iPSC-Derived spinal motor neurons as a model for investigating morphological and functional alterations in Sporadic Amyotrophic Lateral sclerosis (sALS)

Angela Borraccini[1], Matteo Bordoni[2], Paolo Massobrio[1,4], Orietta Pansarasa[2], Martina Brofiga[1,3,5]

1 Department of Informatics, Bioengineering, Robotics and Systems Engineering (DIBRIS), University of Genova, Genova, Italy
2 IRCCS Mondino Foundation, Via Mondino 2, 27100 Pavia, Italy
3 Neurofacility, Istituto Italiano di Tecnologia (IIT), Genova, Italy

Abstract

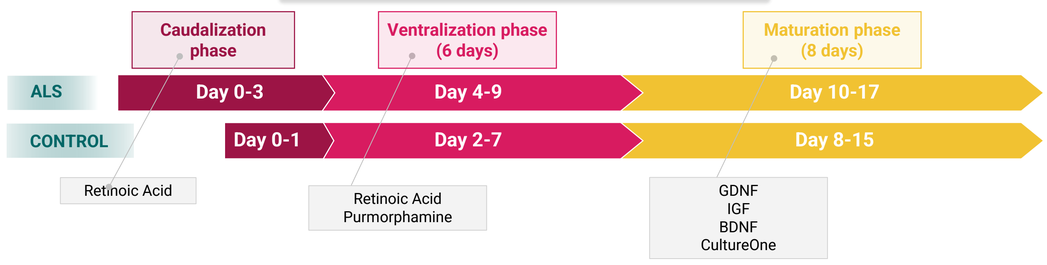
Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease characterized by progressive degeneration of motor neurons. *In vitro* models based on induced pluripotent stem cells (iPSCs) represent an innovative and powerful platform to investigate ALS pathogenesis in a human-relevant context.

This study focuses on the optimization of a differentiation protocol to generate motor neurons (MNs) from iPSCs derived from healthy donors and sporadic ALS patients, with the aim of establishing stable and reproducible long-term cultures. Protocol performance was assessed through morphological analyses, including immunocytochemistry for MN-specific markers and quantitative intensity assessment (data shown below refer to the ALS line).

The optimized protocol is designed to support downstream functional studies. Preliminary results from the control line are presented using calcium imaging to monitor intracellular Ca^{2+} dynamics and microelectrode array (MEA) recordings.

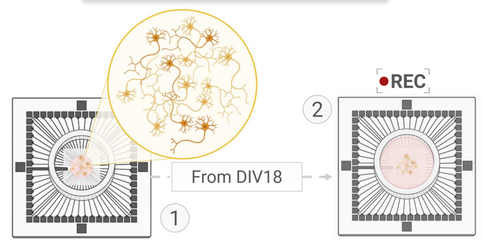
Material and Methods

Differentiation Protocol



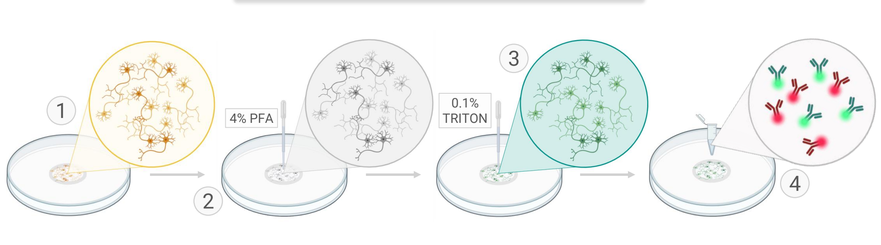
Workflow for Motor Neuron differentiation. Neuronal progenitor cells (NPCs), derived from iPSCs of healthy subjects and sporadic ALS patients, are subjected to three sequential phases: a caudalization phase lasting 2 days for the control line and 4 days for the ALS line, followed by a 6-day ventralization phase, at the end of which immature motor neurons are generated. These are then subjected to an additional 8-day maturation phase using a specific differentiation medium.

REC



Workflow for electrophysiological recordings. Motor neurons were plated on MEA at day 3 of the maturation phase at a density of 1500 cells/mm². Recordings lasted 20 minutes and were performed using a commercial MEA2100 system (MCS).

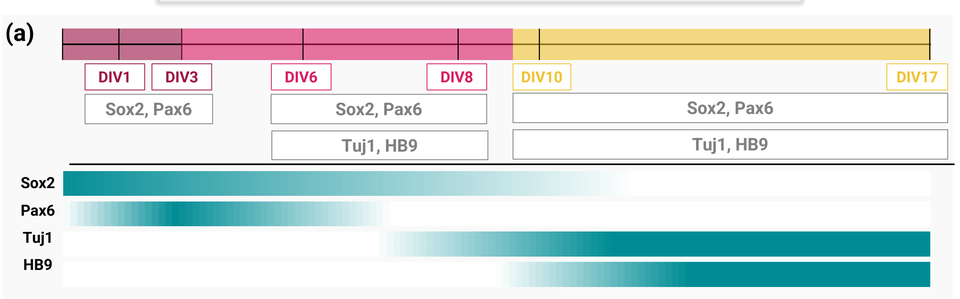
Immunocytochemistry

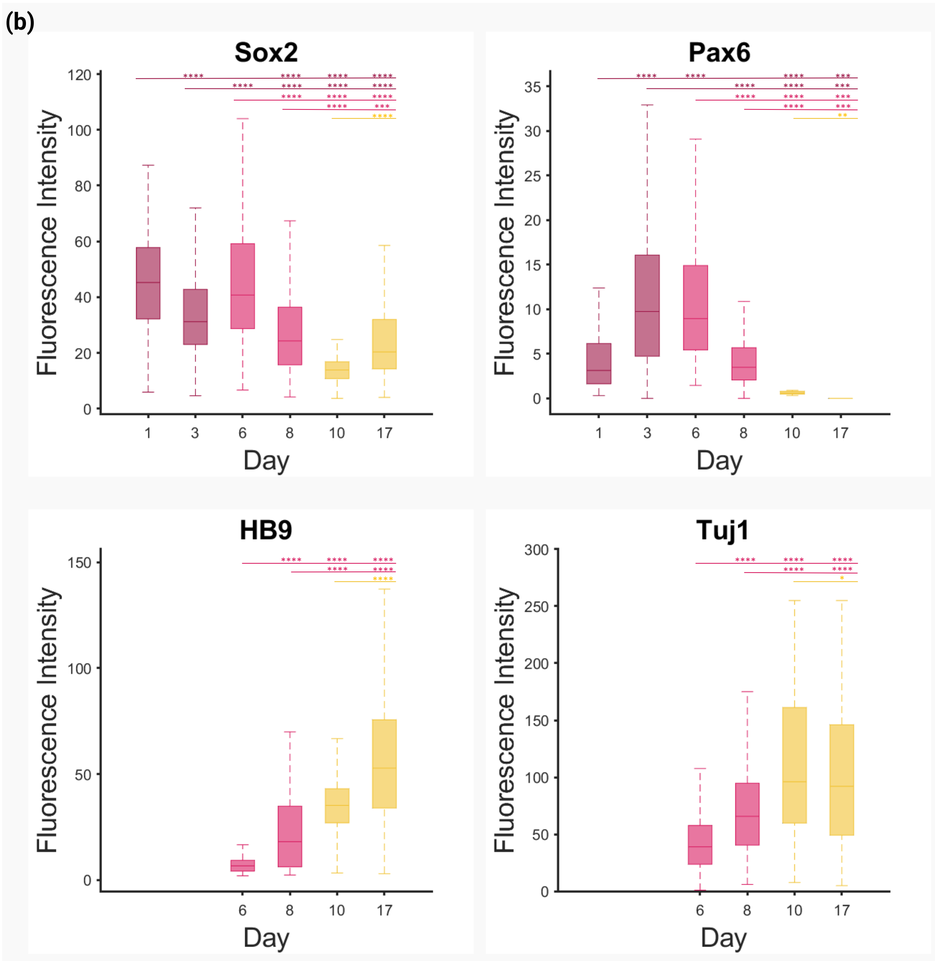


Workflow for immunocytochemistry. Motor neuron samples were fixed at defined time points using 4% PFA for 7 min. This was followed by a permeabilization step with 0.1% Triton X-100 for 10 min and a blocking step to reduce nonspecific binding (5% FBS in PBS for 40 min). Primary antibodies, appropriately selected and diluted, were incubated for 2h at room temperature, followed by incubation with secondary antibodies for 40 min at room temperature.

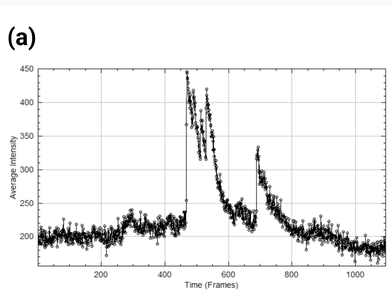
Results

ALS: Fluorescence Intensity Analysis

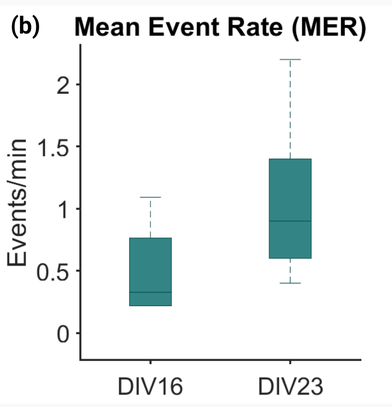




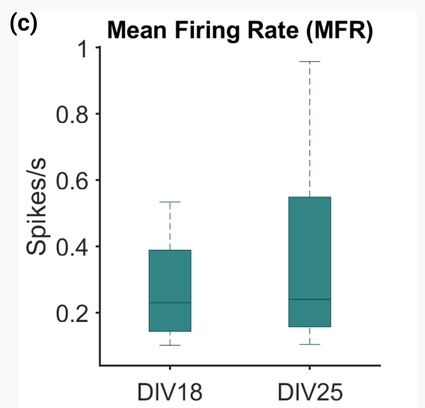
Control: Preliminary functional results



Calcium imaging. Calcium imaging was performed on control motor neurons at DIV16 and DIV23. Samples were incubated for 20 min in a pH-stable (7.4) recording medium containing 4.6 µM of the cell-permeant calcium indicator Fluo-4 AM, followed by a 5 min recording in dye-free medium. Images were acquired at 8000 frames s^{-1} and temporally averaged to an effective sampling rate of 3.65 Hz. Fluorescence intensity traces from detected regions of interest (ROIs) were extracted using the Time Series Analyzer plugin in ImageJ **(a)**. Activity events were identified using a Schmitt trigger algorithm. Macroscopic network activity was quantified as the mean event rate (MER), defined as the average number of events per minute **(b).** Statistical significance was evaluated using the nonparametric Kruskal–Wallis test (p-value of 0.01).

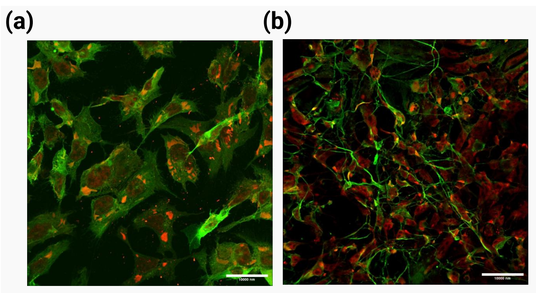


Electrophysiological analysis. Spontaneous activity was recorded using 120-electrode microelectrode arrays (MEAs). The mean firing rate (MFR) of healthy cultures was assessed at DIV18 and DIV25 and calculated as the number of spikes per second. Statistical significance was evaluated using the nonparametric Kruskal–Wallis test (p-value of 0.01).



Fluorescence intensity analysis. a. Schematic representation of the temporal expression of key markers during the differentiation protocol: SOX2, marker of proliferating cells; PAX6, marker upregulated by retinoic acid exposure; Tuj1, early neuronal marker; and HB9, marker of immature motor neurons. **b**. Distribution of fluorescence intensity extracted from ROIs in images acquired under identical imaging conditions at days 1, 3, 6, 8, 10, and 17 of the differentiation protocol. Statistical significance: nonparametric Mann–Whitney U test, with p values of 0.05.

Immunofluorescence staining



Immunofluorescence staining of motor neurons. Representative images of (a) ALS and (b) Control motor neurons at DIV10 and DIV8, respectively, showing Tuj1-positive (green) and HB9-positive (red) cells.

Conclusions

The optimized protocol enabled the generation of motor neurons under both experimental conditions, while highlighting phenotypic differences between the two cell lines. In particular, motor neurons derived from the ALS line predominantly exhibited a more immature state, whereas control cells showed a higher degree of maturation and functional features. Despite the successful induction of neuronal differentiation, the presence of a non-differentiated cell population highlights the need for further optimization of the culture system, in order to promote a more homogeneous maturation across both experimental conditions.

The next steps will focus on the electrophysiological characterization of both populations using non-invasive electrophysiological techniques, in order to investigate key ALS-related processes such as axonal dysfunction, excitotoxicity, glial-mediated neuroinflammation, mitochondrial impairment, and altered neuronal activity, many of which remain poorly understood.

angela.borraccini@edu.unige.it
Via All'Opera Pia 13, 16145, Genova (GE), Italy