



# The Scrib1 Interactome and its relevance for synaptic plasticity & neurodevelopmental disorders

Vera Margarido Pinheiro

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SPÉCIALITÉ: NEUROSCIENCES

Par Vera PINHEIRO

**L'interactome de Scrib1 et son importance pour  
la plasticité synaptique &  
les troubles de neurodéveloppement**

Sous la direction de : Nathalie SANS

Soutenue le 4 Décembre 2014

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Born on 13<sup>th</sup> February 1987 at Portalegre, Portugal

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**The Scrib1 Interactome and its relevance for  
synaptic plasticity & neurodevelopmental disorders**

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Não há regresso.

Há viagens sem regresso nem repetição.

*Miguel Sousa Tavares,*

*No teu deserto*

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The brain is made up of billions of nerve cells, or neurons, which can communicate with each other through functionally distinct structures - the axon and the dendrite. We focused our study on the development and maintenance of dendritic spines, whose changes in morphology are intimately correlated with synaptic plasticity, or the ability to respond to synaptic activity. Dendritic spines originate from motile dendritic filopodia, which mature into spines following axonal contact. However, the spatial and temporal coordination of all the molecular components throughout the formation and maturation of a synapse remains unclear.

Scribble1 is planar cell polarity protein (PCP) classically implicated in the homeostasis of epithelial tissues and tumour growth. In the mammalian brain, Scrib1 is a critical scaffold protein in brain development and function. The main goal of this work was to investigate the molecular mechanisms underlying Scrib1 role in synapse formation and maintenance. Firstly, we depict the importance of Scrib1 PDZ-dependent interactions on glutamate receptors trafficking as well as bidirectional plasticity signalling pathway underlying spatial memory. Secondly, we focus on the functional consequences of a recently identified autism spectrum disorder (ASD) mutation of Scrib1 on neuronal morphology and function. We demonstrated that Scrib1 regulates dendritic arborization, spine formation and functional maintenance via an actin-dependent mechanism. Taken altogether, this thesis highlights the PCP protein Scrib1 as key scaffold protein in brain development and function, playing a plethora of roles from the subcellular to the cognitive level.

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**Keywords:** Scrib1, autism spectrum disorders, hippocampal neurons, synaptic plasticity, dendritic spine, glutamate receptors traffic, actin dynamics.

## **L'interactome de Scrib1 et son importance pour la plasticité synaptique & les troubles de neurodéveloppement**

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Le cerveau contient environ cent milliards de neurones, qui communiquent entre eux par des structures fonctionnellement distinctes – l'axone et la dendrite. Notre étude s'est intéressée au développement et au maintien des épines dendritiques, dont les changements morphologiques sont intimement liés à la plasticité synaptique. Ces épines ont pour origine les filopodes qui évoluent en épines lors du contact axonal. Cette transition implique une myriade de molécules, dont la coordination spatiale et temporelle reste largement méconnue.

Scribble1 est une protéine de polarité cellulaire classiquement impliquée dans l'homéostasie de tissus épithéliaux, la croissance et progression des tumeurs. Scrib1 est aussi une protéine d'échafaudage critique pour le développement et le bon fonctionnement du cerveau. Notre objectif c'est été l'étude des mécanismes moléculaires sous-jacents à un rôle potentiel de Scrib1 dans la formation et le maintien des synapses. On a décrit l'importance d'interactions dépendantes des domaines PDZ sur le trafic des récepteurs glutamatergiques et sur la voie de signalisation de plasticité synaptique. Ensuite, nous avons évalué les conséquences fonctionnelles d'une mutation de Scrib1 récemment identifiée chez un patient humain atteint des troubles du spectre autistique dans la morphologie et fonction des neurones. L'ensemble de ce travail démontre que Scrib1, protéine d'échafaudage clé dans le développement et la fonction du cerveau, joue une multitude de rôle du niveau subcellulaire au niveau cognitif.

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**Mots clés:** Scrib1, troubles du spectre autistique, neurones hippocampiques, plasticité synaptique, épine dendritique, trafic des récepteurs glutamatergiques, dynamique du cytosquelette d'actine.

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## Résumé

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Le cerveau contient environ cent milliards de cellules nerveuses, ou neurones. Ces neurones communiquent entre eux par des structures fonctionnellement distinctes – l'axone et la dendrite – capables d'émettre et recevoir des signaux électriques ou chimiques à partir d'un compartiment présynaptique vers un compartiment, dit post-synaptique. Nous avons focalisé notre étude sur les synapses des neurones hippocampiques, qu'on estime responsables de fonctions cérébrales dites supérieures, comme la mémoire et l'apprentissage. Plus particulièrement, on s'est intéressé au développement et au maintien des épines dendritiques, dont les changements morphologiques sont intimement liés à la plasticité synaptique, autrement dit, capacité de réponse à l'activité synaptique.

Les épines dendritiques ont pour origine les filopodes qui évoluent en épines lors du contact axonal. La transition entre filopode et épine implique une myriade de molécules, dont des récepteurs glutamatergiques, des protéines d'échafaudage et du cytosquelette d'actine capables de recevoir, transmettre et intégrer le signal présynaptique. Cependant, la coordination

spatiale et temporelle de tous ces composants moléculaires au long de la formation et maturation d'une synapse reste largement méconnue.

Scribble1 (Scrib1) est une protéine de polarité cellulaire (PCP) classiquement impliquée dans l'homéostasie de tissus épithéliaux ainsi que dans la croissance et progression des tumeurs. Scrib1 est aussi une protéine d'échafaudage critique pour le développement et le bon fonctionnement du cerveau. Notamment, notre équipe a démontré que Scrib1 pourrait jouer un rôle major dans les troubles du spectre autistique (TSA) chez les souris (Moreau et al., J Neurosci 2010).

L'objectif de cette étude a donc été d'étudier les mécanismes moléculaires sous-jacents à un rôle potentiel de Scrib1 dans la formation et le maintien des synapses des neurones hippocampiques.

Dans un premier temps, on a décrit l'importance d'interactions dépendantes des domaines PDZ sur: (a) le trafic des récepteurs glutamatergiques de type NMDA, en décrivant une nouvelle complex composé par Scrib1, des subunités NR2 et la protéine adaptatrice AP-2; et (b) la voie de signalisation

de plasticité synaptique sous-jacente à la mémoire spatiale impliquant des phosphatases de type PP2A .

Dans un second temps, nous avons évalué les conséquences fonctionnelles d'une mutation de Scrib1 récemment identifiée chez un patient humain atteint des troubles du spectre autistique dans la morphologie et fonction des neurones. Notamment, cette mutation (P592S) est proche des un motif putative de liason à l'actine (KK611) ainsi que des deux sites putatives de liason au complex de nucleation d'actine Arp2/3 (W203 et W386).

En utilisant des cultures des neurones hippocampiques, on a pu démontrer que Scrib1 régule l'arborisation dendritique ainsi que la formation et le maintien fonctionnel des épines dendritiques via un mécanisme dépendent du cytosquelette d'actine. Enfin, le dérèglement de ces mécanismes pourrait être à l'origine du phénotype TSA.

L'ensemble de ce travail met en évidence que Scrib1, protéine d'échafaudage clé dans le développement et la fonction du cerveau, joue une multitude de rôle du niveau subcellulaire au niveau cognitif.

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**Mots clés:** Scrib1, troubles du spectre autistique, neurones hippocampiques, plasticité synaptique, épine dendritique, trafic des récepteurs glutamatergiques, dynamique du cytosquelette d'actine.



## Publications

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- Piguel NH, Fiebre S, Blanc JM, Carta M, Moreau MM, Moutin E, **Pinheiro VL**, Medina C, Ezan J, Lasvaux L, Loll F, Durand CM, Chang K, Petralia RS, Wenthold RJ, Stephenson FA, Vuillard L, Darbon H, Perroy J, Mulle C, Montcouquiol M, Racca C & Sans N. Scribble1/AP-2 complex coordinates NMDA receptor endocytic recycling. *Cell Reports* 9, 1–16, October 23, 2014;
- Hilal M, Moreau MM, Racca C, **Pinheiro VL**, Santoni MJ, Carvalho SS, Piguel NH, Rachel R, Abada YS, Blanc JM, Medina C, Doat H, Papouin T, Panatier A, Borg JP, Montcouquiol M, Olier SHR & Sans N. Scribble 1 scaffolds protein phosphatases 1 and 2A to regulate bidirectional plasticity underlying spatial memory. In preparation;
- Pinheiro VL**, Mehidi A, Fanet H, Piguel NH, Chazeau A, Medina C, Breillat C, Choquet D, Gianonne G, Montcouquiol M & Sans N. Disrupting Scrib1-mediated actin regulation impacts neuronal complexity, synapse formation and maintenance. In preparation;
- Pinheiro, VL**, Racca C & Sans N. Reviewing AMPA receptor trafficking – The Who, What, Why, When, Where & How. In preparation.

## Oral communications

---

- Pinheiro VL**. “Characterization of Scrib1 protein interactions in synapses and their relevance for Autism Spectrum Disorders”. 1<sup>st</sup> SyMBAD meeting, Balatonfüred, Hungary 2011;
- Pinheiro VL**. “The Scrib1 interactome and its relevance for synaptic plasticity”. 2<sup>nd</sup> SyMBAD meeting, Alicante, Spain 2012;
- Pinheiro VL**. “The autism associated Planar Cell Polarity protein Scribble1 regulates AMPA receptor-dependent synaptic plasticity trafficking”. 3<sup>rd</sup> SyMBAD meeting, Stresa, Italy 2013.

## Posters

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- Pinheiro VL**, Piguel NH, Hilal M, Blanc JM, Vuillard L, Montcouquiol M & Sans N. “Characterization of Scrib1 protein interactions in synapses and their relevance for Autism Spectrum Disorders”. Symposium du Neurocentre Magendie, Bordeaux, France 2011;
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- Pinheiro VL**, Piguel NH, Hilal M, Blanc JM, Medina C, Breillat C, Olier SHR, Choquet D, Vuillard L, Montcouquiol M & Sans N. “The PDZ protein Scrib1 regulates the surface expression of AMPA receptors”. Jacques Monod conference, Roscoff, France 2012;
- Pinheiro VL**, Piguel NH, Hilal M, Blanc JM, Medina C, Breillat C, Olier SHR, Choquet D, Vuillard L, Montcouquiol M & Sans N. “The PDZ protein Scrib1 regulates the surface expression of AMPA receptors”. Symposium du Neurocentre Magendie, Bordeaux, France 2012;

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### **Scientific formations**

---

2011 (1w/March): SYMBAD workshop WP1: Cell-permeable peptides: a tool to study synaptic protein complexes in physiology and pathology, Milano, Italy;

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### **Teaching**

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2011 (2m/June- August): tutor of Raphaëlle Hours, ENSTBB 2<sup>nd</sup> year student, "Etude des interactions entre la protéine Scribble (Scrib1) et l'actine et ses conséquences sur la plasticité synaptique", Bordeaux, France;

2013 (1w/September): tutor at ECUBE Bordeaux Summer School "Super-resolution imaging of the Vangl2-Scribble1 complex and its role in AMPA receptor trafficking", Bordeaux, France;

2014 (6m/January-June): co-tutor of Hortense Fanet, Neuroscience M2 at the University of Bordeaux 2, "Etude du rôle de Scribble1 dans la dynamique du cytosquelette d'actine de l'épine dendritique", Bordeaux, France.

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## Abbreviations

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<b>Abi,</b>	Abelson interacting protein
<b>ADF,</b>	actin depolymerisation factor
<b>ADHD,</b>	attention deficit hyperactivity disorder
<b>AKAP,</b>	A-kinase anchor protein
<b>Akt or PKB,</b>	protein kinase B
<b>Ala or A,</b>	alanine
<b>AMPAR,</b>	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
<b>AMPK,</b>	5' AMP-activated protein kinase
<b>ANOVA,</b>	analysis of variance
<b>AP,</b>	adaptor-protein
<b>APC,</b>	adenomatous polyposis coli
<b>aPKC,</b>	atypical protein kinase C
<b>ARF6,</b>	ADF-ribosylation factor 6
<b>Arg or R,</b>	arginine
<b>ArhGEF,</b>	Rho guanine nucleotide exchange factors (GEF)
<b>Arp2/3,</b>	Arp2 and Arp3 complex
<b>ARVCF,</b>	armadillo repeat protein deleted in velo-cardio-facial syndrome
<b>ASD,</b>	autism spectrum disorders
<b>ASD-RD,</b>	ASD-related diseases
<b>ATP,</b>	adenosine triphosphate
<b>BAR,</b>	Bin–Amphiphysin–Rvs domain
<b>CA,</b>	Cornu Ammonis
<b>Ca<sup>2+</sup>,</b>	calcium
<b>CADM1,</b>	cell adhesion molecule 1
<b>CAMKII,</b>	calcium/calmodulin-dependent protein kinase II
<b>CAPON or NOS1AP,</b>	carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein or nitric oxide synthase 1 adaptor
<b>CASK,</b>	calcium/calmodulin-dependent serine protein kinase
<b>Cdc42,</b>	cell division control protein 42
<b>Cdh,</b>	cadherin
<b>Cdk5,</b>	cell division protein kinase 5
<b>cDNA,</b>	complementary DNA
<b>Celsr1,</b>	cadherin, EGF LAG seven-pass G-type receptor 1
<b>chemLTP,</b>	chemical LTP
<b>CIRL,</b>	calcium-independent alpha-latrotoxin receptor
<b>c-myc,</b>	cellular homologue of the viral oncogene v-myc

<b>CNS,</b>	central nervous system
<b>Cntnap or Caspr,</b>	contactin-associated protein
<b>CNVs,</b>	copy number variations
<b>COS-7,</b>	<u>C</u> V-1 (simian) in <u>O</u> origin, and carrying the <u>S</u> V40 genetic material
<b>Crb,</b>	crumbs
<b>Crc,</b>	circletail
<b>Crtam,</b>	cytotoxic and regulatory T cell molecule
<b>Cter,</b>	C-terminal
<b>Ctnnb1,</b>	catenin (cadherin-associated protein), beta 1
<b>Cys or C,</b>	cysteine
<b>DG,</b>	dentate gyrus
<b>Dgo,</b>	diego
<b>DIC,</b>	differential interference contrast
<b>DISC1,</b>	disrupted in schizophrenia 1
<b>DIV,</b>	days <i>in vitro</i>
<b>Dlg,</b>	discs large
<b>DMEM,</b>	Dulbecco/Vogt modified Eagle's minimal essential medium
<b>DNA,</b>	deoxyribonucleic acid
<b>DO,</b>	drop out media
<b>Ds,</b>	dachshund
<b>Dsh,</b>	dishvelled
<b>dSTORM,</b>	direct stochastic optical reconstruction microscopy
<b>eBPAG1,</b>	bullous pemphigoid antigen 1
<b>ECM,</b>	extra-cellular matrix
<b>EE,</b>	enriched environment
<b>EGFR,</b>	epidermal growth factor receptor
<b>EM,</b>	electron microscopy
<b>Eps8,</b>	epidermal growth factor receptor pathway substrate 8
<b>ER,</b>	endoplasmic reticulum
<b>ErbB2 or Her2,</b>	v-erbB-2 avian erythroblastic leukemia viral oncogene homolog 2
<b>ERK,</b>	extracellular signal-regulated kinases
<b>EZ,</b>	endocytic zone
<b>F-actin,</b>	polymerized actin
<b>FBS,</b>	fetal bovine serum
<b>fEPSP,</b>	field excitatory postsynaptic current
<b>FERM,</b>	named after 4.1 protein, ezrin, radixin and moesin
<b>Fj,</b>	four-jointed protein

<b>Fmi,</b>	flamingo
<b>FMRP,</b>	fragile X mental retardation protein
<b>Ft,</b>	fat
<b>Fz,</b>	frizzled
<b>GABAR,</b>	gamma-aminobutyric acid receptor
<b>G-actin,</b>	monomeric actin
<b>GFP,</b>	green fluorescent protein
<b>GIT,</b>	G protein-coupled receptor kinase-interactor
<b>GK,</b>	guanylate kinase
<b>GKAP,</b>	guanylate-kinase-associated protein
<b>Glu or E,</b>	glutamic acid
<b>Gly or G,</b>	glycine
<b>GRIP,</b>	glutamate receptor-interacting protein
<b>GST,</b>	Glutathione S-transferase
<b>GTP,</b>	guanosine triphosphate
<b>GTPase</b>	enzyme that can bind and hydrolyze GTP
<b>GUKH,</b>	GUK-holder
<b>HEK,</b>	human embryonic kidney
<b>HFS,</b>	high-frequency synaptic stimulation
<b>HGF,</b>	hepatocyte growth factor
<b>His or H,</b>	histidine
<b>HRP,</b>	horseradish peroxidase
<b>HSP90,</b>	heat shock protein 90
<b>HTLV,</b>	human T cell leukaemia virus
<b>Itgb4,</b>	integrin, beta 4,
<b>JAK,</b>	Janus kinase
<b>JNK,</b>	c-Jun NH(2)-terminal kinase
<b>K<sup>+</sup>,</b>	potassium
<b>KCl,</b>	potassium chloride
<b>LAP,</b>	LRR and PDZ domains
<b>Leu or L,</b>	leucine
<b>LFS,</b>	low-frequency stimulation
<b>LIM,</b>	named after LIN-11, ISL-1 and MEC-3
<b>LKB1,</b>	liver kinase B1
<b>Llg,</b>	lethal giant larvae
<b>Lp,</b>	looptail
<b>LPP,</b>	lipoma-preferred partner



<b>LRR,</b>	leucine rich repeat
<b>LTD,</b>	long-term depression
<b>LTP,</b>	long-term potentiation
<b>Lys or K,</b>	lysine
<b>MAGUK,</b>	membrane-associated guanylate kinase
<b>MAPK,</b>	Mitogen-activated protein kinases
<b>MARK1,</b>	microtubule affinity-regulating kinase 1
<b>MCC,</b>	mutated in colorectal cancers
<b>MEF,</b>	mouse embryonic fibroblasts
<b>mEPSC,</b>	miniature excitatory postsynaptic current
<b>Mg<sup>2+</sup>,</b>	magnesium
<b>mGluR,</b>	metabotropic glutamate receptor
<b>MINT,</b>	Munc18 interacting protein
<b>miR,</b>	microRNA
<b>mRNA,</b>	messenger RNA
<b>MUPP,</b>	Multiple PDZ protein
<b>MuSK,</b>	muscle, skeletal, receptor tyrosine kinase
<b>NHERF,</b>	Na <sup>+</sup> /H <sup>+</sup> exchanger regulatory factor
<b>Nhs,</b>	Nance-Horan syndrome
<b>Nhs1b,</b>	Nance-Horan syndrome-like 1b protein
<b>NMDAR,</b>	N-methyl D-aspartate receptor
<b>NMJ,</b>	neuromuscular junction
<b>nNOS,</b>	neural nitric oxide synthase
<b>NOS1AP,</b>	see CAPON
<b>NPFs,</b>	nucleation promoting factor
<b>NPRAP,</b>	neural plakophilin-related armadillo protein
<b>Nrlg,</b>	neuroligin
<b>Nrxn,</b>	neuroxin
<b>NTD,</b>	neural tube defect
<b>Nter,</b>	N-terminal
<b>N-WASP,</b>	neural Wiskott-Aldrich syndrome protein
<b>PAK,</b>	p21-Activated Protein Kinase
<b>Par,</b>	partitioning defective
<b>Patj,</b>	pals1-associated tight junction protein
<b>PBS,</b>	phosphate-buffered saline
<b>PCP,</b>	planar cell polarity
<b>PDZ,</b>	named after PSD-95, Dlg1 and ZO

<b>PDZbd,</b>	PDZ-binding domain
<b>PEI,</b>	polyethylenimine
<b>PFA,</b>	paraformaldehyde
<b>PH,</b>	pleckstrin homology domain
<b>PHLPP1,</b>	PH domain and leucine rich repeat protein phosphatases
<b>PI3K,</b>	phosphoinositide 3-kinase
<b>PICK1,</b>	protein interacting with C kinase
<b>Pk,</b>	prickle
<b>PP,</b>	phosphatase
<b>PPIs,</b>	protein-protein interactions
<b>Pro or P,</b>	proline
<b>PSD,</b>	post-synaptic density
<b>PSD-93/95,</b>	postsynaptic density protein 93/95
<b>PTB,</b>	phosphotyrosine-binding
<b>PtdIns,</b>	phosphatidyl inositol
<b>PTEN,</b>	phosphatase and tensin homologue;
<b>Ptk7,</b>	tyrosine-protein kinase-like 7
<b>Rac1,</b>	Ras-related C3 botulinum toxin substrate 1
<b>Raf,</b>	ser/thr-specific protein kinase related to retroviral oncogenes
<b>Ras,</b>	ser/thr-specific protein kinase originally found in rat sarcomas
<b>RE,</b>	recycling endosomes
<b>RER,</b>	rough endoplasmic reticulum
<b>RFP,</b>	red fluorescent protein
<b>RhPV,</b>	Rhesus papillomavirus
<b>RNA,</b>	ribonucleic acid
<b>SALM5,</b>	synaptic adhesion-like molecule 5
<b>SAP-97/102,</b>	synapse-associated protein 97/102
<b>Scrib,</b>	scribble
<b>SD,</b>	synthetic defined media
<b>SDS/PAGE,</b>	sodium dodecyl sulfate polyacrylamide gel electrophoresis
<b>Sec24b,</b>	protein transport SEC24 family member B
<b>SEM,</b>	standard error of the mean
<b>Ser or S,</b>	serine
<b>SER,</b>	smooth endoplasmic reticulum
<b>Sgt1,</b>	ecdysoneless homolog 1
<b>SH,</b>	Src-homology domain
<b>Shank,</b>	SH3 and multiple ankyrin repeat domains protein

<b>Stat,</b>	signal transducer and activator of transcription
<b>Stbm,</b>	strabismus
<b>Std,</b>	stardust
<b>Stgz or Cacng2,</b>	stargazin (TARP $\gamma 2$ ) or voltage-dependent, gamma subunit 2
<b>SV,</b>	synaptic vesicles
<b>TARP or Cacng,</b>	transmembrane AMPAR regulatory protein calcium channel or voltage-dependent, gamma subunit
<b>Tax,</b>	transactivator of pX
<b>Taz,</b>	tafazzin
<b>TFN,</b>	transferrin
<b>Thr or T,</b>	threonine
<b>TIRF,</b>	total internal reflection fluorescence
<b>TM,</b>	transmembrane domains
<b>TRIP6,</b>	thyroid receptor-interacting protein 6
<b>TrkB,</b>	tyrosine kinase B
<b>Trp or W,</b>	tryptophan
<b>TSC,</b>	tuberous sclerosis protein
<b>TSHR,</b>	thyroid stimulating hormone receptor
<b>Tyr or Y,</b>	tyrosine
<b>Val or V,</b>	valine
<b>Vangl,</b>	Van Gogh
<b>Vasp,</b>	vasodilator-stimulated phosphoprotein
<b>VELI,</b>	Vertebrate lin-7 homolog
<b>Vim,</b>	vimentin
<b>WASP,</b>	Wiskott–Aldrich syndrome protein
<b>WAVE,</b>	WASP-family verprolin-homologous protein
<b>Y2H,</b>	yeast two-hybrid
<b><math>\beta</math>Pix,</b>	PAK-interacting exchange-factor

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# Chapter I

## INTRODUCTION

### I.1 General Introduction

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Most, if not all, cell types and tissues exhibit several aspects of polarization. Spatial differences in their shape and/or structure allow a given cell or tissue to carry out specialized functions. Classical examples of cell polarity include epithelial cells with apical-basal polarity or neurons releasing neurotransmitters from pre-synaptic axons to post-synaptic dendrites. Scribble1 (Scrib1) is an apico-basal determinant classically implicated in the homeostasis of epithelial tissues and tumour growth. This LAP (LRR and PDZ domains) family member was, however, lately found to be a critical regulator of brain development and synaptic function as well. Indeed, Scrib1 and other cell polarity proteins have been pointed out as molecular determinants of autism spectrum disorders (ASD) etiopathology in the last few years. Therefore, the study of the molecular mechanisms underlying cell polarity establishment and maintenance in the mammalian brain context emerges as a powerful tool to better understand and develop therapies for brain disorders.

This thesis will start with a brief overview over the LAP family. Next, I will introduce the cell-autonomous apico-basal polarity as well as the planar cell polarity pathway, focusing on Scrib1 role as an apico-basal determinant, tumour suppressor gene and PCP (planar cell polarity) gene, respectively. I will then focus on excitatory synapses of the mammalian brain, and in particular on the development and maintenance of dendritic spines. Scrib1 role in synaptic plasticity and brain function will then be outlined, followed an overview of the current state of the art of ASD etiopathology. Finally, I will present a review on AMPA receptor trafficking.

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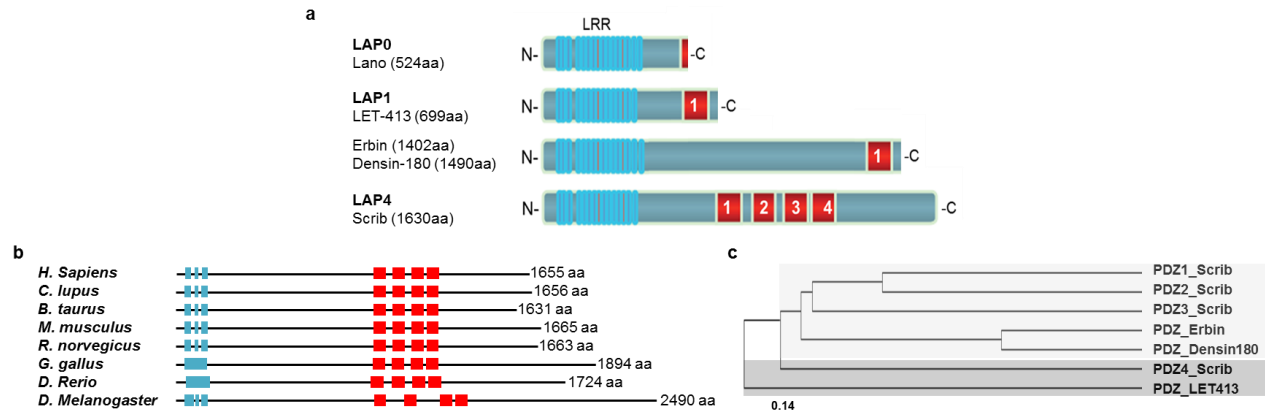
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### I.2 The LAP member Scribble has a conserved gene structure

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Scribble encodes a large cytoplasmic, multidomain scaffold protein from the exclusive LAP family of adaptor proteins. All LAP members - Lano, LET-413, Densin-180, Erbin and Scrib - possess a conserved domain structure, containing 16 (LET-413 and Scrib) or 17 (Lano, Densin-180 and Erbin) N-terminal (Nter) leucine rich repeats (LRRs) and a C-terminal (Cter) PDZ (named after post-synaptic density protein, PSD-95; *Drosophila* disc large tumour suppressor, Dlg1; and zonula occludens-1 protein, ZO-1) binding domain (PDZbd) (LAP0; Lano) or one (LAP1; LET-413, Densin-180, Erbin) to four PDZ domains (LAP4; Scrib) (**Fig. 1 a**). Such multidomain structure was well preserved throughout evolution (Santoni et al., 2002), having a correspondent orthologue from *Drosophila* to human (**Fig. 1 b**). LRR domains, common to all LAPs, are believed to be responsible for their subcellular localization, restricted to the epithelium basolateral membrane or the pre- and post-synaptic compartment in neurons. On the other hand, the distinct Cter PDZ domains (**Fig. 1 c**)

allow each LAP member to be part of a particular multi-protein complex and hence to play distinct roles –  
**Table 1.**



**Figure 1: LRR and PDZ domain family members kept its conserved structure throughout evolution.** LAP family elements are typically composed of 16-17 Nter LRRs (light blue) and a Cter PDZ binding domain (PDZbd) or one to four PDZ domains (red). **(b)** Scrib protein structure shown in several species. **(c)** Multiple alignment between the PDZ domains of LAP1 and LAP4 elements using Fast Fourier transform (Kato et al., 2002). Branch lengths are proportional to the genetic distance between the given sequences. Aligned sequences (Uniprot): LET413\_CAEEL, O61967; LRRC7\_MOUSE, Q80TE7; LAP2\_MOUSE, Q80TH2; SCRIB\_MOUSE, Q80U72.

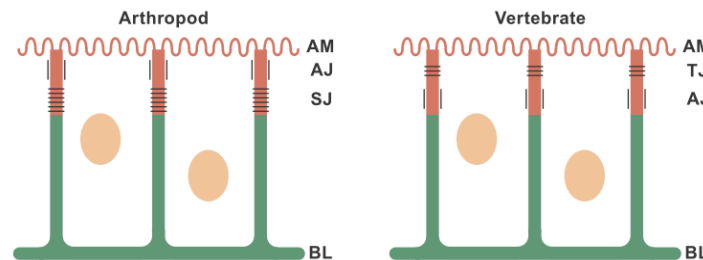
**Table 1: Relevant interaction, localization and function of LAP0 and LAP1 elements.**

LAP protein	Relevant interactions	Localization	Function	Ref.
<b>Lano</b>	PDZ-dependent interaction with hDlg and Erbin	Epithelial basolateral cell membrane	Homeostasis of epithelial tissues and tumour growth	[1]
<b>LET-413</b>	-	Epithelial basolateral cell membrane	Adherens junctions assembly; adaptor in polarizing protein trafficking in epithelial cells	[2-4]
<b>Densin-180</b>	PDZ-dependent interaction with $\alpha$ -actinin/CAMKII and $\delta$ -catenin/NPRAP (N-cadherin); Shank1-3; DISC1 and mGluR5	Synapses in hippocampal neurons; synaptic density (PSD)	Normal synaptic spine architecture and function (LTD)	[5-10]
<b>Erbin</b>	PDZ-dependent interaction with ErbB2/ Her2, PSD-95, MuSK; eBPAG1 and Cter of Itgb4(ErbB2); $\delta$ -catenin and ARVCF; PDZ-independent interaction with TARP $\gamma$ -2	Basolateral cell membrane; myotubes; postsynaptic membranes of NMJ; dendritic shafts in cortical and hippocampal neurons; synaptosomes; parvalbumin-positive interneurons	Recruitment of receptors or complexes to the basolateral membrane and consequent signalling pathway regulation	[11-16]

1. Saito et al., 2001; 2. Legouis et al., 2000; 3. Legouis et al., 2003; 4. Bossinger et al., 2004; 5. Apperson et al., 1996; 6. Strack et al., 2000; 7. Walikonis et al., 2001; 8. Izawa et al., 2002; 9. Quitsch et al., 2005; 10. Carlisle et al., 2011; 11. Borg et al., 2000; 12. Favre et al., 2001; 13. Huang et al., 2001b; 14. Laura et al., 2002; 15. Tao et al., 2013; 16. Simeone et al., 2010.

LAP family members were firstly studied on epithelial cells. These cells are distinctly polarized, being composed by an apical and a basolateral domain separated by specialized junctional complexes (**Fig. 2**). Labouesse and colleagues (Legouis et al., 2000) were the first to show that the genetic loss of the basolateral LET-413 protein in *C. elegans* leads to a disorganization of the epithelial architecture and consequentially to morphogenetic defects. In *Drosophila*, so did the genetic loss of Scrib, normally located in the septate junctions, lead to similar abnormalities (Bilder & Perrimon, 2000), suggesting that LAP proteins create a barrier that restricts apical and basolateral determinants to the respective apical and basolateral domains. Indeed, in

the last years, several studies have shown that LAP elements can efficiently regulate epithelial cell homeostasis by trafficking proteins to the basolateral cell membrane (Borg et al., 2000; Favre et al., 2001; Saito et al., 2001; Laura et al., 2002; Legouis et al., 2003; Bossinger et al., 2004; Yang et al., 2011). Moreover, following the first purification of a LAP member from the postsynaptic density fraction of rat forebrain (Apperson et al., 1996), a growing body of evidences suggest that LAP family proteins can recruit crucial synaptic elements to regulate synaptic architecture and/or function (Apperson et al., 1996; Strack et al., 2000; Huang et al., 2001b; Walikonis et al., 2001; Izawa et al., 2002; Laura et al., 2002; Quitsch et al., 2005; Simeone et al., 2010; Carlisle et al., 2011; Tao et al., 2013).



**Figure 2: Epithelial cell architecture.** Arthropods and vertebrates share a similar distribution of apical and basolateral domains. Both top and bottom are sketched by apical microvilli (AM) and basal lamina (BL), respectively. Adherens junctions (AJ) contain the majority of cell-cell adhesion molecules as well as proteins involved in cell-cell signalling, whereas PDZ-containing proteins and their transmembrane partners are mainly located in tight junctions (TJs). SJ, septate junctions. Taken from Bryant & Huwe, 2000.

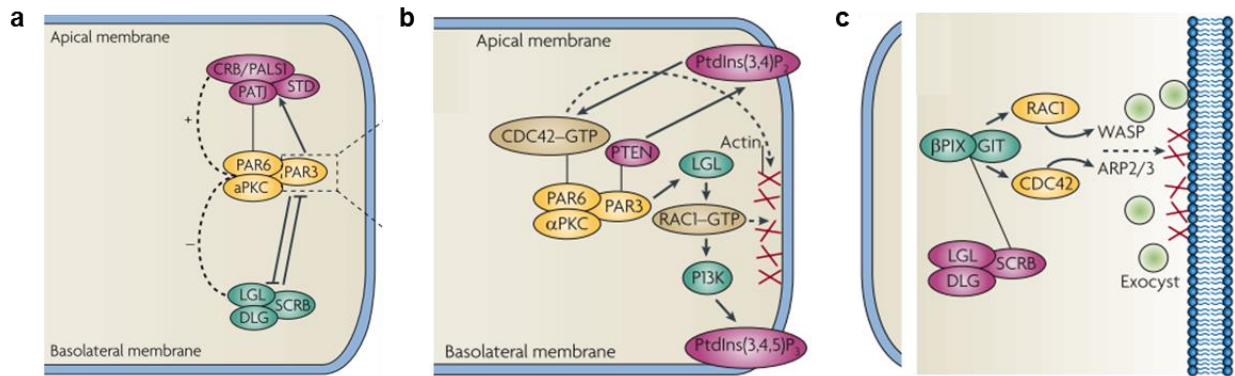
### I.3 Scribble and the apico-basal polarity

#### I.3.1 The cell-autonomous apico-basal polarity

Scrib was firstly identified through the distinctive ‘giant larvae’ phenotype of zygotic mutant animals in *Drosophila melanogaster* together with lethal giant larvae (Llg) and discs large (Dlg) as neoplastic tumour-suppression mutations (Bilder & Perrimon, 2000; Bilder et al., 2000; Brumby & Richardson, 2003; Pagliarini & Xu, 2003). These three proteins – Scrib, Lgl and Dlg, are believed to play a key role in the regulation of polarity at a single cell level (**Fig. 3**). Together they form a signalling complex capable to define and stabilize the basolateral membrane, by mutually antagonizing the apical junctional Par3-Par6-aPKC complex (Bilder et al., 2003; Tanentzapf & Tepass, 2003). Conversely, the Par complex genetically interacts and stabilizes the apical Crb-Std-Patj complex (Roh & Margolis, 2003) (**Fig. 3 a**). This signalling triad is subsequently thought to play a crucial role on the phosphoinositide distribution, actin polymerization and vesicle trafficking (Arimura & Kaibuchi, 2005). Par3 is known to directly bind PTEN regulating local phospholipid synthesis, whereas the direct Par6-Cdc42(GTP) and the indirect Par3-Lgl-Rac1(GTP)-PI3K interactions contribute to the apical PtdIns(3,4)P2 or basolateral PtdIns(3,4)P3 membrane distribution, respectively (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007) (**Fig. 3 b**). Such phosphoinositide asymmetry seems to be crucial to successfully polarize the docking and fusion of Golgi and endosome-derived transport vesicles in such compartments. Moreover, the associated Rho GTPases Cdc42 and Rac1 can also regulate actin polymerization locally, through the activation of the WASP or Arp2/3 (Nishimura et al., 2005; Ridley, 2006) (**Fig. 3 c**).



On what seems to be a rather redundant process, so does the Scrib complex modulate actin polymerization, this time through the  $\beta$ Pix/GIT complex (Zhao et al., 2000; Audebert et al., 2004; Iden & Collard, 2008).



**Figure 3: Scribble takes part on the definition and stabilization of a cell-autonomous asymmetry.** The basolateral membrane is defined by Scrib-DLG-LGL complex, which mutually antagonizes the apical PAR6-PAR3-aPKC complex. In turn, this will interact and stabilize the other apical Crumbs-STD-PATJ complex (a). These signalling complexes can control the phosphoinositide distribution (b) and vesicle trafficking (c) through complex interactions with PTEN, Rho GTPases and the actin polymerization machinery (see text). CRB, Crumbs; PATJ, Pals1-associated tight junction protein; PALS1, protein associated with LIN-7 1; STD, stardust; PAR, partitioning defective; aPKC, atypical protein kinase C; LGL, lethal giant larvae; DLG, disc large tumour suppressor protein; SCRIB, Scribble; CDC42, cell division control protein 42; Rac1, p21-Rac1; PTEN, phosphatase and tensin homologue; PtdIns(3,4)P<sub>2</sub>, phosphatidylinositol-(3,4)-bisphosphate; PtdIns(3,4,5)P<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase;  $\beta$ PIX, PAK-interacting exchange-factor- $\beta$ ; GIT, G protein-coupled receptor kinase-interactor; WASP, Wiskott-Aldrich syndrome protein; ARP2/3, actin-related protein-2/3. Taken from Mellman & Nelson, 2008.

### 1.3.2 Role of Scribble in apico-basal polarity

In the past decade, numerous studies underscored Scrib as an apico-basal polarity determinant, as it was discovered to be involved in several cell polarity events in multicellular structures. The large majority of such complexes rely on protein-protein interactions, dependent on Scrib LRR domain (Eastburn et al., 2012) but mostly on its PDZ domains. The 4 PDZ domains allow Scrib to preferentially interact with proteins containing PDZ domains or Cter PDZbd of proteins involved in a plethora of different cell polarity events in different cell types - **Table 2**. In *Drosophila* neuroblasts, dividing cells precursors of neurons during the embryonic development, Scrib can form a complex with either Dlg/Lgl or LKB1/AMPK to regulate cortical polarity, cell size and mitotic spindle asymmetries (Albertson & Doe, 2003; Albertson et al., 2004; Andersen et al., 2012), whereas in the neural tube of Zebrafish embryos it was found to be implicated in oriented cell division together with  $\alpha$ -catenin (Zigman et al., 2011). It is also known that Scrib plays a key role in the establishment of the anterior-posterior axis, the most ancient of the embryonic axes, interacting with Dlg to regulate *Drosophila* oogenesis (Li et al., 2009) or with the zyxin-related protein LPP at cell-cell junctions to control the conversion-extension of Zebrafish embryonic cells (Petit et al., 2005; Vervenne et al., 2008). In addition, a great number of studies convincingly showed that Scrib is able to form a complex and recruit several proteins to cell-cell junctions from epithelial cells to synaptic sites in neurons. As expected, the cell-autonomous elements Crb and Dlg cooperate with Scrib to maintain cell adhesion (Bilder & Perrimon, 2000; Mathew et al., 2002; Ludford-Menting et al., 2005), as well as interactions with MAGUKs - such as the single PDZ calcium/calmodulin dependent serine protein kinase CASK (Lozovatsky et al., 2009) and the tight junction proteins ZO-1 (Ivanov et al., 2010) and ZO-2 (Métais et al., 2005), or single PDZ proteins - like the human T-cell

**Table 2: Relevant interaction, function and type of cells in which Scrib plays a preponderant role as a PCP gene.**

Cell polarity events	Relevant interaction	PDZ or PDZbd	Function	Type of cells	Ref.
<b>Asymmetric cell division</b>	$\alpha$ -catenin	-MDSI	Clustering of $\alpha$ -catenin during oriented cell division independent of canonical apico-basal and PCP pathways; relevant for cross-midline cell divisions and neural tube morphogenesis	Zebrafish neural tube	[1]
	Dlg/Lgl (LKB1/AMPK)	MAGUK	Regulation of cortical polarity, cell size and mitotic spindle asymmetries	<i>Drosophila</i> neuroblasts	[2-4]
<b>Anterior-posterior patterning</b>	Dlg	MAGUK	Genetic interaction involving the regulation of EGFR, JAK and Notch signalling; relevant for oogenesis	<i>Drosophila</i> oocytes	[5]
	LPP	-STDL	PDZ-dependent interaction at cell-cell junctions; relevant for conversion and extension during development	Zebrafish embryo	[6,7]
<b>Cellular trafficking and cell adhesion maintenance</b>	AP-2	-	Scrib module mutants show defective AP-2 dependent endocytosis; strong genetic interaction between Scrib and retromer components	<i>Drosophila</i> eye discs and follicle cells	[8]
	APC	-VTSV	PDZ1/4-dependent interaction and co-localization at synaptic sites and at the tip of epithelial membrane protrusions	Mammalian epithelial cells	[9]
	$\beta$ PIX/GIT1(ARF6) TSHR	-ETNL -QTAL	PDZ-dependent interaction involved in the vesicle trafficking, receptor endocytosis and recycling	Epithelial, neuronal and thyroid cells	[10,11]
	CASK	MAGUK	Interaction involved in the proper localization of LIN7c and Dlg1/Scrib complex	Mammalian epithelial cells	[12]
	Crtam	-ESIV	Anchoring and coordination of a signalling complex following activation; relevant for the regulation of a late phase of T cell polarity	Mouse T cells	[13]
	Crb	-	Leads to the loss of Scrib function and consequently to the misdistribution of apical proteins and adherens junctions to the basolateral cell surface	<i>Drosophila</i> embryonic epithelia	[14]
	Ctnnb1 (Cdh1,Cdh2/ZO-1)	-DTDL	Phosphorylation-dependent recruitment and complex formation at cell-cell junctions; relevant for cell adhesion, proliferation and proliferation in the neural retina and cardiomyocyte organization	Mammalian epithelial cells	[15-19]
	Dlg (GUKH)	MAGUK	PDZ2-dependent interaction with GUKH, which interacts with the GUK domain of Dlg, implicated in the regulation of synaptic bouton budding; regulation of T cell polarity, morphology during migration and immunological synapse formation	<i>Drosophila</i> synapses and human T cells	[20,21]
	Sec24b	-	Genetic interaction; Vangl2 transport; relevant for NTD and organization of mechanosensory cells in the inner ear	Mouse embryos and inner ears; cultured primary cells	[22]
	Hsp90/Sgt1 ( $\beta$ PIX-PAK)	-MDTV	LRR-dependent localization following HGF stimulation; Sgt1 and HSP90 ensure proper Scrib1 levels; Scrib1- $\beta$ PIX-PAK complex relevant for epithelial morphogenesis	Mammalian epithelial cells	[23]
	Tax	PDZ	PDZ-dependent interaction involved in Tax cytoplasmic localization and NFAT signalling regulation	T cells	[24]
	TRIP6	-TTDC	PDZ3-dependent interaction and co-localization at cell-cell junctions; regulation of the pathway between cell adhesion sites and nucleus shuttle	Mammalian epithelial cells	[25]
	Vangl2 ( $\beta$ catenin/Rho)	-ETSV	Genetic interaction; PDZ-dependent localization, anchoring and complex formation at the basolateral plasma membrane; relevant for lung morphogenesis	Mouse embryos and inner ear cells; mammalian epithelial cells	[26-30]
	ZO-1	MAGUK	Complex formation and recruitment to cell-cell junctions	Human intestinal epithelium	[31]
	ZO-2	MAGUK/ -DTEL	PDZ3/4-dependent interaction and LRR-dependent complex localization at cell-cell junctions	<i>Drosophila</i> epithelial cells	[32]

<b>Directional migration</b>	$\beta$ PIX/GIT/PAK (Cdc42/Rac1)	-ETNL	Recruitment of Scrib complex to the leading edge; polarized distribution of active Rac and Cdc42; regulation of GTPase activity; relevant for lamellipodia and filopodia formation	Mammalian epithelial cells and <i>Drosophila</i> embryos	[33-37]
	MCC ( $\beta$ catenin) NHERF1/2 (Ezrin)	-ETSL 2PDZs/-FSNL/F	PDZ-dependent complex formation and co-localization at the cell membrane; relevant for cell migration independently of Rac1, Cdc42 and PAK activation	Mammalian epithelial cells	[38]
	Vim	-DDLE	PDZ-dependent interaction and localization during the establishment of cell-cell contacts, directional movement and cell aggregation; relevant for Golgi polarization	Mammalian epithelial cells and endothelial human cells	[39]
<b>Neuronal migration</b>	Nhs1b	-	Physical and genetic interaction; co-localization at membrane protrusions; involvement of the Wnt-dependent JNK and ROCK signalling pathway; relevant for neuronal migration	Facial brachiomotor neurons of Zebrafish	[40, 41]

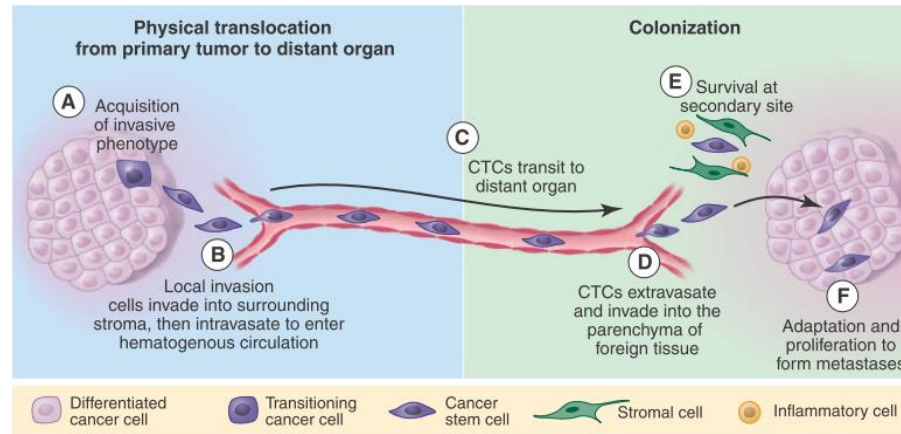
1. Zigman et al., 2011; 2. Albertson & Doe, 2003; 3. Albertson et al., 2004; 4. Andersen et al., 2012; 5. Li et al., 2009; 6. Petit et al., 2005; 7. Vervenne et al., 2008; 8. De Vreede et al., 2014; 9. Takizawa et al., 2006; 10. Audebert et al., 2004; 11. Lahuna et al., 2005; 12. Lozovatsky et al., 2009; 13. Yeh et al., 2008; 14. Bilder & Perrimon, 2000; 15. Navarro et al., 2005; 16. Qin et al., 2005; 17. Yoshihara et al., 2011; 18. Nguyen et al 2005; 19. Phillips et al., 2007; 20. Mathew et al., 2002; 21. Ludford-Menting et al., 2005; 22. Wansleebeben et al., 2010; 23. Eastburn et al., 2012; 24. Arpin-André & Mesnard, 2007; 25. Petit et al., 2005; 26. Montcouquioli et al., 2003; 27. Montcouquioli et al., 2006; 28. Kallay et al., 2006; 29. Courbard et al., 2009; 30. Yates et al., 2013; 31. Ivanov et al., 2010; 32. Métais et al., 2005; 33. Osmani et al., 2006; 34. Dow et al., 2007; 35. Nola et al., 2008; 36. Bahri et al., 2010; 37. Wigerius et al., 2010; 38. Arnaud et al., 2009; 39. Phua et al., 2009; 40. Vivancos et al., 2009; 41. Walsh et al., 2011. Uniprot ref. for PDZbd: P26231, Q61315, Q9ES28, Q68FF6, P47750, Q149L7, Q02248, Q80ZX0, Q9CZP7, Q9DBG9, Q9Z1Y4, P39447, Q9Z0U1, E9PWI3, Q9JHL1, P20152

leukemia virus 1 trans-activating transcriptional regulatory protein Tax (Arpin-André & Mesnard, 2007). On the other hand, Scrib recognizes several Cter PDZbd-containing proteins, whose classification depends on their last Cter four amino acids content. Scrib binds canonical or class I PDZbd (X-S/T-X-L/V, being (x) any amino acid) like the tumour adenomateous polyposis coli protein APC (Takizawa et al., 2006), the GTPase activating protein complex  $\beta$ Pix/GIT1 (Audebert et al., 2004; Lahuna et al., 2005), the thyroid-stimulating hormone receptor TSHR (Audebert et al., 2004; Lahuna et al., 2005), the single pass transmembrane nectin member Crtam (Yeh et al., 2008), the calcium-dependent cell adhesion Ctnnbn1/Cdhs (Navarro et al., 2005; Nguyen et al., 2005; Qin et al., 2005; Phillips et al., 2007; Yoshihara et al., 2011), the transmembrane protein Vangl2 (Montcouquioli et al., 2003; Montcouquioli et al., 2006; Kallay et al., 2006; Courbard et al., 2009; Yates et al., 2013) or the PDZ domain ZO-2 (Métais et al., 2005). Scrib can also interact with class II PDZbd containing proteins (X- $\phi$ -X- $\phi$ , being ( $\phi$ ) any hydrophobic amino acid), such as the co-chaperone Hsp90 protein (Eastburn et al., 2012) and the cell adhesion/nuclear shuttle thyroid receptor-interacting protein TRIP6 (Petit et al., 2005). Lastly, it can also interact genetically with the endocytic adaptor protein AP-2 (de Vreede et al., 2014) or the secretory machinery elements like Sec24b to regulate Vangl2 transport (Wansleebeben et al., 2010). Taking part in such an intricate network, Scrib plays unsurprisingly a multifaceted role in a wide range of molecular mechanisms, from regulating vesicle trafficking, receptor endocytosis and recycling in neurons and thyroid cells (Audebert et al., 2004; Lahuna et al., 2005) to coordinating signalling complexes in epithelial and T cells (Ludford-Menting et al., 2005; Arpin-André & Mesnard, 2007; Yeh et al., 2008), participating in the organization of multicellular structures and morphogenesis of different tissues, from mechanosensory cells in the inner ear, neural retina in eyes, cardiomyocytes to lungs or even the immune system (Montcouquioli et al., 2003, 2006; Nguyen et al., 2005; Phillips et al., 2007; Courbard et al., 2009; Wansleebeben et al., 2010; Pike et al., 2011; Eastburn et al., 2012; Yates et al., 2013). Scrib is also a main coordinator of direc-

tional migration in polarized cells, by recruiting several proteins involved in the cytoskeleton dynamics. As mentioned, Scrib can interact and form a complex with GTPases activating proteins, such as the Rho guanine nucleotide exchange factor 7  $\beta$ PIX or the serine/threonine-protein kinase PAK. The recruited complex can be selectively targeted to focal adhesion sites, where  $\beta$ PIX and PAK can regulate Cdc42 and Rac1 activity and promote the formation of lamellipodia and filopodia (Osmani et al., 2006; Wigerius et al., 2010; Dow et al., 2007; Nola et al., 2008; Bahri et al., 2010). Scrib can be equally engaged in cell migration events independently of Rac1, Cdc42 and PAK activation, by interacting with the colorectal mutant cancer protein MCC in a PDZ-dependent manner (Arnaud et al., 2009). MCC, a former tumour suppressor gene involved in  $\beta$ -catenin-dependent transcription repression (Fukuyama et al., 2008), is also known to bind other PDZ domain containing scaffold proteins NHERF1 and NHERF2. In turn, these  $\text{Na}^+/\text{H}^+$  exchange regulatory co-factors interact with members of the plasma membrane/cytoskeleton interface FERM family, such as Ezrin. Interestingly, MCC interactions appear to be strengthened upon Wnt stimulation (Fukuyama et al., 2008; Arnaud et al., 2009). An additional PDZ-dependent interaction was shown to be crucial in linking Scrib to the cytoskeleton dynamics, this time regarding the type III intermediate filament protein Vimentin (Vim). Vim is long known to support and anchor organelles in the cytosol (Katsumoto et al., 1990), being recently involved in Golgi polarization together with Scrib during the establishment of cell-cell contacts, directional movement and cell aggregation (Phua et al., 2009). Finally, the latest physical and genetic interaction revealed between Scrib and the Nance-Horan syndrome-like 1b protein (Nhsl1b) in Zebrafish underscores an enthralling role of Scrib in neuronal migration (Wada et al., 2005; Vivancos et al., 2009; Walsh et al., 2011). During early stages of development, post-mitotic neurons migrate away from their birthplace to settle in their final destination. Nhsl1b encodes a WAVE-homology domain-containing protein related to human Nance-Horan syndrome protein and *Drosophila* GUK-holder (Gukh). In facial brachiomotor neurons, Nhsl1b co-localizes with Scrib at the membrane protrusions, acting as a neuronal effector of PCP signalling between the epithelium and the migrating neurons.

### 1.3.3 Scribble is a tumour suppressor gene

Cancer can be defined as a complex, multistep process that includes an uncontrolled proliferation of abnormal cells, a failure to resume cell differentiation, invasion and formation of new metastasis (**Fig. 4**). Loss of polarized cell architecture is becoming a well-established hallmark of epithelial cancers, strongly favoring the involvement of cell polarity regulators in suppressing tumorigenesis (reviewed by Dow & Humbert, 2007). It is then with no surprise that already over 30 years ago both Lgl and Dlg were addressed as tumour suppressor proteins in *Drosophila* (Gateff, 1978). Scrib downregulation due to the high risk human papillomavirus (Nakagawa & Huibregtse, 2000) or human epithelial tissues (HPV)-derived E6 oncoprotein overexpression (Dow et al., 2003) was correlated to a loss of apico-basal polarity, pointing the potential link between polarity and tumour progression. Interestingly, the ubiquitin-mediated degradation of PDZ-containing tumour suppressors such as hScrib and hDlg have been long proven to be essential to be determinant in several forms of carcinogenesis, growth of neoplastic lesions and malignant metastasis progression (Gardioli et al., 1999; Nakagawa & Huibregtse, 2000; Nguyen et al., 2003a,b; Grifoni et al., 2004; Nakagawa et al., 2004; Massimi



**Figure 4: The cancer cascade.** Cancer is a complex, multistep process that includes (a) the acquisition of invasive phenotype by a given cell; (b) an uncontrolled proliferation of such abnormal cells into the surrounding matrix and generally towards blood vessels; (c) physical translocation of cancer cells (CTCs) from the primary tumour to a distant organ; (d) invasion of the foreign tissue microenvironment; (e) survival to the innate immune response; and (f) formation of new metastasis. Taken from Chaffer & Weinberg, 2011.

et al., 2004; Navarro et al., 2005; Thomas et al., 2005; Gardiol et al., 2006; Kamei et al., 2007; Wodarz & Nathke, 2007; Vieira et al., 2008; Yamanaka & Ohno, 2008; Wu et al., 2010; Nicolaides et al., 2011). Likewise, PDZ-motifs of the human T cell leukaemia virus type 4 (HTLV-1) derived Tax (Lee et al., 1997; Suzuki et al., 1999; Okajima et al., 2008; Arpin-Andre & Mesnard, 2007), the neurovirulent lethal encephalitis virus NS5 (Werme et al., 2008) or the avian influenza virus NS1 are able to bind hScrib and/or hDlg, affecting their localization, activity and function (Liu et al., 2010; Golebiewski et al., 2011). In primates, so does the Rhesus papillomavirus (RhPV) E7 oncoprotein contain a PDZ-binding motif able to target Par3 (Tomać et al., 2009). The use of an evolutionarily conserved PDZ-dependent mechanism able to target apico-basal complexes by such a broad array of virus does underscore the importance of cell polarity maintenance in tumourigenesis progression. Numerous studies have been trying to elucidate the exact molecular mechanisms underlying Scrib role as a tumour suppressor gene (reviewed by Humbert et al., 2008) - **Table 3**. Overexpression of hScrib was shown to inhibit cell-cycle progression from G1 to S phase in a LRR- and PDZ1-dependent manner (Nagasaka et al., 2006). The consequent cell proliferation blockage might be due to hScrib ability to up- and down-regulate the expression levels of APC and cyclins A and D1, respectively. Conversely, the decreased expression levels and mislocalization of hScrib has been associated with the progression of several cancers, by acting on the proper targeting and expression levels of membranar proteins like E-cadherin (Nakagawa et al., 2004; Ouyang et al., 2010),  $\beta$ -catenin (Kamei et al., 2007), PHLPP1 (Li et al., 2011b) and integrin  $\alpha 5$  (Michaelis et al., 2013). Scrib was also shown to play a determinant role in the renewal of cancer stem cells by regulating Taz protein levels and activity (Cordenonsi et al., 2011). Taz is a Hippo signalling transducer under the inhibition of hScrib. hScrib loss or inactivation upon the induction of epithelial-mesenchymal transition allow Taz to be activated and accumulated in stem-like progenitor cells, resulting in more aggressive tumours. A recent proteomic analysis of human Hippo pathway revealed that Scrib interacts, among others, with PAK, GIT and ArhGEF family members as well as NOS1AP, all known to cooperate with Scrib in cell polarity events (see **Chapters I.3.1 and I.3.2**) (Wang et al., 2014). hScrib was also shown to interact with the cell migration hDlg/Hugl1/syntaxin 4 complex (Massimi et al., 2008). Taken together, these

**Table 3: Relevant direct or complex interaction, function and biological processes that Scrib is involved in.**

Type of interaction	Relevant interaction	Function	Implicated process	Ref.
<b>Direct</b>	APC, cyclin A and cyclin D	Regulation of protein expression relevant for cell cycle progression from G1 to S phase	Cell-cycle progression and proliferation control	[1]
	$\beta$ -catenin	Cytoplasmic accumulation of $\beta$ -catenin	Colon carcinogenesis	[2]
	E-cadherin	Recruitment and co-localization at adherent junctions	Prevention of cancer development	[3-5]
	Hippo pathway	Core kinase cascade able to control the transcription of key tumour suppressor genes	Cell proliferation and apoptosis	[6]
	Integrin $\alpha 5$	Turnover and sorting regulation into and from the plasma membrane	Directed endothelial cell migration and angiogenesis	[7]
	PHLPP1	PHLPP1 recruitment to the membrane and Akt phosphorylation	Proliferation of human embryonic kidney cells	[8]
	Taz	Regulates inhibitory association between TAZ with core Hippo signalling kinases	Self-renewal and tumour-initiation in breast cancer stem cells	[9]
<b>Complex</b>	ArhGEF/GIT/PAK and NOS1AP/Vangl	Co-localization with NOS1AP and Vangl along cellular protrusions in metastatic cells; prevents the establishment of leading-trailing polarity with NOS1AP	Human breast cancer relapse	[6,10]
	hDlg/NOS1AP	Vesicle transport pathway in mammalian cells	Tumour suppressor complex formation and localization	[11]
<b>Oncogenic cooperation</b>	c-myc	Cooperate to regulating apoptosis	Mammary neoplasia	[12]
	miR-296	Regulates Scrib1 gene transcription and protein localization	Angiogenesis	[13]
	Raf, Ras	Act upstream ERK, MAPK, Hippo, Notch and TFN signalling pathways	Cell proliferation and tumour dysplasia	[12, 14-19]
	RasV12 or Stat	Competition over JNK and JAK/STAT signalling	Neoplasia	[20-22]

1. Nagasaka et al., 2006; 2. Kamei et al., 2007; 3. Nakagawa et al., 2004; 4. Navarro et al., 2005; 5. Ouyang et al., 2010; 6. Wang et al., 2014; 7. Michaelis et al., 2013; 8. Li et al., 2011; 9. Cordenonsi et al., 2011; 10. Anastas et al., 2012; 11. Massimi et al., 2008; 12. Zhan et al., 2008; 13. Vaira et al., 2012; 14. Brumby & Richardson, 2003; 15. Dow et al., 2008; 16. Cordero et al., 2010; 17. Vidal, 2010; 18. Nagasaka et al., 2010; 19. Doggett et al., 2011; 20. Pagliarini & Xu, 2003; 21. Wu et al., 2010; 22. Schroeder et al., 2012.

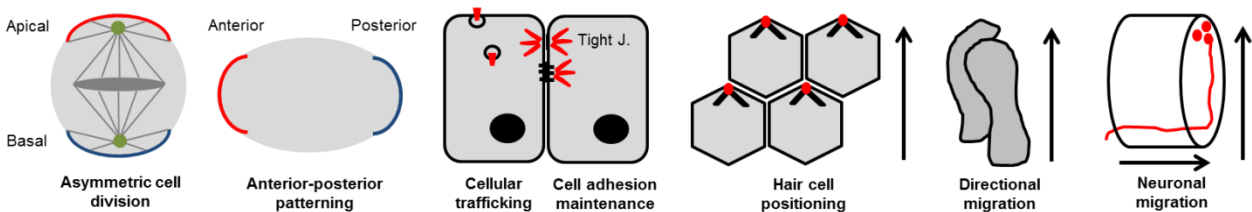
data suggests that Scrib expression levels, localization and activity play a key role in the maintenance of tissue architecture. Finally, it is becoming clear that, as a tumour suppressor gene, Scrib can also cooperate with other oncogenes to regulate several signalling pathways involved in cancer progression. Epithelial tumours of Scrib/Dlg/Lgl *Drosophila* mutants are long known to exhibit all the hallmarks of cancer (Gateff, 1978; Woodhouse et al., 1998; Bilder et al., 2000; Brumby & Richardson, 2005; Grzeschik et al., 2007; Zhao et al., 2008), whereas the same does not necessarily hold true if the mutants are generated in wild type tissues (Brumby & Richardson, 2003; Pagliarini et al., 2003). Moreover, Scrib has been shown to cooperate with several oncogenes, known to be key players throughout cancer progression. Most studies feature a strong oncogenic cooperation between Scrib and several members of the small GTPase Ras family. In both *Drosophila* and human epithelial cells, Scrib and Ras or Raf were shown to act on ERK, MAPK, Hippo, Notch and TNF signalling pathways to promote cell proliferation and tumour dysplasia (Brumby & Richardson, 2003; Dow et al., 2008; Zhan et al., 2008; Cordero et al., 2010; Nagasaka et al., 2010; Vidal, 2010; Doggett et al., 2011; Enamoto & Igaki, 2011). Conversely, Scrib appears to be involved in neoplasia events such as tumour overgrowth and oncogenic cell elimination failure by cooperating with RasV12 (Pagliarini & Xu, 2003;

Wu et al., 2010) or competing with Stat (Schroeder et al., 2012) over JNK and JAK/STAT signalling regulation. Scrib has also been reported to regulate apoptosis by co-acting with the transcription factor c-myc in mammary neoplasia (Zhan et al., 2008). Finally, a recent study shows that Scrib gene transcription and protein localization can be regulated by miR-296, a microRNA also named “angiomir” due to its role in angiogenesis regulation (Vaira et al., 2012). Altogether, these studies suggest that a synergy between a given apico-basal dysfunction and a perturbed oncogenic signalling within a propitious tumour microenvironment might be crucial throughout cancer progression.

## I.4 Scribble and the planar cell polarity pathway

### I.4.1 The planar cell polarity pathway

Most, if not all, cell types and tissues exhibit several aspects of polarization. In addition to the ubiquitous cellular asymmetry seen along the apical-basolateral axis, many tissues and organs are also polarized within the tissue plane. Planar cell polarity (PCP; or historically, tissue polarity) thus refers to the establishment of such cellular asymmetries (reviewed by Goodrich & Strutt, 2011; Gray et al., 2011; Bayly & Axelrod, 2011). PCP can be observed in a vast array of developmental processes, from collective cell movements to tissue organization, and its disruption can lead to uncontrolled cellular proliferation and invasion or entail severe development defects - **Fig. 5**. Moreover, recent studies in the central nervous system have shed light into the importance of “planar polarity” genes in such fundamental aspects as neuronal migration, neuronal polarity, axon guidance, and dendrite morphogenesis (reviewed by Goodrich, 2008), raising the enthralling possibility that PCP can chisel cell morphology in all dimensions.

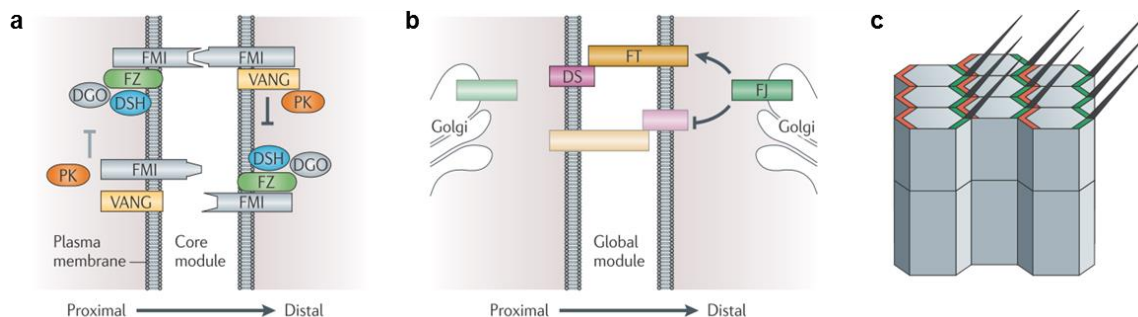


**Figure 5: Examples of planar polarity events at the cell and tissue level.** Cell polarity contemplates spatial differences in shape, structure, and function; while planar cell polarity refers to the polarization at the tissue level. The asymmetric cell division, done within an apico-basal axis, gives rise to two daughter cells with different cellular fates. The anterior-posterior patterning during *Drosophila* morphogenesis is based on a pair-rule graded signalling of genes required for alternative body segments. The cellular trafficking in epithelial cells, essential to the spatial segregation of apico-basal compartments as well as for the cell adhesion maintenance between neighbouring cells. The hair cell positioning, well described in *Drosophila*'s wing, ciliogenesis and the mammalian auditory sensory organ, is essential to such functions as fly, directing fluid flows or ear properly. The directional migration can be observed in a broad range of different cells, from bacteria to immune system cells. In the particular case of neuronal migration, neurons travel from their birthplace to their final destination.

Pioneer genetic and molecular studies in *Drosophila* provided the first models to unravel PCP and its components. PCP is believed to be composed of three key functional modules – a core module, a global directional cue module and an asymmetric core protein localization module - **Fig. 6**. The first module amplifies asymmetry and coordinates polarization between neighbour cells through transmembranar or membrane-



adjoining proteins such as: the seven-transmembrane atypical cadherin Flamingo (Fmi), the serpentine receptor Frizzled (Fz), the four-pass transmembrane protein Van Gogh (Vang; also known as Strabismus, or Stbm), the multidomain protein Dishvelled (Dsh), the ankyrin repeat protein Diego (Dgo), and the LIM domain protein Prickle (Pk). The core module proteins assemble in oppositely oriented complexes between the proximal and distal sides of the adjacent cells, mutually antagonizing each other and thus generating a first local polarized plan (reviewed by Zallen, 2007) (**Fig. 6 a**). The second module provides global directional information in response to both global and core modules signals. This module is believed to be driven by the atypical cadherins Fat (Ft) and Dachshous (Ds), and the Golgi-resident Four-jointed protein (Fj). Ft and Ds are able to form opposite heterodimers at neighbouring cell membranes; while the Fj ectokinase controls their graded expression, being ultimately responsible for the activation of distinct downstream effector modules that set tissue-specific polarization events (**Fig. 6 b**). Finally, the asymmetric core protein localization is made by proximal and distal proteins on opposite sides of cells, leading to a morphological polarity. Such event is classically exemplified by the distally orientated actin polymerisation seen in hair formation during *Drosophila*'s wing development (Adler, 2002) (**Fig. 6 c**). During polarization, the complex Stbm-Pk is mainly found at the proximal side, while the Fz/Dsh/Dgo complex becomes enriched at the distal side, where an actin-based hair will be positioned. Several other studies of vertebrate PCP pathways involved in conversion and extension during neural tube closure and hair cell positioning of the inner ear further reinforce the role of the PCP pathway in generating molecular asymmetries and ensuring polarization in neighbouring cells. Finally, recent work from our group identified a new set of proteins composed of the GTP-binding protein  $\alpha$ -I subunit 3 ( $\alpha_{i3}$ ) and the mammalian Partner of inscuteable (mPins) (Ezan et al., 2013).  $\alpha_{i3}$ /mPins are expressed in an opposite and complementary fashion regarding the aPKC/Par-3/Par-6b domain, controlling cell-autonomous translational polarity through a G-protein-dependent signalling.



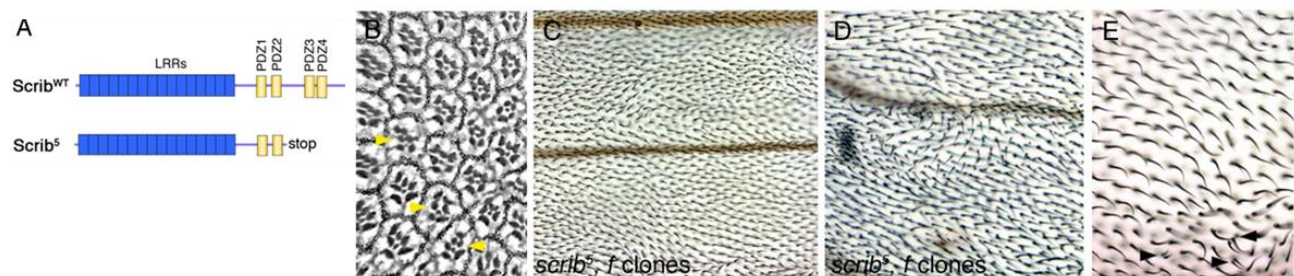
**Figure 6: The three key functional modules of the PCP pathway:** (a) the core module, capable of amplifying asymmetry and coordinating polarization between neighbour cells; (b) the global directional cue module, responsible for activating distinct downstream effector modules; and (c) the asymmetric core protein localization module, which leads to the so called morphological polarity, typically exemplified by the hair formation during the wing formation of the *Drosophila*. Fmi, Flamingo; Fz, Frizzled; Vang, Vang Gogh; Dsh, Dishvelled; Dgo, Diego; Pk, Prickle; Ft, Fat; Ds, Dachshous; Fj, Four-jointed. Taken from Bayly & Axelrod, 2011.

#### I.4.2 Scribble mutants display PCP defects

In metazoans, both apico-basal polarity and PCP are required for the development, morphogenesis and function of the large majority of tissues and organs. The failure to establish one or the other are associated with a vast array of genetic diseases (reviewed by Simons & Mlodzik, 2008). Defects in PCP establishment



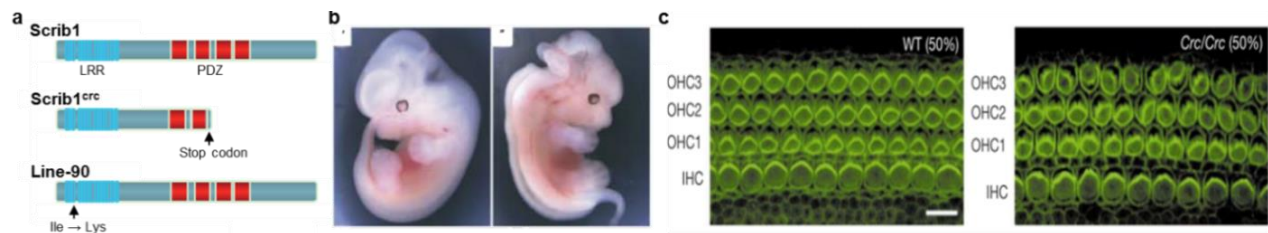
were first described in *Drosophila* external structures, being the distal orientation of wing hairs, the posterior orientation of cellular hairs and the arrangement of ommatidia in the eye the best examples (Adler, 2002; Strutt, 2003; Klein & Mlodzik, 2005). During PCP establishment, all PCP factors are found apically, where they can be engaged in regulatory interactions with apico-basal determinants that dictate the first detectable asymmetries. Montcouquiol and colleagues (2003) were the first to show that Scrib can interact genetically and physically with the core PCP component Stbm/Vang through its third PDZ domain. In addition, *scrib5* mutants lacking the two last PDZ domains fail to establish PCP in *Drosophila* eye and wing (Coubard et al., 2009) (**Fig. 7**), pinpointing Scribble role as a PCP associated gene.



**Figure 7: Scribble mutants fail to establish PCP in *Drosophila*'s eye and wing:** (a) schematic representation of wild-type Scrib (Scrib<sup>WT</sup>) and *scrib5* mutants, missing the two last PDZ domains. (b) Adult eye section of *scrib5* mutants. The asymmetric ommatidial structure is lost (yellow arrows). (c-e) Adult wing of *scrib5* mutants. Larger wing area (c) magnified to show characteristic PCP misorientations (d) and higher magnifications displaying cells with multiple wing hair phenotype (black arrowheads). Taken from Coubard et al., 2009.

Disruption of the PCP pathway has been strongly linked to developmental defects in animals and human pathologies as well. Analogous to the events long elucidated in *Drosophila*, the disorganization of the hair cells in the inner ear and neural tube defects are the best studied events of disturbed PCP signalling in mammals. In the apical surface of each hair cell there is a staircase array of asymmetrically actin-based stereocilia. The hair cell is localized on one side of the cell only, being able to perceive the mechanical deflection of the bundle and subsequently produce an electrical signal capable of regulating hearing and balance (Ezan & Montcouquiol, 2014). Mutations of core PCP proteins were first associated with misorientated stereociliary bundles in the cochleae of Vangl2<sup>Lp/Lp</sup> mouse mutants (Montcouquiol et al., 2003). This spontaneous mouse mutant arises from a destabilization and loss of function of the protein, caused by a missense mutation in Vangl2 Cter domain (Kibar et al., 2001; Montcouquiol et al., 2006). Ever since, mutations in other core PCP genes – the flamingo homolog Celsr1 (Curtin et al., 2003), Fz3/Fz6, the homologs of Disheveled Dvl1-3 (Wang et al., 2006a; Etheridge et al., 2008) and Vangl1 (Torban et al., 2008), were shown to display similar phenotypes. Remarkably, all the aforementioned mutants exhibit neural tube defects as well. Such defects usually occur when apical cells fail to properly integrate the neural epithelium after cell division, forming an ectopic cluster of cells instead. Therefore, the discovery of mice carrying a spontaneous mutation of Scrib1 - circletail (Scrib1<sup>crc</sup>) (Murdoch et al., 2003), which lacks the last 2 PDZ domains - exhibiting severe neural tube defects (NTD) came to strengthen the role of Scrib as an effector during PCP establishment. *Crc* mutants result from a single base insertion whose subsequent frameshift leads to a truncated form of Scrib1

protein without its two last PDZ domains (**Fig. 8 a**). As a consequence, *Scrib<sup>crc</sup>* mutants not only fail to fully complete the neural tube closure process (**Fig. 8 b**) as they also exhibit misorientated actin hair bundles in the inner ear (Montcouquiol et al., 2003) (**Fig. 8 c**). The very same phenotype can be observed in mutations of other PCP proteins like *Vangl2*, *Celsr1* or the single transmembrane domain protein tyrosine kinase *Ptk7* (reviewed by Doudney et al., 2005; Wansleebe & Meijlink, 2011), known to genetically interact with *Scrib* (Rachel et al., 2000; Murdoch et al., 2001a,b; Montcouquiol et al., 2003; Kallay et al., 2006; Savory et al., 2011; Robinson et al., 2012). Moreover, the rare putative mutations in *Scrib1*, *Vangl2* and *Celsr1* are responsible for over 20% of craniorachischisis cases in humans (Juriloff & Harris, 2012). Put together, these data strongly suggests the existence of a candidate gene pathway for NTD, possibly reminiscent in other PCP signalling pathways such as the one implicated in the hair cell orientation in the inner ear. Lastly, a single aminoacid substitution in the third LRR domain of *Scrib1*, leading to the replacement of a hydrophobic isoleucine by a charged lysine in the linker region between the LRR's two  $\beta$ -sheets, was found to be enough to disrupt cortical development (Zarbalis et al., 2004). Homozygous mice for this mutation – also known as line-90, also display craniorachischis, but not the dominant tail defect seen in *crc* mutants (**Fig. 8 a, b''**). Such remark pinpoints the importance of both LRR and PDZ domains of *Scrib1* in the establishment of planar cell polarity.



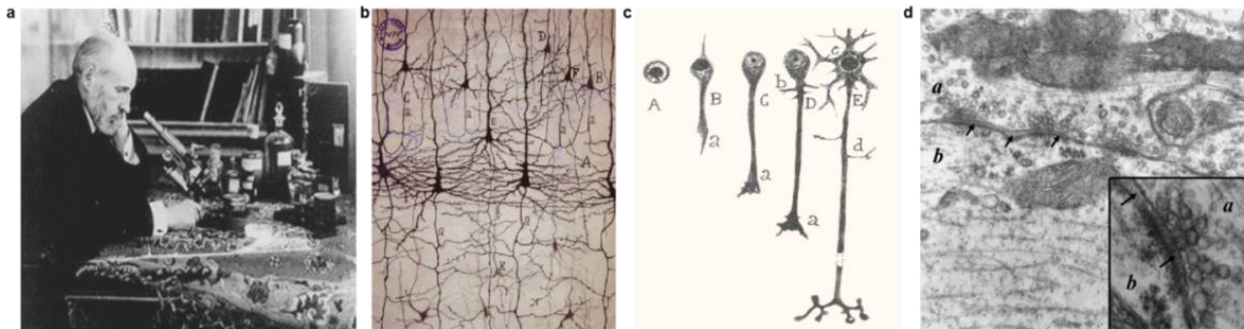
**Figure 8: Scrib1 mutants display severe developmental defects.** (a) Schematic representation of the normal (1665 aa) and mutated *Scrib1* forms – *Scrib<sup>crc</sup>* and line-90). The spontaneous *circletail* (*crc*) mutation results from a single base insertion whose frameshift leads to a truncated form of *Scrib1* without two PDZ domains (971 aa in total). Line-90 results from a single aminoacid mutation (Ile-Lys) in the third Nter LRR domain. (b') *Circletail* mice present craniorachischisis, the most severe form of neural tube defect (NTD), failing to close the cephalic neural tube from the midbrain throughout the spine. (b'') Line-90 mice also display craniorachischisis, but not the dominant tail defect. (c) Alike *Vangl2* homozygous, *crc/crc* mutants display a misorientation of actin hair bundles in the inner ear, a PCP defect. LRR, leucine rich repeats; PDZ, PSD-95, Dlg, ZO-1 domain; OHC, outer hair cell; IHC, inner hair cell. (a) adapted from Moreau et al., 2010; (b) taken from Zarbalis et al., 2004; and (c) from Montcouquiol et al., 2003.

## I.5 Scribble and the central nervous system

### I.5.1 Structure and connexion of neurons: Ramón y Cajal's heritage

More than 100 years ago, Santiago Ramón y Cajal received the Nobel Prize for Physiology and Medicine (1906) "in recognition of his meritorious work on the structure of the nervous system". Ramón y Cajal started by studying the structure of epithelial cells and tissues while in the Universities of Zaragoza and Valencia (López-Muñoz et al., 2006). It was only when he later moved to the University of Barcelona in 1887 that he focused on the study of the central nervous system (**Fig. 9 a**). By using the Golgi staining technique, he obtained detailed descriptions of several cell types associated with neural structures (**Fig. 9 b**). He was the first

to show that nerve cells relate to each other not by continuity – as postulated by Gerlach’s reticular theory (the nerve centres are composed by a diffuse protoplasmic network of grey matter), but rather by contiguity. Such discovery became the foundation for the so called “neuron doctrine”: (a) neurons are the basic units of the nervous system, (b) composed of specialized features such as a cell body, dendrites and an axon (**Fig. 9 c**), and (c) able to communicate with each other to allow transmission. This theory was further consolidated in the following 10 years by the neurohistological work of A. von Kölliker, C. Golgi, F. Nissl, A. H. Waldeyer-Hartz, among others, being later reaffirmed by new technologies such as the electron microscopy (**Fig. 9 d**) and kept barely untouched until today.

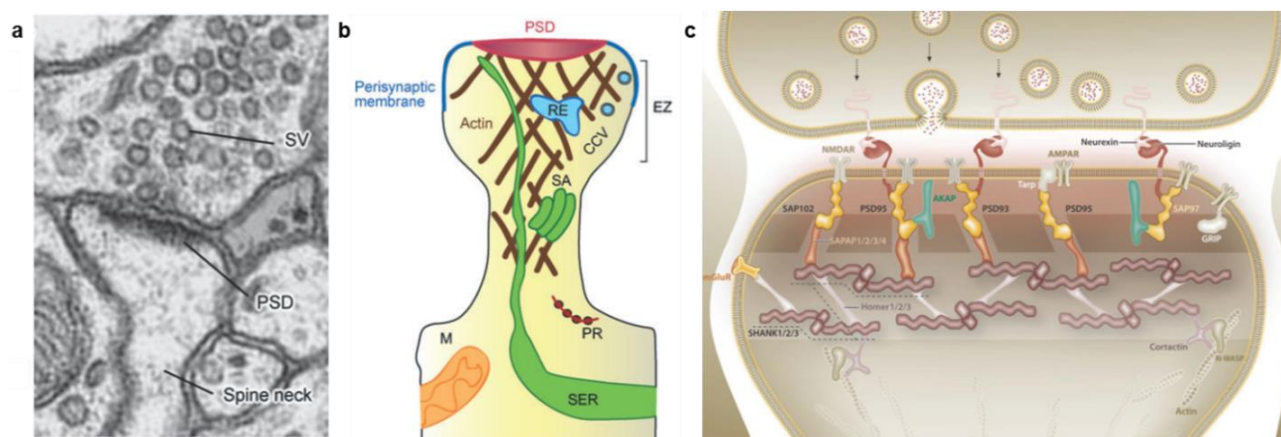


**Figure 9: Neuron theory, the cornerstone of neuroscience.** (a) Santiago Ramón y Cajal in the early 1920's, when the neuron theory was recognized. Cajal's drawings showing (b) a detailed description of the visual cortex of the cat, and (c) the development of a mature neuron, from an immature symmetric cell to a final polarized neuron containing a cell body, an axon and dendrites. (d) Electron microscopy of a synapse between an axon and a dendrite of the anterior horn of the human spinal cord. Taken from López-Muñoz et al., 2006.

### I.5.2 General view of excitatory synapses in the mammalian brain

Following up Ramón y Cajal's work, it is now well accepted that the billions of neurons in by the mammalian brain are able to form synapses (from Greek *synapsis* "conjunction") through distinctive and highly polarized cell junctions – the pre-synaptic axons and the post-synaptic dendrites. A major part of these synapses occur by the propagation of an electrical signal to the post-synaptic cell through the release of glutamate as the excitatory neurotransmitter. Most of these excitatory synapses are received by dendritic spines, small – 0.5-2  $\mu\text{m}$  in length, protrusions located throughout dendrites (Sheng & Hoogenraad, 2007) (**Fig. 10 a**). Dendritic spines are typically composed of a thin spine neck, connected to the parent dendrite, and a bulbous head, containing its own organelles involved in protein synthesis, membrane trafficking, ATP and calcium metabolism, dendritic spines bestow synapse-specific biochemical microcompartments (Kennedy et al., 2005) (**Fig. 10 b**). Moreover, these highly heterogeneous and dynamic structures are controlled by neuronal activity, being their shape – commonly characterized as “mushroom,” “thin,” or “stubby,” a good reflection of different developmental stages and/or synaptic strength changes (Yuste & Bonhoeffer, 2001; Hayashi & Majewska, 2005; Kasai et al., 2003). Excitatory (glutamatergic) synapses in particular are characterized by the presence of a prominent electron-dense, ~200–800 nm wide and ~30–50 nm thick postsynaptic membrane directly opposing the active zone (**Fig. 10 a**), also known as the PSD (post-synaptic density). This outstanding feature is due to the PSD composition itself - a large-scale and dynamic complex of glutamate receptors, associated signalling proteins, and cytoskeletal elements, all assembled by an abundant multiplicity of scaffold

proteins (Siekevitz, 1985; Sheng & Kim, 2002; Kasai et al., 2003). Given their abundance in the brain tissue and the fact that it can be fairly easily extracted out of synaptosome preparations, the biochemical composition of PSD started to be determined 30 years ago (Carlin et al., 1980). In recent years, via several other biochemical techniques (Hunt et al., 1996; Kim & Sheng, 2004; Funke et al., 2005), large proteomic studies (Husi et al., 2000; Walikonis et al., 2000; Husi & Grant, 2001; Satoh et al., 2002; Peng et al., 2004; Jordan et al., 2004; Yoshimura et al., 2004; Chen et al., 2005; Sugiyama et al., 2005; Cheng et al., 2006), 3D structure rendering of individual PSDs (Petersen et al., 2003; Nakagawa et al., 2005), we have a more comprehensive view of this exceptional and yet still puzzling structure that is the PSD. As expected, the great number of identified PSD proteins is involved in a multitude of cellular functions, such as cellular communication and signal transduction (adhesion molecules, surface receptors and channels, GTPases, and kinase/phosphatases), cellular organization (cytoskeleton elements, scaffold, membrane traffic, and motor proteins), energy (mitochondria and metabolism), or even protein synthesis and processing (translation and chaperones). From all these proteins, only a small fraction is now well described in both biochemical and functional terms (**Fig.10 c**).



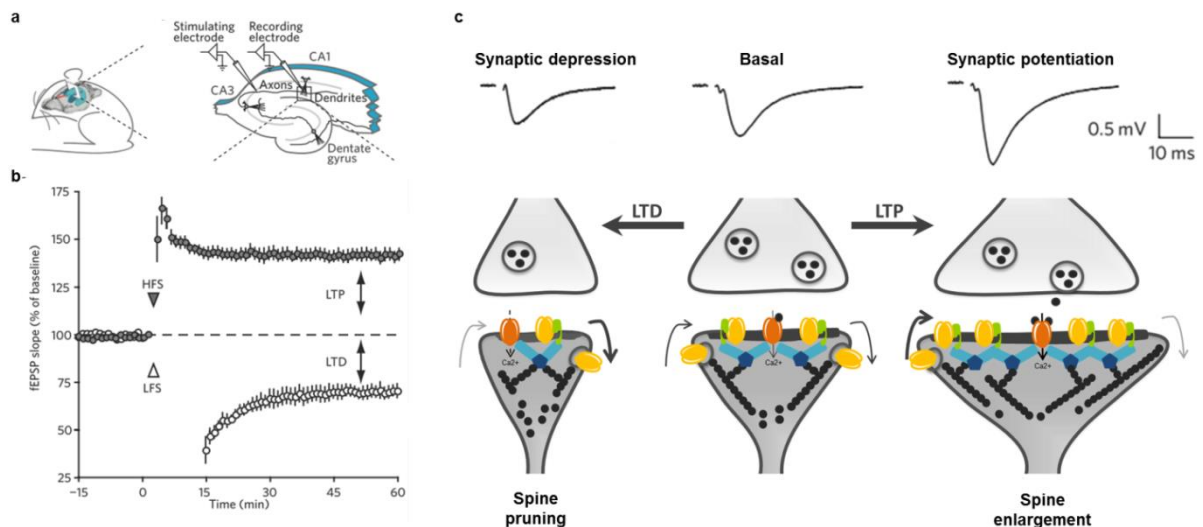
**Figure 10: Morphology and composition of an excitatory synapse.** (a) Electron microscopy of an excitatory synapse. The presynaptic terminal loaded with synaptic vesicles (SVs) containing glutamate faces the postsynaptic density (PSD). (b) Representation of a mushroom-shaped spine, containing a bulbous head and a spine neck. The spine head hosts the (1) PSD; (2) the actin cytoskeleton, which determines spine structure and motility; and (3) the endocytic zone (EZ), where clathrin-coated vesicles (CCV) and recycling endosomes (RE) can be found. Conversely, the spine neck receives several organelles coming from the dendritic shaft, namely the smooth endoplasmic reticulum (SER), the spine apparatus (SA), polyribosomes (PR) engaged in mRNA translation and mitochondria (M). (c) Schematic diagram of the major family members and certain classes of proteins present in excitatory synapses. The pre- and postsynaptic cells are classically coupled by the presence of adhesion molecules, such as the neuroligin and the neuroligin, respectively. The synaptically released glutamate can activate the NMDAR and the AMPAR located in the PSD, or the extrasynaptic mGluR. Surface receptors, adhesion and transmembrane auxiliary proteins such as TARPs can all interact with scaffold proteins, like Shank1/2/3, SAP-97/102, PSD93/95, or GRIP. These can by their turn interact in several degrees with other scaffold proteins (like SAPAP1/2/3/4, Homer1/2/3, or Cortactin), membrane-bound proteins (such as AKAP), or actin cytoskeleton elements (N-WASP, for example), therefore orchestrating a complex and dynamic network. AMPAR,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NMDAR, N-methyl D-aspartate receptor; mGluR, metabotropic glutamate receptor; TARP, transmembrane AMPAR regulatory protein; SAP-97/102, synapse-associated protein 97/102; PSD93/95, postsynaptic density protein 93/95; GRIP, glutamate receptor-interacting protein; SAPAP1/2/3/4, disks large-associated protein 1/2/3/4; AKAP, A-kinase anchor protein; N-WASP, neural Wiskott-Aldrich syndrome protein. (a) and (b) taken from Sheng & Hoogenraad, 2007; (c) taken from Ting et al., 2012.

### I.5.3 Synaptic plasticity: Donald Hebb's legacy

From an evolutionary-biological point of view, the primary function of the brain is to provide coherent control over the actions of an individual. To do so, the brain needs to (1) extract information from the surrounding



environment, (2) process it accordingly to the current needs and memory of past circumstances, and (3) generate a suitable response for the individual's welfare. All these steps require a fine-tuned wiring of the appropriate neural circuits, which essentially dependent on changes in the strength of synaptic contacts. Even though the connection of one neuron to another – or synapse, was thought for a long time to be a relatively static structure, Donald Hebb postulated a theory explaining how neurons adapt during the learning process. According to him, the repeated and persistent stimulation of the post-synaptic cell by the pre-synaptic shall lead to an increase of synaptic strength (Hebb, 1949). In other words, the Hebbian theory introduces the concept of synaptic plasticity, admitting for the first time that a synapse has the ability to change its response capacity to a given stimulus. The concept of synaptic plasticity has quite evolved since Hebb's postulate, being nowadays referred as long-term potentiation (LTP) and long-term depression (LTD) the two major cellular mechanisms believed to underlie learning and memory in the adult brain (Malenka & Nicoll, 1999). LTP refers to a persistent increase in the efficiency of synaptic transmission after inducing a short period of high-frequency synaptic stimulation (HFS); whereas LTD comprehends a decrease of synaptic strength following low-frequency stimulation (LFS; for further reading, see Song & Huganir, 2002; Brecht & Nicoll, 2003; Collingridge et al., 2004). Hippocampal brain slices, composed of stereotyped regions – CA1, CA3 and DG, are classically used to perform electrical recordings and study synaptic activity (**Fig.11 a**). Usually, a stimulating electrode is placed in the region of axons coming from the CA3 region, while a recording electrode is located in a field of activated synapses in the CA1 region. Examples of typical LTP and LTD



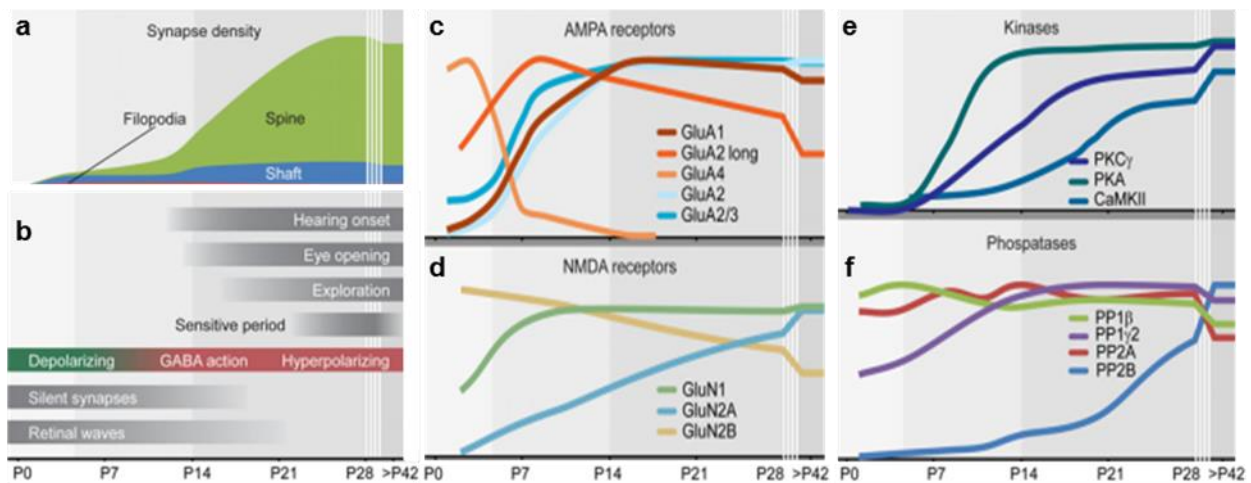
**Figure 11: Synaptic plasticity: functional and structural consequences of long-term potentiation and depression.** (a) The hippocampus, a highly organized structure, is used to perform electrical recording from its well defined regions. A stimulating electrode is placed in the axons coming from the CA3 region, while a recording electrode located in the CA1 region measures the voltage drop due to ions flowing from the electrode to open ion channels in the cell population (also called "field recording"). (b) Typical LTP and LTD response to high-frequency stimulation (HFS; for example, stimulating a population of presynaptic axons 100 times over one second) or low-frequency stimulation (LFS; for example, stimulating 900 times over 15 min). The amplitude or slope of the fEPSP (circles) is measured once every minute. (c) Following NMDA receptor (in orange) activation, Ca<sup>2+</sup> is allowed to go inside the cell and regulate AMPA receptors (in yellow) traffic to the membrane. LTD (left) and LTP (right) trigger the removal or insertion of AMPA receptors by scaffold proteins (in blue) from and to the membrane, respectively. Such functional changes are followed by actin-dependent (black dots) changes of the spine architecture, leading to spine pruning or enlargement, correspondingly. Arrows show the direction of insertion of removal of receptors from the membrane; thin grey and thick black represent slow and fast trafficking, respectively. Examples of field excitatory postsynaptic potentials (fEPSPs) measured from depressed, basal or potentiated synapses are shown above. Adapted from Fleming & England, 2010.

experiments induced after brief HFS and LFS, respectively, are shown in **Fig.11 b**. At the post-synaptic level, synaptic plasticity is predominantly mediated by two subtypes of glutamate-gated ion channels concentrated at post-synaptic sites - AMPA and NMDA receptors. The rapid excitatory synaptic transmission is ensured by AMPARs, while NMDARs regulate the slower component of neuronal activity. NMDARs are blocked by  $Mg^{2+}$  at resting membrane potentials, opening when a sufficient number of AMPARs are activated to depolarize the membrane potential. Once at a positive membrane potential, the  $Mg^{2+}$  blockade is relieved, allowing  $Ca^{2+}$  to flow into the cell through NMDARs. The intracellular  $Ca^{2+}$  can now trigger various signalling cascades that in turn regulate AMPAR trafficking to and from the synaptic membrane, resulting in a potentiated or depressed synapse. Such functional events are believed to be closely linked to actin-dependent changes of the architecture of the spine itself, leading to spine enlargement or pruning, respectively (**Fig.11 c**).

#### **1.5.4 The dendritic spine as the structural and functional output of synaptic plasticity**

The establishment of neuronal circuitry after birth well matches the time course of molecular and physiological stages of synaptic postnatal development - **Fig. 12**. Synaptic partner selection starts early in development, in which most of the existing synapses are located on dendritic shafts or filopodia (Fiala et al., 1998; Nagerl et al., 2007; Zito et al., 2009; Kwon & Sabatini, 2011) (**Fig. 12 a**). The cellular recognition mechanisms are then believed to be independent of neuronal activity (Lohmann & Bonhoeffer, 2008; Lohmann, 2009). The predominant spontaneous network activity is mainly mediated by gap junctions, which allow the developing neurons to properly synchronize their transcription activities (Kandler & Katz, 1995; Khazipov & Luhmann, 2006; Niculescu & Lohmann, 2013). Molecularly, this major period of synaptogenesis coincides with the maximal expression of spontaneous activity-driven GluA4-containing AMPARs trafficking to synapses (**Fig. 12c**). Remarkably, mice lacking GluA4 display schizophrenia-like behaviour (Sagata et al., 2010). Polymorphisms in the GluA4 human gene were also reported to increase susceptibility for schizophrenia (Makino et al., 2003), strongly suggesting that disruptions of early developmental plasticity mechanisms can lead to long-lasting consequences in brain function. During synaptic network formation, there is a gradual increase of the synaptic density (De Felipe et al., 1997; Steward & Falk, 1991), allowing the brain to better interpret and respond to the surrounding environment (**Fig. 12 a, b**). In the case of rodents, this stage coincides with the time when they open their eyes and start hearing. Interestingly, GABAergic transmission shifts its role from depolarizing to hyperpolarizing agent (Rivera et al., 1999; Khazipov & Luhmann, 2006; Ben Ari et al., 2007; Allene & Cossart, 2010). The GABAergic gradual hyperpolarization coincides with a decrease of silent synapses as well as an increase in the expression of key protein kinases and non GluA4-containing AMPAR subunits (**Fig. 12 b, c**). Following the activation of sensory inputs, synaptic density suffers a fast increase until reaching a steady state level in the cortex and in the hippocampus, being only a small proportion of excitatory synapses located on shafts (<10%) and even less in filopodia (Harris et al., 1992). Once the axo-dendritic contact is well established, new spines can turn into functional electro-chemical post-synaptic compartments (Muller & Connor, 1991; Koch et al., 1992; Koch & Zador, 1993; Yuste & Denk, 1995; Sabatini et al., 2002; Chklovskii, 2004; Araya et al., 2006a, b; Grunditz et al., 2008; Bloodgood et al., 2009; Yuste, 2011; Gullledge et al., 2012; Harnett et al., 2012). The majority of synapses is now preferentially lo-

cated on NMDAR-dependent dendritic spines (Boyer et al., 1998; Ultanir et al., 2007) (**Fig. 12 d**), better suited to process and encode all this new information. During this stage, there is a switch from GluN2A- to GluN2B-containing NMDARs, suggesting that GluN2B promotes synaptic stability (Gambrill & Barria, 2011; Gray et al., 2011). In addition, the protein kinases PKC and CaMKII, as well as the phosphatase calcineurin (PP2B) increase within spines (Boyer et al., 1998; Ultanir et al., 2007) (**Fig. 12 e, f**). Such molecular changes are responsible for the decrease of long C-tailed GluA2-containing AMPAR synaptic targeting (Nabavi et al., 2013) (**Fig. 12 c**). Finally, in the mature brain, overall synaptic efficacy, spine density and spine size remain constant or suffer a slight reduction over time (Holtmaat et al., 2005; Loewenstein et al., 2011) (**Fig. 12 a**). This is thought to be mainly due to an increase of individual synapses GluN2A to GluN2B ratio (**Fig. 12 d**). Synaptic structures can nevertheless be quite dynamic while an individual learns, adapts his/her behaviour and/or creates new memories.

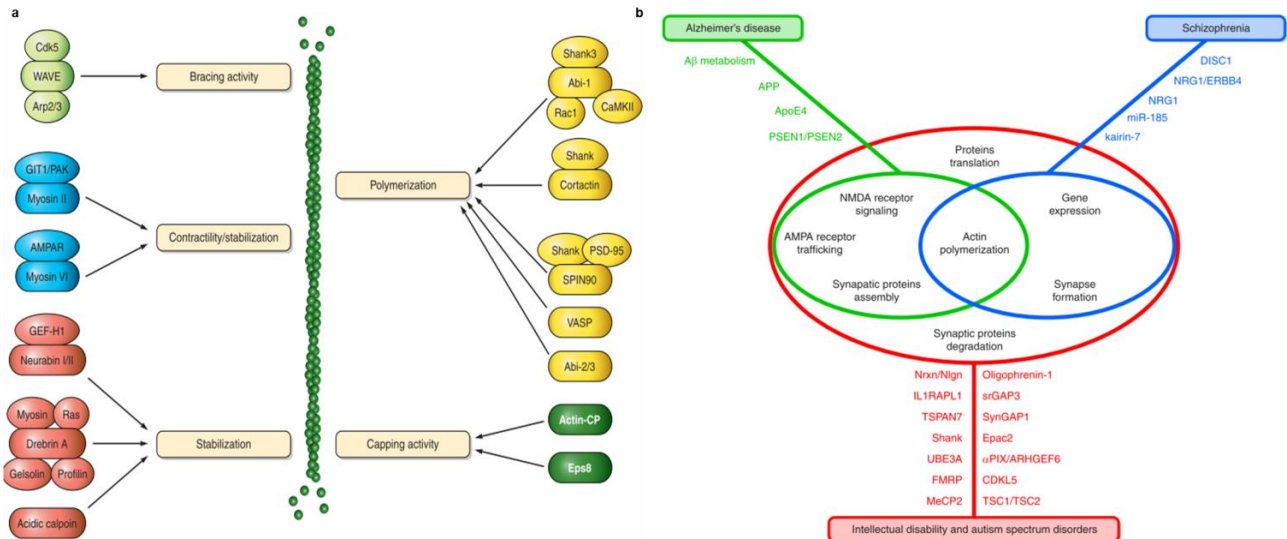


**Figure 12: Spine formation and stabilization during the cellular, network and behavioural development of the brain.** (a) Postnatal development of synapses throughout time. Following the initial induction of a synapse by a filopodium, atypical or “protosynapses” appears, but their ultimate fate is unclear. Filopodia retract and the so called “synapse” can be stabilized at the dendrite shaft level. Synaptic protrusion can then overgrow and give place to a mature spine. (b-f) All these events occur during the cellular, network and behavioural development of the brain (b). Such structural plasticity is believed to be closely followed by changes in the dynamics of key postsynaptic molecules, such as the ionotropic glutamate AMPAR (c) and NMDA (d) receptors, as well as kinases (e) and phosphatases (f). Curves represent the maximal density or expression level percentage (see main text). Taken from Lohmann & Kessels, 2014.

### 1.5.5 Actin cytoskeleton: the missing link between structural and functional plasticity?

Actin is one of the major cytoskeleton proteins whose presence in the neurons is long known (Kelly & Cotman, 1978; Fifkova & Delay, 1982; Drenckhahn & Kaiser, 1983; Kaech et al., 1997). Moreover, actin is highly concentrated in postsynaptic dendritic spines (Matus et al., 1982; Cohen et al., 1985; Fifkova, 1985). Within spines, actin is present as a soluble pool of monomeric G-actin and as polymerized F-actin filaments. The transition between actin polymerization and depolymerisation is responsible for the highly dynamic actin organization in dendritic spines, which is subject to a tight regulation by several actin-binding proteins (Fischer et al., 2000; Matus et al., 2000; Smart & Halpain, 2000; Star et al., 2002) (**Fig. 13 a**). The first functional link between postsynaptic actin filaments and synaptic plasticity dates from 1991, when Pavlik & Moshkov saw for the first time actin bundles formation following a tetanus-induced LTP. Soon after, F-actin

dynamics inhibition was correlated to a decrease in the total number of NMDAR and AMPAR clusters (Allison et al., 1998). Ever since, actin polymerization and depolymerization were repeatedly associated with synaptic plasticity events; whereas disturbing actin dynamics was shown to impact AMPAR-mediated synaptic plasticity and memory formation as well as to be involved in neurological disease ethiopathology (reviewed by Hering & Sheng, 2001; Ethell & Pasquale, 2005; Bellot et al., 2014; Sala & Segal, 2014) (**Fig. 13 b**). In the next sections we will review actin role in dendritic spine development and maintenance as well as on clathrin-mediated endocytosis, two major events linking structural to functional synaptic plasticity.



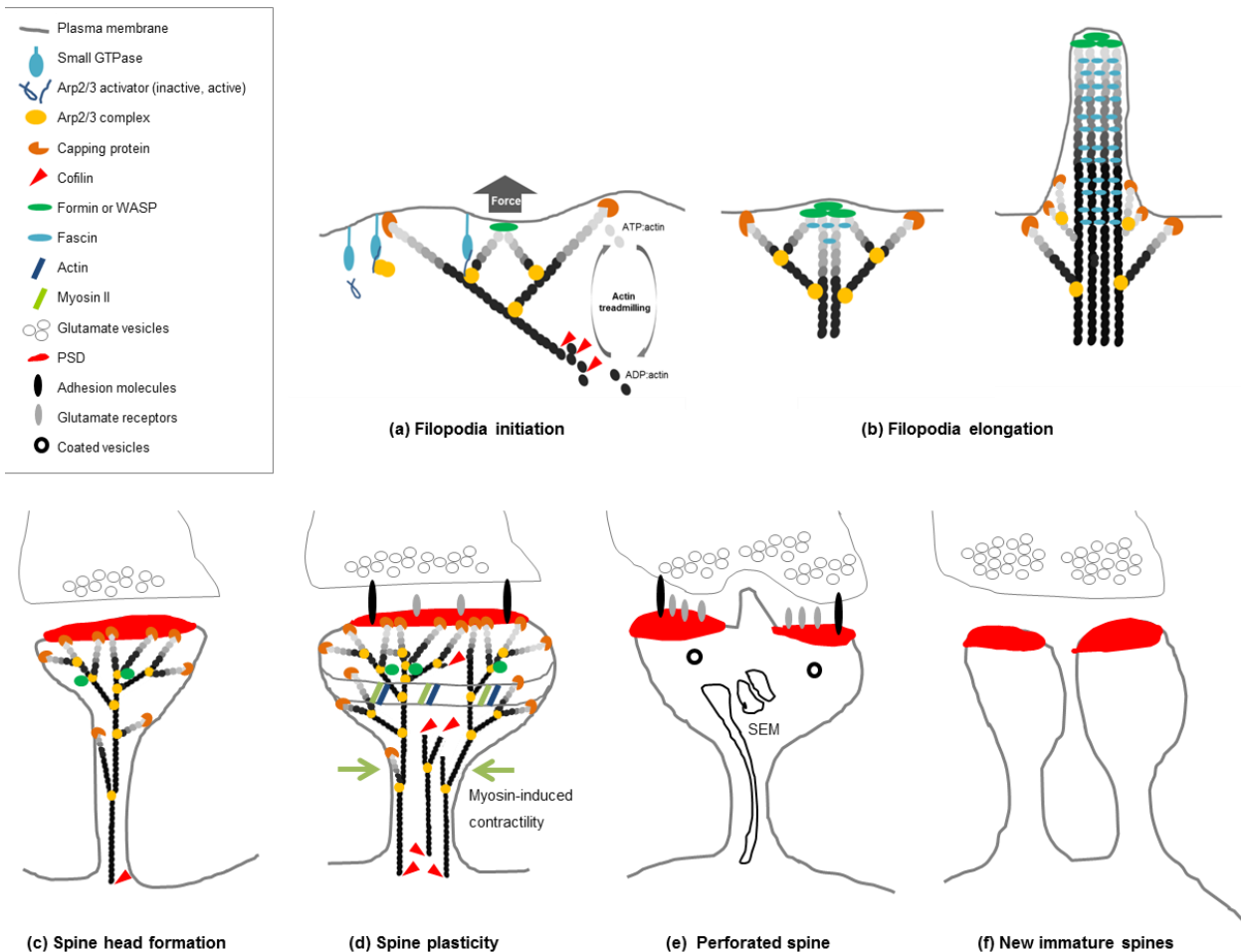
**Figure 13: Regulation of actin polymerization in health and disease.** (a) Actin dynamics is tightly regulated by a wide range of actin-binding proteins. The Arp2/3 complex, Cdk5 and WAVE promote bracing activity (light green), whereas contractibility and/or stability are mediated by GIT/PAK, myosin II and AMPAR, for example (blue). Neurabin I/II, myosin, Ras and drebrin A, among others (red), also stabilize actin filaments. Instead, polymerization is regulated by Shank, PSD-95, Abi1-3, Rac1, CaMKII, cortactin, or VASP (yellow). At last, capping activity is endured by Actin-CP and Eps8 (dark green). (b) Actin-dependent spine morphology deregulation is a central molecular mechanism underlying neurological diseases such as Alzheimer's disease (in green), schizophrenia (in blue) or intellectual disability and autism spectrum disorders (in red). For more details see Sala & Segal, 2014.

### 1.5.5.1 Role of actin cytoskeleton in dendritic spine development and maintenance

Spines are characterized by a high degree of morphological plasticity (reviewed by Edwards, 1995; Muller, 1997; Harris, 1999; Segal et al., 2000; Luscher et al., 2000) – **Fig. 14**. During the early stages of synaptogenesis, filopodia are formed at the dendritic shaft level, often from a small collection of branched actin or lamellipodia (Korobova & Svitkina, 2010) (**Fig. 14 a**). These structures, precursors of dendritic spines, are highly motile, being able to rapidly protrude and retract until they find a pre-synaptic partner (Ziv & Smith, 1996). Once that happens, glutamate release by the pre-synaptic terminal is believed to induce filopodia enlogation, which is NMDA-sensitive and  $\text{Ca}^{2+}$ -dependent (Maletic-Savatic et al., 1999) (**Fig. 14 b**). Fascin binds filaments generated by the actin nucleator Arp2/3 complex to initiate the filament lengthening process, whereas Formin or WASP might participate in the polymerization of actin filaments. After enlogation, filopodia might be stabilized and exhibit a PSD (Prange & Murphy, 2001; Marrs et al., 2001). Recently formed spines are characterized by a thin and long neck with small heads, being its presence associated with high frequency stimulation, pre-synaptic glutamate release or spillover (Engert & Bonhoeffer, 1999;



Know & Sabatini, 2011) (**Fig. 14 c**). Whereas spine neck is thought to be mainly regulated by myosin II; spine head growth occurs essentially through the Arp2/3 complex (Mullins et al., 1998; Welch et al., 1998; Klein, 2009). Arp2/3 branching activity is in turn tightly regulated by Arp2/3 activators, such as cortactin, WAVE or WASP, as well as by inhibitors like cofilin or PICK1 (Hering & Sheng, 2003; Kim et al., 2006). Most of the mature synapses display a single, continuous (simple) PSD per spine, which hosts glutamate receptors and adhesion proteins among other scaffold and signalling proteins crucial to synaptic plasticity events (**Fig. 14 d**). Several electron microscopy (EM) studies suggest that PSD size and spine stability are positively correlated (Harris & Stevens, 1989; Takumi et al, 1999; Holtmaat et al, 2005; Knott et al., 2006), a theory later confirmed by electrophysiology and imaging studies both *in vitro* and *in vivo* (Matsuzaki et al, 2001; Zito et al, 2004; Cane et al., 2014). On the other hand, early EM studies showed that some PSDs can be discontinuous or perforated, exhibiting completely adjacent PSD segments, which are able to invaginate distinct pre-synaptic axon terminals (Geinisman, 1993) (**Fig. 14 e**). Perforated PSDs display larger spine heads and PSD area, a higher proportion of SEM, spine apparatus and coated vesicles as well as an excessive number of surface AMPA receptors, which can contribute to changes in synaptic efficacy (Spacek & Harris, 1997; Geinisman et al, 1993, 1996; Toni et al, 2001; Ganeshina et al, 2004). In fact, perforated

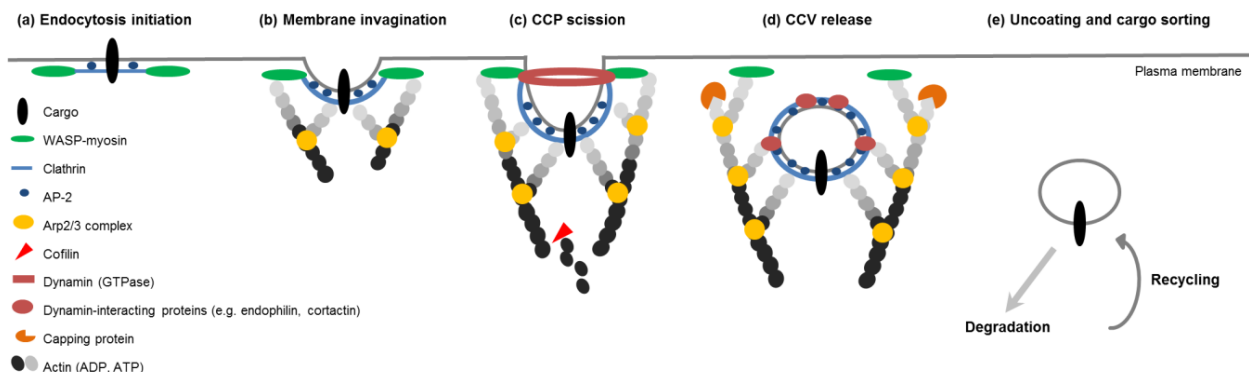


**Figure 14: Actin dynamics during dendritic spine development and maintenance** (see text). Adapted from Yang & Svitkina, 2011b and Soria Fregozo & Perez Vega, 2014.

synapses are believed to be exceptionally efficacious, since this type of spatial arrangement may limit glutamate spillover to adjacent PSD segments and/or prevent glutamate receptors desensitization. Furthermore, as the proportion of perforated spines rises during development and under increased neuronal activity events (Greenough et al., 1978 Nietro-Sampedro, 1982; Carlin & Siekievitz, 1983; Geinisman et al., 1991,1992; Buchs & Muller, 1996; Weeks et al, 1999; Neuhoﬀ et al, 1999), these synapses are thought to precede the formation of newly immature spines (**Fig. 14 f**).

### 1.5.5.2 Role of actin cytoskeleton on clathrin-mediated endocytosis

Another important factor during synaptic plasticity events is the surface turnover of glutamate receptors, which can dictate synaptic efficiency and consequently lead to morphological changes. Actin dynamics has been considered for long as a crucial factor to drive endocytosis in several cell types (reviewed by Engqvist-Goldstein & Drubin, 2003; Merrifield, 2004; Kaksonen et al., 2006). In mammalian cells, the clathrin-dependent endocytosis constitutes the major pathway for internalization of lipids and proteins from the plasma membrane, including glutamate receptors such as the AMPARs (Carrol et al., 1999; Beattie et al., 2000; Ehlers 2000; Lin et al., 2000; Man et al., 2000; Wang & Linden, 2000) - **Fig. 15**. Moreover, several EM studies have shown the existence of clathrin-coated pits, vesicles and proteins mediating endocytosis within dendritic spines (Spacek & Harris, 1997; Cooney et al., 2002; Petralia et al., 2003; Racz et al., 2004). Nowadays, it is believed that there is an endocytic zone segregated from the PSD within dendritic spines, where synaptic proteins can be internalized and recycled, maintaining an available pool of mobile AMPAR required for tuning synaptic transmission (Blanpied et al., 2002; Petrini et al., 2009; Kennedy et al., 2010). Interestingly, clathrin-mediated endocytosis machinery (**Fig. 15**) recruits actin cytoskeleton elements previously implicated in spine development and maintenance (**Fig. 14**), such as actin itself, capping proteins as well as the Arp2/3 complex machinery. Such remark strongly suggests that the regulation of actin cytoskeleton dynamics is crucial for structural and functional plasticity events.



**Figure 15: Clathrin-mediated endocytosis.** (a) Clathrin-mediated endocytosis is initiated by the recruitment of AP-2 to the plasma membrane, thus promoting clathrin assembly. (b) Once formed, clathrin lattices can be invaginated, giving rise to clathrin-coated pits (CCP). (c) Those pits can be further invaginated, forcing the plasma membrane to be pinched off through large GTPases like dynamin. Dynamin is composed by a GTPase domain, a phospholipid-binding pleckstrin-homology domain, a GTPase effector domain and a proline-rich domain (PRD). The PRD domain allows dynamin to interact with several SH3-containing domains like endophilin, Abp1 or cortactin which in turn can directly or indirectly bind the actin cytoskeleton. (d) Following the membrane pinch off, the now clathrin-coated vesicles (CCVs) can move away from the plasma membrane and (e) outbuild their coat and be properly sorted into recycling vesicles or follow the degradation pathway.

### I.5.6 Scrib1 role in synaptic plasticity and brain function

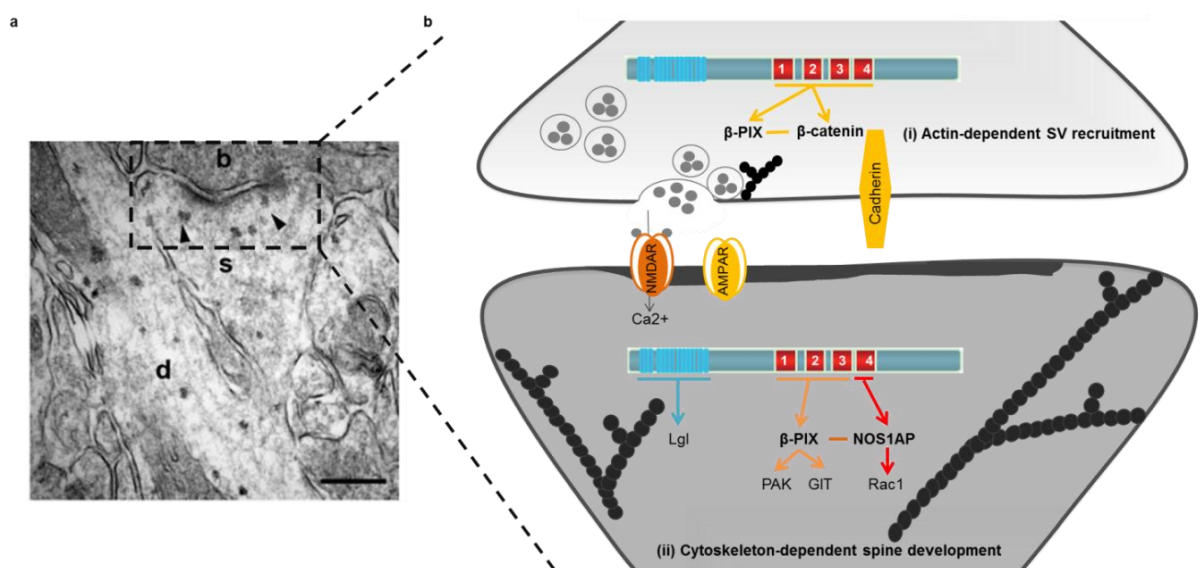
The starting point to unravel Scrib role in the regulation of synaptic plasticity were the same genetic null mutants that featured Scrib1 as a cell polarity determinant and tumour suppression in *Drosophila* (Bilder & Perimon, 2000). Looking at the NMJ of flies, where Scrib had previously been localized (Mathew et al., 2002), Budnik and collaborators (Roche et al., 2002) observed an abnormal thick basal lamina in Scrib mutant spines containing a higher number of synaptic vesicles but less active zones. Such ultrastructural defects translated into a loss of facilitation and post-tetanic potentiation, as well as a faster synaptic depression. These results underscore Scrib as a critical regulator of short-term synaptic plasticity by promoting the recruitment and recycling of synaptic vesicles at their release sites in *Drosophila*. As interestingly rose by the authors, Scrib and Dlg levels display opposite effects at synapses, unlike the functional parallelism seen as apico-basal determinants, suggesting that the PCP signalling pathway in the synaptic context might be ruled differently. Nevertheless, the molecular mechanisms underlying Scrib1 role in mammalian synaptic plasticity started to be unravelled only recently - **Table 4; Fig: 16**. Scrib1 was initially implicated in the coordination of actin-mediated recruitment of synaptic vesicles together with the  $\beta$ PIX/ $\beta$ -catenin/cadherin complex. At first,  $\beta$ -catenin affords a PDZ-dependent recruitment of Scrib1 to the synapse (Sun et al., 2009). Once there, Scrib can bridge the  $\beta$ -catenin/cadherin complex to the Rac/Cdc42 guanine nucleotide exchange factor (GEF)  $\beta$ PIX signalling (Sun & Bamji, 2011), allowing the proper localization of synaptic vesicles at their release site. Scrib1 was then shown to associate the neuronal nitric oxide synthase (nNOS) adaptor protein NOS1AP (or CAPON) both in pre-and post-synapses (Richier et al., 2010; Wang et al., 2014). This association is specifically mediated by the fourth PDZ domain of Scrib1 and the phosphotyrosine-binding (PTB) domain of NOS1AP, allowing Scrib1 to bridge NOS1AP signalling to the  $\beta$ PIX/PAK/Git1 complex (Richier et al., 2010). The PTB domain of NOS1AP alone is able to afford dendritic spine development through Rac activation, suggesting that Scrib1 can regulate actin dynamics by recruiting NOS1AP to the synapse. Interestingly, the contribution of Scrib liaisons with  $\beta$ -catenin,  $\beta$ PIX and NOS1AP to PCP establishment (**Chapter I.3**) and tumour homeostasis maintenance (**Chapter I.3.3**) has been long been established in several cell types and tissues, indicating that this PCP signalling pathway was favoured throughout evolution. Scrib1 role in brain function was first asserted by our group using heterozygote Scrib1<sup>crc/+</sup> mice mutants, which display anatomical and functional deficits in the hippocampal region (Moreau et al., 2010). As aforementioned, homozygote Scrib1<sup>crc/crc</sup> mice die at birth (Murdoch et al., 2003; **Chapter I.4.2**), whereas Scrib1<sup>crc/+</sup> mice, expressing 50%

**Table 4: PDZ-dependent interactions of Scrib1 in glutamatergic synapses.**

Protein	PDZbd	Localization	Function	Ref.
$\beta$ -catenin	-DTDL	Pre-synapse	PDZ-dependent recruitment of Scrib1 to the pre-synapse	[1]
$\beta$ PIX	-ETNL	Pre- and post-synapse	PDZ-dependent macro-complex between Scrib1, $\beta$ -catenin/cadherin and $\beta$ PIX signalling modulates actin-dependent synaptic recruitment; PDZ123-dependent Scrib1 interaction with $\beta$ PIX/PAK/GIT1 implicated in cytoskeleton-driven spine morphology.	[2,3]
NOS1AP	-EIAV	Pre- and post-synapse	PTB-PDZ3 dependent interaction involved in Rac1-induced dendritic spine development	[4]

1. Sun et al., 2009; 2. Sun & Bamji, 2011; 3. Moreau et al., 2010; 4. Richier et al., 2010

of full length protein and 50% of a truncated form missing the last two PDZ domains in the brain, are viable (Moreau et al., 2010). *Scrib1<sup>crc/+</sup>* mutants contain lower protein levels of  $\beta$ PIX, GIT and CaMKII, but a two-fold increase in the activated form of Rac1, impairing activity-dependent actin polymerization. In the hippocampus, *Scrib1<sup>crc/+</sup>* mutants show an increase of synaptic pruning, displaying less but bigger spines. Functionally, the resulting altered basal pyramidal neuronal morphology was correlated with a reduced basal synaptic transmission and impaired LTP, suggesting that Scrib1 controls the number of functional synapses in the CA1 region. At the cognitive level, *Scrib1<sup>crc/+</sup>* mutants exhibit enhanced learning and memory abilities and impaired social behaviour, commonly linked to ASD. Remarkably, a genetic and an exonic *de novo* *SCRIB1* mutation were recently implicated in human autism (Pinto et al., 2010; Neale et al., 2012), strongly suggesting Scrib1 as a potential actor in ASD etiopathology.

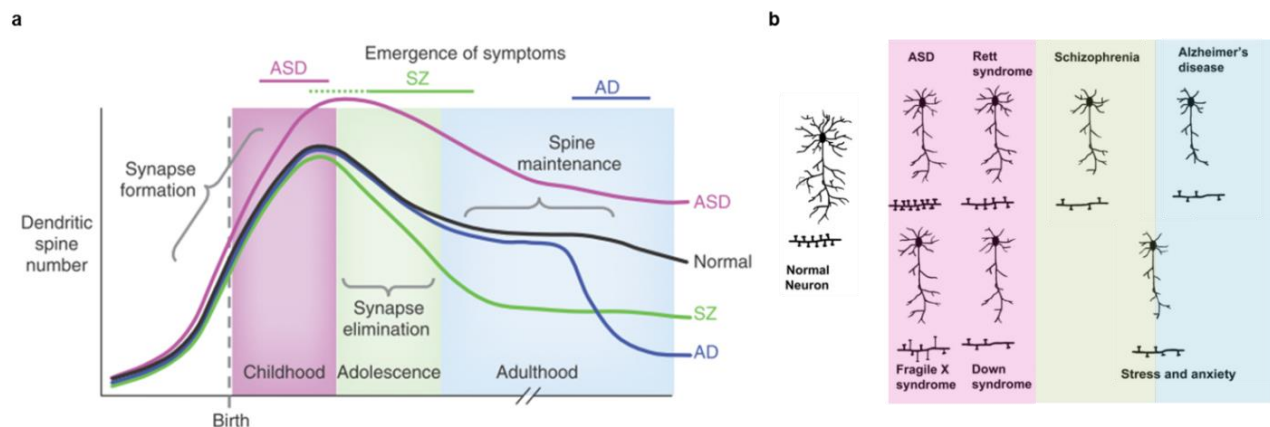


**Figure 16: Role of Scrib1 interactions in glutamatergic synapses.** (a) The PCP protein Scrib1 is present in glutamatergic synapses. EM labeling of Scrib1 in the stratum radiatum of CA1. Note the presence of labelling in the PSD of dendritic spines (s, arrowheads), and within dendrites (d). Scale, 260nm. (b) Scrib1 interactions at pre- and post-synaptic compartments. At the pre-synapse, Scrib1 regulates the actin-dependent synaptic vesicle (SV) recruitment by associating with the  $\beta$ PIX/ $\beta$ -catenin/cadherin complex in a PDZ-dependent manner (i). At the post-synapse, Scrib1 is implicated in cytoskeleton-dependent spine development events, coupling NOS1AP/Rac1 signalling via its PDZ4 to the  $\beta$ PIX/PAK/GIT complex (iii). (a) taken from Moreau et al., 2010.

### I.5.7 Autism Spectrum Disorders

ASD cover classical idiopathic autism, Asperger's syndrome, Rett's syndrome as well as other associated genetic disorders, such as schizophrenia, Down syndrome, Fragile X, mental retardation and tuberous sclerosis (Lord et al., 2000; Pardo & Eberhart, 2007). ASD typically develop before 2-3 years of age, affecting the late phase of brain development, when synaptic formation and maturation is taking place (Courchesne et al., 2007; Schmitz & Rezaie, 2008) (**Fig. 17 a**). Thus, akin to other cognitive disorders, ASD are characterized by dendritic dysmorphologies (**Fig.17 b**), which will affect brain function. ASD commonly feature sociability difficulties, language impairments, a constrained pattern of interests, and/or stereotypic and repetitive behaviours. Others, like epilepsy, learning disabilities or conversely specialized abilities for music or mathematics, for example, can also be observed. About 80% of ASD cases are heritable, strengthening the genetic com-

ponent of such disease. The best characterized mutations in human genes patients occur in genes encoding the cell adhesion complex made of the pre-synaptic neuroxin Nrnx1 (Feng et al., 2006; Szatmari et al., 2007; Kim et al., 2008; Yan et al., 2008; Zahir et al., 2008), and the post-synaptic neuroligin Nlg1, Nlg3 and/or Nlg4 (Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005; Talebizadeh et al., 2006; Tabuchi et al., 2007; Blundell et al., 2010). Moreover, so were exonic deletions, or copy number variations (CNVs), of the *NLG4* locus detected in autistic patients (Chocholska et al., 2006; Macarov et al., 2007; Marshall, 2008; Glessner et al., 2009). Remarkably, linkage studies have correlated mutations in *NRXs* and *NLGs* to Tourette's syndrome, learning disability, schizophrenia and even to addiction (Hishimoto et al., 2007; Lachman et al., 2007; Kirov et al., 2008; Walsh et al., 2008; Lawson-Yuen et al., 2008), pinpointing the couple *Nrx/Nlg* as a hotspot for human cognitive diseases. Conversely, so were multiple mutations and CNVs in autistic patients detected in the gene encoding the intracellular scaffold Shank3 protein (Wilson et al., 2003; Manning et al., 2004; Jeffries et al., 2005; Durand et al., 2007; Moessner et al., 2007; Okamoto et al., 2007a; Marshall, 2008; Waga et al., 2011). Although Shank3 is known to directly interact with Latrophilins - calcium-independent alpha-latrotoxin receptors (CIRLs) similar to *Nrx* (Tobaben et al., 2000) and to indirectly bind *Nlg* through other scaffold proteins (Sheng & Hoogenraad, 2007), the molecular mechanisms underlying ASD are still unclear.



**Figure 17: Formation and maintenance of dendrites in ASD.** (a) Putative dendritic spine number lifetime in ASD (pink), schizophrenia (SZ, green) or Alzheimer's disease (AD, blue) compared to normal subjects (black). Top bars represent the symptomatic period. In ASD, there is an excess of spine number early in childhood, whereas an exaggerated spine pruning occurs during late childhood or adolescence in SZ. Finally, AD is characterized by a rapid spine loss in late adulthood. (b) Representation of dendritic dysmorphologies typically observed in ASD, Rett syndrome, Fragile X syndrome, Down syndrome, schizophrenia, Alzheimer's disease or stress and anxiety. Color code represents the same period of emergence of symptoms as (a). (a) taken from Penzes et al., 2011; (b) adapted from Kulkarni & Firestein, 2012.

### 1.5.7.1 Molecular determinants of ASD ethiopathology

In the past few years, a crescent number of studies have been linking clinical to *in vitro* studies, soughing to explain how the trans-synaptic cell adhesion complex could trigger pre- and post-synaptic signal transduction events that potentially shape synaptic plasticity. Crystal structures provided the first insights on how *Nrx1/Nlg1* complex are formed, by showing that the extracellular LNS (laminin, *Nrx*, sex-hormone-binding globulin) domains of *Nrx1* attach in a  $\text{Ca}^{2+}$ -dependent manner to the lateral sides of *Nlg* extracellular esterase-homology domains (Araç et al., 2007; Fabrichny et al., 2007; Chen et al., 2008). On the other hand, the

intracellular Cter domains of Nr<sub>x</sub> and Nl<sub>g</sub>, composed of distinct PDZbd, are responsible for the polarized targeting and specific interactions at the synapse - **Table 5, Fig. 18**. At the pre-synaptic compartment, class II PDZbd-containing Nr<sub>x</sub>s bind multidomain scaffold proteins like the calcium/calmodulin-dependent serine protein kinase CASK (Hata et al., 1996). CASK is known to impair synaptic function, being its deletion lethal in mice (Atasoy et al., 2007). It takes part in the macro-complex CASK/VELI/MINT (Butz et al., 1998; Borg et al., 1999), interacting with actin nucleators such as 4.1 protein and transmembrane proteins like the contactin-associated proteins Cntnaps (or Casprs) (Biederer & Südhof, 2001). Interestingly, rare mutations in *CNTNAP2* and *CNTNAP5* genes have been recently linked to ASD and ASD-RD (Strauss et al., 2006; Zweier et al., 2009; Pagnamenta et al., 2010; Anderson et al., 2012; Prasad et al., 2012; Vaags et al., 2012; Rodenas-Cuadrado et al., 2013). Caspr proteins belong to the superfamily of Nr<sub>x</sub> composed of several extracellular domains and an intracellular Cter bearing Nr<sub>x</sub>-like class II PDZbd. So far the exact molecular interactions that Caspr elements are engaged to are not well known. Nevertheless, a similar PDZbd was also found in CADM1 (RA175/SynCAM1), suggesting that Nr<sub>x</sub>, Casprs and CADM1 might play similar roles in brain function. CADM1 is an ASD-related immunoglobulin adhesion molecule able to control synapse number and impact LTD-like synaptic plasticity as well as hippocampal-dependent learning (Robbins et al., 2010). In the cerebellum, CADM1 can interact with the multiple PDZ domain protein MUPP (PDZ1-5), which in turn anchors anchor gamma-aminobutyric acid type B receptors (GABBR2; PDZ13) at the synapse (Fujita et al., 2012). At the post-synaptic level, PDZ-dependent interactions rule Nl<sub>g</sub> partnerships as well. The rapid accumulation of Nl<sub>g</sub>1 after a given axo-dendritic contact was shown to induce the recruitment of NMDAR to the synapse, followed by an independent accumulation of PSD-95 (Barrow et al., 2009). PSD-95 over gephyrin recruitment was later described as being dependent on Nl<sub>g</sub>1 Y783 phosphorylation (Giannone et al., 2013). PSD-95-related signal transduction was recently implicated in ASD and Angelman syndrome (Tsai et al., 2012; Cao et al., 2013). Moreover, as a scaffold protein, PSD-95 and related proteins like SAP-102 can interact and form macro-complexes with several other proteins, like the Shank family (Sheng & Hoogenraad 2007). Shank1-3 can directly bind GKAP and Homer, potentially bridging NMDAR/PSD-95/GKAP to the mGluR/Homer complex at the synaptic level (Naisbitt et al., 1999; Tu et al., 1999; Bertaso et al., 2010; Jiang & Ehlers, 2013). Shank1-deficient mice exhibit smaller dendritic spines, decreased basal synaptic transmission but enhanced spatial learning (Hung et al., 2008). Recent work in our lab showed that ASD-related mutations of Shank3 strongly affect synaptic development, morphology and function, impacting growth cone motility and synaptic transmission in mature neurons (Durand et al., 2012). Rare missense variants or mutations correlated with ASD were also found in genes encoding other PDZ-domains containing proteins like the glutamate receptor interacting protein GRIP1 (Meijas et al., 2011) or the LRR-containing cell adhesion protein SALM5 (Xu et al., 2009; de Bruijn et al., 2010; Mitchell, 2010). While GRIP1 is known to impact GluA2/3-containing AMPARs recycling and surface distribution in neurons, SALM5 appears to affect neurite outgrowth and synapse formation. Strikingly, the loss of the LAP member Densin-180, containing both LRR and PDZ domains, was recently correlated with autism and schizophrenia endophenotypes (Carlisle et al., 2011). Densin loss leads to mGluR5 and DISC1 mislocalization in the PSD, impacting mGluR- and NMDAR-

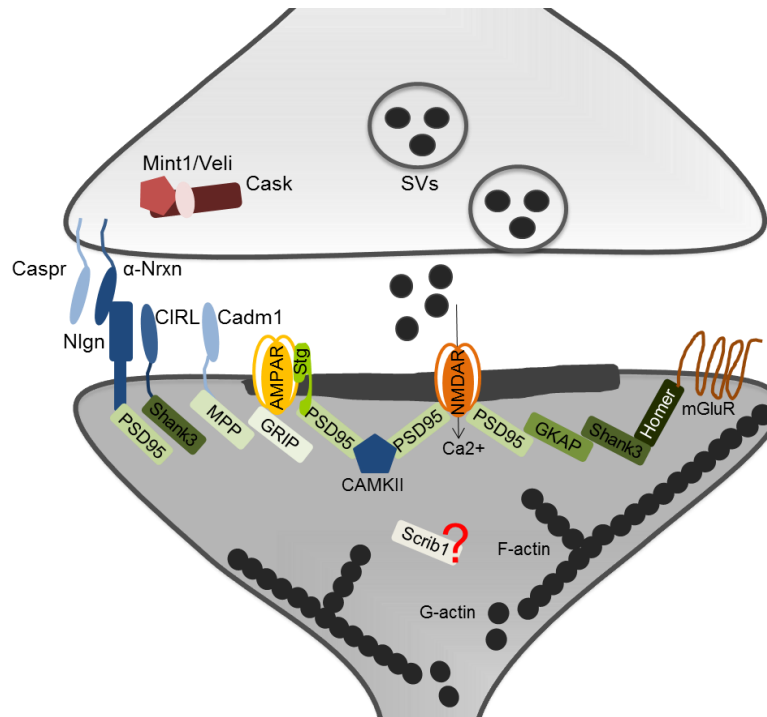
dependent LTD as well as spine morphology. Such results resemble *Scrib<sup>crc/+</sup>* mutant phenotype translated into a functional similarity. Moreover, the implication of well-established binders of Scrib1 in ASD – like the key pre- and post-synaptic coordinator APC (Raedle et al., 2001; Zhou et al., 2007; Rosenberg et al., 2010) and NOS1AP (Delorme et al., 2010), further strengthen Scrib1 potential role as a spine molecular determinant involved in ASD.

**Table 5: PDZ related proteins implicated in autism-spectrum disorders (ASD).**

Protein	Clinical studies	<i>In vitro</i> studies	<i>In vitro</i> model	Ref.
<b>PDZ-domain containing proteins</b>				
Densin-180	Schizophrenia and ASD-like endophenotype	↓ α-actinin; ↓ mGluR5 and DISC1 localization in the PSD; impairment of mGluR- and NMDAR-dependent LTD; change in spine morphology	Mouse forebrain, inc. hippocampus	[1]
GRIP	Autism	GRIP variants (PDZ4-6) associated with altered interactions with GluA2/3; faster AMPARs recycling and ↑ surface distribution	Dissociated hippocampal neurons	[2]
PSD-95 (Dlg4)	ASD and Angelman syndrome	PSD-95 and NMDAR recruitment following NLGN1 accumulation; involved in proteasomal degradation of multiple autism-linked genes; implicated in TrkB-dependent induction and maintenance of LTP	Mouse hippocampus	[3-5]
Shank1-3	Autism, intellectual disability, language disabilities and/or social communication disorders and schizophrenia	Altered PSD protein composition; altered growth cone motility; actin-dependent modification of dendritic spine development and morphology; ↓ basal synaptic transmission; ↑ anxiety and impaired contextual fear memory; ↑ performance in a spatial learning task impaired long-term memory retention; social communication deficits	Mouse hippocampus	[6-14]
<b>Cter PDZ-binding domain containing proteins</b>				
APC -VTSV	ASD	↓ postsynaptic accumulation of S-SCAM and NLG synaptic clusters; ↓ in NRX and active zone proteins; presynaptic terminals are less mature structural and functionally	Chicken ciliary ganglion neurons an brain	[15-17]
CADM1 (or SynCAM1) -EYFI	ASD	↓ excitatory synapses; regulates LTD and spatial learning; destabilization leads to an ↑ GABBR2; ↓ cerebella with ↓ number of synapse of Purkinje cells; impaired ultrasonic vocalization	Mouse hippocampus and cerebellum	[18,19]
Caspr2 -EWLI	ASD, schizophrenia , intellectual disability, dyslexia, ADHD, developmental delay, and language impairment	Ectopic neurons in subcortical white matter (corpus callosum) and mislocalization of neurons within the six-layer cortex; ↓ number of GABAergic interneurons; ↓ of synchronicity of neuronal firing; mice display ASD-like behaviour	Cortex, hippocampus and striatum of cKO mice	[20]
Caspr5 -EYFI	Autism and dyslexia	-	-	[21]
NOS1AP -EIAV	ASD, schizophrenia, and OCD	-	-	[22]
Nrx/Nlg -EYVV/-TTRV	ASD, Tourette's syndrome, learning disability and/or schizophrenia	Nlg1 regulates excitatory and inhibitory scaffold assembly during synapse formation and stabilization; Nlg3 gain-of-function leads to moderate social interactions impairment, ↑ spatial learning capability and ↑ inhibitory synaptic transmission; Nlg4 loss-of-function leads to selective deficits in reciprocal social interactions and communication; impaired LTD and associative fear memory	Mouse hippocampal cultures, somatosensory cortex or amygdala	[23-29]
SALM5 -LESI	Severe progressive Autism, mental retardation and familial schizophrenia	-	-	[30,31]

1. Carlisle et al., 2011; 2. Meijas et al., 2011; 3. Tsai et al., 2012a; 4. Cao et al., 2013; 5. Barrow et al., 2009; 6. Durand et al., 2007; 7. Moessner et al., 2007; 8. Gauthier et al., 2010; 9. Hung et al., 2008; 10. Wöhr et al., 2011; 11. Durand et al., 2012; 12. Lennertz et al., 2012; 13. Sato et al., 2012; 14. Chilian et al., 2013; 15. Raedle et al., 2001; 16. Zhou et al., 2007; 17. Rosenberg et al., 2010; 18. Robbins et al., 2010; 19. Fujita et al., 2012; 20. Penagarikano et al., 2011; 21. Pagnamenta et al., 2010; 22. Delorme et al., 2010; 23. Giannone et al., 2013; 24. Tabuchi et al., 2007; 25. Jamain et al., 2008; 26. Südhof, 2008; 27. Glessner et al., 2009; 28. Kim et al., 2009; 29. Blundell et al., 2010; 30. Xu et al., 2009; 31. de Bruijn et al., 2010.





**Figure 18: Molecular determinants of ASD.** The formation of a NRXN/NLGN-like trans-synaptic macro-complex drives the recruitment of asymmetrically distributed synaptic adaptor proteins to the each side of the synaptic junction. In turn, these scaffold proteins coordinate pre- and post-synaptic signal transduction pathways capable of modeling synaptic architecture, function and wiring. As a consequence, small changes in any of the trans-synaptic macrocomplex localization and /or function lead to massive changes in the neural network and consequently to brain dysfunction.

### 1.5.7.2 Cell Polarity proteins as novel ASD molecular determinants

In the past two decades, an increasing amount of studies have linked cell polarity proteins to ASD – **Table 6**. The first report correlating autism-like features and failure in PCP establishment was provided by Wynshaw-Boris and collaborators (Lijam et al., 1997) describing abnormal social behavior and sensorimotor gating deficits in mice lacking Dvl1, one of the three homologs of *Drosophila* Dsh (**Chapter I.4.1**). Subsequent studies revealed that disruption of Dvl genes can be implicated in several developmental defects (Wynshaw-Boris, 2012) as well as in 1p36 deletion (also known as monosomy 1p36), one of the most common terminal deletions observed in humans characterized by special facial features, mental retardation, heart defects, development delay and epilepsy (Zhu et al., 2013). Moreover, two distinct variants of Pk2, another core PCP gene, were recently found in ASD patients (Sowers et al., 2013). In mouse hippocampal neurons, Pk2 disruption leads to morphological and functional abnormalities. Likewise, apico-basal polarity determinants like Scrib1 were also implicated in ASD etiopathology (**Chapter I.5.4.2**). Mutations in Dlg3 and Dlg4 genes were correlated to ASD like non-syndromic X-linked mental retardation and Angelman syndrome (Tarpey et al., 2004; Barrow et al., 2009; Gilman et al., 2011; Kantojärvi et al., 2011; Tsai et al., 2012a; Cao et al., 2013). The resulting proteins – SAP-102 and PSD-95, respectively, are MAGUKs involved in NMDAR targeting and coordination of several signalling pathways relevant for synaptic plasticity and proper brain function. Deletion of the tumour suppressor phosphatase with tensin homology PTEN, key negative regulator of the PI3K pathway, was also found to cause defects in synaptic architecture, transmission and plasticity (Fraser et al.,



2008; **Chapter I.3.1**) Similar phenotypes were associated to other cell polarity proteins like the key polarity determinant Par-1 protein kinase MARK1 (Maussion et al., 2008) or the tuberous sclerosis protein TSC2 (Choi et al., 2008). Finally, PCP effector genes like Nhs (Burdon et al., 2003; **Chapter I.3.2**) or the ERK1/2 pathway (Fernandez et al., 2010; Levitt & Campbell., 2009) were lately implicated in ASD and related deletion syndromes. Recent work in our lab suggests that a misregulation of the ERK pathway in the hippocampus of *Scrib1<sup>crc</sup>* mutant mice might underlie the observed ASD-like behaviour (Moreau et al., in preparation) (**Chapter I.5.4.2**). Remarkably, the systemic administration of an ERK phosphorylation blocker was able to reverse the social deficits in these mice, identifying the *scrib* gene as a potential novel therapeutic target for ASD-like behaviour.

**Table 6: Cell polarity proteins implicated in autism-spectrum disorders (ASD).**

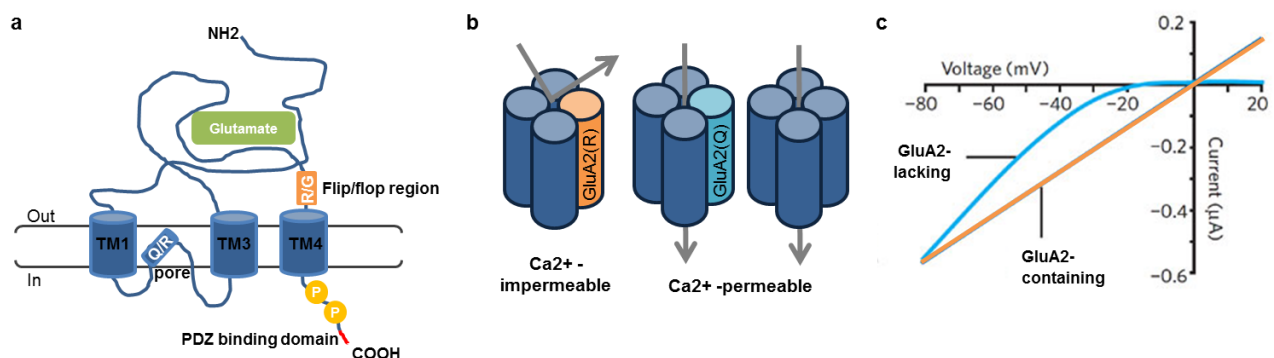
Protein	Clinical studies	<i>In vitro</i> studies	<i>In vitro</i> model	Ref.
<b>Apico-basal polarity determinants (-Cter PDZ binding domain)</b>				
Dlg1-4 (MAGUKs)	ASD, Angelman syndrome, non-syndromic X-linked mental retardation	Disruption of NMDAR targeting and signalling; impairment of TrkB signalling; disturbed proteasomal degradation	Mouse hippocampus	[1-6]
Scrib1	ASD	↓ βPIX, PAK, GIT, CaMKII; ↑ Rac1-GTP; ↑ c-Fos activity; ↑ ERK1/2 phosphorylation; impaired activity-dependent actin polymerization; ↑ synaptic pruning; altered basal neuronal morphology; ↓ basal synaptic transmission; impaired LTP; enhanced learning and memory; impaired social behaviour	Mouse hippocampal neurons and slices	[7-10]
PTEN -ITKV	Autism, chromosome 10 deficiency, Lhermitte-Duclos disease, seizures, ataxia, diabetes, and cancer predisposition	↑ soma size; ↑ neuronal projections calibre; ↑ dendritic spine density; ↑ synaptic structures; myelination defects in the corpus callosum; ↓ synaptic transmission and synaptic plasticity impairment	Mouse cerebellum, cortex, and hippocampus	[11]
<b>PCP genes</b>				
Dvl1-2	ASD, 1p36 deletion syndrome and developmental defects	Defective development, abnormal social behaviour and sensorimotor gating	Mouse	[3,12-14]
Pk2	ASD	↓ dendritic branching, synapse number and PSD size; ↓ frequency and size of spontaneous mEPSCs; altered social interactions, learning abnormalities and behavioural inflexibility	Mouse hippocampal neurons and slices	[15]
<b>Effector genes</b>				
ERK1/2	ASD, 16p11.2 and 22q11.2 deletion syndromes	↑ c-Fos activity and ↑ ERK1/2 phosphorylation in the CA3 and DG of <i>Scrib1<sup>crc</sup></i> mice	Mouse hippocampal neurons and slices	[10,16,17]
Nhs	Severe congenital cataract, dysmorphic features, and mental retardation	-	-	[18]
<b>Others (-Cter PDZ binding domain)</b>				
MARK1	Autism	↓ dendrite length; modified dendritic transport speed	Mouse neocortical neurons	[19]
TSC2 -TEFV	Autism, epilepsy, mental retardation, and tumour predisposition	Lack leads to ectopic axons; overexpression to axon formation suppression; inactivation via upregulation of neuronal polarity SAD kinase	Mouse brain	[20]

1. Tarpey et al., 2004; 2. Barrow et al., 2009; 3. Gilman, et al., 2011; 4. Kantojärvi et al., 2011; 5. Tsai et al., 2012b; 6. Cao et al., 2013; 7. Moreau et al., 2010; 8. Pinto et al 2010; 9. Neale et al., 2012; 10. Moreau et al., in preparation; 11. Fraser et al., 2008; 12. Lijam et al., 1997; 13. Wynshaw-Boris, 2012; 14. Zhu et al., 2013; 15. Sowers et al., 2013; 16. Fernandez et al., 2010; 17. Campbell et al., 2009; 18. Burdon et al., 2003 ; 19. Maussion et al., 2008; 20. Choi et al., 2008.

### I.6.1. Structure, composition and properties of AMPA receptors

$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are tetrameric structures that combine homologous subunits GluA1 to GluA4 in different stoichiometries to form receptor subtypes with distinct properties (reviewed by Wisden & Seeburg, 1993; Hollmann & Heinmann, 1994; Mano & Teichberg, 1998; Rosenmund et al., 1998). Each monomer contains 900 amino acids that share between 68 and 74% of sequence homology (Collingridge et al., 2004). A typical AMPAR subunit is composed of a large extracellular Nter, four hydrophobic domains and an intracellular Cter (Fig. 19 a). The homologous Nter domain hosts the glutamate binding site, which induces conformation changes to the TM segments and triggers opening of the channel upon ligand binding (Gouaux, 2004; Mayer, 2005). Instead, the extracellular loop between TM3 and TM4 contains a flip/flop region shaped by alternative RNA splicing (R/G), creating receptors with different desensitization and endoplasmic reticulum (ER) export kinetics (Sommer et al., 1990; Mosbacher et al., 1994; Coleman et al., 2006). The four hydrophobic domains include three transmembrane domains (TM1, TM3, and TM4) and a specific membrane inserted hydrophobic amino-acid sequence (Q/R) prone to RNA editing that dictates the permeability of the channel pore. The intracellular Cter is quite distinct among different subunits, containing a few phosphorylation sites and a PDZ binding domains, both crucial to the regulation of AMPAR subunit function and protein-protein interactions. Moreover, some subunits, like the GluA1, GluA4 or the alternative splice form of GluA2L have longer, homologous and similar Cter tails, while other, such as the predominant splice form of GluA2, GluA3 and an alternative splice form of GluA4 have shorter cytoplasmic tails with high homology. The majority of AMPARs are a combination of GluA1, GluA3, and GluA4 with GluA2 subunit, responsible for the prototypical calcium-impermeability (Fig. 19 b, first) and the linear current-voltage (I-V) profile (Fig. 19 c, in orange). Nevertheless, there are some AMPAR that either contain an unedited GluA(Q) subunit (Fig. 19 b, middle) or do not possess GluA2 subunits at all (Fig. 19 b, last). These

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**Figure 19: AMPAR structure, regulation and diversity.** (a) An individual AMPAR subunit is composed of a large extracellular Nter, containing the glutamate binding site and the flip/flop region; three transmembrane domains; a hydrophobic loop that determines the channel permeability to calcium; and an intracellular Cter containing phosphorylation sites and a PDZ binding domain. In addition, the Cter can have either a conserved long (GluA1, GluA2L and GluA4) and short (GluA2, GluA3 and GluA4c) tail, prone to be regulated by the same kinases and scaffold proteins. (b) AMPAR assemble as a combination of four subunits –three GluA1/3/4 (in blue) and (1) typically one GluA2 (orange), forming a calcium-impermeable pore; or (2) a unedited GluA2(Q) (in clear blue), or (3) lacking GluA2, being the corresponding AMPAR of both calcium-permeable. (c) Current-voltage (I-V) relationship for AMPAR lacking (in blue) or containing (in orange) GluA2 subunits either rectifying or linear, respectively.

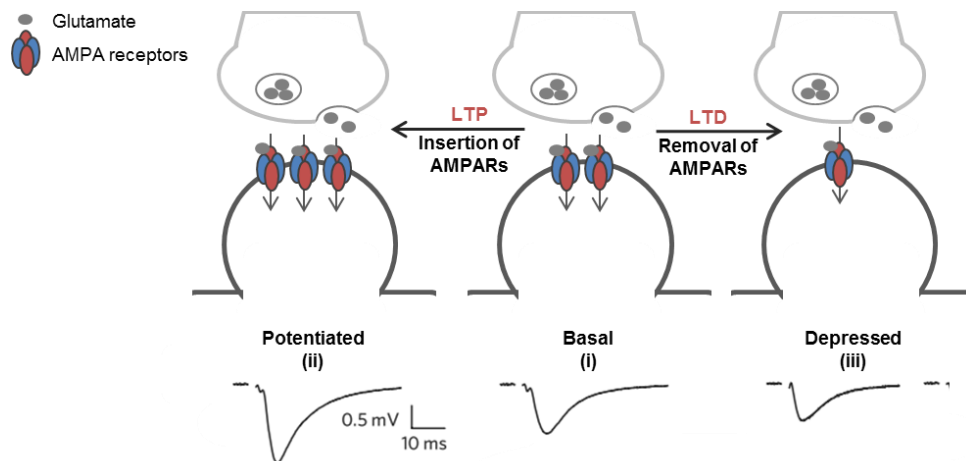
are permeable to calcium and display a rectifying I–V relationship (**Fig. 19 c**, in blue). Interestingly, several studies have been showing that AMPAR composition can be rapidly altered in response to certain inputs and therefore modulate the functional properties of a synapse (Liu & Cull-Candy, 2000, 2002; Bagal et al., 2005; Plant et al., 2006; Goel et al., 2006; Bellone & Luscher, 2006).

### **1.6.2 AMPAR distribution in the brain**

A widespread distribution of AMPAR in the brain was revealed in early studies using *in situ* hybridization, receptor autoradiography of [<sup>3</sup>H]AMPA and [<sup>3</sup>H]glutamate as ligands, and immunocytochemistry of all four GluA subunits. In particular, GluA1-GluA3 subunits are abundant in the hippocampus, outer layers of the cerebral cortex, amygdala, basal ganglia, olfactory regions and lateral septum (Keinanen et al., 1990; Beneyto & Meador-Woodruff, 2004). Conversely, GluA4 subunit is poorly expressed throughout the CNS, being only enriched in the reticular thalamic nuclei and the cerebellum (Petrálie & Wenthold, 1992; Martin et al., 1993; Spreafico et al., 1994). AMPAR can be equally found in glial cells (Gallo & Russell, 1995; Janssens & Lesage, 2001; Lin & Bergles, 2004). Regulation of receptor subunit expression occurs during development (Petrálie et al., 1999; Palmer et al., 2005; Talos et al., 2006). AMPAR mRNA can be detected at early stages of development. In embryonic rat brain, GluA2 is ubiquitously expressed, while the other subunits are differentially expressed (Monyer et al., 1991). Meanwhile, GluA1 protein can be found at E15.5 in rat brain and GluA4 at E11 in mouse brain (Durand & Zukin, 1993; Martin et al., 1998). Protein levels of all GluA subunits gradually increase until the third postnatal week (Insel et al., 1990; Pellegrini-Giampietro et al., 1991; Standley et al., 1995; Arai et al., 1997). It is believed that the first step of AMPAR incorporation into the plasma membrane occurs during neonatal development, when GluA1-containing AMPARs cluster at potential postsynaptic sites. Furthermore, splicing of AMPARs changes during development (Tonnes et al., 1999), as well as the kinetics of channel opening (Koike-Tani et al., 2005; Wall et al., 2002). Unsurprisingly, the subunit composition of AMPAR varies with cell type, development and brain region. In hippocampal pyramidal cells, GluA1/2 and GluA2/3 heteromers are predominant (Wenthold et al., 1996), while Purkinje cells in the cerebellum mainly contain GluA2/3 complexes (Esclapez et al., 1994). A quantitative fluorescence *in situ* hybridization revealed that proximal and distal dendrites of hippocampal cultured neurons contain endogenous mRNAs encoding GluA1 and GluA2 subunits, including clusters of GluA2 mRNA in a significant fraction of synaptic sites (Grooms et al., 2006). All four GluA subunit mRNAs have also been found in the apical dendrites of pyramidal cells in hippocampal and cortical tissue (Muddashetty et al., 2007; Cajigas et al., 2012; Cox & Racca, 2013) as well as in the dendrites of hippocampal GABAergic interneurons (Cox & Racca, 2013). Given that dendrites possess the necessary machine for protein synthesis, these data suggest that local synthesis of AMPAR subunits can afford local regulation of receptor abundance and composition (Steward & Levy, 1982; Kacharina et al., 2000; Tang & Schuman, 2002; Asaki et al., 2003; Muddashetty et al., 2007; Cajigas et al., 2012; Cox & Racca, 2013). Additionally, acute activation of mGluR1, a general depolarization with KCl (Ju et al., 2004), or dopamine receptor activation (Smith et al., 2005) in hippocampal neurons are able to promote GluA1 and GluA2 protein synthesis in dendrites, suggesting that both mRNA transport and dendritic translation are activity-dependent events.

### I.6.3 Role of AMPARs in synaptic plasticity

Long-term potentiation (LTP) and long-term depression (LTD) are nowadays the two major cellular mechanisms believed to underlie learning and memory (reviewed by Malenka et al., 1999). LTP refers to a persistent increase in the efficiency of synaptic transmission after inducing a short period of high-frequency synaptic stimulation; whereas LTD comprehends a reduction in synaptic strength following low-frequency stimulation (reviewed by Song & Huganir, 2002; Brecht & Nicoll, 2003; Collingridge et al., 2004) (**Fig. 20**). Synaptic plasticity is predominantly mediated by two subtypes of glutamate-gated ion channels concentrated at postsynaptic sites - AMPAR and NMDAR (reviewed by Citri & Malenka, 2008). The rapid excitatory synaptic transmission is ensured by AMPAR, while NMDA receptors regulate the slower component of neuronal activity. NMDA are blocked by  $Mg^{2+}$  at resting membrane potential, but when a sufficient number of AMPAR are activated taking the membrane potential to more positive values, the  $Mg^{2+}$  blockade is relieved, allowing  $Ca^{2+}$  to flow into the cell through NMDAR. Intracellular  $Ca^{2+}$  can now trigger various signalling cascades that in turn regulate AMPAR trafficking to and from the synaptic membrane, resulting in a potentiated or depressed synapse, respectively. How, where and when AMPA receptors are inserted or removed will be next discussed.



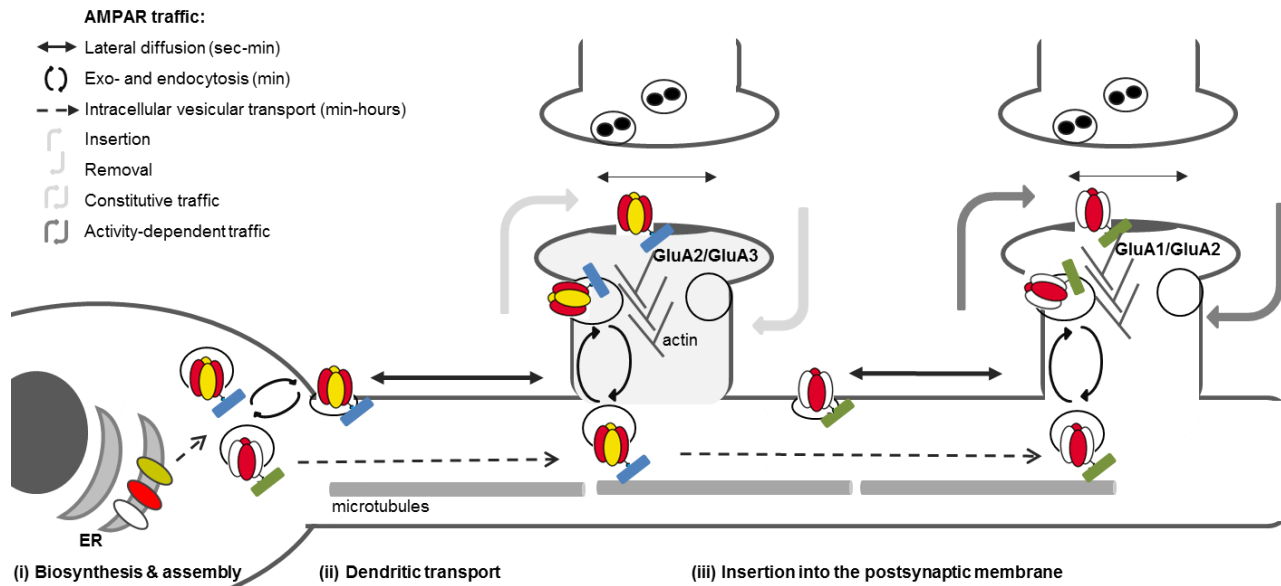
**Figure 20: AMPAR role in synaptic plasticity.** LTP and LTD trigger the insertion and removal of AMPA receptors at synapses, respectively. Examples of field excitatory postsynaptic potentials (fEPSPs) measured from basal (i), potentiated (ii) and depressed (iii) synapses.

### I.6.4 Untangling AMPAR traffic: from the cell soma to the synapse

The traffic of AMPAR in and out synapses is a key, fine-tuned and tightly regulated mechanism in both synaptic development and maturation. Following its biosynthesis and assembly in the cell soma, AMPA receptors are transported to dendrites and later trafficked to the postsynaptic membranes, where AMPAR proper content and composition define synaptic strength – **Fig. 21**.

#### I.6.4.1 AMPARs biosynthesis & assembly

The biosynthesis of oligomeric transmembrane proteins usually takes place at the ER membrane - **Fig. 21 i**. Typically, there is a first step of co-translation insertion of the nascent polypeptide chain through the Sec61



**Figure 21: AMPA receptors trafficking: from the cell soma to the synapse.** Following AMPAR biosynthesis and assembly in the ER, GluA1/2 and GluA2/3 can associate with other proteins, like SAP-97 (in green) and ABP/GRIP (in blue), respectively (i). The complex can be endo/exocytosed to the plasma membrane and diffuse laterally and/or move throughout the microtubules (ii) and trafficked into the plasma membrane (iii). GluA2/3 complex can continuously cycle in and out of the synaptic membrane, maintaining synaptic strength despite protein turnover, whereas the GluA1/GluA2 regulated pathway is transiently set upon induction of synaptic activity.

channel (Clemons et al., 2004), followed by protein folding and sequential assembly in three distinct physico-chemical environments: the cytosol, the RER (rough endoplasmic reticulum) lipid bilayer, and the ER lumen (Netzer & Hartl, 1997). Similarly to  $K^+$  channels (Tu & Deutsch, 1999), AMPAR are believed to be formed as dimers of dimers (Tichelaar et al., 2004; Ayalon & Stern-Bach, 2001; Ayalon et al., 2005). The dimer formation is mostly mediated by the Nter of AMPAR subunits, and the subsequent tetramerization requires the extracellular S2 loop and the TM domains, including the pore loop. Interestingly, these regions are prone to both RNA splicing and editing. The splicing of mutually exclusive exons in all four GluA subunits within the ligand-binding domain is believed to influence desensitization kinetics (Sommer et al., 1990; Seeburg et al., 1998; Lomeli et al., 1994; Grosskreutz et al., 2003). On the other end, RNA editing, Q/R586 at the apex of the pore loop of GluA2 subunits, and the R/G743 in the flip/flop region of all four GluA subunits intimately impact receptor assembly (Greger et al., 2003, 2006). In particular, unedited GluA2 (Q586) forms homomers, while the edited subunit (R586) restricts GluA2 self-assembly, implying that only fully edited GluA2 (R586/G743) produce hetero-tetramers. Remarkably, it was shown that this RNA processing is regulated during development. Furthermore, different tetramer combinations of AMPA subunits can be selectively targeted to specific dendritic subdomains (Toth & McBain, 1998; Rubio & Wenthold, 1997; Gardner et al., 2001), and in response to altered activity patterns (reviewed by Cull-Candy et al., 2006). Following the subunit folding and receptor assembly in the ER membrane, AMPAR are sensed by the quality control machinery at the ER exit (Ellgaard & Helenius, 2003; Grunwald & Kaplan, 2003; Valluru et al., 2005; Mah et al., 2005). The efficiency of all these processes within the ER and its exit will impact on AMPAR export kinetics and consequent number of available channels for synaptic expression. It is believed that the assembled AMPAR

associate with other proteins, like SAP-97 or ABP/GRIP, facilitating both ER export and traffic of AMPAR to the dendritic spine.

#### **I.6.4.2 AMPAR traffic to and in the synapse**

Following biosynthesis and assembly, AMPAR are targeted to a specialized dendritic membrane that constitutes the postsynaptic terminal - **Fig. 21 ii**. Exocytosis of *de novo* AMPAR is not only a requirement during basal conditions as it is during LTP (Lledo et al., 1998; Park et al., 2004). Initial studies suggested that AMPAR could be mobilized from dendritic recycling endosomes and be inserted along the dendritic shaft or directly into the PSD itself (Passafaro et al., 2001; Gerges et al., 2006). However, the turnover rate of synaptic receptors within intracellular pools was proven to be too slow for such a scenario compared to exocytosed receptors at the soma that travel until the synapse by lateral diffusion (Adesnik et al., 2005). Technical advances in the optical field in the last decade or so allowed to better track down the dendritic movement of AMPAR, suggesting the existence of two segregated compartments in a synapse containing distinct population of AMPARs: (1) extrasynaptic AMPARs, which appear to be highly mobile; and (2) synaptic AMPARs, displaying confined movements within microdomains of about 300nm dynamically organized by scaffold proteins (Lee et al., 2002b; Borgdorf & Choquet, 2002; Tardin et al., 2003; Ashby et al., 2004; Sekine-Aizawa & Huganir, 2004). High-density superresolution imaging came to prove that AMPARs can be exchanged between synapses through lateral diffusion and be trapped in the PSD by interacting with several other proteins (Czöndör et al., 2012; Hoze et al., 2012; Nair et al., 2013). On the other hand, single-molecule tracking proved to be better to determine how fast do receptors diffuse at the synapse. Several reports confirmed that receptors are constantly moving, displaying low (below  $10^{-4} \mu\text{m}^2/\text{s}$ ) to high ( $10^{-1}$ - $10^{-2} \mu\text{m}^2/\text{s}$ ) diffusion rates depending where they are trapped or not in the PSD, respectively (Meier et al., 2001; Sergé et al., 2002). In addition, surface mobility of postsynaptic AMPAR, but not NMDAR, was proven to be strongly affected by neuronal activity, being the extrasynaptic population the most affected (Howarth et al., 2005; Chambers et al., 2004). In particular, synaptic activity was reported to restrict AMPAR mobility on a submicron scale within individual synapses, preventing diffusional exchange of GluA1-containing complexes between synaptic and extrasynaptic domains (Ehlers et al., 2007). At last, Heine and collaborators elegantly showed that AMPAR lateral diffusion allows a fast exchange of desensitized receptors for naïve functional ones within the PSD or nearby, thus allowing to fine tune synaptic transmission in the tens of milliseconds range (Heine et al., 2008). One important aspect of lateral diffusion is that it is dependent on spine morphology, being restricted at the spine neck. Synapse specificity is therefore believed to be guaranteed by spine structure, which directly compartmentalizes lateral diffusion, allowing the receptors to spend less or more time in specific synapses (Ashby et al., 2006). Interestingly, LTP-induced actin polymerization was shown to retain AMPAR at perisynaptic sites, which are removed following low-frequency stimulation (Yang et al., 2008). Tracking of individual actin molecules in live spines further corroborated its role as a dynamic element well suited to regulate spatially distinct events throughout several subdomains of the spine (Frost et al., 2010).

#### **I.6.4.3 Constitutive and activity-dependent targeting of AMPARs to the synapse**

The final step of receptor insertion at the synaptic membrane is a tightly regulated event, being dependent on both the receptor composition and specific signals contained in the Cter of the different GluA subunits - **Fig. 21 iii**. Endogenous AMPARs consist mostly on GluA1/2 and GluA2/3 complexes. Surface insertion of individual long-tailed GluA1 and GluA4 occurs slowly in basal conditions, being stimulated by neuronal activity and NMDAR activation (Hayashi et al., 2000). Conversely, the short tailed GluA2 subunit is inserted in rapid and constitutive manner under basal conditions (Passafaro et al., 2001; Shi et al., 2001). When GluA1/2 heteromeric channels are expressed, the activity-dependent GluA1 trafficking signal dominates, while GluA2/3 complex behaves like GluA2 homomers, constitutively trafficking to the synapse. As aforementioned, the differential trafficking of AMPAR subunits depends as well on their Cter tails. In particular, GluA1 Cter has a class I PDZ binding domain able to interact with SAP-97 early in the secretory pathway (Leonard et al., 1998). This interaction is essential for the ER exit but once at the plasma membrane, SAP-97 dissociates from the complex (Sans et al., 2001). Other proteins, like RIL (reversion-induced LIM; Schulz et al., 2004) or 4.1N (Shen et al., 2000), may be involved in actin-dependent trafficking and surface expression of GluA1. Conversely, GluA2, GluA3, and GluA4c contain a class II PDZ binding domain. Several studies showed that GluA2 and GluA3 are also engaged in protein-protein interactions with PDZ domain containing proteins like GRIP (glutamate receptor-interacting protein), ABP (AMPA receptor-binding protein or PICK1 (protein interacting with C kinase) (Dong et al., 1997; Srivastava et al., 1998; Dev et al., 1999; Xia et al., 1999). Furthermore, post-translation modifications within the PDZ binding region of GluA2 are known to prevent its association with GRIP and ABP, but not with PICK1 (Matsuda et al., 1999, 2000; Chung et al., 2000). Altogether, these evidences point to a non-stochastic but rather tuned regulation of AMPAR assembly, traffic and delivery to the synaptic membrane, where they play an essential role in synaptic plasticity.

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#### **I.6.4.4 Post-translational modifications of AMPAR Cter can dictate AMPAR delivery rules**

Calcium influx through the NMDAR is essential for synaptic plasticity and results in the activation of a wide variety of intracellular signalling pathways including several enzymes. On the other hand, the Cter tails of AMPAR subunits contain several phosphorylation, palmitoylation, glycosylation and ubiquitination residues, suggesting that post-translational modifications can be engaged in the regulation of synaptic plasticity – **Table 7; Fig. 22**. The modulation of post-translational events depends not only on the previous history of the synapse, but also on the type of stimulus that the synapse receives. For example, stimulation of naïve synapses leads to an increase of Ser831 phosphorylation, while in previously depressed synapses Ser845 phosphorylation predominates (Lee et al., 1998, 2000). Instead, following a HFS-dependent activation of CaMKII, PKA increases GluA1 phosphorylation to produce LTP (**Fig. 22 a**); whereas LFS activates protein phosphatases like PP1/2A, capable of dephosphorylate Ser845 and reduce the number of GluA1 synaptic clusters, thus promoting LTD (**Fig. 22 b**) (Carroll et al., 1999; Heynen et al., 2000; Huang et al., 2001a; Hu et al., 2007). On the other hand, the same residue can be bidirectionally modulated by the same post-translational modification, be targeted by another type of enzyme or even be primed by a previous modification, suggesting a fine tune regulation of several converging signalling pathways. As a final outcome, post-

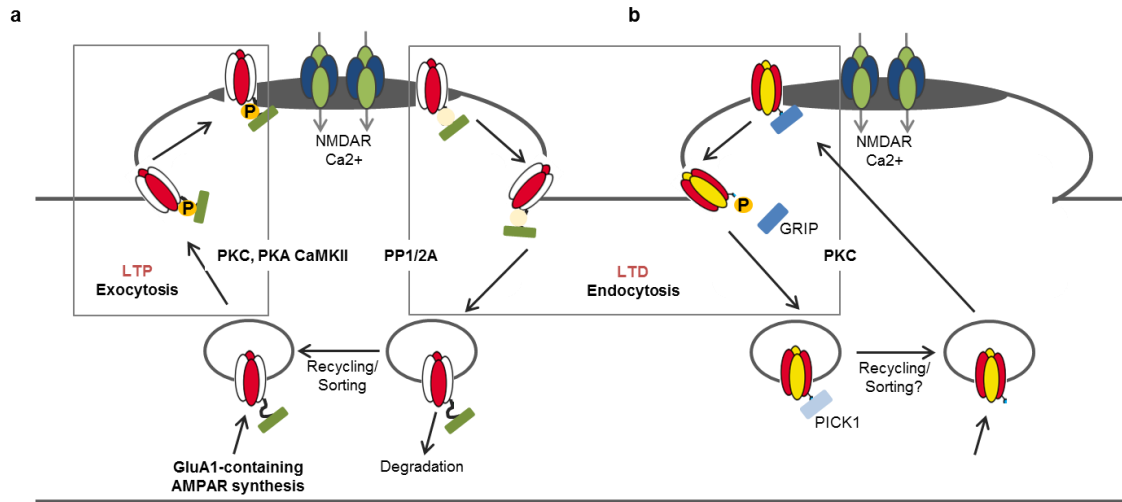
translational modifications can facilitate or disturb interactions with other proteins, orient AMPAR traffic towards internalization, recycling or degradation pathways, ultimately affecting AMPAR surface expression and synaptic transmission.

**Table 7:** Functional effect of the main AMPAR post-translational modifications in AMPAR traffic.

Subunit	Residue	Enzyme	Functional effect	Ref.
GluA1	Cys610	Palmitoyl acyl transferase family	Modulates PKC phosphorylation and interaction with 4.1N	[1,2]
	Ser816	PKC	Promotes interaction with 4.1N, facilitating AMPAR insertion	[2]
	Ser818	PKC	Promotes interaction with 4.1N; essential for LTP-induced AMPAR recruitment to the PSD	[3]
	Ser831	OGT/OGNase	Predicted to be modified during hippocampal LTD	[4,5]
		PKC, CaMKII	Higher channel conductance; promotes synaptic incorporation of receptors following LTP	[6-9]
		PP1	Facilitates LTD by reducing AMPAR surface expression	[10]
	Thr840	OGT/OGNase	Predicted to be modified during hippocampal LTD	[4,5]
		PKC, p70S6	Predicted <i>in vitro</i> PKC site; p70S6 might have a role in LTD	[11,12]
		PP1/2A	Decreased phosphorylation following chemLTD protocol	[11]
	Ser845	PKA	Higher channel open probability; primes AMPAR for CaMKII(Ser831)-dependent synaptic incorporation and efficient LTP	[8,13-15]
		PP1/2A	Reduces AMPAR surface expression and promotes LTD	[10,16,17]
		OGT/OGNase	Predicted to be modified during hippocampal LTD	[4,5]
GluA2	Lysine	E3 ubiquitin ligases	Regulates GluA1 degradation via the proteasome or the lysosome	[18,19]
	Cys811	Palmitoyl acyl transferase family	Modulates interaction with 4.1N	[1,2]
	Cys836	Palmitoyl acyl transferase family	Modulates interaction with 4.1N	[1,2]
	Ser856	OGT/OGNase	Predicted to be modified during cerebellar LTP	[4,5]
	Ser863	PKC	Unknown	[20,21]
	Tyr876	Src family	Modulates interaction with GRIP and PICK as well as AMPA- and NMDA-induced internalization of GluA2	[22]
	Ser880	PKC	Modulates GluA2 internalization and interaction with GRIP and PICK1	[23-25]
		PP1	Stabilizes basal transmission by antagonizing PKC action	[10]
GluA2L	Thr912	JNK1	Controls GluA2L reinsertion following NMDA-dependent internalization	[26]
GluA4	Thr830	PKC	Unknown	[27]
	Ser842	PKC, PKA, CaMKII	PKC leads to increased surface expression; PKA relieves retention signal and modulates surface expression	[8,16,27-29]
	Tyr876	Src	Modulates interaction with BRAG2 and subsequent activation of Arf6; involved in internalization during LTD	[30]
	Ser880	PKC	Favours PICK binding over GRIP; involved in LTD	[31]

1. Hayashi et al., 2005; 2. Lin et al., 2009; 3. Boehm et al., 2006; 4. Din et al., 2010; 5. Kanno et al., 2010; 6. Mammen et al., 1997; 7. Barria et al., 1997; 8. Derkach et al., 1999; 9. Hayashi et al., 2000; 10. Hu et al., 2007; 11. Delgado et al., 2007; 12. Lee et al., 2007; 13. Roche et al., 1996; 14. Banke et al., 2000; 15. Oh et al., 2006; 16. Esteban et al., 2003; 17. Lee et al., 2000; 18. Schwarz et al., 2010; 19. Fu et al., 2011; 20. McDonald et al., 2001; 21. Hirai et al., 2000; 22. Hayashi & Huganir, 2004; 23. Matsuda et al., 1999; 24. Chung et al., 2000; 25. Perez et al., 2001; 26. Thomas et al., 2008; 27. Carvalho et al., 1999; 28. Correia et al., 2003; 29. Gomes et al., 2004; 30. Scholz et al., 2010; 31. Seidenman et al., 2003. OGT, O-GlcNAc transferase; OGNase, O-GlcNAcase; O-GlcNAc, O-linked N-acetylglucosamine.





**Figure 22: AMPAR endocytosis and exocytosis dependent on phosphorylation.** (a) Following NMDA activation, the  $\text{Ca}^{2+}$  influx can activate protein kinases like PKC, PKA or CaMKII and consequent phosphorylation of AMPA receptors containing long tailed GluA1 subunits. They now move to the PSD and increase synaptic strength (LTP). Conversely, if phosphatase actions predominate, AMPAR can be endocytosed, decreasing the synaptic strength (LTD). Once inside the cell, they can recycle back to the membrane or be degraded. (b) Following synthesis, AMPA receptors containing GluA2 subunits can be anchored at the synaptic membrane by interacting with PDZ domain-containing proteins like GRIP. Following a LTD stimulus, PKC is activated and phosphorylates GluA2 short tailed Cter. The interaction between GluA2 and GRIP is disrupted, promoting AMPAR endocytosis. PICK1, insensitive to phosphorylation changes, can now bind and stabilize the newly internalized AMPAR receptors.

#### 1.6.4.5 Molecular determinants of AMPAR synaptic traffic

Targeting, trafficking and insertion of AMPARs into synapses is largely mediated through interactions of the highly complex and specific Cter of each AMPA subunit (**Fig. 19 a**) with multiple proteins that can either be cytoplasmic, such as PSD-95; transmembrane proteins as stargazing; or even secreted proteins like Narp - **Table 8**. As aforementioned, some of these interactions can be affected by post-translational modifications, pinpointing to the existence of a tightly regulated and fine-tuned mechanism orchestrated by several players at each step of the AMPAR traffic from the receptor biosynthesis at the soma until the delivery and surface expression at a specific site and time point.

##### 1.6.4.5.1 Cytosolic proteins

###### 1.6.4.5.1.1 PDZ-containing proteins

The first PDZ domain-containing proteins - the PSD-95/SAP-90 protein, the *Drosophila* discs large tumour suppressor gene Dlg-A, and the epithelial tight junction protein ZO-1 (Woods & Bryant, 1991; Cho et al., 1992; Itoh et al., 1993) - are responsible for the denomination of this family of proteins that typically is involved in the assembly of supra-molecular complexes. PDZ domains are modular protein interaction motifs that can bind to other PDZ domains or to specific sequences in the Cter of several proteins that contain the so called PDZ-binding motifs (reviewed by Sheng & Sala, 2001; Hung & Sheng, 2002). PDZ domains can be classified according to the recognized specific consensus sequence of the last four amino acids of the Cter ligand. Class I PDZ domains recognize the sequence X-S/T-X-L/V (x-any amino acid); whereas Class II PDZ domains identify X- $\phi$ -X- $\phi$  ( $\phi$ -hydrophobic amino acid). PDZ-containing proteins implicated in AMPAR trafficking represented in **Fig. 23** and further described below.

**Table 8:** AMPAR-interacting proteins capable of controlling AMPAR synaptic traffic.

Protein	Interaction	Function	Ref.
<b>Cytoplasmic proteins</b>			
<b>PDZ domain containing proteins</b>			
GRIP/ABP	GluA2/3, GluA4(c)	Membrane surface and intracellular receptor stabilization; implicated mAChR- and mGluR-dependent LTD; and indirectly involved in LTP	[1-9]
PICK1	GluA2/3, GluA4(c)	Receptor clustering, NMDA-induced AMPAR downregulation and internalization; present in recycling endosomes; affects spine dynamics; involved in cerebellar and hippocampal LTD; critical role in bidirectional LTP/LTD switch	[2,10-24]
MAGUKs SAP-97	GluA1	Regulates AMPAR functional number at the synapse and occludes LTP; and $\alpha$ - and $\beta$ SAP-97 differentially regulate GluA1 sub-synaptic localization	[25-27]
PSD-95	<i>Via Stgz</i>	Promotes synaptic clustering AMPARs, as well as AMPAR-mediated currents; anchors AMPAR at the post-synapse by interacting with Stgz; promotes synaptic stability through CaMKII	[28-30]
mLin-10	GluA1	Directs AMPAR localization; and it might regulate AMPA receptor trafficking	[31-32]
SemaF Syntenin	GluA2 GluA1, GluA2(c), GluA3c, GluA4	Unknown Unknown	[33] [2]
<b>Non PDZ domain containing proteins</b>			
4.1N	GluA1, GluA4	Modulates actin cytoskeleton-dependent AMPA trafficking; involved in cerebellar synaptogenesis; and possibly in LTP expression	[34-37]
AKAP79/150	MAGUK/AMPA	Anchors PKA and CaN to MAGUK/AMPA complexes in spines; chem-LTP-induced recruitment promotes Rab11-regulated endosome recycling of AMPARs and spine enlargement	[38,39]
AP2	GluA2	NMDA-induced AMPAR internalization; mediates LTD in the visual and perirhinal cortex	[40-42]
Arf1	PICK/AMPA	Binds PICK1, limiting its action on Arp2/3 activity, synaptic AMPAR internalization and spine shrinkage	[43]
Arp2/3	PICK/AMPA	Binds PICK; critical for NMDA-induced AMPAR internalization	[44]
Cofilin	Actin/AMPA	Links dendritic spine morphology changes to synaptic-related AMPAR trafficking	[45,46]
Dbn1	Actin/AMPA	Contributes to AMPA-mediated spine morphogenesis by organizing F-actin in dendritic spines during synaptic plasticity; relevant for neuro-cognitive diseases and neurodevelopmental disorders	[47-56]
Dynamin	GluA2	Drive the lateral diffusion of newly exocytosed GluA2 and NMDA-evoked GluA2 internalization.	[57]
KIBRA	GluA1/2	Binds the PICK/AMPA complex, regulating NMDA-dependent AMPAR recycling; implicated in long-term synaptic plasticity and memory retention	[58-61]
Lyn	GluA1, GluA2/3, GluA4c	Activation of MAPK and ERK1/2-CREB signalling pathways	[62,63]
NSF	GluA2	Targeting of receptors to the synaptic membrane; mediates GluA2-dependent LTP; and fear memory consolidation in the lateral amygdala	[64-73]
MyoVI	SAP-97/AMPA	Binds the SAP-97/AMPA complex; relevant for AMPAR-mediated synaptic transmission	[74,75]
MyoVa/b	Scaffold/Rab11/ AMPA	MyoVa and MyoVb drive AMPAR, scaffold proteins and Rab11 from the dendritic shaft or recycling endosomes, respectively, into spines; critical in AMPAR's directional transport during activity-dependent synaptic plasticity	[76-79]
PACSIN1-3	GluA1/2	PACSIN members interact with the PICK1/AMPA complex; implicated in PICK1-dependent AMPAR internalization and cerebellar LTD	[80]
PKC $\gamma$	GluA4	Facilitates PKC-driven phosphorylation and membrane targeting of GluA4; involved in LTP, learning, memory and neuropathic pain	[81-85]
Rap2b	Native GluAs	Counterbalances Ras-induced AMPAR delivery during synaptic events; activated in LTD, promoting dendritic pruning and reducing synaptic density	[86-88]
<b>Transmembrane domain proteins</b>			
ADAM22/LGI1	Indirect? -ETS1 :: ?	LGI1 specifically binds ADAM22 at the synapse, promoting the recruitment of new AMPAR to the synaptic surface and increasing the AMPAR-mediated neurotransmission; both implicated in epilepsy	[89-92]

Cntnap1	GluA1	Promotes GluA1 traffic and synaptic content	[93]
Cornichon CINH2/3	Native GluAs	Increase surface expression of AMPARs by assisting the receptor bio-synthesis and trafficking from the ER to the neuronal cell surface; slow deactivation and desensitization kinetics; can functionally interact with TARPs and regulate their number within an AMPA receptor complex	[88,94-110]
CPT-1C & PORCN	Native GluAs	Unknown	[88]
GSG1L	GluA2, inc. native	Unlike TARPs, it slows the recovery from the desensitized state	[88,102]
LRRTMs LRRTM2	Indirect? -ECEV :: PSD-95	Regulates the expression of AMPARs and modulates the strength of evoked excitatory synaptic currents; induces presynaptic differentiation by interacting with NRX; and it specifically promotes synaptogenesis excitatory neurons	[88,103]
LRRTM4 N-cadherin	Native GluA2 GluA2	Unknown. Promotes the synaptic recruitment of GluA2 and consequent synaptic transmission; implicated in recognition mechanisms and regulation of dendritic spine density	[88] [104-106]
NETO <i>Neto1</i>	<i>GluN2A, GluN2B</i> -TTRV :: PSD-95	<i>Involved in the synaptic delivery and/or stability of GluN2AB; implicated in spatial learning and LTP</i>	[107]
<i>Neto2</i>	<i>GluK2</i>	<i>Involved in kainate-mediated neurotransmission.</i>	[108]
NLGN	Indirect? -TTRV :: PSD-95	Mediates synapse formation by interacting with pre-synaptic neuroligins; potentiates NMDAR/AMPA ratios; implicated in autism and other cognitive disorders	[109-114]
Shisa CKAMP44	GluAs, inc. native -EVTV :: ?	Associates with AMPAR complexes in synaptic spines; decreases AMPAR responses by increasing the receptor desensitization without affecting its surface expression; also involved in short-term facilitation	[88,98,115]
CKAMP52	Native GluAs -EVTV :: ?	Unknown	[88]
SynDIG1	GluA1, GluA2	Modulation of GluA1/2 number and function in developing excitatory synapses	[116]
PRRT1,2 TARPs Stgz	Native GluA1 GluA1, GluA2, GluA4, inc. native -TTPV :: PSD-95	Unknown Regulation of AMPA receptors expression levels, synaptic targeting/stabilization and subsequent synaptic strength; substrate for bidirectional control of synaptic strength via phosphorylation and palmitoylation	[88] [26,28,88, 117-126]
<b>Membranar protein</b>			
Neuritin	Native GluAs	Unknown	[88]
<b>Secreted proteins</b>			
Brorin & Brorin-2l	Native GluA2	Unknown	[88]
Corticosterone	GluA2	Increases GluA2 surface mobility and synaptic content; mediates AMPAR-dependent synaptic transmission via intracellular glucocorticoid receptors	[127-130]
Leptin	GluA1	Promotes NMDA-dependent GluA1 cell surface expression through the inhibition of PTEN; relevant for activity-dependent synaptic plasticity in the hippocampus	[131,132]
Narp	GluA1-3	AMPA receptor clustering and excitatory synapse formation; might be involved in enduring forms of neuronal plasticity	[133-137]
Olfm1	GluAs, inc. natives -SDEL :: ?	Unknown	[88,99]
ECM	AMPA receptors	Restricts AMPAR mobility and the availability of naïve receptors at the synaptic, regulating AMPAR-mediated short-term synaptic transmission; crucial to stabilize and maintain synaptic networks in the adult CNS	[138-140]

1. Dong et al., 1997; 2. Hirbec et al., 2002; 3. Braithwaite et al., 2002; 4. DeSouza et al., 2002; 5. Takamiya et al., 2008; 6. Dickinson et al., 2009; 7. Casimiro et al., 2011; 8. Contractor et al., 2002; 9. Hoogenraad & van der Sluijs, 2010; 10. Dev et al., 1999; 11. Xia et al., 1999; 12. Iwakura et al., 2001; 13. Perez et al., 2001; 14. Lee et al., 2001; 15. Nakamura et al., 2011; 16. Matsuda et al., 1999; 17. Matsuda et al., 2000; 18. Chung et al., 2000; 19. Xia et al., 2000; 20. Kim et al., 2001; 21. Seidenman et al., 2003; 22. Citri et al., 2010; 23. Terashima et al., 2008; 24. Xue et al., 2010; 25. Leonard et al., 1998; 26. Rumbaugh et al., 2003; 27. Li et al., 2011a; 28. Chen et al., 2000; 29. Schnell et al., 2002; 30. Taft & Turrigiano, 2013; 31. Stricker & Huganir, 2003; 32. Glodowski et al., 2005; 33. Mayer et al., 2004; 34. Shen et al., 2000; 35. Coleman et al., 2003; 36. Douyard et al., 2007; 37. Lin et al., 2009; 38. Dell'Acqua et al., 2006; 39. Keith et al., 2012; 40. Lee et al., 2002b; 41. Yoon et al., 2009; 42. Griffiths et al., 2008; 43. Rocca et al., 2013; 44. Rocca et al., 2008; 45. Gu et al., 2010; 46. Rust et al., 2010; 47. Harigaya et al., 1996; 48. Hatanpää et al., 1999; 49. Hayashi & Shirao, 1999; 50. Shim & Lubec, 2002; 51. Kobayashi et al., 2004; 52. Aoki et al., 2009; 53. Takahashi et al., 2009; 54. Kojima et al., 2010; 55. Counts et al., 2012; 56. Lee & Aoki, 2012; 57. Jaskolski et al., 2009; 58. Makuch et al., 2011; 59. Sacktor et al., 1993; 60. Drier et al., 2002; 61. Büther et al.,



ley, 2010), being some of them, like GRIP1-a and -b, targets of palmitoylation, thus capable of membrane and protein interactions (Yamazaki et al., 2001). GRIP is enriched in post-synaptic densities and it can be found associated with the rough endoplasmic reticulum, the Golgi apparatus and recycling endosomes (Burette et al., 1999; Lee et al., 2001), pointing to a key role in AMPA receptor traffic to dendrites. Moreover, as a scaffold protein it can interact with many other proteins, including other scaffold proteins, thus building a complex and dynamic network in the PSD. Through Shank2, GRIP links AMPARs to mGluRs in the cerebellum (Uemura et al., 2004); while through liprin, GluR2/3 complexes can signal receptor tyrosine phosphatases, GTPases, and motor proteins, ultimately modulating synaptic maturation (Wyszynski et al., 2002; Ko et al., 2003). Also, GRIP-associated proteins allow AMPAR to associate with activity-dependent Ras signalling (Ye et al., 2000), and GRIP itself was shown to interact with the kinesin heavy chain and microtubule-associated protein-1B (Setou et al., 2002; Seog, 2004), further supporting the involvement of GRIP in AMPAR trafficking. The functional consequence of such regulation in terms of synaptic plasticity remains unclear. Some reports suggest that GRIP might be implicated in LTP events, either in the mossy fibres through its binding to the EphB receptor (Contractor et al., 2002); or indirectly via a neuron-specific effector of the small GTPase Rab4 – the coiled-coil protein GRIP-associated protein-1 (GRASP-1), key component in both the AMPA receptor recycling and maintenance of spine morphology (Hoogenraad & van der Sluijs, 2010). On the other hand, a first study using GRIP1/2 double knock-out Purkinje cells showed that the resulting blocked LTD expression was fully rescued by GRIP1 and partially by GRIP2 (Takamiya et al., 2008), suggesting that the GRIP family can play a role in cerebellar LTD. In addition, the muscarinic acetylcholine receptor-dependent LTD (mAChR-LTD) is known to be driven by the activation of protein tyrosine phosphatase (PTP) and subsequent AMPA receptor endocytosis, whose modulation lies on GluA2, GRIP and liprin-alpha interactions (Dickinson et al., 2009). Finally, in CA1 hippocampal neurons, the activation of NMDARs is known to endorse endocytosis of rapidly cycling surface AMPARs not directly associated with GRIP1/2, whereas mGluR activation with DHPG induces the endocytosis of non-cycling GRIP-bound surface AMPARs (Casimiro et al., 2011), suggesting that GRIP interactions can play a role in the segregation of the two receptors pathways by driving the endocytosis of distinct populations of AMPARs.

**PICK1.** Protein interacting with C kinase (PICK1) possesses a unique PDZ domain capable of binding to GluA2/3, GluA4c, or GluA4 (Dev et al., 1999; Hirbec et al., 2002), like GRIP/ABP. PICK1 was firstly described as a binder of the catalytic domain of PKC alpha and a substrate for PKC (Staudinger et al., 1997), although their interaction is independent on PICK1 phosphorylation. Instead, the same PICK1 domains interact with both PKC and GluA2 (Xia et al., 1999). At excitatory synapses, PICK1 can homo-oligomerize via its PDZ domain, allowing PKC to crosslink with GluA2/3 and thus conferring specificity on AMPA receptor phosphorylation. NMDAR-dependent PKC activation increases Ser880 phosphorylation, releasing GluA2 from GRIP binding while inducing PICK1 to traffic to synapses (Chung et al., 2000; Iwakura et al., 2001; Dev et al., 2004). Importantly, the interaction between PICK1 and several other proteins, including NSF, can potentially disrupt its binding to GluA2 (Hanley et al., 2002; Meyer et al., 2004) as well as the NMDA-dependent GluA2 recycling (Lin & Huganir, 2007). In fact, PICK1 was shown to be located in recycling endosomes (Lee

et al., 2001), possibly explaining the role of PKC-dependent internalization of GluA2-containing AMPAR receptors in cerebellar and hippocampal LTD (Matsuda et al., 1999, 2000; Xia et al., 2000; Kim et al., 2001; Perez et al., 2001; Seidenman et al., 2003; Citri et al., 2010). PICK1 was also suggested as a novel regulator of both structural and functional plasticity, coupling the regulation of AMPAR trafficking to the spine dynamics via its interaction with the Arp2/3 complex (Nakamura et al., 2011). In particular, the induced spine shrinkage following NMDA receptor activation is blocked by knocking down PICK1 or overexpressing a mutant form unable to bind Arp2/3. Nevertheless, a recent study from the same group pointed out a possible involvement of PICK1 in the early stages of LTP. Jaafari and colleagues (2012) showed that, in contrast to the rapid insertion of GluA1 immediately after LTP induction, GluA2 subunits are restricted from trafficking to the cell surface via their interaction with PICK1. However, such interaction can be disrupted after 5-20 min of elevated glycine stimulation, allowing GluA2-containing AMPARs to be inserted into the synaptic plasma membrane. Therefore, PICK1 seems to regulate both the total number of AMPARs at the synapse as well as the transient switch to calcium-permeable AMPARs in the early phase of LTP. Results from other groups further support the scenario where PICK1 plays a critical role in bidirectional LTP/LTD switch, which is believed to rely on its PDZ domain (Terashima et al., 2008; Thorsen et al., 2010; Xue et al., 2010).

## MAGUKs

PSD-MAGUKs are mammalian homologs of the *Drosophila* protein discs large tumour suppressor protein (Dlg), known regulators of epithelial cell polarity and tumorigenesis Scribble/Dlg/Lgl polarity module (see Humbert et al., 2008 for a review), which are present at excitatory synapses, and particularly at the PSD micro-domain. The PSD-95-like subfamily of MAGUKs (PSD-MAGUKs) includes SAP-97 (Dlg1), PSD-93 (Dlg2 or chapsyn-110) and SAP-102 (Dlg3). All of them share a common domain structure containing three Nter PDZ (PSD-95/discs large/zona occluden-1) domains, a Src-homology 3 (SH3) domain, and a Cter enzymatically inactive guanylate kinase (GK) domain.

**SAP-97.** Synapse-associated protein-97 (SAP-97) contains an extra Nter L27 domain (Lin et al., 1997) and within the brain, it can be found at the PSD but also in the cytoplasm and the pre-synaptic compartment (Aoki et al., 2001). SAP-97 is readily extracted by Triton X-100 like AMPARs, contrasting other MAGUKs and NMDARs (Leonard et al., 1998). It is not surprisingly then that SAP-97 is the only MAGUK that can interact directly with the class I PDZ binding motif (-ATGL) of GluA1 via its second PDZ domain (Cai et al., 2002). Nevertheless, it is still unclear how this occurs, since deflecting the PDZ domain of GluA1 does not affect AMPAR-mediated synaptic transmission (Kim et al., 2005). GluA1-containing AMPA receptors can then associate with several other proteins recruited by the multiple interaction domains of SAP-97, such as the kinases PKA, PKC and CaMKII, the phosphatase calcineurin (Paarmann et al., 2002), the ionotropic glutamate receptors NMDA and kainite receptors (Bassand et al., 1999; Mehta et al., 2001; Gardoni et al., 2003), the transmembrane AMPAR regulatory protein stargazin (Ives et al., 2004), the A-kinase anchoring protein AKAP79/150 (Colledge et al., 2000), and the actin-based motor myosin VI (Wu et al., 2002; Hasson, 2003). These multiple interactions support the idea of SAP-97 as an anchoring protein (Karnak et al., 2002; Lee et al., 2002a; Feng et al., 2004), capable of controlling synaptic targeting of AMPARs. Indeed, SAP-97-GluA1

interaction is believed to take place early in the secretory pathway (Sans et al., 2001), with the CaMKII-dependent synaptic targeting of SAP-97 (Mauceri et al., 2004) promoting the stabilization and synaptic insertion of GluA1-containing AMPA receptors (Valtschanoff et al., 2000; Nakagawa et al., 2004). Overexpression of SAP-97 increases the functional number of AMPARs at the synapse and occludes LTP (Rumbaugh et al., 2003). However, SAP-97 does not seem to be implicated in NMDA-dependent internalization of GluA1 (Sans et al., 2001). Instead, the interaction between SAP-97 and AKAP79 leads to a basal phosphorylation of GluA1 Ser845 via PKA; the same residue can be the target of LTD-dependent activated phosphatases (Tavala et al., 2002). Finally, a recent study suggests that the alternative splicing forms of SAP-97 - the palmitoylated  $\alpha$ SAP-97 and the L27-domain containing  $\beta$ SAP-97 can differentially regulate the sub-synaptic localization of GluA1 subunits of AMPA receptors and subsequent capacity to experience synaptic plasticity (Li et al., 2011a). Recordings from pairs of synaptically coupled hippocampal neurons showed that  $\alpha$ SAP-97 can occlude LTP by increasing the synaptic pool of AMPAR; whereas  $\beta$ SAP-97 blocks LTP, possibly by regulating the extra-synaptic pool of both AMPA and NMDA receptors.

**PSD-95.** The postsynaptic density protein 95, also known as synapse associated protein 90 (SAP-90) or disk large homolog 4 (Dlg4) is the prototypical scaffold present at excitatory synapses (Elias & Nicoll, 2007). The high abundance of PSD-95 in the PSD, corresponding to ~2% of the total PSD mass (Chen et al., 2005b), ensures plenty of slots to bind numerous partners, such as the NMDARs, NLGN, or TARPs/AMPARs. Even though PSD-95 does not directly interact with AMPA receptors, its overexpression in cultured hippocampal neurons was reported to promote synaptic clustering of AMPARs, but not NMDAs, as well as increasing both the frequency and the amplitude of AMPAR-mediated currents (Schnell et al., 2002). This may occur through the interaction of PDZ1 and PDZ2 domains of PSD-95 with the Cter PDZ binding domain of Stargazin, which directly anchors AMPAR at the post-synapse (Chen et al., 2000; reviewed by Opazo et al., 2012). Conversely, knockdown of PSD-95 or PSD93 leads to a significant decrease (~50%) in the synaptic transmission mediated by AMPAR, without affecting the NMDAR-mediated one (Elias et al., 2006; Schlüter et al., 2006). Interestingly, PSD-95/PSD93 double knockout mice show an even stronger reduction (~75%) of AMPAR-mediated currents, in a presynaptic-independent manner, suggesting that AMPAR are excluded from the synapse in the absence of these two PSD-MAGUKs. On the other hand, SAP-97 and SAP102 were proven to rescue AMPAR-mediated synaptic transmission caused by knockdown of PSD-95 or PSD93, suggesting that MAGUKs can compensate each other in the PSD (Howard et al., 2010). These results are in good agreement with PSD-MAGUK role as synaptic organizers, making use of distinct PPIs to regulate a homeostatic pool of synaptic AMPA receptors at the PSD (Sun & Turrigiano, 2011), in a particular synapse and in a determined developmental stage (Sans et al., 2000; Beique et al., 2006; Elias et al., 2008).

**mLin-10.** The mammalian homolog of the *C. elegans* receptor targeting protein LIN-10 is also known as amyloid beta A4 precursor protein-binding family A member 1 (APBA1), neuron specific adapter protein X11alpha (X11) or neuronal Munc18-1-interacting protein 1 (Mint1). Lin-10 belongs to the MAGUK family, containing: an Nter that binds Munc18-1 and Lin2/CASK (Okamoto & Sudhof, 1997; Tabuchi et al., 2002),

playing a role in polarized protein localization pathways in both neurons and epithelia (Rongo et al., 1998); a phosphotyrosine-binding domain (PID/PTB) that mediates the interaction with the Cter of beta-amyloid precursor protein (APP), being responsible for APP's trafficking and processing in neurons (Borg et al., 1998); and two PDZ domains that are believed to anchor proteins to the plasma membrane. mLin-10 is enriched in the trans-Golgi network and it can be found in dendrites and spines, suggesting an active role in protein sorting and synaptic delivery (Stricker & Huganir, 2003). Indeed, mLin-10 was first shown to bind to the motor protein KIF17 via its PDZ domains (Setou et al., 2000). Importantly, this interaction took place within a large scaffolding protein complex that also contained NR2B subunits of the NMDA receptor. And recently, mLin-10 was reported to directly bind GluA1 through a PDZ domain interaction as well, possibly directing GluA1-containing AMPA receptors trafficking and proper localization in neurons (Glodowski et al., 2005).

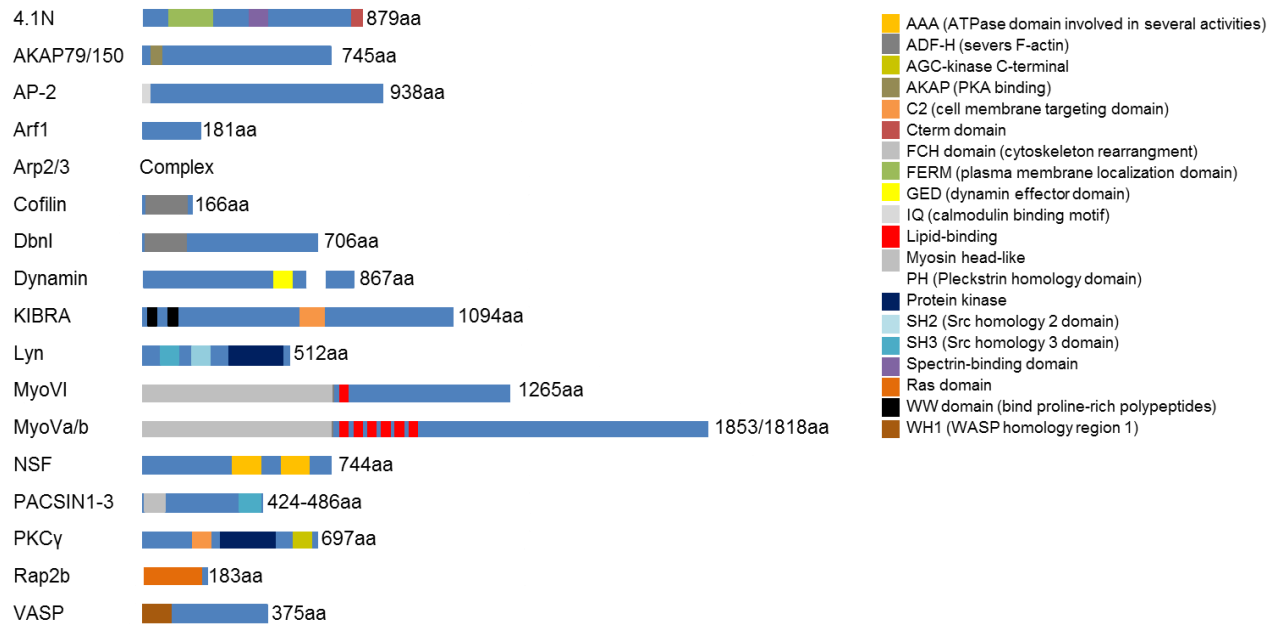
**SemaF.** SemaF is a synapse-associated E3 ubiquitin-protein ligase also known as semaphorin cytoplasmic domain-associated protein 3 (SEMACAP3) or PDZ domain-containing RING finger protein 3 (PDZRN3). It contains a RING-finger motif in its Nter, two PDZ domains in its central region and a consensus PDZ binding motif at its Cter (Kato & Kato, 2004). It was first identified *in silico* as a homolog of the ubiquitin ligase LNX1 (or SEMCAP1) that binds the membrane protein Semaphorin 4C. A yeast two-hybrid screen identified SemaF as a potential binder of GluA2 Cter (Meyer et al., 2004), though the functional implications of such interaction remain unknown. Nevertheless, SemaF was reported to regulate the surface expression of MuSK (muscle-specific receptor tyrosine kinase), the key organizer of postsynaptic development at the mammalian NMJ, by accelerating its endocytosis (Lu et al., 2007a). Also, a phage screen of brain cDNA products showed that the first PDZ domain of *D. rerio* Pdzrn3 can bind Kidins220/ARMS (Kinase D-interacting substrate of 220 kDa/Ankyrin Repeat-rich Membrane Spanning), an adaptor of neurotrophin receptors, thus suggesting that SemaF might play a role in neurogenesis (Andreazzoli et al., 2012).

**Syntenin.** The adaptor protein syntenin contains 2 PDZ domains that recognize class I, II and non-conserved PDZbds, as well as dimerize (Koroll et al., 2001). It is also known to interact with GluA1, GluA2(c), GluA4 and GluA3c, although its function is still not clear (Hirbec et al., 2002). Moreover, syntenin can directly bind the phosphatidylinositol biphosphate component of the plasma membrane (Zimmermann et al., 2002), and syndecans (Grootjans et al., 1997; Zimmermann et al., 2001), suggesting that it might play a role in the developmental regulation of neuronal membrane architecture (Hirbec et al., 2005). Also, syntenin PDZ2 was shown to regulate the plasma membrane insertion of the glycine transporter subtype 2, thus contributing to its trafficking and/or presynaptic localization in glycinergic neurons (Ohno et al., 2004).

#### **I.6.4.5.1.2 Non-PDZ domain-containing proteins**

In the recent years, a vast array of other type of proteins containing distinct structural domains were related to the regulation of AMPAR trafficking. Interestingly, the large majority is either a cytoskeleton member or do play a role in cytoskeleton-mediated events - **Fig. 24**.





**Figure 24: Cytosolic non PDZ domain-containing proteins known to regulate AMPAR trafficking** (see text).

**4.1N.** Protein band 4.1 was first isolated as a cell actin cytoskeleton protein in erythrocytes. The neuronal homolog - 4.1N - was then identified, existing as several splice variants and it can be detected in the PSD of some neurons (Walensky et al., 1999). 4.1N contains an Nter FERM domain, typically involved in the protein proper localization, a spectrin-actin binding region, and a Cter domain known to interact with GluA1 and GluA4 Cter membrane proximal domain (Shen et al., 2000; Coleman et al., 2003). Moreover, the 4.1N/GluA1 interaction was proven to be essential in the actin cytoskeleton-dependent AMPA trafficking. The same group also showed that the 4.1N/GluA1 interaction can be regulated by post-translation modifications of GluA1 Cter. While GluA1 Cter palmitoylation decreases 4.1N/GluA1 interaction, promoting AMPA receptors internalization (Hayashi et al., 2005), PKC phosphorylation of GluA1 Ser816 and Ser818 residues enhances such interaction, facilitating GluA1 insertion at the plasma membrane (Lin et al., 2009). Moreover, palmitoylation of GluA1 C811 was proven to modulate PKC-dependent AMPAR trafficking and possibly LTP expression. Nevertheless, the role of 4.1N in synaptic plasticity remains controversial, since mice containing only 12% of 4.1G/4.1N protein levels in the hippocampus present no changes in basic glutamatergic synaptic transmission and LTP (Wozny et al., 2009). On the other hand, 4.1N together with SAP-97 might be determinant components of rat cerebellar synaptogenesis, by differentially regulating GluA1 traffic and insertion in neurons and Bergmann glia cells (Douyard et al., 2007). Lastly, a similar protein band 4.1-like 3 (4.1B), is known to recruit NMDARs to SynCAM1 (synaptic cell adhesion molecule 1) adhesion sites, promoting an increase in the frequency of NMDAR-mediated mEPSCs (Hoy et al., 2009). In the same study, 4.1N was also shown to interact with SynCAM1 in COS7 cells, specifically affecting AMPAR recruitment.

**AKAP79/150.** A-kinase anchor proteins (AKAPs) are a group of structurally diverse proteins, whose common feature is the ability of binding the regulatory subunit of protein kinase A (PKA), thus confining the holo-

enzyme to discrete locations within the cell. AKAP79/150 in particular can anchor both PKA and CaN to MAGUK/AMPA complexes in spines (Dell'Acqua et al., 2006). The Nter of AKAP79/150 was proven to be essential in the proteins' dendritic targeting, allowing it to form a complex with phosphatidylinositol-4,5-bisphosphate (PIP2), F-actin, and actin-linked cadherin adhesion molecules. In addition, chemLTP-induced recruitment of AKAP79/150 to dendritic spines was related to an increase of Rab11-regulated endosome recycling of AMPARs as well as spine enlargement (Keith et al., 2012)

**AP2.** The clathrin adaptor complex AP2 is known to play a pivotal role in many vesicle trafficking events within the cell. A first study reported that NMDAR-dependent activated AMPARs co-localize with AP2 (Carroll et al., 1999), suggesting that the endocytic membrane trafficking mechanism could modulate synaptic neurotransmission. Shortly after, AP2 was reported to bind GluA2 within a region that overlaps the NSF binding site (Lee et al., 2002b). Using specific blocking peptides for each interaction, the same group found that AP2 is involved in NMDAR-induced, but not ligand-dependent, internalization of AMPA receptors and is essential for hippocampal LTD. In fact, AP2 can form a complex with the neuronal  $\text{Ca}^{2+}$  sensor hippocalcin, coupling NMDAR-dependent activation to regulated GluA2-containing AMPAR endocytosis following LTD (Palmer et al., 2005). Finally, the use of blocking peptides disrupting AP2 binding to GluA2 further confirmed that such interaction was responsible for the AMPAR internalization events underlying the loss of synaptic strength upon sensory deprivation in the visual cortex (Yoon et al., 2009) or the expression of LTD responsible for visual recognition memory in the perihinal cortex (Griffiths et al., 2008).

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**Arf1.** The small GTPase ADP-ribosylation factor 1 (Arf1) is a GTP-binding protein involved in the modulation of vesicle budding and uncoating within the Golgi complex. Arf1 was recently found to bind PICK1 as well, limiting its action on Arp2/3 activity and, consequently, affect synaptic AMPAR internalization and spine shrinkage (Rocca et al., 2013). This study strengthens, once more, the link between scaffold PDZ protein domains and the vesicle trafficking machinery in the modulation of structural and functional plasticity.

**Arp2/3.** The actin polymerization promoter Arp2/3 complex promotes actin polymerization by creating a new nucleation core, which constitutes the first step in the formation of F-actin. Actin nucleation is activated by the Wiskott-Aldrich syndrome family protein members (WASP, N-WASP, WAVE and WASH). In the last years, Arp2/3 was shown to be inhibited by the F-actin binding PICK1 protein during AMPAR-mediated LTD and spine morphogenesis events (Rocca et al., 2008; Nakamura et al., 2011). In particular, PICK1 can inhibit actin polymerization by competing with the N-WASP complex over Arp2/3 binding. Arp2/3 interaction is based on a single residue on PICK1 Cter (W413), which was proven to be critical for NMDAR-induced AMPAR internalization (Rocca et al., 2008).

**Cofilin.** The actin depolymerisation factor (ADF) domain containing protein cofilin severs F-actin and binds actin monomers (Fig.4). Cofilin was recently related to AMPAR trafficking during synaptic potentiation, linking dendritic spine morphology changes to functional-related postsynaptic parameters (Gu et al., 2010; Rust et

al., 2010). Cofilin is inactivated by LIM kinase and reactivated by the Slingshot phosphatase (Agnew et al., 1995; Huang et al., 2006). Dephosphorylated cofilin can bind and sever F-actin, promoting actin depolymerisation. Interestingly, LIMK has been linked to abnormal spine morphology, impaired synaptic plasticity and mental retardation (Meng et al., 2002), whereas Slingshot was recently showed to impact AMPAR-mediated synaptic plasticity and surface expression of AMPAR at synapses (Yuen et al., 2010).

**Dbn1.** The developmental-regulated brain protein Drebrin (Dbn1) contains an ADF domain like cofilin. This F-actin-binding protein is known to couple microtubule to F-actin in growth cone filopodia by interacting with the EB3 protein, thus playing an important role in neuronal migration and neuritogenesis (Geraldo et al., 2008; Mizui et al., 2009; Dun et al., 2012). It has also been involved the organization of F-actin arrangement in dendritic spines during synaptic plasticity (Hayashi & Shirao, 1999; Aoki et al., 2009), contributing to AMPAR-mediated spine morphogenesis as well (Takahashi et al., 2009). Dbn1 has been long implicated in several neurocognitive diseases and neurodevelopmental disorders, such as Alzheimer's disease or Down's syndrome (Harigaya et al., 1996; Hatanpää et al., 1999; Shim & Lubec, 2002; Kobayashi et al., 2004; Kojima et al., 2010; Counts et al., 2012; Lee & Aoki, 2012) suggesting that it does play a key role in brain function.

**Dynamin.** Dynamin is a microtubule-associated force-producing protein involved in vesicular trafficking processes such as the receptor-mediated endocytosis. It is composed by a PH (Pleckstrin homology) domain and a GED (GTPase effector domain) and domain, capable of binding inositol phosphates present in the plasma membrane, self-assembling into 'collars' at the necks of invaginated coated pits. Jaskolski and colleagues have shown (2009) that the dynamin GTPase activity is required not only to drive the lateral diffusion of newly exocytosed GluA2-containing AMPAR complexes at the dendritic shaft membrane, but also during NMDAR-evoked GluA2 internalization.

**KIBRA.** The kidney and brain protein KIBRA is highly expressed in kidney and in memory-related brain regions (Kremerskothen et al., 2003; Papassotiropoulos et al., 2006; Johannsen et al., 2008). It can interact with the polarity protein PATJ, contributing to the regulation of directional migration in podocytes (Duning et al., 2008). KIBRA can also play a role in the Hippo signalling pathway together with Merlin (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010) as well as to participate in intracellular trafficking by interacting with the dynein light chain 1 (Rayala et al., 2006; Rosse et al., 2009; Traer et al., 2007). In mammals, KIBRA has been implicated in long-term synaptic plasticity and memory retention (Sacktor et al., 1993; Drier et al., 2002; Büther et al., 2004). KIBRA was recently found to bind the PICK/AMPA complex, being crucial in NMDAR-dependent AMPAR recycling (Makuch et al., 2011). Moreover, KIBRA deficient mice display impaired LTP and LTD, as well as deficits in contextual fear learning and memory, pinpointing KIBRA as a key regulator of AMPAR trafficking and synaptic plasticity.

**Lyn.** The src tyrosine kinase Lyn contains an Nter SH3 domain, followed by a SH2 domain and a Cter protein kinase domain. Initial studies showed that Lyn can immunoprecipitate with GluA1, GluR2/3, and GluR4c

AMPA receptor subunits through its SH3 domain in the cerebellum (Hayashi et al., 1999). Following AMPAR stimulation, these interactions are believed to enable Lyn activation and subsequent trigger of the MAPK pathway, which would ultimately result in increased levels of BDNF mRNA, suggesting that Lyn can play a role in synaptic plasticity by regulating BDNF expression levels. In addition, a positive modulation of AMPA receptors was shown to prevent downregulation of GluA2 expression and activate the Lyn-ERK1/2-CREB signalling pathway in rat brain ischemia, suggesting that Lyn can be involved in neuro-protective events (Zhang et al., 2010).

**Myosins.** Myosins are actin-based motor molecules with ATPase activity, being the so called unconventional myosins involved in intracellular movements. Myosin VI (MyoVI) was the first to be directly linked to synaptic plasticity events. Myo6-deficient mice display a decreased number of hippocampal synapses, abnormally short dendritic spines and astrogliosis (Osterweil et al., 2005). Moreover, these mice display a significant deficit in AMPAR, but not NMDAR, internalization possibly due to the disruption of the MyoVI interaction with the AMPAR/SAP-97 complex (Nash et al., 2010). On the other hand, the  $\text{Ca}^{2+}$ -sensitive myosin motors Va and Vb were shown to drive AMPAR traffic from the dendritic shaft or recycling endosomes, respectively, into spines (Lise et al., 2006; Correia et al., 2008; Wang et al., 2008). MyoV motors can bind both the cargo binding domains of AMPAR, scaffold proteins like PSD-95 or GKAP, and the small GTPase Rab11, catalyzing AMPAR directional transport during activity-dependent synaptic plasticity. Lastly, MyoVa mutant mice display impaired glutamate synaptic development and disrupted mature plasticity in visual cortex (Yoshii et al., 2013), strongly suggesting that MyoVa plays a pivotal role on localizing and organizing mature glutamate synapses.

**Nrbl.** The F-actin binding protein neurabin I (Nrbl or Pp1r9a) was shown to control AMPAR surface expression at specific PSD microdomains by targeting the protein phosphatase 1 (PP1) (Hu et al., 2007). During basal synaptic transmission, Nrbl interferes with the consensus PKC phosphorylation site Ser880 of GluA2 Cter, known to favor GluA2 interaction with PICK1 over ABP/GRIP (Lu & Ziff, 2005). On the other hand, Nrbl-targeted PP1 seems to facilitate LTD by dephosphorylating Ser845 and Ser831 of GluA1, without affecting GluA2. These results suggest that Nrbl can fine tune the traffic of specific AMPAR subunits in response to distinct synaptic activities.

**NSF.** N-ethylmaleimide-sensitive factor (NSF) is an AAA ATPase typically involved in intracellular membrane fusion events. NSF functions as a SNAP receptors (SNARE) chaperone, binding SNARE complexes through soluble NSF attachment proteins (SNAPs). It then uses the energy of ATP hydrolysis to disassemble them thus facilitating SNARE recycling (reviewed by Zhao et al., 2007). NSF was also demonstrated to interact with the residues lying in between Lys844 and Gln853 of rat GluA2 (Nishimune et al., 1998; Osten et al., 1998). The use of blocking peptides of such interaction resulted in a diminished AMPA receptor-mediated synaptic transmission, a rapid decrease of the amplitude of mEPSCs (Song et al., 1998), as well as a dramatic reduction in surface expression of AMPA receptors (Noel et al., 1999; Ralph et al., 2001), strongly

suggesting that NSF regulates AMPA receptor function. Moreover, GluA2-containing AMPA receptors incapable of binding NSF were endocytosed in a greater extent than wild-type ones following AMPAR- or NMDAR stimulation (Braithwaite et al., 2002). In the same study, GluA2 mutants incapable of binding GRIP/ABP suggested that while the PDZ proteins can afford an intracellular pool of activity-dependent internalized receptors, NSF stabilizes them at the synapse by disrupting such interactions. Also, PICK1, but not NSF, was shown to regulate the formation of extra-synaptic plasma membrane pools of GluA2, which are prone to lateral translocation into synapses upon  $\text{Ca}^{2+}$ -dependent AMPA receptor plasticity (Gardner et al., 2005; Hanley, 2007). Conversely, BDNF stimulation induces a rapid surface translocation of AMPA receptors to the synapse, as well as an enhanced GluA2-NSF interaction in a  $\text{Ca}^{2+}$ -dependent manner (Narisawa-Saito et al., 2002). The use of pHluorin-tagged GluA2 subunits further confirmed that GluA2 insertion at the plasma membrane requires the NSF binding site (Araki et al., 2010). In addition, the NSF/GluA2 interaction was proven to be required and sufficient for synaptic, but not extra-synaptic, incorporation of GluA2-containing AMPA receptors (Beretta et al., 2005), as well as their competence to undergo LTD (Steinberg et al., 2004). On the other hand, the clathrin adaptor complex AP2 was reported to bind GluA2 within a region that overlaps NSF binding site (Lee et al., 2002b). Using specific blocking peptides for each interaction, the same group found that NSF is required to maintain synaptic AMPA receptors responses, but not to mediate NMDA receptor-mediated internalization of AMPARs and LTD. Instead, the NSF/GluA2 interaction seems to be essential for the GluA2-containing AMPAR-mediated LTP (Yang et al., 2010), and it was also suggested to be a target of PKMzeta, an autonomously active protein kinase C isoform known to maintain late-LTP by favouring AMPA receptor insertion at active synapses (Yao et al., 2008). Finally, GluA2/NSF interaction was implicated in lateral amygdala fear memory consolidation, but not retrieval or persistence (Joels & Lamprecht, 2010).

**PACSIN family.** The three members of the protein kinase C and casein kinase II substrate in neurons (PACSIN1-3) were recently shown to interact with the PICK1/AMPA complex (Anggono et al., 2013). Such interaction relies on the phosphorylation state of the variable region of PACSIN members. Phosphorylation of the neuronal-specific PACSIN1 is required for modulating PICK1-dependent AMPAR internalization. On the other hand, PACSIN2, highly expressed in Purkinje cells, was proven to be crucial to cerebellar LTD.

**PKC $\gamma$ .** The gamma isoform of the calcium-phospholipid-dependent protein kinase C (PKC $\gamma$ ) is widely distributed throughout the CNS. It was originally involved in both long-term potentiation (LTP) and learning and memory (Abeliovich et al., 1993a,b) as well as in neuropathic pain (Malmberg et al., 1997), strongly suggesting it can have a determinant role in synaptic plasticity. More recently, PKC $\gamma$  was co-immuno-precipitated with the Cter of GluA4 AMPAR subunit (Correia et al., 2003). Early in development, GluA4 is known to be highly expressed in the hippocampus; being its number dynamically regulated during synapse maturation. In rat hippocampal slices, GluA4 delivery to synapses requires the phosphorylation of Ser842 by PKA or PKC, which promotes GluA4 surface expression. PKC $\gamma$  is believed to assure PKC-driven phosphorylation and membrane targeting of GluA4 (Gomes et al., 2007). PKC $\gamma$  activation was also implicated in the activity-

dependent trafficking of AMPARs in embryonic zebrafish (Patten & Ali, 2009). Importantly, PKC $\gamma$ -driven AMPAR traffic requires AMPA receptors to associate with both PICK1 and NSF in an NMDAR- and SNARE-dependent manner.

**Rap2b.** This small GTPase is a member of the Ras oncogene family, being composed of a Ras domain responsible for its role in signalling transduction inside the cell. An early study suggested that Rap can counterbalance the Ras-induced AMPAR delivery during synaptic events (Zhu et al., 2002). While Ras mediates the NMDAR and CAMKII signalling during the synaptic insertion of AMPAR in LTP, Rap relays their removal during LTD. In addition, activated Rap2 has been found to promote dendritic pruning as well as reduce synaptic density (Zhu et al., 2005; Fu et al., 2007). Rap2-mediated AMPAR removal is believed to be reversed by the MINK (Misshapen/NIKs (Nck-interacting kinases)-related kinase), but not by its closely related TNIK (TRAF2/Nck-interacting kinase) (Hussain et al., 2010). Both these kinases are therefore thought to be required for normal dendritic arborisation and surface expression of AMPA receptors. Finally, it is interesting to note that Rap2b was recently reported as a potential biomarker candidate for schizophrenia (Martins-de-Souza et al., 2010).

**VASP.** The vasodilator-stimulated phosphoprotein (VASP) promotes actin filament elongation by protecting the barbed end of growing F-actin from capping proteins, allowing profilin-bounded actin monomers to be added. In dendritic spines, VASP can couple morphological changes to PSD-scaffolding proteins and AMPA receptors organization, eventually affecting synaptic transmission (Lin et al., 2010).

#### **I.6.4.5.2. Transmembrane proteins**

At the membrane level, so are some proteins involved in AMPAR trafficking. The composition of membrane and transmembrane proteins capable of regulating AMPAR surface expression are represented in **Fig. 25**.

**ADAM22/LGI1.** This complex was originally found alongside with Stgz to associate with PSD-95 in rat brain extracts (Fukata et al., 2006). Interestingly, the three proteins were previously implicated in epilepsy (Sagane et al., 2005; Steinlein, 2004; Nicoll et al., 2006). ADAM22 (disintegrin and metalloproteinase domain-containing protein 22) is a neuronal membrane protein composed by a large extracellular domain composed of a peptidase M12B domain, a desintegrin domain, a Cys-rich region and a EGF-like domain, followed by a transmembrane domain, and finally an intracellular Cter containing a class II Cter PDZbd (-ETSI). On the other hand, LGI1 (leucine-rich glioma-inactivated protein 1) is a secreted neuronal protein made by one LRRNT (leucine-rich repeat Nter) domain, 3 LRR repeats, one LRRCT and 7EAR (epilepsy-associated repeats). Fukata and colleagues (2006, 2010) showed that LGI1 can specifically bind to ADAM22 at the synapse and increase the AMPAR-mediated neurotransmission. It does so by possibly recruiting new AMPAR to the synaptic surface in a GRIP/PICK1 similar way. The epilepsy-related ADAM22/LGI complex is therefore the first description of secreted protein released by the pre-synaptic terminal that can bind a post-synaptic receptor and influence AMPAR trafficking. As such, the role of the relatively large-sized LGI1 (about 60 kDa)

as a neurotransmitter or neuromodulator remains rather controversial.

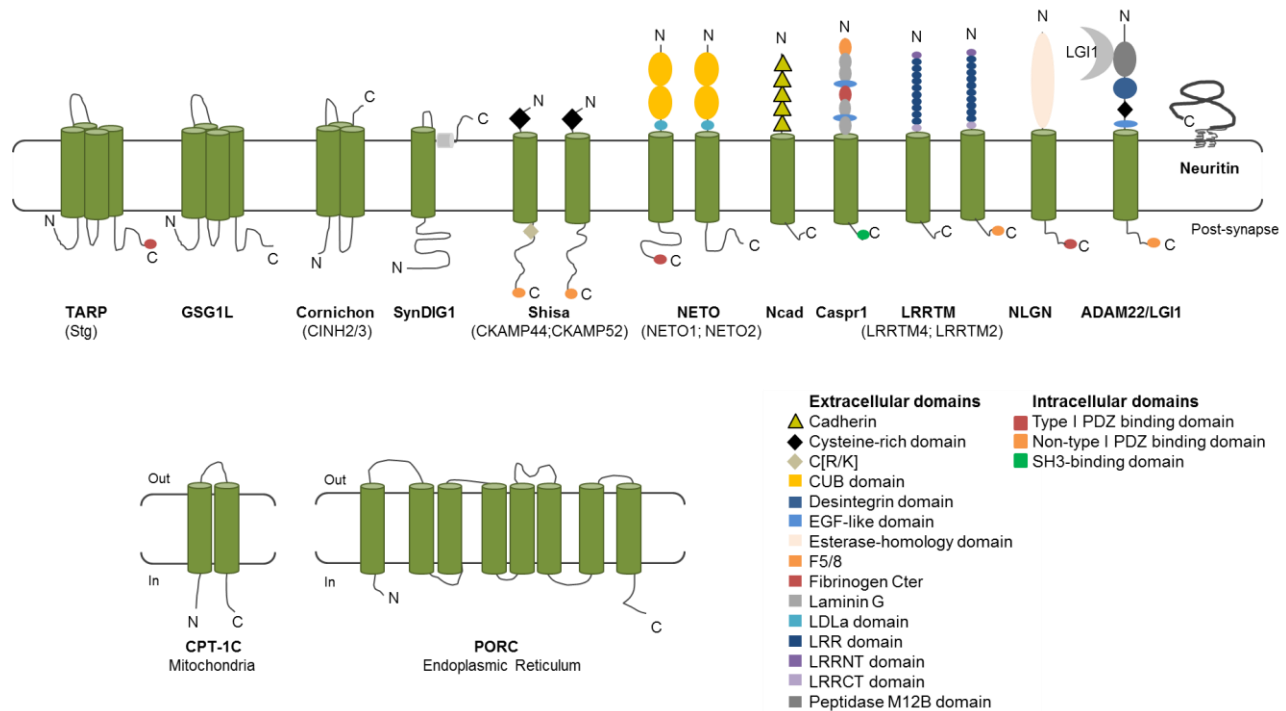


Figure 25: Transmembrane (and membranar) proteins known to regulate AMPAR trafficking (see text).

**Cntnap1.** The contactin-associated protein 1 (Cntap1 or Caspr1), also known as neurexin-4 or paranodin, was first implicated in the formation of functional distinct domains within the axo-glial junction of myelinated nerve fibres (Bhat et al., 2001). In particular, Cntap1 interacts with contactin to properly target potassium channels from the juxta- paranodal into the paranodal domain. Mice lacking Cntap1 exhibit tremor and ataxia, arguing for a pivotal role of Cntap1 in the regulation of peripheral nerve conduction. A recent study showed that Cntap1 is also present in synapses, where it can interact with GluA1, promoting its traffic and synaptic content (Santos et al., 2013).

**Cornichon.** Cornichon proteins are typically composed of a cytoplasmic Nter and three transmembrane domains. They were first studied in *D. melanogaster*, where they act as a cargo receptor recruiting the protein Gurken into COPII vesicles to the oocyte surface (Bökel et al., 2006). In yeast, the cornichon-related Erv14 protein is also involved with the ER export of the majority of transmembrane proteins (Castillon et al., 2009), whereas in the vertebrate CNS, cornichon is known to contribute to both transport and secretion of ErbB4 ligands (Hoshino et al., 2007). The cornichon homologs CNIH2 and CNIH3 were found by proteomic analysis of native AMPAR complexes (Schwenk et al., 2009). The same work showed that CNIH2/3 are present in both synaptic and extra-synaptic sites of the postsynaptic neuron, where they are believed to regulate AMPAR targeting to the synapse as well as their gating properties, alike TARPs. CNIH2/3 are expressed

throughout the rat central nervous system, apart from the cerebellar granule cells, where AMPAR regulation is carried out by Stgz (Chen et al., 2000). Schwenk and colleagues reported that ~70% of AMPARs are co-assembled with CNIH2/3 rather than with TARPs. This percentage is in good agreement with previous studies suggesting that not all AMPARs associate TARPs and that agonist binding can indeed prompt TRAP-AMPAR dissociation (Tomita et al., 2004; Morimoto-Tomita et al., 2009). Similarly to TARPs, cornichons are thought to increase surface expression of AMPARs by assisting the receptor biosynthesis and trafficking from the ER to the neuronal cell surface (Milstein & Nicoll, 2008; Greger et al., 2007; Schwenk et al., 2009; Shi et al., 2010; Harmel et al., 2012). Nevertheless, TARPs and cornichons are structurally distinct, with TARPs intracellular Cter playing a key role in AMPAR synaptic stabilization, as aforementioned. Conversely, although TARPs and cornichons slow AMPAR gating, the last are more effective than the first ones (Milstein et al., 2007). In particular, TARPs promote the recovery from desensitization and increase the response to kainate, whereas CNIH2/3 slow deactivation and desensitization kinetics. CNIH-induced AMPAR desensitization was reported to occur in glia cells as well (Coombs et al., 2012). Recently, cornichon proteins were proven to functionally interact with TARPs (Kato et al., 2010; Schober et al., 2011; Gill et al., 2012) and even regulate the number of TARPs within an AMPA receptor complex (Gill et al., 2011), thus modulating the receptor gating and pharmacology. Nevertheless, the AMPA receptors number capable of co-assembling with one and/or the other accessory proteins as well as the molecular determinants that regulate such mechanism are still unclear.

**CPT-1C & PORCN.** The carnitine O-palmitoyl-transferase 1C (CPT-1C) and the protein-cysteine N-palmitoyltransferase porcupine (PORCN) are multi-pass transmembrane proteins found to interact with native AMPARs (Schwenk et al., 2012). Both of them possess enzymatic activities involved in palmitoylation of cysteine residues, a post-translational modification previously shown to be important in AMPAR trafficking regulations (Hayashi et al., 2005). CPTC-1 is composed by 2 transmembrane domains and intracellular Nter and Cter, being located in the mitochondrial outer membrane where it can influence lipid metabolism and transport. A recent study underscored the importance CPT-1C in regulating the ceramide levels in hippocampal neurons and consequently modulating spine morphology (Carrasco et al., 2012). In particular, CPT-1C KO mice display a greater number of immature filopodia and less mature mushroom and stubby spines. Notably, treatment of cultured neurons with exogenous application of ceramide or overexpression of CPT-1C is enough to reverse this phenotype. CPT-1C KO mice also show a strong impaired spatial learning in the Morris water maze test, pinpointing the role of CPT-1C in dendritic maturation and proper spatial learning. Instead, PORCN can be found in the ER membrane, containing 8 TM domains and intracellular Nter- and Cter as well. Interestingly, PORCN was previously implicated in the serine palmitoylation of Wnt family members, being capable of modulating all Wnt ligand activity (Tanaka et al., 2000; Galli & Burrus, 2001; Proffitt & Virshup, 2012).

**Gsg1l.** The germ cell-specific gene 1-like protein (Gsg1l) is a newly identified binding protein and unique modulator of AMPAR gating found through proteomic studies to bind GluA2 subunits (Shanks et al., 2012;



Schwenk et al., 2012). Akin to its tetraspanning relatives, Gsg1l is composed of four TM domains and intracellular Nter and Cter, being an evolutionarily distant member of the claudin family. However, unlike TARPs, Gsg1l slows the recovery from the desensitized state in a similar way to CKAMP44 (von Engelhardt et al., 2010). This functional difference between structurally similar proteins might reside in the extracellular loop 1 of Gsg1l, known to be essential for the ion channel modulation (Menuz et al., 2008; Tomita et al., 2005), being substantially longer (~50%) and less conserved than Stgz/TARP. Of interest, Gsg1l was also pinpointed as a major candidate present in genetic loci predisposing to colon carcinogenesis in mice (Liu et al., 2012).

**LRRTMs.** The leucine-rich repeat transmembrane protein (LRRTM1-4) family members are single pass transmembrane proteins containing 10 extracellular Nter LRRs, which are highly and dynamically regulated during mouse development (Haines & Rigby, 2007). They are expressed in distinct tissues, especially in the neural tube where each gene is expressed in a unique domain, and they exhibit synaptogenic activity, behaving like synaptic cell-adhesion molecules (reviewed by Wright & Washbourne, 2011). LRRTMs localize to excitatory synapses, being capable of inducing presynaptic differentiation in contacting axons as well as mediate postsynaptic differentiation (Linhoff et al., 2009). Moreover, LRRTMs were reported to bind the same presynaptic receptor - neuroligins (NLGNs) - as neuroligins (NLGNs), likely in a synergistic manner (Ko et al., 2009; Siddiqui et al., 2010). This functional redundancy is thought to occur only during early synapse development, being both LRRTM and NLGN functionally divergent upon synapse maturation (Soler-Llavina et al., 2011). Dual LRRTM/NLGN regulation of synapse formation is believed to be dependent on synaptic activity and mediated by a postsynaptic  $\text{Ca}^{2+}$ /CaM-dependent signalling pathway (Ko et al., 2011). Importantly, LRRTM2 was shown to specifically regulate excitatory synaptogenesis and to interact with the scaffold PSD-95 protein through its class II Cter PDZ binding domain (-ECEV) (De Wit et al., 2009). LRRTM2 regulates AMPA receptors expression, modulating the strength of evoked excitatory synaptic currents. Structure-function studies suggest that LRRTM2 can induce presynaptic differentiation via its binding to NRX1 through the extracellular LRR domains. A similar role is expected for LRRTM4, a newly identified associate of native GluA2-containing AMPA receptors (Schwenk et al., 2012). LRRTM4 is the largest of the four LRRTM members and akin to LRRTM2, its mRNA can be detected from E15, whose expression levels pick at P1 and persist into adulthood (Lauren et al., 2003). In the hippocampus, LRRTM4 protein is highly abundant in the granule cell layer neuron of the DG, and is the only LRRTM member present in neurons of the CA3 pyramidal layer. Of note, human LRRTM1-3 members, but not LRRTM4, are located within introns of  $\alpha$ -catenin genes, suggesting that those LRRTMs can share a common mechanism with such adhesion proteins.

**N-cadherin.** Cadherins are calcium-dependent cell adhesion proteins associated with neuronal recognition mechanisms and the regulation of dendritic spine density (Yasuda et al., 2007). The overexpression of N-cadherin increases the frequency of mEPSCs, possibly through the synaptic recruitment of GluA2-containing AMPA receptors (Saglietti et al., Neuron 2007). The post-synaptic N-cadherin/GluA2 complex is believed to stimulate presynaptic development and function while promoting dendritic spine formation.

**Neto.** A genetic screen of *C. elegans* containing the *lurcher* mutation allowed Zheng and collaborators (2004) to find new AMPAR auxiliary proteins. The suppressor of *lurcher* (Sol1), a single pass transmembrane protein containing four extracellular CUB domains, was able to co-immunoprecipitate and co-localize with the GluA1 AMPAR at the cell surface. Furthermore, Sol1 mutants were reported to lack endogenous glutamatergic synaptic currents (Zheng et al., 2006), endorsing Sol1 role as a modulator of GluA1 gating properties. Besides the Sol1/GluA1 complex, the glutamate-gated currents are believed to be dependent on Stgz1, a *C. elegans* Stgz-like protein (Walker et al., 2006), or on a newly identified auxiliary protein - Sol2, another CUB-domain protein (Wang et al., 2012). Interestingly, Sol2 mutants exhibit a disrupted glutamatergic transmission like Sol1 mutants, with decreased GluA1-mediated currents and modified desensitization as well as pharmacology. Moreover, Sol2 expression seems to be required for the rescue of Sol1 mutant phenotype, pointing to a possible functional redundancy. Recently, the mammalian relatives of Sol1 and Sol2, named neuropilin tolloid-like 1 (Neto1) and 2 (Neto2) were identified in mice. Unlike its nematode homologues, they contain only two CUB domains and an LDLa (low-density lipoprotein receptor class A) domain (Stoohr et al., 2002; Michishita et al., 2003). Neto1 was found to co-immunoprecipitate with GluN2A and GluN2B, as well as PSD-95 in the hippocampal CA1 region, possibly via its Cter PDZ binding domain (-TTRV) (Ng et al., 2009). Neto1 is thought to be involved in the synaptic delivery and/or stability of these NMDA receptor subunits. Additionally, Neto1 KO mice display impaired spatial learning and LTP, further proving Neto1 role in NMDAR-mediated signalling. Conversely, Neto2 was shown to associate with GluK2, but not GluA1 receptors in cerebellar extracts (Zhang et al., 2009a). The cerebellum of GluK2 KO mice exhibit 60% less Neto2, strengthening the causal link between Neto2 and kainate-type glutamate receptors. Contrasting with Sol1 and Sol2, Neto1 and Neto2 seem to finely tune distinct glutamatergic currents – NMDAR and kainite receptor-mediated, respectively. Their involvement in the regulation of AMPA receptors is still not clear, although a previous study in *C. elegans* demonstrated that Sol1 and Sol2 can play a role in the adult nervous system to control AMPAR-mediated currents (Wang et al., 2012).

**NLGNs.** The neuroligin family is composed of four postsynaptic cell-adhesion molecules - NLGN1-4. All of them contain a large extracellular esterase-homology domain, capable of establishing contact with presynaptic NRXs (Ichtchenko et al., 1995; Nguyen & Südhof, 1997), a transmembrane domain, and an intracellular Cter, containing a class I PDZ binding domain (-TTRV) able to interact with PDZ domain-containing proteins (Irie et al., 1997; Meyer et al., 2004). NLGNs were first identified in excitatory synapses (Song et al., 1999), promoting both differentiation and assembly of contacting presynaptic terminals (Scheiffele et al., 2000; Dean et al., 2003) and consequently mediating synapse formation (reviewed by Dean & Dresbach, 2006; Lisé & El-Husseini, 2006; Craig & Kang, 2007). In humans, genetic alterations of NRXs or NLGNs have been implicated in autism and other cognitive disorders (Chubykin et al., 2005; Südhof, 2008), as well as in angiogenesis (Bottos et al., 2009), pinpointing the crucial role of these transmembrane proteins in brain development and cognition. Importantly, NLGN1 overexpression was shown to potentiate NMDAR/AMPA ratios (Chubykin et al., 2007). Conversely, NLGN1 KO mice exhibit a destabilized excitatory synapse organization (Zeidan & Ziv, 2012), as well as decreased levels of synaptic AMPAR and AMPAR-dependent synaptic

transmission (Mondin et al., 2011). On the other hand, NLGN2 overexpression increased inhibitory synaptic responses, suggesting that different NLGNs can specify and validate distinct types of synapses via an activity-dependent mechanism (Chubykin et al., 2007). A recent study further supports this idea, by showing that a Cter mutation of NLGN1 (E740N) significantly reduces the enhancement of AMPAR-mediated EPSCs, whereas a mutation of the homologous residue in NLGN3 (E747N) decreases both AMPAR and NMDAR responses (Shipman et al., 2011). Moreover, this Cter residue seems to specifically modulate excitatory synaptic transmission, since an equivalent mutation of NLGN2 (E740N) has no effect. Notably, an autism-linked point mutation in the Cter of NLGN3 (R704C) was reported to selectively impair AMPAR-mediated synaptic transmission in hippocampus (Etherton et al., 2011). Indeed, AMPA receptors were shown to be recruited into mature synapses and trapped at PSD-95 clusters lying at nascent NRX-NLGN complexes in primary hippocampal neurons (Heine et al., 2008; Mondin et al., 2011). Such enlistment occurs in a rapid and activity-independent manner, allowing calcium to entry in the newly formed synapses through the inserted AMPAR. This result is in good agreement with the fact that NRX-NLGN1 trans-synaptic adhesions induce an initial NMDAR-independent transient morphological stabilization, while persistent and mature synapses require NMDAR activity (Chen et al., 2010a). Finally, NRX-NLGN adhesion complexes appear to be a product of surface insertion and internalization arrest of these proteins in the pre- and post-synapse, respectively (Thyagarajan & Ting, 2010).

**Shisa.** The Shisa family was originally composed of single-transmembrane proteins that possess an Nter cysteine-rich domain and a proline-rich Cter region. But recently, distantly related Shisa homologs were found in vertebrates, giving rise to a new superfamily named STMC6 (single-transmembrane proteins with conserved six cysteines) (Pei & Grishin, 2012). STMC6 proteins - Shisa/Shisa-like, FAM159, KIAA1644, WBP1/VOPP1, CX, DUF2650, TMEM92, and CYR1 - are frequently composed of PY motifs, known to interact with WW-domain-containing proteins such as NEDD4 family E3 ubiquitin ligases. The *Xenopus* Shisa was the first member to be described, promoting head formation by inhibiting Wnt and FGF signalling pathways through the regulation of Frizzles and FGF receptor maturation in the ER (Yamamoto et al., 2005). Several other studies in different species withstand Shisa role in modulating these two PCP pathways (He, 2005; Schlesinger & Shilo, 2005; Katoh & Katoh, 2005; Silva et al., 2006; Filipe et al., 2006; Nagano et al., 2006; Furushima et al., 2007; Hedge & Mason, 2008; Zhu et al., 2008). More recently, a mouse brain-specific Shisa protein containing eight highly conserved Nter cysteines and a Cter PDZ binding motif (-EVTV) was found to associate with AMPA receptor complexes in synaptic spines (von Engelhardt et al., 2010). Within the brain, CKAMP44 (cystine-knot AMPA receptor modulating protein with a predicted mass of 44 kD; also known as mouse shisa homolog 9) is most abundant in the hippocampus, especially in the dentate gyrus granule cell layer. Unlike TARPs, CKAMP44 is believed to decrease AMPAR responses like GSG1L does, increasing the receptor desensitization without affecting its surface expression. Moreover, removing CKAMP44 in the DG enhances short-term facilitation, suggesting that this Shisa protein may set the extent of short-term plasticity. Another cystine-knot AMPAR modulating protein (52kDa) – CKAMP52, was recently suggested to be able to interact with native AMPARs (Schwenk et al., 2012). Also known as protein shisa-6,

CKAMP52 is more closely related to each of the Shisa6-9 members than other Shisa subfamilies, namely Shisa1-3 and 4-5 (Pei and Grishin, 2012). Shisa6-9 proteins main distinctive feature is the lack of cysteines in the juxtaposed TM Cter, suggesting a loss of lipid modification sites. Notably, only Shisa2 and Shisa6-9 proteins possess a Cter PDZ binding motifs (-AVTV and -EVTV, respectively). Indeed, STMC6 members are believed to act as transmembrane adaptors, regulating the surface expression of membrane proteins, like cell surface receptors, through PDZ or PY domain interactions.

**SynDIG1.** The synapse differentially induced gene 1 (SynDIG1), also known as DSPC2 (dispanin subfamily C member 2) or Tmem90b (transmembrane protein 90B), is a single transmembrane protein containing a cytoplasmic Nter and an extracellular Cter. SynDIG1 was found to interact with both GluA1- and GluA2-containing AMPA receptors at developing synapses in dissociated rat hippocampal neurons (Kalashnikova et al., 2010). Furthermore, SynDIG1 levels were shown to modulate both AMPA receptors number and function in developing excitatory synapses. SynDIG1-mediated synapse development is believed to be regulated by neuronal activity and to depend on the interactions of its extracellular Cter with the AMPA receptors. SynDIG1-dependent synaptic AMPAR targeting is believed to occur either by a direct SynDIG1/AMPA interaction that would promote their traffic to the synapses or, alternatively, by a more indirect role of SynDIG1, capable of priming newly formed synapses to receive AMPARs through other auxiliary proteins, like TARPs (reviewed by Diaz, 2012). Of note, two structurally related proteins were recently found in a high resolution proteomic study. The proline-rich transmembrane proteins 1 and 2 (PRRT1/2), also known as SynDIG4 or DSPD1 (dispanin subfamily D member 1), associates specifically with native GluA1-containing AMPA receptors (Schwenk et al., 2012). PRRT1/2 are composed of two transmembrane and domains, with both extracellular Nter and Cter. Their involvement in AMPAR regulation is still unknown.

**TARPs.** The neuronal voltage-dependent calcium channel  $\gamma$ -2 subunit (also known as stargazing, Stgz) is a member of the extended family of transmembrane AMPAR regulatory proteins (TARP). Stgz was the first isolated transmembrane protein found to interact with GluA1, GluA2, and GluA4 AMPAR subunits (Chen et al., 2000). The Stgz mutant mouse, or stargazer, is characterized by absence seizures and cerebellar ataxia, endorsed by the lack of functional AMPARs in cerebellar granule cells (Schnell et al., 2002). Together with  $\gamma$ -3,  $\gamma$ -4 and  $\gamma$ -8, Stgz is a type I TARP, as it can rescue AMPAR-mediated currents. On the contrary, type II TARPs -  $\gamma$ -1,  $\gamma$ -5,  $\gamma$ -7, and claudin-1 - fail to do so (Tomita et al., 2003). TARPs are widely expressed in the CNS (Burgess et al., 1999, 2001; Klugbauer et al., 2000; Moss et al., 2002), although the different members are strictly segregated in terms of time and place of expression, suggesting distinct roles for TARPs isoforms (Tomita et al., 2003; Fukata et al., 2005; Lein et al., 2007). For example, Stgz occurs mainly in the cerebellum,  $\gamma$ -3 in the cerebral cortex,  $\gamma$ -4 in the olfactory bulb, and  $\gamma$ -8 is dramatically enriched in the hippocampus. In addition,  $\gamma$ -4 is expressed in both neuronal and glia cells; while  $\gamma$ -8 is strictly present in neurons. Also, while  $\gamma$ -2,  $\gamma$ -3, and  $\gamma$ -8 reach high expression levels in adulthood,  $\gamma$ -4 peaks at P6, decreasing with time (see Jackson & Nicoll, 2011 for a complete review). TARPs are typically composed by, regardless of the type, a rather conserved intracellular Nter, four transmembrane domains and a variable intracellular Cter. In fact, this

last feature is responsible for the temporal and spatial specificity of the family members as well as their traffic and gating properties of AMPA-selective glutamate receptors (Tomita et al. 2004, 2005b, Turetsky et al., 2005, Bedoukian et al., 2006; Sager et al., 2009; Milstein & Nicoll, 2009). Type I TARPs possess a rather conserved Cter class I PDZ binding motif (-RR/KTTPV), able to bind to several PSD abundant scaffold proteins like the PDZ domain-containing protein PSD-95 or related members of the MAGUK family (Dakoji et al., 2003; Deng et al., 2006). Moreover, all these proteins are known to regulate AMPAR trafficking (Kim & Sheng, 2004; Elias & Nicoll, 2007), but they cannot directly bind to AMPARs, strengthening TARPs intermediary role in anchoring and stabilizing synaptic targeted tetrameric AMPA receptors. Further, other AMPAR Cter-interacting proteins, like GRIP, PICK1 or NSF, were undetectable in native AMPAR complexes, suggesting that such interactions are less stable and/or more transitory than the bona fide AMPAR auxiliary subunit Stgz (Vandenberghe et al., 2005a). The Stgz/PSD-95 interaction is by far the best characterized regulator of AMPAR clustering at the synapse, being its modulation finely tuned through posttranslational modifications. PKA phosphorylation of Stgz Thr321 residue within the PDZ binding motif prevents Stgz binding to PSD-95, inducing a loss of synaptic AMPAR clusters (Chetkovich et al., 2002; Choi et al., 2002). Conversely, synaptic NMDAR activity can trigger Stgz phosphorylation via CaMKII and PKC or dephosphorylation through the PP1/PP2B pathway, thus inducing a bidirectional control over LTP or LTD, respectively (Kessels & Malinow, 2009; Opazo et al., 2010). On the other hand, PSD-95 de-palmitoylation is required for a rapid glutamate-mediate AMPA receptor internalization, regulating synaptic strength as well (El-Husseini et al., 2002). Indeed, growing evidences support the idea in which the Stgz/PSD-95 complex transiently stabilizes laterally diffusing AMPARs in the PSD (Bats et al., 2007; Sainlos et al., 2011).

#### **I.6.4.5.3 Membrane-associated protein**

**Neuritin.** Initially it was identified as CPG15 (candidate plasticity gene 15) in a screen for plasticity-related genes in the hippocampus (Nedivi et al., 1993). This activity-regulated gene encodes a glycosylphosphatidylinositol (GPI)-linked protein able to promote dendritic arbour growth of neighbouring neurons via an intercellular signalling mechanism both in development and adulthood. (Nedivi et al., 1998; Corriveau et al., 1999; Javaherian & Cline, 2005). It was recently proved to associate with native AMPA receptors (Schwenk et al., 2012). This finding is in good agreement with a previous study, where CPG15 was shown to stimulate synaptic maturation by recruiting AMPARs to the post-synapse, coordinating both the number and the strength of synaptic contacts (Cantalops et al., 2000). It does so by acting like an immediate early gene, being induced by the  $\text{Ca}^{2+}$  influx through activated NMDA receptors and L-type voltage-sensitive calcium channels and requiring the activation of both CaMK and MAPK pathways (Fujino et al., 2003). Finally, CPG15 KO mice exhibit delayed developmental maturation of both axonal and dendritic arbors (Fujino et al., 2011). Loss of CPG15 reflects in hindered synaptic maturation and ultimately in poor learning, but sustained memory.

#### **I.6.4.5.4 Secreted proteins and ECM**

Recent studies are bringing to light a possible role of newly described secreted proteins, known hormones or even the extracellular matrix in the regulation of AMPAR trafficking – **Fig. 26**.



**Figure 26: Secreted proteins known to regulate AMPAR trafficking** (see text).

**Brorin & Brorin-2l.** The brain-specific chordin-like protein (Brorin) or CR (chordin-like cysteine-rich) domain-containing adhesive protein or von Willebrand factor C domain-containing protein 2 (Vwc2) and its close relative Brorin-2l (Vwc2l) were recently found to interact with native GluA2-containing AMPA receptors (Schwenk et al., 2012). Both these secreted protein contain 2 Cter VWFC domains, which are usually present in blood plasma proteins like the complement factors or collagen. The best known so far is Brorin, predominantly expressed in the brain, being present only in neurons but not in glial cells (Koike et al., 2007). Firstly described as bone morphogenetic antagonists, both are believed to play a role in neural development as well (Miwa et al., 2009; Ohyama et al., 2012).

**Corticosterone.** The stress hormone corticosterone is a corticosteroid known to regulate excitatory transmission and synaptic plasticity (Karst et al., 2005; Wiegert et al., 2006). Within hours, it is able to preferentially increase AMPAR-mediated transmission via intracellular glucocorticoid receptors, impairing synaptic potentiation (Kim et al., 2002; Karst & Joels, 2005). Single quantum-dot imaging in live rat hippocampal neurons revealed that corticosterone promotes the surface mobility of GluA2-containing AMPAR as well as its synaptic content in an activity-dependent manner (Groc et al., 2008).

**Leptin.** Leptin is a hormone that can cross the blood-brain barrier and influence several brain functions, including activity-dependent synaptic plasticity in the hippocampus (Harvey, 2007; Shanley et al., 2001; Dura-kogluligil et al., 2005; Moulton et al., 2009). Leptin was recently reported to promote GluA1 cell surface expression through a mechanism that requires NMDAR activation and subsequent inhibition of the lipid phosphatase PTEN (Moulton et al., 2010).

**Narp.** The neuronal activity-regulated pentraxin (Narp) is a secreted immediate-early gene whose expression is regulated by synaptic activity (Tsiu et al., 1996). Narp contains a Cter pentaxin domain, belonging to the pentraxin family, whose elements are known to self-multimerize and form pentamers and/or decamers. Narp was initially shown to immunoprecipitate with GluA1-3 AMPA receptor subunits in HEK 293T cells (O'Brien et al., 1999). In neurons, presynaptic Narp expression promoted the clustering of post-synaptic AMPA receptors (O'Brien et al., 2002). Moreover, Narp overexpression significantly increased the number of excitatory synapses. Remarkably, Narp secretion by hippocampal axons selectively targeted the aggregation of AMPARs in interneurons (Mi et al., 2002). Recent studies suggest that Narp might be involved in enduring forms

of neuronal plasticity (Reti & Baraban, 2000; Pacchioni & Kalivas, 2009; Pacchioni et al., 2009; Chang et al., 2010).

**Olfm1.** The neuronal olfactomedin-related ER localized protein (Olfm1), also known as pancortin-1 or noelin was recently co-purified with AMPARs (von Engelhardt et al., 2010; Schwenk et al., 2012). Olfm1 belongs to the Pancortin family, whose elements (1-4) are differentially expressed in the brain and during development, suggesting that they might perform different functions in neural development (Nagano et al., 1998, 2000). Indeed, even though all the member of the Pancortin family possess a Cter olfactomedin-like domain, only Olfm1 seems to contain a class I PDZ binding domain (-SDEL). Interestingly, Olfm1 was shown to regulate the retinal axon growth in zebrafish, acting like a Nogo A receptor (NgR1) ligand (Nakaya et al., 2012), and modulating the Wnt signalling pathway (Nakaya et al., 2008).

**ECM.** Once the mature synaptic circuitry is established, most of the synapses are wrapped by a dense extra-cellular matrix (ECM) mainly composed by glycoproteins and proteoglycans (Köppe et al., 1997; John et al., 2006). ECM is believed to play a key role in stabilizing and maintaining synaptic networks in the adult CNS (Celio & Blumcke, 1994; Pizzorusso et al., 2002). Frischknecht and colleagues (2009) elegantly showed that, due to its net-like structure, ECM is able to compartmentalize the neuronal surface, restricting AMPAR mobility and in particular the availability of naïve receptors at the synaptic surface. Moreover, the enzymatic removal of ECM could not only increase the extra-synaptic diffusion and synaptic exchange of AMPARs, but also increase AMPAR-mediated short-term synaptic transmission.

## Chapter II

# AIM OF THE STUDY & PROJECT OUTLOOK

### Aim of the study

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Scribble is a large multidomain scaffold protein from the LAP family, composed by 16 Nter LRRs (leucine-rich repeats) and 4 Cter PDZ (PSD-95/Discs-large/ZO-1) domains. Scrib is a well-established key regulator of apico-basal polarity, presynaptic architecture and short-term synaptic plasticity in *Drosophila* (Bilder & Perrimon, 2000). Its mammalian homolog Scrib1 has been shown to play a crucial role in both developing and adult central nervous system (Murdoch et al., 2003; Moreau et al., 2010). Heterozygous transgenic mice in which 50% of expressed Scrib1 lacks PDZ3 and PDZ4, have aberrant hippocampal dendritic spines and deficits in synaptic plasticity and behavior that have been linked to ASD (Moreau et al., 2010). The main goal of my work was to further investigate the molecular mechanisms underlying Scrib1 role in synaptic formation and maintenance.

### Project outlook

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I started by identify and validate potential specific protein interactions of the missing Scrib1<sup>crc/+</sup> PDZ3 and PDZ4 domains that could, at the molecular level, explain the Scrib1-dependent autistic phenotype (**Chapter III**). I present two works in which PDZ3 and PDZ4 domains interactions were involved, respectively, in NMDA receptors trafficking (**Chapter IV**) and bidirectional plasticity signalling pathway underlying spatial memory (**Chapter V**). As my main project, I assessed the structural genomic consequences of the recently identified Scrib1 *de novo* mutation c.1774C>T in human autism (Neale et al., 2012) (**Chapter VI**). I showed that disruption of Scrib1-mediated actin regulation impacts neuronal complexity, dendritic spine formation and maintenance. Since Scrib1 can bind AMPA receptors through transmembrane auxiliary proteins (TARPs), I show preliminary results regarding Scrib1 role in subunit-specific AMPAR endo- and exocytosis as well (**Chapter VII**). Taken altogether, this thesis highlights the PCP protein Scrib1 as key scaffold protein in brain development and function, playing a plethora of roles from the subcellular to the cognitive level.





## Chapter III

### Protein-protein interactions of Scrib1 PDZ3 and PDZ4 domains

#### III.1 Context and problematic

Circletail (*Scrib1<sup>crc/crc</sup>*) mice, expressing a truncated form of Scrib1 lacking PDZ3 and PDZ4 domains, display neural tube defects (NTD), a lethal form of PCP defect (Murdoch et al., 2003) (**Chapter I.3.3**). On the other hand, previous work in our lab showed that heterozygous mice (*Scrib1<sup>crc/+</sup>*), expressing only 50% of this truncated protein form exhibit ASD-like deficits (Moreau et al., 2010) (**Chapter I 5.4.2**). We started then by identify and validate potential protein interactions of the missing Scrib1<sup>crc</sup> PDZ3 and PDZ4 domains that could, at the molecular level, explain the Scrib1<sup>PDZ3/PDZ4</sup>-dependent NTD, PCP and ASD phenotypes. PDZ domains were originally identified as small regions (80-90 aminoacids) of sequence homology found in diverse signalling proteins (Cho et al., 1992; Wood & Bryant, 1993; Kim et al., 1995). PDZ consists of 6  $\beta$ -strands and 2  $\alpha$ -helices folded into a compact globular manner, having its N- and Cter close to each other (reviewed by Harris & Kim, 2001). Distinct sequence variations in the residues that line within the PDZ pocket dictate its size, shape, and consequently its preference for specific PDZ binding domains or other PDZ domain (reviewed by Lim & Harris, 2001; Sheng & Sala, 2001; Kim & Sheng, 2004; Subbaiah et al., 2011). Given the great diversity of PDZ binding specificities, PDZ-based protein complexes are highly specialized, enhancing the rate and fidelity of signal transduction within the complex. The characterization of our final selected Scrib1 interacting candidates will be further described in the following Chapters.

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#### III.2 Material and Methods

*Yeast two-hybrid (Y2H) screen.* Scrib1 PDZ3 or PDZ4 domains (990-1079 aa and 1086-1180 aa, respectively from Q80U72-3) were subcloned into a pGBTK7 vector (Clontech) in-frame with the DNA-binding domain of GAL4 and used as a bait for the screening as previously described (Sans et al., 2003). Y2H screening was performed accordingly to the Matchmaker™ Gold Yeast Two-Hybrid System protocol (Clontech). AH109 cells expressing Scrib1<sup>PDZ3</sup> or Scrib1<sup>PDZ4</sup> were combined with Y187 cells expressing a P10 mouse brain cDNA library. The mating mixture was plated on SD/Ade<sup>-</sup>/Trp<sup>-</sup>/Leu<sup>-</sup>/His<sup>-</sup> (4DO media) plates. A total of  $3.5 \times 10^3$  (Scrib1<sup>PDZ3</sup>) and  $1.5 \times 10^3$  (Scrib1<sup>PDZ4</sup>) colonies obtained 5 days after transformation were rescued according to the manufacturer's instructions (RPM® yeast plasmid isolation kit, MP Biomedicals), amplified (PuReTaq Ready-To-Go PCR, GE Healthcare), sequenced (BigDye® Terminator v1.1, Applied Biosystems) and identified through the bioinformatic tools BLAST and Expasy translation. From those, only 98 (**Table 9**) and 56 (**Table 10**) candidates, respectively, were confirmed to be positive interactions.

**TABLE 9: General characterization of the final yeast-two hybrid candidate interactions of Scrib1 PDZ3 domain:** function, cellular localization, presence of PDZ binding domains (PDZbd) or PDZ domains, relevance for neural tube defect (NTD), planar cell polarity (PCP), autism spectrum disorders (ASD) or ASD related disorders (ASD-RD).

Protein	Protein name	Function	Localization	PDZbd or PDZ domain	NTD, PCP, ASD, ASD-RD
<b>Aamp</b>	Angio-associated migratory protein	Other	Other	-	-
<b>Abcb8</b>	ATP-binding cassette, sub-family B (MDR/TAP), member 8	Transmembrane transporter	Mitochondria	-	-
<b>Abhd11</b>	Abhydrolase domain containing 11	Other	Mitochondria	-	-
<b>Actb</b>	Actin, beta, cytoplasmic	Cytoskeleton organization	Axon	-	ASD-RD
<b>Aldh9a1</b>	Aldehyde dehydrogenase 9, subfamily A1	Cellular metabolism	Mitochondria	-	ASD-RD
<b>Alkbh4</b>	AlkB, alkylation repair homolog 4 (E. coli)	Cellular metabolism	Nucleus	-	-
<b>Ankrd50</b>	Ankrin repeat domain 50	Other	Other	-	-
<b>Anks1b</b>	Ankyrin repeat and sterile alpha motif domain containing 1B	Receptor binding	PSD membrane	-	-
<b>Ap2m1</b>	Adaptor protein complex AP-2, mu1	Protein traffic and transport	Recycling vesicle	-	-
<b>Apba1</b>	Amyloid beta (A4) precursor protein binding, family A, member 1	Cell signaling	Intracellular	Class I PDZbd	-
<b>Arcn1</b>	Archain 1	Protein traffic and transport	Recycling vesicle	-	-
<b>Arf5</b>	ADP-ribosylation factor 5	Cytoskeleton organization	Intracellular	Internal PDZ	-
<b>Arhgef7</b>	Rho guanine nucleotide exchange factor (GEF7)	Cytoskeleton organization	Cell junction	-	PCP, ASD-RD
<b>Asb6</b>	Ankyrin repeat and SOCS box-containing protein 6	Cell signaling	Intracellular	-	-
<b>Atp1b1</b>	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, beta 1 polypeptide	Transmembrane transporter	Apico/basal membrane	Class I PDZbd	-
<b>Atp2b1</b>	ATPase, Ca <sup>++</sup> transporting, plasma membrane 1	Cell signaling	Apico/basal membrane	Class I PDZbd	-
<b>Atrn</b>	Attractin	Receptor activity	Transmembrane	-	-
<b>B4galt2</b>	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 2	Cellular metabolism	Transmembrane	-	-
<b>C1qc</b>	Complement component 1, q subcomponent, C chain	Other	Other	-	-
<b>Cacng4</b>	Calcium channel, voltage-dependent, gamma subunit 4	Transmembrane transporter	PSD membrane	Class I PDZbd	-
<b>Cdk5</b>	Cyclin-dependent kinase 5	Receptor binding	Cell junction	-	NTD
<b>Centa1</b>	Centaurin, alpha 1	Cytoskeleton organization	Intracellular	-	-
<b>Chst1</b>	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	Cellular metabolism	Transmembrane	-	ASD-RD
<b>Clptm1</b>	Cleft lip and palate associated transmembrane protein 1	Other	Extracellular	-	-
<b>Clstn1</b>	Calsyntenin 1	Cell adhesion	Cell junction	Class I PDZbd	-
<b>Cltc</b>	Clathrin, heavy polypeptide	Protein traffic and transport	Recycling vesicle	-	-
<b>Cnp</b>	2',3'-cyclic-nucleotide 3'-phosphodiesterase isoform 1	Kinase	Intracellular	-	ASD-RD
<b>Cnrip1</b>	Cannabinoid receptor interacting protein 1	Other	Other	-	-
<b>Comm3</b>	COMM domain containing 3	Other	Other	-	ASD-RD
<b>Cst3</b>	Cystatin C	Other	Other	-	-
<b>Ctnnb1</b>	Catenin (cadherin associated protein), beta 1	Cell adhesion	Cell junction	Class I PDZbd	ASD-RD
<b>Ctnnd2</b>	Catenin (cadherin associated protein), delta 2	Cell adhesion	Cell junction	-	ASD-RD
<b>Ctsd</b>	Cathepsin D	Cellular metabolism	Extracellular	-	ASD-RD

<b>Dmwd</b>	Dystrophia myotonica-containing WD repeat motif	Other	Other	-	ASD-RD
<b>Dnm1l</b>	Dynamin 1-like	Cytoskeleton organization	Intracellular	-	ASD-RD
<b>Dscr3</b>	Down syndrome critical region gene 3	Other	Nucleus	-	ASD-RD
<b>Eif3i</b>	Eukaryotic translation initiation factor 3, subunit I	mRNA	Intracellular	-	-
<b>Eno1</b>	Enolase 1, alpha non-neuron	Cellular metabolism	Extracellular	-	-
<b>Gabarapl1</b>	Gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	Receptor binding	Dendrite	-	-
<b>GAPDH</b>	Glyceraldehyde-3-phosphate dehydrogenase	Cellular metabolism	Mitochondria	-	-
<b>GluN2A</b>	Glutamate receptor, ionotropic, NMDA2A (epsilon 1)	Receptor activity	Transmembrane	Class I PDZbd	ASD, ASD-RD
<b>Grina</b>	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	Other	Transmembrane	-	-
<b>Gusb</b>	Glucuronidase, beta	Cellular metabolism	Lysosome	-	ASD-RD
<b>Inpp5j</b>	Inositol polyphosphate 5-phosphatase J	Phosphatase	Cell junction	-	NTD, PCP
<b>Itgb1bp1</b>	Integrin beta 1 binding protein 1	Cell adhesion	Intracellular	Class I PDZbd	-
<b>Itm2a</b>	Integral membrane protein 2A	Other	Transmembrane	-	-
<b>Klh20</b>	Kelch-like 20 (Drosophila)	Protein degradation	Intracellular	-	-
<b>Klh5</b>	Kelch-like 5 (Drosophila)	Other	Other	-	-
<b>Ldha</b>	Lactate dehydrogenase A	Cellular metabolism	Mitochondria	-	-
<b>Lphn2</b>	Latrophilin 2	Cell signaling	Transmembrane	Class I PDZbd	-
<b>Lphn3</b>	Latrophilin 3	Cell signaling	Transmembrane	-	-
<b>Lss</b>	Lanosterol synthase	Cellular metabolism	Endoplasmic reticulum	-	-
<b>Mapk12</b>	Mitogen-activated protein kinase 12	Kinase	Intracellular	-	-
<b>Metap2</b>	Methionine aminopeptidase 2	Other	Intracellular	-	ASD-RD
<b>Mink1</b>	Misshapen-like kinase 1 (zebrafish)	Kinase	Cell junction	-	PCP, ASD-RD
<b>Mmd2</b>	Monocyte to macrophage differentiation-associated 2	Receptor activity	Transmembrane	-	-
<b>Morn4</b>	MORN repeat containing 4	Other	Other	-	-
<b>N6amt1</b>	N-6 adenine-specific DNA methyltransferase 1 (putative)	Other	Other	-	-
<b>Narfl</b>	Nuclear prelamin A recognition factor-like	DNA	Other	-	-
<b>Ndr3</b>	N-myc downstream regulated gene 3	Other	Other	-	-
<b>Nfyc</b>	Nuclear transcription factor-Y gamma	DNA	Nucleus	-	-
<b>Nob1</b>	NIN1/RPN12 binding protein 1 homolog (S. cerevisiae)	RNA	Nucleus	-	-
<b>Npdc1</b>	Neural proliferation, differentiation and control gene 1	Other	Transmembrane	-	-
<b>Pdk2</b>	Pyruvate dehydrogenase kinase, isoenzyme 2	Cellular metabolism	Mitochondria	-	-
<b>Phb2</b>	Prohibitin 2	Receptor activity	Mitochondria	-	-
<b>Pik3r2</b>	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta)	Cell signaling	Intracellular	-	-
<b>Plek</b>	Pleckstrin	Cell signaling	Intracellular	-	ASD-RD
<b>Polr2c</b>	Polymerase (RNA) II (DNA directed) polypeptide C	DNA	Nucleus	-	-
<b>Pp1r12c</b>	Protein phosphatase 1, regulatory (inhibitor) subunit 12C	Phosphatase	Intracellular	-	-
<b>Psap</b>	Prosaposin	Cell signaling	Other	-	ASD-RD

<b>Ptbp2</b>	Polypyrimidine tract binding protein 2	RNA	Nucleus	-	-
<b>Pycrl</b>	Pyrroline-5-carboxylate reductase-like	Cellular metabolism	Other	-	-
<b>Rab11b</b>	RAB11B, member RAS oncogene family	Protein traffic and transport	Recycling vesicle	-	-
<b>Rps6ka1</b>	Ribosomal protein S6 kinase polypeptide 1	Kinase	Intracellular	-	ASD-RD
<b>Rpusd1</b>	RNA pseudouridylate synthase domain containing 1	RNA	Other	-	-
<b>Sdhb</b>	Succinate dehydrogenase complex, subunit B, iron sulfur (lp)	Cellular metabolism	Mitochondria	-	-
<b>Sh2d3c</b>	SH2 domain containing 3C	Cell signaling	Intracellular	-	-
<b>Sin3b</b>	Transcriptional regulator, SIN3B (yeast)	DNA	Nucleus	-	-
<b>Sirt3</b>	Sirtuin 3 (silent mating type information regulation 2, homolog) 3 (S. cerevisiae)	Other	Mitochondria	-	-
<b>Slc7a5</b>	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	Transmembrane transporter	Transmembrane	-	ASD, ASD-RD
<b>Src</b>	Rous sarcoma oncogene ,	Cell signaling	PSD membrane	-	NTD, ASD-RD
<b>Stard7</b>	START domain containing 7	Other	Mitochondria	-	-
<b>Suclg1</b>	Succinate-CoA ligase, GDP-forming, alpha subunit	Cellular metabolism	Mitochondria	-	-
<b>Syf2</b>	SYF2 homolog, RNA splicing factor (S. cerevisiae)	RNA	Spliceosome	Class I PDZbd	-
<b>Syt11</b>	Synaptotagmin XI	Cell signaling	Cell junction	-	ASD-RD
<b>Syt2</b>	Synaptotagmin II	Cell signaling	Cell junction	-	-
<b>Tcf25</b>	Transcription factor 25 (basic helix-loop-helix)	DNA	Nucleus	-	-
<b>Tgfb1i1</b>	Transforming growth factor beta 1 induced transcript 1	Cell signaling	Cell junction	-	-
<b>Timm17b</b>	Translocase of inner mitochondrial membrane 17b	Transmembrane transporter	Mitochondria	-	ASD-RD
<b>Tle3</b>	Transducin-like enhancer of split 3, homolog of Drosophila E(spl)	DNA	Nucleus	-	-
<b>Tmed4</b>	Transmembrane emp24 protein transport domain containing 4	Cell signaling	Endoplasmic reticulum	-	-
<b>Tmem63b</b>	Transmembrane protein 63b	Other	Transmembrane	-	-
<b>Trim35</b>	Tripartite motif-containing 35	Other	Intracellular	-	-
<b>Trim9</b>	Tripartite motif protein 9	Cell signaling	Dendrite	-	-
<b>Tubb2a</b>	Tubulin, beta 2a	Cytoskeleton organization	Intracellular	-	ASD-RD
<b>Uhmk1</b>	U2AF homology motif (UHM) kinase 1	Kinase	Cell junction	Class I PDZbd	PCP, ASD-RD
<b>Uqcrc1</b>	Ubiquinol-cytochrome c reductase core protein 1	Transmembrane transporter	Mitochondria	-	ASD-RD
<b>Yif1a</b>	Yip1 interacting factor homolog A (S. cerevisiae)	Protein traffic and transport	Endoplasmic reticulum	-	-
<b>Zfp282</b>	Zinc finger protein 282	Other	Other	-	-

**TABLE 10: General characterization of the final yeast-two hybrid candidate interactions of Scrib1 PDZ4 domain:** function, cellular localization, presence of PDZ binding domains (PDZbd) or PDZ domains, relevance for neural tube defect (NTD), planar cell polarity (PCP), autism spectrum disorders (ASD) or ASD related disorders (ASD-RD).

Protein	Protein name	Function	Localization	PDZbd or PDZ domain	NTD, PCP, ASD or ASD-RD
<b>4933434E20Rik</b>	RIKEN cDNA 4933434E20 gene	Cellular metabolism	Transmembrane	Class I PDZbd	
<b>Abr</b>	Active BCR-related gene	Cytoskeleton organization	Intracellular	-	ASD-RD
<b>Actb</b>	Actin, beta	Cytoskeleton organization	Axon	-	ASD-RD

<b>Actg1</b>	Actin, gamma, cytoplasmic 1	Cytoskeleton organization	Intracellular	-	ASD-RD
<b>Alg2</b>	Asparagine-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase)	Cell signaling	Intracellular	-	-
<b>Aptx</b>	Aprataxin	DNA	Nucleus	-	-
<b>Arhgap44</b>	Rho GTPase activating protein 44	Cell signaling	Intracellular	Class I PDZbd	-
<b>Arv1</b>	ARV1 homolog (yeast)	Cellular metabolism	Transmembrane	-	-
<b>Atp1b1</b>	ATPase, Na+/K+ transporting, beta 1 polypeptide	Transmembrane transporter	Apico/basal membrane	Class I PDZbd	-
<b>Atp2b3</b>	ATPase, Ca++ transporting, plasma membrane 3	Transmembrane transporter	Transmembrane	Class I PDZbd	ASD-RD
<b>Atp5a1</b>	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	Transmembrane transporter	Mitochondria	-	ASD-RD
<b>Atp6v1a</b>	ATPase, H+ transporting, lysosomal V1 subunit A	Transmembrane transporter	Apical membrane	-	ASD-RD
<b>Ccdc163</b>	Coiled-coil domain containing 163	Other	Other	Unknown PDZbd	-
<b>Clip3</b>	CAP-GLY domain containing linker protein 3	Protein traffic and transport	Recycling vesicle	-	-
<b>Cnn2</b>	Calponin 2	Cytoskeleton organization	Cell junction	-	NTD, ASD-RD
<b>Cnp</b>	2',3'-cyclic nucleotide 3' phosphodiesterase	Kinase	Intracellular	-	ASD-RD
<b>Caspr5a</b>	Contactin associated protein-like 5A	Cell adhesion	Transmembrane	Class II PDZbd	ASD, ASD-RD
<b>Cox7a2</b>	Cytochrome c oxidase, subunit VIIa 2	Cellular metabolism	Mitochondria	-	-
<b>Cpt1c</b>	Carnitine palmitoyltransferase 1c	Cellular metabolism	Mitochondria	-	-
<b>Crtc1</b>	CREB regulated transcription coactivator 1	DNA	Nucleus	-	ASD-RD
<b>Cst3</b>	Cystatin C	Other	Other	-	-
<b>Ctsk</b>	Cathepsin K	Other	Other	-	ASD-RD
<b>Dpysl2</b>	Dihydropyrimidinase-like 2	Other	Cell junction	Unknown PDZbd	NTD, ASD-RD
<b>Eif1b</b>	Eukaryotic translation initiation factor 1B	RNA	Spliceosome	-	-
<b>Eif3i</b>	Eukaryotic translation initiation factor 3, subunit I	RNA	Spliceosome	-	-
<b>Fech</b>	Ferrochelatase	Other	Mitochondria	-	-
<b>Galk1</b>	Galactokinase 1	Kinase	Intracellular	-	ASD-RD
<b>Lamtor1</b>	RIKEN cDNA 2400001E08 gene	Protein traffic and transport	Recycling vesicle	-	-
<b>Ldha</b>	Lactate dehydrogenase A	Cellular metabolism	Mitochondria	-	-
<b>Morn4</b>	MORN repeat containing 4	Other	Other	-	-
<b>Npc2</b>	Niemann Pick type C2	Protein traffic and transport	Lysosome	-	-
<b>Nudt13</b>	Nudix (nucleoside diphosphate linked moiety X)-type motif 13	Other	Mitochondria	-	-
<b>Pctk1</b>	PCTAIRE-motif protein kinase 1	Kinase	Other	-	-
<b>Pink1</b>	PTEN induced putative kinase 1	Kinase	Mitochondria	-	ASD-RD
<b>Pkp4</b>	Plakophilin 4	Cell adhesion	Cell junction	Class I PDZbd	ASD-RD
<b>Polr2c</b>	Polymerase (RNA) II (DNA directed) polypeptide	DNA	Nucleus	-	-
<b>Ppp2ca</b>	Protein phosphatase 2A, catalytic subunit, alpha isoform	Phosphatase	Intracellular	Class II PDZbd	ASD-RD
<b>Psap</b>	Prosaposin	Cell signaling	Other	-	ASD-RD

<b>Rein</b>	Reelin	Other	Cell junction	-	ASD, ASD-RD
<b>Rit1</b>	Ras-like without CAAX 1	Cytoskeleton organization	Intracellular	Unknown PDZbd	PCP
<b>Rnd2</b>	Rho family GTPase 2	Cytoskeleton organization	Recycling vesicle	-	-
<b>Rnf114</b>	Ring finger protein 114	Other	Intracellular	-	-
<b>Rpl18a</b>	Ribosomal protein L18A	RNA	Intracellular	-	-
<b>Scarb1</b>	Scavenger receptor class B, member 1	Cell adhesion	Transmembrane	Class I PDZbd	-
<b>Smad7</b>	MAD homolog 7 (Drosophila)	Cell signaling	Nucleus	-	PCP, ASD-RD
<b>Solh</b>	Small optic lobes homolog (Drosophila)	Other	Intracellular	-	-
<b>Spcs2</b>	Signal peptidase complex subunit 2 homolog (S. cerevisiae)	Other	Endoplasmic reticulum	-	-
<b>Ssbp3</b>	Single-stranded DNA binding protein 3	DNA	Nucleus	-	-
<b>Stxbp1</b>	Syntaxin binding protein 1	Protein traffic and transport	Membrane	-	ASD-RD
<b>Tecpr1</b>	Tectonin beta-propeller repeat containing 1	Other	Transmembrane	-	-
<b>Trf</b>	Transferrin	Protein trafficking & transport	Apico/basal membrane	-	-
<b>Trp53</b>	Transformation related protein 53	DNA	Nucleus	-	NTD, PCP
<b>Tst</b>	Thiosulfate sulfurtransferase, mitochondrial	Other	Mitochondria	-	-
<b>Vdac1</b>	Voltage-dependent anion channel 1	Transmembrane transporter	Mitochondria	-	ASD-RD
<b>Vtn</b>	Vitronectin	Cell adhesion	Extracellular	-	-

### III.3 General characterization of Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> protein interactions

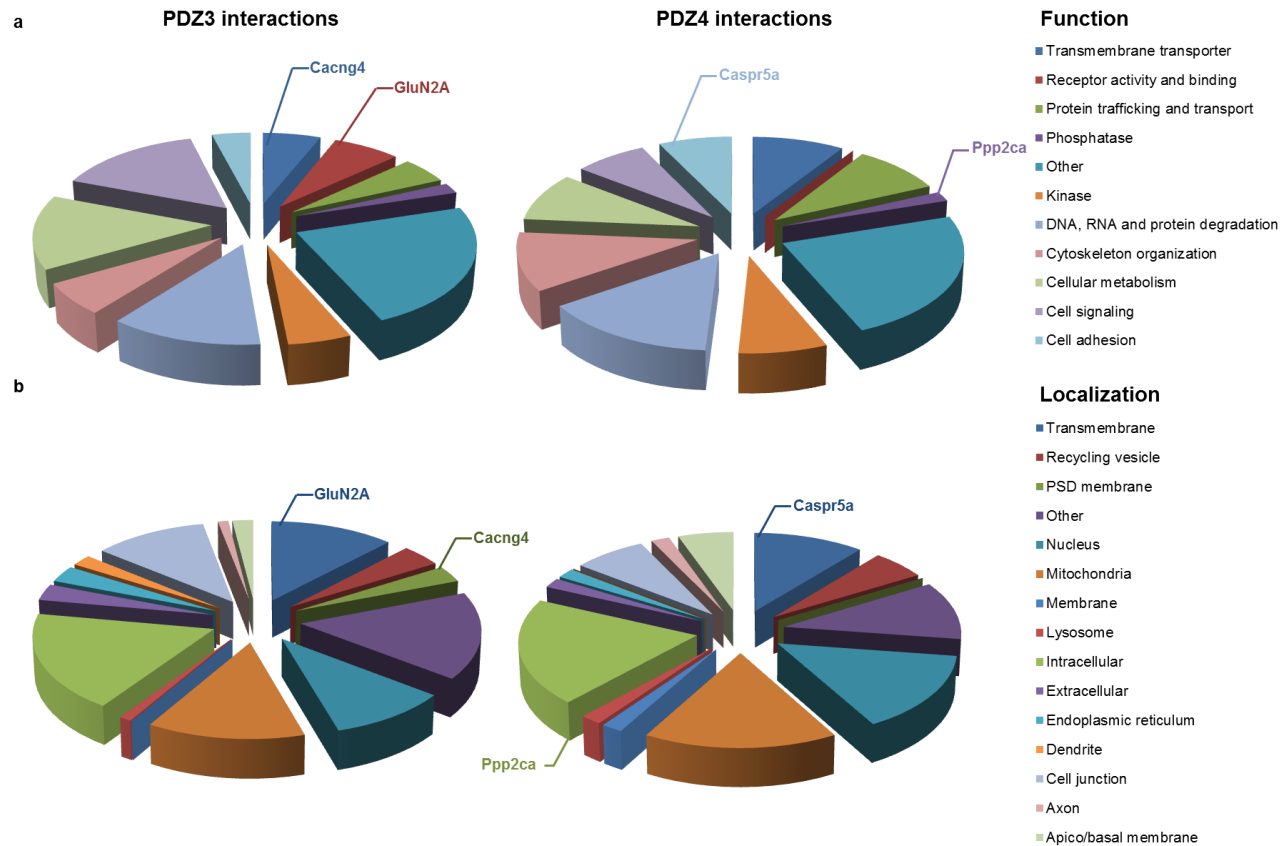
A Yeast Two-Hybrid screen was used to look for potential proteins that could interact with Scrib1<sup>PDZ3</sup> or Scrib1<sup>PDZ4</sup> (bait) from a cDNA library of P10 mouse brain (prey). The two-fold difference between the obtained mating colonies (**Table 11**) might be explained by a greater phylogenetic homology shared by the Scrib1 PDZ3 domain with its first two PDZ domains as well as with Erbin and Densin-180 (**Chapter I.2**) when compared to the Scrib1 PDZ4 domain (**Fig. 27**). Although PDZ3 is highly conserved among different species (Cai et al., 2014), Sidhu and colleagues showed that Scrib1 PDZ1-3 domains are highly promiscuous (Zhang et al., 2006). Interestingly, the same study obtained no specific binding clone for Scrib1 PDZ4, potentially due to its less stable nature. We also classify Scrib1 PDZ3 and PDZ4 positive interactions according to their function or localization based on the available curated NCBI database (**Fig. 28**). Both PDZ domains seem to interact with proteins displaying similar functions (**Fig. 28 a**) and localization (**Fig. 28 b**).

**Table 11: Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> Y2H screening throughout the selection criteria process.** Identified proteins following high stringency conditions are further described in Table 8 and 9, respectively. Proteins containing PDZbd or PDZ domains are in Table 10.

Selection criteria	Scrib1 <sup>PDZ3</sup>	Scrib1 <sup>PDZ4</sup>
Large scale (total in 4DO media)	~ 3.5 x 10 <sup>3</sup>	~ 1.5 x 10 <sup>3</sup>
Positive interactions	98	56
Proteins containing PDZbd or PDZ domains	12	8
Final selection (presented in this thesis)	2	1



**Figure 27: The three first PDZ domains of Scrib1 share a greater homology than PDZ4 domain.** Multiple alignment between Scrib1 PDZ domains using MAFFT (Multiple Alignment using Fast Fourier Transformation) Branch lengths are proportional to the genetic distance between the given sequences. Aligned sequences (Uniprot): SCRIB\_MOUSE, Q80U72.



**Figure 28: Function (a) and localization (b) of Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> interacting proteins.** Highlighted are the proteins chosen for this project.

#### III.4 Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> protein interactions implicated in NTD, PCP, ASD and ASD-RD

PDZ domains are known to specifically recognize short Cter peptide motifs (the so called canonical PDZ-binding domains or PDZbd), internal sequences that structurally mimic a terminus or other PDZ domains. This first criteria allowed us to narrow down our 98 (**Table 9**) and 56 (**Table 10**) candidate lists to 12 and 8 Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> interacting proteins containing PDZbd or PDZ domains, respectively - **Table 12**. Interestingly, 2 out of 12 Scrib1<sup>PDZ3</sup> domain interaction candidate proteins contain PDZ domains (Apba1 and Psap), whereas Scrib1<sup>PDZ4</sup> candidates display no PDZ domain. All Scrib1<sup>PDZ3</sup> screen candidates owning a Cter PDZbd are classified as canonical or class I (X-S/T-X-L/V, being (x) any amino acid), including Scrib1 known partner Ctnnb1 (Navarro et al., 2005; Nguyen et al 2005; Qin et al., 2005; Phillips et al., 2007; Yoshihara et al., 2011). Conversely, even if the majority of Scrib1<sup>PDZ4</sup> screen candidates exhibit a class I PDZbd



Cter, 2 out of 8 (Caspr5a and Ppp2ca) are class II PDZbd ( $X-\phi-X-\phi$ , being ( $\phi$ ) any hydrophobic aminoacid). Such results support the initial argument in which Scrib1 PDZ4 domain shares the lesser phylogenetic homology with the remaining Scrib1 PDZ domains (**Fig. 27**). In addition, the majority of Scrib1<sup>PDZ3</sup> (8/10) and Scrib1<sup>PDZ4</sup> (7/8) interaction candidates own a potential phosphoryable residue in their Cter PDZbd -2 position (underlined in **Table 12**). PDZ-peptide interactions are known to be modulated by phosphorylation events (reviewed by Sheng & Sala, 2001), suggesting that Scrib1 might serve a post-translation multi-complex platform, capable of assembling kinases and/or phosphatases close to their substrates at a given time and space, suitable for reversible PDZ-dependent interactions. Finally, as the lack of Scrib1 PDZ3 and PDZ4 domains was previously implicated in NTD (**Chapter I 3.3**) as well as in ASD (**Chapter I 5.4.2**), we decided to narrow our final selection to proteins implicated in NTD (DeGreene et al, 2009; Copp & DeGreene, 2010), PCP (Gerdes et al, 2007; Simons & Mlodzik, 2008; Harris & Juriloff, 2010), ASD (NIMH Project) and/or ASD-related diseases (ASD-RD), such as attention deficit hyperactivity disorder and attention (ADHD), bipolar disorder, Down syndrome, epilepsy, Fragile X, mental retardation, Rett syndrome and schizophrenia (Genotator) (**Table 12**; light and dark grey). Remarkably, the same percentage of Scrib1<sup>PDZ3</sup> (3 out of 12; Cacng4, GluN2A and Umhk1) and Scrib1<sup>PDZ4</sup> (2 out of 8; Caspr5a and Ppp2ca) interacting proteins are involved in NTD, PCP, ASD and ASD-RD. The chosen candidates (**Table 12**; dark grey) will be presented in the following Chapters – the Scrib1<sup>PDZ3</sup> interacting proteins containing class I PDZbd glutamate ionotropic receptor subunits GluN2 (**Chapter IV**) and AMPA auxiliary protein Cacng4 (**Chapter VI**), as well as the Scrib1<sup>PDZ4</sup> interacting protein containing class II PDZbd, the catalytic subunit of protein phosphatase Ppp2ca (**Chapter V**).

**TABLE 12: Final Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> domain interaction candidates:**  
protein name, Cter, PDZbd class or PDZ domain and known PDZ interactions.

	Protein name	Cter	PDZbd/domain	Known PDZ interactions
PDZ3	<b>Apba1</b>	Amyloid beta precursor pro., family A, member 1	PDZ domain	SAP-97-CASK-Veli-Kir2
	<b>Atp2b1</b>	ATPase, Ca++ transporting, plasma membrane 1	Class I	Anks4b, Slc4a7, SAP102
	<b>Atp1b1</b>	ATPase, Na+/K+ transporting, beta 1 polypeptide	Class I	MAGI1
	<b>Cnrip1</b>	CB1 cannabinoid receptor-interacting protein 1	Class I	-
	<b>Cacng4</b>	Calcium channel, voltage-dep., gamma subunit 4	Class I	MAGI1
	<b>Ctnnb1</b>	Catenin (cadherin associated protein), beta 1	Class I	PSD-95, Scrib
	<b>GluN2A</b>	Glut.receptor, ionotropic, NMDA2A (epsilon 1)	Class I	PSD-95, SAP102, PKC...
	<b>Itgb1bp1</b>	Integrin beta 1 binding protein 1	Class I	Slc9a3r1/2 (internal PDZ)
	<b>Lphn2</b>	Latrophilin 2	Class I	Anks4b
	<b>Psap</b>	Prosapsin	PDZ domain	-
	<b>Syf</b>	SYF2 hom., RNA splicing factor (S. cerevisiae)	Class I	Prr7
	<b>Umhk1</b>	U2AF homology motif (UHM) kinase 1	Class I	Igsf9, MPDZ
PDZ4	<b>Abr</b>	Active BCR-related gene	Class I	Arhgef16, Kcne3, PSD-95
	<b>Atp2b3</b>	ATPase, Ca2+ transp., plasma membrane 3	Class I	Anks4b, Slc4a7, SAP102
	<b>Arhgap44</b>	Rho GTPase activating protein 44	Class I	Tshr
	<b>Ccdc163</b>	Coiled-coil domain containing 163	Class I	-
	<b>Caspr5a</b>	Contactin associated protein-like 5A	Class II	Erythrocyte p55, MAGI
	<b>Pkp4</b>	Plakophilin-4	Class I	MAGI1, Erbin
	<b>Ppp2ca</b>	Protein phosphatase 2, catalytic sub. alpha isoform	Class II	-
	<b>Scarb1</b>	Scavenger receptor class B, member 1	Class I	Slc9a3r1, PDZK1

## Chapter IV

# Scribble1/AP2 Complex Coordinates NMDA Receptor Endocytic Recycling

### IV.1 Context and problematic

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N-methyl D-aspartate receptors (NMDARs) are major excitatory neurotransmitter receptors widely distributed in the brain, where they are believed to play a crucial role in synapse development, synaptic transmission and plasticity (Montgomery et al., 2005). NMDARs are tetramers made of two GluN1 subunits and two switchable GluN2 (A-D) and/or GluN3 (A or B) subunits. In the hippocampus, NMDAR are mainly composed by GluN2B subunits at early stages and a complex of GluN2A and GluN2B subunits at mature stages (Traynelis et al., 2010). In addition, NMDARs can also be differently composed accordingly to their specific location. GluN2A subunits are more stable and can be preferentially found within synapses, whereas GluN2B subunits are more mobile and are usually located at extra-synaptic sites (Rumbaugh & Vicini, 1999; Tovar & Westbrook, 1999; Prybylowski et al., 2002; Groc et al., 2006; Thomas et al., 2006; Zhao et al., 2008). The cellular trafficking of NMDARs from their synthesis in the cell body up to a distant specific synaptic membrane is therefore critical in the modulation of synaptic changes underlying information acquisition and storage in the brain. Traffic of glutamatergic receptors has been long related to PDZ proteins, especially MAGUKs like PSD-95 or SAP-102, the major synaptic scaffold protein anchoring NMDAR at glutamatergic synapses (reviewed by Groc & Choquet, 2006; Sheng & Hoogenraad, 2007; Elias & Nicoll, 2007). The main goal of this study was then to investigate if the PCP protein Scrib1, containing 4 PDZ domains and previously implicated in the cellular traffic of numerous proteins (**Chapter I.3.2**) was able to modulate NMDARs cellular traffic.

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### IV.2 Results summary

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We first showed that there is a spatio-temporal co-localization of Scrib1 and NMDARs in the brain. Scrib1 and NMDARs were found to be highly expressed in hippocampal pyramidal neurons, and particularly in dendritic spines. Next, Scrib1 was found to bind the Cter class I PDZbds of both GluN2A and GluN2B subunits in a PDZ-dependent manner. In particular, Scrib1 prevents GluN2A from lysosomal trafficking and degradation, increasing its recycling to the plasma membrane upon stimulation. Thus, alike other PDZ proteins (Losi et al., 2003; Sans et al., 2003; Chung et al., 2004; Mauceri et al., 2007; Howard et al., 2010) Scrib1 is able to modulate GluN2A/GluN2B ratio at the synapse. Moreover, the PSDs of Scrib<sup>crc/+</sup> mice, expressing 50% of a truncated form of Scrib1 missing PDZ3 and PDZ4 domains, possess less surface GluN2A-, but not GluN2B-containing NMDARs, pinpointing Scrib1's role in fine-tuning excitatory synapses. Finally, Scrib1 was shown

to interact with the adaptor protein AP2 through the specific motif YxxxR. Taken together, our data proposes that the newly described Scrib1-AP2 complex signalling plays a crucial role in regulating NMDARs levels and composition at the synaptic membrane.

### IV.3 My contribution

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Through a Yeast Two-Hybrid screen, I found the glutamate ionotropic receptor subunits GluN2 as a potential interacting protein of Scrib1<sup>PDZ3</sup> domain, as well as the adaptor protein AP-2 (**Chapter III**). I used a directed yeast two-hybrid assay with GluN2A, GluN2B, and Scrib1 constructs (Fig. 1 A) to confirm the first interaction.























































































## Chapter V

# Scribble 1 scaffolds protein phosphatases 1 and 2A to regulate bidirectional plasticity underlying spatial memory

### V.1 Context and problematic

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Spatial memory formation is a complex process that transforms newly-acquired information into long-lasting memories by an encoding followed by a consolidation phase. At the cellular level, memory formation is believed to rely on a fine balance between two forms of synaptic plasticity: one referring to a persistent increase in the efficiency of synaptic transmission after inducing a short period of high-frequency synaptic stimulation (HFS) - long-term potentiation (LTP); and another concerning a decrease of synaptic strength following low-frequency stimulation (LFS) - long-term depression (LTD) (**Chapter I.5.3**). At the molecular level, both events require the activation of NMDARs (Citri & Malenka, 2008; Collingridge et al., 2010). Nevertheless, LTP and LTD signalling pathways differ by entailing the preferential activation of kinases, such as CaMKII and PKA, or phosphatases, such as calcineurin and PP1/PP2A, respectively (Lüscher & Malenka, 2012). The appropriate activation of either signalling pathway is known to be orchestrated by scaffold proteins, able to couple glutamate receptors to downstream signalling molecules (Sheng & Kim, 2011). The following study aims to examine the role of Scrib1, a PDZ-based scaffold protein located in the PSD of excitatory synapses, in bidirectional synaptic plasticity and spatial memory formation.

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### V.2 Results summary

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We have previously showed that Scrib1 does play an interesting role in the morphology and function of glutamatergic synapses (Moreau et al., 2010). Nonetheless, this study was conducted using heterozygote Scrib1<sup>crc/+</sup> mice, in which Scrib1 levels are reduced by 50% in both excitatory and inhibitory neurons as well as in glial cells. As a result, we generated conditional Scrib1 knock-out mice (CaMK-Scrib1<sup>-/-</sup>), targeting the postnatal excitatory neurons of the hippocampus. First, we showed that Scrib1 loss not only affects the maturation of hippocampal glutamatergic synapses, as it also impacts hippocampus-dependent long-term memory consolidation. CaMK-Scrib1<sup>-/-</sup> mice displayed a reduced synaptic transmission and a compromised CA3-CA1-dependent bidirectional plasticity. We found as well that Scrib1 interacted directly with PP1/PP2A phosphatases, known to take part in the bidirectional plasticity signalling. Finally, we were able to re-establish abnormal bidirectional plasticity and spatial memory due to loss of Scrib1 by exposing CaMK-Scrib1<sup>-/-</sup> mice to enriched environment (EE). Altogether, our data pinpoints Scrib1 as a key PSD-based scaffold protein linking functional maturation of hippocampal synapses with their capacity of expressing bidirectional plasticity necessary for memory formation.

### V.3 My contribution

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Through a Yeast Two-Hybrid screen, I found the catalytic subunit of phosphatase PP2A (Ppp2ca) as a potential interacting protein of Scrib1<sup>PDZ4</sup> domain (**Chapter III**). I used a directed yeast two-hybrid assay with Ppp2ca and Scrib1 constructs (Fig. 6 A) to confirm such interaction. I also use COS-7 cells to follow Pp1c and Ppp2ca co-localization with Scrib1 (Fig. 6 C, D). Finally, I participated in the preparation of endogenous CoIP of Scrib1, Pp1 and Ppp2ca from hippocampal tissue from WT and CaMK-Scrib1<sup>-/-</sup> mice (Fig. 6 H) as well as in the preparation of PSD fractions (Fig. 6 H, I).

# Scribble 1 scaffolds protein phosphatases 1 and 2A to regulate bidirectional plasticity underlying spatial memory

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## SUMMARY

Spatial memory formation is a complex process that depends on bidirectional synaptic plasticity in the hippocampus. Underlying molecular mechanisms require specific pathways involving complex interactions between receptors, enzymes and scaffold proteins at excitatory synapses. In the present study, we examined the role of Scribble1 (Scrib1), a member of the planar cell polarity pathway belonging to the LAP (LRR And PDZ domain) protein family that plays crucial roles in regulating hippocampus-dependent memory in both developing and adult central nervous system. Using a conditional genetic approach combined with morphological, molecular, and electrophysiological analyses, we showed that loss of Scrib1 in CA1 hippocampal pyramidal neurons led to impaired spatial memory consolidation associated with functionally immature synapses and altered bidirectional plasticity. Molecularly, we revealed a direct interaction between Scrib1 and PP1/PP2A phosphatases that are central for synaptic plasticity. Finally, we showed that environmental enrichment exposure rescues synaptic transmission and bidirectional plasticity, which restores memory formation in CaMK-Scrib1<sup>-/-</sup> mice. Our study identifies Scrib1 as a key scaffold protein linking functional maturation of hippocampal synapses with their capacity to express bidirectional plasticity necessary for memory formation.

## HIGHLIGHTS

- Maturation of hippocampal glutamatergic synapses depends on Scrib1
- Scrib1 scaffolds PP1/PP2A phosphatases to ensure bidirectional plasticity expression
- Scrib1 modulates spatial memory consolidation
- Enriched environment rescues abnormal bidirectional plasticity and spatial memory due to loss of Scrib1

## INTRODUCTION

Spatial memory formation is a complex process that transforms newly acquired information into long-lasting memories by, first, an encoding and then, a consolidation phase. At the cellular level, memory formation relies on a fine balance between different forms of synaptic plasticity processes such as long-term potentiation (LTP) and long-term depression (LTD), which are believed to represent the prototypic forms of synaptic plasticity at glutamatergic synapses in the hippocampus (Griffiths et al., 2008, Citri and Malenka, 2008; Collingridge et al., 2010, Nabavi et al., 2014). In NMDA receptor (NMDAR)-dependent forms of LTP and LTD, LTP signaling pathway entails activation of kinases such as CaMKII and PKA, while phosphatases such as calcineurin and PP1/PP2A are activated during LTD (Lüscher and Malenka, 2012). Appropriate activation of either signaling pathways involves scaffold proteins that couple glutamate receptors to downstream signaling molecules (Sheng and Kim, 2011).

Scribble1 (Scrib1) is a scaffold protein that belongs to the LAP (LRR And PDZ domain) protein family that combines both LRR (Leucine Rich Repeats) at their N-terminus and one to four PDZ (PSD-95/Dlg/ZO-1) domains in their structure (Bilder et al., 2000). Beside Scrib1, the LAP family also comprises Densin-180, Erbin and Lano (Apperson et al., 1996; Murdoch et al., 2003; Saito et al., 2001). Most members of this family are found in the postsynaptic density (PSD), a highly organised cytoskeletal structure found adjacent to the postsynaptic membrane of excitatory synapses. For instance, Densin-180 was among the first scaffold proteins identified in the PSD and has been shown to bind directly to CaMKII, Shank, and NR2B subunit (Apperson et al., 1996). Through these protein-protein interactions, Densin-180 has been shown to regulate dendritic arborisation, spine morphology and LTD in hippocampal pyramidal neurons (Quitsch et al., 2005; Robison et al., 2005; Carlisle et al., 2011). Erbin has been shown to be enriched in cortical inhibitory neurons where it regulates synaptic transmission through the control of the Transmembrane AMPAR regulatory protein (TARP)  $\gamma$ -2 levels and AMPAR ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) surface expression (Tao et al., 2013). Recently, we unraveled a regulatory role for Scrib1 in dendritic arborisation, spinogenesis and synaptic function of hippocampal neurons by studying a spontaneous mutant for Scrib1 (Moreau et al., 2010). The truncation of Scrib1 after its second PDZ domain gives rise to the circletail mutant mouse (Scrib1<sup>crc</sup>) (Murdoch et al., 2003). Homozygote Scrib1<sup>crc/crc</sup> mice die at birth, whereas heterozygote scrib1<sup>crc/+</sup> mice have a 50% reduction in the level of Scrib1 in the brain, and anatomical and functional deficits in the hippocampus. Unexpectedly, we showed that Scrib1<sup>crc/+</sup> mice exhibit enhanced learning and memory abilities (Moreau et al., 2010). Recently, we suggested that the truncated form of Scrib1 in this animal is a dominant negative form of the protein (Moreau & Sans, unpublished). Thus, these enhanced performances of scrib1<sup>crc</sup> mutants were not the simple reflection of Scrib1 loss of function in the adult brain, but the integrated results of a mix of Scrib1 proteins (full length and truncated) and compensatory mechanisms. In this study, we developed conditional knock-out mice for Scrib1 (CaMK-Scrib1<sup>-/-</sup>) that target postnatal excitatory neurons of the hippocampus. Using these CaMK-Scrib1<sup>-/-</sup> mice, we investigated the consequences of the loss of Scrib1 on spatial memory formation and morpho-functional changes in postmitotic CA1 pyramidal neurons. We found that absence of Scrib1 at excitatory hippocampal neurons led to impaired hippocampus-dependent long-term memory. Functionally, synaptic transmission was reduced and bidirectional plasticity

was compromised at CA3-CA1 synapses in CaMK-Scrib1<sup>-/-</sup> mutants. At the molecular level, we unveiled a direct interaction at the PSD between Scrib1 and PP1/PP2A phosphatases that are involved in bidirectional plasticity signalling. Interestingly, exposure to enriched environment (EE) re-established memory formation in CaMK-Scrib1<sup>-/-</sup> mice by recovering normal synaptic function and plasticity. Taken together, this study reveals a scaffolding role for the polarity protein Scrib1 in the glutamatergic synaptic function underlying spatial memory formation in the hippocampus.

## RESULTS

### Postnatal Scrib1 deficiency causes no gross defect in hippocampus morphology

To prevent the lethality and brain damage observed in Scrib1<sup>crc/crc</sup> mice, we created conditional knock-out mice for scrib1 gene. Using the cre-loxP system, we restricted the excision of scrib1 to CaMKII-expressing neurons of the postnatal forebrain, specifically to the CA1 pyramidal cell layer in the hippocampus. The generation of conditional knock-out allele is detailed in **Figure S1A** (also see, Yamben et al., 2013). Scrib1<sup>f/f</sup> control and Scrib1<sup>f/f,CaMKII-cre</sup> (CaMK-Scrib1<sup>-/-</sup>) mice developed normally and were able to breed. The CaMK-Scrib1<sup>-/-</sup> mice did not exhibit any gross abnormalities in the morphology of young (data not shown) and adult brains stained with cresyl violet, although a slight lateral ventricle enlargement was observed along the rostrocaudal axis (**Figure 1A**). Scrib1<sup>f/f</sup> gene was detected by PCR at all ages (2, 5, and 10 weeks) and in all tissues (tail, cortex and hippocampus) of Scrib1<sup>f/f</sup> control and CaMK-Scrib1<sup>-/-</sup> mice (**Figure S1C**). In contrast, Cre-recombinase and Scrib1<sup>Δf/f</sup> were detected, as early as 2 weeks, only in CaMK-Scrib1<sup>-/-</sup> forebrain structures (**Figure S1C**). Both intact Scrib1<sup>f/f</sup> and excised Scrib1<sup>Δf/f</sup> were detected in CaMK-Scrib1<sup>-/-</sup> hippocampus and cortex because excision of scrib1 takes place only in CaMKII positive neurons. The spatial pattern of Cre/loxP recombination in the CaMK-Scrib1<sup>-/-</sup> mouse line was examined by crossing this line with an Ai6 ZsGreen1 reporter mouse (Madisen et al., 2010) and brain sections from the progeny were stained with DAPI and NeuroTrace Fluorescent Nissl Stains. The Cre/loxP recombination was first detected at 2/3 weeks in the DG, CA3 and CA1 area of the hippocampus (data not shown). At 10 weeks of age, strong green fluorescence certifying the recombination occurred in virtually all pyramidal cells in the hippocampus (**Figure 1B**). As a result, Scrib1 protein levels decreased in the hippocampus of CaMK-Scrib1<sup>-/-</sup> mice as early as 2 weeks postnatally (n = 4, p < 0.05) (**Figure S1C**), and by 5 weeks old (5 w), Scrib1 was reduced by 70% compared to control littermate, and up to 80% decrease at 10 weeks (p < 0.05). Residual Scrib1 (~20%) detected in the hippocampus could be mainly due to the expression of Scrib1 in interneurons, glia and endothelial cells in which Scrib1 down-regulation does not occur. In regions outside the forebrain, like the cerebellum, where CaMKII-cre is not expressed, no decrease of Scrib1 was observed (n = 3, p > 0.10) (**Figure S1D**). We used immunohistochemistry staining with an antibody against Scrib1 (Montcouquiol et al., 2006) to localize Scrib1 in the hippocampus and confirmed the selective deletion of the scrib1 gene in the hippocampus of the CaMK-Scrib1<sup>-/-</sup> mice resulting in loss of the Scrib1 protein (**Figure 1C**). In summary, we generated a mouse model in which Scrib1 is specifically eliminated in the excitatory neurons of the hippocampus.

### Normal short-term but impaired long-term spatial memory in CaMK-Scrib1<sup>-/-</sup> mice

In order to determine whether the loss of Scrib1 affects hippocampal-dependent spatial learning, we investigated the behavioural phenotype of CaMK-Scrib1<sup>-/-</sup> mice. We first investigated locomotor activity and emotional state of CaMK-Scrib1<sup>-/-</sup> mice to avoid biased interpretations of their performances during memory tests (**Figure S2**). Scrib1<sup>fl/fl</sup> controls and CaMK-Scrib1<sup>-/-</sup> mice displayed normal locomotor activities in photocell-based chambers that were adequately correlated to circadian cycle (Scrib1<sup>fl/fl</sup>:  $n = 6$  and CaMK-Scrib1<sup>-/-</sup>:  $n = 9$ , genotype effect  $p = 0.64$ ) (**Figure S2A**). Anxiety levels were measured in elevated plus maze (Dawson & Tricklebank 1995) in which time spent in open arms did not differ between Scrib1<sup>fl/fl</sup> controls ( $53.2 \pm 9.2$ ,  $n = 13$ ) and CaMK-Scrib1<sup>-/-</sup> mice ( $52.9 \pm 8.9$ ,  $n = 14$ ,  $p = 0.97$ ) (**Figure S2B**). Using spontaneous arm alternation measures in the Y-maze paradigm, spatial working memory was found to be normal in CaMK-Scrib1<sup>-/-</sup> mice ( $62.5 \pm 4.4\%$ ,  $n = 6$ ) compared to Scrib1<sup>fl/fl</sup> controls ( $62.4 \pm 2.3\%$ ,  $n = 7$ ,  $p = 0.70$ ) (**Figure S2C**). Altogether these data show that simple form of spatial recognition is preserved in CaMK-Scrib1<sup>-/-</sup> mice. Then, we proceeded to evaluate hippocampus-dependent short-term and long-term spatial reference memory in CaMK-Scrib1<sup>-/-</sup> mice using Morris water maze test (**Figure 2A**). Acquisition of the spatial task during the training phase showed that both Scrib1<sup>fl/fl</sup> and CaMK-Scrib1<sup>-/-</sup> mice learned the task ( $n = 12$  and  $n = 17$  respectively, days effect  $p < 0.001$ ). However, CaMK-Scrib1<sup>-/-</sup> mice had slower spatial learning compared to Scrib1<sup>fl/fl</sup> mice revealed by their significantly different latencies to find the hidden platform over the 8-day acquisition phase (genotype effect  $p < 0.01$ ) (**Figure 2B**). Similar genotype effect was also found by analysing the distance travelled before reaching the platform ( $p = 0.01$ , **Figure S3A**). Scrib1<sup>fl/fl</sup> controls improved their performances by decreasing the latency to find the platform in day 8 (D8), ( $14.6 \pm 2.1$  sec) compared to D1 ( $44.0 \pm 4.5$  sec,  $p < 0.0001$ ). Similarly, CaMK-Scrib1<sup>-/-</sup> mice exhibited spatial learning reflected in the reduction of latency to find the platform in D8 ( $20.8 \pm 2.8$  sec) compared to D1 ( $49.9 \pm 2.9$  sec,  $p < 0.0001$ ). Importantly, performances of both groups were equivalent by the end of the acquisition phase (D7;  $p = 0.15$  and D8;  $p = 0.41$ ). The velocity of the animals in the water maze during the training phase was not significantly different (genotype effect  $p = 0.84$ ) (**Figure S3B**). Both groups performed equally well in the visible platform task that requires the comprehension of the water-maze rule (find a refuge platform) as well as normal sensorimotor abilities (genotype effect  $p = 0.84$ ) (**Figure S3C**). Short-term retrieval probe test was conducted on half of the trained animals at one hour (1h) after the last session of acquisition (D8). Scrib1<sup>fl/fl</sup> and CaMK-Scrib1<sup>-/-</sup> mice spent equivalent time in target quadrant (Scrib1<sup>fl/fl</sup>:  $49.8 \pm 3.7\%$ ,  $n = 6$ , and CaMK-Scrib1<sup>-/-</sup>:  $45.9 \pm 3.7\%$ ,  $n = 9$ ,  $p = 0.27$ ) (**Figure 2C**) and the number of annulus crossings was similar (Scrib1<sup>fl/fl</sup>:  $4 \pm 0.7$  and CaMK-Scrib1<sup>-/-</sup>:  $3.8 \pm 0.7$ ,  $p = 0.84$ ) (**Figure 2D**) reflecting precise localisation of the platform in both groups. However, when these same animals were tested with a probe test at 24h post-training, CaMK-Scrib1<sup>-/-</sup> mice displayed impaired long-term memory. This was apparent in the time spent in the target quadrant, which was significantly decreased in CaMK-Scrib1<sup>-/-</sup> mice ( $40.2 \pm 5\%$ ,  $n = 8$ ) compared to Scrib1<sup>fl/fl</sup> control littermate ( $58.4 \pm 4.3\%$ ,  $n = 6$ ,  $p < 0.05$ ) (**Figure 2E**). A significant decrease in the number of annulus crossings was also observed for CaMK-Scrib1<sup>-/-</sup> ( $2.1 \pm 0.2$ ) compared to Scrib1<sup>fl/fl</sup> controls ( $4.3 \pm 0.7$ ,  $p < 0.01$ ) (**Figure 2F**). Absence of the platform during the probe test at 1h can initiate a new phase of memory extinction in which animals “learn” that the platform is no longer present. Thus, accelerated memory extinction in CaMK-Scrib1<sup>-/-</sup> mice due to the probe test at 1h can



also be translated in impaired performances at 24h probe test. To exclude this possibility, we directly tested the second half of the trained animals at 24h post-training of acquisition. CaMK-Scrib1<sup>-/-</sup> mice exhibited similar decrease in time spent in the target quadrant ( $44.8 \pm 7.4\%$ ,  $n = 8$ ) compared to Scrib1<sup>fl/fl</sup> controls ( $67.5 \pm 3.7\%$ ,  $n = 6$ ,  $p < 0.05$ ) (**Figure S3D**). A similar difference was observed for the number of platform crossings (CaMK-Scrib1<sup>-/-</sup>:  $1.9 \pm 0.4$  and Scrib1<sup>fl/fl</sup>:  $5.8 \pm 0.6$ ,  $p < 0.001$ ) (**Figure S3E**). Taken together, these experiments reveal impairment in long-term, but not short-term, spatial memory in CaMK-Scrib1<sup>-/-</sup> mice that is not due to altered sensorimotor abilities or anxiety levels.

### **Increased dendritic arborisation and immature synapses in the stratum radiatum of CaMK-Scrib1<sup>-/-</sup> pyramidal neurons**

To analyse the cellular mechanisms underlying this spatial memory impairment, we examined whether the deletion of Scrib1 in pyramidal neurons alters their neuronal morphology and function. For that, we focused on CA1 region which has been firmly established to play an important role in the acquisition and retention of spatial reference memory (Martin and Clark, 2007). For morphological analysis, apical and basal dendritic arbours of Scrib1<sup>fl/fl</sup> and CaMK-Scrib1<sup>-/-</sup> CA1 pyramidal neurons stained using Golgi Cox stain were reconstructed as shown in **Figure 3A**. Basal dendrites displayed equal complexity reflected by total intersections (Scrib1<sup>fl/fl</sup>:  $n = 9/3$  mice and CaMK-Scrib1<sup>-/-</sup>:  $n = 12/3$  mice,  $p = 0.84$ ) and dendritic length ( $p = 0.90$ ) in both genotypes (**Figure 3B and 3C**, respectively). In contrast, total apical dendritic intersections was increased by ~37% ( $p < 0.001$ ) and apical length by ~35% ( $p < 0.01$ ) in CaMK-Scrib1<sup>-/-</sup> CA1 neurons compared to Scrib1<sup>fl/fl</sup> controls (**Figure 3D and 3E**, respectively). Distributions of length and intersections according to distance from soma revealed that the medial region of CA1 apical dendrites laying in the stratum radiatum was mostly affected in CaMK-Scrib1<sup>-/-</sup> mice. Electron microscopy was used to investigate whether spinogenesis in CA1 stratum radiatum was also affected by the deletion of scrib1 gene (**Figure 3F and 3H**). The spine density expressed as the number of spines /  $\mu\text{m}^2$  on apical dendrites of Scrib1<sup>fl/fl</sup> CA1 pyramidal neurons was similar in Scrib1<sup>fl/fl</sup> controls ( $0.542 \pm 0.018$  spines/ $\mu\text{m}^2$ ) and CaMK-Scrib1<sup>-/-</sup> neurons ( $0.614 \pm 0.025$  spines/ $\mu\text{m}^2$ ,  $n = 1892/4$ ,  $p = 0.06$ ). We also measured PSD size in stratum radiatum of CA1 neurons that displayed shifted cumulative probability curve toward lower PSD thickness (Scrib1<sup>fl/fl</sup> controls:  $41.950 \pm 0.904$  nm and CaMK-Scrib1<sup>-/-</sup>:  $41.051 \pm 1.1428$  nm,  $n = 1892/4$ ,  $p = 0.001$ ) and PSD length values (Scrib1<sup>fl/fl</sup> controls:  $216.142 \pm 3.026$  nm and CaMK-Scrib1<sup>-/-</sup>:  $204.022 \pm 2.825$  nm,  $n = 1892/4$ ,  $p = 0.01$ ) (**Figure 3G and 3I**, respectively). Specifically, PSDs of CaMK-Scrib1<sup>-/-</sup> CA1 neurons showed an increased probability (38.9%) of being shorter than 170 nm compared to those of Scrib1<sup>fl/fl</sup> CA1 neurons (33.6%). These data indicate that although the spine density is similar in both genotypes, CaMK-Scrib1<sup>-/-</sup> CA1 neurons display a larger portion of small immature PSDs in the stratum radiatum.

### **Reduced number of active CA3-CA1 synapses in CaMK-Scrib1<sup>-/-</sup> mice**

Small PSDs (less than 170 nm) have been previously described as deprived from AMPARs (Takumi et al., 1999). This points towards an increased population of synapses that are non-functional during basal synaptic transmission in CaMK-Scrib1<sup>-/-</sup> CA1 neurons. If this is the case, both basal synaptic transmission at CA3-CA1

synapses and the number of active synapses should be both reduced in CaMK-Scrib1<sup>-/-</sup> CA1 neurons. To test this possibility, field excitatory postsynaptic potentials (fEPSPs) were measured to evaluate CA3-CA1 basal glutamatergic transmission. Input-output relationship was found to be altered in CaMK-Scrib1<sup>-/-</sup> slices in comparison to Scrib1<sup>fl/fl</sup>, with a maximal slope of fEPSPs ~65% smaller (n = 12/6 mice; p < 0.001) (**Figure 4A**). This severe decrease in glutamatergic transmission could be due to distinct pre- and/or postsynaptic processes including a reduction in the probability of glutamate release (p) and/or in quantal size (q). To test whether a change in p could account for the reduction in synaptic transmission, we examined short-term facilitation at CA3-CA1 synapses at four different intervals of 25, 50, 100 and 200 ms. For all these intervals, paired pulse ratio (PPR) was found to be similar in Scrib1<sup>fl/fl</sup> controls and CaMK-Scrib1<sup>-/-</sup> acute slices (n = 6, p > 0.35 for all four intervals) (**Figure 4B**). These data indicate that the probability of glutamate release (p) at CA3-CA1 synapses is not altered in CaMK-Scrib1<sup>-/-</sup> mice. Thus, reduced input-output relation can be due to a decrease in quantal size (q) or quantal content (n.p). We hence measured AMPARs-mediated miniature excitatory postsynaptic currents (mEPSCs) using whole-cell patch clamp recordings in the presence of TTX (**Figure 4C**). Average amplitudes of mEPSCs in CA1 neurons was similar in Scrib1<sup>fl/fl</sup> controls and CaMK-Scrib1<sup>-/-</sup> mice indicating normal quantal size (13.38±0.76 and 11.88±0.42 pA, respectively, n = 5, p = 0.22) (**Figure 4D**). Importantly, mEPSC frequency was reduced by more than 70% in CaMK-Scrib1<sup>-/-</sup> (0.33±0.06 Hz) compared to Scrib1<sup>fl/fl</sup> littermate (0.97±0.23 Hz, n = 5, p < 0.05) (**Figure 4E**). Decrease in quantal content shown by mEPSCs frequency can be due to a change in the probability of release (p) and/or the number of active synapses (n). PPR analysis revealed (p) to be unaltered in CaMK-Scrib1<sup>-/-</sup> synapses, indicating that the number of active synapses (n) must be decreased in CaMK-Scrib1<sup>-/-</sup> CA1 neurons. Since the spine density was similar in both genotypes, we conclude that decrease in (n) is due to an increase in the number of inactive or immature synapses in CaMK-Scrib1<sup>-/-</sup> neurons. This is in agreement with our morphological analysis revealing an increased population of immature synapses in the stratum radiatum of CaMK-Scrib1<sup>-/-</sup> CA1 neurons (**Figure 3F to 3I**).

### Enhanced LTP and abolished LTD in CaMK-Scrib1<sup>-/-</sup> synapses

We next investigated whether the overall decrease in synaptic transmission due to the loss of Scrib1 impacted long-term synaptic plasticity in the hippocampus. We examined NMDAR-dependent LTP at the Schaffer collaterals using a high frequency stimulation (HFS) consisting of 2 trains at 100 Hz. This yielded a significant increase in LTP amplitudes at CaMK-Scrib1<sup>-/-</sup> synapses (145±0.6%, n = 6/5 mice) compared to Scrib1<sup>fl/fl</sup> synapses (121.1±0.7%, n = 5, p < 0.05) (**Figure 5A**). Application of D-AP5, a specific NMDAR antagonist, completely blocked LTP induction, indicating that it was NMDAR-dependent (107±1.37%, n = 6, p = 0.44) (data not shown). Since the threshold to induce LTP seemed to be affected in CaMK-Scrib1<sup>-/-</sup> mice, LTD was also examined using low frequency stimulation (LFS) protocol of 15 minutes at 1 Hz. Synaptic strength was significantly depressed to 85.6±1.1% of initial baseline following LTD induction in Scrib1<sup>fl/fl</sup> slices (n = 6/5 mice, p < 0.05) (**Figure 5B**). This LTD was NMDAR-dependent as it was completely blocked by APV (102.5 ± 0.85 %, n = 4, p = 0.12) (data not shown). Surprisingly, rather than inducing LTD, LFS of Schaffer collaterals yielded significant LTP at CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses (112.2±1.5%; n = 7/6 mice, p = 0.01).

Also, a weaker stimulation protocol at 0.5 Hz did not induce any change in Scrib1<sup>fl/fl</sup> or CaMK-Scrib1<sup>-/-</sup> mice (103.4±0.6%, n = 5, p = 0.31 and 110±0.8%, n = 4, p = 0.13, respectively) (**Figure 5C**). Long-term depotentiation, which is a form of LTD induced by LFS at potentiated synapses, was also impaired in CaMK-Scrib1<sup>-/-</sup> compared to Scrib1<sup>fl/fl</sup> synapses (**Figure 5D**). Indeed, in response to LFS, potentiated Scrib1<sup>fl/fl</sup> synapses were depotentiated (101.6±7.5%, n = 6, p < 0.05) whereas CaMK-Scrib1<sup>-/-</sup> synapses remained potentiated (128.9±8.7%, n = 6, p = 0.84). In conclusion, we found that LTP was enhanced whereas LTD and depotentiation were both abolished at CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses.

### **Scrib1 scaffolds PP1/PP2A at the PSD for appropriate LTD-signaling**

CaMK-Scrib1<sup>-/-</sup> synapses not only did not express LTD but instead exhibited LTP following LTD-inducing protocol (LFS) as shown in **Figure 5B**. To identify potential interacting partners of Scrib1 that could mediate this inappropriate intracellular signaling, portions of Scrib1 were used as baits to screen for potential binding partners in a P10 brain library using yeast two-hybrid screening. We focused on PDZ3 and PDZ4 (aa 990-1079 and aa 1086-1180, respectively of Q80U72-3), as they are lost in the circletail mutant that displays dramatic brain phenotypes (Moreau et al., 2010). One of the 53 clones identified as interacting with Scrib1 encoded a portion of the catalytic subunit of protein phosphatase/PP2 (Ppp2ca). Because phosphatases are important for LTD expression (Mansuy & Shenolikar 2006), we further investigated into the interactions of Scrib1 with members of this phosphoprotein phosphatase family. We analysed the binding properties of Ppp2ca in a yeast two-hybrid assay and found that it was indeed interacting with PDZ4 (**Figure 6A**). In addition, independent yeast two-hybrid screens of a human mammary gland epithelial library using Scrib1 (**Figure 6B**), Erbin or LANO (Borg & Santoni unpublished) as baits, identified Pp1 alpha, beta and gamma as partners of all members of the LAP family. Interestingly, in a secondary screen we observed that the interaction between LAP family members and Pp1 proteins was conserved during evolution. Indeed, Scribble from *Drosophila* or LET-413 (Erbin homologue) from *C. elegans* were able to interact with PP1 (Borg & Santoni unpublished). To assess whether Scrib1 and putative interacting phosphatases colocalize in heterologous cells, we expressed a green fluorescent protein (GFP)-tagged Scrib1 and a Myc-tagged Pp1γ or Ppp2ca. We observed in COS7 cells colocalization of both phosphatases with Scrib1 at the membrane and in intracellular structures (**Figure 6C and 6D**). Then, biochemical approaches were undertaken to ascertain the Pp1-Scrib1 interaction. First, we produced recombinant GST-Pp1 fusion proteins and performed GST pull down assays. We expressed either full or truncated HA-tagged versions ([aa 1-724] NH2- and [aa 717-1630] carboxy-terminal) of Scrib1 in COS7 cells and assayed their binding to recombinant GST-Pp1. Bound proteins were revealed with specific anti-HA antibodies. Scrib1 full length interacted with GST-Pp1, but neither with GST alone nor GST-C-terminal. We identified the NH2-terminal of Scrib1 region as responsible for the interaction with PP1 (**Figure 6E**). Second, we decided to map the regions within Pp1 proteins involved in this interaction. PP1 was truncated or mutated within its catalytic domain at sites shown to decrease or abrogate its phosphatase activity (Y272A, Y272Delta:Y272D) or involved in the binding to I2 (M290K-C291A), an inhibitor of Pp1, or implicated in the binding with PNUTS and spinophilin (Y272Delta: Y272D), two Pp1 binding partners (**Watanabe et al., 2001**). We also introduced a point mutation analogous to a mutation known to affect

the role of glc-7-10 (the yeast Pp1 homologue) in cell polarity processes (mutant F136L; Andrews and Stark, 2000) (**Figure 6F**). Loss of binding was obvious with the mutants affecting the catalytic activity of Pp1 while a small carboxy-terminal truncation (N302Delta: N302D) had no effect on PP1 binding to Scrib1. The Y272A mutation that decreases the phosphatase activity of PP1 did not affect the interaction with hScrib1 (**Figure 6F**). Based on these results, the Pp1-Scrib1 interaction was narrowed down to a PDZ-binding domain – PDZ domain interaction. We used modelling to assess important amino-acids involved in the interaction. In the Scrib1-PP2A complex model, the C-terminus of Ppp2ca is fixed in the hydrophobic pocket formed between helix B and strand B of Scrib1 PDZ4 domain (**Figure 6G**) as is commonly the case between PDZ domains and their targets (**Lee and Zheng, 2010**). Also, the residue D306 of PP2A C-terminal is favourably positioned to form a salt bridge with R1102 and/or K1124 residues of Scrib1. Hence, both hydrophobic interaction and a salt bridge should allow the interaction between the two partners. The generated model with the NetPhos 2.0 server (**Blom et al., 1999**) showed the presence of two serines at the contact zone, one of which (S1100) can potentially be phosphorylatable, although this remains to be confirmed. This serine, S1100, is ideally positioned to disrupt the interaction between Scrib1 and Ppp2ca, as the steric bulk of the added phosphate could prevent the formation of a salt bridge between D306 (PP2A) and R1102/K1124 (Scrib1). We further demonstrated that both endogenous PP1 and Ppp2ca interacted with Scrib1 in hippocampal lysates by coimmunoprecipitation using an antibody against Scrib1 (**Figure 6H**). Taken together, these data suggest that Scrib1 acts as a scaffold protein for PP1 and PP2A at the synapse and led us to investigate their expression levels in the PSDs of CaMK-Scrib1<sup>-/-</sup> synapses lacking Scrib1. Pp1 and Ppp2ca levels were found to be decreased by ~15 and 40%, respectively, at CaMK-Scrib1<sup>-/-</sup> PSDs compared to Scrib1<sup>+/+</sup> control synapses (n = 3, p < 0.05) (**Figure 6I**). On the other hand, levels of the kinase CaMKII were slightly increased in CaMK-Scrib1<sup>-/-</sup> mice compared to Scrib1<sup>+/+</sup> controls (n = 4, p = 0.63) (**Figure 6I**). In other words, Scrib1 interacts directly with PP1 and PP2A phosphatases to ensure their proper localisation at the synapse. In the absence of Scrib1, reduced levels of PP1/PP2A entail a decrease in LTD signaling pathway following LFS, which would favor the competing pathway downstream of CaMKII resulting in LTP induction instead of LTD. If this is the case, we should be able to rescue this phenotype by saturating the competing LTP pathway to enhance the probability of recruiting PP1/PP2A-dependent pathway during LTD. In agreement with this hypothesis, previous work in mutant mice has reported that high (saturated) levels of active CaMKII (phosphorylated T286) prevent LTP and favor LTD (Bejar et al., 2002). To saturate the LTP signaling pathway, we induced three strong consecutive HFS at 2x100 Hz, and then delivered the LTD-inducing protocol (LFS) at 1 Hz for 15 minutes. Under these conditions, saturated CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses were able to express long-term depotentiation similarly to Scrib1<sup>+/+</sup> synapses (81.2±0.4% and 82.5±0.6% respectively, n = 5, p = 0.94) (**Figure S4**). Hence, by saturating the LTP signaling pathway, we were able to rescue long-term depotentiation in CaMK-Scrib1<sup>-/-</sup> synapses.

### **Exposure to enriched environment rescues synaptic function and memory consolidation in CaMK-Scrib1<sup>-/-</sup> mice**

Finally, we aimed at achieving a more physiological rescue of LTD and spatial memory using enriched envi

ronment (EE) exposure (**Figure 7A**). Indeed, EE has been documented to increase synaptic transmission, enhance synaptic plasticity and improve spatial memory (Chancey et al., 2013; Foster and Dumas, 2001; van Praag et al., 2000). Importantly, EE exposure restored basal glutamatergic transmission illustrated by normal input-output relationship in EE CaMK-Scrib1<sup>-/-</sup> mice compared to EE Scrib1<sup>fl/fl</sup> mice (n = 5 and n = 6 respectively, p = 0.51) (**Figure 7B**). Following EE exposure we were also able to induce LTD using LFS in EE CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses (90.4±3%, n = 7/6 mice, p < 0.05) that was not different from LTD at EE Scrib1<sup>fl/fl</sup> synapses (93.5±5.8%, n = 7/6 mice, p = 0.60) (**Figure 7C**). This correlated with a cognitive rescue of spatial learning in Morris water maze revealed by equivalent latencies to find the hidden platform observed in EE Scrib1<sup>fl/fl</sup> and EE CaMK-Scrib1<sup>-/-</sup> mice (n = 15, genotype effect p = 0.08) (**Figure S5A and 7D**). Both genotypes improved their performances by decreasing the latency to find the platform (Day effect p < 0.001) and similar results were found by analysing the distance travelled to reach the platform (genotype effect, p = 0.15, Day effect p < 0.0001, **Figure S5B**). As expected, at short-term (1h) probe-test, no difference was observed in the time spent in the target zone (p = 0.25) (**Figure S5C**) and the number of platform crossings (p = 0.76) (**Figure S5D**) between EE Scrib1<sup>fl/fl</sup> mice (41.3±2.8% and 2.7±0.25, respectively) and EE CaMK-Scrib1<sup>-/-</sup> mice (46.6±3.5% and 2.8±0.35, respectively). More strikingly, long-term memory impairment observed at 24-hour probe-test was recovered in EE CaMK-Scrib1<sup>-/-</sup> mice. Indeed, similar time spent in target zone was found in both genotypes (EE Scrib1<sup>fl/fl</sup>: 45.1±3.8% and EE CaMK-Scrib1<sup>-/-</sup>: 45.9±4.5% p = 0.90) (**Figure 7E**) as well as equal number of platform crossings (EE Scrib1<sup>fl/fl</sup>: 3.7±0.5 and EE CaMK-Scrib1<sup>-/-</sup>: 3.4±0.45, p = 0.63) (**Figure 7F**). In summary, exposure to EE restores synaptic transmission as well as LTD expression leading to a rescue in spatial learning and memory formation in CaMK-Scrib1<sup>-/-</sup> mice.

## DISCUSSION

Elucidating the identity and role of proteins involved in synaptic plasticity is fundamental for understanding the neurobiology of memory formation and memory impairment. In this study, we were able to identify some of the cellular mechanisms at excitatory hippocampal neurons by which Scrib1 regulates hippocampus-dependent memory, using new conditional knock-out mice. According to our data, Scrib1 is required for functional and morphological synaptic maturation of CA1 pyramidal neurons. Molecularly, Scrib1 scaffolds PP1/PP2A phosphatases at the PSD of these synapses to ensure proper bidirectional plasticity signaling. Importantly, by rescuing glutamatergic synaptic function and plasticity using EE exposure, we were able to restore the capacity of normal spatial memory formation and functionally compensate Scrib1 absence. In summary, this study identifies Scrib1 as one of the fundamental scaffold proteins that enable the hippocampal glutamatergic synapse to mature and to express bidirectional plasticity required for memory formation process.

### Functional maturation of hippocampal synapses depends on Scrib1

Apical dendritic arborisation in the stratum radiatum region was increased in CaMK-Scrib1<sup>-/-</sup> CA1 region, a result consistent with previous data showing significant perturbation of the cytoskeleton in other tissues from

circletail (Scrib<sup>crc</sup>) mutants (Montcouquiol et al., 2003; Moreau et al., 2010; Yates et al., 2013). Also, basal glutamatergic synaptic transmission was dramatically reduced at CA3-CA1 synapses, despite normal AMPARs-mediated miniature currents in CaMK-Scrib1<sup>-/-</sup> CA1 neurons. These data are consistent with our recent findings reporting no impact of acute knock-down of Scrib1 on AMPARs EPSCs in transfected CA1 neurons (Piguel et al., 2014). Rather, the decrease in basal synaptic transmission observed in CaMK-Scrib1<sup>-/-</sup> CA1 synapses was due to a significant increase in the number of immature synapses that do not participate in basal synaptic transmission (Isaac et al., 1995; Liao et al., 1995). Interestingly, we recently revealed that Scrib1 is involved in the NMDAR-subunit switch that is associated with synapse maturation (Piguel et al., 2014, Yashiro & Philpot, 2008). We hence suggest that Scrib1 is necessary for appropriate connectivity and morpho-functional maturation of glutamatergic synapses through a mechanism that involves both cytoskeleton remodelling and an NMDAR-subunit switch (Yao et al., 2006). Similarly, the scaffold protein PSD-95, whose mutation in mice leads to a deficit in the NMDAR-subunit switch, has been reported to convert immature silent synapses into functional ones (Beique et al., 2006, Sanz-Clemente et al., 2013, Stein et al., 2003). Finally, new silent synapses formed following synaptic inactivity in CA1 neurons have been shown to be recruited during LTP induction leading to enhanced potentiation (Arendt et al., 2013). We can hypothesize then that the observed enhanced LTP at CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses is due to the increased immature synapses population that is potentially recruited during LTP induction. These results, together with our previous studies, highlight the importance of a regulation of Scrib1 levels, not only during development, for dendritic arborisation, but also in mature neurons, for functional synapses.

### **Scrib1-dependent bidirectional plasticity at hippocampal synapses**

In our study, loss of Scrib1 not only abolished LTD expression, but it generated significant LTP following LTD-inducing protocol and enhanced LTP at CA3-CA1 synapses. Our search for interacting partners of Scrib1 that could mediate such a phenotype in the absence of Scrib1 led to the identification of PP1/PP2A phosphatases. We notably show that Scrib1 directly interacts with PP1 and PP2A, and, also, that in its absence, PP1 and PP2A levels are strongly reduced at the synapse. This original interaction in mammalian neurons is in agreement with previous work demonstrating a direct interaction between hScrib and PP1 in heterologous cells (Nagasaka et al., 2013). PP1/PP2A has been reported to be necessary for both LTD and long-term depotentiation expression at CA3-CA1 synapses in mice, and partial inhibition of PP1 alters bidirectional plasticity by inducing a shift that favours potentiation in the hippocampus (Jouvenneau et al., 2003, 2006). Interestingly, altered bidirectional plasticity similar to the one observed in CaMK-Scrib1<sup>-/-</sup> mice was obtained in Neurogranin knock-out mice (Krucker et al., 2002). Neurogranin regulates the equilibrium between kinases and phosphatases activities by binding available Ca<sup>2+</sup>/calmodulin at basal levels. In normal conditions, a modest increase of Ca<sup>2+</sup> activates preferentially calcineurin instead of CaMKII and, consequently, PP1/PP2A initiates a series of substrate-specific dephosphorylations and triggers LTD. If PP1/PP2A levels are reduced at the synapse, as it is the case in our mutants, the resulting dephosphorylation process is slowed down compared to the competing phosphorylation process by CaMKII (Xia & Storm 2005, Lisman & Zhabotinsky 2001). This leads to the activation of LTP-signaling pathway instead of LTD at modest Ca<sup>2+</sup> lev-

els, as observed in CaMK-Scrib1<sup>-/-</sup> mice. Consistent with this model, we were able to rescue long-term depotentiation expression at CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses by in vitro saturation of LTP signaling pathway. Altogether, these results are consistent with a model where Scrib1 ensures bidirectional plasticity at hippocampal synapses by localising PP1/PP2A phosphatases in the immediate vicinity of NMDARs. This hypothesis is supported by recent data from our group demonstrating a direct interaction between Scrib1 and NMDAR-subunits in hippocampal neurons (Piguel et al., 2014). Based on these findings, we suggest that Scrib1 acts as a scaffold protein, bridging the calcium influx of NMDARs to specific downstream signaling phosphatases necessary for synaptic plasticity. Other proteins with multiple PDZ domain, such as the scaffold protein PSD-95, have also been reported to regulate bidirectional plasticity in a way that favours LTP induction (Migaud et al., 1998), while, another LAP protein, the scaffold protein Densin-180, binds directly to CaMKII to contribute to its basal targeting to spines and regulates LTD at CA3-CA1 synapses (Robison et al., 2005; Hell, 2014; Carlisle et al., 2011).

### **Spatial memory consolidation requires Scrib1 at hippocampal excitatory neurons**

Loss of Scrib1 in excitatory hippocampal neurons led to an impairment in long-term, but not short-term, spatial memory. These results argue for an important role of Scrib1 in regulating hippocampus-dependent spatial memory consolidation. Learning and memory processes have been reported to be regulated by PP1 and PP2A phosphatases (Mansuy and Shenolikar, 2006). PP2A has been shown to be necessary specifically in the consolidation phase starting 50 minutes after the learning session in chicken (Bennett et al., 2001). Also, pharmacological administration of an inhibitor of PP1/PP2A results in impaired LTD as well as spatial reference memory in rodents (He et al., 2001). Moreover, inhibiting LTD expression by blocking AMPARs endocytosis results in impairment in early consolidation (> 1h post-training) of spatial reference memory (Ge et al., 2010). Finally, down-regulation of protein phosphatases as the one observed in CaMK-Scrib1<sup>-/-</sup> mice is often associated with cognitive decline and dementia such as in Alzheimer's disease (Tian et al., 2002). These observations suggest that deficits in spatial memory consolidation in CaMK-Scrib1<sup>-/-</sup> mice involve LTD impairment due to an alteration in Scrib1-PP1/PP2A interaction and their downstream targets. In favour of this hypothesis, rescue of LTD expression at CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses by EE exposure led to the recovery of normal spatial memory consolidation. Our results are consistent with the postulate that PSD proteins mutations disrupting synapse development and function are associated with cognitive defects or mental disorders in humans (Durand et al., 2007; Bayés et al., 2011). Moreover, our findings among others highlight the importance of LAP proteins at the PSD in regulating signaling pathways underlying normal cognitive function. Indeed, knock-out of the LAP protein Densin-180 in mice leads to abnormal behaviours often considered as phenotypes of schizophrenia and autism spectrum mouse models (ADD REF.). Also, Scrib1 spontaneous mutation in Scrib<sup>crc</sup> mice leads to emotional and cognitive phenotype related to autism spectrum disorder (Moreau et al., 2010). In this study, we report specific alterations in spatial memory formation in Scrib1 conditional knock-out mice that might be considered as a mouse model for intellectual disabilities present in certain patients with autism (Seese et al., 2014).

### Beneficiary effect of environmental enrichment during development

In this study, we were able to overcome the absence of Scrib1 at hippocampal synapses using EE exposure, which yielded the rescue of synaptic basal transmission as well as LTD expression at CA3-CA1 synapses. EE has been documented to increase synaptic transmission, enhance synaptic plasticity and improve spatial memory (Chancey et al., 2013; Foster and Dumas, 2001; van Praag et al., 2000). In CaMK-Scrib1<sup>-/-</sup> mice, EE exposure that rescued glutamatergic synaptic function also restored memory formation, suggesting a causal link between altered synaptic plasticity and memory impairment observed in the absence of Scrib1. Other studies have also reported reversed learning and memory impairments by EE exposure in different memory-deficient animal models (Dahlqvist et al., 2004, Frick & Fernandez 2003, Need et al., 2003). EE rescue in CaMK-Scrib1<sup>-/-</sup> mice is independent of Scrib1 expression, and likely involves compensatory phenomena to overcome synaptic dysfunction and memory impairment. EE exposure has been reported to modulate the expression of several genes involved in synaptic plasticity and signaling pathways in the hippocampus (Rampon et al., 2000). For instance, expression of the scaffold protein PSD-95 is increased following 2 weeks of EE exposure. Since disruption of either Scrib1 or PSD-95 leads to similar alterations in synaptic function, a possible mechanism for the rescue by EE exposure in CaMK-Scrib1<sup>-/-</sup> mice can be an up-regulation of other proteins to compensate for the loss of Scrib1. In conclusion, our study demonstrates that EE exposure is a powerful tool sufficient to prevent and treat synaptic dysfunction associated with memory impairment caused by a genetic mutation.

### Conclusions

In this study, we were able to unravel cellular mechanisms by which Scrib1 at excitatory hippocampal neurons regulates hippocampus-dependent memory. We show that loss of Scrib1 leads to impaired spatial memory consolidation by generating immature glutamatergic synapses and abolished bidirectional plasticity at CA3-CA1 synapses. Early exposure to an enriched environment rescued spatial memory formation by restoring synaptic function and plasticity despite the genetic mutation of Scrib1. This work highlights the importance of the LAP protein Scrib1 as a key scaffold protein that ensures normal synaptic maturation and synaptic plasticity required for hippocampus-dependent memory formation.

### EXPERIMENTAL PROCEDURES

Yeast two-hybrid screening. The PDZ3 and PDZ4 portion of rat Scrib1 (aa 990-1070 and 1086-1180, respectively) were subcloned into pGBTK7 vector (Clontech) in-frame with the DNA-binding domain of GAL4 and used as a bait for the screening as previously described (Sans et al., 2003). Yeast two-hybrid screening and assays were performed accordingly to the Matchmaker™ Gold Yeast Two-Hybrid System protocol (Clontech). AH109 cells expressing Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> were combined with Y187 cells expressing a P10 mouse brain cDNA library (Yi Z et al., 2007). The mating mixture was plated on SD/Ade<sup>-</sup>/Trp<sup>-</sup>/Leu<sup>-</sup>/His<sup>-</sup> plates. From 1.5 x 10<sup>3</sup> colonies obtained 5 days after transformation, 56 passed high stringency conditions. Library plasmids from those colonies were rescued, amplified by PCR and sequenced, including Ppp2ca. Interactions between Pp1c and Ppp2ca phosphatases and Scrib1 constructs transfected into the haploid



yeast strain AH109 were tested through mating of the two yeast strains. For additional yeast two-hybrid screens, baits were cloned in pBTM116 to express proteins fused to LexA-BD which carries Trp1. A human mammary gland epithelial library cloned in pACT2 (Clontech) which carries Leu2 as a selection marker, was screened with Scrib1, Erbin or LANO as baits. L40 Trp<sup>+</sup>Leu<sup>+</sup> cotransformants were grown on plates with supplemented minimum medium that lacked tryptophan, leucine, histidine and contained 10 mM 3-aminotriazole (3-AT) and then tested for the b-galactosidase activity by the filter method.

COS-7 cell culture, transfection and immunocytochemistry. Cells were cultured in DMEM, supplemented with 10% (v/v) fetal calf serum (Invitrogen), 2 mM L-glutamine, and penicillin/streptomycin (50 U/mL) and transfected as previously described (Sans et al. 2005). hScrib1 GFP (green fluorescent protein) and HA-tagged Ppp1c or Ppp2ca cDNA samples were generously provided by I. Macara and David Brautigan (University of Virginia, Charlottesville, VA, USA), respectively. 48h after transfection, cells were fixed with 4% PFA, washed and permeabilized with 0.25% Triton X-100. After blocking, cells were incubated with the following primary antibodies: anti-green fluorescent protein (GFP) (1/1000; JL-8, Clontech), anti-HA (1/1000; MMS-101P, Covance). Following washout, cells were incubated with Alexa Fluor 488, 568 and phalloidin-A647 conjugated antibodies (Invitrogen). Images were obtained by the Zeiss Axiolmager Z1 microscope.

Pulldown. For GST pull down assays, either full or truncated HA-tagged versions (NH2- and carboxy-terminal) of hScrib were expressed in COS7 cells and assayed for binding to recombinant GST-PP1 fusion proteins. Cells were washed twice with cold PBS and lysed in lysis buffer (50 mM HEPES [pH 7.5], 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, and 1 mM EGTA) supplemented with 1 mM phenylmethylsulphonylfluoride [PMSF], 10 µg/ml aprotinin, and 10 µg/ml leupeptin. After preclearing, cellular lysates were incubated with the appropriate GST-tagged proteins bound to agarose beads for 2 hr. Protein complexes bound to beads were recovered and washed once with lysis buffer and twice with HNTG buffer (same as lysis buffer with 0.1% Triton X-100 final). Beads containing complexes were boiled in 1x sample buffer, separated on 7.5% SDS-PAGE and transferred on nitrocellulose for Western blot analysis with the appropriated antibody. GST pull down performed with wild type and different mutant PP1 were done as mentioned above using CaCo2 cell lysate. Anti-HA 3F10 monoclonal and anti-Scrib (C20) antibodies are from Roche Molecular Biochemicals and Santa Cruz Biotechnology respectively. The hScrib constructions are described in Audebert et al. (2004). PP1 mutations were done with the Quick Change Kit (Stratagene) according to the manufacturer's instructions.

Interaction model. The PDZ domains are small domains whose structure is highly conserved. There are many structures in the PDB showing an interaction between a PDZ domain and the C-terminal peptide of a partner. In these known structures, the C-terminal of the target peptide comes to be fixed to a hydrophobic pocket. To build an interaction model between residues 1082 to 1179 of Scrib1 (PDZ4 domain) and 304 to 309 of Ppp2ca, we used the known structure of the Scrib1 PDZ4 domain (PDB: 1UJU). We searched the structure of a PDZ domain in complex whose the protein sequence is most similar to Scrib1 PDZ4 domain,

and found the PDB 1MFG (Birrane et al., 2003). We aligned the two PDZ domain structures one on the other, and residues 304 to 309 of the peptide Ppp2ca on the peptide of the PDB 1MFG. The C-terminal residues of Ppp2ca are thus placed in the hydrophobic pocket of Scrib1 PDZ4 domain. This new PDB file was used as the basis for this interaction model. We used Coot (Emsley & Cowtan, 2004) to build the model and MolProbity (Chen et al., 2010) to test its quality. The final model did not show clashes between PP2a peptide and the Scrib1 PDZ4 domain. I used PyMOL to prepare the figures (DeLano, 2002).

Animals. Scrib1 conditional knock-out (cKO) mice were generated by crossing Scrib1<sup>ff</sup> mice that carry a floxed Scribble1 gene and CaMKIIα<sup>Cre/+</sup> mice that express Cre recombinase in principal neurons of the brain (Casanova et al., 2001). To generate the Scrib1 flox line a mouse BAC containing 11994 bp of Scrib1 genomic DNA was identified by screening a 129-based BAC library (CJ7 ES cell DNA, CITB, Research Genetics). After generating an intermediate targeting allele (TA) with loxP sites upstream of Scrib1 exon 2 and downstream of exon 8, and frt sites that flank a positive selection neomycin (NEO) marker, mice containing the TA were mated to mice expressing Flpase, which removed the neo marker and generated the floxed (f/f) allele. In Figure S1, arrows indicate locations of PCR primers F (forward), R1 (reverse 1) and R2 (reverse 2). Scrib1 mutant mice were genotyped by PCR using the following primers: F-gcacactgggtatcatggcta; R1-gcaatctccagagccttacaga; R2-cccttggaacctacatcccaa. The amplified products were 437 bp for WT band (F+R1), 541 bp for flox band (F+Wt); 193 bp for cKO band (F+R1) used to identify if exons2–8 had been deleted (Figure xx). Cre genotyping was done using the following primers: F-CGGCATGGTGCAAGTTGAATA; R-GCGATCGCTATTTTCCATGAG. The resultant band was 300 bp (Figure S1A). To profile CaMKIIα-Cre-directed gene expression throughout the mouse brain, we crossed homozygote B6.Cg-Gt(ROSA)26Sortm6(CAG-ZsGreen1)Hze/J (thereafter called Ai6 mice) with CaMKIIα-Cre/+ mice, in order to obtain a compound Ai6f/+,CaMKIIα-Cre/+. Adult Scrib1<sup>ff</sup> and CaMK-Scrib1<sup>-/-</sup> male mice were housed collectively in groups of five to eight in polypropylene cages for biochemical experiments and enriched environment behavioral experiment and in individual cages for others behavioral experiments. Room temperature (RT) was set at 23 ± 1°C, lights were on from 7:00 a.m. to 7:00 p.m. and food and water were available ad libitum. Male mice littermates were used in all experiments. This study was performed in full accordance with recommendations of the European Communities Council Directives (86/609/EEC), the French national Committee (87/848) and the requirements of the United Kingdom Animals (Scientific Procedures) Act 1986, AWERB Newcastle University (ID: 374).

Coimmunoprecipitation. For coimmunoprecipitation experiments, one month old Sprague Dawley rat hippocampal tissues were used. Soluble extracts of 0.5-1 ml were incubated with 25 µl of PPI or 10 µl of anti-Scrib1 rabbit antibody (Montcouquiol et al., 2006). Samples were immobilized on Protein A/G agarose beads and beads were then pelleted by centrifugation and resuspended in 2X SDS sample buffer (Sans et al., 2003, 2005). Samples were finally analyzed by SDS/PAGE and immunoblotted using 1/500 Scrib1 anti-rabbit polyclonal antibody; 1/100 anti-PP2A (BD Biosciences), 1/400 anti-PP1 (Santa-cruz).

PSD preparation. Ten male mice of each genotype were used for the preparation of subcellular and PSD fractionation. Detailed procedure performed at 4°C is presented in Moreau et al., 2010. Western blots were performed essentially as described in Sans et al. (2000). Rabbit polyclonal antibody Scrib1 was diluted at 1/500, mouse monoclonal CaMKII (C265, Sigma-Aldrich) at 1/5000, PP2A at 1/250 and PP1 at 1/400. Each experiment was repeated three times, and representative blots are shown.

Histology & Immunohistochemistry. Mice were anesthetized using a Phenobarbital i.p injection (lethal dose) Physiological saline followed by paraformaldehyde 4% in 0.1M PBS at pH 7.4 was next perfused transcardially and fixed brains were removed and stored in paraformaldehyde 4% at 4°C for one week. Serial brain sections of 40 µm were obtained using a vibratome and stained with cresyl violet. Cre-recombinase expression in the hippocampus was detected using AI-6 transgenic mice. Fixed adult-brain sections of 40 µm were accordingly to the manufacturer's instructions in the BrainStain Imaging kit (Life Technologies). Neuronal staining was obtained using NeuroTrace 530/615 red fluorescent Nissl stain (1/300; Life Technologies) and was coupled with DAPI (4',6-diamidino-2-phenylindole) a nucleic acid stain (1/300). For Scrib1 immunofluorescence staining, non-specific sites were blocked with 3% normal goat serum (NGS) / 0.3% Triton X-100 in PBS for 2 hours at RT. Anti-Scrib1 rabbit polyclonal antibody (Montcouquiol et al., 2006, 1/300) was incubated with brain sections for 48 hours at 4°C. Following washing, secondary Alexa Fluor 488-coupled anti-rabbit (1/500; Life Technologies) and DAPI (1/10000) in 0.2% Triton X-100 / 5% NGS in PBS were applied for 2 hours at RT. Labelled brain sections were mounted on slides using Prolong Gold Antifade (Life Technologies) and conserved at 4°C for analysis with fluorescence microscope (Zeiss). No fluorescence was found in control experiments in which primary antibody was omitted.

Golgi Cox impregnation method. Adult mouse brains fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4 were removed and stored in the same fixative for 2 h before slicing 100-µm thick coronal sections. Brain sections were first treated for 30 min with PB containing 1% osmium tetroxide then incubated overnight in 3.5% potassium dichromate, followed by 2% silver nitrate solution for 6 h. After gradual dehydration in alcohol, sections were infiltrated with Entellan and mounted. For morphological analysis, neurons were reconstructed by a trained experimenter blind to the conditions and analyzed using camera lucida at a magnification of x100 (DMLS Microscope, Leica). Neurons within the CA1 region used for analysis were chosen from slices that were between 1.58-1.82 anterior to Bregma. For each neuron, all branches of basal and apical dendritic trees were reconstructed. Dendritic length and dendritic complexity were measured by Sholl analysis with respect to growing distance from the cell body. Dendritic complexity was quantified by counting the intersection number between virtual concentric rings of 10 µm and dendritic branches. Electron microscopy analysis of CA1 hippocampal synapses. Adult male mice were terminally anesthetized by a brief inhalation of isoflurane (0.05% in air) and intramuscular injection of ketamine (100mg/kg) and xylazine (10 mg/kg), and then transcardially perfused with 4% PFA and 2% glutaraldehyde in 0.1 M PBS, pH 7.2. The brains were dissected and stored in 4% PFA in PBS, at 4°C, overnight. Vibratome sections of 100 µm thick were cut, collected in PBS, postfixed in 1% osmium tetroxide, dehydrated in ascending scale of

ethanol, infiltrated, and flat embedded in Durcupan epoxy resin as previously described (Moreau et al., 2010). CA1 hippocampi were trimmed and embedded in resin blocks for further semithin and ultrathin sectioning with a Leica UC6 ultramicrotome. Ultrathin sections (70–90 nm) were counter-colored with uranyl acetate and lead citrate, and visualized with a Philips CM100 transmission electron microscope (FEI) at 100 kV. The images were captured with an AMT XR40 4 megapixel side mounted CCD camera at a magnification between 7,900 and 92,000x. Only identified synapses on dendritic spines of apical dendrites of pyramidal cells in CA1 stratum radiatum were included in the analysis. No tangentially cut synapses were analyzed. To determine the spine density (number of spine/ $\mu\text{m}^2$ ), we utilized 14–17 images per animal (7,900x magnification, single image area =  $176.95 \mu\text{m}^2$ ) in which we identified the spines as in Fiala and Harris (Fiala and Harris, 1999), and, then, quantified them using the cell counter tool in ImageJ (<http://rsb.info.nih.gov/ij>) (Sfakianos et al., 2007). The average thickness of the PSDs was measured as described by Dosemeci et al. (2001). Results in *Scrib1<sup>ff</sup>* and mutant animals are presented as the mean  $\pm$  SEM. The measurements were all performed by experimenters blind to the genotype.

Electrophysiological recordings. Littermate male mice between postnatal day 29 and P34 were used for all electrophysiological recordings. To record activity of CA3-CA1 synapses, we electrically stimulated Schaffer collateral fibers and recorded CA1 field excitatory postsynaptic potentials (fEPSPs). Basal transmission was evaluated by increasing the intensity of stimulation from 0 to 10mV in 1 mV steps. Paired-pulse ratio was examined by delivering two stimulations at the same intensity separated by 25, 50, 100 or 200 ms. LTP protocols consisted of 2 or 3 trains at 100 Hz for 1 second and LTD protocol consisted of 900 pulses delivered every second at 1 Hz during 15 minutes. Averages of the last 10 min were compared between *Scrib1<sup>ff</sup>* and *CaMK-Scrib1<sup>-/-</sup>* mice, unless mentioned otherwise. During extracellular field recordings, at least 20 minutes of stable fEPSP slopes (baseline) were recorded before starting any experiment. aCSF contained the following drugs: 10  $\mu\text{M}$  strychnine, 100  $\mu\text{M}$  picrotoxin, and 100  $\mu\text{M}$  RS-MCPG ( $\alpha$ -methyl-4-carboxyphenylglycine). D-AP5 was used when indicated to test LTP dependence on NMDARs. For AMPAR-mediated miniature EPSCs experiments, CA1 cells were voltage clamped at -70 mV in the presence of TTX (0.5  $\mu\text{M}$ ). Amplitude and frequency of at least 200 continuous mEPSC events per cell were analyzed. Recorded data were amplified by Multiclamp 700B (Axon instruments) and recorded on the hard disk using pClamp9 (Axon instruments). All drugs were purchased from Tocris Bioscience. All experiments were performed without prior knowledge of mice genotype.

Behavioural testing. Behavioural experiments were conducted on adult *Scrib1<sup>ff</sup>* and *CaMK-Scrib1<sup>-/-</sup>* male mice littermates. All animals had been weighed twice a week from 8 to 18 weeks of age. Mice were tested in activity cages for their locomotor activity (see Moreau et al, 2010), in the Plus maze test to measure anxiety-like behavior as well as in the Y maze to tested spontaneous alternation. All behavioral tracking images were analyzed with Viewpoint video tracking. During the plus maze test, mice were placed in the center of the maze and allowed free access to all arms for 5 min (60 lux in the center). The percentage time spent in the open arms was computed. During the Y-maze test, mice were placed at the end of one of the arms of the

maze and allowed free access to three arms for 5 min (60 lux in the center). The sequence of arms entries, the total number of arm entries and the number of triads are recorded in order to calculate the percentage of alternation. Morris water maze testing took place in a circular pool (diameter, 150 cm) filled with water (19-20°C) rendered opaque by a nontoxic white cosmetic adjuvant. Mice were trained to locate a submerged platform to escape from the water (14 cm diameter, 1.5 cm below the water surface) using spatial cues placed on the walls. Three daily trials with 5 minute inter-trial interval and a cutoff at 60 seconds were conducted during training phase. Each subject was placed by the tail into the water, immediately facing the perimeter, at one of the cardinal compass points. Mice were released from a different starting point at each trial, and sequence of these starting points was randomized from day to day. One or 24 hours after the last session of acquisition, a probe test for spatial discrimination was conducted. For that, the hidden platform was removed and each subject was placed into the water diagonally opposite to the target quadrant. Time spent in the target quadrant (% of total time, chance level = 25%) in addition to the number of the platform location crossings (where the platform was located during training) were measured over 60 seconds. Visible platform task was also performed in the same water maze using a 15-minute inter-trial interval and a total of 6 trials.

Enriched environment. Environment enrichment started during prenatal period and continued postnatally for at least 4 weeks before the onset of experiments. Enriched cages consisted of large (46 x 37 x 21 cm) cages that contained at least 5 mice per cage and equipped with running wheels, small houses and several toys. Position of running wheels and small houses changed on a daily basis whereas toys were daily rearranged and some replaced with new ones of different shapes, textures and colours and to stimulate animals' exploratory behaviour. All mice received standard lab chow and water ad libitum. The experimenters were blind to genotype throughout electrophysiological and behavioural testing.

Statistical analysis. Statistical analyses performed for each experiment are summarized in Table S1 that indicates chosen statistical test, n and p values, as well as degree of freedom and F/t values. Data were tested for normality using D'Agostino & Pearson omnibus normality test where appropriate and all graphs represent mean  $\pm$  SEM. GraphPad prism software was used for statistical analysis and  $p < 0.05$  was considered as statistically significant.

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## FIGURE LEGENDS

**Figure 1. Generation of *Scrib1* conditional knock-out mouse: *CaMK-Scrib1*<sup>-/-</sup>.** (A) Coronal slices stained with cresyl violet showing normal brain gross anatomy in *Scrib1*<sup>fl/fl</sup> and *CaMK-Scrib1*<sup>-/-</sup> mice (scale bar, 1mm). (B) Neurostain visualized with Cy3 (red) and DAPI staining showing expression in a 10-week-old *Ai6f/+;CaMKIIα-Cre/+* doubletransgenic mouse in vibratome coronal sections (B<sub>a</sub>) with a higher magnification of CA1 pyramidal layer (B<sub>b</sub>). (scale bar, 40μm) (C) *Scrib1* expression in dorsal hippocampus of adult *Scrib1*<sup>fl/fl</sup> (C<sub>a</sub>) and *CaMK-Scrib1*<sup>-/-</sup> (C<sub>b</sub>) mice. \*\* *p* < 0.01. (scale bar, 50μm)

**Figure 2. *CaMK-Scrib1*<sup>-/-</sup> mice exhibit normal short-term but impaired long-term spatial memory.** (A) Experimental timeline of hidden platform water maze test showing an 8-day training phase followed by two probe tests at 1 and 24 hours after the last training session. (B) Slower spatial learning in *CaMK-Scrib1*<sup>-/-</sup> (red; *n* = 17) compared to *Scrib1*<sup>fl/fl</sup> mice (black; *n* = 12) revealed by mean latency to reach the hidden platform in Morris water maze over 8 days of training (*p* < 0.001). (C) Equivalent time spent searching in each quadrant during a 60-second probe trial performed without the platform at 1h (*n* = 6 and 9). (D) Same number of platform crossings and representative swim trace patterns during probe test at 1h for *Scrib1*<sup>fl/fl</sup> (up) and *CaMK-Scrib1*<sup>-/-</sup> (down); a circle marks target location. (E) Time spent searching in target quadrant during probe test at 24h was decreased in *CaMK-Scrib1*<sup>-/-</sup> (red; *n* = 8) compared to *Scrib1*<sup>fl/fl</sup> mice (black; *n* = 6). (F) Fewer platform crossings were counted for *CaMK-Scrib1*<sup>-/-</sup> mice indicating imprecise localisation of the hidden platform and representative swim trace patterns during probe test at 24h for *Scrib1*<sup>fl/fl</sup> (up) and *CaMK-Scrib1*<sup>-/-</sup> (down); a circle marks target location. \* *p* < 0.05, \*\* *p* < 0.01.

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**Figure 3. Increased dendritic arborisation and immature synapses in the stratum radiatum of *CaMK-Scrib1*<sup>-/-</sup> pyramidal neurons.** (A) Representative reconstructions of dendritic arborisation of *Scrib1*<sup>fl/fl</sup> (black) and *CaMK-Scrib1*<sup>-/-</sup> (red) CA1 pyramidal neurons. (B and C) Sholl analysis of basal dendrites revealed equal total number of intersections (B) and similar total length (C) in CA1 *CaMK-Scrib1*<sup>-/-</sup> neurons (red; *n* = 12/3 mice) and *Scrib1*<sup>fl/fl</sup> neurons (black; *n* = 9/3 mice). (D and E) Sholl analysis of apical dendrites showed increased total number of intersections (D) and total length (E) in particular in the median region of CA1 *CaMK-Scrib1*<sup>-/-</sup> neurons compared to *Scrib1*<sup>fl/fl</sup> neurons. (F and H) Representative electron micrographs of hippocampal CA1 stratum radiatum region of *Scrib1*<sup>fl/fl</sup> (F) and *CaMK-Scrib1*<sup>-/-</sup> (H) mice and a higher magnification of a spinous synapse in each (Fi and Hi, respectively). (G and I) Shifted distribution of PSD thickness (G) and PSD length (I) towards smaller PSDs (less than 200 nm) in the stratum radiatum of *CaMK-Scrib1*<sup>-/-</sup> (red) compared to controls (black) (*n* = 946/2 mice). \*\* *p* < 0.01, \*\*\* *p* < 0.001.

**Figure 4. *CaMK-Scrib1*<sup>-/-</sup> mice display reduced number of active CA3-CA1 synapses.** (A) Input-output relationship was reduced in *CaMK-Scrib1*<sup>-/-</sup> slices (red) with respect to *Scrib1*<sup>fl/fl</sup> (black) (*n* = 12/6) (*p* < 0.0001). (B) Similar paired-pulse ratios at 25, 50, 100 or 200 ms in *Scrib1*<sup>fl/fl</sup> and *CaMK-Scrib1*<sup>-/-</sup> slices (*n* = 6). (C) Representative mEPSCs traces from *Scrib1*<sup>fl/fl</sup> (C) and *CaMK-Scrib1*<sup>-/-</sup> (Ci) CA1 neurons. (D) Similar average am-

plitudes of mEPSCs at Scrib1<sup>ff</sup> and CaMK-Scrib1<sup>-/-</sup> CA3-CA1 synapses (n = 5). **(E)** Average frequency of mEPSCs was decreased in CaMK-Scrib1<sup>-/-</sup> compared to Scrib1<sup>ff</sup> CA3-CA1 synapses (p < 0.05).

**Figure 5. Loss of Scrib1 Enhances LTP and abolishes LTD in CaMK-Scrib1<sup>-/-</sup> synapses.** **(A)** Enhanced LTP induced by HFS consisting of 2 trains at 100 Hz at CA3-CA1 CaMK-Scrib1<sup>-/-</sup> synapses (red; n = 6/5) compared to Scrib1<sup>ff</sup> synapses (black; n = 5) (p < 0.01). **(B)** Abolished LTD in CA3-CA1 CaMK-Scrib1<sup>-/-</sup> synapses (n = 6/5) that generated LTP following LFS at 1 Hz stimulation in contrast to Scrib1<sup>ff</sup> synapses (n = 7/6) that induced LTD (p < 0.001). **(C)** A weaker stimulation protocol at 0.5 Hz did not induce any change in Scrib1<sup>ff</sup> (n = 5, p = 0.31) or CaMK-Scrib1<sup>-/-</sup> mice (n = 4, p = 0.13). **(D)** Long-term depotentiation at CA3-CA1 synapses, potentiated with HFS consisting of 3 trains at 100 Hz followed by LFS at 1 Hz delivery, was found to be impaired in CaMK-Scrib1<sup>-/-</sup> (n = 5) compared to Scrib1<sup>ff</sup> mice (n = 6) (p < 0.05). **(E)** Saturation of LTP pathway rescues long-term depotentiation in CaMK-Scrib1<sup>-/-</sup> synapses. Three strong protocols of LTP consisting of 3 trains at 100 Hz were delivered to saturate synapses and followed by LFS to induce long-term depotentiation, which was similar in Scrib1<sup>ff</sup> and CaMK-Scrib1<sup>-/-</sup> slices (n = 5). p = 0.94

**Figure 6. Scrib1 scaffolds PP1 and PP2 phosphatases.** **(A)** Directed yeast two-hybrid assays with Scrib1 and PP2A catalytic subunit (Ppp2ca) constructs. Schematic domain structures of Scrib1<sup>PDZ</sup>, Scrib1<sup>PDZ3</sup> and Scrib1<sup>PDZ4</sup> used as baits. Ppp2ca binds Scrib1<sup>PDZ</sup> and Scrib1<sup>PDZ4</sup>, but not Scrib1<sup>PDZ3</sup>. An empty bait plasmid or a pGAD-Scrib1 constructs were used as negative control. **(B)** Directed yeast two-hybrid assays with Scrib1 and PP1 constructs. Pp1a,b,c can bind Scrib1. **(C, D)** Scrib1<sup>WT</sup> co-localizes with Pp1c **(C)** and Ppp2ca **(D)** catalytic subunits. COS-7 cells transiently co-transfected with hScrib1-GFP (green) and HA-tagged Pp1c (red) **(C)** or hScrib1-GFP (green) and Ppp2ca (red) **(D)**. Phalloidin is shown in blue. Bottom white rectangles show in higher magnification individual or merged staining at the plasma membrane and cytoplasm level. Scale bars, 15µm. **(E)** Three N-terminal HA-tagged constructs that contain either the full length (Full), N-terminal (N-term) or C-terminal (C-term) truncated form of Scrib were expressed in COS cells. Lysates from the different transfected cell populations were pulled-down with GST alone (GST) or GST-PP1 (PP1) and submitted to SDS-PAGE and western blot analysis with an anti-HA antibody. The input of each experiment was also loaded (TL). **(F)** Schematic representation of PP1: with the catalytic core domain spanning from amino-acid residue 42 to 269 and the binding domain from 270 to 330. The different point mutations (F136L, Y272A, M290K, C291A) and truncations (Y272Delta: Y272D, N302Delta: N302D) are mentioned with their corresponding positions. CaCo2 cell lysate was used for GSTpull-down experiments with wild type or mutant versions of PP1 indicated GST fusion protein or GST alone as a negative control. Resulting complexes were analyzed by anti-Scrib antibody western blot. **(G)** Model of the interaction of Scrib1-PDZ4 K1124 (red) and R1102 (orange) residues with the T304 to L309 residues from Ppp2ca C-term (green) and overall view of the model of the interaction (top right) between the alpha helix B (blue) and Beta strand B (red) of Scrib1-PDZ4 (Gray) and Ppp2ca C-terminus (green). The residue S1110 (yellow) is adequately positioned to prevent interaction between PP2a and Scrib1-PDZ4 when phosphorylated. **(H)** Endogenous CoIP of Scrib1, Pp1 and

Ppp2ca from the hippocampus. The 100,000 x g detergent supernatants were immunoprecipitated with Scrib1 antibodies. The precipitates show positive immunoblotting for Pp1 and Ppp2ca subunits. **(I)** Immunoblot analysis of PSD fractions from Scrib1<sup>f/f</sup> controls and CaMK-Scrib1<sup>-/-</sup> mice and quantitation of the various proteins as indicated on the left the blots normalized to WT on the right (100%; black histograms).

**Figure 7. Exposure to enriched environment rescues synaptic function and memory consolidation in CaMK-Scrib1<sup>-/-</sup> mice.** **(A)** Example of enriched environment (EE) cage. **(B)** Input-output relationship was restored in EE CaMK-Scrib1<sup>-/-</sup> (n = 5) with respect to EE Scrib1<sup>f/f</sup> slices (n = 6). **(C)** Normal LTD expression following LFS at 1 Hz stimulation in both EE CaMK-Scrib1<sup>-/-</sup> (red) and EE Scrib1<sup>f/f</sup> CA3-CA1 synapses (black) (n = 7/6). **(D)** Spatial learning was normal in EE Scrib1<sup>f/f</sup> and EE CaMK-Scrib1<sup>-/-</sup> mice revealed by mean latency to reach the hidden platform in Morris water maze over 9 days of training. **(E)** Equivalent time spent searching in each quadrant during long-term probe test performed at 24h after the last session of training (n = 15). **(F)** Same number of platform crossings and representative swim trace patterns during probe test at 24h for EE Scrib1<sup>f/f</sup> (up) and EE CaMK-Scrib1<sup>-/-</sup> mice (down); a circle marks target location.

**Figure S1 (related to Figure 1): Generation of Scrib1 conditional knock-out mouse.** **(A)** The BAC DNA construct used for generation of a conditional Scrib1 allele was made by recombining technique as described in Yamben et al., 2013. **(B)** DNA fragments were detected by PCR in hippocampus, cortex and tail fractions of 2, 5, and 9 weeks old Scrib1<sup>f/f</sup> (a) and Scrib1<sup>f/f,CaMKII-cre</sup> (b) mice. The excised Scrib1 fragment (Scrib1<sup>Δf/f</sup>) was only observed in structures expressing the Cre-recombinase of Scrib1<sup>f/f,CaMKII-cre</sup> mice (hippocampus and cortex). Interleukin and β Actin were used as control genes. **(C)** Histograms show the relative amount of protein (in percent of 2W CaMK-Scrib1<sup>-/-</sup>). Levels were measured by densitometric scanning of Western blots. In the hippocampus of 5 and 10 weeks old mice, Scrib1 levels were reduced by ~80% (n = 4). **(D)** No difference in Scrib1 levels was detected in the cerebellum of 2, 5 and 10 weeks old mice (n = 3).

**Figure S2: Normal locomotor activity, anxiety levels and spatial working memory in CaMK-Scrib1<sup>-/-</sup> mice.** **(A)** Activity episodes in photocell-based chambers, recorded in bouts of 1 hour during 72 hours revealed normal locomotor activity in control (black; n = 6) and CaMK-Scrib1<sup>-/-</sup> mice (red; n = 9). **(B)** Similar anxiety levels in control (n = 13) and CaMK-Scrib1<sup>-/-</sup> mice (n = 14) demonstrated by equivalent time spent in anxiogenic open arms of the elevated plus-maze. **(C)** Equal spontaneous arm alternations for control mice (n = 7) and CaMK-Scrib1<sup>-/-</sup> mice (n = 6) suggesting normal spatial working memory in the Y-maze.

**Figure S3 (related to Figure 2): CaMK-Scrib1<sup>-/-</sup> mice exhibit normal short-term but impaired long-term spatial memory.** **(A)** Distance travelled to reach the hidden platform in Morris water maze over 8 days of acquisition revealed slower spatial learning in CaMK-Scrib1<sup>-/-</sup> (red; n = 17) compared to control (black; n = 12). **(B)** Swim speed measures over the 8-day training phase were similar in control and CaMK-Scrib1<sup>-/-</sup> mice. **(C)** Normal sensory-motor and visual functions in CaMK-Scrib1<sup>-/-</sup> mice during the visible platform Morris water maze training over 6 trials. **(D)** Probe test at 24h (without probe test at 1h) showed a decrease in the

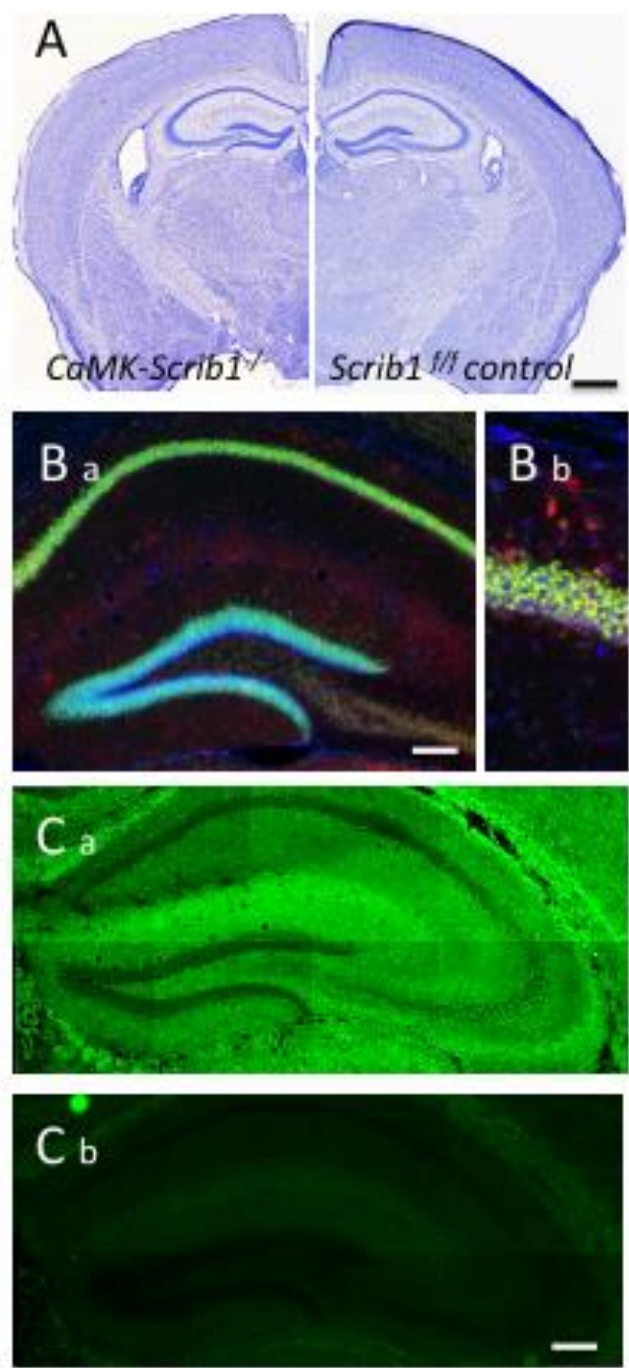
time spent searching in target quadrant in CaMK-Scrib1<sup>-/-</sup> (n = 8) compared to Scrib1<sup>fl/fl</sup> mice (n = 6). **(E)** Decreased number of platform crossings in CaMK-Scrib1<sup>-/-</sup> and representative swim trace patterns during probe test at 24h for control (top) and CaMK-Scrib1<sup>-/-</sup> (bottom); a small circle marks target location. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0,001.

**Figure S4 (related to Figure 8): Exposure to enriched environment rescues spatial learning in CaMK-Scrib1<sup>-/-</sup> mice.** **(A)** Experimental timeline of hidden platform water maze test showing a 9-day training phase followed by two probe tests at 1 and 24 hours after the last training session. **(B)** Distance travelled to reach the hidden platform in Morris water maze over 9 days of acquisition depicted normal spatial learning in EE CaMK-Scrib1<sup>-/-</sup> (red) and EE control (black) (n = 15). **(C)** Probe test at 1h showed equivalent time spent searching in target quadrant in EE CaMK-Scrib1<sup>-/-</sup> compared to EE control mice. **(D)** Similar number of platform crossings in EE CaMK-Scrib1<sup>-/-</sup> and representative swim trace patterns during probe test at 1h for EE control (top) and EE CaMK-Scrib1<sup>-/-</sup> (bottom); a small circle marks target location.

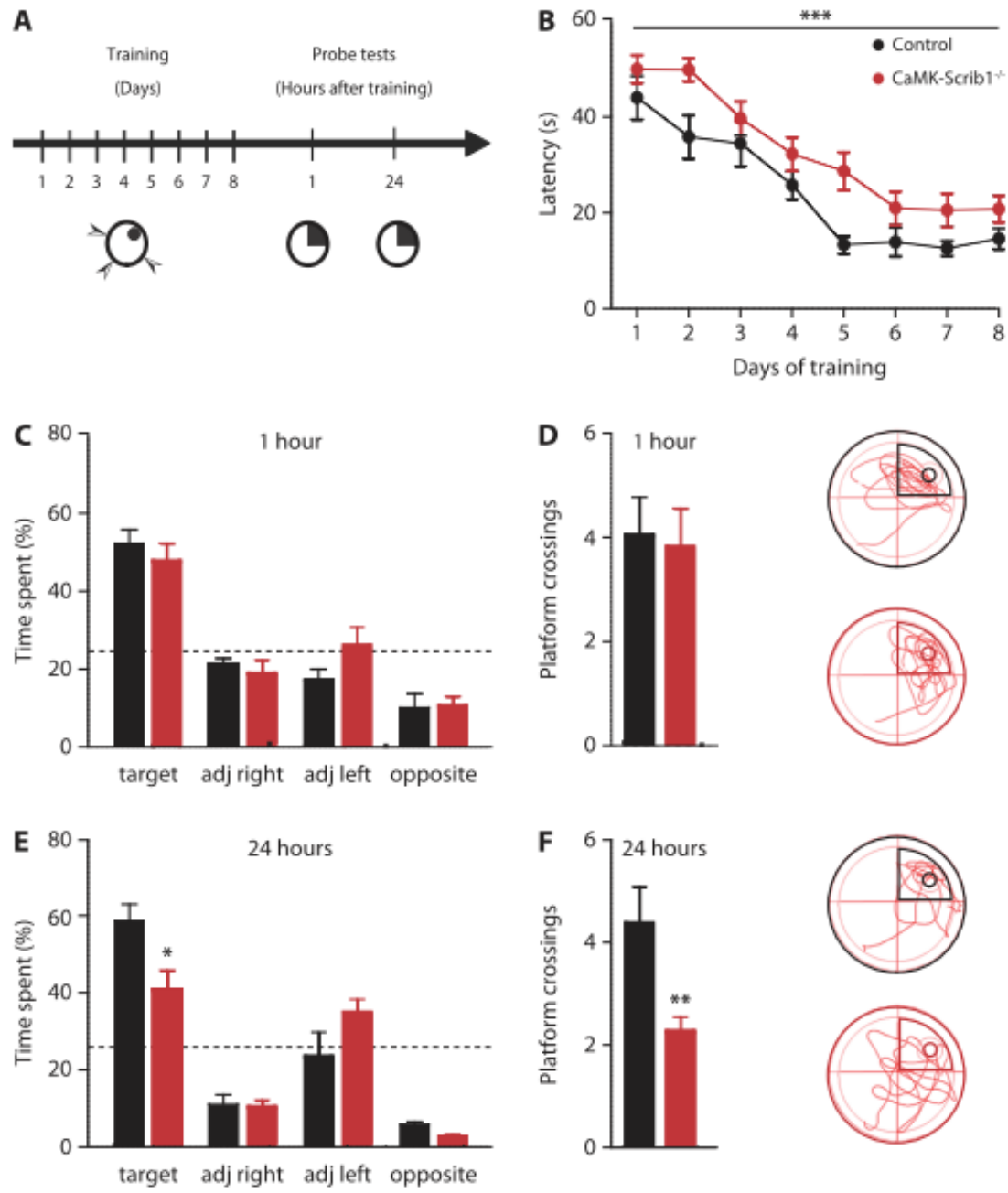
**Table S1: Summary of statistical analysis performed for each experiment.**

Single n values represent the same value for each group (control and CaMK-Scrib1<sup>-/-</sup>). Different n values are represented in black for control and in red for CaMK-Scrib1<sup>-/-</sup> group.

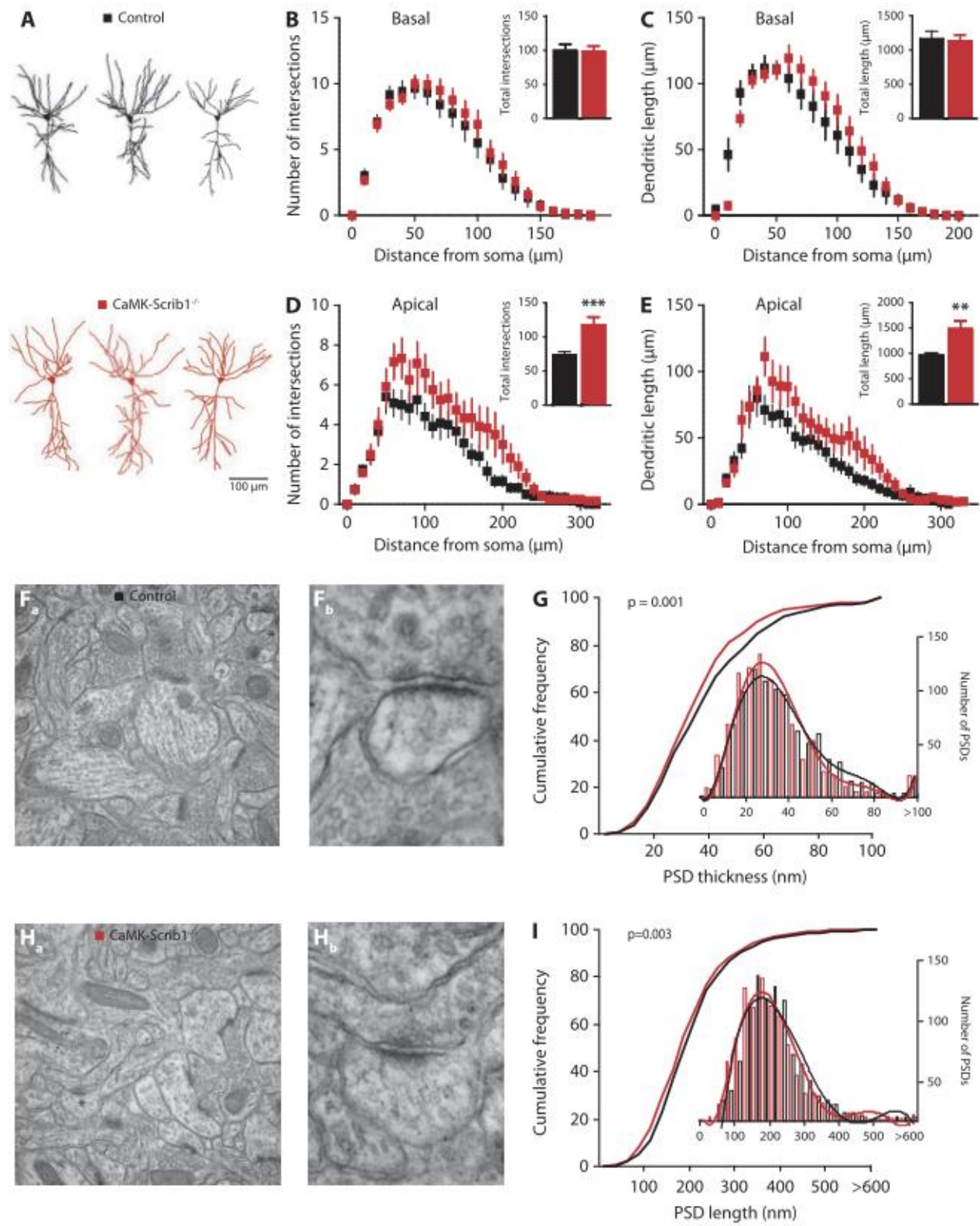
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FIGURE 1



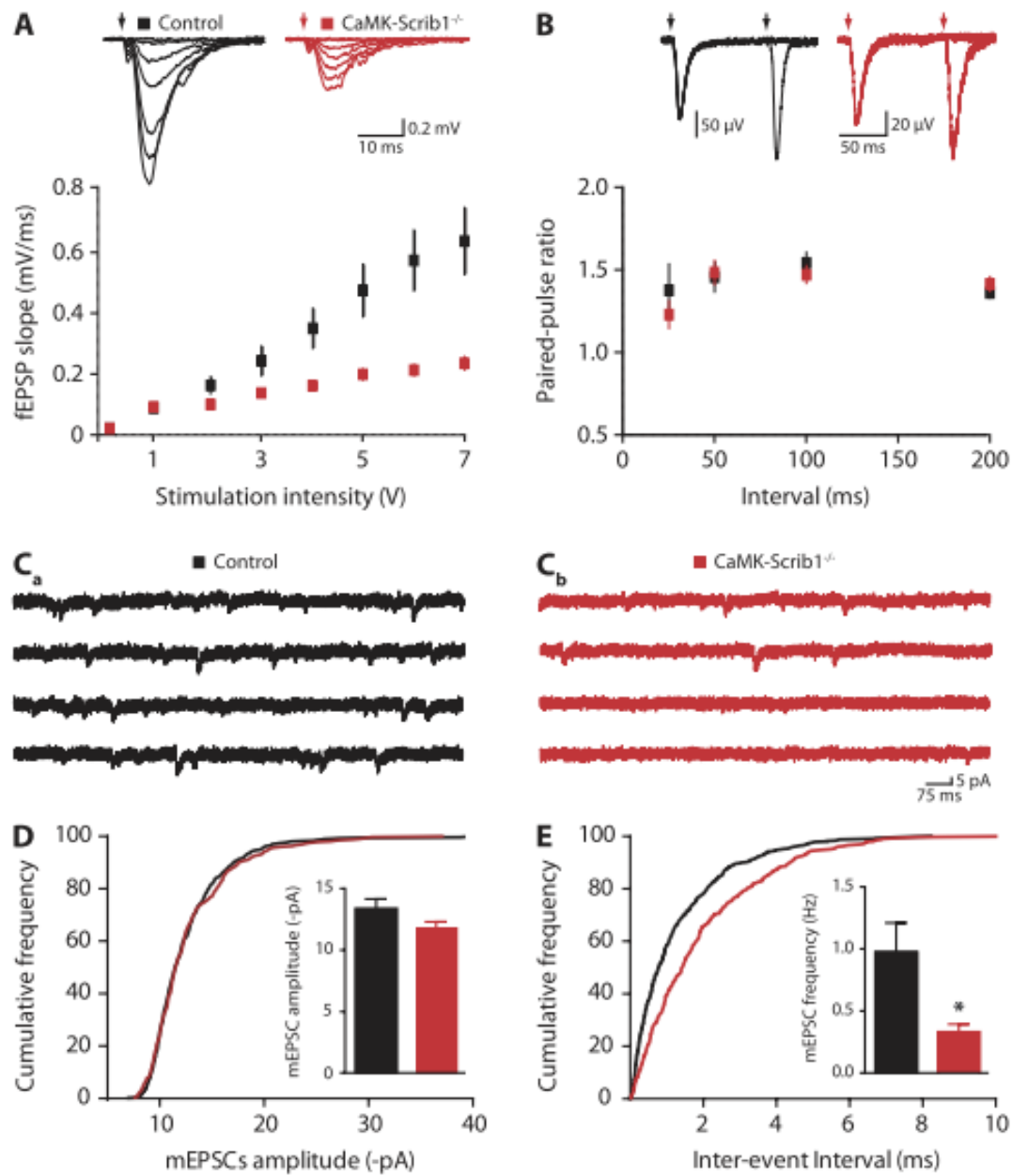
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FIGURE 2



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FIGURE 3

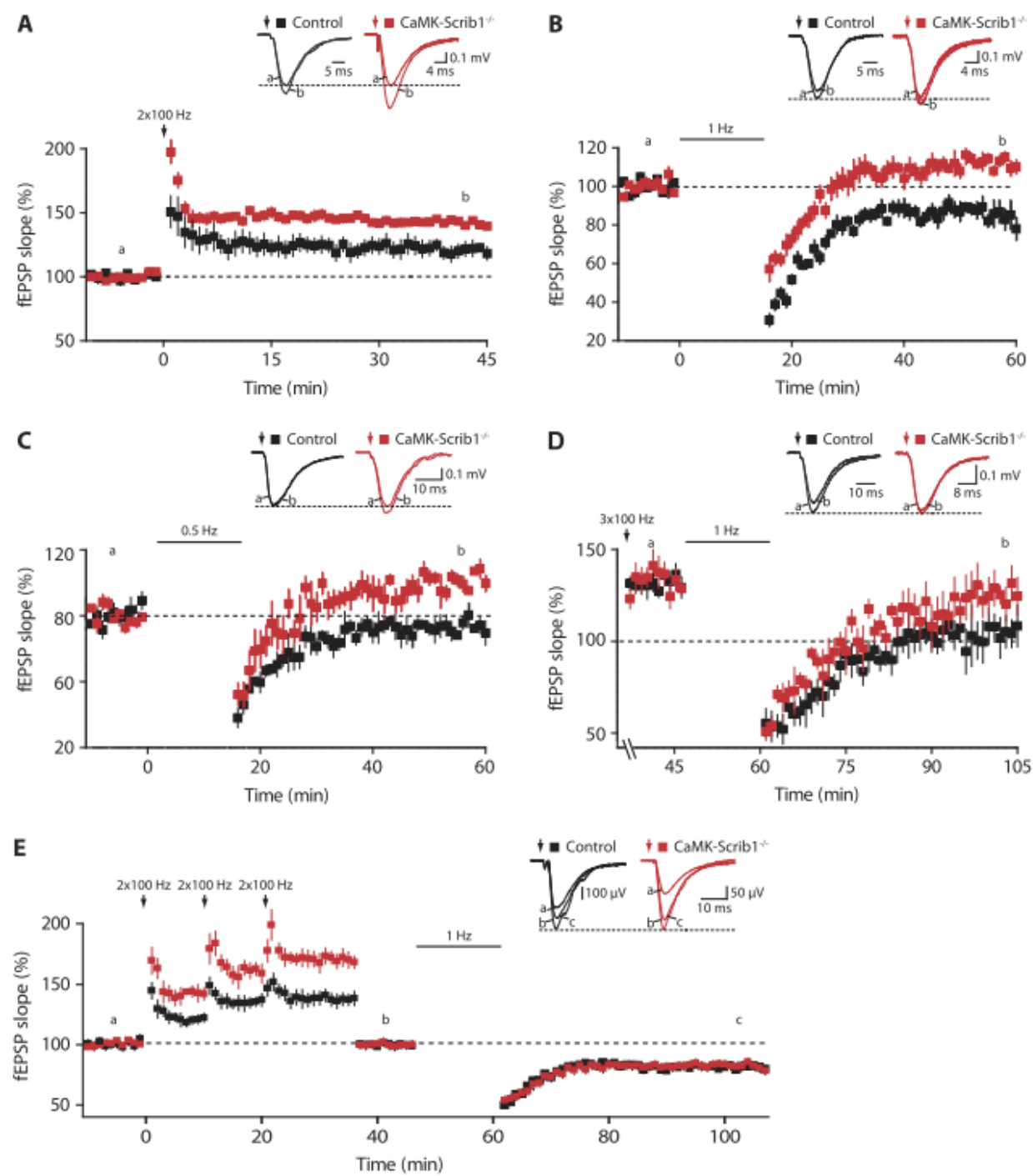


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FIGURE 4

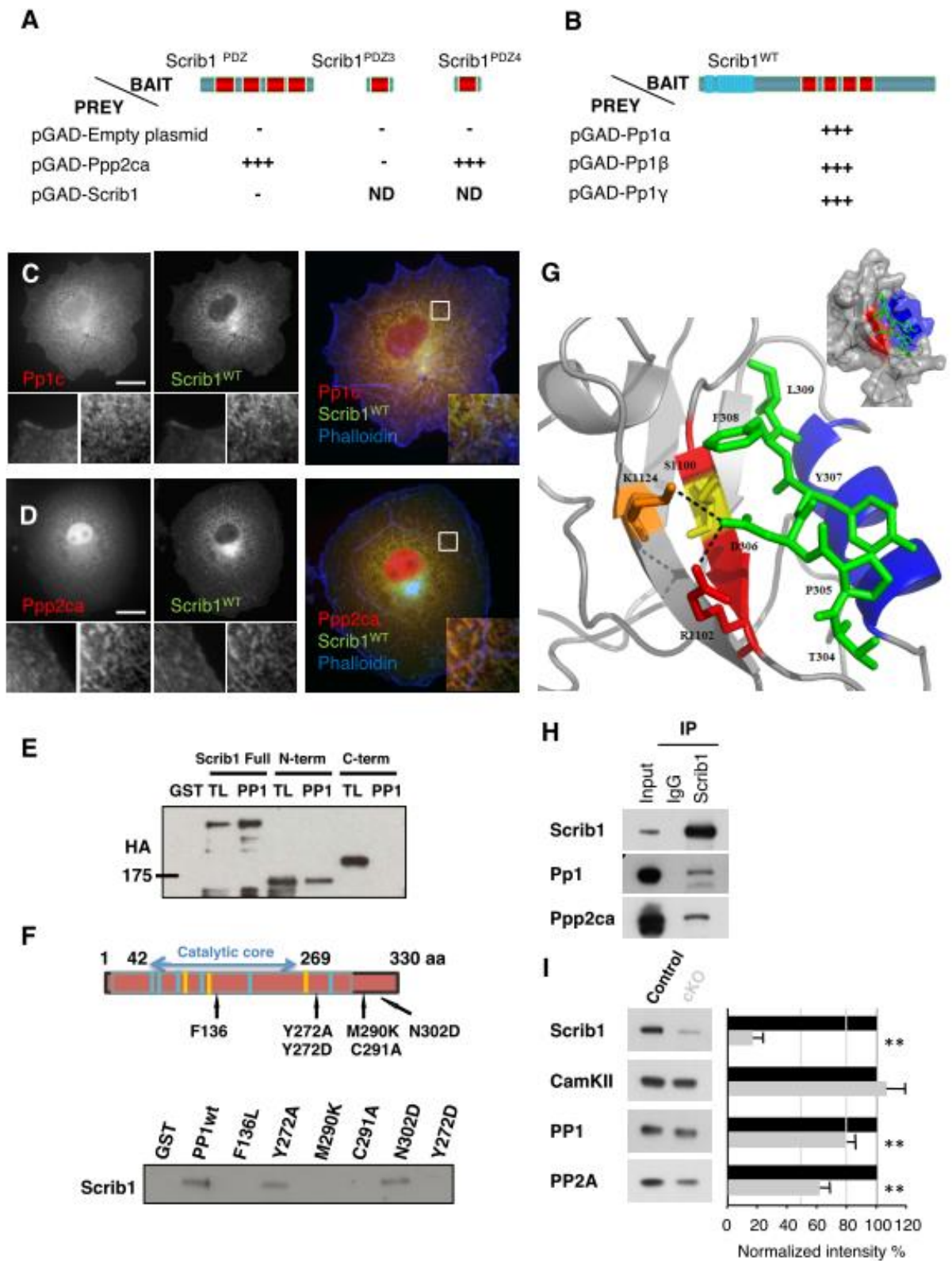




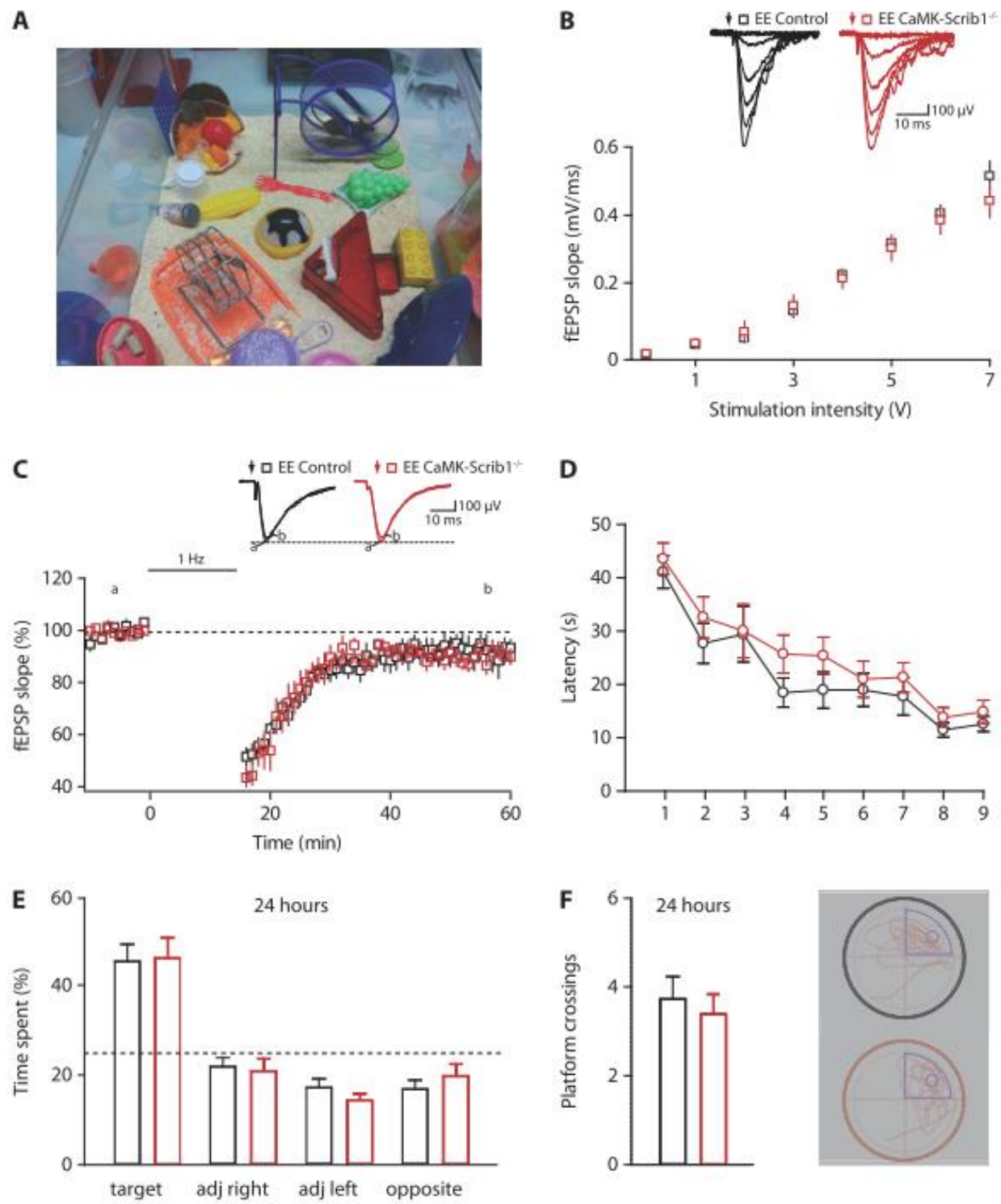
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FIGURE 5

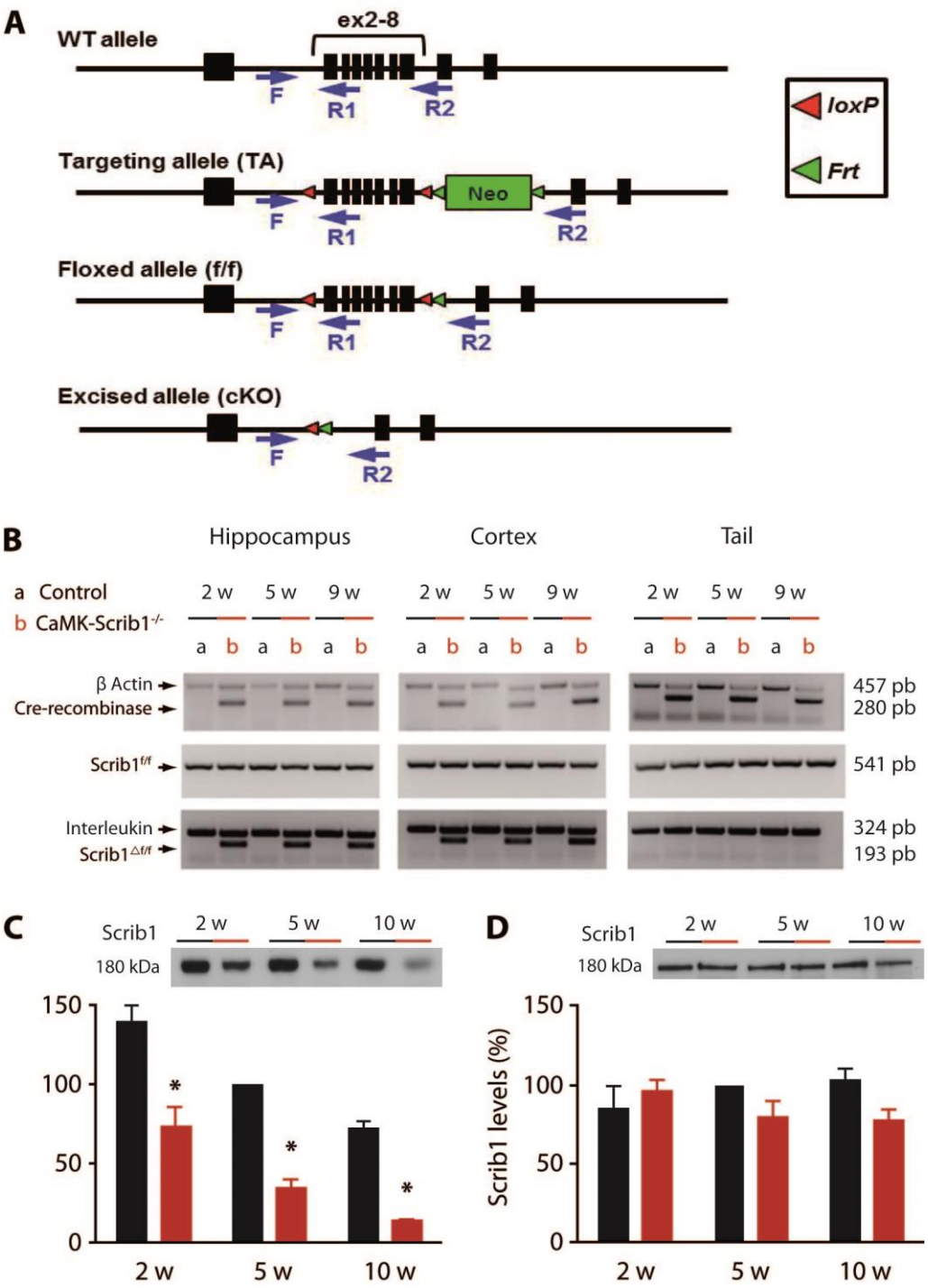


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FIGURE 6

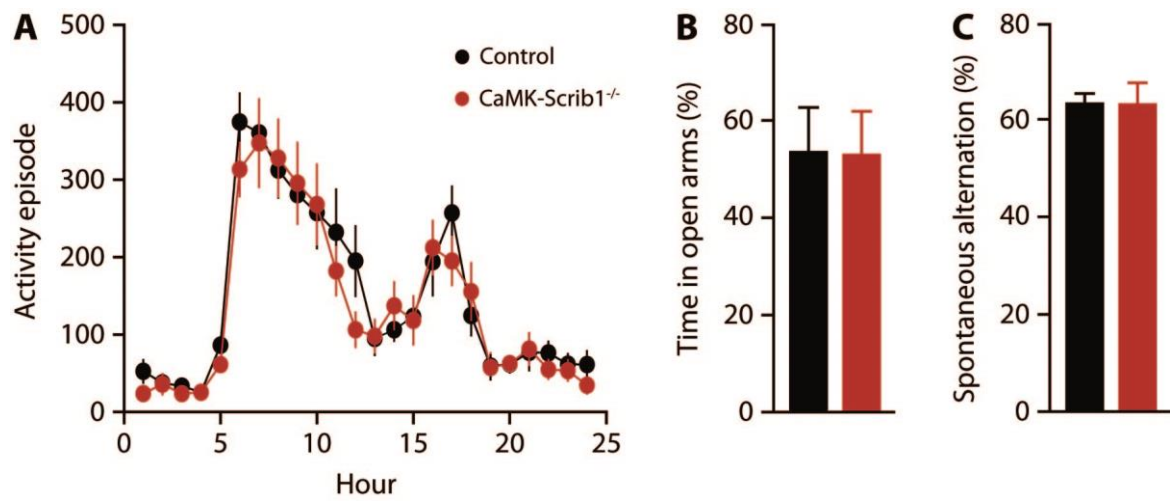


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FIGURE 7

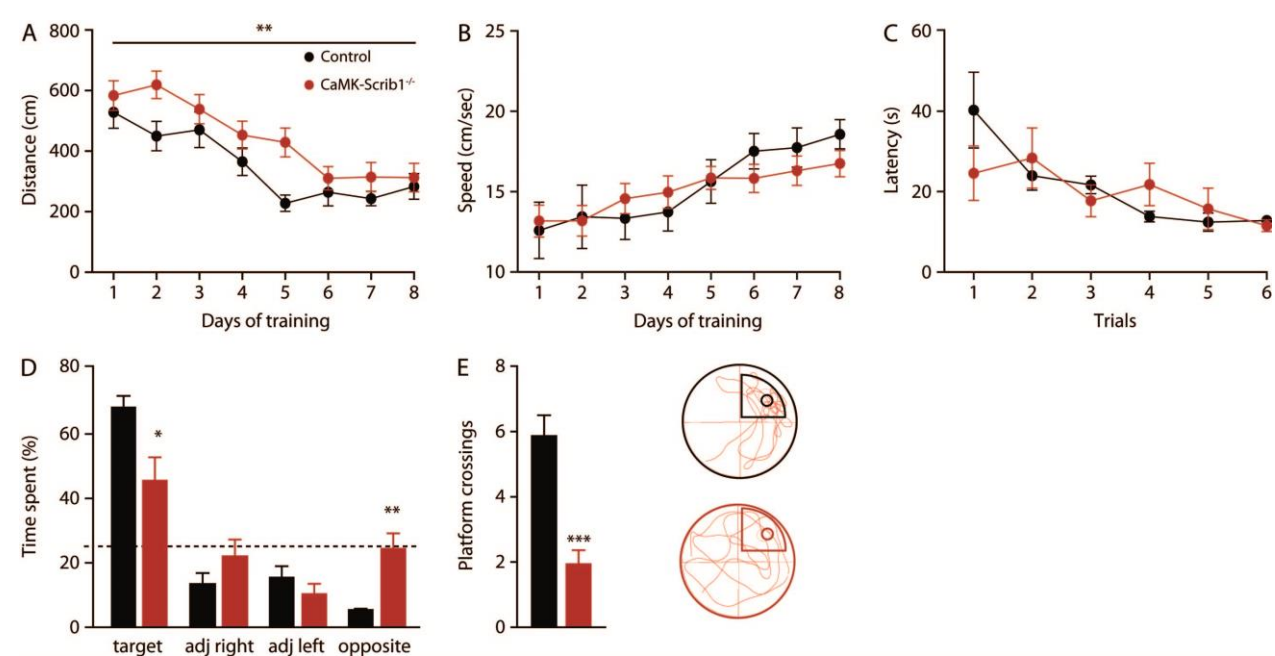


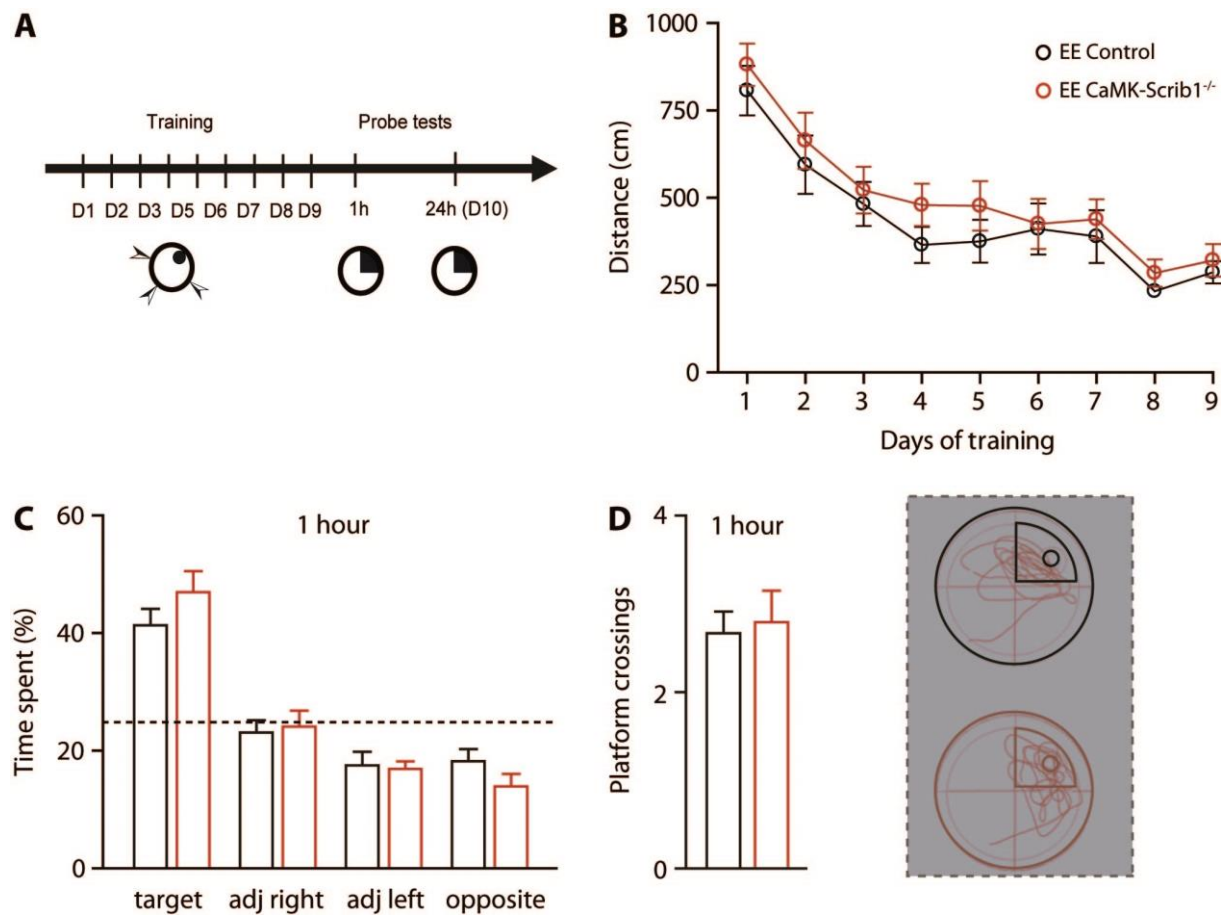


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SUPPLEMENTARY FIGURE 2



Hilal et al.,  
SUPPLEMENTARY FIGURE 3







## SUPPLEMENTARY TABLE 1

	TEST USED		n		P VALUE	DEGREE OF FREEDOM & F/t VALUE
FIGURE NUMBER	WHICH TEST ?	PAGE	EXACT VALUE	DEFINED	VALUE	VALUE
Figure S1C	Mann Whitney test	4	4	batches	p = 0.03	
Figure S1C	Mann Whitney test	4	4	batches	p = 0.03	
Figure S1C	Mann Whitney test	4	4	batches	p = 0.03	
Figure S1D	Mann Whitney test	5	3	batches	p = 0,90	
Figure S1D	Mann Whitney test	5	3	batches	p = 0,10	
Figure S1D	Mann Whitney test	5	3	batches	p = 0,10	
Figure S2A	Two-way ANOVA	5	6 and 9	mice	p = 0.64	F(1,299) = 0.23
Figure S2B	Mann Whitney test	5	13 and 14	mice	p = 0.97	U=90
Figure S2C	Mann Whitney test	5	7 and 6	mice	p = 0.70	U=18
Figure 2B	Two-way ANOVA	5	12 and 17	mice	p < 0.001	F(7,189) = 33.4
Figure 2B	Two-way ANOVA	5	12 and 17	mice	p =0.004	F(1,27) = 9.76
Figure S3A	Two-way ANOVA	5	12 and 17	mice	p =0.01	F(1,30) = 6.9
Figure 2B	paired t-test	5	12	mice	p < 0.0001	t(11) = 6.26
Figure 2B	paired t-test	5	17	mice	p < 0.0001	t(16) = 7.92
Figure 2B	Unpaired t-test	5	12 and 17	mice	p = 0.15	t(27) = 1.48
Figure 2B	Unpaired t-test	5	12 and 17	mice	p = 0.41	t(27) = 0.83
Figure S3B	Two-way ANOVA	5	12 and 17	mice	p = 0.84	F(1,28) = 0.04
Figure S3C	Two-way ANOVA	6	12 and 17	mice	p = 0.84	F(1,12) = 0.04
Figure 2C	Unpaired t-test	6	6 and 9	mice	p = 0.27	t(13) = 1.15
Figure 2D	Unpaired t-test	6	6 and 9	mice	p = 0.84	t(13) = 0.20
Figure 2E	Unpaired t-test	6	6 and 8	mice	p = 0.04	t(12) = 2.27
Figure 2F	Unpaired t-test	6	6 and 8	mice	p = 0.008	t(12) = 3.15
Figure S3D	Unpaired t-test	6	6 and 8	mice	p = 0.03	t(12) = 2.46
Figure S3E	Unpaired t-test	6	6 and 8	mice	p = 0.0002	t(12) = 5.17
Figure 3B	Mann Whitney test	7	9 and 12	neurons from 3 mice	p = 0.84	U=51
Figure 3C	Mann Whitney test	7	9 and 12	neurons from 3 mice	p = 0.90	U=52
Figure 3D	Mann Whitney test	7	9 and 12	neurons from 3 mice	p < 0.0005	U=3
Figure 3E	Mann Whitney test	7	9 and 12	neurons from 3 mice	p < 0.002	U=8
DNS	Mann Whitney test	7	946	spines from 2 mice	p = 0.06	U=383
Figure 3G	Mann Whitney test	7	946	synapses from 2 mice	p =0.001	?
Figure 3I	Mann Whitney test	7	946	synapses from 2 mice	p =0.003	?
Figure 4A	Two-way ANOVA	7	12	slices from 6 mice	p < 0.001	F(7,160)=6.16
Figure 4B	Mann Whitney test	7	6	mice	p > 0.35	U>7
Figure 4D	Mann Whitney test	8	5	mice	p = 0.22	U=6
Figure 4E	Mann Whitney test	8	5	mice	p = 0.02	U=1
Figure 5A	Mann Whitney test	8	5 and 6	slices from 5 mice	p = 0. 01	U=1



## SUPPLEMENTARY TABLE 1 (cont.)

FIGURE NUMBER	TEST USED		n		P VALUE	DEGREE OF FREEDOM & F/t
	WHICH TEST ?	PAGE	EXACT VALUE	DEFINED	VALUE	VALUE
DNS	Wilcoxon matched-pairs test	8	5	mice	p =0.06	
Figure 5B	Wilcoxon matched-pairs test	8	6 and 7	slices from 5 and 7 mice	p =0.001	
DNS	Wilcoxon matched-pairs test	8	4	mice	p =0.12	
Figure 5B	Wilcoxon matched-pairs test	8	7	slices from 6 mice	p =0.01	
Figure 5C	Wilcoxon matched-pairs test	8	5	mice	p =0.31	
Figure 5C	Wilcoxon matched-pairs test	8	4	mice	p =0.13	
Figure 5D	Wilcoxon matched-pairs test	9	6	mice	p = 0.03	
Figure 5D	Wilcoxon matched-pairs test	9	6	mice	p =0.84	
Figure 5E	Mann Whitney test	9	5	mice	p = 0.94	U=12
Figure 8B	Two-way ANOVA	10	6 and 5	slices from 6 mice	p =0.51	F(1,72)=0.43
Figure 8C	Wilcoxon matched-pairs test	10	7	slices from 6 mice	p 0.03	
Figure 8C	Mann Whitney test	10	7	slices from 6 mice	p =0.60	U=20
Figure 8D	Two-way ANOVA	10	15	mice	p = 0.08	F(1,28) = 3.26
Figure 8D	Two-way ANOVA	10	15	mice	p < 0.001	F(7,196) = 14.61
Figure S4B	Two-way ANOVA	10	15	mice	p = 0.15	F(1,28) = 2.14
Figure S4B	Two-way ANOVA	10	15	mice	p < 0.0001	F(7,196) = 16.95
Figure S4C	Unpaired t-test	10	15	mice	p = 0.25	t(28) = 1.16
Figure S4D	Unpaired t-test	10	15	mice	p = 0.76	t(28) = 0.31
Figure 8E	Unpaired t-test	10	15	mice	p = 0.90	t(28) = 0.13
Figure 8F	Unpaired t-test	10	15	mice	p = 0.63	t(28) = 0.48

## Chapter VI

# Disrupting Scrib1-mediated actin regulation impacts neuronal complexity, dendritic spine formation and maintenance

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## INTRODUCTION

Autism spectrum disorders (ASD) cover a plethora of heterogeneous neurodevelopmental disorders diagnosed such as the classical idiopathic autism, Asperger's syndrome or the Fragile X syndrome (Lord et al., 2000; Pardo & Eberhart, 2007). ASD commonly feature sociability difficulties, language impairments, a constrained pattern of interests, and/or stereotypic and repetitive behaviours. About 80% of ASD cases are heritable, being typically developed before 2-3 years of age and mainly affecting the late phase of brain development, when synaptic formation and maturation is taking place (Courchesne et al., 2007; Schmitz & Rezaie, 2008). In the last years, a growing number of molecular determinants of ASD etiopathology has been identified, ranging from cell adhesion complexes like neuroligins and neurexins (Südhof, 2008), to intracellular scaffold proteins like PSD-95 (Cao et al., 2013), Shank3 (Durand et al., 2007) or the recently identified Scribble1 (Scrib1) (Pinto et al., 2010; Neale et al., 2012). Scrib1 is a multidomain protein composed by 16 Nter leucine-rich repeats (LRRs) and 4 PDZ domains. Scrib1 belongs to the LAP (LRR and PDZ domains) family, whose members were previously implicated in the homeostasis of epithelial tissues and tumour growth (Saito et al., 2001; Legouis et al., 2003), cellular trafficking (Borg et al., 2000; Tao et al., 2013) as well as synaptic architecture and function (Apperson et al., 1996; Quitsch et al., 2005; Carlisle et al., 2011). Scrib1 has been described as an apico-basal determinant, playing a pivotal role in establishing and maintaining planar cell polarity (PCP) from invertebrates to mammals (Bilder & Perrimon, 2000; Murdoch et al., 2003; Montcouquiol et al., 2003, 2006). In the past years, our group has shown that Scrib1 plays a critical role in brain development and function (Moreau et al., 2010; Hilal et al., in preparation). In dendritic spines, Scrib1 can recruit PCP downstream signalling complexes (Moreau et al., 2010) and polarize NMDAR transport (Piguel et al., 2014). Herein, we assess for the first time the functional consequences of the newly identified Scrib1 *de novo* mutation c.1774C>T (Neale et al., 2012). Such missense mutation results into a Scrib1 protein form bearing a serine instead of a proline (Scrib1<sup>P592S</sup>) nearby a putative actin-binding motif and two Arp2/3 binding sites. We used an overexpression approach in cultured cells to investigate the molecular mechanisms underlying Scrib1 role in synapse formation and maintenance. Our results show that Scrib1 regulates dendritic arborization, spinogenesis, spine architecture and synaptic content via an actin-dependent mechanism, whose disruption might underlie the ASD Scrib1<sup>P592S</sup> mutant phenotype.

## EXPERIMENTAL PROCEDURES

**DNA constructs.** hScrib1 GFP (green fluorescent protein) or HA-tagged cDNA samples were generously provided by I. Macara (University of Virginia, Charlottesville, VA, USA) and J. P. Borg (Centre de Recherche en Cancérologie de Marseille, Marseille, France), respectively. HA-Scrib1<sup>crC</sup> was previously described (Moreau et al., 2010). Mutated forms of GFP-Scrib1 were generated using the QuickChange site-directed mutagenesis kit (Stratagen, La Jolla, CA, USA). Stg2-GFP, myc-tagged GluA1 and GluA2, and the red fluorescent protein (RFP)–actin constructs were kindly provided by S. Tomita & D. Bredt (Yale University School of Medicine, CT, USA), P. Osten (Cold Spring Harbor Laboratory, NY, USA), and C. Gauthier-Rouvière (Centre de Recherche en Biochimie Macromoléculaire, Centre National de la Recherche Scientifique Montpellier, France), respectively.

*Antibodies.* The following primary antibodies were used in this study: anti-Scrib1 (C20, Santa Cruz Biotechnology) and non-commercial anti-Scrib1 (Montcouquiol et al., 2006); anti-PSD-95 (MA 1-046, Thermo Fischer Scientific); anti-GluA1 Nter (MAB2263, Millipore); anti-GluA2/3 extracellular (MAB397, Millipore); anti-synapsin 1/2 (106103, Synaptic Systems); anti-Cacng2 (Stgz) Cter (07-577, Millipore); anti-green fluorescent protein (GFP) (JL-8, Clontech); anti-DsRed (Clontech); anti-myc mouse 9E11 (Covance); anti-HA (MMS-101P, Covance); and anti-GST (Chemicon). Secondary antibodies used as follows: Alexa Fluor 488, 532, 568 and 647 and Atto647N-conjugated phalloidin antibodies (Invitrogen); and HRP conjugated antibodies (GE Healthcare).

*Cell cultures.* Immortalized MEF (Giannone et al., 2004), HEK-293 and COS-7 cells (Sans et al. 2005) were cultured in DMEM with 10% FBS. Mixed (Sans et al., 2003, 2005) or Banker cultures (Kaech & Banker, 2006) of hippocampal neurons were obtained from E18.5 rat embryos. MEF and HEK-293 were transiently transfected with nucleofection (Lonza) or 1  $\mu\text{g}/\mu\text{L}$  PEI (Polysciences), respectively. COS-7 and mixed or Banker hippocampal neurons were transfected using the calcium phosphate method (Sans et al. 2005) or following Effectene's manufacture protocol (Qiagen) (Nair et al., 2013), respectively. MEF spreading essays were performed on 10 mg/ml fibronectin as previously described (Dubin-Thaler et al., 2004).

*Immunocytochemistry.* For surface staining, mixed hippocampal neurons were briefly fixed with 0.5% PFA and incubated with native AMPAR or anti-myc primary antibodies, respectively. Excess antibodies were removed by consecutively washing neurons with warmed neurobasal medium and PBS, and fixed for 5min with 4% PFA in PBS. After blocking, neurons were incubated with secondary antibodies. For intracellular staining, cells were fixed for 15min with 4% PFA (MEF, COS-7 and mixed neurons) or PFA 4% sucrose in PBS (Banker neurons), washed and permeabilized with 0.25% Triton X-100. After blocking, cells were incubated with primary antibodies. Following washout and incubation with secondary antibodies, coverslips were conserved at 4°C in PBS or mounted on ProLong® Gold Antifade Reagent.

*Low-resolution microscopy and quantitative analysis.* Cells were observed on the 2D stage inverted motorized microscope (Nikon Eclipse TiE) equipped with a 100X/1.45 NA PL-APO objective and a perfect focus system. Atto647 and GFP-expressing cells were detected using a mercury lamp (Nikon Xcite) and the following filter sets (SemROCK). Neuronal and spine morphology analysis as well as AMPAR surface number experiments were done on 16-17 DIV neurons 4d after transfection as previously described (Moreau et al., 2010; Durand et al., 2012). For neuronal morphology, at least 9 neurons per condition were acquired by a Zeiss AxioImager Z1 equipped with an AxioCam MRm camera, using a 40 X magnification. For each neuron, the dendritic tree, including all branches, was reconstructed using Neurolucida software (MBF). The dendritic surface was quantified by counting the number of branches at each order from the cell body by Scholl analysis and by counting the number of ring intersections using an overlay of concentric rings (30  $\mu\text{m}$  between rings). Spine morphology images were acquired with a Leica DMR TCS SP2 AOBS confocal microscope (Leica, France), using the 60 X objective. Each image was a z-series projection taken at 0.02  $\mu\text{m}$  deep inter-

vals. Spine morphology measurements were done using Velocity analysis software (Perkin Elmer, MA, USA). Spine density was measured by averaging 6 different 10  $\mu\text{m}$  length regions per neuron. Spine length was measured by taking the distance from the base of the neck to the spine head. Spine width corresponded to the maximal width of the spine head perpendicular to the length. Images of surface AMPAR and COS-7 cells were acquired by the Zeiss AxioImager Z1 microscope using a 63 X oil-immersion on and analysed by MetaMorph 7.5 software (Universal Imaging). Surface AMPA receptors were quantified by counting the number of puncta and the average intensity from 5 different 20  $\mu\text{m}$  length regions per neuron on isolated dendrites. All conditions in individual experiments used identical acquisition parameters and value thresholds. Each experiment was performed on at least four independent neuronal preparations. Images from endogenous Scrib1/Stgz/AMPA complex were obtained by the Zeiss AxioImager Z1 microscope using a 40 X magnification.

*Dual colour dSTORM and dispersion analysis.* dSTORM images of native PSD-95 and endogenous or GFP-transfected Scrib1 were obtained from 18 DIV Banker hippocampal neurons. Imaging was performed in an extracellular solution containing reducing agents and oxygen scavengers at pH 7.2-7.5. Dual-colour sequential dSTORM images were acquired by an inverted Leica SR GSD 3D microscope equipped with a special 160x objective and a sensitive EMCCD iXon ANDOR camera (Evolve, Photometric). As described (Nair et al., 2013), ensemble fluorescence of Alexa647 was first converted into dark state using a 642 nm laser at 50-100  $\text{kW}/\text{cm}^2$  intensity. Once the ensemble fluorescence converted into the desired density of single molecules per image, the laser power was reduced to 15-30  $\text{kW}/\text{cm}^2$  and streaming acquisition was performed at 50-100 Hz for 20,000-40,000 frames. Laser powers were constantly adjusted to keep a specific optimal level of stochastically activated molecules. The same protocol is done for the fluorophore Alexa532 using a 532 nm laser. The totality of the fluorescence was collected by an ensemble of double dichroic cubes (405+488 / 405+532 / 405+642). The acquisition and localization sequences were driven by Metamorph software Molecular Devices). We used multicolor fluorescent micro-beads (Tetraspeck, Invitrogen) as fiduciary markers to register long-term acquisitions and correct for lateral and chromatic shifts. Each acquisition leads to sets of 20,000-40,000 images (LASAF software, Leica), which were next analysed by the dedicated custom-made image analysis Palm-Tracer Metamorph software (Molecular Devices). A super-resolution time-lapse sequence (frame rate 5s, 500 images) was generated from which high density zones of localizations were identified using wavelet-based segmentation method (Racine et al., 2007; Izeddin et al., 2012; Kechkar et al., 2013). For each spine, the distance between endogenous or GFP-labelled Scrib1 high density zones was quantified with respect to the centroid of PSD-95-A647 zones. The same analysis was done by comparing the distance of each molecule to its own centroid. Each experiment was performed on at least four independent neuronal preparations.

*Yeast two-hybrid screen and assay.* Scrib1<sup>PDZ3</sup> (990-1079 aa, Q80U72-3) was subcloned into pGBTK7 vector (Clontech) in-frame with the DNA-binding domain of GAL4 and used as a bait for the screening as previously described (Sans et al., 2003). Yeast two-hybrid screening and assays were performed accordingly to

the Matchmaker™ Gold Yeast Two-Hybrid System protocol (Clontech). AH109 cells expressing Scrib1<sup>PDZ3</sup> were combined with Y187 cells expressing a P10 mouse brain cDNA library. The mating mixture was plated on SD/Ade<sup>-</sup>/Trp<sup>-</sup>/Leu<sup>-</sup>/His<sup>-</sup> plates. From 3.5 x 10<sup>3</sup> colonies obtained 5 days after transformation, 98 passed high stringency conditions. Library plasmids from those colonies were rescued, amplified by PCR and sequenced, including  $\gamma$ -4 TARP. Interactions between  $\gamma$ -4 TARP and each PDZ domain transfected into the haploid yeast strain AH109 were tested through mating of the two yeast strains.

*Immunoprecipitation and GST binding assays.* For immunoprecipitation experiments, antibodies were immobilized on Protein A/G agarose beads and incubated with either P35 rat brain extracts or with cleared transfected HEK-293 cell extracts overnight at 4°C (Sans et al., 2003, 2005). Washed beads were eluted with sample buffer. Experiments were done more than 3 times. Samples were analyzed by SDS/PAGE and immunoblotting. For GST binding assays, Scrib1-GST fusion proteins containing PDZ1 (714-801), PDZ2 (848-936), PDZ3 (990-1079), PDZ4 (1086-1180), PDZ1-2 (714-936), PDZ3-4 (990-1180), and PDZ1-4 (714-1180) were expressed in BL21-strain *E. coli* using pGEX-4T-1 and purified directly from bacterial extract on glutathione–Sepharose-4B beads. A pull-down assay was performed with S-protein-tagged myc-GluA1 or Stgz-GFP overexpressed in *E. coli*. 10  $\mu$ g of immobilized GST or specific Scrib1-GST fusion proteins were incubated with recombinant S-protein-tagged myc-GluA1, -GluA2 or Stgz-GFP for 2h at 4 °C. After extensive washes with binding buffer, the bead pellets were resuspended in SDS sample buffer. Samples were analysed by SDS/PAGE and immunoblotting.

*Data representation and statistical analysis.* All data is represented as mean  $\pm$  SEM. Statistical analyses were performed using Sigmaplot11 (Systat Software) by two way ANOVA (MEF and neuronal morphology), one way ANOVA followed by Dunnett's Multiple Comparison Test (spine number and morphology; number of surface AMPARs) or unpaired Student t-test (dSTORM dispersion analysis). All data is referred in the text as mean  $\pm$  SEM; \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  were considered statistically significant.

## RESULTS

### ASD Scrib1 mutant loses Scrib1-mediated localization at the lamellipodia

Previous work from our group implicating Scrib1 involvement in ASD suggested a link between this PCP protein and the actin dynamics regulation (Moreau et al., 2010). We started this study by establishing Scrib1 presence in lamellipodia, cytoskeletal enriched actin projections on the mobile edge of cells (Pollard, 2003) (**Fig. 1**). GFP-coupled control (C3) or Scrib1<sup>WT</sup> plasmids were transfected in mouse embryonic fibroblasts (MEF) cells, which were fixed 1h30 after a spreading assay (**Fig. 1 A**). Using phalloidin to stain F-actin, we obtained a ratio GFP/phalloidin starting from outside towards the interior of the cell, including the described leading lamellipodia dendritic bush of about 1  $\mu$ m width (Svitkina & Borisy, 1999; Giannone et al., 2004) (**Fig. 1 B**). Compared to the control, Scrib1<sup>WT</sup> is particularly enriched within this region (C3, 0.54  $\pm$  0.03, 13 measurements from 8 different cells; Scrib1<sup>WT</sup>, 0.87  $\pm$  0.11, 7/4; \*\*,  $p < 0.01$ , One Way ANOVA, Dunnett's Multiple Comparison Test) (**Fig. 1 C**). Moreover, Scrib1<sup>WT</sup> maximal expression is located within the leading edge (C3,

2.51 ± 0.24 μm; Scrib1<sup>WT</sup>, 1.73 ± 0.04 μm; \*,  $p < 0.05$ ) (**Fig. 1 D**), pinpointing Scrib1 as a potential cytoskeleton modulator. Next, we sought to evaluate the recent discovered human ASD Scrib1<sup>P592S</sup> mutant (Neale et al., 2012). Unlike Scrib1<sup>WT</sup>, the ASD Scrib1<sup>P592S</sup> mutant does not accumulate in lamellipodia (Scrib1<sup>P592S</sup>, 0.56 ± 0.07, 7/4; ns,  $p > 0.05$  vs. C3 and  $p < 0.05$  vs. Scrib1<sup>WT</sup>, respectively) (**Fig. 1 A-C**). Instead, Scrib1<sup>P592S</sup> is preferentially placed outside the lamellipodia (Scrib1<sup>P592S</sup>, 3.54 ± 0.28 μm; \*\*/°°,  $p < 0.01$  vs. C3 and  $p < 0.001$  vs. Scrib1<sup>WT</sup>) (**Fig. 1 D**), suggesting that a disruption in actin dynamics might underlie Scrib1<sup>P592S</sup>-mediated ASD etiopathology.

### Scrib1 lamellipodial localization depends on its binding to the Arp2/3 complex and actin

The leading edge of lamellipodia is characterized by a dense network of actin filaments (Svitkina & Borisy, 1999). Lamellipodia initiation involves side branching and recruitment of actin nucleation and elongation factors like the Arp2/3 complex and VASP, respectively (Henson et al., 2002; Lai et al., 2008; Vinzenz et al., 2012). We looked for potential actin-, Arp2/3- and/or VASP- binding motifs in Scrib1 sequence (**Fig. 2**). Through a *in silico* screen, we found one WH2-like motif (Scrib1<sup>KK611,612</sup>) (**Fig. 2 A**) and two putative Arp2/3 binding sites (Scrib1<sup>W203</sup> and Scrib1<sup>W386</sup>) (**Fig. 2 B**), previously found in actin monomer-binding proteins and/or regulators of the Arp2/3 complex, respectively. We mutated these putative actin- (Scrib1<sup>KK611EE</sup>) and Arp2/3- (Scrib1<sup>W203A</sup> and Scrib1<sup>W386A</sup>) binding sites to evaluate their role on Scrib1-mediated localization at the lamellipodia (**Fig. 2 C**). In contrast to Scrib1<sup>WT</sup>, none of the three mutants is enriched in the lamellipodia (**Fig. 2 D**), indicating that Scrib1 localization in lamellipodia depends on its ability to bind actin and the Arp2/3 complex. Moreover, actin- (Scrib1<sup>KK611EE</sup>, 0.55 ± 0.03, 7/7; °°,  $p < 0.01$ , One Way ANOVA, Dunnett's Multiple Comparison Test) and both Arp2/3-binding mutants (Scrib1<sup>W203A</sup>, 0.58 ± 0.06, 9/4; Scrib1<sup>W386A</sup>, 0.62 ± 0.02, 7/5; °,  $p < 0.01$  for both) display a reduced lamellipodial GFP/phalloidin ratio (**Fig. 2 F**) similar to Scrib1<sup>P592S</sup> (**Fig. 2 E**). Taken together, these results suggest that Scrib1 enrichment in the leading edge of lamellipodia depends on its ability to bind actin and Arp2/3.

### ASD and actin Scrib1 mutants display aberrant dendritic trees

Neuronal complexity, specifically dendritic branching and morphology, consents individual neurons to accomplish specialized brain functions and cognitive behaviours. Dendritic arborisation is known to be dependent on cytoskeleton dynamics (Jan & Jan, 2010). We decided then to evaluate Scrib1 role in dendritic complexity and length by transfecting hippocampal neurons at 16-17 DIV (**Fig. 3**). Compared to the control, Scrib1<sup>WT</sup> neurons exhibit dendrites with a higher number of intersections (C3, n=26; Scrib1<sup>WT</sup>, n=22; \*\*\*,  $p < 0.001$  for interaction, distance, and genotype, Two Way ANOVA) (**Fig. 3 A, B**), but similar total length (C3, 1214 ± 131.6 μm; Scrib1<sup>WT</sup>, 1166 ± 79.93 μm; ns,  $p > 0.05$ , One Way ANOVA, Dunnett's Multiple Comparison Test) (**Fig. 3 C**). Instead, Scrib1<sup>P592S</sup> mutant displays a significant low dendritic intersection number (Scrib1<sup>P592S</sup>, n=21, \*\*/°°°  $p < 0.01$  vs. C3 and  $p < 0.001$  vs. Scrib1<sup>WT</sup>) (**Fig. 3 A, B**) and length (Scrib1<sup>P592S</sup>, 744.1 ± 50.23 μm; \*\*/°°°  $p < 0.01$  and  $p < 0.001$ ) (**Fig. 23 C**). PCP downstream effectors, such as the actin polymerization activating signalling cascade composed by Rac1, Pak1, N-WASP and Arp2/3 was previously implicated in neurite outgrowth and remodelling (Rashid et al., 2001; Ng et al., 2002; Sin et al., 2002; Ka-

kimoto et al., 2006; Hayashi et al., 2007; Pinyol et al., 2007). We assessed then the impact of Scrib1 actin- and Arp2/3- binding mutants on dendritic morphology (**Fig. 3 D-F**). Scrib1-mediated dendritic complexity is dependent on its binding to actin (Scrib1<sup>KK611EE</sup>, n=20; °°°  $p < 0.001$ ), but not to the Arp2/3 complex (Scrib1<sup>W203A</sup>, n=15; Scrib1<sup>W386A</sup>, n=19; ns,  $p > 0.05$  for both) (**Fig. 3 D, E**). Moreover, Scrib1 actin (Scrib1<sup>KK611EE</sup>,  $684.2 \pm 63.80 \mu\text{m}$ ; °°  $p < 0.01$ ), but not Arp2/3-binding mutants (Scrib1<sup>W203A</sup>,  $1069 \pm 99.61 \mu\text{m}$ ; Scrib1<sup>W386A</sup>,  $1051 \pm 116.5 \mu\text{m}$ ; ns,  $p > 0.05$  for both) display a reduced dendritic length (**Fig. 3 D, F**). Taken together, this data set suggests that Scrib1<sup>P592S</sup>-mediated aberrant dendritic complexity might be due to a disrupted Scrib1 interaction with actin.

### Scrib1 mutants show aberrant filopodia and dendritic spine morphogenesis

Scrib1 was previously found inside dendritic spines (Moreau et al., 2010; Piguel et al., 2014), whose formation, maturation and elimination is long known to be dependent on actin dynamics (Caceres et al., 1983; Tada & Sheng, 2006). To examine the potential role of Scrib1 in spine formation and morphology, hippocampal neurons were transiently co-transfected with the different Scrib1 constructs together with mRFP-actin, to visualize such dendritic structures (**Fig. 4**). We started by investigating Scrib1 role on filopodia number and length (**Fig. 4 B, C**). Compared to the control, Scrib1<sup>WT</sup> overexpression decreased filopodia number (C3,  $1.37 \pm 0.12$ , 147/17; Scrib1<sup>WT</sup>,  $0.97 \pm 0.10$ , 134/29; One Way ANOVA, Dunnett's Multiple Comparison Test, \*  $p < 0.05$ ) (**Fig. 4 B**) and length (C3,  $2.14 \pm 0.11 \mu\text{m}$ , 184/17; Scrib1<sup>WT</sup>,  $1.44 \pm 0.07 \mu\text{m}$ , 196/29; \*\*  $p < 0.01$ ) (**Fig. 4 C**). Regarding spine number and morphology, Scrib1<sup>WT</sup> induced more (C3,  $7.67 \pm 0.27$ , 144/17; Scrib1<sup>WT</sup>,  $8.96 \pm 0.36$ , 132/29; \*\*  $p < 0.01$ ) (**Fig. 4 D**) and wider spines (C3,  $0.81 \pm 0.008 \mu\text{m}$ , 1100/17; Scrib1<sup>WT</sup>,  $0.88 \pm 0.01$ , 1126/29; \*\*  $p < 0.01$ ) (**Fig. 4 E**), without changing spine length (C3,  $1.28 \pm 0.02 \mu\text{m}$ , 1100/17; Scrib1<sup>WT</sup>,  $1.31 \pm 0.02$ , 1126/29; ns,  $p > 0.05$ ) (**Fig. 4 F**). In contrast, the ASD Scrib1<sup>P592S</sup> mutant displayed more ( $1.30 \pm 1.59$ , 161/27; °,  $p < 0.05$  vs. Scrib1<sup>WT</sup>) (**Fig. 4 B**) and longer filopodia ( $2.37 \pm 0.13 \mu\text{m}$ , 211/27; °°°,  $p < 0.001$ ) (**Fig. 4 C**). Furthermore, Scrib1<sup>P592S</sup> induced less ( $3.35 \pm 0.21$ ; 162/27; \*\*/°°°,  $p < 0.01$  vs. C3 and  $p < 0.001$  vs. Scrib1<sup>WT</sup>) (**Fig. 4 D**), but wider ( $1.06 \pm 0.03$ ; 542/27; \*\*/°°°,  $p < 0.01$  vs. C3 and  $p < 0.001$  vs. Scrib1<sup>WT</sup>) (**Fig. 4 E**) and longer ( $1.59 \pm 0.04$ ; 542/27; \*\*/°°°,  $p < 0.01$  vs. C3 and  $p < 0.001$  vs. Scrib1<sup>WT</sup>) (**Fig. 4 F**) dendritic spines. Since ASD and actin mutants showed a similar aberrant dendritic tree arborisation (**Fig. 3**), we evaluated the effect of Scrib1 binding to actin and Arp2/3 complex in spinogenesis as well (**Fig. 4 H-N**). Compared to Scrib1<sup>WT</sup>, Scrib1 actin- and Arp2/3- mutants display more (Scrib1<sup>KK611EE</sup>,  $1.78 \pm 0.16$ , 113/19; Scrib1<sup>W203A</sup>,  $1.67 \pm 0.16$ , 95/16; Scrib1<sup>W386A</sup>,  $1.89 \pm 0.15$ , 149/25; °°,  $p < 0.01$  for all) (**Fig. 4 I**) and longer filopodia (Scrib1<sup>KK611EE</sup>,  $1.85 \pm 0.08 \mu\text{m}$ , 202/19; Scrib1<sup>W203A</sup>,  $1.90 \pm 0.09 \mu\text{m}$ , 207/16; Scrib1<sup>W386A</sup>,  $1.81 \pm 0.08 \mu\text{m}$ , 291/25; °°,  $p < 0.01$  for all) (**Fig. 4 J**). Mutating Scrib1 actin- and Arp2/3-binding sites induced fewer spines (Scrib1<sup>KK611EE</sup>,  $5.28 \pm 0.32$ , 113/19; Scrib1<sup>W203A</sup>,  $7.14 \pm 0.39$ , 96/16; Scrib1<sup>W386A</sup>,  $7.28 \pm 0.34$ , 150/25; °°,  $p < 0.01$  for all) (**Fig. 4 K**) as well. Interestingly, mutating Scrib1<sup>KK611</sup> and Scrib1<sup>W203</sup> residues (Scrib1<sup>KK611EE</sup>,  $0.82 \pm 0.01 \mu\text{m}$ , 617/19; Scrib1<sup>W203A</sup>,  $0.82 \pm 0.01 \mu\text{m}$ , 860/16; °°,  $p < 0.01$  for both), but not Scrib1<sup>W386</sup> (Scrib1<sup>W386A</sup>,  $0.88 \pm 0.01 \mu\text{m}$ , 1091/25; ns,  $p > 0.05$ ), resulted into smaller spines (**Fig. 4 L**). Instead, Scrib1<sup>KK611EE</sup> and Scrib1<sup>W386A</sup> mutants (Scrib1<sup>KK611EE</sup>,  $1.41 \pm 0.03 \mu\text{m}$ , 617/19; Scrib1<sup>W386A</sup>,  $1.40 \pm 0.02 \mu\text{m}$ , 1091/25; °°,  $p < 0.01$  for both), but not Scrib1<sup>W203A</sup> ( $1.31 \pm 0.02 \mu\text{m}$ , 860/16; ns,  $p > 0.05$ ) showed



longer spines (**Fig. 4 M**). Taken altogether, these data suggests that Scrib1 plays a key role in filopodia-to-spine transition by binding to actin and the Arp2/3 complex (**Fig. 4 N**). In particular, Scrib1-mediated Arp2/3-dependent actin nucleation is necessary for filopodia formation, elongation and spine formation. Moreover, the two different Scrib1 Arp2/3-binding sites seem to be specifically employed to regulate spine width and length. Accordingly, disrupting Scrib1-mediated actin regulation might lead to abnormal spinogenesis (**Fig. 4 G**).

### Scrib1 ASD mutant exhibits a fragmented PSD

Abnormal spine number and/or architecture in synaptic pathologies are often followed by a disturbed recruitment of proteins to the PSD (Kulkarni & Firestein, 2012). As Scrib1 seems to be a critical regulator of dendritic spine morphology, we wanted to unravel the precise Scrib1 localization within that structure (**Fig. 5**). Given the micrometric size of dendritic spines, we used dSTORM (direct stochastic optical reconstruction microscopy), a super-resolution microscopy technique capable of detecting single particle molecules at a ~ 20 nm resolution (van de Linde, 2011) (**Fig. 5 A**). We started by analysing the endogenous localization of Scrib1 taking the localization of PSD-95, the most abundance post-synaptic scaffold protein in the post-synaptic density (PSD), as landmark (**Fig. 5 B**). The obtained images in confocal microscopy suggested a co-localization between Scrib1 and PSD-95 within the dendritic spine (**Fig. 5 B'**). The use of dSTORM allowed however a more detailed nanometric organization (**Fig. 5 B''**). First, Scrib1 and PSD-95 did not co-localize. Second, Scrib1 presented a more homogeneous puncta-like localization within the dendritic spine compared to the central clusterised PSD-95 (PSD-95,  $206.2 \pm 10.90$  nm; Scrib1,  $323.3 \pm 14.68$  nm;  $n=24$  spines; unpaired t test \*\*\*,  $p < 0.001$ ) (**Fig. 5 C, D**). Next, we evaluated the impact of Scrib1 mutations into the nano-architecture of dendritic spines (**Fig. 5 E-J**). We started by comparing the endogenous distribution of PSD-95 to overexpressed GFP-Scrib1<sup>WT</sup> (**Fig. 5 E, G**). Super resolution images of dendritic spines showed a central cluster of PSD-95 surrounded by a more homogenous punctiform Scrib1<sup>WT</sup> (PSD-95,  $243.3 \pm 17.68$  nm; Scrib1<sup>WT</sup>,  $309.6 \pm 15.96$  nm;  $n=16$ ; unpaired t test \*\*  $p < 0.01$ ) (**Fig. 5 E'', G**). Scrib1<sup>WT</sup> dispersion values are analogous to the native condition, thus validating the use of this super-resolution microscopy technique to study the nano-architecture of transfected dendritic spines. As previously, Scrib1<sup>P592S</sup> overexpression resulted into bigger spines, visible in both confocal (**Fig. 5 F, F'**) and dSTORM images (**Fig. 5 F''**). The dispersion analysis showed again a less dispersed PSD-95 compared to Scrib1<sup>P592S</sup> (PSD-95,  $249.5 \pm 15.14$  nm; Scrib1<sup>P592S</sup>,  $360.3 \pm 17.64$  nm;  $n=14$ ; unpaired t test \*\*\*  $p < 0.001$ ) (**Fig. 5 H**). On the other hand, the average distance of Scrib1<sup>P592S</sup> regarding its own centroid is significantly higher than that of Scrib1<sup>WT</sup> (Scrib1<sup>WT</sup>,  $309.6 \pm 15.96$  nm; Scrib1<sup>P592S</sup>,  $360.3 \pm 17.64$  nm; unpaired t test \*\*\*  $p < 0.001$ ) (**Fig. 5 I**), indicating that the ASD form of Scrib1 affects its distribution within the spine. We then wonder if the ASD mutant form could affect PSD-95 distribution as well. Although both conditions present similar mean values of PSD-95 average distance reported to its centroid, super resolved images clearly show that PSD-95 is no longer centred in a single cluster but rather fragmented in Scrib1<sup>P592S</sup> transfected spines (**Fig. 5 F''**). Indeed, while the majority (84%) of native spines contained PSD-95 distributed in a unique cluster, Scrib1<sup>WT</sup> overexpression resulted into a hyper-clusterisation (100%) of PSD-95 (**Fig. 5 J**). Instead, only 21% of Scrib1<sup>P592S</sup> transfected

spines showed a PSD-95 organized into a single cluster, highlighting Scrib1 as a key organizer of the dendritic spine nano-architecture. We are currently performing the same analysis on Scrib1 actin- and Arp2/3-binding mutants.

### **Scrib1 forms a PDZ-dependent complex with TARP/AMPARs**

PSD-95 enriched domains were recently shown to concentrate synaptic AMPARs clusters (Nair et al., 2013). Targeting, trafficking and insertion of AMPARs into the synaptic membrane are known to be largely mediated by PDZ-dependent interactions of AMPAR Cter tails. We probed the missing PDZ domains of the Scrib1<sup>crc</sup> mutant, previously implicated in ASD (Moreau et al., 2010), searching for potential AMPAR traffic regulators (**Fig. 6**). We found that the transmembrane auxiliary regulatory protein (TARP)  $\gamma$ -4 was able to interact with Scrib1 PDZ3 domain in a yeast two hybrid screen using a P10 mouse cDNA library as prey (**Fig. 6 A**). Type I TARPs like  $\gamma$ -4 or Stargazin (Stgz or  $\gamma$ -2), its best known member, possess a conserved Cter class I PDZ-binding domain (PDZbd: -RR/KTTPV) (**Fig. 6 B**). Given the high similarity between Cter PDZbd of  $\gamma$ -4 and Stgz, we looked for an association between native Scrib1, Stgz and AMPAR in brain extracts. Using hippocampal lysates from P35 rats, we were able to co-immunoprecipitate Stgz with Scrib1 and GluA1 (**Fig. 6 C**). Scrib1 and Stgz direct interaction was further confirmed by immunoprecipitating overexpressed tagged HA-Scrib1 and Stgz-GFP in HEK-293 cells (**Fig. 6 D**). Instead, HA tag was unable to immunoprecipitate with myc-GluA1 (**Fig. 6 E**), suggesting that Stgz mediates the interaction between Scrib1 and AMPAR (**Fig. 6 F**). We followed then the spatial localization of the individual components of this complex by immunostaining COS-7 cells (**Fig. 6 G-L**). When expressed alone, Scrib1 was uniformly distributed in the Golgi, ER, cytoplasm and plasma membrane, while Stgz was mainly confined to the Golgi, ER and plasma membrane (**Fig. 6 G**). Instead, GluA1 (**Fig. 6 G**) and GluA2 (not shown) staining was present in the ER and the Golgi. As expected, Stgz and both GluA subunits (**Fig. 6 H**) perfectly co-localized within large clusters at the ER and Golgi level. When co-expressed with Scrib1, Stgz was redistributed to large clusters containing Scrib1 at the cell body and within the plasma membrane (**Fig. 6 I**). Scrib1 was able to alter the distribution pattern of GluA1 (**Fig. 6 J**) and GluA2 (not shown). In particular, GluA1 became uniformly distributed within cytoplasmic clusters, which sparsely co-localize with Scrib1 (**Fig. 6 J**), whereas GluA2 was distributed at the cytoplasm level, being rather concentrated at the ER, Golgi and plasma membrane (not shown). This difference in Scrib1-dependent redistribution and co-localization argues in favour of Scrib1 playing a distinct role on the traffic regulation of different AMPAR subunits. Finally, we wondered if the missing PDZ domains of the Scrib1<sup>crc</sup> mutant form could affect Scrib1 co-localization with the AMPAR/Stgz complex (**Fig. 6 K, L**). In COS-7 cells co-transfected with Scrib1, Stgz and GluA1 (**Fig. 6 K**) or GluA2 (not shown), we observed a perfect co-localization of the complex into large ER, Golgi and cytoplasmic clusters. However, when co-transfected with the ASD-associated Scrib1<sup>crc</sup> mutant, such co-localization was completely lost (**Fig. 6 L**), pinpointing Scrib1 as an active component of the AMPAR trafficking by forming a PDZ-dependent complex with type I TARPs. The native immunolabelling of mature hippocampal neurons revealed that the Scrib1/TARP complex can indeed co-localize with both GluA1- (**Fig. 6 M**) and GluA2-containing AMPARs (**Fig. 6 N**).

### Scrib1 mutants show aberrant AMPAR synaptic targeting

Since Scrib1 is able to form a complex with TARP and AMPA receptors (**Fig. 6**), we evaluated its potential role as AMPAR traffic modulator. Four days after hippocampal neuron transfection, we stained native GluA1- (**Fig. 7 A**) and GluA2-containing AMPA receptors (**Fig. 7 B**), using synapsin as a pre-synaptic marker. Compared to control conditions, Scrib1<sup>WT</sup> overexpression does not change GluA1 nor GluA2 average intensity (C3,  $13.95 \pm 0.91$ ,  $n=22$ ; Scrib1<sup>WT</sup>,  $14.49 \pm 1.54$ ,  $n=19$ ; and C3,  $15.75 \pm 1.14$ ,  $n=15$ ; Scrib1<sup>WT</sup>,  $16.02 \pm 1.34$ ,  $n=15$ , respectively; One Way ANOVA, Dunnett's Multiple Comparison Test, ns,  $p > 0.05$  for both) (**Fig. 7 E, K**), total surface number (C3,  $15.46 \pm 0.76$ ; Scrib1<sup>WT</sup>,  $15.91 \pm 0.98$ ; and C3,  $15.17 \pm 0.75$ ; Scrib1<sup>WT</sup>,  $14.87 \pm 0.86$ , respectively; ns,  $p > 0.05$ ) (**Fig. 7 F, L**), or synaptic ratio (C3,  $0.66 \pm 0.03$ ; Scrib1<sup>WT</sup>,  $0.69 \pm 0.03$ ; and C3,  $0.57 \pm 0.03$ ; Scrib1<sup>WT</sup>,  $0.62 \pm 0.02$ , respectively; ns,  $p > 0.05$ ) (**Fig. 7 G, M**). Overexpressing the ASD Scrib1<sup>P592S</sup> mutant resulted in no difference in AMPAR average intensity ( $14.42 \pm 1.19$  and  $16.20 \pm 1.39$ , respectively; ns,  $p > 0.05$ ) (**Fig. 7 E, K**) nor total surface number either ( $15.93 \pm 1.32$  and  $15.53 \pm 0.65$ , respectively; ns,  $p > 0.05$ ) (**Fig. 7 F, L**). Nonetheless, it induced a significant increase of GluA1 (Scrib1<sup>P592S</sup>,  $0.77 \pm 0.03$ ,  $n=19$ ; \* $p < 0.05$  vs. C3 and Scrib1<sup>WT</sup>) (**Fig. 7 G**), but not GluA2 synaptic ratio (Scrib1<sup>P592S</sup>,  $0.48 \pm 0.03$ ,  $n=19$ ; ns,  $p > 0.05$ ) (**Fig. 7 M**), suggesting that a subunit-specific mistargeting of AMPA receptors might play a role in ASD. Actin cytoskeleton modulators, including Arp2/3, were previously implicated in AMPAR traffic as well (Rocca et al., 2008, 2013; Gu et al., 2010; Rust et al., 2010). We evaluated then the effect of Scrib1 actin- or Arp2/3-binding mutants on AMPAR traffic to the cell surface and to the synapse (**Fig. 7 B, D, H-P**). Compared to Scrib1<sup>WT</sup>, none of the three mutants affected AMPAR average intensity (Scrib1<sup>KK611EE</sup>,  $14.52 \pm 1.08$ ,  $n=20$ ; Scrib1<sup>W203A</sup>,  $15.35 \pm 1.36$ ,  $n=18$ ; Scrib1<sup>W386A</sup>,  $14.34 \pm 1.21$ ,  $n=19$ ; and Scrib1<sup>KK611EE</sup>,  $15.69 \pm 0.76$ ,  $n=15$ ; Scrib1<sup>W203A</sup>,  $15.20 \pm 1.09$ ,  $n=19$ ; Scrib1<sup>W386A</sup>,  $15.66 \pm 1.59$ ,  $n=15$ , respectively; ns,  $p > 0.05$  for all) (**Fig. 7 H, N**) nor its total number at the cell surface (Scrib1<sup>KK611EE</sup>,  $14.64 \pm 0.53$ ; Scrib1<sup>W203A</sup>,  $16.34 \pm 1.05$ ; Scrib1<sup>W386A</sup>,  $15.37 \pm 1.19$ ; and Scrib1<sup>KK611EE</sup>,  $14.28 \pm 0.74$ ; Scrib1<sup>W203A</sup>,  $15.54 \pm 0.95$ ; Scrib1<sup>W386A</sup>,  $16.60 \pm 0.80$ , respectively; ns,  $p > 0.05$  for all) (**Fig. 7 I, O**). On the other hand, mutating Scrib1 actin- (Scrib1<sup>KK611EE</sup>,  $0.50 \pm 0.03$ ; °°  $p < 0.01$  vs. Scrib1<sup>WT</sup>), but not Arp2/3-binding sites (Scrib1<sup>W203A</sup>,  $0.65 \pm 0.03$ ; Scrib1<sup>W386A</sup>,  $0.66 \pm 0.03$ ; ns,  $p > 0.05$ ) (**Fig. 7 J**), induced a significant decrease of synaptic GluA1-containing AMPARs. Instead, GluA2 synaptic targeting is reduced in Scrib1<sup>W386A</sup> ( $0.48 \pm 0.03$ ; °°  $p < 0.01$ ), but not Scrib1<sup>KK611EE</sup> ( $0.54 \pm 0.02$ ; ns,  $p > 0.05$ ) nor Scrib1<sup>W203A</sup> ( $0.56 \pm 0.03$ ; ns,  $p > 0.05$ ) (**Fig. 7 P**), strongly suggesting that AMPAR targeting to the synapse is done in a subunit-specific manner.

### DISCUSSION

Here, we described for the first time the molecular mechanism underlying ASD-related Scrib1<sup>P592S</sup> mutation effect on synapse formation and maintenance. Our results show that the PCP protein Scrib1 is able to regulate dendritic spine structure and neuronal function via a coordinated Arp2/3-mediated actin nucleation and the formation of a Cter PDZ-dependent complex with TARP/AMPA.

### Scrib1 regulates actin dynamics via the Arp2/3 complex

Actin dynamics is known to be tightly regulated by a plethora of proteins able to bind, cross link, nucleate,

cap or sever actin (Pollard, 2003). Our data on MEF cells show that Scrib1 is preferentially accumulated in the lamellipodia due to its ability to bind actin and the Arp2/3 complex. The Arp2/3 is a 220 kDA complex composed by seven subunits, including the nucleating Arp2 and Arp3 subunits (Martin et al., 2005). These subunits display an intrinsic weak nucleation activity due to their distance within the inactive complex. Arp2/3 activity depends on conformational changes induced by nucleation promoting factor (NPFs) such as WAVE/Scar and WASP (Kelly et al., 2006; Boczkowska et al., 2008), stabilizers like Cortactin (Hering & Sheng, 2003), or inhibitory proteins as the BAR-PDZ PICK1 protein (Rocca et al., 2008; Nakamura et al., 2011), the trans-Golgi network/endosomally localized adaptor protein (AP)-1-associated adaptor protein Gadin (Maritzen et al., 2012) or Arpin (Dang et al., 2013). Unlike the aforementioned Arp2/3 regulators, Scrib1 displays one actin-binding motif and two Arp2/3-binding sites. One or both residues might bind Arp2 and/or Arp3 nucleation subunits (Padrick et al., 2011; Ti et al., 2011; Xu et al., 2012), potentially enhancing actin assembly through its dimerization (Wang et al., 2014). Alternatively, Scrib1<sup>W203</sup> and Scrib1<sup>W386</sup> might compete over Arp2/3 binding like N-WASP and Cortactin (Weaver et al., 2002), possibly underlying distinct Scrib1-mediated actin assembly roles. Additional structural and/or biochemical studies will be needed to elucidate how does Scrib1 bind the Arp2/3 complex and what is its effect on actin dynamics.

### **The PCP Scrib1 promotes neuronal complexity via an actin-dependent mechanism**

Neuronal complexity, specifically dendritic branching and morphology, consents individual neurons to accomplish specialized brain functions and cognitive behaviours. In the last years, an increasing number of evidences suggest that PCP genes play a crucial role in establishing neuronal cell polarization (Shima et al., 2004; Rosso et al., 2005; Goodrich, 2008). We show for the first time that the recently identified ASD-related Scrib1<sup>P592S</sup> mutant displays a decreased dendritic branching. This result is in good agreement with one of the earliest reports of pathological changes in autism (Raymond et al., 1996) as well as to previous work in our group regarding ASD-related Scrib1<sup>cr</sup> (Moreau et al., 2010) or Scrib1 loss (Hilal et al., in preparation), depicting Scrib1 as a key player in dendritic morphogenesis regulation. As Scrib1, Densin-180 and Erbin increase dendritic branching of hippocampal neurons (Quitsch et al., 2005; Arikath et al., 2008), suggesting that the LAP family fosters dendritic complexity. We show that Scrib1 mediates dendritic branching complexity via an actin-dependent mechanism. Interestingly, Scrib1<sup>KK611EE</sup> prevents Scrib1-mediated dendritic complexity in a similar way as PICK1<sup>KK251EE</sup> precludes PICK1 knockdown effect (Rocca et al., 2008), suggesting that Scrib1 and PICK1 exert antagonizing roles. Scrib1 effect on actin-dependent dendritic morphology seems, however, to be independent on its ability to bind the Arp2/3 complex. Indeed, dendritic formation and arborisation involves the coordination of several neuronal cytoskeleton actors (Jan & Jan, 2010). Besides actin, microtubule dynamics can also shape the dendritic arbour (Georges et al., 2008). Scrib1 has been repeatedly correlated to microtubules through genetic interactions (Schlüter et al., 2009; Cho et al., 2011; Andersen et al., 2012; Mohseni et al., 2014), as well as to microtubule-binding proteins like APC (Zumbrunn et al., 2001; Kita et al., 2006) and dynein-based microtubule motors (Wang et al., 2013). Additional studies will be needed to decipher Scrib1 potential role on microtubule-dependent neuronal arborisation.

### **Scrib1 promotes spine maturation through Arp2/3-mediated actin nucleation**

Scrib was originally discovered in *Drosophila* basolateral junctions (Bilder & Perrimon, 2000). In mammals, Scrib1 has been shown to be involved in the actin-mediated recruitment of synaptic vesicles via the  $\beta$ PIX/ $\beta$ -catenin/cadherin complex (Sun et al., 2009; Sun & Bamji, 2011). Together with a previous report from our group (Moreau et al., 2010), we show that Scrib1 mutations related to ASD lead to an altered spine number and morphology, further highlighting Scrib1 as a key player in spinogenesis. During the early stages of synaptogenesis, filopodia arise at the dendritic shaft level from a small collection of Rac-dependent Arp2/3-mediated branched actin (Korobova & Svitkina, 2010). Ensuing Arp2/3 recruitment, elongation factors like formins and Ena/VASP are gradually enriched at the filopodia tip (Hotulainen et al., 2009; Lee et al., 2010; Goh et al., 2012; Disanza et al., 2013). Concurring, we showed that mutations of Scrib1 actin and Arp2/3-binding sites reverse Scrib1 effect on filopodia formation and elongation. Following axonal contact, dendritic filopodia can transform into morphologically mature spines. Live imaging studies found that the filopodia-to-spine transition requires the swelling and shortening of the filopodia tip (Marrs et al., 2001; Yoshihara et al., 2009). Whereas spine head expansion most likely involves Arp2/3 complex-dependent actin filament branching (Mullins et al., 1998; Welch et al., 1998; Hering & Sheng, 2003; Klein et al., 2009), filopodia shortening depends on spine neck-enriched myosin II (Ryu et al., 2006; Korobova & Svitkina, 2010). Accordingly, we show that Scrib1-mediated spine enlargement depends on Scrib1<sup>KK611</sup> and Scrib1<sup>W203</sup> binding to actin and Arp2/3 complex, respectively. Strikingly, PICK1-induced spine width restriction is blocked in neurons expressing PICK1<sup>W413A</sup> mutation, pinpointing Scrib1<sup>W203</sup> and PICK1<sup>W413</sup> as key antagonistic residues in Arp2/3-mediated spine remodelling. Other Arp2/3 complex regulators are known to compete over Arp3 binding, resulting into distinctive Arp2/3 activities (Weaver et al., 2002). Unlike Cortactin and actin-binding protein (Abp1) (Hering & Sheng, 2003; Haeckel et al., 2008), Scrib1 has no effect *per se* on spine longitudinal growth. Nevertheless, mutating Scrib1<sup>KK611</sup> and Scrib1<sup>W386</sup> residues result into longer spines, implying the involvement of Scrib1<sup>W386</sup>-mediated Arp2/3 complex nucleation into the filopodia-to-spine transition. In fact, actin cytoskeleton in spine head and neck were previously shown to be differently structured (Frost et al., 2010; Korobova & Svitkina, 2010). While the first is supported by an axial bundle of short actin filaments, reminiscent of dendritic patches; spine neck is composed by a longitudinal stretched network of branched and roughly aligned linear filaments of different length. It is tempting to speculate that a synaptic network made of shorter actin filaments might optimize both the temporal and spatial response to neural activity (Star et al., 2002; Okamoto et al., 2004; Cingolani & Goda, 2008; Penzes et al., 2008). As Scrib1 W203 and W386 residues are preferentially engaged in spine widening or spine lengthening, respectively, we hypothesize that Scrib1 coordinates the assembly of spine head or neck actin filaments via distinct Arp2/3-binding sites.

### **Scrib1 ensures PSD nano-architecture and function**

Synaptopathologies underlying abnormal spine number and/or morphology are often associated to a disrupted recruitment of critical proteins to the PSD as well as to an inability to structurally respond to synaptic activity (Kulkarni & Firestein, 2012). As previous works from our group regarding Scrib1 loss (Hilal et al., in preparation) or truncation (Moreau et al., 2010), we show that Scrib1 ASD mutants display an aberrant PSD struc-

ture and composition, identifying Scrib1 as a key player in PSD architecture and function. Furthermore, we show that Scrib1 effect on spine number and enlargement is linked to a hyper-clusterisation of PSD-95, a fiduciary effective marker of the PSD structure (Okabe et al., 1999). PSD-95 clusters were previously shown to make their first appearance in dendritic shafts or dynamic spine precursors, before migrating to their ultimate position in mature spine heads (Friedman et al., 2000; Marrs et al., 2001; Okabe et al., 2001). Although PSD acquisition can increase the survival rate of new spines *in vivo*, spine stability is mainly correlated to the PSD size (Cane et al., 2014). Consistent with Scrib1 playing a role in spine stabilization, our group showed that Scrib1 truncation (Moreau et al., 2010) or loss (Hilal et al., in preparation) resulted into reduced basal transmission and impaired expression of long-term potentiation (LTP) or long-term depression (LTD). These two major cellular mechanisms are believed to underlie learning and memory in the adult brain (Malenka & Nicoll, 1999), being mainly mediated by NMDA and AMPA receptors. We showed recently that Scrib1 can bind and modulate the traffic of GluN2-containing NMDARs (Piguel et al., 2014). Herein, we found that Scrib1 can bind transmembrane auxiliary regulatory proteins (TARPs), known to regulate AMPAR expression levels, synaptic targeting, stabilization as well as synaptic strength (Chen et al., 2000). In agreement with a recent work from our group showing that Scrib1 does not affect the amplitude of pharmacologically isolated AMPAR-mediated EPSCs (Piguel et al., 2014), we found no difference in the total surface number of AMPA receptors at the cell surface or at the synapse following Scrib1 overexpression. This data reflects a dynamic and coordinated reorganization of existing constituent scaffold proteins and receptors inside the PSD (Blanpied et al., 2008). Nonetheless, we did find that the ASD Scrib1<sup>P592S</sup> mutant displays a higher content of GluA1-containing AMPARs at the synapse. Strikingly, an excessive number of surface AMPA receptors was previously associated to a segmented PSD configuration (Ganeshina et al., 2004). As these spines are believed to have a greater synaptic efficacy (Spacek & Harris, 1997; Geinisman et al., 1993, 1996; Toni et al., 2001; Ganeshina et al., 2004), we are currently investigating if the ASD Scrib1<sup>P592S</sup> mutation contribute to changes in basal transmission. On the other hand, mutating Scrib1 actin- or W386-mediated Arp2/3-binding sites lead to a significant decrease of synaptic GluA1 or GluA2 subunits, respectively. Although latrunculin has been shown to slowly reduce synaptic AMPAR number (Allison et al., 1998; Kim & Lisman, 1999), EM and high-resolution imaging studies show that actin filaments are rather sparse inside the PSD (Fifkova & Delay, 1982; Capani et al., 2001; Frost et al., 2010). Accordingly, AMPARs are believed to link actin through a variety of intermediates, from cell adhesion (Südhof, 2008) or transmembrane proteins (Chen et al., 2000) to scaffold (Dong et al., 1997) or actin-binding proteins (Shen et al., 2000; Lin et al., 2010), including the Arp2/3 complex (Rocca et al., 2008). AMPAR involvement with such intricate network might explain the difference between ASD and actin-related Scrib1 mutants, as Pro592Ser-mediated conformational changes potentially affect more than one interaction. Taken altogether, our data favours a model whereby Scrib1-mediated actin dynamics regulation participates in the distribution of subunit-specific AMPA receptors at the synapse.

## CONCLUDING REMARKS

Despite the high heritability observed in ASD, disease-causing mutations involve a limited number of genes

(Persico & Bourgeron, 2006). On the other hand, loss or disruption of PCP genes (Lijam et al., 1997; Fraser et al., 2008; Wynshaw-Boris, 2012; Sowers et al., 2013; Zhu et al., 2013) or PDZ-dependent protein-protein interactions (Südhof, 2008; Carlisle et al., 2011; Durand et al., 2012; Cao et al., 2013), including Scrib1 (Moreau et al., 2010; Hilal et al., in preparation), have been previously linked to abnormal dendritic complexity, mistargeting of proteins and/or inability to properly form a synapse, strongly favoring an overlap in the genetic etiopathology in ASD. We show that disrupting Scrib1-mediated actin dynamics regulation impacts neuronal complexity as well as dendritic spine maturation and function, highlighting the importance of structural genomic rearrangements in ASD etiopathology.

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## NOTES

Author contributions: NS, GG and VP designed research; VP, CM, HF and AM performed all cell cultures experiments; VP performed the yeast-two hybrid screens and analysis; VP and NP performed biochemistry experiments; VP, HF, AM and NS analyzed data; VP and NS wrote the paper. Competing financial interests: The authors declare no conflict of interest.

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## FIGURE LEGENDS

**Figure 1: The ASD Scrib1<sup>P592S</sup> mutant loses Scrib1<sup>WT</sup>-mediated localization at the lamellipodia. (A)** MEF cells transfected with C3, Scrib1<sup>WT</sup> and Scrib1<sup>P592S</sup> GFP-coupled plasmids (green) and phalloidin (red). Scale bar, 5  $\mu$ m. On the bottom side, magnification of selected lamellipodia. **(B)** GFP/phalloidin intensity ratio plotted along the lamellipodia. Grey area highlights Scrib1<sup>WT</sup> enriched domain. **(C)** Average lamellipodial GFP/phalloidin intensity ratio. Scrib1<sup>WT</sup> is strongly expressed within the lamellipodia, while the Scrib1<sup>P592S</sup> mutant displays a ratio similar to control. **(D)** Coordinates of maximal GFP/phalloidin ratio. Scrib1<sup>WT</sup> is preferentially located near the lamellipodia, whereas the ASD mutant concentrates further away. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ . One Way ANOVA, Dunnett's Multiple Comparison Test vs. C3 (\*) or Scrib1<sup>WT</sup> (°) corresponding to 7-14 measurements from 4-8 different cells.

**Figure 2: Scrib1 localization at the lamellipodia depends on its ability to bind actin and the Arp2/3 complex. (A)** Sequence alignment of Scrib1<sup>KK611,612</sup> with known WH2-like motif-containing proteins. Conserved Lys (K) and Arg (R) residues are shown in red. ASD-related Scrib1<sup>P592S</sup> is highlighted and underlined in green. **(B)** Sequence alignment of Scrib1<sup>W203</sup> and Scrib1<sup>W386</sup> with known Arp2/3 regulators. Conserved Trp (W) residues are shown in orange. **(C)** MEF cells transfected with Scrib1<sup>KK611EE</sup>, Scrib1<sup>W203A</sup> and Scrib1<sup>W386A</sup> GFP-coupled plasmids (green) and phalloidin (red). Scale bar, 5  $\mu$ m. On the bottom side, magnification of selected lamellipodia. **(D)** GFP/phalloidin intensity ratio plotted along the lamellipodia. Grey area highlights Scrib1<sup>WT</sup> enriched domain. **(E)** Average lamellipodial GFP/phalloidin intensity ratio vs. Scrib1<sup>WT</sup>. Dotted line represents Scrib1<sup>WT</sup>. The three mutants are no longer enriched within the lamellipodia. °°,  $p < 0.01$ ; °,  $p < 0.05$ . One Way ANOVA, Dunnett's Multiple Comparison Test vs. Scrib1<sup>WT</sup> corresponding to 7-9 measurements from 4-7 different cells.

**Figure 3: Scrib1 ASD and actin mutants display aberrant dendritic trees. (A, D)** Representative reconstruction of neuronal morphology of control, Scrib1<sup>WT</sup> and Scrib1<sup>P592S</sup> (A) or Scrib1<sup>KK611EE</sup>, Scrib1<sup>W203A</sup> and Scrib1<sup>W386A</sup> (D) transfected neurons using the software Neurolucida. **(B, E)** Scholl analysis showing the corresponding number of dendritic intersections according to the distance from the soma. Compared to the control, Scrib1<sup>WT</sup> shows an increase neuronal morphology, which is lost upon Scrib1<sup>P592S</sup> (B) and Scrib1<sup>KK611EE</sup> (E) expression. **(C, F)** Total dendritic length ( $\mu$ m). Scrib1<sup>WT</sup> does not change the neuronal dendritic length. Instead, Scrib1<sup>P592S</sup> (C) and Scrib1<sup>KK611EE</sup> (F) mutants display a reduced dendritic tree length. Dotted line represents Scrib1<sup>WT</sup>. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ . Two Way ANOVA vs. C3 (\*) or Scrib1<sup>WT</sup> (°) corresponding to the genotype variation of 21-26 neurons from 4 independent experiments (interaction and distance: \*\*\*,  $p < 0.001$  for all the represented conditions).

**Figure 4: Scrib1-mediated actin regulation plays a key role in spinogenesis. (A, H)** Hippocampal neurons were transiently co-transfected at 16-17 days *in vitro* (DIV) with actin coupled to red fluorescent protein (mRFP) and green-fluorescent protein (GFP) alone (C3) or coupled to Scrib1 variants. At DIV 21, neurons were stained with anti-GFP (green) and anti-DsRED (red). Scale bar, 15  $\mu$ m. On the bottom side, magnifica-

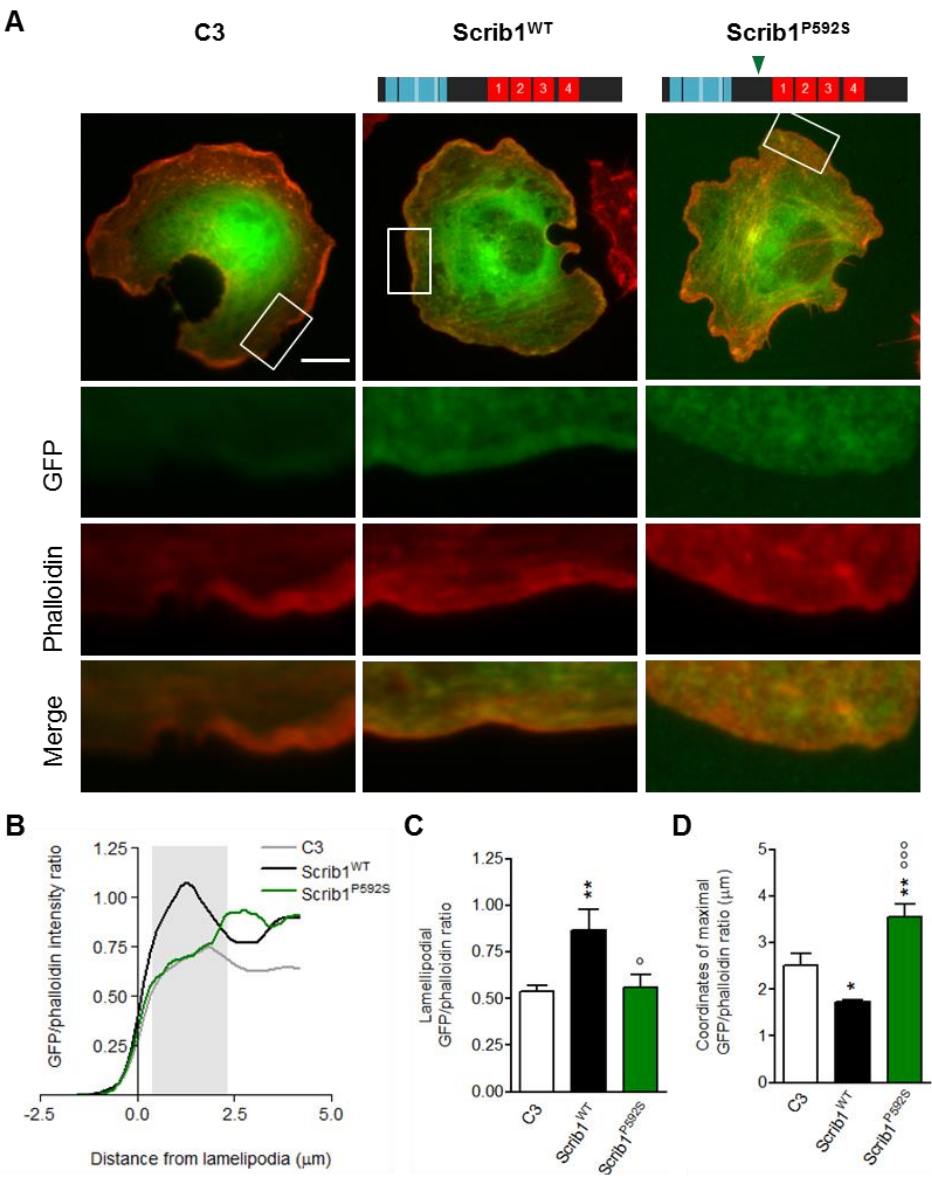
tion of 10  $\mu\text{m}$  dendrite transfected with each construct. **(B, I)** Average number of filopodia per 10  $\mu\text{m}$  of dendritic length in each condition. **(C, J)** Average filopodia length ( $\mu\text{m}$ ). Scrib1-mediated decrease in filopodia number and length is lost in ASD mutant **(B, C)** and actin-related mutants **(I, J)**. **(G)** Example of filopodia (top) and spines (bottom) from transfected neurons with Scrib1<sup>WT</sup> and Scrib1<sup>P592S</sup>. Scale bar, 0.75  $\mu\text{m}$ . **(D, K)** Average dendritic spine number per 10  $\mu\text{m}$  of dendritic length in each condition. Scrib1<sup>WT</sup> increases spine number through a Arp2/3-mediated actin polymerization mechanism **(K)**, which might be impaired in ASD **(D)**. **(E, L)** Average spine width ( $\mu\text{m}$ ) and **(F, M)** average spine length ( $\mu\text{m}$ ). Scrib1-mediated actin-dependent spine enlargement and elongation is dependent on W203 **(L)** and W386 **(M)** residues, respectively. The disruption of such mechanism might underlie ASD aberrant spine morphology **(E F)**. Dotted line represents Scrib1<sup>WT</sup>. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; One Way ANOVA, Dunnett's Multiple Comparison Test vs. C3 (\*) or vs. Scrib1<sup>WT</sup> (°) corresponding to 16-27 neurons from 4 independent experiments.

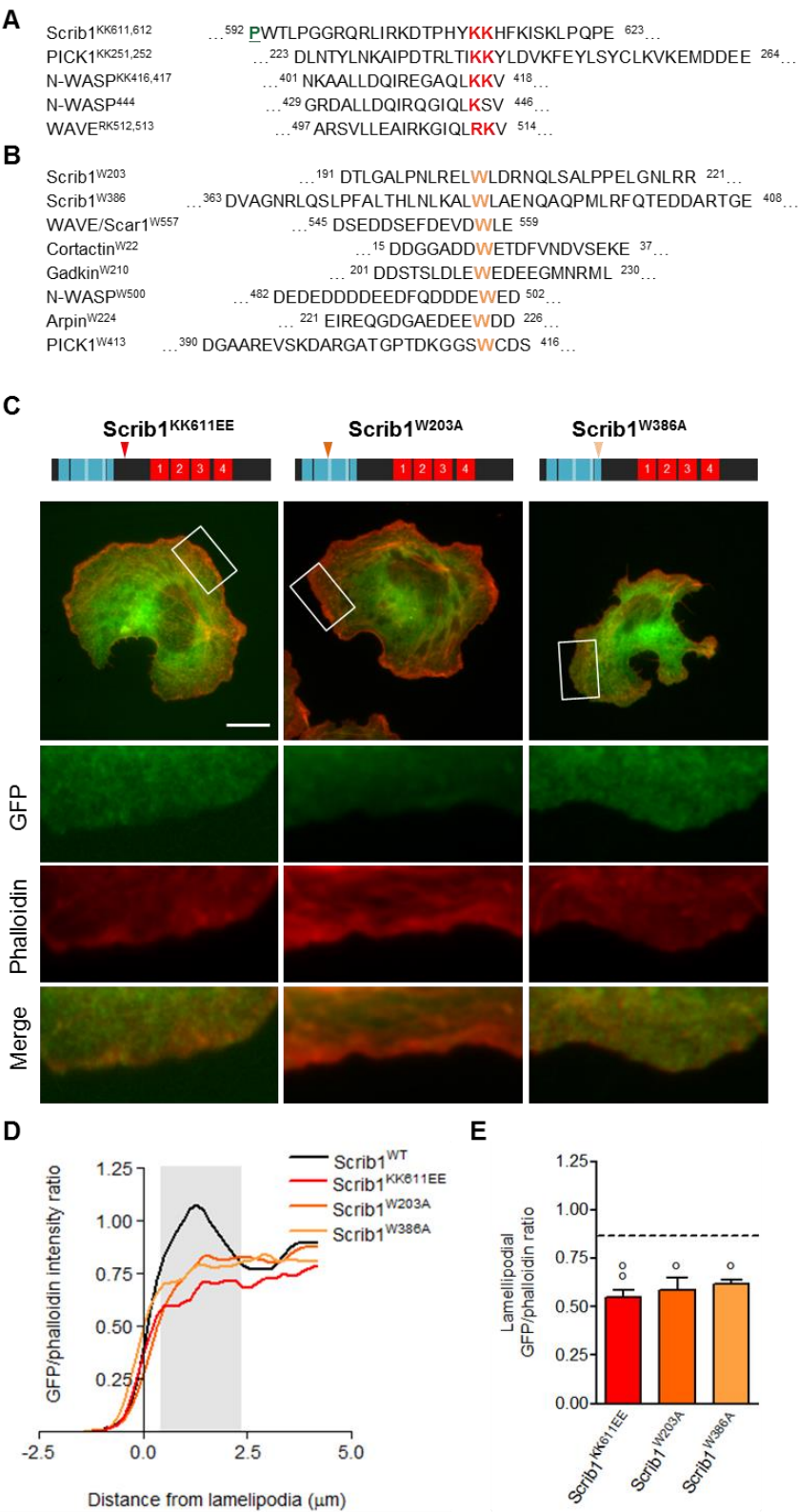
**Figure 5: Scrib1 is a key organizer of the post-synaptic structure.** **(A)** Direct Stochastic Reconstruction Microscopy (dSTORM) principle. The super-resolution microscopy dSTORM technique relies on a fluorophore coupled to a given protein of interest; this fluorophore will be spontaneously quenched from its triplet state to a stable non-fluorescent reduced state (dark state); thus allowing the precise localization and ulterior reconstruction of single particle molecules. The final super-resolution image is obtained by repeating in an iterative and stochastic manner this approach over time (t). **(B)** Hippocampal neurons at DIV 18 were immunolabeled with endogenous PSD-95 (red) and Scrib1 (green). Scale bar, 1  $\mu\text{m}$ . White squares show selected spines, magnified in confocal microscopy **(B')** or dSTORM **(B'')**. Scale bar, 500 nm. **(C)** Schematic representation of average distance quantification for individual molecules (PSD-95 in red and Scrib1 in green) in x and y (in nm) relative to a reference molecule centroid. With the cross represents PSD-95 centroid. **(D)** Average distance of endogenous PSD-95 and Scrib1 relative to PSD-95 centroid (in nm). Native Scrib1 display a higher dispersion compared to the centralized PSD-95. **(E, F)** Hippocampal neurons at DIV 8 were transfected with Scrib1<sup>WT</sup> **(E)** or Scrib1<sup>P592S</sup> **(F)**. At DIV 18 they were immunolabeled with endogenous PSD-95 (red) and anti-GFP (green) antibodies. Scale bar, 1  $\mu\text{m}$ . White squares show selected spines, magnified in low **(E', F')** or super resolution **(E'', F'')**. Scale bar, 500 nm. **(G, H)** Average distance of Scrib1<sup>WT</sup> **(G)** or Scrib1<sup>P592S</sup> **(H)** relative to PSD-95 centroid (in nm). Both Scrib1 forms display similar dispersion values as the native condition. **(I)** Average distance of Scrib1 in Scrib1<sup>WT</sup> or Scrib1<sup>P592S</sup> conditions. Scrib1<sup>P592S</sup> has a higher dispersion than Scrib1<sup>WT</sup>. **(J)** Percentage (%) of dendritic spines containing a cluster- (black) or fragmented-like (spotted grey) PSD-95 organization in native (Scrib), Scrib1<sup>WT</sup> or Scrib1<sup>P592S</sup> conditions. Scrib1<sup>WT</sup> overexpression induces a hyperclusterisation of PSD-95, whereas Scrib1<sup>P592S</sup> promotes PSD-95 fragmentation. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$  unpaired t test corresponding to 14-24 dendritic spines from 2-5 neurons from independent experiments.

**Figure 6: Scrib1 forms a PDZ-dependent complex with AMPAR via type I TARPs.** **(A)** Yeast two hybrid assay between two genetically engineered yeast strains containing a DNA-binding domain (BD or bait) or an activating domain (AD or prey) coupled to the Scrib1<sup>PDZ3</sup> and a P10 mouse brain cDNA library, respectively.

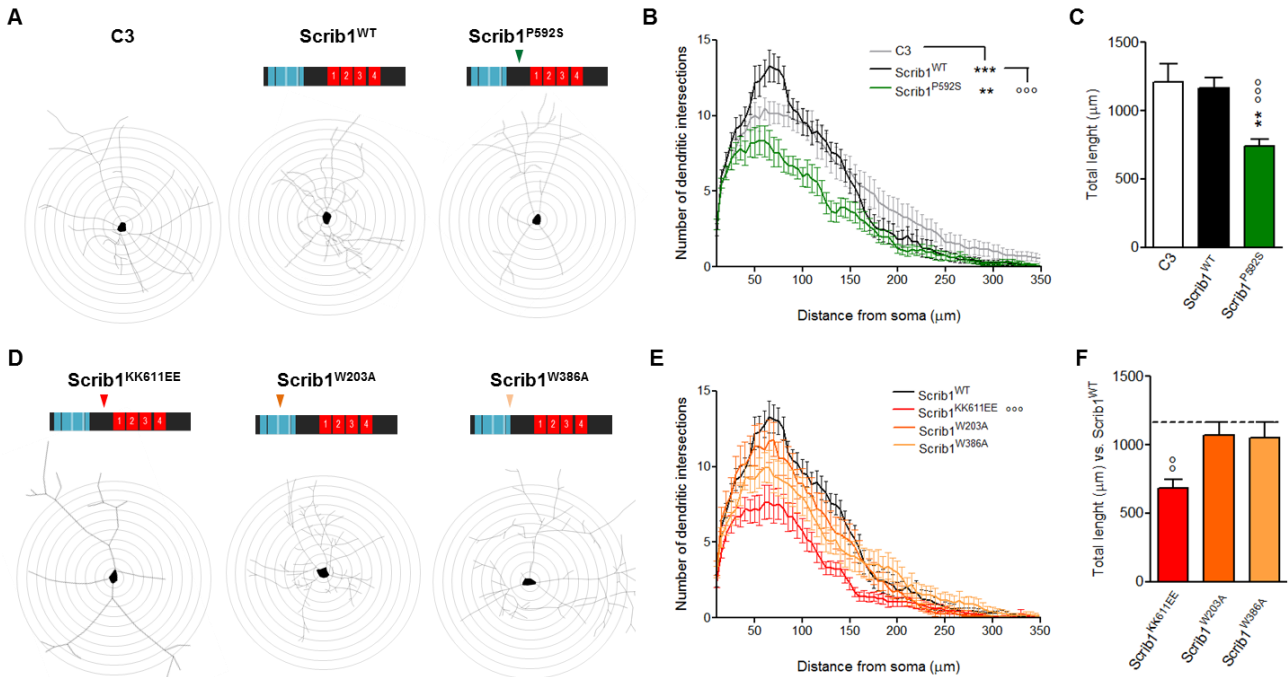
If the bait and the prey proteins are able to interact, the AD will be in close proximity to the BD, which in turn will activate the transcription of the reporter gene producing a change in the cell phenotype. The transmembrane auxiliary regulatory protein (TARP)  $\gamma$ -4 was found to bind Scrib1 PDZ3 domain. **(B)** Dendrogram and bar diagrams illustrating the approximate phylogenetic relationships between type I and type II TARPs. TARPs are composed by four transmembrane domains, one to several N-glycosylation sites, one phosphorylation site and a Cter PDZ binding domain (PDZ-BD). Sequence alignment showing that the Cter of type I TARPs, including Stargazin (Stgz or  $\gamma$ -2) and  $\gamma$ -4 share a great homology. (\*) identical amino acids; (:) conserved substitutions; (.) semi-conserved substitutions. Dendrogram and alignments done by ClustalW2 using the following Uniprot sequences: O88602, Q9JJV5, Q9JJV4, Q8VHW2, Q544Q4, and P62956. **(C)** Co-immunoprecipitation of endogenous Scrib1 and GluA1 with Pan-Stgz antibody from P35 rat hippocampal lysates after Triton-X-100 solubilisation. **(D, E)** Immunoprecipitation of Scrib1, Stgz and GluA1. Lysates from HEK-293 cells, showing that Scrib1 immunoprecipitates Stgz-GFP **(D)**, but not myc-GluA1 **(E)**. Scrib1 forms a complex with AMPAR through Stgz **(F)**. **(G-L)** COS-7 cells transiently co-transfected with HA-Scrib1, Stgz-GFP, and myc-GluA1 display a perfect co-localization along the shown line draw **(K)**. Such co-localization is lost when Stgz and GluA2 are co-expressed with Scrib1<sup>crc</sup> form missing the Cter PDZ3 and PDZ4 domains **(L)**. Plots represent intensity (x axis) vs. position (y axis). Scale bars, 15 $\mu$ m. **(M, N)** Scrib1 and Stgz co-localize with GluA1- **(M)** and GluA2-containing AMPAR **(N)** in hippocampal neurons. 21 DIV dissociated neurons were immunolabelled with endogenous antibodies against Scrib1 (green), Stgz (or  $\gamma$ -2, red), and GluA1 or GluA2 (blue). White rectangle show separated channels in higher magnification at the right. Scale bar, 15  $\mu$ m. Full and empty arrows indicate full or partial co-localization of Scrib1 with Stgz and AMPA receptors in inside dendritic spines, respectively.

**Figure 7: Scrib1 mutants show aberrant AMPAR synaptic targeting.** **(A-D)** Hippocampal neurons were transiently transfected at 16-17 DIV with green-fluorescent protein (GFP) alone (C3) or coupled to Scrib1 variants. Four days after, native Cter GluA1- **(A, B)** or GluA2-containing AMPA receptors **(C, D)** were stained using synapsin as a pre-synaptic marker. Scale bar, 15  $\mu$ m. Bottom white rectangles show in higher magnification surface receptors (red), synapsin (blue) and dendrites (green) from the selected area. **(E, H, K, N)** Average intensity, **(F, I, L, O)** average surface number and **(G, J, M, P)** respective synaptic ratio of GluA1 or -GluA2 subunits. Dotted black line represents Scrib1<sup>WT</sup>. Scrib1<sup>WT</sup> does not change AMPAR surface number nor its targeting towards the synapse. Instead, ASD **(G)** and actin **(M)** or W386-mediated Arp2/3 binding **(P)** mutants show an altered synaptic content of GluA1 or GluA2 subunits, respectively. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; One Way ANOVA, Dunnett's Multiple Comparison Test vs. C3 (\*) or vs. Scrib1<sup>WT</sup> (°) corresponding to 15-22 neurons from 4 independent experiments.



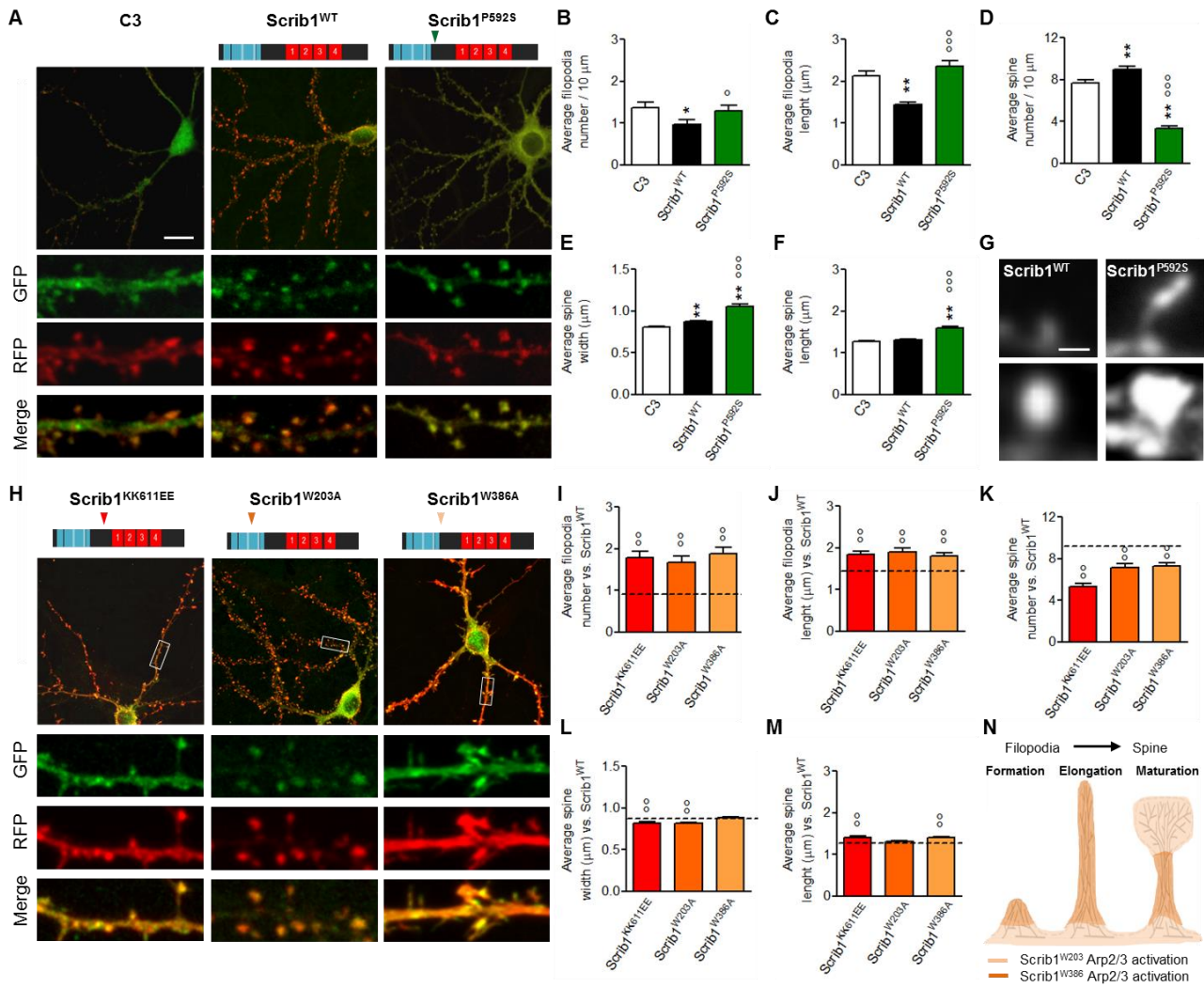


Pinheiro et al.,  
Figure 3

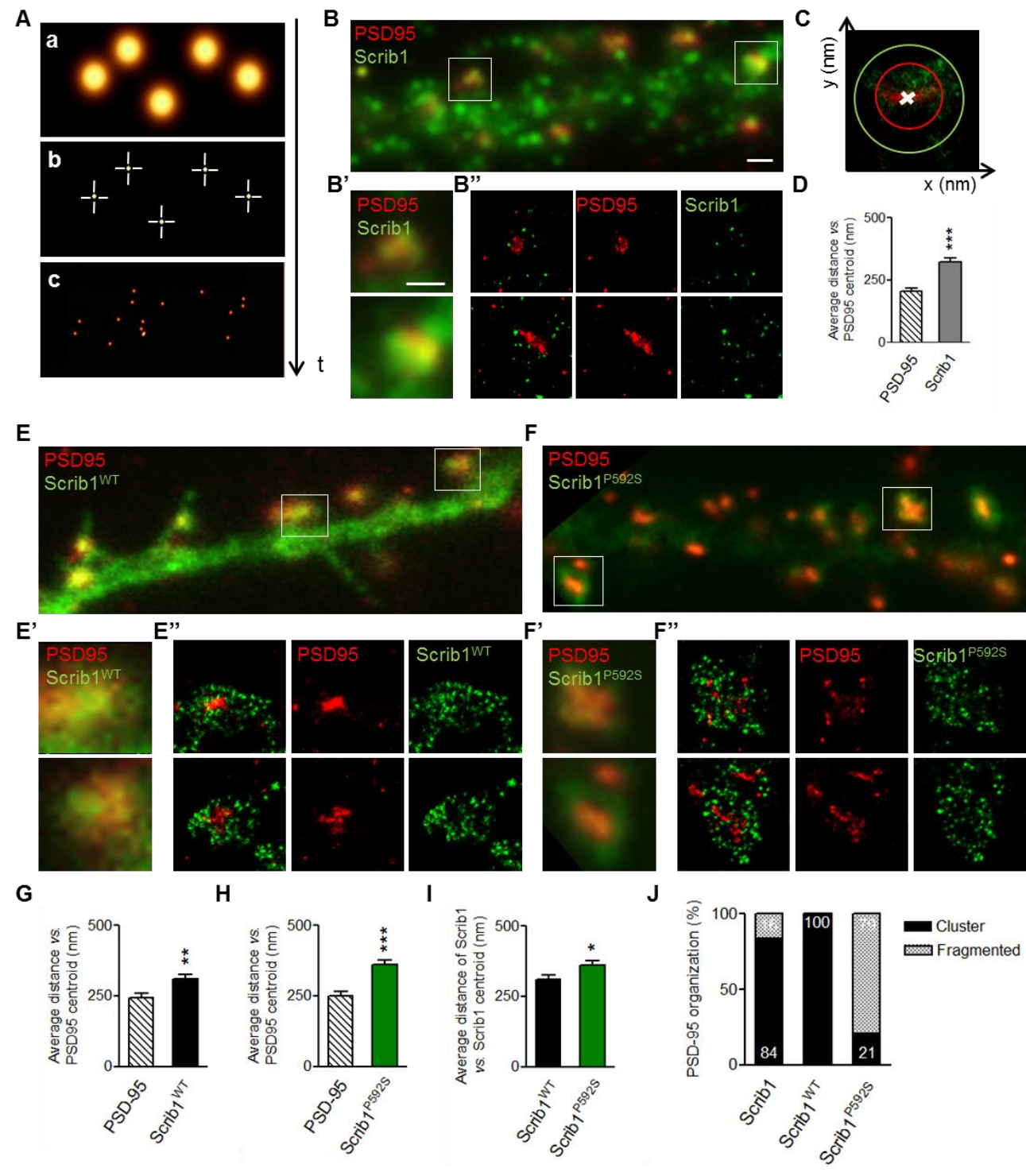




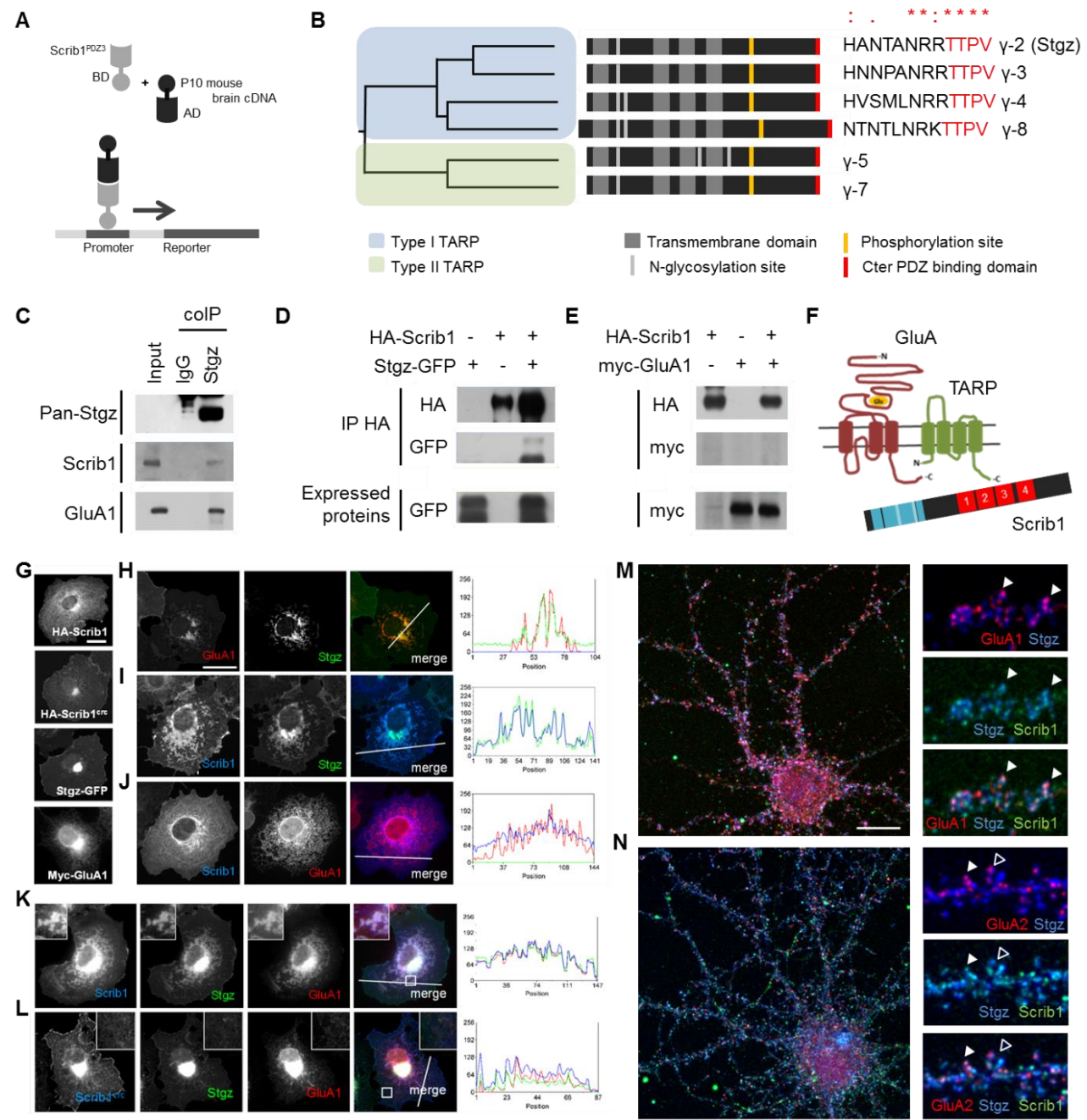
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Figure 4



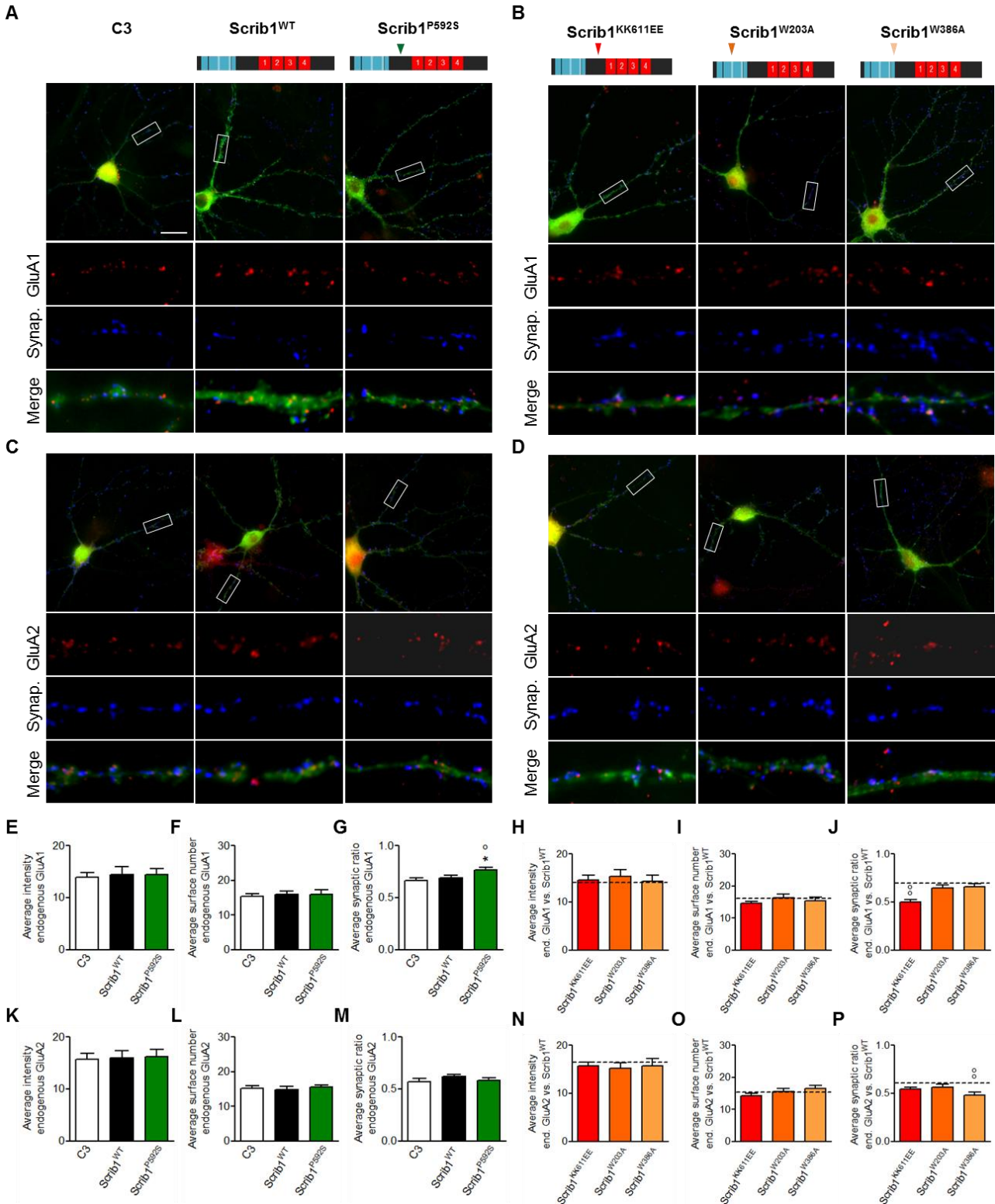
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Figure 5



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Figure 6









## Chapter VII

# Supplementary Experiments: Scribble1 regulates GluA1-, but not GluA2-containing AMPAR endo- and exocytosis

### VII.1 Context and Problematic

Together with NMDA, AMPA receptors are main players within the main CNS excitatory neurotransmitter circuit. AMPARs are tetramers composed of four GluA1-4 subunits, being their composition dependent on the cell type, development and brain region. In hippocampus, GluA1/2 and GluA2/3 heteromers are predominant (Wentholt et al., 1996). Even though both are composed by GluA2 subunits, GluA2/3 complexes are constitutively trafficked to and from the cell membrane, while GluA1/A2 complex is trafficked in an activity-dependent manner. The differential trafficking of AMPARs subunits is dictated by the distinct AMPAR-interacting proteins capable of controlling AMPAR targeting to the synapse (**Chapter I.6**). Previously, we showed that Scrib1 can form a PDZ-dependent complex with type I transmembrane AMPAR regulatory proteins (TARPs) (**Chapter VI**). TARPs are critical regulators of AMPA endocytosis and exocytosis, key events in cellular mechanisms underlying learning and memory (Chen et al., 2000). Moreover, since Scrib1 was recently implicated in an AP-2 dependent NMDAR traffic mechanism (Piguel et al., 2014), we investigated if Scrib1 could modulate AMPAR endo- or exocytosis as well.

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### VII.2 Material and Methods

*DNA constructs and antibodies.* hScrib1 GFP-tagged (green fluorescent protein) and myc-tagged GluA1 and GluA2 cDNA samples were generously provided by I. Macara (University of Virginia, Charlottesville, VA, USA) and P. Osten (Cold Spring Harbor Laboratory, NY, USA), respectively. The following primary antibodies were used in this study: anti-green fluorescent protein (GFP) (JL-8, Clontech), anti-myc mouse 9E11 (Covance) and anti-synapsin 1/2 (106103, Synaptic Systems). Secondary antibodies used as follows: Alexa Fluor 488, 568 and 647 conjugated antibodies (Invitrogen).

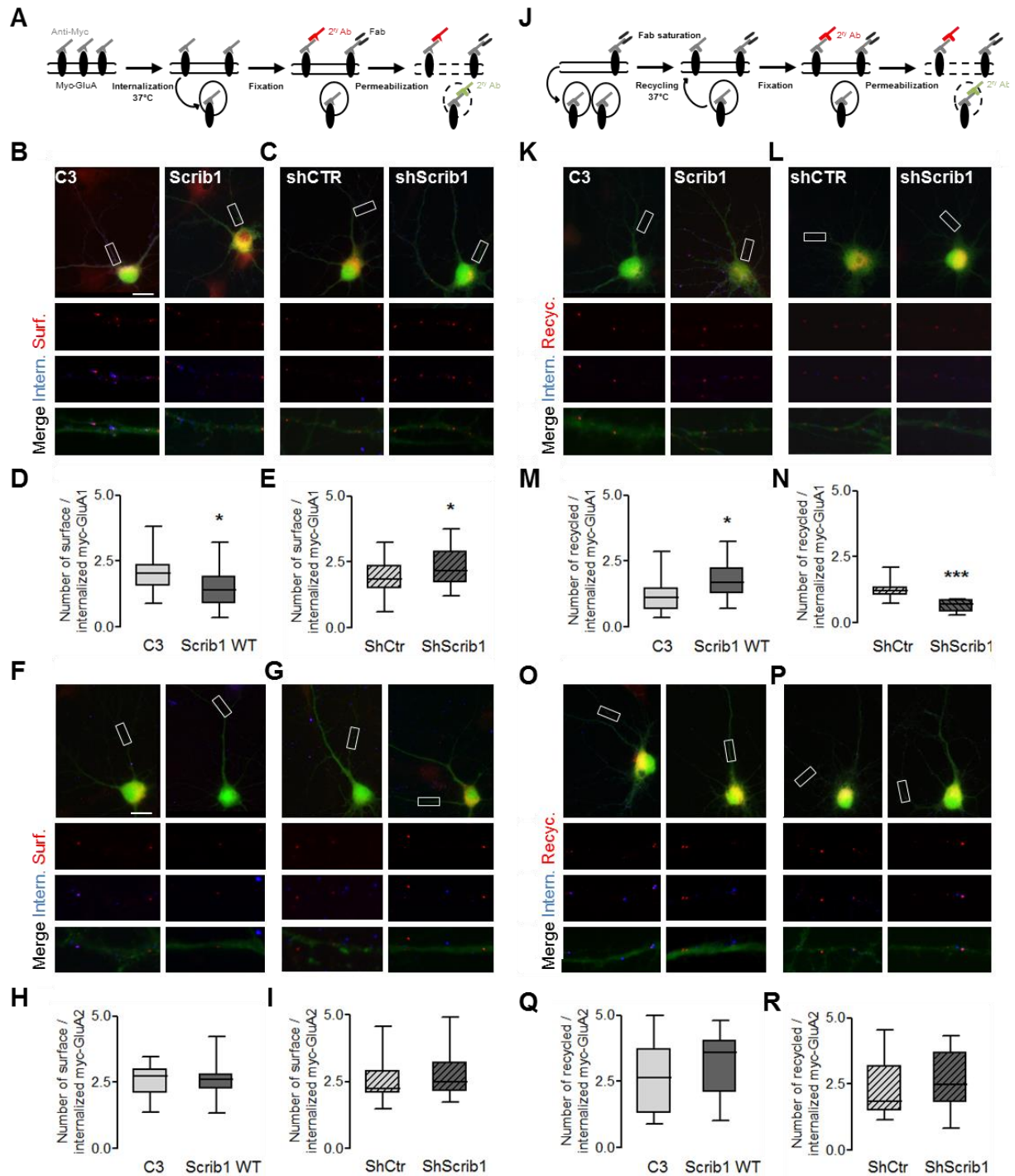
*Neuron cell culture, transfection and immunocytochemistry.* Hippocampal neurons were obtained from E18.5 rat embryos and transfected using the previously described calcium phosphate method (Sans et al. 2005). Internalization and recycling assays of AMPA receptors were done by adapting protocols from previous studies (Suh et al., 2010; Piguel et al., 2014).

*Quantitative analysis and data representation.* AMPAR trafficking experiments were done on 14-16 DIV neurons 2d after transfection. Images were acquired by the Zeiss AxioImager Z1 microscope using a 63 X oil-immersion on and analysed by MetaMorph 7.5 software (Universal Imaging). Surface, internalized and recycled AMPA receptors were quantified by counting the number of puncta and the average intensity from 5

different 20  $\mu\text{m}$  length regions per neuron on isolated dendrites. All conditions in individual experiments used identical acquisition parameters and value thresholds. Each experiment was performed on at least four independent neuronal preparations. All data is represented as box and whiskers plots to put into evidence the median, lowest and highest limits. Statistical analyses were performed using Sigmaplot11 (Systat Software) by unpaired Student t-test. All data is referred in the text as mean  $\pm$  SEM; \*  $p < 0.05$ , \*\* and \*\*\*  $p < 0.001$  were considered statistically significant.

### VII.3 Preliminary Results

To investigate whether Scrib1 could mediate AMPAR endo- and/or exocytosis, we used a similar protocol as Pigué et al. (2014) to follow surface expressed myc-tagged GluA1 or GluA2 subunits in the presence or absence of Scrib1 (**Fig. Suplm. Exp. 1**). 13-14 DIV transfected hippocampal neurons tagged with a primary anti-myc antibody were incubated for 30min at 37°C, during which AMPAR were allowed to internalize (**Fig. Suplm. Exp. 1 A**). A ratio of surface/internalized receptors was obtained by staining surface myc-tagged receptors after fixation and myc-tagged internalized receptors following a subsequent permeabilisation step (**Fig. Suplm. Exp. 1 B-I**). Under basal conditions, overexpressing Scrib1 led to a significant decrease of the surface/internalized myc-GluA1 ratio compared to C3 (C3,  $2.07 \pm 0.13$ ,  $n=32$ ; Scrib1,  $1.55 \pm 0.17$ ,  $n=24$ ; unpaired t-test \*,  $p < 0.05$ ) (**Fig. Suplm. Exp. 1 B, D**), whereas downregulating Scrib1 had the reverse effect (shCTR,  $1.88 \pm 0.10$ ,  $n=37$ ; shScrib1,  $2.28 \pm 0.14$ ,  $n=25$ ; unpaired t-test \*,  $p < 0.05$ ) (**Fig. Suplm. Exp. 1 C, E**), suggesting that Scrib1 does play an active role in GluA1-containing AMPAR endocytosis. Conversely, up- (C3,  $2.59 \pm 0.11$ ,  $n=26$ ; Scrib1,  $2.64 \pm 0.15$ ,  $n=20$ ; unpaired t-test  $p > 0.05$ ) (**Fig. Suplm. Exp. 1 F, H**) or down-regulating (shCTR,  $2.46 \pm 0.17$ ,  $n=19$ ; shScrib1,  $2.77 \pm 0.20$ ,  $n=22$ ; unpaired t-test  $p > 0.05$ ) (**Fig. Suplm. Exp. 1 G, I**) Scrib1 had no effect on the surface to internalized myc-GluA2 ratio. To study Scrib1 effect on AMPARs recycling, we started by saturating surface expressed myc-tagged subunits with FAB fragments (**Fig. Suplm. Exp. 1 J**), allowing AMPARs to be endocytosed and recycle back to the membrane during 1h at 37°C. This time, a ratio of recycled/internalized myc-tagged receptors was done by labelling newly recycled AMPARs after fixation (**Fig. Suplm. Exp. 1 K-R**). As previously, GluA1 recycling rate was dependent on Scrib1 levels, with its up- or downregulation resulting in a substantial increase (C3,  $1.26 \pm 0.12$ ,  $n=31$ ; Scrib1,  $1.74 \pm 0.14$ ,  $n=25$ ; unpaired t-test \*,  $p < 0.05$ ) (**Fig. Suplm. Exp. 1 K, M**) or decrease (shCTR,  $1.26 \pm 0.10$ ;  $n=16$ ; shScrib1,  $0.66 \pm 0.08$ ,  $n=19$ ; unpaired t-test \*\*\*,  $p < 0.001$ ) (**Fig. Suplm. Exp. 1 L, N**) ratio of recycled/intracellular GluA1 subunits, respectively. Instead, Scrib1 presence (C3,  $2.62 \pm 0.33$ ;  $n=14$ ; Scrib1,  $3.11 \pm 0.31$ ,  $n=14$ ; unpaired t-test  $p > 0.05$ ) (**Fig. Suplm. Exp. 1 O, Q**) or absence (shCTR,  $2.37 \pm 0.22$ ;  $n=23$ ; shScrib1,  $2.68 \pm 0.23$ ,  $n=22$ ; unpaired t-test  $p > 0.05$ ) (**Fig. Suplm. Exp. 1 P, R**) had no effect on GluA2 recycling rate. Taken together, these results suggest that Scrib1 is a key player in GluA1, but not GluA2, endo- and exocytosis under basal condition



**Figure Supplementary Experiments 1: Scrib1 levels modulate endo- and exocytosis of GluA1-, but not GluA2-containing AMPARs.** (A, J) Timeline of endocytosis (A) and recycling (J) experiments. Myc-tagged GluA1 or GluA2 transfected neurons were incubated with a primary anti-myc antibody for 30min at 37°C, during which some receptors undergo endocytosis. (A) Endocytosis was evaluated by labeling surface receptors following fixation and internalized receptors after permeabilisation. (J) Recycling was assessed by saturating myc-tagged AMPARs with FAB fragments before incubating the neurons for 1h at 37°C, during which some receptors are internalized and recycled back to the membrane. Recycled receptors are labeled after fixation. (B-I) Surface and internalized or (K-R) recycled and internalized myc-GluA1 (B-E; K-N) and -GluA2 (F-I; O-R) subunits when co-transfected with pEGFP control (C3) or coupled to Scrib1 (Scrib1), shRNA scramble (shCtrl) or a specific shRNA against Scrib1 (shScrib1) in primary hippocampal neuron cultures under basal conditions. Bottom white rectangles show in higher magnification surface or recycled receptors (red), internalized receptors (blue) and dendrites (green) from the selected area. Scale bar, 15  $\mu$ m. (D, E) Ratio of surface to internalized or (M, N) recycled to internalized myc-GluA1 or (H, I; Q, R) myc-GluA2 receptor puncta. Scrib1 regulates GluA1, but not GluA2, endo- and exocytosis. \*,  $p < 0.05$ ; unpaired t test corresponding to 14-37 neurons from 4 independent experiments.





## Chapter VIII

# GENERAL DISCUSSION & PERSPECTIVES

### VIII.1 Scrib1 and neuronal complexity

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Neuronal complexity, specifically dendritic branching and morphology, consents individual neurons to accomplish specialized brain functions and cognitive behaviours. Abnormal dendritic tree arborisation and the consequent altered functional capabilities are therefore common hallmarks for many neuropsychiatric disorders, such as ASD and schizophrenia (Kulkarni & Firestein, 2012). We show that Scrib1<sup>WT</sup> overexpression increased the dendritic complexity *in vitro*, whereas Scrib1 loss (**Chapter V**) or disruption (Moreau et al., 2010; **Chapter VI**) leads to a selective increase of the apical or basal dendritic branching, respectively, depicting Scrib1 as a key player in neuronal cell polarization. In the last years, an increasing number of evidences suggest that “planar polarity” genes play a crucial role in establishing neuronal cell asymmetries (Goodrich, 2008). In the hippocampus, at least one homologue of PCP core gene is present throughout the different development stages (Tissir & Goffinet, 2006), including Scrib1 (Piguel et al., 2014). Furthermore, PCP proteins and downstream effectors have been repeatedly implicated in neurite outgrowth and remodeling (Rashid et al., 2001; Ng et al., 2002; Sin et al., 2002; Shima et al., 2004; Rosso et al., 2005; Kakimoto et al., 2006; Hayashi et al., 2007; Pinyol et al., 2007). Unpublished data from our lab show that Vangl2<sup>Lp/Lp</sup> mutants display altered growth cone and actin dynamics, possibly due to a decrease in Pak1 protein levels (Landmann, Piguel & Moncouquiol, personal communication), strengthening the importance of PCP signalling pathway in neuronal morphology. LAP family and other PDZ domain-containing proteins have been shown to affect dendritic arborisation as well (Quitsch et al., 2005; Charych et al., 2006; Vessey & Karra, 2007; Arikath et al., 2008; Rocca et al., 2008). Strikingly, Scrib1 overexpression (**Chapter VI**) and PICK1 downregulation (Rocca et al., 2008) induced an increase in projections near the cell body and proximal dendrites as well as a reduction of neurite length, favouring an antagonizing relationship as Densin-180 and Shank (Quitsch et al., 2005) or PSD-95 and cypin (Charych et al., 2006). Moreover, mutating Scrib1 actin-binding site (Scrib1<sup>KK611EE</sup>) prevented Scrib1-mediated dendritic branching complexity in a similar way as PICK1<sup>KK251EE</sup> precluded PICK1 knockdown effect (Rocca et al., 2008). Altogether, our data favours a model whereby Scrib1 and PICK1 exert opposite roles on dendritic morphology.

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### VIII.2 Scrib1 and dendritic spine morphogenesis

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Previous reports from our group highlighted Scrib1 presence within dendritic spines (Moreau et al., 2010; Piguel et al., 2014), whose formation, maturation and elimination is long known to be dependent on actin dynamics (**Chapter I.5.5.1**). During the early stages of synaptogenesis, filopodia arise from a small collection

of Arp2/3-mediated branched actin at the dendritic shaft level (Korobova & Svitkina, 2010). We found that Scrib1 plays a negative role in filopodia formation and elongation through its interaction with actin and Arp2/3 complex (Moreau et al., 2010; **Chapter VI**). Moreover, we show that Scrib1-mediated filopodia number and length reduction was abrogated in ASD-related Scrib1<sup>P592S</sup> mutated form. Interestingly, Scrib1 partner (**Chapter I.3.2**) and ASD-implicated (**Chapter I.5.7.1**) APC was recently shown to play a critical role in filopodia formation and elongation (Breitsprecher et al., 2012). Preliminary data from our lab suggests that Vangl2, another PCP protein known to interact with Scrib1 (**Chapter I.3.2**), might be involved in filopodia formation as well (Carvalho dos Santos & Montcouquiol, personal communication). Additional studies will be needed to unravel the exact molecular mechanisms underlying PCP signalling pathway in filopodia morphogenesis. Several proteins involved in actin dynamics regulation have been linked to spine formation and maturation as well (**Chapter I.5.5.1**). These include actin binding (Cortactin, Hering & Sheng, 2003; Dbr, Kobayashi et al., 2007), cross-link ( $\alpha$ -Actinin-2, Hodges et al., 2014) or capping proteins (Eps8, Menna et al., 2013; Stamatkou et al., 2013), NPFs (WAVE, Grove et al., 2004; Abi, Kim et al., 2006; Soderling et al., 2007) or the Arp2/3 complex (Mullins et al., 1998; Welch et al., 1998; Hering & Sheng, 2003; Klein, 2009). Together with a previous work from our group (Moreau et al., 2010), we found a positive correlation between Scrib1 and dendritic spine morphogenesis (**Chapter VI**). We also show that disrupting Scrib1 binding to actin and the Arp2/3 complex phenocopies ASD-mediated spine loss. Although histological examinations of ASD patients, Fragile X or FMR1 mouse models show an increase in spine density (Comery et al., 1997; Irwin et al., 2000; Hustler et al., 2007), spine loss was formerly observed in schizophrenic patients and in a mouse model of Angelman syndrome (Law et al., 2004; Kolomeets et al., 2005; Dindot et al., 2008). These data suggests that Scrib1<sup>crc/+</sup> (Moreau et al., 2010) and Scrib1<sup>P592S</sup> (**Chapter VI**) forms induce an intermediary phenotype between ASD and other ASD-RD, such as schizophrenia. Finally, Scrib1<sup>P592S</sup> spines display an aberrant large head and long neck (**Chapter VI**). The same phenotype was previously linked to an abnormal activity-dependent actin polymerization and irregular Rac activity at the PSD of Scrib1<sup>crc/+</sup> mice (Moreau et al., 2010), as well as to constitutively active forms of Rac1 and Pak1 (Hayashi et al., 2004; Tashiro & Yuste, 2004). Altogether, these results pinpoint Scrib1 as a key hub protein acting upstream the Rac1 signalling pathway, whose malfunction might underlie aberrant spine formation and maturation in the ASD etiopathology.

### VIII.3 Scrib1 and PSD functional architecture

Abnormal spine morphology in brain disorders is often linked to a mistargeting of critical proteins to the PSD and, consequently, to a failure to correctly form synapses (Kulkarni & Firestein, 2012). Our group has shown that Scrib1 loss (**Chapter V**) or disruption (Moreau et al., 2010; **Chapter VI**) results into altered PSD structure, composition and function, pinpointing Scrib1 as a key player in establishing synaptic transmission. In the past few years, the advent of super-resolution light microscopy helped to elucidate how neurotransmitter receptors, scaffold proteins and actin regulatory proteins are held within in the PSD within a 10–100 nanometer range (Dani & Huang 2010; Fukata et al., 2013; MacGillavry et al., 2013; Maglione & Sigrist, 2013; Nair et

al., 2013; Chazeau et al., 2014). PSD is currently seen as a rather dynamic structure, whose individual components are continuously repositioned and exchanged (Kuriu et al., 2006; Blanpied et al., 2008; Sturgill et al., 2009; Kerr & Blanpied, 2012). Unlike PSD-95, which accumulates in dense, subsynaptic clusters (DeGiorgis et al., 2006; Swilius et al., 2010), Scrib1 presents a homogeneous puncta-like distribution within the dendritic spine (**Chapter VI**). Moreover, Scrib1 and PSD-95 staining do not overlap, supporting the existence of a well-structured pattern of scaffold-cytoskeleton element modules inside the spine (MacGillavy et al., 2013). Nonetheless, Scrib1<sup>WT</sup> overexpression induces a PSD-95 hyperclusterisation, whereas ASD-related Scrib1<sup>P592S</sup> mutant shows a robust fragmentation of PSD-95 clusters (**Chapter VI**). Interestingly, overexpression of a constitutively active form of Rac1 induced a similar effect (Chazeau et al., 2014). Furthermore, fragmented or perforated PSDs are believed to precede the formation of newly immature spines (**Chapter I.5.5.1**), strongly suggesting that Scrib1-mediated Rac1 signalling pathway is a key player in dendritic spine remodelling. On the other hand, PSD-95 enriched domains are known to concentrate synaptic AMPARs clusters (Ehlers et al., 2007; Heine et al., 2008; Kerr & Blanpied, 2012; MacGillavy et al., 2013; Nair et al., 2013). Subsynaptical AMPAR positioning relies on AMPARs interaction with, among many others, PSD-95 via TARPs (Chen et al., 2000; Schnell et al., 2002; Bats et al., 2007). We found that Scrib1 can form a PDZ-dependent complex with AMPAR via type I TARPs (**Chapter VI**). Such interaction is lost upon Scrib1<sup>cr</sup> overexpression, suggesting that AMPARs synaptic mistargeting might underlie ASD abnormal synaptic transmission. Indeed, although Scrib1 upregulation does not change AMPAR synaptic content *per se* (Piguel et al., 2014; **Chapter VI**), the ASD Scrib1<sup>P592S</sup> mutant displays abnormally high levels of synaptic GluA1-containing AMPARs. An excessive number of AMPAR was previously reported by EM studies to be linked to larger spine heads and segmented PSDs (**Chapter I.5.5.1**), further strengthening Scrib1 role in spine development and maintenance.

#### VIII.4 Scrib1 and glutamate receptors traffic

Dendritic spines are known to contain spatially diverse subdomains of actin and actin-binding proteins (Racz & Weinberg, 2008; Frost et al., 2010; Urban et al., 2011; Willig et al., 2014). Whereas the PSD is seen as a confinement zone, bringing in close proximity receptors, regulating scaffold proteins and actin cytoskeleton; the perisynaptic area is thought to allow spine distortion during structural rearrangements as well as to position receptor traffic machinery (Blanpied et al., 2002, 2008; Kennedy et al., 2010). Besides the PSD, we also found Scrib1 in endocytic zones (EZs) (Piguel et al., 2014), suggesting that Scrib1 might play a role in glutamate receptors traffic. The majority of hippocampal dendritic spines contain the full set of endocytic machinery (Blanpied et al., 2002; Racz et al., 2004; Lu et al., 2007b). Glutamate receptors are known to be endocytosed through a dynamin-dependent pathway (Carroll et al., 1999; Luscher et al., 1999). Our Y2H screen revealed a potential link between Scrib1 and the adaptor complex AP-2 (**Table 9; Chapter III**), recently implicated in Scrib1-dependent recycling regulation in *Drosophila* (De Vreede et al., 2014). We showed that Scrib1 can stabilize GluN2A-containing NMDARs at the synapse during basal conditions, whereas it favours

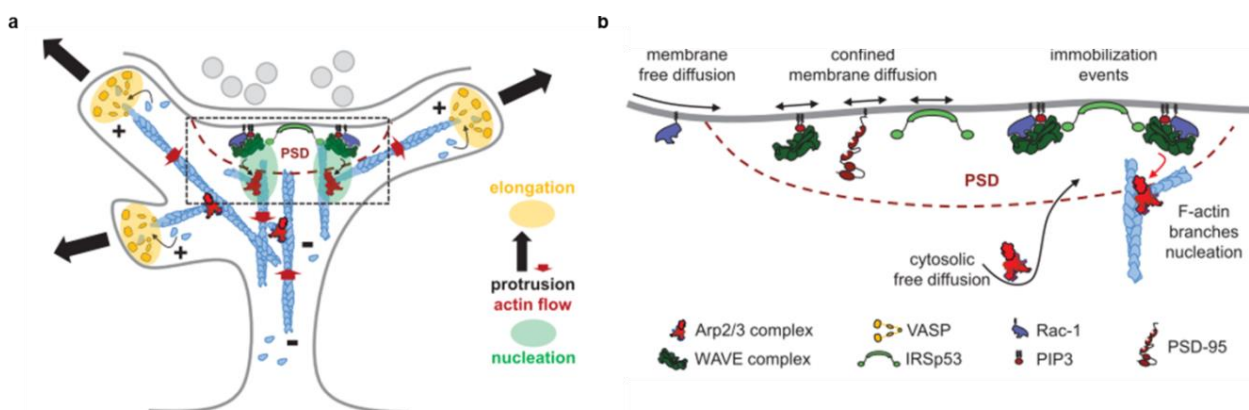
GluN2A over GluN2B recycling upon activation through a direct interaction with the AP-2 binding motif YEKL (Piguel et al., 2014). YEKL was previously shown to regulate synaptic GluN2-containing NMDARs (Scott et al., 2004; Prybylowski et al., 2005). A similar AP-2 binding motif, YHEL, is used by the PCP protein Dishevelled to engage Frizzled receptor's internalization upon Wnt activation as well (Yu et al., 2007), favouring the involvement of canonical apico-basal polarity genes in the regulation of endocytic traffic (Shivas et al., 2010; Dukes et al., 2011; Zeigerer et al., 2012; De Vreede et al., 2014).

Instead, Scrib1 involvement in AMPAR traffic is less clear. We show that Scrib1 is able to form a complex with AMPARs via type I TARPs (**Chapter VI**). Type I TARPs possess a conserved Cter class I PDZbd able to bind several PDZ domain-containing proteins present in the PSD (**Chapter I.6**). Even though all these proteins are able to regulate AMPAR traffic (Kim & Sheng, 2004; Elias & Nicoll, 2007), they are undetectable in native AMPAR complexes (Vandenberghe et al., 2005a), suggesting a less stable and/or more transitory interaction with AMPARs than the bona fide AMPAR auxiliary TARP subunits. Our preliminary data shows that Scrib1 levels specifically affect GluA1, but not GluA2 endo- and exocytosis under basal conditions (**Chapter VII**). Instead, Scrib1 upregulation has no effect on AMPAR total or synaptic number (Piguel et al., 2014; **Chapter VI**), suggesting that Scrib1 might regulate AMPAR subunit-specific endocytic traffic in a similar way as it regulates NMDAR (Piguel et al., 2014). On the other hand, mutating Scrib1 actin- or W386-mediated Arp2/3-binding sites significantly decreases the synaptic targeting of GluA1- or GluA2-containing AMPARs, respectively (**Chapter VI**). These data suggest that Scrib1-mediated actin dynamics regulation might participate in the synaptic insertion of subunit-specific AMPARs. Actin cytoskeleton was previously reported to promote glutamate receptors positioning without directly anchoring them at the plasma membrane (Kerr & Blanpied, 2012). Consistent with a model whereby PSD is a highly dynamic matrix, yet composed by a preserved topology (Blanpied et al., 2008), our data suggests that Scrib1 links structural to functional plasticity by bridging the AMPAR/TARP complex signalling to the downstream actin cytoskeleton. Additional studies are needed, however, to unravel at what level of the secretory pathway does Scrib1 and/or TARP bind GluA1 or GluA2 subunits. Is the complex formation dependent on synaptic activity and in particular on NMDAR activation and/or Scrib1-mediated AMPAR Cter post-translational modifications? Where are AMPAR inserted and/or removed; directly at the PSD or rather at the EZ, being laterally diffused to the PSD after? Are the different AMPAR subunits differently inserted, stabilized and/or removed at/from the synapse? To what extent do other Scrib1 Y2H screen candidates, involved in protein traffic or receptor binding (**Chapter III**), affect ionotropic glutamate receptors traffic as well?

### VIII.5 Scrib1 and synaptic plasticity

Synaptopathologies associated to a failure to correctly form synapses are further characterized by an inability to structurally respond to synaptic activity (Kulkarni & Firestein, 2012). As expected, reduced basal transmission in both Scrib1<sup>crc/+</sup> (Moreau et al., 2010) and CaMK-Scrib1<sup>-/-</sup> (**Chapter V**) mice models was further linked to a failure to properly express synaptic plasticity, most likely due to altered levels of CaMKII and PP1/

PP2A. LTP and LTD signalling pathway is well-known to induce spine head enlargement and shrinkage, respectively (Matsuzaki et al., 2004; Okamoto et al., 2004; Zhou et al., 2004). The densely packed PSD architecture (Sheng & Hoogenraad, 2007) is believed to act as a physical barrier forcing F-actin barbed ends to grow away. Accordingly, a recent study proposed PSD as a convergent zone where proteins involved in branching F-actin meet with a high probability, whereas F-actin elongation occurs at the tips of transient membrane protrusions away from the PSD (Chazeau et al., 2014) (**Fig. 29**). Indeed, membrane juxtaposed to the PSD can retain WAVE complex elements and IRSp53, which directly or indirectly interact with PSD components, such as PSD-95, Shank and CaMKII (Hering & Sheng, 2003; Bockmann et al., 2002; Choi et al., 2005; Proepper et al., 2007; Park et al., 2012). Arp2/3 complex, on the other hand, is immobilized in the PSD domain upon Rac1 activation, representing the final step of a series of well synchronized events occurring at the membrane and involving prenylated Rac1-GTP, PIP3, the WAVE complex and IRSp53 (Miki et al., 2000; Lebensohn & Kirschner, 2009; Chen et al., 2010b; Chazeau et al., 2014). We therefore favour a model whereby Scrib1 ensures bidirectional plasticity expression by localizing the appropriate intracellular signalling in the immediate vicinity of NMDARs, entailing the correspondent spine morphology changes by coordinating a transient nanoscale re-localization of branched F-actin regulators (**Fig. 29 a**). Following enhanced synaptic activity, NMDAR activation triggers CaMKII, releasing WAVE elements that are now available to activate Arp2/3 (Park et al., 2012). On the other hand, Scrib1 can activate Rac1 through the  $\beta$ PIX/GIT complex (Moreau et al., 2010), potentially allowing Arp2/3 complex immobilization and consequent WAVE- and IRSp53-mediated activation in the PSD (**Fig. 29 b**). Long lasting Rac1 enhancement can then delocalize the WAVE complex from the PSD, allowing spine enlargement. It is tempting to speculate that at low levels of glutamate, PP1/PP2A signalling is preferentially activated, probably consenting WAVE sequestration by CaMKII. Inactive CaMKII bundles and stabilizes F-actin, precluding F-actin remodelling (Okamoto et al., 2007b; Lin & Redmond, 2008). Concomitantly, low levels of activated Rac1 and/or high levels of RhoA might allow cofilin



**Figure 29: Scrib1 links structural to functional plasticity.** (a) The PSD is seen as a confinement zone, bringing in close proximity receptors, regulating scaffold proteins and actin cytoskeleton elements, which together promote F-actin nucleation. Instead, the perisynaptic area endorses spine distortion during structural rearrangements, by allowing F-actin elongation at the tip of the membrane protrusions. (b) Inside the PSD, a series of events are needed to properly activate F-actin nucleation, including the recruitment and sequential activation of Rac1 and Arp2/3 complex. Unlike PSD-95, Scrib1 presents a more homogenous distribution inside the dendritic spine, having a closer distribution to VASP or Arp2/3. Moreover, Scrib1 is known to activate Rac1 via the  $\beta$ PIX/GIT1 complex. We therefore hypothesize that Scrib1 is freely diffusing in the cytosol as with Arp2/3, being recruited to the PSD upon synaptic activation. (a, b) taken from Chazeau et al., 2014.

activation through LIM-K (Luo, 2000), promoting F-actin actin severing and depolymerisation activation. Additional studies need to be done to understand whether Scrib1 is freely diffuse in the cytosol together with Arp2/3 complex and/or if both are similarly recruited to the PSD upon synaptic plasticity events.

## VIII.6 Scrib1 and brain function

In the last years, our group has outlined the consequences of Scrib1 mutation or loss in the context of brain function (**Table 13**). Both Scrib1<sup>crc/+</sup> (Moreau et al., 2010) and CaMK-Scrib1<sup>-/-</sup> (**Chapter V**) mouse models share an altered long-term spatial learning and memory, strongly suggesting that Scrib1 plays an important role in spatial memory formation. The early stabilization of long-term spatial memory is known to depend on NMDAR activity and downstream effectors (McDonald et al., 2005); whereas the consolidation phase requires a coordination of early PP1/PP2A activity and CaMKII-mediated gene transcription (Bennett et al., 2001; Von Herten & Giese, 2005; Irvine et al., 2006). Interestingly, block of AMPAR endocytosis or pharmacological inhibition of PP1/PP2A was shown to hinder LTD expression as well to impair spatial memory (Ge et al., 2010; He et al., 2001), both prone to be rescued by enriched environment (EE) exposure (van Praag et al., 2000; Foster & Dumas, 2001; Chancey et al., 2013). We showed that EE can induce higher Scrib1 levels and alter NMDAR subunit expression (Piguel et al., 2014), as well as to rescue CaMK-Scrib1<sup>-/-</sup>-mediated LTD impairment and memory formation (**Chapter V**). Taken altogether, our findings stress the importance of PCP signalling pathway in normal cognitive function. Previous work in our lab showed that Scrib1<sup>crc/+</sup> mice display ASD-like behaviour, featuring enhanced learning and memory but an impaired social behaviour (Moreau et al., 2010). These findings were further corroborated by two recent genetic studies implicating Scrib1 in human ASD (Pinto et al., 2010; Neale et al., 2012). Several PDZ-dependent protein-protein interactions (**Chapter I.5.7.1**) and PCP signalling pathway proteins (**Chapter I.5.7.2**) were previously implicated in ASD and ASD-RD, strongly favouring an overlap in the genetic etiopathology of several neurological disorders. For instance, disruption of the PCP protein Pk2 leads to autism-like behaviours and hippocampal synaptic dys-

**Table 13:** Mice models used by our lab to dissect Scrib1 role in brain development and function from the cellular to the cognitive level.

	Scrib1 <sup>crc/+</sup> (Moreau et al., 2010;* Chapter VI)	CaMK-Scrib1 <sup>-/-</sup> (Chapter V)
<b>Mouse model</b>	50% full-length vs. truncated Scrib1 lacking PDZ3-4; No temporal or local specificity;	Full excision at 5 weeks old; Specific for major neurons of the postnatal forebrain;
<b>Level</b>		
<b>Cognitive</b>	Enhanced learning and memory; Impaired social behaviour.	Slower and inflexible spatial learning; Impaired long-term consolidation.
<b>Functional</b>	↓ basal synaptic transmission; Impaired LTP;	↓ basal synaptic transmission; Enhanced LTP and impaired LTD;
<b>Cellular</b>	↑ synaptic pruning (larger PSDs); ↓ synapse density and number; Altered basal neuronal morphology;	↑ small immature PSDs; = synapse density and number; Altered apical neuronal morphology;
<b>Molecular</b>	↓ βPIX, GIT, CaMKII levels at the PSD; ↑ Rac1-GTP activity; Impaired activity-dependent actin polymerization; Loss of co-localization with the TARP/AMPA complex*.	↓ PP2A and PP1 levels.

function (Sowers et al., 2013). Instead, abnormal behaviours often considered as phenotypes of ASD and schizophrenia were similarly found in mice lacking Densin-180, another LAP member (Carlisle et al., 2011). Strikingly, small-molecule inhibitor of Pak1, a downstream effector of Scrib1, was recently reported to ameliorate schizophrenia-associated dendritic spine deterioration (Hayashi-Takagi et al., 2014). ASD and schizophrenia share indeed common features, such as impaired social cognition and failure to communicate, key higher-order functions dependent on the human neocortex. Miswiring of neocortical circuits often lead to anomalous inter-hemispheric connections (reviewed by Kwan, 2013; Chance, 2014) and consequently to changes in the corpus callosum area, known to harbour millions of contralateral axonal projections (Brambilla et al., 2003; Hrdlicka, 2008). Recent work in our lab showed that Scrib1 loss leads not only to a mistargeting of key determinants of neocortical circuit assembly, but also to corpus callosum agenesis (Ezan et al., in preparation). Other PCP proteins, such as Fz3 and Celsr3, show similar cortical disorganization and axon guidance defects (Tissir et al., 2005; Wang et al., 2006b). In addition, Cdk5, a neuron-specific Rac1 effector found in our Y2H screen (**Table 9; Chapter III**) was also linked to neuronal migration and the laminar configuration of the cerebral cortex (Nikolic et al., 1998), stressing PCP pathway role in brain development. Noteworthy, altered physiological traffic and localization of NMDARs represent a common event in several CNS disorders, like Parkinson's disease or neuropathic pain (reviewed by Mellone & Gardoni, 2013), whereas down regulation of protein phosphatases are often associated with cognitive decline and dementia (Tian et al., 2002). PP2A, for instance, seems to play a crucial role in the onset of Alzheimer's disease (reviewed by Braithwaite et al., 2012). A key residue of PP2A catalytic subunit (Ppp2ca) Cter PDZbd (Tyr307) binding to Scrib1 PDZ4 (**Chapter V**) was recently linked to the abnormal hyper-phosphorylation of tau in brains of patients with Parkinsonism-dementia of Guam, a neurodegenerative disease with Parkinsonism and early-onset Alzheimer-like dementia (Arif et al., 2014). Since Scrib1 binds both NMDAR subunits (Piguel et al., 2014) and Ppp2ca (**Chapter V**), it will be interesting to investigate if Scrib1 could be involved in other neuropathological diseases, like Parkinson or Alzheimer's disease.

## VIII.7 The importance of targeting Scrib1 PDZome: making protein-protein interactions druggable

### VIII.7.1 Scrib1 PDZ domains: tell me who you go with and I'll tell you who you are

In the first part of this study, we identify specific ligands for Scrib1 PDZ3 and PDZ4 domains (**Chapter III**). PDZ domains are often found together with other functional domains, like the SH3 and GuK domains in MAGUKs, or LRR in the case of the LAP family members. Extensive data from epithelial cells long suggested a common functional pathway shared by MAGUK and LAP members, both crucial in the establishment of cellular architecture (**Chapter I.4**) and gatekeeping malignancy (**Chapter I.3.3**). *Scrib* itself was originally found alongside with *Dlg* to be a neoplastic tumour-suppressor gene in *Drosophila* (Bilder et al., 2000). In mammalian cells, several studies have been suggesting the existence of a common signalling pathway shared by MAGUK and LAP members. For instance, Erbin can bind PSD-95 and Stgz (Huang et al., 2001b;

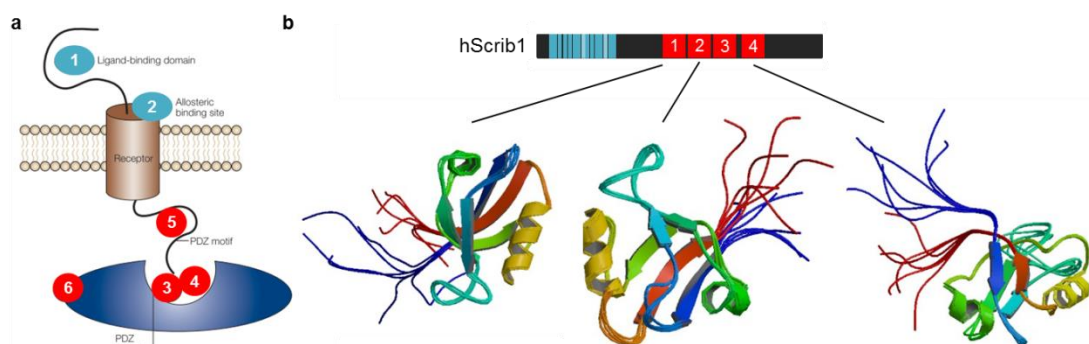


Tao et al., 2013). As PSD-95, the MAGUK protein *par excellence*, Scrib1 binds to NMDAR (Piguel et al., 2014) and AMPAR through type I TARPs like Stgz as well (**Chapter VI**). In addition, both PSD-95 and Scrib1 are known to be involved in the nNOS signalling pathway (Brenman et al., 1996; Richier et al., 2010). Finally, PSD-95 and Densin-180 can form a complex with CaMKII in dendrites, thus regulating the signalling transduction pathway downstream NMDARs (Gardoni et al., 2006; Walikonis et al., 2001). Strikingly, disruption of MAGUK- or LAP-mediated cell signalling has been repeatedly implicated in ASD and ASD-RD like mental retardation or schizophrenia (Tarpey et al., 2004; Hayashi et al., 2007; Barrow et al., 2009; Delorme et al., 2010; Moreau et al., 2010; Pinto et al 2010; Carlisle et al., 2011; Gilman, et al., 2011; Kantojärvi et al., 2011; Neale et al., 2012; Tsai et al., 2012b; Cao et al., 2013; Hayashi-Takagi et al., 2014), strongly favouring an evolutive link between the two families in establishing brain development and function. On the other hand, a recent hypothesis favours a scenario whereby intramolecular combination or cooperation of PDZ domains can function synergically (Grootjans et al., 2000; Raghuram et al., 2001; Zhang et al., 2001). Indeed, 18% of the human PDZ-domain containing proteins possess three or more tandem PDZ domains. Unpublished data from our group concerning isothermal titration calorimetry experiments between Scrib1 and some of its Y2H potential interactors show a higher affinity of most the ligands towards a tandem PDZ34 when compared to the isolated PDZ domains (Pinheiro, Blanc & Sans). The strong phenotype induced by the Scrib1<sup>crc</sup> mutation in mice (Murdoch et al., 2003; Moreau et al., 2010) or the loss of Scrib1/TARP/AMPA interaction in COS-7 when Scrib1<sup>crc</sup> form is overexpressed (**Chapter VI**) pinpoints the importance of Scrib1 tandem PDZ3-4 domains.

### VIII.7.2 Making Scrib1 interactions druggable: a potential solution for NTD and ASD?

The modulation of protein-protein interactions (PPIs) involved in disease pathways has become an attractive strategy for developing drugs in the last decades. Hitting more than one disease-related pathway simultaneously could be proven more efficacious simply by targeting a single type of protein interaction, rather than a single type of protein. PDZ domains, engaged in numerous PPIs with so many and distinct proteins in such a variety of cellular contexts, constitute, therefore, auspicious targets for developing novel treatments. In addition, disruption of PDZ-dependent interactions is a common strategy employed by viruses to enhance their viral activity (Javier & Rice, 2011), indicating that this strategy is biologically feasible. The present work has highlighted Scrib1 role in several biological contexts, from epithelial cell polarity lost in the tumorigenesis context to its function in the proper brain development and maturation. Modulating Scrib1 PDZ domain-mediated interactions can therefore constitute a novel window of therapeutical treatment of PCP defects, including tumorigenesis, NTD, ASD and ASD-related diseases, such as mental retardation or schizophrenia. Nonetheless, this promising strategy is based on the well-defined nature of PDZ domain binding sites (reviewed by Grillo-Bosch et al., 2013) (**Fig. 30 a**). So far, only three out of four Scrib1 PDZ domain structures are known (Protein Data Bank, RCSB), either by solution NMR (hScrib1 PDZ1, 1X5Q; hScrib1 PDZ2, 1WHA; hScrib1 PDZ4 1UJU) or x-ray diffraction (hScrib1 PDZ1, 2W4F) (**Fig. 30 b**). High-throughput screens like our Y2H are therefore helpful in establishing Scrib1 specific PDZ-dependent interactions, possibly contributing to the rational design development of Scrib1 PDZ-mediated interaction inhibitors. Even so, much work concern-

ing known PDZ-dependent Scrib1 interactions highlights the potential therapeutic use of such approach. For example, the use of TAT-coupled sequences successfully occluded the effect of PDZ-dependent GluN2 interactions on NMDAR traffic (Gardoni et al., 2009; Bard et al., 2010) and spine morphology regulation (Vastagh et al., 2012). On the other hand, competing peptide-based ligands able to prevent NMDAR-dependent neurotoxicity without blocking the receptor activity (Aarts et al., 2002) were used in primates as a potential stroke treatment (Cook et al., 2012) (**Fig. 30 b5**). Likewise, tumorigenesis-mediated Wnt signalling pathway was efficiently inhibited by a peptide-based ligand approach targeting Dvl2 PDZ domain (Zhang et al., 2009b), highlighting the potential therapeutic range of disrupting PDZ-mediated interactions. Finally, small, potent plasma-stable peptidomimetic compounds like N-cyclohe-xylethyl-ETA(S)V were shown to specifically disrupt the NMDAR/PSD-95 interaction (Bach et al., 2011) (**Fig. 30 b6**), signposting the possibility of further and more clinically suitable PDZ domain mediated-interactions inhibitors.



**Figure 30: Potential modulation of Scrib1 PDZ domain interactions.** (a) Example of how to modulate a PDZ-mediated receptor activity. Besides the traditional approaches, such as the extracellular binding of an agonist or antagonist to the ligand binding site (1) or an allosteric site of the receptor (2), intracellular inhibition of PDZ domain-mediated interactions might be proven efficient. Blocking peptides (3) or drugs (4) are able to inhibit the receptor's PDZbd. Cter peptide-based ligands (5) can mask and/or alter the PDZbd conformation. Natural or synthetic molecules that allosterically bind the PDZ domain (6) can alter its conformation and interaction properties. (b) Solution NMR structures of Scrib1 PDZ1, PDZ2, and PDZ4 domains. Protein Data Bank numbers (RCSB database): 1X5Q, 1WHA, and 1UJU, respectively. (a) adapted from Dev et al., 2004.



## Chapter IX

### CONCLUDING REMARKS

In this study, we depicted Scrib1 role in hippocampal morphology and function. We found that Scrib1 is a key player in establishing neuronal complexity as well as in dendritic spine development and maintenance. We further strengthen Scrib1 role in PSD architecture and function, emphasizing the importance of Scrib1-mediated interactions and signalling in synaptic plasticity. Finally, our Scrib1 PDZome study opens up thrilling new potential roles for Scrib1 in other brain functions and disorders. Ultimately, our work reinforces the upcoming idea that studying the molecular mechanisms underlying cell polarity establishment and maintenance in the mammalian brain context is a powerful tool to better understand and develop therapies for brain disorders.



# Chapter X

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