Tolerance to Oxygen Glucose Deprivation in Differentiated Adult Hippocampal Neuronal Stem Cells Obtained from Arctic Ground Squirrels (*Spermophilus parryii*)

K.L. Drew, M.S. Wells[1], S.L. Christian, and J.A. Kelleher-Andersson[1]. Univ. of Alaska Fairbanks, Alaska Basic Neuroscience Program, Fairbanks, AK; [1]Neuronascent, Inc., Clarksville, MD.

Introduction

Arctic ground squirrels (AGS), a hibernating species, tolerate global cerebral ischemia *in vivo* and oxygen glucose deprivation *in vitro* without neuronal cell death (Dave et al., 2006; Ross et al., 2006). To test the hypothesis that genetic factors contribute to ischemia tolerance we minimized the influence of environmental factors by using hippocampal neurons differentiated from neural stem cells obtained from adult AGS. We report that these neurons are less susceptible to ischemic-like insult than other neural stem cell species.

Materials and Methods

Arctic ground squirrels (AGS; *Spermophilus parryii*) were wild trapped, transported and housed at 2ºC; 4:20 L:D at UAF. NSC were obtained from an AGS > 2 years of age that had hibernated normally and was euthanized 36 days after final arousal at end of the hibernation season with a body temperature of 37ºC.

Adult hippocampal NSCs were obtained similar to methods described previously [1] . The cells were then grown in the presence of DMEM/F12 +B27 + bFGF for a number of passages to expand the cells.

AGS Neurons were prepared from adult hippocampal NSCs (now available through Lifeline Cell Technology, MD). NSCs were thawed and seeded onto a T-75 flask treated with poly-L-ornithine. Cells were allowed to proliferate using a DMEM/F12 growth media in the presence of bFGF (10ng/ml) till approximately 75% confluency. The cells were then passaged and seeded onto 96-well plates (Biocoat, poly-L-Lysine coated, Becton Dickenson) at a low density (10,000-20,000 cells/well). The cells were grown in differentiation media (-bFGF) for four days before switching to Neurobasal™ neuron maintenance media. The neurons were used in experiments only when they reached a minimum of 14 DIV.

Culture grown in DMEM/F12 +B27+ bFGFis >95% NSC (Nestin positive)

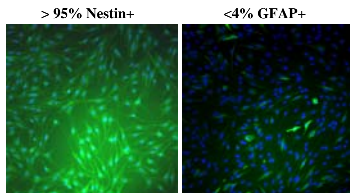

Fig. 1. Adult Hippocampal AGS Neural Stem Cells (NSC) grown for less than a week at late passage. Analyses of numerous fields per well of AGS Neural stem cells using the Cellomics Arrayscan and software show that the culture is > 90% Nestin+ and < 5% GFAP+.

Modeled Ischemia AGS neurons were differentiated from AGS NSCs for a period of 2-3 weeks. 80% of the media was changed from DMEM (25mM glucose) to DMEM (5.5mM glucose) (Invitrogen) and plates were placed in normoxia (a non-sealed Billups-Rothenberg chamber in an incubator at 37ºC and 95% air/5% CO_2) or hypoxia (a sealed Billups-Rothenberg chamber flushed with 95% N_2/5% CO_2 until O_2 in the chamber was below 0.7% (vs 20% O_2 for normoxia). In one experiment (Fig. 3) oxygen, but not glucose was re-introduced as modeled reperfusion. In another experiment (Fig. 4) glucose was added back to the DMEM low glucose to reach 25mM. Following the hypoxic/normoxic period the plates were removed from their respective chambers and placed in an incubator at 37oC and 5% CO2 for 24 hrs. Following this modeled reperfusion period cells were assessed for respiration and neuron number. Human neural stem cells (Clonexpress, MD) were treated in a similar manner and compared to the AGS cells for tolerance to 48hrs OGD + 24hrs oxygen reperfusion.

Neurons differentiated from adult hippocampal AGS NSC

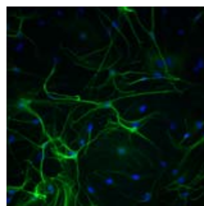

Fig. 2. Cells differentiated for two weeks in the absence of mitogens and maintained in Neuro-basal™ media were stained for neuron specific βIII tubulin (TUJ1, Covance). More than 70% of cells were neurons.

AGS neurons tolerate 48h of modeled ischemia and 24h of modeled reperfusion better than human neurons

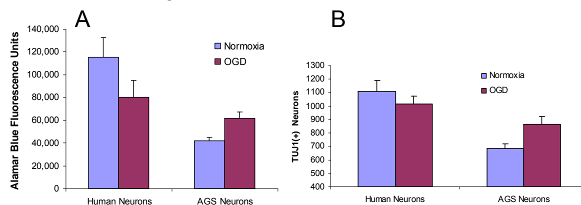

Fig. 3. Cellular respiration monitored with alamar blue (A) and neuron counts (B) after modeled ischemia in neurons differentiated from human neuronal precursor cells and adult hippocampal AGS neural stem cells. Cells were treated with DMEM low glucose for 48hrs in an unsealed chamber exposed to 95% air / 5% CO_2, the normoxic condition and in a sealed chamber with 95%N_2 / 5%CO_2 the hypoxia condition. Both the normoxic and hypoxic plates were removed to an incubator for another 24hrs (oxygen reperfusion) in 95%air / 5%CO_2.

(A) Alamar blue was added to each well for 2hrs and then half of the media removed to another 96-well plate for analysis of respiration. Each bar represents the mean ± SD of 15 wells.

(B) Cells were fixed and stained with antiTUJ1 and Hoechst dye. The values are the average number of TUJ1-positive neurons per treatment condition ± SD.

Alamar Blue™ (Biosource) was used to determine respiration over a two hour time period and this measure was directly related to cell number. Following treatment, 20µl of Alamar Blue solution was added to 200ul of media in each well of the 96 well plate. Following 2hrs 37ºC and 95% air/5% CO_2 and in the presence of Alamar Blue, respiration was determined in the cells by removing 100ul of media into an empty 96-well plate to measure the increase of a reduced fluorescent product using a Fluorescent plate reader. **Neuronal numbers** were determined using the Cellomics Arrayscan II instrument and Neuronal Profiling software. Cells in 96-well plates following Alamar Blue assay were fixed for 20min with 4% paraformaldehyde. The cells were rinsed numerous times with PBS (Ca, Mg free), the cells were blocked with Triton X-100 containing blocking buffer and then β-tubulin polyclonal antibody TUJ1 (Covance Cat# PRB-435P) was added to each well overnight at a 1/2000 dilution. The cells were again rinsed several times with PBS then secondary antibody (Alexa 546 goat anti-rabbit IgG (H+L) (Molecular Probes, Cat #A11010)) was added along with Hoechst dye 33342 (Molecular Probes Cat.# H3570). The plates were then measured using the neuronal profiling software of the Cellomics Arrayscan instrument to assess total number of cells within each field (Hoechst positive cells) and number of neurons within each field with a neuronal process (TUJ1-positive and Hoechst-positive cells). These determinations provide exact neuronal survival as opposed to Alamar Blue, which is a measure of average respiration across all cells in the well.

AGS neurons tolerate 72h of modeled ischemia and 24h of modeled reperfusion

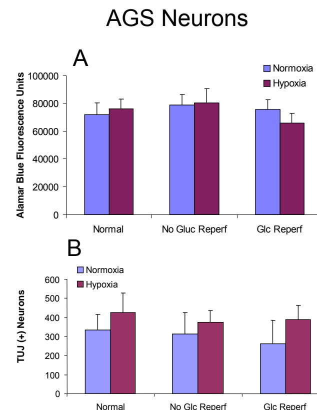

Fig. 4. Cellular respiration monitored with alamar blue (A) and neuron counts (B) after modeled ischemia in neurons differentiated from adult hippocampal AGS neural stem cells. Cells were treated with DMEM low glucose for 72hrs as in Fig. 3 except that a 3rd group was added where glucose was re-introduced with O_2 for 24hrs of modeled reperfusion

(A) Alamar blue was added to each well for 2hrs and media analyzed as index of respiration. Each bar represents the mean ± SD of 15 wells.

(B) Cells were fixed and stained with antiTUJ1 and Hoechst dye. The values are the average number of TUJ1-positive neurons per treatment condition ± SD.

Tolerance to modeled ischemia is upstream to antioxidant defense since AGS neurons are susceptible to H_2O_2

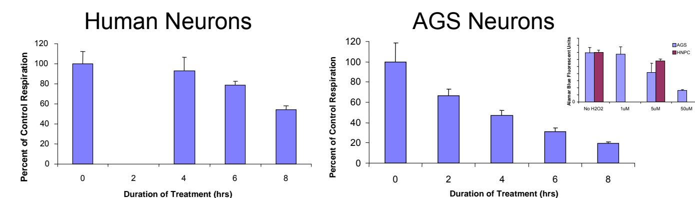

Fig. 5. Cells were differentiated for two weeks and then media changed to Neurobasal without B27. Cells were then treated with 50µM H_2O_2. After 2 to 8 hrs of H_2O_2 the cells were treated with Alamar blue for determination of cellular respiration.

In conclusion, tolerance to modeled ischemia in neurons differentiated from AGS neural stem cells suggest that tolerance is due, in part, to genetic factors.

[1]Palmer TD, Takahashi J, Gage FH. The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci.* 1997;8:389-404

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