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NEW CONCEPT: AGING AS MAJOR REGULATOR OF ALS PATHOLOGY

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Susanne Elise Baars

December 2012

Image on front page:
Researchers suggest that stimulation of cell signaling delays the onset of ALS, by causing motor neurons 'humming'.
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HUBRECHT INSTITUTE

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Final report:

Period: 12-11-2012 – 15-01-2013

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ACKNOWLEDGEMENT

I would like to express my very great appreciation to Professor N. Geijsen, my supervisor, for his enthusiastic and patient guidance and useful assessment of this thesis. I would also like to extend my thanks to the technician of his laboratory of the department Pluripotent stem cells in development and disease, for her help in learning me the basics of working with induced pluripotent stem cells (iPSCs). My grateful thanks are also extended to Professor R.J. Pasterkamp, for willing to be second reviewer.

ABSTRACT

Amyothrophic lateral sclerosis (ALS, Lou Gehrig's disease) is a severe adult-onset neurodegenerative disease, characterized by progressive premature loss of upper and lower motor neurons. Eventually the disease leads to death due to respiratory failure[1]. ALS is the third most common neurodegenerative disease[2] and cause of adult mortality[3]. It appears to be caused by a complex interaction among genetics and environmental factors[2]. While approximately 10% of ALS cases are familial, the remaining cases are believed to be sporadic. However, the exact working mechanism underlying the selective motor neuron death in ALS is not resolved yet. Since a wide variety of cellular processes are involved in ALS, a general, systemic principle might underlie it.

Recent data provides compelling evidence for a major role of aging in the development of ALS[4,5,6,7.] This hypothesis was enhanced by the discovery that reduced Insulin/IGF signalling (IIS) correlates with lifespan extension[8] and a decreased risk to develop ALS[9]. Its pathway is not only regulating lifespan, metabolism and stress resistance[4], TDP-43 has also showed to be part of it, a well known ALS gene[9]. TDP-43 is primarily a nuclear protein that participates in common heteromultimeric complexes, which is involved into diverse RNA processes and stress granule formation[16,10]. Furthermore, TDP-43 is identified as the major component of insoluble cytoplastic inclusions in both sALS as fALS, and associated with the oxidative stress response. It seems likely that this TDP-43 protein mediates longevity through activation of the IIS pathway under stressfull conditions[11]. Besides the activation of various cellular cascades, there is a vicious circle in which TDP-43 expression becomes upregulated. Interestingly, it has been found that during stress, the threshold for TDP-43 phosphorylation is lowered. This lead to increased proteotoxicity[12]. This in turn seems to cause and/or to accelerate motor neuron degeneration.

However, besides aging, also heavy exercise is in part mediated by the IIS pathway and involved in ALS development[13]. During heavy exercise, enormous amount of ROS are generated which causes oxidative stress and disturb the epigenetic codes[14].

Interestingly, age-related changes seemed to be determined by the age of the systemic environment, rather than by the cell-autonomous age[15]. Therefore, it has been suggested that signals from the systemic environment drive age-related, intrinsic changes, mediated by the IIS pathway. This activates a cascade of events that eventually can lead to ALS pathology. However, current animal models are not suitable to further investigate this postulation. Not only did the obtained results fail when translated to the human situation, also are fundamental genetic and anatomical differences between both species in age-related pathways found[16,17]. Therefore, new and improved models are needed. In vitro cell based therapies seems to be the first feasible model to investigate the disease mechanism for both sALS and fALS by utilizing induced pluripotent stem cells (iPSCs)[18]. iPSCs can give us insights into disease mechanisms, drug discovery, cell therapy and a potential new diagnostic method for patients with ALS[19,20,21]. Furthermore, will this technique enable the identification of a systemic regulation of aging on ALS pathology in patient derived iPSCs.

ABBREVIATIONS

ALS	Amytrophic lateral sclerosis
MN	Motor Neurons
UMN	Upper Motor Neurons
LMN	Lower Motor Neurons
FTLD	frontotemporal dementia
sALS	Sporadic ALS
fALS	fALS
ER stress	endoplasmic reticulum stress
UPR	unfolded protein response
IGF1	insulin-like growth factor 1
TNFα	tumour necrosis factor
BBB	blood brain barrier
BSCB	blood spinal cord barrier
MP2	myelin protein 2
MMp9	matric metallopeptidase 9
iPSC	induced pluripotent stem cells
ESCs	Embryonic stem cells
Human iPSC	Human Induced Pluripotent Stem Cell

INTRODUCTION

Amyothrophic lateral sclerosis (ALS, Lou Gehrig's disease) is a severe adult-onset neurodegenerative disease, characterized by progressive premature loss of upper and lower motor neurons (MNs) in the motor cortex, the anterior horn of the brainstem and spinal cord which eventually leads to death due to respiratory failure[1]. Next to being third most common neurodegenerative disease with a worldwide prevalence of 4-6 per 100.000[22], ALS is also one of the most common causes of adult mortality in this disease group. The peak age of onset is between 50 and 70 years[2].

The broad variety of disease manifestations in ALS appears to be caused by a complex interaction among genetics and environmental factors. Approximately 10% of ALS cases are familial, while the remaining cases are believed to be sporadic[23]. Although the enormous efforts put into unravelling the underlying disease mechanism, no efficacious drug or therapy has been developed yet[24]. Numerous hypothesises have been proposed to underlie ALS, but none of them is able to fully explain the mechanism of pathogenesis.

AIM OF THIS THESIS

Within this thesis I will review the proposed disease mechanisms of ALS by discussing the current hypothesizes and models that are used to investigate it. Furthermore, I will propose a new concept with respect to ALS pathology and outline a research proposal for future investigation.

ALS PATHOLOGY AND DIAGNOSIS

ALS pathology is characterized by loss of motor neurons in the anterior horns of the spinal cord, motor nuclei of the brain stem and motor cortex, which together make up the corticospinal tract (with a relative sparing of sensory neurons)[25],[23]. Signals are transmitted from motor neurons in the brain (upper motor neurons, UMN) to motor neurons in the spinal cord (lower motor neurons, LMN). From there they are passed through to particular muscles. Degeneration of these motor neurons ceases signal transmission, which result in muscle weakness, spasticity, fasciculation's and atrophy throughout the body[26]. Eventually progressively loss of voluntary movement is observed[27]. In the contrary to other anterior horn diseases and motor neuropathology's, such as spinal muscular atrophy or primary lateral sclerosis, ALS shows degeneration of both upper and lower motor tracts[28]. The primary clinical symptoms and signs of ALS include impairment affecting limb, bulbar, axial and respiratory function. Specific UMN signs are hyperreflexia, spasticity, extensor plantar response (up going toes), and positive jaw jerk, while LMN signs include weakness, muscle atrophy, cramps and fasciculation's. However, differences in site of onset, pattern and speed of spread as well as the degree of upper motor neuron (UMN) and/or lower motor neuron (LMN) dysfunction produce a remarkably variable disorder[29]. Notably, also frontotemporal dementia (FTLD) is seen in 5% of the cases[30].

Although, the clinical diagnosis is primarily based on clinical manifestations, it is supported by laboratory studies, electrodiagnostics and imaging (Supplementary Table.1). At molecular and cellular level, a wide range of processes are associated with ALS as well, such as protein misfolding and aggregation (ubiquitinated and neurofilamental inclusions) and mitochondrial damage. Also energy deficit, ER stress[31], changes in calcium homeostasis[32] and excitotoxicity are seen[33]. Furthermore, specific vulnerability of motor neuron degeneration is determined by its characteristics (for a overview about motor neuron vulnerability features, see supplementary table.2). Additionally, degeneration of motor neurons is often accompanied by reactive gliosis[34].

These processes have all been intensively investigated and will be shortly reviewed in the next sections.

CAUSES OF ALS

ALS seems to be a multifactorial disease, which appears in a broad variety of disease manifestations caused by a complex interaction among genetics and environmental factors. People of all races and backgrounds are affected[35], but there appears to be a slight male predominance[36]. Although the exact working mechanism remains unclear, ALS is most often seen in elderly individuals, which indicates that aging might be an important risk factor[5].. Furthermore, multiple external risk factors have been associated with ALS, such as: ingestion of high concentrations of β-methylamino- L-alanine[37], use of cholesterol-lowering medication[38], intensive physical exercise[39] including football playing[13,40] and service in the USA Army[41]. The latter possibly links to intermittent work-related hypoxia[42] or to head injury[43, 44]. Other environmental factors include exposure to heavy metals[45], cigarette smoking[46], and pesticides or herbicides[47]. Although exposure to toxics initially seemed to be important risk factors, no additional evidence has been found in the last 25 years[48]. Lastly, associations are found between ALS, viral infections (HIV)[49] and prion disease[50].

GENETICS

Besides environmental factors, several genes have been associated with ALS as well. Approximately 90% of patients[51] suffering from ALS don't have a family history, which is defined as sporadic ALS (sALS)[23]. The residual cases are inherited termed familial ALS (fALS). Whereas fALS is known to be caused by mutations in at least 10 different genes[52], sALS do not show a defined genetic profile. Genes which are associated with ALS include a wide range of cellular processes, namely: involvement into antioxidant response[53], axonal and vesicular transport[54], angiogenesis, endoplasmic reticulum (ER) stress and unfolded protein response (UPR) and RNA metabolism[33].

fALS is inherited as a dominant trait with different penetrance and expressivities among ALS patients[23]. Mutations in the SOD-1 gene are the most common cause of fALS (20%), followed by mutations in TDP-43 (5%), FUS (5%), and ANG (<1%). Due to recent developments, more genes become associated with this disease. Mutant SOD-1 was the first gene associated with fALS and many animal models have been developed to further investigate the working mechanism of ALS. However, since this account only for about 20% of the familial cases, other genes are still under intense investigation. However, the cause of sporadic ALS (sALS), in addition to fALS, remains unsolved[55]. Therefore, environmental causes are thought to play an important role. However, many genes, which are found in fALS, are also affected in sALS, such as SOD1 mutations (5%). Also, similar conformational changes within the wild-typ SOD-1 and TDP-43/FUS gene have been discovered within sALS patients compared to their mutants. This suggests that modified wild-type SOD-1 and TDP-43/FUS are able to contribute to the same disease mechanisms.

Moreover, fALS is clinical indistinguishable to sporadic sALS[56], since they share the same symptoms and pathological phenotype, what highlights the similarity between both forms of the disease[57].Even survival of affected family members which harbours the same mutation in the same gene have showed to be highly variable[58]. This variability is thought to be caused by genetic modifying factors. However, further research is needed to elucidate the role of genetics in ALS.

ALS DISEASE MECHANISM AND MODELS

This section will discuss the disease mechanism of ALS and the most commonly used models with their application.

DISEASE MECHANISMS

The exact working mechanism underlying the selective motor neuron death in ALS is not resolved yet, although numerous hypothesizes have been proposed. These hypothesizes include proteotoxicity, glutamate excitotoxicity, oxidative stress (mitochondrial dysfunction), proteasome inhibition (aggregate formation and ER stress), non-cell autonomous function mediated by astrocytes and glial cells, compromised axonal transport, inflammation, neurovascular and RNA metabolism. The SOD-1 transgenic animal model has been most widely used in ALS research, since this gene was first identified to be associated with significant subpopulation of both fALS as sALS.

This section will briefly outline the core principles of each hypothesizes. Interestingly, although they all seem plausible, either one of them can be the primary cause of the development of ALS, but also be a consequence of an earlier underlying event. People have always considered ALS as a pure motor disorder. However, subsets of patients have emphasizes the involvement of other cell types and working mechanisms as well. The multitude of contributing factors does underline the complexity of the disease. Nowadays, ALS is more seen as a multisystem disorder in which motor neurons appear to be the most severely affected[59].

PROTEOTOXICITY

Accumulation of aggregated proteins is the major phenotypical hallmark of ALS pathology [60, 61]. Interestingly, this is seen in almost all neurodegenerative diseases, including Alzheimer's disease[62], Parkinson's disease[62], frontotemporal dementia (FTLD) and Huntington's disease[63], but also in prion disease[64]. These protein aggregates consist of damaged and misfolded proteins that cannot be removed by the normal protein degradation mechanisms[65]. TDP-43 was identified as the major component of these insoluble inclusions in both sALS as fALS. After this discovery, FUS has also been identified to be components of protein aggregations. TDP-43 and FUS are both primarily nuclear proteins that participate in common heteromultimeric complexes which are enrolled in RNA transcription, translation, splicing, nucleo-cytoplasmic shuttling, transport for local translation, and stress granule formation[16,10]. Research suggests that they interact with other proteins by binding glycine-rich domains. Under pathological conditions, they are redistributed to the cytosol where they form abnormal protein-protein associations[66]. Notably, SOD-1, another well known ALS causing gene, has also been identified in protein aggregations[50]. However, toxicity caused by this gene seems not correlated to its aggregation potential. Therefore, further research proceeded on the function of aggregate prone proteins and their proteotoxicty in relation to ALS.

Proper protein folding has shown to be crucial for cellular function and viability[67]. During cell division, damaged and oxidized proteins are sequestered and incorporated by the mother cells. This mechanism enables daughter cells to consist of an undamaged proteome[68]. However, neurons, which are post-mitotic cells, do need another mechanism to ensure protein quality[67]. Many proteins do fold spontaneously but they do need assistance to correct their structures by a specialized set of chaparones, which perform a quality-control process as well. Nonetheless, aggregation-prone proteins challenge these systems, by making them fail to handle them. Also, protein homeostatic system becomes less efficient and less effective during aging[69].

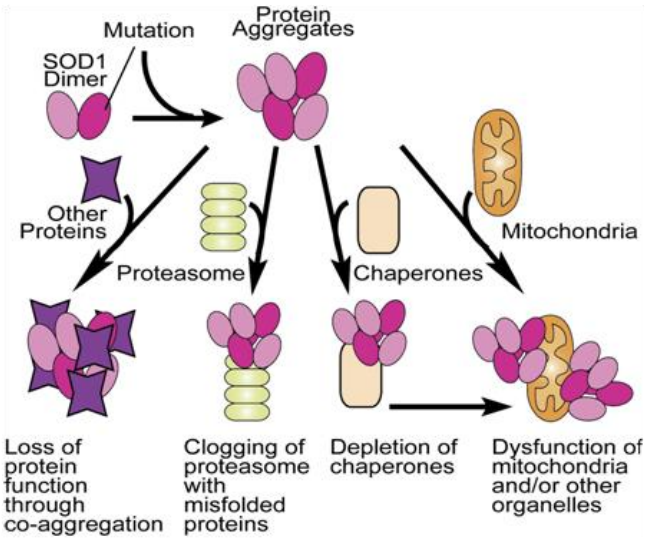


Fig.1: Supposed mechanism of protein aggregation. Aggregation prone proteins causes co-aggregation of other proteins and impairment of proteasome activity. This lead to distortion of other cellular proteins, such as for chaperones and mitochondrial dysfunction. *(Image adapted from Boillée et al, 2006).*

Unfortunately, a failure to properly fold one protein may result in protein aggregations, which can destabilize the proteome even further. This may lead to uncontrolled aggregation of other proteins as well and causes proteotoxicity[70]. Moreover, when misfolded or damaged proteins are not removed, this disturbs cellular process and can trigger mitochondria dysfunction and the unfolded protein response (UPR), which lead to endoplasmatic reticulum (ER) stress. Beside, studies have shown that mutant SOD-1 activates components which mediate ER stress[31]. Eventually ER stress can cause cell death through caspases-mediated apoptosis. Furthermore, protein aggregation have also shown to cause mitochondrial dysfunction (Fig.1)[71]. Nonetheless, there is no universal agreement as to what function they have in disease pathology.

Moreover it is still under debate if protein aggregates could either directly cause neuronal dysfunction and death, or that they arise as a harmless by-product, or if they arise to protect cells by sequestering of toxic proteins. Major evidence for protein aggregation as primary cause is given by the fact that almost all ALS models feature prominent, cytoplasmic, intracellular inclusions in motor neurons as well as in the astrocytes surrounding them[72]. They are highly immunoreactive and develop often before onset of clinical symptoms, as an early sign of ALS[73]. Aggregative proteins have showed to behave in a dose dependent manner, with enhanced aggregations during disease progression, and to generate motor neuron degeneration[11].

Several mechanisms have been suggested to cause toxicity in motor neurons. On the one hand, mutant SOD-1 aggregates may assemble other proteins, which are normally required for neuronal function[74]. On the other hand, aggregations are caused by a reduction of protein-folding chaperones, which are needed to catalyze folding of other proteins as well[75]. Therefore, mutant SOD-1 aggregates causes proteosomal dysfunction, which lead to disrupted protein turnover. Furthermore, imbalance of protein synthesis and degradation compromises its ability to degrade other critical components as well. Finally, almost all aggregates seems to co-express ubiquitin. This was an interestingly finding, since ubiquitin targets proteins to the proteasome degradation pathway for clearance[71]. This implies that accumulation of ubiquitinated, misfolded proteins, may affect the proteasome and impair normal protein degradation of proteins, such as SOD-1.

However, recent research has provided evidence consistent with each of the above-mentioned mechanisms. While it was previously assumed that high molecular mass aggregates are the cause of ALS, recent data in this field implies that small oligomeric aggregative structures, which are highly toxic to the cell, may underlie pathology[76,77]. Therefore the production of less-toxic large aggregates seems to be a protective, compensatory mechanism to detoxify the cell as much as possible[78,79]. It has now been suggested that during lifespan, the cell's capacity to remove these small toxic aggregates become reduced. This could be either the result of an increased amount of small toxic aggregations during lifetime or to a diminished clearance capacity of the cell during disease progression. In either way, more toxic aggregations appear, which causes damage that may lead to neuronal death. This mechanism seems especially important in low turnover cells (i.g. motorneurons), since protein aggregates are not diluted as in high turnover cells due to cell divisions. Moreover, mother cells retain aggregates during early divisions and buddying, which protect new cells from damage.

Taken together, aggregates are seen in almost all neurodegenerative diseases. This accumulation of misfolded proteins appears to disrupt the proteasome degradation pathways and induces ER-stress. The selective vulnerability of aggregates on motor neurons in ALS has to be elucidated.

Current literature has linked multiple protein aggregation to ALS pathology[9,62,80]. However, until now, only SOD-1 and TDP-43/FUS protein aggregations have been found to contribute to ALS pathology. Although proteotoxicity shows clear indications for the development of ALS pathogenesis, it could be argued if this is the primary cause of ALS, or that these proteins form aggregates upon stimulation of another mechanism. Initiation of protein misfolding includes random processes, caused by a reduction of protein folding chaperones, proteosomal dysfunction or by the formation of abnormal protein-protein interactions[80, 81.] Therefore, alterations in other proteins are expected to be involved into ALS pathology as well. Questions arise about the working mechanism of proteotoxicity in ALS, since protein aggregations of only certain proteins has been observed to cause ALS[82].

Protein aggregations have been associated in numerous neurodegenerative diseases. For instance, the accumulation of hyperphosphorylated tau protein, which is involved into the pathogenesis of Alzheimer Diseases (AD)[83]. Interestingly, certain tau isoforms have been reported in other neurodegenerative diseases as well, such as in ALS[84]. However, not all patients that expresses tau protein aggregates develop ALS pathology[85]. This creates doubts about the specificity of the aggregate protein and may suggests that proteotoxicity is mediated by another mechanism rather than causing ALS itself. In this case, protein inclusions and aggregates may represent an end stage of a molecular cascade, while earlier steps in this cascade may be more directly involved into pathogenesis.

Nevertheless, studies support the idea that at least one aspect of toxicity may arise by processes mediated by misfolded aggregated proteins or by loss of essential components as consequence of protein aggregate formation[72].

GLUTAMATE EXCITOTOXICITY

Glutamate is the main excitotoxin in the brain, albeit being major excitatory neurotransmitter in the mammalian CNS[57]. During normal homeostasis, glutamate can be increased in the synaptic cleft, but this level will rapidly decrease. In ALS pathology, high glutamate levels are maintained due to increased glutamate release or an impaired re-uptake mechanisms (Fig.2)[32]. These high levels of glutamate stimulate presynaptic glutamate receptors, which subsequently enhance the release of additional glutamate via a positive feed forward mechanism. This causes increased intracellular Ca2+ levels, which lead to excitotoxicity and eventually cell death. Interestingly, excitatory amino-acid transporters (EAAT2) are often down regulated in the motor cortex and spinal cord of ALS patients[71]. These EAAT2 receptors are the major terminator of excitatory signals through re-uptake of glutamate from the synapse into glial cells and neurons. Eventually opening of mitochondrial permeability pores will occur due to high calcium concentrations[86]. Consequently mitochondria swell and release reactive oxygen species (ROS) along with other proteins that stimulate apoptosis.

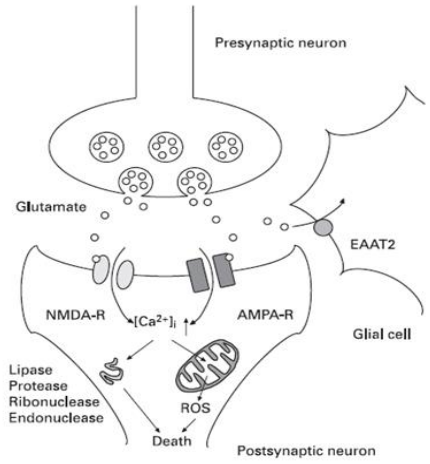


Fig.2: Excitotoxicity in ALS. Excessive glutamate lead to enhanced receptor stimulation. This causes increased glutamate release and inadequate removal and re-uptake. Therefore, electrochemical gradients change and the receptor function converse. Increased levels of calcium activated enzymes that are toxic to the cell. Subsequently, reactive oxygen species (ROS) is produced, which impairs mitochondrial function. This viscious circle eventually lead to excitotoxicity and degeneration of motor neurons in ALS. (Image adapted from Van dan Bosch, et al., 2006)

The pore can also stimulate the mitochondria to release even more calcium, to inhibit the production of adenosine triphosphate (ATP) and to activate ATP synthases, which hydrolyses ATP instead of producing it. Elimination of energy metabolism may distores the electrochemical gradients of certain ions. These gradients are needed to remove glutamate from the extracellular space by activation of glutamate transporters. However, when the electrochemical gradient changes, this causes a reversal in transporter function: instead of removing glutamate they now act as active secreting glutamate channels, which enhance excitotoxicity even more(fig.2)[87]. Moreover, besides the influence of calcium influx on excitotoxicity, it has recently been noted that extra-synaptic NMDA receptor activation stimulates CREB protein inactivation. This results in loss of mitochondrial membrane potential and apoptosis. Additional research on selective motor neuron vulnerability, showed extremely sensibility of motor neurons to excitotoxicity. This selective sensibility is caused by a low Ca2+ buffering capacity and a high number of Ca2+ permeable AMPA receptors[32].

All in all, this hypothesis shows one of the most robust mechanisms and is supported by much evidence. Moreover, the only drug available on the market to slow disease progression is riluzole, which has anti-excitotoxic properties. However, how this excitotoxicity arises remains to be questioned.

It could be argued that if protein aggregates, formed by mutant gene products (as discussed in the previous paragraph), might assemble in motor neurons and/or decrease glutamate uptake in the surrounding glial cells[57]. Interestingly, recent research has found that glutamate hyperstimulation leads to the formation of microaggregates in iPSC-derived neurons. However, further research is needed to investigate if excitotoxicity may underly proteotoxicity as well. Also in this context, it remains unclear why this process would especially involve SOD-1, TDP-43/FUS protein aggregation.

OXIDATIVE STRESS

Oxidative stress derives from an accumulation of reactive oxygen species (ROS), which arise as by-products of aerobic metabolism[88],[87]. With aging, oxidative stress accumulates within the neuron and may cause a reduction in the ability of the biological system to eliminate or to repair ROS-induced damage.

Cellular ROS is caused by a leakage of electrons from the mitochondrial respiratory chain, due to an imbalance between the production and the removal of reactive oxygen. Particularly, the early components from the electron transport chain, complexes I and III, have showed to be prone to leak electrons to molecular oxygen[89]. Therefore mitochondria are the major source of ROS production, followed by the endoplasmic reticulum.

Under normal conditions, the cell has multiple anti-oxidant defence mechanisms to remove ROS and to prevent oxidative stress. One of these mechanisms includes the catalytic removal of ROS by superoxide dismutase, which is mainly regulated by SOD-1. However, mutations in SOD-1 disrupt enzyme function and leads to increased ROS production, which results in enhanced oxidative stress (Fig.3)[90,73]. Moreover, research showed reduced proteasomal activity in both, mutant SOD1 and TDP43/FUS expressing mice, which is caused by aggregations that disrupt the ubiquitin-proteasome system[91]. Under normal conditions this degradation pathway protects the cell by taking up damaged, misfolded and unfunctional proteins. Dysfunction of the proteasome and lysosome systems eventually lead to mitochondrial dysfunction with increased oxidative stress[92]. The process by which ROS creates oxidative stress is as followed: Firstly, reactive oxygen causes incomplete reduction of molecular oxygen during oxidative phosphorylation and lead to the production of hydrogen peroxide (H2O2) and the superoxide radical anion (O2-). Subsequently, ROS will react with radical nitric oxide (NO) and will form the oxidant peroxynitrite (ONNOO-)[93,94], and H2O2 will eventually form the highly reactive hydroxyl radical, OH. These radicals causes damage to proteins, lipids and DNA[53]. This can alter protein conformations and change the active site of the enzymes, change membrane properties by oxidation, and introduce mutations into DNA/RNA. Notably, some components of the electron transport chain are encoded by mitochondrial DNA. Mitochondrial DNA is already exposed to a higher mutation rate compared to nuclear DNA[92]. However, once oxidative stress is present, other mechanisms become involved as well, such as excitotoxicity. Excitotoxicity causes increased intracellular calcium levels, which enhance ROS production. Nonetheless, ROS seems to inhibit glutamate uptake through loss of EAAT2 transporter expression and enhanced expression of calcium permeable AMPA receptors in glial cells. Furthermore, activation of microglial results in secretion of cytokines and further ROS. Lastly, mutant protein aggregates inhibit neurofilament assembly and cytoskeletal transport.

Thereby, the central nervous system appears to be more susceptible to oxidative damage than other tissues. This is caused by the accumulation of metal ions throughout the CNS, which favor ROS production[33]. The selective vulnerability of motor neurons may be caused by their high

metabolic input. Motor neurons have a relative big size and long axon length. This high-energy demand goes along with many mitochondria, with the side effect of increased oxidative stress.

Taken together, oxidative stress regulates multiple mechanisms, but in light of ALS, neuronal degeneration may result from a complex interaction of ROS, excitotoxic stimulation, genetics and dysfunction of proteins and organelles (e.g. mitochondria, ER) [53]. Furthermore, the neurotoxic effects of ROS are not only limited to neurons, but could also affect glial cells. However, further research has to be performed to determine the causative role of oxidative stress in ALS manifestation.

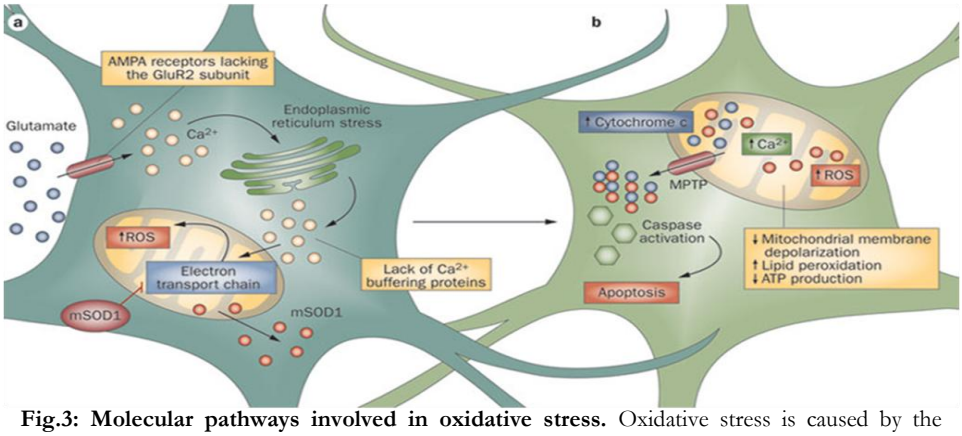


Fig.3: Molecular pathways involved in oxidative stress. Oxidative stress is caused by the enhancement of ROS, excitotoxicity and protein aggregates. (Image adapted from Ferraiuolo, L., et al. 2011) [57].

COMPROMISED AXONAL TRANSPORT

While previous stated mechanisms share many aspects and seem to be related to each other, compromised axonal transport includes a distinct mechanism.

Since motor neurons have extending axons that can be more than a meter in length, efficient transportation systems are needed. In both, patients and animal models of ALS, alternations in large (myelinated) axonal structure by misaccumulation of neurofilements have been reported. Also the two forms of axonal transport, kinesin-mediated (anterograde) and dynein-mediated (retrograde), are decreased [90]. Notably, transgenic mice which express a mutation in the NF-L subunit of neurofilament, which is similar in human, develop motor neuron degeneration[95]. However, deletion of this mutation reversed the phenotype partly and caused prolonged survival[96],[97]. Consistently, presymptomatic deficits of slow axonal transport has been described in mutant SOD1 mice[98], which showed similar misaccumulation of neurofilement.

Furthermore, genetic evidence implicates axonal transport defects as an important mechanism in the development of motor neuron degeneration. Altered gene and protein expressions have been found in multiple genes associated with transport, like: loss of kinesin motor proteins KIF1Bβ[99], KIF5a[100], KIF21A[101], VAPB[54], Rab7[102], and Vps54[103]. Also, mutant SOD-1 mice showed significant inhibition of retrograde axonal transport before onset of the disease[90].

Although many of these features are similar to human ALS pathology, abnormalities in neurofilaments could be either causal or just being a harmless byproduct of neuronal degeneration of mutant SOD1 protein. Moreover, similar as with other protein aggregations,

accumulation of neurofilaments in motor neuron cells could also arise from a protective view of perspective: to protect the neuron against SOD1-mediated damage by buffering diverse components. Nonetheless, it has been shown that particular motor neurons, are highly sensitive to defects in axonal transport[90]. This may be due to their extreme polarization. So while protein aggregation, glutamate hyperstimulation and oxidative stress are linked, axonal transport seems to be a separate potential cause.

ASTROCYTES AND GLIAL: NON-CELL AUTONOMOUS DEATH OF MOTOR NEURONS

Although most research is performed on previous outlined hypothesizes, there is growing evidence for a non-cell autonomous death of motor neurons. This means that the degeneration is not caused by the motor neurons themselves but rather through extracellular influences. Research showed that glial cells play a particular important role in this. On the one hand, astrocytes support neurons by providing them with nutritional factors and regulate neurotransmitter concentrations, metabolic or ionic homeostatis[104]. While on the other hand microglial cells clear damaged and dead cells from the environment. Gliosis is an early process seen in pathogenesis in both the brain and the spinal cord[105]. Although ALS is caused by motor neuron degeneration, neighbouring cells may mediate this in a non-cell autonomous manner. Consistently, all known familial forms are caused by mutations in genes that are ubiquitously expressed (i.g.*SOD1* and *VAPB*) throughout the body or at least expressed in multiple cell types (i.g. *VEGF* and *ANG*). Therefore, researchers started to examine the role of glial cells.

To investigate this in detail, chimeric animals were developed that express mutant SOD-1 selectively in motor neurons, microglial cells or astrocytes [106]. Results showed that expression in all three cell types were needed to cause ALS. After this, they removed 30-50% of the mutant SOD-1 gene in motor neurons and found a delayed onset while disease progression remained the same[107]. Animals who expressed a combination of mutant and wild type SOD-1 showed a positive correlation with the proportion of surviving mutant SOD-1 expressing motor neurons and lifespan of the mice. In addition, research with C.elegans has confirmed this. Glial cell activation also increases after motor neuron injury. After damage, motor neurons seem to release mutant porteins, fragments or distressed signals to the extracellular environment. It has been proposed that glial cells then start to secrete protective components. However, if the stimulation becomes chronically, toxic substances are released instead. These released proteins are involved in the production of nitric oxide or inflammation, to activate other glial cells. However, it is not known how they become activated in the first place, neither how this especially involves motor neurons. It could be that this results from specific signals released from the motor neurons after injury as stated above. Nonetheless, it seems that this active dialogue between motor neurons and glial cells contributes to disease progression.

Since fALS and sALS show similar disease manifestation, it is suggested that a more general principle is causing the disease. A non-cell autonomous mechanism might underlie this principle[107]. However, existing disease models do not give the possibility to research a general mechanism for fALS and sALS. Therefore, induced Pluripotent Stem Cells (iPSCs), an upcoming in vitro technique, used more and more to further decipher the influence of astrocytes and glia on disease progression[107]. Evidence deriving from this novel technique will be dicussed in more detail in a later chapter.

INFLAMMATION: VIRAL INFECTION AND IMMUNE IMBALANCE

A common feature of ALS is the presence of neuroinflammatory reactions consisting of activated glial and T cells. Until recently, this feature has been viewed as a consequence rather than a possible cause of motor neuron degeneration[108]. However, focus has now been put on inflammation as a target to treat ALS[109]. Thus, instead of non-cell mediated motor neuron degeneration caused by intrinsic properties of glial cells, a primary role of inflammation has also been proposed.

Upon injury, microglial accumulate. Investigators have discovered a positive correlation between this microgliosis and disease progression[110]. Although it was already known that microglia becomes increasingly activated after motor neuron injury, research now also showed activation of microglia before motor neuron degeneration and thus before clinical disease-onset[109]. Animal models were used to further investigate the potential link between inflammation and motor neuron degeneration.

Microglia are the macrophages in the nervous system. Next to monitor the extracellular environment, they closely interact with astrocytes and neurons. This is mediated by the M1 activation pathway. They can be identified as CD11b expressing cells and are activated by a range of different signals. Futhermore, they act as the first line of defence against infection or injury. When activated, they acquire an amoeboid like appearance and start to secrete proinflammatory molecules, like: tumour necrosis factor TNFα, interferon-γ, and interleukin 1-β. Furthermore they also upregulate NO and O_2. This mechanism protect against invading organisms and clears hazards. Besides this, activated microglia also mediate T-cells infiltration into the CNS and modulate the neuroinflammatory reactions. To regulate the extant of the immune response neighbouring astrocytes and inflammatory T-cells release anti-inflammatory molecules via the M2 activation pathway, e.g. insulin-like growth factor 1 (IGF1), interleukin 4 and 10.

In more detail, T-cells seemed to be the critical components in mediating disease progression. They are protective prior to onset of disease, but when the disease proceeds, T-cell number increase and their cytotoxic influence outweigh their neuroprotective effect. It has been suggested that the absence of this neuroprotective effect, mediated by the immune system, determines disease onset[111]. Research have found aberrant proinflammatory cytokines and an increased number of proinflammatory mediators in patients with ALS. Moreover, anti-inflammatory drugs have showed to be efficacious on several aspects of ALS in mutant SOD-1 mice. Thereby, inflammation also mediates exitotoxicity, which causes increased Ca^{2+} into motor neurons, which lead to neuronal death. In healthy conditions, astrocytes reduces the Ca^{2+} permeability of AMPA-type glutamate receptors by enhanced expression of Glur2 in motor neurons[112].

Although there seems to be a concededly effect of gliosis on ALS onset and disease progression, the influence could be mediated by other processes as well. So are oligodendrocytes, ependymal and subependymal cells proposed to involved in neuroinflammation as well. However, their function in ALS is poorly understood[113]

NEURO-VASCULAR SYSTEM: A NEUROVASCULAR DISEASE

After the discovery of the influence of local inflammation, researchers started to investigate systemic regulated inflammation[114]. As discussed above, glial cells seem to be activated in patients with ALS. Recent research has discovered that neuroinflammation is regulated by the peripheral immune system, rather than through local responses. Particularly T-cells seem to be prone to be transferred from the systemic regulation into the brain and spinal cord parenchyma.

However, this field is still standing in its infancy. Therefore, it is important to further investigate the systemic influence on changes that occur in the brain during disease progression and to unravel the exact interaction between the peripheral and the local immune responses. Up-regulation of 32 cellular adhesion molecules has already been found. Adhesion molecules may responsible for the arrest of circulating leukocytes and diapedesis into the brain parenchyma[114]. Controversially, tight junction proteins emerge to be down-regulated, which leads to increased vascular permeability[115]. From this point of view, it is interesting to further focus on the role of the blood brain barrier (BBB) and the blood spinal cord barrier (BSCB), as these are the separations between systemic and local immune responses connected by the vascular system. Various structural and functional alternations of the BBB/BSCB have been found in ALS patiens (Fig.4)[114]. Major hallmarks of BBB/BSCB impairment are endothelial cell and astrocyte end-feet degeneration, modified basement membrane composition, tight junction and transporter system impairment, vascular protein leakage, extensive extracellular edema, as well as myelin protein (MP2) and matric metallopeptidase 9 (MMp9) activation[116]. These processes eventually allows entry of blood burne substances into the brain, which might set up a cascade which may lead to direct and/or indirect motor neuron degeneration. Furthermore, high levels of IgG, albumin and complement C3a deposits have been found in the spinal cord and motor cortex, which implies vascular ruptures. These deficits appear to occur before expression of inflammatory molecules (i.e. before macrophage activation) in the brain and before motor neuron degeneration[117]. Interestingly, pre- and post- symptomatic mutant SOD-1 mice have showed decreased capillary length, capillary diameter and capillary[115]. This may imply ischemia or a similar event, that underlie this pathology. However, inconsistencies have been found between studies in both human as in mice.

In conclusion, motor neuron degeneration does not appear to be intrinsic. Systemic regulation seems to mediate neuronal survival and death, by regulation of various cell types (i.e. glial cells) and components within the CNS. Impairment of the BBB/BSCB may be the primary event prior to ALS onset. Furthermore, local BBB/BSCB impairment at the spinal cord or brainstem may cause the selective motor neuron degeneration in ALS. Nonetheless, it remains unclear how big the systemic influences are with respect to the severity of changes in glial cells (M1 and M2 activity) and their influence on disease progression. However, some controversial data has been found. Therefore, additional research is needed to unravel these mechanisms further. Though, this field of research showed promising results and may lead to future therapy on maintaining, repairing, strengthening the BBB/BSCB and reducing inflammation[114].

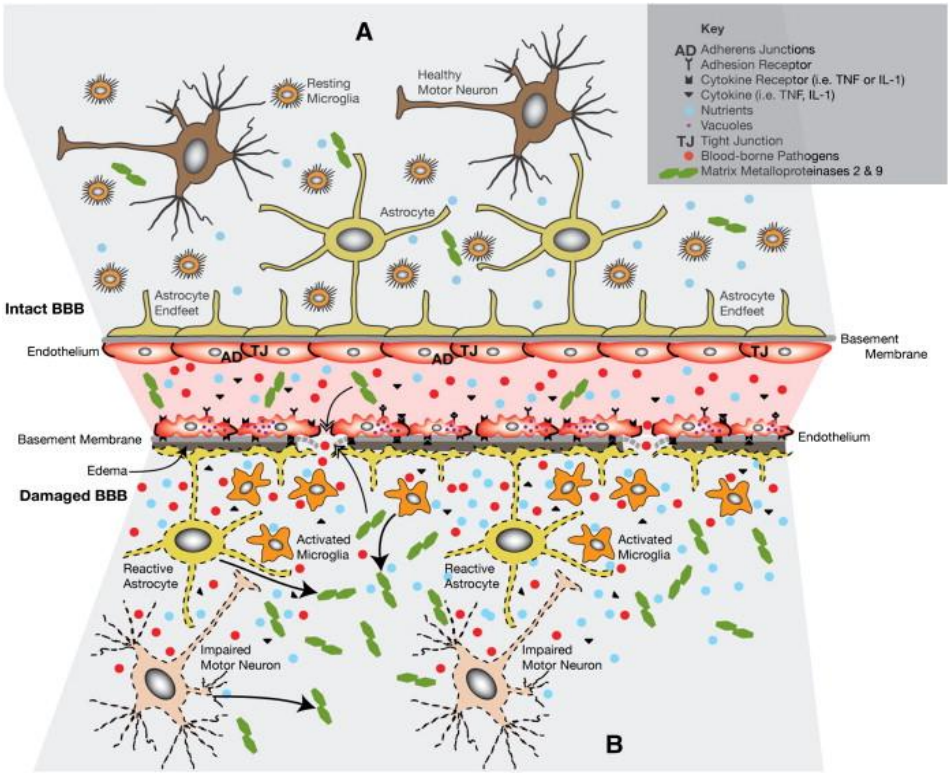


Fig.4: ALS a neurovascular mechanism. A. Represent a healthy condition; the BBB/BSCB maintains homeostasis through selective regulation of the blood circulation within the spinal cord and brain. This process includes endothelial cells and their tight/adherens junctions, astrocyte end-feet, perivascular macrophages, pericytes, and the basement membrane. They are all needed for providing a barrier. **B** Sturctural and functional impairment of the BBB/BSCB allows entry of blood-borne substances, by endothelial cell and astrocyte end-feet degeneration, modified basement membrane composition, tight junction and transporter system impairment, vascular protein leakage, extensive extracellular edema, and MMP-2 and MMP-9 activation Additionally, cytokines (i.e. TNF and IL-1) are released from activated microglia and reactive astrocytes. (Image is adapted from Garbuzova-Davisa, S., et al,. 2011) [107].

RNA METABOLISM

Recently, another disease mechanism has been suggested to play an important role in ALS as well. Currently, an increasing number of mutations, which affect proteins involved in RNA processing, have been identified in ALS. These mutations imply disrupted RNA dependent mechanisms (Fig.5). Evidence for this modifications in epigenetics, transcriptomics, and proteomics have been found in both animal models as in humans suffering from ALS[33]. On the one hand modified expression of genes involved in cytoskeletal dynamics, protein degradation system and mitochondrial dysfunction has been found in motor neurons[118]. Similar altered gene expression have found in muscle. On the other hand alternations in the insulin-like growth factor-1 and the RNA-binding protein ROD1[119] occur in glial cells. Therefore, altered control of gene expression might be the overarching mechanism of ALS[24].,

Epigenetic modifications showed to underlie altered gene expression, due to a disparity between histone acetyl transferases (HATs) and histone deacetylases (HDACs). The function of HATs is to modify core histone tails, which enhance DNA accessibility to transcription factors (TFs). In addition, HDACs activity results in transcriptional repression and gene silencing. However, many of the transcription initiation complex TF are themselves substrates to HATs and HDACs. Interestingly, ALS linked proteins, TDP-43 and FUS/TLS, control the expression of certain HDACs [120].

Another way in which epigenetic control of transcription may be modified is by methylation of DNA methyltransferases (DNMTs) or histone methyltransferases (HMTs). Both use S-adenosyl-methionine (SAM) as methyl donor. In eukaryotes, DNA methylation occurs through covalent modifications of cytosine residues in CpG dinucleotides leading to gene silencing. Histones or TF can be methylated by lysine or guanidinyl residues according to their function (i.e. activate or repress transcription). These processes may have enormous impact on ALS.

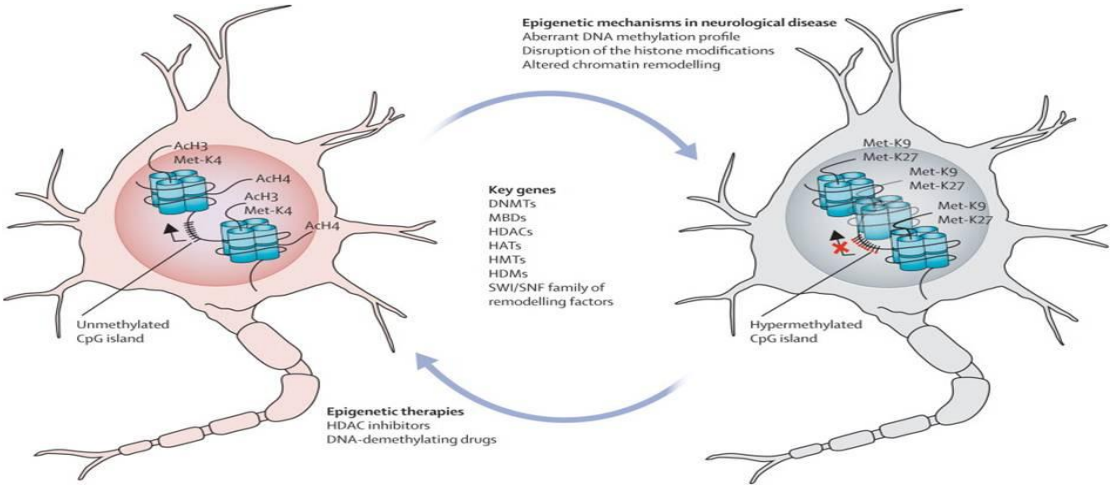


Fig.5: Epigenetic mechanisms. Healthy neurons or glial cells (left) express mRNA of a gene in occupancy from an unmethylated promoter, CpG island and a set of histone modifications. During ALS development altered DNA methylation, histone modifications and chromatin remodeling appear. (Image adapted from Urdinguio, R.G, et al. 2009)[114].

Another hypothesis is that RNA dysmetabolism could underlie the altered gene expression as well (Supplementary fig.1)[120]. Genes, which have already been identified in patients with ALS, appeared to be involved in the following processes: pre-mRNA splicing, mRNA transport, translational regulation and mRNA decay. Nonetheless, additional research has to be performed to investigate the specific motor neuron vulnerability to mutations in RNA-binding proteins.

In general, the function of a RNA protein is dependent on its associated proteins (i.e. RNA binding proteins, RBP) that together forms the ribonuclear particle (RNP). These complexes regulate RNA processes, such as alternative splicing. Alternative splicing is responsible for the generation of multiple mRNAs from a single transcript. Changes in the secondary structure of a protein subsequently lead to functional alterations. Moreover, various genes have already been found to express multiple splice variants.

Although the protein is still unknown, the C90RF72 gene is most likely involved in RNA processing. Recently it has been associated with an extension of a noncoding GGGGCC hexanucleotide repeat in fALS patients. When transcribed, this repeat forms intracellular accumulations of RNA fragments in cells[121]. This sequence may represent a potential binding site for RNA-binding proteins as hnRNP A2/B1 (TDP-43 interactor). To date, it has identified as most common genetic defect found in ALS patients[122].

Another interesting player in RNA processing is TDP-43. Next to the fact that it has already been identified as a major component in ubiquitinated inclusions[11], it showed to be an

ubiquitously expressed RNA-DNA binding protein. Research found that TDP-43 is implicated in multiple aspects of RNA processing, including: transcriptional regulation, alternative splicing and microRNA processing. Pathologic cytoplasmic TDP-43 inclusion are seen in both motor neuron and glial cells in various animal and human ALS patients[59]. This could imply different cascades in these genetic differ phenotypes. Most mutations in TARDBP are located on a glycine-rich domain involved in protein-protein interactions and nuclear transport.

Additional evidence that dysregulated RNA processing may contribute to motor neuron injury in ALS arises from the detection of biomarkers of RNA oxidation[34]. Furthermore transcriptional repression also occurs within motor neurons in the presence of mSOD1[123]. This may provide a possible link between epigenetic modifications with mitochondrial damage and oxidative stress in ALS.

Oxidative stress and mitochondrial dysfunction often occur together. Excessive ROS production negatively influences the functionality of the organelles that in turn would produce more ROS. Presumably, an interaction between oxidative stress, redox signalling and epigenetic modulations may cause ALS outcome. In this vicious circle, oxidative stress modulates gene expression by alternations of DNA accessibility. Additionally it is also a modulator of TFs. The latter implies that ROS and HDACs may coincide within the same mechanism. Next to being involved in regulation of proteins in redox reactions, HDACs also modulate alternative splicing and the activity of TFs. Furthermore, mitochondrial damage causes also alternations in selected splicing variants and RNA metabolism. This is associated with SOD-1 linked ALS as a consequence of mitochondrial stress[123]. Eventually, this may causes proinflammatory responses.

Overall, ALS may arise from RNA dysmetabolism, which explains the broad diversity of modified processing within the brain and muscle. Furthermore, altered expression of genes involved into RNA metabolism has been found in motor neurons. But besides mutations, which affect RNA transcription or processing, RNA proteins, also needs to be correctly transported along the axon to the neuromuscular junction (NMJ). Axonal inclusions may block axonal transport, which may underlie ALS disease onset. Therefore, the primary site of damage in an ALS patient might determine the severity, age of onset and progression[33]. Moreover, different alternations of gene and/or RNA expression may cause the different outcomes on disease pathology. However, RNA dysmetabolism may also be caused by oxidative stress. For the future, this field of research should definitely be further investigated.

DISEASE MODELS

Since the wide variety of disease manifestations, research is performed on many different aspects of ALS. To investigate the cellular and molecular basis of ALS, a wide variety of model organisms have been used, including nematodes (Caenorhabditis elegans), arthropods (Drosophila melanogaster), Wsh (zebraWsh, Danio rerio), rodents (mouse, Mus musculus and rat, Rattus norvegicus) as well as non-human primates (rhesus monkey, Macaca mulatta)(See the supplementary table.3 for an overview)[16]. These animal models are particular important for certain pathological and therapeutic studies which are impossible to perform in human ALS patients. Nonetheless, postmortem tissue remains also very important for investigation of the disease.

To establish a good working model, much research has been performed on human genetics. To mimic the disease in model organisms, a pattern of inheritance is important. Linkage studies have provided knowledge of chromosomal loci and sequencing of putative loci has identified multiple

genetic changes[16]. With this knowledge, transgenic systems are generated to model the disease or aspects of the altered gene function. These systems can be used to further confirm the genetic basis and aid to further investigation of the cellular and molecular disease phenotypes.

Nowadays, the most widely used model includes the SOD-1 transgenic animal model[3]. Much knowledge is obtained from this model about the ASL disease mechanism (Supplementary Fig.2). However, the utility of the (mutant) SOD-1 model is questioned due to recent controversial results.

There is growing evidence that overexpressed wild-type SOD-1 can also be neurotoxic. Research has showed that overexpression of wild-type SOD-1 result in SOD-1 misfolding and aggregate formation that lead to motor neuron degeneration[72,124]. This neurotoxicity seems to result from a gain of function, rather than through loss of SOD-1 activity[125,34]. It might be that conformational changes in wild type SOD-1 mimic structural features of fALS mutant forms of this gene (13-15). These conformation changes might either be caused by altered post-translational modifications or through induction of covalent aberrant modification to wild-type SOD-1. Interestingly, similar results were recently been found in the TDP-43/FUS gene in Drosophila and transgenic mice[126]. However, another hypothesis states that environmental and lifestyle related factors are able to induce the wild-type SOD-1 or TDP-43/FUS to cause ALS[9].

However, alterations within this genes may also be more universally involved in ALS pathology[60]. These data might imply that aberrantly modified wild-type SOD-1 and TDP-43/FUS share a similar mechanism as fALS. Furthermore it would also be important to investigate whether overexpression of these proteins occurs in human ALS patients, too. Perhaps these mechanisms would underlie sALS patients.

If SOD-1 and TDP-43/FUS are involved into a more general mechanism that causes ALS, more research should be done to reveal the exact role or function of these genes. Therefore, previous experiments on various processes may have to be repeated as well. This may lead to new insights about ALS. Nonetheless, these observations suggest that sALS and fALS might share common pathogenic mechanisms.

In general, our lack of knowledge about ALS disease mechanisms and the absence of drugs or therapies reflects the limitations of existing model systems[24]. It has now been recognized that the biology and progression of ALS differs in humans compared to animal models. Also, positive effects, which are observed in animal models, seem to have little or no effects on the disease in humans. Therefore, evolutionary conservation on anatomical as well as cellular levels has to be taken into account to improve experimental studies.

Anatomical divergence is most notably in the rostral regions, such as the cerebellum and the forebrain, but also the position of the corticospinal tract[127]. This would argue for a different evolutionary process that may also govern patterning of cortical motor neuron precursors[128]. This has also been supported by the observation of more subtle differences between human and lower primates or vertebrates, like the direct synaptic contact between the lower and upper MN and the more complex axonal projections of the upper motor neurons[129,25].

There are multiple differences at cellular level as well. Firstly, human transcription factors binding sites have showed to be functional different from rodents[130]. Secondly, loss or gain of function of genes has been observed. For instance, the expression of certain genes, like the recently identified gene sulfiredoxin, contributes to increased anti-oxidant defence in rodents. However, this function has been lost in the primate lineage. To overcome these differences, newly humanized

models have to be developed. Currently, in vitro based technology, with special emphasizes on induced pluripotent stem cells (iPSCs), seems to be the solution[131,132]. This technique uses patient derived somatic cells that can easily be reprogrammed to pluripotent cells and then differentiated to specific cell types.

In conclusion, therapeutic strategies that were thought to be efficacious in animal disease models have proved to be unsuccessful when translated to human trials[24]. Our lack of knowledge about the disease mechanism and the evolutionary differences between animal and human hinder the process. Therefore, the development of new humanized models is needed to overcome these differences. iPSC technology may provide the solution[131].

DISCUSSION

After decades of research on ALS pathology, various disease mechanisms have been postulated (Fig.6)[4,31,53,120,71,114,71]. Interestingly, much coherence is seen between these mechanisms in a complex integrative manner.

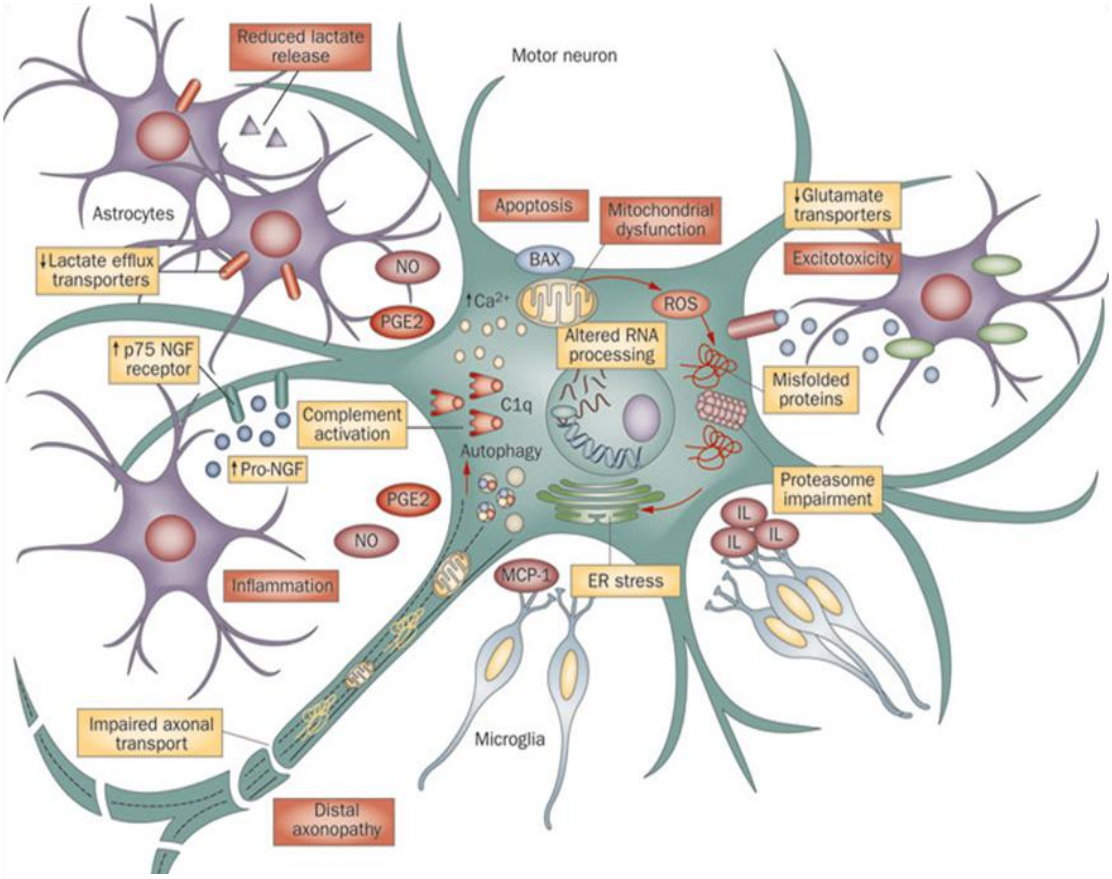


Fig.6: All mechanisms that are involved in ALS pathology: proteotoxicity, excitotoxicity, oxidative stress, impaired axonal transport, non-cell autonomous influences of glial cells and RNA metabolism. Only with exception of inflammation and immunity and neurovascular regulation. *(Image adapted from Ferraiuolo, L. et al. 2011)[57].*

Considering that the age of onset, start of deterioration and disease progression differ greatly among patients, it is an apparent question whether ALS might not be one disease[133]. Instead it could be that what we call ALS, might be an overarching term for diseases where motor neurons degenerate due to different causes[133]. This leads to three hypothesises. Firstly, it may be that different mechanisms can lead to ALS. Secondly, ALS may be caused by different mechanisms

that all results into the same outcome. Or finally, knowing that all the suggested disease mechanisms are related to the vulnerability of motor neurons, it raises the question which comprehensive cause might explain all hypotheses, and therefore, be the most likely cause of ALS.

Considering all aspects of the disease, it is striking that many of these take also place in "healthy" aging, albeit later and in a less pronounced manifestations [134,135]. A more general mechanism, such as aging, explains the natural variation in symptoms, even as a variation in disease onset. In the next chapter, this novel hypothesis will be described and discussed in greater detail.

A NEW CONCEPT: AGING

The incidence of ALS is highest among the elderly and occurs most often in individuals between 50 and 70 years[2]. Therefore, aging was already seen as a risk factor. Though there was no knowledge about the function of aging itself. For decades, aging was thought to be a stochastic, uncontrolled degradation process independent of gene regulation[136]. But this thought has been changed in the past ten years and much knowledge is obtained about the aging process. Therefore, it is tempting to further investigate aging as a potential cause of ALS.

Interesting findings on the role of aging have been identified by the use of parabiotic mice models[137,138]. Parabiotic mice are mice that have been sutured together, side-by-side, along the lateral aspect of the body. Once blood vessels have connected with each other, the mice will share a common blood supply. This model can be used to determine whether the effect of aging in one, is affected by a blood-bourne mediator in the other. Therefore, heterochronic (Young and old) pairs were compared with isochronic (young to young or old to old) pairs. These data show that exposure of young serum to old mice increase the rejuvenation and regeneration capacity of the old mice, which also showed that the effects of aging on regeneration capacity are reversible[137]. These Age-related changes seemed to be determined by the age of the systemic environment, rather than by the cell-autonomous age. Notably, when young serum became mixed with old serum, the regenerative potential was inhibited, suggesting a dominant influence of old serum factors.

Moreover, aging has showed to be dependent on the regulation of classical signaling pathways, which are influenced by environmental and physiological cues. It has now been revealed that at least three independent metabolic pathways are involved in regulation of the aging process. Firstly, dietary restriction showed to extend the lifespan in multiple animal models[136,139]. Secondly, the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway has been associated to be an active regulator of longevity in worms[140], flies[141,142] and mice[143,144]. Lastly, also

the rate of mitochondrial respiration was found to influence lifespan. Among these pathways, the insulin/insulin-like growth factor 1 signaling (IIS) pathway is the most well-known pathway and has already been intensively studied. Therefore, we will focus on how this pathway contributes to aging in relation with ALS pathology. But first, the function of aging during the evolution will be shortly discussed.

EVOLUTION

Research has been performed on mammalian species to investigate to which extend normal brain aging is conserved through evolution. Knowledge about its conservation has important implications for the use and/or the development of animal models of ALS. Genome-wide gene expression studies in C. Elegans, Drosophila melanogaster, mice, rat, chimpanzees and humans have revealed functional conserved genes with age-dependent expression changes[5]. Notably, most genes are associated with reduced mitochondrial functioning. Furthermore, an increased expression of genes that are involved into the stress-response pathway have been identified. . Resistance of stress during aging may be effective to protect the brain against pathology. However, research on evolutionary conservation of aging genes has showed a shift between rodent and human lineages, as rodents showed repression rather than activation of many of these age associated genes during aging (Fig.7). When working with animal models, this should be taken into account.

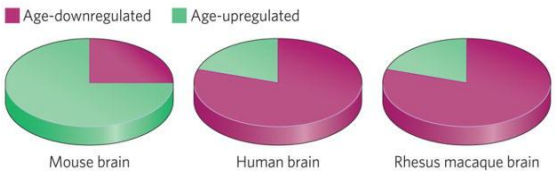


Fig.7: Evolutionair age-related gene expression shift in the primate lineage. Age-related changes are downregulated (green) in Rhesus macaque and human in contrast to downregulation (green) in the mouse. *(Image adapted from Bishop, N.A., 2010)* [138].

THE IIS PATHWAY

The strongly conserved Insulin/IGF signaling (SII) pathway was the first pathway discovered to be involved into aging. This pathway showed to be a major regulator in numerous processes including lifespan, metabolism and stress resistance. Moreover, has recently been associated with neurodegenerative diseases as well[9]. On the one hand, reduced insulin/IGF-1 signaling seems to correlate with lifespan extension[8] and thereby decrease risk to develop ALS. On the other hand altered expression of these factors may result in a broad cascade of cell-protective mechanisms, which extend or shortens lifespan. Multiple polymorphisms within genes involved into this signaling pathway, have been observed in ALS models of worms, flies and mammals, which are associated with longevity[145].

While invertebrates seem to have only one receptor that binds components like insulin and IGF-1, mammals contain different kind of receptors for these factors. IGF-1 mainly controls growth, whereas insulin regulates metabolism. Defective signaling of the latter results in insulin resistance and diabetes, while defective signaling of the former leads to protein breakdown and muscle degeneration. IGF-I overexpression reduces various, age- associated organ dysfunction and increase muscle reparation. Controversially, reduced insulin seems to extend lifespan, particularly in tissue which expresses low IGF-1[146]. Therefore, specific tissue modulation by different signaling pathways might obstruct aging in humans. This contradiction between their neuroprotective effect versus their deleterious effect resembles the complexity of this signaling pathway.

In general, this pathway becomes activated when a insulin like ligand binds to the insulin/IGF-1 receptor DAF-2[140],[147]. Subsequently, DAF-2 recruits insulin receptor substrates and the phosphoinositide 3-kinase (PI3K) adaptor/regulatory subunit (the human orthologoques of IST-1 and AGE-1 in C.elegans)[148]. This mediates the generation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3). Next, members of the AKT kinase family in a pyruvate dehydrogenase kinase (PDK)-dependent way (37,38) are activated. However, dissociation of PtdIns(3,4,5)P3 and reduction of AKT activation is negatively regulated by PTEN (the phosphatase DAF-18 in C. elegans)[149]. Upon activation of AKT, the downstream protein DAF-16 (a FOXO forkhead transcription factor)[150] becomes phosphorylated. This protein in turn is not able to enter the nucleus. Therefore DAF-16 is negatively regulated by the IIS pathway. Nonetheless, this system becomes even more complex when taking the other FOXO transcription factors, the silent information regulator (SIRT) protein deacetylases (sirtuins) and heat-shock factor 1 (HSF-1) into account.

On the one hand FOXO transcription factors initiate stress-induced cell death (apoptosis) in damaged cells. On the other hand they can also upregulate antioxidant defence and DNA repair facilitating genes[70]. Sirtuins belong to a class of proteins that are involved in aging, stress resistance, apoptosis and energy metabolism. SIRT-1 is able to deacetylate FOXO transcription factors. This enhances FOXO dependent resistance to oxidative stress and cell cycle arrest, but inhibits FOXO regulated apoptosis[151]. SIRT1 can also deacetylate the p53 tumour suppressor protein, which diminishes its transcriptional activity and inhibits stress-induced apoptosis and cellular senescence (irreversible cell cycle arrest)[63]. However, it seems to be difficult to decide whether enhanced FOXO or SIRT1 would increase or decrease longevity. The complexity of this system is probably due to protection of the organism against cancer. That would also explain why yeast, flies and nematodes, which don't develop cancer, contain only one kind of receptor in this signalling pathway. Notably, many malignancies are caused by Igf-1 and Igf-2 signalling, which supports the dual role of this pathway. Moreover, research has shown that certain mutations in insulin, IGF-1 or their receptors greatly extend lifespan. This has been clearly depicted in research in C.elegans, in which the IIS pathway has been shown to play an important role on development[152]. IIS is postulated to regulate the developmental switch, which determine whether the development of the larva will arrest, a condition called dauer, or if the larva will become adult. Notably, regulation of lifespan in C.elegans occurs during the reproductive adulthood, which is distinct from the dauer switch during development. Inhibition of DAF-2 (the IIS receptor) in worms prolonged lifespan and protected them from protein aggregation(Fig.8)[82,153.]

Next to FOXO and SIRT1, also HSF-1 has showed to be crucial in longevity[154]. HSF-1 regulates multiple processes, ranging from development[155], the stress response[4], the circadian rhythmicity, innate immunity, to hypoxia. Recently it has been associated with the IIS pathway as well[4]. Although the exact working mechanism remains unclear, it has been suggested that apart from DAF-16, reduced IIS promotes enhanced stress resistance by activation of HSF-1. Remarkably, Sirt-1 has also been identified to activate HSF-1 in mammals[156,69].

Its target genes include additional small HSPs and chaperone proteins. Interestingly, a link between aging and ALS-like protein aggregations has been found in C.elegans. In this model, PolyQ aggregations showed to be reduced by DAF-16 and HSF-1 expression, which is regulated by reduced IIS. Additional evidence was found by a worm model of Alzheimer Diseases which showed that reduced IIS protects against proteotoxicity[82]. Also knockdown of hsf-1 led to a huge increase of soluble toxic aggregates and increased high molecular mass aggregates[82]. This may suggest that the IIS pathway has two distinct counter activities, namely: protective aggregation of high molecular mass aggregates regulated by DAF-16 and disaggregation of the more toxic oligomeres by HSF-1. In general, reduced IIS signalling has been associated with longevity and stress resistance in mice[143].

However, in contradiction with previous findings, increased IIS appears to be protective against proteotoxicity and polyQ-mediated toxicity [157]. On the one hand, stimulation of the IIS pathway by injection of IGF-1 results in decreased Aβ aggregates in Alzheimer's disease (AD) models. On the other hand activation of this pathway also leads to phosphorylation of the polyO gene, dependent on the IGF/AKT pathway. Eventually it will be cleared via autophagy, which has previously been implicated as a crucial regulator of aging[158].

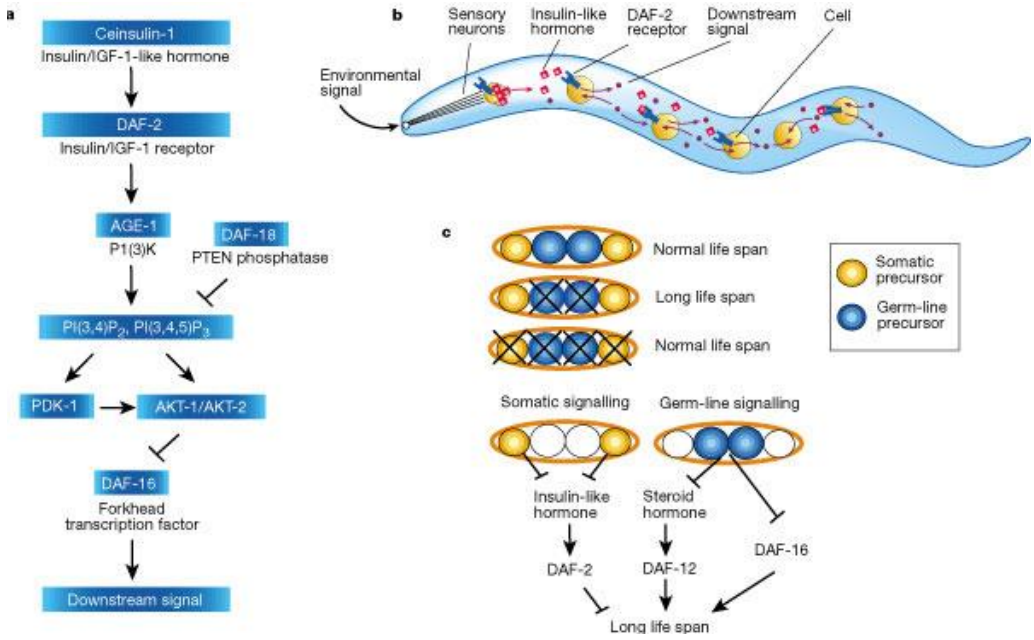


Fig.8: Involvement of the IIS pathway in longevity in C.elegans. **a**, The insulin/IGF-1-like signalling pathway regulates ageing in *C. elegans* (further information can been found into this chapter above). **b**, **c** The lifespan of C. elegans has showed to be regulated by an elaborate endocrine system that involves at least four hormones. Surprisingly, lifespan seems to be very plastic. This process is modulated by two systems: thesensory system (registrates environmental conditions) and its reproductive system. Both systems affect lifespan by activation of components of the DAF-2 insulin/IGF-1 pathway. **b**, Modulation of lifespan regulated by the sensory system. Environmental signals such as food or a pheromones causes sensory neurons to release insulin/IGF-1-like DAF-2 ligands. Rather, release of DAF-2 ligands could also be regulated by other cells. Upon activation of DAF-2, cells either produce (as shown in the picture) or repress production of downstream signals or hormones, which acts through an unidentified pathway to aging. C, Modulation of lifespan regulated by reproductive cells. DAF-2 has found to function in somatic gonad signaling. Furthermore, both DAF-12 and DAF-16 has been found to be required for germ-line ablation to extend lifespan. However, it remains unclear if these proteins function in the same pathway or in parallel (as shown in the picture). Assuredly, sensory neurons, the insulin/IGF-1-like hormone, germ cells or somatic gonad cells could activate or repress hormone production. For instance, somatic gonad cells may produce an insulin/IGF-1 like hormone, which in turn may act as an antagonist..(Image adapted from Guarente L., and Kenyon C. (2000)

Autophagy deficient mice show neuron degeneration with accumulation of ubiquitylated protein aggregates, similar as in human ALS patients[159]. Downregulation of multiple genes involved in autophagy has been observed during aging and may explain enhanced susceptibility to the toxic effects of protein aggregates[153]. Interestingly, autophagy can also be promoted by increased IIS through FOXO-mediated factors.

Nonetheless, more research is needed to fully unravel these mechanisms. Above stated contradictions emphasise the complexity of the IIS pathway. However, it seems plausible that signals downstream of IIS diverge and might counter proteotoxicity through multiple mechanisms.

TDP-43/FUS AS LINK BETWEEN AGING AND THE CELLULAR STRESS RESPONSE

Another very recent breakthrough came from the result of a research in which TDP1, the C.elegans ortholoque of TDP43, showed to have a complex role in lifespan and the cellular stress-response via the IIS pathway[9]. TDP43 seems to regulate longevity and the oxidative stress response downstream the forkhead transcription factor DAF16/FOXO3a through IIS receptor activation. In normal conditions, activated DAF-16 stimulates multiple genes that are involved in

prolonged lifespan and enhanced stress resistance[154]. This makes them resistant to oxidative, osmotic and thermal stress as well as proteotoxicity[160]. However, in response to stress there is DAF-16-dependent upregulation of TDP-43. This initially protects the cell by upregulation of various genes, which combat the effects of stress. Only, prolonged cellular stress showed to cause chronic upregulation of TDP-43, which is neurotoxic and decreases lifespan. Controversially, deletion of TDP-1 has shown to rescue mutant TDP-43 and FUS proteotoxicity in C.elegans after a prolonged period of stress. These results imply that even chronic induction of wild-type TDP-1/TDP-43 may stimulate neurodegneration and reduce lifespan. Whereas TDP-1 has already been associated with proteotoxicity and endoplasmatic reticulum (ER) stress, involvement of the IIS pathway may be the missing part to connect them to each other. However, little is known about the separate functions of the components involved into the IIS pathway.

TDP-43 is a constituent of stress granules. This protein arises in response to environmental stress, such as oxidative and osmotic stress, heat shock, oxidative and osmotic stress[136,139]. Previous research on C.elegans investigated if TDP-43 participates in the cellular stress response and longevity pathways. Furthermore, it was researched if TDP-43 controls age-dependent proteotoxicity. Results showed that tdp-1 is required for the expression of DAF-16 under low IIS conditions (Fig.9)[9]. In case of the presence of oxidative stress, increased TDP-1 protein levels were observed which is dependent on DAF-16. These processes are mediated by a significant altered cellular distribution of TDP-43 upon stress and/or low IIS. These finding suggest that TDP-43 toxicity may activate the ER unfolded protein response (UPR). In turn, experimentally induced ER stress in C.elegans increased expression of TDP-1 compared to their untreated controls[91]. However, this was only the case in worm expressing wild-type TDP-1 and not in mutants. Similar findings were seen when they investigated FUS[9], the counterpart of TDP-43 with similar function. Overall, TDP-1 mutants showed the same age-dependent degeneration of motor neurons as TDP-43/FUS mutants. Therefore, a possible loss of function of TDP-43/FUS can cause protein aggregation and cellular stress. This is mediated by the IIS pathway and results in reduced lifespan.

The ER stress response functions through clearance of misfolded proteins, mediated by ER-associated degradation (ERAD). This facilitates transportation of proteins from the ER lumen towards the cytoplasm to become degraded by the ibiquitin proteasome. Only, this mechanism is redox intense and causes a high production of oxidative stress[92]. Moreover, ER stress causes DAF-16 dependent upregulation of TDP-43 expression, which further reduce neuronal function and lifespan. If protein misfolding would be the primary step in neurodegeneration, this may lead to toxicity by: 1) primary toxicity from damaged or misfolded proteins; 2) secondary toxicity from enhanced oxidative stress or lastly 3) toxicity mediated by stress induced TDP-43 expression. Notably, overexpression of wild-type TDP-43 has also been observed in both animals as human ALS patients. Accumulation of this protein in general may therefore actively contribute to neurodegernation.

Moreover, this model represent the two hit hypothesis for ALS disease in which TDP-43 represents the trigger for the development of pathogenesis[82]. Remarkably, fALS patients mostly harbor mutations within genes that results in aggregate prone proteins. In the contrary, patients with sporadic ALS don't carry those mutant genes and produce less toxic aggregates. This would explain why fALS patients develop the disease earlier in life. Interestingly, the ER stress response showed to be activated in a cell non-autonomous manner. Research found that heat shock

protein-4p (HSP-4p) was mainly expressed in the worms intestinal cells, while mutant TDP-43/FUS appears to be particular expressed in motor neurons of the central nervous system[9]. Therefore, it has been suggested that ER stress is able to signal to other cells and/or tissue types by functioning in a coordinated-organism wide response. Moreover, such a coordinated response has already been identified for mitochondrial stress and the heat shock reseponse[161].

In conclusion, proteostasis is crucial for survival, but protein control systems fail during aging what lead to accumulation of TDP-43[154,147]. Thereby, this mechanism becomes positively regulated upon stressful conditions by expression of TDP-43. This results in pathogenesis. However, more research is needed to further elucidate the role of proteotoxicity in longevity mediated by the IIS pathway. This model shows that alternations in signaling can have multiple effects and implies a very complicated role of IIS in ALS. However, the involvement of this pathway in ALS pathology has found strong scientific support, as this will become emphasized in the proceeding chapters.

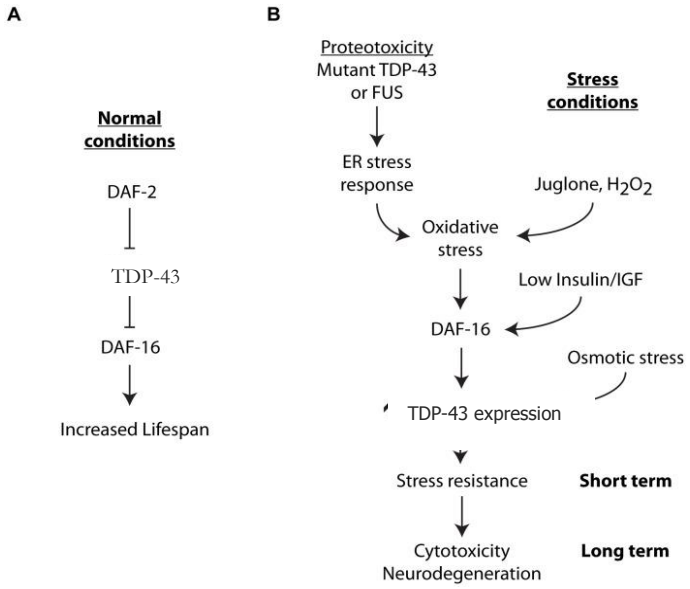


Fig.9: The role of TDP-43 in the IIS pathway. TDP-43 showed to have multiple contributions to lifespan. A. Under normal conditions TDP-43 might function upstream from DAF-16 to regulate longevity (right). B. However, under stressful conditions, TDP43 expression is further induced by oxidative stress and/ or through activation of the IIS pathway. This induction is dependend on DAF-16 and is then regulated in a vicious circle. Higher expression of TDP-43 causes aggregates, which activate the unfolded protein response (UPR). As a consequence, generation of oxidative stress appears. This in turn enhance TDP-43 expression. On the one hand, this mechanism provide stress resistance in acute stress conditions. On the other hand, chronic induction of TDP-43 has negative consequences for the cell, such as neurodegeneration. *(Image adapted from Vaccaro, A., et al. 2012) [9].*

ATHLETES

Heavy exercise has also been supposed to be a strong risk factor for ALS since the seventies or eighties[162]. Early case control studies tried to unravel the relationship between ALS and sports. However, no clear results were found[163]. Interestingly, Mr. Raffaele Guariniello analysed the cause of mortality of 24000 Italian football players within the period of 1960 till 1996[164]. During the course of this study, 375 individuals died, of which 8 due to ALS. The expected number of ALS-caused deaths was 0.69. This indicates a ten-fold higher risk for ALS in soccer players[13]. Typically, these individuals suffered from early onset ALS (in the 40'ties), while mean age of

onset for sALS is around 60-70 years [164]. Interestingly, although the study was completed, more participants developed ALS. Nevertheless, this stimulated researchers to further investigate the relationship between heavy exercise on ALS development. In 2005, Chio et al, analysed a large Italian cohort of 7325 male professional football players in the period 1970 till 2001[13]. Since those men were engaged by teams from the First or Second Division they were subject to very intense exercise. During this period five ALS patients were identified with a mean age of onset of 43.4 years. Again this exceeded the expected number of 0.77 patients[13]. In fact, the percentage of ALS cases would have been even higher if 13 participants hadn't been excluded, due to ethnical origin or above threshold exercise. Furthermore, a dose-response relationship has been found between the duration of professional football activity and the risk to develop ALS[13].

Direct support of the involvement of excessive exercise also derives from the following case. Three men that next to playing soccer on a high level, also grew up under comparable conditions in the same village, developed ALS in the same year. Interestingly, between two of them, the disease progression was similar as well[40]. Therefore, a high level of exercise is most likely the overarching factor, together with the other environmental influences they shared.

Taken all possible contributing factors together, this lead to the following hypothesises: (1) ALS is simply related to participation in sport or heavy physical exercise, and therefore not specifically limited to soccer, (2) Micro trauma, caused by i.g. heading the ball, micro trauma of the legs or muscle lead to an increased change to develop ALS; (3) ALS is related to either the use of illegal drugs or toxic substances to improve performance or to higher dosages of therapeutic drugs; (4) ALS is associated with environmental toxins used on soccer fields. Studies on athletics show an abnormal high amount of individuals with bulbar onset ALS[13]. This could be linked to headers[13],[165]. Though, most individuals develop early onset, which suggest that one or more toxic substances may have triggered ALS in patients with a susceptible genotype[40]. However, it is not known whether and how genetics can interact with environmental toxicants contributing to ALS. Furthermore, no substances have been identified to be involved[166,167,168].

Interestingly, recent research on C.elegans has discovered involvement of the IIS pathway in ALS, which links the insulin pathway to longevity in ALS. Remarkably, two of the three soccer playing friends, even as a significant amount of football players, showed to suffer from diabetes[40]. Prior to the development of diabetes, high levels of insulin are present due to insulin resistance (IR). Because of IR, the capacity to respond to a given amount of insulin drops. Therefore, higher levels of insulin are necessarily to create the same effects. However, this still needs to be investigated.

To further unravel the direct role of exercise on ALS, follow-up studies have been performed in transgenic mice. However, these studies found contradictory results. Mice which over express wild-type SOD-1 show no clear contribution of exercise on ALS[169], while mutant SOD-1 mice showed that high-intensity endurance exercise accelerate motor degeneration and death, but seemed not to affect onset of ALS[170]. In addition, other research showed that low intensity exercise may even is protective by a prolongation of survival in ALS mice. This suggests that the effects of exercise on ALS pathology are dependent on the training intensity[170]. Intense physical exercise seems to facilitate molecular mechanisms of oxidative stress[171] by the enormous generation of reactive oxygen species[164,172]. Research found that 15 minutes of high intensity exercise is already enough to cause an increased oxidant production. This causes a rapid, transient reduction in muscle protein thiol content that lead to increased amounts of SOD-1, catalase and heat shock proteins[172]. It is suggested that high respiratory activity drives the high production of

ROS during intense activity[173]. Additionally, research on C.elegans with knockout of the clk-1 gene showed reduced respiratory rates and longer lifespan. The clk-1 gene is required for ubiquinone, which is involved in mitochondrial respiration by affecting the electron transport chain[174],[160],[160]. However, this seems to be strictly dose dependent, because severe reduction of respiration appears to shorten lifespan[175]. Moreover, reduced expression of strongly conserved mitochondrial genes is observed in organisms ranging from C. elegans to humans[5,176,177]. Research showed that diverse mitochondrial DNA mutations, which causes impaired electron transport chain function (and enhanced ROS), accelerate aging and shorten lifespan[178]. Decreased activity within complex I, III, IV and V in of the electron transport chain showed a 40% life extension in C.elegans[160]. Besides, an increased anti-oxidant function, caused by catalase, seems to prolong lifespan[173]. Although these studies don't reveal the underlying pathways, they do show the importance of mitochondrial function on aging. Motor neurons have a high bio-energetic demand and mitochondrial dysfunction makes them vulnerable to degeneration (as outlined in previous section; oxidative stress). Very intensive exercise may accelerate mitochondrial dysfunction and thereby motor neuron degeneration.

It has now been suggested that a modestly amount of ROS acts as signaling molecule to activate survival pathways, which promote longevity through the induction of stress resistance by regulation of other genes[179]. The initial decline of mitochondrial gene expression during brain aging may therefore be part of an compensatory mechanism that increases stress resistance. Additionally, chronic stress may act as an positive feedback cycle stimulating further decline in mitochondrial function which eventually leads to irreversible damage within the cell (Fig..)[53].

Besides, excessive ROS also results in a disturbed epigenetic modifications, for instance by the silencing of specific gene-promotors[14]. Gene silencing includes a protective mechanism to exclude the expression of DNA damaged regions, rather than undergoing apoptosis. This mechanism may be a transition towards a more repressive transcriptional state[180] in which gene expression becomes totally altered through changes in histone modification patters. SIRT-1 is known to regulate epigenetic gene silencing and has found to be a mediator of the IIS pathway, by regulation of FOXO transcription factors and HSF-1 in response to cellular stress[181]. SIR1 is able to deacetylate FOXO, which enhances FOXO dependent resistance to oxidative stress and cell cycle arrest, but inhibits FOXO regulated apoptosis[151]. Therefore, increased SIRT-1 activity seems initially to have a protective role against cellular damage. However, SIRT-1 contributes to the cellular stress response and chronic activation negatively regulates lifespan. Overall, it seems that epigenomic dynamics are conserved during aging and are controlled by oxidative stress. Interestingly, yet another lifespan-regulating transcription factor similar to DAF-16, SKN-1, have been found to modulate longevity by regulation of genes involved in protection against oxidative stress[182]. Yet these mechanisms have to be further investigated.

ASSOCIATION BETWEEN ATAXIN-2 AND TDP-43/FUS

As previous outlined, the TDP-43/FUS gene have been suggested to play a crucial role in disease pathogenesis, by regulating longevity and oxidative stress[9]. Moreover, these genes have showed to be the major proteins found in ubiquitinated cytoplasmic inclusions in neurons of patients with ALS and frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-TDP)[183,11,44]. However, still little is known about how loss of function or how a (toxic) gain of function, of these proteins would lead to ALS. Until recently, genetic modifiers of TDP-43/FUS were unknown, Therefore, a genetic screen in yeast was performed to define modifiers of TDP-43 /FUS toxicity[184]. Ataxin (ATXN2), a polyglutamine (polyQ) protein was identified. Notably, it is a dose-sensitive modulator of TDP-43 toxicity within Drosophila, Yeast and human[185],[186].

Furthermore, ataxin-2 has already been associated in different kinds of neurodegenerative diseases[187]. Depending on the different polyQ expansions of ataxin-2, ultimately distinct phenotypes develop (Fig.10)[187]. For instance, long-length polyQ expansions in this gene causes spinocerebellar ataxia type 2 (SCA2)[188,189]. Follow-up investigation of polyQ respeats within 900 sporadic and familial ALS patients showed a significant association between intermediate-length polyQ expansions in the ATAXIN-2 gene and ALS pathology[184]. Subsequently, the mechanism by which these polyQ expansions might confer pathology became investigated. ATAXIN-2 is normally localised in a diffuse pattern throughout the cytoplasm of spinal cord cells. However, in ALS its localization is altered and moved towards cytoplasmic accumulations.

Both ATAXIN-2 and TDP-43/FUS are RNA binding proteins and showed to be associated in a complex that depends on RNA[190]. The intermediate-length polyQ expansion within ATAXIN-2 may enhance ataxin-2 stability or prevent its degradation[191]. This could cause increased ataxin-2 concentrations, which further stimulate TDP-43 pathology. In turn TDP-43 regulates longevity in interaction with the IIS pathway[190],[184]. Additionally, overexpression of Ataxin-2 lead to increased caspase activity and apoptosis, which causes increased cell death[192],[157]. Upon cellular stress, ataxin-2 enhance caspase activation. In particular caspase 3 showed to cleave TDP-43, which then facilitates and enhances the sequestrates within cytoplasmic stress granules[184]. Stress granules contain ubiquitin-modifying enzymes, kinases, proteases and phosphatases[193,194,195]. Moreover, they seem to be crucial in making the decision whether or not to enter apoptosis as response to extended stress. Under stressful conditions, ataxin-2 is proposed to lower the threshold for TDP-43 phosphorylation[12],[196] and thereby increases ALS pathology.

Although there will certainly be other routes that lead to TDP-43 pathology as well, these data provide strong support for its causative role in ALS pathology. Recent research reported that ALS cases with intermediate-length ataxin 2 polyQ expansions are characterized by distinct TDP-43 pathological features compared to TDP-43 mutations[191]. Perhaps this distinct pathology is the result of a slightly different mechanism that both result in TDP-43 pathology. Therefore, it is likely that there are multiple independent mechanisms (i.e. mutations in TDP-43, FUS, SOD-1, polyQ respeats), which includes the same target pathways leading to motorneuron dysfunction and eventual degeneration and death[187].

These data have provided important information about the ALS disease mechanism and implies that TDP-43-ATAXIN-2 may be an important genetic risk factor for ALS in both sALS and fALS. Furthermore, this provides crucial knowledge and indications with respect to previous findings about TDP-43 and the IIS pathway.

Effect of ataxin 2 intermediate-length polyQ expansions

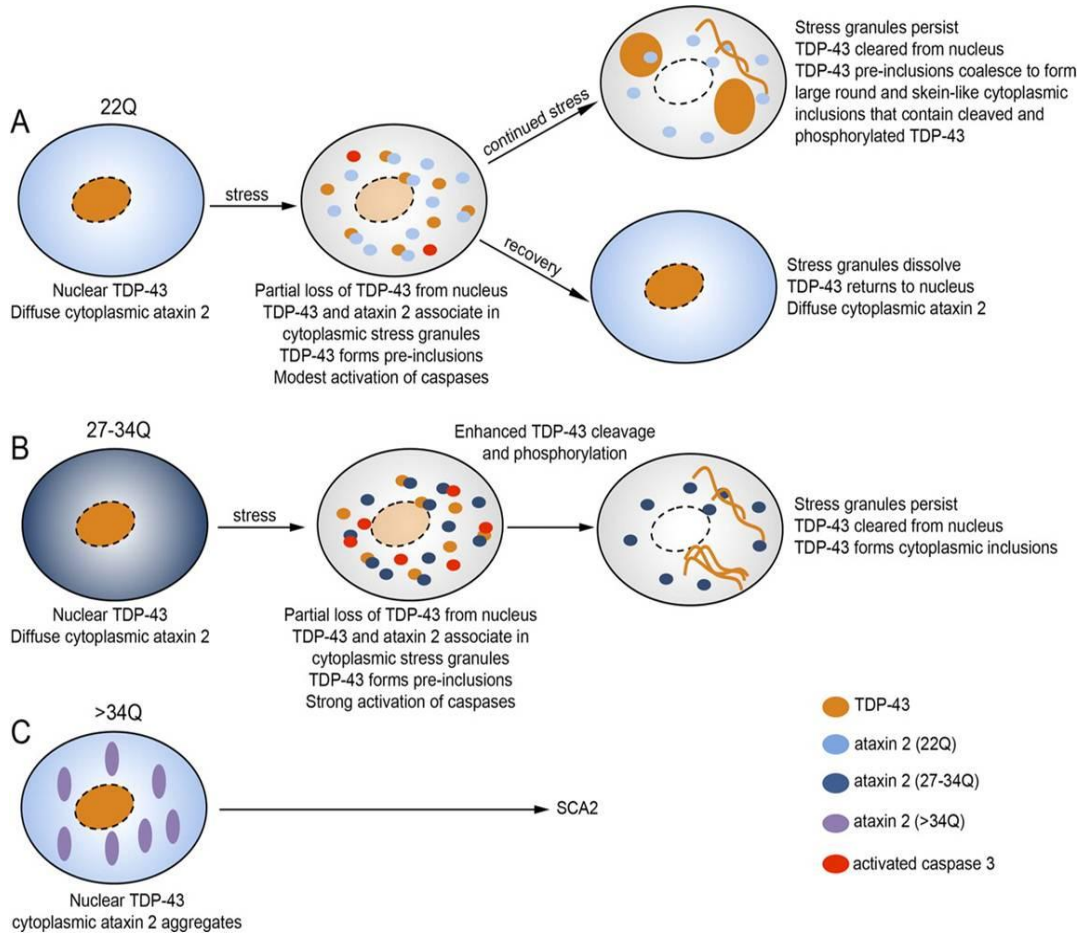


Fig. 10: Effect of ataxin 2 intermediate-length polyQ expansions on TDP-43. This model explains the effect of ataxin-2 intermediate length polyQ expansions on TDP-43. A. During healthy conditions, ataxin-2 is diffusely in the cytoplasm, while TDP-43 is located in the nucleus. Upon cellular stress, TDP-43 is transported into the cytoplasm. TDP-43 and Ataxin-2 both associated within stress granules by activation of caspase 3. After stressfull conditions, the cell recovers, stress granules are dissolved and TdP-43 returns to the nucleus again. However, chronic stress results in persistent stress granules and increased TDP-43 clearance from the nucleus leading to large cytoplastmic aggregates. B. Cells that express Ataxin-2 probably have a lower threshold for activation of caspases upon stress. Therefore, increased caspase activity results in increased TDP-43 which eventually lead to enhanced cytoplasmic aggregation. Notably, since the pathology of TDP-43 aggregation may be slight different than ALS pathology caused without ataxin-2. C. Other evidence from a pathological function of Ataxin-2 resulted from patients whith spinocerebellar ataxia 2 (SCA2), which harbour long ataxin 2 polyQ expansions. Aggregation of ataxin-2 in the cytoplasm causes a toxic-gain of function effect that might be unrelated from the normal function. (*This image is adapted from Hart, M.P., 2010)*[185].

DISCUSSION

Research on aging mechanisms have revealed new insights into ALS pathology. Many features which have previously been associated with ALS have showed to be involved into 'healthy' aging processes, albeit in less extreme form (Fig11)[4,187,154,9]. These features includes, proteotoxicity, oxidative stress, ER stress and RNA metabolism (Fig.12 and Table1; overview). Together, these findings support the idea that premature motor neuron aging, is the common mechanism, underlying ALS

Interestingly, ALS most often occurs in relative healthy individuals: Athletes or individuals without a disease or extraordinary disease history. . Because of the high prevalence among athletics that develop ALS, the influence of heavy exercise on ALS pathology has been investigated. Interestingly, these studies have found a link between heavy exercise and ALS, which is mediated by the aging process[179,181]. High intensity exercise strongly increases the production of ROS and thereby oxidative stress[170,173]. This mechanism has previously been associated with the development of ALS[9,33,9]. Moreover, high cellular ROS concentrations seems to mediate the IIS pathway as well[197,124]. Besides that heavy exercise is supposed to be a strong risk factor for ALS, these results also validate a causal role of both the IIS pathway and oxidative stress into ALS development[40,13].

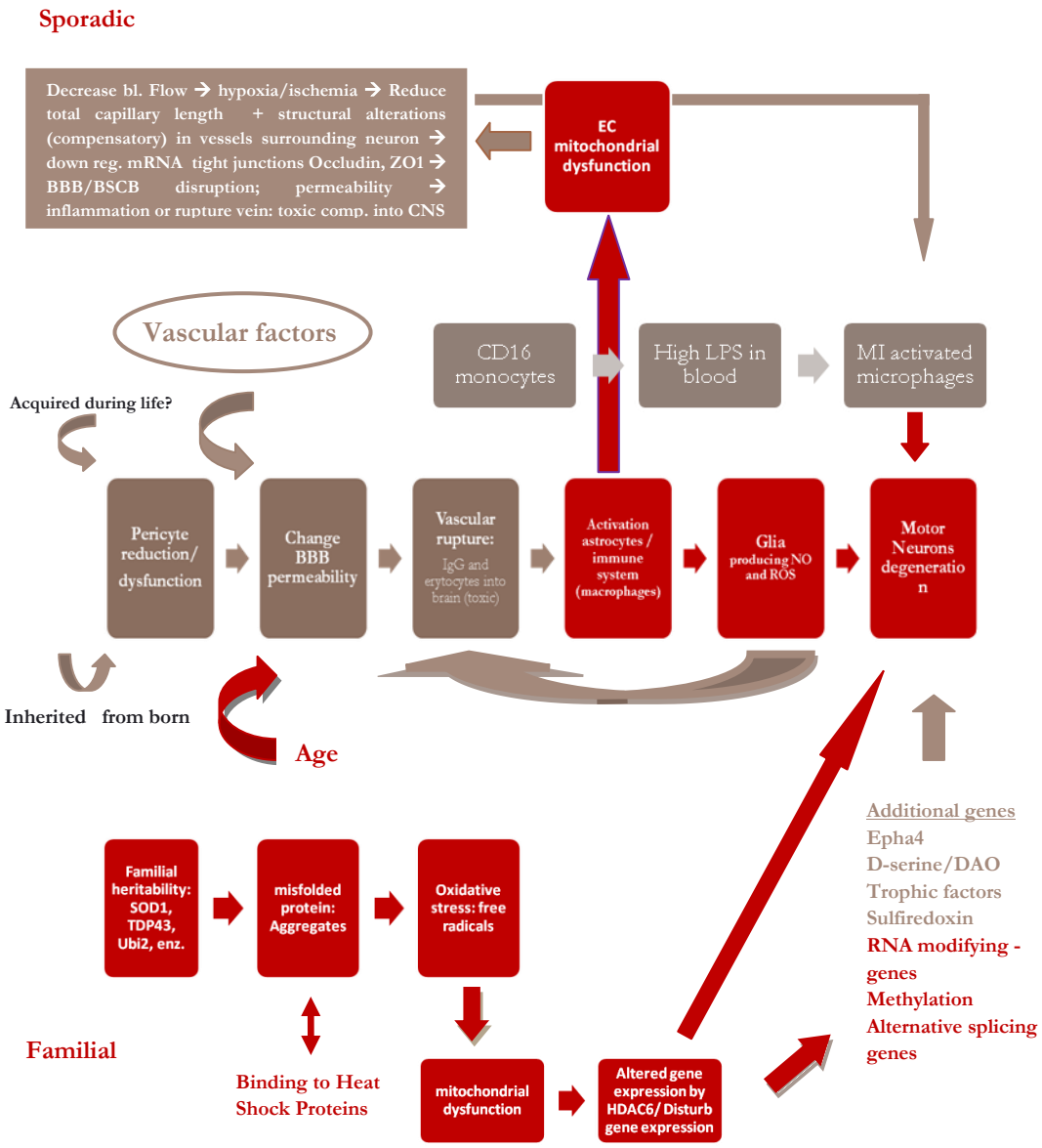
It may be hypothesizes that mutant or excessive SOD-1 in motor neurons cause ALS through a vicious circle. Motor neurons do already have increased levels of SOD-1 to protect them against their own high metabolic demands (see supplementary table.2)[198]. It might be that exercise further stimulates the generation of SOD-1. This toxic gain of function negatively impacts motor neuron function, causing further deterioration.

Another remarkable fact is that a significant number of ALS patients have showed to suffer from diabetes prior to onset of ALS[4,199,40]. Especially diabetes type 2, which is caused by insulin resistance, would be interesting to investigate. Insulin resistance causes the circulation of high levels of insulin in blood. Obviously this can stimulate the IIS pathway excessively. Therefore, this might include the same mechanism in which other components, like TDP-43 or exercise, causes cellular stress and eventually lead to ALS[9]. Consequently, it would be interesting to investigate the occurrence of ALS in diabetes type 1 vs. type 2 patients.

In spite of the evidence that excessive exercise might cause higher prevalence of ALS in athletics, it has also been suggested that trauma underlies the disease[165,200,201,44,]. Although numerous reports have showed evidence of trauma in physically active patients with ALS, the association between physical exercise, trauma and the disease itself is undefined[202]. Nevertheless recent research showed for the first time that ALS patients show a more frequent history of repeater or severe compared to the normal population[200]. In particular this is found in headers.

Nonetheless, research on aging, exercise and head trauma is still in its infancy. Detailed investigation into this relationship might provide crucial evidence on the pathology of ALS. Also the specific role soccer and football need further research, since other sports are not (yet) implicated as risk factors.

An overview of features which has been associated with ALS



Disease mechanisms involved into ALS which are associated with aging:	Disease mechanisms involved into ALS which aren't associated
Immune system astrocyte-glial function mitochondrial dysfunction Altered gene expression fALS age RNA metabolism	Pericyte dysfunction neurovascular: BBB and vascular rupture

Figure 11: Overview of mechanisms which have already been identified by the use of iPSCs (red) compared to all mechanisms which has previous been proposed by the use of animal models (gray and red).

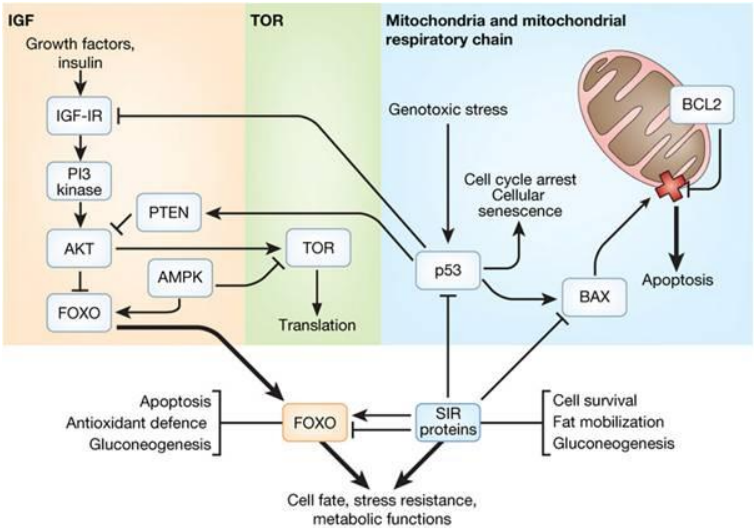


Fig. 12: Integrative aging pathways. These include the three major signalling pathways that reflects the major interactions which are involved into the aging: IIS Pathway (right), TOR (mid) and mitochondrial function (left). Together they regulate the complex interconnections between the IIS – TOR – oxidative stress signalling pathways. Notably, TDP-43 is known to activate FOXO transcription factors , with Ataxin-2 as a possible mediator. *(Image adapted from Vijg, J. & Campisi, J., 2008)* [16].

Pathway	Effects on ageing of model organism	Effects on mammalian brain ageing
Insulin/IGF-1 signalling	Decreased signalling promotes increased stress resistance and lifespan	Decreased signalling promotes decreased Alzheimer's disease pathology; paradoxically, increased signalling may be neuroprotective
TOR signalling	Decreased signalling causes increased lifespan, increased autophagy and decreased protein translation	Regulation of autophagy and protein homeostasis may modulate toxic-protein aggregation in neurodegenerative disease
Mitochondrial function	Severely decreased function causes decreased lifespan, but modestly decreased function can cause increased lifespan	Progressively decreasing function during ageing contributes to decline and pathology. However, preliminary evidence suggests that modestly decreased function may engage beneficial pathways
Sirtuins	Can increase or decrease lifespan in different contexts	Can be neuroprotective or detrimental to neurons, depending on context
Caloric restriction	Optimal caloric restriction causes increased lifespan	Increased preservation of cognitive function during ageing

Table 4: Signaling pathways that influence ageing in model organisms and brain ageing in mammals (adapted from Bishop, N.A. 2010)[203] .

AGING AND STEM CELLS

In the previous chapter we hypothesized that accelerated motor neuron aging may be the underlying cause of ALS pathology. The IIS pathway showed to be a crucial determinant of lifespan and seemed to be regulated by TDP-43 and (oxidative) stress[9]. To further investigate the influence of aging, there has been a lot of research on stem cells. It has already been shown that tissue and organ aging and/or rejuvenation are closely linked to stem cell function[7]. Aging in stem cells is reflected in their self-renewal capacity. It has therefore been suggested that the process of aging impairs self renewal and differentiation of stem cells, either through alternations that affect them directly or through alternations of their niches[204]. In this chapter we will investigate the influence of stem cells on aging and rejuvenation by discussing adult stem cells, the stem cell niche and intrinsic aging. Finally, the influence of stem cells on ALS will be discussed.

INTRINSIC AND EXTRINSIC STEM CELL PROPERTIES

An important question when talking about stem cell is which are their properties and how do they change during aging? Knowledge about both intrinsic and extrinsic properties of stem cells contribute to our understanding of how stem cells are able to the regenerate and rejuvenate tissues and organs

EXTRINSIC PROPERTIES IN GENERAL

Recent data suggest that extrinsic cues, as the systemic and local organ environment, causes multiple changes during aging, such as decreased activation of organ stem cells, inhibition of repair-specific molecular signaling which together lead to diminished tissue repair[15]. Interestingly, there is now evidence that suggests that stem cell based tissue replacement therapies might be rejected in older people. Replaced cells seem to age very rapidly and fail to contribute to organ repair after transplantation of the cells in vivo. Research in animal models has confirmed that the age of the host environment regulates the regenerative capacity[137]. For instance, when skeletal muscle explants were injected into animals, they were only effectively regenerated in young animals[205]. Furthermore, parabiotically paired young and old mice showed that the regenerative potential of muscle and liver was affected by the age of the systemic environment[15]. These data suggest that external factors are regulating the regenerative capacity of stem cells, rather than losing their regenerative capacity irreversibly[138]. Nonetheless, stem cells also showed to be important in the regulation of the replacement of cells that are lost during exercise and aging[128].

However, during all kinds of regeneration, their response includes a rapidly increased cellular turnover. During aging, this regenerative capacity gradually declines. In muscles, this may be witnessed by muscle fiber atrophy and a general loss of muscle bulk and strength[15]. On the one hand, it has been implied that declined stem cell function might be due to intrinsic aging of the resident stem cells. This makes them less responsive to environmental cues. On the other hand, this may be due to an age-dependent decline of environmental signals that regulate these cells[206].

Various researches have showed changes in developmental regulatory pathways, which are thought to regulate stem cell function. Pathways that are identified to promote the regenerative responses include: Activation of IGF-1, Notch, or MAPK/pERK pathways and reduction of TGF-β and Wnt signalling (recently reviewed by Conboy, I.A., 2011)[137,207,208,209]. Most knowledge about stem cell functioning has been gained from the MAPK/pERK pathway. This pathway showed to upregulate Delta and to stimulate Notch signalling. During development, Notch signaling stimulates proliferation and tissue renewal. After stem cell activation, the Notch ligand, Delta[207], showed to be upregulated, which lead to increased cell proliferation. Additional research

showed that when Notch inhibitors were injected into damaged muscle, this causes a dramatic impairment of muscle regeneration similar to the declined response seen in aged muscle[207],[207]. These results showed to be reversed by injection of pseudo-ligands that activate Notch signalling.

In conclusion, recent researchers have found supportive data about the influence of altered environmental cues as cause of declined stem cell regenerative capacity[210]. However, further identification of these cues and their function are needed.

STEM CELL NICE:

The stem cell niche is the microenvironment in which the stem cells reside. It varies in nature and location depending on the tissue type. Stem cells have their own intrinsic properties, though environmental regulation has shown to be important to direct stem cell proliferation and differentiation[204]. Studies in C.elegans and Drosophila have provided important knowledge into the molecular pathways which controls the niche of a stem cell[211]. Both, invertebrates and mammals display an asymmetric niche structure. Among cell divisions, one daughter cell stays in the niche as a stem cell, where the other leaves the niche to proliferate and differentiate[210]. The niche is responsible for maintaining homeostasis and for anchoring stem cells, regulated by evolutionary conserved pathways. These pathways include the Notch, Wnt, TGF-B/BMP and Shh pathways[204]. Evidence for involvement of these pathways shows that activation of developmental regulatory pathways, e.g. IGF-1, Notch or MAPR/pERK pathways, and reduced TGF- β and Wnt signalling, are involved into the regenerative responses in aged tissue (as outlined into the previous chapter).

Interestingly neural stem cells (NSCs) were found in various regions in the adult brain and peripheral nervous system. Here, they regulate the generation of neurons during adulthood. This so called neurogenesis seems to occur in direct contact with blood vessels and astrocytes(Fig.13)[204]. These stem cells undergo self-renewal and generate daughter cells which become granule neurons[210]. Furthermore, endothelial cells seems to play a crucial role in the NSC niche with respect to the generation of signals that control stem cell self-renewal and lineage commitment[210]. Moreover, various external signals regulate neuronal fate and number, such as BMP and Noggin[204]. In general, the BMP and Wnt signal pathways seems most involved in self-renewal properties and lineage fate, while activation of Notch seems to be crucial for tissue genesis and for control and maintenance of stem Cells. Recent work suggests that some of these pathways are controlled by micro-RNAs, with special emphasizes on IGF-1 and FoxO3.

Altogether, developmental regulatory pathways seems to regulate stem cell functioning [212]. There seems to be a correlation between stem cell maintenance and general tissue aging. Molecular identification of molecules, which regulates the formation of the aging niches, will broaden our knowledge about the process of aging itself. Interestingly, the neural stem cell niche is in close proximity of blood vessels. If there would be a systemic influence on aging, this might explain the special vulnerability of neurons to aging. Moreover, there are less stem cells in the adult brain compared to other tissues. This might explain why aging-mediated decline in stem cell function damages first the brain. In addition, since NSC niches reside next to capillaries, neurons and glial cells are directly exposed to high insulin and IGF levels. This may lead to enhanced stimulation of the stem cell, which results in earlier senescence. However, it could also be that neuronal stem cells are more sensitive to regulatory signals as a result of increased receptors or an altered binding affinity to those components. However, further research is needed to further unravel this working mechanism.

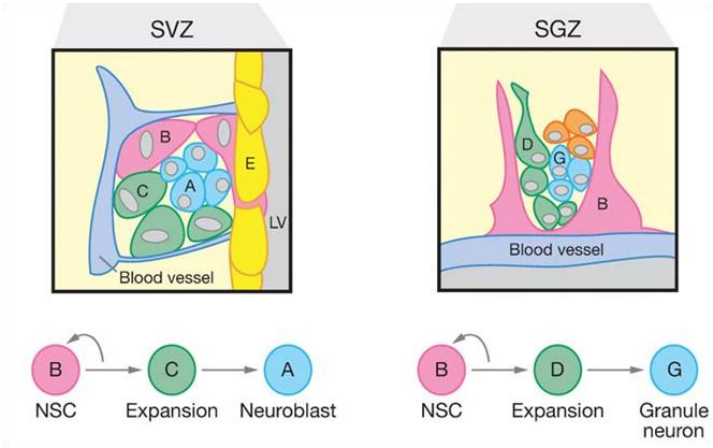


Fig.13: Structural and functional morphology of the neuronal stem Cell Niche. Neuronal stem Cells are always positioned next to a neighboring blood vessel. This is needed to maintain their homeostasis. The numbers in the image each represent: A. neuroblasts, B. Neuronal stem cells, C. expansion of NSCs, E. endothelial cells and LV. the lumen of the vascular blood vessel. *(Image adapted from Li, L., & Xie, T., 2005)* [194].

INTRINSIC AGING

As above mentioned, stem cells are pluripotent, which means that they resist factors that do causes age-related changes in replicative or postmitotic progeny in adult stem cells[128]. They behave as a self-renewing population, which may have sustainable periods of quiescence[213]. Furthermore, they have defense and repair mechanisms[213]. Continuously renewing tissues, like skin cells, undergo a high number of cell divisions to maintain normal tissue homeostasis[214]. However, each replication cycle contributes to intrinsic aging: shortening of the telomere[215], chromosomal rearrangements and mutations.[216] Eventually this may results in cellular senescence[214]. Studies have shown that adult stem cells do have a finite replicative lifespan[206]. However, this was investigated using experimental manipulations and it remains to be questioned whether replicative aging is enough to cause declined stem cell function in long-lived animals[206]. Besides, adult stem cells are also subject to chronological aging (when being in quiescence), which takes place in non-dividing cells, such as neurons[206]. Chronological aging is caused by the accumulation of damaged macromolecules, like proteins, lipids and nucleic acids that cause cellular toxicity[217].

CELL FATE

Another intrinsic feature of stem cells is their cell fate (Fig.14)[206]. Based on the tissue, an adult stem cell could be unipotent, bipotent or multipotent[218]. Functional tissue homeostasis and regeneration are regulated by the ability of stem cells to produce a relevant repertoire of tissue specific progenitors. Cell fate is mainly determined by their epigenome[219]. This epigenomic program is influenced by environmental factors and regulated by developmental signaling pathways[220]. These pathways seemed to be involved in organogenesis during development and include Wnt, Notch, Hedgehog pathways and TGFb (see for more information the following reviews))[10,221,209,206,]. For instance, a research by Carlson et al., (2009) showed that Wnt signalling restored the youthfulness of cells, whereas increased TGF-β cytokine levels are associated with aging[222]. Intrestingly, in a study by Conboy, et al., (2005), researchers demonstrate that premature activation, which occurs when yong stem cells are placed in an old environment, results in aberrant stem cell progeny[15]. Exceedingly, this age related interruption of lineage fidelity can be restored if stem cells are placed in a youthful environment[206].

Notably, when stem cells lose their ability to self-renew and/or if senescence pathways become activated, this can lead to the depletion of the stem cell pool[223]. However, this decline could also be a result from loss of self-renewal activity which seems to be regulated by Bmi-1, HMGA2 and the longevity associated transcription factor FoxO3 in mice[224]. Bmi-1 and HMGA2 proteins regulate NSC self-renewal by inhibition of the expression of the cell cycle inhibitors, p16INK4a

and p19ARF[225]. Interestingly, FOXO proteins, which has previous been associated with ALS, seemed to regulate NSC homeostasis by regulating the expression of cell cycle regulatory proteins as cyclin D1, inhibitor of DNA binding 1 and polo-like kinase 2.

Furthermore, an increased amount of stem cells undergo senescence (growth arrest) during aging, which has a negative impact on tissue homeostasis and regenerative capacity[226],[225] . This seems to b caused by genomic instabilities and/or loss of transcriptional regulation, which affect the accessibility of large chromosomal regions during aging[220]. DNA damage is caused by changes in chromatin-remodelling proteins, loss of nucleosome occupancy and redistribution of histone modifying enzymes on the chromatin in old cells compared to young. Moreover, it would be interestingly to investigate whether and to what degree, rejuvenation of old stem Cells[137] is mediated by restoration of the balance among various chromatin remodelling complexes. This could be performed using the parabiotic mice model. Furthermore, even as in somatic cells, alternations of the proteome can lead to aging as well.

Early removal of improperly folded or damaged proteins is required to maintain normal cellular function[69]. Therefore, autophagosomes, chaperones, lysosomes and the ubiquitin-proteasome system are needed to sense and remove misfolded or damaged proteins in cells[82].

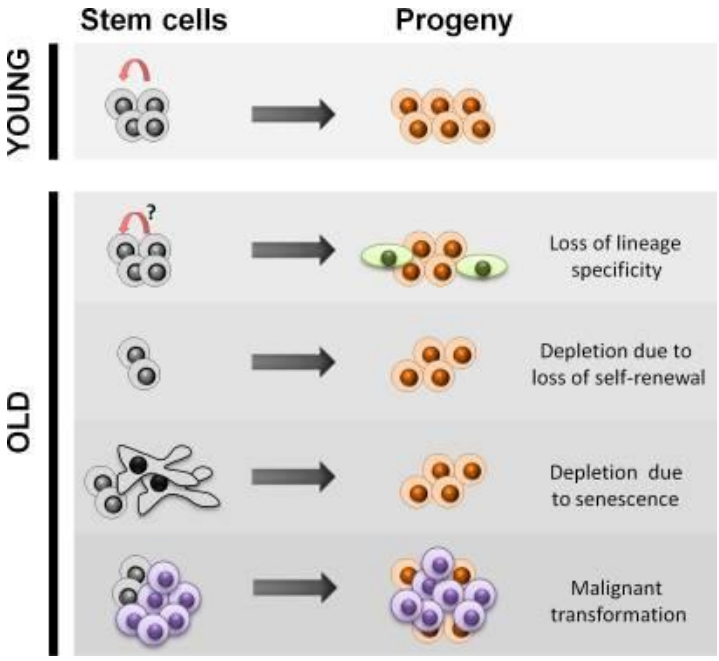


Fig.14: Cell fate. In young organisms, stem cells behave like a indefinitely dividing, self-renewal population. They give rise to lineage specific differentiation during tissue homeostasis or regeneration. However, during lifespan stem cells lose their lineage specificity and contributes to nonfunctional progeny. This results in loss of tissue integrity and a decline of physiological functions. *(Image adapted from Liu, L., 2010)* [196].

EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) are pluripotent stem cells, derived from the inner cell mass of the blastocyst and contain the ability to replicate indefinitely. They are able to differentiate into any other cell type by asymmetric cell divisions. This means that one daughter cell will maintain stemness, while another differentiates into a particular tissue type[7]. Because of their tremendous replication potential, much research is focused on the replacement of damaged tissues and organs. This replacement is mediated by their regenerative capacity and rejuvenation. Cell replacement approaches expand human ESCs and then differentiate them (in vitro). This way patient specific cells can be produced and used to replace their dysfunctional counterparts in vivo. The extent of this method is huge and includes many age-related disorders that are currently incurable. More details about ESCs, and the use of these cells to model ALS can be found in the final chapter; In vitro cell based techniques.

LINK BETWEEN STEM CELL FUNCTION, AGING AND ALS.

Although there are already multiple pathways identified in stem cells and/or in their environment that are associated with aging, these factors do not link the influence of embryonic cells directly to ALS.

It could also be hypothesised that a decreased regenerative capacity of stem cells during aging may be the underlining reason for neurodegenerative late-onset diseases, such as ALS. In this perspective, it may be that the aging process itself causes damage to motor neurons mediated by the IIS pathway. Than it could be a matter of time before the damage will outweigh the protective and regenerative capacity of the motor neurons itself. More damage will accumulate within cells during aging and whenever normal functioning becomes severely impaired this could be the onset of the disease. From that point, a rapid progressive degeneration takes place. The amount of damage increases, while protective and regenerative mechanisms are totally diminished.

This hypothesis has been supported by the discovery of very small embryonic-like stem cells (VSELs) in adult murine tissues[227]. Their proliferations seems to be negatively regulated by epigenetic changes of certain imprinted genes, namely Igf2-H19 locus, Igf2R and RasGRF1[220]. These genes are involved in the IIS pathway. These data support previous findings, in which increased insulin-like growth factor-1 (IGF-1) has been associated with reduced lifespan. Furthermore, a direct link between IGF-1 plasma concentrations, aging and the number of VSELs, residing in adult organ tissues, was found[228],. Depletion of these pluripotent stem cells in an IIS-dependent manner causes a decreased number of VSELs and a reduced regenerative potential that directs rejuvenation of tissues[228]. Moreover, stressful conditions, such as organ injury, increase VSELs number and enhance their cellular mobility in mice as humans[228]. This supports the assumption that they have a potential role in rejuvenation and tissue regeneration. Additional experiments showed that isolated VSELs from young mice were able to expand to all three germ-cell layers both in vitro as in vivo[227], while older mice (>2 years) failed[229,230]. This observation could either be the result of different paracrine influences or due to epigenetic changes in imprinted genes related to the insulin factor signalling in adult tissues[220].

Adult tissue seems to be able to activate a quiescent VSEL state during aging. This cellular mechanism may have been evolved as protection against spontaneous growth of teratomas[220]. Research has found that VSELs downregulate Insulin growth factor-2 (Igf2) expression. Igf2 is highly expressed during the embryonic development and causes upregulation of the IGF-2 receptor (IGF-2R)[231]. Subsequently IGF-2 is able to bind Insulin growth factor-1-receptor (IGF-

1R). However, as the result of epigenetic alterations during aging, VSELs show a decrease in IGF-2 and RasGRF1 expression and an overexpression of IGF-2R[232]. This causes quiescent in adult tissues. Nonetheless, chronic exposure to IGF-2 or insulin seems to accelerate premature depletion of VSELs. To further investigate involvement of the IIS pathway in stem cell functioning, more research was performed using the dwarf mice as model[233]. Dwarf mice harbor a deficiency of growth hormone (GH) receptor, and as a consequence of this deficiency they show a reduction in IGF-1 plasma levels as well. Their number of VSELs showed to be 3-4 fold higher than in normal mice during aging even as their average lifespan[228]. This confirms the influence of the IIS pathway on VSELs appearance.

Interestingly, also calorie intake has previous been associated with the IIS pathway, by regulation of IGF-1 levels in serum[145]. Now, it has been found that high calorie intake causes a reduced number of VSELs during aging[228]. This confirmed that high IGF-1 plasma levels (either autonomous regulated or influenced by calorie intake)[234] may cause premature depletion of stem cells and may thus be the cause of premature aging.

Above findings show, that the rejuvenation and the regenerative capacity of VSELs can be influenced by IGF concentration. Interestingly, chronic exposure to it causes premature depletion in these stem cells[232]. During aging exactly this happens. IGF concentrations are high and therefore can directly influence the regenerative capacity of VSELs. Since the same pathway is implicated in ALS, it might be possible that the loss of VSEL regeneration diminishes the regeneration of MN as well. Therefore it might influence the pathology observed in these patients.

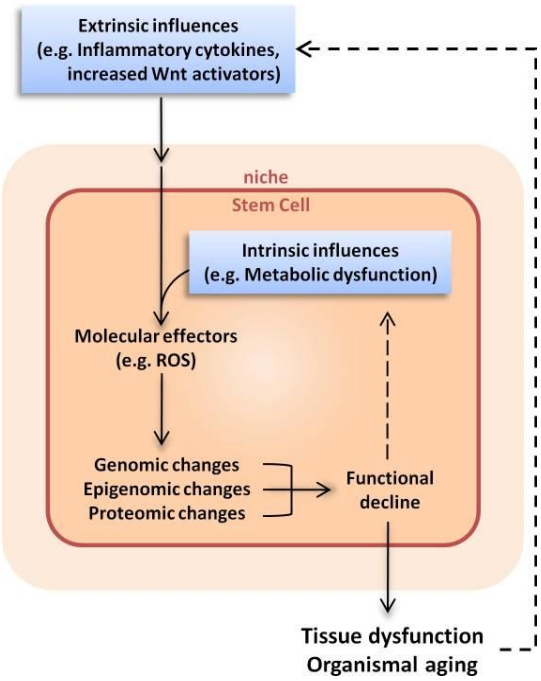


Fig.15: Extrinsic and intrinsic influences on stem cell function. Age-related changes in the systemic milieu might result in altered signaling cascades or molecular changes within the cell. This can be causes by a change in the systemic blood circulation or by changes within the stem cell niche. In contrast, intrinsic changes do also appear during aging, such as mitochondrial dysfunction. These signals might integrate and influence downstream molecular effectors. This causes genomic, epigenomic and proteomic changes in the cell. and subsequently lead to decreased cellular functioning. Decreased stem cell function exacerbates the extrinsic influences of aging. Thereby they contribute to aging. *(Image adapted from Liu, L., 2011)* [196].

DISCUSSION

Stem cell research has provided important knowledge about aging and rejuvenation[137]. Diverse studies have found an association between the IIS pathway, aging and stem cell function[228],[7]. This again confirmed involvement of the IIS pathway in aging. These findings might have important implications for ALS research.

Recent studies suggest that neurodegenerative diseases may be dependent of the rejuvenation and regenerative capacity of stem cells[15,138,206]. During aging, this rejuvenation and regenerative capacity of stem cells declines[203]. This decline may be the result of environmental changes[212]. Research on parabiotic mice showed that the regenerative potential of organ function was affected by the age of the systemic environment[15]. These data suggest that external factors are regulating the regenerative capacity of stem cells, rather than losing their regenerative capacity irreversibly[138]. This implies a systemic role on the regulation of stem cell function[235].

Parabiotic studies have showed the need of increased IGF-1, Sonic Hedgehock, Notch, MAPR/pERK pathway signalling, and a reduced TGF-β and Wnt signalling, during regenerative responses[212,222,236,237,238]. Moreover, researchers have found atrophy of the motor cortex, termed motor cortex thinning, in ALS patients[239] (validated by recent research; preliminary data). Interestingly, a fundamental study into the working mechanism of TGF-β1 by Piras F. et al.. (2012), has discovered a correlation between TGF-β1 serum concentrations and cortical thickness. In healthy individuals, high serum levels of TGF-b1 has been associated with increased cortical thickness in the cingulate and right frontal cortex[240]. This implies that a reduced serum level of TGF-β1 might be involved into ALS pathogenesis. However, this result is in contradiction with the above mentioned studies, in which reduced TGF-β seems to be required for regeneration of cells and tissues[222,209]. Therefore, more research is needed to unravel the mechanism behind motor cortex thinning and its development, to exclude if this is a cause or a consequence of ALS.

Interestingly, recent research discovered that signals from the systemic environment drive age-related, intrinsic changes in oligodendrocyte precursor cells that cause demyelination of neurons which leads to demyelinating diseases such as multiple sclerosis (MS)[137]. Although this finding involves another type of neurodegeneration, it supports the hypothesis that the systemic environment might be able to drive age related changes leading to ALS. Moreover, previous research has observed increased mobility of stem cells in response to damage[241]. This makes us questioning if the presence of stem cells in the skin or intestine could have an influence on ALS pathology (e.g. damage) in the brain? Besides the question about the intrinsic function of stem cells, it would therefore be interesting to further investigate to what extend environmental factors contribute to ALS pathology. Consequently, if systemic factors would be able to influence the regenerative capacity of stem cells, they might enhance the regenerative capacity of somatic cells as well. Nevertheless, additional investigation have to be done to identify the exact location of embryonic stem cells in the brain, since their effect may only affect the local environment.

To further investigate stem cell function in relation to aging and ALS, various aspects have to be considered. For instance: What is the role of stem cell rejuvenation and regenerative capacity on damage induced by oxidative stress and proteotoxicity as caused by ALS? In which way does oxidative stress and proteotoxicity interfere or reinforce stem cell function in relation to the aging process? Oxidative stress and proteotoxicity can either change environmental influences or they can cause direct damage to stem cells or their niche (Fig.15)[206]. To summarize, it is not clear yet to which extend stem cell function is involved in ALS onset and disease progression.

In conclusion, age-related changes seem to be determined by the age of the systemic environment, rather than by the cell-autonomous age[206]. To further investigate these systemic influences and or environmental influences that contribute to a declined stem cell function and/or ALS pathology during aging, a new promising technique, the iPSC model, could be used to obtain a deeper understanding[131]. In addition, these models could then also be used to investigate, whether and to what degree, rejuvenation of old stem cells is mediated by restoration of the balance among various chromatin remodelling complexes, since they have showed to regulate activation of the IIS pathway[137]. In the next section a new ALS model will be introduced, which can be used to investigate the influence of aging. Consequently, the final chapter will suppose a future research suggestion.

IN VITRO BASED THERAPEUTIC STRATEGIES

Previous chapters have suggested an causative role of aging on the development of ALS. To confirm this, more research is needed. Yet, tractable models to test this hypothesis in human neural system are scarce. Much knowledge into the pathophysiology of ALS is obtained from animal models, which displays familial disease forms of ALS. They have been implemented by the use of highly overexpression of mutant genes, such as SOD-1[60]. However, more than 90% of ALS patients suffer from a sporadic forms[23] due to complex interaction between genetics and environmental factors. Therefore, these models may not completely mimic the actual disease[107]. Moreover, a complete recapitulation of ALS is not possible, because of fundamental genetic and anatomical differences between animals and human. This became particularly clear after repetitive failure of translating animal data to the clinical settings[24]. Therefore, new models are needed.

Novel stem cell technologies offer exciting new opportunities to study human neural development and disease in the laboratory. They may provide the first feasible models available to investigate the disease mechanism for sALS by utilizing induced pluripotent stem cells (iPSCs)[18] . Nowadays, the challenge is to evaluate differences and similarities between fALS and sALS. This chapter will introduce the concept of human embryonic stem cells, induced pluripotent stem cells and their purposes.

HUMAN EMBRYONIC STEM CELLS (HESCS)

Human embryonic stem cells were discovered in 1998 and afterwards used for various purposes[242],[243]. They have the advantage that a human-situation can be investigated directly, without further manipulation. Therefore, hESCs would be the perfect model to investigate diseases. However, this technique uses human embryos. Of course this raises several ethical concerns and hinder their application. iPSCs resemble the use of embryonic stem cells (ESCs) in that they are both pluripotent and share the ability to replicate indefinitely. Since there are no ethical concerns with respect to iPSC, this method has obtained increased interest over the past ten year. Though hESCs are still important to investigate fundamental questions and basic biology, but also to further optimize iPSC modelling by unravelling the intrinsic properties of pluripotency and differentiation[244]. Therefore, this model is needed for the development and optimization of other in vitro cell based methods, like iPSCs, for good disease modeling.

HUMAN INDUCED PLURIPOTENT STEM CELLS (HUMAN IPSCS)

IPSCs are adult somatic cells that have been genetically reprogrammed to an embryonic stem cell[19]. They provide enormous potential to model ALS because of their competence to developmental signals that permits their specification of functional, regional subtype-specific neurons[24]. In the case of ALS, this includes the directed differentiation of spinal cord, midbrain and motor cortex neurons[237]. Studies have showed that this technique reliable recapitulates the spatio-temporally regulated, developmental responsiveness to specific extrinsic morphogenetic signals. Subsequently, the next challenge is to understand how these refined subregion specific human neuronal and glial diversity is produced. There are multiple motor neuron subtypes, each with different disease vulnerability[245]. In addition, currently in vitro based stem cell models are already quite competent to do this[246]. Furthermore, iPSCs enables the investigation of patient specific lines that includes disease causing mutations, which is not able with hESCs[247].

The first iPSC, generated from fibroblasts, has been discovered in mice in 2006 and in human in late 2007[248],[249],[249]. These patients'-derived iPSCs carry the exact package of genetic information that is associated with patients' pathology. Therefore iPSCs allows the study of living cells and

provides insight into their intrinsic properties, their susceptibility to environmental influences and their interactions with other cell types[250].

However, the process of reprogramming is low (typically 1% of transfected fibroblasts change into a iPSC) and often incomplete[248, 248]. It has been suggested that numerous cells undergo the process of reframing, but not all finish the process completely. However, it is beyond the scope of this review to provide an overview of the many studies which have investigated this (more details are provided by Papapatrou, E., 2009)[250]. In contrast, one of the main advantages of the use of iPSC is its simplicity and reproducibility[248]. It has been found, that only four transcription factors (TF), namely Oct4, Sox2, Klf4 and c-Myc, are needed to induce pluripotency[251],. This can be performed on both terminally differentiated or postmitotic cells[250],[252]. Furthermore, it has been hypothesized that all somatic cells have a potential to become iPSCs, although with different efficiencies[253].

Another point of debate is whether differentiation of iPSCs are functionally different from ESCs[254]. In vitro directed neural differentiation showed that more than 90% of ESCs became successfully differentiated, compared to 10-50% of iPSCs[255]. Though, other research found much higher differentiation efficacies in iPSCs, which again emphasizes the conflicting results regarding the similarity between those cells[19]. Besides reprogramming efficacy, the reprogrammed cells of iPSC have showed to be similar to ESC in their morphology, expression of marker genes and their ability to form teratomas (tumours compromised of cells from all three germlayers)[254]. Transcriptomics have shown a more fetal state of the neurons rather than an adult one[256]. This may limit the modelling of the disease. However, research confirmed that human iPSCs use the same transcriptional network to obtain specific cell types. This takes place over the same developmental time period as hESCs, mediated by the same morphogenesis (i.g. morphogenetic protein (BMP), Sonic Hedgehog (Shh) and Notch)[254].

Moreover, the biggest question still remains if iPSCs, besides the above-mentioned features, are exactly similar to ESCs with respect to epigenetic reprogramming and cellular memory? Research have showed contradictory results, some have found persistent expression of donor cell genes, while others found differences in DNA methylation pattern and or epigenetic memory, while others haven't found any differences at all[257];[258].

IPSC DISEASE MODELLING OF ALS PATHOLOGY

IPSC technology is an interesting technique to further investigate the ALS pathology. The recent discoveries that emphasize a major role for IIS-pathway-mediated aging and rejuvenation on motor neuron degeneration had made the use of former disease models difficult. Nonetheless, iPSC technology can be used to investigate these disease mechanisms[259]. Although, the question if induced pluripotent stem cells can be produced directly from elderly patients with ALS remains. Their cells have been exposed to disease causing components all their life. However, recent data showed successful results of motor neurons, generated from iPSCs from skin fibroblasts from a 89 and 82-years old patient who suffered from fALS[260]. These patients were among the oldest living individuals with mutant SOD1 alleles. This makes us questioning about the role of SOD-1 in disease pathology, since inherited mutations usual causes early onset of disease. However, more research is needed to further investigate this.

One of the key challenges of iPSC disease modelling is assess to what extend fALS and sALS differ from each other[107]. For instance, recent research has investigated if astrocytes release neurotoxic factors which affect motor neuron degeneration by the use of post mortem spinal

cord neural progenitor cells of both sALS and fALS patients[107]. Motor neurons which were treated with sALS and fALS conditioned media showed a 50% higher reduction of motor neurons compared to non-ALS controls[261]. This glial mediated toxicity has showed to be similar in sALS and fALS and again confirm a systemic or environmental role on motor neuron degeneration. Furthermore, a human iPSC model of TDP-43 proteinopathy in motor neurons has showed that mutant TDP-43 causes increased soluble and detergent resistant cytoplasmic TDP-43 levels, which led to decreased lifespan under basal conditions. Interestingly, mutant TDP-43 motor neurons seems to be more susceptible to PI3K inhibition, which is a crucial signalling pathway in neurons[262]. However, this susceptibility showed to be independent of the MAPK/ERK pathway or the induction of ER stress. Notably, both pathways are part of the IIS pathway, which may link this observation to aging[9]. Yet another study has showed that TDP-43 protein mediated toxicity appears in both sALS and fALS[263]. ALS patients specific iPSCs which harbour mutant TDP-43 have showed to be more vulnerable to cellular stressors compared to non-ALS control patients[263]. They also turned to be more sensitive to oxidative stress which resulted in an increased amount of insoluble TDP-43 and thus enhance aggregation[264]. Moreover, they found various altered gene expressions.

IPSCs could also be used to obtain insight into both development and disease of motor neurons at the RNA level, with high throughput approaches, such as exome- and RNA-sequencing[120],[24]. These techniques have showed various changes in gene expression and alternative splicing events (i.e. C90RF72 gene)[24]. In addition, another research created iPSC lines from patients with mutations in the VAPB gene[265], another gene that has recently been associated with ALS, as well as from non-ALS controls. Reduced level of VAPB protein were observed in patients with mutant VAPB gene compared to controls[54]. Subsequently, they showed a gradual increase of VAPB during cellular differentiation in controls, but this was not found in patients with mutant VAPB. This implies that mutant VAPB causes a failure of VAPB protein upregulation during the induction of motor neurons[266]. This is likely due to posttranslational processing of VAPB, since there has no mRNA level differences been found between control and mutant VAPB patients[267]. Moreover, posttranslational processes have already been identified, though this has not been intensively investigated yet.

In general, iPSCs have showed major potential for disease modelling. Therefore, further research has to be performed to further identify ALS disease mechanisms and to discover candidate drugs for future therapy(For an overview see Table.1) [264].

Disease	Genetic defects	iPSC derived cell types	Disease phenocopied in iPSCs or differentiated cells	Drug or functional tests	Reference
ALS	Mutation in SOD-1	Motor Neurons	NA	No	268, 269, 261
	Mutation in VAPB (vesicle-associated membrane protein)-associated protein B	Motor neurons	Yes	NO	266
	Mutation in TDP-43/FUS	Neurons and motor neurons	Yes	Yes	270, 263

Table.1: Overview of all mechanisms which has already been found in ALS using iPSCs

DRUG SCREENING AND DEVELOPMENT

Besides elucidation of ALS disease pathogenesis, motor neurons produced from patient derived iPSCs may provide also an important tool for screening of drug candidates[264,270]. iPSC serves as a platform to discover novel compounds by making use of molecular dissection of the disease process[218]. Currently, this technique enables to test for efficacy and toxicity of small compounds in patient specific populations. It can now exactly be determined which drug or which drug combinations are effective in human patients[218,132,21]. However, the molecular and cellular phenotypes first have to be identified. Subsequently, the generation of high quantities of specific neurons or glial cells enables high throughput drug screening[271]. On this moment, ALS patients specific iPSCs which harbour mutant TDP-43 have showed to be more vulnerable to cellular stressors compared to non-ALS control patients[263]. They also turned to be more sensitive to oxidative stress which led to an increased amount of insoluble TDP-43 and thus the formation of aggregates[264]. Moreover, they found various altered gene expressions. However, further research has to be performed to further identify candidate drugs for future therapy[264].

CELL THERAPY

Generation of iPSCs from ALS patients allow the production of large numbers of immune matched cells. This technique enables cell replacement and neuroprotection strategies in patients with ALS. Major results have already been observed with rodent and human iPSC derived neurons in various neurodegenerative diseases, such as in Parkinson's Diseases[272,273]. Besides, glial cells could been used to support cells, rather than as replacement of motor neurons[274,218]. However, in spite of the major potential of iPSCs, several challenges have to be overcome before cell therapy could be clinically applied[218].

Firstly, integrating viral vectors, which carry oncogenic genes, like retroviruses, are used to reprogram cells. However, this causes an increased risk to develop cancer. However, currently research has already developed new mechanisms to reprogram cells, without the use of viral vectors[238,275], like the use of microRNAs or transcription activator-like effector nucleases (TALENs).

Secondly, purification of the desired cells, by differentiation of iPSCs to specific cell types is required for objective measurements. This will be established by increasing the understanding of directed differentiation and to optimise or develop improved protocols, which eventually enables highest possible cell specificity. High cell specificity is needed to unravel the exact disease mechanism, but this also lead to a better immune matched cell for future cell transplantations[274].

Thirdly, the exact disease mechanism, of each genetic defect, has to be unravelled before they can be used for cell therapy. This is the hardest point to challenge with respect to ALS. Intrinsic pathogenic mechanisms of ALS may be delayed, due to the relative short lifetime of iPSCs. Since ALS is the result of a lifetime accumulation of disease pathology, iPSC derived motor neurons and glial cells are conducted to external stressors to facilitate late-onset ALS phenotype[276]. As stressor, l-glutamate-induced excitation of iPSCs derived neurons are often used to generate the ALS phenotype[264,277]. This causes CA^{2+} dependent proteolysis, which results in micro aggregate formation. However, the question remains how relevant these iPSC neurons actually are for the investigation of ALS pathology. Notably, heavy exercise has been supposed to be a strong risk factor for ALS by facilitation of oxidative stress and ROS production[171,164,172]. A similar principle could underlie the effect of l-glutamate-induced excitation of iPSC-derived neurons.

Finally, the main goal of cell replacement therapy in ALS disease is to replace degenerated (motor) neurons. Yet, to make them functionally similar to autonomous (motor) neurons, they have to acquire the ability to extend their projections to the proper target structures. This is often mediated by non-cell autonomous signalling regulated by glial cells[218]. Therefore, glial cells need to be taken into account as well. However, further research should be done to further investigate this complexity and to overcome these challenges.

DISCUSSION

The discovery of iPSCs offers very exciting and unprecedented opportunities for the use of ALS disease modelling[264,132,263]. This technique has to potentially decipher the molecular and cellular mechanisms underlying ALS pathology. We hypothesised that accelerated motor neuron aging may be the underlying cause of ALS pathology. iPSCs now enables investigation of aging on the development of ALS in humans.

This technique has several important advantages. Firstly, iPSC technology is the first method, which enables investigation of sALS instead of fALS (Fig.16). Besides, iPSCs derived motor neurons could be co-cultured with human derived glial cells as well[278,269]. Secondly, iPSCs are efficiently converged to neural lineages by the establishment of different protocols. This allows the investigation of early progenitors that were first inaccessible in patients. Lastly, this technique enables the production of a big number of cells that can be used for cell-based toxicity screening and future therapy. This can close the gap between animal studies and clinical settings[279]. iPSCs may even have the potential to establish personalized drug development using high-throughput screening[280]. Though, several challenges have to be overcome before iPSCs can be clinically applied, which mainly includes safety issues and differentiation specificity. However, due to the many developments in this field in recent years, these challenge will certainly be overcome in the near future.

In conclusion, iPSC technology offers huge potential for the investigation of ALS pathology. iPSCs are live functioning, human, patient derived specific cells, which will give us insights into disease mechanisms, drug discovery, cell therapy and a potential new diagnostic method for patients with ALS[19,20,21]. It would be a very tempting purpose to further investigate the influence of IIS-pathway-mediated aging and rejuvenation on motor neuron degeneration.

Experimental set-up

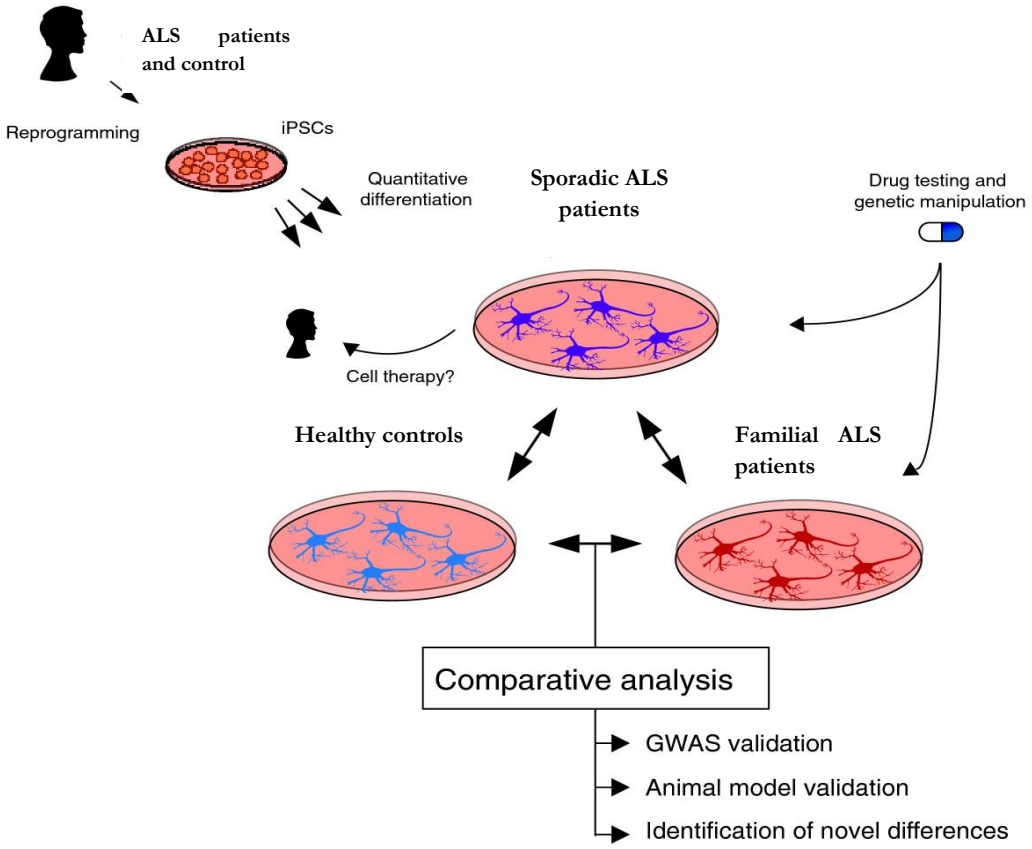


Fig.16: iPSC potential in ALS disease. Patient derived iPSCs could be differentiated in motor neurons and used for disease modeling and drug screening. This technique enables the investigation of disease pathology of sALS in comparison to the disease pathology of fALS. These results could be verified by the use of Genome Wide Association Studies (GWAS) and animal models.

PROPOSAL FOR FURTHER RESEARCH

Recent research strongly supports that accelerated motor neuron aging may be the underlying cause of ALS pathology[9]. Data have showed that age-related changes seemed to be determined by the age of the systemic environment, rather than by the cell-autonomous age[15]. Therefore, it has been suggested that signals from the systemic environment drive age-related, intrinsic changes, mediated by the IIS pathway. This activates a cascade of events that eventually can lead to ALS pathology. However, current animal models are not suitable to further investigate this postulation. Not only did the obtained results fail when translated to the human situation, also are fundamental genetic and anatomical differences between both species in age-related pathways found[16,17]. Therefore, new and improved models are needed.

As stated in the previous chapter, in vitro cell based therapies seems to be the first feasible model to investigate the disease mechanism for both sALS and fALS by utilizing induced pluripotent stem cells (iPSCs)[18,19,20,21]. Therefore, it would be a very tempting purpose to use this iPSCs to investigate the influence of IIS-pathway-mediated aging and rejuvenation on motor neuron degeneration.

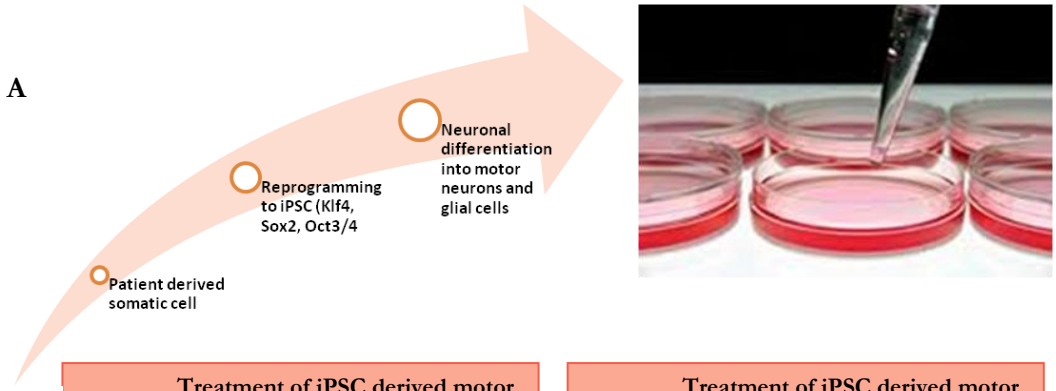
Studies on parabiotic mice, have already indicated a major influence of the environment on aging[7,137,138,212]. Parabiotic mice are mice that have been sutured together, side-by-side, along the lateral aspect of the body. Once blood vessels have connected with each other, the mice will share a common blood supply. This model has been used to determine whether a blood-borne mediator from one, affects the effect of aging of the other. Therefore, heterochronic (young and old) pairs were compared with isochronic (young to young or old to old) pairs. In the study of Conboy, et al. (2005), researchers demonstrated that exposure of young serum to old mice increase the rejuvenation and regeneration capacity of the old mice. Likewise, this research showed that effects of aging on regeneration capacity are reversible. Moreover, these age-related changes seemed to be determined by the age of the systemic environment, rather than by the cell-autonomous age. Notably, when young serum became mixed with old serum, the regenerative potential was inhibited, suggesting a dominant influence of old serum factors[7]. Further research on which systemic factors might influence aging, found that increased IGF-1, Notch, MAPR/pERK pathway signaling or reduced TGF-β or Wnt signaling, increases the regenerative responses in aged tissue[222,207,206]. Interestingly, recent research by Conboy et al. (2011) on multiple sclerosis showed that signals from the systemic environment drive age-related, intrinsic changes in oligodendrocyte precursor cells[6]. This process seems to underlie demyelination of neurons, which leads to multiple sclerosis (MS)[137]. This supports the above mentioned hypothesis, suggesting that the systemic environment is able to drive age related neurodegenerative diseases. Hence, it would be very interesting to identify which systemic factors might contribute to the influence of aging on ALS pathology.

To accomplish this, iPSCs-derived motor neurons from sALS and fALS patients can be cultured in serum from younger non-ALS individuals (Fig.17A). As a control 'old' non-ALS patient derived iPSCs can be used. On the other hand, old serum from both sALS and fALS patients will be exposed to motor neurons, which are derived from non-ALS individuals. This will be compared with iPSCs from sALS and fALS patients as a control (Fig.17B). Since a non-autonomous cause has been proposed, the same experiments can also be performed with iPSCs-derived motor neuron and glial cell co cultures. Additionally, to further investigate the influence of the IIS pathway, an experiment that uses insulin/IGF blockers and agonists can be performed (Fig.17C). The same approach can also be used to investigate the effect of oxidative stressors or if being a diabetic functions as a risk factor.

Furthermore, this model can be used to investigate the role of stem cell functioning on the development of ALS as well. Research showed that human stem cells are responsible for rejuvenation and regeneration of tissues cells. It could be hypothesised that a decreased regenerative capacity of stem cells during aging may be the underlining reason for neurodegenerative late-onset diseases, such as ALS. In this perspective, it may be that the aging process itself causes damage to motor neurons mediated by the IIS pathway. To investigate this, co-cultures of postnatal stem cells with motor neurons of different maturity from the above-mentioned patients would be exposed to the different sera (Fig.17D).

Considering these aspects, it would be a unique approach to investigate the influence of aging on ALS, since this would be the first experiment, on this subject, that uses human derived cells. Importantly, research into the effect of aging on human neurodegenerative disease would have clear implications for future therapies as well.

Experimental set-up



B

Treatment of iPSC derived motor neurons from ALS patients with serum from a young healthy individual

- Cell morphology and properties compared to cells which are treated with patient derived ALS serum (control)
- Disease rescue? Regeneration?

* co-culture with both MN and glial Cells

Treatment of iPSC derived motor neurons from young healthy patients with serum from ALS patients

- Cell morphology and properties compared to cells which are treated with healthy derived young serum (control)
- Disease onset - Manifestation?

*co-culture with both MN and glial Cells

C

Insulin/IGF receptor blockers

Treatment of iPSC derived cells from ALS patients

- Cell morphology and properties compared to cells which are treated without blockers
- Disease rescue? Regeneration? Or slower progression?

Insulin/IGF receptor agonists

Treatment of iPSC derived cells from young healthy individual

- Cell morphology and properties compared to cells which are treated without agonists.
- Introduction of ALS Phenotype?

D

Role Stem cell function

- Treatment of iPSC derived motor neurons from ALS patients with serum and post natal stem cells from a young healthy individual
- Cell morphology and properties compared to cells which are treated with patient derived ALS serum (control)

Disease rescue? Regeneration?

Role Stem cell function

- Treatment of iPSC derived motor neurons from young healthy patients with serum and post natal stem cells from ALS patients
- Cell morphology and properties compared to cells which are treated with healthy derived young serum (control)

Disease onset - Manifestation?

Fig.17: Proposed future experiments

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SUPPLEMENTARY FIGURES

Table 1: Pathological changes in ALS (Table adapted from *Ferraiuolo, L. et al. (2011)*[59].

Gross pathological changes	Microscopic changes
Atrophy of the precentral gyrus	**Depletion of ≥50% of the spinal cord motor neurons**
Shrinkage, sclerosis and pallor of the corticospinal tracts	**Diffuse astrocytic gliosis of spinal gray matter**
Thinning of the spinal ventral roots and hypoglossal nerves	**Atrophic and basophilic changes in surviving motor neurons**
Atrophy of the somatic and bulbar muscles	**Presence in surviving lower motor neurons of ubiquitinated inclusion bodies with**
	thread-like, skein-like or compact morphology
	Variable depletion of giant pyramidal neurons (Betz cells) in the motor cortex
	Variable astrocytic gliosis in the gray matter of the motor cortex and underlying subcortical white matter
	Evidence of microglial activation in pathologically affected areas
	Cytoplasmic aggregate inclusions within glial cell

Table 2: Aspects which make motor neurons more vulnerable for degeneration compared to other cell types (Tabel adapted from *Ferraiuolo, L. et al. (2011)*)[59].

Motor neuron vulnerability
Large cell size and long, large-volume axonal compartment
High reliance on a robust cytoskeleton and axonal transport mechanisms
High metabolic demands
High reliance on optimal mitochondrial function
Increased generation of reactive oxygen species (ROS) and a tendency towards oxidative stress **Possible motor neuron-specific and/or spinal cord-specific mitochondrial properties**
High physiological reliance on signaling through ROS and reactive nitrogen species
High intrinsic oxidative stress
Vulnerability to excitotoxicity and dysregulation of intracellular calcium homeostasis
High expression of calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors lacking the GluR2 subunit
Low expression of calcium-buffering proteins
High reliance on efficient synaptic glutamate reuptake transport mechanisms
Reduced capacity for heat shock response and chaperone activity
Possibly reduced proteasome function
High physiological expression of particular proteins, such as superoxide dismutase 1 (SOD1)
Greater vulnerability to toxicity in the presence of mutant SOD1

Table 3: ALS models which has previously been used to investigate ALS mechanisms and pathology (Table adapted from Gama Sosa, M., et al (2012))[16].

Animal model	Strategy	Pathology	Reference
Caenorhabditis elegans	Pan neuronal expression of wt human TDP-43	Motor neuron dysfunction, abnormal motor neuron synapses, and uncoordinated phenotype	[281]
Fruit Fly Drosophila melanogaster	Motor neuron and photoreceptor GAL4-UAS driven expression of wt and mutant hSOD1	Neither loss of motor neurons nor retinal degeneration was observed, although motor neuron expression of either wt or mutant hSOD1 led to progressive motor dysfunction. Earlier attempts at expressing hSOD1 in motor neurons increased longevity and could rescue the shortened lifespan of a dSod null mutant	[282, 283, 125]
ZebraWsh Danio rerio	In vitro transcribed RNAs of TDP-43 (TARDBP wt and mutants G348C, A382T, A315T) were injected into 1–4 cell stage blastulae	Preferential vulnerability of motor neurons to toxicity of TDP43 mutants; shorter motor neurons axons, premature and excessive branching, and swimming deficits	[284]
Mouse Mus musculus	Pan-neuronal constitutive overexpression of TDP-43 or mutant TDP-43 (A315T) via pronuclear injection of transgenic cassettes using PrP and Thy1.2 promoters	Expression of TDP-43 wt and mutant proteins results in variable pathological phenotypes depending on the animal model	[285, 286]
	Forebrain-targeted Tet-OV conditional expression of TDP-43. Bigenic mice were created with cassettes harboring the Camk2a promoter driving the expression of Tet-OV tTA and the tetracycline-responsive promoter to drive hTDP-43 (tetO-hTDP). hTDP-43 was preferentially induced (in the absence of doxycycline) in forebrain neurons	Selective degeneration of hippocampal dentate gyrus and neocortical neurons along with dysregulation and loss of endogenous TDP-43 and presence of rare phosphorylated and ubiquitinated TDP-43 inclusions in aVected neurons. Transgenic mice develop a motor phenotype associated with spasticity	
	Pronuclear injection of a human SOD1 transgene containing the G93A substitution associated with familial ALS	Hemizygous transgenic mice develop motor neuron disease associated with progressive limb paralysis and death by 5–6 months of age	
	CD11b-TKmt-30 mice contain a transgenic cassette in which the	After ganciclovir treatment, selective ablation of proliferating microglial cells occurs. Ablation of	

	HSV-1 TKmt-30 gene is expressed under the control of the CD11b promoter, which directs high levels of expression in macrophages. Proliferating microglial cells in the brain can be selectively eliminated by ganciclovir treatment	proliferating microglia does not aVect motor neuron degeneration in a mouse model of ALS but causes increased amyloid deposition in a mouse model of AD, suggesting that microglia play a critical role in restricting AD associated senile plaque formation
Rat Rattus norvegicus	Transgenesis with a 22-kb normal and retroWtted-mutant (M337 V) human TDP-43 gene extracted from a BAC	Constitutive expression of the mutant TDP-43 causes early postnatal death in transgenic rats. Founders develop weakness in the limbs and do not survive past 29 days
	Tet-OV inducible system for TDP-43 expression. tTA protein expression controlled by a CMV enhancer fused to the chicken ・ actin promoter (CAG). Normal and mutant TDP-43 cDNAs were expressed under the control of a tTA-dependent promoter constructed by the juxtaposition of multiple t etracycline-responsive elements (TRE, tet-O) upstream from the minimal CMV promoter	Overexpression of mutant TDP-43 but not wt protein induce progressive widespread neurodegeneration aVecting predominantly the motor system with denervation atrophy of skeletal muscles

Figure 1: Processes in which TDP-43/FUS seems to be involved

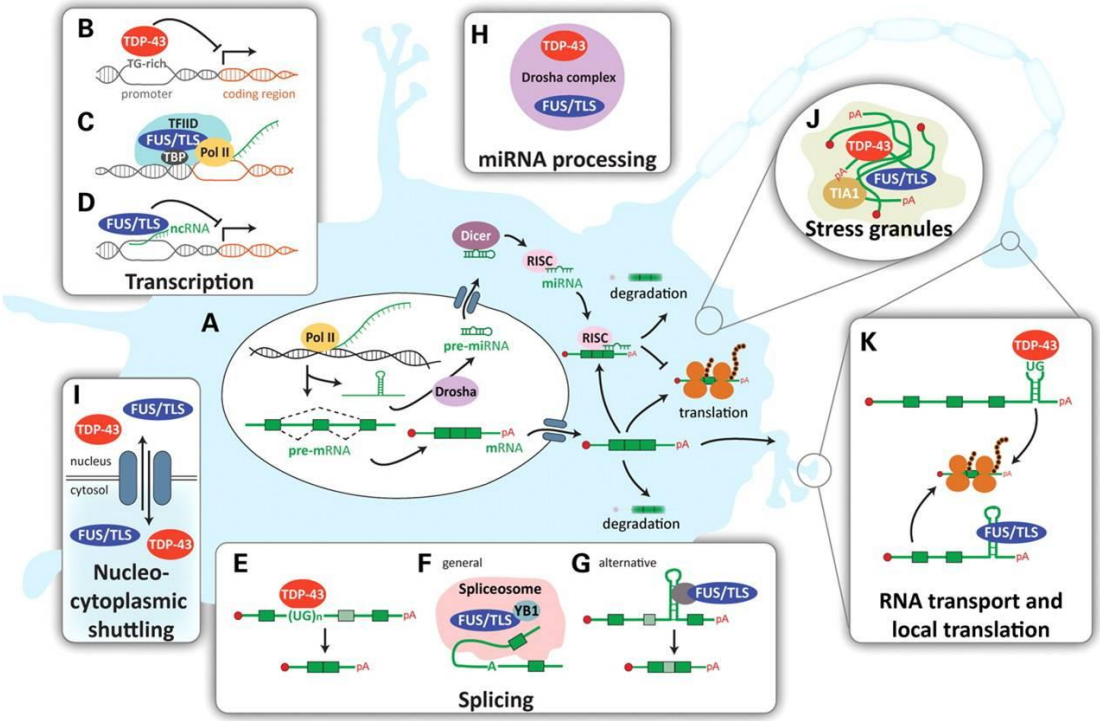

Fig.1: Proposed roles of TDP-43 and FUS/TLS. (A) Overview of crucial steps of RNA processing from transcription to translation or degradation. (B) TDP-43 binds single-stranded TG-rich elements in the promoter regions. This causes transcription of the downstream gene to stop. (C) FUS has been associated with general transcriptional machinery processes. (D) In response to DNA damage, FUS is recruited in the promoter region of cyclin D1 (CCND1) through sense and antisense non-coding RNAs (ncRNAs). This respresses CCND1 transcription. (E) TDP-43 binds towards UG area's in intronic regions prior to alternatively spliced exons. This enhances their exclusion. (F) FUS is identified to bind the spliceosome, and (G) was shown to promote exon inclusion in H-ras mRNA by binding to regulatory elements positioned on the downstream intron (H) Both proteins are associated withmiRNA processing and (I) both shuttle between the nucleus and the cytosol. (J) In the cytosol they form complexes with mRNAs and other RNA binding proteins. (K) TDP-43 and FUS are both involved in the axonal transport of mRNAs to dendritic spines and/or the axonal terminal where they might facilitate local translation. *(Image adapted from Lagier-Tourenne, C., et al. 2010)* [264].

Figure 2: Proposed mechanisms of SOD-1 mediated ALS

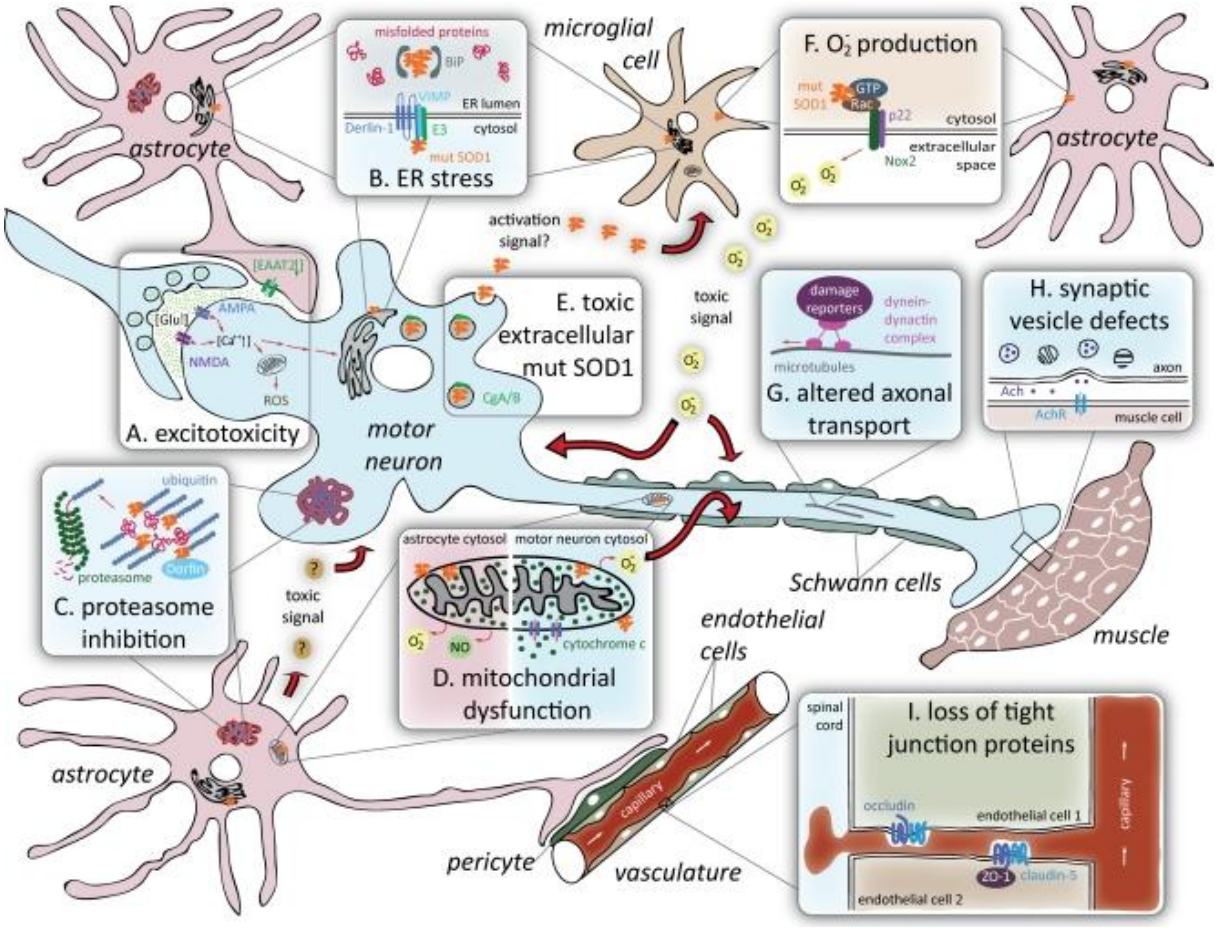

Fig.2: Proposed mechanisms of SOD1-mediated ALS. A. Excitotoxicity B. ER stress is induced by protein aggregations(see text for details). C. Proteasome dysfunction caused by "overload" of the proteasome degradation pathway. This is caused by protein aggregations and ubiquitinated misfolded protein and lead to damage astrocytes and motor neurons. D. Mitochondrial dysfunction. E Toxic secretion of mutant SOD-1 into the extracellular environment. F. Non-cell autonomous regulation from microglia or astrocytes can damage neighboring motor neurons. G. Altered axonal transport: Retrograde transported stress-related proteins were reported in mutant SOD1-expressing motor neurons. H. Synaptic vesicle defects. I. Loss of tight junction proteins within capillary endothelial cells lead to disruption of the BBB/BSCB. This may cause vascular ruptures. *(Image is adapted from Ilieva, H., et al., 2009)* [264].