Amyotrophic Lateral Sclerosis: A Scientific Odyssey

by

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Abbreviations:
ALS, Amyotrophic Lateral Sclerosis
AO, aldehyde oxidase
CAT, catalase
CuZnSOD, copper zinc superoxide dismutase
DDC, diethyldithiocarbamate
FALS, Familial Amyotrophic Lateral Sclerosis
GALS, Guamanian Amyotrophic Lateral Sclerosis
JFALS, Juvenile Familial Amyotrophic Lateral Sclerosis
MnSOD, manganese superoxide dismutase
ODN, oligodeoxynucleotides
SALS, Sporadic Amyotrophic Lateral Sclerosis
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Abstract:

Amyotrophic lateral sclerosis is a late onset neurodegenerative disorder that is progressive and ultimately lethal. One of the forms of ALS that is most interesting is the familial form or FALS. This form has mutation in the gene encoding CuZnSOD and causes a malfunction in the enzyme although the extent of this effect is not completely elucidated. Even though there is not much known about the cause of any of the forms of ALS there is a great deal of interest and research looking into the causes and hopefully the eventual treatment and prevention of the disease. This paper will look at FALS, what is known about it, what research is being done and the future directions of research into this disease.

Introduction:

Amyotrophic lateral sclerosis is a late onset neuromuscular disease that is progressive and leads to an ultimately fatal degeneration of motor neurons, which leads to paralysis. Amyotrophic lateral sclerosis is designated ALS and will be referred to as such in the remainder of this paper. ALS became a better-known disease when a famous Yankee first baseman was stricken with the disease. This baseball player was Lou Gehrig and that is why ALS is more commonly known to most people as Lou Gehrig’s disease. This disease is so devastating because it affects the motor neurons and since muscle fibers only have a single motor neuron the degeneration of that neuron is very destructive [3]. ALS afflicts approximately one in one hundred thousand people [18]. There are multiple forms of ALS, which will be discussed later, but the predominant form is the sporadic form. Familial form of ALS represents about ten percent of the ALS cases diagnosed each year. This paper will mostly deal with the familial
form of ALS and how oxidative stress may play a role in the initiation and progression of this form of ALS.

**Background on ALS:**

Motor neurons are the major target of ALS. ALS is a chronic and progressive disease. There is a death of the motor neurons in the motor cortex, brainstem and spinal cord [14]. The average age of ALS onset is 57 years old [14]. The disease begins with muscle weakness in the extremities accompanied with weak muscle tone [14]. This weakness leads to muscle atrophy, which is followed, by speech problems, difficulty swallowing and loss of muscle use [14]. This disease is devastating to the person afflicted with it because there is no cure and because the brain is unaffected, the patient is fully aware of what is happening to them.

**Sporadic ALS:**

The most prominent form of ALS is the sporadic form or SALS. This type of ALS afflicts approximately 85% of the ALS cases diagnosed every year and men are twice as likely to develop this form than women [14]. The second most common form of ALS is familial ALS or FALS. This form makes up about 10% of the cases of ALS. Women and men are equally affected with this form of FALS [14].

Since the fundamental lesion of ALS is the degeneration of motor neurons in the anterior horn cells the measurement of the varying forms may give clues to the cause of degeneration. SALS is sporadic, there are no known ways to determine whom this disease will affect or how these patients will get the disease. SALS patients have no specific features in the degeneration of the motor neurons. Since there are no common characteristics of this degeneration there is no clear indication of the cause or reason the disease strikes [15]. SALS has no significant changes
in the antioxidant enzymes such as copper zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD) and catalase (CAT) [14]. Oxidative damage is shown to occur in this sporadic form. Recent studies have shown that SALS is associated with CuZnSOD through posttranslational modifications [7]. Measured serum levels of CuZnSOD activity are decreased in SALS patients. In post-mortem studies of SALS patients the CuZnSOD activity level in the cerebrospinal fluid is also decreased, demonstrating that the modification of the enzyme may have something to do with the disease [7]. Studies have also shown that SALS patients like all ALS patients have increased levels of calcium in their blood stream. The interesting thing about SALS is that these patients have antibodies to both the L and P types of calcium channel [9]. These channels are instrumental in helping increase the calcium entry into the neurons. This information could be a good direction for study into the prevalence of SALS.

**Familial ALS:**

FALS is clinically indistinguishable from SALS. FALS patients have degeneration of the motor neurons as well but the features of this degeneration are intracytoplasmic inclusions, which are found in all patients with FALS [15]. FALS is an age independent autosomal dominant disorder where mutations in the \textit{SOD1} gene (the gene encoding CuZnSOD) have been linked to between 20\% and 50\% of the families with FALS [5]. There are several other mutations associated with FALS but the most prevalent one are the \textit{SOD1} mutations [14]. FALS is a hard disease to study as well as making general statements about FALS because each FALS family has a different set of symptoms and a slightly different form of FALS [16].

The \textit{SOD1} gene is encoded on chromosome 21 and produces a 153 amino acid metalloenzyme that dismutes superoxide via reaction 1 [5].
Many missense mutations occur in CuZnSOD that substitutes one amino acid for another [12]. Most of the FALS causing missense mutations are in exon 4 of the SOD1 gene [11]. The most common mutation in CuZnSOD in FALS cases in the United States is the alanine 4 being changed to valine [14]. This mutation seems to cause enzyme instability by blocking the interface interaction between the dimers but also blocks the entry of O$_2^•$ into the active site of the SOD [5,23]. A representation of the CuZnSOD homodimer is shown in Figure 1.

![Figure 1: A computer simulation of human copper-zinc superoxide dismutase homodimer. Copper ions are represented by the large dark circles (blue) and the zinc ions are represented by the large light circles (yellow). Adapted from [7].](image)

There do not seem to be any cases of FALS where there is a deletion of the SOD1 gene so the mutant protein seems to be required in the FALS phenotype [12]. The SOD1 gene, which
encodes the CuZnSOD protein, is proximal to 21q22, which is also where the FALS locus denoted ALS1 is located [10,18]. The location of the ALS1 locus is shown in Figure 2.

![Figure 2: Location of ALS1 and SOD1 gene on chromosome 21, arm q and location 22 [21].]

**Guaminian ALS:**

A third type of ALS was found only in people in the western pacific islands. It is known as Guaminian ALS or GALS. This form of ALS is extremely rare and is believed to be caused by a toxin called N-methyl-D-aspartate (NMDA) that is found in the diet of these people. NMDA is an excitotoxin and is shown to be toxic in cell culture experiment to neuronal cells [2]. NMDA is found in the cycad nut, which is a source of food for the people of this region. The incidences of GALS have decreased since the populations of these islands have started rinsing the flour made from these nuts, which seems to dilute out the effect of the NMDA [2,15]. Not much is known about GALS but the degeneration of motor neurons is mostly due to neurofibrillary tangles [15]. These tangles found in GALS are similar to those found in Alzheimer’s disease.

**Juvenile FALS:**

A fourth form of ALS is heritable like FALS but strikes young people between the ages of 5 and 20 years. This form is juvenile familial amyotrophic lateral sclerosis or JFALS and is an autosomal recessive trait causing similar intracytoplasmic inclusions in the degenerating
motor neurons [15,18]. Like FALS, JFALS has been linked to a genetic mutation. This mutation is not as clearly understood but is located on chromosome 2q33 along with the JFALS locus $ALS2$. The $ALS2$ locus is shown in Figure 3.

![Figure 3: Location of the $ALS2$ gene on chromosome 2, arm q and location 33 [21].](image)

$ALS2$ is linked to a gene that encodes aldehyde oxidase (AO). AO is an enzyme that controls oxygen ($O_2$) [18]. Aldehyde oxidase is one of the four human molybdenum hydrolase enzymes and has been implicated in oxygen radical diseases [18]. A mutation in its function could explain why JFALS behaves so much like FALS.

**Oxidative Stress in FALS:**

Normal functioning SOD can dismute superoxide into hydrogen peroxide, however, another reaction is competing for the superoxide. That reaction is the reaction of superoxide with nitric oxide. These normal reaction are shown in reaction 2.
Superoxide Dismutase:

These reactions are what happen when SOD is functioning normally, in patients with FALS there is a mutant SOD activity and SOD has diminished affinity for copper and zinc. This reduced affinity causes free metals in the system. Since CuZnSOD is located in the cytosol there is a great chance for these mutations to cause reaction that are not appropriate [27]. These FALS mutant reactions are shown in reaction 3, 4 and 5.

\[
\text{SOD Cu}^{2+} \text{Zn} \rightarrow \text{SOD} + \text{Cu}^{2+} + \text{Zn} \quad (3)
\]

\[
\text{SOD Cu}^{2+} \text{Zn} \star \\
\text{ONOO}^{-} + \text{H} - \text{Tyr} \rightarrow \text{NO}_{2} - \text{Tyr} + \text{OH}^{-} \quad (4)
\]

\[
\text{H}_2\text{O}_2 + \text{SOD Cu}^{1+} \text{Zn} \star + \text{R} \rightarrow \text{R(OH)} + \text{SOD Cu}^{2+} \text{Zn} \star + \text{OH}^{-} \quad (5)
\]

As is seen in reaction 3 the release of the copper and zinc will cause toxic reaction in neurons. Reaction 4 shows that with the concentrations of zinc decreased, the SOD has a greater chance to react with peroxynitrite to form a nitronium anion, which can transfer nitrate groups to tyrosines, which can also cause injury in neurons [8]. Reaction 3 shows what happens when copper in the active site is reduced leading to an increase in hydroxyl radicals because the SOD now acts like a peroxidase [8].

There are many different mutations that have been reported in the \textit{SOD1} gene through studies with FALS individuals. These mutations cause a 50% or more reduction in the activity of SOD enzyme activity [5]. FALS mutant enzymes revealed maintenance of enzyme activity
when the glycine 85 changed to arginine mutation is present [5]. However, if the glycine 37 changed to arginine mutation was the one that was noted there was fully activity of the SOD but a two fold reduction in the stability of the protein [5]. If these mutations cause a chronic reduction in SOD activity there is a correlation to the toxic effect seen in motor neurons [23]. This reduction in CuZnSOD activity may give a hint as to why FALS causes degradation of the motor neurons. There are problems determining the SOD activity in human and animal models. There is a two to three fold variability between the different measurements depending when the serum was collected. Studies have shown that the SOD activity variability can account for the observed change in FALS patients and that the activity was decreased by 38.8% [5]. This number fits with what we know about FALS because in SALS patients there is no significant change in the SOD activity level [4]. An example of these activity measurements are shown in Figure 4.

Figure 4: Specific activity of SOD1 polypeptides. A - SOD1 activity assay gels. Lane 1 = vector without HuSOD1 cDNA, Lane 2 = vector with wild type HuSOD1 cDNA, Lanes 3-8 = vectors encoding mutant HuSODs as labeled below the gel. B - Immunoblot detection of SOD1 polypeptides. Extracts are as in part A. Adapted from [5].
As can be seen in Figure 4 by western blot there is SOD protein and it is mostly all active but not as active as the wild type. The activity gel shows different levels of activity but also shows that some of the mutants are not active. The G85R mutation doesn’t seem to be active and also does not have the correct level of protein suggesting the mutation has caused a misfold in the protein. As SOD activity decreases in FALS patients the complex 1 activity of the electron transport chain increases [4]. This suggests that there is more leakage of electrons on the mitochondrial electron transport chain, which leads to the formation of more superoxide that cannot be removed leading to oxidative damage in the neuronal cells.

While most of the studies into FALS focus on CuZnSOD, there have been some studies done with MnSOD in ALS. It has been shown that MnSOD is produced in normal patient neurons, this means that oxygen radicals occur. Age does not seem affect the expression of this enzyme. Studies done with immunostaining show that MnSOD seemed to be induced in SALS patients neurons while the number of cervical motor neurons in these patients was decreased. There was found to be no induction of MnSOD in FALS patients [27]. Therefore it seems that FALS patients disease state comes from a mutant form of CuZnSOD while SALS may have something to do with MnSOD although it is unclear if the induction that is seen in MnSOD is a cause of the ALS or just an effect of the actual cause of the disease.

**Free Radicals and Oxidative Damage:**

The fact that some cases of FALS can be linked to CuZnSOD suggests there is a place in the disease for Free Radicals. This is why some researchers have come up with the free radical hypothesis of ALS as seen in Figure 5.
This figure demonstrates the free radical theory, once the receptors are activated a cascade is initiated that causes the production of superoxide and in FALS patients there is not a good way to remove this superoxide. The $O_2^\cdot-$ then accumulates and causes the death of the motor neuron [20]. This production of superoxide can be produced from metabolism because it has been noted that there is an increase in the activity of complex 1 [27]. This accumulation of superoxide causes damage to other cells and tissues but since the neuronal cells are highly auto-oxidizable this increase in superoxide causes oxygen toxicity [27]. The release of copper as seen in reaction 3 may also involve free radical mechanisms because copper ions promote lipid peroxidation, which aids in formation of highly reactive hydroxyl radical [24]. Since FALS patients generally
have some CuZnSOD activity there is some H$_2$O$_2$ made and this reacting with free copper can cause DNA damage to cells such as strand breaks and base changes [24].

There is also a gain of function theory to ALS. This theory is based on the increase in interaction of H$_2$O$_2$ and an increase in nitrination. Increased levels of H$_2$O$_2$ cause inactivation of CuZnSOD, which has low activity in FALS to begin with. However, some of the CuZnSOD mutations do not allow its inactivation by H$_2$O$_2$ [17]. This mutation allows reduction at the active site and this is where the nitrination occurs because the peroxynitrite can enter causing reaction 6 [3,17]. H$_2$O$_2$ also reacts to generate the hydroxyl radical, which causes oxidative damage in the cell. With a decrease in the activity of SOD there is more superoxide available to react with nitric oxide as shown in reaction 2. Interneurons surrounding motor nuclei produce NO$^*$ to help modulate synaptic plasticity [3]. This NO$^*$ may indirectly contribute to motor neuron destruction because of the lower activity of the CuZn SOD. As seen in reaction 2 superoxide reacts with nitric oxide to for ONOO$^-$ (peroxynitrite) with a rate of $k = 6.7 \times 10^9$ M$^{-1}$ s$^{-1}$ [3]. The formation of peroxynitrates has previously be linked with neurotoxicity and this could possible be a cause of some of the toxicity found in FALS patients [13]. As was also discussed earlier the affinity of metals to these mutant SODs is low and peroxynitrite can react with CuZnSOD to form a nitronium like intermediate as seen in reaction 6.

$$\text{ONOO}^- + \text{SOD-Cu} \rightarrow \text{SOD-CuONO}_2 \quad k = 10^5 \text{ M}^{-1} \text{ s}^{-1} \quad (6)$$

Therefore SOD mutations may cause increases in the peroxynitrite formation because of the inability of CuZnSOD to scavenge the superoxide effectively thus causing the nitrination of cellular targets and leading to slow injury of the motor neurons [3]. This is probably due to the fact that SOD1 mutations cause the CuZnSOD protein to act as a peroxidase instead of a dismutase once it loses affinity for the copper.
A deficiency of DNA repair enzymes and an increase in DNA damage because of the increase in radicals may also be a factor in the degeneration of FALS [6]. Degeneration of neurons may cause the leak of dopamine and the interaction of dopamine with DNA causes massive base damage [24]. This could be another cause of the mechanism of neuronal damage in ALS. Figure 6 shows the kind of base damage that can be found in cells exposed to dopamine.

![Figure 6: Effect of increasing copper ion concentrations on 8-hydroxyguanine concentrations in DNA in the presence of H₂O₂ and L-DOPA, dopamine and 3-O-methyl-DOPA all at 1mM concentrations. Adapted from [24].](image)

This figure shows that there is increasing amount of 8-hydroxy guanosine (the Y axis), which is a marker for DNA base damage in the cells exposed to the different forms of dopamine. The X-axis is with increasing amounts of copper; since mutated CuZnSOD causes free metals to be available this is an important comparison.

**Apoptosis:**

Apoptosis is also an issue with FALS. Chronic SOD1 inhibition, or lack of CuZnSOD activity, appears to cause the apoptotic death of neurons [8,23]. In the mutations of SOD1 that seem to have a gain of function the active site is changed and the copper is allowed to be
chelated leading to apoptosis [8]. So the gain of function FALS mutations seem to have a pro-
apoptotic phenotype because of this more open active site. In experimental models these
neuronal cells that are going toward apoptosis have been rescued. Some of the SOD mutated
cells have shown decrease in apoptosis when supplemented with antioxidants [23]. Whereas
adding neuronal growth factor (NGF) and Bcl2 have been shown to rescue neurons from
apoptotic death [23].

**Current Studies in FALS:**

One of the current studies involves using rat spinal cord slices to measure the toxicity to
spinal neurons. The investigators inhibit CuZnSOD activity by using antisense
oligo deoxynucleotides (ODN) or metal chelating agents like diethyldithiocarbamate (DDC). DCC
has been shown to increase oxygen radical induced toxicity in other experiments. DCC was also
measured to more effectively inhibit SOD activity *in vitro* by increasing the dose the cells
received. These treatments will cause a slow but chronic inactivation of the *SOD1* gene thereby
reducing the activity of CuZnSOD. Measurements of death were done by counting spinal
neuronal cells and measuring choline acetyltransferase activity (ChAT activity) which is largely
restricted to motor neurons in the spine [23]. Some of the results follow in Figure 7.
In Figure 7A by measuring the ChAT activity it is seen that the spinal neuronal cells with higher treatment doses of DCC die off much sooner as a function of dose. This implies that the shut downs of the SOD activity has caused the death of the spinal neurons. In Figure 7B the chronically treated cells had fewer numbers as time went on. This supports the hypothesis that inhibiting SOD activity increases the toxicity to neuronal cells. The investigators next decided to see if inhibiting the SOD with antisense would have the same effect on these neuronal cells [23]. These experiments with ODN antisense are shown in Figure 8.
In Figure 8A the treatment on these spinal neuronal cells with antisense and sense ODN show that the SOD activity is decreased in the antisense treated cells vs. the sense treated cells. This demonstrates that the treatment with antisense causes decrease in SOD activity. In Figure 8B the ChAT activity of the antisense treated cells was greatly decreased in the antisense treated cells showing that the decrease in SOD activity has led to the increase in death of the spinal motor neuron cells. Figure 8C shows the decrease in neuronal number of the antisense treated cells again showing that the decrease in SOD activity increase death of the neuronal cells [23]. These two experiments support the hypothesis that the decrease in CuZnSOD activity leads to the motor neuronal death that is associated with FALS.

Besides the cell culture experiments there are also animal experiments that have been done that suggest that ALS is not due to the lack of CuZnSOD protein but that the SOD that is
generated is somehow mutated and that is what causes the toxicity to the neurons. These mutant forms as was mentioned before lose affinity for the metals and the release of these metals may partially be to blame for the neurotoxic effect that is seen in FALS [24]. Transgenic mice have been made that have some of the qualities of FALS but not all the similar qualities to the human form of FALS.

Some experiments have been done on potential treatments of FALS. These researchers were trying to see if vitamin E would be beneficial in slowing down the effects of FALS or even stopping them. These results are in Figure 9.

![Figure 9: Top- Disease onset in G1H/+ mice either untreated or fed vitamin E. Bottom- Survival of G1H/+ mice either untreated or fed vitamin E. Adapted from [12].](image)

In this experiment the G1H/+ strain of mice were used, these mice carry a mutant human CuZnSOD. The mice were fed vitamin E in their diets. The top figure shows the onset of the
disease in these mice when they are treated and untreated. The vitamin E treated mice show later onset of the symptoms of ALS compared to the untreated. The bottom part of the figure shows the survival data of the treated and untreated mice. There was no difference in either group’s survival. This implies that treatment with the antioxidant vitamin E delays onset of the disease but does not prolong the survival of these mice [12].

There are therapeutic trials attempting to treat FALS in patients using antioxidants like vitamin E, D and B$_{12}$ and chelating agents to try and modify the neurotoxic effects of the mutant CuZnSOD. So far none of these trials have shown any benefit in treatment or preventing symptoms of FALS [25].

**Future Direction in Research of FALS:**

Since quite a few studies seem to point to mutant *SOD1* gene and therefore mutant activity of the CuZnSOD this would seem to be the direction to move in for future research. The research question is whether the activity of the CuZnSOD is a gain of function or loss of function and will there be a way to prevent the neurotoxic effects of this mutant activity on the neuronal cells.

*In vitro studies:*

One method for further study of FALS would be to use differentiated human neuronal cells. These cells would need to be treated with retinoic acid to cause the differentiation. These cells would then be treated with the antisense ODN of any of the mutant CuZnSOD that have been discussed to reduce the SOD activity as in the above mouse experiment. Since rat neuronal cells responded as expected to the antisense and caused a decrease in SOD activity this would give us a good indication whether human neuronal cells will behave similarly. We can then
move forward with the FALS-like cells. The next treatment would be to attempt to rescue the neuronal cells from this neuronal death by treatments with different chemical agents such as a CuZnSOD mimic drug. Problems with this type of experiment would be that the mimic causes more toxicity to the cells leading to more harm instead of benefit. This toxicity could come from the mimic releasing the copper and zinc ions from the active site.

Making a CuZnSOD mimic is an area that should be explored, this would require a drug that is catalytically active that contains both the copper and the zinc metals. The mimic drug would need to be tested extensively to see that it has similar activity to the wild type human CuZnSOD and the metals in the drug did not fall out of the active site. Once this drug is manufactured it should be tested on normal cells for toxicity. This drug could be used for experimentation on cells that were treated with ODN antisense to see if the cells can be rescued as mentioned in the previous experiment. It could be tested in ALS transgenic mice to see if the effects of the neuronal degeneration can be slowed or even stopped. Using this drug on mice could show toxicity from the drug itself or from the drug reaction with other tissues and cells in the body besides the intended motor neurons.

Other pharmaceutical agents could be created to replace the effects of CuZnSOD and scavenge the radicals that are degenerating the neuronal condition of the animals and patients. The drugs would be tested in similar ways to the above-mentioned CuZnSOD mimic. It would be beneficial if these drugs could cross the blood brain barrier to help control the degradation of the motor neurons.

Adenovirus studies in the mutant SOD cells would tell us if adding back wild type CuZnSOD is beneficial to the cells and stop the degeneration of the neuronal cells. As with all
adenovirus experiments there is a danger of reversion of the virus if tested in animals. This can be avoided by using the proper calculations and doses of adenovirus.

**In Vivo Studies:**

Animal models have been made of ALS however, all the features are not the same as in human FALS but these are good places to start in animal model research. These mice are designated wobbler mice because as they age the muscles become weak and atrophied and the movements they make give a wobbly appearance [26]. Using these mice to study the different pharmaceutical agents like the CuZnSOD mimic could lead to greater understanding of what could happen if you give back some of the activity of CuZnSOD. These experiments would use control and treatment mice to measure onset of disease, longevity of the mice and progression of the symptom. A system of how much drug to give and how often to give it would be another possible experiment. A good system of delivery is also needed so it is the least toxic to the animal while maximizing the availability of the drug to the neuronal cells for treatment. These wobbler mice could be used to test other treatments such as adenoviral treatment of the mice to introduce wild type CuZnSOD back and see if the effects of the ALS like symptoms decrease and the degeneration of the neuronal cells halts.

Making a more accurate transgenic mouse to give the FALS phenotype would also be a useful tool for research. Injecting the different mutations of *SOD1* that are known to be associated with FALS would be helpful in the search for treatment of FALS in people.

**Patient Trials:**

If the CuZnSOD mimic drug turned out to reverse some of the effects of ALS in mice the next step would be to treat patients with FALS. These studies would involve treating the patient with the mimic and seeing if there is any halt to the progression of the disease. Since not enough
is known about what causes ALS a cure may not be possible from the mimic type drug. However, the delay in onset, the prolongation of life or the decrease in the symptoms could be helpful to the patient and their family. These experiments would need to be done in the traditional Stages. Stage I being the toxicity to the patient trial. There is always a danger in these beginning trials because of the severity of the disease in the patients that participate in the Stage I trials. If Stage I is successful the experiment would move along to Stage II and Stage III trials.

**Summary:**

Because of the multiple forms of ALS there is not much if anything known about what causes ALS or how all of the symptoms develop. There is research going on to discover what the causes of the degeneration and the onset of the disease are but there is a lot more work that needs to be done. Making and using models of ALS is going to help us research it more thoroughly in the future. There seems to be more known about FALS because of the SOD1 gene mutations in about half the cases of FALS. Studies into this form of ALS can only help the fight for understanding the mechanisms of the other forms of ALS.
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