Investigating the role of OCRL, the gene mutated in Lowe syndrome, in neurons

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OCRL resides at both excitatory and inhibitory synaptic boutons where participates in the regulation of SV recycling

SV recycling at presynaptic side is affected by the amount of PtdIns(4,5)P₂, whose levels are regulated by OCRL in addition to synaptojanin 1

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Abstract

Lowe syndrome, also known as Oculo-Cerebro-Renal syndrome of Lowe, is a rare X-linked condition that primarily affects the eye, the nervous system and the kidney.

OCRL, the gene mutated in Lowe syndrome, encodes an homonymous protein called OCRL which is an inositol polyphosphate 5-phosphatase acting preferentially on PtdIns(4,5)P2. OCRL is known to exert several relevant functions in both non-neuronal and neuronal cells. In neurons, PtdIns(4,5)P2, by binding its effectors, acts as an important regulator of processes indispensable for synaptic transmission such as exocytosis, endocytosis and recycling of synaptic vesicles. Whereas the roles of OCRL in non-neuronal cells have been widely explored over the last years, studies focused on its roles in neuronal cells are still missing. Hence, the pivotal role of PtdIns(4,5)P2 in neurons together with the main neurological features of Lowe syndrome (intellectual disability and epilepsy) prompted us to investigate the role of OCRL in the brain. Here I show that in the absence of OCRL neurons exhibit impaired recycling of synaptic vesicles accompanied by an imbalance between excitatory and inhibitory functions in OCRL-depleted hippocampi. Moreover, under neuronal stimulation an increase in the levels of PtdIns(4,5)P2 at OCRL-depleted synapses is observed. These defects, which can be attributed to dysfunctional PtdIns(4,5)P2 metabolism due to the absence of OCRL, result in an increased number and size of endocytic structures with a decrease in the number of synaptic vesicles available for neuronal functions. These findings shed light on the role of OCRL in neurons, which, by co-operating with other lipid phosphatases such as synaptojanin 1, ensures a correct balance
between PtdIns(4,5)P$_2$ synthesis and degradation at synapses, thus controlling crucial mechanisms in neurotransmission.
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Chapter 1

Introduction

1.1 Phosphoinositides (PIs) are determinants of organelle identity

Eukaryotic cells can perform their functions thanks to a complex system of membrane-bound compartments (organelles) engaged in specialized activities. Two major factors contribute to define organelle identity: the ultrastructural architecture establishing morphological identity, and specific lipids and proteins enriched on the cytosolic face defining the biochemical identity. Among these, the phosphoinositides (PIs) cooperate with two classes of GTPases, the Arf and the Rab proteins, thus playing a pivotal role in providing organelles with a unique identity (Behnia and Munro, 2005). Phosphatidylinositol (PI) is the basic building block for the PIs. In vivo, PIs are enriched at the cytosolic surface of biological membranes: the lipid portion (diacylglycerol, DAG) is directly inserted into the lipid bilayer and the hydrophilic headgroup protrudes into the cytosol where it is accessible for the PI regulatory enzymes and for various cytosolic proteins that use PIs to be recruited onto the membranes and perform their function (Lemmon 2008). The headgroup of PI can be reversibly phosphorylated by specific PI kinases forming three of the five free hydroxyl groups on the inositol ring (D3, D4 and D5) thus generating seven PI species, each of which has unique functions, subcellular localization and abundance (Fig. 1.1).

Although they are minor components of membrane phospholipids (~10%), PIs can regulate numerous cellular functions. This is possible thanks to their versatile nature, which is in turn determined by their fast metabolic interconversion operated by distinct PI kinases and phosphatases.
Figure 1.1 Major PI lipid kinase and phosphatase reaction pathways. Diagram from (Dickson and Hille 2019). Red arrows represent the PI lipid kinases and blue arrows represent PI lipid phosphatases.

Indeed, PI metabolism is guaranteed through the controlled recruitment and activation of PI metabolizing enzymes, so that distinct PIs can be enriched in specific membrane compartments (Fig. 1.2).

The nervous system is extremely enriched in lipids and PIs have key regulatory functions in synaptic transmission and synaptic vesicle (SV) trafficking and recycling. Hence, before going into detail on the role of PIs [in particular PtdIns(4,5)P_2] in synaptic transmission, I will introduce in the next section (1.2) SV exocytosis, endocytosis and recycling topics.
1.2 Synaptic transmission and type of synapses

Synaptic transmission is the biological process by which the communication between neurons occurs. It is extremely important for various functions to be accomplished (such as coordination of movement and cognitive functions) and occurs at synapses, highly specialized structures where the movement of chemical or electrical signals takes place. The flux of electrical signals is known as electrical neurotransmission and occurs at electrical synapses (Huxley & Hodgkin, 1952) where two neurons are tightly connected through gap junctions...
(Meier and Dermietzel 2006). Conversely, the flux of chemical signals, namely chemical neurotransmission, takes place at chemical synapses (Del Castillo & Katz, 1954; Fatt & Katz, 1952). The chemical synapses are functional units where a presynaptic and a postsynaptic neuron can communicate without being physically connected. Indeed, the axonal presynaptic and the dendritic postsynaptic compartments are separated by the synaptic cleft, a space which is about 20 nanometers (nm) wide across which the chemical signals flow. The transmission of the chemical signals is accomplished by chemical messengers called neurotransmitters. These molecules are stored in synaptic vesicles (SVs) and their release is ensured by the fusion of SVs with the plasma membrane (exocytosis). Once released, neurotransmitters diffuse across the synaptic cleft, thus reaching the postsynaptic membrane, where they are selectively bound by specific receptors leading to the activation or the inhibition of the postsynaptic compartment (Nachmansohn, 1959). Depending on the function exerted, synapses are classified in two main classes: excitatory (or glutamatergic) and inhibitory (or GABAergic) synapses, whose functional balance ensures physiological synaptic transmission. These two main synapse types were first analyzed from an ultrastructural point of view almost 60 years ago (Colonnier 1968; Gray 1969). More recently, combined cryo-Electron Tomography (ET)-correlative fluorescence microscopy has provided a more detailed characterization of synaptic ultrastructure and post-synaptic density (PSD) (Tao et al. 2018). Excitatory synapses were previously defined as “asymmetric” due to the presence of dense material on the postsynaptic side, whereas inhibitory synapses lacked prominent PSD and were considered “symmetric” (Peters et al., 1992). Together with a dense PSD at excitatory synapses, consistent with results already reported (Colonnier, 1968; Peters et al., 1992), Chang-Lu and colleagues
observed what they called a thin sheet-like PSD in inhibitory synapses (a feature not previously reported for these synapses). Nevertheless, the main feature of excitatory and inhibitory synapses is the presence of specific neurotransmitters packaged into SVs: glutamate and acetylcholine are the main excitatory neurotransmitters whereas $\gamma$-aminobutyric acid (GABA) and glycine are the main inhibitory neurotransmitters (Mattson and Kater, 1989).

1.2.1 The synaptic bouton: where SVs reside and traffic

The highly specialized cellular compartment dedicated to synaptic transmission is the synaptic bouton. This is the site where SVs filled with neurotransmitters fuse with the plasma membrane at the active zone (AZ) (releasing their content into the synaptic cleft) and then are recycled back, thus being available for a new round of release. The synaptic bouton has recently been studied in detail by Wilhelm and colleagues (Wilhelm et al., 2014) who combined different approaches (electron microscopy, super-resolution microscopy, biochemical and proteomic analyses) to generate a three-dimensional (3D) model of the synaptic bouton in which number, size and position of the organelles as well as abundance and localization of the proteins were described (Fig.1.3). According to data from Wilhelm’s work, it is possible to conclude that the synaptic boutons have a unique composition, with proteins enriched at their site of action, where their abundance correlates with the efficiency of the process in which they participate.

SVs, whose diameter defined by EM is almost 40 nm (Peters et al., 1992), comprise two main classes of proteins: transport proteins, responsible for the acidification of SVs and the uptake of neurotransmitters, and trafficking proteins, which coordinate the SV trafficking.
Considering the predominant role of SV proteins in governing the dynamics and trafficking of SVs, a rigorous regulation of protein turnover is crucial to avoid synaptic dysfunction. In recent years, different processes relevant for the degradation of SVs have been dissected (Binotti et al., 2014; Fernandes et al., 2014; Uytterhoeven et al., 2011), such as a mechanism providing an activity-dependent degradation of damaged SV proteins mediated by Rab35 and the ESCRT pathway (Sheehan et al., 2016). However, relatively recently it became more clear that synaptic transmission relies on an age-dependent control of SV components (Truckenbrodt et al., 2018). Indeed, to ensure reliable neurotransmission, synapses activate specific mechanisms, resulting in differential participation of younger (metabolically younger being composed of new proteins) and older (metabolically older being composed of older proteins) SVs in the SV cycle (Fig.1.4), with the former preferentially used in multiple
release cycles and the latter used only in the absence of newly synthesized vesicle proteins (Truckenbrodt et al. 2018).

**Fig. 1.4 Synaptic vesicle cycle.** Synopsis from Truckenbrodt et al., 2018 showing the SV cycle, from generation to degradation

In the synaptic bouton area, the SVs (estimated to be between several dozen and a thousand) are not randomly dispersed but are spatially and functionally organized in SV pools. Over the past years different criteria have been proposed to classify the different pools: a classical “distribution” criterion (which classifies SV pools depending on their distribution in the synaptic area); an “experimental” criterion (which defines different pools taking into account the approaches used to probe SVs and the methods used to mobilize the different pools) (Park et al., 2012; Shtrahman et al., 2005; Willig et al., 2006); a “functional” criterion aimed at functionally identifying the different pools. However, none of these approaches are complete enough to take into account all the properties, morphological features and molecular functions of each pool. This is due to several reasons: SVs appear to be spatially intermixed (Denker and Rizzoli 2010) rather than unambiguously localized in different pools precisely distributed in the synaptic
space; the “experimental” criterion suffers from a bias due to the fact that the results largely vary depending on the experimental approach used for the analysis; the functional classification, instead, does not envisage that SVs belonging to the same pool may have different characteristics or, conversely, that SVs may reside in different functional pools although they have similar characteristics. Thus, an “ideal” criterion should provide a comprehensive classification of the SV pools based on their spatial distribution, their mobilization in response to different stimuli, but also their molecular characteristics, all of which determine a crucial feature for SV pools: the release probability. According to these requirements, a model has been proposed (Denker and Rizzoli 2010; Rizzoli and Betz 2005) in which SVs belong to three different pools: the Ready Releasable Pool (RRP) composed of SVs already docked at the active zone (Denker et al., 2009); the Recycling Pool (RP) located immediately behind the RRP, but always in the AZ area; and the Reserve Pool, occupying an area distant from the AZ (Denker and Rizzoli, 2010). The RRP and the RP together form the so-called total recycling pool. Since the RRP is composed of already docked SVs [almost 10 in the hippocampal synaptic bouton, (Dobrunz and Stevens 1997; Murthy and Stevens 1999)], it has the highest release probability, being rapidly depleted during physiological stimulation or upon an osmotic stimulus (Fatt and Katz, 1952). The RP (including almost 20% of the total SVs) is mobilized immediately after the RRP during physiological stimulation. In the end, the reserve pool, comprising 50% or even more of the total SVs, which acts as a buffer for proteins involved in SV recycling (Denker and Rizzoli, 2010), is recruited only when recycling is inhibited in vivo (Denker et al., 2011), upon prolonged low-frequency or intense high-frequency stimulation (Fernandez-Alfonso and Ryan, 2008; Ikeda and Bekkers, 2009), or upon inhibition of cyclin-dependent kinase 5
(CDK5), which, together with calcineurin, participates in controlling the availability of the resting pool and the conversion of non-recycling SVs into actively recycling vesicles (Kim and Ryan, 2010). The resting pool architecture is controlled by other proteins, among which a dominant role is executed by synapsin 1, a SV protein already implicated in the maintenance of synaptic cluster composition during synaptogenesis (Bonanomi et al., 2005) and recently found to determine the formation of a distinct liquid phase in the aqueous synaptic environment (Milovanovic et al., 2018).

1.2.2 SV exocytosis: the process responsible for neurotransmitter release

When an action potential reaches the presynaptic terminal it causes Ca$^{2+}$ channel opening followed by a local increase of Ca$^{2+}$ that binds and activates synaptotagmin 1 (syt 1), a synaptic vesicle membrane protein acting as a calcium sensor [this role in SV exocytosis has been demonstrated also for syt 2 and syt 9, which trigger fusion but with distinct kinetics (Xu et al., 2007)]. Syt 1 is anchored to the SV membrane by its N-terminal transmembrane domain whereas two cytoplasmic C2 domains (C2A and C2B) form binding sites for Ca$^{2+}$, thus acting as Ca$^{2+}$-dependent membrane targeting modules (Davletov & Sudhof, 1993; Sugita et al., 1996). Once bound to Ca$^{2+}$, syt 1 acquires the ability to bind phospholipids (Schiavo et al., 1998) and concomitantly interacts with SNARE (Soluble N-ethylmaleimide-NSF-Attachment Protein Receptor) proteins (Hui et al., 2005; Li et al., 1995; Mohrmann et al., 2013; Schiavo et al., 1997; Sugita et al., 2002; Sutton et al., 1999). The three SNARE proteins forming the complex essential for fusion are the vesicular SNARE (v-SNARE) VAMP2 (Vesicle Associated Membrane Protein 2) in the vesicle membrane and two target SNAREs (t-SNAREs) syntaxin1 and SNAP25 (Synaptosome Nerve-Associated
Protein 25) in the plasma membrane. These proteins assemble into stable and parallel four-helix bundles (Jahn and Fasshauer 2012; Jahn and Scheller 2006; Rizo and Südhof 2012; Südhof and Rothman 2009) composed of two SNAP25 motifs and one VAMP2 and syntaxin1 motif. SNARE complex assembly is promoted by some accessory proteins such as Munc18, Munc13, complexin (Dawidowski & Cafiso, 2016; Ma et al., 2013; Xue et al., 2010), and α-synuclein, the latter binding directly to VAMP2 (Burré et al. 2010). Thus, as also shown in Fig. 1.5, upon Ca$^{2+}$ increase at the active zone and Ca$^{2+}$ sensing by syt 1, fusion is driven by the SNARE complex, which facilitates the formation of the fusion pore and thus neurotransmitter release.

Fig. 1.5 SNARE/ SM protein cycle. Diagram from Sudhof & Rizo, 2012 showing the SNARE/SM (synaptic membrane) protein cycle composed of 4 main steps: SNARE-complex assembly, fusion-pore opening, fusion-pore expansion and SNARE-complex disassembly after fusion.
These processes have also been recently studied in a reconstituted fusion assay (Bao et al. 2018). Immediately after fusion the SNARE complex disassembles in an NSF- and SNAPs-mediated process (Zhao et al. 2015).

1.2.3 Coupling of exocytosis and endocytosis at the synapse

A process to retrieve SV membranes and protein components after exocytosis is of fundamental importance to ensure synaptic homeostasis and efficient neurotransmission. This process, known as compensatory endocytosis (explained in 1.2.4), occurring at specialized endocytic sites called endocytic zones (EZs), is highly controlled and tightly coupled with the exocytic process (occurring at the AZs). The regulation of this coupling is mainly due to the presence of proteins and membrane lipids that play a role in both processes. Among these factors, some proteins are noteworthy: the calcium sensors synaptotagmin 1 and calmodulin (CaM) [indeed, it has been demonstrated that Ca$^{2+}$ levels as high as (Hosoi et al., 2009; Kochubey et al., 2011; Lou et al, 2005), with the former regulating both exocytosis and endocytosis in a Ca$^{2+}$-dependent manner (Nicholson-Tomishima & Ryan, 2004; Poskanzer et al., 2003; Yao et al., 2012) and the latter interacting with the protein phosphatase calcineurin (Sun et al., 2019), thus forming a complex able to dephosphorylate and regulate, in response to local Ca$^{2+}$ increase, different proteins involved in SV endocytosis such as dynamin 1 (Armbruster et al., 2013) synaptojanin 1 (Bauerfeind et al., 1997; Lee et al., 2004), PIPK1γ (Di Paolo, Pellegrini, et al. 2002; Sang et al. 2005), and endophilin A (Myers et al., 2016).

Other important players in exo-endocytic coupling are the SNARE proteins VAMP2, syntaxin1 and SNAP25 [involved in SV endocytosis in addition to their role in fusion (Deák et al., 2004; Xu et al., 2013; Zhang et al., 2013); the synaptic
proteins synaptophysin (Gordon et al., 2011) and endophilin A known to regulate endocytosis through recruitment of synaptojanin 1 and dynamin 1 (Milosevic et al. 2011; Verstreken et al. 2003). Because of its presence at both AZs and EZs, actin is also considered an essential matching factor for exo-endocytosis. Furthermore, PtdIns(4,5)P$_2$ is considered a crucial factor in coupling exocytosis and endocytosis for multiple reasons: first, it is enriched at both AZs and EZs (Van Den Bogaart et al. 2011); second, it is bound by proteins involved in exocytosis as well as endocytosis, as syntaxin 1 (Lam et al., 2008); third, when its synthesis-metabolism cycle is deregulated, both exocytosis and endocytosis are affected (Cremona et al., 1999; Di Paolo et al., 2004; Harris et al., 2000; Milosevic et al., 2005); fourth, PtdIns(4,5)P$_2$ at the synapse is mainly synthesized by PIPK1$\gamma$, whose activation is under the control of the CaM/Calcineurin-Cdk5 (cyclin-dependent kinase 5) system (Di Paolo et al., 2002; Sang et al., 2005).

However, some crucial points regarding the coupling between exocytosis and endocytosis remain unaddressed. How does fusion of SVs with the plasma membrane trigger endocytosis and SV retrieval? How is exo-endocytosis coupling regulated? How does the exocytosis rate determine the efficiency of the compensatory endocytosis? Lastly, how is PtdIns(4,5)P$_2$ regulated during SV recycling and how does this lipid contribute to exo-endocytic coupling?

### 1.2.4 Compensatory endocytosis: the process responsible for SV recycling

This process, immediately following SV exocytosis, is indispensable for ensuring recycling of SVs (allowing SV regeneration and reformation of an actively recycling pool), retrieval of membranes (with restoration of plasma membrane tension) and clearance of the AZ (making it available for a new round of exocytosis), thus supporting neuronal activity at high levels. The endocytic
processes identified so far can be essentially grouped in two main categories: clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE). CME is the best characterized endocytic route as well as the predominant mechanism through which vesicles are recycled in response to low intensity stimuli (Granseth et al., 2007; Heuser & Reese, 1973). The retrieval, following fusion at the AZs, occurs at distal sites (Fig. 1.6a) and is relatively slow, requiring almost 30 seconds to be completed (Ehrlich et al., 2004; Taylor et al., 2011). In addition to clathrin and clathrin adaptors (AP2, AP180, CALM), this process largely depends on the function of endocytic factors such as PtdIns(4,5)P$_2$, synaptojanin 1, dynamin and endophilin. In particular, CME is initiated by PtdIns(4,5)P$_2$ (enriched at the plasma membrane), which recruits clathrin and clathrin adaptors to form coated pits, followed by arrival of dynamin and endophilin, and lastly by the fission of the coated vesicles. At the end of this process, PtdIns(4,5)P$_2$ is dephosphorylated by synaptojanin, allowing clathrin disassembly and reformation of SVs ready to be used for a new round of release (Cao et al., 2017; Ferguson et al., 2007; Milosevic et al., 2011; Newton et al., 2006; Perera et al., 2006; Yamashita et al., 2005).

<table>
<thead>
<tr>
<th>(a) Clathrin-mediated endocytosis (CME)</th>
<th>(b) Clathrin-independent endocytosis (e.g. UFE) &amp; Clathrin-dependent SV reformation</th>
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<td><img src="image" alt="Diagram of CME-independent process" /></td>
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<tr>
<td>~20 sec</td>
<td>~1 sec</td>
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<td>(c) Kiss-and-Run</td>
<td>(d) Activity-dependent bulk endocytosis (ADBE)</td>
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<td><img src="image" alt="Diagram of Kiss-and-Run process" /></td>
<td><img src="image" alt="Diagram of ADBE process" /></td>
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<td>~1-2 sec</td>
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**Fig. 1.6 Synaptic vesicle recycling modes.** Modified from Soykan et al., 2016.
Several recycling modes independent of clathrin have been identified and characterized over the years: kiss-and-run (K&R) (Ceccarelli et al., 1973), activity-dependent bulk endocytosis (ADBE) (Clayton & Cousin, 2009; Nicholson-Fish et al, 2015), and ultrafast endocytosis (UFE) followed by clathrin-dependent SV reformation (Watanabe et al. 2013). K&R (SV endocytosis occurs at the same site of exocytosis resulting in reformed SVs that should be identical to those previously fused with the plasma membrane) is not convincingly accepted in the field because it has been demonstrated that the SVs reformed through endocytosis do not have the same protein makeup as those that have undergone exocytosis (Gimber et al., 2015; Wienisch & Klingauf, 2006). ADBE is activated as a repair mechanism in response to prolonged high intensity stimuli causing a strong exocytic response and thus the arrival at the plasma membrane of huge amounts of SV membranes and components. It occurs at distal sites, is a relatively slow process (requiring up to 60 seconds to be completed) and is strictly dependent on calcium and thus on the pathways activated by calcium at the synapse, such the CaM-calcineurin system (Cheung and Cousin 2013).

UFE was identified relatively recently by Watanabe and colleagues, who used an innovative electron microscopy (EM) approach (Watanabe et al. 2013), called “flash and freeze” coupled with optogenetics. They used optogenetics to evoke an action potential in hippocampal neurons and then froze the samples, thus being able to visualize exocytosis (15-30 msec after the light pulse) and, immediately following fusion, endocytosis (starting 50 msec after the light pulse) (Fig 1.7). They also demonstrated that endocytosis depends on exocytosis to start and also on actin and dynamin to be completed (Watanabe et al. 2013).
Clathrin at endosomes after 10 stimuli

The acute inhibition of clathrin function by Pitstop 2, a potent inhibitor.

...not significant. Error bars, s.e.m.

100 ms (eration of synaptic vesicles from endosomes (Extended Data Fig. 8; see...respectively. Arrows outlined in red indicate the plane of the image shown in...the rate of CME by clathrin knockdown to 30% of that in controls

Deep pits

Number of shallow pits, deep pits and large vesicles in the...100 nm

Figure 1.7 Ultrafast endocytosis. Micrographs from Watanabe et al., 2013 showing fusing vesicles at 15 ms (c) and 30 ms (d) after the light pulse. e) image showing two exocytic intermediates in the active zone.

A year later, the same group demonstrated that SVs that reform with ultrafast endocytosis pass through synaptic endosomes and that the reformation of SVs from these endosomes is clathrin-dependent (Watanabe et al. 2014) (Fig 1.8).

Figure 3. Reduced SV Density and Increase in number of...0.31, Tmx 5 Hz: 10.82 ± 1.18, p < 0.0001, EtOH

Fig. 1.8 Ferritin uptake in control and clathrin knockdown neurons. Electron micrographs from Watanabe et al., 2014 showing control neurons, where ferritin is present in large endocytic vesicles after stimulation and in synaptic vesicles (a) and clathrin KO,
where ferritin appears trapped in endosomes (c) at different time points after stimulation. Black arrows indicate ferritin-positive structures.

Simultaneously, Haucke’s group demonstrated that, despite the fact that UFE retrieval of SVs is clathrin-independent, clathrin and the clathrin adaptor AP2 play a major role in the reformation of SVs from endocytic intermediates, that they called endosome-like vacuoles (Fig 1.9).

![Image of electron micrographs showing endosome-like vacuoles in clathrin knockdown neurons.](image)

**Fig. 1.9 Endosome-like vacuoles in clathrin knockdown neurons.** Electron micrographs from Kononenko et al., 2014 showing SVs in synaptic terminals from control neurons (scr; Ai and Bi) and SVs together with endosome-like vacuoles (ELVs, arrows) in synaptic terminals from clathrin heavy chain knockdown neurons (shCHC; Aii and Bii) challenged with 200 APs at 5 Hz (Ai and Aii) or 40 Hz (Bi and Bii) and fixed immediately thereafter.

UFE thus became, particularly interesting in the recent years because its activation in response to intense stimuli has been proved. However, the mechanisms involved in this recycling route have not been completely elucidated yet (Kononenko et al. 2014).
1.3 Phosphoinositides (PIs) in neuronal communication

1.3.1 Roles of PIs in neurons

Kinases and phosphatases (Table 1.1) work to maintain a balance between the synthesis and metabolism of all PI species to avoid pathological conditions associated with an accumulation or decrease of PIs. As in other cells, PIs exert their functions in neurons by cooperating with protein molecules, thus acting as second messengers and driving synaptic functions.

<table>
<thead>
<tr>
<th>Kinases</th>
<th>H. sapiens</th>
<th>D. melanogaster</th>
<th>C. elegans</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIK3C/CB/CD/CG</td>
<td>PK3026/CG414</td>
<td>PK3026</td>
<td>PK3026</td>
</tr>
<tr>
<td>PIK3C2/2B/2G</td>
<td>PK3669/CG11621</td>
<td>PK3669</td>
<td>PK3669</td>
</tr>
<tr>
<td>PIK3C3</td>
<td>PK3684</td>
<td>PK3684</td>
<td>PK3684</td>
</tr>
<tr>
<td>PIK4C/2A/2B</td>
<td>PK3028/CG310260</td>
<td>CELE_Y729A2.4/NSP_49370.1</td>
<td></td>
</tr>
<tr>
<td>PIK4CB/2K/1B</td>
<td>fwo/CG37004</td>
<td>fwo</td>
<td>fwo</td>
</tr>
<tr>
<td>PIS5K/1A/1B</td>
<td>PIS5KB/CG3682</td>
<td>PIS5KB</td>
<td>PIS5KB</td>
</tr>
<tr>
<td>PIS5K/1F/4Y</td>
<td>Fab1/CG3682</td>
<td>Fab1</td>
<td>Fab1</td>
</tr>
<tr>
<td>PIS5K/4A/2B/5C</td>
<td>dppR/CG17471</td>
<td>dppR</td>
<td>dppR</td>
</tr>
</tbody>
</table>

**Table 1.1 PI kinases and phosphatases in Homo sapiens, Drosophila melanogaster and Caenorhabditis elegans.** From (Raghu et al., 2019).
Indeed, even though PIs are relatively low abundant membrane lipids, PI-binding proteins are able to recognize them thus localizing to the membrane compartments where the recognized lipid is enriched (Table 1.2). The most abundant PIs in neurons are PtdIns4P and PtdIns(4,5)P₂, but other PIs have also been found to play a role in the nervous system. These roles have been clearly characterized in the presynaptic endo-lysosomal pathway. As shown in Fig. 1.10, PIs are enriched in distinct sub-compartments, contributing to functional sub-compartmental differences (Di Paolo and De Camilli 2006).

### Table 1.2 Neuronal PI-binding proteins, with binding domains and functions. From (Ueda 2014).

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Binding domain</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEAI</td>
<td>Prk/Prk</td>
<td>FYVE</td>
<td>Excitatory synaptic transmission</td>
</tr>
<tr>
<td>PKCε</td>
<td>Prk/Prk</td>
<td>FYVE</td>
<td>Degradation of Ca(V)1.2 channels, endocytic cycling of AMPAR</td>
</tr>
<tr>
<td>SNX13</td>
<td>Prk/Prk</td>
<td>PX</td>
<td>Neuronal development</td>
</tr>
<tr>
<td>Profilin</td>
<td>Prk/Prk</td>
<td>Positive electrostatic potential</td>
<td>Spine morphology</td>
</tr>
<tr>
<td>Cofilin</td>
<td>Prk/Prk</td>
<td>40 % surface of cofilin</td>
<td>AMPAR trafficking</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Prk/Prk</td>
<td>PH</td>
<td>Development of dendritic spines and synapses</td>
</tr>
<tr>
<td>Golgin</td>
<td>Prk/Prk</td>
<td>N and C termini</td>
<td>C²⁺ channel and NMDAR activity</td>
</tr>
<tr>
<td>Syntaxin</td>
<td>Prk/Prk</td>
<td>FERM</td>
<td>Molecular interactions underlying synaptic junctions</td>
</tr>
<tr>
<td>Ezrin</td>
<td>Prk/Prk</td>
<td>FERM</td>
<td>Filopodial protrusion formation, growth cone guidance</td>
</tr>
<tr>
<td>Radixin</td>
<td>Prk/Prk</td>
<td>FERM</td>
<td>Filopodial protrusion formation, growth cone guidance</td>
</tr>
<tr>
<td>Munc</td>
<td>Prk/Prk</td>
<td>FERM</td>
<td>Filopodial protrusion formation, growth cone guidance</td>
</tr>
<tr>
<td>Fimbrin</td>
<td>Prk/Prk</td>
<td>Amino acid basic region</td>
<td>Migration of neurons</td>
</tr>
<tr>
<td>α-Actinin</td>
<td>Prk/Prk</td>
<td>Amino acid basic region</td>
<td>Spine morphology, NMDAR activity</td>
</tr>
<tr>
<td>MARCKS</td>
<td>Prk/Prk</td>
<td>Amino acid basic region</td>
<td>Maintenance of dendritic spine, LTP</td>
</tr>
<tr>
<td>Cofilin</td>
<td>Prk/Prk</td>
<td>Amino acid basic region</td>
<td>Dendritic spine formation, growth cone</td>
</tr>
<tr>
<td>Spectrin</td>
<td>Prk/Prk</td>
<td>PH</td>
<td>Spine morphogenesis, axonal transport of mitochondria</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Prk/Prk</td>
<td>PH</td>
<td>Neuronal migration</td>
</tr>
<tr>
<td>Synaptogrin</td>
<td>Prk/Prk</td>
<td>C2</td>
<td>Ca²⁺ sensor for the exocytosis</td>
</tr>
<tr>
<td>Doc2</td>
<td>Prk/Prk</td>
<td>C2</td>
<td>Ca²⁺ sensor for asynchronous exocytosis</td>
</tr>
<tr>
<td>Munc13</td>
<td>Prk/Prk</td>
<td>C1</td>
<td>Priming factor for synaptic vesicle</td>
</tr>
<tr>
<td>Rabphilin 1A</td>
<td>Prk/Prk</td>
<td>C2</td>
<td>Priming factor for synaptic vesicle</td>
</tr>
<tr>
<td>CAPS</td>
<td>Prk/Prk</td>
<td>C2</td>
<td>Priming factor for synaptic vesicle</td>
</tr>
<tr>
<td>Mnt</td>
<td>Prk/Prk</td>
<td>PTB</td>
<td>Priming factor for synaptic vesicle</td>
</tr>
<tr>
<td>Pucko/ actinin</td>
<td>Prk/Prk</td>
<td>C2</td>
<td>Priming factor for synaptic vesicle</td>
</tr>
<tr>
<td>RIM</td>
<td>Prk/Prk</td>
<td>C2</td>
<td>Priming factor for synaptic vesicle</td>
</tr>
<tr>
<td>Syntaxin 1</td>
<td>Prk/Prk</td>
<td>SNARE, hydrophobic</td>
<td>SNARE protein for synapse fusion</td>
</tr>
<tr>
<td>Epin</td>
<td>Prk/Prk</td>
<td>AENTH</td>
<td>Creating membrane curvature</td>
</tr>
<tr>
<td>AP180</td>
<td>Prk/Prk</td>
<td>AENTH</td>
<td>Clathrin-mediated Endocytosis</td>
</tr>
<tr>
<td>CALM</td>
<td>Prk/Prk</td>
<td>AENTH</td>
<td>Clathrin-mediated Endocytosis</td>
</tr>
<tr>
<td>AP-2</td>
<td>Prk/Prk</td>
<td>Electrostatics and hydrophobic</td>
<td>Clathrin-mediated Endocytosis</td>
</tr>
<tr>
<td>Arf 6</td>
<td>Prk/Prk</td>
<td>Electrostatics and hydrophobic</td>
<td>Clathrin-mediated Endocytosis</td>
</tr>
<tr>
<td>Dynamin</td>
<td>Prk/Prk</td>
<td>PH</td>
<td>Scission of vesicles at presynaptic side, and AMPAR cycling at endocytic zone in spine</td>
</tr>
<tr>
<td>PLD</td>
<td>Prk/Prk</td>
<td>PH</td>
<td>Neuritic outgrowth</td>
</tr>
<tr>
<td>Prone</td>
<td>Prk/Prk</td>
<td>FERM</td>
<td>Spine morphology and dendritic outgrowth</td>
</tr>
<tr>
<td>KCNQ</td>
<td>Prk/Prk</td>
<td>Electrostatics and hydrophobic</td>
<td>Regulate cell membrane potential and excitability in neurons</td>
</tr>
<tr>
<td>Fodrin</td>
<td>Prk/Prk</td>
<td>PTEN domain</td>
<td>Polarization of neurites</td>
</tr>
<tr>
<td>ARNO</td>
<td>PIP3/PIP3</td>
<td>PH</td>
<td>Regulation of dendritic development</td>
</tr>
<tr>
<td>SRC</td>
<td>PIP3/PIP3</td>
<td>PTB</td>
<td>Neuronal migration, Regulation of hippocampal synaptic plasticity</td>
</tr>
<tr>
<td>Ten1</td>
<td>PIP3/PIP3</td>
<td>PH</td>
<td>Dendritic spine development</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>PIP3/PIP3</td>
<td>PH</td>
<td>LTP induction</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>PIP3/PIP3</td>
<td>PH</td>
<td>Dendritic arborization</td>
</tr>
<tr>
<td>Protrudin</td>
<td>PIP3/PIP3</td>
<td>FYVE</td>
<td>Neuritic outgrowth</td>
</tr>
</tbody>
</table>
Fig. 1.10 Distribution of PIs. Modified from (Wenk and De Camilli 2004). Vesicle traffic in nerve terminals and putative relationship to phosphoinositide metabolism. Membranes are color-coded based on their putative content in specific phosphoinositide species.

PtdIns(3,4,5)P$_3$ is a low abundant lipid, generated from PtdIns(4,5)P$_2$ by class I phosphatidylinositol 3-kinase (PI3K) (Cantley, 2002; Ghigo et al., 2015; Margaria et al., 2019) and degraded either to PtdIns(4,5)P$_2$ by the 3-phosphatase PTEN (Downes et al. 2007) or to PtdIns(3,4)P$_2$ by 5-phosphatases such as the Oculo-Cerebro-Renal-Lowe syndrome protein (OCRL) and the SH2 Domain-Containing Inositol 5-Phosphatases SHIP-1 and SHIP-2 (also known as INPPL1 and INPP5D, respectively). PtdIns(3,4,5)P$_3$ is present at both the presynaptic and postsynaptic terminals. At the presynapse it is enriched at release sites and participates in Syntaxin1 clustering and neurotransmitter release (Khuong et al. 2013), whereas at postsynaptic membranes it is indispensable to control AMPA receptor clustering and to maintain long term potentiation (LTP) through binding to Pleckstrin Homology Like Domain Family B Member 2 (PHLDB2) (Arendt et al. 2010; Xie et al. 2019).

PtdIns(3,4)P$_2$ is mainly generated from PtdIns(3,4,5)P$_3$ through OCRL, SHIP1 and SHIP2-mediated degradation or from PtdIns4P through class II PI3Ks-mediated synthesis (Gulluni et al, 2019). In neurons it is mainly enriched at the plasma membrane and at early endosomes and has been found to be primarily
involved in the regulation of actin aggregation and neuritogenesis during development (Zhang et al., 2017).

**PtdIns(3,5)P$_2$** is mainly enriched on late endosomes and lysosomes (Sbrissa et al. 2007). It is produced from PtdIns3P by the PtdIns3P-5 kinase PIKfyve (Sbrissa et al., 1999) and degraded back to PtdIns3P (Sbrissa et al. 2007) by SAC3 (also known as FIG4) or to PtdIns5P (Schaletzky et al. 2003) by myotubularin (MTM) family 3-phosphatases. In neurons, this lipid has been associated with the regulation of glutamate receptor trafficking and recycling during synaptic depression (McCartney et al. 2014; Seebohm et al. 2012), but also in neuron protection from excitotoxicity (Tsuruta et al., 2009).

**PtdIns3P** is present in axons as well as in dendrites and is associated with early/recycling endosomes, whence it primarily regulates post-synaptic trafficking of GABA receptors at inhibitory post-synapses (Papadopoulos et al. 2017).

**PtdIns4P** is highly abundant at presynaptic terminals being mainly enriched at SVs where it is synthesized from PI by phosphatidylinositol 4-kinase 2α (PI4K2α) (Guo et al. 2003). It is also produced either through degradation of PtdIns(4,5)P$_2$ mainly performed by the 5-phosphatases synj1 (McPherson et al., 1994; McPherson et al., 1996) and OCRL or through degradation of PtdIns(3,4)P$_2$ by the 3-phosphatase PTEN (Downes et al. 2007). Although the role of PtdIns4P at the Golgi complex in non-neuronal cells has been clearly elucidated in the last decade (De Matteis & Godi, 2004; Szentpetery et al., 2010; Venditti et al., 2019), the PtdIns4P functions at the presynaptic terminal in neuronal cells have been strongly associated with PtdIns(4,5)P$_2$ metabolism [PtdIns4P is indeed both a precursor of PtdIns(4,5)P$_2$ and its degradation product]; hence its functions, considered associated with PtdIns(4,5)P$_2$ functions, will be discussed in the next paragraph.
1.3.2 PtdIns(4,5)P$_2$ in neurons: a localization which mirrors its functions

At the beginning of 2000s PtdIns(4,5)P$_2$ had already been associated with a number of functions but no information on its distribution in neurons was available. Hence, Micheva and colleagues started investigations in this direction and, using the pleckstrin homology (PH) domain of the phospholipase C (PLC) δ1 as a probe for PtdIns(4,5)P$_2$, found this lipid enriched at the plasma membrane and absent from synaptic boutons in resting neurons. However, upon neuronal stimulation PtdIns(4,5)P$_2$ decreased on the plasma membrane and increased in the synaptic bouton presumably as a consequence of SV endocytosis following exocytosis in stimulation conditions. It was the first evidence that PtdIns(4,5)P$_2$ enriches at the synapse during stimulation and that its levels at the synaptic bouton depend on neuronal activity (Micheva et al., 2001). Some years later, in 2011, it was demonstrated, through a super-resolution approach, that PtdIns(4,5)P$_2$ forms microdomains with a diameter of almost 73 nanometers (nm) at the inner-leaflet of the plasma membrane and that these microdomains promote syntaxin 1 clustering at release sites (Van Den Bogaart et al. 2011). These observations clearly indicated that PtdIns(4,5)P$_2$ is enriched at the synaptic bouton during stimulation and that it forms microdomains in proximity of the release site where exocytosis is promoted.

1.3.3 PtdIns(4,5)P$_2$ in neurons: the requirement for a metabolic balance

Considering its enrichment at synaptic boutons and the relevant functions that PtdIns(4,5)P$_2$ exerts (explained in the following paragraph), it is absolutely necessary that its levels are strictly regulated during synaptic transmission. The main route for PtdIns(4,5)P$_2$ synthesis in neurons is PtdIns4P phosphorylation by phosphatidylinositol phosphate kinase type 1γ (PIPK1γ)
whose predominant role in PtdIns(4,5)P₂ synthesis in neurons was identified by De Camilli’s group in 2001 (Wenk et al. 2001). The p90 subunit of PIPK1γ (PIPK1γ-p90) has the ability to interact with talin, a protein linked to the presynaptic membrane that acts as an adaptor between integrins and actin (Morgan et al. 2004). PIPK1γ-p90 is under the activity-dependent control of the CaM/Calcineurin-Cdk5 system (Di Paolo et al., 2002; Sang et al., 2005). In resting neurons, Cdk5 phosphorylates a PIPK1γ-p90 serine residue (S650) located in the talin-binding site, resulting in the inhibition of the PIPK1γ-p90 interaction with talin. When an action potential causes local Ca²⁺ increase, it activates calcineurin which drives the S650 dephosphorylation favoring the PIPK1γ-p90 interaction with talin and thus PtdIns(4,5)P₂ synthesis, observed upon stimulation (Wenk and De Camilli 2004). Given the increase in PtdIns(4,5)P₂ synthesis at the synapse upon stimulation, it is extremely important to maintain a balanced phosphorylation-dephosphorylation cycle. This equilibrium is ensured by two main 5-phosphatases: synaptojanin 1 and OCRL. The functions and properties of OCRL will be discussed in the section dedicated to Lowe syndrome and the OCRL protein (sections 1.4 and 1.5). Synaptojanin 1 is concentrated at synaptic terminals (Haffner et al., 1997; McPherson et al., 1994) where it acts as the main 5-phosphatase responsible for PtdIns(4,5)P₂ dephosphorylation back to PtdIns4P during endocytosis. Its role in SV recycling through control of PtdIns(4,5)P₂ metabolism was discovered by De Camilli and co-workers in 1999. Indeed, they found neurological defects in synj1 knockout (KO) mice with enhanced synaptic depression and accumulation of clathrin-coated vesicles at the presynaptic terminal together with PtdIns(4,5)P₂ increase (Cremona et al. 1999). Synj 1-KO neurons also show defects in the internalization of postsynaptic AMPA receptors (Gong and De Camilli 2008) meaning that synj 1 is involved not only in
presynaptic but also in postsynaptic endocytosis. Like PIPK1γ, synj 1 activity is controlled by the CaM/Calcineurin-Cdk5 system: in resting neurons synj 1 is inhibited by Cdk5, which phosphorylates a synj 1 serine residue (S1140) both in vitro and in intact synaptosomes. S1140 phosphorylation inhibits synj 1 catalytic activity because the phosphorylation site is located in a region flanking a domain with high affinity for endophilin 1, the main synj1 partner at the synapse. This situation is reverted upon stimulation by calcineurin, which dephosphorylates synj1, favoring the interaction with endophilin1, which recruits synj1 to membranes when clathrin-coated pits have already formed (Ringstad et al., 1997). Indeed, synj1 catalytic activity is facilitated on highly curved membranes, such as SV membranes (Chang-Ileto et al. 2011; Lee et al. 2004). Another route for PtdIns(4,5)P₂ consumption during endocytosis is provided by phospholipase C (PLC), which uses PtdIns(4,5)P₂ as a substrate to produce diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3), the latter working as a second messenger to mobilize intracellular calcium (Berridge and Irvine 1984).

The reasons of this fine tuning of PtdIns(4,5)P₂ levels at the synapse can be found in the pivotal functions that this lipid accomplishes, especially in controlling SV exocytosis, SV endocytosis and presumably the coupling between the two processes.

1.3.4 PtdIns(4,5)P₂ in neurons: one lipid with multiple functions

As already mentioned in 1.3.3, PtdIns(4,5)P₂ plays a major role in regulating SV recycling. This role is mainly associated with its ability to recruit proteins to its site(s) of enrichment. As shown in Table 1.2, it mainly binds exocytic and endocytic proteins, acting as a ligand for both exocytic and endocytic machineries. PtdIns(4,5)P₂ promotes exocytosis in a dual way, it binds:
synaptotagmin (Schiavo et al. 1998) controlling Ca^{2+} sensing step; Ca^{2+}-dependent activator protein for secretion (CAPS) and rabphilin (Chung et al. 1998; Schiavo, et al. 1998) mediating the SV priming step; syntaxin 1 (Lam et al. 2008), driving the fusion step. Different studies have pointed out the consequences of the dysfunction in PtdIns(4,5)P_2 metabolism during exocytosis. PIPK1γ-KO mice, with a decrease in PtdIns(4,5)P_2 synthesis, show a reduced RRP together with defective vesicle trafficking at the presynapse and defects in neurotransmitter release (Di Paolo et al. 2004). Also in neuroendocrine cells the PtdIns(4,5)P_2 enriched at the plasma membrane was shown to regulate RRP size and vesicle exocytosis (Milosevic et al. 2011).

PtdIns(4,5)P_2 also works in endocytosis thanks to its ability to bind and recruit the clathrin adaptor AP2, AP180, CALM and epsin (Ford et al., 2001; Ford et al., 2002; Gaidarov et al., 1996; Rohde et al., 2002; Zhang et al., 1998) but also the endocytic protein dynamin (Zheng et al. 1996). As for exocytosis, dysfunction in PtdIns(4,5)P_2 metabolism results in several anomalies including accumulation of clathrin-coated SVs at the synapse (Cremona et al. 1999; Harris et al. 2000), defects in neurotransmission associated with a loss of synj1 resulting in defective conversion of PtdIns(4,5)P_2 to PtdIns4P at nerve terminals and thus a failure in clathrin-coat disassembly on SV membranes (Lüthi et al. 2001); a reduced rate of SV endocytosis and recycling (Di Paolo et al. 2004; Sankaranarayanan and Ryan 2000) due to loss of PIPK1γ leading to low levels of PtdIns(4,5)P_2 at nerve terminals.

To better understand the role of PIs (and therefore of the enzymes responsible for the regulation of their metabolism) in biological processes, one possibility is to study a disease resulting from a dysfuction in PI-metabolizing enzymes, a chronic condition characterized by either the accumulation or the reduction of one
or more PI species, leading to an easier comprehension of the biological mechanisms contributing to a certain phenotype. One of the historical interests of our laboratory is Lowe syndrome, whose features will be explained in the next paragraph.

1.4 Lowe syndrome

Lowe syndrome is also referred to as Oculo-Cerebro-Renal syndrome of Lowe (OCRL) due to the involvement of the eye, the kidney and the nervous system (Bökenkamp and Ludwig 2016; Loi 2006). It is a rare genetic disease with an estimated prevalence of approximately 1 in 500,000 (according to the Lowe Syndrome Association) and is characterized by an X-linked pattern of inheritance (Richards et al., 1965), meaning that males are exclusively affected although rare cases of females with X-autosome translocations have been described (Cau et al. 2006). It was described for the first time in 1952 by Lowe and colleagues as a unique syndrome displaying organic aciduria, decreased renal ammonia production, hydrophthalmos and intellectual disability (Lowe et al., 1952). Only a couple of years later, a renal Fanconi syndrome was found to be associated with Lowe syndrome (Bickel and Thursby-Pelham 1954). The gene responsible for the disease is OCRL, identified in 1997 by Nussbaum and co-workers, who had previously identified the locus thanks to two female patients presenting the typical picture of Lowe syndrome associated with a balanced X-autosome translocation involving the Xq26 region (Attree et al. 1992). Later, Nussbaum cloned the causative gene, thus identifying the OCRL gene (Xq25-q26.1) (Nussbaum et al., 1997). OCRL is composed of 23 exons and encodes a type II PtdIns(4,5)P₂ 5-phosphatase protein, known as OCRL.
To date, many pathogenic mutations have been described for \textit{OCRL}: nonsense, missense, frameshift and splice-site mutations (Fig. 1.11).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{ocrl_geno.png}
\caption{Genomic organization of the \textit{OCRL} gene. \textit{OCRL} is composed of 23 exons and 22 introns. Exons 2–5 encode the pleckstrin homology (PH)-like domain, exons 9–15 encode the 5-phosphatase catalytic domain and exons 16–18 encode the ASPM, SPD-2, Hydin (ASH) and RhoGAP-like domains of the \textit{OCRL} protein. Specific mutations that cause Lowe syndrome are shown above the gene whereas mutations that cause Dent disease 2 are shown below. The highlighted mutations (821T>C, 952C>T, 1568G>A and 1567G>A) have been identified as causing both Lowe syndrome and Dent disease 2; their corresponding amino acid changes are shown in brackets. From (De Matteis et al., 2017).}
\end{figure}

These mutations can occur throughout the gene, but while those occurring in the exons 8-23 are causative of Lowe syndrome, those located in the exons 1-7 cause Dent disease 2, a milder condition characterized exclusively by renal Fanconi syndrome (De Matteis et al., 2017).
1.4.1 Clinical signs of Lowe syndrome

The symptoms characteristic of Lowe syndrome affect the eye, the kidney and the nervous system. However, other signs can also be present, including undescended testis, chronic constipation, scoliosis, dental abnormalities, joint dislocation with joint swelling and arthritis (De Matteis et al., 2017).

1.4.1.1 Eye: congenital cataracts and glaucoma

Almost all patients with Lowe syndrome are born with congenital bilateral cataracts probably due to a defective migration of the crystalline embryonic epithelium (Tripathi et al., 1986). Nearly 50% of patients develop glaucoma within the first year of life. Sight sharpness is compromised by aphakia, which together with retinal dysfunction might be responsible for nystagmus (Kruger et al., 2003; Walton et al., 2005).

1.4.1.2 Kidney: renal Fanconi syndrome

Renal symptoms are often absent at birth but develop rapidly within the first months of life. These symptoms, including renal bicarbonate, salt and water wasting, worsen with age until evolving into the classical picture of renal Fanconi syndrome: low-molecular weight (LMW) proteinuria, proximal renal tubular acidosis, renal phosphate wasting (Bockenhauer et al., 2008; Charnas et al., 1991; Monnens & Levchenko, 2008; Norden et al., 2004; Sliman et al., 1995; Vilasi et al., 2007) mainly due to a defective reabsorption by proximal tubular cells normally occurring through receptor-mediated endocytosis (the main receptors involved are megalin and cubilin) and clathrin-mediated endocytosis (De Matteis et al. 2017). The symptoms described become even worse during the second
decade of life, evolving into chronic renal failure, which is the most frequent cause of death.

The severity of renal impairment is variable and for this reason each patient has different needs, requiring a specific supplementation. The most common therapies include supplementation of citrates, sodium bicarbonate to treat renal tubular acidosis and potassium citrate to treat nephrocalcinosis. In some cases vitamin D is supplemented to prevent hypophosphataemic rickets. In the case of growth retardation (due to growth hormone deficiency caused by deficient reabsorption), human growth hormone is used. Polyuria, when present, requires water and fluid supplementation.

1.4.1.3 Nervous system: central hypotonia, epilepsy and intellectual disability

Patients with Lowe syndrome have severe hypotonia at birth, often with loss of deep-tendon reflexes (Bökenkamp and Ludwig 2016; Loi 2006). Motor development is delayed, with only 25% of patients presenting autonomous gait within 6 years of age. Intellectual disability can range from mild to severe, with only 10% of patients showing a slight disability whereas the majority show a severe disability together with an Intelligence Quotient (IQ) of 50 or even less (Charnas et al., 1988; Kenworthy et al., 1993). About 90% of patients show other behavioral abnormalities such as compulsive behavior, aggressiveness, irritability and unintentional stereotyped or self-abusive behavior (Charnas et al., 1988; Lewis et al., 2012; Loi, 2006). Unfortunately, febrile convulsions are common and seizures develop in almost 50% of patients (Mueller et al. 1991; Recker et al. 2015). When available, brain magnetic resonance imaging (MRI) shows myelination defects and periventricular white matter cysts (Allmendinger et al., 2014; Carroll et al., 1993; De Carvalho-Neto, et al., 2009; Demmer et al.,
The pharmacological treatments used for patients with severe behavioral problems mainly comprise antidepressant and antipsychotic drugs, whereas epilepsy remains difficult to treat.

1.5 The OCRL protein

The OCRL gene is located on chromosome Xq 25-26 and encodes a 105 kDa protein, which exists in two isoforms: the a isoform (901 amino acids) and the b isoform (893 amino acids), the latter being shorter due to the absence of an eight amino acid sequence (EDSFLEKE) (Nussbaum et al. 1997). The a isoform is ubiquitously expressed, whereas the b isoform is expressed in all tissues except for the brain (Johnson et al. 2003).

The encoded protein is a multi-domain protein composed of 4 conserved domains: a central inositol polyphosphate 5-phosphatase domain, an N-terminal pleckstrin homology (PH) domain, and C-terminal ASPM, SPD-2, Hydin (ASH) domain and a RhoGAP-like domains (Fig. 1.12).

![Protein architecture of OCRL](image)

**Fig. 1.12 Protein architecture of OCRL.** Organization of the OCRL domains in the full-length protein. The N-terminal PH domain is connected by a linker region to the 5-phosphatase domain, which recognizes PtdIns(4,5)P₂ on the membrane. The ASH domain
and the RhoGAP-like domain have sites that mediate the interactions of OCRL with its partners (for example, Rab GTPases, proteins involved in endocytosis such as APPL1 and Ses1/2, and proteins associated with actin polymerization such as Cdc42 and Rac1). The sites of interaction with clathrin and AP2 are indicated. From (De Matteis et al., 2017).

1.5.1 The 5-phosphatase domain
This domain is responsible for binding and dephosphorylation of PtdIns(4,5)P$_2$, PtdIns(3,4,5)P$_3$ and Ins1,4,5P$_3$ (Erdmann et al. 2007; Suchy et al. 2009), with a higher affinity for PtdIns(4,5)P$_2$. Two motifs located in this domain are responsible for substrate binding and catalysis: WXGDXN(F/Y)R and P(A/S)W(C/T)DRIL. These motifs are conserved from yeast to mammals and their mutations cause the loss of the phosphatase activity.

1.5.2 The PH domain
This domain is composed of seven $\beta$-strands and one $\alpha$-helix formed by the first 119 N-terminal residues. The $\beta$-strands form 2 perpendicular sheets whereas the $\alpha$-helix is located in the gap between the two $\beta$-sheets (Mao et al. 2009). The folding of this domain is similar to that of PH domains of other proteins, such as PLC$\delta$, but unlike this protein, the OCRL PH domain is unable to bind phosphoinositides because of the absence of a positively charged pocket able to accommodate PtdIns(4,5)P$_2$ (K. M. Ferguson et al. 1995). However, a clathrin-binding sequence ($^{73}$LID$^{76}$) has been identified within this module and associated with the arrival of OCRL to clathrin-coated pits (CCPs) (Mao et al. 2009).

1.5.3 The ASH domain
This domain, shared by OCRL and INPP5B, was identified by a sequence analysis study aimed at searching for similarities among ASH domains in different
organisms (Ponting, 2006). This domain is located between the 5-phosphatase domain and the RhoGAP-like domain and is composed of about 200 amino acids. It owes its name to three proteins (all three associated with hydrocephalus) in which this domain was first identified: the spindle-like microcephaly-associated (ASPM) protein; the spindle defective 2 (SPD-2) protein and hydin (Bond et al. 2003; Kemp et al. 2004; Robinson et al. 2002). The ASH domain is important for the binding of Rab proteins (Rab 5) and Rab effectors (APPL1) (Erdmann et al. 2007; Fukuda et al. 2008).

1.5.4 The RhoGAP-like domain

Similar to the ASH domain, the RhoGAP (GTPase Activating Protein) domain was identified in OCRL by a multiple sequence alignment in which the p50 RhoGAP domain was aligned to multiple sequences in the human genome and 53 RhoGAP-containing proteins were identified (Peck et al. 2002). Since several Lowe syndrome-causing mutations of OCRL have been mapped to the RhoGAP domain (Lin et al. 1997, 1998; Satre et al. 1999) and different X-linked mental retardation have been associated to mutations in genes involved in the Rho pathway, a great number of studies have been carried out to clarify the functional role of the OCRL RhoGAP domain: differently from the other RhoGAPs, this domain does not function as a GTPase-activating domain. This is mainly due to the replacement of the critical catalytic arginine with a glutamine in the OCRL RhoGAP domain. Indeed, rather than being a RhoGAP domain with GAP activity, the OCRL domain has been defined as a RhoGAP-like domain, which, differently from the other RhoGAP domains, has the ability to bind both the active and the inactive form of Rac1 (Faucherre et al. 2003), as well as other small GTPases of the Arf family, such as Arf1 and Arf6 (Lichter-Konecki et al. 2006). The OCRL
RhoGAP-like domain contains a type I clathrin-binding module ($^{702}$LIDLE$^{706}$), conferring the ability to bind the terminal domain of the clathrin heavy chain on both OCRL isoforms (Choudhury et al. 2005; Ungewickell et al. 2004), whereas only the a isoform possesses an additional clathrin-binding motif located in a loop inserted in the PH domain (the clathrin-binding sequence is $^{73}$LIDIA$^{77}$). These clathrin binding motifs provide sites of direct binding to clathrin, in addition to the indirect binding mediated by the clathrin adaptor AP2 (the AP2-binding sequence is $^{151}$FEDNF$^{155}$) (Mao et al. 2009).

1.6 OCRL interacting partners and localization

As already mentioned above and shown in Fig. 1.12, OCRL contains two clathrin-binding sites (in the PH-domain and in the RhoGAP-like domain); an AP2-binding sequence within the N-terminal region; a Rab-binding motif in the ASH domain and a binding sequence for APPL1 and IPIP27A and IPIP27B in the RhoGAP-like domain. Thanks to the presence of these sites, OCRL can interact with a number of different proteins and is also localized to different cell compartments: plasma membrane (PM), clathrin-coated pits (CCPs), clathrin-coated vesicles (CCVs), early endosomes (EEs), the trans-Golgi network (TGN), the primary cilium and also lysosomes (Fig. 1.13). As illustrated in Fig. 1.13, the binding of OCRL to several member of the Rab family is responsible for the recruitment of OCRL to sites such as the Golgi complex, the primary cilium, but also endolysosomal compartments and the cellular bridge during cell division. The strongest interactions are reported for the Golgi-associated Rab1 and Rab6, for the endosomal Rab5 (Hyvola et al. 2006), as well as for Rab35, which directly interacts with OCRL and is responsible for its recruitment to the intracellular bridge during cytokinesis (Dambournet et al. 2011) and to newborn endosomes
(Cauvin et al. 2016). As already mentioned, the binding to the clathrin heavy chain and to clathrin- adaptors (including AP2) is important for OCRL localization to CCVs and CCPs that occurs immediately before uncoating (Choudhury et al. 2005; Ungewickell et al. 2004). The interaction with APPL1, a Rab5 effector, is crucial for OCRL recruitment to early endocytic compartments positive for Rab5 (McCrea et al. 2008). Conversely, the interaction with IPIP27A and IPIP27B (also known as Ses1 and Ses2) targets OCRL to endosomal structures distinct from those labeled by APPL1 and Rab5 (Swan et al. 2010). Finally, OCRL can also localize to the plasma membrane, in particular upon epidermal growth factor (EGF) stimulation thanks to its interaction with Rac1 (Faucherre et al. 2003, 2005).

**Fig. 1.13 Intracellular distribution and interactors of OCRL in proximal tubular cells.**

The localization of OCRL to different cell compartments is mainly due to its interactions. In the yellow boxes are grouped the compartment-specific OCRL interactors. From (De Matteis et al., 2017).
1.7 Cellular functions of OCRL

The interaction of OCRL with a variety of proteins contributes to its localization to different cellular compartments where it exerts its diverse roles that are always associated with its function as a PtdIns(4,5)P$_2$ metabolizing enzyme.

At the plasma membrane, OCRL maintains a low level of the PtdIns(4,5)P$_2$. Indeed, cells lacking OCRL show a high level of PtdIns(4,5)P$_2$ together with actin polymerization and the formation of actin comets (Allen 2003; Nández et al. 2014). OCRL is recruited to CCVs during budding where the levels of PtdIns(4,5)P$_2$ are high and clathrin and clathrin adaptors are already assembled. The arrival of OCRL causes a lowering of PtdIns(4,5)P$_2$ levels allowing clathrin uncoating. Indeed, cells with dysfunctional OCRL exhibit an abnormal accumulation of CCVs as a consequence of the inefficient uncoating process (Nández et al. 2014). OCRL downregulates PtdIns(4,5)P$_2$ at early endosomes as well as at CCVs that allows the proper progression of the endosomes through the endocytic pathway. The lack of OCRL causes an accumulation of PtdIns(4,5)P$_2$ on early endosomes together with actin polymerization resulting in defective trafficking of some receptors through early endosomes, such as the mannose 6-phosphate receptor (M6PR) to the Golgi complex, EGF to lysosomes, and also megalin that in physiological conditions is recycled back to the plasma membrane (Choudhury et al. 2005; Ungewickell et al. 2004; Vicinanza et al. 2011). In 2016 a role for OCRL at lysosomes was uncovered in our lab. OCRL is recruited to lysosomes during a lysosomal cargo response, immediately after the arrival of PtdIns(4,5)P$_2$-synthesizing enzymes responsible of a burst in PtdIns(4,5)P$_2$ levels on lysosomes. Once recruited, OCRL restores the physiological PtdIns(4,5)P$_2$ levels allowing proper autophagosome-lysosome fusion (De Leo et al. 2016). Another important role is exerted by OCRL at the primary cilium, a
specialized structure present in some cells such as the kidney proximal tubular cells. The arrival of material and cargo to the primary cilium during its elongation occurs through vesicular trafficking controlled by Rab5 and Rab8 together with OCRL, which is an effector of both of these Rabs (Luo et al. 2012, 2014). The cellular functions of OCRL are shown in the Fig. 1.14.

Fig. 1.14 Cellular functions of OCRL and alterations resulting from its dysfunction.

a) Cellular roles of OCRL in the endolysosomal pathway in physiological conditions.
b) Defects in the endolysosomal pathway induced by loss of functional OCRL. From De Matteis et al., 2017.

1.8 Animal models of Lowe syndrome

The mouse model used for the present work is depleted of murine Ocrl and of murine Inpp5b (the enzyme that compensates for the absence of Ocrl in mice) and expresses a humanized INPP5B (INPP5B cannot compensate for the
absence of Ocrl in humans). This model was generated by Nussbaum’s group in 2011 (Bothwell et al. 2011) and shows proximal tubular dysfunction with low molecular weight proteinuria and aminoaciduria and reduced post-natal growth. This model was generated after a first attempt that failed in 1998: the mice depleted of only Ocrl were normal, fertile and without any kind of defects (Jänne et al. 1998). Nussbaum and coworkers then discovered that the absence of a phenotype was due to the presence of Inpp5b, a PtdIns(4,5)P₂ metabolizing enzyme with a sequence identity of almost 50% with OCRL, that is able to compensate in mice for the lack of Ocrl. The researchers also discovered that this compensatory function is not exerted by INPP5B in humans because of differences between humans and mice in the splicing of INPP5B. Furthermore, the lack of only Inpp5b causes sterility in mice and the depletion of both Inpp5b and Ocrl is embryonic lethal. For these reasons a mouse model with a double knock-down for Ocrl and Inpp5b was generated together with the expression of a humanized INPP5B. In 2017 a conditional mouse model in which Inpp5b was specifically inactivated in kidney tubules of a global Ocrl knockout mouse was generated resulting in low molecular weight proteinuria, phosphaturia and acidemia (Inoue et al. 2017). Another model for Lowe syndrome is a zebrafish model, generated in 2012 by Lowe’s group by a retroviral insertion into the Ocrl promoter. The transgenic embryos are susceptible to heat-induced seizures and also show cystic lesions in the brain. At the cellular level, reduced cell survival due to increased apoptosis and reduced proliferation was registered, whereas in the pronephric tubule impaired fluid phase and clathrin-mediated endocytosis were measured (Ramirez et al. 2012).
Chapter 2

Materials and Methods

2.1 Mouse model

Experiments were conducted on Ocrl\(^{Y+/+}\);Inpp5b\(^{-/-}\) and Ocrl\(^{Y/-}\);Inpp5b\(^{-/-}\) mice harboring BAC-INPP5B expression in equal copies (BAC1) (129S/SvEv) (Bothwell et al. 2010, 2011; Jänne et al. 1998). Animals were housed and studied according to the FELASA (Federation of European Laboratory Animal Science Association) statement for the Care and Use of Laboratory Animals.

2.2 Genotyping

Genomic DNA was isolated from phalanx biopsies from Ocrl\(^{Y+/+}\); Inpp5b\(^{-/-}\);BAC-INPP5B and Ocrl\(^{Y/-}\);Inpp5b\(^{-/-}\);BAC-INPP5B mice in lysis buffer (100 mM EDTA, 100 mM NaCl, 1% SDS, 50 mM Tris-HCl pH8, 20 µg/µl proteinase K). PCR amplification was performed with the AmpliTaq Gold™ 360 Master Mix according to the manufacturer’s instructions. PCR conditions were: 40 cycles of 95°C for 20 sec, 55°C for 25 sec and 72°C for 45 sec. Two sets of primers were used: one for the wild-type allele, the other for the knock-out allele.

Ocrl wild-type, 250 base pairs (bp): forward (fw) primer 5’-CCCTTTTCATCTGTAGGAGAAATC-3’ (located at the junction of intron 18 and the 5’ end of exon 19); reverse (rev) primer 5’-GCATGGTTAAACGCACTATGTGG-3’ (located in intron 19, which is deleted in the Ocrl\(^{Y/-}\) line).

Ocrl knock-out, 480 bp: forward (fw) primer 5’-GCCCTTTGATTCTAATCCCTTTTCATC-3’ (located in the intron preceding exon 19); reverse (rev) primer 5’-TCT GAGCCCAAGAAGCAGAAG- 3’ (located in the PGK promoter, which is part of the neo-cassette gene targeting vector). The PCR
products were separated on a 1.7% agarose gel and visualized using the ethidium bromide analogue SYBR Safe DNA gel stain (Invitrogen).

2.3 Biochemical procedures

2.3.1 Tissue harvesting

Anesthetized mice (2 months old) were subjected to perfusion with PBS (Gibco), followed by removal of the brain. The brain was placed in a Petri dish resting on ice that was full (almost 1 cm deep) of ice-cold HBSS (Thermo Fisher) pH=7.4, in order that the brain was immersed for the most part in HBSS. Meninges were removed using forceps and the brain was divided in the two hemispheres with a razor blade that were either frozen immediately in liquid nitrogen (for immunoprecipitation experiments as well as WB analysis of LC3-SQSTM1 and SNARE-complexes) or subjected to dissection into different brain regions (cortex, hippocampus, thalamus, striatum, midbrain, cerebellum) (for WB analysis of OCRL expression in the brain regions).

2.3.2 Preparation of brain lysates

Lysates from mouse brain (from either total brain or brain regions isolated as above) were obtained as follows: the tissue was placed in Lysis Buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP40) supplemented with protease inhibitors cocktail (Roche) and homogenized with a 5 mm bead in a Tissue Lyser for 2 min at 50Hz. The suspension was shaken for 30 min at 4°C and then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected, centrifuged again, and the final supernatant was collected and analyzed by SDS-PAGE.
2.3.3 SDS-PAGE and western blot analysis

To assemble polyacrylamide gels, two 16 x 15 cm glass plates were assembled to form a chamber using two 1.5 mm plastic spacers lined on the lateral edges of the plates. This chamber was fixed using two clamps and mounted on a plastic base that sealed the bottom (Hoefer Scientific Instruments, Germany). The polyacrylamide gel was then prepared using one solution of the desired polyacrylamide concentration (10% or 12% polyacrylamide).

Samples were prepared by adding sample buffer (0.125 M Trizma base, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 6.8) and boiling for 5 min at 95 °C before loading onto the gel. The end wells were loaded with 3 μl of pre-stained molecular weight standards (Sigma- Aldrich). The chamber was then assembled into the electrophoresis apparatus (Hoefer Scientific Instruments, Germany) and electrophoresis was carried out under a constant current of 8 mA (for overnight runs) or 30-40 mA (for 4 h runs).

To perform western blot (WB) analysis the polyacrylamide gel was soaked for 15 min in transfer buffer, placed on a sheet of 3 MM Whatman paper and covered by a nitrocellulose filter (Schleicher & Schuell, USA). The filter was covered by a second sheet of 3 MM paper to form a "sandwich" that was subsequently assembled into the blotting apparatus (Hoefer Scientific Instruments, Germany). Protein transfer occurred at 400 mA for 4-5 h. At the end of the run, the “sandwich” was disassembled and the nitrocellulose filter was soaked in 0.2% (w/v) red Ponceau in 5% (v/v) acetic acid for 5 min to visualize the protein bands and then rinsed with 5% (v/v) acetic acid to remove the excess of the unbound dye. Nitrocellulose (or PVDF) filters were then cut with a razor blade into strips. Strips containing the proteins of interest were incubated for 30 min at RT in blocking buffer (TTBS (0,05% (w/v) Tween 20, 150 mM NaCl, 20mM Tris-HCl pH 7.5) with
1% (w/v) BSA). The strips were subsequently incubated with fresh blocking buffer containing the primary antibody at its working concentration (see Table 2.1 for the antibody dilution used).

After 2-3 h of incubation at RT, or overnight at 4 °C, the primary antibodies were removed and the strips washed 3 times with TTBS (10 min each wash). Strips were incubated for 1 h with the appropriate HRP-conjugated secondary antibody (diluted 1:10,000 for ECL development) and washed 3 times (10 min each wash) with TTBS and one time with PBS. After washing, strips were incubated with the developing solution: strips were incubated with the ECL (Euroclone, UK) developing solution for 1 min at RT and visualized by chemioluminescence. The quantification of the bands of interest was performed by densitometric analysis with the ImageJ software.

2.3.4 Quantification of higher order SNARE complexes

Total brain samples from OcrlY/+ and OcrlY/− mice (lysates were obtained as described above) were prepared in parallel as follows: 30 µg per sample were mixed with sample buffer and loaded onto a 12% polyacrylamide gel without boiling. To confirm that complexes observed were sensitive to boiling, identical samples were boiled for 5 min at 95°C and processed for western blot analysis at the same time. Following separation by SDS-PAGE, the proteins were transferred to nitrocellulose and the SNARE complex together with the protein monomers were revealed using antibodies against the three SNARE proteins: SNAP25, Syntaxin1 and VAMP2 (Table 2.1). The antibody incubation and ECL development procedures are described above. SDS-resistant SNARE complexes were defined as the immunoreactive material above 40 kDa that was absent from boiled samples.
2.3.5 Sample preparation for proteomic analysis by mass spectrometry

16 day-old Ocrl<sup>Y+</sup> and Ocrl<sup>Y−</sup> male mice (5 animals for each genotype) were euthanized by decapitation (without perfusion) and the brains were separated into the two hemispheres by a sagittal cut and dissected into 6 brain regions: cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum and olfactory bulbs, thus having for each region (e.g. the hippocampus) 2 halves: one half coming from the right hemisphere and the other from the left hemisphere. In order to use the two halves for different purposes (transcriptomics and proteomics analysis), I kept and stored them separately (Fig. 3.21); hippocampal regions were used to prepare samples for mass spectrometry analysis. Hippocampal lysates were obtained as follows: hippocampi were added to 250 µl lysis buffer (described in 2.3.2) and triturated with a p200 pipette until a homogenous lysate was obtained. The lysates were shaken for 30 min at 4°C and then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and underwent a second centrifugation and samples were delivered to the Mass Spectrometry Unit, where they were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) by Paolo Grumati and Michele Santoro at TIGEM. The resulting data were then analyzed by Paolo Grumati, Michele Santoro, Rossella De Cegli and Carmine Cirillo at TIGEM.

2.3.6 Immunoprecipitation for mass spectrometry analysis

All the following steps were performed on ice or at 4°C, using ice-cold solutions. Total brain lysates from 2 month-old Ocrl<sup>Y+</sup> and Ocrl<sup>Y−</sup> male mice (obtained as described in section 2.3.2) were incubated with anti-OCRL Ab (Consorzio Mario Negri Sud; 6µg Ab/18 mg lysate) overnight at 4°C. Immune-complexes were collected by incubation for 1 h at 4°C with protein A Sepharose (Sigma- Aldrich,
Germany), previously equilibrated with lysis buffer. The immunoprecipitates were washed 6 times in lysis buffer and twice in the same buffer without detergent by 2 min centrifugation at 500xg. Proteins were eluted adding 2X sample buffer (SB) and denaturating at 95°C for 5 min. Eluates were sent to the Central Proteomics facility (South Parks Rd., Oxford) for mass spectrometry analysis. The data from this analysis were subjected to bioinformatics analysis performed by Mario Failli at TIGEM.

2.3.7 Co-immunoprecipitation
All the following steps were performed on ice or at 4°C, using ice-cold solutions. Total brain lysates from 2 month-old Ocr1Y/+ and Ocr1Y/- male mice were prepared as in 2.3.2 and incubated with anti-OCRL Ab (3μg Ab/9 mg lysate) overnight at 4°C. Immune-complexes were collected and eluted as described in 2.3.6. Input, flow through and immunoprecipitate (eluate) were analyzed by SDS-PAGE (12%) and western blotting with anti-OCRL, anti-APPL1, anti-Syntaxin 1 and anti-VAMP2 antibodies (Table 2.1).

The antibodies used in the biochemical analyses are listed in Table 2.1.

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<th>Animal source</th>
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</tr>
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</table>

*Table 2.1 List of antibodies used for western blot in this study*
2.4 Primary neuronal culture procedures

2.4.1 Dissection of the hippocampus

Primary cultures of dissociated hippocampal neurons were prepared from newly born (p0) or 1-3 days old (p0-p3) mice as indicated in (Kaech and Banker 2006). The pups were euthanized by decapitation and a midline incision was made at the skin surface and at the skull by using fine scissors, the brain was then removed and placed in a Petri dish containing ice-cold HBSS (pH 7.4) under a dissecting microscope. The meninges were removed, the brain was separated in the two hemispheres by a sagittal cut and the hippocampus was dissected out from each hemisphere using forceps. The hippocampi were placed in a 15-ml centrifuge tube containing 10 ml ice-cold HBSS.

2.4.2 Dissociation of hippocampal neurons

Hippocampi were washed three times with HBSS, resuspended in 4.5 ml HBSS + 0.5 ml of 2.5% Trypsin-EDTA (Invitrogen) and then incubated for 20 min at 37°C in a 5% CO₂ humidified atmosphere. After incubation, the tissues were washed three times (5 min each wash) with HBSS, resuspended in 1 ml pre-warmed plating medium [MEM (Thermo), 10% horse serum (Thermo), 1% glutamine (Thermo), 1% penicillin/streptomycin (Thermo), 0.6% glucose (Sigma-Aldrich)] and carefully triturated with a p1000 pipette to gently dissociate the cells and obtain a homogenous cell suspension. A 10 µl aliquot of the cells was used to estimate the cell density, then the cells were diluted in plating medium to have 10^6 cell/ml, plated (at a density of 150,000 cells/each coverslip) onto poly-D-lysine coated (1 mg/ml, Sigma-Aldrich) 14-mm glass coverslips (placed in a 24-well plate) in plating medium and incubated at 37°C, in a 5% CO₂ humidified atmosphere. 4 h after plating, when the cells had settled on the substrate, the
plating medium was removed and replaced with pre-warmed serum-free maintenance medium (1 ml/well) (Neurobasal, 1% glutamax, 1% penicillin/streptomycin, 2% B27, all from Thermo). 36 h after plating, 1 μM cytosine arabinoside (araC; 1-b-d-arabinofuranosylcytosine) was added to the culture medium to inhibit the proliferation of dividing non-neuronal cells; 48 h after araC addition, the araC-containing medium was removed and replaced with fresh pre-warmed maintenance medium. The neurons were cultivated in maintenance medium at 37°C, in a 5% CO₂ humidified atmosphere for up to 3 weeks (corresponding to 21 D.I.V., days in vitro). Twice a week, half the medium from each well was removed and replaced with fresh pre-warmed maintenance medium. All the experiments were performed at least 14 days after plating.

2.5 Lipofectamine2000-based transfection of neurons

For the expression of DNA constructs in cultured hippocampal neurons (between the 3rd and the 10th D.I.V.), a lipid-based transfection protocol was used (Kaech and Banker 2006). 2.5 ml Lipofectamine 2000 (Invitrogen) was diluted into 100 μl minimal essential medium (MEM) in a sterile microfuge tube; in a second microfuge tube, 1.5 μg plasmid DNA was diluted into 100 μl MEM. Both mixtures were shaken and incubated at room temperature (RT) for 5 min. Then, the lipid solution was mixed with the DNA solution and incubated at room temperature (RT) for 30 min. Meanwhile, half the medium from each well was removed (stored in a sterile tube and added with a comparable volume of fresh maintenance medium) and replaced with fresh maintenance medium. The lipid-DNA complexes were gently distributed (drop by drop) over the neurons and the plate was then returned to the incubator. After 90 min, the entire medium from each well was removed and replaced with the conditioned medium previously
prepared. The culture was maintained as described above, in the primary neuronal culture procedures.

2.6 Hippocampal neuron stimulation

A protocol of depolarization evoked by high potassium was used for all experiments requiring neuronal stimulation. 90 mM KCl evokes maximal SV exocytosis and thus is considered a strong stimulus (like high frequency stimulation), where membrane retrieval occurs via endocytic intermediates (Harata et al. 2001; Wu et al. 2014).

Prior to stimulation, hippocampal neurons were incubated for 10 min at 37°C in a 5% CO₂ humidified atmosphere in 2 mM KCl Tyrode’s solution (150 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, 10 mM Glucose) (low concentration of K⁺ with a corresponding high concentration of Na⁺) and then were stimulated by incubation for 2 min with 90 mM Tyrode’s solution (64 mM NaCl, 90 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, 10 mM Glucose) (high concentration of K⁺ with a corresponding low concentration of Na⁺).

2.7 Immunofluorescence confocal microscopy

2.7.1 Immunofluorescence procedures

Neurons were fixed in a fixing solution composed of 4% paraformaldehyde, 4% sucrose for 15 min at RT and washed three times in PBS. Neurons were then permeabilized with 0.1% Triton-X-100 in PBS for 10 min and subsequently washed three times in PBS. Blocking solution (5% (w/v) bovine serum albumin (BSA) in PBS) was added to the neurons for 1h, followed by an overnight incubation with primary antibody in staining solution (1% w/v BSA in PBS) (see Table 2.1 for the dilutions used). After the overnight incubation, the neurons were
washed extensively with PBS and incubated with the secondary antibody (1:400) for 45 min, diluted in staining solution. After immuno-staining, the neurons were washed three times in PBS and once in sterile water to remove salts. The coverslips were then mounted on glass microscope slides (Carlo Erba, Italy) using Mowiol.

2.7.2 Staining for PtdIns(4,5)P$_2$ and PtdIns4P

The staining for PtdIns(4,5)P$_2$ and PtdIns4P was performed as described by (Hammond, Schiavo, and Irvine 2009) with some modifications. Briefly, neurons in 500 µl medium (or Tyrode’s solution) were fixed by the addition of 300 µl of paraformaldehyde (4%) to a final concentration of 2%, and incubated for 15 min at RT. All steps were performed at room temperature. After removing the paraformaldehyde with three rinses in PBS containing 50 mM NH$_4$Cl, the neurons were permeabilized for 5 min by the addition of 20 µM digitonin in Buffer A (20 mM Pipes pH 6.8, 137 mM NaCl, 2.7 mM KCl) for 5 min. Digitonin was removed by three rinses in Buffer A, and neurons were blocked for 1.5 h with buffer A supplemented with 5% (v/v) FBS and 50 mM NH$_4$Cl. Primary and secondary antibodies were applied in blocking buffer for 1h and 45 min, respectively. Cells underwent post-fixation for 5 min in 2% paraformaldehyde, washed with PBS containing 50 mM NH$_4$Cl, washed once with water and then mounted with Mowiol.

The antibodies used in these studies are listed in table 2.2.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company or other source</th>
<th>Animal source</th>
<th>Dilution</th>
</tr>
</thead>
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<tr>
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<td>rabbit</td>
<td>1:1000</td>
</tr>
<tr>
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<td>Abcam</td>
<td>rabbit</td>
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<tr>
<td>Golgin97</td>
<td>G. Di Tullio CMNS</td>
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<tr>
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<td>Vendor</td>
<td>Species</td>
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<td>----------</td>
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</tr>
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</tr>
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<tr>
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<td>Synaptic Systems</td>
<td>guinea pig</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

**Table 2.2** List of antibodies used for immunofluorescence in this study

### 2.7.3 Image processing and quantification of signals on fixed neurons

For quantification experiments, 10-15 randomly chosen fields were scanned with the same microscope settings (i.e. laser power and detector amplification) below pixel-saturation.

Colocalization analysis: the colocalization analysis was performed using the ImageJ plugin JACoP (Just Another Colocalization Plugin). Mander’s coefficient was used to quantify the degree of colocalization between two channels.

PtdIns(4,5)P₂/PtdIns4P intensity analysis: the PtdIns(4,5)P₂ [stained with the anti-PtdIns(4,5)P₂] fluorescence intensity was determined using the ImageJ software as follows: a mask using the synaptic marker (synaptophysin/vGLUT1/vGAT) was generated for each field and the mean intensity of both PtdIns(4,5)P₂ and the synaptic marker was measured in those regions. The PtdIns(4,5)P₂ values were normalized singularly using their own synaptic marker values. The same procedure was used to quantify the PtdIns4P fluorescence intensity.
Transferrin intensity analysis: transferrin uptake by dendrites and axons and the total transferrin uptake fluorescence analysis was quantified by measuring transferrin fluorescence intensity: a mask using the neuronal marker (MAP2/Tau/βIII-Tubulin) was generated for each field and the mean intensity of transferrin was measured in those regions.

FM 1-43fx intensity analysis: a mask using the synaptic marker (vGLUT1, vGAT) was generated for each field and the mean intensity of FM 1-43fx was measured in those regions.

2.8 FM 1-43fx assay

This assay was used to monitor synaptic vesicle recycling in hippocampal neurons (composed of both SV exocytosis and compensatory endocytosis with SV retrieval). To image SV recycling the FM 1-43fx dye (the fixable version of the FM 1-43 dye, from Molecular Probes) was used and followed the protocol described in (Gaffield and Betz 2007) with some modifications. Prior to stimulation, hippocampal neurons were incubated for 10 min at 37°C in a 5% CO₂ humidified atmosphere with 2 mM KCl Tyrode’s solution (the composition is described in the section 2.6). To allow the trapping of the dye into SVs during the compensatory endocytosis which follows exocytosis during depolarization, SV exocytosis was stimulated by incubation of the neurons for 2 min with 90 mM Tyrode’s solution (the composition is described in the section 2.6) added with 10 µM FM 1-43fx. The stimulating solution containing the dye was then washed away and replaced with a rinsing solution (1 mM Advasep-7 in 2 mM KCl Tyrode’s solution) containing Advasep-7 (Sigma-Aldrich), a dye scavenger able to bind to and remove the background dye. The neurons were washed three times (1 min each wash) with rinsing solution and either fixed (in a fixing solution composed of
4% paraformaldehyde, 4% sucrose for 15 min at RT) to measure endocytosis (T0\textsubscript{endo}) or incubated again with 90 mM Tyrode’s solution (added with 1 mM Advasep-7 to remove background) for 2 minutes (T2\textsubscript{exo}) or 10 minutes (T10\textsubscript{exo}) to stimulate a second exocytic round. The quantification of the intensity of the dye retained at synaptic terminals after this second exocytic stimulus is indicative of the rate and amount of SV exocytosis. Fig. 2.1 reports a schematic work-flow of the experiment.

After fixation, the neurons were processed for immune-staining as described in 2.7.2 and the fluorescence intensity analysis was performed as described in 2.7.3.

![Schematic work-flow of the FM 1-43fx assay](image)

**Figure 2.1** Schematic work-flow of the FM 1-43fx assay

### 2.9 Transferrin uptake assay

To evaluate basal endocytosis, a transferrin uptake assay was performed as described in (Kononenko et al. 2014), with some modifications: Ocrl\textsuperscript{Y+/+} and Ocrl\textsuperscript{Y/-} hippocampal neurons were incubated in serum-free Neurobasal A for 1h at 37 °C, and then kept in serum-free Neurobasal A containing 75 μg/ml transferrin-Alexa488 (Molecular Probes) at 37 °C for 5, 15 and 30 min. Neurons were washed twice in PBS, fixed and processed for immuno-staining (with anti-Tau, anti-MAP2 and anti-βIII-Tubulin), as described in 2.7.1. The intensity analysis was performed as described in 2.7.3.
2.10 Electron microscopy techniques

The presynaptic ultrastructure of Ocr\(^{Y-}\) hippocampal neurons was analyzed by transmission electron microscopy (TEM), using Ocr\(^{Y+}\) neurons as control. The neurons used for this experiment were at 15 D.I.V. and were processed while settled on the coverslip, at the end of these procedures the glass coverslip was removed.

I performed the following experiments together with Elena Polishchuk at TIGEM.

2.10.1 HRP internalization

Prior to stimulation, hippocampal neurons were incubated for 10 min at 37°C, in a 5% CO\(_2\) humidified atmosphere with 2 mM KCl Tyrode’s solution (the composition is described in the section 2.6). Neurons were then incubated for 3 min at 37°C with 90 mM Tyrode’s solution (the composition is described in the section 2.6) containing 10 mg/ml horseradish peroxidase (HRP, Sigma-Aldrich), 10 \(\mu\)M CNQX (Tocris) and 50 \(\mu\)M AP-V (Tocris). The solution containing HRP was washed out and the neurons were incubated for 30 min at 37°C in HRP-free 2 mM KCl Tyrode’s solution containing 10 \(\mu\)M CNQX and 50 \(\mu\)M AP-V. The cells were then fixed in 1.25% glutaraldehyde (Electron Microscopy Science) in 0.2 M Hepes for 30 min at 37°C, the fixing solution was removed, and the cells were then washed twice in 0.2 M Hepes. a DAB (3,3-diaminobenzine) HRP substrate kit (vectorlabs) was used after chemical fixation to develop the HRP signal: the DAB forms a brown reaction product in the presence of the HRP enzyme. Neurons were incubated at RT in the DAB working solution (prepared according to the manufacturer’s instructions) and after 3 min incubation, when a brown substrate developed, the reaction was stopped by washing the neurons in cold distilled H\(_2\)O.
2.10.2 Post-fixation of samples

The neurons were post-fixed for 30 min at 4°C with 1% OsO$_4$ (Electron Microscopy Science) in 100 mM phosphate buffer and then washed 3 times in H$_2$O. To enhance the contrast of lipid-containing membranes, 1% thiocarbohydrazide (prepared in H$_2$O) was added for 5 min; the neurons were washed 3 times with H$_2$O and incubated in a mixture of 1% OsO$_4$ and 1.5% potassium ferrocyanide (K$_4$Fe(CN)$_6$) for 30 min at 4°C. After 3 washes in H$_2$O, the neurons were incubated overnight at 4°C in 0.5% uranyl acetate (UA) diluted in H$_2$O.

2.10.3 Dehydration and embedding of samples

UA was removed and the cells were washed 6 times in H$_2$O. The dehydration of the samples was achieved by passing them through sequential solutions of 50%, 70% and 90% ethanol (every 10 min) and then 100% ethanol for 30 min (changing the 100% ethanol every 10 min). A mixture of Epon and 100% ethanol (1:1) was added to the samples, which was replaced after 1.5 h with only Epon. Finally, the samples (in this case the neurons on the coverslips) were covered with Epon and incubated for 72h at 60°C to allow the resin to polymerize.

2.10.4 Preparation of samples for acquisition and Image acquisition

Once the resin polymerized, the glass coverslips were treated with hydrofluoric acid to corrode the glass surface on which the neurons were settled, thus leaving the neurons attached to the Epon surface only. The samples were, then cut into 60 nm-thin sections at the Leica EM UC7 microtome and EM images were acquired from thin sections using a FEI Tecnai-12 electron microscope.
2.10.5 Quantification

The ultrastructural analysis was performed using the iTEM software. Synapses were selected based on the presence of an active zone. Number and size (diameter) of SVs and endosomes (Ends) (both HRP-positive and HRP-negative) were measured from 20 Ocrl\textsuperscript{Y/+} and 30 Ocrl\textsuperscript{Y/-} synapses. HRP-labeled structures were recognized thanks to the strong electrondense reaction product made by the interaction between DAB and HRP. Area and perimeter of the synapses were also measured. Structures with a diameter smaller than 40 nm were considered as synaptic vesicle (SVs), whereas structures with a diameter larger than 40 nm were considered as endosomes (Ends).

2.11 QuantSeq 3’ mRNA sequencing

Brain regions were obtained as described in 2.3.5. Total RNA was extracted from 5 Ocrl\textsuperscript{Y/+} and 5 Ocrl\textsuperscript{Y/-} hippocampi and the samples were delivered to the Next Generation Sequencing (NGS) facility at TIGEM, where they were processed for QuantSeq 3’ mRNA sequencing as follows: total RNA was quantified using the Qubit 2.0 fluorimetric Assay (Thermo Fisher Scientific); libraries were prepared from 100 ng of total RNA using the QuantSeq 3’ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen GmbH); the quality of the libraries was assessed using screen tape High sentisivity DNA D1000 (Agilent Technologies). Libraries were sequenced on a Nova Seq 6000 sequencing system using an S1, 100 cycles flow cell (Illumina Inc.). The resulting QuantSeq data were then analyzed by Rossella De Cegli at TIGEM. Illumina novaSeq base call (BCL) files were converted in fastq file through bcl2fastq1 (version v2.20.0.422). Sequence reads were trimmed using bbduk software2 (bbmap suite 37.31) to remove adapter sequences, poly-A tails and low-quality end bases (regions with average
quality below 6). Alignment was performed with STAR 2.6.0a3 on mm10 reference assembly obtained from cellRanger website (Ensembl assembly release 93). The expression levels of genes were determined with htseq-count 0.9.15 using mm10 Ensembl assembly (release 93) downloaded from the cellRanger website. All genes having < 1 cpm in less than n_min samples and Perc MM reads > 20% simultaneously were filtered out. Differential expression analysis was performed using edgeR6.
Aims

Lowe syndrome, caused by mutation of the phosphatidylinositol polyphosphate 5-phosphatase OCRL, is characterized by anomalies affecting the central nervous system, the eye and the kidney. There has been tremendous progress in understanding the role of OCRL in the endo-lysosomal pathway in epithelial cells that may be relevant for understanding the renal symptoms of the disease, but whether these underlie the neuronal pathology or if OCRL might have neuronal-specific functions is largely unexplored. Furthermore, the preferential substrate of OCRL is PtdIns(4,5)P$_2$ which is known to regulate important functions in neurons such as exocytosis and endocytosis of synaptic vesicles. Maintaining neuronal PtdIns(4,5)P$_2$ homeostasis in thus crucial and is ensured through a balance in its production mainly by PIPK1$_\gamma$ and its consumption mainly by 5-phosphatases. However, to date it is unknown if OCRL controls PtdIns(4,5)P$_2$ metabolism in neurons together with synaptojanin 1, the main 5-phosphatase responsible for the consumption of plasmalemmal PtdIns(4,5)P$_2$. Thus, considering the roles of OCRL already described in non-neuronal cells and considering also the neuronal pathology of Lowe syndrome, the aims of my project were to:

- investigate the role of OCRL in neurons;
- clarify the mechanisms through which OCRL may control synaptic functions.
Chapter 3

Results

3.1 Defining the expression and localization of OCRL in the brain

3.1.1 OCRL expression in different brain regions

It has been shown previously that OCRL is expressed in almost all tissues (the a isoform is ubiquitously expressed and the b isoform is expressed in all tissues except for the brain) (Johnson et al. 2003; Nussbaum et al. 1997). While its role in epithelial cells together with its interactors and localization has been studied extensively, its function in neuronal cells and tissues had not been clearly determined.

I therefore began the present study by analyzing the expression of OCRL in different regions of the brain (Fig. 3.1). I first validated the OCRL antibody (see Methods) in brain lysates after dissecting, isolating and processing by western blot six different brain regions (cortex, hippocampus, thalamus, striatum, midbrain and cerebellum) from Ocrl\(^{+/+}\) and Ocrl\(^{+-}\) mice. The antibody detected a specific band with the expected molecular weight (105 kDa) in the Ocrl\(^{+/+}\) brain lysates that was absent from the Ocrl\(^{+-}\) lysates, and OCRL expression was judged to be comparable in the different brain regions. This antibody was used for subsequent experiments in the present study.

![Western blot analysis of brain lysates from Ocrl\(^{+/+}\) and Ocrl\(^{+-}\) mice (6 different regions were dissected and analyzed). The figure shows OCRL expression in different brain regions.](image)

*NS*
lysates (50 µg) were separated by SDS-PAGE (12%) gel. The asterisk indicates a non-specific (NS) band recognized by the OCRL antibody. Actin was used as a loading control.

3.1.2 Production of primary neuronal cultures

I proceeded to investigate the role of OCRL in mature hippocampal neurons. As already described in the section 2.4, primary hippocampal neurons were cultured for at least 14 days in vitro (D.I.V.) in order to allow the development of synaptic contacts. Neuronal development was monitored during the 14 days in order to evaluate the grade of differentiation and possible differences between the two genotypes (OcrI^Y/+ and OcrI^Y/-).

As shown in Fig. 3.2, already at the 10th D.I.V., the neurons appeared to be well differentiated (mainly in terms of length of the neuronal processes) but no differences were appreciable between the two genotypes.

![Figure 3.2 In vitro differentiation of OcrI^Y/+ and OcrI^Y/- neuronal cultures. Hippocampal neurons were isolated from OcrI^Y/+ and OcrI^Y/- mice, cultured for 14 days in vitro (D.I.V.) and their differentiation state was monitored by phase-contrast microscopy at D.I.V. 3, 4, 7 and 10.](image)

3.1.3 OCRL is localized at synaptic boutons

To study the role of OCRL in hippocampal neurons, I analyzed the localization of both endogenous and GFP-tagged OCRL using different synaptic proteins as
synaptic markers and quantifying the colocalization between OCRL and each marker used to label the synaptic bouton. As shown in the relative images and confirmed by the quantification (Fig. 3.4 C), both GFP-tagged OCRL (Fig. 3.3) and the endogenous OCRL (Fig. 3.4 A, B) colocalized with Vesicle Associated Membrane Protein 2 (VAMP2; also called synaptobrevin 2), a synaptic vesicle integral membrane protein, which specifically localizes at synaptic boutons. The enrichment of OCRL at the synapse is particularly evident compared with the OCRL signal at the cell body (left panel Fig. 3.4 A) that appeared diffuse.

**Fig. 3.3 GFP-tagged OCRL colocalizes with the synaptic marker VAMP2.** Confocal microscopy images showing the colocalization (right panel) between the GFP-tagged OCRL (left panel) and the synaptic marker VAMP2 (middle panel). Hippocampal neurons overexpressing (72 hours) OCRL were fixed and stained with an antibody against the synaptic protein VAMP2. Two cropped images are reported to facilitate the appreciation of synaptic boutons. The level of colocalization is shown in Fig. 3.4 C.
**Fig. 3.4 Endogenous OCRL colocalizes with the synaptic marker VAMP2. (A,B)**

Confocal microscopy images showing the colocalization between endogenous OCRL (green, upper panel) and the synaptic marker VAMP2 (red, middle panel). Hippocampal neurons were fixed and immunostained for OCRL and VAMP2. A cropped image is reported to facilitate the appreciation of synaptic boutons. **(C)** The level of colocalization between endogenous OCRL and VAMP2 was evaluated with the ImageJ plugin JACoP (Just Another Colocalization Plugin) and compared with the value relative to the localization between GFP-OCRL and VAMP2 (the difference between the two values shown in the graph is not significant). Unpaired t-test was calculated with the GraphPad software. ns= not significant.
Other synaptic markers were used to test for OCRL localization at the synapse. Synaptic boutons were labeled with antibodies against synaptophysin (a presynaptic integral membrane glycoprotein), synapsin (a vesicle-associated protein important for the regulation of the dynamics of synaptic vesicles) and vGLUT1 (a vesicular glutamate transporter associated with excitatory synapses) and colocalization with GFP-tagged OCRL (Fig. 3.5 A) or endogenous OCRL (Fig. 3.5 B and for synapsin and vGLUT1, Fig. 3.6 A,B) was evaluated. As confirmed by the quantifications (Fig. 3.5 C and 3.6 C), the OCRL signal (both endogenous and GFP-tagged) colocalized in part with the synaptic signal.
Fig. 3.5 Endogenous and GFP-tagged OCRL colocalize with the synaptic marker synaptophysin. Confocal microscopy images showing the colocalization between GFP-OCRL (A) or endogenous OCRL (B) and the synaptic marker synaptophysin (red, middle panel). Hippocampal neurons transfected with GFP-OCRL for 72h were fixed and immunostained for synaptophysin (A) or untransfected cells were fixed and immunostained for OCRL and synaptophysin (B). A cropped image is reported to facilitate the appreciation of synaptic boutons. C) The level of colocalization between OCRL and synaptophysin was evaluated with the Imagej plugin JACoP. Unpaired t-test was calculated with the GraphPad software. ns= not significant. In both conditions, OCRL in part overlaps with the synaptic marker synaptophysin.

Fig. 3.6 Endogenous OCRL colocalizes with the synaptic proteins synapsin and vGLUT1. Confocal microscopy images showing the colocalization between endogenous
OCRL (red, upper panel) and the synaptic markers synapsin (A) and vGLUT1 (B) (green, middle panel). Hippocampal neurons were fixed and immunostained for OCRL and synapsin or vGLUT1. A cropped image is reported to facilitate the appreciation of synaptic boutons. C) The level of colocalization between endogenous OCRL and the two synaptic markers was evaluated with the Imagej plugin JACoP. OCRL in part overlaps with both synapsin and vGLUT1. The quantification of the colocalization of OCRL with VAMP2 is reported again in this graph in order to compare colocalization values for the different synaptic proteins. Unpaired t-test was calculated with the GraphPad software. *P<0.05

I concluded that OCRL is associated with synaptic boutons in hippocampal neurons since both the GFP-tagged and the endogenous protein were found to colocalize with synaptic bouton markers.

3.1.4 OCRL is localized at excitatory and inhibitory synaptic boutons

Having identified a synaptic localization for OCRL, I performed the following experiment to determine whether OCRL was differentially distributed at excitatory and inhibitory synapses, the most abundant synapse types. As shown above, OCRL colocalizes with vGLUT1, the major excitatory neurotransmitter. Unfortunately, it was not possible to label the inhibitory synapses with the anti-vGAT antibody (vesicular GABA amino acid transporter, important for the transport of the inhibitory neurotransmitter γ-aminobutyric acid into synaptic vesicles) due to antibody incompatibility. I therefore labeled all the synaptic boutons (excitatory and inhibitory) with the anti-VAMP2 antibody and evaluated the colocalization of endogenous OCRL with both synaptic markers (vGLUT1 for excitatory synapses and VAMP2 for all the synapses) in resting conditions and upon stimulation. As shown in Fig. 3.7, OCRL overlaps with both vGLUT1 and VAMP2. To estimate the degree of OCRL localization at inhibitory synapses I subtracted the number of synapses labeled with vGLUT1, specific for excitatory
synapses, from the total number of synapses, labeled with VAMP2. I used the ImageJ functions to select the vGLUT1 channel and made a selection based on this channel and then applied this selection to the VAMP2 channel and subtracted the vGLUT1 (excitatory synapses) signal from the VAMP2 signal. Using these two values I measured the degree of colocalization with OCRL by calculating the Manders colocalization coefficient, which expresses the percentage of excitatory or inhibitory synapses containing OCRL (Fig. 3.7 B).
Fig. 3.7 OCRL localizes at both excitatory and inhibitory synaptic boutons. (A) Confocal microscopy images showing the triple colocalization of endogenous OCRL (green), the marker of excitatory synapses vGLUT1 (red) and the marker of total synapses VAMP2 (blue). Hippocampal neurons were fixed in resting (left panel) or stimulation conditions (right panel) and immunostained for OCRL and vGLUT1 or VAMP2. A cropped image is reported to facilitate the appreciation of synaptic boutons. (B) Schematic representation of the strategy used to process the images and separate the signals relative to excitatory and inhibitory synapses. (C) Graph summarizing the colocalization results: the level of colocalization between endogenous OCRL and the two synaptic markers was evaluated with the Imagej
plugin JACoP. OCRL signal overlaps with both excitatory and inhibitory signals. Unpaired t-test was calculated with the GraphPad software. ns= not significant.

As also highlighted by the quantification (Fig. 3.7 C), almost 55% of the total excitatory synaptic boutons contained OCRL, whereas almost 40% of the total inhibitory synapses contained OCRL, a little less than the excitatory synapses, and for both synapse types the situation appeared unchanged upon stimulation. All together these experiments suggest that OCRL localizes at excitatory and inhibitory synapses.

3.2 Autophagy does not appear to be affected in the brain in the absence of OCRL

A relationship between OCRL and the endolysosomal pathway has been reported in the kidney where the autophagic flux is impaired in the absence of OCRL (Festa et al. 2019; De Leo et al. 2016). To test whether a similar defect could be observed in brain tissues in the absence of OCRL, I processed total brain lysates from Ocrl<sup>Y</sup>/<sup>+</sup> and Ocrl<sup>Y</sup>/<sup>-</sup> mice and probed them for LC3, a central protein in the autophagy pathway. LC3 levels were comparable in the Ocrl<sup>Y</sup>/<sup>-</sup> brain lysates and the Ocrl<sup>Y</sup>/<sup>+</sup> lysates (Fig. 3.8 A, C). I also monitored the autophagy substrate SQSTM1/p62 in Ocrl<sup>Y</sup>/<sup>-</sup> mice using as a positive control MPS IIIA (Mucopolysaccharidosis IIIA, characterized by an autophagy impairment) lysates and, as shown in Fig. 3.8 B and supported by the quantification (Fig. 3.8 D), SQSTM1 was unchanged in the two Ocrl genotypes, whereas, as expected, it
was increased in MPS IIIA lysates as compared to the wild-type (WT) control.

**Fig. 3.8 LC3 and p62 levels are not increased in Ocrl<sup>Y−</sup> brains.** Western blot analysis of brain lysates from Ocrl<sup>Y+</sup> and Ocrl<sup>Y−</sup> mice showing the levels of (A) LC3 I and II and (B) of SQSTM1/p62. Total lysates (50 µg) were separated by SDS-PAGE (12%). Actin was used as loading control. **C,D** Densitometric quantification of the immunoblot signals of LC3 and SQSTM1.

These results suggested that the autophagic pathway is not influenced by the absence of OCRL in the brain, possibly due to its physiological and functional differences from other organs.
3.3 OCRL is required for a proper exocytosis-endocytosis cycle of SVs

3.3.1 Ocrl\textsuperscript{Y-} synaptic terminals exhibit a reduced recycling rate of SVs

In order to estimate the efficiency of the SV recycling process in Ocrl\textsuperscript{Y-} hippocampal neurons, I used an approach based on the use of the dye FM 1-43fx, a fixable version of the FM 1-43 dye, which allowed the monitoring of the exo-endocytic rate in Ocrl\textsuperscript{Y+} and Ocrl\textsuperscript{Y-} neurons (Gaffield and Betz 2007). FM 1-43fx exhibits green fluorescence that increases immediately after a first “loading” stimulus (the measurement of the fluorescence increase gives an estimation of the amount of the dye uptake by the synaptic terminals and thus of the efficiency of endocytosis) and decreases after a second “unloading” stimulus given to neurons that have already taken up the dye (the measurement of the fluorescence decrease gives an estimation of the efficiency of exocytosis). I followed the protocol illustrated in the Fig. 3.9 A (for details see section 2.8), consisting in a chemical stimulation (2 minutes with a 90 mM KCl Tyrode’s solution) used to load the dye into synaptic terminals (90 mM KCl Tyrode’s solution depolarizes neurons stimulating exocytosis, immediately followed by compensatory endocytosis with the trapping of the dye into synaptic vesicles), followed by the removal of the stimulation solution (plus the dye) and the application for 3 minutes of 2 mM KCl Tyrode’s solution plus ADVASEP-7 (a dye scavenger, used to bind and remove the background dye). Then, I fixed the preparations to have an estimation of the endocytic rate (T\textsubscript{0\textsubscript{endo}}: loading) or stimulated them with a second depolarizing stimulus to have an estimation of the exocytic rate (T\textsubscript{2\textsubscript{exo}}: unloading and T\textsubscript{10\textsubscript{exo}}: unloading).
Fig. 3.9 Ocrl\textsuperscript{Y/} synaptic terminals exhibit a reduced FM 1-43fx uptake. (A) Schematic representation of the experimental work-flow: Ocrl\textsuperscript{Y/+} and Ocrl\textsuperscript{Y/-} hippocampal neurons were depolarized in the presence of FM 1-43fx, washed and fixed (T\text{0\_endo}: loading) or depolarized again for 2 minutes (T\text{2\_exo}: unloading) or 10 minutes (T\text{10\_exo}: unloading) and then fixed. (B) Confocal microscopy images showing FM 1-43fx in Ocrl\textsuperscript{Y/+} (upper panel) and Ocrl\textsuperscript{Y/-} (lower panel) synaptic terminals at the three time points evaluated. (C) Quantification of the mean fluorescence intensity of the dye at the three time points evaluated. The intensity results are expressed as percentage of the Ocrl\textsuperscript{Y/+} T\text{0\_endo} value. Unpaired t-test was calculated with the
GraphPad software. ***P<0.001, ns= not significant. Values are mean ± SD of four independent experiments.

3.3.2 Clathrin-mediated endocytosis (CME) is not significantly altered in Ocrl<sup>Y<sub>−</sub></sup>-neurons

To test whether the impairment observed in Ocrl<sup>Y<sub>−</sub></sup>-synaptic terminals might be due to an impairment in CME, I used a transferrin uptake protocol to monitor the CME rate in Ocrl<sup>Y<sub>+</sub></sup> and Ocrl<sup>Y<sub>−</sub></sup>-hippocampal neurons.

**Fig. 3.10 CME is not altered in Ocrl<sup>Y<sub>−</sub></sup>-neurons.** (A) Confocal microscopy images showing the uptake of fluorescent transferrin (green) by Ocrl<sup>Y<sub>+</sub></sup> and Ocrl<sup>Y<sub>−</sub></sup>-neurons. Transferrin uptake
was allowed for 30 minutes in resting conditions and then the neurons were fixed and
immunostained for the axonal marker Tau (red), the dendritic marker MAP-2 (blue) and the
β-III tubulin (grey). (B) Quantification of the fluorescence intensity of dendritic transferrin
(colocalizing with MAP-2), axonal transferrin (colocalizing with Tau), and total transferrin
(colocalizing with β-III tubulin). ImageJ software was used for the quantification. Unpaired t-
test was calculated with the GraphPad software. ns= not significant. Values are mean ± SD
of three independent experiments.

Fluorescent transferrin uptake was allowed to proceed for 30 minutes under
resting conditions (Kononenko et al. 2014), the neurons were then fixed, and
dendrites were labeled with anti-MAP-2 antibody, axons with anti-TAU antibody,
and neuronal microtubules with anti-β-III tubulin.
The dendritic labeling allowed the quantification of the transferrin uptake in
dendrites, the axonal labeling allowed the quantification of the transferrin uptake
at the axons and the total labeling (with anti-β-III tubulin) allowed the
quantification of the total transferrin uptake. As shown in Fig. 3.10 (A,B) no
differences where detectable in the transferrin fluorescence intensity between the
Ocr1Y/+ and Ocr1Y/- neurons.
I then considered that 30 minutes might be too long a time to capture differences
between Ocr1Y/+ and Ocr1Y/- neurons in terms of general endocytic rate, so I
repeated the experiment including two earlier time points (5 minutes and 15
minutes) (Fig. 3.11). No significant differences between the two genotypes could
be observed (Fig. 3.11 B).
Figure 3.11

A

<table>
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<th>5 min</th>
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Fig. 3.11 CME is not altered in Ocrl\textsuperscript{Y\textdaggerdbl} neurons. (A) Confocal microscopy images showing the uptake of fluorescent transferrin (green) by Ocrl\textsuperscript{Y+} and Ocrl\textsuperscript{Y\textdaggerdbl} neurons at three different time points. The transferrin uptake was allowed for 5, 15 and 30 minutes in resting conditions and then the neurons were fixed and immunostained for the axonal marker Tau (red), the dendritic marker MAP-2 (blue) and the β-III tubulin (grey). (B) Quantification of the fluorescence intensity of dendritic transferrin (colocalizing with MAP-2), axonal transferrin (colocalizing with Tau), and total transferrin (colocalizing with β-III tubulin) taken up at the three time points. ImageJ software was used for the quantification. Unpaired t-test was calculated with the GraphPad software. ns= not significant. Values are mean ± SD of two independent experiments.

3.3.3 OCRL inhibition causes a reduced recycling rate of SVs

At this point, a question emerged: is the defect observed in SV recycling linked to the 5-phosphatase catalytic activity of OCRL? To address this question, I repeated the FM 1-43fx experiment described in section 3.3.1, this time treating Ocrl\textsuperscript{Y+} neurons with a specific inhibitor of OCRL (YU142670) (Fig. 3.12 A).

This small molecule was identified by De Camilli’s group in 2014 (Pirruccello et al. 2014) in a high-throughput screening study and selected for its capacity to
directly interact with the catalytic domain of OCRL and to inhibit its phosphatase activity, resulting in reduced PtdIns(4,5)P\textsubscript{2} dephosphorylation by OCRL. Since the concentration used in De Camilli’s paper was 50 \( \mu \)M (a concentration capable of inducing a reduction of PtdIns(4,5)P\textsubscript{2} dephosphorylation, followed by an accumulation of F-actin foci in proximity of PtdIns(4,5)P\textsubscript{2}-rich sites) for their analysis in human skin fibroblasts and COS-7 cells, I decided to use the same concentration in hippocampal neurons. Ocrl\textsuperscript{Y/+} neurons were pre-incubated for 3 hours with 50 \( \mu \)M YU142670 or with DMSO before addition of FM 1-43fx.
Fig. 3.12 OCRL inhibition causes reduced FM 1-43fx dye uptake. (A) Schematic representation of the experimental work-flow: OcrL Y/+ hippocampal neurons pre-incubated for 3 hours with 50 µM YU142670 (or DMSO), were depolarized in the presence of FM 1-43fx, washed and fixed (T0endo: loading) or depolarized again for 2 minutes (T2exo: unloading) or 10 minutes (T10exo: unloading) and then fixed. (B) Confocal microscopy images showing FM 1-43fx staining in neurons treated with YU142670 (lower panel) or with DMSO (upper panel)
at the three time points evaluated. (C) Quantification of the mean fluorescence intensity of
the dye at the three time points evaluated. The results are expressed as percentage of the
DMSO T0endo value. Unpaired t-test was calculated with the GraphPad software. ****P<
0.0001, **P≤ 0.01 ns= not significant. Values are mean ± SD of three independent
experiments.

As shown in Fig. 3.12 (B,C) neurons treated with the OCRL inhibitor YU142670
exhibited a reduction of almost 50% (50.6 ± 9.3%) in FM 1-43fx fluorescence
relative to control neurons incubated with DMSO at the time point T0endo. No
difference was observed at subsequent time points (T2exo and T10exo) during
exocytosis. These results mirror what was observed in OcrlY/- neurons (section 3.3.1): when
OCRL is absent or pharmacologically inhibited the endocytic step of the recycling
process is strongly impaired, and thus OCRL is required to ensure proper SV
recycling.

3.3.4 The stability of the SNARE complex is not altered in the absence of OCRL
To further analyze the contribution of exocytosis to the defect observed in
neurons depleted of OCRL and in neurons in which OCRL is pharmacologically
inhibited, I evaluated the stability of the SNARE (Soluble NSF Attachment Protein
Receptor) complex. In neurons this complex is composed of the vesicular SNARE
(v-SNARE) protein VAMP2 and the two target SNARE (t-SNARE) proteins
syntaxin 1 and SNAP25. Upon an exocytic stimulus, the three SNARE proteins
interact, forming a complex that drives the fusion of the SVs with the plasma
membrane (Fig. 3.13 A). If the SNARE complex is unstable, neuronal exocytosis
can be defective. To evaluate the stability of the SNARE complex, I used a
biochemical assay based on the resistance of this complex to SDS at room
temperature and its liability upon boiling. Ocrl$^{Y/+}$ and Ocrl$^{Y/-}$ total brain lysates were boiled or not boiled, separated by SDS-PAGE and probed with antibodies against the three SNARE proteins (for further details, see section 2.3.4). As shown in Fig. 3.13 (B,D) the stability of the SNARE complex was similar in Ocrl$^{Y/+}$ and Ocrl$^{Y/-}$ brain lysates.

Fig. 3.13 The absence of OCRL does not alter the SNARE complex stability. (A) Schematic representation for functioning of the SNARE complex in driving membrane fusion and neurotransmitter release. The image was adapted from (Südhof, 2013). (B) western blot analysis of the SNARE complex. Total brain lysates (30 µg per sample)
were prepared from Ocr\textsuperscript{Y/+} and Ocr\textsuperscript{Y/-} brains in parallel and boiled (95\(^\circ\) for 5 minutes) or not as indicated. Following separation by SDS-PAGE (12\%), the SNARE complex and the corresponding protein monomers were revealed using antibodies against the three SNARE proteins as indicated. (C) western blot analysis of the total levels of the three SNARE proteins in Ocr\textsuperscript{Y/+} and Ocr\textsuperscript{Y/-} brains. Actin was used as loading control. (D) Quantification of the levels of the SNARE complex performed with the ImageJ software. The results are expressed as percentage of the Ocr\textsuperscript{Y/+} levels. (E) Quantification of the levels of the SNARE proteins performed with the ImageJ software. The results are expressed as percentage of the Ocr\textsuperscript{Y/+} levels. Unpaired t-test was calculated with the GraphPad software. ns= not significant. Values are mean ± SD of three independent experiments.

I also quantified the total levels of the three SNAREs in the boiled samples and found no significant differences between the two Ocr genotypes (Fig. 3.13 C,E).

3.4 OCRL depletion causes an increase in synaptic PtdIns(4,5)P\textsubscript{2} levels

3.4.1 PtdIns(4,5)P\textsubscript{2} metabolism in WT neurons is efficiently controlled

Having assessed that the defect (mostly endocytic) observed in Ocr\textsuperscript{Y/-} synaptic terminals is linked to the catalytic activity of OCRL and also taking into account that the main substrate of OCRL [PtdIns(4,5)P\textsubscript{2}] in neurons can regulate processes such as endocytosis and exocytosis, I decided to evaluate the abundance (and possible fluctuations) of this lipid.

As already discussed in chapter 1 (see the relative section 1.3.3) it is widely accepted that synaptic PtdIns(4,5)P\textsubscript{2} levels change upon neuronal depolarization, but whether these levels are regulated exclusively by synaptojanin 1, the main neuronal 5-phosphatase, or by other 5-phosphatases, such as OCRL, is still a matter of debate. Thus, I performed experiments to assess PtdIns(4,5)P\textsubscript{2} regulation at the synapse. In these experiments, I evaluated PtdIns(4,5)P\textsubscript{2} (or PtdIns4P) levels at synaptic boutons in resting conditions and
under stimulation. To stimulate neurons, I used 90 mM KCl Tyrode’s solution and to stain for PtdIns(4,5)P$_2$ (or PtdIns4P), I adopted the protocol published by Hammond and colleagues in 2009 (Hammond et al., 2009), following the section dedicated to the staining of internal structures (see section 2.7.2). First, I evaluated the PtdIns(4,5)P$_2$ levels in Ocr$^{Yore}$ synaptic terminals under resting conditions or after stimulation (Fig. 3.14). Three experimental conditions were used: resting (neurons in 2 mM KCl Tyrode’s solution), stimulation (neurons in 90 mM KCl Tyrode’s solution) and recovery after stimulation (neurons first depolarized in 90 mM KCl Tyrode’s solution and then recovered in 2 mM KCl Tyrode’s solution for 30 sec). To consider only the synaptic PtdIns(4,5)P$_2$, I measured and quantified its intensity by using a selection produced on the synaptophysin signal (Fig. 3.14 B). No significant differences in PtdIns(4,5)P$_2$ levels were observed in the three conditions considered, meaning that in WT neurons the PtdIns(4,5)P$_2$ regulation mechanisms (ensured by kinases and phosphatases) are perfectly balanced and thus that the activity-dependent burst in PtdIns(4,5)P$_2$ synthesis by PIPK1$_{\gamma}$ is strictly coupled with its dephosphorylation back to PtdIns4P.
Fig. 3.14 PtdIns(4,5)P$_2$ levels measured at the synaptic boutons do not change during depolarization. (A) Confocal microscopy images showing the presence of PtdIns(4,5)P$_2$ (green) at the synaptic boutons labeled with anti-synaptophysin (red) in resting condition (upper panel), stimulation condition (middle panel) and recovery (lower panel) 30 sec after stimulation. Hippocampal neurons were fixed and immunostained for PtdIns(4,5)P$_2$ and synaptophysin. A cropped image is reported to facilitate the appreciation of synaptic boutons. (B) The PtdIns(4,5)P$_2$ intensity at the synaptic boutons was evaluated with the ImageJ software: a selection produced on the synaptophysin signal was applied to the PtdIns(4,5)P$_2$ channel and the PtdIns(4,5)P$_2$ intensity was measured. The resulting values (expressed in the graph as a mean value) were normalized to the synaptophysin intensity values. Unpaired
t-test was calculated with the GraphPad software. ns= not significant. Values are mean ± SD of two independent experiments.

3.4.2 Synaptic PtdIns4P levels increase during neuronal stimulation as a consequence of PtdIns(4,5)P$_2$ dephosphorylation

Independently of the identity of the 5-phosphatase responsible for the PtdIns(4,5)P$_2$ dephosphorylation at synaptic terminals, PtdIns4P (the product of the PtdIns(4,5)P$_2$ dephosphorylation) is reported to be present on the presynaptic side (and enriched at SVs), where it plays an important role in neuronal functions both as a precursor and a product of PtdIns(4,5)P$_2$ hydrolysis (Guo et al. 2003).

For this reason, I evaluated in WT neurons the changes in PtdIns4P levels resulting from PtdIns(4,5)P$_2$ regulation during synaptic activity (Fig. 3.15) and, again, I considered only the synaptic PtdIns(4,5)P$_2$ and PtdIns4P. To investigate if the Golgi complex, a major site for PtdIns4P synthesis, might be affected under the stimulation conditions, the cells were also labeled with anti-Golgin97. No alterations in the Golgi morphology were observed (Fig. 3.15). Consistent with the previous experiments, PtdIns(4,5)P$_2$ levels at synaptic boutons do not significantly change in WT neurons under stimulation (13.4± 6.5 a.u. in stimulated neurons 18.5± vs 1.5 a.u. in resting neurons) (because of the perfect coupling between synthesis and degradation (as discussed above). Regarding PtdIns4P intensity at synaptic boutons, I could register values increasing by 2-fold as compared to the resting intensity levels (65.3± 12.8 a.u. in stimulated neurons vs 32.95± 1.2 a.u in resting neurons) (Fig. 3.15 B), confirming again that PtdIns(4,5)P$_2$ synthesized during stimulation is consumed mainly through dephosphorylation with the consequent increase in PtdIns4P levels. Of course, PtdIns4P increase during stimulation might be also due to PtdIns4P neo-synthesis in addition to PtdIns(4,5)P$_2$ dephosphorylation back to PtdIns4P, but
unfortunately with this experiment I cannot quantify the contribution of these two processes to PtdIns4P increase. Further experiments are required to clarify this point.

**Fig. 3.15** PtdIns4P levels at synaptic boutons increase during depolarization. (A) Confocal microscopy images showing the presence of PtdIns(4,5)P2 and PtdIns4P (green) at the synaptic boutons labeled with anti-synaptophysin (red) in resting condition (left side)
or stimulation condition (right side). Hippocampal neurons were fixed and immunostained for synaptophysin (red), golgin-97 (G-97, blue) and PtdIns(4,5)P$_2$ or PtdIns4P (green). (B) The intensities of PtdIns(4,5)P$_2$ and PtdIns4P at synaptic boutons were evaluated with ImageJ: a mask generated using the synaptophysin signal was applied to the PtdIns(4,5)P$_2$/PtdIns4P channel and then the PtdIns(4,5)P$_2$ or PtdIns4P intensities were measured. The resulting values (expressed in the graph as a mean value) were normalized to the synaptophysin intensity values. Unpaired t-test was calculated with the GraphPad software. **P £ 0.01 ns= not significant. Values are mean ± SD of two independent experiments.

These first two experiments confirmed that PtdIns(4,5)P$_2$ levels are tightly regulated during neuronal activity and that PtdIns(4,5)P$_2$ metabolism is associated with PtdIns4P production at the synapse.

3.4.3 Synaptic PtdIns(4,5)P$_2$ levels are higher in stimulated OCRL-depleted neurons

The above results indicated that PtdIns(4,5)P$_2$ undergoes a phosphorylation-dephosphorylation cycle, which could be a mechanism relevant to control the effective PtdIns(4,5)P$_2$ levels at the synapse. Therefore, I continued my study assessing the levels of this lipid in OCRL-depleted hippocampal neurons. Ocrl$^{Y/+}$ and Ocrl$^{Y/-}$ synaptic terminals were compared under resting conditions and after stimulation. While PtdIns(4,5)P$_2$ was essentially unchanged after stimulation in Ocrl$^{Y/+}$ neurons as compared to the resting condition (41.5± 6 a.u. in stimulated neurons vs 38.3±10.9 a.u in resting neurons) (indicating that its phosphorylation-dephosphorylation cycle was efficiently controlled), PtdIns(4,5)P$_2$ intensity increased by almost 50% in Ocrl$^{Y/-}$ synaptic terminals after stimulation as compared to the resting conditions (44.9± 7.1 a.u. in stimulated neurons vs 31.1±3.7 a.u in resting neurons) (Fig. 3.16). This increase in synaptic
PtdIns(4,5)P$_2$ levels upon depolarization clearly indicated a deregulation of the phosphorylation-dephosphorylation cycle of this lipid.

**Fig. 3.16** PtdIns(4,5)P$_2$ levels measured at the synaptic boutons increase upon depolarization in the absence of OCRL. (A) Confocal microscopy images showing the presence of PtdIns(4,5)P$_2$ (red) at the Ocrl$^{Y/+}$ and Ocrl$^{Y/-}$ synaptic boutons labeled with anti-synaptophysin (green) in both resting and stimulation conditions. Hippocampal neurons were fixed and immunostained for PtdIns(4,5)P$_2$ and synaptophysin. A cropped image is reported to facilitate the appreciation of synaptic boutons. (B) The PtdIns(4,5)P$_2$ intensity at synaptic boutons was evaluated with ImageJ: a selection produced on the synaptophysin signal was...
applied to the PtdIns(4,5)P₂ channel and the PtdIns(4,5)P₂ intensity was measured for each condition. The resulting values (expressed in the graph as a mean value) were normalized to the synaptophysin intensity values. Unpaired t-test was calculated with the GraphPad software. **P ≤ 0.01, ns = not significant. Values are mean ± SD of two independent experiments.

3.4.4 OCRL depletion causes the accumulation of clathrin at synaptic boutons

Thanks to its ability to recruit different endocytic factors, such as clathrin and clathrin adaptors, PtdIns(4,5)P₂ is considered an important signaling molecule (particularly during endocytosis). Indeed, it is crucial first for clathrin cage assembly on SVs and then for clathrin uncaging of SVs, which starts once the dephosphorylation of PtdIns(4,5)P₂ is completed. For this reason, I continued the characterization of the behavior of the Ocrl⁻/⁻ neuronal cultures by examining the clathrin levels at the synaptic terminals. Clathrin levels were measured in Ocrl⁻/⁻ and Ocrl⁺/⁺ synaptic boutons under resting conditions and after stimulation by quantifying the number of synaptic boutons (identified by labeling with the anti-synaptophysin antibody) positive for the clathrin signal in each condition (Fig. 3.17). In Ocrl⁺/⁺ neuronal cultures, almost 30% of synaptic boutons showed a colocalization with clathrin under resting conditions that increased to almost 40% after stimulation. In Ocrl⁻/⁻ neuronal cultures, the number of synaptic boutons colocalizing with clathrin appeared higher (almost 50%) already under resting conditions and remained high after stimulation.
Figure 3.17

A

Resting

Ocrl Y+/+

Ocrl Y-/

Stimulation

Ocrl Y+/+

Ocrl Y-/

B

Synaptophysin positive structures colocalized with clathrin (Manders coefficient)

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** ns

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Fig. 3.17 Clathrin levels measured at the synaptic boutons increase in the absence of OCRL. (A) Confocal microscopy images showing the colocalization between clathrin (red) and the synaptic marker synaptophysin (green) in Ocrl\(^{Y/+}\) and Ocrl\(^{Y/-}\) hippocampal neurons in both resting condition and stimulation condition. The neurons were fixed and immunostained for clathrin and synaptophysin. (B) The colocalization between clathrin and synaptophysin in each experimental condition was evaluated with the ImageJ plugin JACoP and expressed as Manders coefficient (indicating the percentage of synaptic boutons positive for the clathrin signal). Unpaired t-test was calculated with the GraphPad software. ***\(P \leq 0.001\), **\(P \leq 0.01\), ns= not significant. Values are mean ± SD of two independent experiments.

These results indicate an enrichment of clathrin at synaptic terminals of Ocrl\(^{Y/-}\) neurons possibly due to a dysregulation of PtdIns(4,5)\(_2\) turnover.

3.5 Excitatory and inhibitory synapses are influenced differently by the absence of OCRL

3.5.1 Excitatory as well as inhibitory Ocrl\(^{Y/-}\) synaptic terminals have increased PtdIns(4,5)\(_2\) levels after stimulation

As mentioned previously, there are two main populations of neurons in the neuronal network: excitatory and inhibitory neurons. The balance between excitatory and inhibitory currents is crucial to allow a correct function of neuronal networks. Indeed, many psychiatric disorders, including autism and schizophrenia, are caused by an imbalance between excitatory and inhibitory functions. This is also the case of epilepsy (caused by impaired inhibitory function or expanded excitatory function), which often accompanies psychiatric disorders. Epilepsy is also detected in patients with Lowe syndrome and this observation, together with the fact that I found OCRL expressed in both excitatory and inhibitory synapses (section 3.1.4), motivated me to explore the behavior of these two types of synapses in the absence of OCRL. Excitatory and inhibitory
synapses can be readily distinguished using specific antibodies: anti-vGLUT1 for excitatory synapses and anti-vGAT for inhibitory synapses. To explore possible differences in OCRL-dependent PtdIns(4,5)P<sub>2</sub> levels in excitatory and inhibitory synapses, Ocrl<sup>y/+</sup> and Ocrl<sup>y/-</sup> neurons were immunostained for both synapse markers and for PtdIns(4,5)P<sub>2</sub> under resting conditions and after stimulation (Fig. 3.18).
Fig. 3.18. PtdIns(4,5)P$_2$ levels measured at both excitatory and inhibitory synaptic boutons increase upon depolarization in the absence of OCRL. (A) Confocal microscopy images showing the presence of PtdIns(4,5)P$_2$ at the Ocrl$^{Y/+}$ and Ocrl$^{Y/-}$ synaptic boutons (excitatory and inhibitory) in both resting conditions and stimulation conditions. Ocrl$^{Y/+}$ and Ocrl$^{Y/-}$ hippocampal neurons were fixed and immunostained for PtdIns(4,5)P$_2$ (green), the excitatory marker vGLUT1 (red) and the inhibitory marker vGAT (blue). A cropped image is reported to facilitate the appreciation of synaptic boutons. (B) The PtdIns(4,5)P$_2$ intensity at the synaptic boutons (excitatory and inhibitory) was evaluated with the ImageJ software: a selection produced on the vGLUT1 (or vGAT) signal was applied to the PtdIns(4,5)P$_2$ channel and then the PtdIns(4,5)P$_2$ intensity was measured for each condition. The resulting values (expressed in the graph as a mean value) were normalized to the vGLUT1 (or vGAT) intensity values. Unpaired t-test was calculated with the GraphPad software. *P ≤ 0.05, ns= not significant. Values are mean ± SD of two independent experiments.

Interestingly, even though the PtdIns(4,5)P$_2$ content in Ocrl$^{Y/+}$ synaptic boutons was the same in resting conditions and after stimulation, it dramatically increases in stimulated excitatory and inhibitory synaptic boutons in Ocrl$^{Y/-}$ neurons (Fig. 3.18 B).
3.5.2 *Excitatory as well as inhibitory synapses behave differently in the absence of OCRL in terms of SV recycling rate*

At this point, I used again the FM 1-43fx dye to assess the recycling of SVs in Ocrl<sup>Y−</sup>- excitatory and inhibitory synapses. I applied the protocol already described in section 3.3.1. Briefly, I used a chemical stimulus (90 mM Tyrode’s solution) to load all the synapses with the FM 1-43fx dye (allowing the trapping of the dye into the SVs undergoing compensatory endocytosis) and then I applied a second chemical stimulus to unload the synapses (allowing the release of the dye previously trapped into SVs). As in the experiment described above (paragraph 3.5.1), I identified the excitatory and inhibitory synaptic boutons (by immunolabeling with anti-vGLUT1 and anti-vGAT antibodies, respectively), and evaluated the intensity of the fluorescence emitted by the dye either at the time point T<sub>0<sub>endo</sub></sub> (indicative of the amount of the dye trapped in SVs and thus of the endocytosis efficiency) or at the time point T<sub>2<sub>exo</sub></sub> (indicative of the amount of the dye released and thus of the exocytosis efficiency). As shown in Fig. 3.19 A and quantified in Fig. 3.19 B, at time point T<sub>0<sub>endo</sub></sub> the FM 1-43fx fluorescence intensity in both excitatory and inhibitory Ocrl<sup>Y−</sup>- synapses was almost 35% lower than in Ocrl<sup>Y+/</sup> synapses, indicating that when OCRL is absent, both synapse types exhibit reduced uptake of the dye (indicative of an endocytic defect). For the time point T<sub>2<sub>exo</sub></sub>, I found that the calculation of the intensity of the fluorescence retained at the synapse after the exocytic stimulus would have been more informative: indeed, looking at the relative graph (Fig. 3.19 C), it is possible to appreciate that in Ocrl<sup>Y+/</sup> excitatory and inhibitory synapses the fluorescence decreased at the time point T<sub>2<sub>exo</sub></sub> by almost 50%. Ocrl<sup>Y−</sup> synapses behave differently: the excitatory synapses show almost 30% of fluorescence retained (meaning a loss of fluorescence of almost 70%, that is faster exocytosis as compared to the
controls). As regards inhibitory synapses, a slight increase in the fluorescence retained was measured, but it was not significant.

![Image of fluorescence images showing excitatory and inhibitory synapses with FM 1-43, vGLUT1, vGAT, Ocrl, T0_endo: loading, T2_exo: unloading]
Fig. 3.19 Excitatory and inhibitory synapses exhibit different exo-endocytic rates in absence of OCRL. A) Confocal microscopy images showing the FM 1-43fx trapped into OcrlY/+ and OcrlY/- synaptic terminals (excitatory and inhibitory) at the two time points evaluated. OcrlY/+ and OcrlY/- hippocampal neurons were depolarized in the presence of the FM 1-43fx dye, washed and fixed (T0endo: loading) or depolarized again for 2 minutes (T2exo: unloading) and then fixed. The neurons were then immunostained for the excitatory marker vGLUT1 (red) and the inhibitory marker vGAT (blue). B) Quantification of the mean fluorescence intensity of the dye at the two time points evaluated. C) Ratio between the fluorescence intensity value measured at T2exo and the fluorescence intensity value measured at T0endo, indicative of the fluorescence retained at the synapse after the exocytic stimulus. Unpaired t-test was calculated with the GraphPad software. ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05 ns = not significant. Values are mean ± SD of two independent experiments.
These data suggest that in the absence of OCRL both excitatory and inhibitory synapses show an increase in PtdIns(4,5)P$_2$ content and that the two synapse types respond differently (in terms of exo-endocytic rate) to this increase. These experiments where performed to investigate the possibility that the absence of OCRL might differentially impact the two main synapse types (excitatory and inhibitory) resulting in a functional imbalance that might explain some neurological features of Lowe syndrome such as epilepsy. However, measuring the PtdIns(4,5)P$_2$ content and the exo-endocytic rate in excitatory and inhibitory synapses only partially addresses this point. Indeed, a question that emerges is whether the PtdIns(4,5)P$_2$ enrichment at the synapse would be sufficient to generate an imbalance between the functions of excitatory and inhibitory neurons. This question remains unaddressed and will be dealt with more exhaustively in the discussion.

3.6 OCRL depletion alters the synaptic ultrastructure and the maintenance of SV pools

In view of a possible implication of OCRL in SV endocytosis at mature synapses, I analyzed the presynaptic ultrastructure of Ocr$^{Y/-}$ hippocampal neurons, using Ocr$^{Y/+}$ neurons as control. I also wanted to investigate whether the endocytic defect that I detected in Ocr$^{Y/-}$ synaptic terminals was only due to defective endocytosis of SVs or if it might also be due to impairment in SV recycling. Therefore, I conceived the following experiment aimed at examining presynaptic ultrastructure on the one hand, and evaluating, once again, the endocytic function on the other. To assess the endocytic function, before proceeding with the conventional Transmission Electron Microscopy (TEM) experiment, neurons were stimulated chemically (with 90 mM KCl Tyrode’s solution) in the presence
of soluble horseradish peroxidase (HRP) to visualize the formation of endocytic intermediates and recognize possible anomalies involving these structures. I also exploited the fact that 90 mM KCl evokes maximal SV exocytosis and thus is considered a strong stimulus (such as high frequency stimulation), whereby membrane retrieval occurs via endocytic intermediates (Harata et al. 2001; Wu et al. 2014). Briefly, Ocrl\(^{Y/+}\) and Ocrl\(^{Y/-}\) synaptic preparations were stimulated in the presence of HRP, fixed after 30 minutes of recovery in the absence of HRP (a time estimated enough to allow the reformation of SVs from endosomal intermediates) and prepared for conventional TEM. At the end of the sample preparation, images were acquired and analyzed for each condition (for further details see section 2.10). The analysis was carried out by considering as synaptic vesicles (SVs) structures with a diameter smaller than 40 nm, and as endosomes (Ends) structures with a diameter larger than 40 nm. Different parameters were measured: area (\(\mu m^2\)) and perimeter (\(\mu m\)) of the synapse; density (number/\(\mu m^2\)) of SVs and Ends negative for HRP (HRP-neg), density (number/\(\mu m^2\)) of SVs and Ends positive for HRP (HRP-pos); mean diameter (nm) of SVs and Ends negative for HRP (HRP-neg), mean diameter (nm) of SVs and Ends positive for HRP (HRP-pos). HRP-positive SVs and Ends were considered, according to the literature (Koo et al. 2015), indicative of active cycling during stimulation.

The images and the quantifications (Fig. 3.20) revealed a number of different interesting aspects. The synaptic area and synaptic perimeter were comparable in control and OCRL-depleted neurons (Fig. 3.20 D), meaning that SV retrieval from the plasma membrane is not defective. However, Ocrl\(^{Y/-}\) synapses were characterized by a marked decrease (almost 65% reduction) in the density of HRP-neg SVs accompanied by a strong increase (almost 75%) in the density of
HRP-neg Ends (Fig. 3.20 B) (which might be due to a defect in the recovery of SVs from the endosomal intermediates).
Fig. 3.20 Electron microscopy analysis shows ultrastructural alterations in absence of OCRL. **(A)** Ocrl<sup>Y/−</sup> and Ocrl<sup>Y/+</sup> hippocampal neurons were stimulated in presence of HRP, fixed and processed for conventional TEM. **(B)** Quantification of the density (number/µm<sup>2</sup>) of SVs and Ends (both HRP-neg and HRP-pos) for each genotype (results expressed as mean values). **(C)** Quantification of the diameter of SVs and Ends (both HRP-neg and HRP-pos) for each genotype (results expressed as mean values). **(D)** Quantification of the synapse area and perimeter for each genotype (results expressed as mean values). Unpaired t-test was calculated with the GraphPad software. ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05 ns= not significant. Unpaired t-test was calculated with the GraphPad software. ****P ≤ 0.0001, ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05, ns= not significant. Scale bars= 0.1 µm.

The Ocrl<sup>Y/−</sup> synaptic terminals also displayed a decrease in the density of HRP-pos SVs (indicative of a lower number of SVs recovered by the endosomal intermediates), whereas HRP-pos Ends were not significantly changed (Fig. 3.20 B). Moreover, the diameter of all the structures examined (SVs and Ends both positive and negative for HRP) was significantly higher in Ocrl<sup>Y/−</sup> synapses relative to the control synapses (Fig.3.20 C), with the exception of Ends positive for HRP, whose increase in diameter was not statistically significant.

In summary, there is a decrease in the density of SVs HRP-neg (83±50 SVs/µm<sup>2</sup> in the KO vs 233±88 SVs/µm<sup>2</sup> in the WT) and HRP-pos (14±9 SVs/µm<sup>2</sup> in the KO vs 28,8±11 SVs/µm<sup>2</sup> in the WT) accompanied by an increase in endosomal density (24±9 Ends/µm<sup>2</sup> in the KO vs 6,6±3 Ends/µm<sup>2</sup> in the WT) and size (55±6 nm in the KO vs 45±5 Ends/µm<sup>2</sup> in the WT) in Ocrl<sup>Y/−</sup> synapses. These ultrastructural data clearly indicate that there is a severe endocytic defect partially (if not exclusively) associated with a longer retention of SVs in endosomal structures that originate from the fusion of SVs to early endosomes in OCRL-depleted synaptic terminals upon a strong depolarizing stimulus. Furthermore,
these data are in line with the other data discussed above, delineating a role for OCRL in the regulation of SV recycling.

3.7 Ocrl\textsuperscript{Y/+} and Ocrl\textsuperscript{Y/-} hippocampi have different transcriptomic and proteomic profiles

In order to identify a more general role for OCRL in hippocampal neurons (which may justify the cellular phenotypes mentioned above and the neurological features of the disease), I decided to perform a transcriptomic analysis coupled with a proteomic analysis. As explained in detail in the section 2.3.5, I isolated 6 different regions (cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum and olfactory bulbs) from Ocrl\textsuperscript{Y/+} and Ocrl\textsuperscript{Y/-} brains from 16 day-old mice (it is known that neurological features appear in the early developmental stages of the brain: older mice will be analyzed in the future). Briefly, I selected 5 Ocrl\textsuperscript{Y/+} and 5 Ocrl\textsuperscript{Y/-} mice and dissected the six regions from both hemispheres, thus having for each region (e.g. the hippocampus) 2 halves: one half coming from the right hemisphere and the other from the left hemisphere. In order to use the two halves for different purposes (transcriptomics and proteomics analysis), I kept and stored them separately (Fig. 3.21). To be as consistent as possible with other experiments performed in this study, I decided to begin with the processing of the hippocampi for the two different experiments (to facilitate the understanding, I’m referring to each of the two halves of the hippocampus saying simply hippocampus).
3.7.1 Ocrl\textsuperscript{Y/+} and Ocrl\textsuperscript{Y/-} hippocampi have different gene expression profiles

Total RNA was extracted from 5 Ocrl\textsuperscript{Y/+} and 5 Ocrl\textsuperscript{Y/-} hippocampi and the samples processed for QuantSeq 3' mRNA sequencing (for further details, see section 2.11). The resulting QuantSeq data were then analyzed by Rossella De Cegli at TIGEM. The segregation of the two genotype transcriptomes into two different populations is shown in the Fig. 3.22.
The 5 Ocrl\textsuperscript{Y−} transcriptome profiles (EXP1) were compared to the 5 Ocrl\textsuperscript{Y+} profiles used as controls (CTR1). Differential expression was performed using the edgeR (PMID:19910308) statistical package. By using a False Discovery Rate (FDR)<0.05 (corresponding to a probability of 5% to have false positive results) as threshold for statistical significance, we obtained a total of 209 differentially expressed genes (DEG), 120 transcripts being induced and 89 inhibited in Ocrl\textsuperscript{Y−} hippocampi. Using a less stringent threshold (FDR <0.1), we obtained 219 induced genes and 186 inhibited genes in Ocrl\textsuperscript{Y−} hippocampi. We decided to proceed using this second less stringent analysis. The Ocrl transcript was one of the top down-regulated ones (logFC= -1.222); the fact that the Ocrl expression (white square in the Fig. 3.24 A) was not completely absent was not surprising because, according to what is reported in the papers illustrating the generation of the mouse model (Bothwell et al. 2011; Nussbaum et al. 1997), the approach
used by Nussbaum and colleagues to target and disrupt Ocrl could result either in the complete loss of transcript and protein or in the formation of products unstable and rapidly degraded. As shown in the Fig. 3.23 A (up-regulated genes) and 3.24 A (down-regulated genes), the results were ranked by logFC values (FDR<0.1).

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Fig. 3.23 Isolation of up-regulated transcripts following OCRL-depletion in hippocampi. A) List of up-regulated transcripts in Ocrl<sup>Y<sup>−</sup></sup>/ hippocampi, ranked as decreasing logFC (Ocrl<sup>Y<sup>−</sup></sup>/ hippocampi i.e.EXP1 versus Ocrl<sup>Y<sup>−</sup></sup>/ hippocampi, i.e. CTR1). The light blue and the yellow boxes in the FDR column refer to the FDR values using FDR<0.1 or FDR<0.05 as threshold. B) KEGG pathway analysis on 219 up-regulated transcripts (FDR<0.1).
were performed to evaluate the biological processes associated with the altered

Then bioinformatics analyses using the Kyoto Encyclopedia of Genes and Genomes (KEGG pathway) and the Gene Ontology (GO) functional analysis were performed to evaluate the biological processes associated with the altered

Fig. 3.24 Isolation of down-regulated transcripts following OCRL-depletion in hippocampi. A) List of down-regulated genes in Ocr\(^{Y^1/2}\) hippocampi, transcripts in Ocr\(^{Y^1/2}\) hippocampi, ranked as increasing logFC (Ocr\(^{Y^1/2}\) hippocampi i.e.EXP1 versus Ocr\(^{Y^1/2}\) hippocampi, i.e. CTR1). The light blue and the yellow boxes in the FDR column refer to the FDR values using FDR<0.1 or FDR<0.05 as threshold B) KEGG pathway analysis on 186 down-regulated transcripts (FDR<0.1).
mRNA expression. The KEGG pathway analysis performed on the 219 up-regulated genes showed a significant enrichment of categories such as “neuron projection” (32 genes), “neuron part” (36 genes), “dendrite” (18 genes), “synapse part” (18 genes), “anterograde trans-synaptic signaling” (15 genes), “neuron-neuron synaptic transmission” (7 genes) (Fig. 3.23 B). Restricting the output to the cellular compartment (CC_FAT) terms in the category “neuron part” (GO:0097458~neuron part) (Fig. 3.25) from the GO analysis we considered interesting the following genes (yellow squares in Fig. 3.23 A): Adgrl2/3 encoding latrophilin 2/3: G-protein coupled receptors (specific for the excitatory neurotoxin latroxin), reported as regulators of neuronal exocytosis and implicated in some neuronal disorders (Silva and Ushkaryov 2010); Adora2a encoding the A2AR receptor, which controls excitatory glutamatergic synapses (Gomes et al., 2011); Drd1 encoding the dopamine receptor D1, which has been implicated in neuronal disorders caused by an imbalance of excitatory-inhibitory functions (Gangarossa et al. 2011). Regarding the 186 down-regulated genes, the KEGG analysis returned categories such as “axon development” (18 genes), “neuron development” (24 genes), “ion binding” (64 genes), “GABAergic synapse” (5 genes), “synapse” (23 genes), “neuron part” (30 genes) (Fig. 3.24).
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Fig. 3.25 Gene Ontology analysis of a category of up-regulated genes and down-regulated genes. A) The 36 genes annotated in the GO:0097458~neuron part category resulted from the Gene Ontology (GO) analysis restricting the output at the cellular level.
compartment (CC_FAT) terms. B) List of the 30 genes annotated in the GO:0097458~neuron part category resulted from the Gene Ontology (GO) restricting the output at the CC terms.

Again, a GO analysis was performed on the category “neuron part” (Fig. 3.25 B). Some genes caught our attention (blue square in the Fig. 3.24 A): Mt2 encoding metallothionein 2, a metal binding protein with neuroprotective effects and, when down-regulated, associated with neuronal damage and ROS-induced neurodegeneration (Juárez-Rebollar et al., 2017); LRP1 encoding the lipoprotein receptor-related protein 1, already associated with behavioral and motor defects (May et al. 2004) and also member of the LDL receptor-related protein family together with LRP2/megalin [a receptor whose trafficking is known to be regulated by OCRL (Vicinanza et al. 2011)], the latter, interestingly, responsible for the LDLR-mediated endocytosis of metallothioneins (produced by astrocytes) into neurons; Gabra2, Gabrb1 and Gad2, all associated with GABAergic synapses, thus inhibitory functions (Butler et al. 2018; Galanopoulou 2008).

In total, this transcriptomics experiment indicates a clear expansion of the excitatory functions coupled with an impairment of inhibitory functions in Ocr1Y-/ hippocampi.

3.7.2 Ocr1Y+/ and Ocr1Y-/ hippocampi have different proteomic profiles

I then implemented a high resolution mass spectrometry (MS)-based approach on the remaining 5 Ocr1Y+/ and 5 Ocr1Y-/ hippocampi as described in (Sharma et al. 2015). The MS-experiment was performed by Michele Santoro and Paolo Grumati at TIGEM. Briefly, the 5 biological replicates of the Ocr1Y+/ and Ocr1Y-/ hippocampi were lysed, digested, fractionated and the resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The resulting MS data were then analyzed by Michele Santoro, Rossella De Cegli
and Carmine Cirillo at TIGEM. Among the total proteins detected (4,430 proteins), only those showing a FC > 1.5 (Ocrl\textsuperscript{Y-} vs Ocrl\textsuperscript{Y+}) and a significant q-value were considered (Fig. 3.26).

**Fig. 3.26 Analysis of differentially expressed proteins in OCRL-depleted hippocampi.**

**A)** List of up-regulated proteins in Ocrl\textsuperscript{Y-} hippocampi, scored for FC (fold-change in the Ocrl\textsuperscript{Y-} relative to Ocrl\textsuperscript{Y+}). **B)** List of down-regulated proteins in Ocrl\textsuperscript{Y-} hippocampi, scored for FC (fold-change in the Ocrl\textsuperscript{Y-} relative to Ocrl\textsuperscript{Y+}).

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<th>Fold Change</th>
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<th>Fold Change</th>
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<td>Chd2, Chd1</td>
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**A)** List of up-regulated proteins in Ocrl\textsuperscript{Y-} hippocampi, scored for FC (fold-change in the Ocrl\textsuperscript{Y-} relative to Ocrl\textsuperscript{Y+}). **B)** List of down-regulated proteins in Ocrl\textsuperscript{Y-} hippocampi, scored for FC (fold-change in the Ocrl\textsuperscript{Y-} relative to Ocrl\textsuperscript{Y+}).
We obtained 94 proteins enriched (Fig. 3.26 A) and 37 reduced (Fig. 3.26 B) in the Ocrl<sup>Y</sup>- proteome compared to control (Ocrl<sup>Y/+</sup>). The total proteins resulted from the analysis were then searched in the transcriptomic dataset (Fig. 3.27). Interestingly, we found 2 proteins down-regulated and 5 proteins up-regulated also at the transcriptomic level. Among the up-regulated proteins, we focused our attention on the SLC35F1 (Solute Carrier Family 35 Member F1) protein (with FC= 8.72), a solute carrier already reported to be enriched in the brain, which appeared to be particularly interesting because very recently it has been associated with highly dynamic vesicles, positive for the recycling endosome marker Rab11 (Farenholtz et al. 2019), supporting thus the idea that the absence of OCRL might cause a SV recycling defect mediated by endosomal intermediates. Further analyses will be performed to assess the functional relevance of the other proteins found differentially expressed in the Ocrl<sup>Y</sup>- hippocampus.

Comparative analysis: PROTEOME vs TRANSCRIPTOME

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<tr>
<th>Symbol</th>
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<td>-1.00118256</td>
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<td>Mdn1</td>
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<td>S1c35F1</td>
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<td>Cirbp</td>
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<td>0.98648564</td>
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<td>0.00835155</td>
<td>2.868827184</td>
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**Fig. 3.27 Comparative analysis of proteins and transcripts differentially expressed in OCRL-depleted hippocampi.** List of up-regulated (red) and down-regulated (green) genes and proteins resulted from the comparative bioinformatic analysis. The results were scored for FC, but also FDR and Student’s T-Test values were considered.
3.8 OCRL interacts with different synaptic proteins.

To gain a deeper insight into the mechanism by which OCRL might control SV cycling, a different MS approach was used to identify putative brain-specific OCRL interactors. Ocr¹⁺ and Ocr¹⁻ brains were lysed, immunoprecipitated with the OCRL antibody (Fig. 3.29 A) and sent to the Central Proteomics Facility (South Parks Rd, Oxford) for MS analysis. The data from this analysis were subjected to bioinformatics analysis (performed by Mario Failli at TIGEM). First, the background (consisting in the proteins found in the samples immunoprecipitated with pre-immune immunoglobulins, IgG, as controls) was removed for each sample (Ocr¹⁺ and Ocr¹⁻). Then, proteins that were common in the Ocr¹⁺ and Ocr¹⁻ immunoprecipitates (number) were subtracted from the Ocr¹⁺ list of proteins (number) resulting in 313 OCRL-specific brain interactors exclusively found in Ocr¹⁺ immunoprecipitates. As shown in Fig. 3.28 B, the OCRL-specific interactors were organized according to their protein score (a measure derived from the ion scores, indicating the sum of the highest ion score for each distinct sequence). On the top of the list we found OCRL, as expected (protein score: 3276) (light blue-filled box). Going down the list we found proteins already known to interact with OCRL (orange-filled boxes), such as the endosomal proteins APPL1 (protein score: 134); the endosomal RAB35 (protein score: 92), reported to interact and recruit OCRL on recycling endosomes in epithelial cells (Cauvin et al. 2016) and implicated in both axonal elongation and SV turnover in neurons (Sheehan & Waites, 2017; Sheehan et al., 2016; Villarroel-Campos et al., 2016); the Golgi-associated RAB13 (protein score: 81), already reported to localize at neurites and growth cones, where it supports neurite growth by regulating the transport of membrane-containing vesicles from
the Trans Golgi Network to recycling endosomes (Sakane et al., 2010); the endolysosomal Rab14 (protein score: 49) involved in regulating clathrin-coated vesicle trafficking and recycling routes in neurons (Mignogna and D'Adamo 2018); CDC42 (protein score: 29), known as a regulator of neuronal morphology, by controlling the reorganization of the actin cytoskeleton (Chen et al., 2012). We also found other RAB proteins and proteins associated, in general, with SV trafficking (green-filled boxes): the ER-Golgi associated RAB1A (protein score: 107) (Takamori et al. 2006); the ER-related RAB33b (protein score: 44), already known to regulate axonal outgrowth by mediating anterograde synaptophysin-positive vesicle trafficking (Huang et al., 2019); Syntaxin 1A/1B (protein scores: 26 and 89, respectively), known to be important in SV exocytosis (see section 1.2.2); Endophilin A1 (protein score: 54) involved in clathrin-coated vesicle uncoating through its interaction with the 5-phosphatase Synaptojanin 1 (the interaction occurs via binding of the SH3 domains of Endophilin A1 with the proline-rich region of Synaptojanin1, a region present also in the OCRL sequence) and also in the regulation of actin depolarization at dendritic spines (see section 1.2.3); Synapsin 1/3 (protein scores: 37 and 23, respectively), important for the maintenance of SV pools and dynamics; VAMP2 (protein score: 31), the v-SNARE associated with synaptic vesicles and widely used in this study as a synaptic marker (see section 1.2.2).
OCRL interacts with synaptic proteins. A) OCRL was immunoprecipitated from Ocr1Y+ and Ocr1Y- brains with the α-OCRL antibody. IgG: immunoglobulins used as controls.
IP: immunoprecipitations; FT: flow-through. 180 µg proteins were loaded as Input and FT. The western blot analysis shows that the OCRL-specific band (detected by the α-OCRL antibody) at 105 kDa is clearly enriched in the OcrlY/+ IP and absent in OcrlY/− IP and IgG samples. B) Table listing the proteins resulting from the analysis of the OCRL interactome, together with their protein score (on the right) and protein accession number (on the left).

The OCRL interactors were also subjected to a functional analysis (REACTOME pathway analysis and GO analysis for cellular components). The REACTOME analysis (Fig. 3.29 A) of the cellular pathways associated with the proteins found in the OCRL interactome returned pathways such as “neurotransmitter release cycle”, “neuronal system” and “transmission across synapses”. The GO analysis of the cellular components (Fig. 3.29 B) returned categories such as “clathrin-coated vesicles”, “membrane-bound vesicle”, “cytoplasmic membrane-bound vesicle”.

A
REACTOME pathway analysis

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<th>Pathway name</th>
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</table>

B
Gene Ontology analysis: cellular components

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Fig. 3.29 Functional analysis of the OCRL interactome. A) Table summarizing the main pathways resulted from the REACTOME pathway analysis. B) Table summarizing the main categories resulted from the GO analysis focused on cellular components.
To corroborate the interactome data, OCRL was immunoprecipitated from Ocrl<sup>Y/+</sup> and Ocrl<sup>Y/-</sup> brains and some of its interactors were confirmed. In particular, we confirmed the interaction with APPL1, Syntaxin 1 (even though a weak band in the Ocrl<sup>Y/-</sup> precipitates is visible) and VAMP2. SNAP25 was used as a negative control because it did not result as interactor from the OCRL interactome analysis (Fig. 3.30).

**Fig. 3.30 OCRL immunoprecipitation confirms some OCRL interactions.** OCRL was immunoprecipitated from Ocrl<sup>Y/+</sup> and Ocrl<sup>Y/-</sup> brains with the α-OCRL antibody. IgG: immunoglobulins used as controls. IP: immunoprecipitations; FT: flow-through. 180 µg proteins were loaded as Input and FT. The western blot analysis shows that the OCRL-specific band (detected by the α-OCRL antibody) at 105 kDa is clearly enriched in the Ocrl<sup>Y/+</sup> IP and absent in Ocrl<sup>Y/-</sup> IP and IgG samples. The α-APPL1 and α-VAMP2 antibodies detect the respective bands at the expected molecular weight (100 and 19 kDa respectively) in Ocrl<sup>Y/+</sup> IP, that result absent in Ocrl<sup>Y/-</sup> IP and IgG samples. The α-Syntaxin1 antibody detects a band at the expected molecular weight (33 kDa) that is absent in the IgG samples, but present, even if very weak, in the Ocrl<sup>Y/-</sup> IP.

These data support a role for OCRL in regulating SV cycling at the pre-synapse.
Chapter 4
Discussion

**OCRL resides at both excitatory and inhibitory synaptic boutons where it participates in the regulation of SV recycling**

The role of OCRL as well as its localization in epithelial cells has been described in some detail thanks to several studies performed in our and other laboratories (see section 1.5).

However, when this thesis work began there was no information in the literature regarding either its localization or its functions in neurons. Hence, experiments were devised in order to address this issue (see aims). For all experiments reported in this work a mouse model constitutively depleted of OCRL (Bothwell et al. 2011) was used which represents a reliable model for Lowe syndrome. I first assessed that OCRL localizes at both excitatory and inhibitory synaptic boutons in hippocampal neurons (section 3.2) and then proceeded to understand whether SV recycling might be affected by the absence of OCRL. Using the dye FM 1-43, which is widely employed to monitor exo-endocytosis at synaptic terminals upon neuronal stimulation (Gaffield and Betz 2007; Iwabuchi et al. 2014), I found that Ocrl<sup>−/−</sup> neurons exhibited a clear SV recycling defect, with the endocytic step undoubtedly more affected by the absence of OCRL than the exocytic step (section 3.3.1). Using KCl for neuronal stimulation [which is considered a strong stimulus comparable to high frequency stimulation where neurons retrieve SVs through CIE rather than CME (see section 1.2.4)] and having also demonstrated that CME is not altered when OCRL is absent (section 3.3.2), I could confirm that the endocytic defect observed in the FM 1-43 experiments is associated with clathrin-independent compensatory endocytosis.
This process is strictly coupled to SV exocytosis and is activated when a strong stimulus causes the arrival of large amounts of SV membranes and components to the plasma membrane. Compensatory endocytosis is thus of fundamental importance to ensure the rapid retrieval and recycling of SV membranes and components (see section 1.2.4). This process was found to be defective in the absence of OCRL.

**SV recycling at the presynaptic side is affected by the amount of PtdIns(4,5)P₂, whose levels are regulated by both OCRL and synaptojanin1**

Since OCRL is a 5-phosphatase acting preferentially on PtdIns(4,5)P₂ to produce PtdIns4P (see section 1.3.3) and since PtdIns(4,5)P₂ controls synaptic transmission, participating in several processes such as exocytosis, endocytosis and also actin polymerization (see section 1.3.4), I hypothesized that efficient SV recycling would require the catalytic activity of OCRL rather than other functions of OCRL. This hypothesis was tested in a number of ways. I first evaluated SV recycling upon pharmacological inhibition of OCRL and found that the neurons treated with the OCRL inhibitor YU142670 showed the endocytic defect previously observed in OcrlY⁻/⁻ neurons (section 3.3.3), meaning that the catalytic activity of OCRL can control SV recycling. I then focused my attention on PtdIns(4,5)P₂. In OcrlY⁻/⁺ neurons I found that the PtdIns(4,5)P₂ levels in stimulated presynaptic boutons were comparable to those measured in resting boutons, indicating a functional synthesis/degradation cycle for PtdIns(4,5)P₂, while OcrlY⁻/⁻ neurons showed higher PtdIns(4,5)P₂ levels under stimulatory conditions compared to resting conditions, meaning that the PtdIns(4,5)P₂ synthesized during depolarization is not efficiently consumed in the absence of OCRL. These findings corroborated the hypothesis that, together with synaptojanin 1, OCRL
controls PtdIns(4,5)P₂ levels at the synapse. Consequently, the absence of OCRL may cause a dysfunction in PtdIns(4,5)P₂ metabolism resulting in PtdIns(4,5)P₂ accumulation at the synapse and thus in defects in SV recycling.

Since clathrin and clathrin adaptors are the main PtdIns(4,5)P₂ partners in mediating synaptic vesicle reformation from endocytic intermediates (see section 1.2.4), I evaluated clathrin levels at synaptic boutons and found that while clathrin increases at Ocr[Y/+] synaptic boutons only under stimulation, Ocr[Y/-] synaptic boutons exhibited an enrichment in clathrin already under resting conditions (see section 3.4.4). Together with the analysis described above, these results indicate that there is an accumulation of PtdIns(4,5)P₂ together with its partner clathrin at OCRL-depleted nerve terminals.

An imbalance in excitatory and inhibitory functions accompanies the SV recycling defect observed in the absence of OCRL

Considering the clinical signs of Lowe syndrome and in particular epilepsy (see section 1.4.1.3), we hypothesized that such manifestations could be caused by an imbalance between excitatory and inhibitory functions (see section 1.2). These functions were assessed using multiple approaches in the presence and absence of OCRL (the latter being a condition leading to the accumulation of PtdIns(4,5)P₂, as described before).

I measured PtdIns(4,5)P₂ content at excitatory and inhibitory synapses and could find an increase in PtdIns(4,5)P₂ levels under stimulation in both synapse types in the absence of OCRL (in comparison with control synapses) (see section 3.5.1). Additionally, using the SV recycling assay I found that both synapse types showed defective endocytosis. Moreover, inhibitory synapses showed slower exocytosis whereas exocytosis appeared faster in excitatory synapses (see
section 3.5.2). Future studies will be aimed at verifying whether PtdIns(4,5)P$_2$ may differentially contribute to exo-endocytic functions in the two different synapse types. To obtain complementary information on excitatory and inhibitory functions in our mouse model, I performed a combined transcriptomic-proteomic approach on wild-type (WT) and knock-out (KO) hippocampi, which revealed a clear expansion of the excitatory functions coupled with a strong impairment of the inhibitory functions (see sections 3.7.1-3.7.2).

*The absence of OCRL causes SV cycle stalling at endocytic intermediates*

To substantiate our data indicating a defect in SV recycling associated with an accumulation of PtdIns(4,5)P$_2$ in synapses depleted for OCRL, I analyzed the ultrastructure of Ocrl$^{Yc}$ hippocampal neurons and found a strong reduction in the number of SVs in OCRL-depleted synapses together with an increase in the number and size of synaptic endosomes, meaning that during recycling the SVs arrive at an endosomal intermediate but, differently from the control neurons, in absence of OCRL SVs cannot reform from this endocytic intermediate. I also found that SVs are efficiently retrieved from the plasma membrane because neurons depleted of OCRL did not show an increase in the perimeter of the active zone (which would be indicative of defective retrieval of SVs that had arrived at the plasma membrane) (see section 3.5). These findings, together with the “interactome” results indicating that OCRL interacts with different endocytic proteins such as APPL1, Rab35, endophilin 1, syntaxin and VAMP2 in neurons (see section 3.7), provide strong evidence that OCRL is involved in the regulation of SV reformation from the endocytic intermediates that form at the synaptic boutons during intense stimulation.
In conclusion, my thesis work unravels a novel function for OCRL in SV recycling at the synapse. Following intense stimulation, a readily releasable pool and a recycling pool of SVs are mobilized, arrive at the plasma membrane where they fuse and release neurotransmitters. Immediately following exocytosis, SVs are retrieved through CIE and arrive at endocytic intermediates from which they reform in a clathrin and PtdIns(4,5)P$_2$-mediated mechanism. In this context OCRL, acting as a negative regulator of PtdIns(4,5)P$_2$ levels, guarantees clathrin uncoating and efficient SV reformation from endocytic intermediates, thus the progression of the SV cycle resulting in the return of SVs to a specific pool, where can be used for a new round of release. Indeed, when OCRL is absent, synapses show a defect in SV recycling together with accumulation of PtdIns(4,5)P$_2$ and clathrin. Moreover, the absence of OCRL seems to have different consequences in excitatory and inhibitory synapses, causing an imbalance between the two functions, but other studies are required to further confirm these results.
List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADBE</td>
<td>activity-dependent bulk endocytosis</td>
</tr>
<tr>
<td>AP-V</td>
<td>D-2-amino-5-phosphonovalerate</td>
</tr>
<tr>
<td>AP180</td>
<td>Adaptor protein 180</td>
</tr>
<tr>
<td>AP2</td>
<td>Adaptor protein 2</td>
</tr>
<tr>
<td>araC</td>
<td>cytosine arabinoside</td>
</tr>
<tr>
<td>AZ</td>
<td>active zone</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CALM</td>
<td>clathrin assembly lymphoid myeloid leukemia protein</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CAPS</td>
<td>Ca$^{2+}$-dependent activator protein for secretion</td>
</tr>
<tr>
<td>CDK5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CIE</td>
<td>clathrin-independent endocytosis</td>
</tr>
<tr>
<td>CME</td>
<td>clathrin-mediated endocytosis</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>D.I.V.</td>
<td>days in vitro</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EZ</td>
<td>endocytic zone</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>FELASA</td>
<td>Federation of European Laboratory Animal Science Association</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>K&amp;R</td>
<td>kiss and run</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LMW</td>
<td>low-molecular weight</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>mA</td>
<td>milliAmpere</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTM</td>
<td>myotubularin</td>
</tr>
<tr>
<td>NGS</td>
<td>Next Generation Sequencing</td>
</tr>
<tr>
<td>nm</td>
<td>nanometers</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PHLDB2</td>
<td>Pleckstrin homology like domain family B member 2</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P₃</td>
<td>phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PtdIns(3,4)P₂</td>
<td>phosphatidylinositol 3,4-bisphosphate</td>
</tr>
<tr>
<td>PtdIns(3,5)P₂</td>
<td>phosphatidylinositol 3,5-bisphosphate</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PtdIns3P</td>
<td>phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PI4K</td>
<td>phosphatidylinositol 4-kinase</td>
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<tr>
<td>PtdIns4P</td>
<td>phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PtdIns5P</td>
<td>phosphatidylinositol 5-phosphate</td>
</tr>
<tr>
<td>PIPK₁γ</td>
<td>type I gamma phosphatidylinositol phosphate kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PSD</td>
<td>post-synaptic density</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RP</td>
<td>recycling pool</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>RRP</td>
<td>ready releasable pool</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 Domain-Containing Inositol 5-Phosphatases</td>
</tr>
<tr>
<td>SNAP25</td>
<td>synaptosome Nerve-Associated Protein 25</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-NSF-Attachment Protein Receptor</td>
</tr>
<tr>
<td>SV</td>
<td>synaptic vesicle</td>
</tr>
<tr>
<td>synj</td>
<td>synaptojanin</td>
</tr>
<tr>
<td>syt</td>
<td>synaptotagmin</td>
</tr>
<tr>
<td>TBS</td>
<td>150mM NaCl, 50 mM Tris-HCl pH 7.5</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethyl-ethylenediammine</td>
</tr>
<tr>
<td>TTBS</td>
<td>0.05% Tween20, 150 mM NaCl, 50mM Tris-HCl pH 7.5</td>
</tr>
<tr>
<td>UFE</td>
<td>ultrafast endocytosis</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>VAMP2</td>
<td>vesicle associated membrane protein 2</td>
</tr>
<tr>
<td>vGAT</td>
<td>vesicular GABA transporter</td>
</tr>
<tr>
<td>vGLUT1</td>
<td>vesicular glutamate transporter 1</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
</tr>
</tbody>
</table>


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