Neuroprotective Efficacy and Therapeutic Window of the High-Affinity N-Methyl-D-aspartate Antagonist Conantokin-G: In Vitro (Primary Cerebellar Neurons) and In Vivo (Rat Model of Transient Focal Brain Ischemia) Studies

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ABSTRACT
Conantokin-G (Con-G), a 17-amino-acid peptide derived from marine snails and a potent N-methyl-D-aspartate (NMDA) antagonist, was evaluated for its neuroprotective properties in vitro and in vivo. In primary cerebellar neurons, Con-G was shown to decrease excitotoxic calcium responses to NMDA and to exhibit differential neuroprotection potencies against hypoxia/hypoglycemia-, NMDA-, glutamate-, or veratridine-induced injury. Using the intraluminal filament method of middle cerebral artery occlusion as an in vivo rat model of transient focal brain ischemia, the neuroprotective dose-response effect of Con-G administration beginning 30 min postocclusion was evaluated after 2 h of ischemia and 22 h of reperfusion. In the core region of injury, an 89% reduction in brain infarction was measured with significant neurological and electroencephalographic recovery at the maximal dose tested (2 nmol), although mild sedation was noted. Lower doses of Con-G (0.001–0.5 nmol) were significantly neuroprotective without causing sedation. Postinjury time course experiments demonstrated a therapeutic window out to at least 4 to 8 h from the start of the injury, providing a 47% reduction in core injury. The neuroprotective effect of Con-G (0.5 nmol) was also evaluated after 72 h of injury, where a 54% reduction in core brain infarction was measured. Critically, in both recovery models (i.e., 24 and 72 h), the reduction in brain infarction was associated with significant improvements in neurological and electroencephalographic recovery. These data provide evidence for the potent and highly efficacious effect of Con-G as a neuroprotective agent, with an excellent therapeutic window for the potential intervention against ischemic/excitotoxic brain injury.

The major consequences of a focal disturbance of blood flow after brain ischemia involve increased levels of extracellular glutamate (Martin et al., 1998), in part causing excitatory neurotransmission through the overstimulation of excitatory amino acid receptors, of which the N-methyl-D-aspartate (NMDA) receptor plays a prominent role (Nicotera and Lipton, 1999). The resulting excitotoxicity is due to an influx of calcium along with the downstream activation of phospholipases, proteases, and both reactive nitrogen and reactive oxygen intermediates (Koroshetz and Moskowitz, 1996; Nicotera and Lipton, 1999). Furthermore, ischemia compromises mitochondrial metabolism, resulting in a decrease in high-energy phosphate compounds available to the affected cells (Koroshetz and Moskowitz, 1996). If the ischemic damage is severe enough, an irreversible loss of mitochondrial membrane potential results in the early necrotic death of cells, seen mainly in the core of the injured brain areas (Martin et al., 1998). Due to the loss of internal ionic homeostasis, necrotic cells swell and burst, releasing their internal constituents and causing further damage as a result of an evoked inflammatory response (Pantoni et al., 1998; Nicotera and Lipton, 1999). Surviving neurons that are able to maintain at least some mitochondrial function may still die later due to ATP-dependent apoptotic processes involving the outer perinuclear regions of the injury (Eguchi et al., 1997; Barinaga, 1998; Martin et al., 1998). Varying degrees of both pathways lead to an apoptotic/necrotic continuum of cellular

ABBREVIATIONS: NMDA, N-methyl-D-aspartate; MCAo, middle cerebral artery occlusion; EEG, electroencephalogram; Con-G, conantokin-G; TTC, 2,3,5-triphenyltetrazolium chloride; H/H, hypoxia/hypoglycemia; MABP, mean arterial blood pressure; HR, heart rate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; [Ca2+]i, intraneuronal calcium concentration.
death influenced by glutamate toxicity (Cheung et al., 1998; Martin et al., 1998).

NMDA antagonists have been extensively studied as a therapeutic intervention to ameliorate the damaging cascades after injury such as stroke. However, initial studies with several NMDA antagonists showing efficacious effects in vivo animal models have been terminated in clinical trials due to insignificant results or the emergence of toxic side effects (Muir and Lees, 1995; De Keyser et al., 1999). Despite these failures, NMDA receptors remain a potential target for neuroprotection drug development (Whetsell, 1996). However, their therapeutic profile must be better assessed in the laboratory. Furthermore, clinical trials are being designed to better evaluate the neuroprotective effects of these and other candidate drugs (De Keyser et al., 1999; Muir and Grosset, 1999).

This study evaluated the potential neuroprotective effects of conantokin-G (Con-G), a 17-amino-acid peptide derived from a marine snail, Conus geographus. Con-G is a high-affinity NMDA antagonist (Skolnick et al., 1992) that is selective for the NR2B subunit, with binding activity distinct from that of any other competitive NMDA antagonists (Donevan and McCabe, 2000). Using four different primary neuronal culture models of in vitro excitotoxicity, we describe a differential potency profile for Con-G-induced neuroprotection against NMDA-, hypoxia/hypoglycemia (H/H)-, glutamate-, or veratridine-mediated injury. In addition, we have shown Con-G to block intraneuronal calcium ([Ca\(^{2+}\)]\(_i\)) influx due to NMDA excitotoxicity in these same cultures. Finally, in vivo experiments using acute (i.e., 24 h) and delayed (i.e., 72 h) recovery models of rat focal cerebral ischemia and reperfusion injury delineated the pharmacodynamics of "postinjury" injections of Con-G to decrease brain infarction and improve functional recovery associated with ischemic brain injury.

Materials and Methods

Surgical Procedures. One hundred ninety-two male Sprague-Dawley rats (270–330 g; Charles River Labs, Raleigh, VA) were used in this study. Anesthesia was induced by 5% halothane and maintained at 2% halothane delivered in oxygen. Body temperature was maintained at 37°C throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA). Food and water were provided ad libitum before and after surgery, and the animals were individually housed under a 12-h light/dark cycle. The facilities in which the animals were maintained were maintained and fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.

The i.c.v. catheters were stereotaxically placed into the right lateral ventricle (1 mm posterior and 1 mm right lateral to bregma). Two cortical electrodes (epidural stainless steel screw electrodes, 0–80 × 1/8 inches) were permanently implanted and fixed to the skull using dental acrylate cement (Tortella et al., 1997). Twenty-four hours after the surgical procedures described above, the rats were reanesthetized and prepared for temporary focal ischemia using the filament method of middle cerebral artery occlusion (MCAo) and reperfusion as described elsewhere (Tortella et al., 1999). Briefly, the right external carotid artery was isolated and its branches were coagulated. A 3-0 uncoated monofilament nylon suture with a rounded tip was introduced into the internal carotid artery via the external carotid artery and advanced (approximately 22 mm from the carotid bifurcation) until a slight resistance was observed, thus occluding the origin of the MCA. Once the filament was in place, a drop in amplitude of the cortical electroencephalographic (EEG) recording was used to indicate a successful occlusion. The endovascular suture remained in place for 2 h and then was retracted to allow reperfusion of blood to the MCA. After MCAo surgery, animals were placed in recovery cages with ambient temperature maintained at 22°C. During the 2-h ischemia period and the initial 6-h postischemia period, 75-W warming lamps were also positioned directly over the top of each cage to maintain body temperature normothermic throughout the experiment.

MCAo Experiments. Before each injection, the body temperature was recorded and a neurological examination was performed (see later). EEG samples were recorded in the anesthetized rat before MCAo surgery, prereperfusion surgery, and at the indicated end point. Importantly, the 2-h injection was given immediately after recovery from anesthesia from the reperfusion surgical procedure so as not to compromise the results of the functional neurological examination conducted immediately before the reperfusion. At the conclusion of the experiment (either 24 or 72 h after MCAo), rats were euthanized by decapitation, and their brains were removed for quantification of infarction.

Physiological Experiments. In separate experiments, femoral artery catheters were placed into the right femoral artery using MRE-25 tubing (BrainTree Scientific, Braintree, MA). Mean arterial blood pressure (MABP) and heart rate (HR) were continuously monitored from the femoral artery catheter in awake freely moving rats using a DigiMed blood pressure analyzer (MicroMed, Louisville, KY). Baseline blood samples were taken before and 30 min after each i.c.v. injection of 0.5 nmol of Con-G to measure blood gases using an ABL5 blood gas system (Radiometer A/S; Copenhagen, Denmark) in normal uninjured rats. Physiological analysis was also performed on injured vehicle- and Con-G-treated rats after 2 h of MCAo and reperfusion with injections at 30 min and 2, 4, and 6 h after MCAo. The same time points were analyzed after Con-G administration in both groups (normal and injured).

Infarct Analysis. For each rat brain, analysis of ischemic cerebral damage, including total and core infarct volumes and hemispheric infarct size (calculated as percentage of infarcted tissue referred to the corresponding contralateral uninjured cerebral hemisphere, to exclude the possible contributing effect of hemispheric edema to infarct volume), was achieved using 2,3,5-triphenyltetrazolium chloride (TTC) staining from seven coronal sections (2-mm thick). Brain sections were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the corticocerebellar junction. Computer-assisted image analysis was used to calculate infarct volumes and has been described in detail elsewhere (Tortella et al., 1999). Briefly, the posterior surface of each TTC-stained forebrain section was digitally imaged (Loats Associates, Westminster, MD) and quantified for areas (in square millimeters) of ischemic damage. Core injury was defined as brain tissue completely lacking TTC staining, whereas total injury was specified as all ipsilateral tissue showing a loss of stain compared with the contralateral, uninjured hemisphere. Sequential integration of the respective areas yielded total and core infarct volumes (in cubic millimeters). Similarly, ipsilateral and contralateral hemispheric volumes were measured where hemispheric swelling (edema) was expressed as the percentage increase in size of the ipsilateral (occluded) hemisphere over the contralateral (uninjured) hemisphere. As seen in Fig. 1, penumbral areas were defined as the total (green outline) minus the core (yellow outline) infarct volume and correlated to light pink-staining brain regions. The percentage of penumbral infarct is presented as the penumbral infarct volume over the total infarct volume.

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Neurological Examination. A neurological examination was performed on each rat immediately before MCAo and again before each injection. Neurological scores were derived using a 10-point sliding scale. Each animal was examined for reduced resistance to lateral push (score, 4), open field circling (score, 3), and shoulder adduction (score, 2) or contralateral forelimb flexion (score, 1) when held by the tail (Tortella et al., 1999). Rats extending both forelimbs toward the floor and not showing any other signs of neurological impairment were scored 0. Using this procedure, maximal neurological severity was measured as a score of 10. In the present study, all rats subjected to MCAo either exhibited a neurological score of 10 when examined 2 h after ischemia or immediately before reperfusion or were dropped from the study.

EEG Recovery. Brain EEG activity was sampled while the animals were anesthetized. EEG recordings were taken before MCAo surgery, during insertion of the filament, and at the conclusion of the experiment (immediately before euthanasia). Computer-assisted spectral analysis (Tortella et al., 1999) was used to calculate the percentage increase in EEG power scores collected at the conclusion of the experiment compared with the power scores taken immediately before the initiation of brain reperfusion at 2 h after MCAo. Briefly, the drop in EEG power immediately after MCAo is representative of the massive drop in the amplitude of the recorded analog EEG signal. We used this to confirm a successful filament placement and MCA occlusion and quantified the postinjury return in the amplitude of the recorded EEG wave form as a functional measure of brain recovery and correlation to recovery (Philips et al., 2000).

Cell Culture Preparation. Enriched neuronal cultures were prepared from 15-day-old Sprague-Dawley rat embryos. Using aseptic techniques, the rat embryos were removed from the uterus and placed in sterile neuronal culture medium. Using a dissecting microscope, the brain tissue was removed from each embryo, with care taken to discard the meninges and blood vessels. The cerebellum was separated by gross dissection under the microscope, and only cerebellar tissue was used for the culture. Cells were dissociated by trituration of the tissue and were plated at a density of $5 \times 10^5$ cells/well onto 48-well culture plates precoated with poly(l-lysine). Cultures were maintained in a medium containing equal parts of Eagle’s basal medium (without glutamine) and Ham's F-12k medium supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, 600 μg/ml glucose, 100 μg/ml glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. After 48 h, 10 μM cytosine arabinoside was added to inhibit non-neuronal cell division. Cells were used in experiments after 7 days in culture.

In Vitro Neuronal Injury. Four in vitro models of excitotoxicity were studied. Cells were either exposed to H/H conditions for 2 h (see later) or treated for 45 min with either NMDA (100 μM), glutamate (80 μM), or veratridine (20 μM). All cells were cotreated with Con-G (0.0001–1000 nM) in Locke’s solution. At the conclusion of the respective excitotoxic exposures, the condition medium (original) was
replaced; 24 h later, the morphological and cell viability (MTT measurements) assessments were made. Cell damage was quantitatively assessed using a tetrazolium salt colorimetric assay with 3-(4,5-di- methylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma Chemical Co., St. Louis, MO). Briefly, the dye was added to each well (final concentration, 1.5 mg/ml), cells were incubated with MTT-acidified isopropanol (0.1 N HCl in isopropanol), and the absorbance intensity (540 nm) of each sample was measured in a 96-well plate reader.

Values are expressed relative to vehicle-treated control cells that were maintained on each plate, and the percentage change in cell viability was calculated. H/H was induced by incubating the cells in a humidified airtight chamber saturated with 95% nitrogen, 5% CO₂ gas for 2 h in Locke’s solution without added glucose. Cells were treated with either vehicle or Con-G (0.1–1000 nM) during 2-h H/H, and their morphological and viability (MTT measurements) assessments were made 24 h later. Values are expressed relative to control (normoxic) cells, and the percentage change in cell viability was calculated. Differences in the cell viability among treatment groups were determined using one-way ANOVA and the Newman-Keuls test.

Neuronal Calcium Dynamics. Changes in intraneuronal free calcium concentrations were determined using the fluorescent calcium-sensitive dye fluo-3. Neurons were loaded with the membrane-permeable acetoxymethyl ester form of the dye by exposure to a reduced calcium medium containing fluo-3-AM (5 μM) for 1 h (37°C). They were then washed and maintained in Locke’s solution at 37°C. Fluorescence changes in individual neurons were monitored using the Insight Plus confocal scanning laser microscope system (Meridian Instruments, Okemos, MI). Sequential image scans of fields containing 10 to 50 neurons (250×250 μm²) were collected every 10 s to construct the kinetic profiles of the effects of NMDA on [Ca²⁺]i, in the presence and absence of Con-G. To verify adequacy of dye loading and neuronal viability, the calcium ionophore ionomycin (2 μM) was added 1 to 2 min before the end of each experiment. Neurons not responding or responding very weakly to the treatment with ionomycin were not counted in the study.

Data Analysis. Data are presented as mean ± S.E. Statistical analysis of single dose responses (n = 6–10/group) was made by planned comparisons using independent Student’s t test with a modified Bonferroni correction for multiple tests. Changes in physiological parameters (n = 3 or 4/group) were evaluated by ANOVA followed by Dunnett’s post hoc analysis. These statistical analyses were calculated using Minitab Statistical Analysis software program. Potency comparisons were performed with the percentage of neuroprotection dose-response data. For the neuroprotection ED₅₀ values, the criteria for identifying a positive responder were defined as previously described (Tortella et al., 1999). Potency and ED₅₀ calculations were performed using the Pharmacological Calculations Computer Programs described by Tallarida and Murray (1987).

Compound and Treatment Protocol. Table 1 summarizes the dosing and injection schedules for the various in vivo experiments using both the 24- and 72-h recovery models. Con-G was received from Cognetix, Inc. (Salt Lake City, UT). The compound was dissolved in distilled-deionized water immediately before testing and administered i.c.v. in a 5-μl volume without handling or disturbing normal animal behavior.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Injection Schedule</th>
<th>Dose Range</th>
<th>End Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose-response</td>
<td>0.5, 2, 4, 6</td>
<td>0.001–2.0</td>
<td>24</td>
</tr>
<tr>
<td>Therapeutic window</td>
<td>0.5, 2, 4, 6</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>2, 3, 4, 6</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>4, 5, 6, 8</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>8, 9, 10, 12</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>Single-dose study</td>
<td>0.5, 2, 4, 6, 24</td>
<td>0.5</td>
<td>72</td>
</tr>
</tbody>
</table>

Fig. 2. Dose-dependent decrease in cerebral infarction of both total and core areas for each coronal brain section. Data are presented as mean ± S.E.
at 72 h, regardless of treatment group, with no significant differences in body weight loss between groups. In the vehicle control animals, MCAo caused a transient, mild hyperthermia (37.3 ± 0.3°C) that returned to normal (36.2 ± 0.2°C preocclusion) by 4 to 6 h postocclusion (36.0 ± 0.2°C), which was similar to our earlier findings (Tortella et al., 1999). Interestingly, at 24 to 72 h postinjury, control injured animals exhibited a mild hypothermia (35.5 ± 0.1 and 35.8 ± 0.1°C, respectively). At all doses and time points, temperature measurements from Con-G-treated animals were not significantly different from those of the corresponding control, vehicle-treated animals.

**24-h Recovery**. Post-treatment with Con-G (administered i.c.v. starting at 30 min postocclusion) significantly reduced ischemic infarction uniformly throughout the brain measured at 24 h postocclusion (Figs. 1 and 2). A significant decrease in infarct volume was seen (Fig. 3) corresponding to a neuroprotection in the total and core regions of 89 ± 7 and 64 ± 10%, respectively, at the highest dose tested (2.0 nmol). The neuroprotection ED50 value (95% confidence limits) based on reduction of core infarction was 0.008 nmol (0.002–0.043 nmol). Importantly, with increasing dose, there was a significant and progressive increase in the volume of penumbral tissue as a percentage of the total infarct volume (Fig. 4). Furthermore, the overall percentage of hemispheric infarction compared with the contralateral hemisphere, which takes into account the effects of edema, was also significantly reduced with Con-G to a 2 ± 1% core infarction and 12 ± 3% total infarction at the highest dose (2.0 nmol). The effects of Con-G in altering hemispheric swelling due to edema after MCAo were not statistically significant.

A significant improvement in neurological score was seen with all doses of Con-G at 24 h postocclusion (Table 2). Also, a progressive increase in the percentage EEG recovery was measured in all drug-treated groups at 24 h compared with the vehicle-treated group, although only the highest dose was significantly different from the vehicle group (Table 2). The maximal recovery with the 2-nmol dose of Con-G corresponded to a 70% increase in EEG power compared with the vehicle-treated group.

When injections of Con-G were delayed by an additional 1.5 or 3.5 h, significant reductions in core infarction were still measured compared with the vehicle group (Fig. 5). The resultant neuroprotection (47 ± 16%) was time-dependent and significant out to 4 h postocclusion. Again, coincident

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**Table 2**

Dose-dependent effect of Con-G on percentage EEG recovery and neurological score after 2 h of MCAo and 24-h recovery

<table>
<thead>
<tr>
<th>Dose (nmol i.c.v.)</th>
<th>EEG Recovery</th>
<th>Neurological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>23 ± 9</td>
<td>8.8 ± 0.6</td>
</tr>
<tr>
<td>0.001</td>
<td>33 ± 8</td>
<td>7.7 ± 1.5</td>
</tr>
<tr>
<td>0.01</td>
<td>52 ± 16</td>
<td>6.0 ± 0.9*</td>
</tr>
<tr>
<td>0.5</td>
<td>81 ± 30</td>
<td>5.2 ± 1.0*</td>
</tr>
<tr>
<td>2.0</td>
<td>93 ± 18**</td>
<td>4.7 ± 1.5*</td>
</tr>
</tbody>
</table>

*Percentage recovery of EEG power compared with pre-MCAo recording.*

**Figure 3.** Effect of increasing doses of Con-G, compared with vehicle group, to reduce infarct volume after 24 h of temporary MCAo. Data presented as mean ± S.E. *P < .05, **P < .01 compared with vehicle-treated group (independent t test).

**Figure 4.** Percentage of total infarct volume that is penumbra as opposed to core injury, with increasing doses of Con-G compared with vehicle group. Data are presented as mean ± S.E. *P < .05, **P < .01 compared with vehicle-treated group (independent t test).

**Figure 5.** Effect of delayed treatment (from time of MCA occlusion) with Con-G, compared with vehicle group, to reduce infarct volume after 24 h of temporary MCAo. Data are presented as mean ± S.E. *P < .05, **P < .01 compared with vehicle-treated group (independent t test).
with the decreases in core infarct volume, there was a significant increase in penumbral volume (Table 3). There also was an improvement in neurological recovery with all delayed treatment protocols (Table 3). Interestingly, by delaying the first treatment 8 h, the neurological score at 24 h was $3 \pm 1.4$, with one animal showing complete recovery and 43% of the remaining animals having a score of 1. The delayed treatment groups also showed significantly improved percentage recovery in EEG power at 24 h when Con-G administration started 0.5 and 4 h postocclusion, although there was no strong linear relationship to the time delay (Table 3).

**72-h Recovery.** Using a single dose of Con-G (0.5 nmol) and starting injections at 30 min postocclusion, the resulting cerebral infarction was also significantly reduced when evaluated after 72 h of MCAo-reperfusion (Fig. 6). Total and core infarct volumes were reduced to $241 \pm 49$ and $105 \pm 39 \text{ mm}^3$, respectively, corresponding to a significant neuroprotection in both total (54 ± 17%) and core (38 ± 13%) regions. Similarly, the overall percentage of hemispheric infarction was significantly reduced (core, 11 ± 10%; total, 26 ± 12%). A significant increase in the penumbral region as a percentage of the total infarct volume was also identified (vehicle, 41 ± 5%; Con-G, 66 ± 10%). No significant effect on cerebral edema (6.5 ± 1.5%) was measured compared with the vehicle group. Neurological recovery (1.5 ± 0.5) and percentage recovery in EEG power (21 ± 7%) were also significantly improved at 72 h, with 66% of the animals exhibiting a score of 1 or less.

**Physiological Studies.** The effects of Con-G on physiological parameters, including MABP, HR, blood gases ($\text{pO}_2$ and $\text{pCO}_2$), and blood pH, are shown in Table 4. All measurements were taken 30 min after each i.c.v. injection of Con-G or vehicle using the same injection schedule as the MCAo dose-response experiments. Baseline values between groups were not statistically different. Con-G injections without MCAo produced a mild rise in MABP out to 6 h after the initial injection that reached significant levels only after the third (4.5 h) and fourth (6.5 h) injections. All other parameters were not statistically different from baseline values. After MCAo in vehicle-treated animals, we also noted a moderate increase in MABP that was transient and returned to normal by 4 h. No differences were measured among the other parameters. Interestingly, in MCAo rats treated with Con-G, a consistent but nonsignificant increase in MABP was measured after occlusion. Importantly, there were no significant physiological effects measured at any time after MCAo in Con-G-treated rats.

**TABLE 3**

<table>
<thead>
<tr>
<th>Time Delay of First Injection</th>
<th>Penumbral Infarct Volumea</th>
<th>EEG Recoveryb</th>
<th>Neurological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>37 ± 5</td>
<td>23 ± 9</td>
<td>8.8 ± 0.6</td>
</tr>
<tr>
<td>30 min</td>
<td>78 ± 6**</td>
<td>81 ± 30</td>
<td>5.2 ± 1.0*</td>
</tr>
<tr>
<td>2 h</td>
<td>70 ± 6**</td>
<td>63 ± 9**</td>
<td>5.9 ± 1.1*</td>
</tr>
<tr>
<td>4 h</td>
<td>63 ± 8*</td>
<td>82 ± 19**</td>
<td>6.0 ± 1.5</td>
</tr>
<tr>
<td>8 h</td>
<td>36 ± 5</td>
<td>29 ± 7</td>
<td>3.0 ± 1.5**</td>
</tr>
</tbody>
</table>

*a Percentage penumbral infarction compared with the total infarct volume.

*b Percentage recovery of EEG power compared with pre-MCAo recording.

**In Vitro Studies.** Regardless of the excitotoxic insult, the four injury models produced similar morphological changes, including cell shrinkage, fragmented cellular membrane, and loss of neuronal processes, which were all reduced significantly with cotreatment of Con-G. As shown in Fig. 7, increasing doses of Con-G produced complete (i.e., 100%) neuroprotection against all four types of neuronal injury. However, the neuroprotection potencies (EC50 and 95% confidence limits) were different for the respective injury mechanisms. Con-G exhibited extremely potent and similar levels of neuroprotection against NMDA [77 $\mu\text{M}$ (42–141)] and H/H [54 $\mu\text{M}$ (13–91)]. However, much lower neuroprotection potencies were measured when the injuries was induced by glutamate [819 $\mu\text{M}$ (346–1937)] or veratridine [2136 $\mu\text{M}$ (1508–3026)].

Typical of high-affinity NMDA antagonists, a neuroprotective dose of Con-G was also effective in reducing neuronal [Ca$^{2+}$]i mobilization induced by NMDA toxicity (Fig. 8). Real-time images of [Ca$^{2+}$]i changes in 12 individual neurons exposed to NMDA revealed consistent and sustained increases in [Ca$^{2+}$]i, persisting throughout the 5-min observation period (Fig. 8A), which could be completely prevented by pre-exposure with Con-G (Fig. 8B).

**Discussion**

We used the model of focal cerebral ischemia to produce a 2-h temporary occlusion of the MCA in the rat followed by 1 or 3 days of reperfusion of blood to the ischemic area. This model closely resembles clinical stroke due predominantly to acute focal ischemic attacks (Ringelstein et al., 1992; Kaufmann et al., 1999). The resulting pathological state produced by this injury involves a core area of infarction surrounding the immediate territory of the MCA at the point of occlusion and a surrounding penumbral area of less-compromised tissue (Gill et al., 1995). In this study, histopathological brain damage was measured using a TTC stain that is reduced to a red-formazan product in the presence of active mitochondrial oxidative enzymes. The result is a “core” infarcted region of white (non-TTC-stained) tissue, which is consistent with the necrotic cell damage seen with other histopatholog-
Determined by MTT assay. Data are presented as mean ± S.D. against H/H, glutamate (Glu), NMDA, or veratradine (Vera), as shown in the injured rat. Moreover, there was a clear separation in the ipsilateral hemisphere and neurological recovery still observed with treatment delays of 8 h at least 4 h, with significant improvements in neurological recovery still observed with treatment delays of 8 h.

Our initial in vivo studies revealed that Con-G dose-dependently decreased infarct volume and improved both the EEG activity of the ipsilateral hemisphere and neurological recovery in the injured rat. Moreover, there was a clear separation between behavioral sedation and neuroprotection where doses of less than 0.5 nmol were non-sedative and only the highest dose of Con-G tested (i.e., 2.0 nmol) produced behavioral sedation. Importantly, with increasing doses of Con-G, there was a progressive transformation of the infarct to include a greater volume of penumbral tissue compared with core infarct volume. This is critical because it is expected that the penumbral regions contained viable mitochondria as indicated by the reduced TTC stain present. However, because penumbral regions are also likely undergoing delayed energy-dependent apoptosis (Eguchi et al., 1997; Barinaga, 1998; Nicotera and Lipton, 1999) and inflammation (Phillips et al., 2000), the need for well-designed combination drug therapies should be considered and tested (De Keyser et al., 1999; Nicotera and Lipton, 1999).

After an ischemic attack, brain infarction tends to spread circumferentially from the core of the infarct with an increase in the extracellular levels of excitatory amino acids (EAA) and glutamate (GLU). After an ischemic attack, brain infarction tends to spread circumferentially from the core of the infarct with an increase in the extracellular levels of excitatory amino acids (EAA) and glutamate (GLU).

Critical to the study, this rat MCAo model has been shown to be highly sensitive to pharmacological intervention with various other neuroprotective drugs, including NMDA antagonists, α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid ligands, antioxidants, N-acetylated alpha-linker acidic dipeptidase inhibitors, and anti-inflammatory treatments, reducing cerebral damage and/or improving functional deficits. (Clemens and Panetta, 1994; Kawasaki-Yatsugi et al., 1994; De Keyser et al., 1999; Tatlisumak et al., 1998; Slusher et al., 1999; Tortella et al., 1999; Phillips et al., 2000). For example, in rat MCAo models similar to the one used in this study, the NMDA antagonist dextromethorphan reduced infarction a maximum of 47%.
mum of 41% at its optimal neuroprotective dose (Tortella et al., 1999), whereas the N-acetylated alpha-linked acidic dipeptidase inhibitor 2-(phosphonomethyl)pentanedioic acid elicited approximately 54% neuroprotection (Slusher et al., 1999). Similar but less effective (i.e., approximately 30%) reductions in infarction have been reported for MK801 (Margail et al., 1996). By comparison, at its optimal neuroprotective dose, the effect of Con-G in reducing cortical infarction approached 75% in our 24-h injury model and 54% in the 70-h injury model. In our experience, only AHN649, an analog of dextromethorphan, has produced comparable reductions in cerebral infarction (Tortella et al. 1999).

Although physiological changes may be occurring for several weeks after ischemic injury, results from permanent MCA occlusion in rats have established that the infarction reaches a maximal volume and spread by 72 h postocclusion (Garcia et al., 1993). Using diffusion-weighted imaging, a similar progression of infarct volume has been seen in stroke patients (Schwamm et al., 1998). Therefore, once the potency and efficacy of Con-G were established in the 24-h injury model, an optimal (i.e., maximal neuroprotection without behavioral side effects) neuroprotection dose of 0.5 nmol was chosen for further studies in the 72-h recovery period. After 2 h of MCAo and 70 h of reperfusion, we observed a significant 54% neuroprotection in the core infarct region and a 25% increase in the percentage of penumbral region compared with the vehicle-treated animals. This improved pathology also correlated with significant recovery in both EEG function and neurological performance.

In either recovery model, it is unlikely that these neuroprotective effects of Con-G were due to simply to hypothermia, because rectal temperatures never fell below the normal range during or after the MCAo surgery. Furthermore, it is important to note that the effects were not due indirectly to sedation because there were no signs of sedation at the 0.5 nmol (or lower) dose, which was still highly efficacious in reducing the injury.

We also evaluated cardiovascular changes after Con-G injections. In normal rats, Con-G (0.5 nmol) induced moderate, delayed increases in MABP out to 6 h after the initial injection. However, toxicity studies in rats or dogs after i.v. injections of Con-G (10 mg/kg) showed no increase in MABP (R. T. McCabe, unpublished data). In vehicle-treated rats, MABP was also significantly increased but only during the initial 2 h after MCAo. Interestingly, in Con-G-treated MCAo rats, there was no initial spike in MABP but rather a progressive increase in MABP similar to the Con-G response in normal rats. However, at no single time point was there a statistically significant increase from baseline values. HR, blood gases, and pH were also not statistically different from the baseline values.

Con-G has been shown to be a potent and selective antagonist of NMDA-evoked currents in murine cortical neurons (IC50 = 440 nM), and this effect is due to a competitive action at the NMDA binding site (Skolnick et al., 1992; Donevan and McCabe, 2000). Furthermore, Con-G is a selective antagonist of the NR2B subunit expressed in Xenopus oocytes but does not share a common mechanism with phenylethanolamines (e.g., ifenprodil), which also bind at this site (Donevan and McCabe, 2000). Because this and other evidence indicate that Con-G possesses distinct kinetic properties for the NMDA receptor (Zhou et al., 1996; Blandl et al., 1998; Chen et al., 1998; Prorok and Castellino, 1998), we attempted to use functional in vitro assays of neuronal excitotoxicity to confirm its selectivity for NMDA. Our in vitro analysis of Con-G showed it to be neuroprotective in each of the four injury models studied. However, its potency against nonselective ligand-gated channel activation (i.e., glutamate) or sodium channel voltage-gated depolarization (i.e., veratridine) was 11 to 27 times lower compared with injury induced by selective activation at the NMDA postsynaptic channel complex (i.e., NMDA). As additional support for a site of action at the NMDA receptor, Con-G was also demonstrated to ameliorate NMDA-induced intraneuronal Ca2+ mobiliza-
tion with on-off kinetics similar to that reported for other NMDA antagonists (Klattice et al., 1997). Finally, the in vitro neuroprotective potency of Con-G against H/H injury (i.e., an in vitro ischemia model) is consistent with its described in vivo effect to reduce injury caused by cerebral ischemia.

In conclusion, we have shown that in vivo, the administration of Con-G reduces infarct volume and increases both neurological recovery and EEG power scores when administered even after 4 h after occlusion of the MCA. These effects appear to be independent of change in body temperature, cardiovascular dynamics, or behavioral side effects. In support of these data, we have shown that Con-G possesses potent neuroprotective properties against multiple forms of neuronal injury in vitro and that this action involves blockade of NMDA-induced calcium signaling. Furthermore, because the higher doses of Con-G produced more of a penumbral type of injury as opposed to a core injury, Con-G may represent an excellent adjunct treatment with drug therapy targeting the penumbral tissue.

References

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