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Analysis of Syntaxin-1 Chaperones During Synaptic Activity

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ABSTRACT

Neuronal communication is based on synaptic vesicle exocytosis, which is strongly regulated. The release of neurotransmitters from presynaptic nerve terminals requires cycles of protein-protein interactions. SNARE and SM proteins are universally involved in all intracellular membrane fusion reactions, and reside either on the target membrane (syntaxin-1 and synaptosome-associated protein of 25kDa (SNAP-25)) or on the synaptic vesicle (synaptobrevin-2).

Recent studies have identified chaperones for two SNARE proteins: synaptobrevin-2 and SNAP-25. Since these SNARE chaperones seem essential for the long-term functioning of synapses, the question arises which molecule(s) may chaperone syntaxin-1. Previous studies have suggested that Munc-18 and SNAP-25 may chaperone syntaxin-1. Furthermore, it has been shown that chemical modification of syntaxin-1 or mutation on cysteine residue 145 increases its stability. To investigate a possible chaperone function of Munc-18 and SNAP-25 for syntaxin-1, I aimed to clarify whether this chemical modification inhibits syntaxin-1 degradation, whether the C145S mutation reproduces this modification, and whether this cysteine is normally involved in ubiquitination and degradation of syntaxin-1.

To approach these aims, HEK-293T cells and neuronal cultures from wild-type mice were used in combination with overexpression of syntaxin-1 full-length, several truncations and its mutant C145S. Chemical agents were used to monitor syntaxin-1 levels. These experiments were analyzed by immunoprecipitation, immunoblotting or immunocytochemistry.

Results suggest that munc-18 chaperones syntaxin-1, based on the following observations: 1) it increases syntaxin-1 levels and inhibits syntaxin-1 degradation in co-transfected HEK cells; 2) C145S mutation significantly stabilizes syntaxin-1 levels and results in less degradation products. C145S also dramatically reduces ubiquitination of syntaxin-1; 3) syntaxin-1 may be degraded via the lysosome. Lysosomal inhibitors revealed a trend towards stabilization of syntaxin-1 whereas proteasomal inhibitors

showed no change. Yet, further experiments are needed to understand the precise role of C145S in the degradation mechanism of syntaxin-1.

KEY WORDS: SNARE complex; Syntaxin-1; Munc-18; SNAP-25; chaperones.

RESUMO

O sistema nervoso apresenta como órgão central o cérebro, constituído por neurónios que comunicam entre si através de impulsos nervosos e libertação de neurotransmissores. A libertação de neurotransmissores para a fenda sináptica ocorre devido à fusão da vesícula sináptica com a membrana do neurónio pré-sináptico. Esta fusão intracelular ocorre como resposta a um potencial de acção que origina a abertura dos canais de Ca^{2+}.

Duas famílias de proteínas estão universalmente envolvidas no processo de fusão intracelular, SNARE (*Soluble N-ethyl-maleimide Sensitive Factor Attachment Protein Receptor*), fonte de energia para a fusão entre as duas membranas, e SM (*sec1/munc-18*).

O complexo SNARE é formado por três proteínas: uma proteína vesicular (v-SNARE): *vesicle-associated membrane protein-2 (VAMP-2 ou synaptobrevin-2)* e duas proteínas transmembranares (t-SNARES): *syntaxin-1 e synaptosome-associated protein* of 25 kDa (SNAP-25). As proteínas SNAREs apresentam uma sequência conservada de ~60 a ~ 70 resíduos fortemente reactivos que formam o complexo SNARE através de uma quadrupla hélice. *Syntaxin-1* e *synaptobrevina-2* apresentam apenas um motivo SNARE, contrariamente, a proteína SNAP-25 é constituída por dois motivos SNARE.

As sinapses nervosas transmitem sinais a elevada frequência, assim sendo, as proteínas SNAREs alternam continuamente entre um estado fortemente reactivo e um estado menos reactivo (formação do complexo *vs* não formação do complexo). Estas alterações conformacionais são apontadas como a possível causa para a evolução de *chaperones* tais como CSPα e *α-synuclein*, que mantêm as proteínas SNAREs estáveis durante a vida do neurónio. Enquanto a proteína *α-synuclein* aumenta a formação do complexo SNARE por meio da interação com *synaptobrevin-2*; o complexo CSPα/Hsc70/SGT actua como *chaperone da proteína* SNAP-25 estabilizando-a.

A proteína *synatxin-1* é constituída por: uma região transmembranar (ancoragem da proteína à membrana do neurónio); um motivo SNARE (local de ligação entre proteínas SNARES) e um domínio H_{abc} (local de ligação à SM proteína: *munc-18*). A proteína *syntaxin-1* alterna entre uma conformação aberta, onde forma o complexo SNARE e uma conformação fechada onde se liga à proteína *munc-18*.

A descoberta de *chaperones* específicos para duas das três proteínas SNAREs (*synaptobrevin-2* e SNAP-25) aponta para a hipótese de existir(em) *chaperone(s)* que estabilizem/modifiquem a proteína *syntaxin-1*. Assim sendo, os objectivos da presente tese incluem a: (1) identificação de possíveis *chaperone(s)* da proteína *syntaxin-1*: *munc-18* e/ou SNAP-25; estudo dos domínios responsáveis pela interacção/estabilização; (2) estudo da mutação na cisteína 145 para serina (C145S). Resultados recentes apontam para a estabilização da proteína *syntaxin-1* através do resíduo C145. Deste modo, pretende-se investigar se: (a) modificações químicas inibem a degradação da proteína *syntaxin-1*; (b) se a mutação C145S reproduz essas modificações; (c) se este resíduo está envolvido na ubiquitinação e degradação da proteína *syntaxin-1*.

Resultados anteriores revelaram que em cérebro homogenado NEM (**N**-**E**thyl**m**aleimide) aumenta os níveis de *syntaxin-1*, não alterando os níveis do complexo SNARE. Uma vez que o NEM actua no grupo *thiol* da cisteína, a mutação na cisteína 145 foi generada. De forma a dar resposta aos objectivos propostos, variantes da proteína *synatxin-1 wild-type* e mutante (C145S) foram clonadas em diferentes vectores (pCMV5, FUW e FSW) com diferente *tags* (myc e HA) e diferentes domínios presentes: 1-264, 180-288 e 180-264. As diferentes variantes da proteína *syntaxin-1* foram expressas em linhas celulares HEK 293T de modo a verificar se a proteína SNAP-25 e/ou *munc-18* aumentam os níveis de *syntaxin-1*, bem como identificar os locais de ligação. As amostras foram analisadas por *immunobloting*, immunoprecipitação e imunocitoquímica.

Os resultados obtidos demonstram que ambas as proteínas aumentam os níveis de *syntaxin-1*. Contudo, na presença de *munc-18* os níveis de *syntaxin-1* são mais elevados. Concluindo-se ainda que a variante C145S da proteína *syntaxin-1* é

mais estável que a variante *wild-type.* Os níveis de expressão da variante C145S são similares aos níveis de expressão da proteína *syntaxin-1 wild-type* quando *munc-18* está presente.

A análise das proteínas *syntaxin-1* $^{180\text{-}264}$ e *syntaxin-1* $^{180\text{-}288}$ permitiu concluir que: (a) o domínio H_{abc} é necessário para que a proteína *munc-18* estabilize a proteína *syntaxin-1*; (b) na presença do motivo SNARE os níveis de *syntaxin-1* aumentam drasticamente quando SNAP-25 está presente; (c) a região transmembranar é importante para estabilizar a proteína *syntaxin-1* sendo os níveis desta proteína reduzidos na ausência desta região.

De forma a avaliar se a estabilidade da proteína *syntaxin-1* é alterada na presença ou ausência da proteína *munc-18*, bem como se esta estabilidade é diferente quando a mutação C145S está presente, células HEK 293T foram transfectadas e sujeitas a tratamento químico com cicloheximida (inibidor da tradução) às 0h, 6h, 12h e 24h. Os resultados obtidos sugerem que a proteína *syntaxin-1* C145S é significativamente mais estável que *syntaxin-1 wild-type*. Por outro lado, quando *syntaxin-1* é expressa na presença de *munc-18*, um aumento na estabilidade desta proteína na variante *wild-type* é observado.

De forma a inferir se a estabilidade da proteína *syntaxin-1* é dependente da actividade sináptica, culturas neuronais foram incubadas com silenciadores (APV e TTX) e potenciadores da actividade sináptica (Ca^{2+} e K^{+}). Os resultados obtidos mostram uma tendência para a diminuição dos níveis da proteína *syntaxin-1* quando a actividade sináptica é aumentada. Quando a actividade sináptica é bloqueada os níveis da proteína *syntaxin-1* não sofrem alteração. O facto de, durante a actividade sináptica ocorrer a fusão de várias vesículas e consequente reciclagem, pode explicar os níveis reduzidos de *syntaxin-1*. Estudos em culturas neuronais que não expressem *munc-18* são sugeridos como trabalho futuro, de forma a clarificar o papel de *munc-18* na estabilidade da proteína *syntaxin-1* durante a actividade sináptica.

A análise dos produtos de degradação da proteína *syntaxin-1*, demonstram que a variante *wild-type* apresenta níveis mais elevados de degradação do que a variante

C145S; da mesma forma, quando a proteína *syntaxin-1* é transfectada com a proteína *munc-18* os produtos de degradação diminuem significativamente. Assim sendo, o passo seguinte foi estudar se o resíduo C145 tem um papel activo na degradação da proteína *syntaxin-1* e se está envolvido na ubiquitinação. Desta forma, ensaios de imunoprecipitação com *syntaxin-1*, foram efectuados e os níveis de ubiquitina analisados. Os resultados mostram que a proteína *syntaxin-1 wild-type* é significativamente mais ubiquitinada do que a proteína mutada. Este resultado pressupõe duas hipóteses: (1) *syntaxin-1* é ubiquitinada no resíduo C145, ainda que a ubiquitinação nos resíduos de cisteína não seja termodinamicamente favorável; (2) este resíduo é importante para sinalizar à célula que a proteína deve ser degradada.

Sendo a proteína *syntaxin-1* uma proteína membranar e sendo esta ubiquitinada, a questão coloca-se: é a proteina *syntaxin-1* degradada via lisossoma ou proteossoma? Culturas neuronais incubadas durante 36 horas com inibidores do proteosoma (*Epoxomicin*, MG132, *Clasto-lactocystin*) e com inibidores do lisossoma (leupeptina/pepstatina; PMSF) sugerem que a proteína *syntaxin-1* é degradada via lisossoma, contudo os resultados não são conclusivos.

Em suma: os resultados apresentados sugerem *munc-18* como chaperone da proteína *syntaxin-1*, sendo os níveis desta proteína mais elevados e os produtos de degradação menores quando *syntaxin-1* é transfectada na presença de *munc-18*. Futuras experiências em culturas neuronais que não expressem a proteína *munc-18* são essenciais para confirmação dos resultados. A mutação C145S estabiliza a proteína *synatxin-1*, aumentando significativamente os níveis de expressão da proteína; diminuindo os produtos de degradação; bem como os níveis de ubiquitinação.

Os resultados obtidos sugerem que o mecanismo de degradação da proteína *syntaxin-1* se processa via lisossoma, contudo futuras experiencias são necessárias.

Palavras chave: Complexo SNARE, *Syntaxin-1*, *Munc-18*, SNAP-25, *Chaperone*.

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ABBREVIATIONS

APS	**A**monium **p**er**s**ulfate
APV	(2R)-**a**mino-**5**-**p**hosphonovaleric acid
ATCC	**A**merican **T**ype **C**ulture **C**ollection
BCA	**B**icin**c**honinic **a**cid
BSA	**B**ovine **S**erum **A**lbumin
C	**C**ysteine
$CaCl_2$	**C**alcium **c**hloride
CO_2	**C**arbon Di**o**xide
CHX	**C**yclo**h**e**x**imide
C145S	**C**ysteine **145** **S**erine
DIV	**D**ays **i**n **v**itro
DMEM	**D**ulbecco's **M**odified **E**agle **M**edium
DMSO	**Di**m**e**thyl **S**ulf**o**xide
DNA	**D**eoxyribo**n**ucleic **A**cid
dNTP	**D**eoxy**n**ucleoside **t**ri**p**hosphate
DPBS	**D**ulbecco's **P**hosphate **B**uffered **S**aline
DTT	**Di**thio**t**hreitol
ECL	**E**nhanced **C**hemi**l**uminescence
E. coli	***Escherichia coli***
EDTA	**E**thylene**d**iamine **t**etraacetic **a**cid
FBS	**F**etal **B**ovine **S**erum
GFP	**G**reen **F**luorescent **P**rotein
HEK	**H**uman **E**mbryonic **K**idney
HEPES	4-(2-**h**ydroxy**e**thyl)-1-**p**iperazine**e**thane**s**ulfonic acid
HIV	**H**umane **i**mmunodeficiency **v**irus
HRP	**H**orse**r**adish **p**eroxidase
IC	**I**mmuno**c**ytochemistry
IRES	**I**nternal **r**ibosome **e**ntry **s**ite
IP	**I**mmuno**p**recipitation
KCl	Potassium Chloride
Leup/Pept	**Leu**peptin/**Pep**s**t**antin
LB	**L**ysogeny **b**roth
MDRS	**M**ethylation-**d**ependent **r**estriction **s**ystems
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NEM	**N**-**E**thyl**m**aleimide
NSF	**N**-ethylmaleimide-**s**ensitive **f**actor

PAGE	**P**oly**a**crylamide **g**el **e**lectrophoresis
PBS	**P**hosphate **B**uffer **S**aline
PCR	**P**rotein **C**hain **R**eaction
PMSF	**P**henyl**m**ethyl**s**ulfonyl **f**luoride
RNA	**R**ibo**n**ucleic **A**cid
RRE	**R**ev-**r**esponsive **e**lement
RT	**R**oom **T**emperature
S	**S**erine
SDS	**S**odium **D**odecyl **S**ulfate
shRNA	**S**mall **h**airpin **RNA**
SM	**S**ec/**M**unc-18
SNARE	**S**oluble **N**SF **a**ttachment **re**ceptor
SNAP-25	**Sn**aptosome-**a**ssociated **p**rotein of **25**kDa
Stx-1	**S**ynt**ax**in-**1**
Syb2	**Sy**napto**b**revin-**2**
TEMED	**Te**tra**m**ethyl**e**thylene**d**iamin
TBS-T	**T**ris–**b**uffered **s**aline **T**ween- 20
TTX	**T**etrodo**t**o**x**in
TX-100	**T**riton-**X 100**
VAMP2	**V**esicle-**a**ssociated **m**embrane **p**rotein-**2**
VCP	**V**asolin **c**ontaining **p**rotein
VSV-G	**V**esicular **s**tomatitis **v**irus – **g**lycoprotein
WB	**W**estern **B**lot
WT	**W**ild **T**ype

CHAPTER 1

INTRODUCTION

"The brain is the human body's most mysterious organ. It learns, it changes, it adapts, it tells us what we see, what we hear, it let us feel love, I think it holds our soul."

Shonda Rhimes

1.1 Nervous System

The brain is the center of the nervous system; it weighs approximately 1.3 kg in an adult human. There are about 10^{11} nerve cells, called neurons[1]. Neurons communicate with each other through direct contacts (electrical synapses) and mostly through non-continuous connections known as chemical synapses, the principal computational unit of the nervous system[2]. Neurons are classified according to: 1) function (e.g. motor, sensorial, interneuron), 2) localization (e.g. cortical, spinal), 3) shape (e.g. pyramidal, granule, mitral), and 4) nature of the transmitter synthetized and released (e.g. excitatory, inhibitory, neuromodulatory). In the human brain, neurons are connected by 10^{14} synapses[1]. Synaptic transmission occurs when an actions potential triggers neurotransmitter release from a presynaptic nerve terminal, resulting in synaptic vesicle exocytosis[3]. Thus, many pre-synaptic and post-synaptic proteins are needed to transmit the information from one neuron to another.

1.1.1 Synaptic vesicle cycle

Membrane fusion is one of the vital processes in life, and happens when two separate membranes merge into a continuous bilayer. Fusion can occur as constitutive

intracellular organelle fusion, or as precisely timed fusion of synaptic vesicles (Figure 1) with the presynaptic plasma membrane in neurons[4], which happens in response to an action potential that induces the opening of Ca^{2+} channels[5, 6]. SNARE (soluble NSF attachment protein receptor) and SM (sec1/munc-18) proteins are two conserved protein families that are universally involved in all intracellular membrane fusion reactions[4, 7].

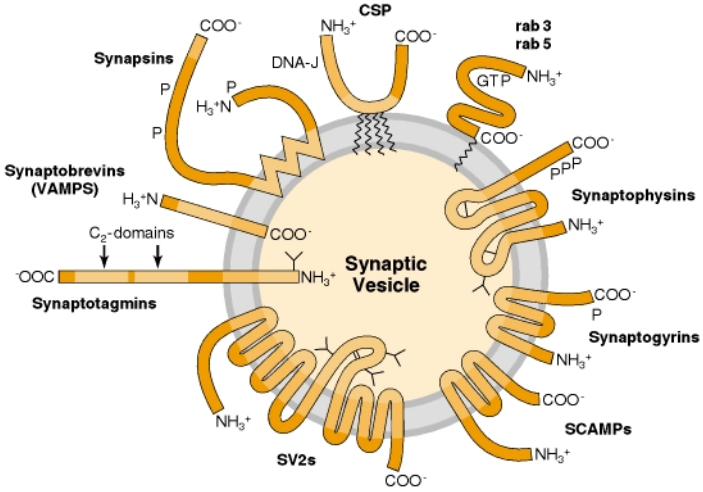


Figure 1- Vesicle proteins (from[8]).

Classical neurotransmitter release is based on the synaptic vesicle cycle that starts when synaptic vesicles are filled with neurotransmitters by active transport and form the vesicle cluster. A vesicle filled with neurotransmitters docks at the active zone and becomes primed; this reaction makes the vesicles competent for Ca^{2+}-triggered fusion-pore opening. Following fusion pore opening, synaptic vesicles undergo endocytosis and can recycle through three different pathways: 1) local reuse (kiss-and-stay), 2) fast recycling without an endosomal intermediate (kiss-and-run) and 3) clathrin-mediated endocytosis (with recycling via endosomes)[3] (Figure 2).

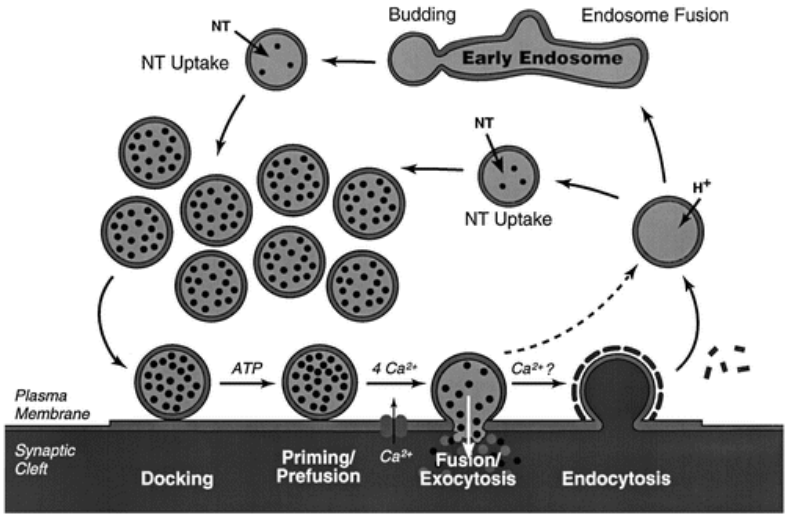


Figure 2 - Trafficking of synaptic vesicles in the nerve terminal (from[9]).

1.2 SNARE proteins

SNARE (Soluble N-ethyl-maleimide Sensitive Factor Attachment Protein Receptor) proteins assemble into a tight core complex (SNARE complex), which mediates vesicle fusion with target compartments[4].

The SNARE protein superfamily includes a group of small proteins, 24 in yeast and more than 35 in mammals[10]. These proteins mediate membrane fusion by bringing two membranes into close proximity and thereby providing the energy for membrane fusion. SNARE proteins were identified to be receptors for NSF (N-ethylmaleimide-sensitive factor) and SNAPs (soluble NSF attachment proteins) which are required for disassembly of the SNARE complex after the fusion event has taken place[5, 11].

Syntaxin-1, synaptosome-associated protein of 25 kDa (SNAP-25) and vesicle-associated membrane protein-2 (VAMP-2 or synaptobrevin-2) are the three proteins responsible for neuronal SNARE complex formation. SNARE proteins can be divided into target or t-SNAREs and vesicular or v-SNAREs according to their location within the cell. Thus, syntaxin-1 and SNAP-25 are synaptic SNARE proteins residing on the

presynaptic plasma membrane; synaptobrevin-2 resides on the synaptic vesicle membrane[4, 7, 11].

The importance of SNARE proteins for neurotransmission became apparent in studies using botulinum toxins (BoNT), proteins produced by the bacterium *Clostridium botulinum*. They are considered to be the most powerful neurotoxins ever discovered and specifically cleave SNARE proteins, preventing synaptic vesicles from docking/fusing with pre-synaptic membranes and therefore blocking neurotransmitter release[12, 13].

SNARE motifs can also be structurally distinguished into R-SNAREs and Q-SNAREs. The Q encodes for the amino acid arginine; thus, Q-SNARE proteins have an arginine residue as central amino acid in the SNARE domain, whereas R-SNAREs have a glutamine residue in the center of the SNARE motif. According to the position of their SNARE motif-containing domains within the SNARE complex and by their sequence similarities, Q and R SNAREs can be distinguished into four classes: 1) R-SNARE motif (VAMPs), 2) Qa-SNARE motif (syntaxins), 3) Qb-SNARE motif (homologs of the N-terminus of SNAP-25), 4) Qc-SNARE motif (homologs of the C-terminus of SNAP-25). Since R-SNAREs correspond to v-SNAREs, and Q-SNAREs correspond to t-SNAREs, all SNARE complexes contain one member of each class[4, 10].

SNARE proteins contain a conserved ~60 to ~ 70 residues SNARE repeat, a highly reactive sequence that assembles into the SNARE complex by forming a four helical bundle[4, 7] (Figure 3).

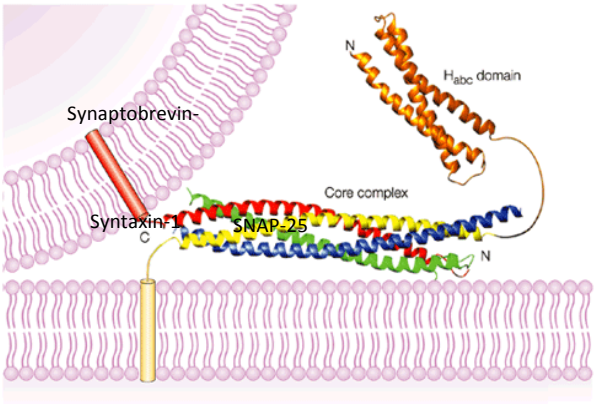


Figure 3 – SNARE complex (from[14])

Most of the SNARE proteins contain one SNARE motif, except for SNAP-class SNAREs which contain two SNARE motifs[4,7]. Synapses transmit signals at high frequencies. Thus, SNARE proteins continuously cycle through a highly reactive, non-assembled state, and a less reactive, assembled state[15]. These conformational changes are probably the reason for the evolution of chaperones such as CSPα and α-synuclein, which keep SNARE proteins stable throughout the life of a neuron[6]. While α-synuclein increases SNARE complex assembly by binding to the v-SNARE synaptobrevin-2[15], the CSPα/Hsc70/SGT chaperone complex binds to monomeric SNAP-25 and stabilizes this protein[16].

1.2.2 Synaptobrevin-2

Synapobrevin-2 is essential for fast synaptic vesicle endocytosis; absence of synaptobrevin-2 in synapses reveal an altered shape and size of synaptic vesicle, and stimulus-dependent endocytosis was delayed[17].

Synaptobrevin-2 has one transmembrane region and one SNARE motif that is unfolded in the monomeric form, but forms an alpha helix upon interaction with the t-SNAREs: SNAP-25 and Syntaxin-1 to form the SNARE complex[17] (Figure 4).

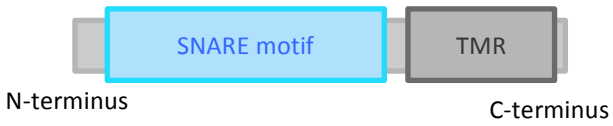


Figure 4 - Synaptobrevin-2 structure (adapted from [7])

1.2.3 SNAP-25

SNAP-25 (synaptosome-associated protein of 25 kDa protein) is associated with regulation of synaptic Ca^{2+} responsiveness. Thus, SNAP-25 knockout mice show that Ca^{2+} triggering was abolished[17]. Heterozygous deletion of the *SNAP-25* gene in

mice results in a hyperactive phenotype similar to attention deficit hyperactivity disorder (ADHD)[18].

SNAP-25 protein has two SNARE motifs and is palmitoylated at cysteine residues between the SNARE motifs, allowing SNAP-25 to anchor to the plasma membrane, since this SNARE protein does not have a transmembrane region[17] (Figure 5).

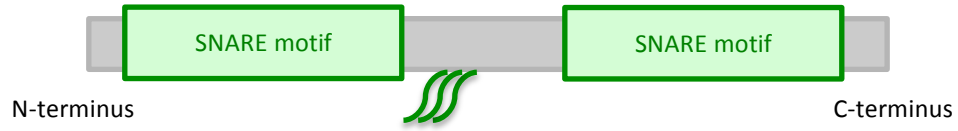


Figure 5 - SNAP-25 structure (adapted from[17]).

1.2.3 Syntaxin-1

Syntaxin-1 (Stx-1) expression starts in early embryonic development, and its levels are intensely up-regulated during synapse formation and brain maturation[19]. Syntaxin-1 has been linked to long-term potentiation, learning and memory, and it has been associated with several neurodegenerative and psychiatric diseases such as schizophrenia, Alzheimer's disease, and Creutzfeldt-Jakob disease[19].

Syntaxin-1 contains an N-terminal H_{abc} domain which has been shown to bind to munc-18[4] and which is connected to the SNARE motif by a short linker sequence. The protein is membrane-anchored by its C-terminal transmembrane region (TMR). SNARE motif and TMR occupy less than half of the sequence[4, 10] (Figure 6).

The N-terminal part of the protein, with the three helix bundles is flexible and allows syntaxin-1 to alter between two conformations: a "closed" conformation, where the H_{abc} domain and SNARE motif bind intramolecularly and thereby prevent its engagement into the SNARE complex, and an "open" conformation where the SNARE motif is exposed and can participate in SNARE complex formation[4,10].

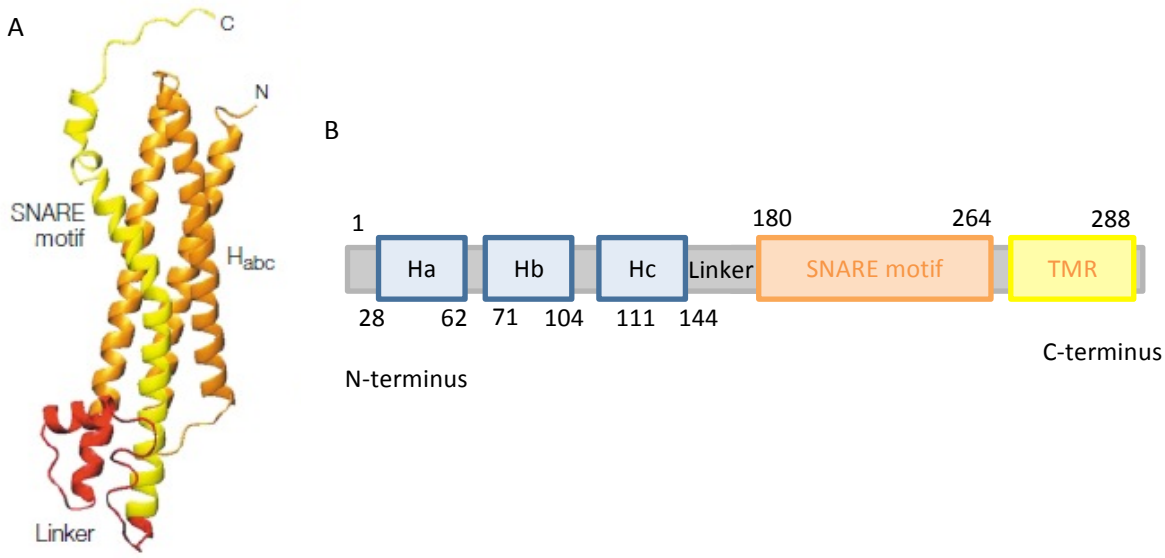


Figure 6 – A) Closed conformation of syntaxin-1 (from[14]), B) Syntaxin-1 structure; (Adapted from[14]).

1.3 SM Proteins

The SM protein superfamily is composed of only a few proteins: 4 conserved subfamilies have been described in eukaryotes, which are essential for exocytosis (Sec1/Munc18); endocytosis (Vps45); protein biosynthesis (Sly1); degradation (Vps33)[20]. SM are highly conserved among different organisms and show a highly conserved overall fold[20].

SM proteins are hydrophilic proteins of 60-70kDa that share homology evenly throughout their sequence, indicating that no particular domain is associated with their primary function. I.e., it is not clear how specificity for vesicle attachment or fusion is mediated[7,10,20]. SM proteins fold into an arch-shaped “clasp” structure containing three domains (called domains 1-3)[20], with a large cavity on one side, and a deep groove on the opposite side[7,10,20] The arch-shape as well as the deep groove have been implicated in interactions with SNARE proteins [20].

SM proteins are part of all membrane fusion reactions and are as essential as SNARE proteins for the fusion process[14]. The reduced number of SM proteins

compared to SNARE proteins suggests that these proteins are versatile fusion agents that function in multiple reactions[10].

SM proteins associate with SNARE proteins in several ways, including clasping both the v-SNARE and t-SNARE components of zippering SNARE complexes[4] (Figure 7). It has been suggested that SM proteins organize trans-SNARE complex spatially and temporally, but it is unknown how SM proteins cooperate exactly with SNARE complexes for fusion[1].

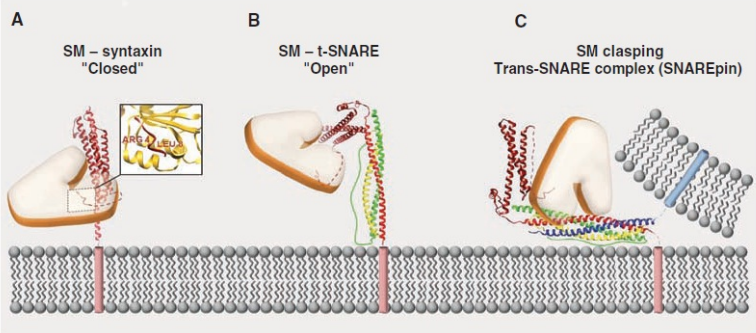


Figure 7 – A) Binding of the SM protein munc-18 to the "closed" conformation of syntaxin-1. B) The "open" conformation of a t-SNARE complex, consisting of a t-SNARE and its cognate SM protein bound to the N-peptide of syntaxin's H_{abc} domain. C) SNARE and SM proteins form the universal fusion machinery (From[4]).

1.3.1 Munc-18

Munc-18 is a cytosolic SM protein and is conserved throughout the eukaryotic kingdom[20]. Munc-18 binds tightly to syntaxin-1 in the "closed" conformation, preventing the formation of the SNARE complex[11]. Munc-18 was also reported to bind to the syntaxin-1/SNAP-25 heterodimer, from which it is released upon synaptobrevin-2 binding[7].

Although munc-18 stabilizes syntaxin-1 and allows proper targeting during the secretory pathway, the physiological function of the munc-18/syntaxin-1 interaction is still unknown. Yet, munc-18 may have local effects on syntaxin-1 stability or targeting within the presynaptic terminal or temporal effects on SNARE complex formation[11].

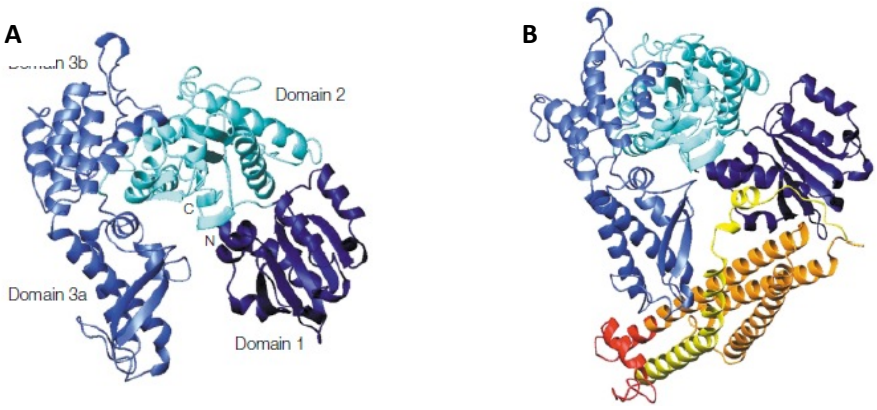


Figure 8 – A) Structure of Munc18-1; B) Structure of syntaxin-1/Munc18 complex. (From[14])

1.4 SPECIFIC AIMS

In the midst of the discovery of specific chaperones for synaptobrevin-2 and SNAP-25, it seems likely that there might be also a chaperone for syntaxin-1 that stabilizes/modifies syntaxin-1. Syntaxin-1 levels are reduced by 70% in absence of Munc-18-1, due to a strong enhancement of the stability of newly synthesized syntaxin-1[11]. I therefore aim to clarify whether munc-18 or SNAP-25 chaperone syntaxin-1 and which protein domains are responsible for this proposed stabilization. Furthermore, I aim to investigate if a previously identified mutation, which appears to be more stable than the wild-type (wt) version C145S inhibits syntaxin-1 degradation and if this cysteine is normally involved in degradation of syntaxin-1 by ubiquitination and proteasomal or lysosomal degradation. I also aim to investigate whether syntaxin-1 stability is activity-dependent, and whether overexpression or knockdown of munc-18 alters its stability and therefore synaptic transmission.

To approach this hypothesis, the following specific aims are proposed:

a) Do munc-18 and/or SNAP-25 chaperone syntaxin-1? I will analyze, whether each of these two proteins are able to stabilize syntaxin-1 protein levels and/or prevent degradation of syntaxin-1. I will also map the binding interface of syntaxin-1 and its putative chaperone.

b) What are the molecular changes in syntaxin-1 caused by the C145S mutation, i.e. is syntaxin-1 C145S more stable than wild type syntaxin-1? First, I aim to analyze whether the cysteine to serine mutation of syntaxin-1 is more stable than the wild-type. Then, I aim to clarify whether degradation of syntaxin-1 happens via the ubiquitin/proteasome pathway or the lysosomal pathway, and whether expression of munc-18 alters this process. Finally, I will analyze if the cysteine 145 is involved in the degradation process of syntaxin-1, e.g. prevents or slows down degradation of syntaxin-1.

c) Does syntaxin-1 stability depend on synaptic activity? What happens to synaptic transmission in presence or absence of the postulated chaperones?

CHAPTER 2

MATERIALS AND METHODS

2.1 Molecular Biology

2.1.1 Plasmid Vectors

A c-myc (**N-EQKLISEEDL-C**) epitope with a linker (**AA**) was added to the N-terminus of a rat syntaxin-1A cDNA to generate myc-tagged syntaxin-1 wild-type (WT) and syntaxin-1 cysteine to serine mutation (C145S), using as a template an already existing HA-tagged (**YPYDVPDYA**) syntaxin-1A cDNA. The two different tags are needed in order to distinguish between WT and C145S when transfected in the same cell. For syntaxin-1A truncation constructs, a stop codon was introduced at residue 265. Syntaxin-1A WT and C145S full length and 1-264 truncations were cloned into pCMV5, FUW and FSW vectors, respectively. Rat SNAP-25 cDNA and rat munc-18-1 cDNA constructs were cloned into FUW and FSW vectors. The following other constructs I used were already generated in the Sudhof lab: pCMV5 HA-syntaxin-1 $WT^{180-264}$, pCMV5 HA-syntaxin-1 $WT^{180-288}$, pCMV5-SNAP-25, pCMV5-munc-18-1 and lentiviral munc-18 shRNA constructs. Syntaxin-1 constructs are shown in Table 1 and 2.

Table 1 – Syntaxin-1 constructs

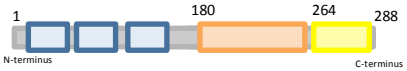
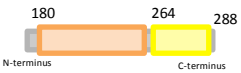
Vectors/tags	Syntaxin-1	Structure	Included domains
pCMV5-HA or myc FUW-myc FSW-myc	WT and C145S full length		Habc domain SNARE motif Transmembrane domain
pCMV5-HA	WT 180-288		SNARE motif Transmembrane domain

Table 2 - Syntaxin-1 constructs (continuation)

pCMV5-HA	WT 180-264		SNARE motif

2.1.2 DNA amplification

Syntaxin-1, Munc-18 and SNAP-25 cDNA was amplified by polymerase chain reaction (PCR), using with the following reagents: 1) PfuUltra™ HF DNA polymerase (Roche); 2) Dimethyl sulfoxide (DMSO) was used in the PCR reaction in order to inhibit secondary structures within the DNA template or within the primers, minimizing interloping reactions[22]; 3) Bovine serum albumin (BSA) (Sigma-Aldrich) works as a stabilizing agent in enzymatic reactions and enhances enzymatic activity[23]; 4) Deoxynucleoside triphosphates (dNTPs) (NEB) are necessary for DNA polymerase to synthetize new DNA; 5) DNA template; 6) Primers. Number of cycles, annealing temperature and primers used are listed in Table 3.

Table 3 – PCR primers, annealing temperature and number of cycles

Protein	Primer Sense	Primer Anti-sense	T / cycles
Myc-Stx-1A 1-288	5'GATCGCCACCATGGATGGAGCAGAAGCTGAT CAGCGAGGAGGACCTGGCCGA AAGGACCGAACCCAGCT 3'	5'GTCGAATTCCTATCCAAAG ATGCCCCCGAT 3'	50°C cycles: 30x
Myc-Stx-1A 1-264	5'GATCGCCACCATGGATGGAGCAGAAGCTGAT CAGCGAGGAGGACCTGGCCGA AAGGACCGAACCCAGCT 3'	5'GTCGAATTCCTACTTCCTG CGTGCCTT 3'	52°C cycles: 33x
SNAP-25	5'CTAGGAATTCACCGCCATGGCCGAAGACGCA GACATG3'	5'GTCGAATTCTTAACCACTT CCCAGCATCTTTG 3'	50°C cycles: 30x
Munc-18	5'CTAGGAATTCACCGCCATGGCCCCCATTGGC CTC3'	5'GTCGAATTCTTAACTGCTT ATTTCTTCGTC 3'	50°C cycles: 30x

PCR program

1) Denaturation (hot start): melting of DNA by disruption of hydrogen bonds; hot start PCR reduces nonspecific amplifications caused by slow heating of DNA with the primers;	94°C	5 min	
2) Denaturation: melting of DNA by disruption of hydrogen bound by complementary bases leading to a single stranded DNA;	94°C	30 sec	* cycles
3) Annealing: annealing the primers to the single DNA strand;	*°C	30 sec	
4) Extension: DNA polymerase synthetizes a new DNA strand complementary to the DNA template by adding dNTPs;	72°C	90 sec	
5) Final Extension: to ensure that all single-stranded DNA is amplified;	72°C	7 min	
6) Final Hold: stop PCR reaction;	4°C	∞	

* According to table 2

PCR products were separated on 1% agarose (Phenix Research Products) gels containing SYBR® Safe DNA Gel Stain (Invitrogen) that stains the DNA by binding to nucleic acids. Stained DNA fragments were detected using a standard UV transilluminator, a visible blue-light transilluminator (BIO-RAD). In order to confirm the presence of the right DNA, molecular markers 100bp and 1kb (NEB) were used. PCR products were purified with a QIAquick® PCR Purification Kit (QIAGEN) according to the manufacturer's protocol.

2.1.3 Ligation

Syntaxin-1A, munc-18, and SNAP-25 cDNA as well as the vectors pCMV5, FUW and FSW were digested with EcoRI (NEB) at 37°C for 2h since both, vectors and PCR fragments contain a 5' and 3' EcoRI restriction site (GAATTC), introduced by PCR. After digestion, vectors were incubated for 1h at 37°C with alkaline phosphatase (Roche) in order to dephosphorylate the 5' ends of the vector and avoid self-ligation. After this step, samples were separated on a 1% agarose gel (Phenix Research

Products) in order to isolate the cut DNA, followed by gel extraction and purification using QIAEX II ® Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. For incorporation of cDNA into the vector, cut vector and PCR fragments were ligated for 1h at room temperature using: 1) 1 µl T4 DNA ligase (NEB); 2) 1 µl 10x DNA ligation buffer; 3) 8 µl insert plus vector at a ratio of insert:vector = 3:1.

2.1.4 Transformation

The bacterial strain used for molecular biology was *Escherichia coli* DH10B. This strain was designed for the propagation of large insert DNA library clones, which takes advantage of properties such as high DNA transformation efficiency and maintenance of large plasmids, the lack of methylation-dependent restriction systems (MDRS), and colony screening via *lac*Z-based α-complementation[24-25].

Transformation, a process that allows DNA to enter the cell[25], was done by heat-shock. First, bacteria strain *E. coli* DH10B was incubated with DNA for 20 minutes on ice, followed by a heat-shock at 42°C for 45 sec and recovery for 2 min on ice, allowing the DNA to enter the bacteria. To allow bacteria to express the ampicillin resistance introduced by the vector, bacteria were incubated in LB medium (Lysogeny broth Medium (1% tryptone (BD); 0.5% yeast extract (BD); 0.5% NaCl (BD)) at 37°C for 1h. Bacteria were plated on LB plates containing ampicillin (1% tryptone (BD); 0.5% yeast extract (BD); 0.5% NaCl (BD); 1.5% agar (BD); 100ug/mL ampicillin (Sigma)) overnight at 37°C to select for clones carrying the ampicillin resistance introduced by transformation. Inoculation of a single colony was performed in LB medium with 10 µg/ml ampicillin (Invitrogen) over night at 37°C.

2.1.5 Analytical Restriction and DNA sequencing

In order to obtain pure DNA that can be used for expressing proteins in cells, a miniprep was done using QIAprep® spin Miniprep Kit (QIAGEN), followed by an

analytical restriction to ensure that the selected colonies carry the correct DNA, and carry the DNA in the right orientation since the same restriction enzyme was used for 5' and 3' insertion. The following reagents were mixed in a total volume of 20 µl: 1) 5 µl mini-prepped DNA; 2) 1 µl restriction enzyme: BamHI (NEB) was used for syntaxin-1A and XmaI (NEB) for SNAP-25 and munc-18 restriction analysis; 3) 2 µl 10x buffer (according to NEB catalogue); 4) 2 µl 1mg/mL BSA; each performed for 1h at 37°C. (Attachment A: vector illustrations).

ElimBio Company performed DNA sequencing with primers listed in table 4.

Table 4 – Sequencing primers

Name	Sequence	Direction	Vector
C1P	5' GCAAATGGGCGGTAGGCG 3'	Forward	pCMV5
C2	5' CCAAGGCCAGGAGAGGCAC 3'	Reverse	pCMV5
FUW	5' ATTGTCCGCTAAATTCTGG 3'	Forward	FUW
FUGW rev	5' GCAGCGTATCCACATAGGG 3'	Reverse	FUW
FSW forw	5' ACTCAGCGCTGCCTCAGTCT 3'	Forward	FSW
FSW rev	5' AGAATACCAGTCAATCT 3'	Reverse	FSW

2.2 Eukaryotic Cell Culture

Human embryonic kidney (HEK) 293 T cells (ATCC – American Type Culture Collection) were used as model system since these cells do not express SNARE proteins. HEK 293T were maintained in Dulbecco's Modified Eagle Medium High Glucose 1x (DMEM) (Invitrogen), containing 4.5 g/L D-glucose, L-glutamine and 110mg/L sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO_2 atmosphere, and were passaged every two days.

For cell passaging, HEK 293 T cells were washed twice with 1x Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen), and were then incubated with 0.05% trypsin-EDTA (Invitrogen) for 2 min to detach cells from the culture dish by digesting cellular integrins. Trypsin digestion was stopped by addition of DMEM. After dissociation, cells were resuspended and redistributed in 6 or 24 wells plate, 10cm plates or T75 flasks.

2.3 Recombinant protein expression in HEK 293T cells

HEK 293T cells were transfected using FuGene-6 (Roche), a reagent with cationic polymers that binds to negatively charged DNA, making a complex that is taken up by the cell via endocytosis. Transfection was performed at a ratio of DNA to Fugene of 1:3.

1) Syntaxin-1A expression experiments: HEK 293T cells (ATCC) were co-transfected with pCMV5-syntaxin1A (*wt*, C145S or truncations), pCMV5-munc-18-1, pCMV5-SNAP-25 or pCMV5-α-synuclein$^{1-95}$ (α-synuclein$^{1-95}$ is used to balance the number of munc-18 or SNAP-25 plasmids transfected into HEK 293T cells (ATCC)) and pCMV5-emerald to control for transfection efficiency. Transfections were performed at a ratio of 1:3:1 for syntaxin-1:munc-18/SNAP-25/ α-synuclein$^{1-95}$:emerald.

2) Cycloheximide chase experiments: HEK 293T cells (ATCC) were co-transfected with: a) pCMV5-syntaxin1A (*wt* or C145S) and pCMV5-α-synuclein$^{1-95}$ at a 1:1 ratio; b) pCMV5-syntaxin1A (*wt* or C145S), pCMV5-munc-18 and pCMV5-α-synuclein$^{1-95}$ at a 1:5:1 ratio.

3) Immunoprecipitations (IP): HEK 293T cells (ATCC) were co-transfected with pCMV5-syntaxin-1A (*wt*, C145S or truncations) and pCMV5-munc-18 at a 1:1 ratio.

4) Immunocytochemistry: HEK 293T cells (ATCC) were transfected with pCMV5-syntaxin-1 *wt* or C145S.

HEK 239T cells (ATCC) were harvested 48 hours after transfection; except for cycloheximide experiments where cells were harvested at different time points (48h plus 0h, 3h, 6h, 12h, 24h and 36h). For harvesting, cells were washed 3x with PBS and solubilized with 0.1% Triton-X 100 (TX-100) (Sigma). After solubilization, insoluble material was removed by centrifugation for 20 min at 10,000g. The supernatant was collected and 5% Laemmli sample buffer (10% sodium dodecyl sulfate (SDS); 5% glycerol; 0.006% bromophenol blue in ethanol; 0.4M Tris-Cl pH 6.8; 77mg/ml dithiothreitol (DTT)) was added. To disrupt SNARE-complexes into SNARE protein monomers, samples were boiled for 20 min at 100°C.

2.4 Cortical neuronal cultures from mice

Mouse cortical neurons were cultured from mouse pups at P0 (< 24hours after birth). Brain regions were dissected on ice, and were incubated in ice-cold Hank's Balanced Salt Solution (HBS) with Hanks Balanced Salts without calcium chloride, magnesium sulfate and sodium bicarbonate (Sigma), pH 7.4. This buffer contains 350mg/L sodium bicarbonate (NaHCO3) and 1mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) to stabilize the pH. Brains were digested in 2% papain solution with 0.5M EDTA pH 8.0 and 1M $CaCl_2$ in HBS for 20 min at 37°C to dissociate cells. Brains were then triturated with a pipette in plating medium (MEM) (Invitrogen) supplemented with 10% FBS (HyClone), 0.2M L-glutamine solution (Invitrogen), 0.25g/L insulin (Sigma). Cells were plated either onto a 12mm coverslip coated with 1mg/mL poly-L-lysine (Sigma) in 0.1M borate buffer (3.1g/L boric acid, 4.8g/L sodium tetraborate, pH 8.5) for imaging, or in a 24well plastic dish for biochemical experiments. After 1 day, plating medium was replaced with growth medium (0ARA-C) containing 5% FBS (HyClone), 0.2M glutamine solution (Invitrogen), 2% B-27 supplement (Invitrogen). Neuronal cultures were kept in growth medium (2ARA-C) containing 5% FBS (HyClone), 0.2M glutamine solution (Invitrogen) 2% B-27 supplement (Invitrogen), 2 uM cytosine arabinose (Sigma).

2.5 Lentivirus production

Lentivirus is a class of retrovirus that can introduce a significant amount of genetic information into animal cells by insertion of their DNA into the host chromosomal DNA, thereby increasing the efficiency by which a modified gene can be stably expressed in animal cells. Lentivirus is the only one among the retrovirus class, which is able to replicate in non-dividing cells[1].

Before transfection, HEK 293T cells (ATCC) were washed twice with DPBS (Invitrogen), and medium was changed to neuronal growth medium 0ARA-C. For overexpression of proteins, FUW and FSW vectors containing cDNA for munc-18 and synaxin-1A variants were co-transfected with Δ8.9 vector (human immunodeficiency virus (HIV-1) packing vectors that are highly efficient vehicles for *in vivo* gene delivery[26] and carry all the major genes except for the major viral envelope protein[1]) and VSV-G (a vector carrying the gene for the glycoprotein of the vesicular stomatitis virus, an envelope glycoprotein which can readily replace the normal lentivirus envelope protein[1]) in a 1:1:1 molar ratio into HEK 293T cells (ATCC) using Fugene-6 (Roche) as described in 2.1.

A third generation lentivirus was used to generate munc-18 knockdown virus. Here, lentiviral L309 vector carrying the shRNA and the two packing vectors REV and RRE (Rev-responsive element) were co-transfected with a vector carrying the envelope protein VSV-G in a 1:1:1:1 molar ratio into HEK 293T cells (ATCC) using Fugene-6 (Roche) as described in 2.1.

Medium containing the viral particles was collected 48 hours later and centrifuged for 5min at 500rpm to remove any cellular debris. The supernatant containing the virus was added to cortical neuronal cultures at 5 days *in vitro* (DIV). For the L309 vector, the expression of the recombinant proteins could be monitored using GFP fluorescence since this vector contains an IRES (internal ribosome-entry site)-driven GFP.

2.6 Brain Homogenate and Lysate

A 8-10 week old stripped mouse brain (Pel-Freez) was homogenized in ice-cold phosphate buffered saline (PBS) (Sigma) with protease inhibitors and was then incubated for 2h at 4°C in 1% Triton-X 100 (Sigma) in PBS (Sigma) with protease inhibitors to solubilize membranes. The brain lysate was centrifuged for 20 min at 10,000g at 4°C to remove TX-100 insoluble material. In order to do quantitation of total protein present, a detergent-compatible formulation based on bicinchoninic acid (BCA) was used which is based on a colorimetric detection (Pierce® BCA Protein Assay Kit; Thermo SCIENTIFIC).

2.7 Pharmacological treatments

2.7.1 Protein degradation

N-ethylmaleimide (NEM) is an irreversible inhibitor of all cysteine peptidases with the capability of blocking vesicular transport. NEM was used to treat neuronal cultures for 5min at 5mM final concentration (Sigma). Neurons were then dissolved directly in 2x Laemmli sample buffer.

2.7.2 Protein turn-over

Cycloheximide (CHX), a protein synthesis inhibitor, blocks eukaryotic translation in the elongation phase, by blocking peptidil transferases[27]. CHX at 0.1g/L (Sigma) final concentration was added to HEK 293T cells 12h after transfection, and cells were harvested 0h, 6h, 12h and 24h after treatment. In neuronal cultures, CHX 0.1g/L (Sigma) was added at different time points (0h, 3h, 24h, 48h, 72h) starting at 11DIV. Neurons were dissolved directly in 2x Laemmli sample buffer, HEK293T cells were washed 3x with PBS and solubilized with 0.1% Triton-X 100 (TX-100) (Sigma).

After solubilization, insoluble material was removed by centrifugation for 20 min at 10,000g. The supernatant was collected, and 5x Laemmli sample buffer was added.

2.7.3 Silencing and enhancing synaptic activity

Cultured cortical neurons were incubated at 12 DIV for 36 in 0.5μM tetrodotoxin (TTX) (Calbiochem), which blocks action potentials in neurons by binding to the voltage-gated, fast sodium channels[28]. Alternatively, neurons were incubated in 20μM AP5 (Sigma), a selective NMDA receptor antagonist that competitively inhibits the interaction between glutamate and NMDA receptors[29]. In order to enhance synaptic activity, neurons were incubated in medium containing 25mM KCl or 4mM $CaCl_2$.

2.7.4 Protease inhibition

Leupeptin (Sigma) is a protease inhibitor that inhibits cysteine, serine and threonine peptidases and was used at 10mg/L final concentration. Pepstatin (Sigma), is a potent inhibitor of aspartyl proteases and was used at a final concentration of 10 mg/L. MG132 (Sigma) is a specific and reversible proteasome inhibitor, which reduces the degradation of ubiquitin-conjugated proteins by the 26S complex without affecting its ATPase or isopeptidase activities, and was used at a final concentration of 10 μM. Clasto-lactacystin β-lactone (Calbiochem) is a highly specific inhibitor, does not affect cysteine or serine proteases, but appears to be the active inhibitor that reacts with the N-terminal threonine of the proteasome β-subunit X[30].This chemical was added at a final concentration of 10 μM. All chemicals were added to neuronal cultures at 12 DIV for 36h. Phenylmethylsulfonyl fluoride (PMSF) (Sigma) is a serine protease inhibitor that binds specifically to the serine residue in the active site of serine proteases. It does not bind to any other serine residues in the protein. This chemical was used at a final concentration of 4 mM. Epoxomicin, a natural occurring selective proteasome inhibitor with anti-inflammatory activity, was added at a final concentration of 10 μM.

2.8 Protein Separation, Immunoblotting and Protein Quantification

2.8.1 Protein Separation

Gel electrophoresis was used in order to perform macromolecular separation of proteins from HEK 293T cells (ATCC) and neuronal culture samples. Molecular separation is based on gel filtration and on electrophoretic mobility of proteins: proteins are separated as a function of the length of a polypeptide chain or molecular weight, due to the binding of sodium dodecyl sulfate (SDS) which gives identical charge per unit mass[1]. Samples were subjected to polyacrylamide gel electrophoresis (PAGE) using the mixtures described in Table 5.

Table 5 – Components of polyacrylamide gels

For 6 gels	15% separation gel	4% stacking gel
30% acrylamide (Bio-Rad)	22.5mL	2.5mL
Water	5.4mL	5.1mL
Tris-Cl pH 8.8 (Sigma)	16.8mL	n.a.
Tris-Cl pH 6.8 (Sigma)	n.a.	7.5mL
20% SDS (Sigma)	225 µL	75 µL
Tetramethylethylenediamin (TEMED) (Sigma)	45 µL	15 µL
10% Amonium persulfate (APS) (Sigma);	225 µL	75 µL

Protein separation occurs by application of an electric field (120milivolts) and the negatively-charged proteins migrate towards the anode (positive electrode). Each protein moves differently through the gel, according to its size: small proteins migrate more, since they fit more easily through the pores; larger proteins encounter more resistance; thus, they migrate less[1].

2.8.2 Coomassie Brilliant Blue Staining

Acrylamide gels were stained for 15min at RT in an orbital shaker with Coomassie Brilliant Blue R-250 solution (1g/L R-250 Comassie (Sigma); 50% methanol (Sigma); Water). Coomassie binds non-specifically to hydrophobic amino acids and thereby stains the proteins in the gel[1]. To decrease background staining, the gel was destained for 1-2 days in a solution with 5% methanol (Sigma) and 7,5% acetic acid (Sigma); Water).

2.8.3 Western Blot

For western blotting, acrylamide gels were transferred onto 0.45µm pore size nitrocellulose membranes (Whatman). For experiments with pCMV5-α-synuclein$^{1-95}$, a small protein (10kDa), the nitrocellulose membrane was completely dried after transfer and then incubated for 15 min at room temperature in 0.2% glutaraldeyde (TCI America) in PBS (Sigma) in order to fix the proteins to the membrane.

After transfer, membranes were incubated with 0.5% Ponceau-S (Sigma) in 1% acetic acid (Sigma) in water to visualize that the proteins have been transferred to the membrane. In order to block non-specific binding of antibody to the nitrocellulose, membranes were incubated in an orbital shaker for 30min at room temperature in 3% non-fat dried milk in Tris–buffered saline containing 0.1% Tween- 20 (TBS-T) (Sigma) supplemented with 2% FBS (HyClone). Three series of 5min washes were done in TBS-T. Afterwards, the blots were incubated in primary antibody in 1% BSA in PBS (Table 6 e 7) for 1h-2h at room temperature or overnight at 4°C, followed by 3 washes with blocking solution. Washed membranes were incubated in blocking solution containing either an anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (MP biomedicals 1:5000) for 2h at room temperature. HRP catalyzes the oxidation of luminol to 3-aminophthalate via several intermediates. This reaction is accompanied by emission of low-intensity light at 428 nm. The intensity of light is a measure of the number of enzyme molecules reacting and thus of the

amount of hybrid[1]. Modified phenols can be used as enhancers of light emission - enhanced chemiluminescence (ECL) (GE healthcare).

To quantitate the levels of proteins, the blots were incubated with ^{125}I-labeled secondary antibody (Perkin Elmer, 1:1000) overnight at room temperature, followed by a series of TBS-T washes. ^{125}I blots were exposed to a phosphorimager screen (Amersham) for 1-2 days and scanned using a Typhoon scanner (GE healthcare), followed by quantification with ImageQuant software (GE healthcare). In order to have accurate values, the background was subtracted.

2.9 Immunoprecipitation

Immunoprecipitations were performed with lysates from transfected HEK 293T cells (ATCC) or from brain homogenate (Pel-Freez). Triton X-100 solubilized HEK 293T cells or solubilized mouse brain were incubated at 4°C for 1h with 15 µl primary antibodies according to Table 6 e 7. As a negative control, samples were incubated without antibody for immunoprecipitations with monoclonal antibodies, or with pre-immune serum for polyclonal antibodies. Then, samples were incubated for 2h at 4°C with 50 µl protein-G sepharose (GE healthcare) (for monoclonal immunoglobulins) or 50 µl protein-A sepharose (GE healthcare) (for polyclonal rabbit sera). Sepharose was washed 5 times with 1% TX-100 in PBS (Sigma) and bound proteins were eluted with 2x Laemmli sample buffer containing DTT. Samples were analyzed by SDS-PAGE and immunoblotting.

2.10 Immunocytochemistry

Immunocytochemistry was performed either on HEK 293T cells (ACCT), which were transfected with syntaxin-1A *wt*, and C145S. Cells were washed 3 times with 37°C-warm PBS (Sigma) supplemented with 1 mM $MgCl_2$, and were then fixed for 15 min at room temperature in 4% paraformaldehyde (Thermo Scientific) in PBS. Fixed

cultures were washed three times with PBS with 1 mM $MgCl_2$ and permeabilized for 5min in 0.1% Triton X-100 (Sigma) in PBS. Cells were then washed 3x with PBS with 1 mM $MgCl_2$ and were blocked in 5% BSA (sigma) in PBS for 30 min at room temperature. Cultures were incubated with primary antibodies (Table 6 e 7) in 1% BSA in PBS overnight at 4°C. The next day, cultures were washed 3x in PBS and were blocked in 5% BSA (sigma) in PBS for 30 min at room temperature. Then, anti-mouse Alexa-488 and anti-rabbit Alexa-633 secondary antibodies (each 1:500 in 1% BSA in PBS) were added for 1h in the dark. Finally, cells were washed 3x in PBS, and coverslips were mounted on glass slides in Fluoromount-G (SouthernBiotech) and stored at 4°C. Laser scanning confocal microscopy was performed to compare localization, with serial excitation at 633nm and 488nm, on a Leica TCS SP-2 inverted microscope.

2.11 Statistical Analyses

Statistical analyses were performed using Prism software. Quantitative results are shown as means +/- SEM of n observations. In order to compare two sets of data, an unpaired *Student's t test* was used.

Table 6 – Primary Antibodies

Antibody	Clone	Company		Dilution	Protein size (kDa)
β-actin	AC-74	Sigma	Monoclonal	1:1000	45
c-myc	9E 10-a	Santa Cruz	Monoclonal	1:1000	n.a.
GFP	JL-8	Clontech	Monoclonal	1:2000	27
Golgi (GM-130)	EP892Y	Abcam	Monoclonal	1:500	130

Table 7- Primary Antibodies (continuation)

HA.11	16B12	Convance	Monoclonal	1:1000	n.a.
Munc-18	31	BD	Monoclonal	1:1000	68
Munc-18	K329	Made in house	Polyclonal	1:1000	68
Rab3	T957	Made in house	Polyclonal	1:1000	23
Rab3a	42.1	Synaptic Systems	Monoclonal	1:1000	23
SNAP-25	71.1	Synaptic Systems	Monoclonal	1:1000	25
SNAP-25	P913	Made in house	Polyclonal	1:1000	25
Synaptobrevin-2	69.1	Synaptic Systems	Monoclonal	1:1000	18
Synaptogamin-1	41.1	Synaptic Systems	Monoclonal	1:1000	65
Syntaxin-1	HPC-1	Synaptic Systems	Monoclonal	1:1000	35
Syntaxin-1	438B	Made in house	Polyclonal	1:1000	25
Ubiquitin	P4D1	Santa Cruz	Monoclonal	1:200	7.5
VCP	K331	Made in house	Polyclonal	1:1000	100

CHAPTER 3

RESULTS

3.1 NEM

3.1.1 NEM: Previous results

Unpublished results have shown that out of the three neuronal SNARE proteins, NEM (**N**-**E**thyl**m**aleimide) specifically increases the levels of monomeric syntaxin-1. Despite this more than two-fold increase in syntaxin-1 levels, levels of SNARE complexes were unaffected (Figure 9).

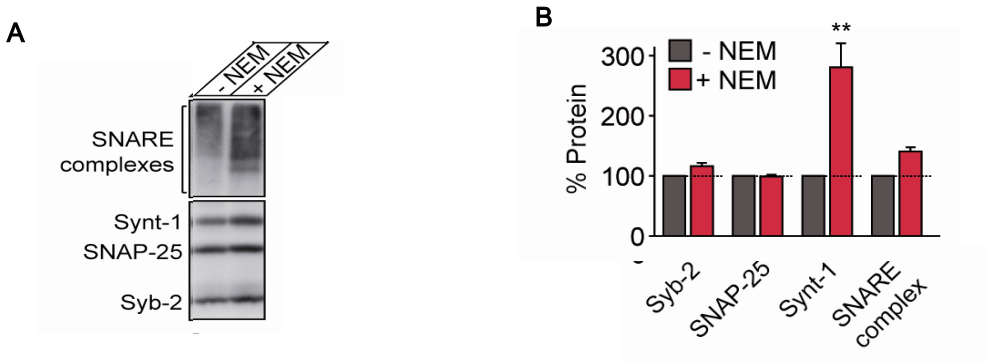


Figure 9 – Effect of NEM on synaptobrevin-2, SNAP-25, syntaxin-1 and SNARE complex levels in brain homogenate. Equal amounts of brain homogenate were treated with 2 mM NEM or vehicle control (ethanol) over night at 37°C. Reaction was stopped by addition of 5x laemmli sample buffer containing DTT. A) Samples were analyzed by immunblotting for levels of SNARE-complexes, syntaxin-1 (Synt-1), SNAP-25 and synaptobrevin-2 (Syb-2). B) Protein levels were quantitated using ^{125}I-labeled secondary antibody and were normalized to the vehicle control. ** $p < 0.01$ using student's T-test (n = 3).

NEM is an alkylating agent, which contains an imide functional group, and irreversibly modifies thiol groups of molecules[31]. NEM is an irreversible inhibitor of all cysteine peptidases, with alkylation occurring at their active site (Figure 10). It has also the capability of blocking vesicular transport, and has been used as an inhibitor of deubiquitinases[31].

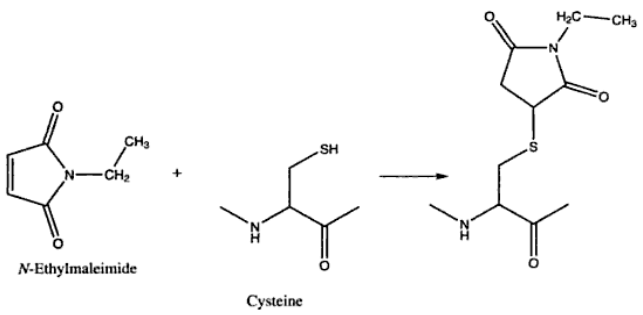


Figure 10 – NEM reaction with the thiol group of a cysteine.

Syntaxin-1A has only 3 cysteine residues: C145 in the linker, and C171 and C172 in the transmembrane region. A C145S point mutation was generated based on the following aspects: 1) cysteine 145 is conserved among species except yeast (Figure 11) and 2) this cysteine is the only one that is "exposed" to NEM during the treatment and can thus play a role in stability, degradation, and/or ubiquitination of syntaxin-1.

```
rat           QSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFASGIIMDS--
mouse         QSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFASGIIMDS--
human         QSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFASGIIMDS--
orang-utan    QSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFASGIIMDS--
cow           QSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFASGIIMDS--
dog           QSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFASGIIMDS--
frog          QSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFSSGIIMDS--
chicken       QTDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFSSGIIMDS--
rabbit        QSDYRERCKGRIQRQLEITGRTTTSEELEDML----------
zebra fish    QSEYRERCKGRIQRQLEITGRKTTKEELETILESDNPSIFTTGVFMDC--
C.elegans     QVDYRDGCKKRLQRQMEITGRATTNEELEDMLESGNPAIFTQGIITDT--
fly1          QTDYRERCKGRIQRQLEITGRPTNDDELEKMLEEGNSSVFTQGIIMET--
bee           QTDYRERCKGRIQRQLEITGRTTTNEELEEMLEQGNPAVFTQGIIMET--
aphid         QTDYRERCKGRIQRQLEITGRTTTNDELEEMLEQGNPAVFTQGIIMET--
yeast1        AKESLEASEMANDAALLDEEQRQNSS--KSTRIPGSQIVIERDPINNE--
yeast2        QNNYLKFLNKDDLKPIRNKASAENTLLLDDEEEEAAREKREGLDIEDYSK

Hc domain
```

Figure 11 - Syntaxin-1A sequence alignment. Yellow boxes highlight the evolutionary conservation of C145 among different species.

Interestingly, C145S mutation in syntaxin-1 abolished the NEM-mediated effect on syntaxin-1 levels (Figure 12). Furthermore, the cysteine to serine mutation resulted in syntaxin-1 levels similar to NEM-treated WT syntaxin-1 (Figure 12). This points to a de-stabilization of WT syntaxin-1 in absence of NEM, which can be abolished by introduction of the cysteine to serine mutation.

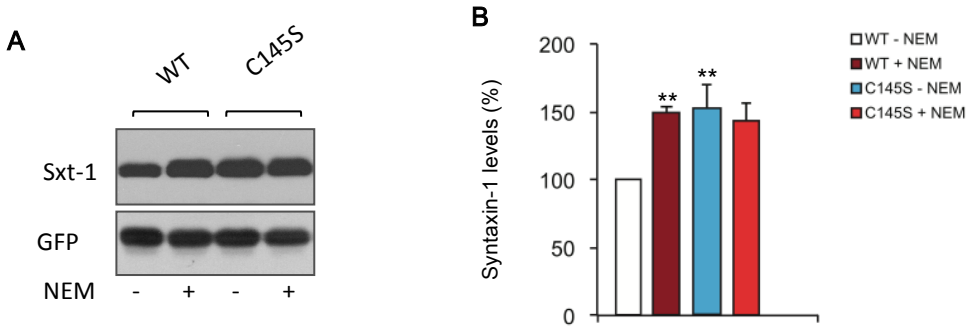


Figure 12 – Effect of NEM on levels of syntaxin-1 WT and mutated C145S in transfected HEK cells treated with 5 mM NEM for 5min at 37°C. Reaction was stopped by washing cells with PBS and solubilization in 0.1% Triton-X 100 (TX-100). Insoluble material was removed by centrifugation for 20 min at 10,000g. Supernatant was collected and 5% Laemmli sample buffer containing DTT was added. A) Samples were analyzed by immunblotting for levels of syntaxin-1 WT and C145S and GFP, which was used as transfection control. B) Protein levels were quantitated using ^{125}I-labeled secondary antibody and were first normalized to GFP levels and then to syntaxin-1 WT without NEM treatment. ** $p < 0.01$ using student's T-test ($n = 3$).

3.1.2 NEM experiments in neuronal cultures

In order to analyze the effect of NEM *in situ,* neuronal cultures were incubated with a range of NEM concentrations between 10 µM to 5mM. In this range, NEM concentrations do not show differences in syntaxin-1 levels (Figure 13).

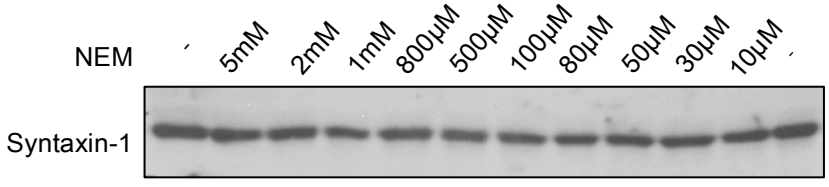


Figure 13 - Effect of different NEM concentrations on syntaxin-1 levels in neuronal cultures treated with a NEM concentration of 10 µM to 5mM. Neuronal cultures at 14 days *in vitro* were incubated for 5 min with NEM or vehicle control (ethanol). Syntaxin-1 levels were analyzed by immunblotting.

Since a NEM concentration of 5 mM does not seem to affect neuron health, a series of 9 NEM experiments were performed in neuronal cultures treated with a final concentration of 5mM NEM and normalized to EtOH (ethanol; vehicle control) since NEM was diluted in EtOH. First, experiments reveal no significant difference between untreated neurons and neurons treated with EtOH. Second, a significant decrease in syntaxin-1 levels of 33% was observed (Figure 14).

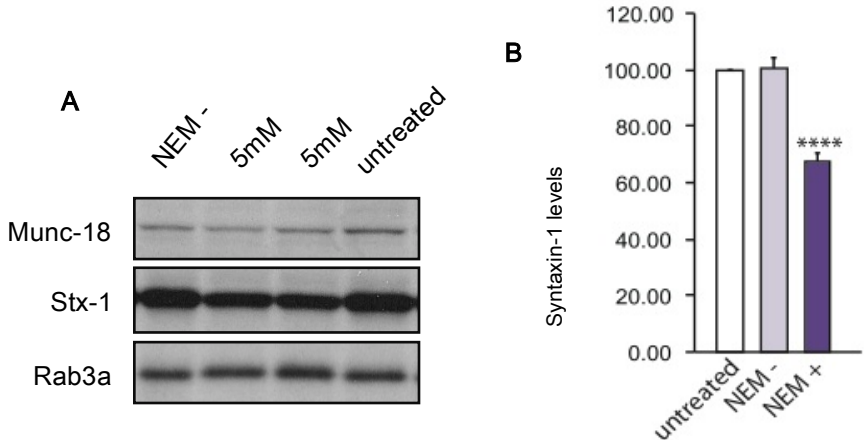


Figure 14 – Effect of vehicle control and 5 mM NEM on syntaxin-1 levels in neuronal culture. Neuronal cultures at 14 days *in vitro* were incubated for 5 min with 5 mM NEM, vehicle control or were kept untreated. B) Munc-18, syntaxin-1 (Stx-1) and rab3A levels were analyzed by immunblotting; B) Proteins levels were quantitated using ^{125}I-labeled secondary antibodies. Syntaxin-1 levels were normalized to rab3A levels and quantitated as percent of levels of untreated neurons. **** $p < 0.0001$, using Student's T-test ($n = 9$).

3.2 Syntaxin-1 wild-type and C145S

In order to check if syntaxin-1 C145S is correctly folded and targeted to membranous compartments, immunocytochemistry on transfected HEK cells was performed. As shown in Figure 15 no difference between C145S and syntaxin-1 WT targeting was observed.

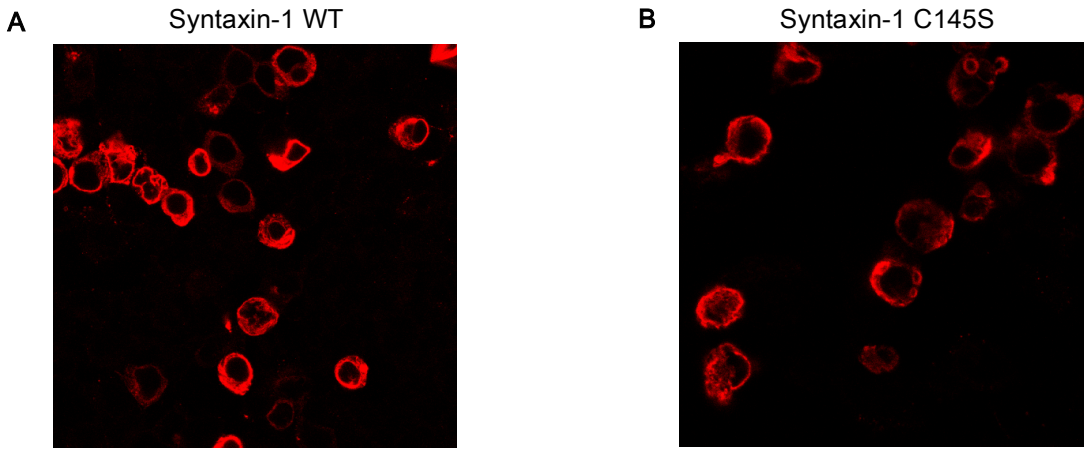


Figure 15 – Targeting of syntaxin-1 wild-type and C145S mutation in transfected HEK cells. 48h after transfection, HEK cells were washed fixed for 15 min in 4% paraformaldehyde and permeabilized for 5min in 0.1% Triton X-100. Cells were then blocked in 5% BSA and were incubated with primary antibody HPC-1 in 1% BSA overnight at 4°C. Cultures were washed and blocked, and an anti-mouse Alexa-488 secondary antibody (1:500 in 1% BSA) was added for 1h in the dark. Coverslips were mounted on glass slides in Fluoromount-G. Laser scanning confocal microscopy was performed with excitation at 488nm on a Leica TCS SP-2 inverted microscope.

3.3 Syntaxin-1 levels in presence of Munc-18 and SNAP-25

In order to analyze a possible chaperone for syntaxin-1, munc-18 or SNAP-25 were co-transfected with syntaxin-1 wild type and C145S. α-synuclein$^{1\text{-}95}$ was used in syntaxin-1 transfections in order to balance the amount of transfected munc-18 or SNAP-25 DNA.

3.3.1 Syntaxin-1 wild-type and C145S full length

For full-length syntaxin-1, a significant increase of 76% can be observed when munc-18 is present. As shown previously, syntaxin-1 C145S reveals higher expression levels than syntaxin-1 wild-type (a significant increase of 90%). An increase is also observed when SNAP-25 is co-transfected; however, this increase is lower when compared to transfections with munc-18 (Figure 16).

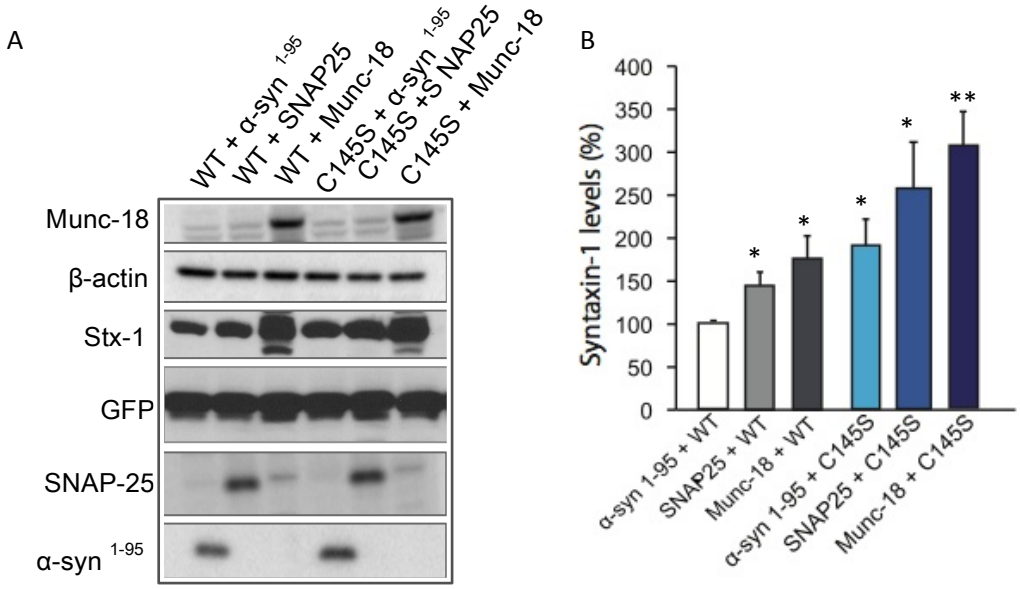


Figure 16 – Syntaxin-1 levels in co-transfected HEK cells. HEK cells were transfected with syntaxin-1 (Stx-1) wild-type (WT) or C145S full length and with α-synuclein$^{1-95}$, SNAP-25 or munc-18. 48hours after transfection, cells were solubilized with 0.1% Triton-X 100 (TX-100) and insoluble material was analyzed by immunblotting for levels of syntaxin-1 WT and C145S (A). B) Protein levels were quantitated using ^{125}I-labeled secondary antibody and were normalized to β-actin and GFP. * p <0.05 ** p <using student's T-test (n = 3).

3.3.2 Syntaxin-1 wild-type and C145S lacking the transmembrane region

To analyze the effect of the transmembrane region, syntaxin-1 was truncated to residues 1-264. Using this construct, a significant increase in syntaxin-1 levels can be observed in presence of munc-18. Interestingly, syntaxin-1 C145S lacking the transmembrane region is less expressed than wild type when transfected without any possible chaperone (Figure 17).

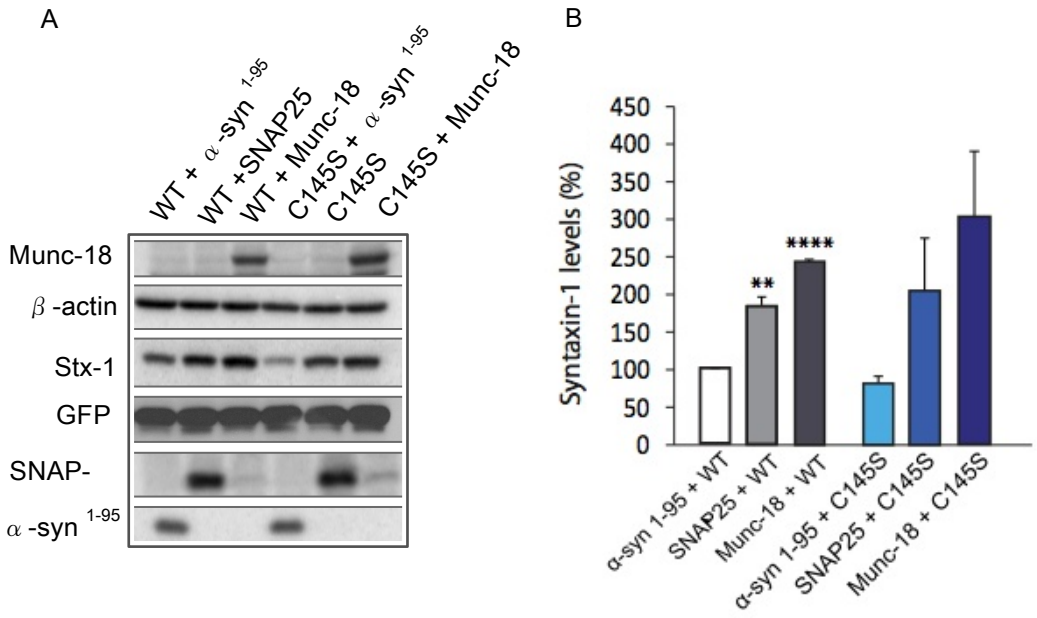


Figure 17 – Syntaxin-1 levels in co-transfected HEK cells. HEK cells were transfected with syntaxin-1 (Stx-1) wild-type (WT) or C145S $^{1-264}$ and with α-synuclein$^{1-95}$, SNAP-25 or munc-18. 48hours after transfection, cells were solubilized with 0.1% Triton-X 100 (TX-100) and insoluble material was analyzed by immunblotting for levels of syntaxin-1 WT and C145S (A). B) Protein levels were quantitated using ^{125}I-labeled secondary antibody and were normalized to β-actin and GFP. ** $p < 0.01$; **** $p < 0.0001$ using student's T-test (n = 3).

3.3.3 Syntaxin-1 lacking the N-terminal H_{abc} domain

Munc-18 has been shown to interact with the Habc domain of syntaxin-1. To analyze syntaxin-1 stability without H_{abc} domain, syntaxin-1 was co-transfected with SNAP-25 or munc-18. Results show a 419.9% increase of syntaxin-1 levels when SNAP-25 is present. No changes were observed in presence of munc-18 (Figure 18).

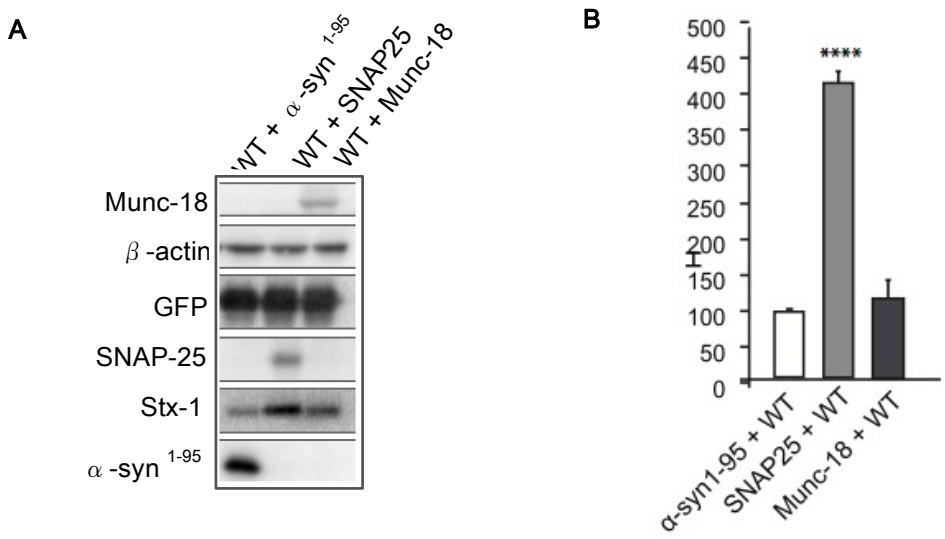


Figure 18 – Syntaxin-1 levels in co-transfected HEK cells. HEK cells were transfected with syntaxin-1 (Stx-1) wild-type (WT) or C145S $^{180\text{-}288}$ and with α-synuclein$^{1\text{-}95}$, SNAP-25 or munc-18. 48hours after transfection, cells were solubilized with 0.1% Triton-X 100 (TX-100) and insoluble material was analyzed by immunblotting for levels of syntaxin-1 WT and C145S (A). B) Protein levels were quantitated using ^{125}I-labeled secondary antibody and were normalized to β-actin and GFP. **** $p < 0.0001$ using student's T-test (n = 3).

3.3.4 Syntaxin-1 WT lacking the H_{abc} domain and the transmembrane region

To investigate the effect of a lack of Habc domain and transmembrane region, syntaxin-1$^{180\text{-}264}$ was generated. This truncation construct expresses the SNARE motif only. Results show that this truncation is only expressed when SNAP-25 is present resulting in an increase of syntaxin-1 levels to 882.2% (Figure 19).

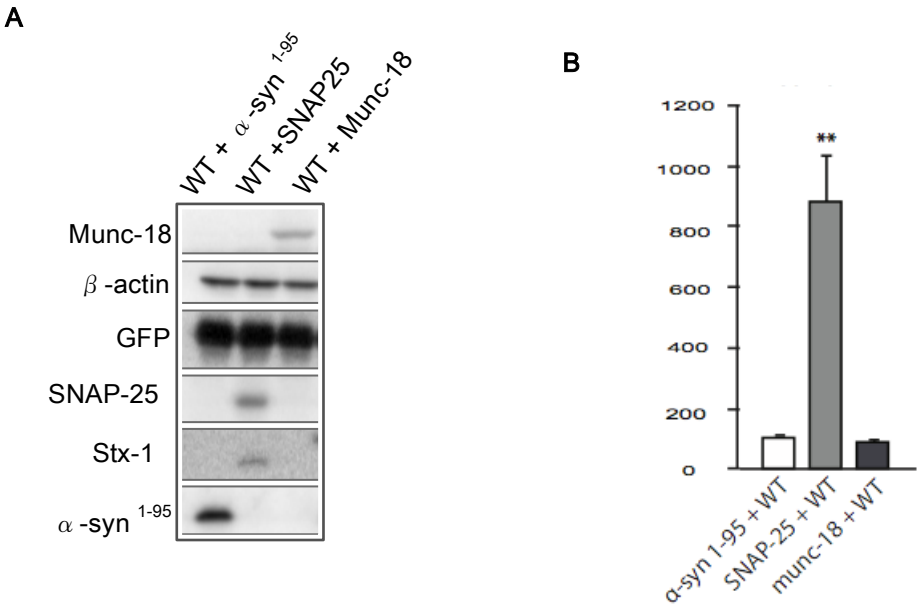


Figure 19 – Syntaxin-1 levels in co-transfected HEK cells. HEK cellswere transfected with syntaxin-1 (Stx-1) wild-type (WT) or C145S $^{180-264}$ and with α-synuclein$^{1-95}$, SNAP-25 or munc-18. 48hours after transfection, cells were solubilized with 0.1% Triton-X 100 (TX-100) and insoluble material was analyzed by immunblotting for levels of syntaxin-1 WT and C145S (A). B) Protein levels were quantitated using ^{125}I-labeled secondary antibody and were normalized to β-actin and GFP. ** $p < 0.01$ using student's T-test (n = 3).

3.4 Interaction between syntaxin-1 and SNARE proteins

Immunopreciptitation in brain homogenate was performed in order to probe for possible interactions between munc-18 and syntaxin-1 or between SNAP-25 and syntaxin-1. As the immunoprecipitations reveal, syntaxin-1 interacts with SNAP-25, possibly in the SNARE-complex. Also, syntaxin-1 shows a robust binding to munc-18 (Figure 20).

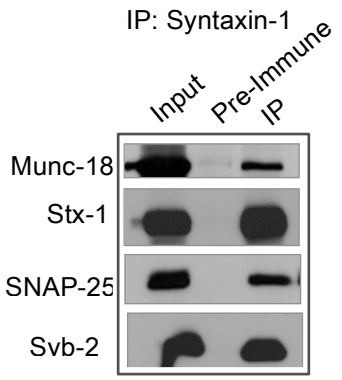


Figure 20 – Immunoprecipitation of SNARE proteins and munc-18 in brain lysate. Solubilized mouse brain was incubated at 4°C for 1h with 15 µl syntaxin-1 antibodies. As a

negative control, samples were incubated with pre-immune serum. Samples were then incubated for 2h at 4°C with 50 µl protein-A sepharose. Sepharose was washed 3x with 1% TX-100 and bound proteins were eluted with 2x Laemmli sample buffer containing DTT. Samples were analyzed by SDS-PAGE and immunoblotting for munc-18, syntaxin-1 (Stx-1), SNAP-25 and synaptobrevin-2 (Syb-2).

3.4 Interaction between syntaxin-1 and munc-18

According to the results shown above, syntaxin-1 full-length and 1-264 levels are increased when munc-18 is present. However, when the same experiment is performed with a truncation that lacks the H_{abc} domain, the levels of syntaxin-1 do not increase. Immunoprecipitations from brain lysate have shown that munc-18 and SNAP-25 interact with syntaxin-1. Yet, these experiments do not reveal a direct interaction, since binding could be mediated by a bridging protein. To probe for a direct interaction and to map the syntaxin-1/munc-18 binding interface, a series of immunoprecipitations (IP) was performed.

The results of the immunoprecipitation reveal an interaction for munc-18 with syntaxin-1 wild-type or C145S full length, as well as for the truncation that lacks the transmembrane region (1-264). Separate immunoprecipitations were performed with antibodies to syntaxin-1 or munc-18 to corroborate the result. For truncations lacking the H_{abc} domain and hence an epitope for the antibody to bind, the immunoprecipitation was performed using an HA antibody. As expected from the stabilization data, truncation 180-288 does not bind to munc-18. Truncation 180-264 that lacks the H_{abc} domain and transmembrane region was not expressed in presence of munc-18 (Figure 21).

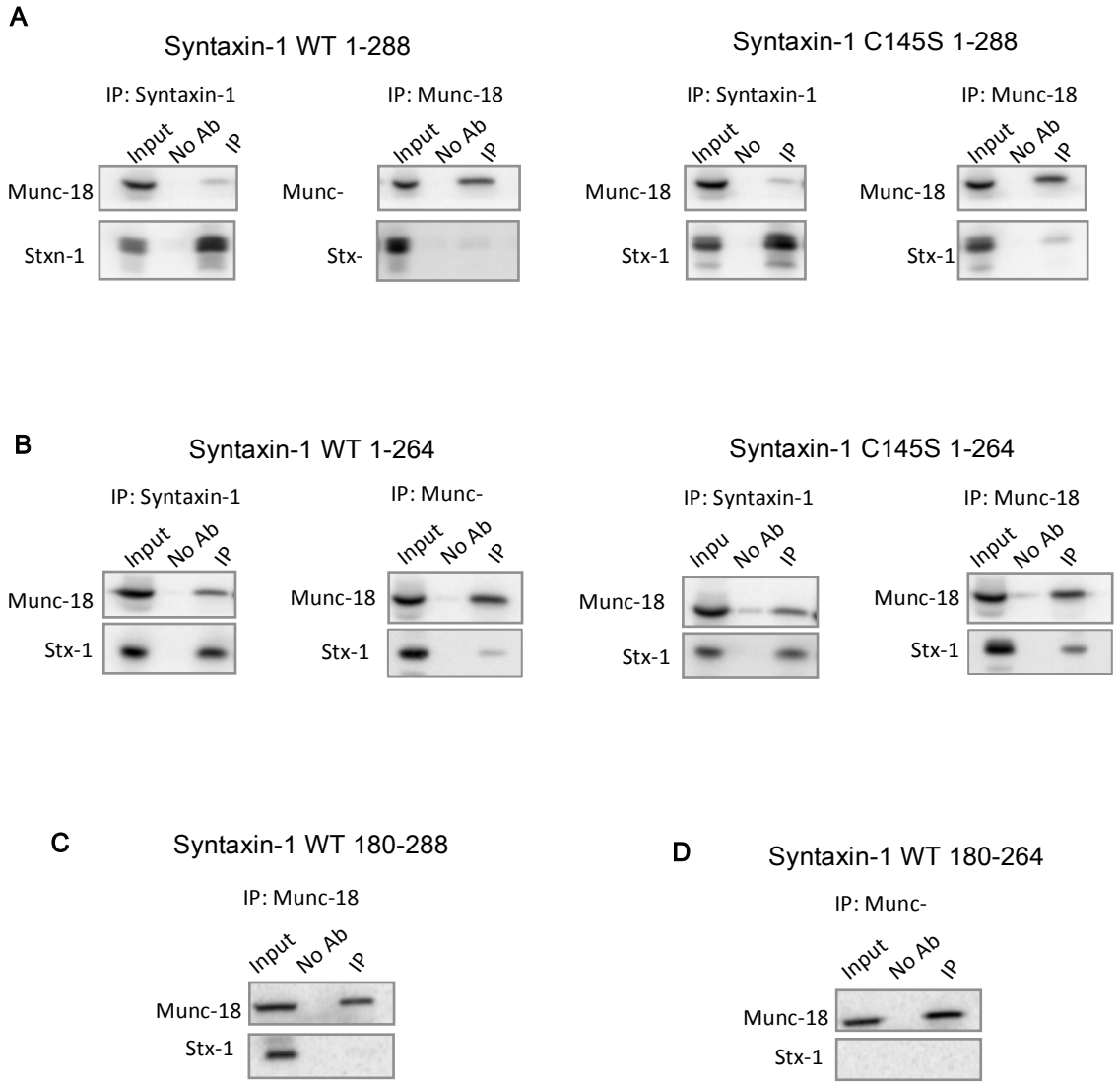


Figure 21 – Immunoprecipitations of transfected HEK cells to map the binding domain of munc-18 and syntaxin-1. HEK cells were co-transfected with munc-18 and A) full length syntaxin-1 wild-type (WT) and C145S, B) syntaxin-1 wild-type and C145S lacking the transmembrane region, C) syntaxin-1 wild-type lacking the H_{abc} domain, and D) syntaxin-1 wild-type lacking the H_{abc} domain and the transmembrane region. Solubilized HEK 293T cells were incubated at 4°C for 1h with 15 µl primary antibodies. As a negative control, samples were incubated without antibody for immunoprecipitations in case of monoclonal antibodies, or with pre-immune serum for polyclonal antibodies. Samples were then incubated for 2h at 4°C with 50 µl protein-G sepharose (for monoclonal immunoglobulins) or 50 µl protein-A sepharose (for polyclonal rabbit sera). Sepharose was washed with 1% TX-100 and bound proteins were eluted with 2x Laemmli sample buffer containing DTT. Samples were analyzed by SDS-PAGE and immunoblotting for munc-18 and syntaxin-1 (Stx-1).

3.5 Syntaxin-1 stability

3.5.1 HEK 293T cells

Since syntaxin-1 levels were increased in presence of the mutation C145S, a cycloheximide chase was performed for 24 hours in HEK cells transfected with syntaxin-1 wild-type and α-synuclein$^{1-95}$ or syntaxin-1 C145S and α-synuclein$^{1-95}$ in order to check for stability differences between syntaxin-1 wild-type and C145S. α-synuclein$^{1-95}$ was added as a control since this protein is not very stable so cycloheximide effects would be evident. After 24 hours, wild-type syntaxin-1 shows a reduction of 36.1%. In contrast, syntaxin-1 C145S levels are not decreasing, even after 24 hours. Yet, appearance of syntaxin-1 degradation products can be observed. When compared after 24 hours, levels of syntaxin-1 wild-type and C145S reach a significant difference upon cycloheximide treatment (Figure 22).

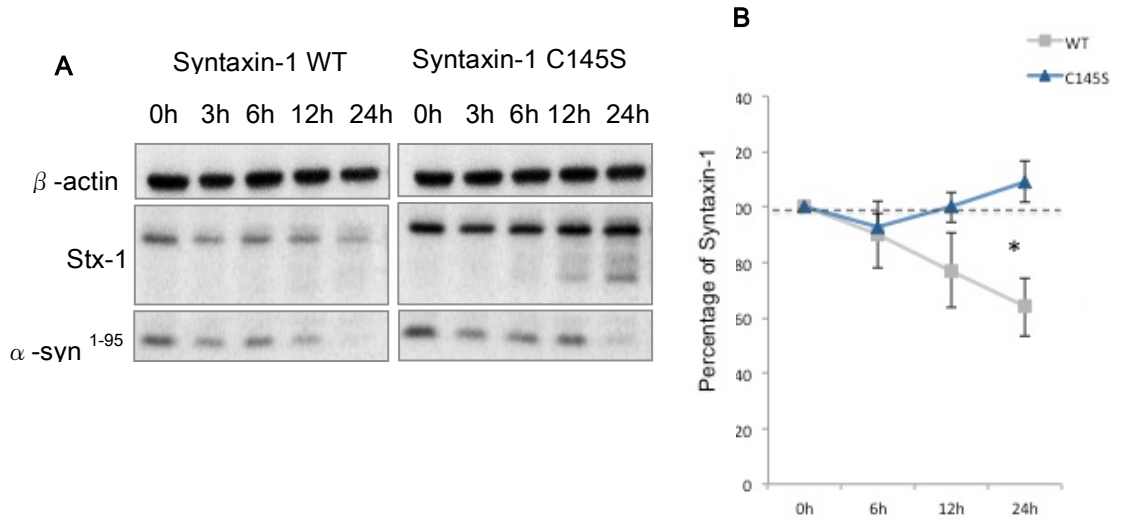


Figure 22 – Analysis of syntaxin-1 stability using a cycloheximide chase experiment. HEK cells co-transfected with syntaxin-1 (Stx-1) wild-type (WT) or C145S and α-synuclein$^{1-95}$ (a-syn$^{1-95}$) were treated with 0.1g/L cycloheximide and analyzed 0h, 6h, 12h, and 24h after treatment. Cells were solubilized with 0.1% Triton-X 100 and soluble material was analyzed by SDS-PAGE and immunblotting for syntaxin-1 (A). B) Protein levels were normalized to beta actin levels and were quantitated using ^{125}I-labeled secondary antibody. * $p < 0.03$ using student's T-test (n = 3).

Does munc-18 change syntaxin-1 stability, and does it affect only wild-type or wild-type and C145S similarly? To address this, HEK cells 293T were co-transfected with munc-18 and syntaxin-1 wild-type or C145S. An increase in syntaxin-1 wild-type levels was observed in presence of munc-18. In contrast, syntaxin-1 C145S stability shows no munc-18 dependent changes, although the levels of degradation products decrease (Figure 23).

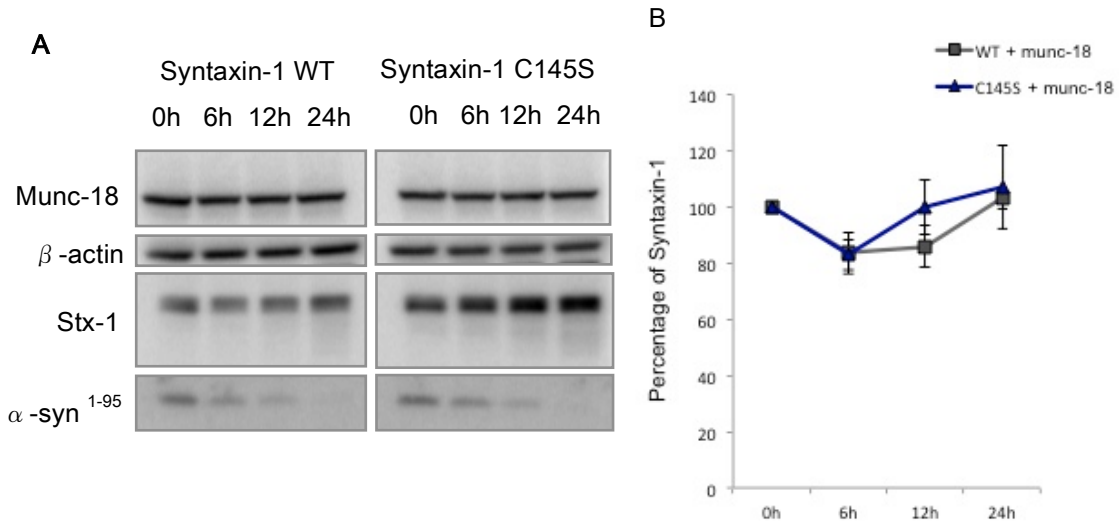


Figure 23 – Analysis of syntaxin-1 stability using a cycloheximide chase experiment. HEK cells co-transfected with syntaxin-1 (Stx-1) wild-type (WT) or C145S, α-synuclein$^{1-95}$ (a-syn$^{1-95}$) and munc-18 were treated with 0.1g/L cycloheximide and analyzed 0h, 6h, 12h, and 24h after treatment. Cells were solubilized with 0.1% Triton-X 100 and soluble material was analyzed by SDS-PAGE and immunblotting for syntaxin-1 (A). B) Protein levels were normalized to beta actin levels and were quantitated using ^{125}I-labeled secondary antibody. p < n.s. using student's T-test (n = 3).

3.5.2 Neuronal Cultures

To replicate the effect seen in transfected HEK cells, a cycloheximide chase was performed for 72 hours in neuronal cultures. This experiment reveals that the syntaxin-1 half-life is approximately 50 hours (Figure 24).

To analyze munc-18 dependent syntaxin-1 stability, cycloheximide chase experiments should have been performed with overexpression or knockdown of munc-

18. Due to time limitations and due to problems with virus production, this aim could not be achieved.

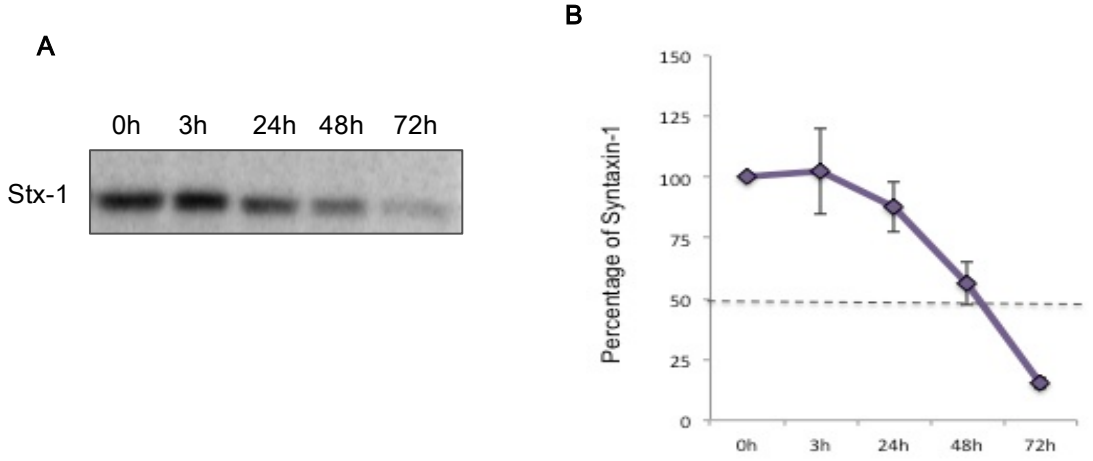


Figure 24 – Analysis of syntaxin-1 half-life in neuronal culture. Neuronal cultures at 11 days *in vitro* were incubated with 0.1g/L cycloheximide. (A) Syntaxin-1 (Stx-1) levels at 0h, 3h, 24h, 48h, and 72h were analyzed by immunblotting (B) and were quantitated using ^{125}I-labeled secondary antibodies, normalized to the 0h levels (n=3).

3.6 Dependence of syntaxin-1 levels on synaptic activity

Syntaxin-1 is part of the SNARE complex and therefore shuttles between a folded conformation in the SNARE complex and an unfolded and highly unstable conformation as a monomer. Its levels may thus be influenced by synaptic activity. To investigate this, neuronal cultures were treated with synaptic activity blockers (APV and tetrodotoxin - TTX) and synaptic activity enhancers (K^+; Ca^{2+}). The results reveal a trend towards an increase in syntaxin-1 levels with synaptic silencing and a decrease in syntaxin-1 levels with synaptic activity, but do not reach significance (Figure 25). Also here, it would have been interesting to analyze whether lack of munc-18 enhances this effect. However, due to reasons give above, this experiment was not performed.

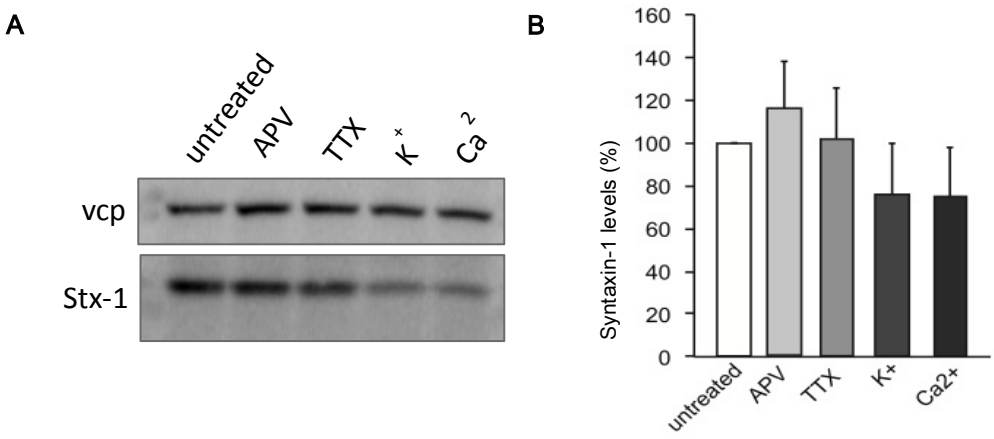


Figure 25 - Effect of synaptic activity on syntaxin-1 levels. Neuronal cultures at 12 days *in vitro* were incubated for 36h with 20 µM APV, 5 µM tetrodotoxin (TTX), 25 mM K^+, 4mM Ca^{2+} or were kept untreated. Syntaxin-1 (Stx-1) and valosin-containing protein (VCP) levels were analyzed by immunblotting (A) and were quantitated using ^{125}I-labeled secondary antibodies (B). Syntaxin-1 levels were normalized to vcp and quantitated as percent of levels of untreated neurons(n = 3).

3.7 Degradation of syntaxin-1

To analyze whether degradation is enhanced in wild-type syntaxin-1 compared to the C145S mutant, syntaxin-1 wild-type and C145S were transfected into HEK cells and degradation products of syntaxin-1 (bands below the full length immunosignal) were quantitated. Analysis results in a significant difference between syntaxin-1 wild-type and C145S, with the wild-type giving rise to ~10-fold more degradation products than C145S (Figure 26 A, B e D).

In order to understand what may happen to syntaxin-1 degradation in presence of munc-18, wild-type syntaxin-1 was co-transfected with munc-18 into HEK cells. In presence of munc-18, syntaxin-1 degradation products are reduced about 2-fold (Figure 26 A, C, D).

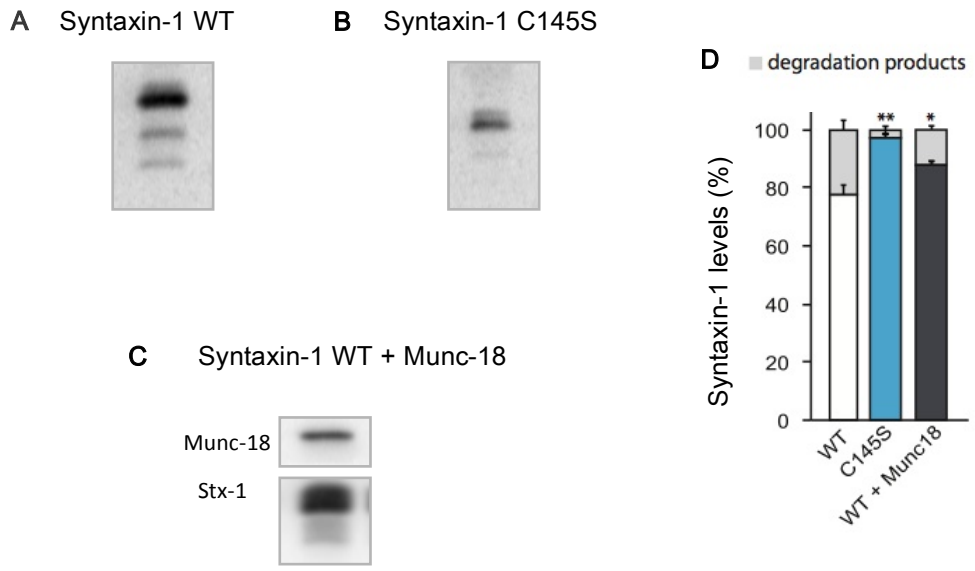


Figure 26 – Analysis of syntaxin-1 degradation A) Syntaxin-1 wild-type (WT), B) Syntaxin-1 C145S; C) Syntaxin-1 WT plus munc-18; were transfected into HEK cells. 48h after transfection, cells were solubilized with 0.1% Triton-X 100 and soluble material was analyzed by immunblotting for syntaxin-1. D) Full-length and degraded protein levels were quantitated using ^{125}I-labeled secondary antibody, with degradation products expressed as percent of total syntaxin-1 protein. * $p < 0.03$ and ** $p < 0.01$ using student's T-test (n = 3).

3.8 Ubiquitination of syntaxin-1

Next we asked whether syntaxin-1 degradation happens via ubiquitination. To approach this question, transfected HEK cells were subjected to an immunoprecipitation with syntaxin-1 antibody and samples were probed for ubiquitin. Analysis shows a significant difference in ubiquitination levels between wild-type syntaxin-1 and C145S (Figure 27).

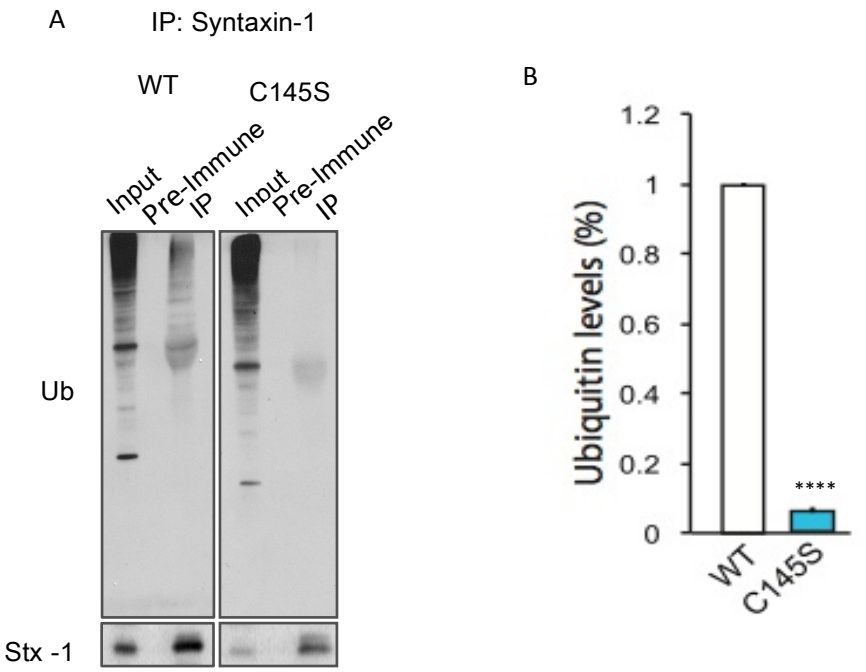


Figure 27 – Analysis of ubiquitination in HEK cells transfected with syntaxin-1 wild-type and C145S. Immunoprecipitations were performed with lysates from HEK 293T cells transfected with syntaxin-1 (Stx-1) wild-type (WT) or C145S. Solubilized HEK 293T cells were incubated at 4°C for 1h with 15 µl primary antibody (438B). As a negative control, samples were incubated with pre-immune serum. Samples were then incubated for 2h at 4°C with 50 µl protein-A sepharose. Sepharose was washed with 1% TX-100 and bound proteins were eluted with 2x Laemmli sample buffer containing DTT. A) Samples were analyzed by SDS-PAGE and immunoblotting for ubiquitin (top) and syntaxin-1 (bottom). B) Ubiquitination was quantitated using ^{125}I-labeled secondary antibodies, normalized to the efficiency of the syntaxin-1 immunoprecipitation. **** $p < 0.0001$ using Student's T-test ($n = 3$).

3.9 Is syntaxin-1 degraded via the lysosome or proteasome?

Ubiquitination can result in protein degradation via the lysosome or proteasome. In order to analyze which pathway is mediating syntaxin-1 degradation, neuronal cultures were incubated with 5 different inhibitors, 3 proteasome inhibitors (MG132, epoxomicin, clasto-lactacystin) and 2 lysosome inhibitors (leupeptin/pepstatin and PMSF). A trend towards a stabilization of syntaxin-1 using lysosomal inhibitors can be observed. However, this change does not reach significance yet ($n = 2$). In contrast, proteasome inhibitors reveal only very modest changes (Figure 28).

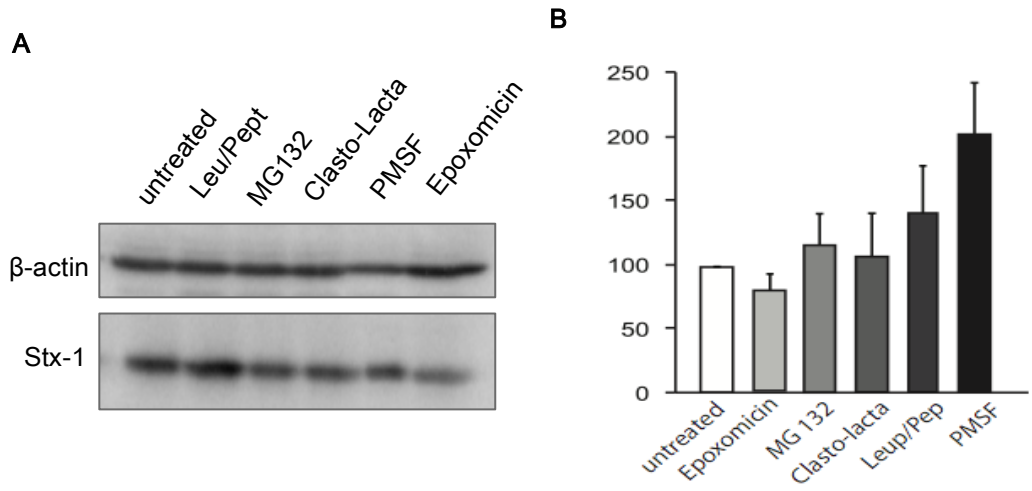


Figure 28 - Effect of proteases inhibitors on syntaxin-1 levels in neuronal culture. Neuronal cultures at 12 days *in vitro* were incubated for 36h with 10 µM Epoxomicin, 10 µM MG132, 10 µM Clasto-lactacystin, 10 mg/L Leupeptin/Pepstatin, 4 mM PMSF or were kept untreated. Syntaxin-1 (Stx-1) and β-actin levels were analyzed by immunblotting (A) and were quantitated using ^{125}I-labeled secondary antibodies (B). Syntaxin-1 levels were normalized to β-actin and quantitated as percent of levels of untreated neurons (n = 2).

CHAPTER 4

DISCUSSION AND CONCLUSION

Although NEM experiments in brain homogenate showed a reliable increase of syntaxin-1 levels in presence of NEM, NEM experiments performed in neuronal cultures revealed that independently of the concentration used (10μM to 5mM), NEM reduced the levels of syntaxin-1. The reduction in syntaxin-1 levels observed with 5mM NEM is neither due to NEM toxicity nor neuronal death. In fact, the observed decrease can be due to the fact that neuronal cultures where made from P0 mice and the chemical treatment was performed at day 14 whereas previous results in brain were performed on old mice. Possibly, NEM shows its effect on syntaxin-1 levels only in later stages when the entire SNARE machinery may be more compromised due to heavy usage, like reported for SNAP-25 and synaptobrevin-2[15,16]. Consequently, an option would be to perform NEM treatment on neuronal cultures at a later time point, e.g. at day 21. Additionally, since munc-18 interacts with and stabilizes syntaxin-1, it may be worthwhile to perform the NEM treatment on neuronal cultures upon knockdown of munc-18. In this case, syntaxin-1 may be more unstable and the stabilizing effect of NEM may be more prominent.

Experiments performed in transfected HEK cells for the analysis of syntaxin-1 levels and interaction domain mapping reveal that both syntaxin-1 wild type and C145S full-length bind to munc-18. Due to this interaction munc-18 increases syntaxin-1 levels, which makes munc-18 a very strong candidate for a syntaxin-1 chaperone. On the other hand, mutation of cysteine 145 to serine results in a stabilization of syntaxin-1 levels. Consequently, levels of syntaxin-1 C145S are similar to levels of syntaxin-1 wild-type when co-transfected with munc-18.

It is reported in the literature that SNAP-25 binds syntaxin-1 in the SNARE motif[17]. This is in agreement with results presented here which show that the interaction between SNAP-25 and syntaxin-1 increases syntaxin-1 levels. This increase is not as high as the increase observed when munc-18 is present; yet, at this point, SNAP-25 cannot be excluded as a syntaxin-1 chaperone.

Even in absence of the transmembrane region (syntaxin-1 $^{1-264}$), munc-18 and SNAP-25 increase the levels of syntaxin-1. Since syntaxin-1 $^{1-264}$ still has the SNARE motif and the H_{abc} domain, munc-18 and SNAP-25 can bind to syntaxin-1 and increase syntaxin-1 levels. Also here, munc-18 has a bigger effect on syntaxin-1 levels compared to SNAP-25, making munc-18 a more likely chaperone for syntaxin-1.

Compared to wild-type levels, syntaxin-1 C145S lacking the transmembrane region reveals a decrease in syntaxin-1 levels. This decrease can be due to the fact that cytosolic syntaxin-1 is per se more unstable and the C145S mutation by itself may be actually more unstable than the wild-type when lacking the membrane anchor.

Analyses of syntaxin-1 truncations that do not express the H_{abc} domain (180-264 and 180-288) show no stabilizing effect by munc-18. This reveals that munc-18 needs the H_{abc} domain to chaperone syntaxin-1, supported by the fact that this domain is the binding interface for munc-18 as shown by the immunoprecipitations. Interestingly, when these two constructs were co-transfected with SNAP-25, syntaxin-1 levels increased with both constructs (180-264 and 180-288), presumably because the SNARE motif was still present.

The observed difference between syntaxin-1 $^{180-264}$ and syntaxin-1 $^{180-288}$ constructs reveal, that the transmembrane region is important for syntaxin-1 stability. Thus, when syntaxin-1 is cytosolic and not membrane anchored, syntaxin-1 $^{180-264}$ (SNARE motif only) expression is observed only in presence of SNAP-25. In contrast, when syntaxin-1 $^{180-288}$ (SNARE motif plus transmembrane region) is expressed, the transmembrane region stabilizes syntaxin-1 and this syntaxin-1 truncation is expressed not only when SNAP-25 is present but also in presence of munc-18 or α-synuclein$^{1-95}$.

To summarize: 1) The C145S mutation increases syntaxin-1 levels; 2) The transmembrane domain is important for the stability of syntaxin-1; 3) Munc-18 increases syntaxin-1 levels only when the H_{abc} domain is present; 4) When the SNARE motif is present, SNAP-25 increases syntaxin-1 level.

Since munc-18 shows a predominant effect on syntaxin-1 levels, further experiments were performed only with munc-18. However, more experiments addressing the syntaxin-1/SNAP-25 interaction and stabilization should be performed. Possibly, even SNAP-25 may stabilize syntaxin-1 levels and act as a syntaxin-1 chaperone.

To further analyze the effect of munc-18 and the C145S mutation on syntaxin-1 levels, cycloheximide chase experiments in HEK 293T cells were performed, showing that syntaxin-1 wild-type is less stable than syntaxin-1 C145S. Thus, this cysteine has an important role in stabilization of syntaxin-1. Furthermore, when munc-18 is present, syntaxin-1 wild-type levels are stable even after 24 hours. An explanation for the observation that syntaxin-1 levels reach values higher than 100% after adding a translation inhibitor cannot be due to different levels of syntaxin-1 at the starting point, since the cycloheximide treatment starts at the same time for all cells. Thus, the increase in syntaxin-1 levels can only be due to HEK 293T cell division during the chase. Additionally, cycloheximide allows a first round of translation before blocking protein synthesis[27], which may also result in levels higher than 100%. One way to overcome this problem would be to increase the cycloheximide concentration. However, increasing cycloheximide concentration resulted in cell death after 24hours (data not shown). Additionally, increasing the time course of cycloheximide treatment up to 36h also resulted in cell death (data not shown). Therefore, cycloheximide chase experiments should be performed in neuronal cultures with knockdown or overexpression of munc-18 together with analysis of endogenous syntaxin-1 levels. Moreover, to investigate the effect of the C145S mutation, both, tagged syntaxin-1 wild-type and C145S should be expressed, together with knockdown or overexpression of munc-18.

Syntaxin-1 is a pre-synaptic protein with an active role during synaptic activity, and is repeatedly folded and unfolded during the SNARE cycle, which may make it vulnerable regarding synaptic activity. The results investigating changes in syntaxin-1 levels during synaptic activity are not conclusive. Yet, they indicate a trend for a decrease in syntaxin-1 levels when synaptic activity is enhanced. During synaptic activity, more vesicle fusion occurs and therefore more vesicles are recycled. As a consequence, syntaxin-1 changes very fast between a SNARE complex form and an unfolded and highly reactive monomeric form, making it more prone to degradation. On the other hand, when synaptic activity is blocked, syntaxin-1 levels remain stable. Here, synaptic vesicles are primed on the pre-synaptic membrane, waiting for the Ca^{2+}-trigger. Syntaxin-1 folding/unfolding cycle is stopped and therefore, the levels of syntaxin-1 do not change. In order to understand if syntaxin-1 stability is affected by the presence or absence of munc-18, neuronal cultures with overexpression and knockdown of munc-18 are proposed as a future plan.

Analysis of syntaxin-1 degradation products corroborated previous results showing that C145S stabilizes syntaxin-1: degradation products are less in syntaxin-1 C145S compared to wild-type syntaxin-1. Munc-18 did not only stabilize syntaxin-1 but reduced also its degradation, leading to the next question: is this degradation due to ubiquitination?

Cysteine residues can be modified in numerous ways: oxidation, glutathionylation, nitrosylation, acylation, and ubiquitination, resulting frequently in modulation of protein activity. Since cysteines have a thiol group, they can form thiol esters, which in turn can be ubiquitinated. Although protein ubiquitination at cysteine residues has been described[32-34], it is thermodynamically the least favorable event. Also, a thiol ester bond appears not to be the most efficient way to link ubiquitin to a protein, unless the aim is to create an activated form of ubiquitin[32]. Therefore, the result showing that syntaxin-1 wild-type is significantly more ubiquitinated than syntaxin-1 C145S could mean that syntaxin-1 is ubiquitinated on that specific residue of cysteine, or that this cysteine residue is important for signaling that the protein should be degraded. In this case, ubiquitination would occur on lysine residues, for instance on

lysine residue 155, which is next to cysteine 145. In order to clarify if ubiquitination is occurring on this specific cysteine residue, as a future plan, mass spectrometry should be performed.

Since syntaxin-1 wild-type is more ubiquitinated than syntaxin-1 C145S and since syntaxin-1 is a transmembrane protein and consequently probably sorted to the inner membranes of endosomes and lysosome after ubiquitination as many membrane proteins[35], neuronal cultures were treated with proteasome inhibitors and lysosome inhibitors in order to understand if syntaxin-1 protein is degraded via lysosome or proteasome. Results obtained are not conclusive yet, and more experiments have to be performed. However a slight increase with lysosome inhibitors pepstatin/leupeptin and PMSF was observed. In contrast, no changes were observed upon application of proteasome inhibitors epoximicin, clastro/lactocystin, and MG132, suggesting that syntaxin-1 may be degraded via the lysosome.

To conclude: 1) Altogether the results shown in this study suggest munc-18 as a chaperone for syntaxin-1. It increases syntaxin-1 levels when co-expressed and it inhibits the degradation of syntaxin-1 in HEK cells when co-transfected. Yet, experiments in neuronal cultures upon knockdown and overexpression of munc-18 are essential for a final confirmation; 2) Mutation of C145 to serine significantly stabilizes syntaxin-1 levels. Syntaxin-1 levels are higher, syntaxin-1 is degraded much slower and less degradation products can be observed. Also, replacement of the cysteine by serine dramatically reduces ubiquitination of the protein. However, the precise role of this residue in the degradation pathway needs further experiments; 3) Syntaxin-1 may be degraded via the lysosome. Lysosomal inhibitors revealed a trend towards stabilization of the protein, whereas proteasomal inhibitors showed no change. However, more experiments, e.g. analyzing proteasomal and lysosomal inhibitors during synaptic activity, are needed to confirm this hypothesis (Figure 29).

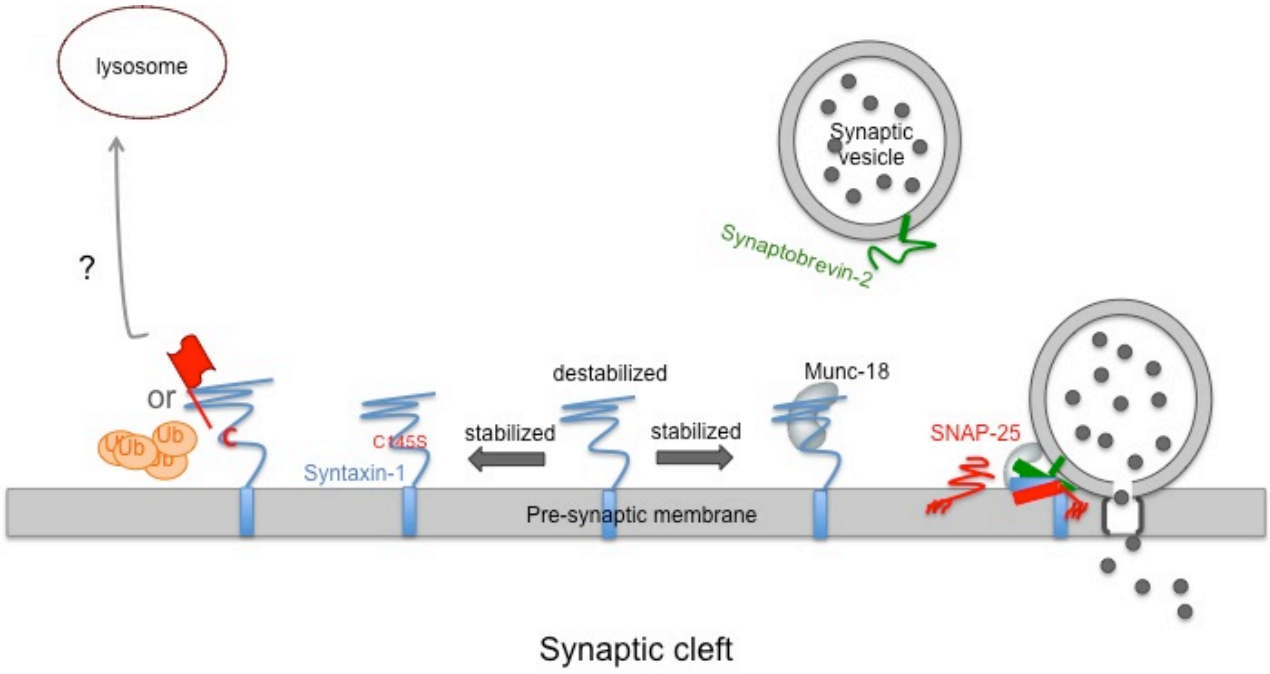


Figure 29 - Model depicting the stabilization of syntaxin-1 by munc-18 and the C145S mutation. Syntaxin-1may be degraded via ubiquitiniation and the lysosome. Ubiquitination of syntaxin-1 may happen on the cysteine residue mutated in the C145S mutant, or this cysteine reside may signal the cell to degrade syntaxin-1.

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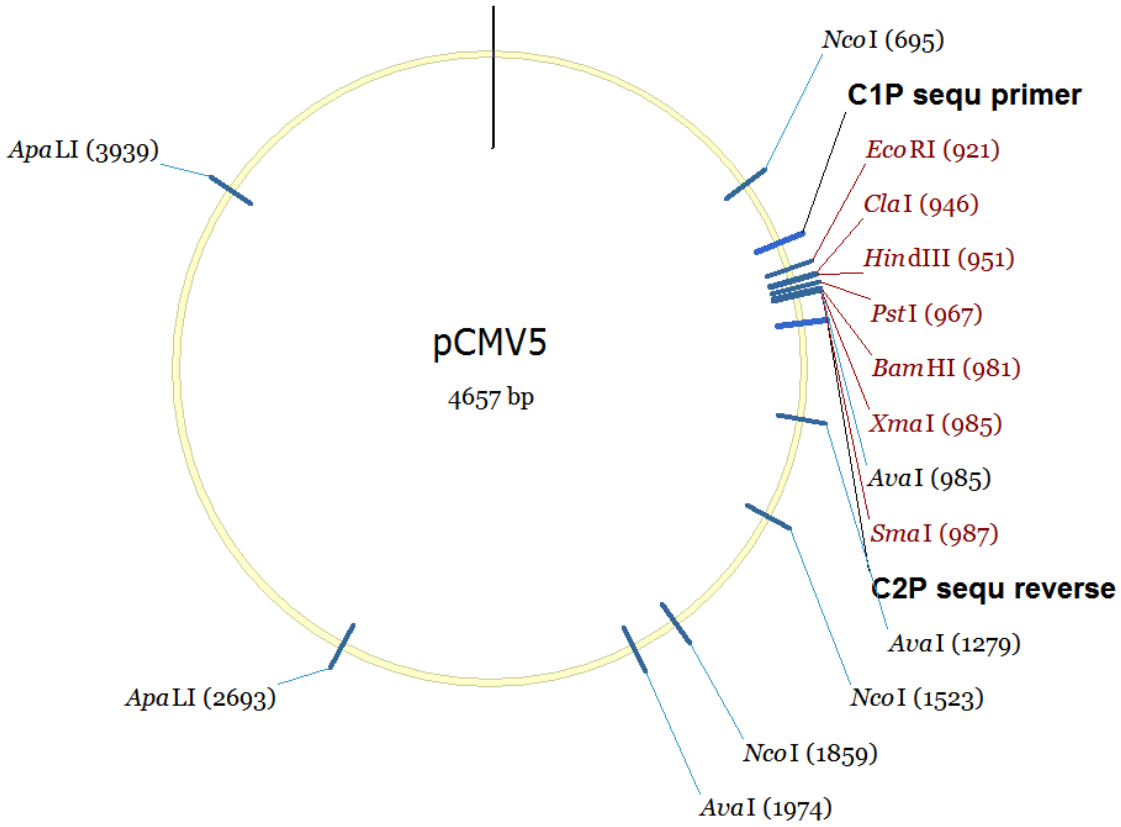
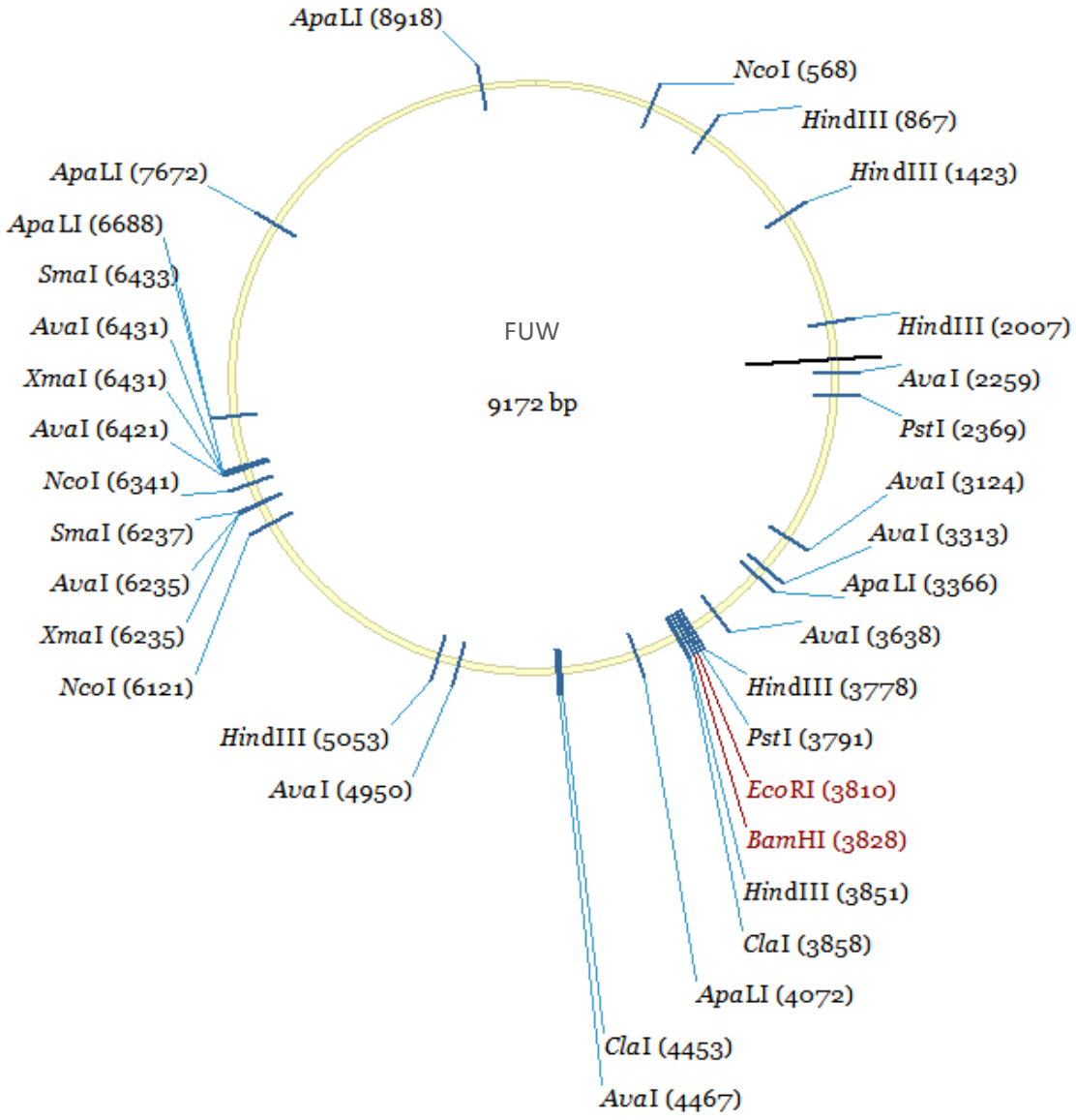
ATTACHMENTS

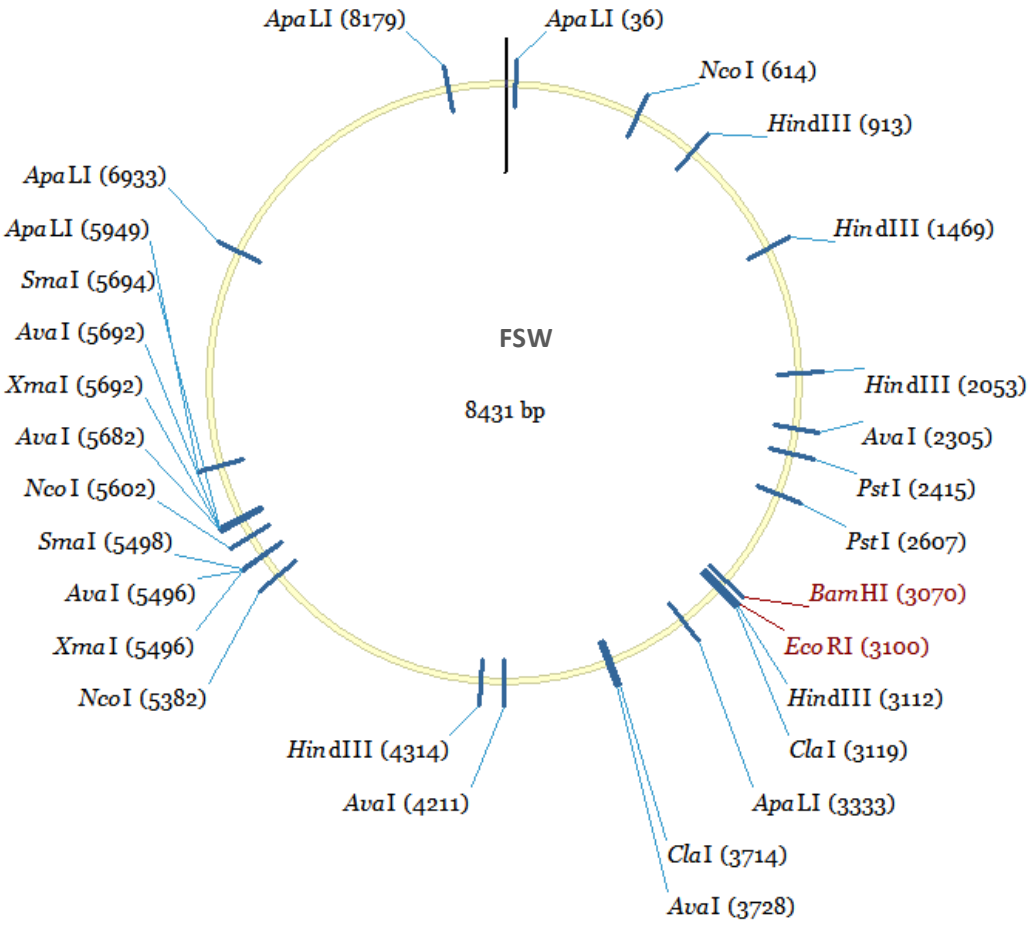
Plasmid Vector maps



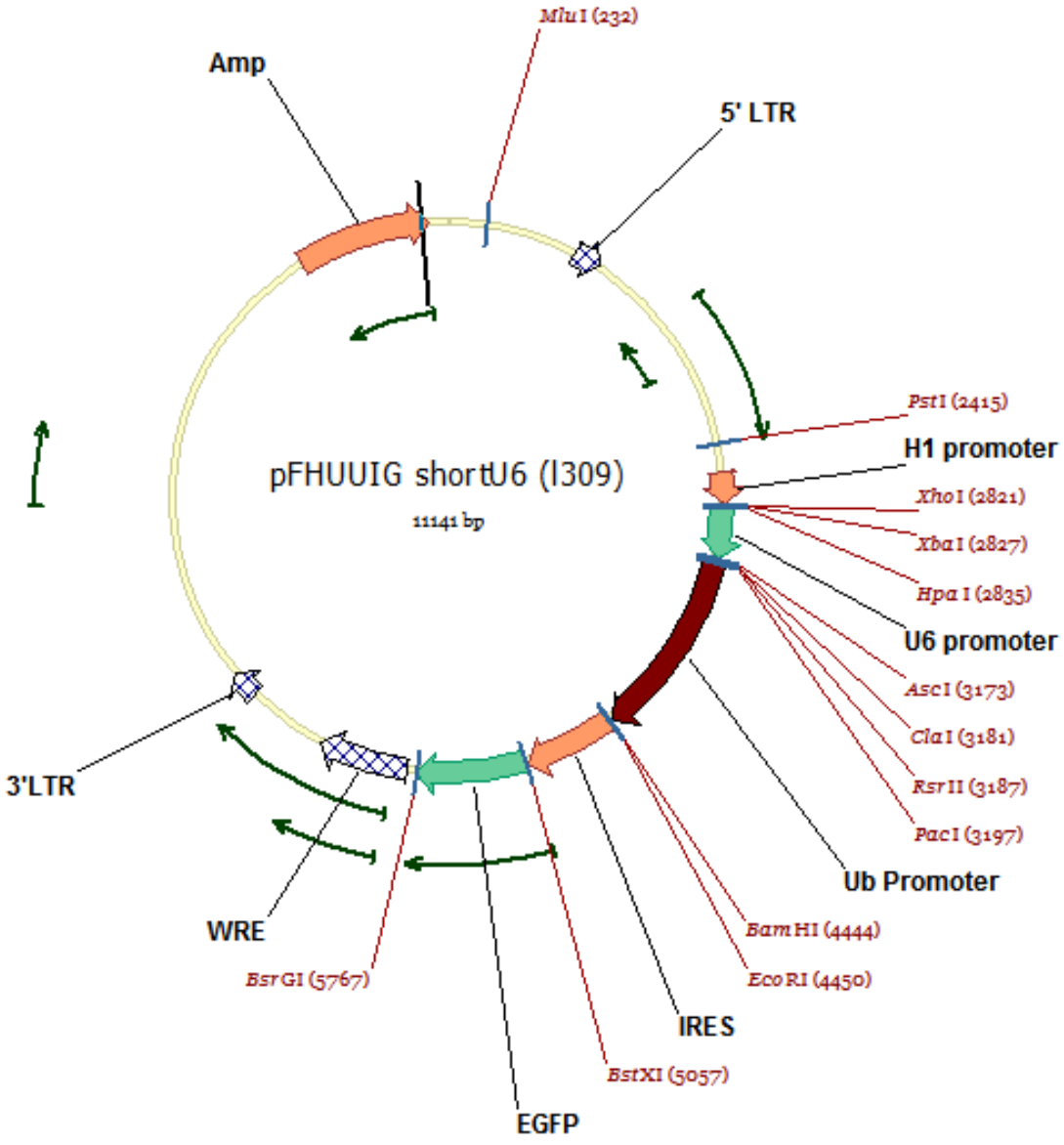
pCMV5 plasmid vector showing EcoRI site



FUW plasmid vector showing EcoRI site



FSW plasmid vector showing EcoRI site



L309 plasmid vector showing it relevant elements and EcoRI site