KU-Leuven
Group Biomedical Science
Faculty of Medicine
Department of Human Genetics
Laboratory of Behavioral and Developmental Genetics

FUNCTIONAL ANALYSIS OF APP IN WNT MEDIATED SIGNALING

Tengyuan Liu

Jury

Supervisor: Prof. Patrick Callaerts

Co-supervisor: Prof. Bassem Hassan

Chair examining committee: Prof. Tassos Economou

Chair public defense: Prof. Jan Cools

Secretary: Prof. Joris de Wit

Jury members: Prof. Bart De Strooper

Prof. Joris de Wit

Prof. Thierry Galli

Prof. Carlos Parras

Doctoral thesis in Biomedical Science

Leuven, 2022

Dissertation presented in partial fulfillment of the requirements for the degree of Doctor in Biomedical Sciences

TABLE OF CONTENTS

TABLE OF CONTENTS I
ABBREVIATIONS IV
SUMMARY VIII
SAMENVATTING IX
CHAPTER 1 INTRODUCTION 1
1.1 APP and Alzheimer's disease (AD) 1
1.2 APP structure and expression 1
1.3 APP expression during development 3
1.4 APP trafficking and processing 4
1.5 Trafficking route of APP after internalization 6
1.6 Amyloidgenic processing of APP 7
1.7 Roles of APP in CNS development 8
1.7.1 APP in neurogenesis 9
1.7.2 Neuronal Migration 10
1.7.3 Neurite outgrowth and synapse formation 10
1.8 Wnt Signaling 12
1.8.1 General introduction to Wnt signaling pathway 12
1.8.2 Wnt structure 12
1.8.3 Intracellular modification processing and transportation 13
1.8.4 Secretion 14
1.8.5 Canonical Wnt signaling pathway 15
1.8.6 Non-canonical (beta-Catenin independent) Wnt signaling pathway 17
1.9 Wnt/PCP pathway 19
1.9.1 The establishment of PCP framework 19
1.9.2 Initiation of PCP signaling 21

1.9.3 Signaling downstream of Wnt/PCP signaling 21
1.10 Wnt/Ca^{2+} pathway 22
1.11 Wnt signaling in CNS development and disease 22
CHAPTER 2 AIMS OF THIS STUDY 27
CHAPTER 3 MATERIALS AND METHODS 28
3.1 Primary cortical neuron culture, virus transduction and plasmids transfection 28
3.2 Amino acids sequence of deleted CRD 29
3.3 Wnt and inhibitor treatment in primary neuron 30
3.4 Quantitative real-time PCR (qRT-PCR) 31
3.5 Western Blots 31
3.6 Immunoprecipitation 31
3.7 Immunofluorescence 32
3.8 Aβ 40/42 detection 33
3.9 Statistical analyses 33
CHAPTER 4 RESULTS 34
4.1 APP is a putative Wnt5a receptor through the Cysteine Rich Domain (CRD) 34
4.2 Wnt5a treatment affects APP trafficking and expression in maturing mouse primary cortical neuron 36
4.3 mAPP is binds Wnt3a via its CRD and stabilizes fl-mAPP 42
4.4 The CRD is required for Wnt-mediated regulation of mAPP trafficking and expression 48
4.5 CRD is critical for APP to regulate neurite outgrowth and complexity .54

4.6 Wnts regulate neurite development in a CRD dependent manner.....63

CHAPTER 5 DISCUSSION.....69

5.1 APP and Wnt signaling 69

5.2 APP is a putative Wnts receptor conserved cross species.....71

5.3 CRD is critical for Wnts to regulate APP trafficking, expression and processing.....74

5.4 CRD of APP mediates Wnts induced neurite morphogenesis in cultured cortical primary neurons.....77

5.5 General conclusion.....79

CONTRIBUTION.....80

REFERENCES.....81

CURRICULUM VITAE.....99

ACKNOWLEDGMENTS.....102

ABBREVIATIONS

AcD	acidic domain
ACI	Axon Complexity Index
ADAM	metalloproteinase family
AD	Alzheimer's disease
Aβ	Amyloid Betta
AICD	APP intracellular domain
AIS	Axon Initial Segment
APP	Amyloid Precursor Protein
APPL	APP homolog in *Manduca sexta*
aPKC	atypical protein kinase C
APH-1	anterior pharynx-defective 1
BACE1	type-1 membrane bound Aspartyl protease
CamKII	Calcium-dependent Kinase II
CAM	cell Adhesion molecules
CBD	collagen binding domain
CE	convergent extension
CNS	central nervous system
CRD	Cysteine Rich Domain
CuBD	copper binding domain
Dgo	Diego
DISC1	disrupted-in-schizophrenia 1
Dsh	Dishevelled
DAG	Diacylglycerol
Dvl 1	Dishevelled 1

Dkk1	Dickkopf-1
DMSO	Dimethyl sulfoxide
ER	endoplasmic reticulum
ECM	extracellular matrix
ExD	extension domain
Fmi	Flamingo
Fz	Frizzled
flAPP	full length Amyloid Precursor Protein
Ft/Ds	Fat/Dashsous
GC	growth cone
GCs	granule cells
GFLD	growth factor like domain
GABABR1a	γ-aminobutyric acid type B receptor subunit 1
hESCs	human embryonic stem cells
hAPP	human Amyloid Precursor Protein
HBD	Heparin binding domain
iPSCs	induced pluripotent stem cells
IP	immunoprecipitation
JMR	juxtamembrane region
KPI	Kunitz-type-protease inhibitor
LDLR	Low density lipoprotein receptor
LPR	lipoprotein receptor-related protein
LTP	long-term potentiation
mAPP	mouse Amyloid Precursor Protein
MBOAT	membrane associated O-acyl transferase
MVBs	multi-vesicular bodies

nAChR	nicotinic acetylcholine receptor
NGF	Nerve growth factor
NSCs	neural stem cells
NPCs	neural progenitor cells
NMJ	neuromuscular junction
PCP	Planar Cell Polarity
PEN2	presenilin enhancer 2
PFA	paraformaldehyde
PTMs	post-translational modifications
PM	plasma membrane
PLC	Phospholipase C
PKC	Protein Kinase C
Pk	Prickle
Porcn	O-acyltransferase Porcupine
PSD95	postsynaptic density protein 95
PS1/2	presenilin-1/2
ROK	Rho associated Kinase
RER	rugose endoplasmic reticulum
RT	room temperature
SVZ	subventricular zone
sAPPα	A large soluble fragment of APP
Swim	Secreted Wg-interacting Molecule
TCF/LEF	T-cell factor/lymphoid enhancer factor
TM	trans-membrane
TGN	Trans-Golgi Network
Vang	Van Gogh

WntD	a member of *Drosophila* Wnt family
WLS	Wntless/Evi/Sprinter

SUMMARY

The precise molecular function of Amyloid Precursor Protein (APP) has long been an intensively studied and highly controversial topic. APP and its homologues are transmembrane proteins which have all the major features expected from a cell surface receptor, but whether it is indeed a receptor and what its ligand may be are unclear. Here we show that APP binds the Wnt ligands Wnt3a and Wnt5a and that this binding regulates APP protein levels. Wnt3a binding promotes full length APP (flAPP) recycling and stability. In contrast, Wnt5a promotes APP targeting to lysosomal compartments and reduces flAPP levels. A conserved Cysteine Rich Domain (CRD) in the extracellular portion of APP is required for Wnt binding, and deletion of the CRD abrogates the effects of Wnts on flAPP levels and trafficking. Finally, loss of APP results in increased axonal and reduced dendritic growth of mouse embryonic primary cortical neurons. This phenotype can be cell-autonomously rescued by full length, but not CRD-deleted, APP and regulated by Wnt ligands in a CRD dependent manner.

SAMENVATTING

De precieze moleculaire functie van Amyloïd Precursor Proteïne (APP) is lange tijd een intensief bestudeerd en zeer controversieel onderwerp geweest. APP en zijn homologen zijn transmembraanproteïnen die alle belangrijke kenmerken vertonen die van een celoppervlakte-receptor verwacht worden, maar of het inderdaad een receptor is en wat zijn ligand kan zijn, is onduidelijk. Hier tonen we aan dat APP bindt aan de Wnt liganden Wnt3a en Wnt5a en dat deze binding de APP eiwit niveaus reguleert. Binding met Wnt3a bevordert de recycling en stabiliteit van APP op volledige lengte (flAPP). Daarentegen bevordert Wnt5a APP targeting naar lysosomale compartimenten en vermindert het flAPP gehalte. Een geconserveerd Cysteine Rich Domain (CRD) in het extracellulaire gedeelte van APP is nodig voor Wnt binding, en deletie van het CRD heft de effecten van Wnts op flAPP niveaus en trafficking op. Tenslotte resulteert verlies van APP in verhoogde axonale en verminderde dendritische groei van muizen embryonale primaire corticale neuronen. Dit fenotype kan celautonoom hersteld worden door APP van volledige lengte, maar niet door APP met CRD-deletie, en wordt gereguleerd door Wnt-liganden op een CRD-afhankelijke manier.

CHAPTER 1 INTRODUCTION

1.1 APP and Alzheimer's disease (AD)

During the past three decades since Amyloid Precursor Protein (APP) was cloned[1], it has been intensively researched due to the Amyloid Betta (Aβ) sequences which exists exclusively in APP and its abnormal accumulation is considered to be one of the critical hall marker for the Alzheimer's disease (AD)[2]. AD is a neurodegenerative disease which was first described and named

by the psychiatrist and pathologist Alois Alzheimer in 1906[3]. This disease is characterized by progressive cognitive decline, memory loss and impairment of functional abilities[4]. According to epidemiological surveys, about 50 million people suffer from dementia globally, 60-70% of which are composed by AD patients, and the number is predicted to increase over time[5]. A lot of attentions and efforts were focused on AD treatment based on the amyloid cascade hypothesis, but clinical trial with drugs targeting Aβ have not been promising so far, indicating the complexity of the onset of the disease and challenging the simpler version of the Aβ hypothesis. Although much evidence supports the central role of Aβ in the pathology of Alzheimer's disease recently, increasing efforts are focused on exploring the physiological function of APP in the brain, as a better understanding of APP's function under normal conditions could bring new insights into AD treatment.

1.2 APP structure and expression

The Amyloid Precursor Protein (APP) is an ancient highly conserved type I one transmembrane protein. APP and its homologs are found across species from fly to human. In human and mice, APP family has three members: APP, APLP1 and APLP2. The numbers of APP homologs are different across species. *Drosophila* has only one APP homolog named APPL, and zebrafish harbor two homologues called

APPa and APPb. Interestingly, APP and its homologs seem to have evolved along with the appearance of organisms with a nervous system, as genes coding for APP-like proteins were not identified in plants, yeast and prokaryotes[6]. In mammals, there are 18 exons encoding APP, and alternative splicing creates several isoforms including APP770, APP751 and APP695. Expression of APP isoform 770 and 751 is detectable in different organs and tissues all over the body, while APP695 expression is brain-specific, mainly in neurons[7].

As a type I transmembrane protein, APP contains a large N-terminal domain targeted to the extracellular side of the membrane, one helix transmembrane domain and a short C-terminal domain. The large ectodomain of APP could be further divided into several subdomains: the E1 region, acidic domain (AcD), the E2 region and juxtamembrane region (JMR) (Figure 1). The E1 region contains a growth factor like domain (GFLD) and a copper binding domain (CuBD). Within the GFLD domain, the heparin binding site is known to have a function in neurite outgrowth[8] . The AcD domain, consisting of a high percentage of acidic amino acids, acts as a connector for E1 and E2. The E2 region harbors a second heparin binding site, a collagen binding domain (CBD) and a pentapeptide RERMS which has been reported to have a function in promoting neurite extension and fibroblast growth[9,10].

C-terminal to the E2 region is the JMR, which functions as a linker to connect E2 to the transmembrane region. In addition to the four regions mentioned above, regions like Kunitz-type-protease inhibitor (KPI) sequence (between AcD and E2) are present in longer APP isoforms like APP770 and APP751, but not in APP695 (Figure 1). One report showed that KPI was required for low-density lipoprotein receptor-related protein (LPR) mediated APP internalization[11]. The trans-membrane (TM) region connects JMR with the intracellular domain of APP. Little is known about the TM except for its helical structure and the presence of cleavage sites for

γ-secretase. The APP intracellular domain (AICD) is composed of 49 amino acids including a highly conserved YENPTY motif reported to interact with several docking proteins and plays an important role in regulating neuronal migration and synaptogenesis[12–16].

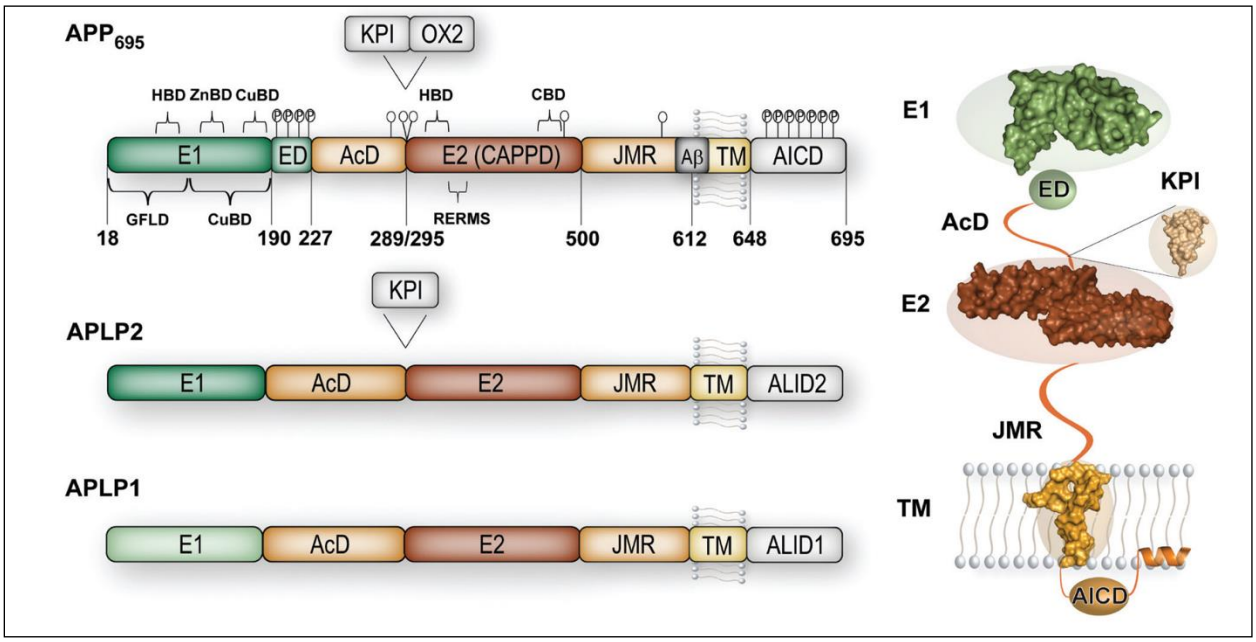

Figure 1. Domain architecture of the APP family members. (Coburger, Ina et al., 2014)

In mammals, APP, APLP1 and APLP2 harbor similar domain structures: (a) a large ectodomain including 1) conserved E1 region consist of growth-factor-like-domain (GFLD) and copper-binding-domain (CuBD), 2) the acidic domain (AcD), 3) E2 region compose heparin binding domain (HBD), RERMS and collagen binding domain (CBD) and 4) juxtamembrane region (JMR). (b) trans-membrane helix region. (c) Short intracellular domain (AICD). The extension domain (ED) is only present in APP. Phosphorylation and glycosylation sites are shown as P in circles and empty circles, respectively. Aβ sequence only present in APP. OX2 only in present in APP770, while Kunitz-type-protease inhibitor (KPI) presents in both APLP2 and APP770 APP751(longer isoform of APP due to the alternative splicing).

1.3 APP expression during development

Consistent with the correlation of the appearance of nervous systems in evolution, APP is expressed early during development of the central nervous system (CNS). In *Drosophila*, expression of APPL begins in embryonic stages and overlaps with

neurogenesis[17,18]. In mice, APP mRNA is detected at gastrulation using a LacZ reporter [19]. In rat, APP mRNA expression is observed at embryonic day 9 and its expression increases as development advances. In addition, regional specific expression of APP isoforms has also been observed in late developmental stages. For example, APP695 mRNA is specifically expressed in the derivatives of neuroectoderm including neural tube, neurogenic placed and neural crest, while APP770 mRNA expression is detected in regions derived from mesoderm and endoderm[20,21]. Human derived induced pluripotent stem cells (iPSCs) differentiated into cortical neurons *in vitro* show continuous expression of APP throughout the differentiation stages: from iPSCs to cortical neurons[22]. Those findings indicate an important role for APP during development, especially in the CNS.

1.4 APP trafficking and processing

Site specific proteolytic processing of APP generates the Aβ peptide in what is termed "the amyloidogenic pathway". In the Aβ cascade hypothesis, abnormal accumulation of Aβ is considered to play a central role in AD. Therefore, much effort has focused on studying APP trafficking and processing, which has led to a better understanding of the different aspects of the functions of APP's proteolytic products physiologically and pathologically[23].

In vitro experiments reveal that the half-life of full length APP (flAPP) is short, ranging from ~1 hour to 4 hours[24], indicating that the quick turn over of membrane flAPP requires a dynamic balance among synthesis trafficking, processing, secretion and degradation. APP is generated on endoplasmic reticulum (ER)[25], before reaching the cell surface through Trans-Golgi Network (TGN), APP goes through several post translational modifications including phosphorylation and glycosylation at the Golgi apparatus[26,27].

At the cell surface, APP can be cleaved by α-secretase before being internalized, in what is reffered to as “non-amyloidogenic processing” (Figure 2). The cleavage site for α-secretase resides within Aβ sequence between amino acid residue Lys16 and Leu17. A large soluble fragment of APP (sAPPα) will be released to the extracellular environment after cleavage by α-secretase, while the intracellular part of APP (CTFα or c83) remains attached on the membrane and can be further processed by γ-secretase after being endocytosed[28,29]. α-secretase activity is found in a set of membrane metalloproteinase, members of a disintegrin and metalloproteinase (ADAM) family, such as ADAM9, ADAM10 and ADAM17, all of which were shown to have the function as α-secretase[30]. In cultured neurons ADAM10 was reported to be the major enzyme that exerts the function of α- secretase, as a significant drop of the fragment released by α-secretase was observed in ADAM 10 but not in ADAM9 or ADAM17 knock out primary neurons [28,31].

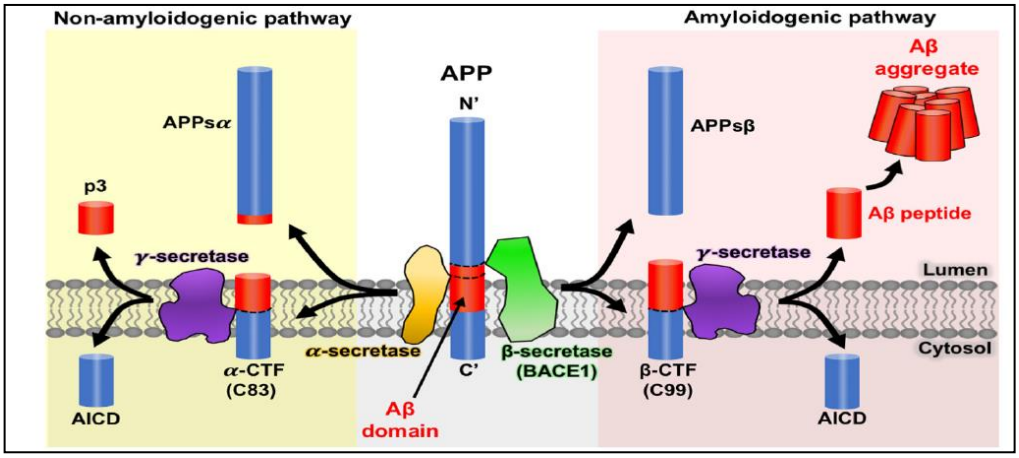

Figure 2. Cleavage of APP. (Tan, Jing et al., 2019)

Cleavage of APP mediated by three enzymes could be divided into two pathways: The non-amyloidogenic pathway shown in the left yellow background, in which APP first is cut by α-secretase on the site within the amyloid-β (Aβ) domain to release the soluble APPα (APPsα) fragment, and the remaining C-terminal fragment (α-CTF/C83) will be cleaved by γ- secretase liberating P3 and APP intracellular domain (AICD). In the amyloidogenic pathway, APP is first cleaved by β-secretase (BACE1) to release the APPsβ, and followed by the release of Aβ and AICD after the processing of the β-CTF (C99) by γ- secretase.

1.5 Trafficking route of APP after internalization

Internalization of nascent APP happens soon after it arrives at the cell surface[32]. This process is clathrin dependent[33]. The YENPTY motif of APP has been proven to be important for regulating APP endocytosis, as mutations in this motif prevent APP endocytosis and Aβ production[34]. Internalized APP is then transported to the endosomes, where APP encounters β- and γ- secretase initiating the amyloidogenic cleavage[35]. In addition to processing and degradation after delivery to the lysosome[36], fractions of APP can be transported back to the plasma membrane from the early endosome through the recycling pathway, or back to the TGN through the retrograde pathway[37,38] (Figure 3).

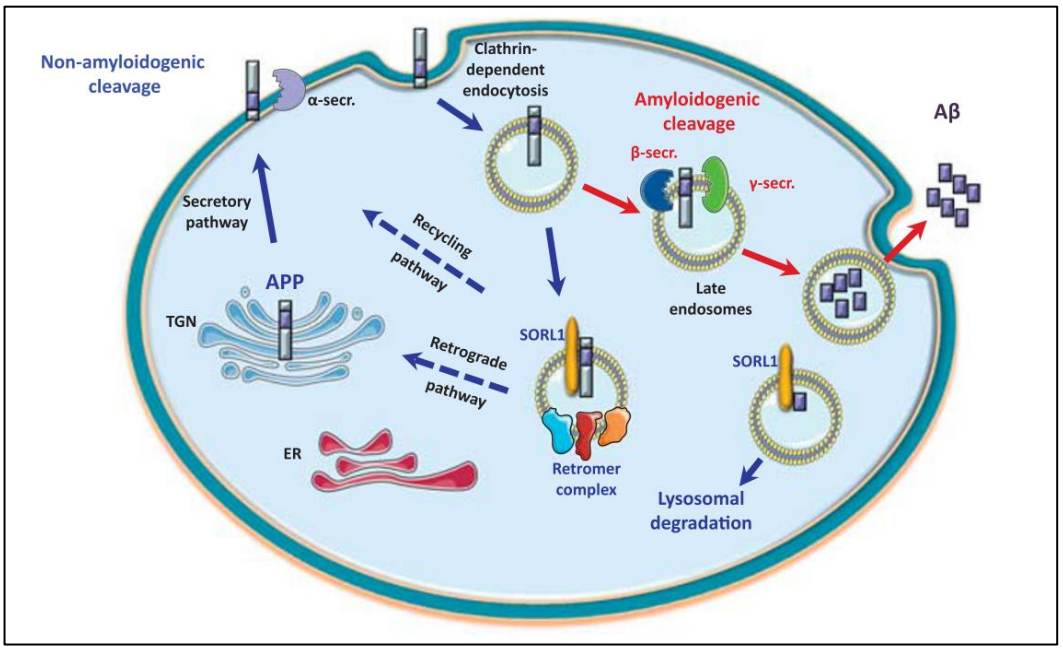

Figure 3. Schematic of APP trafficking and processing. (D Campion et al., 2016)

APP is translocated to plasma membrane (PM) after being modified at the Golgi through the secretory pathway. On the cell surface, APP can be processed by α-secretase (leads to a non-amyloidogenic cleavage followed by CTFα cut by γ- secretase) or internalized by clathrin dependent endocytosis to endosomes. In early endosomes, APP could be recycled directly back to PM or transported back to Trans-Golgi Network (TGN) through retrograde pathway. Alternatively,

APP could also sort to late endosomes where APP undergoes amyloidogenic cleavage mediated by β- and γ-secretase, fractions of released Aβs peptide can be secreted to the extracellular environment while other fractions are degraded in lysosome.

1.6 Amyloidgenic processing of APP

β-secretase plays a central role in APP amyloidgenic processing by initiating site specific cleave of APP and generating the Aβ peptide following cleavage by γ-secretase. The enzyme that possesses the activity of β-secretase is a type-1 membrane bound Aspartyl protease named BACE1[39]. The trafficking route of BACE1 is similar to APP, making it possible for BACE1 to encounter APP in different intracellular compartments[40]. However the cleavage is reported to take place in endosomes or TGN in which the acidic environment provides an optimal pH for the activity of BACE1[41–43]. After cleavage by BACE1, sAPPβ will be released. The and C-terminal fragment (CTFβ or C99), still tethered to the membrane, will be further cleaved by γ- secretase to generate the Aβ peptide (Figure 2)[26].

γ- secretase is a multiprotein complex made by 4 transmembrane subunits including presenilin-1 (PS1) or presenilin-2 (PS2), anterior pharynx-defective 1 (APH-1), presenilin enhancer 2 (PEN2) and nicastrin.[44]. PS harbors the core proteolytic function of the complex, while the other proteins are needed for the stabilization and maturation of the complex[45]. γ-secretase could be detected on TGN, endosome/lysosome systems and ER, and the cleavage of APP CTFs could happen in multiple places[46–48]. Aβ and P3 peptide are generated from the cleavage of APP CTFβ and α, respectively, by γ-secretase. This is accompanied by the release of the APP intracellular domain (AICD) from both CTFs (Figure 2)[49]. Abnormal aggregation of Aβ is considered to be the causative factor of AD in the Amyloid cascade hypothesis[50]. The length of Aβ sequence is different (ranging from 38 to 43 residues) as a result of continuous cleavage on multiple site by γ- secretase[49]. Aβ40

is the most abundant form in secreted Aβ, while Aβ42 is more toxic as this peptide is more prone to form oligomers[51,52].

Trafficking and processing of APP are affected by various adaptor molecules and its own post translational modification. Phosphorylation on SerS655 (based on APP695 numbering) was reported to enhance retrograde transfer of APP from early endosome to TGN, while dephosphorylation on this site promotes lysosomal targeting of APP[53–55]. SORL1, a member of low density lipoprotein receptor (LDLR) family, was shown to link APP with the retromer complex which directs APP sorting to the TGN, and mutations in SORL1 favor amyloidgenic processing[56–58] of APP. In another study, the prolyl isomerase Pin1 was shown to bind to APP phosphorylated on Thr668 (APP 695 numbering) and to regulate the conformation of APP, leading to a reduction in Aβ generation[59]. MINT3, a member of the adaptor protein family, is required for the export of APP from TGN to cell surface and mutations in MINT3 cause rerouting of APP to the lysosome[60]. Trafficking and processing of APP play an important role in the hypothesis of Aβ-based AD pathology, and there is increaisng evidence indicating that this neurodegenerative disease is likely initiated by dysregulation of APP trafficking followed by altered processing which results in abnormal Aβ accumulation.

1.7 Roles of APP in CNS development

The potential role of APP during development especially in CNS has been indicted by serval facts as mentioned before: Evolutionarily, the appearance of APP encoding genes coincides with the evolution of the nervous system; temporally, APP mRNA starts to be expressed during early brain development; spatially, specific APP isoforms and homologs are predominantly expressed in neurons. Along with the efforts to explore the pathogenesis and therapeutic strategy for AD, emerging evidence from different species reveals functions for APP and its proteolytic

fragments in many aspects during CNS development including cell proliferation and differentiation, neuronal migration, neurite growth and synapse formation, although inconsistent results from *in vivo* and *in vitro* studies still exist.

1.7.1 APP in neurogenesis

APP is shown to be expressed early during CNS development, indicating that APP may have functions in neurogenesis. Evidence from cultured human embryonic stem cells (hESCs) suggests that the derivatives of APP processing, like sAPPα and sAPPβ, could suppress cell proliferation and drive hESC differentiation towards a neural fate[61,62]. In contrast to the cell proliferation effect observed in hESC, sAPPα was shown to enhance the proliferation of neural stem cells (NSCs) derived from rat fetal neocortex[63,64]. Furthermore, isolated neural progenitor cells (NPCs) from APP knock out mice showed a reduction of cell proliferation rate, while APP overexpression increased cell proliferation compared with controls[65]. In addition, α-secretase blockage leads to a proliferation reduction in NPCs derived from the adult mouse subventricular zone (SVZ) which could be rescued by the recombinant sAPPα[66,67]. In addition to soluble APP fragments, Aβ and AICD were also shown to affect neurogenesis in hNSC. Aβ treatment was reported to inhibit cell division and drive NSC towards gliogenesis instead of neurogenesis in a GSK3β signaling dependent manner[68]. In contrast, another study found that the proliferation of NSC from rat SVZ was enhanced by Aβ addition and led to a path of neurogenesis[69]. This inconsistency could be explained by the use of cells from different species, different timing, or different dosages of Aβ. AICD was shown to inhibit cell division in NPCs, and this effect may come from the suppression of EGFR, which was proven to induce division in NPCs[70,71]. Furthermore, proliferation of hippocampal NPCs was hampered when AICD was introduced in the APP knock-out mouse[72].

1.7.2 Neuronal Migration

As a type I transmembrane protein, the large extracellular domain provides APP a property which resembles cell Adhesion molecules (CAM), like binding with various molecules in the extracellular matrix (ECM) and intracellularly interacting with cytoskeletal proteins[73]. Hence, APP could play an important role in neuronal migration due to CAM is critical for coordinating neuronal integration at the final destination. In transgenic mice with the triple knockout of APP, APLP1 and APLP2, a phenotype with over migration of neurons into the marginal zone has been reported[74]. Paradoxically, APP was also shown to be required for the neuronal migration. Thus knockdown of APP in rodent by *in utero* electroporation hampers NPC migrating from ventricular zone to the cortical plate, and overexpression with hAPP induces an acceleration in cell migration[75]. In addition, several molecules have been proposed to interact with APP in regulating cell migration. For example, DAB1, Fe65 heterotrimeric G protein Go and disrupted-in-schizophrenia 1 (DISC1) have been shown to regulate cell migration by interacting with APP[76–78]. Results from other species, like in *Manduca sexta*, APPL (APP homolog) are critical for the proper neuronal migration by acting as a receptor for Contactin[79].

1.7.3 Neurite outgrowth and synapse formation

There is significant evidence linking APP to the regulation of neurite outgrowth and synaptogenesis. One of the important clues relating APP to neurite outgrowth is that APP is enriched in the axonal growth cone (GC), which is critical for appropriate pathfinding of neuronal process[80–83]. To some extent, results from *in vitro* experiments show contradictory results as to whether APP promotes or inhibits neurite outgrowth. This inconsistency may derive from the use of different cell types, different analysis time points, different culture media and so forth. An *in vivo* study in *Drosophila* showed that APPL is required cell-autonomously for regulating axon

outgrowth in mushroom body in a Wnt/PCP signaling dependent manner[84]. In zebrafish, APPb is required cell-autonomously for axonal process extension in spinal motor neurons. Interestingly, full length human APP is shown to have the ability to rescue the phenotype caused by APPb mutations[85]. In mice, a decrease in both brain weight and the size of forebrain commissures was observed after APP knockout[85]. Later, Rama et al. revealed that APP plays as a co-receptor facilitating netrin-1 binding with APP-DCC complex and induces downstream signaling for proper commissural axon guidance[85].

With respect to synaptogenesis, APP expression increases with synapse formation[85]. Furthermore, homophilic interactions of APP residing at the pre and post synaptic terminals were found to be critical to establish the synapse[86–88]. *In vivo* work in *Drosophila* showed that formation of new synapses is based on the interaction between APPL, X11L and the cell adhesion molecule Fasciclin II[89]. In mice, defective dendritic development followed by loss of synaptic function could be induced by APP knockout[90]. Overexpression of APP could dramatically increase dendritic spines in cultured hippocampal neurons[91], and consistent with this, *in vivo* work in mice indicates that APP overexpression promotes dendritic arborization and elevates spine density in specific regions in both cortex and hippocampus[92].

1.8 Wnt Signaling

1.8.1 General introduction to Wnt signaling pathway

Wnt signaling is an evolutionary conserved pathway that consists of a group of signal transduction pathways. Wnt signaling plays a critical role during embryonic development. It operates in both vertebrates and invertebrates, including humans, mice, zebrafish, *Drosophila* and others. Based on different Wnt ligands and receptors, there are three well characterized Wnt-signaling pathways: the beta-Catenin based canonical Wnt pathway which is commonly activated by the Wnt3a ligand; the non-canonical planar cell polarity pathway which is commonly activated by the Wnt5a ligand; and the non-canonical Wnt/calcium pathway. Wnt signaling pathways regulate various features during development, such as cell proliferation, migration and differentiation[93]. Recently, increasing evidence indicates that the Wnt signaling pathways are involved in APP related Abeta production[94,95], but the precise mode of interaction between APP and the various Wnt pathways remains unclear.

1.8.2 Wnt structure

Wnts constitute a diverse family of conserved secreted lipid-modified signaling glycoproteins 350–400 amino acids in length. Based on the analysis of primary amino acid sequence, all Wnts contain highly conserved 23-25 cysteine residues[96] that maintain the secondary structure of Wnt through forming disulfide bridges[97]. Glycosylation and posttranslational lipid modifications are reported for most Wnt ligands and are essential for their functions[98,99].

Studies focused on revealing the structures of Wnt family members are relatively few due to the strong hydrophobic property of Wnts, which increases the difficulty of protein purification. However, structures of several Wnts protein have been solved during the past 10 years, including WntD, xWnt8a and hWnt3a[100–102]. The

first description of a crystal structure of xenopus Wnt8 binding with the Cysteine-Rich Domain (CRD) reveals two novel protein structure subdomain and the critical role of Wnt lipidation which contributes to the interaction with its receptor Fzd[100].

1.8.3 Intracellular modification processing and transportation

After translation all Wnts will be targeted to the ER through a signal peptide, whereafter, multiple post-translational modifications (PTMs) of almost all Wnts will take place, except for the WntD (a member of *Drosophila* Wnt family), which was reported not to undergo PTMs like glycosylation or acylation[103]. Processed Wnts then will be transferred to the plasma membrane via the Golgi network[104]. Although many factors responsible for proper Wnts trafficking and secretion have been discovered, how modified Wnts are trafficked intracellularly is not fully understood. Among those factors, the function of Wnt lipid modification is relatively well documented and reported to be indispensable for Wnt trafficking, secretion and singnaling[105]. Lipidation of Wnts occurs at the ER and the O-acyltransferase Porcupine (Porcn) –an ER resident transmembrane protein of the MBOAT (membrane associated O-acyl transferase) family is essential to transfer palmitoleate or myristoleate to a conserved serine of Wnts[98,106,107]. Mutation or inhibition of Porcn has been reported to cause a significant secretion problem due to preventing Wnt transfer out of the ER[98,108,109]. Further studies revealed that the failure of lipidation by Porcn prevents Wnt from binding to WLS (also known as GPR177 in mice or Wntless/Evi/Sprinter in *Drosophila*) in the ER, a multispan transmembrane protein required as a chaperone for Wnt membrane targeting. This is because the palmitoleic acid moiety of Wnt is critical for binding to WLS[104,110–112]. Moreover, correct lipid modification is also important for Wnt signaling, as the recognition and binding between Wnt and its extracellular carriers is based on the fatty acylation at a conserved serine residue, as well as being needed for binding with the Wnt receptor

Frizzled(Fz)[100,113–115]. Beside lipid modification, glycosylation of Wnts is also observed, but the role of glycosylation is not clear compared to lipidation. It is proposed to play an important role in Wnt secretion[116,117], while other studies indicate a minor effect on Wnt, suggesting that it is largely dispensable[118,119].

1.8.4 Secretion

As a secreted protein, Wnts are important for intercellular communication, but one may ask how Wnts are trafficked extracellularly given their hydrophobic property due to lipidation? Fully processed Wnts show long-range diffusion from the cell of origin to the target cells without carriers. Several methods have been proposed to explain how Wnts diffuse and form a signaling gradient. One of the mechanisms is through lipoproteins, monolayer extracellular particles that mediate intercellular exchange of proteins[120]. Co-localization of fly Wg with Lipophorin (Drosophila homologue of lipoproteins) has been reported, and the knockdown of Lipophorin impairs gradient formation of Wg[121]. In mammals, both Wnt3a and Wnt5a were reported to associate with lipoproteins for secretion[122,123]. Exosomes have been proposed as another method to mediate Wnt diffusion. Exosomes are double membraned subcellular structure contained within multi-vesicular bodies (MVBs). After fusion of MVBs with the membrane, exosomes are released and trafficked through the extracellular matrix with their cargoes[124,125]. Data from in vitro experiments showed co-localization of both Wg and Wnt3a with exosome markers suggesting a role of exosomes in aiding the secretion of Wnts[126,127]. Furthermore, in vivo studies in flies showed that Rab11 knock down induced exosome release reduction which could affect the transfer of Wg across neuromuscular junction (NMJ)[126,127]. Beside possible vesicle based Wnts diffusion, it has been proposed that protein chaperones could improve Wnts' solubility by shielding their hydrophobic structure. In Drosophila, a lipocalin protein termed Swim (Secreted Wg-interacting

Molecule) has been shown to interact with and facilitate Wg long-rang diffusion, and knockdown with RNAi reduces the distance of Wg secretion[128]. In human, Afamin, a serum albumin family member, was shown to enhance Wnt3a solubility and secretion by shielding lipids on Wnts[128]. In addition to the diffusion of Wnts via vesicles or chaperones in the ECM, cytonemes (actin based filopodia capable of presenting signaling protein to a distant receiving cells[129–131]) have also been proposed as one of the potential mechanisms. For example, in vertebrate zebrafish, Wnt8a has been shown to be presented on cytonemes to induce signaling by a receptor on a distant cell[132]. The case in Drosophila is reversed in that Frizzled (Fz) containing cytonemes have been reported to extend to bind Wg in donor cells[133]. All these mechanisms are context specific and tissue dependent, and it is not clear if these mechanisms work in a cooperation or independently. Further studies are needed to investigate these questions. In contrast to the idea that Wnt needs to be released to deliver information to target cells at a distance, experiments in Drosophila mutants have shown that flies with membrane tethered Wg are viable but show smaller wings and a delay in development thus challenging the necessity of Wnt secretion for patterning and growth[134].

1.8.5 Canonical Wnt signaling pathway

Canonical Wnt signaling, also known as Wnt/β-Catenin signaling pathway, is one of the most studied Wnt pathways and is characterized by the dependency on β-catenin. Activation of the canonical Wnt signaling pathway could be briefly divided into three steps: first, extracellularly, binding of Wnt ligands with receptor and co-receptor. Several Wnt ligands including Wnt3a, Wnt7a and Wnt8 can trigger β-Catenin dependent pathway have been classified as canonical ligands[135]. Members of the Frizzled (Fz) family serve as receptors for both β-Catenin dependent and independent Wnt signaling pathways, and the conserved cysteine rich domain (CRD)

is recognized and bound by Wnts[100]. In addition, there are several co-receptors like low-density lipoprotein receptor-related proteins 5/6 (LRP5/6) which are mostly reported in regulating the canonical Wnt pathway[136,137]. Second, intracellularly, cytoplasmic signal transduction is initiated after the formation of Wnt, receptor and co-receptor complexes. A so called "β-Catenin destruction complex" is recruited which is used to maintain a certain amount of cytoplasmic β-Catenin by phosphorylation, degradation and disassembling [138,139]. As a consequence, newly synthesized β-Catenin becomes stable and accumulates in the cytoplasm. Third, stabled β-Catenin will be translocated into the nucleus, and binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) by replacing the transcriptional repressor Groucho[140]. Finally the target genes of canonical Wnt pathway will be activatied[141,142] (Figure 4).

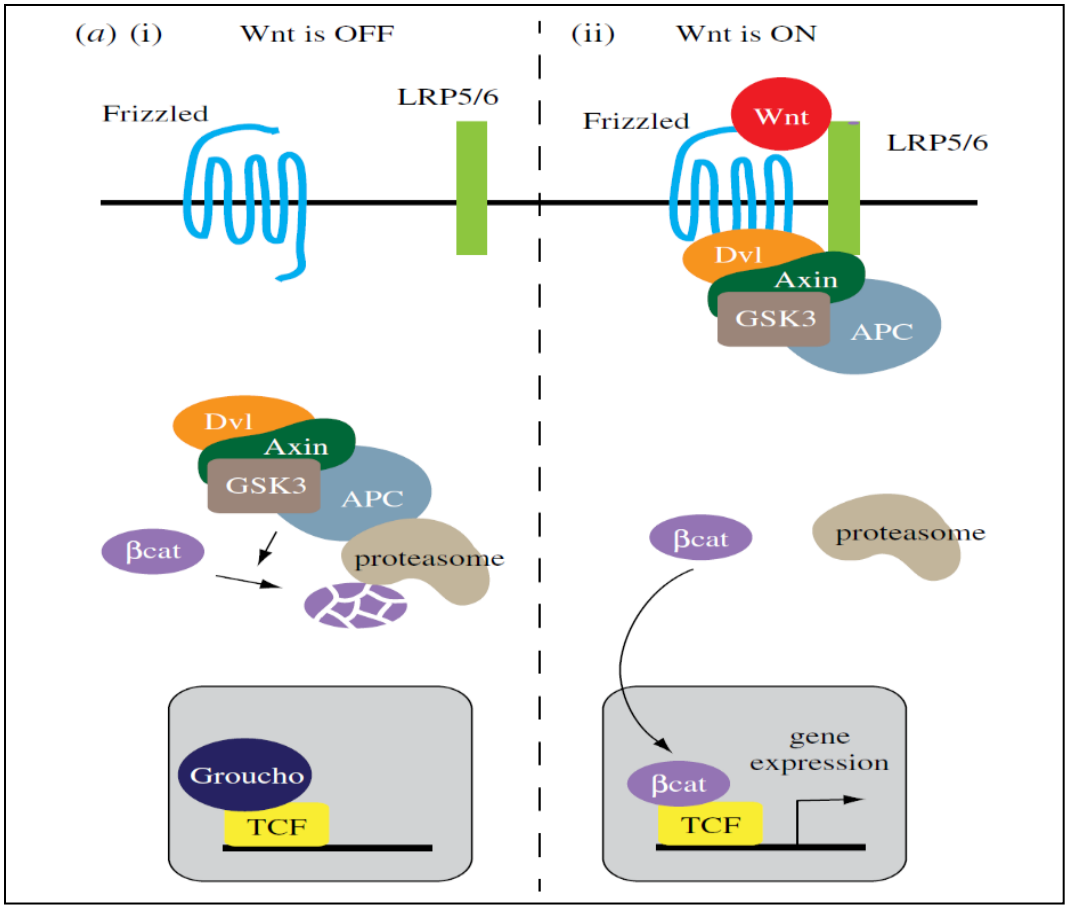

Figure 4. canonical Wnt signaling pathway. (He, Chun Wei et al., 2018)

(i) In the state of Wnts off, first cytoplasmic β-Catenin is phosphorylated by the destruction complex (APC, Axin and GSK3), then be ubiquitinated and degradated by the proteasome. (ii) In the state of Wnts on, β-Catenin is released from degradation and then translated into nucleus to initiate target genes transcription with TCF.

1.8.6 Non-canonical (beta-Catenin independent) Wnt signaling pathway

In addition to canonical Wnt signaling pathway, there are beta-Catenin independent Wnt signaling pathways, which are activated by the Wnts binding to Fz receptors and facilitated by an increasing variety of co-receptors. Wnt4, Wnt5a and Wnt11 are identified as classical non canonical Wnt signaling ligands[143]. Two sub-pathways are

categorized within the scope of non-canonical Wnt signaling: the Wnt/Planar Cell Polarity (PCP) pathway and the Wnt/Ca2^{+} pathway (Figure 5).

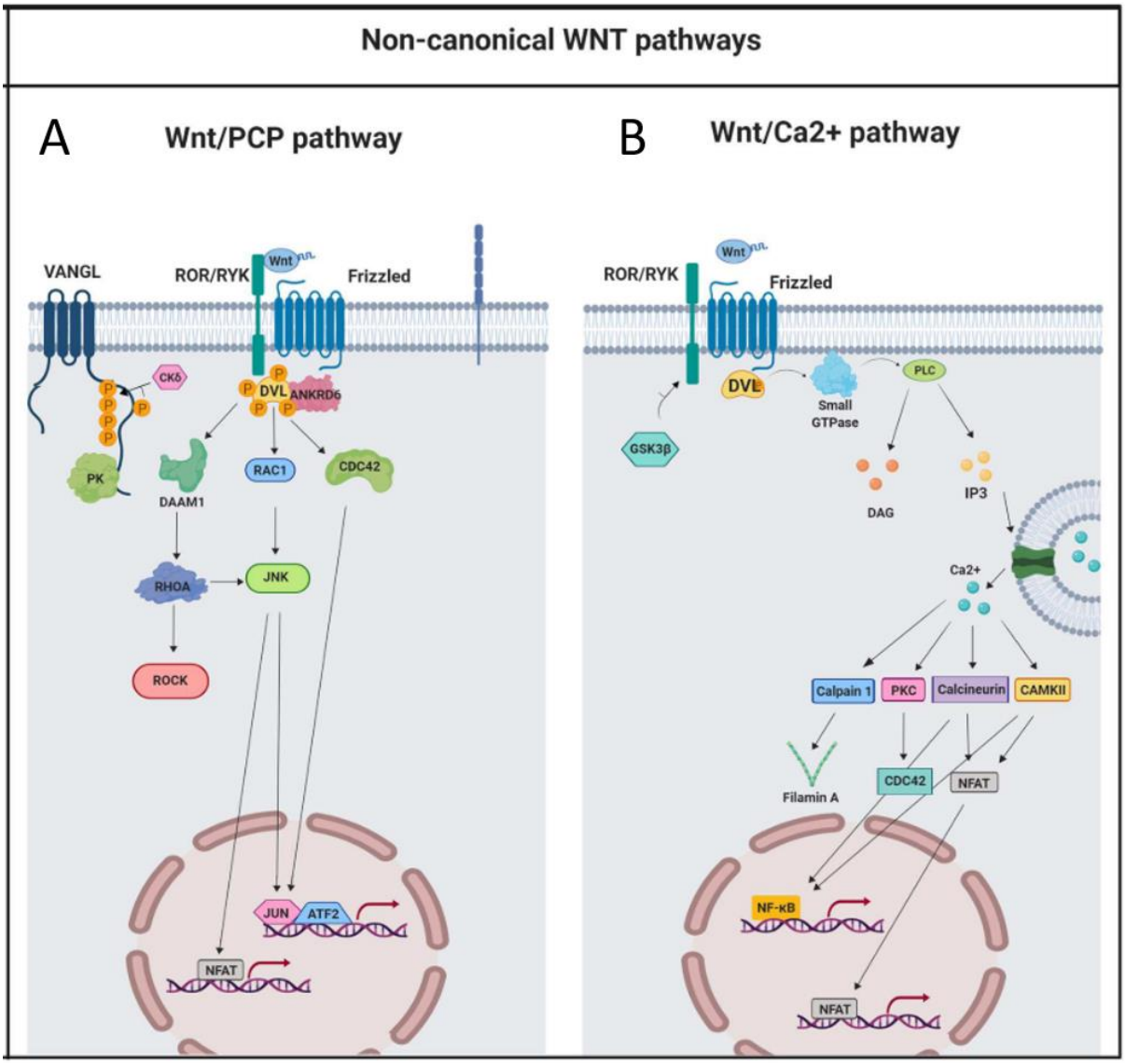

Figure 5. Non-canonical Wnt signaling pathway. (Elena et al., 2019)

(A) Wnt/PCP pathway. After Wnts binding with receptor and co-receptor, Dvl will be recruited and activated followed by binding with small GTPase, then Rock or JNK could be activated by RAC1 and RHO and finally results in cytoskeleton rearrangement or target genes transcription. (B) Wnt/Ca^{+} pathway. After Wnts binding with receptor and co-receptor, Dvl will bind with small GTPase followed by activation of APC, and finally triggers Ca^{+} fluxes and downstream genes transcription or cytoskeleton remodeling.

1.9 Wnt/PCP pathway

In multicellular organisms, almost all cells, tissues and organs show degrees of polarity, which is required for exerting proper function. An obvious instance is the epithelial cells in different tissues, characterized by the apical-basal polarization, which is critical for the patterning, homeostasis and functionality of organs[144,145]. During the morphogenesis of specific tissues or organs, signaling that regulating cell differentiation and proliferation provides a coarse frame. Achieving the final architecture directional information is needed to regulate the position and polarity of those cells. PCP initially identified in *Drosophila*[146–148] was found critical for regulating cell/tissue polarity. Ensuing studies confirmed that the PCP pathway is also conserved in many aspects during development in vertebrates, including convergent extension (CE), sensory hair cell polarity in the inner ear, limp bud developing, elongation etc[149–154]. In addition to the most common polarization phenomenon observed in the epithelium, PCP has also been reported to play an important role in regulating neuronal migration, polarity, axonal guidance and dendritic branching[155–160].

1.9.1 The establishment of PCP framework

The basic knowledge of molecular and cellular mechanisms underlying PCP came from the studies in *Drosophila*. Two groups of factors were identified to form the PCP framework, the core PCP proteins and the Fat/Dashsous (Ft/Ds) group. The Ft/Ds system is well analyzed in fly, although, it is conserved in vertebrate but less studied. In mammals, mild PCP phenotypes were observed in Ft/Ds system mutants[161,162].

The fly core PCP system consists of six key components. Three are transmembrane proteins including Fz, Flamingo (Fmi; celsr in vertebrate) and Van Gogh (Vang; Vangl in vertebrate), while the other three are cytoplasmic proteins including Dishevelled (Dsh; Dvl in vertebrate), Diego (Dgo; Diversion in vertebrate) and Prickle (Pk)[163]. These six proteins could be further divided into two complexes. Asymmetric localization of these two complexes is a conserved feature of PCP signaling which is observed both in invertebrate and vertebrate. In vertebrates, the situation is more complicated due to the redundancy of multiple members of each core protein[164]. In general, after activation of PCP signaling Fz-Fmi-Dsh-Dgo form a complex located at the distal side of cells, while Vang-Fmi-Pk reside on the opposite side (Figure 6).

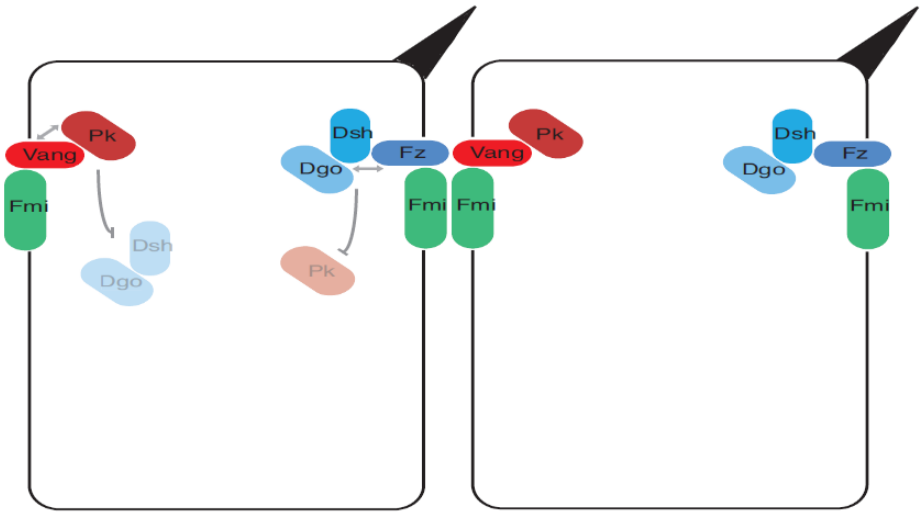

Figure 6. Localization of core PCP components. (A. Humphries et al., 2018)

Upon the activation of PCP signaling, these six core proteins asymmetrically distributed in the opposite side of cells. Fz and Vang locate at distal and proximal side, respectively. Fmi facilitates the interaction between neighboring cells which could be found at both sides and also interacts with Fz and Vang. Dsh and Dgo are recruited by Fz, while Pk is recruited by Vang.

Both inter- and intracellular interactions between the core PCP proteins are required for proper positioning. For instance, membrane recruitment of Dsh towards Fz could

be inhibited by Pk, while Dgo could antagonize Pk's inhibition on Dsh, therefore stabilizing the interaction between Fz and Dsh [165,166]. Intracellularly, Fz has been reported to interact with Vang which may be facilitated by the Fmi-Fmi homophlic adhesion in the neighbouring cell, and Fmi is colocalized with Fz and Vang[167,168].

1.9.2 Initiation of PCP signaling

Core PCP proteins are uniformly spread on the membrane prior to the activation of PCP. Identification of the PCP upstream regulators is a popular research topic in the PCP field. Wnt family members are considered as attractive candidates because they bind to Fz. Based on recent work, an instructive role for Wnts in PCP has been suggested. Wg and dWnt4 were found to redundantly orient PCP proteins in the fly wing[169]. In chick, Wnt11 acts as an instructive cue for PCP to regulate Myogenesis[170]. In mice, Wnt5a gradients regulate elongation by phosphorylating Vangl2[171]. Although increasing evidence supports the instructive role of Wnt family members in PCP signaling pathway, the underlying mechanism of how Wnt affects core PCP proteins asymmetric localization is not well documented.

1.9.3 Signaling downstream of Wnt/PCP signaling

As Wnt may trigger PCP signaling, the question is what is the downstream effect? Studies in flies and vertebrates reveal several downstream genes in Wnt/PCP signaling, including small GTPases such as Rac1 and RhoA which will further activate MAP3K-JNK signal cascade and Rho associated Kinase (ROK), respectively. ROK influences the rearrangement of cytoskeleton[172,173], while the JNK signal cascade triggers transcription of specific gene in the eye of the fly[174]. In vertebrates, Wnt5a/JNK is required for shaping the limb bud and also needed for regulating convergent extension (CE) movement[175,176].

1.10 Wnt/Ca^{2+} pathway

Wnt/Ca^{2+} pathway is a G-protein based Wnt pathway, which is Beta-catenin independent but also shows distinct downstream features compared to PCP signaling, making it a separate branch of the Wnt pathway. As a receptor, Fz is also required to initiate Wnt-Calcium pathway. Wnt5a and Wnt11 are putative ligands to trigger the downstream signaling cascade[177]. G-protein and Phospholipase C (PLC) are activated in membrane after Wnt binds to the receptor leading to the cleavage of PIP2 and the release of IP3 and Diacylglycerol (DAG). IP3 binds to its receptor IP3R, which resides on the rugose endoplasmic reticulum (RER) membrane, with a consequence of increasing Ca^{2+} in the cytoplasm. In parallel, DAG assists the efflux of calcium from RER lumen. Accumulating cytoplasmic Calcium further activates Calcium-dependent Kinase II (CamKII) and Protein Kinase C (PKC). Both PKC and CamKII are reported to regulate transcription factors and transcription of several Wnt target genes.

1.11 Wnt signaling in CNS development and disease

As a highly conserved morphogen, Wnt and its pathways have been considered as one of the most important signals during development and maturation of central nervous system (CNS). A plethora of evidence shows that Wnt signaling is required for diverse neuronal development including neuronal polarization, axonal outgrowth and branching, dendritic arborization, formation of synapse, etc[178,179].

Morphologically, neurons are one of the most polarized cell types, characterized by the two distinct subcellular compartments: axons and dendrites. Functionally, axons and dendrites are fundamental for the unidirectional signal transfer property of neurons[180,181]. Beside the intracellular factors, extracellular signals like Wnt signaling are also required for initial axon differentiation and maturation.

In C. *elegans,* LIN-44 CWN-1 and EGL-20 (three Wnt homologs in C. *elegans*) are needed for proper polarization of mechanosensory neurons, as mutant in those Wnts could cause a reversal neurite polarity in those neurons[182–184]. In isolated rat hippocampal neurons, Dishevelled 1 (Dvl 1) was reported to stabilize atypical protein kinase C (aPKC), which is critical for axon-dendrite polarity by forming tripartite complex with PAR3 and PAR6[185,186]. Mutations in Dvl attenuates axon polarity, while overexpression of Dvl induces multiple axon formation. Furthermore, Wnt5a was showed to be a putative upstream regulator for the axon differentiation through the Dvl-aPKC interaction[187], indicating the participation of Wnt pathway in neuronal polarity. However, *in vivo* experiments in flies showed that Par3, Par6 and aPKC are dispensable for axon polarization[188]. Hence, more *in vivo* experiments are needed to test those interactions and molecules that were found *in vitro*, as redundancy of pathways or molecules may exist.

With respect to neurite guidance, outgrowth and branching, Wnt5a functions as a downstream regulator of Nerve growth factor (NGF) in regulating axon outgrowth and branching in developing sympathetic neurons by activation of PKC[189]. In cultured cortical neurons, Wnt5a was reported to induce axon outgrowth and axonal repulsion, simultaneously. The Wnt receptor, Ryk, is required for axon outgrowth, while both Ryk and Frizzled are needed for repulsive axon turning[190]. In *Drosophila*, components of Wnt/PCP signaling pathway and Wnt5a were reported to play an important role in axon targeting and bifurcation in the mushroom body which is required for learning and memory[191]. Studies carried out in mice revealed that mutations in Celsr3 (key PCP component in mice, ortholog of fly Flamingo) exhibit a similar neural phenotype as Fzd3 mutant mice, namely a defect in axon guidance[192]. Another study showed that Wnt4 is required for commissural axons projection in the

mouse spinal cord in a Fzd3 dependent manner as mutations in Frizzled 3 cause a phenotype of failure of anterior-posterior guidance after midline crossing[193].

Wnt signaling also regulates dendrite outgrowth and morphogenesis. In cultured rat hippocampal neurons, β-Catenin was reported to be critical in regulating dendrite morphogenesis in a depolarization condition induced by high K^+. Overexpression of β-Catenin enhanced dendritic arborization, while sequestering of β-Catenin leads to a decrease in dendritic branch tip number and total dendritic branch length. This effect is not gene transcription related but more likely dependent on the interaction between β-catenin, N-cadherin and αN-catenin. Interestingly, an increment in Wnt expression is observed, indicating that Wnt is involved in this effect[194]. Experiments from another group revealed that depolarization induced by K+ in dissociated rat hippocampal neurons and slices increases VREB-dependent expression of Wnt2, which in turn enhances dendritic complexity[195]. Consistent with these reports, Wnt7b and Dvl were reported to stimulate dendrite morphogenesis by activation of Rac and JNK signaling, which could be blocked by Sfrp1(a Wnt antagonist)[196]. Work in *C.elegans* showed that LIN-44/Wnt through its receptor Lin17/Frizzled acted as an attractive cue for the outgrowth of dendrite during the development of oxygen sensory neurons[197].

With respect to synapse formation and function, Wnt ligands and Wnt signaling have been revealed to participate in the assembly of synaptic structural components and assistance of neural transmission. Wnt7a was reported to be expressed during the period of synapse formation in cerebellar granule cells (GCs), and contributes to the clustering of Synapsin I, a presynaptic protein required in synaptogenesis. Mutation of Wnt7a in mice showed a delay in synapse defferetiation[198,199]. In hippocampal neurons, Wnt7a and Wnt3a are also able to induce the assembly of presynaptic proteins (Synaptotagmin, Synaptic vesicle protein2 and Synaptophysin) and regulate

recycling of synaptic vesicles[200,201]. In addition to the role of Wnts in presynaptic formation, Wnt5a was shown to regulate clustering of Postsynaptic Density Protein 95 (PSD95), which acts as a membrane scaffold for the clustering of receptors or ion channels via non-canonical Wnt/JNK signaling[202]. In another report, Wnt5a was revealed to stimulate the formation of dendritic spines and affect glutamatergic transmission, which is Wnt/Ca^+ signaling dependent[203]. *In vivo* experiments in mice showed that conditional mutations in the key PCP component Celsr3 results in a significant decrease in hippocampal glutamatergic synapses[204].

Wnt signaling is critical in many aspects of CNS development and maintenance. Therefore, links between neurodegenerative disorders and Wnt signaling disruption have been well acknowledged[205]. Alzheimer's disease (AD) is characterized by progressive loss of memory and cognitive ability. Underlying mechanisms of the initiation of AD are not well understood. Accumulating evidence indicates that Wnt signaling pathways are also involved. For instance, in AD patients with mutations in presenilin 1 (PS1), a decrease in β-Catenin levels was observed[206], suggesting a decrease in canonical Wnt signaling. Similarly, an increase of Dickkopf-1 (Dkk1), a competitive inhibitor of Wnt/β-Catenin signaling, was found in both postmortem AD patients and AD models in mice[207,208]. Other studies revealed that exposure to Aβ could enhance Dkk1 expression in cultured neurons, and this is critical in Aβ mediated neurotoxicity[209–211]. Intriguingly, canonical and non-canonical Wnt signaling seem to play a opposite roles in Aβ production[95]. In addition to the Wnt antagonist Dkk1, mutations in LRP6 and APOE genes are considered risk factors for late onset AD, which also exhibits a reduction in canonical Wnt signaling[212]. As components of Wnt signaling are dysregulated in AD, restoring Wnt signaling is considered as a potential therapeutic approach. Activation of canonical Wnt signaling in transgenic mouse AD models was shown to improve the outcome of

cognitive tests, spatial memory and to reduce Aβ production. These data support an important role for the Wnt pathway in AD.

CHAPTER 2 AIMS OF THIS STUDY

As introduced above, Wnt siganling pathway plays an important role during CNS development and APP is expressed throughout CNS development. Therefore, it is interesting to ask if there is an interaction between APP and Wnt signaling pathway during the CNS development. In a previous study from the lab, we showed that APPL (the *Drosophila* APP homolog) functions as a key component of the Wnt/PCP signaling and regulates the axonal outgrowth in fly mushroom body[213]. Our unpublished data showed that the Cystein Rich Domain (CRD) of the APPL extracellular E1 region could be a critical binding site for Wnt5a to regulate fly mushroom body axon outgrowth. The CRD like domain is highly conserved in APP across species, and its sequence resembles the binding site in Wnt receptors like Fz1 Ror2, which makes APP a putative Wnt receptor. Whether the binding of APP to Wnt5a is conserved, whether APP also binds other Wnts and what the cell biological consequences of this binding were unknown. Here we used developing mouse primary cortical neurons as a model to explore the APP-Wnt interactions in developing neurons. In order to explore the nature of the interactions between Wnts and APP, we developed three specific aims.

Aim1: To study if the CRD like domain is critical for APP binding to Wnt.

Aim2: To explore if Wnts interact with APP in maturing primary cortical neurons.

Aim3: To study if the CRD like domain is critical for Wnt to regulate neurite outgrowth.

CHAPTER 3 MATERIALS AND METHODS

3.1 Primary cortical neuron culture, virus transduction and plasmids transfection

The animal experiments were carried out in accordance with animal welfare regulations and have been approved by Ethic Committee and French regulatory authorities of the respective institutes. APP knock out mice were a gift from the De Strooper lab. Cortical primary neuron cultures were prepared from embryonic day 16.5 mice (APP wild or APP mutant), as described previously[214].

Virus (pLv-pSyn1-mAPP-Flag-IRESeGFP, pLv-pSyn1-mAPPΔCRD-Flag-IRESeGFP or pLv-pSyn1- eGFP) transduction was performed during seeding in 24 well plates ($4x10^5$cells/mL). 50 uL (50 uL per well) of the adequate lentiviral dilution in the medium of interest must be ready in tubes. Seed 150uL of the cell preparation to each well. Immediately add 50 ul of the diluted lentiviral preparation to each well (the final multiplicity of infection (MOI) is 2, same MOI used for each group). Mix slowly the cells-lentivirus suspension by pipetting. Incubate 1h at 37°C. Finally add 800 uL of culture medium to each well and incubate for 3 additional days before any analysis.

Plasmids (pLv794_pTrip_PromSynaptin1_GFP_DeltaU3, pLv-pSyn1-MmApp-FLAG-IRES-eGFP or pLv-pSyn1-mAPPΔCRD-FLAG-IRES-eGFP) transfection was performed at the onset of cell seeding ($4x10^5$cells/mL) in 24 wells plates with coverslip coated with PDL 24 hour before. All procedure follows the protocol from Lipofectamine 3000 transfection reagent (Thermofisher Catalog Number: L3000008) with little modified, each well transfection with 500 ng corresponding plasmid, medium was refreshed 5-6 hours after transfection.

3.2 Amino acids sequence of deleted CRD

Amino acids with underscore are deleted in mouse or human APPΔCRD

hApp695 (NCBI Reference Sequence: NP_958817.1):

MLPGLALLLLAAWTARALEVPTDGNAGLLAEPQIAMFCGRLNMHMNVQ
NGKWDSDPSGTKTCIDTKEGILQYCQEVYPELQITNVVEANQPVTIQNWC
KRGRKQCKTHPHFVIPYRCLVGEFVSDALLVPDKCKFLHQERMDVCETHL
HWHTVAKETCSEKSTNLHDYGMLLPCGIDKFRGVEFVCCPLAEESDNVDS
ADAEEDDSDVWWGGADTDYADGSEDKVVEVAEEEEVAEVEEEEADDDE
DDEDGDEVEEEAEEPYEEATERTTSIATTTTTTTESVEEVVRVPTTAASTPD
AVDKYLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWEEAERQAK
NLPKADKKAVIQHFQEKVESLEQEAANERQQLVETHMARVEAMLNDRRR
LALENYITALQAVPPRPRHVFNMLKKYVRAEQKDRQHTLKHFEHVRMVD
PKKAAQIRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQDEVDELLQKE
QNYSDDVLANMISEPRISYGNDALMPSLTETKTTVELLPVNGEFSLDDLQP
WHSFGADSVPANTENEVEPVDARPAADRGLTTRPGSGLTNIKTEEISEVKM
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVITLV
MLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN

MmApp (NCBI Reference Sequence: NP_031497.2):

MLPSLALLLLAAWTVRALEVPTDGNAGLLAEPQIAMFCGKLNMHMNVQN
GKWESDPSGTKTCIGTKEGILQYCQEVYPELQITNVVEANQPVTIQNWCKR
GRKQCKTHTHIVIPYRCLVGEFVSDALLVPDKCKFLHQERMDVCETHLHW
HTVAKETCSEKSTNLHDYGMLLPCGIDKFRGVEFVCCPLAEESDSVDSAD
AEEDDSDVWWGGADTDYADGGEDKVVEVAEEEEVADVEEEEADDDEDV
EDGDEVEEEAEEPYEEATERTTSTATTTTTTESVEEVVRVPTTAASTPDA
VDKYLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWEEAERQAKN

LPKADKKAVIQHFQEKVESLEQEAANERQQLVETHMARVEAMLNDRRRL
ALENYITALQAVPPRPHHVFNMLKKYVRAEQKDRQHTLKHFEHVRMVDP
KKAAQIRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQDEVDELLQKEQ
NYSDDVLANMISEPRISYGNDALMPSLTETKTTVELLPVNGEFSLDDLQPW
HPFGVDSVPANTENEVEPVDARPAADRGLTTRPGSGLTNIKTEEISEVKMD
AEFGHDSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVITLVML
KKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN

3.3 Wnt and inhibitor treatment in primary neuron

Wnt5a (400 ng/ml) (645-WN-010, R&D Systems), Wnt3a (150 ng/ml) (1324-WNP-010, R&D Systems) and PBS/BSA (control) addition performed at DIV 7. In all experiments related to inhibitor, cells will be treated with inhibitor 1 hour after Wnt addition (Bafilomycin A1(100nM, invitrogen, 88899-55-2), LY294002 (10 uM, Sigma, L9908)-as wnt3a or wnt5a treatment could affect APP protein expression clearly 2 hours later (Figure 7)- and a DMSO (0.05%DMSO in culture medium) group will be set as control. Protein or RNA samples collected after 4 hours of Wnt treatment.

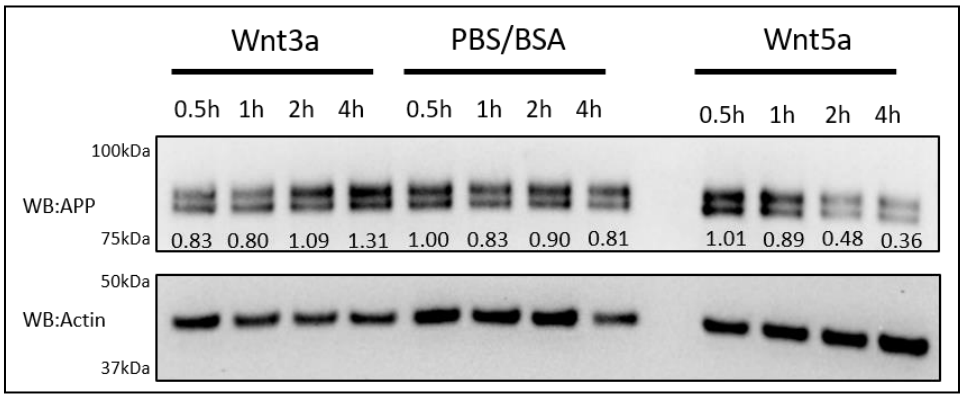

Figure 7. Time course of fl-mAPP after Wnt3a/5a treatment.
Representative western blots for the time course (05h, 1h, 2h, 4h) of fl-mAPP expression after PBS/BSA (ctrl) and Wnt3a/5a treatment at DIV7. Relative expression value of the protein bands normalized to the respective actin were also shown

3.4 Quantitative real-time PCR (qRT-PCR)

Cells were lysed for RNA or protein extraction and then subjected to qRT-PCR or western blots as previously described [215]. The detailed sequence of each primer used in the whole study for qRT-PCR were summarized below： β-actin, sense 5'-TCCATCATGAAGTGTGACGT-3' and anti-sense 5'-GAGCAATGATCTTGATCTTCAT -3', mAPP, sense 5'-CATCCAGAACTGGTGCAAGCG-3' and anti-sense 5'-GACGGTGTGCCAGTGAAGATG -3' GAPDH, sense 5'-GCTGCCAAGGCTGTGGGCAAG-3' and anti-sense 5'-GCCTGCTTCACCACCTTC -3'.

3.5 Western Blots

Western blots were performed follow the user guide of Mini Gel Tank (ThermoFisher, A25977) with little modified. Briefly, Protein samples collected from total cell lysates with RIPA buffer, supernatant were collected after centrifugation, denatured samples were loaded separated on the 4-12% polyacrylamide gels (SDS-PAGE) (ThermoFisher, NW04122BOX) and then transferred to the 0.42um nitrocellulose membranes, blots visualization performed after primary and secondary antibody incubation.

3.6 Immunoprecipitation

HEK293 cells in 10 cm dish (70% confluent) were transfected with pCDNA3-MmApp-FLAG-IRES-eGFP , pCDNA3-mAPPΔCRD-FLAG-IRES-eGFP, pCDNA3-Wnt5a-myc, pCDNA-Wnt3A-V5 or co-transfected APP or APPΔCRD with Wnt3a or Wnt5a. 3 days after transfection, cells were collected with NP-40 lysis buffer, then sample supernatant was collected after >12000 rpm centrifugation

for 20min at 4 degree, 450 ul supernatant was incubated with primary antibody overnight at 4 degree, then Protein G sepharose beads (Thermo Fisher Scientific) were added to the sample to capture protein-antibody complex by rotating 2 hours at room temperature, then washed four times with the lysis buffer, and resuspended with loading buffer, then denatured at 95 degree for 10 mins, blots visualized after western blot procedure as described before.

3.7 Immunofluorescence

At DIV 7, cultured primary neurons in 24 wells were washed once with 1X PBS, then fixed in 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 10 minutes. After 3 times washing with 1X PBS, cells were blocked with 10% normal donkey or goat serum in 1 X PBS for 30 minutes at RT followed by 3 times washing in 1 X PBS. Thereafter, cells were incubated with primary antibodies diluted in 1 X PBS containing 1% normal donkey or goat serum for 2-3 hours at RT. 3 times washing with 1 X PBS, incubated with appropriate secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 (1:500, Invitrogen) in 1 X PBS containing 1% normal donkey or goat serum for 1 hours at RT. Washed with 1 X PBS for 3 times, then counterstained the slides with DAPI (1:2000, Sigma) and mounted by using Vectashield (Vector) after rinsing. Primary antibodies used in this study were rabbit anti-APP (1:100, Synaptic Systems, 127 003), mouse anti-rab5 (1:100, Synaptic Systems, 108011), mouse anti- Golgin-97 (1:100, Invitrogen, A-21270), rat anti-Lamp1 (1:20, Santa Cruz, sc-19992). After staining, images were obtained by using confocal microscope (Olympus FV-1200 or Leica SP8). The percentage of APP or APP-ΔCRD co-localizing with rab5, Golgin-97 and Lamp1 was calculated using JACOP[216] via an automated macro that was written in house.

3.8 Aβ 40/42 detection

For the Aβ 40/42 detection, first, culture medium refreshed with Wnt3a (50 ng/ml or 150 ng/ml) or Wnt5a (100 ng/ml or 400 ng/ml) on DIV7 primary cortical neurons. Then, supernatant was collected after 4 hours Wnt3a/5a treatment for Aβ detection, and refreshed with medium without Wnt3a/5a. At last, the second round of supernatant collection performed after additional 24 hours culture. The procedure of Aβ detection is based on the protocol from V-PLEX Plus Aβ Peptide Panel 1 (6E10) Kit (K15200G, MSD)

3.9 Statistical analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were compared using the t-test, One-way ANOVA and Mann Whitney two-sample test (two-tail) as appropriate.

CHAPTER 4 RESULTS

4.1 APP is a putative Wnt5a receptor through the Cysteine Rich Domain (CRD)

Wnt5 is a member of the large family of Wnt ligands, some of whose receptors and co-receptors harbor a conserved extracellular Cysteine Rich Domain (CRD) thought to be important for Wnt binding[217,218]. Intriguingly, APP harbors a CRD-like domain[219] in its extracellular region that includes 12 cysteine residues conserved across APP paralogs and orthologs (Figure 8A). The distribution of the cysteine residues resembles those present in the CRDs of other PCP receptors such as Fz and Ror-2 (Figure 8B, C). We asked whether the CRDs of APP are potential Wnt binding domains. To test this, we generated forms of mouse APP (mAPP), human APP (hAPP) and APP lacking the CRD (mAPPΔCRD and hAPPΔCRD). Next, we overexpressed a tagged form of Wnt5a together with h/mAPP or h/mAPPΔCRD, respectively, in HEK293 cells and performed co-immunoprecipitation (IP) assays. Wnt5a immunoprecipitated full-length mouse or human APP but not the form that lack the CRD (Figure 9A, B).

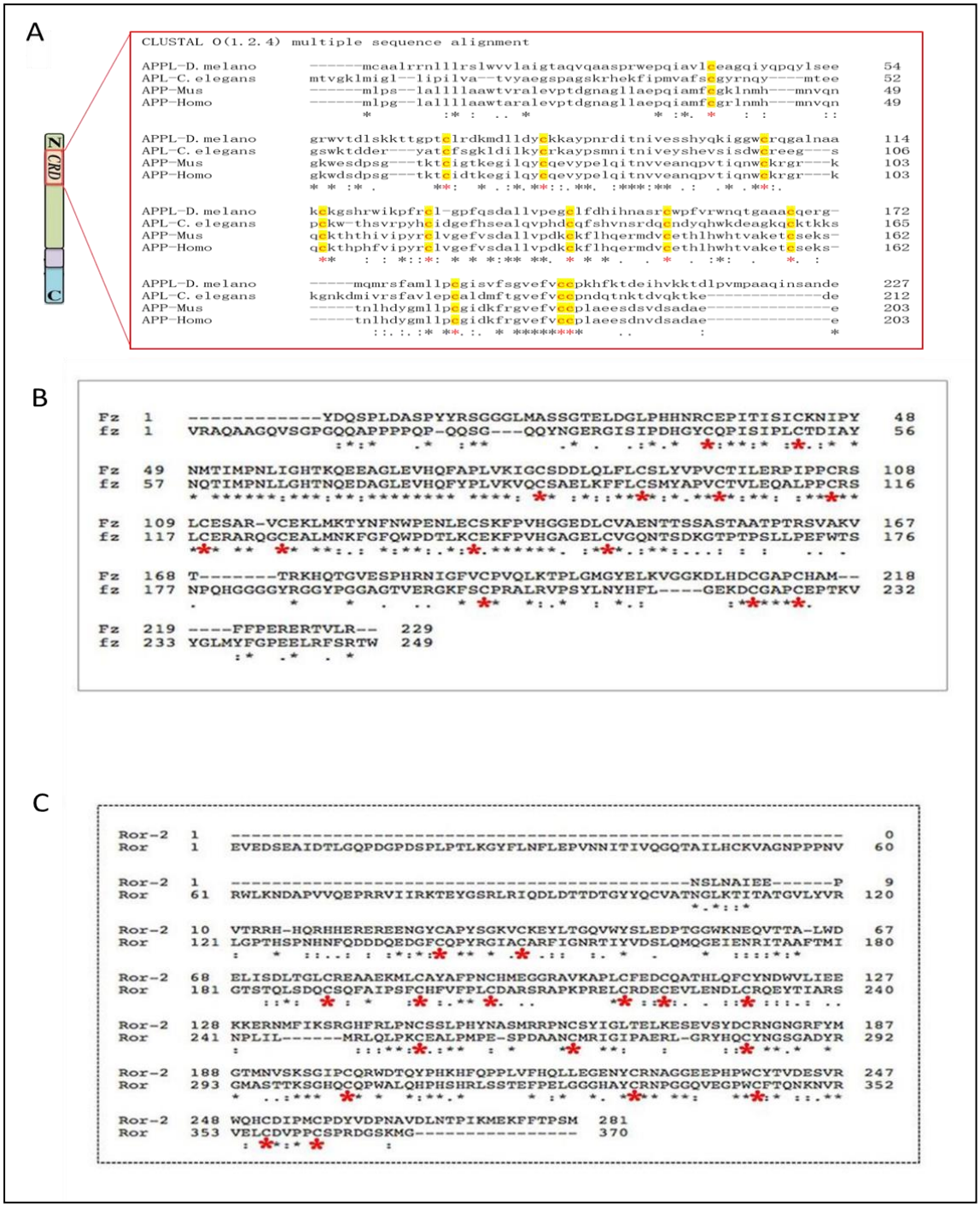

Figure 8. APP extracellular region contains a conserved cysteine rich domain (CRD). (Figure adapt from Maya Nicolas Thesis)

(A) APP extracellular region contains a conserved CRD. The figure shows a CLUSTAL alignment of the CRD of different APP homologs. The 12 cysteine residues (as indicated by the red asterisks with yellow background) are highly conserved across species. (B) CLUSTAL alignment of the ectodomain of Mice Frizzeld-1(Fz-1) and fly Frizzled (Fz). (C) CLUSTAL alignment of the ectodomain of Mice Ror-2and fly Ror. All of the Wnt receptors or co-receptors showed conserved cysteine residues (indicated by red asterisks).

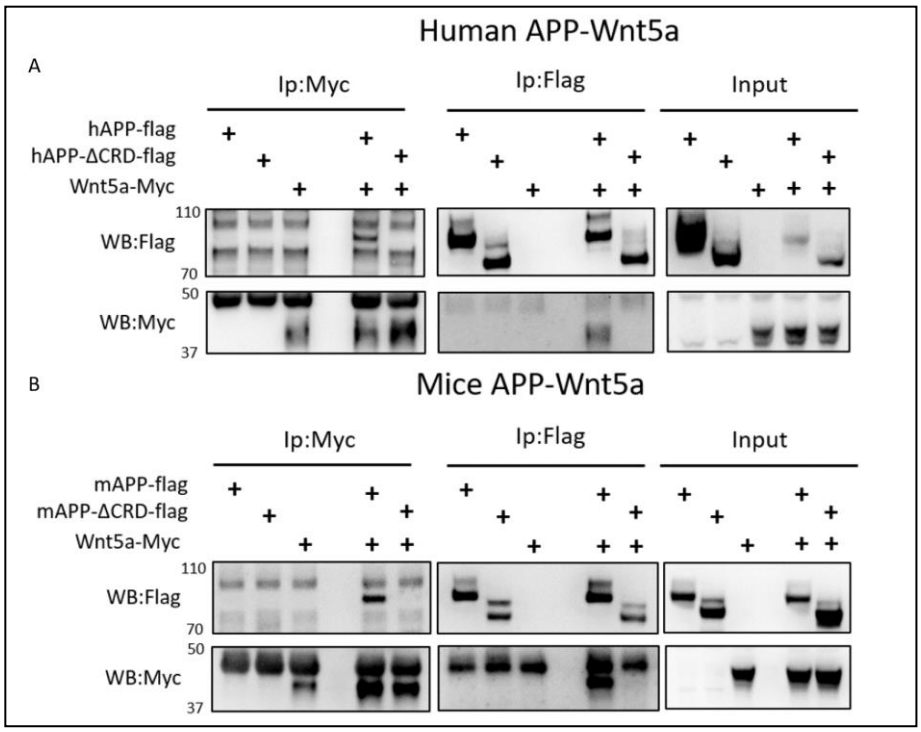

Figure 9. Wnt5a binds APP through its CRD
(A) Co-immunoprecipitation (co-IP) of Wnt5a-Myc with full-length proteins hAPP-flag or hAPP-delatCRD-flag. (B) Co-immunoprecipitation (co-IP) of Wnt5a-Myc with full-length proteins mAPP-flag or mAPP-delatCRD-flag. The tagged proteins were co-expressed in HEK293T cells and immunoprecipitated with ant-flag or anti-Myc antibody, wild type mAPP pulled down Wnt5a and vice versa, while mAPP lacking the CRD showed impaired ability to pull down Wnt5a even with higher protein levels compared to wild type mAPP in the input.

4.2 Wnt5a treatment affects APP trafficking and expression in maturing mouse primary cortical neuron

The findings above suggest that the APP family may represent a new class of conserved Wnt receptors. We sought to investigate this further at a cell biological level using developing mouse embryonic primary cortical neurons as a model system. APP trafficking and processing have been intensively investigated in studies relating to AD, and according to early reports the half-life of APP is quite short, ranging from 1 hour to 4 hours[24,220]. In mouse embryonic (E16) primary neuron cultures, full-length mouse APP (fl-mAPP) expression significantly dropped after 2 hours of

treatment with translational inhibitor (Cycloheximide) (Figure 10A-D), suggesting relatively rapid turnover of mAPP.

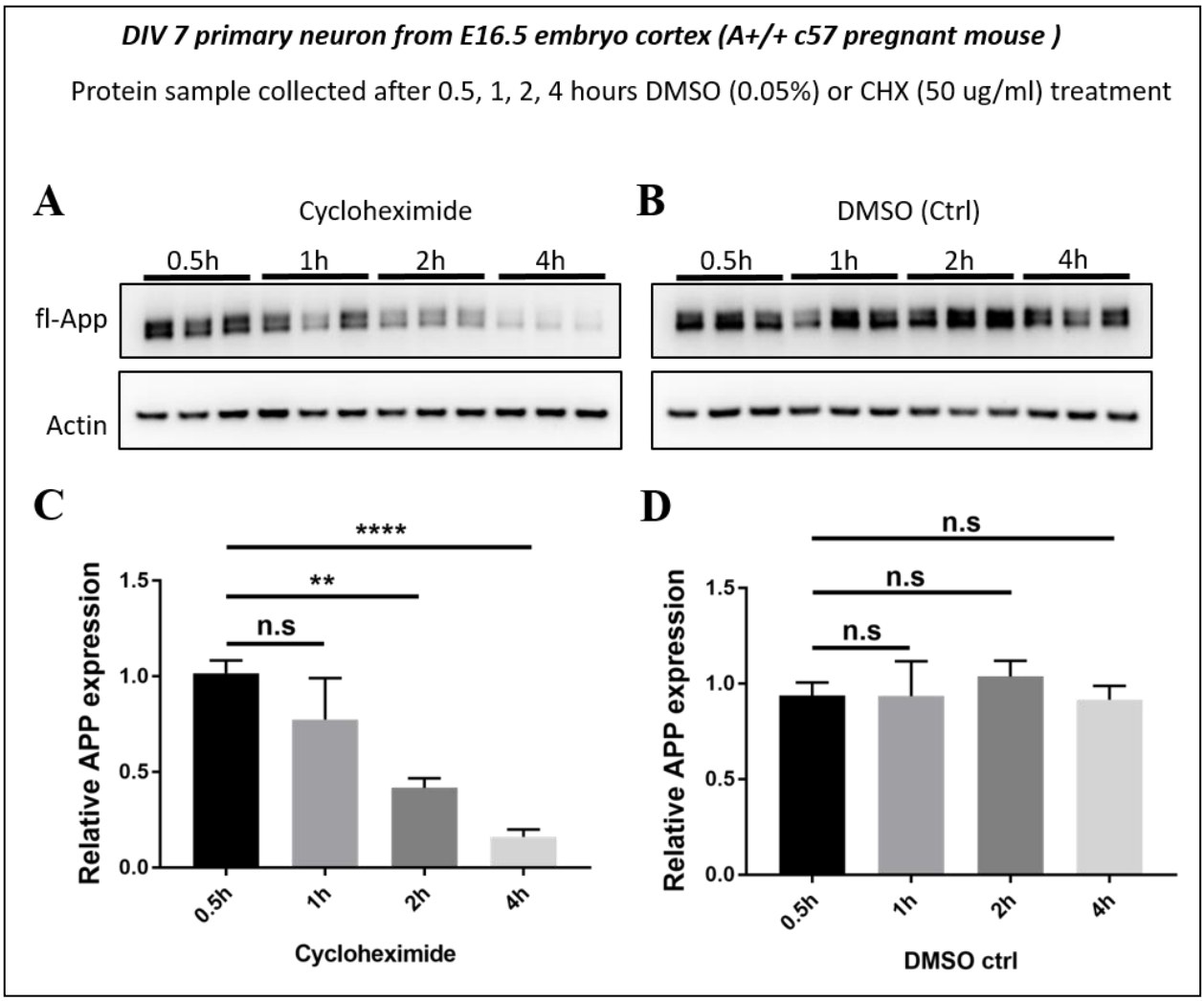

Figure 10. Rapid turnover of fl-mAPP in culture mouse primary cortical neurons.
(A and B) Western blots for the time course (0.5 h, 1 h, 2 h, 4 h) of fl-mAPP expression after Cycloheximide (50ug/ml, A) or DMSO (0.05%, B) treatment at DIV7. (C and D) Quantification of fl-mAPP expression after Cycloheximide (C) or DMSO treatment (D). (n=3 biological independent repeat, one-way ANOVA). Bars represent the mean ± S.E.M. **P<0.01, ****P<0.0001.

Next, we used immunofluorescence to localize mAPP with or without Wnt5a treatment in developing cortical neurons during axonal outgrowth (DIV7). mAPP is modified to maturation in the Trans Golgi Network (TGN) to be subsequently transferred to the plasma membrane where it can be internalized into early endosomes. From the early endosome, mAPP is either recycled back to the TGN through retromer-dependent sorting, or to the late endosome and then lysosome to be degraded [221,222]. We used markers for early endosomes (Rab5), TGN (Golgin97) and lysosomes (Lamp1) to trace mAPP trafficking after Wnt5a treatment. As shown in the Figure 11(A, B), compared to control, the fraction of mAPP co-localizing with early endosomes was not affected by Wnt5a treatment, indicating normal initial internalization of mAPP. However, we found less mAPP in the TGN, and more mAPP in lysosomes (Figure 11A, C and D) suggesting that Wnt5a regulates intracellular targeting of mAPP after internalization. Importantly, the levels of expression of these markers (Rab5 Golgin97 and Lamp1) are not affected by Wnt5a treatment (Figure 12A-E).

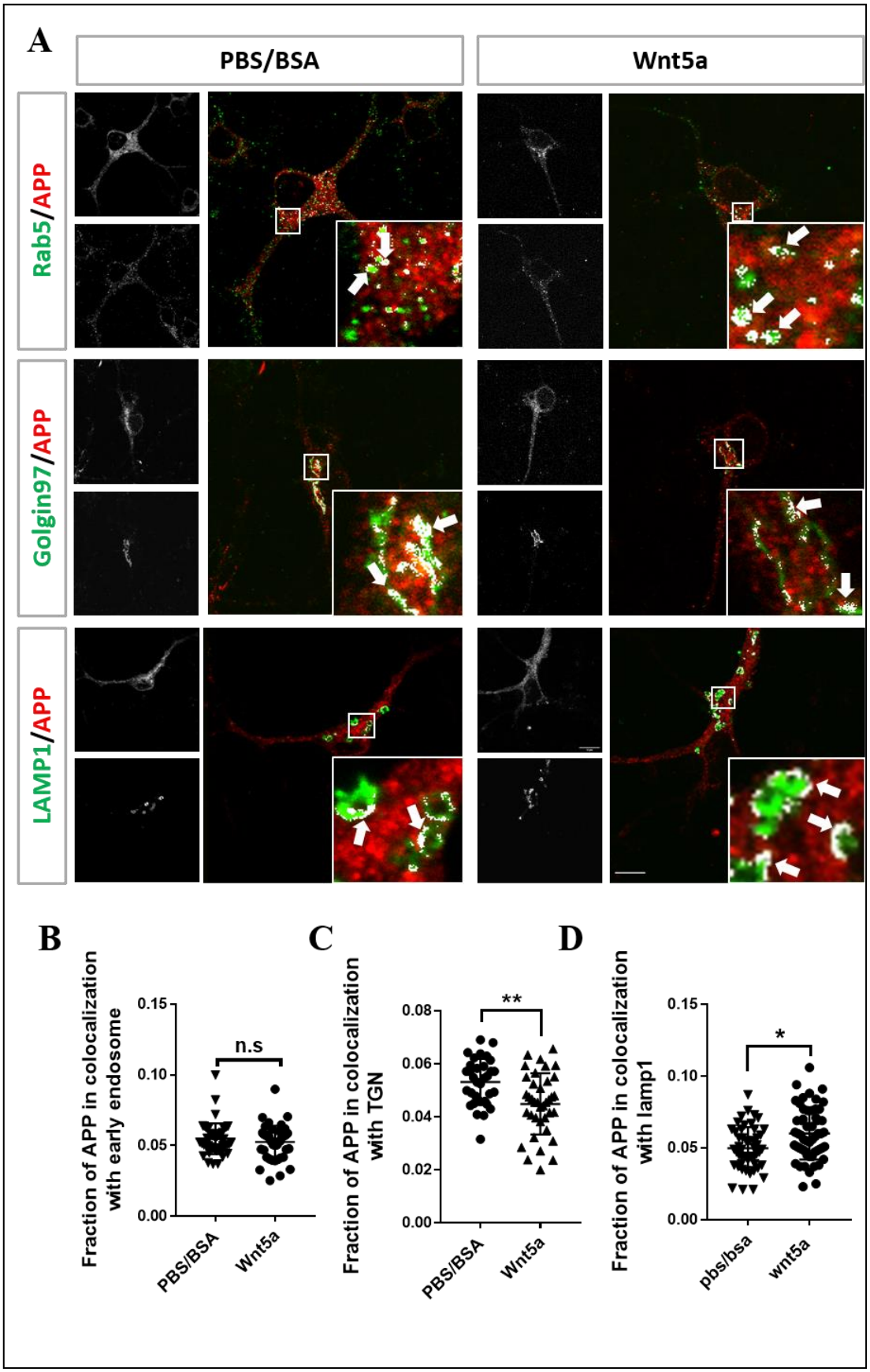
A
PBS/BSA
Wnt5a
Rab5/APP
Golgin97/APP
LAMP1/APP
B
Fraction of APP in colocalization with early endosome
0.15
0.10
0.05
0.00
n.s
PBS/BSA
Wnt5a
C
Fraction of APP in colocalization with TGN
0.08
0.06
0.04
0.02
0.00
**
PBS/BSA
Wnt5a
D
Fraction of APP in colocalization with lamp1
0.15
0.10
0.05
0.00
*
pbs/bsa
wnt5a

Figure 11. Wnt5a affects APP trafficking through its CRD
(A) mAPP localization after 4 hours PBS/BSA or Wnt5a treatment. Immunofluorescence for APP (red), Rab5 (early endosome marker, green), Golgin97 (TGN marker, green) and Lamp1 (lysosome marker, green) revealed mAPP localization in different intracellular compartments. The inset showed high magnification images of the area in the white box and arrows indicated the overlap of mAPP with respective cellular compartment markers. White labels the overlap part between mAPP and respective cellular compartment after image be processed by Fiji co-locolization plugin with same threshold. Scale bar = 10 um. (B-D) Quantification of the overlap of mAPP with early endosome (C), TGN (D) or lysosome (E), respectively, after Wnt5a treatment. (n=33-55 cells, t-test). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05, **P<0.01.

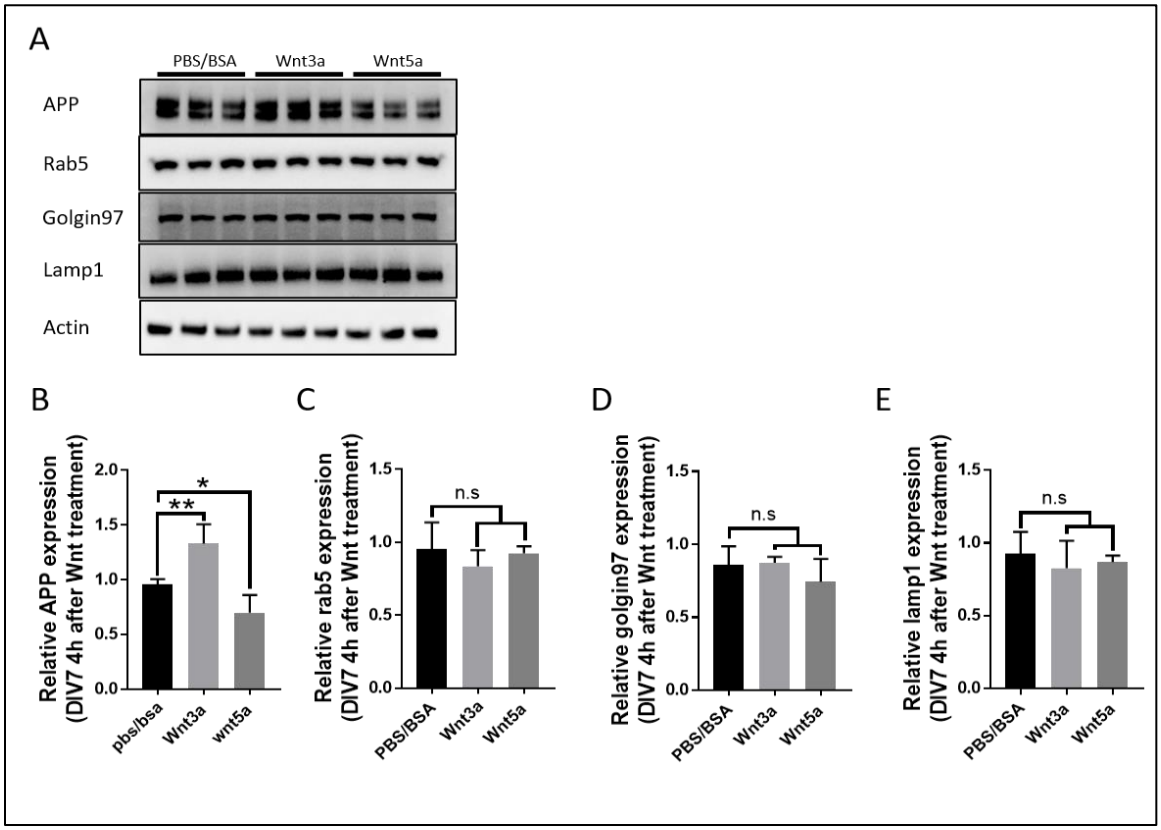

Figure 12. Rab5 Golgin97 and Lamp1 expression after Wnt3a/5a treatment.
(A) Western blots for fl-mAPP, Rab5, Golgin97, Lamp1 and Actin 4 hours after Wnt3a/5a treatment at DIV7. (B-E) Quantification of the western blots results for fig A: fl-mAPP (B), Rab5 (C), Golgin97 (D) and Lamp1 (E). (n=3 biological independent repeat, one-way ANOVA). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05, **P<0.01.

Next, we asked if this altered trafficking affected mAPP levels. We found that the level of fl-mAPP was significantly reduced after 4hrs treatment of Wnt5a, as shown by western blot (Figure 13 A, B) with no effect on mAPP mRNA levels (Figure 13 C). The results of IF and WB suggest that the decrease of the mAPP upon Wnt5a treatment is caused by lysosomal degradation. To confirm this, we used Bafilomycin-A in combination with Wnt5a treatment to inhibit the lysosome and found that this restored mAPP to control levels (Figure 13 D-G). These data suggest that non-canonical Wnt5a-PCP signaling reduces mAPP stability.

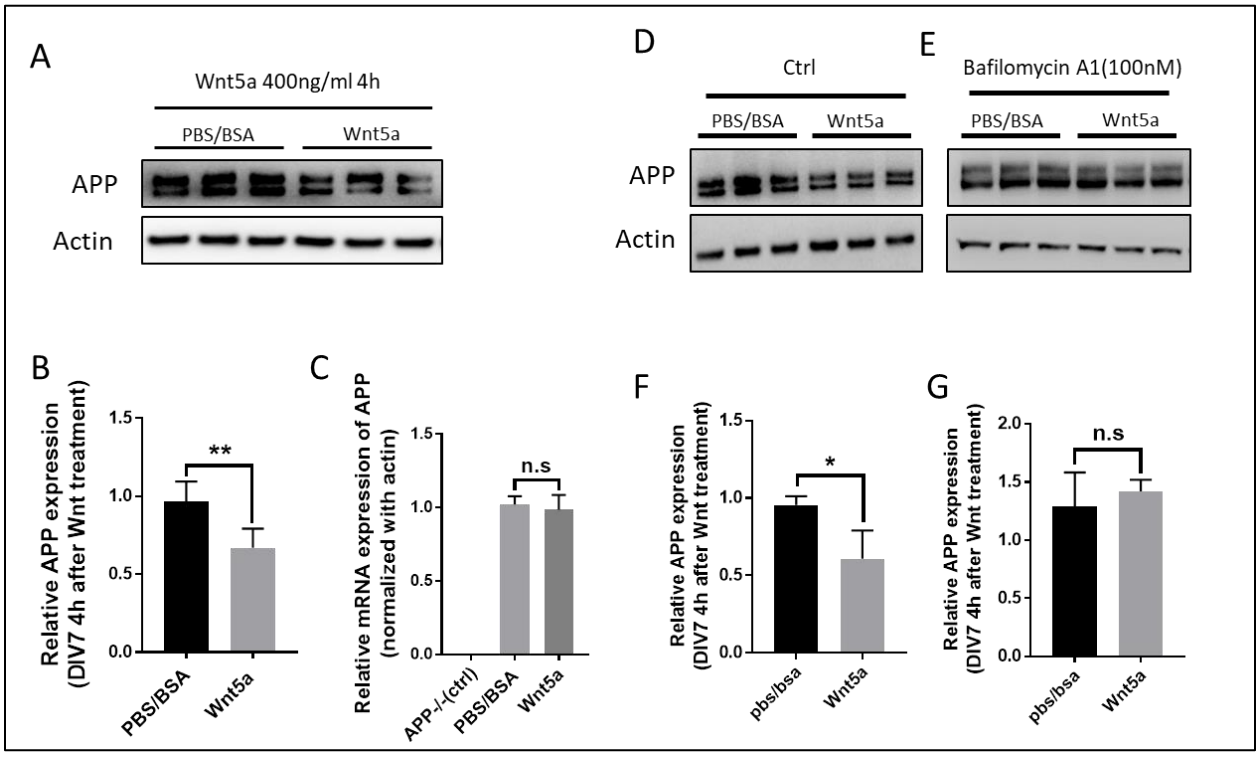

Figure 13. Wnt5a affects APP expression

(A) Western blots of mAPP and Action on the lysates of DIV7 primary cultured cortical neurons showed that mAPP protein expression level was altered after Wnt5a treatment. (B) Quantification of the western blots results for fig F. (n=3 biological independent repeat, t-test). (C) qPCR results showed that mApp mRNA expression was not affected after Wnt5a treatment on DIV7. App-/- mice derived primary neurons were used as a negative control. (n=3 biological independent repeat, one-way ANOVA). (D-E) Western blots for APP showed that the lysosome inhibitor Bafilomycin (E) could rescue Wnt5a-induced mAPP reduction compared with control groups (D). (F and G) Quantification of the western blots result for fig (D and E). (n=3 biological independent repeat, t-test). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05, **P<0.01.

Consistent with this notion, we found that primary cortical neurons from Celsr3 knock out mice, in which neuronal Wnt-PCP signalling is reduced [223], show significantly increased mAPP expression (Figure 14A-C).

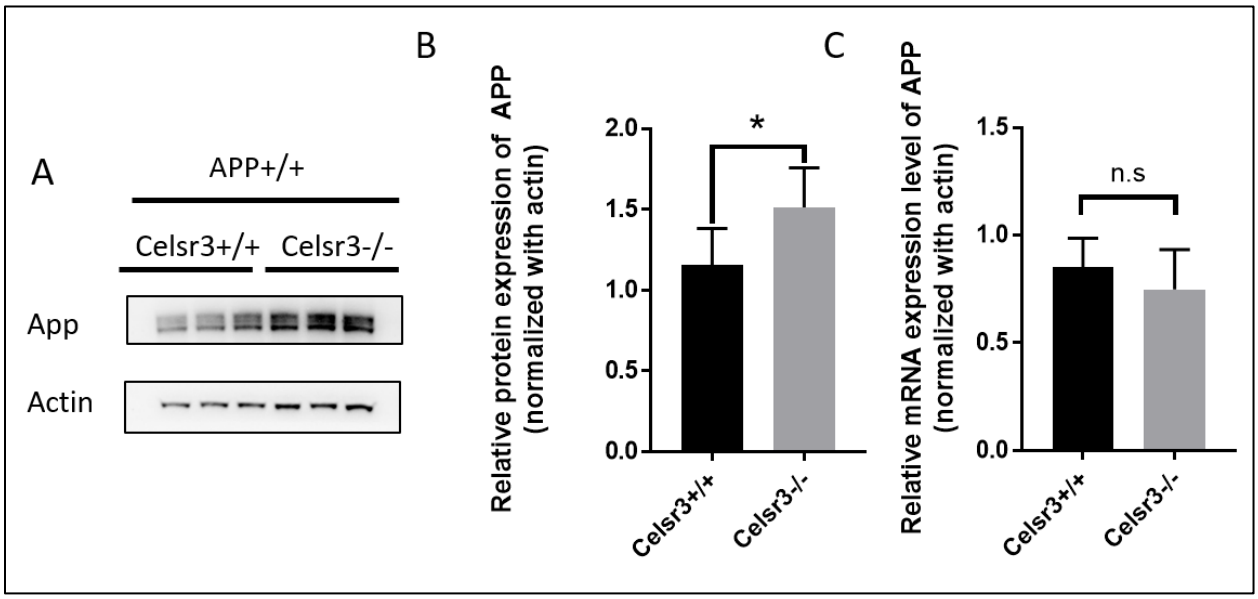

Figure 14. Wnt5a may affect fl-mAPP expression through Wnt/PCP pathway.
Loss of Celsr3 (key component of the Wnt/PCP complex) results in interruption of fl-mAPP. (A) Western blots for mAPP and actin expression at DIV7 on Celsr3 wild type or knock out primary cortical neurons. (B) Quantification of the western blots results from figure A. (C) qPCR for mAPP and actin mRNA expression at DIV7 on Celsr3 wild type or mutant primary cortical neurons. Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05.

4.3 mAPP is binds Wnt3a via its CRD and stabilizes fl-mAPP

We wondered whether APP can also bind other members of the Wnt family of ligands. Wnt3a is one of the 19 Wnt members in mouse and human. During development Wnt3a usually induces β-Catenin signaling pathway which plays an import role in gene expression, cell proliferation and differentiation [93,224]. Recent studies suggest that Wnt3a and beta-Catenin signaling may be involved in AD pathology[225,226]. More interestingly, studies on mouse AD models showed that Wnt3a and Wnt5a interact competitively and antagonistically with regards to APP-mediated synapse loss [94,95]. We therefore wondered whether, like Wnt5a, Wnt3a also

binds to APP through the conserved CRD and regulates its levels. To test this, we performed IP experiments with Wnt3a. We found that both mouse APP and human APP binds with Wnt3a in a CRD-dependent fashion (Figure 15A and B).

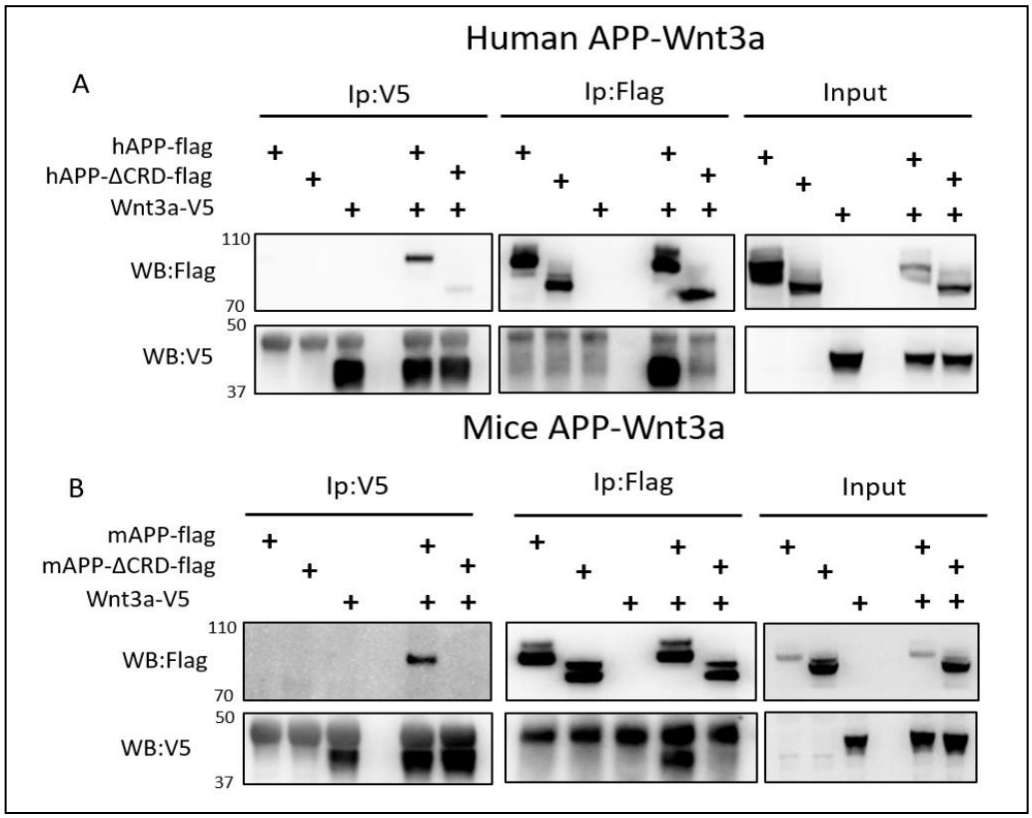

Figure 15. Wnt3a binds to APP through its CRD
(A) Co-immunoprecipitation (co-IP) of Wnt3a-V5 with full-length proteins hAPP-flag or hAPPLΔCRD. (B) Co-immunoprecipitation (co-IP) of Wnt3a-V5 with full-length proteins mAPP-flag or mAPPLΔCRD. The tagged proteins were co-expressed in HEK293T cells and immunoprecipitated with ant-flag or anti-v5 antibody, wild type mAPP pulled down Wnt3a and vice versa, while mAPP lacking the CRD showed impaired ability to pull down Wnt3a even with higher protein levels compared to wild type mAPP in the input.

We next tested the effects of Wnt3a treatment on APP trafficking. As shown in Figure (16A and B), the fraction of mAPP colocalized with early endosomes was not affected. However, more mAPP was present in the TGN compared to controls (Figure16A and C), with no effect on the lysosomal mAPP fraction (Figure16A and

D). The expression levels of Rab5 Golgin97 and Lamp1 are not affected after Wnt3a treatment (Figure 12A-E).

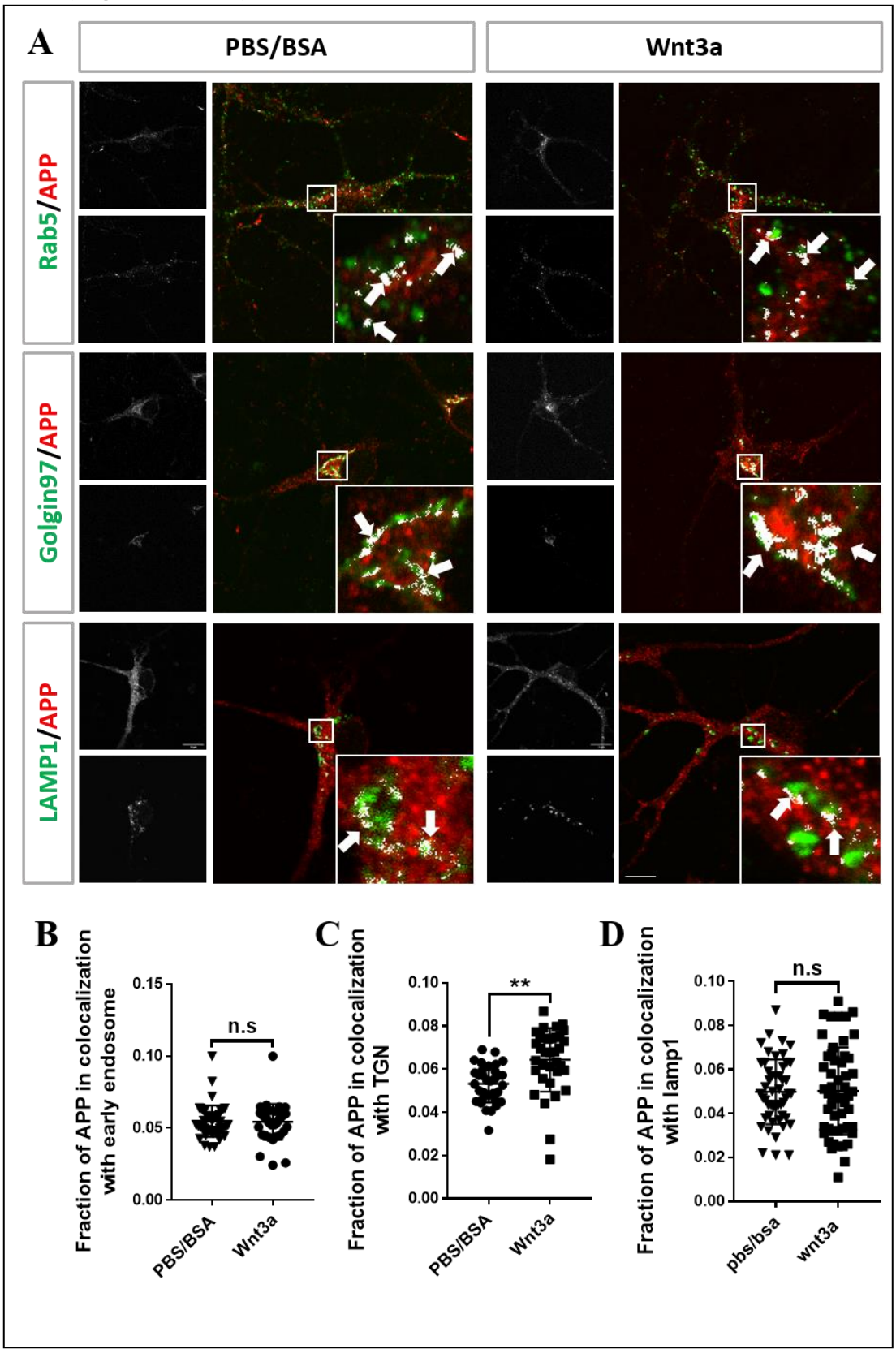

Figure 16. Wnt3a affects APP trafficking through its CRD

(A) mAPP localization after 4 hours PBS/BSA or Wnt3a treatment. Immunofluorescence for APP (red), Rab5 (green), Golgin97 (green) and Lamp1 (green) revealed mAPP localization in different intracellular compartments. The inset showed high magnification images of the area in the white box and arrows indicated the overlap of mAPP with respective cellular compartment markers. White labels the overlap between mAPP and respective cellular compartment after image processing by Fiji co-locolization plugin with same threshold. Scale bar = 10 um. (B-D) Quantification of the overlap of mAPP with early endosome (C), TGN (D) or lysosome (E), respectively, after Wnt3a treatment. (n=33-54 cells, t-test). Bars represent the mean ± S.E.M. for at least two independent experiments. **P<0.01.

Western blots analysis showed increased fl-mAPP upon Wnt3a treatment (Figure 17 A and B), but no effect on mRNA levels (Figure 17C). These evidence showed that mAPP is recycled back to the TGN from early endosomes through the retrograde pathway[222]. To test whether Wnt3a regulates mAPP retrotrafficking to the TGN, we co-treated primary neurons with Wnt3a and a retromer inhibitor (LY294002), which reversed the effect of Wnt3a on mAPP trafficking protein levels (Figure17 D-G).

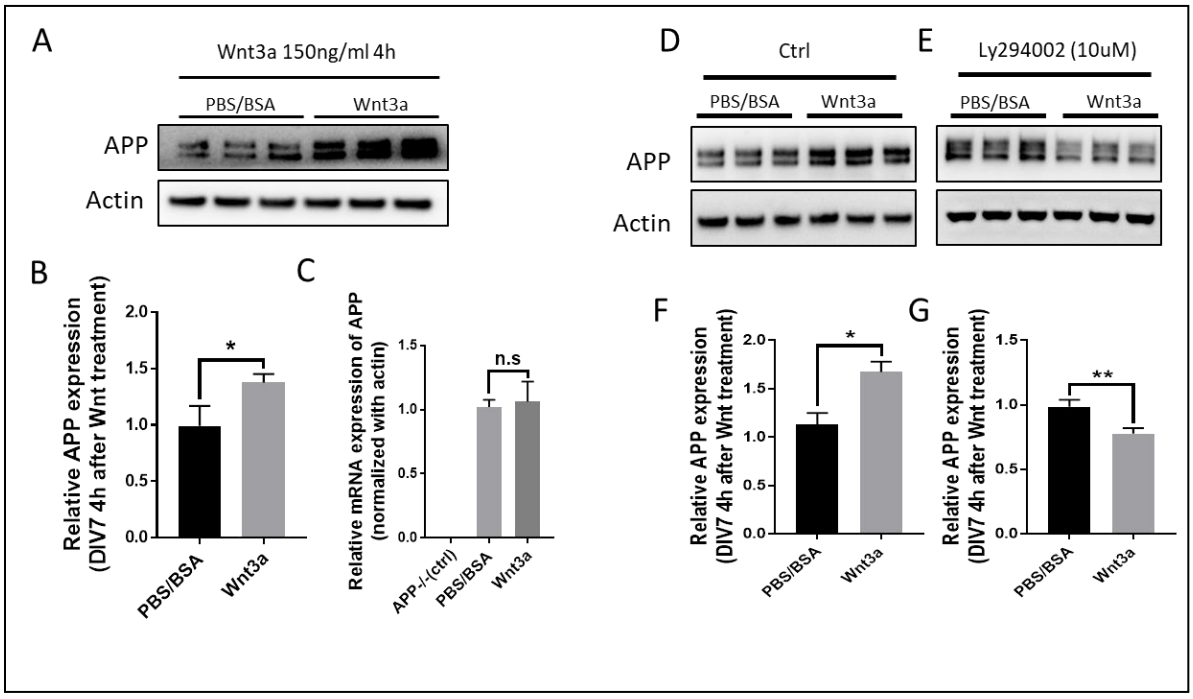

Figure 17. Wnt3a regulates mAPP expression

(A) Western blots of mAPP and Action on the lysates of DIV7 primary cultured cortical neurons showed that mAPP protein expression level was altered after Wnt3a treatment. (B) Quantification of the western blots results for fig A. (n=3 biological independent repeat, t-test). (C) qPCR results

showed that mApp mRNA expression was not affected after Wnt3a treatment on DIV7. App-/- mice derived primary neurons were used as a negative control. (n=3 biological independent repeat, one-way ANOVA). (D and E) Western blots for APP showed that the Retromer inhibitor Ly294002 (E) could rescue Wnt3a-induced mAPP increase compared with control groups (D). (F and G) Quantification of the western blots results for fig (D and E). (n=3 biological independent repeat, t-test). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05, **P<0.01.

Finally, we tested the effects of simultaneous treatment with Wnt3a and Wnt5a. This resulted in no change to APP protein levels compared to controls, suggesting that Wnt3a and Wnt5a neutralize each other's effects on mAPP (Figure18A and B), again with no effects on mRNA levels (Figure 18C).

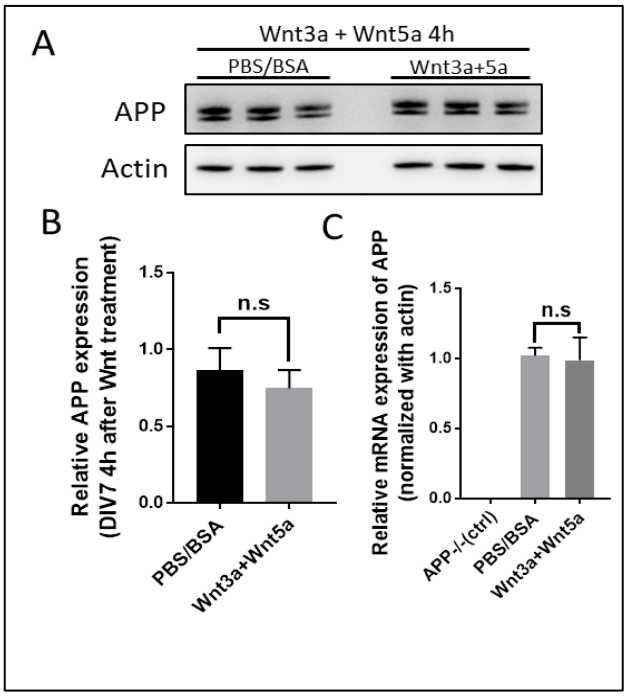

Figure 18. Adding Wnt3a and Wnt5a at the same time abolishes the effects on APP expression induced by solo Wnt treatment.
(A) Western blots for APP expression 4 hours after Wnt3a and Wnt5a treatment at the same time on DIV7. PBS/BSA group acted as control group. (B) Quantification of the western blots results for fig A. (n=3 biological independent repeat, t-test). (C) qPCR results for APP mRNA expression in mApp knockout neurons (negative control), PBS/BSA treated control group neurons and Wnt3a+Wnt5a treated group neurons. (n=3 biological independent repeat, one-way ANOVA). Bars represent the mean ± S.E.M. for at least two independent experiments.

Because Wnts treatment affects APP trafficking and expression, we tested Aβ40 and Aβ 42 production in primary cortical neurons after 4 hours treatment of Wnt3a or Wnt5a on primary cortical neuron at DIV7. Briefly, after a 4-hour treatment with Wnt3a/5a, supernatant was collected at 4 hours and at 24hours for Aβ detection using the MSD kit. Our data show that a 4-hour Wnt3a/5a treatment has a long-lasting effect on Aβ40/42 production and that those effects are antagonistic. Consistent with Wnt3a favoring recycling of APP we found that Wnt3a treatment significantly decrease Aβ40/42 production (Figure 19 A, B). In contrast, Wnt5a treatment, which induces APP internalization into acidic compartments, resulted in an increase in Aβ40/42 production (Figure 19 C, D). Importantly, these data are in accordance with other reports that Wnt/catenin pathway favors non-amyloidogenic APP processing while Wnt/PCP signaling does the opposite[95].

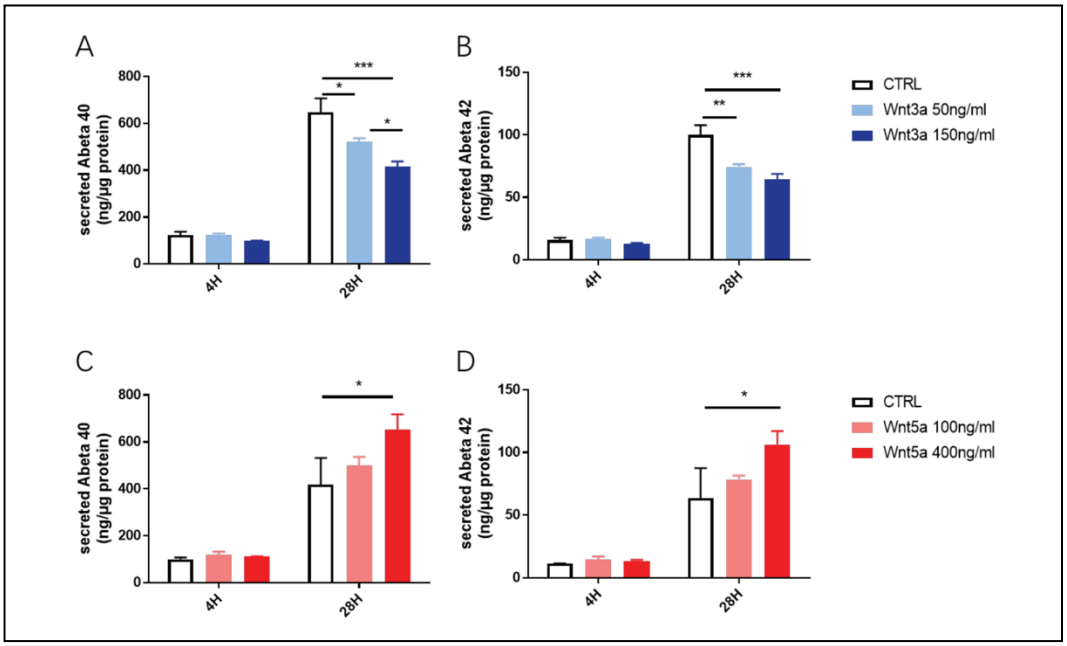

Figure 19. Aβ detection after Wnts treatment on DIV7 primary cortical neurons
(A and B) Detection of secreted Aβ40 (A) and Aβ42 (B) from DIV7 primary cultured cortical neurons treated with Wnt3a (supernatant collected from 4 hours Wnt3a treatment or anther 24 hours cultured with medium refreshed without Wnt3a). (C and D) Detection of secreted Aβ40 (C) and Aβ42 (D) from DIV7 primary cultured cortical neurons treated with Wnt5a (supernatant collected from 4 hours Wnt5a treatment or anther 24 hours cultured with medium refreshed without Wnt5a). Bars represent the mean ± S.E.M., n=3 biological independent repeat, one-way ANOVA. *P<0.05, **P<0.01, ***p<0.001.

Taken together, these data indicate that Wnt3a also binds to mAPP via the CRD and regulates mAPP trafficking and expression and that Wnt5a and Wnt3a act antagonistically to regulate APP protein homeostasis.

4.4 The CRD is required for Wnt-mediated regulation of mAPP trafficking and expression

Our data thus far show that mAPP interacts with Wnts through its CRD and that Wnts regulate mAPP intracellular trafficking and expression. We therefore asked whether the CRD is required for the effects of Wnts on mAPP. To address this question, we created two lentiviral vectors: pLv-pSyn1-mAPP-Flag-IRESeGFP (flag-tagged fl-mAPP) and pLv-pSyn1-mAPPΔCRD-Flag-IRESeGFP (flag-tagged mAPPΔCRD). Primary cortical neurons from APP knockout mice were transduced with the fl-mAPP or mAPPΔCRD vectors, or a control GFP vector (pLv-pSyn1-IRESeGFP) exogenous APP/APPΔCRD could be well detected using either anti-APP or anti-flag antibodies (Figure 20A-C). Virus induced APP/APPΔCRD expression level was on average slightly lower than endogenous (Figure 20B and C).

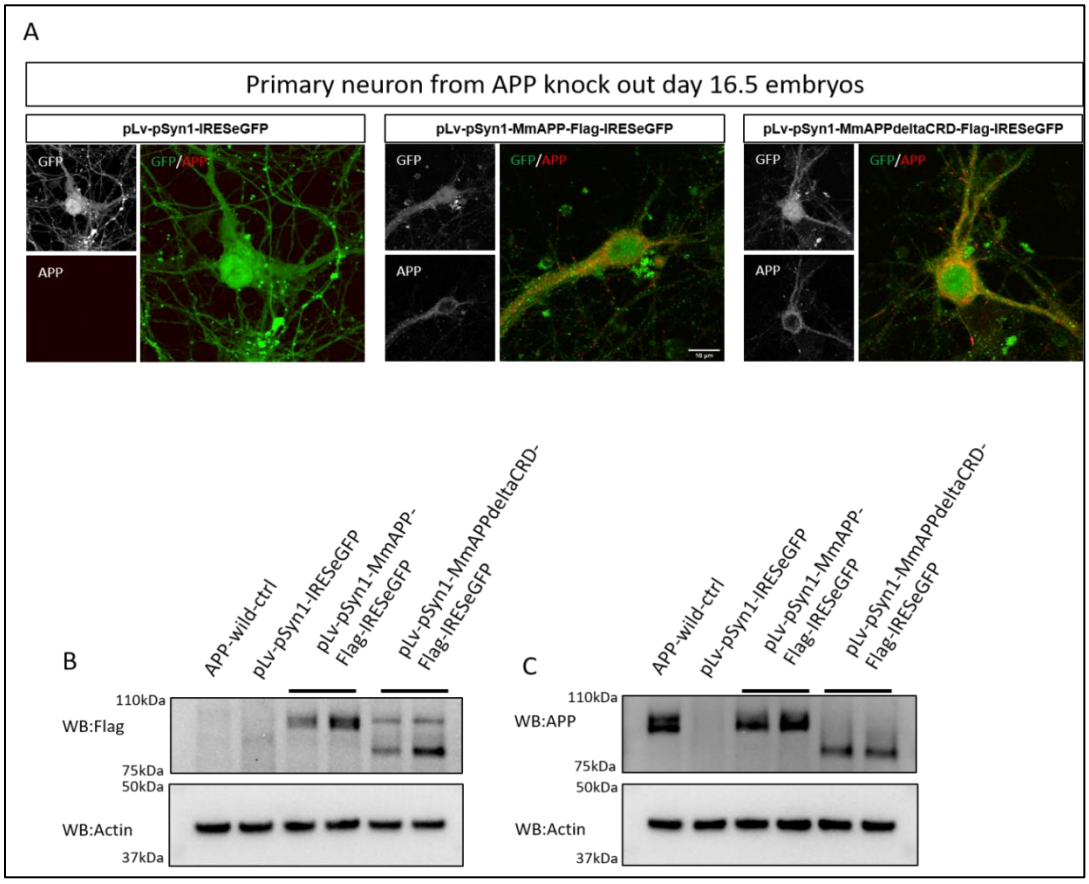

Figure 20. Lenti-virus induced exogenous mAPP expressed in mAPP knock out primary cortical neuron.
(A) Immunostaining of APP (red) after transfected with pLv-pSyn1-IRESeGFP (left panel, negative control), pLv-pSyn1-mAPP-Flag-IRESeGFP (middle panel) and pLv-pSyn1-mAPPΔCRD-Flag-IRESeGFP (right panel) lenti-virus in APP-KO primary cultured neurons. Scale bar = 10um. (B and C) Western blots for the detection of exogenous mAPP expression by using anti-flag (B) and anti-APP (C) antibodies.

In neurons transduced with wild type mAPP we confirmed that mAPP expression could be increased and decreased by Wnt3a and Wnt5a treatments, respectively (Figure 21A and C). In contrast, in neurons transduced with mAPPΔCRD those effects were eliminated (Figure 21B and D).

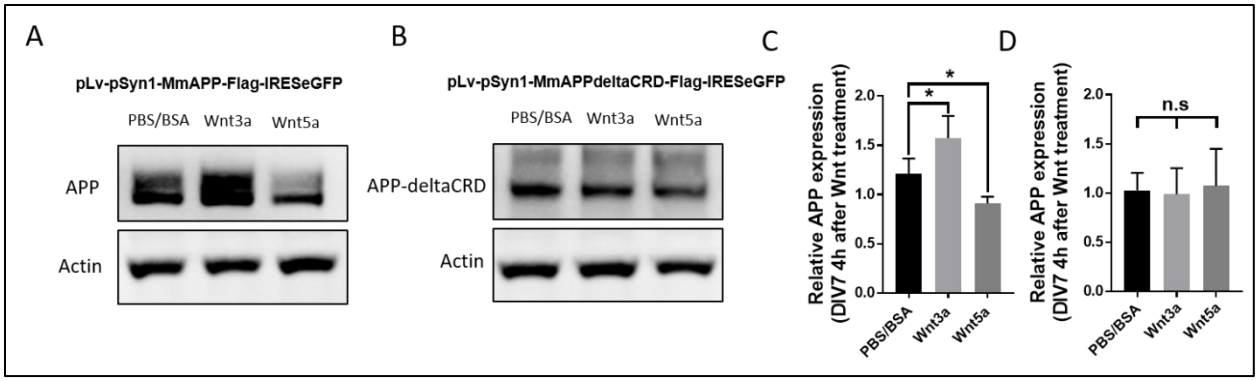

Figure 21. CRD is required for Wnt3a/5a to affect mAPP expression
(A and B) Western blots for the detection of exogenous APP expression 4 hours after PBS/BSA (control), Wnt3a or Wnt5a treatment at DIV7 in APP-KO primary cultured neurons which were transfected with pLv-pSyn1-mAPP-Flag-IRESeGFP (A) or pLv-pSyn1-mAPPΔCRD-Flag-IRESeGFP (B) lenti-virus. (C and D) Quantification of the western blots results for fig A and B, respectively. (n=3 biological independent repeat, one-way ANOVA). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05.

Finally, we performed IF to trace mAPP and mAPPΔCRD localization. Neurons transduced with wild type mAPP showed similar results as wild type neurons with more mAPP in the TGN upon Wnt3a treatment and more mAPP in lysosomes upon Wnt5a treatment (Figure 22A-D).

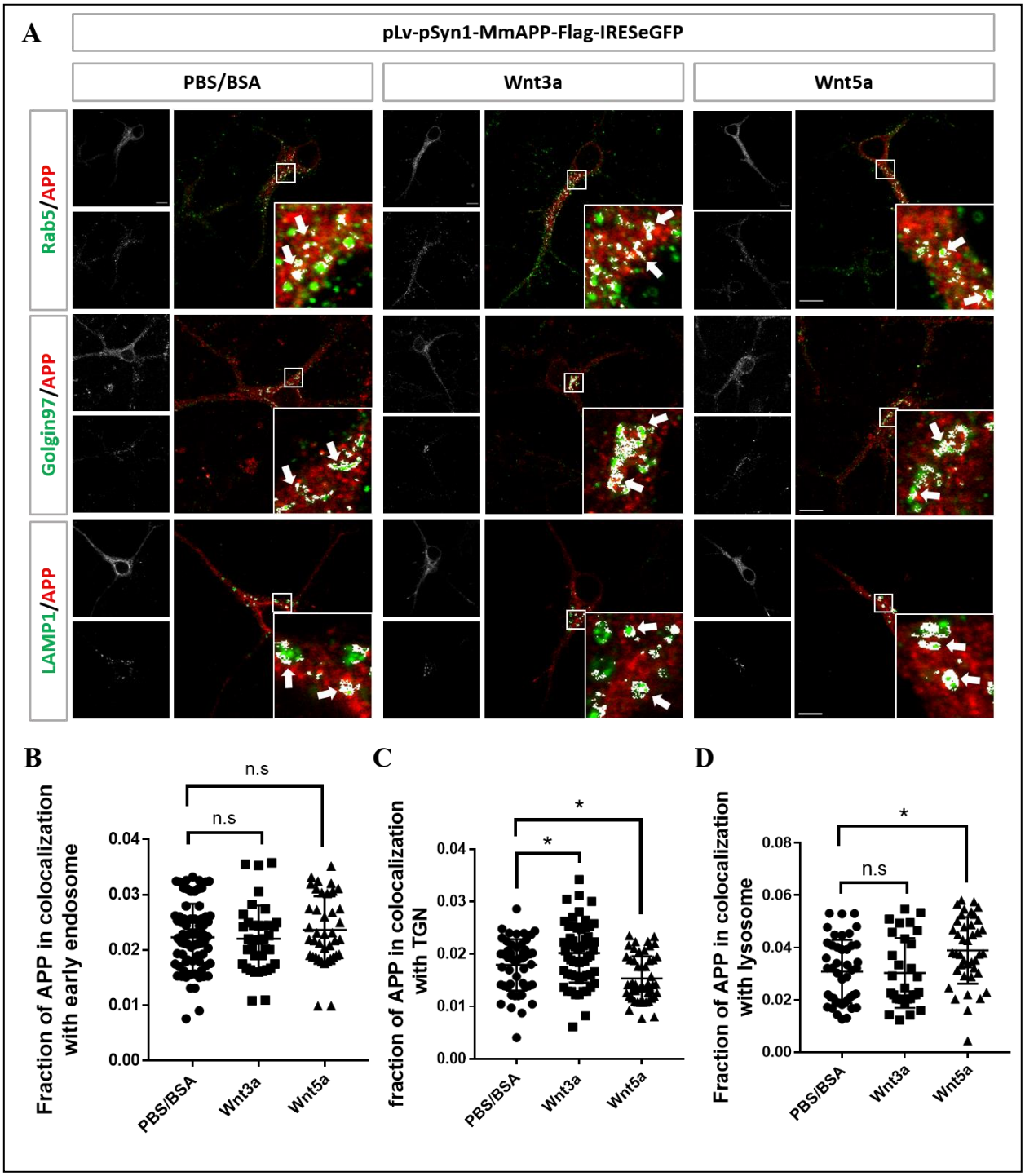

Figure 22. Lenti-virus induced exogenous mAPP interacts with Wnts in mAPP knock out primary cortical neurons

(A) Location of exogenous mAPP in APP knock-out primary cultured cortical neurons 4 hours after Wnt3a or Wnt5a treatment on DIV7. Immunofluorescence for APP (red), Rab5 (green), Golgin97 (green) and Lamp1 (green) revealed mAPP localization in different intracellular compartments. The inset showed high magnification images of the area in the white box and arrows indicated the overlap of mAPP with respective cellular compartment markers. White labels the

overlap between mAPP and respective cellular compartment after image processing by Fiji co-locolization plugin with same threshold. Scale bar = 10 um. (B-D) Quantification of the overlap of mAPP with early endosome (B), TGN (C) or lysosome (D), respectively, after Wnt3a or Wnt5a treatment. Bars represent the mean ± S.E.M., n=37-50 cells, one-way ANOVA. *P<0.05.

In contrast, in neurons transduced with mAPPΔCRD neither Wnt3a, nor Wnt5a treatment showed any significant difference in mAPP localization to early endosomes, TGN or lysosomes compared to controls (Figure 23A-D). In summary, these data strongly suggest that the CRD of mAPP is the critical sequence for Wnt3a/5a binding and mediates the effects of Wnts on mAPP trafficking and expression.

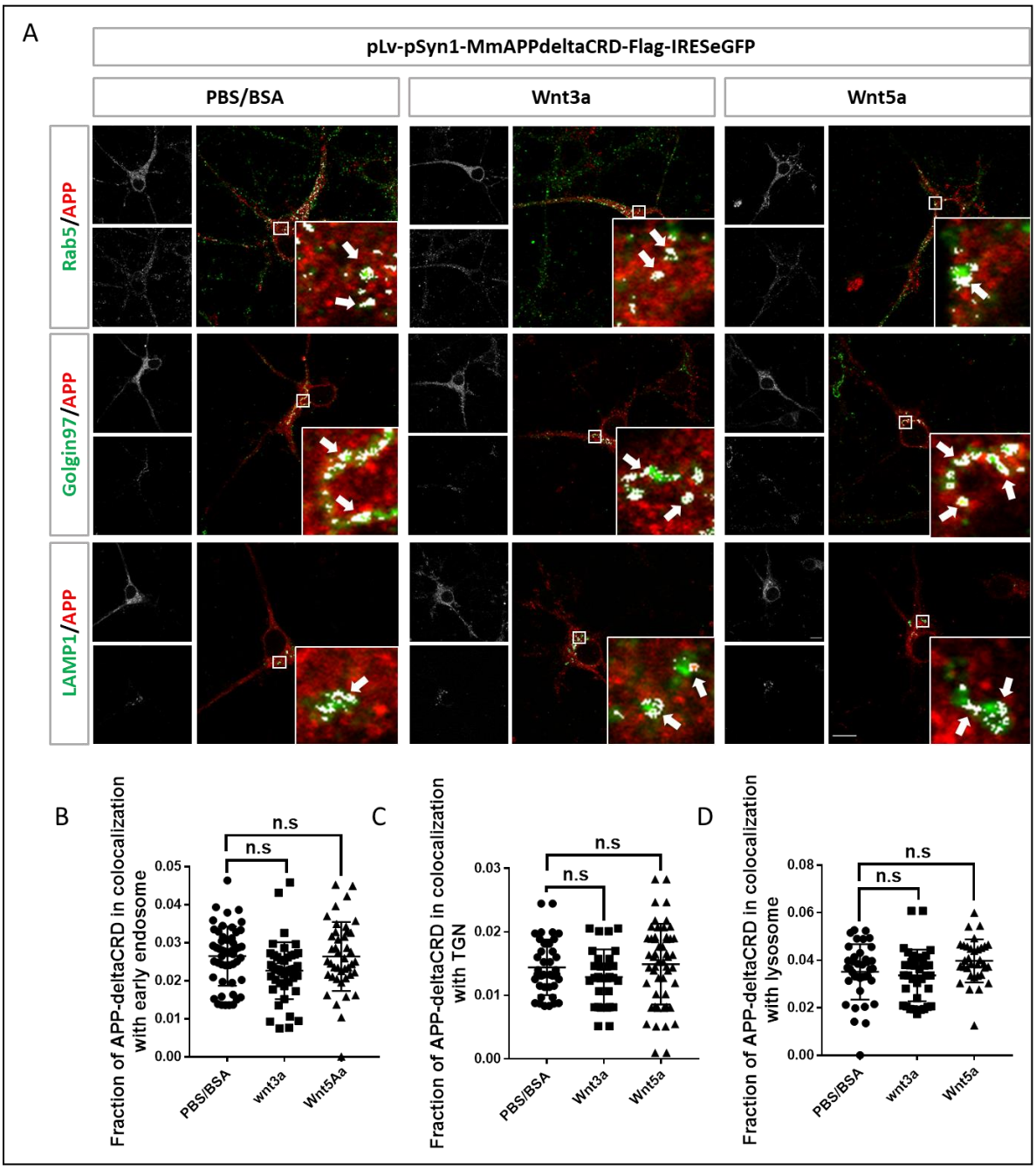

Figure 23. CRD is required for Wnt3a/5a to affect mAPP trafficking and expression

(A) Localization of exogenous mAPP expression in APP–KO primary cultured cortical neurons 4 hours after Wnt3a or Wnt5a treatment. Immunofluorescence for APP (red), Rab5 (green), Golgin97 (green) and Lamp1 (green) revealed mAPP localization in different intracellular compartments. The inset showed high magnification images of the area in the white box and arrows indicated the overlap of mAPP with respective cellular compartment markers. White labels the overlap between mAPP and respective cellular compartment after image processing by Fiji co-locolization plugin with same threshold. Scale bar = 10um. (B-D) Quantification of the overlap of mAPP with early endosome (B), TGN (C), or lysosome (D), respectively, after Wnt3a or Wnt5a

treatment. (n=32-51 cells, one-way ANOVA). Bars represent the mean ± S.E.M. for at least two independent experiments.

4.5 CRD is critical for APP to regulate neurite outgrowth and complexity

APP and its proteolytic products have been reported to affect neurite outgrowth during development [227,228] in different systems. We used primary cortical neuron derived from E16.5 mice embryos to investigate if the CRD of mAPP is required for regulation of neurite outgrowth by mAPP. We examined axonal and dendritic outgrowth (Figure 24) at three developmental stages: DIV2, DIV3 and DIV7.

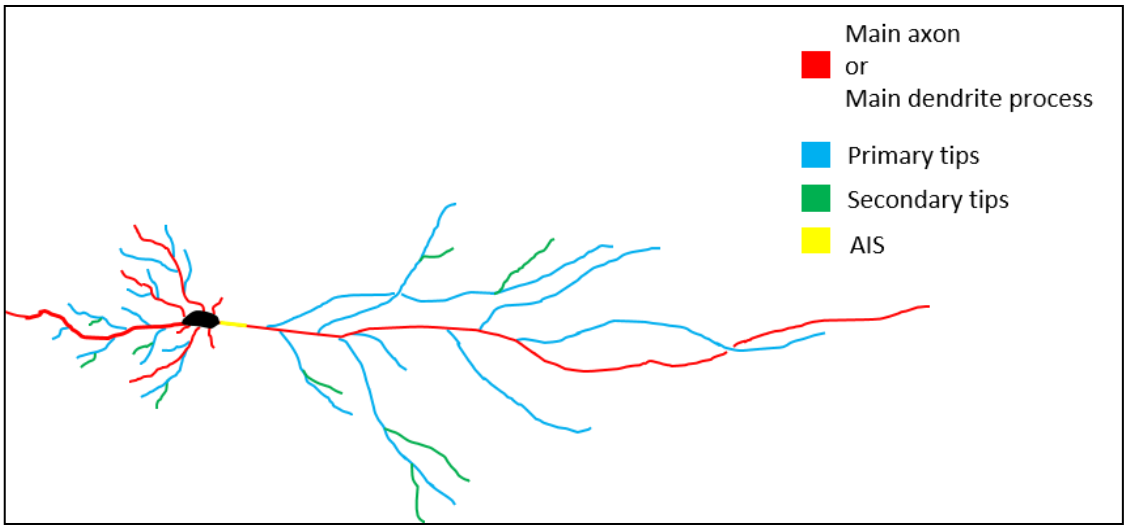

Figure 24. Schematic of a primary neuron.
Schematic of a primary neuron. Colored lines indicate axonal or dendritic branch tips which were quantified. Yellow indicates the Axon Initial Segment (AIS) marked with Ankry G in experiments. Ankry G together with GFP (introduced by plasmid or virus) are used to distinguish axon and dendrite in the following experiments at each stage (DIV2, DIV3 and DIV7).

First, we found no effect on initial outgrowth at DIV2 (Figure 25A-F).

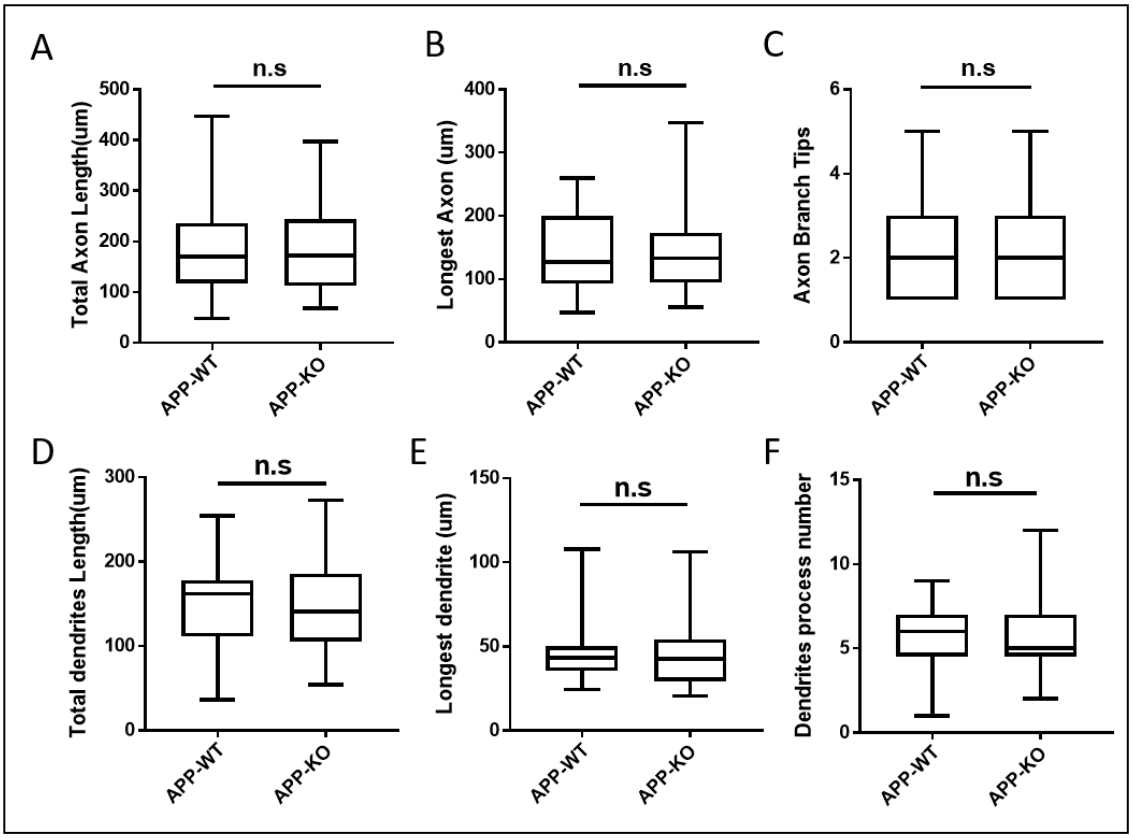

Figure 25. Neurite outgrowth is unaffected in APP knock out neurons at DIV2
(A-C) Quantification of the total axon length (A), the longest axon length (B) and axon branch tips (C) from DIV2 cultured primary cortical neuron. (D-F) Quantification of the total dendrite length (D), the longest dendrite length (E) and dendrite branch tips (F) from DIV2 cultured primary cortical neuron. Bars represent the mean ± S.E.M., n=30-38 cells, t-test.

While at DIV3 mAPP knockout neurons exhibit increased axonal outgrowth compared to controls reflected in three parameters: total axon length, longest axon length and the number of branch tips (Figure 26A-D).

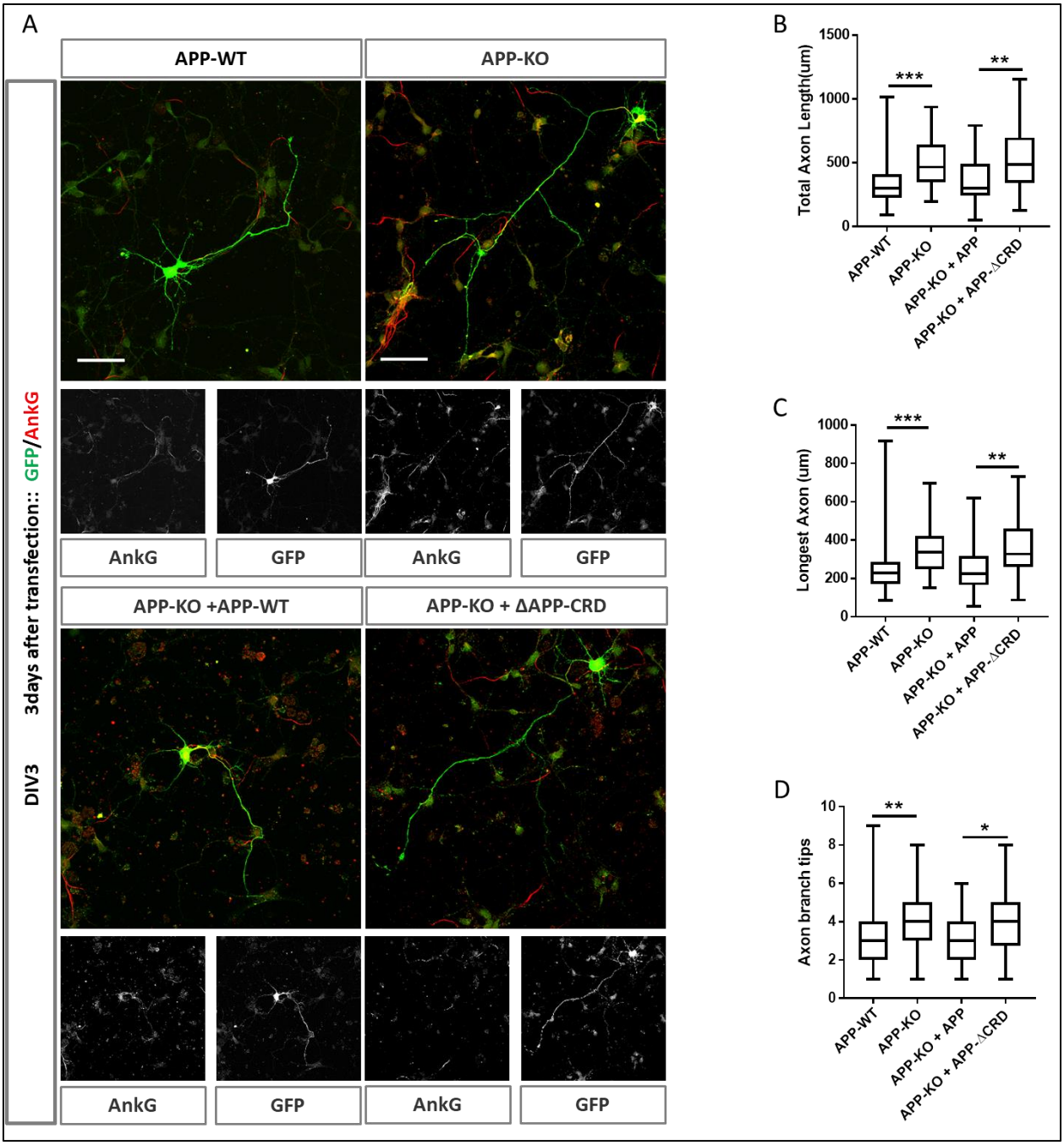

Figure 26. Cysteine Rich Domain is critical for APP to regulate neurite outgrowth at DIV3. (A) Representative confocal images for GFP (green) and AnkG (red) immunostaining at DIV3 on primary cultured cortical neurons of the four genotypes examined: mAPP wild type, mAPP–KO and mAPP–KO rescued with APP or CRD-mutant APP. Transfected plasmids containing GFP alone, mAPP-flag-GFP or mAPPΔCRD-flag-GFP, which was performed at the onset of cell seeding. Scale bar = 50 um. (B-D) Quantification of three parameters on these four genotypes at DIV3: the total axon length (the main axonal process and the branches deriving from the main process, B), the longest axonal length (C) and the total axonal branch tips (D). (n=59-70 cells, one-

way ANOVA). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05, **P<0.01 ***p<0.001.

In contrast, dendritic outgrowth was not different (Figure 27A-C). We asked whether the CRD was required for mAPP function during neurite outgrowth. To this end we transfected mAPP knockout neurons with either fl-mAPP or mAPPΔCRD. Increased axonal length and axonal branch tips were rescued by the fl-mAPP but not by the form lacking the CRD at DIV3 (Figure 26B-E). Next, we analysed axonal branching in greater detail and found that loss of mAPP increased the numbers of both primary and secondary axonal branches at DIV3, an increase that was rescued by fl-mAPP but not or mAPPΔCRD (Figure 28A and B).

Finally, we examined the Axon Complexity Index (ACI) [229], which measures the ratio of branches of different orders to total branch number, at DIV3. At this early stage, the ACI showed a tendency to increase in mAPP knockout neurons that was not significant (Figure 27D), likely because both primary and secondary branches show a similar level of increase. Together these data suggest an overall increase in axonal growth. In contrast to axonal growth, we found no significant alterations on dendritic length or branching (Figure 27A-C and E-G) consistent with the fact that the spur in dendritic outgrowth is largely initiated at DIV4[230,231].

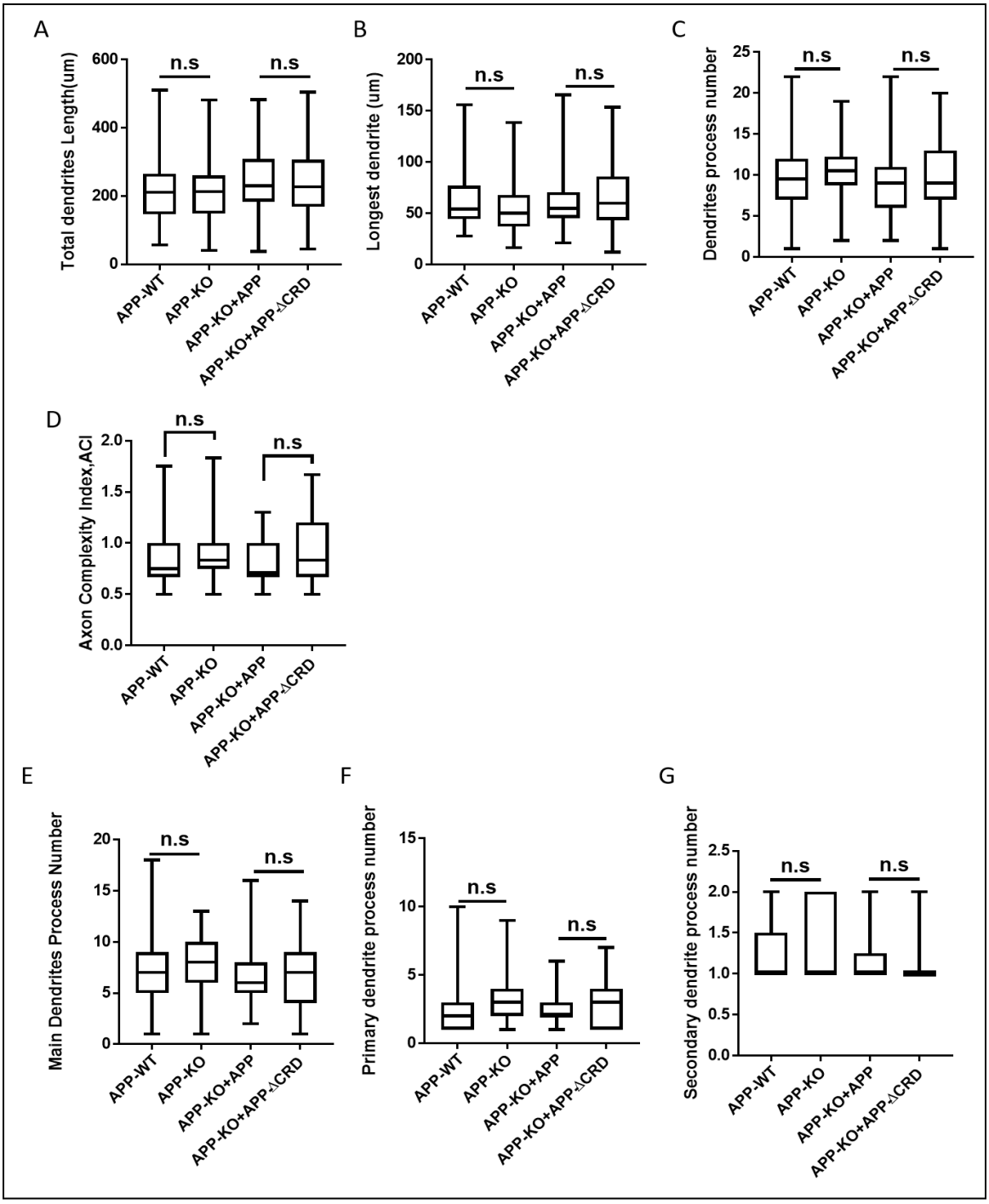

Figure 27. Analysis of dendritic outgrowth and axon complexity index at DIV3

(A-C) Quantification of the total dendrite length (A), the longest dendrite length (B) and dendrite branch tips (C) from DIV3 cultured primary cortical neurons on APP-WT groups, APP-KO groups, APP-KO rescued with APP groups and APP-KO rescued with APP-ΔCRD groups. (n=51 cells, one-way ANOVA). (D) Axon complexity Index (ACI) analysis of axon at DIV3 on each group. (n=59-69 cells, one-way ANOVA). (E-G) Quantification of the main dendrite process (E), the primary (F) and secondary (G) dendrite process numbers on each group (n=51 cells, one-way ANOVA). Bars represent the mean ± S.E.M.

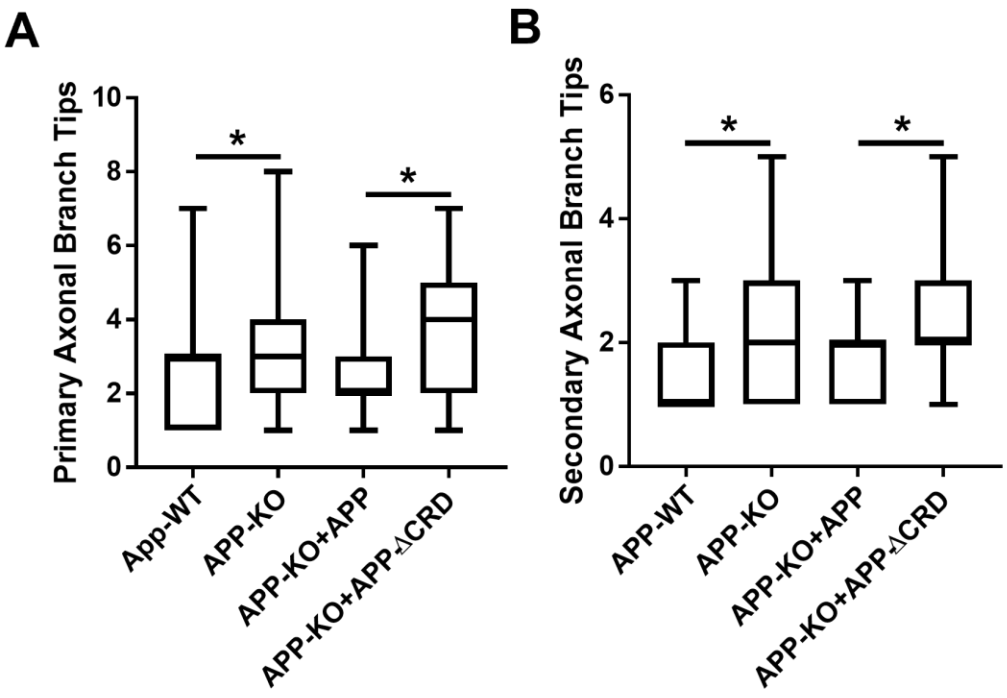

Figure 28. Cysteine Rich Domain is critical for APP to regulate neurite outgrowth at DIV3
(A) Quantification of primary branch tips at DIV3. (B) Quantification of secondary branch tips at DIV3. (n=59-70 cells, one-way ANOVA). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05.

To further analyse neurite outgrowth, we examined axonal and dendritic growth at DIV7. By this stage mAPP knockout neurons showed an increased ACI (Figure 29 A, B). In contrast, total axonal length, longest axon length and the total number of branch tips was not significantly different (Figure 29 A, C-E). The increase in axonal complexity in mAPP knockout neurons was due to a significant reduction in the number of primary branches and a significant increase in the number of secondary branches (Figure 29 F, G). Once again, all phenotypes were rescued by fl-mAPP but not mAPPΔCRD (Figure 29A and C-E).

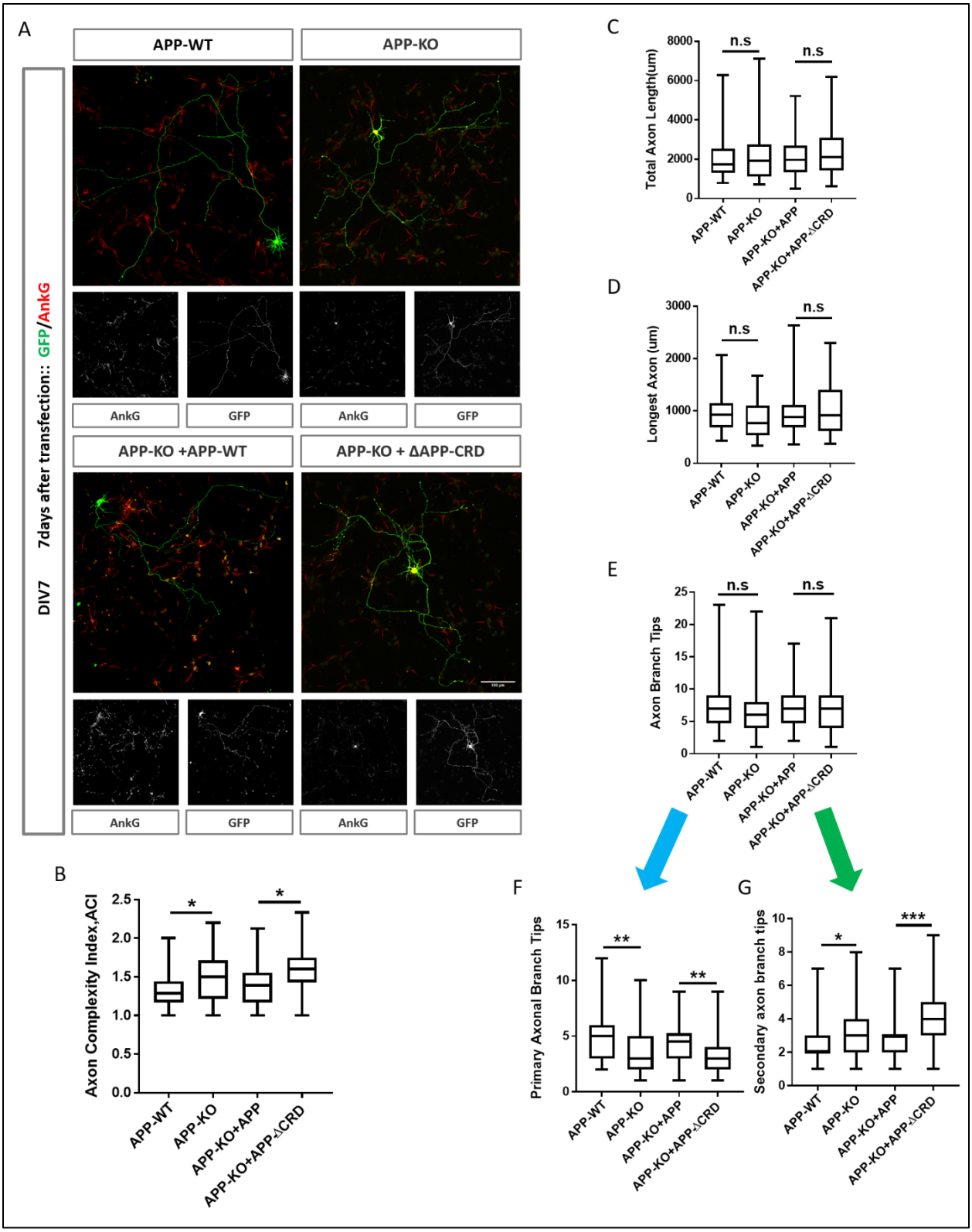
A
APP-WT
APP-KO
DIV7 7days after transfection:: GFP/AnkG
AnkG
GFP
AnkG
GFP
APP-KO +APP-WT
APP-KO + ΔAPP-CRD
AnkG
GFP
AnkG
GFP
B
Axon Complexity Index,ACI
2.5
2.0
1.5
1.0
0.5
0.0
*
*
APP-WT
APP-KO
APP-KO+APP
APP-KO+APP-ΔCRD
C
Total Axon Length(um)
8000
6000
4000
2000
0
n.s
n.s
APP-WT
APP-KO
APP-KO+APP
APP-KO+APP-ΔCRD
D
Longest Axon (um)
3000
2000
1000
0
n.s
n.s
APP-WT
APP-KO
APP-KO+APP
APP-KO+APP-ΔCRD
E
Axon Branch Tips
25
20
15
10
5
0
n.s
n.s
APP-WT
APP-KO
APP-KO+APP
APP-KO+APP-ΔCRD
F
Primary Axonal Branch Tips
15
10
5
0
**
**
APP-WT
APP-KO
APP-KO+APP
APP-KO+APP-ΔCRD
G
Secondary axon branch tips
10
8
6
4
2
0
*
***
APP-WT
APP-KO
APP-KO+APP
APP-KO+APP-ΔCRD

Figure 29. Cysteine Rich Domain is critical for APP to regulate neurite outgrowth and complexity at DIV7.
(A) Representative confocal images for GFP (green) and AnkG (red) immunostaining at DIV7 on primary cultured cortical neurons of the four genotypes examined: mAPP wild type, mAPP- KO and mAPP-KO rescued with APP or CRD-mutant APP. Transfected plasmids containing GFP alone, mAPP-flag-GFP or mAPPΔCRD-flag-GFP, which was performed at the onset of cell seeding. Scale bar = 100 um. (B-G) Analysis of Axon complexity Index (ACI, B), the total axonal length (C), the longest axonal length (D), all axonal branch tips (E), the primary branch tips (F) and the secondary branch tips (G) at DIV7. (n=54 cells, one-way ANOVA). Bars represent the mean ± S.E.M. for at least two independent experiments. *P<0.05, **P<0.01 ***p<0.001.

Finally, we examined dendritic growth at DIV7. We observed no difference in total dendrite length or the size of the longest dendrite (Figure 30 A, B), but observed a significant decrease in the total number of dendritic processes (Figure 30 C). This reduction was due to the presence of fewer main dendritic processes in mAPP knockout neurons but no effect observed on the primary or secondary dendritic branches (Figure 30D-F). All phenotypes were rescued by fl-mAPP but not mAPPΔCRD. Taken together, our results show that the role of APP in neuronal maturation requires the CRD domain.

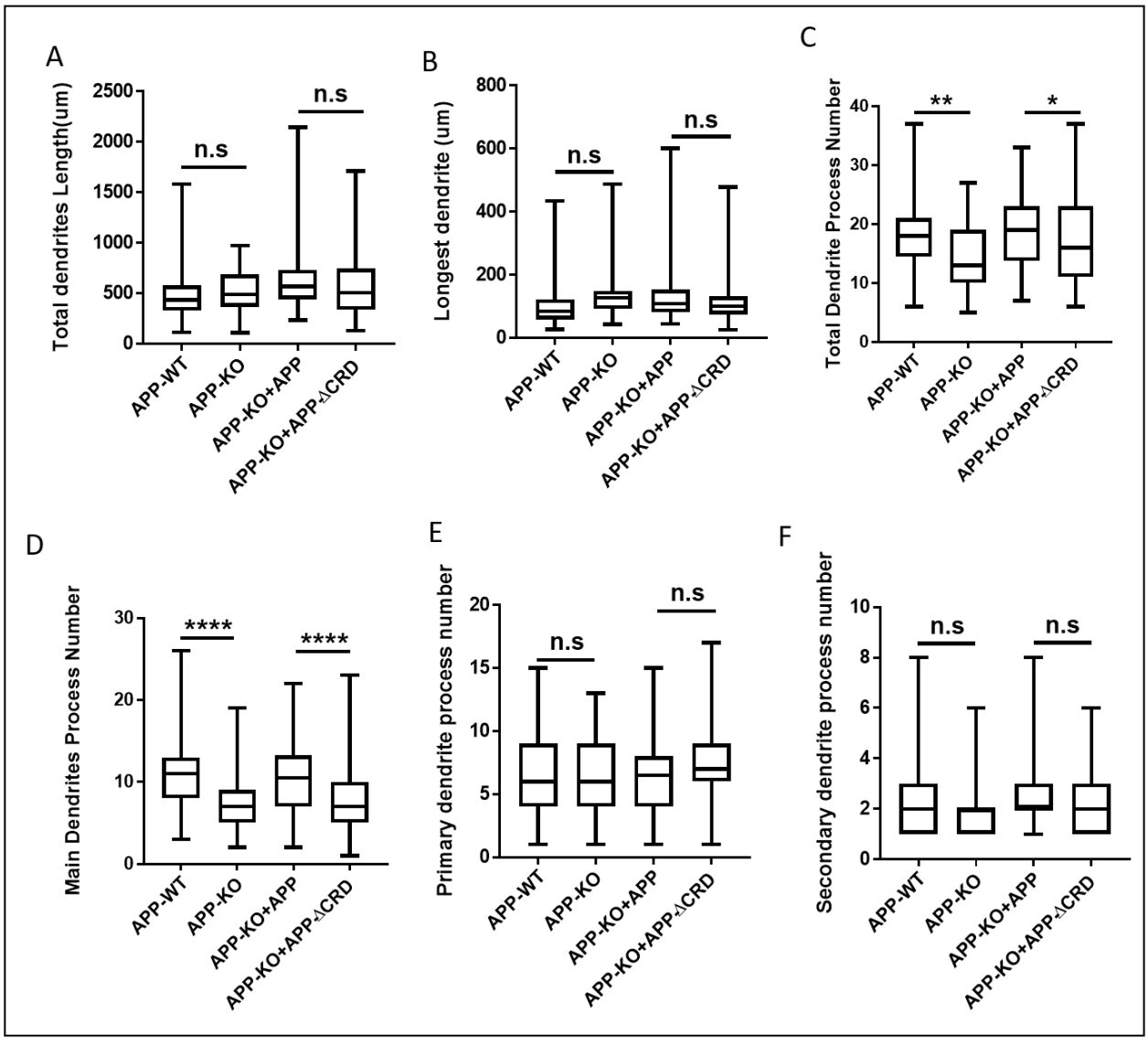

Figure 30. Outgrowth and Complexity analysis of neurite at DIV7

(A-C) Quantification of the total dendrite length (A), the longest dendrite length (B) and dendrite branch tips (C) from DIV7 cultured primary cortical neurons on APP-WT groups, APP-KO groups, APP-KO rescued with APP groups and APP-KO rescued with APP-ΔCRD groups. (n=52 cells, one-way ANOVA). (D-F) Quantification of the main dendrite process (D), the primary (E) and secondary (F) dendrite process numbers from DIV7 cultured primary cortical neurons on each group. (n=52 cells, one-way ANOVA). Bars represent the mean ± S.E.M. *P<0.05, **P<0.01, ****P<0.0001.

4.6 Wnts regulate neurite development in a CRD dependent manner

The Wnt pathway plays an important role in regulating neurite development, therefore it is interesting for us to study if the CRD is important for the interaction between APP and Wnt in regulating neurite outgrowth. To address this question, we analyzed neurite outgrowth in APP-KO primary cortical neurons rescued with APP-WT or APP-ΔCRD plasmids after Wnt3a/5a treatment. At DIV3, Wnt3a treatment significantly increased total axon length, the length of longest axon and the number of axon branch tips in the wild type or APP-WT rescue group but not the APP-KO or APP-ΔCRD rescue group compared to their controls, respectively (Figure 31A-F). While Wnt5a treatment increased the length of the longest axon in both the APP and CRD mutant groups (Figure 31C-D). With respect to dendrites, Wnt5a addition was increased the total dendrite length and the length of the longest dendritic process in a CRD dependent manner compared to controls, while Wnt3a treatment did not significantly affect either of these two parameters in dendrites (Figure 32A-D). Finally, dendrite number was not affected in any of the experimental groups (Figure 32E-F).

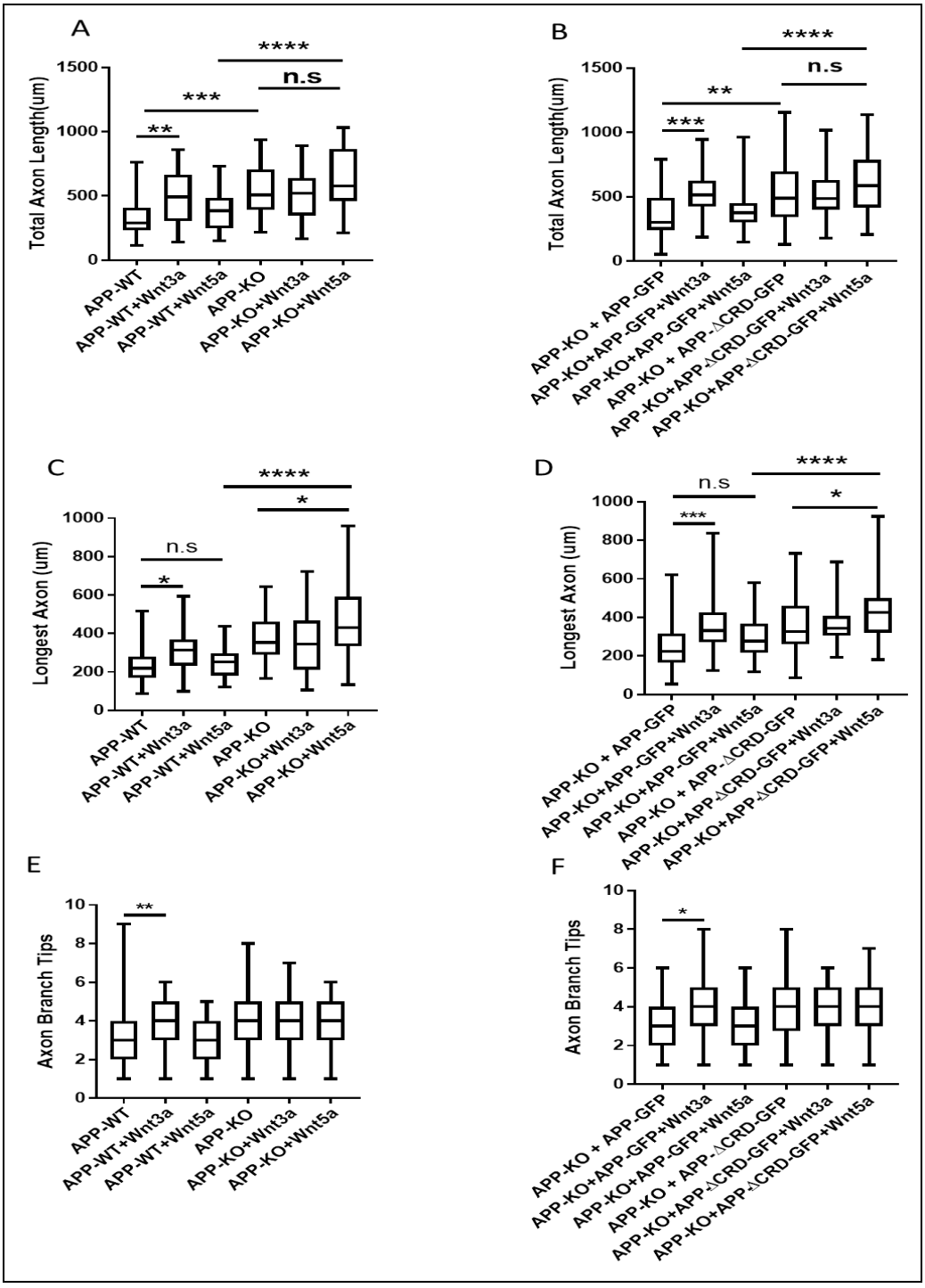

Figure 31. Axonal outgrowth analysis at DIV3 after Wnts treatment

Quantification of (A-B) total axon length, (C-D) length of longest axon and (E-F) axon branch tips from DIV3 cultured primary cortical neurons after Wnts treatment from wild type, APP-KO mice or from APP-KO neurons rescued with APP-WT or APP-ΔCRD, respectively. *P<0.05, **P<0.01 ***p<0.001 ****P<0.0001

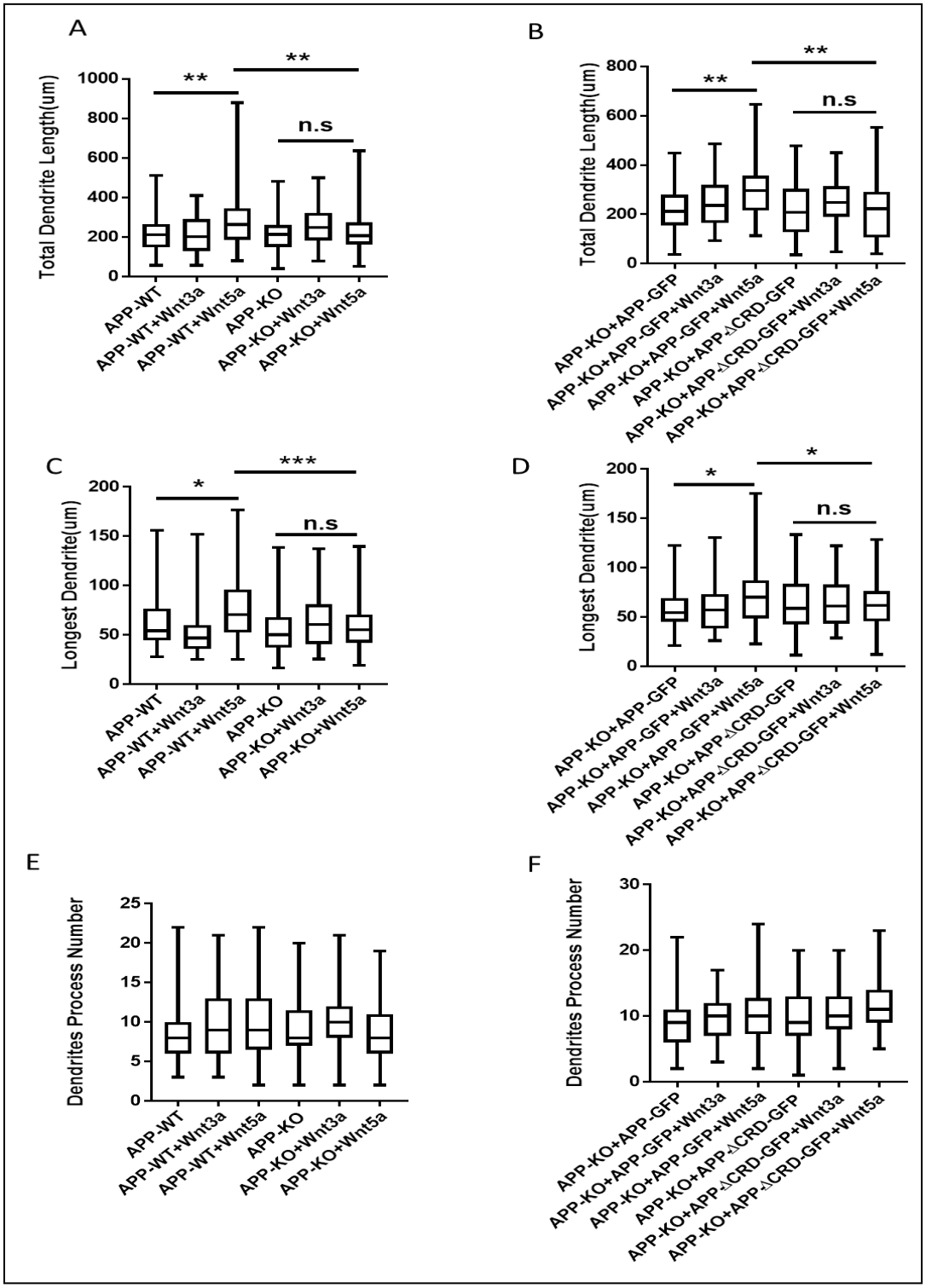

Figure 32. Dendritic outgrowth analysis at DIV3 after Wnts treatment

Quantification of (A-B) total dendritic length, (C-D) length of longest dendritic process and (E-F) dendritic branch tips from DIV3 cultured primary cortical neuron after Wnts treatment from wild type, APP-KO mice or from APP-KO neurons rescued with APP-WT or APP-ΔCRD, respectively. *P<0.05, **P<0.01 ***p<0.001.

At DIV7, Wnt3a was shown to affect the axonal complexity in a CRD dependent manner (Figure 33A and B). Surprisingly Both Wnt3a or Wnt5a treatment significantly affected total axon length, the longest axonal process and axonal branch tips, but not in a CRD or APP dependent manner as all Wnts treatment group showed a significant increment in the three parameters compared to their control groups (Figure 34A-F). This suggest that while Wnt action on individual parameters at DIV7 can be APP independent, the integration of these effects to produce an appropriate level of axonal complexity requires APP and its CRD domain.

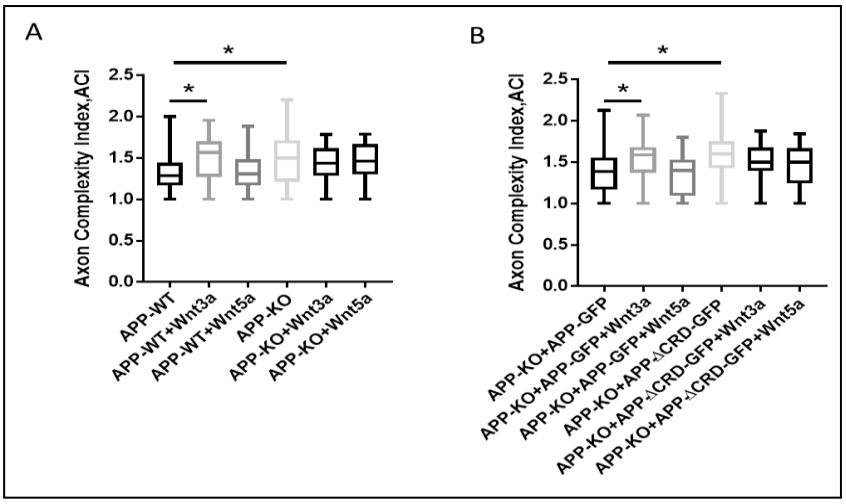

Figure 33. Axon complexity analysis at DIV7 after Wnts treatment
Analysis of (A-B) axon complexity from DIV7 cultured primary cortical neuron after Wnts treatment from wild type, APP-KO mice or from APP-KO neurons rescued with APP-WT or APP-ΔCRD, respectively. *P<0.05.

Regarding dendritic development, we found that both Wnt3a and Wnt5a decreased the main dendritic process number in a CRD dependent manner, where the absence of the CRD even reversed the Wnt3a effect (Figure 35A and B). In contrast, both total dendritic length and length of longest dendritic process were affected after Wnt treatment but did not require the CRD or APP (Figure 35C-F). In summary, these data indicate that Wnt treatment affects different aspects of neurite outgrowth at least in part in a CRD dependent manner.

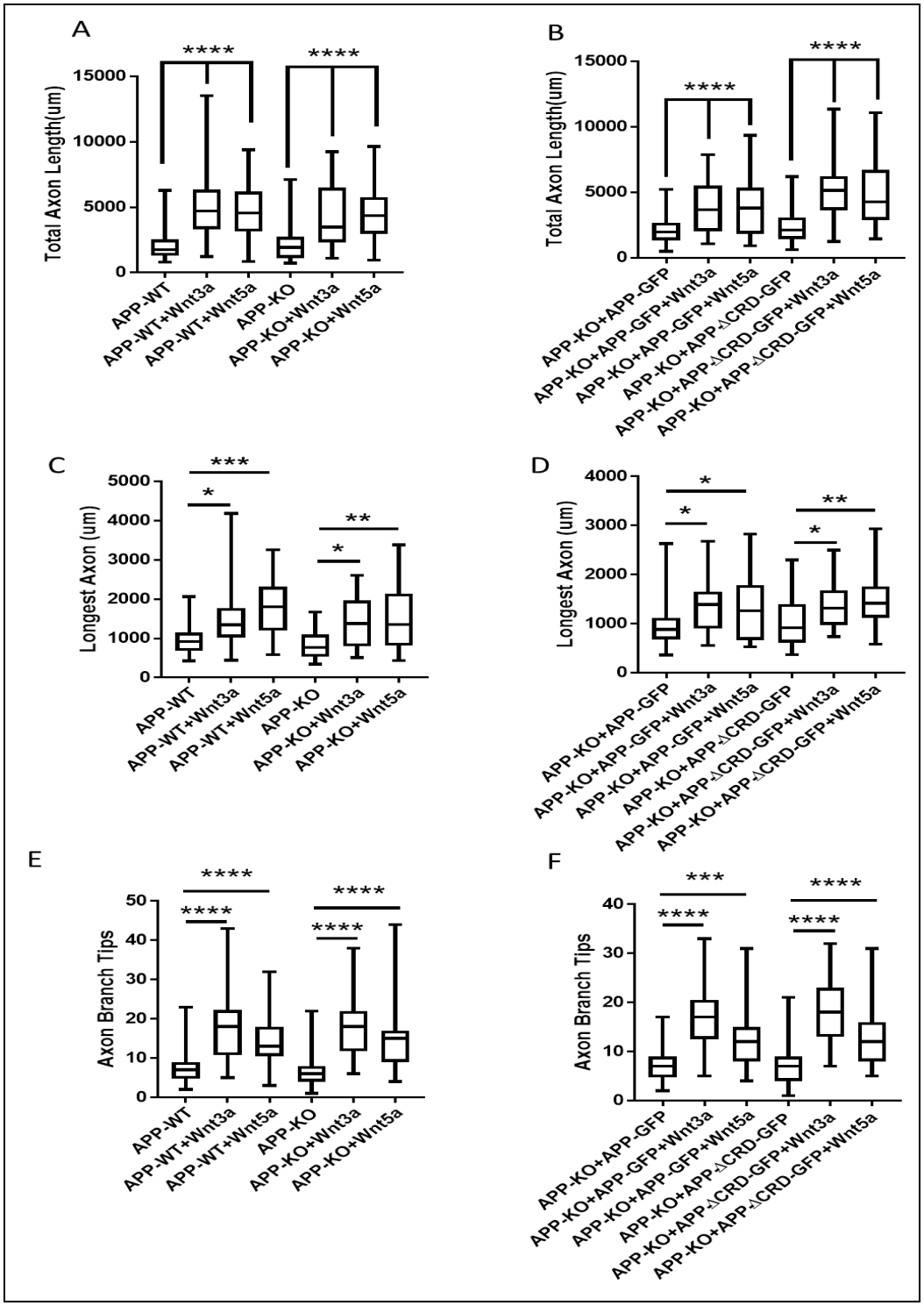

Figure 34. Axonal outgrowth analysis at DIV7 after Wnts treatment

Quantification of (A-B) total axon length, (C-D) length of longest axon and (E-F) axon branch tips from DIV7 cultured primary cortical neuron after Wnts treatment from wild type, APP-KO mice or from APP-KO neurons rescued with APP-WT or APP-ΔCRD, respectively. *P<0.05, **P<0.01 ***p<0.001 ****P<0.0001

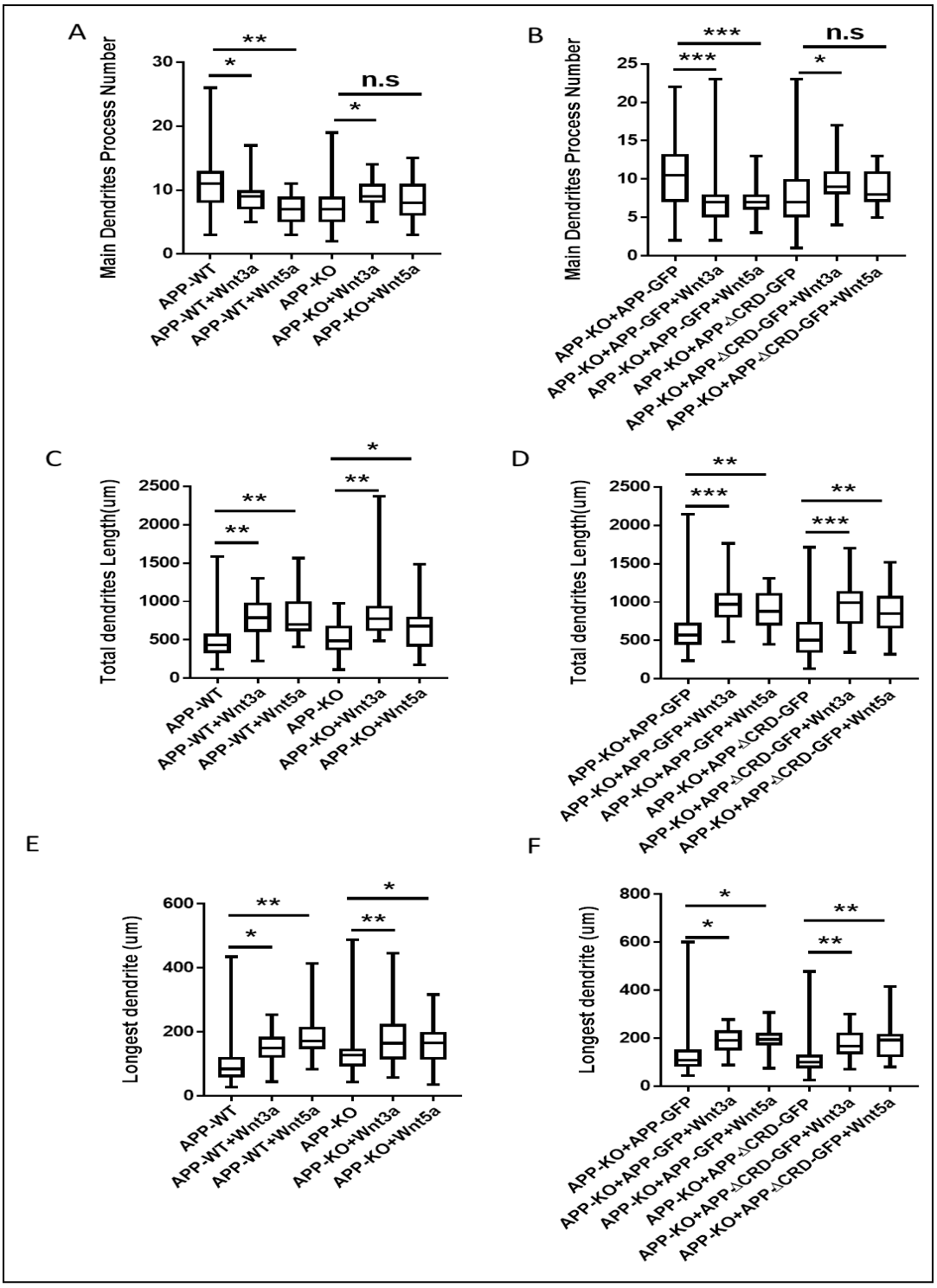

Figure 35. Dendritic outgrowth analysis at DIV7 after Wnts treatment

Quantification of (A-B) main dendritic process number, (C-D) total dendritic length, (E-F) length of longest dendritic process from DIV7 cultured primary cortical neuron after Wnts treatment from wild type, APP-KO mice or from APP-KO neurons rescued with APP-WT or APP-ΔCRD, respectively. *P<0.05, **P<0.01 ***p<0.001.

CHAPTER 5 DISCUSSION

5.1 APP and Wnt signaling

The amyloid precursor protein (APP) was considered to occupy a central position in Alzheimer's disease (AD), due to the proposed amyloid cascade hypothesis, which put forward that the deposition of Aβ, exclusive metabolite of APP, is the causative agent of this neurodegenerative disease[50]. In line with this hypothesis, APP transgenic in mice were shown to reproduce part of the pathological features of AD patients, and further, rescue methods focusing on the inhibition or reduction of Aβ production could significantly reduce neuronal death, synaptic loss and memory impairment[232–234]. In addition, APP is also relevant to another genetic disorder, Down syndrome, characterized by physical and intellectual disabilities, and a significant proportion of Down syndrome patients exhibit AD symptoms in early life. This might be a consequence of the excessive expression and processing of APP due to the third copy of chromosome 21 exist which harbors the gene coding for APP[235]. Although, this hypothesis may well be supported by many experiments performed *in vivo* and *in vitro*, which strongly affected the therapeutic strategy in clinical trials, the negative outcomes of clinical trials targeting on Aβ reopens the question of the causal role of Aβ in AD [236,237]. More recently, increasing effort has shifted to focusing on exploring the normal physiological function of APP and its proteolytic products, as a full understanding of APP could provide a new and effective way in dealing with AD.

Wnt signaling pathways regulates various cellar processes during different stages of life, including tissue morphogenesis during development and tissue homeostasis in aging adults. Disruption of Wnt signaling has been related to cancer, metabolic disease, cardiovascular diseases and neurodegenerative diseases[238]. Therefore,

understanding the underlying mechanisms of Wnt pathways in disease and restoring proper Wnt signaling has been an important strategy to deal with those diseases. With regard to neurodegenerative disease, ageing is one of the risk factors in late onset AD. In the aging brain, synapses are more vulnerable and sensitive to toxic metabolites like Aβ, in parallel components of Wnt signaling complexes are altered during aging which may have important consequence on maintaining the plasticity of synapses. According to the literature, several Wnt ligands and receptors were reported to be downregulated in aged human and rodent brains, while the expression of endogenous canonical Wnt signaling antagonists SFRP1 and Dkk1 were upregulated[239–242]. Therefore, hampered Wnt signaling could be involved in synapse loss in AD. Indeed, in AD transgenic mouse models, mutations in LRP6 (co-receptor of Wnt/β-Catenin pathway) lead to a loss of synapses accelerating AD pathology[243]. In addition, elevated Dkk1 expression has also been observed in both AD mouse models and AD patients[244,245]. DKK1 inhibits Wnt/β-Catenin signaling by directly binding with the co-receptor LRP5/6. More importantly, expression of DKK1 could be induced by Aβ. Furthermore an interesting Aβ feedback loop proposed by Sellers et al. indicating that Aβ induced Dkk1 expression shift the Wnt signaling from canonical branch to the non-canonical PCP pathway which favors amyloidogenic processing of APP and induces synaptic retraction[95]. Based on these reports, both canonical and non-canonical Wnt signaling are implicated in Aβ related AD pathology, but whether the aging related shift of the two branches of Wnt signaling is a cause or consequence of abnormal Aβ accumulation, and how Wnt pathways affect APP processing are still not well documented. A comprehensive understanding of the interaction between APP and Wnt signaling under both physiological and pathological conditions is required.

Given the important role of Wnt signaling and the spatio-temporal expression pattern of APP during CNS development, it is reasonable to speculate that the interaction between APP and Wnt signaling may not only occur in ageing or under pathological conditions but may already start in early development especially in CNS, and may be required for cellular and tissue homeostasis. If so, disease could be viewed as a consequence of the disruption of such an interaction. Evidence from fly experiment has first shown that APPL (APP homolog in fly) is part of the Wnt/PCP complex required for the signal transduction and regulation of axonal outgrowth in the developing mushroom body[246]. Furthermore, a set of *in vitro* experiments published recently has shown that APP may not only be a novel component of Wnt/PCP pathway, but may also be a component of the canonical Wnt/β-Catenin pathway, which could enhance the signaling in both pathways and in turn affect the proteolysis of APP itself[95]. Although the link between APP and Wnt signaling has been established from these studies, the cellular and molecular details of the putative role of APP in Wnt signaling still needs further analysis.

5.2 APP is a putative Wnts receptor conserved cross species

The speculation that APP functions as a cell surface receptor has be proposed for quite a long time[247]. The first strong evidence came from the structure of APP which shares similarity with type I transmembrane receptors. For example the growth factor like domain (GFLD) in the E1 region of APP could act as a ligand binding site, and disulfide bridges within the same E1 area could further facilitate ligand induced signal transduction by stabilizing the structure of APP ectodomain[248–251]. Beside the structural basis, the site-specific proteolytic processing property of APP resembles several membrane receptors and could be the second strong evidence. For instance, both APP and Notch are processed by γ-secretase and release the intracellular domain AICD and NICD, respectively, to induce transcriptional activity

by nuclear transfer of the ICDs[252,253]. Several putative ligands have been proposed including proteolytic products of APP itself. Interestingly, a conserved Cysteine Rich Domain (CRD) within the E1 region of APP makes APP a putative Wnt receptor as the CRD is required for the binding between Wnt and its receptor and co-receptor like Frizzled, Ror-2 and Musk[254]. In this study and part of our fly work, using immunoprecipitation we have shown that both mouse APP and human APP bind to Wnt3a and Wnt5a, the two well-known ligands for triggering canonical or non-canonical Wnt signaling pathways, respectively. Fly APPL and human APP were shown to bind with Wnt5a directly and required for regulating axonal extension (fly data not shown here). Furthermore, all this binding occurs in a CRD dependent manner. Based on these data, we speculate that APP might act as a conserved Wnt receptor as its CRD is highly conserved across species, and that the binding may not be limited to Wnt3a or Wnt5a, but also apply to all other Wnt family members. As Wnt signaling is critical for development and tissue homeostasis, the role of APP as a component or putative receptor or co-receptor in Wnt signaling should be carefully studied. The difficulty to explore the exact role of APP may not only come from the complexity of Wnt signaling, but also from the structural properties of APP itself. For example, the extracellular domain of APP harbors several binding site for the molecules in the extracellular matrix (ECM), such as the Heparin binding domain (HBD) is not only exist in E1 area, a second HBD has been found in the E2 region[255]. And a paper published recently has shown that LRP6, the co-receptor of canonical Wnt signaling, could directly bind to APP[95]. Using peptide Mapping array, this group further revealed several binding sites (20-70 amino acids in length) in the ectodomain of APP, and one of these sites (34 amino acids) is located right inside the CRD. Based on our IP results indicating a Wnt receptor function for APP, an obvious question arises as to whether LRP6 and APP compete for Wnt. Before trying to address this problem, several issues need to be resolved. First, in our IP

experiments the whole CRD (150 amino acids) was deleted, it is not clear if specific sequences inside the CRD are critical for Wnt binding that may or may not overlap with the reported LRP6 binding site. Second, an important point is that the results of the peptide mapping array may not reflect the exact binging site for LRP6 as those short synthesized peptide may lose the information of 3D structure, which is important for recognition between proteins, like the disulfide bridges are formed by Cysteines which may be critical for maintaining the stability of the APP extracellular domain[256,257]. Therefore, we think that more rigorous experiments such as crystal structure analysis are needed to reveal the details of APP binding with Wnt and/or or LRP6.

Another important point is that the binding affinity between APP and Wnt needs to be tested to determine the likely physiological relevance of the direct binding. To address this question, we have tried the solid phase measurements using commercial ELISA kits. Results showed non-specific binding between Wnt3a/5a with the plate, which precludes any further testing with this approach. As Wnts are notoriously "sticky" proteins *in vitro*, using other strategies for example dot blot or biolayer interferometry may not yield easy to interpret results.

Compared with full-length APP, the physiological function of its proteolytic products is another research focus. Studies from the past decades have revealed various functions of secreted APP fragments, including neurogenesis, neurite outgrowth, neuronal migration as mentioned before. More importantly, recent studies have identified secreted APP as a ligand in regulating synaptic activities by interacting with corresponding receptors. Richter et al. have shown that only the sAPP-α but not the sAPP-β could bind to postsynaptic α7 nicotinic acetylcholine receptor (nAChR) and facilitate hippocampal long-term potentiation (LTP) due to the critical 16 amino acids which exist in sAPP-α[258]. In another study, Rice et al.

have found that one of the variants of the γ-aminobutyric acid type B receptor subunit 1 (GABABR1a) is a novel sAPP receptor in regulating GABAergic Neurotransmission[259]. Interestingly, the critical binding region is located in the extension domain (ExD) of APP, which is not present in APLP1 or APLP2, indicating the unique function of sAPP in balancing synaptic plasticity. Furthermore, the 17-mer peptide within ExD is able to induce $GABA_BR1a$ dependent neuronal activity suppression *in vivo*. This finding could provide a potential therapeutic strategy in dealing with several neurological disorders which $GABA_BR$ is involved in[260,261]. Importantly, the authors underline that the influence of full-length APP directly interacting with $GABA_BR$ could not be excluded, indicating that full-length APP and its cleavage products may work in concert for modulation of synaptic transmission. This also provides a new perspective to study APP and its function in Wnt signaling pathway. In most of the known cleavage events, the extracellular domains may be left intact, implying that the released sAPP could act as a Wnt inhibitor by binding to Wnts or to the co-receptor LRP6 to form a feedback loop to modulate Wnt mediated APP trafficking and expression. Whether sAPP separately plays a role in regulating Wnt signaling would be another interesting topic to explore. Accumulating evidence sugegsts that APP and its proteolytic products play an important role in CNS development and the maintenance of brain homeostasis. Therefore, it is important to treat APP and sAPP as a whole when it comes to the study APP or sAPP as a receptor or a ligand.

5.3 CRD is critical for Wnts to regulate APP trafficking, expression and processing

APP trafficking and proteolysis have been intensively researched as they are the core processes to produce Aβ. Disruption in these processes may shift the balance towards AD related amyloidogenic processing of APP. Thus, a full understanding of

these processes could provide an effective therapeutic target for AD. As mentioned before, disrupted Wnt signaling has been related to Aβ generation, but the underlying mechanisms are not well documented. In this study, we have shown that, first, in developing primary cortical neurons Wnt3a or Wnt5a treatment differentially affect full-length APP (flAPP) expression and those influences do not come from the disruption of transcription as Wnt treatment was short and mRNA expression was not affected. Second, our immunofluorescence results indicated that this change in protein expression may come from the altered intracellular trafficking of APP, as Wnt3a treatment transfers more APP to Trans Golgin Network (TGN), while Wnt5a transfers more APP to the lysosome. Furthermore, our data on Aβ generation in cultured primary neurons after Wnts treatment showed a similar outcome to a report published recently[95,262]. Interestingly, Wnt3a and Wnt5a seem to work in a competing manner with regards to APP expression, because treatment with both Wnt3a and Wnt5a at the same time eliminates the influence observed in sole Wnt3a or Wnt5a addition, indicating a common receptor. Finally, by utilizing with lentivirus in APP-knockout primary cortical neuron, we found that the CRD is required for Wnts to regulate APP trafficking and expression. We suggest that our data provide a mechanistic framework for understanding how a shifted balance between canonical and non-canonical Wnt signaling may affect Aβ generation in ageing related AD pathology.

With respect to considering APP as a receptor for Wnts, the physical structure of APP provides the first evidence as mentioned above, and the second clue comes from Wnt triggered APP trafficking and processing, as transducing the extracellular signaling after ligand binding often requires the internalization of cell surface receptors to recruit downstream effectors. On the other hand, endocytosis and processing of receptors is an important way for signal desensitization and decrease

signaling from extracellular circumstance[263–265]. For instance, Dkk1 is able to down-regulate Wnt/β-Catenin signaling by directly binding with the co-receptor LRP6 followed by internalization. Our data indicated that Wnt addition influences APP turnover, but the underlying mechanisms are not fully revealed. For instance, which downstream molecules participate in this process and whether Wnt signaling activation may occur in response to APP trafficking is unclear. To answer these questions, a hint provided from our previously published data showed that APP also binds to Vangl2, a key component of Wnt/PCP signaling pathway[246]. In line with this, a single binding site for Vangl2 was identified in the intracellular domain of APP[95]. Furthermore, part of our unpublished data revealed that in HEK cells Wnt5a addition destabilizes the interaction of *Drosophila* Vang and APPL in a CRD-dependent manner. Taking these observations together, we speculate that Wnt5a treatment disrupts the binding between APP and Vangl, which distablizes APP and increases the chance of internalization and processing. For Wnt3a treatment, the consequence may be opposite. LRP6 was shown to bind with APP on multiple sites in the extracellular domain, which could protect APP from being processed, thereby facilitating full length APP recycling back to the cell surface. Whether LRP6 is internalized when bound to APP still needs to be carefully analyzed.

Limitations of using exogenous APP in this study should also be considered. For example, introduced APP in primary cultured neurons may show a different expression pattern compared to endogenous APP. To test this, additional assay should be performed. For instance, APP could be endogenously tagged with one fluorophore and then neurons with endogenously tagged APP could be transfected with exogenous APP tagged with another fluorophore to quantify the degree of co-localization in various compartments. Another question is whether the trafficking of APP-ΔCRD is already altered before Wnt treatment. To test this the Retention Using

Selective Hooks (RUSH) system[266] could be used in primary cultured neuron. The RUSH system allows synchronous release of proteins of interest in different cellular compartments. Combined with live imaging, this allows the documentation of the trafficking route of APP and APP-ΔCRD before and after Wnt treatment.

5.4 CRD of APP mediates Wnts induced neurite morphogenesis in cultured cortical primary neurons

APP has been extensively reported to be involved in regulating neurite outgrowth [227,228,267–270], with conflicting conclusions as to whether APP promotes or inhibits neurite outgrowth. Our previous data showed that in *Drosophila* the loss of APPL reduced axonal growth[246]. In this study, the comparison axonal outgrowth and branching in primary cortical neurons derived from mAPP wild type or mAPP knock out mice at DIV2, DIV3 and DIV7 results showed that loss of mAPP significantly accelerated axonal maturation. Specifically, we found that the initial phase of axonal growth at DIV2 is unaffected, but axons grow longer at DIV3 without APP expression and also showed increased axon complexity at DIV7. We therefore suggest that the conflicting data in the literature may arise from examining different types of neurons at different time points, where the requirement of APP may differ in a context-specific manner. We speculate that this context specificity may in part be due to the levels and types of Wnt ligands present in the environment.

Wnt signaling has been implicated in regulating different aspects of neuronal development, including neuronal polarity, neurite outgrowth and synapse formation[271]. As APP may act as Wnt receptor, its role in neuronal development makes it interesting to explore the interaction between APP and Wnts in regulating neurite development. In this work, we found that at DIV3 Wnt3a treatment significantly significant increased total axon length, the length of longest axon and

axon branch tips in the presence of fl-APP but not APP-Δ-CRD. These data suggest that APP mediates Wnt3a effects on axonal outgrowth. In contrast to Wnt3a, Wnt5a treatment had no effect in the presence of fl-APP but resulted in increased axonal length when the CRD domain was removed. These data show that APP normally protects axons from the effects of Wnt5a at this developmental stage and that the CRD is also required for this. Thus, APP promotes Wnt3a and antagonizes Wnt5a effects on neurons at DIV 3 via the CRD domain. Similarly, on DIV7 we found a positive Wnt3a effect on axon complexity, which was observed in the presence of fl-APP but not APP-Δ-CRD. In contrast, Wnt5a had no effect. Finally, we found that treatment with either Wnt3a or Wnt5a decreases the number of the main dendrites in the presence of fl-APP and the effect is absent or even reversed in the presence of APP-Δ-CRD. Together, these data show that the CRD of APP is required for Wnt3a/5a to regulate neurite outgrowth in cortical primary neurons. Mechanisms underlying how Wnts together with APP regulate neuronal outgrowth are not fully understood. One of the interesting questions that need to be answered is whether canonical or non-canonical Wnt signaling is activated or required for those effects. Previous reports in *Drosophila* showed that APPL is required for Wnt5 to trigger PCP signaling in regulating axonal outgrowth[246], and a recently published paper indicates that overexpression of APP enhances both canonical or non-canonical signaling under different condition[95]. Another interesting point is if APP together with Wnt signaling plays a role in refinement of the architecture of neural cells in adult brain, as the remodeling and maintenance of the structure of neurites is critical for functional activity. More and more evidence has shown the important role of Wnt signaling in maintaining the plasticity of neural tissue, but less attention was put on APP. This could be quite important as it may shed light on the pathology of neurodegenerative diseases like AD, as the altered Wnt signaling in aging brain may

not only affect Aβ production but also the maintenance of the functional stability of neural cells by regulating neurite architecture through APP.

5.5 General conclusion

In this work, we identify a previously unknown conserved Wnt receptor function for APP proteins. We show that APP binds both canonical and non-canonical Wnt ligands via a conserved cysteine rich domain and that this binding regulates the levels of full-length APP by regulating its intracellular trafficking from early endosomes to the trans-Golgi network versus the lysosome. Furthermore, we show that APP through the CRD regulates neurite growth and axon branching complexity in primary mouse cortical neurons. Our study here not only reveals the normal function of APP in the development of cortical neurons, but also explain part of the mechanisms how Wnt affects APP processing. All these data provide potentially important information for linking APP's physiological function to its role in AD.

CONTRIBUTION

Experiments conceived and designed by Tengyuan Liu and Bassem A. Hassan. APP-KO mice was a kind gift from Bart De Strooper's lab. plasmids used in this work was constructed by Natalia Danda. The work with mice primary cortical neuron culture were performed by Tengyuan Liu and Tingting Zhang. Virus tracduction, plasmids transfection and drug administration were performed by Tengyuan Liu. Hek293T transfection and immunoprecipitation experiments were done by Tengyuan Liu. Quantitative real-time PCR experiments were performed by Tengyuan Liu and Tingting Zhang. Western blots were done by Tengyuan Liu. Supernatant collection and Aβ 40/42 detection were performed by Tengyuan Liu and Lydie Boussicault. Experiments concerning sample immunofluorescence and imaging were performed by Tengyuan Liu and Tingting Zhang. Data analyzed by Tengyuan Liu, Tingting Zhang, Lydie Boussicault, Marie-Claude Potier and Bassem A. Hassan.

REFERENCES

1. Kang, J. *et al.* The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733–736 (1987).

2. Selkoe, D. J. & Hardy, J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol. Med.* **8**, 595–608 (2016).

3. Berchtold, N. C. & Cotman, C. W. Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s. *Neurobiol. Aging* **19**, 173–189 (1998).

4. Tiwari, S., Atluri, V., Kaushik, A., Yndart, A. & Nair, M. Alzheimer's disease: Pathogenesis, diagnostics, and therapeutics. *International Journal of Nanomedicine* vol. 14 5541–5554 (2019).

5. Vaz, M. & Silvestre, S. Alzheimer's disease: Recent treatment strategies. *European Journal of Pharmacology* vol. 887 173554 (2020).

6. Shariati, S. A. M. & De Strooper, B. Redundancy and divergence in the amyloid precursor protein family. *FEBS Lett.* **587**, 2036–2045 (2013).

7. Müller, U. C., Deller, T. & Korte, M. Not just amyloid: Physiological functions of the amyloid precursor protein family. *Nature Reviews Neuroscience* vol. 18 281–298 (2017).

8. Coburger, I., Hoefgen, S. & Than, M. E. The structural biology of the amyloid precursor protein APP - A complex puzzle reveals its multi-domain architecture. *Biol. Chem.* **395**, 485–498 (2014).

9. Ninomiya, H., Roch, J. M., Sundsmo, M. P., Otero, D. A. C. & Saitoh, T. Amino acid sequence RERMS represents the active domain of amyloid β/A4 protein precursor that promotes fibroblast growth. *J. Cell Biol.* **121**, 879–886 (1993).

10. Jin, L. W. *et al.* Peptides containing the RERMS sequence of amyloid β/A4 protein precursor bind cell surface and promote neurite extension. *J. Neurosci.* **14**, 5461–5470 (1994).

11. Kounnas, M. Z. *et al.* LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted β-amyloid precursor protein and mediates its degradation. *Cell* **82**, 331–340 (1995).

12. Ashley, J., Packard, M., Ataman, B. & Budnik, V. Fasciclin II signals new synapse formation through amyloid precursor protein and the scaffolding protein dX11/Mint. *J. Neurosci.* **25**, 5943–5955 (2005).

13. Van Gassen, G., Annaert, W. & Van Broeckhoven, C. Binding partners of Alzheimer's disease proteins: Are they physiologically relevant? *Neurobiology of Disease* vol. 7 135–151 (2000).

14. Ho, A., Liu, X. & Südhof, T. C. Deletion of Mint proteins decreases amyloid production in transgenic mouse models of Alzheimer's disease. *J. Neurosci.* **28**, 14392–14400 (2008).

15. Sullivan, S. E., Dillon, G. M., Sullivan, J. M. & Ho, A. Mint proteins are required for synaptic activity-dependent amyloid precursor protein (APP) trafficking and amyloid β generation. *J. Biol. Chem.* **289**, 15374–15383 (2014).

16. Young-Pearse, T. L. *et al.* A critical function for β-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. *J. Neurosci.* **27**, 14459–14469 (2007).

17. Luo, L., Martin-Morris, L. E. & White, K. Identification, secretion, and neural expression of APPL, a Drosophila protein similar to human amyloid protein precursor. *J. Neurosci.* **10**, 3849–3861 (1990).

18. Torroja, L., Luo, L. & White, K. APPL, the Drosophila member of the APP-family, exhibits differential trafficking and processing in CNS neurons. *J. Neurosci.* **16**, 4638–4650 (1996).

19. Ott, M. O. & Bullock, S. L. A gene trap insertion reveals that amyloid precursor protein expression is a very early event in murine embryogenesis. *Dev. Genes Evol.* **211**, 355–357 (2001).

20. Kirazov, E., Kirazov, L., Bigl, V. & Schliebs, R. Ontogenetic changes in protein level of amyloid precursor protein (APP) in growth cones and synaptosomes from rat brain and prenatal expression pattern of APP mRNA isoforms in developing rat embryo. *Int. J. Dev. Neurosci.* **19**, 287–296 (2001).

21. Sarasa, M. *et al.* Alzheimer β-amyloid precursor proteins display specific patterns of expression during embryogenesis. *Mech. Dev.* **94**, 233–236 (2000).

22. Bergström, P. *et al.* Amyloid precursor protein expression and processing are differentially regulated during cortical neuron differentiation. *Sci. Rep.* **6**, 1–14 (2016).

23. Mañucat-Tan, N. B., Saadipour, K., Wang, Y. J., Bobrovskaya, L. & Zhou, X. F. Cellular Trafficking of Amyloid Precursor Protein in Amyloidogenesis Physiological and Pathological Significance. *Molecular Neurobiology* vol. 56 812–830 (2019).

24. Ayadi, A. El, Stieren, E. S., Barral, J. M. & Boehning, D. Ubiquilin-1 regulates amyloid precursor protein maturation and degradation by stimulating K63-linked polyubiquitination of lysine 688. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13416–13421 (2012).

25. Greenfield, J. P. *et al.* Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer β-amyloid peptides. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 742–747 (1999).

26. Thinakaran, G. & Koo, E. H. Amyloid precursor protein trafficking, processing, and function. *Journal of Biological Chemistry* vol. 283 29615–29619 (2008).

27. Agostinho, P., Pliássova, A., Oliveira, C. R. & Cunha, R. A. Localization and Trafficking of Amyloid-β Protein Precursor and Secretases: Impact on Alzheimer's Disease. *Journal of Alzheimer's Disease* vol. 45 329–347 (2015).

28. Kuhn, P. H. *et al.* ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons. *EMBO J.* **29**, 3020–3032 (2010).

29. Sisodia, S. S. β-Amyloid precursor protein cleavage by a membrane-bound protease. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6075–6079 (1992).

30. Allinson, T. M. J., Parkin, E. T., Turner, A. J. & Hooper, N. M. ADAMs Family Members As Amyloid Precursor Protein α-Secretases. *Journal of Neuroscience Research* vol. 74 342–352 (2003).

31. Jorissen, E. *et al.* The disintegrin/metalloproteinase ADAM10 is essential for the establishment of the brain cortex. *J. Neurosci.* **30**, 4833–4844 (2010).

32. Cheng, H. *et al.* Mechanisms of disease: New therapeutic strategies for Alzheimer's disease - Targeting APP processing in lipid rafts. *Nature Clinical Practice Neurology* vol. 3 374–382 (2007).

33. Nordstedt, C., Caporaso, G. L., Thyberg, J., Gandy, S. E. & Greengard, P. Identification of the Alzheimer β/A4 amyloid precursor protein in clathrin-coated vesicles purified from PC12 cells. *J. Biol. Chem.* **268**, 608–612 (1993).

34. Perez, R. G. *et al.* Mutagenesis identifies new signals for β-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Aβ42. *J. Biol. Chem.* **274**, 18851–18856 (1999).

35. Kinoshita, A. *et al.* Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *Journal of Cell Science* vol. 116 3339–3346 (2003).

36. Chia, P. Z. C. *et al.* Intracellular itinerary of internalised β-secretase, BACE1, and its potential impact on β-amyloid peptide biogenesis. *Traffic* **14**, 997–1013 (2013).

37. Small, S. A. & Gandy, S. Sorting through the Cell Biology of Alzheimer's Disease: Intracellular Pathways to Pathogenesis. *Neuron* vol. 52 15–31 (2006).

38. Choy, R. W. Y., Cheng, Z. & Schekman, R. Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (Aβ) production in the trans-Golgi network. *Proc. Natl. Acad. Sci. U. S. A.* **109**, E2077–E2082 (2012).

39. Vassar, R. *et al.* β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science (80-.).* **286**, 735–741 (1999).

40. Tan, J. Z. A. & Gleeson, P. A. The role of membrane trafficking in the processing of amyloid precursor protein and production of amyloid peptides in Alzheimer's disease. *Biochimica et Biophysica Acta - Biomembranes* vol. 1861 697–712 (2019).

41. Shimizu, H. *et al.* Crystal Structure of an Active Form of BACE1, an Enzyme Responsible for Amyloid β Protein Production. *Mol. Cell. Biol.* **28**, 3663–3671 (2008).

42. Huse, J. T. *et al.* β-secretase processing in the trans-Golgi network preferentially generates truncated amyloid species that accumulate in Alzheimer's disease brain. *J. Biol. Chem.* **277**, 16278–16284 (2002).

43. Huse, J. T., Pijak, D. S., Leslie, G. J., Lee, V. M. Y. & Doms, R. W. Maturation and endosomal targeting of β-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease β-secretase. *J. Biol. Chem.* **275**, 33729–33737 (2000).

44. De Strooper, B. Aph-1, Pen-2, and Nicastrin with Presenilin generate an active γ-Secretase complex. *Neuron* vol. 38 9–12 (2003).

45. Takasugi, N. *et al.* The role of presenilin cofactors in the γ-secratase complex. *Nature* **422**, 438–441 (2003).

46. Niimura, M. *et al.* Aph-1 contributes to the stabilization and trafficking of the γ-secretase complex through mechanisms involving intermolecular and intramolecular interactions. *J. Biol. Chem.* **280**, 12967–12975 (2005).

47. Takami, M. *et al.* γ-Secretase: Successive tripeptide and tetrapeptide release from the transmembrane domain of β-carboxyl terminal fragment. *J. Neurosci.* **29**, 13042–13052 (2009).

48. Tomita, T. Molecular mechanism of intramembrane proteolysis by γ-secretase. *Journal of Biochemistry* vol. 156 195–201 (2014).

49. Haass, C., Kaether, C., Thinakaran, G. & Sisodia, S. Trafficking and proteolytic processing of APP. *Cold Spring Harb. Perspect. Med.* **2**, a006270 (2012).

50. Hardy, J. A. & Higgins, G. A. Alzheimer's disease: The amyloid cascade hypothesis. *Science* vol. 256 184–185 (1992).

51. Haass, C. & Selkoe, D. J. Soluble protein oligomers in neurodegeneration: Lessons from the Alzheimer's amyloid β-peptide. *Nature Reviews Molecular Cell Biology* vol. 8 101–112 (2007).

52. Hilbich, C., Kisters-Woike, B., Reed, J., Masters, C. L. & Beyreuther, K. Aggregation and secondary structure of synthetic amyloid βA4 peptides of Alzheimer's disease. *J. Mol. Biol.* **218**, 149–163 (1991).

53. Bhalla, A. *et al.* The location and trafficking routes of the neuronal retromer and its role in amyloid precursor protein transport. *Neurobiol. Dis.* **47**, 126–134 (2012).

54. Vieira, S. I., Rebelo, S., Domingues, S. C., Cruz e Silva, E. F. & Cruz e Silva, O. A. B. S655 phosphorylation enhances APP secretory traffic. *Mol. Cell. Biochem.* **328**, 145–154 (2009).

55. Vieira, S. I. *et al.* Retrieval of the Alzheimer's amyloid precursor protein from the endosome to the TGN is S655 phosphorylation state-dependent and retromer-mediated. *Mol. Neurodegener.* **5**, 40 (2010).

56. Rohe, M. *et al.* Sortilin-related receptor with A-type repeats (SORLA) affects the amyloid precursor protein-dependent stimulation of ERK signaling and adult neurogenesis. *J. Biol. Chem.* **283**, 14826–14834 (2008).

57. Offe, K. *et al.* The lipoprotein receptor LR11 regulates amyloid β production and amyloid precursor protein traffic in endosomal compartments. *J. Neurosci.* **26**, 1596–1603 (2006).

58. Andersen, O. M. *et al.* Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 13461–13466 (2005).

59. Pastorino, L. *et al.* The prolyl isomerase Pin1 regulates amyloid precursor protein processing and amyloid-β production. *Nature* **440**, 528–534 (2006).

60. Shrivastava-Ranjan, P. *et al.* Mint3/X11γ is an ADP-ribosylation factor-dependent adaptor that regulates the traffic of the Alzheimer's precursor protein from the trans-Golgi network. *Mol. Biol. Cell* **19**, 51–64 (2008).

61. Porayette, P. *et al.* Differential processing of amyloid-β precursor protein directs human embryonic stem cell proliferation and differentiation into neuronal precursor cells. *J. Biol. Chem.* **284**, 23806–23817 (2009).

62. Freude, K. K., Penjwini, M., Davis, J. L., LaFerla, F. M. & Blurton-Jones, M. Soluble amyloid precursor protein induces rapid neural differentiation of human embryonic stem cells. *J. Biol. Chem.* **286**, 24264–24274 (2011).

63. Hayashi, Y. *et al.* Alzheimer amyloid protein precursor enhances proliferation of neural stem cells from fetal rat brain. *Biochem. Biophys. Res. Commun.* **205**, 936–943 (1994).

64. Ohsawa, I., Takamura, C., Morimoto, T., Ishiguro, M. & Kohsaka, S. Amino-terminal region of secreted form of amyloid precursor protein stimulates proliferation of neural stem cells. *Eur. J. Neurosci.* **11**, 1907–1913 (1999).

65. Hu, Y. *et al.* Role of cystatin C in amyloid precursor protein-induced proliferation of neural stem/progenitor cells. *J. Biol. Chem.* **288**, 18853–18862 (2013).

66. Demars, M. P., Hollands, C., Zhao, K. Da & Lazarov, O. Soluble amyloid precursor protein-α rescues age-linked decline in neural progenitor cell proliferation. *Neurobiol. Aging* **34**, 2431–2440 (2013).

67. Demars, M. P., Bartholomew, A., Strakova, Z. & Lazarov, O. Soluble amyloid precursor protein: A novel proliferation factor of adult progenitor cells of ectodermal and mesodermal origin. *Stem Cell Res. Ther.* **2**, (2011).

68. Porayette, P. *et al.* Differential processing of amyloid-β precursor protein directs human embryonic stem cell proliferation and differentiation into neuronal precursor cells. *J. Biol. Chem.* **284**, 23806–23817 (2009).

69. López-Toledano, M. A. & Shelanski, M. L. Neurogenic effect of β-amyloid peptide in the development of neural stem cells. *J. Neurosci.* **24**, 5439–5444 (2004).

70. Zhang, Y. W. *et al.* Presenilin/γ-secretase-dependent processing of β-amyloid precursor protein regulates EGF receptor expression. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 10613–10618 (2007).

71. Ayuso-Sacido, A. *et al.* Activated EGFR signaling increases proliferation, survival,and migration and blocks neuronal differentiation in post-natal neural stem cells. *J. Neurooncol.* **97**, 323–337 (2010).

72. Ghosal, K., Stathopoulos, A. & Pimplikar, S. W. APP Intracellular Domain Impairs Adult Neurogenesis in Transgenic Mice by Inducing Neuroinflammation. *PLoS One* **5**, e11866 (2010).

73. Deyts, C., Thinakaran, G. & Parent, A. T. APP Receptor? To Be or Not to Be. *Trends in Pharmacological Sciences* vol. 37 390–411 (2016).

74. Herms, J. *et al.* Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. *EMBO J.* **23**, 4106–4115 (2004).

75. Young-Pearse, T. L. *et al.* A critical function for β-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. *J. Neurosci.* **27**, 14459–14469 (2007).

76. Sabo, S. L., Ikin, A. F., Buxbaum, J. D. & Greengard, P. The Alzheimer amyloid precursor protein (APP) and FE65, an APP-binding protein, regulate cell movement. *J. Cell Biol.* **153**, 1403–1414 (2001).

77. Young-Pearse, T. L., Suth, S., Luth, E. S., Sawa, A. & Selkoe, D. J. Biochemical and functional interaction of disrupted-in-schizophrenia 1 and amyloid precursor protein regulates neuronal migration during mammalian cortical development. *J. Neurosci.* **30**, 10431–10440 (2010).

78. Ramaker, J. M., Swanson, T. L. & Copenhaver, P. F. Amyloid precursor proteins interact with the heterotrimeric G protein go in the control of neuronal migration. *J. Neurosci.* **33**, 10165–10181 (2013).

79. Ramaker, J. M., Swanson, T. L. & Copenhaver, P. F. Manduca contactin regulates amyloid precursor protein-dependent neuronal migration. *J. Neurosci.* **36**, 8757–8775 (2016).

80. Sabo, S. L., Ikin, A. F., Buxbaum, J. D. & Greengard, P. The amyloid precursor protein and its regulatory protein, FE65, in growth cones and synapses *in vitro* and *in vivo*. *J. Neurosci.* **23**, 5407–5415 (2003).

81. Sosa, L. J. *et al.* Amyloid Precursor Protein Is an Autonomous Growth Cone Adhesion Molecule Engaged in Contact Guidance. *PLoS One* **8**, 64521 (2013).

82. Kolodkin, A. L. & Tessier-Lavigne, M. Mechanisms and molecules of neuronal wiring: A primer. *Cold Spring Harbor Perspectives in Biology* vol. 3 1–14 (2011).

83. Raper, J. & Mason, C. Cellular strategies of axonal pathfinding. *Cold Spring Harbor perspectives in biology* vol. 2 (2010).

84. Soldano, A. *et al.* The Drosophila Homologue of the Amyloid Precursor Protein Is a Conserved Modulator of Wnt PCP Signaling. *PLoS Biol.* **11**, e1001562 (2013).

85. Song, P. & Pimplikar, S. W. Knockdown of amyloid precursor protein in zebrafish causes defects in motor axon outgrowth. *PLoS One* **7**, (2012).

86. Baumkötter, F., Wagner, K., Eggert, S., Wild, K. & Kins, S. Structural aspects and physiological consequences of APP/APLP trans-dimerization. *Experimental Brain Research* vol. 217 389–395 (2012).

87. Ludewig, S. & Korte, M. Novel insights into the physiological function of the APP (GENE) family and its proteolytic fragments in synaptic plasticity. *Front. Mol. Neurosci.* **9**, 161 (2017).

88. Priller, C. *et al.* Synapse formation and function is modulated by the amyloid precursor protein. *J. Neurosci.* **26**, 7212–7221 (2006).

89. Ashley, J., Packard, M., Ataman, B. & Budnik, V. Fasciclin II signals new synapse formation through amyloid precursor protein and the scaffolding protein dX11/Mint. *J. Neurosci.* **25**, 5943–5955 (2005).

90. Tyan, S. H. *et al.* Amyloid precursor protein (APP) regulates synaptic structure and function. *Mol. Cell. Neurosci.* **51**, 43–52 (2012).

91. Lee, K. J. *et al.* Beta amyloid-independent role of amyloid precursor protein in generation and maintenance of dendritic spines. *Neuroscience* **169**, 344–356 (2010).

92. Hoe, H. S. *et al.* Interaction of Reelin with amyloid precursor protein promotes neurite outgrowth. *J. Neurosci.* **29**, 7459–7473 (2009).

93. Mulligan, K. A. & Cheyette, B. N. R. Wnt signaling in vertebrate neural development and function. *Journal of Neuroimmune Pharmacology* vol. 7 774–787 (2012).

94. Sellers, K. J. *et al.* Amyloid β synaptotoxicity is Wnt-PCP dependent and blocked by fasudil. *Alzheimer's Dement.* **14**, 306–317 (2018).

95. Elliott, C. *et al.* A role for APP in Wnt signalling links synapse loss with β-amyloid production. *Transl. Psychiatry* **8**, 179 (2018).

96. Coudreuse, D. & Korswagen, H. C. The making of Wnt: New insights into Wnt maturation, sorting and secretion. *Development* vol. 134 3–12 (2007).

97. Willert, K. & Nusse, R. Wnt proteins. *Cold Spring Harb. Perspect. Biol.* **4**, (2012).

98. Takada, R. *et al.* Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion. *Dev. Cell* **11**, 791–801 (2006).

99. Kurayoshi, M., Yamamoto, H., Izumi, S. & Kikuchi, A. Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochem. J.* **402**, 515–523 (2007).

100. Janda, C. Y., Waghray, D., Levin, A. M., Thomas, C. & Garcia, K. C. Structural basis of Wnt recognition by frizzled. *Science (80-.).* **336**, 59–64 (2012).

101. Chu, M. L. H. *et al.* Structural studies of wnts and identification of an LRP6 binding site. *Structure* **21**, 1235–1242 (2013).

102. Hirai, H., Matoba, K., Mihara, E., Arimori, T. & Takagi, J. Crystal structure of a mammalian Wnt–frizzled complex. *Nat. Struct. Mol. Biol.* **26**, 372–379 (2019).

103. Ching, W., Hang, H. C. & Nusse, R. Lipid-independent secretion of a Drosophila Wnt protein. *J. Biol. Chem.* **283**, 17092–17098 (2008).

104. Yu, J. *et al.* WLS Retrograde transport to the endoplasmic reticulum during Wnt secretion. *Dev. Cell* **29**, 277–291 (2014).

105. Willert, K. *et al.* Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448–452 (2003).

106. Hofmann, K. A superfamily of membrane-bound O-acyltransferases with implications for Wnt signaling. *Trends Biochem. Sci.* **25**, 111–112 (2000).

107. Rios-Esteves, J. & Resh, M. D. Stearoyl CoA Desaturase Is Required to Produce Active, Lipid-Modified Wnt Proteins. *Cell Rep.* **4**, 1072–1081 (2013).

108. Barrott, J. J., Cash, G. M., Smith, A. P., Barrow, J. R. & Murtaugh, L. C. Deletion of mouse Porcn blocks Wnt ligand secretion and reveals an ectodermal etiology of human focal dermal hypoplasia/Goltz syndrome. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 12752–12757 (2011).

109. Biechele, S., Cox, B. J. & Rossant, J. Porcupine homolog is required for canonical Wnt signaling and gastrulation in mouse embryos. *Dev. Biol.* **355**, 275–285 (2011).

110. Goodman, R. M. *et al.* Sprinter: A novel transmembrane protein required for Wg secretion and signaling. *Development* **133**, 4901–4911 (2006).

111. Bartscherer, K., Pelte, N., Ingelfinger, D. & Boutros, M. Secretion of Wnt Ligands Requires Evi, a Conserved Transmembrane Protein. *Cell* **125**, 523–533 (2006).

112. Bänziger, C. *et al.* Wntless, a Conserved Membrane Protein Dedicated to the Secretion of Wnt Proteins from Signaling Cells. *Cell* **125**, 509–522 (2006).

113. Nile, A. H. & Hannoush, R. N. Fatty acylation of Wnt proteins. *Nature Chemical Biology* vol. 12 60–69 (2016).

114. Zhang, X. *et al.* Notum is required for neural and head induction via wnt deacylation, oxidation, and inactivation. *Dev. Cell* **32**, 719–730 (2015).

115. Nile, A. H., Mukund, S., Stanger, K., Wang, W. & Hannoush, R. N. Unsaturated fatty acyl recognition by Frizzled receptors mediates dimerization upon Wnt ligand binding. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 4147–4152 (2017).

116. Komekado, H., Yamamoto, H., Chiba, T. & Kikuchi, A. Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes to Cells* **12**, 521–534 (2007).

117. Kurayoshi, M., Yamamoto, H., Izumi, S. & Kikuchi, A. Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochem. J.* **402**, 515–523 (2007).

118. Mason, J. O., Kitajewski, J. & Varmus, H. E. Mutational analysis of mouse wnt-1 identifies two temperature-sensitive alleles and attributes of Wnt-1 protein essential for transformation of a mammary cell line. *Mol. Biol. Cell* **3**, 521–533 (1992).

119. Tang, X. *et al.* Roles of N-glycosylation and lipidation in Wg secretion and signaling. *Dev. Biol.* **364**, 32–41 (2012).

120. Palm, W. *et al.* Lipoproteins in Drosophila melanogaster—Assembly, Function, and Influence on Tissue Lipid Composition. *PLoS Genet.* **8**, e1002828 (2012).

121. Panáková, D., Sprong, H., Marois, E., Thiele, C. & Eaton, S. Lipoprotein particles are required for Hedgehog and Wingless signalling. *Nature* **435**, 58–65 (2005).

122. Kaiser, K. *et al.* WNT5A is transported via lipoprotein particles in the cerebrospinal fluid to regulate hindbrain morphogenesis. *Nat. Commun.* **10**, 1–15 (2019).

123. Neumann, S. *et al.* Mammalian Wnt3a is Released on Lipoprotein Particles. *Traffic* **10**, 334–343 (2009).

124. Hessvik, N. P. & Llorente, A. Current knowledge on exosome biogenesis and release. *Cellular and Molecular Life Sciences* vol. 75 193–208 (2018).

125. Lo Cicero, A., Stahl, P. D. & Raposo, G. Extracellular vesicles shuffling intercellular messages: For good or for bad. *Current Opinion in Cell Biology* vol. 35 69–77 (2015).

126. Beckett, K. *et al.* Drosophila S2 Cells Secrete Wingless on Exosome-Like Vesicles but the Wingless Gradient Forms Independently of Exosomes. *Traffic* **14**, 82–96 (2013).

127. Gross, J. C., Chaudhary, V., Bartscherer, K. & Boutros, M. Active Wnt proteins are secreted on exosomes. *Nat. Cell Biol.* **14**, 1036–1045 (2012).

128. Mulligan, K. A. *et al.* Secreted Wingless-interacting molecule (Swim) promotes long-range signaling by maintaining Wingless solubility. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 370–377 (2012).

129. González-Méndez, L., Seijo-Barandiarán, I. & Guerrero, I. Cytoneme-mediated cell-cell contacts for hedgehog reception. *Elife* **6**, (2017).

130. Ramírez-Weber, F. A. & Kornberg, T. B. Cytonemes: Cellular processes that project to the principal signaling center in Drosophila imaginal discs. *Cell* **97**, 599–607 (1999).

131. Roy, S., Huang, H., Liu, S. & Kornberg, T. B. Cytoneme-mediated contact-dependent transport of the Drosophila decapentaplegic signaling protein. *Science (80-.).* **343**, (2014).

132. Stanganello, E. *et al.* Filopodia-based Wnt transport during vertebrate tissue patterning. *Nat. Commun.* **6**, (2015).

133. Huang, H. & Kornberg, T. B. Myoblast cytonemes mediate Wg signaling from the wing imaginal disc and Delta-Notch signaling to the air sac primordium. *Elife* **4**, (2015).

134. Alexandre, C., Baena-Lopez, A. & Vincent, J. P. Patterning and growth control by membrane-tethered wingless. *Nature* **505**, 180–185 (2014).

135. Gordon, M. D. & Nusse, R. Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. *Journal of Biological Chemistry* vol. 281 22429–22433 (2006).

136. He, X., Semenov, M., Tamai, K. & Zeng, X. LDL receptor-related proteins 5 and 6 in Wnt/β-Catenin signaling: Arrows point the way. *Development* vol. 131 1663–1677 (2004).

137. MacDonald, B. T., Tamai, K. & He, X. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. *Developmental Cell* vol. 17 9–26 (2009).

138. Cliffe, A., Hamada, F. & Bienz, M. A role of dishevelled in relocating axin to the plasma membrane during wingless signaling. *Curr. Biol.* **13**, 960–966 (2003).

139. Gao, C. & Chen, Y. G. Dishevelled: The hub of Wnt signaling. *Cellular Signalling* vol. 22 717–727 (2010).

140. Flack, J. E., Mieszczanek, J., Novcic, N. & Bienz, M. Wnt-Dependent Inactivation of the Groucho/TLE Co-repressor by the HECT E3 Ubiquitin Ligase Hyd/UBR5. *Mol. Cell* **67**, 181-193.e5 (2017).

141. Nakamura, Y., De Paiva Alves, E., Veenstra, G. J. C. & Hoppler, S. Tissue-and stage-specific Wnt target gene expression is controlled subsequent to β-Catenin recruitment to cis-regulatory modules. *Dev.* **143**, 1914–1925 (2016).

142. van Tienen, L. M., Mieszczanek, J., Fiedler, M., Rutherford, T. J. & Bienz, M. Constitutive scaffolding of multiple Wnt enhanceosome components by legless/BCL9. *Elife* **6**, (2017).

143. Gordon, M. D. & Nusse, R. Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. *Journal of Biological Chemistry* vol. 281 22429–22433 (2006).

144. Tepass, U. The Apical Polarity Protein Network in *Drosophila* Epithelial Cells: Regulation of Polarity, Junctions, Morphogenesis, Cell Growth, and Survival. *Annu. Rev. Cell Dev. Biol.* **28**, 655–685 (2012).

145. Overeem, A. W., Bryant, D. M. & van IJzendoorn, S. C. D. Mechanisms of apical-basal axis orientation and epithelial lumen positioning. *Trends in Cell Biology* vol. 25 476–485 (2015).

146. Yang, Y. & Mlodzik, M. Wnt-Frizzled/Planar Cell Polarity Signaling: Cellular Orientation by Facing the Wind (Wnt). *Annu. Rev. Cell Dev. Biol.* **31**, 623–646 (2015).

147. Simons, M. & Mlodzik, M. Planar Cell Polarity Signaling: From Fly Development to Human Disease. *Annu. Rev. Genet.* **42**, 517–540 (2008).

148. Adler, P. N. The frizzled/stan Pathway and Planar Cell Polarity in the Drosophila Wing. in *Current Topics in Developmental Biology* vol. 101 1–31 (Academic Press Inc., 2012).

149. Montcouquiol, M. *et al.* Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. *Nature* **423**, 173–177 (2003).

150. Tissir, F. & Goffinet, A. M. Shaping the nervous system: Role of the core planar cell polarity genes. *Nature Reviews Neuroscience* vol. 14 525–535 (2013).

151. Guo, N., Hawkins, C. & Nathans, J. Frizzled6 controls hair patterning in mice. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 9277–9281 (2004).

152. Gray, R. S., Roszko, I. & Solnica-Krezel, L. Planar Cell Polarity: Coordinating Morphogenetic Cell Behaviors with Embryonic Polarity. *Developmental Cell* vol. 21 120–133 (2011).

153. Gao, B. *et al.* Wnt Signaling Gradients Establish Planar Cell Polarity by Inducing Vangl2 Phosphorylation through Ror2. *Dev. Cell* **20**, 163–176 (2011).

154. Devenport, D. The cell biology of planar cell polarity. *Journal of Cell Biology* vol. 207 171–179 (2014).

155. Shima, Y. *et al.* Opposing roles in neurite growth control by two seven-pass transmembrane cadherins. *Nat. Neurosci.* **10**, 963–969 (2007).

156. Zhang, X. *et al.* Dishevelled promotes axon differentiation by regulating atypical protein kinase C. *Nat. Cell Biol.* **9**, 743–754 (2007).

157. Ciruna, B., Jenny, A., Lee, D., Mlodzik, M. & Schier, A. F. Planar cell polarity signalling couples cell division and morphogenesis during neurulation. *Nature* **439**, 220–224 (2006).

158. Kim, M. J. *et al.* Synaptic Accumulation of PSD-95 and Synaptic Function Regulated by Phosphorylation of Serine-295 of PSD-95. *Neuron* **56**, 488–502 (2007).

159. Rosso, S. B., Sussman, D., Wynshaw-Boris, A. & Salinas, P. C. Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. *Nat. Neurosci.* **8**, 34–42 (2005).

160. Chen, C.-H., He, C.-W., Liao, C.-P. & Pan, C.-L. A Wnt-planar polarity pathway instructs neurite branching by restricting F-actin assembly through endosomal signaling. *PLOS Genet.* **13**, e1006720 (2017).

161. Mao, Y. *et al.* Characterization of a Dchs1 mutant mouse reveals requirements for Dchs1-Fat4 signaling during mammalian development. *Development* **138**, 947–957 (2011).

162. Saburi, S., Hester, I., Goodrich, L. & McNeill, H. Functional interactions between Fat family cadherins in tissue morphogenesis and planar polarity. *Development* **139**, 1806–1820 (2012).

163. Seifert, J. R. K. & Mlodzik, M. Frizzled/PCP signalling: A conserved mechanism regulating cell polarity and directed motility. *Nature Reviews Genetics* vol. 8 126–138 (2007).

164. Yang, Y. & Mlodzik, M. Wnt-Frizzled/Planar Cell Polarity Signaling: Cellular Orientation by Facing the Wind (Wnt). *Annu. Rev. Cell Dev. Biol.* **31**, 623–646 (2015).

165. Jenny, A., Reynolds-Kenneally, J., Das, G., Burnett, M. & Mlodzik, M. Diego and Prickle regulate frizzled planar cell polarity signalling by competing for Dishevelled binding. *Nat. Cell Biol.* **7**, 691–697 (2005).

166. Tree, D. R. P. *et al.* Prickle mediates feedback amplification to generate asymmetric planar cell polarity signaling. *Cell* **109**, 371–381 (2002).

167. Wu, J. & Mlodzik, M. The Frizzled Extracellular Domain Is a Ligand for Van Gogh/Stbm during Nonautonomous Planar Cell Polarity Signaling. *Dev. Cell* **15**, 462–469 (2008).

168. Strutt, H. & Strutt, D. Differential Stability of Flamingo Protein Complexes Underlies the Establishment of Planar Polarity. *Curr. Biol.* **18**, 1555–1564 (2008).

169. Wu, J., Roman, A. C., Carvajal-Gonzalez, J. M. & Mlodzik, M. Wg and Wnt4 provide long-range directional input to planar cell polarity orientation in Drosophila. *Nat. Cell Biol.* **15**, 1045–1055 (2013).

170. Gros, J., Serralbo, O. & Marcelle, C. WNT11 acts as a directional cue to organize the elongation of early muscle fibres. *Nature* **457**, 589–593 (2009).

171. Gao, B. *et al.* Wnt Signaling Gradients Establish Planar Cell Polarity by Inducing Vangl2 Phosphorylation through Ror2. *Dev. Cell* **20**, 163–176 (2011).

172. Winter, C. G. *et al.* Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. *Cell* **105**, 81–91 (2001).

173. Marlow, F., Topczewski, J., Sepich, D. & Solnica-Krezel, L. Zebrafish Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr. Biol.* **12**, 876–884 (2002).

174. Boutros, M., Paricio, N., Strutt, D. I. & Mlodzik, M. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* **94**, 109–118 (1998).

175. Yamanaka, H. *et al.* JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep.* **3**, 69–75 (2002).

176. Gros, J. *et al.* WNT5A/JNK and FGF/MAPK pathways regulate the cellular events shaping the vertebrate limb bud. *Curr. Biol.* **20**, 1993–2002 (2010).

177. Slusarski, D. C. & Pelegri, F. Calcium signaling in vertebrate embryonic patterning and morphogenesis. *Developmental Biology* vol. 307 1–13 (2007).

178. Rosso, S. B. & Inestrosa, N. C. WNT signalling in neuronal maturation and synaptogenesis. *Frontiers in Cellular Neuroscience* vol. 7 103 (2013).

179. He, C. W., Liao, C. P. & Pan, C. L. Wnt signalling in the development of axon, dendrites and synapses. *Open Biology* vol. 8 (2018).

180. Dotti, C. G., Sullivan, C. A. & Banker, G. A. The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* **8**, 1454–1468 (1988).

181. Tahirovic, S. & Bradke, F. Neuronal polarity. *Cold Spring Harbor perspectives in biology* vol. 1 (2009).

182. Pan, C. L. *et al.* C. elegans AP-2 and Retromer Control Wnt Signaling by Regulating MIG-14/Wntless. *Dev. Cell* **14**, 132–139 (2008).

183. Hilliard, M. A. & Bargmann, C. I. Wnt signals and Frizzled activity orient anterior-posterior axon outgrowth in C. elegans. *Dev. Cell* **10**, 379–390 (2006).

184. Prasad, B. C. & Clark, S. G. Wnt signaling establishes anteroposterior neuronal polarity and requires retromer in C. elegans. *Development* **133**, 1757–1766 (2006).

185. Nishimura, T. *et al.* Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. *Nat. Cell Biol.* **6**, 328–334 (2004).

186. Shi, S. H., Jan, L. Y. & Jan, Y. N. Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* **112**, 63–75 (2003).

187. Zhang, X. *et al.* Dishevelled promotes axon differentiation by regulating atypical protein kinase C. *Nat. Cell Biol.* **9**, 743–754 (2007).

188. Rolls, M. M. & Doe, C. Q. Baz, Par-6 and aPKC are not required for axon or dendrite specification in Drosophila. *Nat. Neurosci.* **7**, 1293–1295 (2004).

189. Bodmer, D., Levine-Wilkinson, S., Richmond, A., Hirsh, S. & Kuruvilla, R. Wnt5a mediates nerve growth factor-dependent axonal branching and growth in developing sympathetic neurons. *J. Neurosci.* **29**, 7569–7581 (2009).

190. Li, L., Hutchins, B. I. & Kalil, K. Wnt5a induces simultaneous cortical axon outgrowth and repulsive axon guidance through distinct signaling mechanisms. *J. Neurosci.* **29**, 5873–5883 (2009).

191. Shimizu, K., Sato, M. & Tabata, T. The Wnt5/planar cell polarity pathway regulates axonal development of the Drosophila mushroom body neuron. *J. Neurosci.* **31**, 4944–4954 (2011).

192. Lyuksyutova, A. I. *et al. Anterior-Posterior Guidance of Commissural Axons by Wnt-Frizzled Signaling Downloaded from*. *Proc. Natl. Acad. Sci. U.S.A* vol. 8 www.sciencemag.org/cgi/content/full/302/5652/1981/DC1 (1969).

193. Lyuksyutova, A. I. *et al.* Anterior-Posterior Guidance of Commissural Axons by Wnt-Frizzled Signaling. *Science (80-.).* **302**, 1984–1988 (2003).

194. Yu, X. & Malenka, R. C. β-Catenin is critical for dendritic morphogenesis. *Nat. Neurosci.* **6**, 1169–1177 (2003).

195. Wayman, G. A. *et al.* Activity-Dependent Dendritic Arborization Mediated by CaM-Kinase I Activation and Enhanced CREB-Dependent Transcription of Wnt-2. *Neuron* **50**, 897–909 (2006).

196. Rosso, S. B., Sussman, D., Wynshaw-Boris, A. & Salinas, P. C. Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. *Nat. Neurosci.* **8**, 34–42 (2005).

197. Kirszenblat, L., Pattabiraman, D. & Hilliard, M. A. LIN-44/Wnt directs dendrite outgrowth through LIN-17/Frizzled in C. elegans neurons. *PLoS Biol.* **9**, (2011).

198. Lucas, F. R. & Salinas, P. C. WNT-7a induces axonal remodeling and increases synapsin I levels in cerebellar neurons. *Dev. Biol.* **192**, 31–44 (1997).

199. Hall, A. C., Lucas, F. R. & Salinas, P. C. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell* **100**, 525–535 (2000).

200. Ahmad-Annuar, A. *et al.* Signaling across the synapse: A role for Wnt and Dishevelled in presynaptic assembly and neurotransmitter release. *J. Cell Biol.* **174**, 127–139 (2006).

201. Cerpa, W. *et al.* Wnt-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. *J. Biol. Chem.* **283**, 5918–5927 (2008).

202. Farías, G. G. *et al.* Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons. *J. Biol. Chem.* **284**, 15857–15866 (2009).

203. Varela-Nallar, L., Alfaro, I. E., Serrano, F. G., Parodi, J. & Inestrosa, N. C. Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 21164–21169 (2010).

204. Thakar, S. *et al.* Evidence for opposing roles of Celsr3 and Vangl2 in glutamatergic synapse formation. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E610–E618 (2017).

205. Libro, R., Bramanti, P. & Mazzon, E. The role of the Wnt canonical signaling in neurodegenerative diseases. *Life Sciences* vol. 158 78–88 (2016).

206. Zhang, Z. *et al.* Destabilization of β-Catenin by mutations in presenilin-1 potentiates neuronal apoptosis. *Nature* **395**, 698–702 (1998).

207. Rosi, M. C. *et al.* Increased Dickkopf-1 expression in transgenic mouse models of neurodegenerative disease. *J. Neurochem.* **112**, 1539–1551 (2010).

208. Caricasole, A. *et al.* Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is associated with neuronal degeneration in Alzheimer's brain. *J. Neurosci.* **24**, 6021–6027 (2004).

209. Sellers, K. J. *et al.* Amyloid β synaptotoxicity is Wnt-PCP dependent and blocked by fasudil. *Alzheimer's Dement.* **14**, 306–317 (2018).

210. Purro, S. A., Dickins, E. M. & Salinas, P. C. The secreted Wnt antagonist dickkopf-1 is required for amyloid β-mediated synaptic loss. *J. Neurosci.* **32**, 3492–3498 (2012).

211. Killick, R. *et al.* Clusterin regulates β-amyloid toxicity via Dickkopf-1-driven induction of the wnt-PCP-JNK pathway. *Mol. Psychiatry* **19**, 88–98 (2014).

212. De Ferrari, G. V. *et al.* Common genetic variation within the Low-Density Lipoprotein Receptor-Related Protein 6 and late-onset Alzheimer's disease. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 9434–9439 (2007).

213. Soldano, A. *et al.* The Drosophila Homologue of the Amyloid Precursor Protein Is a Conserved Modulator of Wnt PCP Signaling. *PLoS Biol.* **11**, e1001562 (2013).

214. Cheng, A. *et al.* Mitochondrial SIRT3 Mediates Adaptive Responses of Neurons to Exercise and Metabolic and Excitatory Challenges. *Cell Metab.* **23**, 128–142 (2016).

215. Liu, T., Zhang, T., Yu, H., Shen, H. & Xia, W. Adjudin protects against cerebral ischemia reperfusion injury by inhibition of neuroinflammation and blood-brain barrier disruption. *J. Neuroinflammation* **11**, (2014).

216. Bolte, S. & Cordelières, F. P. A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy* vol. 224 213–232 (2006).

217. Oishi, I. *et al.* The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes to Cells* **8**, 645–654 (2003).

218. Dann, C. E. *et al.* Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. *Nature* **412**, 86–90 (2001).

219. Bush, A. I. *et al.* A novel zinc(II) binding site modulates the function of the βA4 amyloid protein precursor of Alzheimer's disease. *J. Biol. Chem.* **268**, 16109–16112 (1993).

220. Hunter, S. & Brayne, C. Relationships between the amyloid precursor protein and its various proteolytic fragments and neuronal systems. *Alzheimer's Research and Therapy* vol. 4 (2012).

221. Haass, C., Kaether, C., Thinakaran, G. & Sisodia, S. Trafficking and proteolytic processing of APP. *Cold Spring Harb. Perspect. Med.* **2**, (2012).

222. Vagnozzi, A. N. & Praticò, D. Endosomal sorting and trafficking, the retromer complex and neurodegeneration. *Molecular Psychiatry* vol. 24 857–868 (2019).

223. Chai, G., Goffinet, A. M. & Tissir, F. Celsr3 and Fzd3 in axon guidance. *International Journal of Biochemistry and Cell Biology* vol. 64 11–14 (2015).

224. Rosso, S. B. & Inestrosa, N. C. WNT signalling in neuronal maturation and synaptogenesis. *Frontiers in Cellular Neuroscience* vol. 7 103 (2013).

225. Parr, C., Mirzaei, N., Christian, M. & Sastre, M. Activation of the Wnt/β-Catenin pathway represses the transcription of the β-amyloid precursor protein cleaving enzyme (BACE1) via binding of T-cell factor-4 to BACE1 promoter. *FASEB J.* **29**, 623–635 (2015).

226. Tapia-Rojas, C., Burgos, P. V. & Inestrosa, N. C. Inhibition of Wnt signaling induces amyloidogenic processing of amyloid precursor protein and the production and aggregation of Amyloid-β (Aβ)42 peptides. *J. Neurochem.* **139**, 1175–1191 (2016).

227. Billnitzer, A. J., Barskaya, I., Yin, C. & Perez, R. G. APP independent and dependent effects on neurite outgrowth are modulated by the receptor associated protein (RAP). *J. Neurochem.* **124**, 123–132 (2013).

228. Young-Pearse, T. L., Chen, A. C., Chang, R., Marquez, C. & Selkoe, D. J. Secreted APP regulates the function of full-length APP in neurite outgrowth through interaction with integrin beta1. *Neural Dev.* **3**, (2008).

229. Wong, H. H. W. *et al.* RNA Docking and Local Translation Regulate Site-Specific Axon Remodeling *In vivo*. *Neuron* **95**, 852-868.e8 (2017).

230. Polleux, F. & Snider, W. Initiating and growing an axon. *Cold Spring Harbor perspectives in biology* vol. 2 (2010).

231. Barnes, A. P. & Polleux, F. Establishment of axon-dendrite polarity in developing neurons. *Annu. Rev. Neurosci.* **32**, 347–81 (2009).

232. Puzzo, D., Lee, L., Palmeri, A., Calabrese, G. & Arancio, O. Behavioral assays with mouse models of Alzheimer's disease: Practical considerations and guidelines. *Biochemical Pharmacology* vol. 88 450–467 (2014).

233. Li, C., Ebrahimi, A. & Schluesener, H. Drug pipeline in neurodegeneration based on transgenic mice models of Alzheimer's disease. *Ageing Research Reviews* vol. 12 116–140 (2013).

234. Götz, J. *et al.* Transgenic animal models of Alzheimer's disease and related disorders: histopathology, behavior and therapy. *Molecular Psychiatry* vol. 9 664–683 (2004).

235. Snyder, H. M. *et al.* Further understanding the connection between Alzheimer's disease and Down syndrome. *Alzheimer's and Dementia* vol. 16 1065–1077 (2020).

236. Herrup, K. The case for rejecting the amyloid cascade hypothesis. *Nat. Neurosci.* **18**, 794–799 (2015).

237. Musiek, E. S. & Holtzman, D. M. Three dimensions of the amyloid hypothesis: Time, space and 'wingmen'. *Nature Neuroscience* vol. 18 800–806 (2015).

238. Ng, L. F. *et al.* WNT Signaling in Disease. *Cells* vol. 8 826 (2019).

239. Bayod, S. *et al.* Downregulation of canonical Wnt signaling in hippocampus of SAMP8 mice. *Neurobiol. Aging* **36**, 720–729 (2015).

240. Scott, E. L., Zhang, Q. G., Han, D., Desai, B. N. & Brann, D. W. Long-term estrogen deprivation leads to elevation of Dickkopf-1 and dysregulation of Wnt/β-Catenin signaling in hippocampal CA1 neurons. in *Steroids* vol. 78 624–632 (Elsevier, 2013).

241. Hofmann, J. W., McBryan, T., Adams, P. D. & Sedivy, J. M. The effects of aging on the expression of Wnt pathway genes in mouse tissues. *Age (Omaha).* **36**, 9618 (2014).

242. Folke, J., Pakkenberg, B. & Brudek, T. Impaired Wnt Signaling in the Prefrontal Cortex of Alzheimer's Disease. *Mol. Neurobiol.* **56**, 873–891 (2019).

243. Liu, C. C. *et al.* Deficiency in LRP6-Mediated Wnt Signaling Contributes to Synaptic Abnormalities and Amyloid Pathology in Alzheimer's Disease. *Neuron* **84**, 63–77 (2014).

244. Rosi, M. C. *et al.* Increased Dickkopf-1 expression in transgenic mouse models of neurodegenerative disease. *J. Neurochem.* **112**, 1539–1551 (2010).

245. Caricasole, A. *et al.* Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is associated with neuronal degeneration in Alzheimer's brain. *J. Neurosci.* **24**, 6021–6027 (2004).

246. Soldano, A. *et al.* The Drosophila Homologue of the Amyloid Precursor Protein Is a Conserved Modulator of Wnt PCP Signaling. *PLoS Biol.* **11**, (2013).

247. Kang, J. *et al.* The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733–736 (1987).

248. Reinhard, C., Hébert, S. S. & De Strooper, B. The amyloid-β precursor protein: Integrating structure with biological function. *EMBO Journal* vol. 24 3996–4006 (2005).

249. Kaden, D. *et al.* Subcellular localization and dimerization of APLP1 are strikingly different from APP and APLP2. *J. Cell Sci.* **122**, 368–377 (2009).

250. Stahl, R. *et al.* Shedding of app limits its synaptogenic activity and cell adhesion properties. *Front. Cell. Neurosci.* **8**, 410 (2014).

251. Rossjohn, J. *et al.* Crystal structure of the Nterminal, growth factor-like domain of Alzheimer amyloid precursor protein. *Nat. Struct. Biol.* **6**, 327–331 (1999).

252. Chang, K.-A. *et al.* Phosphorylation of Amyloid Precursor Protein (APP) at Thr668 Regulates the Nuclear Translocation of the APP Intracellular Domain and Induces Neurodegeneration. *Mol. Cell. Biol.* **26**, 4327–4338 (2006).

253. Ables, J. L., Breunig, J. J., Eisch, A. J. & Rakic, P. Not(ch) just development: Notch signalling in the adult brain. *Nature Reviews Neuroscience* vol. 12 269–283 (2011).

254. Niehrs, C. The complex world of WNT receptor signalling. *Nat. Publ. Gr.* **13**, 767 (2012).

255. Deyts, C., Thinakaran, G. & Parent, A. T. APP receptor? To be or not to be…. *Trends Pharmacol. Sci.* **37**, 390–411 (2016).

256. Stahl, R. *et al.* Shedding of app limits its synaptogenic activity and cell adhesion properties. *Front. Cell. Neurosci.* **8**, (2014).

257. Rossjohn, J. *et al.* Crystal structure of the Nterminal, growth factor-like domain of Alzheimer amyloid precursor protein. *Nat. Struct. Biol.* **6**, 327–331 (1999).

258. Richter, M. C. *et al.* Distinct *in vivo* roles of secreted APP ectodomain variants APP sα and APP sβ in regulation of spine density, synaptic plasticity, and cognition . *EMBO J.* **37**, (2018).

259. Rice, H. C. *et al.* Secreted amyloid-b precursor protein functions as a GABA B R1a ligand to modulate synaptic transmission. *Science (80-.).* **363**, (2019).

260. Heaney, C. F. & Kinney, J. W. Role of GABAB receptors in learning and memory and neurological disorders. *Neuroscience and Biobehavioral Reviews* vol. 63 1–28 (2016).

261. Benke, D. GABAB receptor trafficking and interacting proteins: Targets for the development of highly specific therapeutic strategies to treat neurological disorders? *Biochemical Pharmacology* vol. 86 1525–1530 (2013).

262. Sellers, K. J. *et al.* Amyloid β synaptotoxicity is Wnt-PCP dependent and blocked by fasudil. *Alzheimer's Dement.* **14**, 306–317 (2018).

263. Girnita, L., Worrall, C., Takahashi, S. I., Seregard, S. & Girnita, A. Something old, something new and something borrowed: Emerging paradigm of insulin-like growth factor

type 1 receptor (IGF-1R) signaling regulation. *Cellular and Molecular Life Sciences* vol. 71 2403–2427 (2014).

264. Morcavallo, A., Stefanello, M., Iozzo, R. V., Belfiore, A. & Morrione, A. Ligand-mediated endocytosis and trafficking of the insulin-like growth factor receptor I and insulin receptor modulate receptor function. *Frontiers in Endocrinology* vol. 5 (2014).

265. Pandey, K. N. Endocytosis and trafficking of natriuretic peptide receptor-A: Potential role of short sequence motifs. *Membranes* vol. 5 253–287 (2015).

266. Boncompain, G. *et al.* Synchronization of secretory protein traffic in populations of cells. *Nat. Methods* **9**, 493–498 (2012).

267. Araki, W. *et al.* Trophic effect of β-amyloid precursor protein on cerebral cortical neurons in culture. *Biochem. Biophys. Res. Commun.* **181**, 265–271 (1991).

268. Southam, K. A., Stennard, F., Pavez, C. & Small, D. H. Knockout of Amyloid β Protein Precursor (APP) Expression Alters Synaptogenesis, Neurite Branching and Axonal Morphology of Hippocampal Neurons. *Neurochem. Res.* **44**, 1346–1355 (2019).

269. Small, D. H. *et al.* A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *J. Neurosci.* **14**, 2117–2127 (1994).

270. Perez, R. G., Zheng, H., Van Der Ploeg, L. H. T. & Koo, E. H. The β-amyloid precursor protein of Alzheimer's disease enhances neuron viability and modulates neuronal polarity. *J. Neurosci.* **17**, 9407–9414 (1997).

271. He, C. W., Liao, C. P. & Pan, C. L. Wnt signalling in the development of axon, dendrites and synapses. *Open Biology* vol. 8 (2018).

CURRICULUM VITAE

Name: Tengyuan Liu

Date of birth: 30/06/1988

Nationality: China

E-mail: tengyuan.liu@student.kuleuven.be,

liutengy@gmail.com

Tel: +33-0627431578

EDUCATION AND RESEARCH EXPERIENCESE

- **Ph.D of Science** (2014.10-present)

2016.09-2021.08 Laboratory of Brain Development, Institut du Cerveau et de la Moelle épinière – ICM

Laboratory of Behavioral and Developmental Genetics of KU Leuven

Project: Functional analysis of Amyloid precursor protein (APP) in Wnt mediated signaling pathway

2015.10-2017.08 Laboratory of Neurogenetics, VIB and K.U. Leuven School of Medicine

Project: The function of APP and Celsr3 in mice cortical neuron migration during development.

2014.10-2015.10 Laboratory of Glia Biology, VIB and K.U. Leuven School of Medicine

Project: Investigating the molecular mechanisms underlying the function of SynCAM1 in astrocytes

- **Master of Science** (2011.9-2014.3)

School of Biomedical Engineering /Med-X Research Institute, **Shanghai Jiao Tong University**

Thesis project: Adjudin-preconditioned neural stem cells enhance neuroprotection after ischemia reperfusion in mice

- **Bachelor of Science** (2006.9-2010.7)

Major: Biological Science. College of Life Science, **Henan Normal University**

FELLOWSHIP FOR Ph.D STUDY

CSC (Chinese Scholarship Committee): 28/10/2014-28/10/2018

HONORS

National excellent master graduate scholarship (2014)

PUBLICATIONS

Research Articles

1. **Liu T**, Nicolas M, Zhang T, Rice H, Soldano A, Claeys A, Petrova IM, Fradkin LG, Strooper BD, Hassan BA. The Amyloid Precursor Protein is a conserved Wnt receptor. eLife. 2021; 10: e69199.

2. Zhang T, **Liu T**, Mora N, Guegan J, Bertrand M, Contreras X, Hansen AH, Streicher C, Anderle M, Danda N, Tiberi L, Hippenmeyer S and Hassan BA. Generation of excitatory and inhibitory neurons from common progenitors via Notch signaling in the cerebellum. *Cell Rep.* 2021 Jun 8;35(10):109208.

3. Shabani K, Pigeon J, Zariouh M, **Liu T**, Saffarian A, Komatsu J, Liu E, Danda N, Limame R, Bohl D, Parras C, Hassen AH. The Amyloid Precursor Protein regulates human cortical neurogenesis. (In submission: BioRxiv)

4. **Liu T**, Zhang T, Yu H, Shen H, Xia W [*]. Adjudin protects against cerebral ischemia reperfusion injury by inhibition of neuroinflammation and blood-brain barrier disruption. *J Neuroinflammation*. 2014 Jun 14;11:107.

5. Zhang T[#], Yang X[#], **Liu T**, Shao J, Fu N, Yan A, Geng K, Xia W*. Adjudin-preconditioned neural stem cells enhance neuroprotection after ischemia reperfusion in mice. *Stem Cell Res Ther*. 2017 Nov 7;8(1):248. #, co-first author.

6. Shao J, Yang X, **Liu T**, Zhang T, Xie QR, Xia W. Erratum to: Autophagy induction by SIRT6 is involved in oxidative stress-induced neuronal damage. *Protein Cell*. 2017 Apr;8(4):313

7. Yang W[#], Yan A[#], Zhang T, Shao J, **Liu T**, Yang X, Xia W, Fu Y*. Thromboxane A2 Receptor Stimulation Enhances Microglial Interleukin-1β and NO Biosynthesis Mediated by the Activation of ERK Pathway. *Front Aging Neurosci*. 2016 Jan 29;8:8.

8. Zhang T, **Liu T**, Shao J, Sheng C, Hong Y, Ying W, Xia W *. Antioxidant protects synchrotron radiation X-ray-induced blood-testis barrier disruption. *Spermatogenesis*. 2015 Mar 25;5(1):e1009313.

9. Chen Y, Gu H, Zhang DS, Li F, **Liu T**, Xia W. Highly effective inhibition of lung cancer growth and metastasis by systemic delivery of siRNA via multimodal mesoporous silica-based nanocarrier. *Biomaterials*. 2014 Dec;35(38):10058-69.

10. Lei X, Zhang W, **Liu T**, Xiao H, Liang W, Xia W, Zhang J. Perinatal supplementation with omega-3 polyunsaturated fatty acids improves sevoflurane-induced neurodegeneration and memory impairment in neonatal rats. *PLoS One*. 2013 Aug 13;8(8):e70645.

11. Shao J [#], **Liu T**[#], Xie QR, Zhang T, Yu H, Wang B, Ying W, Mruk DD, Silvestrini B, Cheng CY, Xia W [*]. Adjudin attenuates lipopolysaccharide (LPS)- and ischemia-induced microglial activation. *J Neuroimmunology*. 2013 Jan 15; 83-90 . #, co-first author.

12. Xie QR[#], Liu Y[#], Shao J, Yang J, **Liu T**, Zhang T, Wang B, Mruk DD, Silvestrini B, Cheng CY, Xia W [*]. Male contraceptive Adjudin is a potential anti-cancer drug. *Biochem Pharmacol*. 2013 Feb 1; 85(3): 345-551. #, co-first author.

13. Liu Y[#], Xie QR[#], Wang B, Shao J, Zhang T, **Liu T**, Huang G[*], Xia W [*]. Inhibition of SIRT6 in prostate cancer reduces cell viability and increases sensitivity to chemotherapeutics. *Protein & Cell*. 2013 Sep; 4(9): 702-710. #, co-first author.

14. Ma Y, Nie H, Sheng C, Chen H, Wang B, **Liu T**, Shao J, He X, Zhang T, Zheng C, Xia W[*], Ying W[*]. Roles of oxidative stress in synchrotron radiation X-ray-induced testicular damage of rodents. *Int J Physiol Pathophysiol Pharmacol*. 2012;4(2):108-14.

15. Sheng C, Chen H, Wang B, **Liu T**, Hong Y, Shao J, He X, Ma Y, Nie H, Liu N, Xia W, Ying W. NAD(+) administration significantly attenuates synchrotron radiation X-ray-induced DNA damage and structural alterations of rodent testes. *Int J Physiol Pathophysiol Pharmacol.* 2012;4(1):1-9.

Reviews

1. Cheng Y, Xia W, Wong E, Xie QR, Shao J, **Liu T**, Quan Y, Zhang T, Yang X, Geng K, Silvestrini B, Cheng C. Adjudin--A Male Contraceptive With Other Biological Activities. *Recent Pat Endocr Metab Immune Drug Discov*, 2015, 9 (2), 63-73

2. Shao J, Zhang T, **Liu T**, Qian Y, Yang X, Liu J, Li F, Yang J, Xie QR, Xia W[*]. Sirtuin functions in the brain: from physiological to pathological aspects. *J. Shanghai Jiaotong Univ. (Sci.)*, 2014, 19(6): 1-12

3. Zhang T, Xie QR, Shao J, **Liu T**, Liu Y, Xia W[*]. MicroRNAs and stroke: a brief summary of recent research progress, *Journal of Molecular Diagnostics and Therapy*, 2012, 04（1）

ACKNOWLEDGMENTS

Here comes the end of my thesis, also near the end of my study life as a doctoral student. When I look back this was a quite long journey mixed with happiness and sadness that I did not expect. Meanwhile I have learned a lot from this journey and from those people I have met and helped me. Here I would like to show my heartfelt thanks to all of them.

The first person I would like to thank is my supervisor Bassem Hassan, who is one of the most brilliant and kind persons I have ever met. Thank you for giving me the opportunity to join this team as a PhD student, and thank you for the encouragement and great support all the way alone. It is my honor to be part of your group.

I would like to thank Prof. Patrick Callaerts for accepting as my official KU-Leuven supervisor, which was of great help for me.

I would like to thank my internal jury members Prof. Bart De Strooper and Pro. Joris de Wit for the precious time and comments on my project.

I would like to thank Prof. Thierry Galli and Dr. Carlos Parras for being my external jury member.

I would like to thank Prof. Jan Cools for accepting to be the Chairman of my PhD public defense. Thanks for Prof. Joris de Wit to be the secretary

I would like to thank prof. Marie-Claude Potier and her group members Lydie Boussicault and Amal Kasri for supports at the start of my project and in the revision of the paper.

Lot of thanks go to my friends and colleagues or former group members who helps from KU-Leuven to ICM.

A huge thanks to my wife Tingting Zhang who always be there to help, we have shared a lot of happy moments and also went through a very tough time together, she is so optimistic and enthusiastic in work and life, I always believe that she can crush every obstacle in her way and is just unstoppable! I would also like to thank my parents my big brother for their endless loves.

At last, I would like to show my appreciation to my Country, to the China Scholarship Council (CSC) for providing the scholarship that I have the chance to start my PhD life.