Progesterone Treatment in Two Rat Models of Ocular Ischemia

Rachael S. Allen,[1-3] Timothy W. Olsen,[2] Iqbal Sayeed,[1] Heather A. Cale,[1] Katherine C. Morrison,[1] Yuliya Oumarbaeva,[1] Irina Lucaciu,[1] Jeffrey H. Boatright,[2,3] Machelle T. Pardue,[2,3] and Donald G. Stein[1]

[1]Emergency Medicine, Emory University, Atlanta, Georgia, United States
[2]Department of Ophthalmology, Emory University, Atlanta, Georgia, United States
[3]Atlanta VA Center for Visual and Neurocognitive Rehabilitation, Decatur, Georgia, United States

PURPOSE. To determine whether the neurosteroid progesterone, shown to have protective effects in animal models of traumatic brain injury, stroke, and spinal cord injury, is also protective in ocular ischemia animal models.

METHODS. Progesterone treatment was tested in two ocular ischemia models in rats: a rodent anterior ischemic optic neuropathy (rAION) model, which induces permanent monocular optic nerve stroke, and the middle cerebral artery occlusion (MCAO) model, which causes transient ischemia in both the retina and brain due to an intraluminal filament that blocks the ophthalmic and middle cerebral arteries. Visual function and retinal histology were assessed to determine whether progesterone attenuated retinal injury in these models. Additionally, behavioral testing and 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining in brains were used to compare progesterone's neuroprotective effects in both retina and brain using the MCAO model.

RESULTS. Progesterone treatment showed no effect on visual evoked potential (VEP) reduction and retinal ganglion cell loss in the permanent rAION model. In the transient MCAO model, progesterone treatment reduced (1) electroretinogram (ERG) deficits, (2) MCAO-induced upregulation of glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP), and (3) retinal ganglion cell loss. As expected, progesterone treatment also had 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, which may result from mechanistic differences with injury type and the therapeutic action of progesterone.

Keywords: progesterone, retinal ischemia, electroretinogram, middle cerebral artery occlusion, anterior ischemia optic neuropathy

V isual impairment is often a presenting symptom in cerebral stroke resulting from arthrosclerosis of the carotid or ophthalmic arteries.[1,2] 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. Additionally, transient vision loss is often a sign of impending stroke or systemic cardiovascular disease,[3] and neurological imaging shows that an acute brain infarct is present in one of four patients with transient retinal ischemia.[4] Ocular ischemia can also occur independently of cerebral damage. Anterior ischemic optic neuropathy (AION) leads to retinal ganglion cell (RGC) death caused by optic nerve stroke[5] and is the leading cause of sudden vision loss related to optic nerve dysfunction in older adults.[6] No effective treatment currently exists.[7]

During ischemic stroke, reductions in arterial blood flow prevent an adequate supply of oxygen and nutrients from reaching neural tissue, leading to injury and cell death.[8] The overlap between mechanisms involved in cerebral stroke and in retinal and optic nerve strokes is substantial. Both the retinal and cerebral injuries involve excitotoxicity due to increases in extracellular glutamate and calcium influx.[9-11] Other common mechanisms include altered aquaporin expression,[12,13] oxidative stress,[14,15] and increases in proapoptotic and decreases in antiapoptotic markers.[16,17] Increased inflammation occurs in both conditions, including increased activation of glial cells and macrophages,[18,19] increased levels of inflammatory cytokines (IL-6, TNF-α, and so on),[20,21] and nuclear factor kappa-light-chain-enhancer of active B cells (NF-κB) pathway activation.[22,23] The high degree of mechanistic overlap, as well as the presence of central nervous system tissue in both cerebral and retinal/optic nerve ischemia, could allow for treatment crossover between diseases.

The neurosteroid progesterone has been shown to provide protection in several animal models of central nervous system injury, including stroke, traumatic brain injury, and spinal cord injury.[24-28] In cerebral ischemia models, progesterone reduces infarct volume and enhances functional recovery.[29-32] Progesterone also acts 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[29] and to reduce both cerebral[27] and systemic inflammation.[33] Progesterone also

modulates aquaporin expression[34] and decreases oxidative stress,[35,36] apoptotic markers,[37,38] edema, and blood–brain barrier permeability after cerebral ischemia.[34,39] Finally, progesterone attenuates N-methyl-D-aspartate (NMDA)–mediated calcium influx after ischemia[40] and, through its metabolite, allopregnanolone, reduces toxic neurotransmitter release by modulating the gamma-aminobutyric acid ($GABA_A$) receptor in adult animals.[41,42] The substantial overlap between the mechanisms involved in ocular ischemia and the proposed mechanisms of action for 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 rodent AION (rAION) model, which causes permanent thrombosis of the microvasculature within the optic nerve with subsequent RGC death and vision loss,[43] and the transient MCAO model, which produces retinal damage and functional deficits in conjunction with cerebral ischemia.[44–46]

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats ($n = 105$) from Charles River Laboratories (Wilmington, MA, USA) were used in this study. At the time of surgery, rats were approximately 48 days of age for rAION surgery (210–250 g) and 60 days of age (290–330 g) for MCAO surgery. Littermates that did not receive surgery were used as controls for rAION. Littermates given 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 National Institutes of Health guidelines and the ARVO 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.

Progesterone Preparation and Dosing

An 8 mg/kg progesterone dose, previously shown to be most protective after stroke,[47] was made using 2-hydroxypropyl-β-cyclodextrin (25% wt/vol solution in H_2O) as the solvent. For rAION rats, progesterone and vehicle treatments were administered via intraperitoneal injection 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), because this procedure leads to better outcomes.[48,49] Rats were euthanized at 21 days post surgery. For MCAO rats, progesterone and vehicle injections were given 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, group identity was coded with regard to surgery and treatment to prevent experimenter bias.

Methods Utilized in rAION Experiments

rAION Surgery. Monocular rAION surgery was performed with some modifications to a previous description.[43] Briefly, in anesthetized rats (isoflurane: 5% induction, 2% maintenance, 700 mm N_2O, 300 mm O_2), the cornea was anesthetized with topical proparacaine (1%), and pupils were dilated with topical tropicamide (1%) and phenylephrine hydrochloride (2.5%). Immediately following injection of the tail vein with 1 mL/kg of 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 532-nm wavelength laser via the indirect ophthalmoscope (Novus Varia; Lumenis, Salt Lake City, UT, USA) and a 78-diopter condensing lens. This technique differs somewhat from the surgery developed by Bernstein et al.,[43] which involved a yttrium aluminum garnet laser and a slit lamp. However, others have shown that an argon green laser and indirect ophthalmoscope also produce rAION.[50] 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 (i.e., a yellow glow was detected within the vasculature of the optic nerve during laser delivery).

Fundus Photographs. Fundus photographs were used to visualize posterior structures of the eye, including the optic nerve, retina, and retinal vasculature (see Supplementary Methods for details).

Visual Evoked Potential (VEP). The rAION model causes cell death in RGCs only. Thus, VEPs, which are highly correlated with RGC and optic nerve morphology, were used to measure visual cortex activity in response to light stimuli.[51] Briefly, rats were anesthetized, and light-adapted cortical responses to Ganzfeld strobe flashes (137 cd s/m^2) were recorded (see Supplementary Methods for details). Amplitudes and implicit times were measured for P1 and N1 components of the VEP. In a subset of animals ($n = 8$), electroretinograms (ERGs) were used to confirm normal bipolar and photoreceptor cell function.

Immunohistochemistry for Brn3a. Rats were euthanized under deep anesthesia and their eyes enucleated. The RGC-specific marker Brn3a was used to assess RGC death in flat-mounted retinas (see Supplementary Methods for details).

### Methods Utilized in MCAO Experiment

MCAO Surgery. Middle cerebral artery occlusion surgery was performed on anesthetized animals with minor modifications to the previous description.[52] An intraluminal filament was inserted into the internal carotid so that it blocked the ophthalmic and middle cerebral arteries for 120 minutes followed by reperfusion. Laser-Doppler flowmetry (LDF) monitored cerebral blood flow (see Supplementary Methods for details). Following surgery, behavioral tests were performed at 24 hours, ERGs were performed at 48 hours, and rats were euthanized at 72 hours.

Electroretinograms. Electroretinograms 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, and ERG responses to a series of Ganzfeld strobe flashes (0.00039–137 cd s/m^2) were recorded (see Supplementary Methods for details). Amplitudes and implicit times were measured for a-waves, b-waves, and oscillatory potentials.

Neurological Assessment

Grip Strength. The grip strength task is a test of neuromuscular performance. Each rat was held in front of the grip strength device (Columbus Instruments, Columbus, OH, USA) until it grasped the bar. Traction was applied to the rat's tail until the animal released its grip of the bar. The force meter displayed the peak force (in newtons). Three trials were performed on each animal and averaged. 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
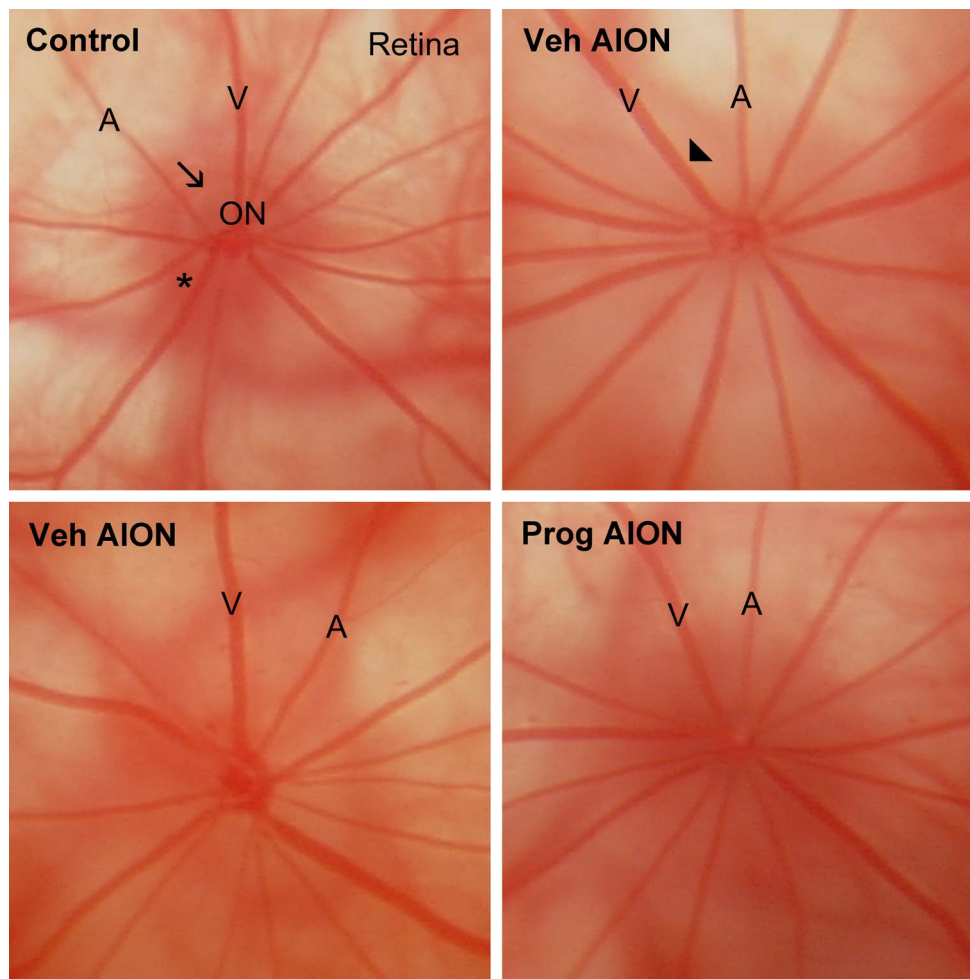
FIGURE 1. Fundus photographs from control, progesterone-treated AION, and vehicle-treated AION rats. In control retinas, distinct boundaries (*arrow*) and a reddish flush indicative of intact microvasculature (*) were observed. Lack of distinct boundaries in AION retinas is a sign of optic nerve edema, and lack of a reddish flush suggests obliteration of microvasculature. Additionally, vascular dilation (*arrowhead*) was observed in AION retinas. No differences were observed between vehicle- and progesterone-treated AION retinas. ON, optic nerve; A, artery; V, vein.

(Glue Dots International, Germantown, WI, USA) was affixed to the bottom of the front left paw, and each 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-Aldrich Corp., St. Louis, MO, USA) to differentiate between metabolically active and stroke tissue (see Supplementary Methods for details).

Retinal Morphology and Immunohistochemistry. Rats were euthanized and their eyes removed. In 5-μm paraffin sections, 0.1% cresyl violet staining was used to assess cell death, and immunohistochemistry was used to label glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) to assess Müller cell reactivity (see Supplementary Methods for details). Cell counts were performed selectively on RGCs, as we have previously shown that this is the only cell type in the retina that is reduced after MCAO.[46]

Statistical Analysis

Results are expressed as mean ± SEM. Electroretinogram and MCAO RGC count results were analyzed using a two-way repeated measures ANOVA followed by Tukey's test for individual comparisons. The ANOVA interaction effect is reported unless otherwise stated. The AION RGC 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. Visual evoked potential results were analyzed using a Kruskal-Wallis one-way ANOVA on ranks. Results for TTC staining 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 as compared with controls (Fig. 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. 2). Electroretinograms performed in a subset of rAION rats ($n = 8$) confirmed normal bipolar and photoreceptor cell function, as expected in an optic nerve stroke model that affects only RGCs (data not shown). Both progesterone- and vehicle-treated rats showed a significant reduction in RGCs (47879 ± 4226 and
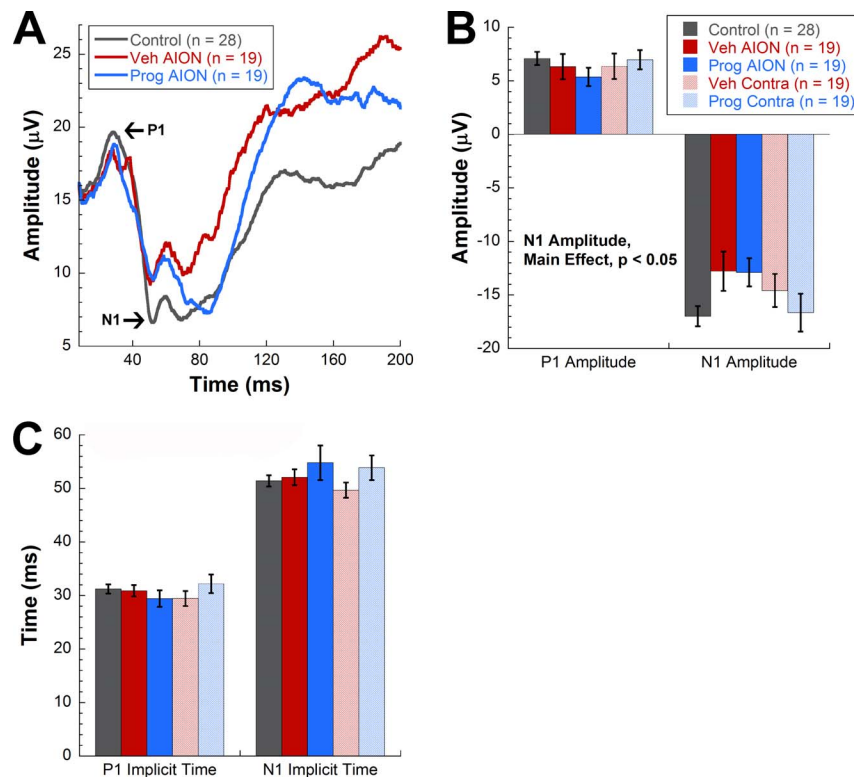
Figure 2. Visual evoked potentials for control and progesterone- and vehicle-treated rAION rats at 3 days post AION. (**A**) Averaged VEP waveforms. *Arrows* designate P1 and N1 for controls. (**B**) Mean P1 and N1 amplitudes at 3 days post AION. A significant main effect of group was observed for N1 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.

44039 ± 4305 cells, respectively) compared with controls (81,750 ± 3876 cells) [ANOVA, $F_{(2, 38)} = 15.621$, $P < 0.001$] (Fig. 3).

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

Progesterone Treatment Protected Against Retinal Ischemia Induced by Transient MCAO

Progesterone Reduced ERG Deficits in MCAO Rats. Figure 4 shows representative ERG waveforms from progesterone- and vehicle-treated MCAO rats and shams at 2 days post MCAO. Vehicle-treated rats had reduced amplitudes in both ipsilateral MCAO and contralateral eyes, and progesterone treatment partially attenuated these deficits, particularly in eyes contralateral to the MCAO.

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. 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 ($P < 0.05$).

Middle cerebral artery occlusion 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. 5C). Additionally, contralateral eyes from progesterone-treated MCAO rats showed a significant recovery in b-wave amplitude (64%) at 0.249 and 4.1 cd s/m² flash stimuli ($P < 0.05$) and a-wave amplitudes that were not significantly different from sham amplitudes (47% recovery).

For ERG implicit times, a significant treatment effect 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. 5). Post hoc analysis revealed significant delays in b-wave and oscillatory potential implicit times only for MCAO eyes from vehicle-treated rats versus 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 (Fig. 6). 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. 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 (Figs. 6, 7). In progesterone-treated rats, MCAO retinas showed significantly reduced levels of GS intensity compared with vehicle-treated rats ($P < 0.05$), and were statistically indistinguishable from shams (Figs. 6, 7).
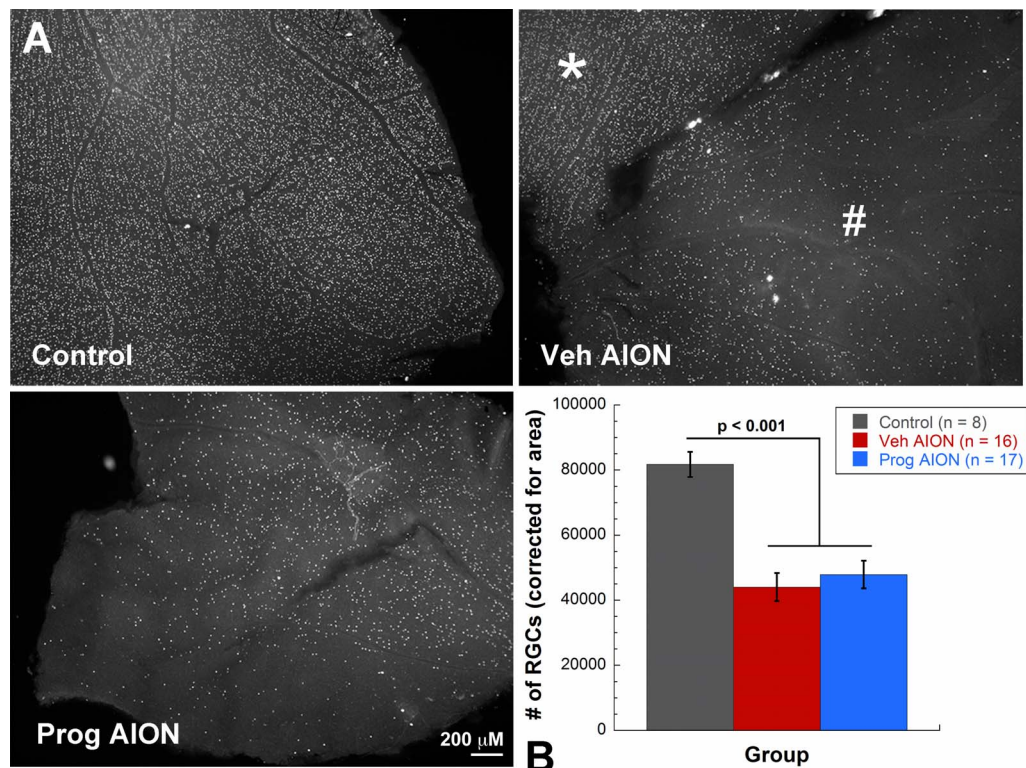
FIGURE 3. Immunohistochemistry with the retinal ganglion cell–specific marker, Brn3a. (A) Representative flat mount petals stained with Brn3a for retinas from control (*top left*), vehicle-treated AION (*top right*), and progesterone-treated AION rats (*bottom left*). Retinas from both progesterone- 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 Reduced Retinal Ganglion Cell Death in Retinas From MCAO Rats. Three days after MCAO, a significant reduction in RGCs was observed in MCAO retinas from vehicle-treated rats compared with shams [repeated measures ANOVA, $F(4, 185) = 44.058$, $P < 0.001$; Fig. 8]. In progesterone-treated rats, MCAO retinas showed greater numbers of RGCs than in vehicle-treated rats ($P < 0.001$) but fewer RGCs than in shams ($P < 0.001$). No differences were observed between sham and contralateral retinas from either treatment group (Fig. 8).

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 on the sticky-tape task ($P < 0.01$) compared to vehicle-treated MCAO rats. Progesterone protection in MCAO rats was statistically indistinguishable from that in shams on both tasks (Fig. 9).

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. 10).

DISCUSSION

Progesterone Treatment Showed Protection in MCAO but Not rAION

Previous studies have identified progesterone[53,54] and progesterone receptors[55-58] in the retina, and progesterone synthesis has been shown to occur in the eye.[59-63] Further
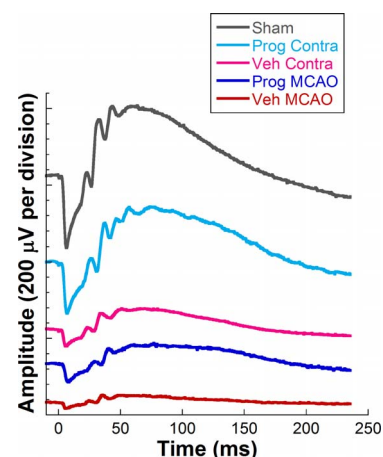
FIGURE 4. Representative ERG waveforms from sham, progesterone-, and vehicle-treated MCAO rats. Vehicle-treated rats show reduced amplitudes in both MCAO and contralateral eyes at 2 days post MCAO in response to 137 cd s/m² flash stimuli. Progesterone treatment reduces these deficits, particularly in contralateral eyes.
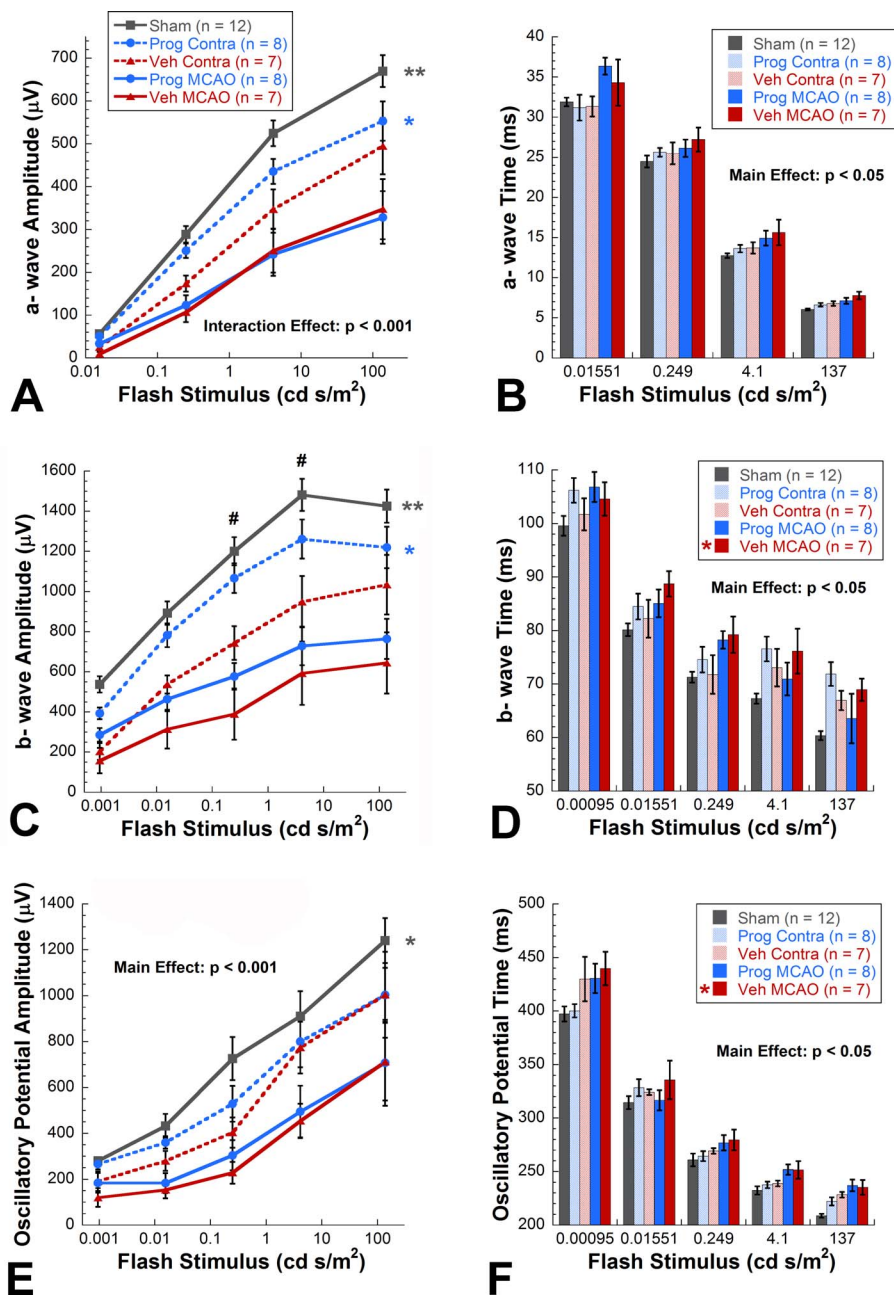
FIGURE 5. Quantification of ERG a-wave, b-wave, and oscillatory potential amplitudes and implicit times. Significant reductions in dark-adapted a-wave (A), b-wave (C), and oscillatory potential amplitudes (E) were observed in MCAO eyes from vehicle-treated rats compared with shams. Significant reductions 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. *$P < 0.05$. Prog, progesterone; Veh, vehicle; Contra, contralateral.

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[64,65] to visual function.[66,67] This idea is supported by previous research demonstrating protective effects of progesterone or progestins in models of light-induced retinal degeneration[68] (Cubilla MA, et al. IOVS 2012;53:ARVO E-Abstract 2565), hereditary retinal degeneration[68] (Araiz JJ, et al. IOVS 2012;53:ARVO E-Abstract 6410;

Sanchez-vallejo V, et al. IOVS 2013;54:ARVO E-Abstract 1787) and pressure-induced retinal ischemia–reperfusion.[69] 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.[70]

In this study, progesterone administration resulted in reduced ERG deficits, reduced GFAP and GS upregulation, and reduced RGC death following MCAO-induced retinal
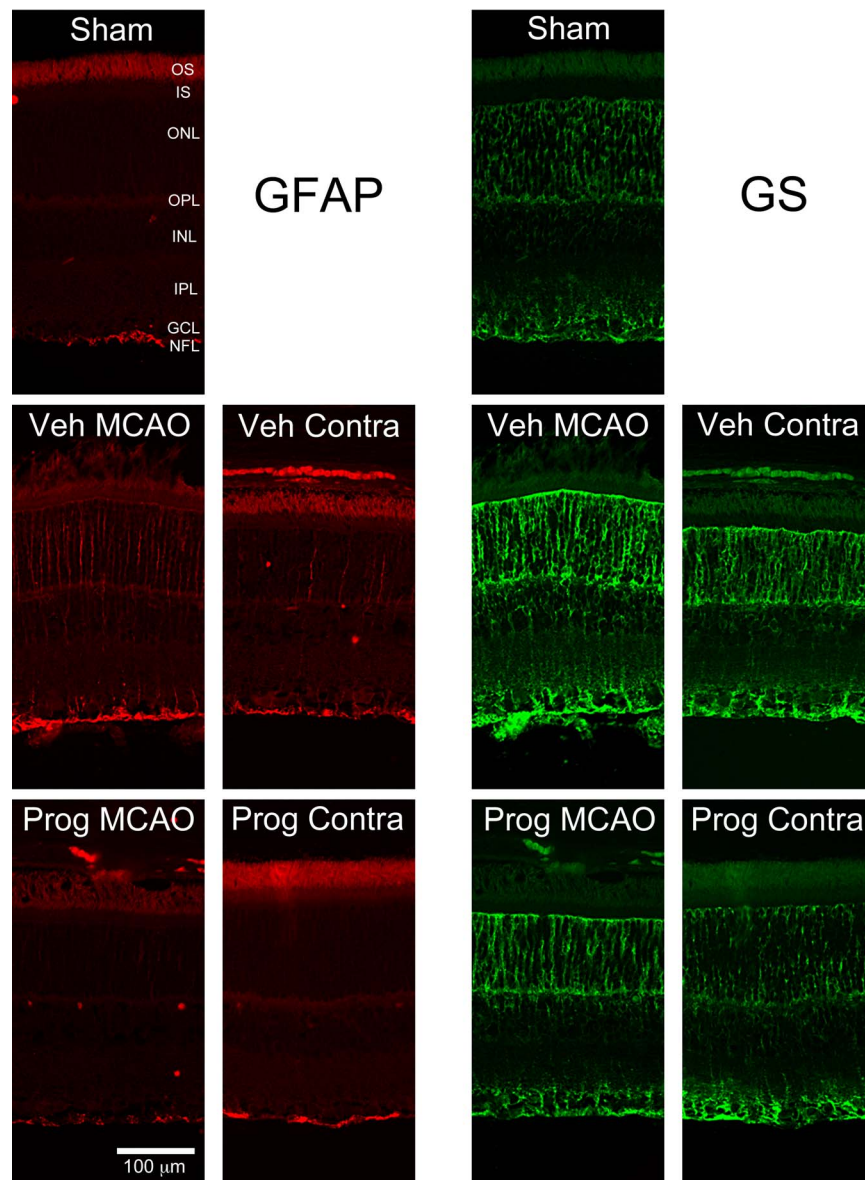
FIGURE 6. Representative photographs from immunohistochemistry with GFAP and GS after MCAO. Three days after MCAO, GFAP was upregulated in Müller cells in MCAO (and some contralateral) retinas from vehicle-treated rats, but not progesterone-treated rats, compared with shams. Glutamine synthetase was 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.

ischemia. The hormone did not produce a protective effect in the rAION model as assessed with fundus photographs, VEPs, and RGC counts.

Differences in methodology should be taken into account when comparing the two injury models. In the MCAO model, the filament used to induce stroke is removed after 2 hours, making the ischemia transient. The rAION model is a permanent ischemia model, and, as expected, greater RGC death occurred in this model. Additionally, a longer survival period was used in the rAION experiments because RGC death is observable by 3 days in the MCAO model, but does not occur until after approximately 2 weeks in the rAION model.[43] Electroretinograms were used in both rAION and MCAO experiments; however, since the ERG reflects the activity of only photoreceptor and bipolar cells, ERG deficits were observed only after MCAO because rAION does not cause

bipolar or photoreceptor cell injury. Thus, in order to make functional comparisons, VEPs from rAION rats are compared with ERGs from MCAO rats. Visual evoked potentials were not performed in MCAO rats because MCAO does not cause enough RGC loss to be detectable by the VEP. While differences in methodology are present, we interpret our data to indicate that progesterone is protective in the eye after MCAO but not rAION.

Retinal ischemia caused by transient MCAO differs from retinal ischemia caused by permanent rAION, and it is possible that the mechanism of injury is different in the rAION model such that progesterone is not effective. In the rAION model, as in other models that involve laser-induced 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.[43]
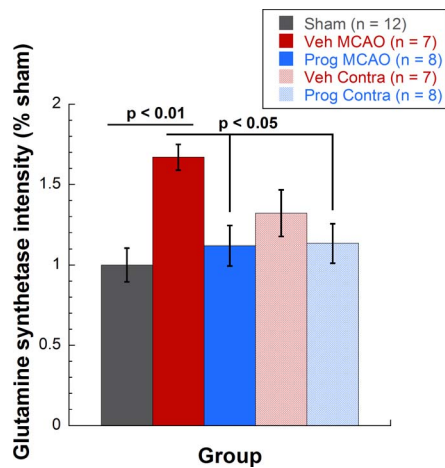
FIGURE 7. Mean GS intensity as percent of sham for vehicle- and progesterone-treated MCAO rats. Significant increases in GS intensity were observed in MCAO retinas from vehicle-treated rats compared with shams ($P < 0.01$), with a trend for an increase in contralateral retinas. Both MCAO and contralateral 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.

However, it is possible that there is another mechanism of injury to the optic nerve ganglion cells in this laser-induced model (i.e., thermal injury), leading to nerve fiber edema and a secondary obstruction of the microvasculature, rather than the originally proposed thrombosis. Meanwhile, transient MCAO cuts off blood supply to the retina almost completely while the filament is in place, with reperfusion occurring after the filament is removed.[44] 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.[8] In the MCAO model, the cause of retinal injury is partially local, due to the filament reducing blood flow to the eye, and partially systemic, due to effects of the infarct in the brain. The systemic effects include a

3-fold increase in glutamate levels,[71] which may explain the increase in GS in contralateral as well as MCAO retinas. In the MCAO model, ERG deficits and increased GS are observed at 2 days post MCAO, suggesting extracellular glutamate as a mechanism. The ERG deficits are found to resolve by 9 days post MCAO, and the GS levels return to normal as well, indicating that GS may have cleared the sublethal extracellular glutamate.[46] Increased extracellular glutamate has also been shown to cause ERG deficits without cell death in mice with inhibited glutamate transport.[72]

We can find no reports of changes in GS or extracellular glutamate following rAION, which highlights another difference between these models. It is possible that MCAO is causing direct retinal damage via increased levels of glutamate, while rAION is causing axonal damage leading to RGC loss (which would not involve direct glutamate release). The glutamate release in MCAO would cause necrotic cell death, while the loss of axonal signaling in rAION would cause apoptotic cell death, and the differences in mechanism may affect the response to progesterone treatment. Progesterone and its metabolite allopregnanolone have been shown to upregulate the expression of the $GABA_A$ receptor[73] and to act as a positive modulator of the $GABA_A$ receptor, causing reductions in excitability.[74] Additionally, progesterone has been shown to protect against glutamate toxicity in primary cultures of hippocampal neurons[75] and spinal cord neurons.[76] It is possible that the extracellular glutamate increases that occur following MCAO, but not rAION, are the key difference influencing progesterone's effectiveness in these two models.

Progesterone Treatment Showed Protection in Brain and Retina Following MCAO

Progesterone treatment has previously been shown to reduce infarct size and functional deficits after cerebral ischemia.[29–32] 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. After MCAO, progesterone had greater effects on reducing ischemia-induced upregulation of GS in Müller cells
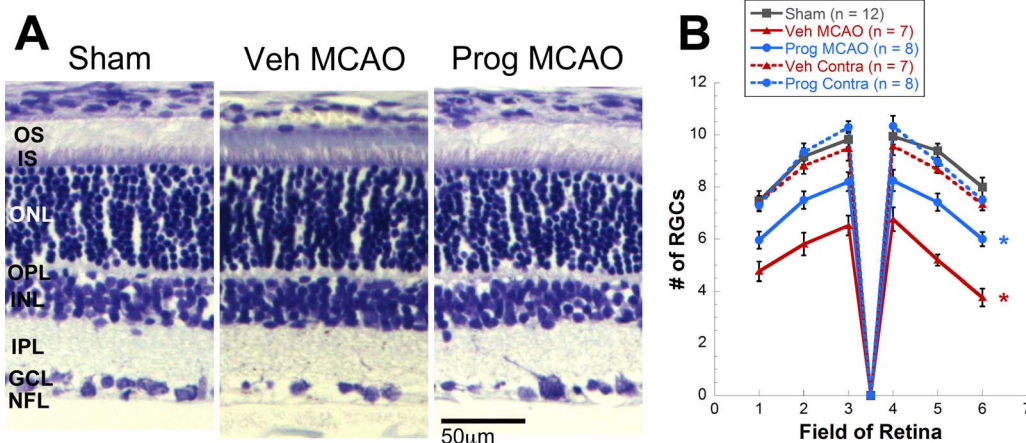
FIGURE 8. Representative photographs of cresyl violet staining and quantification of retinal ganglion cells. (**A**) Cresyl violet staining in retinal sections from sham, vehicle-treated MCAO, and progesterone-treated MCAO rats. (**B**) At 3 days post MCAO, ipsilateral retinas from vehicle-treated MCAO 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.
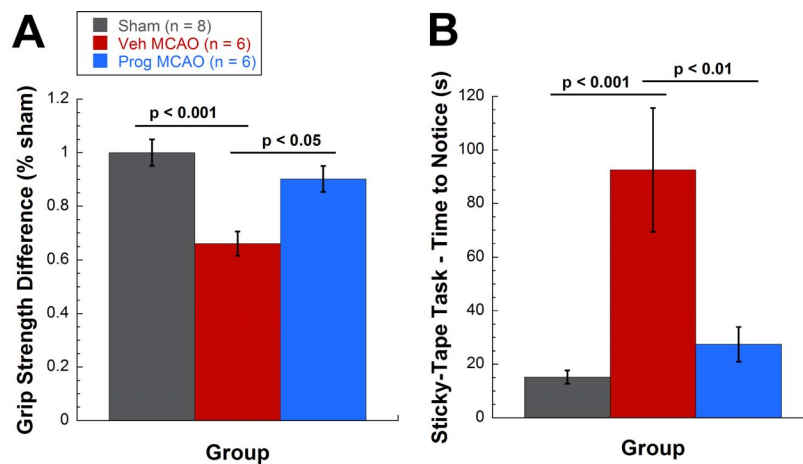
FIGURE 9. Behavioral assessment at 1 day post MCAO. (**A**) Vehicle-treated MCAO rats showed reduced grip strength compared with shams ($P < 0.001$). Progesterone-treated MCAO rats showed significant increases in grip strength over vehicle-treated MCAO rats ($P < 0.05$). (**B**) Vehicle-treated MCAO 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 ($P < 0.01$).
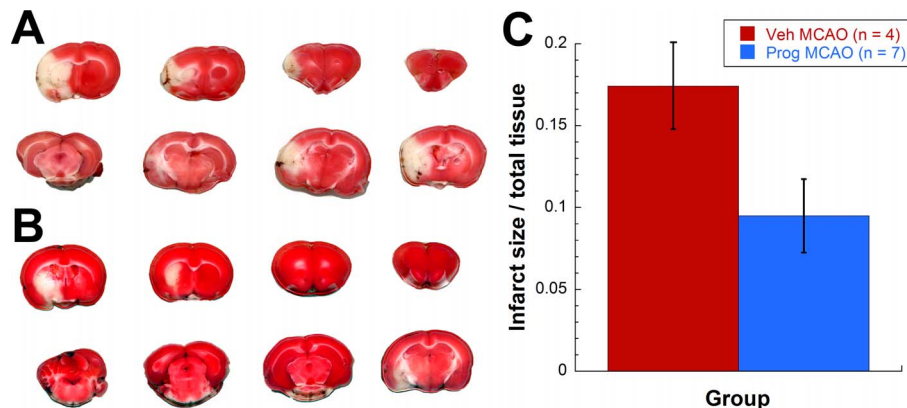
FIGURE 10. Representative TTC staining and quantification at 3 days post MCAO. Representative TTC-stained brain slices for (**A**) vehicle-treated and (**B**) progesterone-treated MCAO rats with ~70% occlusion. (**C**) Reduced infarct size was observed in progesterone- versus vehicle-treated MCAO rats (45%, $P = 0.054$).

than on reducing retinal function deficits or RGC death. GABAergic amacrine and horizontal cells are present in the retina and modulate the glutamatergic pathway of photoreceptor, bipolar, and ganglion cells.[77] Progesterone's positive actions on GABA$_A$ receptors may explain why a smaller reduction in retinal function deficits than in cell death or GS 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.[78]

Optimal progesterone dose, duration, and route of administration of treatment could differ for retina versus brain. For example, in cerebral stroke, 8 mg/kg was found to be the optimal dose,[47] but in traumatic brain injury, 16 mg/kg was found to be a more effective dose.[24] Testing a range of doses in retinal injury and creating a dose–response curve may allow us to better address this question.

While research supports the existence of progesterone receptors in the retina,[55–58] little is known about progesterone receptor localization in retina and the relative levels of progesterone receptor in retina versus brain. Some of progesterone's protective effects are mediated by progesterone acting through classical progesterone receptors,[79–82] while other effects are mediated by progesterone acting as an antagonist at Sigma-1[83–85] and glucocorticoid receptors[86,87]— by progesterone acting as a ligand at the pregnane X receptor,[88,89] or by progesterone's metabolite, allopregnanolone, acting as a positive allosteric modulator at the GABA$_A$ receptor.[74,82] If, as a pleiotropic agent, progesterone acts via all of these pathways in brain but only some of these pathways in retina, this could cause a difference in the extent of neuroprotection seen in retina versus brain. Further research needs to be done to determine the mechanisms by which progesterone is acting in the retina and whether these mechanisms 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 permanent rAION model. Further research is needed to determine why progesterone has differential effects in different tissues and models and how to optimize progesterone treatment in the retina.

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