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mTOR Signaling and Endoplasmic Reticulum Stress in Mixed Glial Cultures

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BY

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Abstract

Oligodendrocytes are the myelinating cells of the central nervous system. The extraordinary protein and lipid synthesis that myelin production entails makes oligodendrocytes especially prone to endoplasmic reticulum (ER) stress. In this experiment, we set out to investigate the dynamics of ER stress in mixed glial cultures, and how the consequences of that stress vary based on the activity of the anabolic PI3K/AKT/mTOR pathway. Previous experiments in isolated oligodendrocyte cultures demonstrated a negative correlation between AKT/mTOR signaling and oligodendrocyte cell viability in the presence of ER stressors. Because ER stress leads to a buildup of improperly processed proteins, decreases in mTOR pathway activity are thought to moderate the excess of misfolded polypeptides. We hypothesized that administration of rapamycin, an inhibitor of mTOR, would ameliorate the effects of ER stress by preventing protein buildup caused by mTOR pathway activity. To test this hypothesis, mixed cultures of oligodendrocytes and astrocytes were isolated from neonatal rat brains and exposed to tunicamycin (TU), an inhibitor of n-linked glycosylation, to induce ER stress. TU was administered alone and after pre-treatment with rapamycin, to investigate how the effects of ER stress change when the activity of that pathway was limited. By assaying cell survival and protein expression, we were able to determine how ER stress, with and without mTOR inhibition, affected cell populations in mixed glial cultures. We found that chronic TU exposure in the absence of rapamycin failed to decrease the percentage of oligodendrocyte-lineage cells, which we attribute to the protective effects of astrocytes. Acute exposure, however, did decrease the percentage of oligodendrocyte-lineage cells in culture. These two trends lead us to speculate that the protective effects astrocytes offer to oligodendrocytes consist of relatively slow-acting growth-factor production and secretion by astrocytes. Adding strength to this contention, we found that the mTOR inhibitor rapamycin did not increase the percentage of oligodendrocyte-lineage cells in cultures exposed to TU. Because mTOR is involved in the function of many growth factors, its inhibition may have hampered the protective functions of astrocytes. While other experiments have investigated the effects of ER toxins on activity of the mTOR pathway, this experiment is more faithful to in vivo conditions because it incorporates meaningful astrocyte-oligodendrocyte interactions. As observed in this experiment, these effects are powerful and serve to improve our understanding of the dynamics of ER stress and mTOR inhibitors in organismal glial cells.

Introduction

The human central nervous system (CNS) – and by extension the mind to which it gives rise – is composed of two categories of cells: neurons and neuroglia. Neurons tend to receive a disproportionate amount of attention, as they execute the electrically-mediated cellular communication that affords sensation, movement, and cognition (Kiernan, 2009). However, neuroglia – a category that includes ependymal cells, microglia, astrocytes, and oligodendrocytes – constitute over half of the CNS cellular population and perform duties that are as diverse as they are essential (Bercury and Macklin, 2015; Kiernan, 2009).

These duties are described in Kiernan's *The Human Nervous System: An Anatomical Viewpoint,* where the author comprehensively reviews the responsibilities of each type of neuroglia (2009). Astrocytes serve to maintain homeostasis, improve synaptic transmission by preventing diffusion of neurotransmitters, and secrete antioxidants and growth factors. Ependyma border the brain's ventricles, allowing for molecular exchange between cerebrospinal fluid and nervous tissue. Microglia function as phagocytes when triggered by inflammation, and thereby protect the nervous system from potential disease. Finally, oligodendrocytes are responsible for the myelination of the CNS.

The importance of the role of oligodendrocytes – and of myelin more specifically – can hardly be overstated. Found to have convergently evolved in a variety of taxa, myelin is a lipid membrane that envelops the exterior of axons (Hartline, 2008). In doing so, it acts as an electrical insulator, preventing signal leakage as an impulse travels from one neuron to another. Additionally, the presence of myelin reduces the radial capacitance of axons, and as a result allows for greater conduction speed (Hartline, 2008). More recent research suggests that myelin also provides metabolic support to axons; when performing aerobic glycolysis, oligodendrocytes

produce lactate that can be metabolized by nearby axons (Bercury and Macklin, 2015). As a consequence of its utility, any absence or damage to myelin – demyelination – can have profoundly negative effects on the brain.

These negative effects have been implicated in a wide variety of disorders. The most infamous, and common, disease associated with demyelination is multiple sclerosis (Mayo *et al.*, 2012). Other diseases associated with dysmyelination include psychiatric disorders like autism spectrum disorder, sensory processing delay disorder, and attention deficit hyperactivity disorder; neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis; and rarer diseases including neuromyelitis optica and acute disseminated encephalomyelitis (Mayo *et al.*, 2012; Bercury and Macklin, 2015). These demyelinating pathologies stem from more factors than damage to oligodendrocytes alone, but that damage can contribute to disease progression. For instance, in animal models of multiple sclerosis, preventing oligodendrocyte cell death improves disease prognosis by reducing demyelination and the degradation of axons (Hussein *et al.*, 2014). Thus, oligodendrocyte survival and health is a key component of outcomes in diseases involving demyelination. Knowing how to treat such disorders will require leveraging knowledge about oligodendrocytes and myelination, and the ways in which the two can be preserved in the face of stressors.

The foundation of this knowledge consists of a wealth of information on oligodendrocyte growth and development. Oligodendrocytes originate as oligodendrocyte progenitor cells, which constitute roughly 5% of all glial cells in the adult brain (Levine *et al.*, 2001). Their subsequent development into mature cells relies on various cellular signaling cascades including the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways (Guardiola-Diaz *et al.*, 2012). The Ras/Raf/MEK/ERK pathway, also known as a mitogen-activated protein kinase pathway, is able

to integrate extracellular signals via G-protein activation, culminating in phosphorylation activity that can alter rates of cellular growth and proliferation (Kolch, 2000). Much like the Ras/Raf/MEK/ERK pathway, the PI3K/AKT/mTOR pathway regulates cellular growth and homeostasis based on the integration of a variety of internal and external signals (Wood *et al.*, 2013). Because each pathway is involved in regulating cellular growth, each plays a role in the life cycle of oligodendrocytes, which is summarized in Figure 1.

The first transition oligodendrocyte progenitors make is from early progenitor cells, which demonstrate a bipolar morphology, into late progenitor cells, which have more extensions and form a multipolar organization (Guardiola-Diaz *et al.*, 2012). In each case, the cells express the lipid antigen A2B5, while only late progenitors express the lipid antigen O4, allowing researchers to distinguish between early and late progenitor cells (Guardiola-Diaz *et al.*, 2012). When researchers inhibited downstream signaling of the Ras/Raf/MEK/ERK pathway using the Mek inhibitor U0126 and used this lipid antigen identification method, they found that the early to late progenitor transition was blocked (Guardiola-Diaz *et al.*, 2012). This blockage did not occur when the PI3K/AKT/mTOR pathway was inhibited with rapamycin, indicating that ERK signaling plays a unique regulatory role in the early to late progenitor transition (Guardiola-Diaz *et al.*, 2012). Following the late progenitor stage, there is postmitotic terminal differentiation to the immature oligodendrocyte stage, again partially regulated by ERK signaling (Guardiola-Diaz *et al.*, 2012). Immature oligodendrocytes then proceed to the mature oligodendrocyte stage – during this process they develop the numerous axonal connections that characterize a terminally differentiated oligodendrocyte (Guardiola-Diaz *et al.*, 2012). This last transition fails to occur when mTOR is inhibited using rapamycin, but occurs despite ERK inhibition, suggesting that the mTOR pathway is the unique regulator of the immature to mature stage of oligodendrocyte

differentiation (Guardiola-Diaz *et al.*, 2012). Interestingly – and usefully – throughout their life cycle, oligodendrocytes express the Olig2 transcription factor, which offers a reliable means of identifying oligodendrocyte-lineage (OL-lineage) cells in every developmental stage (Guardiola-Diaz *et al.*, 2012).

Once at the mature stage, oligodendrocytes are typically quite stable: research in the adult human nervous system using carbon-14 labeling found that only one in 300 oligodendrocytes is replaced over the course of a year (Yeung *et al.*, 2014). The same study found that myelin has a high turnover rate even in adults, indicating that mature oligodendrocytes continue to replenish myelin throughout the lifespan of an organism. This is a formidable task, if only because of the number of connections a myelinating cell makes with the axons around it: mature oligodendrocytes may form up to 50 internodes with adjacent axons (Pfeiffer *et al.*, 1993). These internodes are extensions of the oligodendrocyte's plasma membrane, which envelops neighboring sections of axons. This sheath performs the insulating duties that contribute to greater impulse speed, and its structure and composition are essential for its function: myelin is 70% lipid (Pfeiffer *et al.*, 1993).

The lipid-rich structure of myelin makes its synthesis extremely dependent on certain cellular organelles and processes. In particular, the formation of myelin relies on the endoplasmic reticulum (ER). The ER is an organelle that consists of tubules, sacs, and cisternae that extend from the cytoplasm to the nuclear envelope (Sovolyova *et al.*, 2014). When the ER functions properly, it performs three vital roles (Lin and Popko, 2009). First, it processes the majority of the cell's proteins, governing the post-translational modification and folding that are integral for a protein's structure and therefore its function. Second, it oversees the synthesis of lipid molecules, including steroids and cholesterol. Third, it regulates cytoplasm calcium

concentrations by either taking up or releasing the ion depending on its intracellular concentration. Because of its role in producing the elements that are necessary parts of myelin, a dysfunctional ER limits or eliminates entirely the ability of an oligodendrocyte to myelinate. As a result, demyelinating disorders often stem from or involve the malfunction of the ER (Zhang and Kaufman, 2008). This failure in function can result from various mechanisms, but one of the most common triggers is a phenomenon known as ER stress (Schroder and Kaufman, 2005).

The ER is most likely to become stressed when there is a disturbance in its homeostasis (Lin and Popko, 2009). These disruptions can consist of changes in calcium, glucose, cholesterol, or oxidation status; in elevated rates of protein synthesis; or when glycosylation is inhibited (Lin and Popko, 2009; Yoshida, 2007). If its homeostasis is sufficiently disturbed, the ER loses its ability to properly fold proteins (Lin and Popko, 2009). As a result, misfolded proteins collect in the lumen of the organelle – it is this accumulation that is the ultimate sign of ER stress (Lin and Popko, 2009). For oligodendrocytes, ER stress is mostly likely to occur during periods of myelin development or repair (Hussein *et al.*, 2014). This is because myelination puts an immense load on the protein-folding capacities of the ER, and as this load approaches the capacity of the organelle, stress becomes an increasingly likely outcome.

Fortunately, the ER is not defenseless in the face of stress. Rather, the cell can execute unique responses meant to address and remediate the effects of that stress in a therapeutic manner (Walter and Ron, 2011). These responses consist of several signal transduction pathways and are together known as the Unfolded Protein Response, or UPR, which is summarized in Figure 2 (Walter and Ron, 2011). Each of these pathways begins with one of three transmembrane receptors, described below, which are, in the absence of stress, inactive (Walter and Ron, 2011). But as proteins accumulate in the ER lumen, indicating the onset of stress, each

of these transmembrane receptors is activated (Sovolyova *et al.*, 2014). Their activation induces the downstream signaling cascades that serve as the UPR (Sovolyova *et al.*, 2014).

The consequences of the UPR depend on the intensity and duration of ER stress (Sovolyova *et al.*, 2014). In the presence of mild or short-lived stress, the effect of the UPR is to prolong cell survival (Kapuy *et al.*, 2014). Conversely, when stress is especially exaggerated or long-term, the continued activation of the UPR leads to apoptosis (Kapuy *et al.*, 2014). As these two polar outcomes both rely on the UPR, the ability of the cell to choose the correct path based on cellular conditions is, quite literally, an issue of life and death.

In the event of mild, short-term stress, the UPR exerts five positive effects. First, it prevents the translation of non-essential proteins, given the excess amount already present in the lumen (Schroder and Kaufman, 2005). Second, it ramps up the process of ER-associated degradation (ERAD) in order to destroy some of those excess, misfolded proteins (Duffee *et al.*, 2012). Third, it induces the formation of autophagosomes and upregulates the process of autophagy in order to digest additional unwanted cellular material (Benbrook and Long, 2012). Fourth, it leads to translational changes that increase the number of molecular chaperones, which aid in protein folding (Walter and Ron, 2011). Fifth, it expands the area of the ER membrane in order to facilitate the work of those new chaperones and the creation of new, functional polypeptides (Walter and Ron, 2011).

Three signal cascades are sufficient to produce these potentially cell-saving processes, and the responsibility and mechanism of each has been well-studied. Each of the cascades begins at one of three transmembrane receptors: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), or inositol-requiring enzyme 1 (IRE1) (Walter and Ron, 2011). Each of these transmembrane proteins has a luminal domain that serves to detect ER stress, and a

cytosolic domain that can trigger changes in cellular activity in response to that stress (Cao and Kaufman, 2012).

Of the three transmembrane proteins, IRE1 is the oldest and most conserved (Walter and Ron, 2011). When the lumen becomes crowded with misfolded proteins, luminal binding protein – known as BiP – detaches from the IRE1 protein and binds with those misfolded proteins (Duffee *et al.*, 2012). As a result, IRE1 is activated and catalyzes the removal of an intron from the mRNA that codes for a protein called X-box binding protein 1 (XBP-1). This deletion leads to a frameshift in the coding sequence, producing a different isoform of XBP-1.The new isoform of XBP-1 increases the expression of several different proteins responsible for protein folding and degradation. As a result, the ER is able to fold future polypeptides while disposing of those it has misfolded in the past. Meanwhile, IRE1 activation also activates the c-Jun N-terminal kinase (JNK) pathway, which lipidates microtubule-associated protein 1A/1B-light chain 3 (LC3), altering it from LC3-1 into LC3-II (Szegezdi *et al.*, 2006; Tanida *et al.*, 2008). Lipidated LC3-II is incorporated into the membrane of autophagosomes, which then proceed to increase the amount of autophagy in the cell (Benbrook and Long, 2012).

The second oldest transmembrane protein in the UPR is ATF6 (Duffee *et al.*, 2012). When ER stress occurs, ATF6 is moved into transport vesicles that move it to the Golgi apparatus (Walter and Ron, 2011). There it meets two proteases, S1P and S2P (named for the sites at which they are located), which sequentially remove the ATF6 luminal domain and transmembrane anchor. This leaves a transcriptional domain known as ATF6(N) that moves into the nucleus and upregulates various UPR stress genes, including those that code for molecular chaperones like protein disulfide isomerase (PDI) and glucose-regulated protein 94 (Grp94) (Walter and Ron, 2011; Duffee *et al.*, 2012). ATF6 activation also leads to an increase in the

production of degradation proteins, including ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1) (Duffee *et al.*, 2012). Just as with IRE1, activation of ATF6 allows for the efficient disposal of undesired proteins and the creation of new, fully operational proteins to take their place.

The final, most evolutionarily recent transmembrane protein in the UPR is PERK (Duffee *et al.*, 2012). When activated by the accumulation of misfolded proteins in the lumen, PERK phosphorylates a subunit of the translation initiation factor eIF2α, preventing it from initiating the translation of select proteins from mRNA (Duffee *et al.*, 2012). Thus, activation of PERK leads to a decrease in the translation of non-essential proteins (e.g., those not involved in protein degradation and folding) and gives the ER a critical period during which it can dispose of those already amassed inside of the lumen (Walter and Ron, 2011). Along with the other two signal cascades of the UPR, led by IRE1 and ATF6, PERK serves to give the ER the time and molecular machinery necessary to combat stress.

While the UPR promotes autophagy in cases of mild stress, more intense or enduring stress can cause the UPR to become pro-apoptotic. This switch means that the same transmembrane proteins that previously promoted cell survival become promoters of apoptosis. IRE1, for example, triggers the apoptosis signal-regulating kinase 1 pathway in the event of long-term stress (Duffee *et al.*, 2012). Interestingly, intense stress also turns a positive effect of IRE1 during mild stress – upregulation of the JNK pathway that promotes a healthy level of autophagy – into a negative effect that can lead to apoptosis. This shift occurs through the downstream phosphorylation of a protein known as Bcl-2 (Szegezdi *et al.*, 2006). Bcl-2 usually demonstrates anti-apoptotic activity; phosphorylation inhibits this activity and apoptosis therefore becomes more likely (Szegezdi *et al.*, 2006). The PERK signaling pathway can also

promote cell death; this process occurs via activation of the transcription factor CCAAT/enhancer-binding homologous protein (CHOP) (Lu *et al.*, 2014). In one experiment that assessed the role of CHOP in ER stress, oligodendrocyte progenitor cells isolated from rats were more likely to survive when a chemical inhibitor of CHOP, amiloride, was administered (Kuroiwa *et al.*, 2014). A related study, which used CHOP-knockout mice in order to investigate spinal cord injury recovery, found that the prognosis for CHOP-knockout mice was greatly improved relative to controls, with increased white matter and myelin basic protein testifying to increased oligodendrocyte survival (Ohri *et al.*, 2013). The negative effects of CHOP on cell survival are believed to relate to its upregulation in the transcription of the death receptor 5 gene (Lu *et al.*, 2014). During acute ER stress, this increased transcription is not sufficient to lead to apoptosis, because the IRE1 signal cascade leads to the decay of death receptor 5 mRNA (Lu *et al.*, 2014). However, as stress duration increases the likelihood of apoptosis does as well, since the upregulation of death receptor 5 begins to outpace its destruction.

In addition to the complex cascades described above, the UPR maintains a nuanced relationship with the PI3K/AKT/mTOR pathway (Appenzeller-Herzog and Hall, 2012). mTOR, or mammalian target of rapamycin, is a serine/threonine protein kinase that is often referred to as a master regulator, since it incorporates inputs including growth factors, amino acid and glucose levels, and energy stores in order to control the rates of growth and metabolism in a cell (Wood *et al.*, 2013; Kapuy *et al.*, 2014). Generally, mTOR can be said to have anabolic effects on a cell, which include the upregulation of protein production and the downregulation of autophagy via phosphorylation of a compound known as Atg13, which is required in its dephosphorylated state for autophagosome production (Eisenberg-Lerner *et al.*, 2009; Jung *et al.*, 2010; Sano and Reed, 2013). During very short-term stress, the UPR activates

PI3K/AKT/mTOR signaling via the ATF6 pathway (Appenzeller-Herzog and Hall, 2012). This takes advantage of the role that mTOR plays in promoting growth, allowing the cell to perform certain anabolic processes that are conducive to cell survival, such as ER membrane expansion and the synthesis of molecular chaperones. However, during longer term stress the UPR inhibits the mTOR pathway through PERK-mediated activation of CHOP, which induces the synthesis of an AKT inhibitor known as TRB3, and therefore results in a downstream decrease in mTOR signaling (Appenzeller-Herzog and Hall, 2012). This inhibition helps to prevent apoptosis, since the mTOR pathway would otherwise increase the likelihood of cell death by stimulating high levels of JNK via activation of IRE1 (Appenzeller-Herzog and Hall, 2012). The inhibition of mTOR by the UPR has been shown experimentally: in work with mouse embryonic fibroblasts, Qin *et al.* found that extended UPR activation leads to decreased signaling from the AKT/mTOR pathway (2010).

Lowered mTOR signaling in response to extended UPR activation has the beneficial consequence of increasing autophagy in stressed cells, since active mTOR is anti-apoptotic. This finding has been confirmed both theoretically and experimentally. Using neuroblastoma cells, Ogata *et al.* found that ER stress led to an increase in the rate of autophagosome formation in a cell (2006). In another experiment, this time using human kidney cells, the same upregulation of autophagosome production in response to ER stress was observed (Kapuy *et al.*, 2014). Because this increase in autophagy leads to the digestion of unwanted cellular material and a reversion towards ER homeostasis, the UPR's inhibition of the AKT/mTOR pathway can very well be said to serve as a cytoprotective process.

While mTOR inhibition by the UPR can, along with the other arms of the UPR, help in preventing apoptosis, it sometimes is not a sufficient response. This insufficiency is illustrated in

the high rates of cell death that often accompany ER stress (Sano and Reed, 2013). To try to address this inadequacy, some researchers have investigated the possibility of artificially inhibiting mTOR and thereby effectively accelerating and intensifying the effects of the UPR. Fortunately, there are various chemicals that serve as mTOR inhibitors, including two compounds known as rapamycin and metyrapone (Faivre *et al.*, 2006; Kapuy *et al.*, 2014). Several experiments have investigated the effects of administering these inhibitors in conditions of ER stress. In one such study, administration of rapamycin to stressed human kidney cells led to the onset of autophagy at lower levels of stress than in control cells (Kapuy *et al.*, 2014). Autophagy was also found to continue at higher levels of stress than in control levels, suggesting that artificial mTOR inhibition was delaying apoptosis by prolonging autophagy (Kapuy *et al.*, 2014). In a similar study, Kato *et al.* induced ER stress in rat kidney cells using tunicamycin (TU), which inhibits n-linked glycosylation of secretory and membrane proteins and thus renders the protein-folding process more difficult (2012). Some of the ER stressed cultures had been pre-treated with rapamycin, while others only received the ER stressor. The researchers found that rapamycin-exposed cells were less likely to die than those that were only exposed to TU. Other findings, using monkey kidney cells and mouse β-cells, demonstrate an identical pattern: the upregulation of autophagy can prevent ER stress-induced apoptosis (Ravikumar *et al.*, 2006; Bachar-Wikstrom *et al.*, 2012). Ameliorating ER stress through other means also has positive effects. In oligodendrocyte progenitor cells given TU, cannabidiol treatment prevented apoptosis by blocking ER stress and subsequent activation of CHOP, while promoting expression of Bcl-2, an anti-apoptotic protein (Mecha *et al.*, 2012).

While the literature includes various examples of mTOR inhibition in cases of ER stress, only certain cell lines have been studied. One overlooked cell has been the oligodendrocyte. In

some ways this is especially misguided, since oligodendrocytes are considered the most vulnerable cells in the CNS and are especially vulnerable to oxidative stress and inflammation (Benjamins *et al.*, 2013; Johnstone *et al.*, 2013). Though some research exists that aims to clarify the response of oligodendrocytes to ER stress (e.g. Mecha *et al.*, 2012), far fewer studies assess the potential for artificial mTOR inhibition in those cells.

A further gap in the literature relates to the fact that oligodendrocytes tend to be studied in enriched cultures. An example of these enriched studies can be seen in previous work done by this lab. In this research, enriched cultures of two-day-old oligodendrocyte progenitors were exposed to TU at different concentrations and for different periods (McInnis, 2012). Cell viability was assayed using alamarBlue fluorescence, which offers a measurement of cell survival. Significant drops in cell survival were observed after a 3 hour acute treatment with 10 μg/ml of TU (Figure 3), as well as for 12 hour chronic treatments at 2 μg/ml (Figure 4). However, while enriched culture data tends to be convenient to collect, it fails to approximate *in vivo* conditions, where the presence of other cells influences oligodendrocyte behavior and response to ER stress.

In order to address these gaps in the literature, the present study will study whether ER stress is affected by mTOR inhibition in mixed glial cultures. In this experiment, the effects of rapamycin pre-treatment on mixed glial cultures undergoing ER stress will be analyzed. ER stress will be induced using TU, the inhibitor of N-linked glycosylation discussed above for its role as an ER stressor. This stress will be induced in various time points and concentrations, in order to assess whether any effects of rapamycin are more pronounced for a given stress condition.

We hypothesize that by reducing protein production and stimulating autophagy, artificial inhibition of the mTOR pathway via rapamycin pre-treatment will help to improve oligodendrocyte viability for all mixed glial cultures exposed to ER stressors. If this is the case, it would raise interesting questions about the potential to address certain disorders of myelination that involve ER stress with mTOR inhibitors in order to preserve oligodendrocyte populations. We further hypothesize that overall toxicity as a result of TU exposure will be lowered relative to similar experiments conducted in enriched cultures, such as that of McInnis (2012). We hypothesize that the protective effects exerted by glia, astrocytes especially, will improve oligodendrocyte viability during ER stress. For instance, astrocytes protect oligodendrocyte progenitors from oxidative stress-induced damage and nutrient starvation, while also protecting mature oligodendrocytes in the event of CNS injury (Arai and Lo, 2010; Sofroniew, 2005). Astrocyte presence alters oligodendrocyte fate, and experiments that fail to account for this inherently fail to approximate physiological conditions. Using mixed cultures, on the other hand, will incorporate the contributions of astrocytes to the oligodendrocyte stress response.

Our experimental paradigm, therefore, is more physiologically relevant than those that have preceded it. We believe that our data will allow for more credible extrapolations of our experimental data onto questions concerning how oligodendrocytes behave in an organism. This extension is important, and carries high stakes. Because the activity of oligodendrocytes – and therefore the myelination of the brain – depends on a functional ER, it is critical to understand how oligodendrocytes respond to a stressed ER and whether their response can be helped through the artificial inhibition of certain pathways. By assessing whether rapamycin administration can ameliorate the negative effects of ER stress in oligodendrocytes, we will gain

insight into potential therapies for the otherwise devastating demyelination and dysmyelination that underlie many varied diseases.

However, even if we find that mTOR inhibition lessens some of the damage caused by ER stress, it is important to note that such a solution can only be temporary. This is because mTOR activity is required for much of a cell's most important functions, including myelination. Several studies have shown that mTOR inhibition results in delayed onset of myelination and reduced total myelination (Guardiola-Diaz *et al.*, 2012; Tyler *et al.*, 2009; Lebrun-Julien *et al.*, 2014). Meanwhile, upregulation of mTOR increases overall myelination (Tyler *et al.*, 2009; Lebrun-Julien *et al.*, 2014). While mTOR activity may be fatal for a stressed cell, it is also necessary for a functional one. Any solution that involves mTOR inhibition, therefore, must include a point after which mTOR is allowed to function as normal.

An additional caveat concerns the cascading nature of cellular pathways. The administration of an mTOR inhibitor will produce more widespread effects than mTOR inhibition alone. The mTOR pathway has relationships with various other pathways, which interface with even more pathways, and so on (Hay and Sonenberg, 2004). Therefore, inhibiting mTOR will not simply lower rates of transcription and translation – it will also lead to greater activation of pathways for which mTOR is inhibitory, and less activation of pathways for which mTOR is excitatory (Dai *et al.*, 2014). Therefore, the results produced in this experiment will be the consequence of not only one cellular change, but of one that precipitates many more. Teasing out these changes – in addition to observing the outcome to which they lead – is important, and may well be aided by data from the experiment described herein.

Materials and Methods

Preparation of Mixed Glial Cell Cultures

To establish mixed glial cultures, neonatal rat pups (P2) were sacrificed via rapid decapitation according to IACUC approved protocol. Heads were transferred to a sterile dissection fume hood, where sterile surgical tools were used to remove the brain, which was placed in Hepes-EBSS buffer (HE) on ice.

The hindbrain and olfactory bulb of each brain were removed and the remaining telencephalon was divided into its two hemispheres. The telencephalic hemispheres were rolled on filter paper to remove the meninges and blood vessels, transferred to HE, and minced using a sterile razor held with locking tweezers. The solution of minced tissue was transferred to a 100 ml solution of trypsin-HE and placed in a shaker at 110 RPM for 20 minutes at 37°C.

To terminate trypsinization, 2 ml of trypsin inhibitor (SBTI) was added to the beaker of minced tissue, which was swirled for two minutes at room temperature. Next, 2 ml of $MgSO_4$ was added to the solution, followed by 1 ml of DNAse stock at a concentration of 2 mg/ml. The resulting solution was incubated for five minutes at room temperature with gentle swirling. After this period, the tissue solution was transferred in four 50 ml sterile conical tubes, which were centrifuged for one minute at 2000 RPM. Supernatants were carefully aspirated and discarded, leaving a pellet at the bottom of each tube. Each pellet was resuspended in 5ml of trituration DNAse at a concentration of 80 of μg/ml, then allowed to settle for 5 minutes.

Each of the four resuspensions was transferred into a 15 ml sterile conical tube, and 1 ml of 4% BSA was added to each tube underneath the layer of tissue solution in order to form a density gradient. The four 15ml conical tubes were centrifuged for 8 minutes at 800 RPM. Supernatants were carefully aspirated and discarded, leaving a pellet at the bottom of each tube

covered by a shallow layer of fluid. Pellets were resuspended by tapping the tube and 5 ml of 5% fetal bovine serum (FBS) was added to each tube. The contents of each tube were then added to a sterile 125 ml bottle containing 30 ml of 5% FBS. Using a hemacytometer, the cell density of the solution was calculated. This solution was added to the prepared poly-lysine coated four-well plates – each well was seeded with 500,000 cells, and 5% FBS was added as needed to finalize the volume of each well at 0.5 ml. After each well was seeded, all plates were placed in a sterile incubator at 37°C.

Care of Mixed Glial Cultures and ER Stress Induction

After four days in vitro, during which cells were allowed to divide and proliferate, the media was replaced with 0.5 ml of 5% fetal bovine serum, which inhibits cellular differentiation. Three days after the first media change, a second media change was performed. Three days after the second media change, each well was switched to N2 supplement media, which allows for cellular differentiation. This day was termed differentiation day 0. During this process, each well was divided into one of two groups: one that received N2 media with rapamycin at a concentration of 1 μl/ml (the rapamycin pre-treatment group), and one that received only N2 media (the control group to which rapamycin pre-treatment would be compared). It is important to stress that rapamycin has been shown to not interfere in the differentiation process for early progenitor cells through immature oligodendrocytes (Guardiola-Diaz *et al.*, 2012).

In our control paradigm, N2 media was replaced on differentiation day 1, 24 hours prior to harvest, with rapamycin pre-treatment wells also receiving 1 μl/ml rapamycin. In our chronic stress paradigm, 1 μg/ml TU was added on differentiation day 1, 24 hours prior to harvest, with rapamycin pre-treatment wells again receiving 1 μl/ml rapamycin. In our acute stress paradigm,

10 μg/ml TU was added on differentiation day 2, exactly 3 hours prior to harvest, with rapamycin pre-treatment wells again receiving 1 μl/ml rapamycin. Once the treatment time elapsed, cells were harvested to allow for immunohistochemistry.

Immunohistochemistry

Immunolabeling of cells was performed to determine total number of cells, total number of OL-lineage cells, and ER stress in each culture. Primary antibodies used included anti-Olig2 (1:100; IBLInternational), anti-A2B5 (1:100; EMD Millipore), anti-O4 (1:25; EMD Millipore), and anti-CHOP (1:100; Cell Signaling Technology). Olig2 is a transcription factor expressed in all oligodendrocyte lineage cells, the detection of which requires fixed cells with permeabilized membranes. The lipid antigens A2B5 and O4 are expressed by early to late progenitor oligodendrocytes and late progenitor through mature oligodendrocyte cells, respectively, and together detect all OL-lineage cells when staining is performed in live cells. CHOP is a marker of cell stress and subsequent UPR induction, and antibodies for the protein indicate stressed cells when used in fixed cultures.

During live staining, cultures were blocked in 3% normal goat serum in HE (3% NGS/HE) for 10 minutes, after which they were exposed to the primary antibodies anti-A2B5 (1:100) and anti-O4 (1:25) in 3% NGS/HE for 15 minutes. Cultures were washed in HE, fixed in 4% PFA for 15 minutes, and washed in HE again. Cultures were incubated with Hoechst dye (1:200; Pierce Thermo) and fluorescein isothiocyanate (1:50; Jackson Labs) in 3% NGS/HE for 45 minutes in the dark. Cultures were washed with HE, coverslipped, and stored at -30 °C.

During fixed staining, cultures were fixed in 4% paraformaldehyde for 30 minutes, washed with HE, and incubated with 5% NGS/0.3% Triton X-100 for 60 minutes. Cells were

washed with HE and the primary antibodies CHOP (1:100) and Olig2 (1:100) were added in 3% NGS for 1 hour. Cultures were washed with HE and incubated with Hoechst (1:200), fluorescein isothiocyanate (1:50), and gamma-Cy 3 (1:500; Jackson Labs) for 45 minutes in the dark. Cultures were washed with HE, coverslipped, and stored at -30 °C.

Cell Visualization and Analysis

Cells were visualized using an Olympus IX70 optical microscope equipped with a Carl Zeiss camera. There were a total of 48 wells across the experiment, with between 2 and 10 wells per treatment condition. For each well, ten fields were randomly selected and imaged. Each image was a composite of photographs taken using the FITC, WU, and WG filters (for wells which had undergone live staining, the WG filter was not used). Using Zen 2012 software, the exposure time for each image was standardized.

Image Analysis and Quantification

After all images were taken, blind counts were performed to determine: the number of Hoechst-positive cells per image in live- and fixed-staining conditions, giving the total number of cells in an image; the number of cells expressing A2B5/O4, giving the total number of OL-lineage cells after live-staining; the number of cells expressing CHOP, giving the number of stressed cells after fixed-staining; and the number of cells expressing Olig2, giving the number of OL-lineage cells after fixed-staining. Based on these data, it was possible to investigate how the number of overall cells, OL-lineage cells, and markers of cell stress varied across treatments.

Results

In order to better understand the ER stress response in oligodendrocytes, we artificially induced protein-misfolding – and subsequent ER stress – through the administration of TU to mixed glial cultures grown in the OL differentiation media for a minimum of 24 hours. This exposure was split between chronic – with a lower dosage, for a longer period – and acute – with a higher dosage, for a shorter period – treatments, in order to analyze how cellular response would vary between mild but prolonged stress and intense but brief stress. In order to assess how mTOR inhibition alters the consequences of induced ER stress, the cultures were further subdivided into those given 1 μl/ml rapamycin on differentiation day 0 and controls. Altogether, this paradigm allowed us to ask – and answer – several critical questions about the mechanism and outcome of ER stress in oligodendrocytes.

The Effects of Chronic TU Treatment on Cellular Survival

While our experimental paradigm gave us a variety of data to analyze, we began by investigating the effects of TU exposure in the absence of rapamycin pre-treatment. We further restricted our scope to chronic TU exposure, which consisted of of 24 hours at a concentration of 1 μg/ml. We first quantified whether this exposure affected total cell count, as measured by Hoechst-positive cells. Because Hoechst staining was carried out in both live and fixed conditions, it was possible to cross validate the data as a way of gauging its accuracy. In each case, our results show that chronic TU exposure did not lead to a significant decrease in Hoechst-positive cells (Figure 5). Having established that total cell counts were stable despite chronic TU exposure, we set out to determine whether cell type was changing by quantifying the percentage of all cells that expressed markers of OL-lineage. In this case, staining for both A2B5/O4 and

Olig2 revealed that chronic TU exposure did not reduce the number of OL-lineage cells (Figure 6). More importantly, during chronic TU exposure the *percentage* of all cells that were labeled as OL-lineage did not change from control levels (Figure 7).

The Effects of Acute TU Treatment on Cellular Survival

Having established the relatively innocuous effects of chronic TU exposure on mixed glial cultures, we next examined the effects of acute TU exposure on those cultures. During acute exposure, cell cultures were exposed to TU at a concentration of 10 μg/ml of 3 hours. As before, we initially assayed how that exposure altered total cell counts. Consistent with our findings from chronic TU conditions, the data indicate that acute TU exposure did not lead to a decrease in total cell count (Figure 8). However, the effects of acute TU exposure on OL-lineage cells in particular deviates from those of chronic TU exposure. Based on Olig2 expression in our cultures, acute TU exposure induced a significant drop in the number of OL-lineage cells – from 126.2 to 76.5 (Figure 9) – and in the percentage of OL lineage cells – from 41.2% to 23.2% (Figure 10). Acute TU exposure also led to decreases in A2B5/O4-positive OL-lineage cell count from 66 to 52 (Figure 9), and in the overall percentage of A2B5/O4-positive cells from 26.8% to 17.8% (Figure 10), but these decreases were narrowly insignificant.

The Effects of mTOR Signaling on ER Stress

After investigating the effects of TU exposure in isolation, we proceeded to assess whether rapamycin pre-treatment altered those effects in any way. Half of our cultures received 1 μl/ml of rapamycin prior to, and during, treatment. This allowed us to study whether mTOR inhibition altered the outcome of ER stress. Interestingly, rapamycin pre-treatment was found to

significantly increase the number of Hoescht-positive cells in all conditions for live staining and to significantly increase the number of Hoechst-positive cells in control and acute conditions during fixed staining (Figure 11, Figure 12). In the latter case, chronic cultures also showed an increase in total cell count with rapamycin pre-treatment, but this was narrowly insignificant (Figure 12). All told, these data implicate rapamycin as being a proliferative agent, whether or not cells are given ER stressors; possible mechanisms for this finding will be discussed in more detail below.

After observing the rapamycin-mediated increases in total cell count described above, we analyzed the effects of rapamycin pre-treatment on OL-lineage cells specifically. Our data show that rapamycin pre-treatment does not significantly alter the percentage of all cells that are A2B5/O4-postive, regardless of treatment condition (Figure 13). Olig2 staining largely agreed with these findings: control and chronic TU cultures did not show changes in OL-lineage cell percentages in response to rapamycin pre-treatment (Figure 14). However, acute TU cultures showed a significant decrease in OL-lineage cell percentage in response to rapamycin pre-treatment, dropping from 23.2% to 15.0% (Figure 14). This decrease was unexpected, but one mechanism of how it may have arisen is outlined below.

The Effects of Rapamycin Administration on Cellular Stress

Finally, we attempted to use a measure of ER stress, CHOP, in order to explore how the stress response was affected by rapamycin pre-treatment. We found that there was a high baseline stress in our control controls, with 48% of control cells expressing CHOP (Figure 15). Rapamycin pre-treatment led to an insignificant decrease in this figure, but without more data we cannot determine whether this is a robust finding (Figure 15). Cultures in the chronic TU exposure condition did not significantly differ from controls in their CHOP expression, and again

rapamycin was not shown to significantly change levels of CHOP (Figure 14). Acute TU cultures, however, had significantly fewer CHOP-expressing cells than control cultures – 32.2% relative to 48.3% – and rapamycin pre-treatment lowered that CHOP expression significantly further, to 19% (Figure 15). While further research is needed to understand this phenomenon, it would appear that the differential cellular response to acute versus chronic ER stress alters the effects of rapamycin pre-treatment and mTOR inhibition.

Discussion

Using antigens capable of describing cell populations and stress levels, this study aimed to improve our understanding of the phenomenon of ER stress in mixed glial cultures through TU-mediated stress induction. Through our observations of the differential effects of chronic and acute ER stress and the ways in which those effects are modified by the inhibition of the mTOR pathway, we can offer several insights on the mechanism and outcome of the mixed glial response to ER stress. The following will focus on what our data tell us about the inter- and intracellular response to ER stress, and if and how that response might best be optimized through artificial intervention.

Beginning with total cell count data, we were surprised to find that chronic and acute TU exposure did not decrease the total number of cells – as measured by both live and fixed staining – in cultures relative to controls (Figure 5, Figure 8). Because of its role as an ER stressor, TU has been implicated in elevated rates of cell death (Kato *et al.*, 2012). We had expected to see the same trend in our mixed glial cultures. One possible explanation for this phenomenon is that mixed cultures offer protective intercellular benefits that are not available in enriched settings. Another possibility is that glia are differentially affected by ER stressors. For instance, astrocytes are five times more resilient than oligodendrocytes during oxidative stress, and are known to proliferate in response to inflammation (Johnstone *et al.*, 2013; Penas *et al.*, 2007; Sovrea and Bosca, 2013). Thus, while some cells may have died in response to chronic stress, others may have proliferated and effectively preserved total cell count.

To investigate whether these differential effects may have been relevant in our cultures, we consulted more detailed data on the cellular composition found in each treatment condition. Prior to our data collection, we had expected that TU treatment – acute and chronic alike – would

lead to drops in the percentage of OL-lineage cells, given that they are regarded as the most vulnerable cells in the CNS (Benjamins *et al.*, 2013). However, we found that in our mixed glial cultures, chronic TU exposure did not lead to drops in OL-lineage cell count or percentage (Figure 5, Figure 6). This was especially puzzling given previous work in our lab using enriched OL-lineage cultures. In that research, TU administration at 2 μg/ml – only 1 μg/ml higher than the chronic concentration used in our experiment – significantly lowered cell counts as rapidly as three hours post-administration, with reductions in cell viability growing over the course of the next nine hours (McInnis, 2012; see Figure 4). Furthermore, the negative effects of TU administration were greater for young cells relative to mature ones (McInnis, 2012). Thus, the fact that the relatively immature OL-lineage cells in our cultures retained viability was especially surprising.

The most fundamental difference between this previous research and our own is our decision to use of mixed, instead of enriched, glial cultures. Thus, the fact that chronic TU exposure did not reduce the percentage of OL-lineage cells may have had to do with the glia surrounding those OL-lineage cells. In particular, it is possible that the effects of chronic TU treatment were overcome by the protective actions of astrocytes on OL-lineage-cells. Those protective actions are extensive: astrocytes produce antioxidants that can reduce oxidative stress, regulate glutamate levels that could otherwise induce excitotoxicity, and produce growth factors that are key for oligodendrocyte viability (Spampinato *et al.*, 2015; Chen and Swanson, 2003). In the absence of astrocytes, oligodendrocytes are at elevated risks of damage: in mice, ablation of reactive astrocytes has been shown to lead to worse recovery in the form of more demyelination and reduced oligodendrocyte viability (Faulkner *et al.*, 2004). In the presence of astrocytes, that risk drops: when media taken from enriched astrocyte cultures was added to enriched

oligodendrocyte cultures, the latter experienced less damage associated with hydrogen peroxide-induced oxidative stress, serum starvation, and deprivation of oxygen or glucose (Arai and Lo, 2010). Interestingly, these effects were diminished when the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR signaling pathways were inhibited, suggesting that the astrocyte-mediated protection of oligodendrocytes involves interactions between these pathways and various cellular growth factors (Arai and Lo, 2010).

Indeed, the activity of the growth factors secreted by astrocytes may be a key factor in the survival of oligodendrocytes undergoing chronic TU stress. By providing neurotrophic support, the growth factors produced and distributed by astrocytes – including platelet-derived growth factor (PDGF), neurotrophin-3 (NT-3), nerve growth factor (NGF), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and insulin-like growth factor-1 (IGF-1) – are thought to reduce ER stress (Nair *et al.*, 2008; Kroeger *et al.*, 2012). For instance, IGF-1 enhances the three arms of the UPR and increases the folding capacity of the ER, lowering the likelihood of cellular apoptosis (Novosyadlyy *et al.*, 2008). Meanwhile, NGF and NT-3 each upregulate the Bcl-2 anti-apoptotic pathway, which is also the downstream target of the IRE1 arm of the UPR (Abram *et al.*, 2009). Altogether, the growth factors secreted by astrocytes can help enhance and expand the oligodendrocyte response to ER stress. As a result, when that stress is not excessively severe, the presence of astrocytes can mitigate oligodendrocyte cell death as was seen in cultures given chronic TU exposure.

However, when stress *is* excessively severe, the protective effects of astrocytes on oligodendrocytes can be overwhelmed. This could explain what was seen in our data: while astrocytes may have prevented drops in viability for OL-lineage cells undergoing chronic TU stress, cultures undergoing acute TU stress showed drops in both the number and the percentage

of OL-lineage cells (Figure 9, Figure 10). This finding aligned with previous work our lab has done using enriched OPC cultures, in which a three-hour acute treatment with 10 µg/ml TU induced a significant level of cell death (McInnis, 2012; see Figure 3). It is possible that, in the case of mixed glial cultures, the growth factors secreted by astrocytes cannot provide support rapidly enough to overcome the effects of acute treatment. Growth factor production and secretion is a formidable process, and ramping up synthesis in response to stress is far from an instantaneous process. For instance, one study assessing changes in NGF production in response to hyperosmolar stress found that it took six hours for growth factor levels to increase significantly (Chang *et al.*, 2008). While this finding comes from an experiment that differs from our own in several respects, it underscores the notion that growth factor production is a relatively gradual process. Thus, while chronic exposure offers a window of time during which astrocytes can begin to produce and secrete growth factors to protect oligodendrocytes, acute exposure may occur in too short a period for those measures to come to fruition.

Given that the protective effects of astrocytes can be overwhelmed by intense stress, the potential of mTOR inhibition to improve cell viability as a complementary protective measure is of particular interest. Our data show that rapamycin pre-treatment is generally protective for all treatment conditions, in that it increased total cell count as measured by live and fixed staining (Figure 11, Figure 12). This was somewhat unexpected – while we had hypothesized that rapamycin pre-treatment would be beneficial for cultures exposed to TU, we were surprised to observe that it also increased total cell count for control cultures. Since we anticipated that control cultures would be experiencing very little ER stress, we did not believe that mTOR inhibition would lead to any improvements in cell viability. However, given that both fixed and

live Hoechst stain data stand together in opposing this hypothesis, the proliferative effects of rapamycin appear to be quite robust even in the absence of TU.

One possible explanation of this phenomenon is that control cultures *were* experiencing significant levels of stress. This can be seen in the percentage of cells expressing CHOP, a marker of ER stress and UPR induction, which for control cultures is nearly 50% – a surprisingly high figure (Figure 15). Thus, baseline stress for all cultures may have been relatively high, potentially as a result of incubator variability, media composition, or other minor insults. If this was the case, then rapamycin pre-treatment may have been helpful for controls by helping to dampen the effects of that baseline stress by inhibiting excessive protein production and allowing for a return to equilibrium. Another potential reason for the efficacy of rapamycin pre-treatment is that our mixed cultures may have contained reactive microglia that induced inflammation and stress (Rock *et al.*, 2004). By acting as an immunosuppressant, rapamycin may have limited the damage those microglia were able to cause and improved overall cell survival.

In addition to analyzing the effects of rapamycin of total cell counts, we investigated how rapamycin altered the percentage of cells that were OL-lineage. For A2B5/O4 and Olig2 measures of OL-lineage cells, rapamycin pre-treatment did not lead to alterations in overall percentage for both control and chronic TU treatments (Figure 13, Figure 14). Since rapamycin increased overall cell count without changing the percentage of that cell count that was OL-lineage, this implies that it led to proportional increases in OL-lineage cells and can be said to be a protective factor in these instances. However, while rapamycin pre-treatment did not change the percentage of total cells that expressed A2B5/O4 in acute TU cultures, it did lead to a significant drop in the percentage of cells expressing Olig2 in those cultures (Figure 13, Figure 14). Without a greater variety of metrics, it is difficult to determine why rapamycin pre-treatment

would be less protective of oligodendrocytes in acute stress conditions relative to chronic stress conditions. One possibility is that, much like the protective effects exerted by astrocytes, the benefits due to rapamycin require time. Whereas in cases of chronic TU exposure the inhibition of mTOR may aid the UPR by reducing protein production, in cases of acute TU exposure the inhibition of mTOR may not be a sufficient step to improve cellular viability.

Our study also raises a potential issue concerning the use of rapamycin in combatting ER stress in mixed glial cultures. This particular issue relates to the protective effects of astrocytes that were discussed above, and which we suspect are at least in part exerted through growth factors. The effectiveness of those growth factors relies, at least in part, on both the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways (Ballou and Zin, 2008). For instance, studies have found that the activity of IGF-1 and CNTF, both of which are neurotrophic growth factors secreted by astrocytes, require mTOR signaling (Lau *et al.*, 2012; Yokogami *et al.*, 2000; Kroeger *et al.*, 2012). Thus, when rapamycin is used to inhibit mTOR and prevent protein translation, it might also interfere with the ability of astrocytes to protect oligodendrocytes via growth factor secretion.

As a final step in our attempt to understand the ER stress response of mixed glial cultures, we investigated CHOP expression and how it was altered by mTOR inhibition. Since CHOP is associated with ER stress, it was expected that TU administration – both chronic and acute – would increase CHOP expression relative to control levels, and that rapamycin pre-treatment would decrease CHOP expression by ameliorating ER stress (Kuroiwa *et al.*, 2014). However, the percentage of cells expressing CHOP did not increase in chronic conditions relative to control conditions, and exhibited a significant *decrease* in acute conditions relative to control conditions (Figure 15). This was unexpected, since studies have found that TU

administration significantly increases CHOP expression in oligodendrocytes (Lin *et al.*, 2005; Mecha *et al.*, 2012; Southwood *et al.*, 2002). However, one possible explanation echoes our previous reasoning: during acute TU stress, cells had very little time to respond. In other words, the percentage of cells that expressed CHOP during acute TU stress may have remained low simply because of the brevity of the treatment period. For instance, one experiment that induced ER stress *in vivo* via ischemia found that CHOP mRNA expression began to increase six hours after injury, with production peaking 12 hours post-injury (Tajiri *et al.*, 2004). Another study found that CHOP induction in oxygen- and glucose-deprived astrocytes led to CHOP mRNA induction that became visible at one hour, but which did not reach peak levels until six hours post-insult (Benavides *et al.*, 2005). Thus, it appears that CHOP requires extended treatment periods in order to be produced on a significant scale, and is perhaps an inappropriate measure in cases of acute stress where treatment periods are too brief for CHOP production to reach peak levels.

The data amassed in this experiment allowed us to test several of our hypotheses. First, we found that chronic TU exposure was not sufficient to induce decreases in total cell count or OL-lineage cell count. We believe that this may be due to the protective effects of astrocytes in mixed cultures, which confirms our hypothesis that intercellular interactions in our cultures protected cells from toxicity. We further believe that these protective effects are mediated through growth factors, and thus require a generous amount of time to be felt. By extension, the extended time-course of astrocytic protective mechanisms relates to our findings concerning short-term, severe stress. We observed that acute TU exposure did induce drops in OL-lineage cell percentages. In this case, we believe that stress was intense and abbreviated enough to cause harm to oligodendrocytes before the protective growth factors secreted by astrocytes could be

produced and distributed in order to ameliorate that harm. This adds nuance to our hypothesis on the benefits provided to oligodendrocytes during ER stress by other glia, suggesting that it is only in conditions of modest stress that these benefits can stave off significant cell death. In our investigation of the efficacy of rapamycin inhibition of mTOR, we observed that pre-treatment with rapamycin increased total cell counts and preserved the percentage of cells that were OL-lineage in both chronic and control conditions. During acute TU exposure, however, rapamycin pre-treatment could not prevent a significant drop in OL-lineage cell percentage, perhaps because that stress was too great to be remediated through the downregulation of protein production. Ultimately, we can partly accept our hypothesis on the utility of rapamycin inhibition of mTOR during ER stress, though with the caveat that extreme stress will render rapamycin less potent. Finally, we found that CHOP expression was not upregulated in cells that had been given TU treatments. We speculate that, for chronic TU treatment, astrocytic protection of oligodendrocytes prevented an increase in CHOP; for acute TU treatment, meanwhile, we believe that the time-course was too short to allow for measurable increases in CHOP expression.

While the data collected in our research are far from conclusive, they raise extremely interesting questions about the differences in ER stress response in mixed and enriched cell cultures. Our data suggest a critical role of astrocytes in improving oligodendrocyte viability in the face of long-term, moderate stress – a role that would be neglected in research assessing oligodendrocytes in isolation. In order to better understand how these effects are exerted, more research is needed. In the future, it would be invaluable to perform experiments similar to ours in enriched and mixed glial cultures simultaneously. By controlling for variability between experiments, this methodology would affirm whether or not mixed glial cultures truly

demonstrate improved viability in response to ER stress. Secondly, the mechanism of the protective effects offered by astrocytes during ER stress might better be understood by using growth factor receptor inhibitors. If it is true that astrocytes therapeutically act on oligodendrocytes via growth factors, then inhibiting their receptors should worsen oligodendrocyte viability during ER stress. By exploring these issues, we can better understand the role of astrocytes in oligodendrocyte viability during ER stress. This knowledge, in turn, is critical for understanding how ER stress and the demyelination that results from it might best be treated.

Figures

Figure 1. The development of OL-lineage cells from early progenitors to mature oligodendrocytes. Notice that A2B5 and O4 (combined) or Olig2 staining will label OL-lineage cells at any and all stages in development (Guardiola-Diaz *et al.*, 2012).

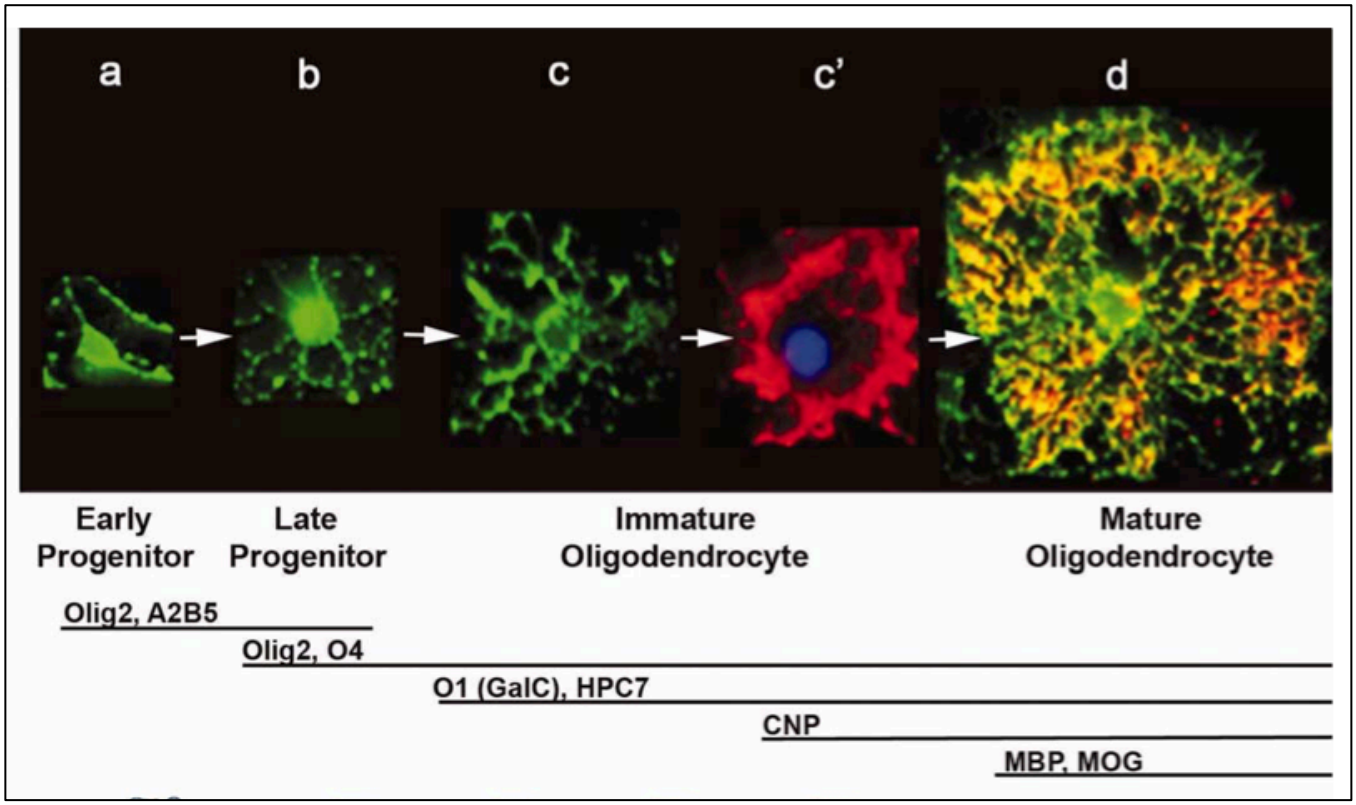

a
b
c
c'
d
Early Progenitor
Late Progenitor
Immature Oligodendrocyte
Mature Oligodendrocyte
Olig2, A2B5
Olig2, O4
O1 (GalC), HPC7
CNP
MBP, MOG

Figure 2. The three signaling pathways of the UPR. The UPR comprises the ATF6 pathway, the PERK pathway, and the IRE1 pathway (Walter and Ron, 2011).

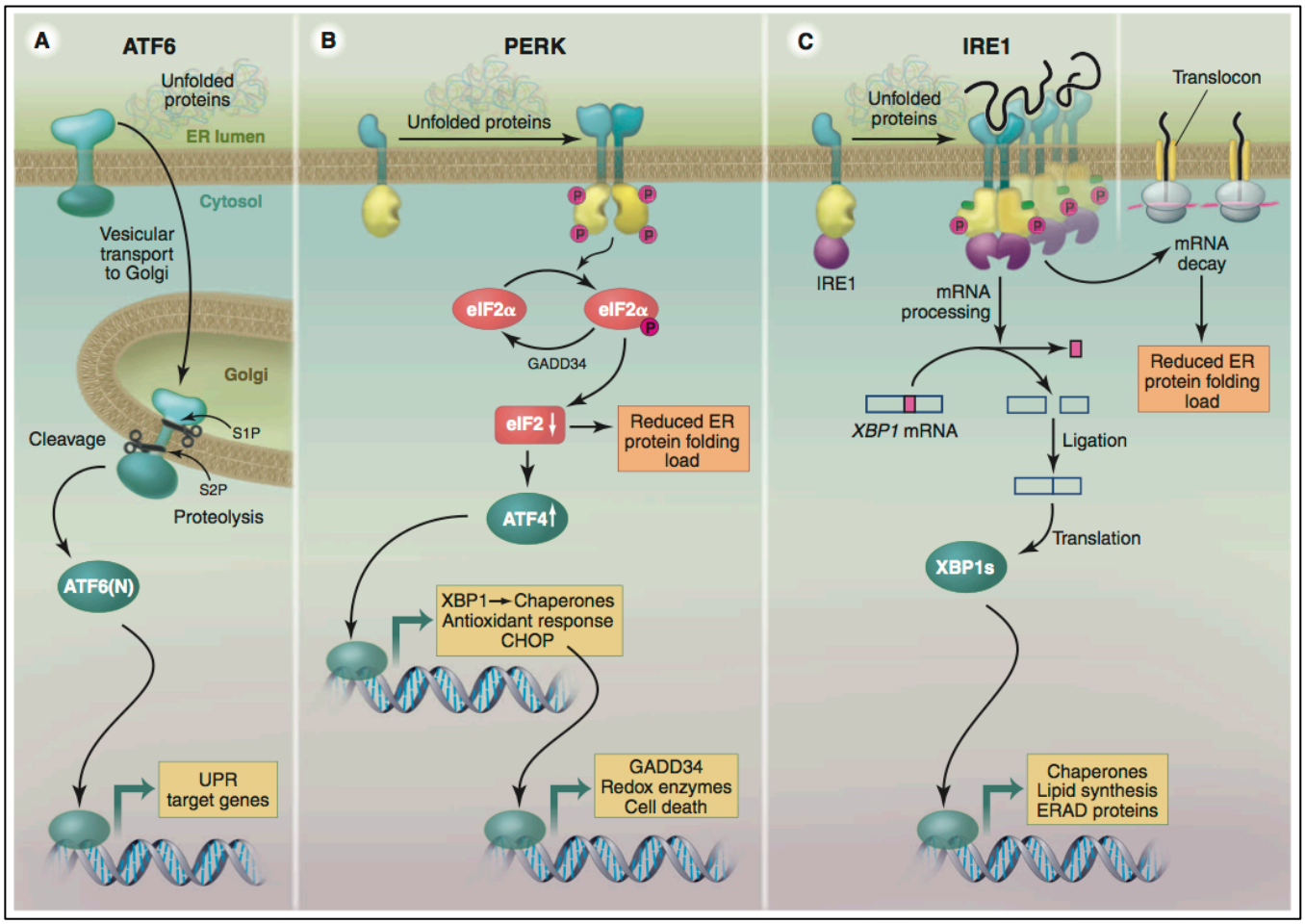

A
ATF6
Unfolded proteins
ER lumen
Cytosol
Vesicular transport to Golgi
Golgi
Cleavage
S1P
S2P
Proteolysis
ATF6(N)
UPR target genes
B
PERK
Unfolded proteins
eIF2α
eIF2α
GADD34
eIF2
Reduced ER protein folding load
ATF4
XBP1→Chaperones
Antioxidant response
CHOP
GADD34
Redox enzymes
Cell death
C
IRE1
Unfolded proteins
Translocon
IRE1
mRNA processing
mRNA decay
Reduced ER protein folding load
XBP1 mRNA
Ligation
Translation
XBP1s
Chaperones
Lipid synthesis
ERAD proteins

Figure 3: The negative effects of three-hour, acute TU exposure at 10 µg/ml on the viability of oligodendrocyte precursor cells in enriched cultures. Greater concentrations of TU are associated with greater drops in cell viability as a result of increased ER stress (McInnis, 2012).

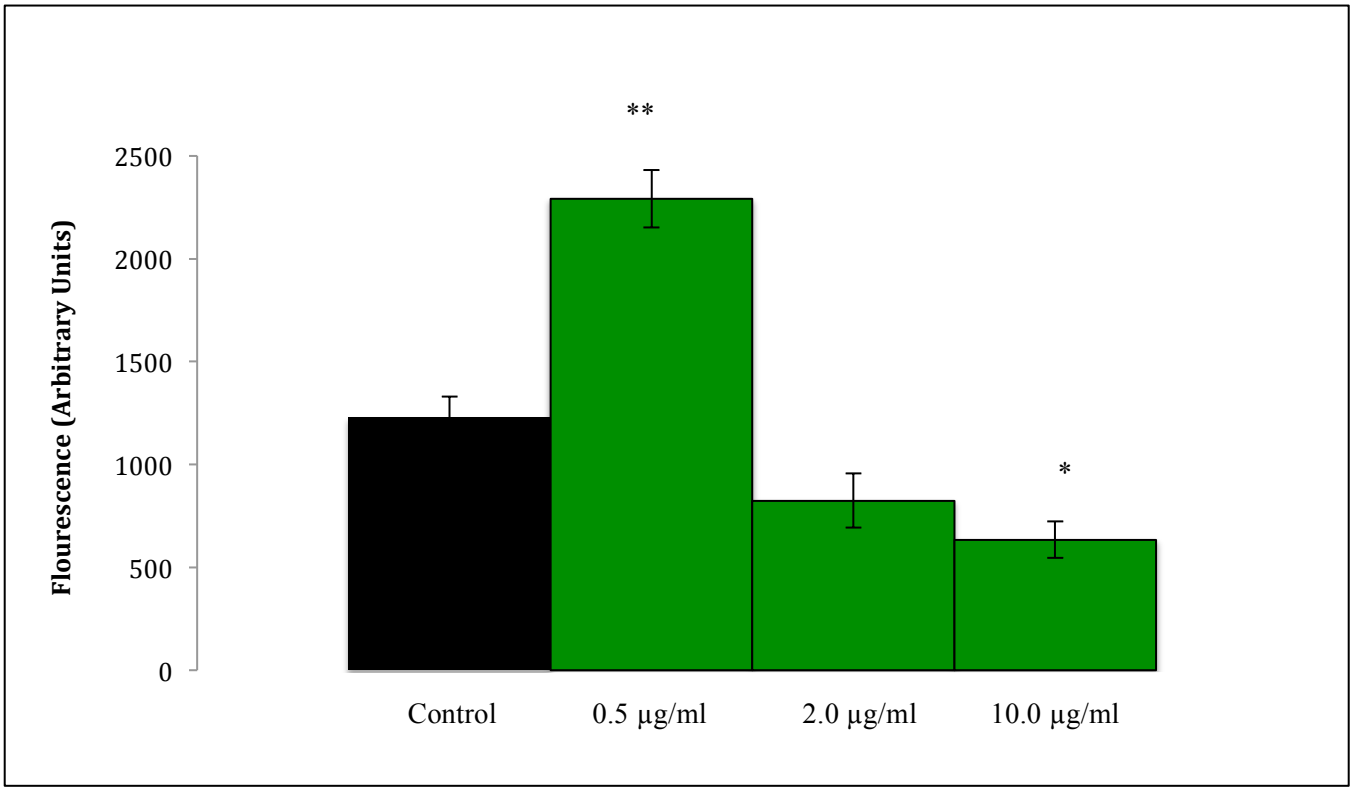

**
*
2500
2000
1500
1000
500
0
Flourescence (Arbitrary Units)
Control
0.5 µg/ml
2.0 µg/ml
10.0 µg/ml

Figure 4: The negative effects of twelve-hour, chronic TU exposure at 2 µg/ml on the viability of oligodendrocyte precursor cells in enriched cultures. Longer durations of TU exposure were associated with greater drops in cell viability (McInnis, 2012).

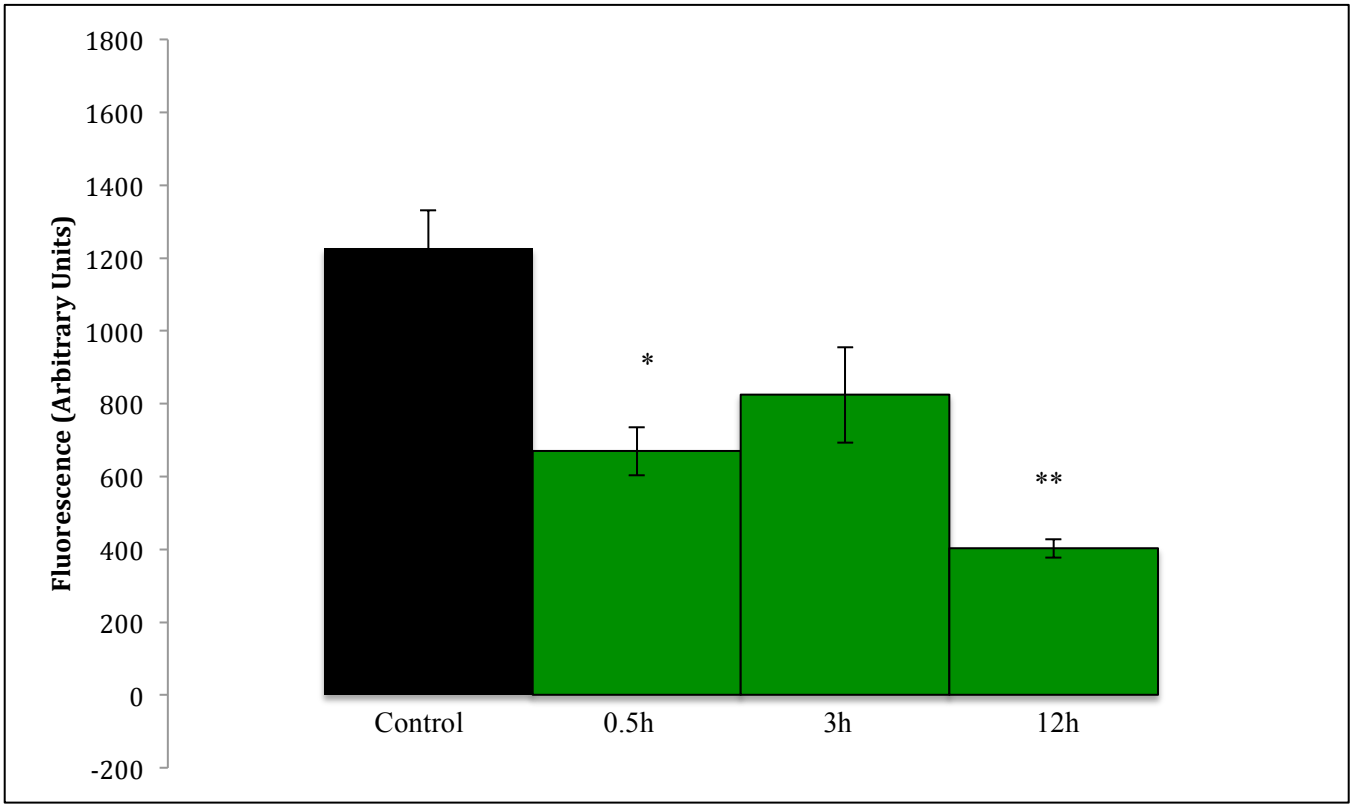

1800
1600
1400
1200
1000
800
600
400
200
0
-200
Fluorescence (Arbitrary Units)
*
**
Control
0.5h
3h
12h

Figure 5. Chronic exposure to TU failed to decrease the total number of Hoechst-positive cells in mixed glial cultures. Mixed glial cultures were grown in the absence (control, n=16) or presence of tunicamycin (TU, 1 μg/ml, n=6) for 24 hours and stained under live (dark) and fixed (light) conditions with Hoechst to determine total cell count. Error bars represent standard error of the mean (SEM).

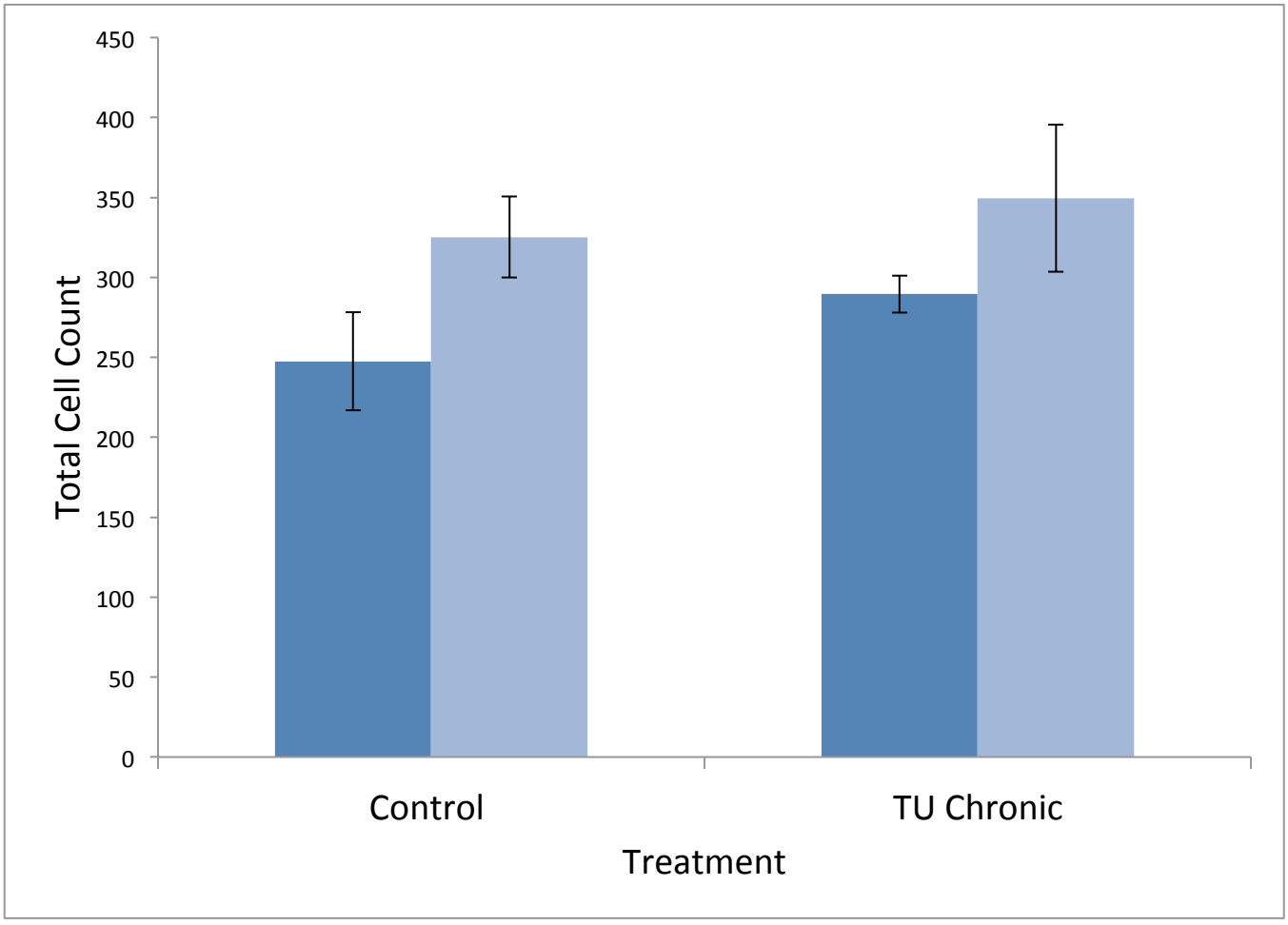

450
400
350
300
250
200
150
100
50
0
Total Cell Count
Control
TU Chronic
Treatment

Figure 6. Chronic exposure to TU failed to decrease the number of OL-lineage cells in mixed glial cultures. Mixed glial cultures were grown in the absence (control, n=16) or presence of tunicamycin (TU, 1 μg/ml, n=6) for 24 hours and analyzed by immunolabeling for A2B5/O4 (dark) and Olig2 (light). Error bars represent SEM.

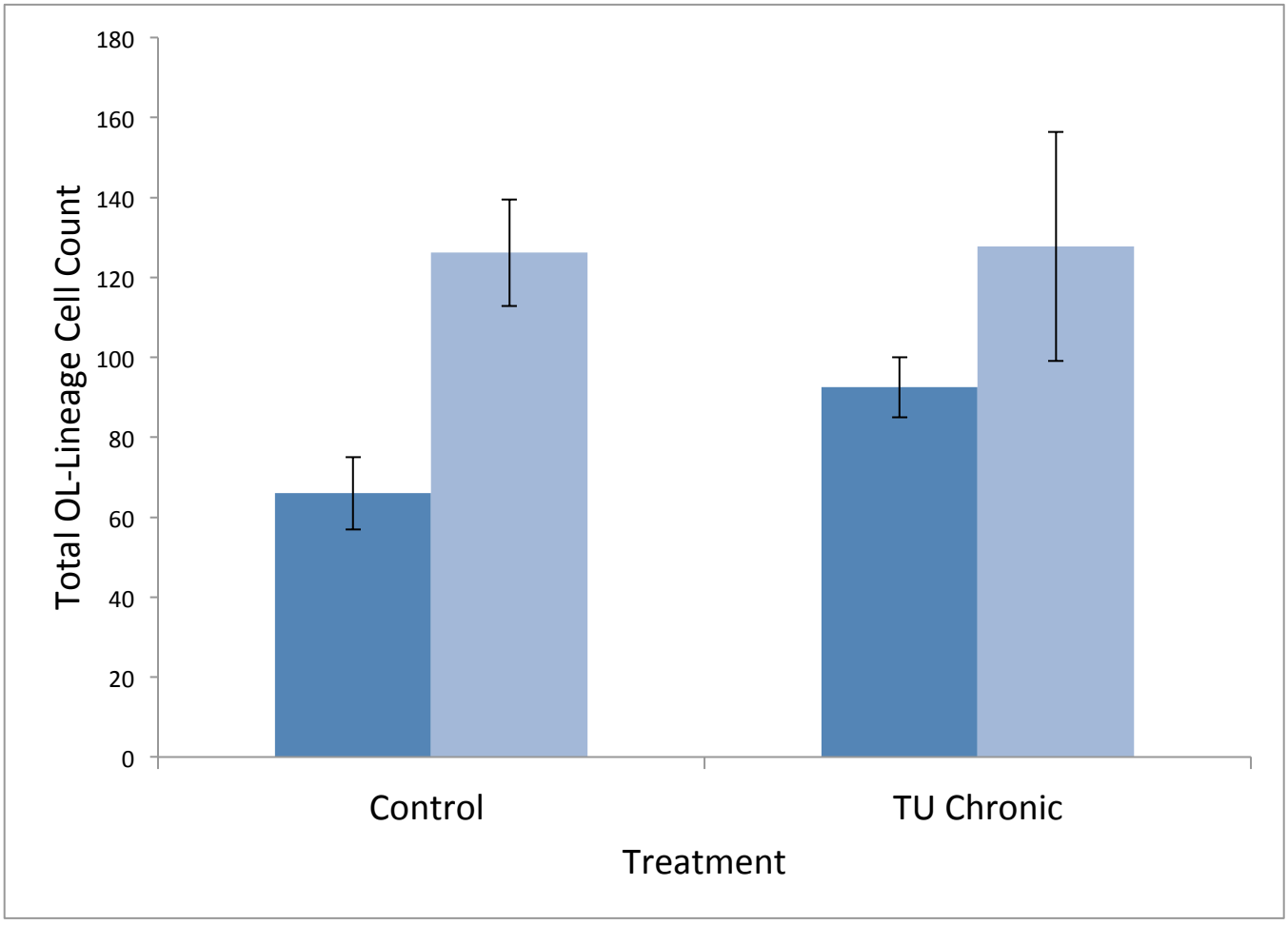

180
160
140
120
100
80
60
40
20
0
Total OL-Lineage Cell Count
Control
TU Chronic
Treatment

Figure 7. Chronic exposure to TU failed to decrease the percentage of total cells that were OL-lineage in mixed glial cultures. Mixed glial cultures were grown in the absence (control, n=16) or presence of tunicamycin (TU, 1 μg/ml, n=6) for 24 hours and analyzed by immunolabeling for A2B5/O4 (dark) and Olig2 (light). Error bars represent SEM.

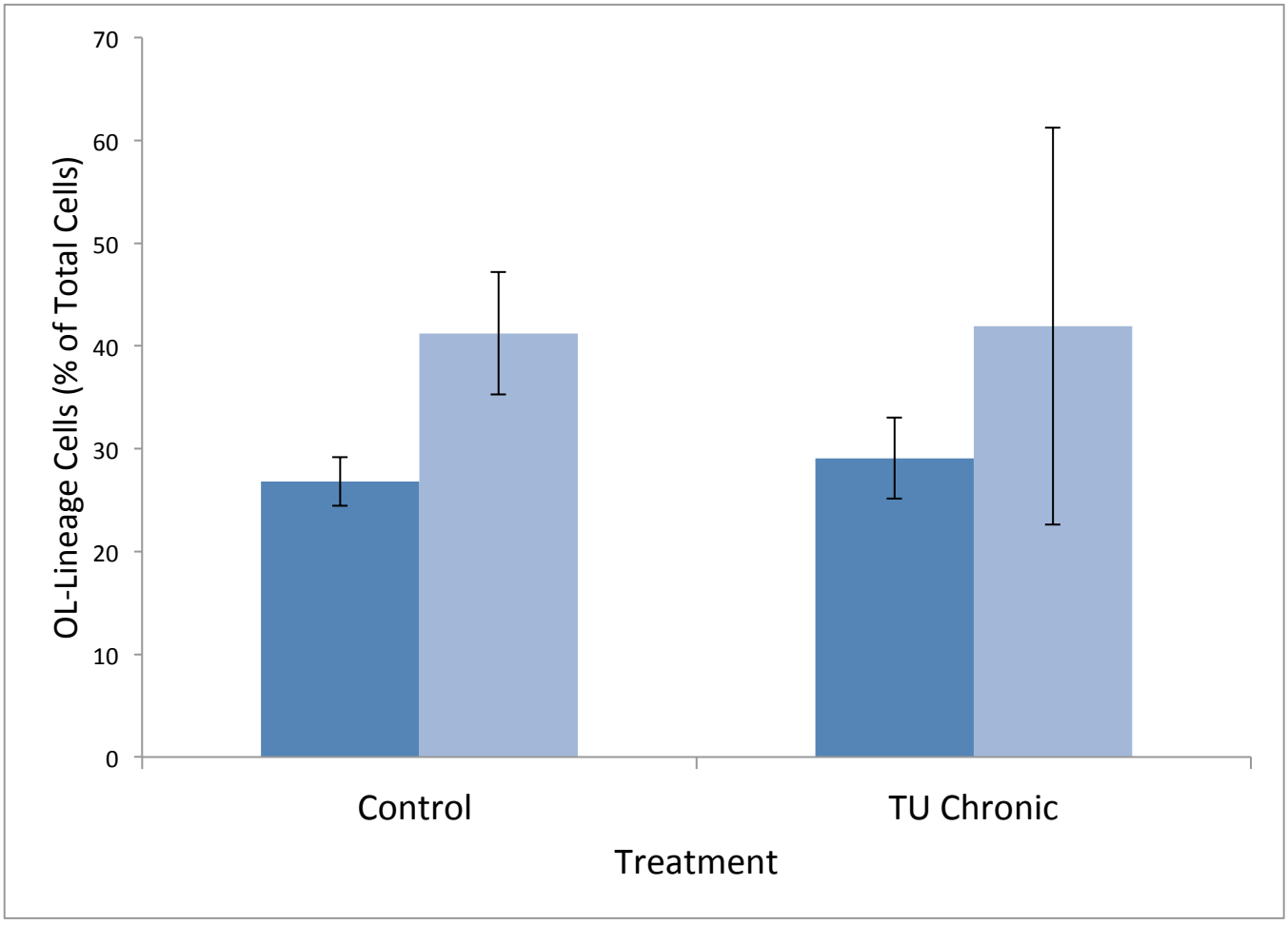

70
60
50
40
30
20
10
0
OL-Lineage Cells (% of Total Cells)
Control
TU Chronic
Treatment

Figure 8. Acute exposure to TU failed to decrease the total number of Hoechst-positive cells in mixed glial cultures. Mixed glial cultures were grown in the absence (control, n=16) or presence of tunicamycin (TU, 10 µg/ml, n=8) for 3 hours and stained under live (dark) and fixed (light) conditions with Hoechst to determine total cell count. Error bars represent SEM.

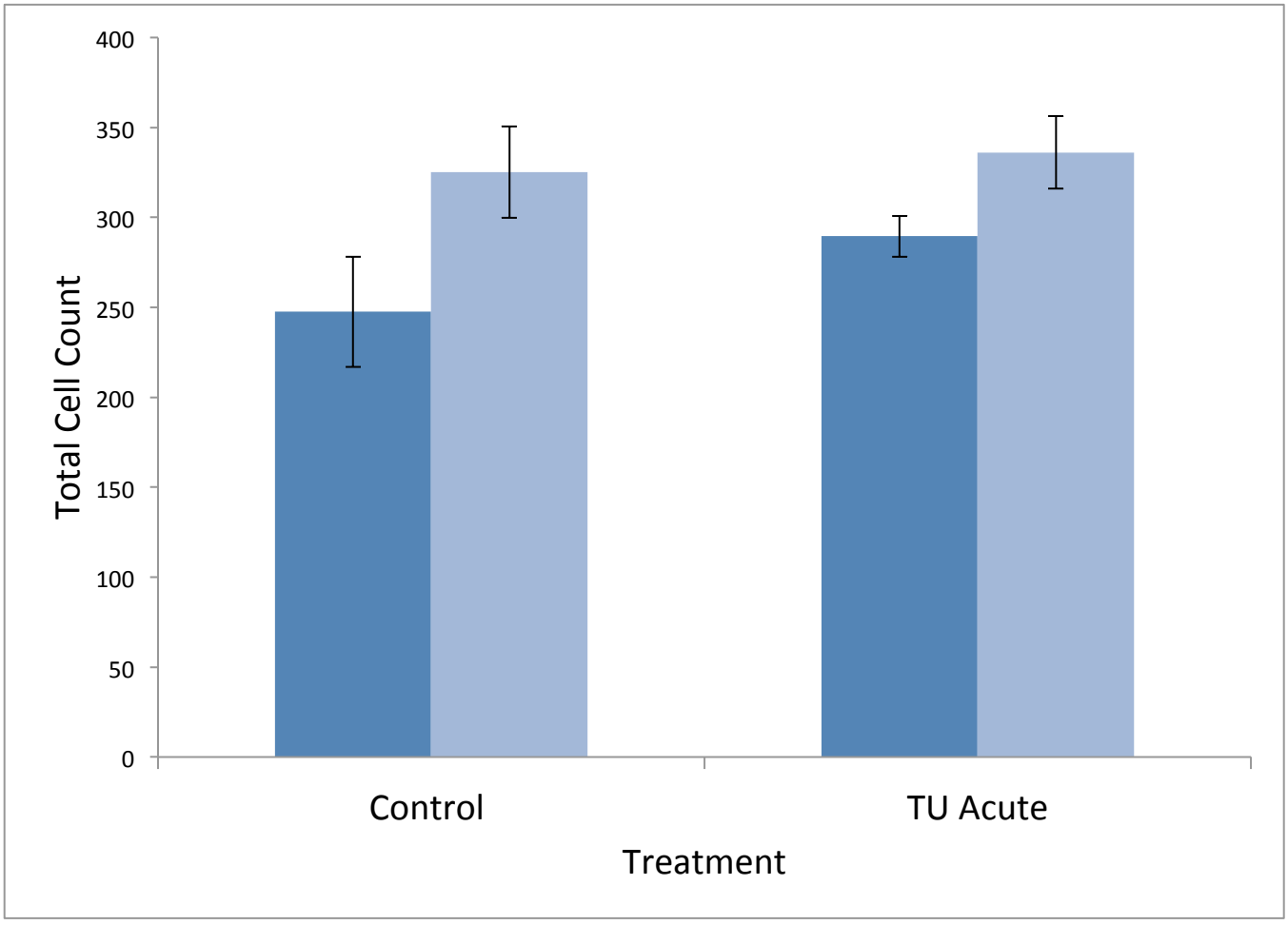

400
350
300
250
200
150
100
50
0
Total Cell Count
Control
TU Acute
Treatment

Figure 9. Acute exposure to TU significantly decreased the number of OL-lineage cells in mixed glial cultures. Mixed glial cultures were grown in the absence (control, n=16) or presence of tunicamycin (TU, 10 μg/ml, n=8) for 3 hours and analyzed by immunolabeling for A2B5/O4 (dark) and Olig2 (light). Acute TU exposure was associated with a significant decrease in Olig2-positive cells ($p=0.0039$) and an insignificant decrease in A2B5/O4-positive cells ($p=0.062$). Error bars represent SEM.

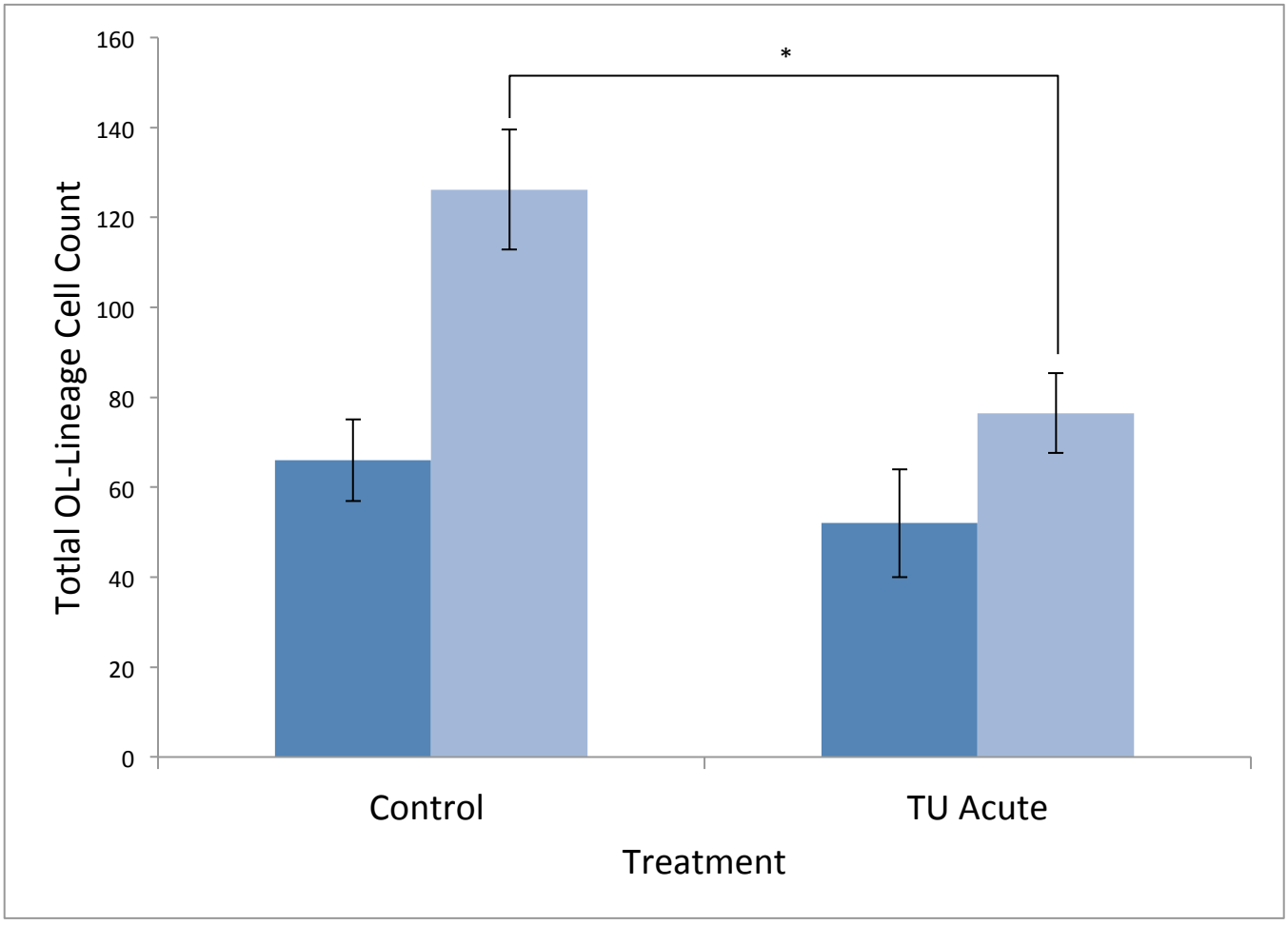

160
140
120
100
80
60
40
20
0
Totlal OL-Lineage Cell Count
*
Control
TU Acute
Treatment

Figure 10. Acute exposure to TU significantly decreased the percentage of OL-lineage cells in mixed glial cultures. Mixed glial cultures were grown in the absence (control, n=16) or presence of tunicamycin (TU, 10 μg/ml, n=8) for 3 hours and analyzed by immunolabeling for A2B5/O4 (dark) and Olig2 (light). Acute TU exposure was associated with a significant decrease in the percentage of Olig2-positive cells signaling ($p=0.01$) and an insignificant decrease in the percentage of A2B5/O4-positive cells ($p=0.079$). Error bars represent SEM.

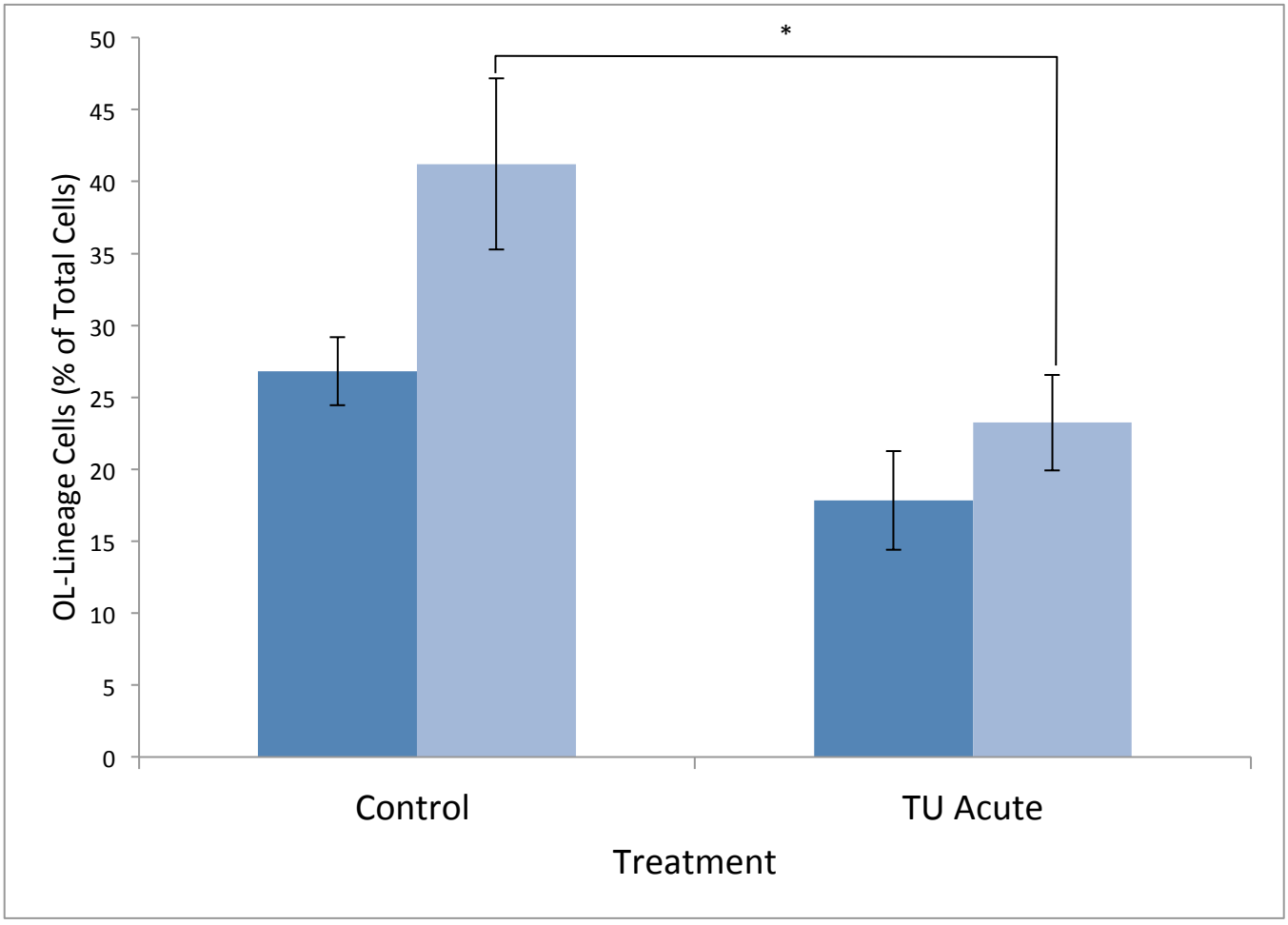

*
50
45
40
35
30
25
20
15
10
5
0
OL-Lineage Cells (% of Total Cells)
Control
TU Acute
Treatment

Figure 11. Rapamycin pre-treatment significantly increased the total number of Hoechst-positive cells in mixed glial cultures for all treatment conditions, as measured by live staining. Mixed glial cultures were grown in the absence (control, n=6 for live staining) or presence of chronic (1 μg/ml for 24 hours, n=2 for live staining) and acute (10 μg/ml for 3 hours, n=2 for live staining) tunicamycin and stained under live conditions with Hoechst to determine total cell count. Some cultures from each condition were pre-treated with rapamycin prior to treatment (control n=2, chronic n=2, acute n=2). Rapamycin pre-treatment resulted in significant increases for control cultures ($p=0.0025$), chronic cultures ($p=0.038$), and acute cultures ($p=0.035$). Error bars represent SEM.

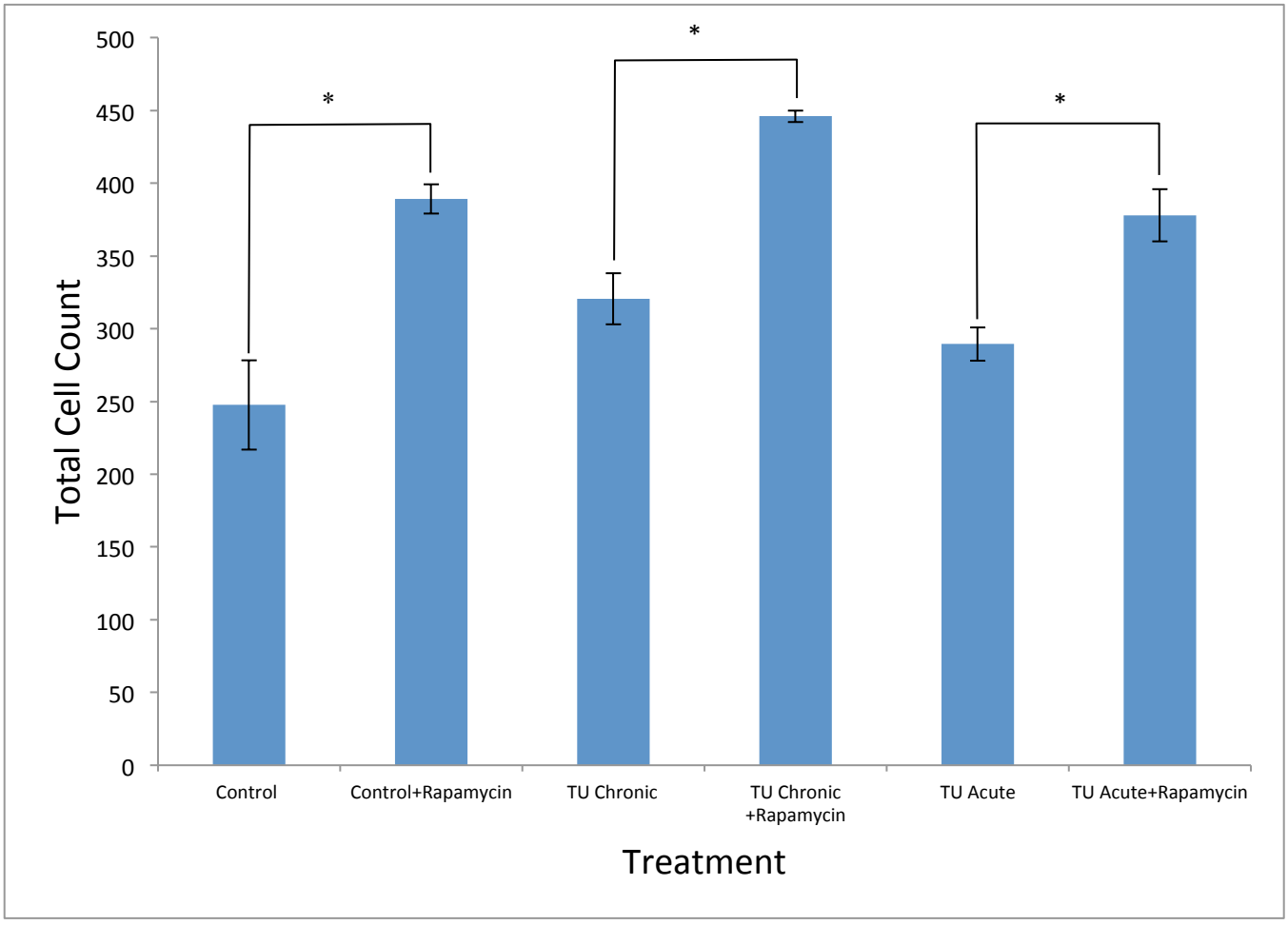

500
450
400
350
300
250
200
150
100
50
0
Total Cell Count
*
*
*
Control
Control+Rapamycin
TU Chronic
TU Chronic +Rapamycin
TU Acute
TU Acute+Rapamycin
Treatment

Figure 12. Rapamycin pre-treatment significantly increased the total number of Hoechst-positive cells in mixed glial cultures for control and acute TU conditions, as measured by fixed staining. Mixed glial cultures were grown in the absence (control, n=10 for fixed staining) or presence of chronic (1 μg/ml for 24 hours, n=4 for fixed staining) and acute (10 μg/ml for 3 hours, n=6 for fixed staining) tunicamycin and stained under fixed conditions with Hoechst to determine total cell count. Some cultures from each condition were pre-treated with rapamycin prior to treatment (control n=6, chronic n=4, acute n=2). Rapamycin pre-treatment resulted in significant increases for control cultures ($p=0.0056$) and acute cultures ($p=0.0023$). Chronic cultures showed an insignificant increase in total cell count in response to rapamycin pre-treatment ($p=0.10$). Error bars represent SEM.

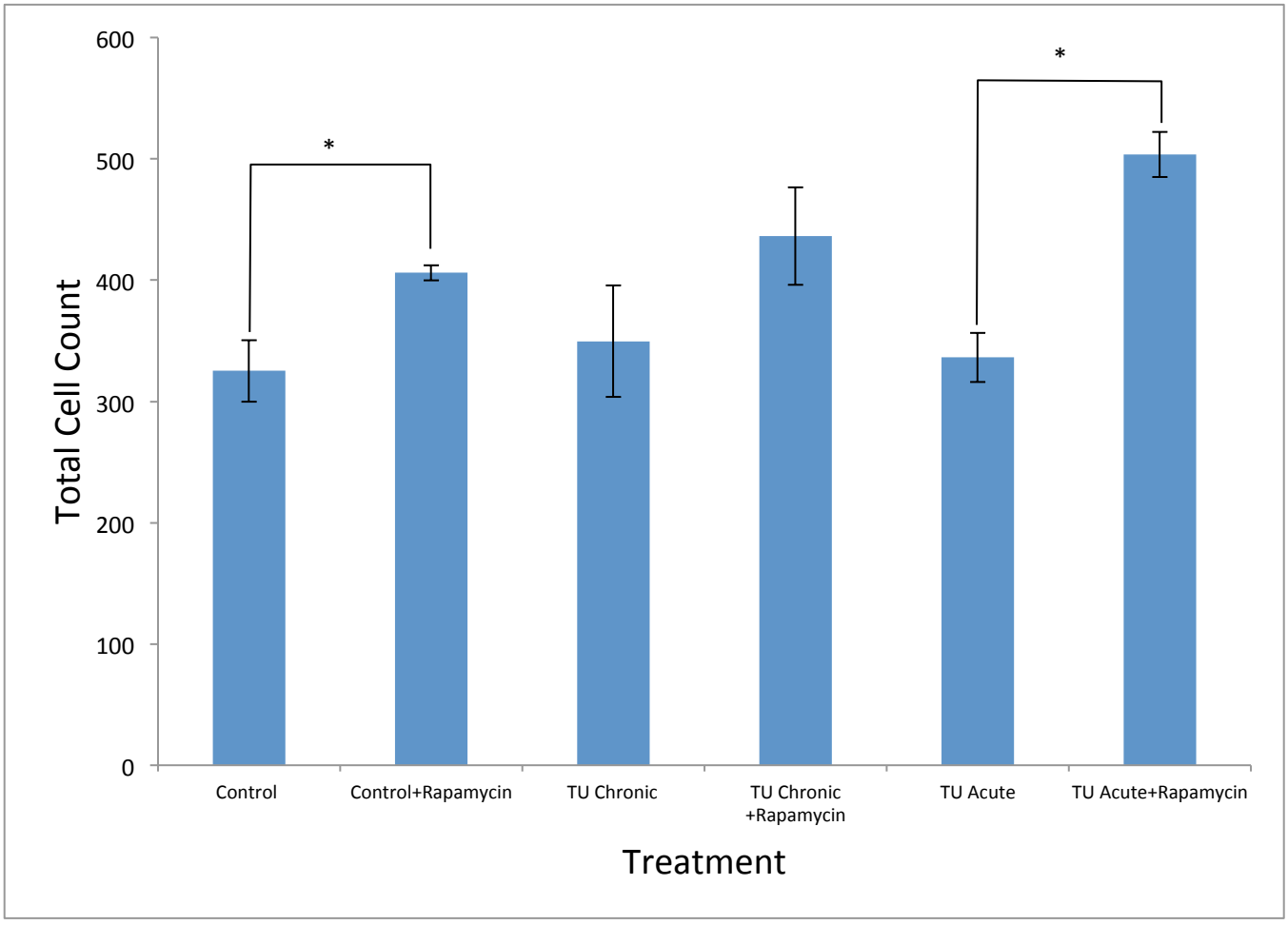

600
500
400
300
200
100
0
Total Cell Count
*
*
Control
Control+Rapamycin
TU Chronic
TU Chronic +Rapamycin
TU Acute
TU Acute+Rapamycin
Treatment

Figure 13. Rapamycin pre-treatment did not significantly alter the percentage of A2B5/O4-OL-lineage cells in mixed glial cultures for any TU treatment condition. Mixed glial cultures were grown in the absence (control, n=6 for live staining) or presence of chronic (1 μg/ml for 24 hours, n=2 for live staining) and acute (10 μg/ml for 3 hours, n=2 for live staining) tunicamycin and analyzed by immunolabeling for A2B5/O4. Some cultures from each condition were pre-treated with rapamycin prior to treatment (control n=2, chronic n=2, acute n=2). No variation in OL-lineage cell percentage was found as a result of rapamycin pre-treatment. Error bars represent SEM.

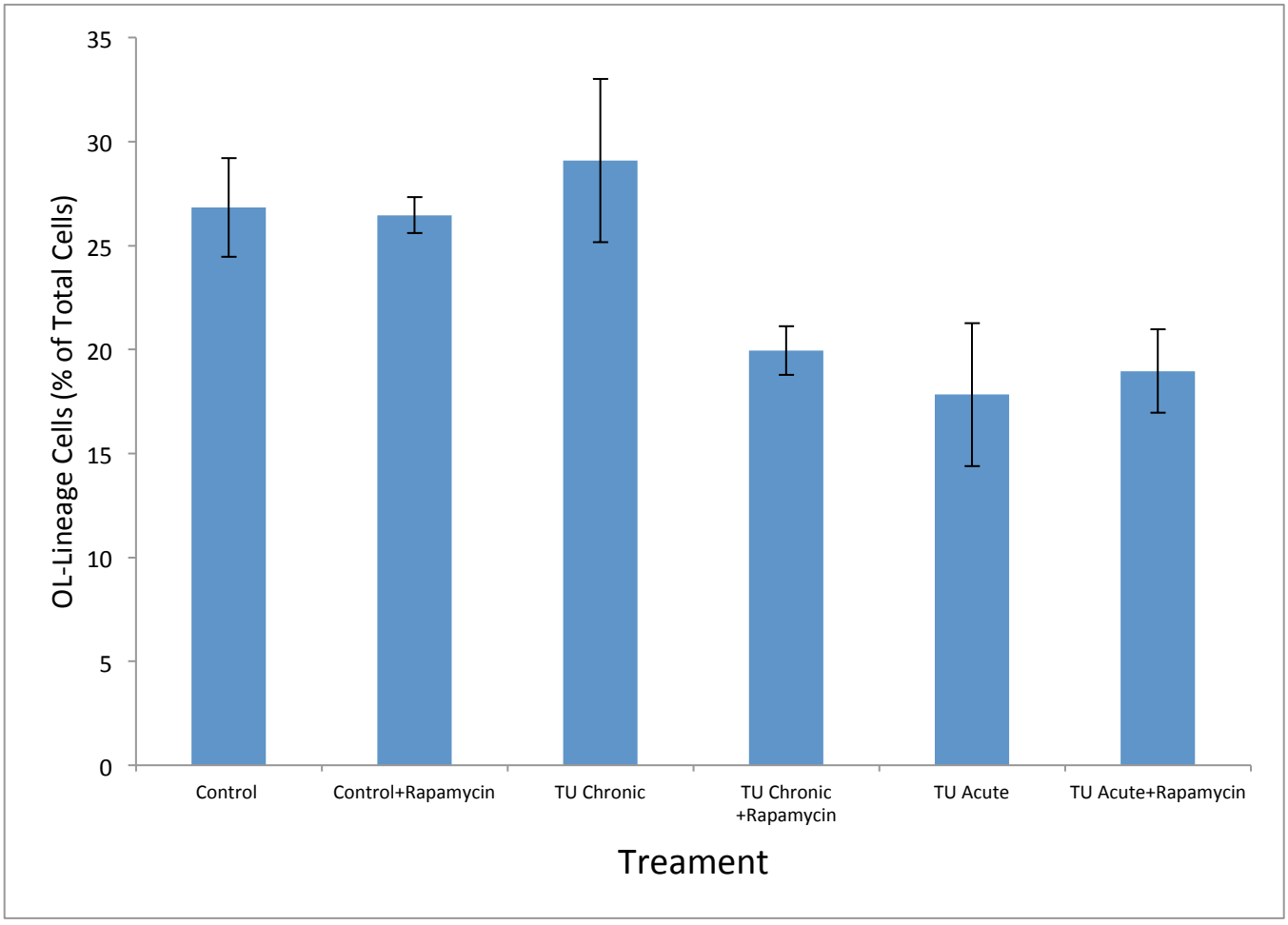

35
30
25
20
15
10
5
0
OL-Lineage Cells (% of Total Cells)
Control
Control+Rapamycin
TU Chronic
TU Chronic +Rapamycin
TU Acute
TU Acute+Rapamycin
Treament

Figure 14. Rapamycin pre-treatment did not significantly alter the percentage of Olig2-OL-lineage cells in mixed glial cultures for control and chronic TU conditions and lowered the percentage for acute conditions. Mixed glial cultures were grown in the absence (control, n=10 for fixed staining) or presence of chronic (1 μg/ml for 24 hours, n=4 for fixed staining) and acute (10 μg/ml for 3 hours, n=6 for fixed staining) tunicamycin and analyzed by immunolabeling for Olig2. Some cultures from each condition were pre-treated with rapamycin prior to treatment (control n=6, chronic n=4, acute n=2). No variation in OL-lineage cell percentage was found as a result of rapamycin pre-treatment for control and chronic conditions, though it was found to lead to a decrease in the percentage of Olig2-positive cells under acute TU conditions ($p=0.028$). Error bars represent SEM.

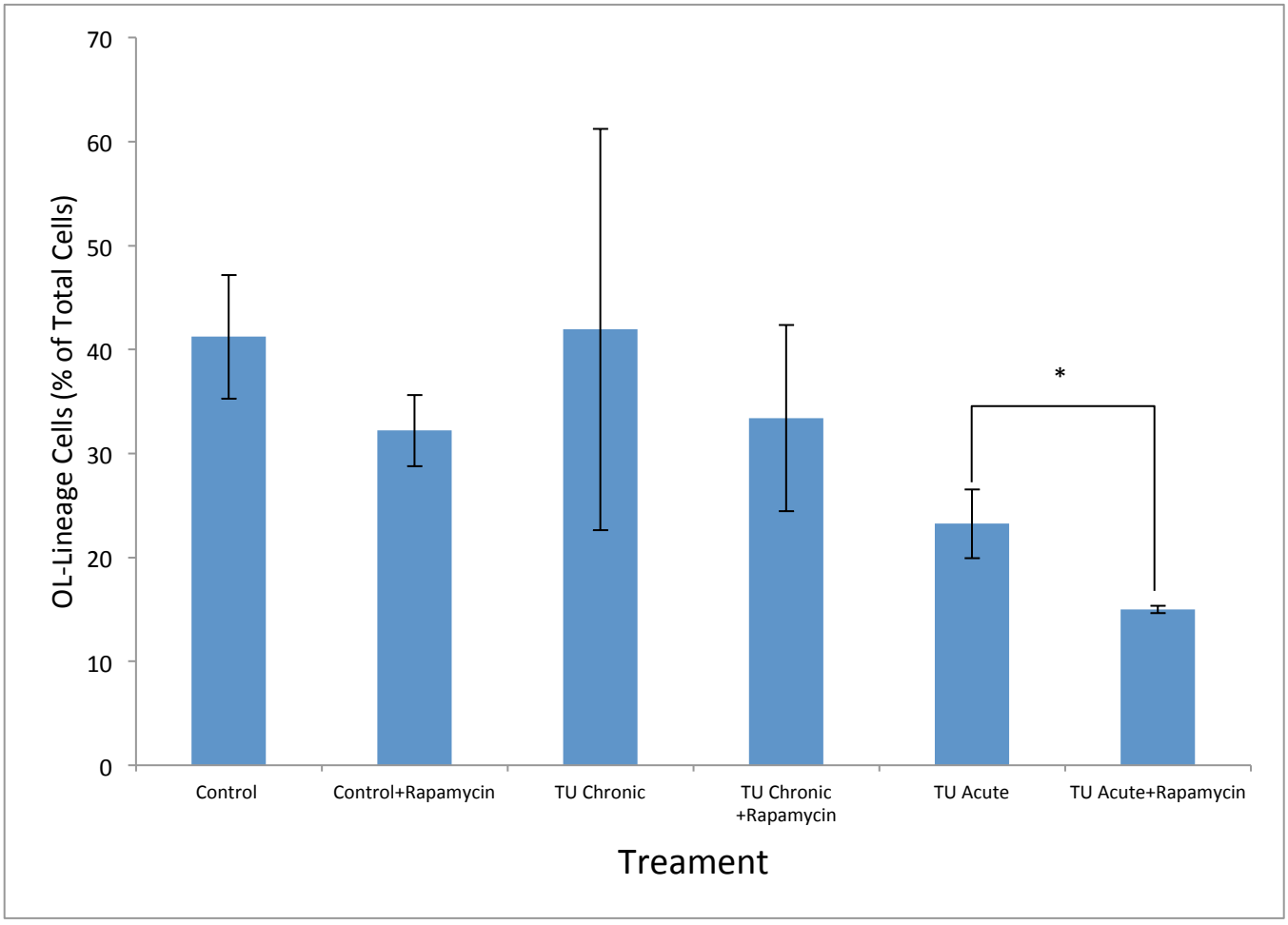
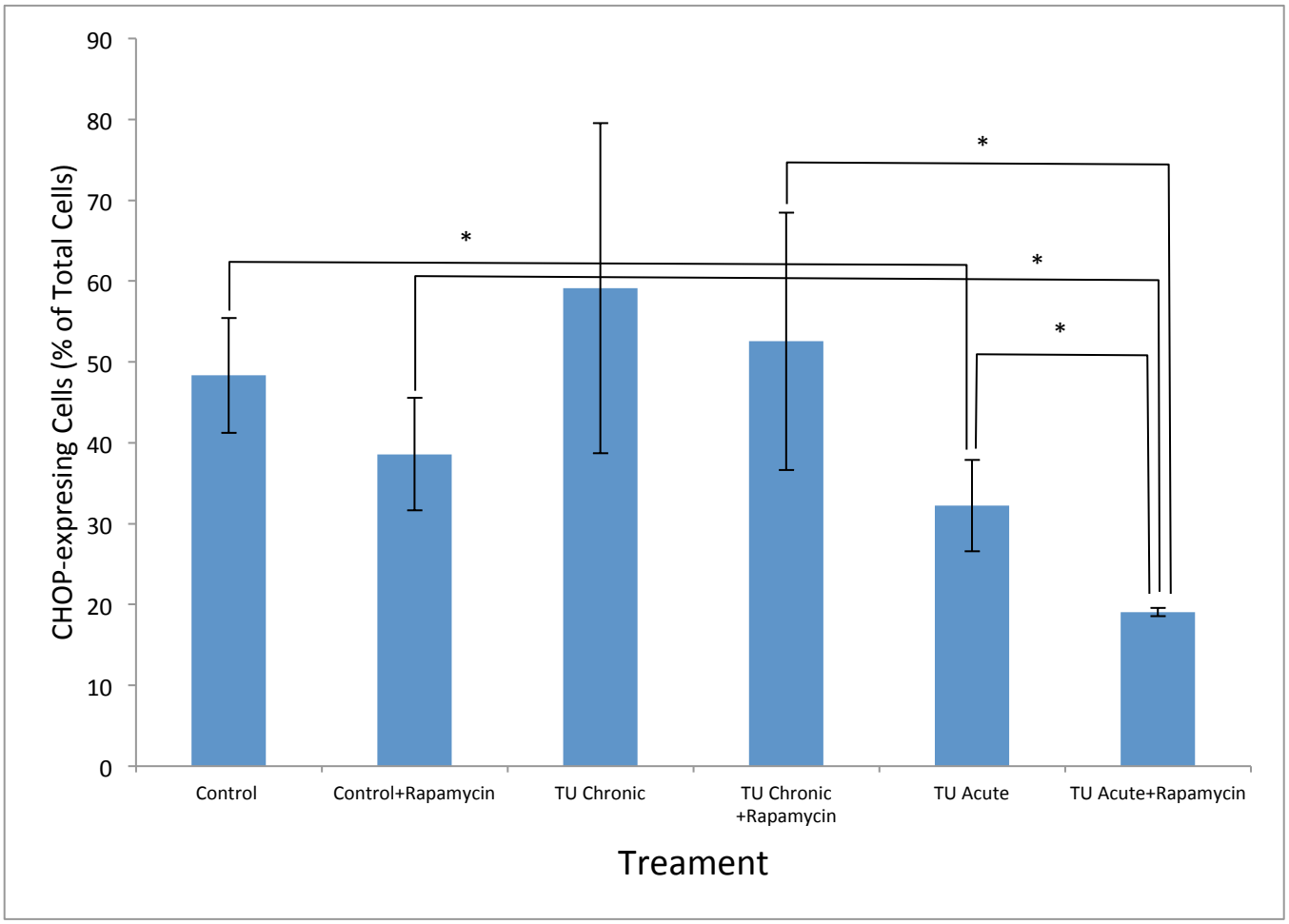

70
60
50
40
30
20
10
0
OL-Lineage Cells (% of Total Cells)
*
Control
Control+Rapamycin
TU Chronic
TU Chronic +Rapamycin
TU Acute
TU Acute+Rapamycin
Treament

Figure 15. Rapamycin pre-treatment reduced CHOP expression under acute conditions, but not under control and chronic conditions. Mixed glial cultures were grown in the absence (control, n=10 for fixed staining) or presence of chronic (1 μg/ml for 24 hours, n=4 for fixed staining) and acute (10 μg/ml for 3 hours, n=6 for fixed staining) tunicamycin and analyzed by immunolabeling for CHOP. Some cultures from each condition were pre-treated with rapamycin prior to treatment (control n=6, chronic n=4, acute n=2). Rapamycin pre-treatment reduced CHOP expression under acute TU conditions ($p=0.033$), while acute TU conditions with rapamycin pre-treatment exhibited less CHOP expression than chronic TU conditions with rapamycin pre-treatment ($p=0.0497$) and control conditions with rapamycin pre-treatment ($p=0.019$). Error bars represent SEM.


90
80
70
60
50
40
30
20
10
0
CHOP-expresing Cells (% of Total Cells)
*
*
*
*
Control
Control+Rapamycin
TU Chronic
TU Chronic +Rapamycin
TU Acute
TU Acute+Rapamycin
Treament

References

1. Abram M, Wegmann M, Fokuhl V, Sonar S, Luger EO, Kerzel S, Radbruch A, Renz H, Zemlin M (2009) Nerve growth factor and neurotrophin-3 mediate survival of pulmonary plasma cells during the allergic airway inflammation. The Journal of Immunology, 182(8): 4705-4712.
2. Appenzeller-Herzog C, Hall MN (2012) Bidirectional crosstalk between endoplasmic reticulum stress and mTOR signaling. Trends in Cell Biology, 22(5): 272-282.
3. Arai K, Lo EH (2010) Astrocytes protect oligodendrocyte precursor cells via MEK/ERK and PI3K/Akt signaling. Journal of Neuroscience Research, 88: 758-763.
4. Bachar-Wikstrom E, Wikstrom JD, Ariav Y, Tirosh B, Kaiser N, Cerasi E, Leibowitz G (2012) Stimulation of autophagy improves endoplasmic reticulum stress-induced diabetes. Diabetes, 62: 1227-1237.
5. Ballou LM, Zin RZ (2008) Rapamycin and mTOR kinase inhibitors. Journal of Chemical Biology, 1: 27-36.
6. Benavides A, Pastor D, Santos P, Tranque P, Calvo S (2005) CHOP plays a pivotal role in the astrocyte death induced by oxygen and glucose deprivation. Glia, 52: 261-275.
7. Benbrook DM, Long A (2012) Integration of autophagy, proteosomal degradation, unfolded protein response and apoptosis. Experimental Oncology, 34(3): 286-297.
8. Benjamins JA, Nedelkoska L, Bealmear B, Lisak RP (2013) ACTH protects mature oligodendroglia from excitotoxic and inflammation-related damage in vitro. Glia, 61: 1206-1217.
9. Bercury KK, Macklin WB (2015) Dynamics and mechanisms of CNS myelination. Developmental Cell Review, 32: 447-458.

10. Cao SS, Kaufman RJ (2012) Unfolded protein response. Current Biology, 22(16): R622-R626.
11. Chang EJ, Im YS, Kay EP, Kim JY, Lee JE, Lee HK (2008) The role of nerve growth factor in hyperosmolar stress induced apoptosis. Journal of Cell Physiology, 216(1): 69-77.
12. Chen Y, Swanson RA (2003) Astrocytes and brain injury. Journal of Cerebral Blood Flow & Metabolism, 23: 137-149.
13. Dai J, Bercury KK, Macklin WB (2014) Interaction of mTOR and Erk 1/2 signaling to regulate oligodendrocyte differentiation. Glia, 62: 2096-2109.
14. Duffee LE, Boatwright JL, Pacha FH, Shockley JM, Pajerowska-Mukhtar KM, Shahid Mukhtar M (2012) Eukaryotic endoplasmic reticulum stress sensing mechanisms. Advances in Life Sciences, 2(6): 148-155.
15. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A (2009) Life and death partners: Apoptosis, autophagy, and the cross-talk between them. Cell Death and Differentiation, 16: 966-975.
16. Faivre S, Kroemer G, Raymond E (2006) Current development of mTOR inhibitors as anticancer agents. Nature Reviews Drug Discovery, 5: 671-688.
17. Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. The Journal of Neuroscience, 24(9): 2143-2155.
18. Guardiola-Diaz HM, Ishii A, Bansal R (2012) Erk1/2 MAPK and mTOR signaling sequentially regulates progression through distinct stages of oligodendrocyte differentiation. Glia, 60: 476-486.

19. Hartline DK (2008) What is myelin? Neuron Glia Biology, 4(2): 153-163.
20. Hay N, Sonenberg N (2004) Upstream and downstream of mTOR. Genes and Development, 18: 1926-1945.
21. Hussein Y, Cavener DR, Popko B (2014) Genetic inactivation of PERK signaling in mouse oligodendrocytes: Normal developmental myelination with increased susceptibility to inflammatory demyelination. Glia, 62: 680-691.
22. Johnstone JT, Morton PD, Jayakumar AR, Johnstone AL, Gao H, Bracchi-Ricard V, Pearse DD, Norenberg MD, Bethea JR (2013) Inhibition of NADPH oxidase activation in oligodendrocytes reduces cytotoxicity following trauma. PLOS ONE, 8(11): e80975.
23. Jung CH, Ro SH, Cao J, Otto NM, Kim DH (2010) mTOR regulation of autophagy. FEBS Letters, 584(7): 1287-1295.
24. Kapuy O, Vinod PK, Banhegyi G (2014) mTOR inhibition increases cell viability via autophagy induction during endoplasmic reticulum stress – An experimental and modeling study. FEBS Open Bio, 4: 704-713.
25. Kato H, Nakajima S, Saito Y, Takahashi S, Katoh R, Kitamura M (2011) mTORC1 serves ER stress-triggered apoptosis via selective activation of the IRE1-JNK pathway. Cell Death and Differentiation, 19: 310-320.
26. Kiernan JA (2009) The human nervous system: An anatomical viewpoint. Philadephia, PA: Lippincott Williams & Wilkins.
27. Kolch, W (2000) Meaningful relationships: The regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochemical Journal, 351: 289-305.
28. Kroeger H, Messah C, Ahern K, Gee J, Joseph V, Matthes MT, Yasumura D, Gorbatyuk MS, Chiang W, LaVail MM, Lin JH (2012) Induction of endoplasmic reticulum stress

genes, BiP and CHOP, in genetic and environmental models of retinal degeneration. Investigative Ophthalmology & Visual Science, 53(12): 7590-7599.

29. Kuroiwa M, Watanabe, M, Katoh H, Suyama K, Matsuyama D, Imai T, Mochida J. (2014) Effect of amiloride on endoplasmic reticulum stress response in the injured spinal cord of rats. European Journal of Neuroscience, 40: 3120-3127.
30. Lau M, Leung PCK (2012) The PI3K/Akt/mTOR signaling pathway mediates insulin-like growth factor 1-induced E-cadherin down-regulation and cell proliferation in ovarian cancer cells. Cancer Letters, 326: 191-198.
31. Lebrun-Julien F, Bachmann L, Norrmen C, Trotzmuller M, Koefeler H, Ruegg MA, Hall MN, Suter U (2014) Balanced mTORC1 activity in oligodendrocytes in required for accurate CNS myelination. The Journal of Neuroscience, 34(25): 8432-8448.
32. Levine JM, Reynolds R, Fawcett JW (2001) The oligodendrocyte precursor cell in health and disease. Trends in Neuroscience, 24(1): 39-47.
33. Lin W, Harding HP, Ron D, Popko B (2005) Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-γ. The Journal of Cell Biology, 169(4): 603-612.
34. Lin W, Popko B (2009) Endoplasmic reticulum stress in disorders of myelinating cells. Nature Neuroscience, 12(4): 379-385.
35. Lu M, Lawrence DA, Marsters S, Acosta-Alvear D, Kimmig P, Mendez AS, Paton AW, Paton JC, Walter P, Ashkenazi A (2014) Opposing unfolded-protein-response signals converge on death receptor 5 to control apoptosis. Science, 345(6193): 98-101.
36. Mayo L, Quintana FJ, Weiner HL (2012) The innate immune system in demyelinating disease. Immunological Reviews, 248: 170-178.

37. Mecha M, Torrao AS, Mestre L, Carrillo-Salinas FJ, Mechoulam R, Guaza C (2012) Cannabidiol protects oligodendrocyte progenitor cells from inflammation-induced apoptosis by attenuating endoplasmic reticulum stress. Cell Death and Disease, 3: e331.
38. McInnis J (2012) The cell signaling response to ER stress in oligodendrocytes. M.Sc. Thesis. Trinity College: Hartford, CT.
39. Nair A, Frederick TJ, Miller SD (2008) Astrocytes in multiple sclerosis: A product of their environment. Cellular and Molecular Life Sciences, 65(17): 2702-2720.
40. Novosyadlyy R, Kurshan N, Lann D, Vijayakumar A, Yakar S, LeRoith D (2008) Insulin-like growth factor-I protects cells from ER stress-induced apoptosis via enhancement of the adaptive capacity of endoplasmic reticulum. Cell Death and Differentiation, 15: 1304-1317.
41. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K (2006) Autophagy is activated for cell survival after endoplasmic reticulum stress. Molecular and Cellular Biology, 26(24): 9220-9231.
42. Ohri SS, Hetman M, Whittemore SR (2013) Restoring endoplasmic reticulum homeostasis improves functional recovery after spinal cord injury. Neurobiology of Disease, 58: 29-37.
43. Penas C, Guzman MS, Verdu E, Fores J, Navarro X, Casas C (2007) Spinal cord injury induces endoplasmic reticulum stress with different cell-type dependent response. Journal of Neuroschemistry, 102: 1242-1255.
44. Pfeiffer SE, Warrington AE, Bansal R (1993) The oligodendrocyte and its many cellular processes. Trends in Cell Biology, 3: 191-197.

45. Qin L, Wang Z, Tao L, Wang Y (2010) ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. Autophagy, 6(2): 239-247.

46. Ravikumar B, Berger Z, Vacher C, O'Kane CJ, Rubinsztein DC (2006) Rapamycin pre-treatment protects against apoptosis. Human Molecular Genetics, 15(7): 1209-1216.

47. Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, Peterson PK (2004) Role of microglia in central nervous system infections. Clinical Microbiology Reviews, 17(4): 942-964.

48. Sano R, & Reed JC (2013) ER stress-induced cell death mechanisms. Biochimica et Biophysica Acta, 1833(12): 3460-3470.

49. Schroder M, Kaufman RJ (2005) The mammalian unfolded protein response. Annual Review of Biochemistry, 74: 739-789.

50. Sofroniew MV (2005) Reactive astrocytes in neural repair and protection. The Neuroscientist, 11(5): 400-407.

51. Southwood CM, Garbern J, Jiang W, Gow A (2002) The unfolded protein response modulates disease severity in Pelizaeus-Merzbacher disease. Neuron, 36: 585-596.

52. Sovolyova N, Healy S, Samali A, Logue SE (2014) Stressed to death – mechanisms of ER stress-induced cell death. The Journal of Biological Chemistry, 395(1): 1-13.

53. Spampinato SF, Merlo S, Mariangela C, Nicoletti F, Sortino MA (2015) Glial metabotropic glutamate receptor-4 increases maturation and survival of oligodendrocytes. Frontiers in Cellular Neuroscience, 8(462): 1-11.

54. Sovrea AS, Bosca AB (2013) Astrocytes reassessment - an evolving concept part one: embryology, biology, morphology and reactivity. Journal of Molecular Psychiatry, 1: 18.

55. Szegedi E, Logue SE, Gorman AM, Samali A (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Reports, 7(9): 880-885.

56. Tanida I, Ueno T, & Kominami E (2008) LC3 and autophagy. Methods in Molecular Biology, 445: 77-88.

57. Tyler WA, Gangoli N, Pradeepa G, Kim HA, Covey M, Levison SW, Wood TL (2009) Activation of the mammalian target of rapamycin (mTOR) is essential for oligodendrocyte differentiation. The Journal of Neuroscience, 29(19): 6367-6378.

58. Walter P, Ron D (2011) The unfolded protein response: From stress pathway to homeostatic regulation. Science, 334: 1081-1086.

59. Wood TL, Bercury KK, Cifelli SE, Mursch LE, Min J, Dai J, Macklin WB (2013) mTOR: A link from the extracellular milieu to transcriptional regulation of oligodendrocyte development. American Society for Neurochemistry, 5(1): e00108.

60. Xu C, Bailly-Maitre B, Reed JC (2005) Endoplasmic reticulum stress: cell life and death decisions. The Journal of Clinical Investigation, 115(10): 2656-2664.

61. Yeung MSY, Zdunek S, Bergmann O, Bernard S, Salehpour M, Alkass K, Perl S, Tisdale J, Possnert G, Brundin L, Druid H, Frisen J (2014) Dynamics of oligodendrocyte generation and myelination in the human brain. Cell, 159: 766-774.

62. Yokogami K, Wakisaka S, Avruch J, Reeves SA (2000) Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR. Current Biology, 10: 47-50.

63. Yoshida H (2007) ER stress and diseases. FEBS Journal, 274: 630-658.

64. Zhang K, & Kaufman RJ (2008) From endoplasmic-reticulum stress to the inflammatory response. Nature, 454(7203): 455-462.

65. Zoncu R, Efevan A, Sabatini DM (2011) mTOR: From growth signal integration to cancer, diabetes, and ageing. Nature Reviews Molecular Cell Biology, 12(1): 21-35.