Effect of Ganaxolone in a Rodent Model of Cerebral Hematoma

Patrick Lyden, MD; Caroline Shin, MA; Catherine Jackson-Friedman, BS; Steven Hassid, BS; Anthony Chong, MD

Background and Purpose—Therapy with γ-aminobutyric acid (GABA) agonists appears to improve outcome after experimental hematoma but with unacceptable side effects. We looked to synthetic GABA agonists, or positive GABA modulators, widely developed as anticonvulsants and anxiolytics, to find compounds that may be effective. Ganaxolone is a synthetic neuroactive steroid that positively modulates GABA. We sought to determine whether ganaxolone was beneficial using a model of intracerebral hematoma.

Methods—We stereotaxically injected varying doses of bacterial collagenase into the caudate nucleus of rats to induce blood-brain barrier failure and hematoma formation. Four hours later, we administered intravenously 15 or 30 mg/kg ganaxolone (n = 23 each group), 20 mg/kg pregnanolone (n = 21), or vehicle (n = 30). Forty-eight hours after collagenase injection, we rated each animal using a standard rodent neurological examination. The ratings were compared with the amounts of injected collagenase using the quantal bioassay procedure. Other sets of animals were tested later for visuospatial learning. Brains were then prepared for histomorphometry, and brain volumes were estimated.

Results—We found that ganaxolone 30 mg/kg significantly increased the ED_{50} in the bioassay, for a potency ratio of 1.8 ± 0.41 compared with vehicle (P < 0.05). Ganaxolone 15 mg/kg and pregnanolone did not affect neurological outcome. Ganaxolone 30 mg/kg did not clearly improve visuospatial learning several weeks after hemorrhage. Ganaxolone exhibited a weak effect on cerebral volumes 48 hours after stroke, but 3 months after hemorrhage no such effect could be detected.

Conclusions—Ganaxolone improves neurological outcome 48 hours after intracerebral hematoma but not visuospatial learning several weeks after intracerebral hematoma. Histological evidence of damage was reduced at 48 hours but not at 3 months. (Stroke. 2000;31:169-175.)

Key Words: cerebral hemorrhage ■ excitotoxicity ■ GABA ■ steroids ■ rats

Many steroids are widely recognized for their genomic actions: stimulation of transcription of genes. Neuroactive steroids are natural or synthetic compounds that affect neuronal membrane excitability through binding sites on neurotransmitter-gated ion channels.1,2 These compounds are devoid of glucocorticoid-like effects. Some neuroactive steroids are potent modulators of the γ-aminobutyric acid (GABA) receptor that allosterically potentiate inhibitory actions of GABA, rapidly altering neuronal excitability.3,4 The neuroactive steroids that most effectively modulate GABA_{A}^- regulated Cl^- flux are A-ring–reduced pregnane steroid metabolites of progesterone and deoxycorticosterone.

The 3α-hydroxy steroid metabolites of progesterone, including pregnanolone (3α-hydroxy-5β-pregn-20-one) and allopregnanolone (3α-hydroxy-5α-pregn-20-one), are fast-acting neurosteroids (endogenously synthesized in brain) that alter membrane ion conductance and neuronal excitability.5 Unfortunately, endogenous neurosteroids, including preg-

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nanolone and allopregnanolone, are readily oxidized at the 3α position, resulting in 3-keto metabolites that are essentially inactive at neuronal membrane receptor sites. Ganaxolone (3α-hydroxy-3β-methyl-5α-pregn-20-one) is a synthetic analogue of allopregnanolone that is methylated at the 3β position, preventing rapid metabolism and offering enhanced bioavailability.3 The 3β substitution does not alter the pharmacological properties of pregnane steroid.

Compounds that enhance GABA_{A} receptor responses bestowed neuroprotection in ischemia and in experimental intracerebral hematoma.6–11 When the GABA_{A} receptor is activated by agonists such as muscimol, neuronal membrane conductance to Cl^- ions increases, resulting in membrane hyperpolarization and reduced neuronal excitability. Since 3α-hydroxy pregnane steroids are potent ligands of the GABA_{A} receptor, functioning as positive GABA modulators,
they may be useful therapeutic agents in stroke. Pregnanolone is one of the most potent positive modulators of GABA, receptors and is without activity at the N-methyl-D-aspartate (NMDA) receptor.12–14 We sought to assess the neurological outcome and neuroprotective potential of the progesterone metabolite pregnanolone and ganaxolone after acute cerebral hematoma.

Materials and Methods

This work was approved in advance by the Animal Use Committee at the Veteran’s Affairs Medical Center, San Diego, Calif, according to all local, state, and federal regulations. Male Sprague-Dawley rats, weighing 250 to 300 g, were anesthetized with 1% halothane in oxygen/nitrous oxide 40:60 by face mask. The head was placed into a stereotaxic frame; after infiltration with 0.3 mL lidocaine (1%, no epinephrine), the scalp was incised over the midline. Through a small burr hole, a calibrated syringe (Hamilton Co) was inserted into the anterior portion of the caudoputamen (1.0 mm anterior to bregma, 3.0 mm right of midline, and 4.0 mm below dura).15 A solution containing collagenase (type 7, Sigma Chemical Co) was infused over 10 minutes. Variable amounts of collagenase were used ranging from 0.1 to 3.0 U, diluted in saline to an infusion volume of 1 μL. After withdrawal of the syringe, the burr hole was filled with paraffin, and the scalp incision was closed with steel surgical staples. Body temperature was not measured and the animal was not heated over 10 minutes. Variable amounts of collagenase were used ranging from 0.1 to 3.0 U, diluted in saline to an infusion volume of 1 μL. After withdrawal of the syringe, the burr hole was filled with paraffin, and the scalp incision was closed with steel surgical staples. Body temperature was not measured and the animal was not heated during surgery because the total anesthetic period was always <20 minutes. Hematoma begins to form slowly over the ensuing few hours, and therefore temperature autoregulation would be intact.16

Four hours after the collagenase infusion, each subject was rated normal or abnormal by an examiner who was unaware of the treatment received by each subject. An abnormal rating was given for any of the following signs: reduced exploration in the cage, circling, asymmetrical forepaw flexion when lifted by the tail, and asymmetrical forepaw grasping. Death was given the abnormal rating. The subjects were also rated at 24 and 48 hours. We chose to use the behavioral rating at 48 hours as a primary outcome variable in this study to avoid confounding sedative effects of the study drugs. If any subject exhibited signs (H) were equivocal, another blinded rater was asked to evaluate that subject. The subject received the abnormal rating if any doubt remained. We have used this scale for 5 years, and the interrater agreement is approximately 95%.9–11

We randomly administered vehicle or test drugs via tail vein 4 hours after collagenase injection because the hematoma is well developed by this time.16 Additionally, the effect of the inhaled anesthetic wears off over 1 hour and therefore does not interact with the test drug; physiological parameters are maintained homeostatically after the unanesthetized subject. We treated 20 subjects with 20 mg/kg pregnanolone and compared them with 21 vehicle-treated subjects. We treated 23 subjects each with 15 or 30 mg/kg ganaxolone (gift of CoCencys Corp, Irvine, Calif) and compared them with 21 vehicle-treated subjects. We treated 23 subjects each with 15 or 30 mg/kg ganaxolone (gift of CoCencys Corp, Irvine, Calif) and compared them with 21 vehicle-treated subjects. The steroids were prepared as solutions with 50% (wt/vol) 2-hydroxypropyl-β-cyclodextrin in saline; concentrations were adjusted so that 1.0 mL of solution, injected into each subject, delivered the drug dose per kilogram noted above. We chose these doses after a pilot study determined that these were the highest tolerated doses without respiratory suppression.

To measure the effect of treatment on clinical outcome, we adapted the quantal bioassay used in ischemia studies.17,18 Comparing the behavioral ratings with the doses of collagenase injected in each group. At lower collagenase doses all subjects are normal, at high doses all subjects are abnormal, and with intermediate doses a fraction of the animals are abnormal. The logistic equation was fit to these data iteratively, using a custom-written computer program17 to generate a location parameter, the ED50, which is the dose of collagenase that renders 50% of the subjects abnormal. Neuroprotective therapy will increase the ED50 by increasing the tolerance to larger hematomas. The quantal bioassay method has proven useful in numerous previous investigations.17,18 The independent samples t test was used to compare ED50 values between groups because no multigroup ANOVA method has been developed yet; Bonferroni correction was used to compensate for multiple comparisons. To rigorously compare treatments, separate control groups were used for each drug tested; this procedure reduces the likelihood of spurious findings due to variation of the control ED50 over time. All assessments were made by an investigator blinded to treatment assignment.

The bioassay, which is rapid and efficient, was used to identify a potent drug and dose schedule for further studies; 30 mg/kg ganaxolone appeared to be most potent. To confirm the findings of the bioassay in separate sets of animals, we used a test of visuospatial learning, the Morris water maze. We included unlesioned control (n = 8), vehicle-treated (n = 11), and 30 mg/kg ganaxolone–treated (n = 9) subjects in this phase of the study; the lesioned subjects (all received 1 mg/kg collagenase) were tested 8 weeks after hematoma and treatment by an examiner who had no knowledge of group assignment. The water maze test of visuospatial learning has been used extensively for assessing stroke outcome.9,10 The maze was a black circular tank (150 cm in diameter), filled with water (at 19.4°C to 21.6°C) to a depth of 50 cm. A 12×12-cm black, submerged (1 cm below the water level) escape platform was placed at a fixed location inside the tank. Four starting points were marked as north, south, east, and west. The sequence of starting positions was randomized daily, and there were 4 trials each day. Each trial began with the rat being placed into the water, facing the tank wall, at a selected starting position. The rat was given 90 seconds to find the platform, then allowed to rest on the platform for 20 seconds between consecutive trials. To test for any motor, visual, or tactile deficits that may cause inaccurate assessments of learning behavior, 2 visible, tan poles were attached to the black platform. This visible platform test was performed for 4 days. Four days after the last day of the visible platform test, a hidden platform test was performed (4 trials per day, for 15 days) to evaluate spatial learning. The 2 tan poles were removed, making the platform invisible to the rat. This required the rat to learn the platform location using visual clues. The rats were completely isolated from the water maze for 10 days before another hidden test was performed to test retention or reference memory.9

We analyzed the water maze data using ANOVA, blinded to group assignment.30 Groups were compared with 1-way ANOVA, with Trial as a covariate. Post hoc comparisons were made with the Newman-Keuls procedure. The retention test was analyzed with 2-way ANOVA using Treatment as the grouping variable and Trial as the factor.

To explore the histomorphometric effects of treatment, selected subjects were anesthetized with halothane and perfused transcardially with 100 mL saline and 100 mL 4% buffered paraformaldehyde. The brains were removed and placed in 4% paraformaldehyde (24 to 48 hours) followed by 30% sucrose (24 hours). Each brain was mounted whole on a freezing microtome stage to cut serial 30-μm sections every 435 μm, which were stained with cresyl violet and eosin and covered. Without knowledge of the treatment assignment, each slide was examined under a microscope with semiautomated image analysis and point counting, an unbiased stereological method we have described in detail elsewhere.10 We computed volume densities after collecting point counts of cortex, white matter, thalamus, hippocampus, basal ganglia, ventricle, and hematoma.21 To compare cerebral volumes among treatment groups, we used a 1-way ANOVA and Newman-Keuls procedure for post hoc comparisons.30 Given the volume of work involved in serial sectioning, we selected 4 groups for this phase of the study: the group treated with 15 mg/kg ganaxolone in the bioassay study (killed 48 hours after stroke) and the group treated with 30 mg/kg ganaxolone in the water maze study (killed 10 to 12 weeks after stroke). We studied all 9 subjects treated with 30 mg/kg ganaxolone and, at random, half (n = 6) of the vehicle-treated subjects after the water maze testing.

From the bioassay study we randomly selected subjects still alive at 48 hours after hemorrhage: 5 vehicle- and 6 ganaxolone-treated subjects. This choice allowed us to compare different doses at different times after treatment, but we could not compare the 2 doses with each other.
given the effect of time. In addition, the mortality skews the results toward smaller lesions.

Results

The results of the bioassay are illustrated in Figure 1. Ganaxolone 15 mg/kg offered no benefit: the ED$_{50}$ for treated subjects was 0.63±0.17 U (n=23) compared with 0.65±0.07 U (n=30) for vehicle-treated subjects, shown in Figure 1A. Ganaxolone 30 mg/kg, however, showed benefit, as evidenced by an ED$_{50}$ of 1.19±0.23 U (n=23; P<0.05) (Student t after Bonferroni) compared with the vehicle group. When these ED$_{50}$ values are compared, the potency ratio is 0.96±0.29 for 15 mg/kg ganaxolone and 1.83±0.41 for 30 mg/kg ganaxolone (P<0.05, Student t after Bonferroni). The endogenous neuroactive steroid pregnanolone 20 mg/kg was tested in the bioassay, but no neuroprotective effect was demonstrated. Higher doses of pregnanolone were not tested because they caused unacceptable side effects.

The results of the visuospatial learning test used to confirm the effect of 30 mg/kg ganaxolone are shown in Figure 2. In Figure 2A, the time required (latency in seconds) to find the escape platform is plotted; the plotted latency each day is the mean±SE of 4 trials. The effect of Trial was compared among Groups, but Trial was not independent of the Day variable, suggesting that the intertrial variance exceeded the learning effect over time. In Figure 2B, the distance traveled to the escape platform is plotted, averaged, and analyzed as for latency. The results of the latency and distance analysis are identical (see below). In confirmation of this, we analyzed the swim velocity and found no differences among groups: mean±SE velocities were 33.9±0.93, 33.5±0.74, and 33.6±1.0 for the unlesioned, vehicle, and ganaxolone groups, respectively.

During the first 3 days of testing, the unlesioned animals performed better than the lesioned animals treated with 30 mg/kg ganaxolone or vehicle, as illustrated in Figure 2. By the fourth day, however, all animals had learned the task and could locate the escape platform in <18 seconds. This
Table 1. Effect of Ganaxolone on Cerebral Compartment Volume Densities 48 Hours After Hematoma

<table>
<thead>
<tr>
<th>Group</th>
<th>Hemisphere Volume, mm³</th>
<th>Compartment Volume Densities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>White Matter</td>
</tr>
<tr>
<td>Ipsilateral to hematoma</td>
<td>432.8±16.9</td>
<td>0.22±0.003</td>
</tr>
<tr>
<td>Control</td>
<td>312.06±8.94</td>
<td>0.24±0.005</td>
</tr>
<tr>
<td>Ganaxolone 15 mg/kg</td>
<td>364.2±9.50*</td>
<td>0.26±0.005</td>
</tr>
<tr>
<td>Contralateral to hematoma</td>
<td>438.4±71.0</td>
<td>0.22±0.004</td>
</tr>
<tr>
<td>Control</td>
<td>357.64±11.8</td>
<td>0.25±0.005</td>
</tr>
<tr>
<td>Ganaxolone 15 mg/kg</td>
<td>408.34±8.2</td>
<td>0.26±0.002</td>
</tr>
</tbody>
</table>

Values are mean±SE; control, n=7; vehicle, n=5; ganaxolone, n=6. Brains were fixed and sectioned 48 hours after cerebral hematoma and treatment with 15 mg/kg IV ganaxolone or vehicle. For comparison purposes only, data from our prior study of unlesioned animals are shown first. The compartment density is the proportion of each hemisphere subsumed by each compartment. There was a protective effect of ganaxolone on white matter and ventricle as well as on total hemispheric volumes (see Results and Discussion).

*Significantly different from vehicle group (P<0.01, ANOVA with Newman-Keuls procedure).

Figure 3. Effect of ganaxolone on retention of the escape platform location. Distance (top) and latency (bottom) are displayed for each of the 4 trials constituting the retention test, which was done several days after the completion of the training period. All subjects appeared to remember the platform location on trial 1 (reference memory) and then to improve over the ensuing trials, confirming intact working memory.

Indicates that all subjects were free of significant motor, sensory, or visual impairments. During the testing phase with the hidden platform, conducted on days 5 to 19, there were differences between the treated groups, as shown in Figure 2. Overall, the main effect of treatment group on latency was highly significant (F1,63=13.09, P<0.001), as was the treatment day covariate (P<0.001). By univariate ANOVA on each day and Dunnett’s post hoc test for significance, the ganaxolone-treated group differed from the unlesioned control group on days 6, 10, 12 (latency but not distance), and 15 (P<0.05 for each test). By the same analysis, the ganaxolone group was not different from the vehicle group on any day.

We conducted a retention test and found that after a 2-week interval, all animals retained the information necessary to find the escape platform (see day 20, Figure 2). The latencies and distances, averaged over the 4 trials, were not different among the groups. Figure 3 illustrates the 4 trials from the retention test separately. All 3 groups exhibited improved performance over the 4 trials, with the 2-way ANOVA Group×Trial interaction term not significant (F1,63=1.44, P=0.24). The main effect for Trial was significant (F1,63=3.0, P=0.03), but the main effect for Group was not (F1,63=2.8, P=0.10). Analysis of distance data yielded the same result (Figure 3).

The morphometric data are presented in Tables 1 and 2. Ganaxolone significantly prevented the reduction of the white matter compartment 48 hours after hemorrhage (Table 1). Ipsilateral to the side of the hematoma, white matter volume was 7% in the group treated with 15 mg/kg ganaxolone but approximately 4% of cerebral volume in the vehicle-treated group (P<0.01). Likewise, contralateral to the hematoma, white matter volume was 8% after ganaxolone but 5% after vehicle treatment (P<0.01). The total volume of the hemisphere containing the hematoma was 364±10 mm³ 48 hours after ganaxolone, compared with 312±9 mm³ after vehicle (P=0.01). In contrast, when the same measurements were made 3 months after treatment with 30 mg/kg ganaxolone, no benefit could be documented (Table 2).

Discussion

The present report is the first investigation of the putative neuroprotective properties of the neuroactive steroid ganaxolone. Higher doses of ganaxolone, 30 mg/kg IV, proved beneficial in the quantal bioassay that we have used extensively in previous studies of both hematoma and ischemia (Figure 1). This behavioral assay, which is based on a global rating of neurological function, indicated the beneficial effect of the drug after cerebral hematoma. Compared with prior studies with other agents, however, the efficacy of ganaxolone is only modest. Since pregnanolone and the lower dose of ganaxolone were not beneficial in the bioassay, they
were not studied further; indeed, one of the principal advantages of the bioassay is its utility as a rapid, efficient, and less expensive screening for putative therapies. To confirm the efficacy of ganaxolone, we used a detailed test of visuospatial learning, in which rats learn to locate an escape platform over 15 days; we could not confirm the bioassay findings. Figure 2 demonstrates no discernible ganaxolone effect using the learning task. Lesioned subjects who received vehicle also did not exhibit a significant learning deficit, suggesting that the lesion used may have been insufficient.

There was a modest effect of ganaxolone on cerebral volumes (Tables 1 and 2): when examined 48 hours after the onset of hematoma, 15 mg/kg ganaxolone resulted in less shrinkage of cerebral white matter. In addition, the ventricular enlargement associated with hematoma was less in the treated animals. However, months after hematoma, 30 mg/kg ganaxolone showed no statistically significant benefits. There is no definite explanation for these disparate findings. In prior hematoma studies, muscimol exhibited cytoprotective effects at 48 hours and 3 months, manifest as preservation of the cortex and hippocampus compartments. To speculate, there may be an effect of ganaxolone on cerebral edema, seen maximally during the first 48 hours after hematoma, that is not evident at longer observation intervals. These findings require further investigations, including time course and dose ranging series, that were beyond the scope of this investigation.

The mechanism of the possible beneficial effect of ganaxolone is not demonstrated by our study. Inhibitory neurotransmitters stabilize the resting membrane potential of neurons and reduce the probability that glutamate stimulation leads to action potentials and calcium influx.22,23 Agonists of GABA receptor are neuroprotective if administered during or after ischemia.7–9,11,18,24,25 Allopregnanolone and its synthetic analogue ganaxolone are active at the GABA_3 receptor.3,26,27 These neuroactive steroids have no glucocorticoid activity and have no effects on the inflammatory system. The ganaxolone effect seen in the bioassay may reflect a number of possible mechanisms, including edema reduction or neuroprotection, but our data cannot differentiate this.

The Morris water maze has been used in several prior studies, and the concordance between maze learning and the quantal bioassay is generally excellent.28 Muscimol and MK-801 preserve visual learning ability after cerebral ischemia.10 This is true even in models that do not involve the hippocampus, the structure typically associated with visuospatial learning disorders. This learning task is sensitive to damage involving unilateral lesions of cerebral cortex, as well as to subcortical structures.28,29 There is a rough correlation between the volume of cortical damage and the degree of learning impairment.28 The lack of beneficial effect for ganaxolone may relate to its weak effect in the bioassay.

Pregnanolone, an endogenously occurring neuroactive steroid, was not neuroprotective when we administered 20 mg/kg. This dose level caused observable sedation, suggesting that adequate quantities entered the brain, consistent with prior studies.27,30,31 Although pregnanolone has positive GABA_3-modulatory properties, in the brain it is rapidly converted into several metabolites, most of which are inactive. Higher doses of pregnanolone caused sedation and respiratory suppression and were not pursued. Ganaxolone is a synthetic derivative of allopregnanolone that is not metabolized in the brain. The parent compound remains active at the GABA receptor to augment chloride flux when GABA occupies its binding domain.

We found that ganaxolone, a neuroactive steroid that acts as a positive GABA modulator, exhibits modest benefit in brain suffering intracerebral hematoma using a global outcome rating and a quantal bioassay. On the other hand, no clear beneficial effects were detected by a visuospatial learning task. Histomorphometry results suggested a benefit at 48 hours but not months after hematoma. The mechanism of this effect is unclear and may not represent neuroprotection. Our results are encouraging, but the potency of ganaxolone appears to be only modest. Higher-potency GABAagonists are needed that are water soluble, readily cross the blood-brain barrier, and have a longer bioactive half-life.
Lyden et al tested the effects of ganaxolone on outcome after intracerebral hemorrhage induced by collagenase injection in rats. Ganaxolone, a positive modulator of GABA_A receptors, improved the tolerance of rats to collagenase injection when administered in a dose of 30 mg/kg 4 hours after the collagenase. The end point was the neurological condition of the rat. It is not exactly clear whether this is a pretreatment, concomitant, or posttreatment administration, because it isn’t certain when the hematoma develops or when any surrounding damage occurs, although the authors suggest that this administration takes place after the hematoma has reached maximal size. The mechanism by which ganaxolone improved the tolerance to collagenase was not investigated in these studies. The therapeutic index does not appear to be very wide for ganaxolone, because 15 mg/kg had no effect and doses over 30 mg/kg caused respiratory depression. A detailed analysis of visual-spatial learning seems to show no significant effect of ganaxolone chronically (2 or 3 months later, depending on where it is mentioned in the manuscript). Measurements of brain volumes showed that 15 mg/kg ganaxolone prevented white matter loss. This dose, however, had no effect on the acute neurological condition, and the effect of the higher dose of ganaxolone on brain volumes was not measured. After 2 or 3 months, though, there was no

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References

effect of ganaxolone on brain volumes. As the authors point out, this study shows a transient improvement in neurological condition 48 hours after treatment with the higher dose of ganaxolone. All surviving rats recovered, so there was no effect on visual-spatial learning 2 or 3 months later. We do not know whether the transient effects were related to the single dose used. The authors speculate that the transient effects of ganaxolone may be result from reduction in brain edema. A pharmacological treatment that reduced or prevented brain edema would be of great clinical interest, but that the effects of ganaxolone have anything to do with edema are purely speculative at this point. Furthermore, even if edema were reduced, further experiments would be necessary to determine whether this was the primary effect or whether reduction of, for example, ischemia, reduced the edema secondarily. As mentioned above and by the authors, the effects of a tolerable dose of the drug were fairly modest and the therapeutic index is narrow. It is agreed that further study of GABA\(_A\) agonists is required before conclusions can be drawn about their potential efficacy in reducing brain damage after intracerebral hemorrhage.

R. Loch Macdonald, MD, PhD, Guest Editor
Section of Neurosurgery
University of Chicago Medical Center
Chicago, Illinois
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