Nuclear phenotypes and astrogliosis in astrocytes derived from ALS SOD1(A4V)-mutated iPSCs

Vincent Soubannier [1,2], Mathilde Chaineau [1,2], Lale Gursu [1,2], Sarah Lépine [1,2], Guy Rouleau [1], Thomas M. Durcan [1,2] and Stefano Stifani [1]

1- Montreal Neurological institute and Hospital, McGill University, Montreal, QC, Canada.
2- Early Drug and Discovery Unit, (EDDU), Montreal Neurological institute and Hospital, McGill University, Montreal, QC, Canada.

Motor neuron (MN) degeneration in ALS involves both cell autonomous processes within MNs and non-cell autonomous mechanisms in glial cells, including astrocytes. We seek to elucidate the contribution of astrocytes to ALS pathology by studying human astrocytes generated from iPSCs derived from ALS and non-ALS individuals. Here we show that astrocytes derived from SOD1 A4V-mutated iPSCs display different cellular and nuclear morphologies, higher nuclear oxidative stress, double DNA breaks, and an increased nuclear/cytosolic ratio of the SOD1 protein when compared to isogenic astrocytes. In addition to these nuclear physiological alterations, SOD1-mutated iPSC-derived astrocytes display detectable signs of activation, such as altered cytoskeletal organization and increased expression of "A2" astrocyte phenotype markers after two months of culture. These findings suggest that the SOD1(A4V) mutation is associated with nuclear damages in human patient-derived astrocytes resulting in a time-dependent activation of reactive astrogliosis.

Background

Amyotrophic lateral sclerosis (ALS) is an adult-onset MN disease that can be caused by dominantly inherited mutations, including mutations in the gene encoding the enzyme Superoxide Dismutase 1 (SOD1). A growing body of evidence suggests an involvement of non-cell autonomous mechanisms of motor neuron toxicity elicited by glial cells. Although animal models have been instrumental in improving our understanding of ALS pathophysiological mechanisms, complementary models based on human cells are needed to further investigate ALS aetiology. We previously described an efficient and streamlined method to generate human iPSC-derived astrocytes with molecular and biological properties similar to physiological astrocytes in the ventral spinal cord (1). In this study, we generated spinal cord astrocytes from human iPSCs harbouring the SOD1(A4V) mutation, and from a matching isogenic iPSC lines, in order to characterize the impact of this mutation on astrocyte physiology.

(1) Soubannier V, Chaineau M. et al. Rapid Generation of Ventral Spinal Cord-like Astrocytes from Human iPSCs for Modeling Non-Cell Autonomous Mechanisms of Lower Motor Neuron Disease. Cells. 2022;11(3):399.

Generation and characterization of iPSC-derived astrocytes

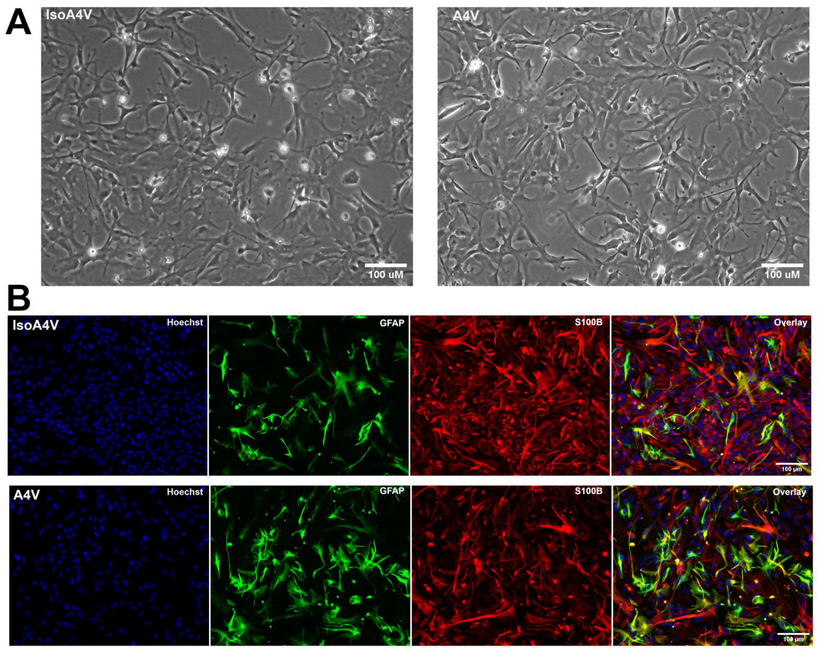

Fig 1. Generation of induced spinal cord astrocytes.
iPSCs from ALS patient cell line carrying the SOD1 A4V mutation (A4V) and matching isogenic cell line with corrected mutation (isoA4V) were submitted to ventralization and caudalization to generate progenitors that were then differentiated into astrocytes as previously described (1).
A. DIC analysis of differentiated cells one month after the start of the differentiation protocol from progenitors.
B. After one month of differentiation, SOD1(A4V) and isogenic astrocytes were analyzed by immunocytochemistry using antibodies against GFAP and S100β. The majority of cells co-express S100β and GFAP. Images show cells expressing high levels of GFAP, with many other cells expressing GFAP at lower levels.

Nuclear oxidative stress in DIV30 SOD1(A4V) astrocytes

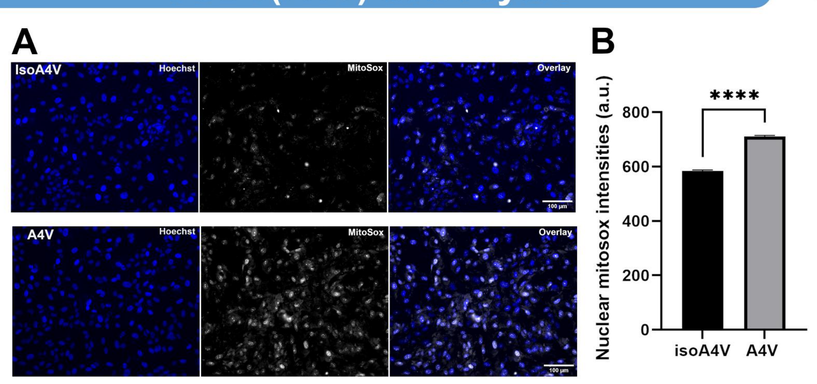

Fig 2. Nuclear oxidative stress in SOD1(A4V) astrocytes at 30 days *in vitro*.
A. Fluorescent microscope images taken after incubation of 1-month old astrocytes for 30 minutes with Hoechst and 2.5 μM MitoSox.
B. Quantification of nuclear MitoSox fluorescence intensities. Individual regions of interest corresponding to the nucleus were obtained from the Hoechst staining and used to measure nuclear MitoSox intensities.
The probe MitoSox was used to measure the concentration of superoxide ions in SOD1(A4V) or isogenic astrocytes. At micromolar concentrations (2.5 μM), MitoSox is exclusively found in the nucleus, allowing estimation of nuclear superoxide ion concentrations. Our studies showed that SOD1(A4V) astrocytes display a higher nuclear superoxide ions concentration than the corresponding isogenic astrocytes at 30 days in vitro.

SOD1 nuclear translocation and increased double DNA breaks in DIV60 SOD1(A4V)

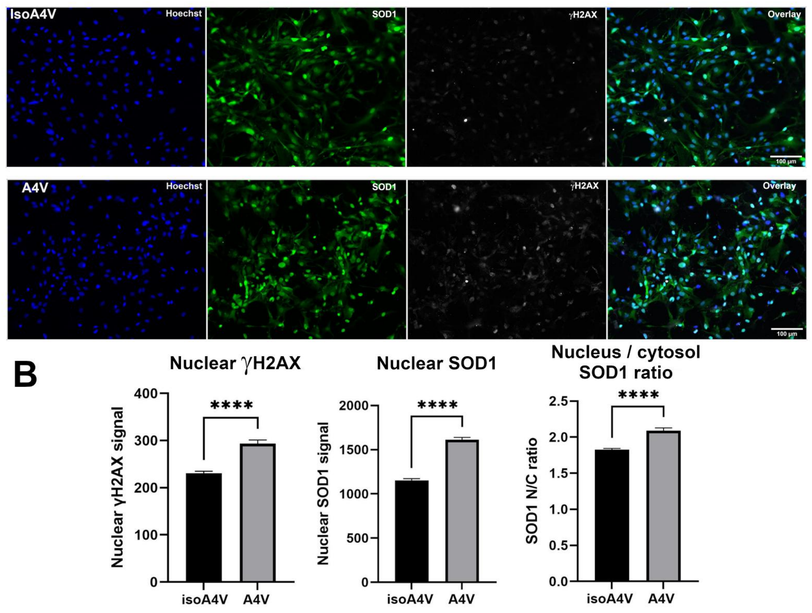

Fig 3. Nuclear damage in SOD1(A4V) astrocytes at 60 days *in vitro*.
A. Fluorescent microscope images of 2-months old astrocytes immunodecorated with antibodies raised against SOD1 and against γH2AX.
B. Quantification of nuclear γH2AX and nuclear SOD1 fluorescence intensities in 2-months old SOD(1A4V) astrocytes compared to isogenic astrocytes. The last graph corresponds to the ratio of SOD1 intensities measured in the nucleus divided by SOD1 intensities measured in the cytosol.
Our studies revealed the emergence of additional phenotypes in SOD1(A4V) astrocytes at later stages of *in vitro* maturation. Specifically, at 60 days *in vitro*, nuclear SOD1 localization was increased in SOD1(A4V) astrocytes, as revealed by the measure of SOD1 signal in the nucleus and by the measure of nucleus/cytosol SOD1 ratio. In parallel, we observed increased γH2AX signal in the nucleus of SOD1(A4V) astrocytes, suggesting an increased occurrence of double DNA breaks. Taken together with the results depicted in Figure 2, these results suggest that increased nuclear oxidative stress leads to SOD1 nuclear translocation and increased nuclear damage in more mature SOD1(A4V) astrocytes.

Cytoskeletal disorganization in SOD1(A4V) astrocytes

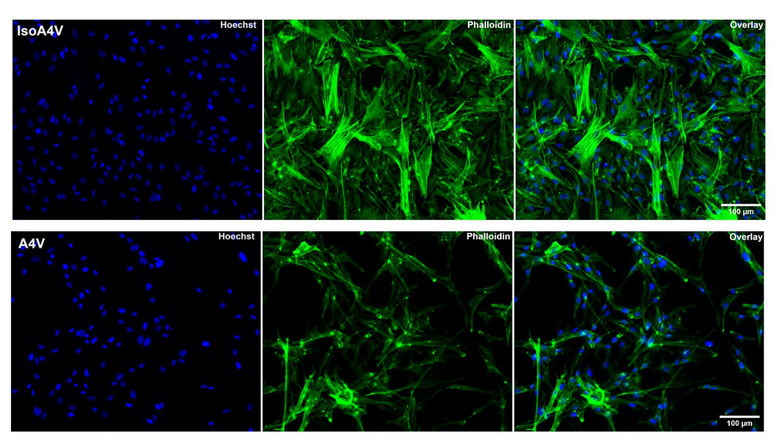

Fig 4. Altered cytoskeletal organization in SOD1(A4V) astrocytes.
Cytoskeletal organization of 2-months old spinal astrocytes was examined by staining with phalloidin Alexa 488. While isogenic astrocytes (isoA4V) displayed the presence of typical F-actin stress fibers, SOD1(A4V) astrocytes exhibited disassembly of the stress fibers and presence of β-actin network characterized by ring-like structures, ruffles and radial actin filament. These observations suggest that SOD1(A4V) astrocytes are more activated than isogenic astrocytes.

Reactive phenotype of SOD1(A4V) astrocytes revealed by expression of astrogliosis markers

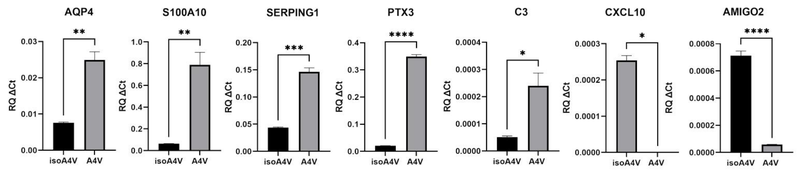

Fig 5. Reactive phenotype of SOD1(A4V) astrocytes revealed by expression of astrogliosis markers.
To further investigate the activated state of SOD1(A4V) astrocytes, we next examined the expression of a number of genes associated with a reactive astrocyte phenotype. Most of them were found to be up-regulated in SOD1(A4V) astrocytes compared to isogenic astrocytes. As reactive astrogliosis is a broad spectrum of states, the additional observation that *PTX3* and *S100A10*, but not *CXCL10* or *AMIGO2*, were up-regulated suggests that, in the absence of other inflammatory cues, the SOD1(A4V) mutation is associated with reactive astrocytes in an anti-inflammatory, protective, state rather than a pro-inflammatory, toxic state.

Increased secretion of astrogliosis-associated cytokines in SOD1(A4V) astrocytes

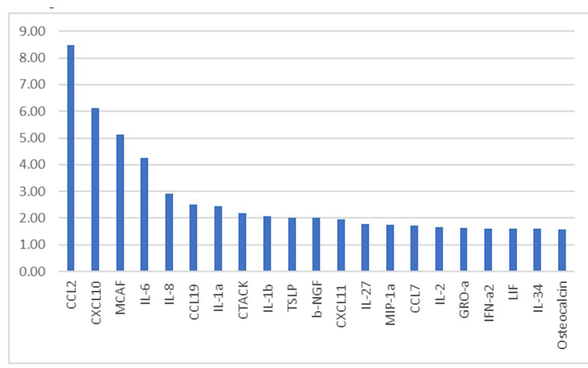

Fig 6. Increased secretion of astrogliosis-associated cytokines in SOD1(A4V) astrocytes.
iPSC-derived astrocytes DIV 90 (both A4V and isoA4V) were cultured in fresh media for three days, and conditioned media were then analyzed by Luminex multiplex protein assay for cytokine/chemokine expression. Ratio between detected amounts were calculated and main fold changes were plotted. This analysis showed that SOD1(A4V) astrocytes over-secrete a number of cytokines and chemokines typical of reactive astrocytes.

Conclusions

Spinal cord astrocytes derived from SOD1(A4V)-mutated iPSCs show signs of nuclear oxidative stress at early *in vitro* stages.

Over prolonged culture, SOD1(A4V) astrocytes display DNA damage and increased nuclear SOD1 levels.

These nuclear phenotypes are associated with increased signs of astrogliosis such as cytoskeletal reorganization and increased expression and secretion of markers of reactive astrocytes.

Together, these observations suggest that the SOD1(A4V) mutation leads to nuclear stress, DNA damage, and a reactive phenotype in astrocytes.

Acknowledgments
This work was supported by ALS Canada / Brain Canada Arthur J. Hudson Translational Team Grant and CQDM on Brain grant.