Using astrocytes and motor neurons with mutated $SOD1^{G93A}$ to create a more realistic *in vitro* co-culture model for amyotrophic lateral sclerosis

Sabine Poux
Briarcliff High School

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal disease that victimizes thousands every year. With ALS, the release of toxic factors in the central nervous system drives widespread upper and lower motor neuron death and degeneration. This cell death promotes muscle weakness and wasting, and eventual organ failure, which leads to decreased quality of life and subsequent death. ALS patients typically do not live more than ten years after diagnosis and as of now there is no known cure.

Within the last decade, scientists have discovered that astrocytes release toxic factors that lead to motor neuron death and are thought to be the main causes of degeneration in ALS patients. This degeneration is most likely a form of programmed necrosis called necropoptosis, and in ALS is specific to motor neurons.

The goal of this research was to study the necroptotic pathway of ALS by creating an *in vitro* coculture model of the ALS degenerative condition that more closely mirrored that of the *in vivo* condition. To do this, we overlaid mutant $SOD1^{G93A}$ primary motor neurons onto mouse astrocyte monolayers. We used the $SOD1^{G93A}$ model because mice that are transgenic for this SOD1 mutation exhibit the phenotypic hallmarks of ALS, making the $SOD1^{G93A}$ model a viable *in vitro* recapitulation of the disease in its familial form. In our results, we saw that the motor neurons only died when exposed to diseased astrocytes, confirming our belief that motor neurons do not release non-cell autonomous factors that contribute to necroptosis in the ALS disease. The significance of this is that diseased motor neurons are not responsible for the death of other motor neurons in ALS. This death is triggered by astrocytes only, as diseased motor neurons will not kill off their neighboring motor neurons if they are plated with nondiseased astrocytes.

Introduction

Amyotrophic lateral sclerosis is a fatal neurodegenerative condition that affects thousands all over the world each year (Jackson et al., 1998). In this disease – also known as ALS and Lou Gehrig's Disease – motor neurons progressively die and the brain loses its ability to send signals to its satellite cells. This cell death promotes cascades of degeneration that can lead to sclerosis, or tissue hardening, as well as paralysis and organ failure (Hefferan et al., 2012). Other symptoms/indicators of ALS are muscle weakness, shortness of breath, and difficulty in breathing and swallowing (Murray et al., 2004; Shaw 2011).

ALS is autosomal dominant in familial cases, meaning a child is extremely likely to receive the disease if one of his/her parents is infected with the familial type. This familial ALS makes up 5-10% of all cases (Mitsumoto et al., 1998). Of these cases, 20% are linked to a mutation in the SOD1 (superoxide dismutase 1) gene. While a necessary enzymatic tool in its wild type, mutated SOD1 can have devastating pathological consequences. In ALS in particular, mutations to the SOD1 gene contribute to 2% of all cases. (Sau et al., 2007). Sporadic ALS, on the other hand, is spurred by an unknown cause, and patients of this type compose 90-95% of all cases (Jackson et al., 1998). ALS affects 30,000 familial and sporadic ALS patients in the United States today, and 6,000 new cases are diagnosed annually (Mancuso et al., 2015). Most of these victims start seeing symptoms when they are over 60 years old, though familial ALS can be early-onset. Due to the fatality of the disease, half of all victims live three or more years after diagnosis, while 10% live 10 or more (Mitsumoto et al., 1998).

For the longest time, scientists looked at ALS from a very neuronal perspective. However, they failed to consider that there are other cell types in the central nervous system (CNS), and mutant proteins are ubiquitous. Scientists discovered within the last decade that the

Motor neuron degeneration in ALS patients is brought on by astrocyte toxicity factors (Yamanaka et al., 2008). These astrocytes tend to be close to the motor neurons and are very abundant in the CNS, since they are the designated glial protectors of the of this area. The discovery of this alternate role of astrocytes has lead to considering targeting astrocytes for cellular therapies and ALS treatments, and since the released toxicity factors affect both familial and sporadic groups, only one treatment will need to be derived for the two (Re et al., 2014).

Cell death that is induced in ALS patients is most likely a form of programmed necrosis called necropoptosis (Re et al., 2014). Necroptopic mechanisms release toxicity factors into the targeted cells, which are able to break apart the cell membrane and irreversibly damage the cell's organelles and the surrounding tissues (Haidet-Phillips et al., 2012). The distinct form of necroptosis/toxicity release that is involved in ALS is specific to motor neurons, meaning only cells involved in body movement are affected by ALS. This excludes sensory neurons (Nagai et al., 2007).

In both familial and sporadic ALS models, astrocyte soluble toxic factors are released and trigger a death cascade that involves the activation of the kinases RIP1, MLKL, and BAX (Nagai et al., 2007). The role of RIP1 in the pathway was confirmed using a shRIP1 knockdown and Necrostatin 1; they also used Necrosulfonamide to inhibit MLKL (Re et al., 2014). GFP^+ motor neurons have also shown that the ALS death cascade is caused by astrocyte solubility factors; when placed in astrocyte-conditioned media (ACM), the motor neurons exhibited significant death. This means that there doesn't need to be cell-to-cell contact to trigger motor neuron death – the release of soluble factors by astrocytes is enough (Re et al., 2014). This death is also shown to be mediated by astrocytes only, because when motor neurons were overlaid on top of non-diseased control (NDC) fibroblasts and sALS fibroblasts and the results were normalized to

those of the NDC, no motor neuron death was observed. These observations were made in both the familial and sporadic ALS models, once again verifying their similarities in pathology and cause (Re et al., 2014).

Additionally, Re's team looked at the cell death markers Fractin, Tunel, and EthD to confirm that motor neurons die via programmed cell death in both familial and sporadic ALS models. Fractin detects caspase-cleaved actin and indicates apoptotic death. When used, it binds to the ends of actin fragments to show the fragmentation of actin happening inside of the degrading cell. Tunel, or terminal deoxynucleotidyl transferase, detects fragmentation of DNA in a degrading cell and also shows that apoptosis is occurring in a cell. EthD, or ethidium homodimer, is an assay that is membrane impermeable. It enters the cell when the plasma membrane is degrading and binds to DNA, showing that the cell is dying. Researchers observed in both mouse (familial) and human (sporadic) models that when motor neurons were exposed to astrocytes for three days, there was increase of each of these markers. Therefore, it seems that in both fALS and sALS cases, motor neurons die via a form of programmed cell death (Nagai et al., 2007; Re et al., 2014). This death is shown to be Bax-dependent and caspase-independent because when researchers plated motor neurons on diseased astrocytes and used V5, a Bax inhibitor, they saw increased motor neuron survival, but when they used zVAD, a pan-caspase inhibitor, they saw relatively little motor neuron survival. These results were confirmed for both the fALS and sALS models (Nagai et al., 2007; Re et al., 2014). Astrocyte characterizations were also used to confirm that the human ALS astrocyte cultures were highly enriched in astrocytes, while microglia, oligodendrocytes or neuronal markers were absent or marginally present. These characterizations were additionally used to observe the similarities in morphology and maturity of the control astrocytes and the ALS astrocytes (Re et al., 2014).

Because of the known connection of mSOD1 to certain fALS cases, the use of mSOD1 transgenic mice has become a useful tool for the study of the pathogenesis of ALS (Ripps et al., 1995). This is mainly because these transgenic mice exhibit the hallmark symptoms of ALS, and, even though the SOD1 mutation only accounts for 20% of fALS cases (2% of all cases), the degenerative cascade is thought to be the same for the sALS model, making the SOD1-mutated astrocytes layered with healthy motor neurons a universal model for *in vitro* motor neuron/astrocyte co-culturing. (Bilsland 2008). The mSOD1 mice that are used for the co-cultures are derived from mice that have the $hSOD1^{G93A}$ mutation. It has also been shown in the past that mice with mSOD1-expressing glial-restricted precursors that are grafted into spinal cords of wild-type rats lead to the degeneration of neighboring motor neurons (Papadeas et al., 2011). These *in vitro* and *in vivo* studies demonstrate that mutSOD1-expressing astrocytes, but not other cell types, can induce motor neuron degeneration.

The current gold standard for the co-culture model comes from a study published by Re et al. in 2014. In this study, drastic motor neuron degeneration occurred in just three days after being overlaied with ALS astrocytes. Re observed that motor neurons are more vulnerable in presence of sporadic astrocytes than in presence of control, chronic obstructive pulmonary disease (COPD) or Alzheimer's disease (AD) astrocytes (Fig. 1). COPD was

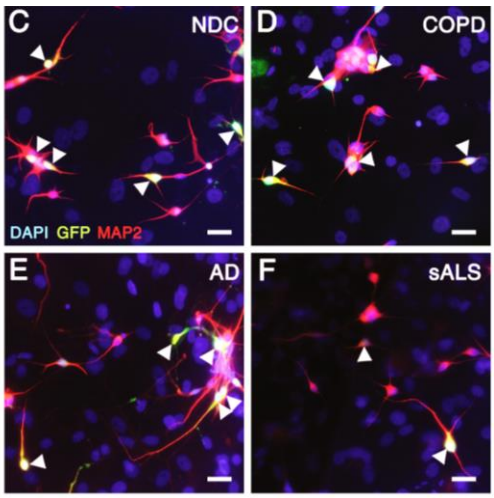

Figure 1: These pictures show a characterization of motor neurons under a fluorescent microscope. GFP, or green fluorescent protein, is a reporter protein that is a product of a transgene inserted under the motor neuron specific HB9 promoter. MAP2, or microtubule-associated protein 2, is a pan-neuronal marker. DAPI is a nuclear stain. (Re et al., 2014)

used as a control to mimic the agonal hypoxia of ALS, while AD was used to mimic the neuroinflammation of ALS. In using COPD and AD as controls, Re was able to rule out the roles of agonal hypoxia and neuroinflammation in motor neuron death (Re et al., 2014).

From her co-culture, Re elucidated observable trends of motor neuron survival for control and diseased astrocyte layers (Fig. 2). While for the controls 70-80% motor neuron survival was common, survival for the sALS layers levelled out at about 50% at day 7. This illustrates how drastic motor neuron degeneration in the ALS patient CNS is after just seven days of being overlaied with ALS astrocytes (Re et al., 2014).

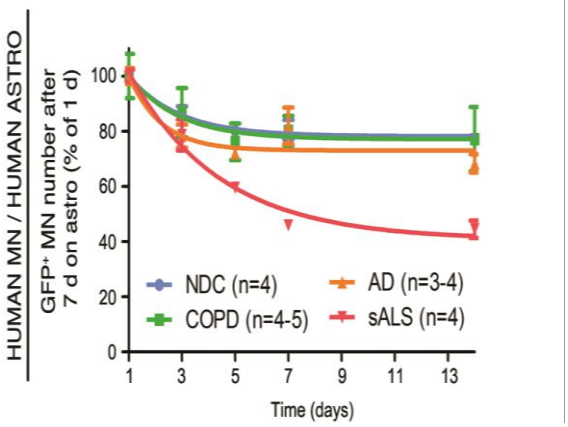

Figure 2: This graph shows the trend of motor neuron survival after 13 days. Significant differences in numbers become especially clear at day 7.
(Re et al., 2014)

Purpose

The purpose of this study was to create a more authentic co-culture model of the ALS disease. Another goal was to rule out the role of motor neuron non-cell autonomous factors as inducers of degeneration in neighboring motor neurons and solidify our belief that ALS is caused by astrocyte non-cell autonomous factors only. To complete these goals, degeneration that occurs as a result of overlaying both transgenic (diseased) and nontransgenic (non-diseased) astrocyte monolayers with mutant $SOD1^{G93A}$ primary motor neurons was quantified. This was done to account for the motor neurons in the surrounding environment that have already died. In this study, the mSOD1 neurons served as these sick neurons. The mutant primary motor neurons we used had a SOD1 mutation in which a glycine was substituted with alanine at the 93rd

position. Human mSOD1 was used because of its ability to mimic the pathological conditions of ALS degeneration.

Materials and Methods

Cell Culture

To start, mutant primary motor neurons (mPMN) were overlaid onto both transgenic and nontransgenic astrocyte monolayers (AMLs) on glass cover slips. These mPMNs were derived from a mouse line that had the human $SOD1^{G93A}$ mutation. The AMLs came from B6SJL mice – the transgenic layers were taken from the tissue of mice that had the disease, while the nontransgenic layers were taken from their healthy littermates. The astrocytes were cultured to confluence, and then the motor neurons were put on top at 15,000 motor neurons/well.

Fixation

After the astrocyte monolayers were co-cultured with motor neurons, the cells were fixed at various time points, particularly at D1 and D7 (one day after co-culturing and seven days after, respectively). This way, the cells could be observed as they were in their D1 states as well as their D7 states. To fix, cells were left 4% paraformaldehyde in DPBS for twenty minutes. After fixing, they were washed twice with DPBS and left overnight.

Immunocytochemistry

To stain the motor neurons that were co-cultured with the AMLs, neurons were blocked in buffer containing 0.1% Triton-X and 5% normal horse serum in DPBS for an hour at room temperature. They were then placed in primary antibodies RbαGFP (1:1000), Invitrogen, and CkαMAP2

(1:2500), Abcam, in 3% normal horse serum. Green fluorescent protein, or GFP, is a reporter protein that is a product of a transgene inserted under the motor neuron specific HB9 promoter. Microtubule-associated protein 2, or MAP2, is a pan-neuronal marker. After an overnight incubation with the primary antibody, they were washed with DPBS and stained with αRb488 and the αCk594 secondary antibodies at 1:400 each in 3% horse serum for one hour. DAPI, a nuclear stain, was also in the mixture at 1:1000. The layers sat in secondary for an hour and were subsequently washed twice with DPBS. Fluorescent mounting media was then used to mount the cover slips onto slides; slides dried overnight.

Microscopy

Next, GFP-positive motor neurons were counted using a confocal fluorescent microscope. The results are below (Fig. 1).

Statistical Analysis

After we calculated the percentages of motor neurons left after seven days, data was analyzed in a chi-squared test. We compared the WT control to the nontransgenic layers, and the $SOD1^{G93A}$ control to the transgenic layers. This is because we wanted to see that the motor neurons were dying because of the factors released by astrocytes and not by those released by the motor neurons. For this chi-squared test, our null hypothesis and alternative hypothesis were as follows:

H_0: There will be no percentage change between the death of motor neurons on the control layers and on the non-control layers.

H_A: There will be a percentage change between the death of motor neurons on the control layers and on the non-controlled layers.

Results

For the controls, we used wild type (WT) motor neurons and $SOD1^{G93A}$ motor neurons on top of fresh media (FM CTL). Though we did not put these motor neurons on top of astrocyte monolayers (AML's,) they had endogenous astrocytes that gave them their nontransgenic and transgenic characterizations, respectively. Each layer of these control cells was plated on one cover slip. For the D1 non-controls there were two cover slips per layer, and for the D7 non-controls there were three cover slips per layer. After counting, we averaged the numbers we got from all two/three slips for each layer together to get a mean number of surviving motor neurons per layer. These results were then graphed to observe trends of survival.

From our chi-squared test we were able to prove our null hypothesis, since our p-value was insignificant ($p = 0.2685$). This meant that the $mSOD1^{G93A}$ status of the motor neurons had no effect on their survival, therefore confirming that there is no non-cell autonomous factor being released by motor neurons that leads to death in the ALS cascade. The graphs below show the specific counts and percentages derived from our data.

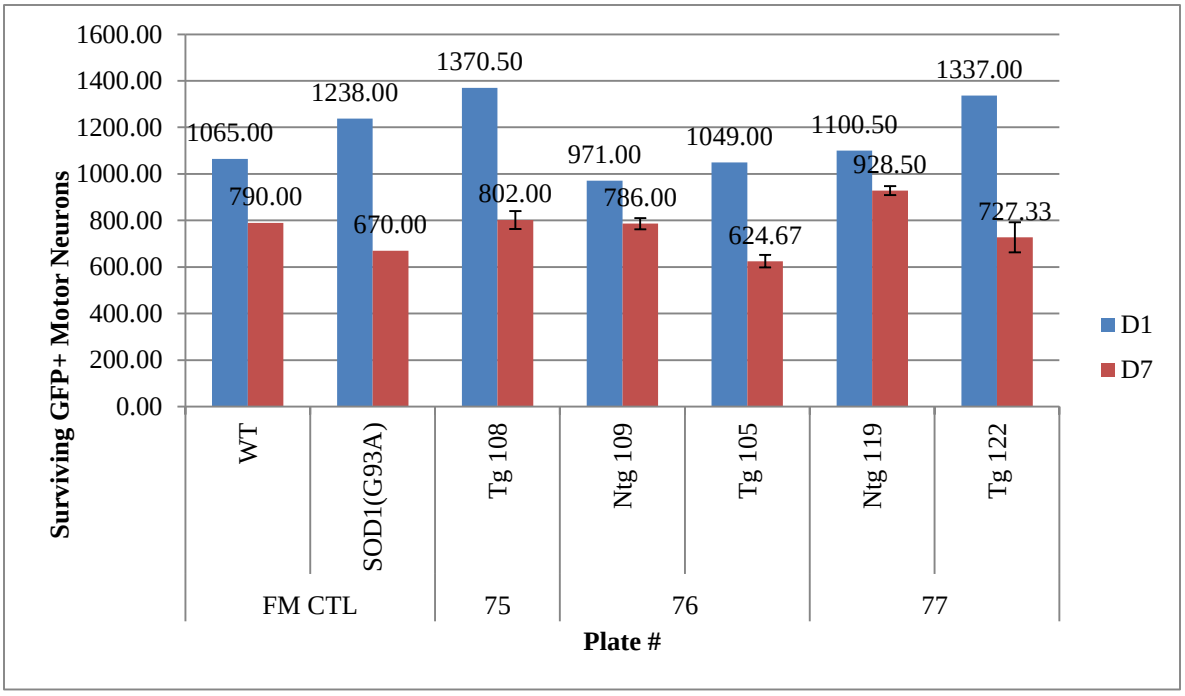

Figure 3: This graph shows the number of motor neurons that were left at D1 and D7 time points. This data was obtained by averaging the number of cells that were counted for each of the layers.

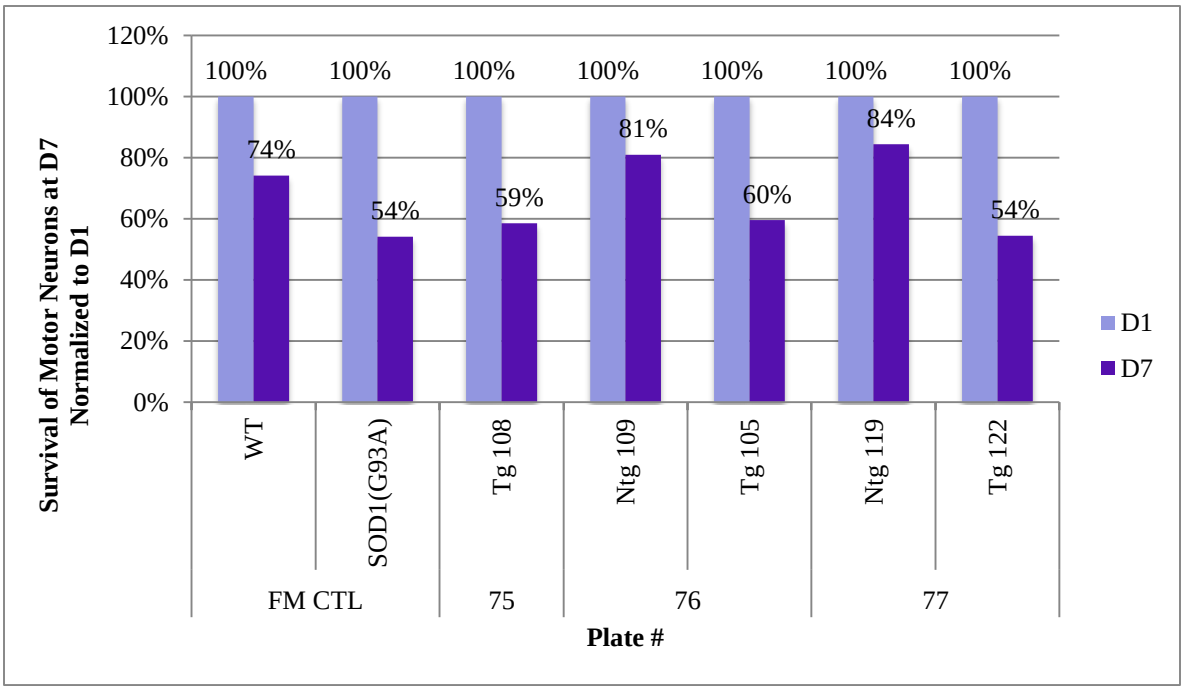

Figure 4: This graph shows the percentages of motor neurons that were left by D7, normalized to the average numbers of motor neurons that were present at D1.
p = 0.2685

Discussion & Future Research

This study confirmed previous findings that there needs to be a toxic astrocyte layer present for the motor neurons to be affected by the mSOD1-induced toxicity. Because the results of the layers closely mirrored the results of the controls (the deviation was insignificant), it is evident that the motor neurons will not degenerate by non-cell autonomous factors released by other motor neurons; there must be a glial factor to trigger death. Plated mSOD1 motor neurons on nontransgenic astrocyte layers saw little death beyond the projected natural decay (~26%), but plated mSOD1 motor neurons on the transgenic layers saw death characteristic of the ALS pathway. This allows us to rule out non-cell autonomous motor neuron factors as key players in the pathological degeneration of ALS. In other words, the existence of diseased motor neurons in a neuronal CNS population does not lead to cell death of surrounding motor neurons.

Additionally, this research created a more realistic *in vivo* model of the ALS disease. The conditions exhibited in this study more accurately represent the conditions in a diseased CNS: in this model, healthy motor neurons are surrounded by other healthy motor neurons as well as diseased motor neurons, while previous studies looked at healthy motor neurons only (Bilsland et al., 2008; Hefferan et al., 2012; Marchetto et al., 2008). To rule out the role of motor neuron non-cell autonomous factors means that the co-culture gold standard is accurate, and in co-cultures going forward the previously-existing sick motor neurons do not need to be accounted for.

Though we are able to recapitulate the fALS model *in vitro*, we are still looking for an avenue in which we can study the sALS model more closely. A model has been developed that involves using human-derived embryonic stem cells converted to motor neurons and astrocytes, but this model is non-specific (Marchetto et al., 2008). An exciting possibility for patient-specific sALS pathogenesis is the use of iPS cells, or induced pluripotent stem cells. These are cells that

can be generated from fibroblasts, allowing neuronal and glial cells to be developed with patient-specific diseased or non-diseased characteristics. In the past, these cells have been able to be transduced into different kinds of neuronal and glial cells. For example, in 2008, Dimos *et al.* discovered that iPSC's generated from ALS patients could be successfully extrapolated and differentiated into motor neurons (Dimos et al., 2008). Other researchers have come to similar conclusions, and have validated the idea that multipotent/pluripotent stem cells can be manipulated into motor neuronal cell form. This proliferation-manipulation can be done by a variety of growth factors, such as basic fibroblast growth factor and epidermal growth factor (Weiss et al., 1996). Forced proliferation can also be assisted by gene insertion, since division patterns are specific (Cashman et al., 2013; Noctor et al., 2004). These motor neurons can be generated from both fALS and sALS models, which proves that the two may have correlated pathways as well as similar cure potentials (Meyer et al, 2014).

In addition, these iPSC's have been differentiated into astrocytes, allowing for disease model astrocytes to use in coculture (Roybon et al, 2013). This holds much clinical potential, and in addition would allow for a more humanized model of ALS while alleviating the need to use controversial human embryonic stem cells for *in vitro* modeling. For example, diseased astrocytes could be generated from specific diseased patients, which would allow for a patient-specific *in vitro* model of the disease to be used for clinical tests such as drug screening. These cells can be derived from the tissues of live patients (Dimos et al., 2008) as well as post-mortem patients (Yagi et al., 2012). In addition, iPSC's hold potential for stem cell therapy, which, if perfected, could help ALS patients regain motor control (Matsui et al., 2012). However, this cell technology is new and volatile, and iPS-derived astrocytes are difficult to characterize in pure cultures. For this reason, the costs of the technology may not outweigh the benefits.

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