Deana protocol testing on cultured human motoneurons *in vitro*

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Introduction

- Glutamate excitotoxicity is a well established phenomenon contributing to ALS pathogenesis
- Thought to cause Ca^{2+} misregulation, leading to stress in the mitochondria
- This in turn upregulates ROS production causing cell damage
- Can trigger activation of the apoptotic cascade, leading to cell death
- Neurofilament malformations known to occur in ALS
- Certain mutations in NF have been found in ALS patients
- Malformations in NF in response to glutamate treatment has been shown previously in rat cells and tissue
- Suggested as a possible mechanism for explaining pathological progression of ALS

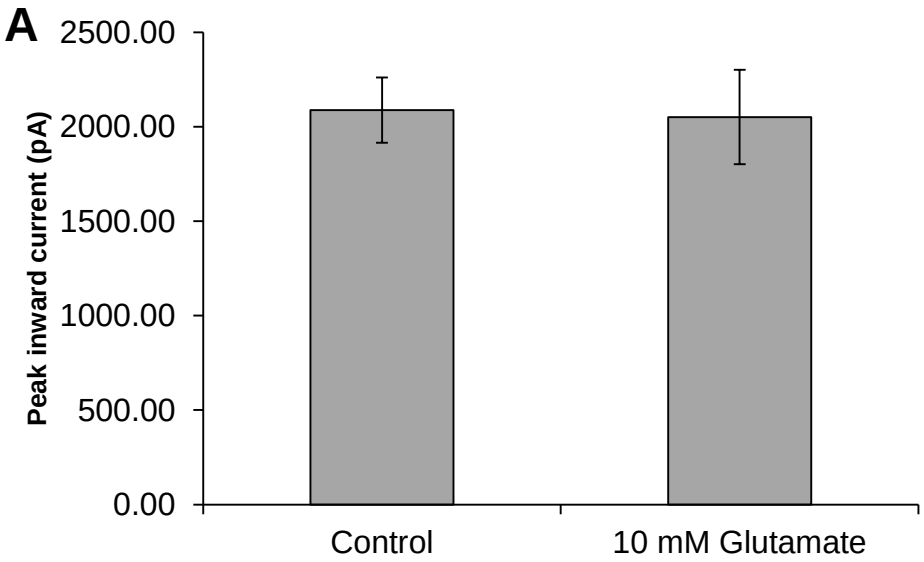
Experimental design

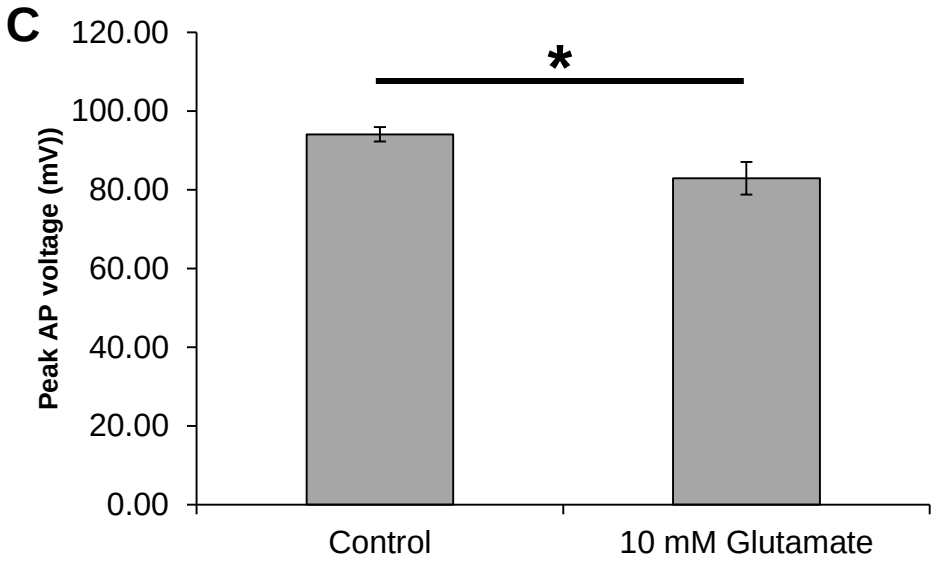
- Human fetal stem cell-derived motoneurons plated on coverslips and maintained *in vitro* for 40 – 45 days

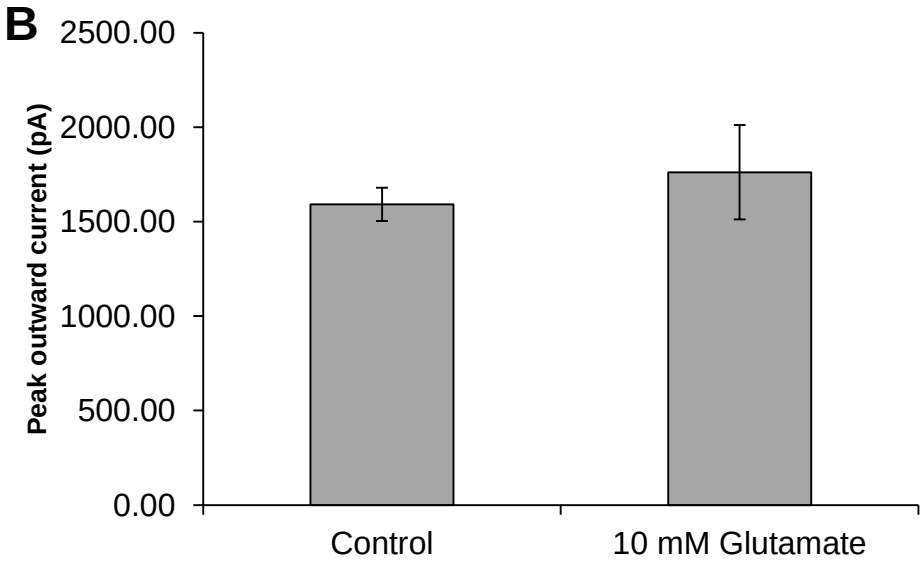
- Cultures were then treated with 10 mM glutamate for 48 hours
  - Treatment dosage was determined based on data from relevant literature and preliminary investigations

- Neuronal functionality and morphology was then assessed using patch clamp electrophysiology, time-lapse microscopy and immunostaining

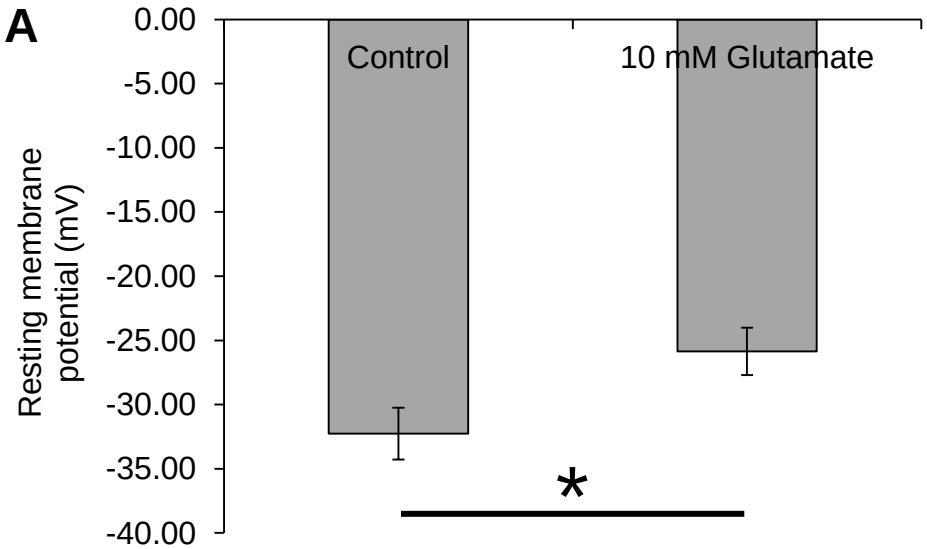
- Human fetal stem cell-derived motoneurons treated with glutamate (10mM) were used to evaluate the benefits of the Deana protocol.

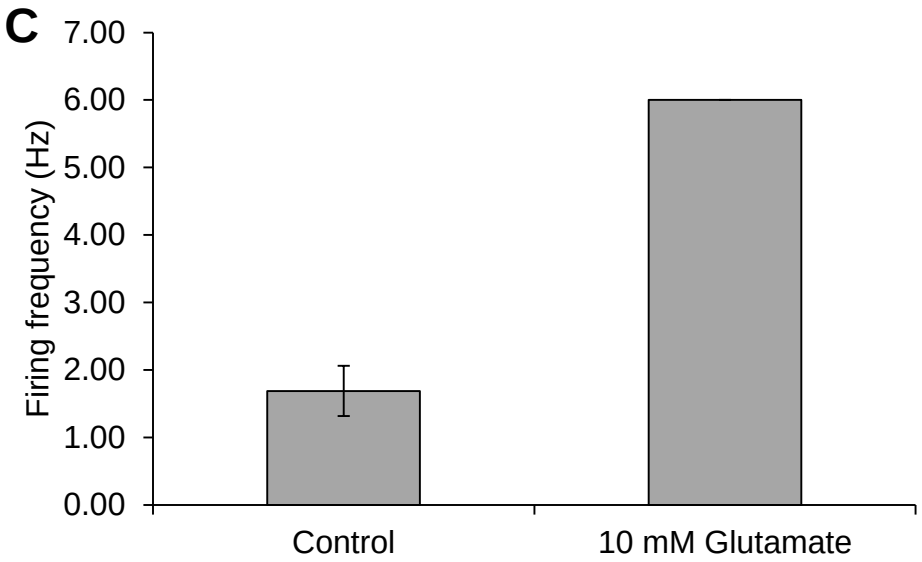


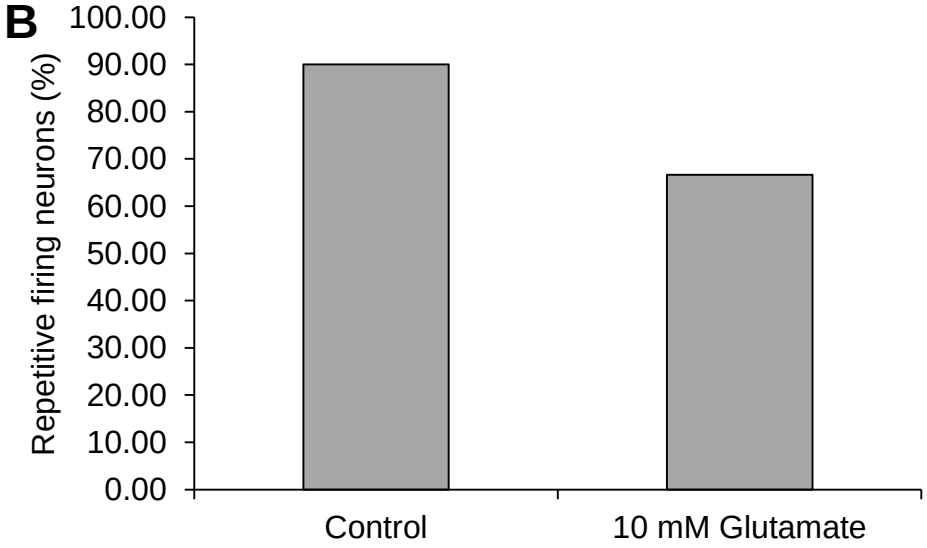




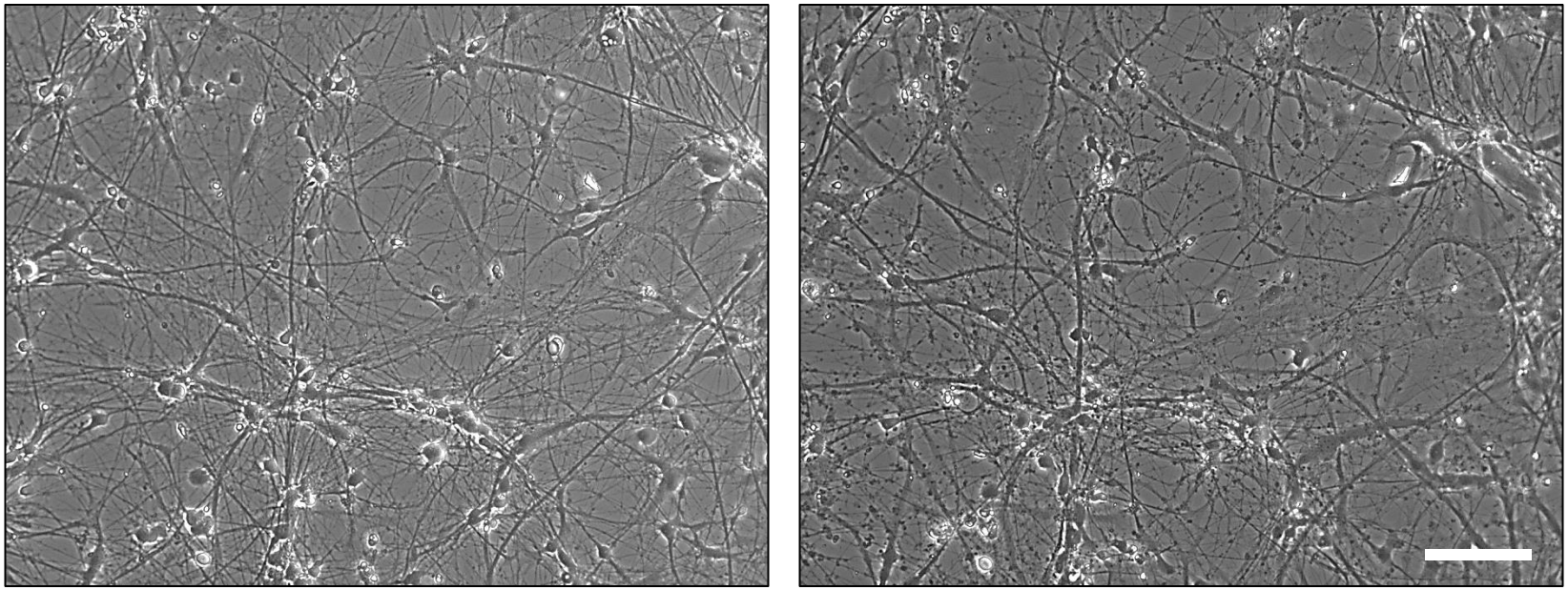
Electrophysiological properties of cultured human neurons in response to glutamate treatment. A. Peak inward sodium currents recorded from firing neurons. **B.** Peak outward rectifying potassium currents recorded from firing neurons. **C.** Peak action potential voltage recorded from firing neurons, *P = 0.03. n = 19 (control) and 13 (10 mM glutamate) from 4 separate cultures. Data represents the mean ± s.e.m.



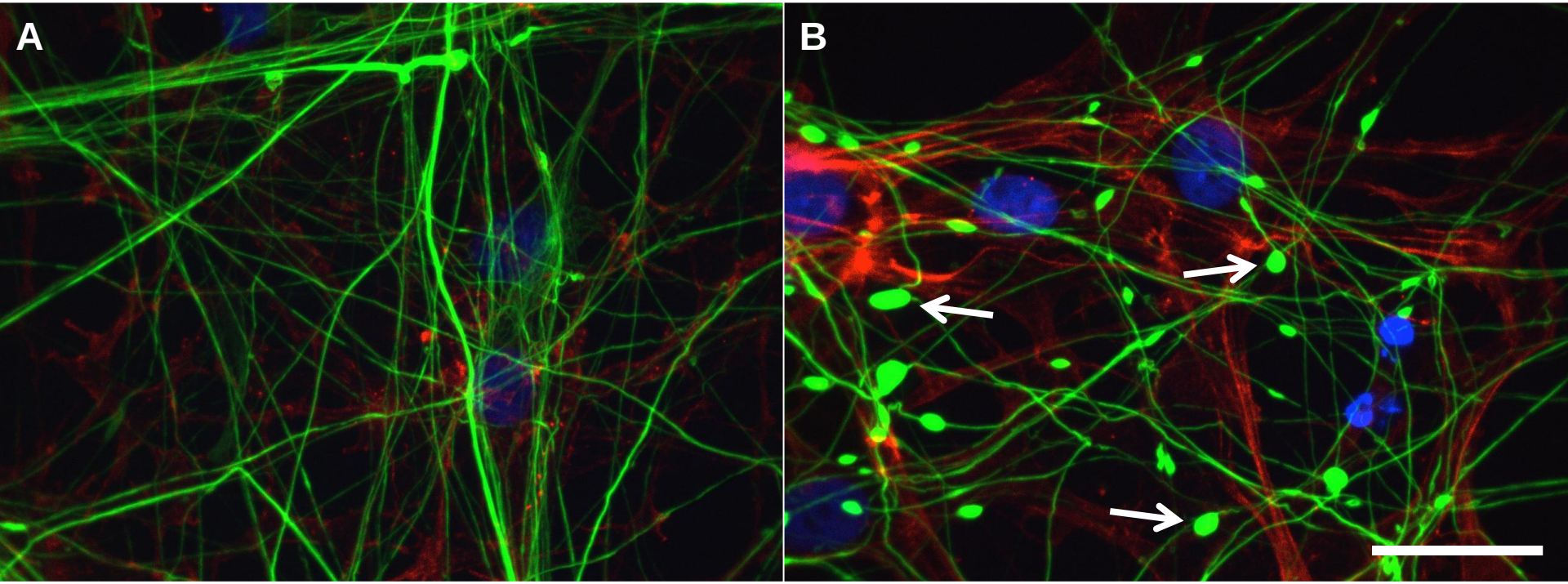




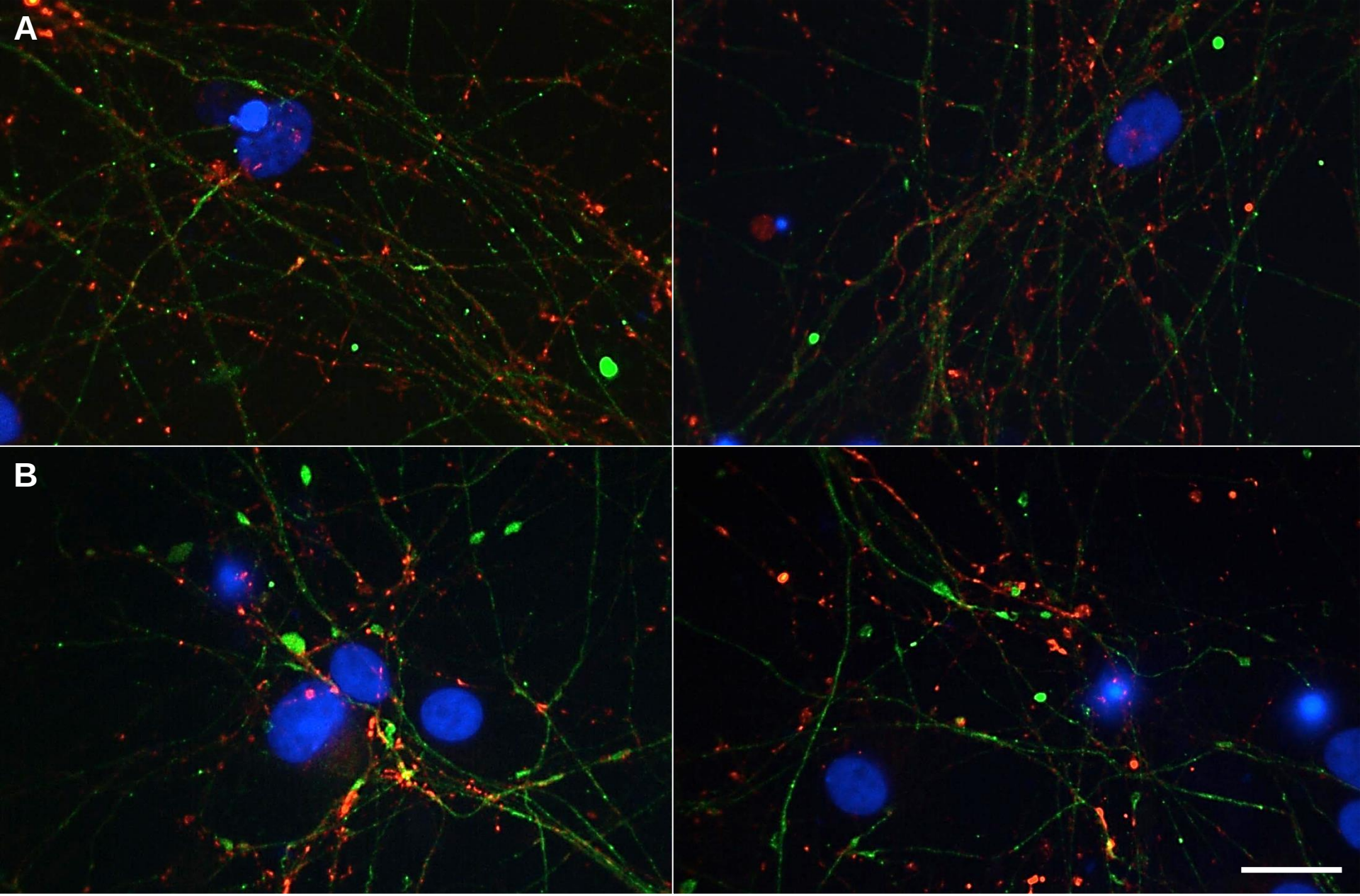
Electrophysiological properties of cultured human neurons in response to glutamate treatment. A. Resting membrane potentials recorded from patched neurons, *P = 0.03. **B.** Percentage of patched neurons displaying repetitive firing behavior. **C.** Spontaneous firing frequency in patched neurons. Note, despite a higher firing frequency, the number of spontaneously active neurons in glutamate treated cultures is substantially lower. n = 19 (control) and 13 (10 mM glutamate) from 4 separate cultures. Data represents the mean ± s.e.m.



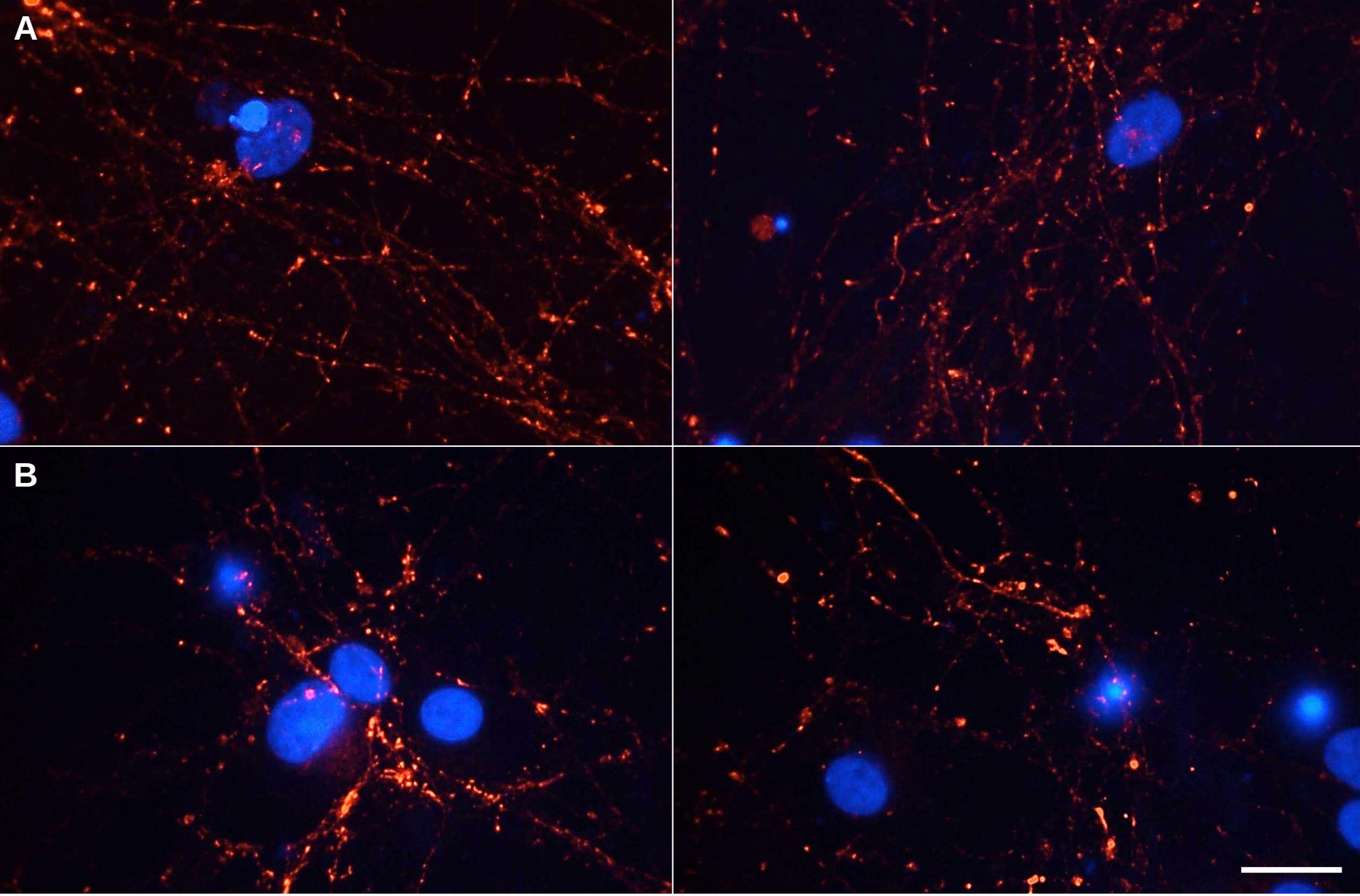
Assessment of morphological changes in response to glutamate treatment. Time-lapse microscopy was used to illustrate the negative effect of glutamate on neuritic morphology and development. Note the increase in axonal varicosities and the trend towards a reduction in axon density between **A.** Time = 0 hours, and **B.** Time = 48 hours. n = 3, scale bar = 60 µm.



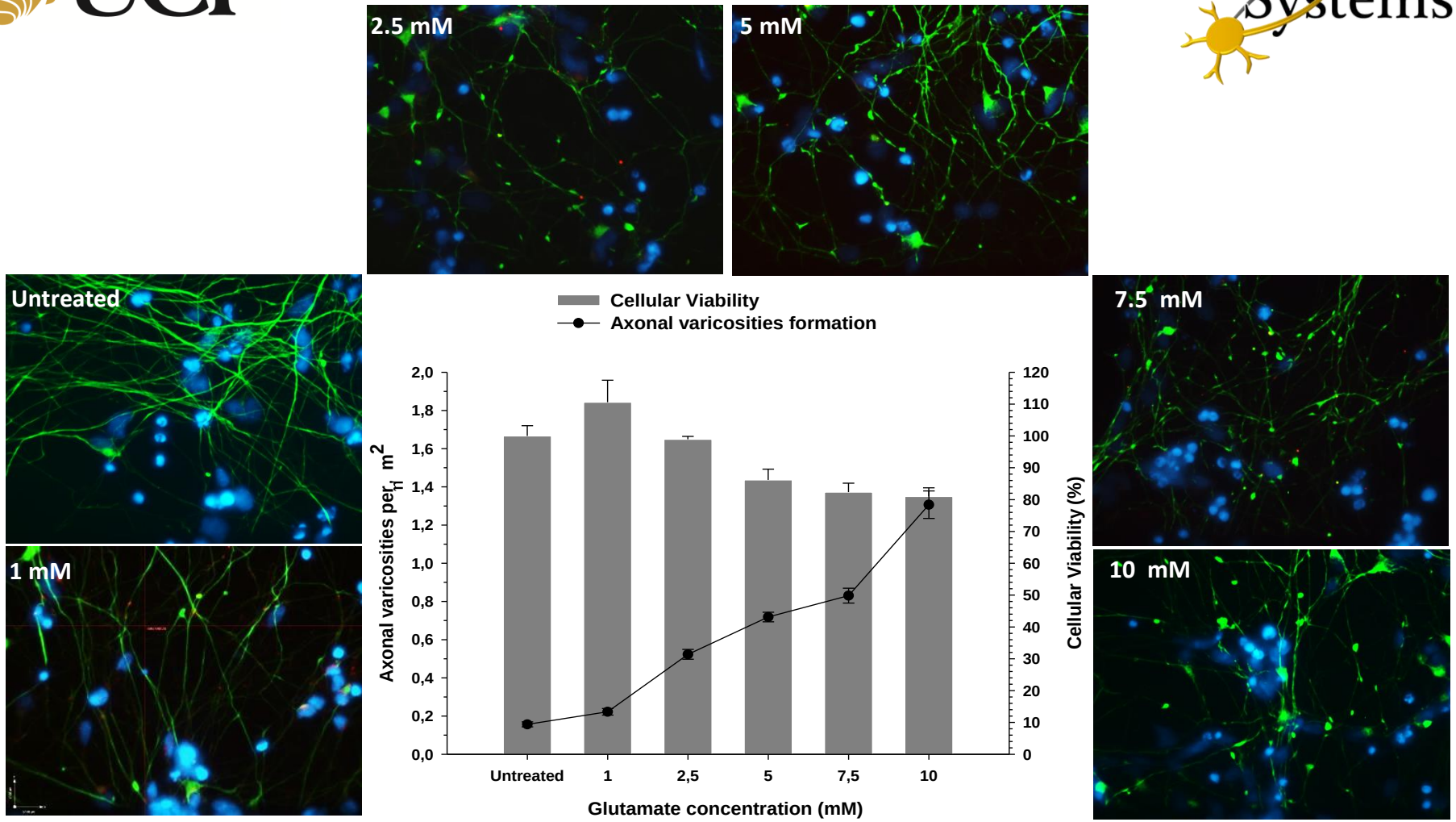
Axonal varicosities form in response to glutamate treatment. Human motoneurons after 40 DIV in culture and stained for neurofilament (green), actin (red) and nuclei (blue). **A**. Control. **B**. + 10 mM glutamate. Note the formation of morphologically aberrant varicosities in the treated condition (white arrows). Scale bar = 30 µm.



Human motoneurons after 40 DIV immunostained for neurofilament (green), SV2 (red) and nuclei (blue). **A.** Control. **B.** 10 mM glutamate for 48 hours. Scale bar = 20 µm.



Human motoneurons after 40 DIV immunostained for SV2 (red) and nuclei (blue). **A.** Control. **B.** 10 mM glutamate for 48 hours. Scale bar = 20 µm.

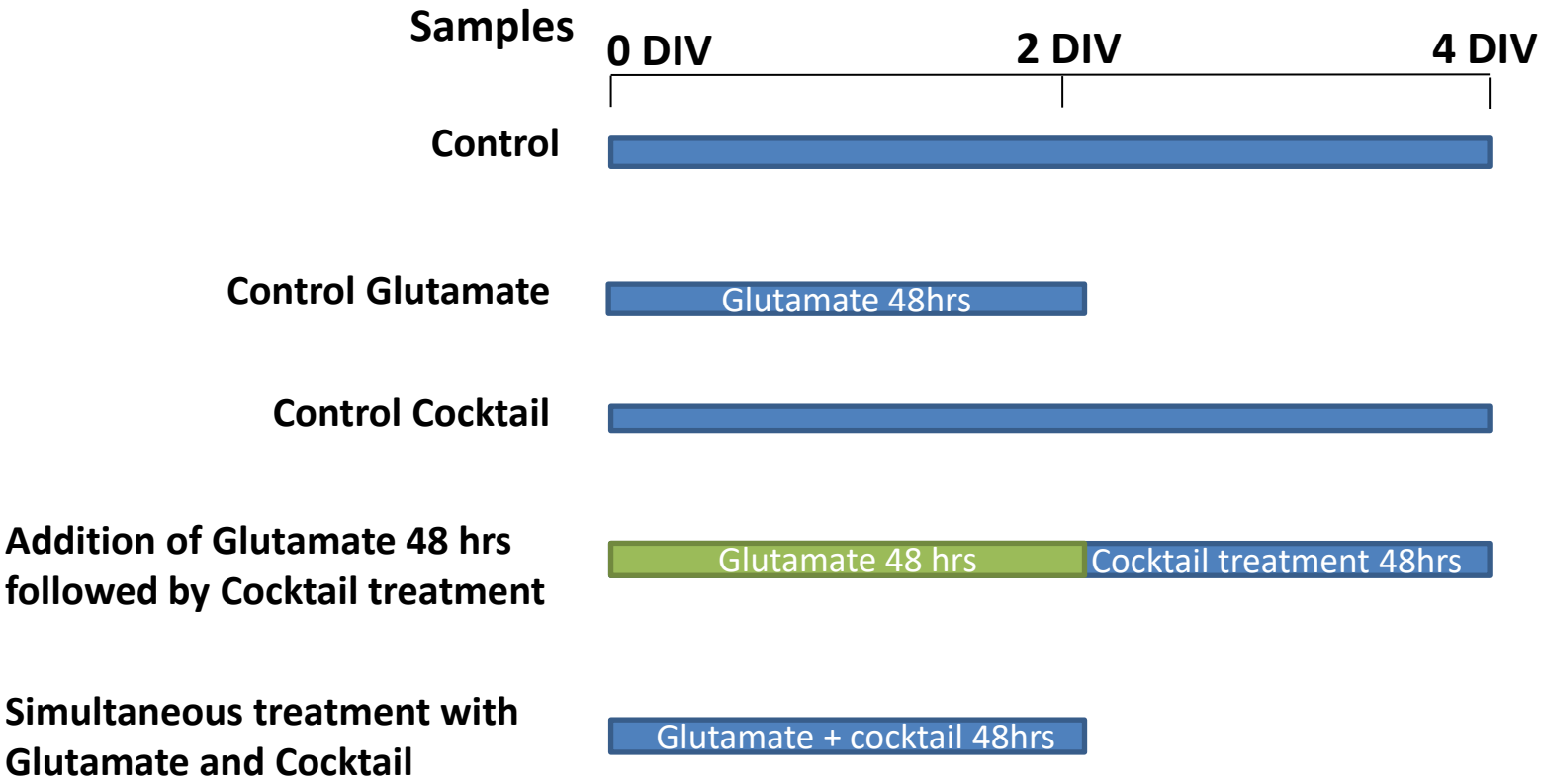


Quantification of axonal varicosity formation *in vitro* by glutamate treatment. Human motoneurons were fixed and stained for neurofilament following 40 days *in vitro* and the number of varicosities per mm2 at 40x magnification was calculated from the collected images. At least 30 images were collected from each condition. n = 3, *P < 0.0001. Data represents the mean ± s.e.m.

Testing of the Deanna Protocol

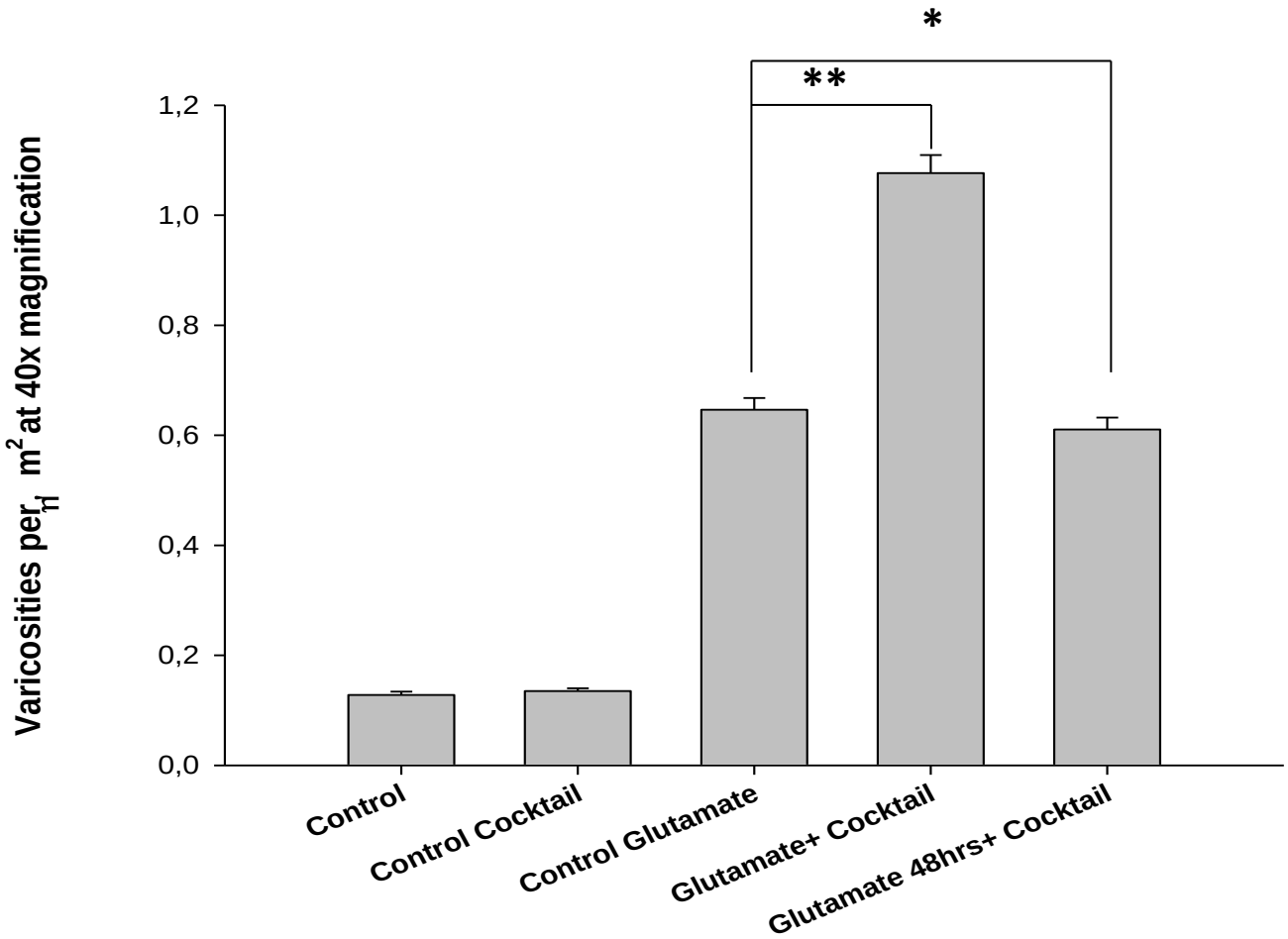
Compound	Effect
L-Arginine/ α-ketoglutarate	Boost protein metabolism/ prevent muscle breakdown
GABA	Inhibits neuronal firing
Coenzyme Q_{10}	Involved in citric acid cycle metabolism/ energy production
Nicotinic acid	Involved in citric acid cycle metabolism/ energy production
5-hydroxytryptophan	Serotonin and melatonin precursors
Glutamate oxaloacetate transaminase	Converts glutamate to glutamine

Experimental design

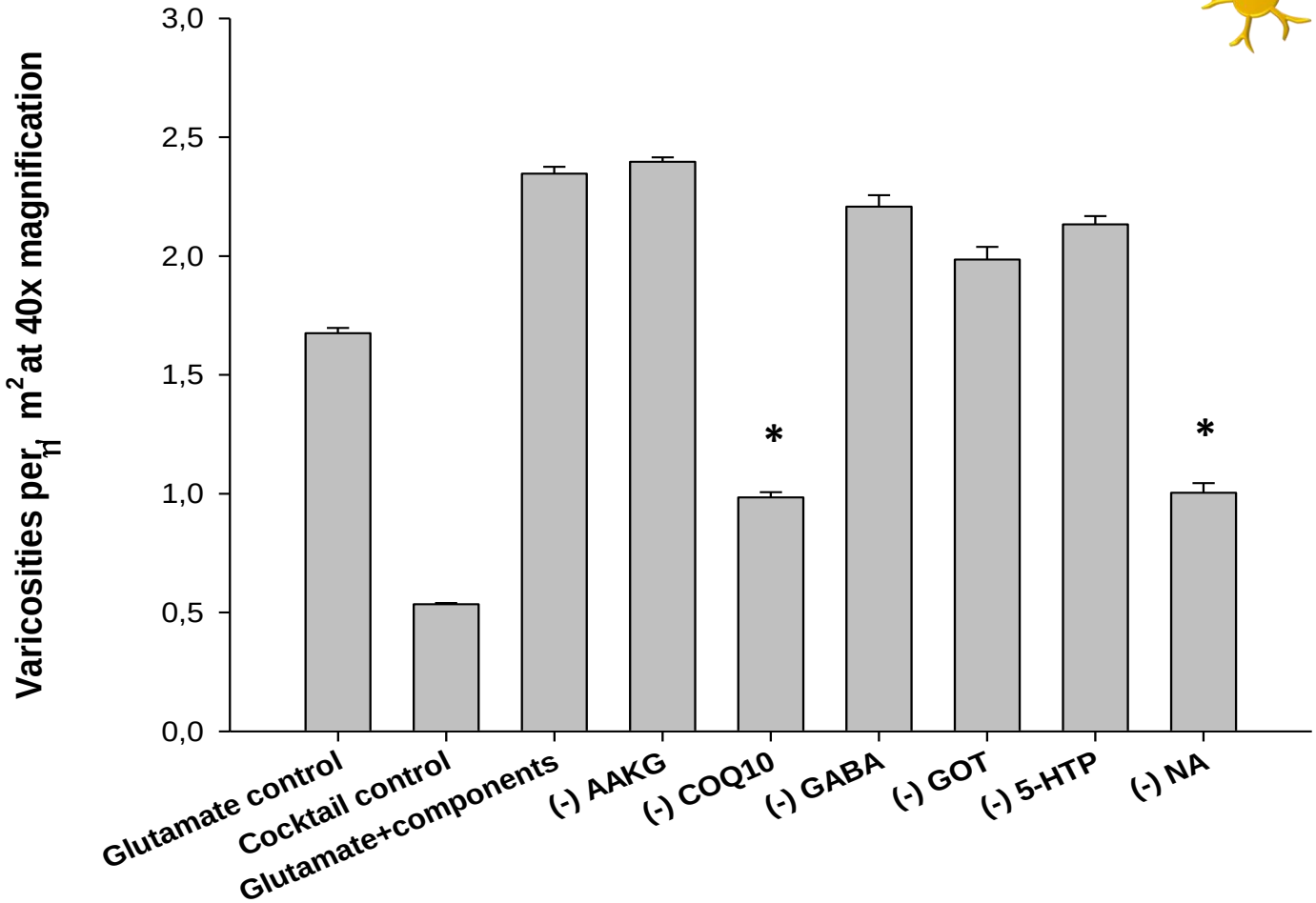


- Human motoneurons were fixed and stained for neurofilament following 40 days *in vitro* and the number of varicosities per mm^2 at 40x magnification was calculated from the collected images.

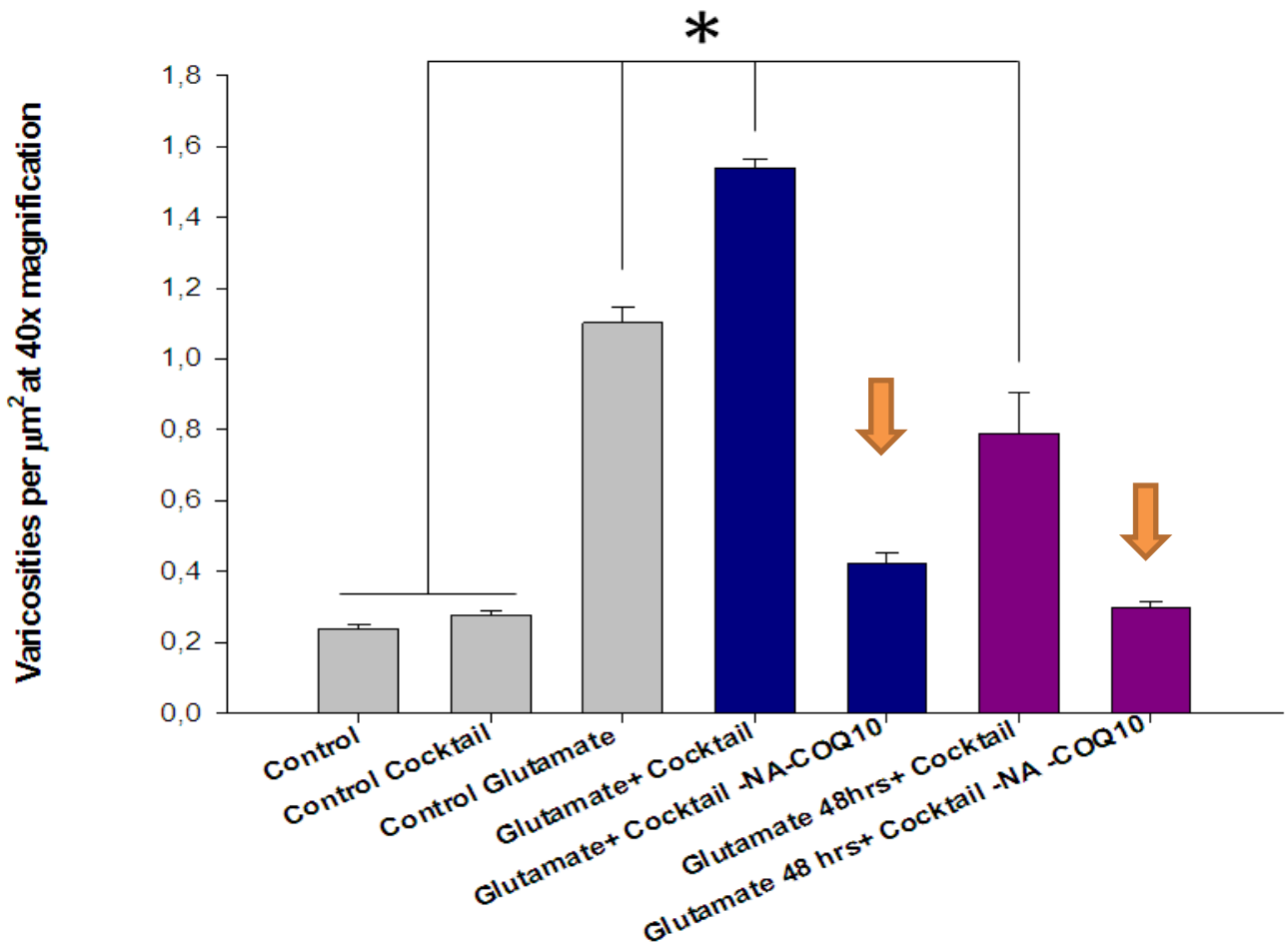
Results



Treatment formulation based on the Deana Protocol. Human motoneurons were fixed and stained for neurofilament following 40 days *in vitro* and the number of varicosities per mm2 at 40x magnification was calculated from the collected images. At least 30 images were collected from each condition. n = 3, *P < 0.0001. Data represents the mean ± s.e.m.



Individual effects of cocktail components in axonal varicosities formation . Human motoneurons were fixed and stained for neurofilament following 40 days *in vitro* and the number of varicosities per mm2 at 40x magnification was calculated from the collected images. At least 30 images were collected from each condition. n = 3, *P < 0.0001. Data represents the mean ± s.e.m.

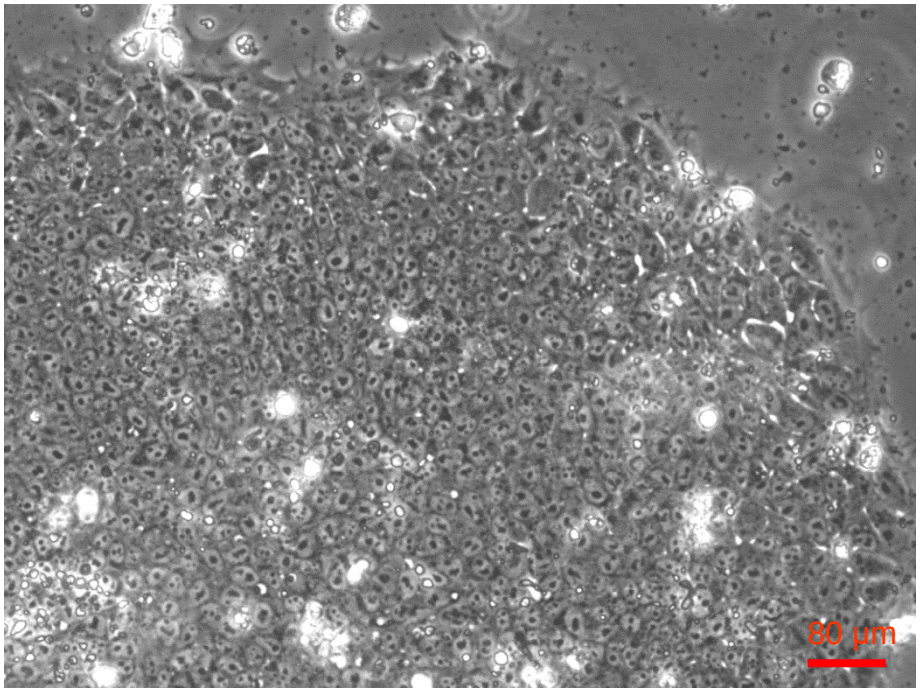


ALS adjusted Formulation E; G
L- Arginine
α-ketoglutarate
GABA
5- hydroxytryptophan
GOT

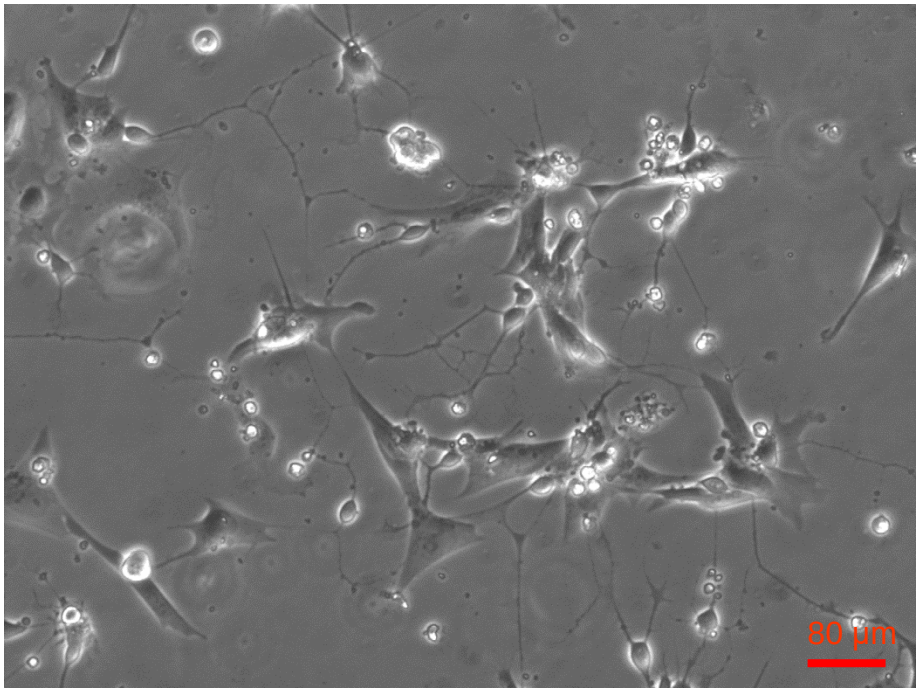
Quantification of axonal varicosities formation following treatment with optimized cocktail formulation . Human motoneurons were fixed and stained for neurofilament following 40 days *in vitro* and the number of varicosities per mm2 at 40x magnification was calculated from the collected images. At least 30 images were collected from each condition. n = 3, *P < 0.0001. Data represents the mean ± s.e.m.

Phase comparison of hiPS cell morphology before and after differentiation

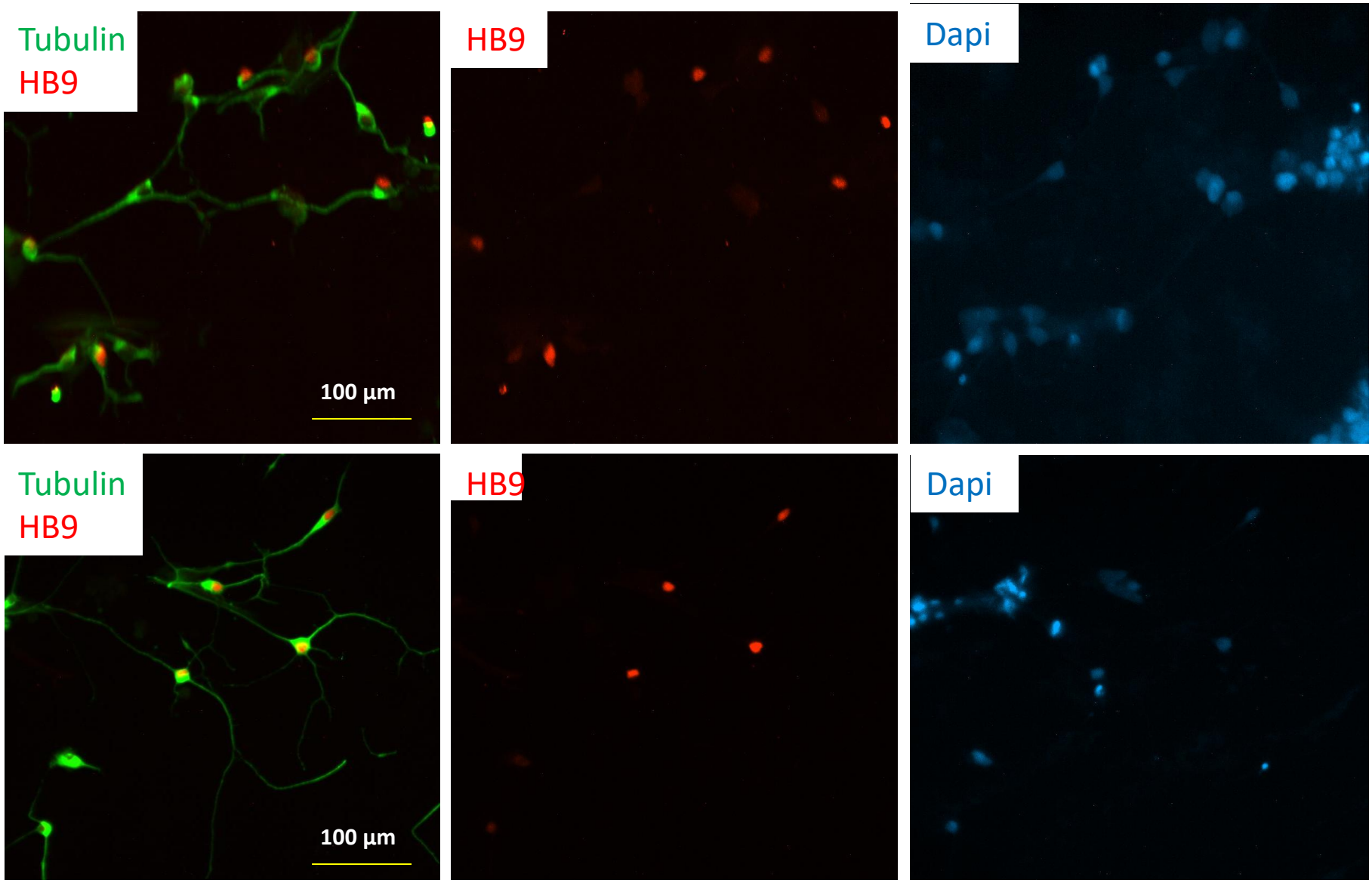
Before Differentiation



13 days After Differentiation



Human Motoneurons were induced in the differentiated culture



Conclusions

- Glutamate treatment causes some small but significant alterations in the electrophysiological function of cultured motoneurons
- More striking is the change in axonal morphology, with a clear increase in neurofilament varicosities caused by glutamate treatment
- Such structures likely inhibit axonal transport and communication between the cell body and the synaptic terminal
- A human assay was used to assess effectiveness of the Deana protocol as therapeutic treatment for ALS in vitro

Conclusions

- The original formulation did not produce alterations in glutamate treated motoneurons but produced significant alterations when performing a simultaneous treatment (glutamate + cocktail)

- The individual assessment of the components of the cocktail revealed that the effects of glutamate were exacerbated by the presence of Coenzyme Q10 and nicotinic acid.

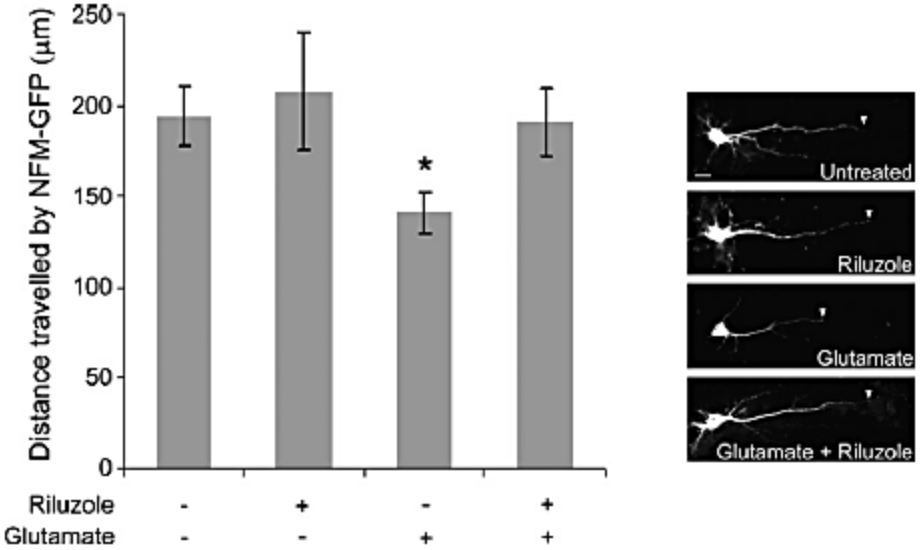
- Removal of Coenzyme Q10 and nicotinic acid from the cocktail formulation had shown a significant reduction on the number of axonal varicosities produced by glutamate treatment.

Neurosci Lett. 2009 Apr 24;454(2):161-4. doi: 10.1016/j.neulet.2009.02.061. Epub 2009 Mar 3.

Riluzole protects against glutamate-induced slowing of neurofilament axonal transport.

Stevenson A[1], Yates DM, Manser C, De Vos KJ, Vagnoni A, Leigh PN, McLoughlin DM, Miller CC.

Riluzole is the only drug approved for the treatment of ALS, but its precise mode of action is not properly understood. **Damage to axonal transport of neurofilaments is believed to be part of the pathogenic mechanism in ALS** and this has been linked to **defective glutamate handling** and increased phosphorylation of neurofilament side-arm domains. Here, we show that **riluzole protects against glutamate-induced slowing of neurofilament transport**. Protection is associated with decreased neurofilament side-arm phosphorylation and inhibition of the activities of two neurofilament kinases, ERK and p38 that are activated in ALS. Thus, the anti-glutamatergic properties of riluzole include protection against glutamate-induced changes to neurofilament phosphorylation and transport.

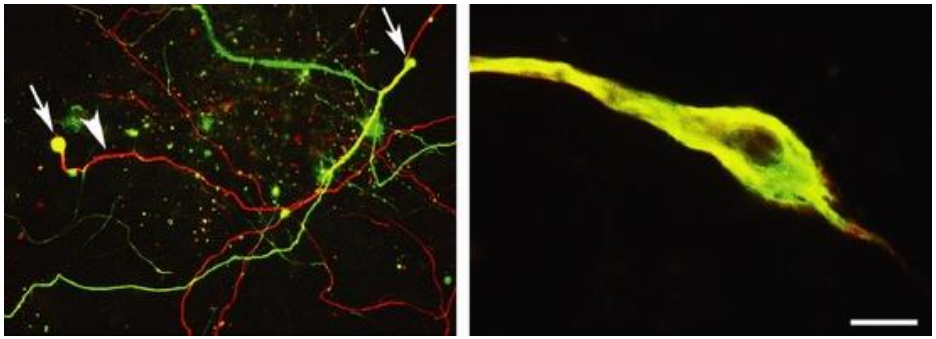


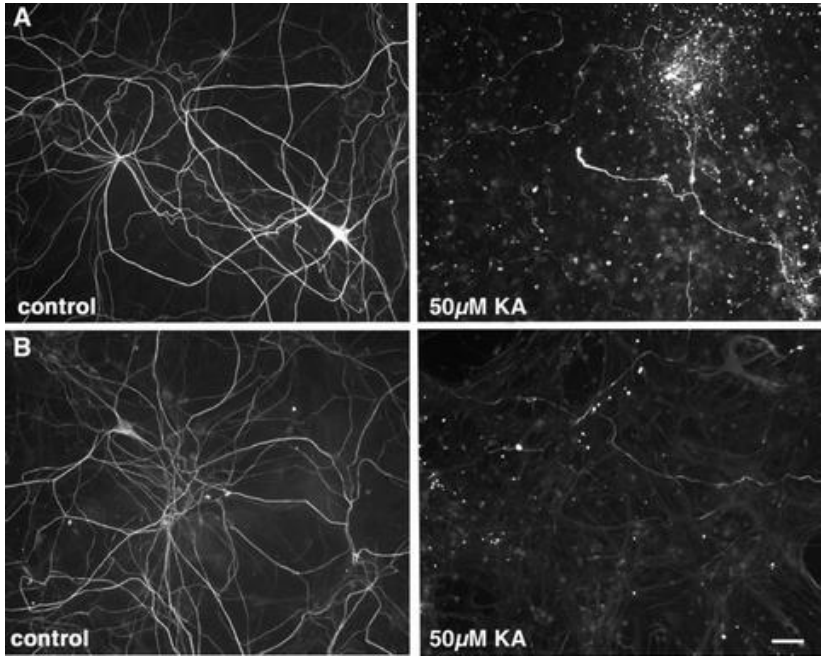
Eur J Neurosci. 2007 Oct;26(8):2151-9. Epub 2007 Oct 1.

Excitotoxicity mediated by non-NMDA receptors causes distal axonopathy in long-term cultured spinal motor neurons.

King AE[1], Dickson TC, Blizzard CA, Foster SS, Chung RS, West AK, Chuah MI, Vickers JC.

Excitotoxicity, mediated principally through non-NMDA receptors, causes an **axonopathy in spinal neurons characterized by cytoskeletal disruption and neurofilament accumulation in distal axonal segments**. This axonopathy was specific for cultured spinal motor neurons relative to cortical neurons, and was linked to the relative maturity of the spinal neurons *in vitro*. Distal axonopathy was associated with the abnormal co-localization of phosphorylated and dephosphorylated neurofilament proteins, indicating **aberrant post-translational processing in the accumulation of filaments and subsequent localized swelling**. The accumulation of neurofilaments within axons following excitotoxicity **mimics the pathological cytoskeletal changes of ALS** and provides a link between excitotoxicity via non-NMDA receptors implicated in this disorder with a related pathological hallmark feature of motor neuron degeneration.





Int J Clin Exp Pathol. 2013 Jul 15;6(8):1505-15. Print 2013.

Effect of intra-cisternal application of kainic acid on the spinal cord and locomotor activity in rats.

Mitra NK[1], Goh TE, Bala Krishnan T, Nadarajah VD, Vasavaraj AK, Soga T.

Intra-cisternal administration of kainic acid was used to study the changes in the morphology of neurons and glial cells of the spinal cord as well as functional evaluation of the movement and nociception. The locomotor activity in the parameter of observed mean action time was reduced on 14th day after administration of KA. Increased GFAP expression, **prominent neurofilament protein** and increased SOD activity in lumbar and cervical parts of the spinal cord **indicated persistent neuronal damage** even after 2nd week of the administration of KA. It can be concluded that the **spinal cord damage with some features similar to ALS can be produced by low dose intra-cisternal administration of KA.**