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Role of Protein Ubiquitination in NFH-LacZ mice

From

Manuel Vestner

Director: Dr. Beat M Riederer

Expert: Dr. Geneviève Leuba

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1 Résumé

Dans les maladies neurodégénératives, on observe des dépôts de produits de dégradations considérés souvent comme marqueurs pathologiques. Les mécanismes sous-jacents à ces processus sont encore mal connus mais une des hypothèses est que le système ubiquitine-protéasome est perturbé dans certaines maladies neurodégénératives. Parmi les facteurs qui jouent un rôle, on trouve le vieillissement, l'oxydation et la formation de radicaux libres, ainsi que des mutations génétiques qui influencent la fonction des protéines produisant leur accumulation anormale, les aggrésomes.

La sclérose latérale amyotrophique, dans laquelle une disfonctionnement de l'enzyme sodium dismutase perturbe le système redox, est caractérisée par une mort neuronale progressive qui découle d'une accumulation d'éléments du cytosquelette dans les motoneurones. Nous supposons que dans cette maladie le système ubiquitine-protéasome est déréglé et nous allons essayer de le démontrer dans cette étude.

Dans ce travail nous avons comparé par électrophorèse et Western blot l'ubiquitination dans des cerveaux et des moelles épinières des souris transgéniques avec ceux des souris contrôles. Ces NFH-LacZ-souris, possédant une protéine neurofilament fusionnées à une β-galactosidase développent une accumulation des NF-protéines et les neurofilaments ne sont plus transportés dans les axones ni les dendrites. L'accumulation de ces agrégats ressemble au phénotype de la sclérose latéral amyotrophique. Les autres points d'investigation étaient l'expression des différents neurofilaments et leur phosphorylation.

Les résultats n'ont pas pu démontrer une perturbation du système ubiquitine-protéasome des souris transgéniques. Par contre, en concordance avec le mécanisme existant chez les souris NFH-LacZ, une diminution des neurofilaments et une hypophosphorylation ont pu être mis en évidence. En conclusion, pour éclaircir le mécanisme pathologique de la sclérose latéral amyotrophique et ainsi faire un pas vers le développement de nouveaux traitements ciblés, il nous faut revoir le mécanisme pathologique des souris transgéniques et réaliser de nouvelles études en utilisant d'autres modèles animaux ou du matériel humain. Une autre possibilité serait d'investiguer d'autres mécanismes de dégradation tel que le système endosomal/lysosomal et mieux définir leur rôle dans la sclérose latéral amyotrophique.

1 Abstract

In neurodegenerative diseases, one can observe deposits of degradation products that represent hallmark structures. Actually, the underlying mechanisms are not well understood, but some hypotheses claim that the ubiquitin-proteasome system is perturbed in neurodegenerative diseases. Some of the influencing factors are aging, oxidation and the formation of free radicals, as well as genetic mutations which affect the function of proteins and result in an accumulation and formation of aggresomes. The amyotrophic lateral sclerosis, in which a malfunction of the sodium dismutase perturbs the redox system, is characterized by the accumulation of elements of the cytoskeleton in motor neurons and a progressive neuronal death. We suppose that in these diseases the ubiquitin-proteasome system is deregulated and try to demonstrate this hypothesis by comparing the ubiquitination of different neurofilaments in brain and spinal cord of transgenic and control mice. These NFH-LacZ mice with a truncated NF-H protein and a ß-galactosidase marker protein induce an accumulation of NF-proteins and neurofilaments are no longer transported into axons or dendrites.

The accumulation of such aggregates resembles the phenotype of amyotrophic lateral sclerosis. Beside the ubiquitination the neurofilament expression and phosphorylation state was investigated.

The results cannot demonstrate a perturbation of the ubiquitin-proteasome system of neurofilaments in transgenic mice. In contrast, in accordance with the mechanism of the NFH-LacZ mice a decrease of high and medium density neurofilaments and a hypophosphorylation were found. In conclusion, to elicit the pathological mechanism of amyotrophic lateral sclerosis and to develop focused treatments, we have to review the pathological mechanism of the transgenic mice and repeat the experiments with other animal models or with human material. Other possibilities would be to focus on other degradation mechanisms, such as the endosome/lysosome system, and to define their role in the amyotrophic lateral sclerosis more clearly.

2 Key Words

Amyotrophic lateral sclerosis, neurodegeneration, ubiquitin-proteasome-system, NFH-LacZ transgenic mice, neurofilament, phosphorylation.

3 Introduction

3.1 Neurodegenerative diseases

Several diseases with a common background in pathophysiology and clinical evolution and similar histological findings are subsumed by the term of neurodegenerative diseases. Alzheimer disease (AD), Huntington disease (HD), Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS) are some of the most important members of this group. These diseases are characterized by a progressive and selective loss of neurons. Although they are genetically and/or environmentally multifactorial, which leads to considerable different clinical patterns and various drug response (Katsuno, et al. 2012), they all show a similar accumulation of misfolded proteins and formation of aggresomes in particular brain structures. In AD misfolded amyloid β peptides accumulate and form senile plaques whereas in inclusions of Lewy body-dementia or Parkinson disease α-synyclein, neurofilaments and ubiquitin can be found. These accumulations of amyloid β peptides and α-synyclein lead to apoptosis by the release of Cytochrom C out of the mitochondria (Hashimoto, et al. 2003). As new studies show, a non-apoptosis programmed cell death seems also to be involved, but the apoptosis pathway remains still an important mechanism for the loss of neuronal structure (E.Bredeson et al., 2006).

The incidence of neurodegenerative diseases increases with extended live expectancy. In the last years especially age-related degenerative diseases reached epidemic rates in developed countries. There can be found more and more mild cognitive impairment with a higher risk for developing dementia (Melo, et al. 2011).

It seems that one of the common pathophysiological mechanisms in neurodegenerative disease development is the generation of reactive oxygen species (ROS) and the accumulation of mitochondrial DNA mutations, resulting in oxidation damage (Lin, et al. 2006) (Melo, et al. 2011). These irreversible oxidations provoke an activation of chaperones to repair damaged and misfolded proteins or to degrade them by the ubiquitin-proteasome system. This is also an argument in favour of the increase of neurodegenerative disorders with elderly people, because progressive

accumulation of oxidative damage and decrease of protein repair mechanisms lead to ubiquitination and formation of aggresomes.

3.2 Amyotrophic lateral sclerosis

The amyotrophic lateral sclerosis is, with a prevalence of 3-5 per 100'000, the most frequent motor neuron disorder (Longo, et al. 2012). It is characterized by the selective loss of upper and lower motor neurons. The motor neuron cytoskeleton is affected and focal enlargements, composed of accumulations of neurofilaments and other proteins can be found in proximal motor axons as well as proliferation of astroglia and microglia, which always accompanies degenerative processes in the central nervous system. With loss of the motor neurons in brainstem and spinal cord the corresponding muscle fibres begin to atrophy. The first part of its name is due to the atrophy of the muscle hence "amyotrophic". The other characteristic, the loss of fibres in the lateral columns and the resulting fibrillary gliosis, explains the rest of the name: "lateral sclerosis".

Remarkable in the development of the disease is the selectivity of neuronal cell death. While there occurs a loss of neurons and atrophy of the whole locomotor system, the sensory apparatus, the regulatory mechanisms for the control and coordination of movement and the cognitive function remain intact, as well as the motor neurons required for ocular motility and the parasympathetic neurons controlling sphincter functions of bladder and bowel rest unaffected.

The first clinical sign of the lower motor neuron dysfunction and denervation is insidiously developing asymmetric weakness in distal limbs, progressive wasting and atrophy of muscles and frequent observed fasciculation of muscle units. If the neuronal impairment affects the bulbar muscles, it results in difficulties to control swallowing and movements of the face and tongue, while corticospinal involvement leads to hyperreflexia and spastic resistance to passive movements.

The evolution of the disease can start with any muscle group, but as time passes, more and more muscles become affected until all regions are symmetrically affected.

ALS is currently untreatable and characterised by a progressive evolution leading to death from respiratory paralysis in about 3-5 years. (Longo et al., 2012) So far, there is no established medication which can stop the underlying pathologic processes in ALS, but the drug riluzole is used to prolong the survival in ALS. Since excitotoxic neurotransmitters, such as glutamate, participate in the death of motor neurons in ALS, riluzole reduces excitoxicity by diminishing glutamate release. Other treatments are in clinical trials, such as ceftriaxone by increasing astroglial glutamate transport and in this way acting antiexcitotoxic, or pramipexole and tamoxifen, two neuroprotective agents. Antisense oligonucleotides which diminish expression of mutant SOD1 protein prolong survival in transgenic ALS mice and are now in trial for SOD1-mediated ALS.

Besides the small opportunities of primary therapy there exist a lot of rehabilitive aids. To mention are: respiratory support, foot-drop splints, and speech synthesizers.

3.2.1 Super-oxide dismutase-1 (SOD1)

About 10% of all ALS cases are familial (FALS) and in another 10 to 20% of these FALS cases, a mutation of the superoxide dismutase (SOD1) can be detected. (Kabashi, et al. 2007) It is an antioxidant protein that transforms highly reactive superoxide anions into hydrogen peroxide. Except for the earlier appearance in a clinical as well as in a pathological way, almost no difference between mutant SOD1-induced ALS (1-2% of all ALS cases) and the classic form of ALS can be detected. Studies show that posttranslational modifications of SOD1 cause the majority of cases of typical ALS.

(Kabashi, et al. 2007) There are more than 120 reported distinct amino acid changes in the SOD1 protein that cause ALS, which shows that any alteration in the structure of SOD1 will cause it to acquire toxic properties that lead to ALS. (Kabashi, et al. 2007) The misfolding of the protein, and not a specific nature or localization of the mutation, leads to a gain of function that is specifically toxic to motor neurons in cortex and spinal cord, cortical interneurons and dopaminergic neurons. The misfolding can be caused by mild oxidation by hydrogen peroxide (the main product of SOD1 catalysis of superoxide anions himself) or another oxidizing reagent leading to ubiquitination, association with chaperones, insolubility and aggregation. Also common in the mutant form, the misfolded SOD1 of wild type mice are secreted in the extracellular space, which leads to death of motor neurons. The role of the glial cells, which secret the misfolded SOD1 into extracellular space seems to be quite important, because reducing levels of SOD1 in motor neurons delays onset but not does improve the evolution of ALS, whereas reducing levels of SOD1 in microglial delays progression of the disease. Candidate genes, which predisposes for ALS seem to be all gene variants who promote oxidation and/or misfolding of SOD1, as well as genes encoding proteins for normal folding of SOD1 (protein chaperones) or efficient removing of misfolded SOD1 (ubiquitin-proteasome and autophagy components) and finally any genes associated with increased free radical production, thus increasing the cellular demand for SOD1 and increasing the production of hydrogen peroxide (Kabashi, et al. 2007).

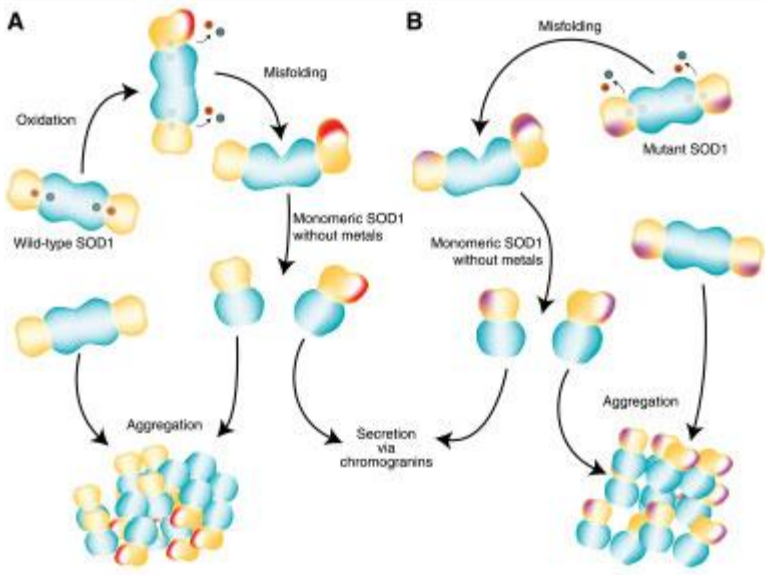


Figure 1 I Misfolded/oxidized wild-type superoxide dismutase-1 (wtSOD1) acquires a comparable toxic gain of function to mutant SOD1 (mutSOD1). (A) After mild oxidation by hydrogen peroxide or another oxidizing reagent, wtSOD1 has been shown to acquire an aberrant conformation that makes it more likely to misfold. The misfolded wtSOD1 dimer can thus lose the copper and zinc ions and dissociate into monomeric units. Further, misfolded wtSOD1 has been shown to possess many of the features that were thought to be exclusive of the mutant protein (B), including ubiquitination, association with chaperones, insolubility, and aggregation. Also, similar to mutSOD1, misfolded/oxidized wtSOD1 may be secreted in the extracellular space, where it would initiate the molecular cascades that lead to death of motor neurons, as has been described for mutSOD1. (Figure and legend from Kabashi et al., 2007)

One hypothetic therapy strategy lowers or blocks the expression of SOD1, by insertion of a virus with RNA against SOD1. Another would be to deliver antisense oligonucleotides to the CNS or the injection of monoclonal antibodies that specifically recognize misfolded or mutant SOD1 and molecules that inhibit monomerization and so aggregation of the SOD1 dimers. Furthermore some

drugs exist, such as arimoclomol that reduces the amount of secretable misfolded SOD1 by upregulating protein-folding chaperones.

3.3 Background Ubiquitin-Proteasome System

For the continuous turnover of intracellular proteins there exist two main regulatory systems: the lysosomal and the ubiquitin-proteasome system (UPS). The function of the ubiquitin-proteasome system (UPS) is to degrade non-useful proteins, such as damaged oxidized, mutant or misfolded proteins (Lehman 2009). These proteins are also implicated in signal transduction systems by turning off signal proteins, in cell-cell communication during development and at the neuronal synapse, regulating of gene transcription via monoubiquitination and deubiquitination of histones, driving circadian clocks and the regulation of cell cycle, progression and apoptosis and functioning of the MHCI-antigen presentation of the immune system. (Lehman 2009) This mechanism is responsible for 80-90% of protein breakdown while the lysosomal proteolysis takes about 10-20%. (Riederer, et al. 2011)

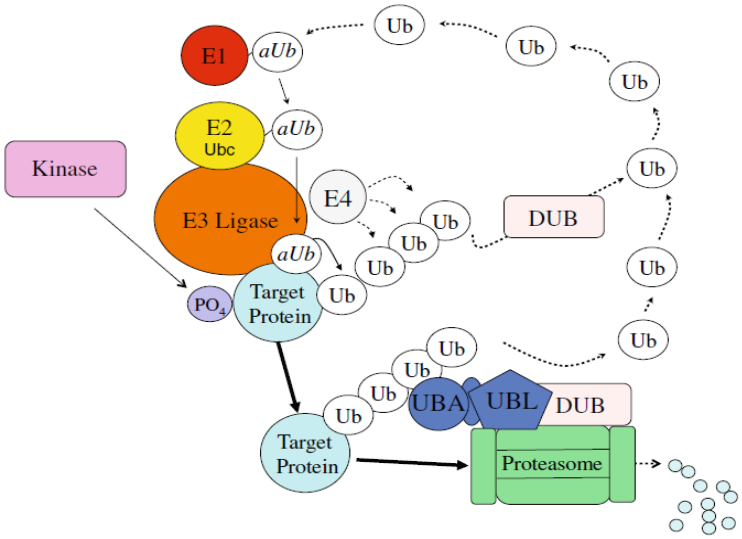


Figure 2 I Ubiquitin activating enzyme (E1) transfer activated ubiquitin to ubiquitin conjugating enzymes (E2) which binds at the ubiquitin ligase (E3), where the target protein binds the ubiquitin. E4 is generating the polyubiquitin chains ready for transfer to the proteasome for cleavage in small peptides and free amino acids. Deubiquinating proteins at the proteasome complex permit recycling of the ubiquitin monomers and reintroduce them into the cycle activating it by E1. (Figure from N. Lehmann, 2009)

The perturbation of the UPS seems to be a cause and a result of neurodegenerative diseases. Molecular chaperones, responsible for protein refolding and repair, form the first line of defence against protein misfolding and aggregation, and the UPS, essential to reduce level of abnormal proteins, is the second line. But a dysfunction of the UPS system may lead to accumulation and an aggregation of ubiquitinated proteins. Furthermore ubiquitinated proteins seem to have an inhibitory effect on the UPS function and cause accumulations of proteins. (Riederer, et al. 2011)

In abnormal cellular inclusions such as neurofibrillary tangles and neuritic plaques, hallmark structures of Alzheimer disease, an accumulation of ubiquitinated and hyperphosphorylated microtubule associated protein tau can be detected. As the p62, a polyubiquitin binding protein that interacts with the UPS, plays a role in preventing the aggregation of polyubiquitinated protein tau in delivering it to the lysosome, relative dysfunction or inhibitory overloading of UPS may contribute to the abnormal accumulation of phosphorylated and ubiquitinated tau (Lehman 2009). Other pathogenic theories exist, such as inflammation of brain tissue leading to oxidative stress and UPS

dysfunction, various inhibitory proteins of the UPS or damage of proteins of the UPS itself. For example the paired helical filaments seem to inhibit the proteasome activity and cause tau accumulation and a general increase in ubiquitinated proteins (Riederer, et al. 2011).

Another example are the Lewy bodies in Parkinson disease, where the UPS is also disturbed. A defect of the parkin, a component of the UPS, seen in familial juvenile onset Parkinson's disease, leads to a lesser ubiquitination of aggregated proteins found in Lewy bodies, such as α-synuclein (α-sp22) and parkin-associated endothelin receptor-like receptor (Pael-R).

In cytoplasmic inclusions in ALS, ubiquitin can also be detected, this suggest dysfunction in ubiquitin proteasome together with a disturbance in the endosome/lysosome system, which might play a more important role than in other neurodegenerative diseases.

Also the aggregated, toxic polyglutamine repeat form of protein huntingtin seen in Huntington disease is degraded by the UPS. Similar to prion diseases misfolded mutant huntigntin protein inhibits the proteasome machinery with the result of toxic over-accumulation of the mutated huntigntin and other proteins.

Finally the UPS is also implicated in the etiology of neurological tumors by missing ubiquitination of certain proteins or the interaction with tumor suppressors.

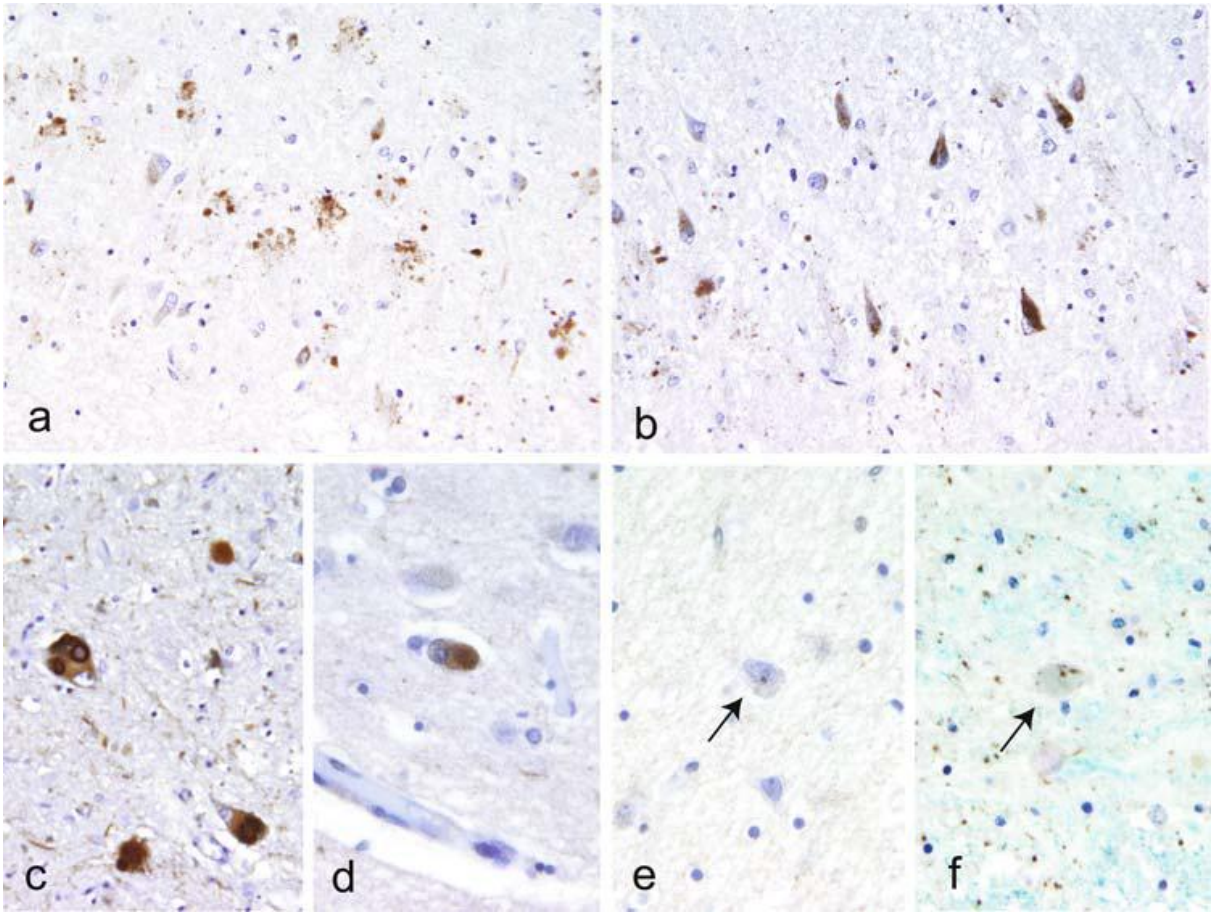


Figure 3 I Anti-ubiquitin immunostaining in neurodegenerative diseases: Ubiquitin positive inclusions are found in hippocampal plaques (a), neurofibrillary tangles (b), brain stem (c) and cortical Lewy bodies (d) of Alzheimer disease, as well as in the nucleus of cortical neurons in Huntington disease (e) and in the cytoplasm of anterior horn neurons in motor neuron disease (f) (Image from L. Lehmann, 2009).

3.4 Role of neurofilaments

Neurofilaments are specific neuronal intermediate filaments and are responsible with other members of the cytoskeleton, as microtubules or microfilaments, for structural integrity, cell shape and cell and organelle motility. The major function of the neurofilaments is to control the axonal calibre related to their phosphorylation state (Q.Liu, et al. 2004). Like all intermediate filaments they have a well-organized coil structure, formed by three neurofilament subunits, defined by their molecular weight: NF-L (light), NF- M (medium) and NF-H (high). In SDS-Page they show a molecular weight as followed: NF-H 200kD, NF-M 160 kD, NF-L 60kD (Q.Liu, et al. 2004).

NF-L is responsible for the precise assembly of neurofilaments. NF-M establishes crosslinks and stabilizes the filament network and has its role in longitudinal extension. NF-H forms cross-bridges as well and interacts with other cytoskeletal elements, such as microtubules and microfilaments.

After their synthesis in the perikaryon the neurofilament-proteins are transported into axons and assemble there into the filamentous structure. Entering into axons there are phosphorylated and by this way the axonal growth is regulated. But alteration in phosphorylation of NF-M and NF-H tail domain is seen in various neurodegenerative diseases as ALS or AD, in which tail domain phosphorylation and neurofilament accumulation occur abnormally in perikarya. Protein inclusions in axons block the transportation of particles through the axon and seem to lead to neuronal death. In contrast, transient phosphorylation of head domain prevents neurofilament assembly and tail domain phosphorylation in perikarya, protecting the neuron from abnormal accumulation of phosphorylated neurofilament-proteins aggregates in cell bodies. (Q.Liu, et al. 2004) Phosphorylation also slows down neurofilament transport within axons and it seems to also have a protective function in protecting the neurofilament-proteins from degradation.

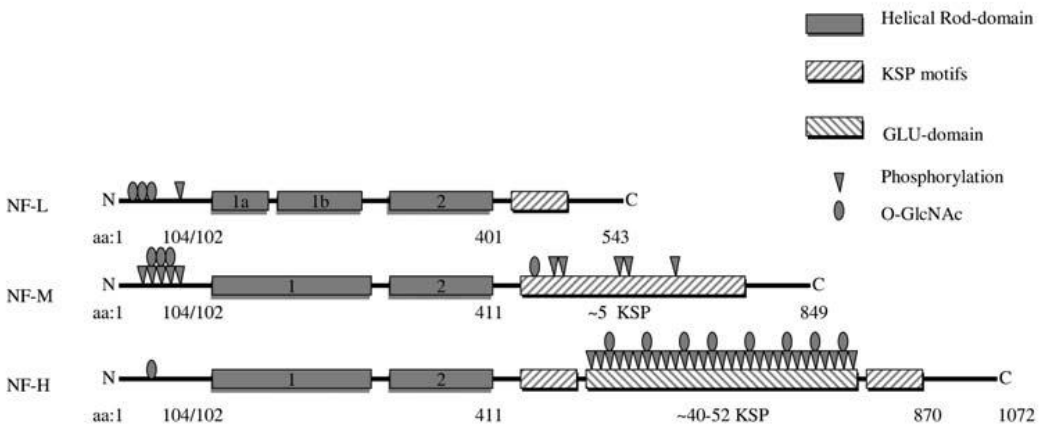


Figure 4 I All intermediate filaments consist of an N-terminal head domain, a central α-helical rod domain and C-terminal domain. A unique character of NF-M and NF-H is that the carboxy terminus contains multiple repeats of Lys-Ser-Pro which are highly phosphorylated. NFH has more than 20 repeats, NFM has 3-4 repeats whereas NFL has 1-2 repeats. (Figure from Q. Liu, 2004)

3.5 NFH-LacZ mice

Transgenic NFH-LacZ mice are created by Eyer and Peterson in 1994 for research in neurodegenerative diseases. These mice are expressing a fusion protein of a truncated high-molecular-weight mouse neurofilament protein (NFH) fused to β-galactosidase (LacZ) of Eschericia Coli. This leads to a decrease in neurofilament triplet protein expression and a loss in neurofilament assembly and abolished transport into axonal and dendritic processes in spinal cord and brain

(Riederer, et al. 2003). In addition, reduced neurofilament phosphorylation may favour increased filament degradation (Eyer und Peterson 1994). Consequently, these mice develop inclusions in neurons throughout the CNS formed by massive filamentous aggregates of all three endogenous neurofilament proteins and the NFH-LacZ fusion protein. These inclusions resemble NF-rich Lewy bodies seen in Parkinson disease or Lewy body dementia and the inclusion formed in motoneurons can be taken as a prototype for amyotrophic lateral sclerosis. These transgenic mice develop not only inclusions in the perikarya and proximal axons of spinal cord motoneurons, but develop also muscular weakness and motoneuron loss similar as in human ALS cases (Tu, et al. 1997).

The accumulation of large neurofilament inclusion leads to a blockage of axonal transport resulting in the loss of spinal motoneurons in these mice. Clinically, there are reduced rearing frequency and poor performance in motor coordination reported. In addition, a reduced phosphorylation in NF-H and NF-M subunits can be detected which is why neurofilaments become less stable and more susceptible to degradation because phosphorylation is essential for neurofilament stabilization and transport (Riederer, et al. 2003).

Due to the fact that ubiquitin activity can be detected in inclusions, the UPS seems to have a role in limiting the size of the inclusions.

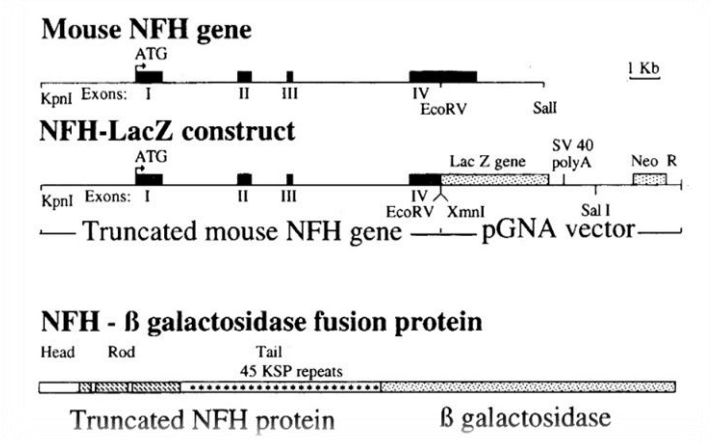


Figure 5 I A β-Galactosidase of Eschericia coli monomer was matched at the carboxy terminus of the NFH permiting the detection of aggregated NFH. (Figure from Eyer and Peterson, 1994)

4 Aim of the project

It is well known that protein mutations are identified by chaperone or heat shock proteins that try to repair damaged or dysfunctioning proteins. Eventually such proteins are tagged by ubiquitin and so destined to degradation via the ubiquitin proteasome system. Under certain circumstances, ubiquitinated proteins escape degradation and accumulate in the cell; such aggregates are also called aggresomes. The NFH-LacZ mice with a truncated NF-H protein and a ß-galactosidase marker protein induce an accumulation of NF-proteins and neurofilaments are no longer transported into axons nor dendrites. The accumulation of such aggregates resembles the phenotype of amyotrophic lateral sclerosis. In many neurodegenerative diseases one observes the formation of protein aggregates that are ubiquitinated (Riederer, et al. 2011) (Al-Chalabi, et al. 2012). The goal of this study is to determine whether the ubiquitin-proteasome system is perturbed in NFH-LacZ mice and hence leads to the formation of aggregates in the soma of motoneurons.

5 Material and Methods

5.1 Transgenic mice

For this research we analysed brain and spinal cord tissue of nine transgenic and ten control mice with the genetic background of C57Bl/6. There were 10 males and 9 females obtained from J.Eyer, Angers France, who describes their generation in Eyer and Peterson, 1994. All animals were anesthetized and perfused with phosphate-buffered saline (PBS) and stored at -80°C.

5.2 Preparation of assay

Brain and spinal cords samples of transgenic and control mice, 10 of each group, were homogenized manually and sonicated in PBS + triton 1‰ + tween + Pic (protein inhibitor cocktail). With a Bradford Method the concentration of protein was determinated and diluated with a SDS-Mix 5x to a final concentration of 2mg/ml. Finally, the samples were heated 10 minutes at 100°C and stored at -20°C for further use.

5.3 Electrophoresis

The main function of electrophoresis is the separation of the charged proteins considering molecular weights and charges of proteins. The most common use is the qualitative analysis of a mixture of proteins. There can be detected differences in molecular weight of a few hundreds of Dalton and differences in an isoelectric point less than 0.1 pH unit. (E.Garvin 2003)

In the Sodium Dodecyl Sulfate Poly Acrylamid Gel Electrophoresis (SDS-PAGE) the goal is to separate the proteins only by their molecular weight. (In comparison with the 2D-electrophoresis, in which the proteins are separated in function of molecular weight and isoelectric point). SDS denatures native proteins and links electrons to the hydrophobic regions, and therefore results in a negative charge in relation to their mass. Because the smaller proteins get faster through the polyacrylamide gel at the end they are closer to the anode than the bigger ones. To define the molecular weight we added a standard protein mix with known molecular weights.

Gels were prepared with acrylamide solution (39% acrylamide stock solution (Merck), 1% N,N'-methylendiacrylamide (Merck)), stacking Buffer (3% Tris (Sigma), 0.2% SDS (BioRad) in a pH of 6.8), Separating Buffer (9.1% Tris (Sigma), 0.2% SDS (BioRad) in a pH of 8.8), ammonium persulfate (APS solution of 10% (Sigma)) and TEMED (Eurobio). The electrophoresis was running with 140V for about 3 hours in a running Buffer containing 0.3% Tris (Sigma), 1.44% Glycine (Biosolve), 0.1% SDS (BioRad) and deionized water. Further on, the gels were either stained over night by Coomassie blue for an unspecific visualizing of the proteins or transferred to a nitrocellulose membrane by Western blot for specific detection by antibodies.

5.4 Western Blot

With the Western Blot, also called protein immunoblot, specific proteins in a homogenate of tissue samples can be detected. In transferring the proteins from the gel to a nitrocellulose membrane they can be detected by attachment of specific antibodies and visualized with secondary antibodies linked to enzymes or chemiluminescence.

The gel from the electrophoresis and the nitrocellulose membrane (Reinforced NC, OptitRan BA-S 85) was placed between two filter papers (GB003, Schleicher & Schuell), submerged in the transfer buffer (24.9mM Tris (Sigma), 191.8mM Glycine (Biosolve), 20% methanol (Brenntag), 0.01% SDS (BioRad), deionized water) and fixed in the blot system (BioRad), assuring that the proteins pass on

the nitrocellulose membrane on their way from the gel toward the anode. The transfer was done with 90V for 2.5 hours.

To detect specific proteins the immune staining was used by adding specific antibodies for searched proteins. To prevent unspecific binding of the nitrocellulose membrane and to reduce background noise and false positives, they were blocked for 30min in 5% dried milk in PBS-Tween. After some washing in PBS the membranes were incubated for 1 hour with a first monoclonal or polyclonal antibody (in PBS with 2.5% dried milk and 0.15%Tween20) followed by washing in PBS. (2x3min, 1x15min, 2x5min). Afterwards, the first antibody was linked with a second, peroxidase-linked antibody by incubating for 1 hour (in PBS with 2.5% dried milk and 0.15%Tween20) and washed again in PBS (2x5min, 1x15min in PBS, 0.15% Tween20, 3x5min). In order to start the chemiluminescence reaction of the peroxidase the membranes were incubated during exactly 1min with a solution of two Western blotting reagents (Amersham TM ECL Western Blotting Detection Reagents (GE Healthcare). Finally, they were exposed to light sensible films (Kodal Biomax MR) for several seconds or a few minutes and developed with the KODAK X-OMAT 1000 Processor.

5.5 Antibodies

antibody	final dilution	second antibody	goal structure
ubiquitin monoclonal	1:2000	anti-rabbit (polyclonal)	ubiquitin
ubiquitin polyclonal	1:2000	anti-mouse (monoclonal)	ubiquitin
BR10	1:100'000	anti-rabbit (polyclonal)	NF-M
M20	1:10	anti-mouse (monoclonal)	NF-M
M9	1:10	anti-mouse (monoclonal)	NF-H
M15	1:10	anti-mouse (monoclonal)	NF-L
SMI-32	1:5000	anti-mouse (monoclonal)	NF-H non phosphorylated
SMI-31	1:5000	anti-mouse (monoclonal)	NF-H phosphorylated
SMI-34	1:5000	anti-mouse (monoclonal)	NF-H phosphorylated

5.6 Quantification

The intensity of bands was evaluated using quantification *software "image J" (Image J 1.41o, Wayne Rasband, National Instituts of Health, USA)*. The films were scanned, the background signal subtracted and the values analysed using a student t-test and shown in a simple excel graph.

6 Results

6.1 Coomassie Blue

Although the main goal of colouring with Coomassie Blue was to adjust the amount of proteins in the different bands, this can also be used for a first analysis of the general protein composition between brain and spine chord samples of NFH-LacZ and controls mice. Colouring of all proteins by Commassie Blue does not permit further specification of searched proteins but provides a first overview.

In Coomassie Blue gels, we can observe that NF-M (130kDa) is missing in transgenic animals and NF-H is reduced. This is more pronounced in spinal cord and less evident in brain tissue *(Figures 6&7)*.

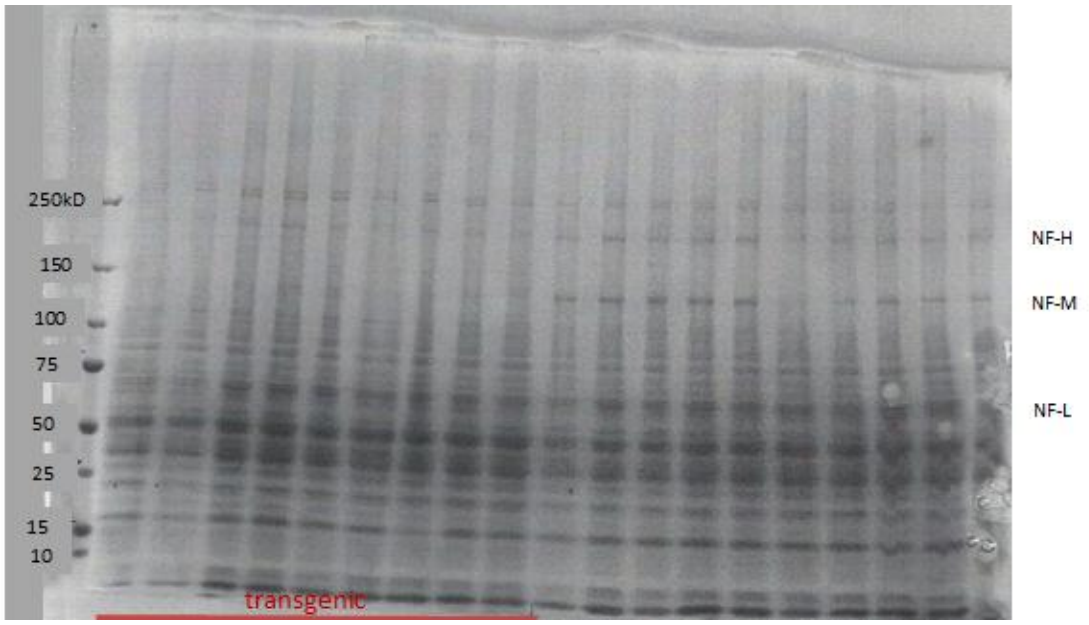


Figure 6 I Spinal cord samples from transgenic and wild type mice were separated on 3.6-15% SDS-PAGE gradient gel and proteins were stained with Coomassie Brillant Blue. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right Transgenic samples are indicated with a red line below the following samples are from control mice I Signal reduction in 130 kDalton and 200 kDalton in transgenic mice reflect decrease of NFM and NFH protein amounts.

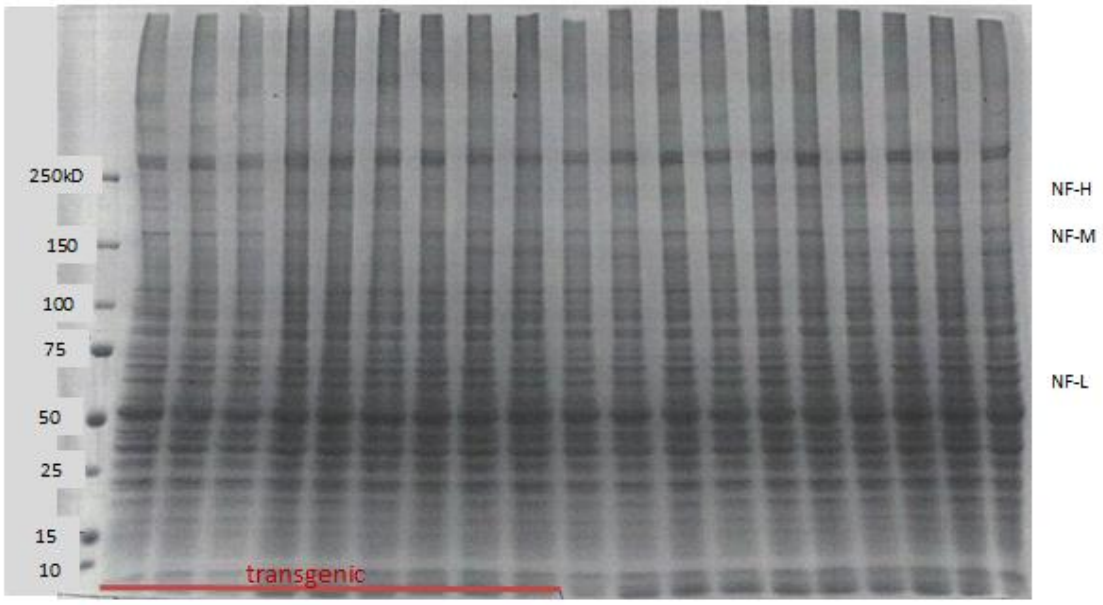


Figure 7 I Brain samples from transgenic and wild type mice were separated on 3.6-15% SDS-PAGE gradient gel and proteins were stained with Coomassie Brillant Blue. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right. Transgenic samples are indicated with a red line below the following samples are from control mice I Signal reduction in 130 kDalton and 200kDalton in transgenic mice reflect decrease of NFM and NFH protein amounts whereas the difference in brain isn't that clear than in spinal cord samples.

6.2 Immunostaining

The immunostaining method for detecting specific proteins allows to compare specific proteins between transgenic and control mice. The first approach was to figure out if our hypothesis that there is a difference in ubiquitination levels between transgenic and control mice was true. In a second step we compared the expression of the neurofilament proteins and thirdly, we analysed the

phosphorylation state of the neurofilament proteins. Listed are only antibodies that show clear results.

6.2.1 Ubiquitination

In contrast to our hypothesis, there was no clear difference in ubiquitination between transgenic and control mice. Having compared the transgenic mice to the control mice, neither in the brain nor in the spinal cord a clear difference in ubiquitination of neurofilaments, reporting a perturbation of the UPS, was detected. Quantification shows a decreased ubiquitination in transgenic mice possibly due to a fast reduction of neurofilaments or a non-recognition of the truncated neurofilaments by the UPS *(Figures 8&9 and Graph 1).*

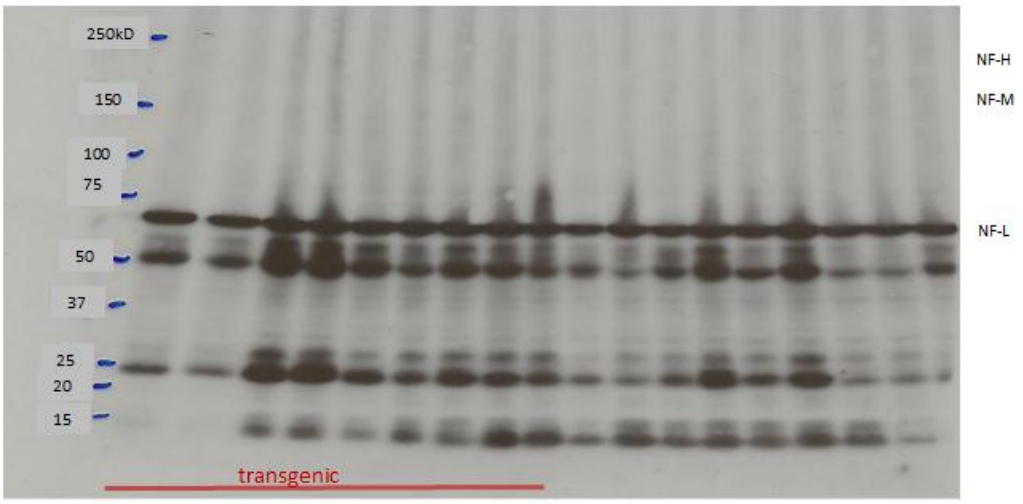


Figure 8 I Spinal cord proteins from transgenic and wild type mice were separated by electrophoresis, transferred to nitrocellulose membrane and specific proteins were detected by Immunoblot. The monoclonal ubiquitin antibody detects a specific epitope of ubiquitin. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right. Transgenic samples are indicated with a red line below the following samples are from control mice I There cannot be shown a difference in ubiquitination of neurofilament proteins in spinal cord of NFH-LacZ and control mice.

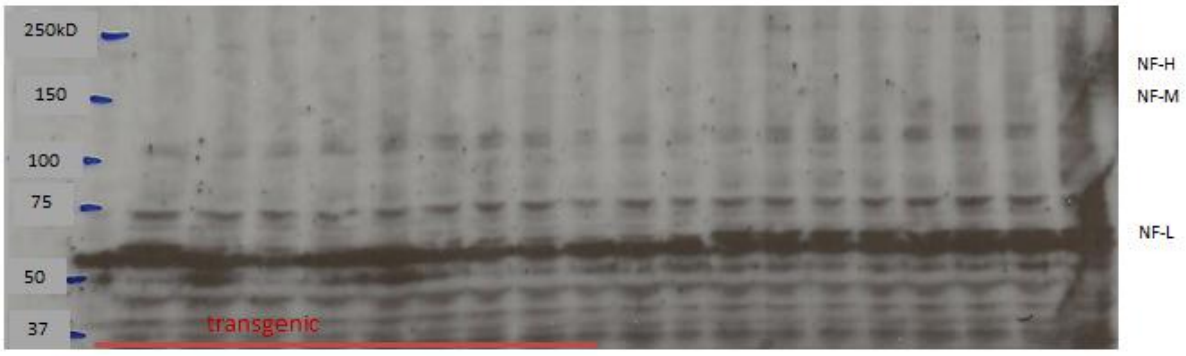
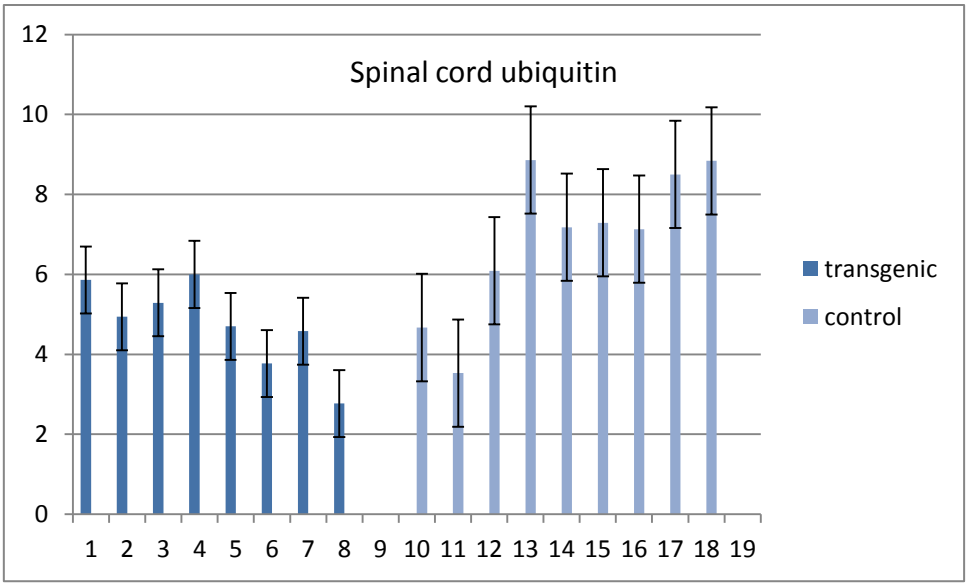


Figure 9 I Brain proteins from transgenic and wild type mice were separated by electrophoresis, transferred to nitrocellulose membrane and specific proteins were detected by Immunoblot. The polyclonal ubiquitin antibody detects different epitopes of ubiquitin. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right. Transgenic samples are indicated with a red line below the following samples are from control mice I There cannot be shown a difference in ubiquitination of neurofilament proteins in brain of NFH-LacZ and control mice.



Graph 1 I Quantification by Image J of an immunoblot of spinal cord stained with ubiquitin polyclonal I We report a significant reduction of ubiquitin in transgenic mice possibly due to a fast reduction of neurofilaments or a non-recognition of the truncated neurofilaments by the UPS. (p-value 0.01)

6.2.2 Neurofilament Expression

By using specific antibodies for each neurofilament, reduction can be shown in brain and spine cord samples. While M20 and BR10 specific for NF-M attest a reduction in NF-M, M15 showed no significant difference in NF-L and the M9 antibody selective for NF-H regrettably showed no clear result *(Figures 10&11 and graph 2&3).* With the antibody SMI32, specific for non-phosphorylated NF-H, a difference in protein composition can be detected. The transgenic mice show a fusion of the bands of the NF-H, possibly due to a bigger amount of NF-H with smaller molecular weight, as well as a hyperphosphorylation of the bigger NF-H proteins at 250 kD *(Figure 12).*

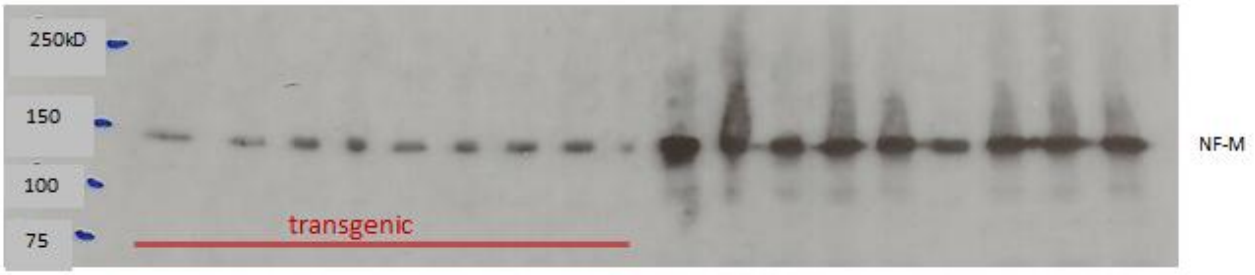
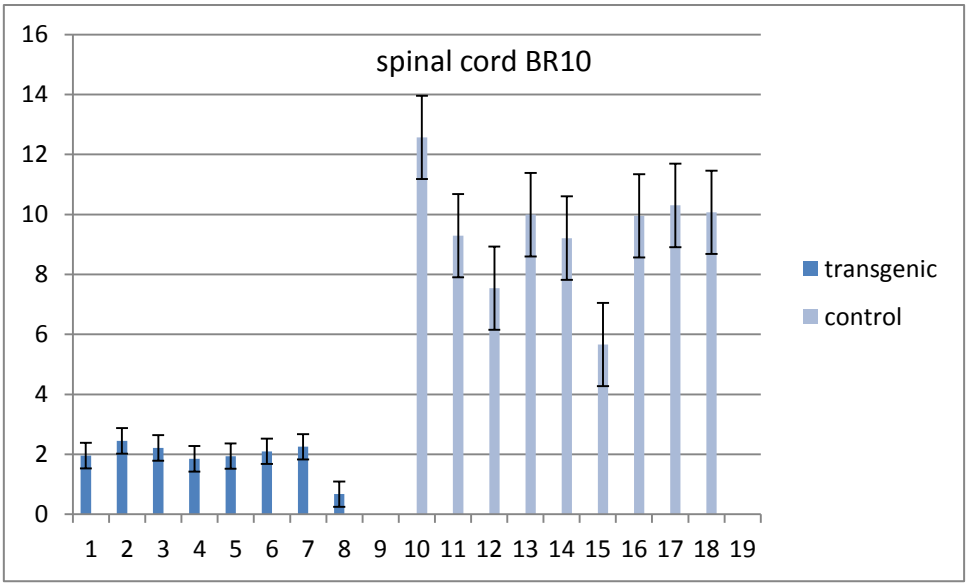
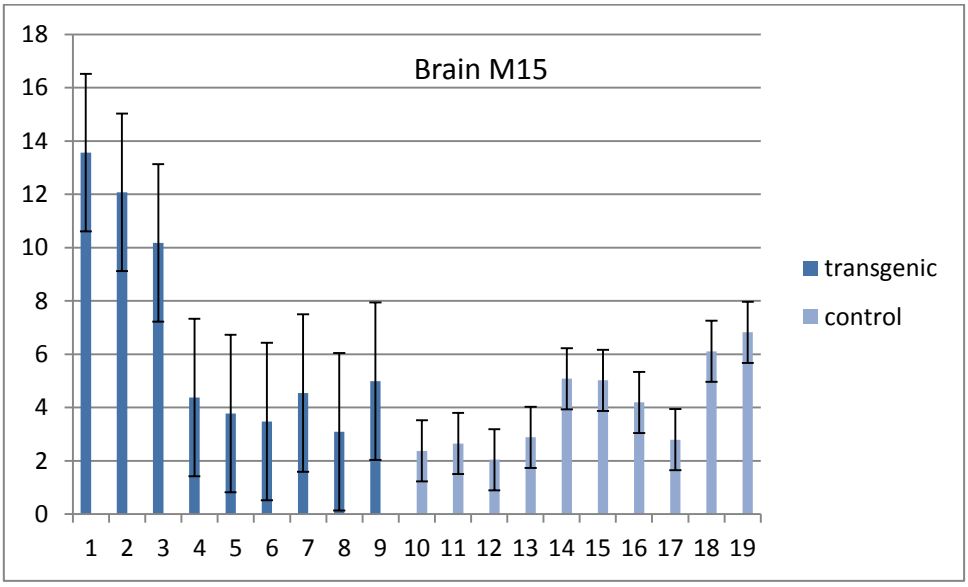


Figure 10 I Spinal cord proteins from transgenic and wild type mice were separated by electrophoresis, transferred to nitrocellulose membrane and specific proteins were detected by Immunoblot. The BR10 antibody is specific for NF-M. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right. Transgenic samples are indicated with a red line below the following samples are from control mice I Fewer signals about 130kD show a reduction of NF-M expression in transgenic mice.



Graph 2 I Quantification by Image J of an immunoblot of spinal cord stained with BR10 I There can be shown a significant reduction of NF-M in transgenic mice (p-value <0.05)

Figure 11 I Brain proteins from transgenic and wild type mice were separated by electrophoresis, transferred to nitrocellulose membrane and specific proteins were detected by Immunoblot. The M15 antibody is specific for NF-L. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right. Transgenic samples are indicated with a red line below the following samples are from control mice I M15 shows no difference in NF-L expressivity.



Graph 3 I Quantification by Image J of an immunoblot of brain stained with M15 I M15 shows no significant difference in NF-L expressivity (p-value 0.09)

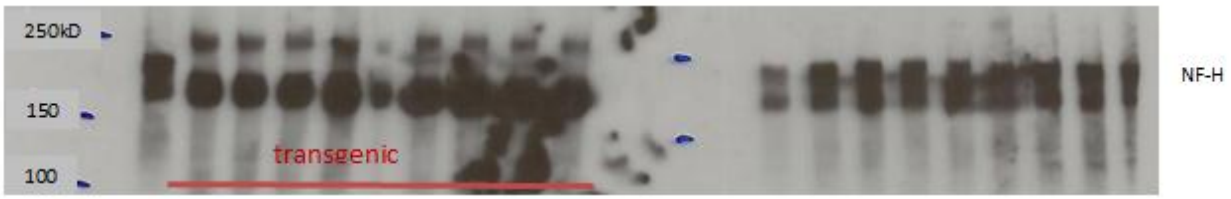


Figure 12 I Brain proteins from transgenic and wild type mice were separated by electrophoresis, transferred to nitrocellulose membrane and specific proteins were detected by Immunoblot. The SMI32 antibody is specific for non-phosphorylated NF-H. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right. Transgenic samples are indicated with a red line below the following samples are from control mice I SMI 32, detects a fusion of the bands of the NF-H in transgenic mice, because of a bigger amount of NF-H with smaller molecular weight, as well as a hyperphosphorylation of the bigger NF-H proteins at 250 kD in transgenic mice.

6.2.3 Phosphorylation

The SMI31 and SMI34 antibodies are specific for phosphorylated NF-H. They both show a reduced signal about 230kD in brain and spinal cord samples of transgenic mice reporting a reduction of phosphorylated NF-H. *(Figure 13)*

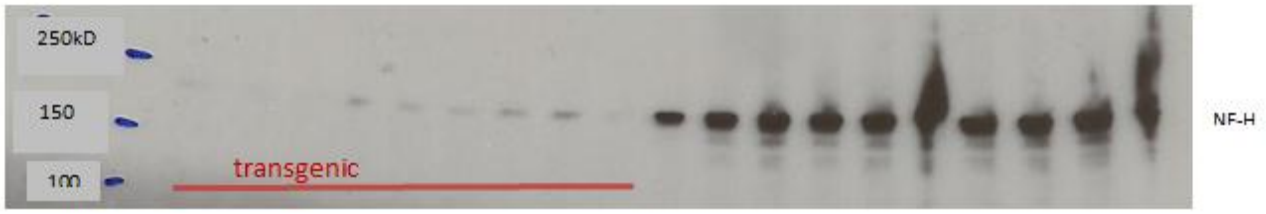


Figure 13 I Brain proteins from transgenic and wild type mice were separated by electrophoresis, transferred to nitrocellulose membrane and specific proteins were detected by Immunoblot. The SMI34 antibody is specific for-phosphorylated NF-H. Reference molecular weights are indicated to the left and the expected level of neurofilaments to the right. Transgenic samples are indicated with a red line below the following samples are from control mice I The SMI-34 antibody shows a clear loss of signal in transgenic mice, reporting a lack of phosphorylated NF-H in transgenic mice.

7 Summary

This work analysed brain and spinal cord samples of 9 NFH/LacZ transgenic and 10 control mice with several antibodies specific to ubiquitin and neurofilaments. The Immunoblot analyses revealed:

- There is no clear difference in ubiquitination of neurofilaments between transgenic and wildtype mice, neither in brain than in spinal cord. *(Figures 8&9 and Graph 1)*
- We detected a reduction of NF-H and NF-M expression in transgenic mice, and no difference in NF-L. *(Figures 10&11 and Graph 2&3)*
- A clear hypophosphorylation of NF-H can be shown in transgenic mice, but has not been quantified *(Figure 13.)*

8 Discussion

The hypothesis that the ubiquitination of the neurofilaments in NFH-LacZ transgenic mice increases has to be renounced in this sample. Increased ubiquitination of neurofilaments cannot be shown, neither in brain nor in spinal cord samples of transgenic mice. This is astonishing because several studies reported ubiquitin immunopositive cytoplasmic inclusions in ALS (Lehman 2009), ubiquinated neuronal hyaline inclusions which contain SOD1, (Okamoto, et al. 2011) and accumulation of ubiquitinated, hyperphosphorylated and truncated fragments of TDP-43 (Neumann, et al. 2007) and even detection of ubiquitin immunoreactivity in the NFH-LacZ inclusions (Tu, et al. 1997).

Different explanations can be given for the negative results. First of all, the NFH-LacZ mice model might not be a perfect example to study the amyotrophic lateral sclerosis. The loss of upper and lower motor neurons in ALS, caused by toxic properties due to a failure of the SOD1 antioxidant protein, may not have the same pathological mechanism as in NFH-LacZ mice, where the loss of spinal motor neurons is caused by blockage of axonal transport by accumulation of large neurofilament inclusions. Despite the fact that the ubiquitin-proteasome system detects and degrades mutant, misfolded, damaged, terminally modified or over-accumulated proteins, it might not recognize the fused NFH-LacZ protein as a non-useful protein that is requiring repair or ubiquitination.

None of the studies mentioned above used a NFH-LacZ model mice, but analysed SOD1-knockout mice or post-mortem material.

It has been well established that in ALS the ubiquitin-proteasome system is affected but the results presented here suggest that the NFH-LacZ model is not the ideal model to study the UPS deficiency.

Therefore further investigations with other models are indicated. Recent gene investigations in ALS described an X-linked form of ALS with a mutation in a gene encoding for ubiquilin-2, suggesting that a malfunction in autophagy and protein recycling plays a role in a minor part of ALS (Al-Chalabi, et al. 2012).

Our results suggest that there must be other pathways perturbed than the UPS that lead to the accumulation of the neurofilament proteins. Studies show that disturbance in the endosome/lysosome system, the other degradation pathway apart the UPS seems to be more important in ALS than in other neurodegenerative diseases. (Lehman 2009).

In contrast, the other findings a decrease of NF-H and NF-M and hypophosphorylation confirm other studies. These studies show a reduction of each neurofilament subunit by more than half the amount in brain as in spinal cord (Riederer, et al. 2003). The only discordance found in this work is that the NF-L expression is unchanged. These findings result of the blockage of axonal transport of neurofilaments caused by fused NFH-LacZ protein and the resulting hypophosphorylation. The same study shows a massive reduction in phosphorylation of NF-M and NF-H, responsible for increased neurofilament degradation, which we confirm with results that show a clear hypophosphorylation of NF-H explained by the lack of transport into axon. Because the phosphorylation of neurofilaments occurs primarily in the axon, the blockage of axonal growth in NFH/LacZ mice leads to hypophosphorylation of mainly NFH (Tu, et al. 1997).

9 Conclusion and Perspectives

In this work it was not possible to distinguish between differences in ubiquitination of neurofilaments from transgenic and wild type mice. The NFH/LacZ mice model seems to be a good model for neurofilament loss and malformative inclusions but might lack congruence in the exact

pathological mechanism of ALS. Regardless of the mechanism behind the amyotrophic lateral sclerosis should be continuously investigated. Only by understanding the exact pathological pathway an effective treatment can be developed to fight the disease.

Aware of the vulnerability of the laboratory methods, the tests should be repeated with other transgenic mice models as SOD1 knockout mice or with human autopsy material affected by ALS to confirm the results. Otherwise the focus should be put on other degradation pathways as the endosome/lysosome system. Until further knowledge of the pathological mechanism between SOD1 disturbance and loss of motor neurons, developing therapies should be focused on a SOD1 blockage. Another approach is the molecular neuropathology that reported several genetic findings in the recent past which allows new therapeutic approaches and advances in disease modelling. (Ludolph, et al. 2012)

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