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GENE THERAPY TARGETING NEUREGULIN FOR THE TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS

Xavier Navarro Acebes
Facultat de Medicina UAB

1. Summary of the project

Objectives

In this project, we aim to promote the survival of motor neurons and the maintenance of muscular innervation in the SOD1G93A mouse, a model of amyotrophic lateral sclerosis (ALS), using gene therapy strategies for over-regulating neuregulin 1 (Nrg1), a neurotrophic factor that favors axonal regeneration and synaptogenesis acting on motoneurons, Schwann cells and muscle fibers. We hypothesize that a combinatorial approach will influence several events involved in the physiopathology of ALS in order to effectively prevent the progression of clinical signs.

This general objective is divided into the following specific objectives:

1. Characterize the expression of Nrg1 and its ErbB receptors in the spinal cord and the muscles of the SOD1G93A mice.

2. Promote the formation of new neuromuscular junctions through over-expression of Nrg1 type I in muscles of SOD1G93A mice.

3. Promote the survival of motoneurons and axonal regeneration by over-expressing Nrg1 type III in the spinal cord by intrathecal injection in SOD1G93A mice.

4. Combine the two previous approaches to modulate different physiopathological mechanisms and to obtain synergistic improvements in the survival of motoneurons and the preservation of neuromuscular junctions in SOD1G93A mice.

5. Investigate the endogenous mechanisms that contribute to the effects of Nrg1 overexpression in the survival and the synaptogenesis of the motoneurons.

Design and Methodology

- Construction of viral vectors: adeno-associated virus (AAV) coding Nrg1-III (AAVrh10-NRG1-III), which will be administered intrathecally to induce over-expression in spinal neurons, and Nrg1-I (AAV1-Nrg1-I), which will be injected into limb muscles.

- Administration of the viral vectors: therapy will be carried out with each of these vectors in groups of transgenic SOD1G93A mice, ALS model, and finally with both combinations. Mice are treated at 8 (presymptomatic stage) and 12 (symptomatic) weeks of age, and will be evaluated serially with electrophysiological and locomotion tests up to 16 weeks. Subgroups of mice will be followed for survival analysis.

- Functional evaluation: electrophysiological tests of motor nerve conduction and motor evoked potentials will be performed to evaluate the function of spinal motoneurons and spinal descending pathways. The locomotive function will be evaluated by means of Rotarod and Digigait tests.

- Histological studies: samples will be obtained at times selected for the analysis of motoneuron survival, glial reaction, maintenance of neuromuscular junctions, and to investigate molecular pathways involved in the Nrg1 signaling.

- Molecular studies: molecular biology techniques (WB, PCR) will be used to analyze the changes in the expression of Nrg1 and of the ErbB receptors, as well as of neuroprotection related pathways.

Work plan

- Characterization of the Nrg1 / ErbB pathways in SOD1G93A mice throughout the evolution of the disease.

- Construction of the appropriate viral vectors.

- Evaluation of the effectiveness of the viral vectors to transduce the expression of Nrg1.

- Treatment of SOD1G93A mice with AAV-Nrg1-I by muscle injection.

- Treatment of SOD1G93A mice with AAV-Nrg1-IIII by intrathecal route.

- Combined therapy in SOD1G93A mice with AAV-Nrg1-I by muscle injection and AAV-Nrg1-III by intrathecal injection. Evaluation of results.

2. Obtained Results

1. Characterization of the Nrg1 / ErbB pathways in SOD1G93A mice throughout the evolution of the disease

Analyses of the expression of Nrg1 and ErbB receptors in the spinal cord of SOD1G93A mice have confirmed that Nrg1-I mRNA levels increase with the progression of the disease, while Nrg1-III mRNA levels are reduced. Nrg1 protein levels were diminished in the spinal cord of the SOD1G93A mice at 16 weeks of age, suggesting a relationship with the progression of the disease. Regarding the ErbB receptors, we observed a progressive reduction in the levels of ErbB2, ErbB3 and ErbB4 in the spinal cord of the SOD1G93A mice. The levels of phosphorylation of the ErbB receptors were also decreased in SOD1G93A mice, indicating a lower functionality. The expression of the ErbB4 receptors in the muscles is reduced throughout the disease in the SOD1G93A mice and has also been found reduced in samples of ALS patients, constituting a possible biomarker.

We also analyzed the presence of proteolytic fragments of ErbB4 (ecto-ErbB4) in cerebrospinal fluid (CSF) and plasma, finding a decrease of the 55 kDa fragment in CSF of ALS patients, and in plasma of the same patients and of transgenic SOD1 mice. These results indicate that the ecto-ErbB4 fragments can be a parameter to evaluate the alterations of the Nrg1-ErbB pathway, as well as a potential biomarker in ALS (López-Font et al. 2019).

Additionally, we have used a spinal cord organotypic culture model to characterize the effect of the exogenous addition of Nrg1 on the survival of motoneurons. The results have shown a neuroprotective effect against the death of motoneurons induced by excitotoxicity (Modol-Caballero et al., Front Cell Neurosci 2018).

2. Design and construction of viral vectors and evaluation of their effectiveness

During the project, we have designed and produced, according to the needs of in vivo gene therapy studies, adeno-associated viral vectors (AAV) that codify the expression of Nrg1 type I or type III, in serotypes AAV1-NRG1-I and AAVrh10-NRG1-III (extracellular domain (ECD) and full domain (FL)), respectively, in addition to the control vectors (mock). We also produced a new vector AAV8-hDes-Nrg1-I for the

specific delivery to skeletal and cardiac muscle of Nrg1 through systemic administration.

Biodistribution and effectiveness of target cell infection were evaluated for the different vectors injected into mice. To verify the efficiency of AAV8 and AAV9 coding under the expression of human desmin, vectors were injected to WT mice and SOD1G93A mice, observing, by bioluminescence techniques and by luciferase activity in extracts, that the desmin promoter allowed a high expression in skeletal and cardiac muscles.

3. Treatment of SOD1G93A mice with AAV-Nrg1-I by muscle injection

We have shown selective preservation of the innervation of the gastrocnemius muscle injected with AAV1-Nrg1-I in SOD1G93A mice. The focal effect of gene therapy was demonstrated by the lack of effect on adjacent non-injected muscles. The electrophysiological study showed that treated mice had a number of motor units similar to untreated mice, but a significant increase in the size of the motor units. Immunohistochemical studies demonstrated a greater number of motor endplates occupied in the gastrocnemius muscle after the injection of AAV-Nrg1-I compared to untreated mice. A larger number of profiles corresponding to axonal branching in the treated muscles were also observed. In a L4 rhizotomy model, the results also revealed that overexpression of Nrg1-I improved the recovery of the partially denervated gastrocnemius muscle, by acceleration of collateral re-innervation (Mancuso et al., Neurobiol Dis 2016).

These results confirm our hypothesis that Nrg1-I would increase the number of neuromuscular junctions by promoting axonal reinnervation. In addition, they suggested the need to design a route of administration capable of transducing most of the muscles of the animal.

4. Treatment of SOD1G93A mice with AAV-Nrg1-I by systemic injection

For this reason, we constructed an AAV for over-expressing Nrg1-I under the promoter of human desmin (hDes), a protein present exclusively in skeletal and cardiac muscle, in order to direct the expression of the therapeutic gene to these muscles through systemic administration. After several studies to optimize the therapeutic vector, we obtained a batch of the viral vector that guarantees the potency of infection. The study with transgenic SOD1 mice has shown that this gene therapy is able to significantly

preserve the neuromuscular function of the muscles of the limb after 16 weeks of life and improve the overall locomotive function compared to the group of mice with non-active control vector, and increase the survival of spinal motoneurons. This trial opens a way of interest for clinical translation, since it allows a single intravenous injection to access the majority of muscles.

5. Treatment of SOD1G93A mice with AAV-Nrg1-III by intrathecal injection

Initial experiments with intrathecal Nrg1 type III-ECD did not demonstrate positive effects on neuromuscular function in SOD1G93A mice, although the number of surviving motoneurons at 16 weeks of age was significantly higher in mice treated with AAV-Nrg1-III-ECD. To complete this task, we used a new AAV that contains the Nrg1-III-FL, since the intracellular domain could play an important role in the Nrg1-III neuroprotective function. Animals injected at 6 weeks have been evaluated at 8 to 16 weeks of age by nerve conduction and rotarod tests. The results in female mice have shown that the overexpression of NRG1-III-FL induces significant improvement of motor function, number of surviving motoneurons, and reduces glial reactivity in SOD1G93A treated mice compared to those receiving AAV-mock. A subsequent study of similar design in male transgenic mice, however, has not produced such clear beneficial results. The possible reasons for differences according to gender in the administered therapy have yet to be clarified.

6. Combined therapy in SOD1G93A mice with AAV-Nrg1-I by muscle injection and AAV-Nrg1-III by intrathecal injection

During the final period of the project, we performed the study of the combined gene therapy, through simultaneous injection of AAV-Desmin-Nrg1-I viral vectors by intravenous route to transfect Nrg1-I into skeletal musculature and AAV-Nrg1-III-FL to produce expression of Nrg1-III in the spinal cord. The electrophysiological analyses indicate that there is no synergistic effect with both simultaneous treatments. The preservation of the motor function that is obtained with each treatment separately is of the same level as with the simultaneous treatment in the SOD1G93A mice. This situation is similar to other simultaneous treatments used by our group (Mancuso et al. Orphanet J Rare Dis 2014). Everything suggests that motor preservation in the ALS model reaches a threshold that is not easy to overcome with several simultaneous therapies.

Summary: In summary, we have found that:

- There is a decrease in the expression of Nrg1 in the spinal cord in ALS and alterations of the expression of its ErbB4 receptors.
- The addition of Nrg1 in culture improves the survival of spinal motoneurons against an excitotoxic damage.
- The increase in expression of Nrg1 type I in skeletal muscle promotes branching of motor axons and maintenance of synaptic connections, both in the ALS model and after spinal root injuries.
- We have developed an AAV vector that under the desmin promoter, allows the transduction of Nrg1 to a large number of skeletal muscles, increasing the viability of systemic gene therapy.
- The increase in expression of Nrg1 type III at the spinal cord level manages to preserve motoneurons, as well as their synaptic connections, and reduce the microglial response, and promotes a significant functional improvement, more potent in female than in male mice.

3. Relevance and possible future implications

The results obtained with the intramuscular injection of an AAV vector producing overexpression of Nrg1-I in the treated muscle have shown an important role of Nrg1-I in the axonal branching and muscle re-innervation process. Overexpression of Nrg1-I in the gastrocnemius muscle of SOD1G93A mice produces significant functional compensation, by promoting collateral re-innervation, thus opening a window for the development of new therapies focused on functional recovery rather than preservation for motoneuron diseases.

The administration of an AAV that induces the expression of Nrg1-I under the desmin promoter has allowed the adequate infection of a large number of skeletal muscles. With this strategy we have been able to produce a beneficial effect systemically, which could prevent the evolution of the loss of neuromuscular connections in ALS. In any case, the expression levels of Nrg1-I administered intravascularly under the desmin promoter, despite encompassing a large number of muscles, are lower compared to those obtained after direct intramuscular injection under the CMV promoter. The

results obtained in the clinical trials of gene therapy in SMA or DMD patients through intravenous administration demonstrate that the viral titers needed to achieve therapeutic effects in motoneurons are by far higher than in other tissues. Subsequent improvements in the efficiency of the viral vector will make it possible to increase the levels of Nrg1 expression to achieve wider beneficial effects without compromising the biosafety of the patient.

The results obtained with AAV-Nrg1 type III show that it allows preservation of the motoneurons as well as their synaptic connections and reduction of the microglial response, and promote significant functional improvement. These results corroborate our initial hypothesis and provide a new type of gene therapy applicable to diseases of motoneurons.

Finally, we performed the study of the combined gene therapy that we had planned, even improved, by simultaneous injection of AAV-Desmin-Nrg1-I viral vectors by intravenous route to transduce Nrg1-I in skeletal musculature and AAV-Nrg1-III-FL to produce expression of Nrg1-III in the spinal cord. The preservation of motor function that is obtained with both treatments separately is similar to that achieved with the simultaneous treatment in SOD1G93A mice. This situation is similar to the results with other combined treatments used by our group. Everything indicates that motor preservation in the ALS murine model reaches a threshold that cannot be overcome easily even if several therapies are combined.

4. Literature produced

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