The role of the Re-1 silencing transcription factor (*Rest*) in the injury response of Müller glia in the murine retina

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A Thesis Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the requirements for the Degree

Master of Science

By

Amanda Darlene Gall, B.S.

August 2014

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ABSTRACT

Purpose: In contrast to their robust regenerative abilities in fish, Müller glia have limited ability to regenerate retinal neurons in the mammalian retina. Based on its function as a developmental switch for neurogenesis vs. gliogenesis, we hypothesize that continued expression of REST in Müller glia may block their neurogenic potential of in the mammalian retina. To test this, Rest was conditionally knocked out in Müller glia and changes in cell proliferation after retinal injury were measured.

Methods: Triple-transgenic mice carrying homozygous or heterozygous *Rest^{flox}*, a Müller glia-specific, tamoxifen-inducible *Rlbp1-CreERT2* and a *Rosa26-tdTomato* Cre-reporter or *Cre*-transgene negative controls received daily intraperitoneal (IP) injections of 4-hydrozytamoxifen (4OHT) from P21 to P25 to induce Cre-expression. Retinal injury was induced with an intraocular injection of N-methyl-D-aspartic acid (NMDA) (0.1M) with contralateral, control eyes injected with PBS. After 2 days, binocular, intravitreal injections of epidermal growth factor (EGF; 1 μg/μl) and 5-ethynyl-2'-deoxyuridine (EdU; 1 μg/μl) were administered to stimulate regenerative response and label S-phase cells respectively. Sections were immunolabeled with anti-glial fibrillary acidic protein (GFAP) or processed for Click-IT detection of EdU. EdU+ cells were counted in frozen retinal sections and scored for co-expression of tdTomato reporter, analyzed using ANOVA and T-tests with Tukey or Bonferroni corrections for multiple comparisons.

Results: NMDA followed by EGF increased GFAP expression in Müller glia and increased the numbers of EdU+ cells in the injured retina vs. contralateral controls

(p=0.006 and 0.016). In *Rlbp1-CreERT2;tdTomato;Rest* $^{flox/flox}$ mice, 4OHT did not increase numbers of EdU+ cells in the retinas compared to controls that lacked the *Rlbp1-CreERT2* transgene. Unexpectedly, *Rlbp1-CreERT2;tdTomato;Rest* $^{flox/+}$ mice had fewer EdU+ cells (p<0.05) in NMDA injured retinas than control or experimental mice homozygous for *Rest* $^{flox/flox}$. In all genotypes, very few Edu+ cells were double labeled with tdTomato.

Conclusions: Despite induction of a gliotic response, the lack of EdU incorporation in *tdTomato*-labeled, *Rest*-knockout cells precluded assessment of direct effects of *Rest* knockout on injury response. Decreased numbers of EdU+ cells in injured retinas of *Rlbp1-CreERT2; tdTomato* mice heterozygous, but not homozygous, for *Rest* ^{flox} may reflect the small sample size or a non-cell autonomous effect.

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INTRODUCTION

In 2012, the World Health Organization reported that 39 million people are blind and 246 million people are visually impaired world-wide. (Visual Impairment and Blindness 2013) Of these, approximately 19 million are affected by degenerative retinal diseases such as *retinitis pigmentosa* (*RP*), age-related macular degeneration (AMD), and glaucoma. Degenerative retinal diseases result in blindness through the loss of neuronal cell death. In 2013, Prevent Blindness America estimated the economic burden of vision loss and eye disorders in the United States to be 139 billion dollars annually ("Cost of Vision Problems: The Economic Burden of Vision Loss and Eye Disorders in the United States" 2013). Although the problem is immense, current therapeutic approaches cannot reverse the damage and vision loss that results from neuronal cell death. A promising new approach is cell regeneration. It has been shown that the fish fully regenerates the retina after injury, whereas in the mammalian retina this regeneration is very rare and not robust enough for regeneration to be effective. To aid in the development and expansion of cell regeneration in the mammal, it is crucial that we identify the contributing factor(s) and understand the network of regulatory pathways involved in suppressing neurogenic potential.

The Retina

The mature vertebrate retina is made up of seven major cell classes: six neuronal cell classes consisting of rod and cone photoreceptors, bipolar cells, horizontal cells, amacrine cells, and retinal ganglion cells and one glial cell type, the

Müller glia (Figure1) (Rodiek 1998). Each cell type can be distinguished from one another by its patterns of gene expression, function as well as their morphology and laminar position within the retina. In the mouse retina the outer nuclear layer contains on average 97% rod photoreceptors and 3% cones. The inner nuclear layer contains approximately 41% amacrine cells, 40% bipolar cells, 16% Müller glia, 3% horizontal cells and the ganglion cell layer contains 41% ganglion cells (Jeon *et al.,* 1998). This differs from the primate retina, which has fewer amacrine cells (28%), and more Müller glia (28%), and an increase in horizontal cells (9%) (Martin and Grunert 1992). Mature retinal cells are derived from retinal progenitors (Turner and Cepko 1987) and studies on birthdating have revealed that retinal cell types are differentiated in a specific, but overlapping histological order, that is similar across all vertebrate species. In the mouse, the ganglion cells, cone photoreceptors, amacrine cells, and horizontal cells are differentiated prenatally, where bipolar cells and Müller glia differentiated postnatally. The most-abundant cell type in the retina are rod photoreceptors which withdraw from the cell cycle over much of retinal development, but do not show morphological or functional differentiation until post-natal development. (Sidman 1961; Young 1985a; Young 1985b).

The retina is made up of three layers of neuronal cell bodies known as the nuclear layers and two layers of synaptic processes called the plexiform layers (Figure 1). The outer nuclear layer (ONL) is located in the back of the eye and contains the rod and cone photoreceptors. The light-sensitive rods and cones contain an outer segment that abuts the pigmented epithelial layer and contains the light-absorbing molecules, an inner segment that contains most of the protein

synthesis machinery, and an axon terminal located in the outer plexiform layer. The outer segments contain opsin and voltage-gate sodium channels. Rods and cones differ by the type and amount of opsin they contain (Shichida and Matsuyama 2009). The outer segment transduces the light stimuli into electrical signals by initiating the phototransduction cascade (Figure 2). This cascade of events alters the receptors membrane potential and the amount of neurotransmitter released by the photoreceptor synapses onto the post synaptic bipolar or horizontal cells. The photoreceptor axon terminal forms synapses with other neurons and releases neurotransmitters. These synapses are found in the outer plexiform layer (OPL). The inner nuclear layer (ONL) contains the bipolar cells, horizontal cells, and amacrine cells. The bipolar cells make synaptic contacts with the dendritic processes of ganglion cells in the inner plexiform layer (IPL). The horizontal cells and amacrine cells make lateral interactions within the retina and are largely responsible for sensitivity to light over a range of intensities. The inner most layer of the retina is the ganglion cell layer (GCL) which contains the ganglion cells. The ganglion cells receive synaptic input from the bipolar cells and serve as the sole output cells of the retina. The ganglion cell axons make up the optic nerve and project visual signals to the rest of the central nervous system (Purves *et al.,* 2001).

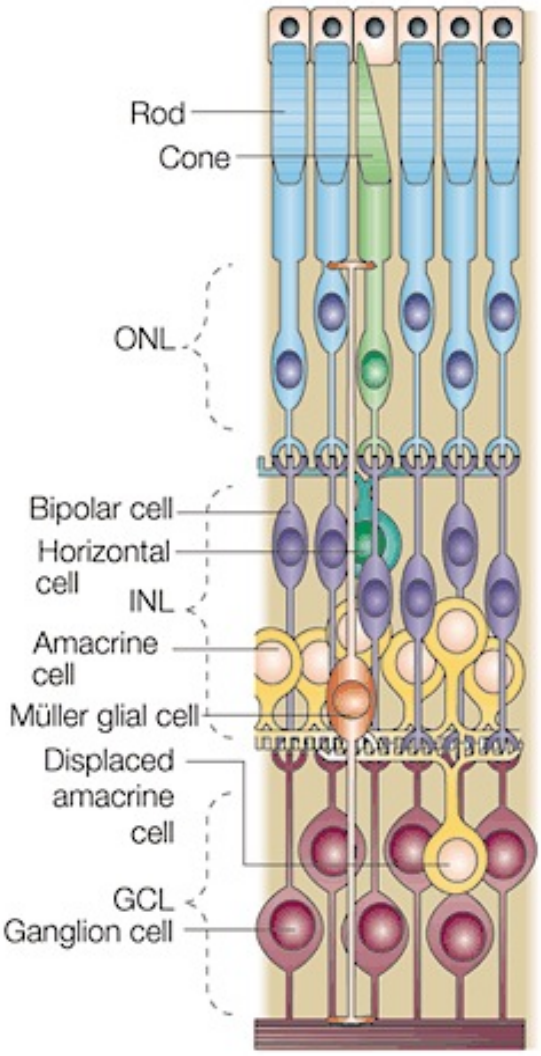


Figure 1: Structure of vertebrate retina
The vertebrate retina consists of seven major retinal cell classes: rod and cone photoreceptors, bipolar cells, horizontal cells, amacrine cells, ganglion cells, and Müller glia and are organized into three cellular layers: outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Figure from: (Dyer and Cepko 2001)

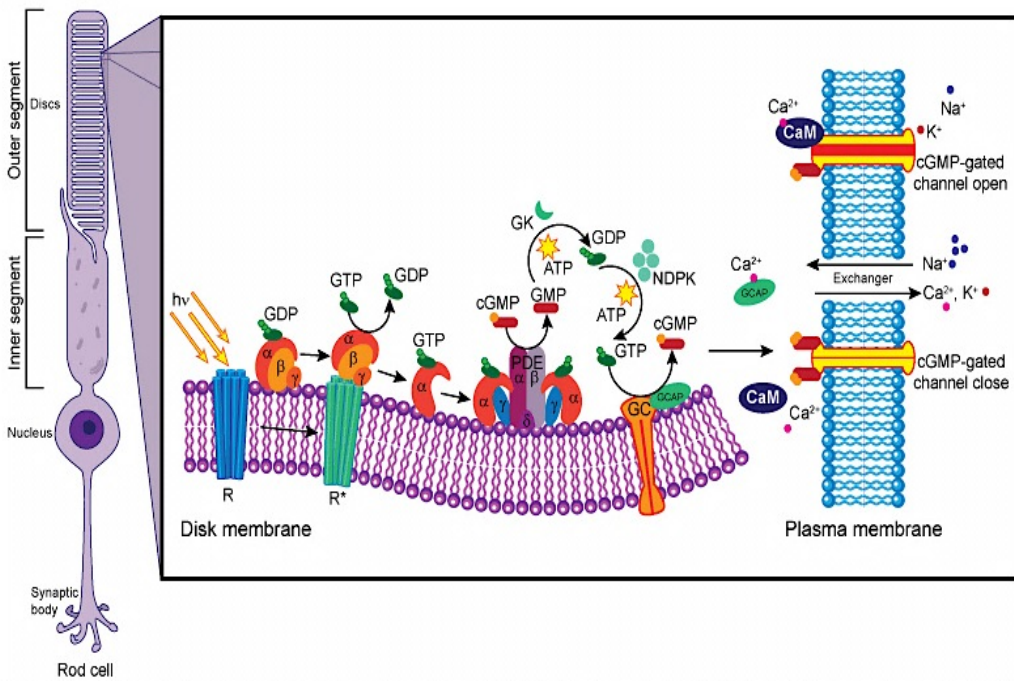


Figure 2: Phototransduction of a rod photoreceptor
In the dark, opsin is bound to 11-*cis*-retinal forming inactive rhodopsin (R), upon the absorption of photons the 11-cis-retinal bound to opsin is isomerized into all-trans-retinal, resulting in rhodopsin activation. The active rhodopsin (R*) transiently binds to transducin (αβγ), a heterotrimeric G protein that alternates between an inactive guanosine diphosphate (GDP) and an active guanosine triphosphate (GTP) bound state. Transducin bound with GDP docks on the R* surface, causing GDP to dissociate from the complex and GTP to bind to transducin. GTP-bound transducin dissociates from R* and interacts with the cyclic GMP (cGMP) phosphodiesterase PDE6 γ subunits and activates the catalytic α or β subunits resulting in hydrolysis of cGMP. Reduction of cGMP leads to the closure of cGMP-gates channels in the plasma membrane and photoreceptor hyperpolarization, which prevents the neurotransmitters from being released (Cai *et al.,* 2009; Ridge *et al.,* 2003; Ridge and K. Palczewski 2007). Figure from: (La Vine 2011)

Müller Glia

In 1851, H. Müller was the first to describe the radial trunks of macroglia that spanned the thickness of the retina. These cells were called Müller glia and are the major intrinsic glial cell of the retina. Müller cell bodies are located in the INL and project irregular thick and thin processes vertically through the thickness of the retina. The end-feet of Müller glia form the inner and outer limiting membranes. Müller cell processes intertwine themselves between neuron cell bodies in the nuclear layers and cover both neuronal processes in the plexiform layers and the retinal vasculature, where they function as a major component of the blood-brain barrier.

Müller glia have a range of functions that are vital for the health of retinal neurons. These functions include: 1) breaking down glycogen during anaerobic metabolism to fuel metabolism in the nerve cells, 2) cleaning up waste products from neurons, 3) protecting neurons from exposure to neurotransmitters, 4) modulating release of neuroactive substances, 5) recycling retinol as well as potentially, synthesizing retinoic acid from retinol (important in development of the eye and nervous system)(Edwards 1994), and 6) controlling homeostasis and general neuroprotection (Reichenbach and Robinson 1995). Müller glia have even been proposed to guide light to the photoreceptors (Franze *et al.,* 2007). They are responsible for structural stabilization of the retina and also modulate immune and inflammatory responses (Bringmann *et al.,* 2009; Reichenbach and Bringmann 2010). Recently, growing evidence suggests that Müller glia have stem- cell like characteristics and can regenerate retinal neurons both *in vitro* and *in vivo*.

Müller Glia and Retinal Regeneration

The retinas of teleost fish are histologically quite similar to the mammalian retina and they also develop from multipotent retinal progenitor cells during embryogenesis. The temporal order of cell birth in the fish retina is generally the same as in mammals. A key distinction between the fish and mammalian retina is that in fish, there is a continuous generation of retinal neurons throughout life. The first discovery of the regenerative ability of the fish retina was in the late 1960s where early experiments induced regeneration by surgical removal of a portion or the entire retina (Lombardo 1968; Lombardo 1972). Later it was discovered that regeneration restores the original architecture, histology, (Cameron and Easter, Jr. 1995; Cameron *et al.,* 1999; Hitchcock and Raymond 1992; Vihtelic and Hyde 2000) and function of the retina (Hitchcock and Cirenza 1994; Mensinger and Powers 1999).

Trying to identify the origin of the new neurons and glia during regeneration led to many hypotheses of potential sources. Initially, the circumferential germinal zone (CGZ) was proposed as the likely source because it was the only area where mitotic figures were observed following injury (Lombardo 1968; Lombardo 1972). Subsequent studies showed no cellular migration from the CGZ to the central retina during regeneration, therefore contracting the CGZ as a potential source (Hitchcock *et al.,* 1992; Hitchcock *et al.,* 1996; Johns 1982; Raymond *et al.,* 1988). Another proposed source was pigmented epithelial cells, as they are the source for retinal regeneration in amphibians (Mitashov 1996; Reh *et al.,* 1987) and embryonic chick retinas (Coulombre and Coulombre 1965). However, no evidence supporting

involvement of pigmented epithelial cells in regeneration of the fish retina was found (Knight and Raymond 1994).

Rod precursors were considered as a possible source for retinal regeneration due to the large amount of mitotic activity in the ONL after injury (Braisted and Raymond 1993; Hitchcock *et al.,* 1992; Hitchcock *et al.,* 1996; Negishi *et al.,* 1987; Raymond *et al.,* 1988). Rod precursors were also supported in the role of regeneration because of the observation that there must be cell loss in the ONL for regeneration to occur, regardless of the extent of cell death in the other retinal layers (Braisted and Raymond 1993; Negishi *et al.,* 1987; Raymond *et al.,* 1988). In the fish differentiated retina, the source of regenerated retinal cells is from a population of proliferating cells. These proliferating cells are modified neruoepithelial cells termed 'rod precursors'. Rod precursors continuously produce only rod photoreceptors and insert new rods into the growing adult retina (Raymond 1991).

In 1996, immunostaining studies on injured retinas of goldfish with Pax6, revealed that after injury cells first appear in the INL (Hitchcock *et al.,* 1996). Pax6 is a developmental regulatory gene that plays a key role in development of the embryonic brain, eye, and retina and is critical for eye development in all species. Additional support for regeneration from cells in the INL came from studies on light-induced retinal degeneration showing that the first proliferating cells in the injured retina were located in the INL (Vihtelic and Hyde 2000). Using long-term systemic labeling of proliferative cells (BrdU) combined with Pax6 immunostaining identified rare spherical Pax6+ cells in the INL that were proposed to be stem cells based on

evidence that they give rise to the rod precursors during normal retinal growth (Otteson *et al.,* 2001). Together, these findings led to the proposal that stem cells in the INL could also be a cellular source for retinal regeneration in the fish.

Subsequent studies have examined Müller glia as the potential source of regeneration from the INL (Figure 3). Proliferating microglia, Müller glia, and retinal progenitors in the INL were found in the lesions of goldfish retinas following laser ablation (Wu *et al.,* 2001). In these studies, the nuclei of both the Müller glia and associated retinal progenitors migrated from the INL to the ONL and the proliferating retinal progenitors regenerated cone photoreceptors and then rod photoreceptors. These results support Müller glial involvement in cone photoreceptor regeneration (Wu *et al.,* 2001). Additional studies confirmed Müller glia as the primary source of the robust retinal regeneration in the zebrafish (Bernardos *et al.,* 2007; Fausett and Goldman 2006; Thummel *et al.,* 2008; Yurco and Cameron 2005). Following several retinal injury models; including surgical removal (Cameron 2000; Cameron and Easter, Jr. 1995; Hitchcock *et al.,* 1992; Hitchcock and Raymond 1992; Hitchcock and Vanderyt 1994; Lombardo 1968; Lombardo 1972), light or laser (Braisted *et al.,* 1994; Vihtelic and Hyde 2000; Wu *et al.,* 2001), puncture (Fausett *et al.,* 2008), injections of metabolic poisons and neurotoxins (Braisted and Raymond 1993; Fimbel *et al.,* 2007; Maier and Wolburg 1979; Negishi *et al.,* 1987; Raymond *et al.,* 1988; Sherpa *et al.,* 2008), or transgene genetics (Montgomery *et al.,* 2010) the Müller glia in the retinas of teleost fish undergo a robust regenerative response that leads to regeneration of all the different neuronal cell types (Fausett and Goldman 2006; Kassen *et al.,* 2007; Qin *et*

al., 2009; Thummel *et al.,* 2008; Vihtelic and Hyde 2000; Wu *et al.,* 2001; Yurco and Cameron 2005). The current understanding of retinal regeneration in fish is that injury stimulates Müller glia to re-enter the cell cycle and dedifferentiate into retinal progenitors, which ultimately generate new neurons.

Simultaneously, it was shown that in developing chicks, Müller glia could generate new neurons following retinal injury (Fischer and Reh 2001). In contrast to the robust regeneration of all retinal cell types observed in fish, in post-hatch chicks, only a limited number of inner retinal neurons are produced (amacrine, bipolar, and ganglion cells). The mammalian retina has an even more limited regenerative response, because the Müller glia do not spontaneously re-enter the cell cycle (Chang *et al.,* 2007; Close *et al.,* 2006; Dyer and Cepko 2000; Sahel *et al.,* 1990; Zhao *et al.,* 2005).

In vitro studies have revealed mammalian Müller glia cells have the capacity to be reprogrammed into a retinal progenitor identity. In isolated Müller glial cell cultures, L-glutamate induced Müller glia to lose their glial phenotype, express progenitor cell markers, and divide (Takeda *et al.,* 2008). A conditionally-immortalized Müller glial cell line (ImM10) demonstrated expression of multiple retinal stem cell genes when cultured as adherent monolayers (Otteson and Phillips 2010). Stimulation of human Müller glia cell cultures with fibroblast growth factor 2 together with NOTCH inhibition resulted in the differentiation of the Müller glia into retinal ganglion cell (RGC) precursors (Singhal *et al.,* 2012). Neural cell cultures from *p53*-null mice gave rise to photoreceptors under a defined culture condition (Zhao *et al.,* 2014).

Studies *in vivo* show that mammalian Müller glia are more resistant to re-entry into the cell cycle after injury or growth factors. Injury with neurotoxins, such as N-methyl-D-aspartic acid (NMDA), resulted in a very few Müller glia re-entering the cell cycle in the adult rodent retina (Dyer and Cepko 2000; Karl *et al.,* 2008; Ooto *et al.,* 2004). Müller glia proliferation following injury can be stimulated by intraocular injection of EGF, FGF1, or the combination of FGF1 and insulin. Experiments in rats revealed that a few of the proliferating Müller glia generated cells that express markers and morphology of neurons (Fischer *et al.,* 2002a; Fischer and Reh 2002; Ooto *et al.,* 2004) and in mouse a small number of growth factors stimulated Müller glia progeny to differentiate into amacrine cells (Close *et al.,* 2006). An important and yet unanswered question is: What inhibits the neurogenic potential of the Müller glia in mammals?

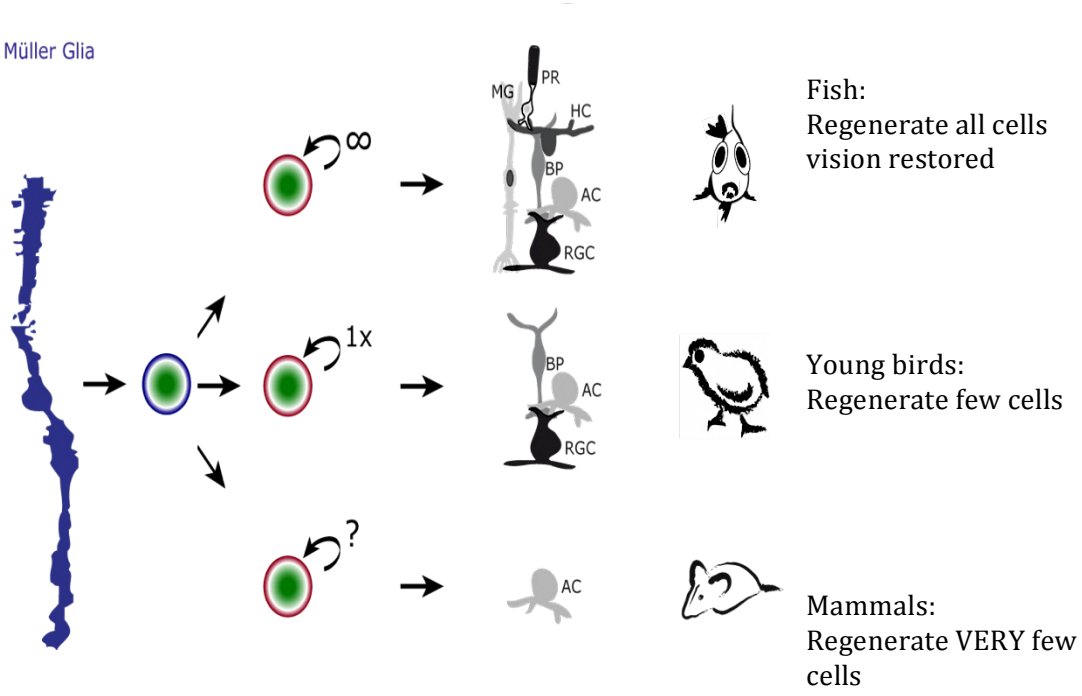


Figure 3: Regeneration by species
In fish, Müller glia can regenerate all cells and fully restore vision. In chicks and mammals, Müller glia cannot regenerate all the cell types and vision cannot be restored following injury. Figure from: (Lamba *et al.,* 2008)

Re-1 Silencing Transcription Factor (REST)

A promising candidate that could be blocking the neurogenic ability of Müller glia is RE-1 silencing transcription factor (REST), also known as the neuronal restricted silencing factor (NRSF). REST was first discovered independently by two groups in 1995 as a factor that could interact with a repressor element (RE-1) and mediate silencing of type II voltage dependent sodium channel and SCG10 genes in non-neuronal cells (Chong *et al.,* 1995; Schoenherr and Anderson 1995).

REST is a 1097 amino acid protein that includes a DNA binding domain containing eight zinc finger motifs, followed by lysine-and proline domains that are sandwiched between two independent repressor domains located at its amino (N-) and carboxyl (C-) termini (Ballas and Mandel 2005; Weissman 2008). In addition to the full length REST, five different splice variants that code for 3 protein isoforms have been characterized (Figure 4) and at least 3 additional isoforms have been shown to exist. Each splice variant is potentially differentially expressed, regulated, and degraded (Lee *et al.,* 2000a; Lee *et al.,* 2000b; Shimojo 2006; Shimojo *et al.,* 1999; Tabuchi *et al.,* 2002).

These isoforms are referred to as REST1 through REST5, with full-length REST, REST1, and REST4 being the major and most characterized isoforms. REST1 is truncated so that is retains only zinc fingers 1-4, blocking its ability to translocate to the nucleus. REST2 and REST3 have an amino acid alteration after the 5^{th} zinc finger and therefore are missing the C-terminal repressor domain. REST4 has three possible splice variants, alternatively referred to as sNRSF, REST-N62, and REST-N4, which all result in a truncation after the fifth zinc finger and an absence of the C-

terminal repressor domain. REST5 has a selective deletion of zinc finger five which contains the nuclear localization signal. Most of the various REST isoforms have not been well characterized but are predicted to have unique affinities for specific RE-1 motifs and/or with distinct patterns of nuclear and cytoplasmic localization that would alter their ability to interact with other proteins, the RE-1 binding site, leading to variable effects on gene regulation.

REST binds to the repressor element 1 (RE-1), which is present in the regulatory regions of target genes (Hara *et al.,* 2009; Johnson *et al.,* 2007; Mortazavi *et al.,* 2006; Palm *et al.,* 1998; Weissman 2008). The RE-1 site is not symmetrical and the canonical RE-1 site (cRE-1) can be divided into two 10 bp sequences with a single basepair (bp) between them (Johnson *et al.,* 2007). Recently studies from genome-wide chromatin immunoprecipitation (ChIP) assays have uncovered a second type of RE-1 site, the non-canonical (nRE-1), that has variable numbers of intervening base pairs, ranging from 0 up to 9 bp (Johnson *et al.,* 2006; Otto *et al.,* 2007).

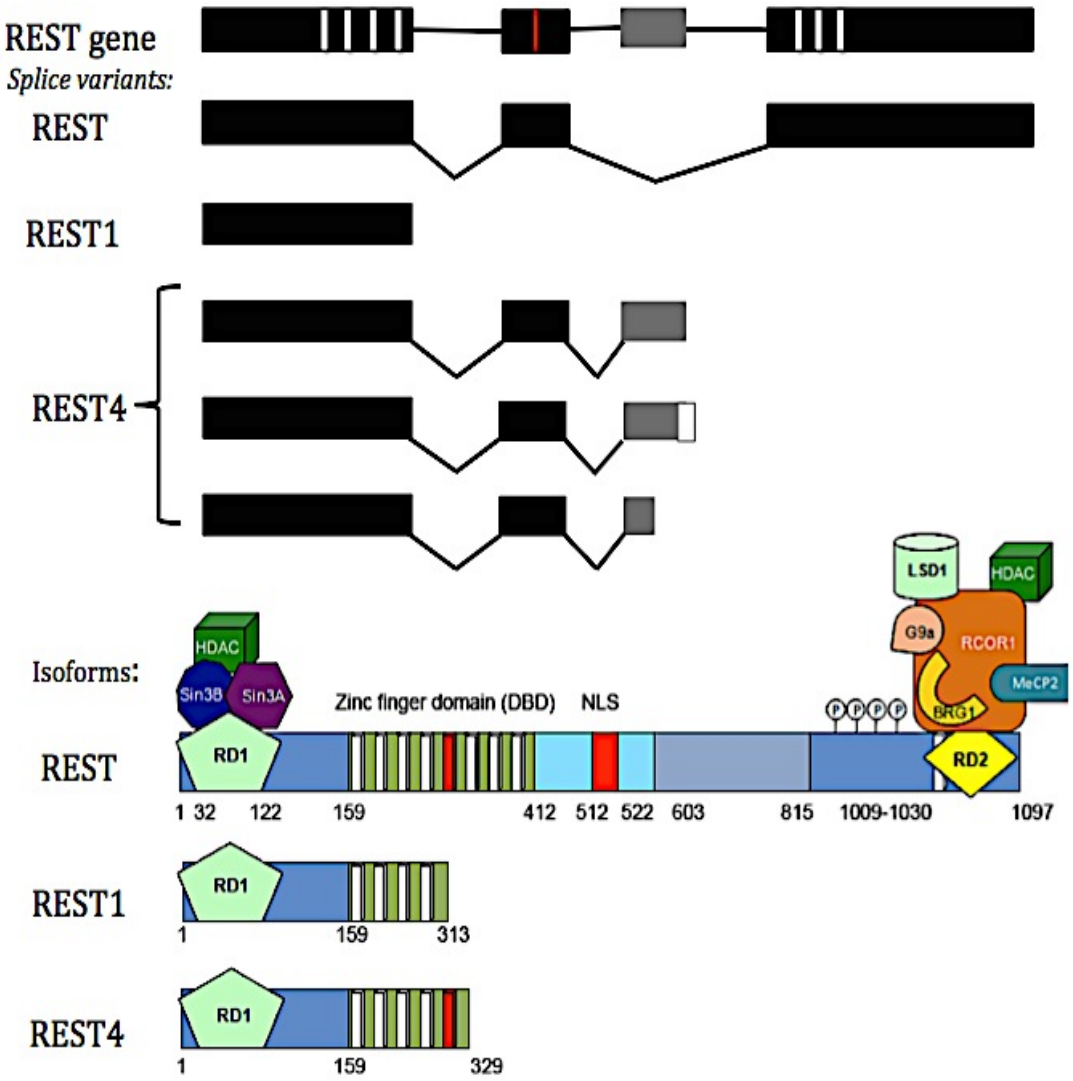


Figure 4: REST alternative splice forms
Top: Diagram illustrating REST mRNA and alternative splice variants. The REST gene has three main exons, an alternative exon (light grey) and varying regions of non-coding (white or not shown). The approximate position of eight zinc fingers are illustrated by boxes, these are associated with DNA binding (white) or nuclear import (red). Five alternative mRNAs are illustrated, grouped according totheir corresponding protein isoforms. Bottom: Diagram illustrating REST protein and alternative isoforms. Figure adapted from: (Faronato and Coulson 2011)

In the human genome, cRE-1 occupancy sites have been mapped to 1946 sites using a large-scale chromatin immunoprecipitation assay (ChIPSeq) based on direct ultrahigh-throughput DNA sequencing (Johnson *et al.,* 2007). Analysis of other vertebrate genomes, including zebrafish, revealed the presence REST orthologs and comparable numbers of cRE-1 sites (Mortazavi *et al.,* 2006). Combined *in silico* and biochemical approaches identified a number of genes whose regulatory regions contained a cRE-1 site and had the potential to be regulated by REST. Many of these genes had functions in neuronal development, such as those encoding ion channels, neurotransmitter receptors, and neurosecrectory factors, but a number of these had functions far removed from neurogenesis (Bruce *et al.,* 2004).

Full length REST contains two major repressor domains located at the N- and C-terminal regions that function to regulate transcriptional repression (Ballas *et al.,* 2005a; Lunyak VV 2005). The N-terminal repressor domain recruits mSin3 protein, which dynamically interacts with a wide range of repressor proteins including the methyl-DNA-binding protein ($MeCP_2$) and histone deacetylases (HDACS) 1 and 2 (Huang *et al.,* 1999; Naruse *et al.,* 1999; Roopra *et al.,* 2000). The N-terminal complex can alternate between repression and activation, therefore, appears insufficient for long-term silencing of neuronal genes without the help of a second repressor domain (Ballas and Mandel 2005). The C-terminal repressor domain recruits CoREST, a specialized co-repressor present only in organisms with a nervous system (Ballas and Mandel 2005). CoREST interacts with large complex of chromatin modifiers for long-term silencing including; HDAC1 and HDAC2, $MeCP_2$; histone demethylase (LSD1), histone methyltransferases G9a and Suv39 h1, zinc

finger proteins ZNF198 and ZNF217, the acetyltransferase Esco2, and components of the SWI/SNF chromatin remodeling complex (BAF57, BRG1, and BAF170) (Battaglioli *et al.,* 2002). Chromatin remodeling complexes generally recognize specific residues of histone tails that are covalently marked by acetylation, methylation, phosphorylation, ribosylation and ubiquitination specific residues of histone tails (Strahl and Allis 2000) and, through ATP hydrolysis, target a region of chromatin to unwrap, mobilize, exchange or eject the nucleosome for DNA binding by other protein complexes (Cosma 2002; Levine and Tjian 2003; Owen-Hughes 2003). The binding of CoREST recruitment of chromatin modifier complex to the c-terminal of REST generally results in permanent silencing.

REST in tissues and organs

Consistent with its proposed role as a neuronal/glial switch, REST mRNA is found in neural progenitors and glial cells, but not in mature neurons (Schoenherr and Anderson 1995). In neural stem cells (NSCs), REST regulates multiple cell type- and developmental stage-specific target genes (Abrajano *et al.,* 2009a), regulates the glial/neuronal switch during neurogenesis (Ballas *et al.,* 2005a), and the maturation and plasticity of neuronal lineages (Abrajano *et al.,* 2009b; Covey *et al.,* 2012). Genome wide promoter occupancy profiles identified many developmental stage- and cell type-specific REST target genes in neural cells. These target genes included many factors known to be involved in a broad array of cell-intrinsic processes, cell-cell communications, and environmental signaling pathways that may similarly be involved in determining aspects of glial cell identity and function (Abrajano *et al.,* 2009a).

REST conditional knockout in mice revealed that inducible deletion of REST in adult neural stem cells triggers an increase in neurogenesis and activation of NCSs to pass through proliferation stages, giving rise to granule neurons (Gao *et al.,* 2011). When neural stem/progenitor cells differentiate, REST remains present in glia (Abrajano *et al.,* 2009a; Dewald *et al.,* 2011), but is largely absent in neurons, allowing the restricted expression of its target genes specifically in neurons (Ballas *et al.,* 2005b). Nitric oxide-induced neuronal to glial fate conversion is dependent on REST. Blocking of REST function resulted in alterations in chromatin modifications to be lost and the neuronal to glial switch was suppressed (Bergsland *et al.,* 2014).

REST is also shown to have many roles outside of the nervous system. During mouse embryogenesis, the REST gene expressed is detectible in high levels in nonneuronal tissues. *In situ* hybridization with RNA probes revealed a pattern of REST mRNA expression wherein REST was abundant in all tissues of the developing embryo except in the neural tube and spinal ganglia (Chong *et al.,* 1995). Northern blot analysis of REST in regulation of fetal cardiac gene expression, RT-PCR, and inhibition of REST revealed REST plays a role in fetal cardiac development and adult heart maintenance (Kuwahara 2013; Kuwahara *et al.,* 2003). Gel mobility shift assays with a novel high-affinity antibody showed that REST in the nuclei of smooth muscles (Cheong *et al.,* 2005; Cheong *et al.,* 2006). Pancreatic islet cell lines (INS-1) have an absence of REST demonstrated by RT-PCR, Northern blot analysis, and gel shift assay. Additionally, the expression of a REST cDNA in INS-1 is sufficient to silence a reporter gene containing a REST binding site (Atouf *et al.,* 1997).

Splice isoforms, REST1-REST5, have been detected in differentiated neurons of

the rat brain (Palm *et al.,* 1998). REST4 and REST5 were only detected in neuronal tissues (Palm *et al.,* 1998; Palm *et al.,* 1999a). However, the biological significance of REST4 and REST5 in neurons remains unclear, as there are lower amounts of each in the adult rat brain than REST. REST4 is likely to have a function distinct from full-length REST. REST4 has an antagonistic effect of REST function and functions as a double negative regulator by competing with REST for DNA binding in neurons (Shimojo *et al.,* 1999; Tabuchi *et al.,* 2002). When REST4 is introduced into cells it can block REST-dependent repression of RE-1-containing genes. In addition REST4 is also found to have an increased expression in many cancers (Aigner and Yeo 2009). All splice isoforms are also commonly found in structures of both neuronal and non-neuronal origin. REST2 and REST3 have the lowest levels of expression in neuronal and non-neuronal structures. In contrast, REST1 is widely expressed in neuronal and non-neuronal structures. To date a function for REST1, REST2, REST3, and REST5 still remained undefined, although all five splicing isoforms are found conserved across the vertebrate species (Palm *et al.,* 1998).

Studies examining the expression, regulation, and function of REST have shown it to have diverse roles in biological processes and various disease states, including neurodevelopmental and neurodegenerative diseases, stroke, epilepsy, cardiomyopathies, and cancer (Ballas and Mandel 2005; Guardavaccaro *et al.,* 2008; Lu *et al.,* 2014; Ooi and Wood 2007; Westbrook *et al.,* 2008; Zuccato *et al.,* 2007). REST is increased in the aging of the human cortical and hippocampal neurons. However, in mild cognitive impairment and Alzheimer's disease REST is almost absent from the nucleus of cortical and hippocampal neurons (Lu *et al.,* 2014).

Chromatin immunoprecipitation analysis revealed that REST represses genes that promote cell death and Alzheimer's disease pathology (Lu *et al.,* 2014). In Huntington's disease, a mutation in huntingtin protein impairs its ability to sequester REST in the cytoplasm of neural cells. This leads to the translocation of REST in to the nucleus where pathological levels of REST cause a loss of wild-type huntingtin–mediated regulatory activity on gene transcription (Zuccato *et al.,* 2003). Ischemia activates REST mRNA and protein expression, which suppresses the promoter of glutamate receptor, *GluR2,* down-regulating gene expression in neurons destined to die. The GluR2 subunit controls biophysical properties of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, the principal mediators of fast excitatory neurotransmission, synaptic remodeling, and exitotoxic cell death. Therefore, changes in GluR2 expression lead to profound effects on synaptic and neuronal survival (Calderone *et al.,* 2003; Tanaka *et al.,* 2000).

REST is also associated with the progression and dysfunction of multiple cancer types. REST was first linked to medulloblastoma (Lawinger *et al.,* 2000), neuroblastoma (Palm *et al.,* 1999b), and small cell lung cancer (Coulson *et al.,* 2000). Medulloblastomas are thought to develop from REST expression being retained or reinitiated in neuroectodermal stem cells followed by a failure to differentiate (Fuller *et al.,* 2005). In small lung cancer, colon cancer, and highly aggressive breast cancer, alternative splicing of REST to REST4 causes a loss of REST repression resulting aberrant expression of its target genes (Coulson *et al.,* 2000; Wagoner *et al.,* 2010; Westbrook *et al.,* 2005). In general, the loss of REST results in the up-regulation of neuronal genes, an imparting of a neuroendocrine phenotype on the

cells, and is linked to malignant progression (Coulson *et al.,* 2000; Wagoner *et al.,* 2010; Westbrook *et al.,* 2005). In small lung cancer cell lines, the regulation of REST by methylation and CREB results in REST behaving like a classic tumor suppressor (Kreisler *et al.,* 2010). A role for REST in tumorigenesis has recently been recognized as it has emerged as one of the first tumor suppressors to be predicted from an unbiased RNA interference (RNAi) library screen (Westbrook *et al.,* 2005).

Rest in the retina

The embryonic retina mainly consists of proliferating progenitors, whereas the postnatal retina composed of differentiated neurons and Müller glia. REST is the primary isoform expressed in early developing retina and REST4 is most abundant in postnatal retina (Mao *et al.,* 2011). This pattern is consistent with the idea that REST is expressed in neural progenitor cells and REST4 in differentiated neurons.

In the retina, REST binding sites are frequently associated with retinal ganglion cell (RGC) genes and REST suppresses expression of RGC-specific genes in retinal progenitor cells (RPCs) (Mao *et al.,* 2011). This study showed that inactivation of *REST* causes abnormal expression of RGC transcription factors in proliferating RPCs resulting in increased RGC formation. Knockout of REST in the developing retina (E10) resulted in increased inner plexiform and ganglion cell layer thickness and a mis-patterning of the retina. REST mutant retinas also had a substantial increase in RGC production resulting from an upregulation in the expression of key transcription factors required for RGC differentiation.

Based on these results, Mao *et al.* (2011), proposed a model in which REST functions in retinal cell fate determination. In early retinal development, REST

suppresses RGC genes in RPCs to prevent premature activation and maintain an appropriate balance of proliferating RPCs and differentiating RGCs. When REST is not present, the RGC gene expression in RPCs is not repressed and additional RGCs are formed. In later stages of retinal development, environment of the retina is no longer conducive to production of RGCs (Mao *et al.,* 2011).

Recently it has been found that REST is expressed in Müller glia (Phillips 2010). Given its role as developmental switch for neurogenesis vs. gliogenesis, the central hypothesis of this study is that continued expression of REST may be blocking the neurogenic potential of Müller glia in the mammalian retina. To examine this hypothesis we will knockout REST specifically in Müller glia in a fully differentiated mouse retina and determine how this alters the injury/regenerative response of Müller glia. To do this, we will use a REST floxed allele mouse line [gift of Dr. C. Mao; (Mao *et al.,* 2011)] was crossed with the tamoxifen inducible *Rlbp1-CreERT2/Rosa26-tdTomato* transgenic [gift of Dr. E. Levine; (Vazquez-Chona *et al.,* 2009)] and knockout REST. Subsequently, intraocular injections with N-methyl-D-aspartic acid (NMDA) will be used to generate a retinal injury followed by stimulation and labeling of regeneration by epidermal growth factor and 5-ethynyl-2'-deoxyuridine (EdU) (Karl *et al.,* 2008).

MATERIALS AND METHODS

Mice

All mice handling procedures were approved by the Institutional Animal Care and Use Committee of the University of Houston and were in accordance with Statement for the Use of Animals in Ophthalmic and Visual Research published by the Association for Research in Vision and Ophthalmology (ARVO). The following strains were used: C57BL/6 (Jackson Laboratories; Bar Harbor, ME), *Rest* $^{flox/flox}$ (generous gift of Dr. C. Mao; MD Anderson;(Mao *et al.,* 2011)), and *Rlbp1-CreERT2;Rosa26-tdTomato* [generous gift of Dr. E. Levine; University of Utah; (Vazquez-Chona *et al.,* 2009)]. Additional lines tested in the optimization of the protocols were *Glast-CreERT2* and *(Cg)-Tg(CAG-Bgeo/GFP)21Lbe/J* (hereafter referred as *Bgeo/GFP* in this thesis) (Jackson Laboratories; Bar Harbor, ME). The breeding schemes for generating triple transgenics used in the optimization and experimental model are illustrated in Figure 5.

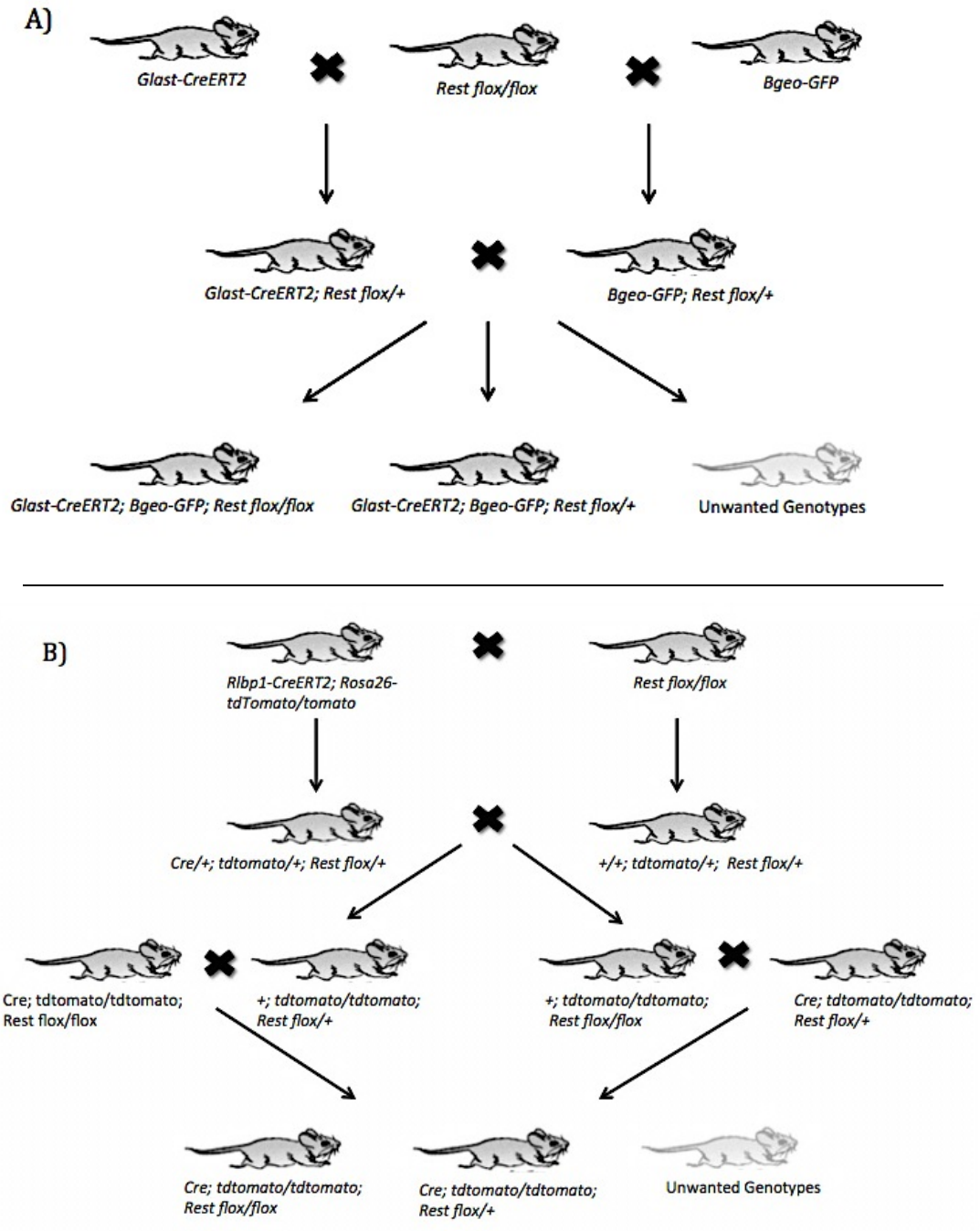


Figure 5: Genetic Breeding Map
A) Generation of genotypes used for optimization of experimental procedures. B) Generation of genotypes used in experimental model. Note: Testing of Cre recombinase and GFP/tdTomato expression were performed crossing the Cre reporter lines to C57Bl/6 instead of *Rest* $^{flox/flox}$.

DNA isolation from tail biopsies

At approximately P15, pups are tagged and tailed. Eartags are inserted in the center of the ear, as close to the head as possible to prevent tearing and loss of tags. The tip of each tail is cut at <5 mm using a separate, new razor blade and placed in an eppendorf tube labeled with the eartag number on ice. To stop bleeding the mouse's tail is dipped in styptic powder (Kwik Stop; Petco) and pressure applied.

For DNA isolation, tails biopsies are placed in 500 µl tail lysis buffer (100 mM EDTA, 1% SDS, 50 mM Tris pH 8.0, 100 mM NaCl, in sterile H_2O up to 50 ml) and 15 µl 100 mg/ml proteinase K, vortexed, and incubated at 65°C overnight. After incubation, tubes are vortexed to ensure that the tail is fully dissociated; if not, more proteinase K is added, the tube is vortexed and incubated at 65°C until no identifiable tail tissue remains. The lysate is then centrifuged at 10,000 rpm for 10 minutes at RT to pellet any debris (e.g. hair, bone). The supernatant is transferred to a clean tube and 500 µl sterile filtered isopropanol is added. The tube is inverted until the DNA is visible and the precipitated DNA is then transferred to a clean tube containing 500 µl of 70% EtOH using a pipet. The DNA is pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. If no DNA is visible with inversion, then the entire lysate is centrifuged at 14,000 rpm for 15 min at 22-25°C to pellet the DNA. After pelleting the DNA, remove the supernatant is removed and discard and the pellet is washed with 500 µl of 70% EtOH as described above. The final DNA pellet is then air dried for 5 min then dissolved in 100-200 µl sterile 1X TE. The tail DNA is incubated at room temperature (RT) for a couple of hours to assist dissolution with occasional vortexing occasionally prior to storage at 4°C.

Genotyping by PCR

Genomic DNA samples were genotyped with primers gene specific for *Cre*, *tdTomato*, and *Rest*$^{flox/flox}$ (Integrated DNA Technologies; Coralville, IA) using goTAQ polymerase (Promega; Madison, WI). Tail DNA samples in TE were vortexed and then 1 µl was added to each PCR reaction mix. PCR reactions were vortexed briefly to fully mix all components, droplets spun down with pulse centrifugation (6000 rpm for 1 min) and tubes were transferred to a thermocycler (Bio-Rad iCycler; Hercules, CA). PCR genotyping primers and conditions are listed in Table 1. PCR products were separated on a 1.5% agarose gel containing a fluorescent stain (GelGreen Phenix; Chandler, NC) at 5 µl gel green/50 ml gel and photographed (ProteinSimple FlourChem HD2; Santa Clara, CA). All mouse genotypes were recorded in a FileMaker Pro mouse colony database (MouSeek; Caleb F. Davis, Baylor College of Medicine).

Table 1: PCR conditions and primers

A) Genotyping primers

Allele	Primers (5'-3')	Product size
Rlbp1-CreERT2	ATG CTT CTG TCC GTT TGC CG CCT GTT TTG CAC GTT CAC CG	*Cre* 270 bp
Rosa26-tdTomato	GGC ATT AAA GCA GCG TAT CC CTG TTC CTG TAC GGC ATG G AAG GGA GCT GCA GTG CAG TA CCG AAA ATC TGT GGG AAG TC	*tdTomato* 196 bp *tdTomato*^{WT} 297 bp
Rest $^{flox/flox}$	CAT GCG AGT ACT GCC ATA CCC CCT CCA GAA GAC TGC CCA TC	*Rest* ^{flox} 264 bp *Rest* $^{+/+}$ 220 bp

B) Master mix concentrations

Master Mix	
5X Buffer	1X
Primer 1 (10 µM)	0.5 µM
Primer 2 (10 µM)	0.5 µM
dNTPs (10mM)	0.2 mM
GoTAQ polymerase (5µ/µl)	1.25 units/rxn
Template DNA	50–100 ng/ µl
H_2O	Up to 25 µl

C) Cycling Conditions

Rlbp1-CreERT2	***Rosa26-tdTomato***	***Rosa26-tdTomato-WT***	***Rest*** $^{flox/flox}$
Hot Start 1 cycle @ 96°C for 2 min 30 cycles: 95°C for 30 s 57°C for 30 s 72°C for 30 s 1 cycle @ 72°C for 2 min 1 cycle @ 4°C for ∞	Hot Start 1 cycle @ 95°C for 2 min 30 cycles: 95°C for 30 s 52°C for 30 s 72°C for 30 s 1 cycle @ 72°C for 2 min 1 cycle @ 4°C for ∞	Hot Start 1 cycle @ 95°C for 2 min 30 cycles: 95°C for 30 s 56.5°C for 30 s 72°C for 30 s 1 cycle @ 72°C for 2 min 1 cycle @ 4°C for ∞	1 cycle @ 95°C for 5 min 9 cycles: 94°C for 50 s 68°C for 1 min 72°C for 30 s 32 cycles: 94°C for 50 s 61°C for 50 s 72°C for 30 s 1 cycle @ 72°C for 5 min 1 cycle @ 4°C for ∞

Preparation and administration of tamoxifen and 4-hydroxytamoxifen

Tamoxifen (TAM) (Sigma/Aldrich T5648; St Louis, MO) and 4-hydroxytamoxifen (4OHT) (Sigma/Aldrich H6278; St Louis, MO) were prepared by adding 500 µl of sterile 100% EtOH to obtain a stock solution of 100 mg/ml, followed by shaking covered at 250 rpm, 42°C until fully dissolved. TAM was then diluted to 10 mg/ml and 4OHT to either 10 mg/ml or 5 mg/ml by addition of corn oil, covered and shaken at 42°C overnight. The TAM/4OHT was distributed into 1 ml aliquots and stored at -20°C. During injection period, aliquots were stored at 4°C for no more than one week. Before injections, TAM or 4OHT was brought to RT and mixed well. Injections were performed in a biosafety cabinet lined with an absorbent pad. Syringes (0.5 ml volume) with an attached needle (Terumo; Somerset, NJ) were loaded through the needle to 0.1 ml and bubbles removed. Mice (P21) were scruffed, their abdomens cleaned with an ethanol wipe, and 0.1 ml was injected intraperitonally. After injection, the mice were placed in a second cage to separate injected from non-injected mice. After all injections were complete, mice were returned to their original cages with standard bedding, food, and water.

Cages were labeled with a biohazard sign and placed on the lowest level of the cage rack to reduce the amount of light entering the cage. To induce Cre recombination mice were injected for 5 consecutive days. At 3 days following the last injection, the mice are transferred to clean cages for 28-30 days after final 4OHT injection to ensure that REST protein levels have decreased following gene knockout. This time point was determined by western blot. Tamoxifen exposed biohazard cages were cleaned with 10% bleach followed by 70% EtOH, and

returned to animal care for autoclaving and washing. Food and bedding were disposed of separately in biohazard bags that were packaged for incineration.

Tissue processing for protein extraction

Mice ages P32-54 were humanly euthanized by CO_2 inhalation and cervical dislocation, retinas were removed using the Winkler method (Winkler *et al.* 1996), and immediately placed in PBS containing 2X protease inhibitors (Roche 11873580001; Indianapolis, IN) on ice. Retinas were homogenized on ice using a pestle, pulse sonicated using a probe sonicator with microtip (Fisher Scientific 60 Sonic Dismembrator; Pittsburg, PA) at an output setting of 5 until no longer viscous (about 6-8 pulses), and the insoluble fraction pelleted by centrifugation at 10,000 rpm for 5 minutes at 4°C. Supernatant was then transferred to a clean eppendorf tube and stored at -80°C.

Western blot

Retinal protein samples for analysis were collected at 7, 14, 21, and 28 days after final 4OHT injection, our control genotype *(+;tdtomato;Rest* $^{flox/+}$ or *+;tdtomato;Rest* $^{flox/flox}$, a control for the effects of Cre (*Cre;tdtomato;Rest* $^{flox/+}$), and the experimental samples to determine the effects of 4OHT on the protein levels of REST (*Cre;tdtomato;Rest* $^{flox/flox}$). Samples were quantified by bichinchoninic acid assay (BCA) (Pierce 23227; Rockford, IL) following the manufacturer's instructions for a 96 well plate. Data from the BCA assay was entered into a data sheet (Excel, Microsoft) designed to calculate sample concentration. Samples were then diluted in 6X Laemmli buffer containing 0.01% beta-mercaptoethanol (BME), and sterile deionized water to a final protein concentration of 0.5 µg/µl and 2X Laemmli buffer.

Protein gels (7.5% polyacrylamide gels; see recipe in Appendix) were placed into the chamber and tested for leaks by adding cold 1X running buffer (see recipe in Appendix) to the upper chamber and allowing it to sit for 15 min. If no leaks were present, the wells of the gel were washed out with running buffer using a 30G needle and syringe. The remaining running buffer was then added to the outer chamber. After the chambers were set up, samples were heat denatured at 99°C for 3 minutes and then loaded at 20 mg/lane into duplicate gels with 5 µl of a protein ladder (BioRad Precision Plus Protein™ Kaleidoscope™ Standards #161-0375; Hercules, CA) loaded as a size marker.

After electrophoresis at 100V, one gel was processed for western blotting and the duplicate gel was stained (Gel-Code Blue, Pierce; Rockford, IL) to verify protein integrity and consistent loading across wells. For western blot, proteins were transferred to nitrocellulose (Whatman N8107; Sigma/Aldrich, St Louis, MO) by immersion electroblotting in cold Towbin buffer (see recipe in Appendix) at 100V for three hours at 4°C, and processed immediately for immunodetection. Blots were washed with diH_20 3X for 5 min on rotator then blocked at RT with rotation for one hour using 5% non-fat dry milk (BioRad 170-5016; Hercules, CA) in TBS-T. The blocking solution was removed and primary anti-REST antibody (ABCam ab2135; Cambridge, MA), diluted 1:800 in 0.05% non-fat dry milk in TBS-T, was applied and incubated overnight at 4°C. Primary antibody solution was removed and blots were washed 3X with TBS-T with rotation at RT. For detection, blots were incubated in anti-rabbit HRP-linked IgG (Pierce 31210; Rockford, IL) at 1:5000 in 0.05% non-fat dry milk in TBS-T for one hour with rotation at RT, followed by 3 washes in TBS-T

and 3 washes in TBS for 10 min each at RT on rotation. Enhanced chemi-luminescence reagent (ECL)(Pierce 32106; Rockford, IL) was applied for 2 min, blots were removed from ECL reagent, held vertically to allow excess solution to drip off and blots were placed between 2 sheets of clear plastic. Blots were exposed on film for one minute, five minutes, overnight and films developed (Kodak RP XoMat M7B).

The REST protein band is 121 kb and results were qualitatively analyzed by comparing the intensity of the sample protein bands to each other. The time point selected for NMDA injection and tissue analysis for subsequent experiments was determined as the time when the intensity of the band no longer decreased from the previous time sample.

Intraocular injections

The retinas were injured using a unilateral injection of NMDA (Sigma/Aldrich M3262; St Louis, MO) at 28-30 days after the last 4OHT injection, according the methods described by Karl *et al.,* (2008). Before injury, the injection station was set up as follows. A raised platform covered with an absorbent pad was placed under the microscope and the microscope adjusted. A heating pad on medium heat setting was placed on the platform to prewarm while the remaining components were prepared. Graded glass micropipettes (World Precision Instruments, Inc. IB100F-6; Sarasota, CA) were made using a glass micropipette needle puller equipped with a box-type filament (Sutter Instrument Co. Model P-87; Novato, CA) with the following settings: Heat: 625, Pull: 150, Velocity: 10, Time: 0. Hamilton syringes (Hamilton Co. 7635-01; Reno, NV) were assembled as follows: the micropipette is

held on the thick portion immediately adjacent to where it starts to thin, the screw cap for the syringe is placed over the thick part of the micropipette followed by (1) a metal washer, made by the University of Houston College of Optometry machine shop and (2) a rubber gasket. A second rubber gasket is placed into the needle attachment opening. The thick section of the micropipette is then carefully inserted into the gasket located within the needle attachment opening with gentle rocking motion, placed onto the syringe and screwed close. Using a ruler, the needle tip (thin part of the micropipette) is cut to 0.7 cm relative to the transition point of the unpulled (thick) and pulled (thinner) needle tip, with distance determined empirically to generate a needle diameter of ~10 microns. The syringe is loaded one of two ways 1) either drawn up through the micropipette very carefully, so as not to break it. It is easiest to do this if the solution to be used is in an eppendorf tube and positioned horizontally (on its side) on an absorbent pad so that both hands can be used to steady the syringe and slowly pull up the plunger to aspirate the solution into the syringe. Alternatively, 2) the solution is loaded into a 0.5 ml syringe with pre-attached needle and then loaded into the Hamilton through the plunger end. Sterile injection solutions were prepared in advance and stored at -80˚C. Once loaded, the syringe was placed into the micromanipulator syringe holder and set aside.

Before starting, the heating pad was moved and placed under an empty cage. Mice were anesthetized by i.p. injection of 60 mg/kg ketamine and 6 mg/kg xylazine determined by the Frishman lab's anesthesia Excel sheet calculator or by isoflurane using the EZ-anesthesia systems isoflurane machine. (Note: further experimental

issues related to the anesthesia are located in sub-section below) To dilate eyes mice received one drop atropine sulfate, and after one minute, excess atropine was removed with a kimwipe and one drop phenylephrine was applied, and after one additional min, the excess was blotted away using a kimwipe. To keep the mouse's eyes from drying out during the procedure, each eye received one drop of lubricant eye drop solution (Refresh Celluvisc; Irvine, CA)

For injections mice were placed with their nose at the edge of the platform, head slightly turned to expose the eye of interest, and the microscope focused to see the eye clearly. Using small forceps, the hair and whiskers around the eye are moved out of the way. The syringe and holder are then moved into place and adjusted to reach the eye. While viewing through the microscope, a 27G needle was used to create a pilot hole into the eye, just posterior to the iris and a little above the line of blood vessels. Hitting the blood vessels makes it harder to locate the pilot hole. If this happened, the excess blood was blotted with a kimwipe. Once the pilot hole was created, the micropipette was lowered at a 90° angle using the controller knobs on the micromanipulator. It is important not go in too deeply, just enough that the solution is going into the vitreous and not flowing out of the hole at the injection site (~0.2 mm). Once inserted, 1 µl was injected by slowly tapping the plunger down; excess speed at this step may cause some of the solution to leak out. After the injection was complete, the micropipette was allowed to remain in the eye for ~30 seconds before it was slowly retracted using the micromanipulator. When repeating procedure for the second eye, lubrication drops were re-applied to both eyes. After injections were finished, additional lubrication drops were applied to each eye and

the mouse was placed in an empty cage on a heating pad set to the medium heat setting and monitored until it is fully revived.

For the injury model, mice received 0.1M N-methyl-D-aspartate (NMDA) (Sigma/Aldrich M3262; St Louis, MO) in the right eye and sterile PBS in the left. Mice were returned to their cages for 48 hours, then re-anesthetized using 60 mg/kg Ketamine and 6 mg/kg Xylazine (See note above), and both eyes received 1 µl of a solution containing 1 µg/µl epidermal growth factor (EGF) (Peprotech; Rocky Hill, NJ) and 1 µg/µl 5-ethynyl-2'-deoxyuridine (EdU) (Sigma/Aldrich T511285; St. Louis, MO). At 4 hours following the last injection, mice were euthanized using CO_2 inhalation followed by cervical dislocation and eyes enucleated for analysis.

Anesthesia using inhalatory isoflourane

When using Ketamine/Xylazine anesthesia, 1 out of 4 experimental mice died between 30 min to 1 hour after the intraocular injections. These mice revived as expected following intraocular injections, but subsequently died. Reducing the dose resulted in some improved survival, but failed to achieve the deep level of anesthesia needed for repeatable and reliable intraocular injections. Therefore, isoflurane anesthesia was tested as an alternative and provided an improved form of anesthesia for intraocular injections. During isoflurane exposure, pups were completely anesthetized and did not start to awaken during the injections. This allowed intraocular injections to proceed at a slower pace into both eyes resulting in more consistent injections. Recovery from isoflurane was rapid and after injections, there were no fatalities.

Isoflurane was administered to the mouse through a nose cone tapped to the raised platform. Mice were initially anesthetized in a chamber with the flow gauge set to 0.5 liters/min and the isoflurane gauge set to 5%. Once mouse was fully anesthetized (as evaluated by slowed breathing and lack of response to toe pinch), it was taken out of the chamber and placed into the nose cone, which maintains the flow at 0.5 liters/min. At this time, the isoflurane flow was reduced to 1.5%. If mouse appeared to be reviving, the isoflurane was increased to 2%. It has been shown that the mouse can safely be on the isoflurane for 3-6.5 hrs (Szczesny *et al.,* 2004), although for intraocular injections, anesthesia time was typically less than 15 minutes.

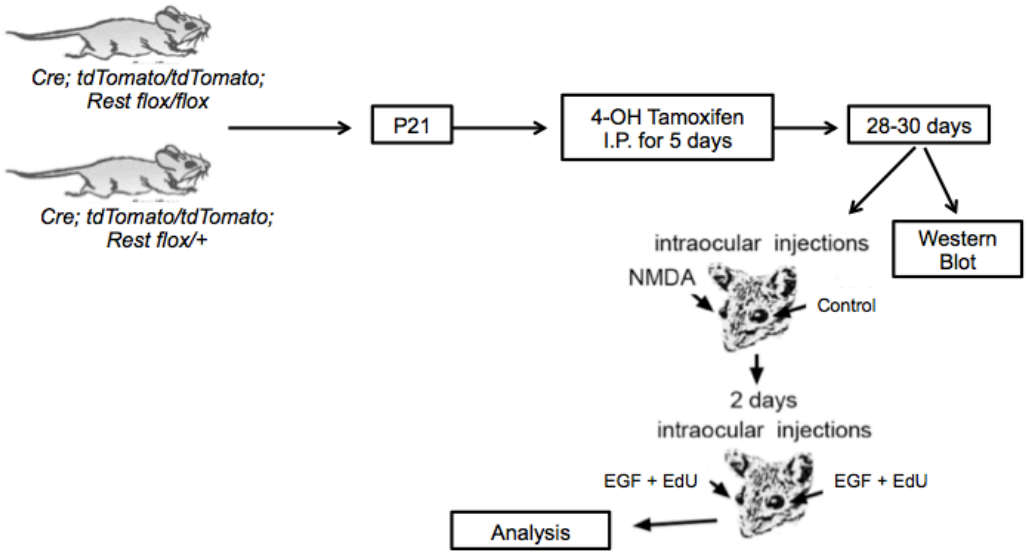


Figure 6: Approach for *Rest* knockout and retinal injury
Experimental genotypes were given 4OHT at age P21 for 5 consecutive days. The NMDA injury model was performed on mice 28-30 days after final 4OHT injection and then analyzed.

Tissue processing for cyrosectioning/histology

Eyes were enucleated using forceps and placed in PBS a 10 ml round bottom tube on ice. After all eyes were in PBS, the cornea and lens were removed using fine iris scissors and eyecups were placed back in PBS on ice. Eyecups were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4-7.8, for 30 min on the rotator at room temperature. The PFA was removed and the eyecups were washed once with PBS. Sucrose infiltrations were done with rotation at 5% sucrose in 0.1M phosphate buffer for 1 hour at RT, 1:1 (5% to 20% sucrose) in 0.1M phosphate buffer for 1 hour at RT, followed by cryoprotection in 20% sucrose in 0.1M phosphate buffer overnight at 4°C. Final infiltration used a 1:1 solution of 20% sucrose to OCT (Tissue-Tek; Sakura Feintek; Torrance, CA) for 1 hour at 4°C. Retinas were then frozen in fresh 1:1 20% sucrose to OCT using liquid nitrogen, wrapped with heavy foil, and stored at -80°C.

Histological sectioning

Frozen blocks were sectioned at 50 μm thickness until the retinal tissue was reached. Eyecups were sectioned at 15 μm using a cryostat (Leica CM1950; Buffalo Grove, IL) set at -20°C and sections were air-dried onto charged microscope slides (VMR SuperFrost Plus; Radnor, PA). Sections were distributed sequentially across 10 slides such that on each slide, the interval between sections was at least 150 μm. Slides were stored at -20°C for no longer than a month prior to analysis. For analysis, sections containing the optic nerve were identified for each eye and a total of eight nonadjacent retinal sections, distributed equally on either side of the optic nerve, were processed for EdU detection or immunohistochemistry.

EdU detection assay

EdU was detected using Click-iT® EdU Alexa Flor® 488 Imaging Kit (Molecular Probes; Grand Island, NY). Kit protocol was adapted for detection using tissue sections. Slides with desired retinal tissue sections were outlined with a PAP pen to keep the solutions from leaking off of the slide during processing, and dried in a desiccator. The tissue was post-fixed by pipetting 300 µl 4% PFA onto the sections for 15 min at RT. The PFA was then removed by tipping the slide and allowing the solution run off onto a kimwipe, which was disposed of in a biohazard waste container. The tissue was washed twice with 300 µl 3% BSA in PBS. Permeabilization of the sections used 0.5% Triton-X in PBS for 20 min then sections were washed twice with 300 µl of 3% BSA in PBS. The Click-it buffer additive was prepared fresh daily by diluting the 10X solution 1:10 in deionized water. The sections received 300 µl of Click-it reaction cocktail (see recipe in Appendix) and incubated for 30 min, protected from light, at RT. Sections were washed once with 300 µl 3% BSA in PBS and twice with 300 µl 1X PBS. Then 300 µl of 1:2000 Hoechst 33342 (Invitrogen 33342; Grand Island, NY) in PBS was applied to the sections for 30 min at RT, protected from light. Sections were washed twice with 1X PBS and then coverslips were mounted using Fluoromount (Sigma/Aldrich F4680; St. Louis, MO). Let dry, protected from light, and then seal with clear nail polish.

Immunohistochemistry

Slides containing retinal sections were thawed and post-fixed in 4% PFA for 10 min at RT. Sections were outlined with a PAP pen and dried in a desiccator. Sections were rehydrated with diH_2O for 5 min and then treated with 1% sodium

borohydride for 2 min, washed for 5 min twice in H_2O and then for 5 min twice in HBSS, and blocked for 2 hours at RT in a moist chamber. Blocking Buffer consisted of 10% goat serum, 0.5% triton-x, 1% fish gelatin, 5% BSA, in HBSS. Primary antibodies for glutamine synthetase (GS) (Millipore MAB302; Billerica, MA), glial fibrillary acidic protein (GFAP) (Millipore MAB360; Billerica, MA), green fluorescent protein (GFP) (ABCam AB290; Cambridge, MA), and CD11b (BD Pharmingen; San Jose, CA) were diluted 1:400, 1:1000, 1:1000 and 1:20, respectively, in blocking buffer and applied to sections overnight, rotating, at 4°C. Primary antibodies were removed and sections washed for 10 min twice in HBSS. Secondary antibodies conjugated to AlexaFlour488 and AlexaFlour543 (Molecular Probes; Eugene, OR) were diluted 1:1000 in blocking buffer, added to sections, and incubated for 1 hour on slow rotation at RT. Following two 5 min washes in HBSS and two 5 min washes in diH_2O, sections were counterstained with Hoechst (Invitrogen 33342; Grand Island, NY) 1:2000 in HBSS and coverslipped with Fluoromount (Sigma/Aldrich F4680; St. Louis, MO). Mounting medias Vectashield and ProlongGold with DAPI were tested, but resulted in rapid fading of tdtomato fluorescence. Specificity of labeling was confirmed by omitting primary antibody. Immunostained tissues were imaged by epifluorescence using an inverted microscope (Olympus IX71) with a monochrome, cooled CCD digital camera (Rolera-XR; Q-Imaging)

Statistical analysis

Cell counts were analyzed using pairwise T-tests with Bonferroni adjustment to correct for multiple comparisons or ANOVA with post-hoc T-tests with Tukey's HSD for multiple comparisons. *p*-values less than or equal to 0.05 were considered statistically significant.

RESULTS

Effects of Tamoxifen on Cre recombination in *Cre-ERT2;Bgeo/GFP* mice

The efficiency of Tamoxifen (TAM) induced Cre recombination in Müller glia was tested in 5 month old mice injected interperitonally with 0.3 ml TAM at a concentration of 10 mg/ml for 5 consecutive days. At 7 days after final injections, mice were euthanized, eyes enucleated, and eye cups processed for histology. *Glast-CreERT2;Bgeo/GFP* mice contain a TAM inducible Cre recombinase and a green fluorescent protein (GFP) reporter gene under control of the *Slc1a3* (solute carrier family 1, glial high affinity glutamate transporter) promoter. Tamoxifen induced Cre expression that will result in targeted deletion of floxed sequences. For the pilot study, Cre removes the LacZ gene replacing it with enhanced GFP expression in Cre expressing cells. Retinal sections were imaged using epifluorescence microscopy to identify cells fluorescing green from the GFP reporter gene (Figure 7). Reporter gene expression was observed in a very small subset of cells, all of which showed correct morphology and retinal position to be Müller glia. GFP labeled cells were verified as Müller glia using immunostaining with antibodies against glutamine syntheses (GS), a marker for Müller glia (Figure 7). The number of GFP expressing cells was unexpectedly low, compared to published results using this Cre strain (de Melo *et al.,* 2012). To test whether the sparse GFP reporter expression resulted from non-fluorescence of the GFP reporter, sections were immunostained with antibodies against GFP. There was no difference in the number of labeled Müller glia (Figure 7) and all GFP immunopositive cells also showed detectible GFP expression. To

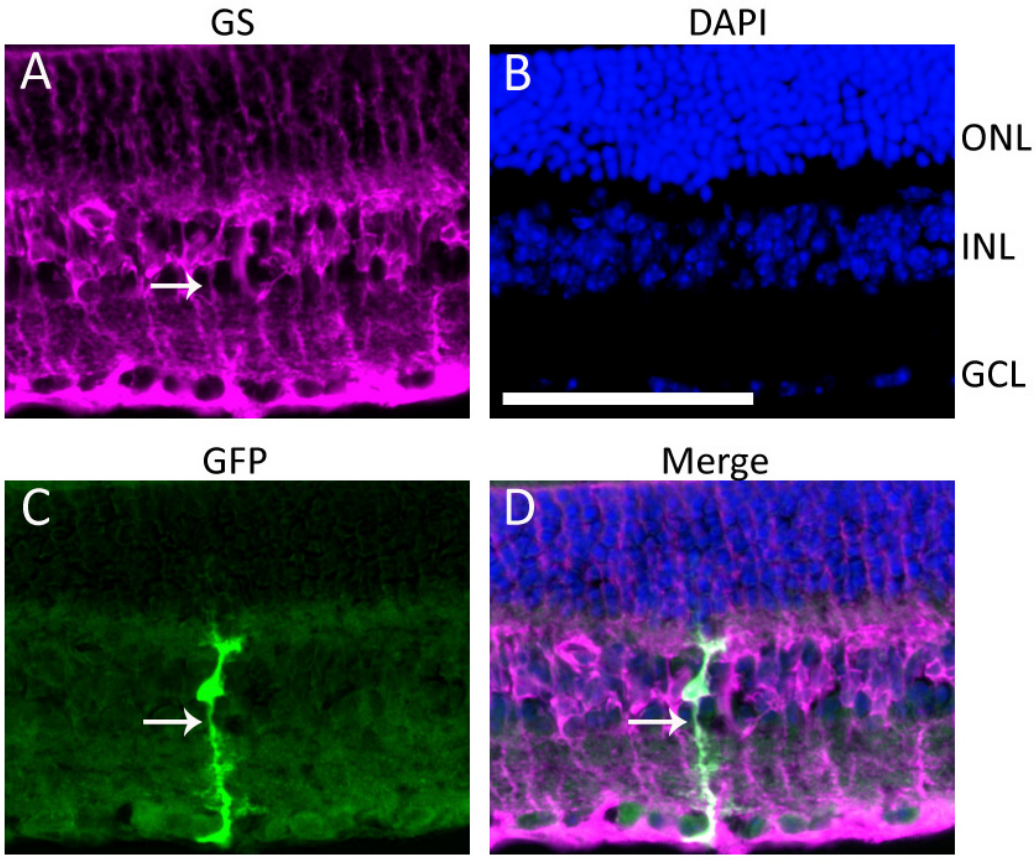


Figure 7: GFP reporter expression in *Glast-CreERT2;Bgeo/GFP* mice
Photomicrographs of *Glast-CreERT2;Bgeo/GFP* mice injected with tamoxifen at 5 months old to induce Cre recombination in Müller glia. (A): Immunostaining for glutamine sythetase (GS) (magenta) shows Müller glia. (B): DAPI (blue) immunostaining for cell nuclei. (C): Immunostaining for green fluorescent protein (GFP) showed no increase in GFP positive cells compared to GFP reporter fluorescence. (D): Merge showing GFP-expressing cells overlap GS immunostaining in Müller glia. Scale bar = 100 microns.

determine if the age of the mice has an effect in the efficiency of Cre recombination, P30 mice were tested using the same paradigm as above. There was no Cre recombination observed (not shown).

Effects of Tamoxifen and 4-hydroxytamoxifen on Cre recombination in *Rlbp1-CreERT2;Rosa26-tdTomato* mice

Since the number of cells with Cre-mediated recombination was so low, *Cre-ERT2;Bgeo/GFP* mice could not be used for experimental analysis. We then obtained a new Cre driver and reporter strain: *Rlbp1-CreERT2;Rosa26-tdTomato* (Gift of Dr. E Levine, (Vazquez-Chona *et al.,* 2009)). *Rlbp1-CreERT2;Rosa26-tdTomato,* under the control of the 3-kb region of mouse genomic DNA encompassing a portion of the *Rlbp1* (retinaldehyde binding protein 1) gene promoter, has the capacity to promote homogenous and robust reporter gene expression in Müller glia (Vazquez-Chona *et al.,* 2009).

The efficiency of Cre recombination and reporter expression was tested in *Rlbp1-CreERT2;Rosa26-tdTomato* mice at age P28 by IP injection of 0.1 ml TAM at 10 mg/ml for 5 consecutive days. At 7 days after final injections, mice were euthanized, enucleated, and eye cups processed for histology. Retinal sections, imaged for tdTomato expression, showed no increase in reporter expression compared to the *Cre-ERT2;Bgeo/GFP*. Published studies using other strains of *CreERT2* transgenic mice were shown to have successful Cre recombination following IP injections of 4-hydroxytamoxifen (4OHT) (Figure 8) (Kim *et al.,* 2004; Lantinga-van Leeuwen *et al.,* 2006). Therefore, 4OHT was tested for induction of Cre recombination in P21 *Rlbp1-CreERT2;Rosa26-tdTomato* mice following the above paradigm. 4OHT injections

resulted in robust tdTomato expression in a large number of Müller glia. Immunostaining with antibodies against GS verified tdTomato expressing cells were Müller glia (Figure 9). Subsequent optimization and experimental manipulations used the *Rlbp1-CreERT2;Rosa26-tdTomato* mice.

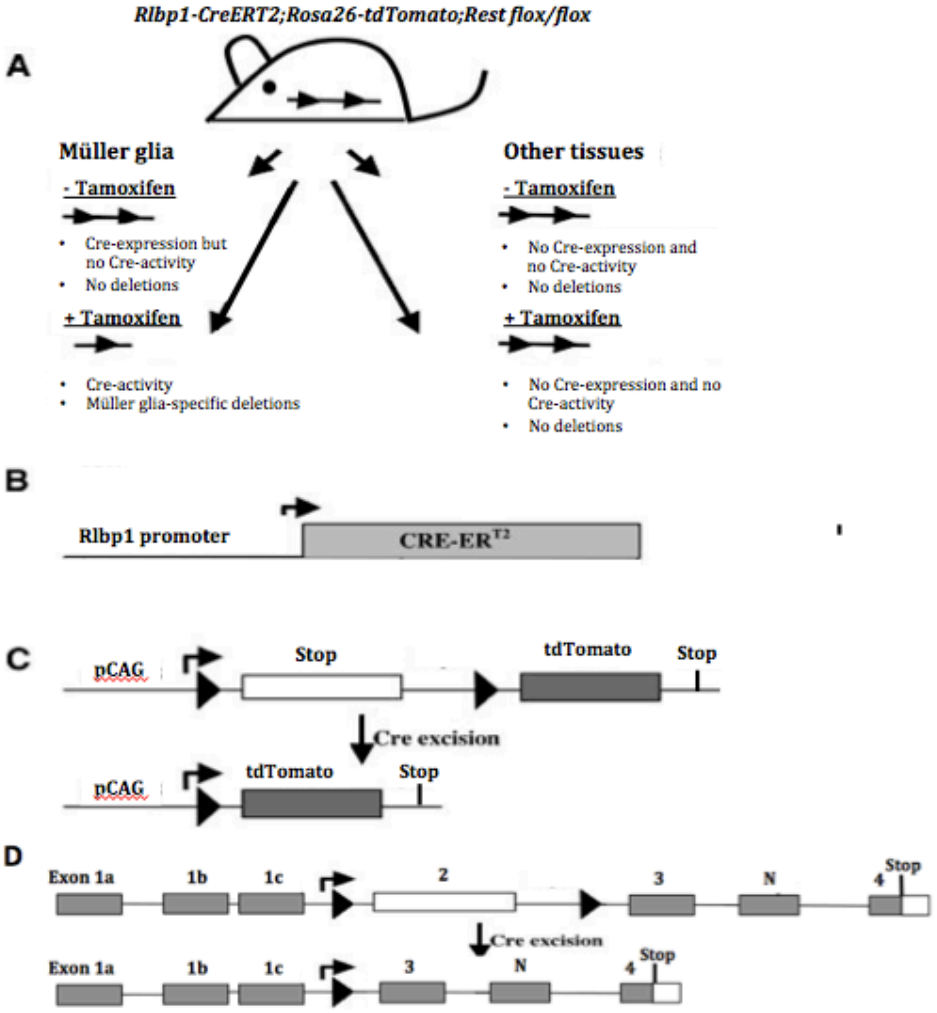


Figure 8: Müller glia specific CreERT2 mediated recombination

Müller glia specific promoter (*Rlbp1*) drives the expression of a tamoxifen-inducible Cre recombinase (*CreERT2*). Transgenic mice carrying this *Cre* transgene are crossed to mice carrying a tdTomato reporter gene and floxed *Rest* gene. In the absence of tamoxifen, Cre is expressed in Müller glia under the Rlbp1 promoter, but Cre protein is not active. Upon tamoxifen-administration, however, Cre is activated and translocated to the nucleus where it can mediate recombination of DNA sequences flanked by loxP sites (▶ ; loxP site). In other tissues, Cre is not expressed, leaving the floxed transgenes intact. (B) Schematic of the *Rlbp1-CreERT2* construct. (C) The Schematic of *Rosa26-tdTomato* construct. Adapted from: (Lantinga-van Leeuwen *et al.,* 2006) (D) Schematic of *Rest* floxed allele. Adapted from: (Mao *et al.,* 2011).

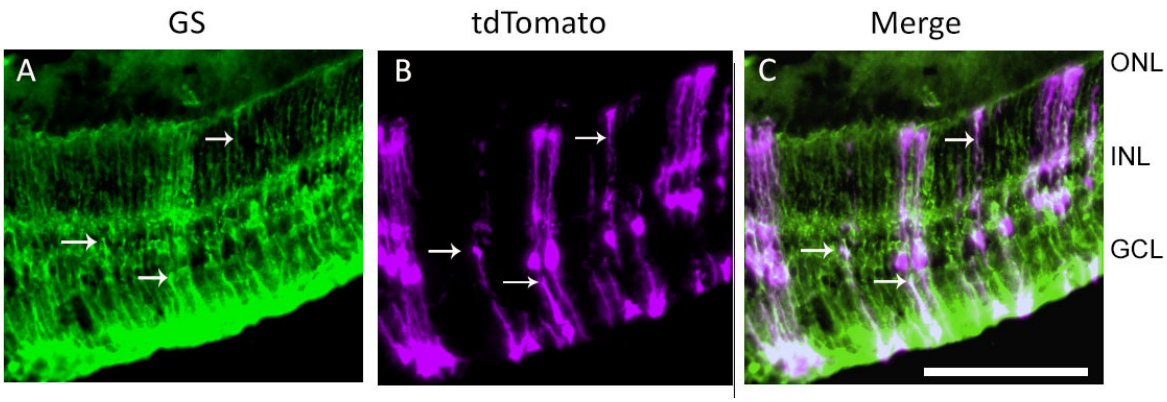


Figure 9: tdTomato reporter expression in *Rlbp1-CreERT2;Rosa26-tdTomato* mice

Photomicrographs of *Rlbp1-CreERT2;Rosa26-tdTomato* mice injected with 4OHT at P21 to induce Cre-recombinase in Müller glia. (A) Immunostaining for glutamine synthetase (GS) (green) shows Müller glia. (B): tdTomato reporter expression (magenta) showing Cre-recombination in a subset of Müller glia. (C): Merge showing tdTomato expressing cells overlap GS immunostaining in Müller glia. Scale bar = 100 microns.

Effects of reduced 4OHT on Cre recombination

A lower concentration and a reduced timecourse for 4OHT injection was tested to determine if this would still be sufficient to induce a robust Cre recombination response. P21 *Rlbp1-CreERT2;Rosa26-tdTomato* mice were injected with 0.1 ml of 5 mg/ml 4OHT for either 3 or 5 consecutive days. At 7 days after final injection mice were euthanized eyes enucleated, and eyecups processed for histology. A decrease in 4OHT to 5 mg/ml triggered a robust Cre recombination, as measured by induction of the tdTomato reporter, with the extent of the response equivalent to that from injections using the higher concentration (10 mg/ml). Mice that received 4OHT for 5 days showed increased numbers of tdTomato expressing cells compared to 3 days (Figure 10). In all cases, tdTomato expression was restricted to cells with a typical morphology of Müller glia.

Validation of NMDA injury in Müller glia

The experimental paradigm used intraocular injections of N-methyl-D-aspartate (NMDA) to elicit an injury response as previously described (Karl *et al.,* 2008). NMDA is a neurotransmitter that activates ionotropic glutamate receptors causing cell death of retinal ganglion cells (RGC) and amacrine cells (Lucas and Newhouse 1957; Siliprandi *et al.,* 1992; Sucher *et al.,* 1997). Karl *et al.,* (2008) showed that, when followed by intraocular injections of epidermal derived growth factor (EGF), NMDA injury induced proliferation of Müller glia. For an initial test of whether NMDA injury in our hands elicited an injury response in Müller glia, retinal sections from NMDA injected eyes were immunostained with antibodies against glial fibrillary acidicprotein (GFAP). In the normal retina, Müller glia processes

contain intermediate filaments, including vimentin, nestin, but very little detectible GFAP, whereas astrocytes at the vitreal surface of the retina are robustly immunoreactive for GFAP. Following trauma to the retina such as NMDA injection, GFAP is quickly upregulated in glial cells and is detected throughout the cell (Bringmann *et al.,* 2013; Humphrey *et al.,* 1993; Sahel *et al.,* 1990). In pilot studies, NMDA intraocular injections induced a widespread upregulation of GFAP in Müller glia (Figure 11).

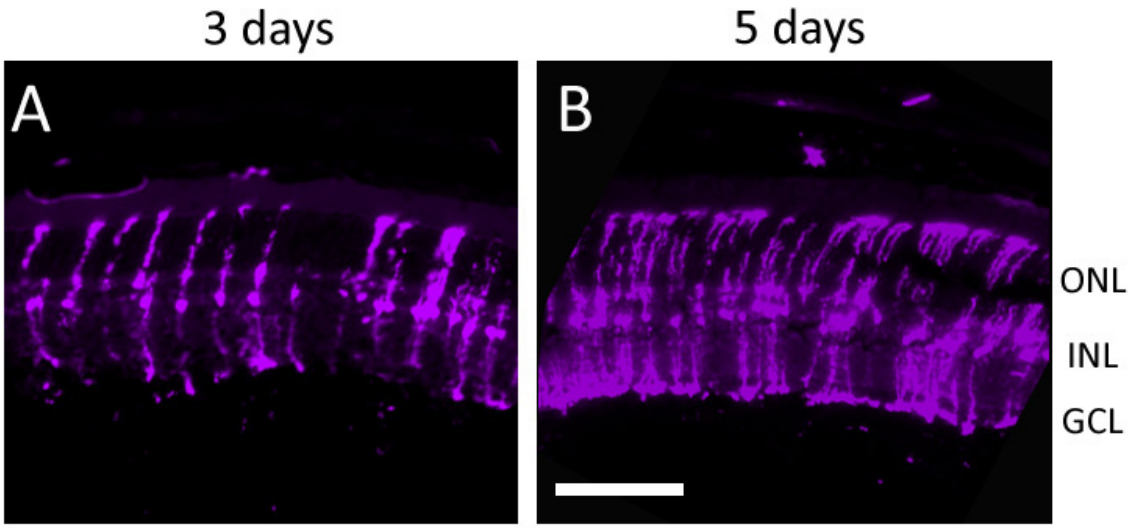


Figure 10: tdTomato expression in 3 vs. 5 day 4OHT injections
Photomicrographs of retinas from P21 mice injected with 5 mg/ml 4OHT for either 3 or 5 consecutive days and analyzed at age P32. (A) Three daily injections of 4OHT resulted in Cre-expression in Müller glia as detected by tdTomato reporter expression. (B) Five daily injections of 4OHT increased the number of Cre-expressing/tdTomato-positive Müller glia. In both cases, there was no evidence of tdTomato reporter expression in astrocytes. Scale bar = 100 microns.

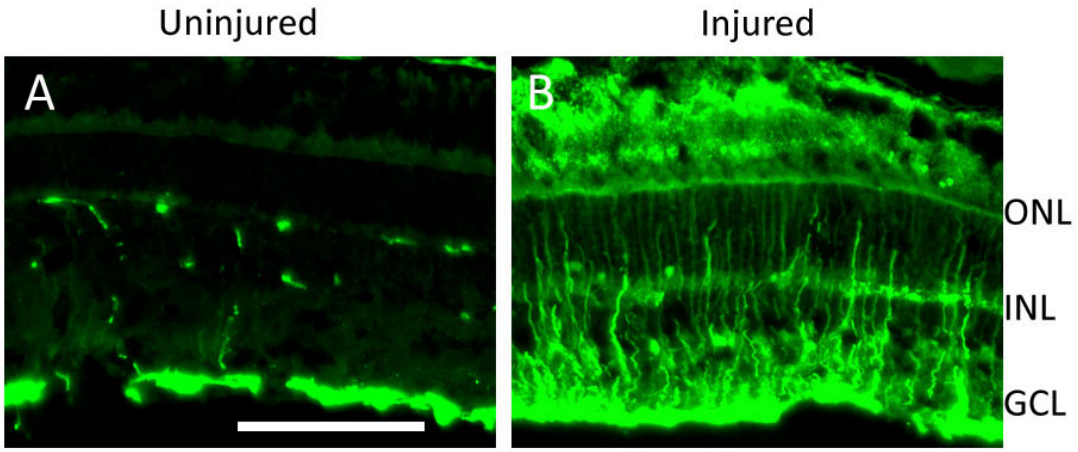


Figure 11: GFAP expression in uninjured and injured retinas
Photomicrographs showing retinal sections from normal (A) and NMDA injected (B) eyes immunostained with antibodies against GFAP (green). (A) In the uninjured retina, GFAP expression is primarily restricted to astrocytes at the vitreal surface of the retina, adjacent to the ganglion cell layer (GCL). Immunoreactivity in the inner nuclear layer (INL) results from cross reactivity of the anti-mouse secondary antibodies with the retinal vasculature. (B) Following NMDA injury, there is a robust increase in GFAP in Müller glia that is evidenced by the immunoreactivity in their radial processes that extend across the retina. The unexpected fluorescence located above the outer nuclear layer (ONL) appears to be associated with photoreceptor outer segments. Scale bar = 100 microns.

Time course of REST protein loss following Cre-mediated knockout in Müller glia

The time point in which REST protein levels in the retina reached the lowest levels following 4OHT induced *Rest* gene knockout was determined using western blot analysis with anti-REST antibodies. Retinal protein was collected from *Rlbp1-CreERT2;Rosa26-dTomato;Rest* $^{flox/flox}$ (further identified in this thesis as *Cre;tdTomato;Rest* $^{flox/flox}$) mice at 7, 14, 21, and 28 days after the final 4OHT injection and additional samples included: *+;Rosa26-tdTomato;Rest* $^{flox/flox}$ or *+;Rosa26-tdTomato;Rest* $^{flox/+}$ (further identified as control) that received 4OHT and was collected at 7 days after final injection to control for the effects of 4OHT, *Rlbp1-CreERT2;Rosa26-tdTomato;Rest* $^{flox/+}$ (further identified as *Cre;tdTomato;REST* $^{flox/+}$) that received 4OHT and was collected 7 days after final injection to control for the effects of Cre recombination, and C57Bl/6 that did not receive 4OHT as a control to verify the normal REST amount found in the retina. The time point between the 4OHT treatment (Cre-induction) and the NMDA injection and tissue analysis for subsequent experiments was determined as the time when the intensity of the band no longer decreased from the previous time sample. Western blot analysis determined this time to be 28 days after the final 4OHT injection (Figure 12).

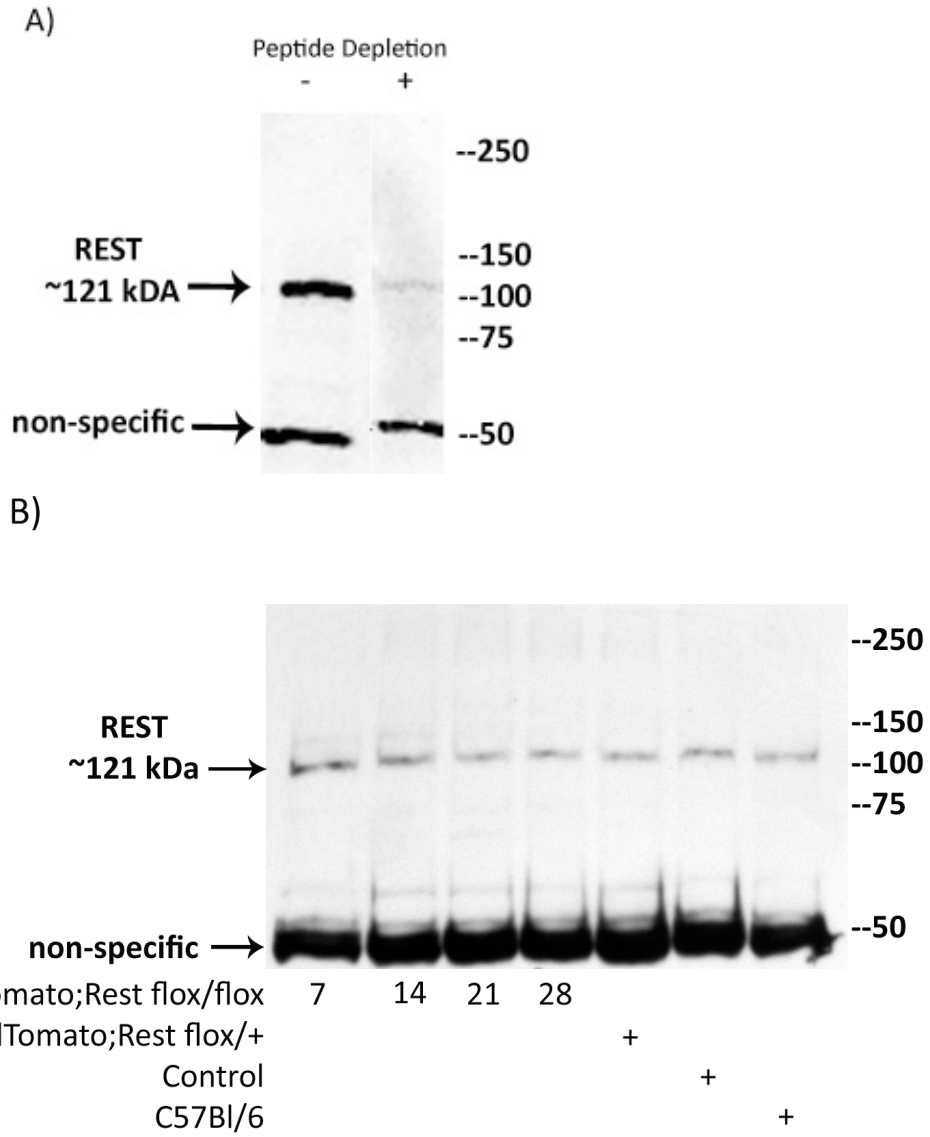


Figure 12: Time course of REST protein reduction in the retina following *Rest* knockout in Müller glia

(A) Western blot verification of REST protein induction and depletion. Courtesy of Bruce Colton, University of Houston College of Optometry. (B) Western blot was used to determine the time point in which there was no reduction of the REST protein band intensity from the time point before. Time point samples tested from *Cre;tdTomato;Rest* $^{flox/flox}$ were at 7,14, 21, 28 days. The control lane is *+;tdTomato;Rest* $^{flox/flox}$ or *Rest* $^{flox/+}$ to test for the effects of 4OHT and the *Cre;tdTomato;Rest* $^{flox/+}$ lane to test for the effects Cre recombination on REST protein levels. C57Bl/6 was used as a baseline for initial REST levels.

Effects of *Rest* knockout in mouse Müller glia followed by retinal injury

Based on its known role in repressing expression of neuronal genes in glial cells, we hypothesized that REST may block the neurogenic potential of Müller glia in the mammalian retina. To begin to test this hypothesis, proliferation in the retina was assessed in mice with Müller glial-specific *Rest* knockout following NMDA injury to the retina. Proliferation was detected using EdU incorporation, which directly measures active DNA synthesis during S-phase of the cell cycle. EdU is an analog of thymidine that is incorporated into DNA during active DNA synthesis (Salic and Mitchison 2008). Following REST knockout, mice received unilateral intraocular injection of NMDA to induce retinal injury, and contralateral eyes received an intraocular injection of PBS as an injection control. This was followed 48 hrs later by bilateral intraocular injections of EGF+EdU (Karl *et al.,* 2008). Retinal sections were stained for EdU and analyzed for EdU+ staining and tdTomato expression.

EdU+ staining was present in the NMDA injured retina, but not in PBS controls. Staining for cell nuclei with DAPI revealed not all EdU+ spots were double-labeled for DAPI and therefore some EdU+ staining was considered to be non-specific (Figure 13). Only cells that were doubled labeled with EdU and DAPI were counted for statistical analysis. Counted cells were further scored for double staining of EdU and tdTomato labeling to identify Müller glia, classified by nuclear size (larger than 10 microns vs. smaller than 10 microns) and counted for statistical analysis (Figure 14).

Pilot studies of Cre recombination induced using 4OHT showed a robust expression of tdTomato (Figure 9). Unexpectedly, when this same treatment

paradigm was used in the final experimental mice, only half of mice (9 out of 19) showed tdTomato expression. Comparing tdTomato positive and tdTomato negative retinas for *Cre;tdtomato;REST* $^{flox/flox}$ and *Cre;tdTomato;Rest* $^{flox/+}$, there were no statistically significant differences in the mean difference in EdU+ cells between NMDA and PBS treated retinas (p=0.847 and p=0.394) (Figure 15). Because the presence of tdTomato expression compared to the absence of tdTomato did not alter the number of EdU+ cells, these groups were combined for further statistical results.

Pairwise comparisons of the number of EdU+ cells/mm retinal length between injured and uninjured retinas within each genotype (control, *Cre;tdTomato;Rest* $^{flox/+}$, and *Cre;tdTomato;Rest* $^{flox/flox}$) showed significant differences. For control mice (Cre-negative), the number of EdU+ cells/mm retinal length for NMDA injected eyes averaged 10.9 ±1.91 (n=5) vs. 0.18±0.25 (n=5) for PBS injected eyes (p=0.006). For *Cre;tdTomato;Rest* $^{flox/flox}$ mice, the number of EdU+ cells/mm retinal length for NMDA injected eyes averaged 6.48±6.4 (n=10) vs. 0.06±0.13 (n=10) for PBS injected eyes (p=0.016). For *Cre;tdTomato;Rest* $^{flox/+}$ *mice,* the number of EdU+ cells/mm retinal length for NMDA injected eyes averaged 2.91±1.44 (n=9) vs. 0.11±0.22 (n=9) for PBS injected eyes, although this difference did not reach statistical significance (p=0.134)(Figure 16). Comparison of the difference in EdU+ cells in NMDA injured retinas vs. PBS control eyes revealed no significant differences between the control and *Cre;tdTomato;Rest* $^{flox/flox}$ or between the *Cre;tdTomato;Rest* $^{flox/+}$ and *Cre;tdTomato;Rest* $^{flox/flox}$. In contrast, there was a

significant decrease between the control and *Cre;tdTomato;Rest* $^{flox/+}$ (p<0.05)(Figure 17).

Two distinctive nuclear morphologies were observed with EdU labeling: some nuclei were small with an irregular morphology and other nuclei were larger, with a round or slightly oblong morphology (Figure 13; Figure 14). There was an unexpectedly low number of double labeled, EdU+ and tdTomato+ cells in the injured retina, although all of these showed the larger nuclear morphology (Figure 14). The small nuclear morphology of the majority of EdU+ cells and their lack of any double labeling with tdTomato reporter suggested that these cells were not likely to be Müller glia. The small nuclear morphology suggested that these cells were potentially microglia or inflammatory cells that migrated into the retina following injury. In an effort to identify the EdU+ cells, retinal sections were immunostained with antibodies against CD11b. Unfortunately, staining results were inconclusive, as the available antibody did not function as expected (data not shown). Due to time constraints, further experiments to identify these cells were not conducted. However, this is a critical area for future analysis.

The subset of EdU+ cells with the larger, round or slightly oblong nuclei were identified as Müller glia and were analyzed independently. Pairwise comparison of the number of large EdU+ cells in retinas from NMDA injected vs PBS injected retinas for each genotypes showed the same pattern as comparisons of total EdU+ cells. In control retinas, the mean difference in large EdU+ cells in NMDA vs. PBS injected retinas was 6.71±1.53 (p=0.017). For *Cre;tdTomato;Rest* $^{flox/flox}$ the mean difference in large EdU+ cells in NMDA vs. PBS injected retinas was 2.39±0.93

(p=0.044). For *Cre;tdTomato;Rest* $^{flox/+}$, the mean difference in large EdU+ cells in NMDA vs. PBS injected retinas was 1.16±0.70, but this difference was not statistically significant (p=0.229) (Figure 18).

The mean differences in EdU+ cells in the retinas of NMDA vs. PBS injected eyes between all genotypes were compared using ANOVA. There was a statistically significant difference between the control and the *Cre;tdTomato;Rest* $^{flox/+}$ (p<0.01) and control and the *Cre;tdTomato;Rest* $^{flox/flox}$ (p<0.005). However, there was no statistical significance in the mean difference in EdU+ cells in the retinas of NMDA vs. PBS injected eyes between *Cre;tdTomato;Rest* $^{flox/+}$ and *Cre;tdTomato;Rest* $^{flox/flox}$ (Figure 19).

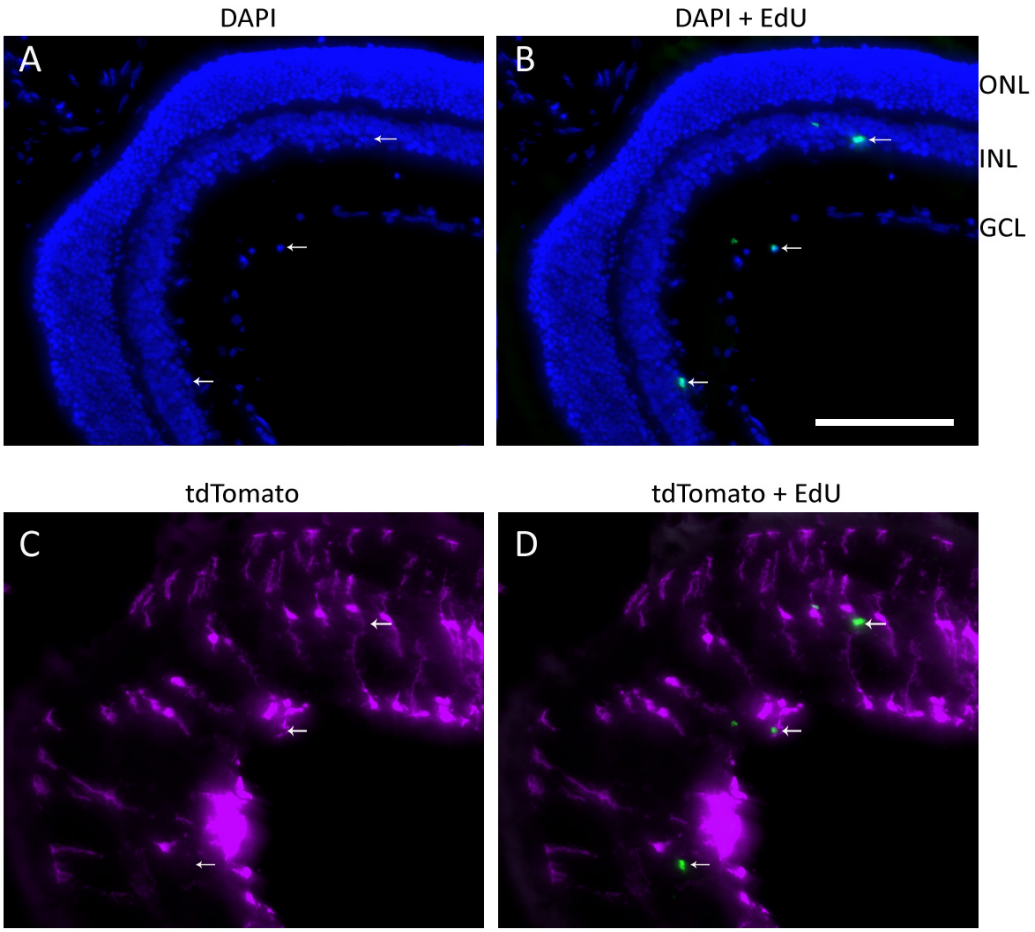


Figure 13: EdU+ cells in the injured retina
Photomicrographs of NMDA injured retinal sections double labeled with EdU (green) and DAPI (blue) and double labeled with EdU (green) and tdTomato (magenta) from *Cre;tdTomato;Rest* $^{flox/flox}$ mice 28 days following 4OHT injections. (A) DAPI immunostaining for cell nuclei. (B) Double labeling for DAPI and EdU+ cells (arrows). (C): tdTomato expression in Müller glia. (D) Double labeling for tdTomato and EdU+ cells (arrows). Scale bar = 100 microns.

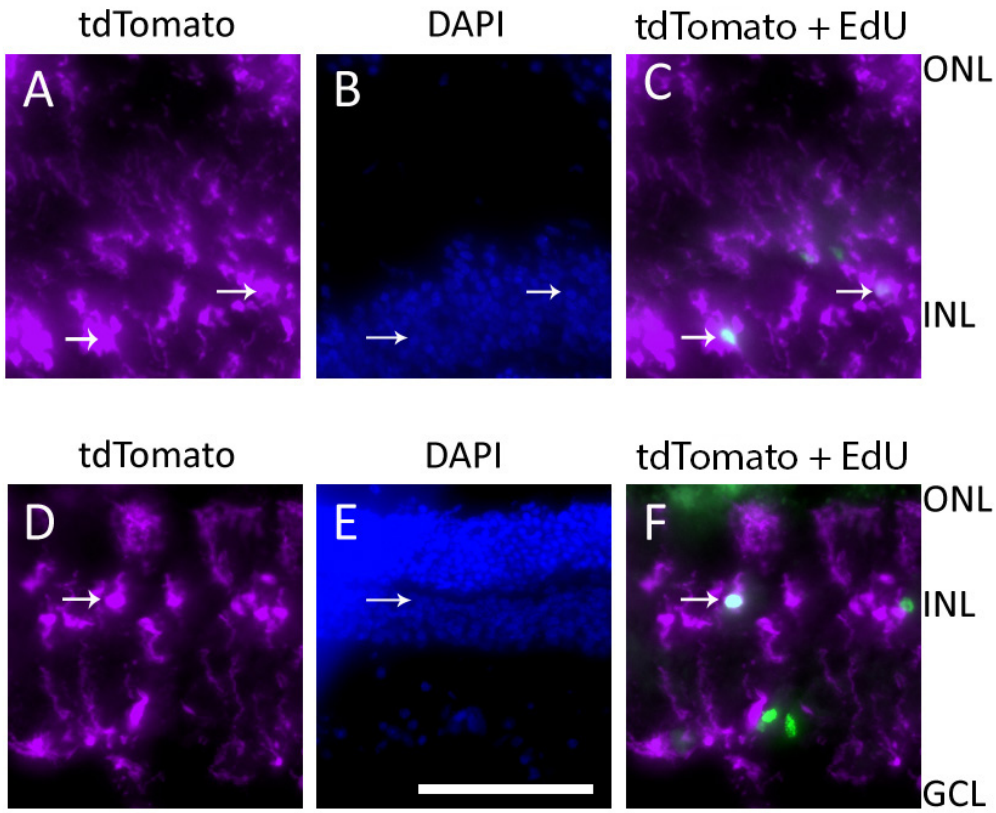


Figure 14: Double-labeled EdU/tdTomato cells
Photomicrographs showing EdU+ cells (green) and tdTomato (magenta) labeling in NMDA injured retinas stimulated with EGF and labeled with EdU from *Cre;tdTomato;Rest* $^{flox/flox}$ mice 28 days following 4OHT injections. (A,D) tdTomato fluorescence. (B,E) DAPI immunostaining for cell nuclei. (C,F) tdTomato and EdU double labeled cells (arrows). Scale bar = 100 microns.

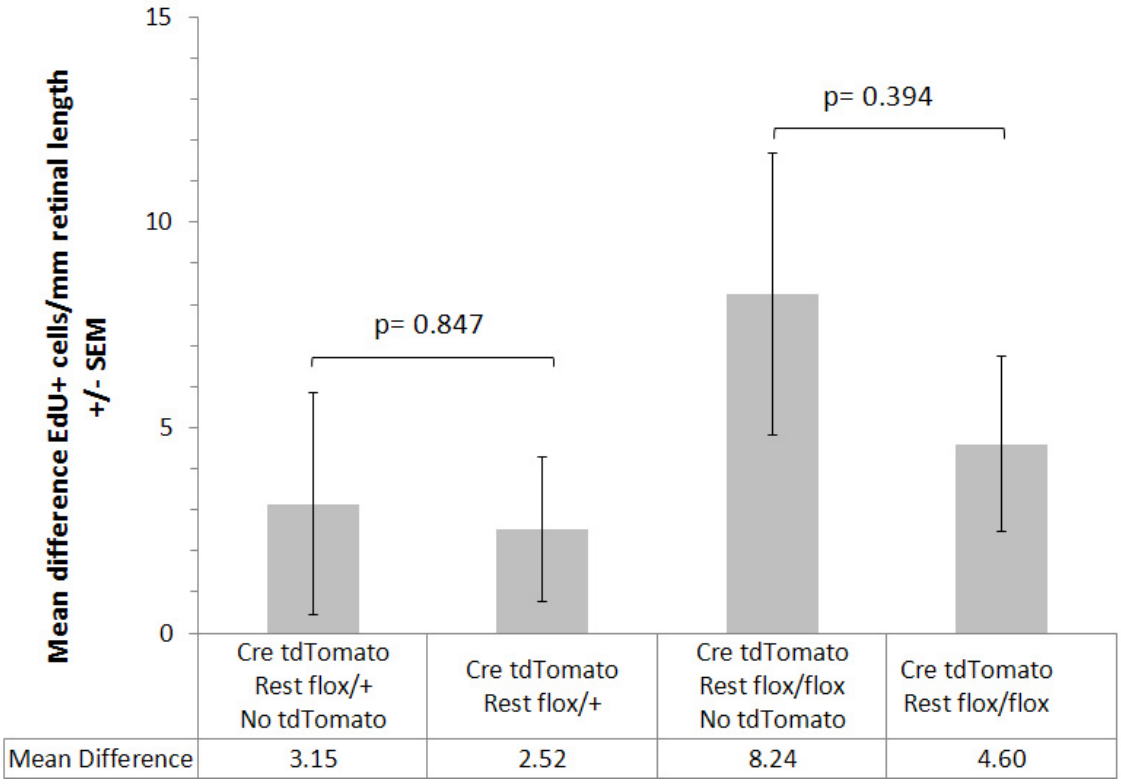


Figure 15: Comparison of EdU+ cell counts in experimental genotypes with and without TdTomato induction

Bar graph showing mean difference of EdU+ cells per millimeter retinal length (+/- SEM) in NMDA injured retina compared to contralateral PBS-control uninjured retina for experimental genotypes with and without tdTomato expression. Comparisons across conditions used ANOVA with post-hoc T tests and Tukey's HSD for multiple comparisons. $p \leq 0.05$ was considered statistically significant.

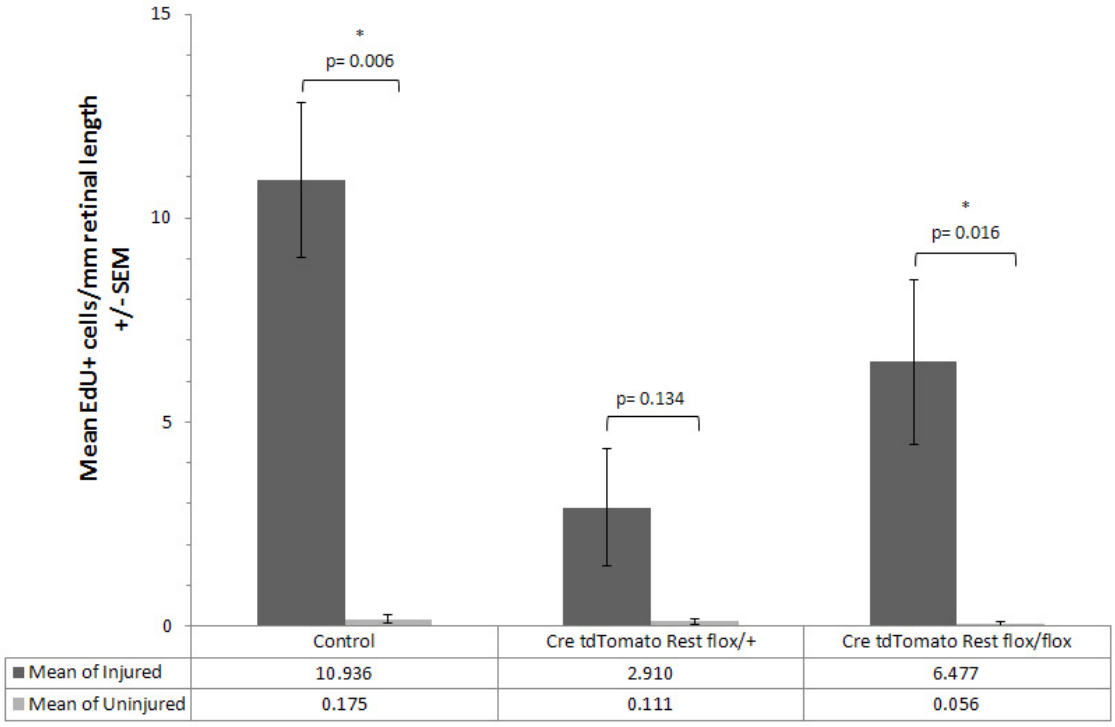
A) Counts for all nuclear morphologies

Genotype	Mean difference (Injured – Uninjured)	SD	SEM	n=
Control	10.9	4.10	1.83	5
Cre;tdTomato;Rest $^{flox/+}$	2.91	4.35	1.45	9
Cre;tdTomato;Rest $^{flox/flox}$	6.48	6.33	2.00	10

B) Counts for larger circular/oblong nuclear morphology only

Genotype	Mean difference (Injured - Uninjured)	SD	SEM	n=
Control	6.71	3.34	1.49	5
Cre;tdTomato;Rest $^{flox/+}$	1.12	2.12	0.71	9
Cre;tdTomato;Rest $^{flox/flox}$	2.33	2.86	0.90	10

Table 2: Data summary for EdU+ cell counts



	Control	Cre tdTomato Rest flox/+	Cre tdTomato Rest flox/flox
Mean of Injured	10.936	2.910	6.477
Mean of Uninjured	0.175	0.111	0.056

Figure 16: Pairwise comparison of cell counts in injured and uninjured retina EdU+ counts, includes all nuclear morphologies

Bar graph showing the mean of EdU+ cells per millimeter retinal length (+/-SEM) in injured retina compared to uninjured retina in each genotype. Data was averaged within genotypes, combining tdTomato expressing and no-tdTomato expressing retinas. Comparisons used pairwise T-tests with Bonferroni adjustment for multiple comparisons. $p \leq 0.05$ were considered statistically significant (*).

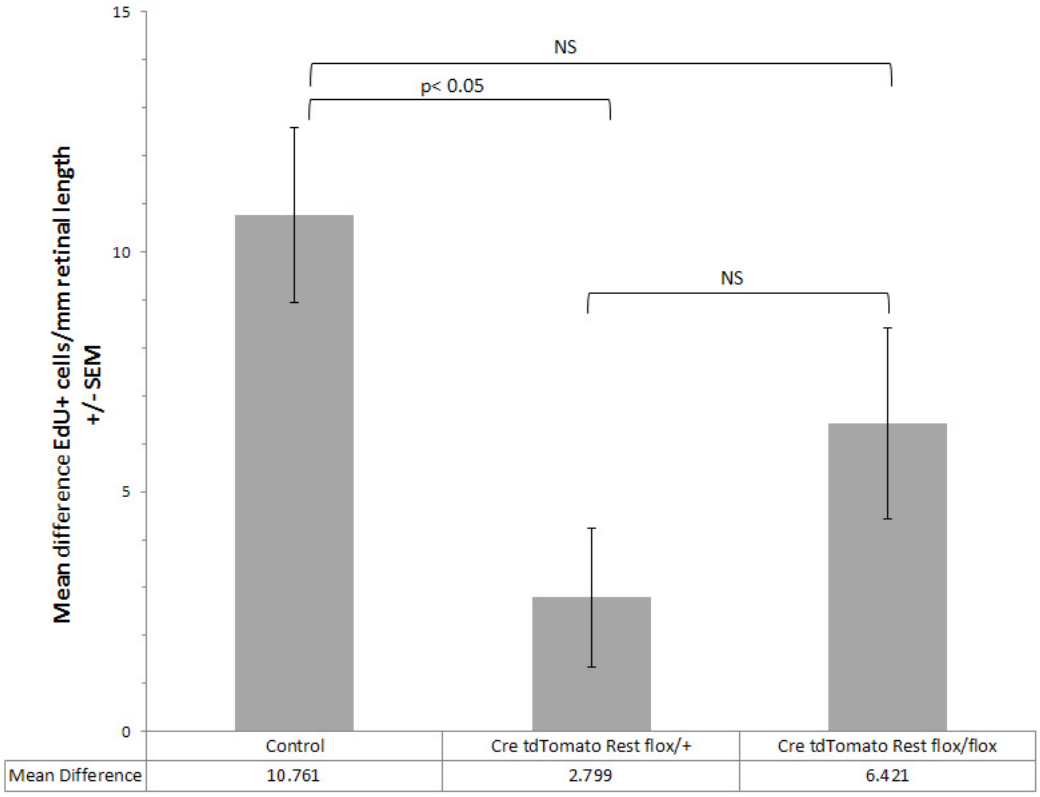
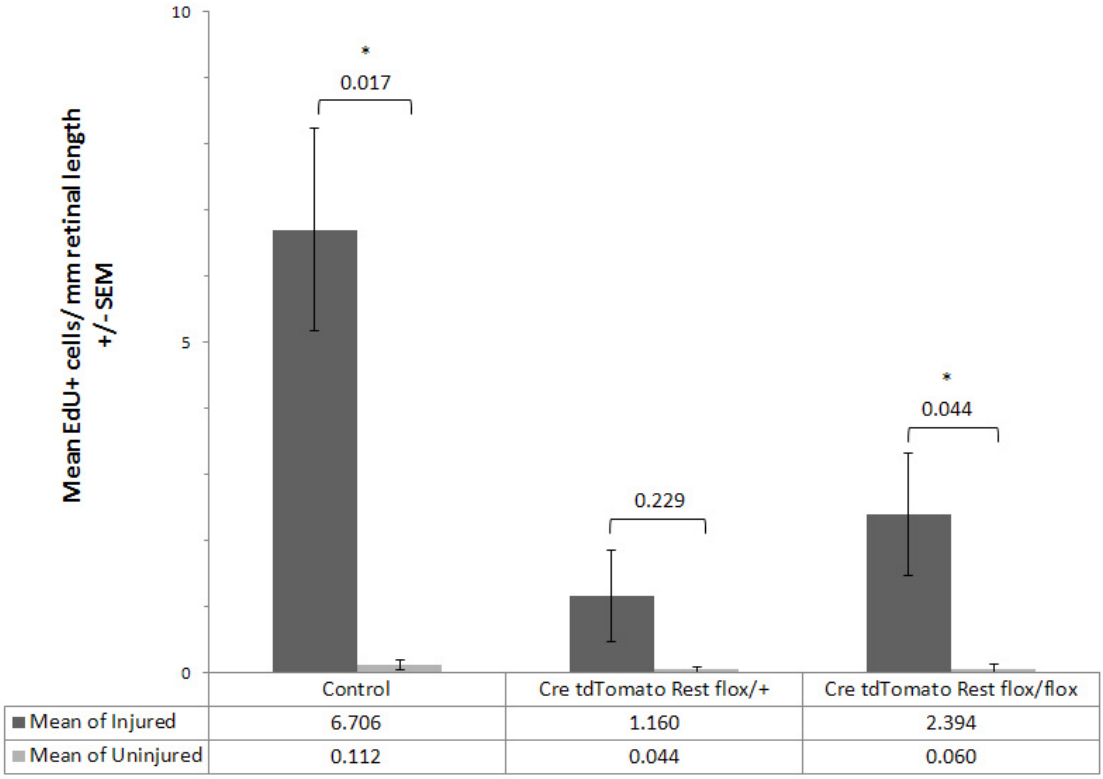


Figure 17: Comparison of mean difference in EdU+ cells across all genotypes
Bar graph showing the mean difference of all EdU+ cells per millimeter retinal length (+/-SEM) in NMDA injured retinas vs. PBS-control uninjured retinas across all genotypes. Data was averaged within genotypes, combining tdTomato expressing and no-tdTomato expressing retinas. Comparisons used ANOVA with post-hoc T-tests and Tukey HSD for multiple comparisons. $p \leq 0.05$ was considered statistically significant (*). NS, not significant.



	Control	Cre tdTomato Rest flox/+	Cre tdTomato Rest flox/flox
Mean of Injured	6.706	1.160	2.394
Mean of Uninjured	0.112	0.044	0.060

Figure 18: Comparison of mean EdU+ cells with large nuclear morphology in injured and uninjured retinas.
Bar graph showing the mean # of EdU+ cells (large nuclear morphology only) per millimeter retinal length (+/-SEM) in NMDA injured retinas vs. PBS-control uninjured retinas across all genotypes. Data was averaged within genotypes, combining tdTomato expressing and no-tdTomato expressing retinas. Comparisons used pair-wise T tests with Bonferroni adjustment for multiple comparisons. $p \leq 0.05$ were considered statistically significant (*).

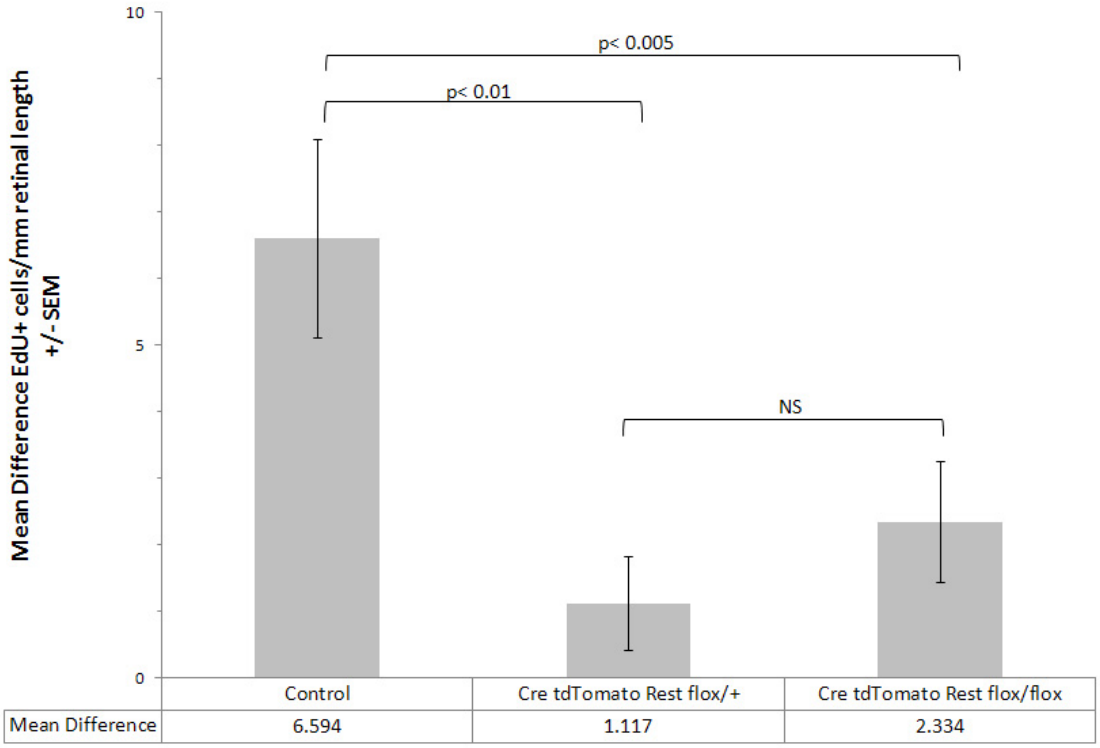


Figure 19: Comparison of large circular cells in injured retina EdU+ counts across all genotypes

Bar graph showing the mean difference in EdU+ cells (large nuclear morphology only) per millimeter retinal length (+/-SEM) in NMDA injured retinas vs. PBS-control uninjured retinas across all genotypes. Data was averaged within genotypes, combining tdTomato expressing and no-tdTomato expressing retinas. Comparisons used ANOVA with post-hoc T-tests and Tukey HSD for multiple comparisons. $p \leq 0.05$ were considered statistically significant (*). NS, not significant.

DISCUSSION

We hypothesized that REST may be blocking the neurogenic potential of Müller glia in the mammalian retina. To test our hypothesis, we used a genetic strategy to conditionally knock out *Rest* specifically in mouse Müller glia in the mature retina using an inducible Cre-lox strategy and determine how this altered the proliferative injury response of Müller glia *in vivo*. The experimental design followed a previously published approach that used intraocular injection of NMDA to cause death of retinal ganglion cells and amacrine cells followed by induction of a proliferative response by EGF (Karl *et al.,* 2008). To assess effects of genotype on injury-induced Müller glial proliferation, cells in S-phase were labeled using the thymidine analogue EdU. In our hands, the NMDA injury did elicit a gliotic response as measured by increased GFAP immunoreactivity in Müller glia. However, in both controls and experimental animals, the proliferative response of the Müller glia never reached the levels that have been previously reported. Furthermore, despite injury-induced increases in proliferation, only a subset of the EdU+ cells in any retinas showed the nuclear morphology or laminar position characteristic of Müller glia. Therefore, conclusions regarding the direct effects of *Rest* knockout on Müller glia could not be drawn from the results of this study.

Initial trials inducing Cre recombination with Tamoxifen (TAM) in 5 month old *Glast-CreERT2;Bgeo/GFP* mice resulted in a minimal amount of Cre recombination and GFP reporter expression (Figure 7). Previous studies have shown that the *Glast-CreERT2* Cre line can each yield robust Cre recombination response (de Melo *et al.,* 2012; Zhang *et al.,* 2005). One possible explanation for the

minimal amount of Cre recombination observed in the *Glast-CreERT2* mice in our hands was the age of animals at the time of TAM injections. Previous reports showed that the *Glast-CreERT2* line shows more robust recombination occurred when TAM was injected in mice P30 and younger (de Melo *et al.,* 2012; Kang *et al.,* 2010; Wang *et al.,* 2012). Previous studies have also shown that the *Bgeo/GFP* reporter line yields a robust reporter expression (Zhang *et al.,* 2005). In addition, reported experiments using the *Bgeo/GFP* reporter in the retina also tend to use younger mice (P21 and younger) (Brzezinski *et al.,* 2013; Ding *et al.,* 2009; Fan *et al.,* 2014), and there is anecdotal evidence that this reporter does not reliably report Cre activity in retinas from mice older than 1 month (Dr. S. Blackshaw, personal communication). However, in our hands, inducing Cre recombination in P30 *Glast-CreERT2;Bgeo/GFP* mice with TAM did not increase Cre recombination and no GFP reporter expression was observed. This outcome demonstrated that *Glast-CreERT2;Bgeo/GFP* would not be suitable for subsequent analysis. Therefore, a new mouse line, *Rlbp1-CreERT2;Rosa26-tdTomato,* was obtained and tested.

Similar to *Glast-CreERT2;Bgeo/GFP* Cre recombination trials, TAM injections in P28 *Rlbp1-CreERT2;Rosa26-tdTomato* mice resulted unexpectedly low Cre recombination, as evidenced by few cells expressing the tdTomato reporter (not shown). This contrasted with Vazquez-Chona, Clark *et al.,* (2009) who showed the *Rlbp1-CreERT2* promoter is sufficient to drive robust reporter expression *in vivo* and could be used to visualize retinal progenitor cells during postnatal development and Müller glia during their differentiation. One difference between previous studies and our experimental design was our use of IP injections of TAM instead of oral gavage.

Unmetabolized TAM has relatively little affinity for its target protein, the estrogen receptor (Desta *et al.,* 2004), although 4OHT, an active metabolite of TAM, has 30-100 times more affinity for the estrogen receptor than either estrogen or TAM (Wang *et al.,* 2004). Since prior studies have shown greater Cre induction in *CreERT2* systems using 4-hydroxytamoxifen (4OHT) (Indra *et al.,* 1999), it is possible that the IP injection route did not result in metabolism of sufficient TAM into 4OHT. Consistent with this, IP injections of 4OHT induced Cre recombination and tdTomato expression robustly in P21 mice (Figure 9), an age when the retina is fully developed. In retrospect, it is possible that the minimal levels of Cre recombination observed in the *Glast-CreERT2;Bgeo/GFP* mice was also a consequence of using TAM and not 4OHT. However, 4OHT was not tested in the *Glast-CreERT2;Bgeo/GFP* mice as the generation of triple transgenics with *Rest flox* had already proceeded using the *Rlbp1-CreERT2;Rosa26-tdTomato* strain.

Retinal injury with NMDA has been shown to induce Müller glia proliferation across the retina when followed by injections of specific growth factors [e.g. EGF, FGF1, FGF2, the combination of FGF1 and insulin, the combination of FGF2 and insulin (Das *et al.,* 2006; Fischer *et al.,* 2002b; Fischer and Reh 2002; Karl *et al.,* 2008; Kohno *et al.,*)]. Injury induced proliferation of Müller glia is associated with upregulation of progenitor markers (Pax6, Notch, Dll1) and with over time, some of the proliferating cells expressed genes typically associated with differentiated neurons (Claretinin, NeuN, Pax6, Prox1, and GAD67-GFP) (Karl *et al.,* 2008). For example, at 30 days after NMDA injury followed by multiple injections of fibroblast growth factor and insulin, Karl *et al.,* (2008) reported that 3.6% of BrdU-labeled

cells (~0.04% of all Müller glia) expressed GAD67, a marker of GABAergic neurons. These studies support that the mammalian retina has the potential to regenerate inner retinal neurons *in vivo*.

In our experiments, retinal injury with NMDA and subsequent EGF injections resulted in proliferation of cells predominantly located in the inner nuclear (INL) and ganglion cell layers (GCL). Among these cells, there were two distinct nuclear morphologies: larger nuclei that were either round or slightly elongated (at least 10 microns) and small nuclei with irregular shaped morphology (10 microns and smaller) (Figure 13; Figure 14). A subset of the larger/round-oblong nuclei located predominantly in the INL were double labeled with EdU and tdTomato, consistent with their identification as Müller glia (Figure 14). In contrast, the smaller/irregular nuclei lacked the typical morphology of Müller glia and were found in all layers of the retina, but were predominately located in the INL and GCL (Figure 13). Because of size, shape and position, the smaller/irregular cells are likely to be associated with an immune response to the injury. Microglia, remain in an inactive state in the inner retina until injury, when they proliferate, migrate, and scavenge dead or dying cells (G.P. Lewis *et al.,* 2005). However, immunostaining for microglia was inconclusive as the CD11b antibody available did not show the typical labeling pattern of retinal microglia (Wang *et al.,* 2011). Retinal pigmented epithelial (RPE) cells also have a large role in regulatory actions in immune responses. The RPE cell is a rich source of both proinflammatory and immunosuppressive cytokines (Detrick and Hooks 2010). Injury induced inflammatory response is likely to recruit immune cells and factors to the retina (Detrick and Hooks 2010; London *et al.,*

2011). Immune response to injury in the mammalian CNS results in an inflammatory cascade with positive and negative effects, some components cause tissue damage, neural death, and reduced regenerative potential (Benowitz and Popovich 2011; Ekdahl *et al.,* 2003; Fitch and Silver 2008; Iosif *et al.,* 2006), whereas others enhance progenitor cell proliferation, promote cell survival and an increase regenerative potential (Benowitz and Popovich 2011; Deierborg *et al.,* 2010; London *et al.,* 2013; Schwartz *et al.,* 2009). Additional studies will be required to characterize the identity of all cell types that proliferate in the injured retina.

Despite optimization of the 4OHT delivery, tdTomato expression was not consistently induced by 4OHT and 50% of retinas from mice carrying Cre and tdTomato had no detectible tdTomato expression. Studies done on fidelity of conditional gene targeting show that many Cre transgenes do not have 100% efficiency in recombination. For example, the tamoxifen inducible Cre transgene *R26-CreERT2* has a wide range efficiency of Cre-excision ranging between 10-90% (Schmidt-Supprian and Rajewsky 2007). In pilot studies, we observed intermediate Cre-recombination with three 4OHT injections, as evidenced by fewer tdTomato expressing cells. Even with 5 daily injections of 4OHT, the number of Müller glia expressing the tdTomato reporter gene expression never reached 100%. However, in experimental mice that received five 4OHT injections, the overall pattern of tdTomato-expressing cells remained relatively consistent between eyes that showed tdTomato expression. Therefore, the complete absence of tdTomato expressing cells did not appear to reflect a partial or mosaic activation of Cre expression.

For each genotype, the number of EdU+ cells present in injured retinas did not differ between the tdTomato expressing and non-expressing retinas (Figure 15). The most parsimonious explanation of the variability in tdTomato expression is that the tdTomato transgene did function as a faithful reporter of *Cre*-recombination and the absence of tdTomato expression indicated insufficient Cre recombinase expression resulting in no Cre-recombination. If this were the case, retinas that lacked tdTomato expression would also lack recombination/deletion of the floxed *Rest* alleles. Thus, any changes in the numbers of EdU+ cells would not be attributable to *Rest* knockout. Alternatively, if the tdTomato reporter did not faithfully report Cre expression, then tdTomato expression would not correlate with knockout of the floxed *Rest* alleles. In this case, *Rest* could be knocked-out in Müller glia, even in the absence of tdTomato expression, and changes in the overall number of EdU+ Müller glia would reflect the effects of *Rest* knockout, regardless of tdTomato expression. Because the tdTomato and Rest flox alleles were independent, there could be variation in the concordance of Cre-driven recombination for the two genes. However, it seems unlikely that there would be complete discordance between the two events. Since the majority of EdU+ cells in NMDA injured retinas did not express tdTomato, regardless of genotype, and only a few tdTomato cells were EdU+, we conclude that any differences in numbers of EdU+ cells following NMDA injury is unlikely to reflect cell autonomous consequences of *Rest* knockout on Müller glial proliferation.

Because there were no statistically significant differences in EdU+ cell counts between retinas with or without tdTomato reporter expression, data from these

retinas were combined for each genotype for further analysis. In paired T-tests, there were significant increases in the total number of EdU+ cells and in the number of large (glial) EdU+ cells between NMDA and PBS injected eyes for *Rlbp1-CreERT2;Rosa26-tdTomato;Rest*$^{flox/flox}$ and the no-Cre control. Surprisingly, *Rlbp1-CreERT2;Rosa26-tdTomato;Rest*$^{flox/+}$ mice (heterozygous for the *Rest*^{flox} allele) had the fewest EdU+ cells in the injured retina and differences in EdU+ cells between NMDA and PBS injected eyes for this genotype did not reach statistical significance. In addition, the mean difference of the total number of EdU+ cells and in the number of the large (glial) EdU+ cells in heterozygous *CreERT2;Rosa26-tdTomato;Rest*$^{flox/+}$ (heterozygous for *Rest*^{flox}) was significantly reduced relative to *CreERT2;Rosa26-tdTomato;Rest*$^{flox/flox}$ or the no-Cre controls. This finding could reflect non-cell autonomous effects of partial *Rest* knockout.

The low number of EdU+/tdTomato+ Müller glia that were observed made unambiguous analysis of the direct effects of Rest knockout on the injury response of Müller glia impossible. There are several potential explanations for the low number of EdU+ Müller glia. First, in our hands, there were problems with the anesthesia that could have led to technical issues with the efficacy of the injections. We did observe more EdU+ Müller glia in mice injected under isoflurane anesthesia compared to ketamine/xylazene anesthesia. We found that ketamine and xylazine anesthesia resulted in fatalities of ¼ of all experimental mice following intraocular injury. It is possible that deaths were a result of neurological problems from the anesthesia in mice that already received 4OHT injections. Although reducing the dose of ketamine and zylazine reduced lethality, it also led to insufficient anesthesia

during the intraocular injections and thus injections were rushed. This could have caused inconsistent or insufficient amounts of NMDA and/or EGF being injected into the eye and reduced injury and/or proliferative responses. Switching to isoflurane improved the effectiveness of the anesthesia and improved the consistency of intraocular injections. Although retinas that were injected using isoflurane appeared to have more EdU+ Müller glia, isoflurane use was not instituted until near the end of the experiment. Therefore, there were not enough animals in this group to be confident of repeatability of this observation.

Another factor that could contribute to the lower-than-expected glial response to the NMDA injury is the mixed genetic background of the mice. A recent report showed that genetic background is a factor in the *in vivo* proliferative response of Müller glia (Suga *et al.,* 2014). Using retinal explants, which caused preferential photoreceptor cell death, authors showed that C57Bl/6 mice have a significantly smaller amount of proliferation compared to 129SvJ. C57Bl/6 mice had lower expression of Cyclin D1, expressed in proliferating retinal progenitors and Nestin, expressed in neural progenitors compared to 129SvJ. C57Bl/6 mice also had increased expression of genes related to immune response. These findings support the idea that C57Bl/6 has an inhibitory environment for regeneration. The mice used in our study were of a mixed genetic background that largely contained the C57Bl/6 line. *Rlbp1-CreERT2;Rosa26-tdTomato* transgenic mice were generated in a C57Bl/6 background (Vazquez-Chona *et al.,* 2009) and the *Rest*$^{flox/flox}$ line was generated in C57Bl/6J-albino and crossed to a 129-derived strain (Mao *et al.,* 2011). All our mouse lines were maintained by crossing to C57Bl/6.

The age of the experimental animals could also contribute to the unexpectedly low proliferative response following NMDA/EGF treatment. The timecourse for injury induction was based on Western blot analysis showing that REST protein levels reached the lowest levels at 28 days following the final 4OHT injection. Thus, mice were 2 months old at the time of NMDA injection. Ueki *et al.,* (2012) show that there is an effect of age effects on Müller glia proliferation in retinal explants, where proliferation declines between P8 and P10, further declines between P10 and P14, and is very low in mice older than P14 (Ueki *et al.,* 2012). However, a variety of age ranges have been used in Müller glia proliferation studies *in vivo* and *in vitro*.

Finally, in these experiments, NMDA injections were timed to occur when REST protein was depleted following knockout. This may not be ideal for promoting stem-like characteristics in Müller-glial derived retinal progenitors. REST plays a fundamental role in the progression of pluripotent cells to neural progenitors (Ballas *et al.,* 2005a; Ballas and Mandel 2005). In neural progenitors REST protein is down-regulated, but is still expressed. The decrease in REST likely allows selective up-regulation of REST target genes. The loss of REST repressor complex from the RE-1 site of neuronal genes induced progenitor differentiation to mature neurons (Ballas *et al.,* 2005a). Therefore, it is possible that a low level of REST expression is needed to stimulate promote retinal progenitor characteristics of Müller glia. This suggests that the time point selected for the NMDA injury may be too late since REST protein had reached its lowest level. To address this, future studies will need to test the injury response at earlier time points.

Control mice in these studies lacked the Cre transgene and thus could not express the tdTomato reporter in the Müller glia. The heterozygous $Rest^{flox/+}$ mice showed a reduction in the number of EdU+ cells compared to control, raising questions as to whether this reflected effects of partial loss of Rest or of Cre expression itself. Therefore, an additional control, *Cre;tdTomato;Rest* $^{+/+}$, should be added to future studies.

To resolve the issues that arose in this study, additional experiments should be undertaken to extend the current findings and determine the role of REST in the regenerative responses of Müller glia. The first step would be to determine the efficiency of Cre-mediated *Rest* knockout in Müller glia. Genotyping DNA from whole retinas is not feasible as Müller glia constitute only 16% of all retinal cells (Jeon *et al.,* 1998). Therefore, tdTomato+ cells from mice that received 4OHT would need to be isolated from dissociated retinas using flow cytometry and DNA isolated for PCR genotyping. Second, the NMDA injury needs further optimization to address the influence of genetic background and the effects of age on injury response. Additional injections of EGF might be needed to increased proliferation following the injury. Studies done by Karl *et al*., (2008), on which our injury model was based, reported that multiple growth factor injections over several days increased the number of proliferating Müller glia. This might prove to be technically challenging as the eye lost intraocular pressure and became flaccid after the two intraocular injections used in the present study.

An alternative approach would be to use retinal explant cultures. In this approach, cutting the optic nerve would serve as the injury stimulus resulting in

death of RGCs. Explant cultures would also limit any immune/inflammatory response to the resident microglia and may enable better evaluation of the intrinsic injury response of Müller glia. Although retinal explant cultures preserve much of the retinal structure, multiple studies have shown that isolated Müller glia *in vitro* have increased proliferative and neurogenic capacity compared to their responses *in vivo*, although the numbers of neurons generated from cultured Müller glia remains low. Thus, Müller glia from the retinas of control and experimental mice could be isolated and grown *in vitro* and treated with 4OHT to induce Cre. Then analyze for Rest knockout and neurogenic response to *in vitro* differentiation procedures (Becker *et al.,* 2013; Bhatia *et al.,* 2011; Otteson and Phillips 2010; Phillips and Otteson 2011; Simon *et al.,* 2012; Singhal *et al.,* 2012; Wang *et al.,* 2013) This approach would also be amenable to transcriptome analysis of the Müller glia under different treatment paradigms and, if successful, could generate cells for future transplantation analysis.

In summary, NMDA injury causes an increase in cell proliferation in the no-Cre control and *Rlbp1-CreERT2;Rosa26-tdTomato;Rest*$^{flox/flox}$ genotypes, but not in the *Rlbp1-CreERT2;Rosa26-tdTomato;Rest*$^{flox/+}$ (heterozygous for the *Rest*^{flox} allele). In newly proliferated cells there were two distinct nuclear morphologies**:** Larger nuclei that were either round or slightly elongated (at least 10 microns) and small nuclei the total number of newly proliferated cells and in the number of newly proliferated large, glial-like cells was significantly reduced in the *CreERT2;Rosa26-tdTomato;Rest*$^{flox/+}$ (heterozygous for *Rest*^{flox}) relative to *CreERT2;Rosa26-tdTomato;Rest*$^{flox/flox}$ or the no-Cre controls. Important questions to be analyzed are:

Does *Rest* knock out promote stem cell switch in Müller glia? What genes do they express? Retinal stem cell-specific markers: Nestin and Pax6? (not found/low in MG). Do any *Rest* knock out cells persist as Müller glia (continued expression of Glutamine synthetase (GS), Glial fibrillary acidic protein (GFAP), and Vimentin)? What is the long-term fate of EdU+/tdTomato+/*Rest* knock out cells after injury? Do they continue to proliferate? Do they migrate? Do they differentiate into neurons? If so, what types?

APPENDIX

Recipes

Tail lysis buffer

Stock (Sterile Reagents)	Volume (ml)	Final concentration
H2O	31.5	--
5M NaCl	1	100 mM
0.5M EDTA	10	100 mM
1M Tris pH 8.0	2.5	50 mM
10% SDS	5	1%

Proteinase K diluent

Reagents	Volume
diH2O	49 ml
CaCl2	0.29 g
1M Tris pH 7.5	1 ml

Mix first three ingredients then add:

Glycerol	50 ml

Sterile filter, store at room temperature.
To make ProtK dissolve in diluent to 100 mg/ml. Aliquot and store -20°C

0.2M Sodium phosphate (Na2PO4) buffer

Make 0.2M dibasic and monobasic solns seperatetly:

Dibasic: dissolve and fill to 1 L with diH2O

If using anhydrous (FW 141.96 g/mol): 28.392 g

If using hydrous (FW 268.07 g/mol): 53.614 g

Monobasic (FW 137.99): 13.799 g and fill to 500 ml with diH2O

Add monobasic to dibasic until pH is 7.5
Divide into bottles and autoclave

4% Paraformaldehyde (PFA)

Mass out 4 g PFA into beaker, add stir bar, and cover with foil
In a separate beaker, heat 40 ml diH2O until just starting to boil
Take to fume hood:

- 4 g PFA powder with stir bar
- 50 ml 0.2M Na2PO4
- 40 ml hot diH2O
- 20-30 ml cold diH2O
- NaOH pellets
- 10% phosphoric acid
- pH strips
- 100 ml graduated cylinder and 150 ml filter unit

In fume hood, pour hot H2O into PFA powder. Stir and add one NaOH pellet until solution becomes clear
Add 50 ml 0..2M Na2PO4
Add 10% phosphoric acid at 0.5 ml incriments to get a pH 7.5
Bring up to 100 ml with diH2O
Sterile filter and store in 4°C, use within a week

Sucrose solutions

Dissolve sucrose in 125 ml 0.2M Na2PO4

- For 5%: 12.5 g sucrose
- For 20%: 50.0 g sucrose

Bring up to 250 ml with diH2O
Sterile filter and store at 4°C

Towbin (Transfer) buffer

Reagents	Volume
Glycine	14.4 g
Tris	3.2 g
MeOH	200 ml
diH2O	Up to 1 L

Check pH after chilled, should be about 8.3

Running buffer

Reagents	Volume
Tris Base	15.15 g
Glycine	71.3 g
20% SDS	25 ml
diH2O	375 ml

Adjust pH to 8.6 with conc HCl. Adjust to 500 ml with diH2O. Stable at RT.

Lower gel buffer

Reagents	Volume
Tris base	1.92 g
20% SDS	2 ml
diH2O	75 ml

Adjust pH to 8.8 with conc HCl. Adjust to 100 ml with diH2O. Stable at RT.

Upper (stacking gel) buffer

Reagents	Volume
Tris base	6.06 g
20% SDS	2 ml
diH2O	75 ml

Adjust pH to 8.8 with conc HCl. Adjust to 100 ml with diH2O. Stable at RT.

7.5% Gel preparation

Set up gel plates
Prepare resolving (lower) gel

Reagents	Volume
30% acrylamide/0.8% bis (3:1)	5 ml
4X lower buffer	5 ml
diH2O	10 ml
10% APS	100 µl
Add when ready to pour gel: TEMED	20 µl

Using a pipet fill carefully fill glass plate without creating bubbles
Gently overlay with 500 µl of diH2O. Cover with plastic and set at least 1 hr at RT
When gel is polymerized, invert apparatus and empty H2O
Prepare stacking (upper) gel

Reagents	Volume
30% acrylamide/0.8% bis (3:1)	1.6 ml
Upper buffer	3 ml
diH2O	7.2 ml
10% APS	60 µl
Add when ready to pour: TEMED	12 µl

7) Using a pipet fill glass plate on top of resolving gel. Place comb into gel. Let set for 45 min

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