The role of presynaptic proteins in maintaining neuronal viability

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The role of Munc18-1 in the spatial separation of APP and BACE1

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Abstract

Spatial separation of amyloid precursor protein (APP) and beta-site APP cleaving enzyme 1 (BACE1) is critical in preventing amyloidogenic processing of APP by BACE1, a key step in the development of Alzheimer's disease (AD) pathology. Here we investigated the role of the presynaptic protein Munc18-1 in the spatial separation of APP and BACE1. We showed that in addition to the reported interaction between Munc18-1 and APP, Munc18-1 also binds to BACE1 when co-expressed in HEK293 cells. In these cells, Munc18-1, BACE1, APP and Mint-1 form a molecular complex. Cyclin-dependent kinase 5 (cdk5) phospho-mimicking and phosphorylation-resistant forms of Munc18-1 both interacted with APP and BACE1. We confirmed the interaction between Munc18-1 and BACE1 in human brain lysate. Furthermore, APP levels were increased in Munc18-1 knockout (KO) mouse brain and during postnatal development in Munc18-1 heterozygote mice. Together, these findings lead us to propose a model in which Munc18-1 enables the formation of a molecular complex, consisting of Munc18-1, APP, BACE1 and Mint-1, independent of Munc18-1 phosphorylation by cdk5. Our data also suggest that Munc18-1 plays a role in the amyloidogenic processing of APP, which may impact the development of pathology related to AD.

Introduction

Alzheimer's disease (AD) is the most common form of dementia. AD is characterized by aggregation of amyloid beta (Aβ) peptides that form extracellular plaques. Aβ peptides are produced by the sequential cleavage of amyloid precursor protein (APP) by β-secretase that cleaves the APP extracellular domain and γ-secretase that cleaves the transmembrane domain[11]. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is the β-secretase that is responsible for the proteolytic cleavage of APP[12,322,323]. The formation of Aβ can be precluded by the alternative cleavage of APP's extracellular domain by α-secretase[13,324,325]. Therefore, the spatial convergence of APP and BACE1 is a critical step in the amyloidogenic processing of APP. However, the mechanism underlying the spatial separation of APP and BACE1 remains unclear.

It was previously suggested that APP binds the presynaptic protein Munc18-1[24]. Munc18-1 is essential for synaptic vesicle (SV) exocytosis in neurons[27]. Overexpression of Munc18-1 interacting protein (Mint) potentiates the retention of APP and reduces Aβ levels in HEK293 cells[326,327]. Munc18-1 synergistically enhances the inhibitory effect of Mint on APP processing in HEK293 cells[326]. Munc18-1 and Mint have been suggested to play a role in the spatial separation of APP and BACE1[24], by proposing that in resting neurons APP associates with syntaxin-1 microdomain through Munc18-1 and Mint. Cyclin-dependent kinase 5 (cdk5) is a neuronal kinase that forms a complex with its activator p35, which is anchored to the plasma membrane through its N-terminus[328-331]. Phosphorylation of Munc18-1 by cdk5 reduces APP-syntaxin-1 association, and increases APP-BACE1 co-localization[24]. Thus, the phosphorylation state of Munc18-1, together with its binding to syntaxin-1, might be important for the mechanism underlying APP-BACE1 spatial separation.

Interestingly, the levels of syntaxin-1, Munc18-1, Mint-1, cdk5 and p35 are elevated in parietal and occipital cortex of AD subjects[22]. Munc18-1 has been suggested to be upregulated as a protective mechanism to decrease Aβ production[22]. However, in frontal cortex, levels of Munc18-1 were reduced and changes in syntaxin-1 levels were not replicated[21,193]. Interestingly, lower levels of the long splice variant of Munc18-1 in inhibitory presynaptic terminals may contribute to cognitive decline[332].

Therefore, the aim of this study was to investigate the role of Munc18-1 in the spatial separation of APP and BACE1. In this study we used HEK293 cells, Munc18-1 wild-type (WT), heterozygous (HZ) and knockout (KO) E18 brain lysates and human brain tissue and performed co-immunoprecipitation and protein quantification by Western blotting. We identified an interaction between Munc18-1 and BACE1 in HEK293 cells and confirmed this interaction in human brain tissue. Furthermore, phosphorylated and non-phosphorylated forms of Munc18-1 were found to bind APP and BACE1 in HEK293 cells. APP levels were increased in Munc18-1 KO mouse brain lysate. Together, these data indicate that, independent of the phosphorylation state, Munc18-1 can facilitate the formation of a molecular complex consisting of Munc18-1, APP, BACE1 and Mint-1. In addition, our data suggest a role of Munc18-1 in regulating APP levels.

Material and Methods

Animals

Munc18-1 KO mice were generated as described previously[27]. Embryonic day 18 (E18) embryos were obtained by caesarian section of pregnant females from timed mating of Munc18-1 heterozygous mice. Munc18-1 KO mice are still born and can be easily distinguished from wild-type and heterozygous littermates. Animals were housed and bred according to the Institutional, Dutch and US governmental guidelines.

Constructs

The following lentiviral constructs were used: APP-mCherry (human wt v3); BACE1-mCherry (wt human v1); Munc18-1 WT; pM18(T574A); pM18(T574A)CMV-3Tag-1; pM18(T574D); pM18(T574D)CMV-3Tag-1clenti; pm18-1(T574P); p35-EGFP; CDK5-EGFP; EGFP-Mint (gift from A. Maximov (The Scripps Research Institute, La Jolla, CA, USA)).

HEK293 cell culture, transfection and protein extraction

HEK293 cells were cultured on plastic culture dishes in Dulbecco's modified Eagle's medium + Glutamax (DMEM+) (Life Technologies) supplemented with 10% FCS, 1% Pen Strep and 1% NEAA (Life Technologies). Cells were transfected at ~ 70% confluence. Medium was changed to Opti-MEM (Life Technologies) 1h before transfection. 200 µL calcium phosphate-DNA precipitate (250 mM $CaCl_2.H_2O$, 2 x HEPES-buffered saline), containing a maximum of 4 µg of DNA, was added per well. Cells were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2. After 5-6h, medium was changed to DMEM+ and cells were returned to the incubator for 2 days. HEK293 cells were lysed 3 days after transfection. After being washed twice with phosphate-buffered saline (PBS), cells were collected and centrifuged for 5 min at 500g at 4°C and lysed in 0.1% Triton X-100 in PBS supplemented with protease inhibitors (PI) (Sigma). After 1h of gentle mixing, lysate was centrifuged for 20 min at 10.000g at 4°C and 5x Laemmli Sample Buffer (LSB) was added to the supernatant.

Co-immunoprecipitation

Co-immunoprecipitations were performed at 4°C. HEK293 cells were lysed in lysis buffer (50 mM Tris pH 7.5, 1% Triton X-100, 1,5 mM $MgCl_2$, 5 mM EDTA, 100 mM NaCl) containing PI (Sigma). After centrifugation (13.2-14.0 krpm), supernatants were incubated with primary antibodies with gentle shaking for 2h. Munc18-1 was immunoprecipitated using a monoclonal anti-Munc18 antibody (clone # 610336, BD Transduction Laboratories) or a polyclonal anti-Munc18 antibody (house made), flag-tagged munc18-1 was immunoprecipitated using a monoclonal anti-flag antibody (clone # F1804, Sigma). APP-mCherry and BACE1-mCherry were immunoprecipitated using a monoclonal anti-mCherry antibody (clone # T515, Signalway Antibody) or a polyclonal anti-mCherry antibody (clone # GTX128508, GeneTex). eGFP-Mint1 was immunoprecipitated using a polyclonal anti-GFP antibody (clone # ab290/50, Abcam). Antibody-bound protein complexes were pulled down using blocked protein-A agarose beads (Sigma). Pellets were washed 5 times with wash buffer, alternating between low

and high salt (50 mM Tris pH 7.5, 0.1% Triton X-100, 1,5 mM $MgCl_2$, 5 mM EDTA, 100 mM/ 200 mM NaCl, 1x PI). Pellets were resuspended in 20 µL 1x LSB and together with their total cell lysates, boiled at ~ 100°C for 5 min and loaded into SDS-polyacrylamide gels. The presence of BACE1-mCherry, APP-mCherry, Munc18-1, Flag-tagged Munc18-1 and EGFP-Mint was detected by immunoblots with the corresponding antibodies.

Mouse brain lysate

Brains for lysate for western blot analysis were homogenized in ice-cold PBS with PI (Sigma) and were then incubated for 2h at 4°C in 1% Triton X-100 (Fisher Scientific) in 1x PBS with PI (Sigma). The brain lysate was then centrifuged for 20 min at 10,000g at 4°C. 1 ml Laemmli Sample Buffer (LSB) was added per 0.1 g of brain and boiled for 20 min at 100°C.

Human brain lysate

Human brain material was collected from patients suffering from left temporal epilepsy. Human brain tissue from left temporal cortex does not originate from the epileptic focus, but instead from the surrounding tissue that needed to be removed in order to access the epileptic focus. Immediately after dissection the tissue was stored in ice-cold solution containing: 110 mM Choline chloride, 26 mM $NaHCO_3$, 10 mM D-glucose, 11.6 mM sodium ascorbate, 7 mM $MgCl_2$, 3.1 mM sodium pyruvate, 2.5 mM KCl, 1.25 mM NaH_2PO_4 (Merck), and 0.5 mM $CaCl_2$ and transported to the laboratory within 15 min. Human brain tissue was homogenized in lysis buffer (50 mM Tris pH 7.5, 1% Triton X-100, 1,5 mM $MgCl_2$, 5 mM EDTA, 100 mM NaCl) with PI (Sigma) and then incubated for 2h at 4°C. The brain lysate was then centrifuged for 20 min at 10,000g at 4°C. After centrifugation, supernatants were incubated with primary antibodies with gentle shaking for 2h. Munc18-1 was immunoprecipitated using a polyclonal anti-Munc18 antibody (house made). Antibody-bound protein complexes were pulled down using blocked protein-A agarose beads (Sigma). Pellets were washed 5 times with wash buffer, alternating between low and high salt (50 mM Tris pH 7.5, 0.1 % Triton X-100, 1,5 mM $MgCl_2$, 5 mM EDTA, 100 mM/ 200 mM NaCl, 1x PI). Pellets were resuspended in 20 µL 1 x LSB, boiled at ~ 100°C for 5 min and loaded into SDS-polyacrylamide gels. The presence of BACE1 was detected by immunoblots with a monoclonal anti-BACE1 antibody (clone # ab89137, Abcam).

Western blot

Samples were loaded into SDS-PAGE gels with 2,2,2-Trichloroethanol (TCE) (Sigma). For visualization of the total proteins, gels were scanned using a Gel Doc EZ imager (Bio-Rad Laboratories) and analyzed with Image Lab (Bio-Rad Laboratories) to correct for input differences per sample. Acrylamide gels were transferred onto 0.45 µm pore size nitrocellulose membranes (Bio-Rad Laboratories). After transfer, membranes were incubated in an orbital shaker for 30 min at RT in 5% Bovine Serum Albumin (BSA) (Thermo Fisher Scientific) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) (Sigma) to block non-specific binding. Primary antibody incubation was done in 1% BSA (Thermo Fisher Scientific) in PBS for 1h-2h at room temperature or overnight at 4°C, followed by 3 washes with TBS-T. Membranes

were then stained with secondary antibody conjugated with alkaline phosphatase (AP) (anti-rabbit IgG from Jackson ImmunoResearch; anti-mouse IgG from Sigma; 1:10000) for 1h at RT. After 3 washes with TBS-T the AP-conjugated antibody was visualized using attophos AP fluorescent substrate (Promega). Membranes were scanned with a Fuji Image FLA-5000 Reader and analyzed with ImageJ software. Primary antibodies used: anti-mCherry (1:500, clone # GTX128508, GeneTex,), anti-APP (1:500, clone # P05067, Covalab), anti-BACE1 antibody (1:500, clone # ab89137, Abcam), polyclonal anti-GFP antibody (1:500, clone # ab290/50, Abcam), anti-Munc18 (1:1000, clone # 610336, BD Transduction Laboratories), polyclonal anti-Munc18 antibody (house made) and anti-VCP (1:1000, clone # K331, gift from T. Südhof, Department of Molecular and Cellular Physiology and Howard Hughes Medical Institute, Stanford University School of Medicine, CA, USA).

Results

Munc18-1 binds APP and BACE1

To test whether the previously suggested binding between Munc18-1 and APP[24] is a direct binding, HEK293 cells were transfected with Munc18-1-flag and APP-mCherry. Co-immunoprecipitation with an anti-mCherry antibody co-precipitated Munc18-1 (Fig. 5.1A). However, the reverse co-immunoprecipitation with an antibody against the flag-tag of Munc18-1 did not co-precipitate APP-mCherry (n=2; Fig. 5.1B). To determine whether Munc18-1 binds BACE1, co-immunoprecipitations were performed in HEK293 cells co-transfected with Munc18-1-flag and BACE1-mCherry. Immunoprecipitation with an anti-flag antibody co-precipitated BACE1, the reverse immunoprecipitation with an anti-mCherry antibody also co-precipitated Munc18-1 (n=2; Fig. 5.1A, B). The Munc18-1 BACE1 interaction was also detected in human brain lysates. Human brain tissue from temporal cortex was lysed and a co-immunoprecipitation with anti-Munc18-1 antibody co-precipitated BACE1 (Fig. 5.1C). These data suggest that, in addition to the reported interaction between Munc18-1 and APP, Munc18-1 and BACE1 also interact in heterologous cells and human brain lysate.

Phosphorylated and non-phosphorylated Munc18-1 binds APP and BACE1

It has been shown that co-localization of APP and BACE1 is increased in Neuro2a (N2a) cells upon co-transfection of cdk5 and p35[24]. This effect was abolished by expression of phosphorylation-resistant Munc18-1, whereas expression of phosphorylation-mimic Munc18-1 resulted in a permanent increase in APP–BACE1 co-localization[24]. To determine whether the phosphorylation state of Munc18-1 affects the binding to APP and/or BACE1, co-immunoprecipitations with phosphorylation-resistant (T574A) and phosphorylation-mimic (T574D) mutants of Munc18-1 were performed in HEK293 cells. In addition, HEK293 cells were transfected with a Munc18-1 mutant (T574P) that has been found in a patient suffering from early onset epileptic encephalopathy[333]. Immunoprecipitation of Munc18-1 (WT, T574A, T574D and T574P) co-precipitated APP (n=2; Fig. 5.2A) and BACE1 (n=2; Fig. 5.2B). In these experiments, the input protein levels of Munc18-1 T574A were lower when compared to Munc18-1 WT, T574D and T574P. When APP or BACE1 were co-precipitated using an

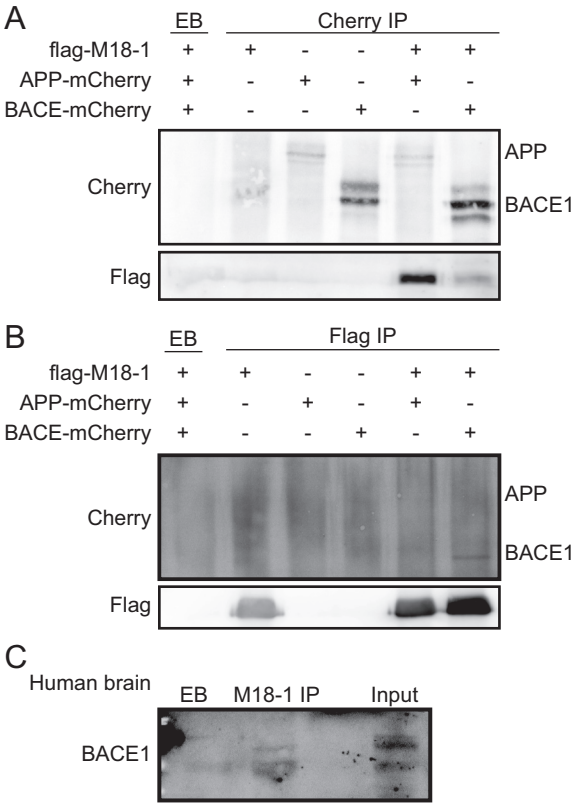

Figure 5.1 - **Munc18-1 binds APP and BACE1.** Munc18-1 (M18-1)-flag, APP-mCherry and BACE1-mCherry were transfected into HEK293 cells. **A**, Munc18-1 immunoprecipitation (IP) (flag antibody) co-precipitated BACE1, but not APP, Empty beads (EB) as control; (n=2). **B,** IP with anti-mCherry antibody against the mCherry-tag of APP and BACE1 co-precipitated Munc18-1, Empty beads (EB) as control; (n=2). **C**, IP with anti-Munc18-1 co-precipitated BACE1 in human brain lysate from left temporal cortex.

anti-mCherry antibody, only Munc18-1 T574D co-precipitated. The effect of Munc18-1 phosphorylation in the binding with BACE1 was further analyzed by co-transfecting HEK293 cells with cdk5 and p35. When cells were transfected with Munc18-1 and BACE1 in the absence or presence of cdk5 and p35, immunoprecipitation of Munc18-1 co-precipitated BACE1 in both conditions (n=1; Fig. 5.3A). These findings suggest that the binding of APP and BACE1 to Munc18-1, in HEK cells, is not dependent on cdk5-dependent phosphorylation of Munc18-1.

Munc18-1 forms a complex with APP and BACE1

It has been reported that Munc18-1 binds Mint-1[334] and that Mint-1 binds APP[327,335]. Therefore, to test whether Munc18-1 can bind APP and BACE1 when both APP and BACE1 are present, either directly or indirectly via Mint-1, HEK293 cells were transfected with Munc18-1-flag, APP-mCherry, BACE1-mCherry and EGFP-Mint. Immunoprecipitation of Munc18-1 with an anti-flag antibody co-precipitated APP and BACE1 in the absence or presence of Mint-1 (n=1; Fig. 5.4A). These findings show that in HEK293 cells, Munc18-1 forms a complex with

APP and BACE1 in the absence as well as presence of Mint-1 and facilitates the formation of a molecular complex consisting of Munc18-1, APP, BACE1 and Mint-1.

Depletion of Munc18-1 leads to an increase in APP levels

In order to examine the biological function of Munc18-1 and APP binding, the effect of Munc18-1 on APP levels was investigated. Therefore, the levels of APP were quantified in Munc18-1 WT, HZ and KO E18 whole brain lysate by Western blotting and normalized to total protein levels. Munc18-1 KO animals had significantly higher APP levels in the brain than WT (APP levels WT 100% ± 0.09; HZ 97.2% ± 0.06; KO 147.4% ± 0.09; * $p < 0.05$ using one-way ANOVA followed by the *post hoc* Bonferroni's test; Fig. 5.5A-C). Furthermore, APP levels where also analyzed in Munc18-1 WT and HZ animals of 4, 10 and 21 weeks old. An increase in APP levels were observed in HZ animals compared to WT animals (APP levels of WT animal at 4 weeks: 100%; 10 weeks: 136.5%; 21 weeks: 166.6%; HZ animal 4 weeks: 250.2%; 10 weeks: 273.5%; 21 weeks: 120.1%; Fig. 5.5D-G). APP levels increased from 4 to 21 weeks in WT animals, while in HZ animals it only increased from 4 to 10 weeks old (Fig. 5.5D-G). These data indicate that the absence of Munc18-1 in the brain results in increased APP levels.

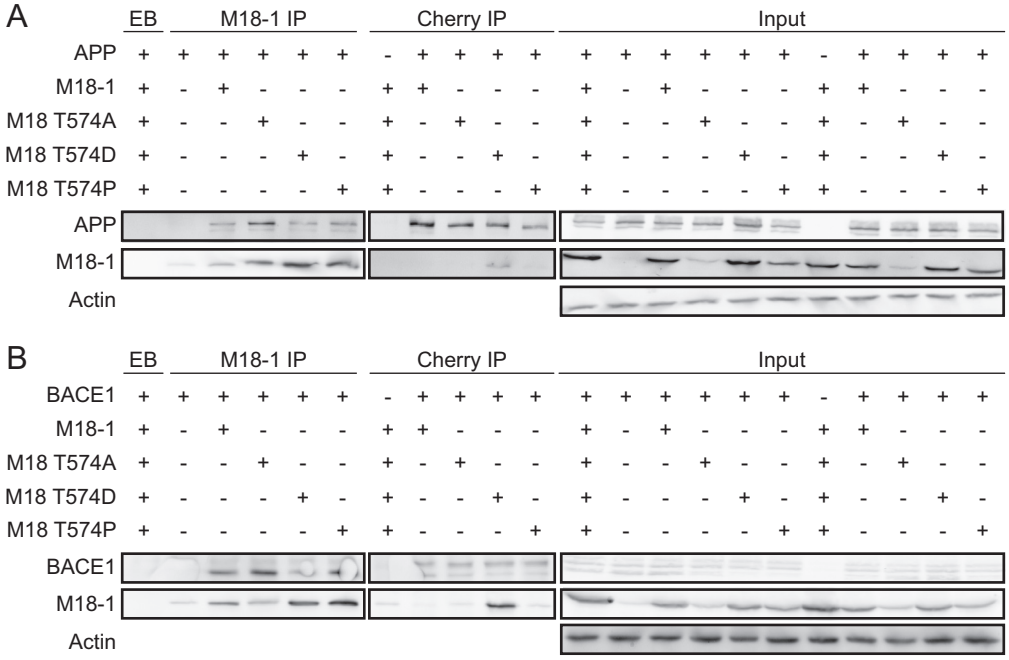

Figure 5.2 - **Phosphorylated and non-phosphorylated Munc18-1 binds APP and BACE1 in HEK293 cells. A**, APP-mCherry (APP) was transfected alone, with Munc18-1 (M18-1), phosphorylation-resistant Munc18-1 (M18 T574A), phosphorylation-mimic Munc18-1 (M18 T574D) or patient mutant Munc18-1 (M18 T574P) into HEK293 cells. Immunoprecipitation (IP) of Munc18-1 co-precipitates APP, whereas APP only co-precipitates Munc18-1 T574D; Input shows the presence of transfected proteins, Empty beads (EB) as control; (n=2). **B**, BACE1-mCherry (BACE1) was transfected alone, with Munc18-1, T574A, T574D or T574P into HEK293 cells. IP of Munc18-1 co-precipitates BACE1, whereas IP of BACE1 with an mCherry antibody only co-precipitates Munc18-1 T574D; Input shows the presence of transfected proteins, EB as control; (n=2).

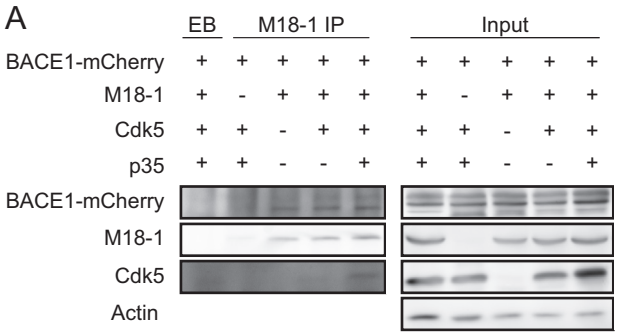

Figure 5.3 – BACE1 binds Munc18-1 upon cdk5 activation by p35. A, HEK293 cells were transfected with combinations of BACE1-mCherry, Munc18-1, cdk5 and p35. Immunoprecipitation (IP) of Munc18-1 co-precipitates BACE-mCherry; Empty Beads (EB) as control; (n=1).

Discussion

In this study, the role of the presynaptic protein Munc18-1 in the spatial separation of APP and BACE1 was investigated and an interaction between Munc18-1 and BACE1 was found. Phosphorylated and non-phosphorylated forms of Munc18-1 bind APP and BACE1 to a similar extent. A molecular complex of Munc18-1, APP, BACE1 and Mint-1 was also observed. Furthermore, APP levels are increased in Munc18-1 KO brains. Our findings indicate that Munc18-1 mediates the formation of a molecular complex (Munc18-1/APP/BACE1/Mint-1) that does not depend on the phosphorylation state of Munc18-1. Finally, our data implicate Munc18-1 in the mechanism of amyloidogenic processing of APP.

Munc18-1 forms a molecular complex with APP and BACE1

We showed that Munc18-1 binds APP in HEK293 cells (Fig. 5.1). These data are in line with previous data showing that APP forms a complex with Munc18-1[24]. The N-terminal MID domain of Mint-1 interacts with Munc18-1[326,334,336] and Mint-1 PTB domain interacts with the C-terminus of APP[335,337]. Thus, it is possible that Munc18-1 interacts indirectly with APP. However, since Mint-1 is not detected in HEK293 cells[338], it is more plausible that Munc18-1 interacts directly with APP. Furthermore, we report an interaction between Munc18-1 and BACE1 in HEK293 cells and human brain (Fig. 5.1). In contrast to our data, co-precipitation of Munc18-1 upon pull-down of BACE1 from BACE1-rich detergent-resistant membrane (DRM) fractions of brain tissue was not observed[24]. One possible explanation is that this interaction might not commonly take place in BACE1-rich DRMs, or that the levels of co-precipitated Munc18-1 might have been too low to be detected. The co-immunoprecipitation data show that Munc18-1 forms a complex with APP, BACE1 and Mint-1 in HEK293 cells (Fig. 5.4), suggesting a role of Munc18-1 in APP processing. Furthermore, the absence of Munc18-1 leads to an increase in APP levels (Fig. 5.5). Thus, our data provide evidence that the formation of this complex might facilitate BACE1-mediated cleavage of APP. Therefore, Munc18-1 might play a role in the amyloidogenic processing of APP and the development of Alzheimer's disease-related pathology.

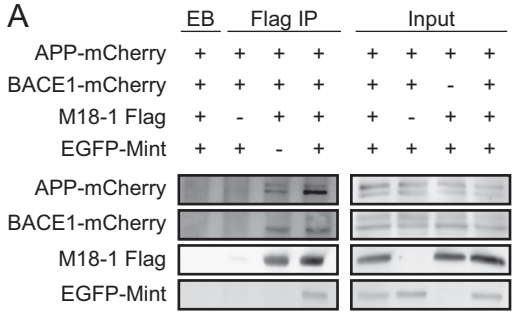

Figure 5.4 - Munc18-1 forms a complex with APP, BACE1 and Mint-1 in HEK293 cells. A, HEK293 cells were transfected with APP-mCherry, BACE1-mCherry, Munc18-1 (M18-1)-flag and EGFP-Mint. Precipitations were performed with anti-flag antibody. M18-1-flag co-precipitated APP and BACE1 in the absence and presence of Mint-1; Empty Beads (EB) as control; (n=1).

Munc18-1 phosphorylation state does not alter Munc18-1 binding to APP and BACE1 in HEK cells

We showed that in HEK293 cells, overexpression of Munc18-1 T574A led to lower levels of Munc18-1 protein, compared to overexpression of other Munc18-1 mutants (Fig. 5.2). A likely explanation is an increased breakdown of Munc18-1 T574A protein. Nevertheless, Munc18-1 T574A co-precipitated high amounts of APP and BACE1. This suggests that non-phosphorylated Munc18-1 has a higher affinity for APP and BACE1 than the other Munc18-1 mutants. However, Munc18-1 T574A might bind APP/BACE1 more efficiently only because the ratio of APP/BACE1 to Munc18-1 T574A is higher for this mutant.

Co-precipitation of APP/BACE1 using an mCherry antibody only co-precipitated phospho-mimic Munc18-1 T574D (Fig. 5.2). Due to a conformational change, Munc18-1 T574D might be more prone to precipitation than the other Munc18-1 variants. Yet, our data show that Munc18-1 WT, phosphorylation-resistant (T574A) and phospho-mimic (T574D) all co-precipitated APP and BACE1 (Fig. 5.2), suggesting that Munc18-1 phosphorylation state does not have a pronounced effect on Munc18-1 binding to APP or BACE1 in HEK cells. Furthermore, we found that Munc18-1 binds BACE1 in the absence and presence of cdk5 and p35 (Fig. 5.3). These data corroborate that phosphorylated and non-phosphorylated forms of Munc18-1 bind BACE1 equally well in HEK293 cells. However, these data do not address whether Munc18-1 was phosphorylated or just bound to cdk5. These findings are in contradiction with a previous study that suggested that Munc18-1 mediates APP processing through microdomain switching that is dependent on cdk5-mediated phosphorylation of Munc18-1[24]. This study showed that when Munc18-1 is phosphorylated, it dissociates from syntaxin-1 and thereby allows the spatial convergence of APP and BACE1, which leads to amyloidogenic processing of APP[24]. In normal conditions, a strong interaction exists between neuronal Munc18-1 and the closed conformation of syntaxin-1[156]. This interaction can be disrupted by phosphorylation of Munc18-1 by cdk5[339]. Our experiments were performed in cells that do not express syntaxin-1. Thus, although in HEK293 cells Munc18-1 phosphorylated state does not seem to play a role in the spatial separation of APP and BACE1, it cannot be excluded that in presence of syntaxin-1, Munc18-1 phosphorylation state might have a different role.

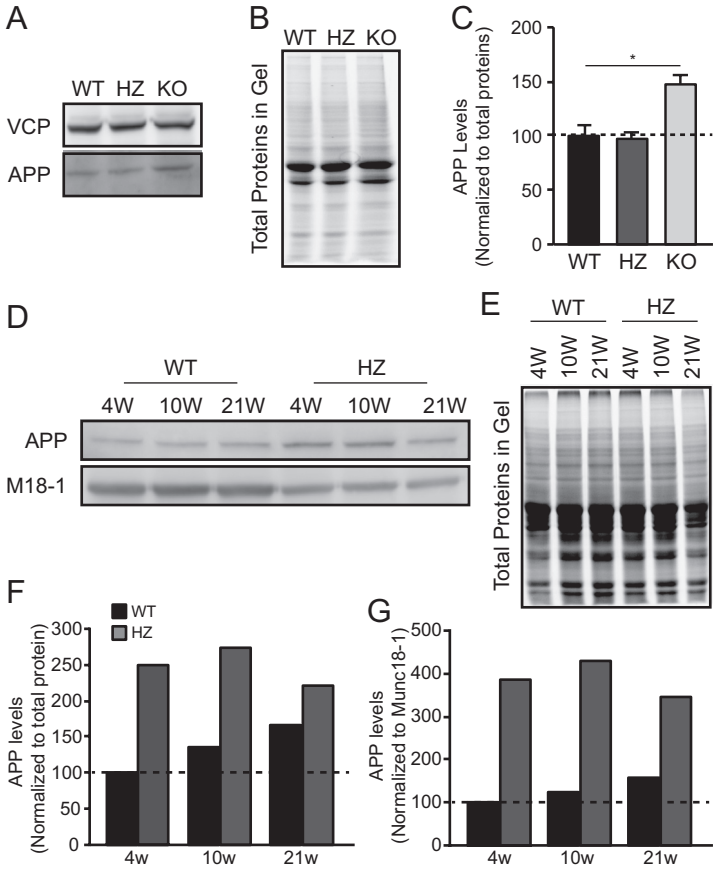

Figure 5.5 - **APP levels are increased 40% in Munc18-1 KO brains.** E18 brain lysates from Munc18-1 (M18-1) wild-type (WT), heterozygote (HZ) and knockout (KO) and WT and HZ brains of 4, 10 and 21 week mice were analyzed by western blotting. **A**, Western blot of APP and VCP, from E18 WT, HZ and KO brain lysates. **B**, Total proteins in the brains. **C**, APP levels were quantified by the ECF method and normalized to total proteins in the gel. * $p < 0.05$ using one-way ANOVA followed by the *post hoc* Bonferroni's test; (n=3). **E**, Western blot of APP and Munc18-1 from WT and M18-1 HZ brain lysates (4 weeks (w), 10w, 21w). **F**, Total proteins in the brain. **G**, APP levels were quantified by the ECF method and normalized to total proteins in the gel (n=1). **H**, APP levels were quantified by the ECF method and normalized to Munc18-1 (n=1).

Munc18-1 regulates APP and BACE1 trafficking possiblly via its role in vesicle release

APP levels are increased in Munc18-1 KO mouse brain, indicating that Munc18-1 promotes APP processing (Fig. 5.5). Similar to Munc18-1, depletion of Mint proteins have been found to decrease beta-cleavage and Aβ production in neurons, whereas in HEK293 cells Mint enhances retention of APP[326,340]. Studies in cell lines show that the Swedish mutant form of APP (APPsw), which is more prone to cleavage by BACE1, enhances Aβ formation[12]. APPsw undergoes BACE1 cleavage in the *trans*-Golgi compartment instead of in the early endosome[341]. Munc18-1 KO neurons have a condensed Golgi (chapter 2). Therefore, it is possible that the synergistic effect of Munc18-1 and Mint-1 on APP processing only becomes apparent when BACE1-mediated cleavage of APP is already high, or that Munc18-1 affects APP processing differently when cleavage preferentially takes place in the Golgi compartment. The microdomain switching

model[24] does not explain the increase in APP observed in Munc18-1 KO, since the model predicts that depletion of Munc18-1 decreases APP-syntaxin-1 co-localization, increasing APP-BACE1 co-localization, thus increasing beta-cleavage and decreasing APP levels. Therefore, we propose that the increase in APP levels in Munc18-1 KO brain might be explained by the formation of a Munc18-1/APP/BACE1/Mint-1 complex, as discussed above, or by the role of Munc18-1 in cellular vesicle release rather than the role of Munc18-1 in molecular microdomain switching.

Previous studies indicate that APP and BACE1 are spatially separated at a cellular level due to their localization in different cellular compartments or vesicles[342-345]. Yet, APP is routed into BACE1-positive compartments upon neuronal activity. Neuronal stimulation increases co-localization of APP and BACE1 as well as the percentage of mobile APP-containing vesicles that is positive for the recycling endosome marker TfR[344]. Interestingly, the convergence of APP and BACE1 observed upon neuronal stimulation can be reversed by inhibiting clathrin-mediated endocytosis (CME) or endocytosis of APP specifically. Inhibition of CME leads to a decrease in Aβ production[346-348]. Munc18-1 KO neurons are synaptically silent[27]. Therefore the APP present at the SVs cannot be routed into BACE1-positive compartments via exo- and endocytosis. This might explain the increase in APP levels observed in Munc18-1 KO mouse brain. Yet, since Munc18-1 KO does not affect the proportion of dense-core vesicle (DCV) release relative to the total DCV pool at DIV 4[349], it would be expected that in Munc18-1 KO neurons exocytosis of APP upon neuronal stimulation is reduced, but not abolished.

In summary, at a cellular level, Munc18-1 might enable the routing of APP into BACE1-containing compartments through regulation of SV exocytosis. At a molecular level, we propose that Munc18-1 mediates the formation of a molecular complex consisting of Munc18-1/APP/BACE1/Mint-1 that might facilitate BACE1-mediated cleavage of APP.