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Progesterone as a Neuroprotective Treatment in the Retina

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Progesterone as a Neuroprotective Treatment in the Retina

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a dissertation submitted to the Faculty of the
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2013

Abstract

Progesterone as a Neuroprotective Treatment in the Retina
By Rachael Stewart Allen

The neurosteroid progesterone has been shown to have protective effects in a number of injury models, including traumatic brain injury and stroke. Progesterone has been shown to reduce inflammation and infarct size in the brain and improve behavioral outcomes after middle cerebral artery occlusion (MCAO), a stroke model thought to affect the retina as well as the brain. Here, the functional, morphological, and inflammatory responses of the retina after MCAO were examined and compared with responses in the brain. While the retina was found to exhibit similar responses to the brain, it showed a reduced susceptibility to the ischemic injury induced by MCAO. Next, we examined progesterone administration in two models of rodent ocular ischemia: the anterior ischemic optic neuropathy model, which causes monocular optic nerve stroke, and the MCAO model, which we showed caused retinal damage, inflammation, and functional deficits in conjunction with cerebral ischemia. Protective effects with progesterone treatment were observed in the retina following MCAO but not anterior ischemic optic neuropathy. While progesterone treatment was protective in both brain and retina after MCAO, greater reductions in inflammation were observed in the brain versus the retina and greater improvements were observed in brain function-based behavioral tasks than in the retina-based electroretinogram. Levels of progesterone receptor were assessed in retina and brain to confirm expression of progesterone receptor at the protein level in the retina. After MCAO, progesterone receptors were upregulated in brain and downregulated in retina, suggesting a potential mechanism for greater progesterone protection in brain over retina. These results demonstrate the promise of progesterone treatment in retinal ischemia and illustrate the importance of progesterone receptors in how injured tissue responds to progesterone treatment.

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Chapter 1:

Introduction

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GENERAL INTRODUCTION

Retinal Anatomy and Visual Processing

The eye has been called the window to the brain. Other neural tissue can be difficult to access, but the retina can be visualized easily and non-invasively. For example, it is the only tissue in which arteriolar blood vessels can be visualized. Due to the eye's unique anatomical characteristics, the retina allows us a glimpse into a portion of both the cardiovascular and central nervous systems (Sharma & Ehinger, 2002). Neuro-ophthalmologists can diagnose diseases by looking at the fundus (Stanford et al., 1978). For instance, the pathophysiological deposits that characterize Alzheimer's disease have also been observed in age-related macular degeneration (Dentchev et al., 2003). In diabetes, high blood glucose levels can lead to damaged blood vessels in the eye (diabetic retinopathy), and retinopathy has been shown to indicate future cardiovascular problems (Gerstein et al., 2013). Additionally, visual deficits are often a warning sign of impending stroke (Falke et al., 1991, Helenius et al., 2012). The unique accessibility of retinal neurons for imaging and functional analysis and the similarities between retina and brain make the retina a useful tool in studying diseases that affect both the eye and brain.

Anatomy and structure

Both the brain and retina derive from neuroectoderm during embryonic development, and both tissues continue to develop well after birth. Both tissues are composed of neurons and glia with specialized functions, with the retina specialized to convert light stimuli into electrical signals that are carried to the brain and also to perform

preliminary visual processing. Light passes through the cornea and pupil and is focused by both the cornea and lens onto the retina (Fig. 1-1A). The photoreceptor cells (rods and cones) contain light sensitive photopigments that allow them to transduce photons of light into graded changes in membrane potential. These signals are processed and modified by the cells of the inner nuclear layer (bipolar, amacrine, and horizontal cells) before they are passed to the retinal ganglion cells (Sharma & Ehinger, 2002) (Fig. 1-1C). Retinal ganglion cell axons form the optic nerve and carry signals from the retina to the brain. Ninety percent of these axons project to the lateral geniculate nucleus of the thalamus, which passes the signal to the primary visual cortex for higher level visual processing, while a smaller number project to the pretectum, superior colliculus, and suprachiasmatic nucleus of the hypothalamus (Wurtz & Kandel, 2000).

In addition to its diverse neuronal cells, the retina and optic nerve also contain four types of glial cells (Fig. 1-1B, C). 1) Glial cells myelinate ganglion cell axons in the optic nerve (Sharma & Ehinger, 2002). 2) Microglia, as in brain, are normally dormant but become phagocytic in response to retinal injury and cell death. Retinal microglia are located primarily in the nerve fiber layer under normal conditions, but migrate to other retinal layers during times of retinal trauma (Sharma & Ehinger, 2002). Additionally, other microglia that originate outside the retina can migrate to the retina in response to injury (Boycott and Hopkins, 1981). 3) Astrocytes are found primarily in the nerve fiber layer. As in brain, retinal astrocytes are star-shaped, express GFAP abundantly, and send out processes that wrap around nerve fibers and capillaries. Retinal astrocytes provide support and homeostatic function for retinal ganglion cells and, along with Müller cells, form the inner blood retinal barrier (Sharma & Ehinger, 2002). Similarly, brain astrocytes

also play a role in support, homeostasis, and blood brain barrier formation (Kandel, 2000). 4) Müller cells are a specialized type of glial cell found only in the retina (Fig. 1-1B). They span most of the retina, from photoreceptor to nerve fiber layer, providing structural support and performing functions that are performed by oligodendrocytes and astrocytes in the brain. Müller cell processes extend to retinal synapses and nerve fibers to provide homeostatic support, and Müller cell endfeet extend to retinal blood vessels as part of the inner blood retinal barrier (Sharma & Ehinger, 2002). Müller cells exhibit diverse responses to retinal stress and injury, including upregulation of GFAP, which is not visualized in Müller cells under normal conditions (Bringmann et al., 2009). Müller cells also remove extracellular glutamate under normal and pathologic conditions, and strongly express glutamine synthetase, an enzyme that converts glutamate to glutamine (Pow and Barnett, 1999, Bringmann et al., 2009).

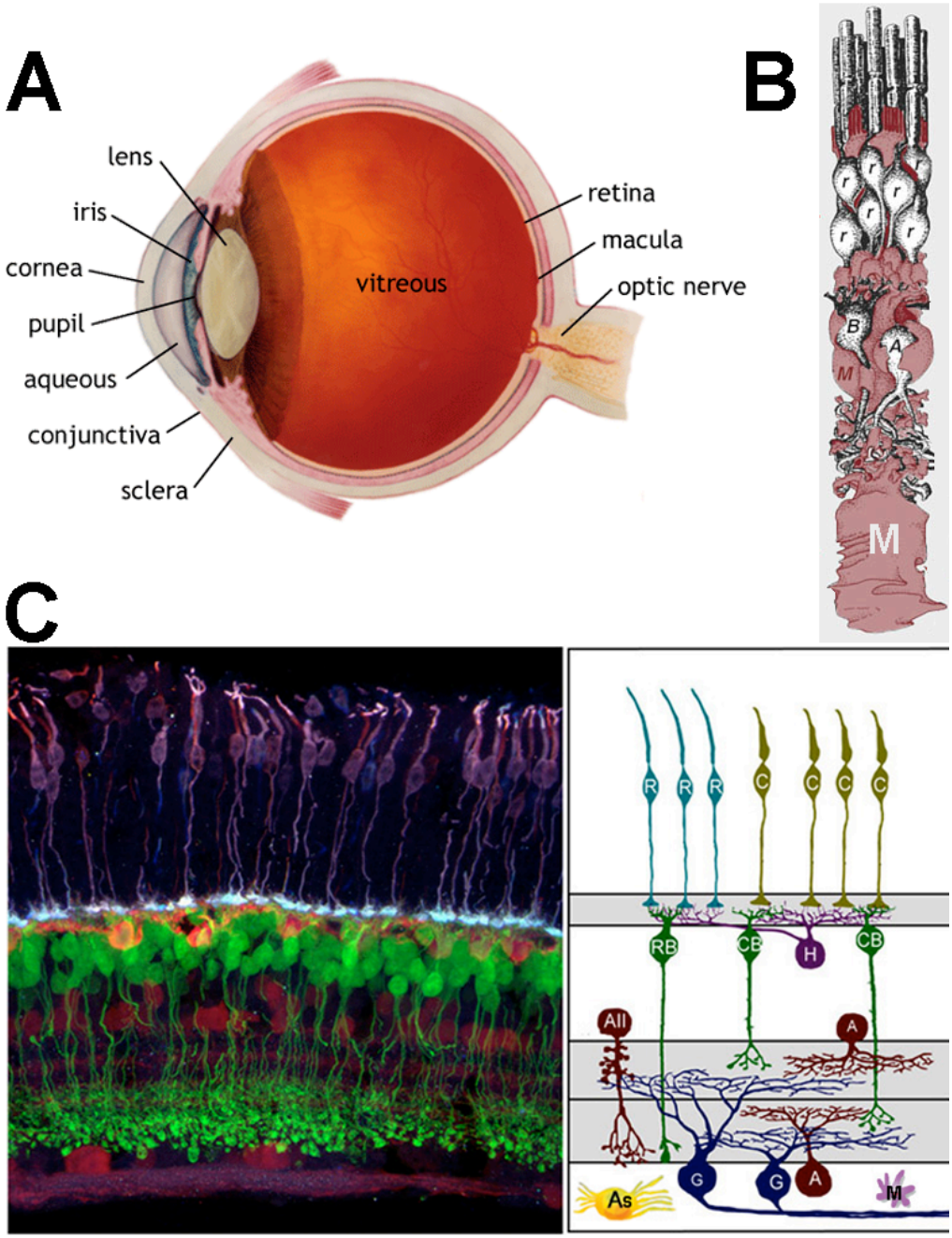

Figure 1-1. Basic ocular and retinal anatomy. A) Anatomy of the eye. Image modified from: National Eye Institutes, National Institues of Health (NEA04) with permission. B) Müller cell morphology. M, Müller cell. Image modified from http://webvision.med.utah.edu/book/part-ii-anatomy-and-physiology-of-the-retina with permission, made available through Creative Commons. C) Retinal anatomy and circuitry. R, rod; C, cone; RB, rod bipolar cell; CB, cone bipolar cell; H, horizontal cell; A, amacrine cell; As, Astrocyte; G, ganglion cell; M, microglia. Image modified from http://webvision.med.utah.edu/book/part-vi-development-of-cell-types-and-synaptic-connections-in-the-retina and http://webvision.med.utah.edu/book/part-xii-cell-biology-of-retinal-degenerations with permission, made available through Creative Commons.

Neurotransmitters

Glutamate is the major excitatory neurotransmitter in both retina and brain (Schwartz, 2000, Sharma & Ehinger, 2002). As the neurotransmitter used by photoreceptor, bipolar, and ganglion cells, glutamate is essential for proper signal conduction in the retina (Massey and Redburn, 1987) (Brust, 2000)(Fig. 1-2A). After glutamate activates receptors, it must be cleared from the extracellular space quickly to avoid constant activation. Glutamate transporters take up the extracellular glutamate into surrounding glial cells, where it can be broken down by enzymes (Schwartz, 2000). In the retina, the Müller-specific glutamate transporter, GLAST (GLutamate ASpartate Transporter) takes up extracellular glutamate into the cell where it is converted to glutamine by glutamine synthetase (Pow and Barnett, 1999) (Fig. 1-2B).

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain (Schwartz, 2000) is also present in the retina. Horizontal cells and approximately 40% of amacrine cells use GABA as their neurotransmitter and play a role in fine-tuning the visual signal (Sharma & Ehinger, 2002) (Fig. 1-2A).

A variety of other neuroactive substances have been detected in retinal neurons as well, including cholingergic and dopaminergic amacrine cells, among others (Sharma & Ehinger, 2002).

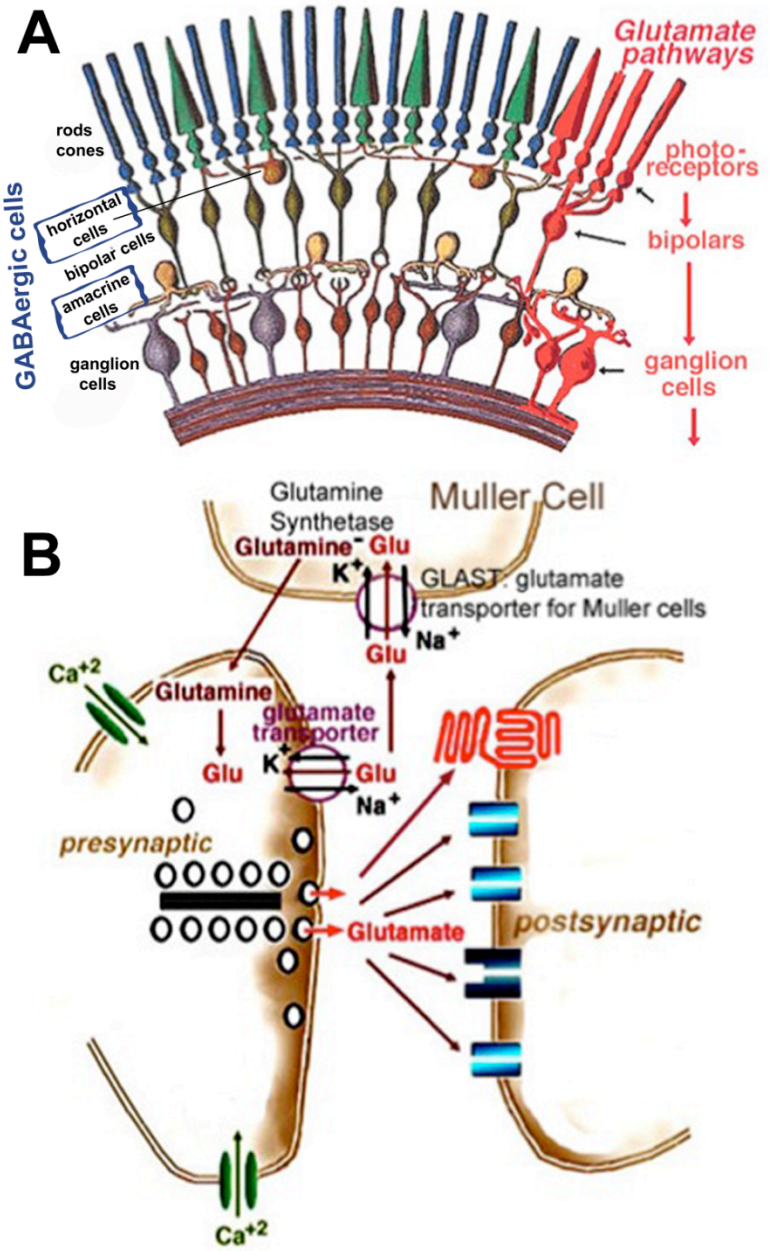

Figure 1-2. Glutamate in the retina. A) Diagram of retinal neurons. Photoreceptor, bipolar, and ganglion cells make up the vertical pathway and are glutamatergic. Amacrine and horizontal cells modulate the pathway and are often GABAergic. **B) Glutamatergic synapse in retina.** Glutamate released by the presynaptic neuron is removed from the extracellular space by GLAST and converted to glutamine by glutamine synthetase. Images modified from http://webvision.med.utah.edu/book/part-v-phototransduction-in-rods-and-cones with permission, made available through Creative Commons.

Blood Supply and Blood Barriers in the Brain and Retina

Two major artery systems supply blood to the central nervous system. The internal carotid arteries carry blood to other arteries that branch to the anterior portion of the brain and also the eye. The vertebral arteries supply blood to the posterior portion of the brain. The internal carotid gives rise to the anterior cerebral and middle cerebral arteries, which, along with other arteries, form a ring called the Circle of Willis at the base of the brain (Fig. 1-3A). The primary blood supply for the cerebral hemispheres and subcortical structures is provided by the anterior cerebral, middle cerebral, and posterior cerebral arteries (Brust, 2000). The ophthalmic artery also branches from the internal carotid and ultimately provides the blood supply for the entire eye, branching into the central retinal artery and posterior ciliary arteries to supply blood to the retina (Smith et al., 2002) (Fig. 1-3B). In both brain and retina, arteries continue to form progressively smaller branches leading to capillary networks. In both tissues, a tight barrier between blood and neural tissue is critical to proper function. Despite these similarities, the retina has seven times the metabolic needs of the brain (Rodieck, 1998).

The inner retina, including the retinal ganglion cells, is supplied blood by capillaries that ultimately branch from the central retinal artery, while the outer retina is supplied by the blood vessel rich choroid layer (Fig 1-3C). The blood brain barrier, inner blood retinal barrier, and outer blood retinal barrier have similar molecular compositions in terms of barrier proteins like occludin, ZO-1, and cell adhesion proteins. All three barriers involve tight junctions, share immunological features, and have similar pharmacological characteristics (Cunha-Vaz et al., 1966, Shlosshauer, 2007). Blood brain and blood retinal barriers also exhibit similar transport characteristics, including

expression of p-Glycoprotein (P-gp), an efflux pump that removes cytotoxic and xenobiotic substances (Bauer et al., 2004, Zhang et al., 2012b).

The blood retinal barrier for the inner retina is nearly identical in structure to the blood brain barrier (Fig. 1-3D). Both originate from mesoderm and follow a similar pattern of development. Both form new capillaries through angiogenesis. Both barriers are composed of continuous endothelial cells with tight junctions resulting in non-fenestrated capillaries. Both are in direct contact with glial cells and both have a high transendothelial resistance. However, the inner blood retinal barrier is composed of Müller processes in addition to astrocytic processes and has a greater density of inter-endothelial cell junctions (Cunha-Vaz et al., 1966, Schlosshauer, 2007). Additionally, pericytes cover a greater portion of retinal capillary circumference compared with brain (Frank et al., 1990).

The outer blood retinal barrier differs more from the other two barriers (Fig. 1-3E). The retinal pigment epithelium is a sheet of cells with tight junctions that forms the interface between the neural retina and fenestrated choroidal vessels (Steuer et al., 2005, Schlosshauer, 2007). The openings in choroidal vessels allow more substances to pass through the capillaries, and in greater amounts, with the retinal pigment epithelium providing the barrier. Additionally, choriocapillaries are larger, allowing a higher rate of blood flow and an increased concentration of oxygen (Rodieck, 1998). In contrast to the blood brain barrier and the inner blood retinal barrier, the outer blood retinal barrier originates from neuroectoderm, is composed of epithelial rather than endothelial cells, and has a resistance that is approximately one-fourth that of the other barriers. The retinal pigment epithelium also has additional properties, for example, supportive functions for

photoreceptor cells, that neither the blood brain barrier or inner blood retinal barrier have (Steuer et al., 2005, Schlosshauer., 2007).

There are also regions of the blood brain and blood retinal barriers that can be thought of as “weak spots” or “defects,” areas that are more permeable due to their structural composition. In the retina, one such weak spot occurs where the optic nerve exits the retina. As a result, the anterior portion of the optic nerve is more permeable to substances that diffuse from the choroid (Cioffi GA, 2002). This permeability increase could contribute to the enhanced susceptibility of the retinal ganglion cells and optic nerve observed in some diseases and animal models, including multiple sclerosis and experimental allergic encephalomyelitis (Schlosshauer, 2007). Brain areas with similar increased permeability due to incomplete blood brain barrier exhibit an earlier susceptibility to inflammation in experimental allergic encephalomyelitis (Schlosshauer, 2007).

Glial and vascular differences between the retina and brain are especially interesting because they could explain differences in how the brain and retina respond to injuries and drug treatment.

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Figure 1-3. Blood supply and blood barriers in the brain and retina. A) Cerebral blood supply. Image modified from http://www.webmd.com/brain/blood-supply-to-the-brain. B) Ocular blood supply. CAs, Ciliary Arteries; CRA, Central Retinal Artery; OphA, Ophthalmic Artery. C) Retinal and choroidal vasculature. Image modified from (Sim and Fruttiger, 2013). D) Visceral capillary (left) vs. capillary in brain and inner retina (right). In capillaries without a blood barrier (for example, the visceral capillary shown in D), the fenestrated endothelium and intercellular clefts allow for nonselective diffusion. Conversely, capillaries in the brain and inner retina are composed of nonfenestrated endothelium connected by tight junctions, creating a selective barrier between blood supply and neural tissue. Image modified from (Laterra, 2000). E) Outer Blood Retinal Barrier. Choriocapillaries are fenestrated, allowing diffusion across Bruch's membrane. The tight junctions of the retinal pigment epithelium (RPE) provide the barrier between blood supply and photoreceptor cells. Image modified from (Shechter et al., 2013).

Systemic and Brain Diseases that Involve the Retina

A number of systemic diseases are known to affect the retina. Stroke and diabetes have well-established retinal components. More recently, research has shown that changes in the retina also accompany diseases historically thought of as "brain diseases," including Alzheimer's disease, Parkinson's disease, and schizophrenia. Here, I review evidence of changes in retinal morphology and function in a variety diseases affecting the brain and/or the whole system. The information presented on diabetes, Alzheimer's disease, Parkinson's disease, and schizophrenia demonstrates the importance of assessing retinal components in disease and injury in general, while the section on ischemic injury in the brain and retina is of particular importance as this topic is a focus of this dissertation.

Ischemic Injury in the Brain and Retina

Ocular ischemia can occur independently of brain damage or as part of a larger injury, such as cerebral stroke or arthrosclerosis. In fact, subclinical visual field defects occur in 57% of minor strokes (Falke et al., 1991), and visual deficits commonly present as a first symptom in stroke and arthrosclerosis (Benavente et al., 2001, Mead et al., 2002). Chronic reductions in ocular blood supply, usually due to severe carotid artery obstruction, can lead to vision loss, as is the case in ocular ischemic syndrome (Sturrock and Mueller, 1984). Other types of ischemia are eye-specific, including retinal artery occlusion (Rumelt et al., 1999) and anterior ischemic optic neuropathy (Johnson and Arnold, 1994), which cause ischemia in the retina and optic nerve, respectively.

During ischemic stroke, reduced arterial blood flow deprives tissue of oxygen and nutrients, leading to injury and cell death (depending on the duration of ischemia) (Kalogeris et al., 2012). There is substantial overlap between mechanisms involved in cerebral stroke and mechanisms involved in strokes of the retina and optic nerve:

- including increases in extracellular glutamate and calcium influx resulting in excitotoxicity (Hillered et al., 1989, Kalloniatis, 1995, Perlman et al., 1996, Kalogeris et al., 2012)
- oxidative stress (Yang et al., 1996, Facchinetti et al., 1998, Liu et al., 2012b)
- altered aquaporin expression (Iandiev et al., 2006, Zador et al., 2009)
- increases in pro-apoptotic and decreases in anti-apoptotic markers (Schmidt-Kastner et al., 2000, Zhang et al., 2005b)
- increases in inflammation, including NF-κB pathway activation (Schneider et al., 1999, Dvoriantchikova et al., 2009), increased levels of inflammatory cytokines (IL-6, TNF-α, etc.) (Hangai et al., 1996, Hill et al., 1999), and increased activation of glial cells and macrophages (Zhang et al., 2005a, Gronberg et al., 2013).

Due to the degree of common mechanisms in cerebral and ocular stroke, treatments that are effective in one system could prove beneficial in the other system.

While the brain and retina exhibit similar pathologies in response to ischemia, it is interesting that their susceptibilities to ischemic injury differ. Differential susceptibility to ischemia is observed in a variety of organs, with the brain being the most sensitive in terms of tolerance time (the point at which ischemia causes irreversible damage)

(Kalogeris et al., 2012) (Table 1-1). Irreversible brain damage occurs after less than 20 minutes of ischemia (Ordy et al., 1993), with some studies reporting tolerance times as low as 3-7 minutes (Weinberger L, 1940, Kabat H, 1941, Brock, 1956). Conversely, the retina exhibits tolerance times that vary from less than 30 minutes to as long as 98 minutes (Reinecke et al., 1962, Fujino and Hamasaki, 1967, Hayreh and Weingeist, 1980, Faberowski et al., 1989, Lafuente et al., 2001, Zhu et al., 2002). Several factors can contribute to tissue susceptibility, including greater vascularization, low levels of antioxidant activity, and high metabolic demand (Kalogeris et al., 2012). However, the retina's metabolic demand is seven times that of the brain (Rodieck, 1998). Perhaps the retina is more resilient because it can rely on stores of glucose and glycogen in the vitreous and the retina itself, whereas the brain is dependent on cerebral vasculature for nutrients. Greater ease of reflow through occluded vasculature in retina vs. brain could also contribute to the retina's resiliency after ischemia (Hayreh and Weingeist, 1980).

Transient vision loss in one or both eyes, a phenomenon known as *amaurosis fugax*, occurs due to temporary retinal ischemia and is often a sign of systemic cardiovascular disease and impending stroke (Slepyan et al., 1975). In addition to visual symptoms, transient ischemic attacks can also include temporary numbness, weakness, and paralysis, among other symptoms. Although symptoms usually resolve in less than an hour (Johnston, 2002), neurological imaging shows that 1 in 4 patients with transient retinal ischemia currently has an acute brain infarct (Helenius et al., 2012). Fundus photography is already used to help diagnose whether patients with visual symptoms have carotid disease and would benefit from endarterectomy (a surgery to remove plaque from an artery) (Stanford et al., 1978). Other research shows that retinal examination

prior to vision loss contributes valuable information in terms of diagnosis and disease prediction. Fundus abnormalities have been found to be the greatest risk factor for cerebral thrombosis (Okada et al., 1976). Changes in retinal vasculature have been correlated with microvascular lesions in the brain (Qiu et al., 2009). And hypertensive retinopathy is associated with organ damage and risk of stroke and heart failure (Bhargava et al., 2012). Clearly, the retina is affected during systemic cardiovascular disease and injury. Information on retinal health and visual function can provide additional information on disease risk and pathogenesis.

Organ	Tolerance Time	Species	Reference
Brain	<20 minutes	Rat	Ordy et al., 1993
	6 minutes	Dog	Kabat et al., 1941
	3.5 minutes	Cat	Weinberger et al., 1940
	3 minutes	Human	Brock, 1956
Heart	20 minutes	Rat	Boengler et al., 2009
Kidney	30 minutes	Human	McDougal, 1988
Intestines	30-60 minutes	Rat	Ikeda et al., 1998
Retina	97-98 minutes	Monkey (Central retinal artery occluson)	Hayreh and Weingeist, 1980
	60-90 minutes	Monkey (elevated IOP)	Fujino and Hamasaki, 1967
	<90 minutes	Cat (elevated IOP)	Reinecke et al., 1962
	<60 minutes	Rat (optic nerve bundle occlusion)	Faberowski et al., 1989
	<30 minutes	Mouse (elevated IOP)	Zhu et al., 2002
	<30 minutes	Rat (Ophthalmic vessel ligation)	Lafuente et al., 2002
Skeletal muscle	1.5-2 hours	Human	Sapega et al., 1985

Table 1-1. Tolerance times of various organs to ischemic injury. Retinal tolerance times adapted from (Hayreh and Weingeist, 1980, Osborne et al., 2004).

Diabetes and Diabetic Retinopathy

Diabetes mellitus is characterized by high glucose levels due to insulin resistance and/or an inability to produce insulin. Diabetes has effects in multiple organ systems, including microvascular complications in the peripheral nervous system (neuropathy), renal system (nephropathy), and retina (retinopathy) (Beisswenger, 1976). Hyperglycemia and duration of disease are risk factors associated with all three complications, (Engerman et al., 1977, Cohen et al., 1987, Engerman and Kern, 1987) and retinal complications are the most common (Deshpande et al., 2008). Early signs of diabetic retinopathy include microaneurysms, intraretinal hemorrhages, and fluid leakage and exudate formation due to increased permeability of retinal vessels. As retinopathy becomes more severe, hemorrhages and vessel occlusions become progressively worse. Retinal ischemia becomes more severe, leading to neovascularization and vision loss (Deshpande et al., 2008).

Interestingly, the diabetic complications of neuropathy, nephropathy, and retinopathy have been found to be significantly associated (Dyck et al., 1993). Microvascular disease can occur in the brain with diabetes as well, and is thought to be the cause of cognitive deficits in diabetic patients (van Duinkerken et al., 2009). The presence of diabetic retinopathy is associated with cognitive deficits and greater amounts of ischemia in brain (Haan et al., 2012). Diabetic patients with one or more complications were also found to have reduced performance on a battery of cognitive tests (Ryan et al., 1993). Early signs of diabetic retinopathy (which may indicate chronic hyperglycemia) were associated with structural changes in brain as well as cognitive deficits, including deficits in fluid intelligence, attention and concentration, and information processing

(Ferguson et al., 2003). Additionally, patients with advanced diabetic retinopathy showed increased cortical atrophy as measured by MRI (Wessels et al., 2006), and diabetic retinopathy may be associated with accelerated cognitive decline with age, particularly in men (Ding et al., 2010). These studies show that diabetic retinopathy is indicative of microvascular disease, and the association between retinopathy and cerebral microvascular disease means that retinopathy could be used as a marker or predictor for pathology elsewhere in the nervous system. Additional support for the retina as an early marker in diabetes is the finding that patients with prediabetes show early signs of diabetic retinopathy (Chen et al., 2012, Tabak et al., 2012).

Alzheimer's Disease and Age-related Macular Degeneration

In addition to diseases that are more commonly thought of to have a retinal component, the retina has also been shown to be involved in a wide spectrum of disorders that we don't generally think of as "eye-related." Alzheimer's disease is the most common cause of dementia in older adults and is characterized by progressive memory impairment and cognitive disturbances (Seligman et al., 2001a). While the precise cause of Alzheimer's disease is not known, neurofibrillary tangles (tau protein fibers that accumulate inside neurons) and plaques (deposits of the amyloid beta protein fragment that build up between nerve cells) are both believed to contribute to neuronal dysfunction and death in Alzheimer's disease pathogenesis (Seligman et al., 2001a).

More recently, amyloid beta has been identified in drusen in patients with age-related macular degeneration (Dentchev et al., 2003). Age-related macular degeneration is the leading cause of irreversible blindness in older adults (Klein et al., 1995). Like

Alzheimer's disease, age-related macular degeneration is progressive, characterized by accumulation of drusen in the macula and a continuous decline in visual function (Penfold et al., 2001). Both diseases share the major risk factors of advanced age, a diet high in fat and cholesterol, and the APOE4 allele (Seddon et al., 2003, Mattson, 2004, Baird et al., 2006, Jager et al., 2008). Both are associated with deposits in the extracellular space that consist of Aβ peptides, apoE protein, complement, and other components (Ding et al., 2011). Treatments that use antibodies or vaccines to target amyloid beta in APP mouse models of Alzheimer's disease also decrease amyloid beta plaques and improve cognitive behavior (Hardy and Selkoe, 2002). Treatment with anti-amyloid beta therapy has also been tested in the APOE4-high fat/cholesterol mouse model, a rodent model of age-related macular degeneration that also results in plaque accumulation in the brain. With anti-amyloid beta treatment, amyloid beta deposits were greatly reduced in both retina and brain, and retinal pathology and visual function loss were reduced as well (Ding et al., 2008, Ding et al., 2011).

In addition to the potential for using Alzheimer's disease therapies in the treatment of age-related macular degeneration, retinal imaging and functional analyses may prove useful in identifying Alzheimer's disease and tracking its progression. Alzheimer's patients have been shown to have deficits in motion detection (Gilmore et al., 1994, Duffy et al., 2000) and contrast sensitivity (Cronin-Golomb et al., 1991), as well as visual field defects (Trick et al., 1995, Armstrong, 1996). Alzheimer's disease has been shown to cause damage to the lateral geniculate nucleus (Leuba and Saini, 1995), and visual cortex (Hof and Morrison, 1990, Gupta et al., 2006) early on in disease pathogenesis, and visual association areas may exhibit damage even before the

hippocampus and other areas involved in memory (McKee et al., 2006). Damage to visual areas may be the reason for loss of retinal ganglion cells (Sadun and Bassi, 1990, Blanks et al., 1996) and loss of nerve fiber layer tissue (Berisha et al., 2007, Paquet et al., 2007, Valenti, 2007, Bouet et al., 2009, Lu et al., 2010) in Alzheimer's disease. Importantly, nerve fiber layer loss has also been detected by optical coherence tomography in patients with mild cognitive impairment (thought to be a pre-Alzheimer's stage) (Paquet et al., 2007) , which may allow retina and optic nerve health to be used as a diagnostic tool in Alzheimer's disease prior to the onset of memory loss (Valenti, 2011).

Parkinson's Disease and the Retina

Parkinson's disease is a neurodegenerative disorder characterized by motor disturbances (tremor, rigidity, bradykinesia) that are caused by the progressive degeneration of dopaminergic neurons in the basal ganglia (Parkinson, 2002, Kempster et al., 2007). Parkinson's disease has historically been viewed as a disease of the motor system, but more recent research shows that Parkinson's disease is a systemic disorder with diverse effects in the body, including changes in the retina and visual function (Archibald et al., 2009). Reduced concentrations of retinal dopamine and reduced immunostaining in amacrine cells by tyrosine hydroxylase (the enzyme that synthesizes dopamine, a marker for dopaminergic cells) have been found in retinas from Parkinson's patients (Nguyen-Legros, 1988, Harnois and Di Paolo, 1990). These changes are ameliorated by dopamine treatment in animal models of Parkinson's disease (Tatton et al., 1990, Naarendorp et al., 1993).

Patients with Parkinson's disease also show abnormalities in visual function, including:

- Reduced contrast sensitivity, which improves after treatment with L-DOPA, the precursor to dopamine (Bulens et al., 1988, Hutton et al., 1993).

- Reduced visual acuity (clinically very important as it is a risk factor for Parkinson's disease-associated visual hallucinations) (Holroyd et al., 2001, Matsui et al., 2006).

- Deficits in color vision (Silva et al., 2005).

- Deficits in motor perception (Trick et al., 1994, Mosimann et al., 2004).

- Delays in visual-evoked potential (Fig. 1-4B) latency (Bodis-Wollner and Yahr, 1978, Ikeda et al., 1994), that are reversed by treatment with L-DOPA (Onofrj et al., 1986).

- Deficits in pattern electroretinogram (Fig. 1-4A) amplitude and latency that are progressive (Ikeda et al., 1994) and improve with L-DOPA treatment (Peppe et al., 1995).

In addition to functional changes, structural abnormalities in the retina have been observed in Parkinson's patients, including swelling of photoreceptor and retinal ganglion cells (Devos et al., 2005) and retinal nerve fiber layer thinning as detected by optical coherence tomography (Inzelberg et al., 2004, Altintas et al., 2008).

Schizophrenia and the Retina

Schizophrenia is a psychological disorder that involves impaired understanding of reality and disturbances in thought, emotion, communication, perception, or psychomotor

behavior (Seligman et al., 2001b). It is well known that patients with schizophrenia see the world differently from other people, but recent research shows that these differences are evident even at the level of the retina. Reduced a- wave amplitudes were detected in patients with schizophrenia during the acute stage of the disease (a period of time, usually lasting several weeks, during which positive symptoms like hallucinations manifest) and a significant inverse correlation was observed between positive symptoms and a- wave amplitude (Balogh et al., 2008). Patients with schizophrenia also show magnocellular pathway deficits (the pathway from the retina to the lateral geniculate nucleus that carries information involving motion detection) (Keri et al., 2004), deficits in smooth pursuit eye movements (Thaker et al., 2003), and differential crossing of foveal projections in the optic chiasm (Korn, 2002). Schizophrenia is generally thought of as a disease of dopamine excess (Seligman et al., 2001b), and it would be interesting to know how dopaminergic amacrine cell function in patients with schizophrenia compares to that in patients with Parkinson's disease.

Additionally, retinal examination may prove useful in predicting schizophrenia and monitoring its progression. Patients with schizophrenia exhibit microvascular abnormalities in both brain and retina. Using retinal imaging techniques, it was shown that not only do schizophrenic patients have wider retinal venules, but these vascular abnormalities may present early in life and are observed even when psychotic symptoms are currently below the threshold of clinical diagnosis (Meier et al., 2013). Patients with schizophrenia also show reduced thickness of the nerve fiber layer and macula and reduced macula volume as measured by optical coherence tomography. These reductions were greater when the disease progression had reached the chronic phase, and duration of

illness was correlated with retinal pathology (Lee et al., 2013). Finally, reductions in rod electroretinogram (in the absence of a cone electroretinogram deficit) were observed in currently healthy children of patients with neuropsychiatric disorders. Following up with the psychiatric health of these children may allow the prediction of schizophrenia based on the retina's response to light (Hebert et al., 2010).

Summary

Based on the research presented here, retinal examination (imaging and functional testing) could contribute to our understanding of non-ocular disease pathogenesis and potentially lead to earlier diagnosis. Fundus examinations can be used in the diagnosis and prediction of stroke (Okada et al., 1976, Stanford et al., 1978, Bhargava et al., 2012) and in the assessment of diabetic retinopathy, which exists in early stages even in prediabetic patients (Chen et al., 2012, Tabak et al., 2012). Pupillary responses enable physicians to identify lesions in the optic nerve and optic tract and the visual cortex. Optical coherence tomography can be used to detect changes in retinal morphology in conjunction with diabetes (Abdelkader, 2013), multiple sclerosis (Parisi et al., 1999), Parkinson's disease (Inzelberg et al., 2004, Altintas et al., 2008), Alzheimer's disease (Iseri et al., 2006), and schizophrenia (Lee et al., 2013). Electroretinogram abnormalities have been reported in diabetes (Abdelkader, 2013), Parkinson's disease (Ikeda et al., 1994), schizophrenia and potentially pre-schizophrenic children (Balogh et al., 2008, Hebert et al., 2010), and patients with seasonal affective disorder, autism, and drug addiction (Lavoie et al., 2014). For many of these diseases, more research is needed to determine the earliest point that retinal changes occur and whether the retina is more or less susceptible than the brain or other affected tissues to the disease.

In most major diseases, multiple systems are affected and could change in response to treatment. For example, treatment with β-secretase in a mouse model of Alzheimer's disease improves pathology in the brain but induces choroidal neovascularization in the retina (Cai et al., 2012). The entire body's response to disease and injury should be considered, with the focus on fixing the whole person rather than just treating symptoms. The retina represents an additional avenue for better understanding and predicting diseases that affect the brain and other systems.

PROGESTERONE AS A NEUROPROTECTIVE TREATMENT IN THE RETINA

Abstract

Introduction: Diseases and injury that affect the eye and cause vision loss are a serious problem worldwide, both in terms of economic impact and patient quality of life. Few effective treatments currently exist to address this significant unmet need.

Areas covered: Here we review research on progesterone treatment in traumatic brain injury and stroke that indicates it is a successful neuroprotective treatment in a variety of animal models. We also review the ocular disorders that we think are the best candidates for progesterone treatment, treatments currently available, and research relevant to bringing progesterone treatment to the eye, including evidence of progesterone and its receptors in the eye, overlap between mechanisms involved in retinal diseases and mechanisms modulated by progesterone, and research on progesterone in the eye thus far.

Expert opinion: Progesterone's pleiotropic properties and its success in pre-clinical models of traumatic brain injury and stroke make it an attractive candidate for the treatment of disorders affecting the retina. This review discusses progesterone as a potential neuroprotective treatment in the retina and optic nerve.

Introduction

Diseases and injury that cause retinal and optic nerve cell death and loss of visual function are a serious problem worldwide, with adult vision problems costing more than $50 billion per year in the United States alone (Prevent Blindness America website). The issue of ocular disease and injury is especially important given our aging population – more than 2.9 million older adults in the United States suffer from some form of visual impairment (CDC). Ocular disorders come in a variety of forms, including acute injuries, such as ocular trauma and retinal and optic nerve ischemias, as well as chronic diseases, such as glaucoma and retinal degeneration. While these diseases take a huge toll in terms of patient quality of life and economic impact, most are without effective (restorative) treatment.

Ocular Trauma

Ocular trauma can be localized to the eye but it often occurs in conjunction with a larger traumatic injury. Most injuries with an ocular trauma component are the result of automobile accidents, sports injuries, and blast injuries (Morley et al., 2010, Dunkin et al., 2011). Approximately 3% of emergency room visits in the United States are due to ocular trauma (Dunkin et al., 2011), with 2.4 million eye injuries occurring annually in 1986 (Parver, 1986). The issue of ocular trauma is especially important with the increase in blast injury incidence due to terrorist attacks and military service. Eye injury occurs in as many as 28% of blast injury survivors of terrorist attacks (Morley et al., 2010) and in 6% of injured veterans returning from Operations Iraqi Freedom and Enduring Freedom.

While eye injuries sustained during peacetime generally affect only one eye, 15-20% of wartime eye injuries are bilateral, some resulting in total blindness (Scott, 2011).

Retinal and Optic Nerve Ischemias

Clinically, ocular ischemia can occur on its own or alongside cerebral stroke or arthrosclerosis of the ophthalmic or carotid arteries, and visual function deficits often present as a first symptom in these systemic diseases (Benavente et al., 2001, Mead et al., 2002). In fact, visual field defects accompany 57% of minor strokes (Falke et al., 1991). Ocular ischemic syndrome, which is usually the result of severe carotid artery obstruction, is characterized by chronic reduction in blood flow to the eye that causes vision loss and has an estimated prevalence of 7.5 cases per million people per year (Sturrock and Mueller, 1984). Ocular ischemia occurs independently of cerebral damage, as well. Among these pathologies are retinal artery occlusion, with an incidence of 8.5 cases per million people per year (Rumelt et al., 1999), and anterior ischemic optic neuropathy (AION), which involves sudden vision loss due to stroke in the optic nerve (Hayreh, 1974) and has an incidence of 23 to 102 cases per million people per year (Johnson and Arnold, 1994).

Retinal Degenerations such as Retinitis Pigmentosa

Retinitis Pigmentosa is a family of hereditary degenerative retinal diseases in which rod and cone photoreceptor cell loss occurs over time (Hartong et al., 2006). Over one million people worldwide are affected by retinitis pigmentosa. Patients begin losing night vision and peripheral vision relatively early in life with central vision deficits

appearing later. By age 40, most patients with retinitis pigmentosa are legally blind (Hartong et al., 2006).

Glaucoma

Glaucoma is the second most common cause of blindness, affecting approximately 2.65% of the over 40 population and 60.5 million people worldwide in 2010, 4.5 million of which are bilaterally blind (Varma et al., 2011). Glaucoma is characterized by progressive degeneration of the optic nerve and retinal ganglion cells, leading to loss of visual field and eventual blindness (Schwartz, 2005, Pang and Clark, 2007). The degeneration often occurs without pain or symptoms, prompting glaucoma's nickname as the 'sneak thief of sight' (Adatia and Damji, 2005). The direct cost of glaucoma to US citizens is an estimated $2.9 billion, and these costs would likely be much higher but approximately half of glaucoma patients have no idea they have the disease (Varma et al., 2011). Glaucoma also has a substantial impact on patient quality of life as the disease progresses and patients lose their ability to drive, read, and perform everyday activities (Varma et al., 2011).

Few Effective Treatments

Despite the impact of ocular injury and disease and the unmet needs for patients, relatively few neuroprotective treatment options exist for any of the aforementioned disorders.

Ocular Trauma

In treating ocular trauma, surgery must be performed as soon as possible (for example, to remove fragments after a blast injury) (Parke et al., 2013). Visual rehabilitation and/or implantation of sensory substitution devices can be performed much later (Scott, 2011), but there is currently no clinical standard for treatment with a neuroprotective drug or other type of intervention immediately after injury. Systemic corticosteroids and surgical decompression have been used to treat ocular trauma, however, these therapies have not been proven to be effective, and in fact, corticosteroids may worsen outcomes (McClenaghan et al., 2011).

Retinal and Optic Nerve Ischemias

Treatment options for ischemia of the retina and optic nerve include aspirin (for AION (Hayreh, 2009, 2011), CRVO – central retinal vein occlusion (London and Brown, 2011)), thrombolytic agents (for CRAO – central retinal artery occlusion (Hayreh, 2011)), vascular endothelial growth factor inhibitors (for AION (Hayreh, 2009), for CRVO (London and Brown, 2011)), laser photocoagulation (for CRVO (London and Brown, 2011)), and systemic corticosteroids (for AION (Hayreh, 2009, 2011), for CRVO (London and Brown, 2011)). A recent clinical trial showed visual acuity improvement in AION with corticosteroid treatment, however the use of corticosteroids in AION is a controversial topic, as most clinicians maintain that corticosteroid treatment is ineffectual (Hayreh, 2011). And while thrombolytic agents are currently the most popular option for CRAO, a recent clinical trial has shown no benefit, and in fact, saw an increase in adverse reactions (Hayreh, 2011).

Retinal Degenerations such as Retinitis Pigmentosa

Currently, retinal degenerative diseases like retinitis pigmentosa are being treated with dietary supplements like Vitamin A, lutein, and omega-3, which have been shown to slow the course of the disease in clinical trials (Hartong et al., 2006). Other areas of treatment research include stem cells, retinal prosthetics, neurotrophic factors, transcorneal electrostimulation, and neuroprotective drugs (Hartong et al., 2006, Ofri and Narfstrom, 2007). Additionally, a big success in this field was in using gene therapy treatments to restore vision in dogs and mice with RPE65 mutations (Acland et al., 2001, Dejneka et al., 2004), although this requires that patients have some remaining photoreceptors prior to treatment (Jacobson et al., 2005).

Glaucoma

While the cause of glaucoma is not yet known, elevated intraocular pressure is the major risk factor in the most common type of glaucoma. The current clinical treatments of medication and surgery focus on reducing intraocular pressure (Schwartz, 2005), however, these treatments are not always successful and compliance rates are low (McKinnon et al., 2008). Additionally, even after treatment, secondary retinal ganglion cell degeneration and visual field loss can continue to occur, and in normal tension glaucoma, retinal ganglion cell death occurs even though the patients have normal intraocular pressure (Acland et al., 2001, Schwartz, 2005). Strategies that target secondary degeneration alone or in conjunction with intraocular pressure are lacking and need to be developed (Acland et al., 2001, Schwartz, 2005, McKinnon et al., 2008).

Research has been conducted on several potential neuroprotective treatments in glaucoma, including memantine, alpha 2 adrenergic agonists, neurotrophins like BDNF, anti-apoptotics, antioxidants, stem cells, and nitric oxide synthase inhibitors (Hartwick, 2001, Schwartz, 2005, Danesh-Meyer, 2011). However, while initial preclinical research was promising, these agents have yet to be proven protective clinically. Memantine, for example, was said to be protective in its first Phase III clinical trial, but a second trial failed to replicate these results (Danesh-Meyer, 2011).

The Nature of the Problem

The bottom line is that while there have been some successes, for most ocular disorders, we are a long way from cures or definitive treatments, and more research into neuroprotection needs to be done.

Due to the complex nature of these disorders, it is unlikely that a treatment that targets any one mechanism would prove effective in even one of these disorders, let alone more than one. Table 1-2 illustrates the many mechanisms involved in each of these disorders as well as which mechanisms have been shown to be modulated by progesterone in other disease models.

Mechanisms implicated in ocular disease and injury	Ocular Trauma	Ocular Ischemia	Retinal Degeneration	Glaucoma	Mechanisms modulated by progesterone
Neurotrophic/growth factor deficits (BDNF, CNTF, IGF-1)	X		X	X	Upregulates BDNF after spinal cord injury
Increases in inflammation (COX-2, TNF-α, IL-6)	X	X	X	X	Inhibits TNF-α production by microglia & macrophages, inhibits cytokine-induced glial activation, reduces levels of IL-1β, TNF-α, IL-6, & COX-2
Activation of astroglial NF-κB pathways	X	X	X	X	Reduces levels of the p65 NF-κB subunit & increases levels of the IκB inhibitor protein
Altered aquaporin expression & glial swelling	X	X	X	X	Modulates aquaporin 4 expression & reduces edema
Oxidative Stress	X	X	X	X	Reduces NO^- production in microglial cells by inhibiting iNOS expression
Glutamate Toxicity	X	X	X	X	Upregulates $GABA_A$ receptor expression, positive allosteric modulator of $GABA_A$ receptor, inhibits the NMDA response
Demyelination	X	X			Promotes myelination in culture & in vivo
Impaired axonal (optic nerve) transport	X		X	X	Restores impaired axonal transport in a mouse model of motoneuron disease
Increased Bax & caspase levels, decreased levels of Bcl-2 family genes	X	X	X	X	Reduces expression of Bax, Bad, & caspase-3, increases expression of Bcl-2 & Bcl-xL

Table 1-2. Overlap between mechanisms involved in ocular disorders and mechanisms modulated by progesterone.

Progesterone as a Candidate for Neuroprotective Treatment of the Retina

The neurosteroid hormone progesterone represents a new avenue of treatment for ocular diseases. While progesterone treatment in the eye has only been investigated in a handful of preliminary studies (discussed in the following section), progesterone's impressive track record in other models and its ability to modulate multiple mechanisms (Schumacher et al., 2007, Sayeed and Stein, 2009, Stein and Wright, 2010) make it an attractive candidate for treatment of eye diseases and injury (Table 1-2).

Progesterone's Effectiveness in Non-Ocular Disease Models

Progesterone synthesis takes place in the brains of both males and females (Baulieu and Robel, 1990, Cherradi et al., 1995, Tsutsui et al., 2000, Tsutsui, 2006) and progesterone is involved in early brain development (Lopez and Wagner, 2009). It may be that progesterone's neuroprotective properties are due its having evolved to protect the fetus during gestation (Stein and Wright, 2010). Progesterone's ability as a neuroprotectant was discovered when female rats were observed to have reduced brain edema and enhanced recovery after traumatic brain injury. The neuroprotective effect in females was found to be caused by increased endogenous progesterone levels (Attella et al., 1987). Since then, exogenous progesterone administration has been tested in traumatic brain injury in both male and female rats with findings of reduced edema, increased neuronal survival, and better functional outcomes (Roof et al., 1992, Roof et al., 1994).

Progesterone's neuroprotective capabilities in treating traumatic brain injury have been studied for over two decades. Progesterone's protective effects have been reported

in over 250 articles by 40 different laboratories in 22+ different animal models. Progesterone has been shown to be protective in two Phase II clinical trials in traumatic brain injury, but Phase III trials have not yet been shown to be effective. In the ProTECT (Progesterone for Traumatic Brain Injury, Experimental Clinical Treatment) trial, traumatic brain injury patients treated with progesterone showed a 50% decrease in mortality (Wright et al., 2007). A separate clinical trial by Xiao et al. (2008) studied traumatic brain injured patients for an extended period of time and found that functional outcomes were significantly improved in addition to mortality rates with progesterone treatment (Xiao et al., 2008). Phase III trials are currently being evaluated and the results will be known in the Spring of 2014.

Progesterone has been shown to exert its protective effects through multiple mechanisms. For example, progesterone upregulates BDNF after spinal cord injury (Gonzalez et al., 2004), promotes myelination in culture (Chan et al., 1998, Ghoumari et al., 2003) and in vivo (Ibanez et al., 2004), and restores impaired axonal transport in a mouse model of motoneuron disease (Gonzalez Deniselle et al., 2005). After traumatic brain injury, progesterone reduces aquaporin 4 expression in astrocytes around the wound site and nearby lateral ventricles, while increasing aquaporin 4 expression in areas distal from the injury, which may act to siphon fluid away from the wound site, thus reducing edema (Guo et al., 2006). Progesterone and/or its metabolites protect neurons against excitotoxicity by upregulating $GABA_A$ receptor expression (Puia and Belelli, 2001), by positively modulating the $GABA_A$ receptor (Reddy et al., 2005), and by inhibiting the NMDA response (Monnet et al., 1995). After traumatic brain injury, progesterone reduces expression of pro-apoptotic proteins Bax, Bad, and caspase-3 and increases

expression of anti-apoptotic proteins Bcl-2 and Bcl-xL (Djebaili et al., 2005, Yao et al., 2005). In microglial cells progesterone reduces TNF-α production and also reduces $NO^{\cdot}$ production by inhibiting iNOS (Drew and Chavis, 2000). Progesterone also reduces TNF-α production in macrophages (Miller and Hunt, 1998). Following traumatic brain injury, progesterone reduces levels of IL-1β, TNF-α, IL-6, COX-2, and the p65 NF-κB subunit, a marker of inflammatory NF-κB activity, while increasing levels of the IκB inhibitor protein, a marker of NF-κB pathway suppression (Cutler et al., 2007).

There is substantial overlap between mechanisms involved in ocular injury and disease and mechanisms modulated by progesterone (Table 1-2). Progesterone's pleiotropic effects, along with its proven history of success in other animal models of injury, its safety profile, and its ease of delivery lead us to recommend progesterone as an option for neuroprotective treatment in injury and disease of the eye.

Progesterone in the Retina

Progesterone Research in Models of Ocular Disease and Injury

Positive Results

While the research is not extensive, the use of progesterone as a neuroprotective agent in ocular disorders has been investigated previously.

Doonan et al. (2011) used Norgestrel, a synthetic progestin, in two animal models that involve severe photoreceptor cell death, a light induced retinal degeneration (LIRD) model and the rd10 genetic model of retinal degeneration. Light induced retinal degeneration mice treated with intraperitoneal injections of Norgestrel (100 mg/kg) 1 hour before and every 3 days after exposure to toxic levels of bright light showed

significantly reduced photoreceptor cell death compared with vehicle-treated controls. rd10 mice treated with intraperitoneal injections of Norgestrel (100 mg/kg) every other day beginning at P18 showed delayed photoreceptor cell death, improved morphology, and decreased retinal function deficits (Doonan et al., 2011).

Neumann et al. (2010) applied steroids to retinal explants from a high intraocular pressure model of ischemia-reperfusion and the STZ model of Type 1 diabetes. Progesterone was found to inhibit glial swelling in the retina, suggesting that it might decrease retinal edema (Neumann et al., 2010).

Similarly, Lu et al. (2008) found protective effects of progesterone in a pressure-induced model of retinal ischemia-reperfusion in vivo. Using intraperitoneal injections of progesterone (4 mg/kg) at 30 minutes before and 2 hours after retinal ischemia in rats, they observed decreases in cellular pathology and reduced thinning of the inner retinal and nerve fiber layers in progesterone-treated animals compared with vehicle-treated controls (Lu et al., 2008).

A few presentations at the Association for Research in Vision and Ophthalmology annual meetings report protective effects of progesterone in the eye as well. Sanchez-vallejo et al. (2013) treated rd1 mice with progesterone (5 mg/kg, oral dose) every other day starting at P5 and found reduced retinal degeneration-induced increases in glutamate concentration in rd1 retinas (Sanchez-vallejo et al., 2013). Cubilla et al. (2012) treated light induced retinal degeneration mice with progesterone (1 mg/kg) and found significant protection, including no TUNEL positive nuclei and no morphological abnormalities in the outer nuclear layer (Cubilla et al., 2012). Araiz et al. (2012) treated rd1 mice with progesterone (5 mg/kg, oral dose) every other day from P5 to P11 and

found that progesterone reduced cell death in the periphery of the retina but did not have an effect on oxidative damage (Araiz et al., 2012).

Negative Results

A few papers report negative results with progesterone treatment in eye disease. Nakazawa et al. (2006) found that following optic nerve transection, ovariectomized rats showed significant decreases in surviving RGC numbers. Intravitreal injection 15 minutes prior to optic nerve transection of 17B-estradiol but not progesterone (100 uM) protected against retinal ganglion cell death (Nakazawa et al., 2006).

Kaldi and Berta did not find differences in ERG deficit or outer nuclear layer thickness with progesterone treatment in a light induced retinal degeneration model in rats using intraperitoneal injections of progesterone (60 mg/kg). However, timing could be an issue, as three of their injections were given prior to light damage and the fourth was given immediately after (Kaldi and Berta, 2004). A second study by O'Steen failed to find a protective effect of progesterone in a rat model of light induced retinal degeneration. Using 2.5 mg of progesterone or progesterone plus estradiol, rats were treated during 14 days of continuous light exposure (route unspecified, precise treatment regimen unspecified), and no significant rescue of retinal thicknesses was observed. Additionally, ovariectomized rats showed reduced light damage (O'Steen, 1977). Yu et al. found protective effects of estradiol but not progesterone in vitro using a hydrogen peroxide model of retinal degeneration (Yu et al., 2004). However, another group has shown that the progesterone receptor functions in intact retina but not cell culture, so this could be the reason (Li et al., 1997).

Dose, timing, and tapering of dose are all critical in successful progesterone treatment, and these issues could be responsible for the negative results mentioned above. We know that pre-treating with progesterone and then abruptly stopping treatment the day of injury actually exacerbates stroke injury caused by middle cerebral artery occlusion (Murphy et al., 2000), a model where progesterone treatment has been shown to be protective in multiple studies (Sayeed et al., 2007, Ishrat et al., 2009, Ishrat et al., 2012, Won et al., 2013, Yousuf et al., 2013a, Yousuf et al., 2013b). Additionally, traumatic brain injured rats that received 5 days of progesterone treatment with progressively halved dosing on days 6 and 7 showed less inflammation, decreased lesion size, and enhanced behavioral recovery compared with rats who received progesterone treatment with acute withdrawal (Cutler et al., 2005, Cutler et al., 2006).

Evidence of progesterone, progesterone receptors, and progesterone synthesis in the retina

Another line of support for progesterone treatment in the eye can be found in studies that show progesterone, progesterone receptors, and progesterone synthesis in the eye and retina. Progesterone itself was found in larger quantities in rat retina than in plasma (Lanthier and Patwardhan, 1987), and in human retina, optic nerve, and occipital cortex for both males and females (Lanthier and Patwardhan, 1986) but not vitreous (Chong and Aw, 1986).

Wickham et al. (2000) found progesterone, androgen, and estrogen receptor mRNA in, among other structures, retina/uvea samples in rats, retina/choroid samples in rabbits, and retinal pigmented epithelium samples in humans. Progesterone receptor

mRNA was found in samples from both males and females, although gender and tissue specific differences were observed (Wickham et al., 2000). Koulen et al. found progesterone receptor expression at the protein level in mouse rod bipolar cells that had been dissociated from the rest of the retina (they did not look at other cell types) (Koulen et al., 2008). Both of the above studies looked at classical progesterone receptors (the classical pathway being that progesterone receptors translocate to the nucleus) (Wickham et al., 2000, Koulen et al., 2008). A third study by Swiatek-De Lange et al., demonstrated expression of membrane-associated progesterone receptor component 1 (PGRMC1, which also goes by the name 25-Dx) in Müller cells and retinal pigmented epithelial cells and showed that progesterone could act through this receptor in addition to classical receptors (Swiatek-De Lange et al., 2007). Not only is the progesterone receptor present in retina, it has been shown to be capable of functioning in intact retina but not primary cultures of Müller cells (Li et al., 1997).

In addition to acting through progesterone receptors, progesterone has been shown to act through other receptors and mechanisms. Progesterone increases the rate of dissociation of glucocorticoids from glucocorticoid receptors (Svec et al., 1980) by binding at a site separate from the agonist (Svec et al., 1989). Progesterone directly influences the activity of the nicotinic acetylcholine receptor (Valera et al., 1992). Progesterone's metabolite allopregnanolone binds to the $GABA_A$ receptor complex and acts as a positive allosteric modulator resulting in increased Cl^- influx and reduced excitability (Reddy et al., 2005). Additionally, progesterone itself upregulates $GABA_A$ receptor expression (Puia and Belelli, 2001). Progesterone acts as an antagonist at the Sigma-1 receptor (Maurice et al., 1998), which colocalizes with the IP3 receptor in the

endoplasmic reticulum to decrease Ca^{2+} release (Su and Hayashi, 2003) and inhibit NMDA-evoked [^{3}H] norepinephrine release (Monnet et al., 1995). Finally, progesterone, pregnenolone (its precursor), and allopregnanolone (its metabolite) activate the pregnane X receptor (PXR) (Kliewer et al., 1998, Langmade et al., 2006), which activates p-Glycoprotein (P-gp), an efflux pump in the blood brain barrier and the retinal pigmented epithelium that removes cytotoxic and xenobiotic substances (Zhang et al., 2012b).

Several studies have shown evidence of progesterone and other neurosteroid synthesis in the retina (Fig. 1-5). Cascio et al. (2007) found that tagged pregnenolone (a precursor to progesterone) gets converted to radiolabled progesterone in the rat retina (Cascio et al., 2007). An in vitro study in rat retina did not find pregnenolone conversion to progesterone, but this could be due to an in vitro/in vivo difference (Lanthier and Patwardhan, 1988). mRNA for the 17β-hydroxysteroid dehydrogenases, a family of enzymes that plays a role in the synthesis and deactivation of steroid hormones, including the conversion of 20α-hydroxyprogesterone into progesterone has been found in human retina (Coca-Prados et al., 2003). 3-β-HSD, another enzyme family that catalyzes diverse reactions, including progesterone from pregnenolone, has been found to be expressed by both retinal ganglion cells and a sub-population of amacrine cells in zebrafish retina (Sakamoto et al., 2001a). Additionally, the actions of 5α-reductase (converts progesterone to the intermediate between progesterone and allopregnanolone) and 3α-hydroxysteroid dehydrogenase (converts the intermediate to allopregnanolone) have been indirectly shown by the conversion of progesterone to 5α-dihydroprogesterone (the intermediate) and then to allopregnanolone (Lanthier and Patwardhan, 1988, Guarneri et al., 1994).

In addition to the research covered above, progesterone has been found to inhibit the activity of enzymes involved in retinoid cycling (Kaschula et al., 2006, Muniz et al., 2006, Muniz et al., 2009), to affect photoreceptor cell excitability in crayfish (Hernandez-Falcon et al., 1997), and to increase retinal ganglion cell survival in culture in the absence of an insult (Lindsey and Weinreb, 1994). We also know that progesterone binds to glucocorticoid receptors in the chick retina with high affinity, possibly acting as an inhibitor (Lippman et al., 1974). Additionally, progesterone's metabolite, allopregnanolone has been shown to increase GABA-activated chloride currents in mouse retinal ganglion cells (Bahring et al., 1994). Clearly, progesterone is capable of playing diverse roles in the retina and may prove to be a valuable neuroprotective treatment.

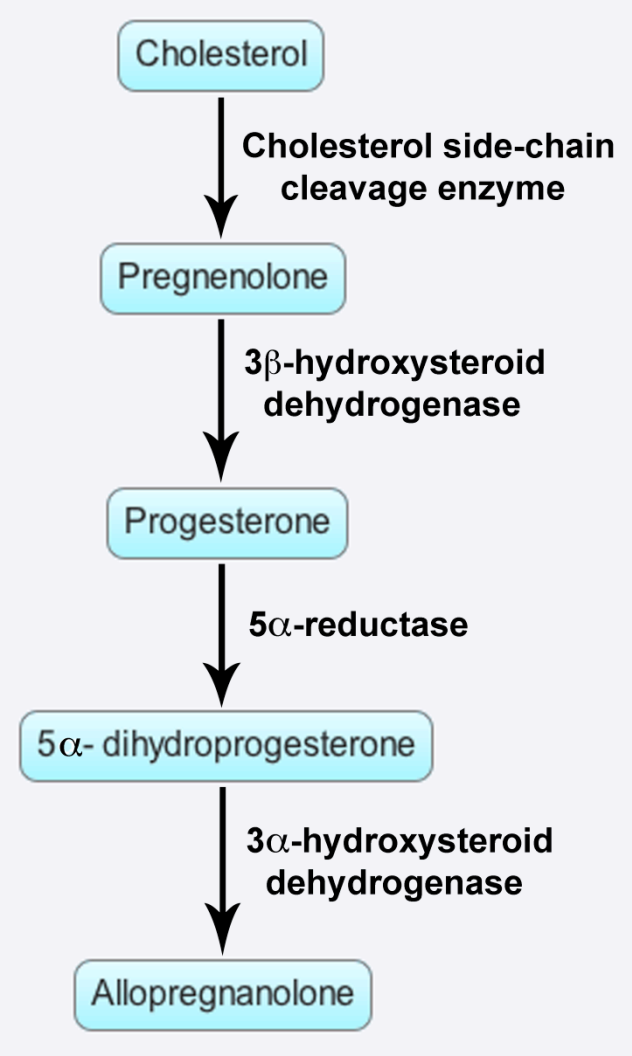


Figure 1-5. Progesterone synthesis and metabolism. Every substance and enzyme in the progesterone pathway has been identified in the retina.

Human Studies and Anecdotal Evidence

Exogenous progesterone has been shown to affect intraocular pressure (Obal, 1950, Posthumus, 1952), lacrimal gland function (Suzuki et al., 2006, Sullivan et al., 2009), and lens permeability (Lambert, 1968). Additionally, a host of ocular changes take place during times of endogenous hormone fluctuation, such as menopause, pregnancy, and the menstrual cycle (Ziai et al., 1994, Yucel et al., 2005, Avitabile et al., 2007, Errera et al., 2013, Grant and Chung, 2013, Panchami et al., 2013). Further, gender differences have been observed in a number of ocular pathologies, including glaucoma, age-related macular degeneration, dry eye, cataracts, and diabetic retinopathy (Wickham et al., 2000).

Much of the human research on the effects of hormones on ocular function and disorders is contradictory and/or uses small sample sizes. Additionally, progesterone and its synthetics are often used without discrimination in studies using oral contraceptives and hormone replacement therapy. For example, one study claimed that progesterone increased the risk for glaucoma, but the compound tested in the study was actually the progestin medroxyprogesterone acetate (Thomas et al., 2003). Progestins are compounds that mimic the effect of natural progesterone in the reproductive system. However, progestins differ from progesterone and from other progestins in chemical structure, metabolism, pharmacokinetics, potency, and biological effects (Thomas et al., 2007). While these synthetics may bind classical progesterone receptors, they do not to bind to a membrane progesterone receptor (Thomas et al., 2007) nor metabolize to allopregnanolone, which has a variety of effects including binding to $GABA_A$ receptor to reduce excitotoxicity (Reddy et al., 2005). Further, their actions in neurons and

vasculature are very different. For example, medroxyprogesterone acetate, the synthetic progesterone most commonly used in hormone replacement therapy, was shown to cause endothelial disruption, monocyte accumulation in the vessel wall, activation of platelets, and formation of clots, while natural progesterone did not (Thomas et al., 2003). Additionally, medroxyprogesterone acetate has been shown to exacerbate rather than protect against excitotoxitcity (Nilsen et al., 2006) and to reduce the protective effects of other neurosteroids (Nilsen and Brinton, 2002, Littleton-Kearney et al., 2005). While sex hormones clearly have effects in human ocular function and disorders, additional research needs to done to determine the precise role each neurosteroid plays and to tease out the actions of progesterone vs. progestins.

Conclusion

There is now substantial literature showing that progesterone acts via multiple mechanisms to reduce cell death and enhance recovery after a variety of injuries. Progesterone's success in injury and disease in both animal models and humans make it a promising candidate for treatment in the eye.

Expert Opinion

In this review, we chose to highlight four ocular disorders in which we think progesterone treatment would prove most promising. Ocular trauma and stroke are natural choices because of progesterone's success in trauma and stroke in the brain (He et al., 2004a, Cutler et al., 2005, Djebaili et al., 2005, Gibson et al., 2005, Yao et al., 2005, Guo et al., 2006, Cutler et al., 2007, Sayeed et al., 2007, Wright et al., 2007, Cai et al.,

2008, Xiao et al., 2008, Ishrat et al., 2009, Ishrat et al., 2010, Liu et al., 2012a, Won et al., 2013, Yousuf et al., 2013a). We discussed retinal degeneration because most of the results showing protective effects of progesterone in the eye have been in retinal degeneration models (Doonan et al., 2011, Araiz et al., 2012, Cubilla et al., 2012, Sanchez-vallejo et al., 2013). Finally, we chose to highlight glaucoma because secondary degeneration is such a prominent issue in glaucoma (Schwartz, 2005, Ofri and Narfstrom, 2007, McKinnon et al., 2008) and progesterone has been shown to reduce secondary degeneration (Schumacher et al., 2007, Sayeed and Stein, 2009). We also discussed glaucoma because of progesterone's potential to act as both a neuroprotectant and an intraocular pressure-reducer (Obal, 1950, Posthumus, 1952, Ziai et al., 1994, Panchami et al., 2013). For all of the disorders discussed there is substantial overlap between mechanisms implicated in the disorder and mechanisms shown to be modulated by progesterone in other non-ocular disease and injury models (Table 1-2).

There are important considerations as research on progesterone in the eye moves forward. First, the progesterone dose must be titrated specifically for the eye and for each disorder. In the brain, a 16 mg/kg dose has been shown to be most effective in traumatic brain injury (Cutler et al., 2007), while an 8 mg/kg dose was most effective in stroke (Wali et al., 2014). Additionally, we know progesterone crosses the blood brain barrier (Sar and Stumpf, 1973), so we believe it crosses the blood retinal barrier as well, but local treatment may be necessary if systemic treatment is not effective. Second, some of the retinal disorders discussed above are acute, while others would require more long-term treatment. Long-term treatment with progesterone is currently being studied in other diseases, including models of Alzheimer's and multiple sclerosis (Nilsen and Brinton,

2002, Wang et al., 2010b, Chen et al., 2011, Hussain et al., 2011, Singh et al., 2012), but would need to be studied in the retina as well.

Third, while progesterone is a promising treatment for the ocular disorders discussed here, more research needs to be done before testing progesterone in retinal diseases that involve increases in VEGF and choroidal neovascularization. Progesterone has been reported to increase VEGF and neovascularization in the brain (Li et al., 2012b, Yousuf et al., 2013b). However, recent studies reveal that increases in VEGF with progesterone may be time point specific (Ishrat et al., 2012) and that at other time points progesterone treatment reduces VEGF (Won et al., 2013). Preliminary research shows that progesterone does not cause growth of new vasculature, but merely prevents existing vasculature from dying after injury (unpublished Stein Lab data). These distinctions are not minor. Teasing out progesterone's actions on VEGF and neovascularization would be very important before testing progesterone in diabetic retinopathy or age-related macular degeneration.

While there have been a handful of studies on progesterone treatment in eye disorders, much work remains to be done before we can bring progesterone treatment of ocular disorders to the clinic. If progesterone is successful in animal models of ocular injury and disease, it is something that could easily be easily translated to the clinic. Progesterone has shown success in Phase II clinical trials although Phase III results are yet to be determined. It is safe, FDA approved, and can be delivered systemically via a number of routes (Stein and Wright, 2010). The great deal of overlap in mechanisms modulated by progesterone and mechanisms involved in disorders of the eye make progesterone a promising candidate for treatment. Disorders of the eye represent an

exciting new branch of progesterone research that could result in a new neuroprotective treatment for injuries and diseases with few clinical options.

SPECIFIC AIMS OF THIS DISSERTATION

By viewing diseases of the brain and retina as separate entities, we miss an opportunity to expand our knowledge of mechanisms of disease, to translate treatments from one system to the other, and to optimize treatments based on a comparison of their performance in both systems. Thus, in the area of cerebral and retinal stroke, my work aims to investigate the common mechanism of inflammation in a stroke model thought to affect both the brain and eye. In addition, I examined whether progesterone, a drug that has been shown to have neuroprotective effects in cerebral stroke, reduces inflammation and preserves function in retinal stroke as well.

To address these questions, I used the transient middle cerebral artery occlusion (MCAO) model, a rodent model commonly used in cerebral ischemia research and thought to cause retinal ischemia as well (Fig. 1-6). In Chapter 2, I report the retinal deficits caused by the transient MCAO model across time. I hypothesized that **occlusion of the ophthalmic artery (ocular stroke) during MCAO will cause retinal damage and retinal function deficits that will correlate with neurological damage in the brain.** To investigate this hypothesis, I assessed retinal function using the electroretinogram (Fig. 1-4A) in adult male rats 2 and 9 days after MCAO surgery. I correlated retinal function with behavioral function, as measured using neuroscores. I also examined retinal cell death and changes in GFAP and glutamine synthetase in the retina after MCAO as well as infarct size in the brain.

In Chapters 3 and 4, I investigated the effects of progesterone treatment in ischemic injury in both brain and retina. In Chapter 3, I used two animal models: one

that causes ischemia in both the brain and the eye (the MCAO model) and one that causes ischemia in the eye alone (the rodent anterior ischemic optic neuropathy or rAION model, Fig. 1-7). My first hypothesis was that **following MCAO, progesterone-treated animals will exhibit reduced retinal function deficits, which will accompany improvements in behavioral outcomes and reduced brain damage.** To test this hypothesis, MCAO rats were treated systemically with progesterone or vehicle. Retinal function was assessed using electroretinogram and compared with behavioral function, as measured using neuroscores, sticky-tape task, and grip strength (Fig. 1-8). I also examined retinal cell death, retinal changes in GFAP and glutamine synthetase, and infarct size in brain. My second hypothesis in Chapter 3 was that **progesterone will protect retinal ganglion cells from ischemic injury in the rAION (Rose Bengal) model, resulting in preserved visual function.** To test this hypothesis, AION rats were treated systemically with progesterone or vehicle. I assessed optic nerve pathology using fundus photography and ganglion cell function using visual evoked potentials (Fig. 1-4B). Retinal ganglion cells were stained with Brn3a, a retinal ganglion cell specific marker, in flat mounts and counted automatically (Fig. 1-9).

In Chapter 4, I investigated the hypotheses that **a) levels of NF-κB and downstream inflammatory cytokines will increase in the retina after MCAO as they do in the brain, and b) progesterone will reduce inflammation in the retina as it does in the brain following MCAO.** To test these hypotheses, I examined levels of NF-κB pathway and other inflammatory markers in brains and retinas from MCAO rats treated systemically with progesterone or vehicle compared with shams. Levels of cytosolic NF-κB (pathway suppressed) vs. levels of nuclear NF-κB and phosphorylated NF-κB

(pathway active) were assessed in retinas and brains at 24 and 48 hours using western blots. Levels of IL-6, TNF-α, CD11b, Progesterone receptor A and B, and pregnane X receptor were measured as well.

In summary, the findings outlined in this dissertation have important clinical implications in optimizing progesterone treatment in retinal ischemia and other ocular diseases. Additionally, this work highlights differences in brain and retinal responses to ischemia that could inform future research in these areas.

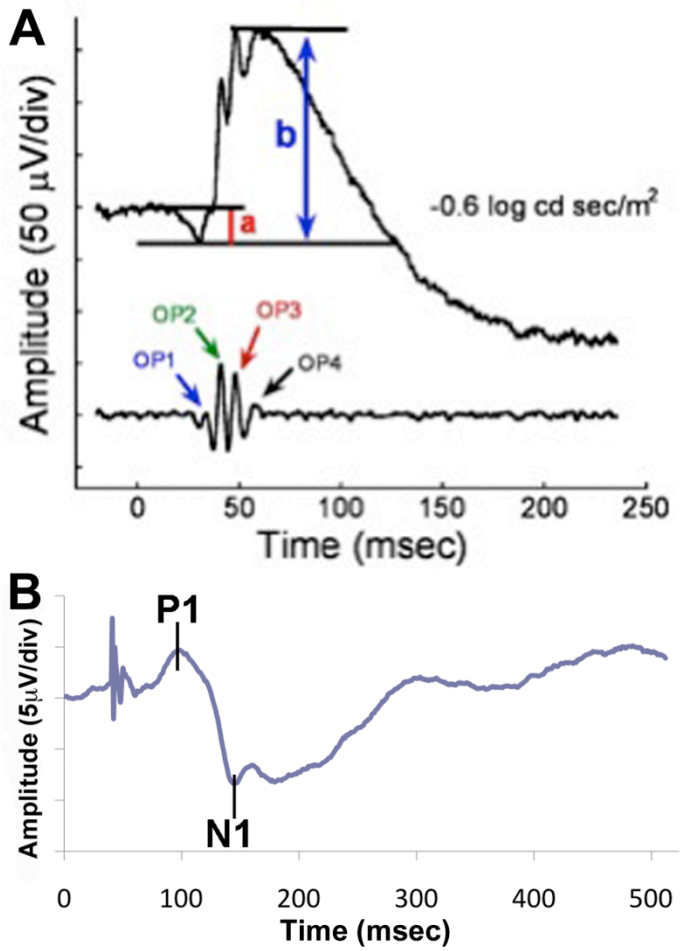


Figure 1-4. ERG and VEP sample waveforms. Light stimuli are delivered by a series of Ganzfeld strobe flashes. Retinal and cortical responses are recorded by electrodes placed on the cornea and over the visual cortex, respectively. **A) A sample ERG waveform (top) and filtered oscillatory potentials (bottom) from an adult rat.** ERGs measure the retinal response to light stimuli. Amplitudes and implicit times are measured for a-waves (baseline to trough, representative of photoreceptor cell function, (Penn and Hagins, 1969, Hood and Birch, 1990), b-waves (trough to peak, representative of bipolar cell function)(Robson and Frishman, 1998), and oscillatory potentials (wavelets which likely represent amacrine and ganglion cell function)(Wachtmeister, 1998). This portion of the figure was created by Dr. Machelle Pardue and reproduced with her permission. OP = oscillatory potential. **B) A sample VEP waveform from an adult rat.** VEPs measure visual cortex activity in response to visual stimuli. Amplitudes and implicit times are measured for P1 (baseline to peak) and N1 (peak to trough). While VEP results are highly correlated with retinal ganglion cell and optic nerve morphology, unlike ERG components, the components of a VEP cannot be mapped to specific retinal ganglion cell populations.

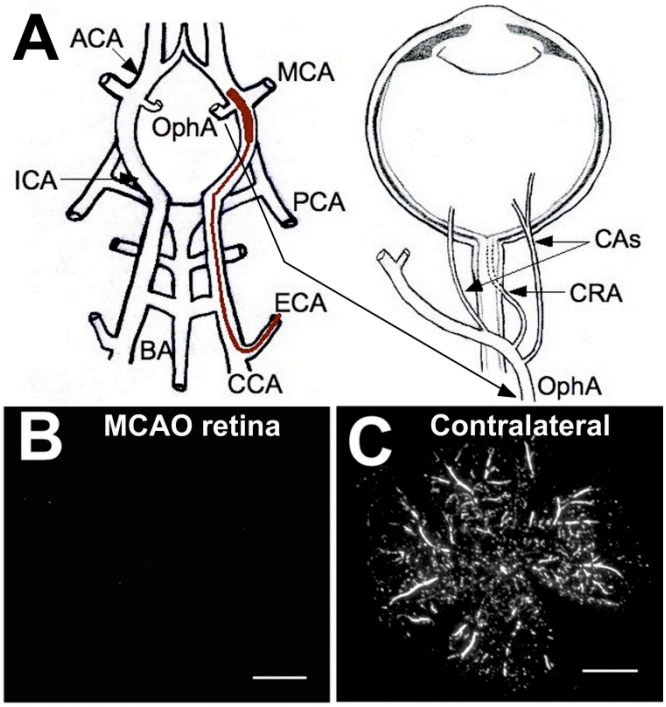


Figure 1-6. Diagram of MCAO filament placement. A) The filament is fed through the external carotid artery (ECA) and into position along the internal carotid artery (ICA) to block the middle cerebral artery (MCA). Note the close proximity of the ophthalmic artery (OphA). CAs, Ciliary Arteries; CRA, Central Retinal Artery; ACA, Anterior Cerebral Artery; PCA, Posterior Cerebral Artery; BA, Basilar Artery; CCA, Common Carotid Artery. B & C) Retinal images following MCAO + injection of fluorescent microspheres into the femoral vein. B) MCAO retina. C) Contralateral retina. Images modified from (Steele et al., 2008) with permission.

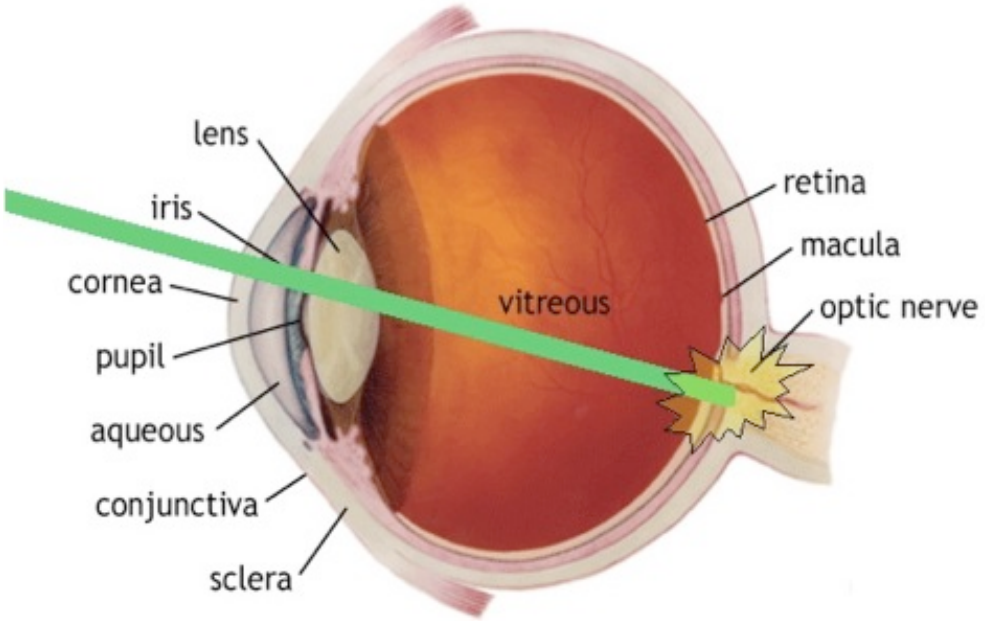


Figure 1-7. Induction of monocular optic nerve stroke using the rodent anterior ischemic optic neuropathy (rAION) model. rAION is induced in anesthetized animals by injecting the tail vein intravenously with Rose Bengal (2.5 mM), and then illuminating the optic nerve with a green wavelength laser (532 nm, 500 μm diameter). The laser photoactivates the dye causing a stroke inside the optic nerve. The advantage of the rAION model is that it actually causes thrombosis of the optic nerve, whereas other models use crush surgeries or pressure to mimic thrombotic damage. Additionally, the surgery is less stressful for the animals (Bernstein et al., 2003). Image modified from: National Eye Institutes, National Institues of Health (NEA04) with permission.

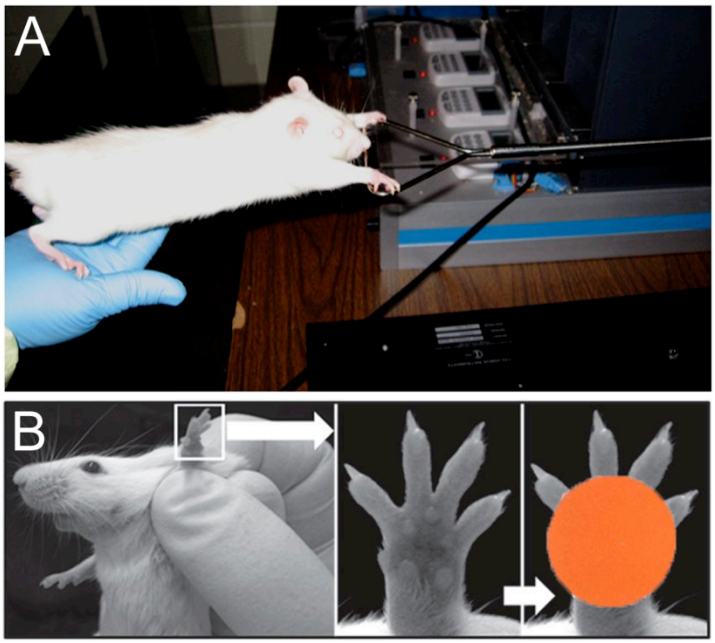


Figure 1-8. Behavioral tests for functional assessment post-MCAO. A) Grip strength task. Rats grip the bar and are gently pulled by the tail until they release. Grip strength is assessed in rats before and after MCAO. **B) Sticky-tape task, a measure of sensorimotor deficits.** A glue dot is adhered to the front paw, and rats are placed in an open field. The time it takes to notice the dot and remove the dot is recorded. Image modified from (Bouet et al., 2009) with permission.

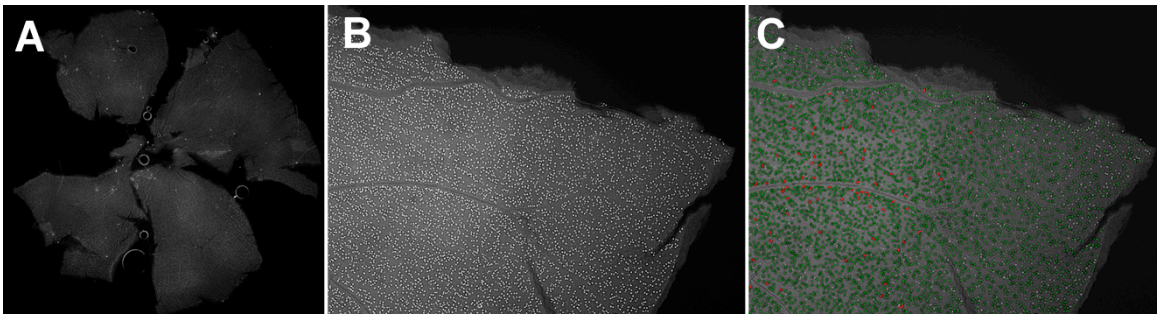


Figure 1-9. Immunohistochemistry with Brn3a, a retinal ganglion cell specific marker. A) Representative flat mount of a control retina stained with Brn3a. B) Close up of a control flat mount showing retinal ganglion cells stained with Brn3a (left) as identified using a cell counting protocol in Image Pro (right).

Chapter 2:

Retina is less susceptible than brain to ischemic injury caused by middle cerebral artery occlusion

This chapter presents, with permission, work published within:

Rachael S. Allen, Iqbal Sayeed, Heather A. Cale, Katherine C. Morrison, Jeffrey H. Boatright, Machelle T. Pardue, Donald G. Stein. Severity of middle cerebral artery occlusion determines retinal deficits in rats. Experimental Neurology. 2014.

ABSTRACT

Middle cerebral artery occlusion (MCAO) using the intraluminal suture technique is a common model used to study cerebral ischemia in rodents. Due to the proximity of the ophthalmic artery to the middle cerebral artery, MCAO blocks both arteries, causing both cerebral and retinal ischemia. While previous studies have shown retinal dysfunction at 48 hours post-MCAO, we investigated whether these retinal function deficits persist until 9 days and whether they correlate with central neurological deficits.

Rats received 90 minutes of transient MCAO followed by electroretinography at 2 and 9 days to assess retinal function. Retinal damage was assessed with cresyl violet staining, immunohistochemistry for glial fibrillary acidic protein (GFAP) and glutamine synthetase, and TUNEL staining.

Rats showed behavioral deficits as assessed with neuroscore that correlated with cerebral infarct size and retinal function at 2 days. Two days after surgery, rats with moderate MCAO (neuroscore < 5) exhibited delays in electroretinogram implicit time, while rats with severe MCAO (neuroscore ≥ 5) exhibited reductions in amplitude. Glutamine synthetase was upregulated in Müller cells 3 days after MCAO in both severe and moderate animals, however, retinal ganglion cell death was only observed in MCAO retinas from severe animals. By 9 days after MCAO, both glutamine synthetase labeling and electroretinograms had returned to normal levels in moderate animals.

Early retinal function deficits correlated with behavioral deficits. However, retinal function decreases were transient and selective retinal cell loss was observed only with severe ischemia, suggesting that the retina is less susceptible to MCAO than the brain. Temporary retinal deficits caused by MCAO are likely due to ischemia-induced increases

in extracellular glutamate that impair signal conduction, but resolve by 9 days after MCAO.

INTRODUCTION

Clinically, ocular ischemia can occur in conjunction with cerebral stroke or arthrosclerosis of the carotid or ophthalmic arteries. Indeed, visual impairments are often a first symptom in these pathologies (Benavente et al., 2001, Mead et al., 2002), and 57% of minor strokes are accompanied by subclinical visual field defects (Falke et al., 1991). Ocular ischemic syndrome, usually caused by severe obstruction of the carotid artery, involves vision loss due to chronically reduced arterial blood flow to the eye (Sturrock and Mueller, 1984). Ocular ischemia can also occur independently of cerebral damage, as is the case in retinal artery occlusion (Rumelt et al., 1999) and anterior ischemic optic neuropathy (Johnson and Arnold, 1994).

Middle cerebral artery occlusion (MCAO) in rodents is a common technique used to study mechanisms and potential treatments of cerebral ischemia. In this model, a filament is inserted into the internal carotid to block the middle cerebral artery (Longa et al., 1989). Due to the proximity of the ophthalmic artery to the middle cerebral artery in rats (Fig. 2-1), the filament blocks both arteries. The ophthalmic artery branches into the ciliary arteries, which provide blood supply to the outer retina via the choroid, and the central retinal artery, which provides blood supply to the inner retina. Since the ophthalmic artery ultimately provides the blood supply for the entire eye (Smith et al., 2002), it is not surprising that several studies have presented evidence of retinal deficits following MCAO in rodents (Block et al., 1997, Kaja et al., 2003, Cheung et al., 2007, Kalesnykas et al., 2008, Steele et al., 2008, Li et al., 2009). Since common behavioral assessments of MCAO in rodents (i.e., Morris water maze (Andersen et al., 1999); radial arm maze (Lee et al., 2003)) depend on successful interpretation of visual cues and can

be affected by visual function deficits (Wong and Brown, 2007), it is imperative to understand how MCAO impacts visual function beyond one week post-ischemia when most behavioral testing is performed.

Block et al. (1997) reported decreased retinal function as measured by electroretinogram (ERG) a- and b-wave amplitudes and delayed b-wave implicit times during MCAO in rats, with reduced b-wave amplitudes persisting to 48 hours after reperfusion. Additionally, glial fibrillary acidic protein (GFAP) expression (a marker for retinal glial cell reactivity) increased in Müller cells following MCAO in rats (Block et al., 1997, Kalesnykas et al., 2008). However, the eyes of these rats did not show cell loss or changes in retinal thickness with Nissl staining (Block et al., 1997, Kalesnykas et al., 2008), although a few apoptotic cells were found with TUNEL staining in the rat retinal ganglion cell layer (Kaja et al., 2003).

It is interesting that there is no cell death in the inner nuclear layer of the rat retina given that 1) the MCAO model causes a ~50% reduction in bipolar cell function as measured by ERG b-wave at 2 days post-MCAO, and 2) MCAO causes extensive cell death in the brain (Longa et al., 1989). The absence of retinal cell death suggests that functional deficits may recover. This idea is supported by the findings of Block et al. (1997) that photoreceptor dysfunction recovers at 2 days post-MCAO as shown by a-wave amplitude recovery (Block et al., 1997).

Thus, we hypothesized that the retina will exhibit reduced susceptibility to MCAO, with retinal function recovering across time. To test this hypothesis, we investigated the temporal response of the retina to injury and whether retinal function and structure correlate with the severity of brain injury as determined by behavioral deficits.

These studies are important in determining whether visual deficits confound behavioral assessments in MCAO rats and in comparing the retinal versus cortical neuron response to MCAO injury.

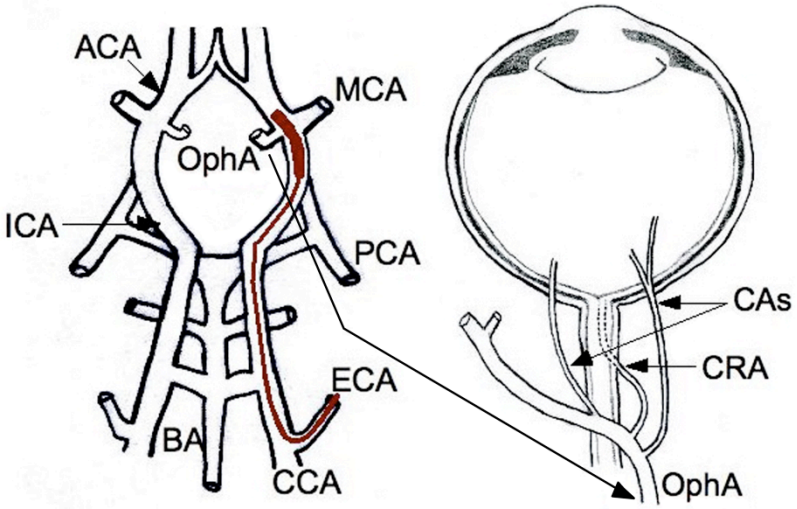


Figure 2-1. Diagram of MCAO filament placement. The filament is fed through the external carotid artery (ECA) and into position along the internal carotid artery (ICA) to block the middle cerebral artery (MCA). Note the close proximity of the ophthalmic artery (OphA). CAs, Ciliary Arteries; CRA, Central Retinal Artery; ACA, Anterior Cerebral Artery; PCA, Posterior Cerebral Artery; BA, Basilar Artery; CCA, Common Carotid Artery. Modified from Steele et al., 2008 with permission.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (n = 31) from Charles River Laboratories were used in this study. Rats were approximately 60 days of age (290-330 grams) at the time of surgery. Littermates that did not receive surgery were used as controls. All animal procedures were approved by the Institutional Animal Care and Use Committee (Emory University protocol #251-2008) and performed in accordance with NIH guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were housed under a 12:12 reverse light:dark cycle with water and food ad libitum and handled daily for at least 5 days prior to surgery. One rat died during surgery, one was excluded due to an incomplete reperfusion, and one control rat was excluded because of functional abnormalities.

MCAO surgery

MCAO surgery was performed with minor modifications to the previous description (Longa et al., 1989). Briefly, animals were initially anesthetized via inhalation of 5% isoflurane (in a N_2/O_2 70%/30% mixture) and remained sedated with inhalation of 2% isoflurane. A pulse oximeter (SurgiVet, model V3304; Waukesha, WI, USA) was used to analyze and sustain blood oxygen saturation (SpO_2) at 90%. Body temperature was monitored via rectal probe and sustained between 36.5°C and 37.5°C with a heating lamp. A laser-Doppler flowmetry (LDF) probe (Moor Instruments, Wilmington, Delaware, USA), an established and reliable system for monitoring changes in cerebral blood flow due to induction of focal cerebral ischemia (Dirnagl et al., 1989),

was used to monitor cerebral blood flow for 5 minutes prior to occlusion through 5 minutes after reperfusion. Because we wanted to investigate correlation between the brain and retina over a range of severities, we did not exclude animals with low % occlusions even though this is a practice commonly used to reduce variability (Sayeed et al., 2006).

A midline incision was made at the ventral surface of the neck, and a 6-0 silk suture was used to separate and ligate the right common carotid arteries. Next, a microvascular clip was used to temporarily occlude the internal carotid and pterygopalatine arteries while a 4-0 silicon-coated monofilament (0.35–0.40 mm long) (Doccol Co., Albuquerque, NM, USA) was inserted through the cut in the external carotid artery and into the internal carotid artery. This filament was pushed an estimated 20 mm distal to the carotid bifurcation to block the opening of the middle cerebral artery and the adjacent ophthalmic artery (Fig. 2-1) and remained in place for 90 minutes, followed by reperfusion. Upon removal of the filament, the wound was sutured, and each rat was transferred to a heating blanket to recover from anesthesia.

Neurological assessment

Neurological deficit scores (herein referred to as neuroscore) were determined as described (Xia et al., 2006). Briefly, scores ranging from 0 (no neurological deficit) to 8 (stroke-related death) were used to assess neurological deficits at 24 and 72 hours post-occlusion. Neuroscores are presented here as an average of both scores. Animals with a neuroscore of less than 5 were classified as “moderate” (n = 11), while animals with a neuroscore of 5 or more were classified as “severe” (n = 5). Moderate animals were euthanized at 3 (n = 6) and 9 (n = 5) days post-MCAO, while all severe animals were

euthanized at 3 days due to IACUC-required endpoints. Animals with a score of 5 “circle or walk spontaneously only to the left” (Xia et al., 2006) and were more likely to be euthanized due to IACUC endpoint requirements.

For rats euthanized at 3 days, brain slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA), which differentiates between metabolically active and stroke tissue. Brains were removed and placed in ice-cold saline. Using a rat brain matrix (Harvard Apparatus, Holliston, MA, USA) and beginning 1 mm posterior to the anterior pole, brains were sliced into 7 serial coronal sections (2 mm thick). Slices were stained with 2% TTC in saline for 15 min at 37°C in the dark and then fixed in 10% buffered formalin. Metabolically active tissue reduces TTC to form a red product, while stroke tissue remains white because its metabolic enzymes are compromised. Stained sections were scanned using a high-resolution scanner (Epson Perfection 2400 Photo).

Electroretinogram (ERG)

ERGs were used to measure the retinal response to light stimuli. Rats were dark-adapted overnight and then anesthetized (ketamine 80 mg/kg and xylazine 16 mg/kg). The corneal surface was anesthetized with proparacaine (1%), and the pupils dilated with tropicamide (1%) and phenylephrine hydrochloride (2.5%). Retinal responses were recorded by placing a DTL fiber in contact with the corneal surface of each eye through a layer of methylcellulose. Platinum needle reference and ground electrodes were placed in the cheek below the eye and in the tail, respectively. Using a commercial amplifier and acquisition system (UTAS-E3000, LKC, Gaithersburg, MD, USA), dark-adapted ERG

responses were recorded to a series of Ganzfeld strobe flashes (with intensities increasing from 0.00039 to 137 cd s/m^2). Interstimulus time increased from 10 to 60 s as light intensity increased, and 3 to 10 responses were averaged per flash stimulus. Amplitudes and implicit times were measured for a-waves (baseline to trough, representative of photoreceptor cell function (Penn and Hagins, 1969, Hood and Birch, 1990), b-waves (trough to peak, representative of bipolar cell function (Robson and Frishman, 1998), and oscillatory potentials (wavelets which likely represent amacrine and ganglion cell function), (Wachtmeister, 1998). Oscillatory potential amplitudes and implicit times are presented as the sum of all 6 oscillatory potentials for each flash stimulus. Reported amplitude averages are for the brightest flash stimulus.

Retinal morphology and immunohistochemistry

Rats were euthanized and their eyes removed. Eyes were fixed in 10% buffered formalin, processed through a series of graded ethanols, and embedded in paraffin. Sections (5 μm) were cut on a rotary microtome. Every 6^{th} slide was stained with 0.1% cresyl violet. Images were captured and inspected for pyknotic nuclei, and counts of total cells (number of cells in the retinal ganglion cell and inner nuclear layers and number of rows of photoreceptor nuclei in the outer nuclear layer) were made in retinal sections containing optic nerve. Three sections were counted and averaged per eye, and 6 fields were counted per section (3 on either side of the optic nerve, spaced approximately 100 microns apart).

To label GFAP and glutamine synthetase (GS) in Müller cells, sections were blocked for 30 minutes in 0.1 M Tris-buffered saline (TBS; pH 7.4) containing 3%

normal serum, then incubated in primary antibodies diluted in the blocking serum overnight at 4°C. Primary antibodies included rabbit anti-GFAP (1:500; Millipore; Billarica, MA, USA; AB5804) and mouse anti-GS (1:1000; Millipore; MAB302). For double labeling, primary and secondary antibodies from different species were used simultaneously. Sections were rinsed 3 times with 0.1 M TBS following primary antibody incubation, then incubated for 1 hour at room temperature with the corresponding fluorophore-conjugated secondary antibody solution (goat anti-rabbit; 1:500; Alexa Fluor 546; A11071 and goat anti-mouse; 1:500; Alexa Fluor 488; A11001; Invitrogen, Grand Island, NY, USA). Sections were then rinsed with 0.1 M TBS, mounted with a DAPI mounting medium, and coverslipped. One image per retina, superior to the optic nerve was acquired. GS images were analyzed using the histogram tool of commercial imaging software (Image Pro©, Media Cybernetics; Rockville, MD, USA) to quantify GS labeling intensity. Measurements were normalized to the brightness of the background for each micrograph.

Fluorescent terminal deoxynucleotidyl transferase-mediated 2’deoxyuridine 5'-triphosphate-biotin nick end labeling (TUNEL) staining was used to assess apoptosis in the retina. Briefly, retinal sections were incubated with TUNEL using a DeadEnd Fluorometric TUNEL kit (Promega; Madison, WI, USA) and counter-stained with a mounting medium containing propidium iodide. A fluorescent microscope (Olympus BX41, Olympus America Inc.) was used to acquire all images and staining intensity was quantified using Photoshop.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). ERG, GS expression, and cell count results were analyzed using a two-way repeated measures analysis of variance (ANOVA) followed by Tukey's test for individual comparisons. All correlations were analyzed using Pearson's correlation.

RESULTS

Retinal function deficits in MCAO animals are transient and correlate with behavioral deficits

Neuroscores ranging from 1 to 7 (mean = 3.9, SEM = 0.4) were observed in MCAO rats. To demonstrate how the behavioral scores compare to neurological damage, Figure 2-2 shows representative brain slices stained with TTC from MCAO rats assigned a score of 3 (Fig. 2-2A) or 6 (Fig. 2-2B). Behavioral deficits measured with the neuroscore were positively correlated with infarct size ($R^2 = 0.805$, $p < 0.005$) (Fig. 2-2C).). Occlusions were designated as severe or moderate based on neuroscore, with animals with a neuroscore of less than 5 being classified as "moderate" and animals with a neuroscore of 5 or greater being classified as "severe". Additionally, % occlusion as measured by LDF was, on average, 15% higher in the severe group versus the moderate group.

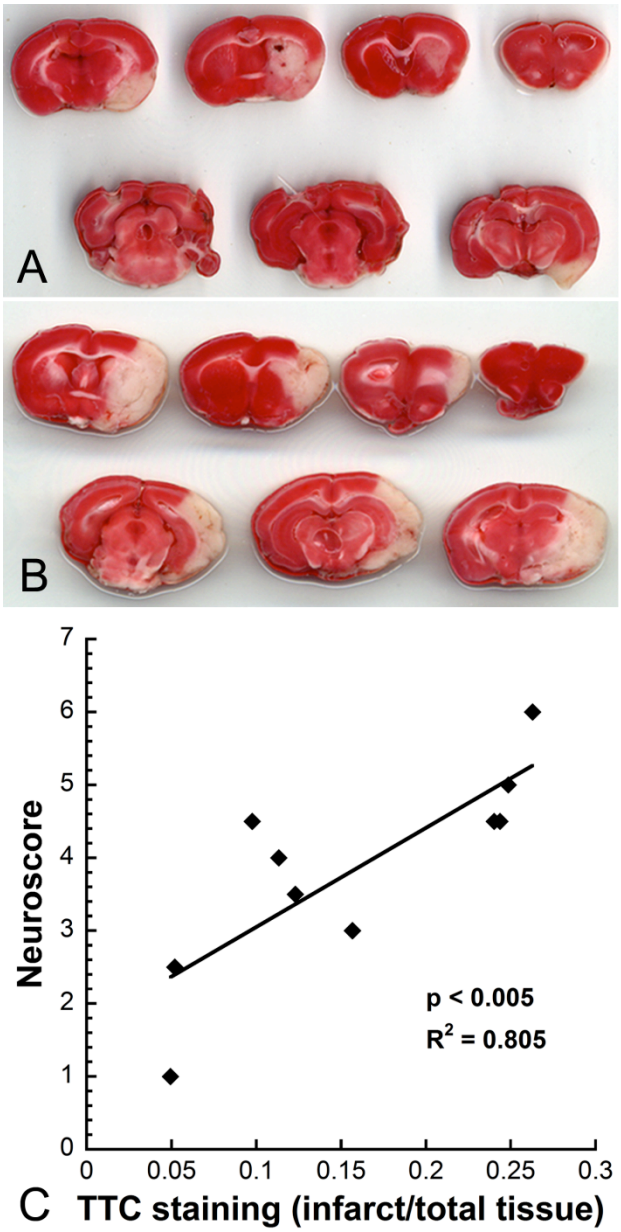


Figure 2-2. Correlation of infarct size with behavioral responses. Representative sequential coronal slices of MCAO brains treated with TTC in an animal that received a neuroscore of 3 (A) or 6 (B). The infarct (white tissue) is larger in animals with higher neuroscores. C) Neuroscores showed a significant positive correlation with infarct size ($R^2 = 0.805$, $p < 0.005$).

We observed different retinal function responses in moderate versus severe stroke animals. Figure 2-3 shows representative ERG and oscillatory potential waveforms from MCAO and control rats 2 days after surgery. Moderate animals showed delays in latency in MCAO eyes, while severe animals showed decreases in amplitude in both MCAO and contralateral eyes.

ERG quantification 2 days after MCAO surgery revealed that rats with severe MCAO showed a trend for reductions (38%) in dark-adapted a-wave amplitudes in MCAO eyes (317 ± 87 μV) and contralateral eyes (388 ± 113 μV), while both moderate MCAO (462 ± 46 μV) and contralateral eyes (476 ± 41 μV) showed similar levels to controls (510 ± 43 μV) [Repeated measures ANOVA, $F(4, 114) = 2.131$, $p < 0.01$] (Fig. 2-4A). Amplitude changes at 2 days after surgery were accompanied by significant delays in a-wave implicit time in MCAO eyes from the moderate group and a trend for delay in MCAO eyes from the severe group compared with all other groups [Repeated measures ANOVA main effect, $F(4, 111) = 5.650$, $p < 0.001$](Fig. 2-4B).

Both MCAO (584 ± 106 μV) and contralateral eyes (644 ± 144 μV) from severe rats had significant reductions in dark-adapted b-wave amplitudes (49% and 43%, respectively) compared with control (1146 ± 84 μV) and moderate contralateral eyes (1106 ± 99 μV) [Repeated measures ANOVA interaction effect, $F(4, 156) = 5.117$, $p < 0.002$] (Fig. 2-4C). For moderate animals, a trend for a decrease (21%) in amplitude was observed in MCAO eyes (911 ± 123 μV) but not contralateral eyes (Fig. 2-4C). For b-wave implicit times, we observed a trend for delay in contralateral and MCAO eyes from the moderate group (Fig. 2-4D).

For dark-adapted summed oscillatory potentials, severe MCAO eyes (502 ± 101 μV) showed significant reductions (43%) in amplitude compared with control eyes (885 ± 70 μV) and moderate contralateral eyes (953 ± 104 μV) while severe contralateral and moderate MCAO eyes showed a trend for a reduction in amplitude [Repeated measures ANOVA main effect, $F(4, 156) = 4.166$, $p < 0.007$](Fig. 2-4E). For summed oscillatory potential implicit times, we observed a significant delay in moderate MCAO eyes versus control and severe contralateral eyes [Repeated measures ANOVA main effect, $F(4,153) = 7.422$, $p < 0.001$](Fig. 2-4F). Delays in light-adapted b-wave implicit times were observed in moderate MCAO eyes as well (data not shown).

By 9 days after surgery, we observed a complete recovery of function in the moderate group (data not shown). There were no significant differences in any ERG parameters at this time point.

A significant correlation was observed between the neuroscore and retinal function measured with the ERG b-wave amplitude to the brightest flash intensity (137 cd s/m^2; $R^2 = -0.478$, $p < 0.05$)(Fig. 2-5), with more reduced b-wave amplitudes occurring in animals with higher (worse) neuroscores.

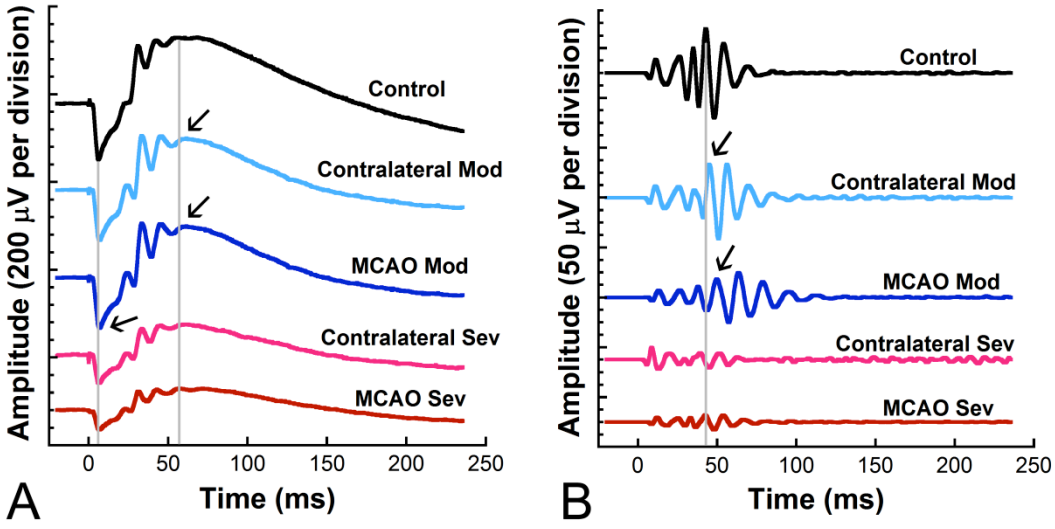


Figure 2-3. Representative waveforms from moderate and severe MCAO eyes, contralateral eyes, and naïve controls at 2 days post-MCAO. For both ERG and oscillatory potential waveforms, severe animals showed decreases in amplitude in both MCAO and contralateral eyes, while moderate animals showed delays in latency in MCAO eyes. A) ERG waveforms in response to 137 cd s/m^2 flash stimuli. The two gray lines mark the control a- wave and b- wave, respectively. Arrows indicate delayed responses. B) Oscillatory potentials (OPs) from filtered ERGs in response to 137 cd s/m^2 flash stimuli. The gray line marks OP4 for the control trace. Arrows indicate delayed responses.

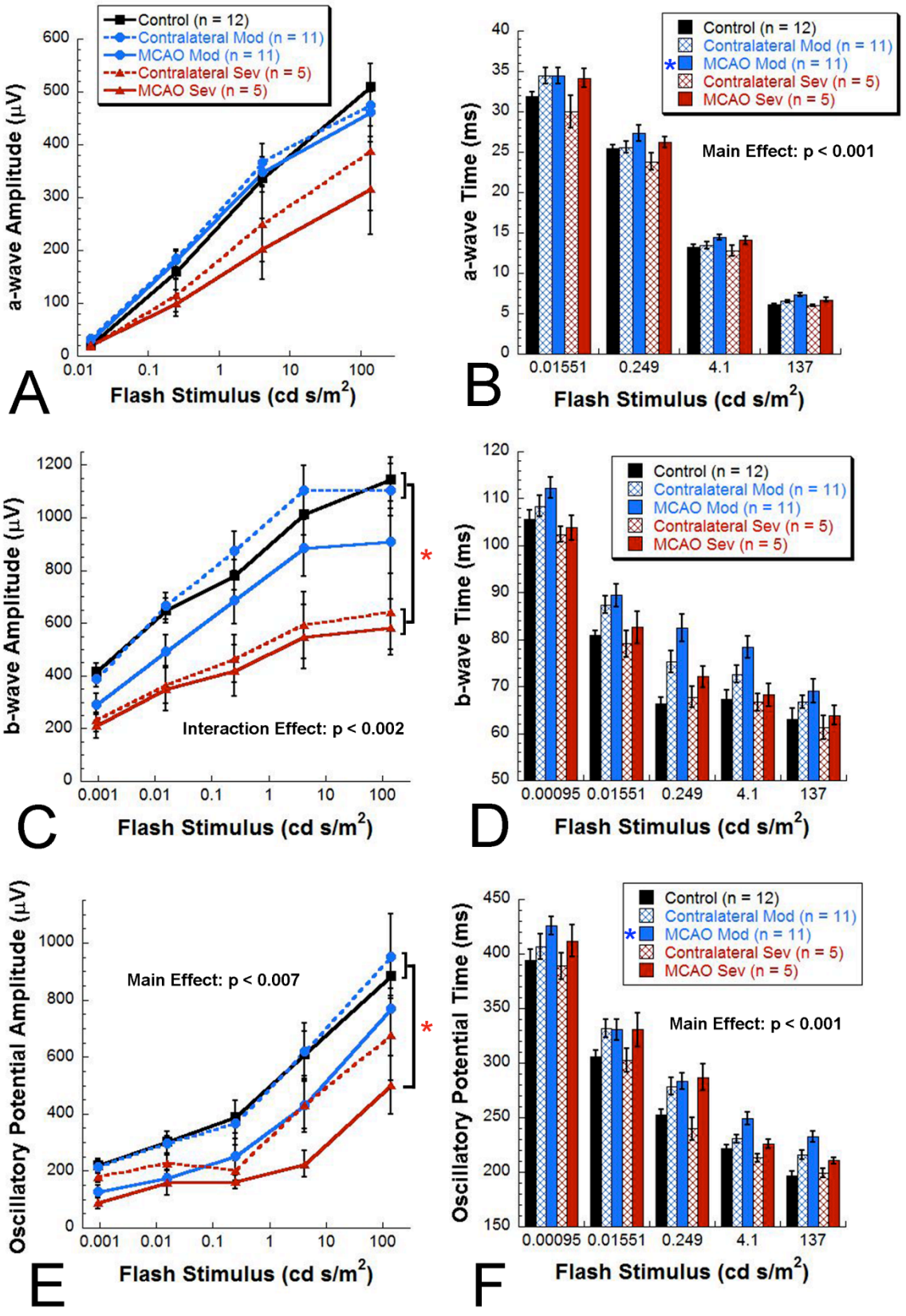

Control (n = 12)
Contralateral Mod (n = 11)
MCAO Mod (n = 11)
Contralateral Sev (n = 5)
MCAO Sev (n = 5)
a-wave Amplitude (μV)
Flash Stimulus (cd s/m²)
A
a-wave Time (ms)
Main Effect: p < 0.001
B
b-wave Amplitude (μV)
Interaction Effect: p < 0.002
C
b-wave Time (ms)
D
Oscillatory Potential Amplitude (μV)
Main Effect: p < 0.007
E
Oscillatory Potential Time (ms)
Main Effect: p < 0.001
F

Figure 2-4. Mean ERG results at 2 days post-MCAO. A) A trend for reduction in dark-adapted a-wave amplitudes was observed in severe MCAO and contralateral eyes [Repeated measures ANOVA, $F(4, 114) = 2.131$, $p < 0.01$]. B) Significant delays were observed in a-wave implicit time in moderate MCAO eyes with a trend for delay in severe MCAO eyes [Repeated measures ANOVA, $F(4, 111) = 5.650$, $p < 0.001$]. C) A significant reduction in dark-adapted b-wave amplitudes was observed for severe MCAO and contralateral eyes while a trend for reduction in amplitude was observed in moderate MCAO eyes [Repeated measures ANOVA, $F(4, 156) = 5.117$, $p < 0.002$]. D) For b-wave implicit times, a trend for delay was observed in contralateral and MCAO eyes from the moderate group. E) A significant reduction in dark-adapted oscillatory potential amplitudes was observed in severe MCAO eyes with a trend for a decrease in severe contralateral and moderate MCAO eyes [Repeated measures ANOVA, $F(4, 156) = 4.166$, $p < 0.007$]. F) For oscillatory potential implicit times, we observed a significant delay in moderate MCAO eyes versus control and severe contralateral eyes [Repeated measures ANOVA, $F(4,153) = 7.422$, $p < 0.001$].

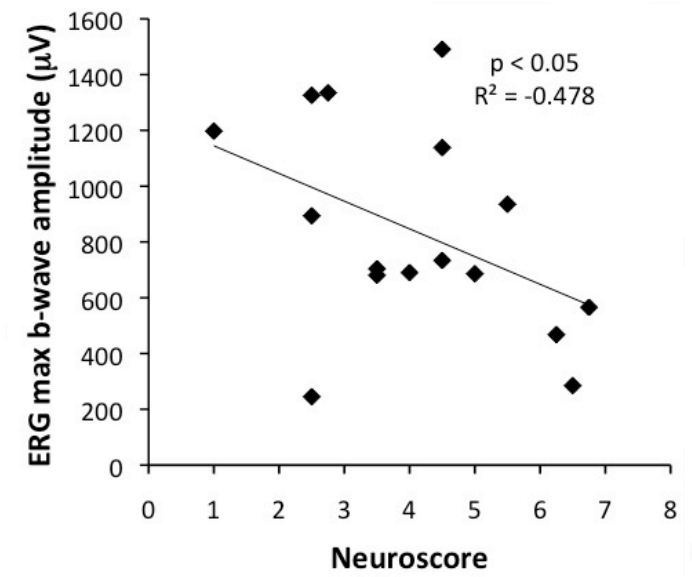


Figure 2-5. Correlation between ERG and neurological deficit scores. ERG b-wave responses at the brightest flash stimulus (137 cd s/m^2) showed a significant correlation with neurological deficit scores (R^2 = -0.478, $p < 0.05$).

Selective apoptosis in retinal ganglion cells from severe MCAO animals

As shown in Figure 2-6, cresyl violet staining revealed no differences in morphology, no pyknotic cells, and no differences in cell numbers between groups in the outer nuclear layer (Fig. 2-6A) or the inner nuclear layer (Fig. 2-6B). However, there was a significant reduction in retinal ganglion cells (Fig. 2-6C) in severe MCAO eyes versus moderate contralateral and control eyes [Repeated measures ANOVA main effect, $F(4, 180) = 2.940$, $p < 0.05$]. Like control eyes, MCAO and contralateral eyes from both moderate and severe MCAO groups all contained a few scattered TUNEL positive cells with no significant differences between groups (data not shown).

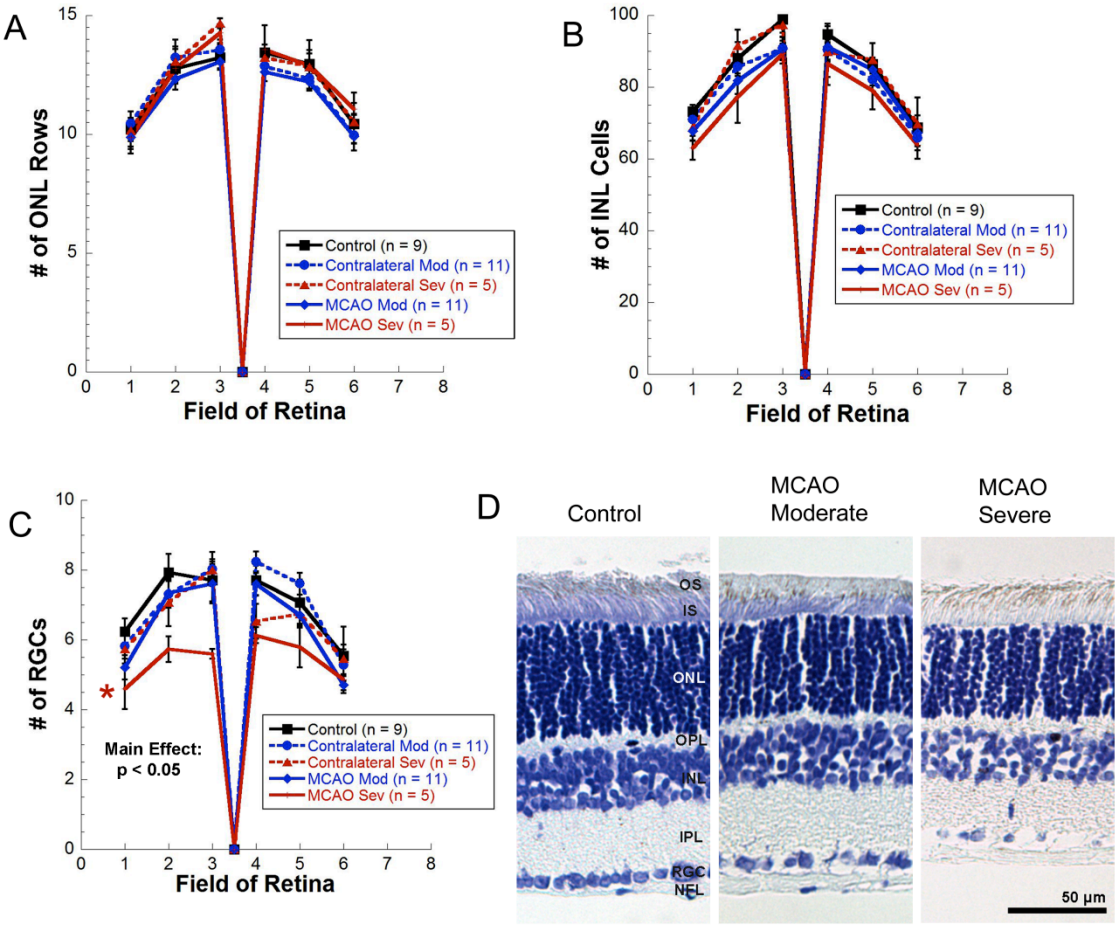


Figure 2-6. Cell counts and representative micrographs from sections stained with cresyl violet. A-C) No significant differences were observed in number of rows of photoreceptor nuclei in the outer nuclear layer (A) or in cell counts in the inner nuclear layer (B). C) A significant reduction in retinal ganglion cells was observed in severe MCAO retinas versus moderate contralateral and controls [Repeated measures ANOVA, $F(4, 180) = 2.940$, $p < 0.05$]; ON, optic nerve. D) Representative micrographs of cresyl violet staining in a control retina, a moderate MCAO retina, and a severe MCAO.

Upregulation of GFAP and Glutamine Synthetase (GS) in MCAO retinas

GFAP and GS were upregulated in the retina following MCAO (Fig. 2-7). GFAP labeling was observed in Müller cells in severe and some moderate MCAO retinas 3 days after surgery. In 9-day MCAO and contralateral eyes and intact controls, GFAP staining was observed only in the nerve fiber layer, where GFAP-expressing astrocytes typically present (Fig. 2-7).

A significant increase in GS intensity was observed between MCAO eyes and contralateral eyes in the moderate 3-day (n = 6) and severe groups (n = 5) but not the moderate 9-day group (n = 5) [Repeated measures ANOVA interaction effect, $F(2, 13) = 8.006$, $p < 0.005$] (Fig. 2-8A). GS intensity was significantly increased in severe MCAO eyes compared with 9-day moderate MCAO eyes and in severe contralateral eyes compared with 3-day moderate contralateral eyes. Additionally, there was a significant inverse correlation between GS staining intensity and ERG max b- wave ($R^2 = -0.627$, $p < 0.05$) (Fig. 2-8B).

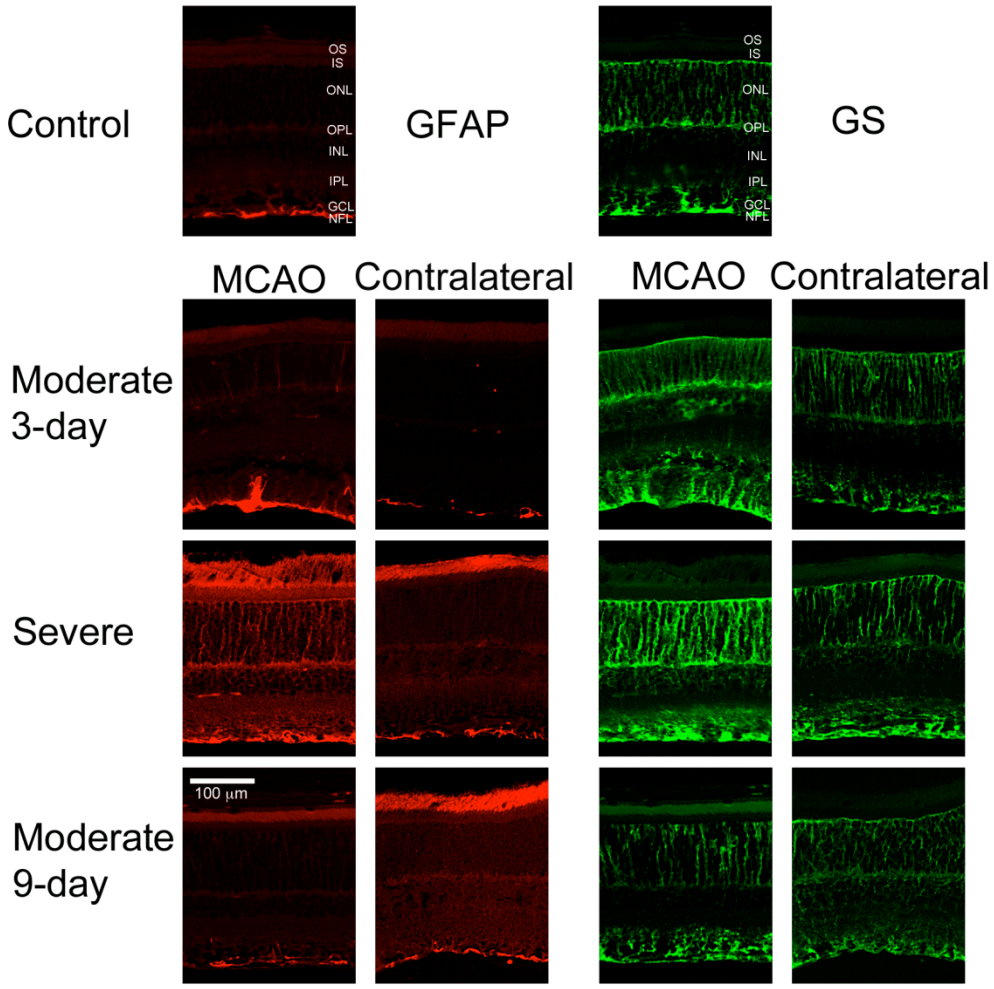


Figure 2-7. Representative micrographs from sections immunostained for GS and GFAP. GFAP and GS were upregulated in the retina following MCAO. GFAP labeling was observed in Müller cells in severe and some moderate MCAO retinas at 3 days. In all other groups, GFAP staining was observed only in the nerve fiber layer (NFL), where GFAP-expressing astrocytes typically present. Increased GS labeling was observed in severe MCAO and severe contralateral eyes and in moderate MCAO eyes at 3 days. Moderate contralateral at 3 days and moderate MCAO and contralateral at 9 days showed levels similar to controls. GCL, Ganglion Cell Layer; IPL, Inner Plexiform Layer; INL, Inner Nuclear Layer; OPL, Outer Plexiform Layer; ONL, Outer Nuclear Layer; IS, Inner Segments; OS, Outer Segments.

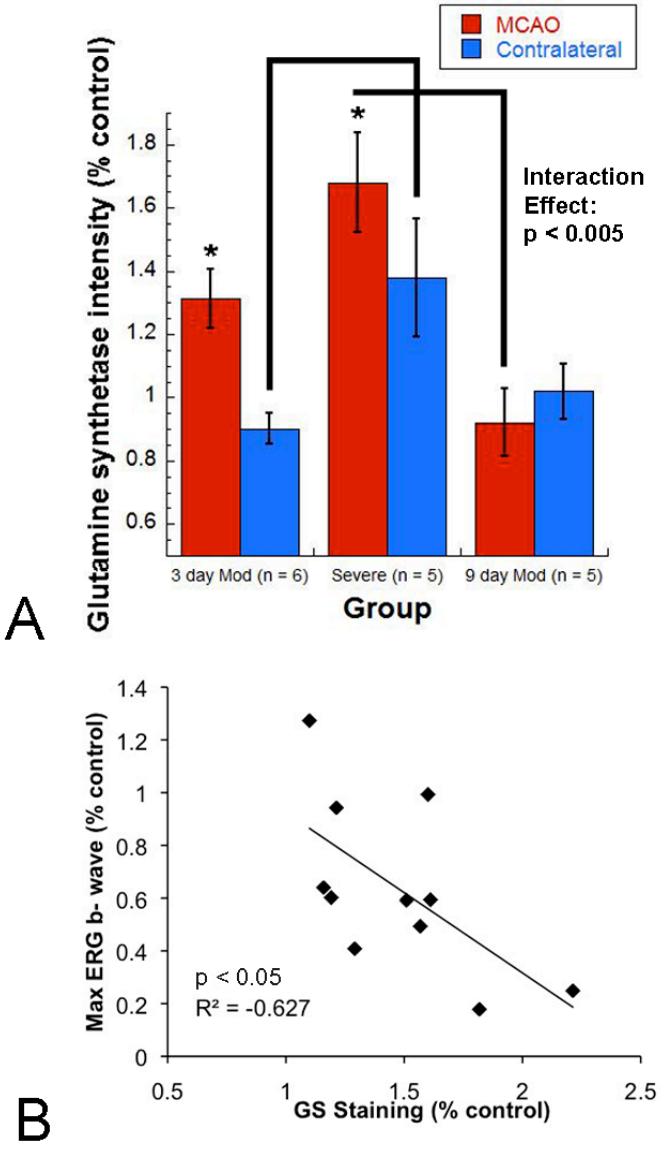


Figure 2-8. Mean GS immunolabeling intensity as percent of naïve control for severe and moderate MCAO eyes vs. contralateral eyes. A) A significant increase in GS intensity was observed between MCAO eyes and contralateral eyes within the severe group and moderate 3-day group but not the moderate 9-day group. Comparisons between severity and time points revealed that GS intensity was significantly increased in severe MCAO eyes compared with 9-day moderate MCAO eyes and in severe contralateral eyes compared with 3-day moderate contralateral eyes [Repeated measures ANOVA interaction effect, $F(2, 13) = 8.006$, $p < 0.005$]. B) A significant inverse correlation was observed between GS staining intensity and ERG b- wave at the bright flash stimulus (137 cd s/m^2; $R^2 = -0.627$, $p < 0.05$).

DISCUSSION

To determine whether neurons in the retina and brain respond similarly to MCAO, and whether MCAO resulted in sustained visual deficits, we investigated retinal function and structure at different times after MCAO with varying severity of stroke and the correlation with brain injury and behavioral deficits.

Retinal dysfunction without cell death with moderate MCAO

We found retinal deficits are dependent upon the severity of the occlusion at 2 days – such that moderate injuries resulted in ERG delays, while severe occlusions caused ERG amplitude reductions. Our observations extended the findings of others (Block et al., 1997, Kaja et al., 2003, Cheung et al., 2007, Kalesnykas et al., 2008, Steele et al., 2008, Li et al., 2009) who found that MCAO surgery resulted in retinal dysfunction, such as decreased ERG amplitude (Block et al., 1997). The Block et al. (1997) study showed recovery of photoreceptor cell function as measured by the a-wave by 2 days after MCAO, while we observed the additional recovery of the b-wave (bipolar cell function) and oscillatory potentials (amacrine and ganglion cell function) by 9 days (i.e., a full ERG recovery) in moderate animals.

While large amounts of pyknotic cells, cell loss, and TUNEL positive cells have been observed in both the retinal ganglion cell and inner nuclear layers in the mouse retina at 1 day following MCAO (Cheung et al., 2007, Steele et al., 2008, Li et al., 2009), rat retinas consistently showed no cell death, no major morphological changes with Nissl staining (Block et al., 1997, Kalesnykas et al., 2008), and very few, if any, TUNEL positive cells in the inner nuclear and photoreceptor layers (Kaja et al., 2003, Kalesnykas

et al., 2008), even in studies showing retinal function deficits (Block et al., 1997). Similarly, in our study the functional and morphological differences following MCAO were not accompanied by retinal cell death in the inner and outer nuclear layers, which are responsible for generating the b- wave and a- wave respectively. We did, however, observe cell loss in the retinal ganglion cell layer in severe MCAO retinas. In addition to differences in retinal cell loss, mice differ from rats in cerebral response after MCAO, requiring greatly reduced occlusion times (30 minutes vs. 90-120 minutes in rats) to induce a comparable cerebral infarct (Carmichael, 2005). These results suggest important species differences in response to ischemia in both the retina and the brain, with mice showing more susceptibility to ischemia in both tissues. Such differences could inform interpretations of studies designed to evaluate the outcome of neuroprotective agents given in the acute stage of an ischemic brain injury.

Transient retinal function deficits may be caused by sub-lethal increases in extracellular glutamate

By investigating retinal function in MCAO rats across time, we were able to show that retinal function deficits following MCAO are transient and recover by 9 days in animals with moderate stroke injury. Since cell death was not detected in the photoreceptor or inner nuclear layers, it appears that these neurons may undergo a transient dysfunctional state. We were unable to examine retinal morphology of the severe group at 9 days, but given that no cell death was detected at 3 days in the photoreceptor or inner nuclear layers, which contain neurons that generate the ERG, it is

likely that the severe group would have experienced an eventual recovery of function as well.

Although retinal cell death did not occur in the inner and outer nuclear layer following MCAO, we observed increases in GFAP at 3 days post-MCAO, which is a general sign of retinal stress and pathology (Bringmann et al., 2009). Increases in GFAP have been observed previously in both rat retinas at 2 and 5 days post-MCAO and mouse retinas at 1 day post-MCAO (Block et al., 1997, Cheung et al., 2007, Kalesnykas et al., 2008). Additionally, we observed increases in GS, a more specific response that occurs following retinal injuries that involve changes in levels of extracellular glutamate (Bringmann et al., 2009). Glutamate is the neurotransmitter used by photoreceptors, bipolar cells, and retinal ganglion cells (Massey and Redburn, 1987), and increased levels of extracellular glutamate could affect the function and/or signal conduction of these cells (Kalloniatis, 1995). When retinal neurons fire, they release glutamate into the synapse. This extracellular glutamate is taken up by nearby Müller cells via the Müller-specific glutamate transporter, GLAST (GLutamate ASpartate Transporter). Then GS, a substrate-regulated enzyme present in Müller cells, converts glutamate to glutamine (Pow and Barnett, 1999).

Ischemia has been shown to induce increases in extracellular glutamate in both the brain (Hillered et al., 1989) and the retina (Perlman et al., 1996) that if uncontrolled can result in excitotoxicity (Beal, 1992, Kalloniatis, 1995). MCAO may cause increased levels of extracellular glutamate in the retina, and the Müller cells respond by upregulating GS, as shown in other retinal injury models that involve increased extracellular glutamate (Bringmann et al., 2009). We hypothesize that even with these

increased levels of GS, enough glutamate remains in the extracellular space to impair signal conduction, resulting in decreased retinal function, but not cell death.

The effects of sub-lethal levels of glutamate on ERG function have been documented previously (Barnett and Pow, 2000). When GLAST is inhibited, extracellular glutamate is increased due to the lack of Müller cell uptake. The result is decreased ERG responses yet no cell death. We postulate that a similar increase in glutamate occurs at 2 days post-MCAO as evidenced by the increased GS. By 9 days post-MCAO, GS levels returned to normal, suggesting that GS may clear all of the excess glutamate and allow for recovery of retinal function.

Similarly, increases in GS have been observed from 3 hours to 3 days after cerebral ischemia (Petito et al., 1992, Tanaka et al., 1992, Hoshi et al., 2006, Verma et al., 2010), and these increases appear to be transient, returning to baseline levels by 5 days (Tanaka et al., 1992). Additionally, treatments that enhance the activity of glutamate transporter (Verma et al., 2010) and GS (Zhang et al., 2011) have been shown to reduce brain injury in ischemia-reperfusion models, providing further support for a protective role of GS in reducing levels of extracellular glutamate after ischemic injury.

We also observed increased GS and decreased retinal function in contralateral eyes as well as MCAO eyes. This result could be an example of diaschisis, that is, the undamaged neural tissue is losing function due to damage to distant, but connected, neural tissue (Stein et al., 1983, Feeney and Baron, 1986). Additionally, the decreased function and increased retinal glutamate in contralateral eyes could be due to indirect effects of the cerebral infarct, such as increased systemic levels of glutamate, which have

been shown to be 3-fold higher after permanent MCAO, a model that causes a less severe stroke than our transient MCAO model (Puig et al., 2000). Furthermore, in the permanent occlusion model, which involves permanent occlusion at a point distal to the internal carotid such that the middle cerebral artery alone is blocked and the ophthalmic artery is unaffected, changes in the retina (i.e., a slight upregulation of HIF-1a) were still detected (Kalesnykas et al., 2008). The fact that retinal changes were observed independent of ophthalmic artery occlusion, coupled with the fact that we observed retinal function deficits in both the occluded and contralateral eye in MCAO rats, suggests that the retinal injury caused by MCAO is at least partially systemic in nature.

Our finding of increased GS and decreased retinal function in the contralateral eye has interesting clinical implications because while it is unlikely that a clot would block both the middle cerebral and ophthalmic arteries in humans, it is possible that increased systemic levels of glutamate are the cause of the transient vision loss that often accompanies stroke in people (Mead et al., 2002).

The retina is less susceptible to MCAO although early retinal function deficits correlate with deficits in brain function

Utilizing a range of stroke severity allowed us to investigate the correlation between retinal function and brain function. We observed a significant correlation between retinal function responses at 2 days and neuroscores (Fig. 2-2), which clearly established similar ischemic effects after MCAO at this early time point. However, while retinal function recovers by 9 days, we know that neurological deficits after MCAO are more lasting (Modo et al., 2000). Further, we showed that cell death occurred in brains

from both moderate and severe animals but only in the retinal ganglion cell layer in retinas from severe animals. Therefore, it appears that the retina (particularly the inner and outer nuclear layers) shows less susceptibility than the brain to the ischemic injury caused by MCAO. It is also possible that the brain and the retina receive different levels of occlusion. One limitation of this study is that we only took laser-Doppler readings from the MCA but not the ophthalmic artery. However, Steele et al. (2008) showed that almost no retinal perfusion occurs during MCAO in the mouse. Future work should compare perfusion to the brain and retina during MCAO in rats.

The retina has been shown to have more or less susceptibility to injury than the brain depending on the type of insult. The endothelial cells of the retina have been shown to be more susceptible than brain-derived endothelial cells to oxidative stress and increased vascular permeability, as measured by glutathione peroxidase activity, superoxide production and superoxide dismutase levels, and junctional protein levels in bovine endothelial cell cultures (Grammas and Riden, 2003). The retina is also more sensitive than either the brain or liver to dietary changes in lipid levels, with the retina but not the brain showing an incorporation of trans DHA (Docosahexaenoic acid) and a reduction in cis DHA as well as ERG defects following a diet high in trans fatty acids (Acar et al., 2006). In the event of ischemia, however, the retina is less susceptible than the brain, showing a much greater tolerance time to ischemic injury. In the brain, permanent damage is caused by 3-7 minutes of global ischemia in both animal models and humans (Weinberger L, 1940, Kabat H, 1941, Brock, 1956, Meyer, 1956), while full retinal recovery is found in monkeys with occlusion of the central retinal artery for 97-98 minutes (Hayreh and Weingeist, 1980) and rodents receiving 8 minutes of increased

intraocular pressure as ischemic preconditioning (Stowell et al., 2010). This resilience could be because the retina and the vitreous have stores of glucose and glycogen while the brain relies on the cerebral vasculature or because reflow through occluded vasculature happens more readily in the retina than the brain (Hayreh and Weingeist, 1980).

We observed a difference in susceptibility among the cell types within the retina as well. Retinal cell types have been shown to have differential susceptibilities previously, with retinal ganglion cells showing more cell death with aging than either photoreceptors or cells of the inner nuclear layer (Lei et al., 2011). It is interesting that retinal ganglion cells, like the cells of the brain, show death in the MCAO model given that the blood supply and blood retinal barrier for retinal ganglion cells is more similar to that of the brain, while the photoreceptors rely on the choroid for blood supply and the retinal pigmented epithelium for blood retina barrier (Cunha-Vaz, 1976).

Conclusions

The transient MCAO model provides a unique opportunity to investigate cerebral and retinal ischemia simultaneously due to the functional deficits that occur in both brain and retina. Moreover, it creates a useful model for the study of mechanisms and treatments of ocular ischemic syndrome in rats. Retinal function deficits occur at 2 days post-MCAO and correlate with behavioral deficits. Decreased b-wave amplitudes occurred in both MCAO and contralateral eyes in severe animals, suggesting the injury is at least partially systemic. With the addition of the 9-day post-injury evaluations, we were able to show that the retinal function deficit following MCAO is transient and

recovers by 9 days with moderate occlusion. This finding suggests that behavioral testing at this time is not confounded by visual deficits.

We have also provided data suggesting that ischemia-induced increases in extracellular glutamate may be the mechanism behind the temporary retinal function reduction after MCAO in rats and this represents a potential mechanism behind temporary retinal function deficits that occur following stroke in humans. Recovery of function may be explained by the transient increase in GS, which may act to overturn sub-lethal levels of extracellular glutamate.

Chapter 3:

Progesterone treatment in two rat models of ocular ischemia

This chapter presents work to be published within:

Rachael S. Allen, Timothy W. Olsen, Iqbal Sayeed, Heather A. Cale, Katherine C. Morrison, Y Oumarbaeva, I Lucaciu, Jeffrey H. Boatright, Machelle T. Pardue, Donald G. Stein. Progesterone treatment in two rat models of ocular ischemia.

ABSTRACT

Purpose: To determine whether the neurosteroid progesterone, which has been shown to have protective effects in animal models of traumatic brain injury, stroke, and spinal cord injury, is also protective in animal models that cause ocular ischemia.

Methods: Progesterone treatment was tested in two models of ocular ischemia in rats: the rodent anterior ischemic optic neuropathy (rAION) model, which induces monocular optic nerve stroke, and the middle cerebral artery occlusion (MCAO) model, which causes ischemia in both the retina and brain due to a filament that blocks the ophthalmic and middle cerebral arteries. Visual function and retinal histology were assessed to determine whether progesterone attenuated ischemic injury in the eye in both models. Additionally, behavioral testing and TTC staining in brains were used to compare progesterone's effectiveness in retina versus brain in the MCAO model.

Results: Progesterone treatment showed no effect on ischemia-induced VEP reduction and retinal ganglion cell loss in the rAION model. In the MCAO model, progesterone treatment reduced ERG deficits, MCAO-induced upregulation of GS and GFAP, and retinal ganglion cell loss. As expected, progesterone treatment also showed significant protective effects in behavioral tests and a reduction in infarct size in the brain.

Conclusions: Progesterone treatment showed protective effects in the retina following MCAO but not rAION injury. Additionally, progesterone had greater protective effects in brain compared with retina in the MCAO model, which could suggest different mechanisms of action by progesterone in different neural tissue.

INTRODUCTION

Clinically, ocular ischemia occurs both as a component of cerebral and systemic disease and as a separate event. Visual impairments often present as a first symptom in cerebral stroke or arthrosclerosis of the carotid or ophthalmic arteries (Benavente et al., 2001, Mead et al., 2002). These visual impairments occur due to temporary retinal ischemia and commonly take the form of transient vision loss in one or both eyes, a phenomenon known as amaurosis fugax. Transient vision loss is often a sign of impending stroke or systemic cardiovascular disease (Slepyan et al., 1975), and neurological imaging shows that an acute brain infarct is present in 1 in 4 patients with transient retinal ischemia (Helenius et al., 2012). Ocular ischemia also occurs independently of cerebral damage. Anterior ischemic optic neuropathy involves retinal ganglion cell death caused by optic nerve stroke (Hayreh, 1974) and is the leading cause of sudden vision loss related to optic nerve dysfunction in older adults (Miller, 1982). No effective treatment currently exists (Fazzone et al., 2003).

During ischemic stroke, reductions in arterial blood flow prevent an adequate supply of oxygen and nutrients to neural tissue, leading to injury and cell death (Kalogeris et al., 2012). The overlap between mechanisms involved in cerebral stroke and in retinal and optic nerve strokes is substantial. Both injuries involve excitotoxicity due to increases in extracellular glutamate and calcium influx (Hillered et al., 1989, Kalloniatis, 1995, Perlman et al., 1996). Other common mechanisms include altered aquaporin expression (Iandiev et al., 2006, Zador et al., 2009), oxidative stress (Yang et al., 1996, Liu et al., 2012b), increases in pro-apoptotic and decreases in anti-apoptotic markers (Schmidt-Kastner et al., 2000, Zhang et al., 2005b), and increases in inflammation,

including increased activation of glial cells and macrophages (Zhang et al., 2005a, Gronberg et al., 2013), increased levels of inflammatory cytokines (IL-6, TNF-α, etc.) (Hangai et al., 1996, Hill et al., 1999), and NF-κB pathway activation (Schneider et al., 1999, Dvoriantchikova et al., 2009). The high degree of overlap in mechanisms involved in cerebral ischemia and retinal and optic nerve ischemia could allow for treatments to be effective in both tissues.

The neurosteroid progesterone has been shown to provide protection against cell death and behavioral impairment in several animal models, including stroke, traumatic brain injury, and spinal cord injury (Cutler et al., 2007, Schumacher et al., 2007, Sayeed and Stein, 2009, Ishrat et al., 2010, Stein and Wright, 2010). Progesterone was also shown to be protective in traumatic brain injury in Phase II clinical trials (Wright et al., 2007, Xiao et al., 2008). In cerebral ischemia models, progesterone has been shown to reduce infarct volume and enhance functional recovery (Sayeed et al., 2007, Ishrat et al., 2009, Wang et al., 2010a, Yousuf et al., 2013b). Progesterone has been shown to act on many of the mechanisms involved in ischemic injury to the brain, retina, and optic nerve. Progesterone treatment following cerebral ischemia has been shown to reduce glial activation (Yousuf et al., 2013b) and to reduce both cerebral (Ishrat et al., 2010) and systemic inflammation (Yousuf et al., 2013a). Progesterone also modulates aquaporin expression (Wang et al., 2013), reduces oxidative stress (Ozacmak and Sayan, 2009, Li et al., 2012a) and apoptotic markers (Ishrat et al., 2012, Espinosa-Garcia et al., 2013), and reduces edema and blood brain barrier permeability after cerebral ischemia (Wang et al., 2013, Won et al., 2013). Finally, progesterone has been shown to reduce NMDA-mediated calcium influx after ischemia (Cai et al., 2008) and, through its metabolite,

allopregnanolone, to reduce toxic neurotransmitter release by modulating the $GABA_A$ receptor in adult animals (Ardeshiri et al., 2006, Knight et al., 2012). The substantial overlap between mechanisms involved in ocular ischemia and mechanisms modulated by progesterone suggest that progesterone treatment could prove protective in ocular as well as cerebral ischemia.

To determine whether progesterone is neuroprotective after ocular ischemia as it is after cerebral ischemia, we tested progesterone treatment in the rAION model, which causes thrombosis of the optic nerve and subsequent retinal ganglion cell death and vision loss (Bernstein et al., 2003), and the transient MCAO model, which has been shown to cause retinal damage and functional deficits in conjunction with cerebral ischemia (Chapter 2) (Block et al., 1997, Steele et al., 2008).

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (n = 105) from Charles River Laboratories were used in this study. At the time of surgery, rats were approximately 48 days of age for rAION surgery (210-250 grams) and 60 days of age (290-330 grams) for MCAO surgery. Littermates that did not receive surgery were used as controls for rAION. Littermates that received sham surgery (incisions in the scalp and neck + vehicle injections) were used as controls for MCAO. All animal procedures were approved by the Institutional Animal Care and Use Committee (Emory University protocols #20001517 and 279-2008) and performed in accordance with NIH guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision

Research. Rats were housed under a 12:12 reverse light:dark cycle with water and food ad libitum and handled daily for at least 5 days prior to surgery. For the rAION experiment, three animals were excluded prior to laser treatment due to poor Rose Bengal injection. For the MCAO experiment, two rats died prior to behavioral testing at 24 hours.

Progesterone preparation and dosing

An 8 mg/kg progesterone dose, previously shown to be most protective after stroke (Wali et al., 2014), was made using 2-hydroxypropyl-β-cyclodextrin (HBC; 25% w/v solution in H_2O; a non-toxic aqueous solution that dissolves progesterone) as the solvent. For rAION rats, progesterone and vehicle treatments were administered intraperitoneally at 1 hour post injury, and then subcutaneously at 6, 24, 48, 72, 96, and 120 hours post-surgery. Doses delivered at 96 and 120 hours were tapered (cut in half over a 2-day period), which has previously been shown to be beneficial (Cutler et al., 2005, Cutler et al., 2006). Rats were euthanized at 21 days post-surgery. For MCAO rats, progesterone and vehicle treatments were administered intraperitoneally at 1 hour post injury, and then subcutaneously at 6, 24, and 48 hours, with rats being euthanized at 3 days post-MCAO. In all experiments, the rats' group identity was coded with regard to surgery and treatment to prevent experimenter bias.

Methods used in rAION experiments

rAION surgery

Monocular rAION surgery was performed with minor modifications to the previous

description (Bernstein et al., 2003). Briefly, in anesthetized rats (isoflurane: 5% induction, 2% maintenance, 700mm N_2O, 300mm O_2), the corneal surface was anesthetized with proparacaine (1%), and the pupils dilated with tropicamide (1%) and phenylephrine hydrochloride (2.5%). Immediately following injection of the tail vein with 1 ml/kg animal weight of Rose Bengal (2.5 mM in PBS), a trained retinal surgeon (TWO) applied 12 seconds of laser energy directly to the optic nerve using 50 mW of 532 nm wavelength laser using the indirect ophthalmoscope (Novus Varia, Lumenis, Salt Lake City, UT, USA) and a 78 diopter condensing lens. Laser applications were consistent and repeatable. The spot size completely covered the optic nerve, and the presence of rose-bengal dye was reliably observed during the laser application.

Fundus Photographs

Fundus photographs are used to visualize posterior structures of the eye, including the optic nerve, retina, and retinal vasculature. Animals were anesthetized via 5% isoflurane inhalation (in a N_2/O_2 70%/30% mixture). The corneal surface was anesthetized with proparacaine (1%), and the pupils dilated with tropicamide (1%) and phenylephrine hydrochloride (2.5%). The camera objective lens was placed in contact with a layer of ultrasound gel on the corneal surface, and images were captured (RetCam II , Clarity Medical Systems, Pleasanton, CA, USA).

Visual Evoked Potential (VEP)

VEPs measure visual cortex activity in response to light stimuli, and are highly correlated with RGC and optic nerve morphology (Price et al., 1988). After rats were

anesthetized (ketamine 80 mg/kg and xylazine 16 mg/kg), the corneal surface was anesthetized with proparacaine (1%), and the pupils dilated with tropicamide (1%) and phenylephrine hydrochloride (2.5%). Cortical responses were recorded using a 1 cm platinum needle electrodes placed subcutaneously over the left and right visual cortex. Ground and reference electrodes were placed in the tail and forehead skin, respectively. Using a commercial amplifier and acquisition system (UTAS-E3000, LKC, Gaithersburg, MD, USA), light-adapted VEPs were recorded three days after rAION for one eye at a time, while the other eye was covered with a patch. Both ipsilateral and contralateral VEPs were recorded, with contralateral VEPs being quantified. 200 responses to Ganzfeld strobe flashes with an intensity of 137 cd s/m^2 were averaged. Amplitudes and implicit times were measured for P1 and N1 components of the VEP.

Immunohistochemistry for Brn3a

The retinal ganglion cell specific marker Brn3a was used to assess retinal ganglion cell death. Eyes were enucleated from euthanized rats and fixed with 10% buffered formalin. Corneas and lenses were removed and the resulting eye cups digested in hyaluronidase (1:500 in 1xPBS, Sigma, 1mg/mL) at room temperature for 30 minutes. Retinas were rinsed, dissected, and permeabilized in PBS/0.5% Triton (PBST) at -80°C for 15 minutes. Retinas were thawed, rinsed in PBS/0.5% Triton, and blocked in 2% donkey serum in PBS/0.25% Triton at room temperature for 30 minutes. Retinas were then incubated with Brn3a primary antibody (Goat polyclonal, 1:500 in PBST, Santa Cruz, sc-31984) over two nights at 4°C. Next, retinas were rinsed in PBST and incubated with Alexa Fluor 488 secondary antibody (donkey anti-goat, 1:500 in PBST, Invitrogen,

A11055) for 1 hour at room temperature. Retinas were rinsed in PBST, dissected into flat mounts, and mounted with aqueous mounting media. Retinal ganglion cells were imaged using a fluorescent microscope (Olympus BX41, Olympus America Inc.) and counted for the whole flat mount using the automatic cell counting tool of commercial imaging software (Image Pro©, Media Cybernetics; Rockville, MD, USA). Retinal ganglion cell counts were corrected for retinal area.

Methods used in MCAO experiment

MCAO surgery

MCAO surgery was performed with minor modifications to the previous description (Longa et al., 1989). Briefly, animals were anesthetized using isoflurance inhalation in a N_2/O_2 70%/30% mixture (5% isoflurane for intial anesthetization, 2% for continued sedation). Blood oxygen saturation (SpO_2) was measured and sustained at 90% using a pulse oximeter (SurgiVet, model V3304; Waukesha, WI, USA). A heating lamp was used to sustain body temperature between 36.5°C and 37.5°C, as monitored using a rectal probe.

A midline incision was made at the ventral surface of the neck, and a 6-0 silk suture was used to separate and ligate the right common carotid arteries. A microvascular clip was used to occlude the internal carotid and pterygopalatine arteries. Next, a 4-0 silicon-coated monofilament (0.35–0.40 mm long) (Doccol Co., Albuquerque, NM, USA) was inserted through the external carotid artery and into the internal carotid artery and pushed an estimated 20 mm distal to the carotid bifurcation to block the opening of the middle cerebral artery and the adjacent ophthalmic artery. The filament remained in place for

120 minutes, followed by reperfusion. Upon removal of the filament, the wound was sutured, and rats were transferred to a heating blanket to recover from anesthesia. Laser-Doppler flowmetry (LDF), an established and reliable system for monitoring cerebral blood flow changes due to induction of focal cerebral ischemia (Dirnagl et al., 1989), was used to monitor cerebral blood flow. Animals with mean ischemic cerebral blood flow greater than 35% of baseline LDF were excluded to reduce variability and ensure relative uniformity of the ischemic insult (Sayeed et al., 2006). The LDF probe (Moor Instruments, Wilmington, Delaware, USA) was placed over the ipsilateral parietal cortex from 5 minutes prior to occlusion to 5 minutes after reperfusion.

Electroretinogram (ERG)

ERGs were used to assess retinal response to light stimuli at 48 hours post-MCAO. Rats were anesthetized (ketamine 80 mg/kg and xylazine 16 mg/kg) after overnight dark adaptation. The corneal surface was anesthetized with proparacaine (1%), and the pupils dilated with tropicamide (1%) and phenylephrine hydrochloride (2.5%). A DTL fiber was placed in contact with the corneal surface of each eye through a layer of methylcellulose to record retinal responses. Platinum needle ground and reference electrodes were placed in the tail and in the cheek below the eye, respectively. Using a commercial amplifier and acquisition system (UTAS-E3000, LKC, Gaithersburg, MD, USA), dark-adapted ERG responses were recorded to a series of Ganzfeld strobe flashes (with intensities increasing from 0.00039 to 137 cd s/m^2). Interstimulus time increased from 10 to 60 s as light intensity increased, and 3 to 10 responses were averaged per flash stimulus. Amplitudes and implicit times were measured for a-waves, b-waves, and oscillatory potentials.

Neurological assessment

Behavioral tests were performed at 24 hours post-MCAO.

Grip strength

The Grip Strength task is a test of neuromuscular performance. Each rat was held near the grip strength meter (Columbus Instruments, Columbus, OH, USA) until it grasped the bar. It was then gently pulled by the tail until it released the bar. The meter displayed the greatest amount of force used during the time that the rat gripped the bar. Three trials were performed and averaged for each rat. Post-MCAO grip strength was calculated as a percentage of pre-MCAO grip strength.

Sticky-tape task

The sticky-tape task is a test of somatosensory function/sensorimotor deficits. A glue dot (Glue Dots International, Germantown, WI, USA) was affixed to the bottom of the rat's front left paw, and the rat was placed in a clear box for 180 seconds. The time to notice the dot and the time to remove the dot were recorded for each rat. One rat froze during the duration of the task, and was excluded based on Grubb's outlier test.

TTC staining

Rats were euthanized and their brains removed for staining with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) to differentiate between metabolically active and stroke tissue. Brains were placed in ice-cold saline upon removal. Beginning 1 mm posterior to the anterior pole and using a rat brain matrix (Harvard Apparatus), brains were sliced into 7 serial coronal sections (2 mm thick). Slices were stained using 2% TTC in saline for 15 minutes at 37°C in the dark and then

fixed using 10% buffered formalin. TTC is reduced by metabolically active tissue to form a red product, while stroke tissue remains white due to its compromised metabolic enzymes. Stained slices were scanned using a high-resolution scanner (Epson Perfection 2400 Photo). A subset of TTC tissue was analyzed due to defective TTC.

Retinal morphology and immunohistochemistry

Rats were euthanized and their eyes removed. Eyes were fixed in 10% buffered formalin, processed through a series of graded ethanols, and embedded in paraffin. Sections (5 μm) were cut on a rotary microtome. Sections containing optic nerve were stained with 0.1% cresyl violet. Images were captured, and retinal ganglion cells were counted in three sections and averaged per eye, with six fields being counted per section (three on either side of the optic nerve, spaced approximately 100 microns apart).

For labeling of GFAP and glutamine synthetase (GS) in Müller cells, sections were blocked in 0.1 M Tris-buffered saline (TBS; pH 7.4) containing 3% normal serum for 30 minutes, then incubated overnight at 4°C in primary antibodies diluted in the blocking serum. Primary antibodies used were rabbit anti-GFAP (1:500; Millipore; Billarica, MA, USA; AB5804) and mouse anti-GS (1:1000; Millipore; MAB302). Primary and secondary antibodies from different species were used simultaneously for double labeling. After primary antibody incubation, sections were rinsed 3 times with 0.1 M TBS. Sections were then incubated in the corresponding fluorophore-conjugated secondary antibody solution (goat anti-rabbit; 1:500; Alexa Fluor 546; A11071 and goat anti-mouse; 1:500; Alexa Fluor 488; A11001) for 1 hour at room temperature. Following secondary antibody incubation, sections were rinsed 3 times with 0.1 M TBS, mounted

with a DAPI mounting medium, and coverslipped. One image per retina was acquired superior to the optic nerve. A fluorescent microscope (Olympus BX41, Olympus America Inc.) was used to acquire all images and a histogram tool (Photoshop, Adobe, San Jose, CA, USA) was used to quantify GS staining intensity in a field of retina 300 pixels wide spanning the length of the retinal Müller cells, which was normalized to background brightness.

Statistical analysis

Results are expressed as mean ± SEM. ERG and MCAO retinal ganglion cell count results were analyzed using a two-way repeated measures ANOVA followed by Tukey's test for individual comparisons. AION retinal ganglion cell count, GS intensity, grip strength task, and sticky-tape task results were analyzed using a one-way ANOVA followed by Tukey's test for individual comparisons. VEP results were analyzed using a Kruskal-Wallis One Way ANOVA on ranks. TTC staining results were analyzed using an unpaired t-test.

RESULTS

Progesterone treatment was not protective in the rAION model

Fundus photographs from both progesterone- and vehicle-treated rats showed optic nerve edema, obliteration of microvasculature, and vascular dilation at 1-day post-rAION compared with controls (Fig. 3-1). At 3 days after rAION, both progesterone- and vehicle-treated rats showed significant deficits in VEP N1 amplitude with no change in implicit time [Kruskal-Wallis One Way ANOVA on Ranks, $p < 0.05$] (Fig. 3-2). ERGs performed in a subset of rAION rats confirmed normal bipolar and photoreceptor cell

function, as expected in an optic nerve stroke model that affects only retinal ganglion cells (data not shown). Progesterone- and vehicle-treated rats also showed a significant reduction in retinal ganglion cells compared with controls [ANOVA, $F(2, 38) = 15.621$, $p < 0.001$] (Fig. 3-3).

Progesterone treatment was tested in additional experiments with extended progesterone dosing (16 days of treatment vs. 5) and smaller rAION injury (6 seconds of laser treatment vs. 12). No neuroprotective effects were found in these additional experiments (data not shown).

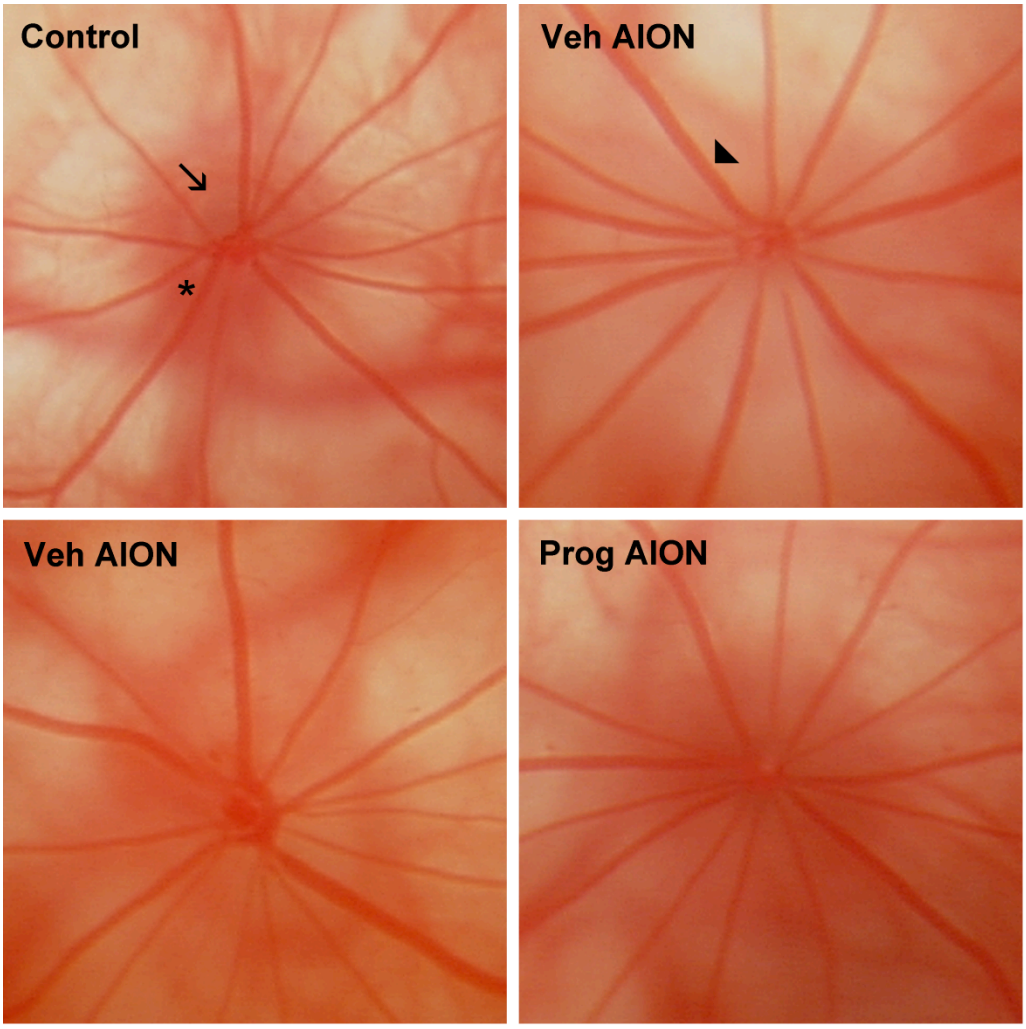


Figure 3-1. Fundus photographs from control, progesterone-treated AION, and vehicle-treated AION rats. In control retinas, distinct boundaries (↘) and a reddish flush indicative of intact microvasculature (*) were observed. Lack of distinct boundaries in AION retinas is a sign of optic nerve edema, and lack of a reddish flush suggests obliteration of microvasculature. Additionally, vascular dilation (◣) was observed in AION retinas. No differences were observed between vehicle- and progesterone-treated AION retinas.

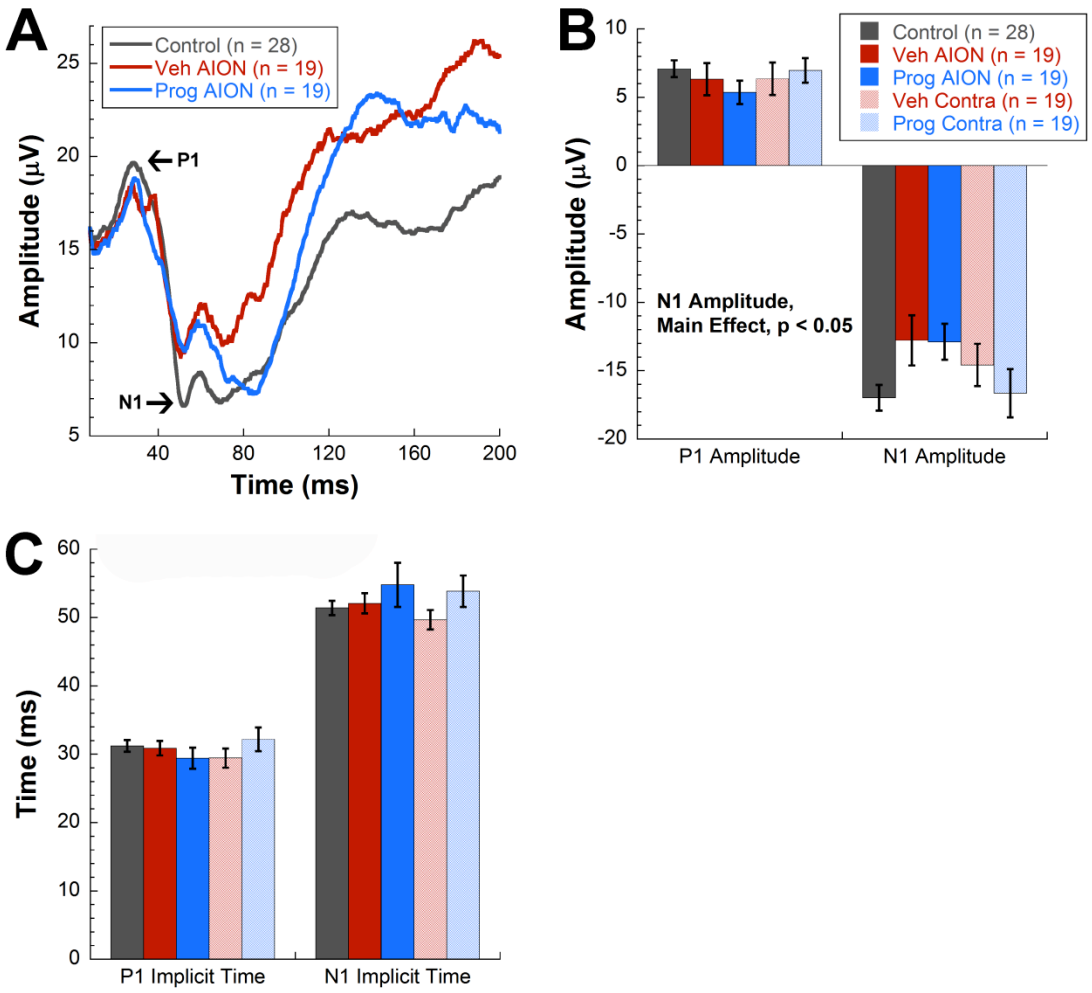


Figure 3-2. VEPs for control and progesterone- and vehicle-treated rAION rats at 3 days post-AION. A) Averaged VEP waveforms. Arrows designate P1 and N1 for Controls. B) Mean P1 and N1 amplitudes at 3 days post-AION. A significant main effect of group was observed for N1 amplitudes [Kruskal-Wallis One Way ANOVA on Ranks, $p < 0.05$], with both progesterone- and vehicle-treated rAION groups showing reduced N1 amplitudes. C) No differences were observed in mean P1 and N1 implicit times at 3 days post-AION.

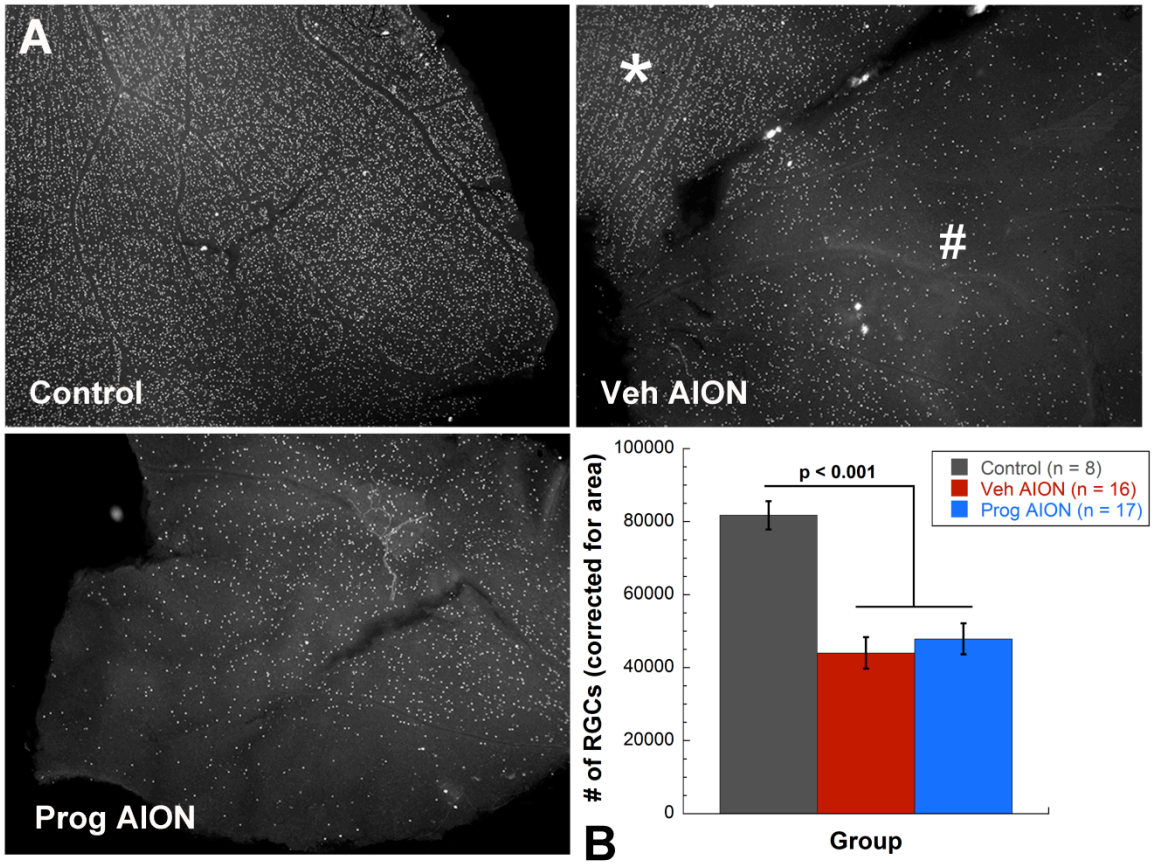


Figure 3-3. Immunohistochemistry with the retinal ganglion cell specific marker, Brn3a. A) Representative flat mount petals stained with Brn3a for retinas from control (top left), vehicle-treated AION (top right), and progesterone-treated AION rats (bottom left). Retinas from both progesterone- and vehicle-treated rats had petals showing extensive retinal ganglion cell loss (#) and petals that appeared "healthy" (*). B) Both progesterone- and vehicle-treated AION retinas showed a significant reduction in retinal ganglion cells as compared with control retinas ($p < 0.001$).

Progesterone treatment protected against retinal ischemia induced by transient MCAO

Progesterone reduced ERG deficits in MCAO rats

Figure 3-4 shows representative ERG waveforms from progesterone- and vehicle-treated MCAO rats and shams at two days post-MCAO. Vehicle-treated rats had reduced amplitudes in both MCAO and contralateral eyes, and progesterone treatment partially attenuated these deficits, particularly in contralateral eyes.

Quantification of ERG data revealed significant reductions in dark-adapted a-wave, b- wave, and oscillatory potential amplitudes in MCAO eyes from vehicle-treated rats compared with shams [a- wave: Repeated measures ANOVA, $F(4, 111) = 11.057$, $p < 0.001$; b- wave: Repeated measures ANOVA, $F(4, 148) = 16.744$, $p < 0.001$; oscillatory potentials: Repeated measures ANOVA, $F(4, 148) = 7.343$, $p < 0.001$] (Fig. 3-5). For a-waves and b- waves, significant reductions in amplitude were also observed in contralateral eyes from vehicle-treated MCAO rats compared with shams ($p < 0.05$).

MCAO eyes from progesterone-treated rats did not differ from vehicle-treated in a- wave or oscillatory potential amplitude, but did show a trend (23%) for b-wave amplitude recovery (Fig. 3-5C). Additionally, contralateral eyes from progesterone-treated MCAO rats showed significant recovery in b-wave amplitude (64%) at 0.249 and 4.1 cd s/m^2 flash stimuli ($p < 0.05$). Contralateral eyes from progesterone-treated MCAO rats showed a-wave amplitudes that were not significantly different from sham amplitudes (47% recovery).

For ERG implicit times, a significant main effect of treatment was found for dark-adapted a- wave, b- wave, and oscillatory potentials [a- wave: Repeated measures

ANOVA, $F(4, 107) = 2.758$, $p < 0.05$; b- wave: Repeated measures ANOVA, $F(4, 147) = 2.990$, $p < 0.05$; oscillatory potentials: Repeated measures ANOVA, $F(4, 144) = 3.414$, $p < 0.05$] (Fig. 3-5). Post-hoc analysis only revealed significant delays in b- wave and oscillatory potential implicit times for MCAO eyes from vehicle-treated rats vs. shams ($p < 0.05$).

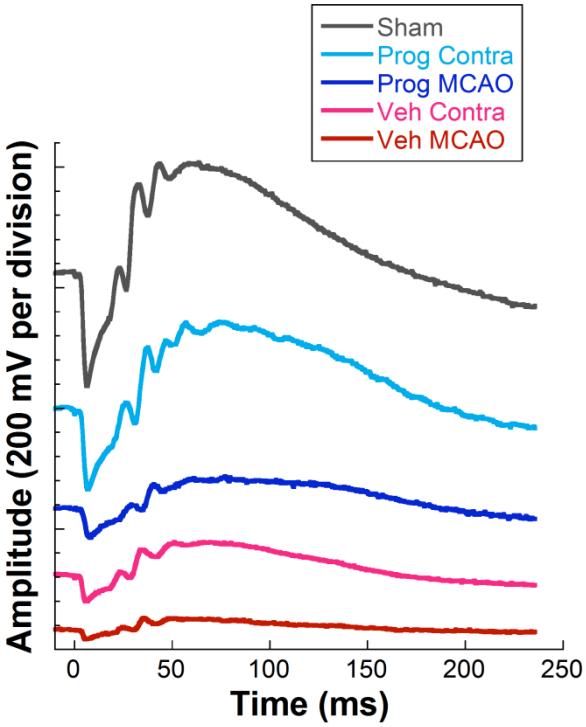
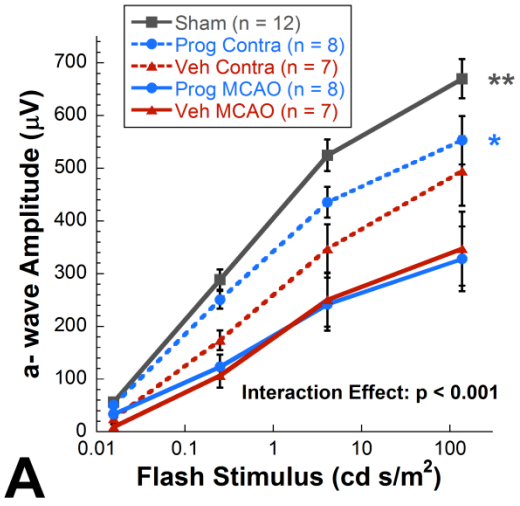
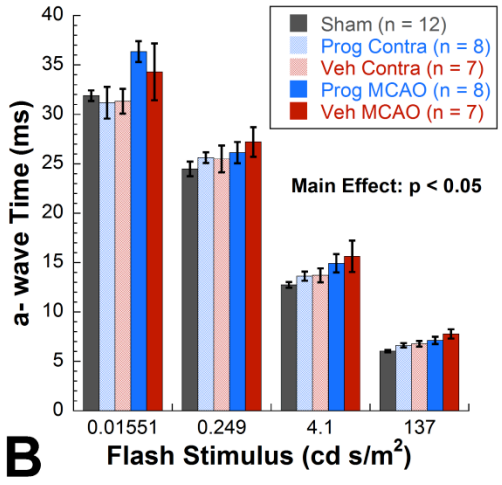
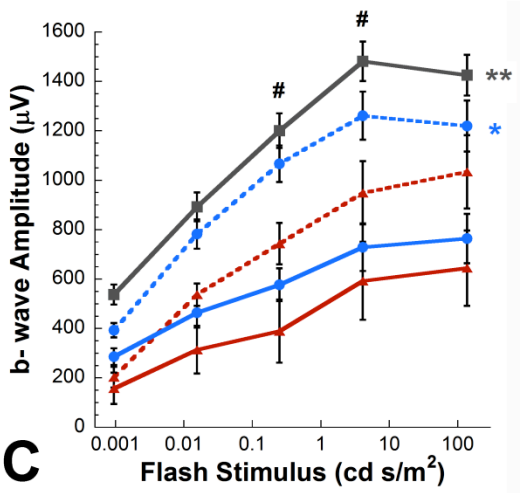
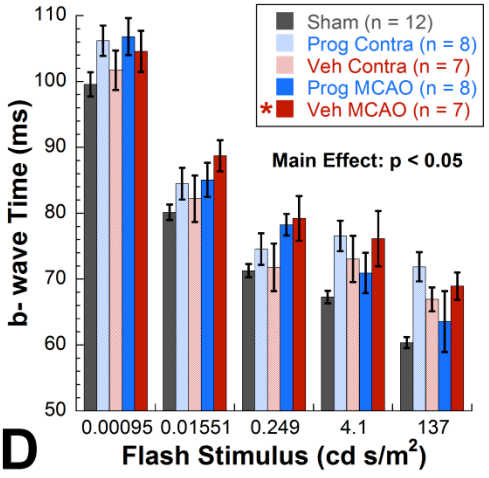


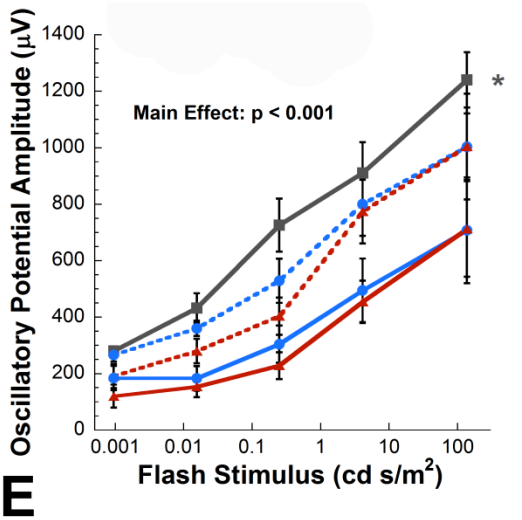
Figure 3-4. Representative ERG waveforms from sham animals and progesterone- and vehicle-treated MCAO rats. Vehicle-treated rats show reduced amplitudes in both MCAO and contralateral eyes at 2 days post-MCAO in response to 137 cd s/m^2 flash stimuli. Progesterone treatment reduces these deficits, particularly in contralateral eyes.


Sham (n = 12)
Prog Contra (n = 8)
Veh Contra (n = 7)
Prog MCAO (n = 8)
Veh MCAO (n = 7)
a- wave Amplitude (μV)
Interaction Effect: p < 0.001
Flash Stimulus (cd s/m²)
A


Sham (n = 12)
Prog Contra (n = 8)
Veh Contra (n = 7)
Prog MCAO (n = 8)
Veh MCAO (n = 7)
a- wave Time (ms)
Main Effect: p < 0.05
Flash Stimulus (cd s/m²)
B


b- wave Amplitude (μV)
Flash Stimulus (cd s/m²)
C


Sham (n = 12)
Prog Contra (n = 8)
Veh Contra (n = 7)
Prog MCAO (n = 8)
Veh MCAO (n = 7)
b- wave Time (ms)
Main Effect: p < 0.05
Flash Stimulus (cd s/m²)
D


Oscillatory Potential Amplitude (μV)
Main Effect: p < 0.001
Flash Stimulus (cd s/m²)
E

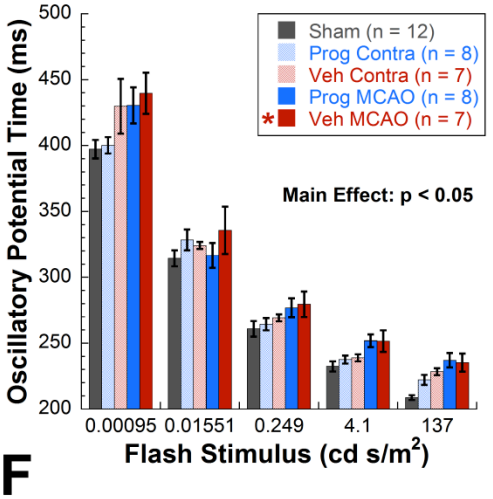

Sham (n = 12)
Prog Contra (n = 8)
Veh Contra (n = 7)
Prog MCAO (n = 8)
Veh MCAO (n = 7)
Oscillatory Potential Time (ms)
Main Effect: p < 0.05
Flash Stimulus (cd s/m²)
F

Figure 3-5. Quantification of ERG a- wave, b- wave, and oscillatory potential amplitudes and implicit times. Significant reductions in dark-adapted a- wave (A), b- wave (C), and oscillatory potential amplitudes (E) were observed in MCAO eyes from vehicle-treated rats compared with shams. Significant reductions in a- wave and b- wave amplitude were also observed in contralateral eyes from vehicle-treated MCAO rats compared with shams ($p < 0.05$). Progesterone-treated MCAO rats showed significant increases over vehicle-treated MCAO rats in b-wave amplitude at 0.249 and 4.1 cd s/m^2 flash stimuli in contralateral retinas ($p < 0.05$) and a trend for an increase in MCAO retinas.
* = significantly different from Veh MCAO and Prog MCAO. ** = significantly different from Veh MCAO, Prog MCAO, and Veh Contra. # = significant difference between Prog Contra and Veh Contra at this flash intensity.

A significant main effect of group was observed for dark-adapted a- wave implicit times (B). Significant delays in ERG implicit times were observed for dark-adapted b- waves (D) and oscillatory potentials (F) for MCAO eyes from vehicle-treated rats compared with shams. * = $p < 0.05$.

Progesterone reduced upregulation of GFAP and GS in retinas from MCAO rats

Three days after MCAO, GFAP was upregulated in Müller cells in MCAO (and some contralateral) retinas from vehicle-treated rats. Little to no GFAP upregulation was observed in MCAO retinas from progesterone-treated rats. In sham retinas and contralateral retinas from progesterone-treated MCAO rats, GFAP staining was apparent only in the astrocytes of the nerve fiber layer (Fig. 3-6).

Three days after MCAO, significant increases in GS intensity were observed in Müller cells in MCAO retinas from vehicle-treated rats compared with shams [ANOVA, $F(4, 37) = 4.729$, $p < 0.01$]. A trend for an increase was observed in contralateral retinas from vehicle-treated MCAO rats (Fig. 3-6 & 3-7). In progesterone-treated rats, both MCAO and contralateral retinas showed significantly reduced levels of GS intensity compared with vehicle-treated rats ($p < 0.05$). Additionally, MCAO and contralateral retinas from progesterone-treated rats did not differ significantly from shams (Fig. 3-6 & 3-7).

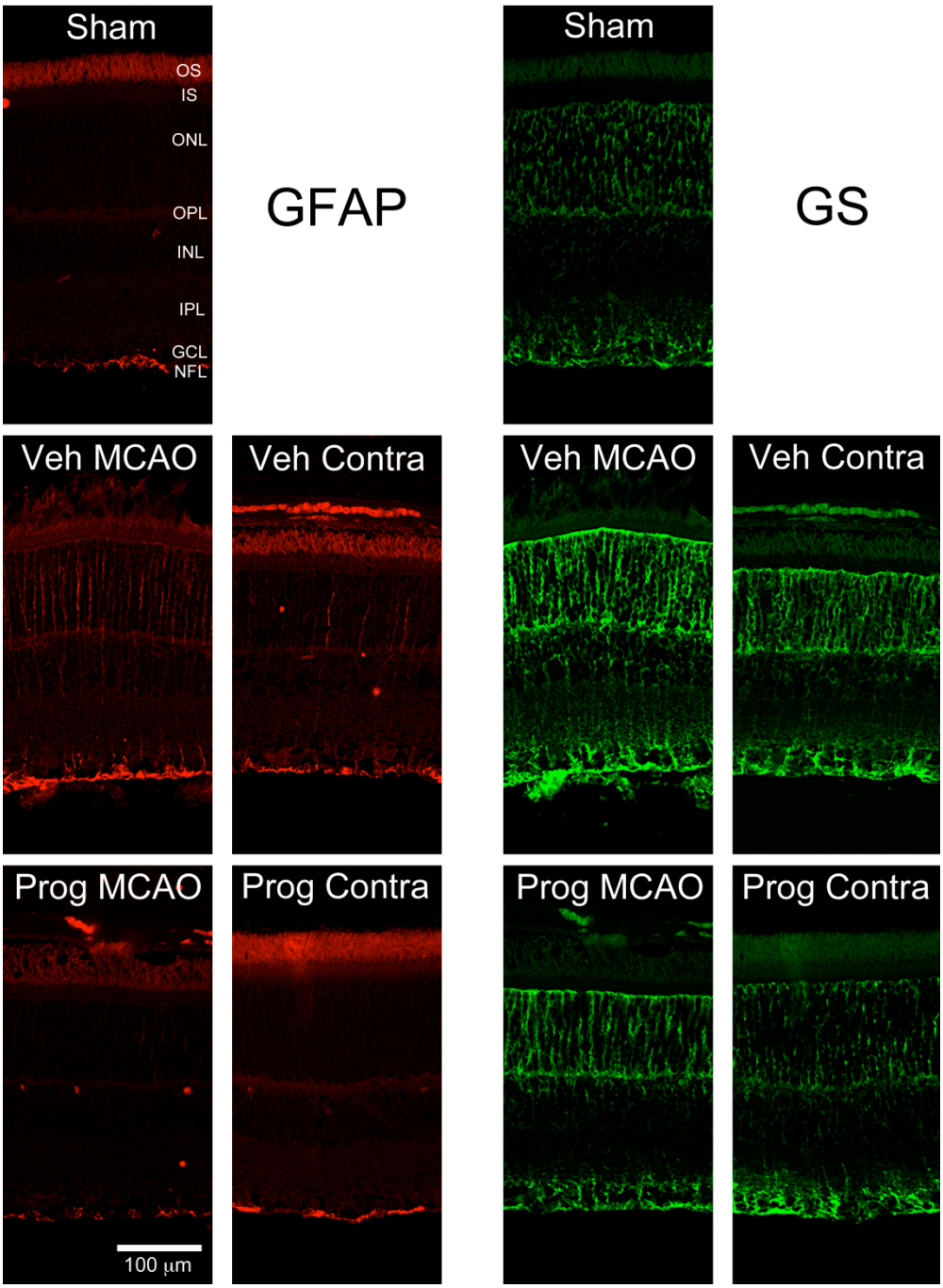


Figure 3-6. Representative photographs from immunohistochemistry with GFAP and GS after MCAO. Three days after MCAO, GFAP was upregulated in Müller cells in MCAO (and some contralateral) retinas from vehicle-treated rats, but not progesterone-treated rats, compared with shams. Glutamine synthetase was upregulated in Müller cells in MCAO and contralateral retinas from vehicle-treated rats, with slight upregulation in MCAO and contralateral retinas from progesterone-treated rats, compared with shams. GCL, Ganglion Cell Layer; IPL, Inner Plexiform Layer; INL, Inner Nuclear Layer; OPL, Outer Plexiform Layer; ONL, Outer Nuclear Layer; IS, Inner Segments; OS, Outer Segments.

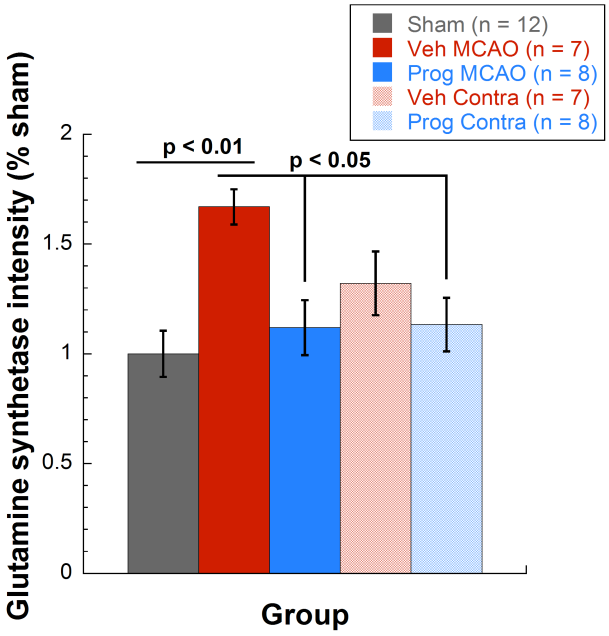


Figure 3-7. Mean GS intensity as percent of sham for vehicle- and progesterone-treated MCAO rats. Significant increases in GS intensity were observed in MCAO retinas from vehicle-treated rats compared with shams ($p < 0.01$), with a trend for an increase in contralateral retinas. Both MCAO and contralateral retinas from progesterone-treated rats showed significantly reduced levels of GS intensity compared with vehicle-treated rats ($p < 0.05$), but did not differ significantly from shams.

Progesterone reduced retinal ganglion cell death in retinas from MCAO rats

Three days after MCAO, a significant reduction in retinal ganglion cells was observed in MCAO retinas from vehicle-treated rats compared with shams [Repeated measures ANOVA, $F(4, 185) = 44.058$, $p < 0.001$; Fig. 3-8]. In progesterone-treated rats, MCAO retinas showed greater numbers of retinal ganglion cells than vehicle-treated rats ($p < 0.001$) but fewer retinal ganglion cells than shams ($p < 0.001$). No differences were observed between sham and contralateral retinas from either progesterone or vehicle-treated MCAO rats (Fig. 3-8).

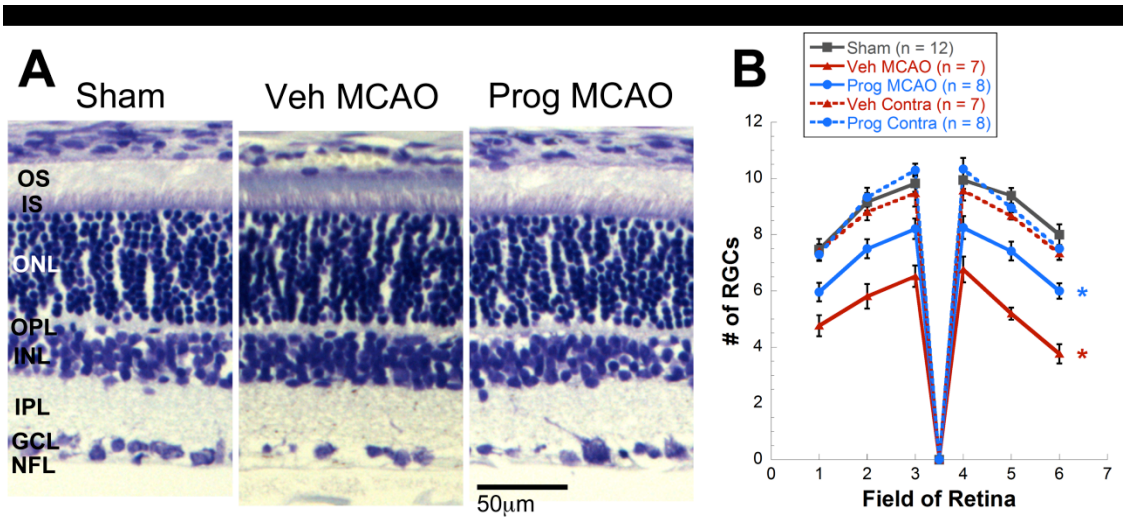


Figure 3-8. Representative photographs of cresyl violet staining and quantification of retinal ganglion cells. A) Cresyl violet staining in retinal sections from sham, vehicle-treated MCAO, and progesterone-treated MCAO rats. B) At three days post-MCAO, ipsilateral retinas from vehicle-treated MCAO rats showed significantly reduced retinal ganglion cell numbers compared to all other groups ($p < 0.001$). Ipsilateral retinas from progesterone-treated MCAO rats showed significant increases in retinal ganglion cell counts over ipsilateral retinas from vehicle-treated MCAO rats ($p < 0.001$), and significant decreases compared with all other groups ($p < 0.001$). GCL, Ganglion Cell Layer; IPL, Inner Plexiform Layer; INL, Inner Nuclear Layer; OPL, Outer Plexiform Layer; ONL, Outer Nuclear Layer; IS, Inner Segments; OS, Outer Segments.

Progesterone treatment protected against cerebral ischemia induced by transient MCAO

Progesterone reduced behavioral deficits in MCAO rats

One day after MCAO, vehicle-treated MCAO rats exhibited reduced grip strength and increased time to notice on sticky-tape task compared with shams [grip strength task: ANOVA, $F(2, 17) = 12.472$, $p < 0.001$; sticky-tape task: ANOVA, $F(2, 23) = 11.312$, $p < 0.001$]. Progesterone-treated MCAO rats showed increased grip strength ($p < 0.05$) and reduced time to notice the tab the on sticky-tape task ($p < 0.01$) over vehicle-treated MCAO rats. A significant difference between progesterone-treated MCAO rats and shams was not observed on either task (Fig. 3-9).

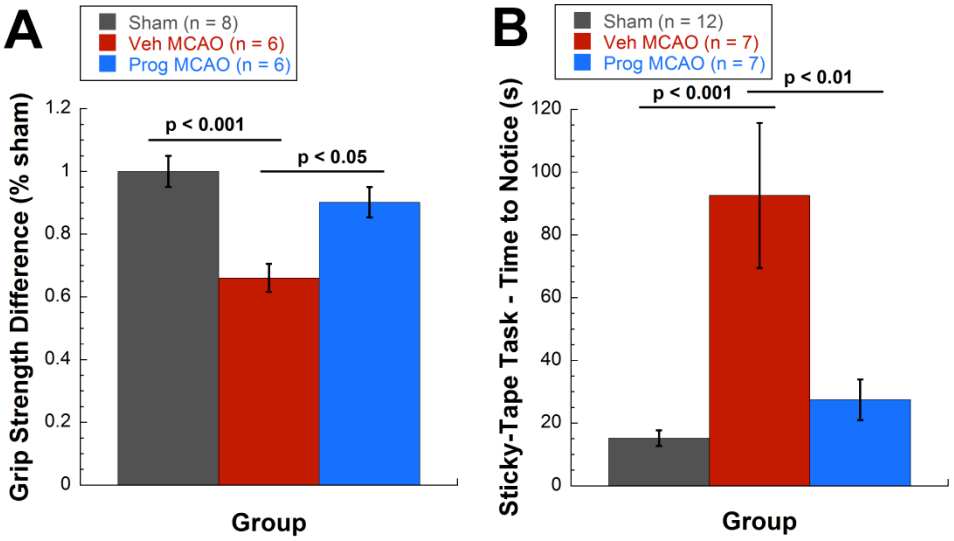


Figure 3-9. Behavioral assessment at 1-day post-MCAO. A) Vehicle-treated MCAO rats showed reduced grip strength compared with shams ($p < 0.001$). Progesterone-treated MCAO rats showed significant increases in grip strength over vehicle-treated MCAO rats ($p < 0.05$). B) Vehicle-treated MCAO rats showed increased time to notice on sticky-tape task compared with shams ($p < 0.001$). Progesterone-treated MCAO rats showed reduced time to notice compared with vehicle-treated MCAO rats ($p < 0.01$).

Progesterone reduced infarct size in MCAO rats

Three days after MCAO, infarcted tissue in the ipsilateral hemisphere was observed in vehicle-treated MCAO rats using TTC staining. Progesterone-treated MCAO rats showed reduced infarct size compared with vehicle-treated MCAO rats (45%, unpaired *t*-test, $p = 0.054$) (Fig. 3-10).

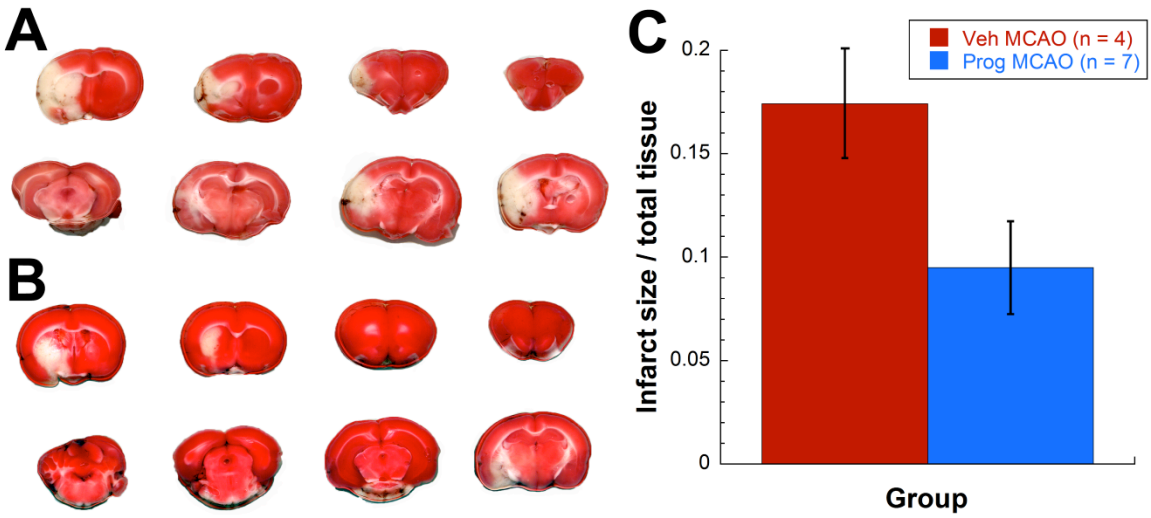


Figure 3-10. Representative TTC staining and quantification at three days post-MCAO. Representative TTC-stained brain slices for A) vehicle-treated and B) progesterone-treated MCAO rats with ~70% occlusion. C) Reduced infarct size was observed in progesterone- vs. vehicle-treated MCAO rats (45%, $p = 0.054$).

DISCUSSION

Progesterone treatment showed protection in MCAO but not rAION

Progesterone administration resulted in reduced ERG deficits, reduced GFAP and GS upregulation, and reduced retinal ganglion cell death following MCAO-induced retinal ischemia, but did not show a protective effect in the rAION model as assessed with fundus photographs, visual evoked potentials, and retinal ganglion cells counts. One possibility is that progesterone is acting systemically, but not locally in the retina. The fact that we see a significant increase in contralateral ERG amplitude with progesterone treatment, but only a trend for an increase in ipsilateral ERG amplitude supports this idea. However, given the similarities between the blood brain barrier and blood retina barrier (Cunha-Vaz et al., 1966, B., 2007), it is likely that progesterone delivered systemically crosses the blood retina barrier as it does the blood brain barrier (Sar and Stumpf, 1973). Previous studies have identified progesterone (Lanthier and Patwardhan, 1986, 1987) and progesterone receptors (Li et al., 1997, Wickham et al., 2000, Swiatek-De Lange et al., 2007, Koulen et al., 2008) in the retina, and progesterone synthesis has been shown to occur in the eye (Lanthier and Patwardhan, 1988, Guarneri et al., 1994, Sakamoto et al., 2001a, Coca-Prados et al., 2003, Cascio et al., 2007). Further support for progesterone's ability to act in the eye is demonstrated by studies showing that changes in endogenous progesterone levels affect a variety of ocular functions in humans from intraocular pressure (Ziai et al., 1994, Panchami et al., 2013) to visual function (Yucel et al., 2005, Avitabile et al., 2007).

Another possible explanation for progesterone protection in MCAO but not rAION is that the mechanism of injury is different in the rAION model such that

progesterone is not effective. This idea is supported by previous research showing protective effects of progesterone or progestins in models of light induced retinal degeneration (Doonan et al., 2011, Cubilla et al., 2012), hereditary retinal degeneration (Doonan et al., 2011, Araiz et al., 2012, Sanchez-vallejo et al., 2013), and pressure-induced retinal ischemia-reperfusion (Lu et al., 2008). Additionally, progesterone has been shown to reduce glial swelling in retinal explants from both the STZ Type 1 diabetes model and the pressure-induced retinal ischemia-reperfusion model (Neumann et al., 2010). In the rAION model, as in other models that involve Rose Bengal photoactivation, superoxide radical formation damages the vascular endothelium, leading to platelet-fibrin thrombosis of the capillaries and obstruction of blood flow to neural tissue (Bernstein et al., 2003). Meanwhile, the transient MCAO model has been shown to cut off blood supply to the retina almost completely while the filament is in place, with reperfusion occurring after the filament is removed (Steele et al., 2008). In this model, as in other ischemia-reperfusion models, lack of oxygen and nutrients during the ischemia phase coupled with robust increases in oxidative stress and inflammation during the reperfusion phase cause cell death via multiple pathways (Kalogeris et al., 2012). While both models involve some type of ischemia, the differences in mechanism and in the extent of the injury itself may result in differential responses to treatment.

Progesterone treatment showed greater protection in brain than retina following MCAO

Progesterone treatment has previously been shown to reduce infarct size and functional deficits after cerebral ischemia (Sayeed et al., 2007, Ishrat et al., 2009, Wang

et al., 2010a, Yousuf et al., 2013b). Our results confirm these findings by demonstrating that progesterone-treated rats had smaller infarcts and performed significantly better on the grip strength (71% recovery) and sticky-tape tasks (84% recovery) after transient MCAO. Additionally, we show that progesterone treatment in MCAO animals results in significant reductions in retinal ganglion cell death and glutamine synthetase upregulation in Müller cells. Progesterone-treated MCAO rats also showed a trend for reduced ERG deficits (23% recovery), but recovery of functional deficits were smaller in retina than in brain. One possibility is that progesterone treatment is less effective in the retina following MCAO because MCAO induces a more severe injury in the brain than the retina. Another possibility is that the optimal progesterone dose is different for retina vs. brain. In cerebral stroke, 8 mg/kg was found to be the optimal dose (Wali et al., 2014), but in traumatic brain injury, 16 mg/kg was found to be a more effective dose (Cutler et al., 2007). Testing a range of doses in retinal injury and creating a dose response curve would allow us to answer this question. Perhaps increasing the number of doses or using a pump to deliver a slow but sustained dose of progesterone would prove more effective for the treatment of retinal injury and disease.

Additionally, while research supports the existence of progesterone receptors in the retina (Li et al., 1997, Wickham et al., 2000, Swiatek-De Lange et al., 2007, Koulen et al., 2008), little is known about progesterone receptor localization in retina and relative levels of progesterone receptor in retina vs. brain. Some of progesterone's protective effects are mediated by progesterone acting through classical progesterone receptors (Ghoumari et al., 2005, Liu et al., 2012a, Labombarda et al., 2013, Schumacher et al., 2013), while other effects are mediated by progesterone acting as an antagonist at Sigma-

1 (Monnet et al., 1995, Maurice et al., 1998, Su and Hayashi, 2003) and glucocorticoid receptors (Svec et al., 1980, Svec et al., 1989), by progesterone acting as a ligand at the pregnane X receptor (Kliewer et al., 1998, Langmade et al., 2006), or by progesterone's metabolite, allopregnanolone, acting as a positive allosteric modulator at the $GABA_A$ receptor (Reddy et al., 2005, Schumacher et al., 2013). If progesterone acts via all of these pathways in brain but only some of these pathways in retina, this could explain the difference in protection in retina vs. brain. Further research needs to be done to determine the mechanisms by which progesterone is acting in the retina and whether these differ from those involved in progesterone protection in the brain.

Conclusions

Progesterone treatment provided protection against both retinal and cerebral ischemia in the transient MCAO model, but not in the rAION model. Protection with progesterone treatment was greater in cerebral vs. retinal ischemia in the MCAO model. Further research is needed to determine why progesterone has differential effects in retina vs. brain and how to optimize progesterone treatment in the retina.

Chapter 4:

Effect of progesterone on inflammation in brain versus retina in a rat model of middle cerebral artery occlusion

This chapter presents work to be published within:

Rachael S. Allen, Iqbal Sayeed, Y Oumarbaeva, Katherine C. Morrison, Paul H. Choi, Machelle T. Pardue, Donald G. Stein. Effect of progesterone on inflammation in brain versus retina in a rat model of middle cerebral artery occlusion

ABSTRACT

Purpose: To determine whether inflammation increases in retina as it does in brain following middle cerebral artery occlusion (MCAO) and to determine whether the neurosteroid progesterone, which has been shown to have protective effects in both retina and brain after MCAO, reduces inflammation in retina as well as brain.

Methods: MCAO rats treated systemically with progesterone or vehicle were compared with shams. Protein levels of cytosolic NF-κB, nuclear NF-κB, phosphorylated NF-κB, IL-6, TNF-α, CD11b, Progesterone receptor A and B, and pregnane X receptor were assessed in retinas and brains at 24 and 48 hours using western blots. Results: The NF-κB pathway showed activation in both brain and retina after MCAO, with significant increases in pNF-κB at 24 hours in both brain and retina and significant increases in nuclear NF-κB at 24 hours in brain and 48 hours in retina. Significant increases were also observed in TNF-α at 24 hours in brain and in CD11b at 24 hours in both brain and retina. Progesterone treatment in MCAO animals significantly attenuated levels of the following markers in brain: pNF-κB, nuclear NF-κB, IL-6, TNF-α, and CD11b, with significantly increased levels of cytosolic NF-κB. Meanwhile, retinas from progesterone-treated animals showed significantly reduced levels of nuclear NF-κB and IL-6 and increased levels of cytosolic NF-κB, with a trend for reduction in other markers. Post-MCAO, progesterone receptor A and B were upregulated in brain and downregulated in retina. Progesterone treatment altered this effect in brain but not in retina.

Conclusions: Levels of inflammatory markers increased in both brain and retina after MCAO, with greater increases being observed in brain. Progesterone treatment reduced inflammation, and these reductions were more dramatic in brain than retina. This

differential effect may be due to differences in the response of progesterone receptor in brain and retina after injury.

INTRODUCTION

Inflammatory responses occur after ischemic injury in both the retina (Hangai et al., 1996, Wang et al., 2006, Dvoriantchikova et al., 2009, Hua et al., 2009, Jiang et al., 2012, Schallner et al., 2012, Ishizuka et al., 2013) and the brain (Hill et al., 1999, Schneider et al., 1999, Stephenson et al., 2000, Berti et al., 2002, Gibson et al., 2005, Ishrat et al., 2010, Tu et al., 2010). The NF-κB pathway plays a key role in post-injury inflammation, and evidence of NF-κB pathway activation and increased cytokine levels have been reported following ischemia in brain (Gibson et al., 2005, Ishrat et al., 2010, Tu et al., 2010). Increases in systemic inflammation after cerebral ischemia have been observed as well (Yousuf et al., 2013a). Increases in NF-κB pathway activation (Schneider et al., 1999, Stephenson et al., 2000, Hua et al., 2009) and increases in levels of inflammatory cytokines (Hill et al., 1999, Berti et al., 2002) are known to occur in the brain after transient MCAO, a model that causes ischemia in both the brain and the retina (Chapter 2) (Block et al., 1997, Steele et al., 2008). A gap in our knowledge is whether these increases occur in the retina after MCAO and how they compare with the brain.

Similar patterns of NF-κB pathway activation and increased levels of inflammatory cytokines (IL-6, TNF-α, etc.) have been observed in other models of retinal ischemia (Hangai et al., 1996, Wang et al., 2006, Jiang et al., 2012, Schallner et al., 2012, Ishizuka et al., 2013). While a couple studies suggest that complete NF-κB inhibition could actually exacerbate retinal injury (Wang et al., 2006, Jiang et al., 2012), selective inactivation of the NF-κB pathway in astrocytes was shown to be protective in retinal ischemia (Dvoriantchikova et al., 2009).

The neurosteroid progesterone (PROG) has been found to have protective effects in a number of animal models, including traumatic brain injury, stroke, and spinal cord injury (Cutler et al., 2007, Schumacher et al., 2007, Ishrat et al., 2009, Sayeed and Stein, 2009, Stein and Wright, 2010). Progesterone treatment has been shown to reduce inflammation, swelling, and neuronal death, and to improve behavioral and functional recovery (Cutler et al., 2007, Schumacher et al., 2007, Ishrat et al., 2009, Ishrat et al., 2010, De Nicola et al., 2013, Yousuf et al., 2013a). Specific effects of progesterone on inflammation include reducing TNF-α production by microglia (Drew and Chavis, 2000) and macrophages (Miller and Hunt, 1998), attenuating NF-κB pathway activation after traumatic brain injury (Cutler et al., 2007), and attenuating systemic increases in TNF-α and IL-6 after post-stroke infection (Yousuf et al., 2013a). Additionally, progesterone suppresses injury-induced increases in levels of inflammatory markers, including IL-1β, TNF-α, IL-6, and COX-2 after traumatic brain injury (Cutler et al., 2007), TNF-α and IL-6 after cerebral ischemia (Ishrat et al., 2010), and TNF-α, iNOS, and CD11b in experimental autoimmune encephalomyelitis (De Nicola et al., 2013). We know that progesterone protects in both brain and retina after transient MCAO and that protective effects are more dramatic in brain than retina (Chapter 3).

Studies on progesterone treatment in the retina (Neumann et al., 2010, Doonan et al., 2011), and more specifically in retinal ischemia (Chapter 3) (Lu et al., 2008), are limited thus far, but progesterone's successes in other models combined with findings of progesterone (Lanthier and Patwardhan, 1986, 1987), progesterone synthesis (Lanthier and Patwardhan, 1988, Guarneri et al., 1994, Sakamoto et al., 2001a, Coca-Prados et al., 2003, Cascio et al., 2007), and progesterone receptor (PR) in the eye (Li et al., 1997,

Wickham et al., 2000, Swiatek-De Lange et al., 2007, Koulen et al., 2008) make it an attractive candidate for neuroprotective treatment in the retina. However, progesterone has been shown to have a smaller protective effect in retina than in brain in the MCAO model (Chapter 3). Thus, the mechanisms by which progesterone provides protection (including reduction of inflammation) in brain and retina should be further studied to determine why the protective effect in brain is greater.

Here, we compared the inflammatory response in retina and brain following transient MCAO model in rats and we tested progesterone's effects on this evoked inflammation. Protein levels of cytosolic NF-κB (pathway suppressed) vs. levels of nuclear NF-κB and phosphorylated NF-κB (pathway active) were assessed in retinas and brains at 24 and 48 hours. Protein levels of inflammatory markers (IL-6, TNF-α, CD11b) were also measured. In order to confirm expression of PR in the retina at the protein level, levels of PR-A and B were assessed in retina and brain. Levels of pregnane X receptor (PXR), another receptor through which progesterone has been shown to mediate protective effects (Kliewer et al., 1998, Langmade et al., 2006), were assessed in retina and brain as well. We hypothesized that levels of NF-κB and downstream inflammatory cytokines would increase in the retina, though perhaps not to the same degree as in the brain. We further hypothesized that progesterone would reduce inflammation in the retina as it does in the brain following MCAO.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (n = 31) from Charles River Laboratories were

used in this study. At the time of MCAO surgery, rats were approximately 60 days of age (290-330 grams). Littermates that received sham surgeries (incisions in the scalp and neck) and vehicle injections were used as controls. All animal procedures were approved by the Institutional Animal Care and Use Committee (Emory University protocol #20001517) and performed in accordance with NIH guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were housed under a 12:12 reverse light:dark cycle with water and food ad libitum and handled daily for at least 5 days prior to surgery. Two rats died during surgery and one was excluded due to an incomplete reperfusion.

MCAO surgery

MCAO surgery was performed as described previously with minor modifications (Longa et al., 1989). Briefly, inhalation of 5% isoflurane (in a N_2/O_2 70%/30% mixture) was used to anesthetize the animals with an inhalation of 2% isoflurane to maintain sedation. Blood oxygen saturation (SpO_2) was analyzed and sustained at 90% using a pulse oximeter (SurgiVet, model V3304; Waukesha, WI, USA). Body temperature was maintained between 36.5°C and 37.5°C. Laser-Doppler flowmetry (LDF) was used to monitor cerebral blood flow, as it has been shown previously to be practical and reliable in detecting changes in cerebral blood flow during focal cerebral ischemia (Dirnagl et al., 1989). To reduce variability and ensure relative uniformity of the ischemic insult, animals with mean ischemic cerebral blood flow greater than 40% of baseline LDF were excluded. An LDF probe (Moor Instruments, Wilmington, Delaware, USA) was inserted over the ipsilateral parietal cortex to monitor blood flow from 5 minutes prior to

occlusion to 5 minutes after reperfusion.

At the ventral surface of the neck, a midline incision was made and the right common carotid arteries were separated and ligated using a 6.0 silk suture. Next, the internal carotid and pterygopalatine arteries were occluded using a microvascular clip to allow insertion of a 4-0 silicon-coated monofilament (0.35–0.40 mm long) (Doccol Co., Albuquerque, NM, USA) through an incision in the external carotid artery. The filament was slid into the internal carotid artery and gently pushed an estimated 20 mm distal to the carotid bifurcation where it blocked the openings to both the middle cerebral and ophthalmic arteries. The filament was removed after 120 minutes at which time reperfusion occurred. Then the wound was sutured, and rats were transferred to a heating blanket until they recovered from anesthesia.

Progesterone preparation and dosing

Progesterone was made in stock solutions using 2-hydroxypropyl-β-cyclodextrin (HBC; 25% w/v solution in H_2O; a non-toxic aqueous solution that dissolves progesterone) as the solvent. An 8 mg/kg progesterone dose was used as this has been shown to be most protective after stroke (Wali et al., 2014). Progesterone and vehicle treatments were administered intraperitoneally at 1 hour post injury, and then subcutaneously at 6 hours post for the 24 hour group and 6 and 24 hours for the 48 hour group. In all experiments, the rats' group identity was coded with regard to surgery and treatment to prevent experimenter bias.

Biochemical Evaluation

Rats were euthanized by overdose of Pentobarbital and their eyes enucleated. Retinas and the penumbral portion of the brain were dissected out for use in western blots. Isolated tissue was frozen immediately on dry ice. The NE-PER kit (Thermo Scientific; Waltham, MA, USA) was used to homogenize the tissue and separate it into nuclear and cytosolic fractions. Western immunoblotting was used to assess brain and retina protein levels of pNF-κB, nuclear and cytosolic NF-κB, IL-6, TNF-α, CD11b, PR-A, PR-B, and PXR (see Table 4-1 for details on antibodies). Protein samples were run on 4-20% Tris-Glycine Criterion Pre-cast TGX gels at 90 V for approximately 2 hours (Bio-Rad; Hercules, CA, USA). Proteins were then transferred to a nitrocellulose membrane at 30V overnight and incubated for 1 hour at room temperature in a 5% BSA/TBS-Tween blocking solution. Blots were then incubated in primary antibody for two nights at 4°C. Blots were rinsed with TBS-Tween prior to incubation with the appropriate secondary antibody for 1 hour at room temperature. Blots were rinsed with TBS-Tween again before a 1-minute incubation in a chemiluminescent solution. Bands were detected using films, scanned, and analyzed using densitometry. Blots were stripped and β-Actin and Histone H3 were used as loading controls for cytosolic blots and nuclear blots, respectively (note: all blots are from cytosolic fraction unless specified as nuclear).

Antibody	Molecular Weight	Dilution	Company	Product #
mouse anti-pNF-κB p65	65 kDa	1:2000	Cell Signaling	3036
rabbit anti-NF-κB p65	65 kDa	1:5000	Cell Signaling	8242S
rabbit anti-IL-6	21-28 kDa	1:5000	Abcam	Ab6672
rabbit anti-TNF-α	23 kDa	1:1000	Abcam	Ab66579
rat anti-CD11b	128 kDa	1:1000	Serotec	MCA 275
rabbit anti-PR	PR-A 81kDa PR-B 116kDa	1:1000	Santa Cruz	SC-539
goat anti-PXR	50 kDa	1:1000	Santa Cruz	SC-7739
rabbit anti-Histone H3	17kDa	1:1000	Cell Signaling	9715L
mouse anti-β-actin	47kDa	1:5000	Sigma	A2228

Table 4-1. Detailed information on antibodies used in this study.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Differences between groups were analyzed using multiple unpaired, two tailed t-tests (four per marker) with the alpha level corrected for with false discovery rate ($p < 0.031$) (Storey, 2003). Mann-Whitney Rank Sum tests were used for groups that failed the normality test.

RESULTS

NF-κB pathway activation increased in both brain and retina following MCAO and progesterone attenuated this increase

Levels of phosphorylated NF-κB (pathway active) showed significant increases in brains (57%, Mann-Whitney Rank Sum test, $T = 54.00$, $p < 0.01$) and retinas (43%, unpaired *t*-test, $t = -2.834$, $p < 0.03$) from vehicle-treated MCAO rats (n = 5) over shams (n = 8) at 24 hours post injury (Fig. 4-1A, 4-1B). Levels of phosphorylated NF-κB were significantly lower in brains from progesterone- vs. vehicle-treated MCAO rats (n = 5/group) at 24 hours post-MCAO (-79%, unpaired *t*-test, $t = 5.601$, $p < 0.001$) (Fig. 4-1A), with retinas showing a trend for lower levels (-19%, Fig. 4-1B). Levels of nuclear NF-κB (pathway active) showed significant increases in brains (65%, unpaired *t*-test, $t = -3.667$, $p < 0.01$) at 24 hours post-MCAO (Fig. 4-1C) and retinas (9%, unpaired *t*-test, $t = -2.534$, $p < 0.03$) at 48 hours post-MCAO in vehicle-treated MCAO rats (n = 5) over shams (n = 8) (Fig. 4-1D). For progesterone- vs. vehicle-treated MCAO rats (n = 5/group), levels of nuclear NF-κB were significantly lower in brains at 24 hours (-31%, unpaired *t*-test, $t = 2.615$, $p < 0.03$) (Fig. 4-1C) and retinas at 48 hours (-13%, unpaired *t*-test, $t = 3.774$, $p < 0.01$) (Fig. 4-1D). Levels of cytosolic NF-κB (pathway suppressed)

showed a trend for a decrease (22%) in brains from vehicle-treated MCAO rats (n = 5) over shams (n = 8) at 48 hours post injury (Fig. 4-1E), with retinas showing a significant decrease at 48 hours (22%, unpaired *t*-test, $t = 8.415$, $p < 0.001$) (Fig. 4-1F). For progesterone- vs. vehicle-treated MCAO rats (n = 5/group), brains showed significantly higher levels of cytosolic NF-κB at 24 hours post-injury (+8%, unpaired *t*-test, $t = -3.681$, $p < 0.01$) and a trend for higher levels at 48 hours post injury (+21%, Fig. 4-1E), with retinas showing significantly higher levels at 48 hours (+12%, unpaired *t*-test, $t = -3.041$, $p < 0.03$) (Fig. 4-1F) (see also Supplemental Figure 4-1).

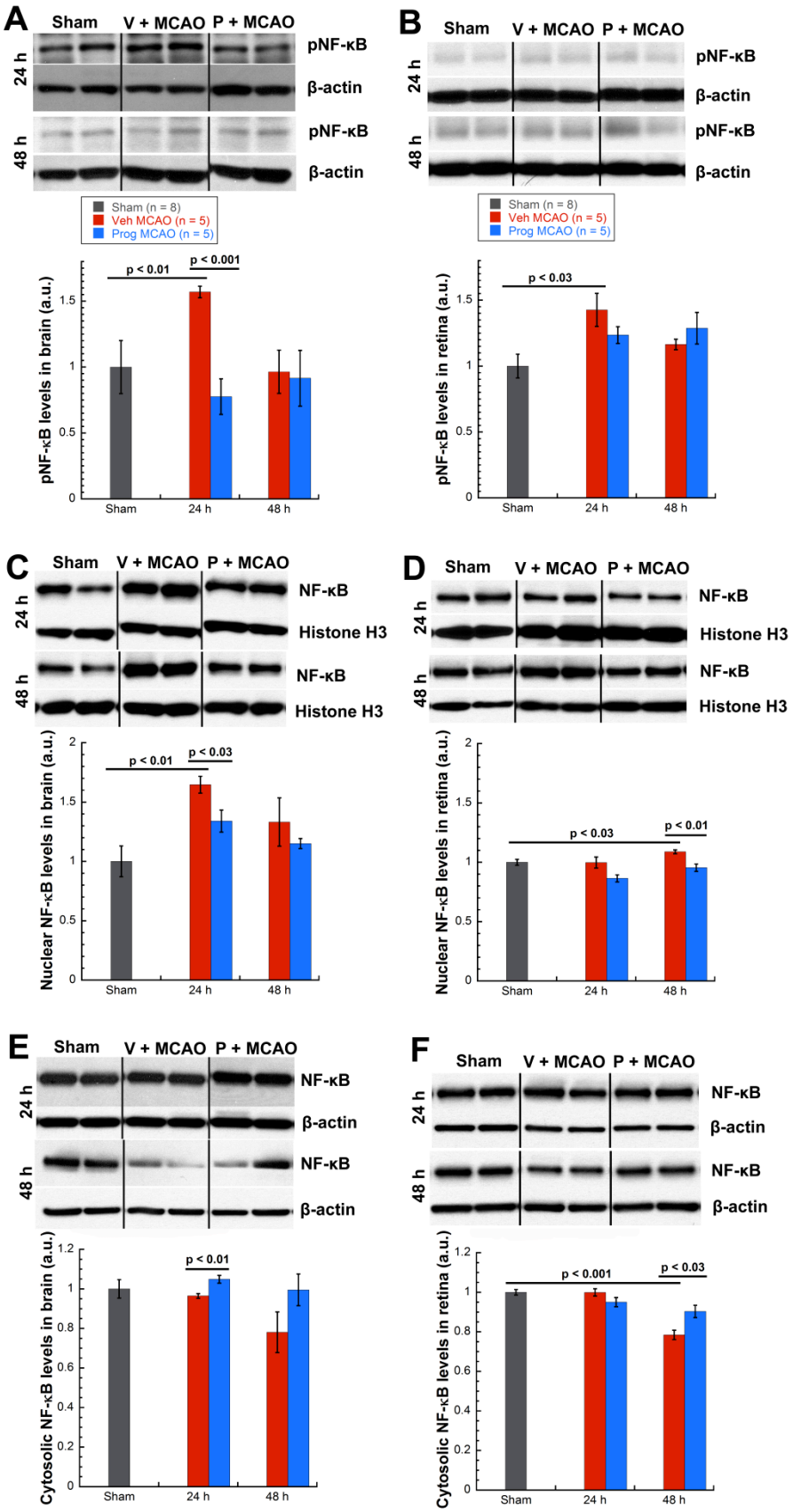
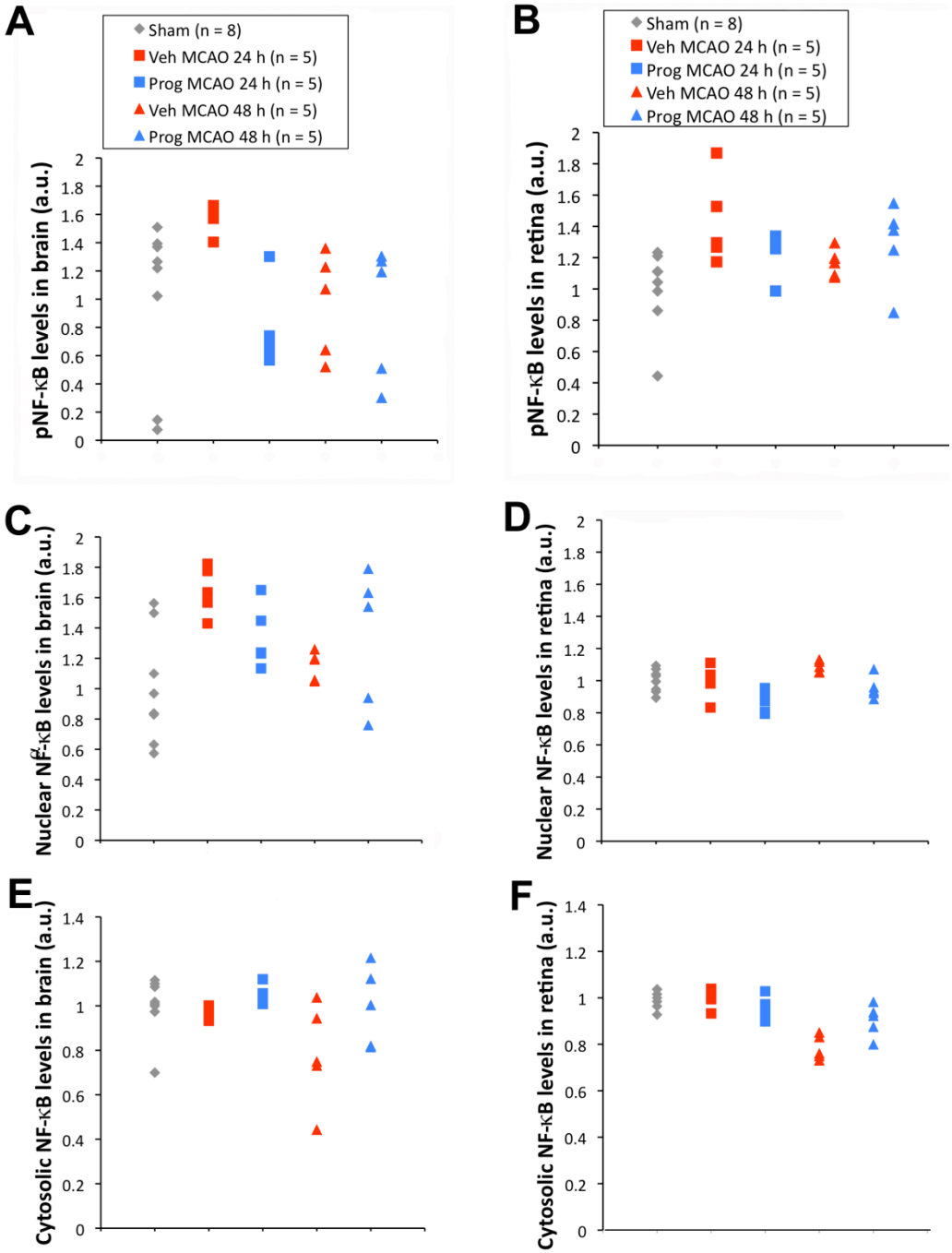

A
Sham
V + MCAO
P + MCAO
24 h
pNF-κB
β-actin
48 h
pNF-κB
β-actin
Sham (n = 8)
Veh MCAO (n = 5)
Prog MCAO (n = 5)
p < 0.01
p < 0.001
pNF-κB levels in brain (a.u.)
Sham
24 h
48 h
B
Sham
V + MCAO
P + MCAO
24 h
pNF-κB
β-actin
48 h
pNF-κB
β-actin
Sham (n = 8)
Veh MCAO (n = 5)
Prog MCAO (n = 5)
p < 0.03
pNF-κB levels in retina (a.u.)
Sham
24 h
48 h
C
Sham
V + MCAO
P + MCAO
24 h
NF-κB
Histone H3
48 h
NF-κB
Histone H3
p < 0.01
p < 0.03
Nuclear NF-κB levels in brain (a.u.)
Sham
24 h
48 h
D
Sham
V + MCAO
P + MCAO
24 h
NF-κB
Histone H3
48 h
NF-κB
Histone H3
p < 0.03
p < 0.01
Nuclear NF-κB levels in retina (a.u.)
Sham
24 h
48 h
E
Sham
V + MCAO
P + MCAO
24 h
NF-κB
β-actin
48 h
NF-κB
β-actin
p < 0.01
Cytosolic NF-κB levels in brain (a.u.)
Sham
24 h
48 h
F
Sham
V + MCAO
P + MCAO
24 h
NF-κB
β-actin
48 h
NF-κB
β-actin
p < 0.001
p < 0.03
Cytosolic NF-κB levels in retina (a.u.)
Sham
24 h
48 h

Figure 4-1. Increased NF-κB pathway activation in brain and retina following MCAO was attenuated by progesterone treatment. Phosphorylated NF-κB expression in A) brain and B) retina. Nuclear NF-κB expression in C) brain and D) retina. Cystosolic NF-κB expression in E) brain and F) retina. Expression of NF-κB pathway markers as determined by western blot and quantified by densitometry. Results expressed as means ± SEM.



Supplemental Figure 4-1. NF-κB pathway western blot data presented as individual data points. Phosphorylated NF-κB expression in A) brain and B) retina. Nuclear NF-κB expression in C) brain and D) retina. Cytosolic NF-κB expression in E) brain and F) retina.

IL-6, TNF-α, and CD11b increased in both brain and retina following MCAO and progesterone attenuated these increases

Levels of IL-6 showed a trend for increases in brains (24% at 24 hours; 48% at 48 hours) (Fig. 4-2A) and retinas (44% at 24 hours; 79% at 48 hours) (Fig. 4-2B) from vehicle-treated MCAO rats (n = 5) over shams (n = 8). For progesterone- vs. vehicle-treated MCAO rats (n = 5/group), levels of IL-6 were significantly lower in brains at both 24 (-26%, unpaired *t*-test, $t = 4.424$, $p < 0.01$) and 48 hours (-62%, unpaired *t*-test, $t = 4.070$, $p < 0.01$) (Fig. 4-2A) post-injury and in retinas at 24 hours (-46%, unpaired *t*-test, $t = 3.350$, $p < 0.01$), with 48 hour retinas from progesterone-treated rats showing a trend for lower IL-6 levels (-61%) (Fig. 4-2B). Levels of TNF-α showed significant increases in brains at 24 hours (60%, unpaired *t*-test, $t = -3.330$, $p < 0.01$) with a trend for an increase at 48 hours (40%) for vehicle-treated MCAO rats (n = 5) over shams (n = 8) (Fig. 4-2C). Retina levels of TNF-α showed a trend for an increase for vehicle-treated MCAO rats (n = 5) over shams (n = 8) at 24 hours (16%) (Fig. 4-2D). Levels of TNF-α were significantly lower in brains from progesterone- vs. vehicle-treated MCAO rats (n = 5/group) at 24 hours post-MCAO (-60%, unpaired *t*-test, $t = 3.918$, $p < 0.01$) (Fig. 4-2C), with retinas showing a trend for lower levels at 24 hours (-17%) (Fig. 4-2D). Levels of CD11b showed significant increases in brains (103%, unpaired *t*-test, $t = -3.903$, $p < 0.01$) (Fig. 4-2E) and retinas (89%, unpaired *t*-test, $t = -4.690$, $p < 0.001$) (Fig. 4-2F) from vehicle-treated MCAO rats (n = 5) over shams (n = 8) at 24 hours post injury, with 48-hour brains showing a trend for an increase (16%). Levels of CD11b were significantly lower in brains from progesterone- vs. vehicle-treated MCAO rats (n = 5/group) at 24 hours post-MCAO (-69%, unpaired *t*-test, $t = 2.738$, $p < 0.03$) (Fig. 4-2E), with retinas

showing a trend for lower levels at 24 hours (-36%) (Fig. 4-2F) (see also Supplemental Figure 4-2).

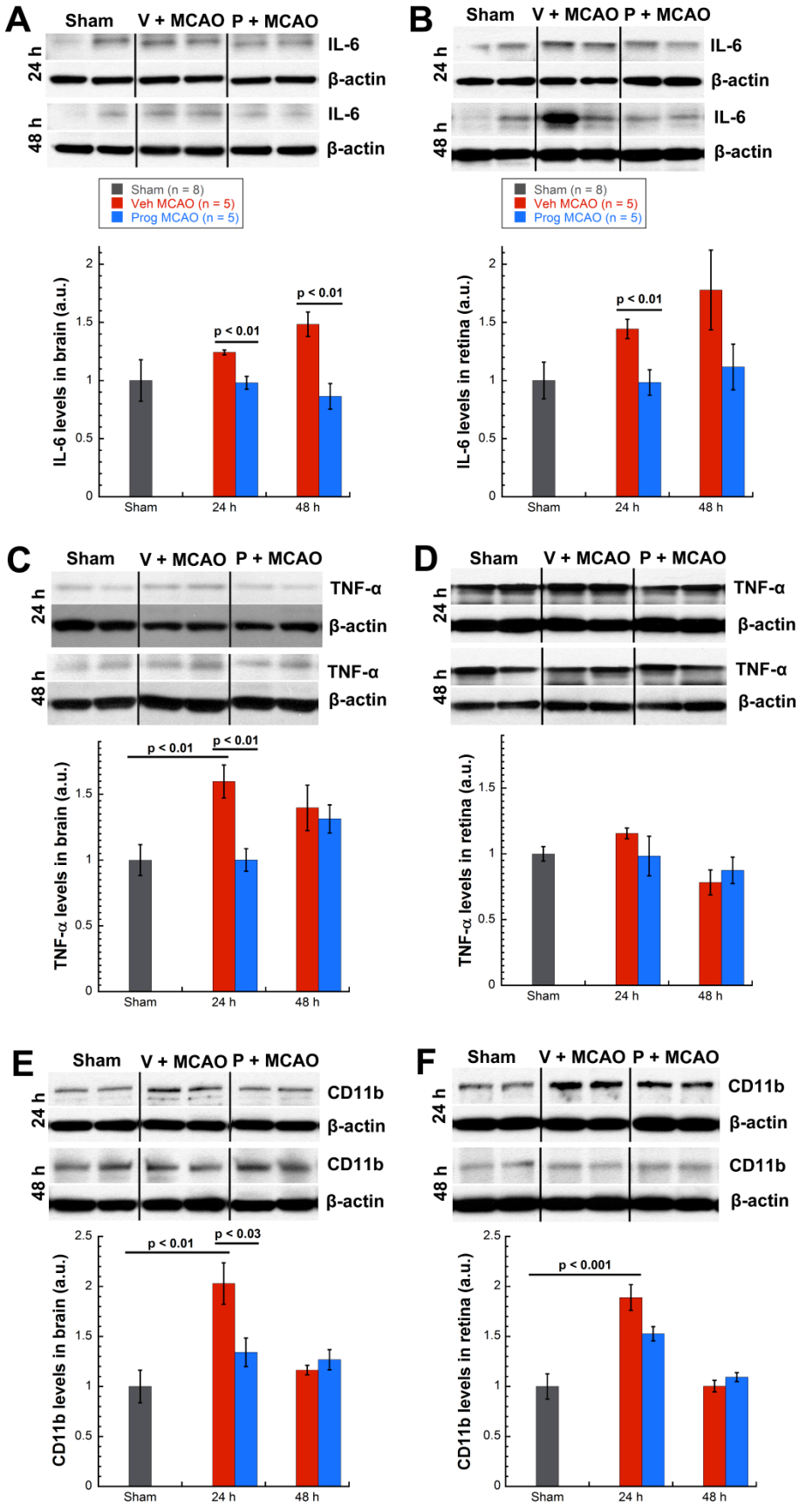
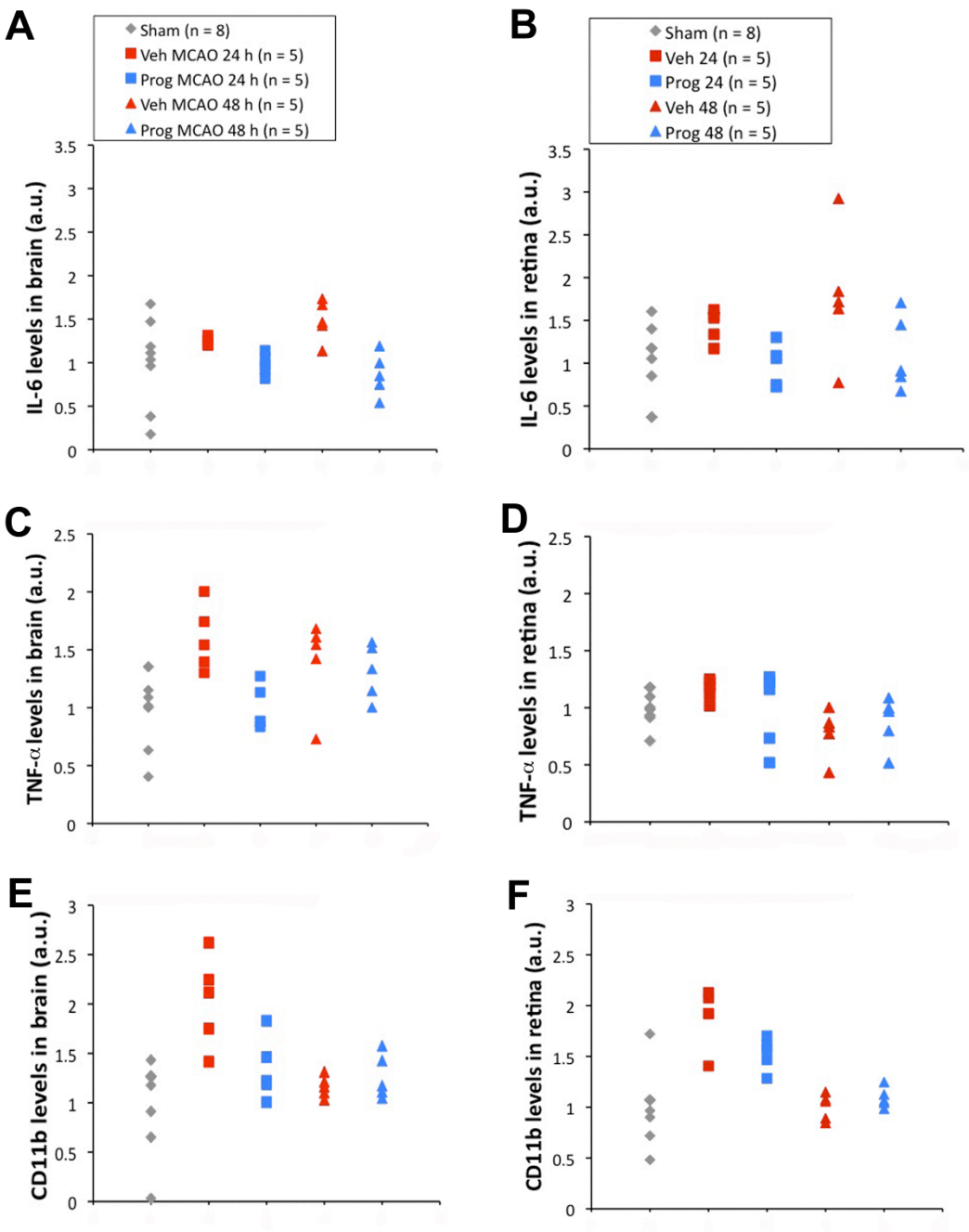

A
Sham
V + MCAO
P + MCAO
24 h
IL-6
β-actin
48 h
IL-6
β-actin
Sham (n = 8)
Veh MCAO (n = 5)
Prog MCAO (n = 5)
IL-6 levels in brain (a.u.)
p < 0.01
p < 0.01
Sham
24 h
48 h
B
Sham
V + MCAO
P + MCAO
24 h
IL-6
β-actin
48 h
IL-6
β-actin
Sham (n = 8)
Veh MCAO (n = 5)
Prog MCAO (n = 5)
IL-6 levels in retina (a.u.)
p < 0.01
Sham
24 h
48 h
C
Sham
V + MCAO
P + MCAO
24 h
TNF-α
β-actin
48 h
TNF-α
β-actin
TNF-α levels in brain (a.u.)
p < 0.01
p < 0.01
Sham
24 h
48 h
D
Sham
V + MCAO
P + MCAO
24 h
TNF-α
β-actin
48 h
TNF-α
β-actin
TNF-α levels in retina (a.u.)
Sham
24 h
48 h
E
Sham
V + MCAO
P + MCAO
24 h
CD11b
β-actin
48 h
CD11b
β-actin
CD11b levels in brain (a.u.)
p < 0.01
p < 0.03
Sham
24 h
48 h
F
Sham
V + MCAO
P + MCAO
24 h
CD11b
β-actin
48 h
CD11b
β-actin
CD11b levels in retina (a.u.)
p < 0.001
Sham
24 h
48 h

Figure 4-2. Increased levels of IL-6, TNF-α, and CD11b in brain and retina following MCAO were attenuated by progesterone treatment. IL-6 expression in A) brain and B) retina. TNF-α expression in C) brain and D) retina. CD11b expression in E) brain and F) retina. Expression of inflammatory markers as determined by western blot and quantified by densitometry. Results expressed as means ± SEM.



Supplemental Figure 4-2. IL-6, TNF-α, and CD11b western blot data presented as individual data points. IL-6 expression in A) brain and B) retina. TNF-α expression in C) brain and D) retina. CD11b expression in E) brain and F) retina.

Progesterone receptor is upregulated in the brain and downregulated in the retina after MCAO

Levels of PR-A showed significant increases in brains from vehicle-treated MCAO rats (n = 5) vs. shams (n = 8) at both 24 (81%, unpaired *t*-test, $t = -4.439$, $p < 0.001$) and 48 hours (63%, unpaired *t*-test, $t = -3.061$, $p < 0.03$) post-MCAO (Fig. 4-3A). In contrast, levels of PR-A showed a trend for decreases in retinas from vehicle-treated MCAO rats (n = 5) vs. shams (n = 8) at 24 (30%) and 48 (37%) hours post injury (Fig. 4-3B). Progesterone-treated MCAO rats (n = 5) showed significantly lower levels of PR-A in the brain compared with vehicle-treated MCAO rats (n = 5) at both 24 (-22%, unpaired *t*-test, $t = 2.711$, $p < 0.03$) and 48 (-85%, unpaired *t*-test, $t = 3.149$, $p < 0.03$) hours post-injury (Fig. 4-3A), however progesterone treatment did not change the PR-A reduction observed in retinas in either direction (Fig. 4-3B). Levels of PR-B showed a trend for increases in brains from vehicle-treated MCAO rats (n = 5) over shams (n = 8) at 24 (38%) and 48 (56%) hours post injury, with brains from progesterone- vs. vehicle-treated animals (n = 5/group) showing a trend for lower levels at 24 hours (-14%) and significantly lower levels at 48 hours post injury (-85%, unpaired *t*-test, $t = 3.369$, $p < 0.01$) (Fig. 4-3C). In contrast, levels of PR-B showed significant decreases in retinas from vehicle-treated MCAO rats (n = 5) vs. shams (n = 8) at 24 hours post injury (52%, unpaired *t*-test, $t = 4.132$, $p < 0.01$), and progesterone treatment did not change this reduction (Fig. 4-3D) (see also Supplemental Figure 4-3).

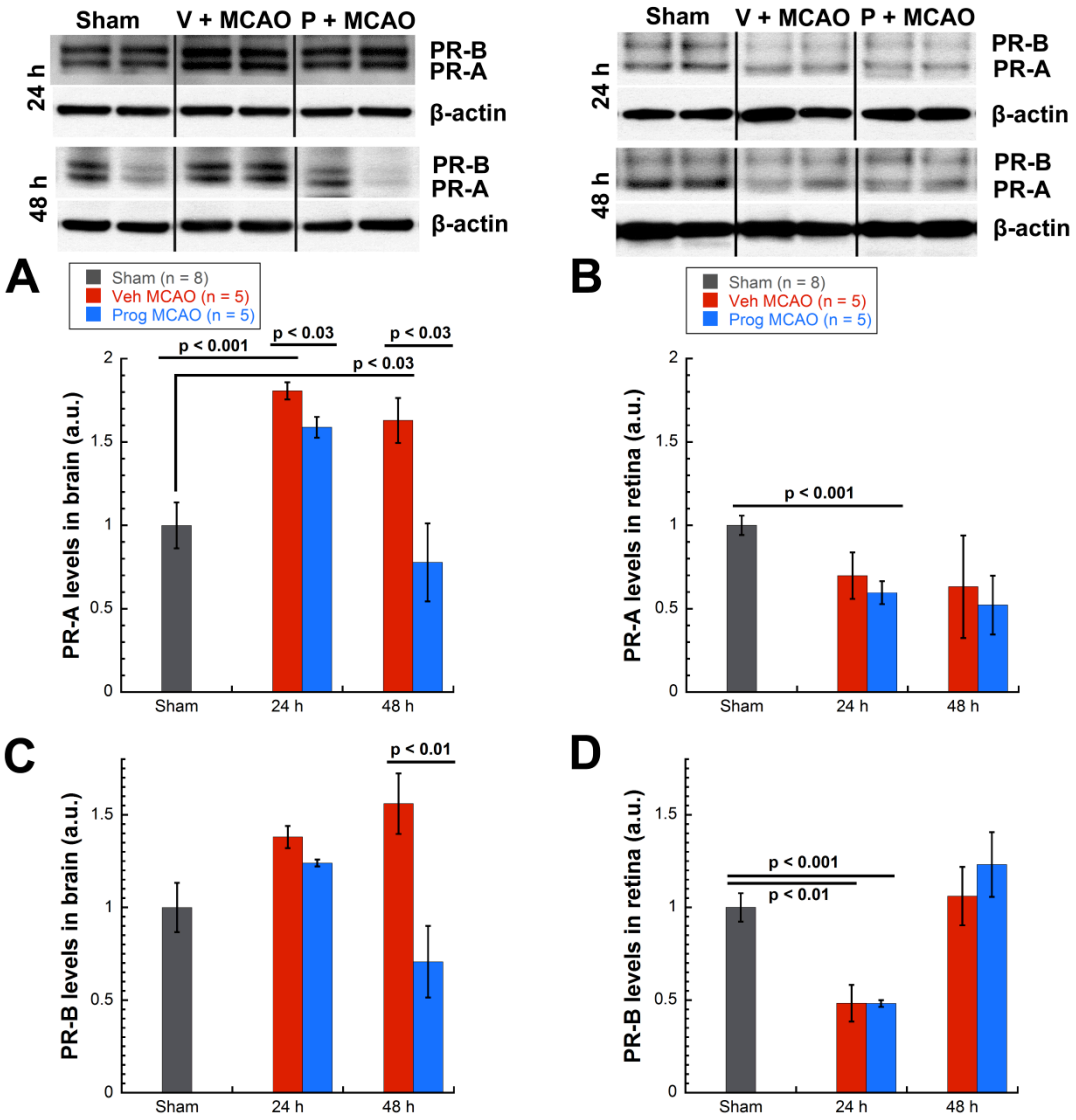
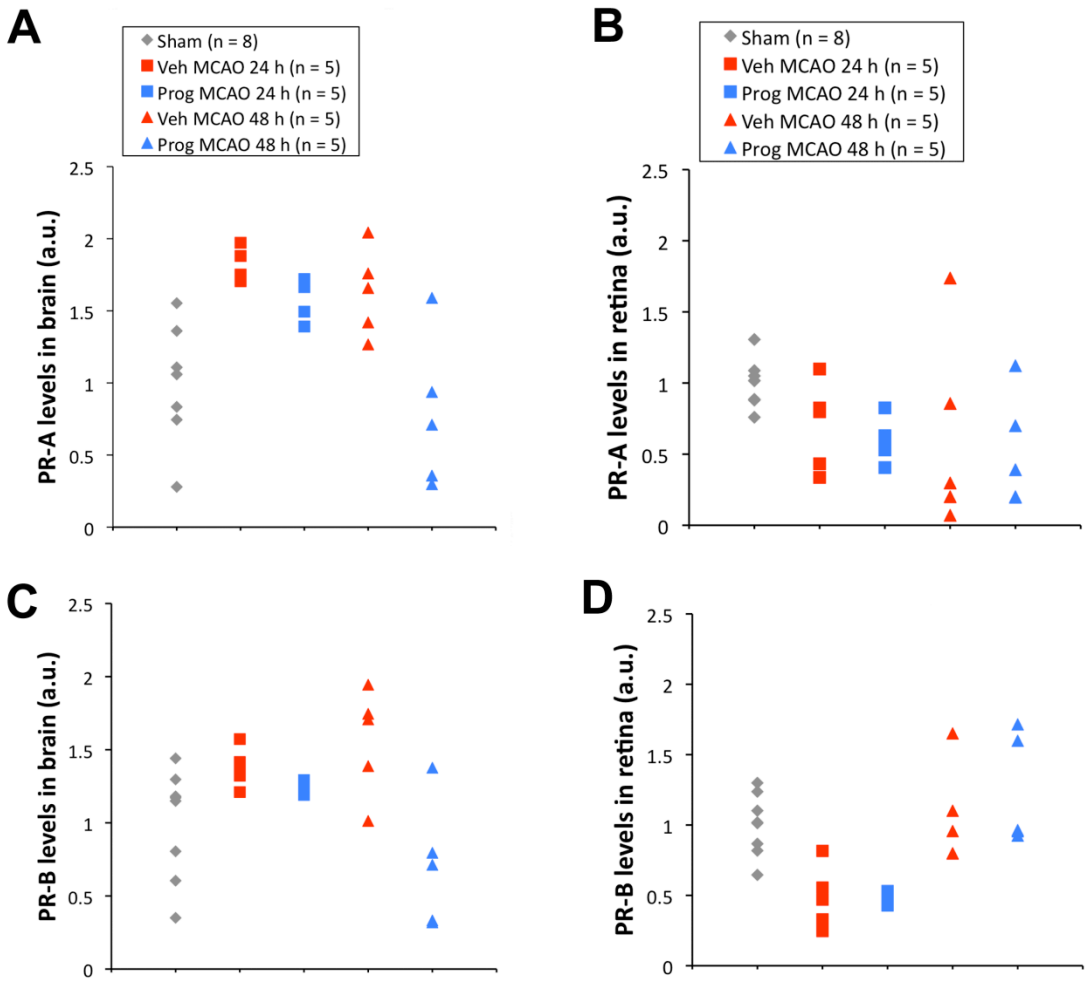


Figure 4-3. Progesterone receptor was upregulated in brain and downregulated in retina after MCAO. PR-A expression in A) brain and B) retina. PR-B expression in C) brain and D) retina. Expression of PRs as determined by western blot and quantified by densitometry. Results expressed as means ± SEM.



Supplemental Figure 4-3. PR-A and PR-B western blot data presented as individual data points. PR-A expression in A) brain and B) retina. PR-B expression in C) brain and D) retina.

PXR was upregulated in brain but downregulated in retina after MCAO

At 24 hours post-MCAO, levels of PXR showed a significant increase in brains from vehicle-treated MCAO rats (n = 5) over shams (n = 8, 75%, Mann-Whitney Rank Sum test, T = 55.000, $p < 0.01$) (Fig. 4-4A) and a significant decrease in retinas (13%, unpaired *t*-test, $t = 2.508$, $p < 0.03$) (Fig. 4-4B). Progesterone treated rats (n = 5) showed significantly lower levels of PXR in brain (-56%, unpaired *t*-test, $t = 3.110$, $p < 0.03$) (Fig. 4-4A) and significantly higher levels of PXR in retinas (+33%, unpaired *t*-test, $t = -5.129$, $p < 0.001$) (Fig. 4-4B) at 24 hours compared with vehicle-treated rats (n = 5) (see also Supplemental Figure 4-4).

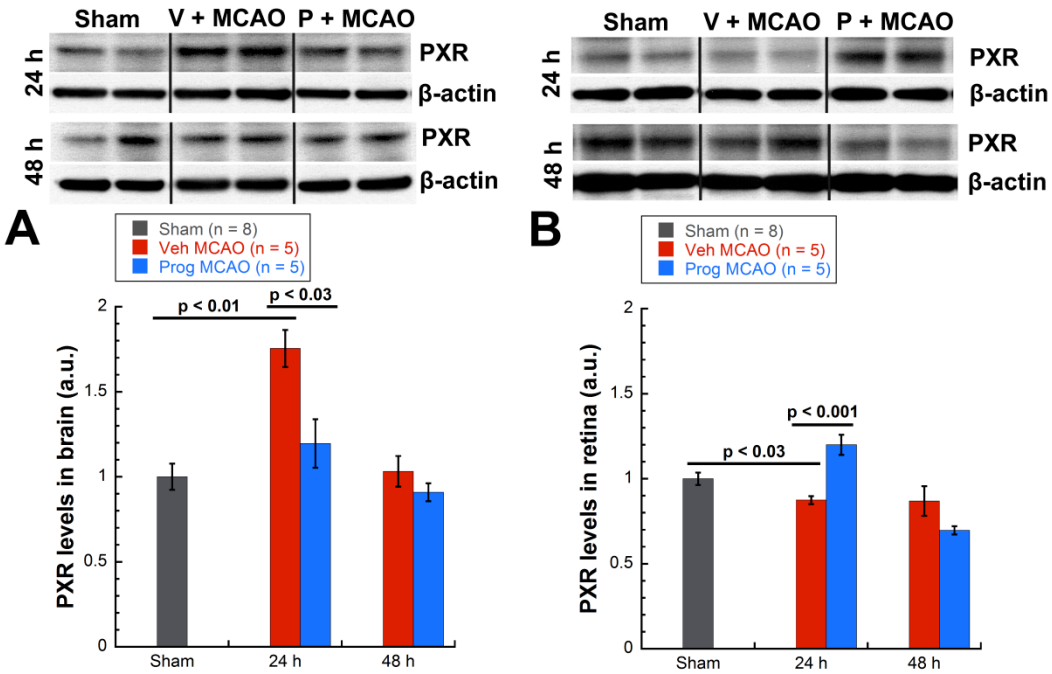
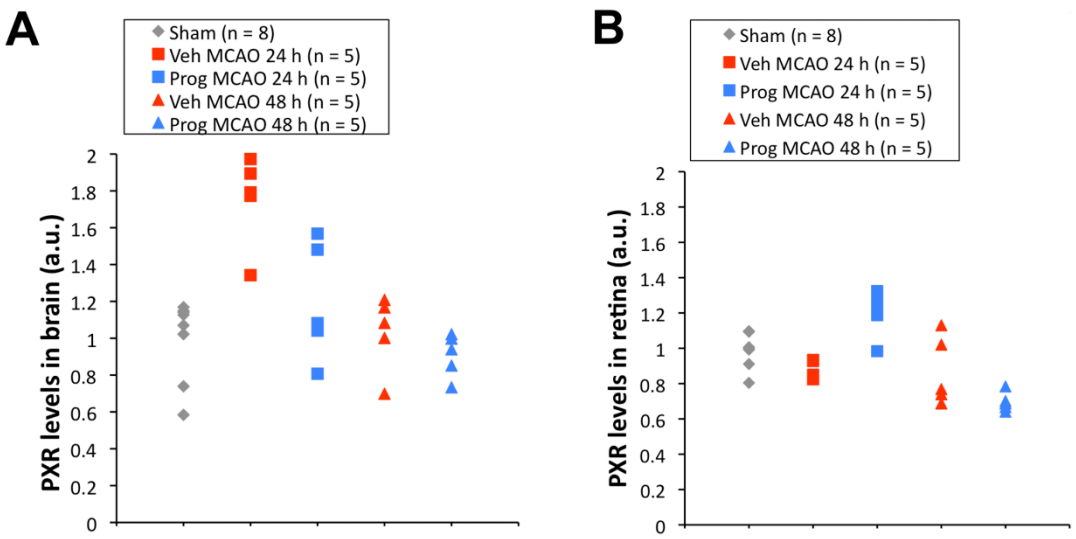


Figure 4-4. PXR was upregulated in brain but downregulated in retina after MCAO. PXR expression in A) brain and B) retina. Expression of PXR as determined by western blot and quantified by densitometry. Results expressed as means ± SEM.



Supplemental Figure 4-4. PXR western blot data presented as individual data points. PXR expression in A) brain and B) retina.

DISCUSSION

NF-κB pathway activation after retinal ischemia

Evidence of NF-κB pathway activation after retinal ischemia includes increases in protein levels of phosphorylated NF-κB (Schallner et al., 2012, Ishizuka et al., 2013), increases in NF-κB DNA binding activity (Schallner et al., 2012), increases in mRNA levels of NF-κB (Wang et al., 2006, Jiang et al., 2012), increases in protein levels of NF-κB in the nucleus (Jiang et al., 2012), and increases in numbers of NF-κB positive cells in the retina (Wang et al., 2006). Additionally, selective inactivation of the NF-κB pathway in astrocytes was shown to be protective in retinal ischemia (Dvoriantchikova et al., 2009). Following retinal ischemia, increased mRNA levels of TNF-α and IL-6 were observed (Hangai et al., 1996, Wang et al., 2006), as well as increases in IL-6 positive cells (Wang et al., 2006). NF-κB inhibition resulted in significantly reduced expression of pro-inflammatory genes like TNF-α, and completely suppressed the upregulation of IL-6 that follows retinal ischemia (Dvoriantchikova et al., 2009). While some suggest that complete inhibition of NF-κB could exacerbate retinal injury (Wang et al., 2006, Jiang et al., 2012), Dvoriantchikova and colleagues concluded that it is chronic NF-κB activation in astrocytes and microglia, rather than NF-κB activation in general, that is problematic following retinal ischemia (Dvoriantchikova et al., 2009).

Similarly, in the transient MCAO model we found increases in NF-κB pathway activation and increases in levels of inflammatory markers in the retina. Increases in inflammation have previously been shown to occur after cerebral ischemia (Hill et al., 1999, Schneider et al., 1999, Stephenson et al., 2000, Berti et al., 2002, Gibson et al., 2005, Hua et al., 2009, Ishrat et al., 2010, Tu et al., 2010), and our results confirm those

findings. Our previous work has shown that the retina is less susceptible than the brain to ischemia induced by the transient MCAO model (Chapter 2), and our results here show smaller increases in inflammation in retina vs. brain (Table 4-2).

	Brain	Retina
pNF-κB	**+57% at 24 h**	**+43% at 24 h**
nuclear NF-κB	**+65% at 24 h** +33% at 48 h	**+9% at 48 h**
cytosolic NF-κB	-22% at 48 h	**-10% at 48h**
IL-6	+24% at 24 h +48% at 48 h	+44% at 24 h +79% at 48 h
TNF-α	**+60% at 24 h** +40% at 48 h	+16% at 24 h
CD11b	**+103% at 24 h** +16% at 48 h	**+89% at 24 h**

Table 4-2. Summary of changes in inflammatory markers expressed as % difference in vehicle-treated MCAO animals over sham. Larger increases were observed in brain vs. retina. **Bold** = significant ($p < 0.031$).

Greater protection with progesterone was observed in brain vs. retina

Progesterone has been shown to provide protection in a number of animal models (Gonzalez Deniselle et al., 2005, Cutler et al., 2007, Schumacher et al., 2007, Ishrat et al., 2009, Sayeed and Stein, 2009, De Nicola et al., 2013, Yousuf et al., 2013a) and in Phase II clinical trials for traumatic brain injury (Wright et al., 2007, Xiao et al., 2008). Progesterone was observed to reduce glial activation and cytokine production both in vitro and in vivo (Miller and Hunt, 1998, Drew and Chavis, 2000, De Nicola et al., 2013) and to reduce NF-κB pathway activation after traumatic brain injury (Cutler et al., 2007). In models of cerebral ischemia, progesterone has been shown to reduce inflammation both in the brain and systemically, to decrease infarct size, and to improve functional recovery (Ishrat et al., 2009, Ishrat et al., 2010, Yousuf et al., 2013a, Yousuf et al., 2013b). Our results confirm previous findings of progesterone protection in cerebral ischemia, showing that progesterone attenuated ischemia-induced increases in NF-κB pathway activation and inflammatory markers in the brain. Additionally, our results demonstrate that progesterone reduces inflammation in the retina following transient MCAO. Interestingly, more dramatic protection with progesterone treatment following MCAO was observed in brain vs. retina. We observed greater reductions in NF-κB pathway markers and inflammatory cytokines with progesterone treatment in brain vs. retina (Table 4-3). These findings concur with our previous observation that progesterone treatment significantly improved function on behavioral tests (71-84% recovery), with a trend for improvement in retinal function as measured by electroretinogram (23% recovery) (Chapter 3).

	Brain	Retina
pNF-κB	**-79% at 24h**	-19% at 24 h
nuclear NF-κB	**-31% at 24h** -18% at 48h	**-13% at 48 h**
cytosolic NF-κB	**+8% at 24 h** +21% at 48 h	**+12% at 48 h**
IL-6	**-26% at 24 h** **-62% at 48 h**	**-46% at 24 h** **-61% at 48 h**
TNF-α	**-60% at 24 h**	-17% at 24 h
CD11b	**-69% at 24 h**	-36% at 24 h

Table 4-3. Summary of changes in inflammatory markers between progesterone- and vehicle-treated MCAO animals expressed as % difference between progesterone- and vehicle-treated rats over sham. Greater protective effects were observed with progesterone treatment in brain vs. retina. **Bold** = significant ($p < 0.031$).

Differences in PR expression in retina and brain may contribute to differences in responsiveness to progesterone treatment

Progesterone and PR have been identified in both brain (Camacho-Arroyo et al., 1994, Guerra-Araiza et al., 2002, Guerra-Araiza et al., 2003, Meffre et al., 2007, Liu et al., 2012a) and retina (Lanthier and Patwardhan, 1987, Wickham et al., 2000, Koulen et al., 2008), and progesterone synthesis occurs in both tissues (Lanthier and Patwardhan, 1988, Guarneri et al., 1994, Cherradi et al., 1995, Tsutsui et al., 2000, Sakamoto et al., 2001a, Coca-Prados et al., 2003, Tsutsui, 2006, Cascio et al., 2007). PR mRNA has been found in retina samples in rats and rabbits (Wickham et al., 2000). Mouse bipolar cells that had been dissociated from the rest of the retina were shown to express PR at the protein level (Koulen et al., 2008). Here, we show that PR-A and PR-B are expressed at the protein level in rat retina, and receptor levels are reduced at 24 hours post-injury. In brain, however, we observed an increase in PR-A and PR-B after injury. With progesterone treatment, we see no change in PR expression in the retina but a reduction in PR expression in the brain at 48 hours.

Progesterone levels are known to increase in serum after traumatic brain injury in humans (Wagner et al., 2011), in brain after traumatic brain injury in rats (Meffre et al., 2007), and in serum and brain after transient MCAO in mice (Liu et al., 2012a). Progesterone levels also increase in response to inflammation and glucose deprivation (Elman and Breier, 1997, Zitzmann et al., 2005). Relatively little is known about how classical PRs respond after neural injury. Increases in PR-A and PR-B expression were observed in brains in an animal model of intra-uterine growth restriction (Palliser et al., 2012). However, another study did not show changes in levels of PR mRNA in brain

after transient MCAO with or without progesterone treatment (Dang et al., 2011). These results in the MCAO model conflict with our own, but this may be due to an mRNA vs. protein difference. Decreases in PR expression were observed after spinal cord injury, and these decreases did not change with progesterone treatment (Labombarda et al., 2003). These findings in spinal cord mirror our findings in retina, possibly suggesting a difference in PR behavior in brain vs. peripheral nervous tissue following injury.

Studies have shown examples of both PR-dependent and independent mechanisms with progesterone treatment in several different injury models. For example, progesterone treatment induced remyelination of demyelinated axons (Ghoumari et al., 2005) and protected against motorneuron loss in injured spinal cord slices (Labombarda et al., 2013) in wildtype mice but not PR knock-out mice. In the transient MCAO model, PR knock out and heterozygote mice were shown to have increased susceptibility to ischemic brain injury at 24 hours. Progesterone treatment reduced infarct size and edema and improved behavioral function in wildtype but not PR knock-out mice after transient MCAO (Liu et al., 2012a). In both the transient MCAO and the spinal cord injury experiments, treatment with allopregnanolone, a metabolite of progesterone, still had a protective effect in PR knock-out mice (Liu et al., 2012a, Labombarda et al., 2013). Allopregnanolone acts independently of the progesterone receptor by binding directly to the $GABA_A$ receptor complex and acting as a positive allosteric modulator, increasing Cl^- influx and reducing excitability (Reddy et al., 2005). Perhaps allopregnanolone treatment would prove more effective in the retina.

A few possibilities could explain the reduction in PR expression in brain following progesterone treatment. 1) Under normal conditions, PR expression is down-

regulated by progesterone treatment in estradiol-inducible areas, including the hypothalamus and some limbic structures, but not in the cerebral cortex, cerebellum, spinal cord, or peripheral nerves (Camacho-Arroyo et al., 1994, Jung-Testas et al., 1996, Guerra-Araiza et al., 2002, Guerra-Araiza et al., 2003, Labombarda et al., 2003). Because the areas damaged in transient MCAO (cerebral cortex and striatum) have been shown not to have PR expression affected by progesterone treatment (cortex) (Camacho-Arroyo et al., 1994, Guerra-Araiza et al., 2002) or have been shown not to be estradiol-inducible areas (striatum) (Parsons et al., 1982), the reduction in PR is probably not caused by a down-regulating effect of progesterone treatment. 2) Our western blots examined PR expression in the cytosolic fraction, and it is possible that these receptors translocated to the nucleus when activated by progesterone. PRs in areas of the brain involved in reproduction (i.e., hypothalamus) generally respond to progesterone binding by forming dimers and translocating to the nucleus (Schumacher et al., 2013). However, much of the PRs in other brain areas are expressed in axons, dendrites, and synapses (not near the nucleus) (Waters et al., 2008) and are thought to act in the cytoplasm or at the plasma membrane by activating kinases or interacting with intracellular signaling pathways (Bagowski et al., 2001, Maller, 2001, Faivre and Lange, 2007, Boonyaratanakornkit et al., 2008, Faivre et al., 2008). 3) Thus, we think it is most likely that PR expression is reduced in progesterone-treated ischemic tissue because progesterone treatment has restored the tissue to a more “normal” condition.

PXR expression in brain vs. retina following transient MCAO

In addition to acting through PRs, progesterone and its metabolites have been shown to bind to and influence the activity of other receptors, including glucocorticoid receptors (Svec et al., 1980, Svec et al., 1989), acetylcholine receptors (Valera et al., 1992), $GABA_A$ receptors (Puia and Belelli, 2001, Reddy et al., 2005), Sigma-1 receptors (Maurice et al., 1998), and pregnane X receptors (PXRs) (Kliewer et al., 1998, Langmade et al., 2006). PXR activates p-Glycoprotein (P-gp), an efflux pump implicated in the removal of cytotoxic and xenobiotic substances in both the blood brain and blood retina barriers (Bauer et al., 2004, Zhang et al., 2012b). PXR has previously been identified in brain capillaries (Bauer et al., 2004) and retinal pigmented epithelium (Zhang et al., 2012a, Zhang et al., 2012b). The literature on PXR expression after injury is not consistent (Souidi et al., 2005, Hartz et al., 2010, Chen et al., 2011).

Here, we show increased levels of PXR in brain at 24 hours post-MCAO, with progesterone treatment resulting in "normal" levels of PXR. Conversely, in retina we observed lower levels of PXR at 24 hours post-injury, and PXR levels increased with progesterone treatment. More research is needed to determine the role of PXR after injury and with progesterone treatment.

Conclusions

Following MCAO, inflammation increased in both brain and retina, with greater increases occurring in brain. Inflammation was reduced post-MCAO with progesterone treatment, and more dramatic protective effects of progesterone were observed in brain than retina. Differences in progesterone protection may be due to differential responses of progesterone receptor after injury in brain and retina.

Chapter 5:

Conclusions, Discussion, and Future Directions

SUMMARY OF RESULTS

The goals of this research were to: 1. Compare ischemic injury, with specific focus on inflammation, in a model of ischemia/reperfusion thought to affect both the brain and the retina. 2. Determine whether progesterone provides protection in retinal ischemia as it does in cerebral ischemia.

In Chapter 2, I demonstrated retinal function (ERG) deficits at 2 days post-MCAO that correlated with behavioral deficits. Retinal function deficits were found to recover by 9 days post-MCAO. Deficits were observed in both MCAO and contralateral retinas, suggesting a systemic component to MCAO-induced retinal ischemia. Upregulation of GFAP and glutamine synthetase was observed at 3 days post-MCAO, and retinal ganglion cell loss was observed in severe MCAO rats. Increased glutamine synthetase levels correlated with ERG deficits, suggesting that sub-lethal increases in extracellular glutamate may be the cause of transient retinal function loss in MCAO and in strokes in humans.

In Chapter 3, I confirmed progesterone's protective effects in the brain in MCAO (as shown by reduced behavioral deficits and infarct size), and demonstrated that progesterone is also protective in retinal ischemia in MCAO (as shown by reduced ERG deficits, reduced GFAP and glutamine synthetase upregulation, and reduced retinal ganglion cell death). While progesterone treatment provided protection in retinal ischemia in the MCAO model, it was not protective in the rAION model. Further, progesterone protection after MCAO was greater in brain than in retina.

In Chapter 4, I demonstrated that increases in NF-κB pathway activation and inflammation occur in the retina as well as the brain after MCAO, with greater activation

in brain. I confirmed that progesterone treatment reduced inflammation after cerebral ischemia and demonstrated that progesterone also reduced inflammation after retinal ischemia in a rat model of MCAO. Progesterone's effects on inflammation were greater in brain than in retina, mirroring our functional results from Chapter 3. Levels of progesterone receptor were upregulated in brain but downregulated in retina after MCAO, which could explain why we see more robust progesterone protection in brain versus retina.

DISCUSSION: PROGESTERONE RECEPTORS IN BRAIN AND RETINA

Expression and function of classical progesterone receptors in the brain

Progesterone has been shown to cross the blood brain barrier (Sar and Stumpf, 1973) and to have diverse effects on behavior and injury in the brain (Schumacher et al., 2013). Some of these effects are mediated through progesterone binding to progesterone receptors (PRs) (Sakamoto et al., 2001b, 2002, Ghoumari et al., 2003, Ghoumari et al., 2005, Hussain et al., 2011). Two isoforms, PR-A and PR-B are referred to as "classical" progesterone receptors, with the "classical" progesterone pathway defined as progesterone binding to PRs which then form dimers, translocate to the nucleus, and act as transcription factors for a variety of genes (Schumacher et al., 2013). While PRs expressed in brain areas that function in reproduction (the hypothalamus, for example) act via the classical pathway (Schumacher et al., 2013), research has shown that PRs elsewhere in the brain are often not localized near the nucleus, and instead are expressed in synapses, dendrites, and axons (Waters et al., 2008). PRs distal to the nucleus may act at the plasma membrane or in the cytoplasm through interaction with intracellular

signaling pathways or kinases (Bagowski et al., 2001, Maller, 2001, Faivre and Lange, 2007, Boonyaratanakornkit et al., 2008, Faivre et al., 2008). Additionally, recent research shows that PR monomers may exhibit even greater activity than PR dimers (Jacobsen et al., 2009, Jacobsen and Horwitz, 2012).

PRs are expressed in a variety of areas throughout the nervous system, including the hypothalamus (Camacho-Arroyo et al., 1994, Guerra-Araiza et al., 2003), limbic structures (Guerra-Araiza et al., 2003), cerebellum (Guerra-Araiza et al., 2002), olfactory bulb (Guerra-Araiza et al., 2002), frontal cortex (Camacho-Arroyo et al., 1994, Guerra-Araiza et al., 2002), tempoparietal cortex (Camacho-Arroyo et al., 1994), occipital cortex (Camacho-Arroyo et al., 1994), striatum (Parsons et al., 1982), spinal cord (Labombarda et al., 2003), and peripheral nerves (Jung-Testas et al., 1996). PR levels in the brain have been shown to change with progesterone administration and injury, and these changes are area-specific (Camacho-Arroyo et al., 1994, Jung-Testas et al., 1996, Guerra-Araiza et al., 2002, Guerra-Araiza et al., 2003, Labombarda et al., 2003, Palliser et al., 2012).

Retinal expression of progesterone and its receptors

Progesterone has been identified in the human retina and optic nerve in both males and females (Lanthier and Patwardhan, 1986), and was observed in the rat retina in larger quantities than in plasma (Lanthier and Patwardhan, 1987). Progesterone synthesis and metabolism has been found to occur in the retina as well (Lanthier and Patwardhan, 1988, Guarneri et al., 1994, Sakamoto et al., 2001a, Coca-Prados et al., 2003, Cascio et al., 2007). PR mRNA has been identified in rat and rabbit retina and human retinal pigment epithelium samples (Wickham et al., 2000). PR has also been identified at the

protein level in mouse bipolar cells that were dissociated from the retina (Koulen et al., 2008). In Chapter 4, I confirmed these results by demonstrating detection of PR-A and PR-B at the protein level in the rat retina (Fig. 5-1).

While PRs were observed in the retina using western blots, PRs were not detected in retinal sections using immunohistochemistry (including frozen vs. paraffin sections, rat vs. mouse, and injured vs. uninjured) (Fig. 5-2). In the brain, PRs are expressed in higher levels in areas like the hypothalamus and olfactory bulb, and are difficult to detect in other areas using immunohistochemistry (Warembourg et al., 1986, Quadros et al., 2007, Lopez and Wagner, 2009). In non-reproductive brain areas, PRs were eventually identified in axons, dendrites, and synapses using immunoelectron microscopy (Waters et al., 2008), and this technique might prove the key to establishing PR localization in the retina as well.

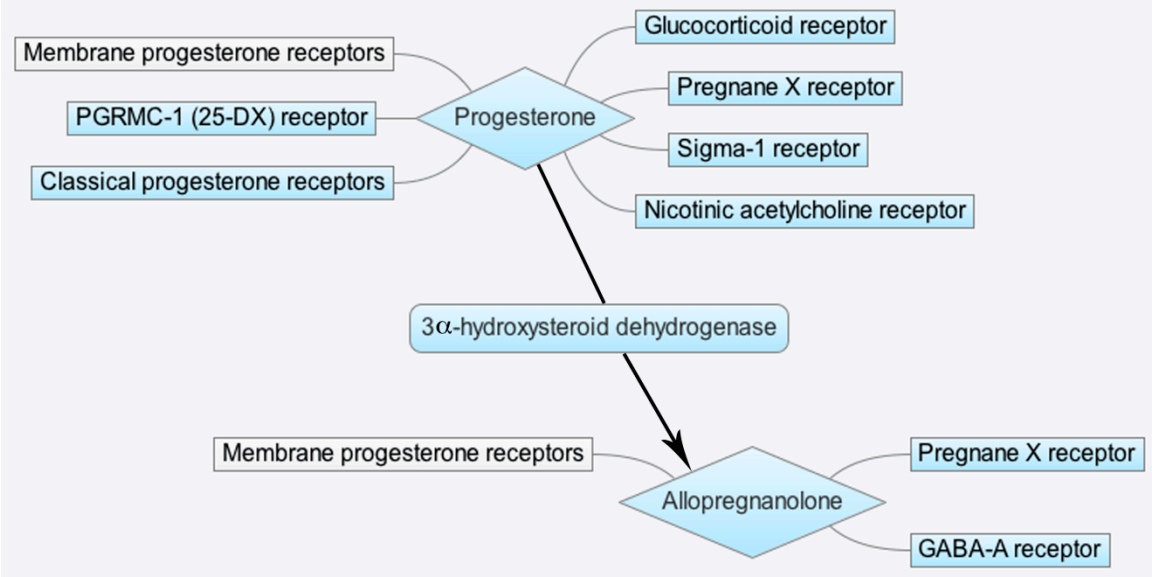


Figure 5-1. Receptors activated or inhibited by progesterone and allopregnanolone. A blue background designates that a receptor has been identified in the retina.

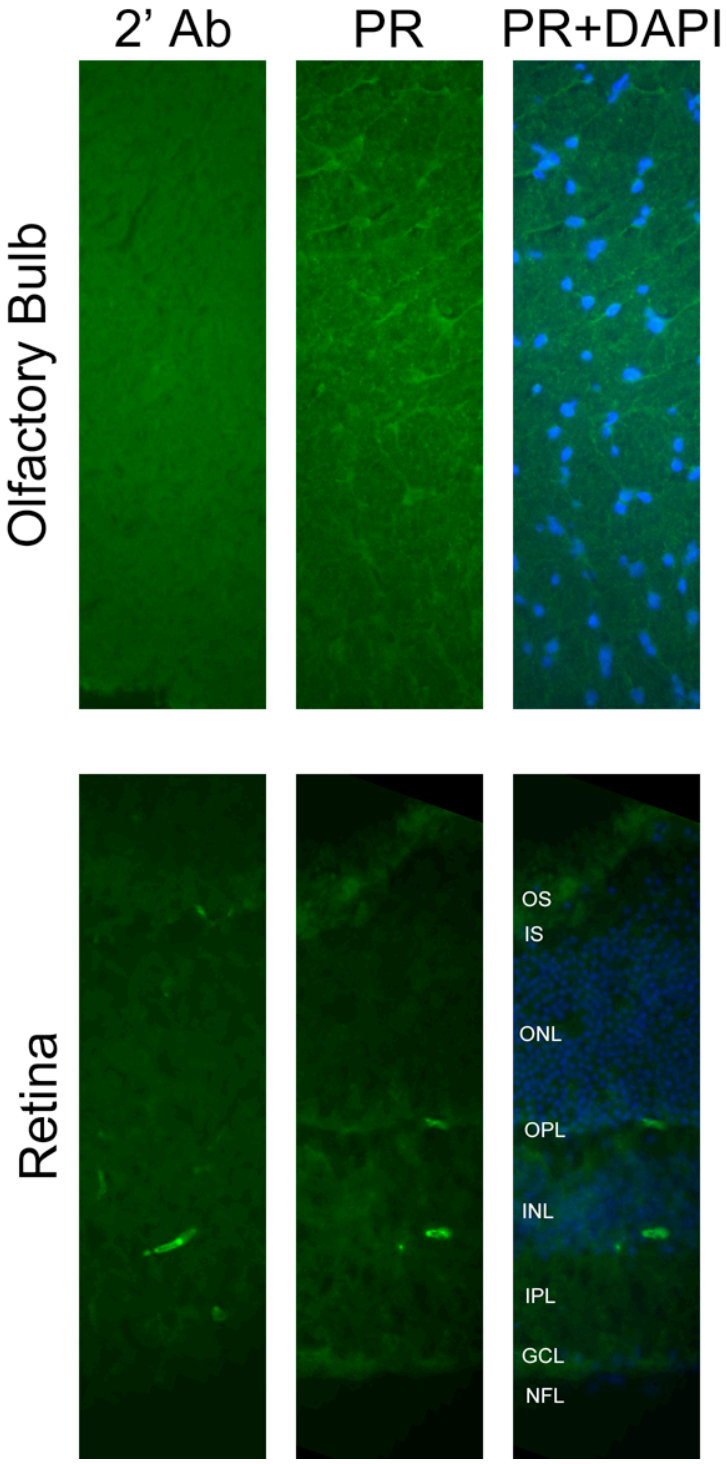


Figure 5-2. Representative micrographs depicting immunohistochemistry for PR in the olfactory bulb (PR staining observed) vs. retina (no PR staining observed). Sections in column 1 received secondary antibody only. Sections in column 2 received both secondary and PR antibody. Column 3 shows sections from column 2 merged with a nuclear stain (DAPI). NFL, Nerve Fiber Layer; GCL, Ganglion Cell Layer; IPL, Inner Plexiform Layer; INL, Inner Nuclear Layer; OPL, Outer Plexiform Layer; ONL, Outer Nuclear Layer; IS, Inner Segments; OS, Outer Segments.

Changes in PR expression with progesterone administration and injury

In the absence of injury, progesterone administration has been shown to down-regulate PR expression in estradiol-inducible areas (areas that show strong upregulation with estradiol treatment). These areas include the hypothalamus and some limbic structures, both of which downregulate PR upon progesterone treatment (Camacho-Arroyo et al., 1994, Guerra-Araiza et al., 2003). Intact brain areas that show no change in PR expression with progesterone administration include the cerebral cortex, cerebellum, spinal cord, and peripheral nerves (Jung-Testas et al., 1996, Guerra-Araiza et al., 2002, Labombarda et al., 2003).

Few studies have investigated the response of classical PRs to neural injury (Schumacher et al., 2013). Progesterone itself has been found to increase in the brain after traumatic brain injury in rats (Meffre et al., 2007), in serum after traumatic brain injury in humans (Wagner et al., 2011), and in both brain and serum after transient MCAO in mice (Liu et al., 2012a). Inflammation and glucose deprivation have also been found to increase progesterone levels (Elman and Breier, 1997, Zitzmann et al., 2005). Further, some central nervous system genes implicated in neuron function have been shown to exhibit sensitivity to progesterone only after injury (De Nicola et al., 2003, Schumacher et al., 2004). One study assessing PR levels in an intra-uterine growth restriction model showed increases in PR-A and PR-B expression in brains (Palliser et al., 2012). Another using the transient MCAO model showed no change in PR mRNA levels in brain after injury or with progesterone administration + injury (Dang et al., 2011). Conversely, in Chapter 4, I demonstrated an increase in protein levels of PR-A and PR-B in brain after transient MCAO. A difference between mRNA levels and protein

levels could explain these differential results. Following spinal cord injury, decreases in PR levels were observed that did not change with progesterone administration (Labombarda et al., 2003). Similarly, in Chapter 4, I demonstrated a decrease in protein levels of PR-A and PR-B in retina after transient MCAO, and this decrease was not affected by progesterone treatment. Combined, these results may indicate a difference in PR behavior after injury in peripheral neural tissue compared with the brain.

Different types of progesterone receptors in the nervous system

PR-A and PR-B expression and regulation in the brain differs by brain region, gender, age, and hormonal status (Kato et al., 1993, Camacho-Arroyo et al., 1994, Szabo et al., 2000, Inoue et al., 2001, Beyer et al., 2002, Guerra-Araiza et al., 2002). In experiments on PRs and reproduction, PR knock-out mice were used to show that PR-A is critical for normal ovarian and uterine function and PR-B is critical for normal mammary gland function and development (Conneely et al., 2001, Mulac-Jericevic and Conneely, 2004). PR-A and PR-B have also been shown to have important and differential effects in reproductive behavior (Mani et al., 2006, Guerra-Araiza et al., 2009). Little is known about differences in PR-A and PR-B effects in the nervous system (Schumacher et al., 2013). One study showed that PR-A and PR-B have different effects on expression of enzymes such as tyrosine hydroxylase and glutamic acid decarboxlyase that are known to play a role in metabolism of neurotransmitters (Gonzalez-Flores et al., 2011).

In addition to PR-A and PR-B, a truncated version of the receptor, PR-C, is non-functional and acts as an inhibitor both by binding available progesterone and by binding

directly to PR-B. An increased ratio of PR-C to other PRs reduces their activity (particularly with respect to PR-B) and may diminish progesterone's effects to prevent labor (Condon et al., 2006). An increased ratio of PR-A to PR-B may also be detrimental, specifically in regulation of inflammation (Equils et al., 2010). Levels of PR-C and ratios of PR-C to other PR isoforms and also PR-A to PR-B under normal conditions and after injury in the retina and brain remain to be studied, and this information could lead to a better understanding of progesterone's actions in the retina and brain.

In addition to classical PRs and PR-C, other receptors for progesterone, including membrane progesterone receptors (mPRs) α, β, and γ, and membrane-associated progesterone receptor component 1 (PGRMC1, also known as 25-Dx), have been identified in the nervous system and elsewhere (Labombarda et al., 2003, Meffre et al., 2005, Guennoun et al., 2008, Labombarda et al., 2010, Meffre et al., 2013). mPR is expressed in both brain and spinal cord and has been found to show differential expression in male and female mice and rats (Labombarda et al., 2010, Meffre et al., 2013). Normally, mPR is expressed only in neurons but after traumatic brain injury, mPR expression is induced in astrocytes, oligodendrocytes, and microglial cells. mPR expression does not change with progesterone treatment in uninjured animals (Meffre et al., 2013). Similarly, PGRMC1 is normally expressed in neurons and ventricular walls and its expression is induced in astrocytes and increased in neurons after traumatic brain injury. (Meffre et al., 2005, Guennoun et al., 2008). In spinal cord injury, however, PGRMC1 expression did not change after injury, but with progesterone treatment + injury was upregulated in neurons at both the mRNA and protein level (Labombarda et al., 2003). These experiments show that in membrane bound as well as classical PRs,

expression changes after injury differ based on tissue and could suggest differences in progesterone action in these tissues. PGRMC1 has been identified in the retina in Müller and retinal pigment epithelium cells (Swiatek-De Lange et al., 2007) (Fig. 5-1). It would be interesting to know whether mPRs are expressed in the retina as well and how mPRs and PGRMC1 respond to retinal injury and progesterone treatment.

DISCUSSION: PROGESTERONE'S MECHANISMS OF ACTION POST-INJURY – INVOLVEMENT OF PR AND OTHER RECEPTORS/PATHWAYS

Progesterone administration after injury has been shown to involve a variety of mechanisms (Schumacher et al., 2007, Sayeed and Stein, 2009, Schumacher et al., 2013). Initially, progesterone's interaction with PR was thought to be responsible for all of its effects (Allan et al., 1992, Leonhardt et al., 2003). Then, studies showed that treatment with progesterone's metabolite allopregnanolone achieved very similar neuroprotective effects (He et al., 2004a, He et al., 2004b, Djebaili et al., 2005, Sayeed et al., 2006). Because of these studies, much of progesterone research has been focused on allopregnanolone and its actions on GABA as the mechanism by which progesterone is neuroprotective (Schumacher et al., 2013). Now, it is thought that progesterone acts through a variety of mechanisms and signaling pathways, some PR-dependent and some PR-independent.

PR-dependent mechanisms

PR has been shown to be critical in mediating some of progesterone's protective effects. Nestorone, a progestin "which selectively activates PR", was found to be

protective in MCAO at low doses (Liu et al., 2012a). Additionally, progesterone treatment was not protective in PR knock-out mice in models of cerebral ischemia (Liu et al., 2012a) and spinal cord injury (Labombarda et al., 2013). While all mechanisms modulated by progesterone have not been investigated for PR-dependence, PR-dependent mechanisms were shown to include stimulation of dendrite and synapse formation and oligodendrocyte proliferation and differentiation using PR antagonists (Sakamoto et al., 2001b, 2002, Ghoumari et al., 2005) and myelination using PR knock-outs (Ghoumari et al., 2003, Hussain et al., 2011).

PR knock-out mice and heterozygotes exhibit increased sensitivity to injury in the MCAO model (Liu et al., 2012a). In PR knock-out mice, PR mRNA is completely absent, and in PR heterozygotes, PR mRNA levels in the cortex, subcortical regions, and hypothalamus are decreased by approximately 60% (Liu et al., 2012a). The finding that PR heterozygotes show diminished responses to progesterone treatment (Ghoumari et al., 2003, Hussain et al., 2011) suggests that levels of PR, and not simply PR presence, are important in the response to progesterone treatment post-injury.

In Chapter 4, I showed that PR-A and PR-B are upregulated in brain and downregulated in retina after ischemic injury. This difference in PR levels likely contributes to the greater effects of progesterone observed in the brain versus retina. I also showed that progesterone treatment had a more dramatic effect in reducing NF-κB pathway activation and inflammation in the brain versus the retina. These results may suggest that reduction of NF-κB pathway activation and reduction of inflammation by progesterone after injury are at least partially mediated by PR.

Research on PRs and inflammation in reproduction and breast cancer also supports PR involvement in NF-κB pathway regulation (Kalkhoven et al., 1996, Allport et al., 2001, Davies et al., 2004, Hardy et al., 2006, Nie et al., 2009, Kobayashi et al., 2010). In human labor, it is thought that NF-κB induces Cox-2 expression, which causes upregulation of prostaglandins in the uterus, inducing labor. High progesterone levels inhibit NF-κB and thus labor (Allport et al., 2001). High PR levels have also been shown to suppress NF-κB pathway activation and labor (Kalkhoven et al., 1996, Allport et al., 2001), and progesterone increases mRNA and protein levels of IκBα, the NF-κB inhibitor (Hardy et al., 2006) and inhibits NF-κB activation of Cox-2 (Hardy et al., 2006, Lei et al., 2012). Both PR-A and PR-B can inhibit the NF-κB pathway by binding to DNA to prevent NF-κB dimers from binding (Davies et al., 2004, Kobayashi et al., 2010), and PRs can bind to certain sequences even when unactivated while ligand binding is required for other sequences (Kobayashi et al., 2010). Additionally, PR-B, but not PR-A, has been shown to induce expression of other factors that inhibit NF-κB activity (Davies et al., 2004) and to bind to the p65 subunit of NF-κB (Kobayashi et al., 2010). PR-A and PR-B ablation increase NF-κB and Cox-2 activity (Hardy et al., 2006), so clearly PRs play a key role in this pathway. However, progesterone also inhibits inflammation via its actions at the glucocorticoid receptor, so multiple mechanisms could be involved (Lei et al., 2012).

PR-independent mechanisms

Allopregnanolone-mediated mechanisms

Allopregnanolone has been shown to have similar protective effects to progesterone in models of traumatic brain injury (He et al., 2004b, Djebaili et al., 2005, Sayeed et al., 2009) and Alzheimer's disease (Wang et al., 2010b, Chen et al., 2011, Singh et al., 2012). Further, allopregnanolone provides greater protection than progesterone following MCAO (Sayeed et al., 2006). Like progesterone, allopregnanolone binds to membrane PRs (Pang et al., 2013) and the pregnane X receptor (Langmade et al., 2006, Zhang et al., 2012b). Additionally, allopregnanolone achieves its protective effects even in PR knock-out mice, suggesting allopregnanolone acts through mechanisms that progesterone is unable to act through (Liu et al., 2012a, Labombarda et al., 2013). Indeed, allopregnanolone, but not progesterone, has been shown to directly inhibit mitochondrial permeability transition pore activity, leading to decreased cytochrome c release (Sayeed et al., 2009). Allopregnanolone's most well-known progesterone-independent effect is through its actions on the $GABA_A$ receptor, where it binds as a positive allosteric modulator (Reddy et al., 2005) Allopregnanolone was shown to protect in hippocampus by acting through $GABA_A$ receptors (Ishihara et al., 2013). Further, allopregnanolone's protective effects in a spinal cord injury model were blocked by a $GABA_A$ antagonist, providing additional support for allopregnanolone's actions being mediated through $GABA_A$ receptors (Labombarda et al., 2013).

In Chapter 3, I demonstrated that after retinal ischemic injury, progesterone had greater effects on reducing ischemia-induced upregulation of glutamine synthetase in Müller cells than on reducing retinal function deficits or retinal ganglion cell death or on reducing inflammation as shown in Chapter 4. GABAergic amacrine and horizontal cells are present in the retina and modulate the glutamatergic pathway of photoreceptor,

bipolar, and ganglion cells (Sharma & Ehinger, 2002) (Fig. 5-1). The robust effect on glutamine synthetase I observed with progesterone occurred even though PR is downregulated in the retina after injury, suggesting this change is mediated by allopregnanolone and that, while PR-mediated mechanisms are not fully responsive in the retina post-injury, allopregnanolone-mediated mechanisms are intact.

Allopregnanolone's positive actions on $GABA_A$ receptors may also explain why a smaller reduction in retinal function deficits than in cell death or glutamine synthetase upregulation was observed post-MCAO. Indeed, treatment with $GABA_A$ agonists has been shown to reduce a- wave and b- wave amplitudes and to completely abolish oscillatory potentials in ERGs in rats (Moller and Eysteinsson, 2003).

Other receptors/mechanisms

Treating with the enantiomer of progesterone, which does not bind to PR or get converted to allopregnanolone (Covey, 2009), still results in progesterone-like protective effects (VanLandingham et al., 2006), suggesting progesterone acts through an additional pathway or pathways that do not involve PRs or allopregnanolone's actions on GABA. Progesterone is known to act as a glucocorticoid antagonist, binding to the receptor at a separate site from glucocorticoids (Svec et al., 1989) and increasing the rate of dissociation (Svec et al., 1980). Progesterone has direct effects on the activity of the nicotinic acetylcholine receptor (Valera et al., 1992). Progesterone may mediate some of its anti-excitotoxic effects by acting as a Sigma-1 receptor antagonist (Monnet et al., 1995, Maurice et al., 1998, Su and Hayashi, 2003). Additionally, both progesterone and allopregnanolone are pregnane X receptor ligands (Kliewer et al., 1998, Langmade et al.,

2006), and are thought to induce pregnane X receptor activation of p-Glycoprotein (P-gp) to pump xenobiotic and cytotoxic substances out of the brain and retina across their respective barriers to provide a measure of functional protection (Zhang et al., 2012b).

In retina, glucocorticoid receptors are expressed in Müller cells (Gorovits et al., 1994), sigma-1 receptors are expressed in Müller and ganglion cells, in the inner nuclear layer and retinal pigment epithelium, and in photoreceptor outer segments (Ola et al., 2001, Jiang et al., 2006), nicotinic acetylcholine receptors are expressed in amacrine, ganglion, and a subset of bipolar cells (Yamada et al., 2003), and pregnane X receptors are expressed in retinal pigment epithelium (Zhang et al., 2012a, Zhang et al., 2012b), suggesting that progesterone has the potential to act through these mechanisms in retina as it does in brain (Fig. 5-1). However, my finding in Chapter 4 that pregnane X receptor is upregulated in brain and downregulated in retina after ischemic injury demonstrates that these receptors, while present, do not necessarily behave the same way in the retina as in the brain post-injury and with progesterone treatment. More research needs to be done to determine whether progesterone acts on all of these receptors in the retina and whether these actions match progesterone's actions in the brain.

FUTURE DIRECTIONS

The ideal progesterone dose and method of delivery must be determined for retinal disorders. Long-term effects of progesterone treatment in the retina should be studied as well. Progesterone's effects on VEGF and neovascularization must be further studied given that choroidal neovascularization causes vision loss. Finally, progesterone's ability to reduce intraocular pressure should be studied because progesterone has the

potential to both reduce intraocular pressure (Obal, 1950, Posthumus, 1952, Ziai et al., 1994, Panchami et al., 2013) and provide neuroprotection in glaucoma.

The research presented in this thesis and discussed in this chapter demonstrates that progesterone is a promising treatment for retinal ischemia. Additionally, by comparing ischemic injury and progesterone treatment in the retina and brain, this research illustrates the importance of PRs in the response of injured tissue to progesterone treatment and presents valuable information that may help us optimize progesterone treatment in retinal disorders and injury.

REFERENCES

Abdelkader M (2013) Multifocal electroretinogram in diabetic subjects. Saudi journal of ophthalmology : official journal of the Saudi Ophthalmological Society 27:87-96.

Acar N, Bonhomme B, Joffre C, Bron AM, Creuzot-Garcher C, Bretillon L, Doly M, Chardigny JM (2006) The retina is more susceptible than the brain and the liver to the incorporation of trans isomers of DHA in rats consuming trans isomers of alpha-linolenic acid. Reproduction, nutrition, development 46:515-525.

Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, Hauswirth WW, Bennett J (2001) Gene therapy restores vision in a canine model of childhood blindness. Nature genetics 28:92-95.

Adatia FA, Damji KF (2005) Chronic open-angle glaucoma. Review for primary care physicians. Canadian family physician Medecin de famille canadien 51:1229-1237.

Allan GF, Tsai SY, Tsai MJ, O'Malley BW (1992) Ligand-dependent conformational changes in the progesterone receptor are necessary for events that follow DNA binding. Proceedings of the National Academy of Sciences of the United States of America 89:11750-11754.

Allport VC, Pieber D, Slater DM, Newton R, White JO, Bennett PR (2001) Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'. Molecular human reproduction 7:581-586.

Altintas O, Iseri P, Ozkan B, Caglar Y (2008) Correlation between retinal morphological and functional findings and clinical severity in Parkinson's disease. Documenta ophthalmologica Advances in ophthalmology 116:137-146.

Andersen MB, Zimmer J, Sams-Dodd F (1999) Specific behavioral effects related to age and cerebral ischemia in rats. Pharmacology, biochemistry, and behavior 62:673-682.

Araiz JJ, Sanchez-vallejo V, Flores-Bellver M, Amal E, Romero FJ, Miranda M. (2012) Progesterone administration effects on an animal model of retinitis pigmentosa. The Association for Research in Vision and Ophthalmology Annual Meeting, USA Abstract No. A303.

Archibald NK, Clarke MP, Mosimann UP, Burn DJ (2009) The retina in Parkinson's disease. Brain : a journal of neurology 132:1128-1145.

Ardeshiri A, Kelley MH, Korner IP, Hurn PD, Herson PS (2006) Mechanism of progesterone neuroprotection of rat cerebellar Purkinje cells following oxygen-glucose deprivation. The European journal of neuroscience 24:2567-2574.

Armstrong RA (1996) Visual field defects in Alzheimer's disease patients may reflect differential pathology in the primary visual cortex. Optometry and vision science : official publication of the American Academy of Optometry 73:677-682.

Attella MJ, Nattinville A, Stein DG (1987) Hormonal state affects recovery from frontal cortex lesions in adult female rats. Behavioral and neural biology 48:352-367.

Avitabile T, Longo A, Caruso S, Gagliano C, Amato R, Scollo D, Lopes R, Pulvirenti L, Toto L, Torrisi B, Agnello C (2007) Changes in visual evoked potentials during the menstrual cycle in young women. Current eye research 32:999-1003.

Bagowski CP, Myers JW, Ferrell JE, Jr. (2001) The classical progesterone receptor associates with p42 MAPK and is involved in phosphatidylinositol 3-kinase signaling in Xenopus oocytes. The Journal of biological chemistry 276:37708-37714.

Bahring R, Standhardt H, Martelli EA, Grantyn R (1994) GABA-activated chloride currents of postnatal mouse retinal ganglion cells are blocked by acetylcholine and acetylcarnitine: how specific are ion channels in immature neurons? The European journal of neuroscience 6:1089-1099.

Baird PN, Richardson AJ, Robman LD, Dimitrov PN, Tikellis G, McCarty CA, Guymer RH (2006) Apolipoprotein (APOE) gene is associated with progression of age-related macular degeneration (AMD). Human mutation 27:337-342.

Balogh Z, Benedek G, Keri S (2008) Retinal dysfunctions in schizophrenia. Progress in neuro-psychopharmacology & biological psychiatry 32:297-300.

Barnett NL, Pow DV (2000) Antisense knockdown of GLAST, a glial glutamate transporter, compromises retinal function. Investigative ophthalmology & visual science 41:585-591.

Bauer B, Hartz AM, Fricker G, Miller DS (2004) Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood-brain barrier. Molecular pharmacology 66:413-419.

Baulieu EE, Robel P (1990) Neurosteroids: a new brain function? The Journal of steroid biochemistry and molecular biology 37:395-403.

Beal MF (1992) Mechanisms of excitotoxicity in neurologic diseases. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 6:3338-3344.

Beisswenger PJ (1976) Neuropathic and vascular complications occurring in diabetes. Postgraduate medicine 59:169-174.

Benavente O, Eliasziw M, Streifler JY, Fox AJ, Barnett HJ, Meldrum H (2001) Prognosis after transient monocular blindness associated with carotid-artery stenosis. The New England journal of medicine 345:1084-1090.

Berisha F, Feke GT, Trempe CL, McMeel JW, Schepens CL (2007) Retinal abnormalities in early Alzheimer's disease. Investigative ophthalmology & visual science 48:2285-2289.

Bernstein SL, Guo Y, Kelman SE, Flower RW, Johnson MA (2003) Functional and cellular responses in a novel rodent model of anterior ischemic optic neuropathy. Investigative ophthalmology & visual science 44:4153-4162.

Berti R, Williams AJ, Moffett JR, Hale SL, Velarde LC, Elliott PJ, Yao C, Dave JR, Tortella FC (2002) Quantitative real-time RT-PCR analysis of inflammatory gene expression associated with ischemia-reperfusion brain injury. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 22:1068-1079.

Beyer C, Damm N, Brito V, Kuppers E (2002) Developmental expression of progesterone receptor isoforms in the mouse midbrain. Neuroreport 13:877-880.

Bhargava M, Ikram MK, Wong TY (2012) How does hypertension affect your eyes? Journal of human hypertension 26:71-83.

Blanks JC, Schmidt SY, Torigoe Y, Porrello KV, Hinton DR, Blanks RH (1996) Retinal pathology in Alzheimer's disease. II. Regional neuron loss and glial changes in GCL. Neurobiology of aging 17:385-395.

Block F, Grommes C, Kosinski C, Schmidt W, Schwarz M (1997) Retinal ischemia induced by the intraluminal suture method in rats. Neuroscience letters 232:45-48.

Bodis-Wollner I, Yahr MD (1978) Measurements of visual evoked potentials in Parkinson's disease. Brain : a journal of neurology 101:661-671.

Boonyaratanakornkit V, Bi Y, Rudd M, Edwards DP (2008) The role and mechanism of progesterone receptor activation of extra-nuclear signaling pathways in regulating gene transcription and cell cycle progression. Steroids 73:922-928.

Bouet V, Boulouard M, Toutain J, Divoux D, Bernaudin M, Schumann-Bard P, Freret T (2009) The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice. Nature protocols 4:1560-1564.

Boycott BB, Hopkins JM (1981) Microglia in the retina of monkey and other mammals: its distinction from other types of glia and horizontal cells. Neuroscience 6:679-688.

Bringmann A, Iandiev I, Pannicke T, Wurm A, Hollborn M, Wiedemann P, Osborne NN, Reichenbach A (2009) Cellular signaling and factors involved in Müller cell gliosis: neuroprotective and detrimental effects. Progress in retinal and eye research 28:423-451.

Brock R (1956) Hypothermia and open cardiotomy. Proceedings of the Royal Society of Medicine 49:347-352.

Brust JCM (2000) Circulation of the brain. In: Kandel ER, Schwartz JH, Jessel TM, eds.

Principles of Neural Science, pp 1302-1316 New York, NY: The McGraw-Hill Companies, Inc.

Bulens C, Meerwaldt JD, Van der Wildt GJ (1988) Effect of stimulus orientation on contrast sensitivity in Parkinson's disease. Neurology 38:76-81.

Cai J, Qi X, Kociok N, Skosyrski S, Emilio A, Ruan Q, Han S, Liu L, Chen Z, Bowes Rickman C, Golde T, Grant MB, Saftig P, Serneels L, de Strooper B, Joussen AM, Boulton ME (2012) beta-Secretase (BACE1) inhibition causes retinal pathology by vascular dysregulation and accumulation of age pigment. EMBO molecular medicine 4:980-991.

Cai W, Zhu Y, Furuya K, Li Z, Sokabe M, Chen L (2008) Two different molecular mechanisms underlying progesterone neuroprotection against ischemic brain damage. Neuropharmacology 55:127-138.

Camacho-Arroyo I, Perez-Palacios G, Pasapera AM, Cerbon MA (1994) Intracellular progesterone receptors are differentially regulated by sex steroid hormones in the hypothalamus and the cerebral cortex of the rabbit. The Journal of steroid biochemistry and molecular biology 50:299-303.

Carmichael ST (2005) Rodent models of focal stroke: size, mechanism, and purpose. NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics 2:396-409.

Cascio C, Russo D, Drago G, Galizzi G, Passantino R, Guarneri R, Guarneri P (2007) 17beta-estradiol synthesis in the adult male rat retina. Experimental eye research 85:166-172.

CDC. The state of vision, aging, and public health in America. Centers for Disease Control and Prevention. Atlanta, GA: U.S. Department of Health and Human Services, 2011. Available at: http://www.cdc.gov/visionhealth/pdf/vision_brief.pdf [Last accessed 27 August 2013]

Chan JR, Phillips LJ, 2nd, Glaser M (1998) Glucocorticoids and progestins signal the initiation and enhance the rate of myelin formation. Proceedings of the National Academy of Sciences of the United States of America 95:10459-10464.

Chen S, Wang JM, Irwin RW, Yao J, Liu L, Brinton RD (2011) Allopregnanolone promotes regeneration and reduces beta-amyloid burden in a preclinical model of Alzheimer's disease. PloS one 6:e24293.

Chen X, Zhao Y, Zhou Z, Zhang X, Li Q, Bai L, Zhang M (2012) Prevalence and risk factors of diabetic retinopathy in Chongqing pre-diabetes patients. Eye (London, England) 26:816-820.

Cherradi N, Chambaz EM, Defaye G (1995) Organization of 3 beta-hydroxysteroid dehydrogenase/isomerase and cytochrome P450scc into a catalytically active molecular complex in bovine adrenocortical mitochondria. The Journal of steroid biochemistry and molecular biology 55:507-514.

Cheung AK, Lo AC, So KF, Chung SS, Chung SK (2007) Gene deletion and pharmacological inhibition of aldose reductase protect against retinal ischemic injury. Experimental eye research 85:608-616.

Chong AP, Aw SE (1986) Postmortem endocrine levels in the vitreous humor. Annals of the Academy of Medicine, Singapore 15:606-609.

Cioffi GA GE, Alm A (2002) Ocular circulation. In: Kaufman PL, Alm A, eds Adler's Physiology of the Eye, pp 319-349 St. Louis, MO: Mosby, Inc.

Coca-Prados M, Ghosh S, Wang Y, Escribano J, Herrala A, Vihko P (2003) Sex steroid hormone metabolism takes place in human ocular cells. The Journal of steroid biochemistry and molecular biology 86:207-216.

Cohen AJ, McGill PD, Rossetti RG, Guberski DL, Like AA (1987) Glomerulopathy in spontaneously diabetic rat. Impact of glycemic control. Diabetes 36:944-951.

Condon JC, Hardy DB, Kovaric K, Mendelson CR (2006) Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. Molecular endocrinology (Baltimore, Md) 20:764-775.

Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ (2001) Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. Molecular and cellular endocrinology 179:97-103.

Covey DF (2009) ent-Steroids: novel tools for studies of signaling pathways. Steroids 74:577-585.

Cronin-Golomb A, Rizzo JF, Corkin S, Growdon JH (1991) Visual function in Alzheimer's disease and normal aging. Annals of the New York Academy of Sciences 640:28-35.

Cubilla MA, Bachor TP, Bermudez V, Marquioni MD, Suburo AM (2012) Progesterone protects photoreceptors from light-induced damage. The Association for Research in Vision and Ophthalmology Annual Meeting, USA Abstract No. D943.

Cunha-Vaz JG (1976) The blood-retinal barriers. Documenta ophthalmologica Advances in ophthalmology 41:287-327.

Cunha-Vaz JG, Shakib M, Ashton N (1966) Studies on the permeability of the blood-retinal barrier. I. On the existence, development, and site of a blood-retinal barrier. The British journal of ophthalmology 50:441-453.

Cutler SM, Cekic M, Miller DM, Wali B, VanLandingham JW, Stein DG (2007) Progesterone improves acute recovery after traumatic brain injury in the aged rat. Journal of neurotrauma 24:1475-1486.

Cutler SM, Pettus EH, Hoffman SW, Stein DG (2005) Tapered progesterone withdrawal enhances behavioral and molecular recovery after traumatic brain injury. Experimental neurology 195:423-429.

Cutler SM, Vanlandingham JW, Stein DG (2006) Tapered progesterone withdrawal promotes long-term recovery following brain trauma. Experimental neurology 200:378-385.

Danesh-Meyer HV (2011) Neuroprotection in glaucoma: recent and future directions. Current opinion in ophthalmology 22:78-86.

Dang J, Mitkari B, Kipp M, Beyer C (2011) Gonadal steroids prevent cell damage and stimulate behavioral recovery after transient middle cerebral artery occlusion in male and female rats. Brain, behavior, and immunity 25:715-726.

Davies S, Dai D, Feldman I, Pickett G, Leslie KK (2004) Identification of a novel mechanism of NF-kappaB inactivation by progesterone through progesterone receptors in Hec50co poorly differentiated endometrial cancer cells: induction of A20 and ABIN-2. Gynecologic oncology 94:463-470.

De Nicola AF, Gonzalez-Deniselle MC, Garay L, Meyer M, Gargiulo-Monachelli G, Guennoun R, Schumacher M, Carreras MC, Poderoso JJ (2013) Progesterone Protective Effects In Neurodegeneration And Neuroinflammation. Journal of neuroendocrinology.

De Nicola AF, Labombarda F, Gonzalez SL, Gonzalez Deniselle MC, Guennoun R, Schumacher M (2003) Steroid effects on glial cells: detrimental or protective for spinal cord function? Annals of the New York Academy of Sciences 1007:317-328.

Dejneka NS, Surace EM, Aleman TS, Cideciyan AV, Lyubarsky A, Savchenko A, Redmond TM, Tang W, Wei Z, Rex TS, Glover E, Maguire AM, Pugh EN, Jr., Jacobson SG, Bennett J (2004) In utero gene therapy rescues vision in a murine model of congenital blindness. Molecular therapy : the journal of the American Society of Gene Therapy 9:182-188.

Dentchev T, Milam AH, Lee VM, Trojanowski JQ, Dunaief JL (2003) Amyloid-beta is found in drusen from some age-related macular degeneration retinas, but not in drusen from normal retinas. Molecular vision 9:184-190.

Deshpande AD, Harris-Hayes M, Schootman M (2008) Epidemiology of diabetes and diabetes-related complications. Physical therapy 88:1254-1264.

Devos D, Tir M, Maurage CA, Waucquier N, Defebvre L, Defoort-Dhellemmes S, Destee A (2005) ERG and anatomical abnormalities suggesting retinopathy in dementia with Lewy bodies. Neurology 65:1107-1110.

Ding J, Strachan MW, Reynolds RM, Frier BM, Deary IJ, Fowkes FG, Lee AJ, McKnight J, Halpin P, Swa K, Price JF (2010) Diabetic retinopathy and cognitive

decline in older people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. Diabetes 59:2883-2889.

Ding JD, Johnson LV, Herrmann R, Farsiu S, Smith SG, Groelle M, Mace BE, Sullivan P, Jamison JA, Kelly U, Harrabi O, Bollini SS, Dilley J, Kobayashi D, Kuang B, Li W, Pons J, Lin JC, Bowes Rickman C (2011) Anti-amyloid therapy protects against retinal pigmented epithelium damage and vision loss in a model of age-related macular degeneration. Proceedings of the National Academy of Sciences of the United States of America 108:E279-287.

Ding JD, Lin J, Mace BE, Herrmann R, Sullivan P, Bowes Rickman C (2008) Targeting age-related macular degeneration with Alzheimer's disease based immunotherapies: anti-amyloid-beta antibody attenuates pathologies in an age-related macular degeneration mouse model. Vision research 48:339-345.

Dirnagl U, Kaplan B, Jacewicz M, Pulsinelli W (1989) Continuous measurement of cerebral cortical blood flow by laser-Doppler flowmetry in a rat stroke model. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 9:589-596.

Djebaili M, Guo Q, Pettus EH, Hoffman SW, Stein DG (2005) The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. Journal of neurotrauma 22:106-118.

Doonan F, O'Driscoll C, Kenna P, Cotter TG (2011) Enhancing survival of photoreceptor cells in vivo using the synthetic progestin Norgestrel. Journal of neurochemistry 118:915-927.

Drew PD, Chavis JA (2000) Female sex steroids: effects upon microglial cell activation. Journal of neuroimmunology 111:77-85.

Duffy CJ, Tetewsky SJ, O'Brien H (2000) Cortical motion blindness in visuospatial AD. Neurobiology of aging 21:867-869; discussion 875-867.

Dunkin JM, Crum AV, Swanger RS, Bokhari SA (2011) Globe trauma. Seminars in ultrasound, CT, and MR 32:51-56.

Dvoriantchikova G, Barakat D, Brambilla R, Agudelo C, Hernandez E, Bethea JR, Shestopalov VI, Ivanov D (2009) Inactivation of astroglial NF-kappa B promotes survival of retinal neurons following ischemic injury. The European journal of neuroscience 30:175-185.

Dyck PJ, Kratz KM, Karnes JL, Litchy WJ, Klein R, Pach JM, Wilson DM, O'Brien PC, Melton LJ, 3rd, Service FJ (1993) The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study. Neurology 43:817-824.

Elman I, Breier A (1997) Effects of acute metabolic stress on plasma progesterone and testosterone in male subjects: relationship to pituitary-adrenocortical axis activation. Life sciences 61:1705-1712.

Engerman R, Bloodworth JM, Jr., Nelson S (1977) Relationship of microvascular disease in diabetes to metabolic control. Diabetes 26:760-769.

Engerman RL, Kern TS (1987) Progression of incipient diabetic retinopathy during good glycemic control. Diabetes 36:808-812.

Equils O, Nambiar P, Hobel CJ, Smith R, Simmons CF, Vali S (2010) A computer simulation of progesterone and Cox2 inhibitor treatment for preterm labor. PloS one 5:e8502.

Kandel ER (2000) Nerve cells and behavior. In: Kandel ER, Schwartz JH, Jessel TM, eds Principles of Neural Science. pp 19-35 New York, NY: The McGraw-Hill Companies, Inc.

Errera MH, Kohly RP, da Cruz L (2013) Pregnancy-associated retinal diseases and their management. Survey of ophthalmology 58:127-142.

Espinosa-Garcia C, Vigueras-Villasenor RM, Rojas-Castaneda JC, Aguilar-Hernandez A, Monfil T, Cervantes M, Morali G (2013) Post-ischemic administration of progesterone reduces caspase-3 activation and DNA fragmentation in the hippocampus following global cerebral ischemia. Neuroscience letters 550:98-103.

Faberowski N, Stefansson E, Davidson RC (1989) Local hypothermia protects the retina from ischemia. A quantitative study in the rat. Investigative ophthalmology & visual science 30:2309-2313.

Facchinetti F, Dawson VL, Dawson TM (1998) Free radicals as mediators of neuronal injury. Cellular and molecular neurobiology 18:667-682.

Faivre EJ, Daniel AR, Hillard CJ, Lange CA (2008) Progesterone receptor rapid signaling mediates serine 345 phosphorylation and tethering to specificity protein 1 transcription factors. Molecular endocrinology (Baltimore, Md) 22:823-837.

Faivre EJ, Lange CA (2007) Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained

activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. Molecular and cellular biology 27:466-480.

Falke P, Abela BM, Jr., Krakau CE, Lilja B, Lindgarde F, Maly P, Stavenow L (1991) High frequency of asymptomatic visual field defects in subjects with transient ischaemic attacks or minor strokes. Journal of internal medicine 229:521-525.

Fazzone HE, Kupersmith MJ, Leibmann J (2003) Does topical brimonidine tartrate help NAION? The British journal of ophthalmology 87:1193-1194.

Feeney DM, Baron JC (1986) Diaschisis. Stroke; a journal of cerebral circulation 17:817-830.

Ferguson SC, Blane A, Perros P, McCrimmon RJ, Best JJ, Wardlaw J, Deary IJ, Frier BM (2003) Cognitive ability and brain structure in type 1 diabetes: relation to microangiopathy and preceding severe hypoglycemia. Diabetes 52:149-156.

Frank RN, Turczyn TJ, Das A (1990) Pericyte coverage of retinal and cerebral capillaries. Investigative ophthalmology & visual science 31:999-1007.

Fujino T, Hamasaki DI (1967) Effect of intraocular pressure on the electroretinogram. Archives of ophthalmology 78:757-765.

Gerstein HC, Ambrosius WT, Danis R, Ismail-Beigi F, Cushman W, Calles J, Banerji M, Schubart U, Chew EY (2013) Diabetic retinopathy, its progression, and incident cardiovascular events in the ACCORD trial. Diabetes care 36:1266-1271.

Ghoumari AM, Baulieu EE, Schumacher M (2005) Progesterone increases oligodendroglial cell proliferation in rat cerebellar slice cultures. Neuroscience 135:47-58.

Ghoumari AM, Ibanez C, El-Etr M, Leclerc P, Eychenne B, O'Malley BW, Baulieu EE, Schumacher M (2003) Progesterone and its metabolites increase myelin basic protein expression in organotypic slice cultures of rat cerebellum. Journal of neurochemistry 86:848-859.

Gibson CL, Constantin D, Prior MJ, Bath PM, Murphy SP (2005) Progesterone suppresses the inflammatory response and nitric oxide synthase-2 expression following cerebral ischemia. Experimental neurology 193:522-530.

Gilmore GC, Wenk HE, Naylor LA, Koss E (1994) Motion perception and Alzheimer's disease. Journal of gerontology 49:P52-57.

Gonzalez-Flores O, Gomora-Arrati P, Garcia-Juarez M, Miranda-Martinez A, Armengual-Villegas A, Camacho-Arroyo I, Guerra-Araiza C (2011) Progesterone receptor isoforms differentially regulate the expression of tryptophan and tyrosine hydroxylase and glutamic acid decarboxylase in the rat hypothalamus. Neurochemistry international 59:671-676.

Gonzalez Deniselle MC, Garay L, Gonzalez S, Guennoun R, Schumacher M, De Nicola AF (2005) Progesterone restores retrograde labeling of cervical motoneurons in Wobbler mouse motoneuron disease. Experimental neurology 195:518-523.

Gonzalez SL, Labombarda F, Gonzalez Deniselle MC, Guennoun R, Schumacher M, De Nicola AF (2004) Progesterone up-regulates neuronal brain-derived neurotrophic factor expression in the injured spinal cord. Neuroscience 125:605-614.

Gorovits R, Ben-Dror I, Fox LE, Westphal HM, Vardimon L (1994) Developmental changes in the expression and compartmentalization of the glucocorticoid

receptor in embryonic retina. Proceedings of the National Academy of Sciences of the United States of America 91:4786-4790.

Grammas P, Riden M (2003) Retinal endothelial cells are more susceptible to oxidative stress and increased permeability than brain-derived endothelial cells. Microvascular research 65:18-23.

Grant AD, Chung SM (2013) The eye in pregnancy: ophthalmologic and neuro-ophthalmologic changes. Clinical obstetrics and gynecology 56:397-412.

Gronberg NV, Johansen FF, Kristiansen U, Hasseldam H (2013) Leukocyte infiltration in experimental stroke. Journal of neuroinflammation 10:115.

Guarneri P, Guarneri R, Cascio C, Pavasant P, Piccoli F, Papadopoulos V (1994) Neurosteroidogenesis in rat retinas. Journal of neurochemistry 63:86-96.

Guennoun R, Meffre D, Labombarda F, Gonzalez SL, Deniselle MC, Stein DG, De Nicola AF, Schumacher M (2008) The membrane-associated progesterone-binding protein 25-Dx: expression, cellular localization and up-regulation after brain and spinal cord injuries. Brain research reviews 57:493-505.

Guerra-Araiza C, Coyoy-Salgado A, Camacho-Arroyo I (2002) Sex differences in the regulation of progesterone receptor isoforms expression in the rat brain. Brain research bulletin 59:105-109.

Guerra-Araiza C, Gomora-Arrati P, Garcia-Juarez M, Armengual-Villegas A, Miranda-Martinez A, Lima-Hernandez FJ, Camacho-Arroyo I, Gonzalez-Flores O (2009) Role of progesterone receptor isoforms in female sexual behavior induced by progestins in rats. Neuroendocrinology 90:73-81.

Guerra-Araiza C, Villamar-Cruz O, Gonzalez-Arenas A, Chavira R, Camacho-Arroyo I (2003) Changes in progesterone receptor isoforms content in the rat brain during the oestrous cycle and after oestradiol and progesterone treatments. Journal of neuroendocrinology 15:984-990.

Guo Q, Sayeed I, Baronne LM, Hoffman SW, Guennoun R, Stein DG (2006) Progesterone administration modulates AQP4 expression and edema after traumatic brain injury in male rats. Experimental neurology 198:469-478.

Gupta N, Ang LC, Noel de Tilly L, Bidaisee L, Yucel YH (2006) Human glaucoma and neural degeneration in intracranial optic nerve, lateral geniculate nucleus, and visual cortex. The British journal of ophthalmology 90:674-678.

Haan M, Espeland MA, Klein BE, Casanova R, Gaussoin SA, Jackson RD, Millen AE, Resnick SM, Rossouw JE, Shumaker SA, Wallace R, Yaffe K (2012) Cognitive function and retinal and ischemic brain changes: the Women's Health Initiative. Neurology 78:942-949.

Hangai M, Yoshimura N, Honda Y (1996) Increased cytokine gene expression in rat retina following transient ischemia. Ophthalmic research 28:248-254.

Hardy DB, Janowski BA, Corey DR, Mendelson CR (2006) Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-kappaB activation of cyclooxygenase 2 expression. Molecular endocrinology (Baltimore, Md) 20:2724-2733.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science (New York, NY) 297:353-356.

Harnois C, Di Paolo T (1990) Decreased dopamine in the retinas of patients with Parkinson's disease. Investigative ophthalmology & visual science 31:2473-2475.

Hartong DT, Berson EL, Dryja TP (2006) Retinitis pigmentosa. Lancet 368:1795-1809.

Hartwick AT (2001) Beyond intraocular pressure: neuroprotective strategies for future glaucoma therapy. Optometry and vision science : official publication of the American Academy of Optometry 78:85-94.

Hartz AM, Miller DS, Bauer B (2010) Restoring blood-brain barrier P-glycoprotein reduces brain amyloid-beta in a mouse model of Alzheimer's disease. Molecular pharmacology 77:715-723.

Hayreh SS (1974) Anterior ischaemic optic neuropathy. I. Terminology and pathogenesis. The British journal of ophthalmology 58:955-963.

Hayreh SS (2009) Ischemic optic neuropathy. Progress in retinal and eye research 28:34-62.

Hayreh SS (2011) Vascular disorders in neuro-ophthalmology. Current opinion in neurology 24:6-11.

Hayreh SS, Weingeist TA (1980) Experimental occlusion of the central artery of the retina. IV: Retinal tolerance time to acute ischaemia. The British journal of ophthalmology 64:818-825.

He J, Evans CO, Hoffman SW, Oyesiku NM, Stein DG (2004a) Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Experimental neurology 189:404-412.

He J, Hoffman SW, Stein DG (2004b) Allopregnanolone, a progesterone metabolite, enhances behavioral recovery and decreases neuronal loss after traumatic brain injury. Restorative neurology and neuroscience 22:19-31.

Hebert M, Gagne AM, Paradis ME, Jomphe V, Roy MA, Merette C, Maziade M (2010) Retinal response to light in young nonaffected offspring at high genetic risk of neuropsychiatric brain disorders. Biological psychiatry 67:270-274.

Helenius J, Arsava EM, Goldstein JN, Cestari DM, Buonanno FS, Rosen BR, Ay H (2012) Concurrent acute brain infarcts in patients with monocular visual loss. Annals of neurology 72:286-293.

Hernandez-Falcon J, Schneider-Ehrenberg O, De la OMA, Campos-Lozada V, Fuentes-Pardo B (1997) Steroids modulate the excitability of crayfish (Procambarus digueti) photoreceptors: long- and short-term actions. General and comparative endocrinology 105:255-269.

Hill JK, Gunion-Rinker L, Kulhanek D, Lessov N, Kim S, Clark WM, Dixon MP, Nishi R, Stenzel-Poore MP, Eckenstein FP (1999) Temporal modulation of cytokine expression following focal cerebral ischemia in mice. Brain research 820:45-54.

Hillered L, Hallstrom A, Segersvard S, Persson L, Ungerstedt U (1989) Dynamics of extracellular metabolites in the striatum after middle cerebral artery occlusion in the rat monitored by intracerebral microdialysis. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 9:607-616.

Hof PR, Morrison JH (1990) Quantitative analysis of a vulnerable subset of pyramidal neurons in Alzheimer's disease: II. Primary and secondary visual cortex. The Journal of comparative neurology 301:55-64.

Holroyd S, Currie L, Wooten GF (2001) Prospective study of hallucinations and delusions in Parkinson's disease. Journal of neurology, neurosurgery, and psychiatry 70:734-738.

Hood DC, Birch DG (1990) A quantitative measure of the electrical activity of human rod photoreceptors using electroretinography. Visual neuroscience 5:379-387.

Hua F, Wang J, Sayeed I, Ishrat T, Atif F, Stein DG (2009) The TRIF-dependent signaling pathway is not required for acute cerebral ischemia/reperfusion injury in mice. Biochemical and biophysical research communications 390:678-683.

Hussain R, El-Etr M, Gaci O, Rakotomamonjy J, Macklin WB, Kumar N, Sitruk-Ware R, Schumacher M, Ghoumari AM (2011) Progesterone and Nestorone facilitate axon remyelination: a role for progesterone receptors. Endocrinology 152:3820-3831.

Hutton JT, Morris JL, Elias JW (1993) Levodopa improves spatial contrast sensitivity in Parkinson's disease. Archives of neurology 50:721-724.

Iandiev I, Pannicke T, Biedermann B, Wiedemann P, Reichenbach A, Bringmann A (2006) Ischemia-reperfusion alters the immunolocalization of glial aquaporins in rat retina. Neuroscience letters 408:108-112.

Ibanez C, Shields SA, El-Etr M, Baulieu EE, Schumacher M, Franklin RJ (2004) Systemic progesterone administration results in a partial reversal of the age-associated decline in CNS remyelination following toxin-induced demyelination in male rats. Neuropathology and applied neurobiology 30:80-89.

Ikeda H, Head GM, Ellis CJ (1994) Electrophysiological signs of retinal dopamine deficiency in recently diagnosed Parkinson's disease and a follow up study. Vision research 34:2629-2638.

Inoue T, Akahira JI, Takeyama J, Suzuki T, Darnel AD, Kaneko C, Kurokawa Y, Satomi S, Sasano H (2001) Spatial and topological distribution of progesterone receptor A and B isoforms during human development. Molecular and cellular endocrinology 182:83-89.

Inzelberg R, Ramirez JA, Nisipeanu P, Ophir A (2004) Retinal nerve fiber layer thinning in Parkinson disease. Vision research 44:2793-2797.

Iseri PK, Altinas O, Tokay T, Yuksel N (2006) Relationship between cognitive impairment and retinal morphological and visual functional abnormalities in Alzheimer disease. Journal of neuro-ophthalmology : the official journal of the North American Neuro-Ophthalmology Society 26:18-24.

Ishihara Y, Kawami T, Ishida A, Yamazaki T (2013) Allopregnanolone-mediated protective effects of progesterone on tributyltin-induced neuronal injury in rat hippocampal slices. The Journal of steroid biochemistry and molecular biology 135:1-6.

Ishizuka F, Shimazawa M, Umigai N, Ogishima H, Nakamura S, Tsuruma K, Hara H (2013) Crocetin, a carotenoid derivative, inhibits retinal ischemic damage in mice. European journal of pharmacology 703:1-10.

Ishrat T, Sayeed I, Atif F, Hua F, Stein DG (2010) Progesterone and allopregnanolone attenuate blood-brain barrier dysfunction following permanent focal ischemia by

regulating the expression of matrix metalloproteinases. Experimental neurology 226:183-190.

Ishrat T, Sayeed I, Atif F, Hua F, Stein DG (2012) Progesterone is neuroprotective against ischemic brain injury through its effects on the phosphoinositide 3-kinase/protein kinase B signaling pathway. Neuroscience 210:442-450.

Ishrat T, Sayeed I, Atif F, Stein DG (2009) Effects of progesterone administration on infarct volume and functional deficits following permanent focal cerebral ischemia in rats. Brain research 1257:94-101.

Jacobsen BM, Horwitz KB (2012) Progesterone receptors, their isoforms and progesterone regulated transcription. Molecular and cellular endocrinology 357:18-29.

Jacobsen BM, Jambal P, Schittone SA, Horwitz KB (2009) ALU repeats in promoters are position-dependent co-response elements (coRE) that enhance or repress transcription by dimeric and monomeric progesterone receptors. Molecular endocrinology (Baltimore, Md) 23:989-1000.

Jacobson SG, Aleman TS, Cideciyan AV, Sumaroka A, Schwartz SB, Windsor EA, Traboulsi EI, Heon E, Pittler SJ, Milam AH, Maguire AM, Palczewski K, Stone EM, Bennett J (2005) Identifying photoreceptors in blind eyes caused by RPE65 mutations: Prerequisite for human gene therapy success. Proceedings of the National Academy of Sciences of the United States of America 102:6177-6182.

Jager RD, Mieler WF, Miller JW (2008) Age-related macular degeneration. The New England journal of medicine 358:2606-2617.

Jiang G, Mysona B, Dun Y, Gnana-Prakasam JP, Pabla N, Li W, Dong Z, Ganapathy V, Smith SB (2006) Expression, subcellular localization, and regulation of sigma receptor in retinal Müller cells. Investigative ophthalmology & visual science 47:5576-5582.

Jiang SY, Zou YY, Wang JT (2012) p38 mitogen-activated protein kinase-induced nuclear factor kappa-light-chain-enhancer of activated B cell activity is required for neuroprotection in retinal ischemia/reperfusion injury. Molecular vision 18:2096-2106.

Johnson LN, Arnold AC (1994) Incidence of nonarteritic and arteritic anterior ischemic optic neuropathy. Population-based study in the state of Missouri and Los Angeles County, California. Journal of neuro-ophthalmology : the official journal of the North American Neuro-Ophthalmology Society 14:38-44.

Johnston SC (2002) Clinical practice. Transient ischemic attack. The New England journal of medicine 347:1687-1692.

Jung-Testas I, Schumacher M, Robel P, Baulieu EE (1996) Demonstration of progesterone receptors in rat Schwann cells. The Journal of steroid biochemistry and molecular biology 58:77-82.

Kabat H DC, Baher AB (1941) Recovery of function following arrest of the brain circulation. Am J Physiol 112:737-747.

Kaja S, Yang SH, Wei J, Fujitani K, Liu R, Brun-Zinkernagel AM, Simpkins JW, Inokuchi K, Koulen P (2003) Estrogen protects the inner retina from apoptosis and ischemia-induced loss of Vesl-1L/Homer 1c immunoreactive synaptic connections. Investigative ophthalmology & visual science 44:3155-3162.

Kaldi I, Berta A (2004) Progesterone administration fails to protect albino male rats against photostress-induced retinal degeneration. European journal of ophthalmology 14:306-314.

Kalesnykas G, Tuulos T, Uusitalo H, Jolkkonen J (2008) Neurodegeneration and cellular stress in the retina and optic nerve in rat cerebral ischemia and hypoperfusion models. Neuroscience 155:937-947.

Kalkhoven E, Wissink S, van der Saag PT, van der Burg B (1996) Negative interaction between the RelA(p65) subunit of NF-kappaB and the progesterone receptor. The Journal of biological chemistry 271:6217-6224.

Kalloniatis M (1995) Amino acids in neurotransmission and disease. Journal of the American Optometric Association 66:750-757.

Kalogeris T, Baines CP, Krenz M, Korthuis RJ (2012) Cell biology of ischemia/reperfusion injury. International review of cell and molecular biology 298:229-317.

Kaschula CH, Jin MH, Desmond-Smith NS, Travis GH (2006) Acyl CoA:retinol acyltransferase (ARAT) activity is present in bovine retinal pigment epithelium. Experimental eye research 82:111-121.

Kato J, Hirata S, Nozawa A, Mouri N (1993) The ontogeny of gene expression of progestin receptors in the female rat brain. The Journal of steroid biochemistry and molecular biology 47:173-182.

Kempster PA, Hurwitz B, Lees AJ (2007) A new look at James Parkinson's Essay on the Shaking Palsy. Neurology 69:482-485.

Keri S, Kelemen O, Benedek G, Janka Z (2004) Vernier threshold in patients with schizophrenia and in their unaffected siblings. Neuropsychology 18:537-542.

Klein R, Wang Q, Klein BE, Moss SE, Meuer SM (1995) The relationship of age-related maculopathy, cataract, and glaucoma to visual acuity. Investigative ophthalmology & visual science 36:182-191.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T, Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 92:73-82.

Knight SR, Davidson C, Young AM, Gibson CL (2012) Allopregnanolone protects against dopamine-induced striatal damage after in vitro ischaemia via interaction at GABA A receptors. Journal of neuroendocrinology 24:1135-1143.

Kobayashi S, Stice JP, Kazmin D, Wittmann BM, Kimbrel EA, Edwards DP, Chang CY, McDonnell DP (2010) Mechanisms of progesterone receptor inhibition of inflammatory responses in cellular models of breast cancer. Molecular endocrinology (Baltimore, Md) 24:2292-2302.

Korn H (2002) Further evidence for a reversed crossing of foveal projections in schizophrenic vulnerability. Medical hypotheses 58:305-311.

Koulen P, Madry C, Duncan RS, Hwang JY, Nixon E, McClung N, Gregg EV, Singh M (2008) Progesterone potentiates IP(3)-mediated calcium signaling through Akt/PKB. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 21:161-172.

Labombarda F, Ghoumari AM, Liere P, De Nicola AF, Schumacher M, Guennoun R (2013) Neuroprotection by steroids after neurotrauma in organotypic spinal cord cultures: a key role for progesterone receptors and steroidal modulators of GABAA receptors. Neuropharmacology 71:46-55.

Labombarda F, Gonzalez SL, Deniselle MC, Vinson GP, Schumacher M, De Nicola AF, Guennoun R (2003) Effects of injury and progesterone treatment on progesterone receptor and progesterone binding protein 25-Dx expression in the rat spinal cord. Journal of neurochemistry 87:902-913.

Labombarda F, Meffre D, Delespierre B, Krivokapic-Blondiaux S, Chastre A, Thomas P, Pang Y, Lydon JP, Gonzalez SL, De Nicola AF, Schumacher M, Guennoun R (2010) Membrane progesterone receptors localization in the mouse spinal cord. Neuroscience 166:94-106.

Lafuente MP, Villegas-Perez MP, Sobrado-Calvo P, Garcia-Aviles A, Miralles de Imperial J, Vidal-Sanz M (2001) Neuroprotective effects of alpha(2)-selective adrenergic agonists against ischemia-induced retinal ganglion cell death. Investigative ophthalmology & visual science 42:2074-2084.

Lambert BW (1968) The effects of progestins and estrogens on the permeability of the lens. Archives of ophthalmology 80:230-234.

Langmade SJ, Gale SE, Frolov A, Mohri I, Suzuki K, Mellon SH, Walkley SU, Covey DF, Schaffer JE, Ory DS (2006) Pregnane X receptor (PXR) activation: a mechanism for neuroprotection in a mouse model of Niemann-Pick C disease. Proceedings of the National Academy of Sciences of the United States of America 103:13807-13812.

Lanthier A, Patwardhan VV (1986) Sex steroids and 5-en-3 beta-hydroxysteroids in specific regions of the human brain and cranial nerves. Journal of steroid biochemistry 25:445-449.

Lanthier A, Patwardhan VV (1987) Effect of heterosexual olfactory and visual stimulation on 5-en-3 beta-hydroxysteroids and progesterone in the male rat brain. Journal of steroid biochemistry 28:697-701.

Lanthier A, Patwardhan VV (1988) In vitro steroid metabolism by rat retina. Brain research 463:403-406.

Laterra JG, G.W. (2000) Ventriular organization of cerebrospinal fluid: Blood-brain barrier, brain edema, and hydrocephalus. In: Principles of Neural Science (E.R. Kandel, J. H. S., & T.M. Jessell ed), pp 1288-1301 New York: McGraw-Hill.

Lavoie J, Maziade M, Hebert M (2014) The brain through the retina: The flash electroretinogram as a tool to investigate psychiatric disorders. Progress in neuro-psychopharmacology & biological psychiatry 48c:129-134.

Lee B, Choi Y, Kim H, Kim SY, Hahm DH, Lee HJ, Shim I (2003) Protective effects of methanol extract of Acori graminei rhizoma and Uncariae Ramulus et Uncus on ischemia-induced neuronal death and cognitive impairments in the rat. Life sciences 74:435-450.

Lee WW, Tajunisah I, Sharmilla K, Peyman M, Subrayan V (2013) Retinal nerve fiber layer structure abnormalities in schizophrenia and its relationship to disease state: evidence from optical coherence tomography. Investigative ophthalmology & visual science 54:7785-7792.

Lei K, Chen L, Georgiou EX, Sooranna SR, Khanjani S, Brosens JJ, Bennett PR, Johnson MR (2012) Progesterone acts via the nuclear glucocorticoid receptor to suppress IL-1beta-induced COX-2 expression in human term myometrial cells. PloS one 7:e50167.

Lei Y, Garrahan N, Hermann B, Fautsch MP, Johnson DH, Hernandez MR, Boulton M, Morgan JE (2011) Transretinal degeneration in ageing human retina: a multiphoton microscopy analysis. The British journal of ophthalmology 95:727-730.

Leonhardt SA, Boonyaratanakornkit V, Edwards DP (2003) Progesterone receptor transcription and non-transcription signaling mechanisms. Steroids 68:761-770.

Leuba G, Saini K (1995) Pathology of subcortical visual centres in relation to cortical degeneration in Alzheimer's disease. Neuropathology and applied neurobiology 21:410-422.

Li SY, Fu ZJ, Ma H, Jang WC, So KF, Wong D, Lo AC (2009) Effect of lutein on retinal neurons and oxidative stress in a model of acute retinal ischemia/reperfusion. Investigative ophthalmology & visual science 50:836-843.

Li XJ, He RF, Li S, Li XJ, Li DL (2012a) [Effects of progesterone on learning and memory and P2X7 receptor expression in the hippocampus after global cerebral ischemia/ reperfusion injury in rats]. Zhongguo ying yong sheng li xue za zhi = Zhongguo yingyong shenglixue zazhi = Chinese journal of applied physiology 28:472-475.

Li YC, Hayes S, Young AP (1997) Steroid hormone receptors activate transcription in glial cells of intact retina but not in primary cultures of retinal glial cells. Journal of molecular neuroscience : MN 8:145-158.

Li Z, Wang B, Kan Z, Zhang B, Yang Z, Chen J, Wang D, Wei H, Zhang JN, Jiang R (2012b) Progesterone increases circulating endothelial progenitor cells and induces neural regeneration after traumatic brain injury in aged rats. Journal of neurotrauma 29:343-353.

Lindsey JD, Weinreb RN (1994) Survival and differentiation of purified retinal ganglion cells in a chemically defined microenvironment. Investigative ophthalmology & visual science 35:3640-3648.

Lippman ME, Wiggert BO, Chader GJ, Thompson EB (1974) Glucocorticoid receptors. Characteristics, specificity, and ontogenesis in embryonic chick neural retina. The Journal of biological chemistry 249:5916-5917.

Littleton-Kearney MT, Klaus JA, Hurn PD (2005) Effects of combined oral conjugated estrogens and medroxyprogesterone acetate on brain infarction size after experimental stroke in rat. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 25:421-426.

Liu A, Margaill I, Zhang S, Labombarda F, Coqueran B, Delespierre B, Liere P, Marchand-Leroux C, O'Malley BW, Lydon JP, De Nicola AF, Sitruk-Ware R, Mattern C, Plotkine M, Schumacher M, Guennoun R (2012a) Progesterone receptors: a key for neuroprotection in experimental stroke. Endocrinology 153:3747-3757.

Liu Y, Tang L, Chen B (2012b) Effects of antioxidant gene therapy on retinal neurons and oxidative stress in a model of retinal ischemia/reperfusion. Free radical biology & medicine 52:909-915.

London NJ, Brown G (2011) Update and review of central retinal vein occlusion. Current opinion in ophthalmology 22:159-165.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke; a journal of cerebral circulation 20:84-91.

Lopez V, Wagner CK (2009) Progestin receptor is transiently expressed perinatally in neurons of the rat isocortex. The Journal of comparative neurology 512:124-139.

Lu N, Li C, Cheng Y, Du AL (2008) [Protective effects of progesterone against high intraocular pressure-induced retinal ischemia-reperfusion in rats]. Nan fang yi ke da xue xue bao = Journal of Southern Medical University 28:2026-2029.

Lu Y, Li Z, Zhang X, Ming B, Jia J, Wang R, Ma D (2010) Retinal nerve fiber layer structure abnormalities in early Alzheimer's disease: evidence in optical coherence tomography. Neuroscience letters 480:69-72.

Maller JL (2001) The elusive progesterone receptor in Xenopus oocytes. Proceedings of the National Academy of Sciences of the United States of America 98:8-10.

Mani SK, Reyna AM, Chen JZ, Mulac-Jericevic B, Conneely OM (2006) Differential response of progesterone receptor isoforms in hormone-dependent and -independent facilitation of female sexual receptivity. Molecular endocrinology (Baltimore, Md) 20:1322-1332.

Massey SC, Redburn DA (1987) Transmitter circuits in the vertebrate retina. Progress in neurobiology 28:55-96.

Matsui H, Udaka F, Tamura A, Oda M, Kubori T, Nishinaka K, Kameyama M (2006) Impaired visual acuity as a risk factor for visual hallucinations in Parkinson's disease. Journal of geriatric psychiatry and neurology 19:36-40.

Mattson MP (2004) Pathways towards and away from Alzheimer's disease. Nature 430:631-639.

Maurice T, Su TP, Privat A (1998) Sigma1 (sigma 1) receptor agonists and neurosteroids attenuate B25-35-amyloid peptide-induced amnesia in mice through a common mechanism. Neuroscience 83:413-428.

McClenaghan FC, Ezra DG, Holmes SB (2011) Mechanisms and management of vision loss following orbital and facial trauma. Current opinion in ophthalmology 22:426-431.

McKee AC, Au R, Cabral HJ, Kowall NW, Seshadri S, Kubilus CA, Drake J, Wolf PA (2006) Visual association pathology in preclinical Alzheimer disease. Journal of neuropathology and experimental neurology 65:621-630.

McKinnon SJ, Goldberg LD, Peeples P, Walt JG, Bramley TJ (2008) Current management of glaucoma and the need for complete therapy. The American journal of managed care 14:S20-27.

Mead GE, Lewis SC, Wardlaw JM, Dennis MS (2002) Comparison of risk factors in patients with transient and prolonged eye and brain ischemic syndromes. Stroke; a journal of cerebral circulation 33:2383-2390.

Meffre D, Delespierre B, Gouezou M, Leclerc P, Vinson GP, Schumacher M, Stein DG, Guennoun R (2005) The membrane-associated progesterone-binding protein 25-Dx is expressed in brain regions involved in water homeostasis and is up-regulated after traumatic brain injury. Journal of neurochemistry 93:1314-1326.

Meffre D, Labombarda F, Delespierre B, Chastre A, De Nicola AF, Stein DG, Schumacher M, Guennoun R (2013) Distribution of membrane progesterone receptor alpha in the male mouse and rat brain and its regulation after traumatic brain injury. Neuroscience 231:111-124.

Meffre D, Pianos A, Liere P, Eychenne B, Cambourg A, Schumacher M, Stein DG, Guennoun R (2007) Steroid profiling in brain and plasma of male and pseudopregnant female rats after traumatic brain injury: analysis by gas chromatography/mass spectrometry. Endocrinology 148:2505-2517.

Meier MH, Shalev I, Moffitt TE, Kapur S, Keefe RS, Wong TY, Belsky DW, Harrington H, Hogan S, Houts R, Caspi A, Poulton R (2013) Microvascular abnormality in schizophrenia as shown by retinal imaging. The American journal of psychiatry 170:1451-1459.

Meyer A (1956) Neuropathological aspects of anoxia. Proceedings of the Royal Society of Medicine 49:619-622.

Miller L, Hunt JS (1998) Regulation of TNF-alpha production in activated mouse macrophages by progesterone. Journal of immunology (Baltimore, Md : 1950) 160:5098-5104.

Miller N (1982) Anterior ischemic optic neuropathy. In: Walsh and Hoyts Neuro-Ophthalmology (Miller, N. R., ed), pp 212-226 Baltimore: Williams and Wilkins.

Modo M, Stroemer RP, Tang E, Veizovic T, Sowniski P, Hodges H (2000) Neurological sequelae and long-term behavioural assessment of rats with transient middle cerebral artery occlusion. Journal of neuroscience methods 104:99-109.

Moller A, Eysteinsson T (2003) Modulation of the components of the rat dark-adapted electroretinogram by the three subtypes of GABA receptors. Visual neuroscience 20:535-542.

Monnet FP, Mahe V, Robel P, Baulieu EE (1995) Neurosteroids, via sigma receptors, modulate the [3H]norepinephrine release evoked by N-methyl-D-aspartate in the rat hippocampus. Proceedings of the National Academy of Sciences of the United States of America 92:3774-3778.

Morley MG, Nguyen JK, Heier JS, Shingleton BJ, Pasternak JF, Bower KS (2010) Blast eye injuries: a review for first responders. Disaster medicine and public health preparedness 4:154-160.

Mosimann UP, Mather G, Wesnes KA, O'Brien JT, Burn DJ, McKeith IG (2004) Visual perception in Parkinson disease dementia and dementia with Lewy bodies. Neurology 63:2091-2096.

Mulac-Jericevic B, Conneely OM (2004) Reproductive tissue selective actions of progesterone receptors. Reproduction (Cambridge, England) 128:139-146.

Muniz A, Betts BS, Trevino AR, Buddavarapu K, Roman R, Ma JX, Tsin AT (2009) Evidence for two retinoid cycles in the cone-dominated chicken eye. Biochemistry 48:6854-6863.

Muniz A, Villazana-Espinoza ET, Thackeray B, Tsin AT (2006) 11-cis-Acyl-CoA:retinol O-acyltransferase activity in the primary culture of chicken Müller cells. Biochemistry 45:12265-12273.

Murphy SJ, Traystman RJ, Hurn PD, Duckles SP (2000) Progesterone exacerbates striatal stroke injury in progesterone-deficient female animals. Stroke; a journal of cerebral circulation 31:1173-1178.

Naarendorp F, Hitchock PF, Sieving PA (1993) Dopaminergic modulation of rod pathway signals does not affect the scotopic ERG of cat at dark-adapted threshold. Journal of neurophysiology 70:1681-1691.

Nakazawa T, Takahashi H, Shimura M (2006) Estrogen has a neuroprotective effect on axotomized RGCs through ERK signal transduction pathway. Brain research 1093:141-149.

National Eye Institutes. National Institutes of Health. Ref# NEA04. Available at: http://www.nei.nih.gov/photo/keyword.asp?conditions=Normal+Eye+Images&match=all [Last accessed 29 March 2014]

Neumann F, Wurm A, Linnertz R, Pannicke T, Iandiev I, Wiedemann P, Reichenbach A, Bringmann A (2010) Sex steroids inhibit osmotic swelling of retinal glial cells. Neurochemical research 35:522-530.

Nguyen-Legros J (1988) Functional neuroarchitecture of the retina: hypothesis on the dysfunction of retinal dopaminergic circuitry in Parkinson's disease. Surgical and radiologic anatomy : SRA 10:137-144.

Nie J, Lu Y, Liu X, Guo SW (2009) Immunoreactivity of progesterone receptor isoform B, nuclear factor kappaB, and IkappaBalpha in adenomyosis. Fertility and sterility 92:886-889.

Nilsen J, Brinton RD (2002) Impact of progestins on estrogen-induced neuroprotection: synergy by progesterone and 19-norprogesterone and antagonism by medroxyprogesterone acetate. Endocrinology 143:205-212.

Nilsen J, Morales A, Brinton RD (2006) Medroxyprogesterone acetate exacerbates glutamate excitotoxicity. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology 22:355-361.

O'Steen WK (1977) Ovarian steroid effects on light-induced retinal photoreceptor damage. Experimental eye research 25:361-369.

Obal A (1950) [Progesterone therapy in glaucoma]. Klinische Monatsblatter fur Augenheilkunde und fur augenarztliche Fortbildung 117:201-203.

Ofri R, Narfstrom K (2007) Light at the end of the tunnel? Advances in the understanding and treatment of glaucoma and inherited retinal degeneration. Veterinary journal (London, England : 1997) 174:10-22.

Okada H, Horibe H, Yoshiyuki O, Hayakawa N, Aoki N (1976) A prospective study of cerebrovascular disease in Japanese rural communities, Akabane and Asahi. Part 1: evaluation of risk factors in the occurrence of cerebral hemorrhage and thrombosis. Stroke; a journal of cerebral circulation 7:599-607.

Ola MS, Moore P, El-Sherbeny A, Roon P, Agarwal N, Sarthy VP, Casellas P, Ganapathy V, Smith SB (2001) Expression pattern of sigma receptor 1 mRNA

and protein in mammalian retina. Brain research Molecular brain research 95:86-95.

Onofrj M, Ghilardi MF, Basciani M, Gambi D (1986) Visual evoked potentials in parkinsonism and dopamine blockade reveal a stimulus-dependent dopamine function in humans. Journal of neurology, neurosurgery, and psychiatry 49:1150-1159.

Ordy JM, Wengenack TM, Bialobok P, Coleman PD, Rodier P, Baggs RB, Dunlap WP, Kates B (1993) Selective vulnerability and early progression of hippocampal CA1 pyramidal cell degeneration and GFAP-positive astrocyte reactivity in the rat four-vessel occlusion model of transient global ischemia. Experimental neurology 119:128-139.

Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M, Melena J (2004) Retinal ischemia: mechanisms of damage and potential therapeutic strategies. Progress in retinal and eye research 23:91-147.

Ozacmak VH, Sayan H (2009) The effects of 17beta estradiol, 17alpha estradiol and progesterone on oxidative stress biomarkers in ovariectomized female rat brain subjected to global cerebral ischemia. Physiological research / Academia Scientiarum Bohemoslovaca 58:909-912.

Palliser HK, Yates DM, Hirst JJ (2012) Progesterone receptor isoform expression in response to in utero growth restriction in the fetal guinea pig brain. Neuroendocrinology 96:60-67.

Panchami, Pai SR, Shenoy JP, J S, Kole SB (2013) Postmenopausal intraocular pressure changes in South Indian females. Journal of clinical and diagnostic research : JCDR 7:1322-1324.

Pang IH, Clark AF (2007) Rodent models for glaucoma retinopathy and optic neuropathy. Journal of glaucoma 16:483-505.

Pang Y, Dong J, Thomas P (2013) Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors delta and {epsilon} (mPRdelta and mPR{epsilon}) and mPRdelta involvement in neurosteroid inhibition of apoptosis. Endocrinology 154:283-295.

Paquet C, Boissonnot M, Roger F, Dighiero P, Gil R, Hugon J (2007) Abnormal retinal thickness in patients with mild cognitive impairment and Alzheimer's disease. Neuroscience letters 420:97-99.

Parisi V, Manni G, Spadaro M, Colacino G, Restuccia R, Marchi S, Bucci MG, Pierelli F (1999) Correlation between morphological and functional retinal impairment in multiple sclerosis patients. Investigative ophthalmology & visual science 40:2520-2527.

Parke DW, 3rd, Flynn HW, Jr., Fisher YL (2013) Management of intraocular foreign bodies: a clinical flight plan. Canadian journal of ophthalmology Journal canadien d'ophtalmologie 48:8-12.

Parkinson J (2002) An essay on the shaking palsy. 1817. The Journal of neuropsychiatry and clinical neurosciences 14:223-236; discussion 222.

Parsons B, Rainbow TC, MacLusky NJ, McEwen BS (1982) Progestin receptor levels in rat hypothalamic and limbic nuclei. The Journal of neuroscience : the official journal of the Society for Neuroscience 2:1446-1452.

Parver LM (1986) Eye trauma. The neglected disorder. Archives of ophthalmology 104:1452-1453.

Penfold PL, Madigan MC, Gillies MC, Provis JM (2001) Immunological and aetiological aspects of macular degeneration. Progress in retinal and eye research 20:385-414.

Penn RD, Hagins WA (1969) Signal transmission along retinal rods and the origin of the electroretinographic a-wave. Nature 223:201-204.

Peppe A, Stanzione P, Pierelli F, De Angelis D, Pierantozzi M, Bernardi G (1995) Visual alterations in de novo Parkinson's disease: pattern electroretinogram latencies are more delayed and more reversible by levodopa than are visual evoked potentials. Neurology 45:1144-1148.

Perlman JI, McCole SM, Pulluru P, Chang CJ, Lam TT, Tso MO (1996) Disturbances in the distribution of neurotransmitters in the rat retina after ischemia. Current eye research 15:589-596.

Petito CK, Chung MC, Verkhovsky LM, Cooper AJ (1992) Brain glutamine synthetase increases following cerebral ischemia in the rat. Brain research 569:275-280.

Posthumus RG (1952) [The use and the possibilities of progesterone in the treatment of glaucoma]. Ophthalmologica Journal international d'ophtalmologie International journal of ophthalmology Zeitschrift fur Augenheilkunde 124:17-25.

Pow DV, Barnett NL (1999) Changing patterns of spatial buffering of glutamate in developing rat retinae are mediated by the Müller cell glutamate transporter GLAST. Cell and tissue research 297:57-66.

Prevent Blindness America. The economic impact of vision problems: The toll of major adult eye disorders, visual impairment, and blindness on the U.S. economy. Chicago, IL, 2007. Available at: http://costofvision.preventblindness.org/downloads [Last accessed 27 August 2013]

Price MJ, Drance SM, Price M, Schulzer M, Douglas GR, Tansley B (1988) The pattern electroretinogram and visual-evoked potential in glaucoma. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie 226:542-547.

Puia G, Belelli D (2001) Neurosteroids on our minds. Trends in pharmacological sciences 22:266-267.

Puig N, Davalos A, Adan J, Piulats J, Martinez JM, Castillo J (2000) Serum amino acid levels after permanent middle cerebral artery occlusion in the rat. Cerebrovascular diseases (Basel, Switzerland) 10:449-454.

Qiu C, Cotch MF, Sigurdsson S, Klein R, Jonasson F, Klein BE, Garcia M, Jonsson PV, Harris TB, Eiriksdottir G, Kjartansson O, van Buchem MA, Gudnason V, Launer LJ (2009) Microvascular lesions in the brain and retina: The age, gene/environment susceptibility-Reykjavik study. Annals of neurology 65:569-576.

Quadros PS, Pfau JL, Wagner CK (2007) Distribution of progesterone receptor immunoreactivity in the fetal and neonatal rat forebrain. The Journal of comparative neurology 504:42-56.

Reddy DS, O'Malley BW, Rogawski MA (2005) Anxiolytic activity of progesterone in progesterone receptor knockout mice. Neuropharmacology 48:14-24.

Reinecke RD, Kuwabara T, Cogan DG, Weis DR (1962) Retinal vascular patterns. V. Experimental ischemia of the cat eye. Archives of ophthalmology 67:470-475.

Robson JG, Frishman LJ (1998) Dissecting the dark-adapted electroretinogram. Documenta ophthalmologica Advances in ophthalmology 95:187-215.

Rodieck RW, ed. (1998) Eyes. In: The First Steps in Seeing. Sunderland, MA: Sinauer Associates, Inc; 20-35.

Roof RL, Duvdevani R, Braswell L, Stein DG (1994) Progesterone facilitates cognitive recovery and reduces secondary neuronal loss caused by cortical contusion injury in male rats. Experimental neurology 129:64-69.

Roof RL, Duvdevani R, Stein DG (1992) Progesterone treatment attenuates brain edema following contusion injury in male and female rats. Restorative neurology and neuroscience 4:425-427.

Rumelt S, Dorenboim Y, Rehany U (1999) Aggressive systematic treatment for central retinal artery occlusion. American journal of ophthalmology 128:733-738.

Ryan CM, Williams TM, Finegold DN, Orchard TJ (1993) Cognitive dysfunction in adults with type 1 (insulin-dependent) diabetes mellitus of long duration: effects of recurrent hypoglycaemia and other chronic complications. Diabetologia 36:329-334.

Sadun AA, Bassi CJ (1990) Optic nerve damage in Alzheimer's disease. Ophthalmology 97:9-17.

Sakamoto H, Ukena K, Tsutsui K (2001a) Activity and localization of 3beta-hydroxysteroid dehydrogenase/ Delta5-Delta4-isomerase in the zebrafish central nervous system. The Journal of comparative neurology 439:291-305.

Sakamoto H, Ukena K, Tsutsui K (2001b) Effects of progesterone synthesized de novo in the developing Purkinje cell on its dendritic growth and synaptogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:6221-6232.

Sakamoto H, Ukena K, Tsutsui K (2002) Dendritic spine formation in response to progesterone synthesized de novo in the developing Purkinje cell in rats. Neuroscience letters 322:111-115.

Sanchez-vallejo V, Benlloch-Navarro S, Alvarez-Nolting R, Romero FJ, Miranda M. (2013) Progesterone administration decreases glutamate concentration in the retina of an animal model of retinitis pigmentosa. . The Association for Research in Vision and Ophthalmology Annual Meeting, USA Abstract No. A0133.

Sar M, Stumpf WE (1973) Neurons of the hypothalamus concentrate (3H)progesterone or its metabolites. Science (New York, NY) 182:1266-1268.

Sayeed I, Guo Q, Hoffman SW, Stein DG (2006) Allopregnanolone, a progesterone metabolite, is more effective than progesterone in reducing cortical infarct volume after transient middle cerebral artery occlusion. Annals of emergency medicine 47:381-389.

Sayeed I, Parvez S, Wali B, Siemen D, Stein DG (2009) Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism for better neuroprotective effects of allopregnanolone over progesterone. Brain research 1263:165-173.

Sayeed I, Stein DG (2009) Progesterone as a neuroprotective factor in traumatic and ischemic brain injury. Progress in brain research 175:219-237.

Sayeed I, Wali B, Stein DG (2007) Progesterone inhibits ischemic brain injury in a rat model of permanent middle cerebral artery occlusion. Restorative neurology and neuroscience 25:151-159.

Schallner N, Fuchs M, Schwer CI, Loop T, Buerkle H, Lagreze WA, van Oterendorp C, Biermann J, Goebel U (2012) Postconditioning with inhaled carbon monoxide counteracts apoptosis and neuroinflammation in the ischemic rat retina. PloS one 7:e46479.

Schlosshauer B (2007) Blood-retina barriers. In: Lajtha, A, ed Handbook of neurochemistry and molecular neurobiology: neural membranes and transport, pp 485-506 Springer-Verlag Berlin Heidelberg.

Schmidt-Kastner R, Truettner J, Zhao W, Belayev L, Krieger C, Busto R, Ginsberg MD (2000) Differential changes of bax, caspase-3 and p21 mRNA expression after transient focal brain ischemia in the rat. Brain research Molecular brain research 79:88-101.

Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M (1999) NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nature medicine 5:554-559.

Schumacher M, Guennoun R, Robert F, Carelli C, Gago N, Ghoumari A, Gonzalez Deniselle MC, Gonzalez SL, Ibanez C, Labombarda F, Coirini H, Baulieu EE, De Nicola AF (2004) Local synthesis and dual actions of progesterone in the nervous system: neuroprotection and myelination. Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society 14 Suppl A:S18-33.

Schumacher M, Guennoun R, Stein DG, De Nicola AF (2007) Progesterone: therapeutic opportunities for neuroprotection and myelin repair. Pharmacology & therapeutics 116:77-106.

Schumacher M, Mattern C, Ghoumari A, Oudinet JP, Liere P, Labombarda F, Sitruk-Ware R, De Nicola AF, Guennoun R (2013) Revisiting the roles of progesterone and allopregnanolone in the nervous system: Resurgence of the progesterone receptors. Progress in neurobiology.

Schwartz J (2000) Neurotransmitters. In: Kandel ER, Schwartz JH, Jessel TM, eds Principles of Neural Science, pp 523-547 New York, NY: The McGraw-Hill Companies, Inc.

Schwartz M (2005) Lessons for glaucoma from other neurodegenerative diseases: can one treatment suit them all? Journal of glaucoma 14:321-323.

Scott R (2011) The injured eye. Philosophical transactions of the Royal Society of London Series B, Biological sciences 366:251-260.

Seddon JM, Cote J, Rosner B (2003) Progression of age-related macular degeneration: association with dietary fat, transunsaturated fat, nuts, and fish intake. Archives of ophthalmology 121:1728-1737.

Seligman MEP, Walker EW, Rosenhan DL (2001a) Late-onset disorders. In: Abnormal Psychology, pp 468-498 New York, NY: WW Norton & Company, Inc.

Seligman MEP, Walker EW, Rosenhan DL (2001b) The Schizophrenias. In: Abnormal Psychology, pp 414-466 New York, NY: WW Norton & Company, Inc.

Sharma RK, Ehinger BEJ (2002) Development and structure of the retina. In: Kaufman PL, Alm A, eds. Adler's Physiology of the Eye. St. Louis, MO: Mosby, Inc.

Shechter R, London A, Schwartz M (2013) Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates. Nature reviews Immunology 13:206-218.

Silva MF, Faria P, Regateiro FS, Forjaz V, Januario C, Freire A, Castelo-Branco M (2005) Independent patterns of damage within magno-, parvo- and koniocellular pathways in Parkinson's disease. Brain : a journal of neurology 128:2260-2271.

Sim D, Fruttiger M (2013) Keeping blood vessels out of sight. eLife 2:e00948.

Singh C, Liu L, Wang JM, Irwin RW, Yao J, Chen S, Henry S, Thompson RF, Brinton RD (2012) Allopregnanolone restores hippocampal-dependent learning and memory and neural progenitor survival in aging 3xTgAD and nonTg mice. Neurobiology of aging 33:1493-1506.

Slepyan DH, Rankin RM, Stahler C, Jr., Gibbons GE (1975) Amaurosis fugax: a clinical comparison. Stroke; a journal of cerebral circulation 6:493-496.

Smith RS, John SWM, Nishina PM (2002) Posterior segment and orbit. In: Smith RS, John SWM, Nishina PM, Sundberg JP, eds Systematic Evaluation of the Mouse Eye: Anatomy, Pathology, and Biomethods, pp 25– 44 Boca Raton, FL: CRC Press.

Souidi M, Gueguen Y, Linard C, Dudoignon N, Grison S, Baudelin C, Marquette C, Gourmelon P, Aigueperse J, Dublineau I (2005) In vivo effects of chronic contamination with depleted uranium on CYP3A and associated nuclear receptors PXR and CAR in the rat. Toxicology 214:113-122.

Stanford JR, Lubow M, Vasko JS (1978) Prevention of stroke by carotid endarterectomy. Surgery 83:259-263.

Steele EC, Jr., Guo Q, Namura S (2008) Filamentous middle cerebral artery occlusion causes ischemic damage to the retina in mice. Stroke; a journal of cerebral circulation 39:2099-2104.

Stein DG, Finger S, Hart T (1983) Brain damage and recovery: problems and perspectives. Behavioral and neural biology 37:185-222.

Stein DG, Wright DW (2010) Progesterone in the clinical treatment of acute traumatic brain injury. Expert opinion on investigational drugs 19:847-857.

Stephenson D, Yin T, Smalstig EB, Hsu MA, Panetta J, Little S, Clemens J (2000) Transcription factor nuclear factor-kappa B is activated in neurons after focal cerebral ischemia. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 20:592-603.

Steuer H, Jaworski A, Elger B, Kaussmann M, Keldenich J, Schneider H, Stoll D, Schlosshauer B (2005) Functional characterization and comparison of the outer blood-retina barrier and the blood-brain barrier. Investigative ophthalmology & visual science 46:1047-1053.

Storey J (2003) The positive false discovery rate: a Bayesian interpretation and the q-value. Ann Statist 31:2013-2035.

Stowell C, Wang L, Arbogast B, Lan JQ, Cioffi GA, Burgoyne CF, Zhou A (2010) Retinal proteomic changes under different ischemic conditions - implication of an epigenetic regulatory mechanism. International journal of physiology, pathophysiology and pharmacology 2:148-160.

Sturrock GD, Mueller HR (1984) Chronic ocular ischaemia. The British journal of ophthalmology 68:716-723.

Su TP, Hayashi T (2003) Understanding the molecular mechanism of sigma-1 receptors: towards a hypothesis that sigma-1 receptors are intracellular amplifiers for signal transduction. Current medicinal chemistry 10:2073-2080.

Sullivan DA, Jensen RV, Suzuki T, Richards SM (2009) Do sex steroids exert sex-specific and/or opposite effects on gene expression in lacrimal and meibomian glands? Molecular vision 15:1553-1572.

Suzuki T, Schirra F, Richards SM, Treister NS, Lombardi MJ, Rowley P, Jensen RV, Sullivan DA (2006) Estrogen's and progesterone's impact on gene expression in the mouse lacrimal gland. Investigative ophthalmology & visual science 47:158-168.

Svec F, Teubner V, Tate D (1989) Location of the second steroid-binding site on the glucocorticoid receptor. Endocrinology 125:3103-3108.

Svec F, Yeakley J, Harrison RW, 3rd (1980) Progesterone enhances glucocorticoid dissociation from the AtT-20 cell glucocorticoid receptor. Endocrinology 107:566-572.

Swiatek-De Lange M, Stampfl A, Hauck SM, Zischka H, Gloeckner CJ, Deeg CA, Ueffing M (2007) Membrane-initiated effects of progesterone on calcium

dependent signaling and activation of VEGF gene expression in retinal glial cells. Glia 55:1061-1073.

Szabo M, Kilen SM, Nho SJ, Schwartz NB (2000) Progesterone receptor A and B messenger ribonucleic acid levels in the anterior pituitary of rats are regulated by estrogen. Biology of reproduction 62:95-102.

Tabak AG, Herder C, Rathmann W, Brunner EJ, Kivimaki M (2012) Prediabetes: a high-risk state for diabetes development. Lancet 379:2279-2290.

Tanaka H, Araki M, Masuzawa T (1992) Reaction of astrocytes in the gerbil hippocampus following transient ischemia: immunohistochemical observations with antibodies against glial fibrillary acidic protein, glutamine synthetase, and S-100 protein. Experimental neurology 116:264-274.

Tatton WG, Kwan MM, Verrier MC, Seniuk NA, Theriault E (1990) MPTP produces reversible disappearance of tyrosine hydroxylase-containing retinal amacrine cells. Brain research 527:21-31.

Thaker GK, Avila MT, Hong EL, Medoff DR, Ross DE, Adami HM (2003) A model of smooth pursuit eye movement deficit associated with the schizophrenia phenotype. Psychophysiology 40:277-284.

Thomas P, Pang Y, Dong J, Groenen P, Kelder J, de Vlieg J, Zhu Y, Tubbs C (2007) Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology 148:705-718.

Thomas T, Rhodin J, Clark L, Garces A (2003) Progestins initiate adverse events of menopausal estrogen therapy. Climacteric : the journal of the International Menopause Society 6:293-301.

Trick GL, Kaskie B, Steinman SB (1994) Visual impairment in Parkinson's disease: deficits in orientation and motion discrimination. Optometry and vision science : official publication of the American Academy of Optometry 71:242-245.

Trick GL, Trick LR, Morris P, Wolf M (1995) Visual field loss in senile dementia of the Alzheimer's type. Neurology 45:68-74.

Tsutsui K (2006) Biosynthesis and organizing action of neurosteroids in the developing Purkinje cell. Cerebellum (London, England) 5:89-96.

Tsutsui K, Ukena K, Usui M, Sakamoto H, Takase M (2000) Novel brain function: biosynthesis and actions of neurosteroids in neurons. Neuroscience research 36:261-273.

Tu XK, Yang WZ, Shi SS, Wang CH, Zhang GL, Ni TR, Chen CM, Wang R, Jia JW, Song QM (2010) Spatio-temporal distribution of inflammatory reaction and expression of TLR2/4 signaling pathway in rat brain following permanent focal cerebral ischemia. Neurochemical research 35:1147-1155.

Valenti DA (2007) Neuroimaging of retinal nerve fiber layer in AD using optical coherence tomography. Neurology 69:1060.

Valenti DA (2011) Alzheimer's disease and glaucoma: imaging the biomarkers of neurodegenerative disease. International journal of Alzheimer's disease 2010:793931.

Valera S, Ballivet M, Bertrand D (1992) Progesterone modulates a neuronal nicotinic acetylcholine receptor. Proceedings of the National Academy of Sciences of the United States of America 89:9949-9953.

van Duinkerken E, Klein M, Schoonenboom NS, Hoogma RP, Moll AC, Snoek FJ, Stam CJ, Diamant M (2009) Functional brain connectivity and neurocognitive functioning in patients with long-standing type 1 diabetes with and without microvascular complications: a magnetoencephalography study. Diabetes 58:2335-2343.

VanLandingham JW, Cutler SM, Virmani S, Hoffman SW, Covey DF, Krishnan K, Hammes SR, Jamnongjit M, Stein DG (2006) The enantiomer of progesterone acts as a molecular neuroprotectant after traumatic brain injury. Neuropharmacology 51:1078-1085.

Varma R, Lee PP, Goldberg I, Kotak S (2011) An assessment of the health and economic burdens of glaucoma. American journal of ophthalmology 152:515-522.

Verma R, Mishra V, Sasmal D, Raghubir R (2010) Pharmacological evaluation of glutamate transporter 1 (GLT-1) mediated neuroprotection following cerebral ischemia/reperfusion injury. European journal of pharmacology 638:65-71.

Wachtmeister L (1998) Oscillatory potentials in the retina: what do they reveal. Progress in retinal and eye research 17:485-521.

Wagner AK, McCullough EH, Niyonkuru C, Ozawa H, Loucks TL, Dobos JA, Brett CA, Santarsieri M, Dixon CE, Berga SL, Fabio A (2011) Acute serum hormone levels: characterization and prognosis after severe traumatic brain injury. Journal of neurotrauma 28:871-888.

Wali B, Ishrat T, Won S, Stein DG, Sayeed I (2014) Progesterone in experiemental permanent stroke: A dose-response and therapeutic time window study. Brain : a journal of neurology (in press).

Wang J, Jiang C, Liu C, Li X, Chen N, Hao Y (2010a) Neuroprotective effects of progesterone following stroke in aged rats. Behavioural brain research 209:119-122.

Wang J, Jiang S, Kwong JM, Sanchez RN, Sadun AA, Lam TT (2006) Nuclear factor-kappaB p65 and upregulation of interleukin-6 in retinal ischemia/reperfusion injury in rats. Brain research 1081:211-218.

Wang JM, Singh C, Liu L, Irwin RW, Chen S, Chung EJ, Thompson RF, Brinton RD (2010b) Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America 107:6498-6503.

Wang X, Zhang J, Yang Y, Dong W, Wang F, Wang L, Li X (2013) Progesterone attenuates cerebral edema in neonatal rats with hypoxic-ischemic brain damage by inhibiting the expression of matrix metalloproteinase-9 and aquaporin-4. Experimental and therapeutic medicine 6:263-267.

Warembourg M, Logeat F, Milgrom E (1986) Immunocytochemical localization of progesterone receptor in the guinea pig central nervous system. Brain research 384:121-131.

Waters EM, Torres-Reveron A, McEwen BS, Milner TA (2008) Ultrastructural localization of extranuclear progestin receptors in the rat hippocampal formation. The Journal of comparative neurology 511:34-46.

Webvision. The organization of the retina and visual system. Eds. Kolb H, Nelson R, Fernandez E, Jones BW. Available at: http://webvision.med.utah.edu/book/ [Last accessed 29 March 2014]

Weinberger LGM, Gibbon JH (1940) Temporary arrest of the circulation to the central nervous system: I. Physiologic effects. II. Pathologic effects. Arch Neurol Psychiat 43:615-634, 961-686.

Wessels AM, Simsek S, Remijnse PL, Veltman DJ, Biessels GJ, Barkhof F, Scheltens P, Snoek FJ, Heine RJ, Rombouts SA (2006) Voxel-based morphometry demonstrates reduced grey matter density on brain MRI in patients with diabetic retinopathy. Diabetologia 49:2474-2480.

Wickham LA, Gao J, Toda I, Rocha EM, Ono M, Sullivan DA (2000) Identification of androgen, estrogen and progesterone receptor mRNAs in the eye. Acta ophthalmologica Scandinavica 78:146-153.

Won S, Lee JH, Wali B, Stein DG, Sayeed I (2013) Progesterone attenuates hemorrhagic transformation after delayed tPA treatment in an experimental model of stroke in rats: involvement of the VEGF-MMP pathway. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism.

Wong AA, Brown RE (2007) Age-related changes in visual acuity, learning and memory in C57BL/6J and DBA/2J mice. Neurobiology of aging 28:1577-1593.

Wright DW, Kellermann AL, Hertzberg VS, Clark PL, Frankel M, Goldstein FC, Salomone JP, Dent LL, Harris OA, Ander DS, Lowery DW, Patel MM, Denson DD, Gordon AB, Wald MM, Gupta S, Hoffman SW, Stein DG (2007) ProTECT:

a randomized clinical trial of progesterone for acute traumatic brain injury. Annals of emergency medicine 49:391-402, 402.e391-392.

Wurtz RH, Kandel ER (2000) Central visual pathways. In: Kandel ER, Schwartz JH, Jessel TM, eds Principles of Neural Science. pp 523-547 New York, NY: The McGraw-Hill Companies, Inc.

Xia CF, Smith RS, Jr., Shen B, Yang ZR, Borlongan CV, Chao L, Chao J (2006) Postischemic brain injury is exacerbated in mice lacking the kinin B2 receptor. Hypertension 47:752-761.

Xiao G, Wei J, Yan W, Wang W, Lu Z (2008) Improved outcomes from the administration of progesterone for patients with acute severe traumatic brain injury: a randomized controlled trial. Critical care (London, England) 12:R61.

Yamada ES, Dmitrieva N, Keyser KT, Lindstrom JM, Hersh LB, Marshak DW (2003) Synaptic connections of starburst amacrine cells and localization of acetylcholine receptors in primate retinas. The Journal of comparative neurology 461:76-90.

Yang CS, Lin NN, Tsai PJ, Liu L, Kuo JS (1996) In vivo evidence of hydroxyl radical formation induced by elevation of extracellular glutamate after cerebral ischemia in the cortex of anesthetized rats. Free radical biology & medicine 20:245-250.

Yao XL, Liu J, Lee E, Ling GS, McCabe JT (2005) Progesterone differentially regulates pro- and anti-apoptotic gene expression in cerebral cortex following traumatic brain injury in rats. Journal of neurotrauma 22:656-668.

Yousuf S, Atif F, Sayeed I, Wang J, Stein DG (2013a) Post-stroke infections exacerbate ischemic brain injury in middle-aged rats: immunomodulation and neuroprotection by progesterone. Neuroscience 239:92-102.

Yousuf S, Sayeed I, Atif F, Tang H, Wang J, Stein DG (2013b) Delayed progesterone treatment reduces brain infarction and improves functional outcomes after ischemic stroke: a time-window study in middle-aged rats. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism.

Yu X, Rajala RV, McGinnis JF, Li F, Anderson RE, Yan X, Li S, Elias RV, Knapp RR, Zhou X, Cao W (2004) Involvement of insulin/phosphoinositide 3-kinase/Akt signal pathway in 17 beta-estradiol-mediated neuroprotection. The Journal of biological chemistry 279:13086-13094.

Yucel I, Akar ME, Dora B, Akar Y, Taskin O, Ozer HO (2005) Effect of the menstrual cycle on standard achromatic and blue-on-yellow visual field analysis of women with migraine. Canadian journal of ophthalmology Journal canadien d'ophtalmologie 40:51-57.

Zador Z, Stiver S, Wang V, Manley GT (2009) Role of aquaporin-4 in cerebral edema and stroke. Handbook of experimental pharmacology 159-170.

Zhang C, Lam TT, Tso MO (2005a) Heterogeneous populations of microglia/macrophages in the retina and their activation after retinal ischemia and reperfusion injury. Experimental eye research 81:700-709.

Zhang W, Miao Y, Zhou S, Jiang J, Luo Q, Qiu Y (2011) Neuroprotective effects of ischemic postconditioning on global brain ischemia in rats through upregulation of hippocampal glutamine synthetase. Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia 18:685-689.

Zhang Y, Cho CH, Atchaneeyasakul LO, McFarland T, Appukuttan B, Stout JT (2005b) Activation of the mitochondrial apoptotic pathway in a rat model of central retinal artery occlusion. Investigative ophthalmology & visual science 46:2133-2139.

Zhang Y, Li C, Sun X, Kuang X, Ruan X (2012a) High glucose decreases expression and activity of p-glycoprotein in cultured human retinal pigment epithelium possibly through iNOS induction. PloS one 7:e31631.

Zhang Y, Lu M, Sun X, Li C, Kuang X, Ruan X (2012b) Expression and activity of p-glycoprotein elevated by dexamethasone in cultured retinal pigment epithelium involve glucocorticoid receptor and pregnane X receptor. Investigative ophthalmology & visual science 53:3508-3515.

Zhu Y, Ohlemiller KK, McMahan BK, Gidday JM (2002) Mouse models of retinal ischemic tolerance. Investigative ophthalmology & visual science 43:1903-1911.

Ziai N, Ory SJ, Khan AR, Brubaker RF (1994) Beta-human chorionic gonadotropin, progesterone, and aqueous dynamics during pregnancy. Archives of ophthalmology 112:801-806.

Zitzmann M, Erren M, Kamischke A, Simoni M, Nieschlag E (2005) Endogenous progesterone and the exogenous progestin norethisterone enanthate are associated with a proinflammatory profile in healthy men. The Journal of clinical endocrinology and metabolism 90:6603-6608.