# Phenotypic discovery and characterization of neuroprotective compounds relevant to ALS

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## Overview

Disease modalities such as Parkinson's, ALS and Alzheimer's represent complex neurological disorders where certain neuronal populations decline via only partially understood mechanisms that frequently involve neuro-inflammation.

**Aim:**

- Identify new and disease-relevant, neuroprotective compounds for ALS.
- Model the non-cell autonomous nature of ALS by integrating stem cell-derived motor neurons, astrocytes and activated microglia

**Method:**

- Screenable phenotypic assay that uses degeneration of motor neurons as primary readout
- Diverse secondary assays for mechanism of action studies

**Results:**

- Identified a small number of neuroprotective compounds in a screen of 11,000 compounds.
- Hit compounds shown to act through multiple mechanisms:
  - inhibition of microglial activation,
  - directed protection of neurons from NO stress
  - glia-specific activation of genes controlled by Nrf2 transcription factor

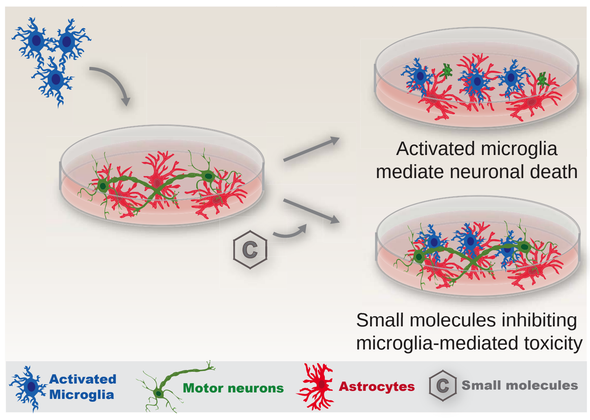



**Figure 1: Overview of the assay concept**

Motor neurons were differentiated from embryonic stem cells, carrying a Hb9-GFP transgene for isolation and visualisation, and plated on neural stem cell-derived astrocytes.

Neuroinflammation was induced by adding microglial cells, activated with lipopolysaccharide and interferon-gamma.

Motor neurons degenerated within 30 hours after addition of microglia.

For screening, compounds were added simultanously with microglia.

## Screening

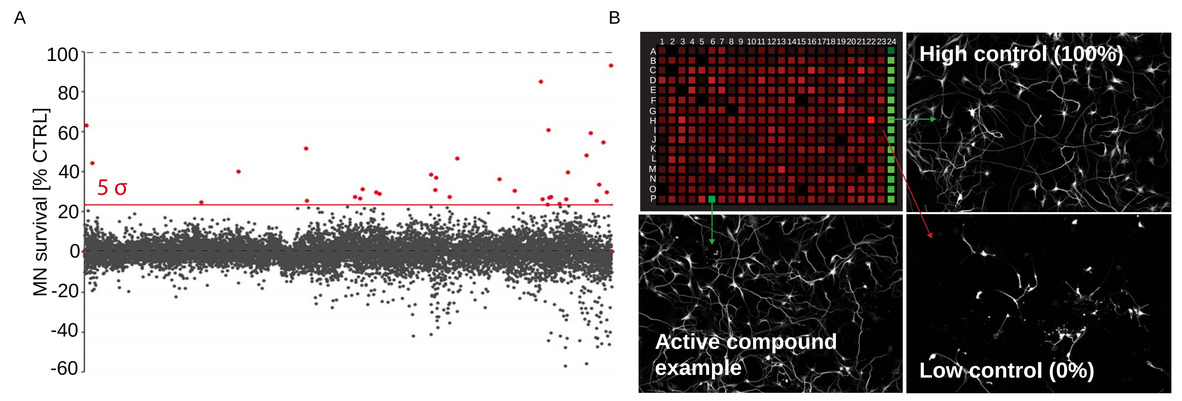



**Figure 2: Identification of Neuroprotective Compounds**

(A) Summary of primary screening results shown as a dot plot of the normalized assay signal values of the primary screening consisting of 11,819 tested compounds. Active compounds, which increased MN survival by at least 5 sigma, are shown in red. Motor neuron survival was quantified by high-content-imaging and defined as the total neurite length of all neurites of the motor neurons in the images captured from one well of the sample normalized to the controls and expressed in percentages.

(B) Example plate from the primary screening, with 100% defined by the "High control", which consisted of non-activated microglial cells, and 0% defined by the "Low control", which included activated microglial cells without any additional small molecules. An active compound example is also shown (well P6), showing 96% neuroprotection.

## Molecular mechanisms

### Neuroprotection via modulation of microglia

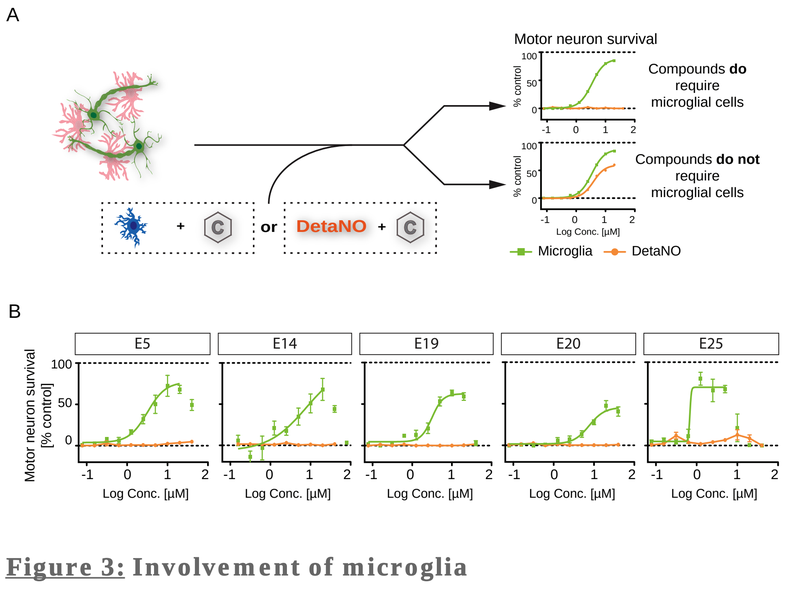
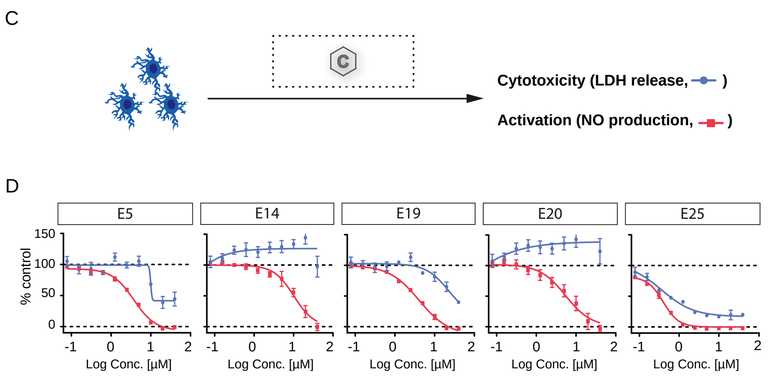



**Figure 3: Involvement of microglia**

(A) Experimental design to assess the necessity of microglia for neuroprotection by hit compounds. Hits that depend on modulation of microglia for neuroprotection are expected to only rescue motor neurons from activated microglia (green line), but not from DetaNO, a chemical nitric oxide (NO) donor (orange line). The astrocyte-motor neuron co-culture was treated for 30 hours with hit compound plus either activated microglia or DetaNO.

(B) Motor neuron survival was evaluated for the indicated hit compounds in the presence of either activated microglia (green line) or with DetaNO (orange line). Hits E5, E14, E19, E20, and E25 all rescue motor neurons from degeneration by acting on microglia.




(C) Hit compounds acting on microglia could either reduce the activation and thus the NO production by microglial cells or selectively kill the microglia. To test this, activated microglia were incubated with hit compounds for 30 hours and tested for NO production and cytotoxicity (LDH release).

(D) Dose-response curves for the indicated hits on microglia for either the production of NO, indicating activation (red line), or the release of LDH, indicating cytotoxicity (blue line). Hit compounds E5, E14, E19, and E20 are neuroprotective by inhibiting the production of toxic mediators (NO) by microglial cells whereas E25 is selectively cytotoxic to activated microglial cells.

All data are shown as mean ± SEM. Significance levels were determined using a two-tailed Student's t test. NS, not significant; p > 0.05; * : 0.05 > p > 0.01; ** : 0.01 > p > 0.001; *** : p > 0.001

### Neuroprotection via Nrf2 activation

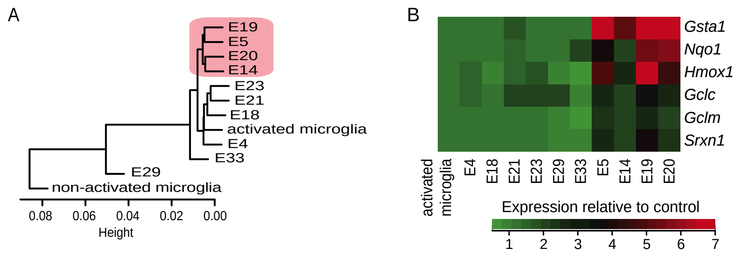



**Figure 4: Hit compounds stimulate Nrf2 target gene expression.**

(A) Hierarchical clustering of whole-genome expression data from non-activated and activated microglia as well as activated microglia treated with individual hit compounds for 4 hours. The cluster of compounds E5, E14, E19, and E20 is highlighted (red box).

(B) Heat map from microarray data demonstrating upregulation of Nrf2 target genes by the hit compound cluster of E5, E14, E19, and E20. The color bar indicates gene expression normalized to activated microglia control.

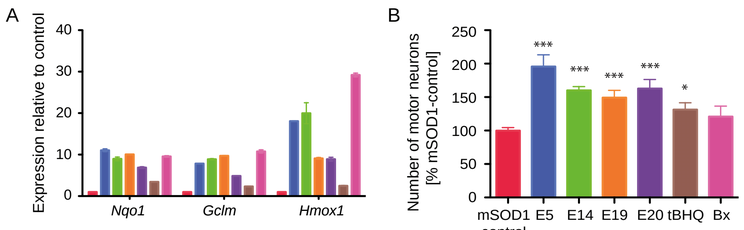



**Figure 5: Hit compounds rescue motor neurons from mSOD1-Astrocytes-mediated toxicity**

The SOD1 G93A mutation (mSOD1) causes ALS in mice and human and mSOD1 astrocytes have been shown to be neurotoxic.

(A) Nrf2 target gene expression in SOD1 G93A primary astrocytes after 24 hours treatment with the indicated compounds. Expression levels are normalized to those of untreated controls.

(B) Co-culture of motor neurons with primary astrocytes expressing mSOD1 in the presence of the indicated compound. The number of motor neurons after 3 days was quantified relative to that of untreated cultures. tBHQ and bx (Bardoxolon) are two known Nrf2 activators.

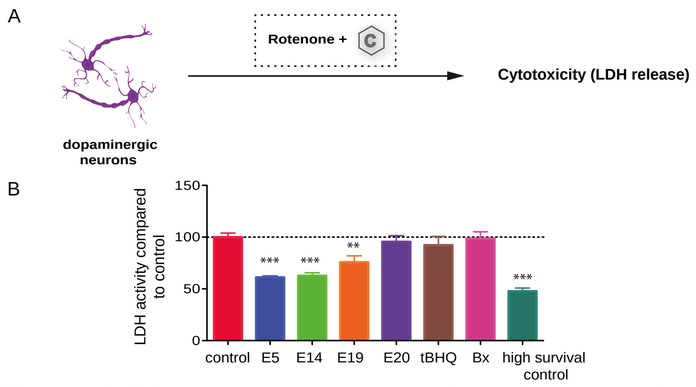
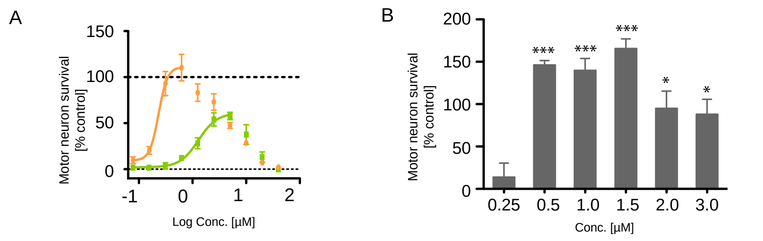



**Figure 6: Direct neuroprotection from Parkinsonian degeneration.**

(A) Experimental design to access the efficacy of hit compounds in a model of parkinsonian degeneration: human dopaminergic neurons were stressed with rotenone and cell death was evaluated by LDH release.

(B) LDH activity was analyzed for the indicated compounds in the presence of rotenone. Control: LDH release in the presence of rotenone with no compound treatment; high survival control: LDH release of unstressed dopaminergic neurons.

### Direct neuroprotection



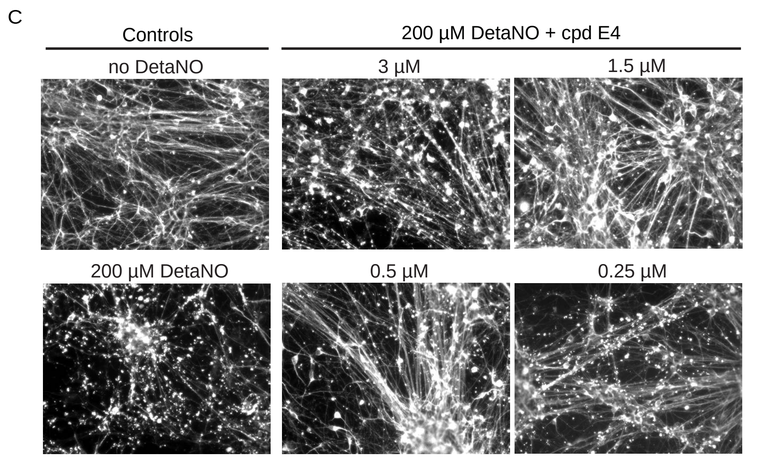




**Figure 7: Direct neuroprotection from NO-induced cell death by compound E4**

(A) Motor neuron survival was evaluated for the hit compound E4 in the presence of either activated microglia (green line) or with DetaNO (orange line). The experimental design is shown in Figure 3A.

(B) Quantification of human neuroprotection.

(C) Validation of direct neuroprotection by compound E4 with human neurons.

## Summary

- Stem-cell-based phenotypic assay
- Suitable for medium throughput screening
- Discovery of small molecules acting in multiple and distinct pathways
- 4 hit classes
  - Nrf2 pathway activation
  - Protection from NO-induced death
  - Inhibition of microglial activation
  - Modulation of astrocytic activation
- Confirmation on human neurons
- Validation in ALS and PD disease models
- In-silico predicted to cross the blood-brain-barrier
- Ideal starting point for the development of new drugs for various neurodegenerative diseases

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- This work has been published: Höing et al., Cell Stem Cell. 2012 Nov 2;11(5):620-32. doi:10.1016/j.stem.2012.07.005.