCE in hippocampal and cortical neurons, while STIM1 prevails as the primary isoform in cerebellar granule and Purkinje neurons (Hooper et al., 2014).

STIM1 has been initially identified as a central required mediator of SOCE (Liou et al., 2005, Roos et al., 2005, Zhang et al., 2005). Alternatively, STIM1 acts as a substrate for γ-secretase, and increased γ-secretase cleavage of STIM1 by familial Alzheimer's disease (FAD) associated mutant presenilin 1 result in compromised SOCE (Tong et al., 2016). STIM2 was reported to play a complex role: it inhibits STIM1 aggregation and SOCE (Soboloff et al., 2006a) while promoting SOCE through ORAI1. Another study found that STIM2 recruits and activates STIM1 when ER calcium levels are not sufficiently low to trigger a STIM1 response (Subedi et al., 2018). In hippocampal neurons lacking STIM2, studies indicate a weak STIM1-Orai1 association (Nelson et al., 2018). STIM2 deficiency in neurons led to reduced SOCE both in the soma and dendritic spines of hippocampal neurons (Sun et al., 2014, Zhang et al., 2015).

The role of STIM1 in spine function remains uncertain, and there is a suggestion that STIM1 may be involved in the formation of new spines (Kushnireva et al., 2020). However, the absence of STIM1 leads to deficits in cerebellar motor behavior, primarily due to its impact on synaptic transmission through metabotropic glutamate receptors (Hartmann et al., 2014). STIM2 co-localizes with calcium/calmodulin-dependent protein kinase II (CaMKII) in mushroom spines, large-headed dendrite spine which is important for memory (Described in Chapter 2.4.1), and STIM2-mediated SOCE was crucial for maintaining appropriate levels of phosphorylated-CaMKII (p-CaMKII) and stabilizing mushroom spines (Sun et al., 2014). Overexpressing STIM2 not only restores p-CaMKII levels but also safeguards mushroom spines from amyloid-induced toxicity, as demonstrated in vitro and in vivo (Sun et al., 2014, Zhang et al., 2015, Popugaeva et al., 2015). Silencing STIM2 reduces dendritic spine density in hippocampal neurons, whereas STIM1 has no detectable effect (Moccia et al., 2015, Garcia-Alvarez et al., 2015). These findings illuminate the intricate role of STIM proteins in regulating neuronal spine dynamics and synaptic plasticity.

2.3.1(b) Calcium release-activated calcium modulator (ORAI)

ORAI proteins, including ORAI1, ORAI2, and ORAI3, are rather small proteins with a molecular weight of about 31 kDa. They possess four transmembrane domains, with their N-termini and C-termini located in the cytoplasm (Zhang et al., 2005). ORAI1 is broadly distributed in various brain regions (Klejman et al., 2009), and is known for its greater efficiency in calcium regulation than the other two (Mercer et al., 2006), making it a focus of extensive research.

Studies using co-expression with STIM1 establish ORAI1 as the definitive pore-forming element of the calcium-release-activated calcium (CRAC) channel. At the same time, the co-expression of STIM1 and ORAI1 significantly enhances CRAC

current, leading to robust initiation of SOCE (Prakriya et al., 2006, Yeromin et al., 2006, Vig et al., 2006, Zhang et al., 2006, Peinelt et al., 2006, Soboloff et al., 2006b), supported by co-immunoprecipitation results indicating that STIM and ORAI interactions are greatly increased under ER depletion state (Sanchez-Collado et al., 2022). However, either ORAI1 or ORAI2 overexpression independently diminished SOCE (Mercer et al., 2006). When co-expressed with STIM1, both ORAI1 and ORAI2 enabled significant calcium entry and current flows, implying a delicate STIM-ORAI protein ratio (Lis et al., 2007, Mercer et al., 2006). It has been reported that the formation of the STIM1/ORAI1 heteromer requires a ratio of approximately (2-4):1 to approach native CRAC channel levels (Mullins and Lewis, 2016).

2.3.1(c) Transient receptor potential canonical (TRPC)

The transient receptor potential canonical (TRPC) family, the initial subset of transient receptor potential (TRP) channels, includes seven mammalian members (TRPC1, TRPC2, TRPC3/6/7, TRPC4/5) that function as calcium permeable nonselective cation channels (Wang et al., 2020a, Curcic et al., 2019). TRPC channels possess intricate structural compositions, including intracellular N- and C-termini, six membrane-spanning helices, and a pore-forming loop. These channels are strategically located both in ER and PM (Dietrich et al., 2014). Of note, TRPC1 is the first member of TRPC channel subfamily to be found as a molecular component of SOCE (Wes et al., 1995, Zhu et al., 1995).

TRPC channels exhibit multiple activation mechanisms, including the classical G-protein-coupled receptor (GPCR)-Gq/Phospholipase C (PLC) pathway (Wang et al., 2020a, Chen et al., 2020). Alternatively, TRPC channels can be activated when ER calcium stores are depleted. However, the involvement of ORAI and STIM proteins in TRPC channel activation is a subject of ongoing debate (Cheng et al., 2013, Liao et al.,

2014). Some research suggests a direct interplay between STIM1 and TRPC channels to initiate activation through electrostatic interaction (Zeng et al., 2008, Worley et al., 2007, Lee et al., 2014). However, a study involving salivary glands proposed that the activation of TRPC1 relies on STIM-ORAI coupling, which facilitates the recruitment of TRPC1 into PM (Cheng et al., 2008). Additionally, it also reported that TRPC channels can be triggered independently of ORAI and STIM through receptor-operated mechanisms, which speculates that TRPC and STIM1/ORAI1 signaling occur within separate domains (DeHaven et al., 2009).

TRPC1 shows a complex interplay in neuronal function and calcium regulation in AD. On one hand, elevated TRPC1 levels in AD models lead to increases in SOCE, contributing to tau phosphorylation, ER stress, and cognitive decline, reflecting the disruptive impact of AD on this process. Conversely, TRPC1 knockout appears to ameliorate these effects (Ye et al., 2020). However, another study reported decreased TRPC1 levels in AD models. Under normal physiological conditions, the absence of TRPC1 didn't affect learning and memory but exacerbated cell death in the presence of amyloid-β (Aβ), a main pathological element of dementia. Furthermore, elevating TRPC1 levels reduced Aβ and apoptosis by binding to β-amyloid precursor protein (APP), suggesting a potential protective role (Li et al., 2018). In addition, overexpression of TRPC1 protects against neurotoxicity caused by Parkinson's Disease (PD) related neurotoxins such as 1-methyl-4-phenylpyridinium ion (MPP^+) (Bollimuntha et al., 2006, Bollimuntha et al., 2005). It is suggested that TRPC channels have a dual role in neurons: they can either protect or harm. Excessive calcium entry, mainly through TRPC1, can cause cell death, while physiological TRPC1 activation can be protective. Maintaining the right calcium balance is crucial (Sukumaran et al., 2017).

2.3.1(d) SOCE regulators

STIM1/ORAI1-interacting proteins were also reported to regulate STIM1 translocation, STIM1–ORAI1 interaction, or SOCE. Store-operated calcium entry-associated regulatory factor is an ER-resident protein that acts as a negative regulator of SOCE. It responds to elevated cytosolic calcium levels after ER calcium refilling, leading to the slow inactivation of STIM2-dependent basal SOCE (Palty et al., 2012). Its overexpression reduces spontaneous intracellular calcium oscillations in interstitial cells of Cajal, while STIM1 overexpression increases them (Park et al., 2018). Microtubule plus end–binding protein 1 binding limits full-length STIM1 access to ER–PM junctions in the resting state and during ER calcium store depletion, preventing excess SOCE and ER calcium overload. Disruption of microtubule plus end–binding protein 1 binding facilitates ORAI1 recruitment, leading to elevated SOCE and ER calcium overload (Chang et al., 2018).

2.3.2 ER calcium uptake and store refiling.

Resting calcium levels are maintained by ATPases pumping calcium into extracellular and internal ER stores. Sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps were found in the ER or in the sarcoplasmic reticulum (SR) of eukaryotic cells. Three isoforms are identified within the SERCA family: SERCA1, SERCA2, and SERCA3. Both isoforms of SERCA2 (SERCA2a and 2b) and SERCA3 are expressed in the brain. These three isoforms share an essentially conserved structural framework (Vandecaetsbeek et al., 2009).

SERCA pumps calcium ions from cytosol into ER, using energy generated by ATP hydrolysis overcoming a substantial calcium concentration gradient. The conventional model, known as the E1/E2 theory, elucidates this process by describing an E1 state with high calcium affinity on the cytoplasmic side and an E2 state with

reduced calcium affinity on the lumen-facing side (Vandecaetsbeek et al., 2009). Thapsigargin (TG), working as an irreversible SERCA inhibitor, acts to stabilize SERCA in the E2 state, thus effectively inhibiting its SERCA activity and causing a decline in ER calcium levels (Sehgal et al., 2017, Rossier et al., 1993). Cytosolic calcium changes have minimal SERCA impact, but ER luminal calcium strongly regulates SERCA. Lower ER calcium increases SERCA uptake velocity, independently of cytosolic levels (Mogami et al., 1998, Solovyova et al., 2002).

Disruptions in SERCA function led to increased ER stress and accumulation of misfolded proteins, involved in neurodegenerative diseases. Age-related neuronal changes are associated with decreased SERCA function (Pottorf et al., 2000a, Pottorf et al., 2000b). Inhibition of SERCA using TG hampers the induction of long-term potentiation (LTP, described in chapter 2.2.2) in hippocampal slices (Behnisch and Reymann, 1995, Harvey and Collingridge, 1992). Manipulating SERCA activity, both pharmacologically and genetically, influences Aβ production, with reduced SERCA levels leading to less Aβ and increased SERCA levels resulting in more Aβ (Green et al., 2008). Presenilin, which functions as the catalytic subunit of γ-secretase in amyloid precursor protein cleavage, has been found to physically regulate SERCA activity, as decreased SERCA expression was observed in presenilin double knockout fibroblasts (Green et al., 2008). However, a study using a novel SERCA activator shows promise in alleviating ER stress and neuronal cell protective function in PD (Dahl, 2017). It also was testified to improve memory and cognitive function in AAP/PS1 mice (Krajnak and Dahl, 2018).

2.3.3 ER calcium release mechanism.

Intracellular calcium release is mediated by two primary channels within the ER: ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP3Rs), both of

which share approximately 40% homology in their putative transmembrane regions, suggesting a common ancestral origin (Santulli et al., 2017). IP3R is a ligand-gated calcium channel consisting of six transmembrane domains (Yule et al., 2010). The IP3R family comprises three distinct isoforms: IP3R Type 1 (IP3R1), Type 2, and Type 3, with varying expression profiles across different cell types and tissues (Wojcikiewicz, 1995). Significantly, IP3R1 is highly prevalent in the brain, particularly in regions such as the cerebellum, cerebral cortex, hippocampus, and striatum, underscoring its prominence (Furuichi et al., 1993). RyR is composed of four 565 kDa monomers and has three subtypes in mammalian tissues: RyR1, RyR2, and RyR3, with RyR1 being the most sensitive to cytosolic calcium (Zalk et al., 2015, Stutzmann, 2005, Lanner et al., 2010). RyR1 and RyR2 are mainly in muscle tissue, while RyR3 has diverse brain locations and is also found in pancreatic islets, leukocytes, and smooth muscle cells. In neurons, IP3R-mediated calcium signals are primarily found in the soma and proximal dendrites (Nakamura et al., 1999, Stutzmann et al., 2003), while RyR-mediated signals are more prominent in dendritic spines and presynaptic terminals (Santulli et al., 2017).

Apart from being activated by inositol 1,4,5-trisphosphate (IP3), calcium serves as a biphasic modulator in regulating IP3R activity. While calcium alone cannot open IP3R channels in the absence of IP3, it does enhance their open probability. Low cytosolic calcium concentrations or elevated ER calcium levels promote its activation (Nunn and Taylor, 1992). Conversely, high cytosolic calcium concentrations inhibit IP3R activity (Thrower et al., 2001, Foskett et al., 2007). Luminal calcium levels influence IP3R sensitivity: full ER stores are most responsive to IP3Rs, but as ER calcium depletes, IP3R sensitivity diminishes, leading to closure despite cytosolic signals (Taylor and Tovey, 2010). A similar biphasic calcium activation mechanism is observed in RyR as well (Hamilton, 2005). Although RyR and IP3R share similarities

in calcium regulation, they display different responses to calcium triggers. RyR responds to calcium influx facilitated by channels like voltage-gated calcium channels (VGCC) and ionotropic glutamate receptor NMDAR. In contrast, IP3Rs are primarily activated by calcium influx mediated by G_q-coupled group I metabotropic glutamate receptors mGluRs (Padamsey et al., 2019, Stutzmann, 2005).

In AD, aberrant calcium signaling involving IP3 (Leissring et al., 1999, Fujii et al., 2000, Kasri et al., 2006) or RyR receptors (Cheung et al., 2008, Chakroborty et al., 2009, Chan et al., 2000, Stutzmann et al., 2007, Stutzmann et al., 2006) disrupts calcium homeostasis within ER, attributed to neurodegenerative pathology and synaptic plasticity.

Presenilin, encompassing both presenilin 1 and presenilin 2, are highly conserved transmembrane proteins predominantly located on the ER. They are implicated in a substantial portion (20-50%) of early-onset FAD cases (Walter et al., 1996, De Strooper et al., 1997). In addition to regulating calcium by affecting SERCA activity (discussed in Chapter 2.1.2), wild-type presenilin functions independently as an ER calcium leak channel (Tu et al., 2006, Nelson et al., 2007, Brunello et al., 2009). This mechanism aligns with the "Ca^{2+} overload" hypothesis in FAD, as presenilin mutations have been associated with elevated ER calcium levels (Nelson et al., 2007, Guo et al., 1997). However, some studies propose that mutations in presenilin 2 increase ER calcium leak and reduce ER calcium levels by inhibiting SERCA (Brunello et al., 2009, Zatti et al., 2006). Additionally, presenilin mutations impact calcium release through RyR or IP3R, although the exact mechanisms remain unclear (Cheung et al., 2008, Wu et al., 2013). This complex interplay highlights the multifaceted role of presenilin in calcium homeostasis and its implications for AD pathology.

2.3.4 ER stress

The unfolded protein response (UPR) is a programmed sequence of events that in the first instance protects cells against death under conditions of metabolic, ionic, and protopathic stress. When excessive protein misfolding occurs in ER, the chaperone protein, glucose-regulated protein (GRP78) coordinates various processes to halt the production of non-essential proteins, preserving cell viability. UPR activation can be triggered by factors such as glucose depletion, calcium imbalances, oxidative stress, and ischemia. Mammalian UPR is controlled by three main ER-resident sensors: inositol-requiring enzyme-1α (IRE1α), protein kinase R-like endoplasmic reticulum kinase (PERK), which inhibits protein synthesis by phosphorylating eIF2α, and activating transcription factor 6α (ATF6α) (Hetz et al., 2020). (Figure 2.2)

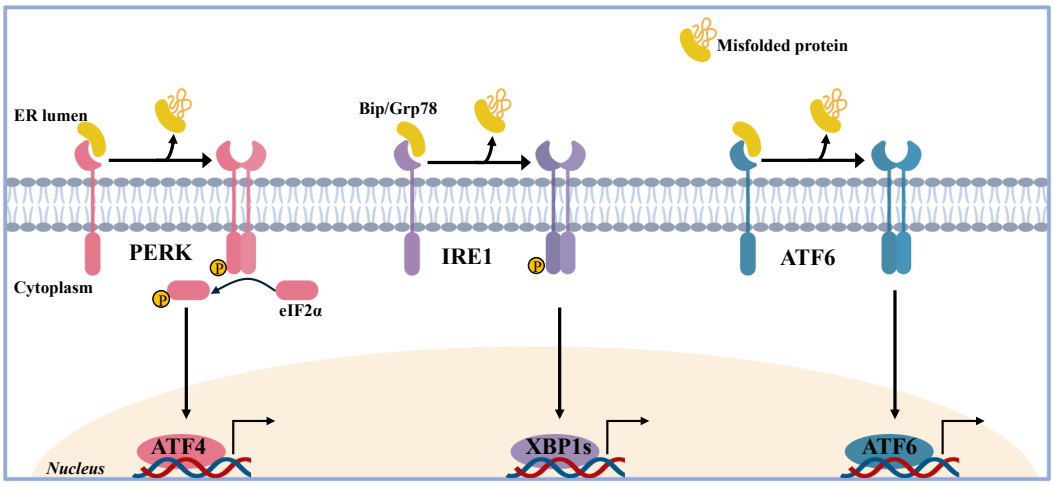

Figure 2.2 Activation of the three unfolded protein response (UPR) pathways is initiated by misfolded protein accumulation in the endoplasmic reticulum (ER). Left: The protein kinase R-like endoplasmic reticulum kinase (PERK) phosphorylates eukaryotic translation initiation factor 2α (eIF2α), which finally leads to increased ATF4 expression and promotes cell survival. Middle: Dissociation of the binding immunoglobulin protein (BiP) from IRE1 activates its endoribonuclease domain, resulting in the production of the transcriptional activator XBP1s. Right: Activated ATF6 enhances protein folding and processing in the ER. Figure created with Biorender.com.

2.4 Synaptic plasticity

2.4.1 The Structure Principle of Synaptic plasticity

Located deep in the medial temporal lobe, the hippocampus is a highly developed brain structure closely linked to learning, emotions, and the process of memory formation (Alam et al., 2018). It consists of two central modules: the dentate gyrus and the cornu ammonis (CA). The dentate gyrus has a trilaminar cortical arrangement, forming a characteristic V or U shape, and is composed primarily of granule cells. Functionally and anatomically, the hippocampus is divided into discrete subfields that house the pyramidal neurons CA1, CA2, and CA3. About 90% of the neurons in the CA1 area are glutamatergic pyramidal cells, the remaining part consists of interneurons (Chauhan et al., 2021).

Granule cells originating from the dentate gyrus extend their axons known as mossy fiber to CA3 region making connections with pyramidal cells located. CA3 pyramidal cells extend their projections to the CA1 region, a process facilitated by Schaffer collateral axons. The pyramidal cells in the CA1 region principally project to the subiculum and innervate entorhinal cortex (Amaral and Witter, 1989). Interestingly, the projection from the dentate gyrus to CA3 ends at the CA3-CA2 junction, marking a distinct divergence. This distinctive boundary underscores the differentiation of CA2 pyramidal cells from their CA3 counterparts, while CA2 shares a large pyramidal cell body and receives Schaffer collateral input from CA3 neurons instead of mossy fiber input from the dentate gyrus (Fanselow and Dong, 2010, Thompson et al., 2008).

The pyramidal neurons in CA1 region represent the main output of the hippocampus. A single CA3 pyramidal neuron makes only one synaptic connection with one postsynaptic CA1 neuron (Stevens and Wang, 1995). This monosynaptic glutamatergic synaptic connection, commonly referred to as CA3–CA1 synapses,

exhibits an all-or-none behavior in which a presynaptic action potential either fails to release the transmitter or can successfully release only a single neurotransmitter quantum (Andersen, 1977, Bliss and Lomo, 1973, Dunwiddie and Lynch, 1978, McNaughton et al., 1978). This unique property contributes to the robust induction properties of synapse-specific long-term potentiation (LTP) or long-term depression (LTD). In electrophysiology studies, NMDAR-dependent LTP in CA3-CA1 synapses has garnered extensive attention due to its strong resemblance to the cellular model of learning. This is supported by compelling evidence suggesting that during learning process, synaptic transmission is strengthened in a manner consistent with LTP. Moreover, many pharmacological and genetic manipulations that disrupt LTP also impair learning and memory (Malenka and Bear, 2004, Baudry et al., 2011).

Dendritic spines, small membranous protrusions extending from neuronal dendrites, play a pivotal role in synaptic function and plasticity by harboring neurotransmitter receptors, organelles, and signaling systems. These structures typically exhibit a morphology characterized by a bulbous head connected to the dendritic shaft via a narrow neck. Classification of postsynaptic dendritic spines is based on their shape and size, leading to three primary categories: mushroom spines, thin spines, filopodial spines, and stocky spines (Sala and Segal, 2014, Peters and Kaiserman-Abramof, 1970, Tonnesen et al., 2014, Bourne and Harris, 2008) (Figure 2.3). Mushroom spines, characterized by their large-headed morphology, serve as stable memory spines that are instrumental in the processes of memory formation and storage. They exhibit remarkable persistence, lasting several months during LTP or learning events (Matsuzaki, 2007). In contrast, thin spines, often mentioned as learning spines, play a distinct role in memory formation (Grutzendler et al., 2002, Bourne and Harris, 2007). Stocky spines might be active forms of mushroom spines with short necks (Holtmaat et

al., 2005). Their structural and functional roles are still inconclusive. These dendritic spine structures are highly dynamic and capable of transforming both physiological and pathological conditions (Matsuzaki et al., 2004), thereby contributing to the anatomical basis of plasticity. Various imaging techniques, including Golgi staining, light microscopy, electron microscopy, and two-photon microscopy, have been developed over the years to visualize the dynamic nature of dendritic spines. Spatial training (Moser et al., 1997) and exposure to enriched environments (Kozorovitskiy et al., 2005) alters the numbers of hippocampal spines. Thus, spine growth is considered a readout for LTP. Postsynaptic density (PSD) is an electron-dense structural thickening along the postsynaptic plasma membrane. Within the excitatory PSD is a complex assembly of proteins that includes a variety of components such as N-methyl-D-aspartate (NMDA) receptors and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and metabotropic glutamate receptors (Yasuda et al., 2022). In addition, the PSD includes scaffolding proteins important for synaptic protein transport during LTP, exemplified by postsynaptic density protein 95 (PSD95), as well as signaling proteins, represented by CaMKII (In section 2.4.4)(Okabe, 2007).

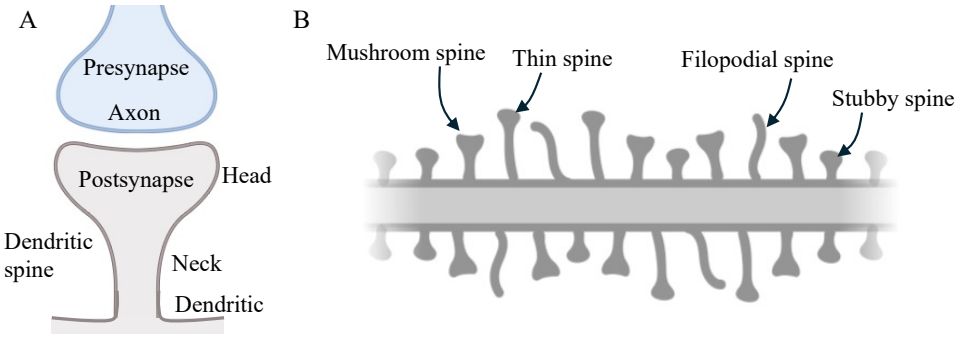

Figure 2.3 Dendritic Spine.
A. Schematic depiction of a synapse. The dendritic spine (postsynapse) is in grey and the presynaptic bouton is in blue. B. Different types of dendritic spines. Figure created with Biorender.com.

2.4.2 The cellular Mechanisms and Phases of LTP

The term "synaptic plasticity" was initially named to describe the persistent and activity-dependent alternations in synaptic strength that occur during neuronal activity. Several persistent forms of synaptic plasticity have been observed in the mammalian central nervous system, with LTP and LTD being the major manifestations of neuronal plasticity. These processes are typically triggered by the activation of postsynaptic NMDARs and are characterized by changes in the number of postsynaptic AMPARs (Morris et al., 1986, Dudek and Bear, 1992). LTP primarily results from an increase in the number and enhanced strength of postsynaptic AMPARs, often accompanied by the enlargement of postsynaptic dendritic spine size (Chang and Greenough, 1984, Tonnesen et al., 2014, Bourne and Harris, 2007). Structurally, LTP was confirmed by electron microscopy, revealing a shift from thin to mushroom-shaped spines (Chang and Greenough, 1984). Conversely, LTD coincides with AMPARs internalization and a sustained reduction in synaptic strength caused by reduced glutamate release, leading to spine retraction or shrinkage (Tonnesen et al., 2014, Bourne and Harris, 2007). As such, both LTP and LTD are extensively explored as cellular models to elucidate the mechanisms underlying learning and memory processes (Bliss and Collingridge, 1993).

In electrophysiology experiments, various induction protocols have been explored to initiate LTP, which includes methods such as high-frequency stimulation (HFS), theta-burst stimulation, and the pairing of presynaptic and postsynaptic facilitation (Luscher and Malenka, 2012). Among them, HFS is the most utilized one for depolarizing neurons, leading to a rapid calcium influx, and thereby initiating LTP, as initially characterized through a single pulse train at 100 Hz for 1 second (Bliss and Lomo, 1973). Additionally, LTP can be triggered chemically by NMDARs agonist glycine in cultured neurons (Lu et al., 2001). Studies on LTP have been conducted in diverse settings, including cultured hippocampal neurons, brain slices, and even in vivo mouse models (Luscher and Malenka, 2012). Typically, LTP induction results in an average enhancement of 150–200% compared to baseline excitatory postsynaptic potential (EPSP) amplitudes (Bliss and Collingridge, 1993, Gustafsson et al., 1987, Sastry et al., 1986). The fundamental mechanism underlying LTP induction involves the synchronized time-locked depolarization of postsynaptic cells with the stimulation of presynaptic afferents, facilitating precise temporal control over both presynaptic and postsynaptic neurons (Blundon and Zakharenko, 2008, Malenka, 2003). LTD, on the other hand, results from a prolonged, less intense calcium influx induced by low-frequency synaptic stimulation, like 1–3 Hz for 5–15 min (Yang et al., 1999) or activation of AMPARs/kainate or mGluRs (Li et al., 2004, Snyder et al., 2001, Kemp and Bashir, 1999, Luscher and Huber, 2010, Palmer et al., 1997).

Both LTP and LTD consist of two phases unfolding on similar timescales. The induction phase of LTP enhances synaptic function but lasts less than an hour, followed by a maintenance phase where the increased synaptic strength stabilizes. Memory formation mirrors this with an early phase of initial learning and a separate late phase for memory consolidation. Specifically, early LTP is initiated by the activation of

diverse protein kinases, notably CaMKII, protein kinase C (PKC), protein kinase A (PKA), and tyrosine kinase (Abel et al., 1997).

These protein kinases orchestrate the phosphorylation of AMPARs and NMDARs, subsequently facilitating the trafficking and insertion of glutamate receptors, specifically AMPARs, into the postsynaptic membrane. Thus, during LTP induction and initial learning phases, existing proteins undergo post-translational modifications but not new protein synthesis at the potentiated synapse (Abel and Lattal, 2001). However, these modifications are temporary, and since proteins degrade over time, both the maintenance and consolidation phases of LTP and memory require new gene expression and protein synthesis (Reymann and Frey, 2007, Abraham and Williams, 2008). These synthesized proteins contribute to the increased number and surface area of dendritic spines and their postsynaptic sensitivity to neurotransmitters (Matsuzaki et al., 2004).

2.4.3 α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPARs)

AMPARs are ionotropic excitatory glutamatergic receptors abundant in the brain (Beneyto and Meador-Woodruff, 2004). Comprising four subunits (GluR1-4, also known GluA1-4), AMPARs typically form tetrameric heteromeric complexes (Lu et al., 2009, Traynelis et al., 2010, Wenthold et al., 1996, Shi et al., 2001), with most containing at least one GluR2 subunit (Luscher and Malenka, 2012). In CA1 pyramidal neurons, GluR1/2 heteromers predominate, accounting for 80% of the AMPARRs, followed by GluR2/3 heteromers (Lu et al., 2009). While GluR4 is mainly expressed during early development and remains at low levels in the adult brain (Henley and Wilkinson, 2013). Long-tailed subunits like GluR1 and GluR4 play a decisive role in AMPAR trafficking, exerting their influence over short-tailed subunits. Consequently, the GluR1/2 tetrameric subunit exhibits surface trafficking properties akin to those of