**Project title:** Role of microglial phagocytosis in ischemic-hypoxic neuronal loss (MIPHISNELO)

**Grant Agreement number:** 251867

**Funding Scheme:** Intra-European Fellowships (IEF); Call: FP7-PEOPLE-2009-IEF

## BACKGROUND

Brain trauma and stroke are two of the main causes of death, morbidity and disability around the globe, and their social, medical and economic burden is still increasing. Stroke is the leading cause of serious, long-term disability, and is responsible for about 10% of all deaths in industrialized countries. Brain trauma is the leading cause of death and disability worldwide among the population under the age of 45 years. Thus, these pathologies have a considerable and growing impact on health, economy and society.

Brain trauma and stroke induce inflammation, which may protect but also damage the brain via various mechanisms. Inflammation in the brain is mainly regulated by resident brain macrophages, known as microglia. Microglia are inflammatory 'activated' by either pathogens or damage via various receptors, and when activated produce pro-inflammatory cytokines, neurotoxic NO, ROS and peroxynitrite, and become highly phagocytic (i.e. capable of eating others cells and debris). Phagocytosis is generally regarded as secondary to apoptosis or necrosis (the primary causes of cell death), i.e. phagocytes are thought to only eat dead cells or cells doomed to die. However, in inflammatory conditions, phagocytosis of live neurons by activated microglia can cause neuronal death (cell death induced by phagocytosis is called 'phagoptosis'). The aim of this project was to investigate whether this form of neuronal death is important in stroke and brain trauma, and whether blocking this process is beneficial.

The specific objectives for this project were as follows:

a) To determine whether traumatic brain injury (TBI) and stroke models ± hypoxia-ischemia can induce neuronal loss without apparent cell death.
b) To test whether induced loss of neurons is mediated by microglia in these disease models.
c) To test whether neuronal loss is mediated by phagocytosis.
d) To test whether inhibition of phagocytosis blocks neuronal loss.

## METHODS

All experiments were performed on cerebellar granule neurons co-cultured with astrocytes and microglia and glia from cortex from 5-7 days postnatal rat brains. The animal lines used were Wistar rats (Harlan) and Royal College Surgeon rats (RCSd), which lack the MerTK receptor required for phagocytosis of cells and cell debris.

## MAIN RESULTS

### ***Hypoxia and hypoglycemia model***

The best hypoxia model was found to be 16 hours of hypoxia plus 4 days of reoxygenation, resulting in a neuronal loss of around 15-20% without apparent neuronal death (Figure 1A). Elimination of microglia by L-leucine-methyl-ester (LME) pre-treatment prevented this neuronal loss (Figure 1A). No prevention of neuronal loss was found by blocking several receptors: cRGD to block the vitronectin receptor (50μM), MRS each 24hrs to block P2Y6 receptor (1 μM) and Annexin V to block exposed phosphatidylserine (100nM). If 2-deoxyglucose (DOG), a glycolytic inhibitor, was also added it caused the death of almost all neurons in hypoxic conditions, whereas in normoxic conditions DOG intriguingly increased the number of neurons by approximately 20% relative to the untreated control. Moreover, DOG caused the disappearance of microglia, and elimination of microglia with LME prevented the effect of DOG. DOG prevented neuronal loss in culture models of brain trauma (Figure 1B) and Alzheimer (250nM aβ treatment).

### ***Brain trauma model***

Brain trauma was modelled by addition of homogenised neurons to neuronal-glial cultures. This induced 30% neuronal loss over 4 days without any significant neuronal necrosis or apoptosis. Elimination of microglia with LME prevented this neuronal loss, and hypoxia enhanced this neuronal loss (figure 1C). Neuronal loss was diminished in RCS dystrophic rats (RCSd), which lack the MerTK receptor. Microglia phagocytosed damaged neurons stained with fluorescent TAMRA (Figure 1D) and this was strongly decreased in microglia from RCSd rats compared to wild type rats (Wistar). No prevention of neuronal loss was found either with phagocytosis inhibitors (cRGD, MRS, AnnexinV).

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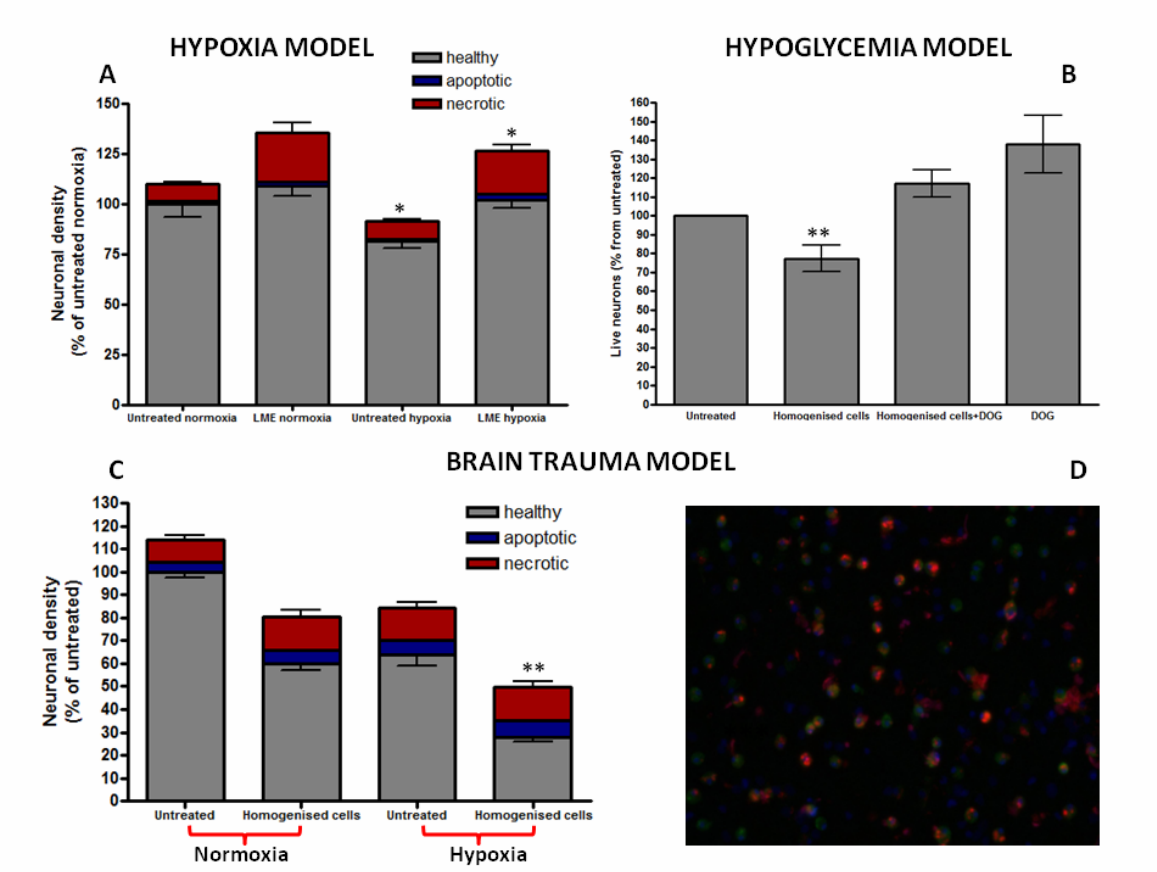


**Figure 1.** Neuronal loss (expressed as % from untreated) in A) Hypoxia model; B) Hypoglycemia model; C) Brain trauma model. D) Phagocytosis of neuronal debris stained with TAMRA (red) by microglia (IB4, green). Stainings used were propidium iodide (necrotic cells), Hoechst (apoptotic cells, condensed nuclei). Live neurons were counted from Hoechst images or phase brightfield images.

## CONCLUSIONS

Treatment of cultures with homogenised neurons is a feasible approach to study TBI *in vitro* and screen new treatments and therapies before they can be applied *in vivo*. Neuronal loss found with this model is microglia dependent and requires the microglial MerTK receptor. Thus, this receptor is a potential new target for drug development to prevent traumatic brain injury. Hypoxia elicits neuronal loss that is dependent on microglia. DOG prevents neuronal loss by killing microglia in models of brain trauma and Alzheimer, when hypoxia is not present. Thus, microglia are involved in neuronal loss induced by brain trauma and stroke, possibly due to phagocytosis of live neurons, so that microglia and phagocytosis are a promising target for therapeutic action. DOG is also a potential drug for treatment of brain trauma and neurodegenerative diseases, but not stroke.

## SOCIO-ECONOMIC IMPACT

The project has examined the roles of inflammation and phagocytosis in culture models of stroke and brain trauma. By understanding the mechanisms of neuronal death in these neurological conditions, and testing which drugs can block these processes and protect brain cells, we have got closer to clinical treatments and trials to prevent such death and disability in affected patients. New drugs can potentially be designed to target different receptors (in particular MerTK) and pathways (phagocytosis and glycolysis) involved in neuronal loss in stroke and TBI. The knowledge obtained from the project will then potentially inform drug design strategies and/or clinical treatment of some of the most important (and growing) diseases in the EU and thus contribute to the health of EU citizens. The culture models of disease developed may also reduce the use of live animals for medical and drug research.