The pathophysiology of amyotrophic lateral sclerosis (ALS) and the role of \textit{C9orf72}

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a disastrous neurodegenerative disorder, where people die within 3-5 years after they were diagnosed, mostly due to respiratory failure. ALS appears with an incidence of 2.6/100,000 per year (Kumar et al. 2016), and normally develops between 50 and 75 years of age, decreasing afterwards (Zufiria et al. 2016).

In ALS, motor neurons in the spinal cord, brainstem, and motor cortex degrade (motor neuron dysfunction). The consequences are motor problems, muscle weakness, and paralysis (Boeynaems et al. 2016). As a result of the involvement of the upper and lower motor neurons, neurological examination confirms a combination of upper motor neuron signs (spasticity, hyperreflexia, and extensor plantar response) and lower motor neuron signs (muscle atrophy, fasciculations and cramps). The clinical phenotypes differ from each other. The classical or spinal forms, with early involvement of the limbs, are most typical (in 65% of cases). In bulbar forms (impairment of the function of the cranial nerves), the disease starts with dysarthria (difficult or unclear articulation of speech), dysphagia (difficulty or discomfort in swallowing) or both (in 30% of cases). 5% of cases starts with early respiratory failure (Zufiria et al. 2016).

ALS is considered to be a complex genetic disorder: there is a combination of multiple genes and environmental exposure needed for the person to be susceptible. Risks include age, male sex, cigarette smoking, physical stress, exposure to pesticides, and excessive sporting (Gordon 2011). In around 10% of ALS patients, the disease runs in the family. These familial cases are caused by a heterogeneous set of genes, mostly TARDBP, SOD1, FUS, and C9orf72. With the gene C9orf72, patients carry a hexanucleotide (GGGGCC) repeat expansions (HRE) in the first intronic region (of chromosome 9 open reading frame 72 gene) (Boeynaems et al. 2016). While healthy people have an average of 2 repeats, with 90% carrying 8 or less, the repeats range from 500 to several thousands among the ALS patients (Kumar et al. 2016). There is no effect of repeat size on clinical presentation.

Since Charcot has described the disease in 1874, the accumulated knowledge has not been enough to find successful therapeutic strategies. Only riluzole, a glutamate agonist, has been described to increase survival by a few months (Zufiria et al. 2016).

Even though there are these new insights, the exact pathophysiological mechanism in ALS is unknown (Boeynaems et al. 2016). In this paper, I will provide further insight into the pathophysiological mechanism of ALS, especially the pathophysiology related to C9orf72.
2. Pathophysiology

In this section, I describe the pathophysiology of mitochondria, axonal transport, oxidative stress, excitotoxicity, inflammation, apoptosis and their relation to ALS.

Morphological and ultrastructural deformities of mitochondria have been found in autopsies of patients with ALS. Aggregates of abnormal mitochondria were shown in skeletal muscle and intramuscular nerves. Raised mitochondrial volume and elevated calcium levels in the mitochondria have been found in muscle biopsies. Moreover, defects in the activities of mitochondrial respiratory chain complex I and IV have been revealed in muscle and spinal cord, indicating that a deterioration of the mitochondrial respiratory chain could be of importance in the pathogenesis of ALS. However, there are also contradictions. Various lines of proof obtained in cellular models reveal that expression of mutant SOD1 (in mice) is not only correlated with mitochondrial morphological changes, but also with mitochondrial dysfunction (Brown et al. 2006). Thus, more research has to be done to find out what the exact correlation between the mitochondria and ALS is.

Axonal transport also has a relationship with ALS. Neurofilaments (NFs) are the most abundant intermediate filaments. NF concentrations in cerebrospinal fluid and blood have been reported to be increased in patients with both familial and sporadic forms of ALS (Brown et al. 2006). Therefore, neurofilament concentration is one of the most promising neurochemical diagnostic and prognostic biomarker candidates to date (Oeckl et al. 2016). To test the relation tau to ALS, mouse models have been created by making changes in the microtubule-associated protein tau. In one of the studies (Lee et al. 2001), there were specifically tau45-230 aggregates present in the spinal cord of people with ALS. Different tau transgenic mice were found to have progressive motor phenotype with muscle atrophy and paresis. The motor axons had dilatations that consisted of an accumulation of NFs, mitochondria, and vesicles (Brown et al. 2006).

In conclusion, a lot is known about the mitochondria: morphological and ultrastructural deformities of mitochondria have been found in autopsies of patients with ALS. Furthermore, there was relationship found between axonal transport and ALS. Increased neurofilament concentrations in cerebrospinal fluid and blood have been reported. However, in each case, the exact relation to ALS is not yet clear.

In this part, I talk about SOD1 mutant mice, meaning mice with a mutation in the SOD1 gene. SOD1, superoxide dismutase (Cu-Zn), is an enzyme that in humans is encrypted by the SOD1 gene, located on chromosome 21. SOD1 is a powerful antioxidant enzyme that defends cells from the detrimental effects of superoxide radicals. This enzyme binds both copper and zinc ions that are straightly involved in the deactivation of toxic superoxide radicals (Brown et al. 2006). Mice with a mutation in SOD1 have progressive motor deficits, hindlimb paralysis, motor neuron degeneration and early death (Joyce et al. 2014). That is
why it is the most used transgenic animal model to study ALS.

ALS has also been related to different processes such as oxidative stress, excitotoxicity, inflammation, and apoptosis. Oxidative stress reflects an imbalance between the systemic expression of reactive oxygen species and a biological system’s ability to readily detoxify the reactive intermediates or to repair the resulting damage. SOD1-mediated oxidative abnormalities are not the primary cause of toxicity (Brown et al. 2006).

The next process is excitotoxicity. Glutamate-induced, AMPA receptor-mediated excitotoxicity adds to the selective motor neuron degeneration in ALS. The most significant argument is that riluzole, which is the only effective drug treatment for ALS patients, interferes with glutamate release (Boeynaems et al. 2016). Furthermore, AMPA receptor antagonists extend survival of mutant SOD1 mice. Also, inhibiting glutamate carboxypeptidase II increased the lifespan in this mouse model (Brown et al. 2006).

So excitotoxicity can be related to ALS, but does inflammation also play a role in ALS? This is not yet clear; however, evidence in human reveals that microglial activation is an extensive phenomenon in patients who have ALS (Brown et al. 2006). The involvement of microglial activation was reported in a transgenic mouse with general overexpression of the cytokine interleukine-3 (IL-3) (Brown et al. 2006). In the figure below (figure 1) you see the different processes I just mentioned. You see that for oxidative stress Nrf2 is needed for survival and vitamin E/A is for disease prevention. In inflammation the most important factors are interleukine-1A, -1B and TNF. And excitotoxicity has to do with glutamate.

Figure 1 Role of inflammation, excitotoxicity and oxidative stress in mutant SOD1 mice (Dennys et al. 2014)
One other process suspected to have a role in ALS is apoptosis. There is growing evidence that apoptosis contributes to neuronal loss in many acute and chronic neurological disease, including ALS. Apoptosis is a highly regulated form of cell death, in which cells die due to activation of a pre-programmed suicide mechanism. It is morphologically characterized by shrinking, collapsing of the cytoskeleton, disassembling of the nuclear envelope, and condensing of the nuclear chromatin. Furthermore, the cell also breaks up into fragments. It is a multistep, energy-dependent process that involves activation of positive and negative regulatory components, for instance the Bcl-2/Bax family of proteins, the p53 tumor suppressor gene, members of the tumor necrosis factor receptors (TNFR) superfamily and cell cycle-related genes (Brown et al. 2006). The argument that apoptosis is related to ALS is that overexpression of Bcl-2 protects against motor neuron death from nerve transection in newborn mice (Sathasivam et al. 2001).

However, there are some arguments against the role of apoptosis in ALS pathogenesis. One of these arguments is that this form of cell death is very rapid and for that reason it cannot be involved in chronic disease in which cells degenerate over a long period of time (Brown et al. 2006).

3. Etiology

Many studies have tried to establish genetic, environmental or lifestyle factors in the etiology of ALS. Data from the UK and the Swedish Twin registries have found that monozygotic twins have a higher risk than dizygotic twins, but both show a higher risk than the control population. The risk of ALS is increased in close relatives, but not in wives, supporting a significant role of genetic influences over unidentified environmental determinants (Zufiria et al. 2016). In this section, the environmental and genetic factors related to ALS will be explained.

There is a wide list of studies of environmental factors such as heavy metals, pesticide contamination electromagnetic radiation, lifestyle and tobacco. It is well known that heavy metals might be the cause of disease, both when they are present in insufficient amounts and also when they are present in toxic concentrations. The potential role of these metals has been extensively studied. However, it is not completely understood yet. Lead, mercury, and selenium have been the most studied ones. (Zufiria et al. 2016).

High lead levels have been described in cerebrospinal fluid, blood and bone samples of ALS in US veterans (Zufiria et al. 2016). The association is especially marked for blood lead. Veterans may be exposed to lead from firing practice and other military-related sources, so the observed lead-ALS association may at least explain the higher risk of ALS found for military service personnel compared with the general population (Kamel et al. 2006).

Another metal that has been associated with an increased risk of ALS is mercury. Mercury has been shown to be neurotoxic in in vitro or in vivo studies and to have accumulated in neural tissues (Wang et al. 2016). Clinical manifestations in patients with long-term accidental exposure to mercury are
very much alike to those reported in classical ALS. ALS mice exposed to mercury show a quicker and more abrupt disease course, implying that this metal may potentiate the development of the disease in patients who are genetically predisposed (Zufiria et al. 2016).

A different metal, selenium, is a metalloid element that has been broadly studied since it was discovered to be both a toxic and an essential trace element for mammals. The first evidences for the association between ALS and selenium came from two epidemiological investigations that documented increased risk of ALS in populations in seleniferous regions. (Estevez et al. 2012).

The last metal associated with ALS is zinc. Zinc is co-released at glutamatergic synapses in the whole central nervous system and acts as a neuromodulator for glutamatergic neurotransmission (Zufiria et al. 2016). Currently, investigators found that zinc exposure may have a neurotoxic effect in motor neurons. Some experiments have confirmed that zinc increases oxidative stress and raises excitotoxicity, as a result of this promoting motor neuron death (Nutini et al. 2011).

Another environmental factor is pesticide contamination. The pesticides, which are the most used, are organophosphates. Organophosphates irreversibly inhibit acetylcholinesterase, thereby inducing brain damage. Acute, extra stimulation of cholinergic receptors causes cholinergic neuronal excitotoxicity and dysfunction. Morahan et al. showed that an impaired ability of sporadic ALS patients to detoxify pesticides could be associated with polymorphisms in the metallothionein family of genes (Zufiria et al. 2016).

A distinct environmental factor is low-frequencies-electromagnetic-fields (EMF). There exists one meta-analysis showing a slight, but significant increase in the risk of developing ALS among intensely low-frequencies-EMF-related occupations. In vitro studies confirm that prolonged exposure to extremely low frequencies-EMF might induce oxidative stress, DNA damage, and apoptosis. However, these results have not been replicated in ALS murine models (Zufiria et al. 2016).

The lifestyle is also considered as a risk factor for ALS. It has been suggested that exercise and good physical conditions, which are usually considered to have advantageous effects on health, could be risk factors for neurodegeneration later in life (Zufiria et al. 2016). There are several case-control studies reporting that intense and powerful physical activity could be associated with the disease. In a study among cross-country skiers, they found that long distance cross-country skiing is associated with a higher risk of ALS, among the best skiers. However, recreational skiers appear to have a largely reduced risk (Fang et al. 2015).

Another environmental factor associated with the risk of ALS is tobacco. The correlation between smoking and ALS has been suggested, but it is not confirmed. Even though several studies found a potential association, other studies didn’t find them (Zufiria et al. 2016).
Among all the factors suspected to cause ALS, the strongest correlations have been made to the different genetics factors, which play a role in ALS. Together, the 4 genes (SOD1, FUS, TARDBP, and C9orf72) account 60-80% of familial ALS cases. C9orf72 expansions serve as about 50% of the mutations found in familial form, but mutations in FUS account for 35% of cases appearing. Though other genes have been related to the Mendelian forms of the disease, its epidemiological importance is nearly neglectable (Millecamps et al. 2012).

4. Role of C9orf72

The C9orf72 gene is very special, because it lies in an intronic region and normally only exons are transcribed, not introns.

So what seems to be the normal function of C9orf72? Bioinformatic studies have predicted that the major structural feature of the C9orf72 protein is a DENN domain. DENN domains are best described as regulators of intracellular membrane traffic through their actions as guanine exchange factors for specific Rab guanosine 5'-triphosphate (GTP)ases. Knockdown of C9orf72 leads to reduced endocytosis and dysregulated autophagy in human neuroblastoma cells (Amick et al. 2016; Mizielsinska et al. 2014). This points out critical membrane traffic functions for C9orf72 (Amick et al. 2016). Amick et al. also found out that C9orf72 interact strongly and significantly with SMCR8 (Smith-Magenis Chromosome Region gene 8), which has been predicted to contain an FCLN-like DENN domain. This interaction is critical for C9orf72 stability (Amick et al. 2016).

ALS has been related to the loss-of-function of the C9orf72 gene, gain-of-function of the C9orf72 gene via RNA repeats, gain-of-function via DPR protein, and finally the formation of G-quadruplexes. In the case of loss-of-function mutations, reduced levels of C9orf72 transcripts may be a cause of hypermethylation of the C9orf72 promoter or increased histone methylation. The resulting lower levels of C9orf72 are also identified in ALS cases without C9orf72 repeat expansion. This means that loss of C9orf72 could be part of a conventional pathway affected in these diseases (Xi et al. 2015). However, there are also some arguments against the loss-of-function role. First, intracerebroventricular delivery to adult mice of antisense oligonucleotides (ASOs) targeting C9orf72 leads to knockdown of C9orf72 of the complete central nervous system, but does not produce any motor or behavioural phenotypes. Secondly, no mutations have been discovered in coding regions of the C9orf72 gene. Finally, a rare homozygous C9orf72 repeat expansion did not show clinical or pathological features outside the normal disease spectrum (Mizielsinska et al. 2014).

The gain of function of the C9orf72 gene was proposed to cause ALS by two mechanisms. The first mechanism associated to the gain-of-function noncoding repeat expansion diseases was toxic function of the repeat RNA. There is sequestration of important RNA-binding proteins into aggregates of
repeat-containing RNA foci. A novel mechanism is discovered, through which expanded CAG repeats are translated in the absence of an ATG initiation codon, which is called repeat-associated non-ATG dependent translation (RAN translation) (Mizielinska et al. 2014).

The manner of action in ALS is by way of sequestration of essential RNA-binding proteins into aggregates of repeat-containing RNA foci, in the nucleus of the affected cell. RNA foci consisting of repeat RNA are present in frontal cortex, hippocampus, cerebellum and spinal cord of C9ALS patients. RNA foci existed in several types of glial cells (astrocytes, microglia, and oligodendrocytes), but are most importantly a neuronal phenotype. Astrocytes derived from familial and sporadic ALS patients can make use of toxicity to motor neurons (Mizielinska et al. 2014). Proteins involved in nuclear mRNA export also bind to G4C2 repeats and are sequestered into foci containing sense RNA transcripts. There has been suggested that antisense transcripts may also cause transcriptional changes, possibly through sequestration of RNA-binding proteins (Kramer et al. 2016).

The other new and potentially toxic species in C9ALS are the DPR proteins formed by RAN translation of the expanded repeat. DPR proteins are translated from all frames of the G4C2 repeat resulting in polymers of glycine-alanine (GA), glycine-proline (GP) and glycine-arginine (GR) in the sense frames and glycine-proline (GP), alanine-proline (AP) and proline-arginine (PR) in the antisense frames. All DPR proteins form extensive neuronal cytoplasmic inclusions in patient’s brain. Poly (GP) and poly (GA) DPR proteins display dot-like neuronal inclusions. Not like RNA foci, DPR inclusions appear to be an exclusively neuronal phenotype, possibly speculative of the clearance ability of mitotic cells (Mizielinska et al. 2014) (see figure 2).
Figure 2 Potential mechanisms of disease in C9FTD/ALS. AP, alanine-proline; DPR, dipeptide repeat; GA, glycine-alanine; GP, glycine-proline; GR, glycine-arginine; PR, proline-arginine.

Healthy neuron: there is a balance between the autophagosomes and the C9orf72
Loss of C9orf72 function: there is a dysregulation of autophagy, meaning a lot of C9orf72 is phagocytosed
Gain of function repeat RNA: there is sequestration of RNA-binding proteins

The final genetical mechanism in ALS involves G-quadruplexes. The C9orf72 hexanucleotide repeat expansions DNA and RNA set up the formation of G-quadruplexes. A G-quadruplex is a guanosine-rich DNA and RNA sequences which easily form a stable four-stranded structure. Two different mechanisms explain the involvement of G-quadruplexes in neurological diseases. First, through repeat expansions of G-rich sequences that are predicted to form G-quadruplexes and are known to cause disease as seen in C9ALS. And secondly, by mutations affecting the expression of G-quadruplex binding proteins. In the case of ALS, the G-quadruplex of the hexanucleotide repeat is expected to be comprimised in determining the proteins it interacts with. It has been demonstrated in myotonic dystrophy, where identical non-coding RNA repeat expansions form nuclear foci, which sequester essential RNA-binding proteins and by that causing functional defects (Kumar et al. 2016).

In the next section, I’m going to describe some therapies. One of the suspects causing ALS are growth factors. A growth factor is a substance, such as a vitamin or a hormone, which is required for the stimulation of growth in living cells. There are strong indications pointing out that hepatocyte growth factor (HGF) improved survival in isolated motor neurons from rat embryos. Vascular endothelial growth factor (VEGF) also plays a role in the pathogenesis of ALS. The normal function of VEGF is to increase the vascular permeability. VEGF also stimulates axonal outgrowth and neuronal survival. In the case of ALS, (Brown et al. 2006), VEGF protects motor neurons from degeneration in ALS animal models. Therefore, it is suspected that VEGF could be used for therapy.
The second group of molecules related to ALS is neurotrophic factors. Neurotrophic factors are a family of biomolecules that support the growth, survival, and differentiation of both developing and mature neurons. Mice with homozygous targeted disruption of the ciliary neurotrophic factor (CNTF) gene demonstrate a loss of 20% of motor neurons. This suggests that CNTF plays a substantial role in ALS. On the other hand, another neurotrophic factor, i.e. glia-derived neurotrophic factor (GDNF), showed a positive effect on the survival of motor neurons. In the study of Oppenheim et al., GDNF was applied to the chorioallantoic membrane of a bird. The goal of the study was to test if there was any motor neuron survival. Indeed, GDNF led to increased motor neuron survival during the critical period of physiological cell death. It also increased the number of surviving sympathetic and sensory neurons (Brown et al. 2006). Therefore, GDNF could be a potential therapy for ALS.

5. Conclusion and future references

This paper describes the pathophysiology of ALS and the role of C9orf72. A lot of different hormones and processes play a role in the pathophysiology. The C9orf72 gene is very special because it lies in an intronic region and normally only exons are transcribed, not introns. The identification of C9orf72 G4C2 repeat expansion is a significant discovery towards understanding the C9ALS. There is only one known medicine, riluzole, which is a glutamate agonist, that increases survival by a few months.

Morphological and ultrastructural deformities of mitochondria have been found in autopsies of patients with ALS. In what way is the mitochondria involved? More research has to be done.

Whether inflammation plays an important role is not yet clear, but the involvement of microglial activation was reported in a transgenic mouse with general overexpression of the cytokine interleukine-3. Further, there is growing evidence that apoptosis contributes to neuronal loss in many acute and chronic neurological disease. All these different processes should be investigated further before we even can make a medicine that interferes with these processes.

G-quadruplex motifs are involved in ALS. Although there is a considerable amount of research executed in this field, much remains to be explored yet. Moreover, the loss-of-function and gain-of-function also might play a role in ALS.

There is an investigation of the role of growth hormones, like HGF and VEGF, in ALS. Isolated motor neurons from rat embryos revealed improved survival in the presence of HGF. If we would do more research, maybe we can find a medicine that interferes with these growth hormones, so that patients with ALS can live longer.
One of the main difficulties in developing new therapies lies in the multiple events that contribute to motor neuron death in ALS. Any intervention aimed at solely reducing any particular pathological condition might not be enough to treat the disease and its symptoms.

6. References


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