

Electrophysiological and immunocytochemical characterization of human stem cell-derived motor neurons

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Introduction

Amyotrophic lateral sclerosis (ALS)
 Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects motor neurons in the brain and spinal cord. A key mechanism mediating motor neuron death is glutamate excitotoxicity.

RNAi screening
 BioFocus' target discovery platform is based on screening proprietary adenoviral knock-down libraries (SilenceSelect®) targeting the druggable human genome. These libraries contain over 1,700 full-length human cDNA and 12,000 human shRNA vectors with a redundancy of three shRNAs per transcript. The combination of adenoviral knock-down technology with screening in human cells is designed to identify critical proteins in disease pathways.

Aim
 As part of an ALS Association-sponsored project (TREAT-ALS), we aim to identify novel therapeutic targets for the treatment of ALS by screening our adenoviral shRNA library (SilenceSelect®) in human motor neurons (MN) exposed to disease-relevant stimuli such as glutamate excitotoxicity. Here we describe the development and characterization of high throughput screening-compatible cultures of motor neurons derived from human embryonic stem cells.

Materials and Methods

- Production of high purity motor neurons from hESCs at CSC**
- Expansion of undifferentiated hES cells on using feeder free/serum free Stemblast and Ectobias media.
 - Differentiation and expansion of neural progenitors and neurospheres in non-adherent conditions using Neuroblast formulation.
 - Continued differentiation of neurospheres into motor neuron progenitors in adherent conditions using Motorblast media.
 - Final differentiation and maturation plating into 96-well plates, coverslips or flasks with CSC substrate and Motorblast media designed for long-term growth.
 - Shipment of live cultures from CSC to BioFocus.

- Electrophysiology**
- Whole-cell patch-clamp recordings were performed at room temperature using an EPC10 amplifier and Pulse v8.76 acquisition software (HEKA). Cells were matured on glass coverslips for 4 weeks.
 - Solutions for outward currents and ligand-gated currents –
 - external solution (mM): NaCl 140, KCl 2.5, MgCl₂ 2, CaCl₂ 2, HEPES 10, D-Glucose 10, Sucrose 23.5 pH 7.4 NaOH.
 - pipette solution (mM): K-methane-sulphonate 140, NaCl 10, CaCl₂ 1, Mg-ATP 3, GTP 0.4, EGTA 0.2, HEPES 10, pH 7.25 KOH.
 - Solutions for inward currents –
 - external solution (mM): NaCl 115, KCl 3, MgCl₂ 1, CaCl₂ 2, HEPES 10, D-Glucose 10, TEA-Cl 30, 4-AP 4, pH 7.25 CsOH.
 - pipette solution (mM): Cs-methane-sulphonate 100, NaCl 10, CaCl₂ 1, Mg-ATP 3, GTP 0.4, EGTA 1, HEPES 10, pH 7.25 CsOH.
 - Inward and outward currents were evoked by a step voltage protocol. Cells were held at -80 mV and stepped for one second in 10 mV increments from -80 to +60 mV.
 - Ligand-induced currents: cells were held at -70 mV and current was measured for 30 seconds. During this time 100 μM glutamate or 100 μM GABA was applied using a Burleigh rapid perfusion system.

Results

Motor neuron marker expression
 Cultures matured in 96-well plates for five weeks were positive for motor neuron markers Islet1, HB9, SMI32 and TUJ1. A minority of cells (<10%) stained positive for nestin (stem cells) and GFAP (astrocytes).

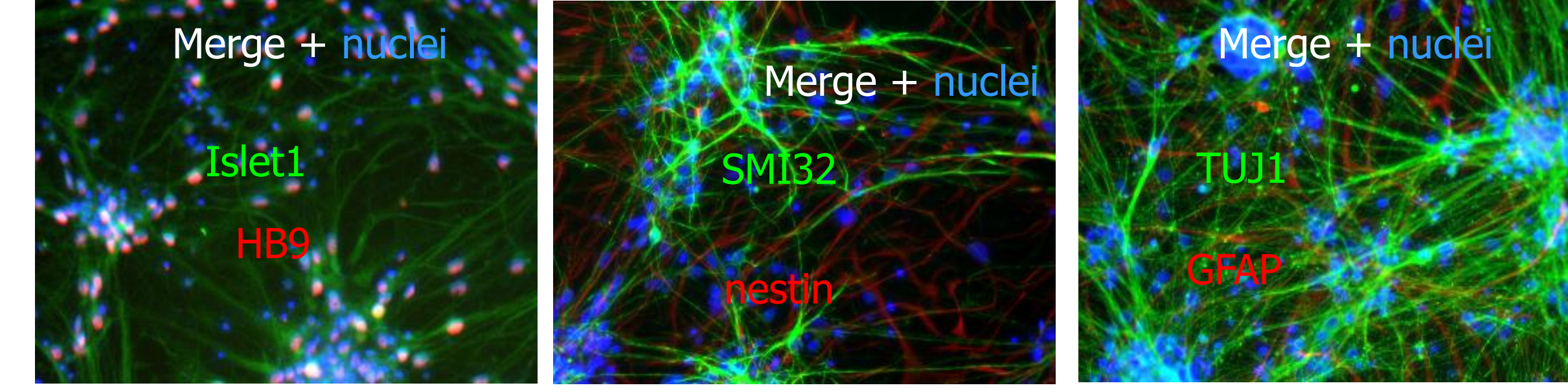


Figure 1. Images acquired using an ImageExpress micro high-content-imager (Molecular Devices)

Transduction with adenovirus
 Human MN cultures grown in 96-well plates were efficiently transduced with adenovirus carrying cDNA for fluorescent reporter proteins AcGFP and ZsGreen. Reporter proteins were clearly detectable by fluorescence microscopy within one day. Expression continued for at least 10 days.

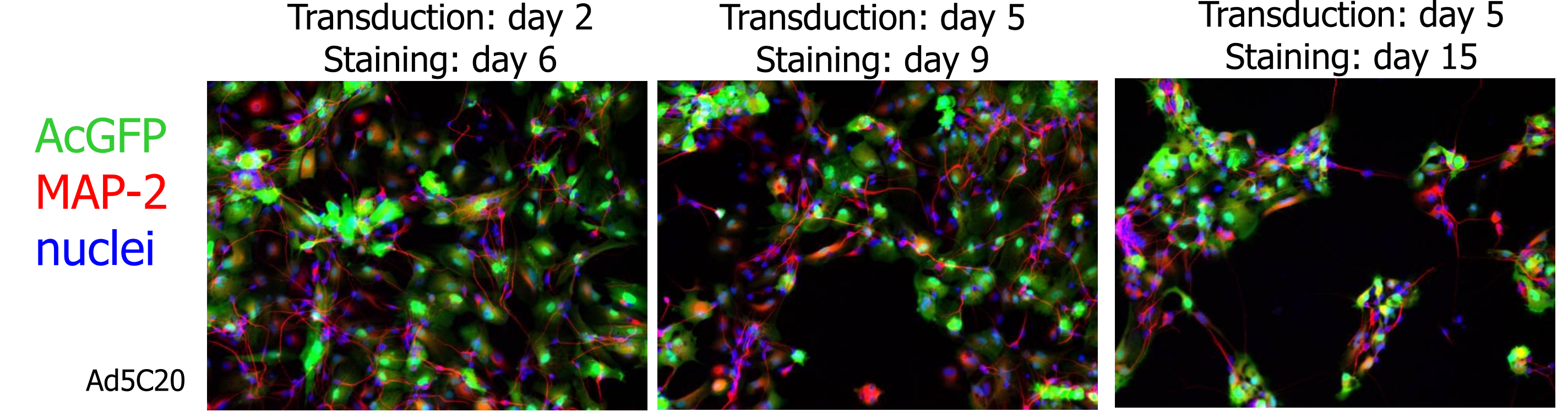
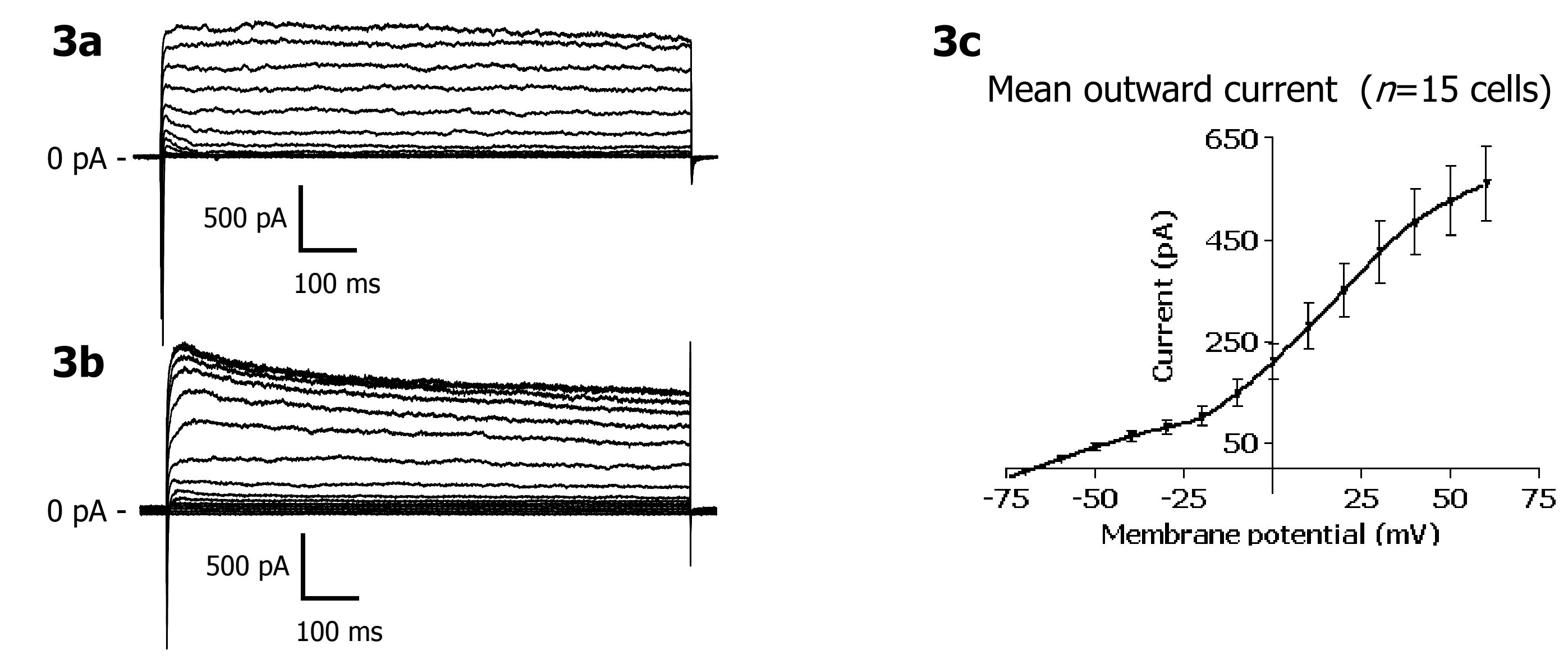
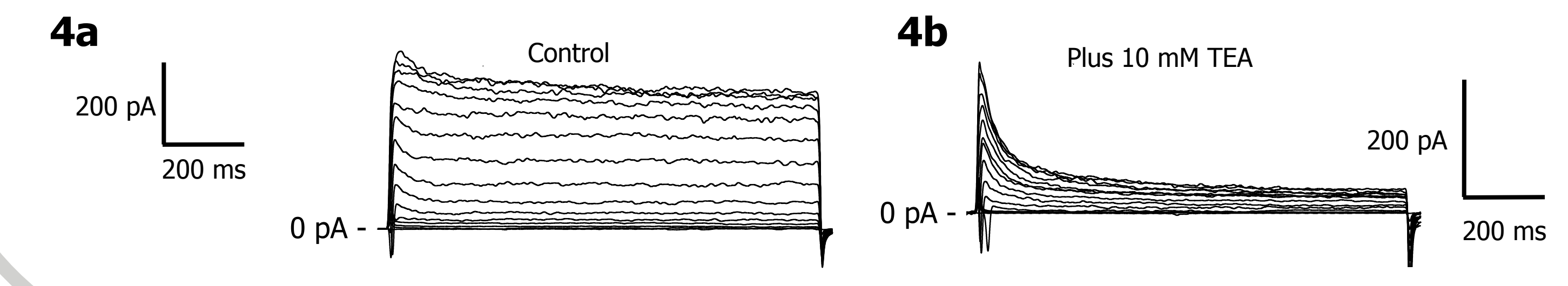


Figure 2. Images acquired using a IN Cell 1000 Analyzer high-content-imager (GE Healthcare)

Electrophysiology: Outward currents
 Heterogeneous outward currents were observed in stem-cell derived motor neurons matured for eight weeks post differentiation. The majority of cells displayed a predominantly non-inactivating current (Figure 3a) although an inactivating component (Figure 3b) was also observed in some recordings. The peak current amplitude at +50 mV was 528 ± 265 pA (mean ± sem, n=15 cells).



The non-inactivating component of the outward current was sensitive to 10 mM TEA (Figure 4b), revealing a neuronal A-type current which in turn was sensitive to 5 mM 4-AP (not shown).



Electrophysiology: TTX-sensitive Na⁺ current
 Inward currents evoked in the presence of TEA, 4-AP and Cs⁺ to block outward K⁺ currents were observed in stem cell-derived motor neurons matured for eight weeks post differentiation. Although expression levels varied, the majority of cells (6/9 cells) displayed a predominantly fast-inactivating current which was sensitive to TTX (Figure 5a). The fast-inactivating inward Na⁺ currents displayed a peak inward current of 243 ± 51 pA (mean ± sem, n=5 cells).

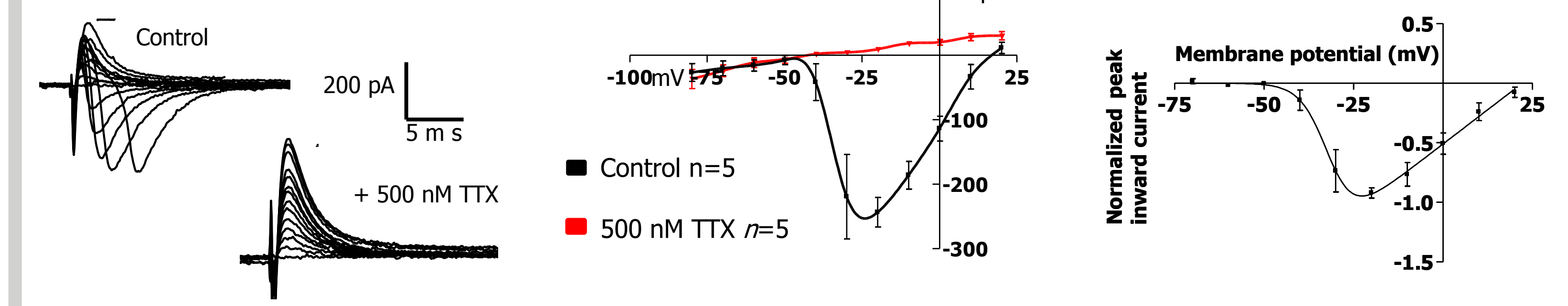
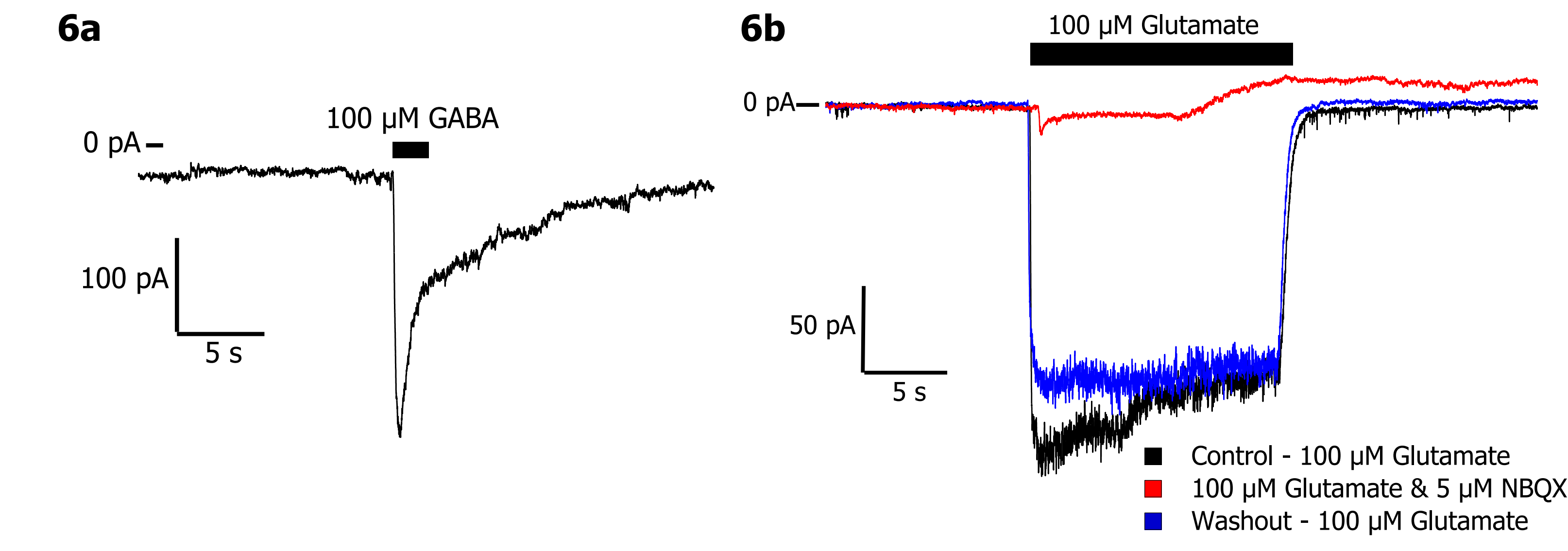


Figure 5a. Current-voltage traces from -80 mV to +50 mV showing typical inward currents in the presence and absence of 500 nM TTX. Figure 5b. Inward current evoked by a series of depolarising steps from -80 mV to +20 mV (black) is completely abolished in the presence of 500 nM TTX (red). Figure 5c. Boltzmann fit to mean normalized data for TTX-sensitive currents $V_{0.5} = -31.7\text{mV}$ n=5 cells (red).

Electrophysiology: Ligand-gated ion channels
 Ligand-gated ion channels were present in a significant proportion of cells with GABA-induced currents seen at eight weeks post differentiation and Glutamate-induced responses seen after 12 weeks post differentiation. GABA responses (Figure 6a) were observed in 60% of cells tested with a mean inward current of -105 ± 55 pA (mean ± sem, n=5 cells). Glutamate-induced responses (Figure 6b) were found in the majority (>85%) of cells with a mean inward current of -120 ± 34 pA (mean ± sem, n=7 cells). Complete inhibition of glutamate-induced response by 5 μM NBQX indicated the presence of GluR's 1-4 (AMPA receptors).



Conclusions

- Cells can be transduced with adenoviral vectors enabling future screening of BioFocus Silence Select® library of shRNAs targeting the human druggable genome.
- Immunofluorescence staining shows the majority of cells express suitable motor neuron markers with a small proportion showing stem cell and astrocyte markers.
- Electrophysiological characterization show the expression of outward K⁺ currents, inward TTX-sensitive Na⁺ currents, and ligand-gated ion channels sensitive to GABA as well as AMPA receptors (GluR's 1-4).
- Stem cell-derived motor neurons are consistent with expression and functional properties of human motor neurons.

Acknowledgements

This work has been financially supported by the ALS Association. The authors would like to acknowledge the BioFocus cell culture team, Carmela Clark, Suzie Clarke for their help and support maintaining the cells.