Allopregnanolone, a Progesterone Metabolite, Is More Effective Than Progesterone in Reducing Cortical Infarct Volume After Transient Middle Cerebral Artery Occlusion

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Study objective: We compare the effects of postinjury administration of allopregnanolone, a metabolite of progesterone, to progesterone in an animal model of transient middle cerebral artery occlusion.

Methods: Focal cerebral ischemia was induced in age-matched, adult, male, Sprague-Dawley rats by using an intraluminal filament and suture method to occlude the right middle cerebral artery. After 120 minutes of middle cerebral artery occlusion, the occluding filament was withdrawn to allow reperfusion. Laser-Doppler flowmetry was used to monitor cerebral blood flow for the entire 2-hour period of occlusion and for 5 minutes after reperfusion. Animals subjected to middle cerebral artery occlusion received injections of allopregnanolone (8 mg/kg, n=6), progesterone (8 mg/kg, n=6) and vehicle (2-hydroxypropyl-β-cyclodextrin, n=7) at 2 hours (intraperitoneally 5 minutes before reperfusion) and 6 hours (subcutaneously) postocclusion. Brains were removed at 72 hours post–middle cerebral artery occlusion, sectioned into coronal slices, and stained with 2,3,5-triphenyltetrazolium chloride (TTC). In a blinded analysis, infarct volume was calculated by using computer-aided morphometry to measure brain areas not stained with TTC.

Results: After progesterone or allopregnanolone treatment, stained sections revealed a significant reduction in cortical, caudate-putamen, and hemispheric infarct volumes (percentage of contralateral structure) compared with vehicle-injected controls. Cortical infarction (percentage of contralateral cortex) was 37.47%±10.57% (vehicle), 25.49%±7.38% (progesterone; $P<.05$ from vehicle), and 11.40%±7.09% (allopregnanolone; $P<.05$ from vehicle; $P<.05$ from progesterone). Caudate-putamen infarction (percentage of contralateral caudate-putamen) was 78.02%±22.81% (vehicle), 48.41%±22.44% (progesterone; $P<.05$ from vehicle), and 50.44%±10.90% (allopregnanolone; $P<.05$ from vehicle). Total hemispheric infarction (percentage of contralateral hemisphere) was 24.37%±6.69% (vehicle), 15.95%±3.59% (progesterone; $P<.05$ from vehicle), and 11.54%±3.71% (allopregnanolone; $P<.05$ from vehicle). No significant differences in cerebral blood flow between groups and time points during ischemia and early reperfusion were observed, suggesting that the relative ischemic insult was equivalent among all groups.

Conclusion: Although progesterone and allopregnanolone are effective in reducing infarct pathology, allopregnanolone is more potent than progesterone in attenuating cortical damage. Our results suggest that both neurosteroids should be examined for safety and efficacy in a clinical trial for ischemic stroke. [Ann Emerg Med. 2006;47:381-389.]

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INTRODUCTION

Stroke is the third leading cause of death in the United States after heart disease and cancer and a leading cause of serious, long-term disability. Each year, about 700,000 people have strokes, either initial or recurrent, and about 160,000 people die.[1] The financial burden of stroke on our society is large, with

Editor's Capsule Summary

What is already known on this topic

The protective effect of progesterone in rodent models of ischemic brain injury is well established. Some studies have indicated that allopregnanolone, a metabolite of progesterone, may have a greater benefit in traumatic brain injury than progesterone. This issue has not been examined in ischemic brain injury.

What question this study addressed

This interventional study of 19 rats compared the neuroprotective effect of progesterone and the progesterone metabolite allopregnanolone to placebo. An established rat model of transient 2-hour middle cerebral artery occlusion was used, and the primary outcome measure was infarct volume. Drugs were given by intraperitoneal injection 5 minutes before reperfusion, followed by a second dose 6 hours later.

What this study adds to our knowledge

The main result is that both progesterone and allopregnanolone treatment reduced infarct size compared with controls. A secondary observation was that allopregnanolone treatment resulted in a greater reduction in cortical infarct volume than progesterone. There are no functional outcome measures.

How this might change clinical practice

This trial demonstrates the potential benefit of allopregnanolone in ischemic brain injury in rats. Further trials are required to delineate its role in the treatment of humans.

estimates of more than $56 billion for 2005.[1] Despite the increasing prevalence of this disease, there are few effective poststroke treatments. Intravenous recombinant tissue plasminogen activator (TPA) initiated within 3 hours of stroke onset remains the only approved and validated therapy, but many hospitals do not have the professional resources to administer TPA within the time needed to benefit patients. Many other therapies have been evaluated, but these trials have been either inconclusive or have had negative results.[2]

Experimental findings have consistently shown that, in a variety of brain injury models, postinjury administration of neurosteroids such as progesterone has substantial beneficial effects on cytologic, morphologic, and functional outcomes.[3–8] Steroid hormones affect neuronal survival, the growth of neurites, and the formation of synaptic connections from early development to the plastic changes observed in the adult nervous system.[9–11] In particular, after injury or disease, steroids exert protective effects on neurons and glial cells and promote neuroregenerative processes.[12–14] Until recently, clinical and basic research has focused heavily on estrogen, but in the past few years, the effects of progesterone and its metabolites in both ischemic and nonischemic (ie, degenerative, traumatic) brain injury have gained greater scrutiny.[3,4,15–19] Studies are beginning to show that progesterone treatment can enhance functional and morphologic recovery after global and focal ischemia.[20–24] Among the advantages of these neurosteroid treatments are: no need for long-term dosing, a large window for treatment without increased risk, effectiveness in the treatment of men and women, ready availability, and ease of administration in emergency situations.[19]

Progesterone and its metabolite allopregnanolone are endogenous and potent positive modulators of central nervous system (CNS) γ-aminobutyric acid A ($GABA_A$) receptor functions.[25,26] The direct GABAergic potentiation mediated by allopregnanolone may enhance its neuroprotective actions by counteracting excitotoxic mechanisms. Some recent traumatic brain injury studies have found that allopregnanolone is more potent than progesterone in facilitating CNS repair.[17,27] However, it is still not clear whether the greater potency of allopregnanolone is due to GABAergic potentiation or to some other receptor mechanism. Previous research by other laboratories has shown that although progesterone can act as a ligand to the classical progesterone receptor, the Sigma-receptor, and the putative progesterone membrane binding site, allopregnanolone has not demonstrated any significant binding or activity at these locations.[28,29] The metabolism of progesterone to allopregnanolone (Figure 1), if it occurs randomly, produces 2 isomers with the pro-GABAergic and 2 isomers with the nonGABAergic effects. In addition, although allopregnanolone can reach an equilibrium state with dihydroprogesterone in a reversible enzymatic reaction, dihydroprogesterone cannot be enzymatically converted back to progesterone. A minor oxidative pathway has been shown to exist,[30] but this would not account for the greater potency of allopregnanolone versus progesterone that has been demonstrated in the treatment of traumatic brain injury.[17,27]

Middle cerebral artery occlusion is the most prevalent form (88%) of all stroke types in humans.[1] For the past decade, intraluminal suture middle cerebral artery occlusion has been the most widely used animal stroke model to investigate the pathophysiology of ischemic stroke and to test the efficacy of neuroprotective agents. The procedure does not require craniotomy, and many aspects of the occlusion model in animals parallel the pathophysiological changes in brain structure and function observed in human stroke.[31,32]

The present study investigated the beneficial effects of allopregnanolone administration and compared them to progesterone on the extent of infarct attenuation after 72 hours of ischemia-reperfusion injury in male rats. To our knowledge, we are the first group to study the effects of allopregnanolone in an animal model of ischemic stroke.

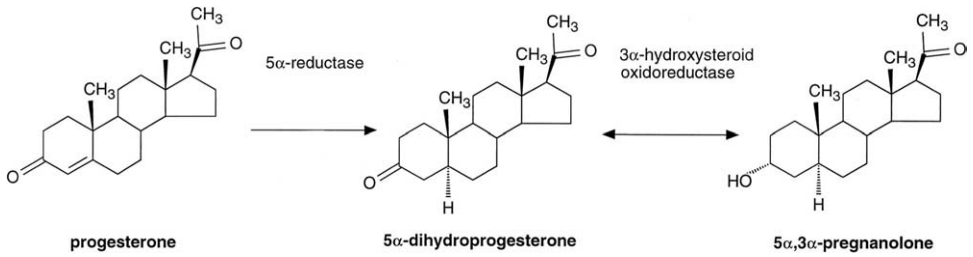


Figure 1. Metabolic pathway for conversion of progesterone to allopregnanolone (5α,3α-pregnanolone).

MATERIALS AND METHODS

Study Design

Male Sprague-Dawley rats, approximately 60 days of age (270 to 300 g) at surgery, were used as subjects (Institutional Animal Care and Use Committee, Emory University protocol 131-2002) and randomly assigned to one of the treatment or control groups. Approved protocols meeting National Institutes of Health (NIH) guidelines require that investigators take all necessary steps to minimize the number of animals needed for statistical analysis and to minimize any unnecessary pain and discomfort caused by the experimental procedures. All animals were housed in a temperature-, humidity-, and light-controlled environment, placed under a 12-h reverse light/dark cycle, and handled daily for at least 3 days before surgery.

A total of 28 rats were used in the present study; 2 died during surgery, and an additional 4 and 3 rats were excluded because of inadequate occlusion and reperfusion, respectively. Criteria for inclusion or exclusion of rats from the study group were based on the laser-Doppler (LD) flowmetry measurement of cerebral blood flow. LD flowmetry is an established, practical, and reliable system for monitoring changes in LD cerebral blood flow because of induction of focal cerebral ischemia.[33] To ensure relative uniformity of the ischemic insult, animals with mean ischemic LD flowmetry greater than 40% of baseline LD flowmetry were excluded from the cohort. This procedure resulted in consistently larger and more uniform infarcts, reducing experimental variablity. Anesthesia was induced by inhalation of 5% isoflurane (in a N_2/O_2 70%/30% mixture) and maintained by inhalation of 2% isoflurane. Using a SurgiVet (model V3304; Waukesha, WI) pulse oximeter, blood SpO_2 was monitored and maintained at levels greater than or equal to 90%. Body temperature was monitored throughout surgery (by rectal probe) and maintained at 36.5 °C to 37.5 °C using a heating blanket (Harvard Apparatus, South Natick, MA). A small incision was made in the skin overlying the temporalis muscle, and the LD probe (Moor Instruments, Wilmington, DE) was positioned on the superior portion of the temporal bone (6 mm lateral and 2 mm posterior from bregma).

Focal cerebral ischemia was induced by occlusion of the right middle cerebral artery, as previously described,[34] but with some minor modifications. Briefly, a midline incision was made on the ventral surface of the neck, and the right common carotid arteries were isolated and ligated with a 6.0 silk suture. The internal carotid artery and the pterygopalatine artery were temporarily occluded using a microvascular clip. A 4-0 nylon monofilament with a heat-blunted tip was introduced into the internal carotid artery through the incision in the external carotid artery. The filament was carefully advanced approximately 20 mm distal to the carotid bifurcation, beyond the origin of the middle cerebral artery. Relative cerebral blood flow was monitored for the entire 2 hours of occlusion. At 5 minutes before the onset of reperfusion, drug treatment was randomly assigned (detailed below). After 120 minutes of middle cerebral artery occlusion, the occluding filament was withdrawn gently back into the common carotid artery to allow reperfusion to take place. Relative cerebral blood flow was monitored for an additional 5 minutes before the wound was sutured and the rats were allowed to recover from the anesthesia.

The rats subjected to middle cerebral artery occlusion incurring ischemic insult less than 40% of baseline LD flowmetry were randomly assigned to receive progesterone (n=6), allopregnanolone (n=6), or vehicle (n=7) treatment. Progesterone (P-0130; Sigma-Aldrich Co., St. Louis, MO) and allopregnanolone (Calbiochem/EMD Biosciences, San Diego, CA) were dissolved in 22.5% 2-hydroxypropyl-β-cyclodextrin and given in the amount of 8 mg/kg by ip injection 5 minutes before the onset of reperfusion to ensure more rapid absorption after injury. One additional sc injection of 8 mg/kg was administered 6 hours after middle cerebral artery occlusion. The 8 mg/kg doses selected were based on previous findings showing the effectiveness of both neurosteroids in both male and female animals.[17,35–37] Rats in the vehicle group underwent the same experimental protocol, except that they received an identical volume and weight of vehicle only.

The rats were killed 72 hours postocclusion with an overdose (75 mg/kg) of Nembutal sodium solution. Brains were removed and sectioned into 7 × 2-mm coronal slices using a commercial rat brain matrix. After sectioning, the slices were subsequently stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma) in saline and kept for 15 minutes at 37 °C in the dark. Stained sections were then fixed in 10% buffered formalin. Both hemispheres of each stained coronal section were scanned using a high-resolution scanner (Epson Perfection 2400 Photo, Epson, Long Beach, CA), and then evaluated by digital image analysis (Image Pro System, Media Cybernetics, Silver Spring,

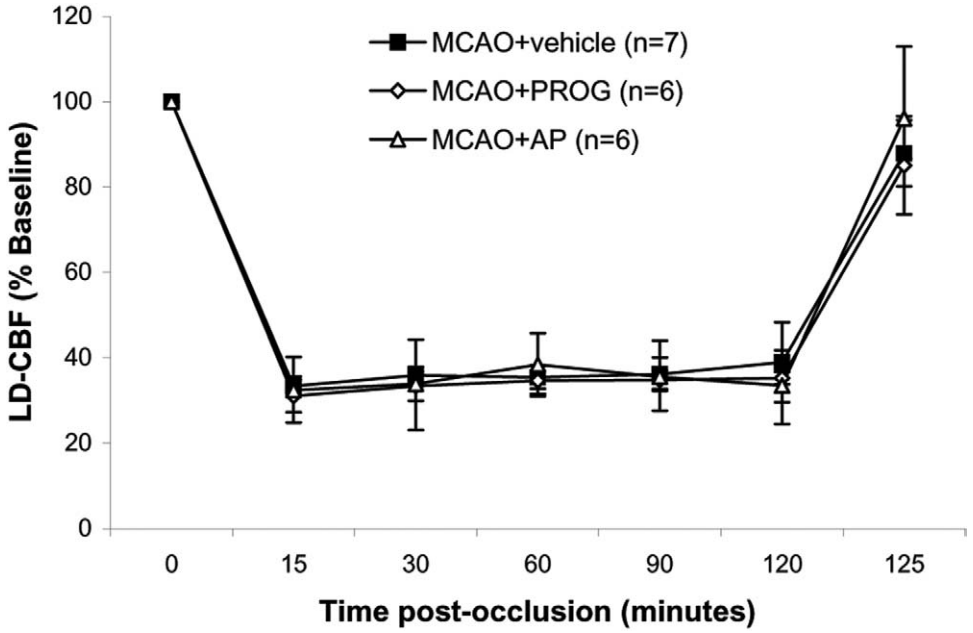


Figure 2. Laser-Doppler measurement of relative cerebral blood flow during 2 hours of MCAO and 5 minutes postreperfusion. Laser-Doppler flowmetry was measured over the ipsilateral parietal cortex and expressed as a percentage of baseline. There were no significant differences between groups at any time points, suggesting that the relative ischemic insult was equivalent among all groups. The data are represented as mean±SD.

MD). The unstained area representing the infarct was integrated across sections, and infarct volume in the cerebral cortex, caudate-putamen, and affected hemisphere was expressed as percentage of the corresponding intact, contralateral structure. A person blinded to the treatment or vehicle groups conducted the measurements and evaluations.

Primary Data Analysis

All results were expressed as mean±SD. The data were tested for normality and homoscedasticity (a measure of the constancy of variance over the levels of the factor under study) before being analyzed. Intraischemic LD flowmetry was subjected to 2-way analysis of variance (ANOVA) and a post hoc Tukey's test for multiple comparisons. Mean ischemic LD flowmetry and lesion volume were analyzed by 1-way ANOVA. If significant differences were observed with 1-way ANOVA, a post hoc Tukey's test was performed. The criterion for statistical significance was set at $P<.05$. The calculations were obtained using SAS 9.1 (SAS Institute, Inc., Cary, NC) and SPSS 11.0 (SPSS, Inc., Chicago, IL) software.

RESULTS

Figure 2 summarizes the intraischemic LD flowmetry expressed as a percent of baseline signal. ANOVA indicated no significant differences in cerebral blood flow between groups and time points during ischemia and early reperfusion, suggesting that the relative ischemic insult was equivalent among all groups. Mean relative cerebral blood flow observed during occlusion in rats treated with either progesterone (33.84%±1.28%) or allopregnanolone (34.76%±7.95%) was not statistically different from that observed in vehicle-treated animals (35.97%±3.14%). Reperfusion, as demonstrated by early recovery of the LD flowmetry signal, occurred in all animals regardless of treatment group within minutes of occluding filament release. There were no significant differences in the increase in mean relative cerebral blood flow in the progesterone-treated (85.05%±11.51%) and allopregnanolone-treated (96.05%±16.86%) animals compared with the vehicle-treated group (87.90%±7.75%), during the 5-minute postreperfusion time.

Figure 3 illustrates the effects of progesterone and allopregnanolone on infarct volume (expressed as percentage of intact contralateral structure). Cortical infarct volumes were significantly reduced in the progesterone (25.49%±7.38%) and allopregnanolone groups (11.40%±7.09%) compared with untreated animals (37.47%±10.57%) ($F_{2,16}=14.705$, $P<.05$). Allopregnanolone attenuated cortical infarct volume significantly compared to the progesterone-treated group. Caudoputamen infarct volumes were significantly reduced by progesterone (48.41%±22.44%) and allopregnanolone (50.44%±10.90%) compared to controls (78.02%±22.81%) ($F_{2,16}=4.655$, $P<.05$). Total hemispheric infarct volumes were also attenuated by progesterone (15.95%±3.59%) and

allopregnanolone (11.54%±3.71%) compared to controls (24.37%±6.69%) ($F_{2,16}$=11.097, P<.05).

LIMITATIONS

Although quantitative lesion volume analysis is useful in the evaluation of a new pharmacologic therapy, interpretation is somewhat limited because structural measures alone cannot provide a prognosis concerning the extent of, and recovery from, cognitive, sensory, and motor deficits. Whereas some studies argue that infarct size correlates well with certain neurologic impairments,[38] this is not always the case.[39–41] Second, this study was conducted using relatively young adult subjects, and much less is known about the effects of neurosteroid treatments for stroke in the senescent subject where stroke is more prevalent.

A third potential limitation to this study is the lack of consistent measures of postoperative temperature and blood pressure. It cannot be completely ruled out that progesterone and allopregnanolone exert some of their protective effects by causing mild postoperative hypothermia. Although the experimental literature is somewhat controversial about the beneficial effects of hypothermia,[42–47] it may be advantageous to determine whether the decreasing, maintaining, or even increasing of brain and body temperature by neurosteroids could affect outcomes. Further, we did not measure postoperative blood pressure. Because neurosteroid administration could lead to vascular dilation and hypotension, and there is evidence to suggest that hypotension may worsen ischemic injury,[48] in future studies measuring postischemic blood pressure will be important in exploring the mechanism of action of these neurosteroids. However, obtaining blood pressure readings will require caution because any restraint for the purpose of measurement could interact with treatment to confound results.

Another potential limitation of the current study is that only males were used as subjects. Previous research has shown, however, that both males and females benefit from neurosteroid treatments in both traumatic brain injury and ischemic stroke models.[49] Also, in a recently completed clinical trial with progesterone in the treatment of traumatic brain injury in both male and female patients, both groups showed salutary effects of the hormone, and an independent data safety monitoring board found no serious adverse events attributable to progesterone administration for either male or female patients.[50]

DISCUSSION

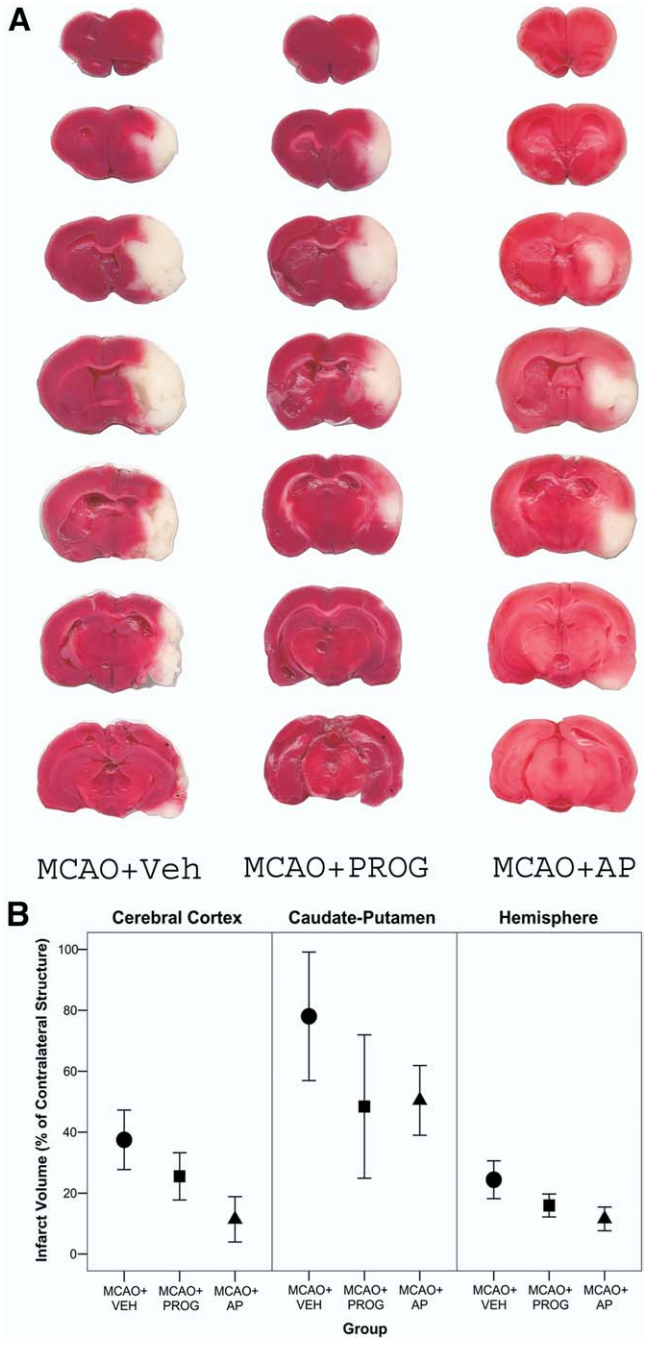
These results strongly suggest that progesterone and allopregnanolone reduce infarct volumes in a transient focal cerebral ischemia model in the adult rat, although, at least with respect to cortical damage, allopregnanolone is significantly more potent than progesterone at similar doses. The finding that progesterone offers neuroprotection after transient ischemic insult is consistent with earlier findings.[22–24,36,37] These investigators used changes in lesion volume as an indicator of reduced damage. Studies have also reported that progesterone improves neurologic outcomes at 2 and 7 days [36,37] and up to 3 weeks [23] after cerebral ischemia.

In the present study, the 8 mg/kg doses selected were based on previous findings showing the maximal effectiveness of both neurosteroids in the treatment of brain injury.[17,35–37,51] In our traumatic brain injury studies, we have reported that allopregnanolone at 8 mg/kg is a better treatment option than 4 mg/kg when behavioral outcomes are measured. Progesterone administered at a dose of 8 mg/kg has been shown to be effective in reducing infarct volume and functional deficits in mice after middle cerebral artery occlusion.[23] In rats, a dose of 8 mg/kg reduces lesion volume,[36,51] whereas lower (4 mg/kg) or higher doses (32 mg/kg) failed to have the same effect [36] after cerebral ischemia. This is an important observation because a higher dose of progesterone would be expected to have a greater impact if indeed it functions solely through its metabolite, allopregnanolone. After administration of a higher chronic dose (30 mg/kg, 7 to 10 days), preinjury progesterone treatment exacerbates striatal injury.[52] In our studies, it was considered more relevant to initiate treatment after, rather than before, the ischemic event.

In our recent work, we found allopregnanolone to be neuroprotective after bilateral cortical contusions in adult rats,[17,27,53] but until now, no study has examined allopregnanolone's effects in an ischemic stroke model. Our observation that allopregnanolone requires a lower dose than progesterone to attenuate infarct volume after ischemic reperfusion injury is consistent with earlier findings showing that both neurosteroids can act to reduce cerebral edema and the expression of inflammatory mediators implicated in secondary neuronal degeneration, apoptosis, and neuronal loss.[17,27,54]

Ischemic stroke is typically characterized by the disruption of cerebral blood flow. This reduction results in energy failure and secondary biochemical disturbances, eliciting a robust, in situ, inflammatory response. There is now abundant evidence that inflammatory processes associated with ischemia and reperfusion contribute to the development of cell injury in stroke.[55–57] Experimental manipulations of some inflammatory factors are yielding insight into therapeutic strategies for ischemic stroke. For example, several studies have shown that progesterone and allopregnanolone suppress the inflammatory response, including the expression of specific proinflammatory cytokines.[24,58–61] Our own results revealed that progesterone and allopregnanolone prevent the initial increase in caspase-3, tumor necrosis factor-α, interleukin-1β mitochondrial RNA (mRNA), and protein after impact injury to the frontal cortex.[53,62] More recently, we have found that in animals given progesterone post-traumatic brain injury, Nuclear Factor (NF)κB p65 and the inflammatory metabolites of C3 (9 kDa and 75 kDa) were decreased in comparison to vehicle-treated animals.[54] In another laboratory using real-time polymerase chain reaction, Gibson et al [24] found that progesterone

treatment suppressed the injury-induced up-regulation of interleukin-1β, transforming growth factor-β(2), and nitric oxide synthase-2 mRNAs in the damaged hemisphere. Taken together, these findings suggest that some of the therapeutic benefits of neurosteroid administration can be attributed to their salutary regulation of inflammatory proteins, preventing the increase in immune cell invasion and reducing cerebral edema.[54] It is likely that administration of progesterone and allopregnanolone, by reducing the actions of these proinflammatory cytokines after ischemic reperfusion injury, results in a smaller cerebral infarction.



There is increasing evidence that some neuronal loss after brain ischemia takes the form of energy-dependent, programmed cell death (apoptosis). This apoptosis is most prominent in ischemic strokes with reperfusion and may contribute to neuronal death in the penumbral region of the infarct.[63–66] Although the literature on progesterone's effects on apoptosis is controversial, the inhibitory effect on apoptosis of its metabolite, allopregnanolone, has been reported in other injury models. For example, pretreatment of human neuron-committed teratocarcinoma NT2 cell line (NT2) neurons with allopregnanolone has been shown to reduce the number of terminal 2′-deoxyuridine-5′-triphosphate (dUTP) nick end labeled–positive cells after *N*-methyl-D-aspartate administration.[67] Caspase-3 has been described as a major cause of apoptotic processes after CNS injury.[68,69] Recently, we showed that both allopregnanolone and progesterone decreased cell death after TBI by reducing caspase-3-activity.[17] It is possible that progesterone and allopregnanolone may interrupt neuronal apoptosis occurring after ischemic episodes, and this would lead to a smaller infarct size and thus a better clinical outcome.

In our experiment, allopregnanolone compared to progesterone treatment reduced the cortical infarct more than the striatal infarct. This differential effect may be related to regional differences in the density and viability of astrocytes, regional expression of GABA, glutamate, and progesterone receptor subunits or subtypes, or to different ischemic mechanisms in the cortex versus the striatum. Alternatively, it could be that even small but significant bypass blood flow supplied through cerebral arteries other than the MCA may be present in the cerebral cortex. It is possible that effects of allopregnanolone might be further amplified when there is bypass blood flow rather than in the condition of complete ischemia or reperfusion (ischemic core).

In conclusion, our study has shown for the first time that allopregnanolone, a metabolite of progesterone, significantly reduces infarct volume after focal cerebral ischemia and appears

Figure 3. Progesterone and allopregnanolone reduce infarct volume. *A,* 2,3,5-Triphenyltetrazolium chloride stained coronal sections from representative animals that were given either vehicle or progesterone or allopregnanolone and that had brains harvested at 72 hours postocclusion. Infarcts are shown as pale (unstained) regions involving striatum and overlying cortex. The infarct area in allopregnanolone-treated animals is substantially reduced. *B,* Infarct volumes after 2 hours' occlusion followed by 72 hours' reperfusion. Compared to vehicle alone, either progesterone or allopregnanolone significantly reduced cortical, caudate-putamen, and hemispheric infarct volumes (percentage of contralateral structure). Allopregnanolone attenuated cortical infarct volume significantly compared to the progesterone-treated group. The data are represented as mean and 95% CI for each group.

to be more potent than progesterone in attenuating cortical infarct at a similar dose. An NIH single-center clinical trial for progesterone in the treatment of moderate to severe TBI has been completed, so there will soon be direct translation of this research to the clinic.[70] Despite initial progress, many of the specific molecular and genomic or proteomic mechanisms accounting for the differences between progesterone and allopregnanolone remain to be determined. Further studies are needed to define dose-response relationships, timed "window of treatment" effects, toxicology, and type, extent, and duration of behavioral recovery in an animal stroke model before evaluation of the hormones in a clinical trial for stroke. The current study is but a first step in replicating the beneficial effects of neurosteroid treatments in stroke that have been demonstrated for traumatic brain injury.[17,35,71,72]

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