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Review

What is the function of neuronal AP-3?

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Neurotransmission requires the proper organization and rapid recycling of synaptic vesicles. Rapid retrieval has been suggested to occur either by kiss-and-stay or kiss-and-run mechanisms, whereas classical recycling is mediated by clathrin-dependent endocytosis. Molecular coats are key components in the selection of cargos, AP-2 (adaptor protein 2) playing a prominent role in synaptic vesicle endocytosis. Another coat protein, AP-3, has been implicated in synaptic vesicle biogenesis and in the generation of secretory and lysosomal-related organelles. In the present review, we will particularly focus on the recent data concerning the recycling of synaptic vesicles and the function of AP-3 and the v-SNARE (vesicular soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) TI-VAMP (tetanus neurotoxin-insensitive vesicle-associated membrane protein) in these processes. We propose that AP-3 plays an important regulatory role in neurons which contributes to the basal and stimulated exocytosis of synaptic vesicles.

Introduction

SVs (synaptic vesicles) are highly specialized secretory organelles which undergo calcium-dependent exocytosis at synapses. After exocytosis, vesicles are rapidly internalized into nerve terminals for further cycles of exo–endocytosis (Ceccarelli et al., 1973; Heuser and Reese, 1973). Several mechanisms of vesicle retrieval, following fusion at the plasma membrane, have been proposed. We will not discuss in the present review the short routes of recycling (Figure 1), i.e. kiss-and-run and kiss-and-stay mechanisms (Galli and Haucke, 2004; Sudhof, 2004), but we will instead focus on the long routes of SV recycling. Two non-exclusive mechanisms of long recycling have been proposed. The first one involves clathrin-dependent endocytosis directly from the plasma membrane and the molecular coat AP-2 (adaptor protein 2). The second involves an endosomal intermediate and AP-3. In the present review, we will particularly focus on recent data on the function of AP-3 in the biogenesis of the SVs.

SV pools and their dynamics

SV pools have been investigated in synapses from several neuronal types: frog and fruit-fly NMJs (neuromuscular junctions), neonatal Calyx of Held neurons and neonatal rodent cultured hippocampal neurons (reviewed in Rizzoli and Betz, 2005). In cultured hippocampal neurons, each bouton contains 100–200 SVs (Schikorski and Stevens, 1997). On the basis of electron microscopy analysis, a small percentage of vesicles are in close apposition to the presynaptic membrane (referred to as 'docked vesicles'), whereas the remainder constitutes a vast homogeneous population clustered throughout. On the basis of electrophysiological recording, SVs can be classified into three different pools: the RRP (readily releasable pool), the 'recycling pool' and the 'reserve pool' (Figure 2). The RRP contains all the vesicles that are immediately available on stimulation.

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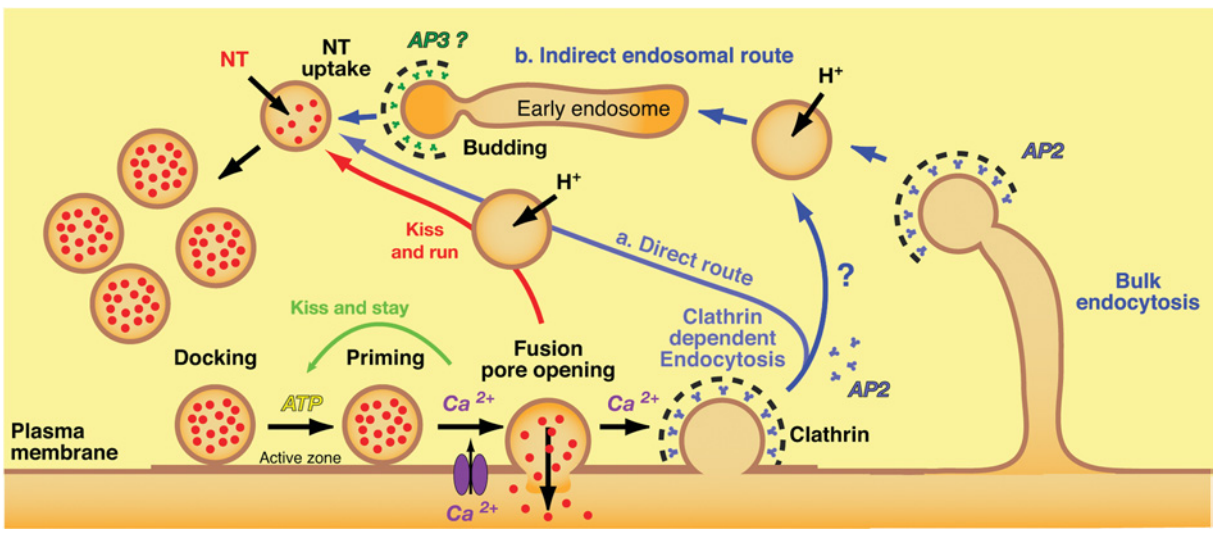
Key words: adaptor protein 3 (AP-3), *mocha*, mossy fibre, synaptic vesicle, tetanus neurotoxin-insensitive vesicle-associated membrane protein (TI-VAMP), vesicle recycling.
Abbreviations used: AP, adaptor protein; ARF, ADP-ribosylation-factor-binding protein; BFA, brefeldin A; BLOC, biogenesis of lysosome-related organelles complex; ClC-3, chloride channel 3; GABA, γ-aminobutyric acid; LAMP, lysosome-associated membrane protein; NMJ, neuromuscular junction; PI4K, phosphatidylinositol 4-kinase; SLMV, synaptic-like microvesicle; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; v-SNARE, vesicular SNARE; t-SNARE, target SNARE; SV, synaptic vesicle; TeNT, tetanus neurotoxin; TGN, *trans*-Golgi network; VAMP, vesicle-associated membrane protein; TI-VAMP, TeNT-insensitive VAMP; VGLUT1, vesicular glutamate transporter 1; VIAAT, vesicular inhibitory amino acid transporter; ZnT3, zinc transporter 3.

Figure 1 | SV cycle at the nerve terminal

SVs dock and are primed at the active zone. Upon stimulation, the SV fuses with the membrane allowing for neurotransmitters (NT) to be released in the synaptic cleft. If the SV does not fuse entirely with the membrane, but forms a transient fusion pore, it can be retrieved by the short routes of recycling, i.e. kiss-and-run (in red) and kiss-and-stay (in green) mechanisms. In the kiss-and-run mechanism, the SV is retrieved by endocytosis at the active zone and rapidly refilled with NT. In the kiss-and-stay mechanism the SV is rapidly refilled without leaving the active zone. If the SV fuses completely with the plasma membrane, then it is retrieved by clathrin-dependent endocytosis. Following strong stimulation, bulk endocytosis has been shown to occur at the nerve terminal and to form large membrane invaginations. Budding from the plasma membrane by clathrin-mediated endocytosis and budding on large invaginations were shown to be positive for AP-2 and clathrin. SVs are then uncoated and follow the direct route ('a'; light blue) to be refilled with NT, or the indirect route ('b'; dark blue) which passes through the endosome. AP-3 mediates budding of SVs from endosomes in PC12 cells.

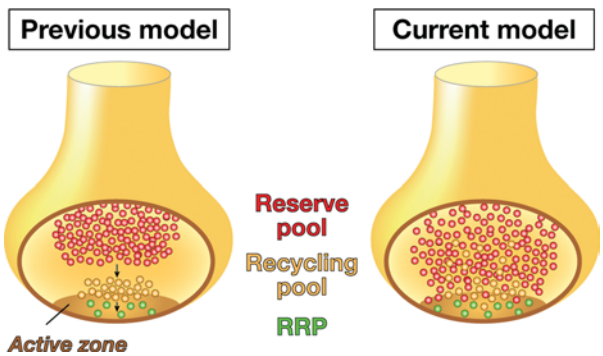


It is rapidly depleted by stimulation (high-frequency stimulation, depolarization or hypertonic shock). In hippocampal slices, Hanse and Gustafsson (2001a, 2001b, 2002) suggested that two vesicles per active zone were released during a short stimulus (10 action potentials at 50 Hz), whereas, on average, only one was immediately available for release. Stimulation by hypertonic shock can release around 5–20 SVs (Schikorski and Stevens, 2001). The recycling pool is the pool that maintains release upon physiological stimulation of moderate intensities. It comprises 10–20% of all of the vesicles (Harata et al., 2001a; reviewed in Harata et al., 2001b). Physiological stimulation allows for the continuous recycling and refilling of the vesicles. The reserve pool contains 80–90% of SVs. These vesicles are reluctant to undergo exocytosis during physiological activity and can be released very slowly in response to an intense or prolonged stimulation.

Until recently, it was assumed that these three SV pools were anatomically segregated in different areas, the RRP being the closest to the membrane, the reserve pool located far away from the active zone and the recycling pool remaining in between (Figure 2). Although it was shown that the SVs of the RRP are generally docked at the active zone, it appears that the recycling pool is actually scattered throughout the nerve terminals in hippocampal boutons (Harata et al., 2001a; reviewed in Harata et al., 2001b). At the frog NMJ, the reserve pool vesicle does not seem to be excluded from near-active zone sites (Rizzoli and Betz, 2004). Therefore, SVs of the RRP are generally docked and primed for release (Schikorski and Stevens, 2001), but some docked SVs are not available for release. The degree of discordance from morphologically docked vesicle and the RRP is not yet fully understood. Other dynamic exchanges do exist between the pools of SVs. For instance,

Figure 2 | SVs are classified in three different pools: RRP, recycling pool and reserve pool

The RRP contains all the vesicles that can be easily mobilized on stimulation. The recycling pool maintains release upon physiological stimulation of moderate intensities. The reserve pool contains the vesicles reluctant to undergo exocytosis under physiological activity. The reserve pool can be drawn on very slowly in response to intense or prolonged stimulation. The previous model proposed that these three SV pools were anatomically segregated in different clusters. It was recently shown that the recycling pool is actually scattered throughout the nerve terminals in hippocampal boutons (current model).



mixing between the RRP and the recycling pool is fast [seconds to minutes (Pyle et al., 2000)], whereas it is slow between the recycling and the reserve pool (Rizzoli and Betz, 2005). Finally, the recycling of the RRP and of the recycling pool is fast (seconds), whereas the recycling of the reserve pool vesicles is longer (minutes). Thus the different pools may reflect different routes of recycling. The precise molecular and physiological characteristics of the different pools of SVs have been studied for only a few synapses and are still lacking for most. Nevertheless, the available data already promote the discussion of several important properties of SV recycling. We will focus in the present review on the role of molecular coats and v-SNAREs (vesicular soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptors) in SV recycling and how they could regulate the dynamics of SV pools.

Clathrin and adaptors

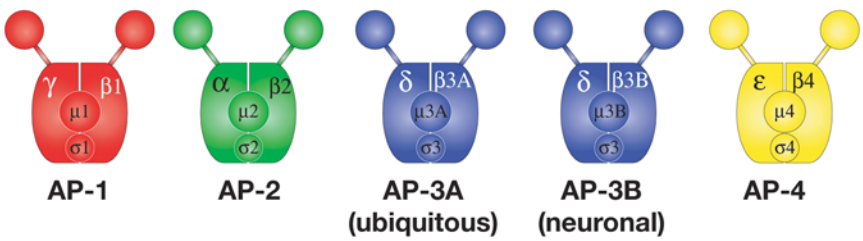
Molecular coats play an important role in the transport between eukaryotic intracellular compartments, particularly clathrin APs which associate with donor membranes and select cargo for inclusion into vesicles. Four adaptors (AP-1, AP-2, AP-3 and AP-4; Figure 3) have been described to date (Robinson, 2004). AP-1 and AP-2 are components of clathrin-coated vesicles. AP-1 is found on the TGN (*trans*-Golgi network) and endosomes, whereas AP-2 is found at the plasma membrane. AP-3 and AP-4 are not significantly enriched in brain clathrin-coated vesicles (Simpson et al., 1997; Hirst and Robinson, 1998), but AP-3 has been detected by proteomic analysis in HeLa cell clathrin-coated vesicles (Borner et al., 2006). Although the clathrin requirement for AP-3 function is still unclear (Simpson et al., 1996; Dell'Angelica et al., 1998; Peden et al., 2002), AP-4 has been localized to non-clathrin-coated vesicles in the area of the TGN (Hirst et al., 1999). AP-3 and AP-4 are localized on the TGN/endosomal membrane, with AP-3 being preferentially localized on endosomes and AP-4 on the TGN.

APs are heterotetrameric complexes formed by two adaptins (large subunits α, β1, β2, β3A, β3B, δ, ε and γ), a medium-sized subunit (μ1A, μ1B, μ2, μ3A, μ3B and μ4) and a small subunit (σ1, σ2, σ3 and σ4) (Figure 3). For example, AP-2 is a heterotetrameric complex composed of α and β2 adaptins, together with μ2 and σ2 subunits. The subunits β3, μ1 and μ3 exist in two isoforms (A and B), which in some cases have cell-specific expression. Indeed, β3A, μ1A and μ3A are ubiquitous, whereas β3b, μ3b are brain-specific and μ1B is present in polarized epithelial cells. Thus the AP3-B complex refers to the neuronal AP-3 complex, which comprises the δ, β3B, μ3B and σ3 subunits. The β subunits are important for clathrin binding and cargo selection – directly in the case of β1 sorting of dileucine-based signals (Rapoport et al., 1998) and indirectly through the binding of clathrin-associated sorting proteins, such as ARH (autosomal recessive hypercholesterolaemia) and β-arrestin in the case of β2 (Edeling et al., 2006). The μ subunits have been implicated to be involved in cargo selection by direct binding (Schmid, 1997). The clathrin-binding site on the AP-3β subunit is also conserved, but there are contradictory reports as to whether AP-3 binds clathrin *in vivo* (Simpson et al., 1997; Dell'Angelica et al., 1998). However, it should be noted that electron microscopy revealed that AP-3 is associated with early endosome tubules and it sometimes co-localized with clathrin (46%) to a smaller extent than it does to AP-1 (91%)

Figure 3 | Subunit composition of APs

APs are composed of two adaptins (one β plus α, δ, ε, or γ), one medium subunit (μ) and one small subunit (σ). The AP-3 complex exists in two isoforms: one is ubiquitous (AP-3A), whereas the other one is specific to neuronal cells (AP-3B).

	Subunits				Type of Vesicle
	large (also called Adaptins)		medium	small	
AP-1	γ	β1	μ1 A (ubiquitous) μ1 B (epithelial)	σ1	Clathrin-coated
AP-2	α	β2	μ2	σ2	Clathrin-coated
AP-3	δ	β3A (ubiquitous) β3B (neuronal) [β-NAP]	μ3 A (ubiquitous) [p47A] μ3 B (neuronal) [p47B]	σ3	Conflicting results
AP-4	ε	β4	μ4	σ4	No clathrin



(Peden et al., 2004). The μ subunits are able to bind to the sorting signal motif YXXΦ (where Φ is a bulky hydrophobic residue) present in the cytoplasmic tails of some transmembrane proteins (Ohno et al., 1995, 1998; Owen and Evans, 1998). This motif can act as an internalization signal at the plasma membrane (in the transferrin receptor) or as an intracellular sorting signal [in LAMP-1 (lysosome-associated membrane protein 1)] (Robinson, 2004). Another type of motif which consists of a pair of leucine residues preceeded by one or more acidic residues [(D/E)XXXL(L/I)] is found mainly in resident proteins of endosomes, lysosomes and related organelles (Bonifacino and Traub, 2003; Janvier et al., 2003).

Molecular mechanism of SV recycling: involvement of endosomal intermediates?

As stated above, two clathrin-dependent mechanisms have been described so far to explain the recycling of SVs. The first mechanism involves direct endocytosis from the plasma membrane and requires the AP-2 complex (Figure 1). This mechanism is thought to ensure the recovery of the recycling pool upon physiological stimulation. When mammalian synapses are subjected to prolonged stimulation (for example, a high depolarizing potassium concentration), slow endocytosis proceeds through the formation of invaginations and involves endocytic intermediates (Takei et al., 1996; de Lange et al., 2003). This may represent the recycling of the reserve pool by bulk endocytosis (Rizzoli and Betz, 2005). Stimulation of brain slices (containing the calyx of Held synapses) with a high concentration potassium solution in the presence of horseradish peroxidase (to label endocytic compartments) resulted in a reduction of the number of SVs and in the appearance of labelled endosomes within several minutes. After returning to a solution of normal concentration, endosomal structures disappeared, whereas the number of SVs increased (de Lange et al., 2003). Similar results have been obtained in cultured hippocampal neurons in which clathrin-coated buds were identified in both the plasma membrane and in intraterminal membrane profiles that had the characteristics of tubulovesicular early endosomes (Takei et al., 1996). Immunocytochemical experiments allowed the identification of clathrin, AP-2 and AP-180 in these buds. Serial section analysis revealed that some endosomal-like profiles were connected to the cell surface. Thus a new model for SVs has emerged (De Camilli and

Takei, 1996; Takei et al., 1996; Cremona and De Camilli, 1997) (Figure 1) and reconciled previous data indicating both bulk, non-selective endocytosis by large vacuoles or selective clathrin-mediated endocytosis. Non-selective bulk endocytosis may prevent the expansion of the cell surface when clathrin-mediated endocytosis is limited after strong stimulation (Murthy and De Camilli, 2003). Thus most SVs may recycle directly without passing through an endosomal intermediate. However, as suggested previously (Sudhof, 2004), several lines of evidence suggest that the endosomal pathway is physiologically relevant for SV recycling under some conditions. Indeed, several proteins involved in endosomal fusion, VAMP4 (vesicle-associated membrane protein 4), Vti1aβ and Rab5, are present in SVs (Fischer von Mollard et al., 1994; Antonin et al., 2000; Rizzoli et al., 2006; Takamori et al., 2006). Furthermore, the fact that endosomes are rarely observed in nerve terminals at a resting state could be explained by the transient nature of these organelles (Wucherpfennig et al., 2003). It could thus be envisioned that SVs generated by endosomes use specific APs to operate.

AP-3 functions and mutants

AP-3 decorates budding profiles on early-endosome-associated tubules (Peden et al., 2004) and is involved in trafficking to late endocytic/lysosomal compartments in non-neuronal cells (Bonifacino and Traub, 2003). AP-3-deficient cultured cells show a missorting defect of certain lysosomal proteins (LAMP-1, LAMP-2, LIMP-2 and CD63) at the level of plasma membrane (Le Borgne et al., 1998; Dell'Angelica et al., 1999, 2000; Peden et al., 2004). The late-endosomal v-SNARE TI-VAMP [TeNT (tetanus neurotoxin)-insensitive VAMP] is also mislocalized to early endosomes in AP-3-deficient cultured cells (Martinez-Arca et al., 2003). Orthologues of AP-3 have been found in the budding yeast *Saccharomyces cerevisiae* and the fruit fly *Drosophila melanogaster* (Table 1). Deletion of AP-3 in yeast results in the mislocalization of the vacuolar proteins alkaline phosphatase and the t-SNARE (target SNARE) Vamp3p – the yeast vacuole being equivalent to the mammalian lysosome (Cowles et al., 1997; Stepp et al., 1997). AP-3 *Drosophila* mutants are eye-colour mutants (*garnet*, *ruby*, *carmine* and *orange*) which belong to the granule group of genes. The *garnet* mutant is characterized by pale eye colour due to a decrease of pigment in granules contained in pigmental cells around the photoreceptors. A major pathway towards the vacuole in the yeast is defined by the VPS mutants [vacuole protein sorting; VPS18 (deep orange), VPS33 (carnation), VPS41 (light)], which are homologues of the *Drosophila* granule gene group. These genes could be involved in trafficking to lysosomes and related organelles (Lloyd et al., 1998). AP-3 has also been implicated to be involved in trafficking of the tyrosinase enzyme, the key enzyme in melanin synthesis, to melanosomes (Huizing et al., 2001; Theos et al., 2005). Again, melanosomes are lysosome-related organelles that co-exist with lysosomes in melanocytes and retinal pigment epithelial cells (Raposo and Marks, 2002). AP-3 has also been implicated in mouse mutants with abnormal hair and eye colour. Mouse coat-colour mutants exhibit defects in synthesis and storage of melanin pigments and prolonged bleeding times due to a platelet-storage-pool deficiency, a disorder caused by decreased numbers of platelet-dense granules. The *mocha* mice (Table 1) are homozygous for a null allele of AP-3δ. They show only trace amounts of any other AP-3 subunits, suggesting that AP-3δ is necessary to the complex stability. *mocha* mice have reduced secretion of lysosomal enzymes in urine and prolonged bleeding, but also neurologic disorders, including seizure, hyperactivity and specific loss of zinc (Kantheti et al., 1998). AP-3δ interacts with the v-SNARE TI-VAMP (Martinez-Arca et al., 2003), and the mislocalization of AP-3δ using dominant-negative form of TI-VAMP has also been shown to be associated with impaired lysosomal secretion (Proux-Gillardeaux et al., 2007). The *pearl* mice have a reduced expression of the ubiquitous AP-3 β3A subunit (Feng et al., 1999). These mice have also colour defects, abnormal lysosomes,

Table 1 | Natural AP-3 mutants and associated diseases in human

AP-3 subunit	Mutants in mouse	Associated disease in humans	Mutants in *Drosophila*
δ	*mocha* (*mh*)	–	*garnet*
β3A	*pear* (*pe*)	Hermansky–Pudak syndrome 2 (HPS2)	*ruby*
μ3	–	–	*carmine*
σ	–	–	*orange*

melanosomes and platelet granules, but apparently no neurological defects. Humans with HPS (Hermansky–Pudlak syndrome) share some of the characteristics of the *mocha* and *pearl* phenotypes: pigment dilution, bleeding disorder and lysosome abnormalities (Jackson, 1998). These manifestations arise from defects in the biogenesis of lysosome-related organelles, including melanosomes and platelet-dense granules. Approx. 12 genes have been implicated in lysosome-related diseases in men and mice. Some of these mutant proteins are components of BLOC (biogenesis of lysosome-related organelles complex)-1, -2 and -3 (Dell'Angelica, 2004). It has been shown that BLOC-1 interacts physically with AP-3 and BLOC-2 (Di Pietro et al., 2006), and that BLOC-1 and AP-3 deficiencies affect the targeting of LAMP1, PI4KIIα (phosphatidylinositol 4-kinase IIα) and TI-VAMP (Salazar et al., 2006; Newell-Litwa et al., 2007). Thus AP-3 may have a complex function that may vary between cell types: biogenesis of lysosome-related organelles in non-neuronal cells and regulation of exocytic organelles and neuronal activity in neurons. AP-3 appears as a good candidate to regulate SV formation at the level of endosomes.

Function of the neuron-specific AP-3 complex

An intriguing feature of AP-3 is that it contains two subunits which are neuron-specific (β3b and μ3b). The AP-3A (δ, β3A, μ3A and σ3) complex is ubiquitous, whereas the AP-3B (δ, β3B, μ3B and σ3) is neuron-specific. *In situ* hybridization and immunochemistry have shown that AP-3δ and AP-3β3B are expressed in the granule cell layer of the dentate gyrus and hippocampal pyramidal cell layer (Newman et al., 1995; Kantheti et al., 1998). AP-3β3B is detected in neuronal soma and nerve terminals as soluble and membrane-bound pools (Newman et al., 1995). The *mocha* mice which do not have AP-3δ, lack both AP-3A and AP-3B, because the δ subunit is used in both isoforms (Kantheti et al., 1998). *mocha* mice showed inner-ear degeneration, leading to balance problems, deafness, abnormal theta rhythms and seizures (Kantheti et al., 1998). They also exhibited a neurological phenotype that included hyperactivity, an epileptiform EEG (electroencephalogram) and changes in the basic function of the hippocampus (Miller et al., 1999). Kantheti et al. (1998) also showed that *mocha* brains have a dramatically decreased staining for ZnT3 (zinc transporter 3), which results in a lack of zinc in presynaptic vesicles in the neocortex and the hippocampus. *pearl* mice, which lack only β3A and therefore AP-3A, still have the neuronal isoform AP-3B and have no obvious neurological abnormalities. Thus the neuronal disorders (deafness, balance problems and seizure) found in *mocha* mice probably result from AP-3B depletion. In order to investigate the role of AP-3B in detail, different strains of mutant mice have been established. AP-3μ3B-deficient mice have been established by Nakatsu et al. (2004). These mice showed spontaneous seizure, but no deafness or balance problems, indicating that these phenotypes were caused by AP-3A/AP-3B double deficiency (Nakatsu and Ohno, 2003). Seong et al. (2005) used β3A- [established by (Yang et al., 2000)] and β3B-deficient mice, and showed that the lack of β3A or β3B does not perturb the assembly of the other adaptor isoforms. Ubiquitous and neuronal AP-3 distribute similarly in hippocampal neuron cell bodies; however, the neuronal AP-3β3B is approx. 4-fold more abundant than the ubiquitous AP-3β3A in axons and dendrites. Thus AP-3B plays an important neuronal function which is impaired in *mocha*, $\mu 3B^{-/-}$ and $\beta 3B^{-/-}$ mice.

AP-3 functions in SLMVs (synaptic-like microvesicles) in PC12 cells and SVs in neurons

Several important studies on the function of AP-3 in SV formation have used the pheochromocytoma cell line PC12 (Faundez et al., 1997). These neuro-endocrine cells contain small SLMVs that have the usual array of SV membrane proteins, but exclude membrane proteins found in other organelles (Clift-O'Grady et al., 1990). To specifically study the recycling and the formation of these SLMVs, an *in vitro* assay was set up to reconstitute the formation of SLMVs from cell lysates (Desnos et al., 1995). Using this assay it was shown that depletion of AP-3 from brain cytosol inhibits SLMV formation from PC12 endosomes *in vitro*. Budding from washed membranes could be reconstituted with purified AP-3 and the small recombinant GTPase ARF1 (ADP-ribosylation-factor-binding protein 1) (Faundez et al., 1998). However, depletion of clathrin, AP-2 and dynamin from brain cytosol had no detectable effect on SLMV biogenesis (Faundez et al., 1997). In PC12 cells, the AP-3-dependent pathway

of SLMV formation was then shown to occur from endosomes, and not from the plasma membrane (Lichtenstein et al., 1998). On the contrary, the AP-2-dependent pathway uses the plasma membrane as a precursor of SLMV (Schmidt et al., 1997) and is BFA (brefeldin A)-insensitive. The AP-2-dependent pathway is inhibited by depletion of clathrin, whereas the AP-3-dependent pathway is not (Shi et al., 1998). The sorting of an exogenous mutant (N49A) of the v-SNARE synaptobrevin 2 into SLMVs was shown to be dependent on AP-3 at the level of endosomes and on AP-2 and clathrin at the plasma membrane (Shi et al., 1998). The targeting of endogenous synaptobrevin 2 is, however, normal in brains of *mocha* flies (Salazar et al., 2004a; Scheuber et al., 2006; Newell-Litwa et al., 2007), thus sorting of synaptobrevin 2 does not probably rely on the presence of AP-3. ClC-3 (chloride channel 3) and ZnT3 were also shown to be packed into the same SLMVs along with AP-3 (Salazar et al., 2004a), whereas synaptophysin was concentrated in different vesicles (Salazar et al., 2004b). These results were confirmed in brain tissue, because both ZnT3 and ClC-3 targeting to SVs was impaired in the absence of the neuronal AP-3B, whereas ZnT3 was increased 2-fold in the absence of the ubiquitous AP-3A (Seong et al., 2005). VGLUT1 (vesicular glutamate transporter 1) is also co-targeted with ZnT3 into SLMVs (Salazar et al., 2005a). The amount of ZnT3 in neuronal SVs is significantly decreased in *mocha* mice in contrast with a slight decrease of VGLUT1. Furthermore, the slight decrease of VGLUT1 is not detectable in *AP-3B*$^{-/-}$ mice (Salazar et al., 2005a). More recently, a membrane fraction enriched in vesicles containing ZnT3 was generated from PC12 cells and was used to identify new components of these organelles by mass spectrometry (Salazar et al., 2005b). PI4KIIα was shown to be enriched in this fraction, and its localization was strongly reduced in nerve terminals of *mocha* hippocampal MFs (mossy fibres). PI4KIIα has thus been proposed to be an AP-3 cargo, similar to ZnT3, ClC-3 and VGLUT1. Interestingly, the down-regulation of PI4KII also triggers a decrease in the intensity of the AP-3 staining (Salazar et al., 2005b). Therefore AP-3 has an important function in generating SLMVs in a BFA-sensitive, ARF1-dependent, clathrin-independent manner and in the sorting of ZnT3, ClC-3 and VGLUT1. To what extent this applies to neurons is still a question, because PC12 cells do not have axons and nerve terminals, the actual site where SVs fuse and are recycled in neurons.

Recent advances in AP-3 function in neuronal SVs

Taking into account the studies on PC12 cells demonstrating a role of AP-3 in SLMV biogenesis and the data on *mocha* mice indicating a role in SV formation, it has been postulated that AP-3B plays a role in SV biogenesis. This hypothesis in neurons has been confirmed by three studies.

Using AP-3 μ*3B*$^{-/-}$ mice, Nakatsu et al. (2004) have shown that hippocampal inhibitory SVs are defective in both morphology and function, indicating that AP-3B plays an essential role in normal synaptic function *in vivo* by regulating the biogenesis of at least a subset of SVs. In the hippocampus, the density of SVs was lower in both excitatory and inhibitory terminals, and the diameter of inhibitory terminals was also reduced. μ*3B*$^{-/-}$ mice suffered from epileptic seizures, and impairment of GABA (γ-aminobutyric acid) release, because of a reduction of the VGAT (vesicular GABA transporter)/VIAAT (vesicular inhibitory amino acid transporter). The levels of synaptophysin, Syb2 and VGLUT1/2 are, however, not affected. Basal GABA or glutamate release was unaffected, but the K^+-evoked release of GABA was impaired, which facilitated the propagation of excitation and the induction of LTP (long-term potentiation). This confirms that the AP-3B-dependent SV formation is of physiological importance.

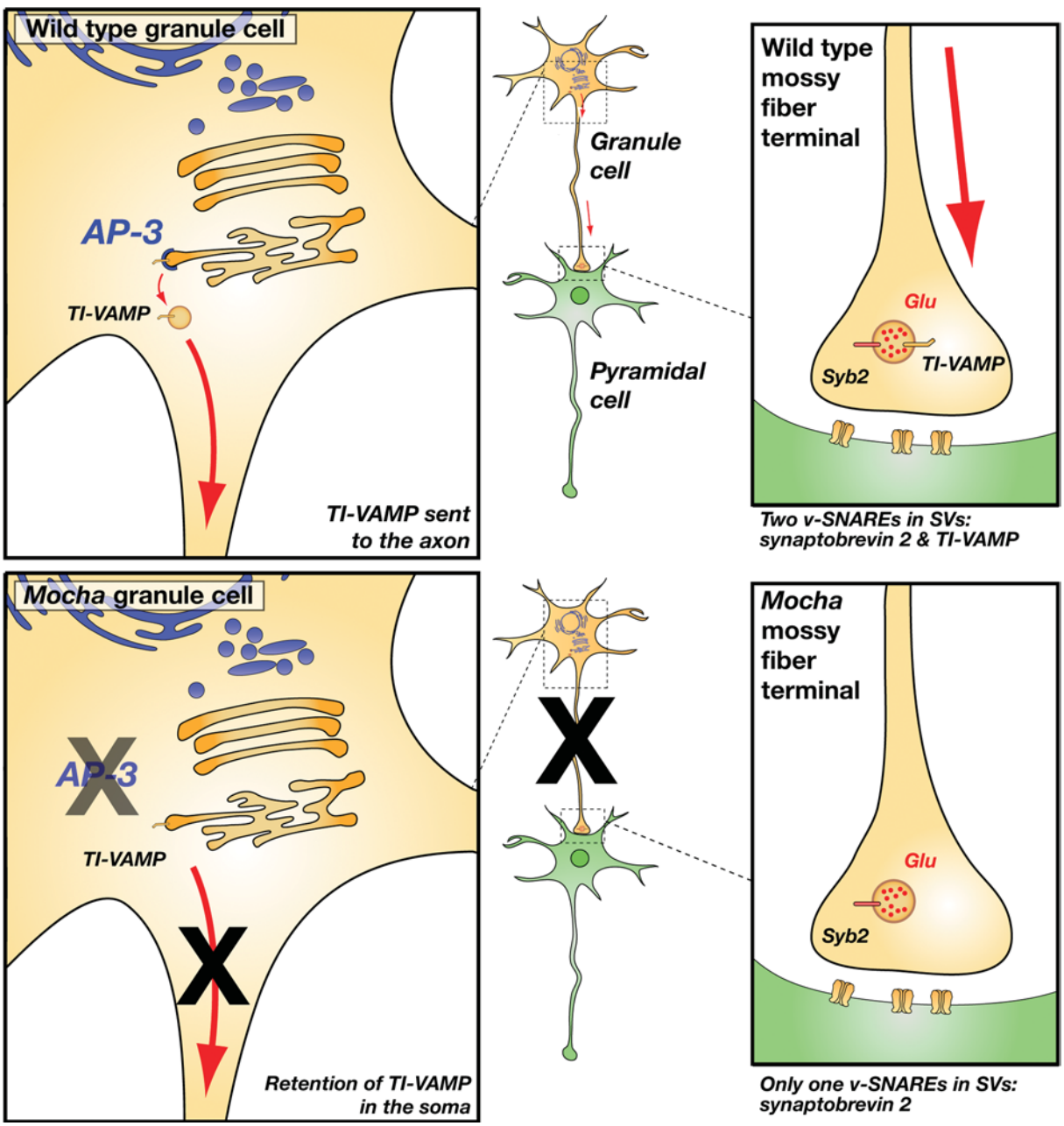
Furthermore, Voglmaier et al. (2006) showed that VGLUT1 can be endocytosed by two pathways, one involving AP-2 and the other one mediated by AP-3. VGLUT1 interacts directly with endophilin, a component of the clathrin-dependent endocytosis via its proline-rich C-terminus. This interaction allows VGLUT1 to recycle more quickly during sustained stimulation. Acceleration of VGLUT1 AP-mediated internalization by endophilin depends on a dileucine-like motif. The sorting of dileucine-like signals may depend on either AP-2 or AP-3. To study VGLUT1 trafficking in real-time, Voglmaier et al. (2006) fused the pHluorin into the large lumenal loop of the transporter, and monitored exocytosis and endocytosis by measuring the fluorescence intensity after stimulation. In the presence of BFA, the recycling of the VGLUT1-ΔPP2 (VGLUT1 without the polyproline motif) is restored to the kinetics of the wild-type protein. These results suggest that BFA diverts VGLUT1

from a slow pathway, most probably mediated by AP-3, to a faster pathway that is likely to involve AP-2 via its interaction with endophilin. Experiments using the *mocha* mice also showed an increased level of transmission during prolonged stimulation. Furthermore, BFA reduces the extent of endocytosis for both wild-type VGLUT1 and VGLUT1-ΔPP2 after stimulation, showing that the slow recycling pathway involving AP-3 contributes to compensatory endocytosis (Voglmaier et al., 2006).

In addition, we have recently suggested an important function of AP-3 in regulating SV exocytosis at hippocampal MF terminals (Scheuber et al., 2006). In hippocampal neurons, two v-SNAREs are known to co-exist in MF terminals: synaptobrevin 2 and TI-VAMP (Martinez-Arca et al., 2000; Proux-Gillardeaux et al., 2005; Galli et al., 2006). The presence of TI-VAMP was not obvious in preparation of SVs (Coco et al., 1999), probably because TI-VAMP is present only in a subset of terminals (Muzerelle et al., 2003). However, the presence of TI-VAMP and AP-3 in SVs has been confirmed by proteomics analysis of the content of rat brain SVs (Takamori et al., 2006). We took advantage of the sensitivity of synaptobrevin 2 to TeNT to distinguish between synaptobrevin-2- and TI-VAMP-dependent secretory components. The v-SNARE TI-VAMP is expressed, together with synaptobrevin 2, in control MF terminals of granule cells that synapse on to CA3 pyramidal cells (Muzerelle et al., 2003). In *mocha* mice, however, TI-VAMP is absent from MF terminals, and this absence correlates with the loss of an asynchronous release which is TeNT-insensitive. Immunofluorescence along with electron microscopy experiments show that TI-VAMP is blocked in the cell body of *mocha* neurons, whereas the pattern of synaptobrevin 2 is unchanged (Figure 4). This indicates that an AP-3-dependent evoked release exists at MF–CA3 synapses and probably involves TI-VAMP as a v-SNARE. We then investigated whether the lack of AP-3 and TI-VAMP may also impact the calcium-independent constitutive release at MF terminals. The frequency of mEPSCs (miniature excitatory postsynaptic currents) was 2-fold higher in *mocha* mice brain slice compared with control slices, whereas the kinetics of fusion pore opening and the number of postsynaptic receptors on CA3 pyramidal cells appeared normal. After cleavage of synaptobrevin 2 by incubating the slices with TeNT, the mEPSC frequency was reduced by 84% in control and by only 44% in *mocha* cultured slices. These results suggest that the absence of AP-3 also reduces the effect of TeNT. Quantification by Western blotting showed that there was 2-fold increase in synaptobrevin 2 that had resisted cleavage by TeNT in *mocha* cultures compared with control. It has been shown that synaptobrevin 2 molecules in assembled SNARE complexes, which may correspond to complexes clamped by complexins *in vivo* (Tang et al., 2006), are TeNT-resistant (Hayashi et al., 1994). Thus we hypothesized that the lack of AP-3 and TI-VAMP in MF SVs could increase the capacity of synaptobrevin 2 to form TeNT-resistant clamped SNARE complexes, thereby enhancing the probability of calcium-independent fusion at *mocha* MF terminals. This hypothesis if further supported by our observation, in *mocha* mice, of the effect of an hypertonic sucrose solution, a treatment known to recruit massively the RRP (Rosenmund and Stevens, 1996) and to stimulate secretion in a calcium-independent manner. Indeed, quantal release evoked by hyperosmotic sucrose was increased in *mocha* cultures. On the basis of these results, we proposed that the AP-3-dependent mechanism decreases the sucrose sensitivity of constitutive secretion. In *mocha* mice, more SVs would be ready to fuse, because of the lack of AP-3 and TI-VAMP (Figure 5). Furthermore, because no other TeNT-resistant v-SNARE was found to concentrate at MF terminals, we suggested that TI-VAMP mediates the asynchronous evoked release as discussed above. Asynchronous release was not observed in TeNT-untreated explants, suggesting that the inactivation of synaptobrevin 2 may be required for the expression of this asynchronous evoked release. In the CA1, however, asynchronous evoked release can be observed and TeNT blocks both synchronous and asynchronous evoked responses. TI-VAMP is absent from CA1 nerve terminals, which suggests that synaptobrevin 2 mediate both synchronous and asynchronous components at these synapses. Our findings are consistent with a scenario where TI-VAMP and synaptobrevin 2 would be present on the same SVs, because otherwise asynchronous evoked release at MFs would have occurred also in the absence of TeNT. Therefore, neurotransmitter release at MF terminals would be ensured by two exocytic v-SNAREs, synaptobrevin 2 and TI-VAMP, and this complexity would have important physiological consequences (Scheuber et al., 2006).

Figure 4 | Targeting of TI-VAMP in wild-type and *mocha* mice

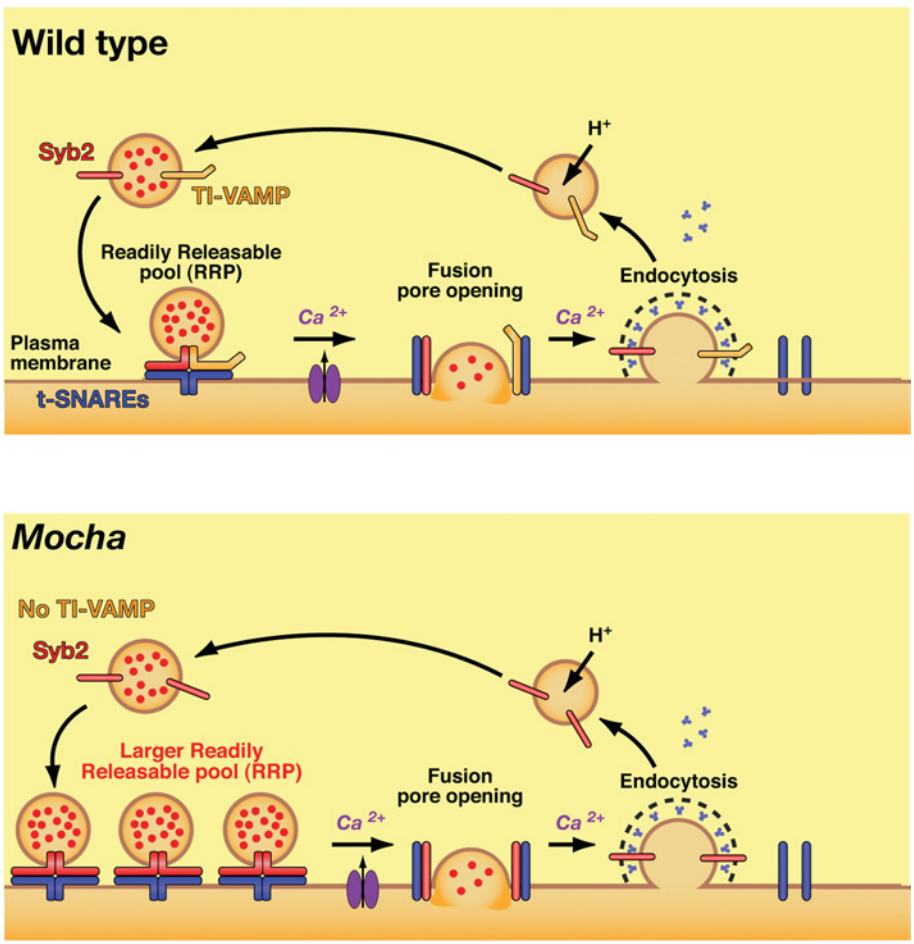
TI-VAMP is expressed in granule cells of rodent dentate gyrus. In wild-type mice, which express AP-3, TI-VAMP is concentrated in nerve terminals contacting on to CA3 pyramidal cells (so-called MF terminals). Thus MF SVs contain two v-SNAREs: synaptobrevin 2 (Syb2) and TI-VAMP. In *mocha* mice, which do not express AP-3, TI-VAMP is blocked in granule cell soma and is absent from MF SVs. We propose that AP-3 is necessary for the export of TI-VAMP from the soma to the SVs present in the axon terminal. Glu, glutamate.



In conclusion, the study of the *mocha* brain confirmed that TI-VAMP is an important cargo of AP-3. It supports the view that AP-3 regulates neurotransmitter release. As several AP-3 cargoes have now been identified, and that all of them have been shown to be absent in MF terminals, we therefore conclude that

Figure 5 | Models of v-SNARE recycling in wild-type and *mocha* mice

In wild-type mice, TI-VAMP and Syb2 are present in SVs. In *mocha* mice, SVs are devoid of TI-VAMP. A TeNT-resistant asynchronous evoked release is lost in *mocha* MF terminals. This component is likely mediated by TI-VAMP. The basal release and the RRP are larger in *mocha* MF terminals. We propose that the presence of TI-VAMP in MF SVs provides a regulation affecting both basal and evoked release. TI-VAMP has a lower capacity to assemble into SNARE complexes than synaptobrevin 2 (Syb2) (Martinez-Arca et al., 2003). This property could, at least in part, be responsible for a weaker docking capacity of control SVs containing TI-VAMP that could lead to smaller RRP in control MF terminals. For simplicity, we only show the clathrin-dependent endocytosis route of recycling (so-called direct route in Figure 1). To date, information is still missing for the different routes of SV recycling in *mocha* MF terminals.



AP-3 is important to target essential regulators to the synapse: vesicular transporters (ZnT3, VGLUT and VIAAT), a kinase (PI4KIIα) and a v-SNARE (TI-VAMP). By regulating the targeting of several proteins, AP-3 is likely to influence both constitutive and evoked release, as well as compensatory endocytosis at central synapse. There are several questions remaining. Does AP-3 function at the level of the TGN or endosomes in the soma? Is AP-3 active in presynaptic terminal at top of the cell body? What explains the specific function seen in MFs given the fact that pyramidal CA3 and CA1 neurons also express AP-3 and its cargos (VGLUT1 and TI-VAMP)? What causes the increased basal release in the absence of AP-3? Further functional and morphological approaches will be needed to answer these questions. Electron microscopy at the level of MF terminal will be very useful to understand if AP-3 is present at the terminal and will provide valuable information to unravel the precise mechanism of AP-3 in neuronal cells.

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