UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

THÈSE

présentée par :

Catherine Estay Ahumada

soutenue le : **2 Décembre 2016**

pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline : Science du vivant

Spécialité : Aspects moléculaires et cellulaires de la biologie

Mécanismes moléculaires du couplage exocytose-endocytose dans les cellules neuroendocrines : rôle des protéines Scramblase-1 et Oligophrénine-1

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Avant-Propos

Après un Master de Neuroscience à l'Université de Valparaiso au Chili, j'ai obtenu une bourse du ministère Chilien de l'éducation dans le cadre de l'appel d'offre « advanced human capital program scholarships » (Becas Chile-CONICYT), pour effectuer un doctorat à l'Université de Strasbourg dans l'équipe du Dr. Stéphane Gasman situé à l'Institut des Neurosciences Cellulaires et Intégratives (INCI, CNRS UPR3212). L'équipe de Stéphane Gasman s'attache depuis de plusieurs années à comprendre les mécanismes cellulaires et moléculaires qui contrôlent la sécrétion neuroendocrine.

Le système neuroendocrinien se compose des organes, tissus et cellules spécialisés qui libèrent des hormones et des neuropeptides dans la circulation sanguine par un processus d'exocytose vésiculaire régulée par le calcium. Ce processus est finement régulé par les protéines SNARE (**S**oluble **N**SF **A**ttachment protein **RE**ceptor), qui permettent la fusion de la membrane des vésicules avec la membrane plasmique, étape ultime de l'exocytose, aboutissant à la libération du contenu vésiculaire. Les mécanismes qui régulent l'exocytose et la fusion membranaire sont étudiés de façon intensive. En revanche, les mécanismes permettant de préserver l'intégrité physique des membranes plasmique et vésiculaire après fusion membranaire, et par conséquent de maintenir l'équilibre fonctionnel de la cellule, ne sont pas connus et restent peu explorés aujourd'hui. Les travaux de l'équipe réalisés dans les cellules chromaffines de la glande surrénale suggèrent que la libération du contenu intra-granulaire est couplée de façon spatiale et temporelle à un processus d'endocytose compensatrice qui permet la recapture de la membrane du granule. Ainsi, nous émettons l'hypothèse selon laquelle la membrane granulaire préserverait son intégrité au sein de la membrane plasmique après l'exocytose avant d'être spécifiquement recapturée avec l'ensemble de ses composés. Cependant, les mécanismes à la base de cette activité d'endocytose compensatrice ne sont pas connus dans les cellules neuroendocrines. Dans ce contexte, le but général de ma thèse fut d'apporter de nouveaux éléments permettant de comprendre comment l'endocytose compensatrice est-elle déclenchée et régulée dans les cellules neuroendocrines et par quels mécanismes est-elle couplée à l'exocytose.

A mon arrivée en doctorat, le Dr Stéphane Ory (qui fût mon encadrant pendant ces trois années et demi) venait de montrer qu'au cours de l'exocytose, la proteine PLSCR1 (Phospholipid Scramblase-1) est capable de redistribuer les phospholipides d'un feuillet à l'autre de la membrane plasmique, perturbant ainsi de façon transitoire l'asymétrie membranaire au niveau des sites d'exocytose. De façon intéressante, Stéphane Ory montre élégamment que cette perturbation membranaire n'empêche pas la sécrétion mais bloque significativement l'endocytose compensatrice des granules de sécrétion. La PLSCR1 m'est alors apparue comme un candidat idéal pour contrôler le couplage entre l'exocytose et l'endocytose. Ainsi, l'un des buts premiers de mon doctorat fut d'essayer de comprendre comment l'activité de la PLSCR1 est régulée et pourquoi un mélange de phospholipides est préalable à la recapture des granules de sécrétion.

En parallèle, je me suis intéressée aux mécanismes de régulation de la sécrétion par une protéine appelée oligophrénine-1 (OPHN1). Cette protéine est particulièrement intéressante. Impliquée dans l'endocytose des vésicules synaptiques, elle possède un domaine « BAR » (Bin, Amphiphysin, Rvs) qui est un senseur de courbure membranaire ainsi qu'un domaine GAP permettant l'inactivation des protéines Rho, une famille de GTPases largement impliquée dans les processus d'exo- et d'endocytose. Au cours de ma première année de thèse, Sébastien Houy un doctorant de l'équipe montrait, en utilisant des souris invalidées pour le gène *Ophn1* que l'oligophrénine participe à la fois à la formation du pore de fusion et à l'endocytose compensatrice de la membrane granulaire. J'ai activement participé à ce projet en essayant notemment de comprendre comment OPHN1 pouvait coordonner son rôle sur l'exocytose avec un rôle dans l'endocytose.

Ce manuscrit fait la synthèse de l'ensemble de mes travaux et s'articule en quatre grandes parties. La première partie introduit de façon générale les connaissances actuelles concernant l'exocytose régulée et l'endocytose compensatrice dans les cellules neuroendocrines. J'y présente le modèle de la cellule chromaffine que nous utilisons au laboratoire et j'y détaille le cycle complet de la vie d'un granule de sécrétion, depuis sa biogénèse jusqu'aux mécanismes permettant son recyclage au cours du processus d'endocytose compensatrice. J'insiste également sur l'implication des protéines et des lipides qui ont été au cœur de mes problématiques de thèse.

La seconde partie est dédiée à mes données sur la régulation de l'activité et le rôle de la PLSCR1 au cours des processus d'exocytose et d'endocytose dans les cellules neuroendocrines tandis que la troisième partie du manuscrit se focalise sur l'implication de la protéine Oligophrénine1. Ces deux parties sont organisées de la même façon. Après un bref rappel du contexte scientifique et des problématiques posées, les données sont exposées sous forme d'article (une ébauche d'article en préparation pour la partie PLSCR1 et un article publié dans Journal of Neuroscience pour la partie sur l'oligophrénine). Je tente ensuite de prendre un peu de recul et de discuter mes données de façon plus globale afin d'élaborer quelques concepts mécanistiques.

Enfin une dernière partie présente les détails des matériels et méthodes utilisés pour mener à bien mes expériences. En annexe, vous trouverez l'ensemble des articles auxquels j'ai pu contribuer de près ou de loin lors de mon doctorat.

L'objectif de ce manuscrit est d'apporter une vision globale des mécanismes régulant la sécrétion neuroendocrine tout en mettant en exergue l'implication des protéines scramblase-1 et oligophrénine-1. Le Français n'étant pas ma langue maternelle, j'ai préféré rédiger ce manuscrit en anglais. J'en profite pour remercier Stéphane Gasman de m'avoir aide à traduire ce prologue.

Je vous souhaite une agréable lecture.

List of abbreviations

AP2: Adaptor protein2

BAR: BIN/Amphiphysin/Rvs

BoNT : Botulinum neurotoxin

Ca^{2+}: Calcium

CME: Clathrin-mediated endocytosis

DAG: Diacylglycerol

DBH: Dopamine β-hydroxylase

ER: Endoplasmic reticulum

F-BARs: Fes/CIP4 homology-BAR

GDI: Gunanine nucleotide Dissociation

Inhibitor GDP: Guanosine Di-Phosphate

GEF: Guanine-nucleotide Exchange Factor

GTP: Guanosine Tri-Phosphate

hPLSCR: Human Phospholipid scramblase

HSCP: Highly Sensitive Calcium-Pool

Inverse-BAR: I-BARs

IRP: Immediately Releasable Pool

ITSN: Intersectin

JMD: Juxta membrane domain

LDCV: large dense core vesicle

L-DOPA: 3,4dihydroxyphenylalanine

Munc13-interacting domains: MID

Na^{+} : Sodium

N-terminal amphipathic helix-BAR: N-BAR

N-WASP: Wiskott-Aldrich syndrome protein

Oligophrenin-1: OPHN1

PC: Phosphatidylcholine

PH: Pleckstrin homology

Phenylethanolamine N-methyltransferase: PNMT

Phosphatidic acid: PA

PIP2: Phosphatidylinositol 4,5-bisphosphate

PKC: Protein Kinase C

PLC: Phospholipase C

PLD1: Phospholipase D1

PLSCR: Phospholipid scramblase

PM: Plasma membrane

PS: Phosphatidylserine

PX: Phox homology

RRP: Readily Releasable Pool

SH3: Src homology 3

SM: Sec1/munc18-like

SNAP: Synaptosomal-associated protein

SNARE: Soluble N-éthylmaleimide-sensitive-factor attachment receptor

SRP: Releasable Pool

STX: Syntaxin

TGN: Trans-Golgi network

TH: tyrosine hydroxylase

TIRF: Total internal reflection florescence

TM: Transmembrane

TMD: Transmembrane domain

t-SNAREs: Target-SNAREs

unc18: uncoordinated18

UPP: Unprimed Pool

VAMP: Synaptobrevin

VGCCs: Voltage-gated Ca^{2+} channels

v-SNARE: Vesicular-SNAREs

I. General introduction

1. The adrenal gland

The adrenal glands are two pyramidal structures located in the upper pole of both kidneys. They produce hormones that help the body to control blood sugar, burn protein and fat, react to stressors like a major illness or injury, and regulate blood pressure.

The adrenal gland is formed by two well defined structures covered by a capsule of connective tissue with different functions and embryological origins: the adrenal cortex derived from the intermediate mesoderm which surround the medulla derived from neural crest cells. The adrenal cortex is devoted to production of steroid hormones, namely aldosterone, cortisol, and androgens. The adrenal medulla is constituted by chromaffin cells, which are able to release hormones and neuropeptides into the bloodstream.

Adrenal gland disorders can be caused by an imbalance in the hormone secretion. For example, Cushing syndrome due to cortex adrenal tumor is caused by an overproduction of cortisol. The Cushing syndrome can lead to diabetes, high blood pressure, and osteoporosis, and other health issues. On the contrary the adrenal insufficiency occurs when the adrenal glands do not make enough cortisol, and aldosterone. The characteristic symptoms include fatigue, muscle weakness, decreased appetite, and weight loss. Another disease associated with this organ is a tumor that arises from the adrenal medulla (Pheochromocytomas). This tumor produces over-secretion of the adrenaline causing of the severe elevation in blood pressure.

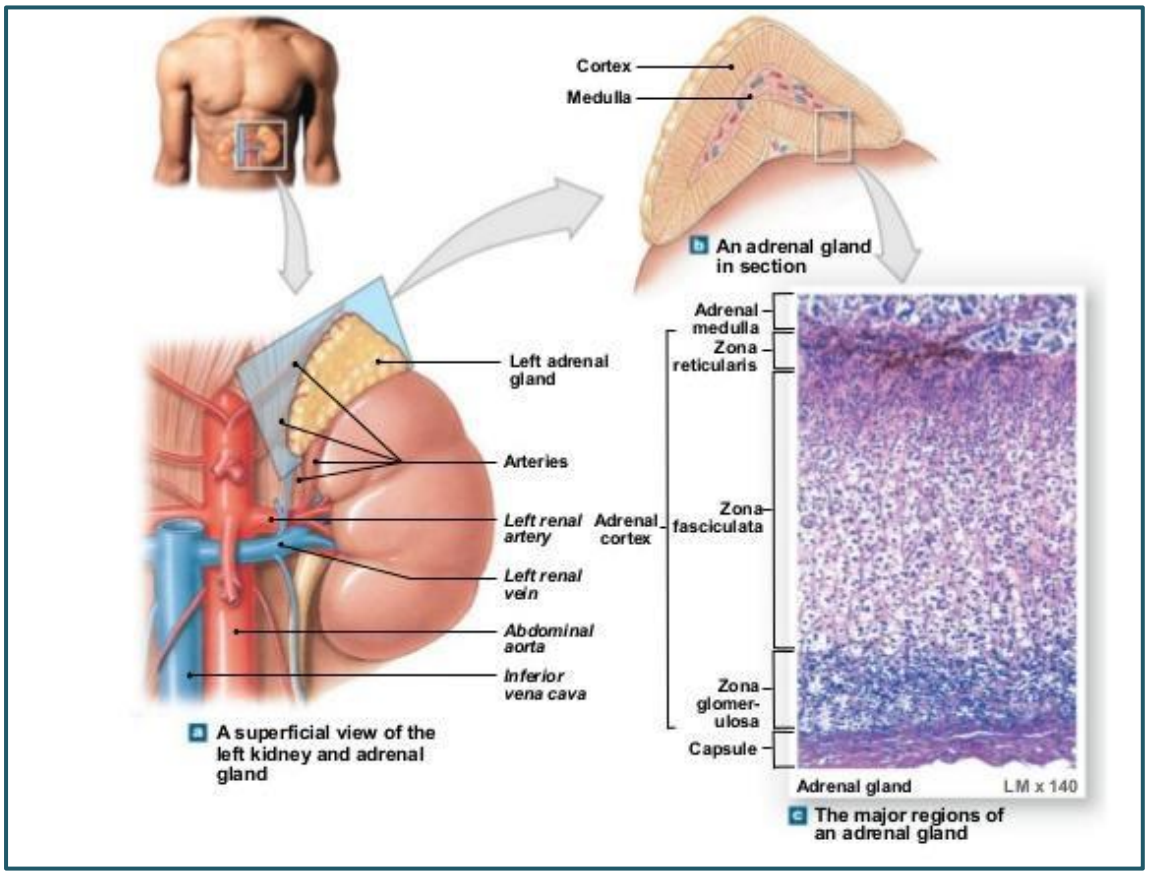

Fig 1: Adrenal gland. A) Representative scheme of the location of the adrenal gland. B) Representative scheme of the adrenal gland section, with the cortex at the periphery and the medulla in the center. C) Amplification of adrenal gland section. (http://www.slideshare.net/gwrandall/163-ch-10lecturepresentation)

2. Calcium regulated exocytosis in chromaffin cells: From the biogenesis to the release

Chromaffin cells are a widely used model to study calcium-regulated exocytosis. They share with neurons the same embryonic origin (neural crest) and both release hormones and neurotransmitters stored in vesicles, by Ca^{2+}-regulated exocytosis

In the adrenal medulla, the splanchnic nerve establishes cholinergic synapse with chromaffin cells. Upon stimulation, acetylcholine is released from presynaptic terminals and nicotinic receptors located on the membrane of chromaffin cells are activated. Nicotinic receptors are cation channels and once activated, they trigger sodium (Na^{+}) entry in the cells. This ionic input induces membrane depolarization, activating voltage-dependent calcium (Ca^{2+}) channels which increase the cytosolic Ca^{2+} concentration, and in turn leads to the release of catecholamine from chromaffin cells.

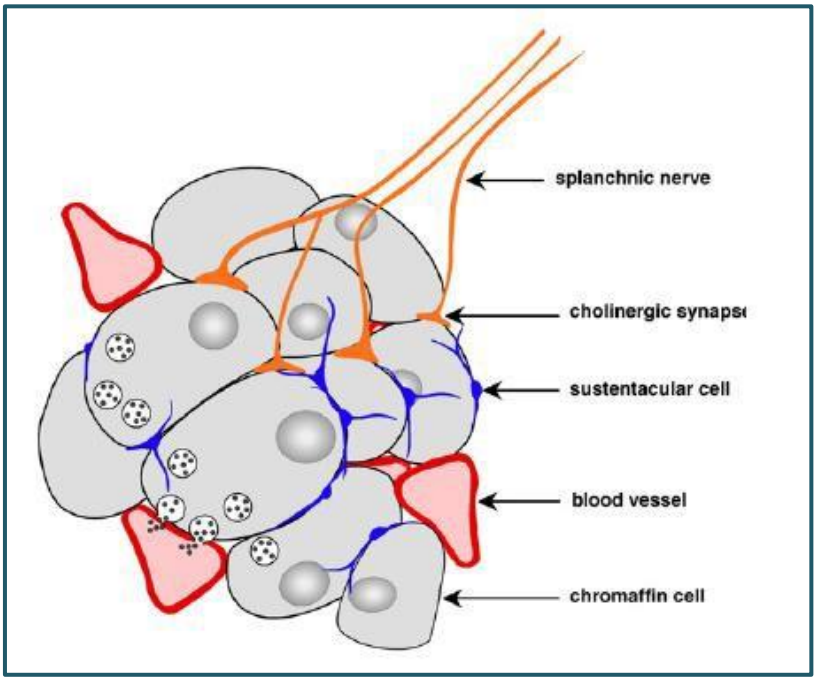

Fig2: Adrenal gland innervation. The splanchnic nerve fibers innervate chromaffin cells, stimulating the release of catecholamine (Colomer et al. 2012).

The main hormones synthesized and released by chromaffin cells are catecholamine (dopamine, noradrenalin and adrenaline). Two types of chromaffin cells are found in the adrenal medulla: adrenergic chromaffin cells secreting adrenaline (or epinephrine) which represent 80% of the chromaffin cells and noradrenergic cells which secrete noradrenalin (or norepinephrine) and represent the remaining 20%.(Kobayashi and Coupland 1993). Chatecolamines are synthesized following a multistep enzymatic cascade. Dopamine is the end product of two enzymatic reactions: hydroxylation of the tyrosine amino acid into 3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH), an enzyme found in dopaminergic cells, and decarboxylation of L-DOPA. These reactions occur in the cytoplasm and dopamine is rapidly transported into specialized organelles, the large dense core vesicle or secretory granule by nonspecific vesicular monoamine transporters (Wimalasena and Wimalasena 2004). Dopamine can be further transformed into norepinephrine (or noradrenalin) by the Dopamine β-hydroxylase (DBH) and epinephrine (or adrenalin) by phenylethanolamine N-methyltransferase (PNMT) (Kuhar et al. 1999).

2.1 Biogenesis of secretory granules

Secretory granules or large dense core vesicles (LDCV) are the main storage unit of chromaffin cells. Although catecholamines are loaded into LDCV once vesicles are formed, proteins found in the matrix of LDCV are involved in the biogenesis of secretory granules (proteins of the granin family) (Kim et al. 2005). Together with the lipid composition of the trans Golgi network (TGN) membranes, they determine the sorting of vesicular components intended either to the constitutive or regulated secretory pathway.

Mechanistically, it has been proposed that the regulated secretory pathway requires a driving force of protein (chromogranin A , B, secretogranin II–IV) and lipid like diacylglycerol, phosphatidic acid, and cholesterol to be generated (Kim et al. 2006). Diacylglycerol (DAG) and phosphatidylcholine can be converted in phosphatidic acid (PA) via DAG kinase and phospholipase D1 respectively. Phosphatidic acid accumulation rather than DAG is a key step in regulating budding of secretory vesicles from the TGN in mammalian cells (Siddhanta and Shields 1998). PA may induce negative curvature of membranes to favor vesicle biogenesis. Additionally, cholesterol form microdomains called lipid rafts from which

the budding of granules occurs (Dhanvantari and Loh 2000). On the other hand, high Ca^{2+} concentration and acidic pH conditions encountered in the TGN promotes aggregation of granins which associate directly or indirectly with lipid rafts to induce budding and formation of the immature secretory granules.(Laurent Taupenot et al.2003; Elias et al. 2012). Different domains of chromogranin have been shown to be important for LDCV formation. For example, a motif consisting of an intramolecular disulfide loop domain bounded by cysteine residues and containing a number of aliphatic hydrophobic residues are necessary and sufficient for the association of the chromogranin B to the TGN membrane and, hence, sorting of chromogranin B to the regulated secretory pathway in the PC12 neuroendocrine cell line (Glombik et al. 1999). This loop is also present in Chromogranin A but does not appear to be necessary for sorting of chromogranin A to the regulated secretory pathway in PC12 cells (Taupenot et al. 2002). It may therefore require additional domain like the region 77-115 which is necessary but not sufficient for trafficking of this protein to the regulated secretory pathway (Hosaka et al. 2002). Conversely, deletion of the segment 48-111 of the chromograninA resulted in missorting of CgA to the constitutive pathway in pituitary (AtT-20) and β-pancreatic (INS-1) cell lines. In addition to their direct role in granule biogenesis, Chromogranins may also help pro-hormones to be sorted into LDCV. Pro-vasopressin, oxytocin and pro-opiomelanocortin are also able to aggregate in condition of high calcium concentration and acidic pH conditions. As they can interact with granin, they participate into sprouting and formation of immature granules (Beuret et al. 2004). This context suggests that the association of granins and prohormone aggregates at lipid rafts is essential to provide the driving force for granule budding at the TGN, whereas lipid components such as DAG, PA, and cholesterol facilitate formation of membrane curvature.

At this stage, secretory granules are immature and may contain proteins not intended to enter the regulated secretory pathway since segregation is not completely efficient. A maturation process takes place which permit the vesicles to sort proteins not supposed to take the regulated pathway. Immature secretory granules (ISG) undergo several steps necessary to convert them into mature granule. The first one is the homotypic fusion of immature granules involving SNARE proteins (Urbé et al.1998; wendler et al. 2001). This granule fusion will induce the increase of their size and also their enrichment in protein necessary for the next steps of maturation like the V-ATPase proton pumps. These latter will

permit the acidification of immature granule and then modify the intragranular pH from 7 to 5 (mature granule). Acidification of the intra-granule content will allow the activation of pro-hormone convertase and carboxypeptidases necessary to the regulated secretory pathway and this acidification is done throughout the transport of the vesicle to the plasma membrane (Wu et al. 2001).

The second step is the removal of lysosomal enzymes, and some membrane proteins that are co-packaged (Kuliawat et al. 1997). This process occurs by budding off of constitutive-like vesicles from the immature granule by a clathrin-dependent mechanism (Kim et al. 2006). It is well known that during this process of maturation, the clathrin coat is removed from the granule. However, the mechanisms regulating this uncoating are still poorly understood (Orci et al. 1985; Tooze and Tooze 1986). Finally, cargo molecules in maturing granules undergo condensation which requires acidification and removal of water by the lipid microdomain-associated aquaporin along with the efflux of Na^+, K^+, Cl (Arnaoutova et al. 2008).

2.2 Transport of secretory granules

Like most intracellular vesicles, secretory granules distribution into the cytoplasm relies on actin filaments and microtubules. Long range transport from the Golgi to the plasma membrane is mediated by microtubules and associated motor proteins whereas short range movement occurring close to the plasma membrane are rather mediated by actin filament and myosins. Although less is known about the role of microtubules in the transport of LDCV compared to actin, interfering with the dynamics of actin or microtubules impair the motility and eventually the release of catecholamine (Neco et al. 2003; Maucort et al. 2014). Interestingly, some evidences suggest that transport of vesicles from the regulated pathway may rely on a subset of proteins represented by SNARE proteins, small GTPases from the Rab family and their regulators. For example, in neuronal cells, Rab27 is required for kinesin1-dependent anterograde movement of Trk-containing (Arimura et al. 2009). In the hippocampal neurite, the SNARE VAMP7 binds to Varp, an activator of Rab21 and this interaction is required for kinesin-dependent anterograde movement of vesicles

(Burgo et al. 2012). In chromaffin cells, although the molecular mechanisms remained to be determined, a subset of proteins from the SNARE and Rab family (VAMP2, Rab3a, Rab27) is also required for efficient transport of secretory granules and fusion at the plasma membrane. It suggests that a general mechanism could be preserved in long range transport of vesicles intended to regulated exocytosis in secretory cells

The actin-based transport of secretory granules has been better studied in chromaffin cells. The first studies proposed that the cortical actin filaments (F-actin) forms a physical barrier that restricts the secretory vesicle access to the plasma membrane. This model was based on the fact that actin depolymerization was observed after stimulation and before massive exocytosis (Trifaró et al. 2000) (Aunis and Bader 1988; M. L. Vitale et al. 1991; Gil et al. 2000) (Nakata and Hirokawa 1992). However, depending on the concentration of actin polymerization inhibitors, both increase (low concentration) or decrease (high concentration) of exocytosis was observed suggesting that actin function was more complex than acting as a physical barrier. Actin can indeed act as a transporter in combination with molecular motors and help directly in the fusion process.

One of this motor, the MyosinVa, plays a crucial role in the control of F-actin dynamics and vesicle displacement. The GTPase Rab27A, located on the granule, interacts with MyRIP (Myosin and Rab interacting protein) and MyosinVa, both bound to actin cytoskeleton. They constitute therefore a link between granules and the actin cytoskeleton (Desnos et al. 2003). Inhibition of MyosinVa function by specific antibody decreases the secretory response in chromaffin cells (Rosé et al. 2003) and the use of MyosinVa dominant negative mutant blocks the traffic of granules at the vicinity of the plasma membrane when granules are entrapped in the actin cytoskeleton in PC12 and β-pancreatic cells (Rudolf et al. 2003; Varadi et al. 2005). This suggests that MyosinVa plays an important role in the granule trafficking and actin is passively required to form “track” for vesicle displacements.

But actin has also an active role by polymerizing at specific site. For example, the actin nucleation promoting factor neural Wiskott-Aldrich syndrome protein (N-WASP) is recruited to plasma membrane upon stimulation and mediate the secretory response in PC12 cells (Gasman S et al. 2004). F-actin forms trails that favor the secretory vesicle motion to the plasma membrane. Forces generated by actin can regulates the expansion of the

fusion pore(Giner et al. 2005; Berberian et al. 2009). Recently, electron microscopy tomography showed that actin filaments can be seen attached to the secretory granule and the plasma membrane to stabilize secretory granule likely in a “ready to fuse” state. Actin filaments have therefore both passive and active function in the transport of vesicle transport and in the regulation of the regulated exocytotic in the chromaffin cells.

2.3 Pools of secretory granules

Biogenesis and maturation of LDCV is a continuous process which supposes that, depending on the maturation step and their distance from the plasma membrane, some LDCV will be ready for fusion with the plasma membrane or not. In chromaffin cells (and in neurons), 4 pools of secretory granule have been described on the basis of their kinetic of release. The Readily Releasable Pool (RRP) in which the vesicles are fusing with the plasma membrane with a time constant 20-40ms after stimulation. The Slowly Releasable Pool (SRP) with a time constant of approximately 200ms. Note that although this pool is made available in the presence of high intracellular Ca^{2+}concentration, a physiological stimulation only generates the release of RRP. Recent studies have shown that RRP can be subdivided into two subgroups: A) The IRP (Immediately Releasable Pool) corresponding to about 25% of the RRP in which the granules are located in the vicinity immediate to the calcium channel (Yang et al., 2002) and B) the HSCP (Highly Sensitive Calcium-Pool) which can be released at lower Ca^{2+} concentrations than the IRP and the RRP. Additionally, another pool was detected in chromaffin cells, the UPP (unprimed Pool). This pool corresponds to the granules located at a maximum distance of 200 nm and which do not belong to the RRP and IRP. The last granule population is the reserve pool where the granules are located at more than 200nm away from the plasma membrane.

2.4 Late phases of exocytosis: from docking to fusion

The exocytosis is the process by which stored neurotransmitters and hormones are released by the fusion of secretory granule with the plasma membrane. This process is dynamic, rapid and spatially restricted in the cells. Exocytosis involved multiple steps including granule trafficking, tethering, docking, priming and eventually fusion. Structural, biochemical and functional studies have allowed the identification of multiple factors and proteins implicated in the exocytosis.

In neurons and neuroendocrine cells, calcium regulated exocytosis is divided in four main steps which are first the tethering of LDCV at the plasma membrane and second, the docking at exocytic sites. At this step, LDCV have to be matured (third step called priming) to be competent for the final and fourth step consisting of fusion and release of intragranular contents (Figure 3).

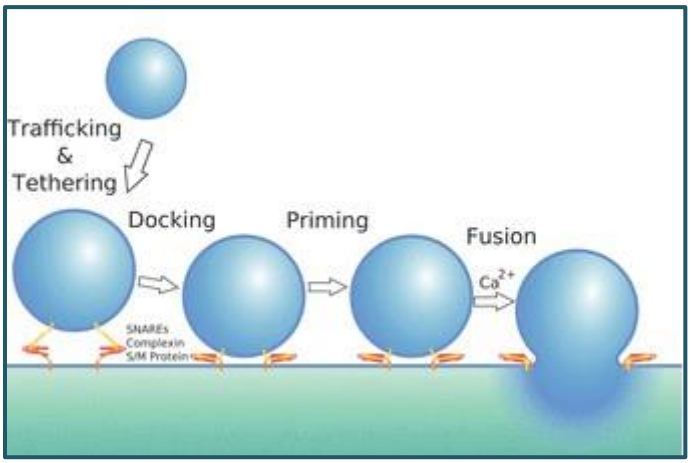

Fig3: Step of exocytosis in the chromaffin cells.

The exocytosis process starts by the recruitment of the granule at the plasma membrane (tethering) and the subsequent docking where Synaptobrevin (a vesicular SNARE) and Syntaxin/SNAP25 (plasma membrane SNARE proteins) interact. During the priming, SNARE proteins are coiled prompting the approach of the granule membrane with the plasma membrane.Finally, the granules fuse with the plasma membrane, releasing their contents.

2.4.1 Docking

Ca^{2+}-triggered release of neurotransmitters and hormones depends on soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE). The SNARE-complex constitutes the minimal machinery needed for the fusion of the secretory granule with the plasma membrane. Within the SNARE proteins involved in regulated exocytosis, we can find the t-SNAREs in the PM and the v-SNAREs located in the vesicular membrane. The SNARE-complex is made up of three SNARE proteins; Syntaxin1 (STX1), synaptosomal-associated protein (SNAP-25) and synaptobrevin (VAMP-2).

Syntaxins were first described as two synaptotagmin-interacting proteins (STX1A and 1B) with a molecular weight of 35 kilo Dalton (kDa) and 84% identical amino acid sequence (Bennett et al. 1992). In adrenal chromaffin cells, STX1A was found to be localized to the membrane as the dominant isoform (Baltazar et al. 2003). This protein present a carboxy-terminal transmembrane domain, SNARE domain (termed H3), a long coiled-coil α-helical structure, and an additional Habc domain with an N-terminal domain containing a short N-peptide (Weimbs et al. 1997). This domain is a linker region, which connects Habc to the H3 domain. The Habc domain (amino acid residues 28-144) is made up of three α helices with high sequence conservation. Habc domain interacts with the H3 domain of the protein which maintains STX1A in a 'closed conformation', preventing its interaction with other SNAREs partner (Misura et al.2001). The closed conformation of STX1A needs to be released to participate in the assembly of the SNARE complex. Point mutations in the linker region of the Habc domain (L165A/E166A) are sufficient to release the interaction between Habc and H3 and bring STX1A in an open conformation able to interact with other partner of the SNARE machinery.

Tight regulation of STX proteins function has to be set up to prevent unexpected release of LDCV contents. The Sec1/Munc-18 like (SM) protein family plays critical function in STX1 regulation. Munc18 was identified as a binding partner to STX and plays an essential role in vesicle fusion from yeast to mammals (Burkhardt et al. 2008), (Südhof and Rothman 2009; Verhage et al. 2000). Munc18 is encoded by the mammalian homologue of the C. elegans gene, uncoordinated18 (unc18) (Hata et al. 1993). In mammals, three isoforms of Munc18 are known: Munc18-1, Munc18-2 and Munc18-3. Knocking out Munc18-1 in mice

leads to deficiency in transmitter release and death at birth due to respiratory defects (Verhage et al. 2000). Munc18, which consists of three D domains (Misura et al.2000), with the third domain divided in D3a and D3b , adopts the form of a horseshoe. D1 and D3a form the bottom part of the horseshoe and D2 and D3b form the upper part. Munc18 binds STX through its central groove and keeps it in a 'closed conformation', preventing STX from binding to SNAP-25 and VAMP2 (Misura et al. 2000). Munc18-1 may stabilize a half-closed conformation of STX1 (Zilly et al. 2006), and forms STX1:Munc18 dimers, serving as stimulators of docking (Gandasi and Barg 2014). Munc18 has a dual function by acting as a chaperone and translocates STX1 to the plasma membrane (Rickman et al. 2007) and as an inhibitor of STX1 activation by maintaining it in a closed conformation (Toonen et al., 2005).

The critical role of Munc18 in docking has been shown in chromaffin cells from Munc18 $^{-/-}$ mice where 90% of LDCV are not docked (Voets et al., 2001). But whether it is a direct role of Munc18 or its function as chaperone has to be defined.

Docking of LDCV implies that STX1 is engaged at least temporarily in the SNARE complex which is formed by the association with SNAP25 localized to the plasma membrane and VAMP2 (or Synaptobrevin2) found at the LDCV membrane. SNAP-25 proteins (SNAP-25a and SNAP-25b) has a molecular weight of 25 kDa and is targeted to the plasma membrane thanks to palmitoylation sites (Bark et al. 1995) (Greaves et al. 2010). In adrenal chromaffin cells, the SNAP-25a isoform is predominating, but both isoforms are expressed (Grant et al. 1999). The role of SNAP25 in docking has been shown with the use of bacterial toxins that cleaves SNAP-25 (Botulinum neurotoxin type A (BoNT/A), C (BoNT/C) or E (BoNT/E)). Treatment of chromaffin cells with these toxins led to reduction of LDCV docking (de Wit et al. 2009) and impairs exocytosis in PC12 cells (Gerona et al. 2000).

Finally the v-SNARE VAMP2 is a protein of 18kDa which is inserted in the LDCV membrane by a single transmembrane (TM) domain. VAMP2 protein presents a juxtamembrane domain able to bridge the negatively charged phospholipid of the plasma membrane. This interaction facilitates the function of the secretory granules with the plasma membrane (Williams et al. 2009).

The structure of the SNARE complex has been resolved in 1998 (Sutton et al. 1998). It is a tightly packed four-helical parallel structure with leucine zipper resemblance. The interaction consists in the carboxyl terminal H3 domain of STX1A (9 kDa) (blue), the cytoplasmic domain of VAMP2 (11 kDa) (green) and the N- and C-terminal portions of SNAP-25B (9 and 10 kDa) (orange), is a cable of four intertwined α-helices with their N-termini at one end and their C-termini at the other protein, as illustrated in figure 4. (Sutton et al. 1998).

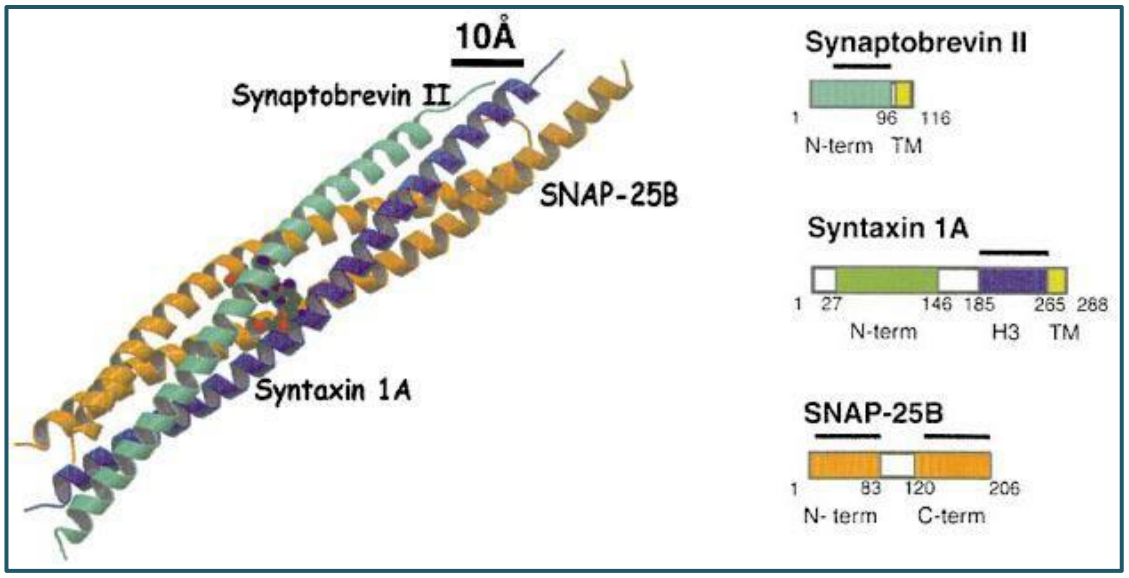

Fig4: Assembly of the SNARE complex (Ybe et al. 2000).

2.4.2 Priming

Once the SNARE complex has formed and docks the LDCV, the priming step will allow these proteins to be wound correctly to bring closer enough the membranes and initiate the formation of the fusion pore after cell stimulation. The molecular details that distinguish the docking from priming is however incomplete but SNARE zippering that could bring LDCV close to the plasma membrane is favored during priming. This is an important step since it will determine the number of vesicles ready to be released when the future intracellular calcium increase will occur.

Munc proteins play also a function in priming. Members of the Munc13 protein family consisting of Munc13-1, -2, -3, and -4 were found to be absolutely required for this priming process (Stevens et al. 2005). During the priming, the SNARE STX1 switch from a closed conformation that binds Munc18-1 tightly to an open conformation within the highly stable SNARE complex. NMR and fluorescence experiments have shown that the Munc13-1 MUN domain, markedly accelerates the transition from the STX1–Munc18-1 complex to the SNARE complex. This activity depends on weak interactions of the MUN domain with the STX1 SNARE motif, and probably with Munc18-1 (Fig5) (Ma et al. 2011).

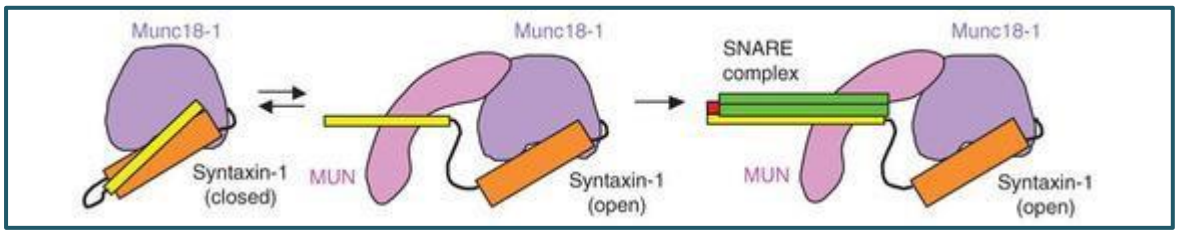

Fig5: Proposed model whereby the MUN domain (purple clear) promotes the transition from the STX1–Munc18-1 complex to the SNARE complex through its weak interactions with the STX1 SNARE motif (from Ma et al. 2011).

Structural and biochemical studies have shown that Munc13 interact with SNARE proteins by a calcium-dependent process because Munc13 present calcium binding C2 domains (Jahn and Fasshauer 2012).

Another protein involved in this process is the $Ca2^{+}$-dependent Activator Protein for Secretion, *aka* CADPS (CAPS). CAPS is a regulator of SNARE complex assembly by creating direct interactions with membrane-associated SNARE STX1 and SNAP25 (Daily et al. 2010). The activity of CAPS in promoting SNARE complex formation was also demonstrated in studies of SNARE-dependent liposome fusion where CAPS markedly increased the rate and extent of fusion between donor VAMP2 liposomes and STX1/SNAP-25 acceptor liposomes (James et al. 2009). Additionally recent work showed that PIP2, STX1 and SNAP25 interactions stabilize the CAPS dimers. The role of the CAPS C2 domain (calcium sensor) in mediating homodimerization was revealed by studies of the mammalian homologous unc-31C2 domain mutants and a C2 domain deletion that exhibits altered dimer formation and loss-of-function in vesicle exocytosis. Lastly, a study suggested that CAPS dimerizes similarly to Munc13-1/2 using conserved homodimerization residues in its C2 domain (Petrie et al. 2016).

The Rab3A, a small G-protein of the Rab family is involved in the priming steps of exocytosis. Recent work showed that Rab3A is essential for the performance of Munc13-1 during vesicle priming. Munc18-1 interacts with Rab3A and promotes Rab3A dissociation from the vesicle membrane; this is downstream of the Rab3A/Munc13-1interaction that regulates vesicle priming (Huang et al. 2011).

After finishing this step, the SNARE complex is mature and the granule is ready to fuse with the plasma membrane.

2.4.3 Fusion

Following stimulation, LDCV either fill up the reserve pool or are recruited to the plasma membrane as a part of the readily releasable pool. Docked LDCV fuse with the plasma membrane. Different release mechanisms have been described in neuroendocrine cells. The *full fusion* corresponds to the complete flattening out of the LDCV into the plasma membrane which led to the total release of intra-vesicular contents. Despite the insertion of the LDCV membrane into the plasma membrane, mechanisms exit to preserve the integrity of the LDCV membrane into the plasma membrane since no intermixing has been observed (Ceridono et al. 2011)(Bittner et al., 2013). The *Kiss-and-Run* corresponds to the formation of a narrow fusion pore between the plasma membrane and the LDCV which allows the release of small compounds of the LDCV like catecholamines. The majority of the granular content is retained and lipids do not intermingle (Gandhi and Stevens 2003). Finally, an intermediate mode of fusion has been described. The *Cavicapture* (cavity recapture or granule recapture) corresponds to partial expansion of the fusion pore, releasing catecholamines and small neuropeptides according to their molecular weight. Like kiss and run, the omega shape of the fusing granule is preserved (Henkel and Almers 1996). (Figure6).

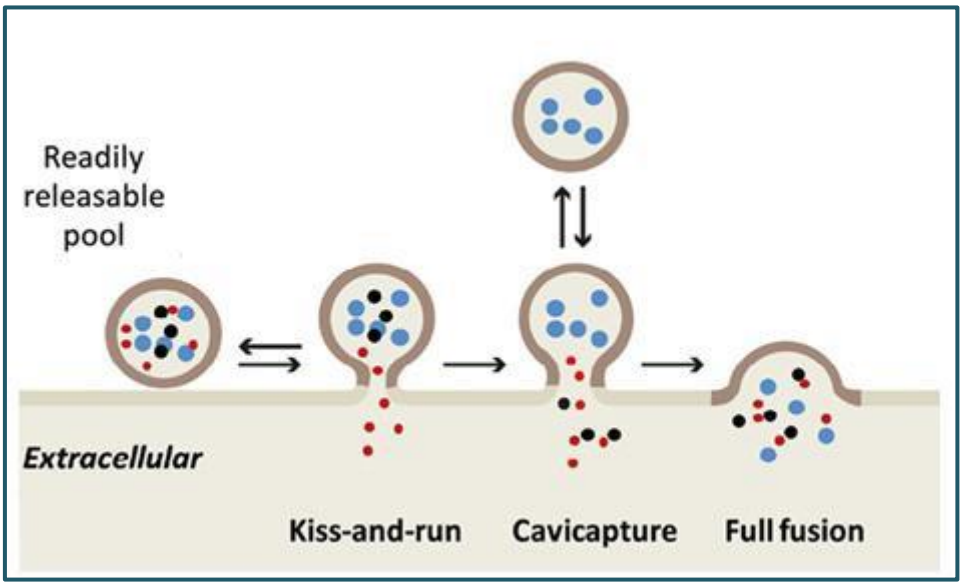

Fig6: Release models in neuroendocrine cells. (Figure modified from Houy et al. 2013)

Mature granules either fill up the reserve pool or are recruited to the plasma membrane as a part of the readily releasable pool. Large proteins (blue dots), small neuropeptides (black dots), and small molecules like catecholamines (red dots) can be released differentially according to the exo-endocytosis mode. During “kiss-and-run” mode, only small molecules are released through a narrow fusion pore, whereas cavicapture (vesicle cavity capture) allows the partial release of small neuropeptides. Note that for these two modes, retrieval of intact granules is easily conceived as the granule shape remains almost intact. During full fusion exocytosis, the intra-granular contents are all released and the granule membrane collapses into the plasma membrane.

3. Role of calcium sensors in regulated exocytosis

In neuroendocrine cells and neurons, the SNAREs proteins are not directly responsible for sensing the Ca^{2+} after stimulation. Important numbers of evidence show that synaptotagmin is the main calcium sensor involved in exocytosis. However, numerous other proteins have been characterized possessing a calcium binding domain and able to regulate the exocytosis in neuroendocrine cells and neurons.

3.1 Synaptotagmin

The synaptotagmin-1 is a granular protein possessing a short N-terminal followed by a transmembrane domain (TMD) and two calcium binding C2 domains (C2A and C2B) (Perin et al. 1991; Herrick et al. 2006). In addition to the Ca^{2+}-binding loops, the C2B domain contains a polybasic region, enriched with lysine residues, that interacts with PIP2 and phosphatidylserine (PS). These interactions are essential for the exocytosis (Araç et al. 2006; Wang et al. 2011) (Honigmann et al. 2013). By interacting with PIP2 clusters formed upon intracellular Ca2+ increase following stimulation, C2 domains reinforced LDCV anchorage. Using various muntants of C2A and C2B domains, it has been proposed that both domains cooperate to bring LDCV membrane and plasma membrane as close as 2 nm distance by interacting with PIP2 at the plasma membrane and PS of the LDCV (Honigmann et al., 2013). In addition to calcium sensing, Synaptotagmin-1 may mechanically promote SNARE zippering to end up with membrane merging (Park et al. 2015).

Synaptotagmin-1 was also involved in docking and priming of vesicles to the plasma membrane in chromaffin cells. Pull down experiments have revealed a possible interaction of Synaptotagmin-1 with SNAP25 and Syntaxin (Mohrmann et al. 2013).

3.2 Munc proteins

As mentioned before, in addition to its function as STX regulators, SM proteins may act as calcium sensors during exocytosis. This is the case of Munc13 (not Munc18). Munc13 proteins have a Ca^{2+}-binding C2-domains (Lipstein et al. 2012). It has been reported that the central C2-domain in Munc13-1 (C_2B) bind phospholipids like $PI(4,5)P_2$ during granules recruitment and release (Shin et al. 2010) suggesting that in addition to help in opening up the so-called "closed" STX1 within STX1/Munc18-1 dimers to allow SNARE complex formation, it may cooperate with Synaptotagmin to bring closer LDCV and plasma membrane. In chromaffin cells, the isoform Munc13-2 plays a fundamental role in the exocytosis (Man et al. 2015). The absence of Munc13-2 decreases significantly the readily releasable pool size and catecholamine release.

3.3 Doc2 proteins

Double C2-domain protein (Doc2) is another calcium-sensing protein. This protein plays an important role in Ca^{2+}-triggered exocytosis. Four members of the protein family that bind Ca2+ are Doc2α, Doc2β, Doc2γ and raphilin-3A. Doc2 proteins possess two C2 domains (C2A, C2B) separated by a linker region, and a N-terminal Munc13-interacting domains (MID) (Sato et al. 2010). The C2 domains have structural similarities, but C2B binds Ca^{2+} with higher affinity that C2A (Giladi et al. 2013). Doc2 proteins were reported as Ca^{2+} sensors in neurotransmission (Groffen et al. 2010) and able to interact with other exocytotic proteins like Munc18-Munc13 and STX. These interactions have been reported in different cell model including β-cells, chromaffin cells and neurons (Ke et al. 2007) (Orita et al. 1997). In neurons, Doc2 is able to translocate to the plasma membrane after stimulation, and the disruption of interaction between Munc13 and Doc2 causes a slow activity in the synaptic transmission (Mochida et al. 1998). In chromaffin cells, Doc2A and Doc2B are recruited to the plasma membrane promoting the increase of granule priming (Voets et al. 2001) suggesting that Doc2 has a regulating role in the priming step.

4. The Rho-family proteins in Ca^{2+}-regulated exocytosis

The Rho-family proteins constitute a major branch of the Ras superfamily of small GTPases. They are of small size (190-250 residues, 21 kDa) and consist in a highly conserved GTPase domain, short N- and C-terminal. The GTP-binding domain has a strong affinity for GDP (Guanosine Di-Phosphate) and GTP (Guanosine Tri-Phosphate) but they are thought to cycle between inactive, GDP-bound and active GTP-bound states. The switch between the two states is catalyzed by regulatory proteins namely Rho GEF (Guanine-nucleotide Exchange Factor) and Rho GAP (GTPase activating) proteins. Despite it stronger affinity for GTP and a lipid anchor, Rho GTPases are mostly found inactive, bound to GDP, and cytoplasmic thanks to Rho GDI (Gunanine nucleotide Dissociation Inhibitor). Upon GDI release, Rho GTPases translocate to membranes where Rho GEF catalyses the exchange of GDP into GTP leading to conformational changes of the Rho proteins and subsequent binding to their effectors. Rho GAP quickly inactivates Rho proteins by increasing their low intrinsic GTPase activity. Rho GDI further “extracts” Rho proteins from membranes. The most studied members of Rho-family proteins are RhoA, Rac1 and Cdc42 and constitute the so-called canonical Rho GTPases as compared to atypical Rho GTPases which mode of regulation depends on degradation or post-translational modification in addition to nucleotide binding (Wennerberg and Der 2004; Aspenström et al 2007).

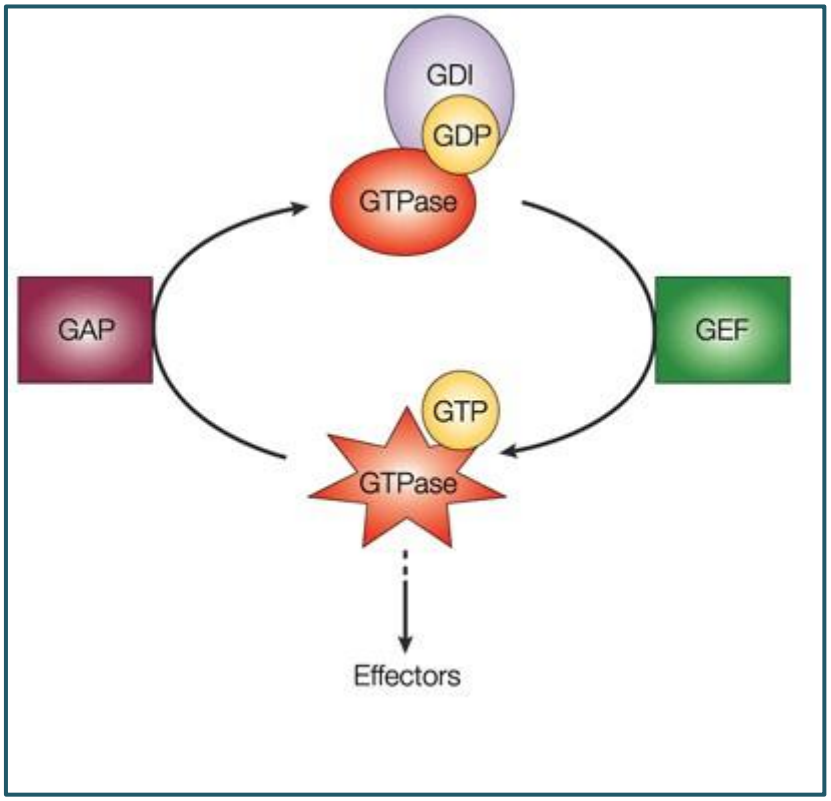

Fig7: The GTPase activation-desactivation cycle. RAS-family proteins are low-molecular-weight guanine-nucleotide-binding proteins. They are inactive when bound to GDP and active when bound to GTP. Regulation of this molecular switch mechanism occurs through a GDP–GTP cycle that is controlled by the opposing activities of guanine nucleotide-exchange factors (GEFs), which catalyze the exchange of GDP for GTP, and GTPase-activating proteins (GAPs), then increase the rate of GTP hydrolysis to GDP (see diagram). In the case of Rho proteins, another layer of regulation is provided by Rho–GDP-dissociation inhibitors (Rho-GDI), which sequester Rho away from the GDP–GTP cycle. GTPases interact with various effector proteins, which influence the activity and/or localization of these effectors; this ultimately influences cell-cycle progression (Coleman et al. 2004).

RhoA, Rac1 and Cdc42 are well-known regulators of actin dynamics and phosphoinositide production in different cell systems (Croisé et al. 2014). As actin play an important role in controlling different phases of exocytosis (LDCV transport, access to the exocytotic site of and expansion of the fusion pore), Rho GTPases appeared as likely regulator of Ca2+-regulated exocytosis. Indeed, depolarization by high concentration of potassium led to the activation of Rac1 and Cdc42, but only Cdc42 was found to induce N-WASP-dependent actin polymerization at the plasma membrane (Frantz et al 2002; Stéphane Gasman et al. 2004; Momboisse et al. 2009). Rac1 instead induced the production of phosphatidic acid (PA), a fusogenic lipid involved in the last step of granule fusion by activating the phospholipase D1 (PLD1). A complex formed by the scaffold protein Scrib and the exchange factor for Rac1 βPIX are crucial to activate Rac1 at the plasma membrane in response to depolarization (Momboisse et al. 2009). RhoA is also involved in that process but unlike Rac1 and Cdc42, RhoA is localized to secretory granules and it has been proposed to regulate PI4Kinase on secretory granules. RhoA inactivation could be a prerequisite to favor exocytosis since, in contrast to Rac1 and Cdc42, the expression of a constitutive mutant inhibit exocytosis in response to depolarization (Gasman S et al. 1999; Gasman S et al. 2004). The precise spatial and temporal regulation of RhoA, Rac1 and Cdc42 have still to be unraveled, but Rho GTPases appeared to act on two fundamental regulators of exocytosis, actin dynamics and lipids biosynthesis.

5. Role of lipids in Ca^{2+}-regulated exocytosis

The secretion in the neuroendocrine cells involve the increase of the intracellular $Ca^{2+}$and the assembly of SNARE complex. Fusion implies merging of two membranes of different origin and it is not so surprising that lipids play an important role in the regulation of exocytosis. Several lipids have been found at exocytotic sites or involved in the regulation of the fusion process which are exemplified below.

5.1 Anionic lipids

The first lipid identified as a regulator of exocytosis is the phosphatidylinositol 4,5-bisphosphate (PIP2) (Martin 2001). Under appropriate conditions, PIP2 can engage in strong electrostatic–based interactions with positively charged molecules (McLaughlin and Murray 2005), and can cluster in small membrane microdomains (Slochower et al. 2014). Phosphoinositide binding proteins can be recruited from the cytosol to cluster of PIP2 to fulfill their function. PIP2 microdomains are required for the docking and fusion of LDCV. In PC12 cells, a spatial correlation between PIP2 microdomains and exocytotic machinery, in particular with syntaxin clusters was observed (Aoyagi et al. 2005). In neurons, loss of plasma membrane PIP2 leads to a decrease in exocytosis and changes in electrical excitability. Restoration of PIP2 levels after phospholipase C (PLC) inactivation which prevent hydrolysis of phospholipid PIP2 into diacylglycerol DAG and the soluble inositol triphosphate (IP3), is therefore essential for a return to basal neuronal activity (Kruse et al. 2016).

Other lipids have been proposed to regulate exocytosis. For example is the DAG, which binds to Munc13-1 through its C1-domain (Rhee et al. 2002) or the Protein Kinase C (PKC), which phosphorylates SNAP25 (Nagy et al. 2002), Munc18-1 (Wierda et al. 2007; Genc et al. 2014) and Synaptotagmin-1 (de Jong et al. 2016), all promoting exocytosis.

In chromaffin cells, the production of Phosphatidic Acid (PA) by phospholipase D1 (PLD1) at exocytotic site is necessary to LDCV (Bader and Vitale 2009) (Zeniou-Meyer et al. 2007) (Vitale et al. 2001). Although we cannot exclude that increased concentration of PA

my also recruit essential proteins for fusion, structural properties of this small lipid which induce negative curvature is privileged and may help in merging membranes.

5.2 Cholesterol

Cholesterol is an abundant lipid in cells and its accumulation at specific sites modify the biophysical properties of the membrane by reducing membrane fluidity and increasing resistance to membrane deformation. These characteristics have led to hypothesize that the cholesterol regulates exocytosis, by modulating membrane curvature, where the spontaneous negative curvature of cholesterol is believed to favor negative curvature regions of the membrane such as the cytosolic side of the fusion pore as illustrated in the Figure 8 (Yang et al. 2016).

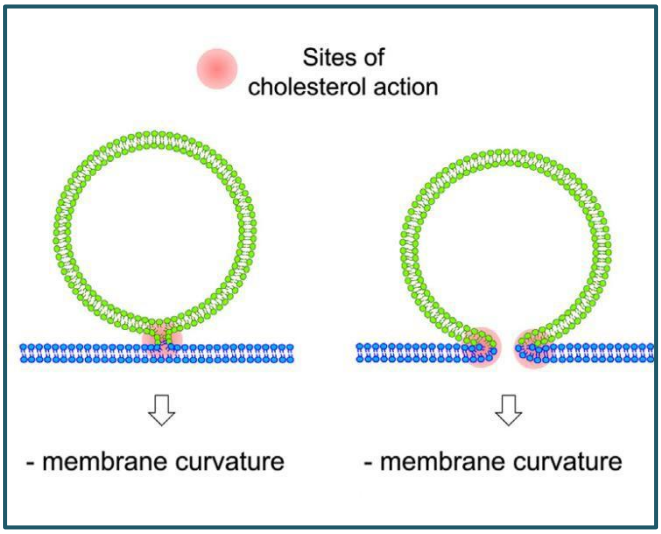

Fig8: cholesterol modulation of the membrane curvature (Figure adapted from S.-T. Yang et al. 2016)

The second role proposed for cholesterol is related to its ability to partition proteins, some proteins accumulating preferentially in cholesterol rich domains. SNARE proteins are preferentially recruited at cholesterol enriched sites in the plasma membrane (Lang 2007; Chasserot-Golaz et al. 2010). During chromaffin cell stimulation, ganglioside GM1/cholesterol/PIP2-enriched lipid microdomains are formed and accumulate at exocytotic site. Cholesterol, PIP2 and GM1 may spatially define the exocytotic sites. Preventing the formation of these microdomains impairs exocytosis. Likewise extraction of cholesterol by methylβ-cyclodextrin treatment affect the actin polymerization (Hissa et al. 2013), alter the molecular organization of synaptic membrane (Chamberlain et al. 2001; Toft-Bertelsen et al. 2016), as well as vesicle motion and docking (Zhang et al. 2009).

To conclude, the lipid distribution and concentration in the exocytotic sites has an important role in the regulation of exocytosis by recruiting, interacting and activating the key proteins to promote the exocytosis in neuroendocrine cells and neurons.

6. Compensatory endocytosis in neuroendocrine cells

Retrieval of membrane proteins or soluble compounds from the extracellular space by endocytosis is a fundamental process critical for cell homeostasis. It is usually separated into two form of endocytosis: the clathrin-dependent or independent pathway. Endocytosis can be either constitutive (best exemplified by the endocytosis of the transferrin receptor for example), or induced by a signal such as the binding of a ligand to its receptor. A third kind of endocytosis can be distinguished: the compensatory endocytosis. This process takes place only when secretory vesicles have fused to the plasma membrane in response to Ca2+ increase. It therefore suggests that a tight coupling should be maintained between regulated exocytosis and compensatory endocytosis to efficiently retrieve transmembrane proteins and lipids coming from the secretory vesicles. Different studies have indeed shown that it is the case in chromaffin cells. Monitoring exocytosis by staining secretory granule membrane components (like dopamineβ-hydroxylase or VMAT2, a transporter of monoamine) inserted into the plasma membrane after full-fusion exocytosis revealed that they remained clustered to help their retrieval by a clathrin-dependent pathway (Ceridono et al. 2011; Bittner et al.,

2013). Although some diffusion of secretory granule proteins have been reported after exocytosis (Sochacki et al., 2012), clathrin is nonetheless important to restrict diffusion since proteins newly inserted into the plasma membrane concentrated to the nearest clathrin-coated pit. The picture is more complicated in neuron where distinct pool of membrane proteins can be retrieved by clathrin-dependent and independent process depending on the cell type and the stimulation (Cárdenas and Marengo 2016).

Recently, the development of a high-resolution and high-throughput fluorescence imaging approach has maped 78 proteins at individual exocytic and endocytic structures in the cell and identified two core groups of proteins that associate with endocytic structures or exocytic vesicles part of which is illustrated in the Figure 9. This approach permited to describe few shared components and several new associations (Larson et al. 2014) suggesting that there are proteins that act independently in each process and others involved in the two process like synaptotagmin-1 (McAdam et al. 2015). This suggests also that these processes occur in a concerted manner in the cell.

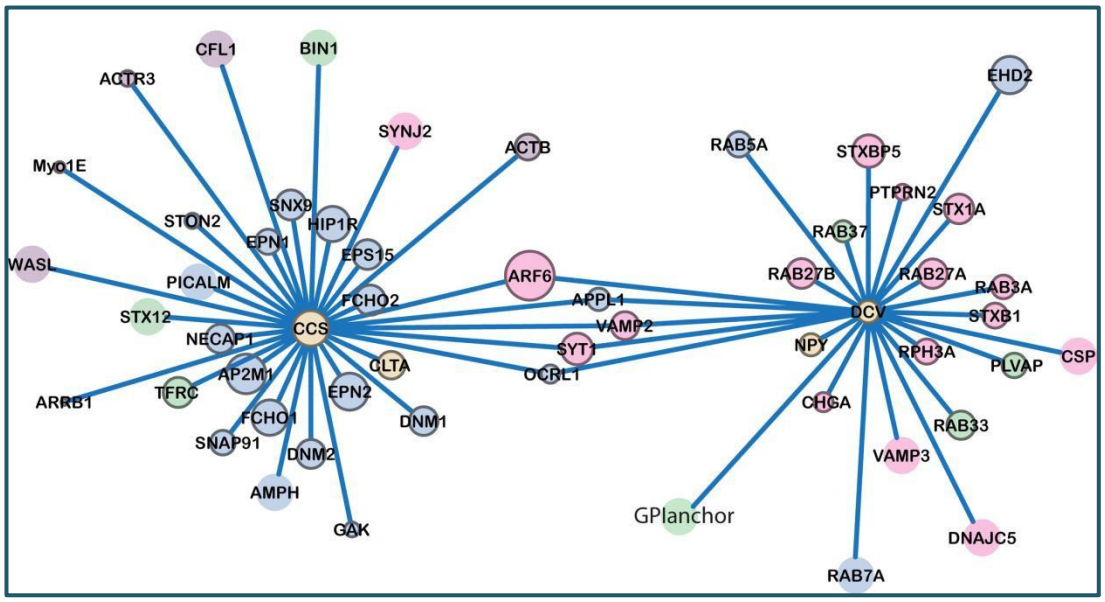

Fig9: Map of proteins associated to endocytic structures CCS and exocytic vesicles DCV.

As stated before, lipids play important function in exocytosis either as biochemical intermediates or by their physical properties. In compensatory endocytosis, they may act as a scaffolding system to maintain specific machinery at restricted site of the plasma membrane. For example, the formation of ganglioside- and PIP2-enriched microdomains at the exocytic sites may prevent or limit proteins and lipids diffusion after the granule have fused (Chasserot-Golaz et al. 2005; Umbrecht-Jenck et al. 2010). On the other hand, a loss of plasma membrane asymmetry has been observed following exocytosis. As it occurs at the rim of the exocytotic site, it might restrict diffusion by creating phase separation into the plasma membrane (Ory et al., 2013). But differences in the way secretory vesicle are released might condition the mode of component retrieval.

6.1 Clathrin-dependent endocytosis

A long lasting question in the field of regulated exocytosis is how proteins and lipids are retrieved by endocytosis after being delivered at the plasma membrane, especially after full fusion, when the secretory granule membrane or the synaptic vesicle collapse into the plasma membrane.

Among the basic pathways involved in endocytosis, clathrin-mediated endocytosis is prominent and consists of embedding a piece of plasma membrane in a coat formed by clathrin. Five steps have been identified to generate clathrin coat vesicles. The first one is the nucleation in which the FCHO proteins bind PIP2-rich zones of the plasma membrane and recruit EPS15 and intersectins which in turn recruit adaptor protein 2 (AP2) to initiate clathrin-coated pit formation (Stimpson et al. 2009; Henne et al. 2010). The second step is the AP2-dependent cargo selection where AP2 recruits several classes of receptors directly through its μ-subunit and σ-subunit. Cargo-specific adaptors (stonin, HRB and Numb for example) bind to AP2 appendage domains and recruit specific receptors to the AP2 hub (Collins et al. 2002; Robinson 2004). Next, clathrin triskelia is recruited by the AP2 hub and polymerizes in hexagons and pentagons to form the clathrin coat around the nascent pit (Boucrot et al. 2010). Once the coat has formed, a scission step occurs to generate a clathrin-coated vesicle. The GTPase dynamin is recruited at the neck of the forming vesicle by

Bin/Amphiphysin Rsv (BAR) domain proteins, where it self-polymerizes and, upon GTP hydrolysis, induces membrane scission by twisting around the neck (Roux et al. 2006) (Roux et al., 2006). The actin machinery module can be added at this stage where actin polymerizes at the neck of the pit and help in vesicle production (Ferguson et al. 2009). The final step consists of the uncoating of the vesicle which is regulated by auxilin or cyclin G-associated kinase (GAK) with the help of the ATPase heat shock cognate 70 (HSC70) to disassemble the clathrin coat and generate an endocytic vesicle containing the cargo molecules (Schlossman et al. 1984; Ungewickell et al. 1995). Synaptojanin probably facilitates this step by releasing adaptor proteins from the vesicle membrane through its PtdIns lipid phosphatase activity, able to hydrolyse PIP2, suggesting that PIP2 cycling is important for the endocytosis (Cremona et al. 1999). It has been proposed that Synaptojanin facilitates also the auxilin recruitment (Massol et al. 2006). After this cycle, components of the clathrin machinery are then available for another round of clathrin-coated vesicle formation (Figure 10).

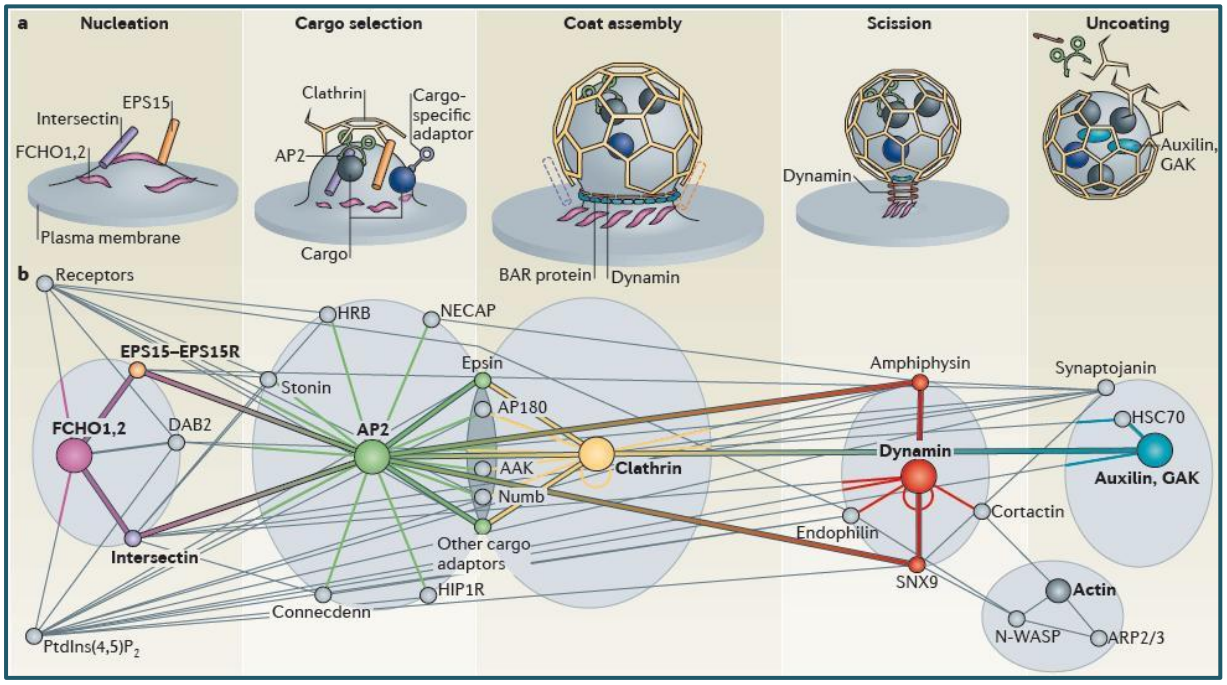

Fig10: Cycle of clathrin-dependent endocytosis. A) Schematic illustrating the five stages of clathrin-dependent endocytosis. The nucleation, cargo selection, coat assembly, section and uncoating. B) The clathrin network. The protein–protein interactions underlying the different stages of vesicle progression are shown. Major hubs are obvious because of their central location in the network and the large number of interacting molecules. They are essential for pathway progression and are denoted by the central colored circles. Possible pathways of progression between hubs are shown with thicker lines.

6.2 Proteins and lipids in compensatory endocytosis

The role of compensatory endocytosis is to refill the vesicle pool that need to be sustained in response to strong and repeated stimulation. As compensatory endocytosis needs exocytosis to occur, proteins participating in both mechanisms would be ideal to couple both. Indeed, in addition to proteins mentioned in the preceding part, the proteins from the exocytic machinery may participate to compensatory endocytosis.

6.2.1 SNARE proteins in endocytosis

Studies using neurons from knock-out animals gave some insight into the role of SNARE proteins in compensatory endocytosis. For example, after depletion of the readily releasable vesicle pool by high K^+ solution stimulation, replenishment of the pool is delayed in hippocampal neurons from VAMP2 knockout mice indicating that slow compensatory endocytosis is altered (Deák et al. 2004). Similarly, treating neurons with tetanus toxin which cleaves and inactivate VAMP2 reduces slow endocytosis. The implication of the VAMP2 in the slow endocytosis was also demonstrated in the hippocampal neurons by capacitance assays (Hosoi et al. 2009; Z. Zhang et al. 2013). Interaction between VAMP proteins and adaptors proteins involved in clathrin-mediated endocytosis have been reported including CALM and AP180. These data suggest a interesting mechanism of SNARE motif-dependent endocytic sorting and identify the ANTH domain proteins AP180 and CALM as cargo-specific adaptors for VAMP2 endocytosis in the central nervous system (Koo et al. 2011).

Contradictory results have been reported regarding the implication of SNAP25 in compensatory endocytosis. Recent studies in hippocampal neurons and PC12 cells showed SNAP 25 plays an important role in endocytosis evidenced by capacitance measurement and dye uptake assay (Z. Zhang et al. 2013). Nonetheless the role of SNAP25 has been

questioned after studies used SNAP25 knock-out which does not show any defect in FM dye uptake into hippocampal boutons after stimulation with high K^+ solution (Bronk et al. 2007).

Finally, the most promising proteins involved in compensatory is the Synaptotagmin-1 (SytI). SytI knock-out mice have a strong defect in Ca2+ regulated exocytosis, but the spontaneous activity is sufficient to monitor compensatory endocytosis which is defective in neuron from $SytI^{-/-}$ mice. Reexpression of calcium binding domains of SytI (C2A and C2B) restored compensatory endocytosis in SytI-/- neurons (Yao et al., 2011). Like VAMP2, SytI can bind to regulators of clathrin-mediated endocytosis including AP-2 (Jarousse et al. 2001).

6.2.2 BAR domain proteins in endocytosis

The BIN/Amphiphysin/Rvs (BAR) domain superfamily of proteins is a central player of endocytosis by linking the plasma membrane to the actin cytoskeleton. The BAR protein family is defined by its membrane-binding BAR domain that folds into a dimeric, tightly inter 6-helix bundle with a curved, crescent-like shape (McDonald and Gould 2016). It forms coiled-coils that dimerize into modules with a positively charged surface able to bind membranes (Shimada et al. 2007). Three kind of BAR domains have been described on the basis of the shape they confer and the way they bind membranes: the “classical” BARs, the F-BARs (Fes/CIP4 homology-BAR), and the I-BARs (Inverse-BAR) (Frost et al. 2009).

BAR Domains		Human BAR Domain Proteins
Classical BAR		Arfaptins, ICA69, PICK1, DNMBP (Tuba)
N-BAR (N-terminal amphiphathic helix-BAR)		Amphiphysins/BINs, endophilins/BIFs, nadrins
BAR-PH (BAR-Pleckstrin Homology)		Oligophrenins, APPLs, GRAFs, centaurin-βs
PX-BAR (PhoX-BAR)		Sorting nexins 1,2,5,6,9
F-BAR (FCH-BAR, Fes/CIP4 Homology-BAR)		Toca-1, FBP17, CIP4 FCHo1, FCHo2, Fes/Fer kinases, syndapins, srGAPs, PSTPIP-1, PSTPIP-2
I-BAR (Inverse-BAR; IMD-BAR, or IRSp53-MIM homology domain)		IRSp53, MIM, IRTKS

Fig11: BAR domain superfamily protein (Frost et al. 2009)

N-BAR domains contains an N-terminal amphipathic helix. Amphiphysin has such a helix, able to insert itself in the plasmatic membrane and induce curvature. The F-BAR domain is able to bind membranes that are already slightly bent leading to the notion that some BAR domains may generate curvature whereas others bind to already curved membrane.The I- BAR domain recognizes curved membrane but with a negative curvature. In association with other membrane binding domains such as PH (plekstrin homology domain) or PX (phox homology domain) domains, BAR domain proteins have different affinity for phosphoinositide which facilitate their recruitment in specific subcellular compartments.

During clathrin-mediated endocytosis, BAR domain proteins control the curvature of membrane. They recruit and connect different binding partners which coordination of the different events during the endocytic process, such as membrane invagination, coat formation, actin nucleation, vesicle size control, fission, detachment and uncoating (Qualmann, Koch, and Kessels 2011).

6.2.3 Role of lipids in endocytosis

During clathrin-mediated endocytosis, the first step which is described as “the nucleation” involves the formation of a membrane invagination called a pit. Clathrin-coated pit initiation was traditionally thought to be triggered by the recruitment of the highly conserved protein AP-2 to the plasma membrane. This can be mediated by the presence in the plasma membrane of specific lipid like phosphatidylinositol-4,5-bisphosphate (PIP2). Two models of membrane deformation in the endocytosis are proposed and both of them require PIP2. The first involves the Epsin protein, which bind to PIP2 by its N-terminal ENTH domain and bends the membrane by partial insertion of an amphipathic helix lying parallel to the membrane (McMahon and Boucrot 2015). The second model proposed consists in recruiting proteins with BAR domains, a banana-shaped domain with a basic charge on the concave surface (Suetsugu et al. 2014). The electrostatic-based binding of this surface to PIP2 can sense membrane curvature, and contributes to membrane deformation. The degree of curvature varies among BAR-domain proteins, suggesting that they may act at different stages of clathrin pit formation. Additionally, in the last step of endocytosis, PIP2 may help scission by recruiting dynamin (Martin 2001).

In summary, PIP2 plays important roles at various stages of CME, suggesting a mechanism by which clathrin-mediated endocytosis is regulated by lipid dynamic, but unfortunately we do not know more details about the role of other lipids in this process. It has been described that cholesterol for example would not be involved in the regulation of endocytosis in PC12 cells (Thiele et al. 2000).

6.3 Proteins with dual functions

6.3.1 Intersectin

Intersectins (ITSNs) are large multidomain proteins that have been mostly involved in clathrin-dependent endocytosis. In vertebrates, ITSNs are encoded by 2 genes (*itsn1* and *2*). The ITSN1 protein is enriched in neurons and neuroendocrine cells whereas ITSN2 is ubiquitously expressed. ITSNs exist in two main isoforms, a short form which presents two N-terminal EH (Eps15 homology) domains followed by a coiled-coil region and five SH3 (Src homology 3) domains a long form (ITSNL) which possesses three additional domains in its C-terminal part: a tandem of Dbl (DH) and pleckstrin homology (PH) domains and a C2 domain. This extension has guanine nucleotide exchange factor (GEF) activities for Cdc42, a small GTPase of the Rho family (Dergai et al. 2016; Hussain et al. 2001).

ITSN1 has been implicated in fast neurotransmitter release in the calyx of held synapse. Electrophysiological recording in cells with genetic deletion of Intersectin 1 showed inhibition decrease in the recruitment of release-ready synaptic vesicles without affecting the rate of membrane retrieval by endocytosis under the same conditions (Sakaba et al. 2013). On the other hand, ITSN1 has been shown to interact with numerous endocytic proteins, including dynamin, AP2, proteins from the Epsin family, and the synaptojanin phosphatase. Its role in the control of endocytosis has been described in different cell types and organisms(O'Bryan and Tsyba et al. 2011). The role of ITSN1 in endocytosis in neurons was further demonstrated in functional assays performed in various model organisms. Studies in neuronal culture by optical assays of endocytosis in the ITSN 1 KO cells suggest a effects on endocytosis (Yu et al. 2008). In addition, it has been reported that ITNS1 is able to interact with the dimer STX:SNAP-25 and dynamin (Okamoto et al. 1999; Peters et al. 2004). This supports the idea that ITSN1 is capable of regulating the coupling between exocytosis and endocytosis.

In PC12 cells silencing of ITSN1 provokes an inhibition of the regulated exocytosis, whereas overexpression of the C-terminal part of ITSN1L (DH-PH-C2 domains) promotes exocytosis and peripheral actin polymerization in neuroendocrine cells (Malacombe et al.

2006; Momboisse et al. 2009). Cdc42 was shown to be activated near the plasma membrane during exocytosis, where it recruits N-WASP and induces actin polymerization. ITSN1-L appeared to be an ideal candidate for Cdc42 activation at docking sites for secretory granules, because it is a specific GEF for Cdc42 and at the same time binds to its effector N-WASP (Hussain et al. 2001). This leads to local polymerization of actin, thereby facilitating exocytosis. ITSN1L colocalized with exocytic sites in PC12 and primary bovine chromaffin cells (Malacombe et al. 2006). In addition, in the neuroendocrine cells, ITSN1 has been reported to interact with SNAP-23 and SNAP-25, but no functional consequence was demonstrated (Okamoto et al. 1999).

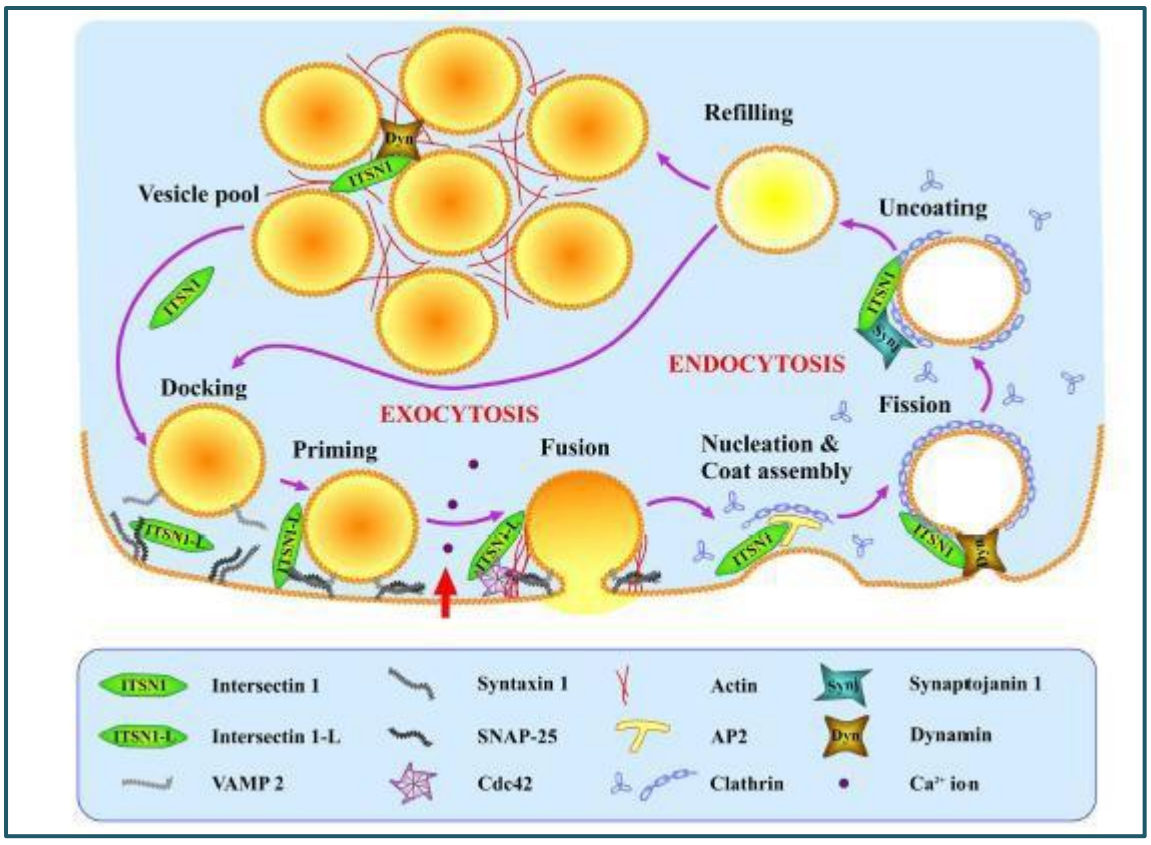

Fig12: Hypothetical model of ITSN1 coupling exo- and endocytosis in neuroendocrine cells (Gubar et al. 2013).

6.3.2 OPHN1

Oligophrenin-1 (OPHN1) is a Rho family GTPase-activating protein (Rho-GAP) that contains, the catalytic GAP domain, N-terminal Bin-Amphiphysin-Rvs (BAR) domain that is able to sense the membrane curvature, a pleckstrin homology domain and three C-terminal proline-rich domains, as illustrated in the figure13 (Billuart et al. 1998; Fauchereau et al. 2003).

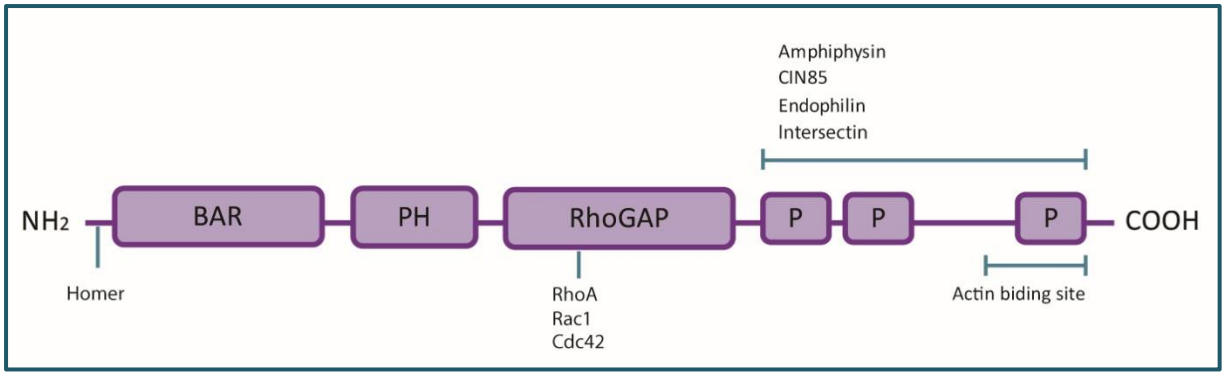

Fig13: Schematic representation of Oligophrenin-1. OPHN1 is a Rho family GTPase activating protein (Rho-GAP) that contains a N-terminal BAR domain, which senses and binds curved membranes and a Pleckstrin Homology (PH) domain, which binds phosphatidylinositol lipids. The catalytic GAP domain inhibits RhoA, Rac1, and Cdc42 in vitro .The C-terminal part, OPHN1 contains an actin-binding site and three Proline-rich sites permitting the interaction with SH3 domain containing proteins including amphiphysinI and II, CIN85, endophilinA1and B2, Homer1 and intersectin1.

The loss of OPHN1 function has been associated with X-linked intellectual disability (Billuart et al. 1998). In vitro studies have shown that this protein is capable of inactivating the GTPases RhoA, Rac1 and Cdc42 with no apparent specificity. This GAP activity is inhibited through an intramolecular interaction between the GAP domain and the N-terminal region (Fauchereau et al. 2003).

In neurons, OPHN1 is expressed both presynaptically and postsynaptically, and is required for the normal dendritic spine morphogenesis in the hippocampal (CA1) neurons (Govek et al. 2004; Khelfaoui et al. 2007). CA1 neurons in which Oligophrenin-1 expression

has been downregulated present a significant shortening of dendritic spines. Re-expressing a constitutively activated form of RhoA recapitulated the defect in spine maturation leading to the idea that Rho inactivation by OPHN1 and inhibition of the contractile force mediated by the RhoA pathway was necessary for normal dendritic spine development. It also indicates that RhoA inactivation may be a requirement for normal synaptic transmission. This is further suggested by the use of Fasudil, an inhibitor of the RhoA effector ROCK, in chronic treatment of mice KO for OPHN1. Fasudil restores some behavior defects like recognition memory in adult mice. However, it can not restore working and spatial memory indicating that the RhoA/ROCK pathway and may be OPHN1 has limited action when synaptic plasticity is reduced (Govek et al. 2004; Meziane et al. 2016).

OPHN1 is also playing a key role post-synaptically in activity-dependent maturation and plasticity of excitatory synapses.OPHN1 localization and function in excitatory synapses depend on synaptic activity and NMDA receptor activation. OPHN1, by its Rho-GAP activity, regulates synaptic structure and function, controlling the stabilization of AMPA receptors. Therefore, decrease in the OPHN1 level and signaling results in destabilization of synaptic AMPA receptors and spine structure, leading to impairment in plasticity and eventually loss of spines and NMDA receptors. Together, this result indicates that OPHN1 are necessary for the normal glutamatergic synapse development (Nadif Kasri et al. 2009).

Additionally, it has been reported that OPHN1 and Homer1b/c interaction contributes to synapse strengthening. Disruption of the OPHN1–Homer1b/c interaction causes a displacement of the endocytic zones from the postsynaptic density, together with impaired AMPAR recycling and reduced AMPAR accumulation at the synapses. It was proposed that OPHN1 is involved in the synapse maturation and plasticity (Nakano-Kobayashi et al. 2014).

OPHN1-dependent AMPA receptor recycling depends on OPHN1 interaction with endophilin A1, another BAR domain-containing protein implicated in compensatory endocytosis and involved in membrane curvature generation during synaptic vesicles retrieval (Nakano-Kobayashi et al. 2009). In addition, OPHN1 inhibits RhoA through its GAP domain. Loss of OPHN1 function in endocytosis is restored by inactivating the RhoA pathway

suggesting that in addition to endophilin binding, RhoA activity needs to be repressed to allow compensatory endocytosis in neurons (Khelfaoui et al. 2009).

7. Regulation of lipid dynamics

Cell membranes are formed by two leaflets of asymmetrically distributed lipids. The maintenance of transbilayer lipid asymmetry is essential for normal cellular function, and disruption of this asymmetry is associated with cell activation or genetic defect (pathologic of hemoglobinopathies) (Daleke 2003; Kuypers 2007). Lipid asymmetry is controlled by three groups of protein with distinct properties and specificity. Flippases and floppases are ATP-dependent transporters. Flippases are highly selective for phosphatidylserine (PS) and transport PS (and to a lesser extent phosphatidyl ethanolamine, PE) from the outer leaflet toward the innerleaflet of the plasma membrane, against the PS concentration gradient. Floppase activity has been associated with the ABC class of transmembrane transporters and transport phosphatidylcholine (PC), sphingomyeline (SM) and cholesterol against concentration gradient in the opposite direction of flippases. Both family of proteins are maintaining plasma membrane asymmetry. Scramblases instead are mixing lipids with no apparent specificity. Its activity is independent on ATP but requires calcium.

In the case of cell organelle, the precise distribution of phospholipids is still unclear but recent studies provide evidences that PS distribution is asymmetric along the secretory pathway. With the development of fluorescent probes with strong affinity and specificity toward PS (C2 domain of lactadherin), it has been shown that from its site of synthesis in mitochondria, PS translocates to the luminal leaflet of the endoplasmic reticulum (ER) through mitochondrial-associated domain (MAM). PS further flips to the cytofacial leaflet at the trans-Golgi network thanks to flippases (Fairn et al. 2011, Leventis, Grinstein, 2010).

In neuroendocrine cells, the leaflet of secretory granules facing the cytoplasm is enriched in PS (Zachowski et al. 1989) upon fusion, the luminal leaflet is exposed to the extracellular space respecting the plasma membrane asymmetry. Nonetheless, PS egress is observed upon fusion and occurs at the periphery of the fusion site indicating that the plasma membrane is locally losing its asymmetry during fusion. This loss of asymmetry does

not alter exocytosis, but rather compensatory endocytosis (Ory et al. 2013). The molecular mechanisms are unknown but changes in PS concentration at the plasma membrane may favor endocytosis (Farge 1995). Therefore, a local change in PS concentration may help membrane curvature to initiate endocytosis. Although flippases and floppases may play an import role in the coupling between exocytosis and endocytosis, no experimental evidences have been reported to my knowledge. I will therefore focus on scramblases.

7.1 Scramblases.

Scramblases are transmembrane proteins that mix lipids in response to Ca^{2+}. Three types of proteins have been reported to have scramblase activity: TMEM16, XKR and phospholipid scramblases (PLSCRs). Historically, PLSCR1, the first form of a family of 5 proteins (PLSCR1 to PLSCR5) was the first to be described. It was purified from platelets and shown to have scrambling activity on artificial liposomes (Comfurius et al. 1996). The role of PLSCR1 in lipid scrambling have been questioned when PLSCR1 $^{-/-}$ mice have been generated. Indeed, despite the critical role of PLSCR1 in PS egress during basic biological function such as apoptosis or blood coagulation, PLSCR1$^{-/-}$ mice did not show any defect in those processes (Zhou et al. 2002). On the other hand, new proteins of the scramblase family have been identified, among them, the TMEM16F scramblase involved in the Scott syndrome, a pathology leading to bleeding disorder due to impaired PS externalization in activated platelets (Zwaal et al. 2004).

7.1.1 TMEM16 and XKR protein

TMEM16 (also called asanoctamin (ANO)), and the XKR family members are recently identified scramblases (Brunner et al. 2016). TMEM16F is localized to the plasma membrane and supports phospholipid scrambling in a Ca^{2+}-dependent manner (Suzuki et al. 2010). TMEM16F has been found mutated in human Scott syndrome, a mild bleeding disorder caused by impaired PS externalization in activated platelets (Zwaal et al. 2004), (Suzuki et al.

2010). TMEM16 is a large family of proteins comprising 10 members that are expressed in a wide range of tissues like brain, skin, ovary, heart among other (Suzuki et al. 2013).

As TMEM16, XKR proteins are localized at the plasma membrane and scramble phospholipids in response to Ca2+ increase. XKR8 and the Caenorhabditis elegans homologue CED-8 were shown to be responsible for PS-externalization during apoptotic cell death, in conjunction with synchronous caspase-mediated inactivation of flippases (Suzuki et al. 2013; Segawa et al. 2014).

7.1.2 Phospholipid Scramblases (PLSCR)

The PLSCR family of proteins is composed of 5 members: *PLSCR1* (318 amino acids), *PLSCR2* (297 amino acids), *PLSCR3* (295 amino acids), *PLSCR4* (329 amino acids) and finally *PLSCR5* (271 amino acids). All PLSCR family members, with the exception of PLSCR2, possess a proline-rich N-terminal region containing PxxP and PPxY domains, a cysteine-rich region, a conserved calcium ion binding domain (EF-hand-like), and a putative transmembrane region enriched in hydrophobic amino acids. (Chen et al. 2005; Zhu et al. 2013).

PLSCR1, PLSCR3, and PLSCR4 isoforms are ubiquitously expressed, whereas PLSCR2 expression seems to be restricted to testis. Intriguingly, this study showed that PLSCR1 is not expressed in the brain and skeletal muscle. (Wiedmer et al. 2000) Recent work from our lab detected PLSCR1 expression in neurons from mouse olfactory bulb by western blot suggesting that PLSCR1 may be expressed in discrete parts of the brain, which indicates that the expression of these proteins in different models and tissues must be re-analyzed.

The subcellular localization of the PLSCRs is variable and depends on the isoforms. For example PLSCR1 and 4 are localized at the plasma membrane where they mediate bidirectional translocation of phospholipids (Bassé et al. 1996; Zhou et al. 1997). PLSCR3 is mostly enriched in the mitochondrial membrane despite the presence of a transmembrane domain. hPLSCR3 localizes to mitochondria and is involved in intrinsic apoptotic pathway and cardiolipin translocation in mitochondria (Wiedmer et al. 2000). In contrast, PLSCR2 is predominantly localized to the nucleus.

In addition to their transmembrane domain, posttranslational modifications regulate the subcellular localization of PLSCR scramblases. The targeting of PLSCR1 to the plasma membrane or PLSCR3 to mitochondria requires an intact palmitoylation motif. In its absence, PLSCR1 and PLSCR3 localized to the cell nucleus thanks to a nuclear localization signal (NLS) (Merrick et al. 2011), which is found in PLSCR1, PLSCR2, PLSCR3, and PLSCR4. Nuclear translocation of PLSCR scramblase may have critical role in cell function. For example, PLSCR1 can regulate the transcription of IP_3R gene (Zhou et al. 2005) and may contribute to DNA replication via its physical interaction with topoisomerase (Wyles et al. 2007). Nuclear PLSCR1 is also involved in the production of mature neutrophils from myeloid progenitors following exposure to G-CSF (Chen et al. 2011). The link between PLSCR function in specific membrane compartment and its activity in the cell nucleus remains elusive. Among PLSCR, PLSCR1 is the best characterized members and I will focus on this isoform in the next parts.

7.1.2.1 Regulation of PLSCR1 activity

The PLSCR1 is a Ca^{2+}-binding, endofacial plasma membrane protein thought to contribute to the transbilayer movement of phosphatidylserine (PS) phosphatidylcholine (PC), phosphatidylethanolamine (PE) (Dekkers et al. 2002). As mentioned above, this protein presents different motifs, like DNA binding motif, EF-hand-like Ca2+ binding , Cysteine-palmitoylation and transmembrane domain (figure 14). Scramblase activity has been reported to be mostly regulated by Ca^{2+}. PLSCR1 possesses and EF-hand-like motif [273-DADNFGIQFPLD-284] which has a relatively weak affinity (mM range) for calcium (Sahu et al. 2007a). EF-hand-like motif form a loop in which amino acids at position 1 (Asp273), 3 (Asp275), 5 (Phe277), 7 (Ile279), 9 (Phe281) and 12 (Asp284) octahedrally coordinate the calcium ion. Mutation leading to the replacement of any amino acids at position 1, 3, 5, 7, 9 or 12 to alanine resulted in a marked reduction of PLSCR1 scrambling activity. Ca^{2+} binding can generate a conformational change in PLSCR1, inducing potential reorientation of helical segments flanking the Ca^{2+} binding loop, that might contribute to the accelerated transbilayer movement of phospholipid in the plasma membrane (Stout et al. 1998; Sahu 2009). When PLSCR1 is inserted into liposomes, addition of Ca^{2+} is sufficient to induce scrambling suggesting that Ca^{2+} is the major regulator of PLSCR1 scrambling activity.

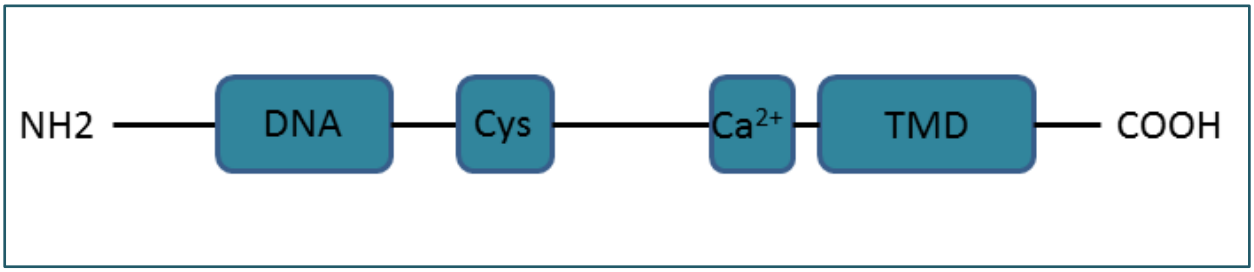

Fig14: Schematic representation of PLSCR1.The PLSCR1, present preset different domain: The DNA biding motif from amino acid 86 to 189 (DNA). Cysteine palmitoylation domain, comprising residues 184-189 (Cys), this motif regulates the trafficking either to nucleus or to the PM (Wiedmer et al. 2003) .The nuclear localization signal (NLS) (residues 257-266) (Sahu et al. 2007b). The Ca^{2+} biding site from amino acid 273 to 284 (Ca^{2+}) and finally PLSCR1 presents a putative transmembrane domain comprising residues 288-306 (TMD).

However, the oligomerization state and lipid composition of the plasma membrane can be as important as calcium for PLSCR1 activity regulation. Indeed, a recent work using human erythrocyte and proteoliposomes have shown that PLSCR1 activity can be triggered in the absence of $Ca2^{+}$ when cholesterol was depleted from membranes. Deletion mutant of PLSCR1 consisting of its transmembrane and EF-hand-like domain was sufficient to scramble PS in the absence of cholesterol. As the transmembrane domain is able to oligomerize, it has been proposed that the transmembrane domain could form a pore in the absence of cholesterol to conduct lipids across the membrane (Arashiki et al. 2016). Oligomer-dependent scramblase activity has also been proposed by Rayala et al. (2014). PLSCR2 has no prolin rich domain (PRD) and no scrambling activity. Adding the PRD of PLSCR1 to PLSCR2 restored scrambling activity and conversely, removing the PRD of PLSCR1 inhibited PLSCR1 scrambling activity. In the absence of PRD, Ca^{2+} canont trigger PLSCR oligomerization indicating that oligomerization is needed for PLSCR1 activity and in that case, the transmembrane domain was not sufficient for oligomerization and scrambling activity. Finally, PLSCR1-dependent scrambling activity can also be forced, in vitro, in the absence of Ca^{2+} if conformational constraint are applied by low pH for example (Francis and Gummadi 2015). Altogether, this data suggest that regulation of PLSCR1 is far more complicated that anticipated and that calcium is not the only regulator of PLSCR1 activity (Rayala et al. 2014).

In cells, the picture could be even more complex since palmitoylation of PLSCR1, which is essential for its trafficking, regulate also phospholipid scrambling activity (Zhao et al. 1998; Wiedmer et al. 2003). Palmitoylation may be important to change the conformation of PLSCR1 during its interaction with $Ca2^{+}$ ion at its EF-hand-like domain [D273–D284] (Wiedmer et al. 2003). This interaction is supposed to reorient a distal segment of the protein back towards the PM. This suggest that palmitoyl group participates in the anchoring of the N-terminal of PLSCR1 during this conformational change during phospholipid translocation (Zhou et al. 1998). Hydroxylamine, a compound that removes palmitoyl groups, reduced the Ca^{2+} dependent scrambling activity of PLSCR1 (Zhao et al. 1998). In addition, palmitoylation determine PLSCR1 localization into membrane raft domains enriched in sphingolipids, cholesterol and glycosylphosphatidyl inositol (GPI) in activated neutrophil (Brown and London 1998; Frasch et al. 2004). Palmitoylation could be therefore a way to localize PLSCR1 and scrambling activity in specific domains of the plasma membrane.

7.1.2.2 Molecular functions of PLSCR1

Analyses of human tissues show ubiquitous expression of PLSCR1, but the majority of the research about the physiologic role of its activity has been restricted to blood cells. For example in activated mast cells, PLSCR1 was found in the lipid rafts, where it associated with tyrosine kinase Lyn and Syk. Knocking down PLSCR1 expression reduces mast cell degranulation. PLSCR1 was reported to be an amplifier of FcεRI signaling that acts selectively on the Lyn-initiated LAT/phospholipase Cγ1/calcium axis, resulting in potentiation of a selected set of mast cell responses (Amir-Moazami et al. 2008). In T cells, PLSCR1 and PLSCR4 interact with CD4 receptor at the plasma membrane, this interaction is regulated by the presence of the secretory leukocyte protease inhibitor (SLPI), which has anti-viral activity against human immunodeficiency virus 1 (HIV-1)(Py et al. 2009). These data suggest a potential role of PLSCR1 in receptor-mediated signaling pathways and receptor internalization. In neutrophils, PLSCR1 was shown to interact with Proteinase 3 (PR3), which is a target of auto-antibodies in Wegener granulomatosis (Kantari et al. 2007). Additionally PLSCR1 is involved in the viral responses, in which it can both assist the viral infection and inhibit viral replication and propagation.

Interestingly, IFNα stimulates expression of PLSCR1 in order to protect cells from staphylococcal αtoxin produced during S. aureus infection. Knockdown of PLSCR1 by shRNA supports a protective role for PLSCR1 following αtoxin exposure (Kusano and Eizuru 2013; Lizak and Yarovinsky 2012).

In chromaffin cells, regulated exocytosis is accompanied by an externalization of PS and PS externalization depends on PLSCR1 activity. PS extermalization is not necessary for regulated exocytosis, but it is required for compensatory endocytosis (Ory et al. 2013).

Altogether, these data indicate that PLSCR1 and more generally PLSCR scramblase activity may alter membrane asymmetry by mixing phospholipids but also participates to complex signaling pathways. The link between membrane remodeling and cell signaling remained to be clarified.

II. Research aims of the thesis

Recent studies in neuroendocrine chromaffin cells have suggested that the secretory granule release is temporally and spatially coupled to a compensatory endocytic process (Ceridono et al. 2011; Ory et al. 2013). Hence, we hypothesized that the secretory granule membrane would preserve its integrity within the plasma membrane after exocytosis before being retrieved as such along with its components. However, the underlying molecular mechanisms of this compensatory endocytic process are largely unknown today. Therefore my thesis project is aimed at addressing the following specific question: What are the different mechanisms triggering, regulating and linking exocytosis and the compensatory endocytosis?

During my PhD, I focused on the role played by PLSCR1 and OPHN1 in the coupling between exocytosis and compensatory endocytosis in adrenal chrommaffin cells.

III. Result

1. PLSCR1 RESULTS

1.1 Research context

Phospholipid remodeling at the exocytotic sites: a key step for coupling calcium-regulated exocytosis with compensatory endocytosis!

Calcium-regulated exocytosis in neurons and chromaffin cells are composed by the same steps which include the recruitment of the secretory vesicle at the plasma membrane, the docking at the exocytotic sites and finally the fusion between the vesicle and the plasma membranes. The release of neurotransmitters, hormones or neuropeptides can occur through different mode including kiss-and-run, full fusion or cavicapture (see description in the introduction section). Independently of the mode of release, exocytosis is always followed by endocytosis. These two processes need to be connected in order to maintain the specific lipid and protein composition of each compartment (e.g. secretory vesicle and plasma membrane), to keep the cell surface constant and of course to allow secretory vesicle recycling. The complexity of this process lies in the spatial and temporal coordination between exocytosis and endocytosis, and obviously the identification of proteins and lipids involved in the balance between these two events is of primary interest.

Studies from our laboratory and other laboratories have suggested that full fusion of secretory granule is temporally and spatially coupled to a compensatory endocytic process in neuroendocrine chromaffin cells in which the granule and plasma membranes seem to maintain their specific protein composition (Ceridono et al. 2011; Bittner, Aikman, and Holz 2013). This implies that secretory granule membrane would preserve its integrity within the plasma membrane (PM) after exocytosis before being retrieved as such, along with its components. If this is true, then the vesicle membrane should remain hermetically sealed without intermixing with the PM lipid bilayer after exocytosis. Altogether, these results raise an important unsolved question: by which mechanisms the vesicle membrane lipids and proteins are maintained together, segregated and sorted-out from the PM during and after exocytosis?

The physical properties of lipids may play fundamental role by acting as scaffolding system to maintain specific machinery at restricted site of the plasma membrane.

When I started my Ph.D, our group was just revealing a new important feature of the plasma membrane lipids in the regulation of compensatory endocytosis. It was found that phosphatidylserine redistributes from the inner layer to the outer layer of the plasma membrane, hence resulting in a loss of phospholipid asymmetry during hormone release in chromaffin cells. While investigating the underlying mechanisms Stéphane Ory and his collaborators demonstrated that the phospholipid scramblase 1 (PLSCR1) controls this process suggesting that most likely bi-directional transport of various phospholipids should occur (Ory et al., 2013). It then appeared that PLSCR1 activity was not required for secretory granule exocytosis, but was pivotal for efficient secretory granule recapture after exocytosis suggesting that PLSCR1-dependent PS egress could contribute to couple exocytosis to compensatory endocytosis in chromaffin cells. Two important questions were raised from these results: how is the activity of PLSCR1 triggered and regulated during exocytosis and why compensatory endocytosis requires the PLSCR1 induced phospholipid redistribution? These two questions became one of the two main issues of my PhD.

While investigating the relationship between PLSCR1 activity, exocytosis and compensatory endocytosis, I observed that secretagogue-evoked PS egress was impaired in chromaffin cells when exocytosis is prevented through cleavage of the SNARE proteins by neurotoxins treatment. These results suggested me that intracellular calcium increase might not be sufficient enough to activate PLSCR1 and/or that PS egress is dependent of the SNARE complex formation. Pull down assays with recombinant PLSCR1 together with co-immunoprecipitation experiments and mass spectroscopy analysis allowed us to identify syntaxin 1A (STX1A) as a potential binding partner for PLSCR1. Interestingly, knocking down STX1A expression by siRNA approach triggered PS egress in resting condition. These data suggest that STX1A might be a regulator of PLSCR1 activity during exocytosis.

In the following parts of this manuscript, I'll describe the main results I have obtained as a first tentative article. Then I'll discuss in more detail these data and propose some conceptual ideas along with additional preliminary data.

Syntaxin-1 regulates phospholipid scramblase-1-induced lipid reorganization during neuroendocrine secretion

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Transient disruption of plasma membrane asymmetry occurs during hormone release in neuroendocrine cells. Indeed, we have previously shown that calcium-regulated exocytosis in chromaffin cells is accompanied by the activation of the phospholipid scramblase-1 (PLSCR1) leading to the redistribution of phosphatidylserine (PS) to the extracellular space. How PLSCR1 activity is regulated during the exocytic process is currently unknown. We found here that both tetanus and botulinum type C neurotoxins significantly inhibit secretagogue-evoked PS egress in bovine chromaffin cells and rat PC12 cells suggesting that PLSCR1 activity is dependent of the SNARE complex formation. Pull down assays and immunoprecipitation experiments coupled to mass spectrometry analysis indentify syntaxin-1A (STX1A) as a binding partner of PLSCR-1. Moreover, down-regulation of STX1A expression by siRNA highly enhanced PS egress suggesting that STX1A is a negative regulator of PLSCR1 activity. These findings reveal for the first time that, through the regulation of PLSCR1 activity, STX1A contribute to the transient remodeling of membrane phospholipids required for the exocytic process in neuroendocrine cells.

Introduction

The secretion of neurotransmitters and hormones from neurons and neuroendocrine cells occurs through calcium-regulated exocytosis, a multistep process that terminates by fusion of secretory vesicle with the plasma membrane through the assembly of the soluble NSF-attachment protein receptors (SNARE) complex. To maintain membrane homeostasis and to ensure secretory vesicle recycling, fusion is followed by a compensatory endocytosis process. Lately, we have focused our work on the molecular mechanisms controlling compensatory endocytosis and its spatial and temporal coupling with exocytosis in neuroendocrine cells (Ceridono et al., 2011; Ory et al., 2013; Houy et al., 2015). In particular, we have recently illustrated the importance of phospholipid distribution between plasma membrane leaflets (Ory et al., 2013). Indeed, dynamic changes of cell membranes lipid

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distribution and asymmetry play an essential role in many aspects of membrane trafficking processes including calcium-regulated secretion (Ikeda et al., 2006).

For example, plasma membrane asymmetry disruption has been observed during calcium-regulated secretion in various cellular models including mast cells (Demo et al., 1999), PC12 cells (Vitale et al., 2001; Malacombe et al., 2006), nerve terminals (Lee et al., 2000). Our previous work performed in chromaffin cells confirmed these data by demonstrating that secretagogue-evoked exocytosis is accompanied by cell surface PS exposure at the close vicinity of the granule membrane transiently inserted into the plasma membrane. Moreover, we have demonstrated that this secretagogue-induced PS externalization requires the phospholipid scramblase-1 (PLSCR-1) activity and that the subsequent cell surface exposure of PS play no role in exocytosis but is rather required for efficient granule membrane compensatory endocytosis (Ory et al., 2013). Combined to the fact that secretory granules membrane and proteins remained clustered after full fusion exocytosis (Ceridono et al., 2011; Bittner et al., 2013), it suggest that PS egress constitutes a signal for granule membrane retrieval and might be an efficient way to synchronize compensatory endocytosis to regulated-exocytosis in neuroendocrine cells. However, the underlying mechanisms remain currently unknown. The aim of the present work is to provide further evidences about the mechanisms regulating PLSCR1 activity during hormone release in neuroendocrine cells.

Materials and Methods

DNA constructs and recombinant protein production. The GFP-tagged mouse scramblase1 constructs (PLSCR-1 WT or D284A) were previously described (Zhao et al., 1998; Ory et al., 2013). The mCherry-tagged Syntaxin1A (mCherry-Syx1A) was a kind gift from Dr R. Duncan (Heriot Watt University, Edingurgh, UK).

N-terminally His tagged constructs were generated by PCR amplification of WT PLSCR1 and Syx1A using forward (5'-CAGATCTGAAAACCACAGCAAGGAAC-3' and 5'-ATATACTCGAGAAGGACCGAACCCAGGAGC-3' respectively) and reverse (5'-GGATTCTTACTGCCATGCTCCTGATC-3' and 5'-ACGCGGCCGCCTATCCAAAGATGCCCCCGATGG-3' respectively) primers. Purified PCR fragments were digested and inserted into pET-28b (Merck Millipore, Molsheim, France) vectors using BglII/EcoRI and XhoI/NotI restriction enzymes respectively. Generated constructs were verified by sequencing.

The transmembrane domain Syx1A (TMD-Syx1A) was amplified by PCR using forward (5'-ATATACTCGAGGCCGTGGACTACGTGGAGC-3') and reverse primers

(5'-ACGCGGCCGCCTATCCAAAGATGCCCCCGATGG-3') and inserted between XhoI and Not1restriction sites of pGEX-4T1 (GE Healthcare, Velizy-Villacoublay, France)

Recombinant proteins were produced in *Escherichia coli* BL21 cells. For the production of His-tagged proteins, cells were cultured in M9 minimal medium (Sigma) supplemented with 20 mM glucose, 2 mM MgSO4 4 mg/ml biotin, 4 mg/ml thiamine, 42 mg of each amino acids and 30 µg/ml kanamycine. Protein expression was induced by 1 mM isopropylthiogalactoside (IPTG) for 3 hours at room temperature. Cells were pelleted and resuspended in lysis buffer (10 mM imidazole pH 7.4 in phosphate-buffered saline (PBS), 300 µg/ml lysozyme, 1 unit/ml DNase I (Thermo Scientific) and protease inhibitor cocktail (Sigma)). The suspension were then centrifuged for 30 min at 20 000 g at 4°C and HisPur Ni-NTA beads (HisPur^{TM} Ni-NTA Resin, Thermo Scientific) were added and incubated for 1 hour at 4°C. Beads were gently pelleted and washed 4 times with washing buffer (300 mM NaCl, 25 mM imidazole pH 7.4 and protease inhibitor in PBS). The amount of proteins bound to beads was estimated using Coomassie stained SDS gel.
The same protocol was used for GST fusion proteins except that lysis buffer was composed of 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 300 µg/ml lysozyme, 1 unit/ml DNase I and protease inhibitor cocktail and proteins incubated with glutathion sepharose beads (GE Healthcare) were extensively washed with 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA.

Cell culture and transfection. Bovine chromaffin cells were cultured as previously described (Gasman et al., 1997). Mammalian expression vectors (3µg) were transfected in the chromaffin cells ($5x10^6$ cells) by Nucleofactor systems (Amaxa Biosystems) according to the manufacturer instructions. Cells were seeded on fibronectin coated coverslips and cultured for 48 h before the experiments. PC12 cells were cultured as previously described (Gasman et al., 2004). 24 hours prior transfection, 5×10^4 cells/cm^2 were seeded on glass coverslips and mammalian expression vectors (3µg) transfected using Lipofectamine (Invitrogen) according to the manufacturer instructions.

Syx1A silencing was achieved by transfecting PC12 cells with Lipofectamine RNAiMax (Invitrogen) and 100 nM of a mix of 4 siRNAs (On Target Plus Smart Pool siRNA, Dharmacon: 5'-GCAAGGAGUAUGCAUGCGA-3', 5'-GACAUUAAGAAGACAGCGA-3', 5'-CACCAAAGGUCUCGGUACA-3', 5'-ACACCAAGAAGGCCGUCAA-3') according to the manufacturer instructions. Cells were cultured for 72 h before the experiments, and Syx1A silencing was estimated and normalized to actin contents by Western blotting.

Pull down assay and co-immunoprecipitation experiments. 10^7 bovine chromaffin or PC12 cells transfected or not with GFP-PLSCR1 were lysed in protein extraction buffer (10 mM Tris HCl ph 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) and centrifuged for 10 min at 20 000 g at 4°C. The concentration of proteins in cell lysate was adjusted to 1 mg/ml protein and 5 µg of recombinant protein linked to beads were added to 500 µl of lysate, incubated for 3 h at 4°C under gentle rotation. When specified, calcium concentration was increased to 5 mM. Beads were then pelleted at 800 g for 5 min and washed four times with 300 mM NaCl, 25 mM imidazole pH 7.4 and protease inhibitor in PBS (His-tagged protein pull-down) or 25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA and protease inhibitors (GST fusion protein pull down). Beads and proteins associated to fusion proteins were eluted using Laemmli sample buffer, spin down using spin cups with paper filter (ThermoFischer scientific). Eluates were analysed by western blotting.
Immunoprecipitation of PLSCR-1 GFP expressed in PC12 cells was performed using the GFP Trap A system (Chrometek) according to the manufacture instructions. Polyclonal anti-GFP (TP401, Clinisciences) or monoclonal anti-Syx1 antibodies (HPC1, Sigma) was used to detect GFP-PLSCR1 or endogenous Syx1A

Mass spectrometry analysis. Adrenal glands from 8 weeks old mice were dissected and cleaned in filtered Locke's solution. The glands were gently opened with tweezers, and medulla was freed from fat and cortex under microscope. Medulla were dissociated and protein extracted in protein extraction buffer for 30 min on ice. Tissue debris were removed by centrifugation (15 min at 20 000 g at 4°C) and lysate incubated for 1 h with Ni-NTA resin for 1 h at 4°C to eliminate protein for unspecific binding. Recombinant His-tagged PLSCR1 bound to resin was then added and recipitated proteins were subjected to SDS-PAGE analysis. Bands from the entire lane were excised, submitted to tryptic digestion, extracted and directly analysed by nano liquid chromatography tandem mass spectrometry (nanoLC-MS/MS). NanoLC-MS/MS was performed using a nanoACQUITY ultra performance liquid chromatography (UPLC®) system (Waters, Milford, MA) coupled to a maXis 4G Q-TOF mass spectrometer (BrukerDaltonics, Bremen, Germany). The system was fully controlled by HyStar 3.2 (BrukerDaltonics). The UPLC system was equipped with a Symmetry C18 precolumn (20×0.18 mm, 5-µm particle size; Waters, Milford, MA) and an ACQUITY UPLC® BEH130 C18 separation column (75 µm×250 mm, 1.7-µm particle size; Waters, Milford, MA). Peak lists in mascot generic format (.mgf) were generated using Data Analysis (version 4.0; Bruker Daltonics, Bremen, Germany).

Induction of apoptosis by staurosporine. To induce apoptosis, PC12 cells were incubated for 4 hours at 37°C in OptiMEM medium (Gibco) containing 2 mM $CaCl_2$ and 1 µM staurosporine (Sigma). The cells were harvested, incubated in extraction buffer for 30 min

at 4°C. Cell extracts were centrifuged at 20 000 g for 10 min at 4°C and protein concentration determined by Bradford assay. 25 µg of proteins were loaded, resolved by SDS-PAGE and processed for western blotting. A polyclonal antibody was used to detect active caspase-3 (AB3623, Millipore).

Immunofluorescence, confocal microscopy, and image analysis. The detection of exocytotic sites using anti-dopamine-β-hydroxylase (DBH) antibodies and PS egress using fluorescent AnnexinV were performed as described previously (Ceridono et al. 2011; Ory et al., 2013). Staining was observed by confocal microscopy (SP5, Leica Microsystems) using a 63x objective (NA 1.40). Images analyses were performed using ImageJ (http://rsbweb.nih.gov/ij/) or Icy freeware (http://icy.bioimageanalysis.org/).

Results

Aborting SNARE complex formation inhibits secretagogue-evoked PS egress in chromaffin cells

We previously reported that plasma membrane asymmetry was disrupted during exocytosis. Although we showed that PLSCR1 was responsible for PS egress at the close vicinity of LDCV fusion during Ca^{2+}-dependent exocytosis in chromaffin cells (Ory et al. 2013), we did not address the importance of LDCV fusion into loss of plasma membrane asymmetry. To do so, we monitored PS egress in bovine chromaffin cells or PC12 cells transfected with plasmid encoding for tetanus toxin (TeNT) or for botulinum neurotoxin type C (BoNT/C), two neurotoxins acting as an endopeptidase on SNARE proteins (Fig.1).
TeNT cleaves vesicle-associated membrane proteins (VAMP) family whereas BoNT/C cleaves syntaxins and SNAPs proteins. Both toxins prevent SNARE complex assembly and LDCV fusion in chromaffin cells (for review see (Humeau et al., 2000). To identify transfected cells, the catalytic domain of the toxins was inserted into a bidirectional vector allowing for GFP or mCherry expression on one side and for the toxin expression on the other side.

We first assessed whether exocytosis was indeed impaired in bovine chromaffin cells expressing TeNT or BoNT/C (Fig.1A). We took advantage of the transient exposure to the extracellular space of the dopamine-β-hydroxylase (DBH) when bovine chromaffin cells undergo exocytosis upon stimulation by a depolarizing K^+ concentration. The DBH is located on the membrane of LDCV and can be detected by incubating living cells with an antibody directed against the luminal domain of the DBH (Ceridono et al. 2011; Ory et al. 2013). Chromaffin cells expressing TeNT or BoNT/C showed a drastic decrease in DBH staining compared to untransfected cells or to cells transfected with empty vector indicating that regulated exocytosis was efficiently blocked by toxin expression (Fig.1A). We next tested for the appearance of PS at the cell surface by incubating living cells with fluorescent AnnexinV (Ory et al. 2013). Fig1B shows that both TeNT and BoNT/C expression drastically reduced AnnexinV binding to cells in response to K^+-evoked stimulation in bovine chromaffin cells and PC12 cells. No AnnexinV staining was seen in resting conditions (not shown). These results indicate that PS egress needs either LDCV fusion or the formation of an intact SNARE complex. Alternatively, toxins expression might alter PLSCR1 localization and prevent phospholipids scrambling at the plasma membrane. To check PLSCR1 localization, cells were co-transfected with plasmid encoding for PLSCR1 fused to GFP and TeNT or BoNT/C fused to mCherry (Fig.2). As previously reported, PLSCR1 localizes at the plasma membrane and on intracellular vesicles in both bovine chromaffin and PC12 cells (Ory et al. 2013). No significant changes in PLSCR1 distribution

were observed upon TeNT or BoNT/C expression indicating that the absence of PS egress in response to stimulation cannot be explained by PLSCR1 mislocalization (graphs in Fig.2).

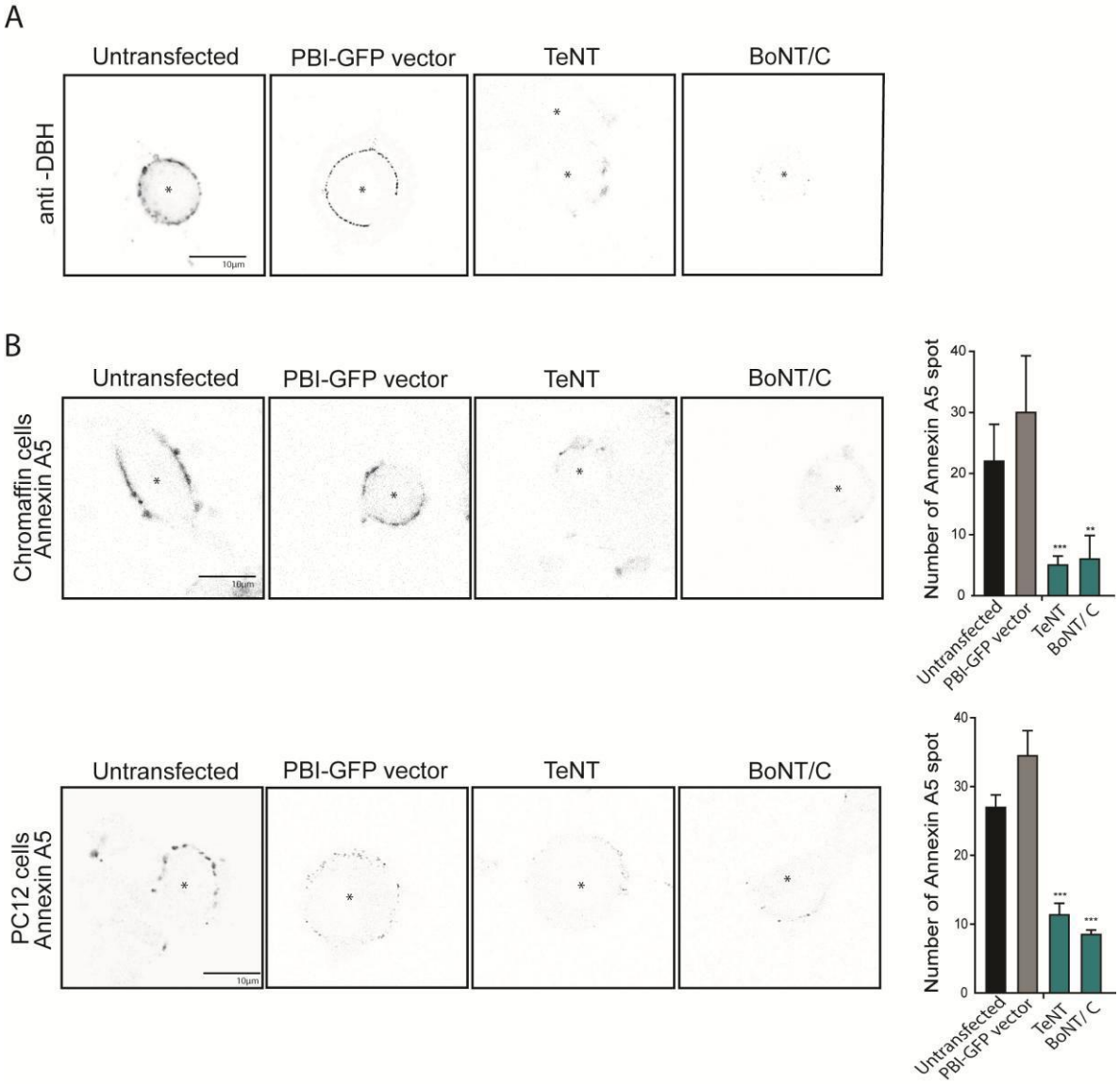

Figure 1. Tetanus toxin and Botulinum toxin type C inhibit PS egress in chromaffin and PC12 cells. Chromaffin cells and PC12 cells expressing TeNT or BoNT/C were stimulated with 59 mM K^+ and incubated for 20 min at 4°C with rabbit anti-DBH antibodies to detect exocytotic sites (A) or incubated for 10 min at 37°C with AnnexinA5 conjugated with alexa-647 to reveal PS at cell surface. Cells were then fixed and DBH was revealed with the Alexa-555-conjugated anti-rabbit antibodies. The graphs show the number of AnnexinA5 spots (n=30 cells), ***$p < 0.001$, **, $p < 0.005$.

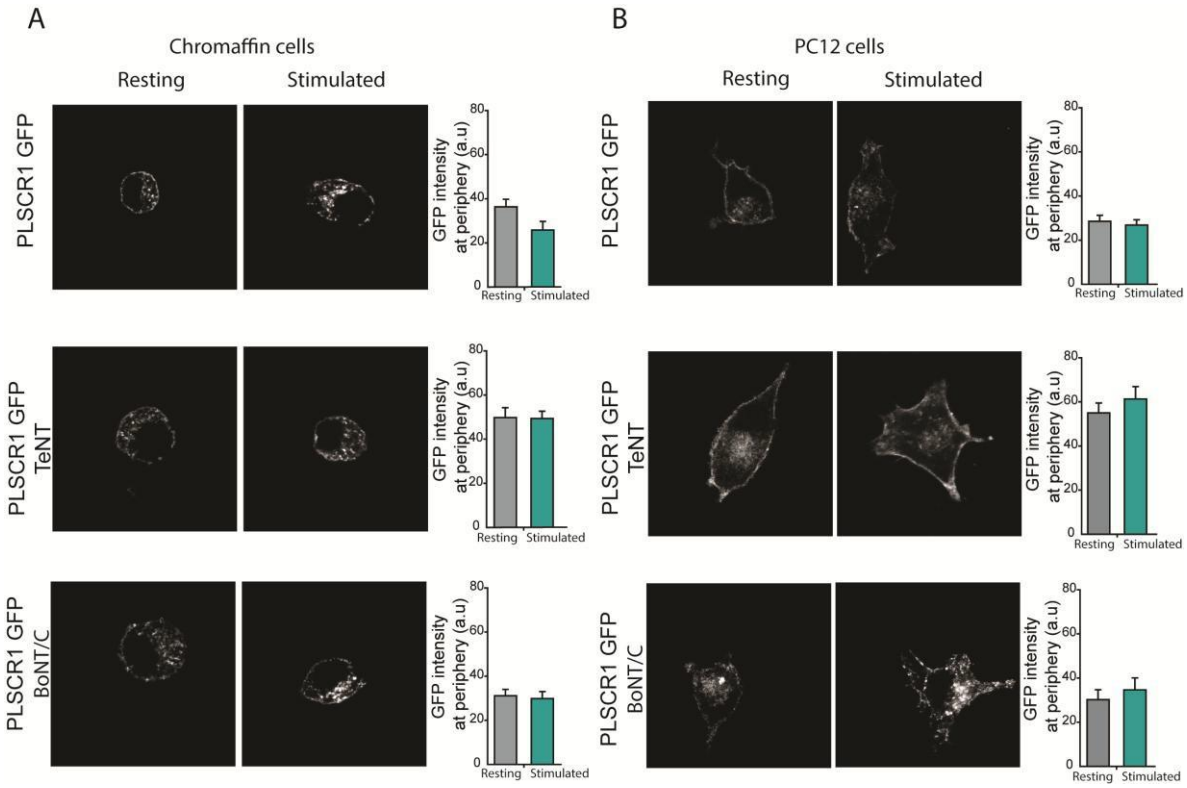

Figure 2. Effet of TeNT and BoNT/C on the localization of exogenously expressed PLSCR1. Construct coding for the GFP-tagged PLSCR1 was co-tranfected in chromaffin cells (A) or PC12 cells (B) with empty vector or vector coding either for tetanus (TeNT) or botulimum type-C (BoNT/C) neurotoxins. 48h after transfection, the cells were maintained in Locke's solution for resting conditions or stimulated for 1min with 59 mM K^+. Cells were then fixed and GFP was observed by confocal microscopy. The graph shows the mean fluorescence intensity of PLSCR1 found in the periphery of the cells (n=20 cells).

PLSCR1 and Syntaxin1A form a complex

Toxin expression has no known effect on Ca^{2+} entry in response to membrane depolarization (Sakaba et al., 2005). Therefore, the toxins-induced inhibition of PS egress indicates that PLSCR1 remains inactive despite intracellular calcium increase upon cell stimulation. This observation suggests that Ca^{2+} by itself is not sufficient to activate PLSCR1 and that a potential inhibition of PLSCR1 should be relieved to allow phospholipid scrambling. We therefore sought for potential PLSCR1 interacting proteins. To do so, we synthesized and immobilized recombinant His-tagged PLSCR1 (His-PLSCR1) on agarose beads and performed pull-down assay experiments using lysates from adrenal medulla tissue. Identification of the proteins pull-downed with His-PLSCR1-enriched beads was carried out by MS/MS analysis. Interestingly, numerous proteins belonging to the SNARE complex were co-purified with PLSCR1 in particular several members of syntaxins (Table 1). Among these proteins, we decided to focus on the isoform 1A of syntaxin involved in calcium-regulated exocytosis in neurons and neuroendocrine cells (Jahn and Fasshauer, 2012).

First, we checked by western blot for the presence of syntaxin-1A (STX1A) in the precipitate from His-PLSCR1 pull-down assays performed with chromaffin cell lysates (Fig. 3A). STX1A co-precipitated with His-PLSCR1 confirming that PLSCR1 and STX1A belong to the same complex in chromaffin cells. Increasing the calcium concentration in the pull-down buffer seems to lower the amount of STX1A precipitated with His-PLSCR1 suggesting a potential role of calcium in this interaction (Fig. 3A). To test this hypothesis, we determined if PLSCR1-STX1A association occurs in cells and whether it is changed by cell stimulation. To do so, we transfected PC12 cells with GFP-PLSCR1 and performed immunoprecipitation using anti-GFP antibodies on lysate from cells maintained in resting condition or stimulated for 10 s with 59 mM K^+ solution. As illustrated in figure 3B, a portion of endogenous STX1A co-precipitated with overexpressed PLSCR1 strengthening the idea that PLSCR1 and STX1 interact directly or indirectly. Even though the amount of STX1A precipitated with PLSCR1 seems to be slightly reduced in response to stimulation, the low level of STX1 immuno-precipitated does not allow us to conclude if the rise of Ca^{2+} modulates this complex formation. Further experiments and quantification are now required.

Short name	Protein identification probability %	Percentage sequence coverage %	Uniprot link	Number of unique peptides
SNAP23	99	11	009044	2
SyntaxinII	99	7	Q9D3G5	2
Syntaxin16	100	8	Q8BVI5	2
Syntaxin18	100	7	Q8VDS8	2
SyntaxinIA	100	11	035526	3
Syntaxin4	100	12	P70452	3
Syntaxin8	100	15	088983	3
Synaptotagmin5	100	8	Q9RON5	1
VAMP7	100	22	P70280	6

Table1: Mass spectrometry analysis

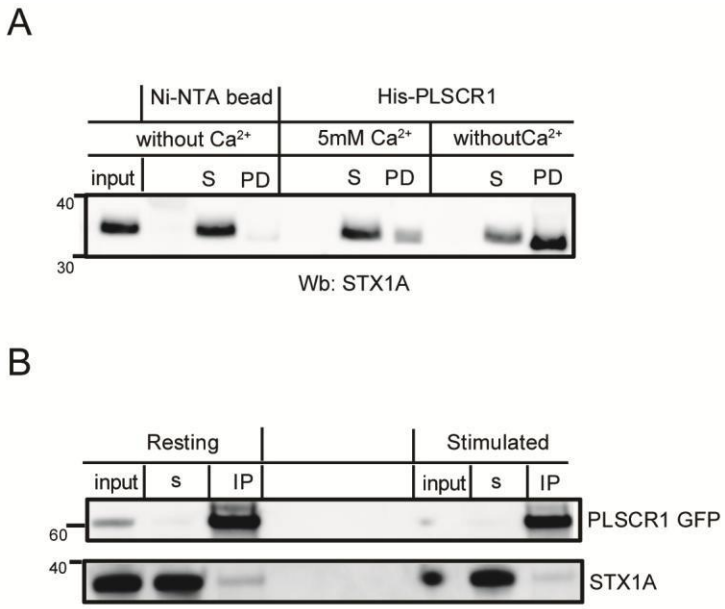

Figure 3. Syntaxin1A from chromaffin cell lysate and PC12 cells lysate precipitates with recombinant PLSCR1 and exogenously express PLSCR1, respectively. (A) Pull-down assay experiments was performed by incubating recombinant His-tagged PLSCR1 (His-PLSCR1) with chromaffin cell lysates. STX1A was revealed by western blot. S corresponds to the supernatant fraction and PD corresponds to the pulldown fraction. (B) Immunoprecipitation assay with GFP-Trap A system (Chrometek) was performed with lysates of PC12 cells expressing PLSCR1-GFP. PLSCR1-GFP (anti-GFP antibodies) and endogenous STX1A were detected by western blot. S corresponds to the supernatant fraction and IP corresponds to the precipitate fraction.

STX-1A knock down triggers PLSCR1-dependent PS egress

The next question we address is whether STX1A is able to modulate PLSCR1 activity. To test this hypothesis, we knocked-down STX1A by siRNA in PC12 cells and analyzed PS exposure to the cell surface by AnnexinV staining. As revealed by western blot analysis, STX1A siRNA consistently exhibited a significant reduction in the level of endogenous STX1A expression (Fig. 4A). Knocking down STX1A resulted in a drastic increase in AnnexinV staining even in resting condition (Fig. 4B). Moreover, no significant additional increase was observed in response to stimulation when STX1A expression was reduced. These observations indicate that preventing STX1A expression enhances PLSCR1 activity and favors PS egress even in absence of calcium increase. To strengthen the inhibitory role of STX1A on PLSCR1 activity, we performed rescue experiments by expressing mouse mCherry-tagged STX1A which is resistant to the siRNAs degradation (due to several mismatches with the rat sequence) as revealed by western blot analysis (Fig. 4C). Co-expression of the resistant mCherry-tagged STX1A with rat STX1 siRNAs in PC12 cells restored PS egress to control levels (Fig.4D) indicating that STX1A is needed to maintain homeostasis of plasma membrane phospholipids. Because PS egress is also a hallmark of cell death by apoptosis, we controlled whether STX1A silencing could activate caspase-3, a predominant caspase in the apoptotic pathway. As a positive control, PC12 cells were treated with staurosporine, an efficient inductor of apoptosis (Olguin-Albuerne et al., 2014). As expected, staurosporine treatment efficiently activated caspase3 in PC12 cells (Fig.5). In contrast, no active caspase 3 was detected in control or in PC12 cells transfected with STX1A siRNA (Fig. 5). Therefore, PS egress in PC12 cells knocked-down for STX1A cannot be attributed to apoptosis.

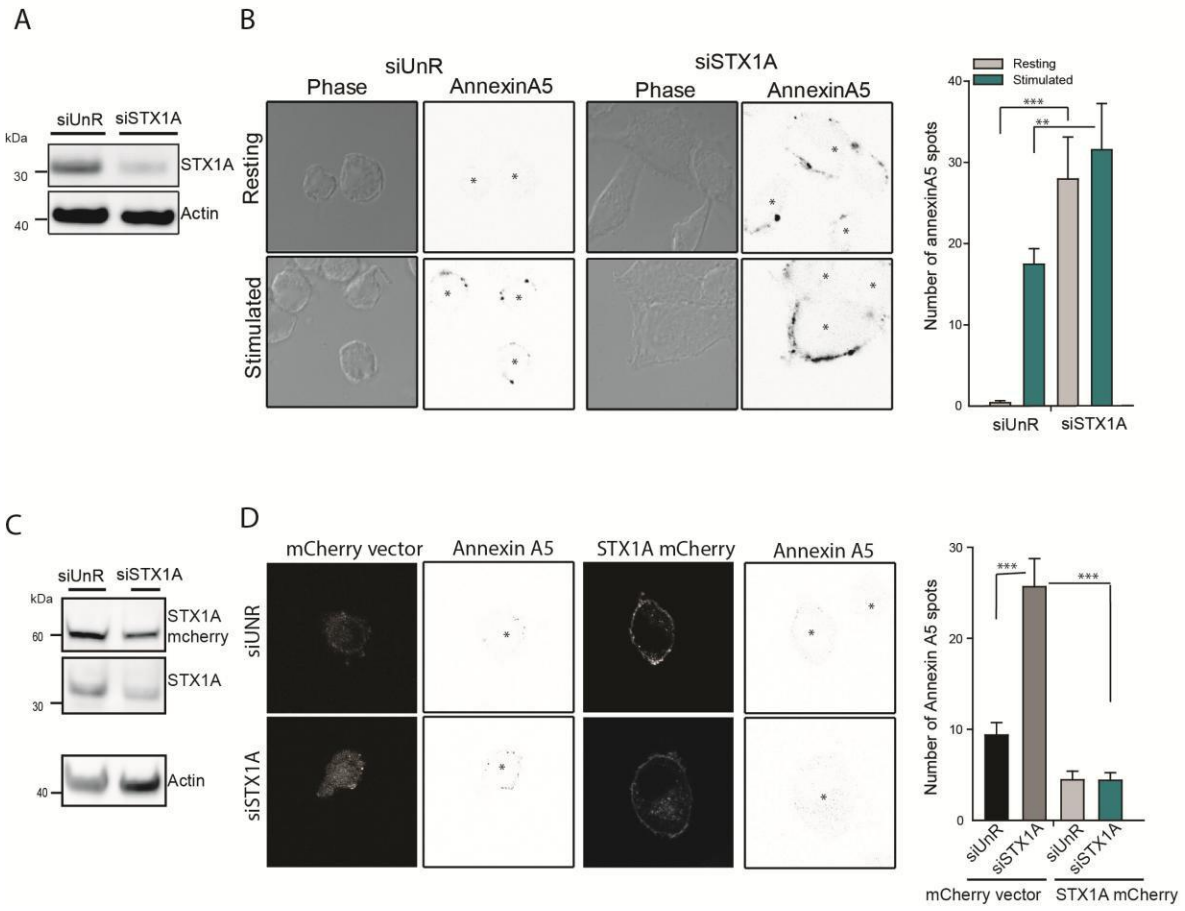

Figure 4. STX1A knock-down triggers PS egress in resting PC12 cells. PC12 cells were transfected with unrelated siRNA (siUnR) or with syntaxin1A siRNA (siSTX1). (A) Cells were lysed 72 h after transfection and processed for western blot analysis using antibodies against STX1 (A) or incubated for 10 min at 37°C with AnnexinA5 conjugated with alexa-647 to reveal PS egress at cell surface in both resting and stimulated conditions (B). Note that knocking-down expression of STX1A significantly stimulates cell surface exposure of PS in resting cells (graph in B, n= 25 cells; **p < 0.005,***p < 0.001). (C-D) Re-expression of STX1A restore normal level of cell surface PS. PC12 cells expressing siUnR or siSyx1 are transfected with a construct coding Syx1a-mcherry which is resistant to the siRNA as shown by western blot analysis (C). The level of cell surface PS was then observed by confocal microscopy and quantified (images and graph in D, n= 30 cells; ***p < 0.001).

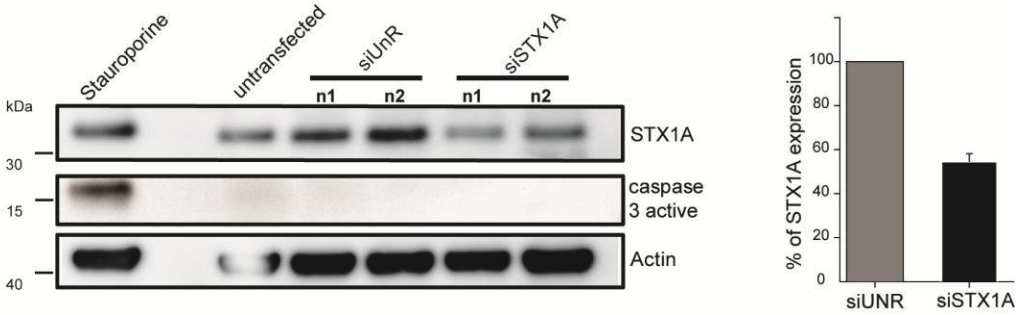

Figure 5. STX1A knock-down does not trigger apoptosis. 72h after transfection of PC12 cells with siUnR or siSTX1A, caspase3 activity and knocking-down efficiency were addressed by western blot analysis using anti-STX1A and anti-caspase 3 active antibodies. Staurosporine treatment (1 µM, 4h) was used as positive control. The level of STX1 expression is quantified (graph).

To determine whether the enhanced PS egress was dependent on PLSCR1 in cells lacking STX1A, we co-expressed the Ca^{2+}-inactive PLSCR1 mutant fused to GFP ($PLSCR1_{D284A}$) with STX1A siRNA. $PLSCR1_{D284A}$ is mutated in the Ca^{2+} binding domain of PLSCR1 and acts as a dominant negative (Ory et al., 2013). As reported previously for bovine chromaffin cells, the expression of $PLSCR1_{D284A}$ in PC12 cells stimulated by a depolarizing concentration of K^+ drastically reduced AnnexinV staining compared to cells transfected with WT PLSCR1. Conversely, PC12 cells silenced for STX1A showed a drastic increase in AnnexinV staining which was unaffected by stimulation when cells expressed WT PLSCR1. Expression of $PLSCR1_{D284A}$ mutant reduced by half the amount of AnnexinV staining when STX1A expression was abolished and no additional increase in PS was observed following cell stimulation (Fig.6). These data suggest that, at least a proportion of the PS exposed to the outer leaflet of the plasma membrane depends on PLSCR1 activity.

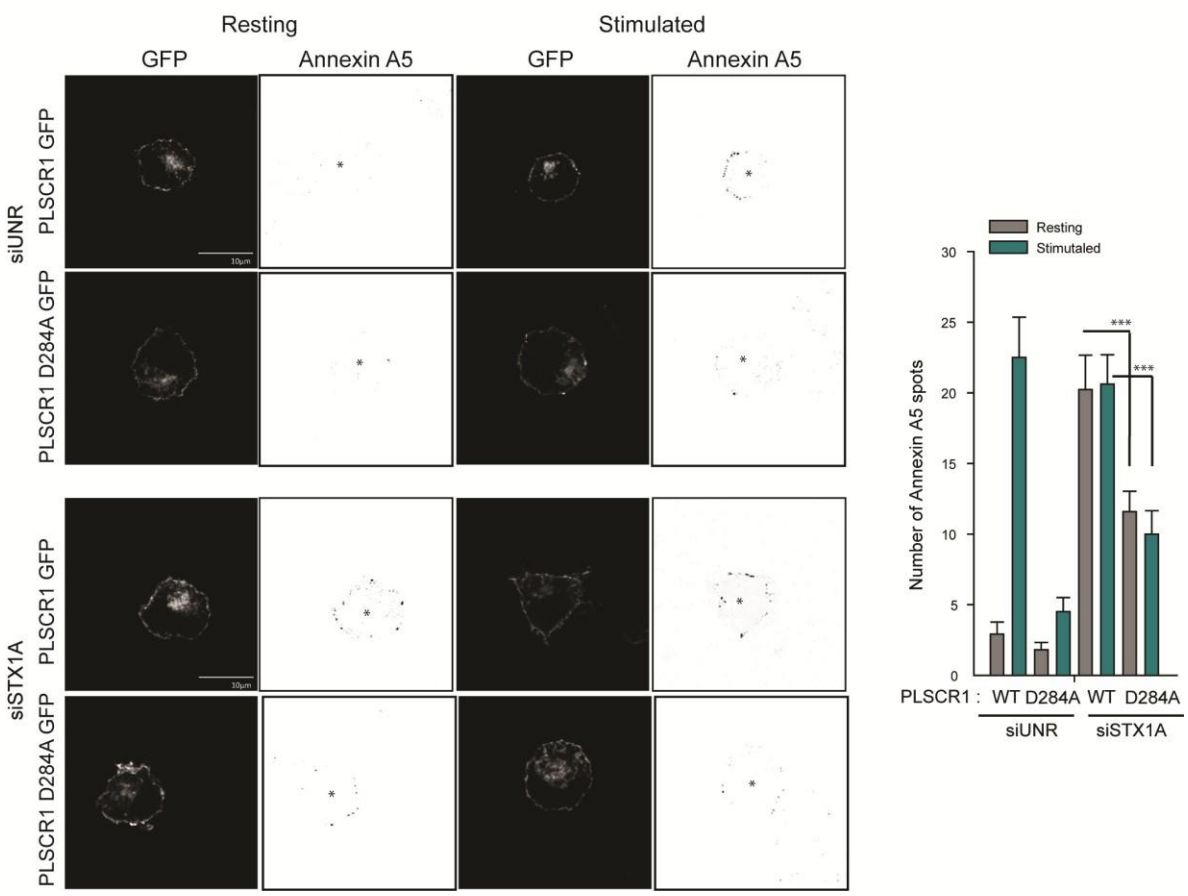

Figure 6. PS egress depends on PLSCR1 in STX1A knock-down cells. PC12 cells expressing siUnR or siSTX1 are transfected with a construct coding either PLSCR1-GFP (WT) or $PLSCR1_{D284A}$-GFP (D284A). Cell surface PS staining was then monitored with fluorescent annexin-A5 in resting and stimulated (59mM of K^+, 10 min) cells. AnnexinA5 spots were quantified (graph; n=30 cells; ***$p < 0.001$).

The transmembrane domain of STX1A interacts with PLSCR1

PS egress is inhibited in cells in which STX1 is cleaved by BoNT/C whereas it is largely enhanced in cells silenced for STX1. How to explain this apparent discrepancy? One possible explanation is that the region of STX1 left at the plasma membrane after toxin cleavage might be sufficient to interact with PLSCR1 and prevent PS egress. Indeed, proteolytic cleavage of STX1A by BoNT/C occurs 12 amino acids before its transmembrane domain (TMD; Fig. 7A). To probe this hypothesis, we generated a GST-fused truncated mutant of STX1A. This mutant contains the TMD and the small part of the extra-membrane domain that is uncleaved by the toxin (GST-TMD-STX1A; Fig. 7A). Pulldown experiment with GST-TMD-STX1A was performed using lysates from PC12 cells expressing GFP-PLSCR1. Figure 7B shows that GST-TMD-STX1A was able to precipitate GFP-PLSCR1 indicating that TMD is required and sufficient for PLSCR1-STX1A complex formation.

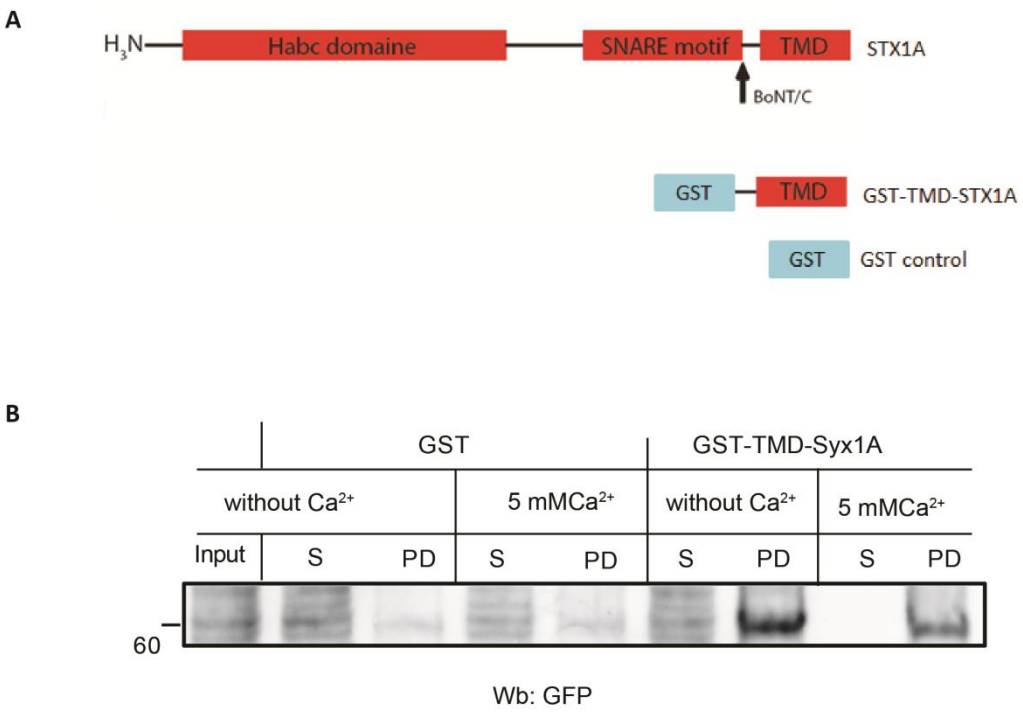

Figure 7. The transmembrane domain of STX1A is required for the interaction with PLSCR1. (A) Schematic representation of STX1A depicting: Habc domain, SNARE motif, transmembrane domain (TMD) and the position of the BoNT/C clivage site. The GST-tagged construct (GST-TMD-STX1A) and the GST construct (GSTcontrol) used are illustrated. (B) Pull-down assay was performed by incubating recombinant GST-TMD-STX1A with PC12 cell lysate expressing PLSCR-1GFP. Anti-GFP antibodies were used to detect PLSCR1-GFP by western blot. S and PD correspond to the supernatant and to the pulldown fractions, respectively.

Discussion

Dynamics of membrane lipids are crucial for a wide variety of membrane trafficking functions. One particular feature of cell membranes is the asymmetric distribution of phospholipid between the leaflets, a notion that has been discovered more than 40 years ago (Bretscher, 1972). The best known example is likely the plasma membrane in which phosphatidylserine (PS) and phosphoethanolamine are enriched in the leaflet facing the cytoplasm whereas phosphatidylcholine and sphyngomyeline are both enriched in the outer leaflet exposed to the extracellular space. Such asymmetry is maintained thanks to active and energy-dependent lipid transports controlled by transmembrane proteins such as P-type ATPases (for comprehensive reviews see (Ikeda et al., 2006; Leventis and Grinstein, 2010; Bevers and Williamson, 2016). However, asymmetrical distribution of plasma membrane phospholipids can be permanently or temporarily disrupted in response to a signal. This is the case for example during apoptosis in which permanent exposure of PS to the outer leaflet of the plasma membrane constitute a recognition signal for clearance of dying cells. Interestingly, such loss of asymmetry has also been observed on a reversible and shorter time scale during Ca^{2+}-regulated exocytosis in neuroendocrine chromaffin cells and in neurons (Lee et al., 2000; Malacombe et al., 2006; Ceridono et al., 2011). Our previous work shown that, in chromaffin cells, full fusion of secretory granules in response to a secretagogue is accompanied by the exposure of PS at the rim of the granule membrane newly inserted into the plasma membrane and that PS egress was dependent on the phospholipid scramblase 1 (PLSCR1) (Ory et al., 2013). Intriguingly, the absence of PLSCR1 or the loss of PLSCR1 activity had no effect on exocytosis but inhibited subsequent membrane and protein retrieval by compensatory endocytosis, suggesting that PS egress constitute a signal for secretory granules components retrieval (Ory et al., 2013). However, the mechanistic details regulating phospholipids scrambling and its downstream molecular cascades to selectively retrieve secretory granules membranes in neuroendocrine cells remain uninvestigated questions. Using chromaffin and PC12 cells, we show here that (1) PS egress during exocytosis requires a functional SNARE complex formation; (2) PLSCR1 and STX1A belong to the same complex; (3) the interaction between PLSCR1 and STX1A is mediated by the transmembrane domain of STX1A; and (4) the interaction between PLSCR1 and STX1A inhibits PLSCR1 activity and the subsequent PS egress. To our knowledge, this is the first study proposing a functional interaction between PLSCR1 and members of the SNARE complex as well as a role of STX1A in the regulation of the plasma membrane phospholipids homeostasis.

The fact that the transmembrane domain of STX1A was involved in the interaction with PLSCR1 let us to suppose that this interaction might be direct and would occurs within the bilayer. However, further experiments are required to prove it. What is the functional role of this interaction remains also a key question. The large increase of PS egress observed

upon STX1A knocking down suggest that STX1A act as a negative regulator of PLSCR1-induced scrambling. Moreover, the inhibition of PS egress by tetanus and botulinum neurotoxins indicate that PLSCR1 activation is dependent of the formation of the SNARE complex. Uncovering the underlying mechanisms and in particular how this interaction is regulated during calcium-regulated exocytosis will be our next challenge. We have previously shown that cell surface exposure occurs exclusively in the close vicinity of secretory granule fusion sites (Ory et al., 2013). One attractive scenario would be that PLSCR1 is recruited at the SNARE complex in formation where it interacts with STX1A. Upon cell stimulation and intracellular calcium increase, the conformational change leading to the SNARE complex zippering triggers the dissociation of PLSCR1 from STX1A. Hence, free PLSCR1 released from the SNARE complex can be activated and scramble phospholipid close to the secretory granules fusion sites.

Interestingly, a high stimulation of PS egress upon STX1A knock-down was observed in resting cells but no additional increase of PS egress occurred in response to stimulation suggesting that intracellular calcium increase was unable to further promote PS egress in absence of STX1A. Since PLSCR1 activity has been proposed to be mostly regulated by Ca^{2+}, it is surprising that PLSCR1 could be activated in resting condition. Hence, these data raise the question of the involvement of calcium on PLSCR1 activity during exocytosis. One possibility is that STX1A silencing increases the steady-state amounts of intracellular Ca^{2+} by altering calcium channel function. Indeed, it has been shown that calcium channel function might be regulated by direct interactions with synaptic proteins such as STX1A through the so-called consensus synprint site that is found on several calcium channels (Rettig et al., 1997; Carbone et al., 2014). Another possibility is that PLSCR1 could be activated in the absence of calcium as it has been shown recently (Francis and Gummadi, 2015; Arashiki et al., 2016).

In conclusion, our findings reveal for the first time that STX1A is able to regulate PLSCR1 activity, hence contributing to the transient remodeling of membrane phospholipids required for the compensatory endocytic process in neuroendocrine cells. The next challenge will be to understand why phospholipids redistribution at the secretory granule fusion sites is pivotal for secretory granule recapture.

NB: ***this discussion will be further developed in the next chapter of my thesis manuscript***

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IV. PLSCR1 Discussion

1. Regulation of PLSCR1 activity

1.1 Calcium or no calcium?

The requirement of calcium for PLSCR1-induced scrambling remains unclear. On one hand, it has been shown that PLSCR1 needs to bind Ca^{2+} for activation, with an apparent affinity of around 10 µM (Stout et al. 1998). Moreover several studies proposed that Ca^{2+} binding leads to conformational changes and/or oligomerization of PLSCR1 that could be important for the scrambling activity (Sahu et al. 2009; Stout et al. 1998). On the other hand, recent in vitro experiments performed in proteo-liposomes suggest that PLSCR1 can stimulate scrambling at physiological intracellular range of calcium concentration (< 0.1 µM) (Arashiki et al. 2016). Moreover, conformational constraints induced by environmental changes (such as pH change for example) might be sufficient to activate PLSCR1 independently of Ca^{2+} (Francis and Gummadi 2015). I have shown that knocking-down the expression of STX1A strongly enhances PLSCR1 activity in unstimulated PC12 cells in which normally no calcium increase occurs. However, at the present stage of my work, I cannot rule out that STX1A silencing does not impact the steady-state amounts of intracellular Ca^{2+} by altering calcium channel function. Indeed, as discussed already in our article in preparation, STX1A is known to interact with and modulate Ca^{2+} channels activity (Rettig et al. 1997; Carbone et al. 2014). In consequence, it will be important first to control the level of calcium in cells knock-down for STX1A and second to evaluate if PLSCR1 can influence a potential function of STX1A/Ca^{2+}-channel interaction.

Finally, one alternative possibility is that knock-down of STX1A bypasses the requirement of calcium. If this hypothesis is true, it would imply that the presence of STX1A is a brake preventing oligomerization and/or conformationnal changes of PLSCR1 and that Ca^{2+} is needed to relieve this brake.

1.2 SNARE proteins as interacting partners for PLSCR1

Pull down assay coupled with mass spectrometry analysis allows us to identify several SNARE proteins including the isoforms 1A, 4, 8, 11, 16 and 18 of syntaxin (STX) as potential binding partners for PLSCR1. It is quite inconceivable that all these STX isoforms interact with PLSCR1 in cell. First of all, the cellular and tissue distribution of these proteins is different (Teng et al. 2001). STX 4, 8, 11, 16 and 18 are expressed ubiquitously whereas STX1 is specifically expressed in (neuro) secretory cell. Regarding the subcellular distribution, STX8 is found in endosomes, STX11 in the trans-Golgi network (TGN) and late endosome, STX16 in the Golgi and TGN and STX18 in the endoplasmic reticulum. Only STX1 and 4 are localized in the plasma membrane which is the compartment where PLSCR1 has been mainly detected in chromaffin cells (Ory et al. 2013). Moreover both STX1 and 4 have functions in the exocytosis pathways. STX1 has been extensively characterized as the STX isoform controlling presynaptic neurotransmitter and hormone release whereas STX4 has been described to control exocytosis of vesicles containing the glucose transporter, Glut4, in adipocytes (Arancillo et al. 2013; Hugo et al. 2013; Volchuk et al. 1996). Therefore, in the context of my study, STX1A appeared as the ideal candidate to interact with PLSCR1 in the course of the secretory granule exo-endo cycle in chromaffin cells. Additional pull-down assays using recombinant PLSCR1 and immunoprecipitation experiments using exogenously expressed PLSCR1 allow me to confirm that PLSCR1 is able to interact with STX1A and to propose that the C-terminal region containing the transmembrane domain (TDM) of STX1A might be required for the PLSCR1-STX1A complex formation.

One remaining important point is to address whether the interaction between PLSCR1 and STX is direct or not. In vitro experiments using recombinants proteins are currently in progress in the laboratory to solve that question. Moreover to visualize directly this possible interaction, Förster Resonance Energy Transfer (FRET) Microscopy using fluorescent PLSCR1 and SXT1A would be an appropriate approach.

Why so many isoforms of STX were trapped in our pull-down assay remain unclear. One possible explanation might be the high sequence homology (around 85%) between the STX isoforms (Teng et al. 2001).

1.3 What is the role of syntaxin1A in PLSCR1 scrambling?

So far, my data indicate that STX1A seems to be a negative regulator of PLSCR1-induced scrambling. The strongest evidence is the large increase of PS egress observed upon STX1A Knocking down. That can be rescue by expression of a siRNA-resistant STX1A construct. To my knowledge, this is the first demonstration of a role of STX1A in the regulation of the plasma membrane phospholipids homeostasis. This begs the question of the underlying mechanisms and in particular how this interaction is regulated during exocytosis? While the experiments I have conducted during my thesis are too preliminary to answer, I'd like to venture two possible hypotheses.

The first hypothesis relies on a mechanism in which PLSCR1 interacts with STX1A within the SNARE complex until a certain threshold of zippering. Once the SNARE complex completely zipped, the PLSCR1 would be dissociated from the STX1A hence triggering conformational changes of PLSCR1 and its subsequent activation (Fig16). This hypothesis fits with the fact that preventing SNARE complex formation by neurotoxins treatment inhibits PS egress.

If PLSCR1 is able to interact with a formed SNARE complex, I should be able to precipitate other SNAREs proteins using recombinant PLSCR1. As illustrated in figure 15, VAMP2 precipitated with PLSCR1 from chromaffin cell lysate in presence of Ca^{2+}. However I was not able to co-precipitate SNAP25 (not shown). Additional experiments are now required to investigate if PLSCR1 is able to interact with SNARE complex. One first simple approach would be to repeat the pull down experiments in which the conditions of electrophoresis allow the preservation of intact SNARE complex. This is possible by lowering the temperature of sample treatment (Kubista et al. 2004). In addition, it would be interesting to try to study the dynamic of PLSCR1/STX1A/VAMP2 interaction by FRET in order to better understand the spatial and temporal regulation of the complex PLSCR1/STX1 dissociation.

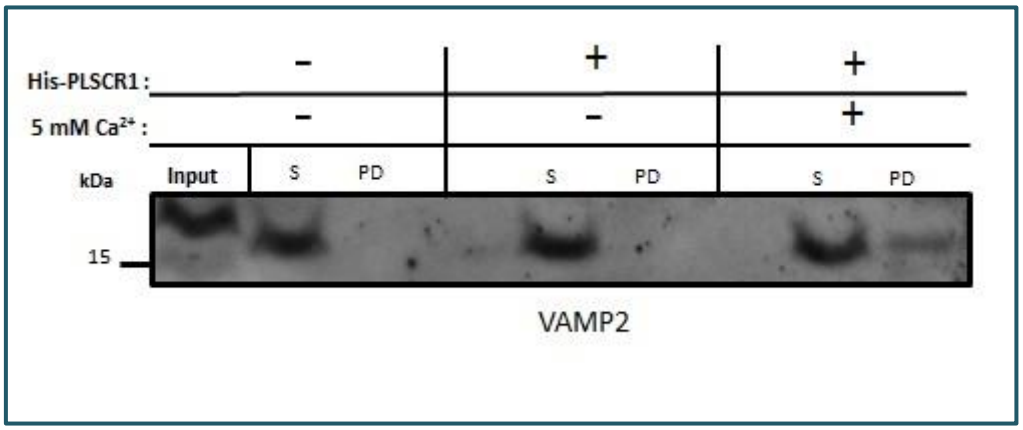

Fig15: VAMP2 from chromaffin cell lysate precipitates with His-tagged PLSCR1 Pull-down assay Recombinant His-tagged PLSCR1 (His-PLSCR1) was incubated with chromaffin cell lysates and pull-down. Precipitated proteins were separated by SDS-PAGE and VAMP2 was revealed by western blot. *S*: supernatant; fraction; *PD*: pulldown fraction.

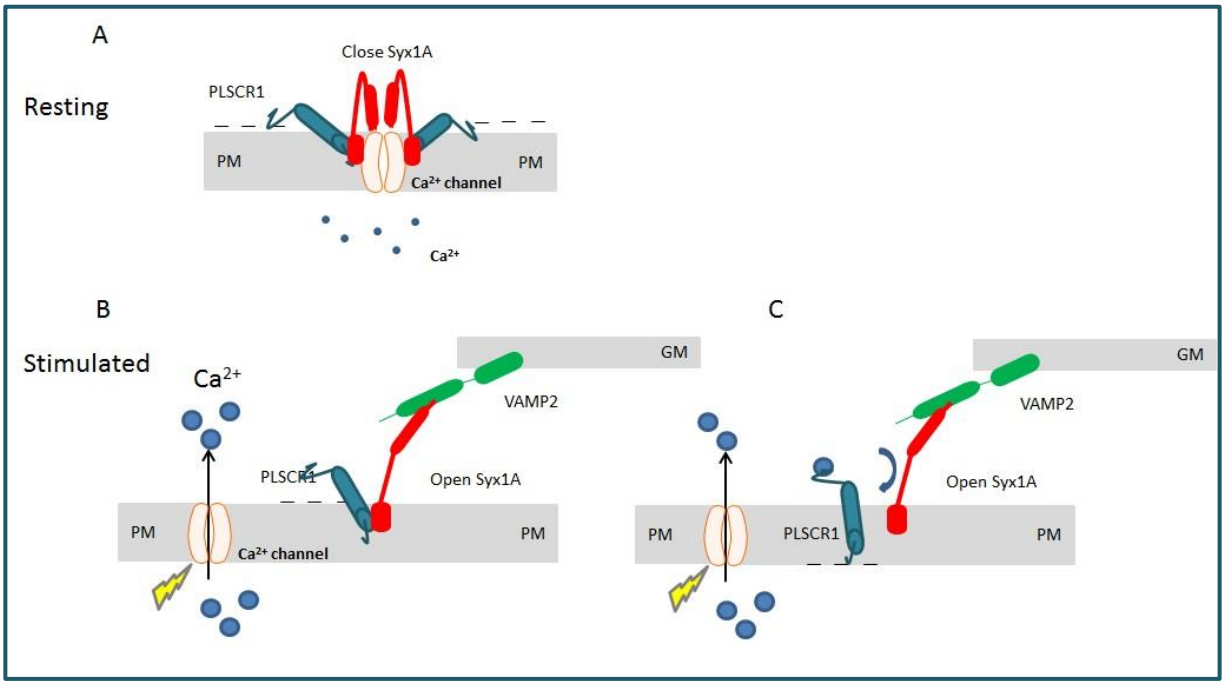

Fig16: Hypothetical model for the complex PLSCR1/STX1A dissociation by SNARE complex zippering. A) In resting condition PLSCR1 forms a complex with STX1A. B) After stimulation STX1A, still coupled to PLSCR1, is recruited to form the SNARE complex. C) The interaction between STX1 and VAMP2 generates a conformational change promoting the dissociation of the complex PLSCR1/STX1 and PLSCR1 activation.

The second hypothesis I'd like to propose relies on the regulation of STX1A clusters at the plasma membrane. STX1A can form homo-cluster in a closed conformation or hetero-cluster STX1A-SNAP25 in chromaffin, PC12 and βcells (Chamberlain et al. 2001; Lang et al. 2001; Toft-Bertelsen et al. 2016; Ohara-Imaizumi et al. 2004). Interestingly, it has been recently been reported that calcium promotes the formation of STX1 domains through the interaction with phosphatidylinositol 4,5-bisphosphate (PIP2) (Milovanovic et al. 2016).

These STX1/PIP2 clusters have been proposed to constitute recognition sites for vesicle docking. In the context of our study, one can imagine that PLSCR1 interact with STX1A cluster in resting condition. Which type of STX1A cluster would be involved in PLSCR1 interaction is currently unknown. Since I was not able to precipitate SNAP25 with PLSCR1 in pull-down experiment, it might suggest that in resting condition PLSCR1 interacts rather with STX1A in a closed conformation. Then, the local increase of PIP2 in secretagogue-stimulated cells could attract STX1A at docking sites. Through a conformational change for example, the interaction between STX1A and PIP2 might dissociate PLSCR1 from STX1. Free PLSCR1 could be then activated to promote PS externalization (fig17). This model fails to explain why tetanus neurotoxin expression prevents PS egress. Whether expression of the toxin itself and/or the cleavage of VAMP2 affect the concentration of PIP2 at the site of exocytosis is currently unknown. One could investigate the level of PIP2 and STX clusters upon tetanus toxin expression using super resolution techniques like stimulated emission depletion (STED) microscopy for example.

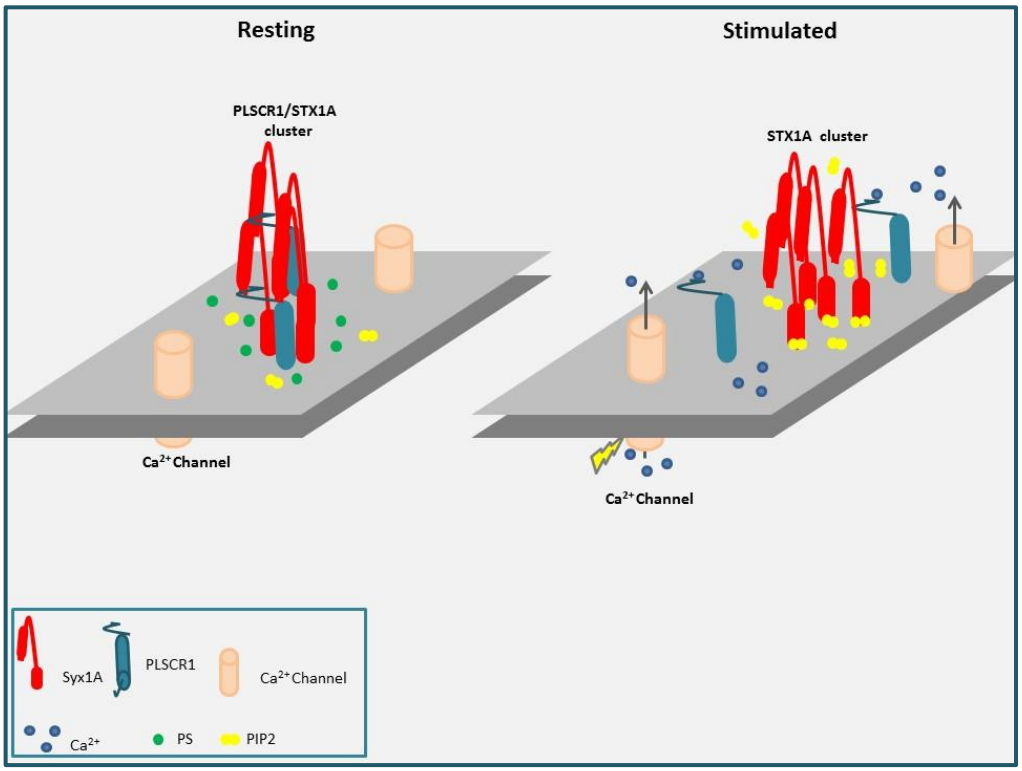

Fig 17: Hypothetical model for the complex PLSCR1/STX1A dissociation by calcium-induced PIP2 domains formation: PLSCR1 interacts with the STX1A in clusters in resting condition. Upon stimulation, calcium increase triggers STX1/PIP2 mesoscale domains formation that could be a signal for the STX1/PLSCR1 dissociation.

1.4 Spatial regulation of PLSCR1-induced PS egress

Previous work performed in our laboratory demonstrated that, upon stimulation of chromaffin cells, PLSCR1-induced PS egress occurs exclusively in the close vicinity of the sites of exocytosis whereas PLSCR1 seems to be homogeneously distributed all along the plasma membrane (Ory et al. 2013). What are the mechanisms restricting PLSCR1 activity around the secretory granule fusion site remains a key unsolved question.

Calcium and/or interaction with STX1 may contribute to the spatial distribution of PLSCR1 activity. The apparent affinity of PLSCR-1 for Ca^{2+} is around 10 µM (Stout et al. 1998), a concentration that is raised only at short distances of the exocytotic hot spots in stimulated chromaffin cells (Klingauf and Neher 1997; Becherer et al. 2003). Moreover, the signal triggering PLSCR1 activation may relies on PLSCR1/STX1 dissociation as I proposed above. It is then conceivable that the release of PLSCR1 from STX1 is spatially restricted around the exocytotic sites if it is triggered either by the recruitment of PLSCR1/STX1 complex to the PIP2 domains of the docking sites, by STX1/VAMP2 interaction or by a certain degree of SNARE complex zippering. Then, one can ask why PS does not diffuse away from the exocytotic sites once it has been translocated to the external leaflet? First of all, it is important to recall here that while we specifically measured PS egress, PLSCR1 is not specific for PS but is able to trigger rapid bidirectional transbilayer movement of various phospholipids like phosphatidylethanolamine, phosphatidylcholine and sphingomyelin across the plasma membrane leaflets. Therefore we need to consider that the loss of PS asymmetry reveals a more profound lipid reorganization around the exocytic sites. Moreover, previous work from our group demonstrated that stimulation of exocytosis in neuroendocrine cells triggers the formation of lipid microdomains in the plasma membrane which are enriched with cholesterol, PIP2 and GM1 at the exocytotic sites (Chasserot-Golaz et al. 2005). These lipid microdomains have been proposed to be necessary for the structural and spatial organization of the exocytotic machinery, including SNARE complex assembly (Chasserot-Golaz et al. 2005). Such mixed cholesterol/phospholipid phase (called liquid-ordered (Lo) phase in contrast to the liquid-disordered (Ld) phase composed only by phospholipid bilayers in absence of cholesterol) reduces the dynamic and the fluidity of the PM. Accordingly, cholesterol has been shown to limit lipids and proteins diffusion (lateral

and rotational) in model membrane system (Yang et al. 2016). Additionally, in vitro experiment performed into monolayers shows an increase of rigidity into PIP2 rich sites caused by the strong electrostatic interaction between PIP2 molecules (by hydrogen bonding) or when PIP2 interacts with Ca^{2+} (Ellenbroek et al. 2011). Such strong electrostatic effect can immobilize lipids in the exocytotic site restricting the movement of molecules out of this site. Altogether, these informations make me think that lipid microdomains might contribute to the spatial and temporal correlation of the exocytosis with compensatory endocytosis. Indeed, it is tempting to imagine that secretagogue-evoked stimulation triggers the formation of lipid microdomains (stabilized by the presence of cholesterol and/or PIP2) in which lateral diffusion of proteins and lipids like PS are limited, hence clustering specific components able to facilitate the membrane retrieval by endocytosis and the temporal and spatial coupling with exocytosis.

1.5 Functional role of PLSCR1-induced PS egress during exo-endocytosis cycle

1.5.1 Does PS egress contribute to exocytosis?

Externalization of PS to the extracellular face during calcium-regulated secretion has been reported in mast cells (Demo et al. 1999), PC12 cells (Vitale et al. 2001; Malacombe et al. 2006), nerve terminals (Lee, Hirashima, and Kirino 2000) and chromaffin cells (Ceridono et al. 2011; Ory et al. 2013). However the link between PS egress and the scramblase activity during regulated exocytosis remains unclear. Using RBL-2H3 rat mast cells, Kato and collaborators propose that phospholipid asymmetry need to be preserved for efficient exocytosis since secretion is inhibited upon PLSCR2 overexpression (Kato et al. 2002). On the contrary, it has been shown that knocking down PLSCR1 expression in these cells significantly diminish the degranulation upon FcεRI aggregation (Amir-Moazami et al. 2008). However PLSCR1 seems to participate to mast cell degranulation through the FcεRI signalling pathway and independently of phospholipid scrambling (Pastorelli et al. 2001; Smrz et al. 2008; Benhamou and Blank 2010). Additionnaly, it has been shown that PLSCR1 plays a modulatory role in the process of neurotransmission at the larval neuromuscular junction in drosophila also independently of a phospholipid scrambling activity (Acharya et al. 2006).

While previous investigations from our laboratory have reported that PS externalization during exocytosis requires PLSCR1 in the neuroendocrine chromaffin cells, a potential role of PLSCR1 and externalization of PS has not been demonstrated in the exocytotic process *per se*. On contrary, the number of exocytic events in chromaffin cells expressing the calcium-insensitive $PLSCR1_{D284A}$ mutant remains unchanged (Ory et al. 2013). However, as I have shown that PLSCR1 interacts with the SNARE protein STX1A, one cannot completely rule out that PLSCR1 could finely tune the late steps of secretory granule exocytosis. To test this hypothesis, I have performed carbon fiber amperometry on chromaffin cells culture from KO PLSCR1 mice. In accordance with our previous data, no change in the number of the exocytotic events was observed in cells lacking PLSCR1. Moreover, the kinetic of secretory granule fusion is strictly similar in $PLSCR1^{-/-}$ cells compared to $PLSCR^{+/+}$ cells (Fig18). These data confirm that PLSCR1 does not modulate, even finely, the exocytotic response in chromaffin cells.

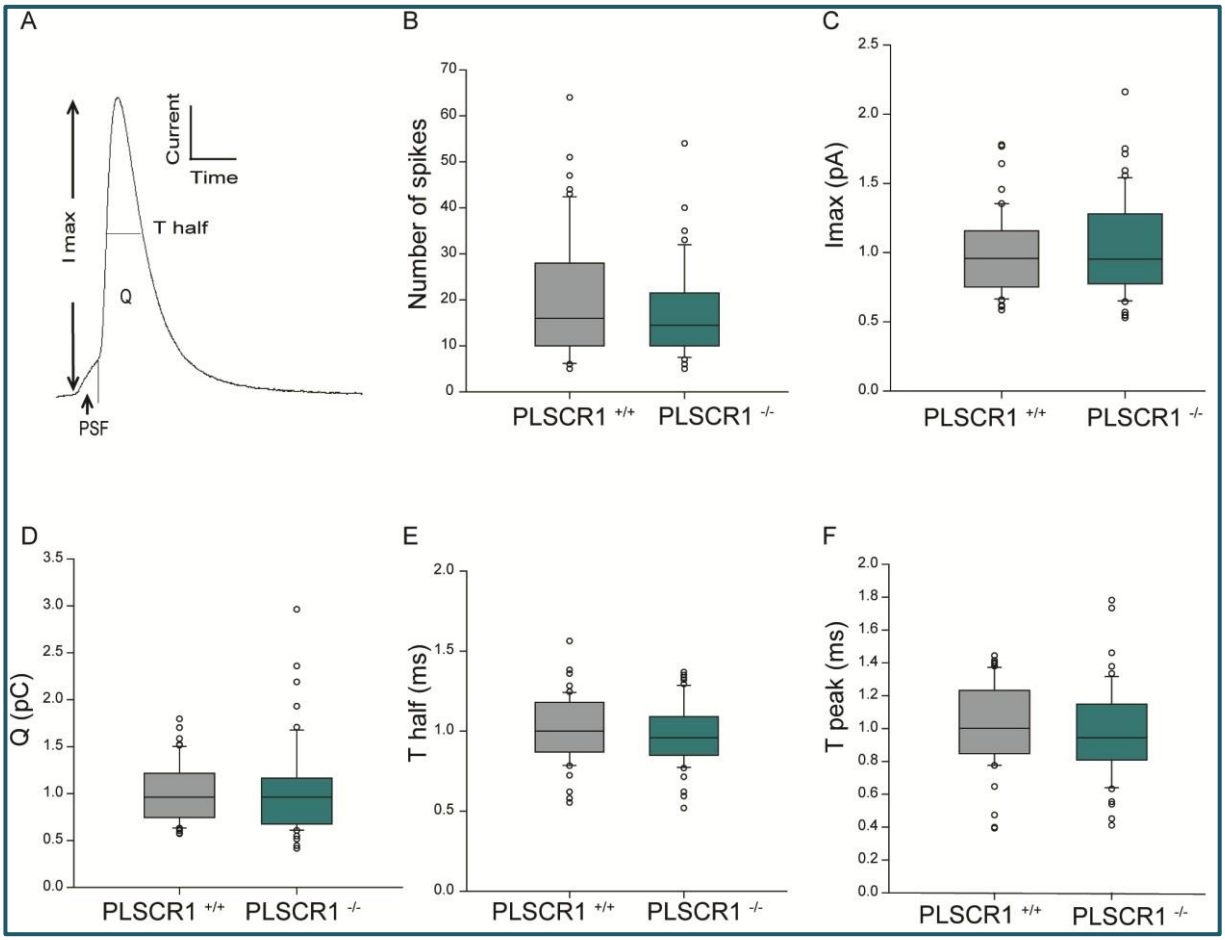

Fig18 : Amperometric analysis of catecholamine secretion from *PLSCR1* knock-out (PLSCR1$^{-/-}$) mice chromaffin cells. (A) Scheme of an amperometric spike describing the following different parameters analyzed: quantal size or charge (*Q*), half-width (*t* half), spike amplitude (*I*max), and PSF signal. Box-and-whisker plot for number of spikes (B), spike amplitude (C), Charge (D), Half-width (E) and time to peak (F). Statistical significance for median values was determined by a Mann–Whitney non parametric median analysis. PLSCR1$^{-/-}$ cells: n= 30; PLSCR1$^{+/+}$ cells: n=30.

1.5.2 How the loss of phospholipid asymmetry might control compensatory endocytosis?

Previous work from our laboratory suggested that PLSCR-1 is specifically involved in secretory vesicle compensatory endocytosis since constitutive endocytotic pathways like receptor-mediated endocytosis of transferrin and EGF or fluid phase uptake are not affected neither in fibroblast (unpublished) nor in chromaffin cells from PLSCR-1 $^{-/-}$ mice (Ory et al. 2013). Moreover, preliminary data obtained by P. Toth and S. Ory indicate that PLSCR1 might also regulate synaptic vesicle recycling in neurons (unpublished) suggesting a common mechanism for the neuro-secretory cells. However, how the loss of phospholipid distribution assymetry controls compensatory endocytosis remains unknow.

As I mentionned earlier, the global lipid remodeling induced by PLSCR1 around the exocytic site might contribute to create microdomain restricting lateral diffusion. Hence, the role of PLSCR1 in endocytosis could be simply to prevent the diffusion of secretory granule membrane components to preserve its identity once it has merged with the plasma membrane after full fusion (Ceridono et al. 2011; Bittner et al. 2013). In absence of PLSCR1, lipids and proteins from secretory granules could freely diffuse into the plasma membrane, hence interfering with an efficient recapture.

Due to the externalization of the anionic phospholipids, PLSCR1 activity will locally change the charge at the cytosolic leaflet. This modification could be a stop signal for exocytosis by promoting the dissociation of PS-interacting proteins known to be involved in exocytosis like for example annexin-A2, rabphilin, Doc2, or synaptotagmin (Stace and Ktistakis 2006). Alternatively, local redistribution of charges across lipid bilayers might be a signal to recruit the endocytic machinery. I have decided to test this hypothesis. Previous work from our group demonstrated that clathrin is efficiently recruited at the exocytotic sites upon cell stimulation (Ceridono et al. 2011). To investigate whether PLSCR1-induced scrambling might be involved in the recruitment of the endocytic machinery, I used chromaffin cells cultured from PLSCR1 knock-out mice. As illustrated in figure 19, recruitment of clathrin is inhibited in PLSCR1$^{-/-}$ cells. Moreover, expression of the PLSCR1$_{D284A}$ mutant in chromaffin cells also inhibits clathrin

recruitment at the exocytic sites upon cell stimulation (Fig20). Another potential player of secretory granule recapture is intersectin which has been shown to colocalized with the PS exit sites in PC12 cells (Malacombe et al. 2006). Albeit less convincing, I obtained similar results indicating that the proportion of intersectin localized at the cell periphery is decreased in chromaffin PLSCR1$^{-/-}$ cells (Data not shown).

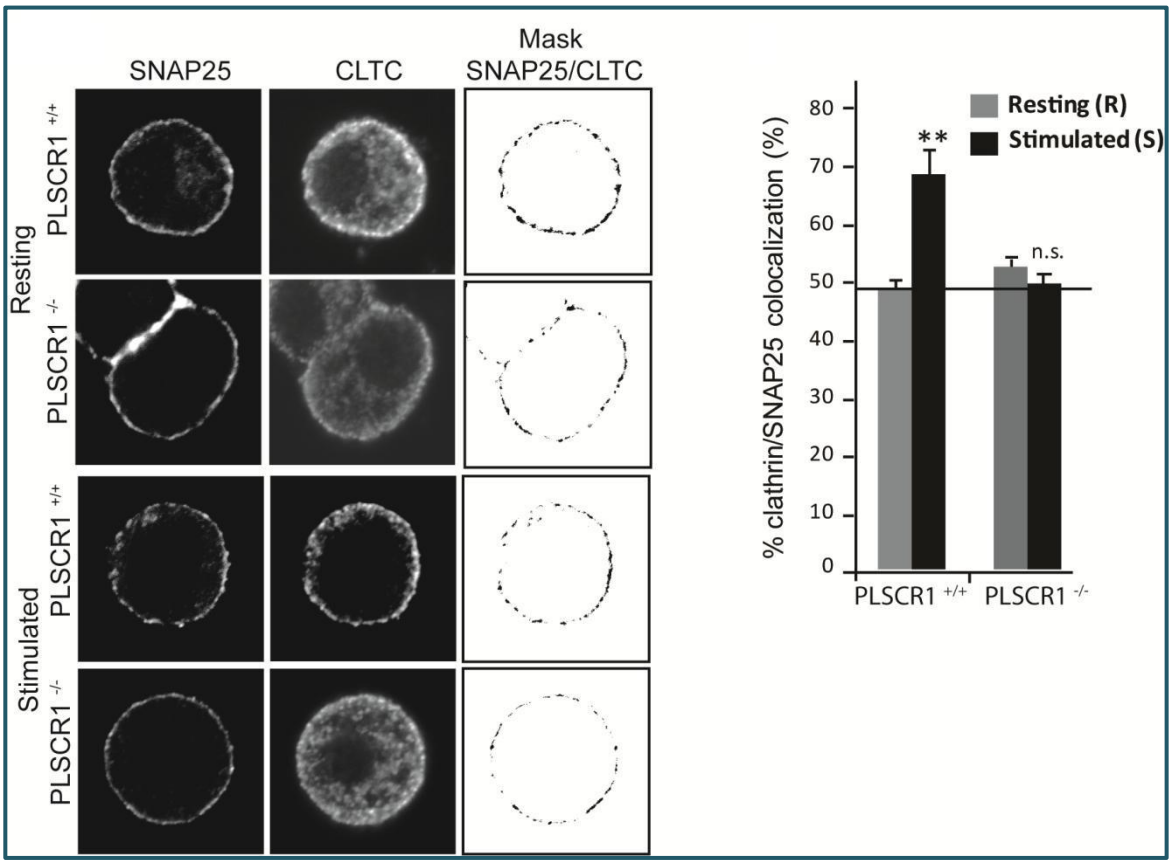

Figure 19: Recruitment of clathrin is inhibited in chromaffin cells lacking PLSCR1. Chromaffin cells from adult PLSCR1$^{+/+}$ or PLSCR1$^{-/-}$ mice were maintained in Locke's solution (Resting) or stimulated 10 min with 59 mM K^+. Cells were then fixed and processed for immunofluorescence with clathrin light chain and SNAP25 antibodies. SNAP25 staining allows to delineate the plasma membran. The graph shows the percentage of clathrin found in the vicinity of the plasma membrane (co-localization with SNAP25 staining) compared to total amounts of clathrin labelling (n= 25 cells).**$p < 0.005$, n.s.: not significant compared to resting conditions.

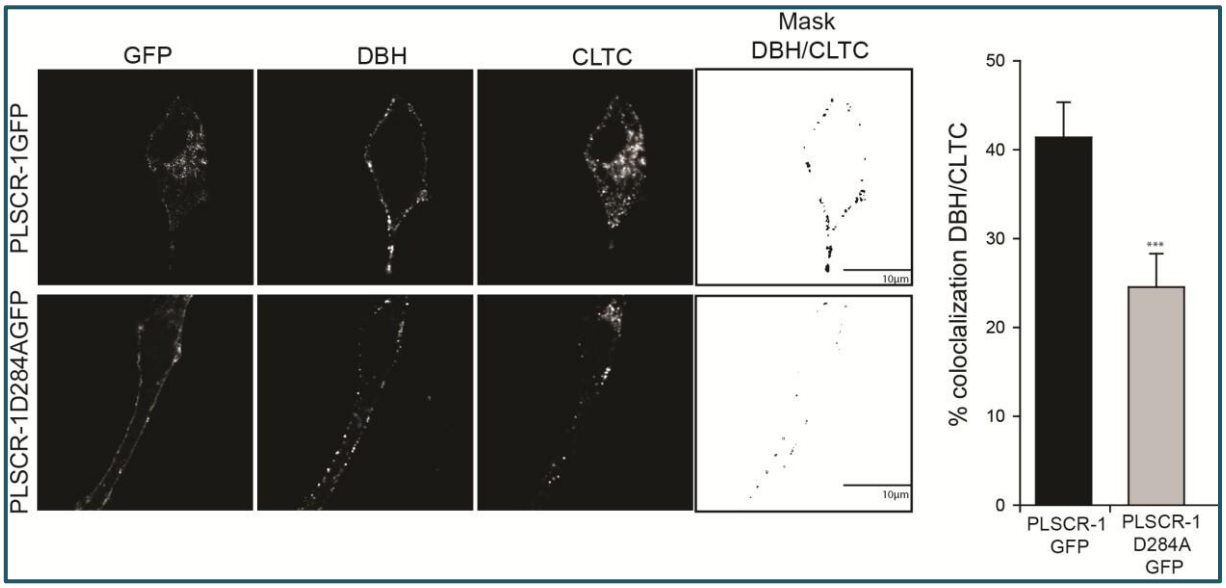

Figure 20: Expression of PLSCR1$_{D284A}$ mutant decreases clathrin recruitment at the exocytic sites in chromaffin cells. Chromaffin cells expressing GFP-tagged PLSCR1 or -PLSCR1$_{D284A}$ were stimulated with 59 mM K^+ and incubated for 20 min at 4°C with rabbit anti-DBH antibodies to detect exocytotic sites. Cells were then fixed and processed for immunofluorescence with the anti-clathrin antibodies. DBH and clathrin were revealed with Alexa-647-conjugated anti-rabbit antibodies and Alexa-555-conjugated anti-mouse antibodies, respectively. The histogram represents a semi-quantitative analysis of the percentage of clathrin co-localized with the cell surface DBH between cells expressing PLSCR-1 and PLSCR-1 D284A proteins. (n=30 cells), ***$p < 0.001$.

These preliminary experiments seem to indicate that PLSCR1-induced PS egress might contribute to the recruitment of the endocytic machinery in the early step of the clathrin-mediated endocytosis. Therefore, we can hypothesize that lipid remodelling induced by PLSCR1 activity enhances localization of intersectin at the secretory granule fusion sites, hence probably facilitating the recruitment of adaptor proteins like AP-2 and consequently the translocation of clathrin to the granule membrane (Fig21)

More experiments are now required to further investigate the sequence of events involved in the recruitment of proteins essential for secretory granule endocytosis. Other candidates need to be studied as well like FCHO, Eps15 and AP2. Ideally, these experiments should be performed in live cell, using TIRF microscopy for example. Moreover ultrastructural analysis by electron microscopy of endocytic proteins localization will be considered.

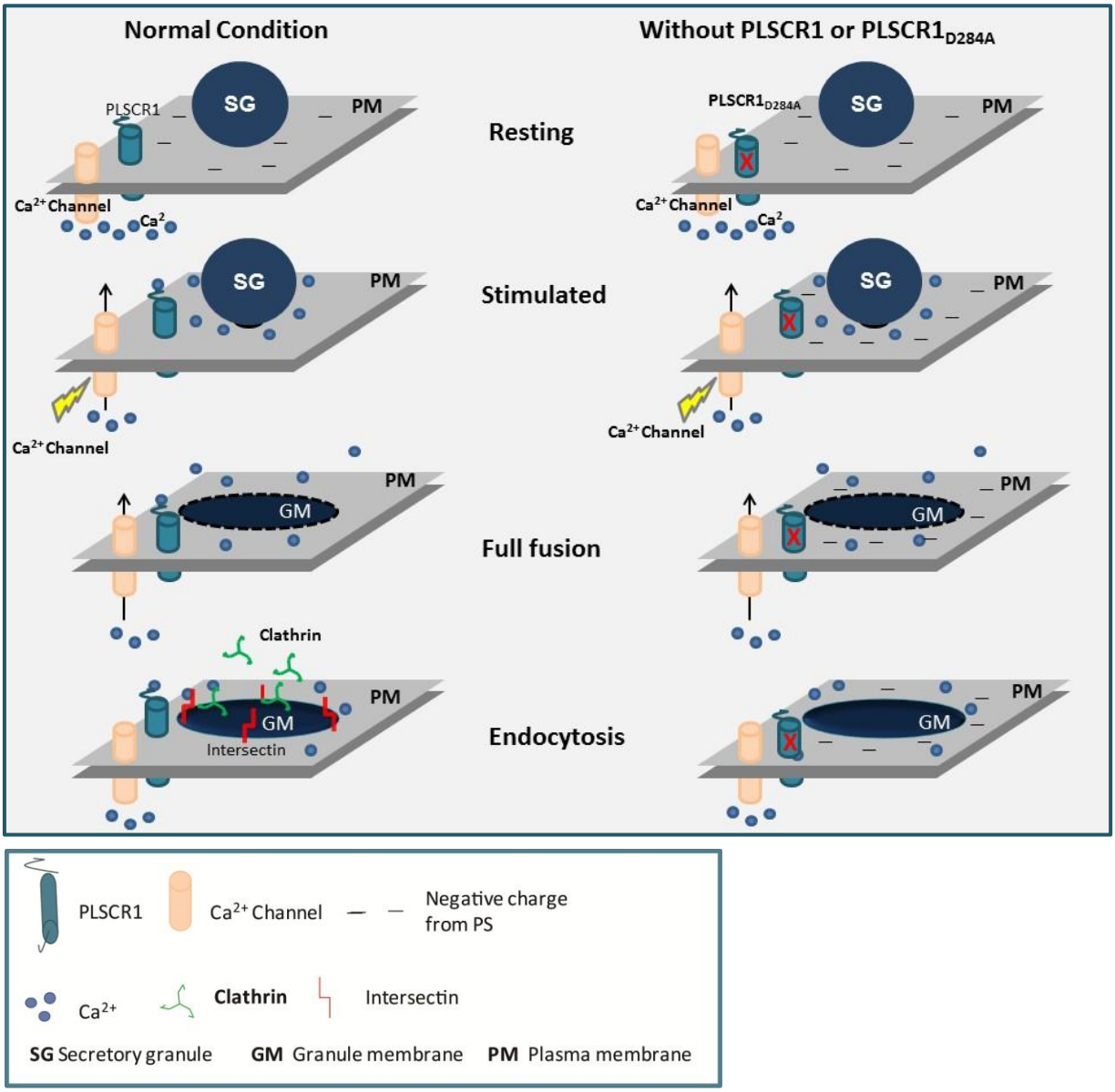

Fig21. Hypothetical model illustrating the recruitment of the endocytic machinery in chromaffin cells in the presence (normal condition) or in the absence of PLSCR1.

In normal condition after cells stimulation, PS externalization occurs (shown as the negative charge loss in the vicinity of the exocytosis site). This enhance the localization of intersectin at the secretory granule fusion sites faciliting the recruitment of adaptor proteins like AP-2 and consequently the translocation of clathrin to the granule membrane. The second condition illustrated on the right of the figure corresponds to a situation where the cell does not express the PLSCR1 or the cells which have been transfected with PLSCR1 D284A, an inactive mutant unable to bind ca^{2+}. In this condition, the normal fusion of the granule with the membrane after the cells stimulation occurs, but the externalization of PS (the negative charges of PS remain in the intracellular layer of the membrane) is impaired. Therefore the recruitment of intersectin and clathrin at the granule membrane site is impaired.

V. OPHN Result

1. Research context

Oligophrenin-1 connects exocytic fusion to compensatory endocytosis in neuroendocrine cells

In the laboratory, one of the main research topic is to investigate the role of the Rho-GTPases on the regulation of hormone release using chromaffin cells. Until now upstream GEF regulators have been characterized (Malacombe et al. 2006;Momboisse et al. 2009; Momboisse et al. 2010), but so far no downstream GAP regulator. At the beginning of my thesis, data obtained by S. Houy another Ph.D students in the laboratory indicated that the protein oligophrenin (OPHN1) could be a potential GAP for RhoA in the course of hormone release in chromaffin cells. The role of OPHN1 on large dense core granule trafficking in neuroendocrine cells was never explored before. In view of its ability to control Rho-GTPase activity, to sense membrane curvature and to regulate synaptic vesicle cycling in neurons, OPHN1 appeared to us as a key candidate to play a pivotal function in large dense core granule exocytosis and endocytosis in neuroendocrine cells. Therefore I dedicated part of my thesis work to study the role of this protein in collaboration with S. Houy. The data are presented as an article published in Journal of Neuroscience last year and in which I signed as a co-first author.

In this study, we show that OPHN1 is expressed and localized at the plasma membrane and in the cytosol in chromaffin cells from adrenal medulla. Using carbon fiber amperometry, we found that exocytosis is impaired at the late stage of membrane fusion in chromaffin cells isolated from OPHN1 knockout mice. Experiments performed with ectopically expressed OPHN1 mutants indicate that OPHN1 requires its Rho-GAP domain to control fusion pore dynamics. On the other hand, compensatory endocytosis assessed by measuring dopamine-beta-hydroxylase (secretory granule membrane marker) internalization is severely inhibited in OPHN1 knockout chromaffin cells. This inhibitory effect is mimicked by expression of a truncated OPHN1 mutant lacking the BAR domain, demonstrating that the BAR domain implicates OPHN1 in granule membrane recapture after exocytosis. These data demonstrate for the first time that OPHN1 is a bi-functional protein

able to couple, through distinct mechanisms, the exocytosis with compensatory endocytosis in adrenal chromaffin cells.

My contribution to this study was to perform and analyze the amperometric recordings and the DBH internalization assays in bovine chromaffin cells overexpressing full length OPHN1 and expressing $OPHN1_{R409L}$ or $OPHN1_{\Delta BAR}$ mutants (Figure 4 and 7A and table1). I also performed the rescue experiments described in figure 5.

Cellular/Molecular

Oligophrenin-1 Connects Exocytotic Fusion to Compensatory Endocytosis in Neuroendocrine Cells

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Oligophrenin-1 (OPHN1) is a protein with multiple domains including a Rho family GTPase-activating (Rho-GAP) domain, and a Bin-Amphiphysin-Rvs (BAR) domain. Involved in X-linked intellectual disability, OPHN1 has been reported to control several synaptic functions, including synaptic plasticity, synaptic vesicle trafficking, and endocytosis. In neuroendocrine cells, hormones and neuropeptides stored in large dense core vesicles (secretory granules) are released through calcium-regulated exocytosis, a process that is tightly coupled to compensatory endocytosis, allowing secretory granule recycling. We show here that OPHN1 is expressed and mainly localized at the plasma membrane and in the cytosol in chromaffin cells from adrenal medulla. Using carbon fiber amperometry, we found that exocytosis is impaired at the late stage of membrane fusion in *Ophn1* knock-out mice and OPHN1-silenced bovine chromaffin cells. Experiments performed with ectopically expressed OPHN1 mutants indicate that OPHN1 requires its Rho-GAP domain to control fusion pore dynamics. On the other hand, compensatory endocytosis assessed by measuring dopamine-[3-hydroxylase (secretory granule membrane) internalization is severely inhibited in *Ophn1* knock-out chromaffin cells. This inhibitory effect is mimicked by the expression of a truncated OPHN1 mutant lacking the BAR domain, demonstrating that the BAR domain implicates OPHN1 in granule membrane recapture after exocytosis. These findings reveal for the first time that OPHN1 is a bifunctional protein that is able, through distinct mechanisms, to regulate and most likely link exocytosis to compensatory endocytosis in chromaffin cells.

Key words: **amperometry; chromaffin cells; compensatory endocytosis; exocytosis; neuroendocrine cells; oligophrenin-1**

Introduction

In neuroendocrine cells, the secretion of hormones and neuropeptides occurs through calcium-regulated exocytosis, a process that involves the docking and fusion of large dense core vesicles (secretory granules) with the plasma membrane. To maintain the specific lipid and protein composition of secretory granules and plasma membranes, and to keep the cell surface constant, exocytosis needs to be rapidly followed by a compensatory endocytosis

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process. Using neuroendocrine chromaffin cells from the adrenal gland, we have previously shown that, after full fusion exocytosis, secretory granule membrane proteins are sorted and segregated together before being recaptured, suggesting a tight spatial and temporal coupling between exocytosis and compensatory endo- cytosis (Ceridono et al., 2011; Ory et al., 2013). However, the molecular mechanisms of granule membrane recapture and its coupling with exocytosis remain largely unexplored. Multido- main or scaffold proteins that are able to control and coordinate multiple cellular functions represent potential candidates.

Oligophrenin-1 (OPHN1) is a Rho family GTPase-activating protein (Rho-GAP) that contains, in addition to the catalytic GAP domain, several domains including a N-terminal Bin- Amphiphysin-Rvs (BAR) domain able to sense membrane cur- vature, a pleckstrin homology domain and three C-terminal proline-rich domains (Billuart et al., 1998; Fauchereau et al., 2003). In neurons, OPHN1 is expressed both presynaptically and postsynaptically, and its loss of function has been associated with X-linked intellectual disability (Billuart et al., 1998). Postsynaptic OPHN1 seems to be important for dendritic spine morphogen- esis and for postsynaptic receptor trafficking (Govek et al., 2004; Khelfaoui et al., 2007; Nadif Kasri et al., 2009; Nakano-Kobayashi et al., 2014). The function of OPHN1 at the presynaptic sites is more puzzling. Short-term plasticity like paired-pulse facilitation is altered in hippocampal neurons from OPHN1 knock-out

mice, suggesting potential changes in the probability of neurotransmitter release, although the underlying molecular aspects linking OPHN1 to exocytosis remained unexplained (Khelfaoui et al., 2007). Moreover, OPHN1 has been shown to regulate synaptic vesicle recycling both through its interaction with endophilin A1, a BAR domain-containing protein implicated in endocytosis, and the inactivation of the RhoA/Rho-associated kinase (ROCK) signaling pathway (Khelfaoui et al., 2009; Nakano-Kobayashi et al., 2009). In view of its ability to control Rho-GTPase activity, sense membrane curvature, and regulate synaptic vesicle endocytosis, we reasoned that OPHN1 could potentially play a pivotal function in coupling exocytosis to endocytosis in neurosecretory cells.

The aim of the present work was to investigate the functional importance of OPHN1 during regulated exocytosis and compensatory endocytosis in neuroendocrine chromaffin cells. Using carbon fiber amperometry and a dopamine-[3-hydroxylase (DBH) antibody internalization assay on chromaffin cells isolated from OPHN1 knock-out mice, we found that both exocytotic fusion and compensatory endocytosis were compromised. Interestingly, OPHN1 appeared to be implicated in these two functions through distinct domains, relying on its Rho-GAP domain to control fusion pore dynamics and requiring its BAR domain to trigger granule membrane endocytosis.

Materials and Methods

DNA constructs, animals, cell culture, and transfection. The bidirectional expression vector pBI-CMV1 (Clontech) was used to simultaneously express the enhanced green fluorescent protein (EGFP) and the OPHN1 proteins in transfected cells. The human wild-type (WT) OPHN1 and R409L mutant (provided by Dr. P. Billuart, Institut Cochin, Paris, France) were amplified by PCR using 5'-TATACGCGTGCCACCATGGGTCATCCCCCGCT-3' and 5'-CATGCGGCCGCTCAACTTTCATCTCCAGGAAG-3' primers. The first 225 aa were deleted from WT OPHN1 to generate the OPHN1 LIBAR mutant using 5'-CGCACGCGTGCCACCATGCAACAGCTCC AACTCAGT-3' and 5'-CATGCGGCCGCTCAACTTTCATCTCCAGG AAG-3' primers. EGFP was amplified using 5'-TATAGATCTCGCCAC CATGGTGAGCAAGGGCGA-3' and 5'-CGCCTGCAGTTACTTGTAC AGCTCGTCCATGC-3' primers. PCR products were ligated into pBI-CMV1 between the MluI and NotI restriction sites in MCS1 (OPHN1), and the PstI and BglII restrictions sites in MCS2 (EGFP).

Nineteen base pair long short hairpin RNA were designed to target the bovine sequence of OPHN1 (GAACCTATCTACCACAGCC). Sense and antisense strands separated by a short spacer were synthesized (Life Technologies), annealed, and cloned between the BglII and HindIII sites in front of the H1 promoter of a pmCherry vector. A vector control was generated by cloning an unrelated sequence (ATTCTATCACTAGCGT-GAC; Randhawa et al., 2004) between BglII and HindIII sites. For rescue experiments, using the QuickChangeII XL Site-Directed Mutagenesis kit (Agilent Technologies), wobble mutations (codon GAA encoding Glu338 to GAG and codon CCT encoding Pro339 to CCC) were introduced into OPHN1, $OPHN1_{R409L}$, and $OPHN1_{LIBAR}$ constructs to make them resistant to OPHN1 short hairpin RNA (shRNA). Forward and reverse primers were, respectively, as follows: 5'-CATGGATGGGAA AGAGCCCATCTACCACAGCCCTA-3' and 5'-TAGGGCTGTGGTA GATGGGCTCTTTCCCATCCATG-3'. All constructs were verified by sequencing.

OPHN1 mice with a C57BL/6N background were described previously (Khelfaoui et al., 2007). All mice were bred, handled, and maintained in agreement with European Council Directive 86/609/EEC and the resulting French regulations. The mice were housed and raised at Chronobiotron UMS 3415.

Mouse chromaffin cells were cultured as described previously (Ory et al., 2013). Briefly, adrenal glands from 8- to 12-week-old males were dissected, and chromaffin cells were purified from papain-digested medulla. Cells were seeded on collagen-coated coverslips and maintained at 37°C, 5% CO_2 for 24–48 h before the experiments. Bovine chromaffin cells were cultured as described previously (Gasman et al., 1997). Mammalian expression vectors (3 μg) were introduced into chromaffin cells (5×10^6 cells) by Nucleofactor systems (Amaxa Biosystems) according to the manufacturer instructions. Cells were seeded on fibronectin-coated coverslips and cultured for 48 h before the experiments.

PC12 cells were cultured as described previously (Gasman et al., 2004). A total of 5×10^4 cells/cm^2 were seeded 24 h before small interfering RNA (siRNA) transfection according to the manufacturer instructions. Lipofectamine RNAiMax (Invitrogen) and an 80 nM mix of four siRNAs directed against *Ophn1* (On Target Plus Smart Pool siRNA; Dharmacon) were used (5'-UGAGAUUAAUAUUGCGGAA-3'; 5'-GGAAGCUG-GUAUAUAGGUU-3';5'-CGGAAGGAACAAAUAGGUU-3'; 5'-CAU GCAAGCUUCCGGGACA-3'). Cells were cultured for 48 h before the experiments, and OPHN1 silencing was estimated and normalized to actin contents by Western blotting.

Real-time quantitative PCR. Total RNA from mouse adrenal medulla and cerebellum were prepared using the GenElute Mammalian total RNA Miniprep Kit (Sigma-Aldrich) and then treated with RNase-free DNaseI (Thermo Scientific). After checking RNA integrity and concentration by spectrophotometry and agarose gel electrophoresis, the template RNA was transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit for real-time quantitative PCR (Thermo Scientific), according to the manufacturer instructions (1 μg RNA/20 μl reverse transcriptase reaction). PCR was performed in 96-well plates using diluted cDNA samples, highly gene-specific primers, and SyberGreen PCR reagents (IQ SYBR Green Supermix; Bio-Rad).

Gene amplification and expression analyses were performed on a MyIQ real-time PCR machine (Bio-Rad) using a three-step procedure (20 s at 95°C; 20 s at 62°C; 20 s at 72°C) followed by a melting curve study to ensure the specificity of the amplification process. PCR efficiency was evaluated by standard curves analysis and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Gene expression in two different samples was compared using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001). Each reaction was performed in triplicate, and the sample was related to GAPDH. The mean LICt (Ct OPHN1 − Ct GAPDH) was calculated for each condition, and expression levels were determined and represented as 2^{-LICt}. Primer sequences used against cDNA of mouse origin (5'–3') were as follows: OPHN1_Fw: CAGGGACCGGTGGACTTAAC; OPHN1_Rv: AGTGATGGTTCCAGGTCTTTCA; GAPDH_Fw: GGC-CTTCCGTGTTCCTAC; and GAPDH_Rv: TGTCATCATACTTGGCA GGTT.

Antibodies, immunofluorescence, and DBH internalization assay. Polyclonal anti-OPHN1 antibody has been described earlier (Fauchereau et al., 2003). Monoclonal anti SNAP25 was from Millipore Bioscience Research Reagents and rabbit polyclonal anti-DBH was as previously described (Ceridono et al., 2011). The mouse monoclonal anti-RhoA (clone 26C4) was from Santa Cruz Biotechnology.

Chromaffin cells were fixed and stained as previously described (Gasman et al., 1998). Cells were observed with a TCS SP5 confocal microscope (Leica Microsystems) using a 63X objective (numerical aperture, 1.40). For the plasma membrane labeling, cells were washed twice with PBS and incubated for 30 min at 4°C with 0.25 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS. Cells were washed, fixed, and processed for immunofluorescence. Biotinylated proteins were revealed using Alexa Fluor streptavidin conjugates (Life Technologies).

Anti-DBH antibody internalization assay was performed as previously described (Ceridono et al., 2011; Ory et al., 2013). Briefly, bovine chromaffin cells were washed twice in Locke's solution and further incubated at 37°C in Locke's solution (resting) or stimulated with an elevated K^+ solution for 10 min. Cells were then placed on ice, washed once in Locke's solution, and incubated for 30 min at 4°C in the presence of polyclonal anti-DBH antibodies. Cells were then washed rapidly with Locke's solution and fixed (stimulated) or further incubated in Locke's solution at 37°C for 15 min (endocytosis) before fixation. Cells were then processed for immunofluorescence. For mouse chromaffin cells, cells were rapidly washed and maintained under resting conditions or stimulated for 10 min at 37°C in Locke K^+ solution in the presence of anti-DBH anti-

bodies. Cells were then washed with Locke's solution and fixed or further incubated at 37°C for 15 min before fixation and immunofluorescence experiments. As previously described, the distribution of DBH-containing granules was analyzed using a Euclidean distance map (Ceridono et al., 2011). Briefly, confocal pictures were segmented using ImageJ (http://imagej.nih.gov/ij/) to isolate DBH-positive vesicles and to generate a corresponding region of interest. The cell periphery was outlined using plasma membrane marker staining, and the cell area was transformed into a Euclidean distance map where each pixel has a value of the minimum Euclidean distance from the cell periphery. The relative positions of vesicles were determined according to the mean gray intensity measured in each region of interest once they were transposed onto a Euclidean distance map. Vesicles were considered internalized when the mean gray value was >10 for bovine and mice chromaffin cells. For more details, see supplemental Figure 1 in the study by Ceridono et al. (2011).

Western blotting and subcellular fractionation. Western blots were performed by chemiluminescence using the Super Signal West Dura Extended Duration Substrate system (Pierce). Immunoreactive bands were detected using the Chemi-Smart 5000 image acquisition system and were quantified using Bio-1D software (Vilber Lourmat).

Subcellular fractionation was performed as previously described (Vitale et al., 1996). Plasma membrane, cytosol, and chromaffin granule membranes were purified from bovine adrenal medulla. Adrenal medullary glands were homogenized in 0.32 M sucrose (10 mM Tris-HCl, pH 7.4) and then centrifuged at 800 × *g* for 15 min. The supernatant was further centrifuged at 20,000 × *g* for 20 min to pellet the crude membrane extract, and the 20,000 × *g* supernatant was centrifuged for 60 min at 100,000 × *g* to obtain the cytosol (supernatant). The crude membrane extract was resuspended in 0.32 M sucrose (10 mM Tris-HCl, pH 7.4) and layered on a cushion sucrose density gradient (1–1.6 M sucrose, 10 mM Tris-HCl, pH 7.4), and centrifuged for 90 min at 100,000 × *g* to separate the plasma membrane (upper fraction) from secretory granules (pellet). The plasma membrane and secretory granule fractions were collected and resuspended in TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT). Secretory granule membranes were recovered by centrifugation for 30 min at 100,000 g. Protein concentration in each fraction was determined by Bradford assay, and 20 μg of protein was resolved by SDS-PAGE, transferred to nitrocellulose, and blotted using anti-SNAP25 (plasma membrane marker), anti-DBH (secretory granule membrane marker), or anti-OPHN1 antibodies.

Amperometry. Chromaffin cells from *Ophn1*$^{-/y}$ and *Ophn1*$^{+/y}$ mice or transfected bovine chromaffin cells were washed with Locke's solution and processed for catecholamine release measurements by amperometry. A carbon fiber electrode of 5 μm diameter (ALA Scientific) was held at a potential of +650 mV compared with the reference electrode (Ag/AgCl) and was approached close to GFP-expressing cells. The secretion of catecholamine was induced by a 10 s pressure ejection of 100 mM K^+ solution from a micropipette positioned at 10 μm from the cell and recorded over 100 s. Amperometric recordings were performed with an AMU130 amplifier (Radiometer Analytical), sampled at 5 kHz, and digitally low-pass filtered at 1 kHz. The analysis of amperometric recordings was performed as previously described (Poëa-Guyon et al., 2013) with a macro (obtained from the laboratory of Dr. R. Borges; http://webpages.ull.es/users/rborges/) written for Igor software (Wavemetrics), allowing automatic spike detection and extraction of spike parameters. The number of amperometric spikes was counted as the total number of spikes with an amplitude of >5 pA within 100 s. The spike parameter analysis was restricted to spikes with amplitudes of 5 pA. The quantal size of individual spikes is measured by calculating the spike area above the baseline (Mosharov and Sulzer, 2005). For a pre-spike foot (PSF) signal, the analysis was restricted to spikes with foot amplitudes of 2 pA. The term "PSF amplitude" refers to the maximal amplitude of the foot. The data for amperometric spikes were averaged by individual cell.

Transmission electron microscopy of wild-type and Ophn1 knock-out chromaffin cells in situ *and secretory granule density analysis. Ophn1*$^{-/y}$ and *Ophn1*$^{+/y}$ mice ($n = 3$ of each genotype) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg), and were transcardially perfused with 0.1 M phosphate buffer, pH 7.3, containing 2% paraformaldehyde and 2.5% glutaraldehyde. The 2-mm-thick slices were cut from the adrenal glands and postfixed in 1% glutaraldehyde in phosphate buffer overnight at 4°C. The slices were then immersed for 1 h in OsO4 0.5% in phosphate buffer. The 1 mm^3 blocks were cut in the adrenal medulla, dehydrated, and processed classically for embedding in Araldite and ultramicrotomy. Ultrathin sections were counterstained with uranyl acetate and examined with a Hitachi model 7500 Transmission Electron Microscope. Secretory granules were counted in 13 and 36 chromaffin cells, respectively, from *Ophn1*$^{-/y}$ and *Ophn1*$^{+/y}$ mice with a visible nucleus randomly selected in ultrathin sections from several blocks (one section/block) from each mouse.

Catecholamine measurement assay. Adrenal glands from 8- to 12-week-old *Ophn1*$^{-/y}$ and *Ophn1*$^{+/y}$ mice were dissected, and medulla was separated from fat and cortex under the microscope. Medulla glands were homogenized in fractionation buffer (10 mM Tris, pH 7.4, 0.32 M sucrose, 4 mM sodium bisulfite, and protease inhibitor cocktail). Crude tissue extract was cleared after centrifugation for 15 min at 800 × *g* to remove unbroken cells and nuclei. Postnuclear supernatant was centrifuged for 1 h at 100,000 × *g* to separate secretory granules and membrane-bound vesicles from the cytosol. Catecholamine contents were measured using the 3-CAT Research ELISA Kit (Labor Diagnostika Nord) according to the manufacturer instructions.

Rho GTPase activity assays. Forty-eight hours after siRNA transfection, PC12 cells were washed twice in Locke's solution at 37°C, and were either left unstimulated (10 s in Locke's solution) or were stimulated for 10 s with 59 mM K^+ solution. Cells were quickly lysed at 4°C, and GTP-bound Rac1 and Cdc42 were measured according to the manufacturer instructions using the G-LISA Activation Assay Kit (Cytoskeleton). Cdc42 and Rac1 activity was normalized to the total amount of proteins in the cell lysate (adjusted to 1 mg/ml). GTP-bound RhoA was measured by pull-down experiments. Cells were lysed for 5 min on ice (25 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2% IGEPAL CA-630, 10 mM $MgCl_2$, and protease inhibitor cocktail), scraped, and centrifuged for 2 min at 20,000 × *g* at 4°C. Aliquots were taken from a supernatant of cleared lysates to determine the total amounts of Rho protein and 30 μg of glutathione Sepharose beads bound to the recombinant GST fused to Rho Binding Domain (Cytoskeleton) were added to an equal volume of each lysate for 1 h at 4°C. Beads were gently spun down and washed four times with 25 mM Tris HCl, 40 mM NaCl, 30 mM $MgCl_2$, and protease inhibitor cocktail. Precipitated proteins were eluted in Laemmli sample buffer and resolved by SDS-PAGE, and the amounts of Rho proteins estimated by Western blotting using anti-RhoA antibodies. Relative Rho activity was determined by normalizing the amounts of precipitated Rho protein to the total amounts of Rho protein in cell lysate.

Data analysis. Data were analyzed using SigmaPlot version 10 software. Column graphs represent the mean ± SEM obtained from at least three independent experiments performed on different cell cultures. *n* represents the number of experiments or the number of cells analyzed, as specified in the figure legends. Box-and-whisker plots represent the first quartile (bottom line), the median (line in the box), the mean (diamond-shaped mark), and the third quartile (top line). Whiskers correspond to the 10th (bottom) and 90th (top) percentiles, and dots represent outliers. Statistical significance has been assessed using the Mann–Whitney test when the data did not fulfill the requirements for parametric tests. Data were considered to be significantly different when the p value was <0.05.

Results

Expression and distribution of OPHN1 in adrenal medullary chromaffin cells

The expression of OPHN1 in adrenal medullary chromaffin cells was first examined by quantitative RT-PCR and Western blot analysis using adrenal medulla tissue and cerebellum tissue as positive controls. Both OPHN1 RNA messenger and protein were detected in the adrenal medulla and cerebellum from *Ophn1*$^{+/y}$ mouse (Fig. 1*A*,*B*), but, as expected, we were unable to detect OPHN1 mRNA (data not shown) or protein (Fig. 1*B*) in tissue from the *Ophn1*$^{-/y}$ mouse. The subcellular distribution of OPHN1 was analyzed by subcellular fractionation of bovine adrenal medulla tissue (Fig. 1*C*). OPHN1 was detected predomi-

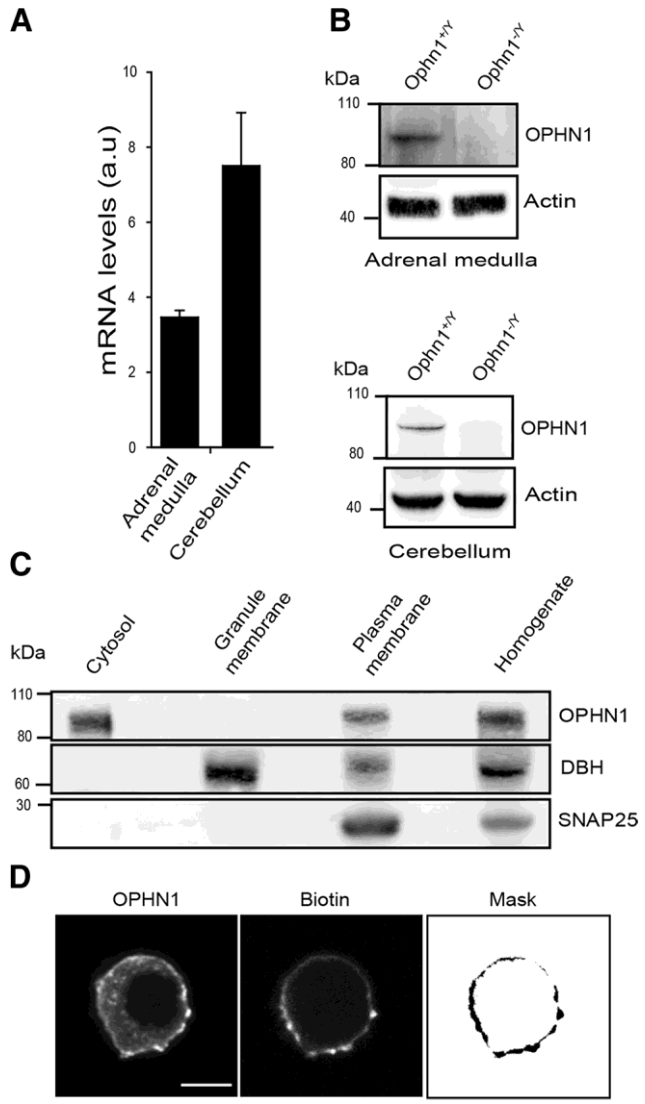

Figure 1. Oligophrenin-1 is expressed at the plasma membrane in adrenal medullary chromaffin cells. ***A***, ***B***, Detection of OPHN1 mRNA by quantitative PCR (***A***) and immunodetection of OPHN1 protein by Western blot (***B***) in adrenal medulla and cerebellum from *Ophn1*$^{+/y}$ and *Ophn1*$^{-/y}$ mice. Actin is shown as the control of loading. ***C***, Subcellular distribution of OPHN1 in bovine adrenal medulla. Fractions obtained by centrifugation on sucrose gradients were col- lected, and subjected to gel electrophoresis (20 μ,g protein/fraction) and immunodetection using anti-SNAP25 antibodies to detect plasma membranes, anti-DBH antibodies to detect chromaffin granules, and anti-OPHN1 antibodies. ***D***, Intracellular localization of ectopically ex- pressed OPHN1 in cultured bovine chromaffin cells. Cells were transfected with the bidirectional expression vector pBI-CMV1 coding simultaneously for EGFP (data not shown) and OPHN1. Plasma membrane was labeled with biotin revealed with Alexa Fluor 633 streptavidin. OPHN1 was detected using anti-OPHN1 antibodies revealed by Alexa Fluor 555-conjugated anti-rabbit antibodies. Mask image highlights the presence of OPHN1 at the plasma membrane. Scale bar, 5 μ,m.

nantly in the cytosol and in plasma membrane-containing fractions revealed by the presence of SNAP25, but it was absent from secretory granule-enriched fractions containing DBH. Because we could not detect endogenous OPHN1 by immunofluorescence with the currently available antibodies, we examined the distribution of exogenously expressed untagged OPHN1 in cultured bovine chromaffin cells labeled with biotin to visualize the plasma membrane. As illustrated in Figure 1*D*, exogenous OPHN1 was mainly present at the cell periphery displaying a staining pattern that colocalized with biotin, confirming the presence of OPHN1 at the plasma membrane in chromaffin cells. OPHN1 staining was also detected as cytosolic puncta. These puncta did not colocalize with DBH, confirming the absence of OPHN1 from secretory granules (data not shown). However, some of them were labeled with EEA1, a specific marker for early endosomes, suggesting a possible recruitment of OPHN1 to early endosomes (data not shown). Note that the distribution of OPHN1 was not modified in chromaffin cells stimulated with a secretagogue (data not shown).

OPHN1 is involved in exocytosis and fusion pore formation

To address the potential role of OPHN1 in exocytosis, we measured catecholamine release from chromaffin cells lacking OPHN1 expression using carbon fiber amperometry to monitor real-time single-granule exocytosis (Mosharov and Sulzer, 2005). Figure 2*A* shows a representative amperometric trace recorded from chromaffin cells cultured from *Ophn1*$^{+/y}$ and *Ophn1*$^{-/y}$ mice. Cells were stimulated with a depolarizing concentration of K^+ for 10 s, and amperometric spikes were measured over a period of 100 s. As illustrated in Figure 2*B*, the number of amperometric events in response to K^+ stimulation appeared to be slightly reduced in *Ophn1*$^{-/y}$ chromaffin cells compared with wild-type cells, indicating that the absence of OPHN1 to some extent affected the number of exocytotic granule fusion events.

Next, we analyzed the shape of the individual amperometric spikes. Each spike represents a single-granule fusion event with the surface area or quantal size being proportional to the amount of catecholamine released per event, with the half-width reflecting the duration of the exocytotic event and the spike height value reflecting the maximal flux of catecholamine (Fig. 2*C*). We found that both the quantal size and the spike amplitude were reduced in knock-out cells compared with wild-type cells (Fig. 2*C*), suggesting that the absence of OPHN1 might affect either the fusion pore formation/expansion or the granule size and/or catecholamine content. Secretory granule catecholamine content was estimated by measuring the levels of epinephrine, norepinephrine, and dopamine in a granule-enriched subcellular fraction prepared from *Ophn1*$^{-/y}$ and *Ophn1*$^{+/y}$ mice adrenal medulla, but no significant differences were observed (Fig. 2*D*). Secretory granules in adrenal glands from *Ophn1*$^{-/y}$ and *Ophn1*$^{+/y}$ mice were also examined by electronic microscopy and morphometric analysis to detect possible morphological changes (Fig. 2*E*). However, neither the intracellular distribution nor the average diameter of large dense core granules were significantly modified in *Ophn1*-deficient chromaffin cells. Thus, the absence of OPHN1 did not modify secretory granule biogenesis and catecholamine storage, suggesting that the reduced amplitude and charge of the amperometric spikes observed in *Ophn1* knock-out cells is likely to reflect a defect in the exocytotic fusion event.

Amperometric spikes are often preceded by so-called PSF currents that are believed to reflect the slow release of catecholamine through an initial narrow fusion pore before its subsequent rapid expansion that gives rise to the spike (Chow et al., 1992; Bruns and Jahn, 1995; Albillos et al., 1997). We assessed whether OPHN1 is involved in the early fusion pore formation by analyzing the PSF currents in wild-type and *Ophn1* knock-out chromaffin cells (Fig. 3). We found that the total PSF charge was largely reduced in cells lacking *Ophn1* (Fig. 3*B*), most likely due to a significant reduction in the maximal foot amplitude (Fig. 3*C*), whereas the foot duration remained unchanged (Fig. 3*D*). PSF amplitude has been correlated to the conductance of the nascent fusion pore (Albillos et al., 1997). Thus, OPHN1 might be implicated in the formation (diameter size) of the initial exocytotic fusion pore.

Altogether, these amperometric results are consistent with a role for OPHN1 in the late stages of large dense core secretory

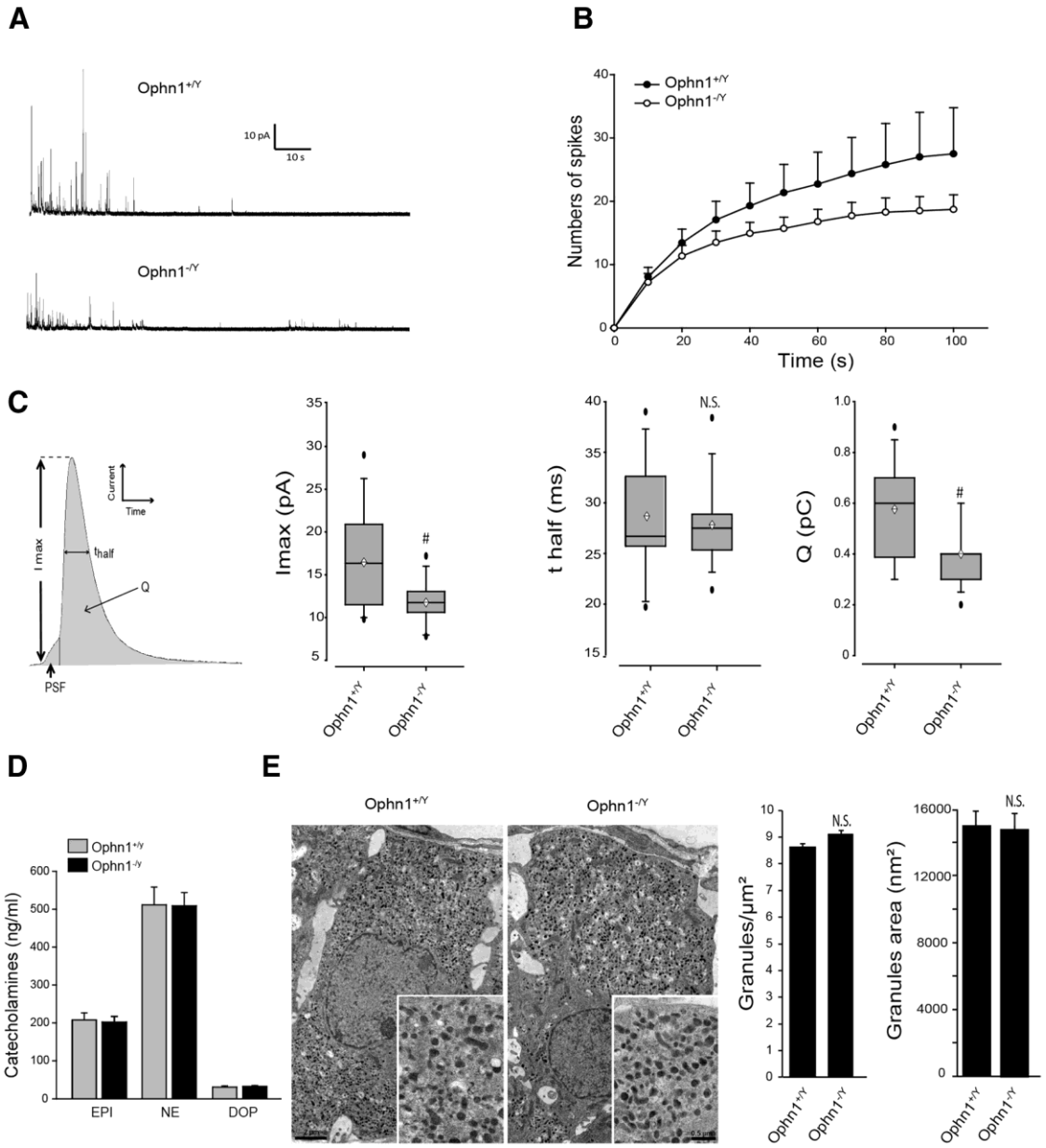

Figure 2. Amperometric analysis of catecholamine secretion from *Ophn1* knock-out mice chromaffin cells. ***A***, Representative amperometric recordings obtained from cultured chromaffin cells from *Ophn1* $^{+/y}$ and *Ophn1* $^{-/y}$ mice. Cells were stimulated for 10 s by a local application of 100 mM K^+. ***B***, Cumulative number of spikes during 100 s of recording *of Ophn1* $^{+/y}$ or *Ophn1* $^{-/y}$ cells. Data are reported as the mean ± SEM. $n = 18$ cells. ***C***, Scheme of an amperometric spike describing the following different parameters analyzed: quantal size or charge (Q), half-width ($t_{1/2}$), spike amplitude (I_{max}), and PSF signal. Box-and-whisker plot for spike amplitude, half-width, and spike charge in *Ophn1* $^{+/}_{y}$ or *Ophn1* $^{-}_{/y}$ cells are shown. Black circles and white diamonds represent outlier observations and mean values, respectively. Statistical significance for median values was determined by a Mann–Whitney nonparametric median analysis. $n = 18$ cells; #$p < 0.01$. n.s., Not significant. ***D***, Catecholamine content of a secretory granule-enriched subcellular fraction prepared from the adrenal medulla of *Ophn1* $^{+/y}$ and *Ophn1* $^{-/y}$ mice was analyzed for total levels of epinephrine (EPI), norepinephrine (NE), and dopamine (DOP) by ELISA (3CAT Assay, Labor Diagnostika Nord). ***E***, Representative transmission electron micrographs of adrenal medulla slices from *Ophn1* $^{+/y}$ or *Ophn1* $^{-/y}$ mice. Average granule surface area and secretory granule density per square micrometer were measured ($n = 60{,}610$ granules, 78 slices, 3 mice for OPHN1 $^{+/y}$; $n = 66434$ granules, 76 slices, 3 mice for OPHN1 $^{-/y}$).

granule exocytosis, possibly controlling the size of the nascent fusion pore and/or its enlargement to full fusion.

OPHN1 functions as RhoA-GTPase-activating protein to regulate fusion pore formation

In addition to its catalytic Rho-GAP domain, OPHN1 contains a N-terminal BAR domain known to sense membrane curvature (Daumke et al., 2014). To determine whether OPHN1 requires its Rho-GAP activity or its BAR domain to function in exocytosis, we transfected cultured bovine chromaffin cells with a bidirec-

tional expression vector, allowing the constitutive and simultaneous expression of EGFP and wild-type OPHN1, the GAP-dead mutant $OPHN1_{R409L}$ (Nakano-Kobayashi et al., 2009), or the BAR domain lacking mutant $OPHN1_{\Delta BAR}$. Bovine chromaffin cells were chosen for these experiments because we failed to transfect mice chromaffin cells. The expression level and distribution of OPHN1 mutants in chromaffin cells are shown in Figure 4*A*. The two OPHN1 mutants displayed an intracellular localization that was similar to that ob- served for wild-type OPHN1 with a predominant distribution in the cytosol and at the plasma membrane.

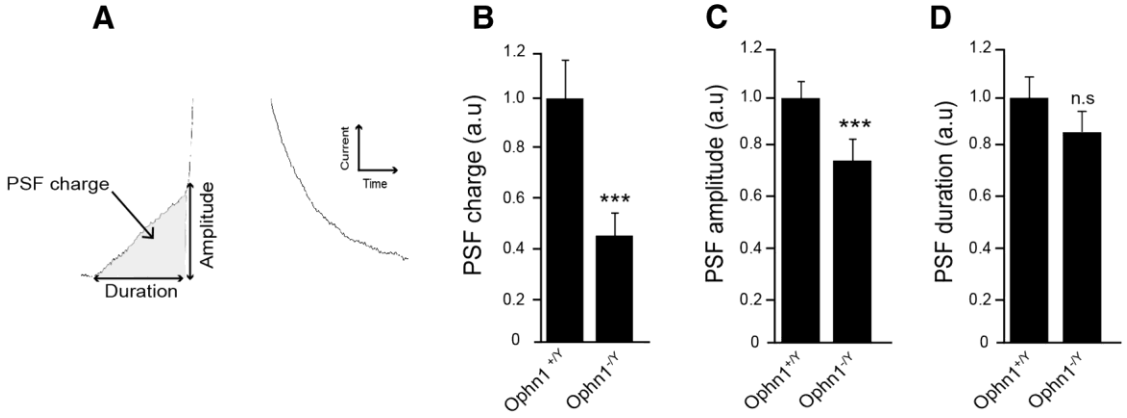

Figure 3. *Ophn1* knock-out reduces the charge and amplitude of the pre-spike foot signal. Cultured chromaffin cells from *Ophn1* $^{+/y}$ and *Ophn1* $^{-/y}$ mice were stimulated by the local application of 100 mM K^+ over 10 s, and secretion was monitored by amperometry. PSF currents recorded from *Ophn1* $^{-/y}$ chromaffin cells (n = 14 cells, 81 PSF currents) were compared with those from *Ophn1* $^{+/y}$ cells (n = 14 cells, 122 PSF currents). ***A***, Scheme of an amperometric PSF describing the following analyzed parameters: amplitude, charge, and duration. ***B***, PSF charge average values. ***C***, PSF amplitude average values. ***D***, PSF duration average values. Data are normalized as percentages of the mean value calculated in *Ophn1* $^{+/y}$ cells and are reported as the mean ± SEM.
***p < 0.001. n.s., Not significant (Mann–Whitney test).

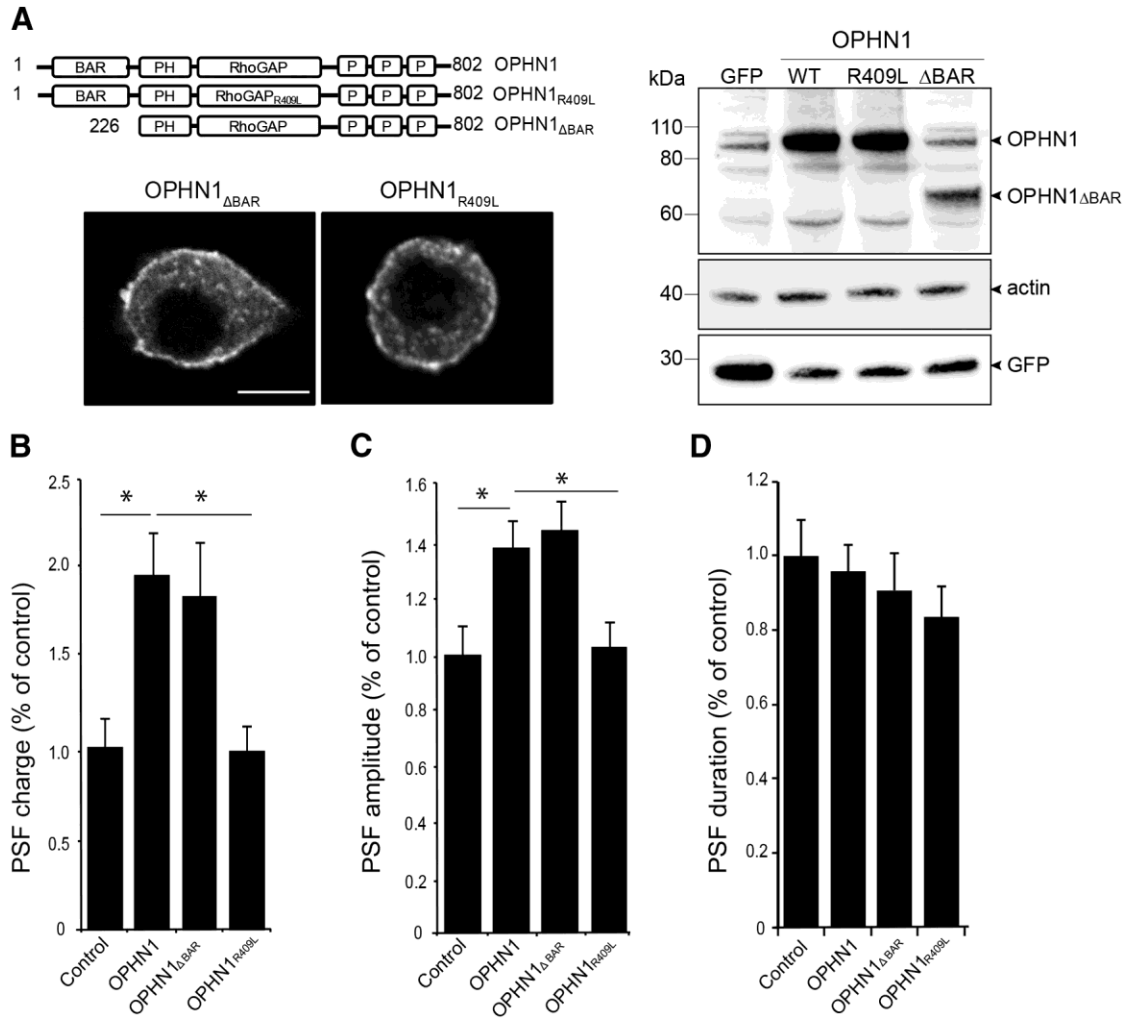

Figure 4. OPHN1 overexpression stimulates fusion pore formation. ***A***, Schematic representation of OPHN1 and the two constructs used in this study depicting the position of the various functional domains. PH, pleckstrin homology domain; P, prolin-rich domain. Bovine chromaffin cells were transfected with a bidirectional expression vector coding simultaneously for EGFP and the indicated OPHN1 constructs. The level of exogenous OPHN1 WT, OPHN1$_{LIBAR}$, and OPHN1$_{R409L}$ expression is analyzed by Western blotting using antibodies against OPHN1, EGFP, and actin. The confocal images show the localization of exogenously expressed OPHN1$_{LIBAR}$ and OPHN1$_{R409L}$ mutants detected by anti-OPHN1 antibodies revealed by Alexa Fluor 555-conjugated anti-rabbit antibodies. Scale bars, 5 μ,m. ***B–D***, Analysis of PSF charge, amplitude, and duration obtained from bovine chromaffin cells expressing EGFP alone (control), OPHN1, OPHN1$_{R409L}$, or OPHN1$_{LIBAR}$. Data are normalized as percentages of control values (considered as 100%) and are reported as the mean ± SEM; control, n = 13 cells, 51 PSF currents; OPHN1, n = 16 cells, 140 PSF currents; OPHN1$_{R409L,}$ n = 22 cells, 61 PSF currents; OPHN1$_{LIBAR,}$ n = 20 cells, 131 PSF currents. *p < 0.05 (Mann–Whitney test). Note that PSF duration remained unchanged in cells expressing OPHN1, OPHN1$_{R409L}$, or

OPHN1$_{LIBAR}$.

Table 1. Table summarizing the amperometric parameters of the exocytotic events recorded from bovine chromaffin cells expressing the indicated OPHN1 proteins

Amperometric parameters	Control	OPHN1	$OPHN1_{LIBAR}$	$OPHN1_{R409L}$
Events per cell (*n*)	22.8±7.5	53.9±10.5*	35.2±9.4	20.7±2.8#
Spikes analyzed (*n*)	191	477	406	307
Q (pC)	1.5±0.2	1.1±0.1	1.3±0.2	1.2±0.1
$t_{1/2}$ (ms)	46.7±2.4	42.1±1.5	43.3±1.9	45.3±2.2
I_{max} (pA)	25.9±2.7	24.7±2.1	27.2±3.3	24.3±2.0
Cells (*n*)	13	16	20	22

Values are given as the mean ± SEM, unless otherwise indicated. Q, quantal size or charge; $t_{1/2}$, half-width; I_{max}, spike amplitude.

*$p < 0.01$ compared with control.

#$p < 0.01$ compared with OPHN1.

Cells expressing OPHN1, $OPHN1_{R409L}$, or $OPHN1_{LIBAR}$ were stimulated with high K^+ and analyzed for catecholamine release by amperometry. Compared with control cells expressing EGFP alone, the overexpression of wild-type OPHN1 increased the number of amperometric events recorded during 100 s but without significantly modifying any of the spike parameters (Table 1). $OPHN1_{LIBAR}$, albeit less effective, enhanced the number of spikes per cell without modifying their shape (Table 1). In contrast, the expression of $OPHN1_{R409L}$ affected neither the number nor the parameters of the amperometric events compared with control cells (Table 1). When PSF currents were analyzed in cells expressing the OPHN1 proteins, OPHN1 and $OPHN1_{LIBAR}$ were found to increase the PSF charge and amplitude without affecting PSF duration, whereas the GAP-dead $OPHN1_{R409L}$ mutant was without effect (Fig. 4 *B*, *C*). These data suggest that OPHN1 requires its Rho-GAP activity but not its BAR domain to modify PSF currents. To strengthen this observation, we have tested the effect of OPHN1 mutant expression on PSF currents in bovine chromaffin cells with OPHN1 expression reduced by shRNA. The rescue OPHN1 (res-OPHN1) constructs used for these experiments (res-OPHN1, res-$OPHN1_{R409L}$, and res-$OPHN1_{LIBAR}$) were generated by introducing two mutations on wobble bases, thus conferring resistance to shRNA degradation (Fig. 5*A*). shRNA-induced reduction of OPHN1 expression did not modify the shape of the amperometric spikes in K^+-stimulated cells (data not shown), perhaps due to the presence of residual OPHN1, but strongly reduced the charge and amplitude of the PSF currents without affecting the duration (Fig. 5*B–D*). Expressing res-OPHN1 or res-$OPHN1_{LIBAR}$ in cells knocked down for OPHN1 restored PSF charge and amplitude, whereas res-$OPHN1_{R409L}$ did not (Fig. 5*B*,*C*), confirming that OPHN1 plays a role in PSF currents through its Rho-GAP domain, but not through its BAR domain. In other words, the function of OPHN1 in fusion pore formation implies at some stage the inactivation of a member of the Rho GTPase family.

In vitro GAP assays indicated that OPHN1 is able to inactivate Cdc42, Rac1, and RhoA (Billuart et al., 1998). To identify the Rho GTPase that might be a target for OPHN1 in the exocytotic machinery, we knocked down endogenous OPHN1 in PC12 cells using a siRNA strategy, and measured the level of activated Rac1, Cdc42, and RhoA in cells stimulated for 10 s with elevated potassium levels. We have chosen PC12 cells for these experiments because 80–90% of the PC12 cell population is efficiently transfected with siRNAs, whereas chromaffin cells in primary culture are resistant to siRNA transfection and efficiently express shRNA in only 10–20% of the cell population.

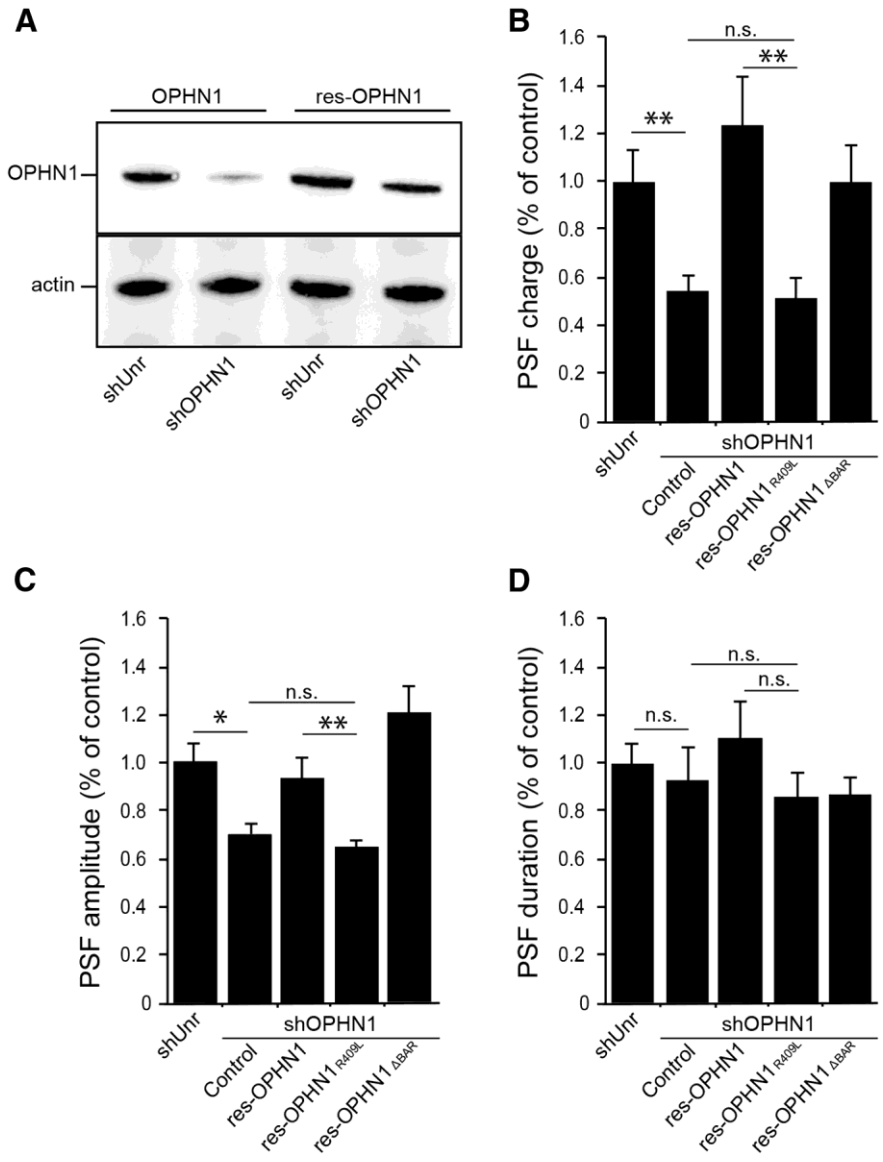

Figure 5. OPHN1 requires its Rho-GAP domain to control fusion pore formation. ***A***, The wobble mutations of OPHN1 cDNA confer resistance to shRNA degradation. Constructs coding for unrelated shRNA (shUnr) or OPHN1 shRNA (shOPHN1) were cotransfected in PC12 cells with vector coding either for OPHN1 or for res-OPHN1. Cells were lysed 48 h after transfection and processed for Western blot analysis using antibodies against OPHN1 and actin. ***B–D***, Analysis of PSF charge, amplitude, and duration obtained from bovine chromaffin cells coexpressing shOPHN1 with EGFP alone (control), res-OPHN1, res-$OPHN1_{R409L}$, or res-$OPHN1_{LIBAR}$. Data are normalized as percentages of control values obtained from chromaffin cells coexpressing EGFP and shUnr, and are reported as the mean ± SEM; shUnr, *n* = 30 cells, 72 PSF currents; shOPHN1/EGFP, *n* = 36 cells, 59 PSF currents; shOPHN1/res- OPHN1, *n* = 24 cells, 52 PSF currents; shOPHN1/res-$OPHN_{R409L}$, *n* = 22 cells, 53 PSF currents; shOPHN1/res-$OPHN_{LIBAR}$, *n* = 36 cells, 87 PSF currents. *$p < 0.05$; ***$p < 0.001$. n.s., Not significant (Mann–Whitney test).

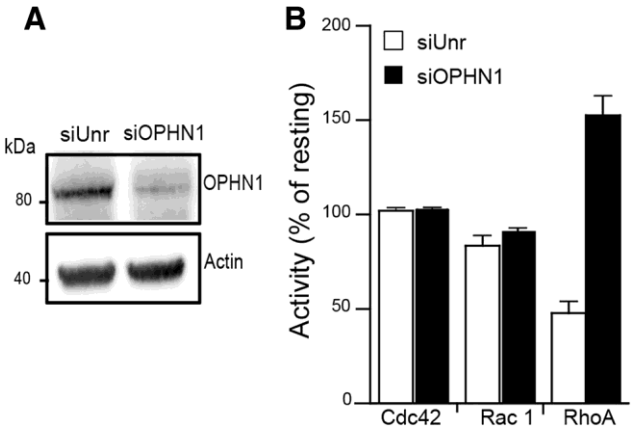

Figure 6. Reduction of the endogenous OPHN1 level affects the RhoA activation/inactivation cycle in secretagogue-stimulated PC12 cells. ***A***, Efficiency of the OPHN1 siRNA. PC12 cells transfected with unrelated (siUnR) or OPHN1 siRNA were lysed 48 h after transfection and processed for Western blot analysis using antibodies against OPHN1 and actin. ***B***, Effect of OPHN1 siRNA on the level of GTP-loaded Cdc42, Rac1, or RhoA in resting and stimulated PC12 cells. PC12 cells transfected with siUnr or OPHN1 siRNA were maintained in resting condition in Locke's solution or were stimulated for 10 s with 59 mM K^+. Cells were then immediately lysed, and the lysates were used for quantification of the level of GTP-loaded Cdc42 and Rac1 by colorimetric-based ELISA assay or by affinity precipitation of GTP-loaded RhoA. RhoA-GTP that was pulled down was detected by immunoblotting using anti-RhoA antibodies and the level of GTP-loaded RhoA quantified by scanning densitometry analysis. Results are normalized as the percentages of the values obtained in resting cells and are reported as the mean ± SEM (n = 3). OPHN1 siRNA did not modify the GTP-loaded RhoA/Cdc42/Rac1 level in resting cells.

Western blot analysis confirmed that PC12 cells transfected with OPHN1 siRNA consistently exhibited a significant reduction (76 ± 5%) in the level of endogenous OPHN1 expression (Fig. 6*A*). In resting PC12 cells, expressing unrelated siRNA (siUnr) or knocking down OPHN1 expression did not significantly modify the steady-state level of GTP-loaded Rac1, Cdc42, and RhoA (data not shown). A 10 s stimulation of control PC12 cells (expressing an unrelated siRNA) with a depolarizing concentration of K^+ did not significantly affect the level of GTP-loaded Cdc42 or Rac1 but inhibited by >50% the level of GTP-loaded RhoA (Fig. 6*B*). Interestingly, the reduction of endogenous OPHN1 strongly increased the level of GTP-loaded RhoA in K^+-stimulated PC12 cells, whereas it did not change the level of GTP-loaded Cdc42 or GTP-loaded Rac1, suggesting that the activation/inactivation cycle of RhoA was significantly affected in K^+-stimulated cells exhibiting a reduced level of OPHN1 (Fig. 6*B*). Thus, OPHN1 appears to be linked to RhoA in the course of calcium-regulated exocytosis.

OPHN1 is implicated in secretory membrane compensatory endocytosis

Since OPHN1 has been described to regulate synaptic vesicle recycling in neurons (Khelfaoui et al., 2009; Nakano-Kobayashi et al., 2009), it is tempting to imagine that it might also be able to play a role in compensatory endocytosis of secretory granules in neuroendocrine cells. We tested this hypothesis by performing an anti-DBH antibody internalization assay in chromaffin cells from knock-out *Ophn1*$^{-/y}$ mice. DBH antibody internalization takes advantage of the transient accessibility of DBH (an intraluminal membrane-associated granule protein) to the extracellular space during exocytotic granule membrane fusion. Granule membrane recapture and compensatory endocytosis can be specifically measured and quantified by following the internalization of the anti-DBH antibodies after exocytosis (Ceridono et al., 2011; Ory et al., 2013). DBH internalization was severely inhibited in the absence of OPHN1 (Fig. 7*A*). We found that 15.9 ± 3.8% of the total DBH was internalized in *Ophn1*$^{-/y}$ cells compared with 59.2 ± 3.3% in *Ophn1*$^{+/y}$ chromaffin cells (Fig. 7*B*), revealing that OPHN1 plays an essential function in the pathway mediating compensatory endocytosis of the secretory granule membrane.

To probe the importance of the BAR domain versus the GAP domain in the function of OPHN1 during compensatory endocytosis, we transfected cultured bovine chromaffin cells to express EGFP and wild-type OPHN1, $OPHN1_{R409L}$, or $OPHN1_{\Delta BAR}$, and examined the effect of these proteins on DBH internalization following K^+-evoked exocytosis. As illustrated in Figure 7, *C* and *D*, DBH internalization remained unchanged in cells expressing wild-type OPHN1 compared with control cells transfected with the empty vector (control). DBH internalization was also unaffected in cells expressing the GAP-deficient $OPHN1_{R409L}$ mutant, suggesting that OPHN1 is not linked to Rho GTPases in the machinery underlying compensatory endocytosis. In contrast, the expression of $OPHN1_{\Delta BAR}$ severely reduced the amount of internalized DBH, which is in line with the idea that the BAR domain of OPHN1 is critical for secretory granule membrane retrieval after exocytosis. Similar results were obtained in bovine chromaffin cells with OPHN1 expression reduced by shRNA, and the expression of res-$OPHN1_{WT}$ or res-$OPHN1_{R409L}$, but not res-$OPHN1_{\Delta BAR}$, significantly rescued endocytotic activity, confirming that the BAR domain of OPHN1 plays an important role in endocytosis (data not shown).

Discussion

Studies from many laboratories have revealed that Rho GTPases are closely connected to vesicular trafficking at the cell surface (for review, see Ory and Gasman, 2011; de Curtis and Meldolesi, 2012; Croisé et al., 2014). In neuroendocrine cells, we and others have described that Rho GTPases control calcium-regulated secretion at diverse crucial stages, including cortical cytoskeletal remodeling and the production of fusogenic lipids at the exocytotic sites (Gasman et al., 1999; Frantz et al., 2002; Li et al., 2003; Gasman et al., 2004; Momboisse et al., 2009a; Wang and Thurmond, 2010; Bretou et al., 2014). Rho GTPases are molecular switches that undergo a tightly regulated activation/inactivation cycle. Activation is under the control of guanine nucleotide exchange factors (GEFs), and inactivation requires GAPs. Rho GEFs have been described in the exocytotic pathway (Malacombe et al., 2006; Momboisse et al., 2009b). In contrast, and despite their potential importance as off signals capable of terminating hormone release, the GAPs triggering Rho GTPase inactivation have so far not been identified. There is only one report proposing that the neuronal Rho GAP nadrin regulates calcium-regulated exocytosis in PC12 cells (Harada et al., 2000), although the physiological relevance of this observation is questioned by the fact that nadrin is not expressed in the adrenal medulla (Harada et al., 2000). In the present study, we report that the Rho-GAP OPHN1 has a pivotal function in chromaffin cells at the crossroads of secretory granule exocytosis and in subsequent compensatory endocytosis. So far, OPHN1 has essentially been described in neuronal dendritic spine development, plasticity, and synaptic vesicle recycling (Govek et al., 2004; Khelfaoui et al., 2007; Khelfaoui et al., 2009; Nadif Kasri et al., 2009; Nakano-Kobayashi et al., 2009). To our knowledge, this is the first report that identifies OPHN1 in the molecular machinery for neuroendocrine secretion.

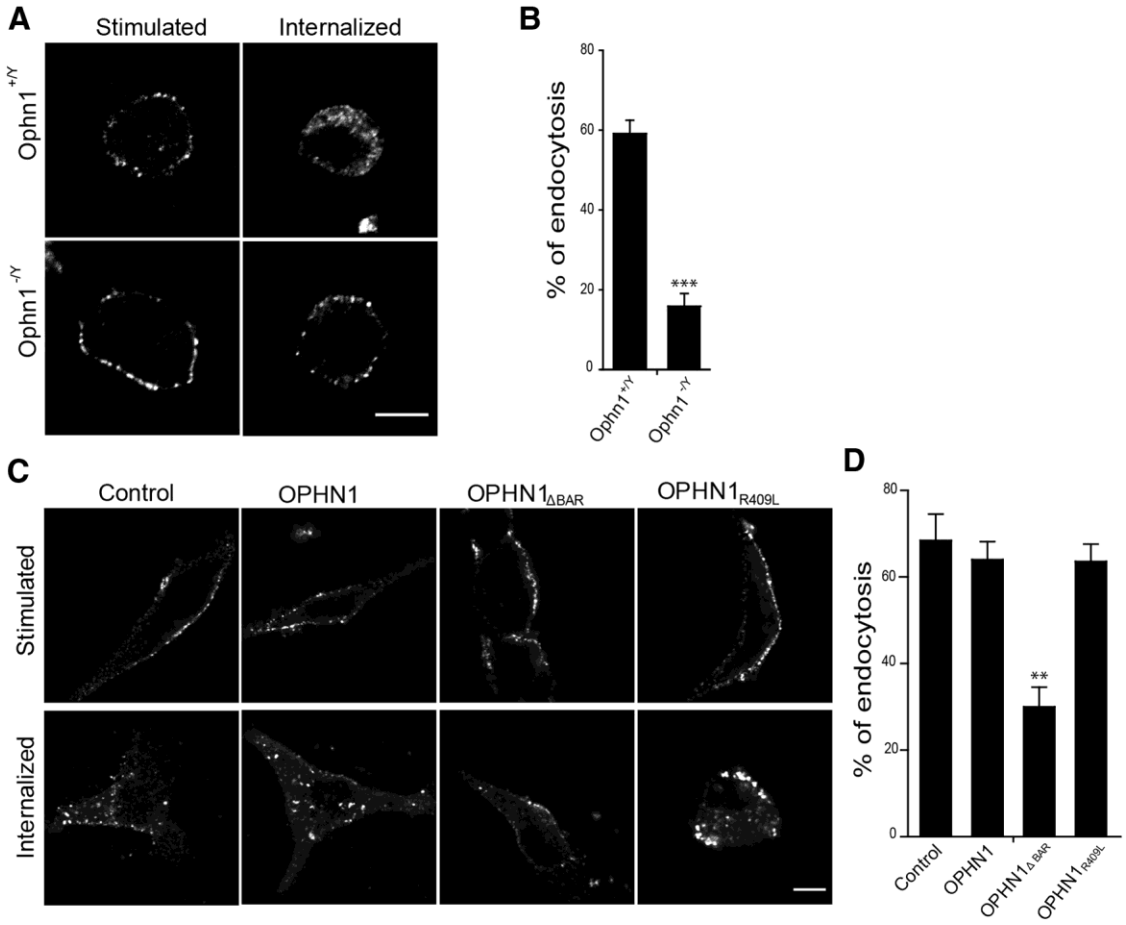

Figure 7. OPHN1 is involved in compensatory endocytosis through its BAR domain. ***A***, Representative confocal images of chromaffin cells from wild-type *Ophn1* $^{+/y}$ and *Ophn1* $^{-/y}$ mice subjected to an anti-DBH antibody internalization assay. Cells were stimulated with 59 mM K^+ for 10 min in the presence of anti-DBH antibodies and fixed (stimulated) or maintained for an additional 15 min period in Locke's solution without antibodies to allow DBH/anti-DBH uptake (internalized). Cells were fixed, permeabilized, and processed for anti-DBH detection using secondary antibodies coupled to Alexa Fluor 555. Scale bar, 5 μm. ***B***, Analysis of DBH-positive vesicle endocytosis using a Euclidean distance map. DBH uptake was reduced by 75% in the absence of OPHN1 (*Ophn1* $^{-/y}$). ***$p < 0.001$ (Mann–Whitney test). ***C***, ***D***, OPHN1 requires its BAR domain to regulate compensatory endocytosis. A anti-DBH antibody internalization assay was performed in bovine chromaffin cells expressing EGFP alone, or EGFP together with OPHN1, $OPHN1_{R409L}$, or $OPHN1_{LIBAR}$. Cells were stimulated with 59 mM K^+ for 10 min and then incubated for 30 min at 4°C in the presence of anti-DBH antibodies. Cells were then fixed (stimulated) or maintained for an additional 15 min period in Locke's solution (internalized) before fixation. Anti-DBH antibody detection was then performed as described above. ***C***, Representative confocal images. Scale bar, 5 μm. ***D***, Analysis of DBH-positive vesicle endocytosis using a Euclidean distance map. Note that only $OPHN1_{LIBAR}$ affects DBH uptake. **$p < 0.01$ (Mann–Whitney test).

Using chromaffin cells from *Ophn1* knock-out mice, we found that individual exocytotic events are affected by the absence of OPHN1. Amperometric analysis of catecholamine secretion revealed a decrease in both the amplitude and charge of pre-spike foots and spikes. This suggests a role for OPHN1 in fusion pore formation and enlargement. Conversely, the overexpression of OPHN1 increased pre-spike foot amplitude and charge, and the number of spikes, but without modifying the shape of the individual spikes. One possible explanation is that other components of the exocytotic fusion machinery might be rate limiting, preventing a further increase of the flux of catecholamines (spike amplitude). As well, OPHN1 expression does not modify the catecholamine content (spike charge) of each secretory granule, which is in line with our observations that granule size and catecholamine storage are apparently unchanged in *Ophn1* knock-out mice. Using OPHN1 mutants, we found that OPHN1 requires its Rho-GAP domain, but not its BAR domain, to play its function in exocytotic fusion, suggesting that a Rho protein is linked to the late stages of exocytosis and needs to be inactivated to somehow release the fusion machinery. Reduction of OPHN1 expression affects essentially the RhoA activation/inactivation cycle in secretagogue-stimulated PC12 cells. Thus, RhoA seems to be a target for OPHN1 in the exocytotic pathway, an idea that receives support from previous reports showing that the expression of a constitutively active RhoA mutant inhibits exocytosis in chromaffin and PC12 cells (Gasman et al., 1998; Bader et al., 2004), and from observations made in neutrophils, indicating that the inactivation of RhoA is a requisite for exocytosis (Johnson et al., 2012). In this context, it is also worth mentioning that the cellular release of vaccinia virus requires the inactivation of RhoA together with cortical actin depolymerization (Arakawa et al., 2007).

How might RhoA be involved in fusion pore formation? In chromaffin cells, the initial fusion pore between secretory granules and plasma membranes has been proposed to be essentially of a lipid nature and controlled by physicochemical laws (Oleinick et al., 2013). However, it is clear that the formation of this initial fusion pore formation requires SNARE proteins (Kesavan et al., 2007; Bretou et al., 2008) and can be influenced by local substructures like the acto-myosin system (Doreian et al., 2008; Neco et al., 2008; Berberian et al., 2009). RhoA might interfere with SNARE functions through the RhoA/ROCK (Rho-kinase) pathway, which has been described to phosphorylate syntaxin1A and/or promote its associa-

tion with negative regulators like tomosyn (Sakisaka et al., 2004; Gladycheva et al., 2007). RhoA/ROCK is also known to phosphorylate myosin light chain and stimulate acto-myosin contraction (Amano et al., 1996; Kimura et al., 1996), which might as well affect fusion pore properties (Doreian et al., 2008; Neco et al., 2008; Berberian et al., 2009). Finally, we cannot currently exclude that, in addition to RhoA, OPHN1 might fulfill its GAP activity towards an as yet unidentified Rho protein involved in the late fusion machinery.

In neuroendocrine cells, secretory granule exocytosis is tightly coupled to compensatory endocytosis, which allows specific granule membrane recapture and maintains cell homeostasis (Ceridono et al., 2011; Houy et al., 2013). We show here that compensatory endocytosis is severely impaired in chromaffin cells lacking OPHN1 expression. In line with this, OPHN1 has been implicated in synaptic vesicle recycling in hippocampal and cortical neurons (Khelfaoui et al., 2009; Nakano-Kobayashi et al., 2009; Powell et al., 2012), as well as in postsynaptic receptor endocytosis (Khelfaoui et al., 2009; Nadif Kasri et al., 2011). Additionally, we found that the expression of the OPHN1 mutant lacking the BAR domain severely inhibited DBH internalization, whereas the expression of the OPHN1 GAP-dead mutant had no effect. These data indicate that OPHN1 through its BAR domain plays an essential function in compensatory endocytosis of large dense core granule in chromaffin cells. BAR domains are known to constrain membranes into specific shapes and sense membrane curvature, and most likely act as scaffolds (Peter et al., 2004; Daumke et al., 2014). As such, BAR domain proteins appear as ideal candidates to regulate endocytotic processes. Experiments performed in neurons revealed that OPHN1 function in synaptic vesicle recycling requires the interaction with endophilin A1, another BAR domain-containing protein involved in endocytosis (Nakano-Kobayashi et al., 2009). Additionally, OPHN1 has been shown to interact with and recruit several other endocytic proteins containing a BAR domain such as amphiphysin and endophilin B2 (Khelfaoui et al., 2009). Whether BAR domain scaffolds are created to sense and/or to generate membrane curvature at the endocytic sites is currently unknown. Moreover, exactly how the OPHN1 BAR domain controls compensatory endocytosis in chromaffin cells and whether it requires additional protein interactions remain to be investigated. The importance of the OPHN1 BAR domain in synaptic vesicle recycling or postsynaptic receptor endocytosis has never been explored. This will be of particular interest because genetic mutations in the *OPHN1* gene leading either to the deletion of the BAR domain or to a nonfunctional BAR domain with a 16 aa in-frame insertion have been detected recently in patients with intellectual disability (Pirozzi et al., 2011; Santos-Rebouças et al., 2014).

To conclude, we describe here for the first time a bifunctional protein, OPHN1, that is involved in both exocytosis and endocytosis in chromaffin cells. As a scaffold multidomain protein, OPHN1 has many assets to tightly coordinate large dense core granule exocytosis to compensatory endocytosis in neuroendocrine cells. Indeed, we found that OPHN1 is linked to the formation of the exocytotic fusion pore through its Rho-GAP domain and controls subsequent granule membrane retrieval through its BAR domain, thereby providing a structural checkpoint to spatially and temporally couple exocytosis and endocytosis in neuroendocrine cells. The next challenging question will be to ask whether OPHN1 might be able to shift the imbalance between exocytosis and endocytosis, and thereby hormone secretory activity in general, and to unravel the upstream regulatory signals. Additionally, from a more physiological point of view, it would be of primary interest to investigate whether, in addition to neuronal defects and associated cognitive disabilities, patients with mutations in the *OPHN1* gene display neuroendocrine disorders.

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VI. OPHN Discussion

1. Oligophrenin-1: a molecular switch between exocytosis and endocytosis of secretory granules

NB: Note that part of this discussion have been published online as a research highlight: *Estay-Ahumada C, Ory S, Gasman S, Houy S. Oligophrenin-1: the link between calcium-regulated exocytosis and compensatory endocytosis in neuroendocrine cells. Neurosci. Commun. 2016; 2: e1251. doi: 10.14800/nc.1251.*

1.1 How OPHN1 regulates calcium-regulated exocytosis ?

As mentioned in the discussion of our article (Houy et al., 2015), our main hypothesis to explain how OPHN1 contributes to the formation of the fusion pore is related to the inhibition of the Rho-kinase (ROCK) pathway, a well known effector of RhoA (Compagnucci et al. 2016). Accordingly, RhoA/ROCK inhibition has been shown to increase insulin secretion in pancreatic-β-cells confirming that the inhibition of RhoA/ROCK pathway is somehow pivotal for (neuro)-endocrine secretion (Liu et al. 2014). I'd like here to re-examine in more detail how inhibition of RhoA pathways might contribute to the dynamic of the fusion pore formation.

One obvious investigative lead is the regulation of the SNARE complex formation which is essential for the initial formation of the fusion pore (Jackson and Chapman 2006). Interestingly, it has been shown that ROCK phosphorylates the t-SNARE Syntaxin1A (STX1A) favoring its interaction with tomosyn (Sakisaka et al. 2004). Tomosyn is a negative regulator of secretion able to reduce the formation of SNARE complex in neuronal model (Sakisaka et al. 2004). Increasing RhoA activity in the absence of OPHN1 might therefore prevent the release of STX1A from tomosyn and reduce the formation of SNARE complex able to drive secretory granule exocytosis (Fig22). To test this hypothesis, we could perform amperometric recording of secretion in OPHN1$^{-/y}$ chromaffin cells transfected with a tomosyn truncated mutant lacking its C-terminal VLD domain. As the VLD domain is responsible for the inhibition of SNARE complex formation, one could expect that tomosyn ΔVLD might compete with endogenous tomosyn and rescue the release from STX1 (Sakisaka et al. 2008).

A second interesting investigative lead is the potential involvement of OPHN1 to the remodeling of the actin cytoskeleton which occurs during exocytosis. Not only can OPHN1 interact directly with actin filaments but the RhoA/ROCK pathway is known to regulate the acto-myosin contraction by enhancing myosin light chain phosphorylation either directly or through the activity of the myosin light chain phosphatase (Amano et al. 1996; Totsukawa et al. 2000). Modulation of the acto-myosin activity in neuroendocrine cells is known to affect several steps of the exocytotic process including granule recruitment at the plasma membrane, docking and fusion (Papadopulos et al. 2015; Bretou et al. 2014; Berberian et al. 2009; Neco et al. 2008). By inhibiting the RhoA/ROCK pathway, OPHN1 can prevent the contraction of actin filaments and thus favoring their relaxation. Interestingly, it has been proposed that the relaxation of the actin cytoskeleton is required for the docking of the granule with the plasma membrane (Papadopulos et al. 2015). Accordingly, a decrease in the number of the exocytotic events, possibly reflecting a docking defect, was also found in chromaffin cells and neurons treated with blebbistatin (Berberian et al. 2009; Miki et al. 2016). In our study, we have observed a slight diminution in the number of the exocytotic events in OPHN1 KO cells. Accordingly, overexpression of OPHN1 significantly increased the number of amperometric spikes, an effect that is reversed by introducing the R409L mutation which inhibits the GTPase activity. Whether, these effects are related to the dynamic of actin filaments through RhoA inhibition remain to be investigated.

Previous studies from our laboratory demonstrated that inactivation of granule-bound RhoA during exocytosis is linked to the organization of the cortical actin network in chromaffin cells (Gasman S. et al. 1997;Gasman S. et al. 1998; Bader et al. 2004). Subsequently, activation of the Rho-GTPase Cdc42 was shown to enhance *de novo* polymerization of actin filaments at granule docking sites (Gasman S. et al. 2004) and annexin-A2 has recently been reported to bundle actin filaments in order to connect the granule membrane with the plasma membrane and regulate the fusion pore dynamics (Gabel et al. 2015). It is tempting to propose that OPHN1-induced inhibition of RhoA is somehow required in this sequence of molecular events. For example, the interaction of OPHN1 with F-actin might allow the specific recruitment of OPHN1 at docking sites in order to inactivate granule-bound RhoA. This inactivation of RhoA could in turn prevent myosin light chain phosphorylation by ROCK, thus modifying the forces generated by the annexin-

A2-induced bundles of filament at the interface between the granule and plasma membranes and affect fusion pore properties.

We have shown that OPHN1 directly regulates the dynamic of the fusion pore formation. Does it involve also actin filaments dynamics? Actin contraction/relaxation can influence membrane tension, known to be one of the driving force for fusion pore dilation (Bretou et al. 2014). Lowering membrane tension with blebbistatin impairs fusion pore expansion in BON cells (Bretou et al. 2014). It would be interesting to compare the membrane tension in the WT versus *Ophn1* knock-out cells, to probe a potential impact of OPHN1 on membrane tension modulation during exo-endocytosis in chromaffin cells.

How could we correlate our data with the studies on OPHN1 performed in neurons which express OPHN1 both pre- and post-synaptically? While a direct function of OPHN1 in neurotransmitter release has not clearly been explored, two studies suggest that it might be the case. It has been shown in hippocampal neurons from *Ophn1* knock-out mice that the size of the readily releasable pool is reduced and that short-term plasticity like paired-pulse facilitation is altered suggesting defects in vesicle availability for exocytosis and the probability of neurotransmitter release (Khelfaoui et al. 2007; Powell et al. 2012). In our study, the number of released vesicle was slightly reduced in knock-out chromaffin cells. However, carbon-fiber amperometry does not provide any indication about the size of the different vesicular pools. To obtain further information about vesicle pool sizes in these cells, capacitance measurements combined with carbon-fiber amperometry are required.

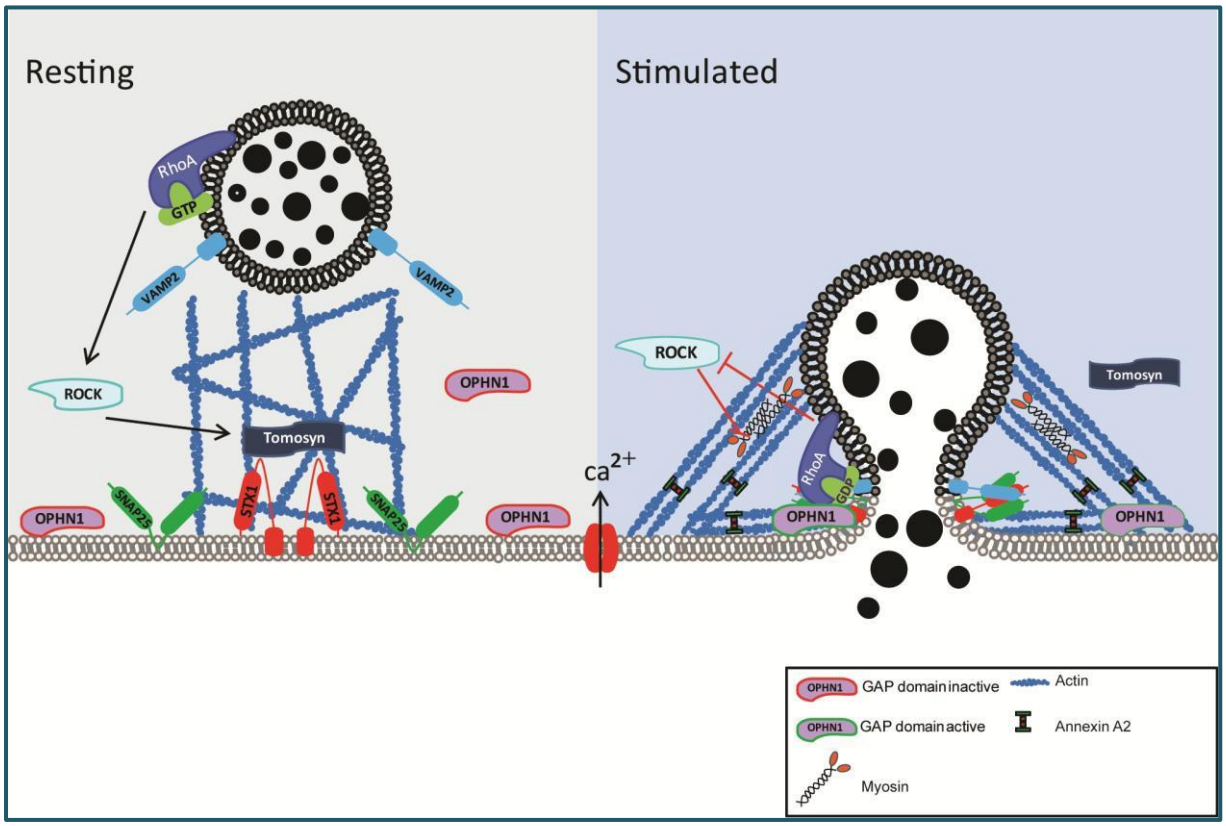

Fig 22: Hypothetical model for the role of OPHN1 in exocytosis in chromaffin cells. In resting condition, the GAP domain of OPHN1 is non-functional maintaining active the granule-bound RhoA. Activated-RhoA might activate its downstream effector, the Rho kinase (ROCK) that subsequently phosphorylates tomosyn enhancing the interaction between syntaxin1 and tomosyn and restrictring the formation of SNARE complex (VAMP-2/Syntaxin-1/SNAP25). In secretagogue-stimulated chromaffin cells, the rise in cytosolic calcium somehow triggers the activation of the OPHN1 GAP activity and the subsequent inactivation of RhoA. Inhibition of RhoA-ROCK pathway might favor the SNARE complex formation by preventing tomosyn-syntaxin1 interaction. Alternatively, it might regulate the level of myosin light chain (MLC) phosphorylation and therefore modulate the myosin-induced forces required for exocytosis.

1.2 How OPHN1 switches from exocytosis to endocytosis ?

The role of OPHN1 in secretory granule recapture is easier to correlate with the data obtained in neurons since OPHN1 has been shown to be pivotal in neurons for synaptic vesicle recycling or post-synaptic receptor endocytosis (Khelfaoui et al. 2009; Nakano-Kobayashi et al. 2009; Nadif Kasri et al. 2011). Nevertheless, the potential implication of the BAR domain of OPHN1 in these processes has never been questioned. On the contrary, it has been shown that OPHN1 is able to recruit and interact with other BAR domain-containing proteins involved in endocytosis including endophilinA1, endophilinB2 and amphiphysin (Khelfaoui et al. 2009; Nakano-Kobayashi et al. 2009). Moreover, an interaction between OPHN1 and endophilinA1 has been proposed to be essential for synaptic vesicle recycling (Nakano-Kobayashi et al. 2009). This clearly raises an issue regarding the exact function of the BAR domain of OPHN1 at synapses and to date, the mechanisms by which OPHN1 regulates compensatory endocytosis in chromaffin cells are open to speculation.

In order to bind and stabilize precise membrane curvature, BAR domains need to form homo- or hetero-dimers leading to the formation of a banana shape. In this context, it is important to note that the interaction with other BAR-domain containing proteins occurs through the C-terminal proline-rich domain of OPHN1 and not directly with its own BAR-domain. Therefore formation of hetero-dimers of BAR domains is a possibility. For example, we can imagine in chromaffin cells that OPHN1, localized in the plasma membrane through its PH domain, could recruit EndophilinA1. Hence, the BAR domains of respectively OPHN1 and EndophilinA1, might associate in heterodimers and form a curve structure, able to stabilize the vesicle that will be internalized. Whether such a conformation occurs in neurons and/or chromaffin cells requires further investigation. Alternatively, OPHN1 could also act as a scaffold protein by recruiting other functional proteins at the place where membranes are curved; in this case where the vesicle will be endocytosed (Mim and Unger 2012; Daumke et al.2014). For example, previous work from our laboratory shown that OPHN1 can interact with intersectin1 (ITSN1) in neuroendocrine cells, a key regulator of endocytosis (Gubar et al. 2012; Gubar et al. 2013). Interestingly, ITSN1 is also a multifunctional scaffold protein able to interact with both proteins of the exocytic machinery (the SNARE SNAP25 for example) and proteins from the endocytic machinery such as dynamin (Okamoto et al. 1999). Therefore,

clustering several scaffold proteins might be a clever way to recruit at the same location all the machinery required for both exocytosis and endocytosis and thus easily couple these two events. But this concept raises an important question: how bifunctional proteins like OPHN1 constantly switch from a role in exocytosis to a role in endocytosis? In other words how does OPHN1 switch from its GAP activity to its BAR activity? Interestingly, the BAR domain itself can interact with the GAP domain leading to the inhibition of the GAP domain (Fauchereau et al. 2003; Eberth et al. 2009). In addition, interaction between the GAP and BAR domains seems to potentiate the BAR-mediated ability of membrane binding (Eberth et al. 2009). An attractive scenario can therefore be considered. OPHN1 is recruited to the exocytotic site in an “open conformation” and regulates fusion pore formation through its Rho-GAP activity. Once the intra-granular contents are released, OPHN1 shifts to an auto-inhibited conformation which blocks the GAP activity and enhances the activity of the BAR domain required for endocytosis. How the BAR domain binds to the GAP domain and how the switch from one conformation to the other is regulated requires further investigations.

VII. General Conclusion

In summary, we can conclude that, i) PLSCR1 and STX1A form a complex in resting chromaffin cells; ii) STX1A and PLSCR1 may dissociate after cell stimulation to perform two separate functions, fusion of LDCV for STX1A and PS scrambling for PLSCR1; iii) STX1A represses PLSCR1 activity. This mechanism may provide a tight synchronization between exocytosis and compensatory endocytosis: STX1A-dependent fusion has to occur to relieve PLSCR1-dependent phospholipid scrambling and provide a cue to initiate compensatory endocytosis. The next challenge will be to understand why phospholipids redistribution at the secretory granule fusion sites is pivotal for secretory granule recapture.

Second, OPHN1 functions as a structural checkpoint that spatially and temporally couples exocytosis and endocytosis in neuroendocrine cells. It appears as a good candidate to ensure a fine tuning of hormone secretory activity. The next challenges will be to decipher the mechanisms by which OPHN1 regulates fusion pore dynamics and to further explore the importance of the OPHN1 BAR domain in endocytic processes. Genetic mutations in OPHN1 gene leading either to the deletion of the BAR domain or to a non-functional BAR domain have recently been reported in patient with an intellectual disability. Along this same line, it would be of primary interest to investigate whether, patients with mutations in the OPHN1 gene display neuroendocrine disorders in addition to neuronal defects and associated cognitive disabilities.

VIII. Materials and Methods

1. Cell culture and Transfection

1.1 Primary culture bovine Chromaffin cells

Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generating Percoll gradients. Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and containing cytosine arabinoside (10 µM), fluorodeoxyuridine (10 µM), streptomycin (50 µg/ml), and penicillin (50 units/ml). Cells were cultured as monolayers either on 24 multiple 16-mm Costar plates (Costar, Cambridge, MA) at a density of 2.5×10^5 cells/well or on fibronectin-coated glass coverslips at a density of 2×10^5 cells and maintained at 37°C, 5% CO2.

1.2 Primary culture mouse Chromaffin cells

Mice were purchased from CDTA (Cryopre´servation, 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 regulations. Mouse chromaffin cells were prepared from 8- to 12-week-old animals of either sex. Adrenal glands were dissected and cleaned in filtered Locke's solution. The glands were gently opened with tweezers, and medulla was freed from fat and cortex under microscope before digestion in 1 ml of papain solution (25 U/ml papain in DMEM supplemented with 0.2 mg/ml L-cystein, 1 mM CaCl2, 0.5 mM EDTA, 0.067 mM -mercaptoethanol, equilibrated in 5%CO2/95% O2) for 30 min at 37°C. The papain activity was inactivated for 5 min by addition of 500µl of DMEM supplemented with 10% heat-inactivated FCS, 2.5 mg/ml albumin, 2.5 mg/ml trypsin inhibitor (Sigma). The solution was carefully removed and replaced by 600 l of complete culture medium (DMEM, containing 0.2% primocin, Amaxa Systems, Lonza; and 1% ITSX, Invitrogen). Medulla were washed twice with complete medium and gently triturated to get a cell suspension in 500µl of complete culture medium. Cells are then seeded on collagen-coated coverslips and maintained at 37°C, 5% CO2.

1.3 Culture of PC12 cells

PC12 cells were grown in DMEM (Dulbecco's Modified Eagle's medium, sigma D5796) supplemented with glucose (4500 mg/l) and containing 30 mM NaHCO3, 5% fetal bovine serum, 10% horse serum, and 100 U/penicillin/streptomycin. The cells are maintained at 37°C, 5% CO2.

2. Transfection

2.1 Transfection of bovine chromaffin cells

Transfection was performed the same day of the primary culture by electroporation (Amaxa Nucleofactor Systems Lonza) according to anufacturer's instructions.

Five million chromaffin cells are centrifuged at 800 rpm for 5min and re-suspended in 100µl of transfection max lonza solution. The cells are then mixed with 3 µg mammalian expression vectors and electroporated with X-001 program. After electroporation, 500 µl DMEM were added in the electroporation tube. The cells were seeded on collagen-coated coverslips, plates or labtek and maintained at 37°C, 5% CO2 for 48 hr before to perform experiments.

2.2 Transfection of PC12 cells

The PC12 cell were transfected with lipofectamin at 50% of confluency. Three µg mammalian expression vectors were mixed with opti-MEM medium (50 µl final volume), and 9µl of lipofectamin were diluted in 41µl of opti-MEM medium. The vector and lipofectamin were mixed and incubated at 37°C for five minutes. Once this complex is formed, it was incorporated in the plates with PC12 cells (with 1-2 ml opti-MEM). The cells were incubated at 37°C, 5% CO2 for 3hr. After transfection the cells were maintained in complete DMEM for PC12 cells at 37°C, 5% CO2 for 48 hr. For transfection with siRNA or siUNR the cells were transfected with RNAmix lipofectamin at 20-25% of confluency. 100pmol of siRNA(siRNASyx1)/siUNR or 80nM siRNA(siRNAOPHN1)/siUNR for OPHN1, were mixed with 12.5 µl lipofectamin in opti-MEM. The cells were incubated at 37°C, 5% CO2 for 8hr. After transfection the cells were maintained in complete DMEM for PC12 cells at 37°C, 5% CO2 for 72 hr.

3. Molecular Biology

3.1 DNA Construct

The N-terminally GFP-tagged mouse scramblase1

The mouse scramblase1 (PLSCR-1) cDNA insert was released from plasmid pMAL-C2-PLSCR1 by double cutting with EcoR1 and SalI, respectively, and then ligated into pEGFP-C2 vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in Escherichia coli strain Top 10, and the orientation and reading frame of the insert were confirmed by sequencing. GFP-PLSCR-1D284A was generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Agilent Technologies).

The pmCherry-C1-Syntaxin1A(Syx1AmCherry) mouse was prepared as previously described (Kavanagh et al. 2014)

N-terminal His and GST tagged PLSCR1

The PLSCR1 cDNA was amplified by PCR using TaqDNA polymerase (Sigma) and specific primers: forward **CAGATCTGAAAACCACAGCAAGGAAC**, and reverse primers: **GGATTCTTACTGCCATGCTCCTGATC**. PLSCR1 amplified was cut by double digestion with restriction enzymes BgIII and EcoR1 (New England Biolab) and then ligated in Pet28b (HIS tag) or pGX (GST tag) vectors. This vector was cut by double digestion using BamH1 and EcoR1. The PLSCR1-HIS and PLSCR1-GST plasmid was amplified from single clones in DH5 bacteria.

HIS- tagged Syntaxin1A

The syntaxin1A was cloned in pMWkan between NdeI and EcoRI restriction sites. The HIS-tagged -syntaxin1A was amplified from single clones in DH5 bacteria.

N-terminal GST tagged Munc18a, Synaptosomal-associated protein 25 and vesicle-associated membrane protein 2.

The munc18, Synaptosomal-associated protein 25 (SNAP25) and vesicle-associated membrane protein 2 (VAMP2) were cloned in pGEX-KG, between BamHI- EcoRI, BamHI and BamHI-HindIII respectively.

OPHN1 vectors

The bidirectional expression vector pBI-CMV1 (Clontech) was used to simultaneously express the enhanced green fluorescent protein (EGFP) and the OPHN1 proteins in transfected cells. The human wild-type (WT) OPHN1 and R409L mutant (provided by Dr. P. Billuart, Institut Cochin, Paris, France) were amplified by PCR using 5'-TATACGCGTGCCACCATGGGTCATCCCCCGCT-3' and 5'-CATGCGGCCGCTCAACTTTCATCTCCAGGAAG-3' primers. The first 225 aa were deleted from WT OPHN1 to generate the OPHN1 ΔBAR mutant using 5'-CGCACGCGTGCCACCATGCAACAGCTCCAACTCAGT-3' and 5'-CATGCGGCCGCTCAACTTTCATCTCCAGGAAG-3' primers. EGFP was amplified using 5'-TATAGATCTCGCCACCATGGTGAGCAAGGGCGA-3' and 5'-CGCCTGCAGTTACTTGTACAGCTCGTCCATGC-3' primers. PCR products were ligated into pBI-CMV1 between the MluI and NotI restriction sites in MCS1 (OPHN1), and the PstI and BglII restrictions sites in MCS2 (EGFP).

Nineteen base pairs long short hairpin RNA were designed to target the bovine sequence of OPHN1 (GAACCTATCTACCACAGCC). Sense and antisense strands separated by a short spacer were synthesized (Life Technologies), annealed, and cloned between the BglII and HindIII sites in front of the H1 promoter of a pmCherry vector. A vector control was generated by cloning an unrelated sequence (ATTCTATCACTAGCGTGAC) between BglII and HindIII sites. For rescue experiments, using the QuickChangeII XL Site-Directed Mutagenesis kit (Agilent Technologies), wobble mutations (codon GAA encoding Glu338 to GAG and codon CCT encoding Pro339 to CCC) were introduced into OPHN1, $OPHN1_{R409L}$, and $OPHN1_{\Delta BAR}$ constructs to make them resistant to OPHN1 short hairpin RNA (shRNA). Forward and reverse primers were, respectively, as follows: 5'-CATGGATGGGAAAGAGCCCATCTACCACAGCCCTA-3' and 5'-TAGGGCTGTGGTAGATGGGCTCTTTCCCATCCATG-3'. All constructs were verified by sequencing.

3.2 Real-time quantitative PCR

Total RNA from mouse adrenal medulla and cerebellum were prepared using the GenElute Mammalian total RNA Miniprep Kit (Sigma-Aldrich) and then treated with RNase-free DNaseI (Thermo Scientific). After checking RNA integrity and concentration by spectrophotometry and agarose gel electrophoresis, the template RNA was transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit for real-time quantitative PCR (Thermo Scientific), according to the manufacturer instructions (1 µg RNA/20 µl reverse transcriptase reaction). PCR was performed in 96-well plates using diluted cDNA samples, highly gene-specific primers, and SyberGreen PCR reagents (IQ SYBR Green Supermix; Bio-Rad).

Gene amplification and expression analyses were performed on a MyIQ real-time PCR machine (Bio-Rad) using a three-step procedure (20 s at 95°C; 20 s at 62°C; 20 s at 72°C) followed by a melting curve study to ensure the specificity of the amplification process. PCR efficiency was evaluated by standard curves analysis and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Gene expression in two different samples was compared using the comparative threshold cycle (Ct) method (Livak and Schmittgen 2001). Each reaction was performed in triplicate, and the sample was related to GAPDH. The mean ΔCt (Ct OPHN1 − Ct GAPDH) was calculated for each condition, and expression levels were determined and represented as $2^{-\Delta Ct}$. Primer sequences used against cDNA of mouse origin (5'–3') were as follows: OPHN1_Fw: CAGGGACCGGTGGACTTAAC; OPHN1_Rv: AGTGATGGTTCCAGGTCTTTCA; GAPDH_Fw: GGCCTTCCGTGTTCCTAC; and GAPDH_Rv: TGTCATCATACTTGGCAGGTT.

3.3 Transformation and culture competent bacteria

The competent DH5α bacteria (50µl) were mixed with 100ng of plasmid and incubated on ice for 30 minutes. Then, bacteria were exposed to a Heat shock at 42°C for exactly 50 seconds without shaking and after incubation the tube was placed on ice for 2 minutes. And then 250µl of pre-warmed (37°C) LB (to make sure this does not contain antibiotics) were added and shaked at 37°C for 1 hour. The bacteria of each transformation were spread onto LB plates with the appropriate antibiotic and incubated at 37°C overnight.

3.4 Plasmid purification

Plasmid Miniprep

The single colony was inoculated in 10 mL of LB medium supplemented with the appropriate antibiotic and incubated overnight at 37°C while shaking at 250 rpm (using a tube or flask with a volume of at least 4 times the culture volume).

The bacterial culture is then centrifuged at 8000 rpm for 5 min at room temperature to obtain a pellet of bacteria. Then, the protocol of plasmid purification thermo scientific GeneJET Plasmid Miniprep kit was followed step by step.

The pelleted cells were resuspended in 250 µl of esuspension solution (Miniprep kit).

Then 250 µl of Lysis Solution were added to the bacterial suspension and mixed thoroughly by inverting the tube 6 times until the solution becomes viscous and slightly clear. After, 350 µl of the Neutralization Solution were added and mixed immediately and thoroughly by inverting the tube 6 times.

A centrifugation for 5 min was performed to the pellet cell debris and chromosomal DNA. The supernatant was transferred to the supplied GeneJET spin column by decanting or pipetting and centrifuge for 1 min. Columns were washed and DNA was eluted with 50µl of elution buffer. DNA concentration was determined and the purified DNA was stored at -20°C.

Plasmid Maxiprep

A single colony inoculated in 500 mL of LB medium supplemented with the appropriate antibiotic and incubated overnight at 37°C while shaking at 250 rpm (using a flask with a volume of at least 4 times the culture volumen).

The bacterial culture was centrifuged at 6000 x *g* for 15 min at 4°C. Then the protocol of plasmid purification from QIAGEN Plasmid Maxiprep kit was followed step by step.

The bacterial pellet was re-suspended in 10ml of buffer P1 and then 10ml of buffer P2 was added. The lysed bacteria were mix thoroughly by vigorously inverting 4–6 times, and incubated at room temperature (15–25°C) for 5 min (the solution will turn blue).Then, 10 ml of pre-chilled Buffer P3 was added and the solution was mix thoroughly, incubated on ice for 20 min.

Centrifugation was performed at 14,000–18,000 x *g* for 10 min at 4°C. Then, equilibration of the QIAGEN-tip 500 was performed by applying 10 ml of buffer QBT. Supernatant was applied to the QIAGEN-tip and centrifuged. QIAGEN-tip was washed with 2 x 30 ml of Buffer QC. DNA was Eluted with 15 ml Buffer QF into a clean 50 ml vessel. DNA is then precipitated by adding 10.5 ml (0.7 volumes) of isopropanol to the eluted DNA. Centrifugation was performed at ≥15,000 x *g* for 30 min at 4°C.

The DNA pellet was washed with 5 ml room-temperature 70% ethanol and centrifuged at ≥15,000 x*g* for 10 min. DNA was redissolved in a suitable volume of appropriate buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).

3.5 Recombinant protein synthesis

HIS-PLSCR1WT, and HIS-Syntaxin1A

The competent rosetta cells (50µl) were mixed with 100ng of plasmid and the protocol of transformation and culture of competent bacteria was followed step by step. A single colony was inoculated in 20ml of M9 minimal medium (200 µl of M9 minimal salts 5X in 777.6ml of sterile water, 20 ml glucose 1M, 2mL MgSO4 1M, 400µl thiamine 10mg/ml, 2ml Biotine 2mg/ml, 42mg each amino acids, 1ml kanamicine 1000X) for 8hrs at 37°C. Then the culture is diluted in 80ml of M9 minimal medium and incubated over night at 37°C. The next morning the bacteria culture is diluted in 900ml M9 minimal medium and incubated at 37°C until reaching to an OD of 0.4-0.6. The following step is the induction of protein, this is acomplish by adding to the culture 1mM IPTG, 20mM proline, 300Mm NaCl final concentration and incubated over night at 18°-20°C. The bacterial culture is centrifuged at 8000rpm, resuspended in 20 ml of equilibrated buffer supplemented with 20µl lisosime, 40µl pic and 20µl DNAse I. Then vortexed and sonicated 3 times. In parallel was washed the HisPur Ni-NTA bead (HisPur^{TM} Ni-NTA Resin Thermo scientific) 2 times at 4°C with equilibrated buffer (20mM sodium phosphate, 300Mm sodium chloride (PBS) with 10mM imidazole; pH7.4 and protease inhibitor) . After sonication, the bacteria were centifugated at 800rpm for 20 min at 4°C. The supernatant is then mixed with the beads and shacked at 4°C for 3hrs.

The beads are then centrifuged at 800rpm, and washed 3 times with washing buffer (PBS with 25mM imidazole pH 7.4) 600mM NaCl and stirring, one time with wash buffer 30mM NaCl and one time with wash buffer 100mM imidazol. The protein is conserved in washing solution (600mM NaCl) at 4°C.

4. Biochemical techniques

4.1 Protein extraction

Untransfected Bovine chromaffin cells

The chromaffin cells are grown in suspension in bacteriological plates. After that they were certifugated at 800rpm for five min and resuspended in cell extraction buffer from invitrogen (FNN0011; 10Mm Tris, pH7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM $Na_4P_2O_7$, 2mM Na_3VO_4,1%Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) supplemented with 1Mm PMSF (phenymethylesulfonyl fluoride) and protease inhibitors (sigma-P8340,500X; 1ml extraction buffer for 10millon of cells). Then the cells were sonicated and incubated on ice for 30 min. The cell lysate is conserved at -20°C.

Transfected bovine chromaffin cells

48 hours after transfection the cells are recovered by trypsination and centrifuged at 800 rpm for 5 min. They were then washed with PBS 1X subsequently centrifuge and resuspended in cell extraction buffer from invitrogen (1ml extraction buffer for 10millon of cells). The cells were sonicated and incubated on ice for 30 min. The cell lysate is conserved at -20°C.

4.2 Pull down assay

For one pull down assay, we use 10 millions of cell lysate, 50µl of beads (His or GST affinity) and 5µg of recombinant protein linked to bead. The first step *was* to make a clear of protein. For that, we incubated the cell lysate with the beads for 1hr at 4°C while shaking.

Then centrifugation was performed at 4000rpm at 4°C for 5min, the supernatant was recovered (taken 20µl for the control). The supernatant was added to a 1.5ml eppendorf tube containing 5µg of recombinant proteins linked to the bead. The mix was incubated for

3hr at 4°C with shaking. For the stimulated condition 5.25µl $CaCl_2$ 1M was added.

After incubation of the protein, centrifugation was performed at 4000rpm at 4°C for 5min (taken 20µl of supernatant for the control), and washed four times minimum with washing buffer (PBS 300mM NaCl ,with 25mM imidazole pH 7.4) for His protein and STE solution for GST protein . Finally the proteins binding to the bead were resuspended in 20µl 5X SDS sample buffer.

Before loading of the samples in the gel, these were heated at 95°C and centrifuged in spin cups paper filter tube (thermo scientific 69700) at 4000rpm at RT for 3min separating the bead of the proteins.

4.3 Co-immunoprecipitation with GFP-Trap A system

For one immunoprecipitacion assay we used 10 million of pc12 cells expressing a PLSCR1WT-GFP. 25µl of GFP Trap A beads were mixed with 500 µl of ice-cold washing buffer (10mM Tris/Cl pH7.5, 150mM NaCl, 0.5mM EDTA) and then centrifuged at 2500g for 2min at 4°C (2 times).

To bind PLSCR1WT-GFP or PLSCR1 D284A –GFP, protein lysate was added to the GFP-Trap bead (saved 20µl of lysate for immunoblot control), and incubated for 1hr at 4°C. Then, the beads were centrifuged at 2500g for 2min at 4°C (saved 20µl supernatant for immunoblot analysis). Afterward, the GFP-Trap A beads were resuspended in 500µl ice-cold dilution buffer and centrifuged at 2500g for 2 min at 4°C. Discarding supernatant and repeating the washing twice. Finally the GFP-Trap A bead were suspended in 50 µl 5X SDS-sample buffer. Before loading the samples into the gel, these were heated to 95°C and centrifuged in spin cups paper filter tube (thermo scientific 69700) at 4000rpm at RT for 3min to separate the beads of the proteins.

4.4 Subcellular fractionation

Subcellular fractionation was performed as previously described (Vitale et al. 1996). Plasma membrane, cytosol, and chromaffin granule membranes were purified from bovine adrenal medulla. Adrenal medullary glands were homogenized in 0.32M sucrose (10 mm Tris-HCl, pH 7.4) and then centrifuged at 800 × g for 15 min. The supernatant was further centrifuged at 20,000 × g for 20 min to pellet the crude membrane extract, and the 20,000 × g supernatant was centrifuged for 60 min at 100,000 × g to obtain the cytosol (supernatant). The crude membrane extract was resuspended in 0.32M sucrose (10 mm Tris-HCl, pH 7.4) and layered on a cushion sucrose density gradient (1–1.6 m sucrose, 10 mm Tris-HCl, pH 7.4), and centrifuged for 90 min at 100,000 × g to separate the plasma membrane (upper fraction) from secretory granules (pellet). The plasma membrane and secretory granule fractions were collected and resuspended in TED buffer (20 mm Tris-HCl, pH 8.0, 1 mm EDTA, 1 mm DTT). Secretory granule membranes were recovered by centrifugation for 30 min at 100,000 g.

4.5 Rho GTPase activity assays

Forty-eight hours after siRNA transfection, PC12 cells were washed twice in Locke's solution at 37°C, and were either unstimulated (10 s in Locke's solution) or were stimulated for 10 s with 59 mM K^+ solution. Cells were quickly lysed at 4°C, and GTP-bound Rac1 and Cdc42 were measured according to the manufacturer instructions using the G-LISA Activation Assay Kit (Cytoskeleton). Cdc42 and Rac1 activity was normalized to the total amount of proteins in the cell lysate (adjusted to 1 mg/ml). GTP-bound RhoA was measured by pull-down experiments. Cells were lysed for 5 min on ice (25 mm Tris-HCl, pH 7.4, 300 mm NaCl, 2% IGEPAL CA-630, 10 mm $MgCl_2$, and protease inhibitor cocktail), scraped, and centrifuged for 2 min at 20,000 × g at 4°C. Aliquots were taken from a supernatant of cleared lysates to determine the total amounts of Rho protein. Then 30 µg of glutathione Sepharose beads bound to the recombinant GST fused to Rho Binding Domain (Cytoskeleton) were added to an equal volume of each lysate for 1 h at 4°C. Beads were gently spun down and washed four times with 25 mM Tris HCl, 40 mM NaCl, 30 mM $MgCl_2$, and protease inhibitor

cocktail. Precipitated proteins were eluted in Laemmli sample buffer and resolved by SDS-PAGE, and the amounts of Rho proteins estimated by Western blotting using anti-RhoA antibodies. Relative Rho activity was determined by normalizing the amounts of precipitated Rho protein to the total amounts of Rho protein in cell lysate.

4.6 Western blotting

Western blots were performed by chemiluminescence using the Super Signal West Dura Extended Duration Substrate system (Pierce). Immunoreactive bands were detected using the Chemi-Smart 5000 image acquisition system and were quantified using Bio-1D software (Vilber Lourmat).

After subcellular fractionation, the protein concentration in each fraction was determined by Bradford assay, and 20 µg of protein was resolved by SDS-PAGE (4-12%), transferred to nitrocellulose, and blotted using anti-SNAP25 (plasma membrane marker), anti-DBH (secretory granule membrane marker), or anti-OPHN1 antibodies. After pulldown or immunoprecipitation, the proteins binding to the bead were re-suspended in 50µl 5X SDS sample buffer, heated for 15 min at 95°C and centrifuged in spin cups paper filter tubes (thermo scientific 69700), after that they were centrifuged at 4000rpm at RT for 3min separating the bead of the proteins. Protein was resolved by SDS-PAGE (gel 4-12% Invitrogen), transferred to nitrocellulose, and blotted using monoclonal antibody anti-Syx1 (HPC1 Sigma), polyclonal antibody anti-GFP (Clinisciences TP401) and polyclonal anti-SNAP25 (Chemicon).

4.7 Induction of Apoptosis by Staurosporine

The PC12 cells were incubated with 1 µM (final concentration) of staurosporine (eg, Sigma S6942) in the Opti-mem medium supplemented with 2mM $CaCl_2$ (final concentration) for 4hr at 37°C. The cells were suspended in cell extraction buffer (InvitrogenFNN0011) and sonicated at 4°C. After 30min of incubation on ice, the extract was centrifuged at 13.000rpm for 10 min at 4°C. The clear lysate (25µg protein) was used for resolving protein by SDS-PAGE

(gel 4-12% Invitrogen), transferred to nitrocellulose, and blotted using polyclonal antibody anti-Caspase 3 active (Millipore AB3623).

4.8 Catecholamine measurement assay

Adrenal glands from 8- to 12-week-old Ophn1$^{-/y}$ and Ophn1$^{+/y}$ mice were dissected, and medulla was separated from fat and cortex under the microscope. Medulla glands were homogenized in fractionation buffer (10 mMTris, pH 7.4, 0.32M sucrose, 4 Mm sodium bisulfite, and protease inhibitor cocktail). Crude tissue extract was cleared after centrifugation for 15 min at 800 × g to remove unbroken cells and nuclei. Postnuclear supernatant was centrifuged for 1 h at 100,000 × g to separate secretory granules and membrane-bound vesicles from the cytosol. Catecholamine contents were measured using the 3-CAT Research ELISA Kit (Labor Diagnostika Nord) according to the manufacturer instructions.

5. Immunofluorescence and microscopy

5.1 Antibodies

Polyclonal anti-OPHN1 antibody has been described earlier (Fauchereau et al. 2003). Monoclonal anti SNAP25 was from Millipore Bioscience Research Reagents and rabbit polyclonal anti-DBH was as previously described (Ceridono et al. 2011) .The mouse monoclonal anti-RhoA (clone 26C4) was from Santa Cruz Biotechnology. For the labeling of endogenous Syx1, the monoclonal antibody anti-Syntaxin1 HPC1 sigma was used. The polyclonal anti-dopamine-β-hydroxylase (DBH) test was performed as described previously (Ceridono et al. 2011). For PS staining AlexaFluor-568-conjugated annexin-A5 (Invitrogen) was used. AlexaFluor-labeled secondary antibodies were obtained from Invitrogen.

5.2 Immunocytochemistry

Chromaffin and PC12 cells grown on fibronectin-coated glass coverslips were washed with Locke's solution and subsequently fixed for 15 min in 4% paraformaldehyde in 1x PBS (Invitrogen), pH 7.0. Then the cells were permeabilized for 10 or 5 min in PBS1X supplemented with Triton X-100 final concentration 0.1%. Following several rinses with PBS solution, cells were pretreated with 3% bovine serum albumin, in PBS to reduce nonspecific staining. Cells were incubated for 2 h at 37 °C with the primary antibodies in PBS containing 3% bovine serum albumin in a moist chamber. Cells were then washed with PBS and subsequently incubated for 1 h at 37 °C with the respective secondary antibodies diluted to 1:1000 in PBS containing 3% bovine serum albumin. Finally, coverslips were extensively washed with PBS, rinsed with water, and mounted in Mowiol 4–88 (Hoechst).

5.3 DBH assay

Anti-DBH antibody internalization assay was performed as previously described (Ceridono et al. 2011; Ory et al. 2013). Briefly, bovine chromaffin cells were washed twice in Locke's solution and further incubated at 37°C in Locke's solution (resting) or stimulated with an elevated K^+ solution for 10 min. Cells were then placed on ice, washed once in Locke's solution, and incubated for 30 min at 4°C in the presence of polyclonal anti-DBH antibodies. Cells were then washed rapidly with Locke's solution and fixed (stimulated) or further incubated in Locke's solution at 37°C for 15 min (endocytosis) before fixation. Cells were then processed for immunofluorescence. For mouse chromaffin cells, cells were rapidly washed and maintained under resting conditions or stimulated for 10 min at 37°C in Locke K^+ solution in the presence of anti-DBH antibodies. Cells were then washed with Locke's solution and fixed or further incubated at 37°C for 15 min before fixation and immunofluorescence experiments. As previously described, the distribution of DBH-containing granules was analyzed using a Euclidean distance map (Ceridono et al. 2011). Briefly, confocal pictures were segmented using ImageJ (http://imagej.nih.gov/ij/) to isolate DBH-positive vesicles and to generate a corresponding region of interest. The cell periphery was outlined using plasma membrane marker staining, and the cell area was transformed into a Euclidean distance map where each pixel has a value of the minimum

Euclidean distance from the cell periphery. The relative positions of vesicles were determined according to the mean gray intensity measured in each region of interest once they were transposed onto a Euclidean distance map. Vesicles were considered internalized when the mean gray value was >10 for bovine and mice chromaffin cells.

5.4 PS staining

To evaluate level of extracellular PS, AlexaFluor-568-conjugated annexin-A5 was used. Chromaffin and PC12 cells were washed two times with 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) and then incubated for 10 min at 37°C in the presence of AlexaFluor-568-conjugated annexin-A5 (Invitrogen) in Locke's solution (resting) or in elevated K^+ solution (Locke's solution containing 59 mM KCl and 85 mM NaCl; stimulated). Cells were then fixed, and annexin-A5 staining was analyzed by confocal microscopy (SP5, Leica Microsystems). Images analyses were performed using ImageJ freeware (http://rsbweb.nih.gov/ij/).

5.5 Confocal microscopy

The acquisitions are performed using confocal laser scanning Leica SP5 with the LAS-AF program (Leica Application Suite Advanced Fluorescence). The proteins fluorescent observed by using different lasers for exciting the fluorophores at wavelengths of 488, 555 and 633nm (argon: λ 488nm; Helium / Neon: λ555nm; and Helium / Neon: λ 633nm).

5.6 Transmission electron microscopy

Ophn1$^{-/y}$ and Ophn1$^{+/y}$ mice (n = 3 of each genotype) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg), and were transcardially perfused with 0.1M phosphate buffer, pH 7.3, containing 2% paraformaldehyde and 2.5% glutaraldehyde. The 2-mm-thick slices were cut from the adrenal glands and postfixed in 1% glutaraldehyde in phosphate buffer overnight at 4°C. The slices were then immersed for 1 h in OsO4 0.5% in phosphate buffer. The 1 mm^3 blocks were cut in the adrenal medulla, dehydrated, and processed classically for embedding in Araldite and ultramicrotomy. Ultrathin sections were

counterstained with uranyl acetate and examined with a Hitachi model 7500 Transmission Electron Microscope. Secretory granules were counted in 13 and 36 chromaffin cells, respectively, from Ophn1$^{-/y}$ and Ophn1$^{+/y}$ mice with a visible nucleus randomly selected in ultrathin sections from several blocks (one section/block) from each mouse.

6. Amperometry

Chromaffin cells from PLSCR1$^{-/-}$, PLSCR1$^{+/+}$, Ophn1$^{-/y}$ and Ophn1$^{+/y}$ mice or transfected bovine chromaffin cells were washed with Locke's solution and processed for catecholamine release measurements by amperometry. A carbon fiber electrode of 5 µm diameter (ALA Scientific) was held at a potential of +650 mV compared with the reference electrode (Ag/AgCl) and was approached close to GFP-expressing cells. The secretion of catecholamine was induced by a 10 s pressure ejection of 100 Mm K^+ solution from a micropipette positioned at 10 µm from the cell and recorded over 100 s. Amperometric recordings were performed with an AMU130 amplifier (Radiometer Analytical), sampled at 5 kHz, and digitally low-pass filtered at 1 kHz. The analysis of amperometric recordings was performed as previously described (Poëa-Guyon et al. 2013) with a macro (obtained from the laboratory of Dr. R. Borges; http://webpages.ull.es/users/rborges/) written for Igor software (Wavemetrics), allowing automatic spike detection and extraction of spike parameters. The number of amperometric spikes was counted as the total number of spikes with an amplitude of >5 pA within the 100 s. The spike parameter analysis was restricted to spikes with amplitudes of 5 pA. The quantal size of individual spikes is measured by calculating the spike area above the baseline (Mosharov and Sulzer 2005). For a pre-spike foot (PSF) signal, the analysis was restricted to spikes with foot amplitudes of 2 pA. The term “PSF amplitude” refers to the maximal amplitude of the foot. The data for amperometric spikes were averaged by individual cell.

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X. Annexes

1. Publication

1. **Catherine Estay-Ahumada,** Stéphane Ory, Stéphane Gasman, Sébastien Houy.Oligophrenin-1: the link between calcium-regulated exocytosis and compensatory endocytosis in neuroendocrine cells. Neuroscience communication. Vol2 2016

2. Houy S, **Estay-Ahumada** C, Croisé P, Calco V, Haeberlé AM, Bailly Y, Billuart P, Vitale N, Bader MF, Ory S, Gasman S. Oligophrenin-1 Connects Exocytotic Fusion to Compensatory Endocytosis in Neuroendocrine Cells.J Neurosci. 2015 Aug 5;35(31):11045-55. doi: 10.1523/JNEUROSCI.4048-14.2015**. (first coauthor)**

3. Croisé P, **Estay-Ahumada** C, Gasman S, Ory S. Rho GTPases, phosphoinositides, and actin: a tripartite framework for efficient vesicular trafficking.Small GTPases. 2014;5:e29469. doi: 10.4161/sgtp.29469. Epub 2014 Jun 10. Review.

2. Publication in preparation

1. **Catherine Estay-Ahumada[1],** Jean-Marc Strub[2], Sarah Cianférani[2], Marie-France Bader[1], Stéphane Ory[1#*] & Stéphane Gasman[1#]. Syntaxin-1 regulates phospholipid scramblase-1-induced lipid reorganization during neuroendocrine secretion. ([#] These authors contributed equally to this work)

2. Marion Gabel, **Catherine Estay Ahumada**, Tamou Thahouly, Nicolas Vitale, Marie-France Bader, Stéphane Gasman and Sylvette Chasserot-Golaz. Tyrosine Phosphorylation switch of Annexin A2 Modulates Calcium-regulated Exocytosis in Neuroendocrine Cells.

3. Charlène Delestre-Delacour[#], Ophélie Carmon[#], **Catherine Estay-Ahumada**, Fanny Laguerre, Maïté Courel, Salah Elias, Juan R. Peinado, Lucie Sengmanivong, Stéphane Gasman, Evelyne Coudrier, Youssef Anouar, and Maité Montero-Hadjadje. Chromogranin A and actomyosin cooperate at the trans-Golgi network to control secretory granule biogenesis. ([#] These authors contributed equally to this work)

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RESEARCH HIGHLIGHT

Oligophrenin-1: the link between calcium-regulated exocytosis and compensatory endocytosis in neuroendocrine cells

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In neuroendocrine cells, hormones and neuropeptides are released from large-dense core vesicles (secretory granules) by calcium-regulated exocytosis. Following exocytosis, compensatory uptake of membrane is required to maintain membrane homeostasis and allow recycling of secretory vesicle membranes. How these cells initiate and regulate this compensatory endocytosis remains poorly understood. Our recent data suggests that oligophrenin-1 (OPHN1) is a link coupling calcium-regulated exocytosis to compensatory endocytosis of secretory granules in the adrenal chromaffin cells (Houy *et al*., 2015, J Neurosci. 2015, 35:11045-55). Here, we highlight the major evidence and discuss how OPHN1 could couple these two processes.

Keywords: calcium-regulated exocytosis; compensatory endocytosis; neuroendocrine secretion; chromaffin cells, oligophrenin1; Rho-GTPases; amperometry

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Introduction

Intracellular membrane trafficking along endocytotic and secretory pathways plays a critical role in diverse cellular functions including developmental and pathological processes. Proteins and lipids destined for transport to distinct locations are collectively assembled into vesicles and delivered to their target site by vesicular fission and fusion. Although much has been learned concerning these mechanisms at donor and acceptor compartments, relatively little attention has been paid to understanding how membrane homeostasis is preserved. This aspect is particularly important in neurosecretory cells in which intense membrane trafficking and mixing occur between the plasma membrane and secretory vesicle membranes during neurotransmission and hormone release.

In neuroendocrine cells, hormones and neuropeptides are stored in large dense-core vesicles (LDCV), the secretory granules. Exocytotic release of hormones and neuropeptides into the blood stream involves four main trafficking steps: i) the tethering of granules to the plasma membrane, ii) their docking at the exocytotic sites through the assembly of SNARE proteins, iii) the priming step rendering the docked granules competent for fusion and iv) the fusion between the granule membrane and the plasma membrane leading to the

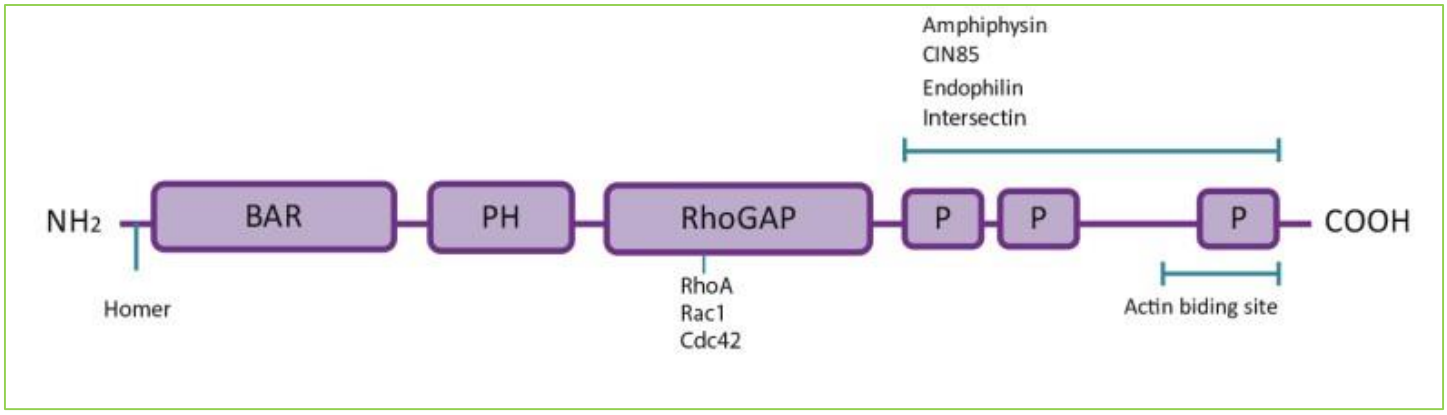

Figure 1. Schematic representation of Oligophrenin-1. OPHN1 is a Rho family GTPase activating protein (Rho-GAP) that contains a N-terminal BAR domain, which senses and binds curved membranes and a Pleckstrin Homology (PH) domain, which binds phosphatidylinositol lipids. The catalytic GAP domain inhibits RhoA, Rac1, and Cdc42 in vitro [2]. Moreover, at the C-terminal part, OPHN1 contains an actin-binding site and three Proline-rich sites permitting the interaction with SH3 domain containing proteins including amphiphysinI and II, CIN85, endophilinA1 and B2, Homer1 and intersectin1 [5, 7, 8, 28].

formation of a fusion pore that expands to release the granule content. While the mechanisms underlying exocytosis per se have been extensively characterized in neuroendocrine cells, how the composition, integrity and functionality of the plasma membrane are maintained after exocytosis is obscure. However, in neuroendocrine chromaffin cells from the adrenal gland, we have proposed that secretory granule proteins cluster together at the plasma membrane after full fusion exocytosis before their recapture by compensatory endocytosis[1]. One of our current research goals is to determine the molecular machinery that enables this sorting, segregation and recapture of secretory granule membrane components.

The characteristics of oligophrenin-1 (OPHN1, figure 1) attracted our attention and suggested it might be a potential candidate for linking the exocytosis and endocytosis in chromaffin cells. OPHN1 was originally discovered as one of the genes implicated in cognitive dysfunctions [2], and has previously been shown to regulate membrane trafficking events linked to synaptic functions, including plasticity, post-synaptic receptor trafficking, and synaptic vesicle recycling [3-9]. It is a multi-domain protein which can interact with membranes through a BAR domain affecting membrane curvature and Pleckstrin Homology (PH) domain which facilitates membrane binding. In addition, OPHN1 is a GTPase activating protein (GAP) of the Rho-family [2] and can also interact directly with cytoskeleton actin filaments and with scaffold proteins with SH3 domains [10]. All these particular features prompted us to examine the role of OPHN1 in exo-endocytosis in chromaffin cells.

Oligophrenin-1, a new actor in calcium-regulated exocytosis

OPHN1 was found to be expressed in adrenal medulla tissue and was localized in the plasma membrane and in the cytoplasm of the adrenal chromaffin cells. Amperometric measurements of catecholamine secretion from cultured chromaffin cells from Ophn1 knock-out mice and from cultured bovine chromaffin cells with knocked down OPHN1 expression revealed that the fusion pore formation is perturbed in the absence of OPHN1. In the bovine chromaffin cells, this phenotype could be rescued by re-expressing OPHN1, but not the GAP-dead OPHN1 mutant ($OPHN1_{R409L}$), indicating that inactivation of a Rho-GTPase by OPHN1 is necessary for fusion pore formation. OPHN1 probably specifically inactivates RhoA during exocytosis because knocking down OPHN1 only significantly increased the level of activated RhoA in stimulated PC12 cells.

Several hypotheses can be proposed to explain how OPHN1 and the RhoA pathways contribute to formation of the fusion pore (figure 2). One possibility is that OPHN1 regulates SNARE complex formation during exocytosis via the RhoA/Rho-kinase (ROCK) pathway. Indeed, deregulation of SNARE complex formation impacts fusion pore formation [11-13]. ROCK-induced phosphorylation of the t-SNARE syntaxin-1A favors its interaction with tomosyn, a negative regulator of secretion [14]. Therefore, OPHN1 may prevent the interaction between syntaxin-1A and tomosyn by inactivating RhoA/ROCK and thereby enhances the formation of the fusion pore. In neurons which express OPHN1 both pre- and post-synaptically, the role of OPHN1 has been studied. While a direct function of OPHN1 in

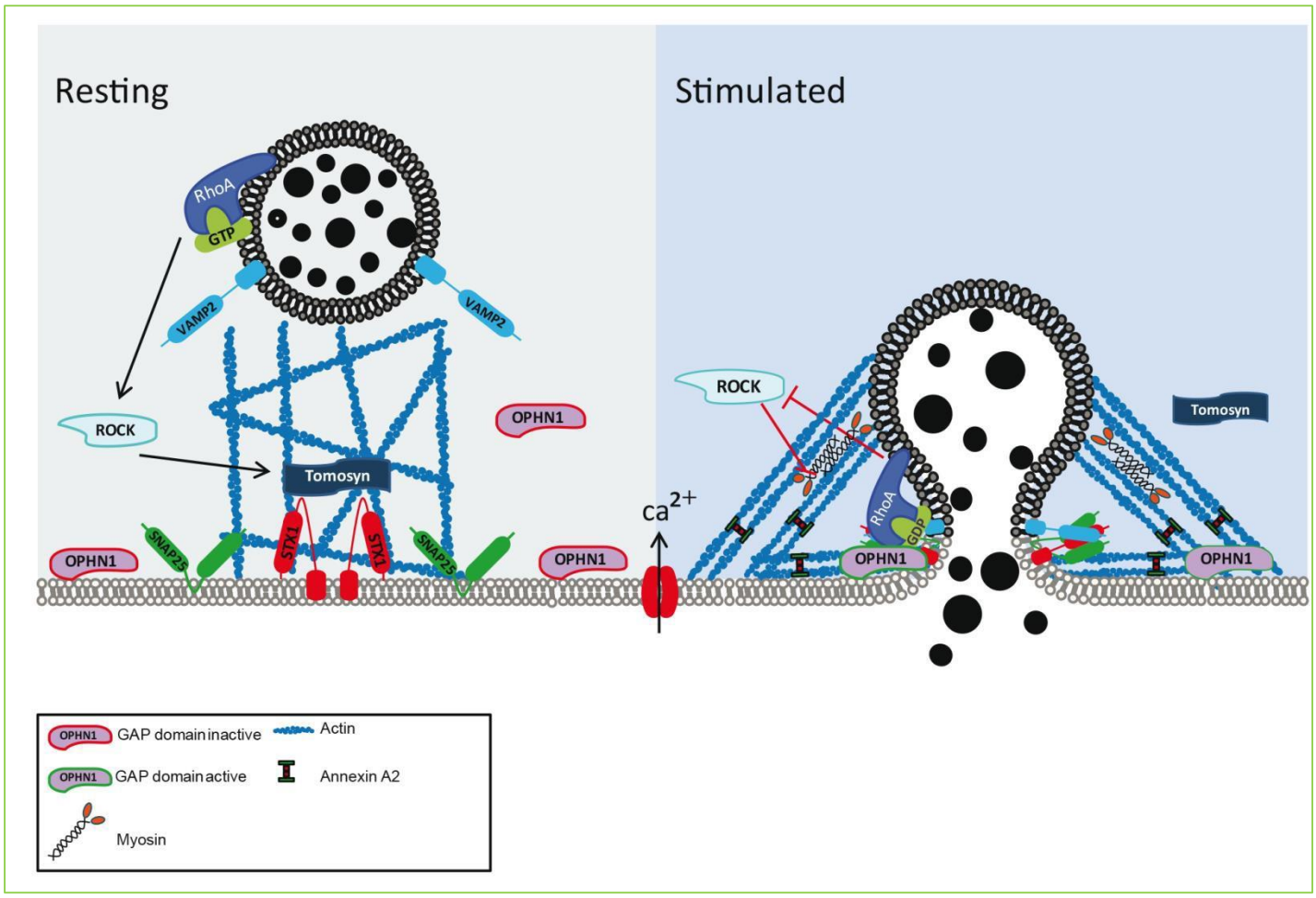

Figure 2. Hypothetical model for the role of OPHN1 in exocytosis in chromaffin cells. In resting condition, the GAP domain of OPHN1 is non-functional which maintains active the granule-bound RhoA. Activated-RhoA might activate its downstream effector, the Rho-kinase (ROCK) that subsequently phosphorylates tomosyn enhancing the interaction between syntaxin-1 and tomosyn and restricting the formation of SNARE complex (VAMP-2/Syntaxin-1/SNAP25). In secretagogue-stimulated chromaffin cells, the rise in cytosolic calcium somehow triggers the activation of the OPHN1 GAP activity and the subsequent inactivation of RhoA. How inactivation of RhoA is linked to fusion pore formation is currently unknown. Inhibition of the RhoA-ROCK pathway might favor the SNARE complex formation by preventing tomosyn/syntaxin-1 interaction. Alternatively, it might regulate the level of myosin light chain (MLC) phosphorylation and therefore modulate the myosin-induced forces required for exocytosis [32]. These two hypotheses are not mutually exclusive and required further investigations.

neurotransmitter release has not clearly been explored, two studies suggest that it might be the case. It has been shown in hippocampal neurons from Ophn1 knock-out mice that the size of the readily releasable pool is reduced and that short-term plasticity like paired-pulse facilitation is altered suggesting defects in vesicle availability for exocytosis and the probability of neurotransmitter release [3, 9]. In our study, the number of released vesicle was slightly reduced in KO chromaffin cells. However, carbon-fiber amperometry does not provide any indication about the size of the different vesicular pools. To obtain further information about vesicle pool sizes in these cells, capacitance measurements combined with carbon-fiber amperometry are required.

Alternatively, OPHN1 may modulate the remodeling of the actin cytoskeleton which occurs during exocytosis. Not only can OPHN1 interact directly with actin filaments but the RhoA/ROCK pathway is known to regulate the acto-myosin contraction by enhancing myosin light chain phosphorylation either directly or through the activity of the myosin light chain phosphatase [15, 16]. Accordingly, modulation of the acto-myosin activity is known to affect fusion pore properties [17-19]. Moreover, previous studies from our laboratory demonstrated that inactivation of granule-bound RhoA during exocytosis is linked to the organization of the cortical actin network in chromaffin cells [20-22]. Subsequently, activation of the Rho-GTPase Cdc42 was shown to enhance de novo polymerization of actin filaments at granule docking sites [23] and annexin-A2 has recently been reported to bundle actin filaments in order to connect the granule membrane with the plasma membrane and regulate the fusion pore dynamics [24]. It is tempting to propose that OPHN1-induced

inhibition of RhoA is somehow required in this sequence of molecular events. For example, the interaction of OPHN1 with F-actin might allow the specific recruitment of OPHN1 at docking sites in order to inactivate granule-bound RhoA. This inactivation of RhoA could in turn prevent myosin light chain phosphorylation by ROCK, thus modifying the forces generated by the annexin-A2-induced bundles of filament at the interface between the granule and plasma membranes and affect fusion pore properties.

Oligophrenin-1 regulates compensatory endocytosis of secretory granules

Using an assay developed in our laboratory to specifically follow the granule membrane recapture after exocytosis (for more details see [1]), we observed a severe decrease (around 70%) of the compensatory endocytosis in chromaffin cells cultured from Ophn1 KO mice. This reveals a major role of OPHN1 in the pathway mediating compensatory endocytosis of the secretory granule membrane. We then demonstrated that overexpression of OPHN1 mutant lacking the BAR domain reproduced the inhibitory effect on granule membrane recapture in bovine chromaffin cells whereas the GAP-dead $OPHN1_{R409L}$ mutant had no effect. These data indicate that the BAR domain of OPHN1 is essential for compensatory endocytosis in chromaffin cells.

The role of OPHN1 in secretory granule recapture is easier to correlate with the data obtained in neurons. OPHN1 is pivotal in neurons for synaptic vesicle recycling or post-synaptic receptor endocytosis [5, 7, 25], yet surprisingly, the potential implication of the BAR domain of OPHN1 in these processes has never been questioned. On the contrary, it has been shown that OPHN1 is able to recruit and interact with other BAR domain-containing proteins involved in endocytosis: endophilinA1, endophilinB2 and amphiphysin [5, 7]. Moreover, an interaction between OPHN1 and endophilinA1 has been proposed to be essential for synaptic vesicle recycling [7]. This clearly raises an issue regarding the exact function of the BAR domain of OPHN1 at synapses. For the moment, the mechanisms by which OPHN1 regulates compensatory endocytosis in chromaffin cells are open to speculation.

In order to bind and stabilize precise membrane curvature, BAR domains need to form homo- or hetero-dimers leading to the formation of a banana shape. Since the interaction with other BAR-domain containing proteins occurs through the C-terminal proline-rich domain of OPHN1, formation of hetero-dimers of BAR domains is a possibility. Whether such a conformation occurs in neurons and/or chromaffin cells requires further investigation. OPHN1 could also act as a scaffold protein by recruiting other functional proteins at the place where membranes are curved; in this case where the vesicle will be endocytosed [26, 27]. For example, we have previously shown that OPHN1 can interact with intersectin1 (ITSN1) in neuroendocrine cells, a key regulator of endocytosis [28].

Conclusion: Oligophrenin-1 is a molecular switch between exocytosis and endocytosis of secretory granules

Altogether, these results clearly demonstrate a role for OPHN1 in the molecular machinery underlying neuroendocrine secretion. In particular, OPHN1 has a bifunctional role both in calcium-regulated exocytosis and compensatory endocytosis. However, an important remaining question is how does OPHN1 switch from its GAP activity required for exocytosis to its BAR activity required for compensatory endocytosis? Interestingly, the BAR domain itself can interact with the GAP domain leading to the inhibition of the GAP domain [10, 29]. In addition, interaction between the GAP and BAR domains seems to potentiate the BAR-mediated ability of membrane binding [29]. An attractive scenario can therefore be considered. OPHN1 is recruited to the exocytotic site in an "open conformation" and regulates fusion pore formation through its Rho-GAP activity. Once the intra-granular contents are released, OPHN1 shifts to an auto-inhibited conformation which blocks the GAP activity and enhances the activity of the BAR domain required for endocytosis. How the BAR domain binds to the GAP domain and how the switch from one conformation to the other is regulated requires further investigations.

To conclude, OPHN1 functions as a structural checkpoint that spatially and temporally couples exocytosis and endocytosis in neuroendocrine cells, and appears as a good candidate to ensure a fine tuning of hormone secretory activity. The next challenges will be to decipher the mechanisms by which OPHN1 regulates fusion pore dynamics and to further explore the importance of the OPHN1 BAR domain in endocytic processes. Genetic mutations in OPHN1 gene leading either to the deletion of the BAR domain or to a non-functional BAR domain have recently been reported in patient with an intellectual disability [30, 31]. Along this same line, it would be of primary interest to investigate whether, patients with mutations in the OPHN1 gene display neuroendocrine disorders in addition to neuronal defects and associated cognitive disabilities.

Conflicting interests

The authors declare that there is no conflict of interest.

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Rho GTPases, phosphoinositides, and actin

A tripartite framework for efficient vesicular trafficking

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Abbreviations: AP-2, adaptor proteins complex 2; BAR, Bin-amphiphysin-Rvs; CLIC, Clathrin-Independent Carrier; CR3, complement receptor 3; CtBP1/BARS, C-terminal-binding protein1/BFA–ADP-ribosylation substrate; CXCR2, chemokine (C-X-C motif) receptor 2; DH, Dbl homology domain; EGFR, epidermal growth factor receptor; FRET, Förster Resonance Energy Transfer; GPI-AP, Glycosylphosphatidylinositol anchored proteins; HGF, hepatocyte growth factor; IGF-1 receptor,
insulin-like growth factor 1 receptor; M-CSF, macrophage colony stimulating factor 1; NGF, nerve growth factor; N-WASP, neural Wiskott-Aldrich syndrome protein; PAK1, p21 protein (Cdc42/Rac)-activated kinase 1; PDGF, platelet-derived growth factor;
PH, pleckstrin homology domain; PI(3)P, Phosphatidylinositol-3-phosphate; PI(3,4)P2), Phosphatidylinositol (3,4)-bisphosphate; PI(3,4,5)P3, Phosphatidylinositol-3,4,5-triphosphate; PI(4)P, phosphatidylinositol-4-phosphate; PI(4,5)P2, Phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PX, Phox homology domain; ROCK1, Rho-associated kinase; TGN, Trans-Golgi network; WASP, Wiskott-Aldrich Syndrome Protein; WAVE, WASP-family verprolin-homologous proteins

rho GTPases are well known regulators of the actin cytoskeleton that act by binding and activating actin nucleators. They are therefore involved in many actin-based processes, includingcellmigration, cellpolarity, andmembrane trafficking. With the identification of phosphoinositide kinases and phosphatases as potential binding partners or effectors, rho GTPases also appear to participate in the regulation of phosphoinositide metabolism. Since both actin dynamics and phosphoinositide turnover affect the efficiency and the fidelity of vesicle transport between cell compartments, rho GTPases have emerged as critical players in membrane trafficking. rho GTPase activity, actin remodeling, and phosphoinositide metabolism need to be coordinated in both space and time to ensure the progression of vesicles along membrane trafficking pathways. although most molecular pathways are still unclear, in this review, we will highlight recent advances made in our understanding of how rho-dependent signaling pathways organize actin dynamics and phosphoinositides and how phosphoinositides potentially provide negative feedback to rho GTPases during endocytosis, exocytosis, and membrane exchange

Introduction

Intracellular membrane traffic governs most aspects of cell homeostasis and behavior by appropriately and accurately transporting vesicles between membranous organelles. The diversity of organelles and the vast array of transported components imply that vesicle delivery has to be stringently regulated to guarantee the fidelity and efficiency of vesicle transport and targeting. Since the early 1990s, two main classes of proteins have been identified as "master regulators" of membrane trafficking: the Rab and Arf subfamilies belonging to the small GTPases of the Ras superfamily, and proteins from the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). Work over the years has demonstrated that these proteins constitute spatial landmarks of vesicular pathways and regulate many aspects of membrane trafficking, including cargo selection during vesicle budding, vesicle transport along actin and microtubule filaments, vesicle tethering to target membranes, and eventually membrane fusion to deliver vesicle contents.[1,2] At the same time, another subfamily of the small GTP-binding proteins of the Ras superfamily, the Rho GTPases, has emerged as new regulator of the actin cytoskeleton, one of the major short range carriers of vesicles in trafficking pathways.[3,4] Therefore, Rho GTPases are also potential regulators of membrane trafficking.

Since the identification of RhoC in 1985,[5] the family of Rho GTPases has expanded to 20 members, divided into 8 subfamilies (Rho, Rac, Cdc42, RhoD/F, Rnd, RhoU/V, RhoH, and RhoBTB),[6] which now tend to be classified into two major groups, the canonical (Rho, Rac, Cdc42, RhoD/F) and the atypical ones (Rnd, RhoU/V, RhoH, and RhoBTB).[7] This classification has evolved from their distinct regulatory modes. The canonical class follow the general scheme of GTP hydrolyzing enzymes, cycling between an inactive GDP-bound and an active GTP-bound form with the aid of guanine nucleotide exchange factors (GEF) and GTPase activating proteins (GAP).[8-10] The GDP-bound form is predominant and mostly found in complex with a guanine dissociation inhibitor (GDI). The GDI stabilizes Rho proteins

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in the cytosol by masking the post-translationally added lipid moiety that serves to anchor Rho GTPases in cellular membranes.[10,11] However, Rho GDI regulation is limited to RhoA, Rac1, Rac2, and Cdc42.[12] In contrast, no GEF, GAP, or GDI have been

Figure 1 (See opposite page). Summary of membrane trafficking pathways regulated by rho GTPases. The figure represents cell compartments and the vesicular pathways in which rho GTPases have a regulatory role. They act by altering the dynamics of the actin cytoskeleton, or the composition in phosphoinositides of the membranes or both. To simplify the scheme, some vesicles are represented with several transmembrane proteins, which are not necessarily present in the same vesicle or even in the same cell type. rhoa, rac1/2, and Cdc42 are mostly localized at the plasma membrane when activated (green circle). Cdc42 and TC10 are also found at the Golgi apparatus and rac1 binds aP1a at the TGN. rhoB, rhoD, and TCL(rhoJ) are enriched in subsets of endosomes or endosome domains encompassing early, late and recycling endosomes. rhoB and rhoD selectively target Src kinases members to the plasma membrane and TCL ensure efficient Tfr recycling. rhoC, rhoF, TCL, rhoU (Wrch-1), and rhoV (Chp) have similar membrane locations as other rho members, mostly endosomes and plasma membrane, but their knock-down impairs constitutive secretion by an unknown mechanism. Despite common subcellular location, rho GTPases differentially regulate endocytosis and exocytosis. Clathrin-mediated endocytosis is blocked by active rhoa and rac1 (cross), but is unaltered by Cdc42, irrespective of its activation state. Macropinocytosis depends on rac1 and rhoG, which cycling are dependent on $PI(3,4,5)P_3$ levels: rhoG for its activation and rac1 for its deactivation (bicolor circle). Both Fcr- and Cr3-dependent phagocytosis require rhoG. Particle engulfment relies on rac1, rac2 (noted as rac) and Cdc42 if bound to Fcr or rhoa if bound to Cr3. Importantly, completion of Fcr-dependent phagocytosis depends on Cdc42 deactivation. Clathrin-independent endocytosis (CLIC/GeeC, DrM, caveolae) depends on different subsets of rho GTPases. although GPI-aP and IL2-r accumulate in cholesterol-enriched membrane domains, their endocytosis is differentially regulated by rho GTPases. Cdc42 is dispensable for IL2r endocytosis, but its activation and/or deactivation cycle is needed for GPI-aP endocytosis. IL2r endocytosis needs $PI(3,4,5)P_3$-dependent rac1 activation by Tiam-1, as well as rhoa activity. During cell migration, some integrin endocytosis is regulated by caveolin, which in turn regulates rhoa, rac1, and Cdc42 activities or expression levels. In addition, rhoG is critical for caveolin-dependent integrin turnover at the plasma membrane promoting efficient cell migration in response to Syndecan-4. Finally, during exocytosis, different subsets of rho GTPases control polarized and regulated exocytosis. TC10 (rhoQ) and Cdc42 are both required for polarized exocytosis and Cdc42 may directly control vesicle fusion by acting on SNare proteins. During regulated exocytosis, differences exist between specialized secretory cells and the type of vesicle released. In response to insulin, GLUT4 exposure to the cell surface of adipocytes relies on rac1, TC10, and Cdc42 with different activation kinetics: TC10 and Cdc42 act early to mobilize vesicles that are docked to the plasma membrane, whereas rac1 recruits vesicle from the storage pool to sustain GLUT4 exocytosis. TC10 deactivation is necessary to complete vesicle fusion. In chromaffin cells and neutrophils,

clearly identified for atypical Rho GTPases and biochemical studies indicate that they are mostly in an active state, bound to GTP and associated with membranes. Their mode of regulation (either positive or negative) is controlled at the transcriptional level and/or by targeted degradation. Compared with canonical Rho proteins, most atypical Rho GTPases possess additional domains that mediate protein-protein interactions and these are likely to be important for their regulation and function.[7,13]

The first evidence of vesicular trafficking controlled by Rho GTPases came from seminal works of Alan Hall's group who demonstrated that active Rac1 stimulated the uptake of extracellular fluid by macropinocytosis.[3] Since then, Rho GTPases have been implicated in many different aspects of membrane trafficking, that rely (or may rely) on Rho-dependent reorganization of the actin cytoskeleton. This occurs through the interaction of Rho GTPases with actin nucleators from the formin and the WASP family, which regulate actin polymerization.[7,14-16] In addition, Rho GTPases interact with various kinases and phosphatases that play a role in regulating actin dynamics and phosphoinositide turnover and both these processes are crucial for membrane trafficking.[17,18] Comprehensive reviews are available on the function of Rho GTPases in the regulation of particular membrane trafficking process.[15,19-23] However, work over the last two decades has shown that Rho GTPases regulate virtually all kinds of exocytic and endocytic processes including constitutive-, polarized-, and regulated-exocytosis, clathrin-mediated endocytosis (CME),[24,25] detergent resistant membrane (DRM)-dependent endocytosis,[26] pinocytosis,[27] macropinocytosis,[3] and phagocytosis.[28] In this review, we compile current evidence indicating how Rho GTPases may control vesicle progression through these trafficking pathways by regulating local actin dynamics, phosphoinositides turnover, or function of complexes involved in vesicle tethering or fusion.

Figure 1 summarizes where Rho GTPases intervene in each type of exo- and endocytosis pathways.

Rho GTPases and Endocytosis

Phospholipid metabolism and actin dynamics in endocytosis

Cell surface proteins, lipids, or extracellular fluids enter endocytic pathways by different mechanisms, which depend on various key molecules, such as specific receptors, clathrin, dynamin, caveolin, or lipid rafts, but also on actin dynamics and phosphoinositide metabolism.[29,30] Although the initial cue and the extent of actin assembly at an endocytic site differ between differentmodesofendocytosis,[31] thegeneralschemeforprogression through endocytic pathways is conserved and requires dynamic actin remodeling and a sequential conversion of phosphoinositides. For example, actin polymerizes extensively to form a large cup around receptor-bound pathogens during phagocytosis or around extracellular fluid during macropinocytosis. In contrast, less polymerized actin is observed during clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis.[31-33] In CME, actin is even dispensable at the initial step of cargo recruitment into coated pits. However, clathrin-coated vesicle maturation, budding, and progression along the endocytic route requires actin reorganization,[31,34-37] indicating that despite differences in amounts of actin polymerization, actin dynamics ensure efficient endocytosis.[34,36-38]

The initial step of most endocytic processes depends on the synthesis of $PI(4,5)P_2$ and its subsequent conversion into different phosphoinositide species ($PI(3,4,5)P_3$, PI(3)P, PI(4)P, and $PI(3,4)P_2$) by phosphoinositide kinases and phosphatases.[18,35,39-44] For example, CME starts with the

recognition of cargo and $PI(4,5)P_2$ by adaptor proteins like AP-2. Clathrin subsequently

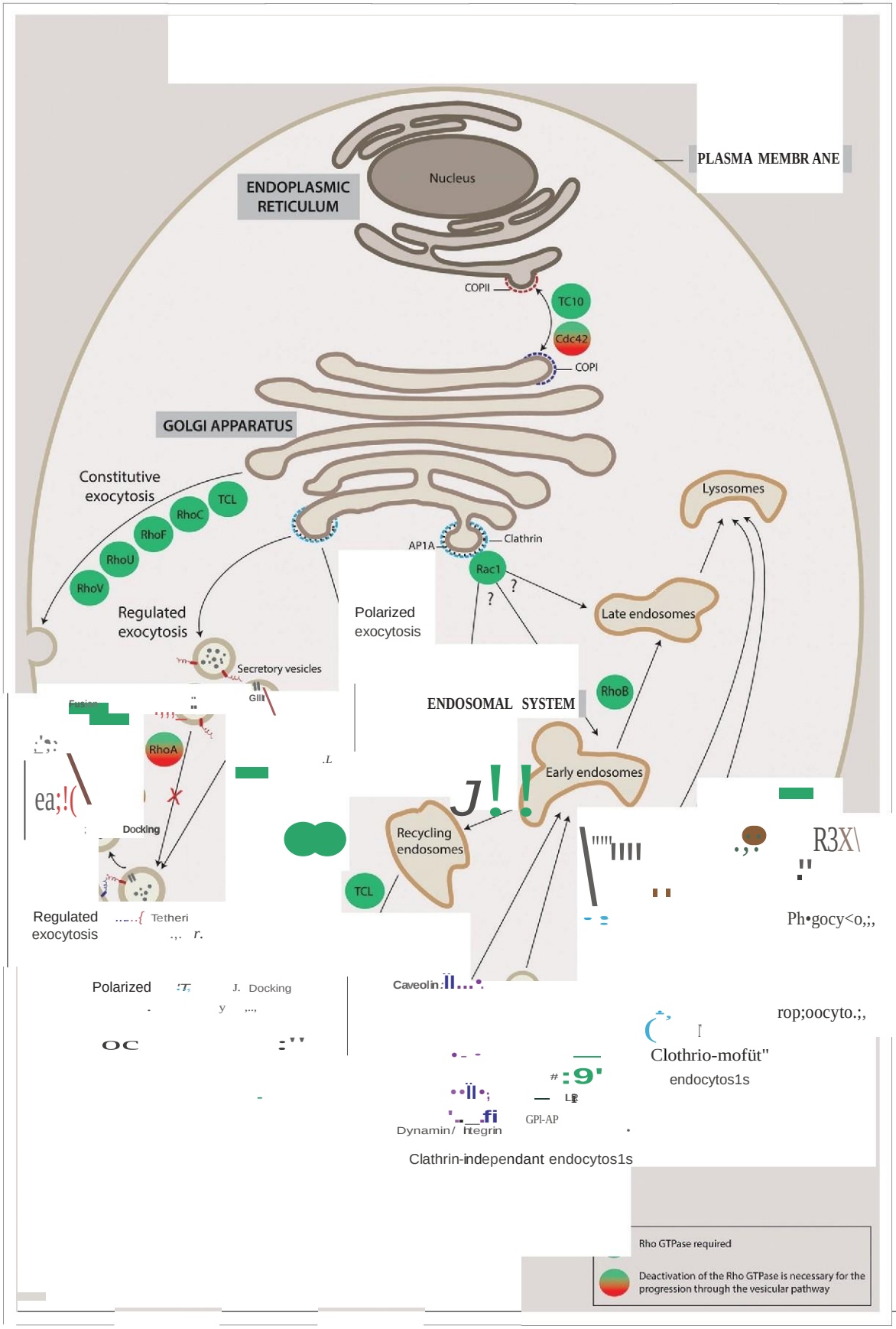

Figure 1. For figure legend, see page 2.

stabilizes the complex and, with the help of accessory proteins (epsins, endophilin, and amphiphysin, for example), the coated membrane bends to form a coated vesicle, which buds until concomitant dynamin-dependent fission and conversion of $PI(4,5)P_2$ into PI(4)P by 5-phosphatases (such as synaptojanin) occur.[45-48] During phagocytosis, the generation of $PI(4,5)P_2$ by type I phosphatidylinositol 4-kinase 5-phosphate (PIP5K) is also required for Fc receptor (FcR) clustering and initiation of particle engulfment.[49] Subsequent reduction of $PI(4,5)P_2$ by PLC, PLD, or PI 3-kinase is necessary for phagocytosis to proceed.[50] Therefore, phosphoinositide switches and actin dynamics constitute major regulatory elements for endocytotic processes.

By binding actin nucleators belonging to the WASp and the Diaphanous formins families, Rho GTPases have been shown to control the formation of large actin-based structures involved in the maintenance of cell shape and the control of cell migration.[19,51,52] In addition to being a signaling intermediate between Rho GTPases and actin, actin nucleators control actin dynamics at discrete steps of endocytic processes.[53-56] Noteworthy, actin binding proteins are also regulated by phosphoinositides, which can form anchoring sites at membranes and/or unfold proteins to locally promote or inhibit actin polymerization.[57,58] Finally, phosphoinositide conversion during endocytosis involves PIP5K,[59,60] class I PI 3-kinase,[61,62] isoforms of the phospholipase C family,[63] and phosphoinositide phosphatases such as synaptojanins[64,65] or OCRL,[66,67] all of which have been shown to bind and/or be activated by RhoA, Rac1, or Cdc42.[68-74] Therefore, by controlling phosphoinositide metabolism and actin polymerization, Rho GTPases are likely to play a critical role in regulating endocytosis. How Rho GTPases interfere with phosphoinositide metabolism and actin dynamics to regulate different endocytic events will be discussed next.

Clathrin mediated endocytosis

The first evidence for a function of Rho GTPases in CME came from studies of transferrin uptake by cells overexpressing constitutively active Rho GTPases. Endocytosis of transferrin (Tf) was blocked by active RhoA and Rac1, but not by active Cdc42, and interestingly, this inhibition occurred independently of actin rearrangement.[24] With the identification of PIP5K and synaptojanin-2 (Synj2) as potential binding partners for RhoA and Rac1,[68,73,75] it was proposed that Rho GTPases control vesicle progression through the endocytic pathway by imbalancing $PI(4,5)P_2$ production. Rac1-dependent recruitment of Synj2 at the plasma membrane was indeed sufficient to diminish CME [73] of Tf by increasing $PI(4,5)P_2$ hydrolysis and knocking down Rac1 by siRNA increased Tf uptake,[76] indicating that Rac1 may negatively regulate CME of Tf by promoting $PI(4,5)P_2$ hydrolysis. However, despite the fact that RhoA and Rac1 stimulate PI(4,5)P production through PIP5K activation[68,75] and that sustained production of $PI(4,5)P_2$ by overexpression of PIP5K is sufficient to increase Tf uptake,[68,75,77] RhoA and Rac1 activation blocked, whereas RhoA and Rac1 inhibition increase CME of Tf. These results are thus inconsistent with the sole function of Rho GTPase to control changes in $PI(4,5)P_2$ levels during CME. Rho GTPases may serve another function, such as regulating the recruitment of accessory proteins at endocytotic sites. For example, the endophilin-A1, a Bin-amphiphysin-Rvs (BAR) domain protein, which is necessary for the completion of clathrin-dependent endocytosis,[78,79] is a substrate for the Rho- associated kinase (ROCK1). When phosphorylated by ROCK1, endophilin-A1 cannot recruit Synj in clathrin-coated pits and results in defective endocytosis of the EGF receptor.[80]

It should be mentioned that most experiments before early 2000s were performed using expression of Rho GTPases locked in either a constitutive active state or a dominant negative form. Although very useful and informative, particularly in the absence of readily available tools, caution has to nonetheless be taken when epitope-tagged GTPases mutants are overexpressed. They may be mislocalized and the extent of overexpression may disturb Rho GTPases pathways.[12,81-84] With the discovery of gene silencing in plants and animals and their use as a tool to knock-down the expression of endogenous proteins in mammalian cells,[85] single gene knock-downs, and also unbiased screen assays using large scale or even genome-wide RNAi libraries have been developed to systematically address the consequence of endogenous protein knock-down in biological processes. For example, the vesicular stomatitis virus (VSV) uses CME to enter the cell, and in an assay designed to identify kinases involved in VSV entry, silencing of PAK1—a well-known effector of Rac1 and Cdc42—was found to increase VSV infection, indicating that by analogy to Rac1 silencing for Tf uptake, Cdc42/Rac1 pathway may have an inhibitory effect on VSV entry.[86]

A genome wide RNAi screening assay was also developed to identify molecular components that regulate endocytosis of both EGF and Tf. This study again highlighted the need for Rac1, but also for RhoD in both of these endocytic pathways. In contrast to the previous cited study,[76] knocking down Rac1 was reported to not increase Tf uptake but, like RhoD, to increase clustering of Tf-positive endosomes close to the nucleus suggesting that these GTPases interfere with vesicle displacement and intervene in endosome maturation process.[87] Interestingly, this assay confirmed that different subsets of adaptor proteins are required for CME of EGF and Tf.[87-89] For example, whereas knocking-down the clathrin heavy chain blocks both Tf and EGF uptake, silencing of AP-2 only inhibits Tf endocytosis. Likewise, knocking-down signaling intermediates or regulators of Rho GTPases usually interferes with both EGF and Tf uptake to similar extents, but some Rho regulators have a selective effect on either EGF or Tf uptake (**Table 1**).[87] This suggests that CME of a given receptor is selectively controlled by specific downstream signaling pathways

dependent on the cycling of Rho GTPases that is determined by
activation of diverse GAPs and GEFs. It remains to be determined whether defects observed in CME are indeed a consequence of an alteration in Rho GTPase cycling. In addition to their conserved Rho GEF or Rho GAP, most regulators of Rho GTPases possess other binding domains that may alter CME independently of Rho GTPase activity. For example, the Cdc42 GEF Intersectin1 and Interectin2 localize to clathrin coated pits through AP-2 binding and regulate both Tf and EGF receptor endocytosis, but the involvement of Cdc42 into CME is still rather elusive.[90-92]

Table 1. effect of knocking-down proteins involved in rho GTPases pathways on transferrin and eGF endocytosis or on endosome distribution (data were extracted from searchable database at http://endosomics.mpi-cbg.de/; Collinet et al., 2010)

Transferrin endocytosis		EGF endocytosis		EGF and Transferrin endocytosis		Endosome redistribution	
Increased	Decreased	Increased	Decreased	Increased	Decreased	Clustered	Dispersed
ARHGEF2	PIK3R1	BCR*	ALS2CL	GMIP	AKAP13	ALS2	PAK1
Chimaerin-2	ARHGAP1*	PLEKHG5*	PREX1	OBSCN	INPP5B	ARHGAP26	PKN1
NET1	*GDI2**	VAV1*	ROCK1		RAC3	ARHGAP6	RACGAP1
PLEKHG1					ARHGEF37**	DOCK4	
Rhophilin-1					DOCK1**	FAM105A	
SH3BP1						RAC1	
VAV2						RHOD	

Proteins are color coded: rho GeF in green, rho GaP in red, rho GTPase in blue, rho effectors in black and rho GDI in black and italicized. *, selective modulation of endocytosis but with accumulation of endosomes in the cell center. **, decrease in eGF endocytosis but increase in transferrin endocytosis.

Macropinocytosis

Unlike CME, macropinocytosis and phagocytosis require extensive actin rearrangements that form a cup at initial steps of the endocytic processes. After internalization, actin depolymerizes. Macropinocytosis is characterized by actin-dependent formation of dorsal membrane ruffles, which occur spontaneously or in response to many growth factors, including PDGF, EGF, M-CSF, or HGF.[93] Dorsal ruffles are different from peripheral membrane ruffles induced by the same growth factors. They depend on different signaling intermediates and their formation is delayed compared with peripheral ruffles. The Rho GTPase Rac1 is involved in both types of membrane ruffles by promoting actin polymerization, but dorsal ruffles relies on WAVE-1-dependent actin remodeling, whereas peripheral membrane ruffles depend on WAVE-2.[53] In addition, unlike peripheral ruffles, dorsal ruffles need functional Rab5 and PI 3-kinase indicating that phosphoinositide production and Rac1 activity may be correlated.[94]

A detailed kinetic analysis of macropinosome formation has provided spatiotemporal insight into the kinetics of phosphoinositide metabolism and Rac1 activation. Upon EGF treatment, $PI(4,5)P_2$ progressively increases in membrane ruffles and PI-3 kinase-dependent production of $PI(3,4,5)P_3$ peaks before cup closure.[95] Monitoring Rac1 activity by FRET microscopy during macropinocytosis showed that Rac1 activity is correlated with $PI(3,4,5)P_3$ production and both reach their maximum in dorsal ruffles prior to macropinosome closure.[96] Interestingly, light-dependent activation of Rac1 is sufficient to trigger macropinosome formation and increase $PI(4,5)P_2$ production, but the macropinosome could not close unless Rac1 is deactivated.[97] Since the cup cannot close in the absence of PI 3-kinase activity, a $PI(3,4,5)P_3$-sensitive Rac GAP may be required for completion of macropinocytosis. Due to the organization of their molecular domain, regulators of Rho GTPases activity are intricately linked to phosphoinositide metabolism. Indeed, the molecular signature of a Rho GEF is a tandem of DH-PH domain (except for GEF of the DOCK family), which confers nucleotide exchange activity toward Rho proteins via the DH domain, and binds to membrane with differential affinity for phosphoinositide species through PX, or BAR domains) in addition to their RhoGAP domain.[98] Up to now, only GEFs for RhoG (SGEF and PLEKHG6) have been found to be sensitive to $PI(3,4,5)P_3$ and involved in macropinocytosis.[100-102] Intriguingly, although stimulation of EGF and PDGF receptors induces Rac1-dependent dorsal ruffles and macropinocytosis, only RhoG is rapidly activated in response to EGF and necessary in this case for dorsal ruffle formation.[101] This raises the question of whether dorsal ruffle formation and macropinocytosis induced by different growth factor are comparable. For example, dorsal ruffles induced by HGF have all the hallmarks of macropinosome initiation: they are dependent on PI 3-kinase, Rab5, and Rac1 activities. However, although macropinocytosis has been shown to be independent of clathrin,[103] HGF stimulation of cells silenced for clathrin did not form dorsal ruffles due to the absence of Rac1 activation on endosomes by one of its exchange factor Tiam-1.[104,105] Since fluid phase uptake was not systematically evaluated in those studies, correlating dorsal ruffle formation and macropinocytosis is difficult. One example of decoupling between membrane ruffles and macropinocytosis has been described for immature dendritic cells, which shows intense membrane ruffling coupled to macropinocytosis. When Rac is inhibited, macropinocytosis is blocked without disturbing membrane ruffling.[106]

Although the correlation between actin rearrangements, phosphoinositides, and macropinocytosis needs to be clarified, Rac1 and RhoG have nonetheless emerged as important Rho GTPases for macropinocytosis. They may also participate in late phases of macropinocytosis, since their common effector PAK1 activates CtBP1/BARS, a potential dynamin counterpart needed for scission of the macropinosome.[107-109]

Phagocytosis

Like macropinocytosis, phosphoinositide turnover and Rho GTPase cycling are critical for phagocytosis, but their involvement depends on the membrane receptor engaged to engulf particles or dead cells. The two best-characterized types, Fc receptor (FcR)- and CR3-dependent phagocytosis, activate different F-actin polymerization pathways triggered by Cdc42/ Rac and RhoA signaling cascades, respectively.[110] During FcR- dependent phagocytosis, PIP5Kα-dependent accumulation

the PH domain.[98,99] Although Rho GAPs have more diverse

domains, they often possess lipid binding domains (PH, C2,

of controls both FcR clustering required for particle $PI(4,5)P_2$

attachment and actin polymerization, which initiates phagocytic

cup formation.[49] In PIP5Kγ knockout cells, RhoA and Rac1 activities are up- and downregulated respectively, and either inhibiting RhoA or activating Rac1 restores particle attachment and phagocytosis.[41,49] This indicates that the defect in $PI(4,5)P_2$ production alters basal levels of Rho GTPase activities and that, although mostly involved in CR3-dependent phagocytosis,[111,112] RhoA may be needed at a discrete step of FcR-dependent phagocytosis for polymerizing actin and promoting initial FcR clustering.[49] FRET experiments designed to examine Cdc42, Rac1, and Rac2 activation showed that their activities are temporally segregated during phagosome cup formation and do not necessarily correlate with actin enrichment. At the time of particle binding and the initiation of particle engulfment, active Cdc42 and Rac1 are localized in the extending pseudopods where actin is enriched. During phagosome cup formation and before closure, active Rac1 and Rac2 are found around the phagosome in regions devoid of actin.[113] PLC, PLD, and PI 3-kinase contribute to the reduction in $PI(4,5)P_2$ levels necessary for phagocytosis to progress.[50] PI 3-kinase is dispensable for Cdc42 and Rac1 activation during phagosome formation, but required for Cdc42 deactivation and phagocytic cup closure,[114] indicating that, a $PI(3,4,5)P_3$-dependent Cdc42 GAP may be required, as observed for Rac1 during macropinocytosis. Interestingly, Rac1 has been recently shown to increase $PI(3,4,5)P_3$ levels by directly binding to the p110β subunit of PI 3-kinase.[70] As the p110β plays a major function in FcR-dependent phagocytosis in macrophages,[115] an intriguing possibility would be that Rac might deactivate Cdc42 by stimulating the production of $PI(3,4,5)P_3$ and thereby ensures phagocytosis progression.

The involvement of Rac1 in FcR-dependent phagocytosis has been recently questioned in RNAi screening assay testing for all Rho GTPases members during FcR and CR3-dependent phagocytosis. In addition to Cdc42, FcR-dependent phagocytosis requires Rac2, but not Rac1. Instead, RhoG has been found to be necessary for phagocytic cup formation and is activated during both FcR and CR3-dependent phagocytosis.[112] Until recently, RhoG was only implicated in apoptotic cell clearance mechanism, triggering Rac-dependent actin remodeling by forming a multimolecular complex with the adaptor ELMO1 and the Rac GEF DOCK180.[116] As RhoG acts upstream of both Rho and Rac,[117] RhoG may have a more general function in phagocytosis by coordinating phosphoinositide signaling and Rho GEF activities in response to the engagement of specific receptors.[118] It remains to be established whether a comparable signaling cascade exists between RhoG and Rac1 during apoptotic cell engulfment and FcR-mediated phagocytosis.

Other clathrin-independent endocytosis: CLIC/GEEC, DRM, and caveolae pathways

Endocytic pathways described in the previous section rely on extensive actin polymerization or clathrin-dependent endocytosis. RhoA, Rac1, Cdc42, and RhoG have been also shown to control clathrin-independent endocytosis,[27,32,119,120] which mainly depends on actin dynamics, caveolae and cholesterol-enriched lipid clusters in the plasma membrane.[29] For example, Cdc42 controls GPI-anchored protein (GPI-AP) endocytosis, which is independent of clathrin, dynamin, or caveolin.[27] GPI-AP and

Cdc42 need to be concentrated into cholesterol-rich nanodomains to promote local actin polymerization and direct GPI-AP into a specific endosomal compartment (GEEC) resulting from the fusion of uncoated tubulovesicular clathrin-independent carriers (CLIC).[27,32] Intriguingly, dominant-negative Cdc42 redirects GPI-AP uptake toward a clathrin-dependent endocytic route.[27] For normal GPI-AP endocytosis, Cdc42 needs to be deactivated by ARHGAP10, which is recruited by Arf1 to nascent endocytic vesicles. GRAF-1, another Cdc42 GAP[119] is necessary for efficient GPI-AP uptake, but unlike ARHGAP10, GRAF1 is mostly located in tubulovesicular structures devoid of Cdc42, and not in Cdc42-positive pinocytic vesicles. The relationship between GRAF1 and Cdc42 is thus unclear, but progression of GPI-AP through the CLIC/GEEC endocytic pathway requires an intact Cdc42 activation cycle.[121,122] These studies also indicate that inhibition of a specific Rho GTPase may divert cargo from their normal route and highlight the versatility of endocytic processes. Together with some other integral membrane proteins such as interleukin receptors, GPI-AP accumulates in detergent resistant membrane (DRM). However, endocytosis of GPI-AP and the interleukin 2 receptor (IL2R) are differentially regulated by Rho proteins. IL2R endocytosis required RhoA and Rac1, but not Cdc42.[26] Interestingly, whereas PI 3-kinase have been shown to be mostly required for large particle or fluid uptake occurring during phagocytosis and macropinocytosis, the p85 subunit of PI 3-kinase appears necessary for IL2R endocytosis by recruiting activated Rac1 to IL2R complex. Rac1 activation is mediated by the $PI(3,4,5)P_3$-dependent recruitment of Vav2 to IL2R endocytic vesicle,[123] and progression through the endocytic pathway is ensured by PAK1-dependent cortactin phosphorylation and formation of a cortactin N-WASP complex.[124,125] This indicates that $PI(3,4,5)P_3$-dependent Rac1 activation and local actin polymerization are essential for IL2R endocytosis.

Caveolins are integral membrane proteins that bind cholesterol and serve as building units for the formation of small (50–80 nm), rounded invaginations in the plasma membrane called caveolae. Caveolae are relatively stable structures, but can detach from the plasma membrane and form endocytic vesicles when triggered by specific signals.[126] Among the protein trafficking controlled by caveolins, the regulation of adhesion molecules such as integrins constitute a major anchorage-dependent growth checkpoint that is overridden in pathological conditions like cell transformation. Upon binding to the extracellular matrix, integrins are well-known activators of Rho GTPases and the absence of integrin engagement deactivates Rac1 and releases it from the plasma membrane.[127] In Caveolin-1 (Cav-1) knockout cells, Rac1 remains active at the plasma membrane even in the absence of integrin engagement indicating that Cav-1-dependent endocytosis regulates Rac1 activity and location.[128,129] Cav-1 may directly reduce Rac1 and Cdc42 activities by increasing Rac1 degradation or acting as a GDI for Cdc42.[130,131] RhoA activity is also indirectly altered by the increased Src activity observed in Cav-1 knockout cells. Src phosphorylates and activates p190RhoGAP, which diminishes RhoA activity.[132] It is thus not surprising that in Cav-1 knockout cells, polarized cell migration is defective due to impaired turnover of adhesion structures.[132]

Interestingly, in response to syndecan-4, cell migration is also impaired when Cav-1 and RhoG-dependent endocytosis of inactive integrin complex is inhibited. This study places RhoG as a downstream effector of Cav-1 necessary to redeploy integrin complexes at the cell surface and subsequent activation of Rac at the leading edge of migrating cells to ensure directed cell migration.[133,134] Cav-1 appears to coordinate both integrin trafficking and Rho GTPase signaling. Thus blocking Cav-1 interaction with Rho GTPases may be an efficient way to reduce metastatic potential of tumor cells.[135]

Finally, among pathogens, many viruses or bacteria produce virulence factors that alter Rho GTPases activities and/or exploit Rho GTPases pathways to infect and invade cells.[136,137] Systematic gene silencing experiments in host-pathogens interaction assays further point out to crosstalk that exist between Rho GTPases signaling and caveolins or cholesterol-rich membrane. For example, the SV40 virus uses caveolins to enter the cell. Systematic silencing of human kinases identified two Rho effectors implicated in SV40 entry. Knocking-down the Cdc42 effector ACK1 blocks whereas knocking-down PAK1 increases SV40 virus entry.[86] The bacteria *Salmonella typhimurium* invades gut tissues by injecting virulence factors into epithelial cells causing diarrhea. Some injected factors activate Rac1 and Cdc42 to trigger membrane ruffles and help bacteria to invade cells. In the absence of the coat protein complex I (COPI), cholesterol- rich membranes are redistributed from the plasma membrane to perinuclear region. As a consequence, Rac1 and Cdc42 are mislocalized and absent from the plasma membrane. Membrane ruffles cannot form and bacteria invasion is prevented.[138] Altogether these studies highlight the role of cholesterol enriched membrane in the control of Rho GTPase activities, which in turn may control the fate of plasma membrane components (proteins or pathogens) or caveolae-dependent endocytic routes.

Endocytic routes and vesicle recycling

Once the vesicle has formed at the plasma membrane, cargo are either recycled back to the plasma membrane or routed toward degradative pathways. It has long been thought that most cargo meet a common population of early endosomes, but recent evidence suggests that instead, early endosomes are a heterogeneous population of vesicles that may condition the fate of the cargo.[139,140] RhoB and RhoD have been localized to early endosomes based on their colocalization with Rab5. They control progression of vesicles through the endocytic pathway by promoting actin polymerization on endosomes that is initiated by Diaphanous-related formins in a Src-dependent manner.[141-143] In addition, RhoB controls the degradation of EGF and CXCR2 receptors, which is necessary to switch off their signaling.[144,145] RhoD has never been shown to direct vesicles toward degradative pathways, suggesting that it might at least in part, target them to a different set of endosomes. This is further emphasized by the subcellular localization of Src kinase family members. They possess different post-translational modifications that target them to different subsets of endosomes. Knocking-down RhoB or RhoD selectively limits the abundance of Src members in the plasma membrane indicating that RhoB and RhoD control and/ or direct them to different subsets of endosomes.[145-147] Other

Rho GTPases have been shown to control the fate of membrane receptors. For example, RhoJ (TCL) is required for Tf recycling to the plasma membrane.[148] The nature of the endosome subsets targeted by the Rho GTPases remains to be characterized.

Rho GTPases and Exocytosis

Vesicle formation at the Golgi apparatus

Membrane pinch-off from a donor compartment is necessary to generate vesicles that will incorporate cargo to be delivered to a target membrane. Coat protein complex I (COPI), complex II (COPII) and clathrin form a cage, which all contain an inner layer of adaptor proteins (AP). These coats drive the budding of vesicles at distinct locations.[149] Whereas clathrin coat function is restricted to post-Golgi membranes (plasma membrane, endosomes, and TGN), COPI and COPII coats act on endoplasmic reticulum (ER) and Golgi to form vesicular carriers that follow bidirectional transport. So far, among Rho GTPases, only Cdc42 has been localized in the Golgi.[150] By binding to COPI subunits, Cdc42 regulates ER to Golgi transport and, importantly, protein exit from the ER depends on Cdc42 cycling between an inactive and an active state.[151] How Cdc42 controls ER to Golgi trafficking is still unclear, but one obvious possibility is the ability of Cdc42 to mediate local actin polymerization in early steps of vesicle formation by recruiting and activating the N-WASP-Arp2/3 complex.[152-154] Interestingly, actin polymerization at the Golgi, as well as the recruitment of Cdc42, requires Arf1, which in turn recruits ARHGAP10[155] and the machinery to promote vesicle scission.[156] As Cdc42-dependent actin polymerization at the Golgi inhibits dynein recruitment to COPI vesicles,[157] these data support a model in which local and transient Cdc42-dependent actin polymerization may help the coatomer bend membrane and form vesicles whereas Arf1-dependent inactivation of Cdc42 may favor vesicle formation and dynein-dependent transport on microtubules. In adipocytes, TC10 (RhoQ), a close relative of Cdc42, may also control secretory vesicle trafficking through N-WASP-dependent actin polymerization and COPI recruitment.[158] There is an intriguing parallel between GPI-AP endocytosis and the formation of secretory vesicles. Both involve Cdc42 deactivation, Arf1 and ARHGAP10 suggesting that this tripartite module may coordinate clathrin-independent endocytosis and secretory pathways. Notably, GPI-AP containing vesicles have been found to be the major membrane supplier for membrane expansion when cells spread during cell adhesion and during phagocytosis[159,160] and Cdc42 is needed for the recycling of major histocompatibility complex of class I.[161]

Clathrin and the AP-1 adaptor mediate the trafficking of specific cargoes from the TGN to the endosomal system.[162] Recently, Rac1 was found to associate with AP-1A and promote actin polymerization at the TGN once activated by βPIX, a known GEF for Rac1.[163,164] These studies further identified a molecular network involving Arf1-dependent activation of Rac1, and subsequent N-WASP-dependent polymerization of actin which was necessary for the biogenesis of clathrin-AP-1 coated carrier formation at the TGN. Intriguingly, Cdc42 is not involved

in this pathway indicating that Rac1 and Cdc42 control actin polymerization and protein transport along different routes of the secretory pathway. In addition to its effects on actin, Rac1 may also regulate the lipid composition of the TGN membrane. Rac1 colocalizes at the TGN with the bifunctional protein OCRL that possesses a RhoGAP domain and a 5-phosphatase activity for $PI(4,5)P_2$.[74] Whether Rac1 modulates OCRL activity or whether OCRL has a bona fide GAP activity toward Rac is unclear,[165] but OCRL and Rac may help to maintain PI(4)P levels at the TGN and enhance the binding of AP-1,[163] which, combined with local actin polymerization, may promote vesicle formation.

Constitutive and polarized exocytosis

The secretory pathway consists of transporting vesicles from the Golgi to the cell surface. The general mechanism to release vesicles containing secretory products is highly conserved, and relies on sequential steps at the plasma membrane, consisting of vesicle tethering, docking, priming, and finally vesicle fusing with the plasma membrane. Whereas constitutive exocytosis occurs constantly and maintains the plasma membrane composition, polarized exocytosis requires the abundant delivery of membrane and proteins to specific spatial landmarks. The first evidence of a role for Rho GTPases in vesicle fusion came from yeast studies in which temperature-sensitive Rho GTPase mutant fail to divide because of defects in bud growth in Cdc42 mutant and vesicle accumulation in the daughter cell in Rho3 mutant. In both cases, post-Golgi vesicles form normally, but do not fuse at budding site leading to a decreased supply of the membrane necessary for bud growth.[166,167] In vitro experiments have unraveled the potential mechanism of Rho function by showing that a blocking peptide against Rho3 and Cdc42 prevents vacuole fusion. Sequential analysis of the fusion reaction revealed that Rho3 and Cdc42 have no effects on vesicle tethering but alter the docking step and subsequent SNARE-dependent fusion events.[168-170] Interestingly, the actin cables necessary for delivering vesicles to budding sites are unaltered in these mutants, however, in vitro, actin polymerization on vacuoles is defective and prevents efficient fusion. Whereas actin cable formation depends on Cdc42 and formins,[171] vacuole fusion rely on Cdc42 and the yeast WASp and WIP homolog, as well as Arp3.[172] This suggests that Rho GTPase-dependent actin polymerization, as well as the type of actin filaments formed at specific sites of vesicle docking regulates vesicle fusion.

Cdc42 has been also shown to control polarized exocytosis in higher eukaryotes through the Cdc42-Par6-αPKC pathway.[173-176] In organs, epithelial cells are the building units of tubes such alveoli and cysts, and they are oriented with their apical surfaces facing the central lumen and their basolateral membranes joining neighboring cells. In a culture system recapitulating cystogenesis, Cdc42 provides the membrane necessary for lumen formation.[177] Interestingly, an unbiased RNAi screen directed against regulators of Rho GTPases has confirmed the importance of Cdc42 and Cdc42 GEFs (Intersectin2 and Tuba) during lumen formation and revealed the requirement for other Rho GEFs known to activate RhoA and RhoG (Lbc/AKAP13 and SGEF respectively).[178] While RhoA controls cell polarity by regulating cell contractility through the actomyosin system and by stabilizing tight junctions,[179,180] no function for RhoG in epithelial cell polarization has been reported yet. The fact that SGEF activity is dependent on $PI(3,4,5)P_3$ and that PTEN phosphatase, which converts $PI(3,4,5)P_3$ into $PI(4,5)P_2$, is required for apical localization of Cdc42 and normal cystogenesis[177] suggest that, like in endocytic processes, $PI(3,4,5)P_3$ levels need to be reduced to establish normal lumen formation. Whether, RhoG controls an early step of cystogenesis by initiating/promoting cell polarity is an interesting possibility, especially since RhoG participates in the establishment of front-rear cell polarity during cell migration.[181]

The exocyst complex, consisting of 8 subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84), mediates the tethering of secretory vesicles at the plasma membrane before SNARE-mediated fusion occurs. This is particularly important for polarized exocytosis in which tethering of secretory vesicles and exocytosis is sustained at a given site to permit cell membrane expansion or polarized secretion. Interactions between Rho GTPases and the exocyst subunits were first reported in yeast. Exo70 and Sec3 form a landmark of bud formation and promote exocytotic-dependent cell surface expansion through their interaction with Cdc42, Rho3, and Rho1.[22,182] The relationship between Rho GTPases and the exocyst complex is conserved and controls different aspects of polarized exocytosis in mammals. It relies mostly on the interaction of Exo70 with two closely related GTPases, Cdc42, and TC10. During neurite outgrowth in response to NGF or IGF, the polarized exocytosis of vesicles at the growth cone requires the integral exocyst complex and TC10 activation.[183-185] Although the relationship between $PI(3,4,5)P_3$ and TC10 activation is unknown, it is noteworthy that PI 3-kinase activation is needed for growth cone expansion in response to IGF-1, and that $PI(3,4,5)P_3$ accumulates in the

distal region to sustain vesicle fusion. This increases IGF-1
receptor exposure to the extracellular medium, which may contribute to self-reinforcement of neurite outgrowth in response to IGF.[184-187] Conversely, the phosphoinositide phosphatase PTEN induces growth cone retraction and neurite collapse.[188] TC10 is homogeneously localized at the plasma membrane, but is activated at sites of growth cone expansion where $PI(3,4,5)P_3$ is enriched and at discrete sites of spine formation where it recruits Exo70. In response to NGF, the TC10-Exo70 complex antagonizes Cdc42-N-WASP-dependent actin polymerization, which is, nonetheless, required for normal neurite growth. This indicates that a subtle balance between Cdc42 and Exo70-TC10 signaling has to be preserved.[184,189] Interestingly, TC10 deactivation by p190RhoGAP-A appears to be necessary for vesicle fusion.[190] Thus, once the vesicle has fused, Cdc42-N- WASP inhibition may be relieved to promote actin-dependent neurite elongation.

Focal exocytosis during phagocytosis or metalloprotease release in invadopodia also requires Cdc42 and a functional exocyst complex.[191,192] Interestingly, during phagocytosis, Rab11 overexpression is able to supplant both Cdc42- and N-WASP- deficiencies and rescue phagocytosis,[191] suggesting that sustaining

the membrane flow toward the plasma membrane may be sufficient to ensure phagocytosis. This mechanism supposes the presence of a functional tethering complex. Knocking- down the Exo70 subunit blocks Rab11-dependent membrane supply for phagocytosis and for transferrin receptor exocytosis, and overexpression of Exo70 is sufficient to override Rab11 knock-down.[191,193] The fact that trafficking and small GTPase pathways are interconnected for efficient delivery of vesicles is further exemplified during cystogenesis in which Rab11 controls the polarized localization of Cdc42 during lumen formation.[194] In addition, in cells knocked-down for PIP5Kγ, polarized exocytosis of integrin β1 at the leading edge of migrating cells is inhibited due to defects in Rab11-dependent supply of vesicles and local PI(4,5)P2 production and exo70 recruitment.[195] The role of Cdc42 has not been addressed in this context, but since knocking-down PIP5Kγ may alter Rho GTPases activity,[49] this provides an additional clue about the existence of a conserved framework in which Rab11, Cdc42 and maybe other Cdc42-like proteins such as TC10, cooperate to regulate membrane fusion at sites of membrane expansion. Finally, Rho GTPases may also control fusion steps by directly regulating the SNARE fusion machinery. For example, syntaxin is phosphorylated by the kinase ROCK, which inhibits neurite outgrowth by preventing vesicle fusion and SNARE complex assembly.[196] A recent siRNA screen has identified several Rho GTPases that may control constitutive secretion of transmembrane proteins. Knocking-down RhoC, RhoF, RhoJ (TCL), RhoU (Wrch-1), or RhoV (Chp) reduces exposure of secreted proteins at the cell surface.[197] Although the molecular mechanisms remain to be explored, this study clearly points out that other less well-characterized Rho GTPases are potential regulators of constitutive secretion.

Regulated exocytosis

In contrast to constitutive exocytosis, regulated exocytosis is triggered by a burst of intracellular calcium in response to an external stimulus. This mode of secretion occurs mostly in specialized secretory cells including neurons, neuroendocrine cells, and granulocytes, to name a few. Most secretory cells possess a dense cortical actin network, which acts as a barrier to prevent inappropriate fusion of secretory granules with the plasma membrane in resting conditions. Upon stimulation, depolymerization of cortical actin, together with local actin repolymerization, is needed for efficient secretion.[198-200] RhoA, Rac1, Cdc42, and TC10 have been shown to control different aspects of regulated exocytosis by modulating actin dynamics, but also phosphoinositide production in different cell systems. In mast cells, serotonin and histamine are stored in secretory granules that are released in response to antigen-mediated cross- linking of IgE. Activated Rac1 and Cdc42 stimulate exocytosis both by PLCγ-dependent production of Ins(1,4,5)P_3, which leads to a rise of intracellular calcium and PAK1-dependent actin remodeling at the plasma membrane.[201-203] In chromaffin cells, knocking-down Rac1, Cdc42 or their respective GEFs, βPIX, or Intersectin-1, inhibits secretagogue-induced exocytosis in PC12 cells.[204,205] Interestingly, whereas membrane depolarization induced by a high potassium concentration activated Rac1 and

Cdc42, only Cdc42 was found to induce N-WASP-dependent actin polymerization at the plasma membrane.[204,206,207] Rac1 instead activates PLD1, which produces phosphatidic acid (PA) at the exocytic site facilitating secretory granule fusion.[204,208] In neurons, actin dynamics is not required for fast neurotransmitter release from synaptic vesicles,[209] but Rac1 and PLD1 are both present on synaptosomes. Since both are required for neurotransmitter release,[210-212] this suggests that, like in chromaffin cells, Rac1 may regulate exocytosis through PA production at the plasma membrane rather than through actin dynamics and remodeling.

Maintenance of glucose homeostasis in the body relies on regulated exocytosis of insulin by β-pancreatic islet cells, which in turn stimulates the translocation of the glucose transporter GLUT4 from intravesicular stores to the plasma membrane in adipocytes and muscle cells. Rho GTPases are implicated in both processes. Insulin release in response to glucose increase requires Cdc42 and Rac1. Interestingly, the kinetics of Cdc42 and Rac1 activation is different with a rapid activation/deactivation of Cdc42 corresponding to the first phase of insulin release mobilizing docked secretory granules for fusion, and a slower Rac1 activation that is required for sustained release of insulin from the storage pool granules.[213,214] Differential activation may be regulated by differential binding and phosphorylation of RhoGDI or Cav-1 that interacts with Cdc42 bound GDP. Upon glucose stimulation, Cav-1 is phosphorylated and allows for β-PIX-dependent Cdc42 activation. Subsequently, Cdc42-activated PAK1 triggers the release of Rac1 from GDI to promote Tiam-1-dependent Rac activation and sustained insulin release.[131,215-217] In contrast to neuroendocrine cells in which Cdc42 and Rac1 have been localized to plasma membrane and Cdc42-dependent actin polymerization facilitates exocytosis, Cdc42 and Rac1 have been localized to secretory granules in β-pancreatic cells and Cdc42-dependent actin polymerization inhibits insulin release. Cdc42 may directly control vesicle fusion of docked vesicles by interacting in its active state with proteins from the SNARE complex,[218,219] whereas a Rac1-dependent actin rearrangement may be required to bring secretory granules close to the plasma membrane.[220]

In adipocytes and muscle cells, insulin triggers the translocation of the GLUT4 transporter from intravesicular store to the plasma membrane. This exocytosis process depends on actin rearrangement and two Rho GTPases, Rac1 and TC10.[221] In adipocytes, insulin stimulation triggers tethering of vesicles carrying GLUT4 to sites of exocytosis via the association of Exo70 with activated TC10.[222] Rac1 and TC10 are necessary for actin remodeling and vesicle translocation, but may act at two different steps dependent on two different classes of PI 3-kinases. Under physiological concentrations of insulin, active Rac1 stimulates GLUT4 translocation to the plasma membrane. Unlike TC10, Rac1 activation, requires ClassI PI 3-kinase and P-REX-1, a PI(3,4,5)P_3-regulated Rac GEF, providing a link between PI 3-kinase and Rac activity.[223] On the other hand, translocation and docking of GLUT4-positive vesicles requires the formation of PI(3)P at the plasma membrane by actvating

a TC10-dependent classII PI 3-kinase.[224,225] Since TC10 can control both the recruitment of vesicles at the plasma membrane by binding to the Exo70 exocyst subunit and actin polymerization through N-WASP activation,[158,184,222,226] this implies a differential role for TC10 and Rac1 in the control of GLUT4 exocytosis. TC10 controls vesicle transport and docking during the initial phase, while Rac1 sustains exocytosis by maintaining high levels of GLUT4 at the plasma membrane. .

Finally, RhoA has been also involved in the control of regulated exocytosis in neuroendocrine cells and neutrophils. Overexpression of activated RhoA induces cortical actin polymerization, but unlike Cdc42, inhibits exocytosis in neuroendocrine cells.[227,228] Similarly, maintaining high levels of RhoA activity by silencing the Rho GAP GMIP in neutrophils prevents myeloperoxidase exocytosis from azurophilic granules,[200] suggesting that RhoA needs to be deactivated for efficient exocytosis. Whether the observed actin depolymerization in response to stimulation is the result of Rho inactivation is still elusive. Alternatively, RhoA has been found to generate PI(4)P by activating PI-4 kinase on secretory granules.[229] As in yeast, PI(4)P may be necessary for myosin-regulated transport of secretory granules to the cell periphery,[230] and reduced RhoA activity may free secretory granules from preexisting actin filaments to favor their docking and fusion with the plasma membrane.

Conclusion

These advances in understanding Rho GTPases function in membrane trafficking emphasize their pleitropic role in endocytosis, exocytosis and vesicle segregation inside the cell. Signaling cascades are gradually being deciphered, and it does not come as a surprise that actin rearrangements constitute the cornerstone of Rho GTPase signaling. However, Rho GTPases also interfere with phosphoinositide signaling, which can then feedback on their own activities. How Rho GTPase signaling, actin rearrangements, and phosphoinositide signaling are spatiotemporally coordinated remains a challenging question. With their multidomain architecture, Rho GEFs and Rho GAPs are well suited for processing and integrating these multiple signaling entries and unraveling their regulatory roles will undoubtedly provide further insight into how this tripartite framework controls membrane trafficking.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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Dix pour cent de la thèse en Français

Avant-Propos

Après un Master de Neuroscience à l'Université de Val Paraiso au Chili, j'ai obtenu une bourse du ministère Chilien de l'éducation dans le cadre de l'appel d'offre « advanced human capital program scholarships » (Becas Chile-CONICYT), pour effectuer un doctorat à l'Université de Strasbourg dans l'équipe du Dr. Stéphane Gasman situé à l'Institut des Neurosciences Cellulaires et Intégratives (INCI, CNRS UPR3212). L'équipe de Stéphane Gasman s'attache depuis de plusieurs années à comprendre les mécanismes cellulaires et moléculaires qui contrôlent la sécrétion neuroendocrine.

Le système neuroendocrinien se compose des organes, tissus et cellules spécialisés qui libèrent des hormones et des neuropeptides dans la circulation sanguine par un processus d'exocytose vésiculaire régulée par le calcium. Ce processus est finement régulé par les protéines SNARE (**S**oluble **N**SF **A**ttachment protein **RE**ceptor), qui permettent la fusion de la membrane des vésicules avec la membrane plasmique, étape ultime de l'exocytose, aboutissant à la libération du contenu vésiculaire. Les mécanismes qui régulent l'exocytose et la fusion membranaire sont étudiés de façon intensive. En revanche, les mécanismes permettant de préserver l'intégrité physique des membranes plasmique et vésiculaire après fusion membranaire, et par conséquent de maintenir l'équilibre fonctionnel de la cellule, ne sont pas connus et restent peu explorés aujourd'hui. Les travaux de l'équipe réalisés dans les cellules chromaffines de la glande surrénale suggèrent que la libération du contenu intra-granulaire est couplée de façon spatiale et temporelle à un processus d'endocytose compensatrice qui permet la recapture de la membrane du granule. Ainsi, nous émettons l'hypothèse selon laquelle la membrane granulaire préserverait son intégrité au sein de la membrane plasmique après l'exocytose avant d'être spécifiquement recapturée avec l'ensemble de ses composés. Cependant, les mécanismes à la base de cette activité d'endocytose compensatrice ne sont pas connus dans les cellules neuroendocrines. Dans ce contexte, le but général de ma thèse fut d'apporter de nouveaux éléments permettant de comprendre comment l'endocytose compensatrice est-elle déclenchée et régulée dans les cellules neuroendocrines et par quels mécanismes est-elle couplée à l'exocytose.

A mon arrivée en doctorat, le Dr Stéphane Ory (qui fût mon encadrant pendant ces trois années et demi) venait de montrer qu'au cours de l'exocytose, la proteine PLSCR1 (Phospholipid Scramblase-1) est capable de redistribuer les phospholipides d'un feuillet à l'autre de la membrane plasmique, perturbant ainsi de façon transitoire l'asymétrie membranaire au niveau des sites d'exocytose. De façon intéressante, Stéphane Ory montre élégamment que cette perturbation membranaire n'empêche pas la sécrétion mais bloque significativement l'endocytose compensatrice des granules de sécrétion. La PLSCR1 m'est alors apparue comme un candidat idéal pour contrôler le couplage entre l'exocytose et l'endocytose. Ainsi, l'un des buts premiers de mon doctorat fut d'essayer de comprendre comment l'activité de la PLSCR1 est régulée et pourquoi un mélange de phospholipides est préalable à la recapture des granules des granules de sécrétion.

En parallèle, je me suis intéressée aux mécanismes de régulation de la sécrétion par une protéine appelée oligophrénine-1 (OPHN1). Cette protéine est particulièrement intéressante. Impliquée dans l'endocytose des vésicules synaptiques, elle possède un domaine « BAR » (Bin, Amphiphysin, Rvs) qui est un senseur de courbure membranaire ainsi qu'un domaine GAP permettant l'inactivation des protéines Rho, une famille de GTPases largement impliquée dans les processus d'exo- et d'endocytose. Au cours de ma première année de thèse, Sébastien Houy un doctorant de l'équipe montrait, en utilisant des souris invalidées pour le gène *Ophn1* que l'oligophrénine participe à la fois à la formation du pore de fusion et à l'endocytose compensatrice de la membrane granulaire. J'ai activement participé à ce projet en essayant notemment de comprendre comment OPHN1 pouvait coordonner son rôle sur l'exocytose avec un rôle dans l'endocytose.

Ce manuscrit fait la synthèse de l'ensemble de mes travaux et s'articule en quatre grandes parties. La première partie introduit de façon générale les connaissances actuelles concernant l'exocytose régulée et l'endocytose compensatrice dans les cellules neuroendocrines. J'y présente le modèle de la cellule chromaffine que nous utilisons au laboratoire et j'y détaille le cycle complet de la vie d'un granule de sécrétion, depuis sa biogénèse jusqu'aux mécanismes permettant son recyclage au cours du processus d'endocytose compensatrice. J'insiste également sur l'implication des protéines et des lipides qui ont été au cœur de mes problématiques de thèse.

La seconde partie est dédiée à mes données sur la régulation de l'activité et le rôle de la PLSCR1 au cours des processus d'exocytose et d'endocytose dans les cellules neuroendocrine tandis que la troisième partie du manuscrit se focalise sur l'implication de la protéine Oligophrénine1. Ces deux parties sont organisées de la même façon. Après un bref rappel du contexte scientifique et des problématiques posées, les données sont exposées sous forme d'article (une ébauche d'article en préparation pour la partie PLSCR1 et un article publié dans Journal of Neuroscience pour la partie sur l'oligophrénine). Je tente ensuite de prendre un peu de recul et de discuter mes données de façon plus globale afin d'élaborer quelques concepts mécanistiques.

Enfin une dernière partie présente les détails des matériels et méthodes utilisés pour mener à bien mes expériences. En annexe, vous trouverez l'ensemble des articles auxquels j'ai pu contribuer de près ou de loin lors de mon doctorat.

L'objectif de ce manuscrit est d'apporter une vision globale des mécanismes régulant la sécrétion neuroendocrine tout en mettant en exergue l'implication des protéines scramblase-1 et oligophrénine-1. Le Français n'étant pas ma langue maternelle, j'ai préféré rédiger ce manuscrit en anglais. J'en profite pour remercier Stéphane Gasman de m'avoir aidé à traduire ce prologue.

Je vous souhaite une agréable lecture.

Résumé

De récentes études ont montré dans les cellules chromaffines que la libération des granules de sécrétion est temporellement et spatialement couplée au processus d'endocytose. Nous avons proposé l'hypothèse que la membrane du granule preserve son intégrité au sein de la membrane plasmique durant l'exocytose avant d'être internalisée ainsi avec ses composants. Cependant, les mécanismes moléculaires de ce processus d'endocytose compensatrice sont encore inconnus. Ainsi; mon projet de thèse vise a répondre à la question suivante : Quels sont les différents mécanismes déclenchant et régulant l'exocytose et l'endocytose compensatrice?

Les propriétés physiques des lipides jouent des rôles fondamentaux dans le trafic membranaire. Ils servent de système d'échafaudage pour maintenir la machinerie spécifique à des endroits précis de la membrane plasmique. Par exemple, la formation de microdomaines de gangliosides et de PIP2 au niveau des sites d'exocytose ou encore le mélange de lipides au sein de la bicouche lipidique représentent des processus attractifs pour permettre cette function au cours des événements d'exo-endocytose dans les cellules neuroendocrines. De plus, en raison de leur implication importante dans les processus d'exo-endocytose ou dans le remodelage des lipides, l'annexine A2, la synaptotagmine 1, l'oligophrénine1 et la scramblase 1 doivent être considérées comme des signaux potentiels pour le déclenchement de l'endocytose de la membrane granulaire.

Au cours de mon doctorat, je me suis intéressée à étudier comment l'exocytose et l'endocytose compensatrice sont régulées par la scramblase1 et l'oligophrénine1 dans les cellules chromaffines de la glande surrénale.

Introduction

Le système neuroendocrinien se compose des organes, tissus et cellules spécialisés qui libèrent des hormones et des neuropeptides dans la circulation sanguine par un processus d'exocytose vésiculaire régulée par le calcium. Ce processus est finement régulé par les protéines SNARE (**S**oluble **N**SF **A**ttachment protein **RE**ceptor), qui permettent la fusion de la membrane des vésicules avec la membrane plasmique, étape ultime de l'exocytose, aboutissant à la libération du contenu vésiculaire. Les mécanismes qui régulent l'exocytose et la fusion membranaire sont étudiés de façon extensive. En revanche, les mécanismes permettant de préserver l'intégrité physique des membranes plasmique et vésiculaire après exocytose et fusion membranaire, et par conséquent de maintenir l'équilibre fonctionnel de la cellule, ne sont pas connus. Nos récents travaux réalisés dans les cellules chromaffines de la glande surrénale suggèrent que la libération vésiculaire est couplée de façon spatiale et temporelle à un processus d'endocytose compensatrice. Ainsi, nous émettons l'hypothèse selon laquelle la membrane granulaire préserverait son intégrité au sein de la membrane plasmique après l'exocytose avant d'être spécifiquement recapturée avec l'ensemble de ses composés. Cependant, les mécanismes à la base de cette activité d'endocytose compensatrice ne sont pas connus à l'heure actuelle.

Dans ce contexte, les questions centrales de ma thèse sont de comprendre comment l'endocytose compensatrice est-elle déclenchée et régulée dans les cellules neuroendocrines et par quels mécanismes est-elle couplée à l'exocytose. Afin d'y répondre, ma thèse fut divisée en deux objectifs spécifiques. Un premier objectif fut d'étudier l'implication potentielle de l'oligophrénine-1, une protéine inactivatrice de type GAP spécifique des GTPases Rho, au cours de la sécrétion neuroendocrine. Mon second objectif fut d'étudier comment le remodelage lipidique au niveau des sites d'exocytose induit par la protéine Phospholipides Scramblase-1 (PLSCR-1) peut contribuer à l'endocytose compensatrice.

Résultats

1) Caractérisation du rôle de l'oligophrénine1 au cours de la sécrétion neuroendocrine.

L'oligophrénine 1 est une protéine à multiples domaines fonctionnels dont l'implication dans les processus de recyclage de vésicules synaptiques et la maturation des dendrites à été démontré dans les neurones. Elle possède un domaine « BAR » (Bin, Amphiphysin, Rvs) qui est un senseur de courbure membranaire et dont l'implication au cours du processus d'endocytose a été mis en évidence pour d'autre protéines telles que l'amphiphysine ou l'endophiline. Elle dispose également d'un domaine GAP permettant l'inactivation des protéines Rho, une famille de GTPases Rho largement impliquée dans les processus d'exo- et d'endocytose. Ainsi, au vu de l'ensemble de ses caractéristiques, l'OPHN1 nous est apparu comme un candidat idéal pour jouer un rôle pivot dans la régulation de l'exocytose et de l'endocytose dans les cellules neuroendocrines.

En collaboration avec un autre doctorant du laboratoire, nous avons montré qu´OPHN1 est exprimée et localisée au niveau de la membrane plasmique et dans le cytosol des cellules chromaffines de la médullo-surrénale (Fig1).

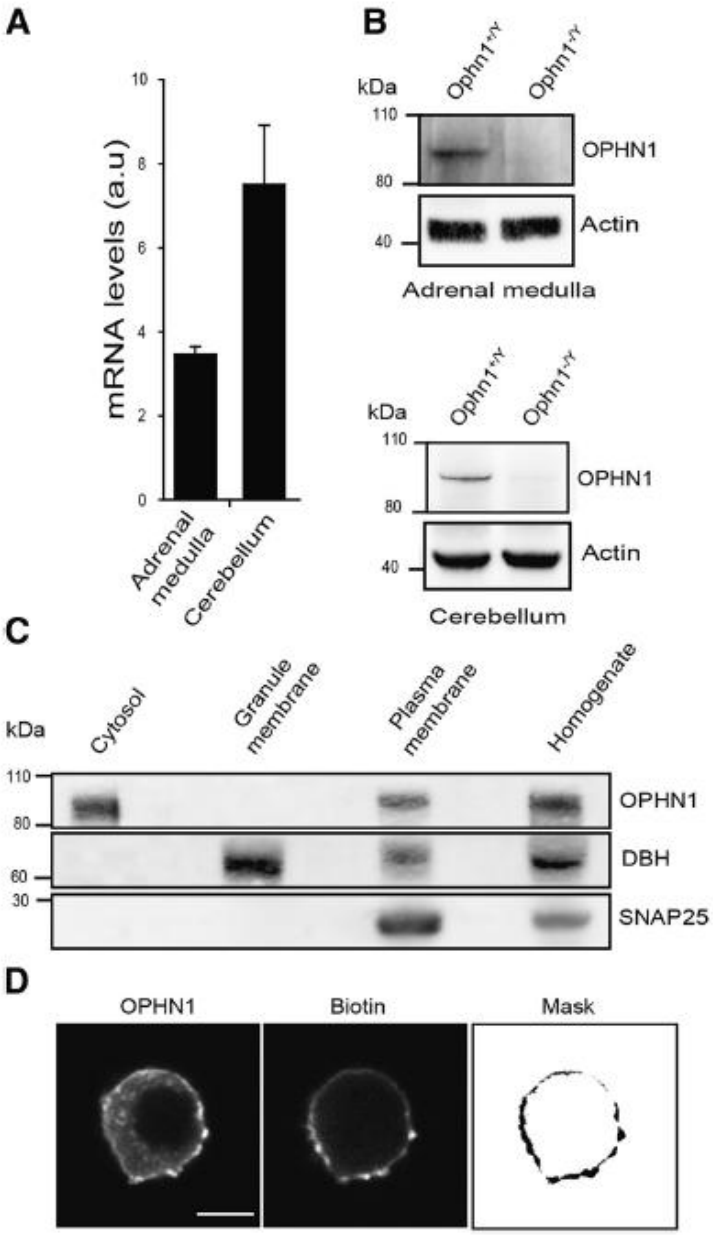

Fig1. L'oligohrénine-1 est exprimée à la membrane plasmique dans les cellules chromaffines de la glande médullo-surrénale. A, B, Détection de l'ARNm de l'OPHN1 par PCR quantitative (A) et immunodétection de la protéine OPHN1 par Western blot (B) dans la médullo-surrénale et le cervelet des souris Ophn1 + / y et Ophn1- / y. L'actine est représentée comme le contrôle du charge. C, Distribution subcellulaire de l'OPHN1 dans la médullosurrénale bovine. Les fractions obtenues par centrifugation sur des gradients de sucrose ont été collectées et soumises à une électrophorèse sur gel (20 ug de protéine / fraction) et à une immunodétection utilisant des anticorps anti-SNAP25 pour détecter des membranes plasmiques, des anticorps anti-DBH pour détecter des granules de chromaffine et des anticorps anti-OPHN1. D, localisation intracellulaire d'OPHN1 exprimée ectopiquement dans des cellules chromaffines bovines cultivées. Les cellules ont été transfectées avec le vecteur d'expression bidirectionnel pBI-CMV1 codant simultanément pour EGFP (données non représentées) et OPHN1. La membrane plasmatique a été marquée avec de la biotine révélée avec Alexa Fluor 633 streptavidine. On a détecté l'OPHN1 en utilisant des anticorps anti-OPHN1 révélés par des anticorps anti-lapin conjugués à Alexa Fluor 555. L'image de masque met en évidence la présence de l'OPHN1 à la membrane plasmique. Barre d'échelle, 5 µm.

En mesurant la sécrétion par la technique d´ampérométrie à fibre de carbone, nous avons observé une diminution des événements d´exocytose dans les cellules chromaffines isolées à partir de souris invalidées pour le gène *Ophn1* (cellules KO Ophn1) (fig2) et une différence au niveau de la dynamique du pore de fusion (fig3). L'expression de différentes formes mutées d'OPHN1 dans cellules chromaffines indiquent que le domaine Rho-GAP d´OPHN1 est nécessaire pour contrôler la dynamique des pores de fusion (fig4). Pour soutenir ces données expérimentale dans cellules sous-exprimant OPHN1 et transfectées avec les constructions OPHN1 Rho-GAP inactifs et delta BAR des expériences d'ampérométrie à fibre de carbone ont été réalisées (fig5). Nous avons trouvé uniquement un effet sur la formation de pores de fusion dans les cellules qui ont été transfectées avec le constriction OPHN1 Rho-GAP inactifs. ce qui implique que la fonction de OPHN1 dans la formation de pores de fusion implique à un certain stade l'inactivation d'un membre de la famille Rho GTPase. Ce est pourquoi nous avons réalisé des expériences dans des cellules dans lesquelles l'expression de OPHN1 est diminuée et on a mesuré l'activité des protéines Cdc42, Rac1 et RhoA.
Cette expérience nous a permis d'identifier une augmentation de l'activité RhoA dans les cellules que exprimant moins la protéine OPHN1 (fig6). Ce qui implique un rôle de régulation négative par OPHN1 sur l'activité de RhoA.

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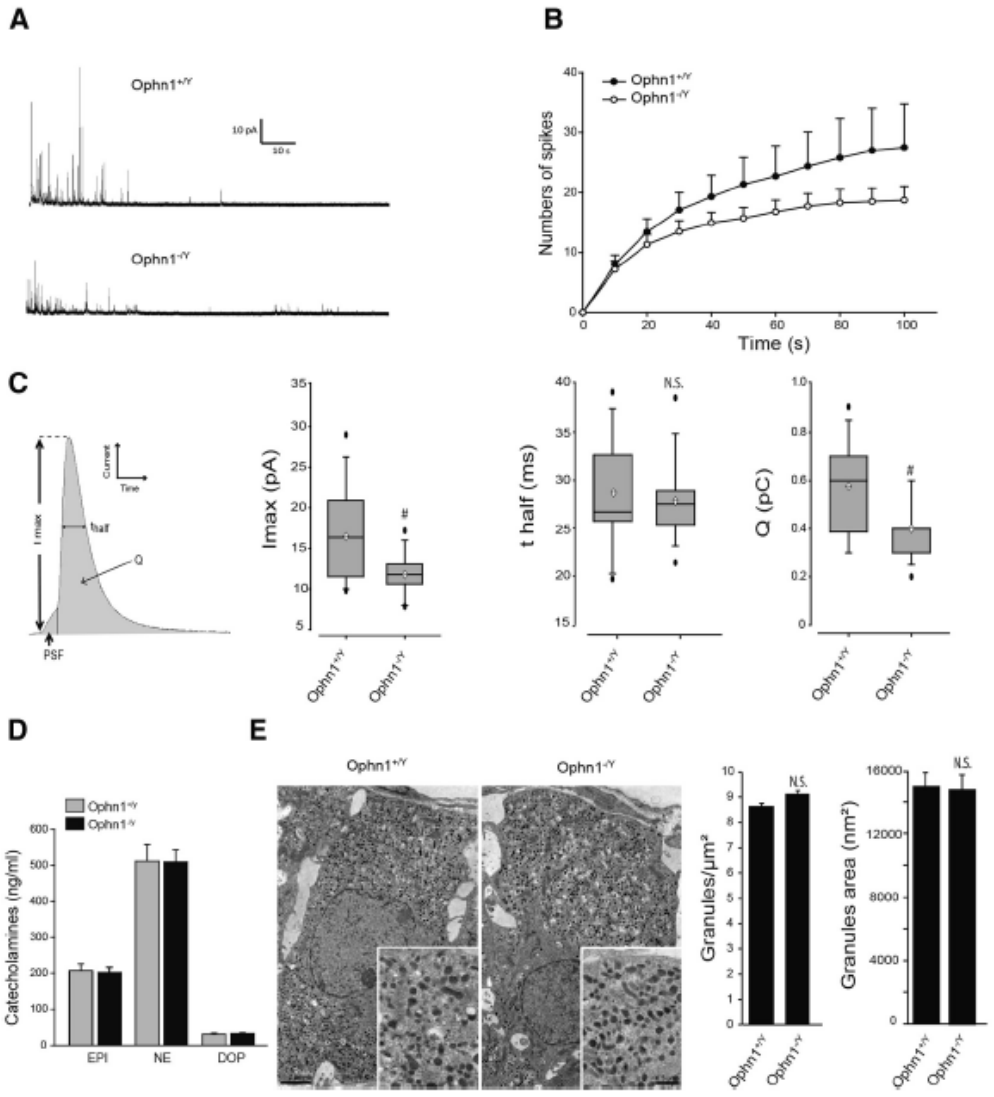

Fig2. Analyse ampérométrique de la sécrétion de catécholamine à partir de cellules chromaffines de souris Ophnl knock-out. A, Enregistrements ampérométriques représentatifs obtenus à partir de cellules de chromaffine cultivées à partir de souris Ophnl + / y et Ophnl- / y. Les cellules ont été stimulées pendant 10 s par une application locale de 100 mm K +. B, Nombre cumulé de pointes pendant 100 s d'enregistrement des cellules Ophn1 + / y ou Ophn1- / y. Les données sont indiquées comme la moyenne ± SEM. N = 18 cellules. C, Schéma d'une pointe ampérométrique décrivant les différents paramètres suivants analysés: taille ou charge quantique (Q), demi-largeur (t1 / 2), amplitude de pic (Imax) et signal PSF. On a représenté un graphique en boîte et en moustaches pour l'amplitude de pic, la demi-largeur et la charge de pic dans les cellules Ophnl + / y ou Ophnl- / y. Les cercles noirs et les diamants blancs représentent respectivement des observations aberrantes et des valeurs moyennes. La signification statistique des valeurs médianes a été déterminée par une analyse médiane non paramétrique de Mann-Whitney. N = 18 cellules; #p <0,01. N.s., Pas significatif. D, la teneur en catécholamine d'une fraction subcellulaire enrichie en granules sécrétoire préparée à partir de la médullosurrénale de souris Ophnl + / y et Ophnl- / y a été analysée pour les niveaux totaux d'épinéphrine (EPI), de norépinéphrine (NE) et de dopamine (DOP) par ELISA (3CAT Assay, Labor Diagnostika Nord). E, micrographies électroniques à transmission représentative de tranches de moelle surrénale de souris Ophn1 + / y ou Ophnl- / y. La surface moyenne des granules et la densité des granules sécrétoires par micromètre carré ont été mesurées (n = 60.610 granulés, 78 tranches, 3 souris pour OPHN1 + / y, n = 66434 granules, 76 tranches, 3 souris pour OPHN1- / y).

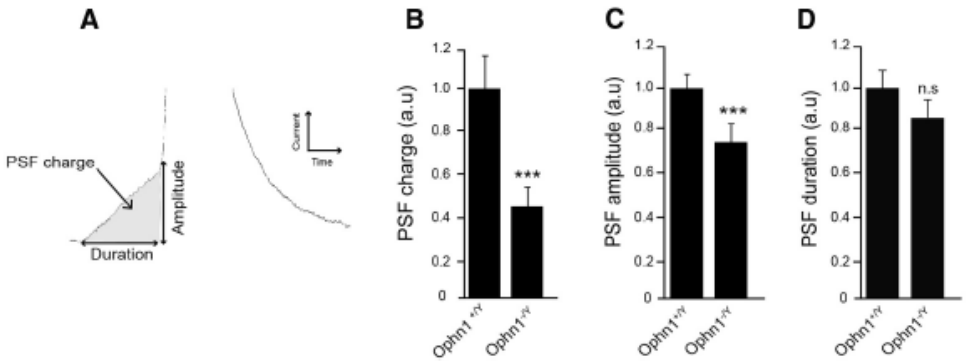

Fig3. Ophn1 knock-out réduit la charge et l'amplitude du signal de pied pré-pic. Les cellules chromaffines cultivées de souris Ophnl + / y et Ophnl- / y ont été stimulées par l'application locale de 100 mm K + pendant 10 s, et la sécrétion a été contrôlée par ampérométrie. Les courants de PSF enregistrés à partir des cellules chromaffines Ophnl / y (n = 14 cellules, 81 courants PSF) ont été comparés à ceux des cellules Ophnl + / y (n = 14 cellules, 122 courants PSF). A, Schéma d'une PSF ampérométrique décrivant les paramètres analysés suivants: amplitude, charge et durée. B, les valeurs moyennes de PSF. C, valeurs moyennes d'amplitude de PSF. D, durée moyenne des PSF. Les données sont normalisées en pourcentage de la valeur moyenne calculée dans les cellules Ophn1 + / y et sont indiquées comme la moyenne ± SEM. *** p <0,001. N.s., Not significant (test de Mann-Whitney).

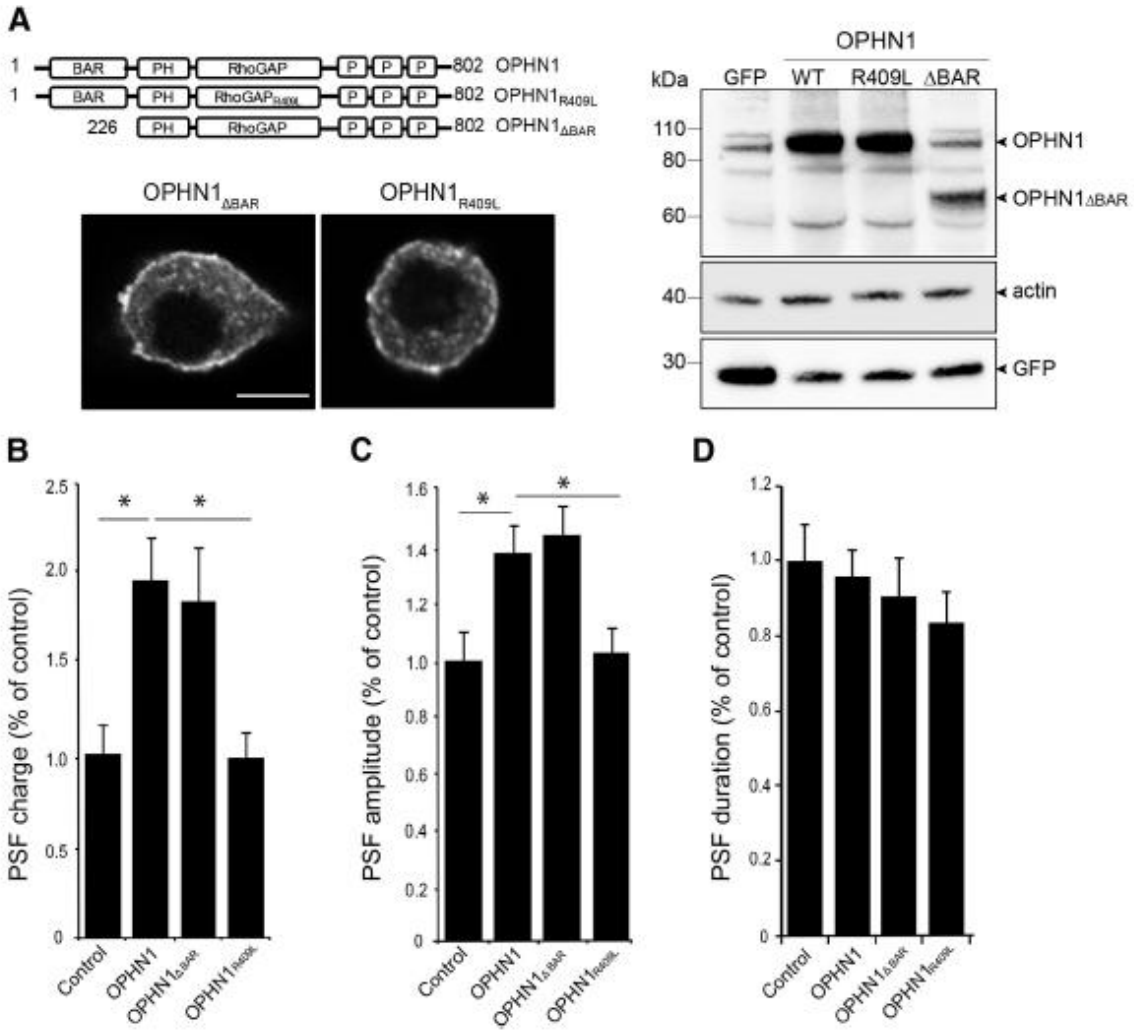

Fig4. La surexpression d'OPHN1 stimule la formation des pores de fusion. A, Représentation schématique de OPHN1 et des deux constructions utilisées dans cette étude représentant la position des différents domaines fonctionnels. PH, domaine d'homologie de la pleckstrine; PH, le domaine riche en prolines. Des cellules chromaffines bovines ont été transfectées avec un vecteur d'expression bidirectionnel codant simultanément pour EGFP et les constructions OPHN1 indiquées. Le niveau d'expression de l'OPHN1 WT, OPHN1ΔBAR et OPHN1R409L exogène est analysé par Western blot en utilisant des anticorps contre OPHN1, EGFP et l'actine. Les images confocales montrent la localisation de mutants d'OPHN1ΔBAR et d'OPHN1R409L exprimés exogène détectés par des anticorps anti-OPHN1 révélés par des anticorps anti-lapin conjugués à Alexa Fluor 555. Barres graduées, 5 µm. B-D, Analyse de la charge, de l'amplitude et de la durée de PSF obtenues à partir de cellules chromaffines bovines exprimant EGFP seul (témoin), OPHN1, OPHN1R409L ou OPHN1ΔBAR. Les données sont normalisées en pourcentage des valeurs de contrôle (considérées comme 100%) et sont indiquées comme la moyenne ± SEM; Contrôle, n = 13 cellules, 51 courants PSF; OPHN1, n = 16 cellules, 140 courants PSF; OPHN1R409L, n = 22 cellules, 61 courants PSF; OPHN1ΔBAR, n = 20 cellules, 131 courants PSF. * P <0,05 (test de Mann-Whitney). On notera que la durée de PSF est restée inchangée dans les cellules exprimant OPHN1, OPHN1R409L ou OPHN1ΔBAR

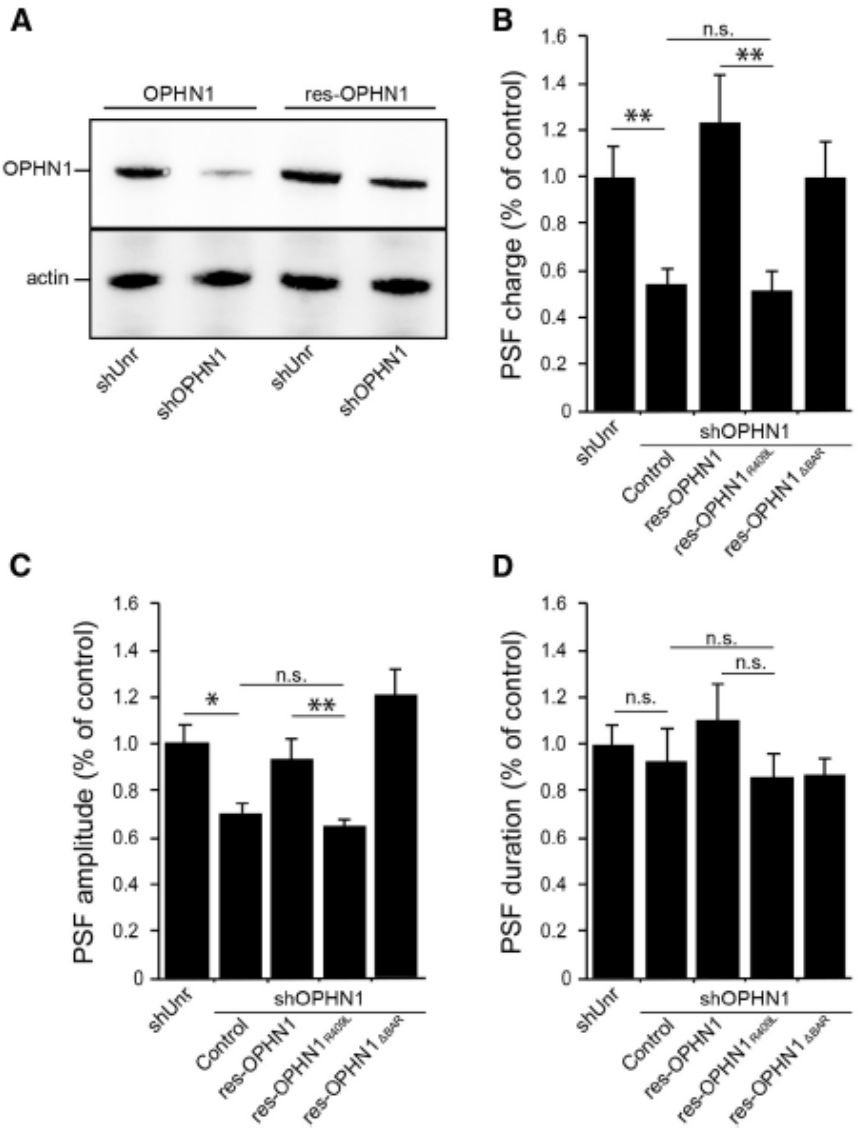

Fig5. OPHN1 nécessite son domaine Rho-GAP pour contrôler la formation des pores de fusion. A, Les *wobble* mutations de l´ADNc de l'OPHN1 confèrent une résistance à la dégradation par le shRNA. Les constructions codant pour le shRNA non apparenté (shUnr) ou shRNA de OPHN1 (shOPHN1) ont été co-transfectées dans des cellules PC12 avec un codage vectoriel pour OPHN1 ou pour res-OPHN1. Les cellules ont été lysées 48 h après transfection et traitées pour analyse Western blot en utilisant des anticorps contre OPHN1 et l'actine. B-D, Analyse de la charge de PSF, de l'amplitude et de la durée obtenues à partir de cellules chromaffines bovines coexprimants shOPHN1 avec EGFP seul (témoin), res-OPHN1, res-OPHN1R409L ou res-OPHN1ΔBAR. Les données sont normalisées sous forme de pourcentages de valeurs témoins obtenues à partir de cellules chromaffines coexprimant EGFP et shUnr, et sont rapportées comme la moyenne ± SEM; ShUnr, n = 30 cellules, 72 courants PSF; ShOPHNl / EGFP, n = 36 cellules, 59 courants PSF; ShOPHN1 / res-OPHN1, n = 24 cellules, 52 courants PSF; ShOPHNl / res-OPHNR409L, n = 22 cellules, 53 courants PSF; ShOPHNl / res-OPHNΔBAR, n = 36 cellules, 87 courants PSF. * P <0,05; *** p <0,001. N.s., Not significant (test de Mann-Whitney).

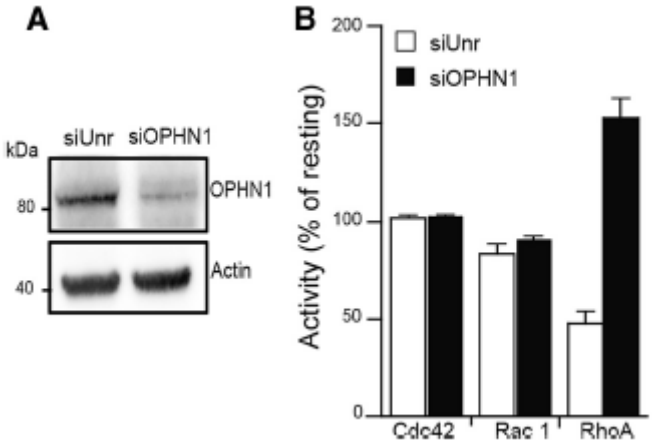

Fig6. La réduction du niveau d'OPHN1 endogène affecte le cycle d'activation / inactivation de RhoA dans des cellules PC12 stimulées par le sécrétagogue. A, Efficacité du siRNA OPHN1. Des cellules PC12 transfectées avec un ARNsi non apparenté (SiUnR) ou OPHN1 ont été lysées 48 h après transfection et traitées pour une analyse par transfert Western en utilisant des anticorps contre OPHN1 et l'actine. B, Effet de l'ARNsi de OPHN1 sur le niveau de Cdc42, Rac1, ou RhoA chargé de GTP dans des cellules PC12 au repos et stimulées. Les cellules PC12 transfectées avec siRNi ou siRNA de OPHNl ont été maintenues en état de repos dans la solution de Locke ou ont été stimulées pendant 10 s avec 59 mm de K +. Les cellules ont ensuite été lysées immédiatement et les lysats ont été utilisés pour la quantification du taux de Cdc42 et de Racl chargés par GTP par dosage ELISA colorimétrique ou par précipitation par affinité de RhoA chargé par GTP. Le RhoA-GTP qui a été retiré a été détecté par immunoblotting en utilisant des anticorps anti-RhoA et le niveau de RhoA chargé par GTP quantifié par analyse de densitométrie de balayage. Les résultats sont normalisés en tant que pourcentage des valeurs obtenues dans les cellules en repos et sont rapportés comme la moyenne ± SEM (n = 3). L'ARNsi de OPHN1 n'a pas modifié le niveau de RhoA / Cdc42 / Racl chargé de GTP dans les cellules en repos

De plus, l'endocytose compensatrice évaluée par la mesure de l´internalisation de la dopamine-bêta-hydroxylase (marqueur membranaire des granules de sécrétion) est fortement inhibée dans les cellules chromaffines KO OPHN1. Cet effet inhibiteur est imité par l'expression d'un mutant tronquée de l'OPHN1, dépourvu du domaine BAR, démontrant l'implication de ce domaine dans la recapture de la membrane du granule après exocytose (fig7).

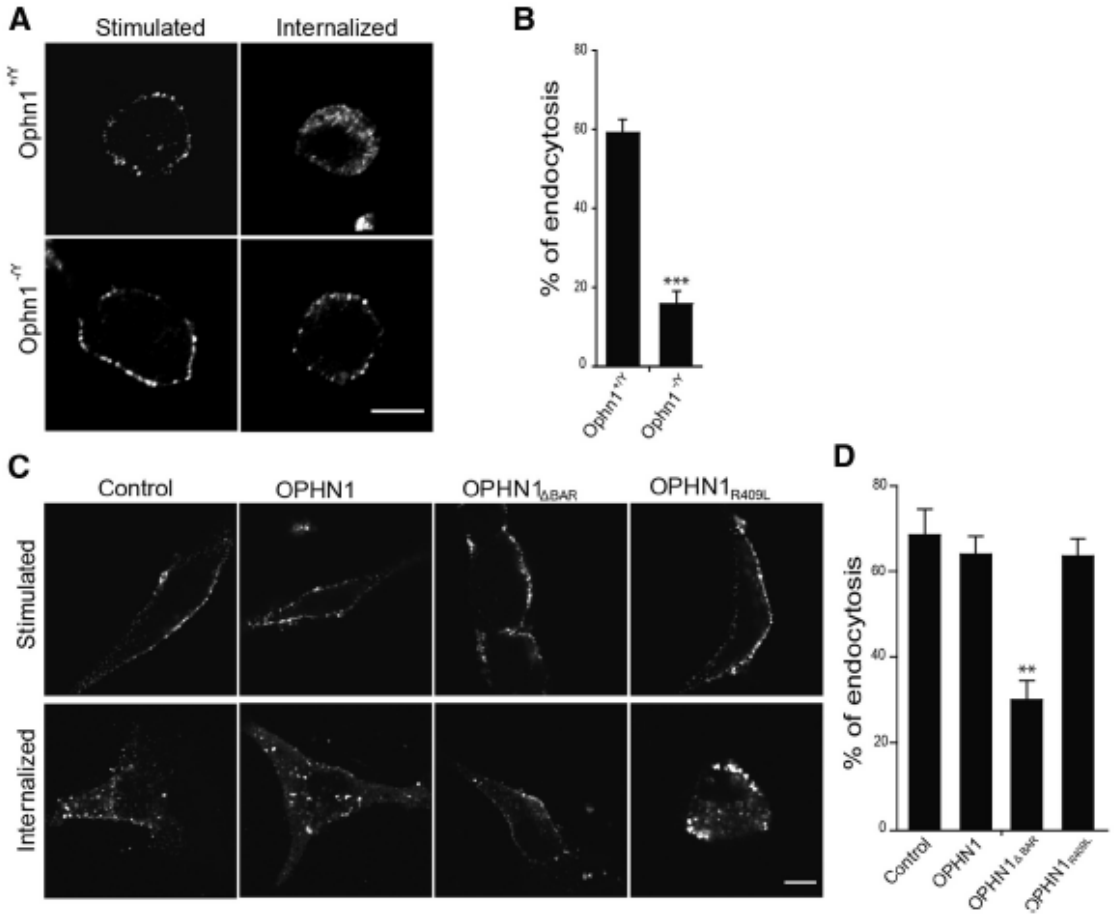

Fig7. OPHN1 est impliqué dans l'endocytose compensatrice par son domaine BAR. A, Des images confocales représentatives de cellules chromaffines de souris sauvages de type Ophnl + / y et Ophnl- / y soumises à un test d'internalisation d'anticorps anti-DBH. Les cellules ont été stimulées avec 59 mm K + pendant 10 min en présence d'anticorps anti-DBH et fixées (stimulées) ou maintenues pendant 15 minutes supplémentaires dans la solution de Locke sans anticorps pour permettre l'absorption de DBH / anti-DBH (internalisée). Les cellules ont été fixées, perméabilisées et traitées pour la détection anti-DBH en utilisant des anticorps secondaires couplés à Alexa Fluor 555. Barre d'échelle, 5 µm. B, Analyse de l'endocytose des vésicules positives au DBH à l'aide d'une carte de distance euclidienne. L'absorption de DBH a été réduite de 75% en l'absence d'OPHN1 (Ophn1- / y). *** p <0,001 (test de Mann-Whitney). C, D, OPHN1 nécessite son domaine BAR pour réguler l'endocytose compensatrice. Un test d'internalisation d'anticorps anti-DBH a été réalisé dans des cellules chromaffines bovines exprimant EGFP seul ou EGFP conjointement avec OPHN1, OPHN1R409L ou OPHN1ΔBAR. Les cellules ont été stimulées avec 59 mm K + pendant 10 min et ensuite incubées pendant 30 min à 4 ° C en présence d'anticorps anti-DBH. Les cellules ont ensuite été fixées (stimulées) ou maintenues pendant une période additionnelle de 15 minutes dans la solution de Locke (internalisée) avant fixation. La détection d'anticorps anti-DBH a ensuite été effectuée comme décrit ci-dessus. C, images confocales représentatives. Barre d'échelle, 5 µm. D, Analyse de l'endocytose des vésicules positives à la DBH en utilisant une carte de distance euclidienne. Notez que seul OPHN1ΔBAR affecte l'absorption de DBH. ** p <0,01 (essai de Mann-Whitney).

Dans l'ensemble, ces données démontrent pour la première fois qu'OPHN1 est une protéine bifonctionnelle capable de coupler, par des mécanismes distincts, l'exocytose avec l'endocytose compensatrice dans les cellules chromaffines de la glande surrénale.

2) Endocytose compensatrice des granules de sécrétion: régulation par la phospholipide scramblase-1 et la syntaxine-1

Sous l'action d'une augmentation de calcium intracellulaire, la protéine phospholipide scramblase-1 (PLSCR-1) stimule le mouvement de phospholipides d'un feuillet à l'autre des bicouches lipidiques. Les travaux antérieurs du laboratoire montrent qu'au cours de l'exocytose, la PLSCR-1, stimule le transport du phosphatidylsérine (PS) du feuillet interne de la membrane plasmique vers le feuillet externe au niveau des sites d'exocytose, conduisant à une perturbation de l'asymétrie de la membrane plasmique. De plus, les données du laboratoire démontrent que l'activité de la PLSCR-1, est essentielle pour l'endocytose compensatrice tandis qu'elle ne semble pas nécessaire pour l´exocytose. Afin de mieux comprendre la relation entre l'activité de la PLSCR-1 et les processus d´exocytose et d´endocytose compensatrice, j´ai analysé la translocation du PS dans les cellules chromaffines qui expriment des neurotoxines bactériennes connues pour altérer l'exocytose en clivant des protéines du complexe SNARE. Les deux toxines tétanique et botulique C bloquent la translocation du PS induite au cours de l'exocytose (fig8). Ces résultats suggèrent que l´augmentation du calcium intracellulaire n'est pas suffisant pour activer la PLSCR-1 et que l´arrimage des granules et/ou la fusion sont également nécessaires.

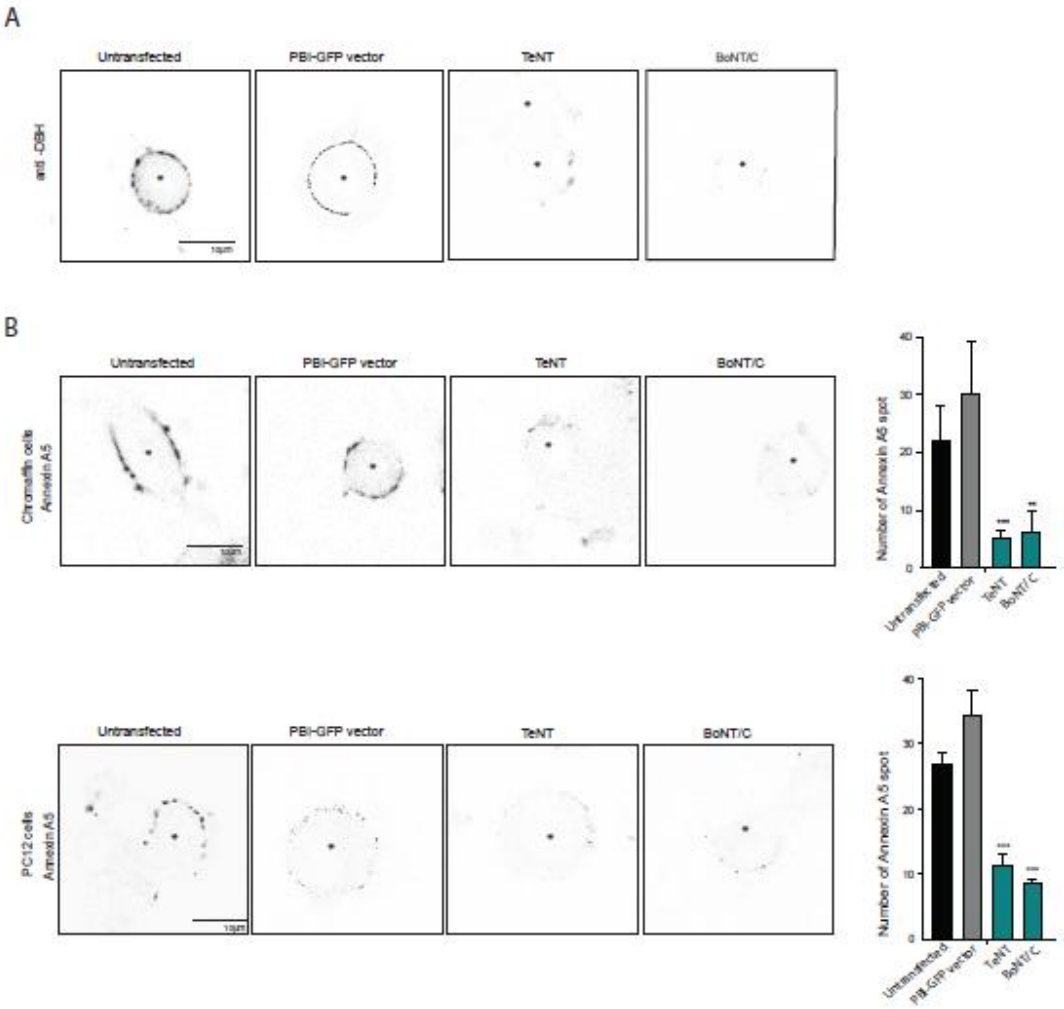

Fig8. La toxine tétanique et la toxine botulique de type C inhibent la sortie de PS dans les cellules chromaffines et PC12. Les cellules chromaffines et les cellules PC12 exprimant TeNT ou BoNT / C ont été stimulées avec K + 59 mM et incubées pendant 20 minutes à 4 ° C avec des anticorps anti-DBH de lapin pour détecter les sites d'exocytoses (A) ou incubées pendant 10 min à 37 ° C avec AnnexinA5 Conjugué avec alexa-647 pour révéler PS à la surface cellulaire (B). Les cellules ont ensuite été fixées et le DBH a été révélé avec les anticorps anti-lapin conjugués à Alexa-555. Les graphiques montrent le nombre de taches d'AnnexinA5 (n = 30 cellules), *** p <0,001, **, p <0,005.

Des expériences de précipitations protéiques avec une PLSCR-1 recombinante analysés par spectroscopie de masse et Western Blot, ainsi que des expériences de co-immunoprécipitation m'ont permis d´identifier la syntaxine-1A (STX1A), une protéine du complexe SNARE, en tant que partenaire protéique de la PLSCR-1 (table1, fig9). De manière surprenante, l´augmentation de la concentration de calcium perturbe l'interaction entre PLSCR-1 et STX1A.

Short name	Protein identification probability %	Percentage sequence coverage %	Uniprot link	Number of unique peptides
SNAP23	99	11	O09044	2
Syntaxin11	99	7	Q9D3G5	2
Syntaxin16	100	8	Q8BVI5	2
Syntaxin18	100	7	Q8VDS8	2
Syntaxin1A	100	11	O35526	3
Syntaxin4	100	12	P70452	3
Syntaxin8	100	15	O88983	3
Synaptotagmin5	100	8	Q9R0N5	1
VAMP7	100	22	P70280	6

Table 1 : analyse spectrométrique de masse

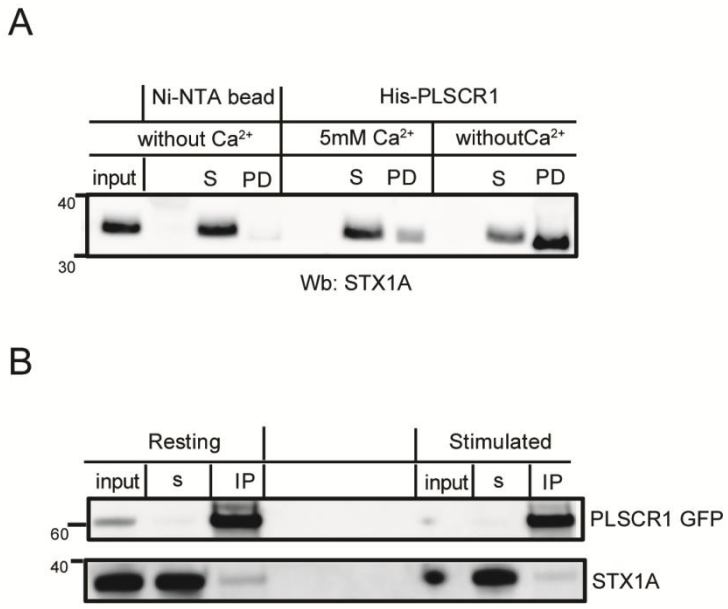

Fig9. La syntaxine 1A à partir du lysat de chromaffin et PC12 cellules précipitent avec PLSCR1 recombinant et exogène PLSCR1, respectivement. (A) Pull-down expériences a été réalisée par incubation de recombinant PLSCR1 marquée par His (His-PLSCR1) avec des lysats de cellules chromaffin. Syx1A a été révélé par western blot. S correspond à la fraction surnageante et PD correspond à la fraction pull- down. (B) Essai d'immunoprécipitation avec GFP-Trap Un système (Chrometek) a été réalisé avec des lysats de cellules PC12 exprimant PLSCR1-GFP. PLSCR1-GFP (anticorps anti-GFP) et endogène STX1A ont été détectés par western blot. S correspond à la fraction surnageante et IP correspond à la fraction précipitée.

J'ai également montré que les cellules dont l'expression de la STX1A est diminuée par ARN interférents présentent une augmentation de l´externalisation du PS en condition non stimulées, suggérant une augmentation de l'activité PLSCR-1 (fig10). J'ai également montré que la sortie du PS dans cette condition expérimentale est dépendante de l'activité de PLSCR1. Ceci a été démontré par des expériences dans les cellules co-transfectées avec le PLSCR1 mutant inactif et siRNA STX1A (fig11).

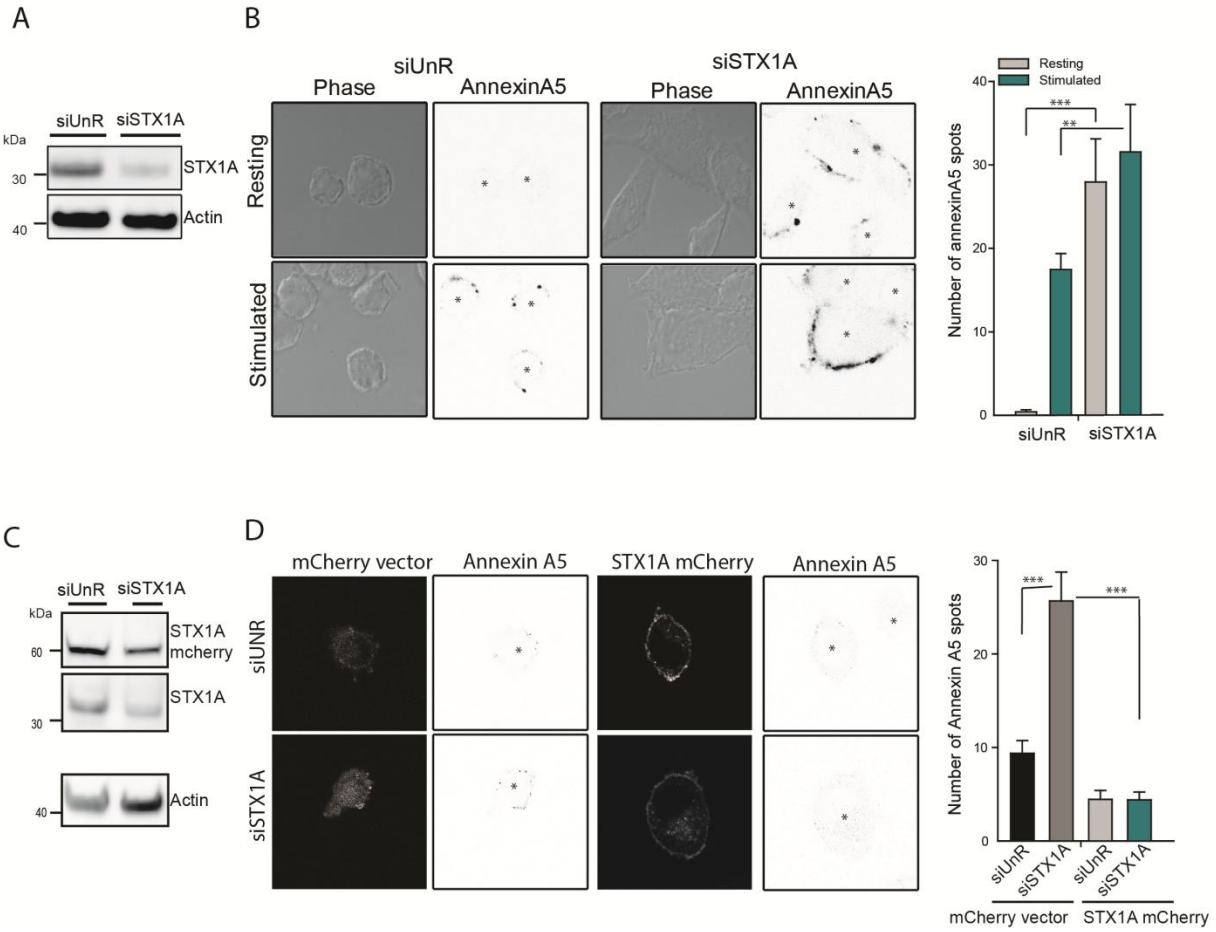

Fig10. STX1A knock-down déclenche la sortie PS dans les cellules PC12 de repos**.** Les cellules PC12 ont été transfectées avec un siRNA non apparenté (siUnR) ou avec un ARNsi de syntaxine 1 (siSTX1). (A) Les cellules ont été lysées 72 h après transfection et traitées pour analyse Western blot en utilisant des anticorps contre STX1 (A) ou incubées pendant 10 min à 37 ° C avec AnnexinA5 conjugué avec alexa-647 pour révéler la sortie PS à la surface cellulaire à la fois en repos et Stimulées (B). On notera que l'expression de knock-down de STX1A stimule de manière significative l'exposition à la surface cellulaire de PS dans des cellules en repos (graphique en B, n = 25 cellules, ** p <0,005, *** p <0,001). Restaurer le niveau normal de la surface cellulaire PS. Les cellules PC12 exprimant siUnR ou siSyx1 sont transfectées avec une construction codant pour Syx1a-mcherry qui est résistante au siRNA comme montré par l'analyse par transfert Western (C). Le niveau de PS de surface cellulaire a ensuite été observé par microscopie confocale et quantifié (images et graphe dans D, n = 30 cellules; *** p <0,001).

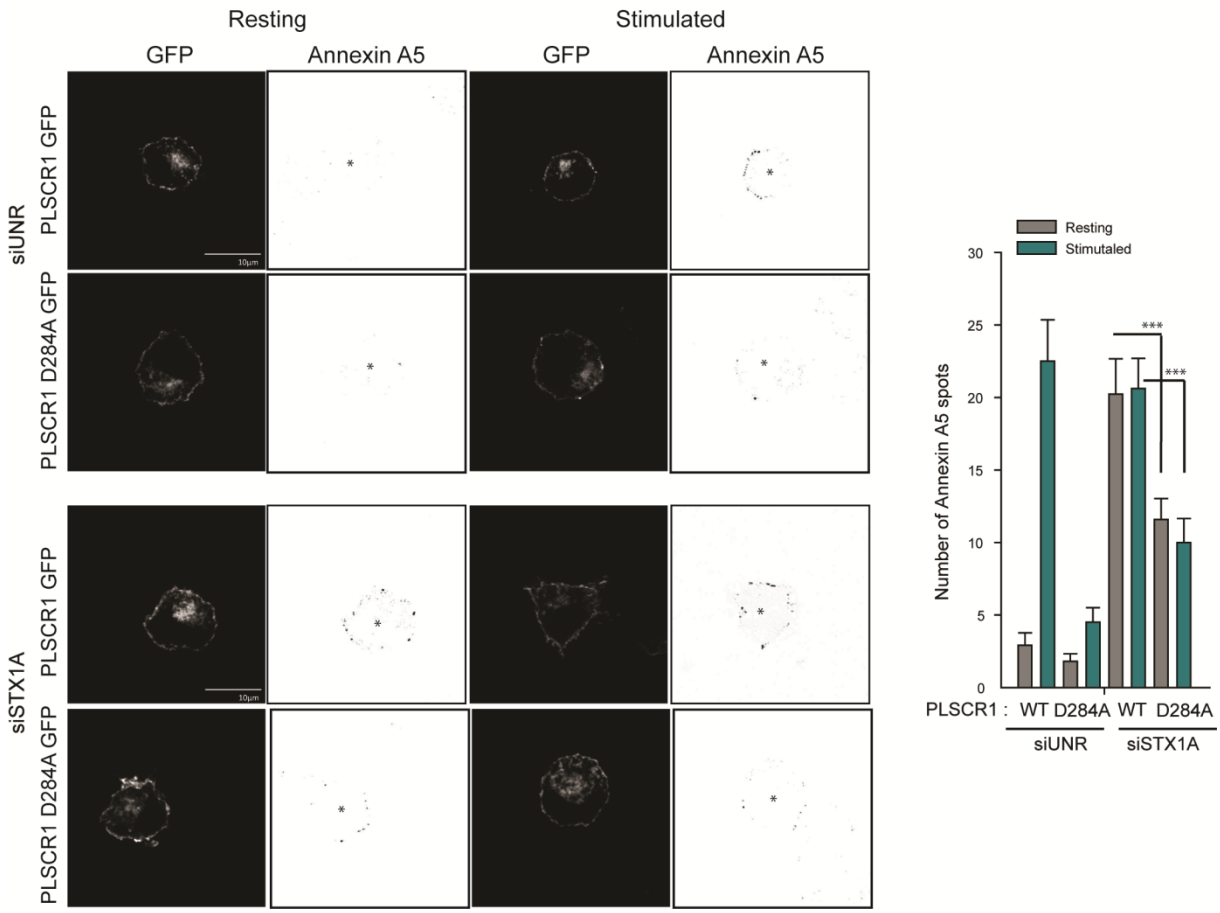

Fig11. Les cellules PC12 exprimant siUnR ou siSTX1 sont transfectées avec une construction codant soit PLSCR1-GFP (WT) soit PLSCR1D284A-GFP (D284A). La coloration de la surface de la cellule PS a ensuite été observée avec de l'annexine-A5 fluorescente dans des cellules de repos et stimulées (59 mM de K +, 10 min). Les taches d'Annexine A5 ont été quantifiées (graphique: n = 30 cellules; *** p <0,001).

Nous proposons que la stimulation des cellules chromaffines perturbe l'interaction de PLSCR-1 et STX1A, ce qui permet d'activer la PLSCR-1 et déclencher le transport de phospholipides entre les feuillets de la membrane plasmique.

D'autre part pourquoi le transport de PS vers le feuillet externe est nécessaire à l'endocytose compensatrice reste une question non résolue. Des analyses d'immunofluorescence m'ont permis de montrer une diminution du recrutement de la clathrine et de l´intersectine à la membrane plasmique en réponse à une stimulation de la sécrétion dans des cellules chromaffines KO PLSCR-1. Ces résultats sont extrêmement intéressants car nous savons que l'endocytose compensatrice est un mécanisme dépendent de la clathrine, finement régulé par des protéines à domaine BAR induisant des courbures membranaire comme FCHO. En effet, cette dernière est capable de recruter la protéine EPS15 qui interagit avec l´intersectine et permet l'initiation de l'endocytose. Ces résultats indiquent que la réorganisation de la distribution des phospholipides d'une face à l'autre de la membrane plasmatique favorise le recrutement de la machinerie moléculaire nécessaire à l'endocytose compensatrice.

Dans leur ensemble, ces résultats nous permettent de proposer l'hypothèse suivante : l'augmentation de calcium intracellulaire induite par la stimulation des cellules provoquerait la dissociation du complexe PLSCR1-SYTX1A et l'activation de PLSCR-1. Une fois activée la PLSCR1 mélange les phospholipides entre les feuillets interne et externe, cassant ainsi l'asymétrie membranaire et permettant ainsi la translocation de PS vers la face externe de la membrane plasmique. Cette redistribution de phospholipide favoriserait le recrutement de l'intersectine et la clathrine, deux protéines clés de l'endocytose compensatrice dans les cellules neuroendocrines.

Conclusion

En résumé, on peut conclure que i) PLSCR1 et STX1A forment un complexe dans les cellules chromaffines au repos; Ii) STX1A et PLSCR1 peuvent se dissocier après stimulation cellulaire pour effectuer deux fonctions distinctes, fusion des LDCV pour STX1A et externalisation du PS pour PLSCR1; Iii) STX1A réprime l'activité PLSCR1. Ce mécanisme peut fournir une synchronisation étroite entre l'exocytose et l'endocytose compensatoire: une fusion dépendante de STX1A doit se produire pour soulager le mélange des phospholipides dépendants de PLSCR1 et fournir une indication pour amorcer l'endocytose compensatrice. Le prochain défi sera de comprendre pourquoi la redistribution des phospholipides au niveau des sites de fusion des granules sécrétoires est essentielle pour la recapture vésiculaire.

Deuxièmement, OPHN1 fonctionne comme un point de contrôle structurel qui spatialement et temporellement couple l'exocytose et l'endocytose dans les cellules neuroendocrines. Il apparaît comme un bon candidat pour assurer un réglage fin de l'activité sécrétoire de l'hormone. Les prochains défis consisteront à déchiffrer les mécanismes par lesquels OPHN1 régule la dynamique des pores de fusion et l'importance du domaine OPHN1 BAR dans les processus endocytaires. Des mutations génétiques du gène OPHN1 menant à la délétion du domaine BAR ou à un domaine BAR non fonctionnel ont récemment été rapportées chez un patient présentant une déficience intellectuelle. Dans cette même ligne, il serait d'intérêt principal d'étudier si, les patients avec des mutations dans le gène d'OPHN1 montrent des désordres neuroendocriniens en plus des défauts neuronaux et des handicaps cognitifs associés.

Catherine Estay-Ahumada

Mécanismes moléculaires du couplage exocytose-endocytose dans les cellules neuroendocrines : rôle des protéines Scramblase-1 et Oligophrénine-1

Résumé :

De récentes études ont montré dans les cellules chromaffines que la libération des granules de sécrétion est temporellement et spatialement couplée au processus d'endocytose. Nous avons proposé l'hypothèse que la membrane du granule preserve son intégrité au sein de la membrane plasmique durant l'exocytose avant d'être internalisée ainsi avec ses composants. Cependant, les mécanismes moléculaires de ce processus d'endocytose compensatrice sont encore inconnus. Ainsi; mon projet de thèse vise a répondre à la question suivante : Quels sont les différents mécanismes déclenchant et régulant l'exocytose et l'endocytose compensatrice?

Les propriétés physiques des lipides jouent des rôles fondamentaux dans le trafic membranaire. Ils servent de système d'échafaudage pour maintenir la machinerie spécifique à des endroits précis de la membrane plasmique. Par exemple, la formation de microdomaines de gangliosides et de PIP2 au niveau des sites d'exocytose ou encore le mélange de lipides au sein de la bicouche lipidique représentent des processus attractifs pour permettre cette function au cours des événements d'exo-endocytose dans les cellules neuroendocrines. De plus, en raison de leur implication importante dans les processus d'exo-endocytose ou dans le remodelage des lipides, l'annexine A2, la synaptotagmine 1, l'oligophrénine1 et la scramblase 1 doivent être considérées comme des signaux potentiels pour le déclenchement de l'endocytose de la membrane granulaire.

Au cours de mon doctorat, je me suis intéressée à étudier comment l'exocytose et l'endocytose compensatrice sont régulées par la scramblase1 et l'oligophrénine1 dans les cellules chromaffines de la glande surrénale

Summary:

Recent studies in neuroendocrine chromaffin cells have suggested that the secretory granule release is temporally and spatially coupled to a compensatory endocytic process. Hence, we hypothesized that the secretory granule membrane would preserve its integrity within the plasma membrane after exocytosis before being retrieved as such along with its components. However, the underlying molecular mechanisms of this compensatory endocytic process are largely unknown today. Therefore my thesis project is aiming to address the following specific question: What are the different mechanisms triggering and regulating exocytosis and the compensatory endocytosis?

Physical properties of lipids play fundamental roles in membrane trafficking. They act as a scaffolding system to maintain specific machinery at restricted site of the plasma membrane. For example, the formation of ganglioside- and PIP2-enriched microdomains at the exocytic sites or the phospholipid scrambling across the bilayer plasma membrane, represent attractive processes to fulfill this function during exo-endocytosis events in neuroendocrine cells. Moreover, in view to their important implication in exo-endocytotic processes or lipid remodeling, annexin-A2, synaptotagmin-1, oligophrenin-1 and phospholipid scramblase-1 have to be considered as potential signal-triggers of the granule endocytosis.

During my PhD, I focused in investigating how exocytosis and compensatory endocytosis are regulated by PLSCR-1 and OPHN1 in adrenal chrommaffin cells.