

The combination of high content screening and stem cells for the treatment of neurodegenerative disorders



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Introduction

Amyotrophic lateral sclerosis (ALS)

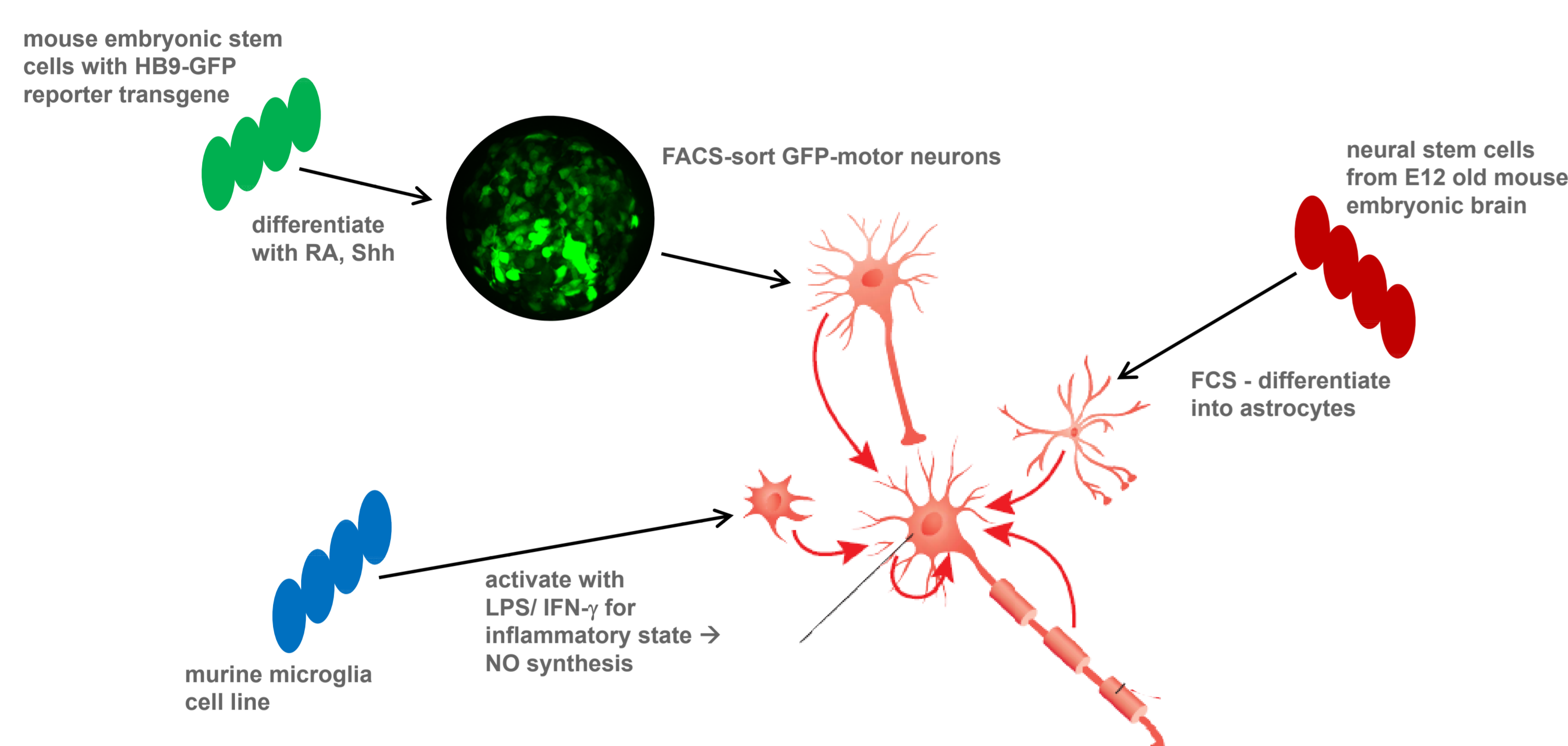
ALS is a progressive neurodegenerative disease that affects motor neurons in the brain and spinal cord. Key mechanisms in motor neuron death in ALS are glutamate excitotoxicity as well as neuroinflammatory processes involving astrocytes and microglia. Activation of microglia by bacterial lipopolysaccharide (LPS) and inflammatory cytokines (e.g. $\text{INF}\gamma$) is an established model to assess the role of microglia in neurodegenerative disease¹.

High content screening (HCS) against ALS

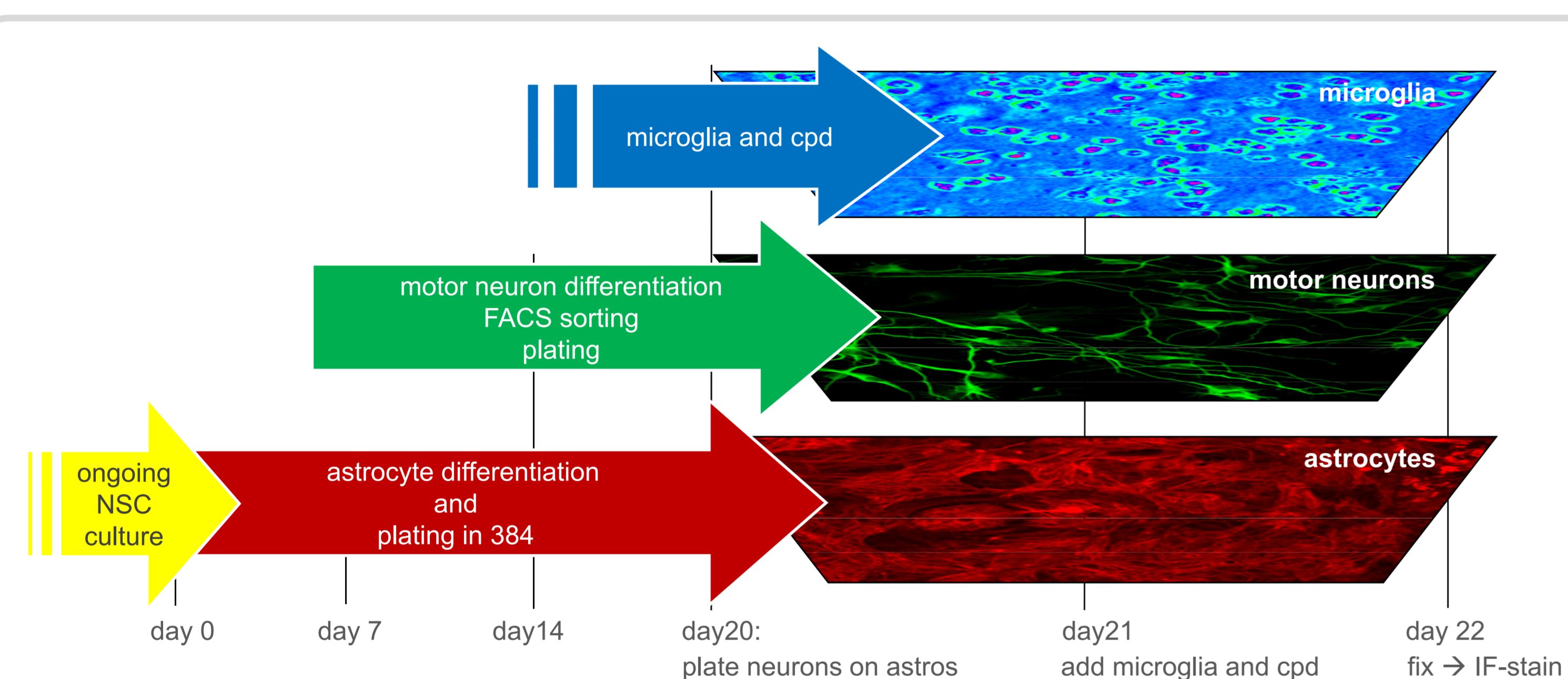
HCS allows the development of cell-type specific phenotypic readouts from co-cultures by a combination of fluorescent markers, automated digital image acquisition and algorithm-based image analysis. By combining stem cell derived motor neurons and astrocytes with LPS/ $\text{INF}\gamma$ -activated microglia we established a High Content screening platform for the detection of novel small-molecule drugs that protect motor neurons from neurite loss and mortality.

Aim and Results

We used the above co-culture assay to identify and profile novel small molecules relevant for the development of new drugs for ALS. A screen of more than 11,000 compounds was conducted and yielded more than 140 primary hits (3s threshold). The 30 most potent hits that were above 5s threshold were selected for hit profiling in 3 assay variants, (i) the motor neuron primary assay, (ii) a cortical neuron variant and (iii) a microglia activation assay. Analysis of the hit population revealed a bias towards inhibitors of microglial activation as opposed to compounds with motor neuron specific protectant properties. Further assays will help elucidating the mechanism of action and cell type specificity.



Cell types involved in ALS and their generation for the high content assay: HB9-GFP transgenic motor neuron differentiation from mouse ES cells via Embryoid Body formation and FACS sorting. Astrocyte generation from primary mouse neural stem cells. BV2 microglia cell line culture and activation with LPS/ $\text{INF}\gamma$.



Assay set up and time lines: Astrocytes differentiation from mNSCs for 3 weeks. Motor neuron differentiation for 2 weeks followed by FACS sorting and plating on astrocytes in 384-well assay plates. Addition of non-activated and activated microglia with and without compounds on day 21 and assay endpoint on day 22.

Methods

Cell culture

- Expansion of primary mouse neural stem cells (mNSCs) isolated from embryonal mouse brain in EGF/FGF2 supplemented defined serum-free medium.
- Differentiation of mNSCs into astrocytes by growth factor withdrawal and addition of fetal calf serum
- Feeder-free expansion of mouse embryonic stem cells derived from a HB9-GFP transgenic line²
- Differentiation and isolation of HB9-GFP expressing motor neurons via embryoid body formation and FACS sorting
- Standard culture of BV2 cells in FCS-containing medium

Motor neuron HCA, cortical neuron HCA

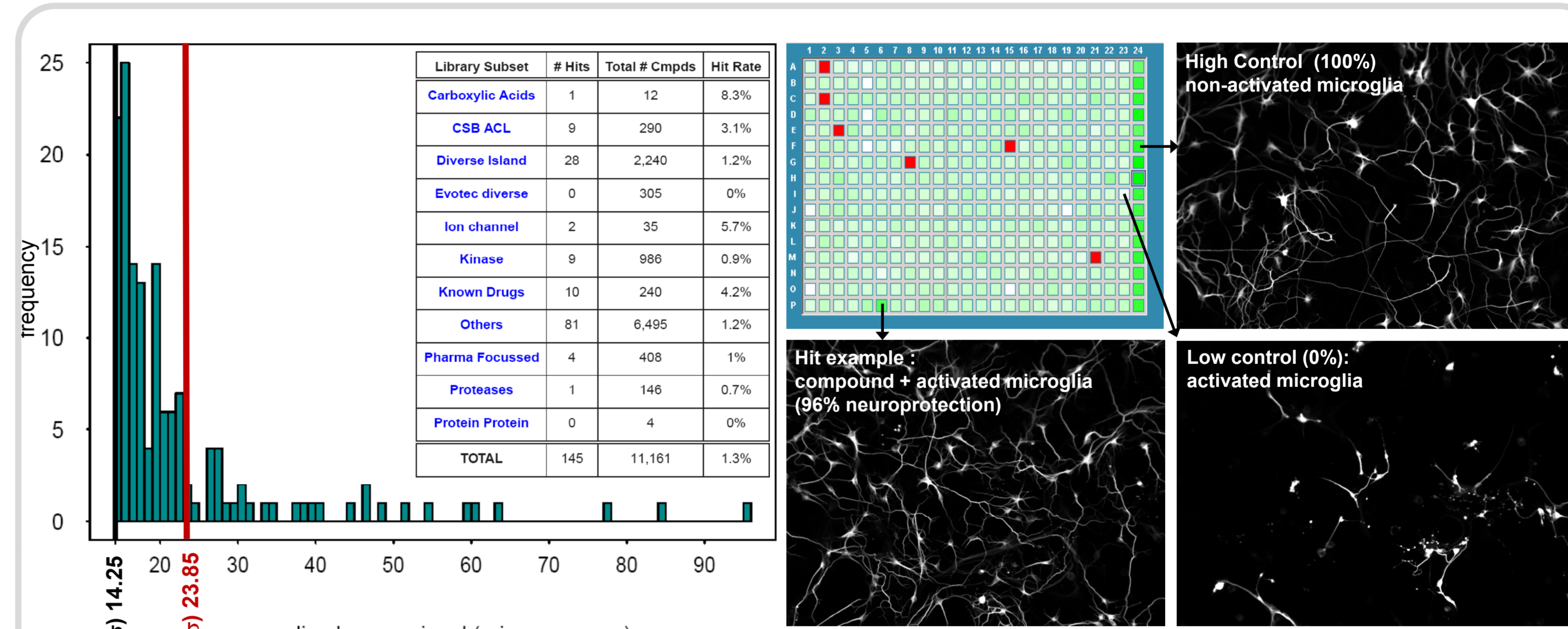
- Plating of astrocytes into 384-well imaging grade assay plates followed by further differentiation up to day 20
- Plating of motor neurons onto astrocytes in defined serum-free assay medium on day 20
- Addition of non-activated BV2 (100% control) and activated BV2 w/o compound (0% control) or with compound (10 μM assay concentration) on day 21 (am), followed by further 30 hrs incubation
- Fixation at assay endpoint on day 22 followed by anti β 3-Tubulin immunofluorescence
- Addition of nuclear stain, imaging on the Opera[®] High Content imaging platform, Acapella[®]-based image analysis
- Mouse primary cortical neurons (from E16 brain) instead of motor neurons were employed in a hit profiling variant

BV2 Nitric Oxide (NO) assay

- Microglial production of Nitric Oxide (NO) was used to quantify microglial activation
- Nitric oxide production was measured as nitrite, using the colorimetric Griess assay³

Primary Screening

A diverse set of more than 11,000 known, well-characterised drugs, and carefully selected small molecules from the Evotec corporate collection was screened at 10 μM against our High Content motor neuron assay. Using a threshold criterion of 3 σ , a hit rate of 1.3% was determined. More stringent thresholding at 5 σ yielded 31 hits (0.3%). These 31 compounds yielded different degrees of neuroprotection, as evidenced in the total lengths of neurite outgrowth. These compounds were therefore selected for concentration response hit profiling.

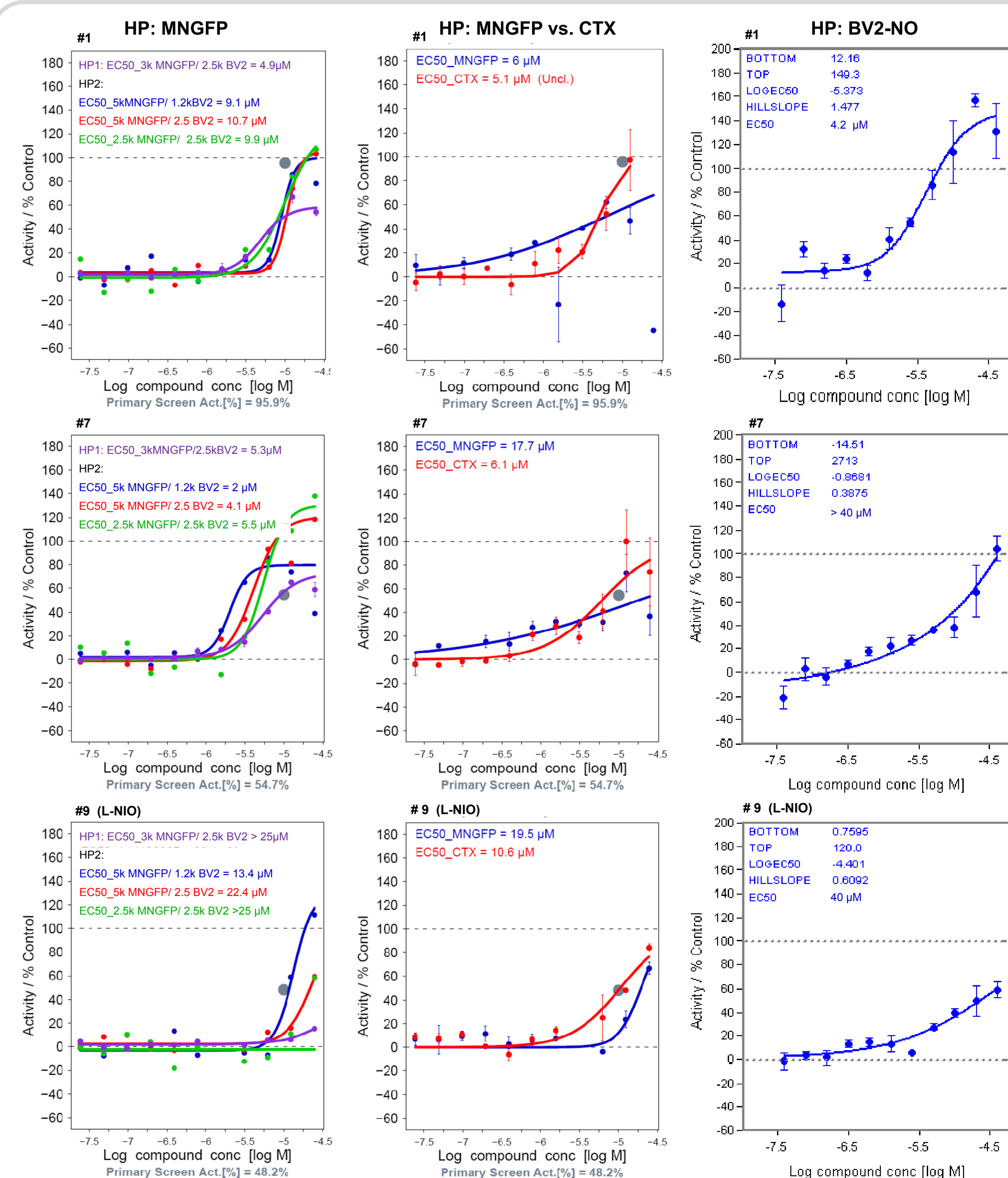


Summary of primary screening results: Histogram of the normalized assay signal values of the 145 (3s threshold) 31 (5s threshold) primary hits amongst 11,161 compounds. Inset showing the segregation of hits into library subsets.

Motor neuron protection assay and hit example: Non-activated (column 24) and activated (column 23) microglia define the 100% and 0% control conditions. A hit example is also shown (well 6P) showing 96% neuroprotection.

Hit profiling

Hit profiling of the 31 most active compounds in 3 different assays demonstrated a strong trend towards inhibitors of microglial activation. A majority of hit compounds protected both motor neurons (MNGFP) and primary cortical neurons (CTX) and reduced NO equivalents in the BV2 NO assay.



Hit profiling, example of 3 compounds: Hit profiling in the main assay (MN-GFP) was done multiple times, varying the number of microglia and the number of motor neurons employed. In HP1, the primary hit results (grey dots) were closely matching the fit curve for the majority of potent compounds. In HP2, BV2 dose dependence of neuroprotection was shown. Protection of cortical neurons (CTX) could be demonstrated for most hit compounds when done in parallel to the MNGFP assay (middle column). The nitrite assay performed on BV2 cells (right column) indicated inhibition of microglial activation for most compounds, as exemplified by the NOS inhibitor L-NIO (compound #7).

Conclusions

We developed and applied a novel stem cell based multicomponent high content screening (HCS) assay platform for the identification and validation of neuroprotective and anti-inflammatory cpds. While further work will be needed to elucidate the mechanism of action of the identified hit cpds and to develop ALS treatments, our cell based phenotypic screening platform can directly identify compounds with the desired outcome, i.e. neuroprotection from inflammatory insult. Furthermore, the assay platform can easily be modified to test other ALS model paradigms, e.g. excitotoxicity. Finally, the stem cell approach, while being complex in protocol, yields quite robust hit data and can easily be upscaled for larger cpds screens.

References and acknowledgments

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- Neurobiology of Disease Volume 37, Issue 3, March 2010, Pages 493-502; Journal of Neuropathology & Experimental Neurology 2004; 63, 964-977; Future Neurology, July 2009, Vol. 4, No. 4, Pages 435-447
 - HB9-GFP from Gensat (<http://www.gensat.org>)
 - Griess Reagent, Sigma G4410 and references therein