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Carlos Manuel Santos Resende

O papel da Ube3a no desenvolvimento neuronal

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Ramiro Daniel Carvalho de Almeida, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

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Dedico este trabalho aos meus amigos pela paciência durante todo este trabalho. À minha namorada Anabela, pelo apoio incondicional.

o júri

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palavras-chave

Síndrome de Angelman, Ube3a, degradação proteassomal, atividade sináptica, sinapses excitatórias

resumo

Síndrome de Angelman é uma doença genética caracterizada por imprinting paternal e deleção maternal da Ube3a. Deste modo, pacientes com Síndrome de Angelman têm níveis reduzidos de expressão da Ube3a em várias regiões do cérebro, incluindo o hipocampo e o cerebelo. Pacientes com Síndrome de Angelman apresentam dificuldades motoras, retardação mental e ausência de fala. Ube3a é uma E3 ligase responsável pela ubiquitinação de proteínas levando a degradação proteasomal dessas proteínas e a perda de função tem sido associada com perda de plasticidade sináptica. Ainda que tenham sido identificados vários substratos desta proteína com um papel pós sináptico importante o seu papel a nível pré sináptico e a correlação com os sintomas encontrados ainda não é clara. A transcrição da Ube3a é induzida por atividade sináptica e libertação de glutamato durante os primeiros estágios de desenvolvimento indicando que a atividade da Ube3a é importante para regular a excitabilidade neuronal. Apesar disso, o papel desta proteína na formação e maturação das sinapses excitatórias é ainda desconhecido. Neste trabalho realizamos uma caracterização da expressão desta proteína em várias regiões dos neurónios do hipocampo de duas espécies de rato (*Mus musculus*) e (*Rattus norvegicus*). Nós observamos que a Ube3a está expressa em elevados níveis nos núcleos dos neurónios em estágio de desenvolvimento iniciais, mas está também expressa no citoplasma e axónios. Os nossos resultados mostram que esta proteína está altamente expressa pré-sinapticamente tendo maior presença em estágios de desenvolvimento inicias seguido de um decréscimo em estágios de desenvolvimento tardios. Além disso, demonstramos que expressar neurónios do hipocampo de Rato com uma proteína cataliticamente inativa perturba a formação e maturação de sinapses. Estes dados indicam que a função catalítica da Ube3a é necessária para promover a formação de sinapses excitatórias. Coletivamente, estes dados podem explicar as alterações cognitivas encontradas em pacientes com Síndrome de Angelman.

keywords

Angelman Syndrome, Ube3a, proteasomal degradation, neurodevelopment, synaptic activity, excitatory synapse

abstract

Angelman syndrome (AS) is a genetic disorder characterized by paternal imprinting and maternal deletion of Ube3a. Therefore AS patients have reduced levels of expression of Ube3a in several regions of the brain including the hippocampus and cerebellum. AS patients have motor impairment, mental retardation and absence of speech. Ube3a is an E3 ligase responsible for the ubiquitination of protein leading to the proteasomal degradation and the lack of function as been associated with loss of synaptic plasticity. Although there's been the identification of several Ube3a substrates with important role in the postsynapse the role of presynaptic Ube3a and the symptoms found in AS patients is still not clear. Ube3a transcription is induced by synaptic activity and glutamate release during early stages of development, indicating that neuronal excitability is important to regulate Ube3a activity. Nonetheless, Ube3a role in excitatory synapse formation and maturation is still not clear. In this work we did a subcellular characterization of Ube3a expression in several regions of Rat and Mouse hippocampal neurons. We observed that Ube3a is expressed at high levels within the nucleus of hippocampal neurons but is also present in the cytoplasm and along the axon. Our results show that Ube3a is highly expressed in the presynaptic compartments of neurons in early stages of development followed by a decrease in the later stages of development. Furthermore, we show that expressing a catalytic inactive form of Ube3a in rat embryonic hippocampal neurons disrupts synapse formation and maturation. Our data suggests that Ube3a catalytic function is necessary for promoting excitatory synapse formation. Collectively, these data contributes to a deeper understanding of the cognitive alterations found in patients with Angelman Syndrome.

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AMPA	α-amino-3-hydrozy-5-methyl-4-isoxazole propionic acid
ARC	Activity-regulated cytoskeleton-associated protein
AS	Angelman Syndrome
ASD	Austism Spectrum disorder
ATP	Adenosine triphosphate
BCA	Bicinchonic Acid
BSA	Bovine Serum Albumin
CNS	Central nervous system
DAPI	4',6'-diamino-2-fenil-indol
DIV	Day in Vitro
DNA	Deoxyribonucleic acid
DUB	Deubiquitylating enzymes
ECL	Enhanced chemiluminescence
GEF	Guanine exchange factor
GFP	Green fluorescent protein
HATS	Histone acetyltransferases
HBSS	Hanks' Balanced Solution
HDAC	Histone deacetylases
HECT	Homologous to the E6-AP Carboxyl Terminus
ICR	Imprinting control Region
KA	Kinurenic acid
Kb	Kilobase
LTP	Long-term potenciation
mRNA	Messenger Ribonucleic acid

mTORC	Mammalian target of rapamycin complex 1
NBM	Neurobasal medium
NMDAR	N-Methyl-D-aspartate
PBS	Phosphate- buffered saline
PBS-Triton	Phosphate buffered saline with triton
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PLL	Poly-L-Lysine
PMSF	Phenylmethylsulfonyl fluoride
PSD	Postsynaptic density
PVDF	Polyvinylidene fluoride
PWS	Prader-willi syndrome
RNA	Ribonucleic acid
RT	Room Temperature
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
SEM	standard error of mean
SNRPN	Small nuclear ribonucleoprotein polypeptide N
SK2	Small condutance potassium channels
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TE	Tris-EDTA buffer
TSC2	
UPS	Ubiquitin-proteasome system
UBE3A-ATS	Ube3a antisense transcript
WB	Western Blot
5-FDU	5-Fluorodeoxyuridine

Introduction

Introduction

Ubiquitin-proteasome function

The ubiquitin proteasome system (UPS) regulates the faith of countless number of proteins. In the process a small protein ubiquitin, a 76 amino-acid peptide with an N-terminus that leads to ubiquitination and allows the binding of several ubiquitin's once the first unit is attached to the substrate. The protein targeted is then transported to the 26S proteasome, localized both in nucleus and cytoplasm of cells, and degradated. The 26S is composed for a 20S subunit and two 19S regulatory subunits, and the 20S subunit is composed by four rings [3]. UPS is responsible for the control of oxidized, mutant and misfolded proteins and this allows a narrow control and destruction of proteins involved in major biological processes like cell cycle and apoptosis. The disruption or mutation of UPS-related proteins can lead to tumours, affect DNA replication and control of mitosis. Degradation of targeted proteins happens in the rings of the 20S subunit where the monomers of ubiquitin are removed by deubiquitinating enzymes and the targeted protein is open [4].

Monoubiquitination leads to modification of the protein function, localization or binding while polyubiquitination acts as mediator of cell signalling, activation of autophagy leading to degradation of the targeted protein. Proteins with Ubiquitin associated domains (UBA) bind to monoubiquitinated proteins protecting them from being polyubiquitinated or from suffering deubiquitination by deubiquitination enzymes (DUBs). In other hand DUB also protect monoubiquitination of proteins through deubiquitination saving them from being targeted to the proteasome [4]. Defects of the UPS contribute to neurodevelopmental disorders associated with loss of cognitive function through the accumulation of defective proteins and this accumulation can lead to progress of diseases.

The activation of ubiquitin is first achieved by an activating enzyme (E1) in an ATP-dependent manner and results in adenylation of the ubiquitin. Ubiquitin is then transferred to a conjugative enzyme (E2), and it's finally associated with a ubiquitin ligase (E3) capable of detecting the substrate. The HECT domain binds to an E2 with the C terminus of activated ubiquitin via cysteine residue. Ubiquitin ligase catalyses the bond of the lysine residue of the targeted protein and the C-terminal of ubiquitin [3].

Ubiquitin protein ligase E3A (UBE3A)

The UBE3A gene (also known as E6-associated protein, E6AP) encodes for Ube3a, an E3 protein ligase. UBE3A is homologous to E6AP HECT domain (Homologous to E6AP carboxyl terminus) that was first described as a protein involved in human papillomavirus mediating degradation of tumoral suppressor p53 and contains the active site of ubiquitin ligase, where ubiquitin is transferred to the subtract. Ube3a is located in the 15q 11.2-13Q locus, a part of a critical region that is frequently deleted or duplicated and has a dual role as ubiquitin ligase and coactivator of transcription. (Figure 1). Ube3a protein has 2 domains, HECT domain that contains the active site for the ubiquitin ligase and a highly conserved amino terminal region of Ube3a named AZUL (Amino- Zn finger of Ube3a Ligase). This domain binds to PSMD4, a 26S proteasome subunit that functions as receptor for ubiquitinated proteins with C-terminal, deleting this domain results in cytosolic accumulation of the target proteins. The N-terminus of some Ube3a isoforms interfere with nuclear retention, this terminus does not interfere with Ube3a binding to PSMD4 but with the retention itself [1].

Chromosomes are located in the nucleus of the human cells and carry the genetic information. Human body cells normally have 46 chromosomes and pairs are numbered from 1 to 22 leaving the sex chromosomes that are X and Y. Males have one X and one Y while females have two X chromosomes. The chromosomes have long and short arm that are designated as Q and P respectively.

The 15q 11.2-13Q region refers to bands (11-13) present in the long arm of chromosome 15[2]. The dual role of Ube3a function as co-activator of transcription and as E3 ligase responsible for targeting proteins for proteasomal degradation it's thought to be involved in the regulation of synaptic plasticity and dendritic spine morphogenesis and altered expression is related to Angleman Syndrome, a neurodevelopmental disorder. This dual function is illustrated in Figure 1.

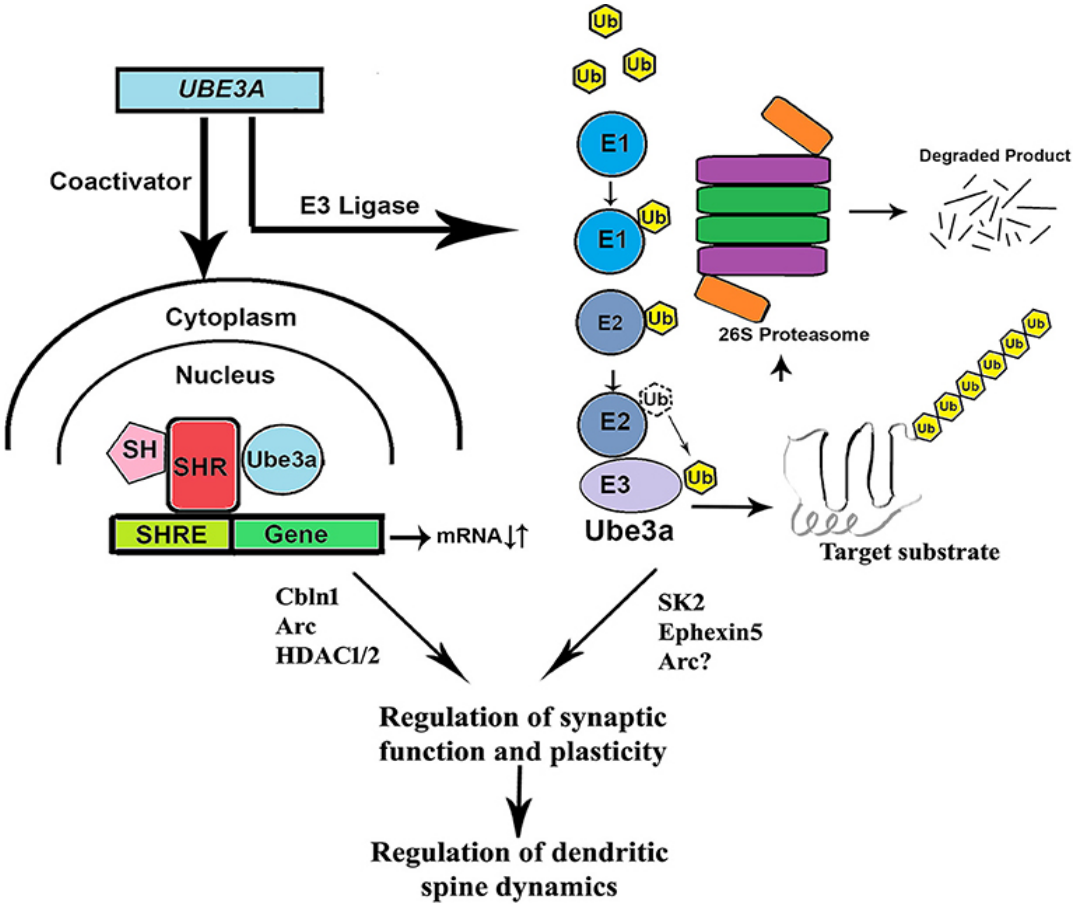

Figure 1. Ube3a has a dual function has a co-activator of transcription and as E3 ligase responsible for catalysing the bond of the lysine of the residue of the target protein and the C-terminal of the ubiquitin. Adapted from [5].

Subcellular localization and alternatively isoforms

Ube3a is highly enriched in the nucleus of human and mouse neurons, but has also been observed in the cytosol, within the dendritic spines and axon terminals with high labelling for growth cones. The localization of ube3a in nucleus and cytoplasm are consistent with its roles in proteasomal degradation and co-activator of transcription. Mice have 3 different isoforms, mUbe3a-Iso3 m-Ube3a-Iso2, the later is mainly localized ti the cytosol and has an additional 21 amino acids at the N-terminus (Table 1) [6]. Alternative polyadenylation of mouse isoform 1 results in a non-coding transcript and has been proposed to be a ligase deficient form[6,7].

In Humans, splicing generates three Ube3a isoforms, hUbe3a-Iso1 and hUbe3a-Iso3 are highly homologous to mUbe3a-Iso3 and mUbe3a-Iso2. Both isoforms are mainly localized in the nucleus.

In addition to these two isoforms, hUbe3a-Iso2 with no homologous in mice is localized to the cytoplasm and is the less abundant one (Table1). Similar to what was previous described in mice, in humans the loss of the most abundant nuclear isoform, hUbe3a-Iso1, results in AS-like phenotype [8]. Both in mice and humans the nuclear isoform accounts for the majority of Ube3a protein in neurons. Importantly, the loss of mUbe3a-Iso3 also results in deficits similar to AS.

Finally in Rat *(Rattus norvegicus)* Ube3a has only one isoform and is localized both in the nucleus and cytosol.

Table 1. *Ube3a isoforms in 3 organisms, Mouse, Human and Rat and subcellular distribution.*

	Rat	Human			Mouse		
Main Features	**Isoform 1 (Inferred)**	**Ube3a isoform 1**	**Ube3a isoform 2**	**Ube3a isoform 3**	**Ube3a isoform 1**	**Ube3a isoform 2**	**Ube3a isoform 3**
Protein Size (Aa)	868	852	875	872	762	870	849
Transcript size (bp)	10041	8544	5319	8728	3837	9224	9037
HECT domain (Aa)	516-866	500-850	523-873	520-870	497-762	518-868	497-847
Subcelullar Organization	Both nuclear and cytosolic	Nuclear [9,10]	Cytosolic [9,10]	Nuclear [9,10]	-----	Cytosolic [9,10]	Nuclear [9,10]
RefSeq mRNA	NM_001191837.4	NM_130838.4	NM_000462.5	NM_130839.5	NM_173010.4	NM_011668.3	NM_001033962.2
Protein RefSeq	NP_001178766.1	NP_570853.1	NP_000453.2	NP_570854.1	NP_766598.1	NP_035798.2	NP_001029134.1

The fact that hUbe3a-Iso3 and mUbe3a-Iso2 differ only in 3 amino acids in the N-terminus led Zampeta and colleagues to investigate the role of the N-terminus in the Ube3a subcellular localization (Figure2). The 3 amino acid change results in different subcellular localization since m-Ube3a-Iso2 has cytoplasmatic localization while hUbe3a-Iso3 is localized in the nucleus. Zampeta shows that adding a short N-terminal tag to human and mice homologous isoforms hUbe3a-Iso1 and mUbe3a-Iso3 results in the translocation to the cytosol. Adding to that, creating a chimeric protein, replacing N-terminal of hUbe3a-Iso3 for mUbe3a-Iso2 leads to an inverse in the localization showing that Ube3a subcellular localization is determined by the N-terminus [10].The existence of different isoforms of Ube3a is not new, but very little is known on how they are generated, their abundance and subcellular localization. In conclusion, several studies indicate that the amino acid sequence of the N-terminal defines the subcellular localization of Ube3a and that alterations in this sequence can lead to translocation to the cytoplasm[6,11].

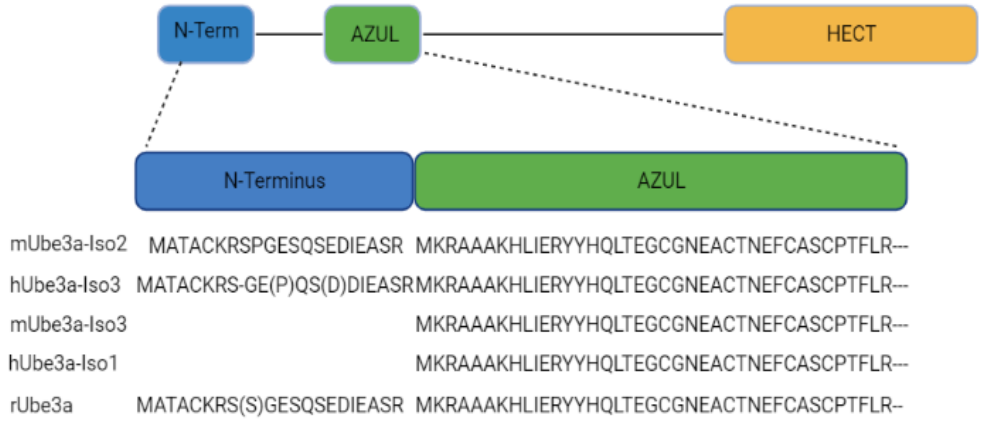

Figure 2. Ube3a Isoforms have different subcellular localization and alterations in N-terminus can interfere with nuclear retention. mUbe3a-Iso2 is homologous to hUbe3a-Iso3 but differences in the N-terminus results in different subcellular localizations. Amino acids change are delimited with parenthesis. Image adapted from [11].

Ube3a substrates

Given the existence of so many neurological phenotypes derived from Ube3a altered expression, one important task is to identify the Ube3a targets and their relevance for neurological diseases. In AS patients, Ube3a expression levels are decreased, this alteration in the expression is predicted to give origin to increased levels of Ube3a subtracts due to Ube3a role in proteasomal degradation. Conversely, when Ube3a levels are increased, there is over-degradation, both events lead abnormal function.

The identification of Ube3a substrates is mostly done by experiments where exists a loss or gain of function in Ube3a using transgenic mice models [12]. The first Ube3a substrate to be identified was p53 requiring not only Ube3a but a viral cofactor E6, giving the original name of Ube3a as E6-associated protein. Ube3a substrates and their alteration in AS patients are indicated Table 2.

Table 2. *Ube3a substrates and altered expression in AS.*

Protein	Function	Substrate	Levels in AS
P53	Cell cycle regulation [13]	yes	Increased
P27	Cell cycle regulation[14]	Yes	Increased
Arc	Endocytosis of AMPARs [15]	Yes	Increased
Ephexin5	Excitatory synapse formation [16]	Yes	Increased
GAT1	GABBA transporter [17]	Yes	Increased
PSMD4	Proteasome [18]	Unknown	Unknown
Ring1B	Transcription repressor [19]	Yes	Increased
SK2	Small-condutance potassium channel Neuronal intrinsic excitability [20]	Yes	Increased
P18	mTORC activity [21]	Yes	Increased
XIAP	Apoptotic cell death [22]	Yes	Increased
Sox9	Transcription factor[23]	Yes	Increased
Pb1/ECT2	Cytoskeleton remodeling/synapse formation [24]	Yes	Increased
Cbln1	Glutamatergic synapse organizer[25]	Yes	Decreased
HHR23A	DNA repair/proteasome [26,27]	Unknown	Increased
TSC2	mTORC inhibitor [28]	Yes	Increased
Huntington	Unknown	Yes	Increased
BMAL1	Circadian rhythmicity [29]	Yes	Increased
ALDH1L2	RA signaling[26]	No change	Yes

Ube3a major signalling pathways

SK2

Nerve cells generate electrical signals to send information over large distances and transmit it to other cells by synaptic connections. Synaptic activity is essential for processing information in the CNS and synaptic activity is modulated by Sk2, small conductance potassium channels important in sleep, learning and memory. SK2 channels are expressed in the postsynaptic membrane of glutamatergic synapses and help regulate the synaptic transmission [17]. A resting potential occurs because the membrane of nerve cells are permeable to K+ resulting in a negative membrane potential at rest. The action potential occurs when there is an increase in Na+ permeability, the increase permeability to Na+ is followed by an increase in k+ permeability repolarizing the membrane. Then the membrane rapidly repolarizes to resting levels due to inactivation of Na+ channels and increase in the K+ permeability and is hyperpolarized by the enhanced permeability to K+[30]. In neurons, the sk2 is enriched at postsynaptic density regions (PSD) and was previously described has colocalizing with PSD95. Postsynaptic density protein 95 is a postsynaptic protein found in excitatory neurons and regulates the trafficking and localization of glutamate receptors like AMPA (a-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate)[31]. The interaction between NMDA and SK2 plays a critical role in the regulation of excitability and induction of long-term potentiation (LTP) through the release of Mg2+ from the NMDA receptors after depolarization allowing glutamate release to induce postsynaptic response[32]. LTP induction regulates levels of synaptic SK2 by triggering endocytosis. SK2 have been previously identified has substrate of Ube3a, suggesting that Ube3a ubiquitinates C-terminus of SK2 and facilitates internalization. The lack of function of Ube3a in AS mouse models has shown to result in higher levels of SK2 in the synapse resulting in NMDAR decreased function, modulating synaptic plasticity and learning performance and could be responsible for intellectual disability found in AS patients [20].

Ephexin5

Ephexin 5 is RhoA GEF that supresses the excitatory synapse development through the activation of RhoA, and this suppression is cancelled upon contact of the axon with the developing dendrite and activation of EphB through contact with EphrinB. Initiation of a synapse depends of local recognition between the presynaptic and postsynaptic membranes.[33] This recognition is followed by accumulation of synaptic vesicles as well as transport vesicles that contain molecular components that contribute for the active zone. The contact between the axon and the developing dendrite is thought to be mediated by cell surface membranes. EphB receptor tyrosine kinases have been described to be localized in the synaptic sites of hippocampal neurons and having an important role in the cell-cell contact crucial for the excitatory synapse formation. Ephs are divided in two classes, EphA and EphB, depending on the ability to bind to EphrinA and EphrinB [16].

EphB is expressed postsynaptically in the surface of dendrites while EphrinB are expressed in axons and developing dendrites. When EphrinB encounters EphB in the developing dendrite, EphB is autophosphorylated increasing his kinase activity. This contact leads to activation of guanine Exchange factors (GEF) originating the actin cytoskeleton remodelling. In hippocampal neurons Ephexin5 limits excitatory synapse by interacting with EphB[34]. After the binding of EphrinB, EphB catalyses the phosphorylation of Ephexin5 leading to its degradation. The low levels of expression of Ephexin 5 help EphB to promote excitatory synapse development by activating several proteins. Ephexin5 is a direct substrate of Ube3a and is abnormally elevated in AS mouse models. Removing Ephexin5 from AS mice rescued deficits in dendritic spine number. The absence of Ube3a in mouse models of AS result in the increased expression of Ephexin5 leading to suppression of EPhB-mediated excitatory synapse formation and could explain cognitive deficits in AS patients [35].

Hdac1/2

Transcription is a highly regulated process and acethylatilation is known to have a role in this regulation. Histone modifications, like acetylation, is one type of modification that is well characterized due to the identification of histones acetyltransferases (HATs) proteins.[36] HATs function by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the amino group of certain lysine side chain within histone basic N-terminal tail region. Histone acetylation is a reversible process and deacetylases are also integral to the cycle of transcription. While acetylation is normally associated with activation lack of acetylation tends to correlate with repression. The correct balance between the activity of HAT and HDAC play a critical role in regulating gene transcription. Histones acetylation is implicated in increased synaptic formation and long-term potentiation and differential histone acetylation has been reported in Prader willi Syndrome [37]

HDAC 2 have been mentioned in literature as a negative regulator of the synaptic plasticity and memory formation. HDAC2 recruits several promotor regions and includes PSD95, Synaptophysin and Arc. HDAC1/2 are Histone deacetylases that are found in increased amounts in AS mice with subsequent diminish in H3(K9) and H4 (K12) acetylation. Although HDAC1/2 are degraded by the proteasome, Ube3a is not responsible for this degradation meanwhile Ube3a is a regulator of HDAC1/2 transcription. Experiments with Ube3a Knockdown and AS mice show that HDAC1/2 transcription levels are increased in the absence of Ube3a. This could explain the increased amounts of HDA1/2 in AS mice. Some HDAC inhibitors are already used as drug treatment, sodium valporate is used as a drug to treat seizures in AS patients and simvastatin as cholesterol lowering drug and both have shown to rescue some behavioural phenotype. [36,37]

Arc

Activity regulated cytoskeletal (Arc) is predominantly expressed in glutamatergic neurons especially in the hippocampus and cortical neurons. Arc is trafficked to dendrites and translated at the synapses where it controls synaptic strength through endocytosis of AMPA receptors, a type of glutamate receptors, and modulates spine morphology. AMPA receptors are mediators of excitatory neurotransmission in the CNS. Arc is sensitive to dysregulations and alteration in the expression have been implicated in many neurodevelopmental disorders including Fragile X and Angelman syndrome leading to cognitive dysfunction [38].

A study shows that Ube3a ubiquitinates Arc leading to its degradation and therefore regulating synaptic plasticity. In AS patient's low levels of Ube3a are found and Arc levels are accumulated in neurons this leading to excessive internalization of AMPA receptors at synapses. This high levels of expression can be an explanation to cognitive problems that are found in AS patients [15].On the other hand there are several studies where authors suggest that Ube3a and Arc do not interact physically but that knockdown of Ube3a expression stimulates Arc gene transcription. Thus, it is difficult to determine if Arc is in fact a Ube3a subtrate[38,39]. Arc and Ube3a levels are very low without synaptic activation while with release of glutamate in the excitatory synapses both Arc and Ube3a levels are rapidly increased and there is endocytosis of AMPAR receptors suggesting that Arc is important to regulate neuronal excitability. Ube3a mRNA is increased with glutamate receptor activation suggesting that Ube3a is regulated by synaptic activity and glutamate release during early life experiences. This could mean that both Ube3a and Arc transcription are induced in response to neuronal activity [40]. Arc Knockdown impairs long term potentiation and memory consolidation and have deficiency in experience dependent plasticity similar to AS mouse model.

Angelman Syndrome

Ube3a has been associated with Prader-willi syndrome, Angelman Syndrome and Autism Spectrum Disorder. Prader-Willi syndrome (PWS) is originated from the deletion of paternal allele, while Angelman syndrome (AS) is caused by deletion of maternal allele, wich associated with genomic imprinting originates low levels of ube3a in the brain. On the other hand, the duplication of 15q11.2-13q has been associated has a genetic cause of Autism spectrum disorder (ASD). Angelman syndrome was first described in 1965 by Dr. Harry Angelman, an English physician that coined this disorder as the "Happy Puppet Syndrome" due to frequent laughter and smiling AS is considered to be extremely rare, it is now estimated to occur at 1 in 15.000 births[2]. AS patients are normally characterized by ataxia, microcephaly, mental retardation, abnormal motor movements and ventricular dilatation, absence of speech and seizures. Children with Angelman syndrome have developmental delays and have significant communication difficulties and most never develop the capability to speak more than a few words. An early trace in children with AS is a movement imbalance due to the difficulty in coordinate voluntary movements. This movement disorders start to be apparent at approximately 6 months of age and some motor milestones like walking are normally delayed. In some severe cases children cannot even walk till they reach 5-10 years of age [41]. Frequent and inappropriate episodes of laughter and excitement are also seen in patients with AS[2]. Several studies link AS to 5 defects in the maternally 15q11.2-13Q locus and the major causes are 15q11.2-13q deletion. Paternal uniparental disomy, genomic imprinting defects and mutations or deletions. Deletion of the chromosomal region including the imprinted domain plus some non-imprinted genes accounts for 65-70% of the cases. The deletions can be divided in serval types depending on the extension. In more severe cases, patients can have a larger deletion including three GABA receptor genes (GABRB3, GABRA5, GABRG3). GABRB3 and NIPA2 are well documented in cases of epilepsy and AS patients with deletion of maternal 15q11.2-13q region have one or both of this genes deleted (Figure 3) [26].

Paternal uniparental dissomy accounts for 1-2% of the cases and results in individuals with two paternal copies. Imprinting defects account for 1-3% of the individuals resulting from imprinting defect affecting the ICR. These patients have two copies of chromosome 15, but lack of expression of maternal Ube3a due to UBE3A-ATS transcript. Ube3a mutations account for 5-10% of the cases and have been found in the entire coding region with more incidence in exon 9 where is located the HECT domain responsible for E3 ligase activity of Ube3a. For last are patients with no abnormalities in chromosome 15 but AS like symptoms. This data suggests that both dose and activity of Ube3a are essential to normal brain function and that changes in the expression can lead to this disorders. The deletion causes severe symptoms while mutation and imprinting defect are normally associated with higher risk of recurrence in the families. The gene is imprinted and it's mainly maternally expressed in cerebellum and hippocampus due to maternal methylation of the imprinting control region (ICR) upstream of SNRPN that has been hypothesized to control the imprint process in the female and male germlines, while lack of methylation of paternal ICR. Paternal expression and transcriptional elongation through the locus results in the expression of an Ube3a antisense transcript (UBE3A-ATS), a noncoding RNA that overlaps Ube3a, silencing the paternal expression of the gene in the brain. In non-neuronal cells, paternal SNRPN transcription does not progress to transcribe the UBE3A-ATS and Ube3a is expressed biallelicaly (Figure 3).

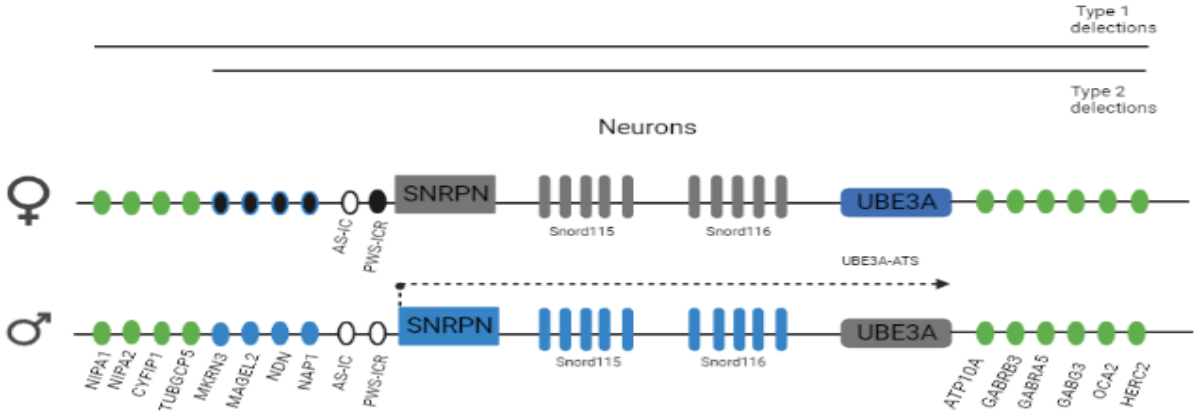

Figure 3. Ube3a gene is paternally imprinted in cerebellum and hippocampus due to maternal methylation of Prader-Willi Syndrome imprinting control region (PWS-ICR) resulting in the expression of an antisense transcript (UBE3A-ATS), a non-coding RNA that overlaps Ube3a silencing the paternal expression of the gene. White circles represent non-methylation while black circles represent the methylation. Grey fill indicates non-expression of the gene. Green circles represent both paternally and maternally expressed genes while blue circles represent only paternally expression. Black fill with blue border indicates paternal expression due to methylation of maternal chromosome. Image adapted from [41]

Mouse models of Ube3a

Animal mouse models are widely used as model organisms to study human biology based in the genetic, physiological similarities, ease of maintaining, breeding and manipulating make them suitable organisms for human biology and disease research. These qualities combined with development of methods for the generation of transgenic and knockout mice have made them powerful tools as model organisms [42]. To the date several mouse models of AS have been developed and there are five lines that are commly used in laboratory research. The first mouse model is the Ube3a (m-/p+) with a deletion of 3 kb of exon 2 in the maternal ube3a gene, which leads to loss of Ube3a expression in a majority of neurons in the brain.

This mutation results in a null allele and animals show motor abnormalities, coordination, cognitive defects and epilepsy.

The Cognitive defects are mainly due to loss of ube3a in hippocampal neurons while motor deficits are due to its absence in the Purkinje cells in the cerebellum. Epilepsy has been suggested in studies to be result of an accumulation of Arc [12,26,43]. The second animal model was generated through replacement of part of exon 15 and the totality of exon 16 of the Ube3a gene with b-galactosidase reporter (ube3a lacZ), wich allows the detection of the allele expression throw lacz staining[44]. This mouse model was created in order to understand the relationship of maternal Ube3a mutations and AS. The mice with mutation of Ube3a and consequent loss of Ube3a ubiquitin protein ligase activity show coordination defects in the bar- crossing test and can be compared to ataxia and coordination defects seen in AS patients [44].

The third mouse model was generated by removing 1.6Mb in the ube3a maternal critical region (15q11.2-13q), mimicking the most common cause of AS with disruption of several genes including GABARB3. In addition to the phenotypes found in the first AS mouse model this mice showed increased vocalizations another phenotype in AS children who have increased laughter and smilling when communicating with adults [12,26]. The fourth mouse model carries a deletion in the IC (Imprinting center) but no behavioural abnormalities have benn reported have been reported so far. Finally, the last mouse model is a ube3a-yellow fluorescent protein reporter mouse originated by fusing YFP to C terminus of the gene, used to determine the subcellular distribution of Ube3a and resulted in the discover of topoisomerase inhibitors now tested in some therapies for unsilencing of the ube3a paternal allele in the brain[26,40]. This mouse model allowed to screen the paternally inherited Ube3a-YFP allele using *in vitro* cultures of cortical neurons. These cells were treated with active compounds that unsilenced the paternally inherited allele and the YFP allowed the detection by fluorescence.

More recently a rat AS model has been generated using CRISPR technology, since rats have more similarities with humans and currently being characterized. [45,46]
Since most cases of AS are originated from large deletion of the maternal Ube3a gene, this rat model includes the deletion of the entire Ube3a gene, this deletion encompasses a 90kb deletion.

Therapies

Gene therapies have been used as an approach to treat AS and even with several approaches being tested the treatment for AS remain symptomatic and include the use of anti-seizure medications for seizures and sedating medications in order to help with sleep disorders. Physical therapy and surgical support may be needed to help walking (National Organization for Rare Disorders).

All patients with Angelman syndrome have at least one paternal copy that is silenced, leading to reduce expression of ube3a in neurons. The activation of this copy could compensate the loss of maternal copy through deletion of maternal copy.

The silence of paternal alelle is consequence of the lack of methylation of the imprinting control region (ICR) and originating a SNHG14 transcript that silences ube3a. One possible strategy would be to provide a diet rich in methyl donors leading to methylation of the paternal ICR. Some molecules have been tested clinically, folic acid and betaine, unfortunately without results. One strategy that was successfully was the use of topotecan, a topoisomerase inhibitor [41]. Since paternal Ube3a is silenced in mature neurons, the use of topoisomerase inhibitors can prevent the production of UBE3A-ATS transcript by inhibiting topoisomerase 1, enabling expression of the paternal Ube3a copy, wich can in turn replace the deleted or mutated maternal copy.[47].

The main problem was that it was shown that it interferes not only with SNHG14 but with long transcripts. Use of topotecan led to expression of ube3a in the hippocampus, neocortex, striatum, cerebellum, spinal cord and in mouse models of AS it was able to improve behavioural deficits. Ube3a unsilenced and restore of ube3a levels in GABAergic neurons has shown to supressed epileptic attacks [17].

Another strategy is the use of RNA interference in order to degrade UBE3A-ATS transcript though antisense oligonucleotides. This approach is being tested, but currently it´s not possible to determine the future outcomes.

Nonetheless, the activation of paternal copy is unlikely to reverse the symptoms in older patients, perhaps the brain of young patients could have the plasticity to recover [41].The study of Ube3a substrates have emerged a potential way of developing new treatments that can ease AS patients symptoms. Sodium valporate is an HDAC inhibitor and is widely known to improve cognitive and behavioural defects in some neurodegenerative disorders. AS patients have altered levels of expression of HDAC1/2 and sodium valporate emerges has a potential treatment. Studies show that AS mice models treated with sodium valporate improve their motor abnormalities and learning and memory behavioural deficits. This treatment was also capable for normalizing HDAC1/2 levels that were increased in AS mice, restoring the acetylation of H3 and H4 histones levels in the hippocampal region of this mice. [37]Simvastatin, a brain penetrating drug, has also been tested has a HDAC inhibitor and the tests show that it reduces HDAC1/2 expression while increases BDNF expression[36]. BDNF is known as a regulator of synaptic plasticity and has decreased levels in the brain of AS mice. This studies suggest that drugs aiming Ube3a substrates could improve cognitive and motor abnormalities found in AS patients and shows potential for the development of drug therapies [36]. Ube3a has been associated with ASD, individuals with an extra copy of maternally inherited chromosome resulting in an increase dosage of Ube3a in the brain [17].

Mice that mimics Ube3a in ASD have tripling gene dosage in the brain and show typical behaviour deficits and increased repetitive behaviour.

Role of Ube3a in translation

mTOR (mammalian target of rapamycin) is a protein with a central role in growth and maintenance of the cells and exists in two complexes, mTORC1 and mTORC2. Abnormal mTOR signalling has been previously associated to neurodevelopmental disorders including ASD (Autism spectrum disorders) and has a critical role in brain development and synaptic plasticity since it integrates several signal pathways including NMDA and glutamate receptors signalling.

Overexpression of Ube3a in neurons results in remodelling of dendritic arborisation and reduction of dendritic number and length [22]. Ube3a degrades TSC2, a member of TSC1/2 complex, a negative regulator of mTOR. Ube3a deficiency results in increased levels TSC2, a negative regulator of Mtorc1, in the cerebellum and the hippocampus. Despite increased levels of TSC2 are found in Ube3a deficient mice, Mtorc1 is over-activated. Nonetheless, TSC2 activity is also regulated by inhibitory phosphorylation at several sites.

AS mice model showed increased levels of phosphorylation of TSC2 at Thr1462, indicating that its inhibitory activity could be reduced.

Over-activation of Mtorc1 results in increased phosphorylation of S6K1 and consequent decrease in Mtorc2. S6K1 negatively regulates Mtorc2 by its inhibitory phosphorylation of rictor, a subunit of Mtorc2 complex[28]. Over-activation of Mtorc1-SK61 pathway can be responsible for synaptic plasticity impairment by increasing translation since this increased activity leads to activation of several substrates that promote mRNA translation initiation including 4EBP1 through its phosphorylation by Mtorc1. The phosphorylation of 4EBP1 from Mtorc1 results in its dissociation from elF4E, e eukaryotic translation initiation factor, allowing translation to occur.

This alterations of Mtorc1 and Mtorc2 in cerebellum result in motor dysfunction while the same alteration in the hippocampus resulted in cognitive deficits in AS mice [21].

Ube3a deficiency and consequent reduced activities of Mtorc2 result in reduced phosphorylation of AKT [48]. AKT has been shown to have an active role in cytoskeleton regulation and reduced phosphorylation of AKT can be responsible for the decreased spine density and length found in Purking cells of AS mice.

Since evidence suggests that Purkinje neuron circuits are involved in coordinating motor learning and synaptic plasticity, decreased spine density and length found in AS mice could be one of the responsible for motor impairment found in AS patients [48].

Treatment of AS mice with rapamycin (inhibitor of Mtorc1) in AS mice resulted in decreased levels of Arc, a protein that has been previously mentioned as a contributor to AS-like phenotypes since it regulates endocytosis of AMPA receptors.

Rapamycin improved spine morphology in Purkinje cells and corrected the levels of both complexes (Mtorc1 andMtorc2). In the hippocampus it rescued LTP and actin polimerization improving learning [28,48]

More recent evidence suggests an additional pathway by which Ube3a can regulate mTORC1 activity in hippocampal neurons suggesting that regulation of mTORC1 activity should be the result of a conjugating factors. In the presence of amino-acids, especially leucine and arginine, mTORC is activated binding to Rag dimers.

Rag dimers are a subfamily of GTPases composed by four Rag proteins that form heterodimers either by RagA or RagB association or by RagC or RagD. These dimers are attached to lysosomal membrane through association with Ragulator complex wich is composed by p18 (LAMTOR1), p14 (LAMTOR2), MP1 (LAMTOR3), C7orf59 (LAMTOR4) and HBXIP (LAMTOR5). The addition of amino acids triggers the change of Rag GTPases to their active stage (GTP-bound form for RagA/B and the GDP-bound state for RagC/D). Active Rag GTPases can bind to Raptor and recruit Mtorc1 to the lysosomal surface where, Rheb, a direct activator of Mtorc1 resides. The absence of amino acids induces the conversion of Rag GTPases to their inactive state (GDP-bound form for RagA/B and GTP-bound state for RagC/D) releasing Mtorc1 from lysosomal surface.[47,49]

Studies suggest that Ube3a ubiquitinates p18 targeting p18 for proteasomal degradation. In the absence of Ube3a p18 levels are increased resulting in reduced degradation of p18. This increase in p18 levels results in over-activation of Mtorc1 since it enhances the lysosomal recruitment of Ragulator-Rag complex, leading to Mtorc1 over-activation. While direct interaction between Ube3a and TSC2 was not established, p18 was found to be a direct subtract of Ube3a. [21]

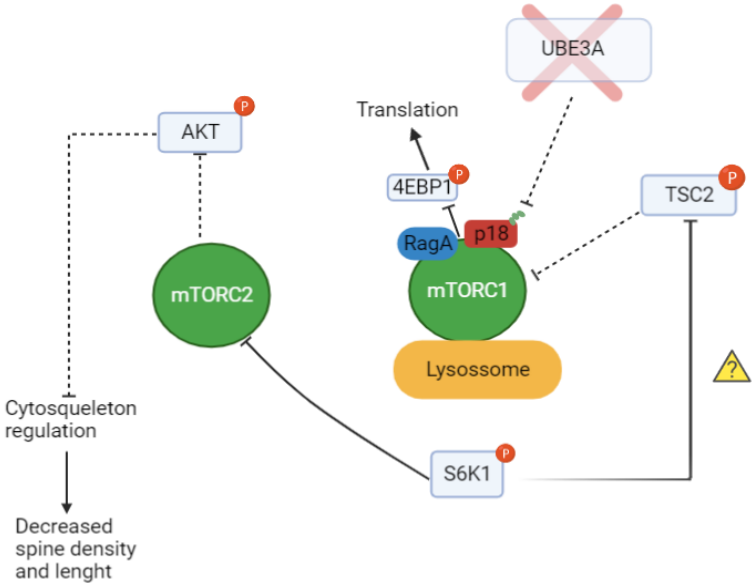

Figure 4. Inhibitory phosphorylation of TSC2 and increased levels of p18 result in Mtorc1 over activation and mTorc2 reduced activity, respectively. AS patients have deficiency in Ube3a in the brain. In the absence of Ube3a TSC2 levels are increased and its inhibitory phosphorylation. P18 is a substrate of Ube3a, and in Ube3a-dificiency p18 levels are increased, wich results in the over activation of Mtorc1. Mtorc1-sk61 increased activation results in phosphorylation o rictor (subunit of Mtorc2 complex) decreasing his activity and having decrease spine density and length. Mtorc1 directly phosphorylates several substrates including 4EBP1 resulting in increasing Translation levels. Dashed lines indicate reduced activity while thin lines indicate increased activity. P represents phosphorylation and question mark indicates the lack of evidence about the phosphorylation of TSC2 from S6K1. Image adapted from [21].

Aims and objectives

Objectives

Angelman syndrome, along with several other neurodevelopmental disorders, is a genetic disorder that primarily affects the nervous system. Individuals with AS have delayed development, intellectual disability and problems associated to the movement (ataxia). The role of Ube3a role in the CNS has been the object of intense investigation in recent years but we still do not understand how ube3a modulates nervous system development and further investigations are needed to clarify the function of Ube3a. Therefore, in this work we aimed to perform a subcellular characterization of Ube3a expression in CNS neurons. Ube3a has been associated with several subcellular compartments, nonetheless a neurodevelopmental characterization of expression is still something not well documented.

We describe Ube3a expression in Rat and Mouse hippocampal neurons and investigated possible changes in its neurodevelopmental expression. Since AS patients have low levels of Ube3a expression the brain we wanted to understand the role of Ube3a catalytic activity in synapse formation and maturation.

Materials and Methods

Cell culture

Pseudo explants culture

For primary mouse and rat hippocampal cultures, the hippocampal region was dissected and meninges removed. The hippocampus were trypsinized in a 10 mL suspension, with 7mL HBSS and 3mL Trypsine (1.5mg/mL) and incubated at 37º C for 15 min. The hippocampus were centrifuged for 1min at 1000 rpm and the trypsin was removed with a sterile Pasteur pipette and the pellet was resuspended in Plating Medium. Hippocampus were centrifuged for 1 min at 1000 rpm and supernatant removed and resuspended in Plating Medium. Cells were mechanically dissociated and transferred into a 1.5mL sterile Eppendorf.

Coverslips were coated with PDL (Poly-D-lysine) for rat hippocampal cultures and PLL (Poly-L-Lysine) for mouse hippocampal cultures. Cells were counted with a hemocytometer and neurons were planted plated as pseudo-explants within 24-well plates at a concentration of 25.000 cells/well in plating Medium for 3h, allowing cells to adhere to coverslips. After 3h plating medium was removed and replaced with NBM supplemented with 25µM of Glutamate. At DIV4 was added 5-FDU (5-fluorodeoxyuridine-1:1000) to prevent the growth of glial cells. After DIV7 cells were supplemented with 1/3 of new Neurobasal medium without Glutamate.

Banker cultures

Two weeks before starting the primary neuronal culture glial cells were unfrozen and distributed in 60mm dishes with 4 ml medium. The medium of glial cells was replaced every 3 days, until 24h prior to use. The cells were cultured at least 7 days in order to achieve confluence.

24h prior to adding neurons the medium of glial cells was exchanged to Neurobasal medium with Glutamate. Banker cultures are low density neuronal cultures used to examine the synapses and were previously described [50].

Dissociated cells from hippocampus were plated into PDL-coated coverslips and prepared with 3 paraffin dots in each coverslip in 60mm dishes at a concentration of 300.000 cells/ 4ml of Plating Medium for 3h to allow cells to adhere on the coverslips.

The coverslips were then transferred and flipped into the feeder layer of glial cells. The culture was maintained in incubator at 37ºC in 5%CO2. After 4 days 5-FDU was added to the culture to prevent astrocyte proliferation. For long term survival after day 7 we exchanged one-third of the existing medium for fresh Neurobasal medium without glutamate every three days.

Cell lysates

For whole cell lysates $1x10^6$ cortical neurons were cultured in PDL coated cell culture dishes until desired developmental time points. The medium was removed and the cells were washed 2 times with ice-cold PBS. Lysates were collected using the RIPA cell lysis reagent (#89900, Thermo scientific) supplemented with 1x proteases (CLAP and PMSF both diluted 1:1000 in RIPA buffer) and 1x phosphatase inhibitors (NaF 50mM and Na_3VO_4 1.5 mM).

Immunocytochemistry

Cells were fixed for 10 minutes in 4%paraformaldehyde, washed 3 times with PBS 1X and permeabilized with PBS-Triton 0.25% and washed with PBS 1X. Cells were then blocked in 3%BSA in PBS for 40 minutes. Antibodies were prepared in 3%BSA in 1X PBS. Antibodies used were the following Anti-E6AP (1:2000, #E8655, Sigma-Aldrich), Anti-Tau (1:500, #AB75714, Abcam), Anti-Synapsin (1:4000, #AB1543P, Merck), Anti-PSD95 (1:1500, #1673450S, Cell signalling), Anti-VgluT (1:1000, #AB5905, Millipore), Anti-MAPK2 (1:5000, #AB5392, Abcam), Anti-EGFP (1:2000, #AB13970, Abcam). All primary antibodies were incubated overnight at 4ºC. Cells were then washed tree times with 1XPBS. Secondary antibodies were diluted in 3%BSA in 1X PBS and incubated for 1h at room temperature protected from the light to avoid losing fluorescence.

The following secondary antibodies were used Goat Anti mouse IgG, Alexa Fluor 488 (1:100, #A11029, Alfagene), Goat Anti Rabbit IgG, Alexa Fluor 568 (1:1000, #A11036, Alfagene) and Goat Anti-chicken IgY, Alexa Fluor 647 (1:1000, #A21449, Alfagene), AMCA Goat Anti Chicken IgY (1:1000, #103-155-155, Jackson immunoresearch), Goat Anti-Guinea Pig IgG, Alexa Fluor 647 (1:1000, #A21450, Alfagene), Goat Anti-Chicken IgY, Alexa Fluor 488 (1:1000, #A11039, Alfagene). Cells were then washed two times with PBS-Triton 0.1% and one time with PBS 1X.

Coverslips were then flipped into mounting media with DAPI for evaluation of Ube3a subcellular distribution and for transfected cells. For axodendrytic synapses cells were flipped into golden anti-fade reagent without DAPI. Coverslips were imaged using Zeiss LSM 880 with Aryscan in 63X oil immersion objective lens. All images were quantified using ImageJ.

Western-Blott

Protein concentration was measured using the BCA Protein Assay Kit (Thermo scientific) and standardized. The gel was loaded with 10ug of protein into 8 well acrylamide SDS-PAGE gel (4%stacking, 10% resolving). Gels were transferred into a PVDF membrane (Millipore). The NZYBlue Protein Marker (MB176, NZYtech) was used as ladder. The PVDF membrane was activated for 5sec in methanol and washed in miliQ

water. The stacking was removed and the gel was transferred to the membrane for 2h with 200mV at 4ºC.

The membranes were removed and washed into Tris-Buffered saline (TBS) and then blocked in 1x Tris-Buffer saline with 0.5% Tween 20 (TBS-T) and 5% non-fat dry milk at room temperature for 1h.

The blots were washed in TBS-T and incubated with the primary antibody, Anti-E6AP mouse monoclonal (E8655-Sigma Aldrich, 1:1000) diluted in 1% non-fat dry milk mixed in TBS-T and left overnight at 4ºC with rotation. After this incubation period, blots were washed 3 times with TBS-T.

The blots were incubated with goat anti-mouse at 1:5000 for 1h at RT. After incubation blots were washed 3 times in Tris-Buffered saline with 0.5% Tween 20 and detected using chemiluminescence in ChemiDoc (Bio-Rad).

For β3tubulin detection blots were washed with TBS-T for 3 times and washed with restore western Blot stripping Buffer (21059, Thermo Scientific) for 1h at Room Temperature. A mouse monoclonal (1:5000, #T8578, Sigma-Aldrich) diluted in 1% non-fat dry milk mixed in TBS-T and used overnight at 4ºC with rotation. Ube3a protein quantification and β3-Tubulin was done using ImageLab.

Co-Tranfection

For co-transfection cells were cultured in pseudo-explants at a concentration of 25.000 cells per well. Cells were transfected at DIV 4 and DIV 7 and analysed at DIV6 and 10, respectively. DNA Precipitates were prepared as follows 2ug per well for Ube3a/C843A plasmids and 0.5ug per well for GFP, was added to TE and this mixture added to $CaCl_2$. The DNA/TE/$CaCl_2$ solution was added to HeBS 2x (pH=7.2). As a negative control cells were incubated cells without DNA. DNA precipitates were incubated at room temperature for 20 minutes and then 50ul of the solution was added to each well. The medium was removed from each well and replaced with Acidic medium (Neurobasal medium, KA 10mM, HCL 1M) and cells further incubated for 20 minutes at 37º.

Acidic medium was removed and cells were washed with neurobasal medium without glutamate and the conditionated medium added to the cells. We allowed cells to express the recombinant DNA for 48 h or 72 h.

Plasmids Preparation

pFlag-CMV2 Ube3a and pFlag-CMV2 C843A plasmids were a kind gift from Dr. Chen Deng, Institute of Basic Medical Sciences [19]**.** DNA was extracted in Biomolecular Water and Transformed in 100ul of E.coli competent cells. The DNA was carefully mixed were mixed with DHα cells followed by an incubation in ice for 30min. Cells were then heat-shocked at 42ºC for 90sec followed by another incubation in ice for 2 min.

Next, 800ul of SOC was added to each transformation reaction and incubated at 37º for 1h at 180 rpm. Cells were then centrifuged at 2.0g for 3 min.

Supernatant was removed until remained approx. 50uL, the pellet was homogenized and spread in selective LB Agar medium plates (with Ampicillin at 75 µg/uL) and left in the bench for some minutes to allow the absorption of the excess. Plates were then inverted and incubated overnight at 37ºC (~15h).

One colony of each transformation reaction was inoculated in 3ml of LB broth (with Ampicillin at 75 µg/mL) and incubated at 37ºC with 180rpm overnight. 1ml of the previously incubated Lb broth was added to 15ml of LB Broth in order to increase the number of transformed cells for extraction. For extraction we used HiPure Plasmid Midiprep Kit (Invitrogen K210005).

DNA was eluted in 100ul of TE and quantified using NanoDrop. In order to evaluate the integrity of the inserts we used electrophoresis separation. Plasmids were digested using restriction enzymes BmHI (#MB064, NZYtech) and HindIII (#MB070, NZYtech) for 1h at 37ºC using NZYBuffer U (#MB110, NZYtech). Elethrophoresis separation was performed in a 1% agarose gel run for 1h30 at 100mA.

3.6. Sequencing

To verify that the DNA used was Ube3a Human isoform 2, we constructed primers for this isoform. The integrity of Ube3a and the catalytic mutant C843A plasmids was done performed by Sanger sequencing (STAB Vida). Coding sequence of Ube3a human isoform 2 has 2628 base pairs, the 4 primers indicated in Table 1 allowed to cover the entire coding region.

Table 3. *Primers for Ube3a/C843A Sanger sequencing*

Primer	**Sequence**	**Lenght (nt)**	**%GC**	**Tm**
pCEP_FWD	AGAGCTCGTTTAGTGAACCG	20	50	54.8
UBE3A/C843A_FWD_1378	GCATGTTCTGCTGCTGCTATGG	22	54.5	59.2
UBE3A/C843A_FWD_2003	GCGAGCTGACACTTCAGGAACT	21	57.1	58.5
UBE3A/C843A_FWD_2690	CCATGGTTGTCTACAGGAAGC	21	52.4	55.6

RESULTS

Results

The Ube3a overall expression is characterized by an increased expression at early stage of development decreasing afterwards.

We first investigated if there was any change in the developmental expression of Ube3a in CNS neurons in vitro. To test this possibility, we performed western Blot analysis using rat embryonic cortical neurons. The cells were lysed at different days in vitro (DIVs) using RIPA supplemented with protease and phosphatases inhibitors, transferred into a PVDF membrane and stained against Ube3a. For normalization membrane was stained against β-3Tubulin, a housekeeping gene, and both Ube3a and β-3Tubulin protein quantity were measured (**Fig5. A**). The observations show that Ube3a expression is increased at Div3 when compared with different days in vitro (**Fig5. B**).

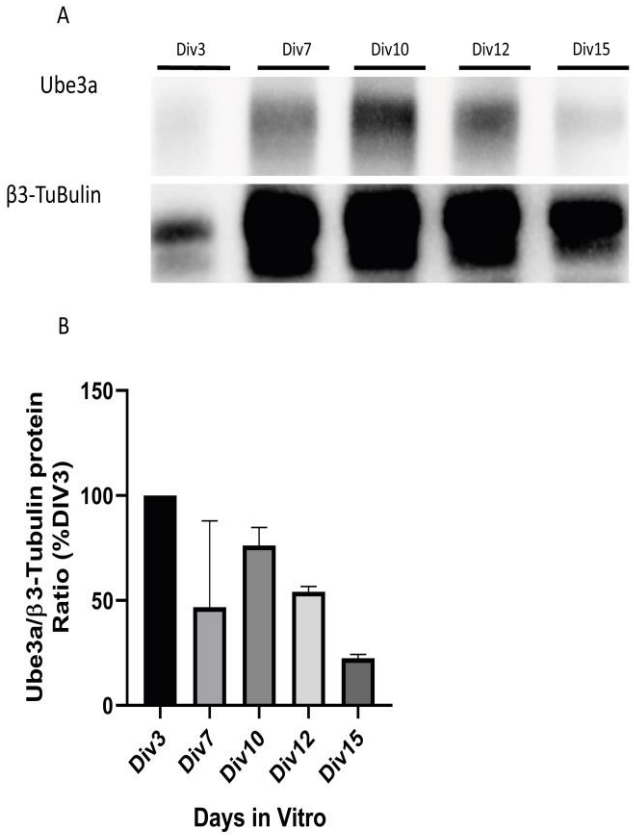

Figure 5. **Ube3a has an increased expression at early stages of development.** (**A, B**)Cortical neurons were cultured until the indicated time-points and then the cells were lysed and expression was evaluated using Western-Blot analysis using antibody against Ube3a and normalized with β-3tubulin (A) Membrane was visualized using chemiluminescence with ECL in ChemiDoc. Quantification was done using ImageLab and Ube3a expression was normalized to B-3Tubulin. Bars and plots represent the mean ± SEM. Results are presented as % of DIV3.

Ube3a is widely expressed in the cell body of Rat and Mouse Hippocampal neurons

We then decided to evaluate Ube3a expression using another experimental approach. In order to confirm the Ube3a expression, we used Rat and Mouse hippocampal cultures. Since the majority of proteins of the neuronal cells are produced in the cellular bodies of neurons evaluation of Ube3a expression in cellular bodies could provide an accurate overview of overall expression in mouse and rat hippocampal neurons. Neurons were stained against Ube3a and DAPI, a nuclear marker and Ube3a signal intensity in the cellular bodies was measured. We observed that Ube3a was highly expressed in the nucleus and cytosol of Rat and Mouse hippocampal neurons (**Fig6. A, B**).

We also observed that Ube3a has increased levels of expression at early stages of development followed by a decrease at the remaining time points both in rat and mouse hippocampal neurons. Ube3a expression decreases abruptly in rat hippocampal neurons from DIV3 to DIV7 stabilizing afterwards. On the other hand, in cell bodies of mouse hippocampal neurons the decrease from DIV3 to DIV7 is lower stabilizing afterwards. These observations confirm our previous western blot results and show that Ube3a has an increased expression at DIV3 followed by a decrease in later stages of development (**Fig6. C, D**).

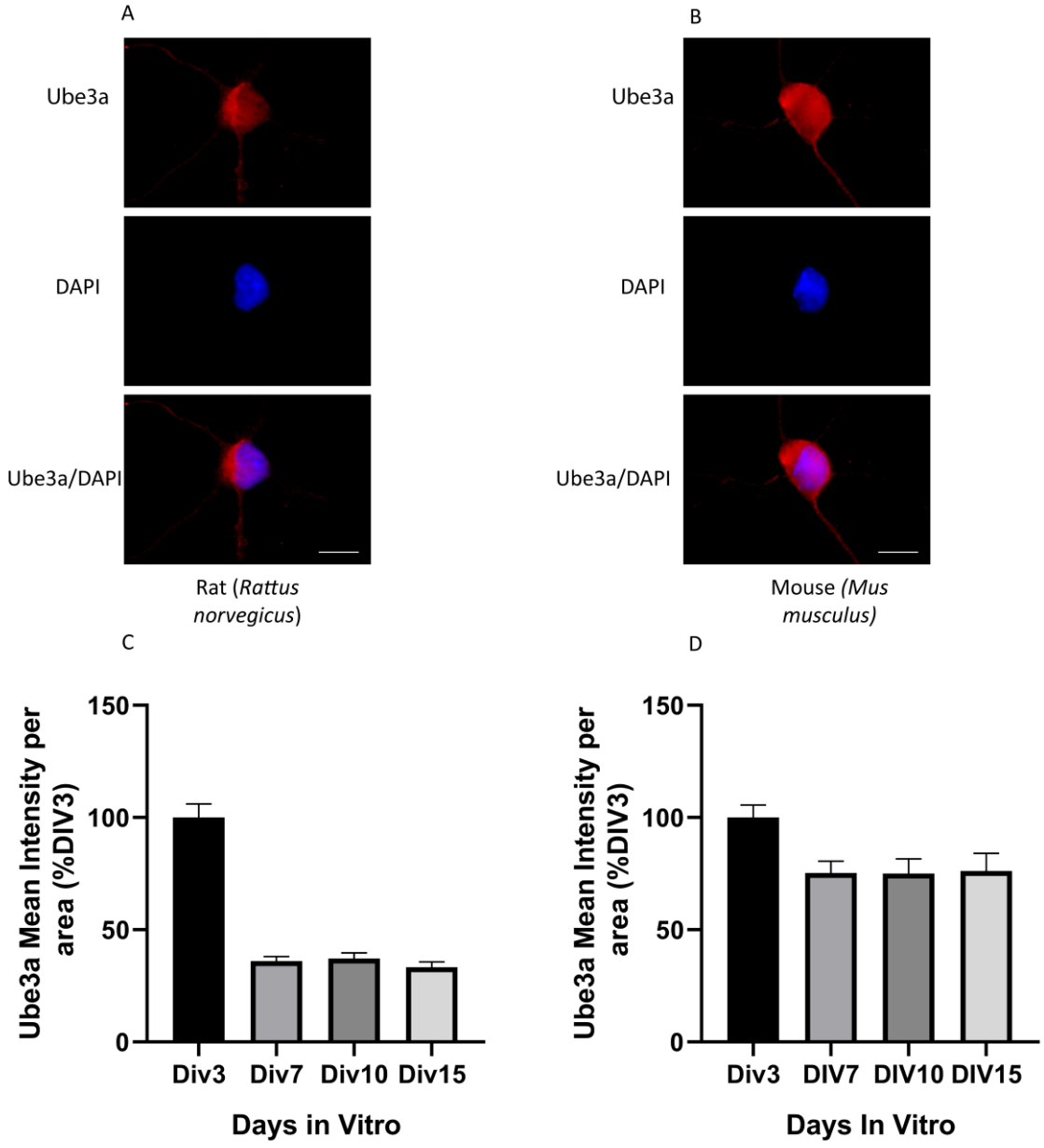

Figure 6. **Ube3a is widely expressed in the cell body of Rat and Mouse Hippocampal neurons. (A, B)** Staining of Ube3a CNS neurons at DIV3.Ube3a signal intensity was measured and divided for

the cell body area, using an antibody against Ube3a, a specific marker for Ube3a. Images were taken from random cellular bodies using a Zeiss LSM 880 with airyscan fluorescent microscope with 63x objective. (**C, D**) Quantification of Ube3a intensity. Results show that Ube3a expression is increased at early stages of development followed by a decrease in the next DIVs, and in addition the decrease is higher in rat hippocampal neurons (**C**) than in mouse hippocampal neurons (**D**). Ube3a signal intensity analysis was performed using ImageJ software. Bars and plots represent the mean ± SEM and are shown as %DIV3. The images are from cellular bodies randomly selected from 2 and 3 independent experiments for mouse and rat neurons. Scale bar 10μm.

Ube3a is present in the axons of Rat and Mouse hippocampal neurons

We next asked if Ube3a was expressed in axons of rat and mouse hippocampal neurons. Rat and Mouse hippocampal neurons were cultured in pseudo-explants allowing the cellular bodies to stay in the center of the coverslips, since axons have a higher growth rate and length, this enable both the isolation of axons in the cultures. Neurons were cultured and immunocytochemistry performed at different time points. At different DIVs cells were fixe and immunostained against the axonal marker Tau.

We observed that Ube3a was present in the axons of rat and mouse hippocampal neurons (**Fig7. A, B**) and like the overall expression, in axons Ube3a had an increased expression at early stages of development. This results show that Ube3a is enriched at Div3 in both Rat and Mouse hippocampal neurons followed by a decrease in the following DIVs, this decrease is stablized until later stages of development in Rat hippocampal neurons while in Mouse hippocampal neurons expression is characterized for a decrease at DIV15 (**Fig7. C-F**).

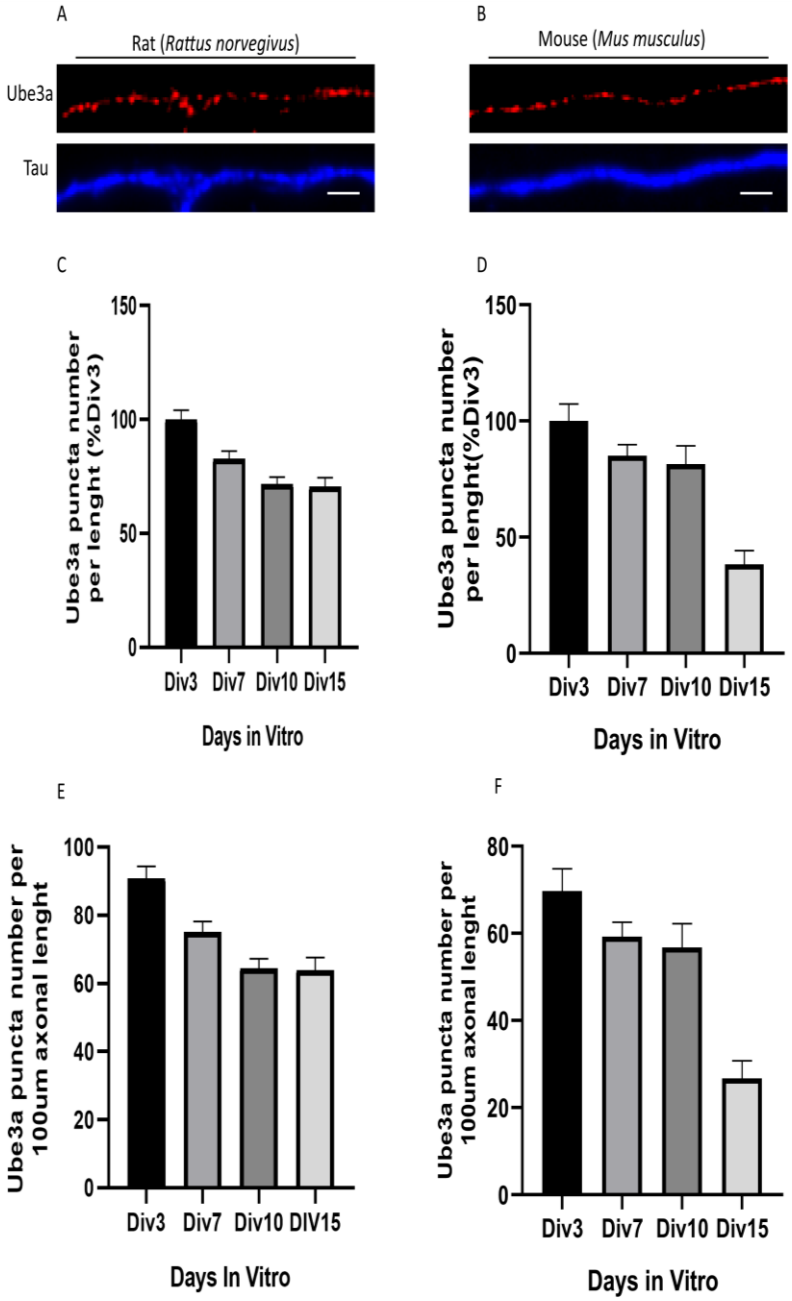

Figure 7. Ube3a is present in axons of rat and mouse hippocampal neurons. (A, B) Ube3a expression in axons of rat and mouse hippocampal neurons at DIV3. Neurons were stained against Ube3a and Tau, an axonal specific marker. Images were taken from random neurons using LSM 880 with aryscan fluorescent microscope with a 63x Objective. (**C-F**) Quantification of Ube3a puncta number in axons of rat and mouse hippocampal neurons. Results show that Ube3a puncta are present in both axons of rat and mouse hippocampal neurons and that this expression is increased at DIV3. Puncta number quantification was performed using Image J software. Bars and plots represent the mean ± SEM of neurons randomly selected from 2 and 3 independent experiments for mouse and rat neurons, respectively. The scale bar is 2.5 µm.

Ube3a is expressed presynaptically in axons of rat and mouse hippocampal neurons

We then sought to investigate ifUbe3a could be expressed in presynaptic sites. Mouse and Rat hippocampal neurons were cultured and immunostained against Tau a specif axonal marker. Synapsin has been previously described has an important molecule in the synapse development and modulation of neurotransmitter release. We analyzed the levels of colocalyzation betwean Ube3a and synapsin puncta. Our results show that Ube3a is expressed presynaptically at low levels with increased expression at DIV3 in both rat and mouse hippocampal neurons (**Fig8. A, B**). We further observed that an increased colocalization between Ube3a and synapsin at early stages of development (DIV3) followed by a decrease until later stages of development (DIV15) (**Fig8. C,D**), in both rat and mouse hippocampal neurons.

This decrease happens until later stages of development (DIV15). Thus, these observations demonstrate that Ube3a is expressed presynaptically at low levels, with a higher expression in rat vs mouse hippocampal neurons, and this levels decreased until later stages of development.

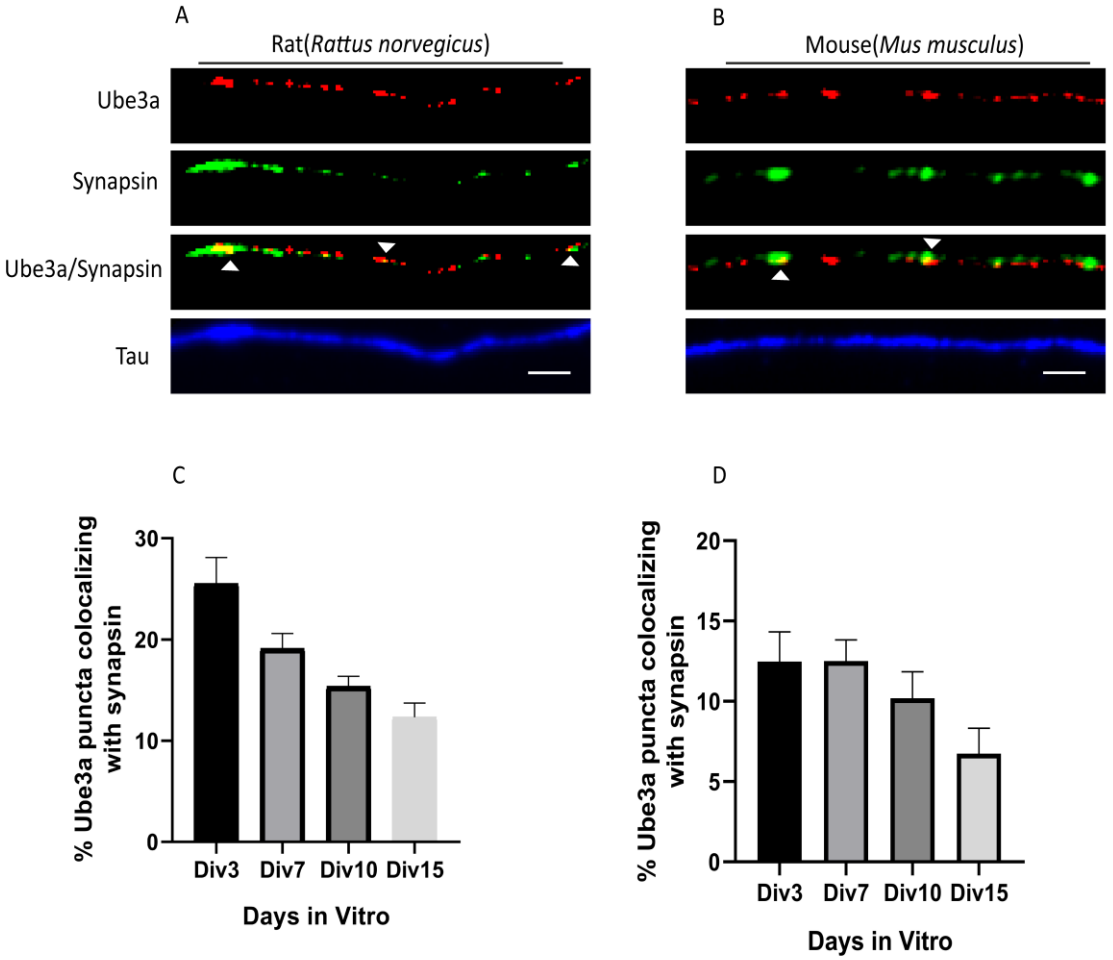

Figure 8. **Ube3a is expressed presynaptically in axons of rat and mouse hippocampal neurons. (A, B)**. Ube3a colocalizing with synapsin, a presynaptic specific marker, in Rat and Mouse hippocampal neurons stained at DIV7. Co-localization of Ube3a and synapsin was assessed by immunocytochemistry using antibody against Tau, Synapsin and Ube3a. Images were taken using LSM 880 with Aryscan fluorescent microscope under 63x objective. (**C, D**) Quantification of Ube3a and synapsin co-localization. Results show that Ube3a is expressed presynaptically at low levels with increased expression at Div3. Quantification of ube3a/synapsin co-localization was performed using Image J software. Bars and plots represent mean ± SEM of 3 independent experiments. Arrowheads represent colocalization of Ube3a and synapsin. Scale bar is 2.5µm.

Loss of catalitic activity of Ube3a impairs synapse formation

In order to express exogenous protein in neurons we needed to optimize the process to reduce cell toxicity and improve the rate of transfection. The transfection with Ca^{2+} phosphate co-precipitation is one of the most used transfected methods for primary neuronal cultures. This technique is based in the formation of DNA crystals with Ca^{2+} ions in a phosphate buffer.

This crystals will precipitate on the cells and enter the cells via endocytosis[51]. There are several advantages of using this technique since it does not require any specialized equipment, is cost-effective and easy to optimize.

However the technique has also some disadvantages since it has a low rate of transfection and if not well optimized it is toxic to the cells[52]. In order to minimize the toxicity to the cells we wanted to optimize the time of precipitate formation since longer times will give rise to big precipitates and these will be harder to be uptake by the cells and will be more toxic. In order to do that we transfected cells with GFP at div 4 and tested different times for the formation of precipitates. We tested 3 different times, 20 min, 30 min and 40 min. After 48h we fixated cells and stained against DAPI and evaluated the efficiency of transfection and cell toxicity through the analysis of live/death cells. Our results show that for Ca^{2+} phosphate co-precipitation the best time for formation of precipitates its 20/30 min. Both of this results show a good amount of transfected cells with low toxicity to the cells. On the other hand, incubation of the precipitates for 40 min will origin low rates of cell death but also low transfection rate. The time of formation of precipitates should be optimized depending on the size of the plasmid DNA since bigger plasmids will need more time to form precipitates.

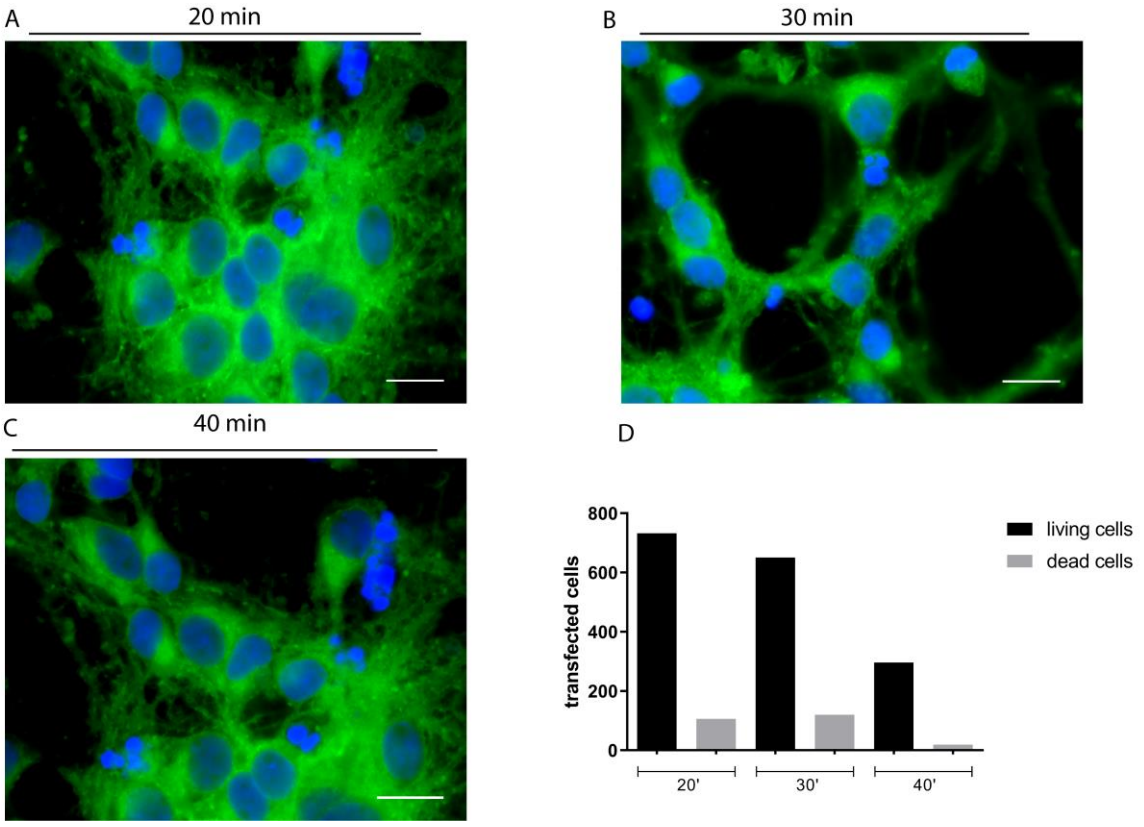

Figure 9. **Optimization of transfection procedure. (A-C)** Incubation of rat hippocampal cells with different times of incubation of precipitates. At DIV4 neurons were transfected and let expression for 48h. Cell death and number of transfected cells was quantified by immunocytochemistry using DAPI, a nuclear marker. Images were taken using LSM 880 with aryscan under 63x objective. (D) Analysis of cell death transfection with GFP. Results show that 20 min of incubation of precipitates origin best rate of transfection and represents less toxicity for the culture. Scale bar 15µm.

It has been previously shown that Ube3a mRNA is increased upon glutamate receptor activation suggesting that Ube3a transcription is regulated by synaptic activity. Ube3a is an E3 ligase with a catalytic HECT catalytic domain. This domain is responsible for binding to the activated ubiquitin via its cysteine residue and catalyses the bond of the lysine residue of a targeted protein and the C terminal of the ubiquitin. Several proteins have been already identified as Ube3a substrates. Some of this proteins are known for their important role in excitatory synapse formation like Ephexin 5, Arc, SK2.

Therefore, we next asked if loss of Ube3a catalytic activity could affect synaptic formation.

In order to answer this question we co-transfected neurons using the Ca^{2+} phosphate co-precipitation method with a Ube3a catalytic inactive form (C843A), where the active site cysteine residue is substituted with an alanine. The WT DNA of the cytosolic human Ube3a isoform 2 (hUbe3a-iso2) was used as a control. As a negative control we transfected the neurons without DNA (CTR-). The co-transfection was done in a proportion of 4:1 (2.0ug of C843A or Ube3a DNA and 0.5ug GFP). After 48h (DIV6) of transfection we immunostained transfected and non-transfected control (CTR-) neurons against Synapsin and VGluT, two distinct presynaptic markers. We observed that neurons transfected with C843A have a significantly reduced number of synapsin (64.14% ± 5.112) (**Fig10. B, D**) and VGluT (63.84% ± 4.553) (**Fig10. B, E**) puncta number when compared to control (**Fig10. A-E**). Transfection of neurons with WT cytosolic Ube3a isoform had no effect in the number of Synapsin (91.65 ± 6.417) (**Fig10. C, D**) and VGluT (97.44% ± 6.202) (**Fig10. C, E**) when compared with non-transfected neurons. These observations demonstrate that loosing catalytic activity of Ube3a leads to a reduction in the number of synapses.

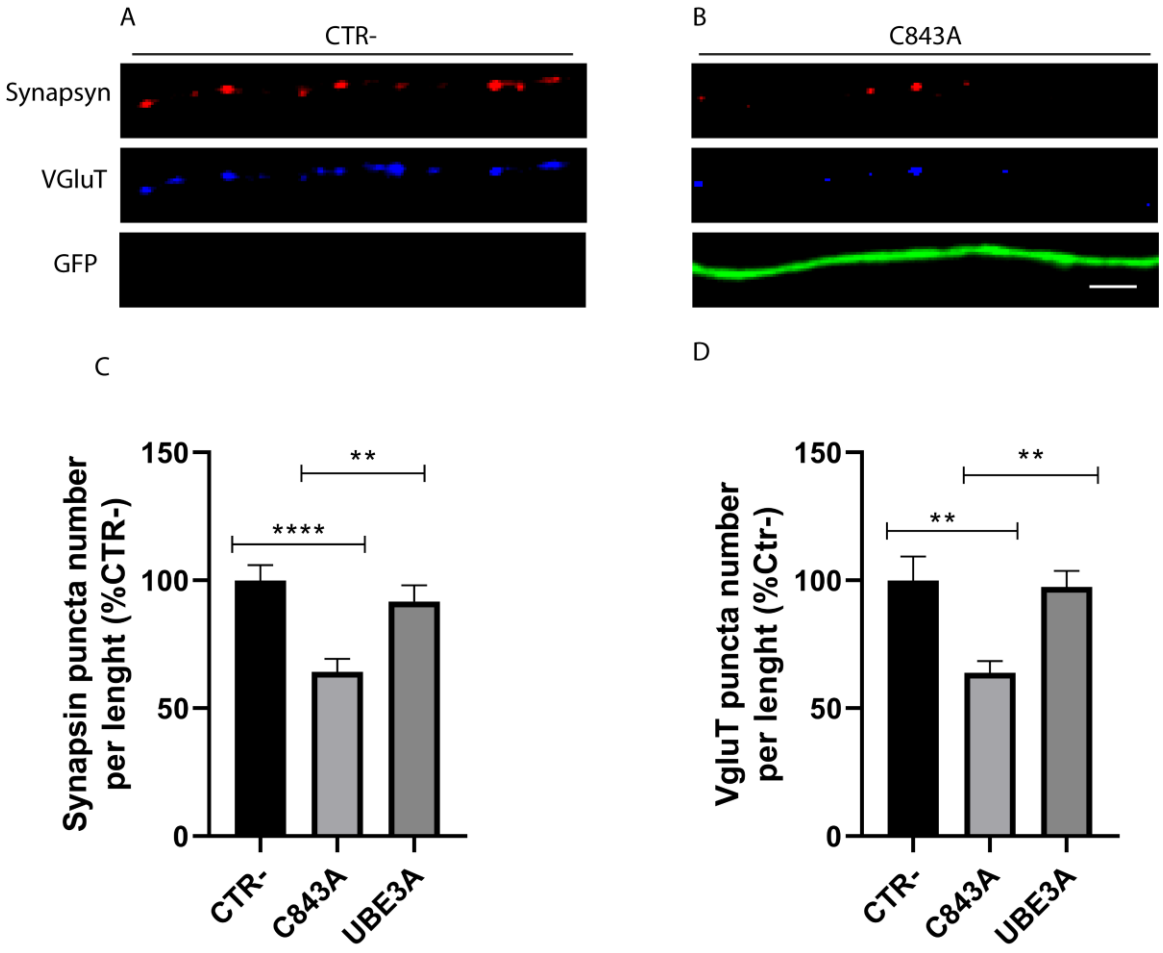

Figure 10. Loss of Ube3a catalytic activity impairs synapse formation in rat hippocampal neurons. (A-C) Synaptic effect of transfection in Rat hippocampal neurons with a catalytic inactive form and human cytosolic isoform Ubbe3a (hUbe3a-Iso2). At DIV4 neurons were transfected for 48h. Number of presynaptic punctas was assessed using antibody against Synapsin and VgluT1; eGFP was used to visualize transfected neurons. Images were taken using LSM 880 with aryscan with 63x objective. (**D, E**) Quantification of Synapsin and VGluT puncta number. Results show that synaptic punctas are significantly reduced when compared with control, demonstrating that loss of E3 ligase activity leads to synaptic impairment in rat hippocampal neurons. Synapsin and VGluT puncta quantification were performed using Image J software. Bars and plots represent the mean ± SEM of neurons selected form 3 independent experiments. (**D**)**** Represents p< 0.0001 (**E**) ** Represents p=0.0037 (**D, E**) ** Represents p=0.0014 and p=0.0034 by Bonferroni's multiple comparisons test when compared to CTR-. The scale bar is 2.5 µm.

Loss of Ube3a catalytic activity reduces the number of mature synapses

Our previous results indicate that Ube3a catalytic activity affects presynaptic differentiation. We next asked if this inactive form of Ube3a would affect synapse maturation. To test this idea we transfected low density hippocampal cultures at DIV7, with C843A mutant and hUbe3a-Iso2 for 72h. Axons were stained at DIV10 against GFP and two presynaptic markers synapsin and VGluT. It was observed that cells transfected with C843A had a significantly decrease in the number of Synapsin (69.13% ± 4.312) (**Fig11. B, D**) and VGluT (65.21% ± 5.047) (Fig6.B, E) puncta number when compared to control (**Fig11. A-E**). Transfection of neurons with WT cytosolic Ube3a isoform had no effect in the number Synspsin (90.16% ± 4. 465) (**Fig11. C, D**) and VGluT (108.6% ± 6.444) (**Fig11. C, E**) puncta. These results indicate that loss of catalytic activity of Ube3a reduces the number of synapses.

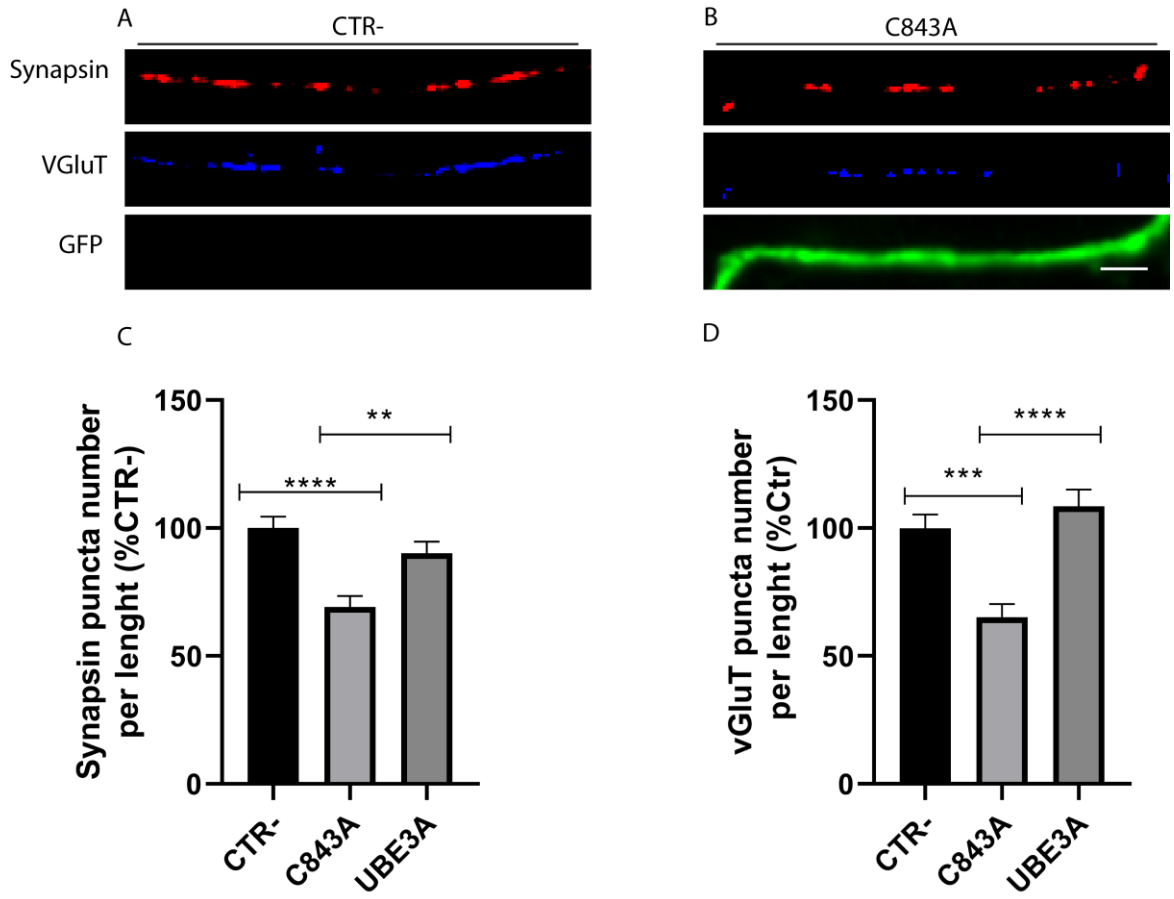

Figure 11. Synaptic effect of the C843A mutant.(A, B) Rat hippocampal neurons were transfected at DIV7 with WT human isoform 2 and the C843A mutant and fixed 72 h later (DIV10). Synapsin and VGluT puncta number was assessed by immunocytochemistry using specific antibodies. Images were taken using LSM 880 with aryscan fluorescent microscope under 63x objective. (**D,E**) Quantification of Synapsin and VgluT puncta number. Results show that C843A, a catalytic inactive form of Ube3a induces a decrease in the number of synapses demonstrating that E3 ligase activity of Ube3a is necessary for synapse development. Quantification of puncta number was performed using ImageJ software. Bars and plots represent the mean ± SEM of neurons transfected from 3 independent experiments. (**D**)**** Represents $p<0.0001$; (**E**) ** Represents $p=0.0020$; (**E**) *** Represents $p=0.0002$ by Bonferroni's multiple comparisons test when compared to CTR-. The scale bar is 2.5 µm.

DISCUSSION

Discussion

In this study we analyzed the subcellular localization and the neurodevelopmental expression of Ube3a in rat and mouse hippocampal neurons. We showed that Ube3a is highly localized in somal compartment[11,53]. To date, several studies concluded that Ube3a is highly enriched in the nucleus, and that this enrichment results from the expression of Ube3a shorter isoform, both in Mouse and Human neurons [8,17,53,54]. However, few studies use rat cells as object of study, since majority of studies were performed in mouse or human cells[45,46]. Given this, and since there´s only been described one Ube3a isoform in rat, a proper characterization of Ube3a expression in Rat needs to be done. Our observations suggest that Ube3a is highly enriched at early stages of development. We showed by western blot of Rat cortical cells lysates and by immunocytochemistry in both Rat and Mouse hippocampal neurons, cultured in vitro, that Ube3a expression is increased at early stages of development. We reported that Ube3a expression is increased at DIV3 followed by a decrease until later stages of development (DIV15). We described that Ube3a expression follows the same pattern in both Rat and Mouse cellular bodies. However, we found that in Rat cellular bodies Ube3a expression had high expression at DIV3 followed by a big decrease by DIV7 stabilizing until later stages of development, while in Mouse cellular bodies the high increased is followed by a small decrease stabilizing until later stages of development. We report that Ube3a expression in mouse hippocampal neurons is increased at later stages of development when compared with the rat neurons.

Several studies report that Ube3a localizes to dendrites and growth cones. The fact that Ube3a expression is followed by a decrease was unexpected. Ube3a has been associated with regulation of synaptic plasticity and its depletion has been associated with loss of synaptic activity[5,15,54,55]

We next performed a neurodevelopmental characterization of Ube3a expression in axons of Rat and Mouse hippocampal neurons using immunocytochemistry. Our observations are in accordance with other studies that report Ube3a presence along the axons[10,54].

Ube3a expression in axons follows the same pattern as the cellular bodies, with higher expression at early stages followed by decrease in the later stages of development. However, Rat hippocampal neurons showed increased expression at later stages of development. Similarly to global expression, in Mouse hippocampal neurons Ube3a expression is highly decreased at later stages of development (DIV15). These results were surprising, since previous studies show that Ube3a mRNA transcription is increased upon glutamate receptor activation suggesting that Ube3a transcription is induced by neuronal activity[15,55]. Thus, it would be expected to see an increased expression upon synaptogenesis (DIV7).

Several studies associate Ube3a with a possible role in the excitatory synapse formation since his E3 ligase activity is linked to ubiquitination and degradation of several subtracts with biological relevance in synapse formation, such as Ephexin 5[16,34], Arc[15,38] and SK2[20,32] channels. The degradation of proteins with biological relevance and presence in the synaptic compartments by Ube3a led us to investigate the local expression of Ube3a in the presynaptic compartments of Rat and Mouse hippocampal neurons. Some studies reported the presence of Ube3a in axons and growth cones. However, a detailed subcellular localization has not been performed. Performing co-localization studies against Ube3a and Synapsin (a presynaptic marker involved in the regulation of neurotransmitter release on the synaptic compartment), we found that Ube3a is partially expressed in the presynaptic compartment this presence was increased at earlier stages of development (around 25% for Rat neurons and 12% for Mouse neurons). Our results are consistent with previous report that situate Ube3a locally in the presynaptic compartments, at least for Mouse neuronal cultures[40,54].

This increased expression was followed by a decrease in the next neurodevelopmental stages. The early enrichment in the expression of Ube3a in axons of Rat and Mouse hippocampal neurons could suggest that Ube3a besides being needed in higher levels to trigger or promote the excitatory synapse formation, may also be required for neurite patterning and axon outgrowth, and Ube3a expression would decrease at later stages but still expressed.

Next, we aimed to understand if Ube3a was also necessary for promoting synapse maturation and if this role was due to its E3 ligase activity and proteasomal degradation.

The association between Ube3a and its putative substrates is dependent on the catalytic HECT domain , which is characterized by a cysteine residue capable of binding to C terminus of activated ubiquitin[3]. Ube3a catalysis the bond of the lysine of the targeted protein and the C terminus of the ubiquitin. Thus, the cysteine residue of HECT domain of Ube3a is necessary for Ube3a activity in proteasomal degradation[55]. We started by co-transfecting neurons prior to synaptogenesis period (DIV4-6) with the catalytic inactive form of Ube3a (C843A) and GFP to track the expression of the exogenous protein, and evaluated the levels of presynaptic markers. Our results suggest that Ube3a catalytic activity is necessary for promoting synapse formation. Co-transfection with hUbe3a-Iso2 show no effect in formation further supporting the hypothesis that lack of function of Ube3a negatively impacts synapse formation. These results, together with the previous results demonstrating increased expression of Ube3a at early stages of development, indicate that Ube3a catalytic activity is necessary for promoting excitatory synapse formation.

Our next goal was to understand if Ube3a activity was also necessary for synapse maturation. To achieve this, we transfected DIV7 low-density hippocampal cultures neurons for 72h with C843A which form extensive axo-dendritic synapses. These results are in concordance with our previous results, showing that Ube3a E3 ligase activity is necessary for synapse maturation and that the loss of this function leads to a reduction in synaptic function.

Overall our results show that Ube3a is expressed in both rat and Mouse hippocampal neurons and is highly enriched at early stages of development. Several studies have linked Ube3a with a role in regulation of excitatory synapse development and being regulated by neuronal activity. However, despite studies showing that Ube3a is present locally in the synaptic sites there is still no link between the local effect of Ube3a at presynapses and the cognitive alteration observed in AS patients.

Our results show that Ube3a is present presynaptically possibly acting locally to modulate synapse formation. Ube3a is thought to ubiquitinate and degradate Arc, a protein responsible for internalization of AMPA receptors, and increased levels of Arc lead to increased internalization of AMPA resulting in synaptic dysfunction [15].

Ube3a is also responsible for ubiquitination of SK2, which increases the levels of SK2 in the post synaptic site [20]**.** Another effect of higher levels of SK2 is the harder activation of NMDAR receptors resulting in the loss of LTP and impairing synaptic plasticity. These studies show that Ube3a catalytic activity is necessary for proper synaptic function. We show that the loss of function results in impaired excitatory synaptic activity and could be responsible for the cognitive defects observed in AS patients.

CONCLUSION AND FUTURE PERSPECTIVES

Conclusion and Future Perspectives

Depletion or mutation of the maternal copy of Ube3a results in Angelman Syndrome, while duplication of the gene has been associated with Autism, suggesting that neuronal development is dependent on a correct level of Ube3a expression. As an E3 ligase, Ube3a is responsible for the ubiquitination of proteins, and targeting them to degradation. Although several proteins and interactions with Ube3a have been identified it's still hard to link this mechanisms with all the clinical finding in AS patients.

To the date, treatment for patients with AS remains symptomatic and include use of anti-seizure medications and physical therapy to help improve the recurrence of seizures and to help patients improving their motor impairments. Even the re-instatement of Ube3a has shown almost no results in improving the recurrence of seizures, nonetheless improvements in rescuing LTP are promising results. Thus, treatments at early stages of development are needed in order to improve the proper shaping of neuronal circuits. We concluded that Ube3a is highly increased at early stages of development and that Ube3a catalytic activity is necessary for the proper synapse formation and maturation. Disruption of Ube3a capability to ubiquitinate substrates at early stages of development reduces the number of synapses. Nonetheless, studies at later developmental stages should be done. Is this reduction of synapses recovered later in development? In addition, Ube3a loss of activity has been associated with hyper activation of Mtorc1 resulting in increased translation levels. The increase in translation levels could lead to increased levels of other proteins that are not Ube3a targets and therefore affect synaptic activity.

Only depletion of most abundant and shorter human Ube3a isoform 1 originates symptoms similar to the ones found in AS patients. Thus, a characterization of the function and interactors of the different isoforms should be done in order to understand if the impairment found in AS are only a result of loss of isoform 1 or a consequence of overall proteostasis regulation.

Despite studies shown that Ube3a is responsible for Ubiquitinating Ephexin5 and SK2, still remains unclear how Ube3a interacts with these proteins with big impact to excitatory synapse formation.

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