UMEÅ UNIVERSITY

Structural Investigation of SOD1 aggregates in ALS

Identification of prion strains using anti-peptide antibodies

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Science is organized knowledge. Wisdom is organized life

- Immanuel Kant

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative syndrome characterized by progressive degeneration of motor neurons that result in muscle wasting. The symptoms advance gradually to paralysis and eventually death. Most patients suffer from sporadic ALS (sALS) but 10% report a familial predisposition. Mutations in the gene encoding superoxide dismutase-1 (*SOD1*) were the first identified cause of ALS. The disease mechanism is debated but there is a consensus that mutations in this protein confer a cytotoxic gain of function. SOD1 aggregates in motor neurons are hallmarks of ALS both in patients and in transgenic mouse models expressing a mutated form of human SOD1 (hSOD1). Recently, our group showed that SOD1 aggregates are present also in sALS patients, thus indicating a broader involvement of this protein in ALS. Misfolding and aggregation of SOD1 are difficult to study *in vivo* since aggregate concentration in the central nervous system (CNS) is exceedingly low. The aim of this thesis was to find a method circumventing this problem to investigate the hSOD1 aggregate structure, distribution and spread in ALS disease.

Many studies provide circumstantial evidence that the wild-type hSOD1 protein can be neurotoxic. We developed the first homozygous mouse model that highly overexpresses the wild-type enzyme. These mice developed an ALS-like syndrome and become terminally ill after around 370 days. Motor neuron loss and SOD1 aggregate accumulation in the CNS were observed. This lends further support to the hypothesis of a more general involvement of SOD1 in human disease.

A panel of polyclonal antibodies covering 90% of the SOD1 protein was developed by our laboratory. These antibodies were shown to be highly specific for misfolded SOD1. Aggregated hSOD1 was purified from the CNS of terminally ill hSOD1 mice. Disordered segments in aggregated hSOD1 could be identified with these antibodies. Two aggregate strains with different structural architectures, molecular properties, and growth kinetics, were found using this novel method. The strains, denoted A and B, were also associated with different disease progression. Aggregates formed *in vitro* were structurally different from these strains. The results gave rise to questions about aggregate development and possible prion-like spread. To investigate this, inoculations of purified strain A and B hSOD1 seeds was performed in lumbar spinal cords of 100-day old mice carrying a $hSOD1^{G85R}$ mutation. Mice seeded with A or B aggregates developed premature signs of ALS and became terminally ill 200 days earlier than mice inoculated with control preparation. Interestingly, a templated spread of aggregates along the neuraxis was concomitantly observed, with strain A and B provoking the buildup of their respective hSOD1 aggregate structure. The phenotypes initiated by the A and B strains differed regarding progression rates, distribution, end-stage aggregate levels, and histopathology.

To further establish the importance of hSOD1 aggregates in human disease, purification and inoculation of aggregate seeds from spinal cords of ALS patients and mice carrying the $hSOD1^{G127X}$ mutation were performed. Inoculation of both human and mouse seeds as described above, induced strain A aggregation and premature fatal ALS-like disease.

In conclusion, the data presented in this thesis provide a new, straightforward method for characterization of aggregate strains in ALS, and plausibly also in other neurodegenerative diseases. Two different prion strains of hSOD1 aggregates were identified in mice that resulted in ALS-like disease. Emerging data suggest that prion-like growth and spread of hSOD1 aggregation could be the primary pathogenic mechanism not only in hSOD1 transgenic models, but also in human ALS.

Keywords: ALS, SOD1, prion, motor neuron disease, neurodegeneration, strain, seeding, protein aggregation, transgenic mice, peptide antibodies

Original papers

This thesis is based on the following papers that are referred to in the text by their Roman numerals:

I. Expression of wild-type human superoxide dismutase-1 in mice causes amyotrophic lateral sclerosis
Graffmo KS†, Forsberg K†, **Bergh J**, Birve A, Zetterström P, Andersen PM, Marklund SL, Brännström T.
Hum Mol Genet. 2013 Jan 1;22(1):51-60.

II. Structural and kinetic analysis of protein-aggregate strains in vivo using binary epitope mapping
Bergh J, Zetterström P, Andersen PM, Brännström T, Graffmo KS, Jonsson PA, Lang L, Danielsson J, Oliveberg M, Marklund SL.
Proc Natl Acad Sci U S A. 2015 April 7;112(14):4489-94.

III. Two superoxide dismutase prion strains transmit amyotrophic lateral sclerosis-like disease
Bidhendi EE†, **Bergh J**†, Zetterström P, Andersen PM, Marklund SL, Brännström T.
J Clin Invest. 2016 Jun 1;126(6):2249-53.

IV. Mutant superoxide dismutase aggregates from human spinal cord transmit amyotrophic lateral sclerosis
Bidhendi EE, **Bergh J**, Zetterström P, Forsberg K, Pakkenberg B, Andersen PM, Marklund SL*, Brännström T*.
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Abbreviations

Ab	antibody
ACh	acetylcholine
AD	Alzheimers's disease
AFM	atomic force microscopy
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASO	antisense oligonucleotide
ATP	adenosine triphosphate
ATR-FTIR	attenuated total reflectance fourier transform infrared spectroscopy
BMAA	β-methylamino-L-alanine
BMI	body mass index
BSE	bovine spongiform encephalopathy
bvFTD	behavioral variant of FTD
C9ORF72	chromosome 9 open reading frame 72
Ccs1	copper chaperone for SOD1
ChAT	choline acetyltransferase
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
DPR	dipeptide repeat
DTPA	diethylenetriaminepentaacetic acid
DTT	dithiothreitol
EAAT2	excitatory amino acid transporter-2
EDTA	ethylenediaminetetraacetic acid
EMG	electromyography
ENoG	electroneurography
ER	endoplasmic reticulum
ERAD	ER-associated degradation
fALS	familiar ALS
FAT	fast axonal transport
FTD	frontotemporal dementia
FTLD	frontotemporal lobar degeneration
FUS	fused in sarcoma
GdmCl	guanidinium chloride
GLT1	glutamate transporter-1
GluR-2	glutamate receptor-2

hALS	hereditary ALS
hSOD1	human SOD1
IAM	1-Iodoacetamide
ICD	International Classification of Diseases
IP	intraperitoneal
IPSC	induced pluripotent stem cell
KLH	keyhole limpet hemocyanin
LMN	lower motor neuron
MCT	monocarboxylate transporter
tcMEP	transcranial electrical motor evoked potentials
MND	motor neuron disease
MRI	magnetic resonance imaging
NCI	neuronal cytoplasmic inclusion
NeuN	neuronal nuclei
NF-κB	nuclear factor-kappa B
NII	neuronal intranuclear inclusion
NMDA	*N*-methyl-D-aspartate
NP40	nonidet P-40
PBP	progressive bulbar palsy
PBS	phosphate buffered saline
PCA	principal component analysis
PD	Parkinson's disease
PDC	parkinsonism-dementia complex
PLS	primary lateral sclerosis
PMA	progressive muscular atrophy
PNS	peripheral nervous system
PrP	prion protein
RAN-translation	repeat-associated non-ATG/AUG translation
ROS	reactive oxygen species
sALS	sporadic ALS
SC	subcutaneous
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SCNA	synuclein alpha
SMA	spinal muscular atrophy
SOD1	superoxide dismutase 1
TBS	tris-buffered saline

TDP-43	transactive response DNA binding protein 43
TLS	translocated in liposarcoma
TMS	transcranial magnetic stimulation
TSE	transmissible spongiform encephalopathies
UMN	upper motor neuron
UPR	unfolded protein response
vCJD	variant CJD
wt	wild-type

Populärvetenskaplig sammanfattning

Amyotrofisk lateralskleros (ALS) är en dödlig nervsjukdom som årligen drabbar cirka 250 personer i Sverige. I världen finns det uppskattningsvis 223,000 patienter med ALS idag, och det antalet ökar för varje år. De flesta som får ALS insjuknar mellan 45 och 75 års ålder men det finns ovanliga former som även drabbar barn. De första symptomen uppträder vanligtvis som en svaghet i ena handen eller foten. Symptomen sprider sig därefter vidare över armen/benet för att sedan drabba den andra kroppshalvan. Svagheten förvärras till förlamning och till slut drabbas andningsmuskulaturen. Detta, i sin tur, leder till andningssvårigheter och död, vanligtvis 2-4 år efter första symptom. Det finns subtyper av ALS där extremiteterna inte drabbas först, eller där överlevnaden är längre än 10 år. Således är ALS-diagnosen att betrakta som ett paraplybegrepp eller syndrom, innefattande flera typer av sjukdomar och sjukdomsförlopp. Alla drabbar de motoriska nerverna som ansvarar för viljemässig rörelse (motorneuron).

När en rörelse initieras skickar nervceller i hjärnans motorcentral (de övre motorneuronen i motorcortex) nervsignaler till de nedre motorneuronen i ryggmärgen. Signalerna skickas vidare till musklerna som drar ihop sig, vilket skapar en rörelse. Vid ALS dör stegvis fler och fler av både de övre och nedre motorneuronen, vilket gör att styrningen av muskulaturen försvinner. Detta, i sin tur, ger symptom som förlamning, men även att musklerna drar ihop sig okontrollerat, till exempel i muskelryckningar (fascikulationer). Det finns inget botemedel mot ALS och bara en bromsmedicin är godkänd i Sverige, Riluzole, med mycket begränsad effekt på sjukdomsförloppet.

Det finns idag endast tre etablerade riskfaktorer: Hög ålder, manligt kön och ALS i familjen. I 90 procent av fallen finns ingen tydlig orsak till insjuknandet, vilket kallas sporadisk ALS. Hos de resterande 10 procenten, de med familjär ALS, finns en ärftlig, genetisk orsak. År 1993 upptäcktes en genförändring (mutation) i superoxid dismutas-1 (*SOD1*) som gav visade sig ge upphov till ALS-sjukdom. Sedan dess har 210 mutationer identifierats i denna gen. *SOD1* är muterat i 15-25 procent hos alla patienter med familjär ALS. SOD1-proteinet finns i alla kroppens celler och dess normala funktion är att bryta ner skadliga fria syreradikaler, som framförallt bildas i samband med att näring omsätts till energi. Idag vet vi att den primära orsaken till att mutationerna i *SOD1*-genen orsakar ALS är att SOD1-proteinet blir instabilt till följd av mutationen. Detta får till följd att proteinet får en benägenhet att inta en felaktig struktur, med andra ord; proteinet felveckas. Detta, i sin tur, gör att SOD1 klumpar ihop sig till stora aggregat, som nervcellen inte kan bryta ner på ett effektivt sätt. Aggregat finns också i majoriteten av de sporadiska fallen, utan mutationer i *SOD1*. Därav finns anledning att misstänka att SOD1-proteinet har en bredare betydelse i ALS-sjukdomens uppkomst. Den bakomliggande mekanismen till att mutationer i *SOD1* ger ALS är fortfarande inte helt klarlagd.

I min avhandling undersöks betydelsen av SOD1-proteinet i ALS-sjukdom. Detta genom att använda musmodeller som uttrycker den humana varianten av SOD1 (hSOD1). Dessa möss utvecklar likt ALS patienterna förlamning, bildar SOD1 aggregat och drabbas av motorneurondöd. Nedan beskrivs kortfattat studiernas innehåll.

Studie I: I den första studien undersöktes huruvida SOD1 utan mutation (vildtyps-SOD1) har potential att orsaka ALS sjukdom. Då många studier har visat att även vildtypsversionen av proteinet kan vara neurotoxiskt, ville vi närmare testa detta i praktiken. Vi utvecklade en ny musmodell där koden för *hSOD1* klippts in på musens båda kromosomer, en så kallad transgen mus. Överuttrycket av hSOD1 gjorde att mössen utvecklade ALS-liknande symptom och blev terminalt sjuka efter 370 dagar (friska möss lever normalt >800 dagar). I likhet med ALS-patienter och andra musmodeller med mutationer i *hSOD1,* observerades uttalad motorneurondöd och hSOD1-aggregat i både ryggmärg och hjärna identifierades i de transgena vildtyps-hSOD1 mössen. Resultatet av denna studie visar att en mutation i genen för SOD1 inte är nödvändig för att proteinet ska bidra till ALS-sjukdom i möss. Detta ger ytterligare stöd för hypotesen att SOD1 har en generell betydelse i ALS-sjukdom hos människa.

Studie II: SOD1 kan bilda aggregat i både provrör och hos patienter med ALS, dock vet vi väldigt lite om orsaken till att proteinet klumpar ihop sig. Detta beror på att koncentrationen av proteinet i människa och försöksdjur är väldigt låg, vilket gör att aggregaten blir mycket svåra att analysera. Faktum är att ytstrukturen av SOD1-aggregaten fortfarande är okänd. Detta gör det bland annat svårt att utveckla mediciner som bryter ned dessa aggregat. I denna studie var målet att utveckla en ny metod för att karaktärisera strukturen av hSOD1-aggregat från möss med terminal ALS-sjukdom.

Metoden som vi utvecklade kallades ”binär mappning av epitoper” (binary epitope mapping). Inom biomedicin används antikroppar, som i kroppen förekommer naturligt för att bekämpa bakterier och virus, för att märka upp specifika proteiner. Med hjälp av flera designade antikroppar riktade mot korta regioner i SOD1-proteinets aminosyrakedja (de byggstenar som ett protein består av) skulle vi kunna få en uppfattning av SOD1-aggregatens unika struktur. Vi identifierade två olika typer av strukturer som vi kallade ”strain A” och ”strain B”. Utöver strukturskillnaderna så skilde aggregaten sig åt med avseende på molekylära egenskaper och förmåga att sprida sig i vävnad. Dessutom påverkades musmodellernas överlevnad beroende på vilket aggregat som hade utvecklats i dess centrala nervsystem. Tillsammans ger detta stöd för att SOD1-aggregatens struktur kan spela en roll i hur sjukdomsförloppet i ALS utvecklas.

Studie III: I flera olika sjukdomar som angriper nervceller (neurodegenerativa sjukdomar), t.ex. Alzheimers eller Parkinsons sjukdom, börjar man misstänka att

den centrala sjukdomsmekanismen involverar prion-liknande spridning av aggregat. En prion är en mycket stabil, infektiös partikel som består av flera felveckade proteiner med en specifik struktur. Creutzfeldt-Jakobs sjukdom och även galna ko-sjukan är klassiska prionsjukdomar. Prioner fungerar som en mall, och provocerar proteiner som är friska att ändra form till samma felaktiga struktur som prionproteinet har, så kallad templatstyrd spridning. Dessa prioner klumpar sedan ihop sig och bildar aggregat. På detta sätt breder sjukdomen successivt ut sig i nervsystemet.

I studie II observerades att de två olika proteinaggregaten påverkade sjukdomsförloppet hos mössen, samt att alla aggregat i varje enskild mus hade samma struktur oavsett om ryggmärgen eller hjärnan undersöktes. Detta fick oss att misstänka att en prionliknande sjukdomsmekanism finns även i ALS.

För att undersöka detta renades strain A- och B-aggregaten fram från ryggmärgen av terminalt sjuka ALS-möss. Dessa aggregat injicerades sedan i ryggmärgen på asymtomatiska, 100 dagar gamla möss, med en hSOD1^{G85R}-mutation. Dessa möss utvecklade sjukdom 100 dagar efter injektionen, hela 200 dagar tidigare än möss som injicerats med kontrollösning. Likt i prionsjukdomarna kunde en templatstyrd spridning av hSOD1-aggregaten ses i det centrala nervsystemet. Injicerades strain A- så utvecklades strain A-aggregat hos mottagarmusen, strain B- gav strain B-aggregat. Dessutom påverkades sjukdomsförloppet och utbredningen av aggregat i centrala nervsystemet beroende på vilken strain som injicerades. Detta tyder på att de två hSOD1-aggregaten (strain A och B) är ALS-inducerande prioner.

Studie IV: I studie IV undersöktes om dessa prionliknande aggregat har relevans för ALS i människa. SOD1-aggregat renades fram, likt ovan, från både en ALS-patient och ALS-möss med en specifik mutation, hSOD1^{G127X}. Det visade sig att även SOD1-aggregat från människa, som dessutom har mycket lägre SOD1-nivåer än försöksdjuren, provocerade fram sjukdom mycket tidigare än hos de möss som inte blivit injicerade med aggregat. De injicerade mössen utvecklade aggregat av strain A-typ, oavsett om dessa injicerats med aggregat från mus eller människa.

Sammanfattningsvis så har denna avhandling bidragit till att ta fram en ny, enkel och kostnadseffektiv metod för att karaktärisera SOD1-aggregatstrukturer i ALS. Förhoppningsvis kan metoden användas för analys av sjukdomsframkallande proteiner hos andra neurodegenerativa sjukdomar i framtiden. Resultaten tyder på att prionliknande spridning av SOD1-aggregat skulle kunna vara den primära sjukdomsframkallande mekanismen i ALS. Detta förhållande bör vara av betydelse vid framtida försök att utveckla nya mediciner mot sjukdomen.

Introduction

Amyotrophic Lateral Sclerosis, an overview

In 1874, the French neurologist Jean-Martin Charcot published the first description of Amyotrophic Lateral Sclerosis (ALS), summarizing 50 years of prior studies on progressive muscular weakness [1, 2]. The name of the disease was a description of histological and physiological symptoms that were observed in the cases. Myos is the Greek name for muscles and throphos is nutrition; thus Amyotrophic means muscle wasting due to loss of trophic signals. Lateral means "on the side" and Sclerosis means hardening of tissue. Lateral Sclerosis is observed in the spinal cord of ALS patients due to loss of the corticospinal axons wiring the upper motor neurons to the lower motor neurons. The remaining connective tissue in this area feels hard. Charcot linked the axonal loss to the death of motor neurons, which today still defines the disease [3].

ALS is also known as Motor neuron disease (MND), Charcot's disease, or in the US as Lou Gehrig's disease. The latter is named for a famous New York Yankees baseball player who died from ALS in 1942, thus giving the disease a public American face. Other well-known people with ALS are the British physician Stephen Hawking, and in Sweden the news reporter Ulla-Carin Lindquist and the journalist and author Maj Fant.

ALS is a progressive, neurodegenerative disease that afflicts specifically the motor neurons. This causes muscle wasting that usually begins focally, but with time spreads relentlessly to involve most muscles including the diaphragm. The cause of death is usually respiratory failure or pneumonia that typically occurs after 2 to 4 years [4, 5], but 5-10% of the patients survive over 10 years [4].

The mechanism(s) behind motor neuron degeneration is unfortunately still not known. This thesis will be a contribution to further the understanding of this devastating disease.

Central nervous system

The symptoms and etiology of ALS originate from the Central Nervous System (CNS) that consists of the brain, cerebellum, brainstem, and the spinal cord. Most regions in the CNS are surrounded by a semipermeable filter – the blood-brain barrier. This filter prevents bigger molecules, potential neurotoxins and infectious agents in the blood from entering the CNS. The motor system of the CNS is divided into upper (UMN) and lower (LMN) motor neurons. The motor cortex, located in the frontal lobe, contains the nerve cell bodies of the UMNs. The UMNs projects it axons down to the LMNs located in the brainstem and spinal cord (Figure 1). For the axons to reach the LMNs located in the lowest part of the spinal

cord, an axon length of over one meter is required. Most UMNs influence the generation of movements by stimulating or inhibiting LMNs directly or indirectly. Lower motor neurons project their axons to several muscle fibers and connect to them by neuromuscular junctions that are located on the surface on each fiber. A motor neuron and its connected fibers is defined as the motor unit. When LMNs are activated, action potentials travel along the axon. When the axon leaves the spinal cord it exits the CNS and enters the peripheral nervous system (PNS). This action potential reaches the neuromuscular junction and activates a release of the neurotransmitter acetylcholine (ACh). The neurotransmitter travels through the synaptic cleft, the space between the axon terminal and motor endplate that together compose the neuromuscular junction (the muscular synapse). This signal activates contraction in each muscle fiber that the neurons are connected to.

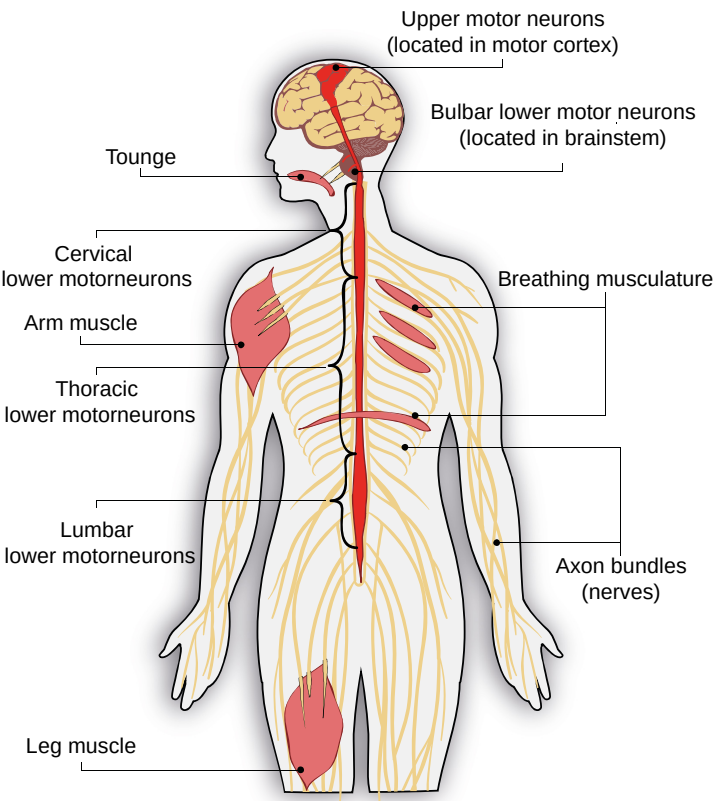

Figure 1. Schematic illustration of the nervous system and its connection to muscles. *The upper motor neurons project their axons down to the lower motor neurons located in the brainstem, cervical-, thoracic- and lumbar spinal cord. The lower motor neurons connect to the muscles through axons located in the peripheral nervous system.*

Organization of the motor system

The LMNs are located in the spinal cord and control peripheral muscles in the arms, legs and torso. LMNs are also found in the brainstem and synapse to muscles in the face, throat, tongue and jaw. This organization plays a role in the clinical classification of ALS (see ALS symptoms and clinical presentation).

The human somatic motor representation of the cortex was investigated in 1937 by the neurosurgeon Wilder Penfield [6]. Penfield and colleagues showed that the motor cortex contain a spatial map of the body's musculature. This later gave rise to the classic cortical homunculus, a caricature of a human with each body part drawn in the size of its representative area size in the motor cortex. Parts with fine and precise movement, such as hands and face, are overrepresented in the cortex. Parts not demanding high precision, like the legs, are represented in a proportionally smaller area in the motor cortex. The neurons in the cortex connect to the LMNs through axons. Numerous axons together form corticospinal and corticobulbar tracts that descend to target neuron(s). The corticobulbar axons synapse to neurons located in the brainstem on the ipsilateral (same) side. On the contrary, the majority of the corticospinal axons project to the contralateral (other) side through the pyramidal decussation in the caudal medulla. This means that the right motor cortex controls the left hand but the right side of the face, and vice versa.

The spinal cord, where LMNs or secondary motor neurons are located, is also divided topographically with concern to movement. The signals to hand and arm musculature originate from the upper part of the spinal cord, i.e. the cervical spinal cord. Muscles of the torso are innervated by thoracic motor neurons and the legs are innervated by motor neurons in the lumbar spinal cord. The area around the genitals is innervated by motor neurons located in the sacral (lowermost) spinal cord.

Cross-sectioning of the spinal cord macroscopically reveals two different kinds of tissues. The central darker tissue contains the nerve cell bodies and the brighter tissue closer to the exterior contains mainly axons; these are termed the gray and the white matter, respectively. The motor neurons are located in the ventral (frontal) horn of the gray matter. There is a somatotopic organization of these neurons as well. The medial neurons innervate axial musculature, whereas the lateral neurons innervate muscles located more distally.

There are three types of LMNs in the ventral horn, and alpha (α), beta (β) and gamma (γ) motor neurons. Gamma-motor neurons are involved in regulating muscle sensory receptors called muscle spindles. These send information to the brain and spinal cord about the length and tension of the muscle. Alpha-motor neurons innervate striated muscles that generate will-controlled forces needed for movements, such as walking and griping. Beta-motor neurons innervate both the muscle spindles and striated muscles, and are less abundant than the other motor neuron types.

The α-motor neuron soma size reflects the number of muscle fibers that is innervated by the specific neuron. A neuronal cell body innervating many muscle fibers is thus relatively big.

In most skeletal muscles the smaller motor unit innervates so called red muscle fibers. The red muscle fibers, also called slow twitch or type I fibers, contract slowly but can be activated for a long time without losing power. These are called slow motor units, and are, for example, important in maintaining an upright posture. Red muscle fibers are dense with capillaries and rich in myoglobin and mitochondria, thus resulting in the red color. On the contrary, fast motions like jumping are initiated by pale muscle fibers that are innervated by large α-motor neurons. The larger motor neuron and its muscle fiber compose the fast fatigable motor unit that generates a high amount of force for a short time. A third class of motor units with intermediate size is called fast fatigue-resistant motor units. These possess properties that lie between the above-mentioned motor units, they are more resistant to fatigue and generate about twice the force of slow motor units.

In a chronic neurodegenerative disease like ALS, denervation of muscles is initiated due to motor neuron death. As muscle fibers lose their innervation, nearby axons from healthy motor neurons compensate for this loss in synapsing to the non-innervated fibers. This leads to enlargement of motor units and the fibers for each motor unit tend to lie next to each other instead of being scattered. In histopathology, this is called fiber type grouping and is indicative of denervation. When this group again loses innervation, the fibers shrink as a group referred to as group atrophy. Not all motor neuron subtypes are equally vulnerable in ALS. Fast-fatigable motor neurons are the first to degenerate in ALS patients [7] and also in mutant superoxide dismutase 1 (SOD1) mice [8]. In mice the fast-fatigable motor unit axons are affected long before symptoms onset. Fast-fatigue-resistant motor unit axons are affected at symptom onset, whereas axons of slow motor units are spared [9]. Gamma-motor neurons, represent approximately one-third of all MNs in limb-innervating motor neuron pools [10]. A recent study using SOD1, transactive response DNA binding protein 43 (TDP-43) and fused in sarcoma (FUS) mouse models suggest that these neurons are spared due to a lack of synaptic contacts from primary afferent fibers, and that surviving γ motor neurons activate α-motor neurons indirectly and thereby contribute to α-motor neuron death [11]. Also, extraocular muscles are relatively spared during the ALS disease course, both in humans and mice [12, 13].

ALS symptoms and clinical presentation

ALS could be considered as a clinical syndrome rather than a single disease. Although the common denominator is motor neuron degeneration afflicting both UMNs and LMNs, the focal area of disease initiation can vary from patient to patient. Also, the presence of subcategories in accordance to genetics might vary the

clinical phenotype. The cardinal symptoms are muscle weakness, muscle atrophy and muscle twitches that progress continuously.

The classification of ALS is complex and can be based on many different clinical phenotypes, for example, ALS with cognitive impairment, the rate of UMN and LMN affection, spinal onset or bulbar onset, and only arm or leg affection are some different classifications used (Figure 2). The classification system is under continuous debate and evolves over time [14]. The division of different clinical phenotypes of ALS discussed in the section below is a classification based on *initial* symptoms, which is the most commonly used taxonomy.

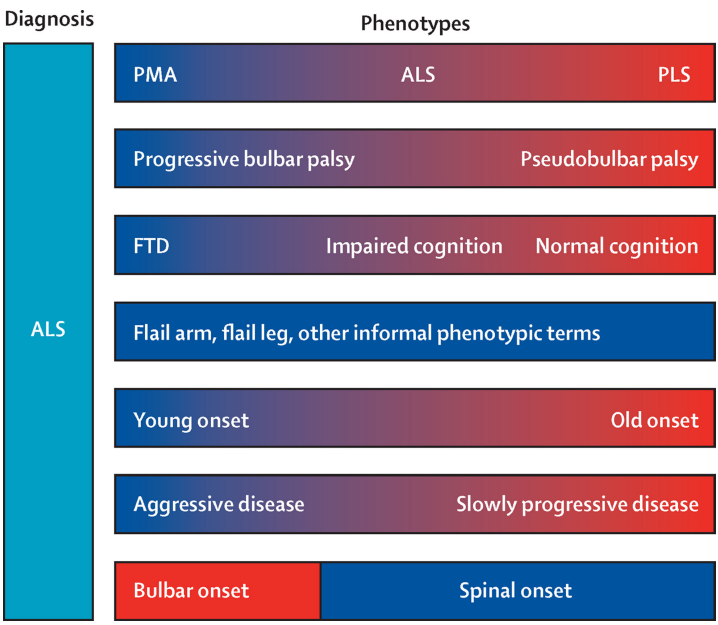

Figure 2. Diagnosis and phenotypes of amyotrophic lateral sclerosis. *Amyotrophic lateral sclerosis is an overarching diagnosis can be sub-classified in many ways. There is no consensus in the field if progressive muscular atrophy (PMA) and primary lateral sclerosis (PLS) is to be considered as phenotypes of ALS or as diseases of their own. Reprinted from Lancet Neurol. [14], Copyright © 2018 Elsevier B.V. Used with permission.*

The most common presentation of ALS is limb onset with an involvement of both upper and lower motor neuron signs initially. This form is usually called “classic ALS”, and constitutes the majority of ALS cases. The typical patient with classic ALS presents with weakness in a foot or a hand with subsequent spread to the contralateral side. Eventually, the symptoms spread to all four limbs and to the diaphragm muscle, thereby impairing breathing function. The mean survival time for this variant is 2-3 years from symptom onset [15, 16]. Cervical or lumbar initiation of classical ALS does affect survival or clinical care requirements [17]. Twenty-

five percent of the patients present with isolated bulbar onset denoted as progressive bulbar palsy (PBP) or pseudobulbar palsy depending on if the symptom onset is originating from the LMNs or UMNs, respectively. Initial symptoms of this form could be slow and nasal speech, tongue muscle wasting with fasciculations (muscle twitches) or spasticity. Bulbar onset ALS has a more aggressive disease course than classical ALS with a mean survival time of 25-27 months [17, 18]. ALS with respiratory onset is uncommon (up to 3 %) [19], and the survival time of these patients are the same as with bulbar onset.

Flail arm and flail leg syndrome is a clinical presentation of ALS with symptoms from either upper or lower extremities with only LMN affection. Many of the patients develop UMN symptoms over time [20]. The debut age is largely the same as classic ALS, but the 5-year survival is much higher in flail arm and leg compared to ALS (52%, 63.9%, and 20%, respectively, reported for populations in the UK and Australia [21]).

Some patients only present signs of LMN dysfunction, generally with an asymmetrical affection of muscle strength. This syndrome is called progressive muscular atrophy (PMA) and the incidence is estimated at 0.02 per 100.000 [22]. Compared to ALS, PMA is more common in males, usually affects an older population and is associated with longer survival. Twenty-two percent of the patients develop UMN signs and then get an ALS diagnosis. Since the clinical symptoms and histopathological picture resemble those of ALS, it has been suggested that PMA should be considered a form of ALS [23]. Another motor neuron disorder, primary lateral sclerosis (PLS), only present with UMN signs. PLS is often symmetrical and progressive in its nature, but with a less aggressive disease course. Patient survival is normally more than 10 years. PLS can also progress to ALS, but four years after symptom onset it is uncommon. [24]

ALS and cognitive impairment

ALS was initially considered a motor neuron only disease, but growing evidence in late 20th century suggested the disease manifestation was more diverse than that. The first study describing ALS and dementia was published in 1950 [25, 26]. In 1981, a review of the relationship between ALS and dementia was published, again highlighting the clear connection [27]. Many publications identified histopathological changes in frontotemporal regions in the brain in ALS patients with dementia [28-31], closely resembling the changes seen in frontotemporal dementia (FTD). The overlap between ALS and FTD was also shown by electrophysiological assessment of 36 FTD patients with no known ALS diagnosis; the finding was that five of these patients met the criteria of ALS [32]. In 2001 the new entity "ALS/FTD" was suggested as a clinical diagnosis [33].

Most cognitive impairment in ALS is attributed to frontotemporal dysfunction. In FTD, which is a clinical diagnosis, the symptoms originate from degener-

ative change of the frontal and temporal lobes. There is also a histological diagnosis describing the decay of frontal and temporal parts of the brain called frontotemporal lobar degeneration (FTLD). FTD is divided into three categories: 1) Behavioral variant of FTD (bvFTD) which is characterized by prominent changes in personality, loss of empathy, and hypersexual behavior. 2) Semantic dementia, where an inability to match words with their meaning occur. Difficulties with naming objects and understanding certain spoken words become problematic; the motoric ability to speak and episodic memory is still intact. 3) Progressive non-fluent aphasia, which is associated with articulatory deficits like stutter and hesitant effortful speech, and ultimately muteness. Of these three, the behavioral variant is overrepresented in ALS [34]. Up to 30 % of the ALS patients are reported to have a mild to moderate cognitive impairment. Eight to fourteen percent of the patients meet the diagnosis criteria of bvFTD [35, 36]. A recent studies show that ALS patients with cognitive deficits and bvFTD patients without any motoric symptoms show similar cognitive deficits [37]. Supporting the above findings, a genetic overlap have been identified between ALS and FTD cases in a genome wide study [38]. Three loci were identified, two of them close to the C9ORF72 gene (described below) and one loci close to UNC13A, a gene involved in both ALS and FTD development. Together these data supports that ALS and bvFTD are similar diseases on a spectrum rather than of single entities.

Clinical investigation

There are no specific tests for the neurologist to pinpoint the ALS diagnosis. ALS is a therefore a diagnosis of exclusion. Several examinations and tests need to be performed to eliminate the possibility that the symptoms originate from other, maybe curable, diseases. The development of symptoms over time is also one of the cornerstones of the progressive ALS disease. Consequently, the diagnostic process often takes valuable time. The patient suffers from being in diagnostic limbo without a clear diagnose and prognosis. A late diagnosis might also worsen the prognosis since the opportunity to start with an early therapeutic intervention is lost. The effect of Riluzole, a disease-modifying treatment of ALS, is more efficient if treatment is initiated early [39]. In addition, early diagnosis makes enrolment to clinical trials easier [40, 41]. Most clinical papers report a 9-12 month delay in setting the diagnosis after the patient shows up at the clinic with the first symptom [42, 43].

For the clinical examination, both UMN and LMN symptoms need to be registered. UMN dysfunction typically gives rise to hyperreflexia, clonus (involuntary, rhythmic, muscular contractions and relaxations), spasticity, and normal reflexes in limbs that underwent muscle wasting [44]. This is due to loss of inhibition on the secondary motor neurons because of degeneration of the first cortical motor neurons. Reflexes specific for UMN are the plantar reflex, jaw jerk, Hoffmann's sign. Affection on the LMNs clinically gives symptoms like fasciculations

(small, local involuntary muscle contractions and relaxations) together with weakness and wasting of muscles [45]. Fasciculations occur due to loss of connections between the secondary motor neuron and the muscle. This leads to less ACh signaling, which in turn upregulates the ACh receptors on the muscle fibers causing hyperexcitability of the muscle, thus giving rise to fasciculations. For the bulbar symptoms the principle is the same, LMN dysfunction leads to wasting of tongue, weakness and fasciculations and UMN symptoms leads to spasticity.

The neurophysiological examinations usually performed are: 1) electromyography (EMG) giving information about LMN damage and to exclude disorders of peripheral nerves and muscles as a cause of symptoms; 2) electroneurography (ENoG) measuring the nerve conductivities, which are usually normal in ALS; 3) transcranial electrical motor evoked potentials (tcMEP) or the newer method transcranial magnetic stimulation (TMS) could give information about UMN involvement.

Blood samples and Cerebrospinal fluid are analyzed to exclude infectious, autoimmune and cancer disease as causes of symptoms. Imaging of the spinal cord and/or brain, depending on symptoms, is usually done with magnetic resonance imaging (MRI) to exclude structural deviations that mimic ALS such as syringomyelia, a fluid-filled cyst within the spinal cord.

Diagnostic criteria for ALS

A scientific-based ALS classification for research purposes was initially developed in 1994 in a magnificent palace-monastery in central Spain called El Escorial. The El Escorial criteria were developed by the World Federation of Neurology Research Group on Motor Neuron Diseases to make international ALS publications more comparable, and to establish a consensus in the scientific field as to which criteria should be used to diagnose ALS [46]. These criteria are commonly used also in clinical practice in lack of other specific tests or definitions. Also, International Classification of Diseases (ICD) moderated by WHO is commonly used in hospital coding systems.

According to the original El Escorial criteria, the diagnosis was based on the identification of UMN and LMN signs within body regions defined as bulbar, cervical, thoracic and lumbar part of the spinal cord. Four levels of diagnostic certainty were initially described: Definite, Probable, Possible and Suspected ALS. The criteria have since then been revised twice and termed Airlie House criteria (2000) [47] and Awaji-Shima criteria (2008) [48]. The former revision was trying to improve sensitivity by removing the Suspected ALS and adding" laboratory-supported probable ALS", and the latter removed that category again to include the recommendation to use electrophysiological data in the diagnosis. Today, for the definite ALS diagnosis, clinical or electrophysiological evidence of UMN and LMN signs in three of four body regions are required. Probable ALS includes UMN and LMN signs in two body regions, with some UMN signs rostral to the

LMN signs. Evidence of UMN and LMN signs in only one region, or UMN signs alone in two or more regions, or LMN signs rostral to UMN signs classifies as possible ALS [14]. The specificity has been improved for each revision, but still there is a problem with delayed diagnosis or misdiagnosis [49], thus leading to delayed medical therapy and perhaps providing bias to scientific studies.

Epidemiology

The incidence of ALS in Europe is 2.08, corresponding to an estimation of 15,335 individuals in Europe per year [50], and the incidence worldwide is 1.75 per 100.000 [51]. The incidence varies between different continents, and the reason for this is still unknown. It is estimated that the number of ALS cases worldwide is around 223,000, and the prevalence is increasing. In 2040 this number has increased to approximately 376,700 ALS patients [52]. The prevalence in Europe is 5.40 per 100.000 [50], which, compared to the incidence, is indicative of the poor prognosis of the disease.

ALS is generally more common in men by a factor between 1.2 and 1.5. Although in younger individuals the men:female ratio can be up to 2.5 [53]. The prevalence of the most common genetic mutations in ALS is, on the other hand, strangely higher in women by a factor of 1.16 [54]. The lifetime risk for developing the disease is 1:350 - 1:400 [55, 56].

Risk factors

Numerous potential risk factors have been investigated in ALS, but to date only three risk factors have been clearly associated with increased risk of ALS, i.e. older age, male gender and family history of ALS [50, 56, 57]. The peak age for ALS-disease is commonly reported as 75 years [55]. Old age is a common risk factor for many diseases and not just ALS. In ALS, several reasons for the increased incidence with age can be discussed. The autophagy-lysosomal and Ubiquitin-proteasome pathway, responsible for breaking down misfolded proteins, is impaired with advancing age, particularly in neurons [58]. Many changes in the immune system come with age. There is a gradual decline of the immune function called immunosenescence but also a gradual increase in pro-inflammatory status, a phenomenon called inflamm—aging [59]. The glial cells are of extreme importance in the neuroimmune system. Activation of both microglia and astrocytes can be observed in ALS, although it is still not clear if this prevents or worsens the disease course, or maybe both at different occasions in the evolvement of disease [60]? In Alzheimer's disease (AD) it has been proposed that inflamm-aging could act as a prodrome to the disease [61]. It has also been suggested that alternations in nuclear-cytoplasmic transport as well as impaired splicing could contribute to the higher incidence of ALS with age [62].

There is no clear explanation why men suffer a higher risk of developing the disease, and the familial connection is closely connected with genetic factors (reviewed below).

Many reports suggest that smoking could be of significance as a risk factor in ALS [63], although some studies fail to show a clear connection [64, 65]. Certain occupations have been reported to potentially increase the risk of ALS. Most of these professions involve exposure to chemicals, metals, pesticides and electromagnetic fields [66]; the mechanisms behind these findings are yet to be discovered. In ALS patients' examinations, increased lead in blood [67] and iron in ventral horn neurons of the spinal cord and in the motor cortex [68, 69] have been observed. Whether these are of importance for the initiation of the disease is still unknown.

In the early 1950s a high incidence of ALS and parkinsonism-dementia complex (PDC) on the island of Guam, in the Western Pacific, was observed [70, 71]. Parkinsonism-dementia complex of Guam was considered a novel disease with both Parkinsonism and dementia, but related to ALS due to similar histopathological findings. Later it was hypothesized that the atypical amino acid β-methylamino-L-alanine (BMAA) could be a part of this high incidence of neurological disease. High concentrations of the amino acid were found on the Micronesian plant *Cycas Micronesia*, which hosted the cyanobacteria that was responsible for the BMAA production [72]. Traditional feasts in the Chamorro culture on Guam included fruit bats as delicacy [73]. The seeds from this plant were a part of the fruit bats diet resulting in high concentrations of BMAA in the bat flesh. Amyotrophic lateral sclerosis and PDC were also detected in western New Guinea [74] and the Kii Peninsula of Japan [75], and was proposed to be an effect of increased BMAA exposure [76, 77]. In addition, cyanobacteria, also called green algae, are found in the Baltic Sea. During hot summers the amounts of the green algae explode due to algae bloom, vastly increasing the BMAA concentration in the water. This exposure was proposed to account for the higher incidence of ALS in the Nordic countries [78]. However, the suggestion of a causal relationship between BMAA and neurodegenerative disease has been highly criticized because the existing data on the subject are very sparse [79].

Metabolic diseases might also affect the development of the disease. A high frequency of the ALS patients are hyper-metabolic [80] and it seems like patients with type 2 diabetes, which is associated with obesity [81], have a lower incidence, thus indicating that high body mass index (BMI) might be a preventive factor. Patients with type 1 diabetes have a threefold increased risk of ALS [82]. The mechanism behind the altered risk of ALS in diabetes is still not known.

Genetics of ALS

The vast majority of the ALS patients do not have a family history of ALS. They develop the disease without any known relatives with motor neuron disease.

About 90-95 % of the ALS cases are classified as sporadic ALS (sALS). The resulting 5-10 % have a family history of the disease and are denoted familiar ALS (fALS). In most studies, the definition for fALS is a first and/or a second-degree relative with ALS, although there is no consensus in the field about this classification [83]. The definitions of sALS and fALS are quite poor and probably lead to a significant underrepresentation of the familial cases. Factors contributing to this could be: inadequate documentation, recessive inheritance, bad family contact, early death due to other causes, incomplete disease penetrance, misdiagnosis and illegitimacy [84]. A classification with three different stages of diagnostic certainty (possible, probable and definite fALS) has been described, but has not yet been implemented in the field [85]. It has also been suggested that fALS and sALS should be regarded as epidemiological classifications only, and a new definition, hereditary ALS (hALS), should be introduced to cover the cases with proven genetic etiology [86]. Around 90% of the fALS patients are offered genetic testing but only 50% of the sALS patients, which make this classification important not only for scientific purposes but also for the individual patient [83].

To date, 37 genes have been described, and four of these has been linked to both ALS and FTD [87] (and Peter Andersen, personal communication; August, 2018). In most affected families, the disease is inherited in an autosomal dominant fashion, although an X-linked [88] and autosomal receive inheritance occur [89-91].

The first gene to cause ALS was identified in 1993 by Rosen *et al* [92]. Since then, *SOD1* has been considered a key player in ALS genetics. Other than *SOD*1 mutations, which represents 15-30% of the fALS cases [93-95], the most frequent genes linked to ALS are chromosome 9 open reading frame 72 (*C9orf72*), transactive response DNA-binding protein (*TARDBP*) and *FUS* that represent 33.7-39.3% [95, 96], 4.1%-5.1% [87, 95], and 2.8-6.4% [95], respectively, of all fALS. The number of mutations varies between different continents with high amounts of *C9ORF72* mutations in Europe, whereas the amount of *FUS* mutations is higher in Asia compared to Europe. Mutations are also found in sALS patients. A recent study scanned 87 sporadic ALS for 31 ALS-associated genes and found that 17.2% of the patients had a probable genetic cause of ALS [97].

Transactive response DNA-binding protein 43 (TDP-43)

In 2006, neuronal inclusions of TDP-43 were found in brains and/or spinal cords of patients with ALS and FTLD [98, 99]. Two years later, the first mutation in a patient with solely a diagnosis ALS was identified [100]. TDP-43 is considered the most common isoform encoded by the *TARDBP* gene. TDP-43 is ubiquitously expressed and is primarily located in the nucleus. The protein plays a critical role in regulating RNA splicing and modulates microRNA [101, 102]. In neurons, TDP-43 is an important component of the dendritic RNA transport granules [103] and in the cytoplasmic stress granule response [104]. Stress granules, as the name implies,

form under conditions with cellular stress, such as oxidative stress or heat. They function as a protective mechanism by silencing mRNA, leaving only the most essential transcripts maintained in active translation [105]. It is not known how mutations in *TARDBP* contribute to ALS pathology. Suggestions involve loss of function [106], decreased binding capacity to other proteins due to hyperphosphorylation of the C-terminus [100, 107], cytoplasmic mislocalization [108], protein aggregation [109, 110], and altered TDP-43 proteostasis [111]. Most sporadic cases of TDP-43 proteinopathies in ALS show no mutation in *TARDBP*, which suggests that wild-type (wt) TDP-43 itself can cause the disease, or that TDP-43 accumulation is a secondary phenomenon. Typically neuronal cytoplasmic inclusions (NCI), skeins, round inclusions and colocalization with Bunina bodies in the spinal cord are seen in ALS-TDP. Many classification systems describing TDP-43 changes exists [112], the most common show four types of histopathological patterns in FTLD-TDP [113]. Various amounts of NCI, dystrophic neurites and neuronal intranuclear inclusions (NII) is divided into four classification groups. These patient groups also present with different clinical phenotypes [113]. The neuronal inclusions are most frequent in the spinal cord, motor nuclei, basal ganglia, hippocampus and frontal cortex [114].

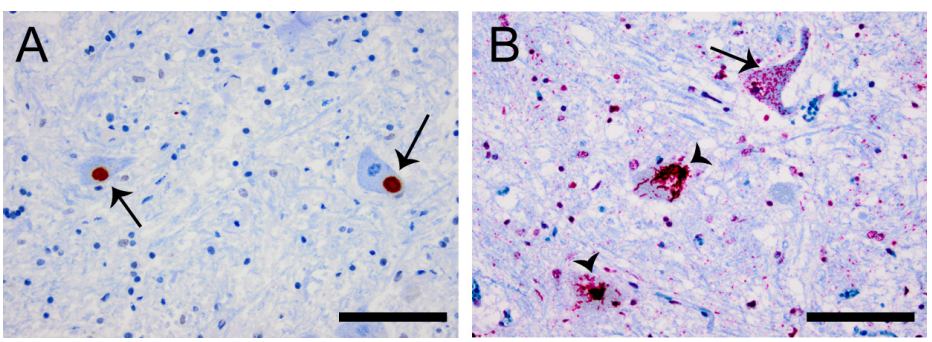

***Figure 3**: **Different types of TDP-43 inclusions in motor neurons located in the spinal cord of C9ORF72 patients with ALS. (A)** Round cytoplasmic inclusions in motor neurons located in the lumbar spinal cord; **(B)** Dot-like inclusions in the cytoplasm and nucleus (arrow), and skein-like inclusions in the cytoplasm in close proximity to the nucleus (arrowhead). Both slides were stained with anti-phospho TDP-43 (Ser409-Ser401) antibody. Staining in A was DAB and in B fast red. (Scale bar = 100 µm)*

Other than ALS and FTLD, immunohistochemical studies have shown abnormal accumulations of the protein in a wide range of different neurodegenerative diseases and other conditions [115]. The inclusions of TDP-43 are hyperphosphorylated, ubiquitinated and abnormally cleaved to generate short C-terminal fragments [116, 117]. An anti-phospo TDP-43 antibody is therefore used to distinguish aggregated TDP-43 from the native form in immunohistochemistry (Figure 3).

In ALS, Immunoblot characterization of the C-terminal part of the TDP-43 protein in different areas of the brain in different cases, proposes there are several distinct strains of the aggregates [118]. Some findings suggest that TDP-43 proteinopathies might exert a prion-like disease mechanism [119, 120]. However, it is unknown how and if the aggregates formed in TDP-43 proteinopathies exert a toxic effect, or are merely a byproduct of other neurotoxic processes. However, recent study showed that aggregates are enriched for components of the nuclear pore complex and nucleocytoplasmic transport machinery [121], which could be cell damaging due to depletion of these components.

Fused in sarcoma (FUS)

FUS or FUS/TLS (Fused in sarcoma/translocated in liposarcoma) was first identified as a chimeric oncoprotein in myxoid liposarcomas [122, 123]. In 2009, two papers linking mutations in *FUS* to ALS were simultaneously published in science [124, 125]. A few months later, FUS pathology was described also in FTLD [126, 127]. *FUS* is located on chromosome 16p11.2, and like *TDP-43* it encodes a multifactorial protein. FUS is involved in DNA repair [128], transcription regulation [129], pre-mRNA splicing [130], and mRNA processing [131-133]. In most cell types FUS is located in both the nucleus and cytoplasm. In neurons, a higher proportion of the protein is found in the nucleus, and in glia it is exclusively nuclear [134]. Most mutations found in this protein are located either in exons 3-6 or more C-terminally in exon 12-15 [135, 136]. The C-terminal part is involved in the nuclear localization of the protein. Mutations located in this part mediate redistribution of the FUS to the cytoplasm and is associated with ALS [137, 138]. Mutations in exon 3-6 appear more infrequent in ALS cases. This region in the N-terminal part is a prion-like domain [139] and bears a critical role in misfolding of FUS proteins [140]. As in TDP-43 proteinopathies, FUS also forms NCI in the brain and spinal cord of ALS and FTLD patients. Tangle like inclusions and round inclusions are most common, and depending on the aggressiveness of the disease, the pathologic picture in ALS-FUS patients can vary [141]. Unlike TDP-43, there has been no proof of FUS inclusions in sporadic patients [142]. In other words, a mutation in *FUS* seems to be required for FUS pathology.

Chromosome 9 Open Reading Frame 72 (C9ORF72)

The most common cause of fALS today is the insertion of an intronic GGGGCC (G4C2) repeat in Chromosome 9 Open Reading Frame 72 (*C9ORF72*). In 2011, two years after the linkage of FUS to ALS, the connection between *C9ORF72* and ALS was established. This time by back-to-back publication in neuron [143, 144]. Information that a locus on 9p21 was associated with ALS and FTD was already known in 2006 [145], but the specific disease causative gene was hard to identify. Most groups were initially searching for point mutations located in the exons and

did not expect an intron repeat to be the cause of the disease, which in turn delayed its discovery. Although repeat sequences are the cause of other neurodegenerative diseases like Huntington's disease [146] (CAG repeats in the huntingtin gene) and Spinocerebellar ataxia [147] (CAG or CTG repeats several different genes), these repeats are located in coding exons and not in introns.

Expansions in *C9ORF72* are not only the most common gene associated with fALS, but are also very common in familial FTD, accounting for about 25% of all cases [148]. Patients in families with this insertion present either with ALS, FTD or with both diseases in combination; this is defined as FTD-MND or ALS-d depending on if dementia or motor neuron symptoms appear first in the disease course [149, 150]. The normal G4C2 repeat size is variable, but in Europe two to ten repeats were reported in more than 90% of the population [143] and are considered normal. Repeat sizes seen in patients are generally several hundred or more commonly thousands of (G_4C_2) repeats [151]. The minimal amount of repeats causing disease is to date not determined [152], and the number of repeats differ between different tissues [153]. The mechanism of neurodegeneration in C9orf72 is unclear and there is no explanation for why some develop FTD and some ALS. However, three major hypotheses exist:

(1) Loss of function/haploinsufficiency – The function of C9ORF72 protein is not clear, it is suggested that it is involved in regulating endosomal trafficking and autophagy through Rab-GTPAse family proteins [154, 155]. Lower levels of the transcript were seen in patients with the repeat expansion, and knockdown of the C9ORF72 in zebrafish caused axonal degeneration [156]. However, knockout mice lacking C9ORF72 specifically in neurons failed to show a neurodegenerative phenotype [157, 158]. A recently published study suggests that loss of function together with a gain of function of the C9ORF72 is required for initiation of neurodegeneration [159].

(2) RNA toxicity – RNA foci were found in the neuronal nuclei of the frontal cortex using $(GGCCCC)_4$ RNA probes in fluorescent in situ hybridization (FISH) [144]. The theory is that long repeat RNA strands form G-quadruplex structures [160] that in turn sequester RNA binding proteins thereby impairing normal RNA homeostasis [161, 162]. The G_4C_2 RNA repeat is also translated into a potentially neurotoxic dipeptide-repeat (DPR) protein which by itself can mediate neurotoxicity [163].

(3) Protein overload - The G_4C_2 repeat is transcribed bidirectionally which is susceptible to an unusual type of translation – repeat-associated non-ATG/AUG (RAN) translation [164, 165]. Since translation is not initiated by the AUG codon, the repeat can be translated in all potential frames, both on sense and antisense strand, leading to five different DPRs (poly-GA, -GP, -GR, -PA, and –PR). These proteins can be toxic for neurons for many reasons, e.g. impaired

proteasome function and altered cellular transport and more (reviewed in [166]). NCI of DPR is mainly found in the hippocampal dentate gyrus and cerebellum. However, the distribution of DPR does not correlate with the clinical phenotype, suggesting that DPR is not the main pathomechanism in C9ORF72 pathogenesis [167, 168].

Other than the DPR pathology, TDP-43 positive inclusions and degeneration are found in the hippocampus, spinal cord, frontal cortex, basal ganglia, substantia nigra, and lower motor neuron nuclei in patients with the C9ORF72 mutation [169]. Patients with FTLD have more cortical pathology and patients with ALS diagnosis alone have more pathology in the spinal cord and brainstem [168]. Since the pathomechanisms in C9ORF72-ALS/FTLD are not known, developing therapeutic strategies is directed against both repeat RNA and DPRs. RNA repeats have recently been inhibited using induced pluripotent stem cell (IPSC) derived motor neurons and cortical neurons from C9ORF72 patients and fruit flies. Small molecules targeting RNA G-quadruples have recently been tested as a potential drug against the RNA toxicity [170]. A reduction of both RNA-foci and dipeptide repeat proteins were seen. Another strategy is antisense oligonucleotides (ASO) that target G_4C_2 RNAs specifically, which also reduces RNA foci, DPR proteins, and phenotypes in C9ORF72 transgenic mice [171] [172]. Inhibition of DPR protein production have also been performed through specific blocking of its translation process [173].

Other mutations in ALS are beyond the scope of this thesis. More information about this topic can be found in published reviews [84, 87].

Superoxide dismutase

Reactive oxygen species (ROS)

The movement of electrons is crucial for all life since this is a way of utilizing energy. Organisms constantly acquire electrons from the environment, which is used to drive energy production through the passage down reduction potentials. Molecular oxygen (O_2) is the perfect molecule to drive this reaction, and the presence of this molecule is essential for all human cells. The paradox is that although cells need O_2, it is also a substrate for cell damaging toxic oxide radicals. Reactive oxygen species (ROS) are generated during the O_2 mediated flux of electrons, which may damage vital cellular components. ROS are oxygen ions like superoxides ($O_2^{\bullet -}$) or oxygen-containing radicals like the hydroxyl molecule ($OH^{\bullet -}$) lacking one electron and are thus highly reactive with other atoms. Most superoxides in animal cells are produced in mitochondria during the electron transport chain [174]. The electron transport chain is needed to produce adenosine triphosphate (ATP), which is the cellular “energy currency”. Superoxides can also be formed through other cellular components, e.g. endoplasmic reticulum bound-enzymes,

and enzymes in the cytoplasm. ROS are important as signaling molecules and as promoters of inflammation, and they play a part in the progression of inflammatory disorders [175]. $O_2^{\bullet -}$ is a reactive compound, but in presence of H_2O_2 or NO it can form the even more oxidizing and toxic compounds $HO^{\bullet -}$ or $ONOO^-$. These highly reactive species can initiate lipid peroxidation and even more complex radicals [176].

ROS toxicity

How can ROS be toxic to cells and organs? ROS can oxidize lipids in a faulty manner, which in turn alters or impairs its function. For example, lipid peroxidation has been linked to cell death through effects on cell membrane components that further down activate inflammatory responses or apoptosis [177]. ROS also modifies DNA through oxidation, which leads to apoptosis or necroptosis [178]. Activation of transcription factors and NO signaling can be altered with high ROS concentrations. For example, a complication of long-term treatment with high levels of O_2 to preterm infants is retinopathy of prematurity. Vascular endothelial growth factor (VEGF), which stimulates vessel growth is inhibited. Together with deficiency of antioxidants, the child might become blind due to excessive amounts of ROS [179]. Also, protein oxidation and nitrosylation can impair a wide range of cellular functions through the loss of protein structural integrity or interruption of regulatory pathways [180]

The major cellular defense against toxic $O_2^{\bullet -}$ is a group of oxidoreductases known as SODs that catalyze the dismutation of oxygen radicals into oxygen and hydrogen peroxide. There are three forms of SOD1 proteins in mammals [181].

SOD1

SOD1 was first described in the late 1940s and was named haemocuprin, since it was identified as a copper-binding protein and purified from bovine erythrocytes [182]. Similar proteins, now called cupreins, with the same molecular weight and copper content were purified from the brain and erythrocytes over the next decades [183, 184]. In 1964, it was suspected that all different cupreins actually were the same protein [185], and in 1969 it was shown that cupreins have the enzymatic function to break down oxygen radicals [186]. From here on, the cupreins were named CuZn superoxide dismutase.

SOD1 is present in almost all eukaryotes and is ubiquitously expressed. The protein is mainly localized in the cytosol, with a smaller fraction in the intermembrane space of mitochondria. The highest expression of both the protein and the RNA is found in the liver. Notably, relatively low amounts of SOD1 are found in muscle tissue and the CNS, the tissues involved in SOD1 related ALS disease [187, 188]. Similar distributions are also found in mice transgenic for human SOD1 [189]. SOD1 also make a great contribution to the total pool of cellular proteins. In the gray matter of the brain, SOD1 represent 0.2% of all proteins [188]. In the spinal

cord, the concentration of SOD1 is around 5-6 µg / g wet weight in a healthy individual [190].

The main function of SOD1 is the dismutation of superoxide radicals following the reactions:

1) $SOD1\text{-}Cu^{2+} + O_2^{\bullet -} \rightarrow SOD1\text{-}Cu^{+} + O_2$
2) $SOD1\text{-}Cu^{+} + O_2^{\bullet -} + 2H^{+} \rightarrow SOD1\text{-}Cu^{2+} + H_2O_2$

With the net reaction:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^{+} \rightarrow O_2 + H_2O_2$$

Through this reaction, the protein protects the cellular environment against reactive oxygen species.

SOD2

SOD2 or MnSOD is a 96 kDa homotetrameric protein containing manganese in the active site that catalyzes the same reaction as shown for SOD1. SOD2 is localized in the mitochondrial matrix, and thus it is the primary defense against oxidative phosphorylation byproducts from the electron transport chain [191, 192].

SOD3

SOD3 (extracellular SOD), as in SOD1, also contain Copper and Zinc, but are an extracellular secretory enzyme [193]. SOD3 is a 135 kDa homotetramer composed of two disulfide-linked dimers [194]. The primary location of SOD3 is in the extracellular matrix and on cell surfaces, and a smaller fraction is found in plasma and extracellular fluids. It is thus the first line of defense against ROS in the extracellular space.

SOD1 structure

Mature SOD1 is a stable homodimer with a molecular weight of 32-kDa, and each monomer is composed of 153 amino acids [195, 196] (figure 4). The dimer interface is held together primarily by hydrophobic contacts. The 3D structure of bovine SOD1 was determined in 1982[197]. This structure was confirmed in 1992 with x-ray crystallography where the human SOD1 molecule was deciphered for the first time [198]. The monomer is folded into an eight-stranded Greek-key β-barrel conformer and the antiparallel β-sheets are connected by seven loops [197] (figure 5). Each monomer contains one copper ion that is essential for enzymatic function and one Zinc ion that is of importance for protein stability [199]. The active site makes up around 10% of the total surface area of the monomer [200]. It is formed as a channel consisting of 18 residues from the zinc-binding (IV) and electrostatic loops (VII), the copper ion represent the floor of the channel [201]. The copper is

coordinated by His_{46}, His_{48}, His_{63} and His_{120}, and the Zinc site is buried in the protein and is coordinated via histidine ligands 63, 71 and 80 and one aspartate residue located on position 83, which is a part of a β-barrel [197]. Zinc stabilizes the enzyme by keeping loop IV in place to form the active site. It also increases the redox potential of the catalytic Cu^{2+} ion [199]. An intrasubunit disulfide bond between Cys_{57} and Cys_{146}, present in all eukaryotes, stabilizes the protein. These types of bonds are unusual in proteins that are primarily cytosolic, such as SOD1, since the cytosol is strongly reducing. This favors Cys-H bonds instead of Cys-Cys. Nevertheless, the disulfide bond binds loop IV covalently. If the metals are not ligated properly, the cysteines will be reduced, and the loop detached, which in turn contributes to SOD1 monomerization [202, 203].

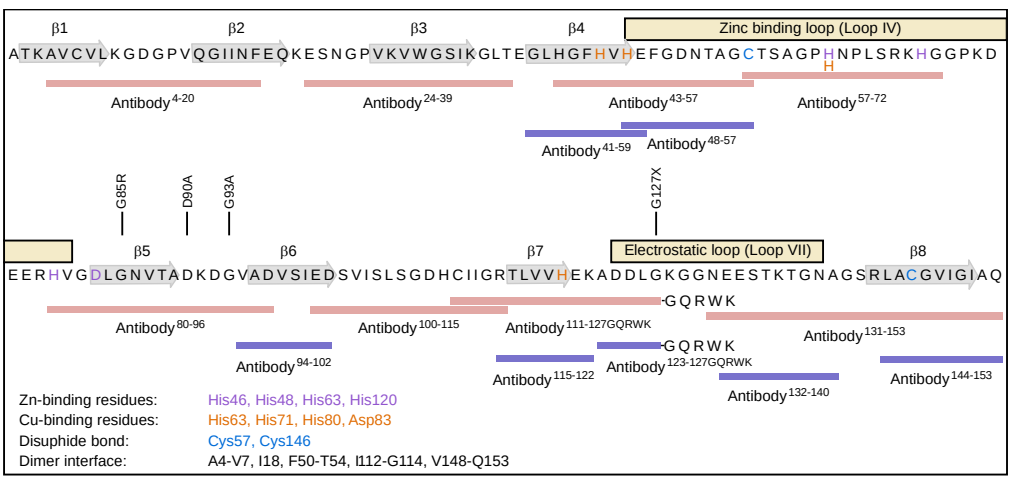

Figure 4. The amino acid sequence and key structural elements of human SOD1. *Structural elements are color-coded: The β-sheets are grey arrows. The zinc and copper binding residues are highlighted as purple and orange, respectively, in the amino acid sequence. Cysteines involved in a disulfide bond are blue. Specific mutations investigated in the thesis are shown. All anti-peptide antibodies directed against SOD1 are marked as boxes. Antibodies used in paper I were 24-39, 57-72 and 131-153; antibodies used in papers II and III were brown and in paper IV blue antibodies were used.*

SOD1 activation and stability

The mature, homodimeric and fully metalated SOD1 protein (holo-SOD1) is a very stable protein with a melting temperature of 92°C. The enzymatic activity is also preserved under strongly denaturant conditions such as 10 M urea and 4% sodium dodecyl sulfate (SDS) [199]. Protein thermal stability is coupled with high lysine residue content and high proportion of β-sheet structures, whilst enrichment of α-helical structures is associated with unstable proteins [204, 205]. SOD1

has 11 lysine residues which is 7.2% of the total amino acid sequence. An analysis of 550,000 proteins showed that the average lysine content for a 150 aa (amino acid) long protein is around 5.5% lysine [206]. Thus, the content of lysine in SOD1 might contribute to its stability. Charged amino acids like lysine increase the internal salt bridges, which is thought to be a factor for keeping the structural integrity of a protein [207]. SOD1 also contains a high proportion of β-sheets enhancing its structural integrity. In addition, the marked stability and activity of the protein are dependent on a series of posttranslational modifications. These maturation steps are in sequence, Zinc and Cupper binding, disulfide bond formation and dimerization. Disruption of any of these steps will result in an unstable, inactive protein. The maturation steps are described below:

1. Zinc insertion

The mechanism of how zinc is inserted into SOD1 is still not clear, although it seems to be independent of Ccs1 (copper chaperone for superoxide dismutase 1), a metalloprotein involved in SOD1 maturation [208]. The addition of zinc is the first maturation step for SOD1 [208, 209].

2. Copper insertion

When zinc is bound to the SOD1 enzyme, Ccs1 is able to dimerize with SOD1. After heterodimer formation, Cu^{1+} is transferred from domain 1 in Ccs1 to SOD1 [210, 211]. The importance of the Ccs1 dependent addition of copper to SOD1 is still under debate since certain organisms, such as *C.elegans* are lacking the Ccs1 protein and still have a fully active SOD1 protein [212]. Human SOD1 has been activated, to a lesser degree than normal, in the absence of Ccs1 through glutathione-dependent delivery of copper [213]. However, multiple studies have shown that deletion of Ccs1 results in a defect SOD1 maturation, which reflects its importance for SOD1 activation [213-215]

3. Disulfide bond formation

Before the disruption of the SOD1-Ccs1 heterodimer a disulfide bond between Cys_{57} and Cys_{146} is introduced. This disulfide bond is formed through oxidation and the involvement of Ccs1 has been shown both *in vitro* and *in vivo* [210, 216, 217].

4. Dimerization

As a fourth and last step of SOD1 maturation, two fully metalated and disulfide oxidized monomers form a homodimer, thereby activating the protein and further increasing the protein stability.

Reduced apo-SOD1 (without the copper and zinc residues) favors a monomeric state, whilst the addition of either zinc or disulfide bond favors the homodimer [218]. It is likely that the monomer undergoes folding before it forms a homodimer. The zinc-binding loop (IV) is a part of the dimer interface (residues 50-54) and is anchored to a β-barrel by the C57-C146 disulfide bond. Disruption of the disulfide bond might thus favor monomerization by increasing the loop dynamics [219]. Removal of loop IV and loop VII provokes Apo-SOD1 to monomerize [220]. Taken together, data show that dimerization occurs late in the SOD1 maturation process.

Apart from the above-described mechanisms, the maturation process is also dependent on oxygen. *In vitro* studies have shown that SOD1 maturation is impaired with O_2 removal, probably through a Ccs1 dependent mechanism [221, 222].

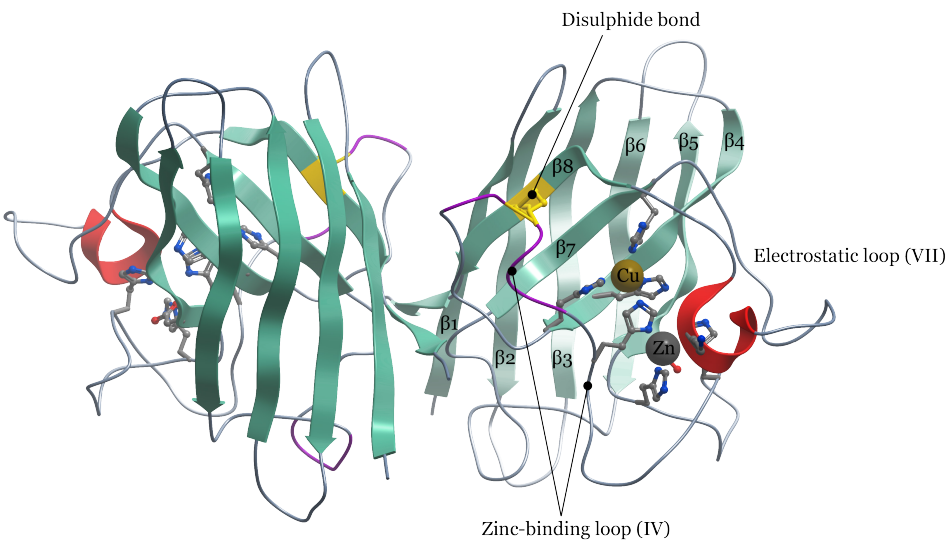

Figure 5. Tertiary crystal structure of dimeric human SOD1*. The model shows the native holo-SOD1 dimeric molecule. B-sheets are colored green, loops are gray, the α-helixes are red and the disulfide bond between Cys57 and Cys146 are colored yellow. Copper and zinc ions are shown as dark yellow and gray spheres, respectively. Metal binding histidines are shown as blue colored pentagons and the aspartic acid are shown as a small red line. The figure was generated with MolSoft Browser (San Diego, CA), using the pdb code 1HL5. Adapted from Işil Keskin (2016)[223], © Işil Keskin. Used with permission.*

Incubation of SOD1 under anaerobic conditions leads to loss of enzymatic activity [224]. There is still not much data concerning cerebral/spinal hypoxia and SOD1 misfolding. Interestingly, in cell culture with ALS patient-derived motor neurons, low oxygen tension does induce misfolding and aggregation of SOD1 [223]. On the contrary, a recent study investigated protein aggregation induced by cerebral ischemia in mice and found an increased aggregation of ALS-associated proteins like TDP-43 and FUS amongst many others. SOD1 aggregation did not seem to be augmented [225]. The SOD1 protein might even itself be a target for oxidation. Oxidative damage of SOD1 has been observed in ALS model mice [226]. The wt-SOD1 was shown to monomerize prior to aggregation due to oxidative damage, implying that oxidation might be an early step in SOD1 pathology [227].

SOD1 in ALS

Since 1874 when Charcot stated the original disease description, the most important breakthrough in ALS research was published in 1993. Mutations in the enzyme Cu/Sn Superoxide Dismutase located on chromosome 21 (21q22.1-22.2) were found clearly associated with the neurodegenerative disease [92]. This discovery provided the first hint of a genetic cause of ALS. Since then more than 28 genes have been directly linked to the disease.

To date, more than 210 ALS-associated SOD1 mutations have been found and located in all five exons (http://alsod.iop.kcl.ac.uk/ [228] and Peter Andersen, personal communication; August, 2018). However, every single mutation might not be clearly pathogenic [229], or might need an additional pathogenic mutation in order to develop disease [230]. SOD1 mutations are found in 2-6% of all ALS patients, in 15-25% of all fALS cases, and in 0-7% of the sALS patients [94, 231]. The majority of the *SOD1* mutations are missense mutations [232], but some are nonsense mutations that introduce a premature stop codon making the protein shorter than normal [233]. Frameshift mutations caused by deletions or insertions have been found, also resulting in a shorter, truncated protein [234]. Most SOD1 mutations are inherited in a dominant fashion, but one recessive mutation where aspartic acid in position 90 is changed to alanine (D90A) have been found [235]. The D90A mutation arose from a single ancient founder [236] and is associated with marked longer survival than that for the general ALS patient (13 years compared to 2-4 years) [237]. In the United States, the most common mutation is A4V that results in an aggressive form of ALS with a survival time of less than two years after disease onset [238].

So how do mutations in SOD1 cause ALS? This is a very complex question that with today's knowledge is impossible to fully answer. Although a lot of research progress is being made, providing more and more data giving the information to formulate at least a partial response to the question.

When SOD1 was first linked to ALS many suggested that a loss of protein function was the neurotoxic mechanism. It was, after all, logical to think that when

such an important antioxidant is mutated, it would be a problem with oxidative stress, resulting in cell damage and neurodegeneration [239, 240]. However, several lines of evidence now show that this is not the case. For example, SOD1 knockout mice do not develop a motor phenotype [241], but transgenic overexpression of SOD1 does, although the enzyme activity is still close to normal [242, 243]. In patients, some SOD1 mutations retain full enzyme activity and still cause ALS disease [244, 245]. It is currently accepted that SOD1 mutations cause a gain of toxic function, rather than a loss of function, although it has been proposed that a loss of function may also play a secondary role in some cases [246].

Mutations in SOD1 reduce the thermal stability of the protein, which in turn makes it more prone to misfolding [247]. The stability of the protein is perturbed in many ways, i.e. by destabilization of the precursor monomers or by weakening of the dimer interface [248], by destabilization of the apo and reduced form of the protein [249], by weakening of the dimer interface that causes monomerization of the protein [250, 251], by reduction of the net repulsive charge, thus decreasing solubility and increasing aggregation propensity [252], or by truncating local conserved salt-links or H-bond networks [253]. Reduced stability of a protein (such as SOD1), in combination with external stress conditions and poor elimination of protein waste, ultimately lead to protein aggregation [254], which today is thought to be the major cause of several neurodegenerative diseases including ALS [255].

Protein aggregation and amyloid formation

Proteins can undergo low energy structures, in addition to its native fold. These structures often organize into aggregates that can exert toxic damage to cells. The low energy structures are often referred to as misfolded proteins. The misfolded proteins are monomeric, associated with loss of function, and are often involved in human disease [256]. Misfolded proteins can bind to each other and form aggregates. There are in fact over 50 disorders that are associated with the misfolding of proteins and aggregate formation [257]. Many neurodegenerative diseases like Alzheimer's (AD), Parkinson's (PD) and Prion diseases form amyloid, a filamentous fibril around 10 nm in width and 0.1-10μm in length [258].

There are two definitions of amyloid. The classic histopathological definition is an extracellular proteinaceous deposit that when stained with the β-sheet binding compound Congo red showed an apple-green birefringence when examined in a microscope with polarized light [259]. A recent more liberal definition is the biophysical description of an amyloid that defines it based on its molecular structure – polypeptide aggregates with a cross-β conformation [258]. With this definition amyloid is best explained as a specific type of aggregate where β-sheets are ordered to give rise to a fibrillar organization. Both aggregates and amyloid have a β-sheet core, and the proportion of β-sheets in an amyloid protein is usually high. The β-sheets are hydrophobic, and in order to reach the lowest global free

energy of the protein, they tightly pack into a growing fibril by intermolecular hydrogen bonds. The Amyloid is actually often the most stable/low energetic conformation a protein can undergo [260]. To illustrate the logic of low free energy, imagine a protein like a cluster of magnets with negative and positive charges on each side attached on a lace. A protein "strives for" having as few repulsive charges (minus-minus) and as many attractive forces (minus-plus) as possible. Proteins with low energy, or amyloid, have found the structure with the most minus to plus bindings as possible, with respect to the lace (protein backbone) elasticity.

So, if aggregated proteins and amyloids have lower energy than proteins in the native state, why do not all proteins aggregate into big clusters of inactive enzymes? First, when proteins are produced by the ribosome, the protein is already starting its folding process to a three-dimensional conformation. Posttranslational modifications like glycosylation, phosphorylation and acetylation then occur. After that, the binding of additional subunits (like in the case of SOD1, zinc and copper insertion) is executed. This process is sometimes driven by chaperones, like Ccs1. Chaperones can also help to refold already misfolded proteins back to their native folded state. If refolding is not possible, the protein is usually recognized as misfolded, and is marked for degradation. If any part of this machinery works incorrectly, the risk of protein aggregation is raised. In conclusion, the posttranslational modifications, in combination with intramolecular interactions between the amino acids, fold the protein in the lowest energy level possible for the given conditions. If the conditions change, e.g. removing a zinc from SOD1, the amyloid form is favored rather than the native state of the protein.

The stability of amyloid is dependent on protein concentration. The more monomers available from a single protein, the more the amyloid state is favored. There is a critical value where the native state and the amyloid state have the same amount of free energy, and therefore the same stability. At monomer concentrations exceeding this value, only the energy needed for structure conversion can hinder the protein from transforming into amyloid; the protein becomes kinetically metastable [260]. Applying energy like heat or oxidative stress might convert the native protein to amyloid. Amyloid formation typically displays sigmoidal growth kinetics [261] (Figure 6). The curve is typically divided into three phases – lag, growth and a final plateau phase. In the lag phase, the aggregation process is starting to initiate, and the formation of fibrils is rather slow. In this phase, the smallest aggregates called nuclei that are stable enough to further grow by addition of monomers are formed [261]. In some literature, the lag phase has been misinterpreted as a "waiting time for the nucleus to form". This is not the case. As soon as nuclei are formed, the addition of monomers on each end of the nuclei starts and the elongation of the fibril begins [261].

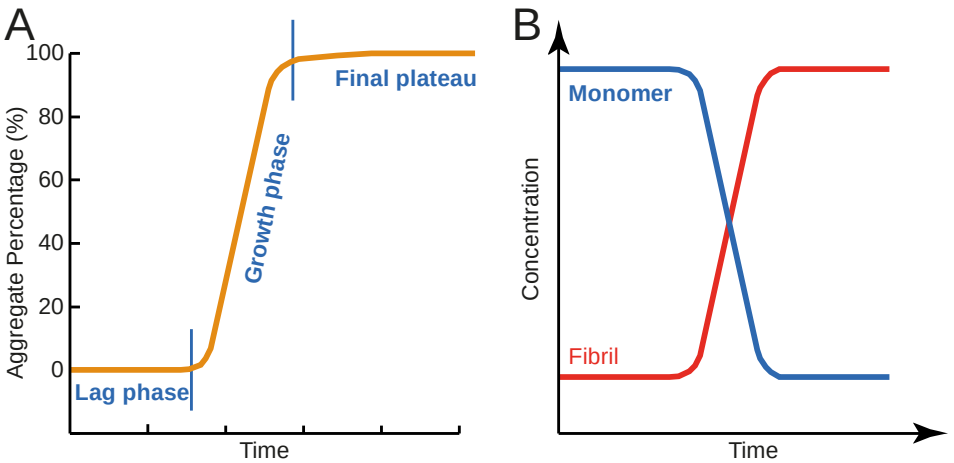

Figure 6. Schematic figure of fibril formation. (A) *A characteristic sigmoidal curve displaying the three phases of fibril formation, lag phase, growth/elongation phase and the final plateau. The fibril concentration is displayed as the aggregate concentration in relation to monomer percentage;* ***(B)*** *Monomer concentration and fibril concentration over time during fibril formation. Over time the curve flattens, an equilibrium between the monomer and fibril concentrations has been reached. Figure adapted from Arosio, P., et al, 2015* [261] *– Published by the PCCP Owner Societies, used with permission*

The next phase is called the growth phase and is characterized by rapid fibril formation. Here the monomers and fibrils are almost in equilibrium. The fibril thus grows through the formation of a nuclei and a primary elongation of these in each end of the fibril. The fibrils can also grow through secondary nucleation where monomers attach to the surface of the fibril to form new nuclei of the same structure. This is separated from elongation, where the monomers only attach to each end of the fibril. When the fibers become too long and fragile, they break. One long fibril becomes two smaller, and two ends become four. This process is called fragmentation, and further speeds up fibril formation. When the monomer quantity runs out, there is no longer raw material for further growth of the fibril. The plateau phase is reached, and the solution is now dominated by fibrils [262].

ALS is defined as a proteinopathy but is not characterized as a classical amyloid disease, at least not with the histopathological definition. Using amyloid dyes, no aggregates has yet been identified in ALS patients [263]. However, SOD1 fibrils are still formed *in vitro*, and recent publications state that SOD1 aggregates are actual amyloids, using the biophysical definition [264, 265]. Nevertheless, the mechanism by which amyloids are formed could potentially provide valuable information about the SOD1 aggregate formation.

Mouse models in ALS

Scientific data from diseased ALS patients are crucial for investigating the disease. Since it is not possible to take biopsies during the disease course of ALS, all biochemical and histopathological investigations are restricted to end-stage patients. This is not enough to explain the background mechanisms for the pathogenesis of the disease. To get more information about the disease development and to be able to manipulate the system to find disease provocative or inhibiting factors, model systems are key for the ability to perform such experiments. Many different ALS models, focusing on different disease-mediating proteins, are available today (reviewed by Van Damme *et al* [266]). In this thesis, the mouse is utilized as a model system for ALS, and this model is reviewed below.

SOD1 mouse models

Shortly after the discovery that mutations in SOD1 could lead to ALS, the first mouse model overexpressing mutant SOD1 was created. The animal model developed motor neuron disease and paralysis in one or more limbs due to motor neuron loss and died after 5-6 months. This model had around 18 copies of human *SOD1* (hSOD1) with a point mutation in amino acid 93 changing Glycine to Alanine (G93A) [267]. This specific SOD1^{G93A} model (Line Gur1, G1) overexpresses hSOD1^{G93A} mRNA 40 times over the murine SOD1, which is normally expressed in mice. The hSOD1^{G93A} protein is expressed proportionally 17 times more than the murine SOD1 protein. There is a strong gene-dosage effect where higher SOD1 expression leads to a more aggressive disease course. For example, Line GurG5 mice with only 4 gene copies did not develop disease [267]. Also, spontaneous loss of copy number in G1 has been shown to extend the survival in a dose-dependent manner [268]. The fact that a transgenic mouse can lose its gene copies has been used to develop new lines of the hSOD1^{G93A} transgenic mice, with later onset and prolonged survival [269]. Copy number dependency is also present in transgenic mice with other SOD1 mutations. The phenotypes are more severe and the disease progression is much faster in mice that have been bred to homozygosity compared to hemizygous mice [270-272]; this is also shown in the first article in this thesis (paper I) .

One of the important advantages for the SOD1 transgenic mice is that their phenotype closely resembles human ALS disease. The SOD1 mouse model is often considered one of the most accurate animal models of all neurodegenerative diseases. The hSOD1^{G93A} mice on C57BL/6 background show progressive hind limb weakness after 120 days and develop terminal disease around 145 days [273], which replicates in a unique way the disease process in the patients, with relatively late disease onset of paresis and a rapid disease course. An even better model with regards to disease onset and protein expression is the SOD1^{G85R} model, where initial symptoms are registered around 330-340 days [274], and the expression of hSOD1 is in level of the murine SOD1 expression [275]. The SOD1 mice also develop

histopathological changes that were also found in ALS patients with SOD1 mutations; Protein aggregates of SOD1 and ubiquitin are detected in motor neurons, prior to symptom onset. Lewy body-like inclusions in both glial cells, neuronal processes and in cell bodies, as well as reactive gliosis occur in both mice and humans with ALS. Axonal and mitochondrial dysfunction and motor neuron loss are also observed in the two [275-277].

The aggregates in the G93A mice are filamentous and about 10-15 nm in diameter, similar to that found *in vitro*. However, the structure of the filaments produced in transgenic mice varies depending on the hSOD1 mutation type [278]. In humans, the G93A mutation was first observed in 1993 [92], and a report of the clinical patient characteristics was published in 2010 [279]. The disease course in this German pedigree carrying the mutation closely resembled the typical phenotype of sALS in terms of site of onset, progression, disease duration and cause of death [279]. The mutation presents as an autosomal dominant trait with high penetrance. The G93A mutation is uncommon in humans and no histopathological examination has been performed to date. Since the G93A mouse was the first transgenic overexpression model for mutated hSOD1, with relatively fast disease onset, it is the most used murine hSOD1 model in the ALS field (a pubmed search on “ALS AND transgenic mouse AND G93A” revealed 1063 hits, whilst G85R had solely 62). Numerous publications over the years have investigated apoptosis, axonal transport, protein properties, inflammation, etc. [280]. Despite this, it is under debate to which extent this model reflects human ALS disease [281].

The mutant SOD1 mouse is, in general, a good model for investigation of ALS disease. Although marked overexpression of hSOD1 in many transgenes, which is not seen in humans, might cause phenotypes that are not relevant for the human disease. For example, high expression rates of hSOD1 cause overloading of the protein in the mitochondria, which in turn might be the reason for the mitochondrial swellings and vacuolar pathology found in D90A, G93A and wt-hSOD1 transgenic mice [282, 283]. Another hard point is that although new therapeutic targets show good effects in prolonging lifespan in the SOD1 murine model, they might not be as beneficial when transferred to clinical studies [281, 284]. This problem do not occur solely in the hSOD1 mice but also in models of AD and PD [285]. Looking past the neurodegenerative field, only a third of the animal studies with a preclinical effect translates to a similar outcome in humans [286]. Over 20 compounds have reached clinical trials in ALS, and only two (Riluzole and Edaravone) are currently approved as a mildly efficacious treatment [287, 288]. A famous example of negative results in humans is the trial with minocycline, an antibiotic with anti-apoptotic and anti-inflammatory effects *in vitro* [289]. This drug showed effects on disease progression and prolonged survival in four separate ALS mouse studies in 2002-2003 [290-293]. Four years later, a phase III clinical trial with more than 400 patients showed that rather than prolonging life, minocycline worsened the symptoms and also showed tendencies of increased mortality during the 9-month long trial [294]. To minimize this kind of failing clinical trials, the preclinical

trials need to improve in study designs with blinded and randomized groups, and also a sufficient number of animals in each study group is required [284, 295].

Mechanisms of SOD1 toxicity

Twenty-four years have passed since SOD1 was coupled with ALS, and the consensus on main toxicity of mutant SOD1 has yet to emerge. Several mechanisms of neurodegeneration and motor neuron death have been proposed and are briefly discussed below.

Excitotoxicity

One of the earliest proposed pathogenic mechanisms of ALS was glutamate excitotoxicity. Defects in the clearance of extracellular glutamate were proposed even before the identification of SOD1 as a causative gene [296]. Glutamate is the main excitatory neurotransmitter of the CNS. It activates the target cell by opening the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors, allowing extracellular Ca^{2+} to enter the cell. Excessive cellular exposure of glutamate will promote a Ca^{2+} overload causing repetitive firing of action potentials, resulting in toxic events such as mitochondrial damage, which in turn results in the formation of ROS and eventually apoptosis [297].

Astrocytes are the principal cells in CNS that are responsible for removal of excessive glutamate. This function is mediated by glutamate transporter 1 (GLT1) also known as excitatory amino acid transporter-2 (EAAT2), which transports glutamate into the astrocyte. The loss of EAAT2 has been observed in SOD1 mutant rodent models of ALS [275, 298] and also in fALS patients [299]. However, there are conflicting results in the treatment efficacy of EAAT2 level restoration in murine models with ALS disease [300, 301].

Astrocytes are also involved in upregulation of the glutamate receptor-2 (GluR-2) subunit located in AMPA receptors found on motor neurons. This upregulation reduces the permeability of calcium and thereby protects against excitotoxicity. Astrocytes that express mutant SOD1 fail to regulate GluR-2 expression *in vitro* and thereby increases the motor neurons vulnerability to excitotoxic damage [302]. In contrast, there are evidence for an increased glutamate efflux from spinal cord nerve terminals in the $hSOD1^{G93A}$ mice [303], speaking in favor of a higher neuronal glutamate release rather than failure of astrocytes to remove the excessive glutamate.

Maybe the main evidence for excitotoxicity in ALS in general is that the antiglutamatergic drug Rilutek® (Riluzole) slows the disease progression of ALS [304], probably by lowering the synaptic glutamate levels and by inhibiting glutamate release [305].

Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an organelle involved in cellular homeostasis and protein quality control. ER stress occurs when misfolded proteins accumulate in the ER lumen due to an overload of newly synthesized polypeptides or disruption of protein quality control. The unfolded protein response (UPR) is activated by the accumulation of misfolded proteins in the ER, thus inducing several adaptive responses to restore the proteostasis and reduce the number of proteins with aberrant confirmation. Failure in the UPR or prolonged ER stress induces apoptotic cell death [306]. Unfolded proteins in the ER are retained until they are accurately folded. If the appropriate confirmation cannot be achieved, the misfolded proteins are subjected to ubiquitination and proteasome-dependent degradation in the cytosol, known as ER-associated degradation (ERAD), a process that is also upregulated by UPR [306].

ER stress have been associated with several neurodegenerative diseases such as Alzheimer's [307], Parkinson's [308] and ALS [309]. ER stress has been suggested to be involved in sporadic ALS [310] and murine transgenic mouse models with a hSOD1 mutation [311, 312]. Mutant SOD1 can trigger dysfunction of ERAD through interaction with Derlin-1, a protein of the ERAD machinery, and thereby cause ER-stress [313]. UPR activation has been observed in transgenic hSOD1 mutant mice at end-stage disease [310]. Moreover, treatment of hSOD1 mice with ER stress-protective agents delay disease progression, and aggravation of ER stress promotes disease [314].

Disruption of axonal transport

In ALS both the upper and lower motor neurons are affected. The motor neuron soma or cell body is located in the motor cortex or brainstem/spinal cord for upper respective lower motor neurons, and the communication between these two is dependent on a functional transport of component from the soma to the synapse in the axonal end. Axons can reach more than one meter in length and comprise the vast majority of the total cell body. Mutant SOD1 has been demonstrated to slow both anterograde and retrograde transport, before any signs of neurodegeneration [315, 316]. In addition, mutated SOD1 has been proposed to shift survival-promoting to death-promoting retrograde signaling [316]. Genetic analysis of a family with slowly progressing motor neuron disease identified a mutation in dynactin, a protein required for dynein-mediated retrograde transport of vesicles and organelles along microtubule [317]. Fast axonal transport (FAT) from the cell body to the neuromuscular junction is mediated through Kinesin, a microtubule-associated transport protein. This anterograde transport is inhibited indirectly through mutant SOD1 polypeptides [318]. Anterograde FAT was also affected by

oxidized wt-hSOD1 as well as in sALS patient-derived wt SOD1, suggesting that SOD1 driven FAT inhibition also occurs in sALS patients [319].

Oxidative stress and mitochondrial damage

Oxidative stress occurs when the amount of ROS generated by aerobic metabolism are higher than the cells ability to remove these toxic species. An imbalance in ROS and antioxidant system leads to oxidative damage to various biomolecules, such as proteins, lipids, and DNA. The mitochondria are the most important organelles for cellular energy production. Energy is stored through phosphorylation of adenosine, to create ATP. Oxidative phosphorylation in the mitochondria is the major source of ROS, and dysfunction of this organelle elevates ROS indirectly [320].

In histopathological investigations of the first hSOD1^{G93A} mice [283], vacuoles in the ventral horns were identified. These vacuoles were later shown to be derived from degenerating mitochondria [321, 322], by SOD1 linked leakage of the outer mitochondrial membrane, and expansion of the intermembrane space [323]. Since then, several reports from other transgenic hSOD1 lines have been shown with similar vacuolar pathology [189, 242, 324]. The relevance of mitochondrial vacuolization for human disease has been questioned for several reasons. Firstly, mitochondrial pathology is rarely seen in human ALS, but have been observed on the odd occasion both in sALS [325] and fALS [326] cases. Secondly, not all SOD1 transgenic mice develop vacuolar pathology. Thirdly, transgenic mice expressing mutated hSOD1 in lower levels, such as G85R, G127X, and H46R, and mice with low expressions of G93A show no vacuolar pathology but still develop ALS phenotypes [189, 275, 283, 327]. Fourthly, mice expressing high levels of mutated SOD1 have a fourfold increase of SOD1 in mitochondria compared to low level expressing mice. Yet, the mice expressing low levels of hSOD1 still develop a deadly motor neuron disease. This indicates an artificial SOD1 overload due to SOD1 overexpression [282]. Although vacuolization seems to be an artifact due to hSOD1 overexpression, other signs of mitochondrial damage are seen in low hSOD1 expressing mice. Mitochondrial calcium loading capacity is decreased early in the disease course in neural tissue but not in the liver [328]. Structural alterations of mitochondria have been observed in hSOD1^{H46R} mice, as well as changes in protein expression indicative of oxidative stress and involvement of the mitochondrial machinery [329].

Oxidative damage was found to be elevated in *post-mortem* tissues of sALS and fALS patients [330, 331], as well as in transgenic hSOD1 mutant mice [332]. SOD1 is an antioxidant protein, and although loss of enzymatic activity does not result

in ALS it is suggested that a loss of function could play a modifying role in the disease [333].

Non-cell-autonomous toxicity

The importance of glial cells in motor neuron degeneration emerged from studies where the expression of mutant hSOD1 was silenced in microglia, astrocytes or oligodendrocytes. Transgenic mice expressing mutant hSOD1 in all cells but glial cells, failed to develop disease [334, 335]. In a later study mutant hSOD1 was expressed solely in neurons, which generated disease only in mice with a high level of hSOD1 expression, and only at very late ages [336]. Disease-modifying silencing of hSOD1 expression showed that expression of hSOD1 within motor neurons is important in early disease, whilst microglial hSOD1 expression is more involved in later disease progression [337, 338]. Replacing hSOD1 expressing microglia with normal microglia using cellular grafts prolonged survival and slowed motor neuron loss [339]. The inflammation regulating protein nuclear factor-kappa B (NF-κB) is upregulated in spinal cords of ALS patients and $hSOD1^{G93A}$ mice. Consistent with previous findings, inhibition of NF-κB signaling in microglia rescued the co-cultured motor neurons from cell death [340]. Microglia has also been proposed to contribute to C9ORF72 associated ALS disease. Patients with the hexanucleotide repeat expansion have a decreased expression of the C9ORF72 protein [341], and C9ORF72 knock-out mice grow normally but develop progressive splenomegaly with altered immune responses in macrophages and microglia [342]. This suggests a loss of function mechanism in addition to the established gain of function pathology in C9ORF72 associated ALS.

Not only microglia are important for hSOD1 pathogenesis. Reduction of the synthesis of mutant hSOD1 into oligodendrocytes, the cells that provide myelin and metabolic support to the motor neuron axons, significantly delays disease onset in $hSOD1^{G93A}$ mice [343]. The energy metabolite lactate is provided to the axon from oligodendrocytes through monocarboxylate transporter (MCT) 1. Mutant SOD1 impairs the expression of MCT1 mRNA in oligodendrocytes in the $hSOD1^{G93A}$ mouse model. In addition, more than 50% reduction of MCT1 and MCT4 protein expression is observed in sALS patients [344]. A recent study suggests that oligodendrocytes contribute to motor neuron death via a SOD1 dependent mechanism. Oligodendrocytes expressing hSOD1 in co-culture with wt motor neurons caused hyperexcitability and death of the neurons. Induced pluripotent stem cells derived from sALS and fALS patients differentiated to oligodendrocytes, also initiated motor neuron death. This effect could be removed through shRNA knockdown of hSOD1 [345]

The involvement of astrocytes in hSOD1 mediated neural toxicity has been suspected for a long time. Inclusions of mutant SOD1 in astrocytes was found in $hSOD1^{G85R}$ mice quite early after the first transgenic mouse model was developed [275]. Expression of mutant hSOD1 in astrocytes alone failed to drive disease [346],

but silencing of mutant hSOD1 expression delayed microglial activation and slowed late disease progression [347], and; it also showed a delay in disease onset [348]. Experiments using cell transplantation have revealed detrimental effects in mutant hSOD1 astrocytes. Focal transplantation-based astrocyte replacement showed a remarkable improvement in phenotype and had a neuroprotective effect in $hSOD1^{G93A}$ rats [349]. In the inverse, transplantation of mutant $hSOD1^{G93A}$ into healthy wt rats induced motor neuron death, as well as forelimb and respiratory dysfunction [350]. Since malfunction of astrocytes are a part of the disease process, groups are now trying to develop intrathecal transplantation protocols with the intent to treat ALS patients [351]

In summary, ALS is not solely a disease of the motor neurons, and none of the major neurodegenerative diseases are non-cell autonomous [352]. This must be considered when developing treatments in ALS.

Prion-like propagation

The first observations of a prion disease were made in sheep in 1732, although investigators were not yet aware of the cause [353]. Scrapie, as the disease later was named, was thought to be an infectious disease transmitted by a virus [354]. The transmissibility of the disease was established in the late 1930s when a healthy sheep were subjected to brain inoculations with CNS material from a diseased sheep. This inoculation induced similar symptoms in the healthy sheep; it had been "infected" with scrapie [355]. In humans, neurologists Hans Gerhard Creutzfeldt and Alfons Maria Jakob described a neurological disorder of unknown etiology that was later named after the two. [356, 357]

Another epidemic neurodegenerative disease, Kuru, was isolated in 1959 from a population in Papua New Guinea [358]. The disease had affected a small tribe that was known for the practice of ritualistic cannibalism. The veterinarian pathologist W.J. Hadlow emphasized a striking neuropathological and symptomatic similarity between Scrapie and Kuru [359]. In the same time period, similarities were noted between Kuru and Creutzfeldt-Jakob disease (CJD) and transmission of both diseases from humans to chimpanzees was done in the second half of the 1960s [360, 361]. The disease-causing agent was still unknown, and theories suggested a slow virus as the disease provoking factor [353].

The term prion was first postulated in a landmark publication by Prusiner in 1982 [362]. Transmissible spongiform encephalopathies (TSE) that include CJD and Kuru, amongst others, were hypothesized to have a protein only etiology. The new term prion was introduced and defined as a "proteinaceous infectious particle that is resistant to inactivation by most procedures that modify nucleic acids" [362]. Prusiner proposed that the pathomechanism of prions could apply to diseases other than TSEs, such as Alzheimer's senile dementia, Parkinson's disease,

and ALS amongst other diseases [362]. Since then, the accumulative evidence speaks in favor of this hypothesis.

A prion is a misfolded protein able to induce abnormal folding of its normal cellular protein, thereby leading to the formation of aberrant multimers. The most well-known prion disease is CJD where the prion protein (PrP) is the disease-transmitting agent. The most common causes of CJD are inherited forms and sporadic forms, whereas infectious CJDs are very rare [363]. In the 1980s, a new form of CJD called variant CJD (vCJD) arose. This form is a well noticed zoonotic prion disease believed to have been transmitted to humans from beef products primarily originating from Great Britain. The infective meat came from cows carrying the prion disorder bovine spongiform encephalopathy (BSE), also called "mad cow disease" [364]. Since then 178 humans have died in the UK from vCJD and millions of cows have been killed [364].

In the laboratory, prion proteins from infected experimental animals carrying TSE can be transferred to a healthy animal to induce disease, a phenomenon called seeding. This has been done early in CJD as previously described, but now also in other neurodegenerative diseases such as AD, PD and Huntington's disease [365-369]. A pathogenic propagation of the protein aggregates across CNS is observed in the latter diseases, as well as in TSEs, and this propagation is coupled with symptom progression. The nomenclature of prion disorders other than the classical human TSEs (CJD, Kuru, Gerstmann-Straussler-Schinker syndrome and fatal familial insomnia), is still under debate, and terms as, prion disease, prion-like disease or prionoid disease are suggested [370-373].

Growing evidence suggest that misfolded SOD1 also had the capacity to propagate similarly as the prion protein. In 2009, it was proposed that motor neuron degeneration starts from a focal point and then spread contiguously throughout the CNS [374], similarly as in TSEs. Two years later the ability of aggregated mutant hSOD1 to spread from cell-to-cell by was established [375]. Aggregates of mutant hSOD1 were shown to penetrate inside cells by macropinocytosis and induce misfolding of the soluble mutated SOD1 in the cytosol; this form of seeded aggregation including SOD1 was described for the first time. Later studies observed that also wt-hSOD1 could be induced to misfold and form multimers in cell cultures transfected with mutant hSOD1 G127X and G85R [376]. In addition, extracellular wt-hSOD1 aggregates released from dying cells were shown to be taken up into neuronal cells by macropinocytosis and exosomes, giving evidence to the spreading hypothesis as well as the potential toxicity of wt-hSOD1. The transmission of misfolded wt-hSOD1 could be blocked by siRNA mediated knockdown of wt-hSOD1 and attenuated by incubation with SOD1 misfolding-specific antibodies [376].

Aggregates of misfolded SOD1 are frequently observed in both fALS and sALS patients [319, 377], and inclusions of wt-hSOD1 in patients with the C9ORF72 expansion has also been shown [376]. Data also suggest that pathological TDP-43 and

FUS proteins can trigger misfolding of wt SOD1, and seed cytotoxic misfolding of endogenous wt-hSOD1 in a prion-like fashion [378].
Together these data implicate that SOD1 could have a more general role in the ALS disease and that a prion-like mechanism is plausible both in patients with the hSOD1 mutation, but maybe also in patients with a sALS pathology. Our aim was to further investigate this theory.

Aims

The field of neurodegenerative diseases is increasingly investigating a prion-like/prionoid mechanism as the main cause of neurotoxicity, albeit in ALS such studies are only in its early stages. SOD1 is associated with familial forms of ALS, but SOD1 pathology is also present in ALS patients with other etiology. The overall aim of this thesis was to investigate the *in vivo* hSOD1 aggregate structure, and to explore if and how the aggregates are contributing to the development of motor neuron disease.

The thesis consists of four papers and the aims of each paper were:

Paper I

- Develop and characterize a transgenic mouse model, highly overexpressing wild-type hSOD1 at a similar rate as for G1H hSOD1^{G93A} mouse model.

Paper II

- Develop a method to characterize the structure of hSOD1 aggregates produced *in vivo.*
- Investigate if the structure of hSOD1 aggregates in the CNS of transgenic mice is shared between different models.
- Describe the distribution of hSOD1 aggregates in the CNS of these mouse models.

Paper III

- Characterize the role of different hSOD1 strains in disease pathogenesis of hSOD1 mice.
- Test the possibility to initiate templated aggregation of mutated hSOD1 in mouse spinal cord, by inoculation of purified hSOD1 aggregates.

Paper IV

- Explore if mutant SOD1 aggregates with prion-like properties exist in human ALS as well as in transgenic mice models.

Materials and Methods

This section describes novel methods and highlights method-diversities between the different papers. For a detailed description of materials and methods, the reader is referred to the Method sections in papers I-IV.

Mouse models

In this thesis, transgenic mouse models used were all backcrossed more than 30 generations in C57BL/6, except for the homozygous Wt-hSOD1 mice. This model was developed through hemizygous C57BL/6 wt-hSOD1 mice backcrossed 8-10 generations into CBA background. Hemizygous wt-hSOD1 CBA mice were in turn crossed for the generation of homozygous wt-hSOD1 mice. The specific characteristics for each mouse model are stated in Table 1.

Characteristics	G93A	G85R	D90A	G127X	wt-hSOD1	wt-hSOD1
Mouse Line	B6SJL-Tg(G93A-SOD1)1Gur/J	Line 148	Line 134	Line 716	N1029	CBA "In-house"
Papers	**I & II**	**I-IV**	**I-IV**	**I & IV**	**I**	**I & II**
Zygosity	Hemizygous	Hemizygous	Homozygous	Homozygous	Hemizygous	Homozygous
Protein aggregation propensity	High [379]	High [379]	High [379]	High [190]	Low	Low
Protein Stability	Stable [189]	Reduced [189]	Stable [189]	Unstable [189]	Stable	Stable
Enzyme activity	Moderate [189, 267]	Inactive [189]	Low [189]	Inactive [189]	Low [189]	Low
Disease progression	Moderate	Fast	Slow	Fast	N/A	Slow
Lifespan in our lab (days)	Short 155 ± 9	Long 397 ± 49	Long 424 ± 60	Moderate 250	No ALS disease	Long 367 ± 56
mRNA level (of G93Agur SOD1)	100%	43 ± 6% [189]	51 ± 8% [189]	63 ± 8% [189]	54 ± 10% (paper **I**)	76 ± 12%
Protein level (related to mouse SOD1)	17 [189]	0.9 [189]	20 [271]	0.45 [189]	N/A	50
Protein Conc. (at 100 days) (µg/g ww)	1200 [271]	60 [189]	1300 [271]	30 [270]	1350 (paper **I**)	3340
Original Publication	Gurney *et al. Science* 1994 [267]	Bruijn *et al. Neuron* 1997 [275]	Jonsson *et al.J Neuropathol Exp Neurol* 2006 [271]	Jonsson *et al. Brain* 2004 [190]	Gurney *et al. Science* 1994 [267]	Graffmo, Forsberg *et al. Hum Mol Genet* 2013 (**paper I**)

Table 1. Mouse models used in this thesis. *Data from homozygous wt-hSOD1 are published in article I. The data in the table is acquired from the papers referred to as numbers in brackets (for detailed information, see reference list).*

Antibodies

The in-house produced SOD1 anti-peptide antibodies used in this thesis are the base for analysis of SOD1 aggregates in all papers. Eight of the antibodies were first described in 2004 [270] and cover over 90% of the entire protein (figure 4). The keyhole limpet hemocyanin (KLH) coupled polyclonal rabbit antibodies were raised against peptides corresponding to amino acids 3-20, 24-39, 43-57, 58-72, 80-96, 100-115 and 131-153 in the human SOD1 sequence. KLH is the most widely used carrier protein for antibody production since the complex structure of the protein is widely distant to any protein found in mammals. That, together with its large size, makes it highly immunogenic, facilitating the rabbit immune response that in turn gives a higher antibody yield [380]. The unique structure of KLH, widely distant from mammalian proteins, greatly reduces the risk of cross-reactivity between antibodies against the KLH carrier and naturally occurring proteins in mammals. In paper IV polyclonal antibodies against amino acids 41-49, 48-57, 94-102, 115-121, 132-140, and 144-153 were used in addition to the other antibodies. These were shorter than the original eight antibodies and could therefore add a better resolution when analyzing the SOD1 aggregate structure.

Two polyclonal antibodies were raised specifically against the neopeptide formed in the G127X mutant corresponding to amino acids 111-127**GQRWK** and 123-127**GQRWK** in the SOD1 sequence, where the latter was only used in paper IV. The bold letters represent the neopeptide, consisting of amino acids; glycine, glutamine, arginine, tryptophan, and lysine. Since the N-terminal part (111-127) is included in the native human SOD1 sequence, this antibody also has the propensity to bind full length SOD1 and not only the G127X mutated SOD1. The antibodies were purified on protein A-sepharose followed by purification on a Sulfolink coupling gel with the respective peptides coupled.

Polyclonal antibodies, in contrast to monoclonal antibodies, cover multiple epitopes on the same antigen. This epitope variation results in relatively high avidity in our assay. For the production of monoclonal antibodies, an exact amino acid sequence has to be determined. If the specific epitope is unknown, the target amino acid sequence have to be estimated. This will most likely result in a bad antibody, with low affinity. In the production of polyclonal antibodies, the resulting antibodies will be directed against many different epitopes on the specific peptide, so at least a part of the antibodies will bind to a epitope if it is available, resulting in a high avidity. Polyclonal antibodies are also more tolerant to minor antigen changes like the heterogeneity of glycosylation or other post-translational modifications. The production of polyclonal antibodies is also relatively quick and inexpensive. This makes the polyclonal antibodies a perfect screening tool for identification of disordered segments within an aggregate. One disadvantage with polyclonal antibodies is the batch-to-batch variability, which does not occur when the antibody production is based on hybridomas as in monoclonal antibody production. However, since the avidity of the polyclonal antibodies used in this thesis

is markedly high, very small volumes of antibody are needed for each experiment, resulting in long-lasting antibody stock. A few antibodies have been replaced with a new batch over the years and I this thesis only the 57-72 antibody has been changed once. The new 57-72 antibody was thoroughly calibrated with respect to avidity and dot blot signaling, to have a consistency in our assay.

During the characterization of the purified polyclonal SOD1 antibodies, we found that all were highly specific for misfolded SOD1 but showed no detectable binding to native SOD1 [377] (and paper II). This is a property that would turn out to be very useful in future studies. The antibodies could be used for staining of SOD1 aggregates in histopathology studies of fALS and sALS patients, something that was not possible with antibodies raised against whole SOD1 [381, 382]. The antibodies were also specific for denatured SOD1 in solution [377]. Many of these antibodies are directed against epitopes clearly available on the surface of the native SOD1 protein (Figure 7). Thus, the inability to bind to the native protein is not caused by epitope concealing in the protein core. The specificity of the antibodies lies in the method used for antibody production. Short peptides have little or no specific conformation when targeted by the antibodies. Therefore the antibody epitopes will recognize disordered structures. A crucial step for the antibody specificity for denatured SOD1 is the sulfolink purification process. Sulfolink gels bind specifically well to exposed sulfhydryls (-SH). Unfolded SOD1 lacks the intrasubunit disulfide bond, as described earlier, and therefore its –SH groups are available for the sulfolink gel. The gel is prepared with each unfolded SOD1 peptide. When the peptides are bound to the gel, their capability of folding is restrained, and antibodies only capable of binding unfolded peptides will be selected from the serum. The resulting antibodies thus only bind SOD1 segments that can freely adapt to the antibody's pattern of recognition. In theory, an antibody will be able to bind globally unfolded SOD1, unfolded monomers and disordered, flexible segments/fringes of a SOD1 aggregate that is not involved in the rigid β-sheet core, the latter exploited in the papers presented in this thesis.

Antibody specificity for different hSOD1 conformational species

To confirm the antibody specificity against disordered/misfolded segments, the different antibodies were immobilized on sepharose gels and incubated with SOD1 that was either native, or had been denatured by exposure to 4 M guanidinium chloride (GdmCl) and 5mM of the chelator diethylenetriaminepentaacetic acid (DTPA). After washing the bound SOD1 was analyzed by Western immunoblot. The native SOD1 was incubated twice with immobilized antibodies to capture all traces of disordered proteins in the first batch. Antibodies had very high reactivity against denatured SOD1 but almost no binding to native SOD1 (paper II, figure 2), except for the C-terminal part of SOD1 (antibody 131-153).

This is probably due to increased flexibility of the C-terminus in the native protein.

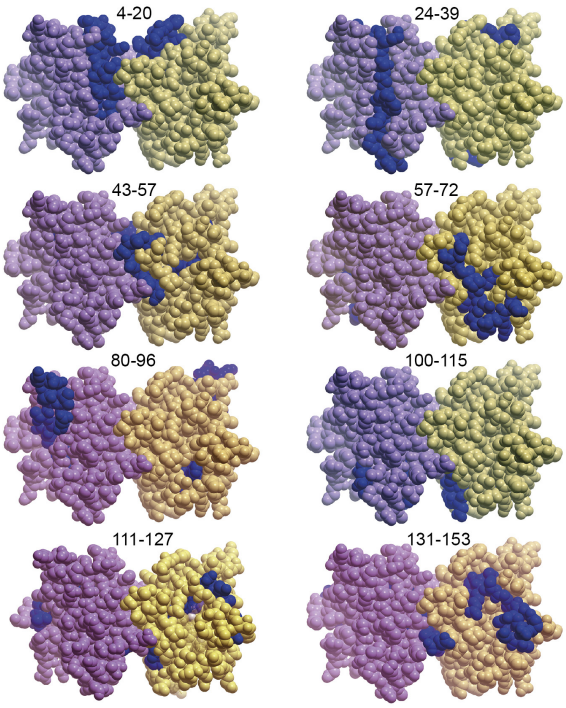

Figure 7. Location of SOD1 peptides used for immunization. *The two subunits of SOD1 are shown in different colors. The segments corresponding to the peptides used for immunization are shown in dark blue. Several of the peptide segments are well exposed to the surface (Paper II).*

Tissue homogenization and binary epitope-mapping assay

Mice were killed with an intraperitoneal (IP) injection of pentobarbital. The brain and spinal cord were divided from the brainstem and cerebellum, flushed out using saline and were directly flash frozen in liquid nitrogen. In papers III and IV, the spinal cord was usually divided into right or left with anterior median fissure as a guide, and was always separated into cervical, thoracic and lumbar spinal segments before storage in freezer or fixation in formalin. Fresh or frozen tissues were homogenized in an ultraturrax apparatus for 30 s and thereafter sonicated for 1 min in 25 volumes of ice-cold phosphate buffered saline (PBS) supplemented with 1.8 mM ethylenediaminetetraacetic acid EDTA, 1 mM dithiothreitol (DTT) and anti-proteolytic cocktail to inactivate aggregate degrading enzymes and to prevent further aggregation. To test which preparation providing the highest aggregate yield in the dot blot analysis, different concentrations of EDTA, DTT and also 1-iodoacetamide (IAM) was added to aggregate solution. The alkylating agent IAM targeting the sulfhydryl groups seemed to reduce the yield of aggregates and was therefore not used further. Instead, DTT was used to reduce disulfide bonds but in a low concentration (1 mM) not to interfere with aggregate composition. This seemed to increase the yield of aggregates. EDTA is a chelating agent and also helps to remove unspecific binding of proteins to the aggregates; here it was

also titrated to a low concentration so as not to dissolve the aggregates. High molecular species of SOD1 are known to be highly resistant to solubilization [383]. There seemed to be no difference in signal pattern when comparing fresh or frozen tissue (not shown). All samples in paper II (which was the only study containing freshly prepared tissue) was analyzed three times. The tissue homogenates were further diluted with 20 volumes (now diluted 520 times) with the buffer described above with the addition of nonidet P-40 (NP40) in order to facilitate lysis of cell membranes. Sonication for 30 s was followed by 200 g centrifugation in 10 minutes (paper I, II and III) and 1000 g in paper IV. The centrifugation speed was increased to reduce clogging of wells during filtering of the aggregates. The supernatants were diluted stepwise 1+1 in PBS and captured on cellulose acetate filters with a pore size of 0.2-µm in a 96-well dot blot apparatus similar to a previous description [383]. Cellulose acetate membranes are perfect for capturing SOD1 aggregates without altering the structure since the membrane is not charged. With no charge, aggregates and proteins in solution are unable to bind to the membrane through electrostatic interactions. Electrostatic binding of proteins would increase the risk of altering of the aggregate structure, and the risk for unspecific capturing of monomeric SOD1 species or other non-aggregated proteins are raised. Studies of polyglutamine expansions in Huntington's disease, a 0.22 µm cellulose acetate filter can clearly separate aggregates from soluble species [384].

After loading of homogenate onto the cellulose acetate filter, washing and blocking with tris-buffered saline (TBS) (50 g/L), dry milk and 0.1 % Tween 20 for 1 hour, the filters were cut in slices to be able to stain the same filter with different peptide antibodies. In paper II typically eight antibodies were used. Since the antibody concentrations and avidity to unfolded SOD1 of the antibodies are different within the group of antibodies, a calibration to soluble misfolded SOD1 was performed (figure S2, paper II). The same number of antibodies was bound to sepharose beads that in turn were incubated with 25,000 x g homogenate of spinal cord from a hSOD1 mouse. In theory, the beads with different immobilized antibodies should bind similar amounts of SOD1, since the solution was saturated with disordered proteins. The bound SOD1 was released by boiling in SDS/PAGE sample buffer and thereafter analyzed by western blot. All antibodies except two bound similar amounts of SOD1 and antibodies were used at a concentration of 0.01 µg/mL. The antibodies 4-20 and 100-115 bound about half as much SOD1, and therefore the antibody concentrations of these were doubled to 0.02 µg/mL. We have earlier determined that changes in SOD1 aggregate- and antibody concentration linearly changes the retrieved staining intensity, i.e. a duplication of the antibody or the SOD1 aggregate concentration will duplicate the staining intensity (not shown).

The blots were thereafter incubated with goat anti-rabbit IgG antibodies recorded with a Chemidoc apparatus and quantified with Quantity One software. To

allow comparison and quantification, a homogenate of several hSOD1^{G93A} spinal cords stained with 57-72 antibody was used as a standard in one or two lanes on each filter. The staining intensity of the dot blot was not linear in wells with high protein concentrations (not shown). This is probably due to aggregate overload on the dot blot filter, which could affect the antibody binding to the aggregates, and in turn, lower the staining intensity. To overcome this problem, the final value was always (when possible) calculated as a mean of the intensities for three dots.

To facilitate comparison of staining patterns, in some data sets the staining intensities for each antibody were normalized against the 57-72 antibody, a region that is always available for staining in the hSOD1 aggregates.

Preparation of the hSOD1 aggregate seeds

Strain A aggregates were prepared from terminally ill hSOD1^{G85R} mice. Strain B aggregates were prepared from a hSOD1^{D90A} mouse with a short lifespan. The shorter the lifespan of this specific model, the higher the proportion of strain B aggregates in CNS (figure 4B, paper II). Seeds from a 100-day old non-transgenic C57BL/6 mouse were used as a mouse negative control. Seeds from an end-stage hSOD1^{G127X} mouse were used in paper IV. Human seeds were prepared using lumbar ventral horn (lamina IX) from a patient carrying the hSOD1^{G127X} mutation (paper IV) that belonged to an ALS family previously described [234]. The human control seed was prepared from a patient that had suffered from epilepsy and had died of an acute myocardial infarction.

Whole spinal cords were homogenized in 25 volumes of PBS containing 1% of the detergent NP40 and 1 M GdmCl using an ultraturrax followed by sonication. The homogenates were centrifuged at 1000 x g for 10 minutes to remove larger debris and the resulting supernatant layered on top of a 4 cm high 13% iohexol density cushion (d = 1074). The tubes were subjected to ultracentrifugation 175,000 x g for 1 hour, and the resulting pellet was again ultracentrifuged as previously, but now with plain PBS. The pellets were suspended in 1 µl PBS per mg of spinal cord and the final suspension contained 5 ng/µl and 10 ng/µl strain A- and strain B-aggregated hSOD1, respectively (paper III). Seeds prepared from transgenic hSOD1^{G85R} and hSOD1^{G127X} in paper IV contained 3 ng/µl each. Under these conditions, only proteinous components with molecular masses >5x10^6 Da will be sedimented into pellets [385]. The seeds will therefore not contain monomeric, dimeric or any oligomeric species.

In paper IV, a second protocol for the human seeds was performed, with half as much GdmCl and one additional centrifugation. By adding longer sonication time the fibrils become smaller, thus providing more fibril ends; this would in

theory improve the protein aggregation potential of the seed. Human seeds contained 0.14 and 0.04 ng/µl respectively.

Inoculation of the seed into the lumbar spinal cord

To become a good surgeon and master a new surgical technique takes effort, time and repeated practice [386]. A new setup of surgical instruments, sedation techniques, and postoperative care are needed to be assembled in order to inoculate the toxic aggregates into the matchstick size of the mouse spinal cord. The surgery also needs to cause the least possible damage to the surrounding tissue. There is always a risk that the surgical procedure is not optimal in 100% of the cases, which in turn might lead to outliers and unexpected results. However, the first spinal cord inoculation done in our laboratory was successful, without postoperative complications. In paper III, About 80% of all inoculations were without complications, 15% of the mice died during anesthesia and the rest got wound infections and were euthanized. Four strain A inoculated mice lived longer than expected, probably because the seed did not reach its target in the spinal cord. One of these mice was among the first to be inoculated and the other three were inoculated later, but all in the same day. However, after gaining more experience as a mouse surgeon, the protocol changes over time, becoming more efficient with fewer errors. The strain B inoculated animals all died early from disease, probably because the surgeons had more experience once the strain B seed inoculations were initiated. The inoculation protocols changed slightly in paper IV compared to paper III. I will briefly go through the surgery procedure and discuss the main parts.

To set up a sedation protocol, the mouse was initially monitored with a Pulse Oximeter (Pulse sense VET, UNO). First Hypnorm® 1.66 µl/gram mouse (0.2 mg/ml fentanyl and 10 mg/ml fluanisone) and Dormicum® 1.66 µl/gram mouse (1 mg/ml Midazolam) were injected IP. This normally gives a sedation time of 20-30 min. An iteration of 0.3 ml/kg subcutaneous (SC) Hypnorm® was done once during the surgery if needed. The surgery time was around 50 minutes for an inexperienced surgeon but was lowered to about 25 minutes after practice. Both Hypnorm® and Dormicum® are known for causing bradycardia and breathing deprivation as a side effect, which was observed during surgery. For the mice to reach an unresponsive state during pain stimulation, without having the oxygen saturation reach under 50% and the heart frequency less than 200/minute (both critical limits for mouse survival), was shown to be problematic during surgery. Mice that were more “hyperactive”, like the hSOD1^{G127X} or wt-hSOD1 mice, responded very poor to this sedation protocol. Therefore, we changed to Ketamine (78 mg/kg) and xylazine (26 mg/kg) IP with the addition of buprenorphine (3 µg SC), which was the sedation protocol used in paper III. Both the heart rate and the breathing frequency were less affected with this protocol. Still, limitations with short sedation time and somewhat unpredictable sedation response in the

CBA strain made us change to continuous sedation by inhalation of isoflurane gas in paper IV. This proved to be a more reliant method superior to IP injections in many perspectives. It is less traumatic, easier to handle, and very short post-surgical anesthesia, to mention a few reasons. Changing to isoflurane sedation markedly lowered the number of mice dying from surgery. For postoperative analgesia, buprenorphine, with an effective analgesia around 12 h was changed to Rimadyl® (a non-steroidal anti-inflammatory drug) that has effect for 24 h.

The goal was initially to accomplish a surgery as non-traumatic as possible. In paper III a laminectomy or partial laminectomy was performed to reach the spinal cord for inoculation. This was changed to an inoculation between two vertebras in paper IV. The inoculation coordinates were determined by measurement of histopathological slides from lumbar parts of hSOD1^{G85R} (n=5) and wt C57BL/6 (n =4) mice around 100 days of age. A mean sagittal distance from midline was determined (around 400µm), and the inoculation depth was set to around 800 µm. The coordinates of the inoculation site could be adjusted manually with the help of a digital display console provided together with the stereotactic frame (model 940-B; Kopf). The precision of the inoculation was controlled by an injection of Fluoro-Gold™ and subsequent analysis under a fluorescence microscope. Labeling in the ventral horn was found, thus confirming that the inoculate reached its intended region.

Supervision of the mice

Weekly weight and supervision of symptoms at least 2 times a week were performed. The symptom onset of the mice was set to uniformly splaying of a hind limb, or difficulties grasping the cage with front limbs. This could be criticized as a subjective determination of symptom onset, but the method is commonly used in the field. However, quantitative methods like grip-strength meter, rotarod performance tests, and locomotor activity tests were performed in our lab to examine if the onset time could be set in a more objective way. These methods had many drawbacks and could not be used comparing injected mice with non-injected mice since the site of symptom onset varied, giving vastly different results in the tests. We therefore abandoned these time-consuming tests in favor of the former method.

Weight is also a common determinant of symptom onset, and usually 10% weight loss from peak weight is used as the time of onset [337]. This definition of symptom onset can only be performed retrospectively and could therefore not be used in this assay.

Histopathology

Fixation of the tissue is critical for good quality images and reproducibility. Ideally, the fixation media (in this thesis 4% paraformaldehyde in phosphate buffer)

should reach all cells at the same time for ideal conservation of the tissue. For obvious reasons, this is not possible, but fast perfusion can be achieved with perfusion fixation. In paper I, all mice analyzed histopathologically were perfusion fixated. In papers III and IV, the CNS from each mouse was separated into right or left where one side generally was frozen for subsequential dot blot analysis while the other side was immersion fixated. Since one side of each mouse was analyzed biochemically as frozen tissue, perfusion with formalin was not possible since it would destroy the frozen sample. Potentially this could slightly affect the epitopes and the tissue integrity of the histopathological samples in papers III and IV. Remaining erythrocytes in the non-perfused animals might also give rise to unspecific antibodies binding, resulting in an increased signal-noise ratio. However, since the mouse CNS is so small, even immersion fixation is relatively rapid. For ideal penetration and fixation of tissue, it is advised to keep tissues thinner than 20 mm for penetration to occur by 24h at 25°C [387], well over the size of the murine CNS.

Staining and counting of motor neurons

There are currently no optimal antibodies specific for motor neurons solely available for immunohistochemistry. The most commonly used antibodies to identify motor neurons are directed against a neuronal nuclear (NeuN) protein, choline acetyltransferase (ChAT) and Islet-1. In this thesis the two former antibodies are used for motor neuron staining. NeuN antibodies are specific for all neurons, with the following few exceptions: it does not stain Purkinje cells, neurons in the retina, inferior olive, and γ-motor neurons [388]. It mainly stains neuronal nuclei but also give a signal in the perinuclear cytoplasm. The advantages with this marker are: 1. the NeuN protein is only expressed in neurons; 2. the protein is not expressed in immature progenitor cells; 3. it is not dependent on the cytoplasmic size since it stains the nuclei, and is therefore a better marker when staining many different cells [388]. ChAT antibodies on the other hand, selectively stain the cytoplasm of cholinergic neurons. Both α- and γ-motor neurons are cholinergic and are therefore often identified using this antibody. Usually soma size is used as one factor when counting neurons, in addition to the immunohistochemical staining. The size of the cell stomata might differ between species and depending on the health of each neuron. The size of a healthy α-motor neuron in mice ranges from around 150-1100 μm^2, and the mean size is~550 μm^2, while the γ-motor neurons are around 100-400 μm^2 with a mean size of ~250-300 μm^2 [389]. In spinal muscular atrophy (SMA) mouse models, a significant decrease around 100 μm^2 of soma size of α-motor neurons was seen in late symptomatic mice, whilst γ-motor neurons did not change in size [389]. In our study, typically we used a 150 μm^2 soma size limit to identify motor neurons. In addition, we performed the analyses using 250 μm^2 and 350 μm^2 limits for all groups, and size charts were checked. These size limits will include both types of motor neurons, when using ChAT

staining but not with NeuN antibody. One caveat using specific markers for motor neurons is that staining methods presupposes that the staining propensity is not affected by the disease process, which might not always be true.

In paper I, stereology was used to manually count the number of neurons in the spinal cord. Stereology is considered the “gold standard” for quantification of amounts of cells in tissue specimens. This is a time-consuming process, and the tissue needs to be cut from exactly the same area of interest since the number of neurons can vary from different segments. To do this, an *in situ* dissection of the spinal cord is crucial since the landmarks (i.e. nerve roots) are needed for guidance. For this reason, stereology could not be performed in papers III and IV since this precision could not be achieved when flushing out the spinal cord. Here the motor neurons were counted usually from 7-10 sections per mouse using ImageJ software. The NeuN positive neurons >150 μm^2 localized in the ventral horn, defined as the area within the grey-matter border and anterior to a frontal plane through the central canal, were subjected for counting. The specificity of the automated counting was controlled by manual counting. Counting of neuropil threads and cytoplasmic aggregates were done on sections stained with 131-153 anti-hSOD1 Ab. The neural cytoplasmic granular aggregates were counted in the area described above, and the number of threads was counted in a box measuring 100 µm x 100 µm placed centrally in the ventral horn. This counting was performed manually by three blinded observers since the automated counting with ImageJ was too erroneous with this setup.

Results and discussion

Paper I

Wild-type SOD1 in ALS

Since 1993 when Rosen *et al* [92] linked SOD1 to ALS, the protein has been under the microscope. Despite decades of research, the specific mechanism(s) of SOD1 pathogenesis in ALS is still not clear. Over 90% of the ALS cases are sporadic, and a majority of these patients lack a known mutation causing the disease [84]. One important question is 'what is/are the disease provoking factor(s) in these cases?' A number of neurodegenerative diseases involve protein aggregation. In some of these patients the gene producing the pathogenic protein is mutated, but in the majority the gene is intact. For example, α-synuclein is a major component of the Lewy bodies, the pathological hallmark of PD, both in sporadic and familial forms. The first mutation identified in PD was synuclein alpha (*SCNA)*, which encodes the protein α-synuclein [390]. In AD, mutations in the amyloid precursor protein (*APP*) is linked to early onset familial AD [391, 392]. Cleavage of *APP* by γ-secretase and β-secretase generates amyloid beta (Aβ), which is included in the extracellular amyloid plaques that are the pathological hallmark of all forms of AD [393]. In ALS, no such specific protein has represented a pronounced link between familial and sporadic cases. However, neural Lewy-body-like hyaline inclusions and astrocytic hyaline inclusions containing aggregated SOD1 are considered a hallmark of ALS patients with the hSOD1 mutation, and in transgenic mice with a hSOD1 mutation [394].

Accumulating data suggest that the hSOD1 protein could be involved in the disease mechanism of patients without a mutation in the SOD1 gene. Pathological TDP-43 or FUS accumulations have been shown to co-localize with misfolded wt-hSOD1 *in vitro* as well as in patient motor neurons in non-SOD1 fALS and sALS [395]. TDP-43 and FUS-induced misfolded wt-hSOD1 can in turn propagate from cell-to-cell and further provoke misfolding of SOD1 [378]. Thus, misfolding of the endogenous wt-hSOD1 might occur secondarily to TDP-43 and FUS pathology.

Using our in-house antibodies specific for misfolded hSOD1 we have demonstrated inclusions in the cell bodies of motor neurons [377] and in the nuclei of glial cells [396], also in sALS patients lacking the SOD1 mutation. Transgenic mice heterozygous for wt-hSOD1 show loss of motor neurons at old age, but the survival time is still normal [189, 397]. It is known that the toxicity in the transgenic ALS models is highly dependent on the amount of overexpression of SOD1. A doubling of protein expression broadly halves the survival time [189, 270]. Studies on mice carrying D90A mutation showed that hemizygous mice do not develop an ALS phenotype whilst the homozygous mice die from terminal disease around 410

days [271]. Interestingly, the hemizygous wt-hSOD1 mouse spinal cord had an aggregate load just in between that of hemizygous and homozygous D90A transgenic mice. To examine if the wt-hSOD1 protein has a potential to initiate ALS symptoms itself, we overexpressed wt-hSOD1 close to the rate of hSOD1^{G93A} mice.

Homozygous wild-type hSOD1 transgenic mice develop terminal ALS disease

The goal was to increase the expression of wt-*hSOD1* close to the rate of the hSOD^{G93A} model. To do this we crossed hemizygous N1029 mice on C57Bl/6 background, but the yield of homozygous pups were markedly low. We therefore changed to CBA genetic background where 21.5% of the pups were homozygous. These pups were born with a lower weight than the non-transgenic and wt-*hSOD1* hemizygous mice, thus indicating that the homozygous mice might be under stress. This weight loss is not found in hSOD1^{G93A} transgenic mice [398], and therefore one might conclude that this phenomenon is not solely a hSOD1 dependent matter. The homozygous wt-hSOD1 mice showed the first symptoms (deficient leg splaying) at around 250 days and developed terminal symptoms (usually hind limb paralysis) at a mean age of 367 ± 56 days. A deviant feature in the homozygous mice, which can also be observed in really old wt C57Bl/6 to a lesser degree, is ataxic staggering gait. This is a phenotype that is not present in other SOD1 mouse models [92, 190, 271, 275]. Gait abnormalities like ataxia commonly originate from cerebellar dysfunction, and cerebellar ataxia has been observed in some ALS cases with SOD1 mutations [237, 399]. We therefore examined the cerebellum separately from other CNS tissues and found a loss of cerebellar Purkinje cells, which is not present in other transgenes. Immunohistochemical staining with the 131-153 anti-hSOD1 peptide antibody showed cytoplasmic staining of misfolded hSOD1 in the Purkinje cells and vacuolization around the remaining cells. The amount of hSOD1 in reduced form (lacking the intrasubunit disulfide bond) in the cerebellum at 100 days of age is similar to the levels in the brain and reaches ¾ of the spinal cord levels in homozygous hSOD1 mice. Taken together, there is evidence of neural pathology and buildup of misfolded hSOD1 in the cerebellum, which could possibly be linked to the staggering gait. Recently, a down-regulation of calbindin 1, a calcium binding protein, in the Purkinje cells of the hemizygous N1029 wt-hSOD1 transgenic mice was observed. These cells were primarily located in the anterior zone of cerebellum, the part which receives somatosensory input [400]. The reason for the Purkinje specific damage in wt-hSOD1 is still unknown.

In the spinal cord ventral horn of homozygous wt-hSOD1 mice, profound hSOD1 pathology was observed. Cytoplasmic inclusions in motor neurons were seen already at 100 days and a massive loss of motor neurons were observed in terminally ill mice. The terminal mice paradoxically showed a significant reduc-

tion of reduced hSOD1. This could be due to motor neuron depletion in the terminal mice leading to less hSOD1 production, perhaps in combination with terminal oxidative stress as earlier observed in the terminal hSOD1 models [401, 402]. Aggregates in the spinal cord and brain were extensive. Capturing of aggregates on filter did not show any aggregates in liver and muscle. In wt-hSOD1 mice, the proportion of aggregated SOD1 in the brain compared to the spinal cord was 40%, much higher than all our other mouse models and 20 times higher than in the D90A mice. By stereology counting of the thoracic spinal cord, it was shown that 41% of all neurons were lost, which is similar to the results in end-stage G93A and D90A mice [271].

We showed that wt-hSOD1 has the ability to cause ALS in paper I. Reviewing all the different mouse models of ALS, only mice transgenic for hSOD1 develop clear motor symptoms and terminal disease [403]. This speaks in favor of the SOD1 protein as the central disease-causing agent in ALS.

Paper II

Analysis of protein-aggregate strains

Superoxide dismutase 1 is a significant protein in ALS pathogenesis, but the mechanism of toxicity is still under debate. Several different mechanisms have been proposed, as reviewed earlier in this thesis. In many neurodegenerative diseases, like such as AD and PD, proteins have the ability to form fibrils [404, 405]. This is true also for mutant and wt-hSOD1 formed *in vitro* [406-408]. These fibrils form insoluble aggregates of different conformations. The structure of *in vivo* formed aggregates is challenging to analyze since the concentration of aggregates in neural tissues are very low. Analysis through conventional methods used on simple protein structures is therefore not applicable. High-resolution methods where crystallization is required cannot be used since the aggregates are too big to form crystals. NMR spectroscopy where proteins are examined in solution require a very high concentration of the desired protein of interest. To by-pass the this problem, protein aggregate-containing material has been extracted from patient tissue and used as seeds to grow fibrils *in vitro*, resembling a similar structure as *in vivo* [409]. Using this method, different Aβ40 fibril strains have been found in two separate AD patients [409] and a structural discrimination has been done between Aβ40 and Aβ42 [410]. The α-synuclein fibril has also been analyzed in a similar fashion [411]. An analogous analysis of the *in vivo* formed SOD1 fibrils still remains to be performed. Another method that stepwise improved its resolution is cryo-electron microscopy, which now is enhanced down to atomic resolution [412]. Although this method is complicated and still have technical challenges a recent study characterized a paired helical tau filament at 3.5 Å resolution from an individual with Alzheimer's disease [413]. To the authors' knowledge, no high resolution characterization of a SOD1 fibril from patient material has yet

been produced. Other methods with lower resolution, e.g. atomic force microscopy (AFM) or attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), might be able to separate two different aggregate structures, but leaves more to be desired in the aspect of aggregate structure characterization [414, 415]. There is still lacking a reproducible and accessible method to determine the structure and quantity of protein aggregates produced *in vivo*. Using our conformation specific anti-peptide antibodies, a novel method to solve this problem was developed.

Binary epitope mapping

Binary epitope mapping is an antibody-based method where binary explains the readout of the method. Either the sequence or epitope targeted by the antibodies are disordered or structured. The antibody binds to disordered flexible parts of the aggregate, while parts involved in the core of the aggregate are sterically hindered to adapt to the antigen binding site. Epitope mapping refers to the panel of antibodies covering 90% of the SOD1 sequence (Figure 4). Antibodies bind misfolded SOD1 but have no reactivity against native hSOD1. Analysis of purified SOD1 aggregate structures, harvested from spinal cords of terminal transgenic SOD1 mice, could now be performed. The aggregates were captured on a cellulose acetate filter that has no ionic interaction with the filtered material. Capturing the aggregates on a cellulose nitrate membrane that binds SOD1 with hydrophobic and electrostatic interactions results in a binding pattern close to SOD1 in solution (see supplementary figure 2B, paper II). By filtering the homogenized mouse tissue, misfolded hSOD1 monomers are removed from the sample. The antibodies do not distinguish disordered monomers from disordered segments on an aggregate, which is why this step is crucial for getting a correct readout. The signal intensity for each antibody on the developed dot-blot together represents a binary fingerprint of the pathological aggregate structure. The novelty in this method rely on a panel of antibodies recognizing the specific 3D structure of the aggregates. Previous studies using similar methods, has been using single peptide antibodies for the identification of aggregated proteins [416, 417]. By using a panel of antibodies, unfolded fringes in different parts of the SOD1 aggregates were characterized. This could be used as a tool to discern different types of *in vivo* produced aggregates from each other.

Two different strains of aggregated hSOD1 in mice

The initial theory was that all hSOD1 aggregates had the same structure, irrespectively of the site of mutation, because of the *in vivo* environment importance for structure formation. We therefore analyzed hSOD1 aggregate structures in $hSOD1^{G93A}$, $hSOD1^{G85R}$, $hSOD1^{wt}$ and $hSOD1^{D90A}$ transgenic mice. Aggregates from the first three mouse models all had similar structures with sequence regions comprising β-strand 1-3 and 5-7 hidden from the antibodies, suggesting

that these areas are involved in the ordered aggregate core. We called this aggregate structure strain A. Surprisingly the hSOD1^{D90A} aggregates had a totally different epitope-mapping pattern, denoted strain B, with region β5 -7 well exposed. These two aggregate structures seemed to differ from previous structural analyses of aggregates produced *in vitro*, although the N-terminal part of SOD1 seemed to be central for the aggregation process also the *in vitro* aggregate [418, 419]. We designed eight different *in vitro* aggregates that were analyzed with epitope mapping. None of these were structurally similar to strain A or B. Strain A and B epitopes were confirmed by a model-free principal component analysis (PCA). More than 96% of the data can be fully explained by these 2 strains, which differed from the *in vitro* produced fibrils.

Further analysis of hSOD1^{D90A} transgenic mice revealed that the ratio of strain A and strain B aggregates increased with age, i.e. in short-lived mice strain B aggregates were predominant. Consequently, there was a linkage between the aggregate structure and the disease progression as similarly seen in prionoid diseases. Strain B aggregates, which are associated with more aggressive disease progression, were also more prone to fragmentation than strain A under denaturing conditions. This follows the principle observed in prion diseases where evidence for a positive correlation between aggregate fragility and neuroinvasiveness/toxicity is present [420].

The high sensitivity of the binary epitope mapping method could also be utilized to identify and quantify aggregate load. In hSOD1^{G93A} mice, aggregates in the spinal cord can be identified from day 14, 26 days earlier than histopathology methods can show evidence of damage [421]. Aggregates underwent an exponential growth with a doubling time of 14 days. This closely resembles the fragmentation-assisted fibrillary growth for apo-hSOD1 monomers *in vitro*, indicating a single mechanism of the kinetics of self-templated aggregation [408, 422]. For hSOD1^{D90A} mice, the growth kinetics is more complex, with both strain A and strain B present. Strain A is growing more slowly than in the other mouse models and first appears around 200 days of growth. Around 250 days strain B aggregates first start to appear. These aggregates then proliferate very fast, raising the total amount of aggregates in the murine CNS. Concomitantly symptom onset generally occurs in the mice.

The aggregate structure within the same mouse is consistent throughout the CNS. There is no difference between aggregates harvested from the spinal cord and brain. This supports the theory that the aggregation process is spreading in the CNS in a templated fashion, as seen in prion diseases [423].

Binary epitope mapping is a fast, straightforward method for analysis of *in vivo* aggregate structure analysis and aggregate growth kinetics, something that today is problematic to investigate. This method has the potential to explore not only the hSOD1 aggregate but might be a useful tool to analyze protein aggregates in other neurodegenerative diseases. In the era of immunotherapy directed

against aggregating proteins, this assay could prove useful when designing therapeutic antibodies.

Recently, a new method for "rationally designed antibodies" has been suggested. This enables the production of monoclonal antibodies directed against any disordered epitope within an aggregate [424]. These antibodies would be interesting to use together with the epitope mapping assay, to further possibly subcategorize different hSOD1 aggregate strains. An antibody scanning strategy, influenced by paper II, has been developed. Here they use a panel of designed antibodies to find sequences in the Aβ42 protein that might be important for aggregate proliferation [425]. Hopefully, the binary epitope mapping will provide more information about neurodegenerative diseases in the years to come.

Paper III

Transmission of two hSOD1 prion strains initiate templated aggregation and fatal motor neuron disease

The data in paper II indicate the high importance of hSOD1 aggregates in phenotype development in transgenic mice. The aggregate structure is the same throughout the CNS and the strains are coupled with different survival times. The motor symptoms usually start in one limb and spread to the contralateral side. This together suggests a focal initiation of the disease dependent with concomitant aggregation of the hSOD1 protein. This resembles the mechanism of prion disorders [426]. To further examine the suspicion that hSOD1 is spreading through a prion-like fashion, we inoculated Strain A and B aggregates into lumbar spinal cords of mice expressing hSOD1. Similar seeding experiments have been done in the AD and PD fields, suggesting the existence of polymorphic prion strains linked to different phenotypes [365-369]. In paper III we indeed show that this is true also in hSOD1 transgenic mice.

Strain A and B aggregates were prepared from spinal cords of hSOD1^{G85R} and young hSOD1^{D90A} mice. Soluble hSOD1 was removed by centrifugation since we aimed to investigate aggregate toxicity only. Ninety-nine and 95% of the hSOD1 in strain A and B seeds were present in aggregates and fibrils; the rest consisted of folded monomers and dimers. No evidence of oligomeric species was found. There were several reasons for choosing hSOD1^{G85R} as the host for inoculations. This model expresses relatively small amounts of mutant hSOD1 [189, 275], thus limiting the potential of artificial phenotypes due to overexpression [282]. The hSOD1^{G85R} mouse model has a long symptom free period with undetectable levels of hSOD1 aggregates, making it easier to discern seeding effects. Sixty-nine days after spinal cord inoculations with minute amounts of strain A seed, the mice showed the first signs of disease. The mouse reached terminal disease stage after 99 days, approximately 200 days earlier than the non-inoculated and control hSOD1^{G85R} mice. This confirms that seeding of hSOD1 aggregates provokes an

ALS phenotype. To test the prion strain hypothesis, we next inoculated the Strain B seed. Unlike strain A aggregates, the strain B structure never forms spontaneously in hSOD1^{G85R} mice (paper II). The disease onset in strain B inoculated mice appeared after 75 days, and the disease progression was markedly slower in this model compared to strain A inoculated mice. The end-stage disease was reached after 124 days. When analyzing the hSOD1 aggregate structure in end-stage inoculated mice, a remarkable similarity was seen between the seed structure and the *in vivo* produced aggregates. Strain A inoculated hSOD1^{G85R} transgenic mice produced strain A aggregates in the CNS, as expected. But strain B inoculated mice produced Strain B aggregates, which is not normally formed in hSOD1^{G85R} mice. This concludes that templated spreading of misfolded and aggregated SOD1 do occur in ALS mice. Strain B aggregates had slower doubling time than strain A (15 and 10 days, respectively), and the spread along the neuraxis was more prominent in those mice inoculated with strain A aggregates. Strain B inoculated mice had a significantly lower concentration of aggregates in the more cranial parts of the CNS compared to the lumbar spinal cord.

In paper II the strain B aggregate was associated with a more aggressive phenotype and a more "explosive" CNS spread. In this setup, it seemed to be the other way around. How is that possible? A speculative answer to this puzzling question might be a "structure-sequence barrier", as observed in the prion field [427]. The hSOD1^{G85R} mouse normally only expresses the strain A aggregates. When introducing strain B aggregates into the spinal cord of these mice, this will serve as a template for aggregation instead. These types of aggregates for some reason are not templating and spreading as good in this host; this may be due to the local environment or that the hSOD1^{G85R} mutation itself favors strain A aggregation rather than strain B. In the prion field it has been shown that amyloid structures have the ability to gradually adapt to its host [428], and become more aggressive in its phenotype. The adaption is thought to occur continuously throughout the host's lifespan. After harvesting aggregates from the already inoculated host and injecting them into a second host, the aggressiveness of the prion disease is increased. This is a phenomenon termed "second passage" [429, 430]. Maybe this mechanism is possible also in second passage strain B inoculated hSOD1^{G85R} mice?

All strain A and strain B-inoculated mice had symptom onset in the hind limbs, and most were first affected on the left side. This corresponds perfectly at the site of inoculation. Amongst the control inoculated mice, seven showed symptoms from the hind limbs and three from the front limbs. Forty percent of the non-inoculated mice had front limb onset. Mice with front limb onset also had a higher amount of aggregates in the cervical spinal cord. Together, these data give further evidence that hSOD1 is the toxic component in these mice, and probably drive the disease in this model.

The histological pictures were different between strain A and strain B inoculated mice. SOD1 immunoreactive granular cytoplasmic inclusion was found in

motor neurons, and neuropil threads were most abundant in the spinal cord. The number of neuropil threads was significantly higher in strain B inoculated end-stage mice, and the amount of cytoplasmic granular aggregates was significantly higher in the inoculated mice in comparison with non-inoculated mice.

In conclusion, the above-described results suggest that the two hSOD1 aggregate strains are ALS-inducing prions. The structures of the strain A and B aggregates are widely different, and they provoke phenotypes that differ in terms of aggregation, disease progression rates, and distribution over the neuraxis, aggregate levels and histopathology.

Paper IV

Relevance of prion strain aggregates in human ALS disease

It is now clear that human SOD1 aggregates from transgenic mice have the potential to transmit disease in a prion-like fashion (Paper III). This is further supported by seeding effects of whole homogenates of spinal cords from end-stage transgenic mice carrying yellow fluorescent protein-fused $hSOD1^{G85R}$ [431]; albeit, a second passage inoculation was needed in order to reach full disease penetration. Initiation of the disease has also been shown from inoculations in peripheral nerves [432]. Moreover, the same group found that homogenates from two patients carrying the $hSOD1^{A4V}$ mutation induce aggregation in spinal cord slices from the above-mentioned transgenic mice, although there was no information of the aggregate structures [433].

Even if the mice express the human version of SOD1, it can still be discussed how relevant these results are for the ALS patients [434]. The above results prompted us to further examine the hSOD1 aggregate strain transmissibility and toxicity, now with aggregates from a human spinal cord. In paper IV, we show that human SOD1 aggregates can seed disease in transgenic hSOD1 mice.

Transmission of mutant hSOD1 aggregates from human patients to transgenic mice

Aggregate seeds were prepared from the ventral horn of the spinal cord from autopsy material belonging to an ALS patient carrying the truncated $SOD1^{G127X}$ mutation. A neopeptide with amino acids GQRWK is inserted at position 127, a mutation that causes severe instability of the native hSOD1. The human as well as mouse $hSOD1^{G127X}$ aggregate seeds were inoculated in mice carrying the $hSOD1^{G85R}$ mutation. The aggregate preparation and the inoculation protocol in paper IV are similar to that in paper III, with slight improvements in aggregate purification and mouse sedation technique. Seven new, shorter polyclonal peptide-antibodies were also added to the binary epitope mapping protocol.

The hSOD1^{G127X} mouse model developed aggregates with a structure closely resembling strain A aggregates. This was further confirmed by the new panel with seven novel antibodies, where the binding pattern was similar to the original eight antibodies. However, the two antibodies raised against the neopeptide GQRWK (111-127GQRWK and 123-127GQRWK) had a markedly high signal in the hSOD1^{G127X} aggregates. The explanation for this is probably an increased flexibility of the C-terminal truncated part including the neopeptide in the hSOD1^{G127X} protein.

The number of aggregates in the murine hSOD1^{G127X} seed was 3 ng/µl, but the human seeds contained only 0.14 and 0.04 ng/µl. To explore if the difference in aggregate concentrations between mice and humans would affect the result, we performed a dose-response study with hSOD1^{G85R} strain A aggregate seeds. There was no difference in survival between mice inoculated with either 1 ng/µl or 3 ng/µl hSOD1^{G85R} seed. These mice developed premature fatal motor neuron disease with a mean survival of 100 days. When reaching down to a concentration of 0.33 ng/µl hSOD1^{G85R} seed per inoculate, a longer survival (mean of 240 days) of recipient mice were observed. This was yet a significantly shorter survival than the non-inoculated mice. At a concentration of 0.11 ng/µl hSOD1 aggregates per seed, no difference in survival time could be observed between the inoculated and non-inoculated mice. The seeding efficiency is thus concentration dependent.

Inoculations of murine hSOD1^{G127X} seed caused end-stage disease 86 days after inoculation. Mice inoculated with human seed I and II (see material methods paper IV) had mean survivals time of 216 and 196 days after inoculation, respectively; these were significantly shorter than mice inoculated with human control seed. Thus, the human hSOD1^{G127X} seeds seemed more potent than seeds from murine hSOD1^{G85R} seeds. This result fails to confirm a presence of a species barrier in this hSOD1 model system, as observed with other proteins in the prion field [430].

The inoculated hSOD1^{G127X} seeds induced strain A aggregation in the hSOD1^{G85R} mice. Previously, studies in AD have utilized the seeding principle *in vitro* to easier identify the molecular structure of Aβ fibrils in patient material [368]. In paper III, we show that hSOD1^{G85R} mice have the ability to develop strain B aggregates when seeded with hSOD1^{D90A} aggregates. If one assumes that the human hSOD1^{G127X} aggregate seed also provoke templated aggregation in this mouse model, this indicates that also ALS patients carrying the SOD1^{G127X} mutation develop strain A aggregates. Confirmative studies need to be performed to assess this fact.

In conclusion, hSOD1 aggregate seeds prepared from the spinal ventral horn of an ALS patient carrying a hSOD1 mutation can initiate fatal motor neuron disease in hSOD1 transgenic mice. The disease was initiated in a cellular environment close to that in human patients. This suggests that prion-like spread of hSOD1 aggregation could be the primary pathogenic mechanism in hSOD1-induced ALS in humans.

Conclusions

- Transgenic mice expressing wt-hSOD1 at a rate close to the commonly studied $SOD1^{G93A}$ ALS model develop a fatal motor neuron disease after 370 days. Thus, wt-hSOD1 has the ability to cause ALS.

- Since wt-hSOD1 is disease causing in transgenic mice, hemizygous wt-hSOD1 mice are not suitable as negative controls.

- We developed a novel, highly sensitive, method called binary epitope mapping capable of determining structure and quantity of SOD1 aggregates produced *in vivo*.

- Two different aggregate strains were identified in hSOD1 transgenic mice, with different structural architectures, molecular properties, and growth kinetics.

- Aggregate strains of hSOD1 transgenic mice, which are different from *in vitro* produced hSOD1 aggregates, seem to be under fragmentation control and are associated with different disease progressions.

- The aggregate strains can be seeded in $hSOD1^{G85R}$ mice, causing templated, spreading aggregation of hSOD1, and concomitant premature fatal motor neuron disease, suggesting the aggregate strains are ALS-inducting prions.

- Human SOD1 aggregate seeds prepared from the spinal ventral horn of an ALS patient carrying a SOD1 mutation can initiate fatal motor neuron disease in hSOD1 transgenic mice.

- Taken together, the results of this thesis suggest that different hSOD1 aggregate strains exist in hSOD1 transgenic models and that a prion-like spread of hSOD1 aggregation could be the primary pathogenic mechanism not only in hSOD1 transgenic models but also in human ALS.

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