Pathophysiology of Synapse Function in Sanfilippo Syndrome

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DEDICATION

This document is dedicated to McGill University, my supervisor, my friends and family, as well as to my laboratory.

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PREFACE AND CONTRIBUTION OF AUTHORS

Dr. Chanshuai Han and Dr. Peter McPherson from the Montreal Neurological Institute collaborated with me on the production, staining, and imaging of neuronal cultures from the several mouse models used. Dr. Graziella Di Cristo from the Ste-Justine Hosptial research center collaborated with me on the confocal imaging of GFP-expressing hippocampal neurons from the mouse models. The previous experimentation performed on the mouse model was completed by the laboratory of Dr. Alexey Pshezhetsky. All of the other experimentation was performed and completed by myself, after being trained by members of the laboratory, Dr. Larbi Dridi and Dr. Carla Martins.

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ABSTRACT

Mucopolysaccharidosis III Type C (MPS IIIC), or Sanfillipo syndrome type C, is an inherited paediatric disease that is manifested through rapid mental and cognitive defects, as well as sleep disorders, hearing loss and ultimately dementia and early death. The disease is caused by impairment of lysosomal catabolism of the glycosaminoglycan, heparan sulfate (HS) due to mutations in the *Hgsnat* gene encoding for heparan sulfate acetyl-CoA: α-glucosamine N-acetyltransferase (HGSNAT) involved in the acetylation of a terminal glucosamine residue of HS, which is required for the subsequent hydrolysis of the remaining chain. In order to study pathophysiology of MPS IIIC, a mouse model of the disease was developed by introducing a ß-geo cassette into intron 7 of the mouse *Hgsnat* gene, rendering the gene inactive. Biochemically, these mice show glucosaminoglycan and ganglioside accumulation in the brain beginning at birth, and significant neuronal loss by 10 months of age. The mice show hyperactivity starting at the age of 6 months, and cognitive memory defects at 10 months. The cause of the major clinical manifestation of the disease, rapid mental decline, is not well understood. Our hypothesis is that the cognitive dysfunction and other manifestations occurring in the MPS IIIC patients and mice before the age when significant neuronal loss is detected are caused by a decrease in neurotransmission due to synaptic defects in cortical and hippocampal neurons. In order to test this hypothesis, synaptogenesis, synaptic spine density and morphology were analyzed at different age in the CA1 region

of the hippocampus of MPS IIIC mouse model that expresses green fluorescent proteins in hippocampal neurons. We found that in the MPS IIIC mice the spine density and maturity were decreased already at the age of 20 days and further declined with age. Similar results were found in the hippocampal neurons of a mouse model of another lysosomal disorder, Tay-Sachs disease indicating that synaptic defects are caused by lysosomal storage in general. The formation and trafficking of synaptic vesicles in the neurons was further analyzed by immunohistochemistry using antibodies against several pre- and post-synaptic markers involved in docking and fusion of synaptic vesicles both in vivo (in brain sections) and in vitro (in E16 cultured hippocampal neurons). The levels of both synapsin and synaptophysin were decreased in cultured neurons from MPS IIIC mice compared to those from wild type mice, as well as the levels of the pre-synaptic proteins VAMP2 and synaptophysin in brain sections. These results suggest the presence of synaptic defects on both hippocampal and cortical neurons, which could lead to the cognitive deficiencies exhibited in patients with the disorder.

Key words: lysosomes, Mucopolysaccharidosis Type IIIC, Sanfilippo syndrome, neurodegeneration, cognitive defects, hippocampal neurons, synapses, synaptic spines, Tay-Sachs disease

ABRÉGÉ

La mucopolysaccharidose de type IIIC (MPS IIIC), aussi appelée syndrome de Sanfilippo, est une maladie héréditaire et infantile qui se traduit par un rapide retard mental et un déclin cognitif aboutissant au décès avant l'âge adulte. Cette maladie est causée par des mutations dans le gène Hgsnat induisant une modification du catabolisme lysosomal des héparines sulfates. Nous avons généré des souris transgéniques déficientes pour le gène codant *Hgsnat*. Ces souris présentent une accumulation d'héparine sulfate dans les neurones, une perte neuronale importante à l'âge de 10 mois et une diminution de la mémoire cognitive à 10 mois. L'hypothèse est que la dysfonction cognitive observée chez les patients et le modèle animal de la maladie MPS IIIC est causée par une diminution de la neurotransmission via un défaut synaptique dans les neurones d'hippocampe. Nous avons généré une souris MPS IIIC qui exprime la protéine fluorescente GFP dans les neurones d'hippocampe et analysé la densité et morphologie des épines dendritiques au niveau de la région CA1. Les résultats montrent que les souris MPS IIIC présentent une diminution du nombre d'épines dendritiques et une morphologie moins mature, à partir du 20ème jour postnatal. Des résultats similaires ont été trouvés dans les neurones de l'hippocampe d'une souris modèle de Tay-Sachs. Nous avons ensuite analysé la formation et le trafic des vésicules synaptiques dans les neurones par immunohistochimie sur des coupes sagittales de cerveau ainsi que dans des cultures de neurones d'hippocampe. Les

résultats montrent que l'expression de la synapsine et la synaptophysine, deux marqueurs synaptiques impliqués dans l'ancrage et la fusion des vésicules présynaptiques, est diminuée dans les neurones en culture issus des souris MPS IIIC. Une diminution de l'expression de la synaptophysine et VAMP2 est aussi observée sur les coupes de cerveau adulte. L'ensemble de ces résultats indique une déficience synaptique dans les neurones d'hippocampe, qui pourrait conduire aux déficiences cognitives observées chez les patients atteints de MPS IIIC.

Mots-Clés: lysosomes , mucopolysaccharidose de type IIIC , le syndrome de Sanfilippo , la neurodégénérescence , défauts cognitifs , les neurones de l'hippocampe , les synapses , épines synaptiques , maladie de Tay-Sachs

ABBREVIATIONS

CNS	Central Nervous System
ER	Endoplasmic Reticulum
GAG	Glucosaminoglycan
EGFP	Enhanced Green Fluorescent Protein
HGSNAT	Heparan sulfate alpha-glucosaminide N-acetyltransferase
KO	Knockout
LAMP	Lysosomal Associated Membrane Protein
LSD	Lysosomal Storage disorder
MPS	Mucopolysaccharidosis
MPSIIC	Mucopolysaccharidosis Type IIIC
SV	Synaptic vesicle
TGN	Trans-Golgi network
t-SNARE	Target-Soluble NSF Attachment Protein Receptor
VAMP1	Vesicle Associated Membrane Protein 1
v-SNARE	Vesicle-Soluble NSF Attachment Protein Receptor
WT	Wild Type

Chapter 1

Introduction and Literature Review

1.1 Lysosomes

Lysosomes are membrane-bound organelles located in the cytoplasm of cells. They contain over 100 hydrolytic enzymes that are used in the catabolism of several substrates such as proteins, lipids, nucleic acids, and non-functional or damaged organelles. These enzymes include phosphatases, sulfatases, proteases, nucleases and lipases among others. The enzymes all function at the acidic pH 5.0, the pH inside the lysosome.

Materials to be degraded in the lysosome are transported there via several mechanisms. One involves the endocytic pathway, in which material from outside the cell is taken into early endosomes. A selection process occurs further where the material to be utilized by the cell is brought back to the plasma membrane via recycling endosomes, and the substrate to be degraded is left to become cargo for the late endosomes. Upon acidification of their lumen these late endosomes fuse with other late endosomes and lysosomes containing acid hydrolases to form multivesicular bodies, which further mature to lysosomes (Saftig and Klumperman 2009). Another mechanism of lysosomal catabolism is autophagy. When organelles such as the mitochondria are defective or in otherwise need of degradation, the organelle is enclosed in a membrane which initially comes from the endoplasmic reticulum creating an autophagosome. This autophagosome then fuses with vesicles carrying lysosomal hydrolases and together they become a mature lysosome, where the defective organelle gets degraded.

The transport of lysosomal hydrolases and other lysosomal proteins to the lysosomes can either be direct, from the trans-Golgi network to the lysosome, or indirect, involving transport of the proteins to the plasma membrane followed by endocytosis (Bonifacino and Traub 2003, Saftig and Klumperman 2009).

1.2.1 Lysosomal Storage Diseases

Lysosomal storage diseases (LSDs) are a group of inherited metabolic disorders involving the lysosome, initially discovered by H.G Hers, who described that an undegraded substrate present in Pompe disease accumulates in the lysosomes (Hers 1972). Most LSDs result from deficiencies of acidic hydrolases (Winchester 2004) and a considerable number are caused by defects in lysosomal membrane transporters or non-enzymatic soluble lysosomal activator proteins (Saftig and Klumperman 2009). In addition, LSD Mucolipidosis II/III (I-cell disease) is caused by multiple deficiencies of soluble lysosomal hydrolases due to the defect in the targeting to the lysosome. The undegraded substrates then accumulate in the lysosome leading to adverse cell response and eventually death.

The storage of the undegraded substrate generally does not occur in all cells, but rather in the specific tissues where the substrate is plentiful (Winchester, Vellodi et al. 2000). For example, since glycosphingolipids are abundant in the brain, LSDs that affect the lysosomal degradation of gangliosides (GM1-gangliosidosis, Tay-Sachs and

Sandhoff diseases, Niemann-Pick Type A and B) cause an accumulation of undegraded glycosphingolipids in neurons, resulting in their subsequent death (Kacher and Futerman 2006). The mechanism by which the storage of these undegraded substrates leads to cell death in the affected tissue is not completely understood. Recently, several studies elucidated mechanisms that may be common for several lysosomal storage diseases. Firstly, LSD patient fibroblasts were analyzed for their mRNA profiles, and a decrease in the expression of the ubiquitin C-terminal hydrolase was discovered in 8 different LSDs. This enzyme is essential for protein degradation pathways in the lysosome and its down-regulation could lead to cell death (Bifsha, Landry et al. 2007). Also, a block of autophagy has been shown to be a crucial component in the pathogenesis of all LSDs (Settembre, Fraldi et al. 2008). Considering that autophagy is one of the main mechanisms by which substrates reach the lysosome, accumulation of undegraded substrates in the lysosome leading to defective fusion between autophagosomes and lysosomes would block the autophagocytic pathway. Because of this block, dysfunctional mitochondria accumulate in the cell, leading to cell death (Settembre, Fraldi et al. 2008).

1.2.2 Classification and incidence of Lysosomal Storage Diseases

The incidences of LSDs vary from about 1:57,000 to 1:4,000,000 live births (Meikle, Hopwood et al. 1999), and 1:5,000-1:10,000 as a group (Staretz-Chacham,

Lang et al. 2009). More than 50 LSDs are known to exist and some are frequent for particular populations. For instance, Tay Sachs disease, which is caused by mutations in the human *HEXA* gene and leads to rapid degeneration of nerve cells and death by age 4, is prominent among those of Ashkenazi Jewish nationality and French Canadians from the lower St-Laurence region (Myerowitz and Costigan 1988, De Braekeleer, Hechtman et al. 1992). Inheritance for LSDs is generally autosomal recessive but several diseases such as Fabry or Hunter diseases are X-linked (Pinto, Vieira et al. 2010). Clinical symptoms and characteristics considerably vary between LSDs, however common symptoms include hepatomegaly (enlargement of the liver), skeletal and muscular deformations, and neurodegeneration. About 20—30% of enzyme residual activity can often be enough to perform normal catabolism of substrates, therefore heterozygotes for LSDs are generally unaffected by clinical symptoms (van de Kamp, Niermeijer et al. 1981). Clinical symptoms generally occur when the residual enzyme activity becomes less than 15%. Severe mutations such as frame-shift and nonsense mutations of lysosomal enzymes causing a complete absence of residual activity normally tend to lead to more severe clinical phenotypes.

LSDs are grouped into several major classes based on biochemical properties of accumulated substrates and manifestations of the diseases (TABLE 1).

Several treatments for lysosomal storage diseases, such as hematopoietic cell (bone marrow) transplantation (Peters and Steward 2003), enzyme replacement

therapy, and gene correction have been shown to be relatively successful in alleviating some of the symptoms (Grabowski and Hopkin 2003), however no cure currently exists.

Disease	Defective Enzyme	Undegraded Substrate
1. Mucopolysaccharidoses		
MPS I (Hurler's syndrome)	α-L-iduronidase	heparan/dermatan sulphate
MPS II (Hunter's syndrome)	iduronate-sulfatase	heparan sulphate
MPS III-A (Sanfillipo A)	heparan N-sulfamidase	heparan sulphate
MPS III-B (Sanfillipo B)	α-N-acetylglucosaminide	heparan sulphate
MPS III-C (Sanfillipo C)	heparan-α-glucosaminide N-acetyltransferase	heparan sulphate
MPS III-D (Sanfillipo D)	N-acetylglucosamine 6-sulfatase	heparan sulphate
MPS IVA (Morquio A)	galactosamine -6-sulfate sulfatase	keratan/chondroitin 6-sulfate
MPS IVB (Morquio B)	ß-Galactosidase	keratin sulphate
MPS VI (Maroteaux-Lamy syndrome)	N-Acetyl galactosamine α-4-sulfate sulfatase	dermatan sulphate
MPS VII (Sly syndrome)	ß-Glucuronidase	heparan/dermatan/Chondroitin 6-sulphate
MPS IX (Natowicz syndrome)	Hyaluronidase	hyaluronic acid
2. Mucolipidoses		
ML I (Sialidosis)	α-neuraminidase I	Sialyloligosaccharides
ML II (I-cell disease)	N-acetylglucosaminyl-1-phosphotransferase	oligosaccharides and lipids
ML III (Pseudo-Hurler's polydystrophy)	N-acetyl glucosamine-1-phosphotransferase	oligosaccharides and lipids
ML IV (Sialolipidosis)	Mucolipin I	Lipids
3. Sphingolidoses		

G_{M1} gangliosidosis	ß-galactosidase	G_{M1} gangliosides
G_{M2} gangliosidosis Type I (Tay-Sachs)	ß-hexosaminidase A	G_{M2} gangliosides
G_{M2} gangliosidosis Type 2 (Sandhoff's disease)	ß-hexosaminidase A+B	G_{M2} gangliosides
G_{M2} activator protein deficiency (AB variant)	G_{M2} ganglioside activator	G_{M2} gangliosides
Niemann-Pick disease, SMPD1-associated	acid sphingomyelinase	Sphingomyelin
Niemann-Pick C	NPC1 and NPC2	cholesterol/glycolipids
Gaucher's Disease	glucocerebrosidase	Glucocerebroside
Farber's disease	Ceramidase	fatty material lipids
Fabry's disease	Trihexosylceramide α-galactosidase	Globotriasylceramide
Metachromatic leukodystrophy	arylsulfatase A	Sulfatides
Mucosulfatidosis	Sulfatase-modifying factor-1	Sulfatases
Krabbe's disease	Galactosylceramide ß-galactosidase	Galactocerebroside
4. Oligosaccharidoses		
Galactosialidosis	Cathepsin A	Sialyloligosaccharides
α/ß-Mannosidosis	α/ß-Mannosidase	mannose/oligosaccharides
Fucosidosis	α-fucosidase	Fucose
Schindler's disease	α--N-acetygalactosaminidase	Oligosaccharides
5. Others		
Pompe disease	α-glycosidase	Glycogen
Salla disease	Sialin	sialic acid
Wolman disease	acid lipidase	cholesterol esters
Batten disease	battenin	Battenin

Table 1 –Different Classes of Lysosomal Storage Disorders

(Adapted from The Merck Manuals)

1.2.3 Tay-Sachs Disease

Tay-Sachs disease is an autosomal recessive genetic disease that is caused by a mutation in the hexosaminidase A gene (Kaback 2000). Although the disease is generally rare, it's prevalence is increased in specific populations such as that of the Ashkenazi Jewish and French Canadian; one in approximately 27 Jewish people in the US carry the Tay-Sachs disease gene (Tegay 2012). It is manifested through rapid neurodegeneration, difficulty swallowing, progressive blindness, as well as cognitive decline and other severe symptoms. The *HEXA* gene codes for the subunit alpha of the lysosomal hydrolytic enzyme hexosaminidase A, which functions in the catabolism of glycolipids, more specifically GM2-ganglioside. Gangliosides play important roles in cell-cell recognition as well as in development and function of the nervous system. If the hexosaminidase A enzyme is deficient, gangliosides and primarily GM2-ganglioside accumulate in the neurones. This interferes with regular neuronal function leading to rapid neurodegeneration and cognitive decline (Rosebush, MacQueen et al. 1995).

1.3 Mucopolysaccharidoses

About 30% of all LSDs belong to a group called mucopolysaccharidoses (MPS), which consist of several storage diseases that are caused by genetic deficiencies of

lysosomal enzymes required for the catabolism of glycosaminoglycans (Muenzer 2011). Thus, glycosaminoglycans that are transported to the lysosome to be catabolised are then accumulated due to the absence of proper enzymatic degradation. These disorders manifest through similar symptoms, which include physical deformations such as rough facial features, short stature, hepatomegaly (enlargement of the liver), as well as joint and bone deformations. Also, neurological issues arise, leading to cognitive and behavioural problems, such as memory loss, reduced fear, increased aggressiveness, and developmental delay. Other symptoms include hearing loss and retinal degeneration. The patients have a very limited lifespan, usually dying prior to adulthood (National Institute of Neurological Disorders and Stroke, 2014).

MPS III (Sanfilippo syndrome) is the most common of the mucopolysaccharidoses, it also causes the mildest of physical abnormalities (Gilkes and Heldermon 2014). MPS III is caused by a lysosomal accumulation of heparan sulfate (HS), a plasma membrane glycosaminoglycan, which consists of long carbohydrate chain of repeating disaccharide units that are attached to a cell surface or matrix proteins. HS is heavily involved in cell-cell and cell-matrix interactions, cell growth and cell differentiation, which are all important in development of bone and connective tissues. When the time comes for its turnover, heparan sulfate is brought to the lysosome via endocytic mechanisms and is subsequently catabolised by a consequent action of eight lysosomal enzymes (Freeman and Hopwood 1992) (FIGURE 1). Defects

in four of these enzymes lead to four different subtypes of MPS III. These four subtypes are: MPS IIIA (heparan sulfamidase deficiency, *SGSH*), MPS IIIB (α-N-acetylglucosaminidase deficiency, *NAGLU*), MPS IIIC (heparan-α-glucosaminide N-acetyltransferase deficiency, *HGSNAT*), and MPS IIID (N-acetylglucosamine 6-sulfatase deficiency, *GNS*). Clinically, there is no significant difference between the 4 types of MPS III, although it has been suggested that MPS IIIA has a more severe course (van de Kamp, Niermeijer et al. 1981). The incidence for all four disorders together is approximately 1 in 70,000 live births (National MPS society 2011).

1.4.1 Mucopolysaccharidosis Type IIIC

Mucopolysaccharidosis Type IIIC (MPS IIIC, Sanfilippo type C) is the second most rare form of MPS III after MPS IIID, with an incidence of approximately 1 in 1,500,000 live births worldwide (Meikle, Hopwood et al. 1999) , and of 0.1-0.2/100,000 live births in Portugal and the Netherlands (Poorthuis, Wevers et al. 1999, Pinto, Caseiro et al. 2004). Heparan sulfate acetyl-CoA: α-glucosamine N-acetyltransferase (HGSNAT) (Fan, Zhang et al. 2006), the enzyme deficient in MPS IIIC is located at the lysosomal membrane and is involved in the acetylation of amino group of a terminal glucosamine residue of HS, which is required for the subsequent hydrolysis of the remaining chain (Kresse, Barttsocas 1976) (**FIGURE 1**). This causes HS to remain undegraded, leading to its accumulation in lysosomes, and cellular damage in many tissues

(Feldhammer, Durand et al. 2009). HS is also excreted in the urine of MPS IIIC patients, which is used as a basic screen for the disease. The disease is manifested through several issues such as sleep disorders, hearing loss, joint stiffness, hepatomegaly, mental retardation and mental decline, among others (Bartsocas, Grobe et al. 1979). The rapid mental decline observed in the patients is not fully characterized, and its cause is unclear

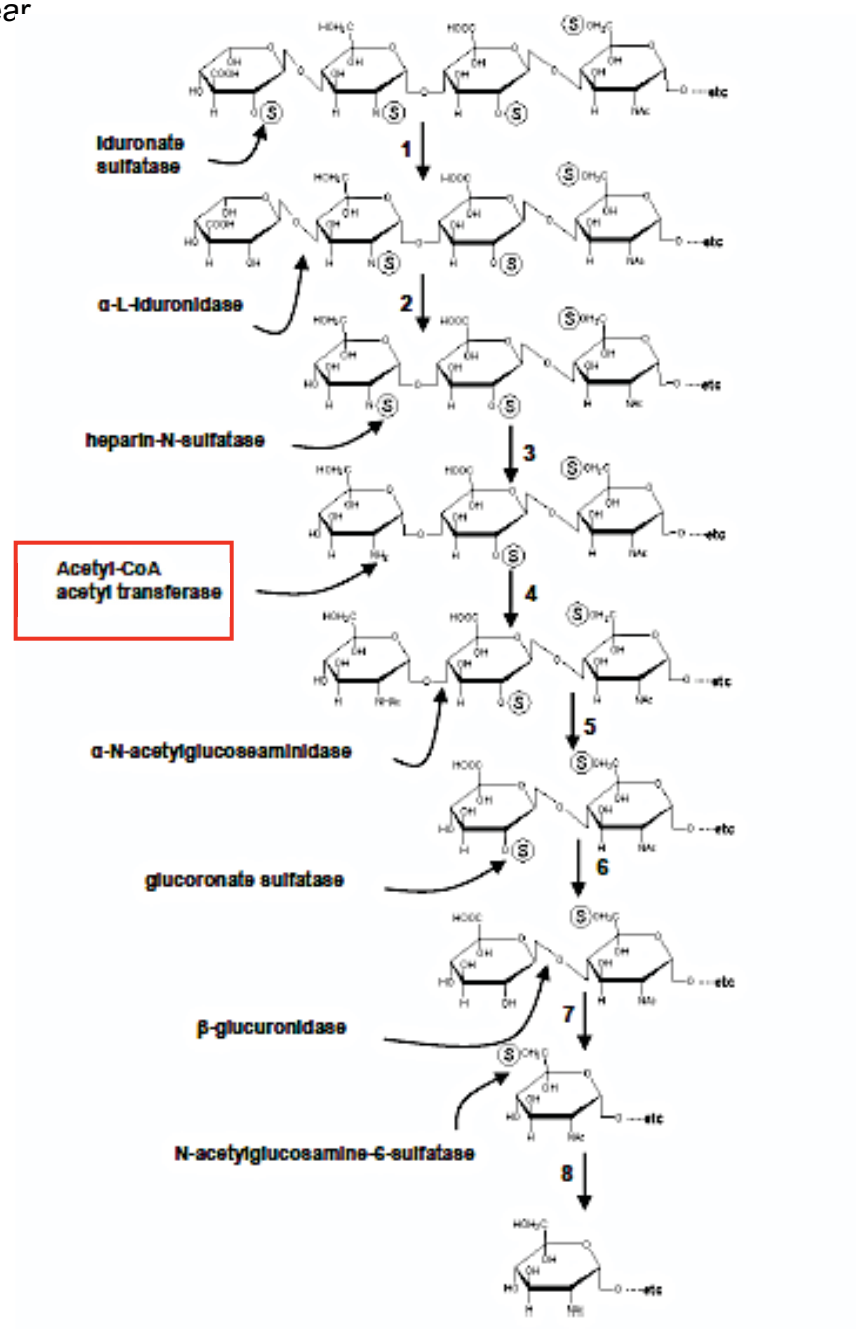


Figure 1 – Catabolic pathway for degradation of heparan sulfate

Heparan sulfate acetyl CoA acetyl transferase (step 4, in red box) is the enzyme which deficiency leads to MPS IIIC or Sanfillippo syndrome. When mutated, this enzyme is unable to acetylate the terminal glucosamine group on the heparan sulfate chain. This leads to accumulation of undegraded heparan sulfate.

1.4.2 Mutations in HGSNAT

Hrebicek *et al.* first described the mutations in the *Hgsnat* gene leading to deleterious effects including 3 frameshift mutations, 4 nonsense mutations, 14 missense mutations and 6 splice-site mutations (Hrebicek, Mrazova et al. 2006). Further studies have elucidated the mutation patterns in specific populations around the world. For example, 3 splice-site mutations, 3 frameshift deletions, 1 nonsense mutation, and 2 missense mutations were discovered in nine alleles from Italian patients (Fedele and Hopwood 2010). In Dutch patients, two splice-site mutations, one frameshift mutation, three nonsense mutations and eight missense mutations were discovered. Two of these missense mutations, p.R344C and p.S518F were most frequent amongst the mutated alleles (Ruijter, Valstar et al. 2008).

1.4.3 Mouse Model of MPSIIIC

A mouse model of MPS IIIC has been developed in order to study the pathophysiology of the disease. This mouse model was generated via gene trap technology (Stanford, Cohn et al. 2001), where the *Hgsnat* gene was effectively inactivated by introducing a ß-geo cassette, a functional fusion between the ß-galactosidase and neomycin resistance genes, into intron 7 of the mouse *Hgsnat* gene. This leads to a splicing of the mouse gene to generate a fusion protein containing Hgsnat amino acid sequence encoded by the exons 1-7 followed by aminoglycoside 3'-phosphotransferase and β-galactosidase. The Btk exon in the trap construct contains termination codons in all reading frames to prevent translation of the downstream Hgsnat exons (Hansen, Markesich et al. 2008). An efficient reduction of *Hgsnat* mRNA expression levels (0.6-1.5% of the normal levels) was demonstrated in multiple mouse tissues including liver, spleen, brain, and muscle. Also, HGSNAT activity in the same tissues was reduced to the levels below 1%, confirming the efficacy and validity of the mouse model. Biochemical analysis of brain and liver of the Hgsnat-Geo mice showed increased levels of glycosaminoglycans in both organs starting from birth, as well as an accumulation of GM2 and GM3-gangliosides in the brain at birth. The MPS IIIC mice have a reduced lifespan of 12-14 months, compared to a wild type mouse's lifespan of

about 2 years. Also, starting from 10 days the MPS IIIC mice show significantly increased levels of inflammatory markers TNFα and MIP1α in the brain. A decrease in neuronal density in somatosensory cortex was demonstrated at 10 and 12 months of age. Behaviourally, the KO mice exhibit hyperactivity beginning at 6 months of age as revealed by the open-field test, as well as cognitive memory defects beginning at 10 months of age as shown by the Morris water maze test. It is unclear however if the cognitive and neurological defects observed in the MPS IIIC mouse model are solely caused by the neuronal loss which could be observed only at 10 months. Similar characteristics were previously discovered in MPS IIIA and MPS IIIB mice (Ohmi, Greenberg et al. 2003, Ausseil, Desmaris et al. 2008).

1.5.1 Synapses and Synaptic Vesicles

The synapse is a structure that allows a neuron to transmit a chemical or electrical signal to another neuron. The pre-synaptic cell, once stimulated by an excitatory or inhibitory signal from another cell, generates and releases synaptic vesicles that are loaded with neurotransmitters. These neurotransmitters activate receptors on the plasma membrane of the nearby post-synaptic cell, which allow either an excitatory or inhibitory signal to be transmitted to the subsequent neuron. In order for the synaptic vesicles to release their neurotransmitters into the synaptic cleft (space

between pre- and post- synaptic cells), they must first form a fusion pore with the pre-synaptic terminal membrane. This fusion pore is formed via intricate protein-protein interaction between proteins on the synaptic vesicle (v-SNAREs) and proteins on the target pre-synaptic membrane (t-SNARES)(Pevsner, Hsu et al. 1994, Hannah, Schmidt et al. 1999). These two sets of proteins form coiled complexes and give the synaptic vesicle enough energy to form a fusion pore with the plasma membrane of the pre-synaptic cell, releasing its contents into the synaptic cleft (Söllner, Bennett et al. 1993).

1.5.2 Synaptic Vesicle Biogenesis and Recycling

Initial synaptic vesicles biogenesis happens in the Golgi apparatus in the cell body of a neuron (Hannah, Schmidt et al. 1999), which can be at a significant distance from the synaptic terminal, sometimes reaching distances of up to 1 meter away (Alberts et al. 2002). Proteins of synaptic vesicles are packaged in vesicles at the Golgi apparatus in the cell body and then brought to the synaptic terminal for use in neurotransmission. It is therefore, essential for the cell to develop synaptic vesicle recycling machinery, where after dissociating via endocytosis (release of neurotransmitters) the components of the synaptic vesicle are recycled and repackaged into another synaptic vesicle. This process occurs near the synaptic terminal so no excess time is spent producing new vesicles (Ryan, Reuter et al. 1993). The endosome

is the organelle responsible for this recycling process. Endosomes gather up the vesicle once it has released its neurotransmitters, and either brings it to the lysosome for degradation, or repackages the components to form another vesicle (Hoopmann, Punge et al. 2010). The proteins involved in these interactions are crucial for successful neurotransmission, as for synaptic vesicle recycling. When defects are introduced into either the synaptic vesicle protein complexes (v-SNARES) or other proteins involved into the recycling pathways, neurotransmission is severely compromised, leading to neurodegenerative symptoms in patients. For example, a mutation in the v-SNARE gene *SNAP29* has been shown to lead to severe neurological defects in patients including symptoms such as cerebral dysgenesis and ichthyosis (Sprecher, Ishida-Yamamoto et al. 2005).

1.5.3 Synaptic Spines

Synaptic spines are small extensions that branch off of neuronal dendrites. These structures act as signal receptors where they process neurotransmitter signals received from a pre-synaptic cell and aid in the transmission of signals to the neuronal cell body (Matsuzaki, Honkura et al. 2004). They also act as contact points between neurons. There are hundreds of thousands of spines present on each neuron and they regularly appear at a frequency of about 5 spines per 1 μm of dendrite in the adult hippocampus (Trommald, Jensen et al. 1995). The spines found on hippocampal and

cortical neurons are generally excitatory. One of their major roles is to help to compartmentalize cellular signals to one specific portion of the dendrite, as this is essential for a more localized cellular response in adult mammalian brain. They are highly dynamic, constantly changing in number and size in order to accommodate the dynamic requirements of memory retention and learning (Engert and Bonhoeffer 1999). Spines are heavily involved in both memory and learning processes in the mammalian central nervous system during which they go through both long-term potentiation (LTP; habituation of neurons to a specific signal) where the synaptic spine density increases in response to the repeated exposure to a signal, and long-term depression (LTD; de-habituation of neurons to a specific signal) where the synaptic spine density decreases in response to the absence of a signal (Toni, Buchs et al. 1999).

Synaptic spines drastically differ in their morphology, some being more mature and dynamic than others. These spines often have a shape of a mushroom with a large bulbous head and a thing stalk, which is the most mature formation. Other subtypes such as thin, with a long stalk and small bulbous head, or stubby, with a large bulbous head and no stalk, are less mature and differentiated (Harris, Jensen et al. 1992). Mushroom formation spines are the most susceptible to signal intake.

1.5.4 Microglia and Synapses

Microglia act as the macrophages of the brain and spinal cord and therefore are considered to be the main form of immune response in the central nervous system (Rivest 2009). They recognize antigens in the central nervous system and rapidly phagocytose them. Microglia are also heavily implicated in synaptic plasticity (Morris, Clark et al. 2013). A major role in biogenesis of synaptic spines belong to microglia cells through a process called synaptic pruning (Paolicelli, Bolasco et al. 2011). Regulating the synaptic network connections that are made by the adult brain is crucial for the correct and efficient wiring of the CNS; if too many or too few connections are made, the central nervous system will not be able to function at full capacity. It has been shown that microglia engulf synapses during the crucial time of synaptic maturation. Also, during synapse maturation, neurons upregulate the production of the chemokine fractaline Cx2cl1, the receptor of which is only expressed on microglia (Parkhurst, Yang et al. 2013). When the receptor is knocked out, more synaptic spines are significantly found in the deficient neurons compared to the wild type, indicating a defect in synaptic pruning. In contrast, if microglia becomes activated, an excess of synaptic pruning may occur, significantly reducing the density of synaptic spines due to over-regulation of synapses. Alternatively, activated microglia secrete TNF-α (tumour necrosis factor alpha), which is a molecule involved in the inflammatory response playing also a significant role in synaptic pruning/scaling (Stellwagen and Malenka 2006). However, at

high enough doses, TNF-α becomes neurotoxic due to increased levels of synaptic pruning, which decreases the density of synapses and synaptic spines to a non-functional level.

1.5.5 Synaptic defects in LSDs

Following exocytosis in the presynaptic nerve terminal, SVs are retrieved from the presynaptic plasma membrane by endocytosis, reloaded with neurotransmitter and become available for additional rounds of neurotransmitter release. Under certain circumstances, SVs fuse with presynaptic endosomes, sorting the SV proteins for degradation instead of reformation of new SVs (Murthy and De Camilli 2003, Uytterhoeven, Kuenen et al. 2011). Although nerve terminals lack ultrastructurally identifiable lysosomes, recent evidence indicates that nerve terminals contain endosomal populations where lysosomal and SV proteins co-exist, along with the adaptor AP-3 (Newell-Litwa, Salazar et al. 2009), which is thought to function in SV reformation from endosomes (Blumstein, Faundez et al. 2001). These endosomal-lysosomal hybrid structures are very likely involved in discrimination between recycling/reformation of SVs and degradation of SV proteins. Therefore, lysosomal storage could potentially affect sorting machineries involved in the generation of SVs.

Several studies by Wilkinson *et al.* and Vitry *et al.* have demonstrated a decrease in the levels of several synaptic proteins, including VAMP2 and synaptophysin, both

involved in synaptic vesicle function, in the cerebral cortex and hippocampus of mouse models of different types of MPS III, specifically MPS IIIA and MPS IIIB (Vitry, Ausseil et al. 2009, Wilkinson, Holley et al. 2012). VAMP2 or synaptobrevin is an integral member of the v-SNARE complex and participates in direct interactions with members of the t-SNARE. When VAMP2 binds to the t-SNARE, the proteins coil together in order to bring the vesicle closer to the pre-synaptic membrane (Melia, Weber et al. 2002). Once the vesicle is in proximity, the vesicle membrane and the target membrane fuse. Synaptophysin is an accessory protein that interacts with proteins such as VAMP2 to aid in the coiling process. If either of these proteins is defective or repackaged incorrectly, the endocytic/neurotransmitter system becomes compromised. The deficiency of both VAMP2 and synaptophysin in MPS III may suggest therefore that defects in synaptic function can also occur in the human patients.

1.6 Hypotheses and Objectives

Our major hypothesis is that the neurological and cognitive decline observed in the MPS IIIC mouse model is not only a consequence of neuronal loss observed at 10 months of age, but also a consequence of a defect in synaptic transmission occurring earlier in life. Defects in synaptic transmission could also explain the behaviour changes (hyperactivity, reduced anxiety) observed at 6 months i.e. before the neuronal loss. By analyzing both pre-synaptic and post-synaptic machinery of neurons in brains

of MPS IIIC mice at different ages, we can define whether synaptic transmission defects are present and when they start.

We therefore propose to study synaptic vesicle proteins as well as synaptic spine density and dynamics in order to elucidate a mechanism for the neurological decline observed in MPS IIIC mice and patients. Both pre- and post-synaptic neurons will be analyzed for the presence of defects that can interfere with regular neuronal function at the synaptic level. In particular we will study proteins that are heavily implicated in synaptic function, as well as morphology of neuronal structures and organelles such as synaptic spines, Golgi, and endosomes. Any defect in these aspects could potentially translate to a compromise of the central nervous system of the MPS IIIC patients. Furthermore, by evaluating cellular processes such as the endosomal cycle and synaptic vesicle cycle or the interactions between neurons and other brain cells, we can elucidate a mechanism through which lysosomal storage of HS can lead to synaptic defects.

Chapter 2

Materials and Methods

2.1 Laboratory animals (Thy1-GFP, Tay-Sachs, HGSNAT)

HGSNAT-Geo mice. A functional murine model of MPSIII C was generated at the Texas A&M Institute for Genomic Medicine using gene trap technology. A selectable marker, ß-geo cassette (a functional fusion between the ß-galactosidase gene and neomycin resistance gene) was introduced into intron 7 of the mouse *Hgsnat* gene to identify successful gene trapping. This insertion leads to the splicing of the *Hgsnat* exon 7 to generate a fusion protein containing Hgsnat amino acid sequence encoded by the exons 1-7 followed by aminoglycoside 3'-phosphotransferase and β-galactosidase. The BTK (Bruton agammaglobulinemia tyrosine kinase) exon in the trap construct contained termination codons in all reading frames to prevent translation of the downstream Hgsnat exons. Targeted embryonic stem cells were injected into blastocysts and the resulting chimeras bred to generate F1 and subsequently F2 generations of mice homozygous for *Hgsnat-Geo* allele.

Thy1-EGFP and Thy-EGFP/Hgsnat-Geo mice. The Thy1-EGFP transgene that leads to expression of enhanced green fluorescent protein (EGFP) in hippocampal neurons was designed using a modified regulatory region of the mouse *thy1.2* gene with the Thy1 promoter that was put upstream of an EGFP gene sequence (http://jaxmice.jax.org/strain/007788.html). The transgene was then injected into C57Bl/6 oocytes. The transgenic mice were subsequently backcrossed with C57Bl/6 mice for ten generations. Heterozygous *Thy1-EGFP* mice were bred with *Hgsnat-Geo mice* in order to generate *Thy-EGFP/Hgsnat-Geo* strain, an MPS IIIC mice that express EGFP in hippocampal neurons (Feng, Mellor et al. 2000)

Thy1-EGFP Tay-Sachs mice. Generation of mouse model of Tay-Sachs disease with targeted disruption of hexosaminidase A gene has been previously described (Yamanaka, Johnson et al. 1994). The strain was backcrossed for at least 10 generations to C57BL/6NCrl strain distributed by Charles River Quebec. Homozygous *Hexa* KO animals were bred to heterozygous *Thy1-EGFP* mice and double-heterozygous animals in the litter intercrossed to produce *Thy-EGFP$^{+/-}$/ Hexa KO* strain.

All mice were bred and maintained in the Canadian Council on Animal Care (CCAC)-accredited animal facilities of the Ste-Justine Hospital Research Center according to the CCAC guidelines. Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity, on a 12

h light: dark cycle. Approval for the animal care and the use in the experiments was granted by the Animal Care and Use Committee of the Ste-Justine Hospital Research Center.

2.2 Genotyping

The genotypes of mice were determined using tail DNA. The clipped tail tip biopsies were incubated in a solution of 500 ml lysis buffer containing 10% SDS, 0.2M EDTA, 1M Tris (pH 7.6), 5 M NaCl and 30 μl of Proteinase K (10 mg/ml) overnight at 55°C. The solution was then centrifuged at 14, 000 RPM for 10 minutes, and the pellet was washed with isopropanol and 70% ethanol, and left to dry. DNA pellets were then re-suspended in 100 μl of water and heated at 55°C for another 10 minutes.

The PCR to amplify the *Hgsnat* gene fragment was performed in a total volume of 50 μl containing 10 pmol of each primer, 0.2 mM dNTPs, 5 U/μl Taq polymerase (Invitrogen) and 50 ng of genomic DNA. Multiplex primers for detection of *Hgsnat* alleles were 5'-AGGCTCCAC ACGTGGTAAGT-3' (2 Forward), 5'-CTTATCTCCACGCGTCAATG-3' (1 Reverse), and 5'-CCAATAAACCCTCTTGCAGTTGC-3' (3 Downstream reverse). Samples were denatured at 95 °C for 2 min, followed by 10 cycles at 94 °C for 15 s, 52 °C for 30 s and 72 °C for 30 s, followed by 30 cycles at 94 °C for 15 s, 52 °C for 1 min and 72 °C for 30 s with a final extension reaction at 72 °C for 5 min.

The PCR to amplify the *EGFP* gene fragment was performed in a total volume of 50 μl containing 20 μM of each primer, 2.5 mM dNTPs, 5 U/μl Taq polymerase (Invitrogen) and 50 ng of genomic DNA. Multiplex primers for detection of *EGFP* alleles were 5'-AAGTTCATCTGCACCACCG-3' (Internal forward), 5'-TCCTTGAAGAAGATGGTG CG-3' (Internal reverse), 5'-CTAGGCCACAGAATTG AAAGATCT-3' (Actin control forward), and 5'-GTAGGTGGAAATTC TAGCATCATCC-3' (Actin control reverse). Samples were denatured at 94 °C for 1.5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min with a final extension reaction at 72 °C for 1 min.

The PCR to amplify the *Hexa* gene fragment was performed in a total volume of 50 μl containing 100 pM of each primer, 0.2 mM dNTPs, 1.5 U/μl Taq polymerase (Invitrogen) and 100 ng of genomic DNA. Multiplex primers for detection of HexA alleles were 5'-GGCCAGATACAATCATACAG-3' (Hexa-F), 5'-CTGTCCACATACTCTCCCCAC AT-3' (Hexa-R) and 5'-CACCAAAGAAGGGAGCCGGT-3' (PGK). Samples were denatured for the first cycle at 94 °C for 1.5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min with a final extension reaction at 72 °C for 1 min.

2.3 Immunohistochemistry

Mice were anesthetised with a pentobarbital solution and transcardiacally perfused with 30 ml of PBS followed by 30 ml of 4% PFA in PBS. Mouse brains were

removed and then incubated in 4% PFA overnight at 4°C. Brains were then placed in a 30% sucrose solution for 2 days, cut into halves sagittaly, frozen in Optimum Cutting Temperature (OCT) compound in plastic organ storage cups and stored at -80°C until used. Brains were cut into 40 μm sagittal sections using a cryostate and stored in PBS in 24 well plates at 4°C. *Thy-EGFP/ Hexa KO* and *Thy-EGFP/ Hgsnat-Geo* brain slices were immediately mounted onto microscope slides using Vectashield and sealed using nail varnish. Tissue sections for immunocytochemistry were blocked in a 5% goat serum -1% Triton X-100-PBS solution for 1 h and subsequently incubated with primary antibodies against synaptophysin (Synaptic Systems, 1:300, rabbit), VAMP2 (Cedarlane, 1:300, rabbit) and NeuN (Millipore, 1:400, mouse) overnight at 4°C. Sections were washed 3 times with PBS, and then incubated with Alexafluor-conjugated secondary antibodies (Alexa 488 goat anti rabbit IgG and Alexa 555 goat anti mouse IgG) for 2 h. Tissue sections were washed three times with PBS, and then mounted onto microscope slides using Vectashield as described before. Stacked images were taken on a Zeiss LSM510 confocal microscope using the 63X oil immersion objective.

2.4 Hippocampal Cultures

Embryos were extracted from pregnant female mice at embryonic day 16-17. The hippocampi were dissected out and kept in Hanks Balanced Salt Solution containing antibiotics. The tissue was incubated with 0.05% trypsin solution (Gibco) at 37°C for 15

minutes, washed 3 times with HBSS, and the cells were dissociated by consecutive pipetting using glass Pasteur pipettes with 3 different opening sizes (3, 2 and 1 mm). The cells were counted using a hemacytometer, and re-suspended in Neurobasal medium with B27, N2, and glutamine supplements. The cells were plated in 12 well plates at 50,000 cells per well on Poly-L-Lysine coated coverslips, left to differentiate for 21 days while changing half of the medium on days 3, 7, 14 and 21, fixed using a 4% PFA, 4% sucrose solution for 20 minutes and then stored in PBS at 4°C.

2.5 Immunofluorescence

The PFA-fixed cells were washed in PBS and permeabilized using 0.1% Triton X-100 in PBS for 5 min. Cells were washed again and then blocked with a 5% BSA-PBS solution for 1 h. Primary antibodies against synapsin (Millipore, 1:200, rabbit), synaptophysin (Sigma, 1:300, mouse), ß-3 tubulin (Sigma, 1:400, mouse), MAP2 (Sigma, 1:2000, chicken), EEA1 (Millipore, 1:200, rabbit), giantin (1:300, rabbit), LAMP2 (1:100, rat), and heparan sulfate (10E4 epitope, USBiological, 1:100, mouse) in 1% BSA-PBS were added and the cells were incubated at 10°C overnight with light shaking. The cells were then washed 3 times with PBS and incubated with secondary antibody (Alexa 555 goat anti mouse/rabbit IgG, Alexa 555 goat anti mouse/rabbit IgG, Alexa 647 goat anti rat IgG) for 2 h. The cells were washed with PBS and the coverslips were

dipped into double-distilled water and mounted onto microscope slides using Prolong Gold anti-fade reagent containing DAPI. Images were taken on a Zeiss LSM510 confocal microscope using the 63X oil immersion objective.

2.6 ELISA

TNF-α levels in media of hippocampal cultures were measured using a TNF-α Mouse ELISA kit from Life Technologies (KMC3011).

2.7 Western blots

Brain and liver tissues from *Hgsnat-Geo* and wild type mice were cut into pieces and mixed with 3 volumes of ice-cold RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 0.1% SDS, 2 mM EDTA, protease inhibitor cocktail, phosphate inhibitor cocktail and PMSF. The tissues were homogenized via sonication (three times for 20 sec). The cells were centrifuged at 4°C for 30 min. The supernatant was collected and analyzed for protein concentration using the Bradford assay. The samples were mixed with Laemmli buffer then boiled for 3 minutes. 40 μg of protein for each sample was loaded into a polyacrylamide gel with 15% resolving gel and 4% stacking gel and electrophoresis was run at 60-80 mV for ~1 h. Kaleidoscope molecular weight ladder (Bio-Rad) markers were used to determine molecular size of

protein fragments. Proteins were transferred onto a nitrocellulose membrane using a Bio-Rad transfer apparatus for 1 h at 70 mV. The membrane was stained with 5% Ponceau red stain to verify the presence of protein bands, and then blocked for 1 h in 5% BSA in TBST. The membranes were washed with TBST and incubated with antibodies for synaptophysin (Synaptic systems, 1:1000), VAMP2 (Cedarlane Laboratories, 1:1000), synapsin (Abcam, 1:1500) and GAPDH (1:10000) overnight at 10^{o}C. Membranes were washed three times for 5 min with TBST, and then stained with either rabbit or mouse HRP-conjugated secondary antibody for 2 h and washed again using TBST. The development was performed with Pierce ECL Western Blotting Substrate (Thermo Scientific) in accordance with the manufacturer's protocol.

Chapter 3

Results

3.1 Thy1-EGFP/MPSIIIC Mouse Shows a Reduction in the Number of Synaptic Spines on Hippocampal Neurons

Since cognitive defects were observed in the MPSIIIC mouse model, we analyzed synaptic spines on hippocampal neurons because of their importance for learning and memory. Due to the expression of GFP in hippocampal neurons of Thy1-EGFP/MPSIIIC mouse the structure of the spines could be directly studied by confocal microscopy without preliminary histochemical or immunohistochemical staining. Initial analysis was performed on brains of 3 months-old mice, i.e. before the age when the first signs of behavioural problems are observed. Mice were perfused with PBS followed by 4% PFA, and their brains extracted and sagittally cut at 80 μm-thick sections using a cryostate. The sections were analyzed for synaptic spine density in the CA1 region of the hippocampus, which is heavily involved in memory processing using a confocal fluorescent microscopy. From 20 to 30 Z-stacked images (1 µm apart) were taken for each field. We detected a significant reduction in the number of synaptic spines present on the MPSIIIC neurons as compared to the WT mice (**FIGURE 2A**).

Also, the MPSIIIC neurons showed an uneven thickness, alternating between a thinning and enlarging of the dendrite, compared to the WT cells which showed mostly regular, uniform dendrites. Overall, the dendrites of MPSIIIC neurons seemed to be defective in both general structure and in synaptic spine density. In order to verify whether this phenotype becomes more pronounced with age, we repeated analysis with brains of 8-month-old mice. We found that similar defects could be seen in hippocampal neurons of the 8 month-old mice: a reduction in synaptic spine density as compared to the WT cells, as well as a structurally inferior overall dendrite structure **(FIGURE 2B)**, but the defects found in 8 month-old mice were more severe than in 3 month-old mice. Finally, in order to establish an onset time for the development of spinal defects, WT and MPSIIIC mouse hippocampal neurons were analyzed at postnatal day 20. We found yet again the synaptic spine and structural defects similar to those seen in the MPSIIIC mice at both 3 months and 8 months of age **(FIGURE 2C)**, although the defects were not as severe. In all cases, large spheroid accumulations were also seen along the dendrites of MPSIIIC hippocampal neurons (**FIGURES 2A-C; white arrows)**. Following quantitative analysis of the synaptic spine density for hippocampal neurons from mice of different age, we could see that the defects in spine density in the KO cells were significant in all cases, and that the defects become progressively worse as the MPSIIIC mouse age increases (**FIGURE 2D)**.

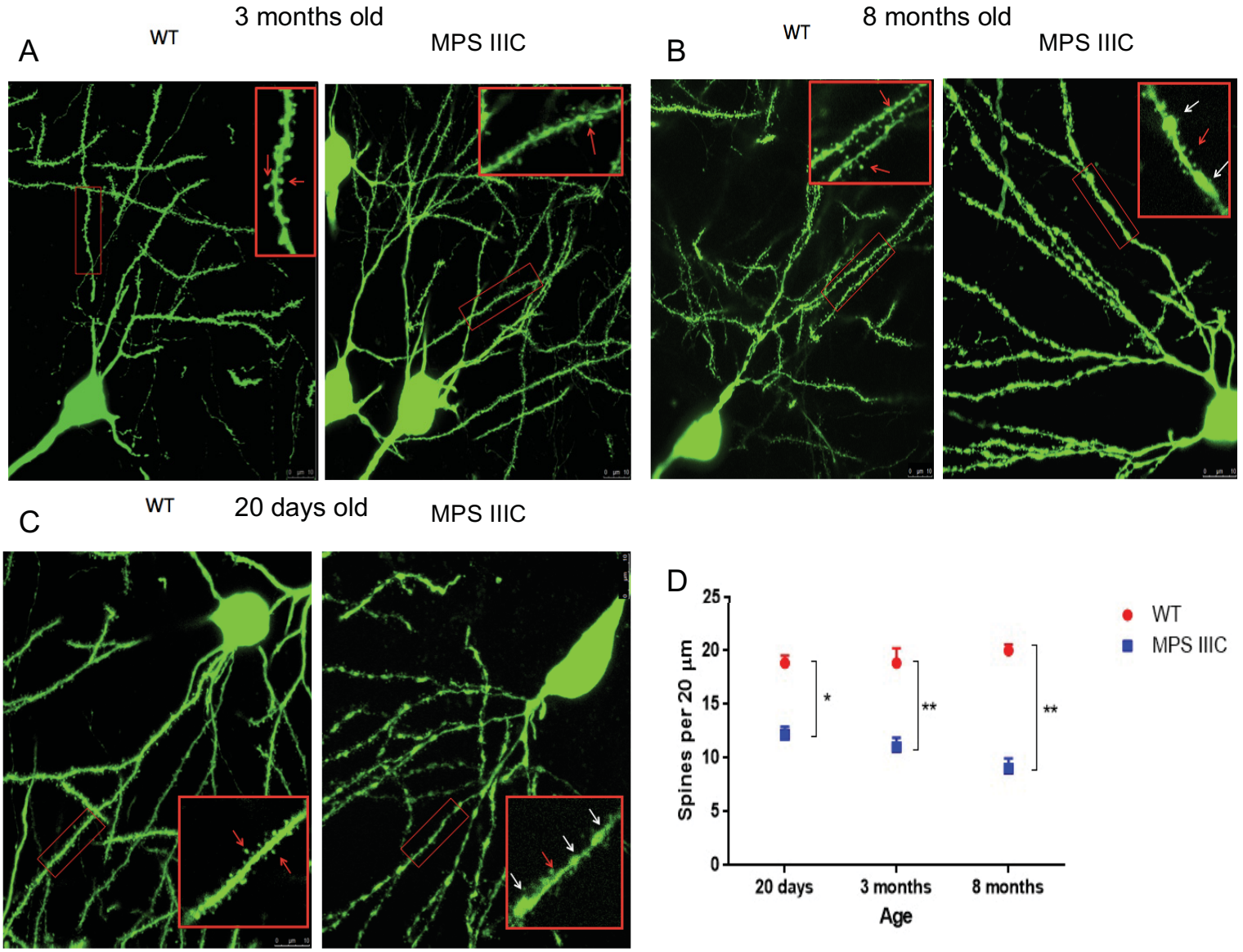


Figure 2. Synaptic Spines on Hippocampal Neurons of Thy1-EFP WT and MPSIIIC KO mice

(A) Confocal microscopy image of neurons in the CA1 region of the hippocampus in brains of 3 month-old (A), 8 month-old (B) and 20 days-old (C) Thy1-GFP WT (left) and MPSIIIC (right) mice.

Dendritic sections ~40 µm away from the soma are marked by red boxes and expanded in high-magnification inserts in each image. Red arrows mark synaptic spines, white

arrows, dendritic spheroids observed in the neurons of MPS IIIC mice. Magnification 63X. Consequent Z-stacks were overlaid in order to obtain an image of the full neuron. Bar equals to 10μm.

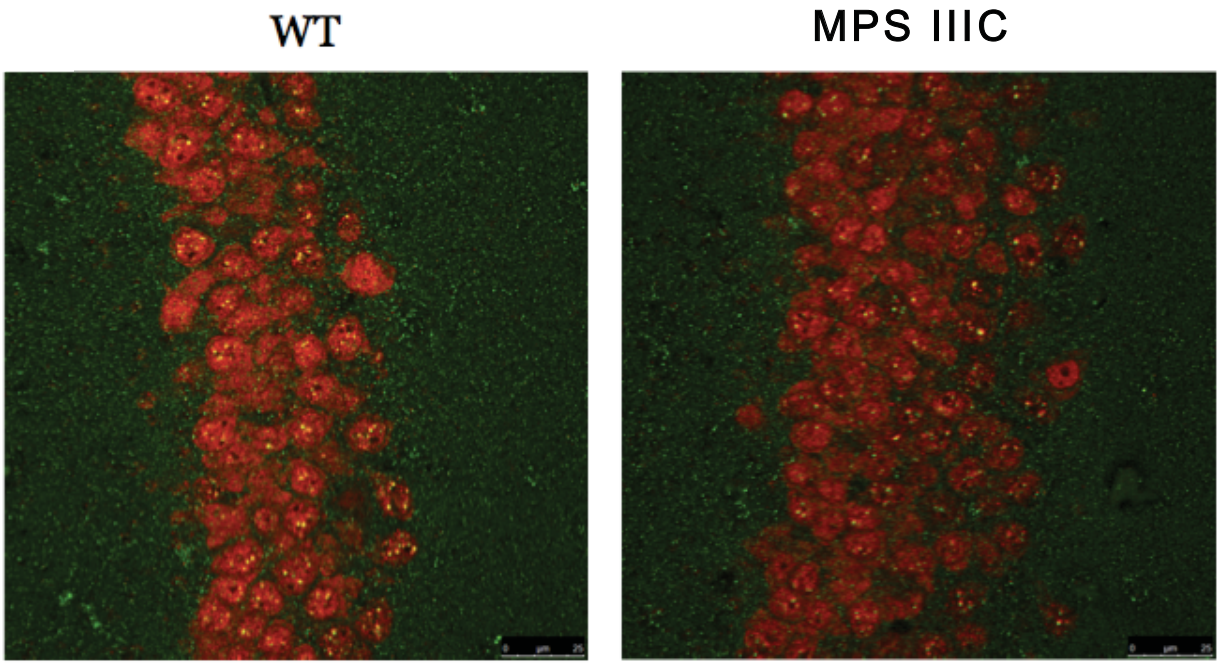
(D) Quantification of images of synaptic spine density for 20 day-old (left), 3 month-old (middle) and 8 month-old (right) WT and HEXA KO hippocampal neurons. Spine density was calculated per 20 μm of dendrite. Quantifications were performed on 3 different neurons per section for 5 different mice per age per genotype. More neurons from additional mice will be analyzed in the future to further validate results. All mice were male. Data show mean values (±SD). Significance via two-way repeated measurements ANOVA was used to test differences between different ages as well as genotype (** $p<0.0001$).

3.2 Synaptic Vesicle Marker Proteins VAMP2 and Synaptophysin Show Reduction in MPSIIIC Mouse Brains

Since post-synaptic defects are often found concurrently with pre-synaptic defects, we have analyzed brain sections of WT and MPSIIIC mice for the levels of the neuronal proteins associated with synaptic vesicles. 40 μm sagittal brain sections from 3 month-old WT and MPSIIIC mice were stained with antibodies against the pre-synaptic

markers synaptophysin (**FIGURE 3A, green)** or VAMP2 (**FIGURE 3B, green)** and co-stained with an antibody against a neuronal marker, NeuN (red). Both pre-synaptic markers were decreased in the MPSIIIC brain sections as compared to the WT brain sections in the CA1 region of the hippocampus, as well as the cerebral cortex. The reduction of these proteins reveals pre-synaptic neuronal defects and suggests a compromise of the entire neuronal network.

A



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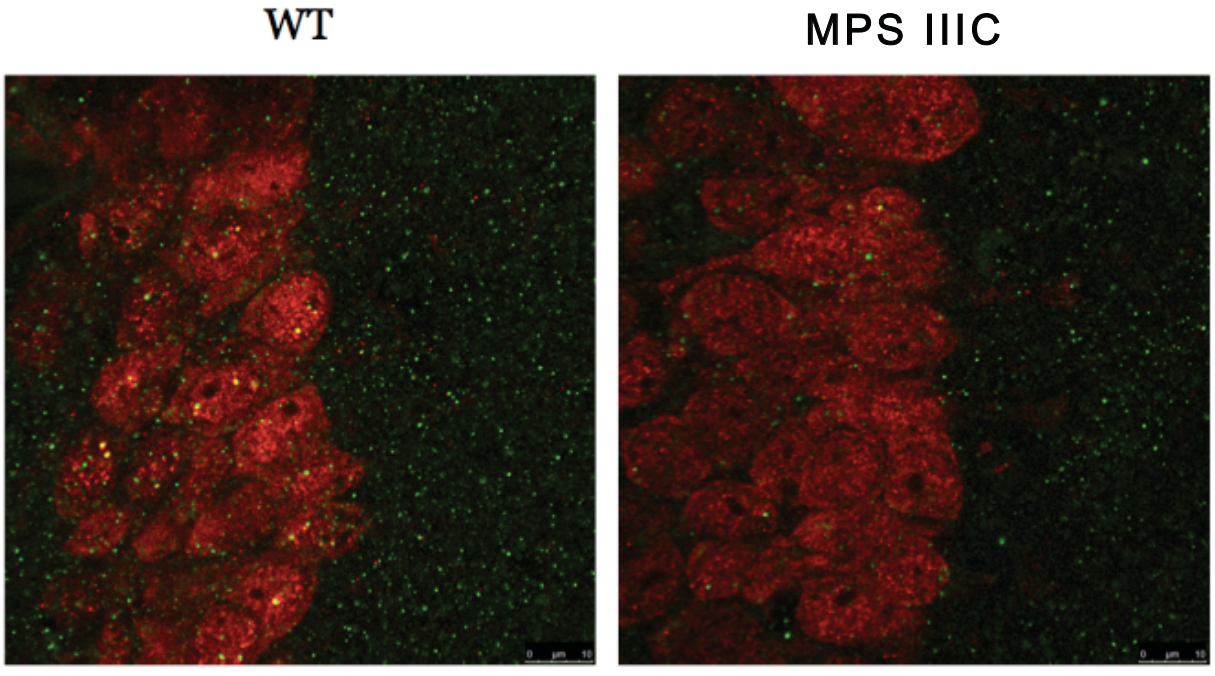

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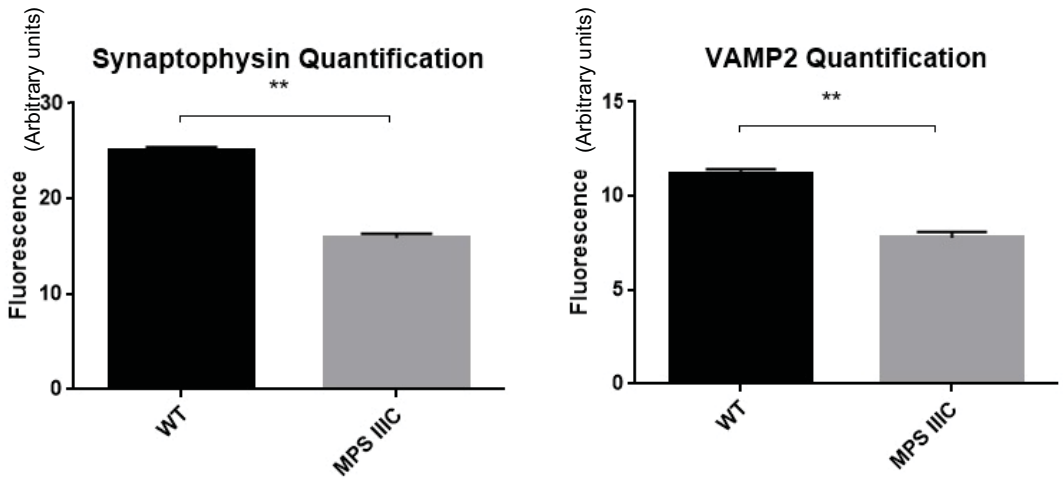


Figure 3. Immunohistochemical analysis suggests reduction of synaptic vesicle proteins in hippocampal neurons of MPS IIIC mice.

(A) Immunohistochemical staining of synaptophysin in the CA1 region of the hippocampus of WT (left) and MPSIIIC (right) 3 month-old mice. Synaptophysin staining is shown in green, and NeuN staining in red.

(B) Immunohistochemical staining of VAMP2 in the CA1 region of the hippocampus of WT (left) and MPSIIIC (right) 3 month-old mice. VAMP2 staining is shown in green, and NeuN staining, in red. Magnification 63X. Bar equals to 10 μm.

(C) Quantification of images of pre-synaptic marker staining in WT and MPS IIIC brain sections. Experiments were performed on 3 different brain sections from 3 different mice per genotype. All mice were male.

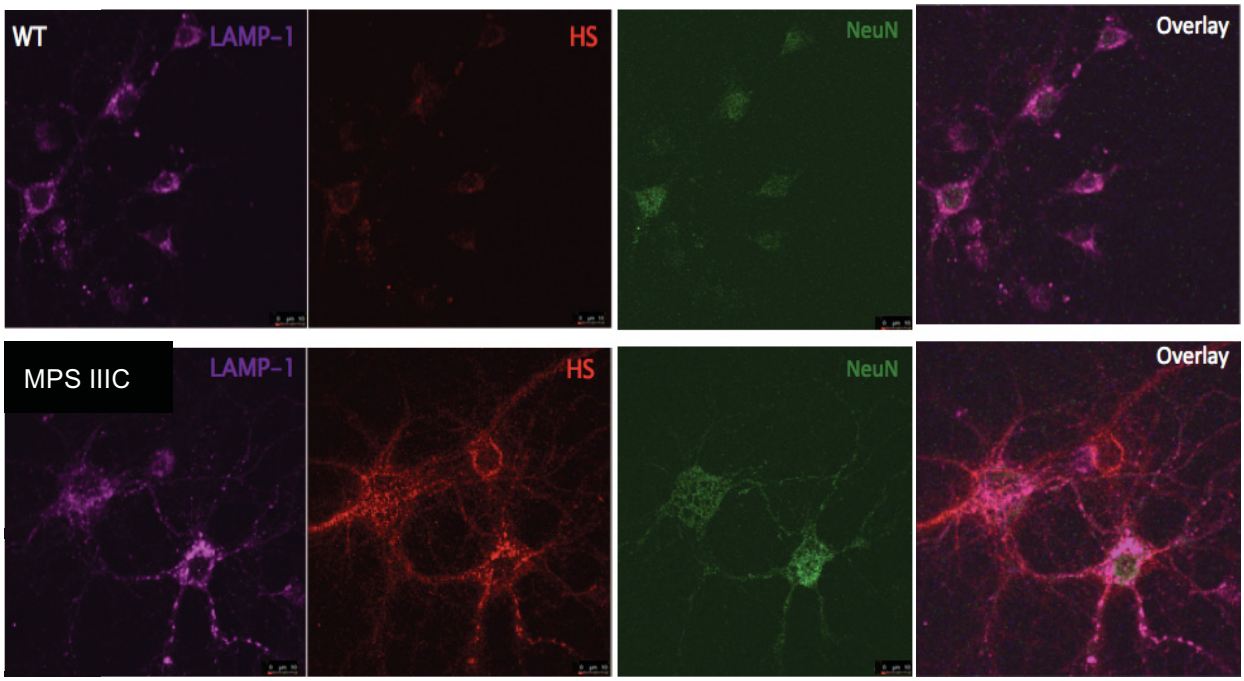
Data show mean values (±SD). Significant differences between the mean values in t-test (** $p<0.01$) are shown.

3.3 Hippocampal MPSIIIC Neurons Maintain in Culture Pathological Alterations Similar to Those Detected in the Mouse Brain

In order to analyze if defects observed in the hippocampal neurons in vivo would occur in cultured neurons, hippocampal cultures were established from both WT and MSPIIIC mouse embryos at embryonic day of 16-17. This is the age when the hippocampi are developed and are large enough to dissect from the brain. The cells were then left to differentiate in culture for 21 days, previously shown to be the optimal period to study synapses in vitro (Grabrucker, Vaida et al. 2009). Of note all the previous data showing pathological changes and lysosomal storage in MPSIIIC mouse neurons have been collected using mouse brains and not neurons in culture. Therefore it was necessary to first establish whether the hippocampal neurons in culture store the same materials, namely glycosaminoglycans (heparan sulfate) and gangliosides as the neurons in mouse brain sections. Cultures from WT and MPSIIIC mice were triple stained with antibodies against LAMP1, a lysosomal membrane protein (purple), heparan sulfate, (red), and the neuronal marker NeuN (green) (**FIGURE 4A).** The confocal images reveal that there is indeed an accumulation of heparan sulfate in the lysosomes of the hippocampal neurons from MPS IIIC mice (shown in pink in the overlay images) and therefore the neurons may be used for further analysis. Furthermore, the MPS IIIC hippocampal neurons show accumulation of secondary

storage material, GM2-ganglioside, similar to the accumulation observed in MPS IIIC mouse brain sections (**FIGURE 4B**).

A



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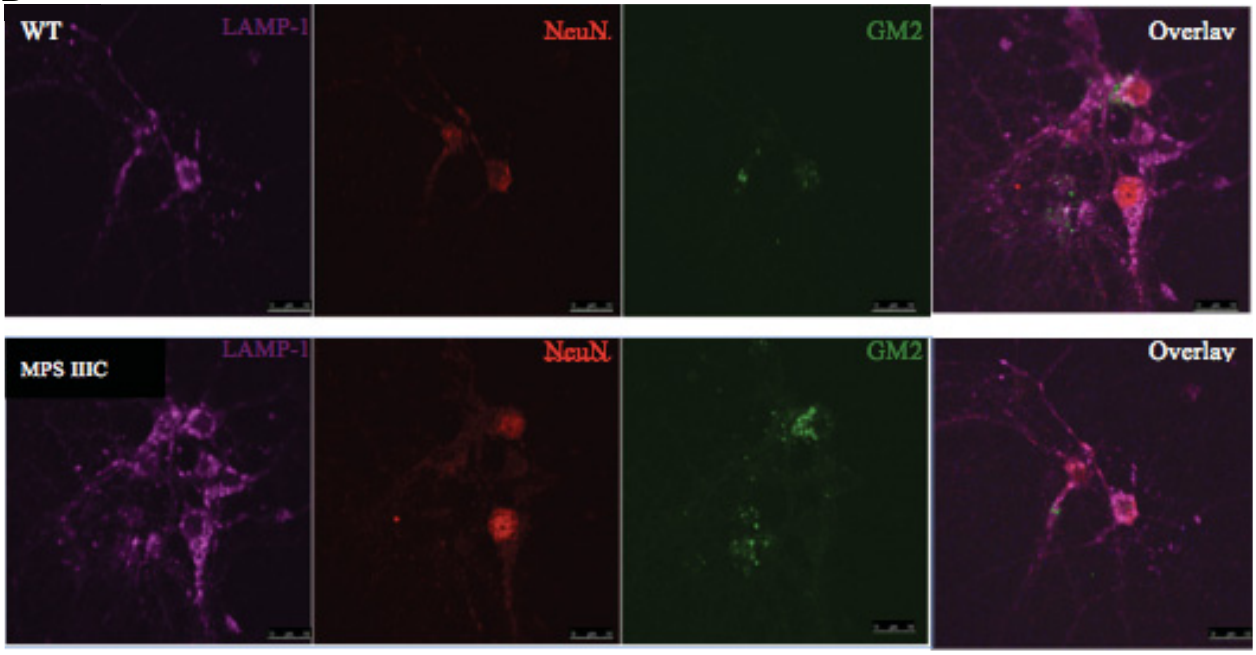


Figure 4. Lysosomal storage of heparan sulfate in cultured hippocampal neurons from MPSIIIC mice

(A) Confocal microscope images taken for WT (top) and MPS IIIC (bottom) hippocampal cultures that were triple stained with antibodies against LAMP1 (left column, purple), heparan sulfate (second column, red) and NeuN (third column, green). An overlay of all three stains is shown in the fourth column. Magnification 63X. Bar equals to 10 μm. Panels show representative images of at least 5 neurons studied for 3 Hgsnat-Geo and 3 WT cultures.

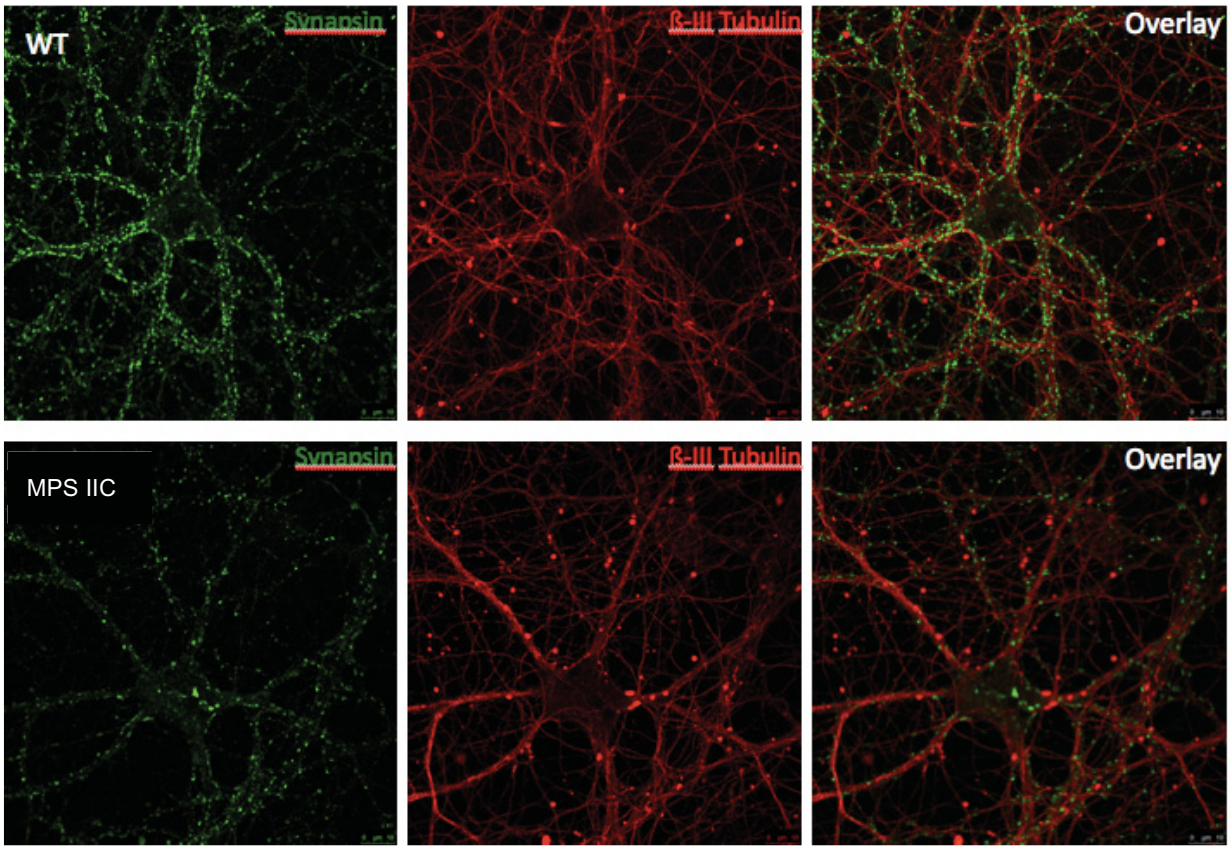
(B) Confocal microscope images taken for WT (top) and MPS IIIC (bottom) hippocampal cultures triple stained with antibodies against LAMP1 (left column, purple), NeuN (second column, red) and GM2 (third column, green). An overlay of all three stains is shown in the fourth column. Magnification 63X. Bar equals to 10 μm. Panels show representative images of at least 5 neurons studied for 3 Hgsnat-Geo and 3 WT cultures

3.4 Synaptic Vesicle Levels are Reduced in MPSIIIC KO Hippocampal Neuron Cultures

The cultured neurons were analyzed for the levels of protein markers of synaptic vesicles. Two main pre-synaptic proteins were studied: synapsin, an integral member of the V-SNARE complex, and synaptophysin, an accessory protein of the V-SNARE complex. Hippocampal cultures were co-stained for synapsin (green) and ß-30 tubulin

(red), a dendritic and axonal structural marker (**FIGURE 5A).** Additional co-staining was done with synaptophysin (green) and another dendritic marker, MAP2 (red), (**FIGURE 5B**). Quantification of these markers show that there is a significant decrease in both synapsin and synaptophysin levels in the MPS IIIC cultures as compared to the WT cultures **(FIGURE 5C).** The synaptic vesicles shown as green punctate are well positioned and numerous along the dendrite in WT cells, as shown by the overlay between synapsin/synaptophysin and ß-3 tubulin/MAP2 staining, whereas in the MPS IIIC cells only a small fraction of synapsin/synaptophysin-stained vesicles is visible along the dendrites.

A



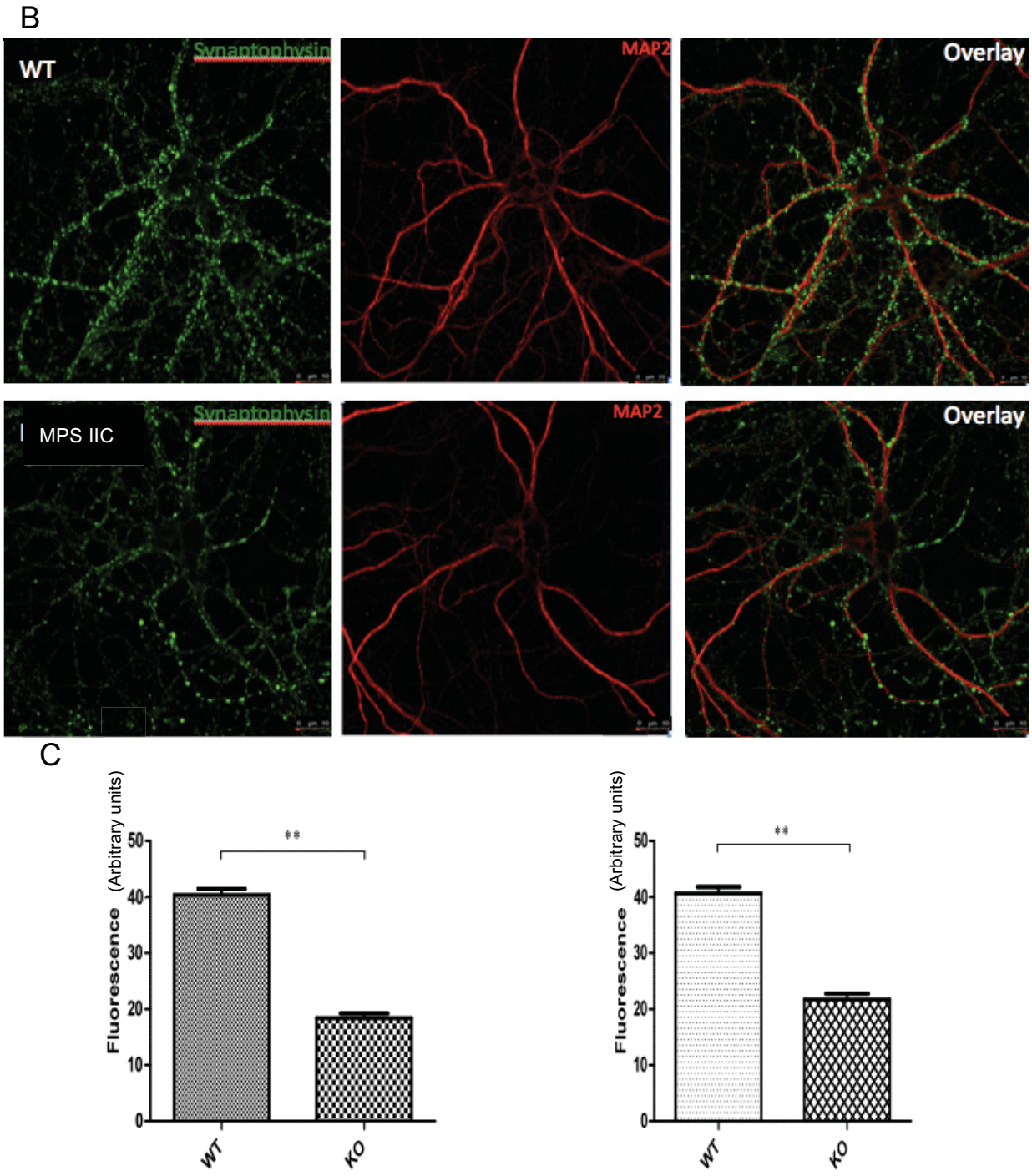


Figure 5. Reduced levels of pre-synaptic markers synapsin and synaptophysin in cultured hippocampal neurons from MPSIIIC mice.

(A) Confocal images of WT (top) and MPSIIIC (bottom) hippocampal cultures stained for the pre-synaptic marker synapsin (green, left column) and the neuronal marker ß-3-tubulin (red, middle column).

(B) Confocal images of WT (top) and MPSIIIC KO (bottom) hippocampal cultures stained for the pre-synaptic marker synaptophysin (green, left column) and the neuronal marker MAP2 (red, middle column).
Magnification 63X. Bar equals to 10 μm. Panels show representative images of at least 5 neurons studied for 5 Hgsnat-Geo and 3 WT cultures.
(C) Quantification of synapsin (left) and synaptophysin (right) levels in the hippocampal cultures. Data show mean values ± SD for quantification obtained for 5 different cultures (5 neurons for each culture were analyzed). Significant differences between the mean values in t-test (** $p<0.001$) are shown.

3.5 Alterations in Post-synaptic Structure in MPSIIIC KO Hippocampal Neuron Cultures

As post-synaptic structures (synaptic spines) were previously shown to be defective in mouse brain sections, we studied post-synaptic structures in hippocampal cultures. Neurons were stained with an antibody against a crucial post-synaptic membrane protein neuroligin-1, important in synapse formation, neuronal maturation and synaptic spine dynamics (green, **FIGURE 6**). In WT cells neuroligin-1 is associated with punctate structures along dendrites, most likely representing spines. This pattern has been previously reported for both cultured neurones and in vivo (Barker, Koch et al. 2008). In contrast, majority of neuroligin-1 in MPS IIIC hippocampal neurons is accumulating in perinuclear structures in the cell body.

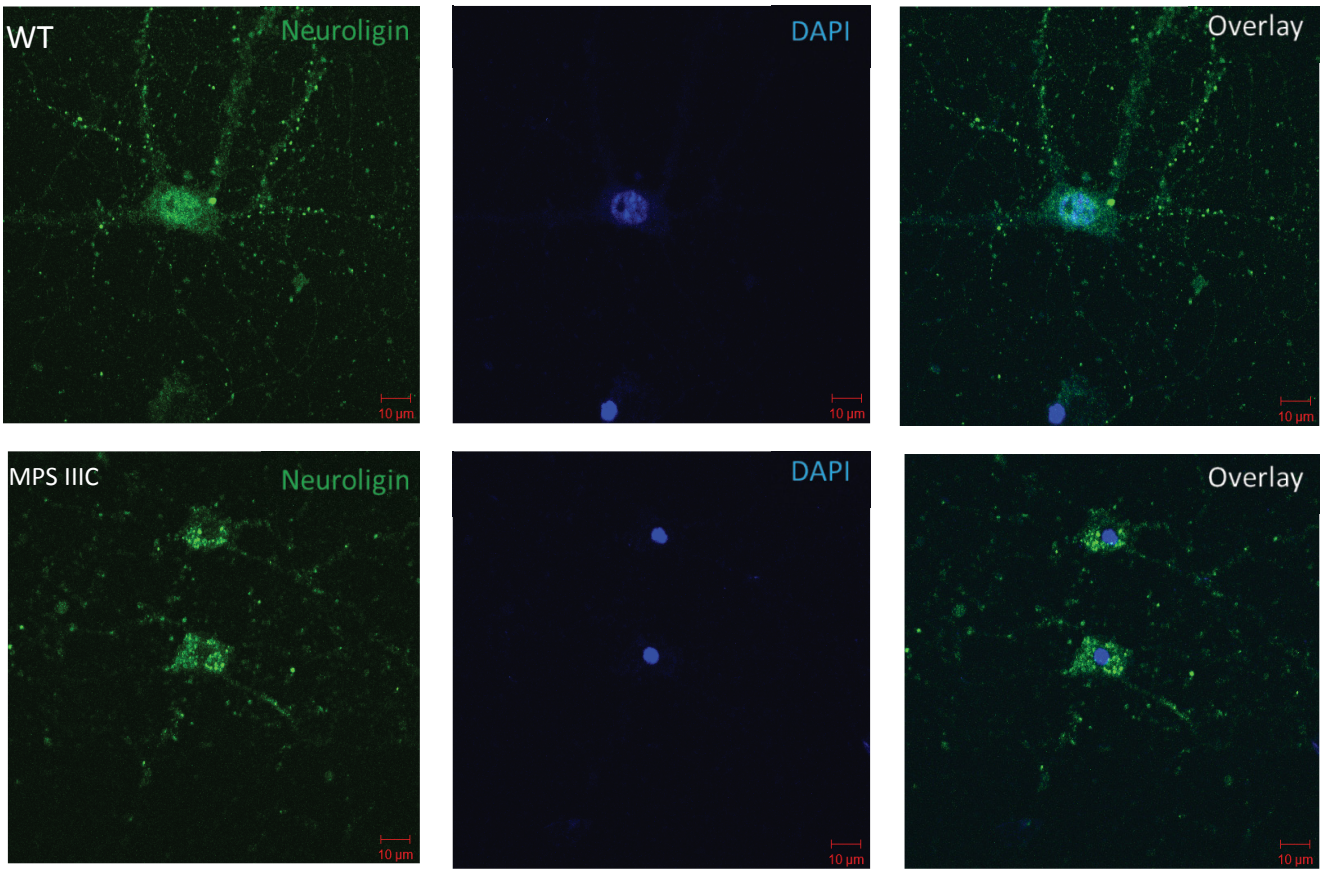


Figure 6. Altered Post-synaptic Structure in MPS IIIC hippocampal neurons

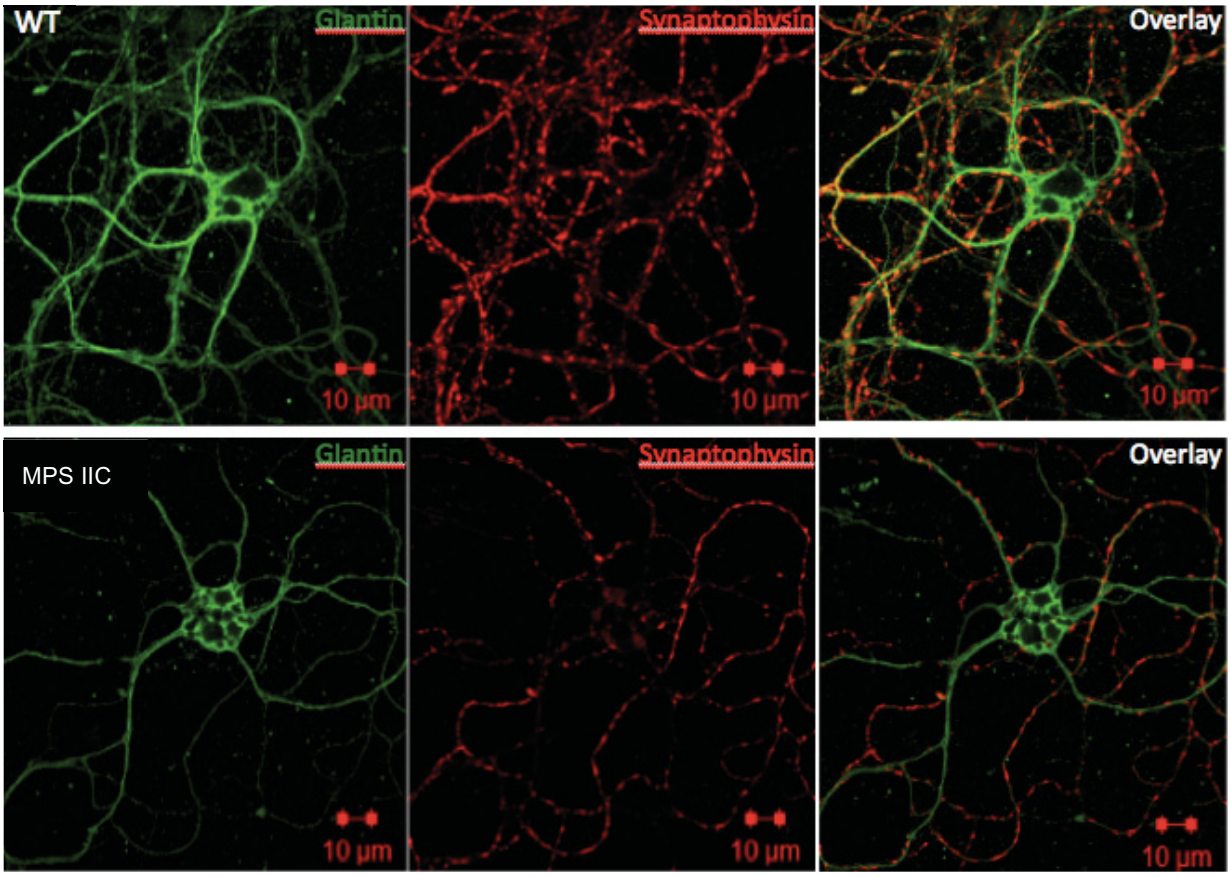
Confocal images of WT (top) and MPSIIIC (bottom) of hippocampal cultures stained for the post-synaptic marker Neuroligin-1 (green) and the nuclear stain DAPI (blue). Magnification 63X. Bar equals to 10 µm. Panels show representative images of at least 5 neurons studied for 3 MPS IIIC and 3 WT cultures. More neurons from additional mice will be analyzed in the future to validate the results.

3.6 No Alterations to the Trans-Golgi Network are Detected in Cultured Hippocampal Neurons from MPS IIIC Mice whereas Accumulations of Early Endosomes are Detected along the Dendrites.

Cumulatively, the results described above provide evidence for the presence of synaptic defects in the neurons of MPSIIIC KO mice. Both number of synaptic spines and synaptic vesicle levels, i.e. post-synaptic and pre-synaptic structures, were reduced in MPS IIIC cultured hippocampal neurons as compared to the WT cells. In order to establish a mechanism elucidating the genesis of these defects, we analysed structures of cellular compartments involved in generation and recycling of synaptic vesicles. The reduction in the levels of synaptic vesicle proteins can occur due to a defect in the trans-Golgi network, which is involved in synaptic vesicle production, or in early endosomes, which are involved in synaptic vesicle recycling once they have released their neurotransmitters. In order to analyze the trans-Golgi network, WT and KO hippocampal cultures were co-stained with the antibodies against the Golgi network tethering protein giantin (green) and the pre-synaptic protein synaptophysin (red) (**FIGURE 7A).** The giantin was found ubiquitously throughout the cell as it has been previously reported for developing neurons (Zhong 2011) and no difference in the Golgi structure was found between the WT and MPS IIIC cells. Secondly, hippocampal cultures were co-stained with the marker of early endosomes, EEA1 (green) and synaptophysin (red) (**FIGURE 7B**). There were no overall changes in the levels of the

EEA1 marker, however differences in its intracellular localization in the WT and MPS IIIC cells were present. In the WT cells EEA1 was found exclusively in the soma, whereas the MPS III cells contained multiple EEA1 accumulations along the dendrites most probably corresponding to early endosomes accumulating storage materials. This result suggests that there could be a defect in the endocytic pathway at the endosome level, and this could lead to an arrest in the recycling of synaptic vesicle proteins, leading to a decrease in synaptic vesicle protein levels as discussed earlier.

A



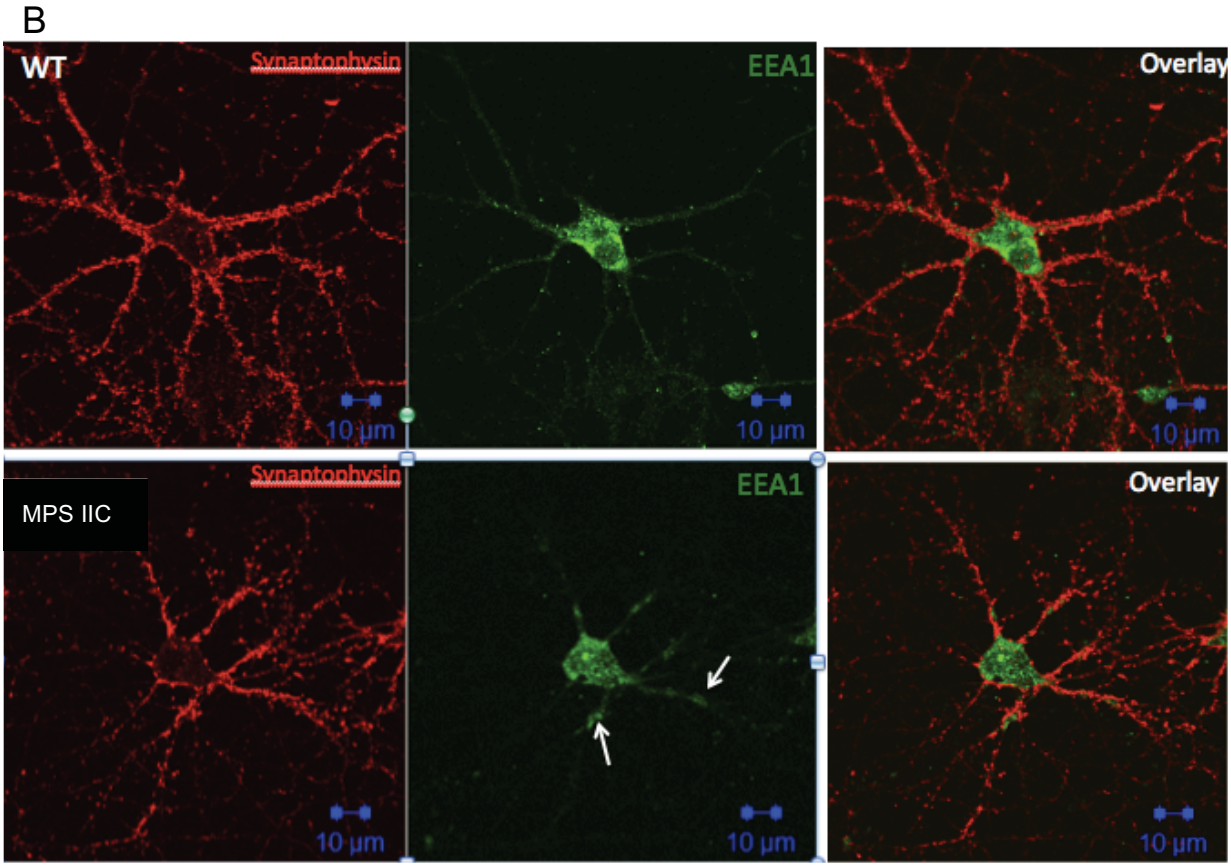


Figure 7. Immunofluorescence analysis suggests no alterations of Trans-Golgi network but reveals accumulation of early endosomes in MPSIIIC hippocampal neurons

(A) Confocal images of WT (top) and MPSIIIC (bottom) hippocampal cultured neurons stained for the trans-Golgi network tethering protein, giantin (green, left column) and pre-synaptic marker synaptophysin (red, middle column). Magnification 63X. Bars are equal to 10 µm. Panels show representative images of at least 5 neurons studied for 5 Hgsnat-Geo and 3 WT cultures.

(B) Confocal images of WT (top) and MPSIIIC (bottom) hippocampal cultures stained with antibodies against the early endosome marker EEA1 (green, left column) and the pre-synaptic marker synaptophysin (red, middle column). Magnification 63X. White

arrows represent accumulations of EEA1 along the dendrite. Bars are equal to 10 μm. Panels show representative images of at least 15 neurons studied for 5 Hgsnat-Geo and 3 WT cultures.

3.7 Microglia and Secreted TNF-α do not Affect Neurons in Culture

Besides the accumulation of endosomes and recycling defects the complex interaction between microglia and neurons can also be considered as a contributing factor to the cause of the synaptic defects in MPSIIIC mouse brains. Microglia play an important role in the refining of synaptic connections and the regulation of the neuronal network (Morris, Clark et al. 2013). Microglia can either physically interact with a neuron (Paolicelli, Bolasco et al. 2011), or be a source for secretion of regulatory cytokines such as the cytokine TNF-α. TNA-α has a regulatory role changing the number of synaptic connections but in excess, TNF-α can mortally damage neurons (Stellwagen and Malenka 2006). Previous studies in MPS IIIC mice demonstrated presence of activated microglia and 10-fold increased TNA-α expression levels in the mouse brains. Since activated microglia could be also present in hippocampal cultures leading to increased amounts of TNF-α in the cell culture medium and synaptic defects we examined the TNF-α levels by ELISA assay. We found however that the amount of TNF-α was similar in cell culture media from WT and MPS IIIC mice (**FIGURE 8).** Also, immunofluorescence analysis using the activated microglial marker ILB4 (isolectin

B4) revealed no activated microglia to be present in culture with the neurons (data not shown). These results together demonstrate that the influence of activated microglia is not a factor causing the synaptic defects seen in the MPSIIIC neurons.

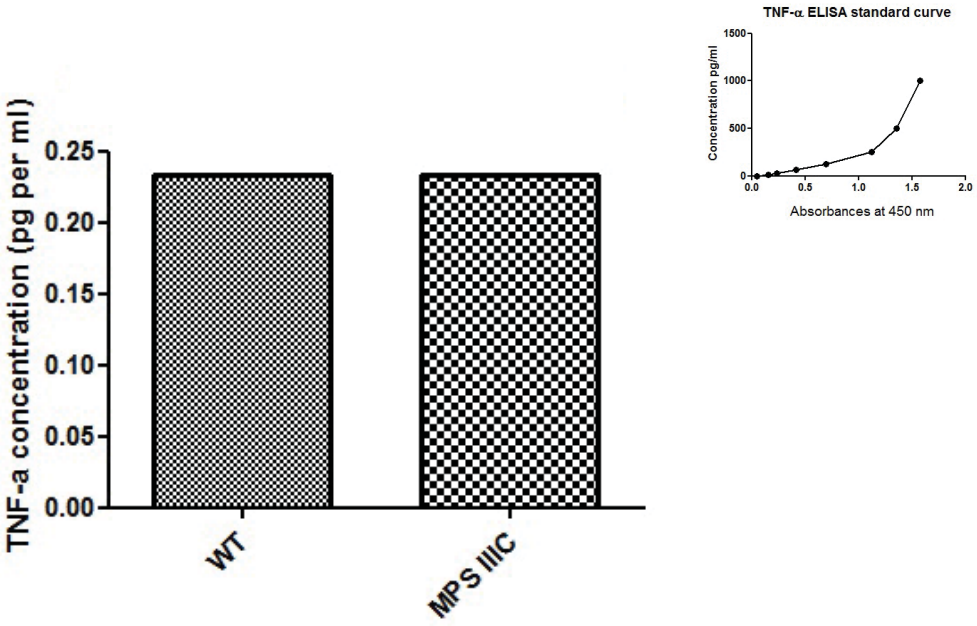


Figure 8. TNF-α concentrations are similar in WT and MPSIIIC KO cell culture medium

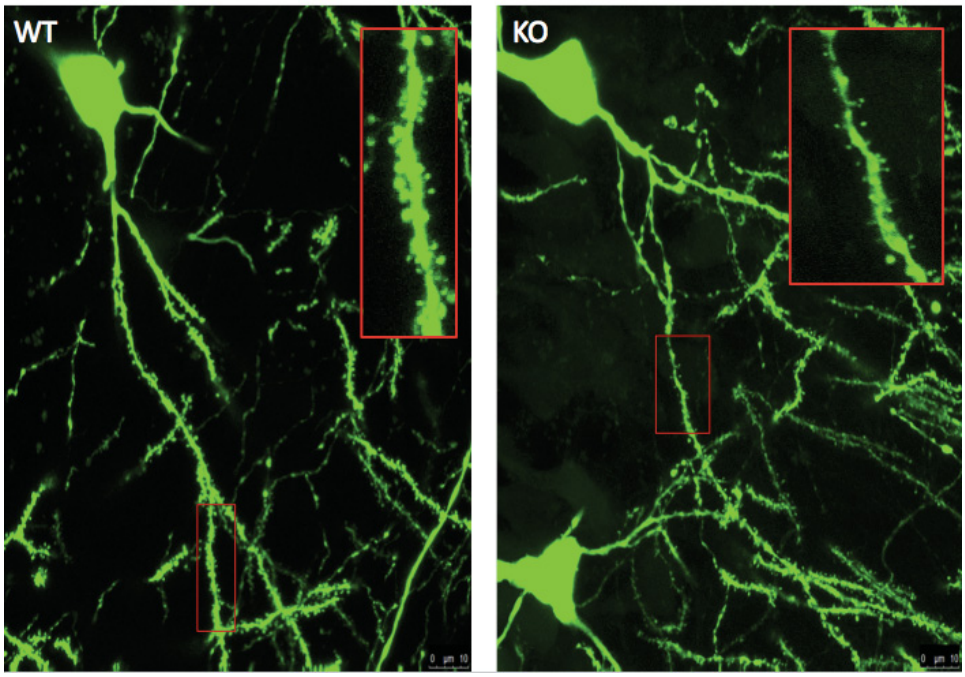
Concentrations of TNF-α measured in WT and MPSIIIC culture medium. Absorbance values were plugged into the trendline equation obtained from the standard curve to calculate unknown TNF-α concentrations. 4 samples of WT and KO cultures were studied. Inset: standard curve for TNF-α ELISA control solutions with increasing concentrations (pg/ml) of TNF-α. Error negligible.

3.8 Synaptic Spine Defects in Hippocampal Neurons of Tay-Sachs mice

Overall, the previous results show that there are indeed synaptic defects present in MPSIIIC neurons. In order to verify whether synaptic defects are also present in other lysosomal storage diseases we have analysed mice with the KO *HexA* gene, encoding for alpha subunit of β-hexosaminidase A. In humans HexA defects in Tay-Sachs disease cause deficiency of β-hexosaminidase A, accumulation of GM2-ganglioside, and neuronal death (Jeyakumar, Thomas et al. 2003). In contrast to human patients *HexA* KO mice retain some ability to catabolize GM2 due to a metabolic bypass catabolized by neuraminidase, so the disease in mice develops slower than in humans (Igdoura, Mertineit et al. 1999). By 10 months they however show gait and sight problems, spasticity, wait loss and progressive myoclonus (Miklyaeva, Dong et al. 2004). As we did for the MPS IIIC mice the *HexA* KO mice were crossed with Thy-EGFP line to visualize hippocampal neurons. At the age of 3 months, i.e. long before any signs of neurological deterioration could be observed behaviourally, mice were sacrificed and their fixed brain slices analyzed by confocal Z-stacks imaging like we have previously done for MPS IIIC mice. Our results demonstrated that hippocampal neurons in the brains of 3 month-old *HexA* KO mice showed a reduction in the number of synaptic spines as well structural defects in the dendrites similar to those found in MPS IIIC mice (**FIGURE 9A).** A quantification of the synaptic spine levels showed that

the reduction in synaptic spines in the KO mice was significant (**FIGURE 9B).** These results suggest that the synaptic defects found in MPSIIIC mice can be a general phenomenon for all LSD.

A



B

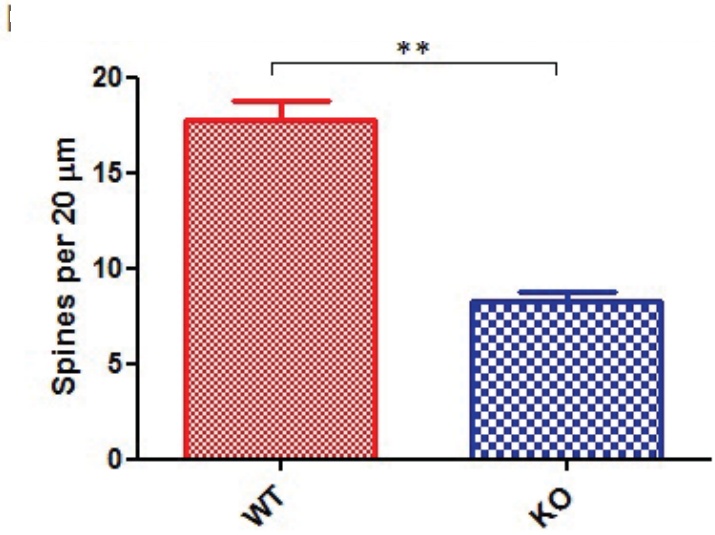


Figure 9. Synaptic Spines are decreased on Hippocampal Neurons of Thy1-EGFP / HEXA KO mice

(A) Confocal microscopy image of neurons in the CA1 region of the hippocampus in brains from 3 month-old Thy1-GFP WT (left) and HEXA KO (right) mice. Dendritic sections ~40 μm away from the soma are marked by red boxes and expanded in high-magnification inserts in each image. Magnification 63X. Consequent Z-stacks were overlaid in order to obtain an image of the full neuron. Bar equals to 10μm.

(B) Quantification of images of synaptic spine density for 3 month-old WT and HEXA KO hippocampal neurons. Spine density was calculated per 20 μm of dendrite. Experiments were performed on 3 different neurons from 5 different male mice. Data show mean values (±SD). Five mice were analyzed for each genotype. More neurons from additional mice will be analyzed in the future to further validate results. Significant differences between the mean values in t-test (** $p<0.01$) are shown.

Chapter 4

Discussion and Conclusions

Our studies represent the first attempt to understand if defects in synaptic transmission and synaptogenesis contribute to neurodevelopmental and behavioural problems as well as neurological decline observed in patients with MPS IIIC, or Sanfillippo type C syndrome. In order to accomplish this goal, we studied synapses on hippocampal neurones of MPS IIIC mice with inactivated *Hgsnat* gene and undetectable levels of HGSNAT activity. We also studied MPS IIIC mice that expressed EGFP in hippocampal neurons (MPS IIIC-Thy1-EGFP), enabling their direct analysis by fluorescent microscopy.

First, we studied morphology and density of synaptic spines, important post-synaptic structures that are heavily involved in synaptic transmission and long-term potentiation/depression, phenomena implicated in learning and memory. We observed a significant, ~30% reduction in the number of synaptic spines on hippocampal neurons of 3 month-old MPS IIIC-Thy1-EGFP mice as compared to neurons of WT mice. This reduction became even more profound with age. By 8 months of age, there was an

approximately 55% decrease in the number of spines in the MPS IIIC mice. Also, the MPS IIIC neurons showed an irregular dendritic structure, alternating between thinning and enlargement of the dendrite, the latter appearing as spheroidal accumulations. These dendritic spheroids are commonly found in LSDs and in particularly in MPS (Walkley 1998). It has been previously shown that storage bodies along the dendrites may cause a blockage in transport of endocytic vesicles or other cargo to and from the cell body, causing adverse effects to cellular morphology, including enlargement and thinning of the neuronal dendrites as shown in MPS IIIC neurons (Jeyakumar, Dwek et al. 2005, Bellettato and Scarpa 2010). Importantly, the synaptic spine defects could be observed much earlier in life of the MPS IIIC mice than any other pathological defect (as early as at 20 postnatal days). It has been reported that such early synaptic defects could lead to late-onset cognitive decline later in life (Brunson, Kramar et al. 2005) and we suggest that this can be occurring in MPS IIIC mice where cognitive impairment is observed approximately 10 months of age (Martins, Hulkova et al., in press).

To study further the origin of post-synaptic defects we established cultures of hippocampal neurones from MPS IIIC and WT mice and studied post-synaptic localization of neuroligin-1 protein. Neuroligin-1 is a post-synaptic membrane protein that plays a very important role in the formation of synapses as well as in synaptic spine formation and dynamics. It interacts with the pre-synaptic protein ß-neurexin to form a functional synaptic connection. This connection is not only important for synapse

formation, but also for synaptic function (Dean and Dresbach 2006). Neuroligins interact with proteins involved in neurotransmission such as PSD95 and gephyrin, important for excitatory and inhibitory synaptic scaffolding, respectively (Giannone, Mondin et al. 2013). With these proteins, neuroligins recruit receptors for neurotransmitters to the post-synaptic membrane, such as NMDA-type glutamate receptors, which is important for NMDA-receptor-specific synaptic plasticity (Budreck, Kwon et al. 2013). Staining of cultured MPS IIIC hippocampal neurons with neuroligin-1 antibody showed that neuroligin-1 is accumulated in perinuclear structures in the soma instead of being localized in synaptic spines as it occured in WT cells (Barker, Koch et al. 2008). This difference in localization of neuroligin-1 may therefore provide insight into the mechanism behind the reduced synaptic density in MPS IIIC neurons. Also, since as neuroligin-1 plays an important role in synaptic spine formation, it's abnormal localization observed in the MPS IIIC neurons may explains the reduction in the number of spines on MPS IIIC neurons. Due to the blockage of the vesicular transport along the dendrite potentially caused by spheroidal accumulations as discussed in the previous section, neuroligin-1, initially being produced at the cell body, could be prevented from being transported to it's rightful localization along the dendrites and into dendritic spines, causing its accumulation in perinuclear structures in the cell body. Further experiments must be performed to identity these neuroligin-1-positive structures but it is tempting to speculate that they could be lysosomes or late endosomes.

Post-synaptic defects are often found to occur in parallel with pre-synaptic defects. In order to verify whether this is the case with MPS IIIC mice, pre-synaptic vesicles were analyzed. Brain sections of 3 months-old MPS IIIC and WT mice were stained with antibodies against pre-synaptic vesicle proteins, VAMP2 and synaptophysin. VAMP2 and synaptophysin are both members of the v-SNARE complex, composed of synaptic vesicle proteins involved in docking synaptic vesicles to the pre-synaptic terminal to release neurotransmitters into the synapse. We found a significant reduction in the signals for both proteins in the MPS IIIC brain sections, notably in the hippocampus and cerebral cortex, suggesting the impairment in generation of synaptic vesicles. Both Wilkinson *et al.* and Vitry *et al.* discovered similar reductions in VAMP2 and synaptophysin in brains of MPS IIIA and MPS IIIB mouse models, respectively (Vitry, Ausseil et al. 2009, Wilkinson, Holley et al. 2012).

To investigate this further at the level of single neurones we established and studied cultured hippocampal neurons produced from MPS IIIC and WT embryonic brains sections. Importantly, MPS IIIC hippocampal neurons showed the biochemical defects previously discovered in the neurons in MPS IIIC postnatal brain sections (Martins, Hulkova et al., in press). In particular, we detected increased number of lysosomes and their enlargement revealed by staining for lysosomal membrane protein, LAMP-1 as well as accumulation of primary storage material heparan sulphate and secondary storage material GM2 ganglioside in the lysosomes. MPS IIIC and WT

hippocampal neurons were stained with antibodies against synaptophysin and another pre-synaptic protein, synapsin, also a member of the v-SNARE complex. Dendrites were stained with antibodies against MAP2 and ß-III tubulin. A significant reduction in both synapsin and synaptophysin was observed in MPS IIIC neurons as compared to the WT neurons, suggesting that both post-synaptic defects and pre-synaptic defects were present in the MPS IIIC neurons in culture potentially compromising the whole synaptic network.

In order for synaptic connections to maintain integrity as well as specificity, the neurons must constantly fire signals between one another. This synaptic transmission between neurons strengthens the synaptic network and also heavily regulates synaptic spine dynamics (synaptic plasticity). Therefore it is tempting to speculate that a reduction in the amount of synaptic vesicles docking to the pre-synaptic membrane in MPS IIIC neurones potentially causing a reduction in the amount of neurotransmitter release and therefore synaptic transmission, could eventually lead to changes in formation of post-synaptic spines (Figure 2). On the other hand post-synaptic defects could be independent event related to observed alterations of neuroligin-1 trafficking towards the dendrite. This latter hypothesis is supported by the previous work showing that neuroligin trafficking is crucial for recruitment of neurotransmitter receptors to the membrane and formation of the spines (Budreck, Kwon et al. 2013, Giannone, Mondin et al. 2013).

Upon documenting the presence of synaptic defects in MPS IIIC neurons, we concentrated on studies pertaining to the mechanism and origin of these defects. We have considered two possible mechanisms underlying synaptic defects in MPS IIIC neurons. The first mechanism suggests that defects originate in the neuron itself, and are not caused by their interactions with other type of brain cells. In this case the reduction of synaptic vesicle proteins could be caused either by a reduction in the rate of synaptic vesicles biogenesis or by a defect in the recycling of synaptic vesicles after they have released neurotransmitters. The biogenesis and recycling of synaptic vesicles are crucial components of the endocytic cycle, which involves input from the Golgi apparatus, early, late and recycling endosomes, as well as lysosomes. Synaptic vesicles are created in the cell body, and the Golgi apparatus plays an important role in the initial assembling of synaptic vesicle proteins and lipids into the final synaptic vesicle (Alberts et al 2002). However, considering the distance and time a synaptic vesicle takes to travel to the pre-synaptic terminal (up to 1 m away from the cell body in certain types of human neurons) (Alberts et al 2002), in order to maintain efficient synaptic transmission levels, once synaptic vesicles release their neurotransmitters into the synaptic cleft, they are recycled locally by early endosomes which repackage the synaptic vesicle proteins into the vesicle. This occurs much faster then the do-novo production of the synaptic vesicles. Synaptic vesicle proteins are therefore constantly in flux between these organelles in the neurons, being transferred to the lysosome for final

degradation. Thus, if there is a defect or accumulation at any one of the participating organelles in the endocytic pathway, this could cause an obstacle for the correct formation of synaptic vesicles, leading to the reduction of their number described above. In order to test this hypothesis, cultured MPS IIIC and WT hippocampal neurons were stained with antibodies against a Golgi tethering protein giantin essential for maintaining Golgi structure as well as against the early endosomal antigen 1 (EEA1). We found no significant difference in Golgi structure or giantin localization between WT and MPS IIIC cells. This result comes in contrast to what was discovered by Vitry *et.al*, who showed alterations in Golgi complex protein localization in MPS IIIB neurons compared to WT neurons, where Golgi proteins such as GM-130 are being stored in storage vesicles, causing irregular Golgi structure (Vitry, Bruyere et al. 2010). Further studies are necessary to understand the nature of the discrepancy between our results and those of Vitry *et al.*, but it is possible that they van be related to the nature of the biochemical defects occurring in MPS IIIC mice and MPS IIIA mice studied by Vitry *et al.*

In contrast to Golgi structure, which was not affected by lysosomal storage, we observed substantial differences in the EEA1 localization pattern between the MPS IIIC and WT neurons. In the MPS IIIC neurons, EEA1 accumulated both in the soma and in the spheroidal structures in the dendrite, whereas in the WT neurons it was found in cytoplasmic punctate exclusively in the soma. Early endosomes frequently merge with lysosomes in order to transfer any endocytosed material, which needs to be degraded.

Since the lysosomes are known to be defective and accumulate in MPS IIIC, this may cause a build up of endosomes along the dendrite. Such a mechanism is well described for impaired autophagy observed in the cells with LSD (Settembre, Fraldi et al. 2008) and is caused by storage-related changes in the composition of lysosomal membrane impairing SNARE complexes involved in lysosome-autophagosome fusion and a blocking of the autophagic flux (Sarkar, Carroll et al. 2013). As a consequence, autophagy substrates, such as polyubiquitinated and aggregated proteins, p62/SQSTM1, and dysfunctional mitochondria, are accumulated causing general defects of cellular homeostasis. Such phenomena are especially pertinent for neurons, which rely on autophagy for survival. It has been reported that spheroidal accumulations observed in neuronal dendrites and axons mainly contain autophagosomes (Vitry, Bruyere et al. 2010). We speculate that they also contain endosomes accumulating storage materials and that this would prevent synaptic vesicle recycling, leading to the decrease in synaptic vesicle levels as shown on Figure 5. Altogether our data suggest that the synaptic defects begins at the endosomal level, leading to decreased synaptic vesicle recycling, decreased synaptic vesicle docking, and consequently reduced synaptic transmission.

The second mechanism through which the synaptic defects could potentially occur involves the interaction between neurons and microglia, which have important regulatory function during synaptogenesis. As previously stated, microglia have the

ability to refine synaptic connections in the process called synaptic pruning, where inter-neuronal connections are regulated in order to meet the ideal standards for synaptic transmission (Paolicelli, Bolasco et al. 2011). Activated microglia can either interact with neurons directly eliminating unwanted synaptic spines via phagocytosis or through the secretion of the inflammatory cytokine TNF-α, which can also regulate synapse dynamics. In contrast, too high level of TNF-α or microglia-neuronal contacts was shown to lead to an excess of synaptic and physical defects in the neurons. In order to test whether microglia could cause synaptic defects in the MPS IIIC cultured hippocampal neurons, a TNF-α ELISA kit was used to analyze the levels of TNF-α present in the culture medium. We found however no significant difference between the levels of TNF-α in MPS IIIC neuronal cultures and the WT neuronal cultures. The amounts of TNF-α in both cultures were below the detection level, suggesting that the secretion of toxic levels of TNF-α from activated microglia, a phenomenon observed in postnatal brain sections of MPS IIIC mice, is not a contributing factor to the synaptic defects observed in MPS IIIC hippocampal cultures. Moreover immunohistochemistry revealed that microglia were not present in the neuronal cultures from WT or MPS IIIC mice, although astrocytes, which also have the ability to secrete TNF- α, were found in both cultures (data not shown). Cumulatively, the data rule out effects caused by microglia and favours the mechanism involving accumulation of storage endosomes as a leading factor in synaptic defects in MPS IIIC neurons.

Finally, if this data are extrapolated to other lysosomal storage diseases, it could provide insight into a general mechanism for cognitive and mental decline in all patients with neurological forms of LSDs. This would have important consequences due to the fact that a common therapeutic approach could potentially be used to alleviate the mental symptoms manifested in all LSDs. We initiated such study and analyzed synaptic spines in a mouse model of Tay-Sachs disease that expressed GFP in hippocampal neurons (HEXA KO – Thy1-EGFP). Spine density reduction of ~50% observed in brain sections from 3-month-old HEXA KO mice compared to the WT mice was similar to that observed in the MPS IIIC neurons. Also, similar dendritic structure defects were found in HEXA KO mice. These data suggest that synaptic spine density reduction may be common to other LSDs, pending further investigation of pre-synaptic vesicles in HEXA mice, as well as analyses in other LSDs.

In summary, our study has revealed malfunctions in the synaptic system, both pre-synaptic and post-synaptic, in MPS IIIC mouse hippocampal neurons, as well as a potential cellular mechanism underlying these defects. The synaptic defects preclude severe cognitive and mental symptoms observed in MPS IIIC mice later in life. They also may be present in human MPS IIIC children hippocampal neurons and can be responsible for the various behavioural manifestations occurring in the patients. For example, Rumsey *et al.* discovered autism spectrum disorder-like behaviour in children aged 3-4 with early-onset MPS III such as social inactivity and lack of eye contact and

facial expressions (Rumsey, Rudser et al. 2014). Mutations that cause misfolding and mislocalization of neuroligin and ß-neurexin can lead to cognitive diseases such as autism (Sudhof 2008) due to the function of these two proteins in synaptic transmission. Autism is a genetic neurodevelopmental disorder caused by reduced brain activity, and several studies showed this to be related to synaptic dysfunction (Levy, Mandell et al. 2009). Since neuroligin-1 has abnormal localization in MPS IIIC cells, its necessary connection with ß-neurexin may not being formed, causing reductions in synaptic activity and brain function. Effective methods were described for alleviating these symptoms (de Ruijter, Valstar et al. 2011, Meijer, van Vlies et al. 2013), mostly involving pharmaceuticals affecting synaptic transmission such as therapeutic doses of amphetamines, which increase neurotransmission via increases in dopamine. Otherwise, some monoamine-based antidepressants can increase synaptic transmission by increasing AMPA receptor levels (Popoli, Diamond et al. 2014). These therapies can potentially be used as a platform for drug development to reverse the behavioural symptoms and to improve cognitive function in MPS IIIC patients.

Conclusions

1) We have revealed post-synaptic defects in the hippocampal neurons of MPS IIIC mice at the synaptic spine level as early as at 20 postnatal days. The severity of the defects in synaptic spine density progresses with age.

2) We have identified decreases in the levels of pre-synaptic proteins VAMP2, synaptophysin, and synapsin in the hippocampal neurons of MPS IIIC mice, both in culture and in brain sections, suggesting existence of pre-synaptic defects at the synaptic vesicle level.

3) We speculate that post and presynaptic defects originate when early endosomes are accumulated while attempting to fuse with lysosomes containing storage materials. This leads to a decrease in synaptic vesicle recycling and therefore synaptic transmission, leading to cognitive and mental decline.

4) Microglia and the secreted cytokine TNF-α do not play a role in the compromise of the synaptic system in MPS IIIC mice.

5) The synaptic defects found in MPS IIIC mice are similar to those in the mouse model of Tay-Sachs disease and can be also present in other LSDs.

Chapter 5

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