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Mécanismes moléculaires du recyclage des vésicules synaptiques : rôle de la Phospholipid Scramblase-1 (PLSCR1)

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“The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science”

Albert Einstein (1879-1955)

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Abbreviations

5-HT (5-hydroxytryptamine)

AAK1 (A2-associated protein kinase 1)

ABC (ATP binding cassette)

ABDE (Activity-dependent bulk endocytosis)

ADBE (Activity-dependent bulk endocytosis)

Ads (Appendage domains)

AP (Action potential)

AP180 (Assemble/adaptor protein 180)

AP2 (Adaptor protein 2)

AZ (Active zone)

BAR (Bin/amphiphysin/Rvs)

CaCC (Ca^{2+}-activated ion channels)

CaPLases (Ca^{2+}-activated phospholipid scramblases)

CBD (Calcium-binding domain)

CCPs (Clathrin-coated pits)

CME (Clathrin-mediated endocytosis)

CME (Clathrin-mediated endocytosis)

DRG (Dorsal root ganglion)

DRMs (Detergent resistant membrane fractions)

DSMs (Detergent soluble membrane fractions)

EAPs (Endocytic accessory proteins)

EM (Electron microscopy)

EMCV (Encephalomyocarditis virus)

EPL (External plexiform layer)

EPSCs (Excitatory post synaptic currents)

ER (Endoplasmic reticulum)

fMLP (Formylated Met-Leu-Phe)

FVF (Full vesicle fusion)

GABA (γ-aminobutyric acid)

GCL (Granular cell layer)

GFAP (Glial fibrillary acidic protein)

GL (Glomerular layer)

Gla (γ-carboxyglutamic acid)

GPI (Glycosylphosphatidylinositol)

GPL (Glycerophospholipds)

GPMVs (Giant plasma membrane vesicles)

GrC (granular cell)

GSK3 (Glycogen synthase kinase 3)

GUVs (Giant unilamellar vesicles)

HSV (herpes simplex virus)

IFN (Interferon)

IP (Intermediate pool)

IP_3 (Inositol 1,4,5 phosphate)

iSCAT (Interferomic scattering microscopy)

KD (Knock-down)

KNR (Kiss and run)

Ld (Disordered liquid-phase)

Lo (Ordered liquid-phase)

MAMs (Mitochondria associated membranes)

MAP2 (Microtubule associated proteins 2)

MCL (Mantle cell lymphoma)

MCL (Mitral cell layer)

MCSs (Membrane contact sites)

MLCK (Myosin light chain kinase)

MP (Microparticles)

N-WASP (Neural Wiskott-Aldrich syndrome protein)

NBD (Nucleotide-binding domain)

NCD (N-terminal cytosolic domain)

NLS (Nuclear localization signal)

OB (Olfactory bulb)

p (Probability of vesicular release)

PA (Phosphatidic acid)

PALM (Photoactivated localization microscopy)

PC (Phosphatidylcholine)

PE (Phosphatidylethanolamine)

PG (Phosphatidylglycerol)

PI (Phosphatidylinositol)

PI3KC2a (Phosphatidylinositol 3-kinase C2a)

PIP (Phosphoinositides)

PIP_2 (Phosphatidylinositol 4,5-bisphosphate)

PLD1 (Phospholipase D1)

PLSCR1 (Phospholipid scamblase 1)

PM (Plasma membrane)

PPR (Paired-pulse ratio)

PRD (Prolin rich region)

PS (Phosphatidylserine)

RP (Reserve pool)

RRP (Ready-releasable pool)

SCR-1 (Scramblase 1)

SLBs (Supported lipid bilayers)

SLPI (Secretory leucocyte protease inhibitor)

SM (Sphingomyelin)

SNAREs (Soluble N-etylmale-imide-sensitive factor attachment protein receptors)

SNX9 (Sorting nexin 9)

STED (Stimulated optical depletion)

SV (Synaptic vesicles)

Syp-pH (Synaptophysin-pHluorin)

Syt (Synaptotagmin)

TLR9 (toll-like receptor 9)

TM (Transmembrane segments)

TMD (Transmembrane domain)

TMEM16 (Transmembrame protein 16)

TNF (Tumor necrosis factor)

TREM2 (Triggering receptor expressed on myeloid cells 2)

UFE (Ultra-fast endocytosis)

VAMP (Vesicle-associated membrane protein)

VGCC (Voltage gated Ca $^{2+}$ - channels)

VGLUT1 (Vesicular glutamate transporter)

VSV (Vesicular stomatitis virus)

WT (Wild type)

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Avant-propos

Les neurotransmetteurs contenus dans les vésicules présynaptiques sont libérés par exocytose dans la fente synaptique suite à la fusion des vésicules avec la membrane plasmique. La capacité des neurones à recycler les composants vésiculaires pour restaurer le stock de vésicules (endocytose compensatrice) permet de maintenir l'activité synaptique et assure la fidélité du transfert d'information entre les neurones. Bien que ces défauts de couplage exocytose endocytose aient été longtemps sous-estimés, ils apparaissent aujourd'hui de plus en plus souvent associés à des pathologies incluant l'épilepsie, l'autisme ou le retard mental. L'une des propriétés de la membrane plasmique est d'être asymétrique avec les aminophospholipides (AP) de type phosphatidylserine (PS) et phosphatidyletanolamine (PE) enrichis sur le feuillet interne et les phosphatidylcholine (PC) et sphingomyélines sur le feuillet externe. Bien que les processus de fusion et de scission vésiculaire impliquent des remaniements des lipides membranaires, les études se sont essentiellement focalisées sur les protéines assurant la fusion vésiculaire et l'endocytose compensatrice incluant les protéines de la famille SNARE et la clathrine par example.

Dans ce contexte, l'équipe a développé, il y a quelques années, un projet de recherche qui s'intéresse au rôle de la PhosphoLipid ScRamblase-1 (PLSCR1), une protéine membranaire qui mélange les lipides en présence de Ca^{2+} durant l'exocytose régulée. Forte de son expertise dans les mécanismes de la sécrétion neuroendocrine et da sa connaissance de la cellule chromaffine, l'équipe a choisi d'utiliser cette dernière comme modèle expérimental. Les travaux antérieurs de l'équipe, dans les cellules chromaffines, ont montré que l'exocytose des granules de sécrétion s'accompagne d'une perte d'asymétrie membranaire transitoire qui dépend de la PLSCR1. La PLSCR1 n'étant pas requise pour l'exocytose mais pour l'endocytose compensatrice, cela indique que la perte d'asymétrie observée au moment de l'exocytose pourrait constituer un signal d'endocytose. Ce mécanisme n'a cependant jamais été décrit dans les neurones et pourrait constituer un nouveau mode de régulation de la transmission synaptique.

Depuis ma licence j'ai été très passionnée pour les neurosciences avec un intérêt particulier pour les mécanismes à la base de la neurotransmission. J'ai suivi un cursus d'études basé sur la neurophysiologie, au cours duquel j'ai eu l'opportunité d'effectuer deux stages, respectivement de 6 et 3 mois dans deux laboratoires de neurosciences différents de l'Université de Naples. Ainsi ce

projet sur la PLSCR1 dans le couplage exocytose-endocytose dans les neurones répondait parfaitement à mes attentes et m'a toute de suite plu. J'ai intégré l'équipe des Drs Gasman/ Vitale à l'Institut des Neurosciences Cellulaires et Intégratives de Strasbourg pour mon stage de M2 en Erasmus afin d'étudier la fonction de la PLSCR1 dans les neurones. L'intérêt pour ce projet m'a fait postuler et obtenir une bourse ministérielle qui m'a permis de commencer une thèse dans le même laboratoire l'année suivante. Les résultats préliminaires ont été obtenus assez rapidement au début de ma thèse mais j'ai été toutefois confrontée à des limitations techniques (imagerie, transfections, méthodes de stimulation, perfusion) que j'ai voulu lever en effectuant un séjour d'un mois au « Centre for discovery brain sciences » chez le Dr Cousin à Edimburgh, UK, qui utilise ces méthodes en routine. Après avoir maitrisé des techniques clés pour l'avancement de mon projet, je les ai ainsi mises en place au laboratoire et j'ai optimisé les protocoles d'analyse. Dans l'ensemble, ces techniques m'ont permis d'évaluer le rôle de la PLSCR1 dans l'endocytose compensatrice des neurones de cervelet suite à une stimulation à haute fréquence et aussi de déterminer le domaine minimal de la protéine PLSCR1 supportant cette fonction.

La première partie de mon manuscrit consiste en une introduction générale dans laquelle je présente la distribution asymétrique des phospholipides dans la membrane plasmique ainsi que les principaux transporteurs de lipides qui la régulent, avec une attention particulière à ma protéine d'intérêt, la PLSCR1. Par la suite, je m'intéresse au rôle de la dissipation de l'asymétrie membranaire, irréversible et non, dans divers processus biologiques tels que l'apoptose, la coagulation et l'endocytose compensatrice dans les cellules chromaffines. J'enchaine avec une description détaillée de l'exocytose et de l'endocytose dans la terminaison synaptique en décrivant les différents types que l'on retrouve dans les neurones. Je termine en pointant l'intérêt fonctionnel du couplage exo-endocytose qui impliquent diffèrent facteurs, dont les micro-domaines lipidiques, les protéines SNARE, le calcium, les protéines de la zone active ainsi que le cytosquelette.

La deuxième partie de mon manuscrit présente les résultats obtenus au cours de ma thèse. Tout d'abord, je présent les résultats préliminaires sur le rôle de la PLSCR1, obtenus en utilisant les cultures neuronales faites à partir du bulbe olfactif de souris. Ensuite je présente les limitations techniques que j'ai rencontrées et qui m'ont incité à améliorer les protocoles expérimentaux.

Les résultats principaux ont été obtenus sur les cellules en grain de cervelet et ils sont présentés sous la forme d'un article scientifique en préparation. La troisième partie consiste en une discussion générale qui inclut des résultats complémentaires. Enfin, dans le dernier chapitre de cette thèse je décris les matériels et méthodes utilisés pour les expériences qui ne sont pas

mentionnés dans l'article. Le Français n’étant pas ma langue maternelle, j’ai préféré écrire ce manuscrit en anglais.

Je vous souhaite une agréable lecture,

A. Introduction

1. Lipid-membranes

Lipid membranes constitute the cell boundaries, and the boundaries of organelles within the cells. They are formed by an oriented lipid bilayer, a very complex and highly dynamic structure containing thousands of different phospholipids, with a variety of saturation and length of acyl chains, membrane proteins and sugars. First, membrane represents a physical barrier that separates two different environments to guarantee the maintenance of cellular homeostasis. Beside its role in compartmentalization, the lipid membrane carries out specific functions. Despite lipids maintain the membrane structure, they also play many functional roles in various cellular functions, notably in the membrane curvature formation, in the recruitment/anchoring of many proteins and further, their metabolism is involved in the cellular energy production, cell mobility and membrane trafficking.

1.1 Structure and dynamics of lipid membranes: an overview

In 1972, Singer and Nicholson proposed the *"fluid mosaic model"* to describe the dynamic and fluid structure of the plasma membrane (PM), substituting the earlier "protein-sandwich" model of Davson and Danielli (1935) in which the lipid layer was sandwiched between two protein layers (Fig.1A). The model is based on the thermodynamic factors in which non-covalent interactions are the major driving forces in the formation of lipid bilayers. Lipids spontaneously arrange so that the hydrophobic "tail" are isolated from the surrounding water while the hydrophilic "head" faces the intracellular (inner leaflet) and extracellular faces (outer leaflet) of the resulting bilayer (Singer & Nicolson, 1972). Transmembrane proteins, integral or not, are placed inside the lipid bilayer and expose their charged residues towards aqueous medium (Fig.1B). Thanks to its structure, cell membrane is impermeable to ions and polar molecules and the arrangement of the lipid bilayer prevents polar solutes (eg. amino acids, nucleic acids, carbohydrates, proteins, and ions) from diffusing across the membrane. This affords the cell the ability to control the movement of these substances through transmembrane protein complexes such as pores, channels, and gates.

According to the Singer and Nicolson's model, the membrane is a uniform fluid lipid mosaic in which both lipids and proteins are in constant motion. Indeed, in physiological conditions, *Rotation* motions occurs around the lipid/protein axis perpendicular to the plane of the membrane while *Translational* (or lateral) diffusion occurs within the plane of the bilayer. This model emphasizes the membrane fluidity and the ability of the lipids and proteins to diffuse across it; however, it does not consider any presence of membrane sub-compartmentalization.

Diverse data have been collected regarding the structure and dynamics of membranes and the current view highlight the importance and roles of specialized membrane domains, such as "lipid rafts" and protein/glycoprotein complexes, which provide specific functional properties.

In 1973, a complete analysis of PM lipid composition has been reported for intestinal cells. Glycosphingolipids were enriched while the phosphatidylcholine was 2-3-fold depleted in the exoplasmic leaflet of apical membrane compared to the basolateral one. In addition the amount of phosphatidylethanolamine, as a percentage of total lipid, was quite equal in the two domains (Forstner & Wherrett, 1973) indicating that specific lipids may be selectively enriched in membrane. Apical enrichment of the sphingophospholipid and sphingomyelin was also observed in other cell types e.g. liver, and renal cells (Molitoris & Simon, 1985) (Meier et al., 1984). Later, study of epithelial cells revealed that they were morphologically and functionally polarized and with heterogeneity in their membrane lipid composition. This heterogeneity resulted from selective lipid delivery, generated by lipid-sorting in the trans-Golgi network, to their apical or basolateral sides. The apical side was enriched in sphingolipids and sphingomyelin and the basolateral one in the phosphatidylcholine (van Meer & Simons, 1986, 1988). This new view of membrane structure led to develop a new model of PM organization, called the "lipid rafts model" (Simons & Ikonen, 1997) (Fig. 1C). Based on this model, sphingolipids are laterally associated with one another and any voids between them are filled by cholesterol molecules which behave as spacers; these sphingolipid - cholesterol clusters, restricted within the exoplasmic leaflet, have been identified because of their insolubility in the detergent Triton X-100 at 4°C, in which they make glycolipid-enriched complexes (Brown & Rose, 1992). The so-called lipid rafts act as platforms for the attachment of proteins, such as glycosylphosphatidylinositol (GPI)-anchored proteins, transmembrane proteins (Sargiacomo et al., 1993) and doubly acylated tyrosine kinases of Src family (Casey, 1995), and are involved in some biological events like signal transduction and membrane trafficking pathways. This novel model adds new layers of complexity and hierarchy, but the concepts described in the Singer and Nicolson's model are still applicable today. In these updated versions, more emphasis has been focused on the mosaic nature of the

macrostructure of cellular membranes, where many proteins and lipids are limited in their rotational and lateral motions in the membrane plane.

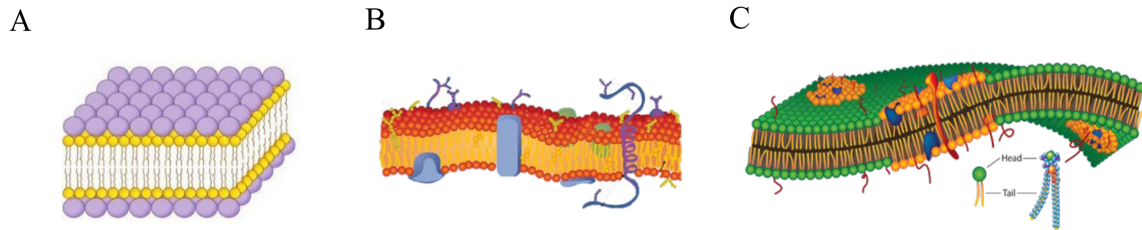

Fig.1 Models of biological membrane structure. **(A) Sandwich model of cell membrane.** The picture shows an old view of membrane structure with a phospholipid bilayer that lies between two layers of globular proteins. **(B) The fluid mosaic membrane model**. The picture represents a section view of a cell membrane with integral and peripheral membrane proteins randomly distributed in the plane of a completely fluid bilayer. The membrane does not show membrane domains of different compositions. **(C) The lipid raft model.** The picture represents a modern view of membrane structure which exhibits lateral heterogeneities with membrane micro-domains (in yellow), within the membrane plane. Adapted *From Oriana De Vos et al., 2018* (Center for molecular modelling)

1.2 Lipid composition of cellular membranes

In physiological concentration, the ability of lipid to assemble in different structures (lamellar, micellar, and cubic) is defined as “lipid polymorphism” and depends on the volume of their polar head groups and fatty acyl chains (Frolov et al., 2011). However, since it is energetically more favorable, the biological membranes are mostly lamellar. Cellular membranes are formed by diverse sets of lipids and proteins, present in different amounts and proportions. Despite the strong protein crowding, the lipid bilayer remains the “core” of bio-membranes, given that the area occupied by proteins in membranes does not exceed 20% (Dupuy & Engelman, 2008). The high lipid diversity is common to all eukaryotic cells and their requirement in the barrier formation is not enough to explain the innumerable degrees of diversity of lipid structure. A possible reason for this lipid complexity is to guarantee a robust apparatus even in presence of local environmental changes, such as pH and osmolarity. The most abundant lipids in cellular membrane can be grouped in three classes: the glycerophospholips, sphingolipids, and sterols (mainly cholesterol in mammals) (Harayama & Riezman, 2018). I will briefly review the major biophysical and biochemical properties of these membrane lipids.

- Sterols

Sterols represent the main non-polar lipids of the PM, which do not participate directly in the structure of lipid bilayer. The most abundant sterol in mammalian membrane is the cholesterol (Fig. 2), which accounts for 30 % of the total lipid content. It can insert perpendicularly into the hydrophobic core of the bilayer and can regulate membrane fluidity by packing the acyl chains. It is enriched especially in lipid rafts, where it binds sphingomyelin molecules thanks to its greater affinity for saturated acyl chains instead of unsaturated ones (McConnell & Radhakrishnan, 2003). According to the "umbrella model", cholesterol preferentially mixes with sphingolipids because it relies on polar sphingolipid headgroups to avoid any contact with water, thus reducing the unfavorable free energy (Huang & Feigenson, 1999). This characteristic of cholesterol molecules leads to an increase in membrane rigidity, resulting in lower permeability.

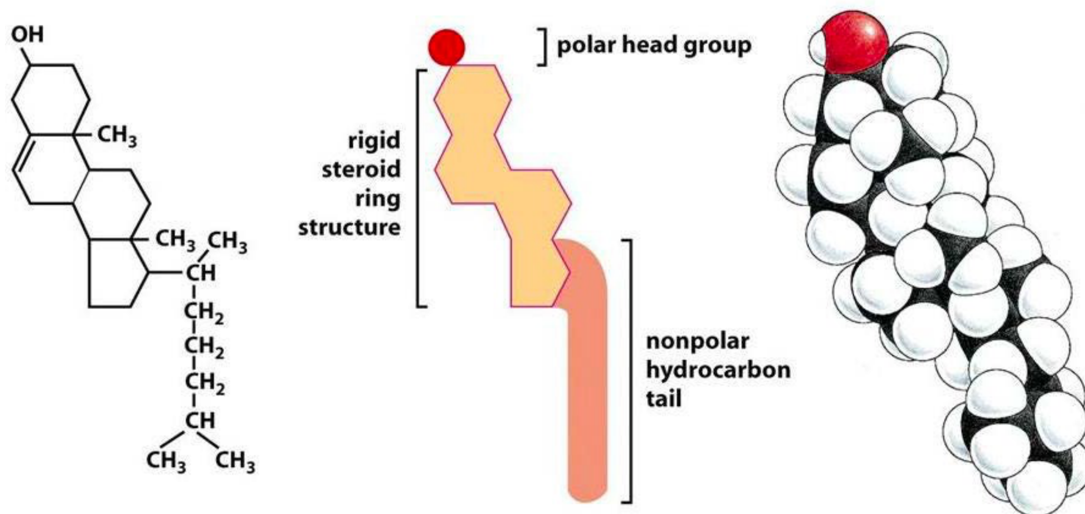

Fig. 2 Cholesterol. The formula, the schematic representation, and the space-filling model of cholesterol. *From Alberts et al., 2002*

- Sphingolipids

Sphingolipid molecules are built on ceramide backbone, which is composed of sphingosine and a fatty acid. The N-acyl chains of sphingolipids are generally more saturated and longer than the acyl chains of glycerophospholipids, therefore are enriched in lipid rafts being able to form specific interaction with cholesterol. The major sphingolipid in mammalian cells is sphingomyelin (SM) (Fig. 3), which has a phosphocholine headgroup (Fahy et al., 2005). It has been especially found in neuronal cell membranes with a higher concentration on the outer than the inner leaflet.

Moreover, it is a component of myelin, the structure that surrounds axon neuronal membranes, and it plays significant roles in signaling pathways.

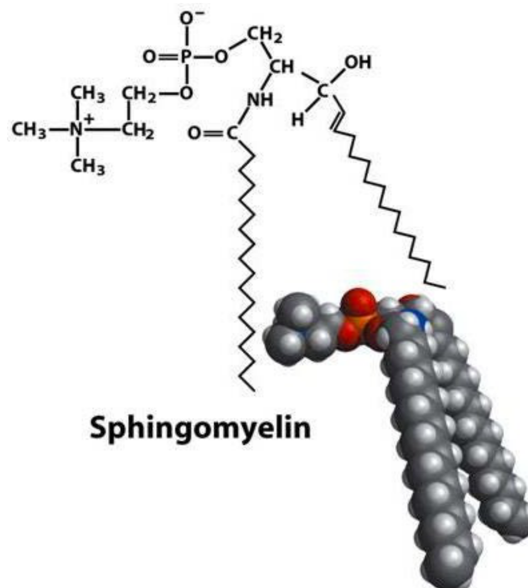

Fig. 3 Sphingomyelin. The formula and the space-filling model of the SM. *From Nelson et al, 2013*

- Glycerophospholipids

The glycerophospholipids (GPLs) are ubiquitous in nature and are key components of the lipid bilayer of cells. They have a glycerol backbone with two fatty acids at C-1 and C-2 position and the glycerol molecule, at C-3 position is esterified with at least one phosphate group, resulting in Phosphatidic acid (PA). As a matter of definition, the head group consists of a phosphate, and an alcohol, which defines the GPL name (Table 1), such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) and the latter two, in turn, can be metabolized to generate PA and phosphoinositides (PIPs).

Table 1. Head polar groups of major GPLs

GPL	Head group substituent
PtdCholine (PC)	Choline
PtdEthanolamine (PE)	Ethanolamine
PtdSerine (PS)	Serine
PtdInositol (PI)	Inositol
PtdGlycerol (PG)	Glycerol
Phosphatidic acid (PA)	-

The main phospholipid contained in biological membranes, in erythrocytes for example, is PC (Fig. 4), which accounts for >50% of the phospholipids in most eukaryotic membranes with one cis-unsaturated acyl chains, which renders the molecule fluid at room temperature (van Meer et al., 2008).

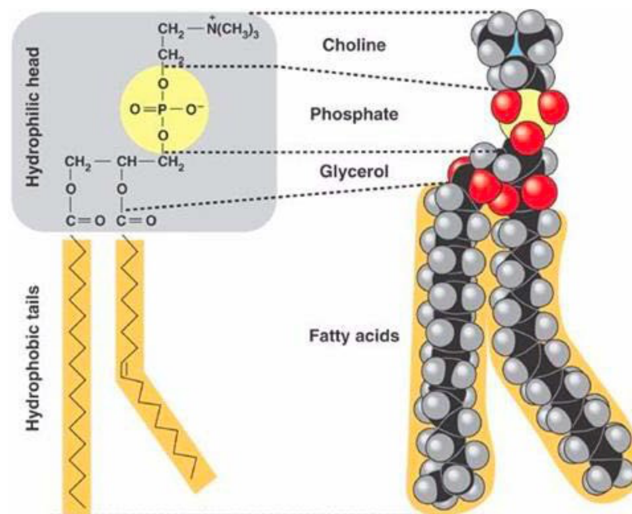

Fig. 4 Phosphatidylcholine. The formula and the space-filling model of PC. *From Rodriguez-Berdini and Ferrero, 2016*

The classification of glycerophospholipids relies on their shapes. For example, they can be cylindrical, conic or inverted-conic (Fig.5). The major factor determining the shape of phospholipids is the nature of their hydrophobic tail, in particular the fatty acid residues linked to

the glycerol backbone. In general, at physiological temperatures, the length of a phospholipid molecule is directly proportional to the number of carbon atoms and inversely proportional to the number of double bonds present in its fatty acid chains. Moreover, the phospholipid shape within the bilayer is influenced either by the surrounding environment or by the compatibility between the size of its polar head group and that of its hydrophobic tail. The lipid has a cylindrical form (when the head and the tail have a similar size), a conical form (when the tail is larger) or inverted conical form (when the head is larger). Basically, in an aqueous environment, cylindrical lipids (PC and PS) determine flat regions, whereas conical (PA and PE) and inverted-conical lipids (PIPs) produce monolayers with negative or positive curvature, respectively (Piomelli et al., 2007) (Fig.5).

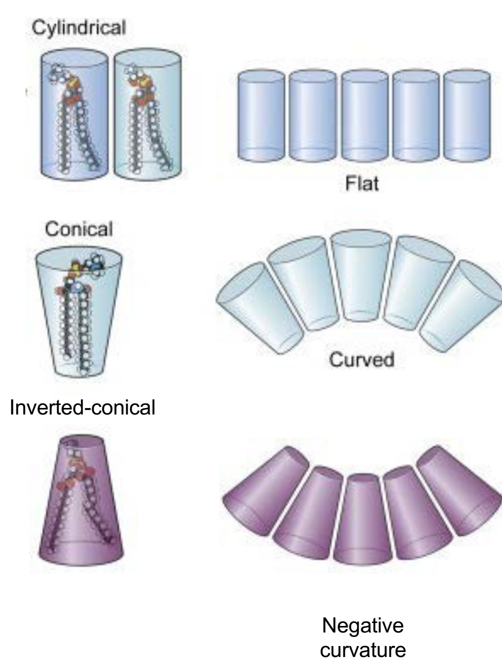

Fig.5 Lipid polymorphism and their assembly in monolayers. Schematic representation depicting shape of various lipids. Lipid with a cylindrical form prefer a flat layer arrangement. Conical-shaped and inverted conical-shaped lipids determine positive or negative membrane curvature, respectively. *Modified from Peetla et al., 2014*

1.3 Compositional lipid diversity of membranes

The high lipid diversity is common to all eukaryotes, highlighting its essential role in many cellular functions. Two types of lipid diversity can be defined: the **chemical** diversity and the **compositional** diversity. The chemical diversity of lipids depends on their chemical properties such as the head polar group, the fatty acid residues, the acyl chain length, the number and the position of double bounds and the hydroxylation. It allows classifying lipids as briefly described before. The **compositional** diversity depends on the ratio of different lipids and it can be observed between species (Hannich et al., 2011, 2017), between cells/tissues within the same organism (Harayama et al., 2014), between the organelles (Antonny et al., 2015), between membrane leaflets and also in membrane sub-domains (Sezgin et al., 2017). To mention some examples of compositional diversity between organelles, we can refer to the organelle-specific lipids such as cardiolipin in mitochondria (Gaspard & McMaster, 2015) and lysobisphosphatidic acid in late endosomes (Chevallier et al., 2008). In addition, different PC species containing diverse fatty acid in C-2 position, are differentially enriched among mouse tissues (Harayama et al., 2014). Other examples of compositional diversity are given by the different lipid composition in the apical and the basolateral PM in polarized cells (as mentioned above) or by the presence of PM microdomains, as well as by the asymmetric distribution of certain lipids between membrane leaflets (J. Suzuki et al., 2010a).

In this context, we will focus on compositional lipid diversity within the PM, deepening the concepts of lateral and transversal cell membrane asymmetry.

1.3.1 Lateral membrane asymmetry (membrane rafts)

As previously mentioned, the raft hypothesis has been developed to explain the apical enrichment of glycosphingolipids in polarized epithelial cells (van Meer & Simons, 1988).
The current view defines membrane rafts as heterogeneous, small (10-200 nm) and highly dynamic (in terms of both lateral mobility and association-dissociation). Cholesterol-sphingolipid-enriched domains are able to segregate proteins and compartmentalize cellular processes (Pike, 2006). As a consequence of certain stimuli, these small rafts can form larger platforms upon clustering (> 300 nm) thanks to lipid-lipid and lipid-protein interactions (Simons & Toomre, 2000).

In the last fifty years, several biochemical and biophysical techniques have been developed and used to prove the presence of such domains in cells, highlighting the key role of the membrane

heterogeneity in various cellular functions.

The first evidence for the membrane lateral heterogeneity came from the identification of differential solubilization of membrane lipids and proteins by non-ionic detergents (Yu et al., 1973). Indeed, under certain conditions, membranes can be fractionated into detergent-soluble (DSMs) and detergent-resistant membrane fractions (DRMs), enriched in cholesterol, sphingolipids (Hanada et al., 1995) and GPI- anchored proteins (Schroeder et al., 1994). In parallel to these biochemical tools, several membrane models with different degrees of complexity (i.e. Supported lipid bilayers (SLBs), Giant Unilamellar Vesicles (GUVs) and Giant Plasma Membrane Vesicles (GPMVs)), have been developed and used to study the liquid-liquid phase separation and to understand the physical principle underlying lipid raft formation (Sezgin & Schwille, 2012). At a given temperature, a lipid bilayer can exist in a more fluid disordered liquid-phase (Ld), containing mainly the unsaturated lipids, or in an ordered liquid-phase (Lo), enriched in saturated lipids and cholesterol (Veatch & Keller, 2003). The difference between these two phases depends on the fluidity of the hydrophobic domains: ordered state lipids have fully extended and closely spaced hydrocarbon chains while fluidized lipid hydrocarbon chains are randomly oriented and fluid. Due to its high molecular packing and enrichment in sterols and saturated lipids, the Lo is considered as the model for the lipid rafts.

In addition, in the last decade, diverse optical microscopy tools such as photoactivated localization microscopy (PALM) (Sengupta et al., 2011), stimulated optical depletion (STED) (Owen et al., 2012), interferomic scattering microscopy (iSCAT) (de Wit et al., 2015) as well as computational models have been successfully applied to observe the dynamics of ordered lipid domains in membrane models.

However, because of their small size and short lifetime, the microscopy detection of lipid rafts and the direct measurement of their properties in living cells remains a major challenge.

- *Cellular functions of membrane rafts*

Although the relevance of membrane rafts *in vivo* is hard to study, their best-described function is to compartmentalize specific elements to regulate their interactions with other membrane components and control their activity. Moreover, it has been shown that rafts, enriched in cholesterol and glycosphingolipids, might also influence protein conformation and their bioactivity (Lingwood et al., 2011).

In 1995, IgE-mediated signaling was the first signaling pathway identified in which lipid rafts were involved. In this pathway, specialized membrane domains were directly involved in the coupling of Fc epsilon RI-receptor aggregation (the high-affinity IgE receptor) to the activation of signaling downstream events (Field et al., 1995). Since this evidence, several studies have been carried out in this field. Indeed, many key immune receptors such as IgE receptor, T-cell receptor and B-cell receptor were found in DRMs only after receptor activation, suggesting that their translocation to membrane rafts is associated with active signaling (Beck-García et al., 2015) (Sproul et al., 2000).

Additionally, in the host-pathogen interaction context, recent data showed that both virus and bacterial products (such as toxins) preferentially bind to highly ordered PM regions to penetrate the cell. This could be due to the raft enrichment of toxin receptors (Iwabuchi, 2015) and viral receptors (Teissier & Pécheur, 2007), such as glycosphingolipids for the cholera toxin or CD4 for HIV virus.

Membrane rafts are also implicated in membrane trafficking. For example, the caveolae, a particular type of rafts enriched in proteins called caveolins and lipids like cholesterol and sphingolipids, seem to play a role in the clathrin-independent endocytic pathway. The caveolae are small PM invaginations (50-100 nm) by which cells internalize several membrane components and extracellular ligands.

Moreover, lipid micro-domains have been proposed to play specific roles in regulated exocytosis. Depletion of cholesterol in neurons impaired the calcium-dependent release of vesicle content at synapse (Linetti et al., 2010) and lipid rafts enriched in cholesterol, SM and PIP_2 is necessary to organize the exocytotic machinery and trigger vesicle fusion (Chasserot-Golaz et al., 2005).

Additionally, membrane rafts have a function in endocytosis process. Indeed, stimulation of chromaffin cells triggers the formation of phosphatidylinositol 4,5-bisphosphate (PIP_2)-enriched lipid domains in the close vicinity of docked granules (Umbrecht-Jenck et al., 2010). These lipid rafts recruit diverse components involved in the clathrin-mediated endocytosis (Cremona & De Camilli, 2001) and thus, constitute preferential hot spots for exo- and endocytosis process.

1.3.2 Transversal membrane asymmetry

The main feature of cell membranes is their lipid asymmetry. The first hypothesis on the asymmetric distribution of lipids across the membrane appeared in 1972, only two weeks after the publication of Singer and Nicolson's paper, by Bretscher (Bretscher, 1972) and was based on experiments performed on red blood cells. Subsequently, several works by many other groups supported Bretscher's hypothesis and showed that the trends in phospholipids distribution in PM of red blood cells seems to have general validity for all animal cells (Op den Kamp, 1979; Zachowski, 1993) and that the transverse distribution of lipids differs considerably between the two leaflets. This appeared to be a common feature for all forms of life and, to various degrees, for all cellular or organelle membranes except for the endoplasmic reticulum (ER) membranes.

The classic picture of lipid distribution across plasma membrane states that SM and PC are mainly present in the outer leaflet whereas aminophospholipids (PS and PE), and phosphoinositides (PI and its phosphorylated derivates such as PIP_2) are essentially restricted to the cytosolic inner leaflet (Fig.6). This asymmetric distribution of phospholipids contributes to the differences in the electrical charges of the two leaflets. The exofacial leaflet is neutral or zwitterionic, and the cytofacial leaflet is more negatively charged due to the enrichment of the anionic phospholipids, PI and PS.

The distribution of cholesterol across the PM is still debated. Although the cholesterol is more soluble in membranes containing phospholipids with large headgroup and saturated acyl chains (such as SM and PC, enriched in the outer leaflet), which can act as "umbrella" to hide its hydrophobic core from water, it has been proposed to be enriched in the inner leaflet in synaptic plasma membrane (Wood et al., 2011). The mechanism responsible for the inner positioning of cholesterol is not completely clear. However, several works have proposed that PS is essential for retaining cholesterol in the inner leaflet of the PM (Hirama et al., 2017; Maekawa & Fairn, 2015). In fact, in PSB-2 cell line that has a low PS synthase activity resulting in 80 % decrease in PS content, more cholesterol was retained in the exofacial leaflet of PM. Then, by supplementing cell medium with PS, cholesterol content was largely restored in the cytosolic leaflet (Maekawa & Fairn, 2015). In addition, a more recent study suggests that the long hydrocarbon chains of common C24 sphingolipids penetrate into the cytoplasmic layer, reducing cholesterol-SM interactions in the outer leaflet cholesterol. Indeed, it has been observed that cholesterol enriches in the inner leaflet (80%) in Hela cells if C24 sphingomyelin is in the outer leaflet. Similarly, in human erythrocytes, where C24 sphingolipids are naturally abundant in the outer leaflet,

cholesterol essentially resides in the cytoplasmic leaflet (Courtney et al., 2018). Cholesterol distribution seems therefore highly dependent on the leaflet lipid composition.

The genesis of phospholipid asymmetry already starts during membrane synthesis and depends both on the localization of biosynthetic enzymes of lipids and mainly on the activity of phospholipids translocases (discussed in the section named "Control of lipid transversal remodeling by translocases"). Several studies, using fluorescent probes with strong affinity and specificity toward PS (C2 domain of lactadherin), showed that PS is asymmetrically distributed along the secretory pathway. PS is synthesized in regions of the endoplasmic reticulum which is in contact with mitochondrial membranes, called MAMs (mitochondria associated membranes) (Stone & Vance, 2000), and then translocated to the luminal leaflet of the ER. It can be next imported either into mitochondria for PE synthesis by phosphatidylserine decarboxylase, or transported to the trans-Golgi network where it flips into the cytofacial leaflet thanks to lipid transporters (Fairn et al., 2011; Leventis & Grinstein, 2010).

SM and ceramide-based glycolipids are mostly synthesized in the luminal leaflet of the Golgi apparatus. When entering the secretory pathway, they are positioned in the luminal leaflet of the secretory vesicles and addressed to the outer layer of the PM after fusion of the vesicles with the PM. Most phospholipids composing biological membranes are synthetized on the cytosolic side of ER membrane (Harayama & Riezman, 2018). Indeed, phospholipid-producing biosynthetic enzymes have their catalytic sites facing the cytoplasmic side of the ER membrane bilayer. After synthesis, phospholipids translocate to the luminal side of ER to generate a uniform and symmetric bilayer. Afterwards, phospholipids are transferred from ER membranes to other compartments through vesicular and non-vesicular pathways (Gillon et al., 2012; Lev, 2010). In particular, the Golgi apparatus acts as a central sorting station to supply the PM with lipid-loaded vesicles but a large part portion of lipids are transferred in a non-vesicular manner. This transfer occurs at specific sites where membranes are in close contact with each other, named "membrane contact sites" (MCSs) (Quon & Beh, 2015). Whilst the classical vesicular pathway between the ER and the PM involves other organelles, such as the Golgi, the so-called PM-ER membrane association represents a direct linkage between the two cell compartments and plays a fundamental role in the lipidic composition of the plasma membrane.

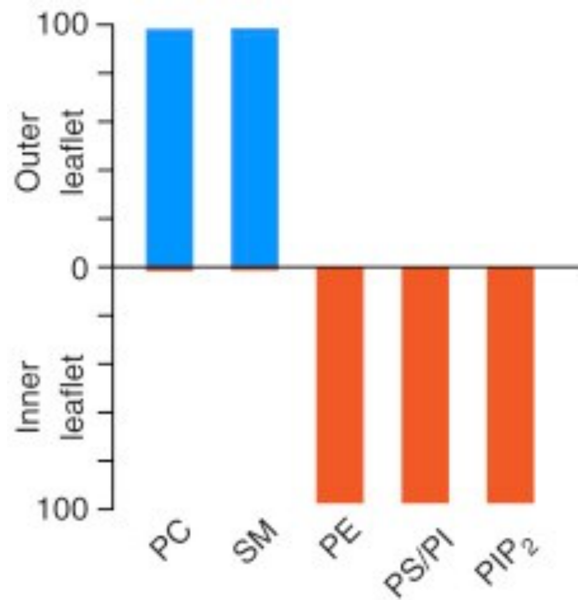

Fig.6 Current picture of lipid asymmetry in plasma membrane. PC and SM (in blue) are majority located in the outer leaflet while aminophospholipids and phosphoinositides (in red) are mostly restricted in the inner leaflet. *From T. Kobayashi and A. K. Menon, 2018*

Besides the asymmetric distribution of phospholipid headgroups, a striking asymmetry is also observed for phospholipid acyl chains between the two PM layers. Lorent and co-workers, using mass spectrometry and fluorescent reporters of membrane packing, have observed that the cytoplasmic leaflet appears to be twofold more unsaturated than the exoplasmic leaflet in two mammalian cell lines (i.e. rat basophilic leukemia and 3T3 fibroblasts) and that plasma membrane leaflets have distinct biophysical properties (Lorent et al., 2020). Indeed, the outer PM leaflet appears to be more rigid and less diffusive than the inner leaflet in which, the higher level of polyunsaturated lipids decreases membrane-bending rigidity favoring different processes such as membrane curvature and fission (Pinot et al., 2014).

- *Physiological roles of plasma membrane lipid asymmetry in "steady-state" condition*

A common hallmark of plasma membrane is its lipid asymmetry resulting in almost exclusive confinement of charged lipids within the cytosolic leaflet. Because of the slow spontaneous flipping of lipids in bilayers, it supposes that transmembrane proteins responsible for lipid flipping act on all cellular membranes to maintain this non-equilibrium situation. The amount of energy consumption is therefore important, indicating that plasma membrane asymmetry should have a critical role in cell function.

The first consequence of the segregation of amino phospholipids in the inner leaflet leads to an imbalance of surface charges between the two membrane faces, which have a direct effect on the insertion and orientation of transmembrane proteins. Actually, positively charged residues of proteins are highly over-represented in cytosolic intracellular regions. For instance, the Na^+ /K^+ ATPase and the gastric H^+ /K^+ ATPase have lysine-rich N-termini located on the cytoplasmic surface of protein, probably because this orientation facilitates their proximity to the negatively charged cytosolic leaflet (Entova et al., 2018). This so-called "positive-inside rule" of membrane proteins, points to a potential key role for membrane asymmetry in determining their topology. In addition, the negative charge of the inner surface of plasma membrane also regulates the organization of peripheral membrane proteins with surface-exposed polybasic stretches, providing a strong electrostatic attraction for their addressing to the plasma membrane (Kapus & Janmey, 2013; Platre & Jaillais, 2017). Obviously, protein distribution is also regulated by phosphorylation of relevant protein motifs which can disrupt electrostatic attractions and modify their final localization.

Unlike the inner leaflet, where most phospholipids possess one or more unsaturation in their acyl chains, the extracellular layer of PM is enriched in high-melting lipids possessing chains that are either fully saturated or contain a trans double bond. As a result, the outer PM sheet is more rigid and ordered than its cytoplasmic counterpart (Lorent et al., 2020). This asymmetric composition contributes to PM capacity to act as physical barrier, determining a low passive permeability of several solutes (Hill & Zeidel, 2000). Indeed, Manni and co-workers have demonstrated that symmetric polyunsaturated bilayers are highly permeable to solutes compared to the asymmetric ones (Manni et al., 2018). Furthermore, it has been shown that polyunsaturated phospholipids, enriched in the inner leaflet, can support endocytosis. Exogenous treatment of neurons with polyunsaturated fatty acids facilitates SNARE assembly (Darios et al., 2006) and synaptic vesicles recycling (Mazra et al., 2008). Molecular dynamics simulations combined to biochemical measurements on liposome and GUVs showed that polyunsaturated phospholipids facilitate dynamin self-assembly and synthetic membrane deformation to promote vesicle

formation by endophilin (Pinot et al., 2014), suggesting that polyunsaturated phospholipids can favor endocytic events dependent on endophilin and dynamin.

In addition, also the asymmetric distribution of cylindrical, conical, and inverted-conical lipids between the two leaflets influences the curvature of plasma membrane. For example, the stability of membrane surface, composed mainly by cylindrical lipids like PC, could be changed by the synthesis and/or arrival of non-cylindrical lipids. Indeed, if these latter are present in a sufficient amount in the membrane, these will lead to a local membrane curvature, which promotes vesicles budding or fusion (Chernomordik & Kozlov, 2008).

The aforementioned functions of lipid transverse asymmetry represent only few examples of how the "steady-state" transversal asymmetry can be involved in various cellular processes, like cell shape. Indeed, although the major contributor to the cell shape is the cytoskeleton, it has been shown that PS, located in the inner layer, interacts with cytoskeletal proteins improving the mechanical stability of red blood cells for example (Manno et al., 2002).

2. Control of lipid transversal remodeling by translocases

When phospholipids are dispersed in aqueous solution *in vitro*, they spontaneously self-assemble in a symmetric lipid bilayer. It has been a long-standing issue limiting the value of artificial lipid membrane to mimic plasma membrane lipid asymmetry (London, 2019). Therefore, how can be plasma membrane lipid asymmetry maintained in living cells?

- Lipid transporters: a brief historical overview

In 1985, protein-mediated lipid transport across the membrane was first demonstrated by Bishop and Bell. They studied the transport of PC across membrane of microsomal vesicles derived from the endoplasmic reticulum by using the homolog "dibutyrolphosphatidylcholine" and, they found that PC transfer was inhibited by proteases, suggesting that tis transport must be mediated by proteins (Bishop & Bell, 1985). Since the process was ATP independent, this protein presumably facilitated lipid movement in both directions and, in the current view it could refer to the so- called "scramblases transporters" (Clarke et al., 2020). The ATP requirement for the lipid transfer was, for the first time, documented in Seigneuret and Devaux's study, demonstrating that the PS/PE rapid diffusion in the inner leaflet was abrogated after ATP depletion (Seigneuret & Devaux, 1984). Nevertheless, we need to wait until 1996 when X. Tang's work reports the decisive evidence that amino phospholipids translocation toward the cytosolic leaflet is due to a P-type ATPase (Tang et al., 1996).

Instead, the movement of lipids from the cytoplasmic to exoplasmic layer is a well-known role of ATP-binding cassette (ABC) transporter floppases. In 1992, in a multidrug resistance context of cancer cells, the ABCA1 (or P-glycoprotein 1) was the first ABC lipid-transporter discovered, able to translocate phospholipids such as PC and glycosphingolipids (Savas et al., 1992).

2.1 Membrane Lipid transporters

Transporters that regulate lipid transfer across membranes, play a key role to maintain membrane asymmetry. These proteins can be categorized in three main classes: Flippases, Floppases and Scramblases (Fig 7). The formers two have an ATP-dependent activity; notably, Flippases move specific lipids "in" (i.e., to the cytoplasmic layer) (flip) whereas Floppases push lipids "out" (into the outer leaflet) (flop). Scramblases facilitate an ATP-independent and

bidirectional movement (flip-flop) of a broad range of lipids (Fig 7). All participate in the regulated maintenance of specific lipid distributions in PM. However, translocation mediated by scramblases can occur at rates faster than 10,000 seconds^{-1} (Marx et al., 2000), while ATP-driven transporters would reach turnover rate not higher than ~100 seconds^{-1} (Coleman et al., 2012), meaning that the lipid asymmetry is much faster to break than to re-establish.

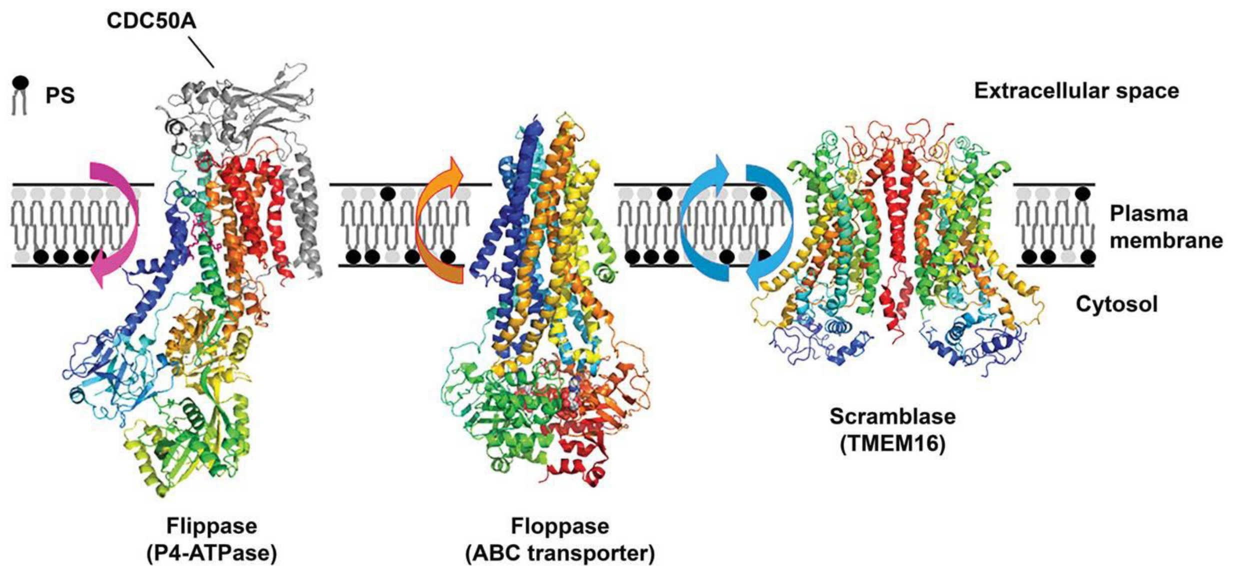

Fig.7 The three classes of phospholipid translocases. Flippases with Cdc50a, transport amino phospholipids (PS and PE) from the outer to the inner leaflet. Floppases transport a broad variety of lipids from the inner to the outer leaflet. Both Flippases and Floppases contribute to maintain the transverse membrane asymmetry. Scramblases mix lipids in both directions, abolishing membrane asymmetry. *From Hye-Won Shin & Hiroyuki Takatsu, 2020)*

2.1.1 Flippases and Floppases

The coordinated activity of ATP-dependent translocators, which include Flippases and Floppases, determine the resulting membrane asymmetry. Current evidence indicates that these proteins are members of P-type and ABC families of transporters, respectively.

- *Flippases*

The P-type ATPase superfamily, which include the well-known Na^{+} /K^{+} -ATPases and Ca^{2+}-ATPases, consists of a large number of transmembrane pumps, divided in five sub-families (P1-P5) with different substrate specificities (Palmgren & Axelsen, 1998).

P4-ATPases, also called Flippases, represent the largest P-type ATPase sub-group and drive the active transport of phospholipids from exoplasmic to cytosolic side of cell membranes.
All P4-ATPases consist of a large polypeptide of approximately 120 kDa (Fig.8). It possesses four main domains. The three cytoplasmic domains involved in the ATPase catalytic cycle (indicated as (A) activator domain, (N) nucleotide binding domain, (P) phosphorylation domain in the Fig 8). Then, the (M) membrane-domain serves as the pathway for the translocation of lipid substrates across cell membranes and is predicted to contain 10 transmembrane analogous segments. Relatively short segments join the transmembrane region on the exoplasmic side of the membrane, whereas large domains and segments connect some of the transmembrane regions on the cytoplasmic side (Fig 8). The transmembrane segments M1–M6 form the principal unit to transport phospholipids across membranes with M7–M10, playing a supporting function. P4-ATPases also contain regulatory domains that modulate the lipid transport activity. These domains are present along the cytoplasmic C-terminal segment of P4-ATPases (Fig.8), but they are poorly characterized for mammalian P4-ATPases (Andersen et al., 2016).

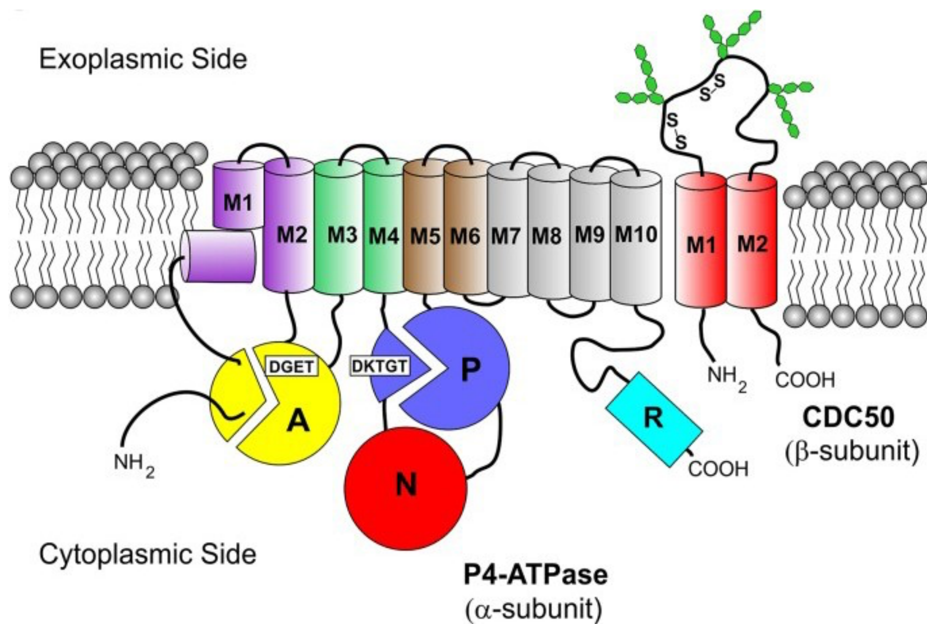

Fig.8 Schematic representation of P4-ATPases. The three cytoplasmic domains of the α-subunit are represented as colored circles. The 10 transmembrane regions (M1-10) are shown as cylinders. The C-terminal region contains a regulatory domain (R) represented, colored in cyan. The β-subunit is also shown. *Adapted from Andersen et al., 2016*

The primary function of P4-ATPase-dependent amino phospholipids flipping is the generation and maintenance of asymmetric lipid distribution with PS and PE largely confined to the cytosolic leaflet. Loss of the yeast plasma membrane P4-ATPases Dnf1p and Dnf2p causes an aberrant exposure of endogenous amino phospholipids at cell surface (Pomorski et al., 2003). It has also been demonstrated that the asymmetry of post-Golgi secretory vesicle is disrupted when Golgi-associated P4-ATPases (Drs2p and Dnf3p) are removed (Alder-Baerens et al., 2006).

Nevertheless, not all mammal P4-ATPases are amino phospholipids transporters. Initial biochemical evidence indicated that P4-ATPases flip only amino phospholipids. Later, several studies have revealed that P4-ATPases differ in their lipid specificities. For example, ATP8B1, initially characterized as a translocase for amino phospholipids and cardiolipin, translocates PC rather than PS upon overexpression in cell lines with a low endogenous phospholipid translocase activity (Takatsu et al., 2014). Similarly, it has been shown that ATP8B2 and ATP10A were specific PC transporters (Takatsu et al., 2014).

Most of P4-ATPases form a heterodimeric complex with a beta subunit from Cdc50/Lem3p proteins (Fig. 8). These beta subunits act as chaperones which is required for the P4-ATPases to leave the ER (Saito et al., 2004). Moreover, the beta subunits may play a role in lipid translocation activity. It has been suggested that the lipid flipping might occurs at the interface between P4-ATPases and its Cdc50 partners. Cdc50 contributes also to the lipid transport specificity (Puts & Holthuis, 2009). In yeast, the trans-Golgi ATPases Drs2p and Dnf3p, which flip different phospholipids, interact with different Cdc50 proteins, Cdc50p and Crf1p respectively (Alder-Baerens et al., 2006). In contrast, the plasma membrane P4-ATPases Dnf1p and Dnf2p, which bind the same beta subunit (Lem3p) (Furuta et al., 2007) (Saito et al., 2004), share the same substrate specifity (Pomorski et al., 2003). The role of Cdc50 in lipid flipping has been recently documented in neurons. Kriegstein and Piao's group, have shown that Cdc50a knowdown leads to a PS exposure at synapse suggesting the murine Cdc50a assists flippases to internalize PS into the inner leaflet. Whether all P4-ATPases require a Cdc50 partner is actually unknown.

- *Floppases*

Floppases belong to the family of ABC transporters, a completely unrelated family to that of P4-ATPase flippases, which transport diverse chemical substrates across membranes preferentially from "inside to outside". They consist of four domains: two transmembrane domains (TMD) surrounding a single central cavity, involved in the substrate transport, and two

ATP-binding cassettes or nucleotide-binding domains (NBD), which supply the energy for substrate transport through the binding and hydrolysis of ATP (Fig.9).

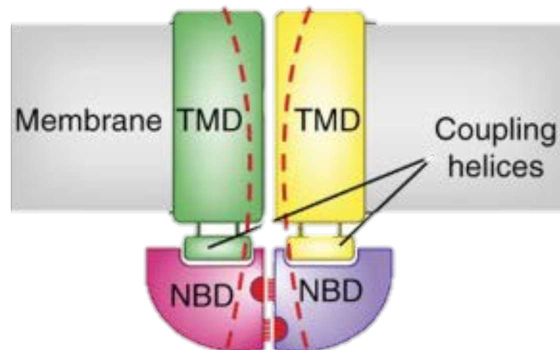

Fig.9 Scheme of ABC transporters: ABC transporters consist of two TMDs, represented as colored cylinder and two ATP-binding sites (NBD). Coupling helices transmit conformational changes between the NBDs and the TMDs. *Modified by P. Locher, 2016*

ABC transporters can be divided in seven sub-groups and several members of these subfamilies have been implicated in a non-specific transport of lipids or lipid-like molecules, such as cholesterol, bile acid, phospholipids, and sphingolipids. The net reaction catalyzed by ABC transporters is fundamentally opposite to that of P4-ATPases. If the high content of amino phospholipids in the inner membrane leaflet is maintained by P4-ATPase Flippases, the enrichment of PC and SM in the extracellular leaflet is guaranteed by their outward pumping via ABC transporters. The principle members implicated in this process are ABCA1, ABCA3, ABCA4, ABCA7, ABCB1, ABCB4 and ABCC1 (Neumann et al., 2017). Improvement in purification and reconstitution of these ABC members provide evidence for their ability to catalyze phospholipid transport. For example, it has been shown that ABCC1 (also known as MRP1) is able to transport fluorescent PC after reconstitution into liposomes (Huang et al., 2004). Furthermore, it has a key role in the maintenance of the outward orientation of endogenous choline-containing phospholipids in erythrocyte membrane (Dekkers et al., 2000). Additionally, studies on fluorescent-lipid transport by ABCA1 and ABCA7 into liposomes have shown that ABCA1 can export PC, PS and SM (with a higher affinity for PC) from the inner to the outer leaflet, whereas ABCA7 preferentially export PS (Quazi & Molday, 2013). ABCA1 and ABCA7 activities mediate the outward movement of phospholipids and, in turn, cholesterol to lipid apolipoprotein acceptors like apolipoprotein A1 (Apo-A1). This represents a key step in the production of HDL cholesterol. Indeed, the loss of ABCA1 function in humans leads to Tangier

disease; a disorder associated with HDL deficiency and, in turn, increased risk of atherosclerosis (Quazi & Molday, 2013).

The lipid-transporter function of ABC proteins is largely conserved also in prokaryotes. MsbA, an ABC lipid transporter expressed in Gram-negative Escherichia coli, is responsible for the export of the Lipid A core to the bacterial outer membrane, and mutation in this protein completely abrogate the lipid translocation from the inner to the outer leaflet in bacterial cells (Doerrler et al., 2001).

It is worth mentioning that, apart from outward-directed transporters, reverse working ABC transporters exist as well. These transporters catalyze lipid movements from the outer leaflet towards the inner one. It is the case of the ABCA4 transporter in mammals, expressed in retinal photoreceptors and associated to Sturgardt macular degeneration. This transporter is an importer that flips the N-retinylidene-phosphatidylethanolamine as well as PE, from the lumen to the cytoplasmic leaflet of disc membranes to remove toxic retinoic compounds from photoreceptors (Quazi et al., 2012).

2.1.2 Scramblases

Given the multiple roles of the PM, it is not surprising that dissipation of PM asymmetry can have profound consequences for the cell and that, this asymmetry disruption is, in turn, tightly controlled. Unlike the P4-ATPase and ABC transporters, phospholipid scramblase activity is involved in the loss of transverse membrane asymmetry, mediating transbilayer movement of lipids in a non-selective and energy-independent manner. This activity is crucial for several cellular functions such as blood coagulation, apoptosis, synaptic pruning as well as compensatory endocytosis, which will be discuss in the next section of this manuscript.

In this paragraph we will mainly focus on the plasma membrane phospholipid scramblases (PLSCR, TMEM16 and XKR families) and their molecular functions.

- Phospholipid scramblases (PLSCRs)

The first molecule exhibiting scramblase activity was identified by Wiedmer's group in 1996. They purified a type II transmembrane protein from erythrocytes that, when reconstituted into liposome, was able to scramble PS in a calcium-dependent manner (Bassé et al., 1996). After the purification of this 37 kDa protein, the encoding cDNA was isolated, and the gene named PLSCR1

(phospholipid scramblase 1). PLSCR1 is a proline-rich, transmembrane protein with a single transmembrane domain (TMD) at its C terminus. It was shown, by immunoblotting analysis, that PLSCR1 was 10-fold more abundant in platelets than in erythrocytes. Whereas incubation with Ca^{2+} ionophore caused a marked transbilayer movement of PM phospholipids in both platelets and erythrocytes, the rate of lipid scrambling in platelet exceeds that in erythrocyte by 10-fold, in good agreement with the higher abundance of PLSCR1 in platelets (Zhou et al., 1997). Furthermore, by Northern blotting, PLSCR1 mRNA was also found in multiple human tissues (i.e. spleen, thymus, prostate, testis, uterus, intestine and colon) and human cancer cell lines (such as promyelocytic leukemia HL-60, epithelial cancer Hela S3, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW480) (Zhou et al., 1997). However, whether mRNA levels correlate with the PLSCR1 protein expression in tissues was not evaluated.

Later on, four additional cDNAs with high homology to PLSCR1 were cloned and the proteins named PLSCR2 (297 aa), PLSCR3 (295aa), PLSCR4 (329aa) and PLSCR5 (271aa) (Wiedmer et al., 2000).

The five members composing now the PLSCR family of protein share similar domains. With the exception of proline-rich domain at the N-terminus region, which is not well conserved, all PLSCR family members possess a DNA-binding domain, a cysteine-rich region, a nuclear localization signal, a conserved calcium ion binding domain (EF-hand-like) and a TMD enriched in hydrophobic amino acids at the C-terminal (Chen et al., 2005) as represented in Fig.10 for the PLSCR1.

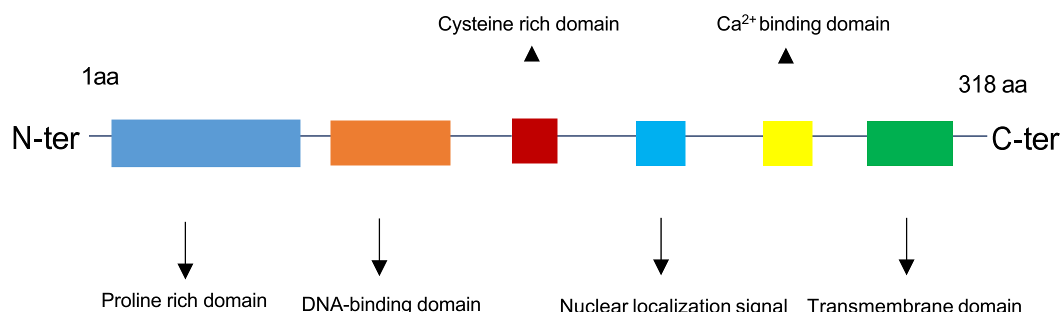

Fig.10 Schematic representation summarizing the known PLSCR1 domains. PLSCR1 contains: a proline-rich region at the N-teminus, a DNA-binding region (aa 86-118), a cysteine- rich region (palmitoylation motif), a NLS (aa 257-266), a conserved Ca^{2+} binding domain and a transmembrane α-Helix at the C-terminus

The tissue distribution of the PLSCR family of proteins was evaluated by Northern blotting with ^{32}P-labeled probes, specific for hPLSCR(1-4) by Wiedmer and co-workers. PLSCR1,

PLSCR3, and PLSCR4 isoforms were found ubiquitously expressed, whereas PLSCR2 expression seems to be restricted to testis. Moreover, mRNA PLSCR1 was below the limit of detection in brain and skeletal muscle (Wiedmer et al., 2000) but later Zhou and co-workers detected the PLSCR1 protein in the mouse brain by Western blot analysis (Zhou et al., 2005).
In the next sections, I will principally focus on PLSCR1, the best-studied member of the family.

- *Subcellular localization of the PLSCR1*

In agreement with the identified domains constituting PLSCRs proteins, their subcellular localization is variable and alternates between the plasma membrane and the nucleus. Although regulatory signal conditioning their distribution is largely unknown, PLSCRs distribution appeared regulated by their structural domains (Blanc et al., 2015), mostly the transmembrane domain, the cysteine-rich domain and its NLS. In 2003, Sim's group identified a Cys-rich sequence CCCPCC in PLSCR1 that can be palmitoylated. Palmitoylation is a reversible post-translational modification, which increases the hydrophobicity of proteins and promotes their association with membranes. Mutations in these five cysteines or treatment of cells with 2-bromo-palmitate, which inhibits palmitoylation, redistribute PLSCR1 from the plasma membrane to the nucleus (Wiedmer et al., 2003). Like PLSCR1, PLSCR4 is mostly found associated to the plasma membrane where it mediates bidirectional translocation of phospholipids (Py et al., 2009). In contrast, PLSCR3 is mostly enriched in mitochondrial membrane, where it is involved in movement of cardiolipin, a lipid required for mitophagy. Although they do not share the same localization, preventing PLSCR3 or PLSCR4 palmitoylation redistributes both PLSCR3 and PLSCR4 to the nucleus.

Altogether, these experiments indicates that cysteine rich region contributes to PLSCRs membrane localization (Lott et al., 2011) and in particular, PLSCR1 nuclear localization can occur only when cysteine-rich region palmytoilation is prevented (Wiedmer et al., 2003).

Diverse nuclear functions of PLSCR1 have been identified. PLSCR1 has at least two known binding activities: first, in response to interferon or other cytokines, PLSCR1 could be translocated into the nucleus where it acts as a modulator of IP3 receptor expression (Zhou et al., 2005), second, it can also contribute to DNA replication via its direct interaction with two DNA topoisomerases II (Wyles et al., 2007). Moreover, a direct role of the nuclear-located PLSCR1 in mediating mitotic expansion of the granulocyte precursors upon G-CSF stimulation has been reported (Chen et al., 2011). PLSCR1 contains a DNA-binding domain and an atypical NLS, able to bind importins α/β to mediate its nuclear import (Chen et al., 2005). Mutation of the minimal

NLS of unpalmitoylated PLSCR4 impedes its accumulation in the nucleus (Lott et al., 2011) confirming that PLSCRs have a functional NLS and a potential role in the nucleus.
Controversial findings have been reported about the role of TMD in regulating PLSCR1 localization at the PM. It has been proposed that palmitoylation rather than its TMD is the primary plasma membrane anchorage motif for PLSCR1 since preventing palmitoylation may lead to accumulation of PLSCRs protein into the nucleus. In addition, by searching for homology in protein database (HHpred server), Söding's group found that hPLSCR1 shared good homologies with the At5g01750 protein, a member of the DUF567 family from *A.Thaliana (homology of 99% after* removing the N-terminal proline-rich stretch of 70 amino acids from human PLSCR1) which structure has been resolved (Bateman et al., 2009). The C-terminal helix of At5g01750 analogous to the TMD of PLSCR is buried within the protein core and is protected from any contacts and cannot contribute to PM localization (Bateman et al., 2009). Consequently, they proposed that the structure of At5g01750, and therefore by similarity the PLSCR1, is a 12- stranded β-barrel that encloses a hydrophobic central C-terminal α-helix (Bateman et al., 2009). However, no direct study has been carried out to determine the structure of PLSCR1 protein and *in vitro* experiments propose an alternative model. Indeed, Francis and co-workers showed that the TMD was required for membrane insertion (Francis et al., 2013). The expression of hPLSCR1 deleted from its transmembrane domain (ΔTMD-hPLSCR1) in human embryonic kidney cells increased its cytoplasmic and nuclear localization, but abolished PM distribution. This domain contains a well-known binding site for cholesterol which can help its insertion in lipid raft domains (Posada, Fantini, et al., 2014). In agreement with the previous data, PLSCR1 mutant lacking the last 28 aa residues, corresponding to the TMD domain, completely lose the capacity to bind membranes (Posada, Sánchez-Magraner, et al., 2014).

Taken together, these results indicate that TMD is as important as palmitoylation in regulating PLSCR1 distribution. Furthermore, although surprising, these observations suggest that PLSCRs may have a functional dimorphism in the cell: acting as a scramblase at the plasma membrane in a palmitoylated form where it modifies lipids distribution or as a transcription factor in the nucleus in a nonacylated form.

- *Regulation of PLSCR1 activity*

Thanks to its EF-hand like calcium-binding domain (CBD), PLSCR1 scramblase activity has been reported to be mostly regulated by Ca $^{2+}$. *In vitro* experiments showed that, when PLSCR1 was reconstituted into liposome, the addition of Ca $^{2+}$ was sufficient to trigger lipid scrambling (Comfurius et al., 1996). The Ca^{2+} binding motif has a micromolar range affinity for Ca^{2+} and is essential for phospholipid scrambling (Bateman et al., 2009; Sahu et al., 2009; Stout et al., 1998; Zhou et al., 1998). In cells, scramblases activation occurs in response to cell injury, apoptosis and during regulated-exocytosis (Heemskerk et al., 2002; Martin et al., 1995; Ory et al., 2013), where Ca^{2+} intracellular concentration is increased by 1000-fold (Pozzan et al., 1994). In platelets, using continuous fluorescence-based assay, intracellular calcium was shown to act as a switch for the scrambling machinery (Williamson et al., 1995).

Yet, Ca^{2+} -mediated PLSCR1-activation may be modulated by the transmembrane domain (TMD) of the protein. Indeed, as they are juxtaposed, there is an intricate relationship between the CBD and the TMD. The truncated mutant ΔTMD PLSCR1 has a much lower affinity (i.e. 50 % decrease) than the full length PLSCR1 (Francis et al., 2013; Sánchez-Magraner et al., 2014a) which may be related to the fact that the TMD domain favors a more compact conformation of the PLSCR1 protein, keeping residues involved in Ca^{2+} coordination close together (Andraka et al., 2017). In parallel, Ca^{2+} binding induces conformational changes of the TMD that is required for PLSCR1 scramblase activity (Francis et al., 2013). Therefore, depending on the intracellular calcium concentration, the conformational changes of the TMD may in turn affect binding of Ca^{2+} to PLSCR1 and influence its activity.

Although Ca^{2+} is thought to be the principal regulator of PLSCR1 activity, other factors, including membrane cholesterol and the proline rich domain (PRD) of the PLSCR1 can influence PLSCR1 activity. Interestingly, in proteo-liposomes reconstituted with full-length hPLSCR1, depletion of cholesterol is sufficient to induce PS redistribution to the outer surface in a Ca^{2+} -independent manner. Conversely, PLSCR1-induced lipid scrambling was strictly dependent on Ca $^{2+}$ when cholesterol was present, suggesting that membrane cholesterol acts as a powerful scramblase inhibitor in the absence of Ca^{2+} (Arashiki et al., 2016). Because PLSCR1 consists of a single transmembrane domain compared to other phospholipid transporters that are multipass transmembrane proteins, doubt has been cast about the ability of PLSCR1 to transport phospholipids. Nevertheless, like other pore-forming peptides such as magainins (Matsuzaki et al., 1996), Ca^{2+} binding may induce oligomerization of PLSCR1 monomers, either by connecting or by inducing a conformational change in the polypeptide that in turn promotes self-association

(Sahu et al., 2009; Stout et al., 1998). Interestingly, Arashiki and co-workers found that, after cholesterol depletion, PLSCR1 in liposome can assemble into oligomers forming an integral “channel” that allows the phospholipid hydrophilic head regions to pass independently of Ca^{2+} (Arashiki et al., 2016). It indicates that the membrane cholesterol might regulate the conformation of PLSCR1 in the membrane, which in turn, affects its oligomerization state.

Finally, by either altering the PLSCR1 conformation or its oligomerization state, the PRD may regulate PLSCR1 scrambling activity. First, ΔPRD PLSCR1 truncated mutant lose its scrambling activity *in vitro.* Second, PLSCR2, which is mostly localized into the nucleus and does not contain a PRD (Yu et al., 2003), does not show scrambling activity *in vitro*. Third, when the PRD of the PLSCR1 was fused to PLSCR2, this latter gains the capacity of mixing lipids on liposomes in response to Ca^{2+} increase *in vitro* (Rayala et al., 2014), Interestingly, using dynamic light scattering approach, Rayala and co-workers demonstrated that only PLSCR1 with a PRD domain was forming oligomers.

Taken together, these studies provide evidence that the scramblase activity may depend on conformational changes and oligomerization state of PLSCR1, both regulated by Ca^{2+} binding and the lipidic environment.

- *PLSCR1 functions*

Dependent or not of its scrambling activity, PLSCR1 has been involved in different cell functions such as innate immune response, viral entry, apoptosis, or compensatory endocytosis. The best-known functions of PLSCR1 are related to hematopoietic cells, where PLSCR1 is highly expressed. In plasmacytoid dendritic cells, a specialized subset of immune cells that link innate to adaptive immunity, PLSCR1 interacts with toll-like receptor 9 (TLR9), which acts as a receptor recognizing foreign and pathogenic proteins/nucleic acids, to regulate TLR9-mediated production of IFN- α (Talukder et al., 2012).

In addition to inducing IFN production, the PLSCR1 is itself, a α/β interferon-inducible protein that mediates antiviral activity against RNA viruses. In HepG2 and HuH7 cell lines, the upregulation of PLSCR1 expression induced by IFN- α reduces Hepatitis B virus replication (Yang et al., 2012). Consistent with this, in A549 cells, IFN-α stimulates PLSCR1 expression to protect cells from α-toxin, produced during *S. Aureus* infection (Lizak & Yarovinsky, 2012). Moreover, $PLSCR1^{-/-}$ mice, treated with α-toxin via intranasal route, exhibited staphylococcal

pneumonia sooner than WT mice, indicating that PLSCR1 deficiency increases sensitivity of mice to α-toxin (Lizak & Yarovinsky, 2012).

Collectively these data indicate that PLSCR1 is strongly involved in viral and innate immune signaling by modulating cytokine response. However, the mechanism by which the PLSCR1 acts and, if it is related to scrambling activity remains completely unknown.

PLSCR1 has also been involved in processes implying membrane remodeling. It acts either by recruiting signaling partners or supposedly, by its scrambling activity. For example, PLSCR1 has been involved in autophagy. In MCL (Mantle cell lymphoma) upon IFN-α stimulation, PLSCR1 is upregulated and acts as a negative regulator of autophagy. It can interact with ATG12, an ubiquitin-like protein involved in the autophagosome formation, preventing ATG16L1 recruitment and its full activation (Mastorci et al., 2016).

PLSCR1-mediated PS distribution has been involved in diverse cellular processes. In *C.Elegans* the SCR-1, ortholog of hPLSCR1, promotes PS egress which provide an "eat me" signal in apoptotic cells (Hsu & Wu, 2010). In chromaffin cells, PLSCR1-dependent PS egress at the plasma membrane occurs around exocytic sites following exocytosis of secretory granules. This PS externalization is required to retrieve proteins newly exocytosed by compensatory endocytosis (Ory et al., 2013).

Finally, a recent study demonstrated that following Herpes simplex virus (HSV) infection of human cervical epithelial cells (CaSki) or keratinocytes (HaCAT), PLSCR1 was activated and induces PS and Akt translocation towards the external leaflet of the plasma membrane. Interestingly, virus entry requires PS externalization which promotes a complex signaling cascade (Cheshenko et al., 2018).

Overall, these data allow us to understand how complex and diverse the PLSCR1 activity is. To summarize, PLSCR1 is implicated in both signaling and membrane trafficking pathways, most of which initiate at the cell surface. If in some of these processes, the lipid remodeling activity of PLSCR1 is clearly required, in others this remains to be investigated.

- TMEM16 and XKR families

Although the earliest studies implicate PLSCR1 in redistributing phospholipids between membrane leaflets, PLSCR1 scrambling activity was cast into doubt. Indeed, it was found that PLSCR1 levels do not correlate with apoptosis-induced PS exposure in six different cell lines and

PLSCR1 overexpression by retroviral transduction of PLSCR1 or treatment with IFN-α in Raij cell line (where PLSCR1 is little expressed) failed to confer the capacity to expose PS in response to apoptotic stimuli (Fadeel et al., 1999). Doubts were reinforced with the generation of PLSCR1$^{-/-}$ mice (Zhou et al., 2002). The Scott syndrome is a bleeding disorder caused by defective scrambling of membrane phospholipids (Zwaal et al., 2004) and it was expected that PLSCR1$^{-/-}$ showed defects in blood coagulation. However, PLSCR1$^{-/-}$ mice were normal and showed normal PS exposure in platelets, red blood cells and lymphocytes. In addition, PLSCR1 extraction from erythrocytes of Scott syndrome patients reconstituted into liposomes showed normal scramblase activity (Stout et al., 1997). The search for additional scramblases lead to the identification by Suzuki and co-workers of two proteins: the TMEM16F protein which is directly responsible for Ca^{2+} -dependent PS egress in erythrocytes and mutated in Scott patients, (J. Suzuki et al., 2010b) and the XKR8 which causes PS exit in response to apoptotic stimuli (J. Suzuki et al., 2013). It therefore indicated that despite their common role in lipid scrambling, different scramblase could be engaged in cell process relying on phospholipid scrambling, smoothing out doubts about PLSCR1scramblase function.

Since then, TMEM16 and XKR protein are extended and regroup several members. The intracellular membrane proteins TMEM16E and TMEM16K, the plasma membrane proteins i.e., TMEM16C, TMEM16D, TMEM16F, TMEM16G, TMEM16J as well as XKR4, XK8, XK9 are known to belong to “phospholipid scramblase family” (Shin & Takatsu, 2020).

- *TMEM16 proteins*

The TMEM16 family, also known as Anoctamins, consists of 10 members (TMEM16A-K) where we can distinguish: Ca^{2+}-activated ion channels (CaCC) and, Ca^{2+}-activated phospholipid scramblases (CaPLases) that passively flip-flop phospholipids between the two leaflets of the membrane bilayer. They are expressed in a wide range of tissues like brain, skin, ovary, heart among other. A functional TMEM16 protein is a dimer with a double-barreled architecture. Each TMEM16 monomer consists of 10 TM segments preceded by a long N-terminal cytosolic domain (NCD) and followed by a short C-terminal extension of TM10. Besides the NCD, the transmembrane region is formed by the “pore-gate” and “supporting” domains, respectively constituted by TM3-8 and TM1, 2, 9, 10 as described in the Fig.11. TM7 and TM8 do not completely traverse the membrane and together with TM6, form two highly conserved Ca^{2+} binding sites which control protein activity. The supporting domain contains a third

Ca^{2+} binding site that is allosterically coupled to the pore-gate domain and establish the dimer interface within the membrane through interactions between the extracellular regions of TM10.

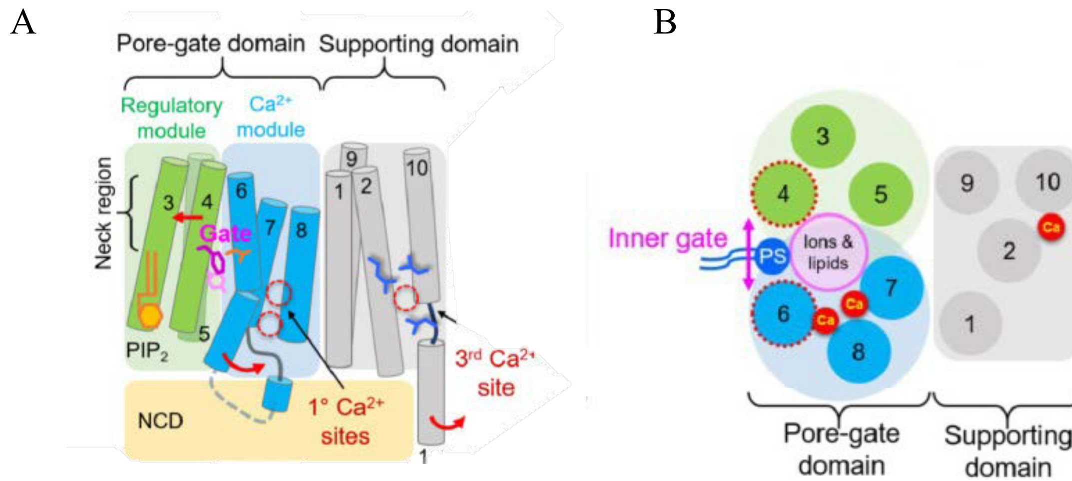

Fig.11 Structure of TMEM16 proteins. A Modular design model of TMEM16, side view. Three sidechains in the middle of the pore represent the inner activation gate residues of TMEM16s. **B** Top view. Calcium induces conformational changes, which lead to TM4 and TM6 separation, allowing the entry of the phospholipid headgroups (PS in blue). *Adapted from Son C. Le et al., 2021*

Although the TMEM16A and TMEM16B members function as chloride channel, most TMEM16 are phospholipid scramblases (Whitlock & Hartzell, 2017). Like passive phospholipid transporters, that do not require energy from ATP hydrolysis to move lipid across PM, TMEM16 can efficiently translocate phospholipids at high speed (4.5×10^4 phospholipids per second for TMEM16F) (Watanabe, Sakuragi, et al., 2018). TMEM16F is localized at the plasma membrane and was the first anoctamin associated with lipid scrambling (J. Suzuki et al., 2010a). It has been shown that PS exposure in activated platelets during blood coagulation is due to its Ca^{2+}-dependent activity and mutations leading to the truncated version of TMEM16F were found to cause Scott syndrome, a rare bleeding disorder (J. Suzuki et al., 2010a). In addition, TMEM16F is essential, in osteoblasts, to the deposition of hydroxyapatite leading to bone mineralization. Indeed, deletion of TMEM16F gene in mice reduce skeleton size and cause skeleton deformities (Ehlen et al., 2013).

Besides the well-known role in blood coagulation and bone mineralization, TMEM16F-mediated PS-egress may play additional functions. In macrophages, TMEM16F is necessary for phagocytosis stimulated by the ATP receptor P2X (Ousingsawat et al., 2015) and microglia lacking TMEM16F shows defects in process motility and phagocytosis (Batti et al., 2016),

indicating that TMEM16F activity can also play a role in membrane trafficking in various cell types.

- *XKR proteins*

The human and mouse XKR protein families consist of nine and eight members respectively, each of which contains 6-10 predicted TM domains. However, only XKR4, 8 and 9 proteins, exhibit a lipid scrambling activity. Murine XKR8 is ubiquitously expressed with higher expression in testis whereas XKR4 and XKR9 are tissue specific, with the former being enriched in brain, eyes and skin and the latter mainly present in the intestine and stomach (Shin & Takatsu, 2020; J. Suzuki et al., 2013). XKR8 is predominantly localized at the plasma membrane and it is consistent with its role in PS externalization in response to apoptotic stimuli (J. Suzuki et al., 2013). Indeed, when murine XKR8, but also XKR 4, were expressed as GFP-fused proteins in cell lines, they were found only located to the plasma membrane (J. Suzuki et al., 2014). Unlike PLSCR and TMEM16 proteins, the C-terminal portion of XKR4, 8 and 9 must be cleaved off by caspases to activate the phospholipid-scrambling activity (J. Suzuki et al., 2013). The caspase recognition sequence differs among XKR4, XKR8, and XKR9. All these XKR proteins were efficiently cleaved by caspases 3 and 7, but XKR4 and, XKR9 were also cleaved by caspase 6. In the brain, a local activation of caspase 6, has been proposed to contribute to the pruning process of axons, dendrites, and synapses which rely on the exposure of PS at the plasma membrane (Cusack et al., 2013) . As XKR4 is enriched in the brain, caspase 6 activation may in turn activate XKR4-dependent externalization of PS on axons, dendrites, and synapses, to trigger microglial phagocytosis, resulting in the remodeling of the neural network. This suggests that XKR protein's function might not be limited to apoptosis regulation.

2.2 Possible mechanisms for lipid flipping

Even though available data support a direct role of Flippases, Floppases and, Scramblases in lipid translocation, it is still controversial how these lipid transporters work. Over the years, diverse mechanisms have been proposed. One of the first models about the flippase mechanism in bacterial membranes suggested that phospholipids were reoriented in its immediate vicinity to give rise to a hairpin-like, a non-bilayer arrangement of phospholipids (Fig.12), and this

arrangement would effectively connect the two membrane leaflets allowing phospholipids to diffuse continuously between leaflets (Langley & Kennedy, 1979).

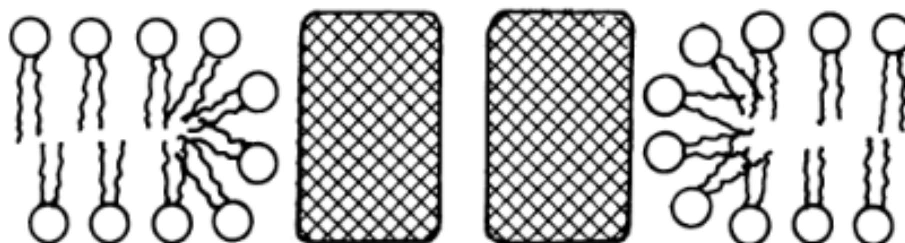

Fig.12 First hypothetical model on Flippase mechanism proposed by Langley and Kennedy. Crosshatched area represents lipid transporter. *From Langley and Kennedy, 1979*

Later, it has been suggested that P4-ATPases could transport substrates by "a classical pathway" with the lipid transported through the interior of the proteins in analogy with the cation transport mechanism of Ca^{2+} and Na^{+} /K^{+} - transporting P2-ATPases. Support for this hypothesis was based on the observation that in HEK293T cells , the exogenously expressed P4-ATPase Atp8a2, mutated in a conserved lysine residue, which is predicted to be near the canonical binding site within transmembrane segment (TM5), showed a reduced affinity for substrate (PS) compared to WT ATP8A2 (Coleman et al., 2012). They hypothesized that lysine neutralizes the phosphate group in the transported phospholipid molecule, facilitating thus the movement (Coleman et al., 2012).

Nevertheless, given that phospholipids are roughly ten times larger than ions this model implies a strong flexibility of P4-ATPases to contain their substrate and mainly the lipid tail during the transport across the PM. The model evolved to consider the constraint linked to amphipathic molecules handling by transporters. In this model, only the head group of the phospholipid makes a direct contact with the protein during flipping, while the acyl chains remain associated with membrane, like the swiping of a credit card in a card reader. Thus, the hydrophilic groove of the "card-reader" provides a low energy path for the lipid headgroup by sequestering it from the unfavorable hydrophobic environment of the membrane interior (Fig.13). Evidence for such potential hydrophilic grooves has been provided for several lipid transporters.

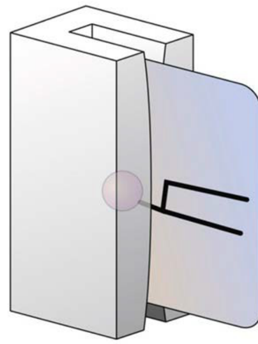

Fig.13 Credit-card mechanism. Schematic representation of the "credit-card model" for lipid flip–flop. *From Pomorski T. et al., 2006*

For instance, structural homology modeling and molecular dynamics stimulation on the mammalian P4-ATPase ATP8A2 showed that isoleucine 364 residue and other adjacent residues in TM4 act as hydrophilic gates that separate the entry and the exit sites of lipid. It generates a cavity formed by the TM1, TM2, TM4, TM6, which contains water-filled pockets, thereby enabling transport of the hydrophilic phospholipid head group in a groove outlined, with the hydrocarbon chains following passively and remaining in the membrane lipid phase (Vestergaard et al., 2014). ATP8A2 mutants in which I364 was replaced by other residues, showed a defective substrate affinity when expressed in HEK293T cells (Vestergaard et al., 2014). Consistent with this model, studies of yeast P4-ATPases, Dnf1p and Dnf2p, identified some amino acid residues involved in substrate specify at the luminal side of TM1 and the cytosolic side of TM1, TM2 and TM4 (Baldridge & Graham, 2012).

Similarly, also scramblases seem to display these potential hydrophilic grooves. X-ray structure of a TMEM16 homologue from *Nectria haematococca* (nhTMEM16) showed that each subunit of the homodimeric protein TMEM16 contains ten transmembrane helices and a hydrophilic membrane-traversing cavity, exposed to the lipid bilayer as a potential site of catalysis. This groove is ~1 nm wide and exhibits polar character, thus it could accommodate the headgroup of a transiting phospholipid (Brunner et al., 2014).

3. Loss of lipid membrane asymmetry in biological processes

In physiological conditions, the aminophospholipids, especially PS, are restricted to the cytoplasmic leaflet of cell membranes leading to an asymmetric lipid distribution. Exposure of the PS at the cellular surface has several consequences. It may serve as a potential extracellular signal and overrides the plasma membrane asymmetry, triggering various biological processes that determine the cell fate. The outward movement of the PS is commonly correlated to apoptosis or blood coagulation processes. The hallmark of these latter events is the massive and irreversible exposure of PS on the cellular surface. However, increasing evidence emphasizes the importance of a located and/or reversible PS exposure in physiological contexts. In the next paragraphs, we will make an overview of the consequences of both irreversible and transient loss of the plasma membrane asymmetry, focusing on the role of scramblases described above.

3.1 Irreversible loss of membrane asymmetry: Apoptosis and blood coagulation

- *Apoptosis*

In 1992, Fadok and co-workers observed for the first time that PS exposure at the plasma membrane of lymphocytes represented a specific recognition signal for macrophages to mediate their own removal (Fadok et al., 1992). PS exposure is now accepted as a general hallmark of cells in apoptosis. Apoptosis, or programmed cell death, is a physiological step of the cellular life cycle and ensures a balance between cell proliferation and cell death, in order to keep the tissue homeostasis. Apoptosis is triggered by two different pathways: the extrinsic pathway, initiated by binding of some tumor necrosis factors (TNF) to receptors on plasma membrane; the intrinsic one, called also mitochondrial pathway, where an increase of mitochondrial membrane permeability, causes the release of cytochrome c into the cytoplasm. Both pathways finally converge to the caspase 3 and caspase 7 activation which, in turn, cleave and activate the scramblase XKR8 (Fig 14) (J. Suzuki et al., 2013, 2014). PS exit mediated by XKR8 is much slower, on the hour timescale, compared to that of TMEM16F, which occurs within minutes. The difference does not lie in the kinetics of lipid transfer, which is quite similar between the two scramblases, but rather to the time required to activate caspases. PS externalization is further enhanced by caspase-dependent cleavage of ATP11A and ATP11C (Fig 14), two major flippases involved in the

transport of PS toward the inner leaflet (Segawa et al., 2014, 2016). The coordinated activation and inactivation of scramblases and flippases respectively results in a loss of membrane asymmetry with dramatic PS exposure, which functions as an “eat me” signal for phagocytes (Fig.14). Indeed, PS exposed on surface membrane can be recognized by PS-receptors on phagocytes, allowing them to them to initiate the engulfment process (Martin et al., 1995).

The cleavage of ATP11A and ATP11C explain in part why PS exposure is irreversible in apoptosis. Moreover, the apoptotic signal, due to PS molecules exposed, depends also on the chemical properties of PS. It has been shown that cytochrome c-mediated PS oxidation could limit PS recognition by flippases, enhancing the strength of the “eat-me” signal (Kagan et al., 2000).

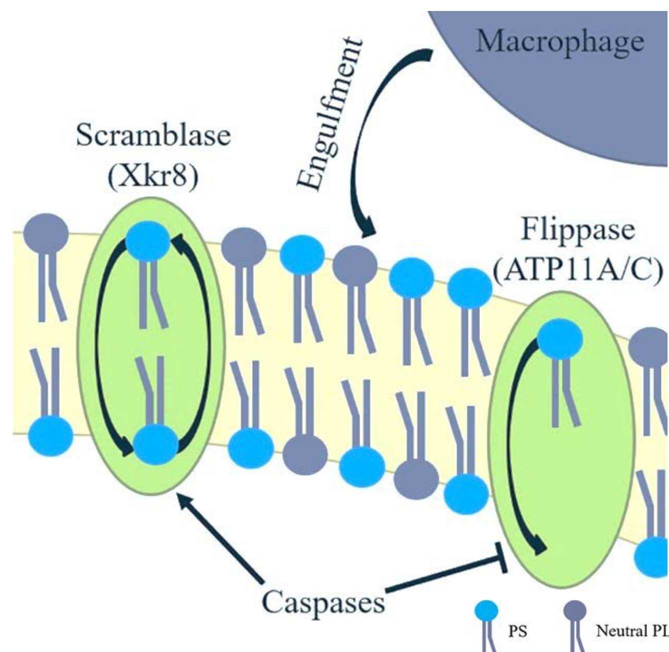

Fig.14 Schematic apoptotic pathway. Caspase activity activates XKR8 and inactivate ATP11A/C, resulting in PS exposure. PS on the extracellular layer provides an “eat me” signal for the cell, which can be recognized and engulfed by macrophages. *Adapted from Clark et al., 2020*

- *Blood coagulation*

When vascular injuries occur, blood coagulation is essential to reduce blood loss and initiate tissue repair. Blood coagulation consists of two subsequent steps: the primary and secondary homeostasis. Initially, to stop bleeding, there is the formation of a weak platelets plug by a mechanism called primary homeostasis. The primary homeostasis consists of four steps:

vasoconstriction, platelets adhesion, platelets activation and finally, platelets aggregation to form a platelet plug which temporary protects from hemorrhage until further stabilization of fibrinogen to fibrin occurs in the secondary homeostasis. During primary homeostasis, the activation of platelets by its interaction with collagen, activates a signaling pathway mediated by glycoprotein IV, phospholipase C and inositol 1,4,5 phosphate (IP_3) (Heemskerk et al., 2002). IP_3, through the activation of IP_3 -receptor Ca^{2+} -channels, allows Ca^{2+} release from the endoplasmic reticulum. Moreover, Ca^{2+} also enters into the cell from extracellular medium via Ca^{2+} - channels located at the plasma membrane, like ATP-gated P2X1 channels, Orai1 store-operated channels and mechanosensitive Piezo1 channels (Ilkan et al., 2017; Mahaut-Smith, 2012). Both extracellular and intracellular fluxes cause an increase in cytosolic Ca^{2+} concentration that activates TMEM16F, which in turn starts mixing lipid and exposes PS on the platelet surface (Varga-Szabo et al., 2009) allowing clotting to proceed in the secondary homeostasis.

In secondary homeostasis, activated platelets shuffle amino phospholipids, especially PS, to the outer leaflet, providing an optimal anionic catalytic surface with 10-15 mol% PS (Heemskerk et al., 2002; R. F. Zwaal et al., 1998) that attracts clotting factors forming a procoagulant complex and promoting the conversion of prothrombin into thrombin (Fig.15).

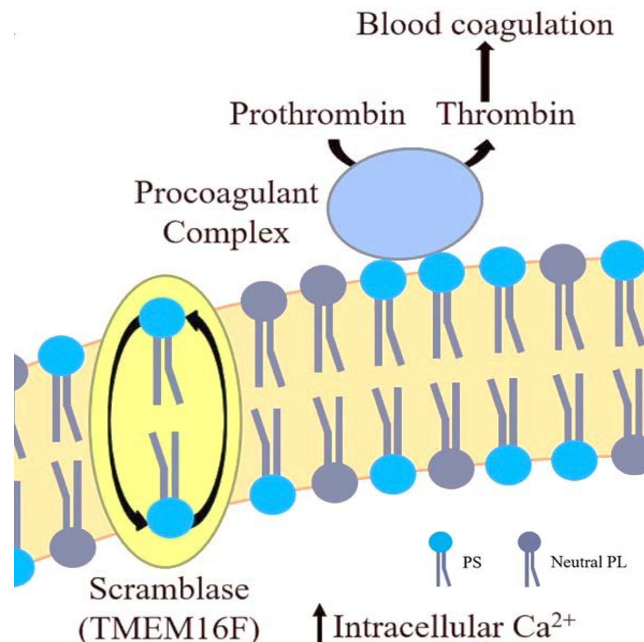

Fig.15 Schematic coagulation cascade activation. An increase in the intracellular Ca^{2+} activate TMEM16F, resulting in PS exposure at the plasma membrane. PS at the external layer attracts clotting factors which form a procoagulant complex promoting the conversion of prothrombin into thrombin. Thrombin catalyzes the formation of fibrin, which makes a stable clot. *Adapted from Clark et al., 2020*

The increase in PS at the platelet cellular surface speeds up coagulation reactions (> thousands of times faster). Moreover, when PE is present, less PS is required for maximum speed. Indeed, Gla (γ-carboxyglutamic acid) containing proteins (such as FVII, FIX, FX, prothrombin, protein C, protein S, protein Z) preferentially bind to PS clusters, and PE helps in grouping PS in these clusters (Neuenschwander et al., 1995). An additional process which occurs on activated platelets is the formation of membrane blebs, resulting in microparticles (MP) or lipid vesicle from the platelets surface (Morel et al., 2011). Microparticles provide additional catalytic PS-containing surfaces which can bind clotting factors and facilitate procoagulant complex assembly and thrombin formation (Zwaal et al., 1992). Thrombin is the key procoagulant enzyme that allows the conversion from soluble fibrinogen to insoluble fibrin clot that reinforces the plug (Davie et al., 1991).

Therefore, the switch which triggers the secondary homeostasis depends on the loss of lipid asymmetry in the platelet plasma membrane and TMEM16F is the specific scramblase involved in this process (J. Suzuki et al., 2010a). Indeed, Scott syndrome, an autosomal bleeding disorder, is caused by deficient Ca^{2+}- dependent PS externalization in activated platelets (Wielders et al., 2009). Scott patients show a homozygous null mutation or complex heterozygous mutations of the tmem16f gene (J. Suzuki et al., 2013), and platelets from Scott patients exhibit defective microparticles release (Sims et al., 1989), suggesting that PS exit and/or TMEM16F activity are also involved in microparticle formations.

3.2 Reversible loss of membrane asymmetry in biological processes

While PS exposure is massive and irreversible in both apoptosis and blood coagulation, increase findings have demonstrated that the loss of PM asymmetry can be also a transient, reversible, and localized cellular response to specific stimuli in living cells. This effect, that involves transient changes in PM asymmetry, plays a key role in a variety of physiological contexts. We will discuss below some examples to better understand the ubiquity of this phenomenon.

3.2.1 PS-mediated cell fusion: Muscle development & Mammalian fertilization

Transient PS exposure is associated with promoting cell-cell contact and fusion. This effect is notable during myogenesis, in which mononucleated precursor cells, called myoblasts, fuse together to form elongated multinucleated syncytia known as myotubes (Abmayr & Pavlath, 2012). In 2001, Van den Eijnde and co-workers have demonstrated that differentiating C2C12 and H9C2 myoblasts reveals a transient expression of PS at the cell surface, mainly at cell-cell contact areas, and this exposure takes place prior to fusion of individual myoblast into multinucleated myotubes. Moreover, myotube formation could be abrogated *in vitro* by masking external PS with AnnexinV, which binds with strong affinity to PS, suggesting that PS on the myoblast cellular surface induces signaling to promote fusion with neighboring myoblasts (van den Eijnde et al., 2001). The mechanism mediating myoblast fusion during muscle growth and regeneration, involves the recognition of PS by PS-receptor, like BAI1 and Stabilin-2 (Hochreiter-Hufford et al., 2013; Park et al., 2016). In addition, the PS scrambling between the two leaflets of myoblast plasma membrane seems to control PIEZO1 activation/deactivation. PIEZO1 is a mechanosensitive plasma membrane Ca^{2+} channel and, acts as a key regulator of myotube formation, preventing uncontrolled fusion of myotubes and governing a proper morphogenesis when activated. The transient PS exposure seems to mediate PIEZO1 deactivation, while the inward translocation of PS, driven by ATP11A, is required for PIEZO1 activation (Tsuchiya et al., 2018). These data indicate that PS flip-flop acts as a switch for PIEZO1 activation/deactivation during myogenesis.

Plasma membrane of mammalian spermatozoa is characterized by an asymmetric distribution of phospholipids like other mammalian cells (Gadella et al., 1999). Upon capacitation, a transient PS exposure was observed on the head region of sperm cells (Gadella & Harrison, 2000). PS is exposed on the head region of viable and motile sperm cells and PS exposure increases during sperm transit through epididymis (Rival et al., 2019). PS scrambling is needed for the sperm-egg fusion and masking PS on sperm completely inhibited the fertilization. Sperm-egg fusion needs PS receptors (such as BAI1, CD36, Tim-4 and Mer-TK), on oocytes to bind PS exposed on sperm cells which triggers a signaling pathway leading to fusion of the two cells (Rival et al., 2019). Intriguingly, it has been shown that PS exposed on sperm cells can be recognized by the BAI1/3 receptors on myoblasts, mediating the fusion between them (Rival et al., 2019). PS externalization and the recognition by its receptors is a general mechanism for cell-cell fusion and it is involved in several processes like in macrophages fusion (Helming & Gordon, 2009), osteoclasts fusion

(Wu et al., 2010), placental syncytium-trophoblast formation from trophoblasts fusion (Lyden et al., 1993) and, myoblasts fusion (Hochreiter-Hufford et al., 2013) as described before.

3.2.2 Immune signaling

Several studies showed that a redistribution of PS on the outer PM leaflet occurs in immune cells upon their activation. For example, following the stimulation with the chemoattractant fMLP, an outward translocation of PS was observed in neutrophils (Frasch et al., 2004; Stowell et al., 2007). Such exposed PS co-localized with PLSCR1 at neutrophil uropod, suggesting that PLSCR1 can be the responsible of phospholipid flip-flop in activated neutrophils. However, although the PS egress appears to be a hallmark of activated neutrophils, the function of this lipid remodeling during their activation is completely unknown.

In mast cells, calcium ionophores, like ionomycin, induced both PS exposure and degranulation of secretory granule suggesting that PS externalization depends on factors downstream of Ca^{2+} and, thus can be potentially related to exocytosis process (Martin et al., 2000). In good agreement with these findings, Amir-Moazmi demonstrated that, knocking-down PLSCR1 expression resulted in a significantly impaired degranulation response in mast cells. Although the underlying mechanism is unclear, these results correlate PS egress, PLSCR1 expression and degranulation in response to stimuli, suggesting a potential role of the PLSCR1-mediated PS-egress in mast cell degranulation.

3.2.3 Synaptic maintenance and pruning

The development and function of nervous system involves the regulation of synapse formation, elimination of supernumerary synapses, correct responses to injury and inflammation and, the maintenance of proper synaptic connections. In most of these processes, a transient and/or localized PS exposure has been observed. For example, in dorsal root ganglion neuronal culture, PS exit occurs locally on injured axons (Shacham-Silverberg et al., 2018) in order to target them for their subsequent removal by phagocytes. The direct blocking of PS accessibility in these neurons, reduces axonal debris engulfment leading to an incorrect removal (Shacham-Silverberg et al., 2018). This ectopic PS exposure is not exclusive to damaged axons, but also occurs at distal dendrites, when injured (Sapar et al., 2018). Therefore, following injuries, such PS redistribution

plays an important role in labeling both damaged axons and dendrites, in order to ensure proper removal of debris.

Synaptic pruning is the process of synapse elimination occurring in the developing brain to refine the synaptic network and neuron connectivity. Alterations in the synaptic pruning are related to various neurodevelopmental diseases, including autism spectrum disorder (ASD), schizophrenia and Alzheimer’s disease (Penzes et al., 2011). Co-culturing microglia and hippocampal neurons results in microglia-mediated synapses reduction, given the lower spine density and excitatory post synaptic currents (EPSCs) frequency (Filipello et al., 2018). Moreover, Filipello and co-workes demonstrated that the supernumerary synapses elimination is mediated by microglial-TREM2 receptor (Triggering receptor expressed on myeloid cells 2). Mice lacking TREM2, a transmembrane receptor that can bind different anionic ligands, displayed enhanced excitatory neurotransmission and increased synapse density (Filipello et al., 2018). This process appears to involve an highly localized PS externalization on the outer leaflet of synaptic membranes, marking them for removal by microglia (Scott-Hewitt et al., 2020). Synapses elimination could be partially decreased by masking the PS exposed with AnnexinV or through microglial loss of TREM2, suggesting that local synaptic PS externalization in a developmentally regulated manner, contributes to microglial TREM2-mediated recognition of neuronal structures and subsequent elimination (Scott-Hewitt et al., 2020). Together, these results demonstrate that locally PS externalization could be a common neuronal signal that occurs during the developmental pruning.

A remaining question is the identification of the upstream mechanism, which might potentially include flippases and scramblases deactivation and/or activation, that trigger PS exposure at the synapse during development. A recent work has shown that CDC50A, a flippase chaperone, is required for synapse maintenance by regulating PS externalization. Indeed, silencing of CD50A by RNAi knockdown in neuronal culture, causes both PS exposure at synapses and an aberrant synapses elimination (Li et al., 2021). Among flippases, ATP8A2 and ATP8B2 are largely present in brain (Segawa et al., 2016) and missense mutations of ATP8A2 are found in cerebellar ataxia, cognitive impairment and hyperkinetic movement disorder (McMillan et al., 2018). Therefore, they could be two potential candidates involved in such mechanism, however further studies are needed to identify the flippase(s) implicated in PS externalization at synaptic plasma membrane during the pruning.

3.2.4 Calcium-regulated exocytosis

In most of aforementioned mechanisms, the PS exposed at the cell surface allows its recognition by receptors or proteins to trigger a cellular response (phagocytosis, synaptic pruning, apoptosis, blood coagulation). PS externalization has been observed in other cellular system in which this kind of “ligand-receptor” function can not be invoked to explain the role of PS egress. It is the case for some excitable cells where a lipid remodeling leading to the loss of the PM asymmetry was observed following cell stimulation. For example, as mentioned before, Martin and co-workers, clearly demonstrated that immune cell degranulation is accompanied by PS exposure to the cell surface (Martin et al., 2000). In neurons, Lee and co-workers showed that approximately 35% of PS in the inner leaflet moved to the outer leaflet of the nerve terminals when stimulated (Lee et al., 2000). Lipid scrambling occurs also upon stimulation of neuroendocrine cells (Ceridono et al., 2011; Malacombe et al., 2006; Ory et al., 2013) indicating that PS egress may have a function during the release of active compounds by these cell types. Functional and genetic evidences support a role for PLSCR proteins in the loss of plasma membrane asymmetry. For example, in RBL-2H3 rat mast cells, Kato et al. have demonstrated that PLSCR1 catalyzes trans-bilayer movement of PS and is required for hexosaminidase release (Kato et al., 2002). In contrast, while PLSCR1 is needed for PS egress in response to stimulation in chromaffin cells, it is not required for exocytosis (Ory et al., 2013).

Although the role of PS egress is completely unknown, these findings suggest that lipid scrambling mediated by lipid scramblases might control regulated secretion and the associated compensatory endocytosis. In the next section, I will summarize the molecular mechanisms controlling exocytosis and endocytosis in neurons, and how both processes are coupled.

4. Exocytosis and endocytosis at the presynaptic terminal

Brain functions, like memory storage and processing of sensory information, depends on the ability of neurons to communicate with each other through synaptic structures that are specialized contact sites between neurons, where neurotransmitter are released following electrical stimuli. Neurotransmitter release is mediated by Ca^{2+} -dependent exocytic fusion of synaptic vesicles (SV) with the pre-synaptic plasma membrane in specific sites defined as active zones (AZs) (Murthy & De Camilli, 2003). Upon stimulation, regulated exocytosis is triggered within milliseconds after membrane depolarization, and compared to constitutive exocytosis in non-excitable cells, regulated exocytosis must be controlled by specialized machinery that enables fast, Ca^{2+} -dependent, and spatially restricted exocytosis. To sustain neurotransmission, it is fundamental that SV fusion is followed by the retrieval of plasma membrane known as compensatory endocytosis. It is crucial to maintain plasma membrane homeostasis, to ensure the correct alignment of the pre- and postsynaptic zones, to replenish the releasable vesicular pool and to clear release sites from exocytic material in a timely manner (Dittman & Ryan, 2009; Kononenko & Haucke, 2015; Murthy & De Camilli, 2003). Despite countless studies, the mechanisms by which SV are recycled remain ill-defined and controversial. Several works suggest that compensatory endocytosis occurs by diverse mechanisms (e.g., clathrin-independent endocytosis, clathrin-mediated endocytosis, bulk endocytosis)(Kononenko & Haucke, 2015; Murthy & De Camilli, 2003) and acts on various time-scales (from hundreds of milliseconds to tens of seconds) depending on synapse and/or stimulus (Delvendahl et al., 2016; Wu et al., 2014).

On the other hand, regulated exocytosis is highly controlled in space and in time and occurs exclusively at the presynaptic AZ, a platform which links Ca^{2+} influx to SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptors)-driven SVs fusion. AZ cytomatrix is a protein dense structure attached to the presynaptic plasma membrane, containing ELKS proteins, Piccolo-Bassoon family, RIM and RIM binding proteins, Munc13 and Liprins (Gundelfinger et al., 2015; Held & Kaeser, 2018; Zarebidaki et al., 2020). Intriguingly, lot of these AZ scaffold proteins are linked directly or indirectly to SV endocytic machinery, suggesting that AZ cytomatrix could be a site to coordinate exocytosis and endocytosis (Gundelfinger et al., 2003; Haucke et al., 2011; Maritzen & Haucke, 2018). However, the space-time coupling of exocytosis and endocytosis is still not completely elucidated. Diverse models have been proposed in the last decades and the emerging one highlights that several factors might be involved in this coupling, including Ca^{2+}, phospholipids, cytoskeleton as well as SNARE proteins. We will discuss about

the coupling of exocytosis and endocytosis and the involvement of these factors, after briefly reviewing the main mechanisms of exocytosis and endocytosis in neurons.

4.1 Calcium-regulated exocytosis

Vesicle exocytosis regulates many biological processes, including the secretion of transmitters from neurons, which mediates synaptic transmission, essential for brain function (Chanaday et al., 2019). The general scheme of Ca^{2+} -regulated exocytosis comprises the translocation of the synaptic vesicles towards the active zone (AZ), their docking, priming and fusion with the plasma membrane in response to Ca^{2+}- influx (Fig.16) (Gundelfinger et al., 2003).

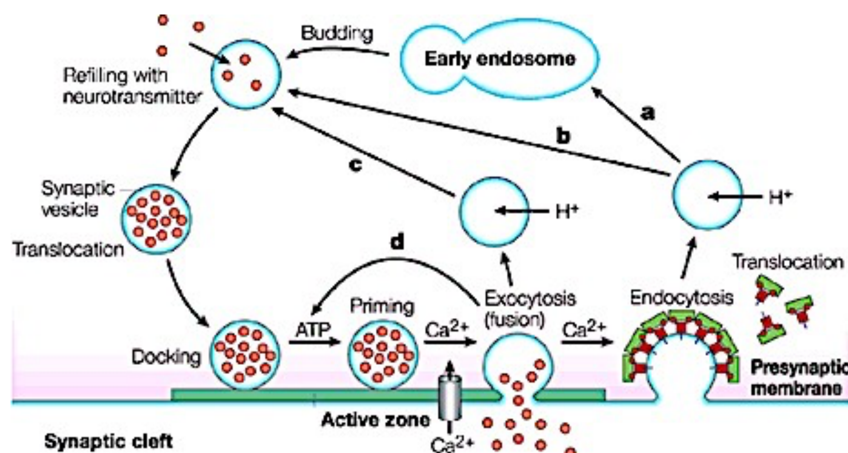

Fig.16 Schematic representation of regulated-exocytosis and subsequent endocytosis at presynaptic terminal. Synaptic vesicles are translocated to the active zone, docked, and primed. In response to Ca^{2+} influx, they undergo exocytosis and release neurotransmitters into the synaptic cleft. Following exocytosis, SVs were retrieved by endocytosis which occurs outside the active zone. *Adapted from Gundelfinger et al.,2003*

- *Synaptic vesicle pools*

Synaptic vesicles can be classified in pools according to their capacity to fuse with the plasma membrane. Ready-releasable pool (RRP) is defined as the first line of vesicles that fuse in response to stimulation. Depending on the synaptic model, the size of the RRP can differs. For example, the calyx of Held shows a very large RRP with 700 to 5000 SVs whereas in GrC

synapses, the RRP consists of 4–8 SVs (Doussau et al., 2017). In small central synapses, like in hippocampal synapses, the size of RRP corresponds to the number of docked vesicles at the AZ. The size of the AZ and the number of docking and release sites correlates with the release probability and synaptic strength (Holderith et al., 2016). Once the RRP is depleted, neurons can release neurotransmitters as fast as the RRP is refilled. The number of released vesicles in response to an AP and the speed of RRP refilling are therefore key determinants of synaptic strength. The second pool of vesicles is the reserve pool (RP), which can fuse in response to mild to sustained stimulation, and conventionally, is considered as the major source for RRP replenishment (Marra et al., 2012). The RRP and reserve pool, which occupy ~ 0,5% and ~10- 60% respectively of the total amounts of vesicles (Marra et al., 2012), constitute the whole releasable pool. It has been proposed that between the RP and RRP, there is another vesicular pool, called the (IP) intermediate pool (~ 2.3 times RRP size), which mediates RRP refilling. Indeed, IP depletion causes a slower RRP replenishment after intense stimulation (Guo et al., 2015). Synaptic vesicles not belonging to the "recycling pool", form the so-called "resting" or "dormant" pool, a group of vesicles that fuses with PM only after strong, high frequency repetitive stimulation or never at all (Fredj & Burrone, 2009). However, the functional role of this inactive vesicular pool is still controversial. It can act as a reserve for neurotransmitter and synaptic proteins (Denker et al., 2011) as well as it can have a potential role in spontaneous release (Fredj & Burrone, 2009) (see section: modes of neurotransmitter release).

Increased findings have shown that an additional pool, called "reluctant pool", is present in high frequency synapses, like Granular cell (GC) – Purkinje cell synapse. It has been demonstrated that this pool is rapidly recruited during intense activity and is essential to sustain stimulation at high frequencies (Doussau et al., 2017). Nevertheless, whether this pool constitute a sub-pool of RRP is still a matter of debate (Neher, 2015; Pan & Zucker, 2009).

While the RRP is located in close vicinity of the AZ, the resting and reserve pools do not appear to have a specific location at the presynaptic terminal, although some studies suggest that the RP is closer to the AZ than the resting pool (Schikorski & Stevens, 2001). Moreover, nanoscale imaging approach combined with advanced imaging analysis in hippocampal synapses, have shown that vesicles can fuse at different AZ sectors depending on the intensity of the stimulation (Maschi & Klyachko, 2017). Under high-frequency stimulation, vesicles fusion mainly occurs in the periphery of the AZ (Maschi & Klyachko, 2017), suggesting that diverse spatiotemporal properties can characterized each synaptic vesicle pool.

4.1.1 Modes of neurotransmitter release: molecular basis of release diversity

There are three primary modes of neurotransmitter release, called synchronous, asynchronous, and spontaneous release. The release modes share key fusion processes (docking, priming and fusion) but rely on different SNARE proteins, Ca^{2+} concentration and Ca^{2+} sensor for SV fusion.

- *Synchronous release*

Neurotransmitter release is triggered by action potential (AP) arrival to presynaptic terminal, where it causes a brief opening of voltage-gated channels, resulting in a local rise of Ca^{2+}. It creates calcium nanodomains, which lead to simultaneous fast neurotransmitter release and rapid post synaptic response (Fig.17).

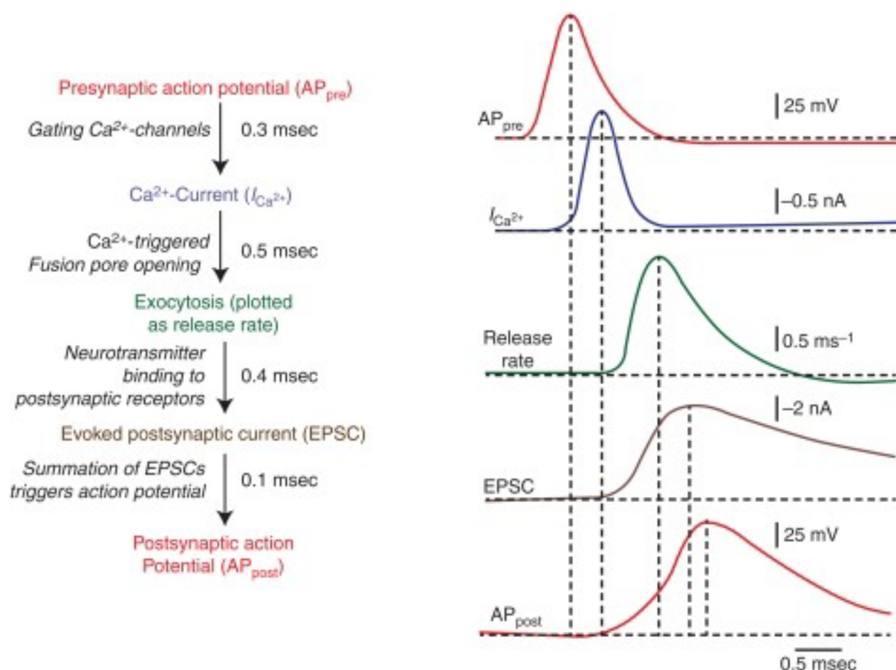

Fig.17 Time course of synchronous release. Schematic representation (left) and time course (right) of synchronous synaptic transmission as measured by pre- and postsynaptic patch-clamp recordings at the synapse of calyx of Held. *From Sudhouf, 2012*

There is a synchronous coupling between the action potential arrival and neurotransmitter release (Kaeser & Regehr, 2014). Synchronous release depends on a complex machinery, which is largely conserved among different neurons and relies on Soluble N-ethylmaleimide sensitive factor Attachment protein REceptor (SNARE) proteins, Synaptotagmins and Sec-1 / Munc18 proteins.

SNARE proteins, that are localized in opposing membranes, drive membrane fusion when two t-SNAREs located at the PM (such as SNAP25 and Syntaxins) form a ternary complex with a v-SNARE located at the vesicular membrane (synaptobrevins or VAMPs) (Fig.18)

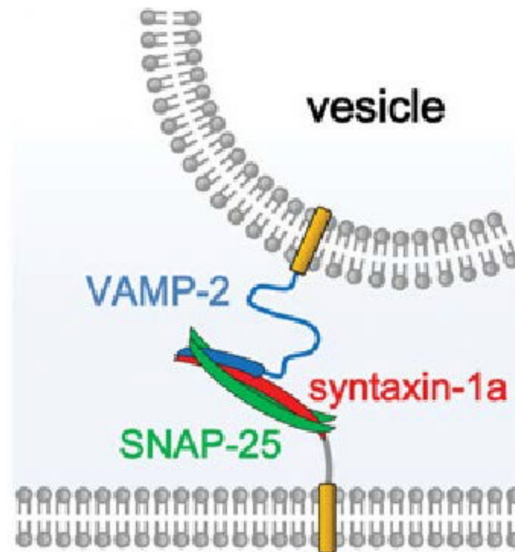

Fig.18 Ternary SNARE complex. *Adapted from Shin et al., 2014*

The SNARE complex bridges the membranes and the free energy released by the formation of the four helix bundle following a zipper-like progression drives membrane fusion (Reubold et al., 2015). The canonical SNARE complex involved in SV fusion with the plasma membrane is formed by the t-SNARE syntaxin-1 (Stx1) and SNAP-25 located at the PM and the v-SNARE synaptobrevin 2 (also called VAMP2) located on the synaptic vesicle. Stx1 consists of a C-terminal transmembrane domain, a SNARE domain (H3 domain), an α-helical structure and a Habc domain with an N-terminal containing a short N-peptide (Weimbs et al., 1997). This latter domain interacts with H3 domain to maintain Stx1 in a "closed conformation" that needs to be released for the assembly of SNARE complex.

The Sec-1 / Munc18 protein family plays a crucial role in Stx1 regulation. Munc18, whose three mammalian isoforms are known (i.e., Munc18-1, Munc18-2, Munc18-3) is required for neurotransmitter release and was found as a binding partner of the plasma membrane Stx1 (Südhof & Rothman, 2009). When Munc18 binds Stx1 through its central groove, it keeps Stx1 in a 'closed conformation', preventing its binding to SNAP-25 and VAMP2 (Toonen et al., 2005). During the priming, Stx1 switch to an "open conformation" to form a highly stable complex with SNAP25 and VAMP2. In 2011, Ma and co-workers have proposed a model according to which the

Munc13-1 MUN domain, accelerates the transition from the Stx1–Munc18-1 complex to the SNARE complex, through its weak interactions with H3 of Stx1 (Ma et al., 2011) (Fig.19). Therefore, both Munc18-1 and Munc13-1 cooperate to tune SNARE complex assembly, allowing an accurate regulation of neurotransmitter release.

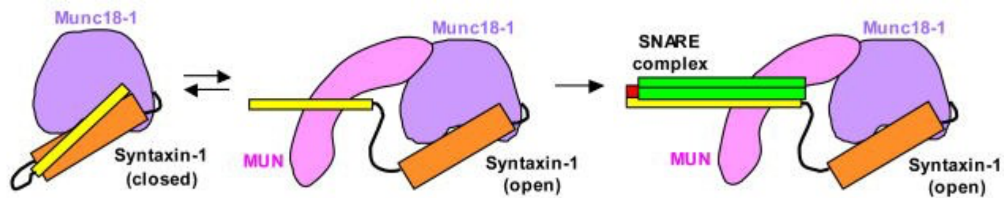

Fig.19 Proposed model for Sxt1 switch to open conformation through MUN domain. MUN domain (pink) promotes SNARE complex formation through its interaction with both Syntaxin-1 and Munc18-1. *Adapted from Ma et al., 2011*

Rab3A, is a small G-protein localized to SV, also required in the late steps of exocytosis (Huang et al., 2011). Several studies have shown that Rab3A, in association with Munc13-1 and Munc18-1, is mainly involved in SV priming. However, the molecular mechanism by which these proteins interact with each other to accomplish this function has remained unclear (Huang et al., 2011).

At the end of the priming, a fusion-ready and highly stable complex (Rizo & Südhof, 2012) awaits the final signal to continue the fusion process. Upon AP arrival at the presynaptic terminal, rapid Ca^{2+} entry triggers the full zippering of the SNARE complex and lead to vesicular and plasma membrane merging. The Ca^{2+}-sensor responsible for coupling between Ca^{2+} sensing and fusion in neurons is Synaptotagmin 1 (Syt1) (Südhof, 2012). SytI is a synaptic vesicle protein composed of a transmembrane domain, a linker sequence and two classical C2 Ca^{2+} -binding domains (C2A and C2B) at the C-terminal, which can also bind to phospholipids, especially PIP_2 and PS (Honigmann et al., 2013). By interacting with PIP_2 clusters that are formed at PM upon intracellular Ca^{2+} increase, Syt1 mechanically promote SNARE zippering leading to membrane merging (Honigmann et al., 2013). Syt1 mutant mice revealed that synchronous release was strongly impaired in hippocampal neurons, whereas asynchronous and spontaneous releases were unaffected (Geppert et al., 1994), indicating that Syt1 represent the Ca^{2+}-sensor responsible for synchronous coupling.

Finally, a fusion pore opens and expands to release the vesicular contents to the extracellular space. The synchronous release of neurotransmitters depends on various critical factors such as

the availability of a readily releasable pool (RRP) as well as the tight spatial organization of the release site containing a fast Ca^{2+} sensor close to presynaptic Ca^{2+} channels.

Concerning the availability of readily releasable vesicles, two classes of molecules are determinant to sequester SV in the RRP: AZ proteins, including Munc13, RIMs, liprins, ELKS and bassoon/piccolo that form a large multi-molecular scaffold complex to attract SV, and SNARE proteins with their regulators which transiently anchors vesicle to the AZ. Evidences come from mice lacking Munc13 for instance, in which glutamatergic hippocampal neurons have highly reduced RRP (Augustin et al., 1999). SNARE complex assembly represents the second critical step for vesicles to enter RRP (Südhof & Rothman, 2009), and is undoubtedly critical for synchronous release, given that deletion of SNAREs dramatically impairs SV exocytosis in response to action potential (Bronk et al., 2007; Schoch et al., 2001; Zhou et al., 2013).

Moreover, the synchronous release is also ensured by a tight spatial organization between Ca^{2+} sensor and presynaptic Ca^{2+} channels. For example, GABAergic presynaptic terminal of hippocampal basket cells represents a good example of a tight spatial organization. Indeed, it has been reported that when Ca^{2+} enters, it forms domain in the range of 10-20 nm (called nanodomains), where one Ca^{2+} channel was directly coupled to a single vesicle to ensure an efficient coupling (Bucurenciu et al., 2008).

To summarize, the “canonical “SNAREs and the Syt1 are mostly involved in synchronous release, since the deletions of one of these, leads to a strong impairment of neurotransmission throughout the central nervous system (Bronk et al., 2007; Schoch et al., 2001).

However, a small fraction of vesicle is still able to fuse, suggesting that non-canonical SNAREs and diverse synaptotagmin isoforms are involved in other modes of release.

- *Asynchronous release*

In most synapses, synchronous release accounts for almost all release (> % 90) at low-frequency stimulation but sustained moderate- to high- frequency stimulation evokes asynchronous release (Atluri & Regehr, 1998; Barrett & Stevens, 1972), which continue for tens to hundreds of milliseconds after the AP, depending on neuron type and on the previous synaptic activity (Goda & Stevens, 1994; Kaeser & Regehr, 2014). Moreover, this mode of release is also prominent in specialized synapses such as in glutamatergic synapses onto magno-cellular neurosecretory cells in the hypothalamus. In these synapses the asynchronous release is important to prolongate glutamate release which, in turn, generates a large and prolonged postsynaptic

depolarization in response to brief high-frequency bursts of presynaptic activity (Iremonger & Bains, 2007).

SV, that fuses through synchronous or asynchronous release, belong to the same releasable pool (RRP + reserve pool) (Otsu et al., 2004), but differ in SNARE proteins and Ca^{2+}-sensors with which they are equipped. Instead of Syt1 for synchronous relase, the major Ca^{2+}-sensor for asynchronous release is Synaptotagmin 7 (Syt7) (Luo et al., 2015). To monitor exclusively asynchronous release, Bacaj and co-workers have performed experiments in hippocampal neuronal knock-downed (KD) for Syt1 expression where synchronous release is blocked. Syt7 KO or KD suppressed asynchronous release in both Syt1-deficient excitatory and inhibitory hippocampal neurons. In WT hippocampal neurons, KD of Syt7 resulted in a decrease in asynchronous release only during extended stimulus train, suggesting that Syt7 is important for asynchronous release during intense stimulation in both excitatory and inhibitory synapses, even in WT neurons (Bacaj et al., 2013).

In hippocampal neurons from mouse embryos, the VAMP4 protein, a non-canonical SNARE, seems to preferentially drive asynchronous release and recycling, independently from VAMP2, complexin and Syt1 (Raingo et al., 2012).

Moreover, in glutamatergic hippocampal neurons, SNAP23/Syt7 complex have been related to asynchronous release, unlike SNAP25/Syt1 for synchronous one, although SNAP25 and SNAP23 function is in part overlapped (Weber et al., 2014). Altogether these findings show that the two release modes involve different isoforms of exocytosis machinery components. How SV acquire different v-SNARE and synaptotagmin is currently unknown but it may result from various modes of SV retrieval and protein segregation which occurs during intense stimulations (Nicholson-Fish et al., 2015a).

- *Spontaneous release*

Spontaneous release is involved in diverse functions, including regulation of neuron excitability as well as synaptic stabilization and long-term forms of synaptic plasticity (Farrant & Nusser, 2005; Reubold et al., 2015). This mode of neurotransmitter release is independent of presynaptic action potential (AP) and exhibit a variable regulation by Ca^{2+} (Kavalali, 2015). Spontaneous synaptic vesicles seem to be morphologically identical from vesicles fused after an AP stimulation. But despite their morphological similarities, the spontaneous and the evoked

vesicle pool identity is conserved after fusion and retrieval (Afuwape et al., 2017; Virmani et al., 2005).

It is indeed assumed that the spontaneous vesicles belong to the so-called "resting or dormant pool" that is normally not mobilized by neuronal activity. Using hippocampal culture, Fredj and co-workers found that, after a total depletion of recycling or releasable pool upon strong stimulation, spontaneous release drew vesicles precisely from the resting pool (Fredj & Burrone, 2009). Moreover, the non-canonical SNARE VAMP7 is enriched in the resting pool (Hua et al., 2011) and regulates selectively spontaneous release of neurotransmitter in a regulatory pathway that involves the Reelin glycoprotein and SNAP25 (Bal et al., 2013). Another non-canonical SNARE, Vti1a, also enriched in vesicles from resting pool, guides vesicles to a robust spontaneous recycling in hippocampal neurons (Ramirez et al., 2012). Furthermore, Vti1a does not interact with the canonical SNARE Stx1, SNAP25 and VAMP2 (Antonin et al., 2000) but binds VAMP4, and syntaxins 6 and 16 (Kreykenbohm et al., 2002), forming a specific SNARE complex for spontaneous release.

The Ca^{2+}-regulation of spontaneous release is still an open question: some studies reveal that a fraction of spontaneous release is Ca^{2+}-independent, but other ones have been shown that spontaneous fusion requires Ca^{2+} (Reese & Kavalali, 2015). The Ca^{2+} source for spontaneous release could come from internal stores (Reese & Kavalali, 2015), or from stochastic openings of voltage gated Ca^{2+}-channels as shown in inhibitory synapses (Ermolyuk et al., 2013). Genetic deletion of the Ca^{2+}-sensors Doc2 and Doc2 related protein family impairs spontaneous but not evoked release in hippocampal neurons, favoring the Ca^{2+} dependency hypothesis for this type of exocytosis (Ramirez et al., 2017).

4.1.2 Modes of vesicle fusion

Following an AP, docked and primed SVs fuse with the plasma membrane to release their content. The two major modes of fusion in neurosecretory cells are the *Kiss and Run* and the *Full fusion* or *collapse,* each of which has different consequences for plasma membrane expansion and cargo secretion. Indeed, the full fusion implies that the fusion pore formed between SV and the PM expands until vesicle flattens into the plasma membrane leading to the full insertion of the vesicle membrane into the plasma membrane. Instead, during kiss and run, the fusion pore does not expand but rapidly closes before the vesicle membrane can flatten into the plasma membrane.

Both processes were first described from electron microscopy observations of the frog neuromuscular junction in early 70's.

The full vesicle collapse represents the major mode of SV fusion in neurons. This model was proposed by Heuser and Reese in 1973. Vesicles with omega-shaped profiles with narrow or wide necks, led Heuser and Reese to propose a model in which fusion pore opening was followed by the full collapse of the vesicle into the plasma membrane (Heuser & Reese, 1973), now referred as *full-vesicle fusion* (FVF). Later, the same group confirmed FVF at the frog neuromuscular junction, mainly based on freeze-fracture electron microscopic (EM) observations. They reported the presence of an increased amount of coated pockets with diameters ranging from 60 to 120 nm (larger than regular SV with diameters of ~50 nm) after the stimulation (Heuser & Reese, 1981), suggesting that such pockets could result from the fully collapse of the SV vesicles into the PM.

A careful quantification of vesicle and synaptic surfaces leads to the hypothesis that a mechanism takes place to retrieve the membrane newly added to the plasma membrane to maintain the synapse area constant. The concept of compensatory endocytosis was born.

In the meantime, another mode of fusion has been proposed, the *Kiss and Run (KNR)*, based on the identification of uncoated omega-shaped membrane profile with a narrow neck connected to the plasma membrane at the active zone of the frog neuromuscular junction (Ceccarelli et al., 1972). Since then, the existence of KNR mode of fusion for SV is highly debated. The first convincing results came from membrane capacitance measurements, which reports the change in surface area on neurons. Observation of capacitance flickers, during which a pore conductance corresponding to a narrow pore of 0.5-3 nm diameter was sometimes detected (Klyachko & Jackson, 2002). Evidences for this mode of fusion are more convincing when looking at the release of neurotransmitters following the fusion of large dense core (LDCV) in neuroendocrine cells. LDCV are larger vesicles (250 nm) allowing both electrophysiological recordings and direct fluorescent imaging of the exocytotic process. By combining amperometry, that measures the content release of a single secretory granule and capacitance, Ales et al. (1999) showed that capacitance flickers (small and short capacitance variation) were associated to the release of neurotransmitters by KNR in chromaffin cells (Fig.20) (Alés et al., 1999).

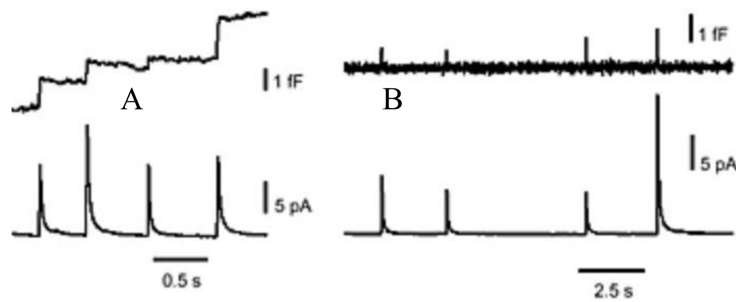

Fig.20 Catecholamine release during fast KNR and FVF in chromaffin cells. Top, capacitance traces. **Bottom**, detection of catecholamines by amperometry. **A.** Irreversible fusion events showing large and stable increases in capacitance (top) associated to neurotransmitter release (bottom) measured by amperometry **B.** Fast kiss-and-run fusion events detected as small and short capacitance variations (top) but still associated to release of neurotransmitter measured by amperometry (bottom). *From Alés et al., 1999*

Additional evidences and maybe the more direct ones came from live cell experiments in which protein in the membrane of secretory granule like phogrin fused to GFP (GFP-Phogrin) was observed by TIRF microscopy during exocytosis. Upon cell stimulation, whereas the content of the secretory granule was released, the GFP-Phogrin did not diffuse into the plasma membrane, indicating that membrane of secretory granule can preserve its shape (Tsuboi & Rutter, 2003). More recently, several modes of exocytosis have been reported for LDCV including various steps between KNR and FVF (Shin et al., 2020). While the presence of the KNR is well established in neuroendocrine cells for LDCV exocytosis, its role is still debated for SV at synapses. The signature of kiss-and-run is a transient fusion pore opening and closing, as detected with the cell-attached capacitance recording technique in endocrine cells. Application of this technique to synapses has two major drawbacks. First, the small size of vesicles makes it more difficult to resolve individual vesicle fusion. Second, the postsynaptic neuron facing the presynaptic release site makes it difficult for the electrode to access the release site. At small hippocampal synapses, KNR has been supposed on the basis of little release of FM dye, a fluorescent molecule that insert into the outer leaflet of phospholipid bilayer membranes and strongly fluoresces in the hydrophobic environment which allows vesicular recycling tracking (Zhang et al., 2009). Indeed, one characteristic of KNR compared to FVF is that it prevents, , the escape of preloaded FM dye molecules following the fusion event. Additional evidences come from live cell imaging of pHluorin-tagged SV proteins. pHluorin is a pH-sensitive GFP. When fused to the luminal side of

SV proteins, it allows for monitoring the kinetics of exo-endocytosis. Acidic lumen of the vesicle quenches the pHluorin and upon exocytosis, pHluorin is unquenched leading to fast rise of fluorescence. pH-dependent fluorescence variation is a good estimate of the kinetics of exocytosis and endocytosis SV cycle. Since fast increase and decrease of pHluorin fused to the SV protein synaptophysin were observed at synapses, authors proposed that fluorescence changes could reflect a transient pore opening and closure, consistent with the KNR fusion (Zhang et al., 2009). Although debated, KNR would be a convenient mechanism to prevent SV pool depletion during stimulation at high frequency. Indeed, the speed of endocytosis might limit the capacity of a synapse to sustain neurotransmission during high AP firing. Consistent with this hypothesis, based on FM dye uptake experiments and imaging, it has been proposed that KNR operates largely on the RRP vesicles in hippocampal synapses, resulting in their local recycling and reuse for multiple rounds of fusion (Pyle et al., 2000). Other imaging studies are not in agreement with this hypothesis. To determine whether endocytosis taking place in hippocampal synapses was a kiss-and-run form, Fernandez-Alfonso and co-workers preloaded with FM 1-43 (FM dye molecule) the releasable SV pool (RP) and then examined the ability of the RP to discharge FM dye under sustained stimulation. Indeed, destaining of FM dye would have been prevented during repetitive stimuli if KNR took place. On the contrary, they observed FM dye destaining during stimulation, indicating that SVs completely collapse into the PM refuting the kiss-and-run hypothesis (Fernández-Alfonso & Ryan, 2004). These discrepancies questioned whether the KNR apply to SV and whether it represents a significant fraction of SV exo-endocytosis at synapses.

4.2 Compensatory endocytosis

To sustain neurotransmission, synapses require a constant supply of SVs filled with neurotransmitters. This supply is kept at the presynaptic terminal through membrane endocytosis, sorting of cargos and rapid neurotransmitter refilling of newly formed SVs. The entire process of SV reconstitution and refilling is called “SV recycling” and the mechanisms underlying SV recycling have been intensely studied but also debated since the 1970s. The current view considers that, at least four mechanisms fulfil this task, distinguished by their molecular machinery and speed: clathrin-mediated endocytosis (CME) which is relatively slow (10-30s), Kiss and Run (< 1-2 s), ultrafast endocytosis (within 50-100 ms) and activity-dependent bulk endocytosis (ADBE) (within 1-2 s to form intracellular endosome). It is likely that all four endocytosis modes co-exist

in nerve terminals and proceed differently depending on activity levels, temperature and type of synapse (Gan & Watanabe, 2018). Regardless of the mechanism, compensatory endocytosis has two crucial functions: first, it removes fused SV membrane from AZ to restore membrane tension and to clear release sites that need to become available for subsequent SVs exocytosis (Hosoi et al., 2009) and second, it replenishes SV pools to sustain exocytosis over long periods of synaptic activity. I will first detail the mechanism of clathrin-mediated endocytosis (CME) and second, I will briefly review the different modes of compensatory endocytosis and the role that they play at synapses.

4.2.1 Mechanism of Clathrin-mediated endocytosis

After full fusion of SVs, proteins and lipids are retrieved by different endocytic pathways (briefly reviewed in the next section) and among these modes, clathrin-mediated endocytosis appears to be the prominent and consists of embedding a piece of plasma membrane in a coated-pit formed by clathrin. This pathway is characterized by five steps: initiation, stabilization, maturation, fission, and vesicle uncoating (Fig.21). The first step of endocytic event, called nucleation or initiation, starts by the clustering of endocytic proteins at the plasma membrane in order to form the "nucleus" for the assembly of vesicle coat. Many endocytic proteins are recruited to the plasma membrane by essentially binding to $PI(4,5)P_2$ enriched domains. FCHo1 and FCHo2, two proteins containing F- Bin/amphiphysin/Rvs (BAR) domains have a shallow curvature that may sculpt the initial budding sites where the clathrin machinery is recruited for coated-clathrin pits (CCPs) formation (Henne et al., 2010). Following FCHo1/2 binding proteins to the plasma membrane, scaffold proteins like Eps15, Eps15R and Intersectins 1 and 2 are recruited, which in turn interact with the C-terminal AP2-μ homology domain (μHD) of the adaptor protein 2 (AP2) (Henne et al., 2010). The heterotetrametric AP2 adaptor complex consists of a core comprising the N-terminal domains of the α- and β2-adaptins in complex with the μ2 and σ2 subunits. Long flexible linkers, referred to as hinge regions, connect the C-terminal appendage domains (ADs) of α- and β2-adaptins to the core (Collins et al., 2002). The core domain has three $PI(4,5)P_2$ binding sites on the α, β2, and μ2 subunits (Jackson et al., 2010), while αAD binds to and can recruit numerous pioneer endocytic accessory proteins (EAPs) like FCHo proteins, Eps15 and AP180 (Praefcke et al., 2004). Moreover, diverse cargo binding sites are located on the μ2 subunit, leading specific transmembrane cargo molecules to be enriched in the forming vesicle and thereby being selectively endocytosed. Also, AP180 (assembly/adaptor

protein 180) function as an adaptor proteins for SNAREs, facilitating their incorporation into CCPs (Koo et al., 2011).

Basically, cytosolic AP2 exists in a closed conformation, which cannot bind clathrin. The interaction with PM-enriched PI(4,5)P_2 is required for triggering an allosteric conformational change leading to AP2 opening which exposes the clathrin binding site on the β2 hinge as well as cargo binding sites (Kadlecova et al., 2017). In addition to protein and lipid interactions, the open conformation of AP2 is also stabilized by phosphorylation via AAK1 (A2-associated protein kinase 1) (Conner & Schmid, 2002; Jackson et al., 2010). Next, AP2 hub recruits clathrin triskelia, which polymerizes in both hexagons and pentagons to form the clathrin coat around the nascent pit (Boucrot et al., 2010). Clathrin and adaptors proteins assembly in liposome generates curved coated pits (Takei et al., 1998), suggesting that clathrin assembly might drive curvature formation during CCPs maturation. However, other studies demonstrated that clathrin assembly *per se* is not sufficient to generate membrane curvature, but EAPs containing N-BAR or ENTH domains are likely candidates (Mettlen et al., 2018). For example, the amphipatic helix of ENTH domain of epsin, targeted to areas of endocytosis by binding PI4,5P_2, directly modifies membrane curvature in conjunction with clathrin polymerization (Ford et al., 2002). Moreover, it has been shown that HSC70, a protein essential for vesicle uncoating after fission, could provide the necessary energy source to remodel clathrin lattice, transforming hexagons into pentagons to favor the change of curvature of the assembling polymeric coat (Schlossman et al., 1984). In addition, in yeast, another model in which actin polymerization drives membrane invagination, has been proposed. According to this model, the actin cytoskeleton can contribute to membrane bending during the formation of CCPs; actin filaments are nucleated by the ARP3/3 complex and polymerize at the plasma membrane generating a flow of actin into the cells. The clathrin coated membrane associated with actin network by protein like Ent1 (homologue of mammalian epsin1) and Sla2 (homologue of mammalian HIP1R), is pulled inwards by the actin network (Carlsson & Bayly, 2014). However, the exact mechanism involved in membrane invagination during endocytosis remains unclear. Once the coat has formed, a scission step occurs to generate a clathrin- coated vesicle.

N-BAR proteins, like endophilin and amphipysin, with highly curved BAR domains are recruited to promote higher curvature and dynamin recruitment (Meinecke et al., 2013).

In hippocampal neurons lacking amphiphysin 1, FM dye imaging showed a smaller size of the recycling pool of SV. This resulted from an impaired compensatory endocytosis in the absence of such endocytic protein (Di Paolo et al., 2002). Similarly, synaptophysin-pHluorin (Syp-pH) imaging in hippocampal neurons lacking the endophilin A1 and A2 isoforms (EndoA DKO),

exhibited higher fluorescence time constant decay which suggests that compensatory endocytosis was slower in their absence (Milosevic et al., 2011).

The recruited GTPase dynamin assembles into a helical collar and upon GTP hydrolysis, induces membrane scission by twisting around the neck of CCPs , and in this role, dynamin can be assisted by actin polymerization (Ferguson et al., 2009). The formation of the endocytic vesicle ends with the vesicle uncoating, which takes place through two key mechanisms: breakage of clathrin lattice by HSC70 and dephosphorylation of PIP(4,5)P_2. The recruitment of HSC70 to clathrin-coated vesicles requires auxilin or cyclin-G-associated kinase. HSC70-auxilin-clathrin complex stimulates the ATPase activity of HSC70 promoting coat disassembly (Eisenberg & Greene, 2007).

Conversion of PIP(4,5)P_2 to PI4P, by the synaptojanin phosphatase, is the second key factor involved in the endocytic vesicle uncoating (McPherson et al., 1996). Neurons lacking synaptojanin 1 showed an accumulation of coated vesicles as well as PIP(4,5)P_2 (Cremona et al., 1999), suggesting that this mechanism acts independently and occurs upstream of the auxlin-HSC70 machinery. Consistent with this hypothesis, it has been proposed that synaptojanin can participate to auxilin recruitment (Massol et al., 2006).
Following this last step, components of the clathrin machinery are then available for another round of clathrin-coated vesicle formation.

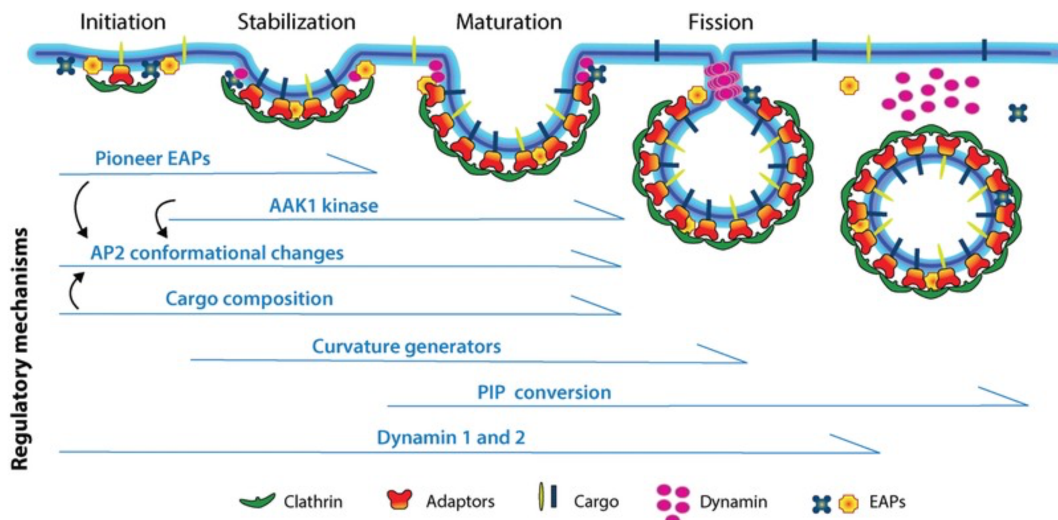

Fig.21 Stages of clathrin-mediated endocytosis and associated regulatory mechanisms. CME consists of different steps involving CCP initiation and stabilization, maturation with increase membrane curvature, fission and the uncoating of vesicle (not shown). *Adapted from Mettlen et al., 2018*

- *BAR domain proteins in endocytosis*

As described above, clathrin-mediated endocytosis relies on the sequential recruitment of proteins containing diverse BAR domains. F-BAR proteins like FCHos are recruited earlier than N-BAR proteins like endophilin or amphiphysin and proteins with BAR domains such as sorting nexin 9 (SNX9) are recruited in between (Posor et al., 2013; Taylor et al., 2011). Temporal recruitment of different BAR proteins seems to be associated to the progressive increase in membrane curvature during the coated pit formation. BAR domain proteins exhibit several roles in the endocytosis, especially at the beginning where they mark the endocytic spots (e.g. FCHo proteins) and in vesicle fission where they recruit dynamin and may serve as a direct player in CCPs scission by the so-called "mechanism friction-driven scission" (Simunovic et al., 2017).

- *Role of lipids in clathrin-mediated endocytosis*

PI(4,5)P2 acts as a spatial landmark and determine the site of endocytosis. It is necessary for the early steps of CME, including, nucleation, maturation, cargo selection and coat assembly. Its conversion into PI4P is key to vesicle scission and uncoating. Remarkably, with the exception of clathrin itself, almost all proteins involved in CME contain PI(4,5)P2 – binding sites.
CME is initiated at sites enriched in PI(4,5)P2 (Fig.22), which is synthetized from PI(4)P by type I PI 4-phosphate 5-kinases (PIPKIs)(Di Paolo & De Camilli, 2006). The first step called "the nucleation" is triggered by recruiting specific PI(4,5)P2-binding proteins such as the heterotetrameric cargo adaptor complex AP-2, FCHo1/2, CALM, and other factors that direct the assembly and invagination of the clathrin lattice (McMahon & Boucrot, 2011). During the maturation of CCPs, a complex topological remodeling of the plasma membrane occurs, from budding to elongation resulting in the formation of a vesicle with constricted membrane neck that provides a substrate for membrane fission by the GTPase dynamin. This strucutal changes are accompanied by a progressive modification of the lipid composition of the plasma membrane by converting PI4P and PI(4,5)P2 in subspecies thanks to lipid kinases and phosphatases. Selective changes in membrane lipid composition enable CME progression through differential protein recruitment. Local conversion of PI(4)P by class II phosphatidylinositol 3-kinase C2a (PI3KC2a) into $PI(3,4)P_2$ is required to progress to late-stage CCPs (Fig.22) (Posor et al., 2013). As observed by electron microscopy, loss of PI3KC2α or enzymatic depletion of $PI(3,4)P_2$ blocks CME at U-

shaped CCPs, a step before dynamin-mediated fission (Posor et al., 2013) indicating that changes in membrane lipid composition are necessary for the completion of endocytosis.

Computational modeling and super-resolution imaging demonstrated that $PI(3,4)P_2$ synthesized by PI3KC2a within the clathrin-coated area of endocytic intermediates, contribute to recruitment of the Phox (PX)-bin-amphiphysin-rvs (BAR) domain protein SNX9 and SNX18 at late-stage CCPs (Fig.22) (Schöneberg et al., 2017). Self-assembly of SNX9 promotes constriction of the endocytic vesicle neck to facilitate endocytic vesicle scission by dynamin, probably in conjunction with other BAR proteins such as endophilin. Meanwhile, Endophilin 1 recruits the predominant brain isoform of the synaptojanin 1 (Synj1), a lipid phosphatase which converts PI(4,5)P2 or PI (3,4,5)P3 into PI4P or PI(4,5)P2 respectively, to endocytic sites at the synapses (Perera et al., 2006). Interestingly, Synaptojanin 1 converts the PI(4,5)P2-to-PI4P selectively at sites of high membrane curvature (Fig.22). Local phospholipid switch favor dynamin-mediated membrane fission (Chang-Ileto et al., 2011) and concomitant auxilin-HSC70 vesicle uncoating. Auxilin, thanks to the so called "PTEN homology domain "is recruited to the clathrin-coated pit through the binding with PI3P, PI4P and $PI(3,4)P_2$. Therefore, Auxilin recruitment occurs only after dephosphorylation of $PI(4,5)P_2$and $PI(3,4)P_2$ by synaptojanin and PI4 phosphatase respectively, when PIP4P and PI3P has accumulated at membrane (Massol et al., 2006). This highlights that $PI(4,5)P_2$ dephosphorylation by synaptojanin 1 occurs upstream of both membrane fission and vesicle uncoating, synchronizing these latter events in time.

In summary, CME progression is highly regulated by changes in lipid dynamics and plasma membrane lipid composition. $PI(4,5)P_2$ metabolism and its regulation pace CME in space and time.

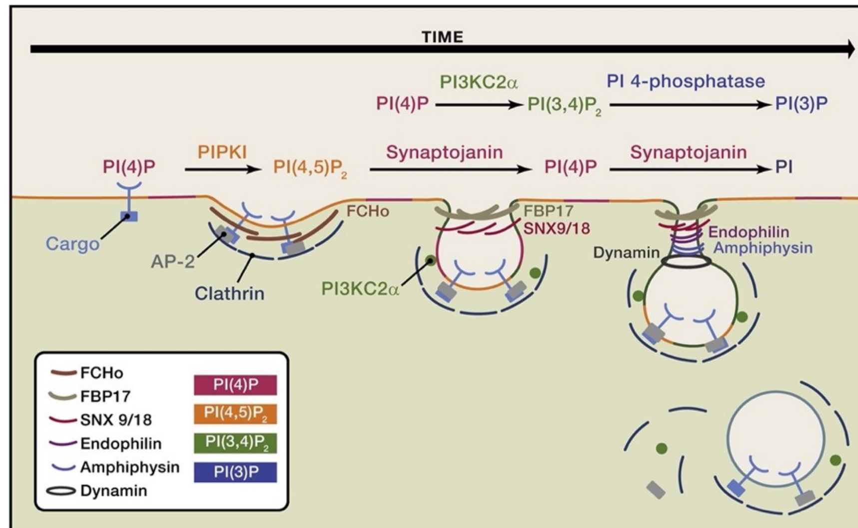

Fig.22 Endocytic Membrane Remodeling during Clathrin-Mediated endocytosis
CME is nucleated by coassembly of the shallowly curved F-BAR domain protein FCHo with endocytic adaptors such as AP-2 and clathrin at $PI(4,5)P_2$-rich membrane. CCPs maturation is accompanied by membrane remodeling and conversion of $PI(4,5)P_2$ (orange) into PI(4)P by Synaptojanin. Then, PI3KC2α catalyzes the phosphorylation of PI(4)P to form $PI(3,4)P_2$, required to recruit the SNX9/18 to maturing CCPs. The BAR domain proteins endophilin and/or amphiphysin assemble around the constricted vesicle neck and aid the recruitment of the dynamin which finally catalyze CCP fission. *From Daumke et al., 2014*

4.2.2 Modes of compensatory endocytosis in nerve terminal

Once SV or LDCV have fused with the plasma membrane, vesicle membrane and proteins are rapidly retrieved to maintain the cell surface constant and restore the vesicle pools. Basically, two kind of compensatory endocytosis can be discriminated: a fast one that has been attributed to KNR and independent on clathrin and a slower one engaging CME (Artalejo et al., 2002; Wu et al., 2009) (He et al., 2008; Lou et al., 2008).

As mentioned before, KNR might be kinetically and energetically advantageous but given the small size of synaptic cleft, all methods used to monitor KNR in neurons are indirect making its experimental existence difficult to prove for SV.

- *Role of Clathrin-medited endocytosis*

The unified view that clathrin and clathrin-associated proteins is the predominant mechanism of SV recycling in synapses has been supported by numerous studies starting with electron microscopy studies from early 1970's which showed an increase in the amount of coated vesicle after intense stimulation at the frog neuromuscular junction reminiscent of clathrin coated vesicle (Heuser & Reese, 1973). Afterward, the role of CME in SV recycling has been confirmed in various synaptic types. At retinal bipolar neurons, disrupting the interaction of clathrin, the AP2 adaptor complex, and amphiphysin selectively inhibited the slow-phase membrane retrieval (Jockusch et al., 2005). At hippocampal synapses, Granseth et al., showed that knocking-down clathrin heavy chain blocks all forms of endocytosis during low neuronal activity (Granseth et al., 2006a). Later, the Syp-pH imaging experiments showed that, in hippocampal neurons treated with the clathrin inhibitor pitstop-2, the Syp-pH retrieval was completely abrogated.

Nonetheless, the predominant role of CME in SV recycling has been questioned recently (Delvendahl et al., 2016; Soykan et al., 2017; Watanabe et al., 2014). After clathrin heavy chain or AP-2 knock-down or pharmacological inhition of clathrin function, endocytosis was shown to proceed normally at hippocampal and cerebellar neuron synapses. Authors argued that some of the discrepancies in the field were related to the experimental temperature. Indeed, comparing the kinetics of SV proteins retrieval in neurons silenced for clathrin showed delayed endocytosis compared to control only when the experiment was conducted at room but not at physiological temperature (Delvendahl et al., 2016; Soykan et al., 2017). In addition, Delvendhal and Watanabe' works revealed that at physiological temperature, single APs (up to 100 APs) was associated to ultrafast endocytosis (within 50 ms) which was clathrin-independent but actin and dynamin dependent. Indeed, neuron treatment with actin and dynamin inhibitors (lantriculin A and dynasore, respectively) reduces the amount of endocytic figure observed by electron microscopy. This fast SV recycling is the so-called ultrafast endocytosis (UFE) (Fig.23). According to this endocytic mode, membrane retrieval generates a large endocytic vesicle (~80 nm) in a clathrin-independent manner. This vesicle is delivered to synaptic endosome about one second after stimulation. From the endosome, in turn, SV are formed via budding in a clathrin-dependent manner (Watanabe et al., 2014), indicating that clathrin is not required for UFE at the plasma membrane but is required for the subsequent SV generation from the endosome. Moreover, Watanabe and co-workers have demonstrated that synaptojanin and its partner, endophilin-A, plays a role in this mode of endocytosis. In hippocampal neurons lacking synaptojanin or endophilin, the membrane is rapidly invaginated but the narrow neck at the base of the pits is not

formed during vesicle budding (Watanabe, Mamer, et al., 2018), indicating that in the absence of lipid conversion and recruitment of endophilin, dynamin can not separate the nascent vesicle from the PM.

In these same works, Delvendhal and Watanabe demonstrated that, when stronger stimulation was applied (30 ms duration) at physiological temperature, UFE was replaced by a slower endocytosis that required clathrin, in addition to actin and dynamin (Delvendahl et al., 2016; Watanabe et al., 2014).

Altogether, these findings indicate that synapses can harbor multiple modes of SV endocytosis depending on the strength of the stimuli and the experimental temperature. Literature must also be reevaluated considering the temperature at which experiments were carried out.

- *Activity-dependent bulk endocytosis*

Neurons can be subjected to a range of stimuli. When subjected to intense stimulation, both CME and UFE are saturated, and another type of clathrin-independent endocytosis takes place at synaptic terminals (Gan & Watanabe, 2018). Indeed, the so-called activity-dependent bulk endocytosis (ADBE) is the dominant mode of synaptic vesicle endocytosis during high-frequency stimulation (400 APs delivered at 40 Hz) (Fig.19). UFE and ADDE shares mechanistic similarities. “Bulk” endosomes formation during ADBE is rapid and synchronous with neuronal activity (Clayton et al., 2008). Inactivation or knockdown of clathrin in both hippocampal and cerebellar neurons does not influence the formation of bulk endosomes. In contrast, inhibition of actin polymerization completely blocked ADBE (Clayton & Cousin, 2009a; Nicholson-Fish et al., 2015a) indicating that clathrin is dispensable but Dynamin-1 activity and actin polymerization are required. Subsequently, SVs are generated from bulk endosomes in a clathrin-dependent manner (Kokotos & Cousin, 2015), indicating that, like in UFE, although clathrin and clathrin- associated proteins are not necessary to initiate bulk endosome at the PM, they are essential at later time for SVs budding from intracellular endosomes.

Intense neuronal stimulation induces an accumulation of Ca2+ because of the build up of residual calcium in nerve termini. This may, in part explain, why different mechanisms are triggered in response to various stimuli. The protein phosphatase Calcineurin has a low, micromolar range, calcium affinity (Klee et al., 1979) and is required for ABDE (Kokotos & Cousin, 2015). Calcineurin dephosphorylates a set of endocytic proteins called dephosphins including Dynamin-1 and synaptojanin (Cousin & Robinson, 2001). During mild neuronal activity, Dynamin-1 interacts in a phosphorylation independent manner with amphiphysin and

this interaction is essential for CME completion (Grabs et al., 1997). During intense neuronal activity, increase in Ca^{2+} concentration triggers both increased SV fusion and activation of calcineurin. Active calcineurin dephosphorylates Dynamin-1 allowing its interaction with Syndapin-1, a protein that can tubulate membrane independently of clathrin (Anggono et al., 2006; Itoh et al., 2005). Calcineurin acts therefore as a switch to orient Dynamin towards bulk endocytosis processes when SV supply is massive. It also indicates that while Dynamin-1 is essential for all modes of SV endocytosis, its dephosphorylation is only required for ADBE. In addition, Syndapin proteins represent an important link between the cortical actin cytoskeleton and the endocytosis because this family of proteins interact with the neural Wiskott-Aldrich syndrome protein (N-WASP), which regulates actin polymerization by stimulating the actin-nucleating activity of the Arp2/3 complex (Kessels & Qualmann, 2002). Since actin-dependent invagination drives formation of bulk endosomes, the phosphorylation-dependent recruitment of Syndapin by dephosphorylated Dynamin-1 may be key to bulk endosome formation (Soykan et al., 2017).

Despite ADBE being the dominant SV endocytosis mode under intense neuronal activity, ABDE sort specific cargo. VAMP2, synaptophysin and glutamate transporter (VGLUT) are not retrieved by ABDE during intense stimulation while the SNARE protein VAMP4 is specifically sorted in bulk endosomes (Nicholson-Fish et al., 2015a). It suggests that cargo possesses sorting determinant to orient them to specific mode of endocytosis and that during intense neuronal activity, other modes of SV retrieval take place to provide a correct sorting of SV cargo proteins.

In conclusion, although the debate on SV recycling is far from resolved, the current view indicates that at lower neuronal activity, SV retrieval could be achieved by three endocytosis modes: KNR in which fusion pore is reclosed after fusion, ultrafast endocytosis which occurs at the edges of AZ and, and CME (Fig.23). At higher neuronal activity, ABDE is the predominant endocytic mode, in which retrieval of large plasma membrane areas generates bulk endosomes from which SVs generates (Fig.23).

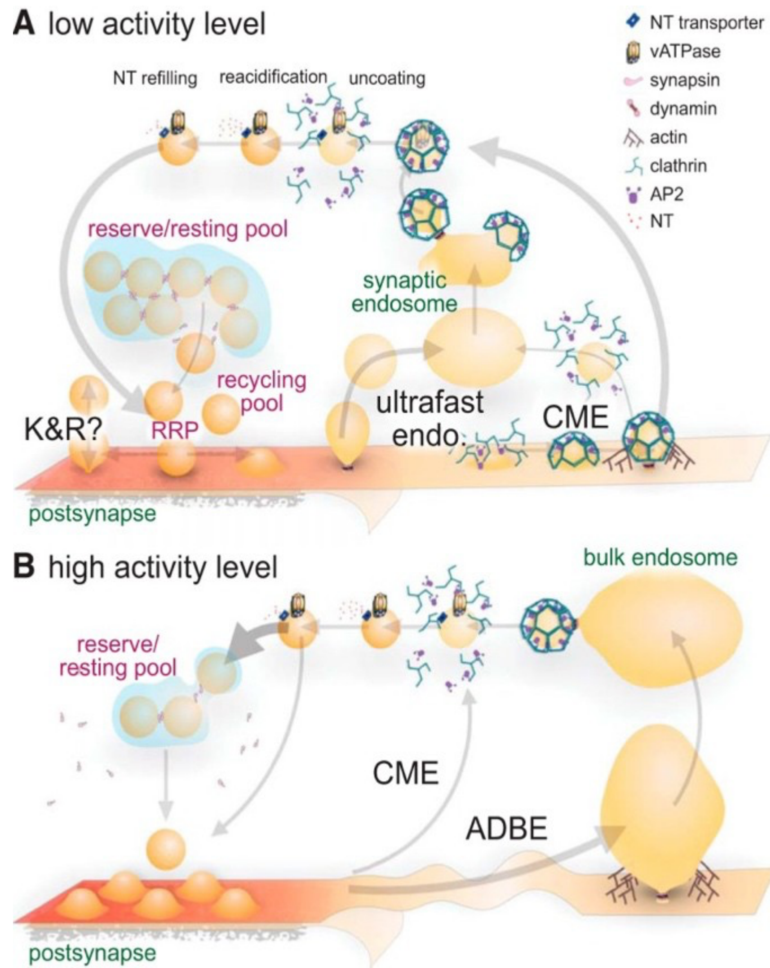

Fig.23 Endocytic modes at the synapse. A At low activity level, three different endocytic mechanisms could take place: KNR, ultrafast endocytosis and CME. **B** At higher activity, ADBE is the predominant mechanism that occurs at the nerve terminal. *From Chanaday et al., 2019*

5. Coupling of exocytosis and endocytosis

Although exocytosis and endocytosis have been largely studied independently, a long-standing question is how these processes are coupled. Upon depolarization, amounts of SV vesicles diminish while clathrin-coated pits and structures related to bulk endocytosis increase near the release sites (Gundelfinger et al., 2003; Hosoi et al., 2009), indicating that exocytosis and endocytosis are tightly coupled. Moreover, studies with vesicular proteins tagged with pHluorin, a pH-sensitive fluorescent protein which allows the direct visualization of exocytosis and subsequent endocytosis in living neurons confirmed the tight coupling, in terms of both timing and amount of SV fusion and retrieval (Ferguson et al., 2007; Yao et al., 2011).

5.1 Lipid microdomains

Phosphatidylinositides represent only a minor fraction of membrane phospholipids. However several studies highlighted their importance as key regulators of membrane trafficking and signaling pathways at the synapse (Cremona & De Camilli, 2001).
Biochemical experiments and super-resolution imaging have shown that $PI(4,5)P_2$ is concentrated at synapses, where it form nanoclusters with a diameter of about 70 nm. It has a crucial role in exocytosis in several systems. In neuroendocrine cells, it is required both for priming and for Ca^{2+} -mediated fusion of granules with the plasma membrane (Cremona & De Camilli, 2001). $PI(4,5)P_2$ is generated during priming by chromaffin granule-associated phosphatidylinositol-4Phosphate-5-kinase activity (PIP5K) (Wiedemann et al., 1996). When PIP5K was blocked by quercetin, $PI(4,5)P_2$ level was decreased and secretion inhibited, indicating that PIP5K acts in exocytosis (Wiedemann et al., 1996). Depletion of $PI(4,5)P_2$ by overexpression of membrane-targeted variant of the $PI(4,5)P_2$-specific phosphatase synaptojanin or knockout of PIP5K1 γ, the main enzyme isoform synthesizing $PI(4,5)P_2$ at the synapse, strongly impairs exocytosis in chromaffin cells and neurons (Di Paolo et al., 2004; Milosevic et al., 2005). It seems that $PI(4,5)P_2$ facilitates exocytosis by regulating vesicular release probability through the activation of some $PI(4,5)P_2$- binding effectors like Syntaxin, Munc13, Synaptotagmin 1, either via the C2 domain or via electrostatic interaction with basic amino acids (Koch & Holt, 2012; Walter et al., 2017).

At the same time, $PI(4,5)P_2$ is also important in the recruitment of endocytic proteins, indeed it depletion via knockout of PIP5K1 γ reduces SV endocytosis (Di Paolo et al., 2004). $PI(4,5)P_2$ provides binding sites for several important endocytic proteins such as dynamin, whose

oligomerization depends on the interaction of its autoinhibitory PH domain with membrane $PI(4,5)P_2$ (Reubold et al., 2015). Moreover, it interacts with Epsin, Bar domain proteins (e.g., endophilin) (Ferguson et al., 2009), clathrin adaptors like AP-2 (Kononenko et al., 2014a), indicating that $PI(4,5)P_2$ is involved in initiation, assembly and scission of clathrin-coated pits, as well as with actin-regulatory proteins like N-WASP, promoting actin remodeling during endocytosis. Finally, hydrolysis of $PI(4,5)P_2$ by phosphatase synaptojanin is required for the uncoating of newly formed vesicles (McPherson et al., 1996).

Reports indicate that, in addition to $PI(4,5)P_2$, downstream of both Ca^{2+} and PIP_2 , DAG might be an another lipid that coordinated exocytosis and endocytosis. DAG facilitates exocytosis by enhancing the activity of Munc13 and protein kinase C, which phosphorylates Munc18 and SNAP25, both of which are essential for SV exocytosis (Puchkov & Haucke, 2013).

In the endocytosis process, phosphorylation of DAG by DGK produces PA, an important co-factor for $PI(4,5)P_2$ synthesis by PIPK1 γ and a facilitator of endocytosis (Di Paolo & De Camilli, 2006). PA plays a crucial role also in exocytosis in neuroendocrine cells. A local synthesis of PA by phospholipase D 1 (PLD1) affects the topology of the membrane inducing a negative curvature that facilitates membrane fusion (Momboisse et al., 2009). Moreover, since DAG induces negative membrane curvature, it has been proposed that DAG microdomains, formed during exocytosis at fusion sites, can reduce the membrane tension favoring the movement of preformed clathrin-coated pits toward fusion sites to mediate clathrin-dependent endocytosis (Yuan et al., 2015).

These data suggest that a potential interplay between $PI(4,5)P_2$ synthesis and DAG/PA conversion could have a role in exocytosis-endocytosis coupling.

5.2 Cytoskeleton

The actin-spectrin network represents a barrier for secretory granules, preventing their access to release sites. For this reason, during the exocytosis, actin filaments disassembly needs to take place to allow the access of selected vesicle to the fusion sites. During regulated exocytosis in chromaffin cells, Ca^{2+} influx triggers the re-organization of cortical cytoskeleton via Ca^{2+}-dependent actin-binding proteins, such as scinderin which exhibit a Ca^{2+} - dependent (filamentous)- or F-actin severing activity during exocytosis (Trifaró et al., 2000). Later, when Ca^{2+} concentration decreases, scinderin also promotes cortical actin re-assembly in a Ca^{2+}-independent manner (Trifaró et al., 2000), which could be important for subsequent endocytosis.

Indeed, in chromaffin cells actin polymerization might guide different steps of endocytosis, including vesicle fission, detachment from plasma membrane and the movement of new formed vesicle towards cytoplasm (Qualmann et al., 2000). In nerve terminals, it has been reported that actin filaments disassemble and re-assemble reversibly during SV recycling (Bernstein et al., 1998), suggesting that actin polymerization/depolymerization timing can play a role in exocytosis-endocytosis coupling.

Furthermore, actin and related factors, such as Cdc42, N-WASP, interact with AZ scaffolding proteins like Piccolo and, organize vesicle trafficking and fusion at AZ. Cdc42 and N-WASP, in turn, interact with Intersectin, a scaffold protein recruited at the early phase of CCP formation, suggesting that actin can act as a bridge between exocytosis and endocytosis (Alabi & Tsien, 2013).

The presynaptic bouton has to be highly organized to enable the coordination of exocytosis and compensatory endocytosis and the cortical cytoskeleton might be essential. Several interactions between cytoskeleton elements and proteins involved in SV fusion have been reported. For instance, it has been demonstrated that Munc13 interacts with a brain specific β spectrin isoform (Sakaguchi et al., 1998) and syntaxin interact with α-fodrin, , a membrane-associated cytoskeletal protein belong to the spectrin family (Nakano et al., 2001). In addition Drosophila spectrin null mutants showed an impairment of neurotransmitter release at neuromuscular junctions without alteration of synapse morphology (Featherstone et al., 2001). These results might indicate that cytoskeleton is important for the localization and SV fusion.

Moreover, at presynaptic bouton in the frog neuromuscular junction, F-actin and β-fodrin appear concentrated at non-release domains (Dunaevsky & Connor, 2000), which could correspond to endocytic spots. Consistent with this, after stimulation, a reorganization of actin filaments associated with forming clathrin-coated pits has been shown in lamprey reticulospinal axons (Shupliakov et al., 2002).

5.3 The active zone cytomatrix

Regulated exocytosis is facilitated by a cytomatrix assembled at the active zone. AZ scaffolding proteins, such as Piccolo, Bassoon, liprins, RIM, RIM-binding proteins (RIM-BPs) and Munc13 form a 3-dimensional network that directs SV to sites of Ca^{2+} -entry at the active zone (Fig. 24). This geometrical organization of release-ready SVs and voltage-gated channels is important for synchronization of Ca^{2+} influx and neurotransmitter release.

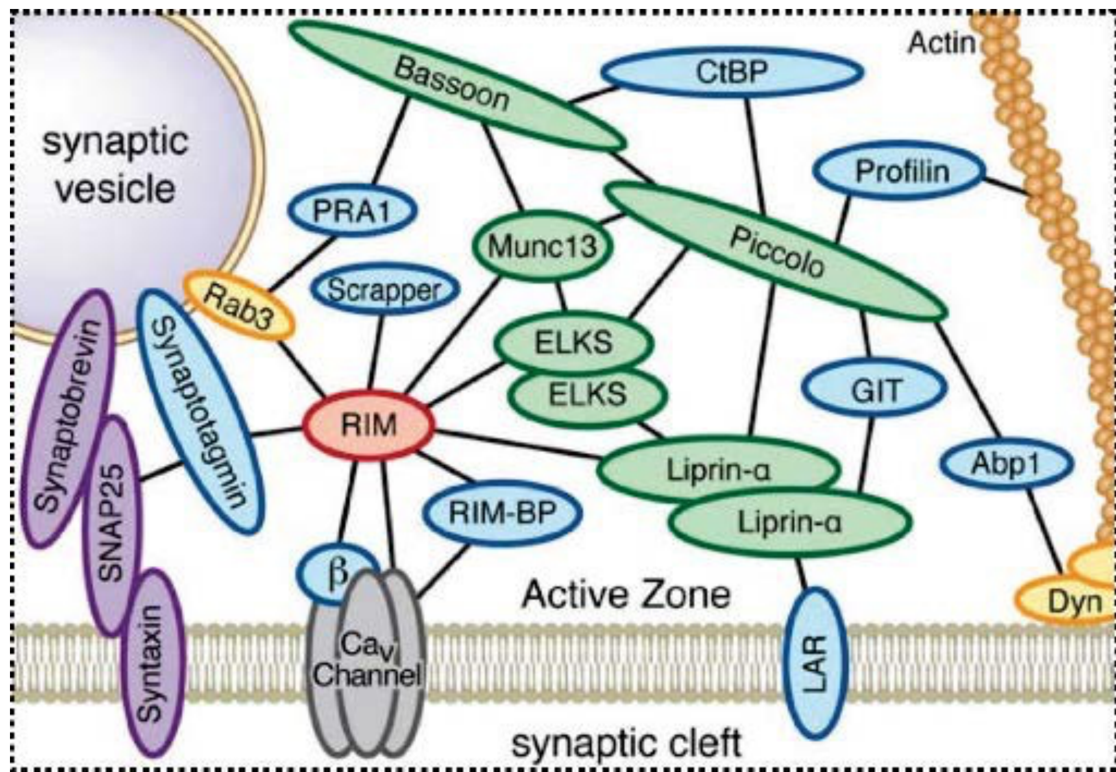

Fig.24 Scheme of the ultra-structural organization of AZ citromatrix. Localization of some AZ proteins and their relation to the synaptic vesicle fusion machinery, Ca^{2+} Channels, and endocytic and actin-binding proteins. The various active zone proteins and their interactions are depicted schematically. *From A.Schulz, 2013*

Moreover, this structure is also important for coupling the exocytosis to endocytosis by the formation of nanodomains for Ca^{2+} signaling. For example, the RIM-binding proteins closely combines with RIM and voltage-gated calcium channel (Fig. 24) in order to stabilize the AZ structure in the presynaptic region and to collect Ca^{2+} ion channels in proximity to SVs (Gao et al., 2021).

Several AZ scaffolding proteins, like piccolo and bassoon, are directly or indirectly associated to endocytic and actin-binding proteins, such as GIT1 and ABP1. The scaffold G protein coupled receptor kinase 2 interacting (GIT1) protein and its D. melanogaster ortholog dGIT mediates the link between piccolo and liprins with the endocytic adaptor stonin 2/stoned B. Drosophila dGIT mutants showed morphological and functional defects in SV recycling (Podufall et al., 2014).

Furthermore, ABP1 binds both F-actin and GTPase dynamin and links the actin cytoskeleton to clathrin-mediated endocytosis. ABP1 localization at AZ is mediated by Piccolo which creates a functional connection between the dynamic actin cytoskeleton and synaptic vesicle recycling (Fenster et al., 2003). Altogether, these results show that the AZ cytomatrix may coordinate exocytosis to SV endocytosis by maintaining close together determinants of exocytosis like Ca2+ channels and the exocytotic machinery as well as the endocytosis and actin regulatory machineries.

5.4 Calcium

While the role of Ca^{2+} is well-known for regulated exocytosis, early studies regarding its role in endocytosis were more conflicting. Capacitance measurements in giant retinal bipolar terminals showed that, elevated Ca^{2+} level delayed endocytic retrieval of membrane following exocytosis (von Gersdorff & Matthews, 1994). Conversely, other studies showed that calcium influx facilitates endocytosis in diverse systems. Increase of extracellular Ca^{2+}, and in turn, Ca^{2+} entry, accelerate slow endocytosis in hippocampal synapses (Sankaranarayanan & Ryan, 2001). By using FM dye imaging, Marks and co-workers demonstrated that after the treatment of synaptosomes with ionomycin, a calcium ionophore allowing calcium entry, the endocytosis was triggered (Marks & McMahon, 1998). The concentration of ionomycin used was unable to elicit glutamate exocytosis, indicating that a lower concentration of Ca^{2+} than that required for exocytosis triggering, was required. Consistent with these results, adding calcium chelator like EDTA in the medium, slowed down fast endocytosis at retinal nerve terminals (Neves et al., 2001). Altogether, these studies evidence that Ca^{2+} influx plays a critical role in compensatory endocytosis. However, how cytosolic Ca^{2+} regulates exo-endocytosis coupling is poorly understood. Studies in synaptosomes indicated that calcineurin, a calcium-dependent phosphatase, is crucial for vesicle membrane internalization, suggesting that Ca^{2+} elevation was required for calcineurin activation, and therefore for the progression of the endocytic process (Marks & McMahon, 1998). Proteins able to bind both Ca^{2+} and CME adaptor proteins constitute also ideal candidates. For example, Syt1 has been shown to bind the endocytic adaptor protein AP-2 and the µ-homology domain of stonin-2 through its C2B domain (Haucke et al., 2000). Studies of Syt-1-dependent endocytosis are complicated because endocytic defects are secondary to the impaired exocytosis caused by Syt1 deficiency. Nonetheless, strategies allowing for acute loss of synaptotagmin-1 during endocytosis slowed SV endocytosis in hippocampal neurons as

well as in Drosophila neuromuscular junctions (Nicholson-Tomishima & Ryan, 2004; Poskanzer et al., 2006) and deletion or mutation of its C2 calcium-binding domains extended the time constant of slow endocytosis (Yao et al., 2011).

Another key role in the exo/endocytosis coupling is related to calmodulin, a calcium-binding protein. Once bound to Ca^{2+}, calmodulin interacts with various targets including kinases and phosphatases. In 2009, Wu and co-workers showed that $Ca^{2+,}$ and calmodulin initiate all form of endocytosis and calmodulin knockdown or application of calmodulin inhibitors impaired SV retrieval in diverse systems (Wu et al., 2009). Calmodulin may also alter actin dynamic during compensatory endocytosis since it activates the myosin light chain kinase (MLCK). MLCK phosphorylates the myosin light chain in a Ca^{2+} and calmodulin-dependent manner to enhance myosin ATPase activity and promote actin-myosin contraction. MLCK inhibition increases the size of the fast-releasing pool of vesicle at the Calyx of Held synapse (Srinivasan et al., 2008) and increases SV endocytosis in response to high-frequency stimulation at mouse neuromuscular junctions (Maeno-Hikichi et al., 2011). Finally, a recent study provides evidences that calmodulin may shape the membrane by interacting with several endocytic N-BAR domain proteins such as endophilins and amphiphysins in mammalian cells (Myers et al., 2016). Active calmodulin may therefore coordinate actin dynamics and endocytosis in response to Ca^{2+} accumulation during high frequency stimulation of neurons.

5.5 SNARE proteins

In addition to calcium and calcium effectors, SV retrieval may also depend on the exocytic machinery, in particular, the SNARE proteins. Indeed, compensatory endocytosis occurs only if exocytosis happened. Botulinum and tetanus neurotoxins are potent inhibitors of exocytosis by cleaving SNARE proteins essential for exocytosis. In neuron treated with these toxins, action potential-mediated Ca^{2+} entry is normal but neither exocytosis nor endocytosis occurs, indicating that intracellular Ca^{2+} increase is insufficient and that exocytosis must occur to trigger endocytosis (Yamashita et al., 2005). Studying the role of SNARE in endocytosis is complicated since altering SNARE function often impact exocytosis and therefore subsequent endocytosis.

In 2004, Deak and co-workers have demonstrated for the first time at hippocampal synapse, that Synaptobrevin-2 (or VAMP-2), a SNARE protein require for calcium-regulated SV exocytosis is also essential for SV fast endocytosis. Indeed, after depletion of RRP by a pulse of hypertonic

sucrose, mice lacking synaptobrevin displayed a severe phenotype with a delayed pool replenishment and an altered shape and size of vesicle (Deák et al., 2004).

Consistent with this experiment, Hosoi et al. set up a protocol in which only partial cleavage of VAMP2 is achieved by tetanus toxin. They evaluated the effect of toxin in endocytosis only in cells where the remaining fractions of intact synaptobrevin elicited exocytosis, making possible to evaluate the involvement of synaptobrevin only in endocytosis. Although membrane capacitance changes revealed that exocytosis was not completely blocked in these cells, no membrane retrieval was observed, highlighting the importance of synaptobrevin-2 in compensatory endocytosis (Hosoi et al., 2009).

With the same approach but using botulinum neurotoxin E and botulinum toxin C that cleaves SNAP25 and syntaxin-1, Xu and co-workers further demonstrated that partial block of SNAP-25 or syntaxin inhibited both fast and slow endocytosis (Xu et al., 2013). Since SNARE proteins mediate exocytosis at all synaptic terminals and in non-neuronal secretory cells, the potential dual role of these three SNARE proteins into exocytosis and endocytosis could be a convenient way to couple both mechanisms. Compensatory endocytosis could only occur when SNARE-dependent exocytosis is achieved, and SNARE-dependent vesicle fusion could constitute a "check point" for compensatory endocytosis. Nevertheless, how SNARE proteins trigger compensatory endocytosis is still unknown. One possibility is that SNARE proteins may interact with endocytic proteins that are present in the periactive zone where clathrin-mediated endocytosis occurs. Indeed, it has been shown by superresolution microscopy that, after SV fusion, SNARE proteins quickly spread outside the active zone to recluster at the level of the periactive zone. The potential role of clathrin endocytic machinery to limit SV proteins diffusion has been exemplified by following Synaptobrevin-pHluorin dynamics in neurons silenced for the endocytic adaptor AP180 or CALM. In that context, newly exocytosed pHluorin molecules occupied a significantly larger area compared to WT neurons, indicating that endocytosis machinery is required to confine synaptobrevin-2 at periactive endocytic zone (Gimber et al., 2015). Other SNARE proteins can potentially interact or associate with the endocytic machinery. For example, SNAP25 has been found in complex with EHSH1/intersectin (Okamoto et al., 1999), which has been recently shown to mediate synaptic clearance after SV exocytosis (Jäpel et al., 2020a) and syntaxin can associate with dynamin (Galas et al., 2000). Altogether these findings suggest that SNAREs could be key in exocytosis-endocytosis coupling by associating with endocytic proteins.

6. Conclusion: membrane lipid remodeling and neurotransmission

Although lipids are primarily structural components of the PM, studies of membrane processes, like exocytosis and endocytosis, mainly focused on the role of proteins. Nevertheless, it is now becoming clear that the lipids dynamics also play a key role in the modulation of neuronal transmission. As presented in the above sections, the plasma membrane lipid microdomains can contribute to ion channel organization, enzyme-mediated lipid signaling and SNARE protein assembly during SV exocytosis (Chamberlain et al., 2001; Chasserot-Golaz et al., 2005; Gil et al., 2005). Moreover, local lipid remodeling may contribute to membrane shape changes including local membrane curvature that facilitates SV fusion and its subsequent retrieval (Devaux, 2000; Hirama et al., 2017). Beside their role in membrane remodeling, the membrane lipids including PIPs play essential roles in vesicular trafficking by recruiting many protein regulators such as synaptotagmin, rabphilin and small GTPases and in endocytosis, since they recruit clathrin adaptors and dynamin (Koch & Holt, 2012).

The importance of lipid metabolism is further exemplified by human pathologies for which it can be responsible. For example, people with Down syndrome have an extra copy of the gene encoding synaptojanin 1, which converts $PI(4,5)P_2$ into PI(4)P (Arai et al., 2002), and patients with Lowe syndrome, who also have mental retardation, lack an inositol polyphosphate 5-phosphatase, which dephosphorylates $PI(4,5)P_2$ (Prosseda et al., 2017). All these findings indicate that lipids play a critical physiological role at synapses and in neurotransmission. However, another "grey area" remains concerning structural phospholipids (PE, PC, PS, sphingomyelin), which could act, unlike PIPs, without triggering measurable signaling pathways, but by altering protein dynamics, microdomains assembly or protein conformation. Evidences exist *in vitro*, but such a function in live cells is rather difficult and challenging to catch.

Neurotransmission is likely one of the biological processes where structural phospholipids may have a critical function since membrane reorganization has to take place at a massive and often high rate. Synaptic transmission rely on fusions of synaptic vesicles with the presynaptic membrane within the active zone (Kaeser & Regehr, 2014). As a result, the membrane and proteins of synaptic vesicles are incorporated into the plasma membrane, altering its structure and potentially impairing its function. The accumulation of vesicular lipids and proteins might jam release sites and impede upcoming fusion (Hosoi et al., 2009; Jäpel et al., 2020a; Park et al., 2018). Therefore, the AZ must be cleared before being re-used. This process, called release site clearance (Neher, 2010), is a key step in ensuring efficient neurotransmission. Structural lipid

reorganization as well as proteins regulating the dynamics of these lipids might have a crucial role in this process, which, today, remains elusive.

B. Aims of the thesis

A previous work of our team provides the first evidence that the exocytosis of secretory granule in neuroendocrine cells triggers a PLSCR-1-dependent outward translocation of PS around the exocytic sites at the plasma membrane. This PLSCR1-dependent lipid reorganization did not appear to regulate the exocytic process *per se* but rather had a functional implication in subsequent compensatory endocytosis. As mentioned in my introduction, although the stimulation of mast cell induces PS egress, discrepancies about the role of PLSCR1 have been reported since PLSCR1 appeared to inhibit exocytosis. In addition, PLSCR1 function has been questioned in several cell systems. Hence, I wanted to further explore the role of PLSCR1 in excitatory cells, especially in neurons. Data on PLSCR1 and PS egress in neurons were sparse. One study reported the detection of PLSCR1 expression in the brain and one other reported that upon stimulation of purified synaptosomes, a Ca^{2+}-dependent increase in PS translocation occurred. Based on these observations, my thesis project aims at answering the following questions:

1. Is the PLSCR1 expressed in neurons? What is its subcellular localization?
2. Is lipid scrambling occurring at synapses when neurons are stimulated? Is the PLSCR1 required for lipid scrambling in neurons?
3. What is the function of PLSCR1-dependent lipid scrambling in neurons and in neurotransmission?

In the next chapter, the results obtained during my thesis will be presented in two different parts. The first section will describe the preliminary results obtained, while the second one will concern the main results presented in form of an article.

C. Results

1. Preliminary data from olfactory bulb neurons

1.1 PLSCR1 expression in mouse brain

At the beginning of my thesis, I first had to define the neuron type where PLSCR1 might be expressed and have a function. Since few data were available in the literature, I referred to the Allen Brain atlas (http://www.brain-map.org/) for information on the relative levels of mRNA expression of PLSCR1 in various brain structures. Olfactory bulb express higher ratio of PLSCR1 (2.9 arbitrary units) compared to hippocampus and cerebellum (0.4 and 0.6 arbitrary units). To evaluate and compare expression levels of PLSCR1 protein in these three areas of the mouse brain, I performed Western blot analysis on brain tissue extracts. Lysate of mouse primary fibroblasts, where PLSCR1 is abundant, was used as a positive control. Culture of fibroblasts and tissues from PLSCR1 $^{-/-}$ (KO) mice were used as negative controls. PLSCR1 protein is well detected in the cerebellum, a little less in the olfactory bulb (OB) and weakly in the hippocampus (Fig.25). Since the olfactory bulb primary culture was already set up in our laboratory, the first experiments of my thesis have been carried out on cultured neurons from mouse OB.

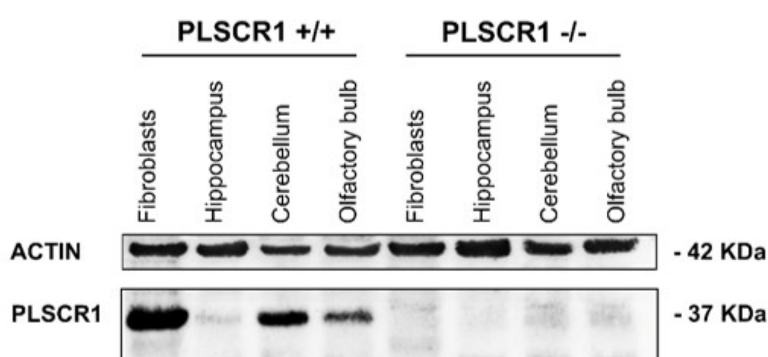

Fig.25 PLSCR1 expression in mouse brain. Detection of PLSCR1 expression in mouse Hippocampus, Cerebellum and Olfactory Bulb by Western Blot analysis. Fibroblasts from PLSCR1 $^{+/+}$ and tissue extracts from PLSCR1 $^{-/-}$ mice were used as positive and negative control, respectively. Actin was used for normalization.

1.2 Characterization of olfactory bulb primary neurons

I decided first to characterize the culture of OB neurons. OB from newborn mice were dissociated and neurons maintained 8 days in culture (Protocols are detailed into the "Supplementary materials and methods" section). Cells were fixed and stained with a neuronal-specific (MAP2) and glial-specific (GFAP) antibodies, in order to evaluate the neuron/glia ratio. Nuclei were labeled using Hoechst. MAP2 immunostaining revealed microtubule associated proteins in neurons and is enriched in dendrites, while GFAP labeling targeted acidic fibrillary proteins of astrocytes. As expected, I observed the presence of two distinct cell populations in our culture positive for either MAP2 or GFAP. MAP2 positive neurons represented approximately 50 % of the total amount of cells (Fig.26)

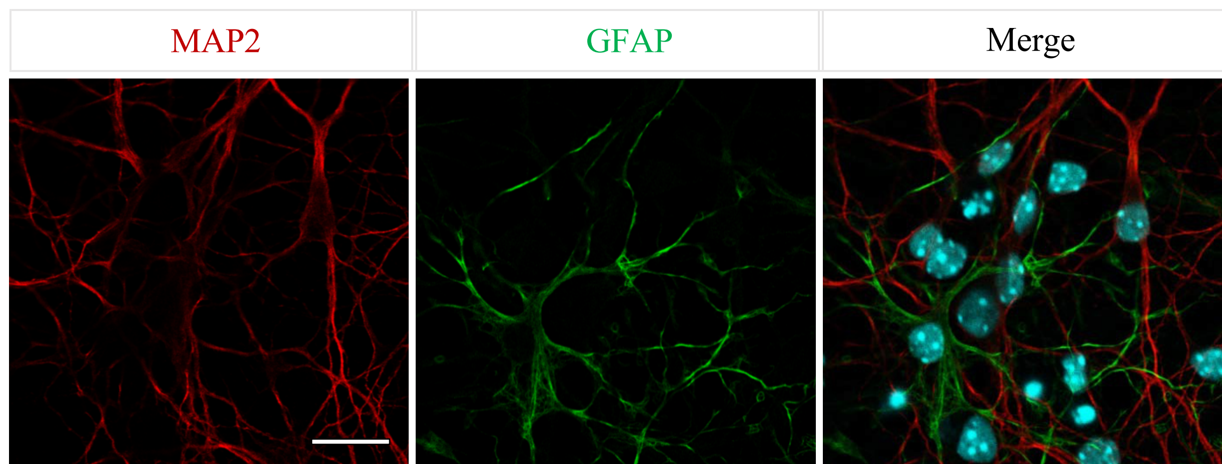

Fig.26 Cell culture characterization. Representative confocal images depicting MAP2 (red), GFAP (green), Hoechst (cyan) immunostainings in olfactory bulb neuronal culture of PLSCR1$^{+/+}$ (WT) mice after 8 days. Scale bar 20 µm

Next, since most of neurons of OB are GABAergic (Parrish-Aungst et al., 2007), to identify neuron subtypes, cultures were fixed and stained with an antibody directed against the main inhibitory neurotransmitter GABAand an antibody against VAMP2, a transmembrane protein found on synaptic vesicles to label synapses (Fig.27). After counting GABA positive cells, I could determine that 37% of total cell (neurons + glial cells) exhibit GABA staining. Since cultures are made of 50 % of neurons, about 74% of neurons after 8 days of culture in *vitro* are GABAergic neurons.

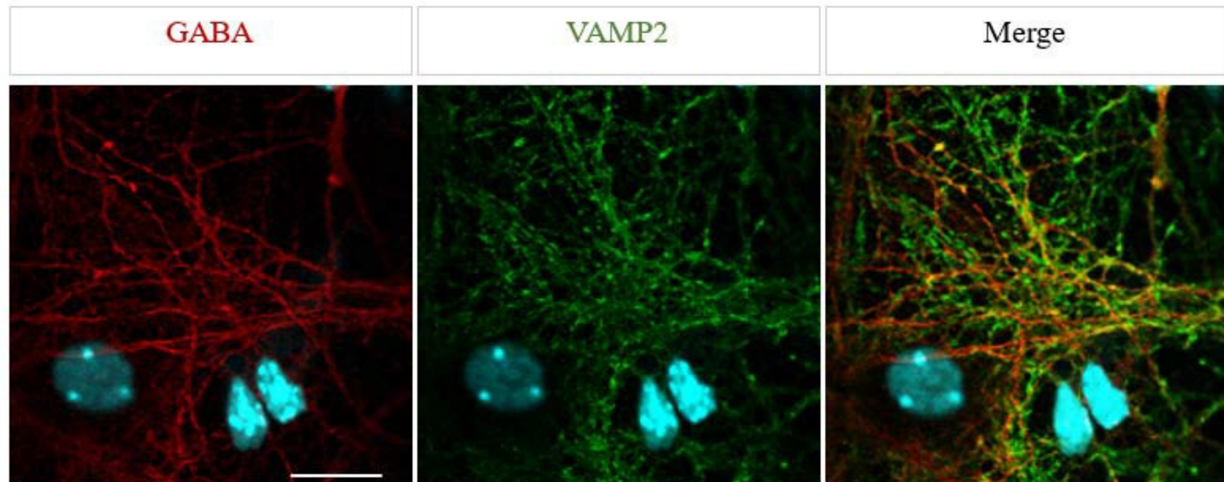

Fig.27 Synapses characterization in OB primary. Representative confocal images depicting GABA (red), VAMP2 (green), Hoechst (cyan) staining are illustrated in OB cell cultures of PLSCR1$^{+/+}$ mice. Scale bar 20 μm

The principal goal was to study the dynamic of exocytosis-endocytosis cycle in primary neurons, I needed to visualize presynaptic vesicles in living cells *in vitro*. To this purpose, I decided to setup experiments using Synaptophysin fused to a pH-sensitive GFP, the pHluorin (Syp-pH). This method is widely used to report SV cycle dynamics (Granseth et al., 2006b; Jäpel et al., 2020b; Nicholson-Fish et al., 2015b). In resting conditions, pHluorin faces the SV lumen and is quenched by the acidic pH of SV and amounts of Syp-pH fluorescence is limited. Upon exocytosis, Syp-pH is exposed to the neutral extracellular medium leading to fast fluorescence unquenching and a dramatic rise in fluorescence intensity. Syp-pH fluorescence intensity rapidly declines as it is retrieved by endocytic vesicles that progressively reacidified. Since this method relies on Syp-pH overexpression, I first checked whether Syp-pH was correctly targeted at the presynapse. Co-localization study with a presynaptic marker (SytI) was then carried out. OB neurons were electroporated with the plasmid coding for synaptophysin-pHluorin (Syp-pH) and cells were maintained 8 days in culture and stained using specific antibodies against GFP and SytI. Anti-GFP antibodies recognize pHluorin and were used since basal fluorescence intensity of pHluorin is low and killed by fixation.

Fig.28 shows that GFP and SytI staining partially overlap especially in small dots (arrows) indicating that overexpressed Syp-pH is targeted to the presynaptic vesicle membrane. To further confirm that Syp-pH localizes at synapse, I labeled inhibitory postsynaptic compartments using an antibody against gephyrin, a central element that anchors, clusters, and stabilizes glycine and

γ-aminobutyric acid type A receptors at inhibitory synapses of the mammalian brain. Again, I detected that Syp-pH co-localizes with gephyrin (data not shown). Therefore, I conclude that overexpressed Syp-pH is correctly addressed to synapses.

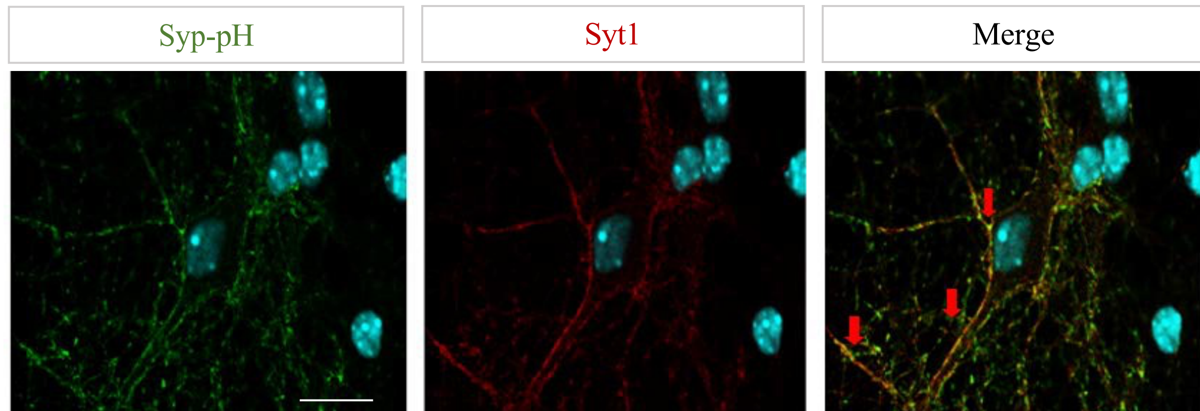

Fig.28 Syp-pH is targeted to the synapses. Representative confocal images depicting Syp-pH (green), Synaptotagmin (red), Hoechst (cyan) staining in olfactory bulb primary neuronal cultures. Scale bar 20 μm

1.3 Evaluation of the optimal KCl concentration to depolarize and stimulate olfactory bulb neurons

The depolarizing concentration of potassium is well known to stimulate exocytosis beacause it activates voltage gated potassium channels and calcium entry. However, depending on the neurons, the resting membrane potential may differ and the potassium concentration to be applied must be adjusted to obtain optimal exocytosis. I thus tested different KCl concentrations and added 15, 50 and 90 mM KCl solution for 10 minutes on primary neurons transfected with Syp- pH. KCl solutions were added directly to the culture medium and compared to control (Locke solution). To detect exocytosis, anti-GFP antibodies were added in the extracellular buffer, at 4°C for 1h. Cells were then washed and fixed. GFP extracellular staining was revealed using a secondary antibody coupled to a fluorescent molecule (Alexa 555). This protocol allowed me to stain only Syp-pH that has been exposed to the cell surface following fusion of synaptic vesicles with the plasma membrane. I could observe that 15 mM of KCl can hardly stimulate neurons since no significant difference could be detected in GFP staining compared to the control. Indeed, only about 5 neurons per well responded to the stimulation (none in the control). At 50 mM KCl concentration approximately 20 neurons showed punctiform labelling corresponding to exocytotic

sites (Fig.29). At 90 mM KCl, only 12 neurons were positive for anti-GFP staining. Thus, we conclude that 50 mM KCl was the optimal concentration to stimulate primary OB neurons and induce exocytosis. This concentration was used in the next experiments.

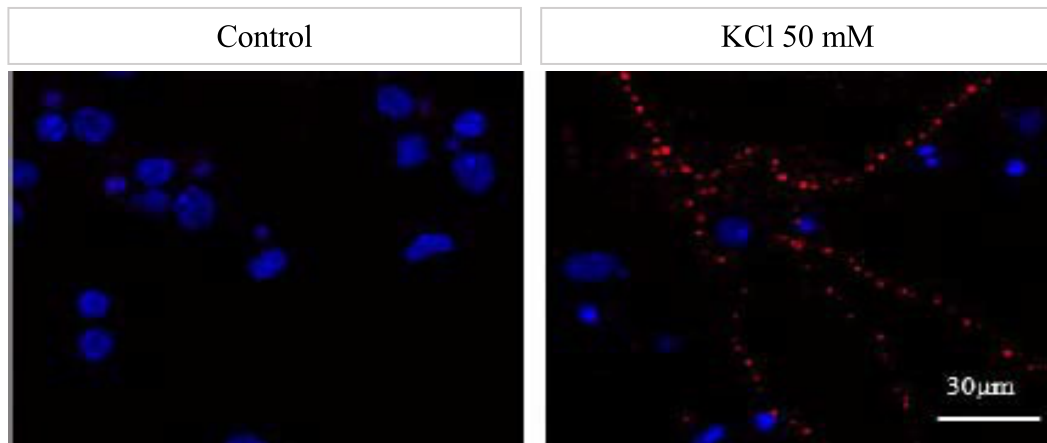

Fig.29 KCl 50 mM is the optimal concentration to stimulate OB neurons. Exocytic sites (red) revealed by an anti-GFP antibody following 50 mM KCl stimulation of olfactory bulb neurons transfected with Syp-pH. Nuclei were stained using Hoechst (blue).

1.4 Analysis of Syp-pH kinetics in living neurons

To address the potential role of PLSCR1 in exocytosis/endocytosis cycle of synaptic vesicles, we used olfactory bulb neurons from PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ mice. Neurons were transfected with Syp-pH and cultured for 8-10 days in microscopy slide chambers. As most of pHluorin fluorescence is quenched at acidic pH, transfected cells are chosen on their faint residual fluorescence. Transfected neurons generally show a punctiform labelling along the axons, due to the overexpression and enrichment of Syp-pH in synaptic vesicles. Few seconds after the beginning of the video recording, transfected cells are stimulated by adding 50 mM KCl solution in bath. Fluorescence intensity variations are then recorded for 3 minutes. To make sure that recorded cells were indeed expressing Syp-pH, 50 mM NH_4Cl was added at the end of the recordings. NH_4Cl blocks proton pumps leading to a fast increase of vesicular and cellular pH. Consequently, pHluorin fluorescence immediately increases.

Fluorescence measurements could be done on 2 PLSCR1 $^{+/+}$ (19 synapses) and 2 PLSCR1$^{-/-}$ (17 synapses) neurons. Fig.30 shows the variation of fluorescence over time in PLSCR1$^{+/+}$ and $^{-/-}$ cells. Interestingly, fluorescence intensity rised and decreased faster in PLSCR1$^{+/+}$ as compared to PLSCR1 $^{-/-}$ neurons. In PLSCR1$^{+/+}$ neurons, the maximum intensity is reached 6 sec after

stimulation whereas it needs more than 30 sec for PLSCR1 $^{-/-}$ cells suggesting that exocytosis in the absence of PLSCR1 was slowed down. Concomitantly, the decrease in fluorescence intensity begins very early (10 seconds after stimulation), and it returns to initial levels after 2 minutes in PLSCR1$^{+/+}$ neurons. In contrast, the decrease of fluorescence is slower in PLSCR1$^{-/-}$ neurons and remains about 50% of the maximum value at the end of the recordings suggesting that compensatory endocytosis in PLSCR1$^{-/-}$ neurons is highly impaired.

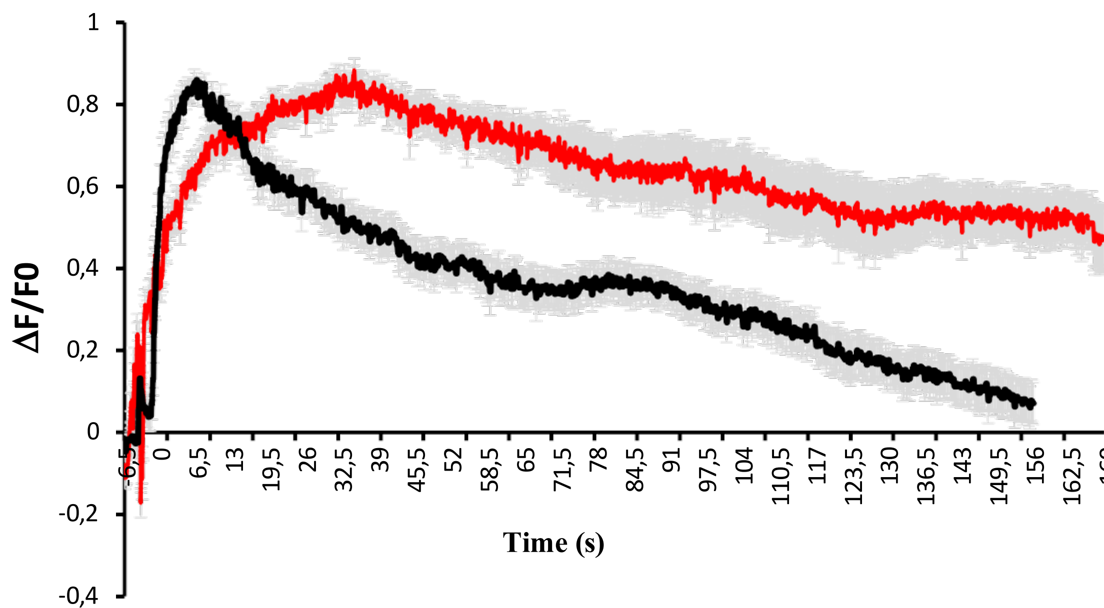

Fig.30 Comparison of over time fluorescence variation of (ΔF/F0) between PLSCR1$^{+/+}$ (black curve) and PLSCR1$^{-/-}$ neurons stimulating with 50 mM KCl solution (red curve). The error bars are shown in grey and result from the analysis of 19 and 17 synapses from 2 WT and 2 KO neurons respectively.

To compare the kinetic of exocytosis and endocytosis between neurons of differentgenotypes, fluorescence increase, and decrease were fitted to logarithmic and mono exponential function respectively and the time constants of each event were determined. Fig.31A represents the distribution of the time constant extracted from the equations. As shown in panel A, time constant was significantly higher in PLSCR1 $^{-/-}$ cells compared to PLSCR1$^{+/+}$ neurons, indicating that fluorescence reaches its maximum at later time point. Time constant median is 0.530 (mean is 0.568 ± 0.096s) for the PLSCR1$^{+/+}$ neurons and 0.230 (mean is 0.197s ± 0.083) for the PLSCR1$^{-/-}$, suggesting that the speed of exocytosis is reduced by a factor of 2.88 in PLSCR1$^{-/-}$ neurons compared to PLSCR1$^{+/+}$. The distribution of median values between the two groups (evaluated with the non-parametric Mann-Whitney test) is significant (P = <0,001). It therefore indicates that

amount of exocytosed Syp-pH is reduced in PLSCR1$^{-/-}$ cells. Concomitantly, time constant of fluorescence decrease was lower in WT compared to KO cells (Fig. 31 B) indicating that the time to return at basal fluorescence levels is shorter in PLSCR1$^{+/+}$ neurons. The logarithm of the time constant median is 2.453 (mean is 2.498 ± 0.368s) for PLSCR1$^{-/-}$ and 1.969 (mean 1.932 ± 0.088s) for PLSCR1$^{+/+}$ neurons, suggesting that the speed of endocytosis is decreased by a factor of 1.29 in the absence of PLSCR1 protein. The distribution of the median values between the two groups (evaluated with the non-parametric Mann-Whitney test), is significant (p= < 0,001). It shows that compensatory endocytosis is faster in PLSCR1$^{+/+}$ compared to PLSCR1$^{-/-}$ cells.

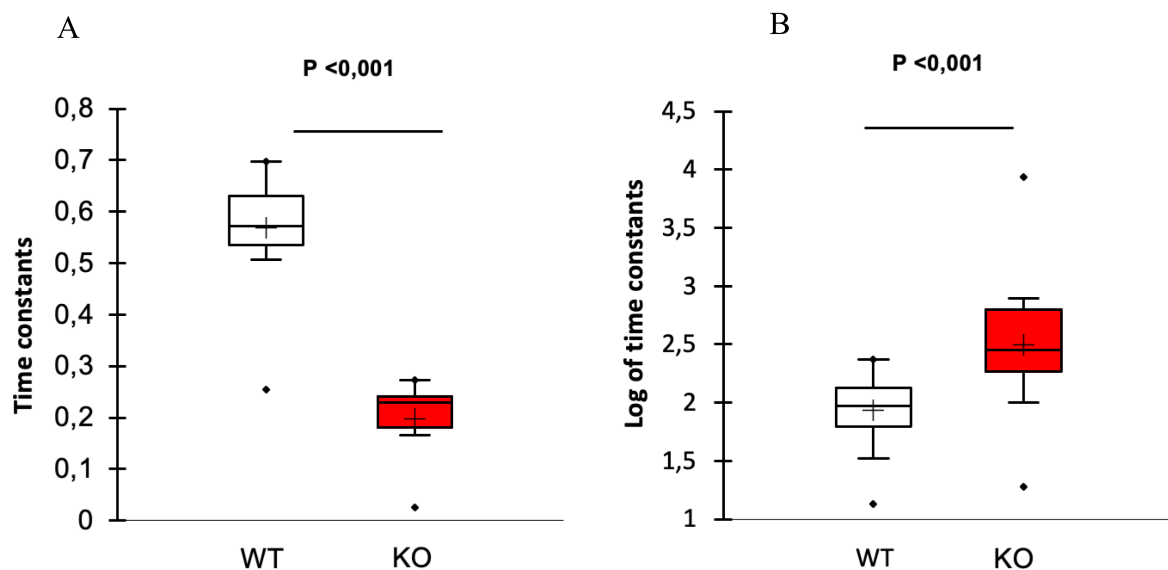

Fig.31 Distribution of time constants of fluorescence variations. A Representation of time constants of the **increase** of fluorescence of PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ neurons by a box and whiskers plot. Fluorescence intensities were fitted to logarithmic curve ($I=I0+ \tau\tau$ x ln (t)) and constant extracted for each synapse. **B** Representation of time constants of the **decrease** of fluorescence of PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ neurons by a box and whiskers plot following the adjustment with a mono- exponential curve $II=II0$ x $(-t/\tau\tau)$, with I = intensity at time t, I0 = intensity at time 0, and $\tau\tau$ = time constant. The black cross represents the mean, and the significance was evaluated with Mann- Whitney test.

1.5 Technical limits

These preliminary data suggest that both exocytosis and endocytosis may be impaired in the absence of PLSCR1 in neurons from mice olfactory bulb. However, despite several tests, we obtained only two usable video recordings (n=2) for each genotype ($PLSCR1^{+/+}$ and $^{-/-}$) for analysis.
This was mainly due to technical limitations related to: 1) the model system that we used, 2) the transfection of Syp-pH reporter by electroporation and 3) the stimulation in bath by KCl 50 mM. Therefore, to better investigate the role of PLSCR1 in the exo/endocytosis coupling, I tried to bypass these technical issues by improving experimental conditions:

- *Model system: from olfactory bulb to cerebellum primary culture*

The olfactory bulb is a complex structure, characterized by several layers (Fig.32) containing mixed neuronal population (Nagayama et al., 2014). The axons of sensory olfactory neuron project into the glomerular layer (GL) where they form synapses with mitral cells. Mitral cells' soma is located in the mitral cell layer (MCL), and tufted cells' soma is located in the external plexiform layer (EPL). Both mitral and tufted cells projects into GL where their activity is regulated by GABAergic interneurons, the so-called periglomerular cells. In the granular cell layer (GCL), GABAergic small granule cells are also found (Nagayama et al., 2014). The histological characterizations of OB sections indicate that it is made up of 40% glial cells and that the 90% of the GABAergic neurons are localized in the granular layer (Parrish-Aungst et al., 2007). Despite the structural complexity of the olfactory bulb, our primary neuron culture seems to be representative of the *in vivo* heterogenous structure. After 8 to 10 days of *in vitro* culture, about 50 % of cells are glial cells and among neurons, 70% are GABAergic, indicating that most of the neurons present in our cultures are granule cells from GCL or interneurons from GL, and the remaining 30% is represented by other neuronal populations.

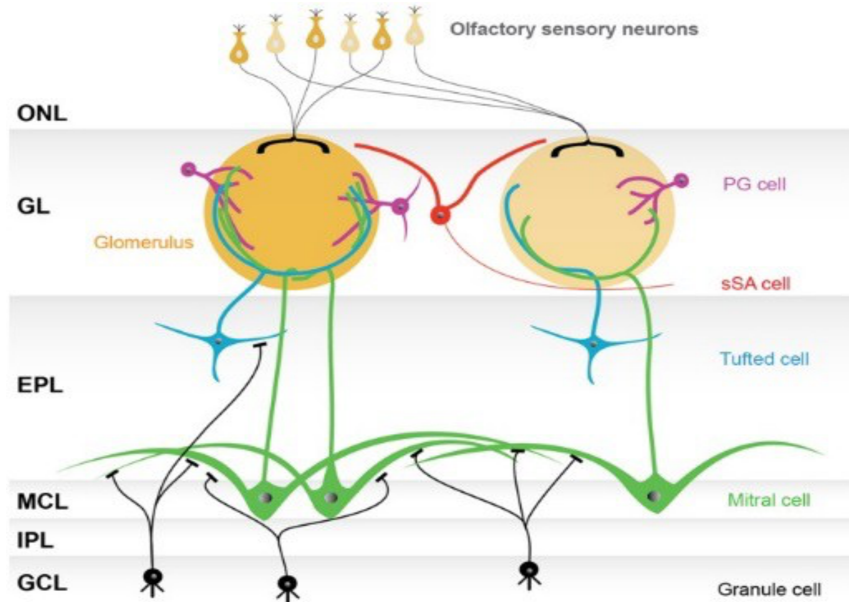

Fig.32 Schematic representation of mouse olfactory bulb. *From Nagayama et al., 2014*

Since PLSCR1 is more abundantly expressed in the cerebellum than in OB (see Fig. 1A in the paper), I performed neuronal primary culture from PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ mice cerebella. Cerebellar neuron cultures have been described to contains 95% of granular cell neurons (GrC) with morphological, biochemical and electrophysiological characteristics of mature neurons (D'Mello et al., 1993; Krämer & Minichiello, 2010). I therefore prepare GrC neuron culture from 6 days old mice and characterized it after 6-7 days in culture. Cells were fixed and stained for Pax6, a transcriptional regulator selectively expressed in GrC, and parvalbumin, an interneuron-specific marker. After quantification and as described, we found that our cultures contain 95 % of GrC and 5 % of interneurons (Fig.33).

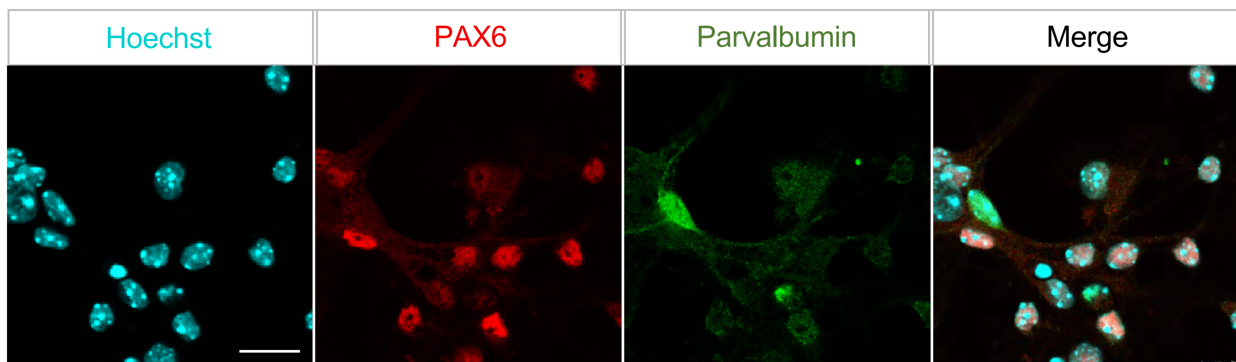

Fig.33 Characterization of primary culture of cerebellar granule cells. Representative confocal images depicting PAX6 (red), Parvalbumin (green) and Hoechst (cyan), immunostainings are illustrated in PLSCR1$^{+/+}$ culture. Scale bar 20 μm

- *Weak efficiency of neuron tranfection by electroporation*

Neuron transfection by electroporation yield low transfection rate in our hand (0.06%) making the identification of transfected neurons difficult and time consuming. Previous work from our team compared the efficiency of various electroporation systems. As reported for chromaffin cells (Covello et al., 2014), electroporation by using Neon system (Invitrogen) still achieved a higher transfection rate and better cell survival in comparison to electroporation with Amaxa system (Lonza) but the rate remained too low. To resolve this issue, we tested and compared different chemical transfection protocols on our primary culture. By using Lipofectamine 2000 as transfection reagent, we reached a transfection rate of 10 % when cells were incubated in 10% CO_2 atmosphere incubator during transfection. Cell survival was improved when the transfection mix was replaced by a conditioned medium instead of fresh culture medium after transfection. This optimized transfection method was then used for the subsequent experiments.

- *Disadvantages of neuron stimulation by KCl*

Neurons were stimulated *in vitro* by the addition to the culture medium of a depolarizing concentration of KCl solution to trigger exocytosis. To limit medium changes, half volume of KCl solution twice concentrated was added to the solution used to maintaincells at rest (Locke buffer). Once stimulated, cells remained all along recordings in a bath containing high concentration of KCl, a type of stimulation that has several disadvantages:

- First, manual addition of the KCl solution to the bath with a pipette introduced a lack of reproducibility both because the exact moment of stimulation and the duration used to add the solution are variable from one experiment to another. Progressive dilution of the concentrated KCl solution does not allow precise control of the stimulation. Consequently, the difference in the observed fluorescence increases between PLSCR1$^{+/+}$ and PLSCR1 $^{-/-}$ could be due to technical issues.
- Second, addition of a relatively large volume of depolarizing solution (50%) to the cell medium often results in a loss of focus impeding video recordings. Fluorescence intensity fluctuation may alter measurements.
- Third, this mode of stimulation results in a strong and continuous depolarization. This creates a prolonged and irreversible neuron excitation, which does not reproduce

physiological conditions where neurons return to the basal state after a transient and controlled stimulus.

- Fourth, although we found that 50 mM KCl was the optimal concentration to evoke exocytosis in OB neurons, we were surprised by the low number of neurons responding to KCl during live cell imaging recordings. This can be attributed to several reasons. First, since we prepared olfactory bulb primary culture from newborn mice, neurons present in our culture were probably still immature (Deshpande et al., 2013) and, in turn, not able to respond to potassium stimulation. Second, OB primary cultures show a heterogenous cellular population, regardless of the degree of maturation of neurons. Therefore, the stimulation with 50 mM KCl could not be appropriate for all type of neurons.

To accurately control neuron stimulation, we decided to switch from KCl depolarizing solution in bath to electrical field stimulation, which allows control of timing, frequency, and strength of the stimulation.

In the next part of my thesis manuscript, I will present my main results in the form of an article ready for journal submission and I will discuss my data in more details to come up with some conceptual ideas I would like to share.

2. Article

Phospholipid Scramblase-1 regulates synaptic vesicle retrieval in cerebellar granule cells

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Abstract

The Phospholipid Scramblase 1 (PLSCR1) has been involved in compensatory endocytosis by controlling the retrieval of secretory granule proteins after exocytosis in neuroendocrine cells. As synaptic vesicles recycling is crucial for sustaining synaptic transmission, we tested the role of PLSCR1 in synaptic vesicle compensatory endocytosis and neurotransmission by using neurons from PLSCR1$^{-/-}$ mice. Here, we report that PLSCR1 is expressed in granular cell of the cerebellum (GrC) and that PLSCR1-dependent phosphatidylserine (PS) egress occurred at synapses in response to neuron stimulation. In the absence of PLSCR1, PS egress is abolished and endocytosis of the fluorescent reporter Synaptophysin-pHluorin or the uptake of anti-Synaptotagmin antibody are dramatically delayed in cultured GrC. Rescue experiments identified the transmembrane domain associated to the Ca^{2+}-binding domain of PLSCR1 as the minimal domain able to restore both PS egress and compensatory endocytosis. Interestingly, endocytosis defect of PLSCR1$^{-/-}$ neurons are associated to increased synapse formation *in vitro* and impaired synaptic transmission at GrC synapses in response to high frequency stimulation. Altogether, this work provides evidences, that PLSCR1-dependent PS egress regulates synaptic vesicle recycling to sustain neurotransmission during high frequency stimulation.

Keywords: lipid scrambling, PLSCR1, compensatory endocytosis, neurotransmission, pHluorin, high frequency stimulation, cerebellum

Introduction

Structural phospholipids (PLs) at the plasma membrane are asymmetrically distributed thanks to energy-dependent transporters, which convey phosphatidylethanolamine (PE) and phosphatidylserine (PS) from the extracellular to the cytoplasmic leaflet (flippases) and, conversely, phosphatidylcholine (PC) from the cytoplasmic to the extracellular leaflet (floppases). PS and PE are therefore virtually absent from the outer leaflet of the plasma membrane (Clarke et al., 2020; Kobayashi and Menon, 2018; Zachowski, 1993). This homeostatic and asymmetric lipid distribution is broken in several biological processes resulting in PS exposure at the extracellular leaflet. Whereas PS exposure at the cell surface is massive and irreversible during apoptosis or platelet activation required for thrombosis (Bevers and Williamson, 2016), PS externalization is limited, restricted to exocytotic sites and transient during regulated exocytosis in immune or neuroendocrine cells (Audo et al., 2017; Kato et al., 2002; Martin et al., 2000; Ory et al., 2013; Rysavy et al., 2014).

Whether massive or limited, the loss of plasma membrane asymmetry is mainly due to the activation of phospholipid scramblases which randomize PLs across the plasma membrane (Daleke, 2003; Kobayashi and Menon, 2018). The phospholipid scramblase-1 (PLSCR1), isolated from erythrocytes, was the first scramblase identified able to reproduce lipid scrambling in response to Ca^{2+} increase when introduced into liposomes (Bassé et al., 1996; Zhou et al., 1997). PLSCR1 was also shown to control PS egress in response to mast cell and chromaffin cell stimulation (Kato et al., 2002; Malacombe et al., 2006; Martin et al., 2000; Ory et al., 2013; Vitale et al., 2001) but preventing PS egress by suppressing PLSCR1 expression led to different functional outcomes. The release by exocytosis of secretory granule contents was reduced in mast cells but unaltered in chromaffin cells (Amir-Moazami et al., 2008; Ory et al., 2013) which instead showed defective compensatory endocytosis preventing secretory granules protein retrieval (Ory et al., 2013). This indicates that the topology of the lipids at the plasma membrane and/or PLSCR1 control exocytosis and/or endocytosis in secretory cells. Doubt has been however cast about PLSCR1 scrambling activities since PLSCR1 is a single pass transmembrane protein that cannot form a channel on its own to transport PLs and the deletion of *Plscr1* gene in mice did not alter plasma membrane PS egress in activated platelets or in response to apoptosis (Zhou et al., 2002). Nonetheless, the identification of additional scramblases, namely TMEM16F and XKR-8 controlling extracellular PS exposure during platelet activation (Suzuki et al., 2010; Yang et al., 2012) and apoptosis respectively (Suzuki et al., 2013) provides evidences that, despite their

common ability to collapse lipid asymmetry, scramblases can regulate distinct mechanisms at the plasma membrane.

Neuronal communications rely on the release of neurotransmitters at synapses, which occurs by Ca^{2+}-dependent exocytosis of synaptic vesicles (SVs) and result in the insertion of SVs membrane into the presynaptic plasma membrane. To support high rates of release during synaptic transmission, SV components have to be retrieved rapidly by compensatory endocytosis to preserve plasma membrane homeostasis, to clear release sites and allow new release-ready SVs to be recruited at exocytic sites and to restore the SV pool. Activity-dependent SV release and retrieval are spatially and temporally coupled within nerve terminals and a dysfunction in the coupling of exocytosis and endocytosis is increasingly associated with pathologies including epilepsy, autism or mental retardation (Bonnycastle et al., 2020). The molecular mechanisms coupling endocytosis to exocytosis have been extensively studied in neurons (Maritzen and Haucke, 2018) but, despite the fact that exocytosis and endocytosis imply successive reorganization of PLs belonging to separate membrane compartments, little attention has been paid to the role of PLs at the plasma membrane. A previous study had shown that upon stimulation of purified synaptosomes, a Ca^{2+}-dependent increase in PLs translocation occurred (Lee et al., 2000) suggesting that PLs might be reorganized at neuronal synapses. Building on the role of PLSCR1-dependent PS egress in chromaffin cells and its requirement for compensatory endocytosis (Ory et al., 2013), we wondered whether SVs cycling in response to stimulation required PLSCR1 and whether it could tune neurotransmission. We first analyzed the expression profile of PLSCR1 in brain regions and found that PLSCR1 was expressed in cerebellar granular cell neurons (GrC). By combining immunofluorescence, electron microscopy and optical live cell imaging of pHluorin probes from wild-type ($PLSCR1^{+/+}$) or PLSCR1 knock-out mice ($PLSCR1^{-/-}$), we found that PLSCR1-dependent PS egress at synapses was required for SV retrieval following neuron stimulation. Rescue experiments in $PLSCR1^{-/-}$ neurons allowed us to identify the transmembrane domain associated to the Ca^{2+} binding site of PLSCR1 as the minimal domain required for SV retrieval. Analysis of the synaptic density revealed that PLSCR1 deficiency increased the number of synapses and lowered the distance between pre-and post-synaptic markers suggesting that synaptic homeostasis was altered in $PLSCR1^{-/-}$ neurons. Interestingly, in acute cerebellar slices, electrophysiological recordings of evoked synaptic currents performed at GrC to Purkinje cell (PC) synapses showed a reduced paired-pulse ratio and a faster depression of synaptic transmission following stimulation of GrC at high frequency. Altogether, our data confirm that PLSCR1 is required for stimulation-dependent PS egress in excitatory cells and

provide the first evidence that PLSCR1-dependent PS egress is needed for efficient SVs recycling able to sustain synaptic activities upon high frequency stimulation.

Material and Methods

Animals and genotyping

Neuronal cells and brain tissues were obtained from Plscr1 KO and wild type mice. PLSCR-1$^{+/-}$ mice were purchased from CDTA (Cryopréservation, Distribution, Typage et Archivage animal) housed and raised at Chronobiotron UMS 3415. All mice were bred, handled, and maintained in agreement with European council directive 86/609/EEC and resulting French regulation. PLSCR1 knock-out and wild-type mice were maintained as heterozygous breeding pairs and genotyped by duplex PCR using 5'-CTACACTGACCTTTAATCAGAGCAG-3', 5'- CCATGTCTGCCCAAGTTCACTCTC-3', and 5'- GCAGCGCATCGCCTTCTATC-3' primers to detect the presence of a 261 bp or a 311 bp fragment for the wild-type or mutant allele respectively. The PCR program was: 95°C for 3 min; 30 cycles (95°C for 30 sec, 60°C for 30 s,72°C for 30 sec) followed by 10 min elongation at 72°C.

Cell culture, transfection and plasmids

Cerebellar granule cultures were prepared as previously described (Cheung and Cousin, 2011). Briefly, primary cultures of cerebellar neurons were prepared from the cerebella of 6-7 days old C57BL/6J mouse of both genotypes. After removal, cerebella were placed in HH solution (Table 1), minced up and then incubated into the Trypsin solution (2T) (Table 1) at 37° for 20 min. After digestion with trypsin, 3 mL of Neutralization solution (N) (Table 1) was added to the suspension and the sample was centrifuged at 150 × *g* for 60 s. The supernatant was removed, and the pellet was suspended in 1 mL of N solution and then triturated using 1000 µl pipette 30-35 times. The cell suspension was centrifuged at 340 × *g* for 2 min and the pellet resuspended in 1,5 mL of DMEM/FCS medium (DFM)(Table 1) and plated as one spot/well at a density of 5–10 × 10^6 cells/coverslip coated with poly-D-lysine diluted in boric acid (100 mM, pH 8.5). After 1 hour, wells were filled up with 2 mL of Neurobasal growth medium (NBK) (Table 1). The following day, cultures were further supplemented with 1 µm cytosine β-D-arabinofuranoside to inhibit glial cell proliferation. 5 days after seeding, cells were transfected with Lipofectamine 2000 as described by Nicholson-Fish et al. (2015). Briefly, cells were preincubated in 2 ml of MEM

(Thermo Fisher) in 10% CO_2 incubator for 30 min at 37°C, and then transfected for 2 h with a complex containing 2 µl of Lipofectamine and 1 µg of the indicated plasmids/well. Cells were subsequently washed with MEM before replacement with conditioned Neurobasal media. Cells were imaged 48 h post-transfection.

Synaptophysin-pHluorin and GFP-PLSCR1 plasmids were as previously described (Nicholson-Fish et al., 2015; Ory et al., 2013). The mCherry-PLSCR1 constructs were obtained by PCR using the mouse PLSCR1 as a template. Amplified fragments were subcloned in pmCherry-C1 vectors between BglII and EcoRI using forward specific primers (see Table2) and a common reverse primer 5' –GGAATTCTTACTGCCATGCTCCTGATC-3'.

Table 1: Solutions for CGN preparation

HH Solution	HBSS Ca^{2+}/Mg^{2+} free (Sigma, H6648) supplemented with 5 mL of 1M sterile Hepes pH 7.3
Trypsin Solution (2T)	1 mL of sterile 5mg/mLTrypsin in 9 mL of HH to make final 0,05% solution
DFM medium	DMEM high glucose (Thermo Fisher, 41966029) supplemented with 10%FCS and 1% Pen/Strep
Neutralization Solution (N)	1 mL of sterile DNase containing 1200 U in 19 mL of DFM to make final 60U/mL solution
NBK medium	Neurobasal (Thermo Fisher 21103049) supplemented with 2% B27, 0.25% 200 mM Glutamine and 1% Pen/Strep

Table 2: forward primers used to generate mCherry-PLSCR1 constructs

Constructs	Forward primers	Fragment size	PLSCR1 amino acids
Full length	5'-CAGATCTGAAAACCACAGCAAGCAAAC-3'	1001 bp	1-328
TmbCa	5'-GGAATTCTTACTGCCATGCTCCTGATCG-3'	160 bp	282-328
Tmb	5'-GCAAGATCTAAGATGAAAGCTGTGATGCTTGG-3'	115 bp	297-328

Immunofluorescence, Confocal microscopy and Image analysis

Neurons were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% v/v Triton x-100 in PBS for 4 min. The cells were washed with PBS, blocked with 3% BSA in PBS and 5% Goat Serum for 1h before being incubated with primary antibodies in 3% BSA in PBS for 1h. Then cells were labelled with secondary antibodies coupled to fluorescent Alexa Fluor dyes. Actin was stained with Tetramethyl-rhodamineB coupled Phalloidin (TMR-phalloidin, 1/1500, Sigma) and nuclei were stained with 1 µg/ml Hoechst (Thermo Scientific, 33342). Cells were observed under a confocal microscope (SP5, Leica Microsystems), using a 63× objective (NA 1.4). Image analyses were performed using Icy software.

Annexin-A5 (AnxA5) staining was performed as described before (Ory et al., 2013). Cerebellar granule cells were incubated for 10 min in the Imaging Buffer (170 mM NaCl, 3.5 mM KCl, 0.4 mM KH2PO4, 20 mM TES (N-tris (hydroxyl-methyl)-methyl-2-aminoethane-sulfonic acid), 5 mM NaHCO3, 5 mM glucose, 1.2 mM MgCl2, and 1.3 mM CaCl2, pH 7.4) to repolarize them before the experiments. Neurons were then maintained for 10 min at 37°C in the presence of AlexaFluor-647-conjugated AnxA5 (1/50, Biolegend #640912) in resting solution (Locke's solution:140 mm NaCl, 4.7 mm KCl, 2.5 mm $CaCl_2$, 1.2 mm KH_2PO_4, 1.2 mm $MgSO_4$, 11 mm glucose, and 15 mm HEPES, pH 7.2; resting) or in stimulation solution containing 59 mM K+ (Locke's solution containing 59 mM KCl and 85 mm NaCl). Neurons were then fixed and counterstained for 30 minutes with TMR-phalloidin. To identify PS egress sites, cells were incubated for 30 min in 30 mM K^+ solution with both AlexaFluor-647-conjugated AnxA5 and anti Synaptotagmin1 (SytI) antibody directed against the luminal domain (Synaptic System, #105103). Cells were then fixed and SytI antibodies revealed with a goat anti-rabbit antibody conjugated to Alexa Fluor 555. AnxA5 and Syt1 staining were observed by confocal microscopy (SP5, Leica Microsystems) and the image analyses were performed with Icy software. HK-means thresholding of the fluorescence intensity of actin was used to delineate neuronal network and mean AnxA5 staining computed from the resulting region of interest.

vGlut1 and PSD95 cluster and distance analysis

Analysis has been performed according to Lagache et al., 2018. Briefly, fixed GrC neurons from PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ mice maintained for 10 days in culture were stained for vGlut1 (Synaptic System, #135304),

PSD95 (Invitrogen, #MA1-045) and Tau (Abcam, #76128). Confocal images were acquired (SP8, Leica Microsystems). Axon masks were obtained by HK-means thresholding applied on Tau staining. The mask is then dilated to cover also postsynaptic PSD95 spots in adjacent dendrites. Wavelet detection was applied to detect spots corresponding to vGlut1 and PSD95 and statistical analysis of the coupling between vGlut1 and PSD95 was performed using SODA. The amounts of spots and paired pairs of spots of each staining in the ROI were calculated.

Synaptotagmin1 antibody uptake assay

To evaluate the efficiency of SV recycling in PLSCR-1 Knock-out and Wild-type neurons, Syt1 Ab uptake assay was performed under basal neuronal network activity. Neurons were incubated for 30 min culture medium in the presence of anti-SytI directed against the SytI luminal domain. After washing, neurons were fixed with 4% PFA in PBS and anti-SytI revealed with fluorescent secondary antibodies.

Preparation of Synaptosomal Fraction

Synaptosomal fractions were prepared as previously described (Dunkley et al., 2008a). Briefly, cerebella (Cb) were collected in an isotonic Sucrose/EDTA buffer (0.32 M Sucrose, 1 mM EDTA, 5 mM Tris, pH 7; Homogenizing Buffer) and homogenized with 13 even strokes. After centrifugation of the homogenates (1000 *g*, 10 min, 4°C), the supernatant (S1, Crude extract) was kept on ice and the pellet was resuspended in homogenizing buffer, subjected to additional 17 even strokes and centrifuged (1000 *g*, 10 min, 4°C). The pellet was discarded and the supernatant S2 was pooled with S1. Protein concentration was measured and adjusted with ice-cold homogenizing buffer to 4-5 mg/mL. Crude extract was loaded onto a discontinuous Percoll gradient (3%, 10%, 15% and 23% Percoll (vol/vol)) and centrifuged at 31.000g for 5 min, at 4°C. Each fraction was individually recovered and Fractions 3 and 4 enriched in synaptosomes were pooled. Synaptosomes were then diluted in ice-cold homogenizing buffer and centrifuged at 20,000g for 30 min at 4°C to concentrate the synaptosomes into the pellet and remove Percoll. Synaptosomes were resuspended in lysis buffer (Cell Extraction Buffer, Invitrogen) supplemented with protease and phosphatase inhibitor cocktail (P8340, Sigma-Aldrich) and further subjected to Western Blot analysis.

Western Blotting

Samples were lysed in cell extraction buffer (10 mM Tris, pH 7,4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1 % Triton X-100, 10 % glycerol,

0,1 % SDS, 0,5 % deoxycholate; FNN0011, Invitrogen) and protease and phosphatase inhibitor cocktail (P8340, Sigma-Aldrich). Cell lysates were cleared at 20,000g for 10 min at 4°C and protein concentration determined using BioRad protein assay. 20 µg of proteins was separated on a Novex 4-12% Bis-Tris gel (ThermoFisher Scientific) and transferred to nitrocellulose membrane. Blots were blocked for 1 h at room temperature in Tris-buffered saline containing 3% (w/v) BSA and 0.1% Tween-20 (TBST; 0.1% Tween, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) and probed with the following anti-PLSCR1, anti-Synaptotagmin1 or anti-PSD95 antibodies. After three washes, blots were incubated with the corresponding secondary antibodies coupled to HRP. Detection was carried out with Prime Western Blotting System (ThermoFisher Scientific) and immunoreactive bands were imaged using Amersham Imager 680 RGB camera System (GE healthcare Life Sciences). Values were normalized to β-actin protein levels.

SynaptopHluorin live cell imaging

Imaging was performed with cerebellar granule neurons DIV6 to DIV7 as reported by (Gordon et al., 2011). Cerebellar neurons were removed from culture medium and incubated in the Imaging Buffer (see above) for 15 min. Coverslips were then mounted in an imaging chamber (Warner RC-21BRFS) supplied with a pair of embedded platinum wires allowing connection to a low impedance field stimulator (Digitimer, D330 MultiStim System). The chamber was placed on the stage of an inverted microscope (Zeiss AxioObserver Z1) equipped with a sCMOS camera (Orca-FLASH4, Hamamatsu) and a control of focus (Zeiss Definite Focus) to prevent drift after stimulation. During recording, cells were continuously perfused with the Imaging Buffer at 37°C. Synaptophysin-pHluorin (Syp-pH) transfected neurons were visualized with a Zeiss Plan Apochromat x40 oil-immersion objective (NA 1.4) at 475 +/- 28 nm excitation wavelength using 525+/- 50 nm emission filter (LED light source SpectraX, Lumencor). When specified, cotransfected mCherry expression was visualized using 555 +/- 28 nm excitation and 605+/- 70 nm emission filters. Neurons were stimulated with a train of action potential (400 action potentials delivered at 40 Hz; 100 mA, 1-ms pulse width) and the acquisition sequence driven by MetaMorph Software (Molecular Devices) at 5 Hz frequency. At the end of the recording, cells were challenged with alkaline imaging buffer (50 mM NH_4Cl substituted for 50 mM NaCl) to reveal total pHluorin fluorescence. Quantification of the time-lapse series was performed using the Time Series Analyzer plugin for ImageJ and only synapses that responded to action potential stimulation were selected for the analysis. A circular region of interest (ROI, 1.8 µm diameter)

was drawn around each spot characterized by a sudden rise in fluorescence. ROI was centered on the maximum fluorescence of the spot. The pHluorin fluorescence change in each spot was calculated as $F\Delta/F_0$, and n refers to the number of individual cells examined. Statistical analyses were performed using Microsoft Excel and GraphPad Prism software.

Statistical analysis

In all cases *n* refers to the number of independent experiments performed, except Syp-pHluorin imaging and electrophysiological recordings where N indicates the individual cells. The analysis were performed by using GraphPad Prism (2018) and SigmaPlot (13.0). All data passed normality test (Shapiro–Wilk test) and variance equality. Data were analysed by one-way analysis of variance (Anova) with Sidak's multiple comparison test or two-way anova with Tukey's multiple comparison *post hoc* test. All data are reported as mean ± standard error of the mean (SEM) with individual data points shown. Significance was set as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Databases

Preliminary information about PLSCR1 distribution in the mouse brain were obtained from the Allen brain atlas (https://mouse.brain-map.org/experiment/show/632487) and the Human brain atlas databases (https://www.proteinatlas.org/ENSG00000188313-PLSCR1/brain)

Results

PLSCR1 is expressed in cerebellar granular cell and localizes at synapses

PLSCR1 is expressed in a wide range of tissue including the brain (Wiedmer et al., 2000; Zhou et al., 2005). Mining in mouse brain databases revealed that PLSCR1 was barely detectable by in situ hybridization (Allen brain atlas, Lein et al., 2007) but expressed in the olfactory bulb, the cerebellum, the pons and medulla when using next generation sequencing, a more sensitive method (Human Protein Atlas, Sjöstedt et al., 2020). To validate the expression profile of PLSCR1 in specific area of the mouse brain, we dissected the hippocampus, the olfactory bulb and the cerebellum to perform Western blots on tissue extracts from PLSCR1$^{+/+}$ mouse. As negative and positive controls, we used mouse brain extracts from PLSCR1$^{-/-}$ and lysates of mouse skin fibroblasts respectively. We confirmed that PLSCR1 was expressed in the cerebellum and the olfactory bulb but barely detectable in the hippocampus (Figure 1A). To further study the function of PLSCR1 in neurons *in vitro*, we turned to the cerebellum, which allows to obtain neuronal cultures of a high purity containing up to 95% of GrC neurons with morphological, biochemical and electrophysiological characteristics of mature neurons (D'Mello et al., 1993; Krämer and Minichiello, 2010). To show that PLSCR1 is expressed in GrC neurons, we cultured cerebellar neurons from 6 days old mice in the presence of 30 mM K^+ to promote mild depolarization necessary for GrC neurons survival (D'Mello et al., 1993) and checked for PLSCR1 expression in neuron lysates after 10 days in culture. Figure 1B shows that PLSCR1 is detected in PLSCR1$^{+/+}$ but, as expected, not in PLSCR1$^{-/-}$ lysates indicating that PLSCR1 is expressed in GrC neurons.

Next, we wanted to analyze the subcellular distribution of PLSCR1 in neurons. First, we prepared synaptosomes from mice cerebellum to determine whether PLSCR1 could localize to synaptic terminals (Dunkley et al., 2008b). Compared to crude extract, PLSCR1 was enriched in synaptosomal fractions containing the post- and pre-synaptic markers PSD95 and Synaptotagmin 1 (Syt1), respectively (Figure 1C). To further show that PLSCR1 can localize to synapses, we performed immunofluorescence on GrC neurons. Because of the lack of reliable antibodies to detect endogenous PLSCR1 by immunofluorescence, we exogenously expressed GFP-tagged PLSCR1 and compared its distribution with specific markers of the pre-synaptic terminal, the vesicular glutamate transporter1 (vGlut1) and Syt1. Like in chromaffin or mast cells, GFP-PLSCR1 was mostly localized at the plasma membrane and in endomembranes of the GrC soma

(not shown). GFP-PLSCR1 decorated axons and colocalized with vGlut1 and Syt1 indicating that GFP-PLSCR1 localized at the synapse (Figure 1D). We next sought to refine the subcellular distribution of GFP-PLSCR1 by performing immunogold electron microscopy (EM) analysis of native plasma membrane sheets of neurons. This method consists of tearing off the membrane at the dorsal surfaces of cells to examine, at high resolution, molecules associated to the plasma membrane (Figure S1; Gabel et al., 2015; Wilson et al., 2000). To determine whether synaptic components could be retrieved by this method, plasma membrane sheets of GFP-PLSCR1 transfected GrC neurons were labeled for GFP and vGlut1 and revealed using a combination of secondary antibodies coupled to 10- (vGlut1) and 25- nm gold particles (Figure 1E). Gold particles of different size were distributed over membrane patches of the size of a synapse (1 μm) and GFP-PLSCR1 localized close to the synaptic marker vGlut1. 73% (+/-13%, n=5) of GFP- PLSCR1 were found close to vGlut1. Altogether, our data indicate that GFP-PLSCR1 is associated to the plasma membrane and most likely enriched at the pre-synaptic terminals were vGlut1-positive docked vesicles are found.

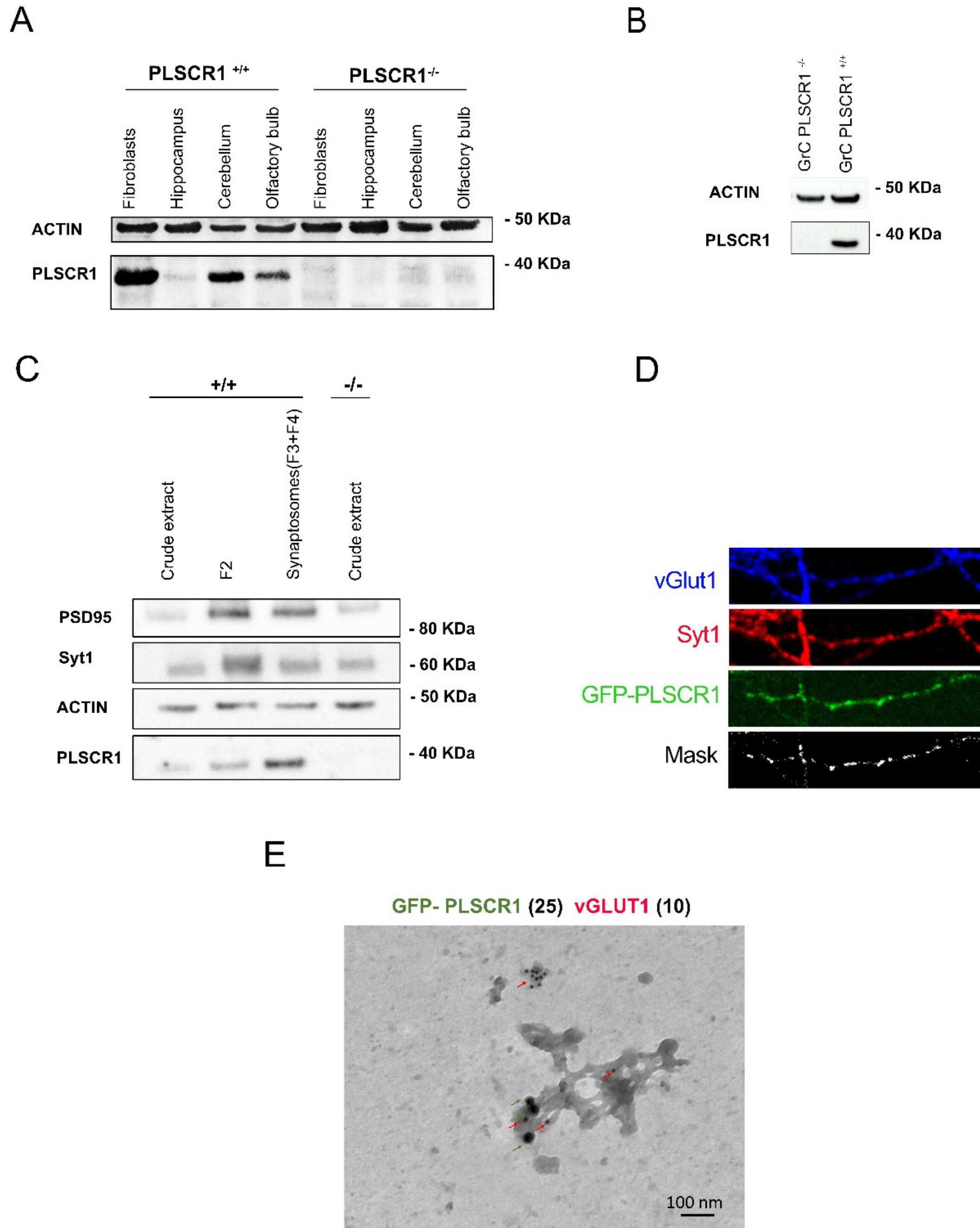


Figure 1: PLSCR1 is enriched at synapses of GrC neurons of the cerebellum. **A**, immunodetection of PLSCR1 protein by western blot in cerebellum, olfactory bulb, hippocampus and skin fibroblasts from PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ mice. Actin is shown as a loading control. **B**, immunodetection of PLSCR1 protein by western blot in cultured GrC neurons. **C**, PLSCR1 is enriched in synaptosome. Cerebella were homogenized, cell body removed by centrifugation and the resultant supernatant layered on a discontinuous Percoll gradient. Fractions were collected and probed for PLSCR1, the presynaptic marker SytI and the postsynaptic marker PSD95. F2 corresponds to the fraction enriched in membranes and fractions F3 and F4 enriched in synaptosomes were pooled and compared to crude extract loaded on gradient. **D**, confocal microscopy of GrC neurons transfected with expression vector coding for GFP-PLSCR1 and

labeled for vGlut and SytI. Mask of synapses containing the three markers is shown. **E**, electron micrograph of plasma membrane sheets prepared from GrC neurons expressing GFP-PLSCR1. Immunolabeling of GFP and vGlut1 were revealed with 25 nm (green arrows) and 10 nm (red arrows) gold particles respectively

PLSCR1-dependent PS egress occurs at synapses upon stimulation

We previously reported that chromaffin cell stimulation induced PLSCR1-dependent PS egress (Ory et al., 2013). Study of synaptosomes prepared from the electric organ of *Narke japonica* electric ray showed that, upon depolarization, rapid transbilayer movement of PLs occurs (Lee et al., 2000). We therefore asked whether stimulation of GrC neurons could lead to disruption of the plasma membrane asymmetry in response to stimulation and whether PLSCR1 could be required for that process. To detect PS exposed to the extracellular leaflet of the plasma membrane, living GrC neurons were stimulated with high K^+ solution containing fluorescent AnnexinV (AnxA5). GrC neurons were then fixed and stained for actin to delineate neuronal processes and to quantify AnxA5 staining associated to neurons. Compared to unstimulated neurons, K^+-dependent depolarization of PLSCR1$^{+/+}$ GrC neurons induced an increase in AnxA5 staining (Figure 2A,B). Interestingly, the increase in AnxA5 staining was abrogated in stimulated PLSCR1$^{-/-}$ GrC neurons (Figure 2A,B) indicating that PLSCR1 was required for the translocation of PS to the extracellular leaflet. AnxA5 staining organized as discrete spots along neuronal processes (Figure 2A) suggesting that PS egress could preferentially occur at the synapse. To test this hypothesis, we took advantages of anti-Syt1 antibodies that recognize the luminal domain of the integral SV protein SytI which is transiently accessible from the extracellular space upon SV fusion with the plasma membrane (Figure 2C). Incubating living GrC neurons with both fluorescent AnxA5 and anti-SytI antibodies showed that AnxA5 partially overlapped with SytI staining indicating that PS egress occurs at active synapses (Figure 2D). We also prepared plasma membrane sheets of stimulated neurons to assess where PS egress occurs. PLSCR1$^{-/-}$GrC neurons were transfected with GFP-PLSCR1 and stimulated in the presence of gold-coupled AnxA5. AnxA5 distribution was analyzed respectively to vGlut1 and GFP on membrane sheets. As shown in Figure 2E, clusters of GFP and AnxA5 gold particles were found close to vGlut1 staining and the number of AnxA5 gold particles associated to vGlut1 was increased in stimulated as compared to resting

GrC neurons expressing GFP-PLSCR1. Altogether, these data indicates that GFP-PLSCR1 localizes at synapses where PS egress occurs following neuron stimulation.

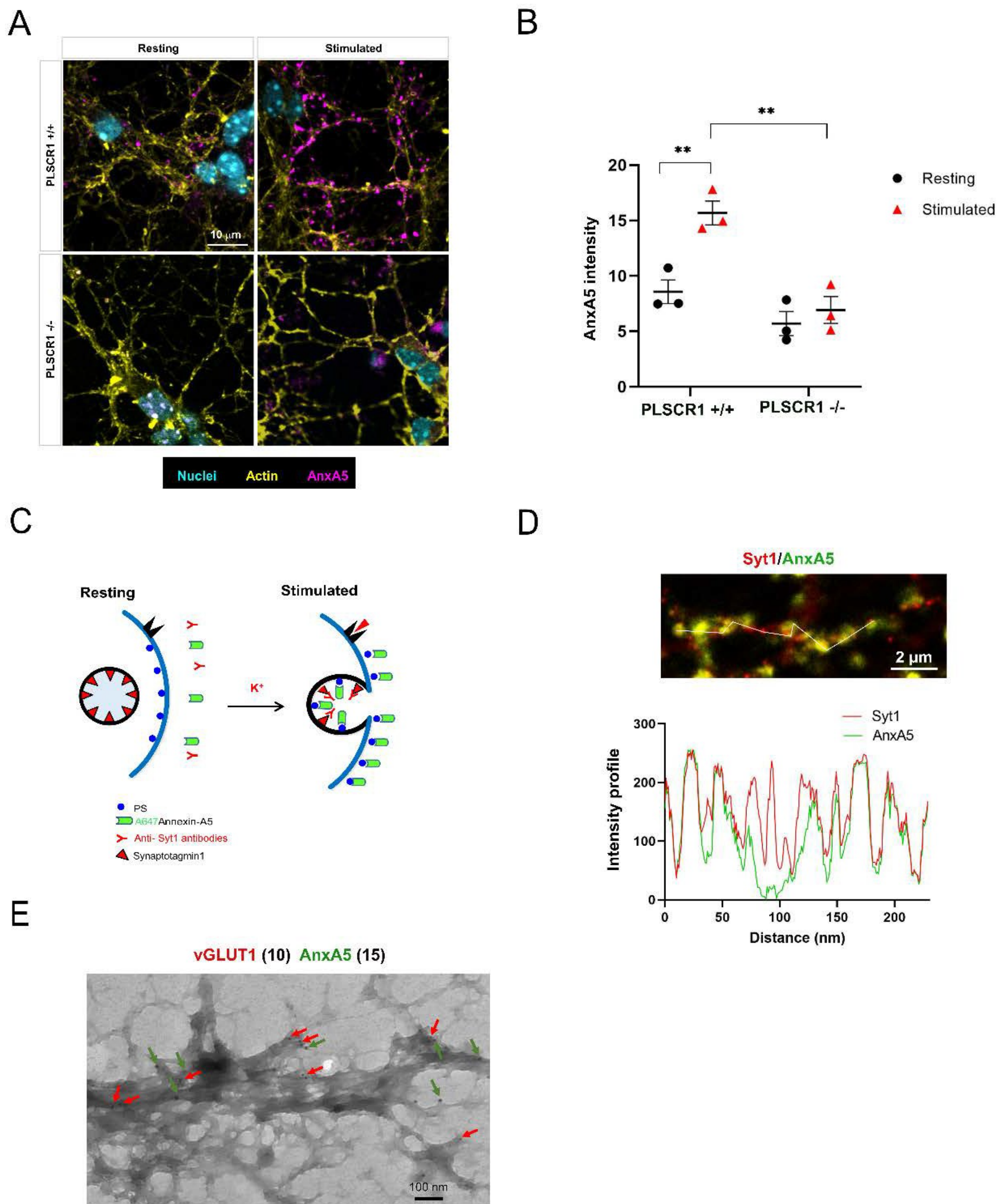


Figure 2: **PLSCR1-dependent PS egress occurs at synapses**. **A**, cultured GrC neurons from PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ mice were stimulated for 10 min with 59 mM K+ or maintained under resting condition in the presence of fluorescent AnxA5. Cells were fixed, counterstained for actin and observed under confocal microscope. **B**, quantification of AnxA5 mean intensity of PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ neurons stimulated for 10 min with 59 mM K+ or maintained under resting condition (10 field of view per experiments, n=3 independent experiments). **C**, scheme of Syt1 staining assay. **D**, Living PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ GrC neurons were maintained in culture

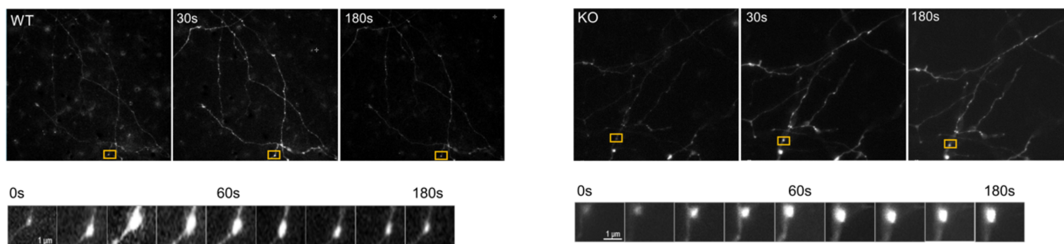
medium containing 30 mM KCl, anti-Syt1 antibodies directed against the luminal domain of Syt1 and fluorescent AnxA5. Cells were fixed and anti-Syt1 antibody revealed with fluorescent secondary antibody without cell permeabilisation to reveal Syt1 at the plasma membrane. Cells were observed under a confocal microscope and intensity profile along the depicted line is shown. **E,** representative electron micrograph of plasma membrane sheets prepared from GrC neurons stimulated for 10 min in the presence of biotin-coupled AnxA5. Immunolabeling of AnxA5 and vGlut1 were revealed with 15 nm (green arrows) and 10 nm (red arrows) gold particles respectively.

PLSCR1 is required for SVs retrieval

Since PLSCR1 was required for compensatory endocytosis in neuroendocrine cells, we sought to monitor the exocytosis and endocytosis SV cycle to determine whether presynaptic function were altered in the absence of PLSCR1. Synaptophysin fused to a pH-sensitive GFP, the pHluorin (Syp-pH), is widely used as a reporter of SV cycle (Granseth et al., 2006; Jäpel et al., 2020; Nicholson-Fish et al., 2015). In resting conditions, as pHluorin faces the SV lumen, Syp-pH is quenched by the acidic pH of SV and emits limited amount of fluorescence. Upon exocytosis, Syp-pH is exposed to the neutral extracellular medium leading to fast fluorescence unquenching and a dramatic rise in fluorescence intensity. Then, the intensity of the Syp-pH fluorescence decreases rapidly as it is retrieved by the endocytic vesicles which progressively reacidify. PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ GrC neurons were therefore transfected with Syp-pH and stimulated with a train of high-frequency action potentials (40Hz, 10s) to evoke robust exocytosis (Nicholson-Fish et al., 2015). Accordingly, a fast rise in fluorescence was observed upon stimulation for both genotypes (Figure 3A,B) and a progressive return to the fluorescence baseline was observed for PLSCR1$^{+/+}$ GrC neurons. In contrast, fluorescence recovery was delayed in PLSCR1$^{-/-}$ GrC neurons (Figure 3A,B). Analysis of the fluorescence intensity profiles showed no differences in the amount of Syp-pH released but a significant block in Syp-pH retrieval (Figure 3B,C) suggesting that Syp-pH exocytosis was not altered but its endocytosis impaired in the absence of PLSCR1. Since the assay relies on the sensitivity of pHluorin to vesicle acidification, a delay in fluorescence decrease could reflect a delay in vesicle acidification and not in endocytosis. To further explore the role of PLSCR1 in endocytosis, we took advantages of GrC neuronal culture conditions which requires K^+-dependent mild depolarization to maintain spontaneous synaptic activity for their survival (D'Mello et al., 1993; Lawrie et al., 1993). To detect spontaneous release of SV and a potential subsequent defect in endocytosis, GrC neurons were incubated with culture medium containing antibodies directed against the luminal domain of Syt1. To reveal the amounts of antibodies

remaining at the cell surface, fluorescent secondary antibodies were added without cell permeabilization and the fluorescence intensity quantified. In agreement with pHluorin experiments, Syt1 staining was increased by about 50% in PLSC1$^{-/-}$ GrC neurons as compared to PLSCR1$^{+/+}$ (Figure 3D,E) providing further evidences that endocytosis of SV proteins was delayed in the absence of PLSCR1. It also indicates that delays in Syp-pH fluorescence decay is likely not due to reduced vesicle reacidification but rather to a defect in compensatory endocytosis in PLSCR1$^{-/-}$ GrC neurons.

A



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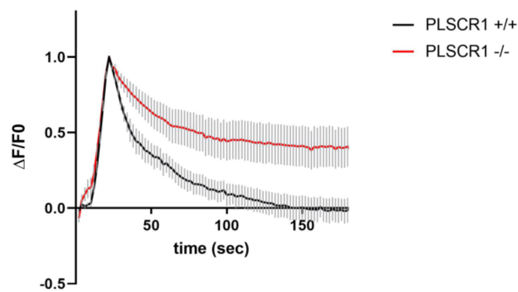

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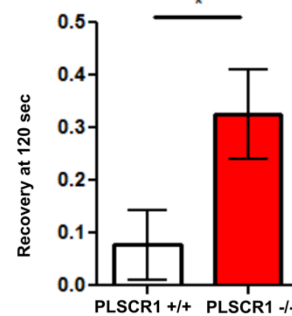

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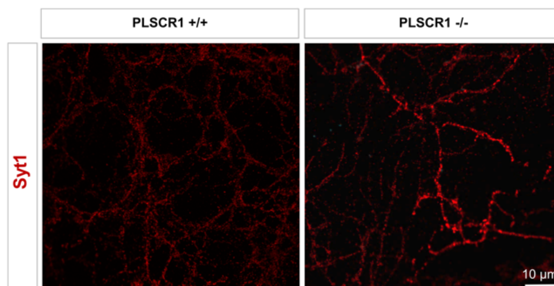

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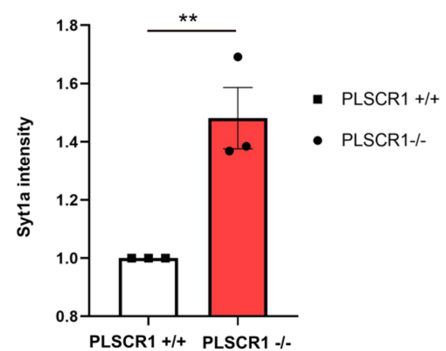

Figure 3: PLSCR1 is required for efficient SVs endocytosis during high frequency stimulation of GrC neurons. A, representative images of GrC neuron transfected with Synaptophysin-pHluorin (Syp-pH) and stimulated with a train of 400 action potentials delivered at 40 Hz. Still images shows PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ GrC neurons before stimulation, 30 s and 180 s after stimulation. Inset shows a representative time course of Syp-pH variation at a single synapse. **B**, average normalized time course of fluorescence response of Syp-pH represented as F/F0 +/- SEM (5 independent experiments, n=17 neurons). **C**, mean normalized intensity of Syp-pH fluorescence remaining after 2 min imaging. **D**, representative confocal images of anti-Syt1 antibodies uptake experiments. PLSCR1+/+ or PLSCR1-/- GrC neurons were incubated in culture medium containing 30 mM KCl and anti-Syt1 directed against the luminal domain of Syt1 for 30 min. Neurons were fixed and stained for Syt1 at the plasma membrane. **D**, quantification of Syt1 fluorescence normalized to PLSCR1$^{+/+}$ (3 independent experiments, 10 field of view per experiment).

The transmembrane and Ca^{2+} binding domains of PLSCR1 are sufficient to restore compensatory endocytosis in PLSCR1 $^{-/-}$ GrC neurons

PLSCR1 possesses a single pass transmembrane and adjacent Ca^{2+} binding site that is critical for its membrane targeting and function. Indeed, a peptide containing both domains retained the capacity to associate with lipid bilayers as well as full scramblase activity *in vitro* (Arashiki et al., 2016; Posada et al., 2014b). To explore further the function of PLSCR1 and investigate the role of its transmembrane domain in neurons, we attempted to restore SV retrieval in PLSCR1$^{-/-}$ GrC neurons by reexpressing the full length (FL) or deletion mutants of PLSCR1 possessing the transmembrane and the Ca^{2+}-binding domain (CBD+TMD) or the transmembrane domain alone (TMD, Figure 4A). To identify transfected neurons and simultaneously monitor the rate of exocytosis/endocytosis, we coexpressed Syp-pH with PLSCR1 constructs fused to mCherry at its N-terminus (Figure 4B). As expected, when a train of action potential was applied to PLSCR1$^{-/-}$ neurons expressing full length PLSCR1, Syp-pH endocytosis was efficiently restored as compared to untransfected PLSCR1$^{-/-}$ neurons (Figure 4C,D). Interestingly, in contrast to TMD mutants, expression of the CBD+TMD mutants in PLSCR1$^{-/-}$ neurons was sufficient to restore Syp-pH endocytosis in response to stimulation (Figure 4C,D). To further determine whether endocytosis restoration by PLSCR1 and PLSCR1 mutant was correlated to PS egress, PLSCR1$^{-/-}$ GrC were transfected with mCherry alone or with PLSCR1 deletion mutants and stimulated with high K^+ solution containing fluorescent AnxA5 to label PS exposed to the extracellular leaflet of the plasma membrane. In contrast to mCherry or TMD transfected PLSCR1$^{-/-}$ neurons, AnxA5 staining increased in CBD+TMD expressing neurons following stimulation (Figure 5A). Plasma

membrane sheets preparation of PLSCR1$^{-/-}$ GrC neurons transfected with GFP-PLSCR1 showed that AnxA5 was found in the vicinity of GFP-PLSCR1 and vGlut1 clusters (Figure 5B). In addition, reexpressing GFP-PLSCR1 into PLSCR1$^{-/-}$ GrC neurons increased amounts of AnxA5 associated to vGlut1 only when GrC neurons were stimulated (Figure 5C). Altogether, our data show that PLSCR1 restore PS egress at synapses when expressed in PLSCR1$^{-/-}$ Grc neurons and that the single transmembrane-spanning domain associated to the Ca^{2+} binding site of PLSCR1 is sufficient to support both compensatory endocytosis and PS egress.

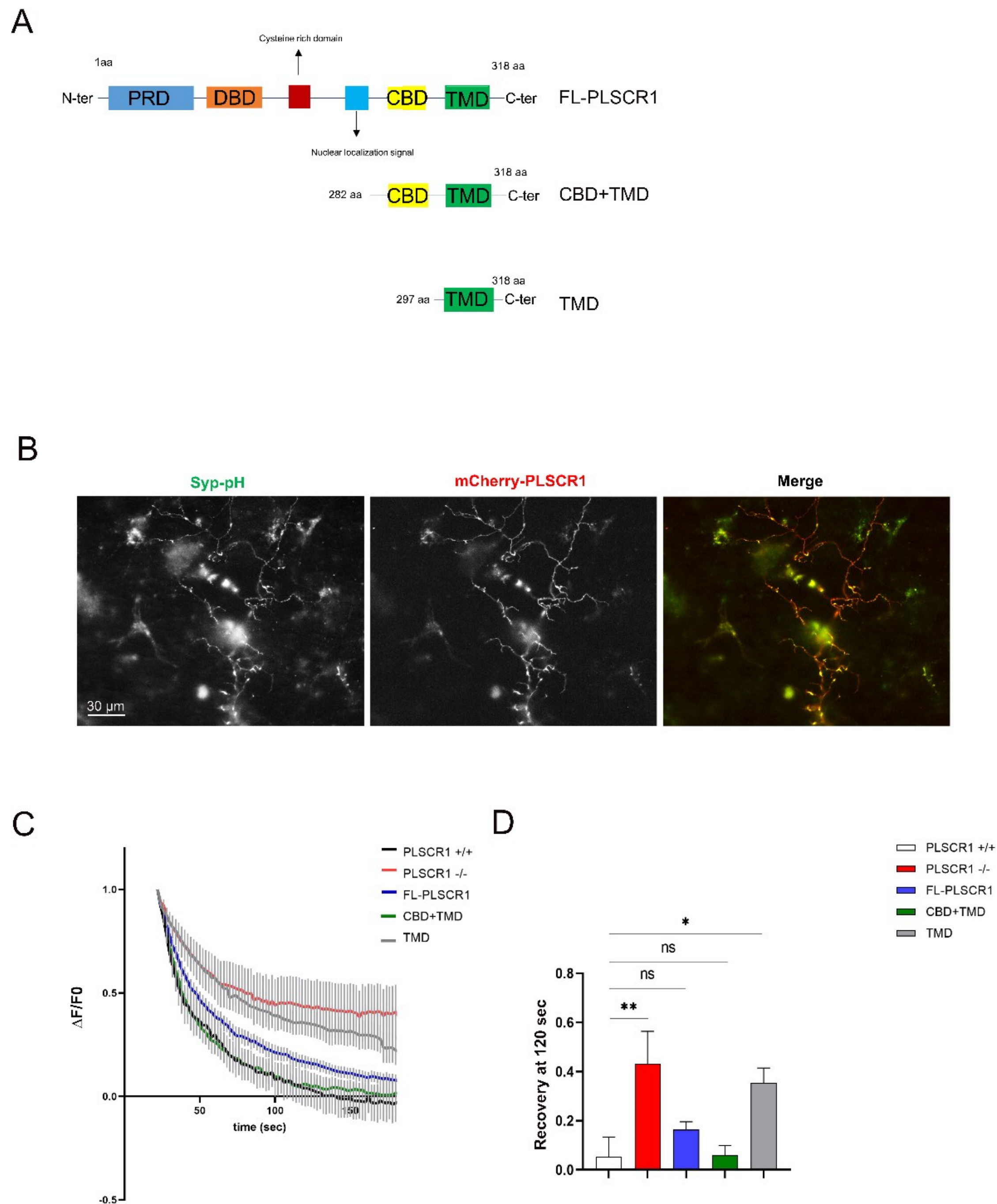

Figure 4: The transmembrane domain associated to the Ca^{2+} binding domain is sufficient to restore SVs endocytosis in PLSCR1$^{-/-}$ GrC neurons. A, schematic representation of PLSCR1 deletion mutants used. FL PLSCR1: full length PLSCR1; PRD: prolin rich domain; DBD: DNA binding domain; CDB: calcium binding domain; TMD: transmembrane domain. **B**, representative image of PLSCR1$^{-/-}$ GrC neurons co-expressing mCherry-tagged FL PLSCR1 and Syp-pH. **C**, average normalized time course of fluorescence response of Syp-pH represented as F/F0 +/- SEM in PLSCR1-/- GrC neurons transfected with the indicated constructs and challenged with an

action potential train (40Hs, 10 s). **D**, mean normalized intensity of Syp-pH fluorescence remaining after 2 min imaging.

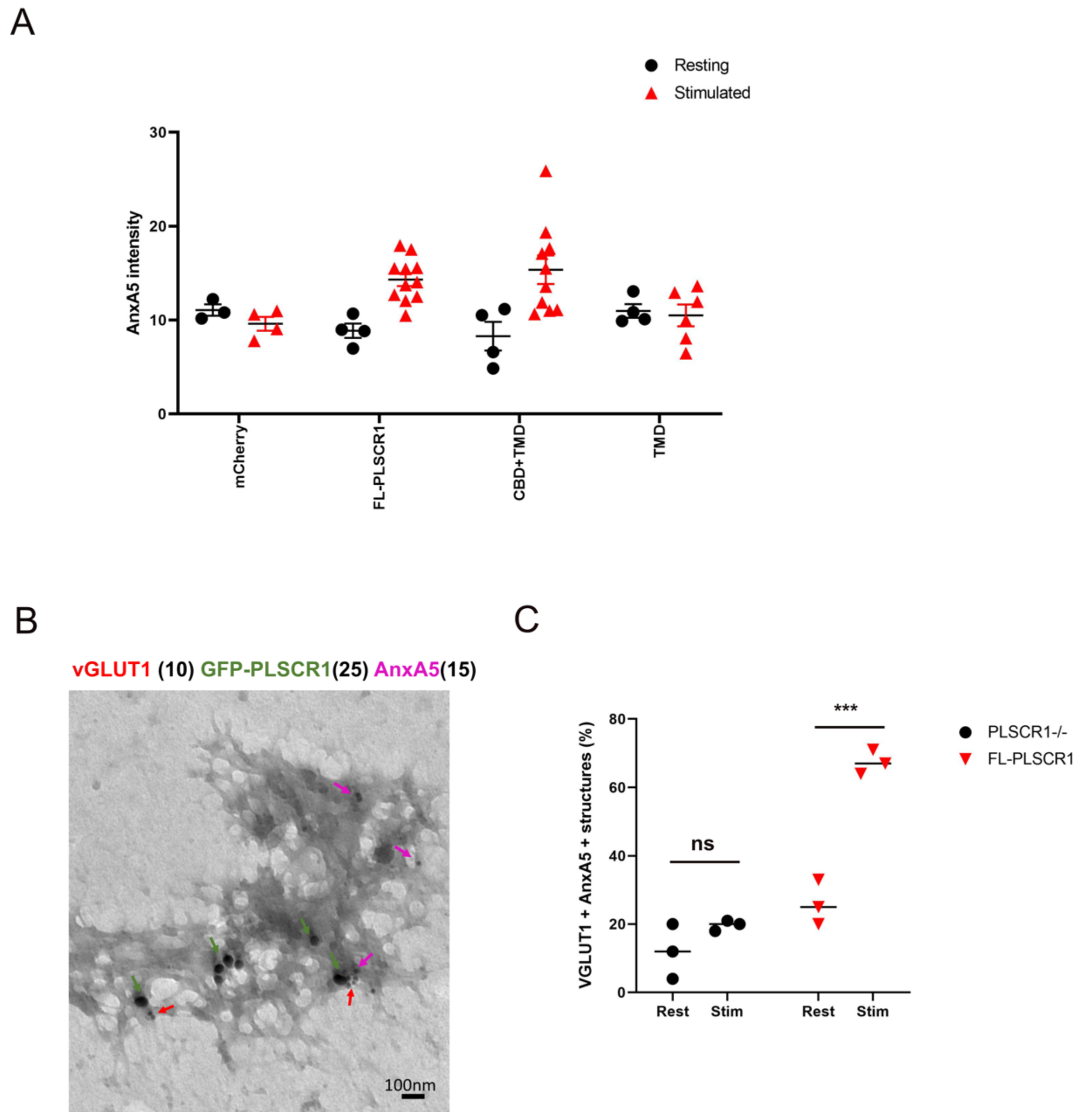

Figure 5: The transmembrane domain associated to the Ca^{2+} binding domain restore PS egress in PLSCR1$^{-/-}$ GrC neurons. A, PLSCR1$^{-/-}$ GrC neurons were transfected with either the mCherry alone, or mCherry-tagged FL PLSCR1, mCherry-CBD+TMD or mCherry-TMD. Neurons were stimulated for 10 min with 59 mM K+ or maintained under resting condition in the presence of fluorescent AnxA5. Cells were fixed, counterstained for actin and imaged under a confocal microscope. AnxA5 staining was quantified and the average intensity in neurons is presented. **B**, representative electron micrograph of plasma membrane sheets prepared from

PLSCR1$^{-/-}$ GrC neurons expressing GFP-PLSCR1. Neurons were stimulated for 10 min with 59 mM K^+ or maintained under resting condition in the presence of biotin AnxA5. Immunolabeling of vGlut1, AnxA5 (anti-biotin antibodies) and GFP were revealed with 10 nm (red arrows), 15 nm (pink arrows) and 25 nm (green arrows) gold particles respectively. Graph represents the % of synapses (vGlut1 positive) closely associated to AnxA5 and GFP beads.

The lack of PLSCR1 induces structural synaptic plasticity *in vitro*

Chronic perturbation of neuronal activity led to homeostatic synaptic plasticity which drives counteracting changes in the network connectivity and/or synaptic strength to restore neuronal activity to baseline levels (Davis, 2013; Pozo and Goda, 2010; Yin and Yuan, 2015). Because GrC neurons are cultured in the presence of 30 mM KCl to prevent cell death, they are chronically stimulated and therefore may adapt to impaired endocytosis in the absence of PLSCR1. To test this hypothesis, GrC neurons from PLSCR1 $^{+/+}$ and PLSCR1$^{-/-}$ mice were cultured for 10 days and stained for vGlut1 and PSD95 to identify the presynaptic bouton and the postsynaptic compartment respectively and for Tau protein to counterstained and identify GrC axons. Molecular arrangement of both markers were mapped using statistical object distance analysis (SODA) of confocal images which consist of detecting stained spot as objects and analyzing the spatial relation between detected objects (Lagache et al., 2018). It allows to determine the amounts of synapses formed when the presynaptic and postsynaptic markers are apposed and to measure the coupling distance between pre- and postsynaptic markers. As shown in Figure 6, the coupling distance between both markers is reduced in PLSCR1$^{-/-}$ GrC neurons (Figure 6A) and the proportion of synapses formed (vGlut1 or PSD95 clusters apposed to its pre-or postsynaptic counterparts relative to total vGlut1 or PSD95 clusters) is increased in PLSCR1$^{-/-}$ neurons (Figure 6B). Next, we analyzed whether increase synapses formation was associated to a change in synapse density along the axon. Interestingly, the density of vGlut1 and PSD95 clusters as well as the density of synapses along axons increase in PLSCR1$^{-/-}$ neurons suggesting that in the absence of PLSCR1, structural synaptic plasticity takes place.

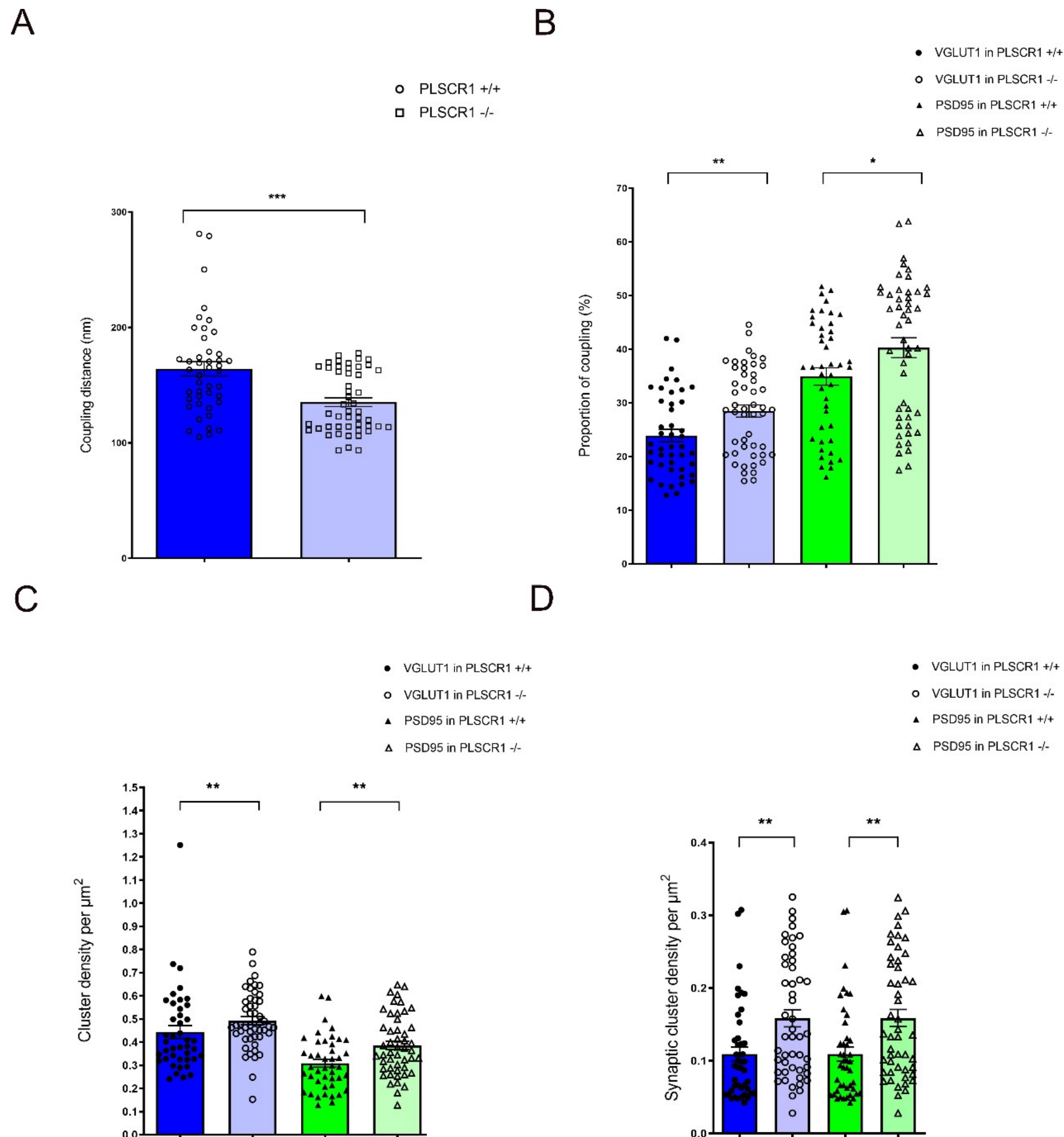

Figure 6: PLSCR1 deletion induces structural synaptic plasticity *in vitro*. A, cultures of PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ GrC neurons were fixed and stained for vGlut1, PSD95 and Tau proteins to counterstain axons. Neurons were imaged under confocal microscope and statistical object distance analysis were performed (SODA) to determine the coupling distance between presynaptic vGlut1 and postsynaptic PSD95 clusters in axons **B**, analysis of the proportion of vGlut1 or PSD95 clusters associated to its pre- or postsynaptic counterpart. Graph represents the percent of statistical relevant coupling of stained clusters (either vGlut1 or PSD95). **C**, analysis of the density of clusters formed either by vGlut1 or PSD95 along axons of PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ GrC neurons. Bar represents the mean +/- SEM. **D**, analysis of the density of synapses relative to either vGlut1 or PSD95 staining along axons of PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ GrC neurons. Data were obtained from 3 independent experiments. Bar represents the mean +/- SEM

PLSCR1 is required to sustain synaptic transmission at high frequency

In the cerebellar cortex, GCs convey high-frequency (HF) information (several hundreds of Hz) to Purkinje cells (PCs) and molecular layer interneurons. A slowing-down of endocytosis may impair the relative size of pools or sub-pools of releasable vesicles and in turn the dynamic of synaptic transmission during HF stimulations. To challenge this hypothesis, we recorded synaptic transmission at GrC to PC synapses in cerebellar acute slices from PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ littermates. To focus on presynaptic mechanisms and to rule out postsynaptic contributions, we pharmacologically blocked the induction of postsynaptic long-term plasticity (LTP and LTD), NMDA-dependent plasticity (Bidoret et al., 2009; Bouvier et al., 2016) and endocannabinoid signalling (Beierlein et al., 2007; Marcaggi and Attwell, 2005). To estimate presynaptic behaviour of GrC -PC synapses, we recorded synaptic current in PCs evoked by stimulations of GrC axons (that is parallel fibers, PFs, Figure 7A) with twin stimuli (paired-pulse stimulation at 50 Hz) or trains of 50 stimuli at 100 Hz (50 pulses) and at near-physiological temperature (34°C). As already reported in our previous works (Doussau et al., 2017), a strong paired-pulse facilitation of synaptic transmission was observed at GrC -PC synapses from PLSCR1$^{+/+}$ mice (Fig 7B). Strikingly, in PLSCR1$^{-/-}$ mice, paired-pulse facilitation was strongly reduced at 50 Hz and 100 Hz (mean paired-pulse ratio: 2.03 ± 0.7 for PLSCR1$^{+/+}$ versus 1.59 ± 0.07 for PLSCR1$^{-/-}$, *t*-test, p = 0.001, n= 7 for PLSCR1$^{+/+}$ and n=8 for PLSCR1 $^{-/-}$). During 100 Hz trains, GrC synapses in PLSCR1$^{+/+}$ mice stood out by a sustained facilitation of synaptic transmission, which was maintained during at least 20 stimuli (Fig 7C). In PLSCR1$^{-/-}$ mice, synaptic facilitation was reduced and synaptic transmission rapidly collapses indicating that GC synapses have lost the capacity to rapidly refill release sites.

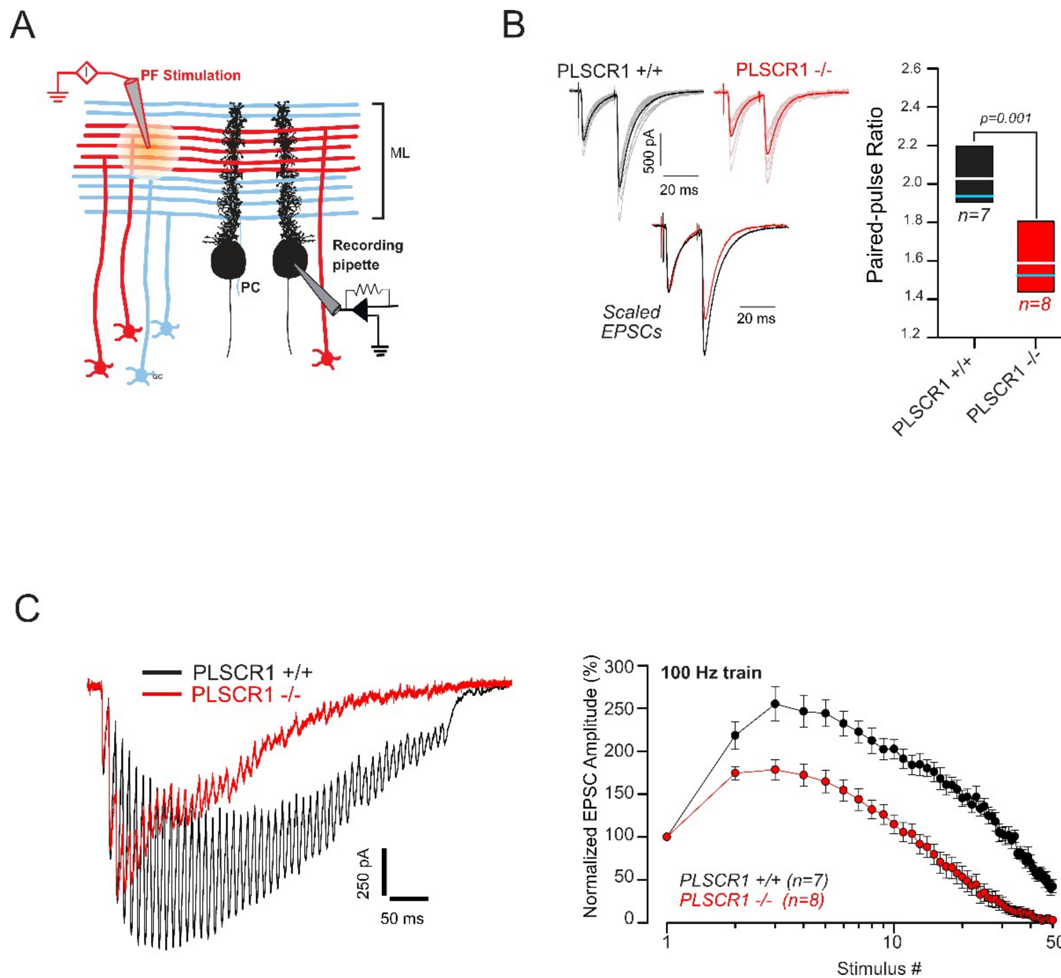

Figure 7: PLSCR1 is required to sustain synaptic transmission at high firing rate. A, Schematic representing the connectivity in the cerebellar cortex and the position of the stimulating and recording electrodes. (GC: granule cell; PC: Purkinje cell, PFs: parallels fibers, ML: molecular layer). **B,** *Left:* representative EPSCs evoked by paired-pulse stimulation (50 Hz) of PFS recorded in WT and PLSCR1 $^{-/-}$ mice acute slices (black and red traces respectively). Thick lines correspond to averaged EPSCs of at least 10 successive EPSCs (thin lines). Lower traces correspond to normalized averaged EPSCs. Right Box-plots showing the values of the paired-pulse ratio obtained in WT and PLSCR1 $^{-/-}$ acute slice. White and blue line corresponds to mean and median values respectively. **C,** *Left:* representative recording traces evoked by PF stimulations at 100 Hz (50 pulses) and recorded in WT and PLSCR1 $^{-/-}$ mice acute slices (black and red traces respectively). *Right:* Mean values of normalized EPSC amplitude elicited by trains of stimulation at 100 Hz and recorded in WT and PLSCR1 $^{-/-}$ mice acute slices (black and red traces respectively).

Discussion

In a previous study, we have shown that PLSCR1-dependent PS egress in neuroendocrine chromaffin cells from the adrenal medulla was not involved in large dense core vesicle (LDCV) exocytosis but was required during compensatory endocytosis. Importantly, whereas the absence of PLSCR1 delayed LDCV proteins retrieval, it did not alter constitutive endocytosis of the transferrin receptor or the uptake of dextran by fluid phase endocytosis indicating that PLSCR1 was specifically mobilized during Ca^{2+}-regulated exocytosis to trigger compensatory endocytosis (Ory et al., 2013). As the molecular mechanisms that control exocytosis and compensatory endocytosis of LDCV and SVs rely on a common set of proteins, we wondered whether PLSCR1 may control SVs exo-endocytosis cycle. Here, we provide evidences that PLSCR1 is dispensable for SVs exocytosis but required for their efficient recycling since Syp-pH and SytI retrieval was altered at synapses of PLSCR1$^{-/-}$ GrC neurons. Interestingly, by the use of deletion mutants of PLSCR1, we established a correlation between the restoration of PS egress and compensatory endocytosis suggesting that randomizing structural phospholipids like PS at the plasma membrane may constitute a key signal to initiate compensatory endocytosis. In agreement with a defect in vesicular pool replenishments, synaptic transmission at GrC to PC synapses in cerebellar acute slices exhausted faster at high frequency in the absence of PLSCR1. We therefore propose that PLSCR1-dependent PS egress controls compensatory endocytosis of excitatory cells and contributes to sustain neurotransmission at high frequency.

Plasma membrane asymmetry disruption and compensatory endocytosis

Probing the asymmetry of the plasma membrane by immunofluorescence and immunogold EM approaches revealed that, in agreement with PLSCR1 enrichment at synaptic terminals, PS egress occurred at synapses in a PLSCR1-dependent manner. In the absence of PLSCR1, PS translocation to the extracellular leaflet is blocked and the retrieval of SV associated Syp-pH is dramatically slowed down after neuron stimulation. Defect in endocytosis was not restricted to Syp-pH since, in condition of mild stimulation, SytI staining at the synapses increased suggesting that the absence of PLSCR1 causes a general delay in compensatory endocytosis. Interestingly, by expressing deletion mutant in PLSCR1$^{-/-}$ GrC neurons, we identified the minimal domain able to restore both PS egress and compensatory endocytosis. Despite the fact that the transmembrane

domain of PLSCR1 can insert into lipid bilayers *in vitro* (Posada et al., 2014a; Venken et al., 2017), it was unable to restore by itself PS egress and compensatory endocytosis unless associated to the Ca^{2+} binding site of PLSCR1. Although PLSCR1 possesses domains able to interact with and modulate signaling proteins (Amir-Moazami et al., 2008; Sun et al., 2002), they are dispensable to convey PS and restore compensatory endocytosis in neurons. Therefore, it indicates that PS transfer from the intracellular to the extracellular leaflet might be sufficient by itself to trigger compensatory endocytosis in response to intracellular Ca^{2+} increase. How the local loss of plasma membrane topology can induce compensatory endocytosis? The inner leaflet of the plasma membrane is negatively charged due to the presence of anionic phospholipids including polyphosphoinositides (PIs) and PS. Compared to PIs, which represent only a minor fraction of the phospholipids present at the plasma membrane, PS is predominant and its redistribution from one leaflet to the other may alter the charge at the surface (Puchkov and Haucke, 2013; Yeung et al., 2008). According to AnxA5 staining, PS egress occurred only locally, at synapses, where PLSCR1 is enriched. Local decrease of negative charges may have several consequences on exocytosis and endocytosis depending on the timing of lipid scrambling relative to vesicle fusion. By reducing the repulsive forces of opposing membrane, local PS depletion may favor fusion between SVs and the plasma membrane (Davletov and Montecucco, 2010). However, amounts of Syp-pH released were not different in PLSCR1$^{-/-}$ as compared to PLSCR1$^{+/+}$ neurons suggesting that exocytosis was unaltered in the absence of PS scrambling. This is in agreement with results obtained in chromaffin cells where LDCV fusion was unaltered in the absence of PLSCR1 (Ory et al., 2013). Local decrease in PS may also reduce the recruitment of cytosolic proteins with positively charged residues (Clarke et al., 2020; Kay and Fairn, 2019). However, rather than a decrease in protein recruitment, endocytosis requires the assembly of a complex network of proteins to initiate endocytosis through hydrophobic and electrostatic interactions. In addition, the synthesis of the negatively charged PtdIns(4,5)P_2 at synapses after cell stimulation is necessary to recruitendocytic proteins and favor membrane bending (Micheva et al., 2001; Puchkov and Haucke, 2013). It is therefore hard to conceive that PS egress by itself would be the adjustment variable for local membrane charge. In contrast, PS egress may modify plasma membrane fluidity. Enriched in tightly packing sphingolipids, the outer plasma membrane leaflet is highly ordered and rigid (Gupta et al., 2020; Lorent et al., 2020). Insertion of SVs that are highly enriched in cholesterol participate to change in the presynaptic plasma membrane (Takamori et al., 2006). Membrane cholesterol content affects lipid lateral self diffusion, confines synaptic vesicle proteins and limits their diffusive behavior (Byczkowicz et al., 2018; Dason et al., 2014; Mercer et al., 2011; Wilson et al., 2020). Sustained synaptic release is limited by the availability of fusion-

competent SVs, but also by clearance of specific release sites in the presynaptic active zone, which provides spatial availability for release-ready SVs. It may be a major rate-limiting step for sustained synaptic transmission especially at synapses with high firing rates (Neher, 2010; Park et al., 2018). Redistributing PS to the outer leaflet of the presynaptic membrane may therefore favor active zone clearance to prepare exocytotic site for the next round of vesicle fusion. Of note, PLSCR1 is not specific to PS and can also scramble PE and PC but not sphingolipids (Dekkers et al., 2002). The redistribution of lipids follows their gradient and should lead to increased PE to the outer leaflet and increased PC to the inner leaflet of the plasma membrane. PE egress may also contribute to increase in membrane fluidity (Wilson et al., 2020). Finally, PS and cholesterol are codistributed in the inner leaflet of the plasma membrane and the local decrease of PS may lead to concomitant decrease in cholesterol contents (Maekawa and Fairn, 2015) contributing to fluidify the leaflet and clear the active zone. The precise role of PLs egress needs to be further explored.

PLSCR1 and GrC neurotransmission

Synaptic plasticity allows for fast adaptation of synaptic strength to neuronal network activity, which is critical for information processing. Synaptic plasticity comes in two main flavors: functional plasticity, which change the strength of the synapse by modifying signal transmission and structural plasticity leading to a variety of changes including synapses number and connectivity between pairs of neurons (Caroni et al., 2012). Interestingly, in Drosophila, mutation or deletion of several proteins associated with vesicle recycling, including endophilin, synaptojanin and dynamin results in homeostatic increases in synapses number that may compensate for a decrease in compensatory endocytosis to stabilize neuronal network activity (Dickman et al., 2006; Goel et al., 2019; Verstreken et al., 2003). Here, we exploited the specificity of in vitro GrC neuron culture, which is achieved by using 30 mM KCl in the culture medium to allow neurons to survive. As a result, GrC neurons are constantly depolarized and activated, mimicking chronic activation conducive to the establishment of synaptic plasticity. In the absence of PLSCR1, GrC neurons form more synapses as revealed by the increased in paired clusters labelled for vGlut1 and PSD95 found on axons *in vitro*. Intriguingly, the distance between vGlut1 and juxtaposed PSD95 clusters was reduced in PLSCR1$^{-/-}$ GrC neurons suggesting that the shape of the synapse is altered and that PLSCR1 may be involved in synapse maturation. Excitatory synapse maturation depends on activity-dependent proteolytic cleavage of synaptic adhesion proteins leading to the release of the ectodomain. Binding of the ectodomain to its

cognate receptor modulates synaptic maturation and allows fine tuning of the mature synapses (Nagappan-Chettiar et al., 2017). Although the molecular mechanism is still unclear, matrix metalloproteinases (MMPs and ADAMs) acts as major sheddases of synaptic adhesion proteins. Interestingly, PS-binding domain have been identified in some ADAMs and transient exposure of PS at the surface of erythrocytes activates ADAM10 (Bleibaum et al., 2019), the protease responsible for neuroligin-1 cleavage and synapse maturation in mice (Suzuki et al., 2012). It is therefore tempting to speculate that, in the absence of PLSCR1 and PS egress, synaptic adhesion proteins cleavage might be reduced and synapse tightened *in vitro*.

In the cerebellar cortex, GrC-PCs synapses have to transmit sensorimotor information emitted at extreme frequencies (several hundred of Hz to kHz, Rancz et al., 2007). To do so, these synapses are endowed with specific presynaptic processes allowing a boost (facilitation) and maintenance of the release of neurotransmitters during HF trains. In GrC terminal, releasable SVs are segregated in two sub-pools differentially poised for exocytosis: a fully-releasable pool that can be released by a single stimulus and a reluctant pool recruited only by HF stimulation. The ultra-fast mobilization of reluctant SVs underlies the large facilitation of glutamate release during paired-pulse stimulation or trains at HF (Doussau et al., 2017; Miki et al., 2016). Here we show that a lack of PLSCR1 strongly affects the boost of glutamate exocytosis during paired-pulse stimulation and train of stimulations elicited at HF suggesting that the contribution of the reluctant pool to neurotransmission is reduced. Hence, in GrC-PC synapses lacking PLSCR1, the slowdown of endocytosis may affect the size of the reluctant pool and alternatively, a defect in PL scrambling during SV exocytosis may impair the clearance of release sites which is required prior to their ultra-fast replenishment with reluctant SVs. Given that for all synapse types the profile of the paired-pulse ratio is shaped by the probability of release (the higher is this probability of release the lower is the paired-pulse ratio; Jackman and Regehr, 2017), we cannot exclude that a compensatory mechanism related to a lack of PLCR1 and increasing the overall probability of release underlies abnormalities in the profile of presynaptic plasticity in PLSCR1$^{-/-}$ miceInterestingly, we detected PLSCR1 in the cerebellum and the olfactory bulb, two brain regions known to sustain high frequency stimulation. In contrast, PLSCR1 is barely detected in hippocampus and absent from the cortex (data not shown), two brain regions that fire at low frequency (Delvendahl and Hallermann, 2016). PLSCR1 appears therefore a good candidate to play a specific role in high-frequency synaptic transmission by scrambling PLs at synapse, a fast way to modify plasma membrane properties.

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Supplementary data

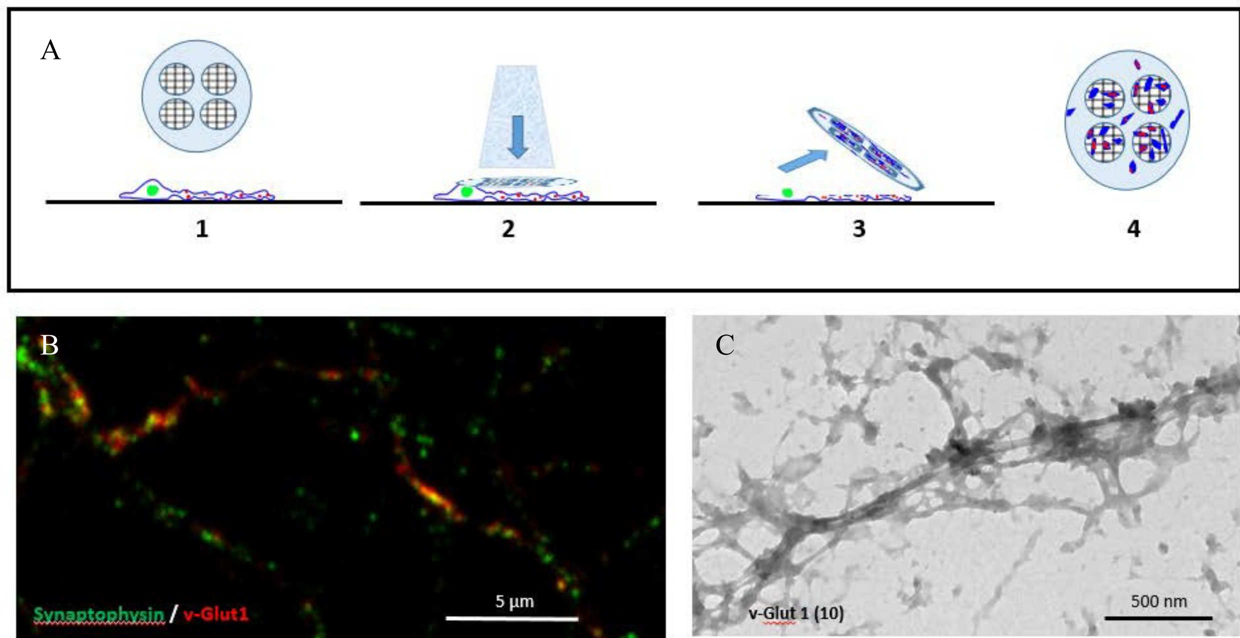

Figure S1: Schematic representation of plasma membrane sheet preparation. **A**, carbon-coated Formwar films on nickel EM grid (1) were inverted onto neurons grown on PLL-coated coverslips and pressure is applied to the grid (2). Grids are then lifted (3), leaving fragments of plasma membrane (blue) and synapses containing docked SVs (red) on the grids. **B**, confocal images of neurons stained for vGlut1 (red) and synaptophysin (green) **C**, representative electron micrograph of plasma membrane sheets prepared from GrC neurons.

D. Discussion

Additional data have been included in the discussion. For the experimental protocols, refer to "Supplementary materials and methods" section.

1. PLSCR1 is necessary for compensatory endocytosis in cerebellar neurons

Several studies have suggested that PLSCR1 is involved in regulated secretory pathways. PLSCR1 controls mast cell degranulation and secretion of vasoactive amines, proteases, and proinflammatory cytokines (Amir-Moazami et al., 2008; Pastorelli et al., 2001). PLSCR1 is indirectly involved in secretion of the amyloid β-peptide through the interaction with β-secretase (β-site amyloid precursor protein–cleaving enzyme) (Kametaka et al., 2003). In Drosophila, Scrm1 and Scrm 2 double mutants, which are the homologues of human PLSCR1 and PLSCR2 respectively, reveal a role for Scramblases in modulating regulated exocytosis at the neuromuscular junction (Acharya et al., 2006).

A previous work of our team has shown that PLSCR1 is expressed in chromaffin cells, where it is involved in compensatory endocytosis of secretory granule proteins. Since constitutive endocytic pathways, such as receptor-mediated endocytosis of transferrin or fluid-phase uptake of dextran were not affected in PLSCR1$^{-/-}$ cells, these results represented the first evidence for a specific role of the plasma membrane-bound PLSCR1 in secretory granule recycling (Ory et al., 2013). In addition, in contrast to immune cells like mast cells (Amir-Moazami et al., 2008), the absence of PLSCR1 did not alter exocytosis in chromaffin cells, reinforcing the idea that PLSCR1 function might be limited to the control of compensatory endocytosis in chromaffin cells and more widely to neurosecretory cells. To further explore the role of PLSCR1 in such cells, we asked whether PLSCR1 may play a role in the synaptic vesicle exo-endocytosis coupling in neurons and, we found an alteration of endocytosis kinetics in PLSCR1 $^{-/-}$ cerebellar neurons, which was completely restored by the re-expression FL-PLSCR1.

1.1 Preferential distribution of PLSCR1 at the plasma membrane of synaptic terminal

At the beginning of my Ph.D, I first decided to check where PLSCR1 was expressed in the brain. Little information was available about the expression profile of PLSCR1 in the mouse brain. Studies realized before I started my thesis have reported that PLSCR1 was expressed in the brain, and more specifically in the human hippocampus (Rami et al., 2003). However, in Rami et al. study, detection of PLSCR1 was obtained only by histochemistry and the specificity of the anti-PLSCR1 antibody was not properly tested. Previous attempt in the team and by myself to detect endogenous PLSCR1 by immunofluorescence using several available anti-PLSCR1 antibodies failed. Especially, I performed immunofluorescence on primary macrophages, a cell type reported to express high level of PLSCR1. I cultured macrophages from PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ mice and I compared immunofluorescent PLSCR1 staining. Despite western blot analysis showing that PLSCR1 expression was, as expected, abolished in PLSCR1-/- macrophages, I could not detect any difference either in fluorescence intensities nor in the staining pattern between PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ samples. It indicates that in the absence of relevant control, the reliability of immunological staining for PLSCR1 should be considered with care. I therefore detected PLSCR1 using western blot analysis and found that PLSCR1 was highly expressed in the mouse cerebellum, the olfactory bulb but barely detectable in the hippocampus or the cortex. I first addressed the role of PLSCR1 in neurons from the olfactory bulb, and despite encouraging preliminary results, due to the heterogeneity of cell responses likely linked to the diversity of neuron types present in the culture (Parrish-Aungst et al., 2007), I decided to use rather neurons from the cerebellum, which led to culture with 95 % of granular cells (GrC). To get insight into PLSCR1 function, it was crucial to know where it was localized in neurons. Indeed, depending on the cell types and/or post-translational modification, PLSCR1 has been found either at the plasma membrane or in the nucleus. Following the expression of a PLSCR1 coupled to mCherry in Neuro-2a cells (neuroblasts isolated from mouse brain), we could observe that exogenously expressed PLSCR1 was exclusively located at the plasma membrane (Fig.34).

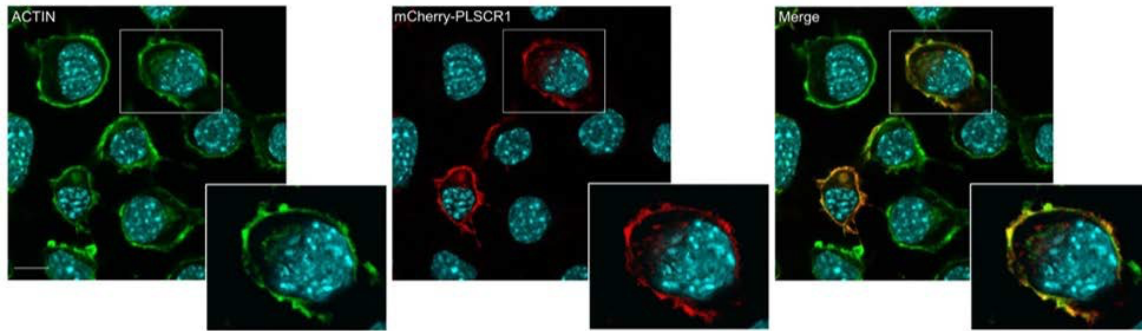

Fig.34 mCherry-PLSCR1 localizes at the plasma membrane in Neuro-2a cells. Representative confocal images depicting mCherry-PLSCR1 (red), ACTIN (green) and Nuclei (cyan). Surrounded areas correspond to the zooms in the bottom. Scale bar 10 µm

It has been shown that PLSCR1 nuclear localization can occur only when PLSCR1 is not palmitoylated. Indeed, both mutation in any of the five cysteines or treatment with a palmitoyltransferase inhibitor (Wiedmer et al., 2003) reduced PLSCR1 targeting to the PM, favoring nuclear localization. In my study, the exogenously expressed PLSCR1 mutant, containing only the Ca^{2+} -binding site and the transmembrane region (referred as PLSCR1-(CBD+TMD)) appears to be preferentially located at the PM in Neuro-2a cells (data not shown). By contrast, my findings indicate that cysteine-rich region *per se* would not be required neither for its localization at the PM nor for its function. Moreover, the re-expression of this mutant in PLSCR1 $^{-/-}$ neurons completely restores compensatory endocytosis. Consistent with my results, Merregaert and co-workers have observed that the majority of eGFP-PLSCR1 expressed in HaCaT cells, in the presence of 2-bromopalmitate, thus in the absence of acylation, was still localized at the cell membrane, and only a small amount was translocated to nucleus. Moreover eGFP-PLSCR1 that is not palmitoylated, displayed a normal scramblase activity (Merregaert et al., 2010). These results suggest that the lack of acylated residues, by itself, does not necessarily lead to the redistribution of PLSCR1 in some cell types and that other cellular elements (proteins or lipids) must control the distribution of PLSCR1.

Furthermore, given that PLSCR1 is a membrane-bound protein, we questioned if it displayed a preferential distribution at neuronal PM. In primary neurons, my data suggest a preferential localization of both endogenous and exogenously expressed PLSCR1 at the plasma membrane of

synapses in neurons. First, endogenous PLSCR1 is significantly enriched in synaptosomes prepared from PLSCR1 $^{+/+}$ cerebella. Second, exogenously expressed GFP-PLSCR1 perfectly colocalized with two pre-synaptic markers, synaptotagmin-1 and vGlut1 along the axon. Third, by preparing plasma membrane sheets of PLSCR1 $^{-/-}$ neurons transfected with GFP-PLSCR1, we detected GFP-PLSCR1 in the vicinity of SNAP-25 at the plasma membrane (data not shown). GFP-PLSCR1 was localized to structures labeled with two pre-synaptic markers, synaptophysin and vGlut1, in agreement with the synaptic enrichment of the endogenous PLSCR1. It appeared therefore that PLSCR1 is mostly found at the plasma membrane and preferentially enriched at synaptic terminals.

However, the main limit of my localization study is that we do not have access to the endogenous PLSCR1 due to the lack of reliable antibodies for immunofluorescence or electron microscopy. We used several other techniques that have their own limitations. Except for the preparation of the synaptosomal fraction, all experiments were performed using exogenously expressed PLSCR1. It is therefore not excluded that overexpression of PLSCR1 may alter its subcellular localization.

Cytoplasmic face-up membrane sheet is a technique that has been set up on cultured human fibroblasts (Sanan & Anderson, 1991) and then adapted for chromaffin cells (Umbrecht-Jenck et al., 2010) which are round and thicker than axons of neuronal cells. To prepare membrane sheets (see materials and methods in the paper), a pressure is applied with a grid for 20 s and then the grid is rapidly lifted up in order to rip off the cell roof. Given the small thickness of the axons compared to the cell body, most of the fragments left on the grid derived from the soma of neurons. Only some pieces of the axonal network are preserved, making it difficult to recognize the morphology of synaptic structures. We could nonetheless find structures labelled for both GFP-PLSCR1 and synaptic markers suggesting that PLSCR1 is localized to synapses. Alternatively, we are also trying to visualize the expressed GFP-PLSCR1 in cerebellar granule neurons by classical transmission electron microscopy. The ongoing experiments would provide more consistent evidence about PLSCR1 localization, and importantly, determine whether PLSCR1 is found in the active zone and/or the peri-active zone of the synapses.

1.2 Is PLSCR1 a determinant of high frequency synapses?

Western blot analysis has revealed that PLSCR1 expression levels differ in different regions of the brain. It is highly expressed in the cerebellum, less in the olfactory bulb and almost not present in both hippocampus and cortex (data not shown). An intriguing possibility would be that PLSCR1 have a potential region-specific function related to specific neuron types able to fire at high frequency. Indeed, the differential expression level observed by Western blot suggest that PLSCR1 is rather highly expressed in the cerebellum and the olfactory bulb, two structures known to sustain high frequency stimulation, and much less expressed in hippocampus or cortex, two structures known to fire only at low frequency (Delvendahl & Hallermann, 2016).
More precisely, in cerebellum, Purkinje cells (PCs) are contacted by granule cells (GrCs) that fire at very high frequencies (several hundreds of Hz to kHz) (Delvendahl & Hallermann, 2016) compared to low frequencies (1 to 20 Hz) from cortical or hippocampal pyramidal neurons (Delvendahl & Hallermann, 2016).

When synapses are activated at high frequencies for an extended period, most of them exhibit frequency-dependent depression, meaning that larger initial post-synaptic responses rapidly depress (Fig. 35A). Conversely, at some synapses, like PC-GrC synapses, facilitation counteracts depression in a frequency-dependent manner. In fact, initial post-synaptic responses are small, but facilitate markedly as a function of firing frequency (Fig. 35 B).

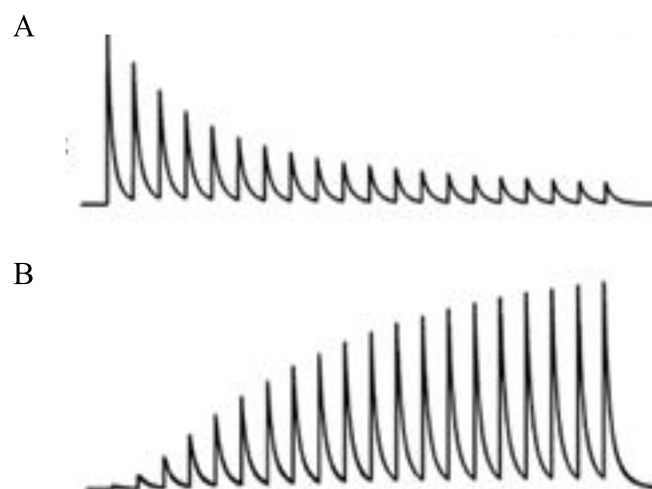

Fig.35 Short-term plasticity: Synaptic depression and facilitation. Representative excitatory postsynaptic currents. A low-frequency synapse produces large initial response that depress rapidly. B High-frequency synapse shows small initial responses but facilitate as a function of firing frequency. Facilitation increases the amplitude of the second response than the first one. *Adapted from Jackman et al., 2017*

Facilitation is observed at synapses with low initial probability of vesicular release (*p*), whereas synapses with high initial probability of vesicular release usually exhibit a frequency-dependent depression. In other words, if the initial *p* is high, the RRP is rapidly depleted and synaptic responses are depressed until the RRP is replenished (Zucker & Regehr, 2002). Conversely, the facilitating synapses, able to sustain high frequency stimulation, possess specialized mechanisms, such as additional SV pools, that boost neurotransmitter release even as the RRP pool decreases (Jackman & Regehr, 2017). Considering this, the question that arises is "How does PLSCR1 activity play a role in high frequency synapses?" Since PLSCR1 is required for compensatory endocytosis, we asked if a defect in the number of SV is present in PLSCR1 $^{-/-}$ mice.

The results obtained by electron microscopy show that the total number of synaptic vesicles, the number of docked vesicles and the vesicles distribution in synapses are normal in PLSCR1 $^{-/-}$ synapses (Fig.36), in agreement with results published in the single Scrm1 null mutant Drosophila (Acharya et al., 2006). Despite using electron microscopy approach, we cannot discriminate the different vesicular pools and we are not able to exclude that some changes in size and/or in composition of pool might occur. It is well established that the RRP represents the fraction of primed vesicles available for immediate release upon increase in cytosolic Ca^{2+}. As the RRP is rapidly depleted, replenishment/recruitment of vesicles is a crucial process to insure continuous and sustained responses in high frequency synapse. Indeed, the main difference between low-frequency and high-frequency synapses relies on an additional pool present only in these latter ones. Diverse studies in high frequency synapses have demonstrated that an additional pool, named in different ways depending on synaptic model or laboratory, is recruited and/or replenish the RRP during the stimulation at high frequency (Miki et al., 2016; Sakaba, 2018). In particular, at the cerebellar GC-PC synapse, Doussau and co-workers have shown that high frequency stimulation triggers the ultrafast recruitment of a vesicular pool, named "reluctant pool," enabling fast recovery and large facilitation of glutamate release (Doussau et al., 2017). More precisely, they propose that the release of glutamate and the presynaptic short-term plasticity at GC-PC synapses are shaped by two pools of releasable SVs, namely fully releasable SVs and reluctant SVs. The former pool can be released by a single AP and is gradually silenced, leading to full blockage of synaptic transmission during low-stimulation frequency at 2 Hz, while the latter one is specifically recruited to sustain stimulation at high frequency (Doussau et al., 2017).

In our electrophysiological recordings, we observed that in the absence of PLSCR1, the paired-pulse ratio (PPR), the ratio between the amplitude of the second excitatory post-synaptic

potential (EPSP) and the first EPSP, is significantly reduced in PLSCR1 $^{-/-}$, meaning that the initial probability of vesicular release probability (*p*), during the first APs is higher in PLSCR1$^{-/-}$. Consequently, PLSCR1 $^{-/-}$ mice show a faster depression during 100 Hz compared to PLSCR1 $^{+/+}$ mice.

These results suggest that, in the absence of PLSCR1, cerebellar granule neurons are less able to sustain high frequency stimulation and display a higher *p* and a faster depression during intense activity compared to PLSCR1$^{+/+}$ neurons. But how can PLSCR1 be important in sustain high frequency synaptic activity?

The synaptic depression in PLSCR1$^{-/-}$ neurons could arise from the faster depletion of synaptic vesicle pools available for fusion during a train of stimuli. One could imagine that the PLSCR1 might have a specific role in SV recycling of the so-called reluctant pool under high frequency stimulation. Thus, in absence of PLSCR1, an impairment in SV recycling of such reluctant pool, during intense activity can lead to a faster depression of synapses, suggesting that PLSCR1 could be required to sustain high frequency stimulation.

To further investigate the role of PLSCR1 in the recycling of reluctant SVs, additional studies at various stimulation frequencies, which recruit RRP or other pools specifically, are required. In addition, it would be interesting to express PLSCR1 in low-frequency hippocampal neurons to evaluate whether transfected neurons would be able to withstand high-frequency stimulations.

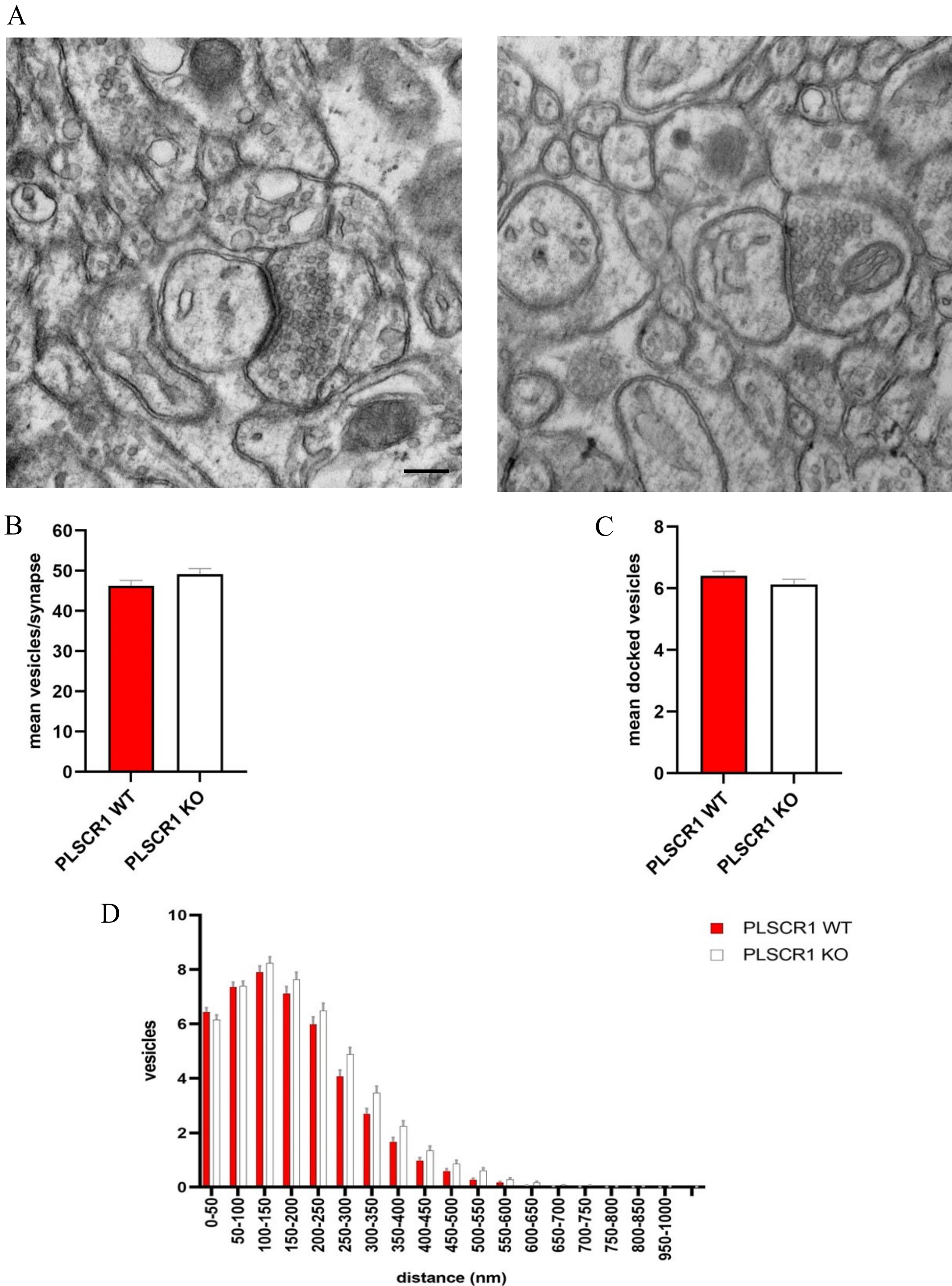

Fig. 36 PLSCR1 $^{-/-}$ mice display no abnormalities in synaptic morphology. Data shown in graphs derive from the analysis of 268 synapses for PLSCR1$^{+/+}$ and 271 synapses for PLSCR1$^{-/-}$, and represent the mean ± SEM. **A** Transmission EM of a synaptic bouton from PLSCR1$^{+/+}$ (left) and PLSCR1$^{-/-}$ (right). Scale bar 100 nm. **B** Mean SVs per synapse (PLSCR1$^{+/+}$, mean is 46,29 ±1,35; PLSCR1$^{-/-}$, mean is 49,14± 1,43). **C** Mean docked SVs per synapse (PLSCR1$^{+/+}$, mean is 6,44 ±0,15; PLSCR1 $^{-/-}$, mean is 6,12± 0,16). **D** Comparison of SVs distribution in PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ boutons.

1.3 Delayed kinetics of Syp-pH in PLSCR1$^{-/-}$ mice: defect of compensatory endocytosis or vesicle reacidification?

The results obtained from Synaptophysin-pHluorin experiments show that, in the absence of PLSCR1, fluorescence decay is delayed suggesting that compensatory endocytosis is impaired in cerebellar granule neurons lacking PLSCR1. Since pHluorin imaging is based on pH variation, we could not fully exclude that the delay in pHluorin decay was the result of an altered re-acidification of synaptic vesicles. However, the Syt1 uptake assay showed that Syt1 remained longer time at the cell surface and confirmed that compensatory endocytosis was altered when PLSCR1 is absent. In addition, since glutamate pumping within synaptic vesicles requires continuous V-ATPase activity and vesicle re-acidification (Carlson & Ueda, 1990), we should have observed the decrease of post-synaptic response by electrophysiology due to reduce release of glutamate. However, the amount of exocytosis measured by peak heights shows no significant difference between PLSCR1 $^{+/+}$ and PLSCR1 $^{-/-}$ neurons (Fig.37).

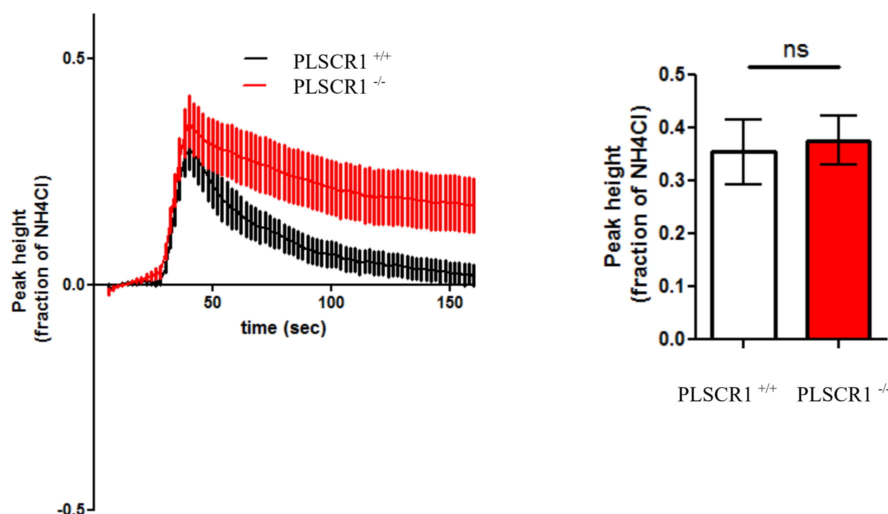

Fig.37 PLSCR1 $^{-/-}$ synapses display a normal SV fusion. (left) Average time course of the Syp-pH response normalized to the total SV pool (revealed by NH_4Cl application). (right) Peak Syp-pH response as a fraction of the total SV pool. n=16 PLSCR1 $^{-/-}$ coverslips and n=17 PLSCR1$^{+/+}$ coverslips from four independent CGN preparations.

However, to completely exclude a defect of newly formed, endocytic vesicle reacidification, we could apply an extracellular acid buffer (pH 5.5) during endocytosis. Syp-pH that is exposed

to the cell surface would immediately be quenched while internalized Syp-pH would be protected. Analysis of pHluorin fluorescence variation in response to acid solution would inform us about the amount of Syp-pH internalized and about a potential defect in endocytic vesicle re-acidification.

1.4 What kind of endocytosis is altered in PLSCR1 $^{-/-}$ neurons?

Our findings evidence that the PLSCR1 protein is required for SV recycling in neurons, but the question of what type of compensatory endocytosis is altered is complex to answer.
Considering the limitations due to the technique used, we first have to wonder what type of endocytosis we are observing. Several parameters must be evaluated, such as: the imaging technique based on tagged Synaptophysin, the stimulation frequency used to trigger endocytosis (40Hz,10sec), and the frequency of image acquisition.

The ADBE is a well-established processus that is triggered by intense neuronal stimulation and represents the most predominant endocytic pathway during strong stimulation (Clayton et al., 2010). As we used a high-frequency stimulation in our experiments, one could imagine that ADBE was triggered too. Nevertheless, Nicholson and co-workers have shown that inhibition of ADBE does not affect the retrieval of diverse SV cargo proteins including synaptophysin (Nicholson-Fish et al., 2015a), suggesting that Syp-pH is not sorted via ADBE. Therefore, Syp-pH imaging experiments do not allow to draw conclusion on the activity of PLSCR1 in the ADBE kinetics. To try to answer that question, we measured dextran-uptake at PLSCR1 $^{+/+}$ and PLSCR1 $^{-/-}$ synapses to compare the number of synaptic terminals performing bulk endocytosis following intense stimulation. Fig.38 shows no significant difference, suggesting that amounts of synapses that perform ADBE is not reduced in PLSCR1 $^{-/-}$.For more accuracy and to further investigate the PLSCR1 involvement in ADBE kinetic, live imaging with VAMP4-pHluorin, a genetically encoded reporter of ADBE (Nicholson-Fish et al., 2015a) is required.

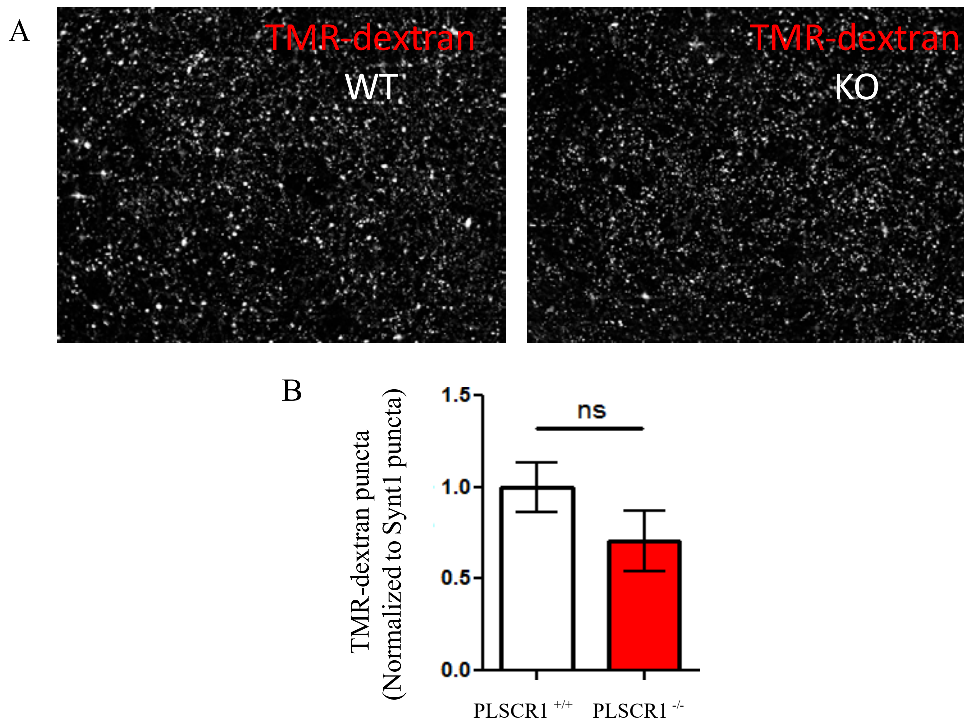

Fig. 38 Synapses performing ADBE are not reduced in PLSCR1 $^{-/-}$. APLSCR1 +/- and -/- neurons incubated with an antibody against Syt1 to label active synapses, and then stimulated with 400 Aps at 40 Hz, in presence of 50 µM TMR-dextran. **A** Representative image of TMR-dextran in PLSCR1$^{+/+}$ and $^{-/-}$. **B** TMR-dextran puncta per field of view, normalized to Synt1 puncta. N= 16 coverslips for each genotype from four independent CGN preparation.

Regarding the fast endocytosis, pHluorin reporter assays have a kinetic resolution that is limited by the rate of SV reacidification (approximately 4-5 seconds) (Atluri & Ryan, 2006) and thus, is unable to report on fast modes of SV recycling like UFE. In addition, the frequency of image acquisition used in our experiments (every 2 seconds) does not allow to image fast endocytosis that occurs within milliseconds.

It has been shown that the majority of SV proteins, including synaptophysin, appears to be internalized on time scale of couple of seconds following AP train stimulation at physiological temperature (Ferguson et al., 2007; Kononenko et al., 2014; Soykan et al., 2017). This endocytic rate is compatible with time constants reported for clathrin-mediated endocytosis (Dittman & Ryan, 2009).

Our results have shown that kinetic of Syp-pH endocytosis, at physiological temperature, occurs within 60 seconds after stimulation. The time course observed in our recordings is in perfect agreement with that obtained from Syp-pH imaging experiments by Nicholson-Fish et al. in both cerebellar and hippocampal neurons during strong stimulation (Nicholson-Fish et al., 2015a). In addition, they observed that inhibition of CME in cerebellar neurons by exposure to the clathrin inhibitor pitstop-2, completely abolished the fluorescence recovery of Syp-pH after stimulation of 40 Hz during 10 seconds at physiological temperature (Nicholson-Fish et al., 2015a). Thus, Syp-pH seems to be predominantely retrieved via CME during intense neuronal activity. Based on these findings, we can speculate that PLSCR1 may be required for clathrin dependent-Syp-pH retrieval.

However, as described in the introduction, the role of clathrin during SV recycling in neurons have been recently questioned. Soykan and co-workers showed that, in hippocampal neurons, SV recycling can occur when clathrin is silenced (Soykan et al., 2017). Similar observations were done at Drosophila neuromuscular junction when photoablation of clathrin was performed (Heerssen et al., 2008). Interestingly, to explain their discrepant results compared to previously studies, Soykan et al. explored various parameters including the role of the transfection reagent and temperature.

They demonstrated that SV endocytosis is completely independent of clathrin at physiological temperature and that the discrepancy with previous results relies on the fact that most experiments were performed on living neurons at room temperature. For example, UFE, which is clathrin independent, is not observed at room temperature but only at physiological temperature (Watanabe et al., 2014) as well as synaptotagmin-pHluorin (Syt-pH) endocytosis was twice more efficient at physiological temperature than at room temperature (25°C).

Intriguingly, clathrin knock-down (KD) resulted in Syt-pH endocytosis delay only when hippocampal neurons were transfected with lipofectamine 2000. By contrast, neurons silenced for clathrin and transfected using calcium-phosphate behaved as the control. They proposed that lipofectamine reduced mitochondrial function (less ATP production) which impaired endocytic vesicle reacidification. Therefore, in the absence of clathrin, larger vesicles are formed, due to budding defect from endosomes, and reacidification is slowed down, suggesting that the previously reported dependence of SV retrieval on clathrin (Granseth et al., 2006a) may result from the use of lipofectamine.

In our experiments, as I compared Syp-pH endocytosis rate from PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ neurons at 37°C, all transfected with lipofectamine 2000, I can rule out any bias due to the temperature and transfection reagent. However, further experiments will be required to determine

whether PLSCR1 is effectively acting CME of SV. If after transfection of PLSCR1$^{+/+}$ neurons with shRNA directed against clathrin, the Syp-pH kinetic (at 37 °) does not change, we could definitely exclude that the endocytosis that we imaged is clathrin-dependent.
Regarding the ADBE and UFE, we do not have sufficient data to exclude or to prove the involvement of PLSCR1 in such mechanisms.

1.5 PLSCR1$^{-/-}$ neuronal network in vitro exhibits a different morphology: structural synaptic plasticity or impaired synapse maturation?

Since PLSCR1$^{+/+}$ and PLSCR1 $^{-/-}$ neurons are cultured in the presence of 30 mM KCl and thus, are continuously subjected to depolarization/ hyperpolarization cycles, we questioned if PLSCR1$^{-/-}$ cultured neurons show a structural adaptation resulting from persistent perturbation of neuronal activity in the absence of PLSCR1. Indeed, similarly to the homeostatic regulation of synaptic strength and/or efficacy, a structural plasticity can take place to modify and adapt the neuron excitability and synaptic network properties to functionally compensate for neurotransmission modification or defects (Yin & Yuan, 2015).

Interestingly, we found that the density of vGlut1 and PSD95 clusters as well as the density of synapses along axons in PLSCR1$^{-/-}$ GrC neurons is increased. In addition, the coupling distance between the pre- and postsynaptic components is reduced in PLSCR1$^{-/-}$ GrC neurons indicating that the lack of PLSCR1 induces synapses and neural network reorganization reminiscent of structural plasticity.

However, as compensatory endocytosis measured by Syp-pH imaging is altered in PLSCR1 $^{-/-}$ cultures, it means that structural synaptic adaptation *in vitro* is not correlated to a functional compensation.

A possible explanation could rely on the fact that, although the absence of PLSCR1 induces a synaptic structural adaptation *in vitro*, this latter is not enough to compensate for the lack of PLSCR1 activity in Syp-pH uptake during high-frequency stimulation (40 Hz, 10 sec). It would be interesting to evaluate and compare the kinetics of the Syp-pH retrieval followinglow frequency stimulation. One could imagine that structural synaptic plasticity can compensate synaptic activity up to a certain range of stimulation, after which, it would not be sufficient. We can also imagine that compensation occurs at the level of the whole network and the increase in the total number of synapses could instead compensate for reduced neurotransmission within the total synaptic network.

A second possibility is that the lack of PLSCR1 causes a defect in synapse maturation *in vitro* and thus, a difference in synaptic stability and/or dynamics. It has been shown that the proteolytic cleavage of cell adhesion molecules (CAMs) can regulate the structural and functional remodeling of glutamatergic synapses during synaptic development and plasticity (Nagappan-Chettiar et al., 2017). The two major classes of extracellular proteases linked to brain development are Matrix Metalloproteinases (MMPs) and A Disintegrin and Metalloproteinases (ADAMs) (Nagappan-Chettiar et al., 2017).

The post synaptic CAM adhesion molecule neuroligin-1 appears to be involved in the formation of synapses by binding to its presynaptic ligand, neurexin. In adult rat brains as well as in neuronal cultures, genetic studies in which the knock-out of diverse *ADAM proteins (i.e., Adam8*$^{-/-}$*, Adam17*$^{-/-}$*, Adam19*$^{-/-}$*, Adam9*$^{-/-}$*, Adam12*$^{-/-}$*, Adam15*$^{-/-}$*,* Adam 10 $^{-/-}$) have been evaluated, lead to the identification of ADAM10 as the major protease responsible for neuroligin-1 cleavage (K. Suzuki et al., 2012). Inhibition of neuroligin-1 shedding due to ADAM10 silencing, lead to an increase in number of spine density in neuronal cultures (K. Suzuki et al., 2012), which might facilitate the formation of synaptic connections. Interestingly, PS-binding domain has been identified in ADAM proteins, including ADAM10. Moreover, the transient exposure of PS at the surface of erythrocytes seems to be required for ADAM10 activation (Bleibaum et al., 2019).

Considering all these findings, one could therefore imagine that the transient exposure of PS mediated by PLSCR1 can influence / activate such extracellular metalloproteinase able to regulate synapse maturation. This might be the cause of the shortening of the pre and postsynaptic distance in PLSCR1-/- neurons.

To assess whether PLSCR1-mediated PS-egress might be required for ADAM10 sheddase activity and in turn, for synaptic network maturation, we could culture PLSCR1-/- neurons in presence of a soluble PS headgroup that cannot be incorporated in cell membrane and act as competitive inhibitor of PS substrate cleavage (Bleibaum et al., 2019). We should expect to reproduce the PLSCR1-/- phenotype by shortening the pre- and postsynaptic distance and increase synapses number in PLSCR1+/+ neurons. Alternatively, we could detect the cleavage product of ADAM10 which should be reduced or absent in PLSCR1-/- neuron cultures.

2. Does the PLSCR1 control compensatory endocytosis via its scrambling activity?

2.1 Is PLSCR1 function linked and/or limited to its scrambling activity?

Cell surface exposure of PS during regulated secretion has been observed in mast cells (Demo et al., 1999), PC12 cells (Vitale et al., 2001), chromaffin cells (Ory et al., 2013) as well as in neurons (Lee et al., 2000).

Our results show that PS egress is abolished in PLSCR1 $^{-/-}$ neurons following their stimulation. It indicates that, despite the doubt regarding the scrambling activity of PLSCR1 (see introduction), in our experimental conditions, the lack of PLSCR1 cannot be bypassed by other PLSCR isoforms or other Ca^{2+}-dependent scramblases, suggesting that the activity of PLSCR1 is specifically required for the PS reorganization during regulated exocytosis in neurons. As expected, the exogenously expressed PLSCR1 restores the outward movement of PS in PLSCR1 $^{-/-}$ cells and compensatory endocytosis, which is dramatically impaired in PLSCR1 $^{-/-}$. These results are in good agreement with a previous work of our laboratory which demonstrated that, upon stimulation of chromaffin cells, PLSCR1-induced PS egress was required for efficient granule membrane compensatory endocytosis (Ory et al., 2013). It further suggests that PLSCR1-dependent loss of membrane asymmetry might be a common feature of neurosecretory cells to trigger compensatory endocytosis. An important question remains: which one of the PLSCR1 expression or PS egress is critical for compensatory endocytosis?

Indeed, we only have a correlation between PS egress, PLSCR1 expression and compensatory endocytosis.

To discriminate between the need of lipid rearrangement at the PM and the requirement of PLSCR1 expression, we could transfect PLSCR1 $^{-/-}$ neurons with a calcium-insensitive PLSCR1 $_{D284A}$ mutant, unable to trigger PS distribution (Zhou et al., 1998). If the compensatory endocytosis is not restored, we could conclude that a non-functional PLSCR1 is not sufficient to restore the wild-type phenotype, meaning that PS egress is required for this mechanism.

Vice versa, we can trigger PS egress independently of the PLSCR1 activity and then, evaluate the effects on the endocytosis kinetic. The TMEM16F is a well-known scramblase activated during blood clotting and a point mutation was described to confer constitutive scrambling activity (TMEM16F Y516F) (Le et al., 2019). I expressed TMEM16F Y516F in neurons to force PS egress independently of PLSCR1 activation. Unfortunately, expressing TMEM16F mutant did not lead to constitutive PS egress in neurons preventing me to test my hypothesis. Another approach would

consist of adding PS in the extracellular medium to mimic PS egress toward extracellular leaflet. This experiment will require careful setup because PS excess on the external leaflet triggers endocytosis by itself in HeLa cells (Hirama et al., 2017).

Due to its ability to translocate into the nucleus of some cell types, PLSCR1 has been shown to act as a transcription factor (Zhou et al., 2005) and interacts with topoisomerase IIα to enhance its decatenation activity (Wyles et al., 2007). PLSCR1 could therefore control expression of proteins critical for compensatory endocytosis. I evaluated the expression of some key endocytic proteins, including clathrin and dynamin, and no difference in the expression levels was found in PLSCR1 $^{-/-}$ as compared to PLSCR1 + / + (Fig.39). At least on proteins tested, I can exclude a transcriptional role of PLSCR1.

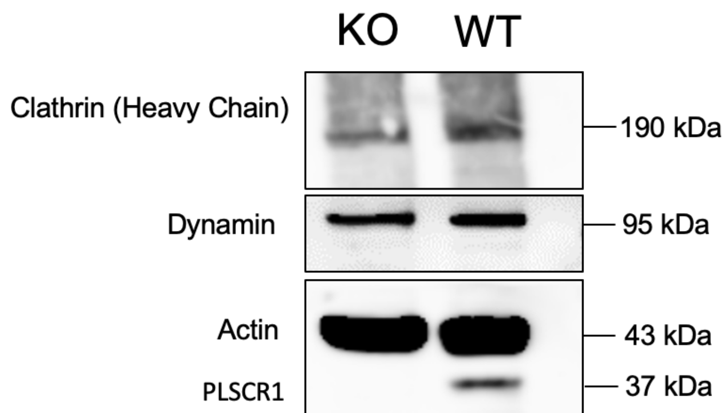

Fig.39 Western blot detection of endocytic proteins (Clathrin and dynamin I) in lysates from PLSCR1+/+ and -/- neuronal cultures. After normalization with actin, no differences in clathrin and dynamin levels were found. The experiment was repeated twice.

2.2 How can PLSCR1 regulate PS egress?

Although our findings clearly show that PLSCR1 controls PS movement in neurons, the exact nature and the underlying mechanism of the lipid remodeling remain to be investigated.

It has been hypothesized that PLSCR1 may not be a true phospholipid scramblase by itself because it consists of only a single transmembrane domain compared to other phospholipid

transporters. PLSCR1 might be indirectly implicated in a signaling pathway leading to a specific PS redistribution. For example, one could imagine that following conformational changes due to Ca^{2+}-activation (Sahu et al., 2009; Stout et al., 1998), PLSCR1 might interact and positively regulate other phospholipid scramblases, resulting in a PS egress. So far, to my knowledge, no interactions between PLSCR1 and other phospholipid transporters have been reported in the literature. Moreover, previous experiments of our laboratory, based on pull down assay associated to mass spectrometry analysis from mouse adrenal gland and bovine chromaffin cells did not detect any phospholipid scramblases as interacting partners for PLSCR1 (Estay-Ahumada et al., unpublished). Furthermore, all results obtained from the insertion of PLSCR1 in liposomes (Arashiki et al., 2016; Francis et al., 2013; Sahu et al., 2007; Sánchez-Magraner et al., 2014a; Stout et al., 1998), indicate that PLSCR1 is able, by itself, to exchange lipids in the presence of Ca^{2+}.

When combining in vitro data and cellular effects of various PLSCR1 mutants, it suggests that PLSCR1 may oligomerize to scramble lipids when bound to calcium. For example, endogenous PLSCR1 extracted in the native state from erythrocyte membranes, migrated at 170–280 kDa, that correspond to an oligomer of five to eight subunits (Arashiki et al., 2016). Oligomerization of PLSCR1 monomers may be induced by a conformational change in the polypeptide once PLSCR1 binds to Ca^{2+} (Sahu et al., 2009; Stout et al., 1998).

But “Which domains of PLSCR1 are necessary for oligomerization and, in turn, for scrambling activity?”

PLSCR1 can bind Ca^{2+} through its EF-hand-like motif [273- DADNFGIQFPLD-284] (Stout et al., 1998). Substitution of critical aspartic acid residues in the above domain resulted in 90% reduction in scrambling activity (Stout et al., 1998), suggesting that CBD is essential for PLSCR1 scrambling activity. In chromaffin cells, the exogenously expressed calcium insensitive $PLSCR1_{D284A}$ mutant behaves like a dominant negative mutant. In fact, when expressed, PS egress is blocked despite the presence of the endogenous PLSCR1 (Ory et al., 2013). One possible explanation is that the endogenous PLSCR1 can oligomerize with $PLSCR1_{D284A}$ monomers and, this latter could prevent the formation of a functional channel, preventing lipid translocation. Indeed, it has been shown that Ca^{2+}-binding may change the folding and/or the tilting of the TMD and this new conformation would allow the membrane lipid scrambling activity (Sánchez-Magraner et al., 2014b). Accordingly, we showed that when TMD was expressed in PLSCR1 $^{-/-}$ neurons, it was not able to mix lipid in stimulated conditions although it appears mainly located at the plasma membrane and in cytosol when expressed in Neuro2A cell line, (Fig. 40). However, adding CBD to PLSCR1-TMD completely restores the scrambling activity during stimulation,

suggesting that the TMD *per se* is not sufficient for the promotion of lipid translocation but CBD could be necessary for PLSCR1
oligomerization and, in turn, for its activity.

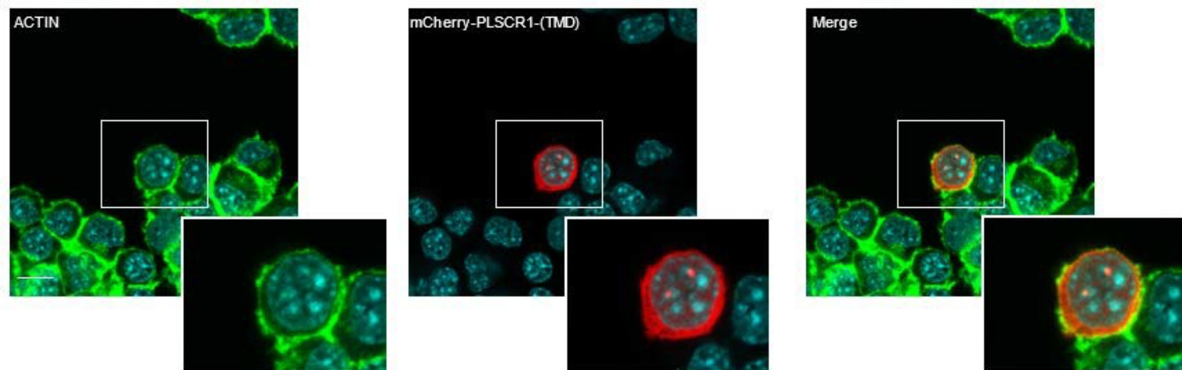

Fig.40 mCherry-PLSCR1-(TMD) localizes both at the PM and in cytoplasm in Neuro-2a cells. Representative confocal images depicting mCherry-PLSCR1-(TMD) in red, Actin in green and Nuclei in cyan. Surrounded areas correspond to the zooms in the bottom. Scale bar 10 µm

This is in agreement with *in vitro* experiments showing that the reconstitution of TMD of hPLSCR1 in liposome does not oligomerize in the membrane (Francis et al., 2013), indicating that it is not able to form oligomers by itself. Indeed, both CBD and TMD domains seem to be necessary for PLSCR1 lipid scrambling. The Scatchard plot analysis, commonly used to determine the affinity of the receptor for its ligand, revealed that TMD deletion in PLSCR1, resulted in a 50% decrease in affinity for Ca^{2+} compared with the PLSCR1 wild-type (Francis et al., 2013). It seems that, in the presence of TMD, the protein adopts a compact conformation which brings protein residues involved in Ca^{2+} coordination closer together (Francis et al., 2013). Altogether, these findings suggest that the mutual presence of CBD and TMD is necessary to trigger the oligomerization that, in turn, is required for lipid translocation.

Yet, Rayala and co-workers have shown that in the absence of PRD, Ca^{2+} cannot trigger ΔPRD-PLSCR1 oligomerization, and in turn, PLSCR1 activity is completely abrogated (Rayala et al., 2014), suggesting that also PRD might be essential for oligomerization of PLSCR1 monomers. In contrast to what it has been observed by Rayala and co-workers, our results show that PLSCR1-(CBD+TMD) mutant exhibit a normal scrambling activity following Ca^{2+} increase. Although in our experiments, we do not directly measure monomers oligomerization, according

to the literature we can speculate that only when PLSCR1-(CBD+TMD) monomers oligomerize, PS egress can occur. Conversely if monomers cannot form a channel, scrambling activity is abolished. Our data results from the direct expression of PLSCR1-(CBD+TMD) in neurons lacking PLSCR1. On the contrary, Rayala and co-workers have monitored Ca^{2+}-dependent formation of PLSCR1 aggregates in suspension by using dynamic light scattering approach (Rayala et al., 2014). Thus, since PLSCR1 is a transmembrane protein, an additional factor to consider when interpreting the above results is that PLSCR1 lacking PRD may be incorrectly folded in suspension compared to the protein inserted into the plasma membrane. One could imagine that the incorrect folding in suspension might prevent oligomerization, therefore it would be necessary to check the capacity of ΔPRD-PLSCR1 to form oligomers and, in turn, to mix lipids, when expressed with membranes.

In conclusion, our results show that CBD and TMD domains are directly implicated in lipid scrambling. Further investigations are needed to directly link their involvement in PLSCR1 monomers oligomerization.

3. PS and compensatory endocytosis

3.1 PS exit occurs at the synapse: Spatial regulation of PLSCR1?

PLSCR1 has been localized at the plasma membrane of most cell type studied, and I show here by combining confocal and electron microscopy study with Annexin-A5 coupled to a fluorophore or to biotin, respectively, that it is enriched at synapses in neurons. In addition, my results demonstrate that PS redistribution is tightly linked to PLSCR1 activity in neurons. Outward translocation of PS is abolished in the absence of PLSCR1 but restored when PLSCR1 is re-expressed in PLSCR1 $^{-/-}$ cells. Importantly, PS egress occurs at active synapses as demonstrated by its colocalization with Syt1 and PS egress was observed only where PLSCR1 was expressed on membrane sheets from stimulated PLSCR1 $^{-/-}$ neurons transfected with PLSCR1. Local egress of PS at synapses indicates that PLSCR1 is restricted and locally activated. Similar observation has been done in chromaffin cells. Despite homogenous distribution of PLSCR1 at the cell surface, PS egress occurred only at the vicinity of the exocytosis sites suggesting that PLSCR1 is locally activated. It was argued that due to its low Ca^{2+} sensitivity, PLSCR1 was activated only where intracellular Ca^{2+} increase reaches a critical concentration which is obtained close to the exocytic site (Ory et al., 2013). Local activation of PLSCR1 is easier to imagine in neurons. Indeed, in nerve terminal, intracellular Ca^{2+} persists for very short periods limited to action potentials duration. It is spatially constrained to the vicinity of opened voltage gated Ca^{2+} - channels (VGCCs) localized at synapses (Neher & Sakaba, 2008). Therefore, since PLSCR1 is enriched at synapses and have a weak affinity for Ca^{2+} (Stout et al., 1998), its Ca^{2+}- dependent activation can occur only at short distances from VGCCs, restricting the PS redistribution to synapses.

We can nonetheless not exclude that activation of PLSCR1 may also depend on other proteins of the presynaptic zone. Previous experiment in chromaffin cells showed that, preventing exocytosis by expressing clostridial or tetanus neurotoxins, which cleaves SNARE proteins, was sufficient to prevent PS egress in response to stimulation (C. Estay-Ahumada, unpublished data). It suggests that intracellular Ca^{2+} increase is not sufficient to activate PLSCR1 in chromaffin cells and that the fusion of secretory granule is required. An obvious possibility to explain fusion requirement would be that PS come from the fused vesicles. According to the topology of the vesicle, PS is enriched in the cytoplasmic leaflet of the vesicle meaning that upon fusion, the PS-

enriched leaflet of the vesicle remains on the cytoplasmic side, whereas the luminal leaflet is exposed to the extracellular space. It is therefore unlikely that PS comes from the vesicle unless an active transport occurs. Up to now, no scramblases have been reported to be present on secretory granules or synaptic vesicles, the best way to promote fast PS translocation. As it seems that PLSCR1 activation and exocytosis might be coupled, SNARE proteins might regulate PLSCR1 activity. It could constitute a checkpoint if we imagine that in resting condition, PLSCR1 would bind a tSNARE that might maintain PLSCR1 inactive, and upon Ca^{2+} increase, when tSNARE is engaged in the fusogenic complex, PLSCR1 would be released and scramble lipids to allow PS exit. Scrambling would therefore occur only when cells are stimulated and able to assemble a functional SNARE complex. Mass spectrometry experiments performed by our team could not validate PLSCR1 interaction with SNARE proteins. However, recombinant PLSCR1 production used in the assay has proven difficult and questions the functionality of the PLSCR1 produced. An alternative approach that uses an engineered biotin ligase genetically tagged to the PLSCR1 could be used. This method allows for proximity labeling by converting an inert small molecule substrate into a highly reactive and short-lived activated ester that diffuses out from the enzyme active site to tag covalently neighboring endogenous species (Branon et al., 2018). It is particularly suitable for insoluble and membranous proteins since it uses the cellular and native environment of the protein of interest. In addition, as the labeling distance is limited to 10-15 nanometers and the substrate molecule designed to contain biotin, tagged molecules representing potential binding and regulatory partners or even weak interactants can be enriched using streptavidin beads and identified by mass spectrometry analysis (Qin et al., 2021; Varnaitė & MacNeill, 2016).

3.2 How might PS egress regulate compensatory endocytosis?

PLSCR1-induced PS exit is a key step for the recycling of secretory granules in chromaffin cells and SVs in cerebellar granule neurons. However, how PS distribution can control compensatory endocytosis remains completely unknown. Here, it is important to recall that PLSCR1 is not specific for PS but is able to trigger rapid non-specific bidirectional trans-bilayer movement of various phospholipids like PE and PC across the plasma membrane. In this thesis project, I specifically measured PS egress, but we need to consider that the loss of PS asymmetry reveals a more complex lipid reorganization at the synaptic terminals.

Based on the literature, I propose diverse hypothesis regarding the mechanisms by which PLSCR1-dependent PLs redistribution can control compensatory endocytosis and, more widely the coupling between exocytosis and endocytosis in neurons during high frequency stimulation.

- *Membrane fluidity: release site clearance defects in the absence of PLSCR1-induced PS egress?*

Several works have demonstrated that lipid rafts could play an important function in regulated exocytosis in both neurons and neurosecretory cells. Lipid rafts have been proposed to be necessary for the clustering of the exocytotic machinery, including SNARE complex assembly (Lang et al., 2001; Salaün et al., 2004). Cholesterol depletion causes dispersion of these clusters, resulting in strong reduction in the rate of secretion (Linetti et al., 2010). Consistent with this, a previous work from our group demonstrated that during regulated-exocytosis in neuroendocrine cells, *de novo* formation of lipid microdomains enriched in GM1, PIP_2 and cholesterol, occurs at the plasma membrane in the exocytotic sites of chromaffin cells (Chasserot-Golaz et al., 2005). Moreover, it has been proposed that Annexin A2, a Ca^{2+} -dependent actin-binding protein, stabilizes PIP_2 microdomains at the plasma membrane during exocytosis (Chasserot-Golaz et al., 2005), which in turn recruit PIP_2-binding proteins acting in the subsequent stages of exocytosis (Grishanin et al., 2004). *In vitro* experiments performed on lipid monolayers have shown that strong electrostatic interaction between PIP_2 molecules or between PIP_2 and Ca^{2+} increase the rigidity of the membrane (Ellenbroek et al., 2011). Additionally, SVs contain high levels of cholesterol (40 mol%) (Takamori et al., 2006) which has been postulated to cluster synaptic vSNAREs (Chamberlain et al., 2001) and to decrease the energy barrier for fusion pore formation (Stratton et al., 2016). Addition of cholesterol at the PM of exocytic sites during SV fusion could increase membrane rigidity.

Such mixed cholesterol/phospholipid phase, called ordered liquid-phase, strongly reduces the dynamics and the fluidity of the PM, resulting in a limited lateral diffusion of SV proteins and lipids during the regulated-exocytosis (Yang et al., 2016).

However, following SVs full collapse, lateral diffusion of the components of the synaptic vesicles is required for the subsequent compensatory endocytosis (Gimber et al., 2015). Indeed, endocytosis processes and SV proteins retrieval occur at specific membrane domains of the presynaptic plasma membrane, called the peri-active zone. The lateral diffusion of SV proteins is also necessary for the so-called release site clearance (Byczkowicz et al., 2018). Indeed, when

SVs fusion occurs, it instantaneously alters AZ structure, thereby impairing the integrity of AZ constitution and function. Before a new vesicle can fuse at the site of a preceding fusion event, this site of fusion must be cleared from vesicular proteins (Byczkowicz et al., 2018).

One could imagine that PLSCR1 can act at a step before endocytosis, by facilitating the lateral movement of SNARE complexes away from the AZ release sites. As mentioned in the introduction, a striking asymmetry is observed for phospholipid acyl chains, with the cytoplasmic leaflet composed largely of highly unsaturated lipids. Obviously, differences in lipid packing are present between the two layers, wherein a tightly packed exoplasmic leaflet apposes a more loosely packed cytoplasmic leaflet.

One could speculate that PLSCR1-mediated PLs scrambling disrupts the packing of polyunsaturated lipids between the two layers in a non-specific manner, increasing the membrane fluidity and favoring the clearance of the release sites.

Furthermore, another important role in this process could be represented by the membrane cholesterol. A recent work has demonstrated that the proper trans-bilayer distribution of cholesterol relies on PS distribution (Maekawa & Fairn, 2015). Therefore, when activated by Ca^{2+} at the synaptic terminal, PLSCR1-dependent PS exit could cause the collapse of lipid raft. This PLs redistribution could also contribute to increase membrane fluidity by disrupting the ordered liquid-phase zone in the PM.

The increase of membrane fluidity facilitates lateral diffusion of proteins and lipids, clearing out the fusion sites (Fig.41). Thus, we can speculate that PLSCR1 could be required for coupling exocytosis to endocytosis by clearance of release sites.

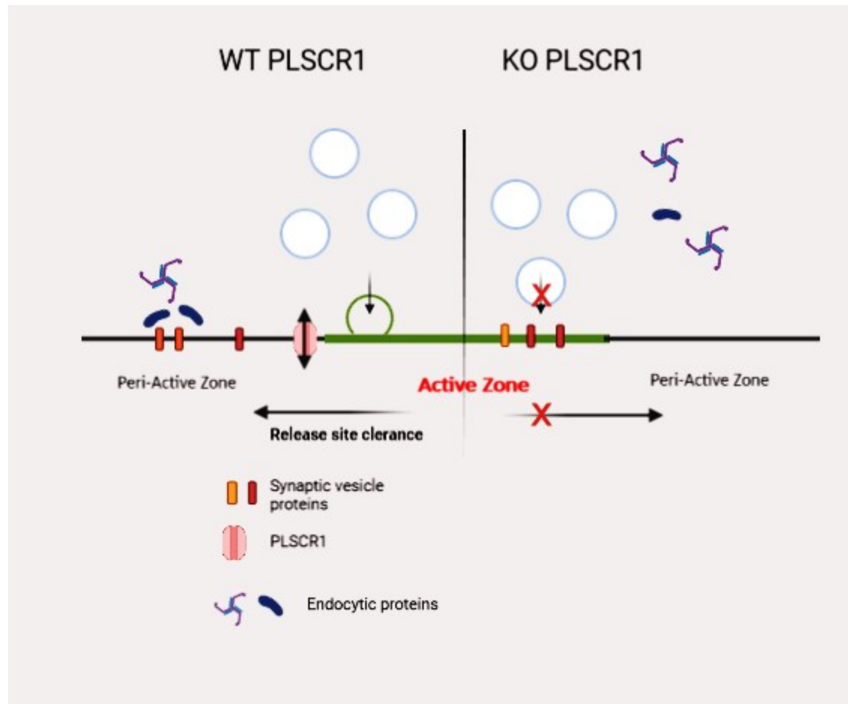

Fig.41 PLSCR1 increases clearance of release sites. PLs redistribution PLSCR1-mediated increases membrane fluidity, favoring lateral diffusion of the vesicular protein from the active zone. When the site of the preceding event fusion is cleared out, new vesicle can fuse.

Moreover, this could represent a crucial overlooked step that is required to sustain high-frequency neurotransmission. Indeed, although SV depletion is known as a main cause of synaptic depression, several studies have suggested that clearance of specific release sites in the presynaptic active zone provides spatial availability for SVs fusion and, this is one of the major rate-limiting steps for sustained synaptic transmission (Neher, 2010). In particular, during high frequency stimulation, the accumulation of SNARE components at the PM, could conceivably constitute a kinetic bottleneck, by blocking recruitment of further SVs to AZ release sites (Jäpel et al., 2020). Therefore, a rapid diffusion of vesicular proteins can be advantageous during sustained synaptic activity, clearing rapidly the way for subsequent fusion events (Jäpel et al., 2020). In good agreement with these findings, one could imagine that removing PLSCR1 would slow down clearance rate and lead to the faster depression that we observed in PLSCR1 $^{-/-}$ mice. Furthermore, the reduced clearance of release sites, due to a slower lateral diffusion of lipids and proteins may cause a delay/impairment in subsequent endocytosis processes. For example, it has been shown that hippocampal neurons knockdown for SCAMP5, a membrane protein found in secretory and endocytic compartments and associated to sporadic autism (Castermans et al., 2010), exhibit synaptic depression during high frequency stimulation and it is accompanied by a slower recovery of the SVs. Such synaptic defects in SCAMP5 KD are due to inefficient release

site clearance of newly exocytosed synaptotagmin from the active zone (Park et al., 2018). However, to further investigate the role of PLSCR1 in regulating synaptic clearance during intense stimulation, we should measure and compare the kinetics of lateral diffusion of Syp-pH at the synapse, between $PLSCR1^{+/+}$ and $PLSCR1^{-/-}$ neurons. For that, it is necessary to label newly exocytosed synaptic vesicles (with Syp-pH) and exogenously express a tagged-protein of AZ (such as Basson, Piccolo) in the living synapse that can be stimulated and visualized by high spatial resolution imaging (STED superresolution microscopy) (Hell, 2007).

- *PS exit during regulated-exocytosis can enhance compensatory endocytosis rate*

As described above, PLSCR1 has a weak affinity for Ca^{2+}. It therefore requires high intracellular Ca^{2+} concentrations during regulated exocytosis. However, thanks to the Ca^{2+} protein buffer, Ca^{2+} is quickly brought back to basal level, causing the rapid inactivation of PLSCR1. The question that arises is "How PLSCR1-mediated lipid remodeling during regulated exocytosis can affect the subsequent compensatory endocytosis?"

One could imagine that PLSCR1 lipid remodeling activity could affect the rate of endocytosis in two different ways, not mutually exclusive. First, as presented before, increased membrane fluidity due to PLSCR1 activity may accelerate the lateral diffusion of SV proteins, enhancing their sorting for subsequent endocytosis at the edges of AZ. Second, PLSCR1 activity could indirectly act as endocytic regulator, generating a PS (and also PE) gradient in the extracellular leaflet that could be necessary to enhance the endocytic process rate. At this point, it is worth to recall that Scramblases activity is much faster than that of the Flippases to transport lipids (10.000 lipids/sec, 1.000 lipids/sec respectively) (Coleman et al., 2012; Marx et al., 2000). Therefore, its activation, albeit temporarily short, determines a transient and massive externalization of the PS during regulated exocytosis. Following SVs fusion, the concomitant inactivation of PLSCR1, due to the lowering of Ca^{2+} levels, and the activation of Flippases, determines the re-entry of the accumulated PS to the internal membrane sheet. In an earlier work, in erythroleukemia cell line K562, it has been demonstrated that the lipid translocation rate is proportional to the concentration of PS in the outer layer. Indeed, lipid translocation was increased by adding PS in the extracellular medium at different concentrations, without any saturation effect (Farge, 1995). In light of this, we could imagine that the increased concentration of PS in the external leaflet, generated by PLSCR1-activity during exocytosis, could speed up the inward movement of PS, mediated by the pumping activity of Flippases. In a subsequent work, Farge and co-workers have shown that there is a linear correlation between the rates of lipids inward translocation and membrane endocytosis. This rate directly depends on aminophospholipid (PS and PE) concentration in the outer membrane sheet (Farge et al., 1999). In fact, they have shown that adding of either PS or PE in living cells, led to enhancement of endocytosis (Farge et al., 1999). Based on these findings, one could imagine that aminophospholipid gradient created by PLSCR1 activity during exocytosis, could be necessary to increase compensatory endocytosis rate during high frequency stimulations, improving exo-endocytosis coupling. However, to investigate whether PS flipping, influences endocytosis rate during high stimulation, we could perform Syp-pH imaging experiments of

PLSCR1 $^{-/-}$ neurons in the presence of increasing PS concentration in the extracellular medium, and then evaluate if Syp-pH uptake is linearly speed up.

On the contrary, following PS egress after the stimulation, we could prevent its flip towards the inner layer in order to check whether the PLSCR1-induced PS gradient is necessary to enhance compensatory endocytosis. To block PS in the external leaflet, we could perform Syp-pH experiments on PLSCR1$^{+/+}$ neurons, in the presence of Annexin V. Indeed, following stimulation, the Annexin A5 can bind the PS in the external leaflet with a high affinity and prevent its re-entry. In these experimental conditions, we could assess whether the kinetics of compensatory endocytosis are slowed down when the inward movement of PS is prevented. These results could provide the first evidence that PLSCR1 activity could be useful in ensuring a more efficient exocytosis/endocytosis coupling, which may be necessary during the high frequency stimulation of the synapses.

- *PLSCR1-induced PS gradient could accelerate compensatory endocytosis by facilitating membrane curvature and endocytic proteins recruitment*

The force leading to membrane internalization in endocytosis process is thought to be generated by the oligomerization of clathrin at the plasma membrane (Takei et al., 1998). On the contrary, several experiments indicate that there is no strict correlation between membrane budding and clathrin oligomerization (Mettlen et al., 2018). These findings, thus, raise the question of what may be the force inducing membrane curvature and vesicle formation. Relying on the physical elastic properties of the PM, it has been proposed that a difference in the surface area between the inner and outer layers of the membrane could potentially drive vesicle formation by inducing membrane curvature (Miao et al., 1991). This surface asymmetry between the two layers of PM, was established in liposome, after the addition of exogenous PS to the outer layer and its specific translocation to the inner layer by the endogenous Flippase (Lopez-Marques et al., 2014). Based on these findings, we could speculate that the Flippase-mediated translocation of aminophospholipids, previously accumulated in the outer leaflet by PLSCR1 remodeling activity, could generate a difference in the surface area between the two leaflets, which rapidly induces a negative curvature of the bilayer, enhancing the endocytic process. Indeed, the electrostatic repulsion of negatively charged lipids, such as PS, increases the surface charge density of the cytosolic leaflet of the PM, leading to enhanced headgroup repulsion and favoring formation of negative membrane curvature. This, in turn, may support and facilitate the initiation of endocytosis (Hirama et al., 2017). Additionally, both local distribution of charge at the cytosolic leaflet and membrane curvature might contribute to a more efficient recruitment of the endocytic machinery in the early step of endocytosis. Of note, BAR domains, such as endophilin and amphiphysin, associate preferentially with highly curved membranes and can interact directly with anionic phospholipids, including PS (Farsad et al., 2001). Indeed, they have a similar NH_2-region with an amphipathic pattern, with a hydrophobic patch and an opposite hydrophilic face, consisting of several basic residues. This feature allows this domain to interact with phospholipid headgroups via the hydrophilic face, and then to partially embed into the bilayer via the hydrophobic patch in a manner which favors membrane deformation (Farsad et al., 2001). Moreover, also the activity of synaptojanin, that regulates membrane fission, is stimulated on highly curved membrane, in part thanks to the interaction with endophilin (Chang-Ileto et al., 2011).

However, to further investigate on the recruitment of proteins essential for SVs endocytosis,

we could use tagged-endocytic proteins (i.e. endophilin, synaptojanin, amphiphilin) and thus, assess and compare their recruitment by using TIRF microscopy in PLSCR1$^{+/+}$ or $^{-/-}$ living cells. Other candidates like FCHo and Eps15 which sense membrane curvature and bind negative lipids, could be also considered.

E. General conclusion

The transfer of information in neuronal networks relies on the ability of presynaptic boutons to convert the firing rate of action potentials into corresponding amounts of neurotransmitter release. Beyond this straightforward relationship, neurotransmission is also dependent on the availability of postsynaptic receptors, their dynamics and the surrounding cells like astrocytes. My thesis work address only part of the mechanism that could be altered by the lack of PLSCR1 and we cannot exclude that PLSCR1 may have postsynaptic function or regulate other cell types such as astrocytes, which play an important role in the building of the glutamatergic synaptic network.

In the cerebellum, the afferent sensory mossy fibers convey interoceptive, proprioceptive, and exteroceptive information on cerebellar granule cells, the only excitatory neurons of the cerebellum located in a layer just below Purkinje cells in the cerebellar cortex (Wagner et al., 2017). From mossy fibers to Purkinje cells, this information is encoded at very high-frequency bursts (up to hundreds of Hz) (Delvendahl and Hallermann, 2016) and to sustain such firing rate, high-frequency synapses of sensory/motor networks have been developed. Although the mechanisms are to be solved, compared to low-frequency synapses like hippocampal pyramidal neurons (Delvendahl and Hallermann, 2016), specific strategies to promote sustained release of neurotransmitter must occur in high frequency synapses.

Doussau et co-workers demonstrated that granule cell-Purkinje cell (GC-PC) synapses are able to sustain high frequency stimulations through the recruitment of the so-called reluctant pool in the millisecond range upon intense synaptic activity (Doussau et al., 2017). My thesis work suggests that PLSCR1 might be a key protein required for tuning fast neurotransmission since the lack of PLSCR1 leads to faster depression of EPSPs, when GrC neurons are stimulated at high frequency rate. The function of PLSCR1 is particularly attractive since it controls probably one of the fastest and versatile biological processes, which is lipid topology and dynamics. Interestingly, we detected PLSCR1 in the cerebellum and the olfactory bulb, two brain regions known to sustain high frequency stimulation (Delvendahl and Hallermann, 2016). Searches in databases like the Allen brain Atlas revealed that mRNA coding for PLSCR1 is also detected in pons and medulla, two precerebellar nuclei known to fire at high frequency. PLSCR1 could therefore constitute a “marker” of high-frequency synapses.

Finally, one of the obvious questions that remained concerned the consequence of Plscr1 gene depletion on mice behaviour. Cerebellum being the centre of locomotors integration, we have only carried out, so far, some general behavioral test including the Open Field and the Rotarod test that did not reveal any motor defects in PLSCR1$^{-/-}$ mice (data not shown). According to the defect observed in vitro or in electrophysiological recordings, it is surprising at first glance that mice present any obvious phenotype. However, electron microscopy analysis of the cerebellum did not show any obvious morphological differences in the synaptic structure of PLSCR1 $^{-/-}$ mice suggesting that the phenotype might be more subtle than initially predicted from *in vitro* experiments. Interestingly, besides its role in the coordination of movement, it is now becoming apparent that the cerebellum is critical for many other functions including the regulation of cognition and the coordination of emotions (Stoodley and Schmahmann, 2010). For example, it has been shown that, by connecting to the thalamus, the cerebellum innervates not only motor areas of the cortex, but also prefrontal and parietal cortex brain areas responsible for several functions including the working memory that allow to hold information temporarily and the sensory perception and integration respectively (Yeganeh-Doost et al., 2011). Neuroimaging studies have suggested the presence of abnormalities in the prefrontal-thalamic-cerebellar circuit in different mental disorders like in schizophrenia (Yeganeh-Doost et al., 2011). As electrophysiological recordings showed an impaired neurotransmission in PLSCR1 $^{-/-}$ mice, one could imagine that the absence of such protein could influence the signals outgoing from the cerebellum, leading to a defective synaptic transmission, observed in various pathologies including schizophrenia. More accurate tests like the prepulse inhibition of the startle reflex (PPI), which represent the most used behavioral test for schizophrenia and other menthal disorders, could provide further information on the phenotype of PLSCR1 $^{-/-}$ mice. Further studies will be required to elucidate the precise role of PLSCR1 and phospholipid scrambling in neuron functions and more widely in brain neurotransmission.

F. Supplementary materials and methods

1. Primary neurons culture from mouse olfactory bulb

Olfactory bulbs (OB) were dissected from new-born mice having C57BL/6 genetic background. Wild-type (WT) or Knock-out (KO) mice for the Phospholipid Scramblase-1 gene (PLSCR1) were scarified independently. After decapitation, the brain was removed from the skull and placed in a Petri dish containing ice cold Hibernate-A medium (BrainBits). Using a binocular microscope OBs were isolated and cleaned from their meninges in sterile conditions. The procedure was repeated mouse by mouse, then all the OBs were pooled in one recipient on ice and covered with Hibernate-A medium. This medium favors neuron survival during the dissection and allows manipulation of cells at ambient O_2 without CO_2. After recovery of all OBs, the tissue was chopped into tiny pieces (~ 1 mm^3) and transferred into a plastic tube (Falcon 15ml) containing 1 ml of Hibernate-A. Tissue was then digested by papain (Worthington, 40 U / ml in Hibernate-A) at 30°C for 25 minutes. At the end DNase (1 mg/ml, Sigma) was added to the mixture for 5 minutes to remove the DNA "filaments" released by the dead cells. Tissue fragments were sedimented for some minutes by natural gravity. The supernatant was discarded, and the pellet was then resuspended in 1.5 ml of Neurobasal-A culture medium supplemented with 2% B27 supplement, a mixture of penicillin / streptomycin (100 U/ml each, Sigma - Aldrich) and glutamine (0.5 mM) (Gordon et al. Al., 2016). Cells were mechanically dissociated by trituration, using a long glass Pasteur pipette that was previously flamed at the tip to obtain blunt and smooth end in order not to damage the cells while they are aspirated in and out of the pipette. After 10 to 20 up and down aspiration cycles a homogenous cell suspension was obtained. Cells were then harvested by centrifugation at 150 g for 4 minutes, counted using Neubauer cell counting chamber and viability was evaluated by Trypan blue staining. Later, one million cells were electroporated (2µg total DNA in 100µl electroporation buffer, Neon System, Invitrogene) and seeded in 8-well glass-bottom plates (IBIDI, ref. 80826 - 20µl of cell suspension / well in 100 µl of culture medium) previously coated with Poly-L-Lysine. Cultures were placed in cell incubator at 37 °C and 95% humidity with 5% CO_2. The culture medium was replaced with fresh one half an hour after cell plating in order to eliminate cellular derbies and not attached, contaminating cells. Cultures were maintained for 8 to 14 days.

2. Olfactory bulb neuron transfection

Primary neuronal cells were transfected by electroporation using the Neon System (Invitrogen). Electroporation was always realized on the day of culture when the cells were in suspension. After dissociation and resuspension of the cells, 10^6 cells were taken up in 100 µl of electroporation buffer and 2 µg of total DNA (Syp-pH plasmid), was added. According to manufacturer's instructions the 100 µl of suspension was aspirated by a capillary (supplied with the system) containing the electrode and placed in the connector recipient where an electric pulse of 1400 V for 30 ms was applied (MCC program, 30 min at 1400 V). Then, 20 µl of the electroporated mixture was seeded in 200 µl of culture medium, previously distributed in 8-well IBIDI plates and heated at 37 °C. These cultures were used for videomicroscopy. For immuno-labeling, 40 µl of electroporated cell suspension was added to 24-well plates containing 500 µl of medium and glass cover slips at the bottom. Cultures were maintained at 37 °C for 7 days to allow growth of neuronal processes and development of fully functional synapses before experiments.

3. K+ stimulation, videomicroscopy and statistical analysis of olfactory bulb neurons

Cells were stimulated by the addition of 150 µl of a 100 mM KCl solution (50 mM final) directly into the well. In such condition, the stimulation was constant and irreversible. Video acquisition was carried out using Metamorph software (Molecular Devices) and a sCMOS camera (Orca-FLASH4, Hamamatsu) at a frequency of 5 Hz during 3 min. The recording was started few seconds before KCl stimulation in order to have a baseline before the fluorescence variation. Image sequences were analyzed with the freely available software Icy (http://icy.bioimageanalysis.org/) as followings. A circular region of interest (ROI, 10 µm x 10 µm) was drawn around each spot, which showed a sudden fluorescence change revealing exocytosis or endocytosis. ROI was centered on the maximum fluorescence of the spot and sized (~1.5 µm diameter) as to make it possible to follow the spot over time even if the spot was laterally moving. Photobleaching was corrected by adjusting the fluorescence decay to a monoexponential function using the first 100 plans that preceded exocytosis (Debleach plugin;http://icy.bioimageanalysis.org/plugin/Debleach). An ROI of the same diameter was positioned outside of any cell to estimate the background in the image. Background was subtracted from fluorescence intensities at each time points, and the mean fluorescence of the 40th plans

preceding exocytosis event was considered as the initial value (F0). Fluorescence intensity variations were calculated as (Ft-F0) / F0 where Ft was the mean fluorescence of the circular ROI at time "t".
To compare the kinetic of exocytosis and endocytosis between both PLSCR1$^{+/+}$ and PLSCR1 $^{-/-}$ cells, fluorescence increase, and decrease were fitted to logarithmic and mono exponential function respectively and the time constants of each event were determined. The distribution of median values between the two groups was evaluated with the non-parametric Mann-Whitney test.

4. Transmission electron microscopy in PLSCR1$^{+/+}$ and PLSCR1$^{-/-}$ slices of cerebellum

3 PLSCR1$^{+/+}$ and 3 PLSCR1$^{-/-}$ 2 months mice were anesthetized (mixture of tiletamine 40mg/kg, zolazepam 40mg/kg (Zoletil ND) and xylazine 16mg/kg (Rompun ND) diluted in NaCl 0.9% and trans-cardially perfused with 0.1 M of phosphate buffer, pH 7.3, containing 2% of paraformaldehyde and 2.5% of glutaraldehyde. Cerebella were collected and then fixed in the same fixative overnight. Vermis sagittal 200µm vibratome sections were cut in phosphate buffer. For synapse sampling, three sections were chosen in left, middle and right vermis respectively of each cerebellum. Later, sections were postfixed in osmium tetroxide before dehydration in increasing concentrations of ethanol followed by 2 baths of propylene oxide and flat embedding in Epon.
70nm ultrathin sections in lobule 9 were cut by ultramicrotome (Leica EM FC7), counterstained with lead citrate and examined with a Hitachi 7500 transmission electron microscope.
Pictures of 30 synapses/section were randomly taken. Vesicle distance to the synaptic cleft was measured with an Icy home-made protocol.

5. Dextran internalization protocol

Dextran internalization assay was carried out by Daniela Ivanova to quantify the number of synapses as previously described (Clayton & Cousin, 2009b). To label active synapses, neurons were preincubated with anti-synaptotagmin 1 antibody (sysy, 105 103C5) dissolved in culture medium for 30 min. Then, cells labeled with Syt1 Ab, were placed in imaging buffer for 15-20 min in order to be repolarized. Tetramethyrhodamine (TMR)-dextran (50uM) was dissolved in

the imaging buffer and applied just before stimulation (400 AP at 40Hz). Dextran was washed away after stimulation and cultures were imaged immediately after these experimental protocols had finished. The following imaging of neurons was performed on an inverted Zeiss Axio Observer Z1 microscope using 40× oil immersion objective (numerical aperture, 1.3) and Colibri 7 light-emitting diode light source (Zeiss). The microscope was equipped with a Zeiss AxioCam 506 camera and controlled by ZEISS ZEN 2 software. Both channels, the one with dextran and the one with synaptotagmin 1 AB, were imaged using DsRed (exciter, 538 to 562 nm; beam splitter, 570 nm; emitter, 570 to 640 nm) and Cy5 (exciter, 625 to 655 nm; beam splitter, 660 nm; emitter, 665 to 715 nm) filter sets respectively. For analysis, 4-5 fields of view were captured for each coverslip of neurons and 16 coverslips from 4 neuronal culture were investigated for each experimental condition. To account for differences in synapse density, the number of TMR-dextran puncta (number of synapses performing bulk endocytosis) was normalized to the number of synapses labeled with Syt1 Ab (active synapses) in the same fields of view. For quantification, the number of TMR-dextran puncta was averaged per coverslip.

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Résumé de thèse

Suite à une exocytose régulée, l'homéostasie membranaire et la régénération des stocks de vésicules sont assurées par une endocytose dite compensatrice. Ce couplage exo-endocytose est le garant du bon fonctionnement de la neurotransmission. Les propriétés biophysiques ainsi que les échanges entre les compartiments membranaires durant ces processus font des lipides des régulateurs majeurs. La membrane plasmique est une bicouche lipidique asymétrique et il a été montré que la protéine Scramblase1 (PLSCR1), impliquée dans le transfert de phospholipides d'un feuillet à l'autre, est indispensable à l'endocytose compensatrice des cellules neuroendocrines. Cependant les mécanismes moléculaires sous-jacents restent à identifier et la conservation de la fonction de la PLSCR1 dans les neurones reste à démontrer. Ma thèse s'est articulée autour de trois objectifs majeurs:

1- Déterminer les régions du cerveau exprimant la PLSCR1 ainsi que la localisation de la PLSCR1 dans les neurones
2- Déterminer si l'asymétrie des membranes était modifiée durant la neurotransmission et si la PLSCR1 intervenait dans ce processus.
3- Étudier la fonction de la PLSCR1 dans le cycle exocytose/endocytose des vésicules synaptiques.
4- Évaluer les conséquences de la perte d'expression de la PLSCR1 dans la neurotransmission et l'organisation des synapses

Les données disponibles dans les banques d'expression donnaient peu ou pas d'expression de la PLSCR1 dans certaines zones du cerveau. Après dissection et analyse par western blot, j'ai pu détecter l'expression de la PLSCR1 dans le cervelet et le bulbe olfactif. J'ai choisi d'utiliser les neurones du cervelet qui permettent d'obtenir des cultures homogènes contenant 90% de cellules en grain. Dans un deuxième temps, j'ai étudié la localisation subcellulaire de la PLSCR1 dans les neurones en utilisant différentes approches (microscopie électronique, microscopie confocale). Malheureusement, comme tous les anticorps commerciaux se sont révélés non spécifiques pour les techniques d'immunomarquages cellulaires, j'ai analysé la distribution de la protéine PLSCR-1 surexprimées, fusionnée à une protéine fluorescente, la GFP. J'ai effectué des co-marquages de la GFP-PLSCR1 avec la Synaptotagmine1a et le transporteur du Glutamate vGlut1 à la fois en microscopie confocale et électronique. J'ai pu ainsi montré que la GFP-PLSCR1 était localisée à

la membrane plasmique et enrichie au niveau de la synapse. J'ai confirmé ces résultats grâce à une approche biochimique en préparant des fractions synaptosomales de cervelet de souris. J'ai pu montrer que la PLSCR1 endogène était enrichie dans les synaptosomes.

Ensuite en utilisant la culture primaire de grains de cervelet comme système modèle, j'ai comparé la cinétique d'exo/endocytose en utilisant comme rapporteur la Synaptophysin-pHluorin. En faisant face au lumen des vésicules, la pHluorine est exposée à un pH acide qui éteint sa fluorescence. A la fusion des vésicules synaptiques avec la membrane plasmique, la pHluorin est exposée au milieu neutre extracellulaire entrainant une augmentation instantanée de la fluorescence qui décroit ensuite en fonction de la vitesse d'endocytose. Associé à un protocole de stimulation électrique, cette technique informe sur la cinétique d'exocytose et d'endocytose. Après avoir passé un mois dans le laboratoire du Dr Cousin à l'Université de Edimbourg, j'ai pu transposer cette technologie dans notre laboratoire. J'ai montré que l'absence de la PLSCR1 ralentissait l'endocytose dans les cellules PLSCR1-/-. De plus, en transfectant mCherry-PLSCR1 WT dans les neurones PLSCR1-/-, j'ai observé que l'endocytose était restauré à un niveau similaire aux cellules WT. Ces résultats suggèrent que PLSCR1 est nécessaire pour l'endocytose compensatrice. Ensuite, par des marquages à l'AnnexinV fluorescente, une protéine à forte affinité pour le phosphatidylserine, j'ai aussi découvert que dans les neurones, l'absence de PLSCR1 est corrélée à une absence de perte d'asymétrie de la membrane plasmique après stimulation.

De plus, en utilisant des mutants de délétion de la PLSCR-1, j'ai pu identifier le domaine minimal fonctionnel, constitué du domaine transmembranaire de la protéine associée au domaine de liaison du calcium, qui nous donne une première vision sur le mécanisme moléculaire sous-jacent. Ce mutant tronqué est ainsi capable de restaurer à la fois l'endocytose compensatrice et la sortie de PS, suggérant que le remodelage des phospholipides dépendant de l'activité de PLSCR1, jouent un rôle dans le processus d'endocytose compensatrice des neurones.

Enfin, en collaboration avec le Dr Doussau (INCI, Strasbourg), nous avons réalisé des expériences d'électrophysiologie sur des coupes de cervelet pour analyser la transmission synaptique entre les cellules en grain du cervelet et les cellules de Purkinje. En accord avec un défaut d'endocytose, nous avons pu voir que l'altération du recyclage des vésicules synaptiques en absence de PLSCR1 entraine une diminution de la réponse synaptique au cours d'une stimulation à haute fréquence.

Dans l’ensemble, les résultats de mes travaux de thèse ont permis de montrer que la PLSCR1 participe au couplage de l’exocytose à l’endocytose dans les neurones. Ce mécanisme est primordial pour la transmission synaptique et son maintien lors de stimulations prolongées. Mes travaux mettent aussi en lumière le rôle potentiel des lipides structuraux de la membrane plasmique dans la fonction neuronale. La localisation subcellulaire de la PLSCR1 suggère qu’une réorganisation des lipides à la synapse serait nécessaire pour assurer un recyclage optimal des vésicules synaptiques durant une activité synaptique intense. Comme l’efficacité de la neurotransmission nécessite des transferts de protéines vésiculaires de la zone active (exocytose) vers la zone péri-active de la synapse (endocytose) après exocytose, la perte d’asymétrie membranaire pourrait modifier la propriété des membranes en facilitant la clearance des sites de relargages et la diffusion latérale des protéines et lipides pour évoquer une endocytose optimale. Dans l’ensemble, mes données ouvrent de nouvelles perspectives quant à la fonction des protéines régulant le transport de lipides d’un feuillet à l’autre de la membrane plasmique dans la fonction neuronale.

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Mécanismes moléculaires du recyclage des vésicules synaptiques : rôle de la Phospholipid Scramblase-1 (PLSCR1)

Résumé

Les défauts de transmission synaptique sont observés dans diverses pathologies telles que l'épilepsie, l'autisme ou la déficience intellectuelle et, en raison de son importance critique dans le maintien de la fidélité de la neurotransmission, la compréhension des mécanismes de régulation qui couplent l'exocytose à l'endocytose des vésicules synaptiques est essentielle pour comprendre ces pathologies. La libération des neurotransmetteurs se produit par une exocytose dépendante du Ca^{2+}, qui résulte de la fusion entre les vésicules synaptiques et la membrane plasmique. Pour maintenir la fonction synaptique et l'homéostasie des organelles, l'exocytose doit être suivie d'une endocytose compensatrice, un processus nécessaire pour préserver l'intégrité de la membrane plasmique et restaurer les stocks de vésicules synaptiques. Bien que les processus de fusion et de scission vésiculaire impliquent des réarrangements de lipides membranaires entre la membrane plasmique et les vésicules, peu d'attention a été accordée au rôle de la dynamique des lipides lors de la transmission synaptique. Mon travail de thèse montre que la PhosphoLipid SCRamblase 1 (PLSCR1), une protéine qui mélange les lipides à la surface cellulaire lorsqu'elle est activée, contrôle le remodelage des lipides et le recyclage des vésicules synaptiques (SV) à la suite de la libération de neurotransmetteurs dans les cellules granulaires du cervelet (GrC). Les synapses chez les souris $PLSCR1^{+/+}$ (WT) se distinguent par une facilitation soutenue de la transmission synaptique lors d'une stimulation à haute fréquence. A l'inverse, la facilitation synaptique est réduite et la transmission synaptique diminue rapidement dans les neurones n'exprimant plus la PLSCR1 indiquant que les synapses GrC ont perdu la capacité de remplir rapidement les stocks de vésicules synaptiques. Mes résultats suggèrent que la PLSCR1 pourrait faciliter le couplage endo- exocytose pour soutenir une activité neuronale intense.

Mots-clés : endocytose compensatrice, remodelage de phospholipides, PLSCR1, couplage de l'exo-endocytose, stimulation à haute fréquence

Abstract

Defects in synaptic transmission are observed in various pathologies such as epilepsy, autism, or intellectual disability. Because of the critical importance in maintaining the fidelity of neurotransmission, insights to the regulatory mechanisms that couple exocytosis to endocytosis of synaptic vesicles are key to understand these pathologies at cellular level. Neurotransmitter release occurs through Ca^{2+} - dependent exocytosis, which rely on the fusion between synaptic vesicles and the plasma membrane. To maintain synaptic function and organelle homeostasis, exocytosis must be followed by compensatory endocytosis, a process required to preserve plasma membrane integrity and restore synaptic vesicle pools. Although the processes of vesicular fusion and scission involve rearrangements of membrane lipids between the plasma membrane and vesicles, little attention has been paid to the role of lipid dynamics during synaptic transmission. My thesis work shows that the PhosphoLipid SCRamblase 1 (PLSCR1), a protein that mixes lipids on the cell surface when activated, controls lipid remodeling and synaptic vesicles recycling following neurotransmitter release in cerebellar granule cells (GrC). Synapses in $PLSCR1^{+/+}$ (WT) mice are characterized by a sustained facilitation of synaptic transmission during high frequency stimulation. On the contrary, synaptic facilitation was reduced and synaptic transmission rapidly depresses in $PLSCR1^{-/-}$ neurons indicating that GrC synapses have lost the capacity to rapidly refill synaptic vesicle pools. These results suggest that PLSCR1 could facilitate the endo-exocytosis coupling to sustain intense neuronal activity.

Key words: compensatory endocytosis, phospholipid scrambling, PLSCR1, exo-endocytosis coupling, high frequency stimulation