Enhancing neurite outgrowth: Use of 96-well electroporation to screen regeneration associated genes

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Introduction

- Postnatal and adult CNS neurons show limited axonal regeneration following injury**.** This is due to a combination of factors including a reduced intrinsic growth state and the presence of growth inhibitory factors such as chondroitin sulphate proteoglycans (CSPG) and myelin associated glycoprotein (MAG) in the extrinsic environment.
- 518 novel regeneration-associated genes were identified. Their expression levels were increased in spinal neurons when they regenerated an axon into a Schwann cell graft implanted after transection of the adult rat thoracic spinal cord.
- Electroporation can be used as a method to transfect cells. It uses an electric field to surpass the capacitance of the cell membrane resulting in the formation of reversible pores (Neumann et al, 1999). The area and degree of membrane permeabilisation can be controlled by varying the electroporation settings (Gabriel et al, 1997).
- We adapted a 96-well electroporation assay (Buchser *et al*., 2006) to transfect CNS neurons and over-express novel regeneration-associated genes.

Aim

Our aim is to enhance the intrinsic growth state of CNS neurons, enabling them overcome an growth-inhibitory substrate.

Methods

- Regeneration-associated genes were purchased pre-cloned into pCMVSPORT6.
- Cerebellar granule neurons (CGNs) were dissociated from P7-9 rat pups.
- 250,000 cells/well were co-electroporated with two DNA plasmids (pCMVSPORT6 and pmaxGFP) at a ratio of 4µg : 1µg (regeneration-associated gene : reporter GFP).
- 35,000 cells/well were plated onto either a non-inhibitory substrate (PLL) or one of two inhibitory substrates; either a confluent layer of MAG expressing CHO cells (CHO-MAG) or a Laminin/CSPG substrate.
- After 48 hours the cultures were fixed, stained for βIII tubulin, Imaged and analysed.
- The neurite length per transfected neuron was measured using the In-Cell Analyser 1000.

Results

Typical field of view

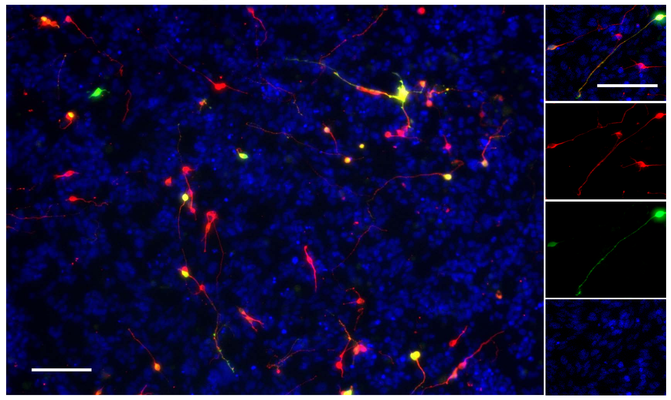

Figure.1: CGNs electroporated with pmaxGFP, cultured on CHO-R2 cells. Green: GFP. Blue: DAPI. Red: βIII tubulin. Scale bars: 100 µm.

Optimal CGN electroporation parameters give a transfection efficiency of ~30%

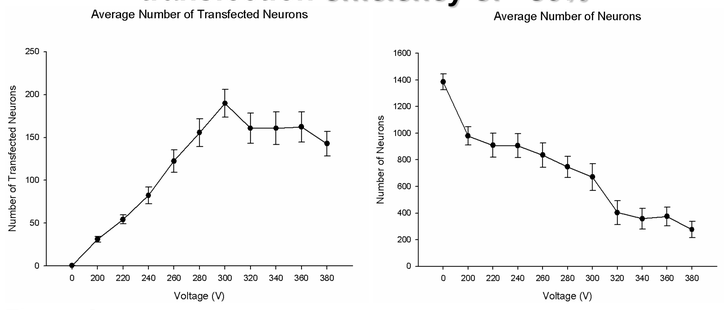

Figure.2: Voltage was varied from 200-380V. The number of transfected neurons increased with voltage up to 300V. Neuronal viability decreased with voltage. (Mean and SEM, n=8).

Optimised and confirmed gene over-expression

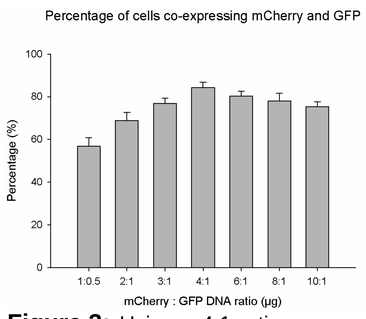

Figure.3: Using a 4:1 ratio we observed 84% co-expression of mCherry and GFP. (Mean and SEM, n=8).

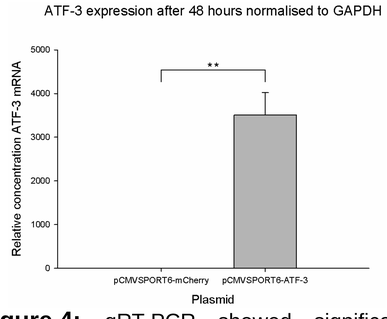

Figure.4: qRT-PCR showed significantly higher levels (2700 fold) of ATF-3 in cells transfected with the ATF-3 plasmid. (Independent samples t-test, **: P<0.01, n=4).

Screening identified a gene that increases neurite outgrowth

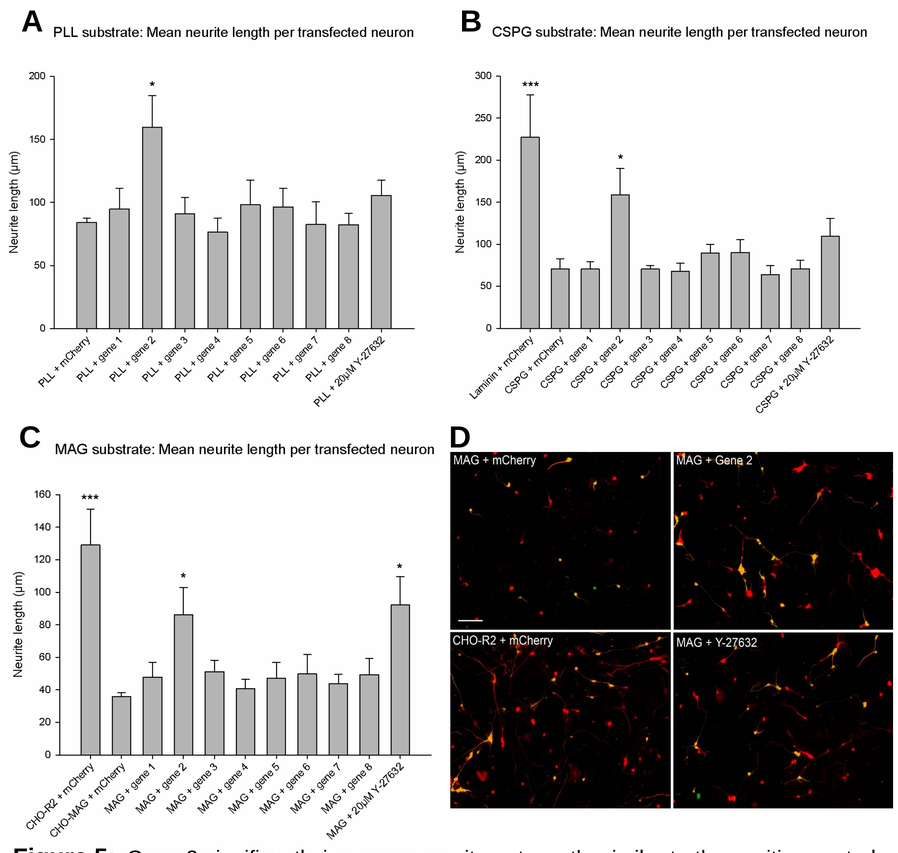

Figure.5: Gene 2 significantly increases neurite outgrowth, similar to the positive control Y-27632 in comparison to a control plasmid (mCherry) on an non-inhibitory PLL (A) and both the growth-inhibitory CSPG (B) and MAG (C) substrates. Electroporated CGNs (green) stained for βIII tubulin (red) overcoming MAG inhibition, scale bar: 100µm (D). (One-way ANOVA, Dunnett's post hoc, *** : P<0.0001, * : P<0.05, n=4).

Conclusions

We have established a medium-throughput electroporation assay, which allows us to investigate the potential of genetic targets to increase neurite outgrowth. Using this we have discovered a novel genetic target (gene 2) which when over-expressed increases CGN neurite outgrowth on both non-inhibitory and inhibitory MAG and CSPG substrates.

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