Reduced copper transporter trafficking and synaptic deficits in iPSC-MNs derived from an ALS patient with a novel variant of ATP7A

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**Introduction**

Dysregulation of copper distribution has been discovered in a number of neurological diseases, including Menke’s disease, Alzheimer’s disease, and a distal motor neuron disease. Due to its role in mitochondrial function, antioxidant activity, and synaptic transmission, changes in copper homeostasis can cause a broad range of cognitive and motor deficits. Mutations in the P-type ATPase copper transporter ATP7A are known to cause Menke’s disease, occipital horn syndrome, and X-linked spinal muscular atrophy type II (SMA). It has previously been reported a novel variant of ATP7A in a male patient with brahial amyotrophic diplegia, a slow-progressing form of motor neuron disease. The possible contribution of this mutation to the patient’s disease pathogenesis is unknown. The non-synonymous M1311V substitution, located near the binding site of the protein, is associated with mislocalization of ATP7A at basal levels and impaired trafficking in response to increased copper when overexpressed in HeLa cells and in patient fibroblasts. No changes in ATP7A transcript or gene expression were observed in fibroblasts. Patient fibroblasts were reprogrammed into induced pluripotent stem cells and differentiated into motor neurons (iPSC-MNs). This model was used to examine ATP7A expression and localization in a more disease-relevant cell type. In addition, we examined morphological and functional parameters commonly affected in ALS and other ATP7A-associated diseases, namely, dendritic morphology, hyperexcitability, and excitotoxicity. Due to copper’s previously established role as a local modulator of glutamate receptor expression, distribution, and permeability changes in transporter trafficking could explain changes in synaptic function. Further work will be required to determine if changes in ATP7A protein expression or trafficking cause a toxic level of copper redistribution capable of contributing to the observed physiological deficits and, ultimately, ALS.

**Proposed Role of ATP7A in Motor Neurons**


**Methods**

**RNA Sequencing**

RNA was isolated using the RNAqueous-PCR-free total RNA isolation Kit. cDNA synthesis and library preparation were performed using a SMARTer and KAPA Hyper kit combination. Demultiplexing and fast generation were performed using CAUSA v.8.4, followed by trimming of 76 from both R1 and R2 using cutadapt v.1.10, as recommended in the SmartRf manual. Reads were then aligned to the genome using STAR v.2.5.2L, and raw counts were calculated with featureCounts (subread v.2.1) using the ensembl57 annotation. Analysis and differential expression testing were performed in R using DESeq2 v.1.41.

**IPSC Motor Neuron Cell Culture Model**

Control and p.M1311V patient-derived fibroblasts were reprogrammed into iPSC, which were then differentiated into motor neurons and expanded (as reviewed). Experiments were conducted on or after day 53 of the differentiation protocol, a time point which has previously proven to generate functional motor neurons.

**ATP7A Dysregulation and Response to Elevated Copper Concentrations**

Control and M1311V fibroblasts and iPSC-MNs were plated on cover slips. Copper (II) chloride was added to the media to reach the given concentration. After 1 hour, the media was removed and cells were fixed and immunostained. Cells were imaged using a Zeiss confocal microscope with a 532 nm laser. Images in analysis software was used to quantify the colocalization of ATP7A and the trans-membrane glycoprotein TGN46. After setting fluorescence thresholds to remove background, the Pearson’s correlation coefficient of the three-dimensional position of above-threshold peaks in the ATP7A and TGN46 channels were calculated.

**Dendritic Length, Sholl Analysis, and Spine Density**

Control and M1311V iPSC-MNs were cultured on astrocytes and transfected with a lentiviral CMV promoter-driven GFP construct. Cells were treated with virus for 24 hours. They were washed and fixed 48 hours after virus removal for immunofluorescence staining. Cells were imaged using a Zeiss confocal microscope with a 405 excitable laser. Images in analysis software was used to quantify the dendritic length and total dendritic length was extracted from these traces. Traces were then uploaded into ImageJ for Sholl analysis. Spine density was calculated by measuring the number of spines in a traced cell and dividing by total dendritic length. Sine subplots were defined as follows: f(100) = total spine length × 3μm, f(1) = total spine length × 3μm, < f(1) = total spine length × 3μm, > f(100) = all others are stubby.

**Micro Electrode Array Analysis**

Control and M1311V iPSC-MNs were cultured on astrocytes and plated on 48-well plates designed for the Axion Biosystems Maestro MSA. Spontaneous spikes (changes in electric field potential > 50 SD above background) were recorded for 5 minutes at multiple time points from day 76 of the differentiation. Mean firing rate, mean bursts, and mean network bursts were analyzed.

**Results**

1. Differentially expressed genes and proteins in ATP7A-M1311V iPSC-MNs


2. ATP7A-M1311V fibroblasts and iPSC-MNs exhibit mislocalization of ATP7A and decreased ATP7A trafficking in response to elevated copper.

3. ATP7A-M1311V iPSC-MNs exhibit morphological deficits.

4. ATP7A-M1311V patient iPSC-MNs show age-sensitivity changes in excitability and increased sensitivity to glutamate toxicity.

**Conclusions**

The M1311V mutation in ATP7A causes mislocalization and impaired trafficking of the protein in response to changes in copper concentration. In addition, M1311V patient iPSC-MNs showed increased expression of ATP7A and decreased expression of ATP7B. ATP7A patient iPSC-MNs show deficits in both synaptic and spine morphology with accompanying changes in excitability and increased sensitivity to glutamate toxicity. Further work is required to determine if the mislocalization and changes in copper transport, which has previously been shown to affect synaptic protein expression and distribution and increase excitotoxicity.

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**References**

(A) Reduced copper transporter trafficking and synaptic deficits in iPSC-MNs derived from an ALS patient with a novel variant of ATP7A

(B) ATP7A-M1311V patient iPSC-MNs exhibit increased expression of ATP7A and decreased expression of ATP7B. ATP7A patient iPSC-MNs show deficits in both synaptic and spine morphology with accompanying changes in excitability and increased sensitivity to glutamate toxicity.

(C) The M1311V mutation in ATP7A causes mislocalization and impaired trafficking of the protein in response to changes in copper concentration. In addition, M1311V patient iPSC-MNs showed increased expression of ATP7A and decreased expression of ATP7B. ATP7A patient iPSC-MNs show deficits in both synaptic and spine morphology with accompanying changes in excitability and increased sensitivity to glutamate toxicity. Further work is required to determine if the mislocalization and changes in copper transport, which has previously been shown to affect synaptic protein expression and distribution and increase excitotoxicity.

(D) ATP7A-M1311V patient iPSC-MNs show age-sensitivity changes in excitability and increased sensitivity to glutamate toxicity.

(E) The M1311V mutation in ATP7A causes mislocalization and impaired trafficking of the protein in response to changes in copper concentration. In addition, M1311V patient iPSC-MNs showed increased expression of ATP7A and decreased expression of ATP7B. ATP7A patient iPSC-MNs show deficits in both synaptic and spine morphology with accompanying changes in excitability and increased sensitivity to glutamate toxicity. Further work is required to determine if the mislocalization and changes in copper transport, which has previously been shown to affect synaptic protein expression and distribution and increase excitotoxicity.